Mechanisms and Contexts of Kin Recognition in Female House Mice

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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For my family...

... because kin deserve recognition

Declaration of Work Conducted

All the work presented in this thesis is my own, except for where it has been stated otherwise in the text.

Thesis Abstract

As relatives share genes that are identical by descent, organisms can gain additional fitness benefits by improving the reproductive success of known kin. There are a number of costs associated with close inbreeding, including an increased likelihood of the expression of recessive deleterious alleles and reduced survivorship. The ability to recognise kin therefore enables individuals to improve their inclusive fitness and avoid problems associated with close inbreeding. Female house mice (*Mus musculus domesticus*) will nest and nurse offspring communally. Choosing an appropriate nest partner is therefore important and competition between nesting females can result in reproductive inhibition and infanticide. Kin selection theory suggests that females could gain inclusive fitness benefits from nesting with relatives.

This thesis explores the mechanisms of kin recognition in female house mice in the contexts of social partner choice and inbreeding avoidance. Female house mice recognised unfamiliar relatives, suggesting a phenotype matching mechanism for kin recognition. Females were presented with maternal and paternal half-siblings to investigate recognition template formation. Females nested with maternal but not paternal half-sisters, suggesting that female house mice may use a recognition template learnt from their mother for social partner choice decisions. However females avoided both maternal and paternal half-brothers suggesting that females may use a match-to-self mechanism for inbreeding avoidance.

Female house mice were able to identify relatives from urine, suggesting that genetic markers are present in urine. To investigate the molecular markers of kin recognition mice were bred to control for the major histocompatibility complex (MHC) and major urinary proteins (MUPs). Females nested with females that matched for MHC or MUP type, suggesting that both gene families may be used for kin recognition between females. A non-significant trend was observed for females to avoid males that matched themselves for MUP type, but females showed no avoidance of males that matched for MHC type.

A small pilot experiment investigated the physiological effects of female social environment. Housing with unfamiliar females resulted in short-term decreases in female body mass and urinary protein concentration but an increase in urinary creatinine concentration. Competition between unfamiliar females may have resulted in a decreased

water uptake and an increase in scent marking which could explain the physiological changes observed.

Together these results suggest that female house mice may use two mechanisms of kin recognition. For social partner choice females may use a match-to-maternal MHC and MUP type mechanism, whilst for inbreeding avoidance females may use a match-to-own MUP type mechanism. The possibility of a single species using two separate mechanisms suggests that kin recognition may be considerably more complex than previously thought.

1 Introduction – Kin Recognition and Female House Mice

Recognition is a crucial aspect of many interactions between (and within) organisms. There are a number of different levels of recognition including self, familiar individuals, relatives, groups, and species. The ability to recognise kin enables individuals to improve their own inclusive fitness and avoid problems associated with close inbreeding. This chapter will present the theory behind kin recognition, provide a background to the mechanisms and molecules potentially involved in kin recognition, and introduce the study species - house mice.

1.1 Kin Recognition

1.1.1 Inclusive Fitness and Kin Selection

In 1964 Hamilton published two papers (1964a, 1964b) outlining the theory of inclusive fitness, thereby opening the door for modern kin recognition research. Hamilton's work provided a theory for the existence of altruism – behaviours whereby an individual acts in a manner that is costly to its own fitness but which helps that of a receiver (Hamilton, 1963). Relatives have genes in common that are identical by descent. By aiding a relative's reproductive success, the chances of those shared genes being passed on to the next generation are increased. An individual's inclusive fitness therefore consists of both its own reproductive success (direct fitness) and that of any relatives that have been affected as a result of its actions (indirect fitness). The evolutionary selection of traits that promote the survival and reproduction of relatives is known as kin selection (Maynard Smith, 1964).

Whilst behaviours that improve the reproductive success of relatives are beneficial, the average benefits gained should outweigh the average costs of these actions for such altruistic behaviours to have evolved. This is summarized by Hamilton's Rule:

$$rb - c > 0$$

where r is the genetic relatedness between the recipient and the individual acting, b is the benefit gained by the recipient, and c is the cost to the individual acting. The more closely related two individuals are, the greater the proportion of genes they will share. Full siblings or parent-offspring have a coefficient of relatedness of 0.5, half-siblings have a coefficient of relatedness of 0.25 and so on. This scale of relatedness led Haldane to joke

that he'd "jump into the river to save two brothers or eight cousins" (quoted in Nowak, 2006, p 1560).

The benefits of inclusive fitness could just as easily be gained by aiding individuals that happen to share the same genotype but which aren't directly related (Griffin & West, 2002). Inclusive fitness and kin selection are therefore subtly different from one another. To investigate this further Queller (2011) expanded the approach of inclusive fitness to include kin, kind (sharing the same phenotypic trait regardless of relatedness) and kith (behaviours such as reciprocity, mutualism or manipulation). Despite this, kinship is the most likely reason for two randomly selected individuals in a population to share the same genotype and inclusive fitness is often referred to in terms of kin selection alone.

Improving the reproductive success of a known relative can increase an individual's own inclusive fitness. It follows that organisms may behave differently according to the relatedness of the individual with whom they are interacting. Examples of differential behaviour towards relatives include reduced aggression (Holmes, 1986; Markman *et al.*, 2009; Lizé *et al.*, 2012); increased amicable interactions (Maher, 2009); reduced odour investigation (Mateo & Johnston, 2000); reduced scent marking (Heth *et al.*, 1998); increased time spent in proximity (Grau, 1982; Werner *et al.*, 1987); increased alarm calling (Davis, 1984); reduced territorial response (Sun & Müller-Schwarze, 1997); increased rates of play (Mateo, 2009); enhanced social learning (Kavaliers *et al.*, 2005); increased tendency to nest communally (Garza *et al.*, 1997); the avoidance of hosts already parasitized by relatives (Lizé *et al.*, 2006); and less competitive resource allocation (Biernaskie, 2011). These different forms of discriminative behaviour based on the level of relatedness suggest that there may be mechanisms for recognising kin.

1.1.2 Inbreeding and Outbreeding

Inbreeding occurs when individuals closely related through a shared ancestry reproduce (Partridge, 1983). Inbreeding depression occurs when deleterious problems associated with inbreeding reduce the fitness of a population. Since closely related individuals are likely to have high levels of genetic similarity, offspring resulting from such pairings have an increased likelihood of homozygosity at a given locus. Increased homozygosity can result in the expression of recessive deleterious alleles that in heterozygous individuals would be hidden by a dominant allele (Bateson, 1983; Partridge, 1983; Charlesworth & Willis, 2009). At certain loci individuals that are heterozygous can have a higher fitness than

individuals that are homozygous (overdominance). This fitness advantage can be reduced as a result of close inbreeding as offspring are more likely to be homozygous at a given locus (Bateson, 1983; Partridge, 1983; Charlesworth & Willis, 2009). Inbreeding depression can also cause a population to be susceptible to extinction as individuals within the population may not be variable enough to cope with habitat changes (Bateson, 1983). Problems associated with inbreeding include a lower survival rate (Chen, 1993; Jimenez *et al.*, 1994; Keller *et al.*, 1994; Meagher *et al.*, 2000) and a reduction in reproductive success (Krackow & Matuschak, 1991; Rosenfield & Bielefeldt, 1992; Chen, 1993; Alberts & Altmann, 1995; Meagher *et al.*, 2000). In many species individuals will therefore avoid reproducing with close relatives, although a number of species actively breed with relatives (Pusey & Wolf, 1996).

Two mechanisms have been suggested to enable individuals to avoid inbreeding. Firstly one or both sexes could disperse. By settling away from the natal territory reproductive encounters between relatives would be limited and any inbreeding costs resulting from the occasional chance encounter would be negated by the proportion of matings occurring between unrelated individuals (discussed in Greenwood, 1980). This mechanism is likely to be most effective in species where individuals are unlikely to meet close relatives after dispersal from the natal area. Alternatively, for those species that do not disperse or where the chance of encountering relatives is high, kin recognition could be used to prevent inbreeding.

Extreme levels of outbreeding can also have deleterious consequences (outbreeding depression). Populations of the same species are often influenced by and adapted to local environmental conditions. Crosses between two such populations could result in offspring with a reduced adaptation to the local environment and therefore a reduced fitness. Additionally, gene complexes are often coadapted and outbreeding could result in these complexes being broken up, leading to reduced fitness (Bateson, 1983; Partridge, 1983; Pusey & Wolf, 1996).

The optimal inbreeding/outbreeding hypothesis predicts that individuals may want to optimise outbreeding by avoiding mating with close relatives and genetically distant individuals (Bateson, 1983). In support of this hypothesis Bateson (1982) showed that Japanese quail (*Coturnix japonica*) prefer first cousins over full siblings and more distantly

related individuals. Kin recognition could therefore be important in mate choice to enable individuals to avoid the deleterious effects of both inbreeding and outbreeding.

1.1.3 Evidence for Kin Biased Behaviour

1.1.3.1 Kin Biased Behaviour in Non-Animals

Evidence for kin recognition (via kin discrimination) has been found in most taxa so far examined. Microbes display a large range of cooperative behaviours and kin selection is likely to be a reason for some of the behaviours observed (for reviews see West *et al.*, 2007; Strassmann *et al.*, 2011). Siderophores are iron-scavenging agents produced by bacteria in response to iron deficiency. Siderophore production is an altruistic act as siderophores are costly to produce and can benefit other nearby bacteria. *Pseudomonas aeruginosa* produce more siderophores when population relatedness is high than when relatedness is low (Griffin *et al.*, 2004). Quorum sensing allows *P. aeruginosa* to communicate and coordinate behaviours such as the formation of biofilms or swarming motility; levels of quorum sensing increase with increasing relatedness (Diggle *et al.*, 2007). Under harsh environmental conditions social amoebae group together to form fruiting bodies and spores that can disperse to potentially more suitable habitats. The individual amoebae that form the fruiting bodies die during this process and this altruistic act allows those amoebae that develop into spores to disperse. When faced with such difficult conditions *Dictyostelium purpureum* prefer to form groups with own relatives, meaning that whilst the individual fruiting body amoeba may die they gain an advantage from the survival of their close kin (Mehdiabadi *et al.*, 2006).

There is some evidence that plants are also able to identify relatedness of nearby plants (reviewed in Biedrzycki & Bais, 2010). When grown in unrelated groups the annual plant *Cakile edentula* allocates more resources to below-ground competition in the form of increasing root mass than when grown in sibling groups (Dudley & File, 2007). A similar result was found in *Ipomoea hederacea* where plants grown in mixed paternity groups allocate more resources to root mass and produce fewer seeds than plants grown in sibling groups (Biernaskie, 2011). *Impatiens pallida* do not increase root mass allocation when grown in proximity to unrelated plants but instead invest more in leaf allocation, increasing above-ground competitive ability (Murphy & Dudley, 2009).

1.1.3.2 Kin Biased Behaviour in Invertebrates

The majority of kin recognition studies have focused on animals. One well known example is the star ascidian tunicate (*Botryllus schlosseri*), highlighted by Grafen (1990) as the single true example of kin recognition in his controversial paper (discussed in Section 1.1.4). In this species planktonic larvae settle on substrate and develop into colonies, with sibling larvae being more likely to settle in close proximity to each other than non-sibling larvae (Grosberg & Quinn, 1986).

In insects, the larvae of *Aleochara bilineata* (a species of rove beetle) parasitize the pupa of cyclorraphous flies. The larvae actively seek out potential hosts and will preferentially super-parasitize hosts that are already parasitized by unrelated larvae over those that are parasitized by siblings, reducing sibling competition (Lizé *et al.*, 2006). In the parasitoid wasp (*Goniozus legneri)* female-female contests are less aggressive when females are more closely related (Lizé *et al.*, 2012). Inbreeding avoidance also occurs in insects as unrelated pairs of male and female parasitic wasps (*Venturia canescens*) are twice as likely to mate as paired full siblings (Metzger *et al.*, 2010). Inbreeding avoidance also influences re-mating time interval as female green-veined white butterflies (*Pieris napil*) re-mate sooner when mated with a full brother than when mated with an unrelated male (Välimäki *et al.*, 2011). Paired sibling bumblebees (*Bombus terrestris*) take longer to mate than paired unrelated individuals, suggesting that relatives are less motivated to mate with each other (Whitehorn *et al.*, 2009).

Whilst less common, some kin discriminative behaviours have been observed among the Arachnida. Juvenile social crab spiders (*Diaea ergandros*) preferentially cannibalise unrelated individuals instead of siblings when starved; subadult females cannibalise unrelated females and sibling males over unrelated males, suggesting both female-female altruism and female inbreeding avoidance (Evans, 1999). Nine month old whip spiders (*Damon diadema*) spend more time in proximity to the odour of their mother than the odour of an equivalent unrelated female (Walsh & Rayor, 2008).

1.1.3.3 Kin Biased Behaviour in Non-Mammalian Vertebrates

In fish, kin recognition has been demonstrated during both altruistic and inbreeding avoidance behaviours (reviewed in Ward & Hart, 2003). Groups of juvenile rainbow trout (*Oncorhynchus mykiss*) display fewer aggressive bouts and gain more weight than groups of unrelated individuals (Brown & Brown, 1993). Arctic charr (*Salvelinus alpinus*) reared

in full sibling groups also gain more weight and have a greater increase in body length than those reared in mixed or unrelated groups (Brown *et al.*, 1996). An advantage of kin association may also be present in cichlids (*Neolamprologus pulcher*), as juveniles preferentially associate with relatives over unrelated individuals (Le Vin *et al.*, 2010). Juvenile zebrafish (*Danio rerio*) prefer the olfactory cues of relatives over those of unrelated individuals suggesting kin affiliation, but on reaching adulthood this preference is reversed and females prefer the odours of unrelated males over those of brothers, suggestive of inbreeding avoidance behaviour (Gerlach & Lysiak, 2006).

Amphibians also display kin recognition behaviours (reviewed in Waldman, 1991). Fire salamander larvae (*Salamandra infraimmaculata*) display fewer aggressive behaviours resulting in fewer injuries as genetic relatedness increases (Markman *et al.*, 2009). Female mountain dusky salamanders (*Desmognathus ochrophaeus*) are more likely to spend time guarding eggs that are more genetically similar to themselves than those that are more distantly related (Masters & Forester, 1995). Cascade frog tadpoles (*Rana cascadae*) prefer to associate with more closely related conspecifics such as full and half-siblings over unrelated individuals (Blaustein & O'Hara, 1982). One reason for this kin preference could be due to benefits during development - common frog (*Rana temporaria*) tadpoles grow larger when reared with close relatives than with unrelated tadpoles under low population density, but show no difference in growth when population density is high (Pakkasmaa & Laurila, 2004). This suggests that the benefits of aggregating with kin are outweighed by the costs of competition between kin when conditions are not suitable.

Whilst most reptiles are not generally social, there are still examples of kin recognition behaviours to be found. In a test arena, related hatchling green iguanas (*Iguana iguana*) group together whilst unrelated hatchlings separate (Werner *et al.*, 1987). Neonate smooth snakes (*Coronella austriaca*) are able to discriminate a difference in the odours of kin and non-kin, as they exhibit more tongue flicks towards the odour of non-siblings than towards siblings (Pernetta *et al.*, 2009). Pregnant female timber rattlesnakes (*Crotalus horridus*) preferentially aggregate with kin at birthing rookeries during the summer gestation period (Clark *et al.*, 2012).

Kin recognition in birds is well studied, although most experiments have examined parent-offspring recognition (reviewed in Halpin, 1991; Komdeur & Hatchwell, 1999; Nakagawa & Waas, 2004). For example, fledgling zebra finches (*Taeniopygia guttata*) show a

preference for odours from the nest in which they were hatched (Krause *et al.*, 2012). Male peacocks (*Pavo cristatus*) display to attract mates in leks and are more likely to establish leks in close proximity to male relatives. This occurs even when male peacocks have had no prior experience of relatives (due to experimental manipulation, Petrie *et al.*, 1999). Long-tailed tits (*Aegithalos caudatus*) breed cooperatively and preferentially help to rear related young. A mechanism for identifying kin in this species could be auditory, as long-tailed tits can discriminate between the contact calls of kin and unrelated individuals (Sharp *et al.*, 2005). Japanese quail (*C. japonica*) of both sexes prefer to spend more time near opposite-sex first cousins over full siblings, third cousins and unrelated individuals, suggesting an avoidance of close inbreeding and outbreeding (Bateson, 1982).

1.1.3.4 Kin Biased Behaviour in Mammalian Vertebrates

A large number of mammals also exhibit kin biased behaviours (reviewed in Halpin, 1991) and there is also some evidence that humans are capable of recognising their kin through olfactory cues (e.g. Porter, 1998). Among primates, chimpanzees (*Pan troglodytes*) are able to recognise individual faces and match close relatives together (Parr *et al.*, 2010); ringtailed lemurs (*Lemur catta*) are able to discriminate between the glandular secretions of related and unrelated conspecifics (Charpentier *et al.*, 2010). Aggressive behaviour between vervet monkeys (*Cercopithecus aethiops*) is more likely to occur if one individual has previously fought with the relative of the other individual (Cheney & Seyfarth, 1986).

Other groups of mammals also exhibit kin biased behaviour. During dyadic interactions European polecats (*Mustela putorius*) are more tolerant of individuals they are reared with, reducing the potential costs of competitive behaviour between relatives (Lode, 2008). Fostering in Antarctic fur seals (*Arctocephalus gazelle*) involves providing milk to young that are not own offspring. In a genotyping study, females were more likely to foster related young than unrelated young (Gemmell, 2003). By providing milk to related but not unrelated young, female Antarctic fur seals may be increasing their own inclusive fitness. Related female bottlenose dolphins (*Tursiops aduncus*) are more likely to associate together than unrelated females in order to share resources and protect young (Wiszniewski *et al.*, 2010). Inbreeding avoidance behaviour is also observed in mammals. Male African elephants (*Loxodonta africana*) direct fewer reproductive behaviours towards closely related females (including those that are only paternally related) than towards unrelated females (Archie *et al.*, 2007).

Among mammals, rodents are the most well studied order for kin recognition (reviewed in Mateo, 2003; Holmes & Mateo, 2007). In many species aggressive and competitive behaviours decrease with increasing relatedness. During paired encounters male white-footed deer mice (*Peromyscus leucopus*) chase unrelated males more than related males (Grau, 1982). Male golden hamsters (*Mesocricetus auratus*) flank mark less in response to flank odours from a brother compared to odours from an unrelated male (Heth *et al.*, 1998). The territorial response in beavers (*Castor canadensis*) is stronger in reaction to the anal gland secretions of unrelated individuals than to those of siblings (Sun & Müller-Schwarze, 1997). Female Belding's ground squirrels (*Spermophilus beldingi*) and Arctic ground squirrels (*S. parryii*) also show less aggression towards sisters than towards unrelated females (Holmes & Sherman, 1982; Holmes, 1986).

Altruistic behaviours often increase with increased relatedness. Male Richardson's ground squirrels (*S. richardsonii*) are more likely to give alarm calls if they expect to have a close relative nearby (Davis, 1984). Amicable interactions between solitary marmots (*Marmota monax*) are more likely when individuals are related than unrelated (Maher, 2009). Social learning also improves with relatedness, as deer mice (*P. maniculatus*) learn how to perform a self-burying behaviour (for parasite avoidance) faster from their siblings than from unrelated individuals (Kavaliers *et al.*, 2005).

Rodent species also display inbreeding avoidance behaviours. Both male and female common voles (*Microtus arvalis*) prefer the odours of unrelated over related opposite-sex individuals (Bolhuis *et al.*, 1988). Female golden hamsters (*M. auratus*) investigate unrelated male odours for longer than related male odours (Mateo & Johnston, 2000); and male bank voles (*Myodes glareolus*) prefer to spend time in close proximity to unrelated females than to sisters (Lemaitre *et al.*, 2012). Female Brandt's voles (*Microtus brandti*) copulate more with unrelated males than with related males (Yu *et al.*, 2004). A number of studies have examined kin recognition in house mice (*Mus musculus domesticus*) and laboratory mice (a combination of *M. m. domesticus*, *M. m. musculus* and *M. m. castaneus*) (e.g. Bowers & Alexander, 1967; Kareem & Barnard, 1986; Barnard *et al.*, 1991; Johnston, 2003). These will be discussed in Section 1.2.

1.1.4 Kin Recognition, Discrimination and Bias

It is important to note that recognition and discrimination are two separate processes (Byers & Bekoff, 1986). Recognition is the neurological process whereby individuals or

templates are recalled from previous encounters. Discrimination is the behavioural process where related individuals are treated differently from those that are unrelated. An apparent lack of discrimination does not necessarily show that recognition has not occurred, merely that it has not been acted upon under the specific circumstances (Barnard, 1991; Tang-Martinez, 2001). Frequently the two distinct processes can be confused, leading to difficulties in terminology that can distract from the biology itself and affecting comparison across different studies.

Whilst kin recognition is often inferred through kin discrimination (Byers & Bekoff, 1986), the observation of behavioural discriminations based on relatives does not automatically imply kin recognition. Kin bias occurs when an organism responds differentially towards relatives compared to non-relatives but not as a result of direct recognition of relatedness (Barnard, 1991; Tang-Martinez, 2001). Observed kin biased behaviour could be interpreted as kin discrimination but could actually be based on other factors that merely correlate with relatedness.

Grafen (1990) asked whether kin are truly recognised or whether observed kin biased behaviour is actually a result of another recognition system. Genetic similarity increases from species to populations to relatives to individuals. He argued that "an experiment showing that a species has an ability to discriminate by genetic similarity does not show that the species has a kin recognition system" (Grafen, 1990, p 53). An individual using genetic similarity for species recognition would identify relatives (that have the closest genetic similarity to self) as a closer species match than unrelated individuals of the same species. Alternatively, observed kin biased behaviour, rather than being based on recognition of relatedness, could instead arise due to behavioural similarities between relatives or a shared requirement for specific resources (Barnard, 1991).

Grafen (1990) argued that for kin biased behaviour to be a result of true kin recognition then the mechanisms of recognition must have evolved specifically for the purpose of identifying kin. Only one study met Grafen's requirements – Grosberg & Quinn's (1986) study of the star ascidian tunicate (*B. schlosseri*), the larvae of which settle in close proximity to siblings. Grosberg & Quinn (1986) showed that siblings settle in proximity to each other; that settlement and colony fusing was due to matching at a polymorphic locus where matching individuals are likely to be close kin; and that colony fusion with a relative provides benefits against cheating. This, argued Grafen (1990), was a true kin

recognition system. However, Barnard (1991) has suggested that matching at the locus itself may convey fitness advantages making the preferential settlement in proximity to siblings a by-product of matching at the recognition locus. In a later paper Grafen (1991b) concluded that whilst a number of species undoubtedly do exhibit strict kin recognition, there is as yet little conclusive proof that behavioural biases observed are as a result of a mechanism that has evolved for the purposes of recognising and differentially responding to relatives.

Subsequently Grafen (1992) has suggested (with reservations) that house mice (*M. m.* domesticus) potentially employ a kin recognition system. Petrie *et al*'s (1999) study of male peacock (*P. cristatus*) leks has also been proposed as a true kin recognition system (Barnard, 2004).

A number of authors have challenged Grafen's (1990) strict definition of kin recognition. Byers & Bekoff (1991) argued that Grafen's definition of kin recognition does not take into account the effects that rearing environment may have on both an individual's phenotype and behaviour. Even if individuals are genetically identical for a recognition cue, kin recognition could still occur based on rearing environment effects. A similar comment was made by Stuart (1991) who argued that, whilst Grafen's definition required kin recognition based on genetic cues, there is no reason why other cue types could not be used if they reliably indicate genetic relatedness. Blaustein *et al.* (1991) agreed with the principles of Grafen's definition but argued that, whilst species recognition may have originally encouraged kin association in many species, the selective advantages of kin association could have led to the further evolution of mechanisms focused primarily on kin recognition. They also argued that a species recognition mechanism would not explain inbreeding avoidance behaviours (Blaustein *et al.*, 1991). In his subsequent replies, Grafen proposed two categories of kin recognition: strict kin recognition (based on his original definition) and loose kin recognition (kin are identified by cues that do not necessarily correlate with genetic relatedness; Grafen, 1991b, 1991a, 1991c). To date the debate remains unresolved and it has been suggested that Grafen's strict definition could "unnecessarily define the topic out of existence" (Penn & Frommen, 2010, p 61).

1.1.5 Individual Recognition and Familiarity

Recognising previously encountered individuals allows animals to adjust their behaviour to account for prior experience. Familiarity is a learning process occurring throughout an

animal's life that enables a previously encountered social or environmental stimulus to be recognized and discriminated from a novel but otherwise equivalent stimulus (Cheetham *et al.*, 2008). Novel individuals are likely to be encountered regularly in species such as house mice and the ability to learn new identities rapidly is advantageous. Through the recollection of previous interactions animals can avoid repeated investigation and costly assessments. An inability to learn individual identities could result in costs including injury from fighting or assessment, energy expenditure and time loss. The benefits of individual recognition can include group defence, warmth, reciprocal altruism, including communal nesting and nursing (Halpin, 1980; Ligon, 1991; Thom & Hurst, 2004).

Individual recognition influences behavioural interactions, social group choice and mating decisions. Pairs of unrelated male Turkish hamsters (*Mesocricetus brandti*) are less likely to fight when they are familiar than unfamiliar (delBarco-Trillo *et al.*, 2009), a trend also seen in male deer mice (Peromyscus maniculatus, Dewsbury, 1988). In meadow voles (*Microtus pennsylvanicus*), paired familiar females show less agonistic behaviour and more amicable acts than paired unfamiliar females, whilst encounters between familiar males produce more agonistic behaviour than between unfamiliar males (Ferkin, 1988). Juvenile red-backed salamanders (*Plethodon cinereus*) grow faster in the presence of a familiar adult than an unfamiliar adult, possibly due to decreased aggression (Liebgold & Cabe, 2008). After 12 days cohabitation female guppies (*Poecilia reticulata*) prefer familiar over unfamiliar individuals when choosing schooling partners (Griffiths & Magurran, 1997). Female prairie voles (*Microtus ochrogaster*) prefer to spend more time with males they have just mated with (and therefore familiar) than with novel males (Shapiro *et al.*, 1986). Female golden hamsters (*M. auratus*) produce larger litters when mated with familiar males compared to when mated with unfamiliar males (Tang-Martinez *et al.*, 1993). Dominant males scent mark close to female burrows more than subordinate males. It is probable that females prefer males whose odours they are already familiar with, as these males are likely to be dominant. Conversely, paired gray-tailed voles (*Microtus canicaudus*) produce fewer litters when pairings are familiar as opposed to unfamiliar (Boyd & Blaustein, 1985). This could be a result of an inbreeding avoidance mechanism as in many species familiar individuals are often also related (due to a shared rearing environment).

1.1.6 Mechanisms of Kin Recognition

In order to gain the benefits of inclusive fitness and to avoid inbreeding, individuals need to identify their relatives. A successful kin recognition mechanism limits two error types: the failure to identify a relative (false exclusion); and the identification of an unrelated individual as related (false assignment). Recognising kin requires cues or labels (such as a spatial or phenotypic cue), the perception of that cue, and then behavioural responses to the recognised cues. Hamilton proposed (1964a, 1964b) four mechanisms by which kin recognition could occur: spatial location; prior association; phenotype matching; and recognition alleles (for reviews see Holmes & Sherman, 1983; Waldman, 1987; Halpin, 1991; Tang-Martinez, 2001; Holmes, 2004; Mateo, 2004). These mechanisms, however, are not mutually exclusive.

1.1.6.1 Spatial Location

The spatial location kin recognition mechanism occurs where individuals within a certain area (such as a nest or territory) are treated as kin and those outside of the area as non-kin (Holmes & Sherman, 1983; Hepper, 1986). Spatial location as a mechanism of identifying kin may be more suitable for species where relatives reliably cluster in a particular area. Spatial location may be less reliable as a mechanism in species where one or both sexes disperse as invading unrelated individuals could otherwise be falsely included. It is possible however, that if one sex disperses (e.g. males) then a spatial location mechanism could be used by the non-dispersing sex (e.g. females). One problem associated with spatial location is that it can be easily exploited. Bird species that are brood parasitized by cuckoos are victims of this form of exploitation – cuckoo chicks are treated as offspring by the owners of the nest they were laid in because they are found in the exact place (the nest) where only relatives are expected to be found (Payne, 1977; Rothstein, 1990).

An example of spatial location occurs in the strawberry poison frog (*Oophaga pumilio*). Mothers deposit tadpoles in small pools of water formed amongst vegetation, returning regularly to provide food in the form of unfertilised eggs. Using artificial pools Stynoski (2009) showed that recognition of offspring was based on the spatial location of the pools, as females fed unrelated tadpoles that were experimentally transferred into the pools but ignored own tadpoles even if they had only been transferred into a pool 2 cm away from the original location. Female strawberry frogs are therefore identifying their offspring indirectly by using the spatial location of the pools.

Spatial location has been rejected by some as a true kin recognition mechanism as relatives are not being specifically identified (Halpin, 1991; Tang-Martinez, 2001), suggesting that individuals in a particular area are treated as kin because there is no mechanism for directly recognizing kin (discussed in Holmes, 2004). As a rule spatial location might provide benefits from inclusive fitness but this does not mean that relatives are specifically recognised. Others have termed spatial location as an indirect mechanism of kin recognition as, whilst kin may not be directly recognised, the consequences of the rule remain evolutionarily adaptive (Mateo, 2003). Spatial location is likely to be employed during the learning phases of prior association and phenotype matching, as young learn the identity or template of those individuals encountered during a critical phase in a specific location (see below).

1.1.6.2 Prior Association

In prior association an individual learns the identity of all the relatives present during a critical period (for example during rearing in the nest). In later life those specific individuals are treated as related whilst all other individuals encountered are treated as unrelated (Tang-Martinez, 2001; Mateo, 2003; Holmes, 2004). This mechanism allows for the future identification of parent(s), and siblings as well as any other individuals that might be present during the learning phase (such as helpers in the nest). Full siblings from the same breeding session may compete for resources or encounter one another during mate choice. Kin recognition via prior association could enable reduced competition between familiar relatives and allow for the avoidance of inbreeding. However, relatives absent during the critical period (such as full siblings from a previous breeding session) would not be recognised, whilst any unrelated individuals that happen to be present would later be treated as related. Since unfamiliar relatives would not be recognised as kin by the prior association mechanism, it has been argued that prior association is not a true kin recognition mechanism (Blaustein, 1983).

Examples of prior association can be found in a number of species. Female parasitoid wasps (*G. legneri*) display less aggression during female-female contests if they have developed on the same host and therefore have encountered each other during development (Lizé *et al.*, 2012). Cross-fostering is often used to demonstrate the similar behavioural treatment of biological and foster siblings. European polecats (*M. putorius*) reared together are more tolerant of each other regardless of relatedness, suggestive of a

prior association mechanism (Lode, 2008). During encounter trials juvenile Arctic (*Spermophilus parryii*) and Belding's (*Spermophilus beldingi*) ground squirrels are equally aggressive towards related and unrelated individuals with which they were reared (Holmes & Sherman, 1982). Experimental cross-fostering has demonstrated that Brandt's voles (*M. brandti*) behave more amicably and less aggressively towards familiar unrelated foster young than towards their biological but unfamiliar offspring (Li & Zhang, 2010).

1.1.6.3 Phenotype Matching

Phenotype matching also has a critical learning phase, but differs from prior association in that, instead of learning individual identities, a phenotypic recognition template is learnt from either self or a known relative(s). In later life novel individuals are compared against this template. If the template and the phenotype of a novel individual encountered are suitably similar then the novel individual is treated as related, otherwise it is treated as unrelated (Blaustein, 1983; Holmes & Sherman, 1983; Tang-Martinez, 2001). Closely related individuals are likely to have the same or similar phenotypes due to genetic or environmental factors, meaning that individuals that match can be treated as kin whilst those that are different can be treated as unrelated. The learnt phenotypic template could come from a specific relative, a collective template compiled from a number of relatives, or from an individual learning its own template (using self as a recognition template was termed the 'armpit effect' by Dawkins, 1982). The timing of this learning phase could be pre-natal in the womb, post-natal during rearing, at or around weaning or emergence from the nest, or during a combination of these times.

Phenotype matching presents a number of advantages over prior association (discussed in Mateo, 2004). It is unlikely that all of an individual's relatives would be present during the learning phase and phenotype matching therefore allows for a wider range of relatives to be recognised. Many species frequently produce multiple litters and the ability to recognise unfamiliar full-siblings would therefore be advantageous if close relatives from separate litters are likely to meet. Additionally, multiple mating means that individuals reared together may be maternal half-siblings, whilst paternal half-siblings may be reared apart. Phenotype matching could allow for these unfamiliar paternal half-siblings to be identified. The inclusive fitness benefits of aiding a full sibling are higher than for aiding a half-sibling, as full siblings are likely to share more genes that are identical by descent than half-siblings. The degree of relatedness between two individuals to be assessed using

a phenotype matching mechanism, allowing preferential treatment of closer relatives over more distant relatives. Phenotype matching could also allow parent-offspring recognition in those species where one or other parent plays no role in rearing. Many species communally rear young, meaning that the rearing environment could contain a range of related and unrelated individuals (Lucas *et al.*, 1997; Geffen *et al.*, 2011). Various forms of inter- and intra-specific parasitism would involve environments of mixed relatedness during a learning phase (e.g. brood parasitism), and therefore phenotype matching could allow individuals to discriminate true siblings from foster siblings.

A potential disadvantage of phenotype matching is that it requires a sufficient amount of variation in the recognition phenotype so that close relatives share or have a similar phenotype but unrelated individuals have a different phenotype. A lack of variation in the recognition phenotype would mean that unrelated individuals that shared the same phenotype would be falsely identified as being related. It has also been suggested that the identification of unfamiliar kin is simply the result of mistaken individual identity (Waldman, 1987): the identities of known relatives are learnt through prior association but unfamiliar relatives that share the same phenotypic marker are mistaken for those same learnt relatives. Strangers are assigned related status because the assessor has confused them with a known relative (discussed in Mateo, 2004). Additionally, phenotype matching is a more complex mechanism than prior association, requiring the ability to recall a memorised template and compare it against a novel phenotype for an assessment of relatedness. The degree with which different levels of relatedness could be assessed is unknown.

Examples of both familial-referent (learning the cues of known relatives) and self-referent phenotype matching are found across a range of species (evidence for self-referent matching is reviewed in Hauber & Sherman, 2001; Hare *et al.*, 2003; Mateo & Johnston, 2003). The larvae of the star ascidian tunicate (*B. schlosseri*) settle in proximity to siblings that match themselves at a specific polymorphic recognition locus (Grosberg & Quinn, 1986). When male peacocks (*P. cristatus*) establish leks in close proximity to male relatives they do so having had no prior experience of other relatives suggesting a self-referent phenotype matching mechanism (Petrie *et al.*, 1999). Tadpoles of the American toad (*Bufo americanus*) preferentially associate with full siblings and maternal half-siblings but not with paternal half-siblings, suggesting learning of maternal phenotypes

from egg jelly (discussed in Waldman, 1991). American coots (*Fulica Americana)* reject parasitic chicks by using the first hatched chicks in a brood as a referent to recognise their own chicks and discriminate against later-hatched parasitic chicks in the same brood (Shizuka & Lyon, 2010).

1.1.6.4 Recognition Alleles

There are two separate mechanisms of recognition alleles, although they are not always clearly distinguished. Both are innate processes whereby all three steps of discrimination (the expression of a phenotypic marker, the ability to recognize the marker, and the behaviour directed at an identified carrier of the marker) are all encoded by the same gene or set of genes (Tang-Martinez, 2001; Barnard, 2004, p 457). The first form of recognition alleles occurs where the marker that is expressed, recognized and acted upon directly signals relatedness. Kin discrimination can therefore occur without the need for learning experience, and this mechanism of recognition alleles has been suggested as simply a "hardwired version of phenotype matching" (Barnard, 2004, p 457). No evidence has been found for this recognition alleles mechanism and many have suggested it unlikely that a gene, or set of genes, could have evolved to code for the three separate processes (Hamilton, 1964b; Dawkins, 1976). Additionally, such a mechanism would also be detrimental if it could not be altered through experience (Hepper, 1991).

The second recognition alleles mechanism is termed Green Beard recognition (Dawkins, 1976). Here a gene or set of genes code the expression of a marker (a green beard), the recognition of the marker and the subsequent behavioural response directed toward the bearer of the marker. The important difference is that green beard recognition involves an allele attempting to ensure the continued survival and replication of itself regardless of genetic similarity at other loci. Whilst it is likely that kin may share the same marker and therefore could benefit from green beard recognition, green beard recognition has not evolved as a mechanism of identifying kin and individuals using this form of recognition allele would behave equally towards related and unrelated bearers of the marker. Red fire ants (*Solenopsis invicta*) appear to possess a green beard gene at the locus *Gp-9* as *Bb* workers recognise an odour produced by *BB* queens and kill *BB* queens whilst allowing *Bb* queens to reproduce (Keller & Ross, 1998).

1.1.6.5 Current Status of Kin Recognition Mechanisms

It is important to remember that none of the potential mechanisms described above are discrete and a number of authors have argued that continuing to treat them as distinct mechanisms complicates the issue (Waldman, 1987; Barnard, 1999). It is possible that some species even use multiple mechanisms. During behavioural interactions juvenile Belding's ground squirrels (*S. beldingi*) do not discriminate between related and unrelated individuals if they are raised with both (suggesting a prior association mechanism, based upon spatial location of relatives within the nest). However after hibernation yearling squirrels are able to recognise their full siblings but do not behave as if they recognise foster-siblings with which they were also reared (suggesting a phenotype matching mechanism; Holmes & Sherman, 1982; Mateo, 2010).

All the recognition mechanisms described above involve the recognition of a cue (spatial or phenotypic) and the subsequent action. The differences between the mechanisms are based on the type of marker and whether discrimination requires a learning phase. A number of authors have used the terms direct and indirect to discriminate between recognition mechanisms, although there is no consensus on the definitions of these two processes (discussed in Mateo, 2004). Waldman (1987) suggested that spatial location is indirect, as it uses non-phenotypic cues for recognition, whilst the mechanisms that use phenotypic cues are direct recognition (prior association, phenotype matching and recognition alleles). In contrast Porter (1988) suggested that direct kin recognition was recognition through individual familiarisation (prior association), whilst indirect recognition was through familiarisation with shared relatives (phenotype matching). Tang-Martinez (2001) has even argued that there is only one mechanism of kin recognition, namely recognition via familiarity and that differences in response are due to direct or indirect familiarity as defined by Porter (1988). This has been disputed by Mateo (2004) who argued that direct and indirect familiarisation could be based on different cues and will result in a different recognition ability (the recognition or not of unfamiliar kin).

Whilst these different perspectives are useful in thinking about the differences and similarities between the various kin recognition mechanisms, the traditional four kin recognition mechanisms are still widely cited. Theoretical issues surrounding the validity of both the spatial location mechanism (vulnerable to the mis-identification of unrelated individuals that have entered the specified location) and the recognition alleles mechanism

(the theoretical likelihood of their existence and the difficulty in proving or disproving their existence) have meant that researchers primarily discuss prior association and phenotype matching as the two main forms of kin recognition. As a result, studies on the mechanisms of prior association and phenotype matching have come to dominate the literature.

1.1.6.6 Imprinting – Behavioural and Genomic

The term imprinting is occasionally used in the kin recognition literature but it has two very different definitions which are discussed here. The mechanism of forming a strong learnt identity during development for recognition (e.g. for the prior association or phenotype matching mechanisms) is often referred to as behavioural imprinting – a normally irreversible social (or environmental) learning process during a specific period of development for an underlying purpose distinct from individual recognition. The term imprinting was first used to describe the irreversible process by which newly hatched birds learn the characteristics of whichever moving object they first see during a critical period, and then react towards it as if it were the rearing mother (Lorenz, 1937). In mammalian kin recognition the term imprinting is less used (e.g. Carter & Marr, 1970; Porter & Etscorn, 1975; Yamazaki *et al.*, 1988). The critical period for behavioural imprinting could be pre-natal in the womb, post-natal during rearing, at or around weaning, post-dispersal, or at several of these occasions for differing reasons (for reviews on imprinting see: Hess, 1959; Moltz, 1960; Bateson, 1979; Bolhuis, 1999).

Behavioural imprinting has been shown to influence sibling recognition, mate choice and odour preference. Under normal circumstances zebrafish (*Danio rerio*) larvae can recognise the odour of unfamiliar siblings, however they are unable to discriminate siblings from unrelated individuals if they are not exposed to relatives 6 days after fertilisation (Gerlach *et al.*, 2008). Male goats (*Capra aegagrus hircus*) reared by ewes (*Ovis aries*) prefer to socialise and mate with ewes, even after several years cohabitation with their own species (Kendrick *et al.*, 1998). Experiments using artificial odours during the rearing of guinea-pigs (*Cavia porcellus*) and spiny mice (*Acomys cahirinus*) have demonstrated a learnt preference for the artificial odours that pups were exposed to (Carter & Marr, 1970; Porter & Etscorn, 1974).

Genomic imprinting occurs where the expression of a particular allele is determined by whether it was inherited from the mother or father (parent-of-origin); one allele being

expressed whilst the other allele remains silent dependent on parent-of-origin (Bartolomei & Tilghman, 1997; Burt & Trivers, 1998; Wilkins & Haig, 2003; Munshi & Duvvuri, 2007). A predominant theory for the origin of genomic imprinting is the kinship theory which suggests that genomic imprinting evolved because of differences in relatedness between maternally and paternally derived alleles at a single locus (Haig, 2000a, 2000b, 2004; Brandvain *et al.*, 2011). Haig (2000a, 2000b) argued that a dam would improve her inclusive fitness if her offspring behaved altruistically towards maternal relatives, but would gain nothing if her offspring directed altruistic behaviour towards their paternal relatives. This behaviour might be advantageous in species with male-biased dispersal, as female groups are likely to be closely related through the matrilineal line (Clutton-Brock & Lukas, 2012).

Genomic imprinting can influence odour preference in laboratory mice. Mice transferred to separate strains whilst embryos spend longer investigating control urine than urine from their maternal strain, but show no difference in investigation time between control and paternal strain urine (Isles *et al.*, 2001; Isles *et al.*, 2002). Genomic imprinting could also explain indiscriminate nursing of pups in communal litters. If males are likely to sire multiple litters in the same nest then it would be in the sire's interest for dams to be unable to identify own pups and therefore to nurse all pups present (Roulin & Hager, 2003; Hager & Johnstone, 2005). Rove beetle larvae (*A. bilineata)* avoid parasitizing *Delia radicum* pupae that are already parasitized by full siblings or paternal cousins, but not those that have been parasitized by maternal cousins (Lizé *et al.*, 2007). Others have suggested that a phenotype itself could be affected by epigenetic effects (Hager *et al.*, 2009) and that phenotypes could be produced by offspring that do not have the corresponding allele (Vrana, 2007).

Imprinting could therefore be important for understanding kin recognition mechanisms through the learning of identities or templates in the nest (behavioural), or by affecting which relatives are recognised (genomic). This thesis investigates behavioural imprinting but will not directly test for genomic imprinting.

1.1.7 Molecular Markers of Kin Recognition

A variety of cues are used for kin recognition and animals have been shown to recognise relatives based on auditory (e.g. recognition of the calls of relatives in long-tailed tits; Sharp *et al.*, 2005) and visual cues (e.g. facial recognition in chimpanzees; Parr *et al.*,

2010). However, olfaction is the most well documented sense for kin recognition and odour discrimination of kin has been shown in insects (e.g. Lizé *et al.*, 2010), arachnids (e.g. Walsh & Rayor, 2008), fish (e.g. Gerlach & Lysiak, 2006), amphibians (discussed in Waldman, 1991), reptiles (e.g. Pernetta *et al.*, 2009), birds (e.g. Krause *et al.*, 2012), and mammals (e.g. Heth *et al.*, 1998). Ideal molecular markers of relatedness require three essential characteristics for effective kin recognition. Firstly, markers should be genetically determined so that close relatives share markers through a common decent whilst unrelated individuals are unlikely to. Secondly, potential markers need to be sufficiently polymorphic that only close relatives share specific markers. This high degree of variation would mean that unrelated individuals should have different markers, preventing the false identification of non-kin. Finally, a molecular marker of relatedness should be consistent throughout an organism's life. Variation in marker pattern or expression due to health, dominance, age or diet could obscure kin signals and prevent efficient identification (Hurst & Beynon, 2004).

1.1.7.1 The Major Histocompatibility Complex (MHC)

By far the most widely studied of potential genetic markers of kin recognition is the major histocompatibility complex (MHC), a highly polymorphic family of genes that are important for self / non-self recognition in the immune system. Known as H-2 in mice and HLA in humans, the MHC family is found in all vertebrates. The first suggestion that MHC might be involved in kin recognition came from observations of male laboratory mice that displayed mating preferences based on MHC type (Yamazaki *et al.*, 1976). A large number of studies have since examined the role of MHC in mouse kin recognition. Both male and female laboratory mice prefer opposite-sex individuals of a different MHC type from either themselves or their parents (Yamazaki *et al.*, 1988; Egid & Brown, 1989). Crosses resulting from laboratory strains and wild house mice similarly show that females prefer males of a different MHC type from the parents that reared them (Penn & Potts, 1998a). Female laboratory mice are able to discriminate their own pups from otherwise genetically equivalent pups based on differences at the MHC locus only (Yamazaki *et al.*, 2000). Using crosses between laboratory strains and wild mice Manning *et al* (1992) showed that females prefer female nest partners that share *Mhc* genes.

The role of MHC in kin recognition has been investigated in a wide range of different species (for a review of MHC and kin recognition in non-model vertebrates see Bernatchez

& Landry, 2003). For example, Arctic charr (S. *alpinus*) prefer water scented by a sibling with the same MHC type as themselves over water scented by a sibling of a different MHC type to themselves (Olsen *et al.*, 2002). Brown trout (*Salmo trutta*) fry preferentially associate with siblings that share more MHC alleles (sharing of two alleles preferred over sharing of one allele preferred over sharing of no alleles; O'Farrell *et al.*, 2012). African clawed frog tadpoles (*Xenopus laevis*) preferentially associate with siblings that share MHC haplotypes over those that share no MHC haplotypes (Villinger & Waldman, 2008).

MHC may also be important for inbreeding avoidance and mate choice. Female seahorses (*Hippocampus abdominalis*) preferentially mate with males of a different MHC type over those of the same MHC type to themselves (Bahr *et al.*, 2012). Female Swedish sand lizards (*Lacerta agilis*) prefer the odour of males of a different MHC type to their own (Olsson *et al.*, 2003). Female Savannah sparrows (*Passerculus sandwichensis*) preferentially pair with MHC-different males over MHC-similar males (Freeman-Gallant *et al.*, 2003). Female bank voles (*M. glareolus*) prefer the odour of males of a different MHC type to self than males of the same MHC type (Radwan *et al.*, 2008). There is even some evidence that MHC might influence mate choice in humans (*Homo sapiens*) as women score the body odour of men that differ from themselves for MHC type as more pleasant than the odour of MHC similar men (Wedekind *et al.*, 1995). Recent studies of inbreeding avoidance in the house mouse (*Mus musculus domesticus*) investigating MHC and other markers however suggest that MHC may not be as integral to kin or individual recognition as previously thought (Cheetham *et al.*, 2007; Sherborne *et al.*, 2007).

1.1.7.2 Major Urinary Proteins (MUPs)

Major urinary proteins (MUPs) are a multigene family of lipocalins and in certain species are excreted in high quantities in the urine or other secretions such as saliva (Beynon & Hurst, 2004). *Mup* genes have been found in a number of mammal species including chimpanzee, orangutan, lemur, pig, horse and dog (Logan *et al.*, 2008). Of the species so far screened, house mice and the Norway rat (*Rattus norvegicus*; in the rat the MUP family was previously known as A2U) have the greatest amount of polymorphism for MUP. Horses and lemurs appear to have more than one *Mup* gene, and humans have only a single *Mup* pseudogene (Logan *et al.*, 2008). Examination of the MUP lineage revealed that the rat and house mouse MUP families expanded to their current degree of polymorphism independently but simultaneously (Logan *et al.*, 2008). Despite evidence of the MUP

family in a number of other rodent species (for review, see Beynon *et al.*, 2008), the majority of research into MUPs has used the house mouse.

From reaching sexual maturity the expressed individual patterns of MUP proteins remain consistent (Beynon & Hurst, 2004). This stability means that the signal produced by MUPs in mouse urine is consistent over time and, given the high degree of polymorphism, MUPs could therefore be used as a reliable signal of individual identity (Robertson *et al.*, 1996; Cavaggioni *et al.*, 1999; Beynon & Hurst, 2003). The importance of MUPs in individual recognition has been demonstrated in wild house mice (Hurst *et al.*, 2001; Cheetham *et al.*, 2007). The high degree of polymorphism of MUPs, along with the reliability of the signal as a marker for individual identity suggests that MUP pattern could also be a marker for genetic kin recognition in house mice. In an enclosure-based study where house mice were allowed to mate freely, Sherborne *et al.* (2007) found a deficit of litters resulting from successful matings between mice that shared both MUP haplotypes. They found no evidence that sharing of MHC type prevented successful mating, suggesting that MUP type maybe more important than MHC during inbreeding avoidance decisions in house mice.

1.1.7.3 Exocrine-Gland Secreting Peptides (ESPs)

It has recently been demonstrated that differences in a third multigene family, exocrine-gland secreting peptides (ESPs), can also be detected by laboratory mice (Kimoto *et al.*, 2005; Kimoto *et al.*, 2007). Initially a male-specific peptide (*Esp1*) was identified and shown to stimulate female vomeronasal sensory neurons (Kimoto *et al.*, 2005). Further examination by Kimoto *et al.* (2007) revealed that this peptide was one of a family of 38 *Esp* genes in mice, including a second sex-specific peptide expressed by females only (*Esp36*). Rats also have a family of *Esp* genes, although on a different chromosome to MHC (Kimoto *et al.*, 2007). A possible *Esp* gene has been observed in the gray short-tailed opossum (*Monodelphis domestica*) but the human genome appears to contain no *Esp* genes (Kimoto *et al.*, 2007).

Whilst there is currently no evidence for ESPs being used during kin recognition, the differences in ESP expression between strains, expansion of the lineage in mice, and evidence of a distinct neural pathway all suggest that non-volatile peptides from this multigene family could be important in scent communication in mice. It is therefore

important to understand further the role of ESPs in mouse scent communication in order to assess whether they might play a role in kin recognition between house mice.

1.1.8 Assays of Kin Recognition

A number of different assay designs have been used to study kin recognition and it is important to understand why they are used and what they show. This is by no means an exhaustive list, and each assay described below has a multitude of variations, but the following section covers the most common assay designs and what they are designed to examine.

1.1.8.1 Cross-fostering

To remove the effects of prior association a number of studies of kin recognition have used a cross-fostering design (reviewed in Todrank & Heth, 2001; Mateo & Holmes, 2004). Cross-fostering involves moving infants from their biological parents to foster parents. Infants could be transferred to unrelated members of their own species (e.g. Holmes & Sherman, 1982) or to a different species (e.g. Kendrick *et al.*, 1998). Either entire litters or individuals can be cross fostered in experimental studies. Often litters are reciprocally cross-fostered so that young are swapped between two sets of parents. The inability of cross-fostered individuals to discriminate kin from non-kin suggests a prior association mechanism, whilst the ability of cross-fostered individuals to recognise own biological kin signals the use of self-referential phenotype matching. The behaviour of cross-fostered individuals towards foster-siblings can also reveal information about the importance of familiarity and the interaction between individual and kin recognition. One flaw with the cross-fostering design is that it is difficult to prevent any learning prior to fostering. Whilst most studies transfer kin within one day of birth, learning could potentially occur during the hours immediately following birth or even pre-natal, subsequently influencing experimental results and interpretation (Hepper, 1991).

1.1.8.2 Scent assays

Olfactory kin recognition has been widely documented and therefore there have been a number of tests designed to examine olfactory discrimination. Habituation-dishabituation tests are often used to demonstrate that an animal can distinguish a difference between two odours. In the habituation phase individuals are presented (often over multiple trials) with the same habituation odour (or samples from the same habituation stimulus animal). The subject's response to the odour decreases over multiple trials as it quickly recognises an

odour with which it is already familiar. In the dishabituation phase, a new odour is presented to the individual instead of the habituation odour. If the response to the new odour is significantly higher than the last habituation odour response then the habituation odour and the test odour are perceived by the subject as being different from each other. This method has been used, for example, to demonstrate the ability of laboratory mice to discriminate between urine samples from different strains (Isles *et al.*, 2002), and those that differ at only one MHC locus (Penn & Potts, 1998b).

Habituation-discrimination tests are a variation of the habituation-dishabituation design. The habituation phase proceeds as normal, but instead of a single dishabituation odour, in the discrimination phase two odours are presented – the same habituation odour (or another sample from the same habituation stimulus animal) and a novel odour. The subject's duration of investigation towards both odours is then measured. If the subject investigates the novel odour significantly more than the habituation odour then the subject perceives that there is a difference between the two odours. Using this design Newman & Halpin (1988) showed that prairie voles (*M. ochrogaster*) can discriminate between the urine of male and female conspecifics.

Odour-genes covariance tests are another variant on the habituation-dishabituation tests (reviewed in Todrank & Heth, 2003). Whilst habituation-dishabituation/discrimination tests are used to demonstrate that two odours are perceived as being different from each other, odour-genes covariance tests assess odour similarities (Heth & Todrank, 2000). During the habituation phase a subject is presented with a habituation odour. The habituation odour is removed and the subject is presented with two novel discrimination odours (this can be simultaneously or consecutively). As both discrimination odours are unfamiliar to the subject, the subject should spend an equal amount of time sniffing each odour. However, if one of the discrimination odours is related to, or otherwise shares a common feature with the habituation odour, then the subject may spend less time investigating the related discrimination odour than the second discrimination odour. The habituation and related discrimination odour can then be said to appear to the subject as more similar to each other than the habituation odour and the unrelated discrimination odour. In a study of golden hamsters (*M. auratus*) by Todrank *et al.* (1998), hamsters perceived more similarity between two sibling odours than between unrelated odours. Similarly, female blind mole rats (*Spalax galili*) treat the odours of more closely related

individuals as more similar compared to the odours of more genetically distant individuals (Tzur *et al.*, 2009).

In the three scent assays described so far the stimulus odours used could be entirely unrelated to the subject, for example laboratory mice have been used to detect odour similarities between related chacma baboons (*Papio ursinus*) (Celerier *et al.*, 2010). A fourth design is often used to assess individual response to the odours of own relatives compared to the unrelated odours – a scent discrimination and/or attraction assay. There is no habituation phase and instead individuals are presented with two odours simultaneously (normally one related to the subject and one unrelated) and their response to each odour is recorded. The assay measures the ability of an individual to discriminate spontaneously between two odours, and whether individuals demonstrate any attraction towards either odour. Individuals are often predicted to spend longer investigating unrelated odours, as related odours may contain elements with which the individual is already familiar. For example, neonate smooth snakes (*C. austriaca*) investigate unrelated odour more than related odour (as measured by the number of tongue flicks directed towards each odour; Pernetta *et al.*, 2009), and female golden hamsters (*M. auratus*) spend longer with their nose in close proximity to unrelated than related male odours (Mateo & Johnston, 2000). Where kin are expected to aggregate, individuals are predicted to show an attraction towards related over unrelated odours. In an odour choice flume assay juvenile zebrafish (*D. rerio*) were found to spend longer on the related side than the unrelated side (Gerlach & Lysiak, 2006). Whilst the first three scent assay designs are therefore useful for understanding how animals perceive similarities and differences between odours, the scent discrimination and attraction assay is useful for understanding how animals spontaneously respond to odours.

1.1.8.3 Proximity/preference assays

Kin aggregation or proximity to kin is often used as an indication of kin recognition. For example, sibling star ascidian tunicate (*B. schlosseri*) planktonic larvae settle in closer proximity to each other than to non-sibling larvae (Grosberg & Quinn, 1986). By giving a subject access to two stimulus animals (or groups), experimenters can measure affiliation and attraction. For example, when given the choice both cascade frog (*R. cascadae*) tadpoles and juvenile cichlids (*N. pulcher*) spend more time in proximity to more closely related conspecifics (Blaustein & O'Hara, 1982; Le Vin *et al.*, 2010). Inbreeding avoidance

studies tend to look for a reverse pattern – a tendency to spend more time in proximity to unrelated individuals of the opposite sex. For example, male bank voles (*M. glareolus*) spend more time in close proximity to unrelated females than to full sisters (Lemaitre *et al.*, 2012).

1.1.8.4 Interaction assays

Social responses often change with relatedness. Researchers often use dyadic interactions to demonstrate kin discriminative behaviours. Two individuals are often placed in a neutral test arena and their behaviours towards each other measured. Kin selection theory predicts that relatives may be less aggressive towards each other than unrelated individuals; for example reduced aggression during related interactions has been shown in parasitoid wasp (*G. Legneri)* (Lizé *et al.*, 2012) and fire salamander (*S. infraimmaculata*) larvae (Markman *et al.*, 2009). Male white-footed deer mice (*Peromyscus leucopus*) chase unrelated males more than related males (Grau, 1982). Conversely, amicable interactions are often predicted to increase with relatedness, as shown in European polecats (*M. putorius*) and marmots (*M. monax*) (Lode, 2008)

1.1.8.5 Inbreeding avoidance assays

A number of inbreeding avoidance studies use assays of the style described above (e.g. Bateson, 1982; Partridge, 1983; Lemaitre *et al.*, 2012). Whilst these studies are informative, measures such as odour attraction or time spent in proximity do not necessarily predict reproductive choice. Therefore a number of studies allow animals to mate and measure pre-copulatory behaviour (e.g. time to mating; Whitehorn *et al.*, 2009), reproductive success (e.g. number of litters; Boyd & Blaustein, 1985), or post-copulatory behaviours (e.g. time taken to re-mate; Välimäki *et al.*, 2011). 'No-choice' studies are often used to examine inbreeding avoidance where individuals are paired and reproductive success is measured. Reproductive success between pairs of individuals with varying relatedness is often then compared (e.g. Barnard & Fitzsimons, 1989). Free-breeding experiments are also used to assess inbreeding avoidance. This is where several animals are allowed to interact and breed freely (often in a semi-natural enclosure). Any resulting offspring are then genotyped to calculate the parentage and therefore reveal the individuals that successfully mated (e.g. Lucia & Keane, 2012). Whilst such studies directly measure inbreeding avoidance, one problem is that they are often expensive in terms of time taken to run and animal costs and can produce excess animals that may have to be culled.

Additionally they also provide limited information on the mechanisms that lead to successful reproduction, including which sex chooses mating partners.

1.2 Study Species

House mice are a useful model with which to study the mechanisms of kin recognition. Female house mice nest communally providing opportunities for both cooperation and competition between females and kin selection theory suggests that females could gain inclusive fitness benefits by nesting with relatives. The costs of inbreeding mean that females may also use kin recognition during mate choice decisions. The life history of female house mice therefore provides separate contexts where the ability to recognise relatives may be beneficial.

1.2.1 House Mice

The house mouse (*M. m. domesticus*) is a small murid rodent species that is distributed across much of the world (Bronson, 1979). House mice can live commensally with humans or survive in feral populations independently of humans (Berry, 1981; Rowe, 1981). Studies of the remains of small mammals trapped in excavated wells in Cyprus suggest that the commensal existence of house mice with humans dates back to at least 8000 BC (Cucchi *et al.*, 2002).

House mice are nocturnal and are mainly active around dawn and dusk, with periods of activity through the night (Mackintosh, 1981). House mice feed on a wide range of different food sources including cereals, seeds, roots and insect larvae (Rowe, 1981). The life expectancy of house mice can vary: in a feral population mice were found to live for an average of 100 days (Berry & Jakobson, 1971); feral and communal mice in Russia have been reported to survive for over 630 days (Berry & Bronson, 1992); whilst a study of a commensal population where predation was restricted, found a life expectancy of 196 days (König & Lindholm, 2012). Under laboratory conditions house mice can live considerably longer (Pennycuik *et al.*, 1986; Miller *et al.*, 2002).

House mice have been described as a weed species (Berry, 1981) as they are adaptable and reproduce quickly, allowing them to easily exploit opportunities. Female house mice reach sexual maturity at about 6 to 8 weeks old and gestation length is approximately 21 days with lactation lasting approximately 24 days (Berry, 1981). Females exhibit post-partum oestrus, meaning that they are able to conceive again almost immediately after giving birth

(König & Markl, 1987). This allows mice to breed approximately every 4 weeks. Litters are comprised of around six to eight offspring and first litters tend to be smaller than second litters (König & Markl, 1987). House mice can breed throughout the year, even during extreme low temperatures, however breeding tends to be influenced by seasonal changes, with lower numbers of pups weaned in winter months (Rowe, 1981; König & Lindholm, 2012). Commensal populations of house mice are also more likely to breed year round than feral populations (Pelikan, 1981).

Within a population of house mice a small number of dominant males will form and defend territories, whilst the remaining subordinate males are non-territorial and restricted in their movements by dominant males (Crowcroft & Rowe, 1963). Territory size can vary greatly with population type, density and other habitat factors; in commensal populations, territories tend to be small and stable and mice tend to have homes ranges of less than 10 m^2 (Bronson, 1979) while in feral populations territories tend to be much larger (up to 1000 m^2) and territory borders are less rigidly defined (Bronson, 1979). Male house mice competitively defend their territories from other males, showing aggression primarily towards males (Hurst, 1987).

A typical male territory consists of a dominant male, one or more breeding females and their offspring, as well as any subordinate or sub-adult males and non-breeding females (Hurst & Barnard, 1992). Females are not necessarily restricted to a single male territory and usually have home ranges that extend over one or more male territories (Hurst, 1990b). Several females will often share the same home range and nest site (see Section 1.2.2). Females are attracted towards territorial males, but are not confined to mating with the dominant male on whose territory they reside (Wolff, 1985; Pusey & Wolf, 1996). Whilst females usually display less frequent and intense aggressive behaviour than males, they will behave aggressively towards both males and females intruding in the nest site (Mackintosh, 1981).

Olfaction is a dominant method of communication in house mice and male mice scent mark in relatively large quantities (Smith, 1981). Scent has an advantage over other forms of communication as a scent mark remains in the environment after it has been deposited (Hurst & Beynon, 2004). Scent marking is important for displaying male status and territory. Dominant male house mice produce a large number of urine marks throughout their territory, however subordinate caged males tend to urinate in large pools (Desjardins

et al., 1973). In naturalistic enclosures dominant males deposit more scent marks on introduced tiles than subordinate or sub-adult males (Hurst, 1990a). Male house mice scent mark in their territories, countermarking any scent marks from other intruding males. Female house mice prefer males that have successfully countermarked intruder scent marks (Rich & Hurst, 1999). Female house mice may also use scent marking as a signal to other females of breeding status (Hurst, 1990b). As well as providing information about competitive ability and breeding status, scent also contains information about gender (Roberts *et al.*, 2010), health status (Ehman & Scott, 2001), diet (Hurst & Beynon, 2004) and identity (Cheetham *et al.*, 2007).

1.2.2 Female house mice

Female house mice share home ranges that overlap the territories of one or more males (Hurst, 1987; Hurst, 1990b). House mice are plural cooperative breeders meaning that several females within the same group will produce and rear young. Females often nest and nurse communally, pooling their young within the nest and nursing indiscriminately (Sayler & Salmon, 1971). In addition to nursing young communally, females also behave cooperatively by babysitting young, increasing nest temperature and contributing to both territory and pup defence (König & Lindholm, 2012).

Communal nesting and nursing provides a number of reproductive advantages (for review see Hayes, 2000). In laboratory mice the growth rate of communally nested young is higher than young from solitary litters (Sayler & Salmon, 1971). The birth weight of young in communal nests is higher compared to solitary litters and communally nesting females are heavier on the final day of weaning than females in solitary nests (König, 1993). Communal nesting in house mice also reduces the level of infanticide (Manning *et al.*, 1995). However, competition between females can also be costly, leading to aggressive interaction, reproductive inhibition and infanticide (Hurst, 1987; König, 1994a; Rusu & Krackow, 2004; Palanza *et al.*, 2005). Therefore nest partner choice is important for reproductive success. Female house mice have been shown to increase reproductive output when paired with a preferred partner (Weidt *et al.*, 2008).

Kin selection theory has been applied to social partner choice in house mice, suggesting that females could gain many reproductive advantages from nesting and nursing communally with relatives. In addition, by helping to rear the offspring of a relative, females would gain additional indirect fitness benefits. Related female groups rear more

litters (König, 1994b, 1994a), of a greater weight (König, 1993), and with a higher survival rate (König, 1994a) than unrelated females nesting communally. Sisters in communal nests breed also more frequently and have a higher pregnancy rate than unrelated females sharing a nest (Rusu & Krackow, 2004). Lifetime reproductive success is not thought to be affected by birth order when sisters communally nest, whereas in nests containing unrelated females the first female to give birth has a tendency to rear more young (König, 1994b). In laboratory strains of mice, females have been shown to devote more care to the pups of related nestmates than those of unrelated nestmates (D'Amato, 1993). The rate of infanticide is also reduced among sister groups communally nesting (Palanza *et al.*, 2005).

The advantages described above suggest that females may choose to nest communally with relatives. Female house mice tracked in the wild are found communally nesting with females that are more genetically similar than would be expected if nest partner choice was random (Wilkinson & Baker, 1988). In semi-natural enclosures where females were given free-choice of nesting partner, females sharing nestboxes are more likely to be sisters than unrelated females (Dobson *et al.*, 2000; Dobson & Baudoin, 2002). Using house mice crossed with laboratory strains Manning *et al.* (1992) found that littermate sisters preferentially nest together.

1.2.3 Laboratory strains of mice

Most laboratory strains of mice are derived from a cross between the subspecies *M. m. domesticus*, *M. m. musculus* and *M. m. castaneus* (Yang *et al.*, 2007; Cheetham *et al.*, 2009). Laboratory mice have been selectively bred for reduced aggression (to enable mice to be housed in groups) and to mate easily with their own relatives. As a result of this there are crucial differences between laboratory strains of mice and house mice, including differences in lifespan, reproductive rate and growth rate (Harper, 2008). Inbreeding over multiple generations has meant that individuals within a specific strain have almost no genetic variation and are homozygous for most loci. There are therefore a number of advantages to using laboratory mice, including control over specific loci. Such precise control over genetic variability allowed Yamazaki *et al.* (1983) to show that laboratory mice can distinguish between the scents of individuals with single genetic differences at the MHC loci. A number of inbreeding avoidance studies have also used laboratory strains of mice (e.g. Yamazaki *et al.*, 1976; Hayashi & Kimura, 1983; Egid & Brown, 1989; Penn & Potts, 1998a; Roberts & Gosling, 2003).

Despite the advantages of using laboratory mice to study kin recognition, there are also a number of disadvantages. Generations of selective breeding means that the behaviour of laboratory mice and house mice can be subtly different, for example laboratory mice often show reduced aggression (Smith *et al.*, 1994). Kin selection predictions suggest that relatives may behave less aggressively towards each other than non-relatives; however this may not be apparent when laboratory strains of mice display limited levels of aggression to start with. Laboratory mice have been selectively bred to mate with full siblings. Studies of inbreeding avoidance using laboratory strains of mice are therefore not necessarily accurate representations of natural mate choice decisions. The genetic uniformity of individual strains also calls into question whether individual laboratory mice recognise unfamiliar kin or whether strange relatives are in reality mistaken for known relatives.

Laboratory mice have been useful in understanding the role of the highly polymorphic MHC locus in kin recognition. However, Cheetham *et al.* (2009) examined MUP variation across 30 strains of laboratory mouse and found only two different individual patterns among the strains examined. This was in stark contrast to house mice where variation in MUP patterns between mice can provide a signal of individual identity (Hurst *et al.*, 2001; Cheetham *et al.*, 2007). Close relatives share genes that are identical by descent, and similarities at a locus that influences odour (such as MHC or MUP) could be used to identify relatives. The absence of a natural level of variation at MUP and potentially many other genes between laboratory strains means that individuals could be attempting to recognise kin without a prominent marker of relatedness. For these reasons laboratory mice, whilst they have a number of other advantages for addressing specific aspects of recognition mechanisms, are often unsuitable for drawing conclusions about behaviours in natural populations. This project will therefore investigate kin recognition in house mice.

1.2.4 Evidence for kin recognition in female house mice

Female house mice gain reproductive advantages from nesting with relatives and have been shown in both wild and semi-natural conditions to preferentially choose to nest with their relatives. This suggests that females employ a kin recognition mechanism to recognise female relatives and choose a communal nesting partner. Evidence from studies of female – female kin recognition using laboratory mice (or crosses between laboratory mice and house mice) is mixed with regards to a possible kin recognition mechanism, suggesting that prior association and/or phenotype matching are important (Manning *et al.*,

1992; D'Amato, 1993; Yamazaki *et al.*, 2000). Studies of house mice have also illustrated the importance of both familiarity and relatedness. Familiar sisters are less aggressive towards each other and are also more likely to cohabit than unfamiliar sisters, suggesting the importance of familiarity (Rusu *et al.*, 2004). Familiarity has also been shown to improve reproductive success, although relatedness is clearly important, as sisters have a higher reproductive success than unrelated females when familiarity is controlled (König, 1994b). A number of questions therefore remain concerning mechanisms of female – female kin recognition in the house mouse.

Female house mice are likely to encounter close male relatives on reaching sexual maturity. Inbreeding has a number of disadvantages (as discussed above) and inbred male house mice have reduced competitive ability compared to outbred male house mice, resulting in reduced survivorship and fewer offspring (Meagher *et al.*, 2000). Full sibling breeding pairs in both laboratory strains and house mice have a lower reproductive success than unrelated pairs (Barnard & Fitzsimons, 1989; Krackow & Matuschak, 1991). Female house mice therefore need to be able to recognise their relatives in order to avoid mating with them, Females make mate decisions based on a number of different male characteristics, including relatedness, and female house mice paired with preferred males have more litters and fitter offspring than females paired with non-preferred males (Drickamer *et al.*, 2000).

Female inbreeding avoidance has been examined in laboratory mice. Females prefer males of an MHC type dissimilar to their own type, however limited genetic variation within strains means that this could instead be a result of behavioural imprinting on the MHC of parents (Egid & Brown, 1989). This theory is supported by a free-breeding study where females resulting from crosses between laboratory and house mice preferred males that differed at MHC from the parents that reared them (Penn & Potts, 1998a). Female house mice prefer the ultrasonic vocalisations of unfamiliar, unrelated males over those of familiar full brothers, although it is difficult to disentangle relatedness from familiarity in this study (Musolf *et al.*, 2010). When presented with equally unfamiliar males, female house mice prefer to associate with unrelated males over related males, suggestive of a phenotype matching mechanism (Winn & Vestal, 1986). Free breeding house mice avoid partners that share both MUP haplotypes, suggestive of a self-reference phenotype matching mechanism using MUP (Sherborne *et al.*, 2007). The study by Sherborne *et al.*

(2007) however was not a direct test of inbreeding avoidance and it is difficult to know whether males or females were responsible for mate choice decisions. It is therefore important to fully understand female inbreeding avoidance in house mice, to further increase our knowledge of kin recognition in mice.

1.3 Thesis Outline

Female house mice appear to discriminate between male and female kin in the contexts of both nest partner choice and inbreeding avoidance. This thesis aims to understand how female house mice recognise and choose to associate with, or avoid, female and male relatives. Chapter two tests whether kin recognition between females is dependent on prior familiarity. Chapter three investigates the formation of recognition templates and whether they are of a maternal or self origin. Chapter four focuses on the role of MHC, MUP or ESP in female-female kin recognition. In chapter five the physiological effects of female social environment are tested using changes in the production of faecal corticosterone, urinary protein and body mass. Chapter six addresses female inbreeding avoidance and familiarity, template formation and the role of MHC, MUP and ESP. In chapter seven the results from the preceding chapters are briefly summarised and discussed in the context of the existing literature.

2 The Importance of Familiarity in Kin Recognition

2.1 Abstract

Kin selection theory predicts several advantages of discriminating relatives from non-relatives, such as altruistic behaviour, reduced aggression and inbreeding avoidance. Individual familiarity can also cause behaviours that appear similar to altruistic kin biased behaviours. It is therefore important to establish whether the apparent kin biased behaviours frequently observed in many species are the result of kin recognition rather than individual familiarity recognition. Female house mice nest and nurse communally, a condition that has potential for both cooperation and conflict. Females are often found nesting with close relatives in the wild and laboratory studies. In this chapter I show that female house mice can recognise and show a nesting partner preference for full sisters over unrelated females. This discrimination was present whether females were familiar or unfamiliar, suggesting that a phenotype matching mechanism is involved in kin recognition between female house mice. Females were able to recognise their full sisters when presented with urine alone, indicating that genetic recognition markers are present in house mouse urine. A comparison of nesting partner choice and degree of major urinary protein (MUP) peak sharing suggests that females may use a match to self mechanism based on MUP peak sharing.

2.2 Introduction

Kin selection theory predicts several advantages of discriminating relatives from non-relatives, such as altruistic behaviour and inbreeding avoidance (Maynard Smith, 1964). Relatives share a proportion of genes that are identical by descent and behaviours that increase the fitness of relatives will also improve an individual's own inclusive fitness (Hamilton, 1964a, 1964b). House mice present an ideal opportunity to investigate the mechanisms of kin recognition as females nest and nurse communally (Sayler & Salmon, 1971), providing opportunity for both cooperation and conflict between females. Whilst much work has been done in this area, particularly in laboratory mice (e.g. Egid & Brown, 1989; D'Amato, 1994), the actual mechanisms of kin recognition remain unclear.

2.2.1 Female House Mice and Kin Recognition

Female house mice nest communally, and will nurse pups present in the nest (Sayler & Salmon, 1971). Communally nesting females have been shown to have a higher lifetime reproductive output (König, 1993) and lower levels of infanticide (Manning *et al.*, 1995) than solitary nesting females (for review see Hayes, 2000). Within this system there is plenty of opportunity for both cooperation and conflict between females which can result in infanticide, aggression and reproductive inhibition (Hurst, 1987; König, 1994a; Rusu & Krackow, 2004; Palanza *et al.*, 2005). Nesting partners should therefore be chosen wisely; indeed unrelated female house mice in preferred partner dyads have a higher reproductive output over 6 months than those in non-preferred dyads (Weidt *et al.*, 2008). However, whilst unrelated females will nest together, kin selection theory suggests that related nesting partners would gain increased inclusive fitness by helping to rear each other's related pups.

During long-term free-breeding studies König has shown a number of advantages of females nesting with sisters. Sister pairs housed with a male rear more young than a monogamously paired female (König, 1989), and the combined weight of weaned offspring is greater for sister pairs compared to pairs of unrelated females or solitary nesting females (König, 1993). Familiar sisters also rear more litters cooperatively, with improved litter survival directly after birth compared to unrelated females that are unfamiliar before pairing (König, 1994a). The birthing order of communally nesting familiar sisters does not affect the lifetime reproductive success of either female, whereas

in other related/familiar combinations the first female to give birth has a tendency to rear more young (König, 1994b).

Competitive costs such as infanticide and reproductive inhibition cause a reduction in reproductive success between paired unrelated females compared to sister pairs of house mice (Palanza *et al.*, 2005). Three sisters nesting communally together in laboratory enclosures breed more frequently and have higher pregnancy rates compared to three unrelated females nesting together (Rusu & Krackow, 2004). In laboratory strains of mice, females have been shown to devote more care to the pups of related nestmates than to the pups of unrelated nestmates (D'Amato, 1993). However in a study looking at female infanticidal behaviour, pairs of sisters nesting together had a lower overall reproductive success than unrelated, unfamiliar females nesting together, although this was in a particularly aggressive laboratory colony of house mice (Vom Saal *et al.*, 1995). Competition between females can result in lowered reproductive output, however no difference in aggression levels are seen between familiar full sisters and unrelated, unfamiliar females in paired laboratory mice (Maestripieri & Rossiarnaud, 1991; Akre *et al.*, 2011). As discussed in the introduction chapter (Section 1.2.3), laboratory strains of mice are often less aggressive and so differing levels of aggression are less likely between pairs of related and unrelated laboratory females.

In general the evidence implies that communal nesting between related female house mice is advantageous, suggesting that female house mice should choose to nest with relatives in the wild in order to maximise their reproductive capabilities. Female house mice tracked in the wild are found communally nesting with females that are genetically more similar across 5 loci than would be expected by random pairing, suggesting that they are nesting with close relatives (Wilkinson & Baker, 1988). Females sharing nestboxes in semi-natural enclosures are more likely to be sisters than unrelated females (Dobson *et al.*, 2000; Dobson & Baudoin, 2002). Crossed lab-wild strains of mice choose communal nesting partners based on MHC type (close relatives are more likely to share MHC type), and littermate sisters preferentially nest together (Manning *et al.*, 1992).

Overall these results suggest that female house mice do gain benefits from communally nesting and rearing offspring with close relatives and in wild, semi-natural and laboratory conditions related females tend to nest together. However, in the majority of the studies previously mentioned, related females were littermates and therefore familiar as well. It is

important then to understand whether these kin biased cohabitation decisions are made on the basis of relatedness per se, or whether they are down to a prior association with relatives. Is kin discriminative behaviour between female house mice based on a previous familiarity with those same individual relatives (prior association) or would the behavioural responses be similar if the females had been unfamiliar (phenotype matching)?

2.2.2 Prior Familiarity and Recognition

The role of familiarity in kin recognition has been examined in a number of species. As previously discussed in the introduction chapter (Section 1.1.6), prior association involves individuals learning the identity of relatives present during development and then treating those previously familiar individuals as related and all other encountered individuals as unrelated (Tang-Martinez, 2001; Mateo, 2003; Holmes, 2004). In phenotype matching, individuals learn a recognition template from relatives or self during development and then compare that template to the phenotype of novel individuals (Blaustein, 1983; Holmes & Sherman, 1983; Tang-Martinez, 2001). If there is a sufficient degree of similarity between the recognition template and the newly encountered phenotype then that novel individual should be treated as related. This mechanism has the advantage that novel relatives can be recognised, and the degree of relatedness could potentially be assessed (discussed in Section 1.1.6.3). Some authors have argued that these two mechanisms differ only in the degree of familiarity and kin recognition occurs either through direct familiarity (prior association) or indirect familiarity (phenotype matching; Porter, 1988).

Among rodents, kin recognition via prior association (irrespective of genetic relatedness) has been shown in a number of species: in meadow voles (*Microtus pennsylvanicus*) during interactions between adult females (Ferkin & Rutka, 1990); in thirteen-lined (*Spermophilus tridecemlineatus*) and Columbian (*Spermophilus columbianus*) ground squirrels during interactions between juveniles (Holmes, 1984; Hare, 1992; Hare & Murie, 1996); in montane voles (*Microtus montanus*) during female development (Berger *et al.*, 1997); in Brandt's voles (*Microtus brandti*) during parent-offspring recognition (Li & Zhang, 2010); and in inbreeding avoidance in prairie (*Microtus ochrogaster*), and gray-tailed (*Microtus canicaudus*) voles (Gavish *et al.*, 1984; Boyd & Blaustein, 1985; Lucia & Keane, 2012).

Conversely, rodent phenotype matching has been demonstrated during odour investigation in mound-building mice (*Mus spcilegus*) and blind mole rats (*Spalax galili*) (Heth *et al.*,

2003; Baudoin *et al.*, 2005; Tzur *et al.*, 2009); in scent marking behaviour in golden hamsters (*Mesocricetus auratus*) (Heth *et al.*, 1998); during adult female interactions in Arctic (*Spermophilus parryii*) and Belding's (*Spermophilus. beldingi*) ground squirrels (Holmes & Sherman, 1982); in beaver (*Castor canadensis*) territorial defence (Sun & Müller-Schwarze, 1997); and in Brandt's voles (*M. brandti*) during inbreeding avoidance (Yu *et al.*, 2004).

Several studies of rodent species have shown the importance of both prior familiarity and genetic relatedness. Social learning of defensive responses in deer mice (*Peromyscus leucopus*) is enhanced when the demonstrator is either familiar and/or related to the observer (Kavaliers *et al.*, 2005). In both Arctic (*S. parryii*) and Belding's (*S. beldingi*) ground squirrels cross-fostered juvenile pups that shared a nest are treated like siblings during interactions despite differing levels of relatedness, but among female pups that were reared apart, sister-sister pairs were less aggressive than pairs of non-related females (Holmes & Sherman, 1982). In Belding's ground squirrels this familiarity kin recognition is lost over winter hibernation and only full siblings appear to be recognised once the squirrels emerge after winter (Mateo, 2010). Sibling recognition between laboratory rat pups (*Rattus norvegicus*) has also been demonstrated both when familiar with one another through a shared rearing environment, and without any prior association (Hepper, 1983).

In laboratory strains and house mice, inbreeding avoidance and male-male interactions tend to dominate studies of relatedness and familiarity in the literature (e.g. Hayashi & Kimura, 1983; Kareem, 1983; Yamazaki *et al.*, 1988; Aldhous, 1989; D'Amato, 1994; Penn & Potts, 1998a; Isles *et al.*, 2002). Fewer studies have looked at relatedness and familiarity between female house mice and evidence for prior association and phenotype matching is mixed. Interaction trials with female laboratory mice suggest that females are unable to recognise full sisters with which they were reared after a separation of 60 days (D'Amato, 1997). Female laboratory mice exhibit more parental care towards the pups when they are already familiar with the pups' mother compared to when the mother is unfamiliar. However, when presented with two familiar females they show more parental care to the pups of related females than those of unrelated females. This suggests the importance of both prior familiarity and relatedness in interactions between laboratory mice (D'Amato, 1993).

In a semi-natural enclosure study of female house mice, familiar full sisters were less aggressive and had a higher spatial cohabitation than unfamiliar full sisters (Rusu *et al.*, 2004) suggesting the importance of familiarity when relatedness is constant. Familiarity between female house mice through cross-fostered prior association also improved the probability of paired females producing and weaning offspring, although when familiarity was constant, sisters had a better reproductive output than equally familiar but unrelated females (König, 1994b). However, there is evidence of mice using phenotype matching to recognise kin as females resulting from crosses between laboratory strains and house mice prefer communal nesting partners of shared MHC type (suggestive of match-to-self phenotype matching; Manning *et al.*, 1992). It therefore remains unclear whether familiarity is required for female-female kin recognition in wild house mice that have a naturally diverse genetic background.

2.2.3 Olfaction and Kin Recognition

Kin discriminative behaviour based on odour has been demonstrated in a wide range of species (e.g. Heth *et al.*, 1998; Gerlach & Lysiak, 2006; Lizé *et al.*, 2010; Krause *et al.*, 2012). Olfaction is a dominant mode of communication in house mice and influences a large range of social behaviours. One of the main odours sources in mice is urine; female house mice scent mark around their home ranges and there is evidence of female scent marking as a signal of breeding status (Hurst, 1990b). House mouse urine can also signal individual identity (Cheetham *et al.*, 2007). It is possible therefore that highly polymorphic markers present in the urine of house mice may enable females to recognise relatives.

A large body of research involving laboratory mice, primarily looking at mate choice, has built up in support of the Major Histocompatibility Complex (MHC) being involved in familiarity-based individual recognition and kin recognition (for reviews see: Penn & Potts, 1999; Beauchamp & Yamazaki, 2003; Yamazaki *et al.*, 2007). Laboratory mice are able to distinguish between the scents of individuals that differ at a single MHC locus (Yamazaki *et al.*, 1983). Further studies suggest that the ability of laboratory mice to discriminate single MHC differences depends on the specific location of the MHC locus and the subsequent effect a difference has on the MHC peptide-binding region (Carroll *et al.*, 2002). Adult female laboratory mice preferentially nest with females of the same MHC type (Manning *et al.*, 1992) but avoid mating with males of the same MHC type (Egid & Brown, 1989; Penn & Potts, 1998a). However, recent work using wild house mice has

failed to find evidence supporting MHC's involvement in individual recognition, and instead indicates that a highly polymorphic set of proteins present in large quantities in mouse urine, the Major Urinary Proteins (MUPs), play a role in recognition (Hurst *et al.*, 2001; Cheetham *et al.*, 2007; Sherborne *et al.*, 2007; Thom *et al.*, 2008b). Importantly, the study by Sherborne *et al* (2007) looked at inbreeding avoidance between house mice and suggested a match-to-self kin recognition mechanism using sharing at both MUP haplotypes. It is therefore important to understand the genetic component of kin recognition between female house mice.

2.2.4 Chapter Aim

In this chapter I investigate the importance of prior familiarity on the ability of female house mice to recognise their female relatives. This was addressed by asking the following questions:

i) Can female house mice discriminate between urine from a full sister and urine from an unrelated female, and do they show any attraction towards either?

ii) Do female house mice preferentially associate with full sisters over unrelated females?

iii) Does a discrimination of and an attraction for full sisters depend on prior association or will females also display a preference for previously unfamiliar full sisters (indicating phenotype matching)?

iv) Does observed female association correlate with differences in urinary MUP peak sharing between subject and stimulus females?

Female house mice were tested for their ability to discriminate full sisters from unrelated females, and whether this recognition depends on prior familiarity. Subject females were given choice tests between a full sister and an otherwise comparable unrelated female. Females were either unfamiliar or highly familiar. A scent discrimination and attraction assay was used to assess whether cues signalling relatedness are present in female house mouse urine. Nest partner choice for related or unrelated females was then established using a remote tracking system. The urine of house mice contains a large concentration of proteins, the majority of which are major urinary proteins (Humphries *et al.*, 1999). Individuals of different MUP genotype express different MUP proteins in their urine

(Hurst *et al.*, 2001). More closely related individuals are more likely to have similar MUP profiles and individual mice could assess the degree of relatedness between two individuals by comparing the similarity in MUP profile. Mass spectrometry established MUP profiles for each female by calculating the intact mass of proteins in female urine. MUP proteins for each triad of females where then compared with observed behavioural response to indicate whether differences in MUP correlate with nest partner choice.

2.3 Methods

2.3.1 Animal Housing and Handling

Subjects and stimuli were captive-bred adult females from an outbred colony of house mice. The colony was established from individuals captured from populations in the North West of England and outbreeding was maintained with regular introductions of wild-caught mice. At weaning females were housed in single-sex groups of 2 - 5 individuals in MB1 cages (45 x 28 x 13 cm, North Kent Plastics, UK). Each cage was lined with Corn Cob Absorb 10 / 14 substrate, and contained paper - wool nesting material (Shredded Nesting International Product Supplier Limited, London, UK) and environmental enrichment such as cardboard tubes (11 cm long, 5 cm diameter), a red plastic mouse house (Techniplast, NJ, USA), and a lid-attached nest box (MPlex, Otto Environmental, WI, USA). Food (Lab Diet 5002, International Product Supplies Limited, London, UK) and water were provided *ad libitum*. Mice were maintained on a reversed 12:12 hours light:dark cycle, with the dark phase beginning at 8 am. All experimental animal handling was carried out during the dark phase under dim red light.

Females were injected beneath the skin at the nape of the neck with radio frequency identification (RFID) tags. Females in the familiar group were injected at weaning and females in the unfamiliar group several weeks before testing commenced, to prevent any potential stress from the injection procedure affecting the mice during behavioural assays. Aside from the RFID tagging process, females were handled using a handling tube (18 cm long, 5 cm diameter, one open end and one end closed with aluminium mesh of 0.5 x 0.5 cm) to minimise stress and anxiety (Hurst & West, 2010).

2.3.2 Subject and Stimulus Females

During all assays a subject female was presented with two stimulus females or their odours. One stimulus female was a full sister of the subject, the other stimulus female was unrelated to the subject. Females were deemed unrelated if they did not share more than one great-grandparent. Two groups of testing females were established, based on differing levels of prior familiarity.

The unfamiliar group consisted of 23 subject females from 8 unrelated litters. One subject female died between the first scent test and the nesting partner choice test. Related stimulus females were full sisters from a previous litter by the same parents, and were

approximately 1 month older than the subject females. Unrelated stimulus females were age matched (within 20 days) and weight matched (0.28 ± 0.46 g (mean ± standard error)) to the related stimulus females. Subject females had not encountered any stimulus females prior to testing and had not been used in any other behavioural tests previously. All subjects were 8 - 10 months old at testing.

In the familiar group subject females (n = 24) were from 16 unrelated litters. Related stimulus females were full sisters from the same litter as the subject female. Subject females were therefore familiar with the related stimulus females from birth. At weaning, 24 days after birth, caged groups of familiar females were established consisting of two age-matched unrelated pairs of littermate sisters. In four instances litters only had 1 female and so cages of three females was established instead (i.e. a single female housed with an unrelated sister pair). Subjects were therefore familiar with the unrelated stimulus females from being 24 days old. Where possible stimulus females were weight matched (0.53 ± 0.83 g (mean ± standard error)). All subjects were 5 - 9 months old at testing, meaning that familiar groups consisting of unrelated sister pairs had cohabited for a minimum of 4 months before tests began.

2.3.3 Urine Collection

Two urine collection techniques were initially tested: scruffing and recovery. In the scruffing method the mouse is quickly picked up and held by the scruff of the neck above a clean 1.5 ml Eppendorf tube (Cheetham *et al.*, 2007). Recovery involves placing the mouse on a metal grill above a clean MB1 cage, with a second empty MB1 cage placed upside down over the first to prevent escape. Any urine produced falls through the grid and can be collected later from the bottom cage. Initial pilot studies suggested that both methods of collection were equally suitable for the scent discrimination and attraction assay and so the recovery method was used as it does not involve direct handling of the mice. Urine from all of the stimulus and subject animals was collected 1 - 2 weeks before any testing commenced and stored at -22 °C.

2.3.4 Behavioural Assay Schedule

Subject females were given three assays and the same triads of females (subject and two stimulus females) were used in each test. First, females were given a scent discrimination and attraction assay designed to observe female response to urine from related and unrelated females before unfamiliar individuals encountered each other. House mice

investigate scent marks to gather information about the owner of the marks (Cheetham *et al.*, 2007). If markers of relatedness are present in female house mouse urine then urine from relatives are expected to contain similar markers compared to urine from non-relatives. Females are therefore expected to spend longer investigating unrelated female urine as the novel components will require longer to gather information about the scent. Scent marks are used by house mice to indicate territory ownership (Hurst, 1990a). Kin selection theory predicts that related females may affiliate and females may therefore display an attraction by spending more time in the location of the related scent mark. Self-grooming behaviour may broadcast information about the state or interest of the grooming individual; for example female prairie voles (*M. ochrogaster*) self-groom more in response to unrelated male odours than to related male odours, and this behaviour could signal sexual motivation and inbreeding avoidance (Paz-Y-Miño C *et al.*, 2002). Self-grooming may also play a role in female – female kin recognition. A bias in self-grooming location may indicate female kin recognition or attraction to urine from a related female.

One week following the completion of all scent assays subject females were tested in the nest partner choice assay. The scent discrimination and attraction assay is a short test aimed at investigating spontaneous response to urine from related or unrelated females. Any scent attraction observed could indicate a preference for females to associate with relatives (as predicted by kin selection theory). The nest partner choice assay was designed to discover whether female house mice exhibit a clear preference for spending time near particular individuals (consistent with nest partner choice). Subject females were placed in a series of three inter-connected cages with restricted access to stimulus females and their cage location was recorded over 72 hours.

Following the nest partner choice assay females were given post-encounter scent discrimination and attraction assays to discover whether encountering the stimulus females in the nest partner choice assay influenced female response to urine marks. This second scent discrimination and attraction assay occurred two days after the nesting preference tests using fresh urine samples collected in the two days following the nest partner choice assay. On completion of all three behavioural assays fresh urine samples were collected from all females and mass spectrometry was performed to reveal the individual MUP peak profiles for each female. Differences in individual MUP peak profiles were compared to the observed nest partner choice results (see Section 2.3.7).

2.3.5 *Scent Discrimination and Attraction*

The first assay was designed to determine whether females are able to spontaneously discriminate the urine of a full sister from an unrelated female, and whether they show an attraction towards either scent. In order to establish the most effective assay, a number of initial pilot designs were tried involving different presentation locations for the urine samples (e.g. on the walls, floor or cage lid). A design modified from Ramm *et al.* (2008) was found to be the most effective (Figure 2.1). Scent assays were performed in modified clean MB1 cages. Metal braces supported a clear acrylic barrier (28 x 13cm) located across the width of the middle of the cage. In the centre of the barrier was a circular hole (diameter 5 cm) that allowed movement from one side of the cage to the other. The cage was covered by a perforated clear acrylic lid.

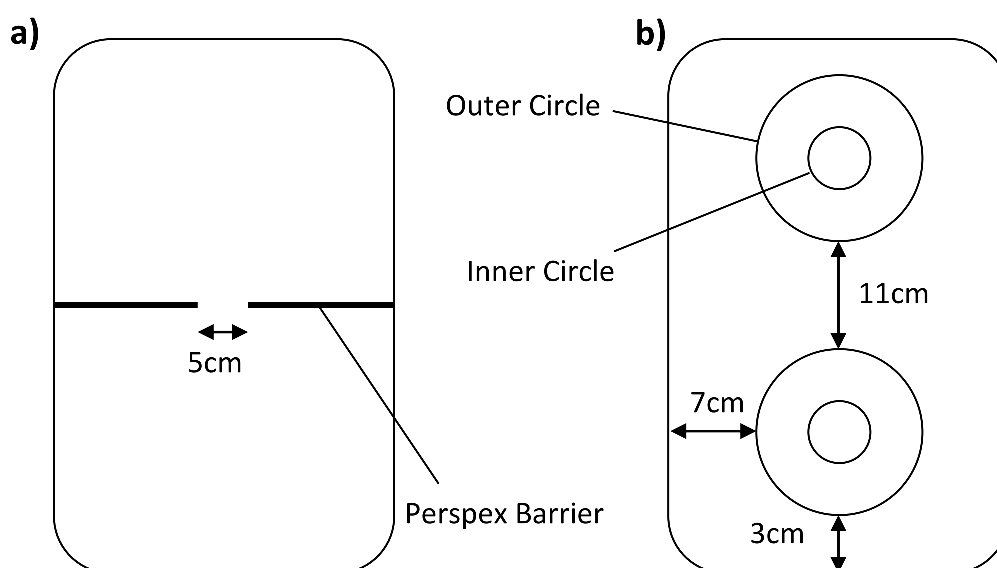


Figure 2.1: Scent discrimination and attraction assay design.
Design for the scent discrimination and attraction modified from Ramm *et al.* (2008). a) Modified MB1 test cage with Perspex barrier with 5 cm diameter circular hole. b) Acrylic test cage lid with outer (14 cm diameter) and inner circles (5.5 cm diameter). Urine (10 μl) from stimulus females is streaked onto Benchkote taped on the undersides of the inner circles.

Females were allowed to habituate to the test cage for 30 minutes with a clean lid before the test commenced. Two inner circles (5.5 cm diameter) were marked 25 cm apart on the underside of test lids, each in the centre of an outer circle (14 cm diameter; Figure 2.1).

Half-circles of Benchkote (5.5 cm diameter) were stuck with Sellotape in the inner circles on the underside of the lid. Urine (10 μl) was streaked onto each of the two pieces of Benchkote in the same orientation. The position of the related and unrelated stimulus urine was randomised but balanced to prevent any side bias. Urine was given two minutes to dry before the lids were swapped and the subject was presented with the test lid.

Trials lasted 20 minutes and female behaviour towards the two scents was recorded remotely to DVD. At the end of each scent trial the female was returned to her home cage. DVDs were watched blind to the position of each scent and behaviours were recorded using an event recorder program (written by R.J.Beynon). Female location was determined by the position of the nose and female position and behaviour was measured (Table 2.1). Female investigation of urine from related and unrelated females was assessed by measuring the amount of time females spent sniffing each scent. Since the urine samples were streaked onto the test lid, females had to stand on their hind legs to investigate the scents, making this behaviour easy to observe. Females were predicted to spend longer sniffing urine from an unrelated female compared to urine from a related female. Two measures were used to assess female attraction: the amount of time they spent within the outer circles, and the total amount of time females spent on each side of the cage barrier. If females do exhibit an attraction towards related females and are able to discriminate between urine from related and unrelated females then they may be expected to spend longer in the area of related urine. The amount of time females spent self-grooming on each side of the test cage was measured to determine if self-grooming was biased by the presence of urine from related or unrelated females.

Table 2.1: Female behaviours measured during scent discrimination and attraction assays. Behaviours watched blind to the location of related and unrelated urine.

Behaviour	Description
Sniff Related Urine	Subject on the related urine side of the test cage, standing on hind legs within the inner circle sniffing at urine mark
Close Proximity To Related Urine	Subject within the outer circle on related urine side of the test cage
Related Side	Subject on the related urine side of the test cage
Related Self-Groom	Subject on the related urine side of the test cage performing self-grooming behaviours (such as rubbing, licking or scratching)
Sniff Unrelated Urine	Subject on the unrelated urine side of the test cage, standing on hind legs within the inner circle sniffing at urine mark
Close Proximity To Unrelated Urine	Subject within the outer circle on the unrelated urine side of the test cage
Unrelated Side	Subject on the unrelated urine side of the test cage
Unrelated Self-Groom	Subject the unrelated urine side of the test cage performing self-grooming behaviours (such as rubbing, licking or scratching)

2.3.6 Nest Partner Choice

To test nesting preference, females were placed in a series of three interconnected MB1 cages (Figure 2.2). The end cages were bisected laterally with an acrylic barrier (45 x 13 cm), along the middle of which was cut a slot (31 x 5 cm) covered on either side with aluminium mesh (0.5 x 0.5 cm). The cages were connected by clear acrylic tunnels (33 cm long, 5 cm diameter). Aluminium covers (27 x 15 cm) were placed over the end cages to give the females shelter during the light phase. The bottom of each cage was lined with Corn Cob Absorb 10/14 substrate, and contained a small amount of paper/wool nesting material (Shredded Nesting International Product Supplier Limited, London, UK). Food (Lab Diet 5002, International Product Supplies Limited, London, UK) and water were provided in each section of each cage *ad libitum* so that each stimulus female had her own separate food and water supply, and the subject female had food and water in each cage.

A stimulus female was placed in each of the end cages, behind the mesh barriers. The subject female was placed in the central cage and allowed to move freely between the three cages. The subject female was able to interact with the stimulus females through the mesh barriers, which allowed for scent, sight and sound contact, as well as limited touch. The barriers prevented females from accessing the other halves of the test cages, however bedding material and substrate was often pulled from one side of the barrier to the other. This material provided additional scent contact between females. Stimulus females were placed into the end cages approximately 1 hour before the subject female was placed in the central cage. The system was started toward the end of the dark phase (4 - 5pm) and allowed to run for 72 hours. Food and water were checked daily and each female was seen to be alive. After 72 hours the females were returned to their home cages and the apparatus was cleaned thoroughly.

The connecting tunnels ran through automated RFID readers between the cages. These were housed in clear Perspex boxes (11 x 12.6 x 11.5 cm; Francis Scientific Instruments (FSI), Cambridge, UK). Each reader also contained an infrared beam and detector. The combination of RFID and infrared detectors meant that each time a female passed through a tunnel the time, date and direction of travel could be recorded. A central computer running custom software (FSI; Thom *et al.*, 2008b) logged this data and the Output files were converted using custom software written by J.L.Hurst in Revolution (a software development environment) and SPSS (version 18.0.2) to calculate the total time spent by

subject females in each cage. If females are able to recognise relatives and preferentially associate with relatives then subject females are predicted to spend longer in the cage containing the related female than the cage containing the unrelated female. If female house mice use a prior association mechanism to recognise kin then it might be expected that females would spend longer in the cage containing the related female compared to the cage containing the unrelated female in the familiar but not the unfamiliar group. If female house mice use a phenotype matching mechanism to recognise kin then females may spend longer in the related female cage than the unrelated female cage in both the familiar and unfamiliar groups.

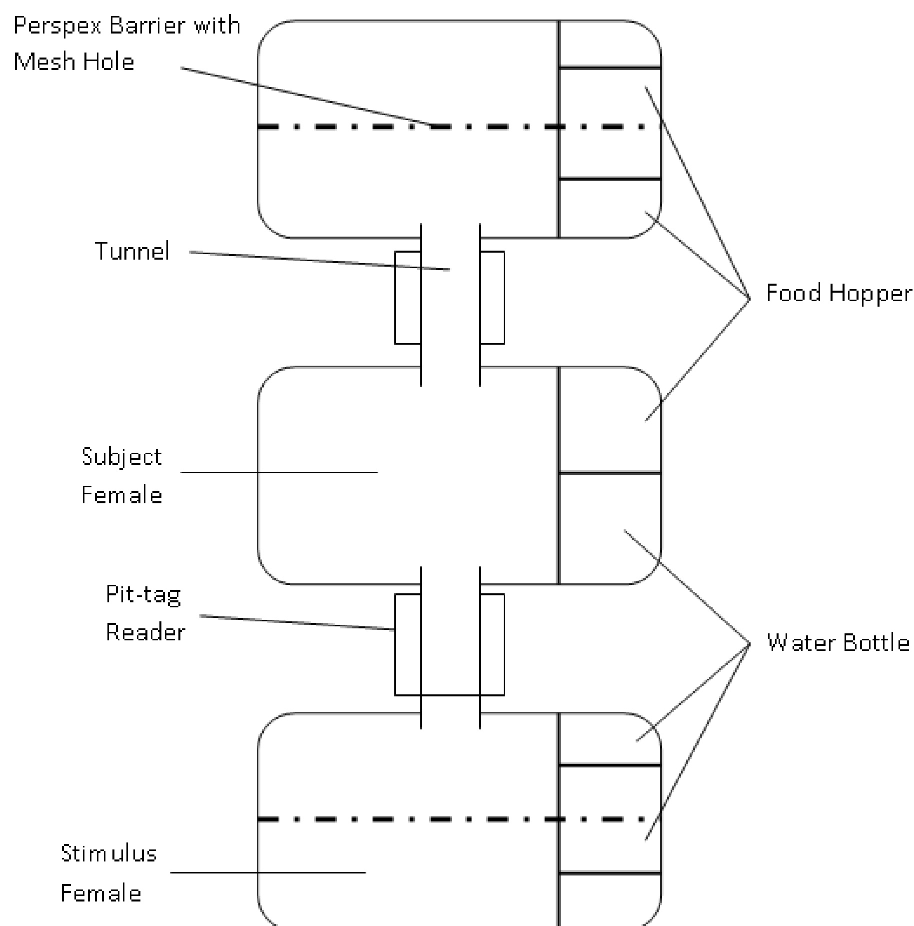


Figure 2.2: Nest partner choice assay design.
Three MB1 cages connected by tunnels. The end cages are bisected laterally by acrylic barriers with large, mesh-covered slots. Stimulus females are housed in the contained halves of the end cages. The subject female is placed in the centre cage and can access all three cages, interacting with both stimulus females through the mesh. The stimulus females cannot interact with each other. Tunnels have automated RFID readers allowing for the subject female's movements to be recorded for 72 hours and the amount of time spent in each cage is calculated.

2.3.7 *Comparison of Major Urinary Protein Profiles*

The degree of MUP pattern similarity between subject and stimulus females was compared against observed nest partner choice behaviour. Urine samples were collected from subject and stimulus females using the recovery method after behavioural testing was completed. In seven instances a sample was not obtained from one individual in a triad meaning that only 20 familiar and 19 unfamiliar female triads were compared. Electrospray ionization mass spectrometry was used to produced the mass profiles of MUP samples following the methods described in Mudge *et al.* (2008). This process established the molecular mass of individual proteins present in a urine sample. Samples were run by Amanda Davidson at the Protein Function Group, University of Liverpool, UK. Samples were run using a Nanoacquity ultra high performance liquid chromatography system (Waters, Manchester, UK) and were processed and transformed to a true mass scale using MazEnt1 deconvolution software (Waters Micromass, Massachusetts, USA). The resulting mass spectra were cropped between the mass range 18600 and 18900 Da, normalised to the most abundant protein and profiles were inspected in SpecAlign (Version 2.4.1; http://physchem.ox.ac.uk/~jwong/specalign/).

A peak profile was established for each female (Figure 2.3). Peaks were considered true if they had a relative intensity of greater than 0.15. This value was chosen as peaks of a lower intensity may not represent true proteins, and any peaks of a lower relative intensity were excluded. Similarity in MUP profile between the subject and each of the stimuli females was calculated using two methods: 1) the number of peaks shared by a subject female and a stimulus female, regardless of the relative intensity of the peaks (Peak Sharing); 2) the number of protein peaks expressed by a subject and a stimulus female of a similar relative intensity (Peak Matching). First the total number different protein peaks expressed by a subject female and a stimulus female were counted.

Peak sharing was the proportion of the total number of different peaks expressed by both females that were expressed by both the subject and stimulus female (Figure 2.3). Peaks were defined as matching if the difference in relative intensity between the two peaks was less than 0.50. Peak matching was calculated as the proportion of the total number of different peaks expressed by both females that were expressed in a matched relative intensity by both the subject and stimulus female (Figure 2.3). The degree of MUP peak sharing and matching was calculated for each pair of subject and stimulus females.

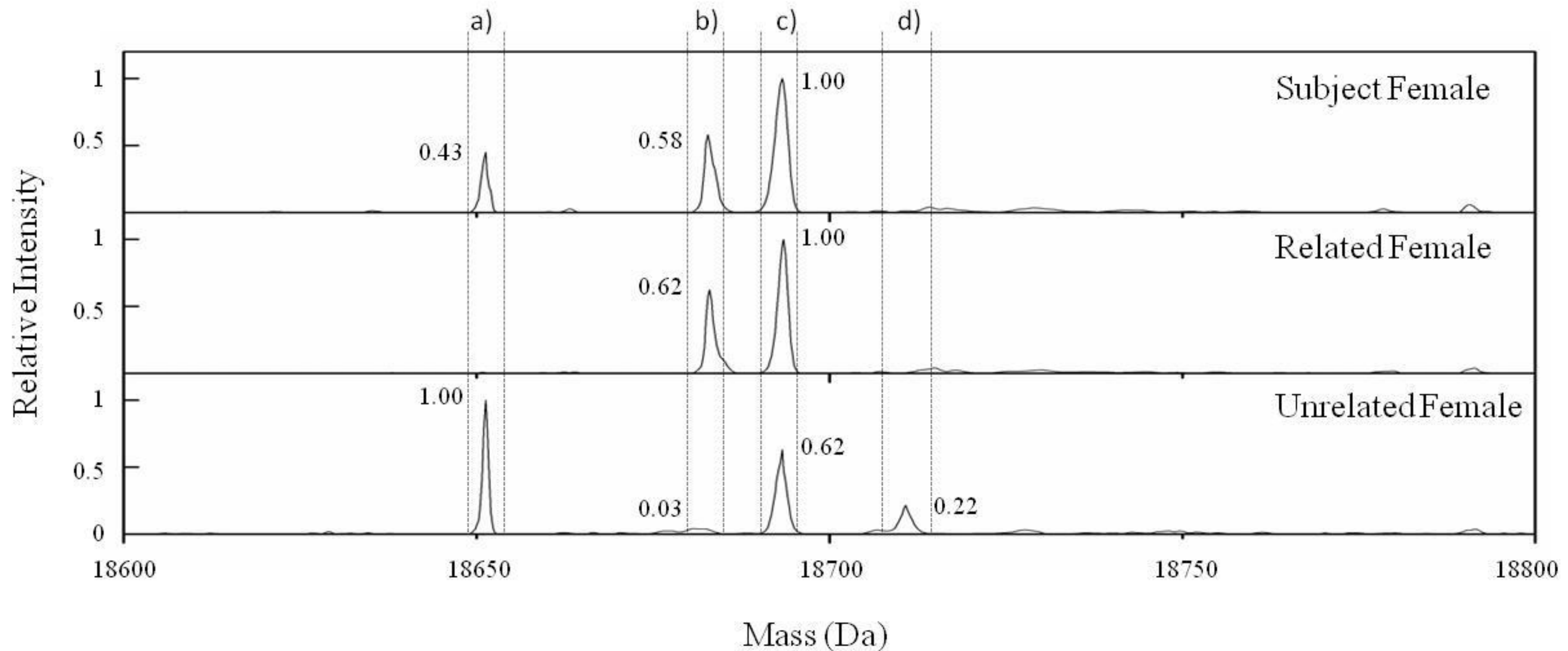


Figure 2.3: Example MUP mass spectra for a test triad of a subject female and two stimulus females.
Example MUP mass spectra for subject female (top) and corresponding related (middle) and unrelated (bottom) stimulus females. The relative intensities for each peak are indicated. Spectra are cut off at a mass of 18800 Da for this example. At mass a) the subject female and unrelated stimulus female share a peak but do not have matching peaks as the difference in relative intensity is greater than 0.50. At mass b) the subject and related females share a peak of matching relative intensity but the unrelated stimulus female does not have a peak that is counted as it has a relative intensity of below 0.15. The subject and both stimulus females share and match the peak at position c). At position d) only the unrelated stimulus female expresses a peak. As this peak has a relative intensity of greater than 0.15 it is counted as a true peak. The subject female and related stimulus female express a combined total of three different protein peaks. Two of these are shared between the subject and related stimulus female, and both are also of a matching relative intensity. This gives a peak sharing score of (2 / 3 = 0.67) and a peak matching score of (2 / 3 = 0.67) for the subject female and the related stimulus female. The subject female and the unrelated stimulus female also express a combined total of three protein peaks. The subject female shares two protein peaks with the unrelated stimulus female (giving a peak sharing score of 2 / 3 = 0.67), but only one peak is of a matching relative intensity (giving a peak matching score of 1 / 3 = 0.33).

2.3.8 Data Analysis

All statistical tests were carried out using the SPSS software package (version 18.0.2). All figures use untransformed data.

2.3.8.1 Scent Discrimination and Attraction Analysis

In the two scent discrimination and preference assays four female behaviours were measured in response to each urine mark: time spent sniffing, time in close proximity, total time on each side of the test cage and the time spent self-grooming on each side of the test cage. It was predicted that subject females would spend longer sniffing urine from unrelated stimulus females than urine from related stimulus females. The behaviour of subject females during the scent discrimination and attraction assays was first analysed across the full 20 minutes of each trial. The data was then divided into two blocks of 10 minutes and re-analysed to investigate how female behaviour changed across the course of the scent assays. Only females that sniffed both urine marks over the full 20 minutes were included in the analysis of the whole assay and the second half of the assay. Only females that sniffed both urine marks during the first 10 minutes were included in the analysis of the first half of the assays.

Where possible data were transformed to meet parametric assumptions (specific transformations used for each data set are recorded in the results). Where data could be transformed, a repeated measures GLM compared the amount of time spent by females sniffing (or in proximity to, on the side of or self-grooming on the side of) urine from the related stimulus female and urine from the unrelated stimulus females. Familiarity group was assigned as a between subject factor.

Where data did not meet parametric assumptions Mann-Whitney U tests compared the ratio of time females spent sniffing (or other recorded behaviours) urine from the related stimulus female compared to urine from the unrelated stimulus female between the familiar and unfamiliar groups. If there was no difference in female response between the groups then data from the two familiarity groups was combined. If there was a difference then the data for the familiarity groups were analysed separately. Wilcoxon Signed Ranks tests compared the amount of time females spent sniffing (or other recorded behaviours) urine from a related female to urine from an unrelated female.

2.3.8.2 Nest Partner Choice Analysis

Data from the nest partner choice assay did not meet parametric assumptions. The proportion of time spent by subject females in the related stimulus female cage (as a proportion of the total assay time) was compared between the familiar and unfamiliar groups using a Mann-Whitney U test. If there was a significant difference between the familiarity groups then female cage choice was analysed separately for each group. If there was no difference between the familiarity groups then the data were combined. The amount of time females spent in each test cage was compared against the amount of time females spent in each of the other test cages using Wilcoxon Signed Ranks tests. The stimulus cage in which each female spent the longest was assigned using the ratio of time spent in the related female stimulus cage compared to the time spent in the unrelated female stimulus cage. If a ratio was above 0.50 then the subject female spent longer in the related stimulus female cage. If the ratio was below 0.50 then the subject females spent longer in the unrelated stimulus female cage.

To establish whether time of day affected female response these tests were repeated using data from the light and dark phases. It was thought that female behaviour may change over the course of 72 hours. To discover whether continued experience of the assay influenced female behaviour the ratio of time females spent in the related cage was compared between each complete dark phase and each complete light phase. The 72 hour period encompasses two complete dark phases and two partial dark phases (the remainder of the first dark phase after the assay starts and the start of the fourth dark phase before the assay is ended). A Wilcoxon Signed Ranks test compared the ratio of time females spent in the related stimulus female cage in the second and third dark phases. The 72 hour assay period encompasses three complete light phases and a Friedman's ANOVA compared the ratio of time females spent in the related cages in the first, second and third light phases.

2.3.8.3 Major Urinary Proteins and Nest Partner Choice Analysis

Subject females were grouped as to whether they spent longer in the related or unrelated stimulus female cage (as assigned by the ratio of time spent in the related female cage compared to the time spent in the unrelated female cage). For subjects that spent longer in the related stimulus female cage Wilcoxon Signed Ranks tests compared the difference in MUP peak sharing and matching between the subject and related stimulus female and the subject and unrelated stimulus female. This analysis was repeated for subjects that spent longer in the unrelated stimulus female cage.

2.4 Results

2.4.1 Pre-Encounter Response to Scent

Subject females were presented with urine from a full sister and an unrelated female and were either familiar or unfamiliar with both females. This assay was designed to assess whether females are able to spontaneously discriminate the urine of a full sister from an unrelated female, and whether they show an attraction towards either scent (the results are presented below and summarised in Table 2.2).

2.4.1.1 Pre-Encounter Scent Discrimination

Female discrimination of urine from related and unrelated females was measured by the amount of time females spent investigating (sniffing) each urine mark. Over the full 20 minutes, a significant difference was observed between the level of female investigation in the familiar and unfamiliar groups ($z = -2.28$, $p = 0.02$, two-tailed; Figure 2.4). As a difference was observed between the familiarity groups, the time females spent sniffing related and unrelated urine marks was analysed separately for each group. Females spent longer sniffing urine from an unrelated female than urine from a related female in both the familiar ($z = -2.03$, $p = 0.02$, one-tailed; Figure 2.4) and unfamiliar groups ($z = -2.54$, $p = 0.004$, one-tailed; Figure 2.4).

To investigate female response to urine from related and unrelated females further, the 20 minute assay was split into two blocks of 10 minutes. During the first half of the assay there was no difference in the level of female investigation between the familiar and unfamiliar groups ($F_{(1,27)} = 0.003$, $p = 0.96$, data square root transformed; Figure 2.4). Females spent longer sniffing urine from an unrelated female than urine from a related female ($F_{(1,27)} = 14.16$, $p = 0.001$, data square root transformed; Figure 2.4).

During the second half of the assay (from 10 to 20 minutes) there was no difference in female investigatory response between the familiar and unfamiliar groups ($z = -0.97$, $p = 0.35$, two-tailed; Figure 2.4). As a result the data for the two familiarity groups was combined and females spent longer sniffing the unrelated than the related urine ($z = -1.63$, $p = 0.05$, one tailed; Figure 2.4).

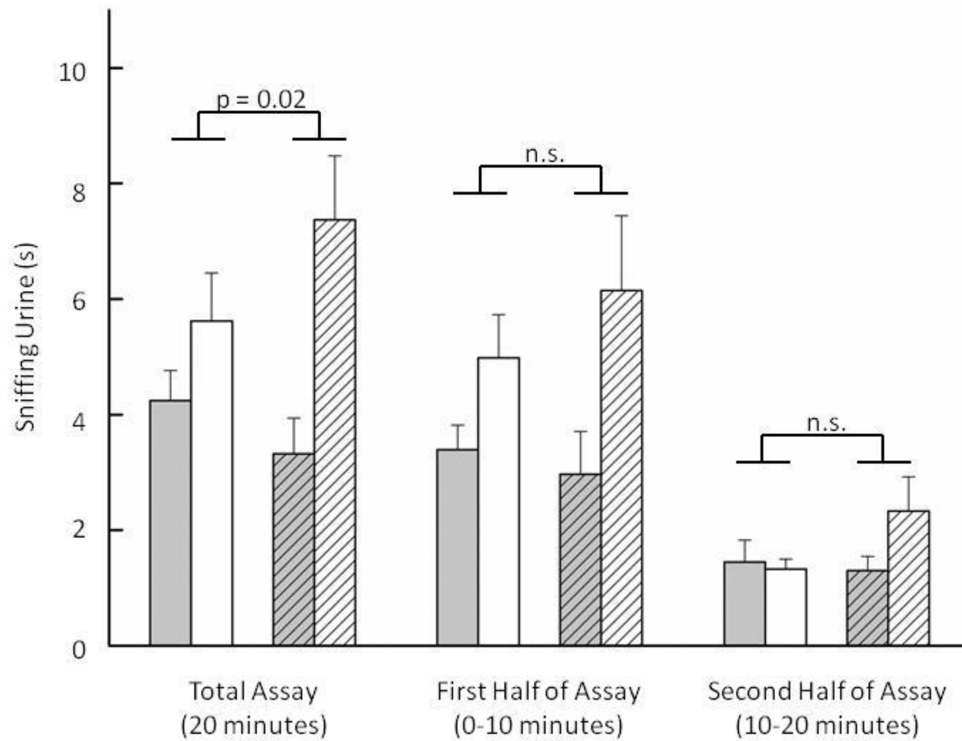


Figure 2.4: Pre-encounter scent discrimination of urine from related and unrelated stimulus females in the familiar and unfamiliar groups.

Time (seconds, mean + standard error) spent by subject females sniffing urine from related (grey bars) and unrelated (white bars) stimulus females in the familiar (open bars) and unfamiliar (hashed bars) groups. Across the total assay (0-20 minutes, familiar n = 18, unfamiliar n = 14) there was a significant difference between the familiarity groups. There was no difference between the familiarity groups during either the first half of the assay (0-10 minutes, familiar n = 17, unfamiliar n = 12) or the second half of the assay (10-20 minutes, familiar n = 11, unfamiliar n = 10).

2.4.1.2 Pre-Encounter Scent Attraction

Female attraction towards urine from related and unrelated females was measured by the amount of time females spent in close proximity to each scent and the total amount of time females spent on each side of the test cage. There was no difference between the familiar and unfamiliar groups in the amount of time females spent in proximity to the related or unrelated odours ($z = -1.25$, $p = 0.22$, two-tailed; Figure 2.5), and females did not differ in the amount of time they spent in proximity to related or unrelated urine ($z = -0.57$, $p = 0.58$, two-tailed; Figure 2.5).

To investigate female response to urine from related and unrelated females further, the 20 minute assay was split into two blocks of 10 minutes. In the first half of the assay there was no effect of prior familiarity on the amount of time females spent in proximity to urine from the related and unrelated stimulus females ($F_{(1,27)} = 0.47$, $p = 0.50$, data square root transformed; Figure 2.5). There was no difference in the amount of time females spent in proximity to the related or unrelated urine ($F_{(1,27)} = 0.24$, $p = 0.63$, data square root transformed; Figure 2.5).

Measures of attraction across the second half of the assay were assessed using females that sniffed both sides over the full 20 minutes. During the second half of the assay (from 10 to 20 minutes) there was no difference in the amount of time females spent in close proximity to either stimulus urine mark between the familiar and unfamiliar groups ($z = -1.33$, $p = 0.19$, two-tailed; Figure 2.5). As a result the data for the two familiarity groups was combined and females did not spend any longer in proximity to either the related or unrelated scent ($z = -0.39$, $p = 0.71$, two-tailed; Figure 2.5).

Female attraction towards urine from related and unrelated stimulus females was also measured using the total amount of time females spent on each side of the test cage. Over the full 20 minutes familiar and unfamiliar females did not differ in the proportion of time they spent on the related urine side ($z = -0.72$, $p = 0.49$. two-tailed; Figure 2.6). There was no difference in the amount of time that females spent on the side with urine from a related female compared to the side with urine from an unrelated female ($z = -0.90$, $p = 0.38$, two-tailed; Figure 2.6).

To investigate female response further the 20 minute assay was split into two blocks of 10 minutes. Over the first half females spent longer on the related urine side than the unrelated

urine side ($F_{(1,27)}$ = 14.16, p = 0.001; Figure 2.6), a preference that was not influenced by degree of familiarity ($F_{(1,27)}$ = 0.003, p = 0.96; Figure 2.6).

Measures of attraction across the second half of the assay were assessed using females that sniffed both sides over the full 20 minutes. Female side attraction did not differ between the familiar and unfamiliar groups (z = -1.52, p = 0.14, two-tailed; Figure 2.6). Females showed no attraction for the side of urine from either a related female or an unrelated female (z = -0.26, p = 0.80, two-tailed; Figure 2.6).

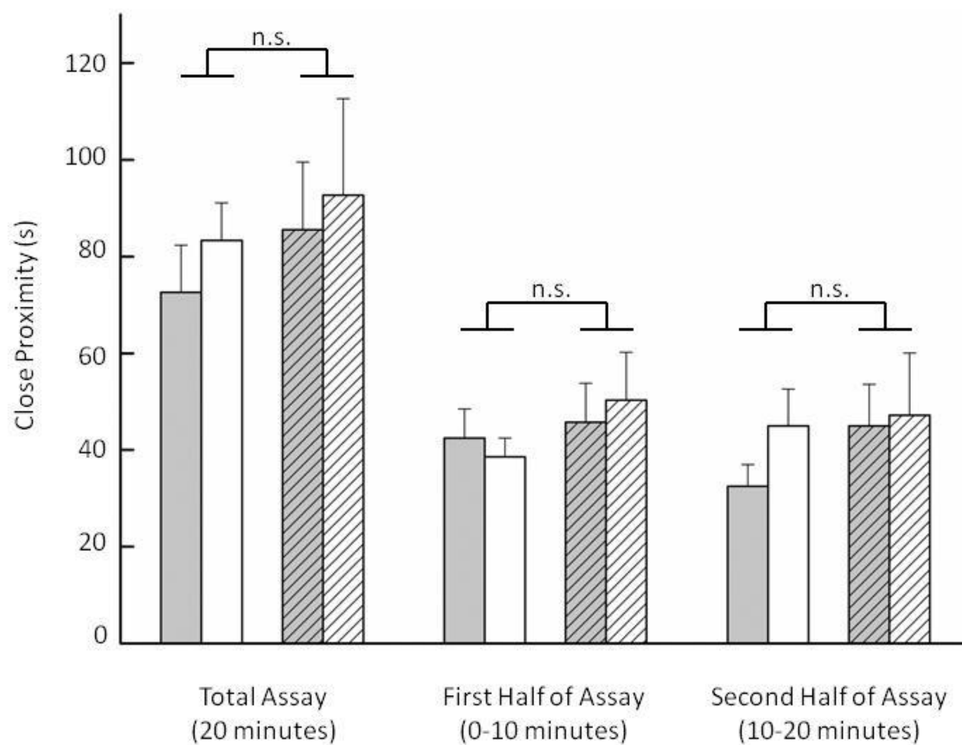


Figure 2.5: Pre-encounter close proximity attraction towards urine from related and unrelated stimulus females in the familiar and unfamiliar groups.
Time (seconds, mean + standard error) spent by subject females in close proximity to urine from related (grey bars) and unrelated (white bars) stimulus females in the familiar (open bars) and unfamiliar (hashed bars) groups. There was no difference between the familiarity groups across the total assay (0-20 minutes, familiar n = 18, unfamiliar n = 14), the first half of the assay (0-10 minutes, familiar n = 17, unfamiliar n = 12), or the second half of the assay (10-20 minutes, familiar n = 18, unfamiliar n = 14).

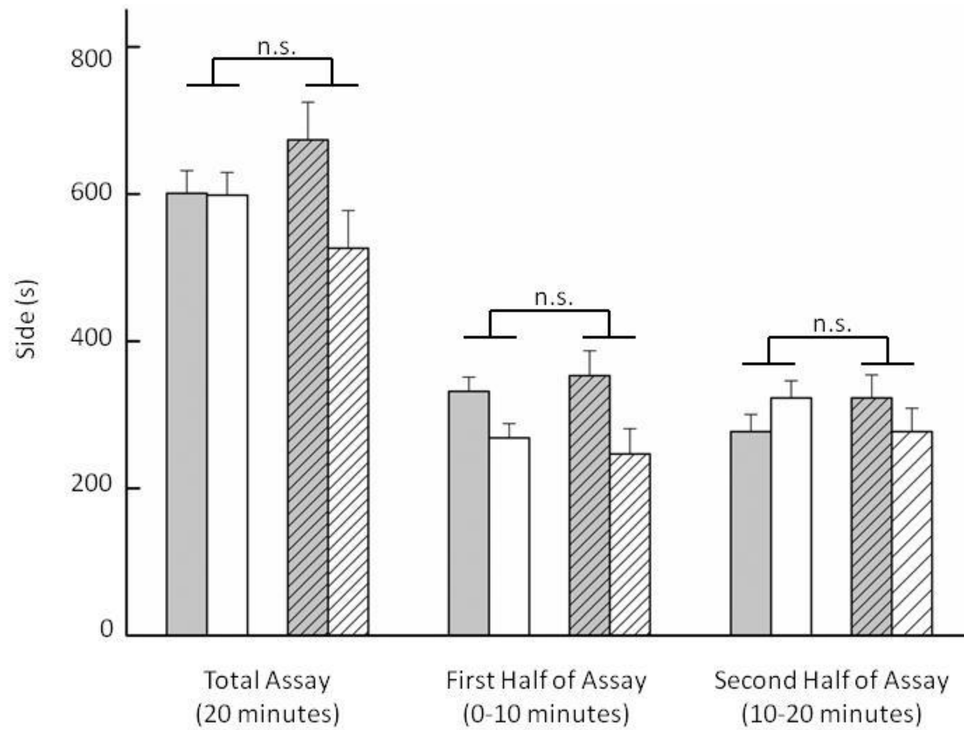


Figure 2.6: Pre-encounter cage side attraction towards urine from related and unrelated stimulus females in the familiar and unfamiliar groups.
Time (seconds, mean + standard error) spent by subject females on the cage side containing urine from a related female (grey bars) and an unrelated female (white bars) in the familiar (open bars) and unfamiliar (hashed bars) groups. There was no difference between the familiarity groups across the total assay time (0-20 minutes, familiar n = 18, unfamiliar n = 14), the first half of the assay (0-10 minutes, familiar n = 17, unfamiliar n = 12), or the second half of the assay (10-20 minutes, familiar n = 18, unfamiliar n = 14).

2.4.1.3 Pre-Encounter Self-Grooming

Subject females may self-groom more in response to urine from a full sister compared to urine from an unrelated female (or visa verse). Over the total assay there was no difference in self-grooming between females in the familiar and unfamiliar groups ($F_{(1,30)} = 3.29$, $p = 0.08$, data square root transformed; Figure 2.7), and females did not differ in the amount of time spent self-grooming on either the related urine side or unrelated urine side ($F_{(1,30)} = 0.93$, $p = 0.34$, data square root transformed; Figure 2.7).

To investigate the female behaviour further the 20 minute assay was divided in half. In the first half of the assay there was no difference between females in the familiar and unfamiliar groups in the amount of time spent self-grooming ($z = -0.11$, $p = 0.92$, two-tailed; Figure 2.7). As there was no difference between the familiarity groups the data were combined. There was a non-significant trend for females to spend significantly longer self-grooming on the related side than the unrelated side ($z = -1.65$, $p = 0.10$, two-tailed; Figure 2.7).

Over the second half of the assay there was no effect of familiarity group on the amount of time females spent self-grooming on either the related or unrelated stimulus urine sides ($z = -0.81$, $p = 0.43$, two-tailed; Figure 2.7). The data was combined and females did not differ in the amount of time they spent self-grooming in the related stimulus urine side and unrelated stimulus urine side ($z = -0.80$, $p = 0.95$, two-tailed; Figure 2.7).

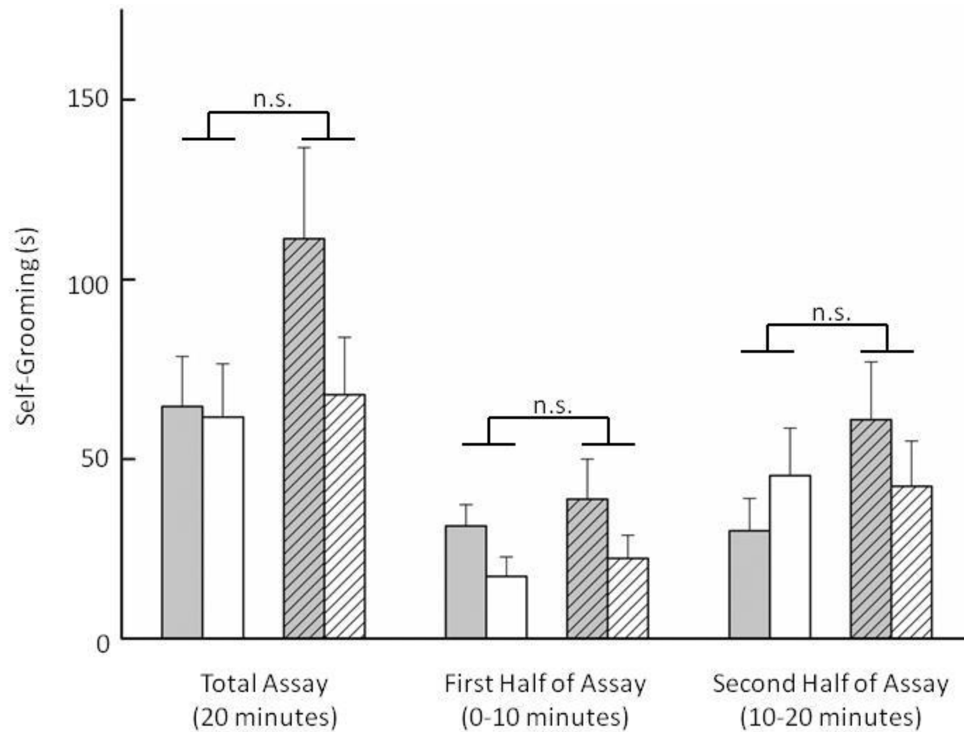


Figure 2.7: Pre-encounter self-grooming on the cage side containing urine from related and unrelated stimulus females in the familiar and unfamiliar groups.
Time (seconds, mean + standard error) spent by subject females self-grooming on the cage side containing urine from a related female (grey bars) and unrelated female (white bars) in the familiar (open bars) and unfamiliar (hashed bars) groups. There was no difference between the familiarity groups during either the total assay time (0-20 minutes, familiar n = 18, unfamiliar n = 14), the first half of the assay (0-10 minutes, familiar n = 17, unfamiliar n = 12), or the second half of the assay (10-20 minutes, familiar n = 18, unfamiliar n = 14).

2.4.2 Nest Partner Choice

Subject females were placed in a series of three interconnected cages with restricted access to stimulus females in the end cages. The amount of time females spent in each cage was compared and the results are presented below and summarised in Table 2.2. There was no difference in the proportion of time subject females spent in the related stimulus female cage between the familiar and unfamiliar groups ($z = -0.45$, $p = 0.66$, two-tailed; Figure 2.8). As a result the data for the two familiarity groups were combined. Females spent longer in the cage that contained the related stimulus female than the cage containing the unrelated female over a 72 hour period ($z = -2.84$, $p = 0.004$, two-tailed; Figure 2.8). To further investigate female cage preference the time spent in the centre cage was compared to the time spent in each stimulus cage. Females spent significantly longer in the cage containing the related female than in the centre cage ($z = -2.25$, $p = 0.024$, two-tailed; Figure 2.8). This is despite the fact that the centre cage presents an accessible area for the subject female twice as large as the related female cage. There was no difference in the amount of time females spent in either the centre cage or the cage containing the unrelated female ($z = -1.18$, $p = 0.24$, two-tailed; Figure 2.8).

The amount of time females spent in the related cage as a ratio of the total amount of time females spent in both stimulus female cages was calculated to examine female choice (Figure 2.9). In the familiar group, 65 % of females chose the related female over the unrelated female, whilst 73 % of females chose the related female in the unfamiliar group.

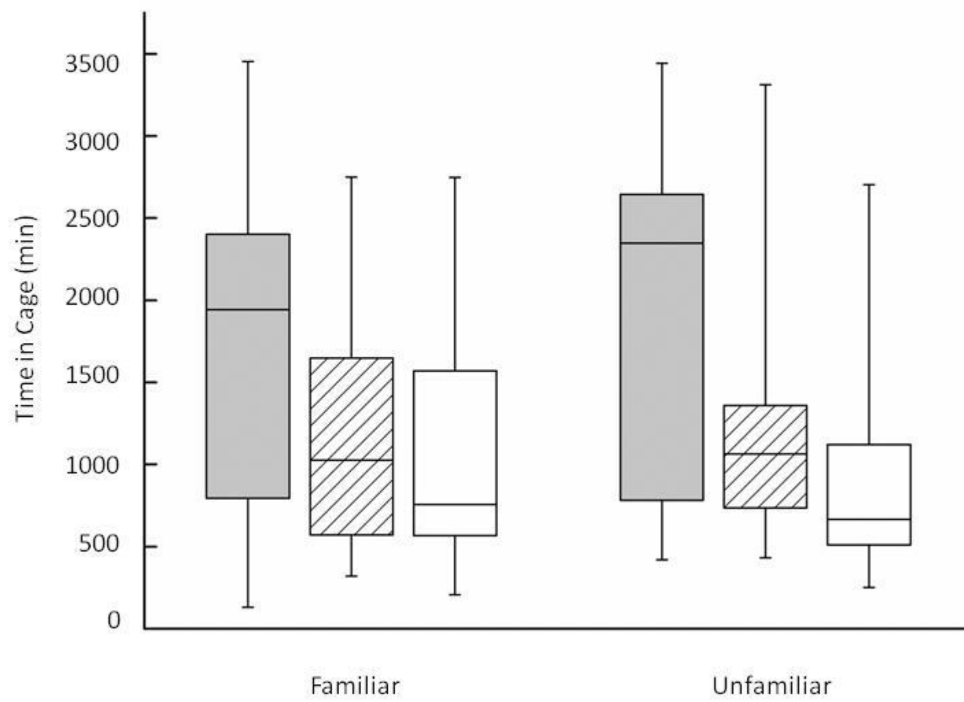


Figure 2.8: Female nest partner choice in the familiar and unfamiliar groups.
Time (minutes) spent in the related female cage (grey boxes), the centre cage (hashed boxes) and the unrelated female cage (open boxes) in the familiar (n = 23) and unfamiliar (n = 22) groups.

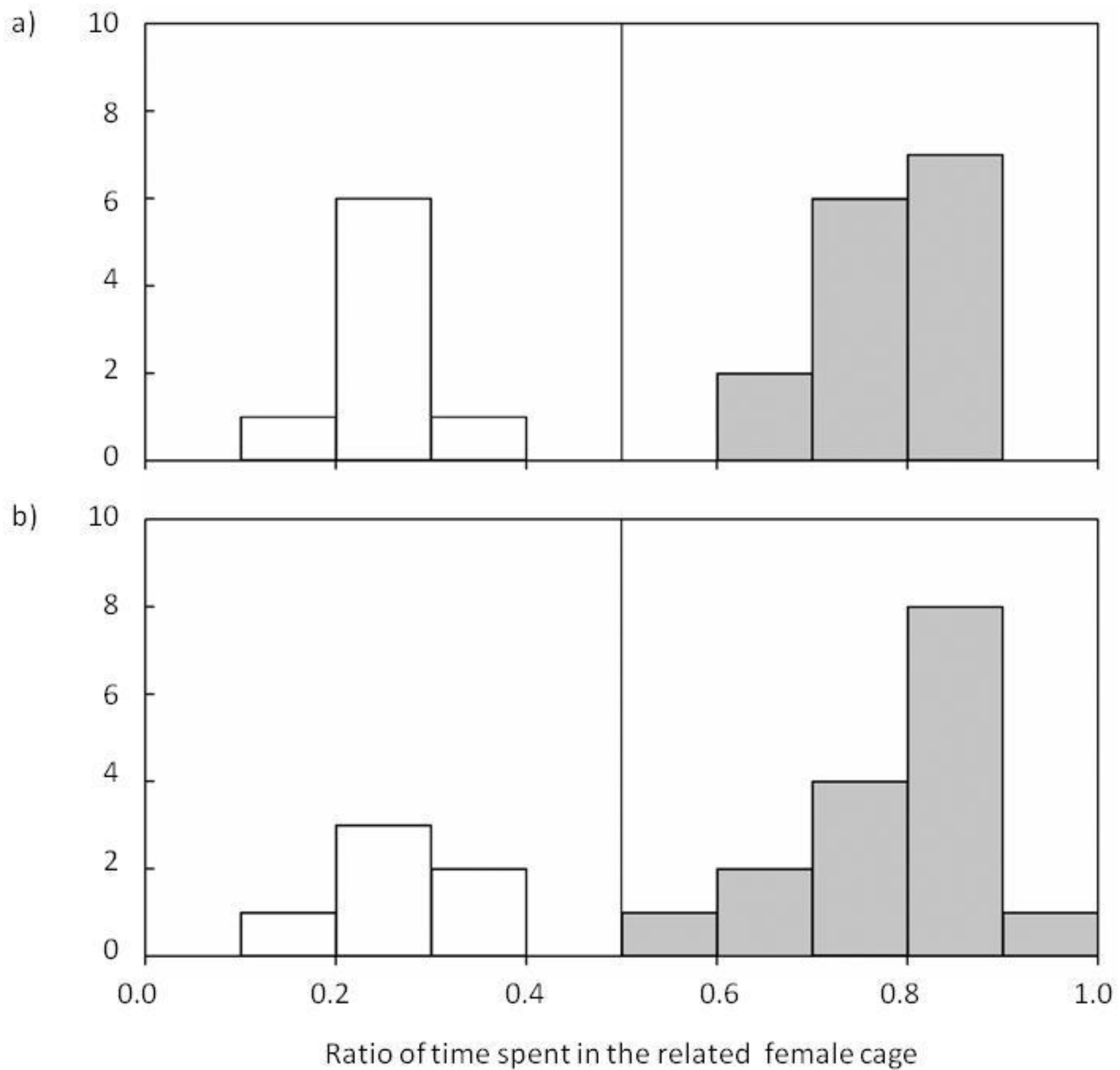


Figure 2.9: Ratio of time spent by subject females in the related stimulus female cage in the familiar and the unfamiliar groups.
a) Ratio of time in the familiar group (n = 23). b) Ratio of time in the unfamiliar group (n = 22). Ratio expressed as the amount of time spent in the related female cage as a proportion of the time spent in both stimulus female cages. A score above 0.50 indicated that a female chose the related side, below 0.50 indicated that females chose the unrelated side. Only one female across both familiarity groups had a score between 0.40 and 0.60.

House mice are nocturnal and so to investigate female response further the data was split into dark and light phases. In the dark phases females spent more time in the related stimulus female cage than the unrelated stimulus female cage ($z = -2.51$, $p = 0.01$, two-tailed; Figure 2.10). There was no effect of prior familiarity on subject female preference for related over unrelated stimuli females in the dark phase ($z = -1.07$, $p = 0.29$, two-tailed). During the dark phase there was a non-significant trend for females to spend more time in the related cage than the centre cage ($z = -1.86$, $p = 0.06$, two-tailed), but there was no difference in the amount of time females spent in the unrelated cage compared to the centre cage ($z = -0.32$, $p = 0.75$, two-tailed).

House mice are less active during the day and therefore female nest partner choice during the light phases may give a stronger indication of female nest partner preference. In the light phase females spent more time in the cage with the related female compared to the cage containing the unrelated female ($z = -2.78$, $p = 0.005$, two-tailed; Figure 2.10). Familiarity did not influence female preference for the related cage during the light phase ($z = -0.14$, $p = 0.90$, two-tailed; Figure 2.10). During the light phase females spent longer in the related cage than the centre cage ($z = -2.22$, $p = 0.02$, two-tailed; Figure 2.10), and there was a non-significant trend for females to spend more time in the centre cage than the unrelated cage ($z = -1.59$, $p = 0.12$, two-tailed; Figure 2.10). The proportion of time females spent in the related cage was compared in the light and dark phases. Females displayed a stronger preference for the cage containing the related stimulus female in the light phase compared to the dark phase ($z = -2.65$, $p = 0.007$, two-tailed; Figure 2.10).

The proportion of time females spent in the related cage was calculated for each of the consecutive dark-light phases to examine how female preference might change over the complete 72 hour period (Figure 2.11). Females did not differ in the proportion of time they spent in the related cage between the two complete dark phases ($z = -0.65$, $p = 0.52$, two-tailed; Figure 2.11). There was no difference in the proportion of time females spent in the related cage across all three complete light phases ($\chi^2_{(2)} = 2.71$, $p = 0.28$; Figure 2.11). This suggests female preference was consistent over time.

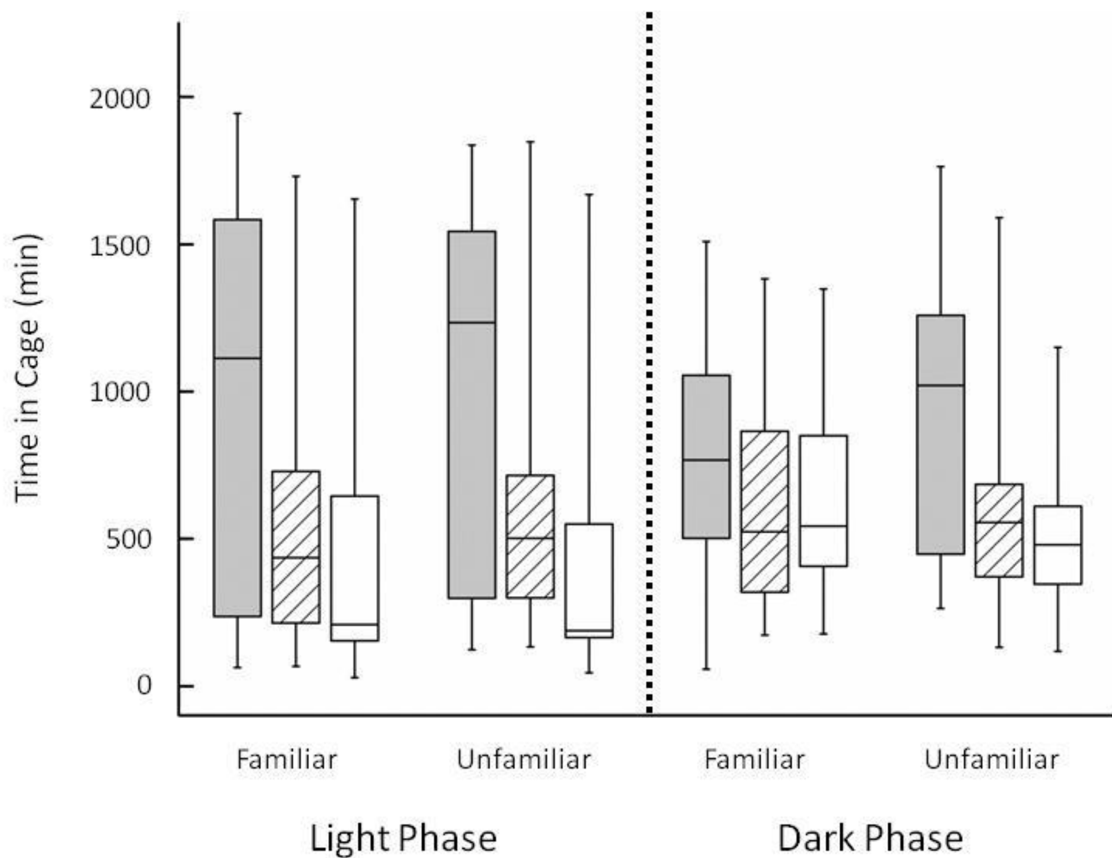


Figure 2.10: Female nest partner choice during the light and dark phases in the familiar and unfamiliar groups.
Time (minutes) subject females spent in the related female cage (grey boxes), the centre cage (hashed boxes), and the unrelated female cage (open boxes) in the familiar (n = 23) and unfamiliar (n = 22) groups.

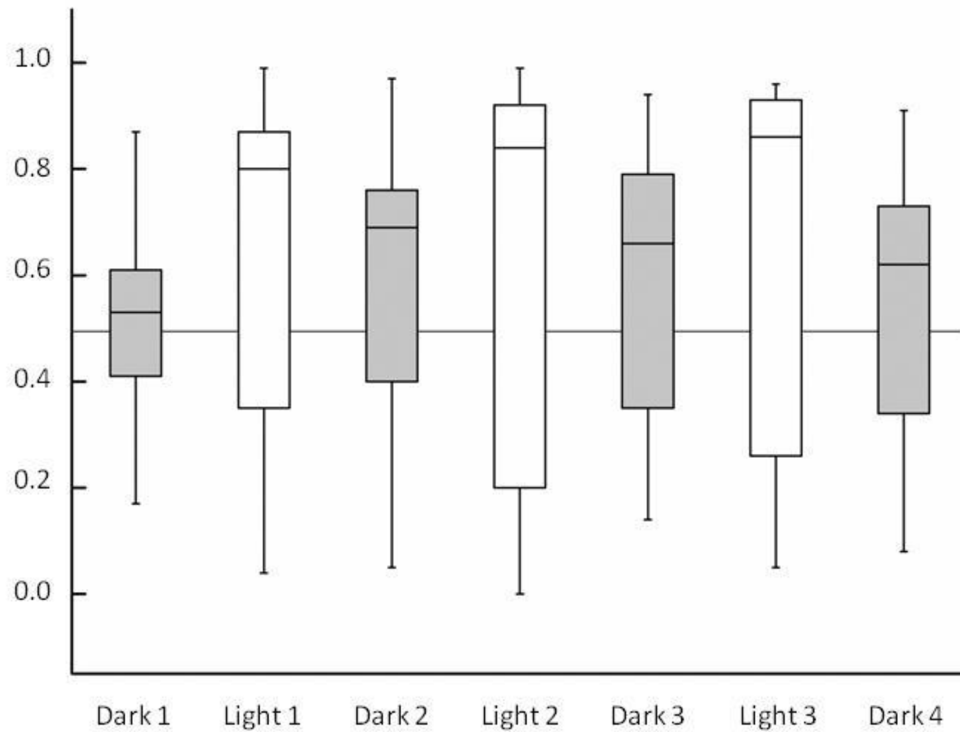


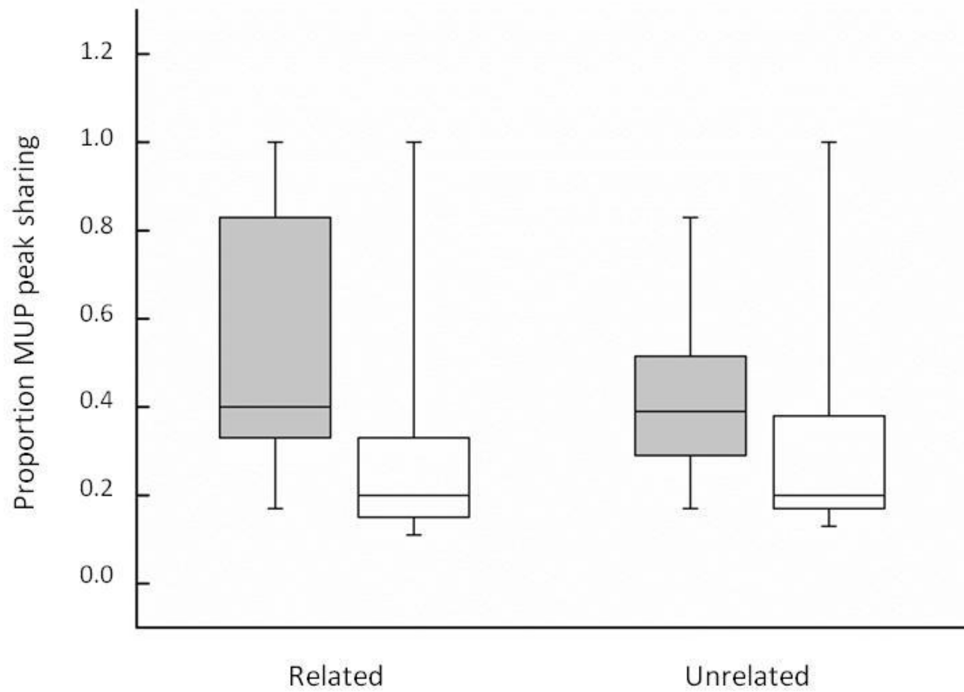
Figure 2.11: Ratio of time spent in the related stimulus female cage across the dark and light phases.
Ratio expressed as the amount of time spent in the related female cage as a proportion of the time spent in both stimulus female cages. Female preference was consistent over the five complete phases. The line at 0.5 represents the point at which females spend an equal amount of time in both stimulus female cages. Values above 0.5 represent more time spent in the related stimulus female cage, whereas values below 0.5 represent more time spent in the unrelated stimulus female cage.

2.4.3 *Major Urinary Proteins and Nest Partner Choice*

During the nesting partner choice test females were assigned a preference based on the ratio of time they spent in the related cage. Ratio was calculated as the amount of time subject females spent in the related female cage as a proportion of the total amount of time they spent in both stimulus female cages. If the ratio was above 0.50 then the subject female spent longer in the related stimulus female cage than in the unrelated stimulus female cage. If the ratio was below 0.50 then the subject female spent longer in the unrelated stimulus female cage than the related stimulus cage. MUP peak profiles were established for females and the degree of MUP peak sharing (both having the same peak regardless of its relative intensity) and MUP peak matching (both having the same peak of a similar relative intensity) between a subject female and both stimulus females was calculated for each triad of females.

Subjects that spent longer in the related stimulus female cage shared more MUP peaks with related stimulus females than with unrelated females ($z = -2.59$, $p = 0.008$, two-tailed; Figure 2.12). This was a non-significant trend when MUP peak matching was considered ($z = -1.91$, $p = 0.06$, two-tailed; Figure 2.12). When subject females spent longer in the unrelated stimulus female cage there was no significant difference in MUP peak sharing ($z = -1.38$, $p = 0.19$, two-tailed; Figure 2.16a) or MUP peak matching ($z = -1.07$, $p = 0.32$, two-tailed; Figure 2.16b) between subject females and related stimulus females and subject females and unrelated stimulus females.

a)



b)

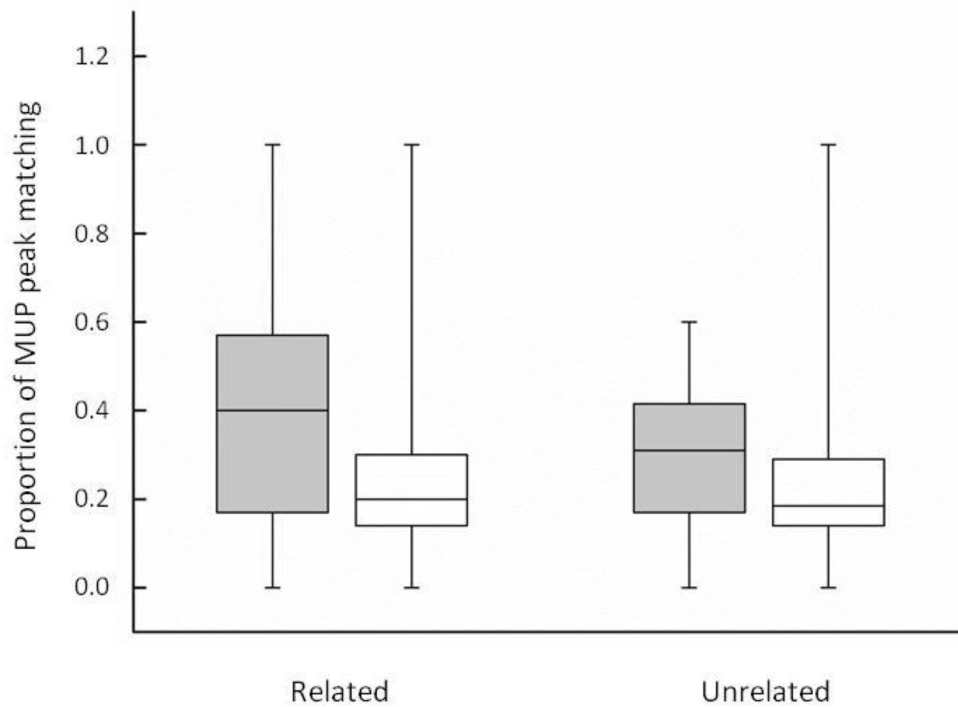


Figure 2.12: Proportion of MUP peak sharing and matching between subject and stimulus females when subject females spent longer in the related and unrelated stimulus female cages. MUP peak sharing (a) and matching (b) between subject and related stimulus female (grey boxes), and between subject female and unrelated stimulus female (open boxes) when subject females spent longer in the related (n = 26) and unrelated (n = 12) stimulus female cages.

2.4.4 Post-Encounter Response to Scent

The second scent discrimination and attraction assay investigated whether encountering the stimulus females in the nest partner choice assay influenced female response to urine marks (the results are presented below and are summarised in Table 2.2).

2.4.4.1 Post-Encounter Scent Discrimination

Across the full 20 minutes of the assay females did not differ in the amount of time they spent sniffing urine from either a related from or an unrelated female ($F_{(1,34)} = 0.06$, $p = 0.81$, data logarithmically transformed; Figure 2.12) and there was no difference in sniffing discrimination between the two familiarity groups ($F_{(1,34)} = 0.04$, $p = 0.84$, data logarithmically transformed; Figure 2.12).

To further investigate female response to urine from related and unrelated stimulus females the 20 minute assay was split into two 10 minute blocks. During the first 10 minutes the amount of time females spent sniffing the related and unrelated scents was not affected by familiarity group ($z = -0.51$, $p = 0.63$, two-tailed; Figure 2.12). As a result the data were combined. Subject females did not spend longer sniffing urine from an unrelated female compared to urine from a related female ($z = -1.18$, $p = 0.12$, one-tailed; Figure 2.12).

Over the second 10 minutes there was no difference between the familiarity groups in the amount of time subject females spent sniffing urine from either a related or an unrelated female ($z = -1.06$, $p = 0.32$, two-tailed; Figure 2.12). Since there was no difference between the familiarity groups the data sets were combined. Subject females did not spend longer sniffing urine from a related female compared to urine from an unrelated female ($z = -0.95$, $p = 0.18$, one-tailed; Figure 2.12).

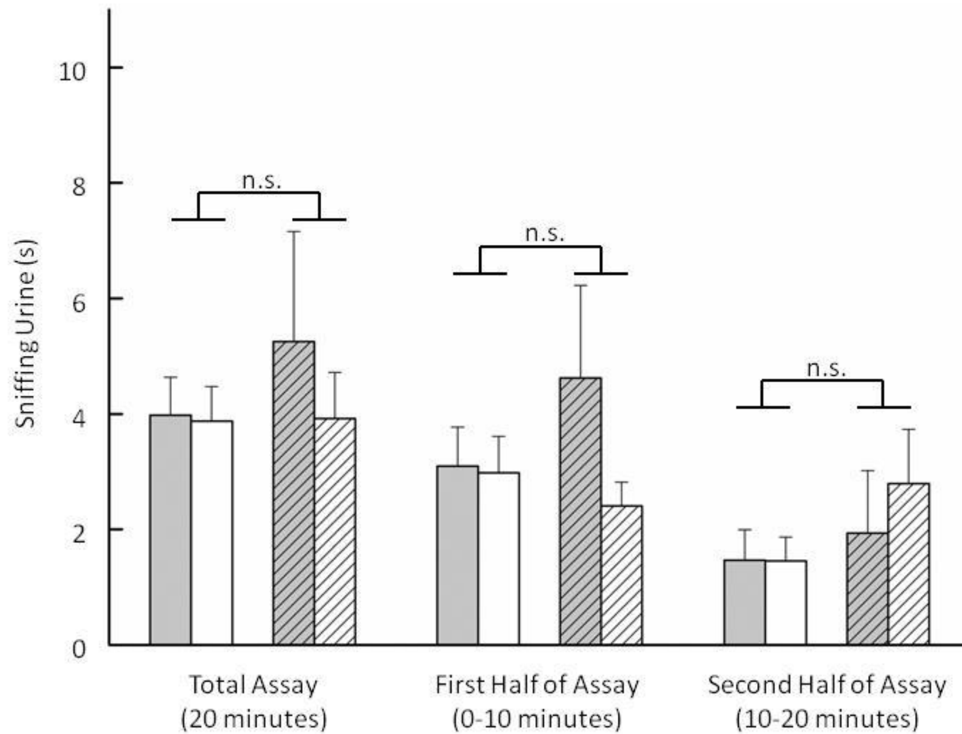


Figure 2.13: Post-encounter discrimination of urine from related and unrelated stimulus females in the familiar and unfamiliar groups.

Time (seconds, mean + standard error) spent by subject females sniffing urine from related (grey bars) and unrelated (white bars) stimulus females in the familiar (open bars) and unfamiliar (hashed bars) groups. There was no difference in female response between the familiarity groups across the total assay time (0-20 minutes, familiar n = 19, unfamiliar n = 17), the first half of the assay (0-10 minutes, familiar n = 17, unfamiliar n = 15), and the second half of the assay (10-20 minutes, familiar n = 10, unfamiliar n = 9).

2.4.4.2 *Post-Encounter Scent Attraction*

Female attraction towards urine from related or unrelated stimulus females was assessed first by measuring the amount of time females spent in close proximity to the stimulus urine. Over the full assay there was no influence of familiarity group on the amount of time females spent in proximity to urine from either a related or an unrelated female ($z = -0.90$, $p = 0.38$, two-tailed; Figure 2.13). As there was no difference between the familiarity groups the data for each group was combined. Females did not to spend longer in proximity to urine from either a related female or an unrelated female ($z = -1.21$, $p = 0.23$, two-tailed; Figure 2.13).

To further investigate female behaviour the full 20 minute assay was split into two blocks of 10 minutes. Over the first 10 minutes female proximity to related and unrelated urine was not influenced by familiarity group ($z = -1.53$, $p = 0.13$, two-tailed; Figure 2.13). As there was no difference between the familiarity groups the data were combined. There was no difference in the amount of time females spent in proximity to either the related or unrelated urine ($z = -0.39$, $p = 0.70$, two-tailed; Figure 2.13).

During the second half of the assay the amount of time subject females spent in close proximity to either urine mark was not affected by familiarity group ($z = -0.14$, $p = 0.90$, two-tailed; Figure 2.13). Data were therefore combined and no difference in the amount of time females spent in proximity to urine from either a related or an unrelated female was found ($z = -0.38$, $p = 0.72$, two-tailed; Figure 2.13).

A second measure of female attraction to urine was measured: the amount of time subject females spent on each side of the test cage. Over the full 20 minutes familiar and unfamiliar females did not differ in the proportion of time they spent on the related urine side ($F_{(1,34)} = 0.02$, $p = 0.90$; Figure 2.14), and there was no difference in the amount of time females spent on either the related urine side or the unrelated urine side ($F_{(1,34)} = 1.39$, $p = 0.25$; Figure 2.14).

During the first 10 minutes there was a suggestion that female preference for either side could be affected by familiarity ($z = -1.68$, $p = 0.10$, two-tailed; Figure 2.14). Data from the familiarity groups were combined. During the first half of the assay there was no difference in the amount of time females spent on either the related urine or unrelated urine side ($z = -0.90$, $p = 0.38$, two-tailed; Figure 2.14).

During the second half of the assay females in the familiar and unfamiliar groups did not differ in the proportion of time they spent on the related urine side (z = -0.90, p = 0.38, two-tailed; Figure 2.14). As there was no difference between the groups female attraction was analysed using data combined from both familiarity groups. There was no difference in the amount of time females spent on either the related or unrelated side during the second half of the assay (z = -0.94, p = 0.36, two-tailed; Figure 2.14).

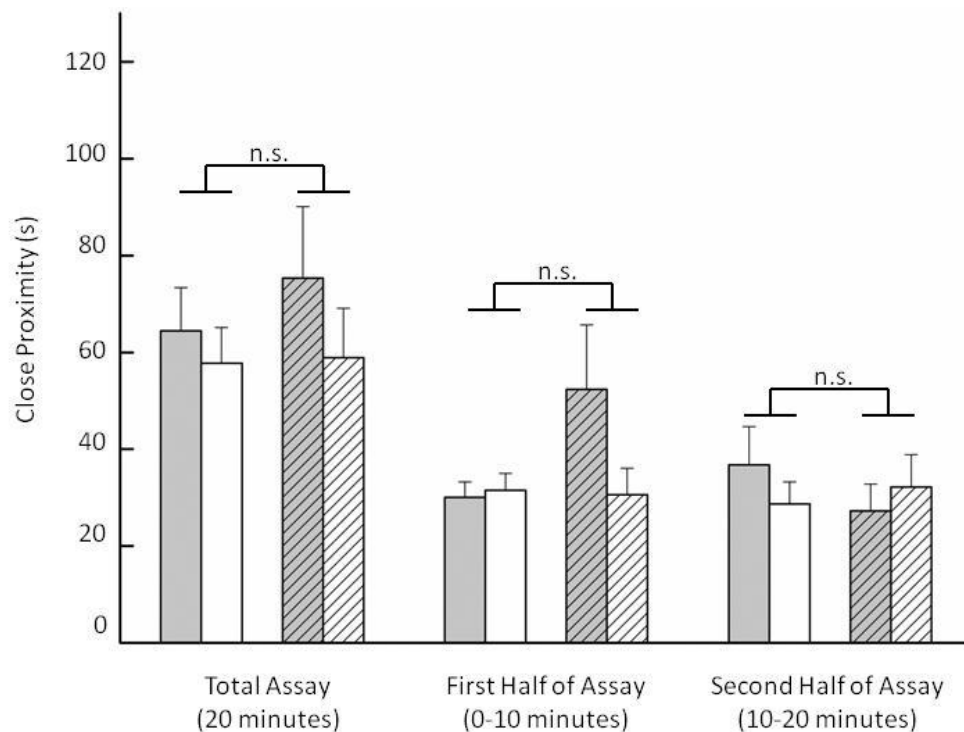


Figure 2.14: Post-encounter close proximity towards urine from related and unrelated stimulus females in the familiar and unfamiliar groups.
Time (seconds, mean + standard error) spent by subject females in close proximity to urine from related (grey bars) and unrelated (white bars) stimulus females in the familiar (open bars) and unfamiliar (hashed bars) groups. There was no difference in female response between the familiarity groups during the total assay time (0-20 minutes, familiar n = 19, unfamiliar n = 17), the first half of the assay (0-10 minutes, familiar n = 17, unfamiliar n = 15), or the second half of the assay (10-20 minutes, familiar n = 19, unfamiliar n = 17).

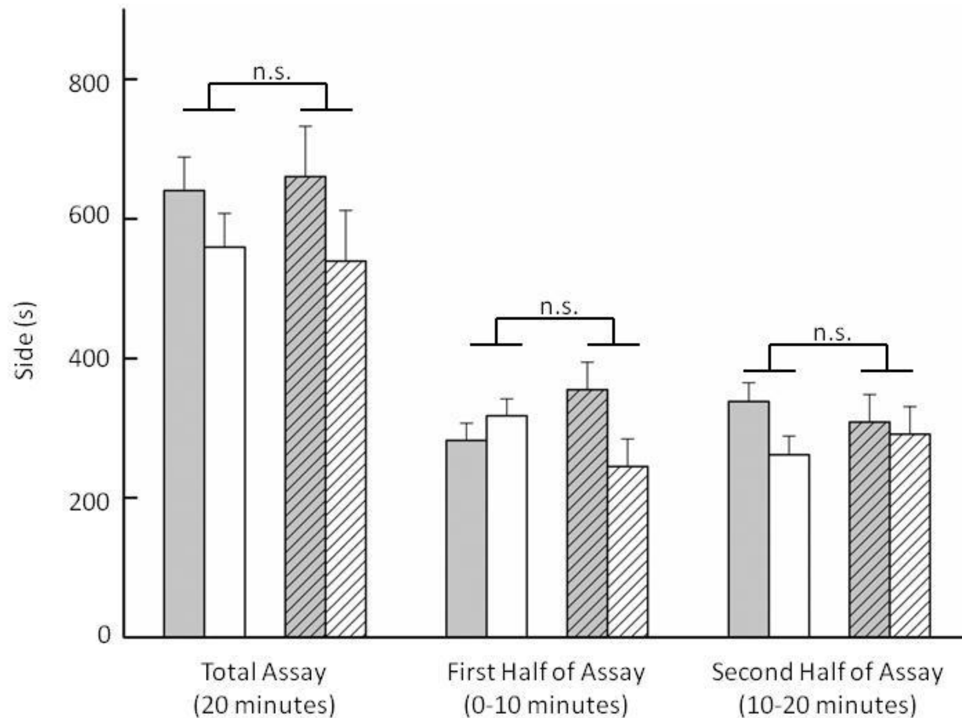


Figure 2.15: Post-encounter side attraction towards urine from related and unrelated stimulus females in the familiar and unfamiliar groups.
Time (seconds, mean + standard error) spent by subject females on the cage side containing urine from related (grey bars) and unrelated (white bars) stimulus females in the familiar (open bars) and unfamiliar (hashed bars) groups. There was no difference in female response towards either familiarity group during either the total assay time (0-20 minutes, familiar n = 19, unfamiliar n = 17), the first half of the assay (0-10 minutes, familiar n = 17, unfamiliar n = 15), or the second half of the assay (10-20 minutes, familiar n = 19, unfamiliar n = 17).

2.4.4.3 Post-Encounter Self-Grooming

To investigate the effect that proximity to female urine has on self-grooming, the amount of time females spent self-grooming either side of the test cage was compared. Over the full 20 minutes there was no difference between the familiarity groups in the amount of time females spent self-grooming on either the related urine side or the unrelated urine side ($z = -0.27$, $p = 0.80$, two-tailed; Figure 2.15). Since there was no difference between the groups, data for both groups were combined. Females showed no difference in the amount of self-grooming behaviour when on the related or unrelated urine side of the test cage ($z = -0.96$, $p = 0.35$, two-tailed; Figure 2.15).

To further investigate female behaviour the 20 minute assay was split into two 10 minute blocks. There was a non-significant trend for female self-grooming response to differ between the two familiarity groups ($z = -1.79$, $p = 0.08$, two-tailed; Figure 2.13). To investigate this trend further the familiarity groups were initially analysed separately. There was no difference in self-grooming in the familiar group ($z = -0.36$, $p = 0.74$, two-tailed; Figure 2.15), however females in the unfamiliar group preferred to self-groom in the side containing urine from a related female compared to the side containing urine from an unrelated female ($z = -1.99$, $p = 0.05$, two-tailed; Figure 2.15). When the familiarity groups were combined there was no significant difference in the amount of time females spent self-grooming between the related urine side and the unrelated urine side ($z = -1.61$, $p = 0.11$, two-tailed; Figure 2.15).

During the second half of the assay there was no difference in the amount of self-grooming females performed between the familiar and unfamiliar groups ($z = -1.57$, $p = 0.12$, two-tailed; Figure 2.15), and females showed no preference for self-grooming in either the related stimulus urine side or the unrelated stimulus urine side ($z = -0.31$, $p = 0.76$, two-tailed; Figure 2.15).

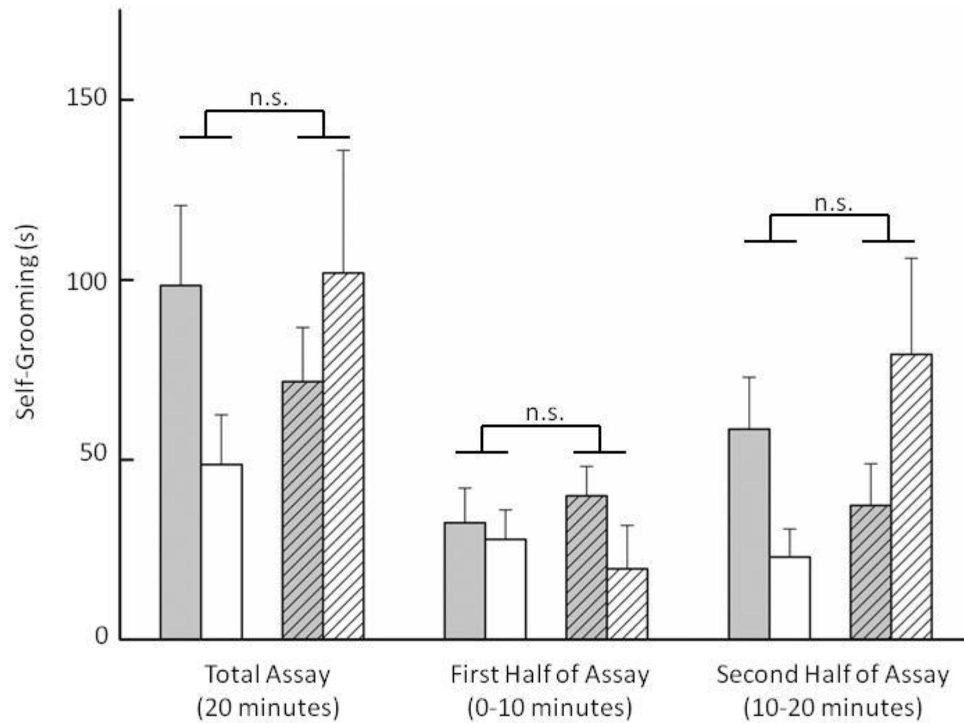


Figure 2.16: Post-encounter female self-grooming on the cage side containing urine from related and unrelated stimulus females in the familiar and unfamiliar groups.

Time (seconds, mean + standard error) spent by subject females self-grooming on the test cage side containing urine from related (grey bars) and unrelated (white bars) stimulus females in the familiar (open bars) and unfamiliar (hashed bars) groups. There was no difference in female response towards stimulus urine between the familiarity groups during either the total assay time (0-20 minutes, familiar n = 19, unfamiliar n = 17), the first half of the assay (0-10 minutes, familiar n = 17, unfamiliar n = 15), or the second half of the assay (10-20 minutes, familiar n = 19, unfamiliar n = 17).

Table 2.2: Summary of familiarity and kin recognition results.

Assay	Behaviour Measured						
		Total Assay (20 Minutes)		**First 10 Minutes**		**Second 10 Minutes**	
		Familiarity	**Relatedness**	**Familiarity**	**Relatedness**	**Familiarity**	**Relatedness**
Pre-Encounter Scent Discrimination and Attraction	Sniff Urine (discrimination)	**p = 0.02**[b]	Fam: **p = 0.02**[c] **(U>R)** Unf: **p < 0.01**[c] **(U>R)**	p = 0.96[a]	**p < 0.01**[a] **(U>R)**	p = 0.35[b]	**p = 0.05**[c] **(U>R)**
	Proximity Urine (attraction)	p = 0.22[b]	p = 0.58[c]	p = 0.50[a]	p = 0.63[a]	p = 0.19[b]	p = 0.71[c]
	Cage Side (attraction)	p = 0.49[b]	p = 0.38[c]	p = 0.96[a]	**p < 0.01**[a] **(R>U)**	p = 0.14[b]	p = 0.80[c]
	Self-Groom	p = 0.08[a]	p = 0.34[a]	p = 0.92[b]	p = 0.10[c]	p = 0.43[b]	p = 0.95[c]
		Total Assay (72 Hours)		**Dark Phases Only**		**Light Phases Only**	
		Familiarity	**Relatedness**	**Familiarity**	**Relatedness**	**Familiarity**	**Relatedness**
Nest Partner Choice	Time in stimulus cage	p = 0.66[b]	**p < 0.01**[c] **(R>U)**	p = 0.29[b]	**p = 0.01**[c] **(R>U)**	p = 0.90[b]	**p < 0.01**[c] **(R>U)**
		Subjects Chose Related		**Subjects Chose Unrelated**			
		Peak Sharing	**Peak Matching**	**Peak Sharing**	**Peak Matching**		
MUP peak profiles and Nest Choice		**p < 0.01**[c] **(R>U)**	p = 0.06[c] (r>u)	p = 0.19[c]	p = 0.32[c]		
		Total Assay (20 Minutes)		**First 10 Minutes**		**Second 10 Minutes**	
		Familiarity	**Relatedness**	**Familiarity**	**Relatedness**	**Familiarity**	**Relatedness**
Post-Encounter Scent Discrimination and Attraction	Sniff Urine (discrimination)	p = 0.84[a]	p = 0.81[a]	p = 0.63[b]	p = 0.12[c]	p = 0.32[b]	p = 0.18[c]
	Proximity Urine (attraction)	p = 0.38[b]	p = 0.23[c]	p = 0.13[b]	p = 0.70[c]	p = 0.90[b]	p = 0.72[c]
	Cage Side (attraction)	p = 0.90[a]	p = 0.25[a]	p = 0.10[b]	p = 0.38[c]	p = 0.38[b]	p = 0.36[c]
	Self-Groom	p = 0.80[b]	p = 0.35[c]	p = 0.08[b]	p = 0.11[c]	p = 0.12[b]	p = 0.76[c]

Difference in subject female response in the familiarity groups (Fam – Familiar, Unf – Unfamiliar) towards stimulus females of differing relatedness (R/r – Related, U/u – Unrelated). Statistical tests performed: a – repeated measures GLM, b – Mann-Whitney U test, c – Wilcoxon Signed Ranks test.

2.5 Discussion

2.5.1 Discriminating Full Sisters

Female house mice discriminated full sisters from unrelated females in both the scent and nest partner choice assays. In the scent assay females displayed an affiliation for the urine of full sisters, whilst in the nest partner choice assay females spent longer in the related stimulus female cage than in the unrelated stimulus female cage. These results suggest that female house mice may be using kin recognition to make behavioural decisions that could in turn affect lifetime reproductive output. These results are consistent with studies showing increased association of genetically similar females in the wild (Wilkinson & Baker, 1988) and sisters under experimental conditions (Manning *et al.*, 1992; Dobson *et al.*, 2000; Dobson & Baudoin, 2002). Subject females investigated urine from unrelated stimulus females more, but displayed an attraction towards urine from full sisters. Female nesting partner choice was consistent over time suggesting that the responses observed here are accurate representations of female behavioural decisions.

2.5.2 The Importance of Early Learning

In the majority of instances female response did not statistically differ between the familiar and unfamiliar groups. However, during the pre-encounter scent discrimination and attraction assay there was a difference between the familiarity groups in sniffing time across the full 20 minutes. Females displayed non-significant trends for increased self-grooming on the related urine side than the unrelated urine side in the unfamiliar group but not the familiar group. Females in both familiarity groups spent longer sniffing urine from an unrelated female than urine from a related female however this difference was larger in the unfamiliar group. Together these results suggest that whilst females in both familiarity groups were responding in a similar manner, the unfamiliar females may have responded more strongly. This is to be expected as the familiar females will already have been very familiar with the two urine samples they were presented with. Importantly there was no difference between the familiar and unfamiliar groups in the nesting partner choice assay. This shows that despite any differences in response to urine, when given the choice between a full sister and an unrelated female with limited interaction, the response of subject females is the same whether familiar or unfamiliar with both.

Females preferred to nest with their full sisters even when highly familiar with both stimulus females. This suggests that kin recognition is an important mechanism in female nesting

partner choice and that familiarity from weaning does not negate the effects of relatedness on recognition. Subject females were familiar with the unrelated stimulus females from weaning at 24 days, whereas they were familiar with the related stimulus females from birth. The initial period of rearing until weaning therefore appears important for establishing female kin relationships. This is suggestive of prior association, with the learning phase occurring before or at weaning.

A number of rodent species have periods of learning during development. Guinea-pigs (*Cava porcellus*) show a greater responsiveness to odours that they were exposed to during the first 6 days after birth than odours they were exposed to from 7 days onwards (Carter & Marr, 1970). Social learning in prairie voles (*M. ochrogaster*) appears to develop around the time of weaning, allowing for inbreeding avoidance in later life (Gavish *et al.*, 1984). Odours introduced into the nests of Belding's ground squirrels (*S. beldingi*) before juvenile emergency appear to be incorporated into juvenile recognition templates (Mateo, 2009). This form of behavioural imprinting during development allows for relatives to be identified in later life. It would be interesting to further investigate the timing of kin learning in female house mice, perhaps by cross-fostering pups at different periods of development on to laboratory females (to reduce the likelihood of pup rejection by the foster mother).

2.5.3 Discriminating Unfamiliar Full Sisters

By itself the fact that females recognised and showed a preference for full sisters over unrelated females when familiar with both is suggestive of a prior association kin recognition mechanism. However, females were also able to recognise and nested with unfamiliar full sisters over unrelated females, suggestive of a genetic matching mechanism. Phenotype matching requires that a recognition template is learnt from known relatives. For house mice there are a number of potential options available. An individual could learn a recognition template from its mother, father, siblings, self, or a combination of phenotypes present during the learning phase. An additional source could be the nest environment itself or some form of maternal labelling may be happening through maternal licking or milk intake such as has been found in goats (*Capra aegagrus hircus*) (Gubernick, 1980). Results from an inbreeding avoidance study suggest a phenotype matching mechanism using match-to-self (Sherborne *et al.*, 2007). The comparison between degree of MUP peak sharing and nest partner choice presented here (Section 2.4.3) also supports the idea that female house mice may be using a match-to-self phenotype matching kin recognition mechanism.

As has been argued by a number of authors, prior association and phenotype matching are not mutually exclusive (e.g. Waldman, 1987; Porter, 1988; Barnard, 1999). The results presented here support the idea that female house mice are able to use phenotype matching to recognise full sisters, but this not mean that females are not also using prior association when presented with familiar full sisters.

2.5.4 Genetic Markers Present in Urine

Females were able to identify and showed a preference for full sisters from urine marks alone. This suggests that genetic markers indicating kinship are present in urine. Both MHC and MUPs have been suggested as possible markers for kin recognition. Here nest partner choice was compared with MUP peak sharing and matching. Females that spent longer in the related stimulus female cage shared more MUP peaks with the related female than with the unrelated female. This finding was not significant when degree of peak matching was compared. Where females spent longer in the unrelated stimulus female cage, there was no significant difference in MUP sharing between the subject-related, and subject-unrelated females. These results suggest that MUPs may be involved in kin recognition between female house mice and agree with previous work in wild house mice that looked at inbreeding avoidance (Sherborne *et al.*, 2007). It is important to remember however that MHC was not measured in this experiment, and this experiment only correlated observed behaviour with levels of MUP peak similarity and did not directly test the role of MUPs in house mouse kin recognition. Both MHC and MUP may therefore be involved in kin recognition in female house mice.

The comparison of MUP peaks with the nest partner choice assay was stronger when females shared rather than matched MUP peaks. This indicates that having a particular MUP peak may be more important for kin recognition than the strength of the MUP peak. When subject females spent the majority of their time in the unrelated cage there was no difference in MUP peak sharing or matching between a subject and the two stimulus females. It could be that a minimum number of peaks need to be shared between two females before recognition can occur, and in those instances where females chose the unrelated side, either the threshold was not reached, or the unrelated female also happened to share a similar number of peaks.

2.5.5 Making a Choice

In the nesting partner choice assay, subject females tended to spend the majority of their time in one of the stimulus female cages. This suggests that female house mice may have been

making a choice of which cage to nest in and then sticking with that choice. Approximately two-thirds of females spent longer in the related stimulus female cage, whilst the remaining females spent longer in the unrelated stimulus cage. There are a number of possible reasons for why some females did not choose to nest with their full sister. It is possible that not all relatives are recognised. A match to self phenotype matching mechanism would mean that only full sisters that matched or shared recognition markers would be identified. Whilst full siblings share half their genome, if both parents are heterozygous then only a quarter of full siblings would match completely at an individual recognition locus. A further half of full siblings would share one allele at a single recognition locus. The remaining quarter of full siblings would share no alleles at a single locus. If a single recognition locus is used then a significant proportion of full sisters may not be recognised.

It is also possible that the unrelated stimulus females were mistakenly identified as related to the subject females. An ideal genetic kin recognition system should allow for recognition of individuals that share genes that are identical by descent, however it is more likely that kin will be recognised on the basis of genes that are identical by state (Paterson & Hurst, 2009). Within a population it is possible that unrelated individuals will share the same polymorphic markers. This may cause confusion during identification and unrelated individuals could be identified as being related. When subject females spent longer in the unrelated stimulus female cage, no difference in MUP peak sharing or matching was found between the subject and the two stimulus females. As the MUP peak profiles of the related stimulus females were not more similar to that of the subject females in comparison, this may provide a reason why these subject females spent more time in the unrelated stimulus female cage.

Finally, other differences between the females could have overridden kin recognition decisions. Female association decisions are likely to be influenced by a range of other factors and it could be that other differences between the stimulus females were larger enough to take priority over differences in relatedness. Both weight and age were controlled for as much as possible but other factors such as dominance, stage of oestrus cycle or hormonal state were not controlled for and could have influenced female nesting partner choice.

2.5.6 Assay Design

The strongest results from the scent discrimination and attraction assay came during the first 10 minutes of observation. This is consistent with other similar studies looking at response to scent in the house mouse (e.g. Ramm *et al.*, 2008). These other studies however have mostly

looked at male scent discrimination or at female-male discrimination. Female responses to females tend to be subtler than male-male or female-male responses, and so the test was extended to 20 minutes to ensure that all important behaviours were observed. Female scent discrimination appeared to disappear in the post-encounter scent assay. Female house mice need initially to contact male urine to learn it, but once they are familiar with the scent they are able to identify it from the volatile component alone (Ramm *et al.*, 2008). Female house mice may also behave similarly towards female urine and it is possible that females were still able to recognise the related urine. However, the scent discrimination and attraction assay was unable to measure this recognition of volatile cues. Additionally, familiarity with the scent discrimination and preference test could explain why females did not respond to the urine samples in the same manner as they did in the pre-encounter scent assay.

Whilst the design of the nesting partner assay did not mean that females were actually observed nesting together, the fact that females spent the majority of their time in the chosen cage suggest that it is likely they did nest there. This was supported by the evidence that the proportion of time females spent in the related stimulus female cage was higher during the light phase, as house mice are less active during the light phase (Mackintosh, 1981). Additionally, when females were removed from the nest partner choice cages, bedding material was often found to have been moved from all the cages to one of the end cages. This anecdotal evidence combined with the amount of time females spent in their chosen cage strongly suggests that females did indeed nest in the cage in which they spent the majority of their time.

Female nesting partner choice was consistent over the 72 hour period. During the initial dark phase after subject females had just entered the cages, females tended to move regularly between all three cages, presumably to explore the cages and interact with the two stimulus females. However, by the time that the first light phase began it appears that females had made their choice and for the remainder of the assay spent the majority of their time in the chosen cage. As predicted for a nocturnal species, female house mice were more active during the dark phase and less active during the light phase, as suggested by the increased proportion of time females spent in their chosen cage during the light phase.

2.5.7 Conclusions

The results of this study (summarised in Table 2.2) suggest that female house mice are able to recognise their full sisters and do choose to associate with them when given a choice between

a related and unrelated female. Both the discrimination of relatives, and an attraction towards full sisters was demonstrated in response to urine alone. Females made these decisions whether familiar or unfamiliar with the stimulus females. When nesting partner choice was compared to similarity in MUP peak patterns between the subject females and the related and unrelated females, the proportion of MUP peaks shared was greater between subject and related females than between subject and unrelated females when the subject had chosen the related side. These results suggest two avenues of questioning: which individuals are used for the creation of a kin recognition template during development (e.g. self, mother); and what markers are being used that allow for identification of relatives through urine alone (e.g. MUPs, MHC)? These questions will be addressed in Chapters 3 and 4.

3 Phenotype Matching and Template Origin in Female House Mice

3.1 Abstract

The phenotype matching kin recognition mechanism allows animals to recognise unfamiliar relatives by comparing the phenotype of a novel individual against a learned phenotypic recognition template. If the template and the phenotype of the novel individual are similar then the individual is treated as a relative, but if there is a difference then the novel individual is treated as being unrelated. There are a number of potential options for where this recognition template may be learned from, however the two most reliable options are from self and mother.

To investigate the origin of phenotype recognition templates, female house mice were tested for their ability to discriminate half-sisters from unrelated females, and whether this discrimination depended on relatedness through the maternal and/or paternal lineage. Using a maternally learnt template would enable individuals to recognise previously unfamiliar maternal relatives, but would mean that paternal relatives may not be identified. Alternatively, using a self template may enable an equal proportion of maternal and paternal relatives to be recognised.

When presented with a half-sister and an unrelated female, female house mice preferentially associated with maternal half-sisters but displayed no preference for paternal half-sisters. This suggests that female house mice may be using a match-to-maternal template kin recognition mechanism for recognising female relatives, and together with the results presented in Chapter 6 suggests that females may be using two different mechanisms for kin recognition depending on the context of recognition. Females were able to discriminate between urine from half-sisters and urine from unrelated females, suggesting that genetic markers of relatedness are present in the urine of half-sisters, however there was no difference between the maternal and paternal groups in response to urine. Females also did not differ in their behaviour during interactions with either maternal or paternal half-sisters, however females did display more competitive behaviour towards unrelated females than towards related females, as predicted by kin selection theory.

3.2 Introduction

The results of the previous chapter suggested that female house mice use a phenotype matching kin recognition mechanism when making nest partner choice decisions. The kin recognition mechanism of phenotype matching requires individuals to acquire a recognition template against which to compare newly encountered individuals. This template may be learnt during early development and could originate from a number of sources. The aim of this chapter is to investigate template formation; specifically do individuals use their own phenotype as a template, or is a template learned from a relative, a combination of relatives, or from the entire nest environment?

3.2.1 Phenotypic Template Options

In the phenotype matching kin recognition mechanism individuals learn a phenotypic template from self, a known relative(s), or from the rearing environment itself (e.g. a nest cue). On encountering a novel individual this template is then compared to the phenotype of the novel individual. If the template and the individual's phenotype are suitably similar then the novel individual is treated as being related, otherwise it is treated as unrelated (Blaustein, 1983; Holmes & Sherman, 1983; Tang-Martinez, 2001). The main advantage of this mechanism is that previously unfamiliar relatives can be recognised (Mateo, 2004). This allows for a greater number of relatives to be recognised than the prior association mechanism, and may even allow individuals to differentially respond according to the degree of relatedness.

The possible options for recognition templates vary from species to species. In a typical species there are a number of options: self, mother, father, siblings, a combination of the phenotypes of several relatives or a combined template from the nest environment. There are two main requirements for the location from which a template is learnt: it should be reliably related to the individual learning and reliably present at the time of learning. Multiple mating means that often the dominant or breeding male present in a rearing environment may not actually be the father of all or even any of the young. Learning a recognition template from this male may not therefore result in the recognition of own kin. Alternatively, relatives may be absent from the rearing environment; in many species males provide little parental care which presents problems for individuals trying to learn a template father's template. Multiple mating also means that siblings in the nest may not be full siblings. Additionally, in communally breeding species breeding nest partners may not always be related. In these

cases learning templates from siblings or other young in the rearing environment means that the template learned may not be an accurate representation of a related template. The two most reliable relative sources are therefore self and mother in many species (particularly mammalian species as mothers provide milk for offspring). In mammals an individual's mother is reliably related to the learning individual and a reliable presence during development, both at pre and post-natal stages. However, an individual is more related to itself than to its mother and self is an even more reliable presence. In addition, using self may not require a long-term memory formation as the template memory could be refreshed regularly.

3.2.2 Genetic Inheritance and Phenotype Matching Templates

An effective kin recognition mechanism should have a high rate of success for recognising relatives, and a low error rate of falsely identifying unrelated individuals as relatives (Paterson & Hurst, 2009). Offspring inherit 50 % of their genome from their mother and 50 % from their father. Therefore at every polymorphic locus an individual will share one allele with their mother. All of a female's offspring will have inherited one of the mother's two alleles at a particular polymorphic locus. Individuals could learn the maternal phenotype resulting from the two alleles at a single recognition locus and then partially match that learned template against newly encountered individuals. All full siblings and maternal half-siblings would have one of the two maternal alleles at the recognition locus and so could be identified. However other unrelated individuals may also happen to have one of the mother's alleles and so could be falsely identified as relatives.

Full siblings have a coefficient of relatedness of 0.5; they inherit 50 % of their genome from their mother and 50 % from their father. However, at a single polymorphic locus full siblings can share none, one or two alleles. Genetic inheritance means that at a single polymorphic locus a quarter of full siblings will share two alleles, half will share one allele, and a quarter will share no alleles. Close relatives are more likely to share both recognition alleles at an individual locus so using a match to self mechanism might reduce the error rate of falsely identifying unrelated individuals. However, using a match-to-self mechanism based on a single recognition locus would mean that a quarter of unfamiliar full siblings mechanism may fail to recognise one another if they share no alleles at that specific recognition locus.

Paterson & Hurst (2009) modelled the success rates of using self and mother as recognition templates and the number of polymorphic loci contributing to the recognition template. Using

a Bayesian model the authors showed that for a single recognition locus a match-to-self mechanism was more effective at assessing relatedness than a partial match-to-maternal mechanism. The match-to-self had a lower error rate for falsely identifying unrelated individuals than the partial match-to-maternal mechanism. However in the match-to-self mechanism for a single recognition locus, a relatively small proportion of relatives were identified (Paterson & Hurst, 2009). By increasing the number of independent recognition loci used the authors were unable to increase the effectiveness of match-to-self recognition without also increasing the error rate of unrelated individuals falsely categorised as related (Paterson & Hurst, 2009). In the partial match-to-maternal model a single recognition allele was not as effective as a high number of unrelated individuals were falsely identified. However increasing the number of recognition alleles reduced the number of false inclusions making a partial match-to-maternal at multiple recognition loci an effective kin recognition mechanism (Paterson & Hurst, 2009). These results suggest that a match-to-self mechanism may be more effective for recognising kin if only a single recognition locus is used, but that a partial match-to-maternal mechanism is more effective if multiple recognition loci are used as fewer false identifications are made. However the ability to use partial matching across multiple independent loci may be difficult and so the match-to-self mechanism could therefore be favoured.

Examples of both match-to-maternal and match-to-self kin recognition mechanisms are found in a number of species. Tadpoles of the American toad (*Bufo americanus*) associate with full siblings and maternal half-siblings but show no affiliation towards paternal half-siblings (Waldman, 1991). This suggests that they use a recognition template based on the maternal phenotype. The larvae of the star ascidian tunicate (*Botryllus schlosseri*) settle in close proximity to siblings that match themselves at a specific polymorphic recognition locus (Grosberg & Quinn, 1986). Male peacocks (*Pavo cristatus*) establish leks in close proximity to male relatives even when they have no prior experience of any relatives (Petrie *et al.*, 1999). This suggests that male peacocks are using themselves as a recognition template to identify male relatives.

3.2.3 House Mice and Template Origin Options

Female wild house mice nest (and often nurse) communally (Sayler & Salmon, 1971). In addition to themselves, their immediate siblings and their mother, developing pups are likely to experience their mother's nestmate(s) (who may or may not be a related) and any of the

nestmate's pups. Other mice may enter the nest environment, including the dominant male in whose territory the nest lies (which may or may not be their father), and other adult, sub-adult and juvenile males and females. Given the range of potentially unrelated individuals that may enter the nest, mother and self are likely to be the most reliable relatives from which to learn a phenotypic template.

Studies of laboratory and house mice for match-to-self and match-to-maternal phenotype matching mechanisms is mixed. Cross-fostered adult house mice spend more time investigating the odours of conspecifics that are genetically more similar to themselves than those that are more distant (Heth *et al.*, 2003). In a study of inbreeding avoidance in free-breeding house mice, female house mice avoided mating with males that shared both MUP haplotypes with themselves, and found no evidence for a match-to-maternal MUP type (Sherborne *et al.*, 2007). The results found in Chapter 2 also suggest a match-to-self mechanism using MUP sharing, as subject females spent longer in the cages of related stimulus females if they shared more MUP peaks with the related stimulus female compared to with the unrelated stimulus female. However in that experiment maternal MUP patterns were not investigated.

In a study using cross-fostered female semi-wild mice (wild crossed with laboratory strains to produce mice with controlled MHC type) females preferred males with different homozygous MHC types from the foster-parents that reared them (Penn & Potts, 1998a). Similar studies using cross-fostered male laboratory mice have demonstrated an avoidance of females that have the same MHC type as the foster-parents that reared them (Beauchamp *et al.*, 1988; Yamazaki *et al.*, 1988). Yamazaki *et al* (2000) demonstrated laboratory mouse pup preference for odours of the MHC-type on which they were reared, however this preference was not wholly reversed by cross-fostering, suggesting that perhaps both self and maternal cues are important. Manning *et al.* (1992) also suggest that both self and maternal cues may be important as females mice resulting from crosses between laboratory strains and wild-caught house mice prefer nest partners that share the same MHC type as themselves, and the same MHC type as their parents, suggesting both match-to-self and match-to-maternal mechanisms.

3.2.4 Chapter Aim

The aim of this chapter is to investigate the origin of phenotypic recognition templates in female house mice. This was addressed by asking the following questions:

i) Can female house mice discriminate urine from a half-sister from urine from an unrelated female, and do they show any attraction towards either?

ii) Do female house mice preferentially associate with half-sisters over unrelated females?

iii) Do female house mice show kin discriminative behaviour when allowed to interact with half-sisters compared to with unrelated females?

iv) Does kin discriminative behaviour for half-sisters compared to unrelated females depend on maternal and/or paternal lineage?

v) Does urine from half-sisters appear more similar to females than the urine from unrelated females?

Female house mice were tested for their ability to discriminate half-sisters from unrelated females, and whether this discrimination depended on relatedness through the maternal and/or paternal lineage. A match-to-self mechanism matches own phenotype against that of encountered novel individuals. An individual's own phenotype is a product of the two alleles, one inherited from the mother and one from the father. The ability to recognise maternal and paternal relatives should therefore be equal. A match-to-maternal mechanism matches a maternally learnt template against that of encountered novel individuals. All maternally-descended relatives will share one allele at every locus with the mother, however all paternally-descended relatives may not share any alleles at any locus. The ability to recognise maternal and paternal relatives should therefore be different if a match-to-maternal mechanism is used, with maternal relatives being discriminated but not paternal relatives. Female discrimination was assessed using a scent discrimination and attraction assay, a nest partner choice assay and a female-female interaction assay. Additionally, the perceived similarity between urine from half-sisters was assessed using an odour-genes covariance assay.

3.3 Methods

Consecutive litters of unfamiliar maternal and paternal half-sisters were bred and females tested for their ability to recognise half-sisters based on maternal or paternal relatedness. A match-to-maternal phenotype mechanism may enable maternal but not paternal half-sisters to be discriminate, whilst equal discrimination to both maternal and paternal half-sisters may be shown using a match-to-self mechanism.

3.3.1 Animal Housing and Handling

Subjects and stimuli were captive-bred adult females from an outbred colony of house mice. The colony was established using individuals captured from populations in the North West of England and outbreeding was maintained with regular introductions of wild-caught mice. Animal housing and handing methods were the same as detailed previously (see Section 2.3.1).

3.3.2 Breeding Programme

3.3.2.1 Outline

Two consecutive rounds of breeding were used to produce mice that were unfamiliar maternal and paternal half-siblings (n = 14 breeding pairs; Figure 3.1). A smaller breeding programme occurred in parallel to the half-sibling breeding programme to produce unfamiliar full siblings for use in Chapters 5 and 6, as well as for additional subject females in the odour-genes covariance assay (n = 8 breeding pairs; Figure 3.1). Full sisters were also used (together with half-sisters) as unrelated stimulus females to expand the pool of available unrelated stimulus females for each subject female. After the first round of litters were weaned pairs of breeding males and females were established with new partners to produce second litters that were unfamiliar maternal and paternal half-siblings of the first litters, or breeding pairs were re-established to produce second litters that were unfamiliar full sisters of the first litters (Figure 3.1). Breeding females were only ever paired with unrelated breeding males. In second round of breeding for half-siblings, no mice were ever paired with partners that were related to their partner in the first round of breeding. This was to ensure that all half-siblings were related through their maternal or paternal side but were unrelated through the other parental side. Individuals were considered unrelated to one another if they did not share any great grandparents.

3.3.2.2 Breeding Protocol

All mice used for breeding were over three months old and weighed above 13 g. All dams were injected beneath the skin at the nape of the neck with radio frequency identification (RFID) tags a minimum of two weeks before females were paired with males. On three occasions over the two weeks prior to establishing breeding pairs for the first round of litters breeding females and males were primed with soiled bedding from opposite and same-sex conspecifics (methods as described in Section 6.3.2.1). Priming served to give mice experience of opposite-sex odours, and ensured normal oestrus cycling in females (Marsden & Bronson, 1964; Cheetham *et al.*, 2007). The final priming session was timed so that dams would be in oestrus when paired with males.

On the day of pairing both dams and sires were weighed to determine a baseline body mass. Breeding pairs were established in MB1 cages (45 x 28 x 13 cm, North Kent Plastics, UK). Each breeding cage was lined with Corn Cob Absorb 10 / 14 substrate, and contained paper - wool bedding material (Shredded Nesting, International Product Supplier Limited, London, UK), a red plastic mouse house (Techniplast, NJ, USA), a lid-suspended nesting box (MPlex, Otto Environmental, WI, USA), and two cardboard tubes (11 cm long, 5 cm diameter). A cage of two mice would normally contain one cardboard tube and a mouse house. Extra environmental enrichment was provided here to ensure that the previously unfamiliar females and males were able to avoid each other if required. Food (Lab Diet 5002, International Product Supplies Limited, London, UK) and water were provided *ad libitum*. Cages were monitored for 90 minutes after introduction for any excessive levels of aggression between the pairs. Bouts of intense aggression lasting over ten seconds were interrupted by placing a hand over the cage. After this initial observation period, several checks were made over the following 24 hours. Pairs were established in the morning to enable checks to be performed through the day.

Breeding pairs were housed in a quiet, designated breeding room and checks performed daily. Female house mouse gestation is approximately 21 days (Berry, 1981). On days 17, 21 and 25 after pairing, mice were weighed and compared to previous baseline measurements. If a female weighed 5 g or more than her initial weight she was recorded as pregnant. Females were also observed in clear acrylic handling tubes (18 cm long, 5 cm diameter, one open end and one end closed with aluminium mesh of 0.5 x 0.5 cm) for signs of pregnancy. Body mass difference was backed up by observations – pregnant females appeared larger and more

rounded, and teats were clearly visible. When females were observed to be pregnant, males were removed from the cage (to prevent pup exposure to own sire) and housed in individual cages (M3, 48 x 15 x 13 cm, North Kent Plastics, UK). Females were then transferred to a clean cage to prevent pups from encountering own sire odours in the cage environment (although any male odours transferred on to the females would still be present). Female cages were checked daily for litters.

On the day of birth the number of pups was recorded and both the breeding female and the combined litter were weighed. Cages were checked daily until weaning at 24 days. At weaning breeding females and combined litters were weighed, the total number of pups counted and pups were sexed. Female pups were then housed in MB1 cages in sister groups of between two and four females, and male pups were housed singly in M3 cages.

After all litters were weaned, breeding females and males were primed with same and opposite-sex soiled bedding. Three days following priming, when females were expected to be in oestrus, baseline weights were measured and pairs were introduced (for half-sisters) or re-introduced (for full sisters) in fresh breeding cages. Breeding protocol for observing pregnancy, breeding and weaning was as before.

3.3.2.3 Breeding Programme Results

In the first breeding round 21 of 22 females produced litters, 13 females assigned to the half-sibling group and all eight females assigned to the full sibling group (Table 3.1). Two pups died or were killed as a result of infanticide in the half-sibling group between birth and weaning. Mean time between dam and sire pairing and the birth of litters was 22.0 days in both the full sibling and half-sibling groups, with a range between 19 and 25 days.

In the second breeding round, 22 out of 22 dams produced litters, 14 in the half-sibling group and eight in the full sibling group (Table 3.1). Six pups died between birth and weaning in the half-sibling group. The dams that lost pups from their first litters kept all pups in the second litters, suggesting that pup death was not due to infanticidal dams. Mean time between dam and sire (re)pairing and the birth of second litters was 22.7 days in the half-sibling group and 20.6 days in the full sibling group, with a range between 19 and 32 days.

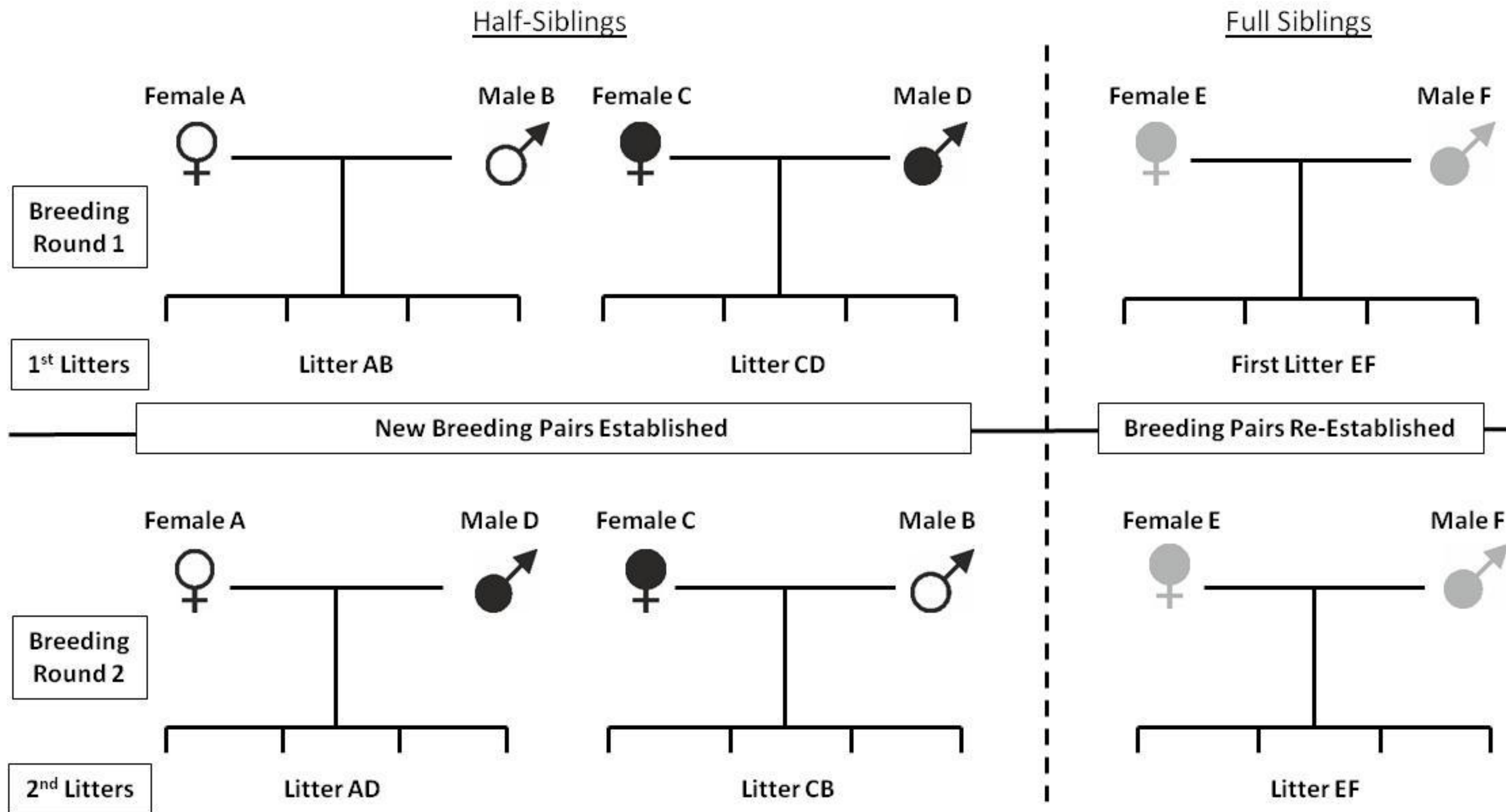


Figure 3.1: Breeding programme for half and full siblings.
Males and females paired for the first litters. When females appeared pregnant males were removed from breeding cages. After the first litters were weaned, paired of males and females were either re-established to produce second litters that were full siblings of first litters (grey symbols), or new pairs were established to produce second litters that were maternal and paternal half-siblings of first litters (open and black symbols).

Table 3.1: Breeding success of first and second breeding sessions for full and half-siblings.

		First Litter	Second Litter
Total number of females	Half-Siblings	37	54
	Full Siblings	24	34
Total number of males	Half-Siblings	38	50
	Full Siblings	25	30
Number of females per litter (mean ± standard error)	Half-Siblings	2.6 ± 0.5	3.9 ± 0.3
	Full Siblings	3.0 ± 0.4	4.3 ± 0.6
Number of males per litter (mean ± standard error)	Half-Siblings	2.7 ±0.4	3.6 ± 0.3
	Full Siblings	3.1 ± 0.5	3.8 ± 0.7
Litter size at birth (mean ± standard error)	Half-Siblings	5.5 ± 0.5	7.8 ± 0.5
	Full Siblings	6.1 ± 0.4	8.0 ± 0.4
Litter size at weaning (mean ± standard error)	Half-Siblings	5.4 ± 0.5	7.4 ± 0.5
	Full Siblings	6.1 ± 0.4	8.0 ± 0.4
Mass (g) of pup on day of birth (mean ± standard error)	Half-Siblings	1.5 ± 0.04	1.5 ± 0.07
	Full Siblings	1.3 ± 0.08	1.4 ± 0.04
Mass (g) of pup at weaning (mean ± standard error)	Half-Siblings	12.2 ± 0.4	11.8 ± 0.3
	Full Siblings	11.7 ± 0.3	11.5 ± 0.4

3.3.3 Behavioural Assay Schedule

Subject females were given three behavioural assays and the same individual triads of females (subject and two stimulus females) were used in each test. First, subject females were given a short scent discrimination and attraction assay designed to test female response to urine from related (either maternal or paternal half-sisters) or unrelated stimulus females. Subject females were then given a nest partner choice assay to establish whether subjects prefer to nest with related or unrelated stimulus females. Finally, subject females were given two consecutive female-female interaction assays, one with a related stimulus female and one with an unrelated stimulus female. This tested whether females behave differently with related or unrelated stimulus females when allowed to interact in a neutral arena over a short time period.

The different behavioural assays were arranged to follow after one another in order of increasing female interaction: females were presented with urine, then given a nest partner choice with limited interaction occurring through a mesh barrier, and finally pairs of females were given a free behaviour interaction trial in a neutral arena. Scent discrimination and attraction assays were run in the morning of the first test day and females were placed in the nest partner choice cages at approximately 4 – 5 pm of that same day. On the morning of the third test day females were removed from the nest partner choice cages and returned to their home cages. Four days later (on the morning of the seventh test day) subject females were given the first interaction trial with either a related or unrelated stimulus female. The following day (test day eight) subject females were given a second interaction trial with the remaining stimulus female.

On completion of all the previous behavioural assays an odour-genes covariance assay tested for whether neutral female house mice can detect a similarity between the urine of half-sisters (maternal or paternal) compared to the urine of unrelated females. Subject females for the odour-genes covariance assay were unrelated and unfamiliar with any of the stimulus females. Urine samples were provided by a triad of stimulus females: an habituation female, a related discrimination female and an unrelated discrimination female. These triads were the same as those tested previously in the scent discrimination and attraction, nest partner preference and female-female interaction assays – previous subject females provided the habituation urine and the previous corresponding related and unrelated stimulus females provided the related and unrelated discrimination urine respectively.

3.3.4 Subject and Stimulus Females

Subject females were six months old when behavioural assays began, and stimulus females were eight months old. Stimulus females were weight-matched to within 2 g (1.08 ± 0.09, mean ± standard error). Subject females were weight-matched to within 3 g of both stimulus females (1.72 ± 0.17, mean ± standard error).

The scent discrimination and attraction assay requires that subject females directly sniff both stimulus urine marks. A number of females often do not sniff both scents and therefore have to be excluded from the analysis. For the nest cage partner choice and female-female interaction assays 44 subject females were tested (maternal n = 22, paternal n = 22). However, to increase the scent discrimination and attraction assay sample size an additional eight triads of females were tested in each lineage group (maternal = 30, paternal = 30). Triads of these additional subject and stimulus females were established according to the same conditions as before. The subject females were only tested in the scent assay. Of the 16 additional subject females in the scent discrimination and attraction assay, seven had previously been used in the scent assay before. Scent assays for the subject females were performed at least four weeks following the completion of the second female-female interaction assay, providing a large amount of time between repeated scent assays for those seven females that had been used previously. Repeated females were not used as subject females in the same lineage group.

All subject females were fur marked at least two days prior to the start of behavioural assays. This allowed for identification of subject females from stimulus females during the female-female interactions. Fur marking was completed using Jerome Russell b Blonde hair dye (Jerome Russell, CO, USA). Powder bleach and peroxide solution was mixed according to the manufacturer's instructions and lightly applied onto the centre of the lower back while the subject was held in a handling tube. Females were then transferred to an empty M3 cage for ten minutes, after which the dye solution was carefully washed off using warm, damp cotton pads. A dry cotton pad was then gently applied to soak up any excess water from the fur. Females were checked to ensure all dye had been washed off before being returned to their home cage. By using a handling tube this process could be completed without having to physically restrain the females and therefore was considered less stressful.

3.3.5 Scent Discrimination and Attraction Assay

Females were initially tested for whether they spontaneously discriminate the urine of an unfamiliar half-sister from the urine of an unfamiliar unrelated female. Related stimulus females were either maternal (n = 30) or paternal (n = 30) half-sisters. The methods were similar to those described in section 2.3.5, based on the design of Ramm *et al.* (2008), although the duration and some behaviours recorded were altered. Briefly, scent assays were performed in MB1 cages bisected by a acrylic barrier (28 x 13 cm) across the width of the cage (Figure 2.1). A circular hole (diameter 5 cm) in the centre of the barrier allowed movement from one side of the cage to the other. Females were given 30 minutes to habituate to the cage with a clean perforated acrylic lid and then tested with a lid onto which two stimulus urine marks (10 µl) were streaked on half-circles of Benchkote. The position of the related and unrelated stimulus urine was randomized but balanced. Urine was collected using the recovery method (Section 2.3.3) at least a week before testing commenced and stored at -22°C. Female position and behaviour was recorded to DVD and watched back blind to the position of each scent using an event recorder program (written by R.J.Beynon).

In the previous study the scent discrimination and attraction assay lasted for 20 minutes (Section 2.4.1). The strongest behavioural responses, however, were observed during the first half. Therefore trials were run for 10 minutes only. Scent has volatile and non-volatile components. The sniff measure previously recorded (where females sniffed urine directly standing on their hind legs) largely focuses on the non-volatile component. To assess whether the airborne component may also enable female house mice to discriminate urine from related and unrelated females a second investigatory measure was recorded: the total amount of time females spent directly under each urine scent – this included the time spent sniffing urine and time spent within the inner circle not sniffing the urine. As subject females did not differ in the amount of time spent in close proximity to either stimulus urine (Section 2.4.1) this measure was not recorded here. Time spent by females self-grooming on either side of the test cage was also not recorded. The behaviours that were recorded are listed in Table 3.2.

In the previous study females spent more time investigating unrelated urine, but spent more time on the related urine side (Section 2.4.1). However in that study related urine came from full sisters – closer relatives than the half-sisters that are tested here. Since half-sisters are less related than full sisters it is predicted that the same responses may be observed, but that they could be weaker.

Table 3.2: Revised female behaviours measured during scent discrimination and attraction assay.
Behaviours watched blind to the location of related and unrelated urine.

Behaviour	Description
Sniff Related Urine	Subject on the related urine side of the test cage, standing on hind legs within the inner circle sniffing at urine mark
Directly Under Related Urine	Subject within the inner circle on related urine side of the test cage – includes time spent sniffing and not sniffing
Related Side	Subject on the related urine side of the test cage
Sniff Unrelated Urine	Subject on the unrelated urine side of the test cage, standing on hind legs within the inner circle sniffing at urine mark
Directly Under Unrelated Urine	Subject within the inner circle on unrelated urine side of the test cage – includes time spent sniffing and not sniffing
Unrelated Side	Subject on the unrelated urine side of the test cage

3.3.6 Nest Partner Choice Assay

Females were given a choice of nest partner preference between unfamiliar maternal (n = 22) or paternal (n = 22) half-sisters and unrelated females. The methods were similar to those described in Section 2.3.6. Briefly, females were placed in a series of three interconnected MB1 cages (Figure 2.2). Stimulus females were housed in the end cages confined in one half by acrylic and mesh barriers. The subject females were placed in the centre cage and could move freely between the cages through acrylic tunnels. Subject females could interact with the stimulus females through the mesh barrier, which allowed for scent, sight and sound contact, as well as limited touch. Stimulus females were placed into the end cages approximately an hour before the subject female was placed in the central cage. Acrylic tunnels passed through automated RFID readers (FSI) working in tandem with infrared

sensors, enabling a central computer running custom software (FSI; Thom *et al.*, 2008b) to track female movement. The output files were converted in SPSS (version 18.0.2) to give the total amount of time females spent in each of the three cages.

Previously it was shown that females made consistent decisions over 72 hours (Section 2.4.2, Figure 2.11), and the proportion of time females spent in the related stimulus female cage did not change with time. As a result run time was reduced; the system was started towards the end of the dark phase (4 – 5 pm) and allowed to run for approximately 41 hours. Food and water were checked daily, and each female was seen to be moving freely. After approximately 41 hours, near the start of the dark phase (10 – 11 am), the females were returned to their home cages and the apparatus was thoroughly cleaned with hot soapy water. To standardise assay time, during analysis the total trial time was reduced to 36 hours – the first light period (from 8 pm on the start day), the first complete dark period, and then the second light period (until 8am on the end day).

Females were predicted to spend more time in the related stimulus cage than in the unrelated stimulus cage, but no prediction was made for the differences in female response between the maternal and paternal lineage groups.

3.3.7 Female-Female Interaction Assay

The scent and nest partner choice assays presented subjects with a choice between two stimulus females, either via urine alone, or by interacting with the stimulus females through mesh barriers. These barriers allowed scent, sight and sound contact, and limited touch, but do not give a full indication of how females might behave towards one another under more natural circumstances. Pairs of females were introduced in neutral arenas (Figure 3.2) and allowed to interact freely to assess subject female behavioural response to stimulus females that were related (either maternal or paternal half-sister) or unrelated females. Subject females had previously encountered both stimulus females in the nest partner choice assay.

Over two consecutive days subject females interacted with two stimulus females: a related stimulus females (either a maternal (n = 22) or paternal half-sister (n = 22)) and an unrelated stimulus female. The behaviour of the subject female towards related and unrelated stimulus females was compared. Subject and stimulus female triads were as described in Section 3.3.4.

Interactions took place in a neutral testing arena (60 x 70 cm; Figure 3.2) with two red plastic mouse houses (Techniplast, NJ, USA) and an acrylic sheet (39 x 30 x 0.2 cm) supported on

two small concrete blocks (20 x 3 x 3 cm). Females could move under the sheet and on top of it. Females were weighed and then were individually habituated to two identically set up habituation arenas for 10 minutes. After habituation, females were placed at opposite sides of the clean testing arena in handling tunnels, and released from the tunnels simultaneously. Trials were monitored for excessive aggression. Sustained aggression lasting longer than ten seconds was interrupted by waving a hand over the testing arena, or by clicking fingers over the arena if aggression continued. If mice had to be broken up three times in a single session then they were separated and the trial abandoned. Two pairs had to be separated in the maternal lineage group, whilst five pairs had to be separated in the paternal lineage group. Trials lasted 15 minutes, and began when the two females first encountered one another. Female behaviour was recorded remotely to DVD. After 15 minutes both females were removed from the testing arena and returned to their home cages. Stimulus female test order and female placement in the test arena were randomised but balanced. In between trials, test arenas were cleaned thoroughly with water, wiped down with 70 % ethanol and allowed to air dry completely. All nestboxes, blocks and sheets were cleaned in hot soapy water, rinsed under running water to remove any soap residue, and allowed to air dry.

DVDs were watched blind to the identity of females and behaviours were recorded using an event recorder program (written by R.J.Beynon). Behaviours recorded are described in Table 3.3. They break down into three categories: investigatory, associative and competitive. Investigatory behaviours involve follow and sniff behaviours. It might be expected that, as in the scent discrimination tests, females will spend longer investigating unrelated stimulus females than related ones. Associative behaviours involve huddling and allogrooming, where females remain in close proximity of each other. These behaviours could indicate an affiliation between females and may be more likely when females are related than when females are unrelated. Competitive behaviours involve chasing, submissive posturing and fighting. Females might be expected to show more agonistic behaviours towards unrelated females than related females.

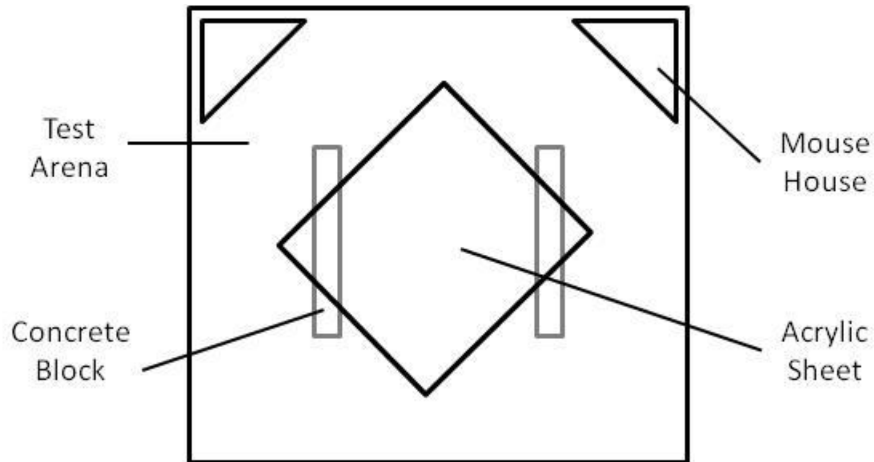


Figure 3.2: Female – female interaction test arena design.
Test arena (60 x 70 cm) with two red plastic mouse houses, an acrylic sheet (39 x 30 x 0.2 cm) supported on two concrete blocks (20 x 3 x 3 cm). Females were able to move on top of and beneath the acrylic sheet. Test arena, houses, sheet and blocks not drawn to scale.

Table 3.3: The three social behaviour types and individual female behaviours recorded during female-female interaction assays.

Social Behaviour Type	Behaviour	Description
Investigatory	Sniff Nose	Subject sniffing nasal and oral region of stimulus female
	Sniff Anogenital	Subject sniffing the anogenital region of stimulus female
	Sniff Elsewhere	Subject sniffing elsewhere on the body of stimulus female
	Follow	Subject tracks stimulus female closely (walking)
Associative	Allogroom	Subject grooming or being groomed by stimulus female
	Huddle	Subject stationary either in contact with or within a body length of stimulus female
Competitive	Chase	Subject rapidly pursuing stimulus female (running)
	Flee	Subject rapidly moving away from stimulus female (running)
	Submissive behaviour	Subject performing submissive posturing, rearing on hind legs
	Tail Rattling	Subject rattling tail at stimulus female
	Fight	Subject biting/attacking stimulus female

3.3.8 Odour-Genes Covariance Assay

Odour-genes covariance tests are used to assess how similar two odours are perceived to be to each other (Heth & Todrank, 2000). A subject is presented with an habituation odour and then given two different discrimination odours. As both discrimination odours are unfamiliar to the subject, the subject should spend an equal amount of time sniffing each odour. However, if one of the discrimination odours is related to, or otherwise shares a common feature with, the habituation odour, then the subject may spend less time investigating the related discrimination odour than the second discrimination odour. The related discrimination odour can therefore be said to appear more similar to the habituation odour than the unrelated discrimination odour.

Subject females (n = 44) were presented with an habituation odour and then presented with two discrimination odours, one related to the habituation odour and one unrelated. All three odours came from donors unrelated and previously unfamiliar to the subject female. These triads were the same as those tested previously in the scent discrimination and attraction, nest partner preference and female-female interaction assays – previous subject females provided the habituation urine and the previous corresponding related and unrelated stimulus females provided the related and unrelated discrimination urine respectively. All urine was collected using the recovery method at least two weeks before testing commenced and stored at -22 °C. Subject females were from the second round of litters.

Females were tested with related discrimination urine from either maternal half-sisters (n = 22) or paternal half-sisters (n = 22). Assays were carried out in a similar clean testing cage as Ramm *et al* (2008). Females were initially habituated to a MB1 testing cage with a clean perforated acrylic lid for 30 minutes. After 30 minutes, the clean lid was replaced with an habituation lid onto which semi-circles of Benchkote had been Sellotaped. On one of these semi-circles was streaked the habituation urine (10 µl), and onto the other was streaked ddH_20 (10 µl). The position and behaviour of the subject female was remotely recorded to DVD for ten minutes, after which time the habituation lid was replaced with the clean lid for two minutes.

After two minutes the clean lid was replaced with a discrimination lid with Benchkote semi-circles. On to each of these semi-circles was streaked a discrimination odour (10 µl urine). Female position and behaviour was remotely recorded to DVD for ten minutes, after which

the subject female was returned to her home cage. Habituation and discrimination lids were as in Figure 2.1.

The side position of all habituation and discrimination odours was randomised but balanced. DVDs were watched back blind to the odour positions and behaviours were recorded using an event recorder program (written by R.J.Beynon). Female position was determined by the nose location. Investigatory behaviours recorded are shown in Table 3.4. Two investigatory behaviours were recorded to discover whether similarity between urine marks could be detected from non-volatile components (sniff) or a combination of both non-volatile and volatile components of urine (time under). Females were predicted to spend longer investigating the habituation urine than the ddH_2O. Perceived similarity between the habituation urine and the related discrimination urine was measured by the different amount of time females spent investigating each of the discrimination urine marks. If females spent significantly longer investigating the unrelated discrimination urine than the related discrimination urine then the related discrimination urine can be said to appear more similar to the habituation urine than the unrelated discrimination urine.

Table 3.4: Female investigatory behaviours recorded during odour-genes covariance assays.

Investigatory Behaviour	Description
Sniff Odour A	Subject standing on hind legs sniffing at odour within the inner circle on Side A
Under Odour A	Subject within the inner circle on Side A, sniffing and not sniffing odour
Sniff Odour B	Subject standing on hind legs sniffing at odour within the inner circle on Side B
Under Odour B	Subject within the inner circle on Side B, sniffing and not sniffing odour

3.3.9 Data Analysis

All statistical tests were carried out using the SPSS software package (version 18.0.2). All figures use untransformed data.

3.3.9.1 Scent Discrimination and Attraction Analysis

Only females that sniffed both stimulus urine marks were included in the analysis. The amount of time females spent sniffing the related and unrelated urine did not meet parametric assumptions. Therefore a Mann-Whitney U test compared the ratio of time subject females spent sniffing urine from related females compared to urine from unrelated stimulus females between the maternal and paternal lineage groups. If there was a difference between the groups then Wilcoxon Signed Ranks tests were used to compare the amount of time females spent sniffing related and unrelated urine for each lineage group. If there was no difference between the lineage groups then the data were combined and a Wilcoxon Signed Ranks test then compared the amount of time subject females spent sniffing urine from related stimulus females compared to urine from unrelated stimulus females. This approach was repeated for the total amount of time subject females spent on each side of the test cage.

The amount of time females spent directly under each urine scent was logarithmically transformed to meet parametric assumptions. A repeated measures GLM then compared the total amount of time females spent directly under urine from the related stimulus female and urine from the unrelated stimulus female, with lineage group as a fixed factor.

3.3.9.2 Nest Partner Choice Analysis

Data from the nest partner choice assay did not meet parametric assumptions. The proportion of time spent by subject females in the related stimulus female cage (as a proportion of the total assay time) was compared between the maternal and paternal lineage groups using a Mann-Whitney U test. If there was a significant difference between the lineage groups then female cage choice was analysed separately for each group. If there was no difference between the familiarity groups then the data were combined. The amount of time females spent in each test cage was compared against the amount of time females spent in each of the other test cages using Wilcoxon Signed Ranks tests.

3.3.9.3 Female-Female Interaction Analysis

Subject females interacted with stimulus females in two interaction assays over consecutive days. The eleven individual behaviours recorded were combined into the three social

behaviour types described in Table 3.3. The amount of time subject females spent performing investigatory behaviours was square rooted to meet parametric assumptions. A repeated measures GLM compared the amount of time subject females spent performing investigatory behaviour towards related and unrelated stimulus females. Lineage group and stimulus order were included as fixed factors, although stimulus order was removed from the analysis if it did not have a significant effect on female behaviour.

Subject female associative behaviour did not meet parametric assumptions. Therefore a Mann-Whitney U test compared the ratio of time subject females spent performing associative behaviours towards the related stimulus females between stimulus order groups, and then between lineage groups. If there was no difference between the both the stimulus order groups and the lineage groups then the data were combined and a Wilcoxon Signed Ranks test compared the amount of time subject females spent performing associative behaviours towards the related stimulus and unrelated stimulus females. If there was a difference between the stimulus order or lineage groups then Wilcoxon Signed Ranks test compared the amount of time spent performing associative behaviours towards the related and unrelated stimulus females separately for each group. This approach was repeated for subject female competitive behaviour which also did not meet parametric assumptions.

3.3.9.4 Odour-Genes Covariance Analysis

Female odour investigation was measured by the amount of time females spent sniffing each odour mark and the amount of time females spent directly under each odour mark. In the habituation assay both time spent sniffing and total time under odour were logarithmically transformed to meet parametric assumptions. A paired t-test then compared the amount of time subject females spent sniffing the habituation urine and the ddH_2O. This was repeated for the amount of time females spent directly under each urine mark. Females were predicted to spend longer investigating the habituation urine than the ddH_2O.

In the discrimination assay both the time spent sniffing and the total time under odour were logarithmically transformed to meet parametric assumptions. A repeated measures GLM compared the amount of time females spent sniffing the related and unrelated discrimination urine marks, with lineage group as a fixed factor. This approach was repeated for the total amount of time females spent directly under the related and unrelated discrimination urine marks.

3.4 Results

The results of the questions examined here are presented below and summarised in Table 3.5.

3.4.1 Scent Discrimination and Attraction

3.4.1.1 Scent Discrimination

Two measures of investigatory behaviour were used to assess subject female discrimination of urine from related and unrelated stimulus females. Females were predicted to spend longer investigating urine from an unrelated female compared to urine from a related female. There was no difference in the ratio of time females spent sniffing related urine between the maternal and paternal lineage groups ($z = -0.25$, $p = 0.80$, two-tailed; Figure 3.3a). The data for the two groups were combined and there was a non-significant trend for subject females to spend longer sniffing urine from an unrelated female compared to urine from a related female ($z = -1.50$, $p = 0.07$, one-tailed; Figure 3.3a).

To establish whether females discriminate between urine from the related and unrelated stimulus females based on both the volatile and non-volatile components of urine, the total amount of time under related and unrelated urine was compared. There was no effect of lineage group on the amount of time females spent directly under urine from the related and unrelated stimulus females ($F_{(1,41)} = 0.57$, $p = 0.46$; Figure 3.3b) however, females spent longer directly under urine from the unrelated female compared to urine from the related female ($F_{(1,41)} = 4.39$, $p = 0.04$; Figure 3.3b).

3.4.1.2 Scent Attraction

Female attraction to urine from related and unrelated stimulus females was measured by the total amount of time spent on either side of the test cage. The ratio of time spent by females in the related stimulus urine side of the cage did not differ between the maternal and paternal lineage groups ($z = -0.93$, $p = 0.37$, two-tailed; Figure 3.4). As a result data from the two groups was combined. There was no difference in the amount of time subject females spent on the related stimulus urine side of the test cage compared to the unrelated stimulus urine side of the test cage ($z = -0.09$, $p = 0.47$, one-tailed; Figure 3.4).

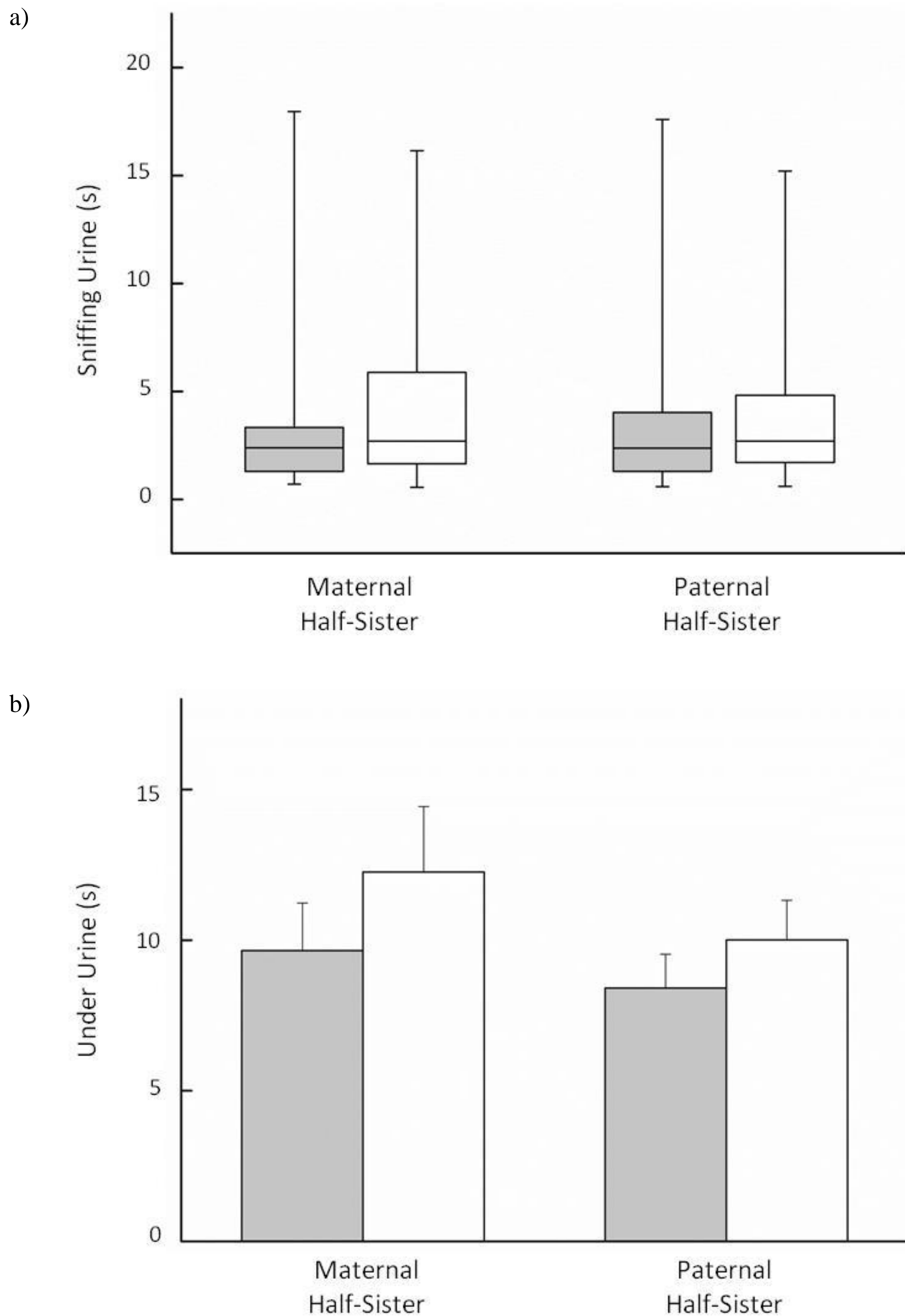


Figure 3.3: Investigation of urine from related and unrelated stimulus females in the maternal and paternal half-sister groups.
Investigatory behaviour towards related (grey boxes/bars) and unrelated (open boxes/bars) urine, in the maternal (n = 20) and paternal (n = 23) half-sister groups. Investigation measured by a) time (s) subject females spent sniffing each urine mark, and b) time (s, mean + standard error) spent by subject females directly under each urine mark.

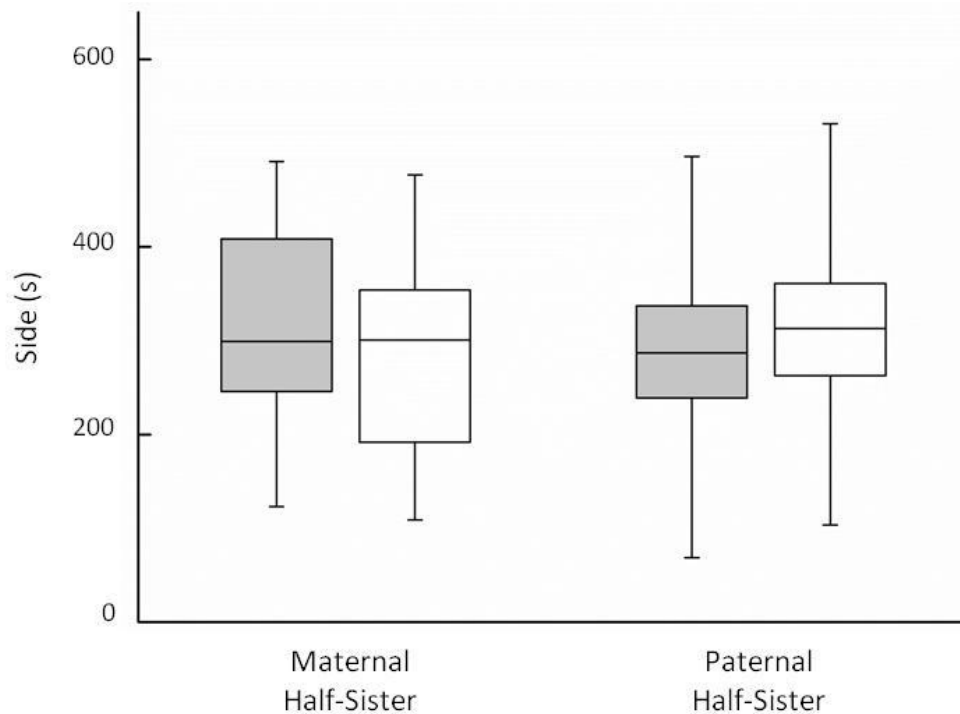


Figure 3.4: Attraction towards urine from related and unrelated stimulus females in the maternal and paternal half-sister groups.
Attraction towards related (grey boxes) and unrelated (open boxes) urine in the maternal (n = 20) and paternal (n = 23) half-sister groups. Attraction measured by the amount of time subject females spent on each side of the test cage (s).

3.4.2 Nest Partner Choice

Subject females were placed in a series of three interconnected cages with restricted access to stimulus females in the end cages. The amount of time spent in each cage was measured over 36 hours. There was a significant difference in subject female response between the maternal half-sister and paternal half-sister groups over 36 hours ($z = -2.00$, $p = 0.05$, two-tailed; Figure 3.5), and therefore the amount of time subject females spent in each of the stimulus female cages was analysed separately for each group. In the maternal lineage group, females spent significantly longer in the related stimulus female cage than in the unrelated stimulus female cage ($z = -3.23$, $p < 0.01$, one-tailed; Figure 3.5). There was no difference in the amount of time females spent in the related and unrelated stimulus female cages in the paternal group ($z = -0.80$, $p = 0.22$, one-tailed; Figure 3.5).

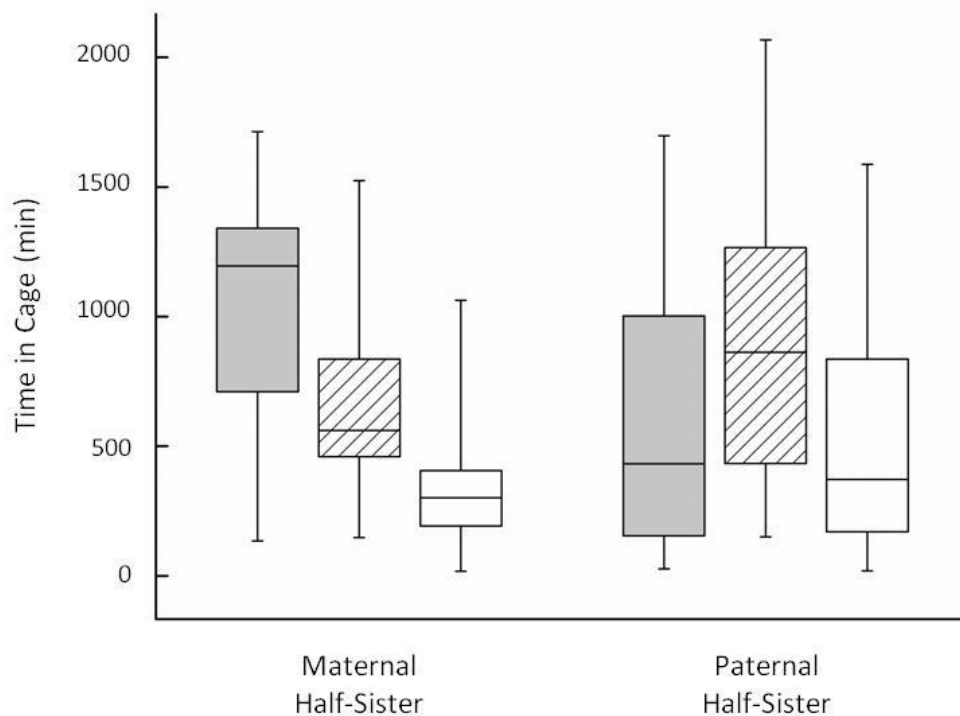


Figure 3.5: Nest partner choice in the maternal and paternal half-sister groups.
Time (minutes) spent by subject females in the related stimulus female cage (grey boxes), the centre cage (hashed boxes) and the unrelated stimulus female cage (open boxes) in the maternal (n = 22) and paternal (n = 22) half-sister groups.

3.4.3 Female-Female Interactions

Subject females were allowed to interact with both stimulus females consecutively over two days. The order with which subject females encountered the related and unrelated stimulus females was randomised. Stimulus order was included in the model initially and then removed if not significant. The amount of investigatory, associative and competitive behaviour performed by subject females was compared when the subject females interacted with a related female and an unrelated female. The related stimulus female was either a maternal half-sister or a paternal half-sister. Subject females had encountered the urine of both stimulus females in the scent discrimination and attraction assay, and had limited interaction with both stimulus females in the nest partner choice assay.

3.4.3.1 Investigatory Behaviour

The amount of time subject females spent performing investigatory behaviours towards related and unrelated stimulus females was compared. Stimulus order was not significant ($F_{(1,33)} = 0.98$, $p = 0.33$) and so was removed from the model. There was a significant interaction between the level of investigatory behaviour and the lineage group ($F_{(1,35)} = 7.77$, $p = 0.01$; Figure 3.6). Investigation of the data revealed that this was due to females in the maternal half-sister group displaying more investigatory behaviour towards the unrelated stimulus female than towards the related stimulus female, whereas in the paternal half-sister group subject females investigated related stimulus females more than unrelated stimulus females. There was no difference in the amount of time subject females investigated either the related or unrelated females ($F_{(1,35)} = 1.25$, $p = 0.27$; Figure 3.6) and no difference between the lineage groups ($F_{(1,35)} = 0.06$, $p = 0.81$; Figure 3.6).

3.4.3.2 Associative Behaviour

The amount of time subject females spent performing associative behaviours towards related and unrelated stimulus females was compared when related females were either maternal or paternal half-sisters. The ratio of time subject females spent performing associative behaviours towards the related stimulus female did not differ with stimulus order ($z = -0.66$, $p = 0.52$, two-tailed). There was no difference in the ratio of time spent performing associative behaviours by subject females towards the related stimulus females between the lineage groups ($z = -1.36$, $p = 0.18$, two-tailed; Figure 3.7). The data from the two lineage groups were therefore combined and the amount of time spent by subject females performing associative behaviours was compared. Subject females did not differ in the amount of time

they spent performing associative behaviours towards either related or unrelated stimulus females (z = -1.15, p = 0.26, two-tailed; Figure 3.7).

3.4.3.3 Competitive Behaviour

The amount of time subject females spent performing competitive behaviours in response to related and unrelated stimulus females was compared when related females were either maternal or paternal half-sisters. There was no difference in the ratio of time females spent performing competitive behaviours towards related females when females were tested with a related or an unrelated stimulus female first (z = - 0.47, p = 0.66, two-tailed; Figure 3.8). Females in the maternal and paternal lineage groups did not differ in the ratio of time spent performing competitive behaviours towards related stimulus females (z = -0.86, p = 0.40, two-tailed; Figure 3.8), and therefore the two lineage groups were combined. Subject females spent longer performing competitive behaviours towards unrelated stimulus females than towards related stimulus females (on average subject females performed 11.57 s competitive behaviour towards related stimulus females and 23.83 s competitive behaviour towards unrelated stimulus females; z = -2.10, p = 0.04, two-tailed; Figure 3.8).

3.4.3.4 Full Sister Interactions

The interaction assay had not previously assessed differences in behavioural response between full sisters and unrelated females. To investigate whether full sisters might show a stronger response than half-sisters interaction trials were run using 14 female triads where related females were unfamiliar full sisters. All methods were as described in Section 3.3.7. Females had been bred during the breeding programme documented in Section 3.3.2. not previously been used in any behavioural assays and were unfamiliar with both stimulus females. Subject females and stimulus females were 8 and 10 months old respectively. The triads of subject and stimulus females were assigned based on the methods described in Section 3.3.4. Two 15 minute trials were run over two days and stimulus female order was randomised. All stimulus females were fur bleached for identification. Assays were recorded to DVD and watched back blind to the identity of the stimulus female. Behaviours recorded are described in Table 3.3.

Investigatory behaviour was square root transformed to meet parametric assumptions. The amount of time subject females spent performing investigatory behaviours towards related and unrelated stimulus females was compared using a repeated measures GLM. There was no difference in the amount of time subject females spent performing investigatory behaviour

towards the related or unrelated stimulus females ($F_{(1,12)} = 2.68$, $p = 0.13$; Figure 3.6) and no effect of stimulus order ($F_{(1,12)} = 0.29$, $p = 0.60$). The ratio of time subject females spent performing associative behaviours towards related stimulus females did not differ with stimulus order ($z = -0.39$, $p = 0.72$, two-tailed, Mann-Whitney test). Subject females did not differ in the amount of time spent performing associative behaviours towards related or unrelated stimulus females ($z = -0.66$, $p = 0.54$, two-tailed, Wilcoxon Signed Ranks test; Figure 3.7). The ratio of time subject females spent performing competitive behaviours towards related stimulus females did not differ with stimulus order ($z < 0.001$, $p = 1.00$, two-tailed, Mann-Whitney test). There was no difference in the amount of competitive behaviour subject females performed towards related or unrelated stimulus females ($z = -0.87$, $p = 0.41$, two-tailed, Wilcoxon Signed Ranks test; Figure 3.8).

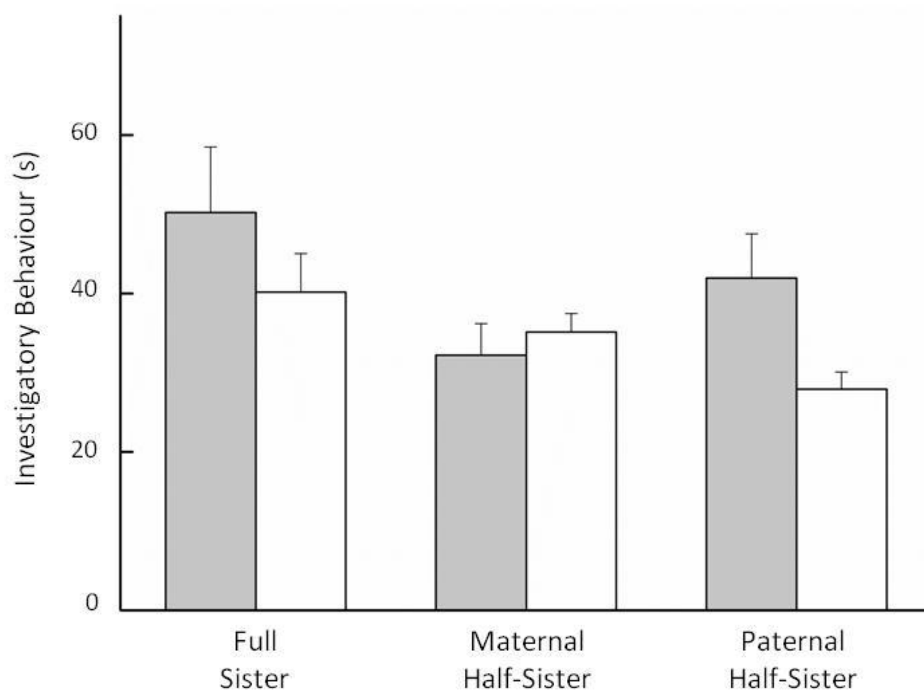


Figure 3.6: Investigatory behaviour in the maternal and paternal half-sister groups, and the full sister group.
Investigation (s, mean + standard error) of related (grey bars) and unrelated (open bars) stimulus females in the full sister (n = 14), maternal half-sister (n = 20) and paternal half-sister (n = 17) groups.

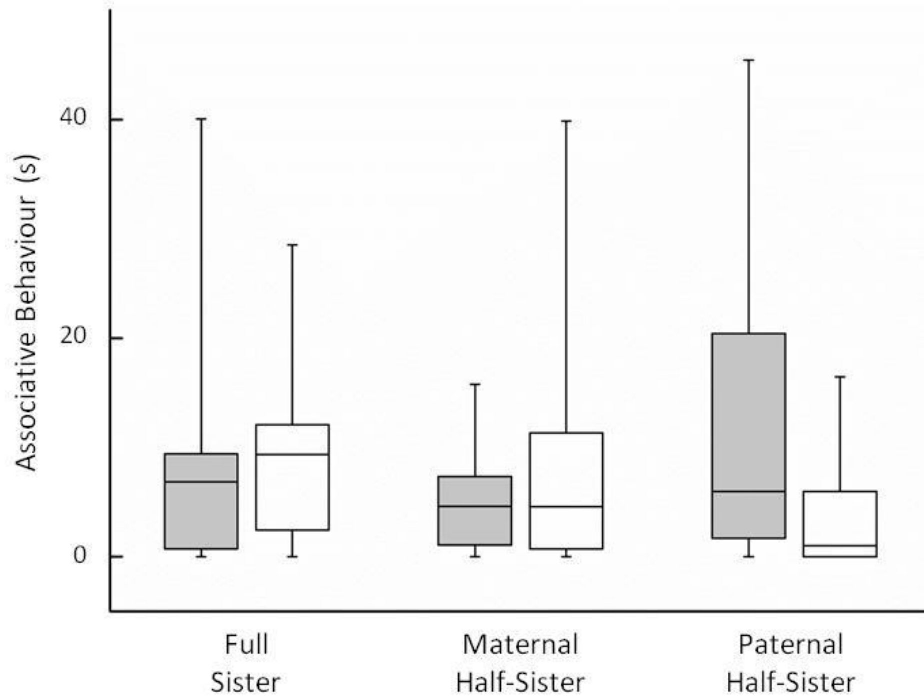


Figure 3.7: Associative behaviour in the maternal and paternal half-sister groups, and the full sister group.
Associative behaviour (s) towards related (grey bars) and unrelated (open bars) stimulus females in the full sister (n = 14), maternal half-sister (n = 20) and paternal half-sister (n = 17) groups.

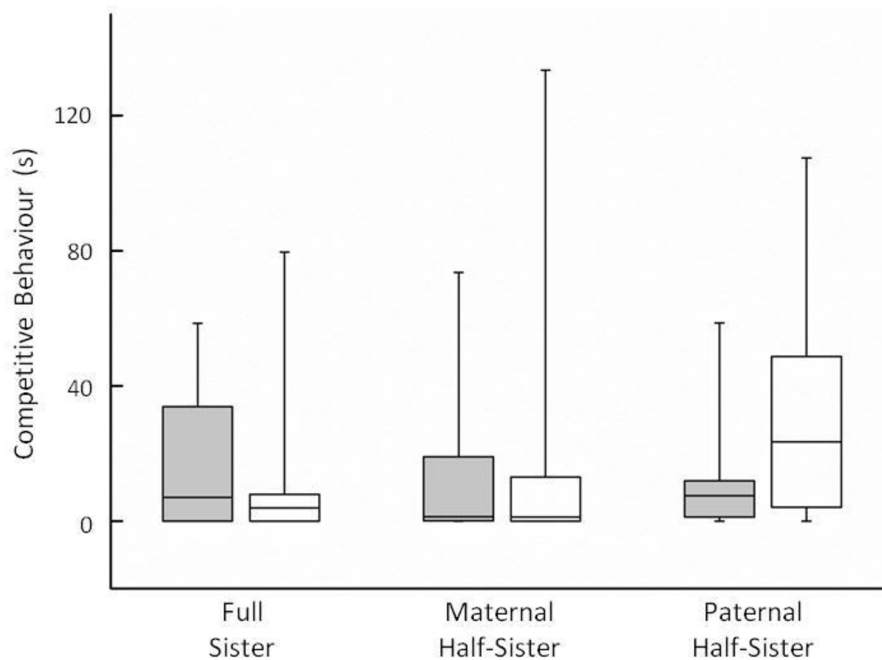


Figure 3.8: Competitive behaviour in the maternal and paternal half-sister groups, and the full sister group.
Competitive behaviour (s) towards related (grey bars) and unrelated (open bars) stimulus females in the full sister (n = 14), maternal half-sister (n = 20) and paternal half-sister (n = 17) groups.

3.4.4 Odour-Genes Covariance

Odour-genes covariance assays are designed to assess whether two odours are perceived as being similar to each other by the subject animal. Subject females given an habituation assay (presented with habituation urine and water). Females were predicted to spend longer investigating habituation urine than water. Subjects were then given a discrimination assay (presented with two discrimination urine marks one from a female related to the habituation urine female and one discrimination urine sample from a female unrelated to either the habituation or related discrimination females). If the related urine samples appeared similar then subject females may spend longer investigating the unrelated discrimination urine compared to the related discrimination urine. Two investigatory behaviours were measured: time spent directly sniffing the urine samples and the total amount of time spent directly under the urine samples. Time spent under urine was measured to include both the time subject females spent directly sniffing the non-volatile components of the urine samples and also time spent also sniffing the volatile components of urine.

3.4.4.1 Habituation Assay

To check that females behaved as predicted in the habituation assay the amount of investigatory behaviour directed at the urine and water marks was compared. Females spent longer sniffing habituation urine than water ($t_{(18)} = 4.57$, $p < 0.01$; Figure 3.9a). Females also spent longer directly under habituation than water ($t_{(18)} = 3.70$, $p < 0.01$; Figure 3.9b).

3.4.4.2 Discrimination Assay

Subject females did not differ in the amount of time they spent investigating either related or unrelated discrimination urine. Females in the maternal group did not differ from females in the paternal group in the amount of time they spent sniffing either discrimination urine ($F_{(1,17)} = 0.34$, $p = 0.57$; Figure 3.9a). Subject females did not sniff the unrelated discrimination urine for longer than the related discrimination urine ($F_{(1,17)} = 1.09$, $p = 0.31$; Figure 3.9a). Time spent under urine did not differ between the maternal and paternal groups ($F_{(1,17)} = 0.10$, $p = 0.75$; Figure 3.9b) and females did not spend longer directly under the unrelated discrimination urine ($F_{(1,17)} = 0.47$, $p = 0.50$; Figure 3.9b).

3.4.4.3 Odour-Genes Covariance using Full Sisters

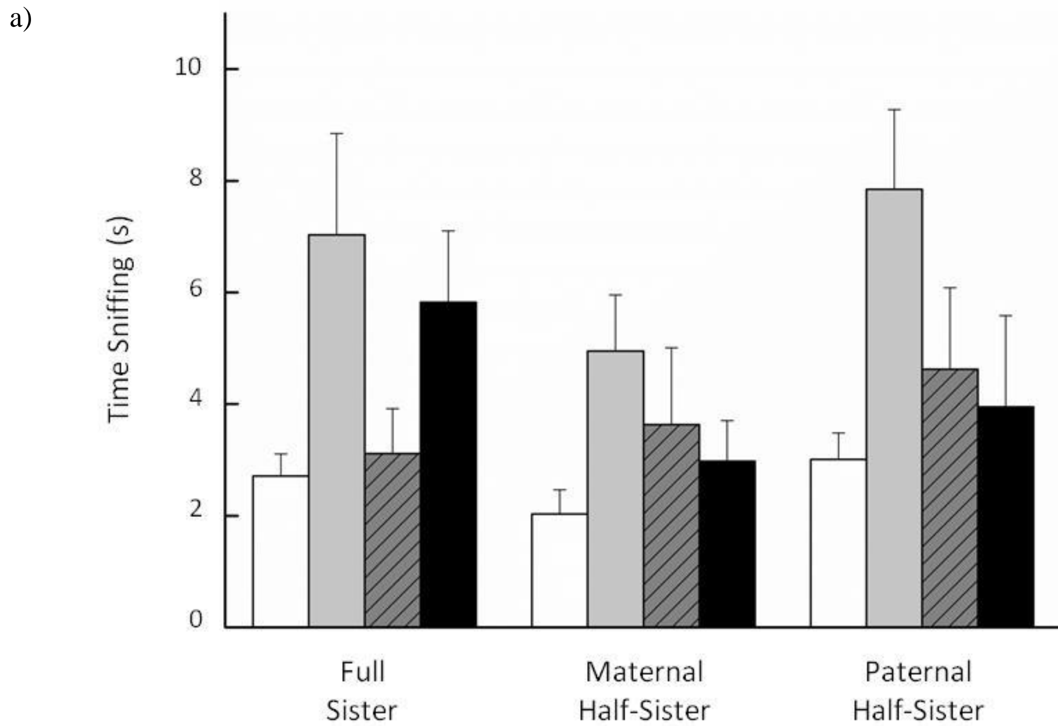
Subject females did not show any difference in the level of investigation of either discrimination odour, suggesting that female house mice do not perceive the urine of two half-sisters to be more similar to each other than the urine of two unrelated females. To

investigate whether more closely related urine may appear similar and to test the assay design the odour-genes covariance assay was repeated with habituation and related discrimination urine being from full sisters. All methods were as in Section 3.3.8. Subject females ($n = 20$) were from the breeding programme described in Section 3.3.2 and were unfamiliar and unrelated to urine-donor females. Subject females were 8 months old, habituation females were 8 months old and discrimination females were 10 months old at the time of testing. The triads of habituation and discrimination females were assigned based on the methods described in Section 3.3.4. Subject females were habituated to the test cage for 30 minutes, followed by the 10 minute habituation assay, a 2 minute gap and then the 10 minute discrimination assay. Assays were recorded to DVD and watched back blind to the position of the habituation and discrimination urine marks. Behaviours recorded are described in Table 3.4. Subject females had to have sniffed the habituation urine and both discrimination urine marks to be included in the analysis.

In the habituation assay subject females spent longer sniffing the habituation urine compared to the water ($t_{(13)} = 2.95$, $p = 0.01$, paired t-test, data logarithmically transformed; Figure 3.9a). Subject females also spent longer directly under the urine sample compared to the water sample ($t_{(13)} = 2.23$, $p = 0.04$, paired t-test, data logarithmically transformed; Figure 3.9b).

In the discrimination assay subject females spent longer sniffing the unrelated discrimination urine mark compared to the related discrimination urine mark ($t_{(13)} = -3.28$, $p < 0.01$, paired t-test, data logarithmically transformed; Figure 3.9a). There was no difference in the amount of time subject females spent directly under either the related or unrelated discrimination urine ($t_{(13)} = -0.94$, $p = 0.36$, paired t-test, data square root transformed; Figure 3.9b).

a)



b)

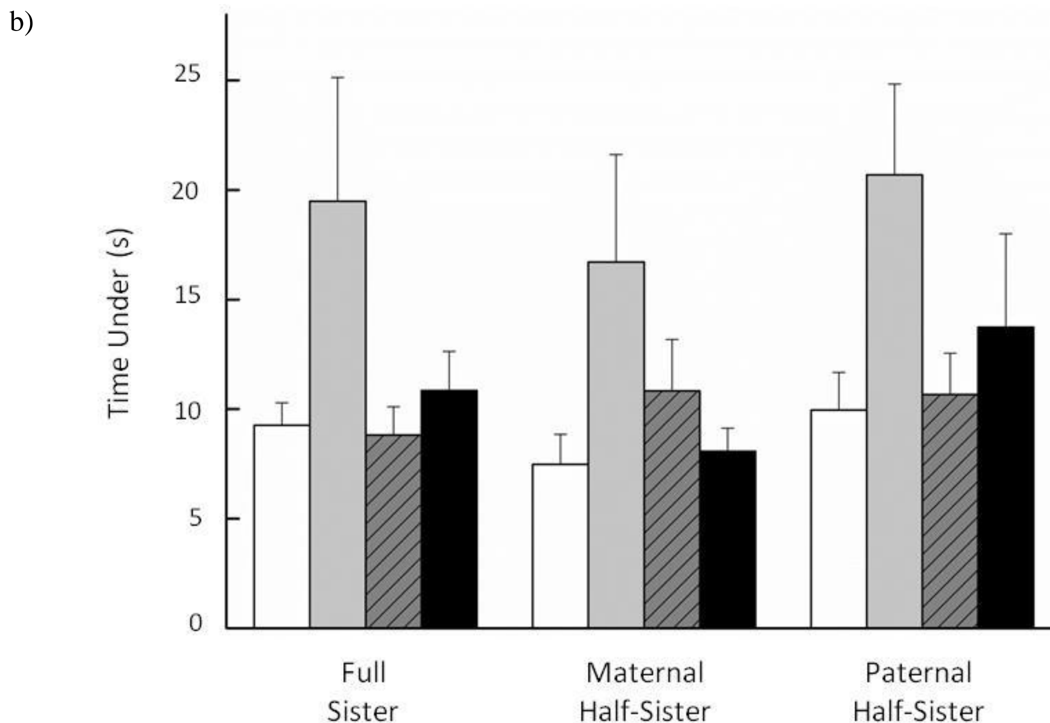


Figure 3.9: Investigation of habituation and discrimination urine when related discrimination females are maternal or paternal half-sisters, or full sisters.
Investigation of water (open bars), habituation urine (light grey bars), related discrimination urine (dark grey hashed bars) and unrelated discrimination urine (black bars) in the maternal (n = 10) and paternal (n = 9) half-sister groups, and the full sister group (n = 14). Investigation measured by a) time spent by subject females sniffing and b) total time spent by subject females directly under urine marks (s, mean + standard error).

Table 3.5: Summary of phenotype matching and template origin results.

Assay	Behaviour Measured	Lineage (Maternal vs. Paternal)	Relatedness (Related vs. Unrelated)	Full Sister Relatedness (Related vs. Unrelated)
Scent Discrimination and Attraction	Sniff Urine (discrimination)	$p = 0.80$[b]	$p = 0.07$[c] (u>r)	
	Under Urine (discrimination)	$p = 0.46$[a]	**$p = 0.04$**[a] **(U>R)**	
	Cage Side (attraction)	$p = 0.37$[b]	$p = 0.47$[c]	
Nest Partner Choice	Time in stimulus cage	**$p = 0.05$**[b]	MHS: **$p < 0.01$**[c] **(R>U)** PHS: $p = 0.22$[c]	
Female-Female Interaction	Investigatory	**$p = 0.01$**[a]	MHS: $p = 0.29$[d] PHS: **$p = 0.01$**[d] **(R>U)**	$p = 0.13$[a]
	Associative	$p = 0.18$[b]	$p = 0.26$[c]	$p = 0.72$[c]
	Competitive	$p = 0.40$[b]	**$p = 0.04$**[c] **(U>R)**	$p = 0.41$[c]
Odour-Genes Covariance	Sniff Urine (discrimination)	$p = 0.57$[a]	$p = 0.31$[a]	**$p < 0.01$**[d] **(U>R)**
	Under Urine (discrimination)	$p = 0.75$[a]	$p = 0.50$[a]	$p = 0.36$[d]

Difference in subject female response in the lineage groups (MHS – Maternal Half-Sister, PHS – Paternal Half-Sister) towards stimulus females of differing relatedness (R/r – Related, U/u – Unrelated), including results from additional full sister tests. Statistical tests performed: a – repeated measures GLM, b – Mann-Whitney U test, c – Wilcoxon Signed Ranks test, d – paired t-test.

3.5 Discussion

3.5.1 A Match-To-Maternal Template Mechanism

A phenotypic matching based kin recognition mechanism uses a recognition template against which to compare newly encountered individuals. The aim of this study was to investigate whether female house mice use self or maternal cues as a kin recognition template by establishing whether female house mice discriminate and show a preference for maternal and/or paternal half-sisters over unrelated females. Discrimination of both maternal and paternal half-sisters would suggest the ability of females to use their self (or both self and maternal) as a template against which to compare novel individuals. If females discriminated only maternal half-sisters then this would suggest that maternal cues may be used as a recognition template. The results of this study are summarised in Table 3.5.

The nest partner choice assay showed a significant difference between the maternal and paternal half-sister groups. Subject females spent longer in the cage of a maternal half-sister than that of an unrelated female, but showed no difference in the amount of time they spent in either a paternal half-sister or an unrelated female. This suggests that female house mice are able to recognise their maternal half-sisters, and choose to associate with them. Whilst no preference was observed between paternal half-sisters and otherwise equivalent females, this does not mean that paternal half-sisters were not recognised. Subject females may have recognised paternal half-sisters but chose not to associate with them. However, given that subject females only associated with maternal half-sisters this suggests that females use maternally learnt cues and not their own cue when making nesting partner decisions.

Paterson & Hurst (2009) found that a maternal comparison kin recognition mechanism was more effective when using more than one recognition locus. The results presented here suggest that female house mice were using a match-to-maternal mechanism during nest partner choice. The results of Chapter 4 suggest that both MHC and MUP may be used by female house mice recognise relatives. MHC and MUP are families of genes; however a MHC or MUP haplotype consists of the set of MHC or MUP alleles on a chromosome. Whilst between different haplotypes there may be variation based on differences in alleles at different loci, a complete MHC or MUP haplotype may act as an allele at a single recognition locus (Paterson & Hurst, 2009). By using a match-to-maternal mechanism with both MHC and MUP female house mice may therefore improve the effectiveness of kin recognition, by

being able to recognise a high proportion of relatives recognised and having a low rate of false inclusion.

Penn & Potts (1998a) also found a suggestion of a match-to-maternal mechanism in female inbreeding avoidance behaviour. This is in contrast to the match-to-self mechanism of inbreeding avoidance suggested by Sherborne *et al.* (2007). In Chapter 6 an experiment similar to that described here examined female inbreeding avoidance. Females avoided cages containing half-brothers of both a maternal and a paternal lineage, suggesting inbreeding avoidance using a match-to-self mechanism in female house mice and supporting the findings of Sherborne *et al.* (2007).

Female house mice may therefore use two phenotype matching mechanisms for kin recognition: a match-to-maternal mechanism for female-female kin discrimination and a match-to-self mechanism for inbreeding avoidance. Individual females gain inclusive fitness advantages from communally nesting with related females (e.g. König, 1993, 1994b, 1994a; Rusu & Krackow, 2004). However females could gain additional advantages if their daughters only nested with females related to each other through the maternal line. Haig (2000a, 2000b) argued that a dam could improve her overall inclusive fitness if her offspring behaved altruistically towards maternal relatives. A dam gains little from her offspring behaving altruistically towards paternal relatives. It is possible then that the conflict of interests between mothers and daughters has resulted in a kin recognition mechanism between females that favours maternal relatives.

The results presented here support the idea that females learn the phenotype of their mother and use that as a template to discriminate female relatives. As discussed in the introduction (Section 1.1.6.6) this learning phase is sometimes called behavioural imprinting. As females were weaned at 24 days and removed from their mothers this learning phase may have occurred within 24 days after birth, or even pre-natal. It would be interesting to investigate the time of learning further, perhaps by removing pups from mothers at earlier stages and fostering them on lactating laboratory mice (as laboratory mice may accept older foster pups more readily) to see at what age behavioural imprinting may occur.

Whilst behavioural imprinting is a likely reason for the discrimination of maternal but not paternal half-sisters observed here, other possible explanations remain. Genomic imprinting occurs where the expression of a particular allele is determined by whether it was inherited

from the mother or father (Bartolomei & Tilghman, 1997; Burt & Trivers, 1998; Wilkins & Haig, 2003; Munshi & Duvvuri, 2007). Genomic imprinting can influence odour investigation in laboratory mice: mice transferred to separate strains whilst embryos spend longer investigating control urine than urine from their maternal strain, but show no difference in investigation time between control and parental strain (Isles *et al.*, 2001; Isles *et al.*, 2002). Epigenetic effects may also influence an individual's phenotype (Hager *et al.*, 2009). Here female house mice spent longer investigating urine from half-sisters compared to urine from unrelated females, regardless whether those half-sisters were maternal or paternal half-sisters. This suggests that epigenetic effects may not have changed female urine odour to enable the recognition of maternal but not paternal relatives.

Another possible reason for the recognition of maternal but not paternal half-sisters is that a maternal cue could be transferred to offspring during rearing (or pre-natal) that would allow individual females to recognise relatives born to that same dam. For instance, this cue could be bacterial gut flora passed to the offspring through milk (Brown, 1995). If bacterial gut flora or a maternal nest cue of some sort was passed from mother to offspring then it is expected that this may cause the odour of urine from maternal half-sisters to appear more similar but the odour of urine from paternal half-sisters to be different (as they were born to different mothers and therefore may have received a different combination of gut flora). As there was no difference between the lineage groups in the scent discrimination and attraction assay and no difference between the groups in perceived odour similarity in the odour-genes covariance assay, this suggests that maternally inherited gut flora was not responsible for the behaviour observed here.

3.5.2 Scent Discrimination

In Chapter 2 females spontaneously discriminated between urine from full sisters and urine from unrelated females. This suggests that there are genetic markers of relatedness present in the urine of female house mice. Here females also spent longer investigating urine from unrelated females compared to urine from half-sisters, regardless whether they were maternal or paternal half-sisters. As females in both lineage groups investigated unrelated urine for longer than related urine this suggests that the markers of relatedness present in female urine are recognisable in the urine of half-sisters. Females showed no attraction towards either the related or unrelated urine. It is possible that the assay is not sensitive enough to detect

differences in attraction when the related urine samples come from more distant relatives than full sisters.

In the odour-genes covariance assay neutral subject females did not differ in the amount of time they spent sniffing the related and unrelated stimulus urine. This suggests that females did not perceive a similarity between the habituation and related urine compared to the unrelated urine. When the related urine was from full sisters, however, females spent longer investigating the unrelated discrimination urine than the related discrimination urine. This suggests that urine from two sisters is more similar than urine from two unrelated females. This similarity is likely to be due to shared components of urine which may be the molecular markers used in kin recognition. As half-sisters are more distantly related than full sisters it is possible that the urine of half-sisters contains fewer similarities than the urine of full sisters. As the assay required that females sniffed the habituation urine and both discrimination urine marks, a number of trials were excluded from the analysis. A larger sample size might reveal a difference in investigatory behaviour and suggest that urine from half-sisters appears similar compared to urine from unrelated females.

3.5.3 Interaction Behaviour

Kin selection theory predicts that relatives may behave more associatively and less aggressively than unrelated individuals (e.g. Maher, 2009; Markman *et al.*, 2009). In the female-female interaction assay pairs of females were allowed to interact for 15 minutes. There was no difference in investigatory or associative behaviours between related and unrelated females, however subject females performed more competitive behaviours towards unrelated stimulus females than towards related stimulus females. More competitive behaviour towards an unrelated individual compared to a related individual is in keeping with kin selection theory, although the full sisters when tested did not exhibit this pattern. It is possible that the smaller sample size of the full sister group meant that a difference was not significant. Further tests could establish whether this is the case.

There was no difference in female behaviour between the maternal and paternal lineage groups, suggesting that maternal and paternal half-sisters may behave similarly when encountering each other. Kareem & Barnard (1986) performed similar behavioural assays using female house mice and also found no difference between maternal and paternal half-sisters. However they also found more associative behaviours between related females

compared to between unrelated females (Kareem & Barnard, 1982, 1986), a trend not observed here.

The interaction assay was only a short behavioural trial and it is possible that differences in behaviour might have been observed over a longer time period. Prolonged or repeated behavioural assays are often needed to observe differences in behaviour between females (L. Bottell, personal communication). Additionally, investigatory behaviour may not have been expected to differ significantly as subject females had already experienced urine from the stimulus females in the scent discrimination and attraction assay, and encountered and had limited interaction with the stimulus females in the nest partner choice assay. Interaction trials using completely novel females may reveal differences in investigatory behaviour. However full sisters used in this test were unfamiliar with each other, yet no difference in investigatory behaviour was observed.

3.5.4 Conclusions

To investigate the origin of phenotype recognition templates, female house mice were tested for their ability to discriminate half-sisters from unrelated females, and whether this discrimination depended on relatedness through the maternal and/or paternal lineage. Female house mice spent longer in the cages of maternal half-sisters compared to the cages of unrelated females, but there was no difference in the amount of time females spent in paternal half-sister cages or unrelated female cages. This suggests that females use a match-to-maternal phenotype matching mechanism for choosing nest partners. There was no difference between the maternal and paternal groups in response to urine suggesting that dams do not influence the phenotype of their offspring. Females were able to discriminate between urine from half-sisters and urine from unrelated females, suggesting that genetic markers of relatedness are present in the urine of half-sisters. Females also did not differ in their behaviour during interactions with either maternal or paternal half-sisters, however females did display more competitive behaviour towards unrelated females than towards related females, as predicted by kin selection theory.

4 Signalling sisterhood: Investigating molecular markers of kin recognition between female house mice

4.1 Abstract

Previous work has shown that female house mice are able to recognise close relatives despite being unfamiliar with them, and that females are able to discriminate relatives from unrelated females when presented with urine alone. This suggests a genetically-based kin recognition mechanism and that cues of relatedness are present in the urine of house mice. The major histocompatibility complex (MHC) and major urinary proteins (MUPs) have both been suggested as potential markers of relatedness. Recently it has been demonstrated that the polymorphic multigene family of exocrine-gland secreting peptides (ESPs) also influence mouse odour, suggesting that ESPs could play a role in individual or even kin recognition in the house mouse.

Here wild-derived house mice were bred and genotyped to provide females where MHC, MUPs and ESP were known and could be independently controlled for to directly test kin recognition assumptions. ESP haplotypes were found to mirror MHC haplotypes and therefore any separate effects of the two markers remains inseparable. Females were presented with a choice of two equally related stimulus females (double cousins), one that matched at either MHC/ESP or MUP, and one that was dissimilar at MHC/ESP or MUP. The marker not being directly tested in each trial was dissimilar to the subject female so that it did not influence female choice, and was therefore held constant between the two stimulus females. Female recognition and preference was then tested using a scent discrimination and attraction assay and a nest partner choice assay. Females showed no discrimination of urine from matching and dissimilar stimulus females, but chose nest partners that matched exactly to self or to maternal cues for either MHC/ESP or MUP type. This is the first evidence that more than one family of genes might be involved in kin recognition between female house mice.

4.2 Introduction

One of the main questions dominating kin recognition research is how a cue of relatedness is transmitted from sender to receiver. Scent is a dominant method of communication in many mammalian species, and a large body of research exists on scent communication in the house and laboratory mouse. In Chapter 2 subject females investigated urine from unrelated females more than urine from related females, suggesting that there might be genetic markers of relatedness present in the urine of female house mice. Ideal molecular markers of relatedness require three essential characteristics for effective kin recognition: firstly markers should be genetically determined so that close relatives share markers through a common descent whilst unrelated individuals are unlikely to; secondly potential markers need to be sufficiently polymorphic that only close relatives share specific markers. This high degree of variation would mean that unrelated individuals should have different markers, preventing the false identification of non-kin; finally a molecular marker of relatedness should be consistent throughout an organism’s life. Variation in marker pattern or expression due to health, dominance, age or diet could obscure kin signals and prevent efficient identification (Hurst & Beynon, 2004).

In Chapter 2 subject females that spent longer in the cage of a related stimulus female shared more major urinary protein (MUP) peaks with the related stimulus female than with the unrelated stimulus female. However, whilst this suggests that MUPs may be important for kin recognition between female house mice, other potential markers of relatedness were not tested. It is therefore crucial to further investigate potential genetic markers of kin recognition by directly testing female response to equally related stimulus females that match or differ at those markers. Additionally, since the results of Chapter 3 suggested that females may use a match-to-maternal phenotype matching kin recognition mechanism when making nest partner decisions, it is important to examine female response to stimulus females that match or are dissimilar to the maternal profile.

4.2.1 The Major Histocompatibility Complex

By far the most widely studied of potential genetic markers of kin recognition is the major histocompatibility complex (MHC), a highly polymorphic family of genes that are important for self / non-self recognition in the immune system. Known as H-2 in mice and HLA in humans, the MHC family is found in all vertebrates. A high degree of polymorphism is found in the MHC region; across only two loci (H-2K and H-2D) up to 100 different alleles have

been observed in wild populations of mice (Klein, 1979). MHC was first linked to kin recognition through observations of males from different laboratory mice strains that displayed mating preferences based on MHC type (Yamazaki *et al.*, 1976). Since then a large number of studies in a range of different species have investigated the role of MHC in scent discrimination, individual recognition and kin recognition (in particular inbreeding avoidance where individuals attempt to avoid mates with the same MHC type or shared MHC alleles as themselves). MHC has been linked to kin recognition in mammals (including humans, Wedekind *et al.*, 1995; Penn & Potts, 1998a; Radwan *et al.*, 2008), birds (Freeman-Gallant *et al.*, 2003; Baratti *et al.*, 2012), fishes (Olsen *et al.*, 1998; Olsen *et al.*, 2002; Bahr *et al.*, 2012; O'Farrell *et al.*, 2012), amphibians (Villinger & Waldman, 2008) and reptiles (Olsson *et al.*, 2003). For a review of MHC and kin recognition in non-model vertebrates see Bernatchez & Landry (2003). Whilst at first glance the evidence for MHC appears overwhelming, in reality few studies do more than correlate observed behaviour with known MHC type. In many cases it is difficult to know whether MHC directly influences kin recognition or whether MHC might correlate with other non-investigated markers.

The role of MHC in kin recognition has been most studied in mice. In house mice MHC is found in the middle portion of chromosome 17 and split into three classes of gene (Klein, 1979). *Mhc* class I and II genes encode large (45 kDa) glycoproteins that bind to the surface of cell membranes and have a groove-shaped peptide binding region (Edwards & Hedrick, 1998). This groove is normally either empty or contains a self-peptide that enables the immune system to recognise that the cell is part of its own body. If the cell becomes infected then a peptide from the virus (an antigen) binds to the groove and is displayed on the surface of the cell. This allows the immune system to recognise that the cell is infected and destroy it to stop the spread of the infection (Brown & Eklund, 1994; Edwards & Hedrick, 1998). The integral role of MHC in the immune system is a likely explanation for the high degree of MHC polymorphism. This high degree of polymorphism may have then allowed for secondary functions such as individual or kin recognition (Tregenza & Wedell, 2000). Alternatively it has been proposed that the kin recognition function of MHC preceded the immune function of MHC (Boehm, 2006).

4.2.1.1 Mechanisms of MHC as a Marker for Kin Recognition

For individuals to use a genetic recognition mechanism based on MHC, they require a mechanism determining MHC type. A number of mechanisms for how MHC might affect

odour type have been suggested. One indirect route could be through metabolites released by bacterial flora found in the gut and/or urinary tract (Howard, 1977). Specific microbes will only be present if the immune system allows and therefore individuals with different MHC types could have a different gut flora, the metabolites of which could create different odour types. Experiments using germ-free laboratory mice however, suggest that this mechanism is unlikely, as urine from germfree mice can still be distinguished by laboratory mice (Yamazaki *et al.*, 1990). A second proposed mechanism is based on the observation that the development of organs appears to be influenced by MHC, and therefore variation in organ development as a result of MHC variation could create different odour profiles (Boyse *et al.*, 1987). The most widely accepted hypothesis for how MHC influences odour type is the 'carrier hypothesis' (Singh *et al.*, 1987; Singh, 1998, 2001). The carrier hypothesis suggests that MHC polypeptides may detach from the cell surface and fragments of these broken down polypeptides are transported to the urine. Once excreted in urine the binding site may relax and release the bound peptide which is subsequently detected as an odour. In an alternative mechanism, the peptide may have been lost before urination and the binding site may have bound other volatile metabolites that are released once excreted in the urine (Pearse-Pratt *et al.*, 1998). A molecular marker of genetic identity should ideally be consistent and not subject to changes as a result of age, health or diet. Infection or changes in metabolism can both influence MHC expression, potentially creating difficulties for reliable recognition of relatedness using MHC alone (Hurst & Beynon, 2004; Willse *et al.*, 2006).

Trained laboratory mice are able to distinguish urine from two inbred congenic laboratory mice that differ only at alleles in the MHC region (Yamazaki *et al.*, 1979; Yamazaki *et al.*, 1983). Additionally, untrained mice from laboratory-wild crosses are able to spontaneously discriminate between urine from two laboratory mice that differ specifically at one MHC locus (Penn & Potts, 1998b). These trials suggest that MHC is important for influencing mouse urinary odour and could therefore be integral to kin recognition. However, the mice from which the urine was sampled were laboratory mice and whilst they controlled for MHC, the background level of genetic variation was otherwise practically identical between the mice, making it unclear whether such specific discrimination would be possible against a natural level of genetic variation. Additionally, in a study by Ehman & Scott (2001) female laboratory mice spent an equal amount of time investigating urine from females that were the same and different for MHC, suggesting that MHC odour differences may not always be

detected (although females were not allowed to directly contact the urine and therefore would only be able to detect difference in the volatile component of MHC).

4.2.1.2 Evidence for MHC as a Marker in Kin Recognition

Evidence for MHC mediated kin recognition comes largely from studies of inbreeding avoidance using laboratory mice. Two mechanisms have been proposed for how animals may avoid inbreeding. Firstly the dispersal of one of both sexes could make reproductive encounters between relatives unlikely (Greenwood, 1980). However, in species where individuals do not disperse or dispersal is local, relatives may be encountered. A mechanism of kin recognition would enable mating between close relatives to be avoided and therefore it is expected that individuals show a preference for a mate that differs from self or parental type. Cross-fostering studies using laboratory strains of mice have demonstrated this and allowed researchers to separate the effects of parental MHC type from those of own MHC type. Yamazaki *et al* (1988) demonstrated male laboratory mice prefer females that differ at MHC from own parents. A free-breeding study using laboratory-wild crosses similarly showed that females preferred males that differed at MHC from the parents that reared them (Penn & Potts, 1998a). In a study without cross-fostering, laboratory female mice were shown to prefer MHC dissimilar-to-self males, although since individual strains of laboratory mice are uniform for MHC this could be as a result of behavioural imprinting on the MHC of parents, which happens also to be the same as own type (Egid & Brown, 1989).

Laboratory mice have also been used to explore other contexts of kin recognition. Dams are able to discriminate their own pups from otherwise genetically equivalent pups based on differences at the MHC locus only (Yamazaki *et al.*, 2000). Using crosses between laboratory strains and wild mice Manning *et al* (1992) showed that females in semi-natural populations preferred nest partners that share *Mhc* genes. This suggests that MHC is important for female – female kin recognition. However genetic background was not controlled for and therefore MHC might not be responsible for female choice, but may simply correlate with markers that are.

Laboratory strains of mice, and crosses between laboratory mice and house mice are useful for controlling specific loci but are limiting for kin recognition studies as they only have a limited amount of background genetic variation. Importantly, even between most strains, there are only two variants of MUP type (Cheetham *et al.*, 2009). Tests for kin recognition using laboratory strains are therefore lack variation in a second highly polymorphic multigene

family that strongly influences mouse urine odour. Recent studies of individual and inbreeding avoidance looking at both MHC and MUPs have suggested the importance of MUPs and found little evidence supporting a role of MHC (Hurst *et al.*, 2001; Cheetham *et al.*, 2007; Sherborne *et al.*, 2007). It is important therefore, to understand the role that MHC plays in kin recognition in house mice.

4.2.2 Major Urinary Proteins

In house mice Major Urinary Proteins (MUPs) are a multigene family of lipocalins that are often excreted in high quantities in the urine or other secretions such as saliva (Beynon & Hurst, 2004). *Mup* genes have been found in a number of mammal species including chimpanzee, orangutan, lemur, pig, horse and dog (Logan *et al.*, 2008). Of the other species screened so far only the horse and lemur appear to have more than one *Mup* gene, and humans have only a single *Mup* pseudogene (Logan *et al.*, 2008). Of the species so far examined, house mice and rats (in the rat the MUP family used to be known as A2U) have the greatest amount of MUP polymorphism. Examination of the *Mup* lineage revealed that the rat and house mouse MUP families expanded to their current degree of polymorphism independently but simultaneously (Logan *et al.*, 2008). Despite evidence of the MUP family in a number of other rodent species (for review, see Beynon *et al.*, 2008), the majority of research into MUPs has used the house mouse.

In house mice, *Mups* are found on chromosome 4 and consist of at least 19 functional genes, with a further 18 pseudogenes (Mudge *et al.*, 2008). The encoded lipocalins range in size from 18 kDa to 20 kDa (Beynon *et al.*, 2002; Hurst & Beynon, 2004; Mudge *et al.*, 2008) and an individual adult mouse will express in its urine 4 – 15 different MUP isoforms (Beynon & Hurst, 2003; Logan *et al.*, 2008; Mudge *et al.*, 2008; Hurst, 2009). MUPs are highly polymorphic in house mice (Robertson *et al.*, 1997), even in a highly geographically constrained population where general background genetic variation is low (Beynon *et al.*, 2002).

4.2.2.1 Mechanisms of MUP as a Marker in Kin Recognition

Whilst some MUPs are expressed in the salivary glands, mammary glands, lacrimal glands and nasal tissue, most MUPs are expressed in the liver and pass through the kidneys into the urine (Shaw *et al.*, 1983; Shahan *et al.*, 1987; Utsumi *et al.*, 1999). MUPs are found in extremely high volume in the urine of both male and female house mice (Robertson *et al.*, 1997; Humphries *et al.*, 1999), although males excrete a concentration of MUPs three to four

times as high as females (Beynon & Hurst, 2004). This volume of excreted protein has been suggested as a substantial cost of scent marking, as synthesising large amounts of protein could be energetically expensive (Gosling *et al.*, 2000). One specific MUP, darcin, is found in male but rarely in female urine, and is a sex pheromone that stimulates female learning and sexual attraction (Roberts *et al.*, 2010). MUPs are not themselves volatile, and require direct contact with the vomeronasal organ in order to be directly identified (Ramm *et al.*, 2008). However, the structure of MUPs provides a central cavity or pocket that binds hydrophobic ligands (Flower, 1996). Once in the environment (deposited as a scent mark) this structure allows volatiles, that would otherwise evaporate rapidly, to be slowly released and so prolongs the duration of the scent signal (Hurst *et al.*, 1998).

MUPs are very stable and individual MUP patterns that are expressed are consistent throughout a mouse's lifetime (Beynon & Hurst, 2004). This stability means that the signal produced by MUPs in mouse urine is consistent over time and, given the high degree of polymorphism, MUPs could therefore be used as a reliable signal of identity (Robertson *et al.*, 1996; Cavaggioni *et al.*, 1999; Beynon & Hurst, 2003). The importance of MUPs in individual recognition has been demonstrated in wild house mice (Hurst *et al.*, 2001; Cheetham *et al.*, 2007). Cheetham *et al.* (2007) also showed that MUPs and not MHC were important in female individual recognition of males.

4.2.2.2 Evidence for MUP as a Marker in Kin Recognition

The high degree of polymorphism of MUPs, along with the reliability of the signal as a marker for individual identity suggests that MUPs could also be an important marker for genetic kin recognition in house mice. However, studies of genetic markers of kin recognition have largely focussed on MHC, and frequently utilised laboratory strains of mice. Whilst laboratory strains of mice present a number of advantages (e.g. controlled genetic background), a recent study has demonstrated that all observed laboratory strains have only two basic MUP patterns (Cheetham *et al.*, 2009). Studies of kin recognition using laboratory strains are therefore lacking a large amount of variation in a highly polymorphic family of proteins that are found in high quantities in mouse urine. By using wild house mice, Sherborne *et al.* (2007) demonstrated that mice may avoid inbreeding by using self-referent matching of MUP patterns, but found no evidence to support MHC-disassociative mate choice. They also found no evidence to support maternal behavioural imprinting being used to avoid mating with close relatives. However, it is important to note that this study was not a

direct assessment of mate choice based on MHC or MUP sharing and was instead a free breeding experiment where successful matings were then compared to known genotypes. No work has as yet looked at the role of MUPs in other contexts of kin recognition such as female nest partner choice.

4.2.3 Exocrine-Gland Secreting Peptides

It has recently been demonstrated that differences in a third multigene family, exocrine-gland secreting peptides (ESPs), can be detected by laboratory mice (Kimoto *et al.*, 2005; Kimoto *et al.*, 2007). Initially a male-specific peptide (*Esp1*) was identified and shown to stimulate female vomeronasal sensory neurons (Kimoto *et al.*, 2005). Further examination by Kimoto *et al.* (2007) revealed that this peptide was one of a family of 38 *Esp* genes in mice, including a second sex-specific peptide expressed by females only (*Esp*36). Rats also have a family of *Esp* genes, although in rats this is on a different chromosome to MHC (Kimoto *et al.*, 2007). A possible *Esp* gene has been observed in opossum but the human genome appears to contain no *Esp* genes (Kimoto *et al.*, 2007).

The ESP family of genes are based on mouse chromosome 17 near the class I MHC region, and between the two regions is a cluster of olfactory receptor genes. Of the 38 identified *Esp* genes 14 appear to be pseudogenes, leaving 24 *Esp* genes that are functional and encode intact proteins (Kimoto *et al.*, 2007). The size range of the proteins encoded by these *Esp* genes is predicted to be between 5 kDa and 15 kDa (Kimoto *et al.*, 2007). Comparison of ESPs between laboratory mice and rats found a number of mice-specific clades, suggesting an expansion of the ESP family during the evolution of house mice (Kimoto *et al.*, 2007). The levels of individual ESP expression also differ between laboratory mouse strains (Kimoto *et al.*, 2007). ESPs are expressed in the extraorbital lacrimal and Harderian glands (released in tear fluid), and in the submaxillary (released in saliva; Kimoto *et al.*, 2007). As yet there is no evidence for ESP being found in mouse urine.

The male-specific peptide, *Esp1*, is currently the most studied of the ESP family. *Esp1* is expressed in the extraorbital lacrimal gland and when a female makes close contact with the facial area of an adult male, or bedding soiled by an adult male, the protein is taken up into the vomeronasal organ and stimulates V2R-expressing vomeronasal sensory neurons (Kimoto *et al.*, 2005; Haga *et al.*, 2010). Those neurons that respond to *Esp1* are different from neurons that respond to MHC or MUPs in mouse urine (Kimoto *et al.*, 2007). ESPs may therefore be separate signals of sex or identity in mice.

Whilst there is currently no evidence for ESPs being used during kin recognition, the differences in ESP expression between strains, expansion of the lineage in mice, and evidence of a distinct neural pathway all suggest that non-volatile peptides from this multigene family could be important in scent communication in mice. It is therefore important to further understand the role of ESPs in mouse scent communication, and assess whether they might play a role in kin recognition between house mice.

4.2.4 Match-To-Self and Match-To-Maternal Templates

In a phenotype matching kin recognition mechanism a template is learnt from a known relative and later compared against the phenotypes of novel individuals. As discussed in Chapter 3 (Section 3.2) this recognition template could originate from one or more relatives, or from the learning individual themselves. Self and mother are often the most reliable options as they are reliably present in the nest and reliably related to the learning individual. Paterson & Hurst (2009) found that a match-to-self mechanism when using a single recognition locus was more effective at identifying relatives than a match-to-self mechanism using multiple loci. In contrast, a partial match-to-maternal mechanism using a single recognition locus is not very effective but as the number of recognition loci used increase the effectiveness of the mechanism improves as a lower proportion of unrelated individuals are falsely identified. Whilst individual MHC, MUP or ESP haplotypes consist of alleles at a number of different loci, a complete MHC, MUP or ESP haplotype may act as a single allele at a single recognition locus (Paterson & Hurst, 2009). Individuals may use one or more markers as a recognition template and this template could originate from self and/or mother (or from elsewhere).

The results of Chapter 3 suggested that female house mice may use a match-to-maternal mechanism when making nest partner choice decisions. Some studies using laboratory and mouse mice to investigate the roles of MHC and/or MUP in kin recognition have however, suggested a match-to-self phenotype matching mechanism. Egid & Brown (1989) found that female laboratory mice prefer males of a dissimilar MHC type to themselves, suggestive of a match-to-self mechanism. Using house mice Sherborne *et al.* (2007) found evidence to suggest that females may use a match-to-self phenotype matching mechanism based on MUP type, as there was a deficit of successful matings between mice that matched for both MUP alleles. The experiments described in this chapter investigate both the molecular markers

used in kin recognition between females, and further investigate whether females use a match-to-self and/or a match-to-maternal template mechanism.

4.2.5 Chapter Aim

There is evidence for a role of both MHC and MUP in kin recognition in the house mouse. The recently discovered ESP family may also be important in the ability of house mice to recognise their relatives. The experiments aim to investigate whether MHC, MUP and ESP are important for kin recognition in female house mice. This was addressed by asking the following questions:

i) Can female house mice discriminate urine from two equally related females that match or are dissimilar for either MHC, MUP or ESP genotype?

ii) Do female house mice preferentially associate with females that match for MHC type over equally related females that are dissimilar for MHC genotype?

iii) Do female house mice preferentially associate with females that match for MUP type over equally related females that are dissimilar for MUP genotype?

iv) Do female house mice preferentially associate with females that match for ESP type over equally related females that are dissimilar for ESP genotype?

v) Does discrimination of, and association towards matching females depend on whether the stimulus female matches the subject or the subject's mother (match-to-self or match-to-maternal)?

To that aim, mice were bred that would have a limited set of MHC, MUP and ESP types but an otherwise natural level of genetic diversity with no inbreeding. In this way subject females could be presented two equally related stimulus females, one that matched (to self or mother) for one marker (e.g. MHC) but differed at the other markers (e.g. MUP and ESP) and one that differed at that same marker (e.g. MHC) but had the same other markers (e.g. MUP and ESP) as the 'matching' stimulus female. As stimulus females were equally related to the subject females any discrimination and association observed would be based on matching for each specific marker alone. To obtain subject and stimulus females, double cousins were bred and genotyped for MHC, MUP and ESP. Females discrimination and attraction were assessed using a scent discrimination and attraction assay and a nest partner choice assay.

4.3 Methods

To assess the importance of MUPs, the MHC and ESP in kin recognition between female house mice, subject females were presented with stimulus females of an equal degree of relatedness but matched or were dissimilar for either MHC, MUP or ESP genotype (matching to self or mother). To produce females that would have a limited set of MHC, MUP and ESP but an otherwise natural level of background genetic variation, double cousins were bred. Double cousins are created when siblings from one parental lineage reproduce with siblings from a separate parental lineage (see Figure 4.1). For instance, two brothers (e.g. A1 and A2) could breed with two sisters that are unrelated to themselves (e.g. B1 and B2). The resulting offspring from litter A1 x B1 and litter A2 x B2 would be double cousins to each other as they are cousins through both their maternal and paternal parental lines. To create double cousins, F0 mice were caught from two geographically separate populations and then paired with opposite-sex individuals of the same population. These pairings were then allowed to breed several litters of full siblings (F1 generation mice) for each population. The resulting full siblings from one population were then crossed with full siblings from the second population to create several litters of individuals that were double cousins of each other but remained completely outbred (F2 generation mice). As no relatives were directly bred to create double cousins, all females were completely outbred.

Additionally, to assess how similar urine from MHC, MUP or ESP matching females appeared, a separate line of mice were bred that were entirely unrelated to the double cousin line. These neutral unrelated females were then used to assess scent similarity in an odour-genes covariance test.

In the match-to-self template groups double cousins (F2) were used as stimulus females. However, as half the genome is inherited from the mother and half from the father, no offspring will match completely the profile of an individual parent in an outbred population. Therefore the sisters of dams (F1) were used as stimulus females in the match-to-maternal template groups (the aunts of subject females).

4.3.1 House Mouse Trapping

For the double cousin line mice were trapped from two locations in the North West of England, UK (Knowsley Safari Park, Prescot, Merseyside and Holme Farm, Ince, Cheshire) between February and December 2010. The caught founder mice were known as the F0 generation. Mice from the same population were bred from to produce an F1 line from each

location, and then the two F1 lines were crossed to produce F2 generation mice, litters of which were double cousins of each other (see Figure 4.1).

For females that were unrelated to mice in the double cousin line, mice were trapped from Hatchwood Farm, Chorley, Lancashire, UK in March 2011, and crossed with outbred wild-derived mice from the Mammalian Behaviour & Evolution group colony (originally established from populations in the North West of England, UK).

Mice were trapped using Longworth small mammal traps (Longworth Scientific Instrument Co., Abingdon, UK; Chitty & Kempson, 1949). Traps were baited with peanut butter and contained paper – wool bedding material (Shredded Nesting, International Product Supplier Limited, London, UK), food (Lab Diet 5002, International Product Supplies Limited, London, UK) and fresh pieces of apple. Traps were set and left overnight and checked for mice the following morning. Mice were quarantined on return to the laboratory and screened for *Lymphocytic choriomeningitis* virus (LCMV) by Amanda Davidson at the Mammalian Behaviour & Evolution Group, University of Liverpool, UK. Mice were also visually checked for signs of mites before the breeding programme commenced.

4.3.2 Animal Housing and Handling

Animal housing and handing methods were the same as detailed previously (see Section 2.3.1). Mice were handled using clear acrylic handling tubes (18 cm long, 5 cm diameter, one open end and one end closed with aluminium mesh of 0.5 x 0.5 cm).

4.3.3 Breeding the Double Cousin Line

In order to produce the maximum number of double cousins (F2) and so allow for the greatest choice in assigning subject and stimulus pairings, several litters of F1 full sibling mice were bred from the founder mice (F0). As a minimum, only four F0 mice were needed: a male and a female from each location. Two pairs of mice from Knowsley Safari Park and three pairs of mice from Holme Farm mice were established and allowed to breed consecutive litters (see Section 4.3.5 for breeding protocol). Of these, only the offspring from the most successful pairing from each location were then used to breed double cousins. Fourteen crossed pairs of F1 Knowsley Safari Park (six males and eight females) and F1 Holme Farm (eight males and six females) mice were established (see Section 4.3.5 for breeding protocol) to produce double cousins (see Figure 4.1 for double cousin breeding diagram).

4.3.4 Breeding the Unrelated Line

Three pairs of Hatchwood Farm mice (F0) were established (see Section 4.3.5 for breeding protocol), and only offspring from the most successful pairing were used for the unrelated line. Six F1 Hatchwood Farm males were paired with six unrelated wild-derived outbred females from the Mammalian Behaviour & Evolution group colony (see Section 4.3.5 for breeding protocol). The offspring of these pairings (F2) were used as the unrelated mice for the odour-genes covariance assay to assess the similarity between the urine from mice that shared MHC, MUPs or ESP.

4.3.5 Breeding Protocol

The breeding protocol for both the double cousin line and unrelated line was the same as for the half-sisters bred in Chapter 3, Section 3.3.2.2. All mice were over 3 months old and weighed over 13 g. All F1 females were RFID tagged a minimum of 2 weeks before introduction. Both females and males were primed with opposite and same-sex bedding for 2 weeks prior to introductions to familiarise mice with opposite-sex odours and ensure regular oestrus cycling in females (Marsden & Bronson, 1964; Cheetham *et al.*, 2007). Additionally, females were primed so as to be in or entering oestrus at the time of introduction to males. All mice were weighed to establish baseline measurement prior to introduction. Pairs of mice were introduced in a MB1 cage (45 x 28 x 13 cm, North Kent Plastics, UK) provided with additional environmental enrichment to enable mice to avoid each other. Cages were monitored for excessive levels of aggression immediately after introductions and regularly checked over the following 24 hours. Females were checked and weighed on days 17, 21 and 25 after pairing to determine pregnancy.

When the paired mice were F0 (wild-caught) the male was left in the cage when the female was discovered to be pregnant with the aim of the male impregnating the female soon after birth to produce successive litters of full siblings. When the paired mice were F1 (and so being bred from to produce double cousins) the male was removed from the cage when the female was discovered to be pregnant. After pregnancy was established cages were checked daily for litters.

On the day of birth the number of pups was recorded and the both the dam and the litter were weighed. At weaning, 24 days after birth, dams and litters were weighed, pups were counted and sexed. Male pups were singly housed in M3 cages (48 x 15 x 13 cm, North Kent Plastics, UK) and female pups were housed in groups of no more than four individuals in MB1 cages.

Litters consisting of five or more females were split so that all females were housed in groups of at least two individuals. Females from single female litters were housed together in pairs with other single females. Pups were a minimum of three months old before being used for breeding or behavioural assays.

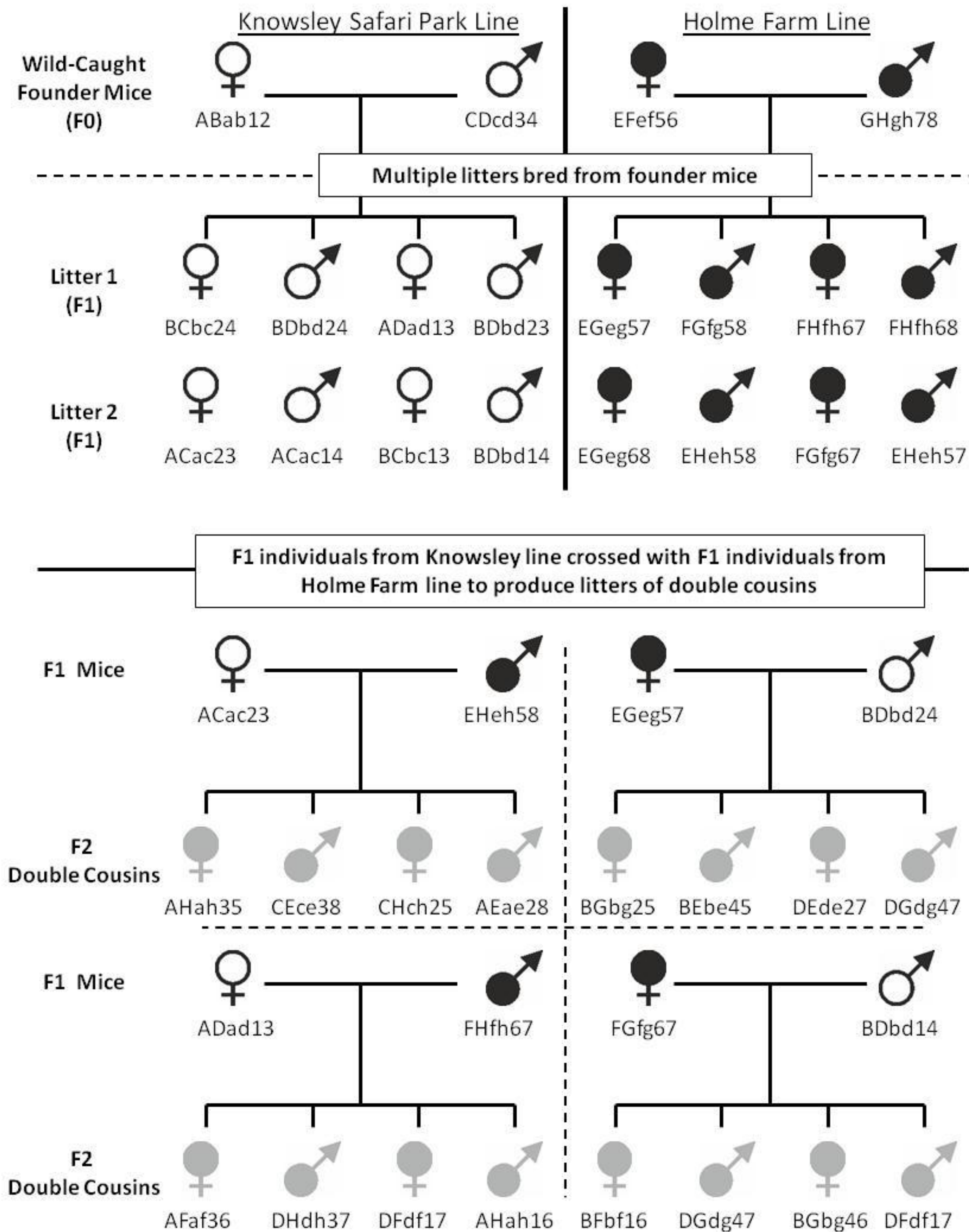


Figure 4.1: Breeding programme for double cousins.
Founder mice (F0) caught from Knowsley Safari Park (open symbols) and Holme Farm (black symbols) were paired with same-population mice and allowed to breed consecutive litters. The offspring from these pairings (F1) were then crossed to breed litters that would be double cousins (grey symbols) of each other (F2). Example MHC, ESP and MUP genotypes are also shown for each individual. MHC is denoted as capital letters (e.g. AB), ESP as lower-case letters (e.g. ab), and MUP as numbers (e.g.12). As MHC and ESP genotypes were found to match (see Section 4.4.3 for results), the MHC and ESP letters are the same.

4.3.6 Breeding Success

4.3.6.1 Founder Pair Breeding

Two pairs of Knowsley Safari Park mice, three pairs of Holme Farm mice and three pairs of Hatchwood Farm wild-caught founder mice were initially established and allowed to breed for multiple litters. Offspring from the most successful pairing from each founder population were then used for crossing for further breeding. The results of the founder pair breeding are presented below in Table 4.1.

Table 4.1: Breeding success of wild-caught founder pairs from each location and the offspring from which pairs were used for the second round of breeding.

Location	**Pair Number (Female-Male)**	**Number of Litters**	**Number of Females**	**Number of Males**	**Further Breeding**
Knowsley Safari Park	9287 - 9290	2	8	6	Yes
Knowsley Safari Park	9292 - 9290	1	2	3	No
Holme Farm	8544 - 8866	3	7	9	Yes
Holme Farm	8513 - 9056	2	5	3	No
Holme Farm	8514 - 9057	2	3	2	No
Hatchwood Farm	9685 - 9687	1	1	7	Yes
Hatchwood Farm	9690 - 9688	1	3	4	No
Hatchwood Farm	9717 - 9715	1	6	1	No

4.3.6.2 F2 Pair Breeding

Fourteen F1 mice from the Knowsley Safari Park line (six males and eight females) were crossed with 14 F1 mice from the Holme Farm line (eight males and six females) to produce 14 litters of double cousins. Six F1 males from the Hatchwood Farm line were crossed with six females from the wild-derived outbred laboratory colony of the Mammalian Behaviour & Evolution group to produce six litters of mice unrelated to the mice in the double cousin line. The results of these pairings are shown in Table 4.2.

Table 4.2: Breeding success of F1 mice in the double cousin line and the unrelated line.

	Double Cousin Line	**Unrelated Line**
Total number of pups	93	40
Total number of females	33	18
Total number of males	60	22
Mean number of females per litter	2.4	3.0
Mean number of males per litter	4.3	3.7

4.3.7 MHC, MUP and ESP Genotyping

4.3.7.1 Genotyping Methods

MHC, MUP and ESP genotype were established for all double cousin F1 and F2 mice using the protocol in Sherborne *et al* (2007), aside from the use of a different DNA extraction kit. Microsatellite markers were used to establish the haplotypes of parents (F1) and offspring (double cousins, F2) for MHC, MUP and ESP.

Mice were anaesthetised using a mix of oxygen and halothane on a Compact Anaesthetic Workstation (Model No. AN001, Vet Tech Solutions Ltd, UK). A 1 – 5 mm tail snip was taken from each mouse as it was unconscious, and an RFID (FSI) chip was injected under the skin in the middle of the back of each female F2 to allow for identification of females and matching to genotype data. The tail snip procedure was carried out by John Waters and Amanda Davidson of the Mammalian Behaviour and Evolution Group, University of Liverpool, UK under Home Office license (Project Licence (PPL) 40/3492). Subsequent DNA extraction and polymerase chain reaction (PCR) amplification were carried out by

Amanda Davidson at the Mammalian Behaviour and Evolution Group and the Protein Function Group respectively.

DNA extraction was carried out using a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, West Sussex, UK), following the manufacturer's instructions precisely. To establish the haplotypes four microsatellite markers from across the MHC region on chromosome 17 were used, six microsatellite markers from across the MUP region on chromosome 4 were used, and three microsatellite markers from across the ESP region on chromosome 17 were used. These were selected from the Mouse Genome Informatics site (MGI 5.1.3) and are displayed in Table 4.3. The forward primer for each of the 13 microsatellite markers was 5'-end fluorescently labelled with 6-FAM, PET, NED or VIC phosphoramidite. This allowed for multiple markers to be pooled into a single run.

PCR amplification was conducted in 10 µl reactions of 20 ng DNA, 0.5 µM primer and 5.0 µl of BioMix Red reaction mix (Biolin, London, UK). The PCR protocol steps were: an initial denaturation for 2 minutes at 95 °C; 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 52 °C – 58 °C (depending on the primer) for 2 minutes, and extension at 72 °C for 30 seconds; and after the 30 cycles were complete a final extension at 72 °C for 10 minutes. The PCR reactions were then diluted to 25- to 50-fold (depending on primer set) and multiplexed in formamide with GeneScan LIZ500 size standard (Applied Biosystems). Haplotype size was determined with an ABI PRISM 3100 DNA analyzer and GeneMapper v3.0 software (Applied Biosystems).

The resulting data was compiled in MS Excel (2007). The output was grouped into MHC, MUP and ESP markers. Individuals had two alleles for each microsatellite marker. Full sibling parents (F1) from each founder population were grouped to enable identification of haplotypes for MHC, MUP and ESP. Offspring (double cousins, F2) haplotypes were then assigned based on parental haplotype and own allele set. This process established the genotypes for MHC, MUP and ESP for each individual (as denoted subsequently as capital letters, numbers and lower case letters respectively). MUP genotypes were then confirmed using MUP phenotypes produced by electrospray ionization mass spectrometry (discussed in Section 4.3.8).

Table 4.3: Microsatellite markers used for MHC, MUP and ESP Genotyping

MHC Marker	Label	Location	Size	Repeat	Forward Primer	Reverse Primer	Comments
D17Mit22	VIC	17 B2 33.9 Mb	216	GT	5'-GCATTAGATAGAGAGTAGATGGGTTG-3'	5'-ATGGATGGCGAGAATGAGAC-3'	Used
D17Mit231	PET	17 B2 34.1 Mb	285	GT	5'-GCCTCAGCAAGACCCTAAAC-3'	5'-ACTCCTCCTTTTCCCTCTCC-3'	Used
D17Mit13	FAM	17 B2 34.6 Mb	239	GT	5'-TGCAGGCAAGATCCAAGAAG-3'	5'-GAAAGAGGGTGTCGATGCTC-3'	Used
D17Mit47	NED	17 B3 35.8 Mb	238	CA	5'-CTGAGACCAGTGCAGTGGAA-3'	5'-TTTTTCAATATGTGAGCATGTGC-3'	Used
MUP Marker	**Label**	**Location**	**Size**	**Repeat**	**Forward Primer**	**Reverse Primer**	**Comments**
D4NDS6	VIC	4 B3 52.8 Mb	240	GT	5'-CGGGGAAGGTTGTTTGTTTG-3'	5'-AGGCCAGCAATGTAGAAAGG-3'	Used
D4Mit139	PET	4 B3 55.2 Mb	149	GT	5'-TCAAACTGGGAAGAGCCAAG-3'	5'-GCCGTAGAAGAGAAGTAATTTTTCC-3'	Used
D4Mit241	FAM	4 C1 55.7 Mb	219	CA	5'-TTTCCAGTGTTGTCCAGAGC-3'	5'-AAGGCAAATCACTAGGTGCTG-3'	Used
D4Mit164	NED	4 C1 59.5 Mb	142	CA	5'-AACACATATATACCAAGGCAGCAC-3'	5'-ATTTCCACCCTGTCCACTCC-3'	Used
D4Mit217	NED	4 C1 59.7 Mb	246	GT	5'-ACTCAATTAGGTTGTTCAGATAGCC-3'	5'-GGCACTTGCTGCCACATC-3'	Used
D4Mit17	FAM	4 C1 62.8 Mb	138	GT	5'-GCCAACCTCTGTGCTTCC-3'	5'-CCTCTGACATCCACACACATC-3'	Used
ESP Marker	**Label**	**Location**	**Size**	**Repeat**	**Forward Primer**	**Reverse Primer**	**Comments**
D17Mit234	NED	17 B1 39.1 Mb	100 - 129	CA	5'-GCAAAGACAAAAATTGAAATGTG-3'	5'-CTGCTTAGCACACATGCTTTG-3'	Used
D17Mit126	VIC	17 B1 41.1 Mb	125 - 137	CA	5'-TATGTGGCATCTCTTTATTCATGA-3'	5'-GCCAAGGATTGTCTGCCTTA-3'	Used
D17Mit263	FAM	17 B1 41.8 Mb	103 - 113	CA	5'-TATACATATGGGCTGTGTCACATG-3'	5'-TTAAGTTATTTATCACTGTGGGGTG-3'	Problems

4.3.7.2 Genotyping Results

Two MHC haplotypes were discovered from the Knowsley Safari Park line and two different MHC haplotypes were discovered from the Holme Farm line (see Table 4.4). The crossing of the lines in the second round of breeding meant that there were four possible MHC genotype combinations.

Table 4.4: MHC haplotypes showing the allele for each microsatellite marker.

	MHC Microsatellite Marker			
Line	**D17Mit22**	**D17Mit231**	**D17Mit13**	**D17Mit47**
Knowsley Safari Park	180	267	230	211
Knowsley Safari Park	196	267	232	236
Holme Farm	176	286	230	227
Holme Farm	206	286	224	227

Five MUP haplotypes were discovered in the Knowsley Safari Park line (four would be expected but the additional one appears to be the result of a crossover) and four different MUP haplotypes were found in the Holme Farm line (see Table 4.5). When the lines were crossed to breed the double cousin litters, 15 different MUP genotype combinations were observed in the resulting offspring.

One of the ESP microsatellite markers, D17Mit263, had amplification problems and so was excluded from the analysis. Two ESP haplotypes were found in the Knowsley Safari Park line, and two ESP haplotypes were found in the Holme Farm line, however one haplotype was found in both lines meaning that only three different haplotypes were found in total (see Table 4.6). When the lines were crossed in the second round of breeding four ESP genotypes were possible (three heterozygous and one homozygous).

Table 4.5: MUP haplotypes showing the allele for each microsatellite marker.

	MUP Microsatellite Marker					
Line	**D4NDS6**	**D4Mit139**	**D4Mit241**	**D4Mit164**	**D4Mit217**	**D4Mit17**
Knowsley Safari Park	224	142	220	76	245	134
Knowsley Safari Park	240	142	220	76	245	134
Knowsley Safari Park	240	127	216	130	245	127
Knowsley Safari Park	240	127	216	130	245	134
Knowsley Safari Park	224	142	216	130	245	127
Holme Farm	224	142	226	87	247	131
Holme Farm	234	142	226	87	247	131
Holme Farm	224	142	226	97	249	131
Holme Farm	234	142	226	97	245	121

Table 4.6: ESP haplotypes showing the allele for each microsatellite marker.

Line	**ESP Microsatellite Marker**	
	D17Mit234	**D17Mit126**
Knowsley Safari Park	118	127
Knowsley Safari Park & Holme Farm	114	136
Holme Farm	122	140

4.3.7.3 Matching of MHC and ESP

In total four MHC genotypes and three ESP genotypes were found. Together this should give a total of 12 different combinations of MHC and ESP genotypes that could be present in the mice. However, only four combinations were observed. Further investigation revealed that each of the four observed MHC haplotypes were always paired with a specific ESP haplotype (see Table 4.7). As the same MHC and ESP haplotypes were found paired together, females were matched according to MHC/ESP and the two separate markers were treated as one for the remainder of the study.

Table 4.7: Matching between MHC and ESP haplotypes.
The four MHC haplotypes were always found to be matched to the same three ESP haplotypes.

	MHC Microsatellite Marker					ESP Microsatellite Marker	
Line	**D17Mit22**	**D17Mit231**	**D17Mit13**	**D17Mit47**	**Line**	**D17Mit234**	**D17Mit126**
Knowsley Safari Park	180	267	230	211	Knowsley Safari Park & Holme Farm	114	136
Knowsley Safari Park	196	267	232	236	Knowsley Safari Park	118	127
Holme Farm	176	286	230	227	Holme Farm	120	140
Holme Farm	206	286	224	227	Knowsley Safari Park & Holme Farm	114	136

4.3.8 Major Urinary Protein Phenotyping

Whilst genotyping revealed the microsatellite MUP haplotypes, it is possible that difference in the actual *Mup* genes may be present that are not detected by microsatellites alone. Between individuals with the same MUP microsatellite genotype there may be difference in the actual *Mup* genes which would express different MUP phenotypes. MUP phenotypes were therefore also examined to ensure that individuals with the same microsatellite haplotypes were expressing the same MUP phenotype.

Urine samples were collected using the recovery method once mice were over 3 months old. Only F1 and F2 mice from the double cousin line were phenotyped. Electrospray ionization mass spectrometry was used to establish the mass profiles of MUP samples (following the methods described in Mudge *et al.*, 2008). This process establishes the molecular mass of individual proteins present in a urine sample. This allows for females to be matched for MUPs according to both the known genotype and the phenotype expressed in female urine. Samples were run by Amanda Davidson at the Protein Function Group, University of Liverpool, UK. Samples were run using a Nanoacquity ultra high performance liquid chromatography system (Waters, Manchester, UK) and were processed and transformed to a true mass scale using MazEnt1 deconvolution software (Waters Micromass, Massachusetts, USA).

The MUP phenotypic profiles of all F1 and F2 double cousin line mice were inspected in SpecAlign (Version 2.4.1; http://physchem.ox.ac.uk/~jwong/specalign/). The mass of all known MUPs falls between 18600 and 18900 Da (Mudge *et al.*, 2008) and therefore mass spectra were cropped to include only this mass range. The protein peaks for each spectrum were then normalised to the intensity of the most abundant protein. This allows for the comparison of mass spectrum profiles from different females, as individual urine samples will contain different concentrations of protein. Peaks were considered true peaks if they had a relative intensity of greater than 0.15. This value was chosen as peaks of a lower intensity may not represent true proteins, and any peaks of a lower relative intensity were excluded.

A MUP phenotype profile was established for each female. Each peak was recorded according to mass and then relative intensity. These profiles could then be compared between females to ensure that subject females were assigned stimulus females that for MUP either matched themselves (for both genotype and phenotype) or were dissimilar to themselves (for both genotype and phenotype). All females that were assigned as matching for MUP shared

MUP peaks of the same mass and of a relative intensity of less than 0.50. All females that were assigned as dissimilar for MUP had at least one significant peak difference in their MUP phenotypic profile: the presence or absence of a MUP peak of a relative intensity of greater than 0.50.

4.3.9 Behavioural Assay Schedule

Females were assigned to two molecular marker groups: the MHC/ESP and the MUP group. Within each of these groups subject females were presented with matching stimulus females that matched either the subject's own genotype or the subject's mother's genotype. Subject females were given two behavioural assays during which they were presented with two stimulus females or the urine from two stimulus females. First, subject females were given a short scent discrimination and attraction assay designed to test whether females spontaneously discriminate between urine from females that match or are dissimilar for MHC/ESP or MUP type. Subject females were then given a nest partner choice assay to establish whether subject females preferentially associate with MHC/ESP or MUP matching stimulus females compared to MHC/ESP or MUP dissimilar stimulus females. The same individual triads of females (a subject and the corresponding two stimulus females) were used in each assay.

All scent discrimination and attraction assays were carried out before the nest partner choice assays. The double cousin breeding programme only produced 33 females, and one of these died. The limited number of females meant that 27 females were used as subject females twice. Females were never used as subject females in the same molecular marker group. One full week was left between first and second scent discrimination and attraction assays. After the second set of scent discrimination and attraction trials were finished, females were given the nest partner choice assay. A maximum of ten subject females were tested at any one time, with a minimum of 24 hours between trials.

A separate odour-genes covariance assay also assessed whether neutral female house mice can detect a similarity between the urine of females that match for either MHC/ESP or MUP compared to urine from females that are dissimilar at MHC/ESP or MUP. Subject females for the odour-genes covariance assay were unrelated and unfamiliar with any of the stimulus females. Subject females were presented with three urine samples: urine from a habituation female, urine from a matching (for MHC/ESP or MUP) discrimination female and urine from a dissimilar (for MHC/ESP or MUP) female. Urine samples were provided by the same triad

of females as those tested in the scent and nest partner assays: previous subject females provided the habituation urine and the previous corresponding matching and dissimilar stimulus females provided the matching and dissimilar discrimination respectively.

As only 18 females were born in the unrelated line, four separate sessions of the odour-genes covariance assays were run, separated by one week. No unrelated female ever encountered urine from the same female more than once, nor experienced the same group (MHC/ESP and MUP; Self or Maternal) more than once. Within these parameters unrelated females were randomly allocated the triad of odours (one habituation and two discrimination odours).

4.3.10 Assignment of Matching and Dissimilar Stimulus Females

The limited number of available females meant that all females expect for one were used as stimulus females more than once. Where females were repeatedly used as stimuli, all attempts were made to ensure that they were used in different MHC/ESP and MUP groups, and where repeated use in the same group occurred females were used as the different stimulus (matching or dissimilar). The mean stimulus usage for each female was 3.88 times, although the median was three uses. No two females repeatedly met to keep familiarity constant.

4.3.10.1 Matching to Self or Maternal Genotype

Whilst the focus of this study was to investigate whether MHC/ESP or MUP are important for kin recognition between female house mice, an additional aspect was to further examine matching to self and maternal cues. Within each marker group subject females were presented with matching stimulus females that matched either the subject's own genotype or the subject's mother's genotype.

4.3.10.2 Match To MHC/ESP

In the MHC/ESP marker group females were presented with a 'matching' stimulus female that had the same MHC/ESP genotype as either the subject female or the subject female's mother but a different MUP genotype, and a 'dissimilar' stimulus female had a different MHC/ESP genotype compared to the subject female or the subject female's mother, but had the same MUP type as the matching stimulus female. In this way female recognition was assessed based on matching of MHC/ESP genotype but against a consistent but different MUP genotype (see Table 4.8).

4.3.10.3 Match To MUP

In the MUP marker group females were presented with a 'matching' stimulus female that had the same MUP genotype as either the subject female or the subject female's mother but a different MHC/ESP genotype, and a 'dissimilar' stimulus female had a different MUP genotype compared to the subject female or the subject female's mother, but had the same MHC/ESP type as the matching stimulus female. In this way female recognition was assessed based on matching of MUP genotype but against a consistent but different MHC/ESP genotype (see Table 4.8).

4.3.10.4 Subject and Stimulus Females

Females were assigned to two marker groups: MHC/ESP (match-to-self n = 15; match-to-maternal n = 15) and MUP (match-to-self n = 15; match-to-maternal n = 15). The same triads of females (subject female, matching stimulus female and dissimilar stimulus female) were used in both the scent discrimination and attraction assay and the nest partner choice assay. These same triads were then used as the habituation and discrimination urine donors in the odour-genes covariance assay, with females from the unrelated line as neutral subjects.

Double cousins were used as stimulus females for the matching to self tests. All stimulus and subject females were 6 to 7 months old at testing and aged within 1 month of each other. Where possible stimulus females were matched according to mass (within 2 g), however the limited numbers of available mice did not always allow for this and in 6 out of 30 pairings the mass difference was between 2 and 5 g.

As offspring from heterozygous parents cannot have the exact same genotype as their dam, the bred double cousins were not suitable for using as stimulus females in the matching to maternal tests. Instead stimulus females were aunts of the subject female – sisters of their dam that shared the exact genotype for one specific marker but not the other. All stimulus females in the match to maternal groups were approximately 4 months older than the subject females, aged within 4 months old of each other and were over 9 months old at testing. Where possible stimulus females were matched according to mass (within 2 g), however the limited numbers of available mice did not always allow for this and in 10 out of 30 pairings the mass difference was between 2 and 5 g.

Table 4.8: Example genotypes for Match-to-MHC/ESP and Match-to-MUP groups, split into match-to-self and match-to-maternal groups.

	MHC/ESP		MUP	
	Self	Maternal	Self	Maternal
Subject Female	**AHah**35	DEde27	CHch**25**	AFaf36
Subject Female Dam	ACac23	**EGeg**57	ACac23	ADad**13**
Matching Stimulus Female	**AHah***16*	**EGeg***68*	*BGbg***25**	*BCbc***13**
Dissimilar Stimulus Female	BFbf*16*	FHfh*68*	*BGbg*46	*BCbc*24

MHC is denoted as capital letters (e.g. AB), ESP as lower-case letters (e.g. ab), and MUP as numbers (e.g.12). As MHC and ESP genotypes were found to match (see Section 4.3.7.3 for results), the MHC and ESP letters are the same. Bold types indicate where subject female and matching stimulus female match at a specific genotype. Italics indicate where the matching and dissimilar stimulus females have an identical genotype. Subject females can therefore be tested with stimulus females that match (to self or maternal-type) for one marker whilst the other genetic marker is held constant. Examples show complete dissimilarity with no haplotypes shared between subject and matching stimulus females for the non-tested marker, and no haplotypes shared between the subject female and the dissimilar stimulus female for the tested marker. Due to limited number of available females this was not always the case, particularly as only four MHC/ESP haplotypes were found.

4.3.11 Scent Discrimination and Attraction Assay

Urine was collected for behavioural assays from all females one to two weeks before testing using the recovery method and stored at -22 °C.

Double cousin line females were tested for their ability to discriminate between the urine of matching and dissimilar stimulus females. The methods were similar to those described in Section 3.3.5, a modification of the design of Ramm *et al* (2008) using a clean MB1 cage that bisected by a central acrylic barrier. Females were given 30 minutes to habituate to the cage with a clean perforated acrylic lid and then presented with a testing lid onto which stimulus urine marks (10 µl) were streaked on half-circles of Benchkote. The side position of urine from the matching and dissimilar stimulus females was randomized but balanced to prevent any side bias. Tests lasted 10 minutes and female position within the cage and behaviour was

recorded to DVD and watched back blind to the position of each scent using an event recorder program (written by R.J.Beynon).

As both MHC and MUP are known to affect house mouse urine odour, it is predicted that females will spend longer investigating the dissimilar stimulus female urine and more time investigating the matching stimulus urine as females should already be familiar with elements of the urine.

4.3.12 Nest Partner Choice Assay

Nest partner choice was measured using the assay design described in Section 3.3.6. Subject females were placed in the centre cage of a series of three interconnected cages. Stimulus females were housed behind mesh and clear acrylic barriers in the end cages. Subject females could move between all three cages through clear acrylic tunnels and interact with stimulus females through the mesh of the barriers. Females were placed in the cages towards the end of the dark phase (4 – 5 pm) and removed two days later near the start of the dark phase (10 – 11 am), after which females were returned to their home cages and the apparatus cleaned thoroughly. Trials lasted approximately 41 hours and were standardised to 36 hours to account for variation in start time. The 36 hour period consisted of two light phases separated by a dark phase.

Subject female movement was tracked using a combination of automated RFID readers (FSI) and infrared sensors connected to a central computer running custom software (FSI; Thom *et al.*, 2008b). Output files were converted using custom software written by J.L.Hurst in Revolution (a software development environment) and SPSS (version 18.0.2) to calculate the total time spent by subject females in each cage. A bias in the amount of time a female spends in one cage suggests a preference for that cage. Females were predicted to spend more time in the cages of the matching stimulus female than the dissimilar stimulus female for both the MHC/ESP and MUP groups. No prediction was made for differences in female response between the matching to self and maternal groups.

4.3.13 Odour-Genes Covariance Assay

Females from the unrelated line were used as subjects for the odour-genes covariance test. Stimulus urine was from the same subject and stimulus female triads that were used in the scent discrimination and attraction and nest partner choice assays. The odour-genes covariance assay design was the same as described in Section 3.3.8. Females were presented

with a habituation urine mark and then presented with two discrimination urine marks, one of which matched the habituation urine at either MHC/ESP or MUP and one that was dissimilar to the habituation urine at either MHC/ESP or MUP. Females were habituated to the test cages for 30 minutes with a clean perforated acrylic lid. Habituation trials lasted 10 minutes, followed by a 2 minute gap, and then a 10 minute discrimination trial. The habituation urine (10 µl) and ddH_20 were streaked onto Benchkote semi-circles on each side of the habituation test lid. The clean lid was used during the 2 minute gap between the habituation and discrimination trials. A discrimination lid was used for the discrimination trial on each side of which urine (10 µl) from the discrimination stimulus females was streaked.

Female cage position and behaviour toward the urine samples during both the habituation and discrimination trials was remotely recorded to DVD for 10 minutes, after which the female was returned to her home cage. Female location was determined by the nose position. The side position of all habituation and discrimination urine was randomised but balanced to remove any cage side bias. DVDs were watched back blind to the odour positions and behaviours were recorded using an event recorder program (written by R.J.Beynon).

The amount of time females spent investigating each discrimination odour was compared. Only trials in which the subject female directly sniffed the habituation odour were used for the analysis. If females spent longer sniffing the dissimilar odour than the matching odour, then the matching odour was perceived as more similar to the habituation odour than the dissimilar odour. Females were predicted to spend less time investigating the matching discrimination urine than the dissimilar discrimination urine. No prediction was made for difference in female investigation between the matching to self and maternal cues groups.

4.3.14 Data Analysis

This chapter addresses whether MHC/ESP and/or MUP are involved in kin recognition between female house mice, and whether females use a match-to-self or a match-to-maternal mechanism based on MHC/ESP and/or MUP. The data for each marker group were analysed separately. Data analysis was performed using the SPSS software package (version 18.0.2). All figures use untransformed data.

4.3.14.1 Scent Discrimination and Attraction Analysis

Females were only included in the analysis if they sniffed both stimulus urine marks. Female investigation of urine marks in the scent discrimination and attraction assay was analysed by

comparing the time spent sniffing each urine mark (just investigation of non-volatile components) and total time directly under each urine mark (to include investigation of both volatile and non-volatile components). In both marker groups the investigatory behaviour data were logarithmically transformed to meet parametric assumptions. In each molecular marker group a repeated measures GLM was used to establish whether females spent longer sniffing from a dissimilar stimulus female compared to urine from a matching stimulus female, with template group (match-to-self or match-to-maternal) as a fixed factor. This was repeated for the total amount of time spent under the urine marks.

Female attraction towards urine from matching and dissimilar females was measured by the amount of time subject females spent on each side of the test cage. In the MHC/ESP marker group the time spent on each side met parametric assumptions without transformation however in the MUP marker group the data were logarithmically transformed. For each group a repeated measures GLM compared the amount of time females spent on each side of the test cage, with template group as a fixed factor.

4.3.14.2 Nest Partner Choice Analysis

Data from the nest partner choice assay did not meet parametric assumptions in either marker group. For each marker group the proportion of time females spent in the proportion of time spent by subject females in the matching stimulus female cage (as a proportion of the total assay time) was compared between the match-to-self and match-to-maternal template groups using a Mann-Whitney U test. If there was a significant difference between the template groups then female cage choice was analysed separately for each group. If there was no difference between the template groups then the data were combined. Wilcoxon Signed Ranks tests compared whether subject females spent longer in the matching stimulus female cage than in the dissimilar stimulus female cage for each marker group.

In each marker group female cage preference was assigned using the ratio of time spent in the matching stimulus female cage compared to the time spent in the dissimilar stimulus female cage. Females with ratios that were above 0.50 spent longer in the matching stimulus female cage, whilst females with ratios that were below 0.50 spent longer in the dissimilar stimulus female cage. The observed number of females that spent longer in the matching stimulus female cage was compared to the number expected by chance using a Binomial test.

4.3.14.3 Odour-Genes Covariance Analysis

Females were only included in the analysis if they sniffed the habituation urine and both discrimination urine marks. In Chapter 3 (Section 3.4.4.1) females showed a strong response in the habituation assay. As predicted females in that experiment spent longer investigating (both sniffing and total time under) the habituation urine compared to the water. In this experiment the habituation trials were watched to ensure that subject females sniffed the habituation urine but no data was recorded. Instead the analysis focuses on the discrimination assay.

In the MHC/ESP marker group investigatory behaviour (time sniffing and time directly under urine) data did not meet parametric assumptions. Mann-Whitney U tests were therefore used to compare whether there was a difference in the ratio of time subject females spent sniffing the dissimilar discrimination urine between the match-to-self and match-to-maternal template groups. A Wilcoxon Signed Ranks test then compared whether females spent longer sniffing the dissimilar discrimination urine than the matching discrimination urine. This approach was repeated for the total time spent directly under urine.

In the MUP marker group both measures of investigatory data were logarithmically transformed to meet parametric assumptions. A repeated measures GLM tested whether subject females spent longer sniffing the dissimilar discrimination urine than the matching discrimination urine, with template group as a fixed factor. This approach was repeated for the total amount of time subject females spent directly under urine marks.

4.4 Results

The results of the questions examined here are presented below and summarised in Table 4.9.

4.4.1 Scent Discrimination and Attraction

4.4.1.1 Match to MHC/ESP

In the MHC/ESP marker group, females were presented with urine from a stimulus female that matched at MHC/ESP (matching) and an equally related stimulus female that was different at MHC/ESP (dissimilar). Female discrimination was assessed by time spent sniffing each urine mark and by the total time spent directly under each urine mark. There was no difference in female investigatory behaviour between the match-to-self and match-to-maternal template groups (sniffing – $F_{(1,18)} = 2.00$, $p = 0.18$ (Figure 4.2a); time under - $F_{(1,18)} = 0.46$, $p = 0.51$ (Figure 4.2b)). Females did not spend longer sniffing dissimilar stimulus female urine compared to matching stimulus urine ($F_{(1,18)} = 0.01$, $p = 0.92$; Figure 4.2a), and females did not spend more time directly under dissimilar urine than directly under matching urine ($F_{(1,18)} = 0.09$, $p = 0.77$; Figure 4.2b).

Subject female attraction to matching or dissimilar urine was measured by the total time spent on each side of the test cage. There was no difference in female attraction between the match-to-self and match-to-maternal template groups ($F_{(1,18)} = 1.42$, $p = 0.25$; Figure 4.3). There was no difference in the amount of time females spent on the matching or dissimilar stimulus urine sides ($F_{(1,18)} = 0.10$, $p = 0.76$; Figure 4.3).

4.4.1.2 Match to MUP

In the MUP marker group, females were presented with urine from a stimulus female that matched at MUP (matching) and urine from an equally related stimulus female that was different at MUP (dissimilar). There was no difference between the match-to-self and match-to-maternal template groups for female investigatory behaviour, as measured by the amount of time spent sniffing urine ($F_{(1,20)} = 0.45$, $p = 0.51$; Figure 4.2a) and the total amount of time females spent directly under urine marks ($F_{(1,20)} = 0.39$, $p = 0.54$; Figure 4.2b). Subject females did not spend longer sniffing urine from dissimilar stimulus females than urine from matching stimulus females ($F_{(1,20)} = 0.07$, $p = 0.80$; Figure 4.2a, nor did they spend longer directly under urine from dissimilar stimulus females compared to urine from matching stimulus females ($F_{(1,20)} = 0.19$, $p = 0.67$; Figure 4.2b).

Subject female attraction to the stimulus females was measured by the amount of time females spent on each side of the test cage. Overall females showed no significant attraction to either the matching or dissimilar stimulus urine side ($F_{(1,20)} = 1.52$, $p = 0.23$; Figure 4.3), however there was a significant interaction between time spent on each side and whether matching was to self or maternal templates ($F_{(1,20)} = 7.66$, $p = 0.01$). Further investigation revealed that in the match-to-self template group females did not spend longer on the matching stimulus urine side than the dissimilar stimulus urine side ($t_{(13)} = 1.38$, $p = 0.19$, paired t-test; Figure 4.3). However in the match-to-maternal template group there was a non-significant trend for females to spend longer in the dissimilar stimulus urine side than in the matching stimulus urine side ($t_{(7)} = -2.21$, $p = 0.06$, paired t-test; Figure 4.3).

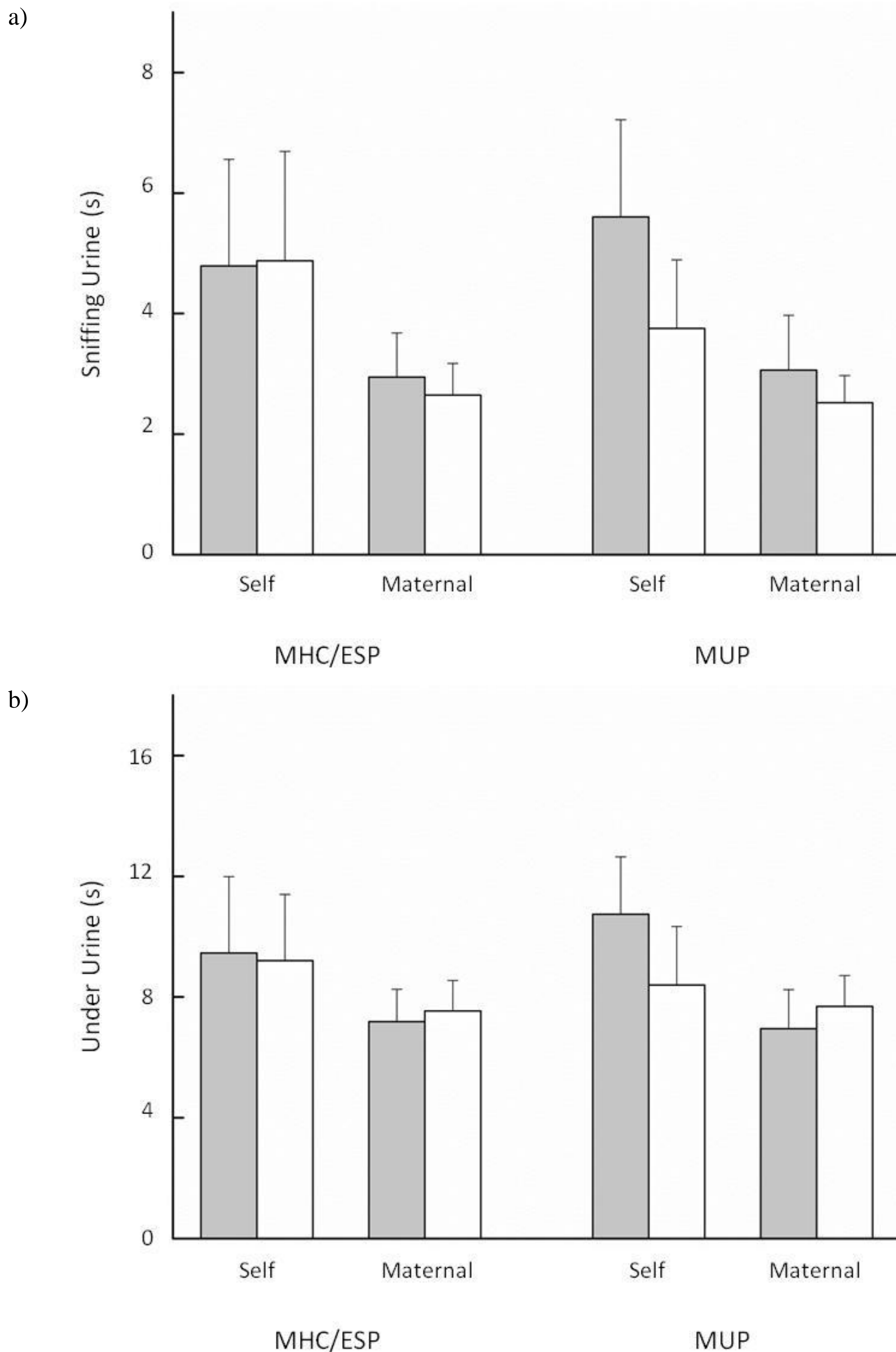


Figure 4.2: Investigation of urine from matching and dissimilar stimulus females in the MHC/ESP and MUP marker groups.

Investigation of urine from matching (grey bars) and dissimilar (open bars) stimulus females in the MHC/ESP (Self n = 8, Maternal n = 12) and MUP (self n = 14, Maternal n = 8) marker groups. Female discrimination ability measured by a) time spent sniffing urine (seconds, mean + standard error) and b) the total time spent directly under urine (seconds, mean + standard error).

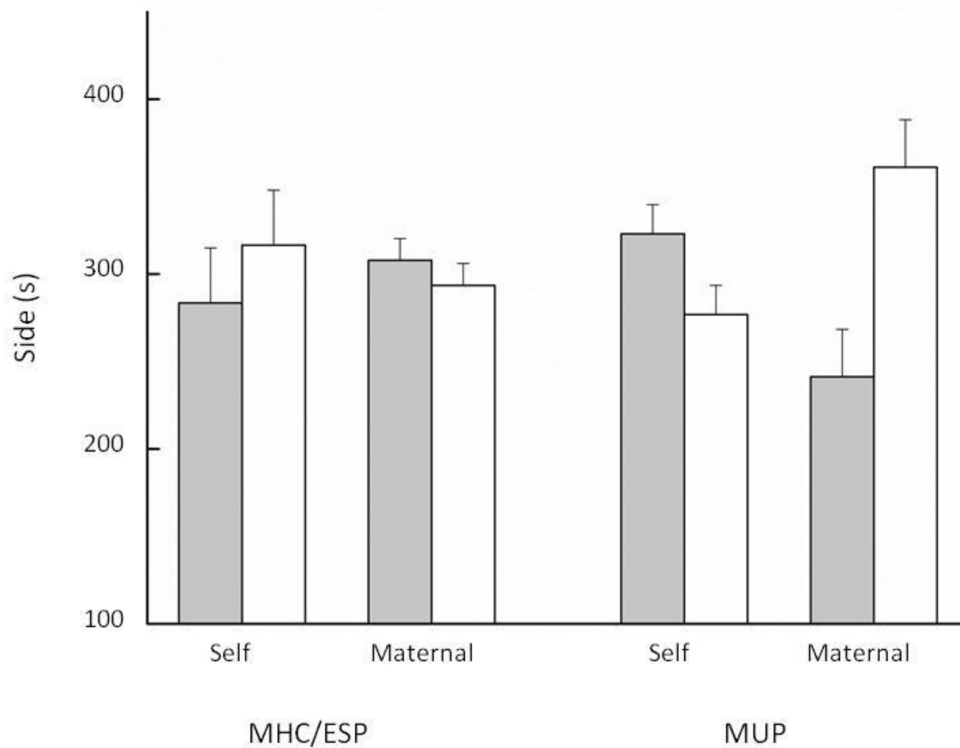


Figure 4.3: Attraction towards urine from matching and dissimilar stimulus females in the MHC/ ESP and MUP marker groups.

Female attraction towards urine from matching (grey bars) and dissimilar (open bars) stimulus females in the MHC/ESP (Self n = 8, Maternal n = 12) and MUP (Self n = 14, Maternal n = 8) marker groups. Attraction measured by the total amount of time spent on each side of the test cage (seconds, mean + standard error).

4.4.2 Nest Partner Choice

4.4.2.1 Match to MHC/ESP

Females were given a choice of nest partner over 36 hours based on matching of MHC/ESP. There was no difference in the proportion of time females spent in the matching stimulus female cage between the match-to-self and match-to-maternal template groups ($z = -0.92$, $p = 0.38$, two-tailed; Figure 4.4). Overall females spent more time in the matching stimulus cage than in the dissimilar stimulus cage ($z = -2.03$, $p = 0.02$, one-tailed; Figure 4.4). There was a non-significant trend for more subject females (68%) to choose the matching stimulus cage than the dissimilar stimulus cage ($p = 0.07$; Figure 4.4).

4.4.2.2 Match to MUP

Over 36 hours females were given a choice of nest partner between a MUP matching stimulus female and a MUP dissimilar stimulus female. There was no difference in the proportion of time females spent in the matching stimulus female cage between the match-to-self and match-to-maternal template groups ($z = -1.57$, $p = 0.13$, two-tailed; Figure 4.4). Subject females spent longer in the matching stimulus female cage than in the dissimilar stimulus female cage ($z = -1.87$, $p = 0.03$, one-tailed; Figure 4.4). More females (73%) chose to nest in the matching stimulus female cage than in the dissimilar stimulus female cage ($p = 0.03$; Figure 4.4).

Inspection of the data suggested that there might be a difference in female response between the match-to-self and match-to-maternal template groups. To investigate this further the groups were analysed separately. Subject females in the match-to-self template group spent more time in the matching stimulus female cage ($z = -1.96$, $p = 0.03$, one-tailed; Figure 4.4). However, females in the match-to-maternal template group did not spend longer in the matching stimulus female cage compared to the dissimilar stimulus female cage ($z = -0.87$, $p = 0.21$, one-tailed; Figure 4.4).

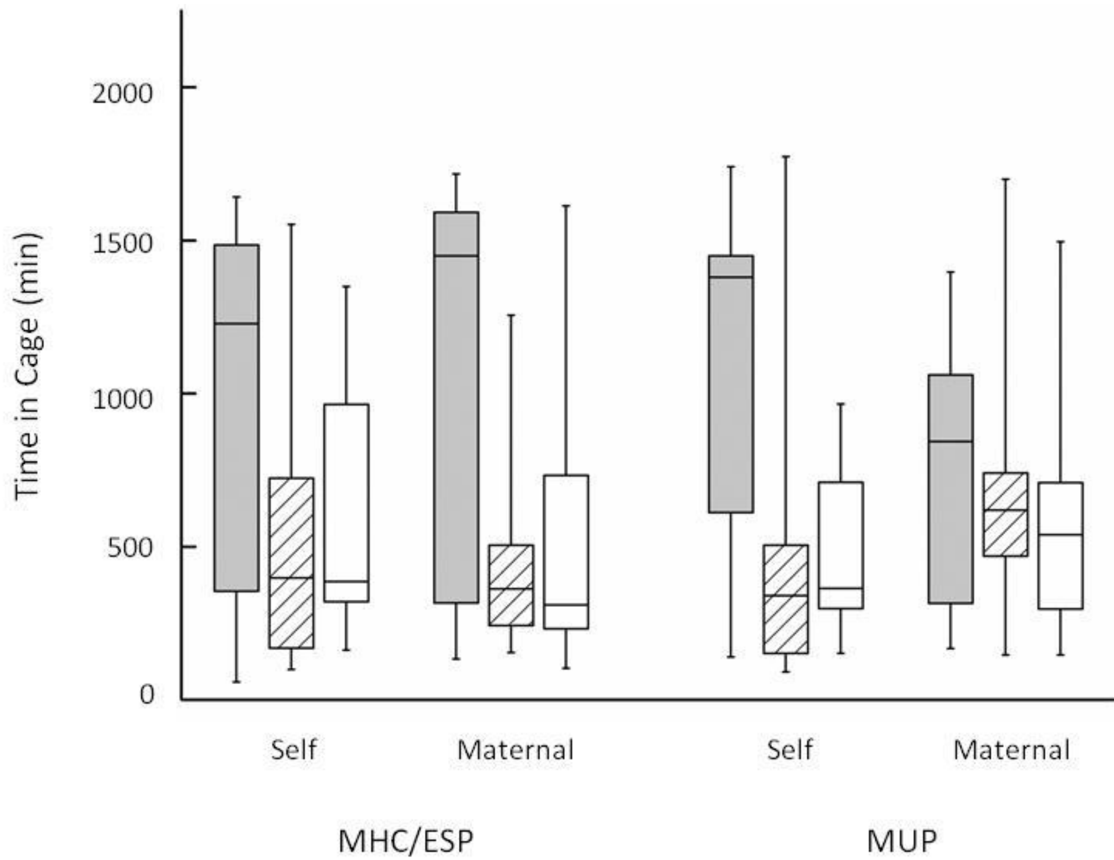


Figure 4.4: Nest partner choice in the MHC/ESP and MUP marker groups.
Time (minutes) spent in the matching (grey boxes), centre (hashed boxes) and dissimilar (open boxes) stimulus female cages in the MHC/ESP (Self n = 12, maternal n = 10) and MUP (Self n = 9, maternal n = 13) marker groups.

4.4.3 Odour-Genes Covariance

4.4.3.1 Match to MHC/ESP

Females from the unrelated line were tested using an odour-genes covariance assay to assess whether neutral females perceive a similarity between urine from females that match for MHC/ESP genotype compared to females that have a dissimilar MHC/ESP genotype. Investigation was assessed by the amount of time females spent sniffing each discrimination urine mark and the total amount of time spent directly under each discrimination urine mark.

There was no difference in the proportion of time females spent sniffing the dissimilar discrimination urine between the match-to-self and match-to-maternal template groups ($z = -1.17$, $p = 0.28$, two-tailed; Figure 4.5a). Subject females did not spend longer sniffing the dissimilar discrimination urine than the matching discrimination urine ($z = -0.73$, $p = 0.25$, one-tailed; Figure 4.5a).

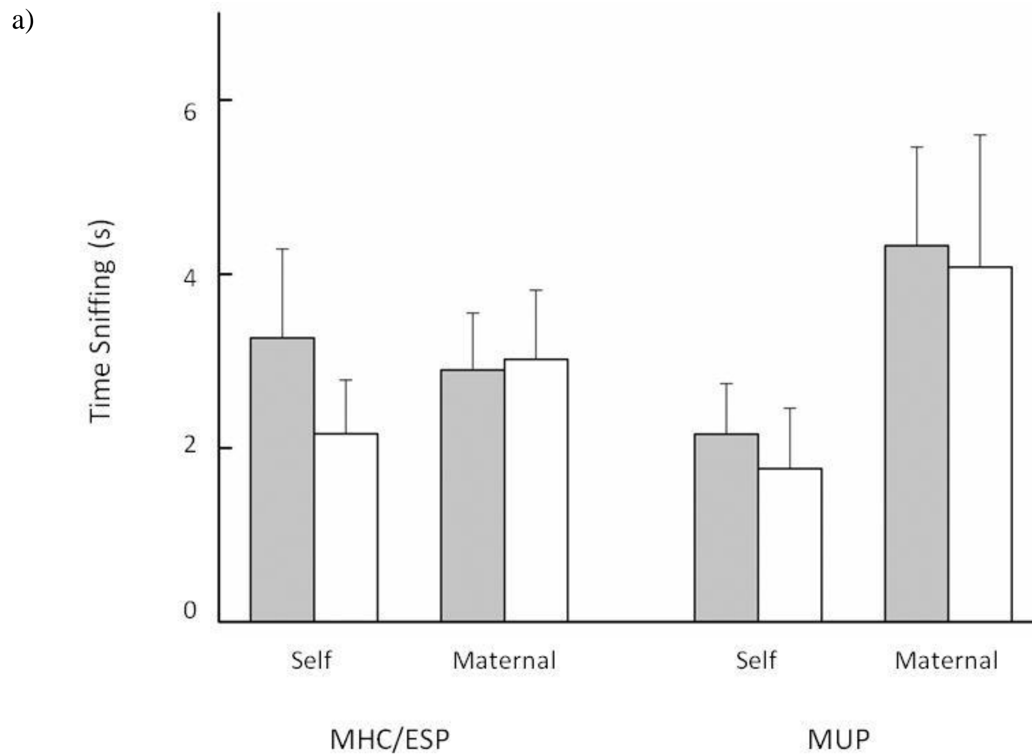
There was a significant difference in the proportion of time females spent directly under the dissimilar urine between the match-to-self and match-to-maternal template groups ($z = -2.20$, $p = 0.03$, two-tailed; Figure 4.5b). The total time spent directly under each urine mark was therefore analysed separately for the match-to-self and match-to-maternal template groups. Females in the match-to-self template group did not spend longer under dissimilar discrimination urine than under matching discrimination urine ($z = -0.84$, $p = 0.23$, one-tailed; Figure 4.5b). However in the match-to-maternal template group there was a non-significant trend for females to spend longer under the dissimilar stimulus urine than under the matching stimulus urine ($z = -1.75$, $p = 0.06$, one-tailed; Figure 4.5b).

4.4.3.2 Match to MUP

In the MUP marker group females did not spend longer sniffing the dissimilar discrimination urine than the matching discrimination urine ($F_{(1,11)} = 0.86$, $p = 0.37$; Figure 4.5a). There was no difference in the proportion of time females spent sniffing the dissimilar discrimination urine between the match-to-self and match-to-maternal groups ($F_{(1,11)} = 0.002$, $p = 0.97$; Figure 4.5a). However, the amount of sniffing behaviour differed between the match-to-self and match-to-maternal template groups and females spent longer sniffing both urine marks in the match-to-maternal template group than in the match-to-self group ($F_{(1,11)} = 4.91$, $p = 0.05$; Figure 4.5a). Similarly, there was a non-significant trend for females to spend longer under both urine marks in the match to maternal cue group than in the match to self cue group ($F_{(1,11)} = 3.78$, $p = 0.08$; Figure 4.5b). Subject females did not spent longer directly under

urine from the dissimilar discrimination female compared to urine from the matching discrimination female ($F_{(1,11)} = 0.70$, $p = 0.42$; Figure 4.5b), and there was no difference in the proportion of time females spent sniffing the dissimilar discrimination urine between the match-to-self and match-to-maternal groups ($F_{(1,11)} = 0.04$, $p = 0.84$; Figure 4.5b).

a)



b)

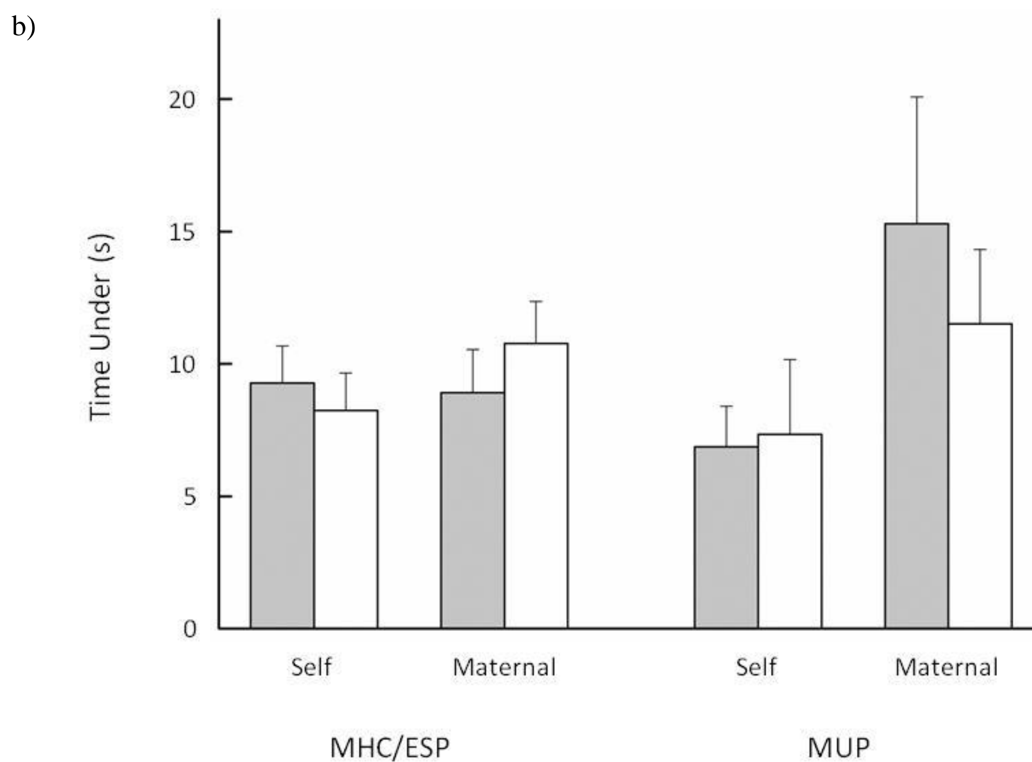


Figure 4.5: Investigation of odour-genes covariance discrimination urine in the MHC/ESP and MUP marker groups.
Total time (seconds, mean + standard error) subject females spent a) sniffing urine or b) under urine from the matching (grey bars) and dissimilar (open bars) stimulus females in the MHC/ESP (Self n = 8, Maternal n = 5) and MUP (Self n = 6, Maternal n = 7) marker groups.

Table 4.9: Summary of molecular marker results.

Marker	Assay	Behaviour Measured	Template Origin (Maternal vs. Self)	Marker Similarity (Matching vs. Dissimilar)
MHC/ESP	**Scent Discrimination and Attraction**	Sniff Urine (discrimination)	p = 0.18[a]	p = 0.92[a]
		Under Urine (discrimination)	p = 0.51[a]	p = 0.77[a]
		Cage Side (attraction)	p = 0.25[a]	p = 0.76[a]
	Nest Partner Choice	Time in stimulus cage	p = 0.38[b]	**p = 0.02**[c] **(M>D)**
	Odour-Genes Covariance	Sniff Urine	p = 0.28[b]	p = 0.25[c]
		Under Urine	**p = 0.03**[b]	Mat: p = 0.06[c] (d>m)
				Self: p = 0.23[c]
MUP	**Scent Discrimination and Attraction**	Sniff Urine (discrimination)	p = 0.51[a]	p = 0.80[a]
		Under Urine (discrimination)	p = 0.54[a]	p = 0.67[a]
		Cage Side (attraction)	**p = 0.01**[a]	Mat: p = 0.06[d] (d>m)
				Self: p = 0.19[d]
	Nest Partner Choice	Time in stimulus cage	p = 0.13[b]	**p = 0.03**[c] **(M>D)**
	Odour-Genes Covariance	Sniff Urine	p = 0.97[a]	p = 0.37[a]
		Under Urine	p = 0.08[a]	p = 0.42[a]

Difference in subject female response between the template origin groups (Mat – Match-To-Maternal, Self – Match-To-Self) towards stimulus females of differing marker similarity (M/m – Matching, D/d – Dissimilar). Statistical tests performed: a – repeated measures GLM, b – Mann-Whitney U test, c – Wilcoxon Signed Ranks test, d – paired t-test.

4.5 Discussion

Female house mice recognise and preferentially associate with unfamiliar full sisters over unfamiliar unrelated females, and show an attraction toward the urine of full sisters (Chapter 2). This suggests that genetic markers of relatedness are present in female house mouse urine. The MHC and MUP multigene families influence mouse odour and both have been proposed as potential molecular markers of kin recognition. Recently it was found that differences in a third polymorphic multigene family, ESP, can be detected by mice. In this study MHC, ESP and MUP were investigated by directly testing female kin recognition ability of females that matched or were dissimilar at each marker. The results (Table 4.9) suggest that all three families may be involved in kin recognition, although due to the mirroring of MHC and ESP haplotypes it is currently impossible to disentangle their separate effects.

4.5.1 Response to MHC/ESP and MUP types

Effects of matching at MHC/ESP and MUP were observed in the nest partner choice assay. Wild-derived female house mice spent longer in the cages of stimulus females that matched for MHC/ESP over those that had a different MHC/ESP type, and this preference was also shown in a non-significant trend for more females to choose the MHC/ESP matching cage. This is consistent with the hypothesis that MHC is used for kin recognition between females. This finding supports that of Manning *et al* (1992) who found that wild-laboratory crossed females preferred nest partners that had the same MHC genotype (as self or parents). The study presented here also complements other kin recognition studies that showed the importance of MHC in inbreeding avoidance and pup recognition (Yamazaki *et al.*, 1988; Egid & Brown, 1989; Penn & Potts, 1998a; Yamazaki *et al.*, 2000). The difference between this experiment and those previous is that wild-derived house mice were used that had background levels of genetic variation that is consistent with natural situations. Female house mice therefore showed a preference for MHC/ESP matching females over MHC/ESP dissimilar females against a variable genetic background.

Females also preferred to nest with, and spent longer in the cages of, stimulus females that matched for MUP type over those that were dissimilar for MUP type, when both stimulus females shared a MHC/ESP type that was different to the subject female. This suggests that MUP is important for kin recognition between female house mice. These results complement the findings in Chapter 2 where females that spent longer in the cage of the related stimulus female shared more MUPs with their full sister than with the unrelated stimulus female.

In contrast to the results presented here, recent studies investigating both inbreeding avoidance and individual recognition using house mice found no evidence in support of MHC, (Cheetham *et al.*, 2007; Sherborne *et al.*, 2007) and instead MUP type appeared to explain both individual recognition and inbreeding avoidance behaviours. This could suggest that MHC is a marker of relatedness used between females but that it does not play a role in other behaviours.

Whilst MHC/ESP differences were detected here against a variable genetic background, MUP type was held constant between the stimulus females (and vice versa). Therefore it is difficult to say whether the discriminative behaviour observed here would occur if the second marker also varied. For instance, the difference in MHC/ESP (or MUP) type between the two stimulus females might be more apparent if they both have the same MUP type, but may be more obscured when MUP type varies between the stimulus females. An important further test is therefore to present subject females with a MHC/ESP type matching stimulus female with a neutral MUP type, and a MHC/ESP type dissimilar stimulus female with a second, different neutral MUP type, and to test the reverse for MUP type matching/dissimilarity.

Not all matching stimulus females were preferred in each marker group. This could be due to differences in the second marker, or other differences between the females. It would be interesting to test whether a higher proportion of females would choose to nest with a female that matched for both markers compared to the proportion of females that nested with females that matched for each marker. At a highly polymorphic locus, close relatives are more likely to share haplotypes than unrelated individuals. If individuals share haplotypes across more than one polymorphic locus this increases the likelihood that they are related. Therefore if a higher proportion of females chose to nest with a stimulus female that matched at both MHC/ESP and MUP compared to a stimulus female that matched at only one then this would support the importance of both MHC/ESP and MUP as markers enabling kin recognition between female house mice.

This study suggests that both MHC/ESP and MUP are involved in kin recognition in the house mouse. As discussed in the introduction all three markers influence mouse odour profile, but MHC/ESP and MUP are on different chromosomes and were inherited independently. Both MHC and MUP are highly polymorphic and influence an individual's odour profile. It has been proposed that MHC and MUP might work in tandem to create an individual's odour profile (Beynon & Hurst, 2004). This hypothesis involves a modification

of the carrier hypothesis for how MHC influences odour profiles: MUPs could act as carriers for a selection of volatile molecules that result from MHC genotype. That is, an odour profile could be the result of both *Mhc* and *Mup* genes, with *Mhc* genes providing volatile molecules, and MUPs binding and transporting a subsection of those volatiles before releasing them in the urine. It is possible that this is occurring here as females were able to identify relatives matching for either MHC or MUP whilst the second marker varied.

4.5.2 Response to self and maternal

As discussed in Chapter 3, female house mice could use two main reliable sources of information as a template for recognising relatives – self and maternal type. The results from Chapter 3 suggest that females used maternally learnt cues when making nest partner decisions. In the experiments reported in this chapter females were presented with two equally related stimulus females that matched or were dissimilar at MHC/ESP or MUP to their own type (self) or the type of their mother (maternal). Overall there appeared to be little difference in female response to stimulus females that matched to self or maternal cues, however in the match to MUP group females appeared to show a stronger response when tested with stimulus females whose MUP profile matched to self rather than maternal cues. Additionally, females appeared to show an attraction to the dissimilar stimulus urine in the match to maternal MUP group.

Individuals inherit half of their genome from their mother and half from their father and therefore in an outbred population offspring will not exactly match their mother for a polymorphic locus – they will partially match at all loci. This however, is different from what was tested here. The reason that the preference for matching to maternal cues was not stronger could be because the matching stimulus female matched exactly at one marker but no other markers. A more accurate test of maternal matching would have been to present females with a stimulus female that partially matched (had one haplotype) at both the MHC/ESP and MUP markers but was completely different to self at those markers, and a stimulus female that matched at neither. It should be possible using the breeding programme described here to find double cousins that fit this description and present them against a dissimilar female that partially matched to mother at neither marker.

Paterson & Hurst (2009) investigated the effectiveness of match-to-self and match-to-maternal kin recognition mechanisms using different numbers of recognition loci. They found that a match-to-self mechanism was more effective at identifying relatives using one

recognition locus than when using more than one recognition locus, as additional loci increased the number of falsely identified unrelated individuals. In the partial match-to-maternal mechanism this pattern was reversed. The use of a single recognition locus was relative weak at successfully identifying relatives, however using more than one recognition locus reduced the error rate, meaning that a lower proportion of unrelated individuals were falsely recognised. The results of Chapter 3 suggest that female house mice may use a match-to-maternal template kin recognition mechanism during nest partner choice decisions as females preferentially nested with maternal half-sisters over unrelated females but showed no preference between paternal half-sisters compared to unrelated females. In this chapter females spent longer in the cages of stimulus females that matched for either MHC/ESP or MUP than dissimilar females, suggesting that both MHC/ESP and MUP may be used for kin recognition. A complete MHC, MUP or ESP haplotype may act as a single allele at a single recognition locus. This supports the theoretical predictions that if a match-to-maternal mechanism is used then it will be more effective with multiple recognition loci compared to with a single recognition loci.

4.5.3 Molecular Markers of Kin Recognition in Urine

Females showed little differentiation of urine from female double cousins that either matched or were dissimilar at MHC/ESP or MUP. The only indication of any difference in response to the urine marks came from a non-significant trend for females in the match to maternal MUP group to spend more time on the dissimilar side than the matching stimulus urine side of the test cage. Female investigation of matching and dissimilar urine in the odour-genes covariance assay also did not suggest any perceived similarity between the matching discrimination stimulus urine and the habituation urine in either the match to MHC/ESP or MUP types groups.

A number of studies have shown that laboratory mice are able to discriminate urine from individuals that differ only at MHC (e.g. Yamazaki *et al.*, 1979; Yamazaki *et al.*, 1983; Penn & Potts, 1998b). In these studies the stimulus urine was from congenic strains of mice that differed only at MHC. Mice were therefore able to discriminate different urine marks based on MHC when the differences in MHC were against limited background variation. Differences in MHC type might therefore be much more obvious in those situations than the experiment reported here. Additionally whilst the second, non-tested marker was held constant between the stimulus females, it is possible that was still novel to the subject female.

This may have resulted in either equal levels of investigation towards both urine marks or a subtler difference that was not detected here. Aside from the specific markers tested here, other differences in background variation between the subject and matching stimulus female may also have caused females to display an equal amount of investigatory behaviour towards matching and dissimilar urine.

Odour-genes covariance assays have been used to demonstrate similarity between the odours of full siblings, cousins and double cousins (Busquet & Baudoin, 2005; Hagemeyer & Begall, 2006). Busquet & Baudoin (2005) also demonstrated with male mound-building mice (*Mus spicilegus*) that odours from double cousins were perceived as more similar to each other than to single cousins. In all cases, however, one discrimination odour was always more strongly related to the habituation odour than the second discrimination odour, and therefore will have been more similar across all loci than the more distantly related discrimination female. In this study the two discrimination odours were equally related to the habituation odour. Therefore, whilst the habituation urine may have appeared more similar to the matching discrimination urine for one loci, other differences in background variation may have caused both discrimination urine marks to appear overall equally similar to the habituation urine mark.

In the odour-genes covariance assay, females from the match to MUP group spent longer investigating both the discrimination odours if they matched to maternal cues than if they matched to self cues. The habituation urine came from virgin F2 individuals from the double cousin breeding line. The discrimination urine in the match-to-self cues groups was also from virgin F2 individual from the double cousin breeding programme, however in the match-to-maternal template group the discrimination urine was from F1 dams that had been used for breeding the double cousin litters – aunts of the habituation females. These females were older than the habituation female, and were reproductively experienced. These differences may have affected aspects of urine odour and so caused the prolonged investigatory behaviour observed here toward the match to maternal cues discrimination urine.

Together these results suggest that female house mice are unable to discriminate between urine from equally related females that differ at a specific locus. However females spent longer in the cages of matching stimulus females than the cages of dissimilar stimulus females in the nest partner choice assay, suggesting that females are able to discriminate between equally related females based on differences at a single polymorphic recognition locus. It is possible therefore that other differences between matching and dissimilar urine

samples are causing the equal levels of investigatory behaviour in a short-term test, whilst over a longer time period females discriminate and show a preference. As females were only used if they sniffed all scents, samples sizes were limited. Increasing the number of scent and odour-genes covariance assays might reveal significant differences in the amount of investigatory behaviours displayed towards matching and dissimilar urine.

4.5.4 Matching of MHC and ESP

In the mice tested here haplotypes of MHC and ESP appeared to be linked – if an individual had a certain MHC haplotype then they would also have a corresponding ESP haplotype. Both MHC and ESP are on chromosome 17 and it is possible that both are inherited together (Kimoto *et al.*, 2007). Only two microsatellite primers were successful for ESP and only four for MHC. The primers may not have therefore picked up the full extent of variation present in the mice tested. Additional genotyping with different microsatellite markers agrees with the numbers of haplotypes found here (personal communication J.L.Hurst). This supports the finding of only three ESP haplotypes and four MHC haplotypes and the hypothesis that MHC and ESP were inherited together.

One interesting aspect of the observed ESP patterns is that the same microsatellite haplotype was observed in both populations, despite the fact that the two populations (Knowsley Safari Park and Holme Farm) are not geographically close to each other. The limited number of microsatellite markers used could mean that the full variation of ESP was not observed in this study and more markers might reveal more variation. Alternatively it is possible that the two populations (or the two mice sampled from each population) both happened to share the same haplotype. Whilst ESP is known to be a polymorphic family, there will still only be a limited number of haplotypes and the two populations could easily share types. It is also possible that ESP is not actually as variable as previously suspected. The limited number of studies investigating ESP makes it at present difficult to know whether this last suggestion is likely or not.

4.5.5 Variation in MHC haplotypes

As predicted by the observed mirroring of ESP and MHC and the limited number of ESP haplotypes identified here, the number of MHC haplotypes discovered was lower than expected. Only four different MHC haplotypes were observed, two from each founder population (Knowsley Safari Park and Holme Farm). At a highly polymorphic locus two unrelated individuals are expected to have more than two haplotypes between them, but this

was not the case here. One possible reason for this could be that the two founder individuals from each population were related to each other. During trapping both populations appeared large and it seemed unlikely that they were inbred. The chances of two closely related individuals happening to be caught separately and then randomly paired for breeding in the laboratory seem low. The MUP genotyping data also suggests that individuals from the same population were unrelated as all founder individuals had different MUP heterozygous genotypes. However, a quarter of all full siblings will share no haplotypes at a given locus and therefore it is still possible that founder mice from one population were closely related to each other. The breeding programme design used here established multiple pairings of mice, but only selected offspring from the most successful pairings for each population. An unintended consequence of this could be that the most successful pairings might have been those that were unrelated to one another whist the least successful pairings may have been unsuccessful as a result of pairing together relatives. Whilst it remains impossible to say at this time whether the founder mice were related or not, the breeding programme design (along with the observed MUP haplotype variation and observations of the populations) support the idea that the founder mice were not related to one another.

Only four microsatellite markers were used to identify MHC haplotypes. As discussed above in relation to the limited observed ESP haplotype variation, it is possible that the small number of MHC microsatellite markers meant that differences in MHC haplotype may have been missed during genotyping. Whilst the MHC microsatellite markers were selected to cover the spectrum of the MHC genome, haplotype variation could still have been missed. Microsatellite markers are used to infer MHC haplotypes, but do not reveal variation in individual *Mhc* genes. It is therefore important to confirm that differences in the MHC microsatellite haplotypes match differences in the actual *Mhc* genes. However, females made nest partner choice decisions based on matching of the MHC haplotypes as defined by the microsatellite markers, suggesting that the microsatellite markers did reveal the MHC variation. Following the experiments presented here additional genotyping results with different microsatellite markers supports the number of MHC microsatellite haplotypes reported (J.L.Hurst, personal communiation).

4.5.6 Results of the Breeding Programme – A Male Biased Sex Ratio

The breeding programmes discussed here produced a double cousin line and an unrelated line for behavioural assays. In the double cousin line, however, the sex ratio of males to females

was heavily in favour of males. Sixty males were born in the double cousin line compared to only 33 females. The mean number of males per litter in the double cousin line was 4.3, whereas the mean number of females per litter was 2.4. In the unrelated line there was less of a male bias, with 22 males (mean per litter 3.7) and 18 females (mean per litter 3.0) bred. The bias in sex ratio meant that the number of females available for use as subject and stimulus females was limited and choice was very limited when forming triads of matching and dissimilar females (whilst also considering mass differences between females). The sample sizes for each category were therefore small and as a result the data presented here are not as strong as it might be with larger sample sizes. It is therefore important to perform additional tests to confirm the findings presented here.

A similar sex ratio was observed in the first litters of the three founder population pairs. The Knowsley Safari Park founder pair had a total of eight females and six males over two litters and in the first litter the pair had three females and four males. The Holme Farm founder pair had three litters and produced seven females and nine males. In their first litter there were two females and four males. The Hatchwood Farm founder pair only had one litter of one female and seven males. Whilst this could be chance, it hints that perhaps sex ratio alters over multiple litters, with the first litter consisting of more males. A possible explanation for this could be that whilst both sexes are likely to disperse on weaning, females are more likely to remain in the nest or in proximity to the natal nest. A strategy of initially producing more males than females could limit competition (for resources or mating) between a mother and large numbers of daughters whilst the mother is still reproductively active. No such trend was observed during the half-sibling breeding programme described in Section 3.3.2 and it is probable that the male sex ratio bias observed here was just unfortunate chance.

4.5.7 Conclusions

Mice were bred that allowed for direct behavioural testing of recognition based on all MHC, MUP and ESP type independently, against a normal level of background genetic variation. MHC and ESP haplotypes were found to mirror each other so that a specific MHC haplotype would always be found with a specific ESP haplotype. It was hypothesised that as they both located close to one another on chromosome 17 that they might be inherited together. When presented with stimulus females that matched or were dissimilar for MHC/ESP type or MUP type, females chose to nest with the matching stimulus in both marker groups. This is the first evidence to suggest that MHC/ESP and MUP could interact to provide information on

relatedness. The results presented here suggest a number of additional questions and tests remain to establish beyond doubt that both MHC/ESP and MUP are markers of relatedness and are used in kin recognition between female house mice.

5 Physiological Changes as a Result of Female Social Environment, Relatedness and Familiarity

5.1 Abstract

Social environment can influence animal behaviour and physiology, and may have positive and negative consequences on different aspects of life history. Here I present the results of a pilot study aimed at investigating the physiological effects of housing female house mice in pairs of varied levels of relatedness and familiarity. The effect of social environment on female house mouse physiology was measured using changes in faecal corticosterone and urinary protein, as well as female body weight. Physiological changes were measured over a short (7 hours) and longer (3 days) time scale. The effect of female social environment was compared by varying either relatedness or familiarity whilst keeping the second factor constant.

Over the short-term time scale (7 hours) there was no change in faecal corticosterone across any of the familiarity or relatedness groups, although females in all unfamiliar groups did exhibit a non-significant increase in corticosterone in the short term. As this suggests that initial housing with unfamiliar females may stressful for female house mice, this trend should be further investigated. Female body mass decreased more in distantly related females than in full sisters, whilst urinary creatinine increased in the unfamiliar groups. A decrease in water intake (as a result of competitive behaviour between females) could explain both the decrease in female body mass and the increase in creatinine levels over the short-term. Urinary protein decreased over the short-term in the relatedness groups, which could be due to increased competition between more distantly related females.

Over the longer-time scale (3 days) female faecal corticosterone did not change in any of the familiarity or relatedness groups. This suggests that housing with females of varied familiarity and relatedness is not stressful for female house mice in the long-term. Female body mass and urinary protein increased over the long-term in all unfamiliar groups group, which may be as a result of competitive behaviour between unfamiliar females.

5.2 Introduction

Social environment can have both positive and negative consequences on a range of different aspects of an animal's life history. Disruptions to rearing environment, periods of social isolation when adult, differences in social group composition, social status, size, density and stability can all affect animal behaviour and physiology (reviewed in Olsson & Westlund, 2007). In many research laboratories animals are housed in a variety of different social conditions depending on cost, welfare considerations, experimental requirements or animal facilities, however housing and social environment are important both for animal welfare and the quality of the results produced by such research (Wurbel, 2001; Olsson & Westlund, 2007). Kin selection theory predicts that animals may behave more amicably towards relatives (e.g. Maher, 2009) or show reduced aggression towards relatives (e.g. Holmes, 1986; Markman *et al.*, 2009). Group housing with kin may therefore be a less stressful environment than group housing with unrelated individuals.

5.2.1 Stress and Corticosterone

When presented with a stressor the body employs a number of mechanisms in an attempt to re-establish homeostasis (the stable internal environment). The hypothalamic-pituitary-adrenal (HPA) axis is one of the two main mechanisms involved in responding to stressors (Sapolsky, 2002). When a stressor is perceived, the hypothalamus releases corticotrophin-releasing hormone which stimulates the release of adrenocorticotropic hormone from the pituitary gland. This in turn stimulates the release of glucocorticoids (steroid hormones) from the cortex of the adrenal gland. In rodents the dominant glucocorticoid is corticosterone (known as cortisol in humans; Sapolsky, 2002). The body's different responses to stress are designed to re-establish homeostasis over the short-term, however long-term stress (and so long-term exposure to glucocorticoids released as a result of the HPA axis) can have a number of adverse affects. Prolonged exposure to glucocorticoids can disrupt bone and tissue repair, inhibit immune defences and disrupt reproduction through the reduction of the secretion of sex hormones and a decreased probability that viable eggs are released (Sapolsky, 2002).

Since glucocorticoids are released in response to physical and psychological stressors, changes in the levels of cortisol (or corticosterone) are often used as a measure of detecting stress (Palme, 2012). The separation of squirrel monkey (*Saimiri sciureus*) infants from their mothers increases blood plasma cortisol levels of both individuals suggesting that separation

is stressful (Levine & Wiener, 1988). Wied's black tufted-ear marmosets (*Callithrix kuhli*) exposed to stressors such as temporary isolation or manual restraint show an increased level of urinary cortisol (Smith & French, 1997), and faecal cortisol levels increase in chimpanzees (*Pan troglodytes*) subjected to anesthetisation (Whitten *et al.*, 1998). Evicted subordinate meerkat (*Suricata suricatta*) females show an increased level of faecal glucocorticoid metabolites (including corticosterone; Young *et al.*, 2006), suggesting that eviction and isolation is stressful. Young female pigs (*Sus scrofa domesticus*) isolated from their social group also show an increase in salivary cortisol concentration (Ruis *et al.*, 2001). Monogamous female Gouldian finches (*Erythrura gouldiae*) have higher levels of blood plasma corticosterone when paired with lower quality males, suggesting that pairing with poor quality males could be stressful for females (Griffith *et al.*, 2011).

5.2.2 House Mouse Social Environment

Mice (both laboratory and house mice) are among the most commonly used species for research studies. Male mice are either housed singly to prevent aggression between males or in groups, whilst female mice are generally housed in same-sex groups. Female house mice nest communally and nest partner choice is important for female reproductive success, as competition between females can lead to aggression, reproductive inhibition and infanticide (Hurst, 1987; König, 1994a; Rusu & Krackow, 2004; Palanza *et al.*, 2005). Female house mice also contribute to territorial defence and will attack female intruders, particularly during pregnancy and lactation (Palanza *et al.*, 1996). Females in preferred nest partner pairs have a higher reproductive success than females in non-preferred pairs (Weidt *et al.*, 2008).

Compared to unrelated females, related female mice nesting together have an improved reproductive success (König, 1993, 1994b, 1994a; Rusu & Krackow, 2004; Palanza *et al.*, 2005). In the wild female house mice have been found nesting with genetically similar females (Wilkinson & Baker, 1988), and under experimental conditions sisters are more likely to nest with each other than with unrelated females (Dobson *et al.*, 2000; Dobson & Baudoin, 2002). The results of chapters 2 and 3 also suggest that female house mice can discriminate and nest preferentially with unfamiliar full sisters and maternal half-sisters over unrelated females, although females also displayed no apparent discrimination or preference for paternal half-sisters.

Measurements of corticosterone levels are often used to assess stress in mice. Levels of corticosterone can be measured in mice using either blood plasma or faecal samples.

Monitoring faecal corticosterone levels presents advantages as obtaining samples is non-invasive and repeated samples can be taken (Palme, 2012). Additionally, blood plasma corticosterone levels can change within seconds as a response to immediate stressors and therefore could rapidly change in response to the sampling process itself (Palme, 2012).

The effects of social group housing on female laboratory mouse corticosterone levels have been investigated with mixed results. Female laboratory mice re-housed regularly in groups of four novel females have an increased plasma corticosterone level compared to those housed constantly in the same groups, suggesting that females find it more stressful living in an unstable social environment (Schmidt *et al.*, 2010). Arndt *et al.* (2009) found that after two weeks of experimental housing groups of three female laboratory mice have higher levels of plasma corticosterone levels than singly housed females, suggesting that housing in groups of three may be more stressful than solitary housing. In contrast, Bartolomucci *et al.* (2009) found no difference in plasma corticosterone levels between female laboratory mice housed in isolation, in groups of three unfamiliar, unrelated females or three familiar sisters for one week. Akre *et al.* (2011) also found no difference in faecal corticosterone levels between paired familiar littermates and paired unfamiliar unrelated female laboratory mice.

Together these results suggest that whilst an unstable environment increases female stress (as measured by corticosterone levels), housing with related or unrelated females does not. However, both Bartolomucci *et al.* (2009) and Akre *et al.* (2011) measured change in corticosterone over a week and only compared measurements from before housing and at the end of housing. It is possible that a short initial stress response as a result of novel social environment may have therefore been missed. Additionally, as discussed in the introduction (Section 1.2.3) laboratory mice behave subtly differently from house mice, and often show reduced levels of aggression (Smith *et al.*, 1994). Female house mice gain reproductive benefits from preferentially nesting with relatives. Kin selection theory also predicts that individuals may behave less aggressively and more amicable towards close relatives than towards unrelated individuals (e.g. Maher, 2009; Markman *et al.*, 2009). In house mice housing with unfamiliar or unrelated females may therefore be stressful and this could be detected by changes in corticosterone level.

5.2.3 Scent Marking and Protein Levels

House mice of both sexes scent mark extensively using urine, and scent can signal territory ownership (Hurst & Beynon, 2004), gender (Roberts *et al.*, 2010), reproductive status, health

(Ehman & Scott, 2001), diet (Hurst & Beynon, 2004), identity (Cheetham *et al.*, 2007) and relatedness (Chapter 2, this thesis). Proteins are present in large amounts in the urine of house mice, and in male mice 99% of these are major urinary proteins (Humphries *et al.*, 1999). Scent marks are useful for signalling as marks remain in the environment after being deposited (Hurst & Beynon, 2004). However, in order to maintain ownership signals, male house mice countermark the signals of any intruding males. Female house mice prefer males that have successfully countermarked intruder marks as this signals territory defence (Rich & Hurst, 1999). Continual countermarking has a cost however as it is both time consuming and the production of large quantities of proteins is likely to be metabolically expensive (Gosling *et al.*, 2000).

Whilst urine from male house mouse contains approximately 3 – 4 times as much major urinary protein that from females, female urine still contains a high proportion of urinary protein (Beynon & Hurst, 2004). Female house mice also invest in territorial defence and use scent marks as a signal to other females of breeding status (Hurst, 1990b). Breeding females also countermark the scent marks of other intruding breeding females (Hurst, 1990b). Reproducing females exposed to territorial intrusions by both males and females have a higher level of urinary protein output than non-territorial reproducing females (Garratt *et al.*, 2011). It is possible therefore that unfamiliar females housed together may increase their scent marking behaviour, which may be reflected in changes in urinary protein output.

5.2.4 Body Mass

Body mass is often linked to social dominance, health and competitive activity (Broom & Johnson, 1993). Weight loss and lack of growth are often used as a measure of stress in laboratory mice (Bartolomucci *et al.*, 2004; Olsson & Westlund, 2007; Bartolomucci *et al.*, 2009). Female laboratory mice lose weight as a result of increased activity in laboratory cages (Whittaker *et al.*, 2012). In addition laboratory mice subjected to experimental stress events have a reduced body mass compared to control mice (Sahin & Gumuslu, 2007). The effects of social housing with a novel partner may therefore influence body mass in wild house mice.

5.2.5 Chapter Aim

This chapter aims to investigate the effects of social housing, relatedness and familiarity on three measures of female physiology. This study asked two questions:

i) Do physiological characteristics change over a short (seven hours) and longer (three days) time period in female house mouse when they are housed with social partners depending on the degree of relatedness between females?

ii) Does housing with a novel female result in a greater physiological change over a short (7 hours) and longer (three days) time period compared housing with a familiar female?

Females were housed in pairs of varying relatedness and familiarity and monitored for changes in faecal corticosterone levels, urinary protein levels and body mass. Housing with unrelated or unfamiliar females was predicted to be more stressful than housing with related or familiar females, and may result in increased faecal corticosterone levels or decreased body mass. Housing with unrelated or unfamiliar females may also increase urinary protein levels as a response to increased competition or marking.

5.3 Methods

Female house mice were assigned to one of five experimental treatments and housed in pairs of: full sister littermates; unfamiliar full sisters; unfamiliar maternal half-sisters; unfamiliar paternal half-sisters; or unfamiliar unrelated females. The effects of housing treatment were assessed by comparing pre-treatment levels (Pre) of female weight, urinary protein output and faecal corticosterone concentration against the same measures taken 7 hours following pairing (Day 1) and 3 days after introduction (Post).

5.3.1 Animal Housing and Handling

Female house mice were housed and handled according to the methods detailed in Section 2.3.1. Prior to testing females were housed in sister groups of 2-4 individuals. Cages were provided with environmental enrichment and food and water *ad libitum*. Mice were maintained on a reversed 12:12 hours light:dark cycle, with the dark phase beginning at 8 am. All animal handling was carried out during the dark phase under dim red light.

5.3.2 Breeding Programme

Female house mice were from the same stock as bred in chapter 3, which were captive-bred from the Mammalian Behaviour & Evolution group's outbred colony of house mice, established using individuals captured in the North West of England, UK. To produce unfamiliar full and half-siblings, two consecutive rounds of litters were bred as detailed in Section 3.3.2. In the full sibling line of breeding, the male – female pairs established for the first litters were the same as those used for the second litters. In the half-sibling line males and females were paired for the first litters and then males were moved to different females for the second round of litters (Figure 3.1).

Breeding pairs of mice were established according to the procedures described in Section 3.3.2. Females were primed with bedding from same and opposite sex house mice prior to being introduced so that females would be in or entering oestrus at the time of introduction (Marsden & Bronson, 1964; Cheetham *et al.*, 2007). This technique also provides experience of same and opposite sex odours. Pairs were then placed in MB1 cages and monitored frequently over the following 24 hour period. Females were checked on days 17, 21 and 25 and weights compared to pre-introduction weight. If females appeared visibly pregnant and had gained over 5 g in mass then males were removed. Litters were weaned 24 days after birth. At weaning male pups were housed singly in M3 cages, and female pups were housed in sister groups of up to four individuals in MB1 cages. Once all first litters had been weaned,

breeding pairs of males and females were re-established (for the full siblings) or new breeding pairs established (for the half-siblings) and the process repeated.

5.3.3 Female House Mice

Females from the first litters were 12 months old at the time of testing, females from the second litters were 10 months old. Females had previously been used in the behavioural assays described in Chapter 3. Paired females were weight matched to within 2 g of each other (0.58 ± 0.14, mean ± standard error) and assigned to one of five treatment groups. Females in the full sister littermate group (n = 14 pairs) were already highly familiar with one another and were the same age. In the remaining four treatment groups paired females were from separate litters and therefore previously unfamiliar, differing in age by 2 months. Females in these four treatment groups were housed in pairs of full sisters (n = 14 pairs), maternal half-sisters (n = 14), paternal half-sisters (n = 14) or unrelated females (n = 14). All females had previously had radio frequency identification (RFID) tags injected beneath the skin at the nape of the neck, as described in Section 2.3.1.

5.3.4 Prior Experience

Prior to the experiment, females were housed in sister groups of two, three or four females. Females had previously been used in behavioural experiments documented in chapter 3. One week before the start of the experiment each female was housed with a single littermate sister in fresh MB1 cages to remove potential confounding odours from other individuals (Hurst, 2005). Females were also primed with same and opposite sex soiled bedding on two occasions prior to the experiment to regulate female oestrus cycles (Marsden & Bronson, 1964; Cheetham *et al.*, 2007) (methods as described in Section 6.3.2.1). The second priming session was 4 days before the start of the experiment so that females would be in or entering oestrus the day before the experimental commenced pairing (Cheetham *et al.*, 2007).

Females were tested for oestrus stage on the day before the experiment to confirm oestrus state using a vaginal smear. Females were handled in a clear acrylic tube and restrained by gently holding their tail up. A plastic loop (1 μl soft, Copan Innovation, Brescia, Italy) was swept just inside the vaginal opening. This process took no more than a few seconds after which the females were returned to the home cage. The loop was rubbed in a drop of 0.1% methylene blue stain on a 76 x 26 mm slide (Menzel-Glaser Superfrost, Thermo Fisher Scientific Oy, Vantaa, Finland) and covered with a 26 x 22 mm coverslip (Deckglaser). Cells were visualised under a light microscope (M75, Vickers Instruments Ltd, York, UK) at x 10

objective. A predominance of clusters of anucleated cornified epithelial cells confirmed oestrus (Caligioni, 2010).

At 15:00 on the day before experimental pairing urine and faecal samples were collected from all females using the recovery method (described in Section 2.3.3). Females were placed individually on cage lids above clear MB1 cages, with inverted MB1 cages placed over the top. Urine and faecal matter could fall between the bars of the cage lids and collect in the MB1 cage below. Females were left for 90 minutes, after which they were weighed, tested for oestrus stage and returned to their home cages. Urine and faecal samples were collected in separate 1.5 ml Eppendorf tubes and stored immediately at -22 °C. These measures provided the pre-treatment levels for female body mass, protein and corticosterone.

5.3.5 Experimental Procedure

On the first experimental day at 08:00 (the start of the dark cycle) females were housed in their experimental pairs, in clean MB1 cages. Each cage was lined with Corn Cob Absorb 10 / 14 substrate and contained paper - wool bedding material (Shredded Nesting, International Product Supplier Limited, London, UK), a red plastic mouse house (Techniplast, NJ, USA), a lid-suspended nesting box (MPlex, Otto Environmental, WI, USA), and two cardboard tubes (11 cm long, 5 cm diameter). A cage of two females would normally contain one cardboard tube and a mouse house. The extra environmental enrichment was provided here to give the previously unfamiliar females the opportunity to avoid each other. Food (Lab Diet 5002, International Product Supplies Limited, London, UK) and water were provided *ad libitum*. Cages were monitored for 90 minutes after introduction for any excessive levels of aggression between the pairs. Bouts of intense aggression lasting over 10 seconds were interrupted by an observer placing their hand over the cage. Females that had to be interrupted on three separate occasions over the 90 minute period were separated and subjects were consequently returned to their home cages and dropped from the experiment. Only one pair of females had to be separated.

To assess the short-term effects of treatment urine and faecal samples were collected at 15:00 on the first experimental day - 7 hours after initial introduction. Corticosterone has been shown to take approximately 4 hours during the dark phase to be released from the body via faecal matter (Touma *et al.*, 2003). Mice were left for 7 hours before sampling in order to limit any changes in corticosterone caused by handling. Urine and faecal samples were collected over 90 minutes using the recovery method and stored in 1.5 ml Eppendorf tubes at

-22 °C. After urine and faecal collection, females were weighed and returned to the experimental cages.

The pairs of experimental females were then left for a period of three days. Every day each female was removed from the test cages and examined in clear acrylic handling tubes. Females that appeared in poor condition (e.g. fur ruffled or missing, bite marks) were separated from their experimental cage, returned to their home cages and excluded from the analysis. Only one pair of females were separated after the introduction day. On the third day at 15:00 urine and faecal samples were again collected over 90 minutes using the recovery method and stored in 1.5 ml Eppendorf tubes at -22 °C. Females were weighed and then returned to their original home cages.

5.3.6 Corticosterone

Corticosterone was analysed from faecal matter as this is a non-invasive method which does not influence stress levels of animals unlike the methodology associated with collecting plasma samples (Palme, 2012). Faecal samples were weighed prior to corticosterone extraction. Approximately 0.1 – 0.5 g of faecal material was placed in an extraction vial and 3 ml of 90% methanol added. Vials were vortexed (CM-1 Cyclone Vortex Mixer, Nickel Electro Ltd, Avon, UK) and placed on a rotator (Stuart™ gyratory rocker SSL3, Sigma-Aldrich, UK) over night (15 – 17 hours). Vials were centrifuged (Eppendorf centrifuge 5702, Eppendorf, Hamburg, Germany) for 20 minutes at 1800 rpm and the supernatant poured into a glass extraction tube (16 x 125 mm, boro rimless, Thermo Fisher Scientific Oy, Vantaa, Finland). Supernatant was dried under air in a 60 °C water bath (JB1, Grant Instruments Ltd, Cambridge, UK) for 30 – 60 minutes. Samples were re-suspended in 1 ml 100% methanol and agitated on a rotator for 15 minutes. Samples were then stored in 1.5 ml Eppendorf tubes at -22°C.

The amount of faecal corticosterone was determined using an enzyme immunoassay (EIA) developed by C. Munro, Department of Population Health and Reproduction at the University of California, USA (Watson *et al.*, 2012). EIAs were run by Amanda Davidson of the Mammalian Behaviour & Evolution Group, University of Liverpool, UK. All samples from the same female were run on one plate. Assay plates (Nunc-Immuno F96 MicroWell™ MaxiSorp™, Thermo Fisher Scientific Oy, Vantaa, Finland) were coated with 50 μl antibody solution (1:11,000, corticosterone Ab (CJM006) diluted with sodium bicarbonate coating buffer) and refrigerated overnight. Plates were washed five times using 1x wash solution

(deionized distilled water) and a Thermo Scientific Wellwash (Thermo Fisher Scientific Oy, Vantaa, Finland). 50 µl per well of standard, sample or control were added in duplicate. Standard values were 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 and 3.9 pg corticosterone/well (1 mg/100ml EtOH corticosterone primary stock diluted in EIA buffer – phosphate buffered solution (PBS) containing 0.1% bovine serum albumin (BSA); pH 7.0). 50 µl CC-horseradish peroxidise (HRP conjugate, 1:65,000) was then added to each well. Plates were then covered and incubated in the dark for 2 hours. After incubation plates were washed five times with wash solution and 100 µl substrate solution (0.5M H_2O_2, 40mM ABTS and 0.05M Citric Acid) was added to each well. Plates were incubated in the dark for 15 minutes and then when blank wells read ~ 0.8 optical density, plates were read. The absorbance of each sample was read at 405 nm in a Thermo Scientific Multiskan FC microplate photometer (Thermo Fisher Scientific Oy, Vantaa, Finland). SkanIt software 3.1 (research edition for Multiskan FC, Thermo Fisher Scientific Oy, Vantaa, Finland) was used to produced a standard curve and the concentration of each sample was calculated by interpolation. Readings of corticosterone for each sample were adjusted to take into account the original faecal mass from which corticosterone was extracted.

5.3.7 Protein

For each female there were three urine samples – a pre-treatment level sample collected prior to the experiment, a sample from the first day of introductions (7 hours after experimental pairing), and an end of experiment sample (3 days after experimental pairing). All samples for each female were run at the same time to correct for any differences between individual assays. The concentration of protein in each urine sample was measured using the Coomassie plus® protein assay reagent kit from Perbio Science UK Ltd. (Cramlington, Northumberland, UK), following the protocol of (Cheetham *et al.*, 2009). Each sample was diluted 1:100 with ddH_20, and 100 µl aliquots pipetted in duplicate to a 96 well microtiter plate (Sterilin Microplate F Well 611F96, Thermo Fisher Scientific Oy, Vantaa, Finland). A stock solution of 2 mg/ml BSA was used to generate a standard curve, with concentrations ranging from 0 – 50 µg/ml made up using ddH_20. To each microtiter plate well 200 µl Coomassie reagent was added. The absorbance of each sample was read at 620 nm in a Thermo Scientific Multiskan FC microplate photometer (Thermo Fisher Scientific Oy, Vantaa, Finland). SkanIt software 3.1 (research edition for Multiskan FC, Thermo Fisher Scientific Oy, Vantaa, Finland) was used to produced a standard curve and the concentration of each sample was calculated by interpolation.

5.3.8 Creatinine

Urinary dilution can influence urinary protein concentrations and therefore confound results. Creatinine is produced and excreted in mouse urine at a constant rate according to muscle mass. Urinary creatinine therefore is therefore a useful indicator of urinary dilution and is often used to correct for the concentration of protein in mouse urine (Beynon & Hurst, 2004). If muscle mass changes over repeated sampling of an individual, however, then urinary protein concentration cannot be adjusted for creatinine concentration as changes in creatinine could be as a result of either the change in muscle mass or the experimental treatment on the individual. Over the short-term, between the pre-treatment and Day 1 it is unlikely that changes in creatinine will be due to changes in muscle mass. Therefore over the short term urinary protein concentration can be adjusted for urine dilution using urinary creatinine concentration. Over a longer time period (pre- to post-treatment) it is possible that female muscle mass may change. It is therefore important to first establish that urinary creatinine does not change between pre- and post-treatment, before urinary protein concentration can be corrected for urine dilution using urinary creatinine concentration.

An alkaline picrate assay (Sigma Chemicals, UK) was used to measure urinary creatinine values (Cheetham *et al.*, 2009). Each sample was diluted 1:50 with ddH_20, and 100 μl aliquots pipetted in duplicate to a 96 well microtiter plate (Sterilin Microplate F Well 611F96, Thermo Fisher Scientific Oy, Vantaa, Finland). A stock solution of 3 mg/dl creatinine was used to generate a standard curve, with concentrations ranging from 0 – 30 μg/ml, made up using ddH_20. To each microtiter plate well 150 μl alkaline picrate reagent (5 ml pictrate colution : 1 ml sodium hydroxide) was added. The absorbance of each sample was read at 492 nm in a Thermo Scientific Multiskan FC microplate photometer (Thermo Fisher Scientific Oy, Vantaa, Finland). SkanIt software 3.1 (research edition for Multiskan FC, Thermo Fisher Scientific Oy, Vantaa, Finland) was used to produced a standard curve and the concentration of each sample was calculated by interpolation. All samples from the same female were run on one plate.

5.3.9 Data Analysis

Only one female from each housed pair was used in the analysis. Females were used only if they were in oestrus when sampled pre-treatment. Creatinine and protein levels were only measured if 10 μl of urine or more was collected on all three sampling days (Pre, Day 1 and Post). Where possible, protein was adjusted for creatinine to correct for urinary dilution (see

Section 5.3.8). Corticosterone levels were only measured if 0.1 g or more faeces was collected on all three sampling days. Data analysis was performed in SPSS (version 20.0.0). Corticosterone data was logarithmically transformed to meet parametric assumptions. Adjusted protein scores were logarithmically transformed to meet parametric assumptions. All figures use untransformed data.

To initially demonstrate that there was no pre-treatment difference between the familiarity groups independent t-tests compared pre-treatment familiar and unfamiliar groups for each physiological measure (e.g. corticosterone, body mass, creatinine and protein). To establish that there was no pre-treatment difference between the relatedness groups ANOVAs compared pre-treatment full sister, maternal half-sister, paternal half-sister and unrelated female groups for each physiological measure.

To investigate the short-term effect of familiarity, repeated measures GLMs compared pre-treatment and Day 1 measurements for each physiological measurement, with familiarity group as a between subjects factor. To investigate the long-term effect of familiarity repeated measures GLMs compared pre-treatment and post-treatment measurements for each physiological measurement, with familiarity group as a between subjects factor. This analytical approach was repeated to investigate the effect of relatedness on physiology over the short- and long-term using relatedness group as a between subjects factor.

5.4 Results

5.4.1 Physiological Changes with Familiarity

Females were experimentally housed for three days with a familiar full sister or an unfamiliar full sister and changes in female faecal corticosterone, body mass and urinary creatinine and protein levels were measured across the short-term (pre-treatment to Day 1) and the long-term (pre-treatment to post-treatment). The results are summarised in Table 5.1. There were no pre-treatment differences between the familiarity groups in faecal corticosterone ($t_{(22)} = 1.11$, $p = 0.28$), body mass ($t_{(22)} = 1.00$, $p = 0.33$), urinary creatinine ($t_{(22)} = -1.12$, $p = 0.27$) or urinary protein ($t_{(22)} = -1.58$, $p = 0.13$).

5.4.1.1 Short-Term Physiological Changes with Familiarity

Over a shorter time period there was no change in faecal corticosterone levels ($F_{(1,22)} = 1.25$, $p = 0.28$; Figure 5.1), and there was no difference in corticosterone change between the familiar and unfamiliar groups ($F_{(1,22)} = 2.74$, $p = 0.11$; Figure 5.1).

There was a non-significant interaction for body mass to decrease between pre-treatment and Day 1 in the familiar group (-0.26 ± 0.14 g, mean ± standard error) but increase in the unfamiliar group (0.15 ± 0.13 g, mean ± standard error; $F_{(1,22)} = 4.07$, $p = 0.06$; Figure 5.2), however overall female body mass did not change in response to short-term housing ($F_{(1,22)} = 0.62$, $p = 0.44$; Figure 5.2).

Urinary creatinine levels did not change in response to housing ($F_{(1,22)} = 0.36$, $p = 0.56$; Figure 5.3). There was no effect of familiarity treatment group on creatinine change ($F_{(1,22)} = 0.91$, $p = 0.35$; Figure 5.3). Female urinary protein concentration did not change between pre-treatment and Day 1 ($F_{(1,22)} = 0.21$, $p = 0.65$; Figure 5.4). There was no interaction between familiarity group and change in urinary protein between pre-treatment and Day 1 ($F_{(1,22)} = 0.12$, $p = 0.73$; Figure 5.4).

5.4.1.2 Long-Term Physiological Changes with Familiarity

There was no change in faecal corticosterone over a longer time period ($F_{(1,22)} = 0.84$, $p = 0.37$; Figure 5.1). Change in corticosterone over a longer time period did not differ with familiarity group ($F_{(1,22)} = 1.71$, $p = 0.20$; Figure 5.1).

Over a longer time period there was a significant interaction between change in body mass and familiarity group ($F_{(1,22)} = 8.40$, $p = 0.01$; Figure 5.2), suggesting that familiar females

decreased in body mass (-0.32 ± 0.19 g, mean ± standard error) and unfamiliar females increased in body mass (0.48 ± 0.20 g, mean ± standard error). Further investigation revealed that unfamiliar females significantly increased in body mass between pre- and post-treatment ($t_{(12)} = -2.45$, $p = 0.03$, paired t-test; Figure 5.2), whilst females in the familiar group did not significantly change in body mass over the course of the treatment ($t_{(10)} = 1.68$, $p = 0.12$, paired t-test; Figure 5.2).

Over a longer time period urinary creatinine levels did not change in response to housing ($F_{(1,22)} = 0.46$, $p = 0.51$; Figure 5.3). There was no difference in creatinine change between pre- and post-treatment according to the familiarity of sisters groups ($F_{(1,22)} = 3.06$, $p = 0.09$; Figure 5.3). As there was no difference in urinary creatinine between pre- and post-treatment, urinary protein concentration could be adjusted for urine dilution. Over the full duration of the treatment female urinary protein increased (familiar 0.55 ± 0.32 mg/mg creatinine, unfamiliar 1.34 ± 0.67 mg/mg creatinine (mean ± standard error); $F_{(1,22)} = 8.13$, $p = 0.01$; Figure 5.4). There was no difference in the change in urinary protein between the familiar and unfamiliar full sister groups ($F_{(1,22)} = 1.12$, $p = 0.30$; Figure 5.4).

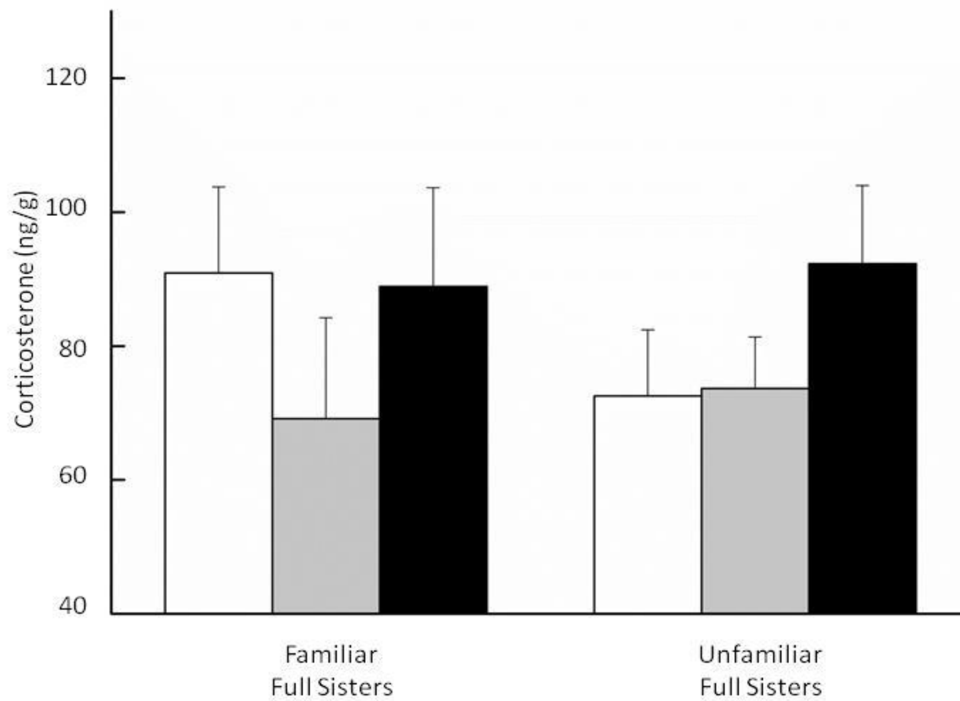


Figure 5.1: Female faecal corticosterone across time in the familiar and unfamiliar groups.
Corticosterone (ng/g, mean + standard error) measured at pre-treatment (open bars), Day 1 (grey bars) and post-treatment (black bars) in the familiar (n = 11) and unfamiliar groups (n = 13).

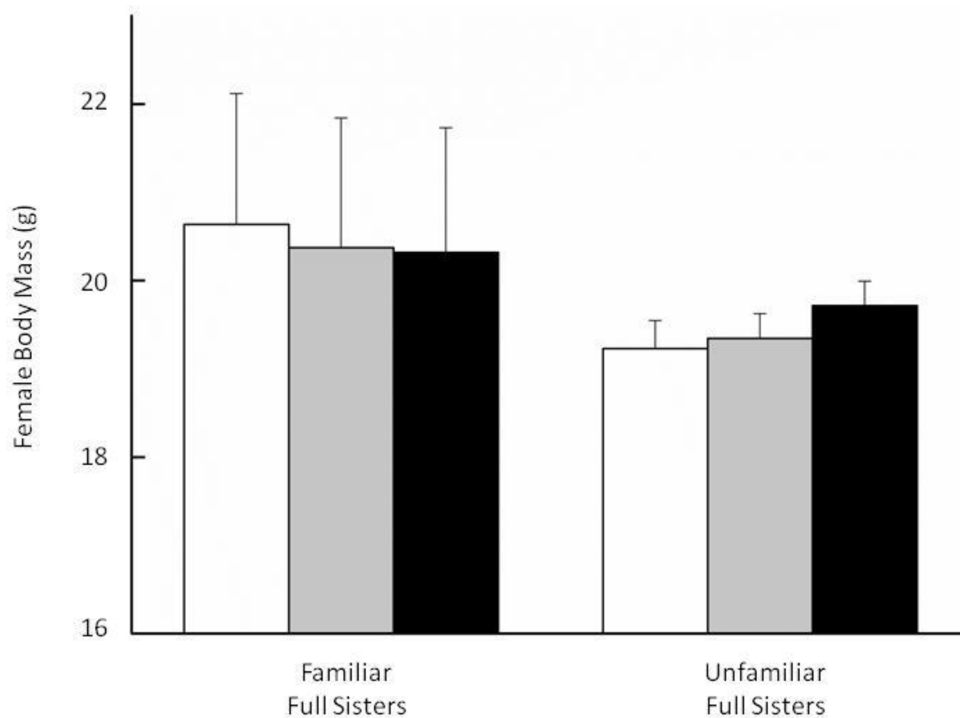


Figure 5.2: Female body mass across time in the familiar and unfamiliar groups.
Body mass (g, mean + standard error) measured at pre-treatment (open bars), Day 1 (grey bars) and post-treatment (black bars) in the familiar (n = 11) and unfamiliar (n = 13) groups.

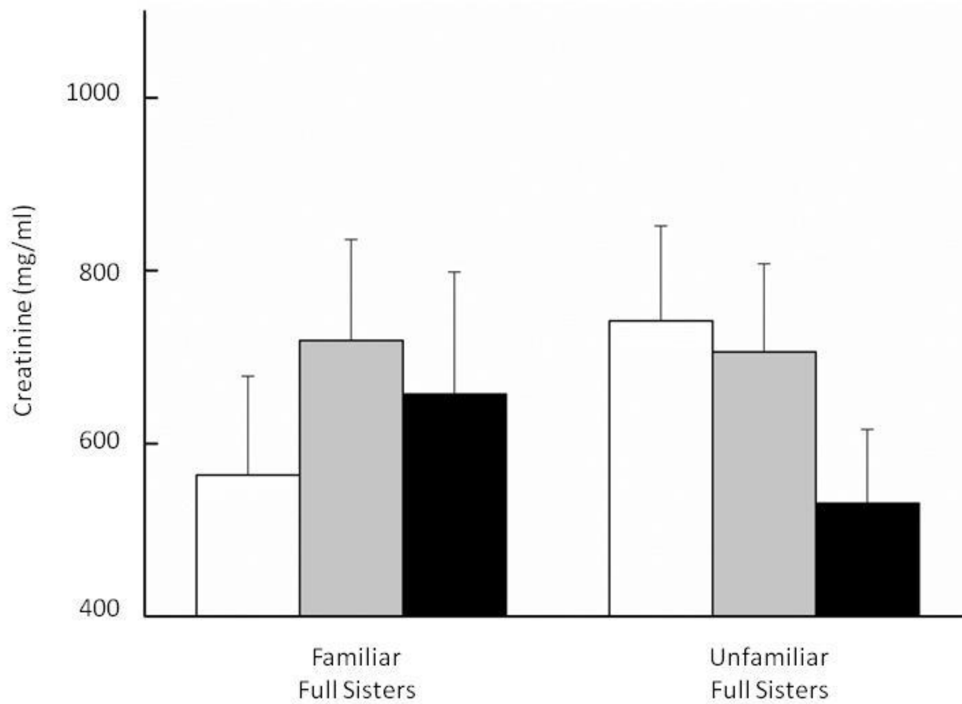


Figure 5.3: Female urinary creatinine concentration across time in the familiar and unfamiliar groups.
Urinary creatinine (mg, mean + standard error) measured at pre-treatment (open bars), Day 1 (grey bars) and post-treatment (black bars) in the familiar (n = 11) and unfamiliar (n = 13) groups.

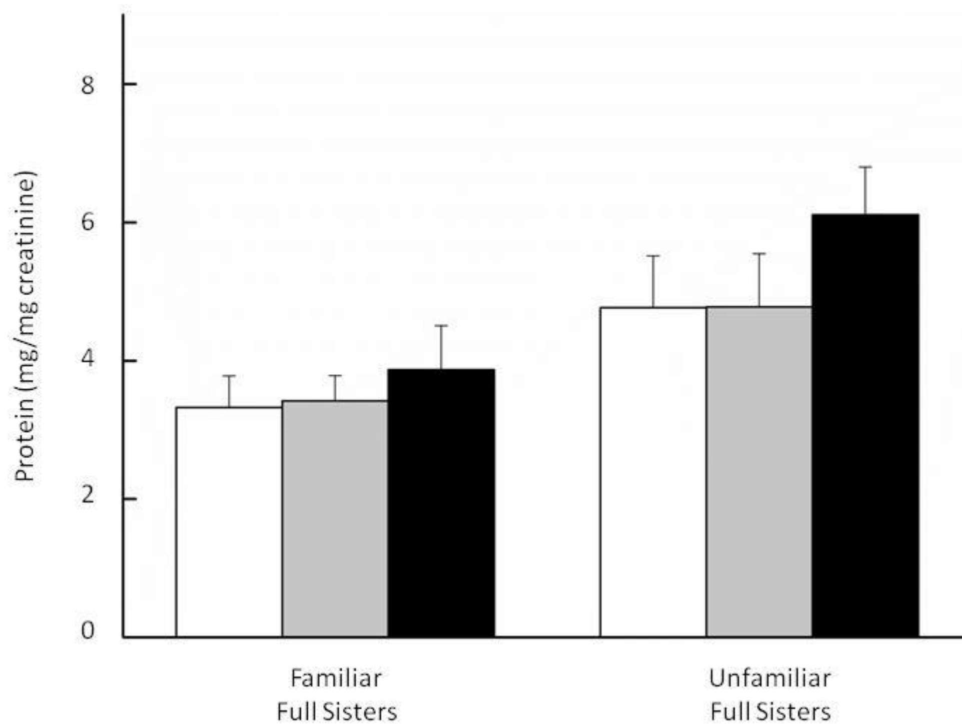


Figure 5.4: Female urinary protein adjusted for urine dilution across time in the familiar and unfamiliar groups.
Urinary protein (mg/mg creatinine, mean + standard error) measured at pre-treatment (open bars), Day 1 (grey bars) and post-treatment (black bars) in the familiar (n = 11) and unfamiliar (n = 13) groups.

5.4.2 Physiological Changes with Relatedness

Females were experimentally housed for three days with an unfamiliar full sister, an unfamiliar maternal half-sister, an unfamiliar paternal half-sister or an unfamiliar unrelated female. Changes in female faecal corticosterone, body mass and urinary creatinine and protein levels were measured over the short-term (pre-treatment to Day 1) and the longer-term (pre-treatment to post-treatment). The results are summarised in Table 5.1. There were no pre-treatment differences between the relatedness groups in faecal corticosterone ($F_{(3,45)}$ = 0.11, p = 0.96), body mass ($F_{(3,45)}$ = 1.63, p = 0.20) or urinary creatinine ($F_{(3,45)}$ = 0.62, p = 0.60). There was no difference between the relatedness groups in creatinine-adjusted urinary protein ($F_{(3,45)}$ = 0.06, p = 0.98).

5.4.2.1 Short-Term Physiological Changes with Relatedness

Over a short time period female faecal corticosterone did not change ($F_{(1,45)}$ = 0.92, p = 0.34; Figure 5.5). There was no difference in corticosterone change between the relatedness groups ($F_{(3,45)}$ = 0.12, p = 0.95; Figure 5.5).

Overall female body mass decreased between pre-treatment and Day 1 measurements ($F_{(1,45)}$ = 15.32, p < 0.001; Figure 5.6), however there was a significant interaction between change in body mass between pre-treatment and Day 1 and relatedness group ($F_{(3,45)}$ = 3.87, p = 0.02; Figure 5.6), although pairwise comparisons revealed no difference in short-term change in body mass between any of the individual groups (all p > 0.05). Further inspection of the data revealed that females did not significantly change in body mass in the full sister group (0.12 ± 0.13 g (mean ± standard error), $t_{(12)}$ = -0.92, p = 0.38, paired t-test) and in the maternal half-sister group (-0.43 ± 0.27 g (mean ± standard error), $t_{(11)}$ = 1.63, p = 0.13, paired t-test). However there was a decrease in female body mass between pre-treatment and Day 1 in the paternal half-sister group (-0.40 ± 0.14 g (mean ± standard error), $t_{(11)}$ = 2.94, p = 0.01, paired t-test), and the unrelated female group (-0.80 ± 0.22 g (mean ± standard error), $t_{(11)}$ = 3.63, p = 0.004, paired t-test).

Urinary creatinine increased over a short time period (148.23 ± 66.52 mg/ml, mean ± standard error; $F_{(1,45)}$ = 5.33, p = 0.03; Figure 5.7). There was no interaction between creatinine and relatedness group ($F_{(3,45)}$ = 1.35, p = 0.27; Figure 5.7).

Over the short-term female urinary protein decreased (-0.85 ± 0.40 mg/ml, mean ± standard error; $F_{(1,45)}$ = 5.14, p = 0.03; Figure 5.8). There was no difference between the relatedness

groups in the urinary protein change between pre-treatment and Day 1($F_{(3,45)} = 1.03$, $p = 0.39$; Figure 5.8).

5.4.2.2 Long-Term Physiological Changes with Relatedness

There was no change in faecal corticosterone between the pre- and post-treatment measurements ($F_{(1,45)} = 0.63$, $p = 0.43$; Figure 5.5). Change in faecal corticosterone between pre- and post-treatment did not differ between relatedness groups ($F_{(3,45)} = 1.18$, $p = 0.33$; Figure 5.5).

Over the longer time measurement female body mass increased (0.28 ± 0.11 g, mean ± standard error; $F_{(1,45)} = 6.44$, $p = 0.02$; Figure 5.6). There was no difference between the relatedness groups and change in body mass over the longer-term ($F_{(3,45)} = 0.75$, $p = 0.53$; Figure 5.6).

Female urinary creatinine did not change between the pre- and post-treatment measurements ($F_{(1,45)} = 2.65$, $p = 0.11$; Figure 5.7) and there was no difference between the relatedness groups in the change in faecal creatinine from pre- to post-treatment ($F_{(3,45)} = 0.75$, $p = 0.53$; Figure 5.7). As there was no difference in urinary creatinine between pre- and post-treatment, urinary protein concentration could be adjusted for urine dilution. Female urinary protein concentration increased between pre- and post-treatment (0.44 ± 0.35 mg/mg creatinine (mean ± standard error); $F_{(1,45)} = 4.37$, $p = 0.04$; Figure 5.8). There was no difference in the change of urinary protein with relatedness group ($F_{(3,45)} = 1.32$, $p = 0.28$; Figure 5.8).

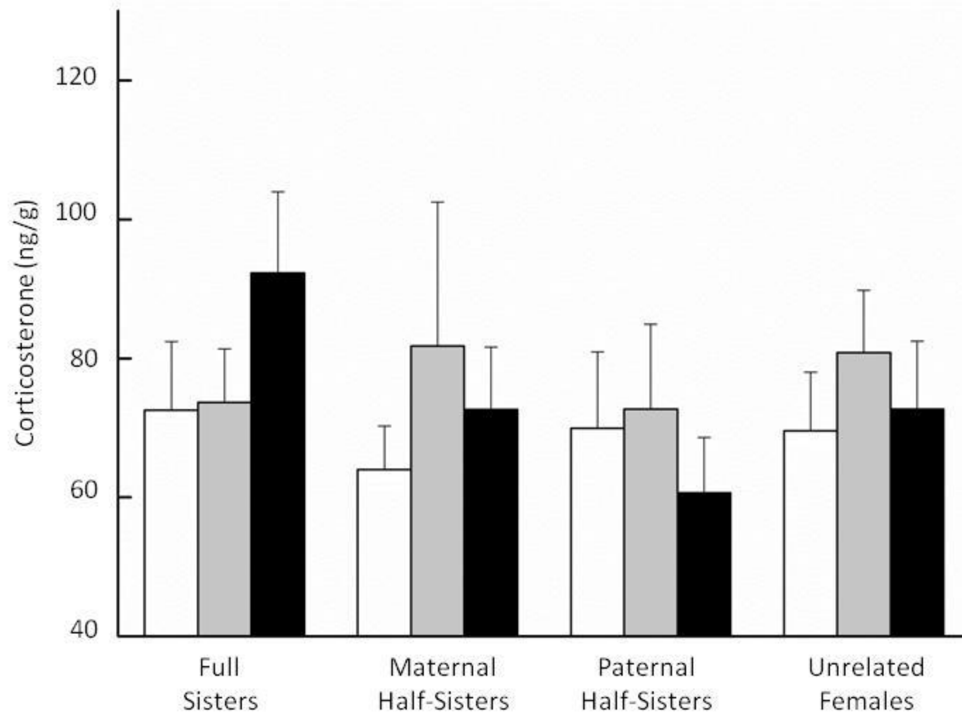


Figure 5.5: Female faecal corticosterone across time in relatedness groups.
Faecal corticosterone (ng/g, mean + standard error) measured at pre-treatment (open bars), Day 1 (grey bars) and post-treatment (black bars) in the full sister (n = 13), maternal half-sister (n = 12), paternal half-sister (n = 12), and unrelated female (n = 12) groups.

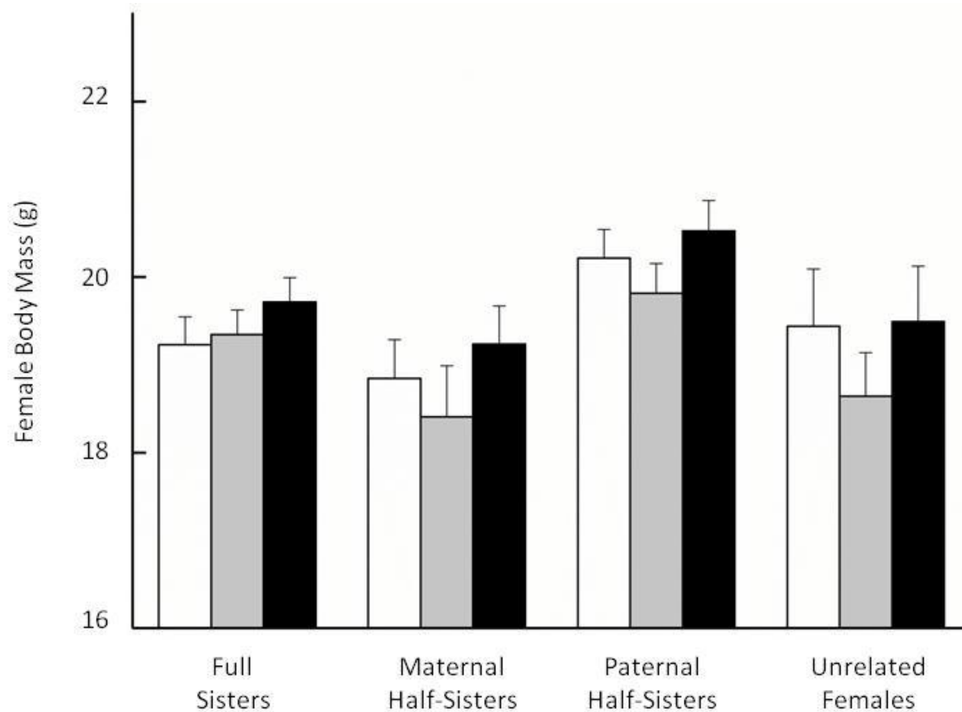


Figure 5.6: Female body mass across time in relatedness groups.
Body mass (g, mean + standard error) between pre-treatment (open bars), Day 1 (grey bars) and post-treatment (black bars) in the full sister (n = 13), maternal half-sister (n = 12), paternal half-sister (n = 12), and unrelated female (n = 12) groups.

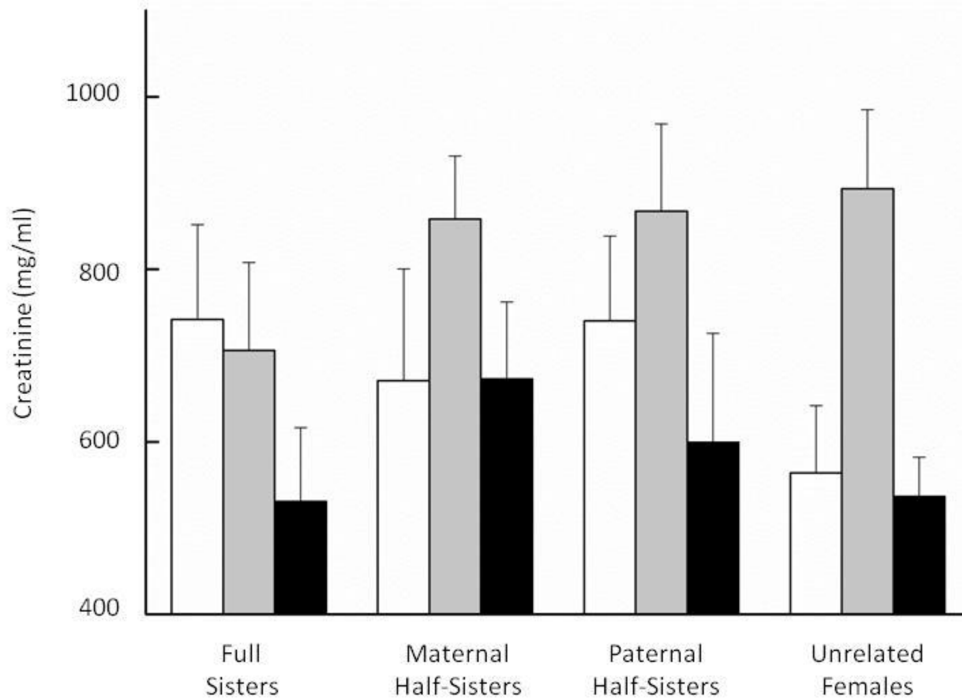


Figure 5.7: Female urinary creatinine concentration across time in relatedness groups.
Urinary creatinine (mg, mean + standard error) measured at pre-treatment (open bars), Day 1 (grey bars) and post-treatment (black bars) in the full sister (n = 13), maternal half-sister (n = 12), paternal half-sister (n = 12), and unrelated female (n = 12) groups.

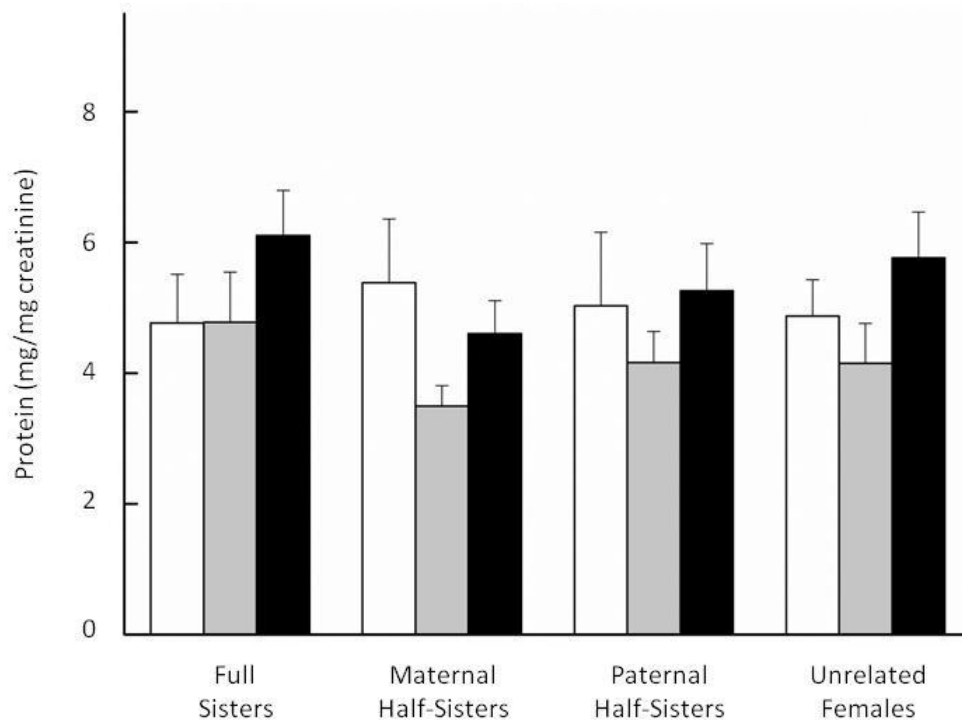


Figure 5.8: Female urinary protein concentration across time in relatedness groups.
Urinary protein (mg/mg creatinine, mean + standard error) measured at pre-treatment (open bars), Day 1 (grey bars) and post-treatment (black bars) in the full sister (n = 13), maternal half-sister (n = 12), paternal half-sister (n = 12) and unrelated female (n = 12) groups.

Table 5.1: Summary of physiological changes and social environment results.

	Physiological Measure	Short Term Change		Long Term Change	
		Familiarity	**Pre-Treatment To Day 1**	**Familiarity**	**Pre- To Post-Treatment**
Familiarity	Faecal Corticosterone	p = 0.11[a]	p = 0.28[a]	p = 0.20[a]	p = 0.37[a]
	Body Mass	p = 0.06[a]	p = 0.44[a]	**p = 0.01**[a]	Fam: p = 0.12[b] Unf: **p = 0.03**[b] **(Post>Pre)**
	Urinary Creatinine	p = 0.35[a]	p = 0.56[a]	p = 0.09[a]	p = 0.51[a]
	Urinary Protein	p = 0.73[a]	p = 0.65[a]	p = 0.30[a]	**p = 0.01**[a] **(Post>Pre)**
		Relatedness	**Pre-Treatment To Day 1**	**Relatedness**	**Pre- To Post-Treatment**
Relatedness	Faecal Corticosterone	p = 0.60[a]	p = 0.34[a]	p = 0.33[a]	p = 0.43[a]
	Body Mass	**p = 0.02**[a]	FS: p = 0.38[b] MHS: p = 0.13[b] PHS: **p = 0.01**[b] **(Pre>D1)** Un: **p < 0.01**[b] **(Pre>D1)**	p = 0.53[a]	**p = 0.02**[a] **(Post>Pre)**
	Urinary Creatinine	p = 0.27[a]	**p = 0.03**[a] **(D1>Pre)**	p = 0.53[a]	p = 0.11[a]
	Urinary Protein	p = 0.39[a]	**p = 0.03**[a] **(Pre>D1)**	p = 0.28[a]	**p = 0.04**[a] **(Post>Pre)**

Physiological changes across the short (pre-treatment to Day 1) and longer (pre- to post-treatment). Pre – Pre-Treatment,D1 – Day 1, Post – Post-Treatment, FS – Full Sister group, MHS – Maternal Half-Sister group, PHS – Paternal Half-Sister group, Un – Unrelated group. Statistical tests performed: a – repeated measures GLM, b – paired t-test.

5.5 Discussion

The effect of social environment on female house mouse physiology was measured using changes in faecal corticosterone and urinary protein, as well as female body weight. Female house mice were housed experimentally in pairs for approximately 79 hours and were sampled pre-treatment, 7 hours after initial introduction (Day 1), and post-treatment. The effect of female social environment was compared by varying either relatedness or familiarity whilst keeping the second factor constant. To assess familiarity females were either paired with a familiar full sister or an unfamiliar full sister. To assess relatedness, unfamiliar females were paired that were full sisters, maternal half-sisters, paternal half-sisters or unrelated to each other. The results are summarised in Table 5.1.

5.5.1 Short- and Longer-Term Changes in Faecal Corticosterone

Corticosterone is a glucocorticoid hormone released by the adrenal gland in response to a perceived physical or psychological stressor (Sapolsky, 2002). Changes in corticosterone (or cortisol) are often used as indicators of stress in animal studies (e.g. Smith & French, 1997; Young *et al.*, 2006). No significant differences in female faecal corticosterone levels were observed over a short or longer time period, and no differences were observed with either familiarity or relatedness. Together these results suggest that, regardless of the degree of familiarity or relatedness between the paired females, introduction and housing with an unfamiliar female is not stressful for female house mice as measured by changes in faecal corticosterone. This result corresponds with studies of female laboratory mice where plasma and faecal corticosterone did not increase as a result of experimental housing with either familiar related or unfamiliar unrelated females (Bartolomucci *et al.*, 2009; Akre *et al.*, 2011).

Whilst female corticosterone levels did not statistically change, the values displayed in Figure 5.5 may suggest a short-term affect of housing with an unfamiliar female as mean measurements of faecal corticosterone increased between pre-treatment and Day 1, whilst in the familiar full sister group faecal corticosterone decreased over the short-term. It is possible therefore that introduction and housing with a novel female is a slight stressor. Alternatively, this change could be as a result of changes in female body mass or water intake. In the relatedness group, urinary creatinine values change between pre-treatment and Day 1, suggestive of a decrease in water intake. This could also explain the observed trend for an increase in faecal corticosterone over a short time period in females paired with unfamiliar females (regardless of relatedness). Further studies could investigate this using both related

and unrelated individuals that are familiar or unfamiliar and an increased sample size. This study, however, found no difference in change in female faecal corticosterone levels in response to housing with females of varied familiarity or relatedness.

A large amount of individual variation in corticosterone levels was observed in female response to experimental housing. For individual females levels of faecal corticosterone increased or decreased across the short- and longer-term. Female interactions and behaviour were not observed, other than monitoring behaviour over the initial 90 minutes following experimental housing when bouts of prolonged aggression were separated. It would have been interesting to understand the nature of the relationship between paired females. In some experimental pairs unfamiliar females may establish a dominance hierarchy, with one female behaving in a more aggressive or competitive manner and the other acting more submissive. In cooperatively breeding species dominant individuals often have higher levels of glucocorticoids (including corticosterone) than subordinate individuals (Creel, 2001). If social hierarchies were formed between pre- and post-treatment stages, corticosterone levels may have increased in dominant individuals only. Alternatively, subordinates may have increased corticosterone levels as a result of competitive behaviours directed towards them by dominant individuals (Creel, 2001). Subordinate male laboratory mice have higher levels of blood plasma corticosterone than dominant males (Louch & Higginbo.M, 1967), a finding echoed by Garratt (2010) who found a trend for male house mice that received more attacks to have higher corticosterone levels. As a result of competitive female behaviour, evicted female meerkats have an increased level of faecal corticosterone (Young *et al.*, 2006). Behavioural observations of paired females could have allowed for the comparison of corticosterone levels with social rank, and so could help to explain some of the individual variation observed here.

5.5.2 Short- and Longer-Term Changes in Female Body Mass

Female body mass changed in response to housing across both the short- and longer-term. Over the longer-term familiar full sisters did not significantly change in body mass whilst unfamiliar full sisters increased in body mass. This difference was observed as a non-significant trend over the short-term. Body mass changed across both the short- and longer-term in the relatedness measurement. In the short-term the significant interaction between body mass and relatedness group suggested that full sisters may increased in body mass whilst unfamiliar maternal and paternal half-sisters and unrelated females decreased in body

mass. Whilst pairwise comparisons did not show any significant differences between the relatedness groups it is possible that a larger sample size may have shown a significant difference between the relatedness groups. Over the longer-term female body mass increased in all unfamiliar relatedness groups.

Overall then, body mass of full sisters appeared constant over the short-term, whilst more distantly related females appear to decrease in body mass. There are a number of possible reasons for this. As urinary creatinine concentration increased over the short-term in the relatedness analysis, this suggests that the reason female body mass decreased may be down to a decrease in water uptake. Close kin are often predicted to behave less aggressively and more amicably towards each other (Maher, 2009; Markman *et al.*, 2009) and it is possible that interactions between more distantly related females (and unrelated females) prevented females from drinking as much water as they usually would.

Additionally, the novel social and physical environment could cause female body mass to decrease as females actively explore the environment, and engage in investigatory and competitive behaviours with their new cagemates. Increased movement around the cage and high levels of interaction with an unfamiliar cagemate immediately following introduction could therefore explain the decrease in body mass observed over the short-term in the more distantly related groups (Whittaker *et al.*, 2012). Alternatively, encountering an unfamiliar environment or unfamiliar female, regardless of relatedness, could be a stressor resulting in a decrease in body mass but not an increase in faecal corticosterone (Sahin & Gumuslu, 2007).

Female oestrus cycle stage affects female food intake in both laboratory mice and rats (Drewett, 1973; Petersen, 1976). In mice the oestrus cycle is 4 to 5 days long, although females have been known to have shorter and longer cycles. All females used in the analysis were in oestrus pre-treatment. At the Post-treatment stage (4 days after pre-treatment) the majority of females should have been in or entering oestrus again. In laboratory mice food intake decreases before oestrus, increases during oestrus and peaks in metoestrus (Petersen, 1976). The degree of food intake is likely to influence body mass. However if food intake as a result of changes in oestrus stage were solely responsible for the changes in female body mass observed here than females might actually have been expected to increase in body mass between pre-treatment and Day 1. This is the reverse of what was observed, suggesting that the changes in female body mass are unlikely to be as a result of the oestrus cycle.

Over the longer-term body mass did not change in familiar full sisters whilst unfamiliar full sisters and more distantly related females increased in body mass. This suggests a difference with familiarity as females in all the unfamiliar groups increased in body mass over the longer-term. The oestrus cycle of the female house mouse is approximately 4 – 5 days and so females may be expected to be in approximately the same stage of oestrus at post-treatment as at pre-treatment. This suggests that an increased food intake as a result of the oestrus cycle was unlikely to have caused the observed increase in body mass. There was no difference in urinary creatinine concentration suggesting that at the post-treatment measurement females were drinking water at a standard rate. The resumption of pre-treatment levels of water intake could account for a return to original body mass, but does not explain why female body mass increased pre- and post-treatment.

The establishment of a social hierarchy means that repeated energetically-expensive highly competitive interactions are no longer necessary. Once the cage environment is no longer novel and female relationships have been established, females would likely make fewer investigatory or competitive behaviours which could also explain a return to original body mass after the initial reduction. However, the increased social stimulation or competition may also promote a compensatory mechanism that results in an increased body mass. A greater body mass may give females a higher competitive ability. As discussed above, it would be interesting to know the relationship between females tested here and correlate the results of behavioural interactions with the observed changes in body mass. Additionally, it would be interesting to observe the change in female body mass over a longer time period to see whether body mass returns to its pre-treatment levels or remains higher than pre-treatment levels.

5.5.3 Short- and Longer-Term Changes in Urinary Creatinine and Protein

House mice excrete large amounts of protein in their urine and extensively mark their territories using scent marks (Beynon & Hurst, 2004). The production and excretion of large quantities of proteins in urine is likely to be metabolically costly (Gosling *et al.*, 2000). Over the short-term there was no change in urinary creatinine or protein in either familiarity group, however protein did increase in both familiarity groups between pre- and post-treatment.

Over the short-term urinary creatinine increased in the relatedness groups meaning that urine samples were more concentrated on Day 1 than pre-treatment. Competitive behaviour between females may have reduced water intake, resulting in urine of a higher concentration.

Additionally, increased scent marking in response to a novel social and physical environment or increased competition between females may have also caused females to deposit small but more concentrated urine marks. In the longer-term creatinine did not change meaning that there was no difference in urinary concentration between pre- and post-treatment.

In the relatedness groups urinary protein decreased over the short-term and increased over the longer-term. This longer-term increase in urinary protein was also observed in the familiarity groups. There are a number of possible explanations for the changes in urinary protein observed here. Female protein output and body mass could be linked with heavier females producing more urinary proteins. The reduction in body mass between pre-treatment and Day 1 could cause females to excrete fewer proteins in their urine, whilst the increase in body mass between pre- and post-treatment could result in a higher level of urinary protein excretion.

Oestrus cycle stage has been reported to influence MUP output in female laboratory mice, with peak MUP production coinciding with the start of the oestrus cycle (Stopka *et al.*, 2007). In mice the oestrus cycle is 4 to 5 days long, although females have been known to have shorter and longer cycles. Pre-treatment females were in oestrus and by post-treatment (4 days following) the majority of females were expected to be entering oestrus again. Female oestrus cycle could therefore explain the pattern of protein change observed here as protein could have peaked at that start of the oestrus cycle shortly before sampling at pre-treatment, decreased by Day 1 and then increased by post-treatment, beyond the level observed at pre-treatment to coincide with the start of the oestrus cycle.

Whilst the oestrus cycle influences levels of MUP expression in female laboratory mice, the levels of MUP expression between laboratory and house mice are considerably different. Female laboratory mice produce an average urinary MUP output of 3.01 (mg / mg creatinine) with a range of 0.7 – 7.6, whilst female house mice produce an average urinary MUP output of 9.5 (mg / mg creatinine) with a range of 4.0 – 20.3 (Beynon & Hurst, 2004). This difference in MUP output between laboratory and house mice means that it is possible that the effects of the oestrus cycle on urinary MUP output are only apparent when base levels are low to start with, and an additional study could examine the relationship between MUP output and oestrus further. A study of the East-European house mouse (*Mus musculus musculus*, a closely related sub-species) revealed no differences in urinary MUP output with oestrus stage (Janotova & Stopka, 2011). Investigation of the data suggested that urinary

protein concentration did not decrease over the short term in either the familiar or unfamiliar full sister groups. Female oestrus stage may therefore not be completely responsible for the short-term decrease in urinary protein level observed in the relatedness groups. Regular monitoring of female oestrus cycle could reveal an effect on urinary protein levels, however repeated use of the vaginal smear test may disrupt females' regular oestrus cycling.

An alternative reason for a short-term decrease in urinary protein could be in response to a novel social and physical environment. House mice scent mark their territories and in the experiment reported here subject females entered a clean cage devoid of any urine marks. In the clean cage environment the females then also encountered novel females. It is possible that the decrease in urinary protein level observed between pre-treatment and Day 1 represents a temporary depletion of urinary protein as a result of high degrees of scent marking (and countermarking) on encountering a clean environment and a potential competing female. Competition between females could also result in a reduced food intake. This could mean that mice did not have the metabolic resources to produce as many urinary proteins as they would normally.

Female laboratory mice increase MUP output in response to social contact (Stopka *et al.*, 2007), and female house mice exposed to territorial invasion from both males and females have a higher level of urinary protein output compared to non-territorial females (Garratt *et al.*, 2011). The social environment presented by encountering a novel female may therefore have caused the increase in urinary protein level observed between pre- and post-treatment. As familiar full sisters are already likely to have an established dominance hierarchy this could explain why protein appeared to only increase slightly in this group compared to unfamiliar full sisters. Protein levels could also have increased between pre- and post-treatment as a result of exposure to the novel cage prompting a flurry of scent marking and causing urinary protein levels to increase. In a novel environment where territory ownership has yet to be established even familiar females may competitively scent mark until dominance and ownership has been re-established and this might explain the small increase in protein observed in the familiar full sisters group.

5.5.4 Conclusions

In this pilot experiment female house mice were housed in experimental pairs to investigate whether relatedness or familiarity influenced female stress and competitive behaviour. Changes in female faecal corticosterone, body mass and urinary protein were measured over a short and longer time period. Over the short-term there was no change in faecal corticosterone across any of the familiarity or relatedness groups, although females in all unfamiliar groups did exhibit a short-term non-significant increase in corticosterone. As this suggests initial housing with unfamiliar females may stressful for female house mice, this trend should be further investigated. Female body mass decreased more in distantly related females than in full sisters, whilst urinary creatinine increased in the unfamiliar groups. A decrease in water intake (as a result of competitive behaviour between females) could explain both the decrease in female body mass and the increase in creatinine levels over the short-term. Urinary protein decreased over the short-term in the relatedness groups. This may be due to increased competition between females.

Over the long-term female faecal corticosterone did not change in any of the familiarity or relatedness groups. This suggests that housing with females of varied familiarity and relatedness is not stressful over the long-term. Female body mass increased in all unfamiliar groups but remained constant in the familiar full sister group. The decrease in body mass may be as a result of competitive behaviour between unfamiliar females, something that would be unlikely to see between highly familiar females. Competitive scent marking may explain the increase in urinary protein observed in all groups. Housing with females of varied familiarity or relatedness does then influence female house mouse physiology and further experiments are needed to establish whether the changes observed here would be found over a longer time period.

6 Female Inbreeding Avoidance in House Mice: Familiarity, Template Formation and Molecular Markers of Relatedness

6.1 Abstract

Breeding with close relatives can cause problems associated with a loss of heterozygous benefits and an increased expression of deleterious recessive alleles. Individuals are therefore expected to avoid breeding with close relatives where other options are available. To avoid inbreeding, however, relatives must first be identifiable. This chapter investigates three aspects of female inbreeding avoidance and mate choice in house mice. Females recognised and avoided unfamiliar full brothers suggesting that a phenotype matching mechanism is used by females to avoid inbreeding. Female house mice also avoided both maternal and paternal half-brothers. As females had not experienced their own father, this suggests that they used a match-to-self mechanism to avoid inbreeding. This is contrary to the findings in Chapter 3, suggesting that female house mice may employ two context-dependent mechanisms for kin recognition: a match-to-maternal cues mechanism for female-female nest partner decisions and a match-to-self mechanism for inbreeding avoidance. Whilst no significant results were found during the investigation of markers used for female inbreeding avoidance, a non-significant trend was observed for females to avoid males that matched themselves for MUP type, whereas no trend was observed for MHC/ESP type. An increased sample size is needed to establish whether this trend represents a significant response to MUP but not MHC/ESP type. A population preference was also observed, with females preferring males derived from one specific population even when they were full siblings. This highlights the importance of other male characteristics in female mate choice, as well as the relatedness level.

6.2 General Introduction

Inbreeding happens when reproduction occurs between individuals that are closely related through a shared ancestry (Partridge, 1983). A number of costs are associated with inbreeding, largely arising from the increased likelihood of homozygosity at a given locus. Deleterious recessive alleles will remain un-expressed when paired with a dominant allele, but inbreeding increases the chances that the resultant offspring are homozygous for such deleterious alleles (Bateson, 1983; Partridge, 1983; Charlesworth & Willis, 2009). Overdominance occurs where individuals that are heterozygous at a particular locus have a higher fitness than those individuals that are homozygous. As inbreeding increases the chances of homozygosity the benefits gained from heterozygosity can be lost (Bateson, 1983; Partridge, 1983; Charlesworth & Willis, 2009).

Whilst less studied, extreme outbreeding can also result in deleterious effects (known as outbreeding depression). If two genetically differentiated individuals cross, their offspring could have reduced adaptation to the local environment, or gene complexes that are coadapted could be broken up (Bateson, 1983; Partridge, 1983; Pusey & Wolf, 1996). Theory predicts that individuals may want to optimise outbreeding by avoiding mating with close relatives and also avoiding genetically distant individuals (Bateson, 1983).

Inbreeding avoidance behaviours have been demonstrated in mammals (e.g. Bolhuis *et al.*, 1988; Keane, 1990; Lemaitre *et al.*, 2012), birds (Bateson, 1982), reptiles (Olsson *et al.*, 2003), fish (Gerlach & Lysiak, 2006), and insects (e.g. Simmons, 1991; Keller & Passera, 1993; Välimäki *et al.*, 2011). There are two main methods by which individuals could avoid breeding with close relatives. Firstly, the dispersal of one or both sexes could make reproductive encounters between relatives unlikely (discussed in Greenwood, 1980). Alternatively, for species where individuals are likely to encounter relatives, kin recognition could be used to prevent inbreeding.

Whilst most male house mice disperse from the natal area, females will also disperse if chances of reproduction are low (Gerlach, 1996), however dispersal is usually local as commensal house mice typically exploit local patches of high resource abundance. House mouse populations are therefore often dense and the chances of encountering close relatives after dispersal are high (Barnard *et al.*, 1991). In male house mice the costs of inbreeding are high. In a free-breeding experiment, inbred male house mice had a lower competitive ability

compared to outbred male house mice, resulting in a reduced survivorship and fewer offspring (Meagher *et al.*, 2000).

Female house mice make mate decisions based on a number of different male characteristics, including relatedness, and female house mice paired with a preferred male have more litters and fitter offspring than females paired with a non-preferred male (Drickamer *et al.*, 2000). Full sibling breeding pairs of laboratory and house mice have a lower reproductive success than unrelated pairs suggesting a pre- or post-copulatory inbreeding avoidance mechanism (Barnard & Fitzsimons, 1989; Krackow & Matuschak, 1991). A large number of studies have investigated inbreeding avoidance and mate choice in laboratory strains of mice. Laboratory mice have been used to demonstrate preferences for mice of different strains (e.g. Yamaguchi *et al.*, 1978; Beauchamp *et al.*, 1988), for familiar and unfamiliar mice (e.g. D'Udine & Partridge, 1981; Hayashi & Kimura, 1983), for odours from relatives (e.g. D'Udine & Partridge, 1981), for mice of a different strain or MHC type to parents (e.g. Yamazaki *et al.*, 1988; Penn & Potts, 1998a), for MHC-associative (e.g. Andrews & Boyse, 1978; Yamazaki *et al.*, 1978) and dissassociative preferences (e.g. Beauchamp *et al.*, 1988; Egid & Brown, 1989; Eklund, 1997a), as well as differences in response between laboratory strains (e.g. Andrews & Boyse, 1978) and the sexes (e.g. Hayashi & Kimura, 1983). As discussed in Chapter 1 (Section 1.2.3) however, laboratory mice have been selectively bred to mate with full siblings. The results of inbreeding avoidance studies using laboratory mice may therefore not necessarily provide an accurate representation of natural mate choice decisions.

In this chapter three aspects of female inbreeding avoidance are investigated: the effect of prior familiarity; the origin of a kin recognition template; and the molecular markers involved in female inbreeding avoidance. In previous chapters subject females were predicted to preferentially associate with related (or matching) stimulus females. To avoid inbreeding females must avoid mating with close relatives and therefore in this chapter females are predicted to avoid stimulus males perceived as related and instead preferentially associate with stimulus males that are perceived as unrelated.

Studies investigating inbreeding have measured avoidance via the duration/number of matings (e.g. Yu *et al.*, 2004), mating success of specifically paired individuals (e.g. Boyd & Blaustein, 1985; Barnard & Fitzsimons, 1989), free-breeding enclosure-based experiments (e.g. Penn & Potts, 1998a; Sherborne *et al.*, 2007), pre- and post-copulatory behaviour (e.g. Simmons, 1991), time spent in proximity to potential mates or their odour (e.g. Bateson,

1982; Keane, 1990; Lemaitre *et al.*, 2012), and re-mating interval time (e.g. Välimäki *et al.*, 2011). Two assay designs are used in this chapter to investigate female discrimination and preference: a scent discrimination and attraction assay to discover whether females can spontaneously discriminate between related and unrelated male urine; and a nest partner preference assay in which female proximity to males is measured by the amount of time spent in stimulus male cages. Whilst neither assay directly tests female inbreeding avoidance by allowing mating decisions and reproduction, inbreeding avoidance is inferred by the amount of time subject females spend in proximity to stimulus males. The assays used here also measure female choice between two comparable males, whilst a free-breeding assay would not reveal whether choice was male or female based.

6.3 The Role of Familiarity in Female Inbreeding Avoidance

6.3.1 Inbreeding Avoidance and Familiarity: Introduction

The mechanisms suggested for recognising relatives during inbreeding avoidance are the same as in other contexts of kin recognition. One way to avoid mating with a close relative would be for an individual to learn its relatives during rearing and then avoid mating with them in later life (prior association). A disadvantage of this would occur in situations where novel close relatives might be encountered after reaching reproductive maturity. By only learning and recognising those individuals present during rearing, inbreeding could occur between unfamiliar close relatives. Inbreeding avoidance based on prior association alone has been observed in a number of vole species. Free-breeding female prairie voles are more likely to breed with an unfamiliar male than a familiar male even when both are either full brothers or unrelated, and neither males nor females recognise opposite-sex siblings with whom they have had no prior contact (Gavish *et al.*, 1984; Lucia & Keane, 2012). Female mandarin voles avoid mating with unrelated males they were reared with, instead preferring unrelated males of which they have no experience (Fadao *et al.*, 2000). Pairs of male and female grey-tailed voles that are reared together produce fewer litters than previously unfamiliar pairs, regardless of the degree of relatedness (Boyd & Blaustein, 1985).

An alternative method would be to learn a family cue or marker and then use that to compare against newly encountered potential mates (phenotype matching). This mechanism would allow novel relatives to be recognised and so prevent inbreeding. However phenotype matching could open up the possibility of recognition errors. Related individuals may be falsely excluded and therefore mating between close relatives could occur. Additionally, unrelated individuals may be falsely identified as related and so preventing mating between compatible partners. Brandt's voles (*Microtus brandti*) demonstrate inbreeding avoidance via phenotype matching as females spend more time copulating with unfamiliar unrelated males than with unfamiliar brothers (Yu *et al.*, 2004). Female golden hamsters (*Mesocricetus auratus*) spend longer investigating and mark more in response to the odours of unrelated males than to half-brothers when both are completely unfamiliar (Heth *et al.*, 1998; Mateo & Johnston, 2000). Adult female zebrafish (*Danio* rerio) also display an odour preference for unrelated males compared to full brothers when both are equally unfamiliar (Gerlach & Lysiak, 2006).

As discussed in the general introduction to this chapter (Section 6.2), a number of inbreeding avoidance studies have used laboratory strains of mice (e.g. Yamazaki *et al.*, 1976; Hayashi & Kimura, 1983; Egid & Brown, 1989; Penn & Potts, 1998a; Roberts & Gosling, 2003). Laboratory strains allow for carefully controlled experiments as individuals of one strain are genetically uniform. Because of this, however, it is difficult to be entirely clear that avoidance of same strain individuals is down to recognition of genetic similarity (relatedness) or a case of mistaken identity. The similarity between individual laboratory mice of the same strain could mean that unrelated individuals appear similar to related individuals and mice are identifying what they think are known relatives when instead they are novel unrelated individuals that appear similar. This would instead be a familiarity-based kin recognition mechanism (prior association).

The importance of familiarity in inbreeding avoidance has also been studied in house mice. In wild house mice pairs of familiar full siblings are less likely to produce litters than pairs of unfamiliar, unrelated mice (Krackow & Matuschak, 1991). Female house mice prefer the ultrasonic vocalisations of unfamiliar, unrelated males over those of familiar full brothers (Musolf *et al.*, 2010). Female house mice also prefer to associate with unrelated males over related males when both equally unfamiliar, suggestive of a phenotype matching mechanism (Winn & Vestal, 1986). However, Winn & Vestal (1986) also showed that females spend more time with a familiar male than an unfamiliar male when both were full brothers of the female, suggesting the important of familiarity when relatedness is constant. In Chapter 2 female house mice discriminated and showed a preference for full sisters over unrelated females when familiar or unfamiliar with both, suggesting that female house mice use a phenotype matching mechanism for recognising female relatives at least. The first experiment aims to address the following questions:

i) Can female house mice discriminate between urine from a full brother and an unrelated male, and do they show any attraction towards either?

ii) Do female house mice prefer unrelated males over full brothers?

iii) Does avoidance of full brothers depend on prior association or will females also display an avoidance of previously unfamiliar full brothers (phenotype matching)?

6.3.2 General Inbreeding Avoidance Methods

Throughout this chapter the same assay designs are repeated for each different experimental question. Mice were housed and handled under the conditions described in Section 2.3.1. Subject females were injected under the skin at the nape of the neck with an RFID (FSI) chip a minimum of one week prior to behavioural assays. RFID chips allowed individual identification of group housed females and enabled female movement to be tracked during the nest partner choice assay. In all cases subject females were presented with two stimulus males, one related or matching themselves and one unrelated or dissimilar to themselves. For each question the same subject – stimulus triads were used for both the scent discrimination and attraction assay and the nest partner choice assay.

6.3.2.1 Priming

Whilst actual mating was prevented, female response to males is more likely to best represent female mate choice if females are in a reproductively receptive state. As oestrus stage is known to affect female preference (Egid & Brown, 1989), females were primed with soiled male bedding before testing to ensure that females were in or entering oestrus at the time of testing. Males were also given experience of female odours by presenting males with soiled bedding from female cages. Additionally, both sexes were primed with same-sex soiled bedding to give experience of unrelated same-sex odours and to suggest reproductive competition. Mice were primed with soiled bedding three days before testing to ensure that females were in or entering oestrus at the time of testing (Marsden & Bronson, 1964; Cheetham *et al.*, 2007).

Soiled paper/wool nesting (Shredded Nesting International Product Supplier Limited, London, UK) and substrate (Corn Cob Absorb 10/14 substrate) was collected from mouse cages at least one week after routine cage cleaning to ensure that the bedding contained sufficient mouse odour. Bedding from each sex was collected from multiple cages. Each cage of subject or stimulus mice received a small handful of soiled bedding material from each sex and two pinches of soiled substrate material. Mice were never presented with bedding from individuals that they would later encounter to prevent any influence of odour familiarity, or with bedding from relatives.

6.3.2.2 Assay Order

In all the experiments described in this chapter, females were given the scent discrimination and attraction assay in the morning of the test day and then placed in the nest partner choice

assay cages in the afternoon of that same day. This ensured that females were in approximately the same reproductive condition for both assays.

6.3.2.3 Scent Discrimination and Attraction Assay

The scent test assay was the same as described in Section 3.3.5 with the exception that male urine was used instead of female urine. This design was a modification of the methods of Ramm *et al* (2008), except with a barrier dividing the cage in half. Urine was collected no more than two weeks before testing using the recovery method (described in Section 2.3.3) and was stored at -22°C. Females were habituated to the test cage for 30 minutes prior to testing. At testing the clean habituation acrylic lid was replaced with a test lid onto which two half-circles of Benchkote had been taped. Stimulus male urine (10 µl) had been streaked onto each half-circle. The side position of each stimulus urine mark was randomized but balanced to prevent any side bias. Female behaviour towards each urine mark and female position in the test cage was recorded remotely to DVD for 10 minutes. DVDs were watched back blind to the position of each scent using an event recorder program (written by R.J.Beynon). Discrimination was assessed by comparing the amount of time females spent sniffing each urine mark, and the amount of time females spent directly under each urine mark (which could also include sniffing of airborne odours). Attraction was measured by the total amount of time females spent in each side of the test cage.

6.3.2.4 Nest Partner Choice Assay

Females were given a choice of nest partner using the same experimental design as described in Section 3.3.6. Subject females were placed in the central cage of three interconnected cages and were able to move through tunnels to access each cage. Stimulus males were placed behind barriers in the two end cages. Females could interact with the males through the mesh barriers, allowing for scent, sight, sound and limited touch communication. Female movement between cages was tracked using a combination of automated RFID readers (FSI) and infrared sensors connected to a central computer running custom software (FSI; Thom *et al.*, 2008b). Trials lasted approximately 41 hours and were standardised to 36 hours (two light phases separated by a dark phase). Output files were converted using custom software written by J.L.Hurst in Revolution (a software development environment) and SPSS (version 18.0.2) to calculate the total time spent by subject females in each cage. A bias in the amount of time a female spent in one stimulus male cage suggested a preference for that cage and therefore the stimulus male in the cage.

6.3.3 Inbreeding Avoidance and Familiarity: Methods

Subject females were presented with a related stimulus male (a full brother) and an unrelated stimulus male, and female recognition and inbreeding avoidance was assessed using assays of scent discrimination and attraction and nest partner choice. Two familiarity groups were created based on whether the subject females were familiar with the related male. In the unfamiliar group females were equally unfamiliar with both stimulus males – the related male was a full brother from a previous litter. As unrelated females and males could not become familiar with one another without risk of mating or cross-fostering after birth, the stimulus males in the familiar group differed for familiarity as well as relatedness. The related male was a full brother from the same litter as the subject female. The unrelated male was completely unfamiliar to the subject female.

6.3.3.1 Subject Females and Stimulus Males

All subject females and stimulus males were F1 mice, offspring of wild-caught mice from four distinct populations in the North West of England, UK (Knowsley Safari Park, Prescot, Merseyside; Holme Farm, Ince, Cheshire; Hatchwood Farm, Chorley Lancashire; and Twycross Zoo, Atherstone, Warwickshire). Once in the laboratory, wild-caught same-population individuals had been paired and allowed to breed multiple litters. These offspring were used as subject females and stimulus males for this experiment. Subject females were presented with two stimulus males, one related (a full brother) and one unrelated male (in all cases from a different founder population to the subject female). Paired males were age-matched to within 1 month of each other, and weight-matched to within 2 g of each other. All females were virgins and were between 7 and 10 months old at the time of testing. Subject females were only used once, 12 out of 42 stimulus males were used twice, but never as the same stimulus type.

In the Familiar group (n = 12) related stimulus males were full brothers from the same litters as the subject females. Related stimulus males were therefore the same age as the subject females. Littermate subject females and related stimulus males were familiar with one another from birth until weaning, but were housed apart after weaning. Subject females had not encountered the related stimulus males for 6 to 9 months before behavioural assays. Females were unfamiliar with unrelated stimulus males.

In the Unfamiliar group (n = 12) related stimulus males were full brothers from a previous litter to the subject females. Stimulus males were 1 to 4 months older than the subject females. Females were unfamiliar with both stimulus males.

Female inbreeding avoidance was assessed in a scent discrimination and attraction assay and a nest partner choice assay. Females were predicted to spend more time investigating the unrelated urine (as it would contain more previously unfamiliar elements) and show an attraction towards the unrelated urine. Females were predicted to choose the unrelated stimulus male cage in the nest partner choice assay and avoid the related male stimulus cage. No difference was predicted for female response in the familiar or unfamiliar groups.

6.3.3.2 Data Analysis

Data for time spent sniffing each urine mark met parametric assumptions and a repeated measures GLM compared time spent sniffing urine from related and unrelated stimulus males, with familiarity group as a between subject variable. This approach was repeated for the total time subject females spent directly under urine marks and for the total amount of time females spent on each side of the test cage. Time spent under each urine mark was transformed using a square root function to meet parametric assumptions. The total time spent by females on each side of the test cage was logarithmically transformed to meet parametric assumptions.

Data from the nest partner choice assay did not meet parametric assumptions, even with transformations. The proportion of time spent by subject females in the unrelated stimulus male cage (as a proportion of the total assay time) was compared between the familiar and unfamiliar group using a Mann-Whitney U test. Female preference for the related or unrelated stimulus male cages was compared using a Wilcoxon Signed Ranks test. Female preference was assigned using the ratio of time spent in the unrelated male stimulus cage compared to the time spent in the related male stimulus cage. Females with ratios that were above 0.50 spent more time in the unrelated stimulus male cage. Ratios that were below 0.50 spent more time in the related stimulus male cage. The observed number of females that spent more time in the unrelated stimulus male cage was compared to the number expected by chance using a Binomial test. All figures use untransformed data.

6.3.4 Inbreeding Avoidance and Familiarity: Results

The results from this section are presented below and summarised in Table 6.1.

6.3.4.1 Scent Discrimination and Attraction

Two measures of female investigatory behaviour were used to assess whether females could spontaneously discriminate between urine from a full brother and an unrelated male: a) time spent directly sniffing each urine mark, and b) total time spent under each urine mark. Subject females did not show any discrimination between related and unrelated male urine through the time spent sniffing ($F_{(1,21)} = 0.004$, $p = 0.95$; Figure 6.1a), or in the total time spent under each urine mark ($F_{(1,21)} = 0.001$, $p = 0.98$; Figure 6.1b). Level of familiarity did not influence the time subject females spent sniffing either male stimulus urine mark ($F_{(1,21)} = 0.02$, $p = 0.89$; Figure 6.1a) or the total time females spent under each urine mark ($F_{(1,21)} = 0.15$, $p = 0.70$; Figure 6.1b).

Female attraction towards either related or unrelated male urine was measured using the total time females spent on each side of the test cage. Subject females spent longer on the side of the cage with urine from a full brother urine than the side of the cage with urine from an unrelated male ($F_{(1,21)} = 4.64$, $p = 0.04$; Figure 6.2). There was no influence of level of familiarity on female side preference ($F_{(1,21)} = 0.15$, $p = 0.70$; Figure 6.2).

6.3.4.2 Nest Partner Choice

Subject female preference was assessed using the nest partner choice assay, during which the time females spent in each of the three test cages was measured. Contrary to predictions females did not spend longer in the unrelated stimulus male cage compared to the related stimulus male cages ($z = -0.21$, $p = 0.42$, one-tailed; Figure 6.3). Additionally, the number of subject females that chose the unrelated stimulus male cage was not greater than the number that chose the related stimulus male cage (57 %, $p = 0.34$, one-tailed; Figure 6.3). There was no difference in the proportion of time subject females spent in the unrelated stimulus male cage between the familiar and unfamiliar groups ($z = -0.80$, $p = 0.45$, two-tailed; Figure 6.3).

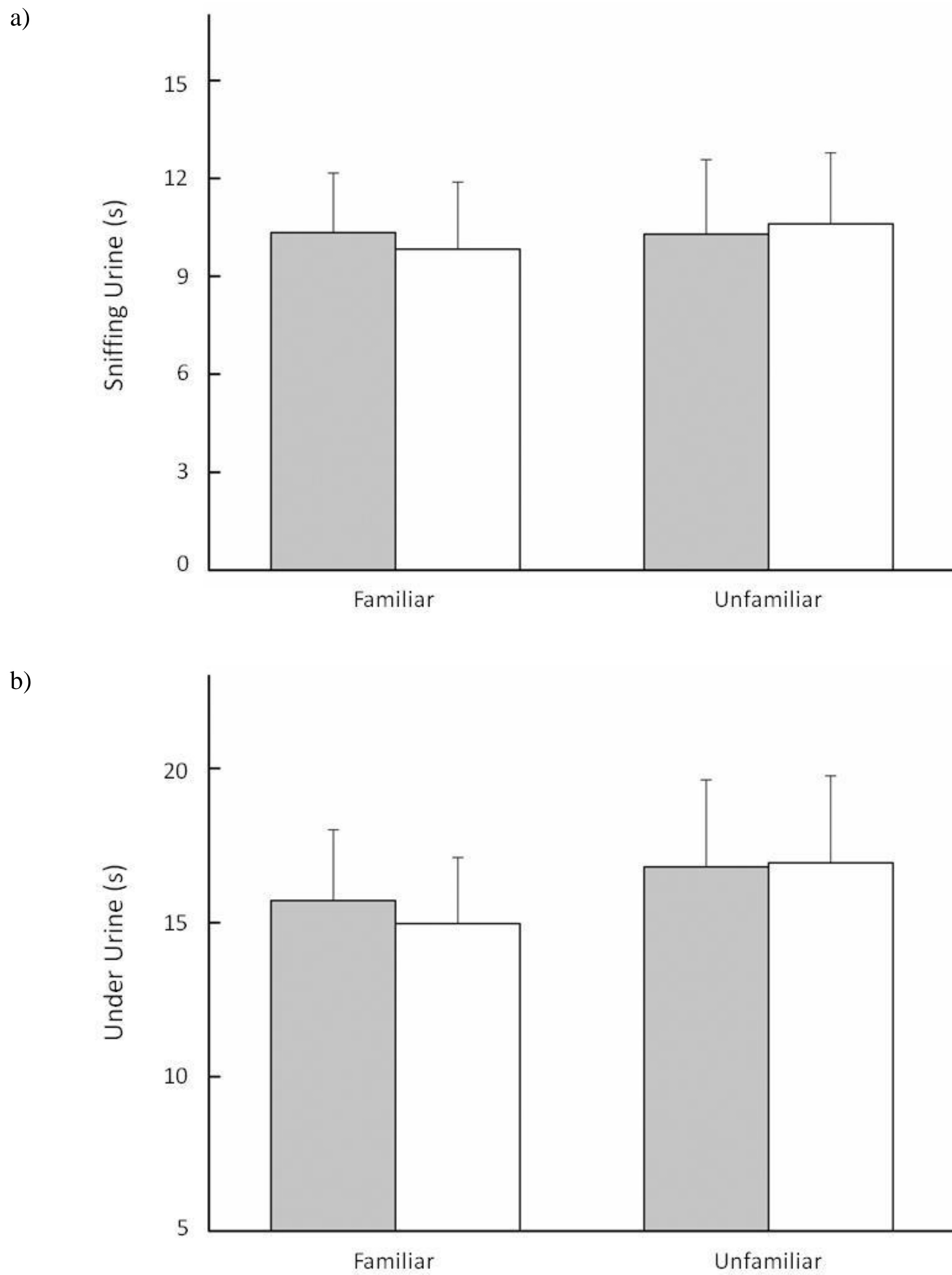


Figure 6.1: Female investigation of urine from related and unrelated stimulus males in the familiar and unfamiliar groups.
Investigatory behaviour towards related (grey bars) and unrelated (open bars) male urine in the familiar (n = 12) and unfamiliar (n = 11) groups. Investigation measured by a) time spent sniffing urine (s, mean + standard error), and b) time spent directly under urine (s, mean + standard error).

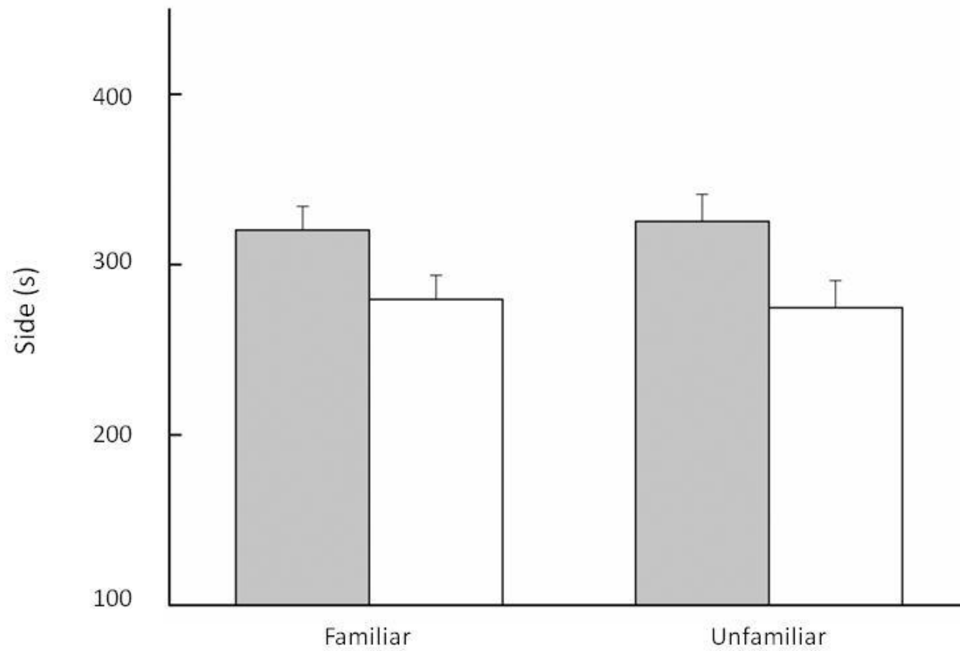


Figure 6.2: Female attraction towards urine from related and unrelated males in the familiar and unfamiliar groups.
Attraction towards related (grey bars) and unrelated (open bars) male urine in the familiar (n = 12) and unfamiliar (n = 11) groups. Attraction measured by the amount of time females spent on each side of the test cage (s, mean + standard error).

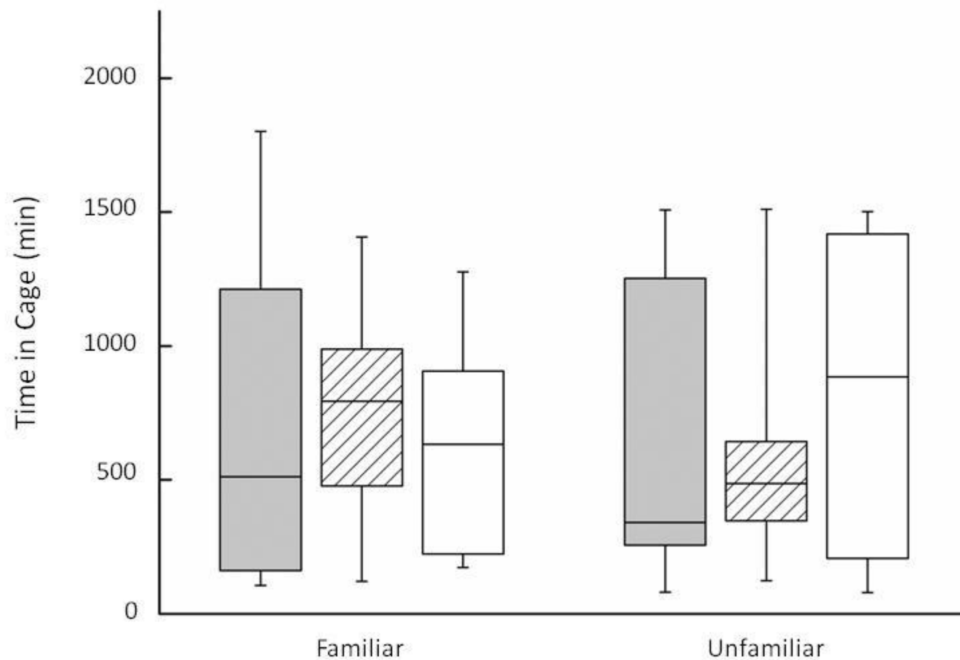


Figure 6.3: Female nest partner choice in the familiar and unfamiliar groups.
Time (minutes) spent in the related stimulus male cage (grey boxes) then centre cage (hashed boxes) and the unrelated stimulus male cage (open boxes) in the familiar (n = 12) and unfamiliar (n = 11) groups.

6.3.4.3 An Attraction for Hatchwood Farm Males

Further investigation of the data revealed an unexpected female bias towards stimulus males of Hatchwood Farm descent. Of the 23 female-male triads, 12 contained Hatchwood Farm-descended subject females and related stimulus males, and 10 contained Hatchwood Farm-descended unrelated stimulus males. To investigate the data further the amount of time females spent in each cage in the nest partner choice assay was analysed using time in Hatchwood Farm stimulus male cage compared to the amount of time females spent in the non-Hatchwood Farm stimulus male cage (other population). Females were found to spend longer in the Hatchwood Farm stimulus male cage ($z = -2.65$, $p = 0.01$, Wilcoxon Signed Ranks test, two-tailed; Figure 6.4), with 73 % of females spending more time in the Hatchwood Farm stimulus male cage than the other population stimulus male cage ($p = 0.05$, Binomial test, two-tailed; Figure 6.4).

No effect of Hatchwood Farm was detected in any of the scent discrimination and attraction assay measures. There was no difference in the amount of time females spent sniffing urine from a Hatchwood Farm stimulus male or a male from a different population ($z = -1.02$, $p = 0.32$, Wilcoxon Signed Ranks test, two-tailed). There was no difference in the amount of time subject females spent under urine from either a Hatchwood Farm stimulus male or a male from another population ($t = -0.48$, $df = 21$, $p = 0.64$, paired t-test on square root transformed data). Whether stimulus urine was from a Hatchwood Farm male or a male from a different population did not influence subject female side preference ($t = -0.30$, $df = 21$, $p = 0.77$, paired t-test on non-transformed data).

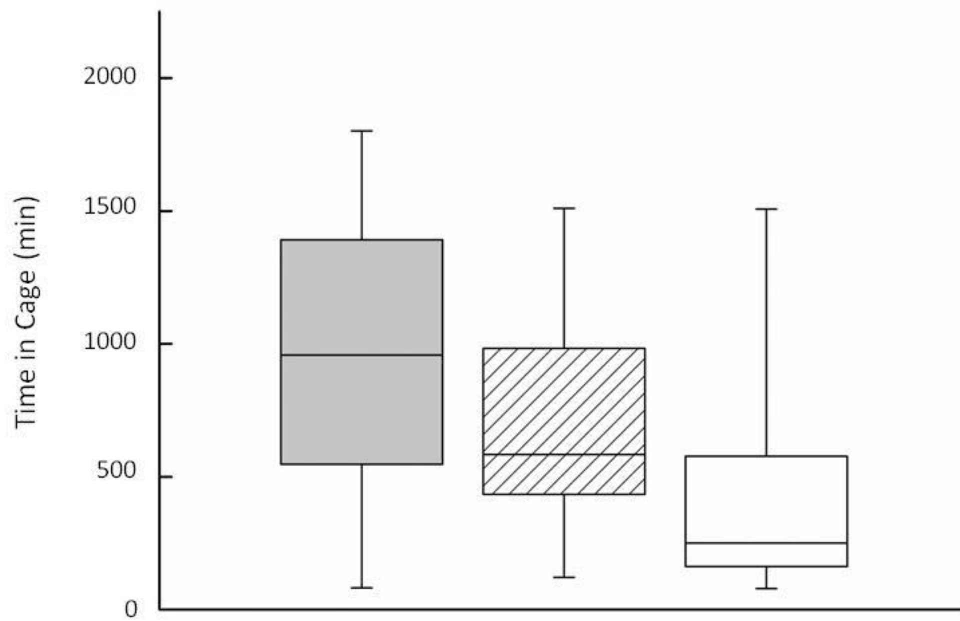


Figure 6.4: Female nest partner choice for Hatchwood Farm derived males compared to males derived from other populations.
Amount of time spent by subject females (n = 22) in Hatchwood Farm stimulus male cage (grey box), centre cage (hashed box) and other population stimulus male cage (open box).

6.3.5 *Female Preference for Hatchwood Farm Males*

Whilst the data from the nest partner choice makes it likely that females displayed a preference for Hatchwood Farm males, it was not a direct test. It is difficult to say for certain that females do prefer Hatchwood Farm males and that this is the reason that female inbreeding avoidance behaviour was not observed here. The next section addresses female preference for Hatchwood Farm males directly.

In order to directly test female preference for Hatchwood Farm males, females were given a nest partner choice assay of two unfamiliar and unrelated males, one descended from Hatchwood Farm and one descended from a different founder population.

6.3.6 *Hatchwood Farm Preference: Methods*

6.3.6.1 Subject Females and Stimulus Males

Subject females were presented with two stimulus males, one of Hatchwood Farm ('HF') descent and one with no Hatchwood Farm ancestry ('other'). Subject females were descended from Knowsley Safari Park (KN, n = 4), Holme Farm (HMF, n = 4) or Twycross Zoo (TZ, n = 4). Subject females were 7 to 9 months old at testing. Subject females were completely unrelated to and unfamiliar with both stimulus males.

Stimulus males were age-matched to within 1 month and weight-matched to within 2g of each other, and were within 4 days to 3 months of subject females. 'Other' population stimulus males were descended from a different population to the subject females (KN (n = 4), HMF/HF (n = 4) or TZ (n = 4)). Due to limited numbers of available mice, the four HMF/HF descended stimulus males were the result of a cross between a Holme Farm female and a Hatchwood Farm male. Whilst they therefore were of Hatchwood Farm descent, this was diluted in comparison to the pure 'HF' stimulus male. These MHF/HF stimulus males were unrelated to the HF stimulus males with which they were paired.

Female preference for HF males was examined using the nest partner choice assay alone (n = 12). It was predicted that females would spend more time in the cage of the HF stimulus male.

6.3.6.2 Data Analysis

Data did not meet parametric assumptions, even with transformations. Female preference for the HF compared to other stimulus male cages was compared using a Wilcoxon Signed Ranks test. Female choice was assigned using the ratio of time spent in the HF male stimulus

cage compared to the time spent in the other male stimulus cage. Females with ratios that were above 0.50 spent longer in the HF stimulus male cage, whilst females with ratios that were below 0.50 spent longer in the other stimulus male cage. The observed number of females that chose the HF stimulus male cage was compared to the number expected by chance using a Binomial test. The figures use untransformed data.

6.3.7 Hatchwood Farm Preference: Results

The amount of time females spent in each of the three test cages was measured over 36 hours. There was no significant difference in the amount of time females spent in either the HF stimulus male cage or the other stimulus males cage ($z = -0.71$, $p = 0.26$, one-tailed; Figure 6.5a). The number of females (67 %) that chose the HF stimulus male cage did not differ from the number of females that chose the other stimulus male cage ($p = 0.12$, one-tailed; Figure 6.5a).

The four females that were tested with an other male that was of HMF/HF descent were removed from the analysis so that the remaining females were given a true test of HF preference. Females spent significant longer in the HF stimulus male cage than in the other stimulus male cage ($z = -2.10$, $p = 0.02$, one-tailed; Figure 6.5b). More females (88 %) chose the HF stimulus male cage over the other stimulus male cage ($p = 0.04$, one-tailed; Figure 6.5b, Table 6.1).

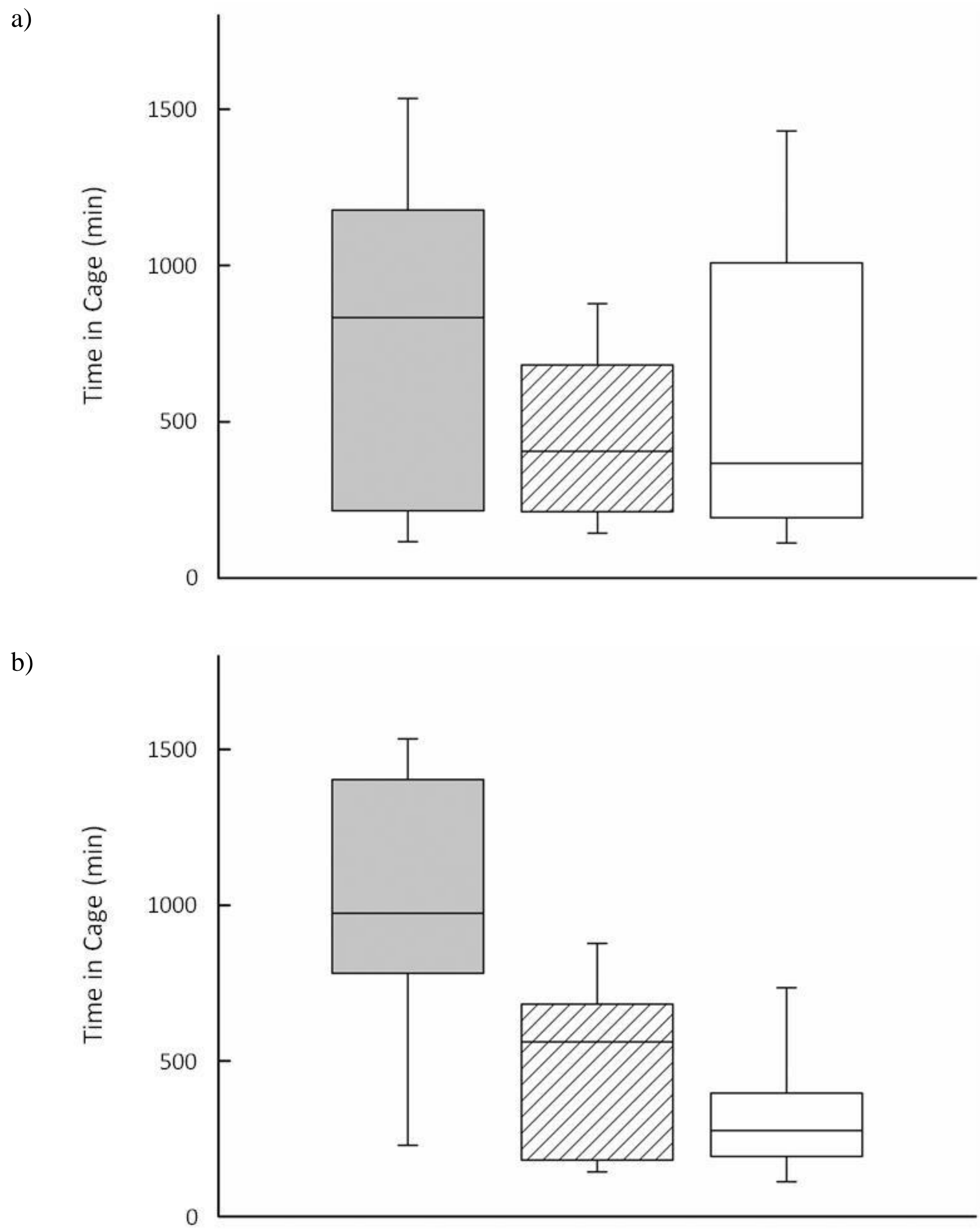


Figure 6.5: Female nest partner choice for Hatchwood Farm derived stimulus males compared to stimulus males derived from other populations.
Time spent by subject females in Hatchwood Farm stimulus male cage (grey boxes), centre cage (hashed boxes) and other population cage (open boxes) for a) all females tested (n = 12) and b) excluding females that were tested with an HMF/HF other stimulus male (n = 8).

6.3.8 Hatchwood Farm Preference: Discussion

In both the first nest partner choice assay and the subsequent direct test some subject females displayed a preference for HF males over males descended from other populations. Some females preferred HF males even when they were full siblings, indicating that the preference for HF males is strong. Interestingly, no discrimination of or attraction towards HF male urine was displayed in the original scent assay, suggesting that HF male behaviour might be the strongest factor for female preference in this study.

There are a number of reasons why males from one population might appear more attractive than males from a different population. Inherent differences between the populations could cause differential attractiveness. Whilst no direct measurements were taken, the Hatchwood Farm source population was captured from a battery poultry farm where high population densities had sometimes previously been observed. Independently it was found that Hatchwood Farm descended males were particularly aggressive in response to handling in comparison to mice from other populations and stock colony mice (M.Timonin, personal communication). Although these observations are anecdotal it is possible that a higher population density led to increased male-male competition which could in turn cause the base level of male competitiveness in one population to be higher than in the others. More competitive males could appear more dominant and therefore more attractive to females causing the population preference observed here (Halliday, 1983).

Parasitic infection is known to influence male house mouse odour attractiveness, with females preferring uninfected male odour over infected male odour (Penn *et al.*, 1998; Ehman & Scott, 2001). Wild-caught mice are screened for *Lymphocytic choriomeningitis* virus on entering the Mammalian Behaviour & Evolution group colony, as well as checked for mites or ticks but no other screening takes place. It is possible that mice from different populations have different levels of infection, with the HF mice being the least infected. This could have caused female preference for HF males.

Genetic heterozygosity could also have influenced female preference. The heterozygosity theory suggests that females prefer males that are heterozygous at a specific locus over homozygous males (Brown, 1997; Thom *et al.*, 2008a). Female house mice prefer to co-inhabit the nest of males that are heterozygous for MUP over those that are homozygous (Thom *et al.*, 2008b). Differences in molecular markers such as MUP or MHC could have caused the preferences observed here. Mice from all lines described here have been

phenotyped by Amanda Davidson, Jonathan Green and Jane Hurst of the Mammalian Behaviour & Evolution group, University of Liverpool. Hatchwood Farm derived males tended to have more MUP peaks than males derived from other populations, including one peak that none of the males from other populations had (J.Green, personal communication). It is possible that female attraction towards HF derived males is due to these MUP differences between males from different populations. If this is the case then females may have been expected to show an attraction towards urine from HF males, which was not observed here.

If population differences in male urine caused the female preference observed in the nest partner choice assay then females might have been expected to show attraction towards HF urine in the scent assay, however no attraction to HF male urine was displayed. There are three possible reasons for this. First, female preference for HF males could be governed by male behaviour or some other characteristic not displayed in urine. Females would therefore not show an attraction towards HF male urine. Second, female attraction could be based on markers present in HF male urine, but any attraction could be confounded by differences in relatedness. Female attraction for HF male urine could have been disguised in the short scent assay but revealed in the longer nest choice assay. Finally, the scent assay may not be sensitive enough to detect female preference for HF male urine. No attraction for unrelated male urine was detected in Section 6.4.3.1 where population was not an issue and females were presented with urine from a related male and an unrelated male. It would have been interesting to have run a direct test of female scent discrimination and attraction to HF and other stimulus male urine to discover whether female preference for HF males was as a result of differences in urine content, such as MHC or MUP differences.

6.3.9 Inbreeding Avoidance and Familiarity Repeat

Having established that there was a preference for Hatchwood Farm mice, I now return to the original questions of this section: Do female house mice prefer unrelated males over full brothers, and does the avoidance of full brothers depend on prior association? The numbers of suitable mice available at the time of testing meant that it was not possible to run the original experimental design without incorporating some mice descended from Hatchwood Farm. Therefore in order to avoid known issues with female preference for Hatchwood Farm males, all subject and stimulus mice were of Hatchwood Farm descent.

6.3.10 Inbreeding Avoidance and Familiarity Repeat: Methods

6.3.10.1 Subject Females and Stimulus Males

All subject females and stimulus males were of Hatchwood Farm (HF) descent – the result of crosses between F1 HF mice and F1 KN, HMF or TZ mice, or crossed with captive-bred adult house mice from the Mammalian Behaviour & Evolution Group's colony of outbred house mice. By ensuring that all mice were of HF descent, there could be no issues of female preference for particular populations of males. Subject females were presented with two stimulus males, one related (full brother) and one unrelated.

Previously subject females were all of the same generation and in the unfamiliar group subject females were younger than stimulus males. Here however, limited numbers meant that subject females were taken from across two set of litters and in the unfamiliar group subject females were either younger or older than the stimulus males. In the familiar group both subject females and stimulus males were from either the first or second litters. Females were between 5 and 7 months old at testing.

In the familiar group (n = 12) related stimulus males were full brothers from the same litters as the subject females. The unrelated stimulus males were age-matched to the related stimulus males to within 1 month of each other, and weight-matched to within 2 g of each other. Females were familiar with related stimulus males having experienced them from birth until weaning, at which point they were housed apart. Females had not encountered related stimulus males for 4 to 6 months before behavioural assays. Females were completely unfamiliar with the unrelated stimulus males.

In the unfamiliar group (n = 12) related stimulus males were full brothers from a previous or subsequent litter to the subject females. Related stimulus males were either 1 to 2 months older or 1 to 3 months younger than subject females. Subject females were unfamiliar with both stimulus males prior to behavioural testing.

Female inbreeding avoidance was assessed in a scent discrimination and attraction assay and then in a nest partner choice assay. Females were predicted to spend more time investigating and show an attraction towards the unrelated urine. Females were predicted to choose the unrelated stimulus male cage in the nest partner choice assay and avoid the related stimulus male cage. No prediction was made for female response in the familiar or unfamiliar groups.

6.3.10.2 Data Analysis

In the scent discrimination and attraction assay the time spent by subject females sniffing each stimulus male urine mark was logarithmically transformed to meet parametric assumptions and a repeated measures GLM compared time spent sniffing related and unrelated urine, with familiarity group as a between subject variable. This approach was repeated for the total time spent under urine and the total time spent on each side of the test cage. The total time spent under urine raw data met parametric assumptions, whilst the total time on each side of the test cage was logarithmically transformed to meet parametric assumptions.

Data from the nest partner choice assay did not meet parametric assumptions, even with transformations. To compare the affect of familiarity on female response the proportion of time spent by subject females in the unrelated stimulus male cage (as a proportion of the total assay time) was compared between the familiar and unfamiliar group using a Mann-Whitney U test. Female preference for the unrelated over the related stimulus male cage was then compared using a Wilcoxon Signed Ranks test. Female preference was assigned using the ratio of time spent in the unrelated male stimulus cage compared to the time spent in the related male stimulus cage. Females with ratios that were above 0.50 spent longer in the unrelated stimulus male cage, whilst females with ratios that were below 0.50 spent longer in the related stimulus male cage. The observed number of females that spent longer in the unrelated stimulus male cage was compared to the number expected by chance using a Binomial test. All figures use untransformed data.

6.3.11 Inbreeding Avoidance and Familiarity Repeat: Results

The results of this study are presented below and summarised in Table 6.1.

6.3.11.1 Scent Discrimination and Attraction

Female discrimination of related and unrelated male urine was assessed by measuring two investigatory behaviours: time spent directly sniffing each urine mark and total time under each urine mark (inclusive of direct sniffing and volatile sniffing). Subject females showed no spontaneous discriminative ability based on sniffing ($F_{(1,20)} = 0.001$, $p = 0.97$; Figure 6.6a), but there was a non-significant trend for subject females to spend more time under the related stimulus male urine mark than the unrelated mark ($F_{(1,20)} = 3.42$, $p = 0.08$; Figure 6.6b). There was no effect of familiarity on the time subject females spent sniffing either

stimulus male urine mark ($F_{(1,20)} = 0.02$, $p = 0.89$; Figure 6.6a) or on time females spent under each stimulus urine mark ($F_{(1,20)} = 0.05$, $p = 0.82$; Figure 6.6b).

Female attraction towards each male urine mark was measured by the total amount of time females spent on either side of the test cage. There was no difference in the amount of time subject females spent on either side of the test cage ($F_{(1,20)} = 0.51$, $p = 0.49$; Figure 6.7). Familiarity group did not affect the time subject females spent on either side ($F_{(1,20)} = 0.29$, $p = 0.60$; Figure 6.7). There was no significant interaction between familiarity group and the amount of time subject females spent on the side with unrelated male stimulus urine or related male stimulus urine ($F_{(1,20)} = 0.66$, $p = 0.43$; Figure 6.7).

6.3.11.2 Nest Partner Choice

In the nest partner choice assay the amount of time females spent in each test cage was measured over 36 hours. Females did not spend longer in the unrelated stimulus male cage compared to the related stimulus male cage ($z = -0.71$, $p = 0.25$, one-tailed; Figure 6.8). The number of females that chose the unrelated stimulus male cage (42 %) was not larger than the number of females that chose the related stimulus male cage ($p = 0.27$, one-tailed; Figure 6.8). There was no difference in the proportion of time females spent in the unrelated stimulus male cage between the familiar and unfamiliar groups ($z = -1.21$, $p = 0.24$, two-tailed; Figure 6.8).

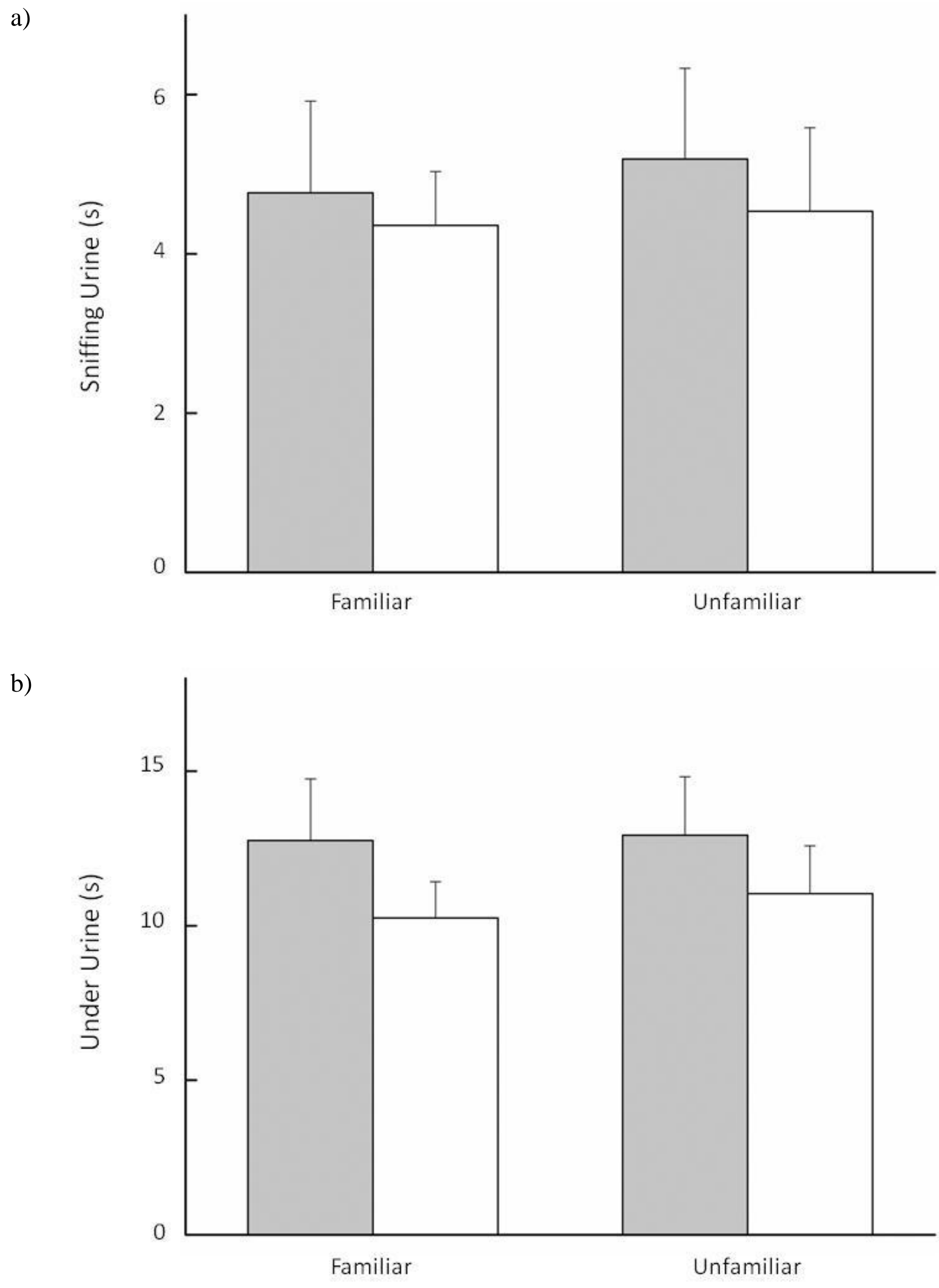


Figure 6.6: Repeated female investigation of urine from related and unrelated stimulus males in the familiar and unfamiliar groups.
Investigatory behaviour towards related (grey bars) and unrelated (open bars) stimulus males in the familiar (n = 11) and unfamiliar (n = 11) groups. Investigation measured by a) time spent sniffing urine and b) time spent directly under urine (s, mean + standard error).

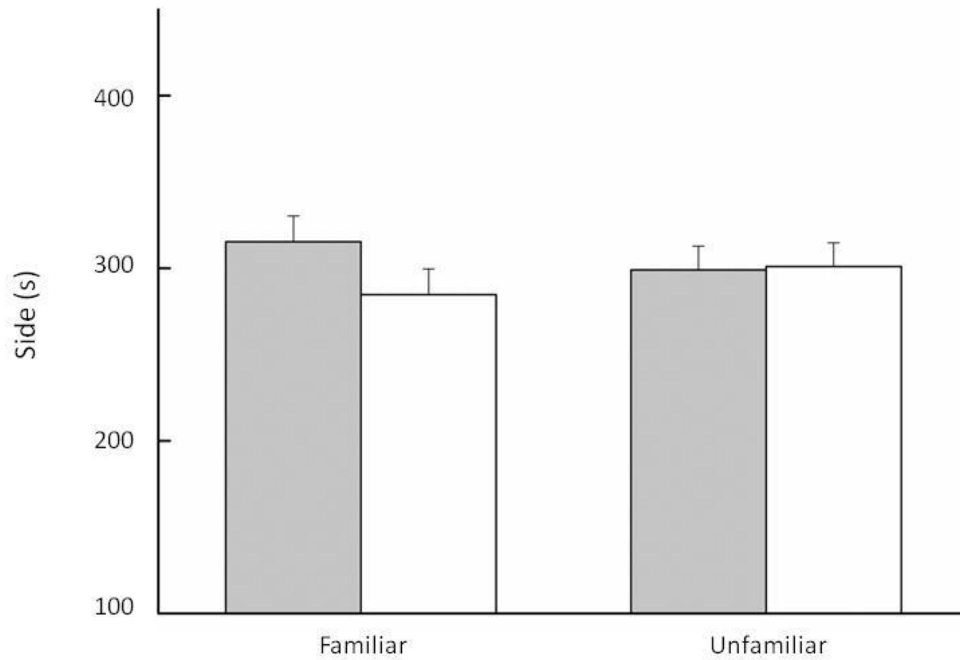


Figure 6.7: Repeated female attraction towards urine from related and unrelated stimulus males in the familiar and unfamiliar groups.
Attraction towards related (grey bars) and unrelated (open bars) stimulus male urine in the familiar (n = 11) and unfamiliar (n = 11) groups. Attraction measured by the amount of time females spent on each side of the test cage (s, mean + standard error).

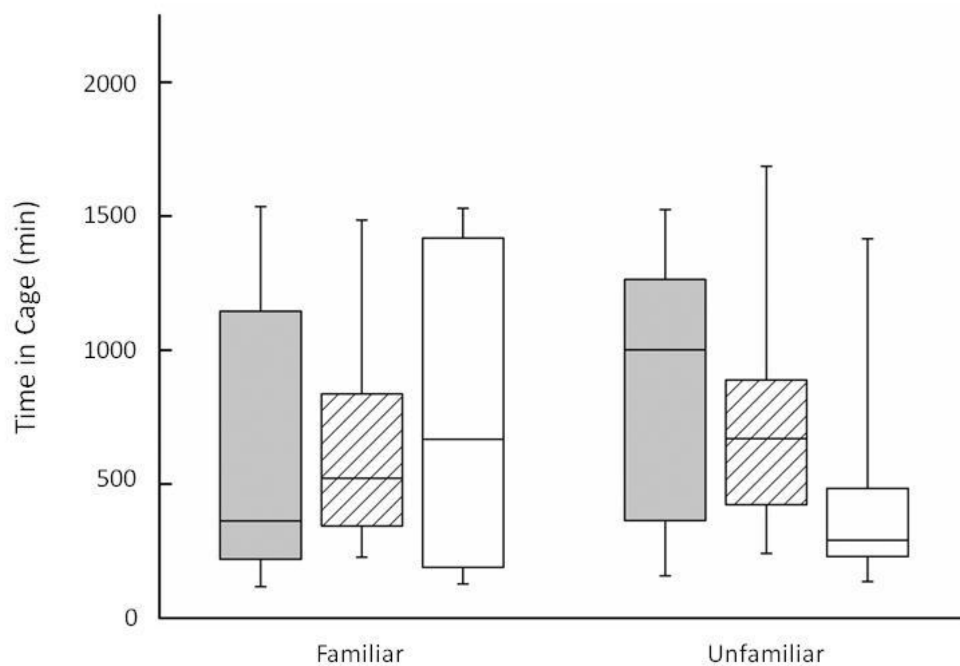


Figure 6.8: Repeated female nest partner choice for related and unrelated stimulus males in the familiar and unfamiliar groups.
Time (minutes) spent by females in the related stimulus male cage (grey boxes), the centre cage (hashed boxes) and the unrelated stimulus male cage (open boxes) in the familiar (n = 12) and unfamiliar (n = 12) groups.

6.3.12 Inbreeding Avoidance and Familiarity: Discussion

In contrast to predictions females displayed no inbreeding avoidance behaviour during the nest partner choice assay (Table 6.1). Additionally, females did not show any discrimination of related from unrelated urine and did not show an attraction towards unrelated male urine. In the first familiarity series of scent assays females spent significantly longer in the side of the cage with the related stimulus male urine than the unrelated stimulus male urine. There was also a non-significant trend for females in the second familiarity series of scent assays for females to spend longer under the related urine than under the unrelated urine. In a scent test using wild-derived house mice Krackow & Matuschak (1991) found no evidence of female discrimination between the soiled bedding of related and unrelated males, suggesting that females may not spontaneously discriminate between the scent of related and unrelated males. However, in Section 6.4 females did show discrimination of related from unrelated urine and therefore it is possible that other factors influenced female behaviour here.

That females did not display any inbreeding avoidance based on the measures used here is surprising. Winn & Vestal (1986) showed previously that female house mice preferred unfamiliar, unrelated males over unfamiliar related males. Musolf *et al.* (2010) demonstrated a female preference for the ultrasonic vocalisations of unfamiliar, unrelated males over those of familiar full brothers. Additionally in Section 6.4, females discriminated full and half-brothers from unrelated males when both males were unfamiliar, and showed a preference for the unrelated males.

It is possible that other differences between the mice have caused the results found here. Other factors are known to influence female mate choice, including male health, social status and genetic heterozygosity (reviewed in Clutton-Brock & McAuliffe, 2009). Whilst age and weight were controlled, other differences between the stimulus males may have disrupted female inbreeding avoidance. Attempts were made to eliminate the bias towards HF males by only using subject and stimulus mice of HF descent. It is possible, however, that the outcome of crossing HF mice with different populations might depend on the population to which the HF was crossed. For instance, HF/KN males may be more attractive than HF/TZ males. Without further experiments designed to look into the reasons for the preferences observed here it is at present difficult to explain why females did not display avoidance of full brothers in the nest partner choice assay when theory and evidence from other studies (including those later in this chapter) suggest that they should.

6.3.13 Inbreeding Avoidance and Familiarity: Conclusions

No evidence of female inbreeding avoidance based on prior association or phenotype matching was observed (Table 6.1). Females did display a strong preference for Hatchwood Farm males, and it is possible that this attraction was based on a specific cue that took priority over cues of relatedness in mate choice decisions. Whilst no inbreeding avoidance was observed, the results of this study highlight the importance of other male attributes that influence female mate choice, although it was not possible to investigation what those attributes might specifically be here. Female house mice then will show inbreeding avoidance when selecting a mate (e.g. Winn & Vestal, 1986; Section 6.5 of this chapter), however these preferences for an unrelated male can and will be over-ridden if other factors influencing mate choice are stronger.

6.4 Inbreeding Avoidance Template Formation

6.4.1 Template Formation: Introduction

Whilst female house mice in the previous study displayed no inbreeding avoidance, Winn & Vestal (1986) demonstrated that females were able to recognise and preferred unfamiliar brothers, suggesting that phenotype matching might be used for female inbreeding avoidance in this species. During phenotype matching a learnt kin template is compared against novel individuals to assess relatedness. An important question to ask is from where this template originates. In house mice a number of individuals are likely to be present during rearing, such as mother, father, siblings, nestmates (the female nest partner of own mother including any of her offspring), and other males and females in the natal area. As discussed in Chapter 3, the two most reliable sources for template formation are self and mother. This is because they are guaranteed to be closely related to the learning individual and they both are a strong presence during gestation and rearing.

The experiments in Chapter 3 showed that subject females spent longer in the cages of maternal half-sister stimulus females than unrelated females, but displayed no preference when the choice was between a paternal half-sister and an unrelated female. As each father had been removed prior to birth, females only experienced their own phenotype, that of their mother and the phenotypes of their littermate full siblings. Therefore, had females discriminated both maternal and paternal half-sisters we would have concluded that they use a recognition template formed from themselves and/or their siblings. As they only discriminated maternal half-sisters it suggested that females may use a match-to-maternal phenotype kin recognition mechanism when making nest partner decisions. The evidence from inbreeding avoidance studies however is mixed as to whether females use maternal or self cues.

A number of studies using laboratory mice have suggested a match-to-maternal/parental mechanism for inbreeding avoidance. Using cross-fostering designs male mice have been shown to prefer females of a different MHC type (Beauchamp *et al.*, 1988; Yamazaki *et al.*, 1988) or a different strain (D'Udine & Partridge, 1981) to the parents that reared them. Laboratory strains of mice are known to have limited genetic variation within and between strains, and have been selected to breed with close relatives, and are therefore far from ideal for studying inbreeding avoidance. However, using wild-laboratory crossed mice Penn & Potts (1998a) found a similar result for female preference – females preferred males with a

different MHC type to the parents that reared them. However, under these controlled conditions both parents were homozygous for the same MHC haplotype which is not a situation realistically found in nature. It is difficult therefore to know whether a match-to-parent mechanism might operate under more naturalistic circumstances.

Sherborne *et al.* (2007) showed in a free-breeding enclosure experiment that pairs of wild-derived house mice were less likely to breed if they shared both MUP haplotypes. In contrast to the previous studies this suggests the use of a match-to-self mechanism for inbreeding avoidance, in this instance using MUP type as the recognition marker. The authors also found no evidence of a maternal matching mechanism, although it is important to note that they did not directly test for this.

In house mice a match-to-maternal mechanism appears to be used in female-female kin discrimination (Chapter 3), however the Sherborne *et al.* (2007) study suggests inbreeding avoidance occurs via a match-to-self mechanism. This hints at the intriguing idea that two context-dependent mechanisms are used during kin discrimination by female house mice. To further investigate this possibility the following questions are addressed in this section:

i) Can female house mice discriminate between urine from related and unrelated males, when related males are unfamiliar full brothers, maternal or paternal half-brothers, and do they show any difference in attraction towards urine from related and unrelated males?

ii) Do female house mice avoid unfamiliar full brothers in preference for unfamiliar, unrelated males?

iii) Do female house mice preferentially associate with an unrelated male when presented with an unrelated male and a half-brother?

iv) Do female house mice use self or maternally learnt cues to avoid inbreeding, as inferred from discrimination of maternal and/or paternal half-brothers?

6.4.2 Template Formation: Methods

Subject females were presented with a related stimulus male and an unrelated stimulus male, both previously unfamiliar. The related stimulus males were a full brother, a maternal half-brother or a paternal half-brother. All subject females and stimulus males resulted from the

breeding programme described in Chapter 3 (Section 3.3.2). Female recognition and inbreeding avoidance was assessed using the scent discrimination and attraction assay and the nest partner choice assay.

6.4.2.1 Breeding Programme

Full and half-siblings (maternal and paternal) were from the same stock as bred in Chapter 3. Subject and stimulus mice were captive-bred from the Mammalian Behaviour & Evolution group's outbred colony of house mice, established using individuals captured in the North West of England, UK. To produce unfamiliar full and half-siblings, two consecutive rounds of litters were bred as detailed in Section 3.3.2. In the full sibling line of breeding, the male – female pairs established for the first litters were the same as those used for the second litters. In the half-sibling line males and females were paired for the first litters and then males were moved to different females for the second round of litters. None of the breeding mice used were of Hatchwood Farm stock.

Pairs of mice were established according to procedures described in Section 3.3.2. Mice were primed with same and opposite sex bedding prior to being introduced so that females would be in or entering oestrus at introduction. Pairs were then placed in MB1 cages and monitored over the following 24 hours. Females were checked on days 17, 21 and 25 and weights compared to pre-introduction weight. If females appeared pregnant then males were removed. Litters were weaned 24 days after birth. At weaning male pups were housed singly in M3 cages and females were housed in sister groups of up to four in MB1 cages. Once all first litters had been weaned pairs were re-established (for the full siblings) or new pairs established (for the half-siblings) and the process was repeated. All mice were over 3 months old before any behavioural assays began.

6.4.2.2 Subject Females and Stimulus Males

Subject females were presented with two stimulus males, one relative and one unrelated. In the full sibling group (n = 16) related stimulus males were unfamiliar full brothers from a subsequent litter. In the two half-sibling groups, related males were either maternal (n = 16) or paternal (n = 16) half-brothers from a subsequent litter. Stimulus males were approximately 2 months younger than subject females. Subject females were 14 months old and stimulus males were 12 months old at testing. Stimulus males were age-matched to within 1 week and were weight-matched to within 2 g of each other. All mice were unfamiliar with one another prior to testing.

Female recognition and avoidance of inbreeding was assessed via a scent discrimination and attraction assay and then a nest partner choice assay. Females were predicted to spend more time investigating the unrelated urine (as it would contain more previously unfamiliar elements) and show an attraction towards the unrelated urine. Females were predicted to choose the unrelated stimulus male cage in the nest partner choice assay and avoid the related cage. No difference in female behaviour was predicted towards the full brother, maternal or paternal half-brothers.

6.4.2.3 Data Analysis

All measures from the scent discrimination and attraction assay were square root transformed to meet parametric assumptions. Repeated measures GLMs compared a) time spent sniffing related and unrelated urine, b) total time under related and unrelated urine and c) total time spent by females on each side of the test cage. For all three measures lineage group was used as a between subject variable.

Data from the nest partner choice assay did not meet parametric assumptions, even with transformations. A Mann-Whitney U test compared whether there was a difference in the proportion of time subject females spent in the unrelated stimulus male cage between the maternal and paternal lineage groups. A Mann-Whitney U test assessed whether the proportion of time subject females spent in the unrelated male cage differed between females presented with a related stimulus male that was a full brother and females presented with a related stimulus male that was a half-brother. A Wilcoxon Signed Ranks assessed whether subject females spent longer in the unrelated stimulus male cage than in the related stimulus male cage.

Female preference was assigned using the ratio of time spent in the unrelated male stimulus cage compared to the time spent in the related male stimulus cage. Females with ratios that were above 0.50 spent longer in the unrelated stimulus male cage, whilst females with ratios that were below 0.50 spent longer in the related stimulus male cage. The observed number of females that spent longer in the unrelated stimulus male cage was compared to the number expected by chance using a Binomial test. All figures use untransformed data.

6.4.3 Template Formation: Results

The results of this section are presented below and summarised in Table 6.1.

6.4.3.1 Scent Discrimination and Attraction

Female discrimination of male urine was measured by two investigatory behaviours: time spent directly sniffing each urine mark and total time spent under each urine mark. Subject female investigatory behaviour did not differ between the full brother, maternal half-brother and paternal half-brother groups for either the amount of time spent sniffing ($F_{(2,37)} = 0.55$, $p = 0.58$; Figure 6.9a) or the total time spent under each urine mark ($F_{(2,37)} = 0.29$, $p = 0.75$; Figure 6.9b). Subject females did not spend longer sniffing urine from unrelated stimulus males compared to urine from related stimulus males ($F_{(1,37)} = 0.24$, $p = 0.63$; Figure 6.9a). However, females spent longer in total under the unrelated stimulus male urine than under the related stimulus male urine ($F_{(1,37)} = 2.92$, $p = 0.01$; Figure 6.9b).

Female attraction to male urine was measured by the total amount of time spent on either side of the test cage. There was no difference in the amount of time subject females spent on either male urine stimulus side of the test cage ($F_{(1,37)} = 0.23$, $p = 0.63$; Figure 6.10), and there was no effect of full, maternal or paternal group on female side preference ($F_{(2,37)} = 0.85$, $p = 0.43$; Figure 6.10).

6.4.3.2 Nest Partner Choice

In the nest partner choice assay the amount of time subject females spent in each of the three cages was measured over 36 hours and female preference for the two stimulus male cages was compared. There was no difference in the proportion of time subject females spent in the unrelated stimulus male cage between the maternal and paternal half-brother groups ($z = -0.11$, $p = 0.93$, two-tailed; Figure 6.11). Data from the half-brother groups were therefore combined. There was no difference in the proportion of time subject females spent in the unrelated stimulus male cage between the full brother and half-brother groups ($z = -1.23$, $p = 0.23$, two-tailed; Figure 6.11). The data for the three groups were therefore combined. Subject females spent more time in the unrelated male stimulus cage than in the related stimulus male cage ($z = -1.94$, $p = 0.03$, one-tailed; Figure 6.11). Additionally, more subject females (69 %) chose the unrelated stimulus male cage than the related stimulus male cage ($p = 0.01$, one-tailed; Figure 6.11).

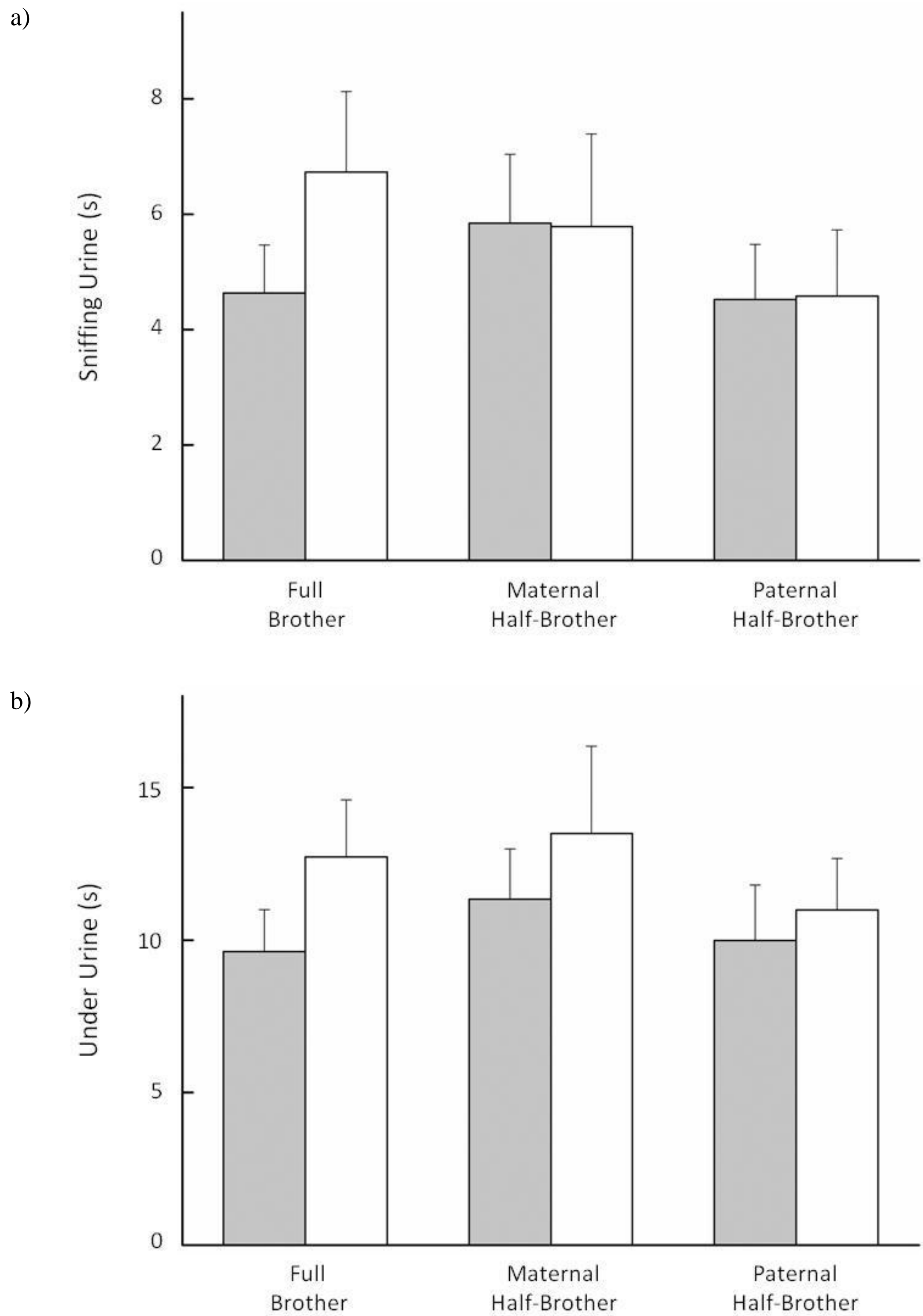


Figure 6.9: Female investigation of urine from related and unrelated stimulus males in the full brother, maternal half-brother and paternal half-brother groups.
Investigatory behaviour towards related (grey bars) and unrelated (open bars) stimulus male urine in the full brother (n = 15), maternal half-sister (n = 16) and paternal half-sister (n = 16) groups. Investigation measured by a) the amount of time spent sniffing urine mark and b) the time spent under urine mark (s, mean + standard error).

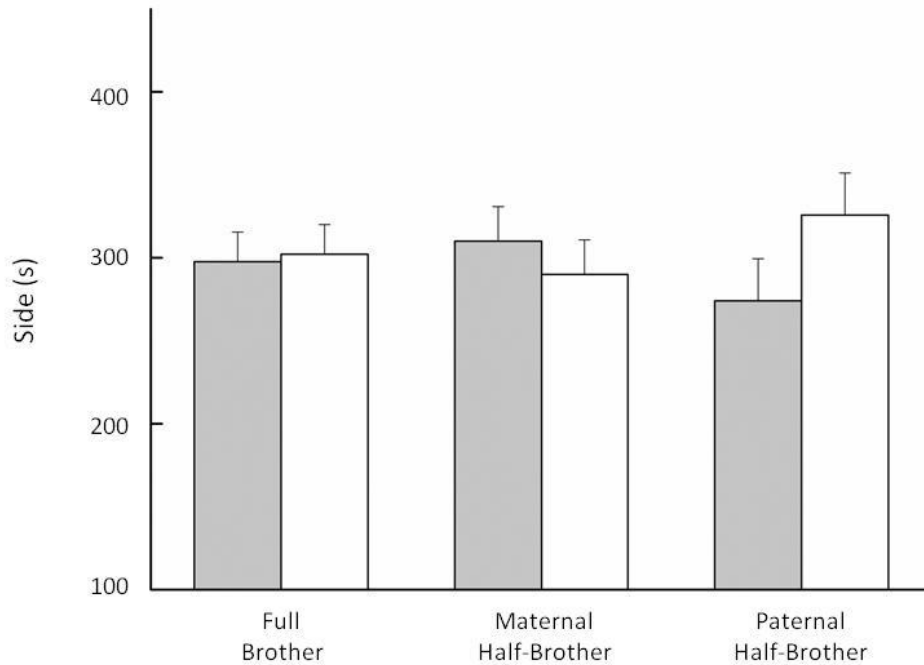


Figure 6.10: Female attraction towards urine from related and unrelated stimulus males in the full brother, maternal half-brother and paternal half-brother groups.
Attraction towards related (grey bars) and unrelated (open bars) stimulus male urine in the full brother (n = 15), maternal half-brother (n = 16) and paternal half-brother (n = 16) groups. Attraction measured by the amount of time females spent on each side of the test cage (s, mean + standard error).

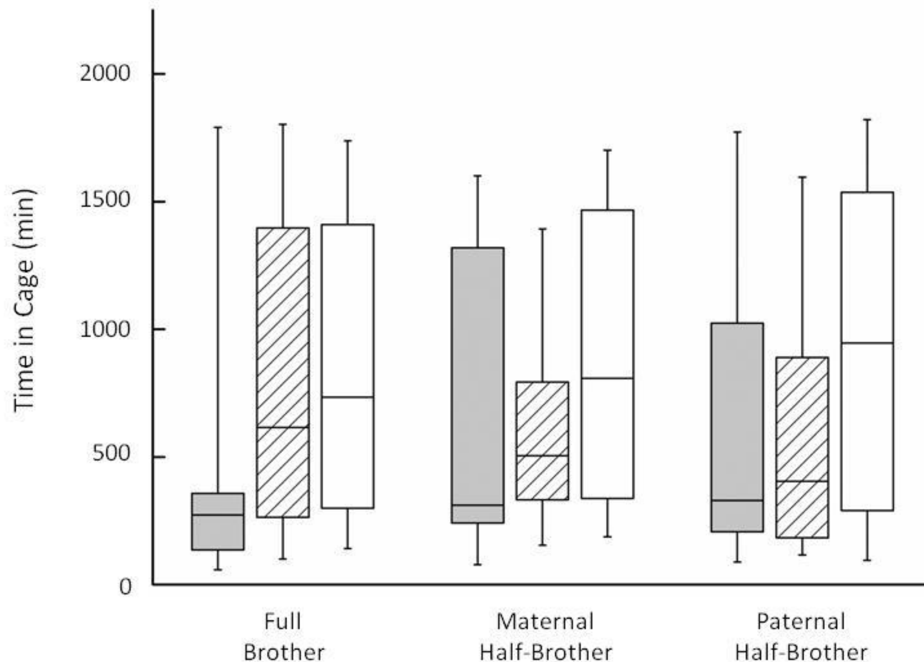


Figure 6.11: Female nest partner choice for related and unrelated stimulus males in the full brother, maternal half-brother and paternal half-brother groups.
Time (minutes) spent by subject females in the related stimulus male cage (grey boxes), the centre cage (hashed boxes) and the unrelated stimulus male cage (open boxes) in the full brother (n = 16), maternal half-brother (n = 16) and paternal half-brother (n = 16) groups.

6.4.4 Template Formation: Discussion

Females avoided full and half-brothers in preference for unrelated males in the nest partner choice assay, and discriminated between related and unrelated urine in the scent assay by spending longer under unrelated urine than related urine (Table 6.1). Whilst the previous section of this chapter found no evidence to support female inbreeding avoidance, here females spent longer in the cages of unrelated males than the cages of full brothers. This agrees with the findings of Winn & Vestal (1986) who also found that female house mice spent longer with unrelated males than either familiar or unfamiliar full brothers. Females paired with full brothers are less likely to produce litters than females paired with unrelated males (house mice; Krackow & Matuschak, 1991) and have smaller litters at birth and weaning (laboratory mice; Barnard & Fitzsimons, 1989). By avoiding full brothers and so avoiding close inbreeding female house mice can ensure that their offspring do not have a lower fitness (Meagher *et al.*, 2000).

Females were able to discriminate urine from related males from that of unrelated males. This is in contrast to the findings of the previous section where females did not discriminate between related and unrelated urine. It also differs from an independent study where females displayed no discrimination between soiled bedding from related and unrelated males (Krackow & Matuschak, 1991). In that study however, male house mice spent longer investigating unrelated female bedding suggesting that the assay design may not have been subtle enough to detect female discrimination. Female house mice are able to discriminate between urine from related and unrelated females (Chapters 2 and 3), and between urine from related and unrelated males (this chapter), suggesting that genetic markers of kin recognition are present in female and male urine.

Female house mice showed an avoidance of related males and an attraction towards unrelated males during the nest partner preference assay. This corresponds with the behaviour of females in the Winn & Vestal (1986) study. Whilst females were not directly tested for an avoidance of inbreeding, time spent in proximity of unrelated males is often used as a proxy for inbreeding avoidance (e.g. Bateson, 1982; Lemaitre *et al.*, 2012). It would be interesting to directly assess female preference by presenting females with both males and testing resulting offspring paternity, however this approach would not be able to tell whether females and/or males were responsible for mate choice. As female avoided relatives, in accordance

with predictions and previously studies, inbreeding avoidance was inferred from time spent in proximity to males.

Females preferred the unrelated male when paired with a full brother, a maternal half-brother or a paternal half-brother. That females discriminated and showed an avoidance of paternal half-brothers suggests that females use a match-to-self mechanism for inbreeding avoidance. This agrees with the findings of Sherborne *et al.* (2007) who found a scarcity of successful matings between mice that shared the same MUP phenotype.

In Chapter 3 female house mice discriminated maternal half-sisters from unrelated females, but showed no discrimination of paternal half-sisters. This suggested that females used a match-to-maternal cues mechanism for female-female kin discrimination, although it was not possible to establish why paternal half-sisters were not recognised. Here females were able to discriminate between both types of half-brother and an unrelated but equivalent male, which suggests that female house mice are using a match-to-self mechanism for inbreeding avoidance. Therefore it appears for these two distinct contexts of kin recognition (female-female altruistic behaviour and female inbreeding avoidance) there may be two different mechanisms employed by female house mice.

Haig (2000a, 2000b) argued that a dam could improve her overall inclusive fitness if her offspring behaved altruistically towards maternal relatives, but would gain little from her offspring behaving altruistically towards paternal relatives. This may explain the evolution of a female-female match-to-maternal kin recognition mechanism. However, breeding with close relatives is known to reduce reproductive success in laboratory and house mice (Barnard & Fitzsimons, 1989; Krackow & Matuschak, 1991), and also reduce offspring fitness in house mice (Meagher *et al.*, 2000). These costs are likely to be the same for mating with both maternal and paternal relatives of the same degree of relatedness. It is therefore in an individual female's interest to avoid mating with close relatives, regardless of whether they are maternal or paternal relatives. Therefore females have two interests in kin recognition – i) to avoid mating with close relatives to prevent deleterious consequences of inbreeding, and ii) for female offspring to preferentially associate with maternal relatives only. The different costs and benefits associated with contexts of recognition could explain why females showed match-to-maternal discrimination in response to female relatives, but match-to-self discrimination in response to male relatives.

Females had no direct experience of paternal odours as dams were moved into clean cages before litters were born. However it is possible that instead of a match-to-self mechanism, females learn the cues of their littermate full siblings and use a combined template. A template formed from a combination of a number of littermate full sibling phenotypes would likely be considerably more complicated to learn and use for recognition than from self alone. A combined template would consist of the phenotypes of multiple individuals. This would increase the chances of unrelated individuals sharing at least some of the same phenotype components of that combined template and increase the chances that those unrelated individuals are falsely identified as being related.

A match-to-combined-littermate mechanism may also falter under communal nesting conditions when more than one litter is present in the nest at any one time, particularly if the nesting dams are not related. Sherborne *et al.* (2007) found a deficit of matings between individuals that shared the same MUP type, supporting the possibility that a match-to-self and not a match-to-combined-littermate mechanism is used for inbreeding avoidance in hosue mice.

6.4.5 Template Formation: Conclusions

Females displayed behaviour consistent with inbreeding avoidance of full brothers and half-brothers. This supports the hypothesis that females make mate choice decisions based on relatedness and actively avoid close male relatives. Females were able to discriminate both maternal and paternal half-brothers, suggesting that they may use a match-to-self phenotype matching kin recognition mechanism for inbreeding avoidance. Previous findings suggested that females use a match-to-maternal cues mechanism for female-female nest partner choice (Chapter 3). Female house mice may therefore use two context-dependent mechanisms for kin recognition.

6.5 Molecular Markers of Inbreeding Avoidance

6.5.1 Molecular Markers of Inbreeding Avoidance: Introduction

In the phenotype matching mechanism a kin template (the phenotype of a known relative(s)) is learnt and then compare against the phenotype of a newly encountered individual. Whilst the previous section (Section 6.4) addressed where this template may originate for female inbreeding avoidance, this section addresses what molecular markers may be important for kin recognition. Females were able to discriminate between the urine of related and unrelated males (Section 6.4.3.1) as shown by females spending longer directly under urine from an unrelated male than from a related male. Females were also able to discriminate between related and unrelated female urine. This suggests that a marker of relatedness is present in the urine of house mice of both sexes.

A good marker of genetic relatedness requires two main characteristics. Firstly a marker should be highly polymorphic, providing enough variation in the population so that only close relatives share the same marker. Close relatives could be then be easily recognised but unrelated individuals would not, as they would be unlikely to share the same marker. Choosing as a mate an individual different at a highly polymorphic marker would strongly decrease the chances of that mate being a relative. Marker-dissassociative mating is therefore expected during inbreeding avoidance studies. However, one problem with a highly polymorphic marker is that not all relatives are likely to share the recognition marker. Secondly, a marker of relatedness should be consistent. Markers that alter with time or are easily influenced by health, diet or the environment are not reliable as changes would make reliable identification difficult. A stable marker allows for the recognition of relatives despite other conditions.

The major histocompatibility complex (MHC) is by far the most commonly cited example of a potential marker of relatedness. Initial evidence in support of a role of MHC in mate choice came from observations of MHC-based mate preferences in laboratory strains of mice (Yamazaki *et al.*, 1976). The role of MHC in the immune system means that dissassortative mating for MHC could improve offspring resistance to pathogens (Penn, 2002), although some doubt remains about this (see Thom *et al.*, 2008a). Since then a number of studies have shown that laboratory mice can discriminate between the odours of MHC-congenic strains (Yamazaki *et al.*, 1979; Yamazaki *et al.*, 1983; Penn & Potts, 1998b).

Evidence for MHC's involvement in mate choice is mixed. Whilst a number of subsequent studies of some laboratory strains of mice have demonstrated MHC-dissassortative mating preferences (Andrews & Boyse, 1978; Yamaguchi *et al.*, 1978; Yamazaki *et al.*, 1978; Beauchamp *et al.*, 1988; Yamazaki *et al.*, 1988; Eklund, 1997a), studies of other strains have shown an MHC-associative mate preference (Andrews & Boyse, 1978; Yamaguchi *et al.*, 1978; Yamazaki *et al.*, 1978), a particular strain preference (Yamaguchi *et al.*, 1978; Beauchamp *et al.*, 1988) or found no influence of MHC on mate choice (Yamazaki *et al.*, 1978; Eklund *et al.*, 1991; Eklund, 1997a; Ehman & Scott, 2001). Mate choice studies using laboratory strains of mice are problematic both because laboratory mice were selected to breed with close relatives, and because of the limited variation both within and between different strains. To counter this a few studies have used mice resulting from crosses between laboratory and wild house mice to produce more naturalistic mice whilst maintaining controlled levels of MHC variation. Whilst these studies support MHC-dissassortative mate choice (Egid & Brown, 1989; Potts *et al.*, 1991; Penn & Potts, 1998a), further studies using wild-derived house mice have found little evidence of MHC's involvement in mate choice (Eklund, 1997b; Eklund, 1998; Sherborne *et al.*, 2007; Thom *et al.*, 2008b). An additional problem associated with the majority of MHC studies is that often only MHC is examined. Mate choice decisions could be made based on other loci that correlate with MHC (Jordan & Bruford, 1998).

Major urinary proteins (MUPs) are a second multigene family that have been suggested as a marker of kin recognition. MUPs are found in high concentrations in house mouse urine (Humphries *et al.*, 1999) and are known to prolong the release of odorous volatile molecules once in the environment (Hurst *et al.*, 1998). Both highly polymorphic and stable, MUPs are an ideal candidate for a kin recognition marker (Robertson *et al.*, 1997; Beynon *et al.*, 2002; Beynon & Hurst, 2004), and are known to signal individual identity in house mice (Hurst *et al.*, 2001; Cheetham *et al.*, 2007). Crucially, only two MUP patterns have been found across all common laboratory mouse strains (Cheetham *et al.*, 2009). If MUPs are important for kin recognition then studies investigating inbreeding avoidance using laboratory mice are missing a large portion of individual variation found in wild mice.

Evidence from studies of wild house mice supports a role of MUP in mate choice and inbreeding avoidance. Thom *et al.* (2008b) showed that female house mice prefer MUP heterozygous males over MUP homozygous males, but found no evidence of female mate

choice based on MHC heterozygosity. Sherborne *et al.* (2007) used a free-breeding enclosure experiment and examined the number of successful matings with known relatedness, MHC type and MUP type. They found a trend for fewer successful matings between full siblings than would be expected by chance. There was no evidence of dissassortative mating based on MHC, but a strong deficit of successful matings between mice that matched for both MUP haplotypes. This reproductive avoidance was not present when mice shared only one MUP haplotype. As close relatives are more likely to share both MUP haplotypes the authors concluded that MUP, and not MHC, sharing between individuals was sufficient to explain inbreeding avoidance in mice. It should be noted, however, that this was not a direct test of female preference, and MUP's sole involvement in inbreeding avoidance remains to be confirmed.

Exocrine-gland secreting peptides (ESP) have also been suggested as a potential marker of relatedness. This polymorphic multigene family has only recently been shown to influence mouse odour (Kimoto *et al.*, 2005; Kimoto *et al.*, 2007). The male-specific peptide *Esp1* stimulates separate neurons from those that respond to MHC or MUP, suggesting that ESP is important for signalling sex and is distinct from the other two families (Kimoto *et al.*, 2007). ESP has been suggested to signal individual identity and therefore could be important for kin recognition. At the moment, however, no studies have investigated ESP's involvement in inbreeding avoidance in mice.

The results of Chapter 4 suggest that both MUP and MHC (and/or ESP) are involved in kin recognition between female house mice, and it is possible that a similar combined recognition system occurs in inbreeding avoidance. The previous section suggested that females use a match-to-self mechanism for inbreeding avoidance, as both maternal and paternal half-brothers were avoided. This section aims to address the following questions:

i) Can female house mice discriminate between urine from equally related males that matches or is dissimilar to themselves for either MHC/ESP type or MUP type, and do they show any difference in attraction towards urine from matching and dissimilar males?

ii) Do female house mice avoid males that share the same MUP type against a different MHC/ESP type?

iii) Do female house mice avoid males that share the same MHC/ESP type when against a different MUP type background?

6.5.2 Molecular Markers of Inbreeding Avoidance: Methods

Subject females were presented with two equally related males (double cousins) that differed from themselves for either MHC/ESP type or MUP type. All subject females and stimulus males were produced as a result of the breeding programme described in Chapter 4. Female recognition and inbreeding avoidance was assessed using the scent discrimination and attraction assay and the nest partner choice assay. Female mate choice was assessed through a scent discrimination and attraction assay and the nest partner choice assay.

6.5.2.1 Trapping

Founder mice were trapped as described in Section 4.3.1, from two separate founder populations in the North West of England, UK – Knowsley Safari Park (Prescot, Merseyside) and Holme Farm (Ince, Cheshire) between February and December 2010.

6.5.2.2 Breeding Programme

The breeding design was described in Section 4.3.3. F0 mice from the two founder populations were paired with same population mice and allowed to breed multiple litters of F1 offspring. F1 full siblings (six males and eight females) from the Knowsley Safari Park lineage were then paired with F1 full siblings (eight males and six females) from the Holme Farm lineage to produce fourteen litters that were double cousins of each other.

The method of pairing mice was described in Section 4.3.5. Mice were primed with same and opposite sex bedding prior to being introduced so that females would be in or entering oestrus at introduction. Pairs were then placed in MB1 cages and monitored over the following 24 hours. Females were checked on days 17, 21 and 25 and weights compared to pre-introduction weight. If females appeared pregnant males were left in when the pair was a F0 founder pair (to allow for multiple litters) and removed when the pair were F1. Litters of F2 mice were weaned 24 days after birth. Sister groups of up to four were housed in MB1 cages. Males were singly housed in M3 cages.

6.5.2.3 Genotyping

Genotyping of males was carried out at the same time as females and using the same methods described in Section 4.3.7, following the protocol of Sherborne *et al* (2007). Tail snips were taken from all F1 and F2 mice (by John Waters and Amanda Davidson of the Mammalian

Behaviour & Evolution Group, University of Liverpool, UK) under home office license, and DNA extracted using a QIAGEN DNeasy Blood & Tissue Kit (QIAGE, West Sussex, UK) by Amanda Davidson following the manufacturer's instructions. Microsatellite markers were used to establish haplotypes – four for MHC, six for MUP and three for ESP (although only two ESP markers amplified successfully). Haplotype size was determined using GeneMapper v3.0 software (Applied Biosystems) and haplotypes assigned to F1 parents in MS Excel (2007). Double cousins were then assigned haplotypes based on parental type and own allele set. MUP genotypes were confirmed using MUP phenotypes produced by electrospray ionization mass spectrometry.

6.5.2.4 MUP Phenotyping

MUP phenotypes were established following the methods described in Mudge *et al* (2008), and here in Section 4.3.8. Urine was collected once mice were over 3 months old to allow for individual profiles to have become stable. Urine samples were run by Amanda Davidson at the Protein Function Group, University of Liverpool, UK using electrospray ionization mass spectrometry on a Nanoacquity ultra high performance liquid chromatography system (Waters, Manchester, UK) and were processed and transformed to a true mass scale using MazEnt1 deconvolution software (Waters Micromass, Massachusetts, USA). Phenotypic profiles of F1 and F2 male and female mice were inspected in SpecAlign (Version 2.4.1; http://physchem.ox.ac.uk/~jwong/specalign/). Mass spectra were cropped to only include the mass range of 18600 to 18900 Da. Each profile was then normalised to the intensity of the most abundant protein (as protein concentrations vary between urine samples) so that multiple profiles could be compared.

A MUP phenotype profile was established for each mouse. Each peak was recorded according to mass and relative intensity. These profiles could then be compared to ensure that subject females were assigned stimulus males that for MUP either matched themselves (for both genotype and phenotype) or were dissimilar to themselves (for both genotype and phenotype). Profiles matched if mice had peaks of the same mass as each other, and a relative intensity of within 0.50 of each other. To be dissimilar from the subject, a stimulus male had to have a peak at a mass where a female didn't have one, or a peak of the same mass but a difference in relative intensity of more than 0.50. The male-specific MUP darcin was not included in the matching/dissimilar measurement as it was naturally absent in all females used (Roberts *et al.*, 2010).

6.5.2.5 Matching vs. Dissimilar

Genotypes for MHC and ESP matched (see Chapter 4 for a discussion of this result), and therefore two groups were established – Match To MHC/ESP and Match To MUP. Subject females were assigned stimulus males that matched or were dissimilar to themselves for MHC/ESP or MUP. The triads of females and males were the same for both behavioural assays. Females were only used once, but 17 stimulus males were used twice. No male was used twice as the same stimulus type.

In the MHC/ESP group (n = 16) females were presented with a stimulus male that matched self for MHC/ESP type but not for MUP type (Matching stimulus male), and a stimulus male that differed for MHC/ESP type but had the same MUP type as the Matching male (the Dissimilar stimulus male). In this way female inbreeding avoidance was assessed according to MHC/ESP type but against a consistent but different MUP type background.

In the MUP group (n = 16) the subject female was presented with a matching stimulus male that matched self for MUP type but not MHC/ESP type, and a dissimilar stimulus male that had a MUP type different to self but a MHC/ESP type the same as the matching stimulus male. Female inbreeding avoidance was assessed according to MUP type against a consistent but different MHC/ESP background.

6.5.2.6 Subject Females and Stimulus Males

Subject females and stimulus males were over 8 months old at testing. To control for male mass, stimulus males were paired to within 2 g of each other. All mice were unfamiliar with one another prior to testing. Female recognition and avoidance of inbreeding was assessed in a scent discrimination and attraction assay and then in a nest partner choice assay. It was predicted that subject females would spend longer sniffing the dissimilar urine (as it would contain elements that were unfamiliar to the females), and show an attraction towards the dissimilar urine.

Females were predicted to preferentially nest in the dissimilar male cage as they would appear least related to the females and therefore the better choice to avoid inbreeding. No predictions were made for any differences between the Match to MHC/ESP and Match to MUP groups.

6.5.2.7 *Data Analysis*

In the scent discrimination and attraction assay time spent by subject females sniffing each stimulus male urine mark was square root transformed to meet parametric assumptions and a repeated measures GLM compared time spent sniffing matching and dissimilar urine, with marker group as a between subject variable. This approach was repeated for the total time spent directly under the urine marks and the time spent on each side of the test cage. Total time directly under the urine marks was square root transformed to meet parametric assumptions. Total time on each side of the test cage met parametric assumptions without transformation.

Data from the nest partner choice assay did not meet parametric assumptions, even with transformations. To compare the affect of marker group on female response the proportion of time spent by subject females in the dissimilar stimulus male cage (as a proportion of the total assay time) was compared between the MHC/ESP and MUP group using a Mann-Whitney U test. Female preference for the matching or dissimilar stimulus male cages was compared using a Wilcoxon Signed Ranks test. Female preference was assigned using the ratio of time spent in the dissimilar male stimulus cage compared to the time spent in the matching male stimulus cage. Females with ratios that were above 0.50 spent longer in the dissimilar stimulus male cage, whilst females with ratios that were below 0.50 spent longer in the matching stimulus male cage. The observed number of females that chose the dissimilar stimulus male cage was compared to the number expected by chance using a Binomial test. All figures use untransformed data.

6.5.3 Molecular Markers of Inbreeding Avoidance: Results

The results of this section are presented below and summarised in Table 6.1.

6.5.3.1 *Scent Discrimination and Attraction*

Female scent discrimination was measured by two investigatory behaviours: time spent directly sniffing each stimulus urine mark, and time spent under each urine mark. There was no difference in subject investigatory behaviour between the marker groups (MHC/ESP or MUP type) for either time spent sniffing ($F_{(1,27)} = 0.54$, $p = 0.47$; Figure 6.12a) or time spent directly under urine marks ($F_{(1,27)} = 0.43$, $p = 0.52$; Figure 6.12b). Females did not display more investigatory behaviour towards urine from dissimilar males compared to urine from matching males in either the time spent sniffing ($F_{(1,27)} = 0.65$, $p = 0.43$; Figure 6.12a) or the total time spent directly under the urine marks ($F_{(1,27)} = 1.51$, $p = 0.23$; Figure 6.12b).

Female attraction towards male urine was measured by the amount of time females spent on either side of the test cage. The amount of time subject females spent on either side of the test cage did not differ between the marker groups ($F_{(1,27)} = 0.93$, $p = 0.34$; Figure 6.13). Females did not show any attraction towards urine from a dissimilar male compared to urine from a matching male ($F_{(1,27)} = 0.19$, $p = 0.67$; Figure 6.13).

6.5.3.2 Nest Partner Choice

The amount of time subject females spent in each of the three nest partner choice test cages was measured over 36 hours. The proportion of time subject females spent in the dissimilar cage was not significantly different between the match to MHC/ESP and match to MUP groups ($z = -0.22$, $p = 0.85$, two-tailed; Figure 6.14). Subject females did not spend longer in the dissimilar stimulus male cage than in the matching stimulus male cage ($z = -0.60$, $p = 0.28$, one-tailed; Figure 6.14). The number of subject females that spent longer in the dissimilar stimulus male cage (57 %) was not greater than the number of females that spent longer in the matching stimulus cage ($p = 0.29$, one-tailed; Figure 6.14).

Inspection of the data suggested that female response might be different depending on whether males matched and were dissimilar for MHC/ESP or MUP (Figure 6.14). The data suggest that females may spend longer in the dissimilar stimulus cage than the matching stimulus cage when the molecular marker tested was MUP, but that females showed no difference in the amount of time spent in either stimulus male cage when the molecular marker tested was MHC/ESP. To investigate this difference further female preference for the dissimilar male was compared separately for each group. Females did not spend longer in the dissimilar stimulus male cage compared to the matching stimulus male cage in either the MHC/ESP marker group ($z = -0.05$, $p = 0.49$, one-tailed; Figure 6.14) or the MUP marker group ($z = -1.01$, $p = 0.17$, one-tailed; Figure 6.14).

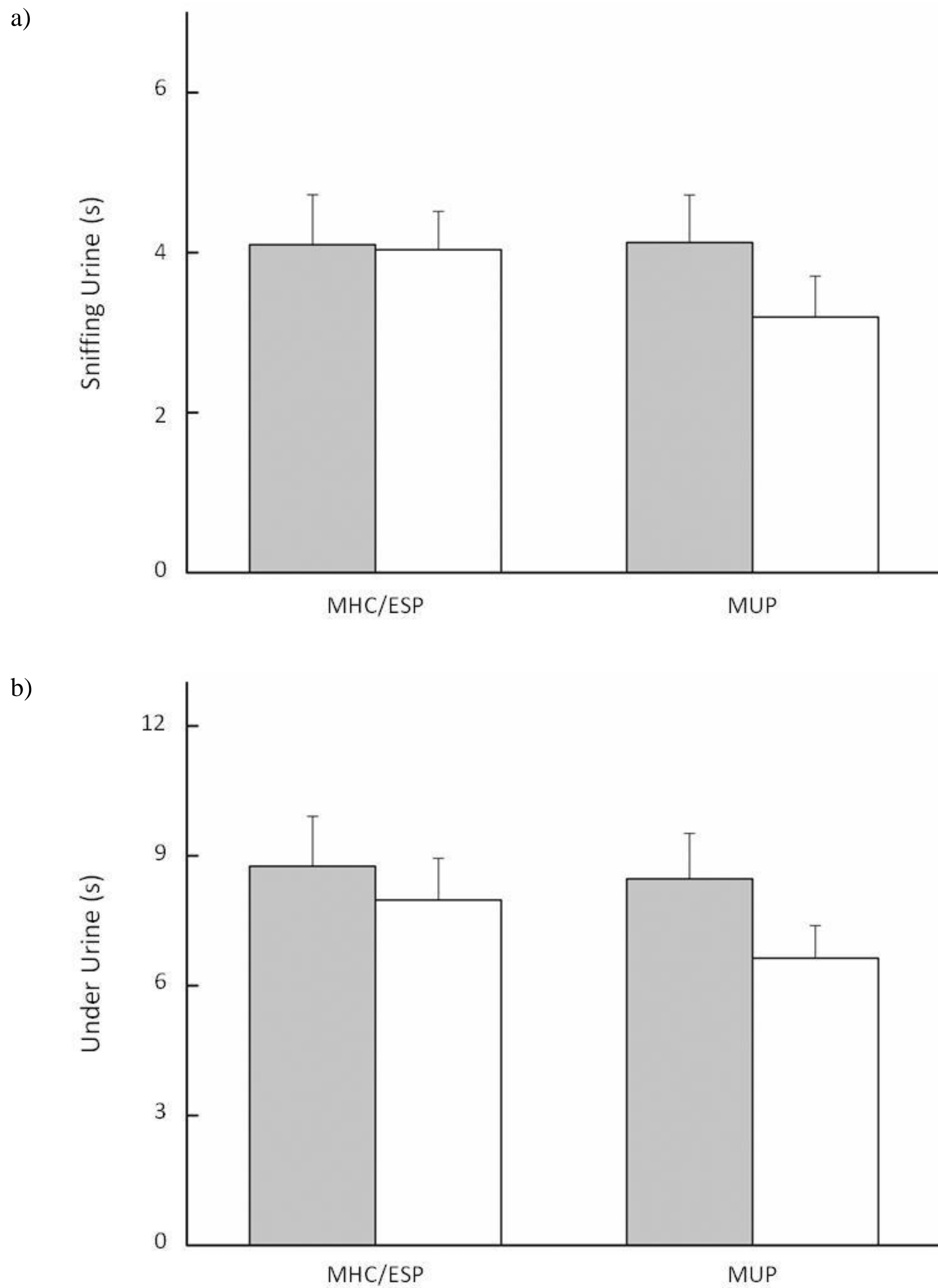


Figure 6.12: Female investigation of urine from matching and dissimilar stimulus males in the MHC/ESP and MUP marker groups.
Investigatory behaviour towards matching (grey bars) and dissimilar (open bars) stimulus male urine in the MHC/ESP (n = 14) and MUP (n = 15) marker groups. Investigation measured by a) the time spent sniffing urine and b) the time spent directly under urine (s, mean + standard error).

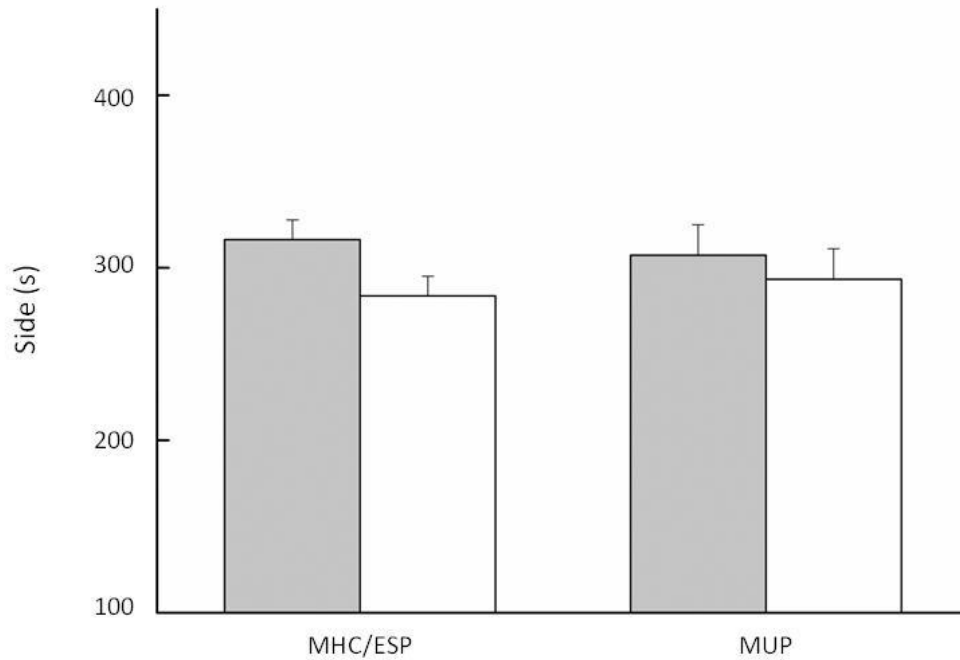


Figure 6.13: Female attraction towards urine from matching and dissimilar stimulus males in the MHC/ESP and MUP marker groups.
Attraction towards related (grey bars) and unrelated (open bars) stimulus male urine in the MHC/ESP (n = 14) and MUP (n = 15) marker groups. Attraction measured by the amount of time females spent on each side of the test cage (s, mean + standard error).

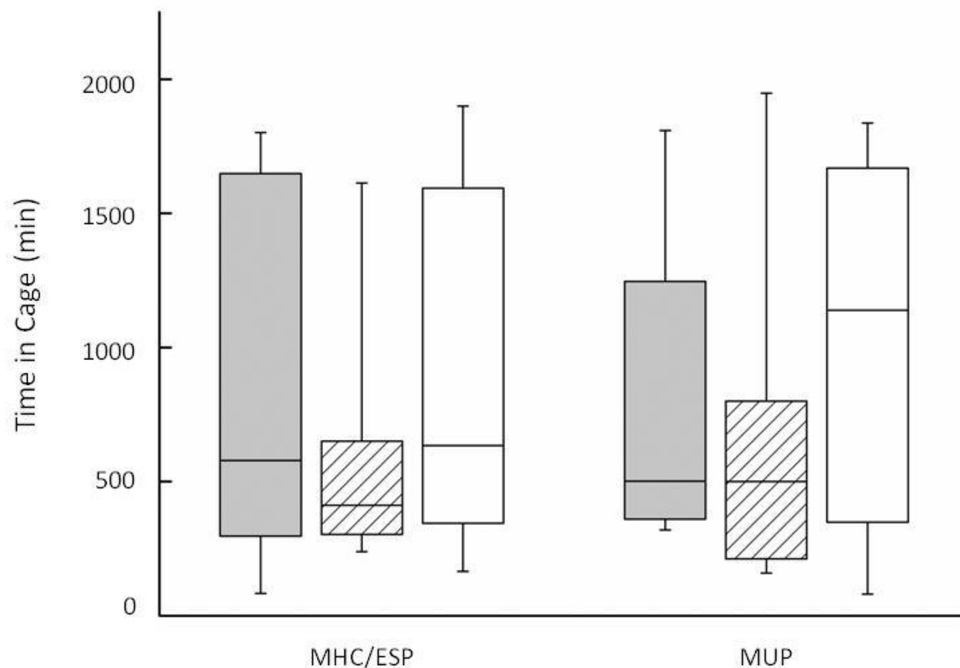


Figure 6.14: Female nest partner choice for matching and dissimilar stimulus males in the MHC/ESP and MUP marker groups.
Time (minutes) spent in the matching stimulus males cage (grey boxes), the centre cage (hashed boxes) and the unrelated stimulus male cage (open boxes) in the MHC/ESP (n = 16) and MUP (n = 13) marker groups.

6.5.4 Molecular Markers of Inbreeding Avoidance: Discussion

No significant avoidance of males that shared the same MHC/ESP or MUP type by subject females was observed here (Table 6.1). Females did not display any discrimination between or attraction towards urine from equally related males that matched or were dissimilar for either MHC/ESP or MUP type. Contrary to findings from other studies (Penn & Potts, 1998a; Sherborne *et al.*, 2007) these results provide no evidence for the involvement of MHC, ESP and MUP in female mate choice and inbreeding avoidance decisions, however the small sample sizes of the present study may limit the conclusions that can be drawn.

Despite an apparent lack of support for MHC, ESP or MUP being important for female mate choice, inspection of the data suggests that further trials should be conducted. The smaller sample size of the MUP group in the nest partner choice assay was created when three trials needed to be removed from the analysis due to two water bottle leaks and a RFID reader error. The MHC/ESP group consisted of 16 subject females whilst the MUP group consisted of 13 subject females. In the nest partner choice assay female response varied. Whilst the majority of females spent more time in the related stimulus female cage, approximately one third of females spent longer in the unrelated stimulus female cage. In the MUP group eight females spent longer in the dissimilar male cage whilst five females spent longer in the matching MUP cage. Of the 13 subject females in the MUP group approximately a third spent longer in the matching cage. In the MHC/ESP group eight females spent longer in the dissimilar stimulus male cage and eight females spent longer in the matching stimulus male cage. These results suggest that a difference between female responses in the marker groups may exist and an increased sample size may demonstrate this. This finding could agree with the work of Sherborne *et al.* (2007) and Thom *et al.* (2008b) who suggested that MUP and not MHC is integral for mate choice and inbreeding avoidance in house mice. Whilst separately analysis of the MHC/ESP and MUP groups did not back this finding, it is possible that a larger sample size might confirm the observed tendency of a difference between the marker groups.

Paterson & Hurst (2009) modelled the effectiveness of a match-to-self mechanism using different number of recognition loci. They found that a single recognition locus to be the most effective. Increasing the number of recognition loci increased the proportion of relatives recognised but at the cost of also increasing the number of unrelated individuals falsely identified. The results of Section 6.4 suggest that female house mice may use a match-to-self

mechanism for inbreeding avoidance, whilst the results presented here lend support to MUP and not MHC/ESP being used as a marker for kin recognition in inbreeding avoidance. Whilst there may be other markers that are as yet untested, this supports the model of Paterson & Hurst (2009) where a match-to-self mechanism was more effective using a single recognition locus than using multiple recognition loci.

Apparent non-significant trends were also observed in the scent discrimination and attraction assay. Inspection of the data suggests that females may have spent longer sniffing MUP matching urine than MUP dissimilar urine. Overall there appeared to be a non-significant attraction towards matching urine over dissimilar urine in both marker groups. At present it is difficult to explain why females may have spent longer investigating and shown an attraction towards matching marker urine. Stimulus males matched or were dissimilar at the tested marker, but differed from the subject female at the non-tested marker. Perhaps no significant discrimination or attraction was observed because the non-tested marker present in both urine marks would be equally unfamiliar to subject females. It is even possible that females spent longer sniffing the matching urine because the non-tested marker was easier to distinguish against an already familiar background, or because the combination of familiar (the tested marker) and unfamiliar (non-tested marker) appeared more novel. Once again the sample sizes were relatively small and an increased sample size may reveal whether the observed trends are significant or not.

It is possible that no strong female choice was observed due to the fact that both stimulus males were equally related to the subject female. Whichever male was chosen females would still be breeding with a close relative, and therefore female choice may have been based on other male attributes. The aim of this experiment was to present females with stimulus males that may appear more or less related (based on matching or differences at MHC/ESP or MUP) but that were actually the same degree of relatedness. In this way females might make decisions based on the molecular marker of relatedness rather than the degree of relatedness itself. As females did not significantly spend more time with matching or dissimilar males this suggests that there may be other molecular markers of relatedness that were not controlled for here.

It is possible that the nest partner choice assay was not sensitive enough to detect female preference in this instance and a different assay design might more clearly display female mate choice when both males were equally related but differed at particular molecular

markers. By allowing females access to both males and then typing litters for paternity female choice might become more apparent, although again this approach would present difficulties about whether females or males were making the mating decision.

Alternatively, a combination of both markers might be required for female inbreeding avoidance. Whilst Sherborne *et al.* (2007) and Thom *et al.* (2008b) found no evidence to support a role of MHC in mate choice, in Chapter 4 females appeared to use both MHC/ESP and MUP during female-female nest partner choice decisions. Further tests could investigate this possibility by presenting subject females with double cousin males that either matched or were dissimilar for both MHC/ESP and MUP.

6.5.5 Molecular Markers of Inbreeding Avoidance: Conclusions

No significant avoidance of males that matched females for either MHC/ESP or MUP was observed and at present it is difficult to draw many conclusions from the data presented here. Clearly more tests are needed to increase the sample size and establish whether females do make mate choice decisions based on either MUP alone or a combination of MUP and MHC/ESP. The trends observed here support previously observed findings in mate choice in house mice (Eklund, 1997b; Eklund, 1998; Sherborne *et al.*, 2007; Thom *et al.*, 2008b) but neither confirm a role of MUP nor completely refute MHC's involvement in female mate choice.

Table 6.1: Summary of female inbreeding avoidance results.

Experiment	Assay	Behaviour Measured	Grouping Variable	Repeated Measure
Familiarity 1			**Familiarity (Familiar vs. Unfamiliar)**	**Relatedness (Related vs. Unrelated)**
	Scent Discrimination and Attraction	Sniff Urine (discrimination)	p = 0.89[a]	p = 0.95[a]
		Under Urine (discrimination)	p = 0.70[a]	p = 0.98[a]
		Cage Side (attraction)	p = 0.70[a]	**p = 0.04**[a] **(R>U)**
	Nest Partner Choice	Time in stimulus cage	p = 0.45[b]	p = 0.42[c]
Population Preference				**Population (Hatchwood Farm vs. Other)**
	Nest Partner Choice	Time in stimulus cage		**p = 0.02**[c] **(HF>O)**
Familiarity 2			**Familiarity (Familiar vs. Unfamiliar)**	**Relatedness (Related vs. Unrelated)**
	Scent Discrimination and Attraction	Sniff Urine (discrimination)	p = 0.89[a]	p = 0.97[a]
		Under Urine (discrimination)	p = 0.08[a]	p = 0.82[a]
		Cage Side (attraction)	p = 0.60[a]	p = 0.49[a]
	Nest Partner Choice	Time in stimulus cage	p = 0.24[b]	p = 0.25[c]
Template Formation			**Lineage (Maternal vs. Paternal)**	**Relatedness (Related vs. Unrelated)**
	Scent Discrimination and Attraction	Sniff Urine (discrimination)	p = 0.58[a]	p = 0.63[a]
		Under Urine (discrimination)	p = 0.75[a]	**p = 0.01**[a] **(U>R)**
		Cage Side (attraction)	p = 0.43[a]	p = 0.63[a]
	Nest Partner Choice	Time in stimulus cage	p = 0.23[b]	**p = 0.03**[c] **(U>R)**
Molecular Markers			**Molecular Marker (MHC/ESP vs. MUP)**	**Marker Similarity (Matching vs. Dissimilar)**
	Scent Discrimination and Attraction	Sniff Urine (discrimination)	p = 0.47[a]	p = 0.43[a]
		Under Urine (discrimination)	p = 0.52[a]	p = 0.23[a]
		Cage Side (attraction)	p = 0.34[a]	p = 0.67[a]
	Nest Partner Choice	Time in stimulus cage	p = 0.85[b]	p = 0.28[c]

Subject female responses towards males of differing relatedness (R – Related, U – Unrelated), population (HF – Hatchwood Farm, O – Other Population), or marker similarity (Matching or Dissimilar). Statistical tests performed: a – repeated measures GLM, b – Mann-Whitney U test, c – Wilcoxon Signed Ranks test, d – paired t-test.

6.6 General Inbreeding Avoidance Conclusions

Three aspects of female inbreeding avoidance and mate choice were investigated here (Table 6.1). Females avoided half-brothers regardless of lineage, suggesting that females use a match-to-self mechanism for inbreeding avoidance. Combined with the results of Chapter 3 this suggests that female house mice may use two context-dependent mechanisms of kin recognition; a match-to-maternal cues mechanism for female-female nest partner decisions, and a match-to-self mechanism for inbreeding avoidance. A non-significant trend was observed for female avoidance of males that matched themselves for MUP type, however the small data set prevents any firm conclusions from being drawn at this time. Females were also observed to display a preference males derived from one specific population over males from other populations. This preference was observed even when these males were full brothers, highlighting the importance of other characteristics in mate choice as well as relatedness. Finally none of the tests discussed here directly measured female inbreeding avoidance. It is possible that clearer results might be more apparent if females were allowed to actively choose reproductive partners and the paternity of resultant litters investigated.

7 General Discussion: Kin Recognition Mechanisms and Contexts

The field of kin recognition has progressed a considerable distance since Hamilton's explanation of inclusive fitness (Hamilton, 1964a, 1964b), and kin biased behaviour has been demonstrated in a large number of diverse species (e.g. Werner *et al.*, 1987; Brown & Brown, 1993; Petrie *et al.*, 1999; Griffin *et al.*, 2004; Lizé *et al.*, 2006; Dudley & File, 2007; Lode, 2008). Evidence from the extensive kin recognition literature suggests that there is not a one-size-fits-all rule for how organisms recognise their relatives. Inclusive fitness benefits and inbreeding costs provide separate contexts where kin recognition expectations are reversed and, within a single species, individuals are predicted to favour their relatives during non-reproductive encounters but shun their relatives when making decisions about mating partners. In this chapter I will provide a brief summary of the main results presented in the preceding chapters and discuss them in the context of the broad themes introduced in Chapter 1. I also discuss some of the limitations of this thesis and possible directions in which this work could be expanded.

7.1 Thesis Summary

One of the key questions of kin recognition is whether novel relatives can be recognised. If unfamiliar relatives are not recognised then a prior association mechanism is often assumed: where only relatives present during development are identified as related in later life (Tang-Martinez, 2001; Mateo, 2003; Holmes, 2004). The ability to recognise novel relatives suggests a phenotype matching mechanism: where a general relatedness template is learnt and then compared against newly encountered individuals (Blaustein, 1983; Holmes & Sherman, 1983; Tang-Martinez, 2001). In Chapter 2 I showed that female house mice preferred to nest with full sisters over unrelated females, when highly familiar or unfamiliar with both (Table 7.1). In Chapter 6 I found that females nested with unfamiliar unrelated males over unfamiliar related males (Table 7.1). Importantly, in both cases this involved outbred wild-stock house mice that have clearly distinguishable individual identity cues, unlike laboratory mice. Females therefore appear to be using a phenotype matching mechanism for both social partner choice and mate choice, and the direction of these results agrees with the predictions of kin selection and inbreeding avoidance respectively.

A phenotype matching mechanism requires that a template is learnt from a known relative(s). Which relative(s) provides the template can alter the types of relatives that are recognised. Chapters 3 and 6 presented females with maternal and paternal half-siblings to assess female

recognition ability. Females nested with maternal but not paternal half-sisters, yet avoided both maternal and paternal half-brothers (Table 7.1). These results suggest that females may be using two different mechanisms for recognising their relatives – a match-to-maternal cues mechanism when making social partner choice decisions and a match-to-self mechanism when making inbreeding avoidance decisions. As discussed previously, this could be due to the different interests of a female and her mother. It is in the mother's interest for daughters to recognise their maternal relatives, but is in both the female and her mother's interest to avoid the deleterious consequences of the female mating with a close relative. Alternatively, a different evolutionary history could be responsible for the different mechanisms for discriminating female and male relatives.

Table 7.1: Female house mouse discrimination of females and males of differing relatedness.

	Preference for Female Relative	Avoidance of Male Relative
Full Sibling	✓	✓
Maternal Half-Sibling	✓	✓
Paternal Half-Sibling	✗	✓

In order to recognise kin there needs to be some form of identifying marker or signal and in mice the dominant mode of communication is olfaction. Results from Chapters 2 and 6 suggest that females are able to recognise relatives based on urine samples alone. Female house mice spent longer investigating unrelated female urine but spent more time in the area of related female urine (Chapter 2). Females also spent more time investigating unrelated male urine than related male urine (Chapter 6), although no scent attraction was observed. An odour-genes covariance assay showed that female house mice perceive urine from two full sisters as more similar than the urine of two unrelated females (Chapter 3). Together these results suggest that a marker of genetic relatedness is present in the urine of house mice, and that this causes urine samples from relatives to appear more similar than those from non-relatives.

The major histocompatibility complex (MHC) has long been proposed as a potential marker of relatedness (Yamazaki *et al.*, 1976). Recently however, major urinary proteins (MUPs) have also been suggested as a possible marker of relatedness in house mice (Sherborne *et al.*, 2007). Additionally, the diversity of the exocrine-gland secreting peptides (ESP), along with their effect on male mouse odour (Kimoto *et al.*, 2005; Kimoto *et al.*, 2007) hints at a role in house mouse scent communication and the possibility of an involvement of ESP in mouse kin recognition. Microsatellite markers revealed a potential link between MHC and ESP types (although further work is needed to investigate this, Chapter 4). As a result of this the effects of MHC and ESP could not be disentangled from each other.

A novel breeding programme was used to produce equally related subject and stimulus females that differed at either MHC/ESP or MUP (whilst the second marker was held constant, Chapter 4). Females spent longer in the cages of those stimulus females that matched (either themselves or their mother) at MHC/ESP or MUP (Table 7.2). A similar experiment was conducted to investigate markers involved in female inbreeding avoidance; however limited numbers meant that no significant avoidance of males based on matching to self for MHC/ESP or MUP was observed (Chapter 6). The data suggested, however, that females avoided males that matched themselves for MUP but not those that matched themselves for MHC/ESP. This would support evidence from Sherborne *et al.* (2007) (Table 7.2) suggesting that MUP, not MHC is important for female inbreeding avoidance.

The results presented over Chapters 3, 4 and 6 suggest that females may employ a match-to-maternal mechanism using both MHC/ESP and MUP for nest partner decisions but may use a match-to-self mechanism using MUP for inbreeding avoidance. This could support the predictions made by Paterson & Hurst (2009) who found that a maternal comparison kin recognition mechanism was more effective for correctly identifying relatives and excluding unrelated individuals if it used multiple recognition loci compared to a single locus; whilst a direct comparison model (match-to-self) was most effective when using complete matching at a single recognition locus compared to multiple recognition loci.

Table 7.2: Female house mouse discriminative ability of females and males that match to self or mother for MHC/ESP or MUP.
Female avoidance of self MUP matching males based on trend observed in Chapter 6 and results of Sherborne *et al.* (2007).

	Preference for Matching Female	Avoidance of Matching Male
Self MHC/ESP	✓	✗
Maternal MHC/ESP	✓	n/a
Self MUP	✓	✓?
Maternal MUP	✓	n/a

Three physiological effects of female social environment were compared in Chapter 5. Females were housed in pairs of differing relatedness and familiarity and changes in faecal corticosterone, urinary protein and body mass were compared. Over a short time period (7 hours) corticosterone did not change. Female body mass decreased in females paired with more distantly related females. This could be due to a reduction of water intake as a result of competitive behaviour between unfamiliar females as urinary concentration increased over the same time period. Urinary protein decreased over the short-term, possibly as a result of competition between females. Over the long term (3 days), corticosterone did not change in any of the familiarity or relatedness groups. However, both female body mass and urinary protein concentration increased in all unfamiliar female groups, possibly as a result of increased competition between females.

7.2 Do Female House Mice Show True Kin Recognition?

In 1990 Grafen proposed a question that has been debated ever since: are kin truly recognised? He argued that for kin biased behaviour to be as a result of true kin recognition then the mechanisms of recognition must have evolved specifically for the purpose of identifying kin (Grafen, 1990). Grafen's exemplar species for kin recognition, *Botryllus schlosseri*, showed that siblings settled in close proximity to each other on the basis of matching at a particular locus, that colonies fused due to matching at this locus, and that relatives gain benefits from fusing (Grosberg & Quinn, 1986).

Grafen later suggested that house mice may also provide an example of true kin recognition, although he retained a number of reservations (Grafen, 1992). Results from the literature, as well as those presented in this thesis, can tell a similar story to *B. schlosseri* for house mice. Female house mice gain reproductive benefits (e.g. König, 1994a; Rusu & Krackow, 2004; Palanza *et al.*, 2005) from choosing to nest with female relatives (Manning *et al.*, 1992; Dobson *et al.*, 2000; Dobson & Baudoin, 2002; Chapters 2 and 3), identified by both MHC and MUP (Manning *et al.*, 1992; Chapter 4). Females also eschew male relatives (Winn & Vestal, 1986; Chapter 6) in order to avoid the costs associated with close inbreeding (Meagher *et al.*, 2000) and this is suggested to be based in part on MUP sharing (Sherborne *et al.*, 2007; Chapter 6). Therefore there is a strong evolutionary incentive for kin biased behaviour in house mice; both positive and negative kin biased behaviour is observed to be performed by house mice in line with theoretical predictions, and the mechanisms involved appear to be designed to identify only close kin. Together these results are suggestive that female house mice do indeed recognise their relatives. Female house mice are therefore an ideal species with which to study multiple contexts of kin recognition mechanisms.

7.3 The Other Mechanisms

Of the four original mechanisms proposed for how individuals recognise their kin, prior association and phenotype matching have come to dominate the literature, and throughout this thesis I have followed suit. Here I will discuss the possibility of spatial location and recognition alleles in playing a role in kin recognition between female house mice.

Spatial location occurs when individuals within a certain area are treated as kin whilst those individuals outside of the specific area are treated as non-kin (Holmes & Sherman, 1983; Hepper, 1986). Both male and (to a lesser extent) female house mice disperse, although not all females disperse the nest and some females remain in the natal area for longer and disperse as adults. Whilst spatial location could potentially be used as a mechanism of kin recognition in these instances, location may not be a reliable indication of relatedness as unrelated individuals may still disperse into the area. Therefore it is unlikely that spatial location is used as a kin recognition mechanism during adult interactions.

It remains possible that spatial location is used by young during rearing however. The results presented in Chapters 2 and 6 showed that females are able to identify unfamiliar kin, suggesting a phenotype matching mechanism, whilst the results presented in Chapter 3 implied that females use maternally learnt cues when making nest partner decisions. It is

likely that these cues are learnt prior to weaning, and that a spatial location mechanism could be used to identify those relatives from which to learn the relevant cues. In a number of species, individuals cross-fostered shortly after birth often display a switched preference (e.g. Hare & Murie, 1996; Li & Zhang, 2010), recognising instead the relatives of their foster parents. In natural conditions it is improbable that litters will spontaneously move to foster parents and it seems likely that for many species spatial location is a reliable mechanism for identifying relatives in the nest.

Throughout this thesis subjects were reared in single female nests, however in communally nesting species such as house mice offspring are likely to encounter not just their own mother and siblings, but also their mother's nesting partner(s) and her offspring (Sayler & Salmon, 1971). Whilst related females appear to preferentially nest together, unrelated females have also been found communally nesting (Dobson *et al.*, 2000; Rusu & Krackow, 2004). Here a spatial location mechanism used for identifying relatives in the nest may be imperfect as offspring could potentially misidentify the unrelated nesting partner female (and her offspring) as being related. In house mice nesting females also communally nurse young, meaning that pups gain a lot of experience of and exposure to their mother's nesting partner(s) (Hayes, 2000). It would be interesting then to see whether females do use the cues of their own mother, or whether they learn the cues of any adult lactating female in the nest. One way to investigate this could be to spike a single female nest with the odours from a second lactating female. This would reveal whether the odours of any lactating female are learnt in the nest. However, nestmate females will nurse young in the nest and the odours alone may not therefore be a strong enough cue. Alternatively, two females could be allowed to communally rear their litters and the resulting offspring could be tested for their ability to recognise unfamiliar relatives of the female that was not their own mother. This would reveal whether interactions with a lactating female in the nest would cause the formation of recognition templates, although nestmates would have to be unrelated females and this may increase the chances of infanticidal behaviour (Palanza *et al.*, 2005).

All house mice used as subject and stimulus animals in the experiments presented in this thesis were reared in nests where only the breeding female was present. This meant that females did not have an opportunity to learn male cues. Whilst males that visit the natal area may not be the sires of offspring in the nest it is still possible that female pups could learn the cues of males and use them for inbreeding avoidance as well as using a match-to-self

mechanism. It would therefore be interesting to further investigate the effect that rearing in presence of males (or their odours) may have on female inbreeding avoidance. A way to investigate this without the risk of males killing pups would be to spike the female nest cages with male odours, either from the father of the developing pups or from unrelated males. Females could then be assessed for their ability to recognise the male relatives of that male.

Recognition alleles are where a gene or set of genes code for the expression of a marker, the recognition of a marker and the action that an individual takes on perceiving the marker (Tang-Martinez, 2001; Barnard, 2004). It remains difficult to prove or disprove the involvement or existence of recognition alleles in kin recognition. However, the difference in behavioural response observed in Chapter 3, where females nested with maternal half-sisters but did not show a nesting preference for paternal half-sisters, lends some evidence against the idea that recognition alleles are being used. If female house mice did use a recognition allele mechanism when choosing female nest partners then it might be expected that both maternal and paternal half-sisters would be selected in an equal proportion. Additionally, MHC is important for self/non-self recognition in the immune system (Brown & Eklund, 1994), whilst MUPs are important for scent communication (including individual recognition; Cheetham *et al.*, 2007). Given the selection pressures they will have been subjected to during the evolution of the immune system and other aspects of scent communication, it seems unlikely that either MHC or MUP could also have evolved as recognition alleles that code for the production of a signal, a recognition and a behavioural response.

7.4 Multiple Mechanisms across Animal Species

For a behavioural trait to have evolved a selective force is required. Benefits from inclusive fitness or costs of inbreeding are two such evolutionary pressures for the evolution of kin recognition, but they are significantly different from each other. Kin recognition for the purposes of nest partner choice or the purposes of inbreeding avoidance will therefore have had different selection pressures, potentially occurring at separate times during the evolution of a species. Whilst the same mechanism may operate for both purposes, it is not entirely unreasonable to suggest that two different mechanisms may be employed, such as match-to-maternal-cues and match-to-self cues in house mice (Chapters 3 and 6). The possibility of multiple kin recognition mechanisms within a single species suggests that it is essential to consider the context of recognition. Studies that differ in their results for a single species

should consider the contexts of their different results. Likewise, studies suggesting that a particular species displays no kin recognition abilities in one context should be aware of other contexts where recognising kin may be beneficial. This thesis has looked at two separate contexts of kin recognition from the perspective of females only. Whilst it seems unlikely that a third mechanism for recognising kin may be present within a single species there is no reason why this could not be the case and male mate choice and male – male interactions and parent-offspring contexts of kin recognition should be further investigated.

The life history of house mice means that females are likely to encounter unfamiliar relatives, both male and female. Since house mice reach sexual maturity rapidly and are capable of having multiple litters in quick succession, populations can quickly reach a high density if the conditions are suitable. Whilst males are the primary dispersers, females will also disperse under the right conditions and are likely to encounter unfamiliar relatives of both sexes. The ability to identify unfamiliar relatives therefore has evolutionary benefits as male relatives can be avoided as mates and female relatives can bring benefits as nest partners.

Other species, however, have different degrees of dispersal. In Belding's ground squirrels (*Spermophilus beldingi*) males disperse but females remain in or close to their natal area. The requirements for kin recognition are therefore different between the sexes. Females are unlikely to encounter many male relatives after male dispersal but will encounter female relatives from one year to the next, whilst dispersing males are unlikely to encounter related males or females. This leads to the prediction that females will evolve to recognise female relatives but not male relatives, whereas males may show little indication of kin recognition. Accordingly, related female-female pairs show fewer agonistic behaviours than unrelated female pairs whilst there is no difference in agonistic behaviours between female-male pairs or male-male pairs (Holmes & Sherman, 1982). This suggests that females use kin recognition to alter behaviour towards related and unrelated females, but it doesn't necessarily mean that females are unable to recognise male relatives or that males are unable to recognise female or male relatives. As different species have diverse requirements and life-history traits, predictions for kin recognition and kin discriminative behaviour are likely to vary.

Life-history traits could also influence the mechanism of kin recognition used. Species that do not disperse or have relatives that are reliably encountered in a particular area may use either spatial location or a mechanism based on spatial location. Prior association may be

more useful in species that are unlikely to meet novel relatives but regularly interact with relatives encountered in the nest. Species that are likely to encounter unfamiliar relatives in later life may use a phenotype matching mechanism to allow individuals to recognise novel kin. It would be interesting to perform a comparative analysis to investigate the interaction between life-history traits such as dispersal and kin recognition mechanism. This may produce a better understanding of the link between life history and kin recognition mechanisms and could allow predictions to be made about species where no direct studies of kin recognition have been performed.

7.5 Molecular Markers used for Kin Recognition

The cues used for kin recognition should be genetically determined and polymorphic enough that close relatives are likely to share markers but unrelated individuals are unlikely to match. Ideally they should also be consistent so that they do not change or alter dramatically with response to environmental factors such as diet or changes in physiology such as those that are associated with age. MHC and more recently MUPs have been suggested as possible markers for kin recognition. MHC is more widespread, appearing in all vertebrates (Edwards & Hedrick, 1998), whilst current knowledge about MUPs suggests that mice and rats have the greatest degree of MUP variation (Logan *et al.*, 2008). In the kin recognition literature MHC is frequently the only marker examined and assumptions are made that because a species appears to discriminate on the basis of MHC, that MHC must therefore be responsible for kin recognition. The results of Chapters 4 and 6 suggest that it could be limiting to think like this. During female – female nest partner decisions both MHC and MUP were found to influence female partner choice. Whilst no significant avoidance of males based on MHC or MUP type was observed, the trend for females to spend more time in the MUP dissimilar male cage (but not the MHC dissimilar male cage) suggests, along with evidence from Sherborne *et al.* (2007), that it is MUP and not MHC that is central for female inbreeding avoidance. This highlights the importance of investigating other markers of kin recognition as well as MHC.

MHC was found to correlate with ESP, as females that had a particular MHC type also had a corresponding ESP type (Chapter 4). Whilst the limited number of microsatellite markers used to type for ESP could have hidden the full diversity of ESP, the proximity of ESP to MHC on chromosome 17 suggests the possibility that MHC and ESP are inherited together. It is crucial to discover whether this in fact is the case. Additional microsatellite markers could reveal whether the number of haplotypes observed here is an accurate representation of the

actual number of haplotypes present. Studies of house mice from different populations would also confirm whether MHC and ESP are linked. It would be interesting to disentangle the roles of MHC and ESP further. The observed possible linkage of MHC and ESP here stresses the importance of investigating other potential markers as well as MHC during studies of kin recognition. One way to investigate this without relying on fortuitous breeding to produce suitable subject and stimulus animals may be to directly manipulate the markers themselves.

7.6 The Importance of Being Related

The research presented throughout this thesis has focused mainly on the mechanisms of how individuals recognise their relatives. As the results of Chapter 6 can attest to however, there is more to life than relatedness. During experiments presented within that chapter, females appeared to prefer males on the basis of their original population, regardless of relatedness. Additionally throughout this thesis, whilst it has often been demonstrated that the majority of females preferred to nest with their female relatives (or avoid male relatives), a small proportion of females have instead nested with unrelated females (or preferred related males). These results serve as an important illustration that whilst relatedness is important, it is often one of a number of different factors that can contribute in mate and nest partner choice decisions.

In Chapter 2 I showed that females preferred to nest with full sisters over unrelated females even after living with both for several months, suggesting that relatedness takes priority over familiarity. However, this does not mean that familiarity is not an important process integral to female interactions. The tests documented in Chapters 2, 3, 4 and 6 were designed to look at female discrimination and preference when familiarity was kept constant. In Chapter 5, however, the difference in female physiological response was compared when relatedness was constant and familiarity varied. As the results showed, there was a difference in response, highlighting the importance of familiarity when relatedness is constant. The tests in Chapter 2 examined two sides of a relatedness-familiarity square – do females nest with related or unrelated females when either familiar with or unfamiliar with both (Figure 7.1)? The other sides of the square are just as important, however, for understanding female behaviour. It would be interesting to discover if females prefer familiar or unfamiliar nest partners when relatedness is held constant, and to complete the square by investigating the interaction between familiarity and relatedness (Figure 7.1).

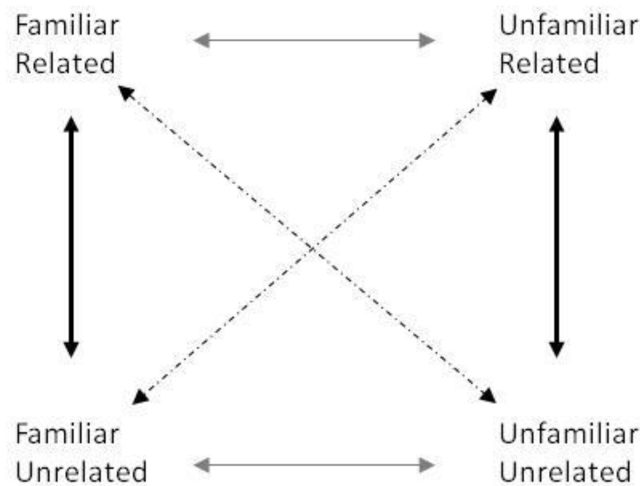


Figure 7.1: Relatedness and familiarity form a square representing possible test pair combinations.
Chapter 2 varied relatedness when subject females were familiar or unfamiliar with the stimulus females (black arrows) and found that subject females associated with related stimulus females whether familiar or unfamiliar with both. The importance of familiarity could be assessed by keeping relatedness constant but varying familiarity (grey arrows). The interaction between relatedness and familiarity could then be investigated by varying both (dashed arrows).

Other factors are also likely to play a role in female mate and social partner choice including dominance, age, weight, reproductive experience and aggression (Rusu *et al.*, 2004; Lemaitre *et al.*, 2012). Whilst it was important in the studies presented here to keep these other factors constant in order to focus on the mechanics of kin recognition, in natural situations females are unlikely to be presented with two females matched for everything except relatedness (or particular markers). It would be interesting to try and understand how these different factors interact, whether there is a hierarchy of importance and whether certain traits take priority over others. It is likely that different species will have different priorities. A series of small enclosure based studies presenting females with a controlled range of different possible nestmates may help to tease apart the different factors more fully, females could be selected to vary for more than one trait and nesting aggregations measured.

When studying kin recognition it is important to remember that the theoretical benefits of kin discrimination may not always be high enough to outweigh any costs. For example, theoretically it may be advantageous for relatives to aggregate but as a result of close kin grouping, competition between relatives for resources may increase, negating the benefits of kin association (Griffin & West, 2002). It is important then not to assume that the theoretical

benefits of kin biased behaviour will have resulted in the evolution of a kin recognition mechanism.

7.7 Concluding Remarks

The importance of kinship and relatedness is apparent throughout human society and culture, from the tragedy of Oedipus (Sophocles, c. 429 BC) to the comedy of errors surrounding Antipholus and Dromio (Shakespeare, c. 1594). Kin biased behaviour has been demonstrated in a wide range of non-human species, from single celled organisms to vertebrates and there are often a number of different reasons why an ability to recognise kin might have evolved, often within the same species. It has been almost 50 years since Hamilton published his explanation of inclusive fitness. The field of kin recognition remains an exciting area of evolutionary and behavioural biology and it is important to understand why and how mechanisms to identify kin have evolved.

This thesis has attempted to progress knowledge of the mechanisms involved in kin recognition, by studying two contexts where kin recognition may be important in the female house mouse. A number of questions remain however and these have been discussed in this and previous chapters. The possibility of a single species using two separate mechanisms suggests that kin recognition may be considerably more complex than previously thought. A combined molecular and behavioural approach, as demonstrated here, is therefore important for understanding the different aspects of kin recognition.

Literature Cited

Akre, A. K., Bakken, M., Hovland, A. L., Palme, R. & Mason, G. (2011). Clustered environmental enrichments induce more aggression and stereotypic behaviour than do dispersed enrichments in female mice. *Applied Animal Behaviour Science*. **131** (3-4), 145-152.

Alberts, S. C. & Altmann, J. (1995). Balancing costs and opportunities - dispersal in male baboons. *American Naturalist*. **145** (2), 279-306.

Aldhous, P. (1989). The effects of individual cross-fostering on the development of intrasexual kin discrimination in male laboratory mice, *Mus musculus* L. *Animal Behaviour*. **37** (5), 741-750.

Andrews, P. W. & Boyse, E. A. (1978). Mapping of an H-2-linked gene that influences mating preference in mice. *Immunogenetics*. **6** (3), 265-268.

Archie, E. A., Hollister-Smith, J. A., Poole, J. H., Lee, P. C., Moss, C. J., Maldonado, J. E., Fleischer, R. C. & Alberts, S. C. (2007). Behavioural inbreeding avoidance in wild African elephants. *Molecular Ecology*. **16** (19), 4138-4148.

Arndt, S. S., Laarakker, M. C., van Lith, H. A., van der Staay, F. J., Gieling, E., Salomons, A. R., van't Klooster, J. & Ohl, F. (2009). Individual housing of mice - impact on behaviour and stress responses. *Physiology & Behavior*. **97** (3-4), 385-393.

Bahr, A., Sommer, S., Mattle, B. & Wilson, A. B. (2012). Mutual mate choice in the potbellied seahorse (*Hippocampus abdominalis*). *Behavioral Ecology*. **23** (4), 869-878.

Baratti, M., Dessi-Fulgheri, F., Ambrosini, R., Bonisoli-Alquati, A., Caprioli, M., Goti, E., Matteo, A., Monnanni, R., Ragionieri, L., Ristori, E., Romano, M., Rubolini, D., Scialpi, A. & Saino, N. (2012). MHC genotype predicts mate choice in the ring-necked pheasant *Phasianus colchicus*. *Journal of Evolutionary Biology*. **25** (8), 1531-1542.

Barnard, C. (1991). Kinship and social-behavior - the trouble with relatives. *Trends in Ecology & Evolution*. **6** (10), 310-312.

Barnard, C. (1999). Still having trouble with relatives. *Trends in Ecology & Evolution*. **14** (11), 448-448.

Barnard, C. (2004). *Animal behaviour: mechanism, development, function and evolution*. Harlow, Pearson Education Limited.

Barnard, C. J. & Fitzsimons, J. (1989). Kin recognition and mate choice in mice - fitness consequences of mating with kin. *Animal Behaviour*. **38**, 35-40.

Barnard, C. J., Hurst, J. L. & Aldhous, P. (1991). Of mice and kin - the functional significance of kin bias in social behavior. *Biological Reviews of the Cambridge Philosophical Society*. **66** (4), 379-430.

Bartolomei, M. S. & Tilghman, S. M. (1997). Genomic imprinting in mammals. *Annual Review of Genetics*. **31**, 493-525.

Bartolomucci, A., Gioiosa, L., Chirieleison, A., Ceresini, G., Parmigiani, S. & Palanza, P. (2004). Cross fostering in mice: behavioral and physiological carry-over effects in adulthood. *Genes Brain and Behavior*. **3** (2), 115-122.

Bartolomucci, A., Parmigiani, S., Gioiosa, L., Ceresini, G. & Palanza, P. (2009). Effects of housing social context on emotional behaviour and physiological responses in female mice. *Scandinavian Journal of Laboratory Animal Science*. **36** (1), 87-95.

Bateson, P. (1979). How do sensitive periods arise and what are they for. *Animal Behaviour*. **27**, 470-486.

Bateson, P. (1982). Preferences for cousins in Japanese quail. *Nature*. **295**, 236-237.

Bateson, P. (1983). Optimal outbreeding. *Mate Choice*. P. Bateson. Cambridge, Cambridge University Press**:** 257-277.

Baudoin, C., Busquet, N., Dobson, F. S., Gheusi, G., Feron, C., Durand, J. L., Heth, G., Patris, B. & Todrank, J. (2005). Male-female associations and female olfactory neurogenesis with pair bonding in *Mus spicilegus*. *Biological Journal of the Linnean Society*. **84** (3), 323-334.

Beauchamp, G. K. & Yamazaki, K. (2003). Chemical signalling in mice. *Biochemical Society Transactions*. **31**, 147-151.

Beauchamp, G. K., Yamazaki, K., Bard, J. & Boyse, E. A. (1988). Preweaning experience in the control of mating preferences by genes in the major histocompatibility complex of the mouse. *Behavior Genetics*. **18** (4), 537-547.

Berger, P. J., Negus, N. C. & Day, M. (1997). Recognition of kin and avoidance of inbreeding in the montane vole, *Microtus montanus*. *Journal of Mammalogy*. **78** (4), 1182-1186.

Bernatchez, L. & Landry, C. (2003). MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? *Journal of Evolutionary Biology*. **16** (3), 363-377.

Berry, R. J. (1981). Population dynamics of the house mouse. *Symposia of the Zoological Society of London*. **47**, 395-425.

Berry, R. J. & Bronson, F. H. (1992). Life-history and bioeconomy of the house mouse. *Biological Reviews*. **67** (4), 519-550.

Berry, R. J. & Jakobson, M. E. (1971). Life and death in an island population of the house mouse. *Experimental Gerontology*. **6** (2), 187-197.

Beynon, R. J. & Hurst, J. L. (2003). Multiple roles of major urinary proteins in the house mouse, *Mus domesticus*. *Biochemical Society Transactions*. **31**, 142-146.

Beynon, R. J. & Hurst, J. L. (2004). Urinary proteins and the modulation of chemical scents in mice and rats. *Peptides*. **25** (9), 1553-1563.

Beynon, R. J., Hurst, J. L., Mirton, M. J., Robertson, D. H. L., Armstrong, S. D., Cheetham, S. A., Simpson, D., MacNicoll, A. & Humphries, R. E. (2008). Urinary lipocalins in rodenta: is there a generic model? *Chemical Signals in Vertebrates 11*. **11**, 37-49.

Beynon, R. J., Veggerby, C., Payne, C. E., Robertson, D. H. L., Gaskell, S. J., Humphries, R. E. & Hurst, J. L. (2002). Polymorphism in major urinary proteins: molecular heterogeneity in a wild mouse population. *Journal of Chemical Ecology*. **28** (7), 1429-1446.

Biedrzycki, M. L. & Bais, H. P. (2010). Kin recognition in plants: a mysterious behaviour unsolved. *Journal of Experimental Botany*. **61** (15), 4123-4128.

Biernaskie, J. M. (2011). Evidence for competition and cooperation among climbing plants. *Proceedings of the Royal Society B-Biological Sciences*. **278**, 1989-1996.

Blaustein, A. R. (1983). Kin recognition mechanisms - phenotypic matching or recognition alleles. *American Naturalist*. **121** (5), 749-754.

Blaustein, A. R., Bekoff, M., Byers, J. A. & Daniels, T. J. (1991). Kin recognition in vertebrates - what do we really know about adaptive value. *Animal Behaviour*. **41**, 1079-1083.

Blaustein, A. R. & O'Hara, R. K. (1982). Kin recognition in *Rana cascadae* tadpoles: maternal and paternal effects. *Animal Behaviour*. **30** (4), 1151-1157.

Boehm, T. (2006). Co-evolution of a primordial peptide-presentation system and cellular immunity. *Nature Reviews Immunology*. **6** (1), 79-84.

Bolhuis, J. J. (1999). The development of animal behavior: from Lorenz to neural nets. *Naturwissenschaften*. **86** (3), 101-111.

Bolhuis, J. J., Strijkstra, A. M., Moor, E. & Vanderlende, K. (1988). Preferences for odors of conspecific non-siblings in the common vole, *Microtus-arvalis*. *Animal Behaviour*. **36**, 1551-1553.

Bowers, J. M. & Alexander, B. K. (1967). Mice - individual recognition by olfactory cues. *Science*. **158**, 1208-1210.

Boyd, S. K. & Blaustein, A. R. (1985). Familiarity and inbreeding avoidance in the gray-tailed vole (*Microtus canicaudus*). *Journal of Mammalogy*. **66** (2), 348-352.

Boyse, E. A., Beauchamp, G. K. & Yamazaki, K. (1987). The genetics of body scent. *Trends in Genetics*. **3** (4), 97-102.

Brandvain, Y., Van Cleve, J., Ubeda, F. & Wilkins, J. F. (2011). Demography, kinship, and the evolving theory of genomic imprinting. *Trends in Genetics*. **27** (7), 251-257.

Bronson, F. H. (1979). Reproductive ecology of the house mouse. *Quarterly Review of Biology*. **54** (3), 265-299.

Broom, D. M. & Johnson, K. G. (1993). *Stress and animal welfare*. The Netherlands, Kluwer Academic Publishers.

Brown, G. E. & Brown, J. A. (1993). Do kin always make better neighbors - the effects of territory quality. *Behavioral Ecology and Sociobiology*. **33** (4), 225-231.

Brown, G. E., Brown, J. A. & Wilson, W. R. (1996). The effects of kinship on the growth of juvenile Arctic charr. *Journal of Fish Biology*. **48** (3), 313-320.

Brown, J. L. (1997). A theory of mate choice based on heterozygosity. *Behavioral Ecology*. **8** (1), 60-65.

Brown, J. L. & Eklund, A. (1994). Kin recognition and the major histocompatibility complex - an integrative review. *American Naturalist*. **143** (3), 435-461.

Brown, R. E. (1995). What is the role of the immune-system in determining individually distinct body odors. *International Journal of Immunopharmacology*. **17** (8), 655-661.

Burt, A. & Trivers, R. (1998). Genetic conflicts in genomic imprinting. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **265**, 2393-2397.

Busquet, N. & Baudoin, C. (2005). Odour similarities as a basis for discriminating degrees of kinship in rodents: evidence from *Mus spicilegus*. *Animal Behaviour*. **70**, 997-1002.

Byers, J. A. & Bekoff, M. (1986). What does kin recognition mean. *Ethology*. **72** (4), 342-345.

Byers, J. A. & Bekoff, M. (1991). Development, the conveniently forgotten variable in true kin recognition. *Animal Behaviour*. **41**, 1088-1090.

Caligioni, C. S. (2010). Assessing reproductive status/stages in mice. *Current Protocols in Neuroscience*, John Wiley & Sons, Inc.

Carroll, L. S., Penn, D. J. & Potts, W. K. (2002). Discrimination of MHC-derived odors by untrained mice is consistent with divergence in peptide-binding region residues. *Proceedings of the National Academy of Sciences of the United States of America*. **99** (4), 2187-2192.

Carter, C. S. & Marr, J. N. (1970). Olfactory imprinting and age variables in Guinea-Pig, *Cavia porcellus*. *Animal Behaviour*. **18** (2), 238-&.

Cavaggioni, A., Mucignat, C. & Tirindelli, R. (1999). Pheromone signalling in the mouse: Role of urinary proteins and vomeronasal organ. *Archives Italiennes De Biologie*. **137** (2-3), 193-200.

Celerier, A., Huchard, E., Alvergne, A., Fejan, D., Plard, F., Cowlishaw, G., Raymond, M., Knapp, L. A. & Bonadonna, F. (2010). Detective mice assess relatedness in baboons using olfactory cues. *Journal of Experimental Biology*. **213** (9), 1399-1405.

Charlesworth, D. & Willis, J. H. (2009). Fundamental concepts in genetics: the genetics of inbreeding depression. *Nature Reviews Genetics*. **10** (11), 783-796.

Charpentier, M. J. E., Crawford, J. C., Boulet, M. & Drea, C. M. (2010). Message 'scent': lemurs detect the genetic relatedness and quality of conspecifics via olfactory cues. *Animal Behaviour*. **80** (1), 101-108.

Cheetham, S. A., Smith, A. L., Armstrong, S. D., Beynon, R. J. & Hurst, J. L. (2009). Limited variation in the major urinary proteins of laboratory mice. *Physiology & Behavior*. **96** (2), 253-261.

Cheetham, S. A., Thom, M. D., Beynon, R. J. & Hurst, J. L. (2008). The effect of familiarity on mate choice. *Chemical Signals in Vertebrates 11*. **11**, 271-280.

Cheetham, S. A., Thom, M. D., Jury, F., Ollier, W. E. R., Beynon, R. J. & Hurst, J. L. (2007). The genetic basis of individual-recognition signals in the mouse. *Current Biology*. **17** (20), 1771-1777.

Chen, X. F. (1993). Comparison of inbreeding and outbreeding in hermaphroditic *Arianta arbustorum* (l) (land snail). *Heredity*. **71**, 456-461.

Cheney, D. L. & Seyfarth, R. M. (1986). The recognition of social alliances by vervet monkeys. *Animal Behaviour*. **34**, 1722-1731.

Chitty, D. & Kempson, D. A. (1949). Prebaiting small mammals and a new design of live trap. *Ecology*. **30** (4), 536-542.

Clark, R. W., Brown, W. S., Stechert, R. & Greene, H. W. (2012). Cryptic sociality in rattlesnakes (*Crotalus horridus*) detected by kinship analysis. *Biology Letters*. **8** (4), 523-525.

Clutton-Brock, T. & McAuliffe, K. (2009). Female mate choice in mammals. *Quarterly Review of Biology*. **84** (1), 3-27.

Clutton-Brock, T. H. & Lukas, D. (2012). The evolution of social philopatry and dispersal in female mammals. *Molecular Ecology*. **21** (3), 472-492.

Creel, S. (2001). Social dominance and stress hormones. *Trends in Ecology & Evolution*. **16** (9), 491-497.

Crowcroft, P. & Rowe, F. P. (1963). Social organization and territorial behaviour in the house mouse. *Proceedings of the Zoological Society of London*. **140** (3), 517-531.

Cucchi, T., Vigne, J. D., Auffray, J. C., Croft, P. & Peltenburg, E. (2002). Passive transport of the house mouse (*Mus musculus domesticus*) to Cyprus at the Early Preceramic Neolithic (late 9th and 8th millennia cal. BC). *Comptes Rendus Palevol*. **1** (4), 235-241.

D'Amato, F. R. (1993). Effect of familiarity with the mother and kinship on infanticidal and alloparental behavior in virgin house mice. *Behaviour*. **124**, 313-326.

D'Amato, F. R. (1994). Physiological evidence for genetically mediated sibling recognition in mice. *Behavior Genetics*. **24** (6), 493-496.

D'Amato, F. R. (1997). Neurobiological and behavioral aspects of recognition in female mice. *Physiology & Behavior*. **62** (6), 1311-1317.

D'Udine, B. & Partridge, L. (1981). Olfactory preferences of inbred mice (*Mus musculus*) for their own strain and for siblings - effects of strain, sex and cross-fostering. *Behaviour*. **78**, 314-324.

Davis, L. S. (1984). Alarm calling in richardsons ground-squirrels (*Spermophilus richardsonii*). *Zeitschrift Fur Tierpsychologie-Journal of Comparative Ethology*. **66** (2), 152-164.

Dawkins, R. (1976). *The selfish gene*. Oxford, Oxford University Press.

Dawkins, R. (1982). *The extended phenotype*. Oxford, Oxford University Press.

delBarco-Trillo, J., McPhee, M. E. & Johnston, R. E. (2009). Nonagonistic familiarity decreases aggression in male Turkish hamsters, *Mesocricetus brandti*. *Animal Behaviour*. **77** (2), 389-393.

Desjardins, C., Maruniak, J. A. & Bronson, F. H. (1973). Social rank in house mice - differentiation revealed by ultraviolet visualization of urinary marking patterns. *Science*. **182**, 939-941.

Dewsbury, D. A. (1988). Kinship, familiarity, aggression, and dominance in deer mice (*Peromyscus maniculatus*) in seminatural enclosures. *Journal of Comparative Psychology*. **102** (2), 124-128.

Diggle, S. P., Griffin, A. S., Campbell, G. S. & West, S. A. (2007). Cooperation and conflict in quorum-sensing bacterial populations. *Nature*. **450**, 411-417.

Dobson, F. S. & Baudoin, C. (2002). Experimental tests of spatial association and kinship in monogamous mice (*Mus spicilegus*) and polygynous mice (*Mus musculus domesticus*). *Canadian Journal of Zoology-Revue Canadienne De Zoologie*. **80** (6), 980-986.

Dobson, F. S., Jacquot, C. & Baudoin, C. (2000). An experimental test of kin association in the house mouse. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*. **78** (10), 1806-1812.

Drewett, R. F. (1973). Oestrous and dioestrous components of the ovarian inhibition on hunger in the rat. *Animal Behaviour*. **21** (4), 772-780.

Drickamer, L. C., Gowaty, P. A. & Holmes, C. M. (2000). Free female mate choice in house mice affects reproductive success and offspring viability and performance. *Animal Behaviour*. **59**, 371-378.

Dudley, S. A. & File, A. L. (2007). Kin recognition in an annual plant. *Biology Letters*. **3** (4), 435-438.

Edwards, S. V. & Hedrick, P. W. (1998). Evolution and ecology of MHC molecules: from genomics to sexual selection. *Trends in Ecology & Evolution*. **13** (8), 305-311.

Egid, K. & Brown, J. L. (1989). The major histocompatibility complex and female mating preferences in mice. *Animal Behaviour*. **38**, 548-550.

Ehman, K. D. & Scott, M. E. (2001). Urinary odour preferences of MHC congenic female mice, *Mus domesticus*: implications for kin recognition and detection of parasitized males. *Animal Behaviour*. **62**, 781-789.

Eklund, A. (1997a). The effect of early experience on MHC-based mate preferences in two B10.W strains of mice (*Mus domesticus*). *Behavior Genetics*. **27** (3), 223-229.

Eklund, A. (1997b). The major histocompatibility complex and mating preferences in wild house mice (*Mus domesticus*). *Behavioral Ecology*. **8** (6), 630-634.

Eklund, A., Egid, K. & Brown, J. L. (1991). The major histocompatibility complex and mating preferences of male mice. *Animal Behaviour*. **42**, 693-694.

Eklund, A. C. (1998). Use of the MHC for mate choice in wild house mice (*Mus domesticus*). *Genetica*. **104** (3), 245-248.

Evans, T. A. (1999). Kin recognition in a social spider. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **266**, 287-292.

Fadao, T., Tingzheng, W. & Yajun, Z. (2000). Inbreeding avoidance and mate choice in the mandarin vole (*Microtus mandarinus*). *Canadian Journal of Zoology-Revue Canadienne De Zoologie*. **78** (12), 2119-2125.

Ferkin, M. H. (1988). The effect of familiarity on social interactions in meadow voles, *Microtus pennsylvanicus*: a laboratory and field study. *Animal Behaviour*. **36** (6), 1816-1822.

Ferkin, M. H. & Rutka, T. F. (1990). Mechanisms of sibling recognition in meadow voles. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*. **68** (3), 609-613.

Flower, D. R. (1996). The lipocalin protein family: structure and function. *Biochemical Journal*. **318**, 1-14.

Freeman-Gallant, C. R., Meguerdichian, M., Wheelwright, N. T. & Sollecito, S. V. (2003). Social pairing and female mating fidelity predicted by restriction fragment length polymorphism similarity at the major histocompatibility complex in a songbird. *Molecular Ecology*. **12** (11), 3077-3083.

Garratt, M. (2010). Oxidative stress, reproductive investment and sexual signalling in house mice. PhD, University of Liverpool.

Garratt, M., Vasilaki, A., Stockley, P., McArdle, F., Jackson, M. & Hurst, J. L. (2011). Is oxidative stress a physiological cost of reproduction? An experimental test in house mice. *Proceedings of the Royal Society B-Biological Sciences*. **278**, 1098-1106.

Garza, J. C., Dallas, J., Duryadi, D., Gerasimov, S., Croset, H. & Boursot, P. (1997). Social structure of the mound-building mouse *Mus spicilegus* revealed by genetic analysis with microsatellites. *Molecular Ecology*. **6** (11), 1009-1017.

Gavish, L., Hofmann, J. E. & Getz, L. L. (1984). Sibling recognition in the prairie vole, *Microtus ochrogaster*. *Animal Behaviour*. **32** (2), 362-366.

Geffen, E., Kam, M., Hefner, R., Hersteinsson, P., Angerbjorn, A., Dalen, L., Fuglei, E., Noren, K., Adams, J. R., Vucetich, J., Meier, T. J., Mech, L. D., Vonholdt, B. M., Stahler, D. R. & Wayne, R. K. (2011). Kin encounter rate and inbreeding avoidance in canids. *Molecular Ecology*. **20** (24), 5348-5358.

Gemmell, N. J. (2003). Kin selection may influence fostering behaviour in Antarctic fur seals (*Arctocephalus gazella*). *Proceedings of the Royal Society B-Biological Sciences*. **270**, 2033-2037.

Gerlach, G. (1996). Emigration mechanisms in feral house mice - a laboratory investigation of the influence of social structure, population density, and aggression. *Behavioral Ecology and Sociobiology*. **39** (3), 159-170.

Gerlach, G., Hodgins-Davis, A., Avolio, C. & Schunter, C. (2008). Kin recognition in zebrafish: a 24-hour window for olfactory imprinting. *Proceedings of the Royal Society B-Biological Sciences*. **275**, 2165-2170.

Gerlach, G. & Lysiak, N. (2006). Kin recognition and inbreeding avoidance in zebrafish, *Danio rerio*, is based on phenotype matching. *Animal Behaviour*. **71**, 1371-1377.

Gosling, L. M., Roberts, S. C., Thornton, E. A. & Andrew, M. J. (2000). Life history costs of olfactory status signalling in mice. *Behavioral Ecology and Sociobiology*. **48** (4), 328-332.

Grafen, A. (1990). Do animals really recognize kin. *Animal Behaviour*. **39**, 42-54.

Grafen, A. (1991a). Development, the conveniently forgotten variable in true kin recognition - reply. *Animal Behaviour*. **41**, 1091-1092.

Grafen, A. (1991b). Kin recognition in vertebrates - what do we really know about adaptive value - reply. *Animal Behaviour*. **41**, 1085-1087.

Grafen, A. (1991c). Kin vision - reply. *Animal Behaviour*. **41**, 1095-1096.

Grafen, A. (1992). Behavioral ecology - of mice and the MHC. *Nature*. **360**, 530-530.

Grau, H. J. (1982). Kin recognition in white-footed deermice (*Peromyscus leucopus*). *Animal Behaviour*. **30** (2), 497-505.

Greenwood, P. J. (1980). Mating systems, philopatry and dispersal in birds and mammals. *Animal Behaviour*. **28**, 1140-1162.

Griffin, A. S. & West, S. A. (2002). Kin selection: fact and fiction. *Trends in Ecology & Evolution*. **17** (1), 15-21.

Griffin, A. S., West, S. A. & Buckling, A. (2004). Cooperation and competition in pathogenic bacteria. *Nature*. **430**, 1024-1027.

Griffith, S. C., Pryke, S. R. & Buttemer, W. A. (2011). Constrained mate choice in social monogamy and the stress of having an unattractive partner. *Proc Biol Sci*. **278**, 2798-2805.

Griffiths, S. W. & Magurran, A. E. (1997). Familiarity in schooling fish: How long does it take to acquire? *Animal Behaviour*. **53**, 945-949.

Grosberg, R. K. & Quinn, J. F. (1986). The genetic-control and consequences of kin recognition by the larvae of a colonial marine invertebrate. *Nature*. **322**, 456-459.

Gubernick, D. J. (1980). Maternal 'imprinting' or maternal 'labelling' in goats? *Animal Behaviour*. **28** (1), 124-129.

Haga, S., Hattori, T., Sato, T., Sato, K., Matsuda, S., Kobayakawa, R., Sakano, H., Yoshihara, Y., Kikusui, T. & Touhara, K. (2010). The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature*. **466**, 118-136.

Hagemeyer, P. & Begall, S. (2006). Individual odour similarity and discrimination in the coruro (*Spalacopus cyanus*, Octodontidae). *Ethology*. **112** (6), 529-536.

Hager, R., Cheverud, J. M. & Wolf, J. B. (2009). Change in maternal environment induced by cross-fostering alters genetic and epigenetic effects on complex traits in mice. *Proceedings of the Royal Society B-Biological Sciences*. **276**, 2949-2954.

Hager, R. & Johnstone, R. A. (2005). Differential growth of own and alien pups in mixed litters of mice: a role for genomic imprinting? *Ethology*. **111** (8), 705-714.

Haig, D. (2000a). Genomic imprinting, sex-biased dispersal, and social behavior. *Annals of the New York Academy of Sciences; Evolutionary perspectives on human reproductive behavior*, New York Academy of Sciences, New York, USA. **907:** 149-163.

Haig, D. (2000b). The kinship theory of genomic imprinting. *Annual Review of Ecology and Systematics*. **31**, 9-32.

Haig, D. (2004). Genomic imprinting and kinship: how good is the evidence? *Annual Review of Genetics*. **38**, 553-585.

Halliday, T. R. (1983). The study of mate choice. *Mate choice*. P. Bateson. Cambridge, Cambridge University Press**:** 3-33.

Halpin, Z. T. (1980). Individual odors and individual recognition - review and commentary. *Biology of Behaviour*. **5** (3), 233-248.

Halpin, Z. T. (1991). Kin recognition cues of vertebrates. *Kin Recognition*. P. G. Hepper. Cambridge, Cambridge University Press**:** 220-258.

Hamilton, W. D. (1963). Evolution of altruistic behavior. *American Naturalist*. **97**, 354-356.

Hamilton, W. D. (1964a). Genetical evolution of social behaviour I. *Journal of Theoretical Biology*. **7** (1), 1-16.

Hamilton, W. D. (1964b). Genetical evolution of social behaviour II. *Journal of Theoretical Biology*. **7** (1), 17-52.

Hare, J. F. (1992). Colony member discrimination by juvenile columbian ground-squirrels (*Spermophilus columbianus*). *Ethology*. **92** (4), 301-315.

Hare, J. F. & Murie, J. O. (1996). Ground squirrel sociality and the quest for the "holy grail": does kinship influence behavioral discrimination by juvenile Columbian ground squirrels. *Behavioral Ecology*. **7** (1), 76-81.

Hare, J. F., Sealy, S. G., Underwood, T. J., Ellison, K. S. & Stewart, R. L. M. (2003). Evidence of self-referent phenotype matching revisited: airing out the armpit effect. *Animal Cognition*. **6** (1), 65-68.

Harper, J. M. (2008). Wild-derived mouse stocks: an underappreciated tool for aging research. *Age*. **30** (2-3), 135-145.

Hauber, M. E. & Sherman, P. W. (2001). Self-referent phenotype matching: theoretical considerations and empirical evidence. *Trends in Neurosciences*. **24** (10), 609-616.

Hayashi, S. & Kimura, T. (1983). Degree of kinship as a factor regulating preferences among conspecifics in mice. *Animal Behaviour*. **31**, 81-85.

Hayes, L. D. (2000). To nest communally or not to nest communally: a review of rodent communal nesting and nursing. *Animal Behaviour*. **59** (4), 677-688.

Hepper, P. G. (1983). Sibling recognition in the rat. *Animal Behaviour*. **31**, 1177-1191.

Hepper, P. G. (1986). Kin recognition - functions and mechanisms a review. *Biological Reviews of the Cambridge Philosophical Society*. **61** (1), 63-93.

Hepper, P. G. (1991). Recognizing kin: ontogeny and classification. *Kin Recognition*. P. G. Hepper. Cambridge, Cambridge University Press**:** 259-288.

Hess, E. H. (1959). Imprinting. *Science*. **130**, 133-141.

Heth, G. & Todrank, J. (2000). Individual odour similarities across species parallel phylogenetic relationships in the *S. ehrenbergi* superspecies of mole-rats. *Animal Behaviour*. **60**, 789-795.

Heth, G., Todrank, J., Busquet, N. & Baudoin, C. (2003). Genetic relatedness assessment through individual odour similarities in mice. *Biological Journal of the Linnean Society*. **78** (4), 595-603.

Heth, G., Todrank, J. & Johnston, R. E. (1998). Kin recognition in golden hamsters: evidence for phenotype matching. *Animal Behaviour*. **56**, 409-417.

Holmes, W. G. (1984). Sibling recognition in 13-lined ground-squirrels - effects of genetic relatedness, rearing association, and olfaction. *Behavioral Ecology and Sociobiology*. **14** (3), 225-233.

Holmes, W. G. (1986). Identification of paternal half-siblings by captive Belding's ground-squirrels. *Animal Behaviour*. **34**, 321-327.

Holmes, W. G. (2004). The early history of Hamiltonian-based research on kin recognition. *Annales Zoologici Fennici*. **41** (6), 691-711.

Holmes, W. G. & Mateo, J. M. (2007). Kin recognition in rodents: issues and evidence. *Rodent Societies: An Ecological & Evolutionary Perspective*. R. J. Wolff & Sherman, P. W. Chicago, University of Chicago Press**:** 216-228.

Holmes, W. G. & Sherman, P. W. (1982). The ontogeny of kin recognition in two species of ground squirrels. *American Zoologist*. **22** (3), 491-517.

Holmes, W. G. & Sherman, P. W. (1983). Kin recognition in animals. *American Scientist*. **71** (1), 46-55.

Howard, J. C. (1977). H-2 and mating preferences. *Nature*. **266**, 406-408.

Humphries, R. E., Robertson, D. H. L., Beynon, R. J. & Hurst, J. L. (1999). Unravelling the chemical basis of competitive scent marking in house mice. *Animal Behaviour*. **58**, 1177-1190.

Hurst, J. L. (1987). Behavioural variation in wild house mice *Mus domesticus Rutty*: a quantitative assessment of female social organization. *Animal Behaviour*. **35** (6), 1846-1857.

Hurst, J. L. (1990a). Urine marking in populations of wild house mice *Mus domesticus rutty* .I. Communication between males. *Animal Behaviour*. **40**, 209-222.

Hurst, J. L. (1990b). Urine marking in populations of wild house mice *Mus domesticus rutty* .II. Communication between females. *Animal Behaviour*. **40**, 223-232.

Hurst, J. L. (2005). Making sense of scents: reducing aggression and uncontrolled variation in laboratory mice. www.nc3rs.org.uk, National Centre for the Replacement, Refinement and Reduction of Animals in Research.

Hurst, J. L. (2009). Female recognition and assessment of males through scent. *Behavioural Brain Research*. **200** (2), 295-303.

Hurst, J. L. & Barnard, C. J. (1992). Kinship and social-behavior in wild house mice - effects of social group membership and relatedness on the responses of dominant males toward juveniles. *Behavioral Ecology*. **3** (3), 196-206.

Hurst, J. L. & Beynon, R. J. (2004). Scent wars: the chemobiology of competitive signalling in mice. *Bioessays*. **26** (12), 1288-1298.

Hurst, J. L., Payne, C. E., Nevison, C. M., Marie, A. D., Humphries, R. E., Robertson, D. H. L., Cavaggioni, A. & Beynon, R. J. (2001). Individual recognition in mice mediated by major urinary proteins. *Nature*. **414**, 631-634.

Hurst, J. L., Robertson, D. H. L., Tolladay, U. & Beynon, R. J. (1998). Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. *Animal Behaviour*. **55**, 1289-1297.

Hurst, J. L. & West, R. S. (2010). Taming anxiety in laboratory mice. *Nat Meth*. **7** (10), 825-826.

Isles, A. R., Baum, M. J., Ma, D., Keverne, E. B. & Allen, N. D. (2001). Genetic imprinting - urinary odour preferences in mice. *Nature*. **409**, 783-784.

Isles, A. R., Baum, M. J., Ma, D., Szeto, A., Keverne, E. B. & Allen, N. D. (2002). A possible role for imprinted genes in inbreeding avoidance and dispersal from the natal area in mice. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **269**, 665-670.

Janotova, K. & Stopka, P. (2011). The level of major urinary proteins is socially regulated in wild *Mus musculus musculus*. *Journal of Chemical Ecology*. **37** (6), 647-656.

Jimenez, J. A., Hughes, K. A., Alaks, G., Graham, L. & Lacy, R. C. (1994). An experimental-study of inbreeding depression in a natural habitat. *Science*. **266**, 271-273.

Johnston, R. E. (2003). Chemical communication in rodents: from pheromones to individual recognition. *Journal of Mammalogy*. **84** (4), 1141-1162.

Jordan, W. C. & Bruford, M. W. (1998). New perspectives on mate choice and the MHC. *Heredity*. **81**, 239-245.

Kareem, A. M. (1983). Effect of increasing periods of familiarity on social interactions between male sibling mice. *Animal Behaviour*. **31** (3), 919-926.

Kareem, A. M. & Barnard, C. J. (1982). The importance of kinship and familiarity in social interactions between mice. *Animal Behaviour*. **30**, 594-601.

Kareem, A. M. & Barnard, C. J. (1986). Kin recognition in mice - age, sex and parental effects. *Animal Behaviour*. **34**, 1814-1824.

Kavaliers, M., Colwell, D. D. & Choleris, E. (2005). Kinship, familiarity and social status modulate social learning about "micropredators" (biting flies) in deer mice. *Behavioral Ecology and Sociobiology*. **58** (1), 60-71.

Keane, B. (1990). The effect of relatedness on reproductive success and mate choice in the white-footed mouse, *Peromyscus leucopus*. *Animal Behaviour*. **39**, 264-273.

Keller, L. & Passera, L. (1993). Incest avoidance, fluctuating asymmetry, and the consequences of inbreeding in *Iridomyrmex humilis*, an ant with multiple queen colonies. *Behavioral Ecology and Sociobiology*. **33** (3), 191-199.

Keller, L. & Ross, K. G. (1998). Selfish genes: a green beard in the red fire ant. *Nature*. **394**, 573-575.

Keller, L. F., Arcese, P., Smith, J. N. M., Hochachka, W. M. & Stearns, S. C. (1994). Selection against inbred song sparrows during a natural population bottleneck. *Nature*. **372**, 356-357.

Kendrick, K. M., Hinton, M. R., Atkins, K., Haupt, M. A. & Skinner, J. D. (1998). Mothers determine sexual preferences. *Nature*. **395**, 229-230.

Kimoto, H., Haga, S., Sato, K. & Touhara, K. (2005). Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature*. **437**, 898-901.

Kimoto, H., Sato, K., Nodari, F., Haga, S., Holy, T. E. & Touhara, K. (2007). Sex- and strain-specific expression and vomeronasal activity of mouse ESP family peptides. *Current Biology*. **17** (21), 1879-1884.

Klein, J. (1979). Major histocompatibility complex of the mouse. *Science*. **203**, 516-521.

Komdeur, J. & Hatchwell, B. J. (1999). Kin recognition: function and mechanism in avian societies. *Trends in Ecology & Evolution*. **14** (6), 237-241.

König, B. (1989). Behavioral ecology of kin recognition in house mice. *Ethology Ecology & Evolution*. **1** (1), 99-110.

König, B. (1993). Maternal investment of communally nursing female house mice (*Mus musculus domesticus*). *Behavioural Processes*. **30** (1), 61-74.

König, B. (1994a). Components of lifetime reproductive success in communally and solitarily nursing house mice - a laboratory study. *Behavioral Ecology and Sociobiology*. **34** (4), 275-283.

König, B. (1994b). Fitness effects of communal rearing in house mice - the role of relatedness versus familiarity. *Animal Behaviour*. **48** (6), 1449-1457.

König, B. & Lindholm, A. K. (2012). The complex social environment of female house mice (*Mus domesticus*). *Evolution of the House Mouse*. M. Macholán, Baird, S. J. E., Munclinge, P. & Piálek, J. Cambridge, Cambridge University Press.

König, B. & Markl, H. (1987). Maternal-care in house mice .1. The weaning strategy as a means for parental manipulation of offspring quality. *Behavioral Ecology and Sociobiology*. **20** (1), 1-9.

Krackow, S. & Matuschak, B. (1991). Mate choice for non-siblings in wild house mice - evidence from a choice test and a reproductive test. *Ethology*. **88** (2), 99-108.

Krause, E. T., Kruger, O., Kohlmeier, P. & Caspers, B. A. (2012). Olfactory kin recognition in a songbird. *Biology Letters*. **8** (3), 327-329.

Le Vin, A. L., Mable, B. K. & Arnold, K. E. (2010). Kin recognition via phenotype matching in a cooperatively breeding cichlid, *Neolamprologus pulcher*. *Animal Behaviour*. **79** (5), 1109-1114.

Lemaitre, J. F., Ramm, S. A., Hurst, J. L. & Stockley, P. (2012). Inbreeding avoidance behaviour of male bank voles in relation to social status. *Animal Behaviour*. **83** (2), 453-457.

Levine, S. & Wiener, S. G. (1988). Psychoendocrine aspects of mother-infant relationships in nonhuman-primates. *Psychoneuroendocrinology*. **13** (1-2), 143-154.

Li, Q. L. & Zhang, L. (2010). Parent-offspring recognition in Brandt's voles, *Lasiopodomys brandti*. *Animal Behaviour*. **79** (4), 797-801.

Liebgold, E. B. & Cabe, P. R. (2008). Familiarity with adults, but not relatedness, affects the growth of juvenile red-backed salamanders (*Plethodon cinereus*). *Behavioral Ecology and Sociobiology*. **63** (2), 277-284.

Ligon, J. D. (1991). Co-operation and reciprocity in birds and mammals. *Kin Recognition*. P. G. Hepper. Cambridge, Cambrudge University Press**:** 30-59.

Lizé, A., Carval, D., Cortesero, A. M., Fournet, S. & Poinsot, D. (2006). Kin discrimination and altruism in the larvae of a solitary insect. *Proceedings of the Royal Society B-Biological Sciences*. **273**, 2381-2386.

Lizé, A., Cortesero, A. M., Atlan, A. & Poinsot, D. (2007). Kin recognition in *Aleochara bilineata* could support the kinship theory of genomic imprinting. *Genetics*. **175** (4), 1735-1740.

Lizé, A., Cortesero, A. M., Bagneres, A.-G. & Poinsot, D. (2010). Kin recognition in the larvae of a solitary insect: the cue is in the plug. *Behavioral Ecology*. **21** (3), 633-638.

Lizé, A., Khidr, S. K. & Hardy, I. C. W. (2012). Two components of kin recognition influence parasitoid aggression in resource competition. *Animal Behaviour*. **83** (3), 793-799.

Lode, T. (2008). Kin recognition versus familiarity in a solitary mustelid, the European polecat *Mustela putorius*. *Comptes Rendus Biologies*. **331** (3), 248-254.

Logan, D. W., Marton, T. F. & Stowers, L. (2008). Species specificity in major urinary proteins by parallel evolution. *Plos One*. **3** (9), e3280.

Lorenz, K. Z. (1937). The companion in the bird's world. *The Auk*. **54** (3), 245-273.

Louch, C. D. & Higginbo.M (1967). Relation between social rank and plasma corticosterone levels in mice. *General and Comparative Endocrinology*. **8** (3), 441-444.

Lucas, J. R., Creel, S. R. & Waser, P. M. (1997). Dynamic optimization and cooperative breeding: an evaluation of future fitness effects. *Cooperative Breeding in Mammals*. N. G. Solomon & French, J. A. New York, Cambridge University Press**:** 171-198.

Lucia, K. E. & Keane, B. (2012). A field test of the effects of familiarity and relatedness on social associations and reproduction in prairie voles. *Behavioral Ecology and Sociobiology*. **66** (1), 13-27.

Mackintosh, J. H. (1981). Behaviour of the house mouse. *Symposia of the Zoological Society of London*. **47**, 337-365.

Maestripieri, D. & Rossiarnaud, C. (1991). Kinship does not affect litter defence in pairs of communally nesting female house mice. *Aggressive Behavior*. **17** (4), 223-228.

Maher, C. R. (2009). Effects of relatedness on social interaction rates in a solitary marmot. *Animal Behaviour*. **78** (4), 925-933.

Manning, C. J., Dewsbury, D. A., Wakeland, E. K. & Potts, W. K. (1995). Communal nesting and communal nursing in-house mice, *Mus musculus domesticus*. *Animal Behaviour*. **50**, 741-751.

Manning, C. J., Wakeland, E. K. & Potts, W. K. (1992). Communal nesting patterns in mice implicate MHC genes in kin recognition. *Nature*. **360**, 581-583.

Markman, S., Hill, N., Todrank, J., Heth, G. & Blaustein, L. (2009). Differential aggressiveness between fire salamander (*Salamandra infraimmaculata*) larvae covaries with their genetic similarity. *Behavioral Ecology and Sociobiology*. **63** (8), 1149-1155.

Marsden, H. M. & Bronson, F. H. (1964). Estrous synchrony in mice - alteration by exposure to male urine. *Science*. **144**, 1469.

Masters, B. S. & Forester, D. C. (1995). Kin recognition in a brooding salamander. *Proceedings of the Royal Society B-Biological Sciences*. **261**, 43-48.

Mateo, J. M. (2003). Kin recognition in ground squirrels and other rodents. *Journal of Mammalogy*. **84** (4), 1163-1181.

Mateo, J. M. (2004). Recognition systems and biological organization: The perception component of social recognition. *Annales Zoologici Fennici*. **41** (6), 729-745.

Mateo, J. M. (2009). The causal role of odours in the development of recognition templates and social preferences. *Animal Behaviour*. **77** (1), 115-121.

Mateo, J. M. (2010). Self-referent phenotype matching and long-term maintenance of kin recognition. *Animal Behaviour*. **80** (5), 929-935.

Mateo, J. M. & Holmes, W. G. (2004). Cross-fostering as a means to study kin recognition. *Animal Behaviour*. **68**, 1451-1459.

Mateo, J. M. & Johnston, R. E. (2000). Kin recognition and the 'armpit effect': evidence of self-referent phenotype matching. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **267**, 695-700.

Mateo, J. M. & Johnston, R. E. (2003). Kin recognition by self-referent phenotype matching: weighing the evidence. *Animal Cognition*. **6** (1), 73-76.

Maynard Smith, J. (1964). Group selection and kin selection. *Nature*. **201**, 1145-1147.

Meagher, S., Penn, D. J. & Potts, W. K. (2000). Male-male competition magnifies inbreeding depression in wild house mice. *Proceedings of the National Academy of Sciences of the United States of America*. **97** (7), 3324-3329.

Mehdiabadi, N. J., Jack, C. N., Farnham, T. T., Platt, T. G., Kalla, S. E., Shaulsky, G., Queller, D. C. & Strassmann, J. E. (2006). Kin preference in a social microbe - given the right circumstances, even an amoeba chooses to be altruistic towards its relatives. *Nature*. **442**, 881-882.

Metzger, M., Bernstein, C., Hoffmeister, T. S. & Desouhant, E. (2010). Does kin recognition and sib-mating avoidance limit the risk of genetic incompatibility in a parasitic wasp? *Plos One*. **5** (10).

Miller, R. A., Harper, J. M., Dysko, R. C., Durkee, S. J. & Austad, S. N. (2002). Longer life spans and delayed maturation in wild-derived mice. *Experimental Biology and Medicine*. **227** (7), 500-508.

Moltz, H. (1960). Imprinting - empirical-basis and theoretical significance. *Psychological Bulletin*. **57** (4), 291-314.

Mudge, J. M., Armstrong, S. D., McLaren, K., Beynon, R. J., Hurst, J. L., Nicholson, C., Robertson, D. H., Wilming, L. G. & Harrow, J. L. (2008). Dynamic instability of the major urinary protein gene family revealed by genomic and phenotypic comparisons between C57 and 129 strain mice. *Genome Biology*. **9** (5).

Munshi, A. & Duvvuri, S. (2007). Genomic imprinting - the story of the other half and the conflicts of silencing. *Journal of Genetics and Genomics*. **34** (2), 93-103.

Murphy, G. P. & Dudley, S. A. (2009). Kin recognition: competition and cooperation in *impatiens (balsaminaceae)*. *American Journal of Botany*. **96** (11), 1990-1996.

Musolf, K., Hoffmann, F. & Penn, D. J. (2010). Ultrasonic courtship vocalizations in wild house mice, *Mus musculus musculus*. *Animal Behaviour*. **79** (3), 757-764.

Nakagawa, S. & Waas, J. R. (2004). 'O sibling, where art thou?' - a review of avian sibling recognition with respect to the mammalian literature. *Biological Reviews*. **79** (1), 101-119.

Newman, K. S. & Halpin, Z. T. (1988). Individual odours and mate recognition in the prairie vole, *Microtus ochrogaster*. *Animal Behaviour*. **36** (6), 1779-1787.

Nowak, M. A. (2006). Five rules for the evolution of cooperation. *Science*. **314**, 1560-1563.

O'Farrell, B., Benzie, J. A. H., McGinnity, P., Carlsson, J., de Eyto, E., Dillane, E., Graham, C., Coughlan, J. & Cross, T. (2012). MHC-mediated spatial distribution in brown trout (*Salmo trutta*) fry. *Heredity*. **108** (4), 403-409.

Olsen, K. H., Grahn, M. & Lohm, J. (2002). Influence of mhc on sibling discrimination in Arctic char, *Salvelinus alpinus* (L.). *Journal of Chemical Ecology*. **28** (4), 783-795.

Olsen, K. H., Grahn, M., Lohm, J. & Langefors, A. (1998). MHC and kin discrimination in juvenile Arctic charr, *Salvelinus alpinus* (L.). *Animal Behaviour*. **56**, 319-327.

Olsson, I. A. S. & Westlund, K. (2007). More than numbers matter: The effect of social factors on behaviour and welfare of laboratory rodents and non-human primates. *Applied Animal Behaviour Science*. **103** (3-4), 229-254.

Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B. & Wittsell, H. (2003). Major histocompatibility complex and mate choice in sand lizards. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **270**, S254-S256.

Pakkasmaa, S. & Laurila, A. (2004). Are the effects of kinship modified by environmental conditions in *Rana temporaria* tadpoles? *Annales Zoologici Fennici*. **41** (2), 413-420.

Palanza, P., Della Seta, D., Ferrari, P. F. & Parmigiani, S. (2005). Female competition in wild house mice depends upon timing of female/male settlement and kinship between females. *Animal Behaviour*. **69**, 1259-1271.

Palanza, P., Re, L., Mainardi, D., Brain, P. F. & Parmigiani, S. (1996). Male and female competitive strategies of wild house mice pairs (*Mus musculus domesticus*) confronted with intruders of different sex and age in artificial territories. *Behaviour*. **133**, 863-882.

Palme, R. (2012). Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. *Animal Welfare*. **21** (3), 331-337.

Parr, L. A., Heintz, M., Lonsdorf, E. & Wroblewski, E. (2010). Visual kin recognition in nonhuman primates (*Pan troglodytes* and *Macaca mulatta*) inbreeding avoidance or male distinctiveness? *Journal of Comparative Psychology*. **124** (4), 343-350.

Partridge, L. (1983). Non-random mating and offspring fitness. *Mate Choice*. P. Bateson. Cambridge, Cambridge University Press**:** 227-255.

Paterson, S. & Hurst, J. L. (2009). How effective is recognition of siblings on the basis of genotype? *Journal of Evolutionary Biology*. **22** (9), 1875-1881.

Payne, R. B. (1977). The ecology of brood parasitism in birds. *Annual Review of Ecology and Systematics*. **8**, 1-28.

Paz-Y-Miño C, G., Leonard, S. T., Ferkin, M. H. & Trimble, J. F. (2002). Self-grooming and sibling recognition in meadow voles, *Microtus pennsylvanicus*, and prairie voles, *M. ochrogaster*. *Animal Behaviour*. **63** (2), 331-338.

Pearse-Pratt, R., Schellinck, H., Brown, R., Singh, P. B. & Roser, B. (1998). Soluble MHC antigens and olfactory recognition of genetic individuality: the mechanism. *Genetica (Dordrecht)*. **104** (3), 223-230.

Pelikan, J. (1981). Patterns of reproduction in the house mouse. *Symposia of the Zoological Society of London*. **47**, 205-229.

Penn, D. & Potts, W. (1998a). MHC-disassortative mating preferences reversed by cross-fostering. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **265**, 1299-1306.

Penn, D. & Potts, W. K. (1998b). Untrained mice discriminate MHC-determined odors. *Physiology & Behavior*. **64** (3), 235-243.

Penn, D., Schneider, G., White, K., Slev, P. & Potts, W. (1998). Influenza infection neutralizes the attractiveness of male odour to female mice (*Mus musculus*). *Ethology*. **104** (8), 685-694.

Penn, D. J. (2002). The scent of genetic compatibility: sexual selection and the major histocompatibility complex. *Ethology*. **108** (1), 1-21.

Penn, D. J. & Frommen, J. G. (2010). Kin recognition: an overview of conceptual issues, mechanisms and evolutionary theory. *Animal Behaviour: Evolution and Mechanisms*. P. Kappeler**:** 55-85.

Penn, D. J. & Potts, W. K. (1999). The evolution of mating preferences and major histocompatibility complex genes. *American Naturalist*. **153** (2), 145-164.

Pennycuik, P. R., Johnston, P. G., Westwood, N. H. & Reisner, A. H. (1986). Variation in numbers in a house mouse population housed in a large outdoor enclosure: seasonal fluctuations. *Journal of Animal Ecology*. **55** (1), 371-391.

Pernetta, A. P., Reading, C. J. & Allen, J. A. (2009). Chemoreception and kin discrimination by neonate smooth snakes, *Coronella austriaca*. *Animal Behaviour*. **77** (2), 363-368.

Petersen, S. (1976). The temporal pattern of feeding over the oestrous cycle of the mouse. *Animal Behaviour*. **24** (4), 939-955.

Petrie, M., Krupa, A. & Burke, T. (1999). Peacocks lek with relatives even in the absence of social and environmental cues. *Nature*. **401**, 155-157.

Porter, R. H. (1988). The ontogeny of sibling recognition in rodents - superfamily *Muroidea*. *Behavior Genetics*. **18** (4), 483-494.

Porter, R. H. (1998). Olfaction and human kin recognition. *Genetica*. **104** (3), 259-263.

Porter, R. H. & Etscorn, F. (1974). Olfactory imprinting resulting from brief exposure in *Acomys cahirinus*. *Nature*. **250**, 732-733.

Porter, R. H. & Etscorn, F. (1975). Primacy effect for olfactory imprinting in spiny mice (*Acomys cahirinus*). *Behavioral Biology*. **15** (4), 511-517.

Potts, W. K., Manning, C. J. & Wakeland, E. K. (1991). Mating patterns in seminatural populations of mice influenced by MHC genotype. *Nature*. **352**, 619-621.

Pusey, A. & Wolf, M. (1996). Inbreeding avoidance in animals. *Trends in Ecology & Evolution*. **11** (5), 201-206.

Queller, D. C. (2011). Expanded social fitness and Hamilton's rule for kin, kith, and kind. *Proceedings of the National Academy of Sciences of the United States of America*. **108**, 10792-10799.

Radwan, J., Tkacz, A. & Kloch, A. (2008). MHC and preferences for male odour in the bank vole. *Ethology*. **114** (9), 827-833.

Ramm, S. A., Cheetham, S. A. & Hurst, J. L. (2008). Encoding choosiness: female attraction requires prior physical contact with individual male scents in mice. *Proceedings of the Royal Society B-Biological Sciences*. **275** (1644), 1727-1735.

Rich, T. J. & Hurst, J. L. (1999). The competing countermarks hypothesis: reliable assessment of competitive ability by potential mates. *Animal Behaviour*. **58**, 1027-1037.

Roberts, S. A., Simpson, D. M., Armstrong, S. D., Davidson, A. J., Robertson, D. H., McLean, L., Beynon, R. J. & Hurst, J. L. (2010). Darcin: a male pheromone that

stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol*. **8**, 75.

Roberts, S. C. & Gosling, L. M. (2003). Genetic similarity and quality interact in mate choice decisions by female mice. *Nature Genetics*. **35** (1), 103-106.

Robertson, D. H. L., Cox, K. A., Gaskell, S. J., Evershed, R. P. & Beynon, R. J. (1996). Molecular heterogeneity in the major urinary proteins of the house mouse *Mus musculus*. *Biochemical Journal*. **316**, 265-272.

Robertson, D. H. L., Hurst, J. L., Bolgar, M. S., Gaskell, S. J. & Beynon, R. J. (1997). Molecular heterogeneity of urinary proteins in wild house mouse populations. *Rapid Communications in Mass Spectrometry*. **11** (7), 786-790.

Rosenfield, R. N. & Bielefeldt, J. (1992). Natal dispersal and inbreeding in the Cooper hawk. *Wilson Bulletin*. **104** (1), 182-184.

Rothstein, S. I. (1990). A model system for coevolution: avian brood parasitism. *Annual Review of Ecology and Systematics*. **21**, 481-508.

Roulin, A. & Hager, R. (2003). Indiscriminate nursing in communal breeders: a role for genomic imprinting. *Ecology Letters*. **6** (3), 165-166.

Rowe, F. P. (1981). Wild house mouse biology and control. *Symposia of the Zoological Society of London*. **47**, 575-589.

Ruis, M. A. W., te Brake, J. H. A., Engel, B., Buist, W. G., Blokhuis, H. J. & Koolhaas, J. M. (2001). Adaptation to social isolation - acute and long-term stress responses of growing gilts with different coping characteristics. *Physiology & Behavior*. **73** (4), 541-551.

Rusu, A., König, B. & Krackow, S. (2004). Pre-reproductive alliance formation in female wild house mice (*Mus domesticus*): the effects of familiarity and age disparity. *Acta ethologica*. **6** (2), 53-58.

Rusu, A. S. & Krackow, S. (2004). Kin-preferential cooperation, dominance-dependent reproductive skew, and competition for mates in communally nesting female house mice. *Behavioral Ecology and Sociobiology*. **56** (3), 298-305.

Sahin, E. & Gumuslu, S. (2007). Stress-dependent induction of protein oxidation, lipid peroxidation and anti-oxidants in peripheral tissues of rats: comparison of three stress models (immobilization, cold and immobilization-cold). *Clinical and Experimental Pharmacology and Physiology*. **34** (5-6), 425-431.

Sapolsky, R. M. (2002). Endocrinology of the Stress-Response. *Behavioral Endocrinology*. J. B. Becker, Breedlove, S. M., Crews, D. & McCarthy, M. M. Cambridge, USA, The MIT Press**:** 409-450.

Sayler, A. & Salmon, M. (1971). Ethological analysis of communal nursing by house mouse (*Mus musculus*). *Behaviour*. **40**, 61-84.

Schmidt, M. V., Scharf, S. H., Liebl, C., Harbich, D., Mayer, B., Holsboer, F. & Muller, M. B. (2010). A novel chronic social stress paradigm in female mice. *Hormones and Behavior*. **57** (4-5), 415-420.

Shahan, K., Denaro, M., Gilmartin, M., Shi, Y. & Derman, E. (1987). Expression of 6 mouse major urinary protein genes in the mammary, parotid, sublingual, submaxillary, and lacrimal glands and in the liver. *Molecular and Cellular Biology*. **7** (5), 1947-1954.

Shakespeare, W. (c. 1594). The comedy of errors.

Shapiro, L. E., Austin, D., Ward, S. E. & Dewsbury, D. A. (1986). Familiarity and female mate choice in two species of voles (*Microtus ochrogaster* and *Microtus montanus*). *Animal Behaviour*. **34**, 90-97.

Sharp, S. P., McGowan, A., Wood, M. J. & Hatchwell, B. J. (2005). Learned kin recognition cues in a social bird. *Nature*. **434**, 1127-1130.

Shaw, P. H., Held, W. A. & Hastie, N. D. (1983). The gene family for major urinary proteins - expression in several secretory-tissues of the mouse. *Cell*. **32** (3), 755-761.

Sherborne, A. L., Thom, M. D., Paterson, S., Jury, F., Ollier, W. E. R., Stockley, P., Beynon, R. J. & Hurst, J. L. (2007). The genetic basis of inbreeding avoidance in house mice. *Current Biology*. **17** (23), 2061-2066.

Shizuka, D. & Lyon, B. E. (2010). Coots use hatch order to learn to recognize and reject conspecific brood parasitic chicks. *Nature*. **463**, 223-226.

Simmons, L. W. (1991). Female choice and the relatedness of mates in the field cricket, *Gryllus bimaculatus*. *Animal Behaviour*. **41**, 493-501.

Singh, P. B. (1998). The present status of the 'carrier hypothesis' for chemosensory recognition of genetic individuality. *Genetica*. **104** (3), 231-233.

Singh, P. B. (2001). Chemosensation and genetic individuality. *Reproduction*. **121** (4), 529-539.

Singh, P. B., Brown, R. E. & Roser, B. (1987). MHC antigens in urine as olfactory recognition cues. *Nature*. **327**, 161-164.

Smith, J., Hurst, J. L. & Barnard, C. J. (1994). Comparing behavior in wild and laboratory strains of the house mouse - levels of comparison and functional inference. *Behavioural Processes*. **32** (1), 79-86.

Smith, J. C. (1981). Senses and communication. *Symposia of the Zoological Society of London*. **47**, 367-393.

Smith, T. E. & French, J. A. (1997). Psychosocial stress and urinary cortisol excretion in marmoset monkeys (*Callithrix kuhli*). *Physiology & Behavior*. **62** (2), 225-232.

Sophocles (c. 429 BC). Oedipus Rex.

Stopka, P., Janotova, K. & Heyrovsky, D. (2007). The advertisement role of major urinary proteins in mice. *Physiology & Behavior*. **91** (5), 667-670.

Strassmann, J. E., Gilbert, O. M. & Queller, D. C. (2011). Kin discrimination and cooperation in microbes. *Annual Review of Microbiology, Vol 65*. S. Gottesman & Harwood, C. S. Palo Alto, Annual Reviews. **65:** 349-367.

Stuart, R. J. (1991). Kin recognition as a functional concept. *Animal Behaviour*. **41**, 1093-1094.

Stynoski, J. L. (2009). Discrimination of offspring by indirect recognition in an egg-feeding dendrobatid frog, *Oophaga pumilio*. *Animal Behaviour*. **78** (6), 1351-1356.

Sun, L. X. & Müller-Schwarze, D. (1997). Sibling recognition in the beaver: a field test for phenotype matching. *Animal Behaviour*. **54**, 493-502.

Tang-Martinez, Z. (2001). The mechanisms of kin discrimination and the evolution of kin recognition in vertebrates: a critical re-evaluation. *Behavioural Processes*. **53** (1-2), 21-40.

Tang-Martinez, Z., Mueller, L. L. & Taylor, G. T. (1993). Individual odors and mating success in the golden-hamster, *Mesocricetus auratus*. *Animal Behaviour*. **45** (6), 1141-1151.

Thom, M. D. & Hurst, J. L. (2004). Individual recognition by scent. *Annales Zoologici Fennici*. **41** (6), 765-787.

Thom, M. D., Stockley, P., Beynon, R. J. & Hurst, J. L. (2008a). Scent, mate choice and genetic heterozygosity. *Chemical Signals in Vertebrates 11*. **11**, 291-301.

Thom, M. D., Stockley, P., Jury, F., Ollier, W. E. R., Beynon, R. J. & Hurst, J. L. (2008b). The direct assessment of genetic heterozygosity through scent in the mouse. *Current Biology*. **18** (8), 619-623.

Todrank, J. & Heth, G. (2001). Rethinking cross-fostering designs for studying kin recognition mechanisms. *Animal Behaviour*. **61**, 503-505.

Todrank, J. & Heth, G. (2003). Odor-genes covariance and genetic relatedness assessments: rethinking odor-based "recognition" mechanisms in rodents. *Advances in the Study of Behavior, Vol 32*. **32:** 77-130.

Todrank, J., Heth, G. & Johnston, R. E. (1998). Kin recognition in golden hamsters: evidence for kinship odours. *Animal Behaviour*. **55**, 377-386.

Touma, C., Sachser, N., Mostl, E. & Palme, R. (2003). Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *General and Comparative Endocrinology*. **130** (3), 267-278.

Tregenza, T. & Wedell, N. (2000). Genetic compatibility, mate choice and patterns of parentage: invited review. *Molecular Ecology*. **9** (8), 1013-1027.

Tzur, S., Todrank, J., Juergens, A., Nevo, E. & Heth, G. (2009). Odour-genes covariance within a natural population of subterranean *Spalax galili* blind mole rats. *Biological Journal of the Linnean Society*. **96** (3), 483-490.

Utsumi, M., Ohno, K., Kawasaki, Y., Tamura, M., Kubo, T. & Tohyama, M. (1999). Expression of major urinary protein genes in the nasal glands associated with general olfaction. *Journal of Neurobiology*. **39** (2), 227-236.

Välimäki, P., Kivelä, S. & Mäenpää, M. (2011). Mating with a kin decreases female remating interval: a possible example of inbreeding avoidance. *Behavioral Ecology and Sociobiology*. **65** (11), 2037-2047.

Villinger, J. & Waldman, B. (2008). Self-referent MHC type matching in frog tadpoles. *Proceedings of the Royal Society B-Biological Sciences*. **275**, 1225-1230.

Vom Saal, F. S., Franks, P., Boechler, M., Palanza, P. & Parmigiani, S. (1995). Nest defense and survival of offspring in highly aggressive wild Canadian female house mice. *Physiology and Behavior*. **58** (4), 669-678.

Vrana, P. B. (2007). Genomic imprinting as a mechanism of reproductive isolation in mammals. *Journal of Mammalogy*. **88** (1), 5-23.

Waldman, B. (1987). Mechanisms of kin recognition. *Journal of Theoretical Biology*. **128** (2), 159-185.

Waldman, B. (1991). Kin recognition in amphibians. *Kin Recognition*. P. G. Hepper. Cambridge, Cambridge University Press**:** 162-219.

Walsh, R. E. & Rayor, L. S. (2008). Kin discrimination in the amblypygid, *Damon diadema*. *Journal of Arachnology*. **36** (2), 336-343.

Ward, A. J. W. & Hart, P. J. B. (2003). The effects of kin and familiarity on interactions between fish. *Fish and Fisheries*. **4** (4), 348-358.

Watson, R., Munro, C., Edwards, K. L., Norton, V., Brown, J. L. & Walker, S. L. (2012). Development of a versatile enzyme immunoassay for non-invasive assessment of glucocorticoids metabolites in a diversity of taxonomic species. *Article submitted for publication.*

Wedekind, C., Seebeck, T., Bettens, F. & Paepke, A. J. (1995). MHC-dependent mate preferences in humans. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **260**, 245-249.

Weidt, A., Hofmann, S. E. & Konig, B. (2008). Not only mate choice matters: fitness consequences of social partner choice in female house mice. *Animal Behaviour*. **75**, 801-808.

Werner, D. I., Baker, E. M., Gonzalez, E. D. & Sosa, I. R. (1987). Kinship recognition and grouping in hatchling green iguanas. *Behavioral Ecology and Sociobiology*. **21** (2), 83-89.

West, S. A., Diggle, S. P., Buckling, A., Gardner, A. & Griffins, A. S. (2007). The social lives of microbes. *Annual Review of Ecology Evolution and Systematics*. Palo Alto, Annual Reviews. **38:** 53-77.

Whitehorn, P. R., Tinsley, M. C. & Goulson, D. (2009). Kin recognition and inbreeding reluctance in bumblebees. *Apidologie*. **40** (6), 627-633.

Whittaker, A. L., Howarth, G. S. & Hickman, D. L. (2012). Effects of space allocation and housing density on measures of wellbeing in laboratory mice: a review. *Laboratory Animals*. **46** (1), 3-13.

Whitten, P. L., Stavisky, R., Aureli, F. & Russell, E. (1998). Response of fecal cortisol to stress in captive chimpanzees (*Pan troglodytes*). *American Journal of Primatology*. **44** (1), 57-69.

Wilkins, J. F. & Haig, D. (2003). What good is genomic imprinting: the function of parent-specific gene expression. *Nature Reviews Genetics*. **4** (5), 359-368.

Wilkinson, G. S. & Baker, A. E. M. (1988). Communal nesting among genetically similar house mice. *Ethology*. **77** (2), 103-114.

Willse, A., Kwak, J., Yamazaki, K., Preti, G., Wahl, J. H. & Beauchamp, G. K. (2006). Individual odortypes: interaction of MHC and background genes. *Immunogenetics*. **58** (12), 967-982.

Winn, B. E. & Vestal, B. M. (1986). Kin recognition and choice of males by wild female house mice (*Mus musculus*). *Journal of Comparative Psychology*. **100** (1), 72-75.

Wiszniewski, J., Lusseau, D. & Moeller, L. M. (2010). Female bisexual kinship ties maintain social cohesion in a dolphin network. *Animal Behaviour*. **80** (5), 895-904.

Wolff, R. J. (1985). Mating-behavior and female choice - their relation to social-structure in wild caught house mice (*Mus musculus*) housed in a semi-natural environment. *Journal of Zoology*. **207**, 43-51.

Wurbel, H. (2001). Ideal homes? Housing effects on rodent brain and behaviour. *Trends in Neurosciences*. **24** (4), 207-211.

Yamaguchi, M., Yamazaki, K. & Boyse, E. A. (1978). Mating preference tests with recombinant congenic strain Balb.HTG. *Immunogenetics*. **6** (3), 261-264.

Yamazaki, K., Beauchamp, G. K., Curran, M., Bard, J. & Boyse, E. A. (2000). Parent-progeny recognition as a function of MHC odortype identity. *Proceedings of the National Academy of Sciences of the United States of America*. **97** (19), 10500-10502.

Yamazaki, K., Beauchamp, G. K. & Daisuke, Y. (2007). Genetic basis for MHC dependent mate choice. *Advances in Genetics*, Academic Press. **59:** 129-145.

Yamazaki, K., Beauchamp, G. K., Egorov, I. K., Bard, J., Thomas, L. & Boyse, E. A. (1983). Sensory distinction between H-2b and H-2bm1 mutant mice. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*. **80** (18), 5685-5688.

Yamazaki, K., Beauchamp, G. K., Imai, Y., Bard, J., Phelan, S. P., Thomas, L. & Boyse, E. A. (1990). Odortypes determined by the major histocompatibility complex in germ-free mice. *Proceedings of the National Academy of Sciences of the United States of America*. **87** (21), 8413-8416.

Yamazaki, K., Beauchamp, G. K., Kupniewski, D., Bard, J., Thomas, L. & Boyse, E. A. (1988). Familial imprinting determines H-2 selective mating preferences. *Science*. **240**, 1331-1332.

Yamazaki, K., Boyse, E. A., Mike, V., Thaler, H. T., Mathieson, B. J., Abbott, J., Boyse, J., Zayas, Z. A. & Thomas, L. (1976). Control of mating preferences in mice by genes in major histocompatibility complex. *Journal of Experimental Medicine*. **144** (5), 1324-1335.

Yamazaki, K., Yamaguchi, M., Andrews, P. W., Peake, B. & Boyse, E. A. (1978). Mating preferences of F2 segregants of crosses between MHC-congenic mouse strains. *Immunogenetics*. **6** (3), 253-259.

Yamazaki, K., Yamaguchi, M., Baranoski, L., Bard, J., Boyse, E. A. & Thomas, L. (1979). Recognition among mice - evidence from the use of a y-maze differentially scented

by congenic mice of different major histocompatibility types. *Journal of Experimental Medicine*. **150** (4), 755-760.

Yang, H., Bell, T. A., Churchill, G. A. & de Villena, F. P. M. (2007). On the subspecific origin of the laboratory mouse. *Nature Genetics*. **39** (9), 1100-1107.

Young, A. J., Carlson, A. A., Monfort, S. L., Russell, A. F., Bennett, N. C. & Clutton-Brock, T. (2006). Stress and the suppression of subordinate reproduction in cooperatively breeding meerkats. *Proceedings of the National Academy of Sciences of the United States of America*. **103** (32), 12005-12010.

Yu, X. D., Sun, R. Y. & Fang, J. M. (2004). Effect of kinship on social behaviors in Brandt's voles (*Microtus brandti*). *Journal of Ethology*. **22** (1), 17-22.

Appendix: New Scientist / Association for the Study of Animal Behaviour Science Writing Prize 2010

Family Matters

Family. It's an odd thing, isn't it? A bunch of people you can adore or absolutely hate but who you'd still probably do anything for. People you can have nothing in common with except other relatives. You only have to look at culture through the ages to see just how important kinship is. Shakespeare used the theme of family repeatedly throughout his works, Hamlet's very first words are "A little more than kin, and less than kind", a fitting introduction to the family tragedy that unfolds. From Oedipus to Blood Brothers, kinship appears again and again in literature of all forms, and in particular a case of mistaken identity can make for fine comedy or delicious tragedy.

Why do relatives matter? In humans your family often consists of a group of people who are there for you no matter what. They provide support and encouragement, give advice and learning and do their best to help you have the best life possible. In nonhumans kin can group together for protection or foraging, can cooperatively care for young, or can simply choose not to fight one another. It doesn't always happen, siblicide and infanticide are common in many species, but there are many occasions when knowing your relatives can help. In particular the two big kin recognition factors are inbreeding avoidance and inclusive fitness: being able to recognise your relatives helps prevent matings between close kin; whilst W.D.Hamilton showed in the 1960s that because relatives have genes that are identical by descent, aiding a relative's reproductive success can improve an individual's own fitness, provided that the costs of giving help are outweighed sufficiently by the benefits gained.

It may not always be beneficial but there is evidence that under certain conditions kin recognition and the differential behaviours that result from it can be advantageous. Whilst self will normally come first, the closer the relative the more likely you are to share genes identical by descent and therefore the more you could gain from helping them. It's rather like the Arabic saying: "I against my brother, I and my brother against our cousin, I, my brother and our cousin against the neighbours, all of us against the foreigner".

Recognising relatives is often an easy task for us humans. Our highly social lifestyle and advanced vocal communication mean that it's rare to meet a relative without being aware of it, and we can normally remember those relations we've been introduced to before. It's a rare situation, but have you ever spotted a resemblance between two people and realised they were related before being told? Or have you noticed similarities between your own family members, a shared physical or behavioural trait perhaps? Depending on who sees us my brother and I could be twins or adopted. One particular observer even claimed that we share a nose but nothing else.

But how do non-humans recognise relatives? A lot of the time it can be a simple rule – those present in the nest with me are related to me, everyone else I should treat as being unrelated to me. In many situations this mechanism no doubt works, it enables the recognition of the closest relatives, parents and siblings. But what about those situations where there is no nest, no initial period of cohabitation that allows relatives to be learnt? What about situations where previous litters have already left the nest? Close relatives could easily be encountered that simply weren't present when the young were taking names.

This is where I find kin recognition really comes together and gets interesting, because there is evidence that many different animals can recognise their relatives, despite never having encountered them before. Indeed, there is evidence of kin recognition in all the major groups from mammals to fish, birds to amphibians, as well as insects, plants and single-celled organisms. I'm sure that I'd be unlikely to recognise most of my relatives had I just encountered them in the street and never been introduced to them, shared nose or not. Yet certain animals are able to recognise unfamiliar kin and change their behaviour towards them accordingly, choosing not to fight, not to breed, to nest or group together or simply to avoid each other. And these are only the behaviours that can actually be observed. Often there's no way of telling whether any recognition has actually occurred. Perhaps many more species can recognise their kin but choose not to respond to them.

There are just so many questions associated with kin recognition. It can occur under different situations, for different reasons, and is perhaps often undetectable. We don't even know much of the time whether the behavioural difference we observe are due to a mechanism designed for recognising kin or whether it is simply part of an overall recognition mechanism reaching from self, through the different levels of relatedness to group recognition and onto species recognition. Debates flare up about particular issues or new bold claims for or against it and

arguments linger about definitions for mechanisms, whether certain ideas would ever really work. Experiments are performed in their hundreds and models created. The modern phase of kin recognition is nearing 50 years old and yet it still seems just as fresh and exciting as it must have done originally. We know so much and yet there's so much left to discover. It can be messy and frustrating and confusing but it's always interesting and it always has a story to tell.