# Proximate mediators of behaviour in cooperatively breeding mammals: applications to breeding programmes for endangered species

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by

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Table of Contents

# Proximate mediators of behaviour in cooperatively breeding mammals: applications to breeding programmes for endangered species

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## General Abstract

Cooperative breeding describes a social group within which helpers, in addition to parents, aid in the rearing of offspring. Many cooperatively breeding mammals are threatened with extinction, and captive breeding programmes are essential for their long-term survival. Although anecdotally these species often do poorly in captivity, the proximate reasons for this are generally not well quantified. The reasons for poor reproductive performance in captivity are likely to be complex, involving multiple interacting factors, such as variation in development, behaviour and physiology influenced by the captive environment, as well as animal husbandry. To explore how these factors might influence the welfare and reproductive success of cooperatively breeding species in captivity, in this thesis I have used a combination of laboratory studies on a model species, wild house mice (*Mus musculus domesticus*), and applied studies of captive African wild dogs (*Lycaon pictus*). This dual approach allows me to explore the behaviour of cooperatively breeding mammals from both mechanistic and applied perspectives. Both lines of investigation required the use of non-invasive hormonal monitoring and the application of enzyme immunoassays (EIAs), to quantify variation in hormone metabolites linked to reproduction, stress and social behaviour. I validated commercially available EIAs to measure oxytocin in mouse urine, serum and the paraventricular nucleus, which were then applied to investigate mechanisms underlying the development of social and anxiety related behaviours under carefully controlled experimental conditions. I investigated whether variation in the early rearing environment (being reared in a communal or single nest) influenced adult behaviour and neuroendocrinology, specifically the neuropeptide system and hypothalamic pituitary axis. For female house mice, being reared in a communal nest did not influence nepotistic behaviour, or urinary oxytocin concentrations. However, variation in nepotistic behaviour was significantly related to urinary oxytocin levels independent of rearing environment. Communally reared adult female house mice were found to exhibit reduced anxiety-related behaviour, in terms of enhanced exploration in a novel open field test, with significantly lower oxytocin receptor and significantly higher AVP receptor-1a expression within central brain regions. Non-invasive hormonal monitoring is an important technique to monitor captive endangered species, however, collecting the excreta from dangerous species can present difficulties, resulting in a lag-time between defecation and faecal collection for hormone analysis. Following validation

of in-house EIAs, I conducted faecal hormone metabolite time degradation studies for corticosterone, testosterone, and progesterone of wild dogs. Measured hormone levels within wild dog faeces remained stable for up to 4 hours and were sensitive to environmental conditions within UK *ex situ* environments. This needs to be considered when using faecal hormones to monitor the endocrine status of captive wild dogs. For captive wild dog populations, I utilised studbook data and a questionnaire to investigate factors influencing reproductive success and aggression in European collections. The juvenile mortality rate for captive wild dogs was 44.7%, with trauma by conspecifics being the most common cause of death. Reproductive success was greater for young dams (less than 2.5 years at parturition) and offspring survival was lower for dams that had previously unsuccessfully reared offspring to post-natal day 30. Results from the questionnaire revealed that 88% of zoological collections that house wild dogs experience aggression to some degree. In conclusion, in addition to other stressors of captivity, unnatural groupings and rearing environments found within captivity could be perturbing adult behaviour resulting in heightened stress, adding to the high aggression and poor reproductive performance seen.

# Chapter 1: General Introduction

Social and anxiety-related behaviours are critical to survival and reproductive success in mammals. The need to manage complex social interactions and attachments to social and sexual partners has driven the evolution of complex brain structures and enlargement of the brain (Dunbar and Shultz, 2007). To gain access to often limited resources, behavioural interactions between group living mammals are a balance between affiliation and competition, and for philopatric females, the balance can tilt towards increased competition (Stockley and Campbell, 2013, West et al., 2001, Clutton-Brock et al., 2006b). The theory of kin selection suggests that interactions between relatives should be more affiliative, as helping a relative to reproduce results in an individual indirectly passing on genes to the next generation (Hamilton, 1964a, Hamilton, 1964b, Hamilton, 1970, West et al., 2002); however, this relies on kin recognition (Mateo, 2017, Mateo, 2004, Green et al., 2015). The development and expression of social behaviour are modulated by changes in the concentrations of neuropeptide and steroid hormones (Soares et al., 2010). These systems are sensitive to the gestational and early post-natal environment (Taborsky, 2017, Curley et al., 2009).

Cooperative breeding, a term encompassing a social system within which members of the social group in addition to the parents assist in rearing young (Solomon and French, 1997), is rare across mammalian taxa (Lukas and Clutton-Brock, 2012a). Unfortunately, as for many species, a large number of cooperatively breeding mammals are classed as threatened with extinction on the International Union for Conservation of Nature (IUCN) red list. Indeed, the natural world is in decline, and we are going through an anthropogenic sixth mass extinction (Ceballos et al., 2020). One example of an endangered cooperatively breeding mammal is the African wild dog (*Lycaon pictus)* (Woodroffe and Sillero-Zubiri, 2020). Captive breeding programmes are important for the conservation of this species (for example see Van der Weyde et al., 2015) and zoos are vital tools to prevent numerous extinctions (Bolam et al., 2020). However, due to the complexity of their social and reproductive behaviours, cooperatively breeding mammals are particularly difficult to manage in captivity as they exhibit high levels of aggression and poor reproductive performance (Van der Weyde et al., 2015, Van der Weyde et al., 2016, Yordy and Mossotti, 2016).

## 1.1. The cooperative breeding system and female competition

Cooperative breeding systems are most commonly found in birds and are limited to less than 5% of all mammalian species (Lukas and Clutton-Brock, 2012a). Cooperative breeding is limited to 34 species within eight families of mammal, all of which are socially monogamous and polytocus, often with short inter-birth intervals (Lukas and Clutton-Brock, 2012b, Lukas and Clutton-Brock, 2012a). Even though rare in mammals, cooperative breeding is distributed across a wide range of mammalian taxa, including rodents and canids (Sayler and Salmon, 1971). Two main categories of cooperative breeding are defined by the degree of reproductive skew. Firstly, singular breeding systems (cooperative), whereby the highest-ranking individual dominates reproduction (high reproductive skew), for example African wild dogs. Caregiving alloparents are nonbreeding adults/sub-adults that act altruistically by expressing delayed dispersal, reproductive suppression and care for another's offspring (Sayler and Salmon, 1971). These obligate cooperatively breeding mammals require help from other group members to successfully reproduce (Clutton-Brock, 2006). The second cooperatively breeding system; plural breeding (communal), is where multiple females reproduce (low reproductive skew), for example wild house mice (*Mus musculus domesticus*). Caregivers are reproductively active adults which share the responsibility of rearing young with all the breeders in that group, including allonursing young (Solomon and French, 1997, Sayler and Salmon, 1971). Cooperative behaviours in these social groups are required to maintain group cohesion (Hayes, 2000).

The evolution of cooperative breeding within mammals is complex. Kin selection theory explains that an individual can gain inclusive fitness benefits by increasing reproductive output of related individuals (indirect fitness), thus passing on a proportion of the helper's genes to the next generation (Hamilton, 1964a, Hamilton, 1964b). This results in individuals behaving altruistically towards related conspecifics with a high number of shared alleles. Indeed, the benefits of helping relatives to rear their offspring may out-weigh the costs of competing with them, suggesting competition between relatives could be maladaptive (Clutton-Brock, 2002). Many cooperative and communal breeders produce highly dependent altricial young requiring costly care (Lukas and Clutton-Brock, 2012b). Cooperation to assist in care may therefore be enforced via manipulation strategies and enforcement by the dominant

individuals; for example those that do not cooperate may be evicted from the group (Cant and Johnstone, 2006).

Competition between kin can arise if related individuals remain within close proximity within the same social group for extended periods, for example due to limited dispersal or captivity (West et al., 2001). Males have more ornaments, secondary sexual characteristics and are generally more overtly aggressive than females (Stockley and Campbell, 2013). Competition between females within a cooperative breeding system has therefore received comparably little attention, but examples include meerkats (*Suricata suricatta*) (Clutton-Brock et al., 2006a) and dwarf mongooses (*Helogale parvula*) (Creel and Creel, 1991, Creel et al., 1992). Competition is likely to vary throughout the reproductive cycle, as access to a mate when in oestrus will cause intra-female competition within synchronised females. Additionally, female competition will be high during gestation and lactation due to heightened calorie requirements, and throughout the post-natal period to protect the offspring (Huchard and Cowlishaw, 2011). In group living species, this competition can result in hierarchical formation with dominant pairs gaining access to the best foraging locations and nest sites. Access to nest sites is a requirement for reproduction in some mammalian species, and females may be unable to reproduce in high density populations with restricted access to nest sites (Hurst, 1987).

Female dominance can be linked to body mass, with larger females being more competitive (Creel, 2001, Clutton-Brock et al., 2001). Less competitive females often are subordinate, leading to reduced food consumption and poor weight gain. Indeed, it is often the largest subordinate females that are likely to successfully compete and win a dominant position (Clutton-Brock, 2006). Age related hierarchies are also common, with older more experienced individuals being more competitive (Rusu et al., 2004, Pusey et al., 1997).

### 1.2. Physiological mediators of affiliative and competitive behaviours

The closely related neuropeptides oxytocin and arginine vasopressin (AVP) are important mediators of social behaviour and the stress response (Veenema and Neumann, 2008) and are highly conserved across species (Donaldson and Young, 2008). Indeed, all vertebrate animals display nanopeptide secreting cells within the hypothalamus and preoptic area (Goodson and Thompson, 2010), and the release of peptides from these modulates social

behaviours in all species examined to date. In addition to neuropeptides, glucocorticoids also mediate social behaviour. Both AVP and oxytocin are intertwined with the Hypothalamic Pituitary Adrenal (HPA) axis (Winter and Jurek, 2019). A stressful event activates the HPA axis resulting in the production of glucocorticoids from the adrenal glands (Creel, 2001) and these circulating glucocorticoids are known to modulate aggressive-like and anxiety-like behaviour (Malkesman et al., 2006, Neumann et al., 2000a). Testosterone and its precursors are widely known for their role in male aggression, however, there is also evidence suggesting that testosterone can modulate female competitive behaviour (Langmore et al., 2002, Gleason et al., 2009), including dominant females having higher plasma testosterone concentrations than subordinate conspecifics in meerkats (Clutton-Brock et al., 2006a).

### *1.2.1. The neuropeptides oxytocin and arginine vasopressin*

Both oxytocin and AVP are synthesised by magnocellular neurons within the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus (Stoop, 2012). Axons from these magnocellular neurons project to the posterior pituitary gland and action potentials then cause AVP and oxytocin release into systemic circulation (Gimpl and Fahrenholz, 2001, Baribeau and Anagnostou, 2015). AVP and oxytocin neurones also project to multiple brain regions to include the bed nucleus of the stria terminalis (BNST), medial amygdala (MA), lateral septum (LS), medial preoptic area (MPOA) in addition to other locations (van Leeuwen and Caffé, 1983, Caffé and van Leeuwen, 1983, Smith et al., 2019, Curley et al., 2009, Dong and Swanson, 2006, Knobloch et al., 2012, Jurek and Neumann, 2018). These long ranging axonal projections into the forebrain are found only in mammals and reptiles (Jurek and Neumann, 2018), and variations in neuropeptide neuronal and receptor distribution between different species is proposed to result in species differences in social behaviour (Stoop, 2012, Goodson, 2008). In central regions, AVP and oxytocin are released from dendrites to act as neurotransmitters to elicit behavioural responses (Baribeau and Anagnostou, 2015, Aguilera and Rabadan-Diehl, 2000, Landgraf and Neumann, 2004, Ross and Young, 2009, Leng and Ludwig, 2016, Leng and Sabatier, 2016, Kompier et al., 2019). Magnocellular projections to the neurohypophysis in addition to axonal projections into forebrain regions can result in both coordinated and independent release of oxytocin into blood and specific central regions (Jurek and Neumann, 2018). Centrally released oxytocin and AVP then bind to G-coupled local receptors in target regions to exert behavioural responses (Jurek and Neumann, 2018, Gimpl and Fahrenholz, 2001, Campbell, 2008).

*1.2.2. Oxytocin and social behaviour*

Oxytocin is known for its role in female reproduction, particularly parturition, milk let down and lactation, and the infant maternal bond (Olff et al., 2013, Folley and Knaggs, 1965, Folley and Knaggs, 1966). Oxytocin is also key in the mediation of prosocial behaviour, for example pair bonding in voles (montane (*Microtus montanus*) and meadow (*Microtus pennsylvanicus*) (Young and Wang, 2004) and increased levels of prosocial behaviours across mammalian taxa (Madden and Clutton-Brock, 2011, Crockford et al., 2014, Crockford et al., 2013, Wittig et al., 2014, Lukas et al., 2011). On the other hand, oxytocin is also important in the neuroregulation of female aggression including maternal aggression towards nest intruders (Bosch, 2013), aggressive social behaviours towards group outsiders, and evicting kin from the group (Anacker and Beery, 2013).

Pair bonding is the term given to the selective preference for a specific mate and is related to the evolution of larger brain sizes (Dunbar and Shultz, 2007). Only found in 3-5% of mammalian species, monogamy is rare in mammals (Kleiman, 1977). There is an array of evidence highlighting oxytocin's role in pair bonding between monogamous prairie voles (*Microtus ochrogaster*) (Carter et al., 1995, Cho et al., 1999, Insel and Hulihan, 1995). The prairie vole unusually exhibits long term preference for one mate and is aggressive towards other prairie voles following mating (Insel and Shapiro, 1992, Young and Wang, 2004). Insel and Shapiro (1992) found species specific variation in oxytocin receptor expression between these monogamous prairie voles and non-monogamous asocial montane voles (*Microtus pennsylvanicus*). This suggests that specific oxytocin receptor distributions (in the case of the monogamous prairie vole, specifically increased oxytocin receptor densities in the nucleus accumbens) are required for monogamy, and variation in receptor locations could influence bonding and therefore affiliative behaviour across species (Insel and Shapiro, 1992, Ross et al., 2009). Furthermore, the central administration of synthetic oxytocin to ovariectomised prairie voles results in significantly increased partner preference (Williams et al., 1994) and inhibiting the oxytocin receptor in the nucleus accumbens of female prairie voles also inhibits bond formation (Keebaugh et al., 2015).

Intrasexual bonding can be important for overall fitness of individuals (Cameron et al., 2009, Silk, 2007) and oxytocin has been found to influence bond formation in same sex pairings. Oxytocin infusions have been found to increase social huddling with partners in female meadow voles (*Microtus pennsylvanicus*) (Beery and Zucker, 2010). A study comparing

oxytocin receptor binding in two closely related mole rat species, the communal naked mole rats (*Heterocephalus glaber*) and the solitary Cape mole rats (*Georychus capensis*) showed that the sociable female naked mole rats had a high level of oxytocin receptor binding in the nucleus accumbens compared to no detectable oxytocin receptor binding in the same location in Cape mole rats. In contrast, intense oxytocin receptor binding was found within the piriform cortex in Cape mole rats, where no such binding was found in the communal naked mole rats (Kalamatianos et al., 2010). Peripheral intramuscular administration of synthetic oxytocin to wild ranging meerkats significantly increased a host of cooperative behaviours including pup guarding, increased time spend on-guard, increased proportion of food given to pups and decreased aggressive behaviours (Madden and Clutton-Brock, 2011).

Urinary oxytocin has been shown to be higher following social interactions including grooming and food sharing in chimpanzees (*Pan troglodytes*) (Wittig et al., 2014, Crockford et al., 2013). Interestingly, a higher concentration of urinary oxytocin was found after grooming events with preferred grooming partners compared to interactions with less preferred partners (Crockford et al., 2013). Similarly, Finkenwirth et al. (2015) found consistent correlations between urinary oxytocin concentration and dyad bonding strength in co-housed family groups of common marmosets (*Callithrix jacchus*), further highlighting the role of oxytocin in social bonding. This included synchronised longitudinal fluctuations in urinary oxytocin concentrations, and this was greater in strongly bonded dyads compared to less strong dyads.

Social recognition is the ability of an animal to distinguish between conspecifics, identify intruders, select mates and form social hierarchies (Choleris et al., 2009). Furthermore, mice have kin recognition capabilities without prior familiarity (Green et al., 2015). Proximate mechanisms involved in social or kin recognition are likely to have varying degrees of overlapping neurobiology (Choleris et al., 2009). Mice have the ability to recognise individuals using olfactory signals via the vomeronasal system (Cheetham et al., 2007). Mouse urine contains large concentrations of major urinary proteins (MUP), species specific genetic kin recognition markers, and female mice prefer to nest with partners that share their own MUP genotype (Green et al., 2015). There are high concentrations of oxytocin receptors within the accessory olfactory bulb in this species (Oettl et al., 2016), which could suggest a pathway for oxytocin and olfaction in the formation of social memory (Ferguson et al., 2000, Engelmann et al., 1998). In a rat experiment, blocking the oxytocin receptor by directly injecting an antagonist into the BNST significantly impaired social recognition in both sexes compared to

controls, and oxytocin treatment (oxytocin injected into the BNST) increased the duration of social recognition in males but not in females (Dumais et al., 2016). Furthermore, female oxytocin knock-out mice additionally show impaired social memory (Caldwell et al., 2017).

The research outlined above has revealed interactions between oxytocin and prosocial behaviour and bonding. However, there is also evidence to suggest that the prosocial actions of oxytocin only occur under specific social situations. Indeed, oxytocin may also have an important role to play in competitive behaviour (de Jong et al., 2014, Lukas and de Jong, 2017) and maternal aggression (Olff et al., 2013). Competition between conspecific females to access resources can be heightened especially during the breeding season or when there are high population densities (Huchard and Cowlishaw, 2011), and maternal aggression towards nest intruders can be mediated by oxytocin (Campbell, 2008, Bosch, 2013, Caughey et al., 2011). de Jong et al. (2014) found significantly lower oxytocin neuron activation in virgin female Wistar rats that aggressively attacked intruder females compared to less aggressive individuals. In female meadow voles, oxytocin administration decreases affiliative behaviour towards strangers (Beery and Zucker, 2010) emphasising the role of oxytocin in agonistic behaviours towards unfamiliar individuals (Beery, 2015). Harmon et al. (2002) found the administration of oxytocin to female Syrian hamsters (*Mesocricetus auratus*) significantly reduced aggression in a dose dependent manner. Maternal aggression is correlated with oxytocin release within the brain (Bosch et al., 2005); it inhibits aggression directed towards pups, however, it also increases aggression directed towards nest intruders (Campbell, 2008).

The response to oxytocin administration can also vary with dosage chronicity; acute intranasal oxytocin administration increases social behaviour, however, chronic intranasal oxytocin administration results in deficient partner preference behaviour in male prairie voles (*Microtus ochrogaster*) (Bales et al., 2013). Further complicating the role of oxytocin, Harrison et al. (2016) found no effect of intraperitoneal injection of synthetic oxytocin on positive or negative behaviours between female house mice during initial introductions. Further studies showed that this exogenous administration of oxytocin increased the time within which two unfamiliar female mice affiliated and successfully reared offspring in a communal nest (Harrison et al., 2017). Additionally, female prairie voles (*Microtus ochrogaster*) that were treated with oxytocin on post-natal day one have been found to exhibit enhanced aggression and decreased social huddling compared to controls (Bales and Carter, 2003). These slightly

contradicting studies suggests more research is required into species and location dependent roles of oxytocin in aggression.

### 1.3. Neuropeptides and the hypothalamic pituitary adrenal axis

A key response to stress is the activation of the Hypothalamic Pituitary Adrenal (HPA) axis resulting in an increase in circulating glucocorticoids (Creel, 2001) (Figure 1.1). Stress exposure, be that physical or social stress, also results in oxytocin release into peripheral circulation (Kasting, 1988). High levels of circulating glucocorticoids have been shown to increase aggressive-like behaviour (Malkesman et al., 2006), are known to disrupt the immune and reproductive systems, and can modulate aggression (Oppong and Cato, 2015, Calogero et al., 1999). It is clear that stress activates the oxytocinergic system, evidenced by central and peripheral oxytocin levels in an array of studies (see Jurek and Neumann (2018) for review). Oxytocin is anxiolytic, reducing glucocorticoid levels and blood pressure (Uvnas-Moberg, 1998) and oxytocin antagonists have the ability to increase HPA axis activation resulting in increased levels of circulating glucocorticoids (Neumann et al., 2000a). Even though oxytocin is known to mediate anxiety-like behaviour and the HPA axis, no individual variation in oxytocin or oxytocin receptor distributions have been found between Wistar rats bred for high anxiety-related behaviour or those bred for low anxiety-related behaviour (Wigger et al., 2004). However, oxytocin knockout female mice display heightened anxiety-like behaviour compared to wild-types (Amico et al., 2004, Mantella et al., 2003). The application of synthetic endogenous oxytocin via local central infusion has anxiolytic effects in female rodents in a brain region specific manner (Bale et al., 2001, Jurek et al., 2012, Sabihi et al., 2014, Neumann et al., 2000b) and reduces plasma glucocorticoid concentration (Windle et al., 1997, Acevedo-Rodriguez et al., 2015).

The principal regulators of the HPA axis are corticotropin releasing hormone (CRH) and AVP from the parvocellular division of the PVN. These act synergistically and are released into hypophyseal portal circulation (Papadimitriou and Priftis, 2009) to initiate adrenocorticotropic hormone (ACTH) release. ACTH is released as the pro-hormones pre-ACTH and pro-opiomelanocortin (POMC) from corticotropes within the pars distalis of the anterior pituitary gland. POMC cleavage products include α-melanocyte stimulating hormone (α-MSH) and β-endorphin. α-MSH triggers dendritic release of oxytocin (Sabatier, 2006, Sabatier et al., 2003), outputs of which feed into the CRH releasing neurons of the PVN (Winter and Jurek, 2019) but

also inhibit oxytocin secretion from magnocellular neurons nerve terminals in the posterior pituitary (Sabatier, 2006, Sabatier et al., 2003). These magnocellular neurons project into the neurohypophysis and secrete both oxytocin and AVP into peripheral circulation via axonal release (Knobloch and Grinevich, 2014, Baribeau and Anagnostou, 2015). Prohormone convertase I (PHC I), found within the pars distalis of the pituitary gland, further cleaves POMC to active ACTH (Papadimitriou and Priftis, 2009). ACTH release is potentiated by oxytocin (Engelmann et al., 2004) and acts directly on the adrenal cortex to elicit the release of glucocorticoids. Oxytocin thereby enhances the negative feedback loop of the HPA, dampening down the stress response (Winter and Jurek, 2019). The key glucocorticoid in many mammals, including humans, is cortisol, however in rodents the principle glucocorticoid is corticosterone (Matthews and Challis, 1996).

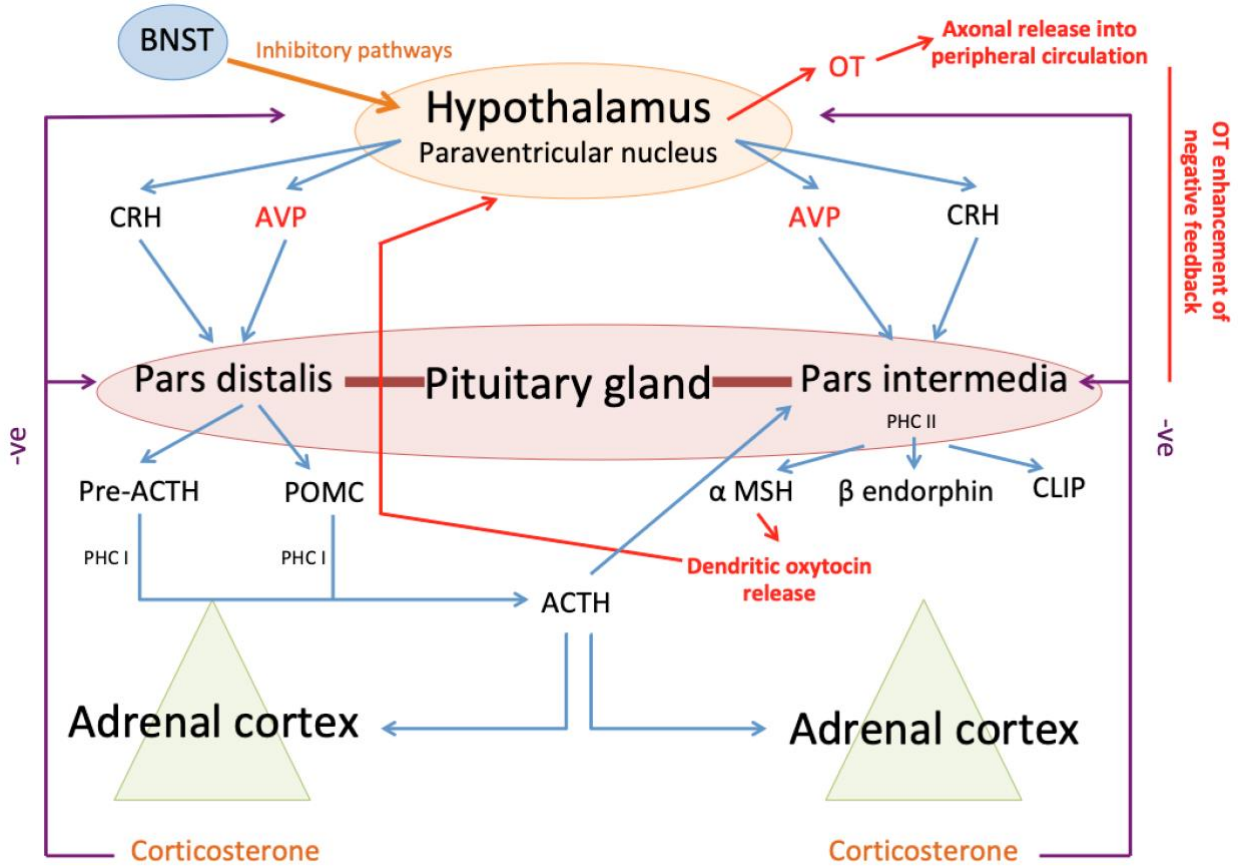


*Figure 1.1: Schematic of the hypothalamic – pituitary – adrenal axis. Corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) are released from nerve terminals in the paraventricular nucleus (PVN) of the hypothalamus into portal circulation. These initiate pro-opiomelanocortin (POMC) and pre adrenocorticotropic hormone (pre-ACTH) release from the pars distalis of the anterior pituitary gland. These are cleaved into active adrenocorticotropic hormone (ACTH) by prohormone convertase I (PHC I). ACTH enters the pars intermedia and is further cleaved into α-melanocyte stimulating hormone (αMSH), β-endorphin ad CLIP. αMSH induces dendritic oxytocin release outputs of which feed into the PVN. ACTH also travels via systemic circulation to the adrenal cortex initiating corticosterone release. Initiation of the HPA axis additionally induces OT axonal release into peripheral circulation which adds to the negative feedback loop, dampening down the stress response. The HPA axis is partially controlled by negative neuronal inputs from the bed nucleus of the stria terminalis (BNST).*

## 1.4. The gestational and early postnatal environment

Due to a period of epigenetic plasticity, the gestational and early postnatal environment can alter adult phenotypes enabling animals to adapt to a changing environment (Taborsky, 2017, Sachser et al., 2013). This is particularly important to consider for endangered species being bred in captivity where conditions vary and are unnatural. Indeed, stressors during late gestation can program the developing foetal HPA axis, leading to an altered adult stress response and therefore the phenotype of F1 and F2 offspring in a laboratory setting (for example see Kapoor et al. (2006)). The main stress hormones, glucocorticoids, pass through the placenta to the developing foetus and can act as transcription factors at glucocorticoid and mineralocorticoid receptors to alter foetal gene expression (de Kloet et al., 2008). This is an adaptive response to enable changes in organogenesis to optimise survival in a challenging environment (Crain et al., 2008). Dantzer et al. (2013) have investigated the effects of increased *in utero* glucocorticoid concentrations on the offspring of wild North American red squirrels (*Tamiasciurus hudsonicus*). Increasing the perceived population density of female red squirrels resulted in an increased maternal faecal glucocorticoid concentration, which in turn resulted in faster growing offspring (Dantzer et al., 2013). Increased competition due to high population densities during nesting has also been found to result in increased growth rates and increased competitive behaviour in F1s in tree swallows (*Tachycineta bicolor*) (Bentz et al., 2013) and increased growth rates in female offspring in guinea pigs (*Cavia perea f. porcellus*) (von Engelhardt et al., 2015). This growth enhancement is likely to be adaptive as larger and quicker growing offspring are more likely to survive in a highly populated, and therefore competitive, environment. However, any extreme in glucocorticoid response: excessive, inadequate or prolonged, can result in negative consequences such as an impaired stress response. For example, high *in utero* foetal glucocorticoids have been associated with perturbed cognitive development, birth weight and anxiety-like behaviour in offspring (Harris and Seckl, 2011). In addition, *in utero* stress can reduce maternal behaviour in the offspring when they reach adulthood (Champagne and Meaney, 2006). As well as alterations of the HPA axis, increased maternal stress has also been shown to reduce oxytocin receptor expression, having consequences for maternal behaviour in F1 offspring (Champagne and Meaney, 2006).

Both the neuropeptide system and the HPA axis are also sensitive to the early postnatal environment (Cushing and Kramer, 2005, Liu et al., 2000); variations in the postnatal environment can alter oxytocin receptor binding. Reduced maternal care can increase

anxiety-related behaviours in F1 offspring including perturbed exploratory behaviours (Modlinska et al., 2018, Meaney, 2001, Caldji et al., 1998). In addition, parental-offspring interactions (for example licking and grooming behaviour) are thought to be very important in shaping F1 phenotypes during the early stages of rapid brain development, and oxytocin and AVP undergo experience-dependent development in early life (Teicher et al., 2003, Bales and Perkeybile, 2012, Hammock, 2015). Indeed, prairie voles (*Microtus ochrogaster*) naturally have broad individual variation in oxytocin receptor expression in the nucleus accumbens, and females with high oxytocin receptor densities are more resilient to negative early life experiences, resulting in normal adult pair bonding. However, females with naturally low oxytocin receptor levels that experience postnatal neglect were unable to form social bonds as adults (Barrett et al., 2015). In a laboratory rat study performed by Champagne and Meaney (2007), F1 offspring from highly attentive mothers housed in impoverished conditions (housed singly in small cages on mesh) had significantly lower oxytocin receptor binding in the MPOA of the brain compared to highly attentive mothers in standard conditions (housed in same sex, same litter groups in larger cages). On the other hand, F1 offspring from poorly attentive mothers (low levels of lick / grooming behaviour) housed in enriched conditions (multilevel, large multiple compartments full of toys), had significantly more oxytocin binding in the MPOA compared to poorly attentive mothers in standard conditions. Furthermore, maternal separation in C57BL/6 mice has been found to decrease oxytocin immunoreactivity in lactating F1s (Veenema et al., 2007). The above suggests that early parental attachment and interactions influence the neural networks required to form social bonds and maternal behaviour as adults (Barrett et al., 2015, Rilling and Young, 2014).

### 1.5. Study species: House mice

The house mouse (*Mus musculus domesticus*) is a small rodent species distributed wildly across the globe (Bronson, 1979). House mice live in social groups which consist of a dominant male, multiple breeding females and their offspring, and the addition of non-breeding females (Hurst, 1987). House mice exhibit both communal and single nest reproductive strategies where relatedness and familiarity of females may improve reproductive success in a communal nest (König and Lindholm, 2012, Stockley et al., 2013, Konig, 1993, König, 1994b, König, 1994a, Sayler and Salmon, 1971). Female mice prefer to nest with related individuals regardless of prior familiarity (Green et al., 2015). These naturally complex social groups result in competition over resources, including nest sites (König and Lindholm, 2012, Stockley et al.,

2013). The natural variation in reproductive strategy may have physiological and behavioural consequences for F1 adult offspring.

For house mice, it is thought that communal rearing of offspring occurs under conditions of increased social competition, providing multiple fitness benefits including cooperative feeding, defence and thermoregulation (Hayes, 2000). It is often the smaller, less competitive females that decide to follow this reproductive strategy (König and Lindholm, 2012, Weidt et al., 2014, König, 1994a, Auclair et al., 2014, Ferrari et al., 2019). It is unknown whether the increased density of offspring and allolactating females within a communal nest causes variation in F1 adult female social / anxiety-related behaviour or physiology in house mice. Indeed, studies on inbred laboratory mouse strains have concluded that a communal nest environment may be a positive socially enhanced environment (increased maternal care and sibling to sibling interactions) resulting in improved anxiety-related behaviour, improved social behaviour and increased maternal behaviour with a corresponding increase in oxytocin receptor expression in specific brain regions (Branchi, 2009, Curley et al., 2009, Branchi et al., 2013a, Branchi et al., 2006a). However, wild house mice differ in reproduction, growth and lifespans compared to inbred laboratory mouse strains, and genetic variability will be greater between unrelated house mice (Harper, 2008). Indeed, male house mice may find the increased population density of a communal nest competitive, resulting in increased competitive behaviour as adults (Fischer et al., 2018). It is currently unknown how communal or single nest rearing environments may affect female F1 house mice in terms of social or anxiety-like behaviour, recognition of kin or corresponding neurobiology.

### 1.6. The African wild dog and *ex situ* institutions

The African wild dog (*Lycaon pictus*) belongs to the family Canidae and unusually for mammals is a cooperative breeder with a high reproductive skew (Kleiman, 1977). They are characterised by highly flexible social organisations with pack members cooperating to raise offspring, hunt and defend territory and pups (Macdonald and Moehlman, 1982, Creel and Creel, 2002). In the wild, wild dog packs produce one litter a year during the time of high prey abundance, and only the dominant female is sure to reproduce (Frame et al., 1979, Malcolm and Ken, 1982).

There are estimated to be fewer than 1500 adult wild dogs in the wild, and therefore they are "one of the world's most threatened carnivores" (Woodroffe, 2004, Woodroffe et al., 2007, Woodroffe and Sillero-Zubiri, 2020). They are classed as endangered on the IUCN red list and numbers are in decline (Woodroffe and Sillero-Zubiri, 2020). Once widespread across sub-Saharan Africa, wild dogs are now found in 14 out of the 39 countries, or 7% of their former range (Woodroffe et al., 2007, Woodroffe and Sillero-Zubiri, 2020). Wild dogs live in low population densities with resultant large home-ranges which are becoming fragmented due to human encroachment, and this encroachment is unlikely to be reversible across the majority of the historical range (Woodroffe and Sillero-Zubiri, 2020). Indeed, human causes may be the most important contributor to adult wild dog mortality due to snaring, road traffic accidents, deliberate shooting, spearing and clubbing. African wild dogs that live outside protected areas have been found to have significantly higher human-related mortality rates than those found inside protected areas (Woodroffe et al., 2007). Furthermore, wild dogs are sensitive to fatal domestic dog diseases including distemper and rabies (Woodroffe, 2012), and to the effects of climate change (Rabaiotti and Woodroffe, 2019), making their future uncertain.

*In situ*, female wild dogs are mono-oestrous seasonal breeders, producing one litter per year during the time of high prey abundance (Malcolm and Marten, 1982, Creel et al., 1997, Monfort et al., 1997). Females reach sexual maturity in their second year (at approximately 23 months of age) (Van Heerden et al., 1985, Creel et al., 1997, Courchamp and Macdonald, 2001) and the gestational length is approximately 70 days with females whelping in underground dens (Creel and Creel, 2002). Generally, only the alpha female produces pups, monopolising 75-96% of reproductive output (Frame et al., 1979, Creel et al., 1997) resulting in a high reproductive skew. Reproductive suppression of female subordinates is evidenced by no observable oestrous cycles (Van Heerden et al., 1985), however, *ex situ* physiological monitoring suggests reproductive suppression is behavioural rather than physiological (ovulation still occurs in subordinate females) (Van der Weyde et al., 2015). When subordinate females do breed, their pups can be killed by the dominant female, or can be reared alongside the alpha's pups in a communal nest (Creel et al., 1997, Frame et al., 1979).

Even though there are monitoring schemes and recovery plans in place for *in situ* populations, numbers of wild dogs are still in decline and *ex situ* conservation breeding programmes are therefore important for species survival. However, captive groups of wild dogs suffer from

high levels of aggression and poor reproductive performance (Van der Weyde et al., 2015, Yordy and Mossotti, 2016). The high level of aggression found within captive packs could be due to high levels of circulating glucocorticoids found in captive wild dogs (Creel, 2001). Indeed, Van der Weyde et al. (2016) found higher levels of faecal glucocorticoid metabolite concentrations in female wild dogs than their wild counterparts, suggesting a heightened stress of captivity.

Modern zoos are focused on conserving genetic diversity through conservation breeding programmes (EAZA, 2013). This has led to approximately one in seven threatened species being housed in zoos and aquaria (Fa et al., 2011). Modern zoos aim to house self-sustaining populations (Conde et al., 2011); however, the stress of captivity may have the potential to reduce reproductive output (Sapolsky, 2005, Creel and Creel, 2002) due to unnatural limited space, no dispersal and human interaction (Sherwen and Hemsworth, 2019, Morgan and Tromborg, 2007). Therefore, it is vital to use an evidenced based management approach to conservation breeding programmes in artificial *ex situ* institutions to both maintain a sustainable breeding group and to achieve the highest level of welfare. However, to date, little is known about aggression and reproductive performance in the European captive populations of wild dogs, and not all management practices are supported by an evidence base (Canessa et al., 2016). The second part of this thesis therefore investigates factors which affect reproductive success and aggression in the European population of African wild dogs to improve best practice guidelines.

## 1.7. Thesis overview

Gaining a broader understanding of proximate factors that promote social tolerance or aggression, or anxiety-related behaviour in group-living species has broad application, especially for animal keepers. It is particularly important for rearing endangered cooperatively breeding species in confined captive settings where replicating a dynamic and complex social group is near impossible but where ensuring reproductive sustainability is achieved.

The first part of this thesis uses the house mouse as a model species to decipher proximate mediators of social and anxiety-like behaviour. Conservation breeding programmes need to ensure genetic diversity is maintained and targeted breeding groups are paired together to achieve this. However, it is not always possible to recreate the natural complexity of social

groups found in cooperatively breeding mammals. The confines of captivity can result in small group sizes with few or no helpers, but it is unknown whether this affects offspring adult phenotypes. As cooperative breeding species often require helpers to successfully raise offspring and communal breeders can naturally rear offspring communally or alone, a key theme here was investigating whether offspring born in communal or single nest environments varied in their behavioural or physiological response to conspecifics or novel environments.

To enable the investigation of specific physiological markers mediating behaviour, validations of biochemical assays are required; therefore, this thesis starts by exploring methods to measure central and peripheral oxytocin in house mice (Chapter 2). Chapter 2 focuses on biochemical validations of a commercially available enzyme immunoassay (EIAs) used for the measurement of oxytocin in paraventricular nucleus homogenate, serum, and non-invasive oxytocin measurements in urine. Due to the small size of the study species and resultant urine volumes, extraction of oxytocin from urine was not possible and the relationship between extracted and un-extracted urinary oxytocin was investigated. These successful validations enabled further exploration into the relationship between peripheral oxytocin concentration and social behaviour in adult female house mice, and whether this was influenced by the early rearing environment (Chapter 3). Chapter 3 examines whether the early rearing environment, specifically being reared in a communal or single nest, influences social responses to unfamiliar conspecifics of varying relatedness, and the role of oxytocin in these social behaviours. Next, we investigated whether being reared in a communal or single nest influenced the behaviour of adult female house mice in response to a novel open field test (Chapter 4). In chapter 4, anxiety-related behaviour was investigated with corresponding physiological markers including oxytocin receptor and AVP receptor gene expression in central brain regions.

The second part of the thesis applies theoretical knowledge to gain greater understanding of problems facing captive endangered cooperatively breeding mammals, with a specific focus on African wild dogs. Social manipulations and invasive physiological monitoring are not possible in zoological settings and therefore non-invasive physiological measuring is required using faeces. Due to zoo management practices for carnivores, faecal samples are not always collected immediately following defecation, and collection times can be further delayed for identifiable samples from specific individuals. As faecal collection times can be so variable,

Chapter 5 investigates the degradation of faecal hormone metabolite concentrations in wild dog faecal samples over time in an *ex situ* environment (summer and winter months). Measured hormone concentrations significantly varied often within four hours for progesterone, glucocorticoids and testosterone.

It is important to ensure management practices for captive endangered species follow guidance that has an evidence base; however, little is currently known about varying management practices across the European population of captive wild dogs. Chapter 6 therefore investigates factors that may contribute to aggression and reproductive success in captive wild dogs. The chapter includes a survey of stud book data to include 20 years of wild dog births, and results of a husbandry questionnaire. Results of this study can be used to make informed management choices to improve breeding sustainability and welfare for this endangered species.

Together, this thesis aims to bring further understanding to factors that affect social and anxiety-like behaviour in cooperatively breeding mammals and to apply this to improve the management and reproductive success of endangered *ex situ* populations.

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## Chapter 2: Measurement of peripheral and central oxytocin concentrations in female house mice (*Mus musculus domesticus*)

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Abstract

Oxytocin is hypothesised to influence a broad range of social behaviours, and there is a need for non-invasive and non-destructive approaches to better understand its role in this context. Application of such approaches to model rodent species would be particularly beneficial to facilitate repeated sampling under naturalistic experimental conditions. However, there is currently a lack of consistency in methodological approaches for measuring peripheral concentrations of oxytocin, and evidence is lacking that peripheral concentrations reflect those at central sites of action. Here we successfully biochemically validate a commercially available enzyme immunoassay (EIA) for measuring oxytocin in the urine, serum, and paraventricular nucleus (PVN) of the hypothalamus of adult female house mice (*Mus musculus domesticus*). Small volumes of urine produced by mice in a single void resulted in concentrations outside the working range of the assay if samples were extracted. However, a significant correlation was found between oxytocin concentrations in raw and extracted urine, providing confidence that raw, non-extracted urinary oxytocin may be used to quantify urinary oxytocin in this species. By contrast, under baseline conditions, no significant correlation was found between peripheral concentrations of oxytocin in the urine or serum and central concentrations in the PVN. Hence we conclude that caution is required when interpreting the results of peripheral oxytocin assays, although correlations between peripheral and central measures of oxytocin are likely to be context dependent, and are not ruled out by this study.

Keywords

Enzyme Immunoassay, urinary oxytocin, non-invasive sampling, social behaviour, *Mus musculus domesticus*, validation.

Highlights

- An assay is validated to quantify oxytocin levels in wild house mice
- Oxytocin is quantified and compared in urine, serum and brain tissue samples
- Non-invasive sampling can be achieved with small volumes of urine
- This opens new opportunities for longitudinal studies of social behaviour

## 2.1. Introduction

Oxytocin and its related peptides are well conserved across the animal kingdom; all vertebrates display nanopeptide-secreting cells within the hypothalamus and preoptic area (Goodson and Thompson, 2010), and the release of these peptides modulates social behaviours in all species examined to date (Goodson, 2008). Oxytocin has been shown to influence social behaviour in a broad range of mammalian species from rodents to primates (Anacker and Beery, 2013, Crockford et al., 2014). For example, it has a role in mediating social bond formation and same sex huddling in prairie voles (*Microtus ochrogaster*) and meadow voles (*Microtus pennsylvanicus*) (Ross and Young, 2009, Beery and Zucker, 2010), maternal aggression in laboratory rodents (Bosch, 2013, Bosch and Neumann, 2012), positive social behaviours in grey seals (*Halichoerus grypus*) (Robinson et al., 2017), grooming and food sharing events in chimpanzees (*Pan troglodytes*) (Crockford et al., 2013, Wittig et al., 2014), cooperative behaviours in wild meerkats (*Suricata suricatta*) (Madden and Clutton-Brock, 2011), and family bonds in common marmosets (*Callithrix jacchus*) (Finkenwirth et al., 2015).

Oxytocin is synthesised by magnocellular neurones in the supraoptic and paraventricular nucleus (PVN) of the hypothalamus (Ross and Young, 2009). There are two mechanisms by which it is released; firstly, axonal release via the posterior pituitary into peripheral circulation to regulate parturition and lactation, and secondly, dendritic release into central brain regions (Baribeau and Anagnostou, 2015, Aguilera and Rabadan-Diehl, 2000, Landgraf and Neumann, 2004, Ross and Young, 2009, Leng and Ludwig, 2016, Leng and Sabatier, 2016). This latter centrally released oxytocin binds to receptors throughout the brain to elicit behavioural responses. Central oxytocin release is triggered by multiple stimuli including visual, auditory and tactile communication from a social partner (Neumann, 2008, Macdonald and Macdonald, 2010).

Investigating the role of oxytocin in social behaviour often requires its quantification under different experimental contexts, and non-invasive or non-destructive approaches may often be preferred, particularly under naturalistic conditions or field studies. To this end, oxytocin may be sampled in peripheral fluids including serum and plasma, or non-invasively in saliva and urine (McCullough et al., 2013). However, the extent to which peripheral oxytocin concentrations reflect central oxytocin activity is often unclear (Valstad et al., 2017). Previous studies have shown parallel oxytocin release both centrally and into peripheral circulation during specific situations, including the shaker stress tests in rats (Nishioka et al., 1998). However, there is also some evidence to suggest that central and peripheral release of oxytocin may be differentially regulated (Engelmann et al., 1999); hence there may be no link between the two (McCullough et al., 2013, Lefevre et al., 2017b). Furthermore, some researchers highlight the limited evidence that oxytocin can cross the blood-brain barrier, suggesting that peripheral oxytocin measures may be inaccurate predictors of central oxytocin concentrations (McCullough et al., 2013, Lefevre et al., 2017b). Nonetheless, non-invasive monitoring, including measuring urinary oxytocin concentration, is increasingly used to investigate the proximate mechanisms behind pro-social and competitive behaviours (Benitez et al., 2018, Crockford et al., 2013, Wittig et al., 2014, Samuni et al., 2017). In such studies, urinary oxytocin concentration is higher during cooperative events and is positively correlated with affiliative behaviour (Crockford et al., 2013, Snowdon et al., 2010). Moreover, the use of non-invasive measures is particularly valuable to facilitate longitudinal studies (Snowdon et al., 2010), where invasive monitoring is not possible.

When investigating non-invasive oxytocin measurements, it is important to conduct thorough validations (Szeto et al., 2011, McCullough et al., 2013). This is especially important when the majority of commercially available immunoassays are designed for human samples (McCullough et al., 2013, Schaebs et al., 2019), but are being applied to samples of different origin or species (Kelley and DeSilva, 2007). For example, it is possible that the matrix surrounding the analyte oxytocin within a sample could interfere with the concentration measured in an assay. Extraction removes interfering substances from the sample matrix, retaining and concentrating the target analyte of interest, and it has been suggested that this process is essential to obtain reliable measurements (Robinson et al., 2014, Szeto et al., 2011). A common protocol advised prior to oxytocin measurement is solid phase extraction, where the sample is applied to a cartridge that contains a hydrophobic reversed phase C18 chromatographic sorbent (Bienboire-Frosini et al., 2017). This retains oxytocin, which is

washed to remove unwanted interfering substances, and then the molecules of interest are eluted using a solvent such as acetonitrile (MacLean et al., 2019). However, both Brandtzaeg et al. (2016) and MacLean et al. (2019) conclude that extraction steps actually remove the majority of oxytocin from a sample by removing the protein to which active oxytocin is bound, possibly removing the active oxytocin of interest from a sample. Furthermore, solid-phase extraction protocols include a drying step which may include a raised temperature (Samuni et al., 2017), which could result in oxytocin degrading due to its labile nature (MacLean et al., 2019).

Given that extraction and elution may result in a loss of measurable oxytocin in a sample, its application could preclude non-invasive testing for small mammals such as mice, for which the small volumes of urine or serum available may result in unmeasurably low concentrations. It is therefore important to test whether extracted and non-extracted oxytocin concentrations are significantly correlated, since this would support analysis of oxytocin from raw matrices where necessary due to limited sample volumes. However, current evidence regarding the relationship between oxytocin concentrations from extracted and raw matrices is mixed (Szeto et al., 2011, Robinson et al., 2014, Michopoulos et al., 2011).

Mice and other rodents are useful models to investigate the physiology underpinning social behaviour (König and Lindholm, 2012, Ross et al., 2009, Beery and Zucker, 2010). To date though, most studies have applied destructive approaches to quantify central oxytocin or receptor levels in rodent models (Curley et al., 2009, Branchi et al., 2013a). To facilitate longitudinal studies of varying oxytocin levels, a reliable approach to measure oxytocin non-invasively would be beneficial. However, published methods for the extraction of oxytocin from urine require a volume ranging from 3ml to 200ul (Samuni et al., 2017, Crockford et al., 2014, Wittig et al., 2014), whereas house mice typically produce as little as 50ul of urine following a behavioural test. As oxytocin concentration within urine needs to be normalised to creatinine concentration to account for urinary dilution (Beynon and Hurst, 2004), the volume of urine left for oxytocin analysis is further reduced. Currently it is unknown whether extracting oxytocin from such small urine volumes is feasible, or whether there is a relationship between extracted and non-extracted urinary oxytocin in this model species. House mice also provide an ideal study species to investigate the relationship between peripheral and central oxytocin concentration within the same individuals. However, it is

currently unknown whether central oxytocin concentration predicts peripheral levels in this species, or *vice versa*.

The aims of this study are: 1) to biochemically validate a commercially available oxytocin EIA for the measurement of oxytocin concentrations in urine, serum and PVN homogenate of female house mice, 2) to determine the suitability of this EIA for the measurement of oxytocin in urine of female house mice, particularly the challenge of performing measurements in small volumes of urine, and 3) to investigate the relationship between central and peripheral oxytocin, by testing whether central oxytocin concentrations within the PVN correlate with peripheral oxytocin concentration in serum and urine.

## 2.2. Materials and Methods

### *2.2.1. Subjects*

Samples were collected from captive bred adult female house mice (*Mus musculus domesticus*), derived from wild populations in the North West of England. Mice were housed in sister pairs in MB1 cages (45 x 28 x 13cm, North Kent Plastics, UK) on Corn Cob Absorb 10/14 substrate, paper wool nesting material (Shredded Nesting International Product Supplies Ltd., London, UK) with *ad libitum* water and food (Lab diet 5002, International Product Supplies Ltd., London, UK). Within the MB1 cages, mice were provided with cardboard tubes or boxes and plastic mouse houses (Techniplast UK Ltd) as enrichment. Animals were housed at 20-21$^{o}$C with 45-65% relative humidity on a reversed 12:12 hour light cycle with lights going off at 0800. Mice were handled using handling tubes to minimise handling stress (Hurst and West, 2010).

### *2.2.2. Sample collection and processing*

To investigate the relationship between peripheral and central oxytocin concentrations, PVN homogenate, serum and urine samples were collected from adult female mice (n = 26). Inadequate sample volume or assay inaccuracies (with inadequate sample volume to repeat) resulted in 24 PVN, 25 urine and 19 serum samples. To collect urine, individual mice were placed on a metal grate above a transparent MB1 cage, and left for a maximum of 1.5 hours, allowing urine to fall through the bars into the clean cage below between 0830 and 1000. Urine was then collected using a pipette, avoiding faecal contamination, transferred into 1.5ml microcentrifuge tubes (Fisherbrand, UK), and stored at -80$^{o}$C until use. To obtain central

oxytocin parameters, mice were humanely killed by a trained animal technician, using an approved Schedule 1 procedure. Cardiac puncture was performed post-mortem using a 5/8" 25G sterile needle (BD Microlance needles, UK) and 1ml sterile syringe. Whole brains were removed within 5 minutes and frozen in hexane on dry ice before storage at -80°C. Whole blood was placed into an ice-cold 1.5ml microcentrifuge tube containing 500KIU of Aprotinin (Sigma, UK) per ml of blood. Blood remained at room temperature for no more than 30 minutes, allowing for clotting, before being centrifuged at 1600xg for 15 minutes at 4°C. Following centrifugation, serum was transferred to a chilled 1.5ml microcentrifuge tube and stored at -80°C.

Whole brains were sectioned using a Leica Cryostat at -20°C, using stereotaxic coordinates (Paxinos and Franklin, 2001) for reference. The PVN was removed at Bregma -0.34mm using a 1mm biopsy punch (Selles Medical, UK), placed into a frozen 1.5ml microcentrifuge tube and quickly transferred to dry ice before storage at -80°C. To homogenise the PVN tissue, 100ul of ice-cold RIPA buffer (ThermoFisher Scientific, UK) containing a 1:100 Halt™ protease inhibitor cocktail (ThermoFisher Scientific, UK) was added to each PVN sample. The sample was drawn up and down using a Gilson pipette (Gilson Pipettes, UK) for 2 minutes on ice until the tissue was homogenised. The samples were then incubated for 15 minutes on ice, centrifuged at 12000xg for 15 minutes at 4°C and stored at -80°C. If a pellet formed, the supernatant was removed and stored at -80°C (Tait et al., 2009, Zhang et al., 2015).

*2.2.3. Urinary oxytocin extraction*

To test for a relationship between extracted and non-extracted oxytocin from urine, 18 urine pools were created by combining urine samples from a different set of adult female mice that were housed under standard conditions in the captive colony (n = 2-8 mice per urinary pool, n = 47 mice in total) collected between 0830 and 1000. Each pool was split into two: one for extraction and one for unextracted urinary oxytocin analysis. Oxytocin was extracted from urine using a modified solid-phase extraction technique (Crockford et al., 2013, Samuni et al., 2017). Accordingly, urine was acidified with 10% 0.5N phosphoric acid (Fisher Scientific, UK), vortexed for 10 seconds and diluted 1:2 with 0.1% trifluoroacetic acid (TFA) (Acros Organics, UK) before being centrifuged at 17,000xg for 15 minutes at 4°C. C18 Sep-Pac columns (Waters, UK) were equilibrated with 1ml 100% methanol (Fisher Scientific, UK) followed by 1ml $ddH_20$. Acidified, centrifuged urine was then added to the C18 column using a 1ml sterile syringe at a flow rate of approximately 1ml per minute. The C18 cartridges were then washed with 1ml

10% acetonitrile (ACN) (Sigma, UK) containing 0.1% TFA in water before the oxytocin was eluted with 1ml 80% ACN into test tubes. Samples were placed in a 50°C water bath and dried down under an air stream. Once dry, samples were reconstituted with 300ul 100% ethanol (Sigma, UK), vortexed for 10 seconds and left at 4°C for 1 hour. Samples were dried down a second time using an air stream at 50°C as described above. Once dry, samples were stored at -20°C until use. To investigate whether oxytocin extraction can be achieved from small volumes of murine urine, a further pool of urine was collected by combining urine samples from adult female mice (n = 10) and placed into aliquots of varying volume ranging from 200ul to 10ul of urine. Oxytocin was extracted from these samples as detailed above.

*2.2.4. Protein assay*

Oxytocin concentration within serum could be influenced by hydration of the subject at the time of sample collection. To ensure serum oxytocin concentration was comparable between different subjects, it was normalised to the serum protein concentration. In addition, since we assume that each 1mm micro-punch of PVN tissue contained slight variation in the precise weight of hypothalamic tissue sampled, oxytocin was normalised to protein concentration within the final eluted PVN homogenate. Prior to quantifying protein concentration accurately, an approximate concentration was estimated for serum and PVN to enable appropriate dilutions to be made prior to conducting the EIA. A 1µl of sample (serum or PVN) was pipetted onto nitrocellulose paper alongside protein (bovine serum albumin) standards (1mg/ml and 2mg/ml). These were left to dry for 5 minutes before Ponceau S stain was added, followed by a further 5-minute incubation period. The Ponceau S stain was then washed off thoroughly with $ddH_20$, and protein content was determined by visual comparison with the standards. The protein concentration within the PVN homogenate and serum samples was then quantified using a Coomassie plus® protein assay reagent kit (Perbio Science, UK), (Cheetham et al., 2007). PVN homogenate was diluted 1:200 and serum was diluted 1:100 with $ddH_20$. Briefly, 100µl of sample was pipetted in duplicate into a 96 well plate (Sterilin Microplate F Well 611F96, Thermo Fisher, Finland), and standards of bovine serum albumin (BSA) (concentration range 0-50 µg/ml) prepared from a stock solution of 2mg/ml were added to the same plate. Following this, 200µl of Coomassie reagent was added to each well and the absorbance was read at 620nm using a Thermo Scientific Multiskan FC microplate photometer (Thermo Fisher, Finland). The protein concentration for each sample was calculated by interpolation from the standard curve. Oxytocin levels in tissue extracts and serum were expressed as pg/mg total protein.

*2.2.5. Creatinine assay*

To account for varying urine concentration, urine creatinine concentrations were measured. Creatinine is excreted in mouse urine at a constant rate and creatinine levels are often used as a reference point to correct for the concentration of substances within mouse urine (Beynon and Hurst, 2004). To determine creatinine concentration, an alkaline picrate assay (Sigma Chemicals, UK) was conducted using a 96 well microtiter plate (Sterilin Microplate F Well 611F96 Thermo Fisher, Finland). 10µl of urine samples were diluted 1:50 with $ddH_20$, and 100µl aliquots were plated in duplicate. A standard curve (0, 5, 10, 15, 20 and 30 µg/ml) produced by serial diluting a stock creatinine solution (0.03mg/ml) was performed in duplicate on the same plate. 150µl of alkaline picrate reagent (0.65% Picric Acid/ 10mM Sodium Tetraborate/ 0.1M Sodium Hydroxide) was then added to each well. Absorbance was read at 492nm using a Thermo Scientific Multiskan DC microplate photometer (Thermo Fisher Scientific, Finland). Urinary oxytocin levels were expressed as pg/mg creatinine.

*2.2.6. Oxytocin enzyme-linked immunosorbent assay*

Oxytocin was measured in serum, PVN homogenate and urine using a commercially available EIA (Enzo, USA) following manufacturer's instructions. Prior to commencing the assay, all reagents were brought to room temperature for 30 minutes. Extracted dry urine samples were reconstituted with 250µl of supplied assay buffer and vortexed for 10 seconds. Briefly, 100µl of standard and sample were plated in duplicate onto a goat anti-rabbit igG Microtiter plates, followed by 50µl of oxytocin conjugate (alkaline phosphatase conjugated to oxytocin) and 50µl of rabbit polyclonal antibody to oxytocin, then left to incubate at 4°C for 18 hours. Next, the plates were emptied and washed three times using the supplied wash buffer before being blotted with lint-free paper. 200µl of para-Nitrophenylphosphate (pNpp) was then added to each well, and the plate was left to develop for 1 hour before 50µl of stop solution was added. The plate was then read immediately at 405nm. All samples were run in duplicate, alongside a standard curve in triplicate (15.6, 31.2, 62.5, 125, 250, 500 and 1000pg/ml). Intra-assay CVs were less than 10% for all samples and inter-assay CVs were 17.15% for high quality control (45.64%b/Bo, 500pg/ml) and 19.25% for low quality control (82.83%b/Bo, 62.5pg/ml).

*2.2.7. Biochemical validation*

First, a serial dilution of pooled sample (PVN, serum or urine) was run in duplicate alongside a serial dilution of synthetic antigen standard (in triplicate) for standard concentrations of

15.6, 31.2, 62.5, 125, 250, 500 and 1000pg/ml. The assay was run following the protocol detailed above. Parallelism was assessed by visual inspection of the displacement curves of antigen within the sample and the standard curve of serial diluted synthetic antigen. This demonstrates that the antigen within the varying samples is immunologically similar to the supplied synthetic oxytocin standard, and the EIA is successfully measuring oxytocin. From this, the correct working dilution of sample was ascertained to achieve approximately 50% binding in the assay (PVN and serum 1:8, urine 1:10). To assess whether components within each matrix were causing non-specific interference, a matrix interference assessment was conducted. Serial dilutions of synthetic oxytocin standard (PVN: 125, 250, 500 and 1000 pg/ml, serum and urine: 15.6, 31.2, 62.5, 125, 250 and 500pg/ml): were spiked with equal volumes of working dilution of sample (PVN and serum 1:8, urine 1:10). Samples were corrected for non-specific background effects and then compared with synthetic standard oxytocin.

*2.2.8. Statistical analysis*

Biochemical assay validation including parallelism and matrix interference were confirmed using linear regression analysis. Data had a non-Gaussian distribution. Correlations were therefore analysed using Spearman's rank tests, and comparisons between extracted and non-extracted urinary oxytocin concentrations were conducted using Wilcoxon signed rank tests.

## 2.3. Results

*2.3.1. Biochemical validation of the oxytocin EIA*

Biochemical validation was achieved using a commercially available oxytocin EIA for samples from female house mice. Successful parallelism was achieved for (A) PVN homogenate over the 37-91% binding range, (B) serum over the 32-87% binding range and (C) urine over the 37-98% binding range, as confirmed by linear regression analysis (Figure 2.1, Table 2.1: $p<0.001$ for all three matrices). No significant interference was found for any of the matrices tested, as confirmed by linear regression analysis (Figure 2.2, Table 2.1: PVN: $p<0.05$, serum: $p<0.001$, urine: $p<0.001$). Recovery was 83.2±17.2% (mean plus SEM) for PVN, 187.6±20.8% (mean plus SEM) for serum and 120.2±8.5% (mean plus SEM) for urine.

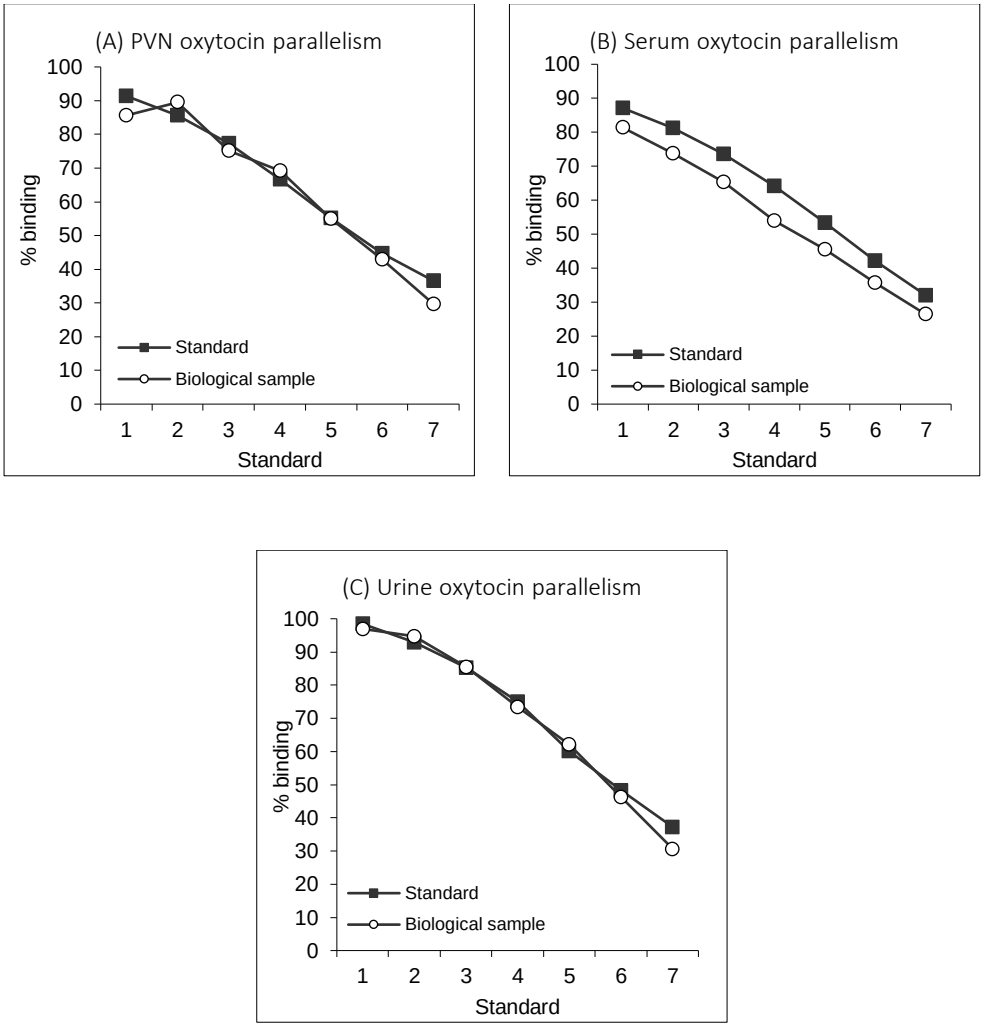


*Figure 2.1: Displacement curves showing significant parallelism of dose response between (a) PVN homogenate oxytocin concentration, (b) serum oxytocin concentration and (c) urine oxytocin concentration, to a standard synthetic oxytocin. Significant parallelism was achieved for all matrices ($p<0.001$).*

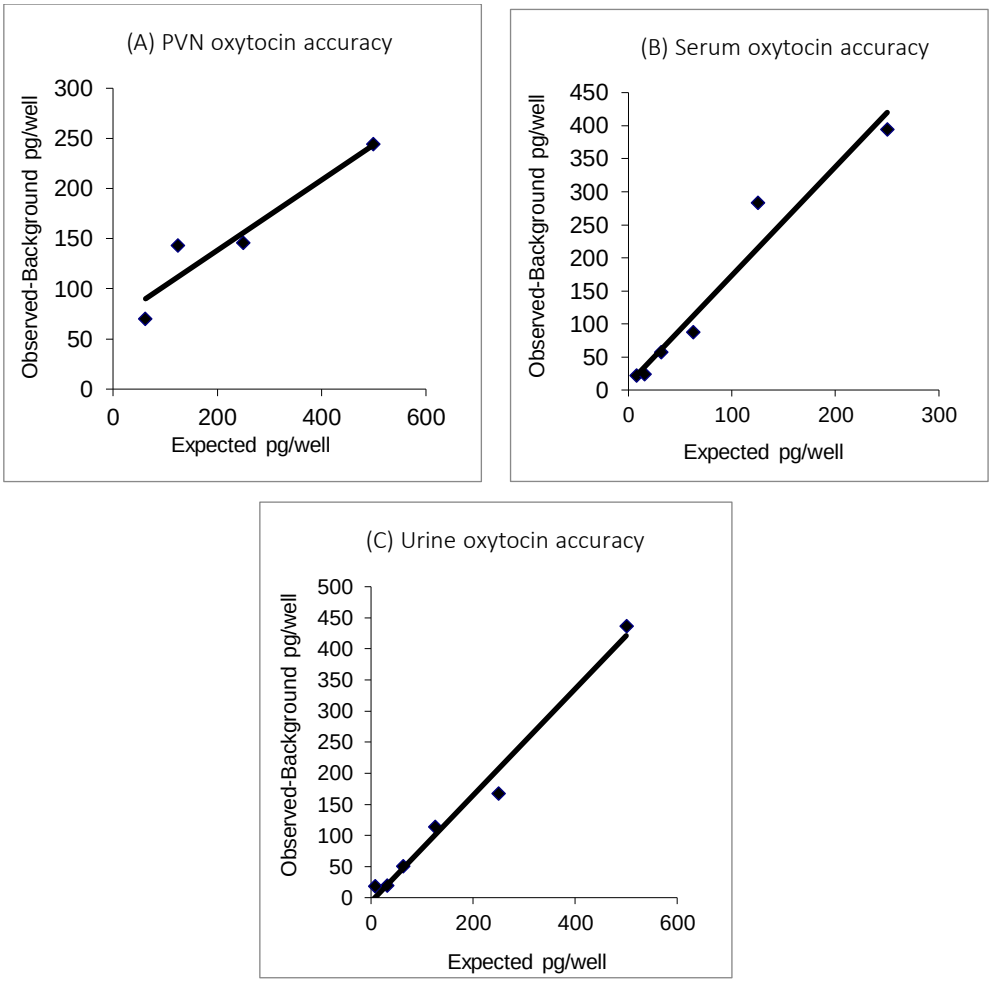


*Figure 2.2: Matrix interference assessment for (A) paraventricular nucleus (PVN) homogenate ($p < 0.05$), (B) serum ($p<0.001$) and (C) urine ($p<0.001$) oxytocin. No significant interference was found for any of the matrices examined, as confirmed by linear regression.*

*Table 2.1: Logistic regression analysis of the successful parallelism and matrix interference assessment for paraventricular nucleus (PVN) homogenate oxytocin, serum oxytocin and urine oxytocin for female house mice.*

| Biological sample | Parallelism | Matrix interference assessment |
|---|---|---|
| PVN homogenate | Sample % binding = 0.88 (standard % binding) + 15.4<br>$R^2 = 0.95$, $F_{1,5} = 89.6$, $p<0.001$ | Observed = 0.35 (Expected) + 68.2<br>$R^2 = 0.91$, $F_{1,2} = 18.9$, $p < 0.05$ |
| Serum | Sample % binding = 0.99 (standard % binding) + 6.0<br>$R^2 = 0.99$, $F_{1,5} = 4735$, $p<0.001$ | Observed = 1.64 (Expected) + 9.4<br>$R^2 = 0.95$, $F_{1,4} = 74.9$, $p<0.001$ |
| Urine | Sample % binding = 1.07 (standard % binding) – 6.4<br>$R^2 = 0.99$, $F_{1,5} = 562$, $p<0.001$ | Observed = 1.09 (Expected) + 11.30<br>$R^2 = 0.99$, $F_{1,5} = 984.1$, $p<0.001$ |

*2.3.2. Urinary oxytocin extraction*

Following the extraction procedure, the measured oxytocin concentration decreased from 1073 ± 510 pg/mgCr to 166 ± 78 pg/mgCr (mean ± SD) (Figure 2.3A: Wilcoxon signed rank test: v = 0, p<0.001, n = 18). This resulted in concentrations of oxytocin outside the working range of the assay for the low volumes of urine produced by mice in one void (assay sensitivity 15pg/ml). However, a significant correlation was found between extracted and non-extracted urinary oxytocin from the same urinary pools (Figure 2.3B: Spearman's rho = 0.56, p < 0.02, n = 18).

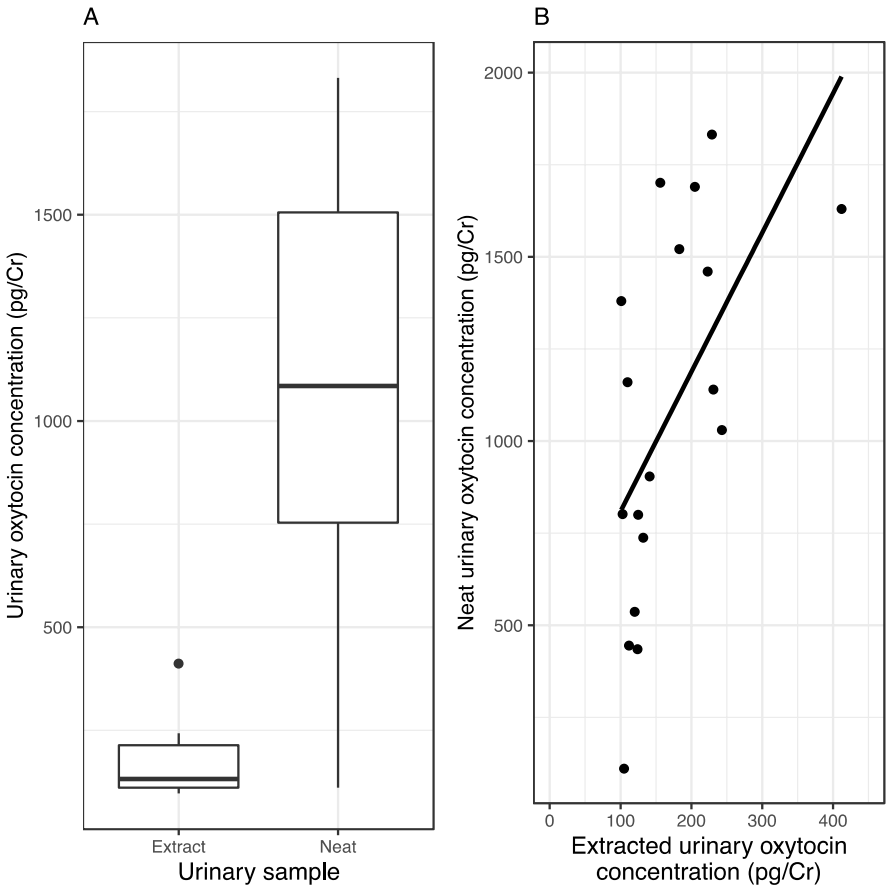


*Figure 2.3: A: Urinary oxytocin concentration was significantly lower in extracted urine samples compared to non-extracted neat urine samples (p < 0.02, n = 18). B: Significant correlation between extracted urinary oxytocin concentration and neat urine oxytocin concentration (p < 0.001, n = 18).*

*2.3.3. Central and peripheral oxytocin concentration*

Mean PVN homogenate oxytocin concentration was 607pg/mg of protein, whereas serum oxytocin concentration was nearly 10 times lower at 62.7pg/mg protein. No significant relationship was found between oxytocin concentration in central PVN homogenate and serum (Figure 2.4A: Spearman's rho = 0.24, p = 0.34, n = 18). Moreover, no relationship was found between oxytocin concentration in urine that was collected 24 hours prior to culling and in PVN homogenate or serum (Figure 2.4B: serum: Spearman's rho = 0.04, p = 0.86, n = 18, Figure 2.4C: PVN: Spearman's rho = 0.293, p = 0.17, n = 23).

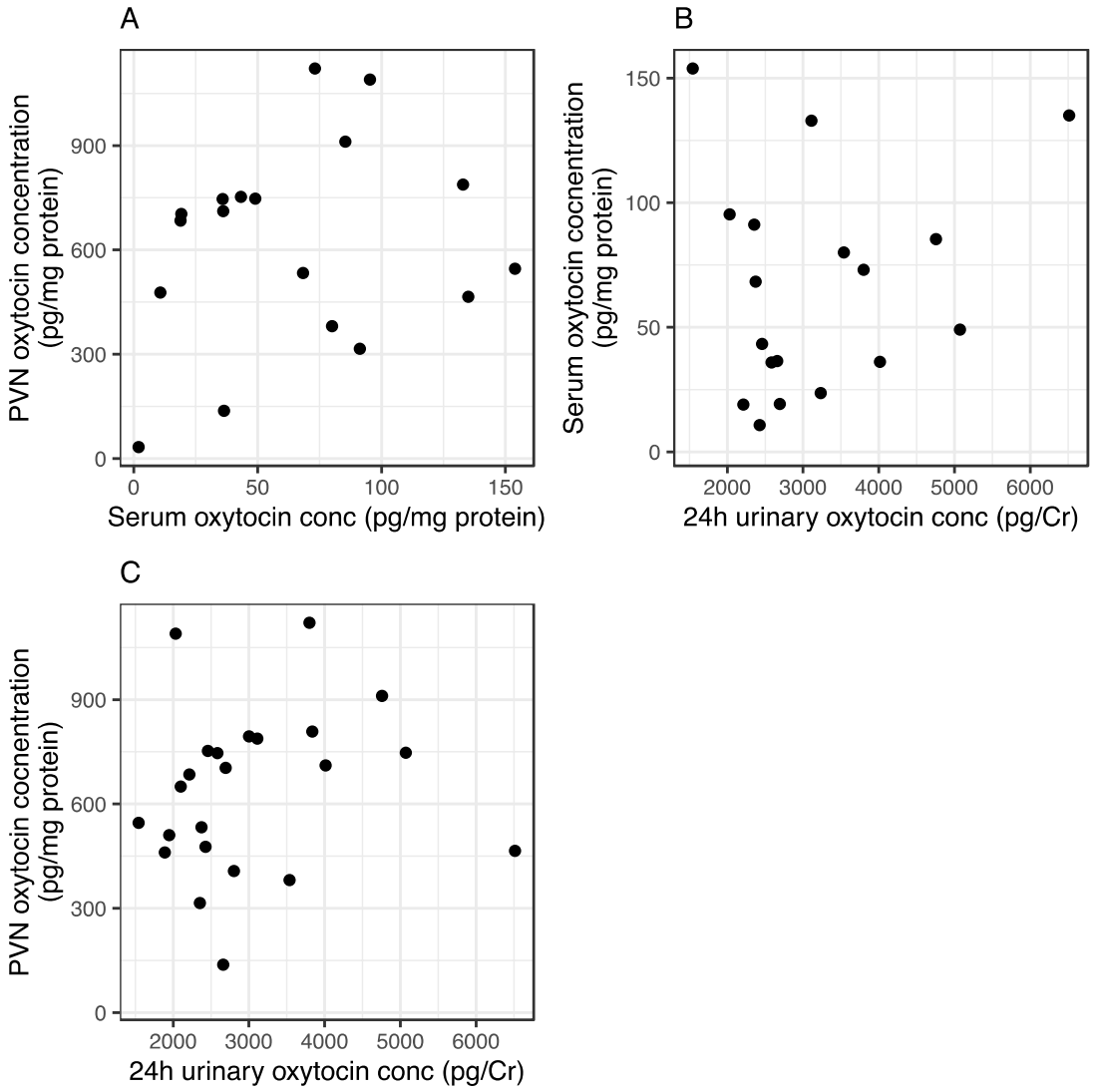


*Figure 2.4: No significant relationship was found between oxytocin concentration in (A) serum and PVN homogenate (p = 0.34), (B) urine that was collected 24 hours prior to culling and serum (p= 0.86) or (C) urine that was collected 24 hours prior to culling and PVN homogenate (p = 0.17).*

## 2.4. Discussion

In this study, we have biochemically validated a commercially available oxytocin EIA to accurately measure oxytocin concentration in samples from adult female house mice. The assay was applied to quantify both central and peripheral oxytocin levels, in PVN homogenate, serum and urine respectively. House mice are a model species for studies of social behaviour (König and Lindholm, 2012, Stockley et al., 2013, Hurst, 1987), and the availability of a non-invasive assay to quantify variation in oxytocin levels in urine is therefore likely to be of particular interest to researchers studying behaviour patterns under naturalistic conditions.

Parallelism is an important validation criterion for immunoassays. The parallelism of the response of pure standard oxytocin with dilutions of samples in the current study indicates that material within the sample is binding to the specific antibody with the same affinity as pure oxytocin, and that the assay is measuring oxytocin within the sample. There is always potential for non-specific interference in binding of the antigen to the antibody, particularly in urine where high salt levels are known to interfere (Leng and Ludwig, 2016). However, here the observed parallelism suggests that the EIA is indeed measuring oxytocin or a component containing an epitope of similar structure to oxytocin. The significant linear regressions and high $R^2$ examining potential matrix effects also suggested that there was no interference. These results show that the EIA can be used to measure oxytocin within each of the different matrices examined.

The physiological validation of immunoassays is difficult for many hormone analytes. Such validation often includes physiological manipulation of the endocrine system, for example by the pharmacological administration of synthetic antigen of interest before measurements are conducted (Polito et al., 2006). An alternative method is therefore a biological validation where a hormone of interest is measured before and after a physiological event that is known to alter the concentration of the analyte. For example, for oxytocin, it is widely accepted that circulating concentrations will be higher during early lactation than under baseline conditions (i.e. during a non-reproductive time) (Folley and Knaggs, 1966), providing a straightforward physiological validation for oxytocin immunoassays. However, in the current study, collecting urine from early post-natal mice was not possible due to the high risk of infanticide in wild house mice if disturbed at this time. Experiments such as this would enable the confirmation that assays are able to detect biological meaningful changes in the oxytocin system (Palme,

2019). In addition, the measurement of oxytocin in the PVN homogenates in the current experiments may not indicate levels of active oxytocin and we may have been measuring inactive, stored PVN oxytocin content, which may not reflect oxytocin release. An alternative method for measuring central biologically active oxytocin levels could be the use of autoradiography to measure receptor-bound oxytocin, this would enable the investigation of total and active (specific oxytocin receptor-bound) within specific brain regions (Curley et al., 2009). Alternatively, measurements of central released oxytocin could be measured within the cerebrospinal fluid (CSF) (Lefevre et al., 2017a). However, due to the method of euthanasia used in the current study (cervical dislocation), it was impossible to collect CRF that was not contaminated with blood.

We found that extracting oxytocin from the small urine volumes typically produced by individual female house mice resulted in concentrations outside the working range of the assay. However, a significant and positive relationship was found between extracted and non-extracted urinary oxytocin. This is consistent with previous evidence for a significant correlation between oxytocin concentrations from extracted and raw matrices (Michopoulos et al., 2011), including urine (Leeds et al., 2018). By contrast, some studies have found no relationship between extracted and non-extracted peripheral plasma samples (Szeto et al., 2011, Robinson et al., 2014). The nearly 10-fold difference in concentration between extracted and non-extracted urinary oxytocin concentrations found in the current study could suggest that oxytocin is metabolised, resulting in other substances within urine also reacting with the assay (Schaebs et al., 2019). Indeed, it is likely that the assay is measuring immunoreactive oxytocin species including protein-bound oxytocin, degraded oxytocin and oxytocin precursors (Brandtzaeg et al., 2016, MacLean et al., 2019). On the other hand, our findings could suggest that the extraction procedure is actually removing important and active oxytocin that is bound to proteins (Brandtzaeg et al., 2016, MacLean et al., 2019), particularly as, unusually, murine urine contains a large amount of protein (Beynon and Hurst, 2003).

It has been questioned whether peripheral oxytocin concentrations are indicative of central oxytocin concentrations (McCullough et al., 2013). Consistent with this, we found no significant relationship between central and peripheral oxytocin concentrations in samples for female mice, although it is important to emphasise that these samples were taken under baseline conditions (i.e. independent of any specific social or other stimulus) and, in the case of urine and PVN/serum, 24 hours apart. Nonetheless, our measure of central oxytocin

concentration in PVN homogenate was not significantly correlated with peripheral measures in serum or urine. Under baseline conditions, it is thought that central and peripheral release of oxytocin may be differentially regulated (Engelmann et al., 1999), and there are both parallel and independent surges of oxytocin released centrally and from neuro-hypophysial terminals into the peripheral circulation (Insel, 2010). However, there is evidence to suggest that, at specific times, central and peripheral oxytocin concentrations are related, although this is context dependent. For example, Nishioka et al. (1998) have shown parallel oxytocin release centrally and into peripheral circulation following shaker stress tests in rats. Similarly, Neumann et al. (2013) found significant positive relationships between central (hippocampal micro-dialysis) and peripheral (plasma) oxytocin concentrations following nasal administration of synthetic oxytocin in rats, although no such relationship was found in mice. Carson et al. (2015) also found significant relationships between cerebrospinal fluid and plasma oxytocin concentration in children undergoing a stressful lumbar puncture. Hence, it's likely that similar correlations were not found in the current study because samples were collected during a baseline period (Valstad et al., 2017). It would therefore be useful to investigate whether a relationship between central and peripheral oxytocin measures is found for female house mice where samples are collected following a stressful event, or more interestingly still, following a social bonding experience, both of which are known to induce oxytocin release. Such an effect may explain why numerous studies have found evidence that urinary oxytocin concentrations are significantly related to specific behaviours which appear to reflect central activity of the hormone (Crockford et al., 2014, Wittig et al., 2014, Samuni et al., 2017, Crockford et al., 2013, Snowdon et al., 2010, Finkenwirth et al., 2015, Benitez et al., 2018, Leeds et al., 2018). The lack of relationship between urinary and serum oxytocin concentration in the present study might also be partly explained by a time difference of 24 hours in sample collection. Nonetheless, previous studies have also found urine and blood oxytocin concentrations to be unrelated (Feldman et al., 2011). Moreover, urinary oxytocin concentration may represent an average over time since the last urine void, rather than the specific time point that blood was collected.

In conclusion, we have shown that a commercially available EIA is able to measure oxytocin concentration in PVN homogenate, serum and urine from adult female house mice. In line with previous literature, no relationship was found between central and peripheral oxytocin concentrations under baseline conditions. Non-invasive measurement of oxytocin in urine is a useful tool to study mechanisms of social behaviour, although the volume of urine produced

by mice is inadequate for successful extraction of oxytocin. Nonetheless, there is a significant relationship between extracted and non-extracted urinary oxytocin, validating the use of this EIA to measure total oxytocin in murine urine.

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Ethical note

Animal use and care was in accordance with the EU directive 2010/63/EU and UK Home Office code of practice for the housing and care of animals bred, supplied or used for scientific purposes.

Declaration of interest

None.

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# Chapter 3: Nepotism in the absence of social familiarity linked to urinary oxytocin levels in female house mice

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Abstract

Behaviour that promotes the fitness of relatives is fundamental to kin selection and may be expressed in the absence of social familiarity through kin discrimination. Proximate factors explaining individual variation in this behaviour are not well understood but in mammals important influences may include early life experience and the oxytocin system. The house mouse (*Mus musculus domesticus*) is an ideal experimental subject to investigate variation in nepotistic behaviour, with an ability to discriminate kin and a complex social system involving cooperative offspring care. Communal nursing of litters by closely related females could influence how offspring respond to unfamiliar relatives in later life, via increased opportunities for behavioural imprinting on familiar kin, or through effects of increased maternal care on the oxytocin system. We tested whether 1) the experience of being reared communally, and 2) urinary oxytocin levels prior to social interaction, explained variation in the expression of nepotistic behaviour by female house mice towards unfamiliar individuals of differing relatedness. Overall, more affiliative behaviour was directed to kin, and more aggressive behaviour to non-kin, consistent with known genetic mechanisms of kin discrimination in house mice. We found no evidence that variation in nepotistic behaviour is related to rearing environment, or that communally reared offspring are better able to discriminate full from three-quarter sisters. However, variation in nepotistic behaviour was significantly related to urinary oxytocin levels, independent of rearing environment. Subjects with higher urinary oxytocin levels directed more affiliative behaviour to full sisters, but not to three-quarter sisters or unrelated females. By contrast, no relationship was found between urinary oxytocin levels and the differential expression of aggressive behaviour. We hypothesise that variation in urinary oxytocin levels may reflect differences in the strength of social bonds formed by subjects with familiar sisters in their home environment. This could explain why females with higher oxytocin levels reacted positively to unfamiliar full sisters, which were likely to most closely resemble their familiar social partner. We conclude that oxytocin-mediated responses to unfamiliar relatives could be an unappreciated factor influencing nepotistic behaviour among species with an ability to discriminate kin and form social bonds.

Keywords

Cooperative breeding, kin discrimination, kin selection, oxytocin, maternal effects.

## 3.1. Introduction

The social behaviour of vertebrate animals spans a broad range of social systems and complexity, with a high degree of behavioural flexibility (Kappeler, 2013). Among group-living vertebrates, social interactions are typically characterised by a tension between affiliative and competitive behaviours. Kin selection theory predicts that interactions with relatives should be more cooperative and less competitive, because they share a relatively high proportion of genes (Hamilton, 1964a, Hamilton, 1964b, Hamilton, 1970, West et al., 2002). Hence by helping a close relative to reproduce, an individual is indirectly passing on some genes to the next generation. These general principles for understanding patterns of social behaviour among kin are particularly relevant to social vertebrates with sex-biased dispersal, where individuals of contrasting relatedness are often in competition for limited resources. In mammals, females are more often the philopatric sex, and intrasexual competition for resources and reproductive opportunities can be intense (Stockley and Campbell, 2013, West et al., 2001, Clutton-Brock et al., 2006b).

Although differential social responses to kin are predicted at an evolutionary level, the factors influencing such responses at a proximate level are poorly understood. Importantly, kin selection requires differential expression of nepotistic behaviour towards relatives, which in turn requires distinguishing kin from non-kin. This can be achieved via a variety of mechanisms (Tang-Martinez, 2001), at least two of which are potentially sensitive to variation in early life experience. Firstly, individuals may learn to recognise individual kin during critical periods of development when close relatives interact (Holmes and Sherman, 1982). For example, cross fostering experiments in house mice suggest early-life familiarity is an important mechanism for promoting cooperation among adult sisters (König, 1994b). Alternatively or additionally, individuals might compare the genotype of an unfamiliar animal with that of familiar relatives, such that kin discrimination is facilitated through behavioural imprinting on the phenotype of close relatives during rearing (Paterson and Hurst, 2009, Beecher, 1982). In this case, the effectiveness of phenotype matching may be influenced by the diversity of relatives encountered during the sensitive period of development. For example, in mammals with communal rearing of young, offspring may be reared by more than one related female and with litter mates of varying relatedness (Hayes, 2000). Hence, compared to individuals reared by their mother only, those reared by a mother and an aunt, with both full and half (or three-quarter) siblings, might be better able to discriminate unfamiliar kin of varying relatedness.

Early life experience is also known to influence the development of social behaviours in mammals (Molet et al., 2014, Branchi, 2009, Dantzer et al., 2013, Meaney, 2001), which in turn might influence interactions with kin. For example, in laboratory mice, subjects reared in a communal nest by two females (as occurs naturally in wild house mice (König, 1994b, König, 2006)) have significantly more oxytocin receptor binding sites in multiple brain regions compared to those reared in a single-nest setting (Curley et al., 2009). This suggests the early rearing environment could have widespread effects on social behaviour, including differential responses to kin, via the oxytocin system, which has an important role in mediating social interactions and social recognition (Young and Wang, 2004, Anacker and Beery, 2013, Beery and Zucker, 2010, Mooney et al., 2014, Lukas et al., 2011).

Oxytocin plays a central role in social bonding behaviour of mammals, facilitating social discrimination by helping to establish remembered social recognition towards odour cues from specific individuals (Brennan and Kendrick, 2006). Although most well studied in relation to bonding between mothers and offspring or monogamous mating partners (Young and Wang, 2004), variation in oxytocin levels or receptivity can also influence pro-social behaviours among other conspecifics (Beery and Zucker, 2010, Mooney et al., 2014). For example, huddling with preferred social partners increases with centrally administered oxytocin in female meadow voles (Beery and Zucker, 2010), and natural levels of oxytocin (measured in urine) are higher in wild chimpanzees after grooming with preferred partners (Crockford et al., 2013). Furthermore, oxytocin is important in the neuroregulation of female aggression, including maternal aggression towards nest intruders (Bosch, 2013), aggressive social behaviours towards group outsiders, and evicting kin from social groups (Anacker and Beery, 2013).

Oxytocin is synthesised in the paraventricular nucleus of the hypothalamus, where it is released dendritically to elicit central behavioural responses (Baribeau and Anagnostou, 2015). Oxytocin is also released into peripheral circulation at the posterior pituitary (Ross and Young, 2009) where it can be measured in plasma / serum (Robinson et al., 2014), or non-invasively in urine (Polito et al., 2006). It is unclear whether measurements of peripheral oxytocin concentrations reflect those centrally (Valstad et al., 2017); however, multiple studies highlight correlations between urinary oxytocin and social behaviour (Crockford et al., 2014, Wittig et al., 2014, Samuni et al., 2017, Crockford et al., 2013, Finkenwirth et al., 2015).

The house mouse (*Mus musculus domesticus*) is an ideal model species to explore how social responses to unfamiliar relatives are influenced at a proximate level. It is a highly social species, typically living in territorial groups consisting of a dominant male and several breeding and non-breeding females (König, 2006, König, 1994b). Females often rear their young communally, with two or more females combining their litters and nursing offspring indiscriminately (Manning et al., 1995, Sayler and Salmon, 1971, König, 2006, König, 1989). However, since not all offspring are reared in communal nests (Ferrari et al., 2019), the early rearing environment experienced by young house mice is variable, with potentially significant consequences for behavioural development (Branchi et al., 2013a, Curley et al., 2009). House mice are found in populations of varying relatedness (König and Lindholm, 2012), and it is common for females to encounter both kin and non-kin within natural environments. Consistent with kin selection theory, females prefer close relatives as nesting partners (König, 1994b), with evidence of kin discrimination based on the use of highly polymorphic genetic markers. Specifically, female house mice prefer nest partners that share their own major urinary protein (MUP) phenotype (Green et al., 2015). Kin recognition is therefore achieved via phenotype matching, regardless of prior familiarity, allowing for potentially widespread differential responses to conspecifics based on relatedness.

Here we use an experimental approach to investigate how the social responses of female house mice when meeting unfamiliar conspecifics are influenced by their early rearing environment and hormone profiles. Specifically, we investigate whether affiliative and aggressive behaviours differ in response to conspecifics of varying relatedness, and whether the extent of these different responses are influenced by the early rearing environment (communal or single nest rearing) and / or are related to naturally occurring variation in peripheral oxytocin levels.

## 3.2. Methods

### *3.2.1. Subjects*

House mice used in this study were from a captive colony derived from wild populations originating from North West England, UK. Subjects had ancestors bred with wild-caught animals in the previous one to three generations. The colony was maintained under controlled environmental conditions (20-21$^{o}$C, 45-65% relative humidity and 12:12 hour light-dark cycle,

with dark commencing at 0800). All animals were provided with *ad libitum* access to water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, St Louis, MO, USA), and housed on Corn Cob Absorb 10/14 substrate with paper wool nest material. Subjects were bred and housed in standard laboratory cages (MB1 cage, 45 x 28 x 13cm, North Kent Plastics, UK), with some modifications (see below). Radio frequency identification (RFID) tags were used for individual identification and animals were handled with handling tubes to minimise stress (Hurst and West, 2010).

*3.2.2. Experimental design and protocols*

Our experiment was designed to compare the behaviour of female subjects produced by the same parents under communal or single nest rearing conditions. To breed subjects from both communal and single nest conditions, parental females (n = 16) and males (n = 8) from the captive colony were grouped into eight breeding trios, each consisting of one full-sister (r = 0.5) pair and an unrelated male. Subjects raised in the same nest were therefore either full siblings (r = 0.5) or three-quarter siblings (i.e. with a shared sire and sisters as dams, r = 0.375). Each breeding trio bred three times (blocks 1, 2 and 3), alternating communal and single nest breeding to control for experience (Table S3.1). Sister pairs and their combined offspring at each breeding attempt (communal or single, see below) are hereafter referred to as family units.

Breeding trios were housed in MB1 cages and primed with each other's scent prior to introduction. Males were removed after a week and sister pairs were randomly allocated to rear litters within either a communal or single nest-rearing environment (Figure S3.1). To facilitate the formation of single nests a mesh divide was inserted to split MB1 cages into two sections. This enabled olfactory and auditory communication between sisters on opposite sides of the divide, avoiding social isolation, while preventing the formation of a shared nest. Sister pairs were otherwise housed under identical conditions and remained in their home cages to rear litters until weaning at approximately post-natal day (PND) 28. In two communal nest treatments only one dam produced a litter, although no subjects from these nests were used in the current study. One further communal nest was formed when females in a single nest treatment chewed through the barrier at post-natal day (PND) 6 (Table S3.1). To reduce the risk of infanticide, nests were not disturbed for inspection until PND 14. Total pup numbers for each family and breeding block are summarised in Table S3.1. As expected, communal nests contained larger litters than single nests (communal = 11.1 ± 0.84; single = 5.6 ± 0.5

[mean litter size ± SE]), although there was no difference in the mean time interval between sisters giving birth under communal or single nest conditions (communal = average 2.2 days apart, range 0 to 5 days; single = average 2.2 days apart, range 1 to 3 days). Mean weaning weights were also similar for both communal and single nest rearing conditions (communal = 12.74g ± 1.88; single = 12.64g ± 1.85 [mean ± SE]). Female offspring were housed in sister pairs at weaning, under standard conditions as described above.

*3.2.3. Direct social interaction behavioural assay*

Behavioural assays were performed to test whether the early rearing environment and / or hormone profile of subjects predict their social response to unfamiliar conspecifics of contrasting relatedness. Subjects (n = 20) were F1 females randomly selected from five communal nests (communal subjects n = 8) and six single nests (single subjects n = 12) produced by the eight breeding trios across both communal and single nest rearing environments (Table S3.1). The behaviour of each subject was measured three times in a randomised order: once when meeting an unrelated female and once with each of two related females. Related females used as stimulus animals were either full or three-quarter siblings, although since their relatedness to subjects was not confirmed until after the behavioural assay there is some variation in the number of each sibling-type tested (Table S3.2). The behaviour of subjects from the first and third breeding blocks was tested in response to meeting stimulus animals from the second breeding block, so that all interactions were between previously unfamiliar animals. Subjects were chosen pseudo-randomly to include individuals from each family, as were stimulus animals, within the constraint of matching the relatedness of stimulus animals to subjects. Subject and stimulus animals were also matched for rearing background and the order of tests was balanced. Since female behavioural responses can vary according to oestrous stage (Huchard and Cowlishaw, 2011), subjects were each induced to be in oestrus prior to the assay. To achieve this, soiled bedding from an unrelated male was introduced into the female's home cage 72 hours prior to starting behavioural assays (Cheetham et al., 2007). Habituation to the test arena and each of three behavioural interactions were then conducted 72 hours apart to follow the oestrous cycle.

Behavioural assays were conducted in a medium density fibreboard (MDF) test arena under red light during the dark phase of the light-cycle (Figure S3.2). Prior to the first test, stimulus animals were marked using a black marker pen to the tail base for identification. Subject and stimulus animals were placed in opposite corners of the test arena and left to interact for up

to 15 minutes. The social encounters were filmed using a tripod mounted night-vision camera (Panasonic CCTV camera WV-BP310/B with TV lens WV-LA4R5C3B 1:1,2, 4.5mm) onto a DVD. An experimenter remained in the room throughout the behavioural tests, watching the interactions through a monitor 3m away from the test arena to minimise interference. If social interactions were observed to be escalating in aggression (see ethical note, S3.1), the behavioural test was interrupted. Behavioural analysis was conducted blind using behavioural observation research interactive software (BORIS) (Friard and Gamba, 2016) following the ethogram in Table 3.1, quantifying total duration of affiliative behaviour and counts of aggressive behaviours.

*Table 3.1: Ethogram used to analyse the behaviour in the social interaction assay. Affiliative behaviour was analysed as combined allosniffing and allogrooming time. Aggressive behaviours were counts of attack, chase fight and tail rattle.*

| | Behaviour | Description |
|---|---|---|
| Affiliative behaviours | Allosniffing | Sniffing the competitor |
| | Allogrooming | Grooming the competitor |
| Aggressive behaviours | Attack | Rushing and leaping at an individual usually with kicks and bites. |
| | Chase | Rapid locomotion to follow the path of a fleeing individual at faster than average walking pace. |
| | Fight | Behaviour of two individuals when they are locked together with kicking, biting and wrestling. Usually animals roll over each other rapidly. |
| | Tail rattle | Fast movement of the tail. |

*3.2.4. Urine sample collection and preparation*

Urine for oxytocin analysis was collected non-invasively at habituation to the test arena, and after each social interaction, although it was not possible to collect adequate volumes of sample at each sample collection point (see Table S3.2 for sample numbers). Urine samples were collected throughout the day (during the red-light period) but balanced for family, treatment group and relatedness of interacting conspecific. Urine samples were collected by placing subjects on a metal grate above a clean, transparent MB1 cage for up to 1.5 hours. Urine contaminated with faeces was avoided. Urine was pipetted into 1.5ml micro-centrifuge tubes (Fisherbrand, UK) and stored at -80$^{o}$C.

*3.2.5. Oxytocin EIA*

Urinary oxytocin was measured using a commercially available EIA (ADI-900-153, Enzo, USA) following manufacturers' instructions. To ensure accurate measurement of analytes was achieved, biochemical validations were conducted. First, a duplicated serial dilution of pooled urine was run with a serial dilution of synthetic antigen standard (15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg/ml). Parallelism was assessed by plotting the displacement curves of the endogenous antigen serial dilution with the serial dilution of the standard antigen and confirmed by a linear regression analysis. Results from diluted standards were significantly parallel to those of diluted urine samples (sample % binding = 1.07 (standard % binding) - 6.42, $R^2$ = 0.99, $F_{1,5}$ = 56, p <0.001). Next, the effect of the background matrix was assessed to confirm there was no interference in the assays. The background matrix of urine at a working dilution of 1:10 added in equal volumes to synthetic oxytocin standard dilutions of 15.6, 31.2, 62.5, 125, 250 and 500 pg/ml had no significant effect on oxytocin results (Observed = 1.09 (Expected) + 11.30, $R^2$ = 0.99, $F_{1,5}$ = 984.1, p<0.001) with a recovery of 120.2±8.5%. All samples were run in duplicate alongside a standard curve in triplicate. Inter-assay CVs were 17.15% for high quality control (45.64%b/Bo, 500pg/ml) and 19.25% for low quality control (82.83%b/Bo, 62.5pg/ml). Intra-assay CVs were less than 10% for all samples.

The oxytocin EIA was performed following kit instructions and using supplied reagents. 100µl of standard and sample were plated in duplicate onto goat anti-rabbit igG Microtiter plates, followed by 50µl of oxytocin conjugate (alkaline phosphatase conjugated with oxytocin) and 50µl of rabbit polyclonal antibody to oxytocin, then left to incubate at 4$^{o}$C for 18 hours. The plates were then emptied and washed three times using the supplied wash buffer before

being dried with lint-free paper. 200µl of para-Nitrophenylphosphate (pNpp) was then added to each well, and the plate was left to develop for 1 hour before 50µl of stop solution was added. The plate was then read immediately at 405nm.

*3.2.6. Genotyping subjects*

As maternal identity could not be established within the communal nests, the relatedness of subjects to stimulus animals was determined by genotyping, following methods previously described (Green et al., 2015). At weaning, F1 females were anaesthetised with isoflurane in oxygen, and an ear snip taken using a 2mm ear punch (K.L.Giddings Ltd., Cambridge). Recovery was monitored before returning subjects to home cages. DNA was extracted from the ear tissue using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, UK) following manufacturer's instructions. Genotyping was conducted using microsatellite markers selected from Mouse Genome Informatics site (MGI 5.1.3). The forward primer for each marker was 5'-fluorescently labelled with 6-FAM, NED, PET, or VIC. The loci were organised into three multiplex loading groups, containing mixed loci from the three regions. PCR amplification was performed on 10µl reaction of 20ng DNA, 0.5µM primer and 5µl of BioMix Red reaction mix (Biolin, London, UK). The PCR conditions were as follows: initially, a 5-minute denaturation step at 94$^{o}$C; followed by 35 cycles of 30 seconds denaturing at 92$^{o}$C, annealing for 30 seconds at 60$^{o}$C (decreasing by 0.1$^{o}$C per cycle to 56$^{o}$C), 30 seconds extension at 72$^{o}$C, followed by a final extension cycle for 10 seconds at 72$^{o}$C, final hold at 10$^{o}$C. PCR reactions were diluted as required (25-50-fold depending on primer set) and multiplexed with GeneScan LIZ500 size standard (Applies Biosystems) in formamide. Haplotype sizes were ascertained using ABI PRISM 3100 DNA analyser and GeneMapper v3.0 software (Applied Biosystems). Results were used to determine maternal identification within communal nests, although this was not possible for all F1 females when dam sister pairs were homozygous.

*3.2.7. Statistical analysis*

To test whether being reared in a communal nest influenced the behaviour or physiology of subjects, rearing background was included as a categorical explanatory variable in all models. The continuous response variables duration of affiliative behaviour and urinary oxytocin concentration were analysed using linear mixed-effect models (LMMs) (lmer; lme4 package, R (Bates et al., 2015)). Aggression count data contained zeros and were analysed in two ways. First, generalised linear mixed models (glmer; lme4) with a binomial family were used to investigate factors predicting whether subjects had an aggressive phenotype or not. An

aggressive phenotype was assigned to subjects that displayed aggression beyond an initial first encounter. Second, for subjects that did display aggression (30% of females), generalised linear mixed models with a Poisson family were used (glmer; lme4) to analyse variation in aggression. Over-dispersion (variance greater than the mean) was corrected using an individual based random effect (Harrison, 2014). Average values of urinary oxytocin concentration (including pre and post-test values) were used to test for differences between communal and single nest reared subjects, and pre-test measures were used to test if urinary oxytocin concentration predicts social behaviour. For all models, subject age, body mass, and number of pups within the natal nest were included as covariates and removed where non-significant. Rearing environment and relatedness of the interacting pair were retained in all models as experimental treatments. P-values were generated using the anova() or summary() function in the lmerTest package. The mouse ID, family ID and breeding block were included as random factors as individuals from the same family could not be considered independent. Residuals and Q/Q plots were plotted and visually inspected for all linear mixed models, and Shapiro and Lillie normality tests performed to confirm that the residuals were normally distributed. Data were transformed (log or square root) if residuals did not meet normal criteria.

## 3.3. Results

### *3.3.1. Effects of early rearing environment*

The early rearing environment, specifically whether subjects were reared in a communal or single nest setting, had no influence on measures of affiliative behaviour (Table 3.2), or aggressive behaviour (Table 3.3) when meeting unfamiliar conspecifics of varying relatedness. Similarly, the early rearing environment had no significant influence on the average urinary oxytocin concentration of subjects measured across the study (n = 47 observations for 17 subjects from 7 families; d.f. = 1, F = 1.25, p = 0.34) (Figure S3.3).

*Table 3.2. Linear mixed models to investigate whether the early rearing environment of female house mice influences expression of affiliative behaviour when meeting (A) unfamiliar kin or non-kin and (B) unfamiliar kin of differing relatedness (full sister, ¾ sister) or non-kin. Affiliative time was square root transformed. Animal ID, family ID and breeding block were included as random factors. Non-significant interactions and covariates were excluded from the model (number of pups in the post-natal nest and subject body mass). A: number of observations for model = 55, subject females = 20, families = 7, breeding blocks = 2. B: number of observations for model = 54, subject females = 20, families = 7, breeding blocks = 2.*

| Affiliative behaviour | | | | | |
|---|---|---|---|---|---|
| Fixed effect | Estimate (SE) | t-value | p-value | Random effect | Variance (SD) |
| A: kin versus non-kin | | | | | |
| Intercept | 6.482 (1.448) | 4.478 | 0.033 | Mouse ID | 1.315 (1.147) |
| Relatedness (kin vs non-kin) | -1.948 (0.615) | -3.168 | 0.003 ** | Family ID | 2.675 (1.636) |
| Rearing environment (communal vs single) | 1.861 | 1.558 | 0.291 | Breeding block | 1.425 (1.194) |
| B: Differing relatedness | | | | | |
| Intercept | 17.586 (4.668) | 3.767 | 0.002 | | |
| Age | -0.050 (0.019) | -2.673 | 0.018 * | Mouse ID | 1.077 (1.038) |
| Full sib vs ¾ sib | 0.084 (0.817) | 0.102 | 0.919 | Family ID | 4.389 (2.095) |
| ¾ sib vs non-kin | 1.733 (0.708) | 2.450 | 0.019 * | Breeding block | <0.001 (<0.001) |
| Non-kin vs full sib | -1.650 (0.730) | -2.259 | 0.030 * | | |
| Rearing environment (communal vs single) | 1.203 (1.847) | 4.850 | 0.544 | | |

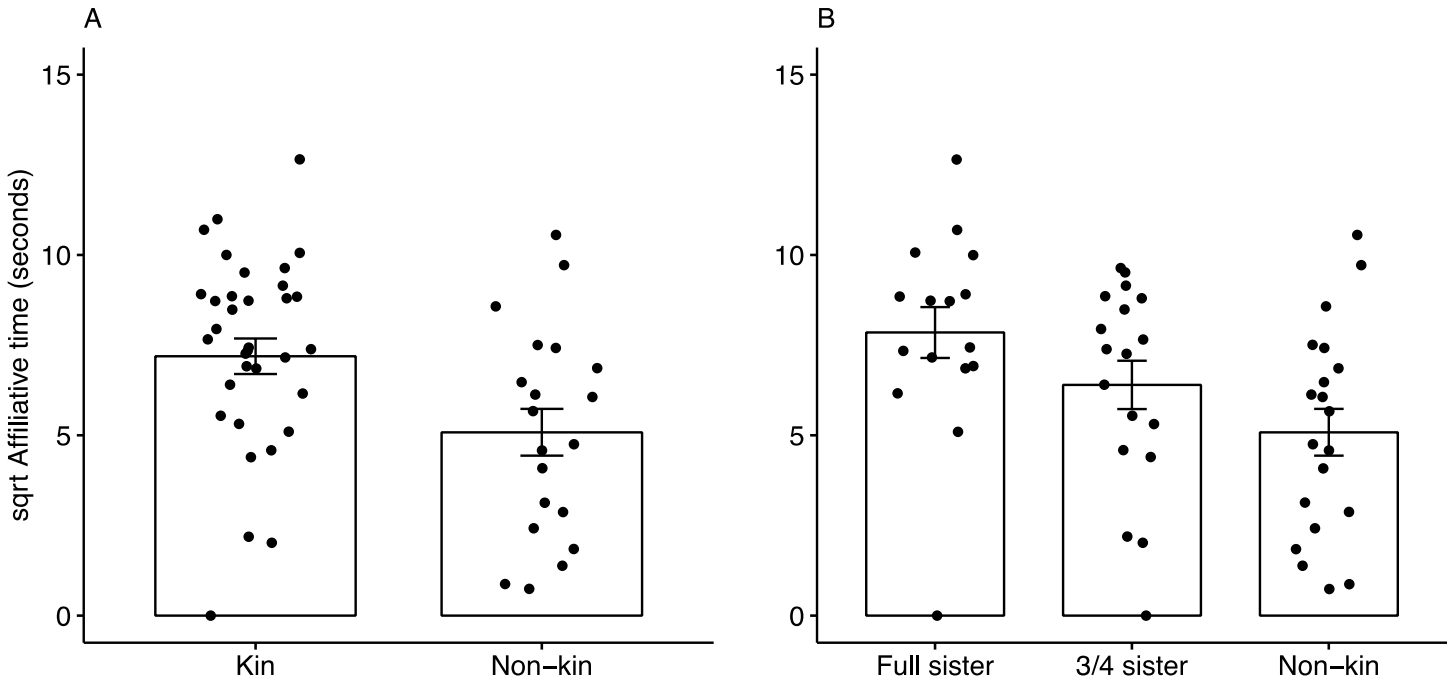
*Table 3.3. Models to test whether the early rearing environment of female house mice influences frequency of aggressive behaviour when meeting unfamiliar conspecifics of differing relatedness. A: Generalised linear mixed model with binomial family to test responses to kin and non-kin. B: Generalised linear model with a Poisson family to test responses to kin or non-kin only for subjects that displayed any aggression. C: Generalised linear model with a Poisson family to test responses to conspecifics of differing relatedness (full sister, ¾ sister or non-kin) only for subjects that displayed any aggression. Age was log transformed. Non-significant covariates (number of pups in the post-natal nest and subject body mass) were excluded from all models and subject female ID, family ID and breeding block were included as random factors. A: number of observations = 54, subject mice = 20, families=7, breeding block = 2. B: number of observations = 29, subject mice = 15, family ID = 7, breeding blocks = 2. C: number of observations = 28, subject mice =14, families=6, breeding block = 2.*

| Aggressive behaviour | | | | | |
|---|---|---|---|---|---|
| Fixed effect | Estimate (SE) | z-value | p-value | Random effect | Variance (SD) |
| A: Binomial - kin versus non-kin | | | | | |
| Intercept | -0.377 (0.837) | -0.450 | 0.653 | Mouse ID | <0.001 (<0.001) |
| Relatedness (kin vs non-kin) | 1.175 (0.695) | 1.691 | 0.091 | Family ID | 1.227 (1.108) |
| Rearing environment (communal vs single nest) | -1.365 (1.165) | -1.172 | 0.241 | Breeding block | 0 (0) |
| B: Poisson - kin versus non-kin | | | | | |
| Intercept | -146.660 (32.693) | -4.486 | <0.001 *** | Mouse ID | 0.734 (0.857) |
| Age | 27.625 (6.063) | 4.556 | <0.001 *** | Family ID | 0.138 (0.372) |
| Relatedness (kin vs non-kin) | 0.622 (0.143) | 4.353 | <0.001 *** | Breeding block | 6.877 (2.623) |
| Rearing environment (communal vs single nest) | -0.703 (0.656) | -1.073 | 0.283 | | |
| C: Poisson - differing relatedness | | | | | |
| Intercept | -154.980 (33.657) | -4.605 | <0.001 *** | Mouse ID | 0.715 (0.846) |
| Age | 29.035 (6.235) | 4.657 | <0.001 *** | Family ID | 0.095 (0.308) |

| | | | | | |
|---|---|---|---|---|---|
| Full sib vs non-kin | 1.076 (0.273) | 3.941 | <0.001 *** | Breeding block | 6.220 (2.494) |
| Full sib vs ¾ sib | 0.596 (0.309) | 1.931 | 0.053 * | | |
| Non-kin – ¾ sibling | 0.480 (0.162) | 2.964 | 0.003 ** | | |
| Rearing environment (communal vs single nest) | -1.143 (0.705) | -0.203 | 0.839 | | |

*3.3.2. Affiliative behaviour and relatedness of conspecifics*

When meeting unfamiliar conspecifics of contrasting relatedness, subjects displayed significantly more affiliative behaviour to kin than to non-kin (Table 3.2A, Figure 3.1A, $p = 0.003$). Further investigation revealed that affiliative behaviour was directed differentially towards full sisters, ¾ sisters and non-kin (Figure 3.1B, $d.f = 2$, $F = 4.105$, $p = 0.024$), with no difference in affiliative time directed towards full or ¾ sisters (Table 3.2B, $p = 0.919$).


A
B
sqrt Affiliative time (seconds)
15
10
5
0
Kin
Non−kin
Full sister
3/4 sister
Non−kin

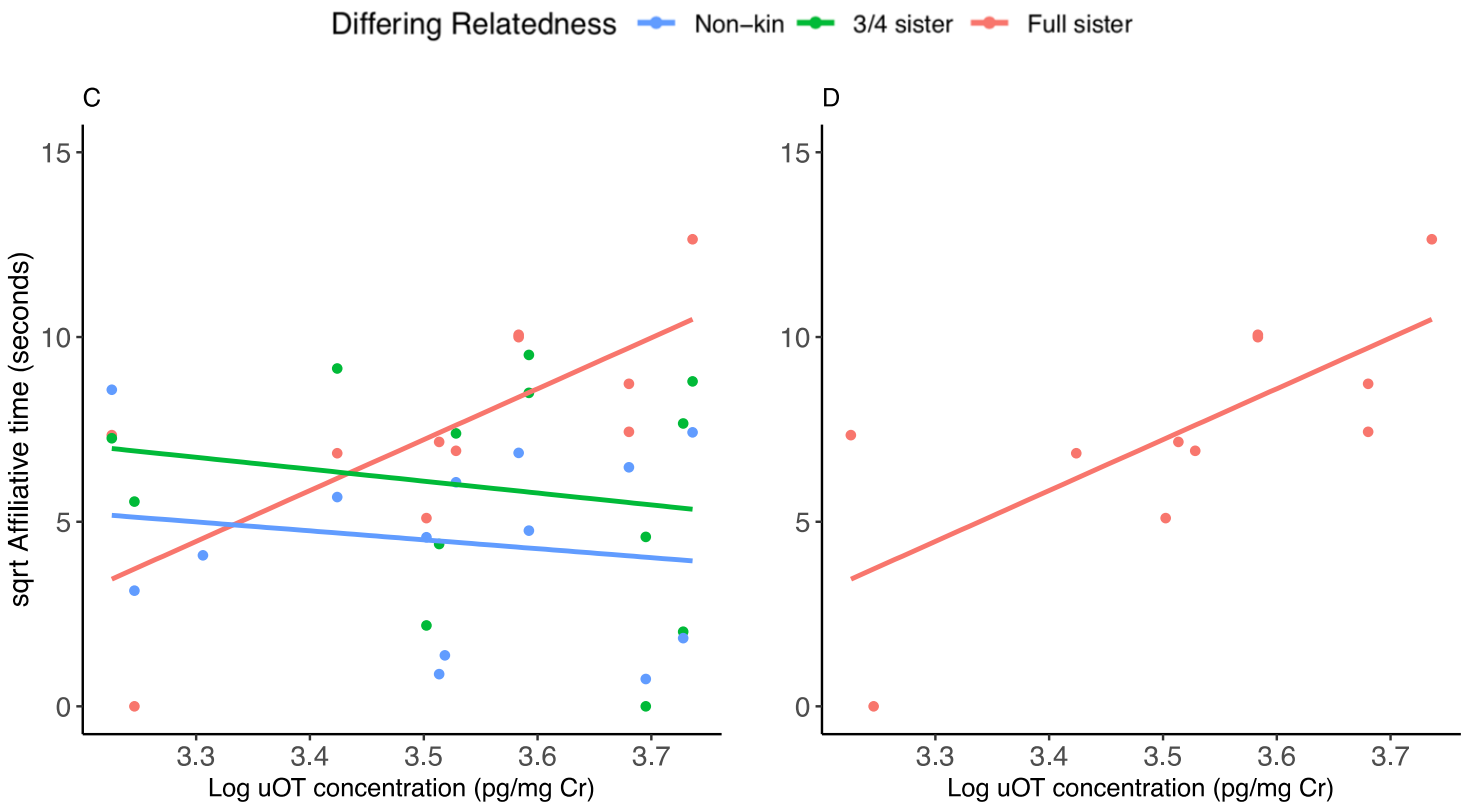



*Figure 3.1. Effect of relatedness (A and B) and urinary oxytocin concentration (C and D) on affiliative behaviour displayed by adult female house mice when meeting an unfamiliar conspecific. A. Subjects displayed significantly more affiliative behaviour towards kin than non-kin (p = 0.003, observations n = 55, subjects n = 20). B: Differing relatedness (full sibling, ¾ sibling, non-kin) of unfamiliar conspecifics influenced the duration of affiliative behaviour expressed (p = 0.02, observations n = 54, subjects n = 20), but there was no significant difference in affiliative behaviour when meeting full and ¾ sisters (p = 0.9). C. A positive interaction was found between affiliative behaviour directed towards conspecifics of differing relatedness (full sibling, ¾ sibling and non-kin) and pre-test urinary oxytocin concentration (p = 0.04, observations n = 38, subject n = 14). D. A significant positive relationship was found between pre-test urinary oxytocin concentration and affiliative time directed towards full siblings (p = 0.03, observations n = 11, subjects n = 9).*

Pre-test urinary oxytocin concentration did not predict the duration of affiliative behaviour directed towards kin or non-kin (Table 3.4A, p = 0.69). However, when kin of different relatedness were differentiated, there was a significant interaction between urinary oxytocin concentration and affiliative behaviour directed towards competitors of differing relatedness (unrelated, ¾ sisters and full sisters) (Table 3.4B, Figure 3.1C, p = 0.04). That is, urinary oxytocin concentration was significantly and positively related to affiliative behaviour directed towards full sisters (Table 3.4C, Figure 3.1D, p = 0.03). By contrast, there was no similar relationship between urinary oxytocin concentration and affiliative time with ¾ sisters or non-kin (3/4 sisters: n = 13 observations for 10 subjects from 5 families; d.f = 1, F = 2.230, p = 0.171; non-kin: n = 14 observations for 14 subjects from 7 families; d.f = 1. F = 0.403, p = 0.540).

*Table 3.4. Linear mixed models to investigate whether pre-test urinary oxytocin concentration of female house mice predicts the duration of affiliative behaviour when meeting unfamiliar (A) kin or non-kin, (B) conspecifics of differing relatedness (full sisters, ¾ sisters and non-kin) and (C) full sisters only. Affiliative time was square root transformed, and urinary oxytocin concentration was log transformed. Non-significant covariates were excluded from the model (A: number of pups in the post-natal nest and subject body mass, B and C: number of pups in the post-natal nest, subject body mass and age). Subject ID, family ID and breeding block were included as random factors in all models. A: number of observations for model = 39, subject females = 14, families = 7, breeding blocks = 2. B: number of observations for model = 38, subject females = 14, families = 7, breeding blocks = 2. C: number of observations for model = 11, subject females = 9, families = 5, breeding blocks = 2.*

| Affiliative behaviour | | | |
|---|---|---|---|
| Fixed effects | df | F-value | p-value |
| A: Kin versus non-kin | | | |
| Relatedness (kin vs non-kin) | 1 | 9.448 | 0.006 ** |
| Rearing environment (communal vs single nest) | 1 | 0.094 | 0.771 |
| Urinary oxytocin concentration | 1 | 0.169 | 0.689 |
| B: Differing relatedness | | | |
| Rearing environment (communal vs single nest) | 1 | 0 | 0.995 |
| Urinary oxytocin concentration | 1 | 0.448 | 0.518 |
| Relatedness: urinary oxytocin concentration | 2 | 3.671 | 0.041 * |
| C: Full sisters only | | | |
| Rearing environment (communal vs single nest) | 1 | 0.104 | 0.758 |
| Urinary oxytocin concentration | 1 | 14.124 | 0.029 * |

*3.3.3. Aggressive behaviour and relatedness of conspecifics*

For all subjects, when meeting unfamiliar female conspecifics of contrasting relatedness, there was no significant difference in levels of aggression directed to kin and non-kin (Table 3.3A, $p = 0.09$). However, for mice that displayed aggression, significantly more was directed towards non-kin than kin (Table 3.3B, Figure 3.2A, $p<0.001$). In addition, among subjects that displayed aggression, significantly more was directed to non-kin than to full siblings ($p<0.001$), and to non-kin than to ¾ siblings ($p = 0.003$), with a trend for higher levels of aggression directed towards ¾ siblings compared to full siblings (Table 3.3C, Figure 3.2B, $p = 0.053$).

There was no overall relationship between pre-test urinary oxytocin concentrations and aggressive behaviour directed towards kin or non-kin (Table 3.5, $p = 0.44$). Further analysis of the relationship between urinary oxytocin and aggression directed towards conspecifics of differing relatedness was not possible due to a limited sample size (full siblings, $n = 6$).

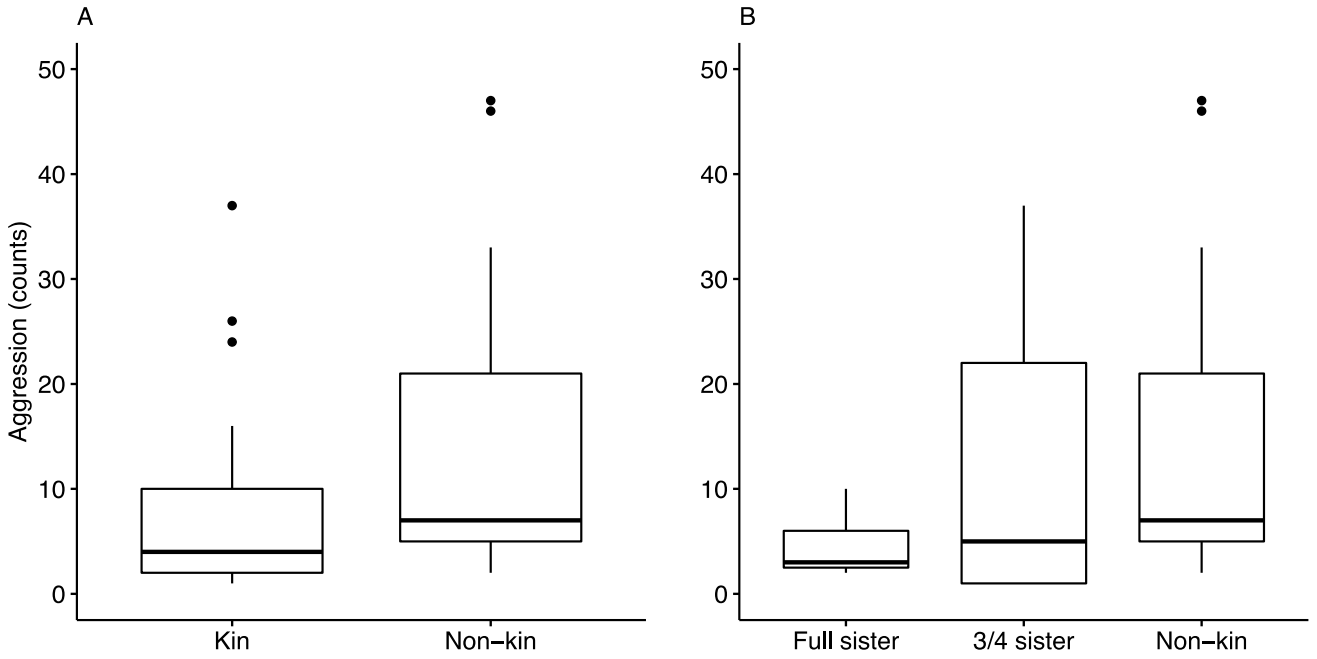



*Figure 3.2. Aggressive behaviour directed to unfamiliar conspecifics of varying relatedness by subjects that displayed any aggression. Subjects showed (A) significantly less aggressive behaviour to kin than to non-kin ($p<0.001$, observations n = 29, subjects n= 15) and (B) significantly more aggressive behaviour to non-kin than to full siblings ($p<0.001$, observations n = 39, subjects n = 15), and to non-kin than to ¾ siblings ($p = 0.003$, observation n = 29, subject n = 56) and between full and ¾ siblings ($p = 0.05$, observations n = 29, subjects n = 15).*

*Table 3.5. Generalised linear models with a Poisson family to test if pre-test urinary oxytocin concentration of female house mice predicts frequency of aggressive behaviour when meeting unfamiliar kin or non-kin. Urinary oxytocin and age were log transformed. Non-significant covariates (number of pups in the natal nest and subject body mass) were excluded from the models. Subject female ID, family ID and breeding block were included as random factors. Number of observations for model = 20, subject females = 11, families = 7, breeding blocks = 2.*

| Aggressive behaviour | | | | | |
|---|---|---|---|---|---|
| Fixed effect | Estimate (SE) | z-value | p-value | Random effect | Variance (SD) |
| Urinary oxytocin concentration | | | | | |
| Intercept | -35.177 (9.279) | -3.791 | <0.001 *** | Mouse ID | 0.343 (0.586) |
| Age | 0.143 (0.030) | 4.834 | <0.001 *** | Family ID | 0.375 (0.613) |
| Relatedness (kin vs non-kin) | 0.404 (0.155) | 2.601 | 0.009 ** | Breeding block | 10.391 (3.224) |
| Rearing background (communal vs single nest) | -0.391 (0.729) | -0.536 | 0.592 | | |
| Urinary oxytocin concentration | 1.722 (2.235) | 0.770 | 0.441 | | |

## 3.4. Discussion

We found that the early life experience of female house mice, in terms of whether they were reared in a communal or single nest, had no significant influence on their social responses when meeting unfamiliar conspecifics, or on their urinary oxytocin levels. However, the expression of social behaviours when meeting unfamiliar conspecifics differed significantly according to the relatedness of stimulus animals, consistent with previous studies showing evidence of kin discrimination in wild house mice (König, 1989, Ferrari et al., 2019, König, 1994b, Green et al., 2015). More affiliative behaviour was directed to related than to unrelated conspecifics, and affiliative responses to full sisters were significantly correlated with variation in urinary oxytocin concentrations. Our findings therefore suggest that affiliative behaviour directed towards close kin may be mediated by the oxytocin system under certain conditions, independent of prior familiarity and social bonding with the individual encountered.

Female house mice in our study demonstrated an ability to discriminate kin, a response that most likely relies on the highly polymorphic major urinary proteins (MUPs) used in scent communication (Hurst et al., 2001, Beynon and Hurst, 2003, Beynon et al., 2002). Previous studies have shown evidence that these signals are used to discriminate kin in other contexts such as mate choice or choosing communal nesting partners (Green et al., 2015, Kaur et al., 2014, Roberts et al., 2018). In the current study, female subjects were able to distinguish unfamiliar kin and non-kin, directing significantly more affiliative and less aggressive behaviour to kin than to non-kin. It is likely that mice with higher relatedness coefficients share more similar MUP profiles (and other potential genetic markers of relatedness) (Green et al., 2015), and hence that full sisters may be more easily recognisable to one another than ¾ sisters. Consistent with this expectation, significantly more aggression was directed towards ¾ sisters compared to full sisters, although no difference was found between affiliative behaviour directed to full or ¾ siblings. We did however find that affiliative behaviour directed to full sisters, but not to ¾ sisters or unrelated conspecifics, was predicted by urinary oxytocin levels. This novel finding, if confirmed more widely, could have broad implications for understanding the mechanistic basis of kin selection in social vertebrates.

Although the role of oxytocin in facilitating social recognition and affiliative behaviour is well established in the context of social bonding between conspecifics, this relates to contexts

where interacting individuals are familiar. Here we have shown that oxytocin appears also to predict affiliative behaviour towards unfamiliar conspecifics, but only where they are closely related to the subject. Prior to testing, subjects in our study were each housed with a female littermate and may therefore already have formed a social bond with a familiar sister. Hence it is possible that the response to unfamiliar relatives in our behavioural assay might reflect an extrapolation of social bonds formed with familiar sisters in the home cage, with social recognition based on odour cues that are similar for related individuals. If relatively strong social bonds with familiar sisters in the home cage, perhaps based on similarity of odour cues, led to both higher urinary oxytocin concentrations and a pre-disposition for affiliation to conspecifics with similar odour cues, this could explain the pattern we report here. Consistent with this hypothesis, the olfactory bulb in mice has a high concentration of oxytocin receptors and could be a potential site for the action of oxytocin in kin recognition and social memory formation (Ferguson et al., 2001, Ferguson et al., 2000, Ferguson et al., 2002, Oettl and Kelsch, 2018). Alternatively, subjects with higher urinary oxytocin levels may show a greater affiliation for unfamiliar sisters for reasons unrelated to the relationship with their familiar partner. For example, they could be more anxious and attracted to social contact with a readily identifiable relative. By contrast to the results found here based on natural variation in endogenous oxytocin, previous studies have found no effect of intra-peritoneal injection of synthetic oxytocin on positive or negative behaviours between female house mice during initial introductions (Harrison et al., 2016). However, pharmacological studies often use supraphysiological concentrations and behavioural responses can vary depending on the dose of oxytocin received (Bales et al., 2007).

Interpretation of our findings for urinary oxytocin depends on the assumption that peripheral oxytocin levels are related to central levels, which as yet is undetermined (Valstad et al., 2017). Centrally, oxytocin is synthesised by magnocellular neurons in the supraoptic and paraventricular nuclei (PVN) of the hypothalamus, and is both released directly into the bloodstream via the neurohypophyseal oxytocin system at the posterior pituitary (Ross and Young, 2009), and dendritically within the PVN (Baribeau and Anagnostou, 2015). Since a simultaneous central and peripheral oxytocin response can be coordinated or independent (Insel, 2010), a relationship between measures of each may not occur consistently. Nonetheless, pharmacological studies have shown that peripheral oxytocin administration results in expected behavioural changes, and hence that oxytocin can cross the blood brain barrier (Madden and Clutton-Brock, 2011, Olff et al., 2013). Moreover, relationships between

social behaviour and peripheral oxytocin levels have been reported in several studies. For example, urinary oxytocin was higher following social interactions including grooming and food sharing in wild chimpanzees (*Pan troglodytes*) (Wittig et al., 2014, Crockford et al., 2013), and a higher concentration of urinary oxytocin was found after grooming events with preferred grooming partners compared to interactions with less preferred partners (Crockford et al., 2013). Similarly, Finkenwirth et al (2015) found consistent correlations between urinary oxytocin concentration and dyad bond strength in family groups of common marmosets (*Callithrix jacchus*). Our results for female house mice may therefore be explained if those subjects that have strong social bonds with a familiar sister in their home cage also have higher urinary oxytocin concentrations, and a greater propensity for affiliative behaviour with conspecifics that smell similar to their familiar sister.

Female aggression, particularly the physiology mediating aggression, is far less well studied than that of males (Stockley and Campbell, 2013). Here, consistent with our findings for affiliative behaviour, we found that aggressive behaviour of female house mice, when expressed, is differentially directed towards unrelated conspecifics. Oxytocin is known to mediate female aggression in certain contexts (Campbell, 2008, Bosch, 2013, Beery, 2015) but we found no evidence here for a relationship between urinary oxytocin and aggressive behaviour.

Despite differences in social behaviour linked to peripheral oxytocin, we found no evidence for a role of the early life experience of female house mice in influencing social behaviour or oxytocin levels. Under natural conditions, wild house mice rear young both in communal nests and individually in single nest environments (König, 1994b, Ferrari et al., 2019). The early rearing environment is known to influence adult offspring behaviour and physiology as a result of epigenetic plasticity (Taborsky, 2017, Branchi, 2009), and can affect the oxytocin system (Branchi et al., 2013a). For example, previous research on Balb/c laboratory mice has shown that communal nest reared F1 females display significantly less aggression towards intruder males, reduced anxiety-like behaviour and increased oxytocin receptor expression in certain brain regions (Curley et al., 2009). Communal rearing has also been shown to influence behavioural traits of male house mice, including some aspects of scent marking and behaviour that may influence propensity to disperse (Fischer et al., 2018). In the context of the current study however, it appears that unknown sources of variation in oxytocin had a more important influence on behaviour than early rearing conditions. Moreover, although our results are

consistent with a genetic kin discrimination mechanism used by female house mice, we found no evidence that communally reared subjects were more successful in discriminating part-sisters, as might have occurred if exposure to more diverse relatives during development facilitates more effective phenotype matching.

In conclusion, although we found no evidence for an effect of communal rearing on the response of female house mice to unfamiliar conspecifics, we have shown that urinary oxytocin concentration can predict nepotistic behaviour directed to close kin in the absence of social familiarity. This novel finding offers potential new insights for understanding the proximate basis of kin selection in vertebrate animals and is worthy of further detailed experimental investigation.

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Ethical note

Animal use and care was in accordance with the EU directive 2010/63/EU and UK Home Office code of practice for the housing and care of animals bred, supplied or used for scientific purposes.

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Declaration of interest

None.

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3.6. Supplementary Material

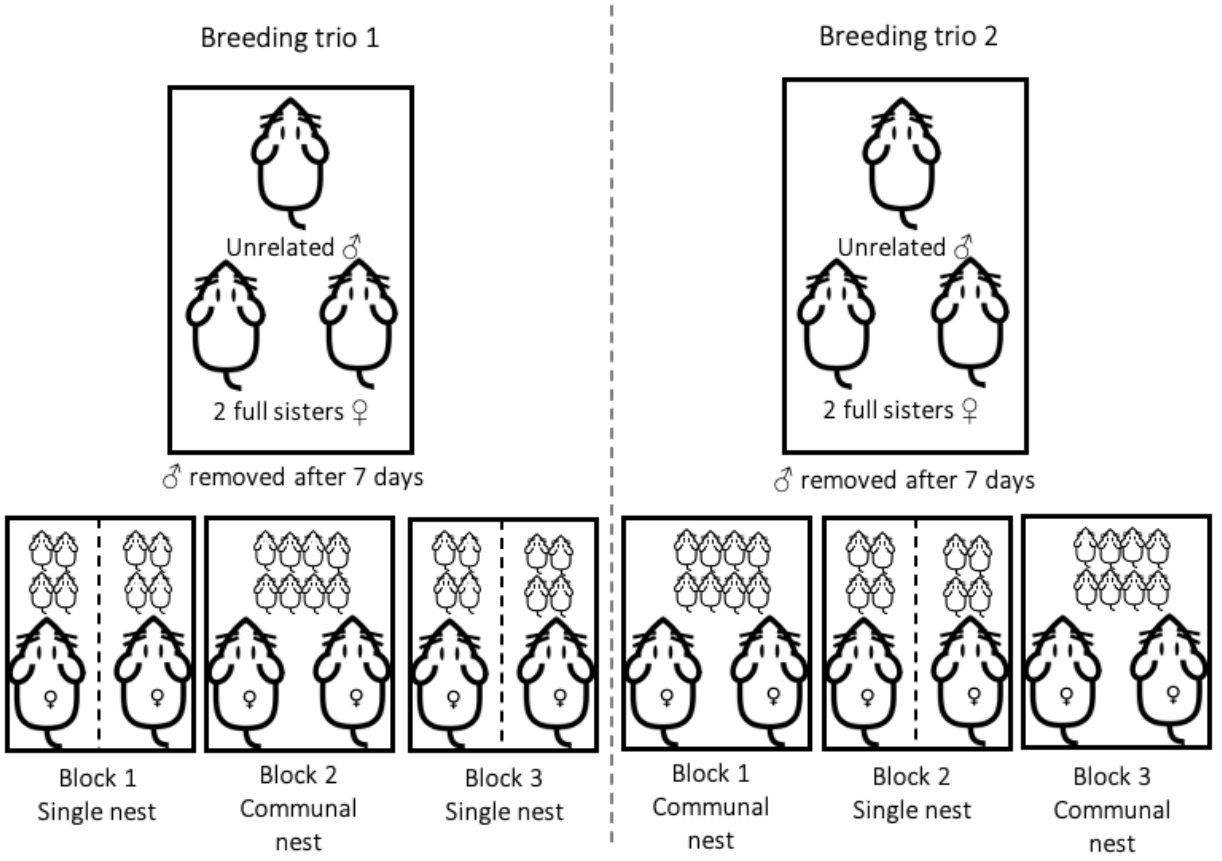


*Figure S3.1. Breeding set up. Each breeding trio consisted of one unrelated male and two full sisters (n = 8 breeding trios). After seven days, males were removed, and sister pairs were randomly allocated into single or communal nest breeding environments. Single nest sister pairs were separated using a mesh divide. Each breeding trio had the opportunity to breed three times, alternating the breeding environment between communal and single nest.*

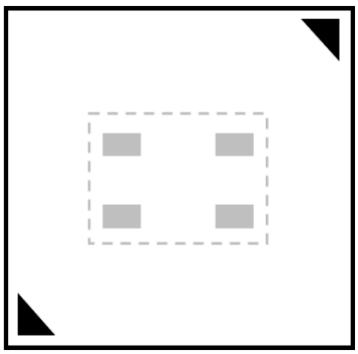

*Figure S3.2. The test arena consisted of a MDF enclosure (70 x 60 x 55 cm) containing a clear Perspex sheet (grey, dashed rectangle) balanced on four plastic boxes (grey rectangles). Two clear plastic nest boxes (15 x 11 x 7.7 cm, Techniplast, NJ, USA), each with two escape routes, were placed on opposite sides of the arena (black triangles).*

*Table S3.1. Summary of litters produced during the experiment over three breeding blocks, and the number of female subjects used in the direct interaction behavioural assay. Each breeding trio (1-8), consisting of two related females and one unrelated male, had the opportunity to breed three times, however, not all females bred in each round. Abbreviations: Tx: breeding treatment, CN: communal and SN: single nest rearing environment. The number of pups (# pups) in each single nest within one breeding cage is separated by |. Animals bred in round 2 were used as unfamiliar stimulus animals in behavioural tests.*

| Breeding trio | Round 1 | | Round 2 | | Round 3 | | Round 1 subjects | Round 3 subjects |
|---|---|---|---|---|---|---|---|---|
| | Tx | # pups | Tx | # pups | Tx | # pups | | |
| 1 | CN | 14 | SN | 8\|6 | SN[2] | 6\|0 | 2 CN | 0 |
| 2 | CN | 13 | SN | 6\|8 | | 0 | 2 CN | 0 |
| 3 | CN | 8 | | 0 | | 0 | 2 CN[3] | 0 |
| 4 | | 0 | SN | 7\|0 | CN | 9 | 0 | 0 |
| 5 | CN[1] | 11 | CN | 15 | | 0 | 2 CN | 0 |
| 6 | SN | 7\|4 | CN | 12 | SN | 7\|1 | 2 SN | 4 SN |
| 7 | SN | 3\|0 | SN[2] | 5\|0 | | 0 | 2 SN | 0 |
| 8 | SN | 4\|8 | CN | 13 | SN | 5\|0 | 2 SN | 2 SN |

[1] Initially a single nest treatment, females chewed through the central divide creating a communal nest at postnatal day 6.

[2] Designated into the communal nest treatment group but only one female bred resulting in a single nest.

[3] No related stimulus females available to conduct behavioural interactions between kin.

*Table S3.2. Relatedness of interacting pairs and sample numbers for each interaction. Genotyping to ascertain maternal identity was conducted after the social interaction assay, hence there is some variation in numbers of interaction type. Genotyping was not successful for all subjects, resulting in two interactions of unknown relatedness. Urinary samples were collected for analysis of oxytocin concentration before (at habituation) and after social interactions. The number of samples differs due to variation in success at collecting adequate urine volumes for analysis.*

| Social interaction | Number of social interactions | Number of samples prior to interaction | Number of samples post-interaction |
|---|---|---|---|
| Full sisters | 16 | 10 | 12 |
| ¾ sisters | 18 | 13 | 13 |
| Unrelated | 20 | 17 | 15 |
| Unknown | 2 | 2 | 2 |

*S3.1 Ethical Note*

An aggressive behaviour that lasts for more than 10 seconds (a stopwatch will be started as soon as an aggressive interaction is observed (attack, fight, chase on ethogram)) will be interrupted by the experimenter by placing a hand over the test arena (predatory escape response induced). If this doesn't work, the experimenter instead will click their fingers over the test arena. If the observer has to intervene twice within the 15-minute observation period, the trial will be stopped, and mice returned to their home cages

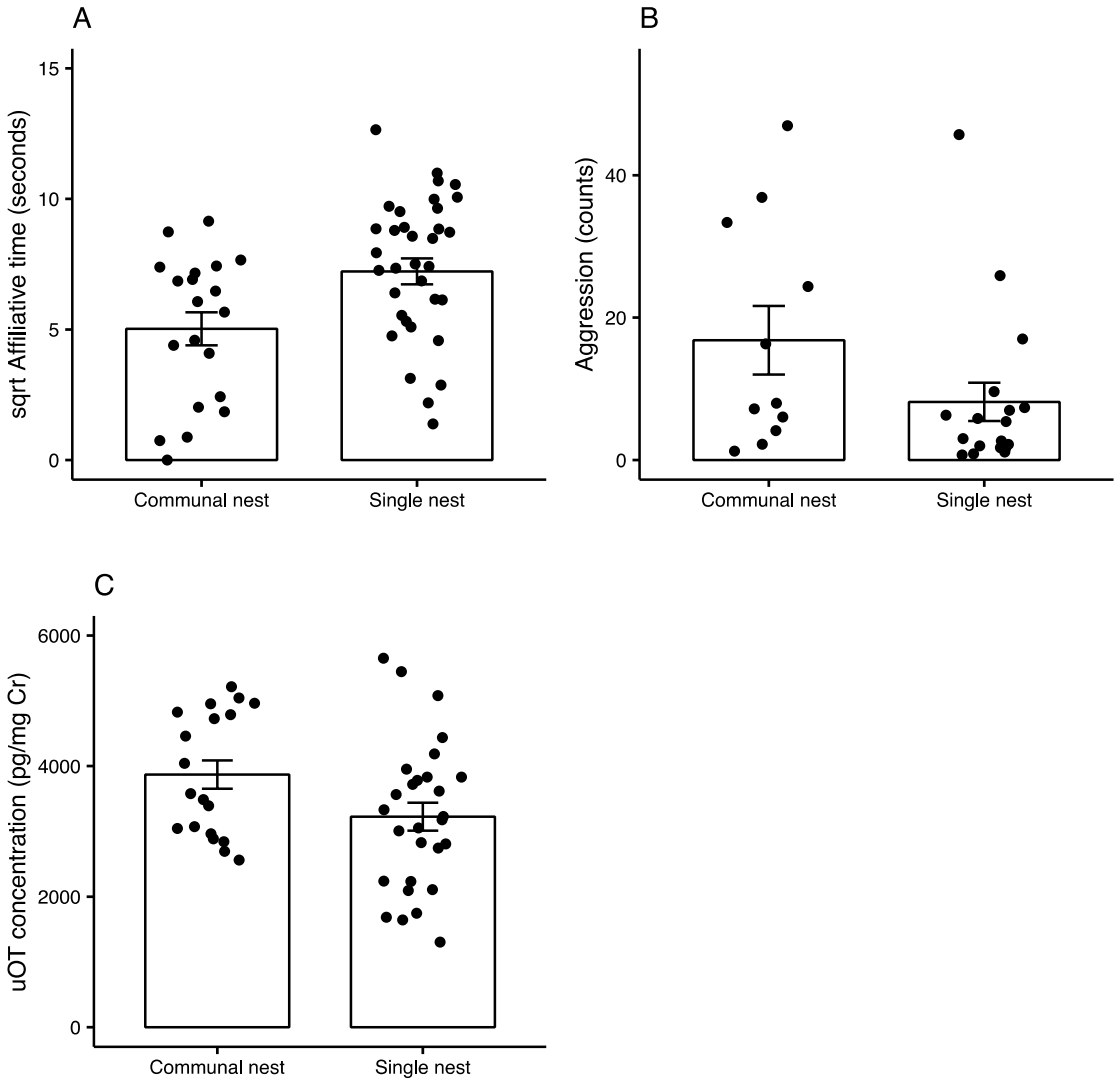


*Figure S3.3. Effects of early rearing environment (communal or single nest) on social behaviour and urinary oxytocin levels of adult female house mice. A. Total duration of affiliative behaviour, and B. total frequency of aggressive behaviours expressed when meeting unfamiliar female kin (full or ¾ sisters) or non-kin. Rearing environment refers to whether subjects were reared in communal or single nests. Data on duration of affiliative behaviour is presented for all subjects, whereas data for counts of aggressive behaviour are presented only for a subset of subjects that displayed aggression (see also Table 3 for analysis including response of all subjects). Rearing environment had no significant influence on affiliative (A: p = 0.3, observations n = 55, subjects n = 20) or aggressive behaviour (B: Poisson: p = 0.3, observations = 29, subjects n = 15) or average urinary oxytocin concentrations (C: p = 0.3, observations n = 47, subjects n = 17).*

## Chapter 4: Effects of communal rearing on anxiety-like behaviour and neuropeptide receptor expression in female house mice

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Abstract

The stress response of mammals is modulated by an intertwining relationship between oxytocin and arginine vasopressin (AVP) networks (the neuropeptide network), and the hypothalamic pituitary adrenal (HPA) axis. Both the neuropeptide network and HPA axis are sensitive to early life experience due to a period of neuroplasticity. Hence the level of parental care received can affect anxiety-related behaviour and physiology via the neuropeptide system. However, although such effects have been demonstrated in laboratory rodents, the extent to which they may apply under more naturalistic conditions remains largely unexplored. Wild house mice show natural variation in offspring care, choosing either to rear their offspring in a communal nest, with shared offspring care, or on their own. Here, in order to investigate how communal rearing affects offspring anxiety-related behaviour, we tested 1) whether the communal rearing influences (a) behavioural responses to a novel open field test, and (b) oxytocin and AVP receptor expression levels in central brain regions, and 2) how variation in anxiety-related behaviour linked to early life experience might be mediated by the neuropeptide system. The early rearing environment (single versus communal nest) may have the potential to influence anxiety-related behaviour: communally reared females travelled significantly further distances in a novel open field test, suggesting lower anxiety, although no difference was found for time spent in the centre of the test arena. Since the arginine vasopressin receptor 1a (AvpR1a) and the structurally similar oxytocin receptor (OxtR) are both closely related to stress-responsiveness these were investigated. Communally reared females also had significantly lower OxtR expression in the bed nucleus of the stria terminalis (BNST), and significantly higher AvpR1a expression in the medial pre-optic area (MPOA). The early rearing environment had no effect on urinary corticosterone concentration. Although evidence that the behaviour of communally reared subjects was mediated by differences in receptor expression was mixed, we found that both the total distance travelled and time spent in the centre of the test arena increased with AvpR1a expression in the MPOA of subjects. Our findings thus suggest that higher AvpR1a expression linked to early life experience may be involved in mediating the lower anxiety-related behaviour of communally reared mice. In conclusion, our findings suggest that natural variation in the reproductive strategy of wild house mice may influence anxiety-related behaviour of their offspring via the neuropeptide system.

Keywords
Communal rearing; anxiety-related behaviour; *Mus musculus domemticus*; oxytocin, arginine vasopressin, receptor expression.

## 4.1. Introduction

The stress response of mammals is modulated by an intertwining relationship between the neuropeptide system (e.g. oxytocin and arginine vasopressin (AVP) networks) and the hypothalamic pituitary adrenal (HPA) axis (Neumann and Landgraf, 2012). In mammals, both the neuropeptide system and the HPA axis are sensitive to the gestational and post-natal environment (Bosch et al., 2007, Dantzer et al., 2013, Champagne and Meaney, 2006, Provencal and Binder, 2015, Sakhai et al., 2016, Curley et al., 2009). The early rearing environment may therefore influence how an individual responds to stressful and novel experiences via these systems.

Stress induces neuropeptide release, which modulates the HPA axis (Winter and Jurek, 2019, Mak et al., 2012). A well-balanced relationship between oxytocin and AVP is vital for normal hypothalamic homeostasis and therefore stress regulation (Neumann and Landgraf, 2012, Landgraf and Neumann, 2004). Oxytocin is released during the stress response and regulates glucocorticoid production via negative feedback loops, dampening down the stress response (Neumann et al., 2000a, Winter and Jurek, 2019, Windle et al., 1997), and thereby reducing anxiety-like behaviour (Neumann et al., 2000a, Waldherr and Neumann, 2007). AVP also modulates the stress response via the HPA axis, resulting in the release of adrenocorticotropic hormone (ACTH) (Winter and Jurek, 2019), which then acts on the adrenal glands to promote glucocorticoid release (Antoni, 1993), thereby increasing anxiety-related behaviour. Indeed, exogenous AVP administration has been shown to significantly increase anxiety-like behaviour in wild rodents (Freeman et al., 2018). Thus, to date, research suggests that oxytocin has an anxiolytic effect (decreasing anxiety) whereas AVP is anxiogenic (increasing anxiety) (Neumann and Landgraf, 2012, Amico et al., 2004, Windle et al., 2004, Neumann et al., 2000a, Neumann and Slattery, 2016, Mak et al., 2012).

Early life experience, particularly in relation to levels of parental care received, is known to affect anxiety-related behaviour and physiology in laboratory rodents. For example, reduced maternal care can result in offspring displaying increased anxiety-related behaviour (Modlinska et al., 2018, Caldji et al., 1998, Meaney, 2001). In species with communal care of

offspring, young may be reared by multiple dams sharing offspring care (Ferrari et al., 2019, König and Lindholm, 2012), potentially resulting in increased levels of care. Consistent with this expectation, communally reared BALB/c mice display reduced anxiety-like behaviour in a novel open field test compared to single nest reared offspring, and have significantly higher oxytocin receptor binding and significantly lower AVP receptor binding in specific brain regions (Curley et al. (2009). High levels of peer-to-peer interactions within the communal nest have also been shown to increase oxytocin receptor expression within the amygdala of CD-1 Swiss-derived mice (Branchi et al., 2013b). These studies suggest that a communal nest, with increased maternal and peer to peer interactions (Branchi et al., 2013a, Branchi et al., 2006a), might be regarded as a socially enriched environment, at least for the laboratory strains of mice tested to date (Curley et al., 2009). However, for wild house mice, the communal nest might also be regarded as predictive of a competitive environment (Fischer et al., 2018), which could potentially result in heightened anxiety-like behaviour (Branchi et al., 2006b). However, as yet it is unknown how communal rearing influences anxiety-related behaviours of wild house mice, and if their responses to early rearing experience are mediated by the oxytocin and AVP systems in a similar way to laboratory rodents.

The house mouse (*Mus musculus domesticus*) rears offspring in both communal and single nest environments under natural conditions (Ferrari et al., 2019, König and Lindholm, 2012, Sayler and Salmon, 1971, Konig, 1993, Weidt et al., 2014), making this an ideal model wild rodent to investigate how the early rearing environment influences offspring anxiety-related behaviour and corresponding physiology. However, communal rearing of young may not always be a beneficial strategy for female house mice (Ferrari et al., 2015). Indeed, females often choose to rear their offspring in single nests (Weidt et al., 2014), and communal rearing of young has been associated with competitive conditions (Schradin et al., 2010, Ferrari et al., 2019). Here we investigate the effect of communal rearing during early life on anxiety-related behaviour and physiology of adult female house mice. Under controlled laboratory conditions, mice were bred in communal or single nest environments to test (1) whether the different rearing tactics influences: (a) behavioural responses to a novel open field test, and (b) oxytocin receptor (OxtR) and arginine vasopressin receptor 1a (AvpR1a) expression in specific central brain regions, and (2) how variation in anxiety-related behaviour linked to early life experience is mediated by the neuropeptide system.

## 4. 2. Methods

### *4.2.1. Subjects*

House mice used in this study were from a captive colony derived from wild populations in Northwest England. The colony was housed under controlled environmental conditions (20-21$^{o}$C, 45-65% relative humidity and 12:12 hour light-dark cycle, with dark commencing at 0800) in standard laboratory cages (MB1 cage, 45 x 28 x 13cm, North Kent Plastics, UK). Mice had access to *ad libitum* water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, St Louis, MO, USA) and were housed on Corn Cob Absorb 10/14 substrate with paper wool nest material. Subjects were bred in standard MB1 cages with some modifications (see below) and individuals were identified using radio frequency identification (RFID) tags. To minimise stress all mice were handled with handling tubes (Hurst and West, 2010).

### *4.2.2. Experimental design*

To investigate whether a communal or single nest rearing environment influences anxiety-like behaviour and physiology (specifically oxytocin and arginine vasopressin receptor 1a expression in the medial preoptic area (MPOA) and bed nucleus of the stria terminalis BNST), subjects were bred under controlled experimental conditions. Adult females (n = 16) and males (n = 8) from the captive colony were allocated to eight breeding trios, each of which bred under both communal and single nest rearing conditions to produce offspring with contrasting early life experience (Table S4.1). Each breeding trio had three breeding attempts, alternating between the communal and single nest treatment. Maternal pairs and their combined offspring across each breeding attempt are hereafter referred to as family units. Not all breeding attempts were successful, and in two communal nests, only one dam produced offspring. These litters were therefore re-classified as resulting from a single nest (Table S4.1). However, only two subjects from these litters were used in the current experiment, and the conclusions of the study are not affected by the classification of these as originating from a single or communal nest. The females within one single nest-allocated cage chewed through the mesh divide at post-natal day (PND) 6, creating a communal environment.

Prior to introducing the breeding trios, all mice were primed with each other's scent to reduce the possibility of aggression. Males were introduced to the maternal sister pairs for one week in MB1 home cages and following male removal, maternal pairs were randomly allocated to

the communal or single nest treatment group. To ensure single nests were formed in the single nest treatment group, a mesh barrier was placed into the MB1 cage, dividing the cage into two identical sections preventing social isolation but allowing for olfactory and auditory communication. Maternal sister pairs were otherwise housed in identical conditions and remained in their home MB1 cage until their litters were weaned at approximately PND 28. To prevent infanticide, nests were not disturbed until PND 14 and pup numbers for each breeding round can be found in Table S4.1. Communal nests contained larger litters than single nests (CN = 11.1 ± 0.84; SN = 5.6 ± 0.5 [mean litter size ± SE]), although there was no difference in the mean time interval between sisters giving birth under CN or SN conditions (CN = average 2.2 days apart, range 0 to 5 days; SN = average 2.2 days apart, range 1 to 3 days). Mean weaning weights were also similar for both CN and SN rearing conditions (CN = 12.74g ± 1.88; SN = 12.64g ± 1.85 [mean ± SE]). Following weaning, F1 female subjects were housed in sister pairs under the standard conditions described above in sister pairs.

*4.2.3. Open field test*

A novel open field test was conducted to investigate whether the early rearing environment or hormone profiles of subjects predict anxiety-like behaviour in adult female house mice (n = 21). Adapted from Paylor et al. (2006), subjects were placed into the centre of a rectangular medium density fibre test arena (70 x 60 x 55 cm) and were left to freely explore the area for 15 minutes during the dark phase of their light cycle. The test was filmed using a ceiling mounted night-vision camera (Panasonic CCTV camera WV-BP310/B with TV lens WV-LA4R5C3B 1:1,2, 4.5mm) onto a DVD. The experimenter left the room once the mouse was placed into the test arena and observed the subject remotely from a monitor. To control for behavioural and hormonal differences throughout the oestrous cycle and circadian rhythm, single and communal nest reared subjects were balanced throughout the test period. Videos were analysed blind using BORIS video analysis software (Friard and Gamba, 2016). For analysis, the open field test arena was split into 16 squares (Figure S4.1). The total number of lines crossed was counted (only when all four feet had crossed the distance markers was it counted) and time spent in the central region (highlighted in grey, Figure S4.1) was recorded. Five subjects displayed stereotypic behaviour and were therefore removed from analysis. Stereotypic behaviour is defined as a repetitive, invariant behaviour pattern with no obvious goal or function. In this case, the stereotypic mice were defined as those that continuously and quickly ran the full perimeter of the test arena for the entire test period (Mason, 1991).

*4.2.4. Sample collection and processing*

To investigate whether the early rearing environment influences the glucocorticoid response of adult female house mice, urinary corticosterone was measured. Urine was non-invasively collected from each subject following the open field test by placing mice on a metal grate above a clean, transparent MB1 cage for a maximum of 1.5 hours. Each urine void fell through the grate and was pipetted into 1.5ml microcentrifuge tubes (Fisherbrand, UK). Samples were stored at -20$^{o}$C until analysis. Urine contaminated with faeces was avoided. Sample numbers can be found in Table S4.1 (n = 21). To control hormonal differences due to circadian rhythm, single and communal nest reared subjects were balanced throughout the test period between 09:30 and 16:30 during the red-light period.

To investigate whether the early rearing environment influences the expression of the oxytocin receptor or AVP receptor 1a, and whether receptor expression influences the anxiety-like behaviour of adult female house mice, subjects were humanely killed by a trained animal technician using an approved Schedule 1 procedure (n = 34). Brains were removed and frozen in hexane on dry ice before being stored at -80$^{o}$C prior to microdissection. Whole brains were micro-dissected using a Leica Cyrostat at -20$^{o}$C using The Mouse Brain in Stereotaxic Coordinates for reference (Paxinos and Franklin, 2001). Brains were sectioned at 40µm in the horizontal plane and samples removed using a 1mm punch biopsy needle (Selles Medical, UK). The medial preoptic area (MPOA) and bed nucleus of the stria terminalis (BNST) were dissected out at Bregma +0.26mm. Punch biopsies were placed into frozen autoclaved 1.5ml Eppendorf tubes and placed on dry ice until storage at -80$^{o}$C.

*4.2.5. Corticosterone enzyme immuno-assay*

Subject urinary corticosterone was measured using an in-house EIA at Chester Zoo (corticosterone antiserum CJM006; Caroline Munro, University of California Davis, CA, USA) following methods previously published (Watson et al., 2013, Munro and Stabenfeldt, 1984). Prior to determining hormone concentrations, biochemical validations of the EIA were conducted. Significant parallelism was achieved between the serial dilution of standards (3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 and 500 pg/ml) in triplicate and a serial dilution of pooled urine in duplicate (Sample % binding = 0.737 (standard % binding) + 17.213, $R^2 = 0.988$, $F_{1,6} = 492.0$, $p < 0.001$). No significant matrix interference was detected on the addition of a 1:20 dilution of urine to synthetic standard concentrations of 31.2, 62.5, 125, 259 and 500 pg/ml (Observed = 1.180 (expected) + 22.948, $R^2 = 0.999$, $F_{1,7} = 15431.0$, $p<0.001$). Recovery was 177.2 ± 27.2%.

Intra-assay CVs were less than 10% for all samples and inter-assay CVs were 15.9% for high quality control (19.01%b/Bo) and 13.0% for low quality control (53.84%b/Bo). Urinary corticosterone was normalised to creatinine to account for urine dilution. To determine creatinine concentration, an alkaline picrate assay (Sigma Chemicals, UK) was conducted. 10µl urine samples were diluted 1:50 with $ddH_20$, and 100µl aliquots were plated alongside a standard curve (range 0 to 30 µg/ml) produced by serial diluting stock creatinine (0.03mg/ml). 150µl of alkaline picrate reagent (0.65% Picric Acid/ 10mM Sodium Tetraborate/ 0.1M Sodium Hydroxide) was then added to each well. Absorbance was read at 492nm using a Thermo Scientific Multiskan DC microplate photometer (Thermo Fisher Scientific, Finland). Samples and the standard curve were plated in duplicate.

*Biological validation*

To ensure the assay measuring urinary corticosterone concentration was able to detect biological meaningful changes in adrenocortical activity, urinary corticosterone concentrations collected after the novel open field test were compared between mice that displayed stereotypic and non-stereotypic behaviours. Stereotypic mice had near significantly higher urinary corticosterone concentration than non-stereotypic mice (Figure, 4.1, paired t-test, t=-2.32, df = 4, p = 0.08).

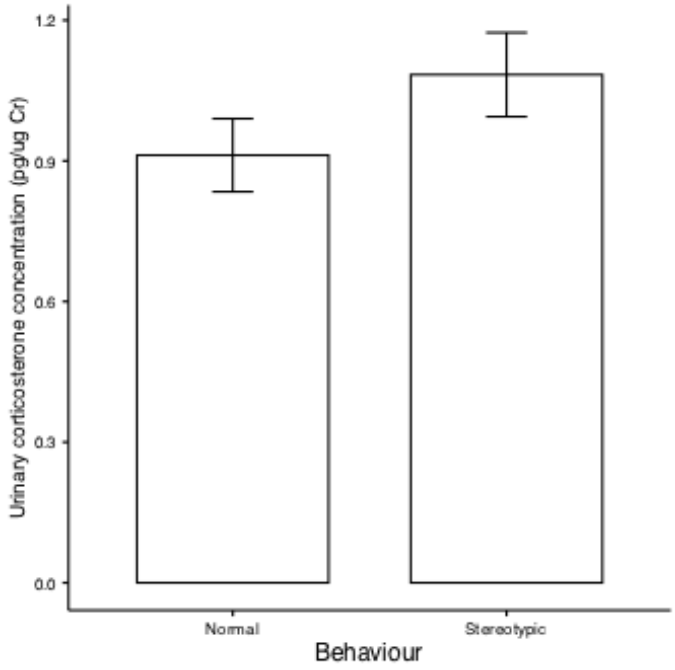



*Figure 4.1. Stereotypic mice had near significantly higher urinary corticosterone concentrations than mice that did not display stereotypic behaviours to the open field test (p = 0.08).*

*4.2.6. Gene expression*

RNA was extracted and purified from brain samples using a Qiagen RNeasy Mini Kit (Qiagen UK) with the addition of an on-column DNAse digestion step (Invitrogen, UK), and homogenised using a TissueLyser (Qiagen, UK) for 40 seconds at 30Hz following manufacturer's instructions. RNA was assessed for concentration and purity using a NanoDrop (NanoDrop Technologies inc., ThermoFisher Scientific). For each sample, cDNA was reverse-transcribed from 30ng of RNA using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen, UK) following manufacturer's instructions using random hexamer primers. The cycling conditions were: 7.5 minutes 25°C, 50 minutes 50°C, 5 minutes 85°C and storage at -20°C. Relative quantification was completed with a Roche LightCycler® 480 Real-Time PCR Instrument using 1.5ng/uL of cDNA in a 20uL reaction using TaqMan® Fast Advanced Master Mix and Applied Biosystems' gene expression assays: oxytocin receptor (OxtR) (Assay ID Mm01182684_m1), arginine vasopressin receptor 1a (AvpR1a) (Assay ID Mm00444092_m1) using peptidylpropyl isomerase A (Ppia) (Assay ID Mm02342430_g1) and hypoxanthine phosphoribosyltransferase 1 (Hprt1) (Assay ID Mm00446968_m1) as exogenous reference genes (AppliedBiosystems, UK) (Timaru-Kast et al., 2015, Lesse et al., 2017). The following conditions were used: uracil-N-glycosylase (UNG) incubation 50°C for 2 minutes, polymerase activation 95°C for 20 seconds, 40 PCR cycles of 95°C for 3 seconds and 60°C for 30 seconds. Ninety-six well plates (StarLab, UK) with Advanced Polyolefin StarSeal (StarLab, UK) were run with no template controls and samples run in duplicate. Cycle threshold (CT) values were created using the supplied Roche software and were converted into relative expression ratios using the ΔCt method ($Ct_{target}$-$Ct_{reference}$) where $Ct_{reference}$ was the mean of both reference cycle threshold values (White et al., 2017).

*4.2.7. Statistical analysis*

To investigate whether being reared in a communal versus a single nest environment influenced anxiety-like behaviour or physiology, rearing background was included as an explanatory variable in all models. Total distance travelled was a count of the total number of distance markers crossed within the 15-minute test, and therefore was analysed using generalised linear mixed models (GLMMs) (glmer; lme4 package, R) with a Poisson distribution. Including all subjects resulted in over-dispersion of data and therefore an individual-based random effect was included in these models (Harrison, 2014). Time in the centre of the test arena was analysed using linear mixed-effect models (LMMs) (lmer; lme4 package, R) (Bates et al., 2015). For anxiety-like behaviour models, the following covariates

were tested and removed where non-significant: age at test, number of pups in the post-natal nest and subject weight. Receptor expression data were analysed using LMMS (lmer() function) and the covariates subject mass at death and number of offspring in the post-natal nest were removed where non-significant. Breeding block and family ID were included as random factors in all models. P-values were generated using the summary() function in the lmerTest package and $p<0.05$ was deemed significant. Residuals and Q/Q plots were plotted and visually inspected, and Shapiro and Lillie normality tests performed to confirm the residuals were normally distributed. Time in centre of the test arena was log transformed to achieve homogeneity of residuals.

## 4.3. Results

### *4.3.1. Effects of early rearing environment on anxiety-related behaviour*

The early rearing environment, in terms of being reared in a communal or single nest, was found to significantly influence anxiety-like behaviour, quantified as total distance travelled in the open field test. Communal nest reared females travelled significantly longer distances (Figure 4.2A, Table 4.1A, $p = 0.037$, $n = 21$). However, the early rearing environment had no influence on time spent in the centre of the test arena (Figure 4.2B, Table 4.1B, $p = 0.440$, $n = 21$).

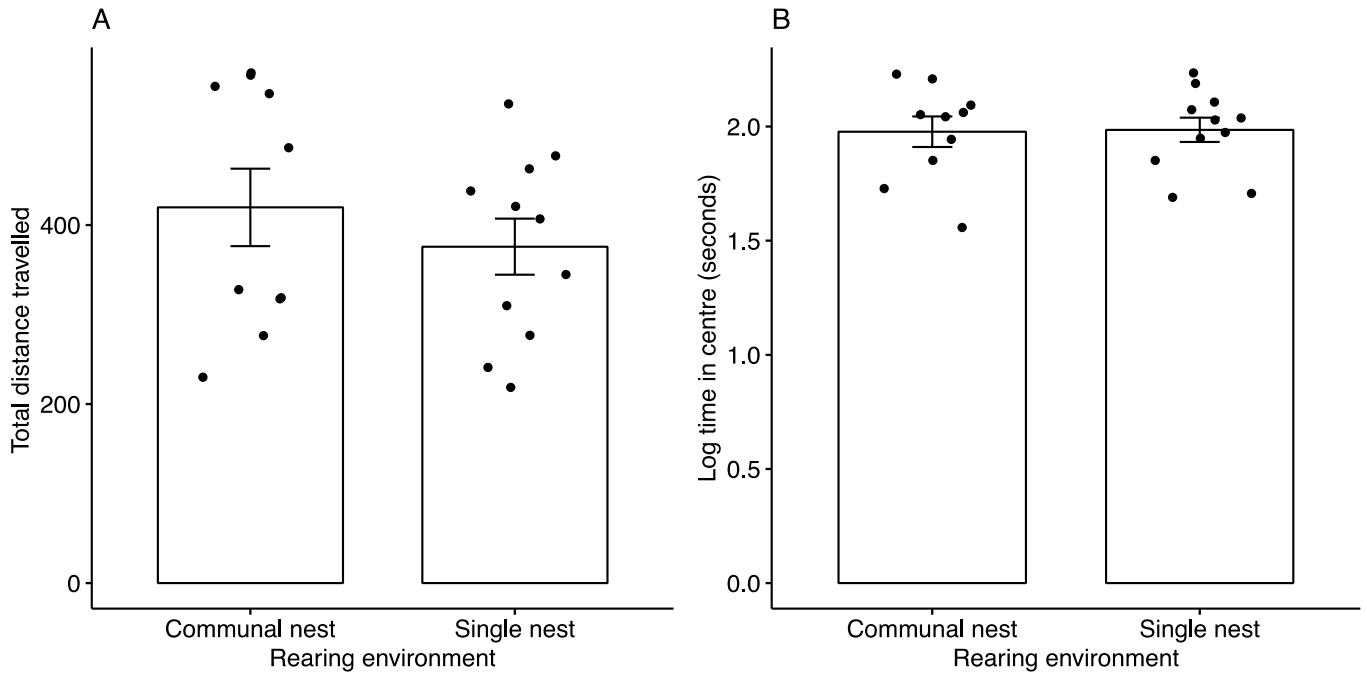


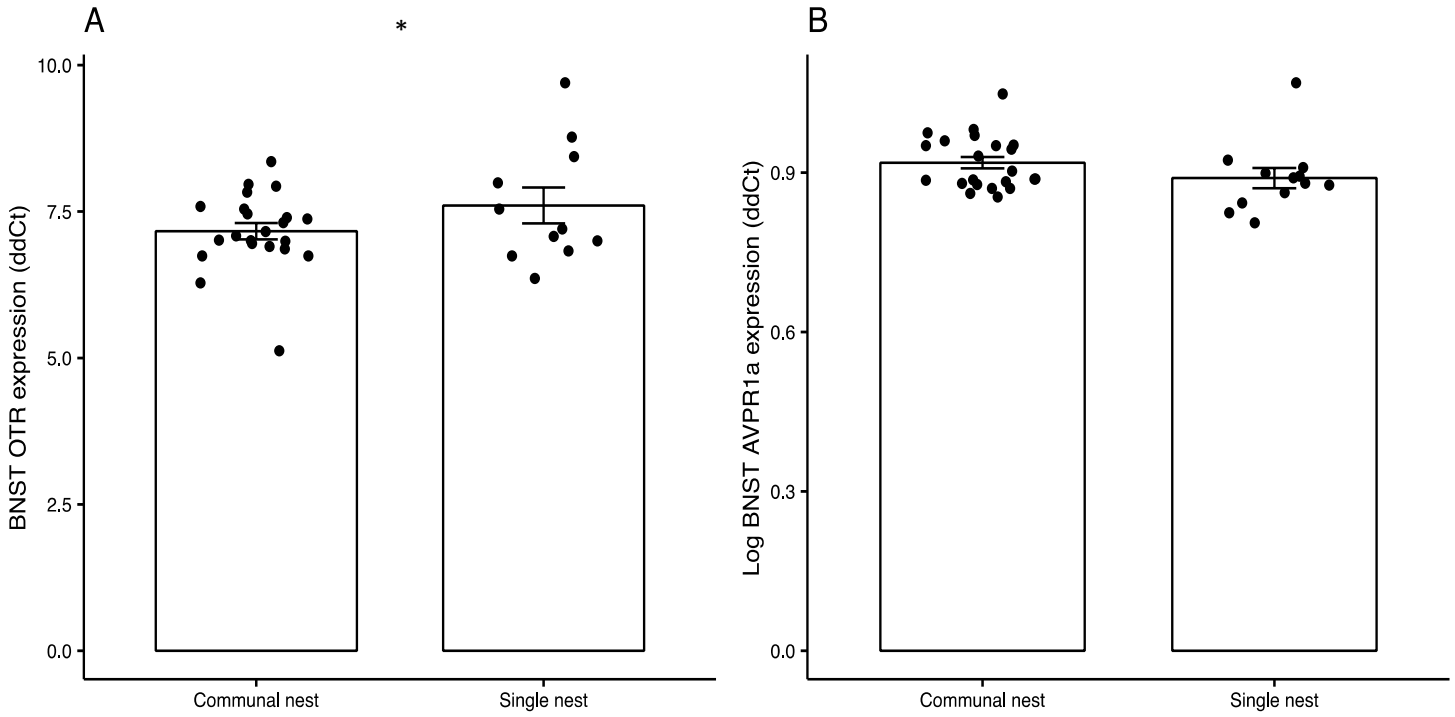

*Figure 4.2: Effects of early rearing environment (communal or single nest) on anxiety-like behaviour of adult female house mice during a novel open field test (mean ± SEM). A: Total distance travelled, and B: Total time spent in the centre of the test arena. Communally reared mice travelled significantly longer distances (A: p = 0.037, n = 21). Rearing environment had no influence on time spent in the centre of the test arena (B: p = 0.440, n = 21). Time spent in the centre of the test arena was log transformed.*

*Table 4.1: Linear mixed effect models to investigate whether the early rearing environment of adult female house mice influences the expression of anxiety-like behaviour during an open field test in terms of (A) total distanced travelled and (B) total time spent in the centre of the test arena. Total distance travelled was analysed using the glmer() function with a Poisson distribution and mouse ID was included as a random factor due to over-dispersal. Time spent in the centre of the test arena was analysed using the lmer() function. Family ID and breeding block were included as random factors. Non-significant interactions and covariates were excluded from all models (A: subject body mass and number of pups in the post-natal nest, B: age at test, subject body mass and number of pups in the post-natal nest). A and B: subjects = 21, family ID = 8, breeding blocks = 3.*

| Fixed effect | Estimate (SE) | z-value | p-value | Random effect | Variance (SD) |
|---|---|---|---|---|---|
| A: Total distance travelled | | | | | |
| Intercept | 3.391 (0.423) | 8.015 | <0.001 *** | Family ID | 0.026 (0.162) |
| Rearing environment | 0.057 (0.027) | 2.084 | 0.037 * | Block | 0.011 (0.103) |
| Subject age | 3.126 (0.542) | 5.766 | <0.001 *** | | |
| Fixed effect | Estimate (SE) | t-value | p-value | Random effect | Variance (SD) |
| B: Time in centre of arena | | | | | |
| Intercept | 101.509 (21.774) | 4.662 | 0.011 * | Family ID | 562.50 (23.720) |
| Rearing environment | 9.945 (12.530) | 0.794 | 0.440 | Block | 800.80 (28.30) |

*4.3.2. Effects of early rearing environment on physiological parameters*

Communal nest reared females were found to have significantly lower OtR expression in the BNST than single nest reared females (Figure 4.3A, Table 4.2A, $p = 0.01$, $n = 32$). However, the early rearing environment had no influence on OtR expression levels in the MPOA (Figure 4.3C, Table 4.2C, $p = 0.866$, $n = 34$). Similarly, no effect of early rearing environment was found on AvpR1a expression in the BNST (Figure 4.3B, Table 4.2C, $p = 0.093$, $n = 34$), however, significantly higher AvpR1a expression was found in the MPOA of communal nest reared females (Figure 4.3D, Table 4.2D, $p = 0.034$, $n = 34$). The early rearing environment had no influence on urinary corticosterone concentration (Table 4.3, $p = 0.882$, $n = 21$).


A
*
B
BNST OTR expression (ddCt)
10.0
7.5
5.0
2.5
0.0
Communal nest
Single nest
Log BNST AVPR1a expression (ddCt)
0.9
0.6
0.3
0.0
Communal nest
Single nest

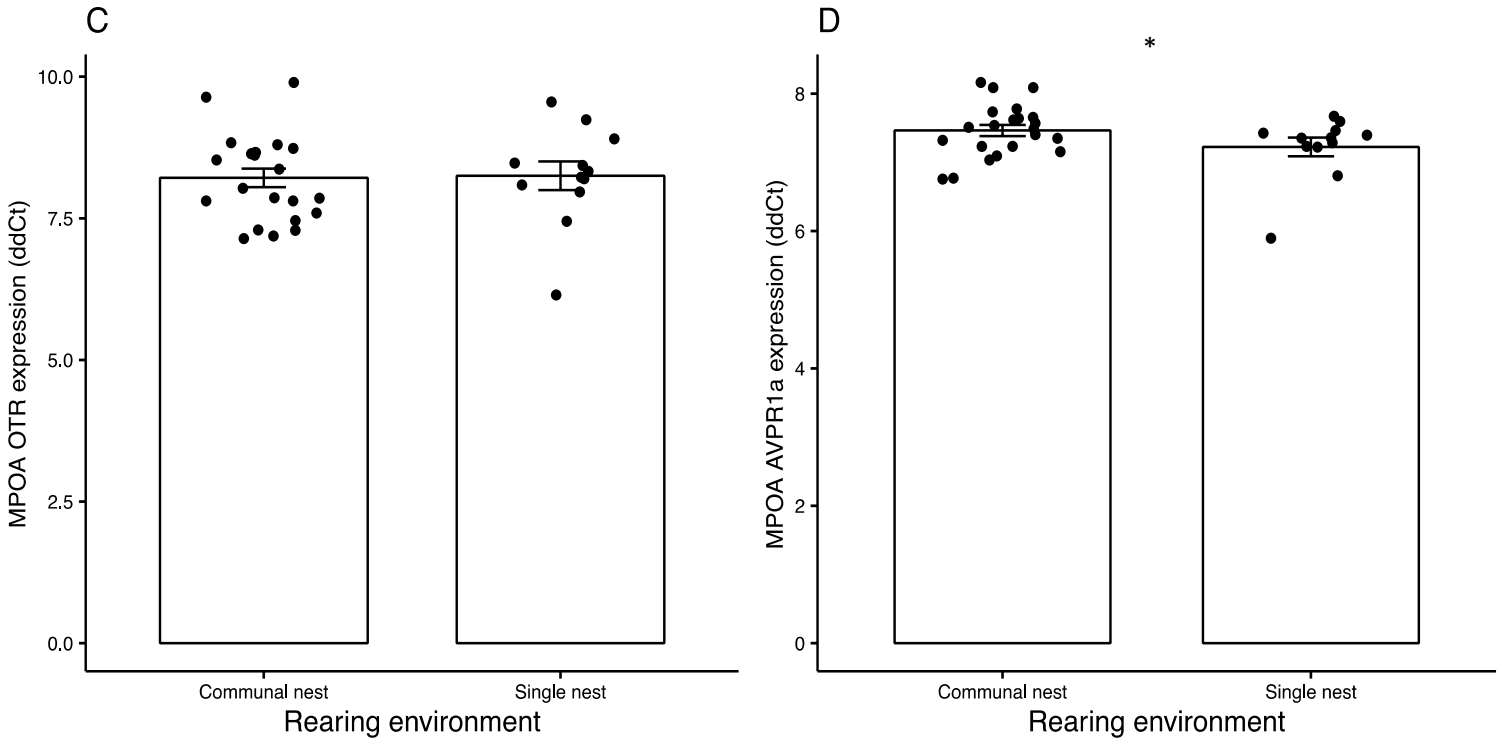



*Figure 4.3: The effect of early rearing environment on oxytocin receptor (OtR) and arginine vasopressin receptor 1a (AvpR1a) in the bed nucleus of the stria terminalis (BNST) and medial pre-optic area (MPOA) in adult female house mice (mean ± SEM). Communal nest reared females had significantly lower OtR expression in the BNST (A: p = 0.032, n = 32) but no difference in OtR expression levels was found in the MPOA (C: p = 0.866, n = 34). AvpR1a expression in the BNST was not influenced by the early rearing environment (B: p = 0.093, n = 34) however, AvpR1a expression was significantly higher in the MPOA of communally reared females (D: p = 0.034, n = 34). BNST AvpR1a data were log transformed to achieve homogeneity of residuals.*

*Table 4.2: Linear mixed effect models to investigate whether the early rearing environment of adult female house mice influences oxytocin receptor (OtR) and arginine vasopressin receptor 1a (AvpR1a) expression in the bed nucleus of the stria terminalis (BNST) or medial pre-optic area (MPOA) of adult female house mice. Rearing environment refers to whether subjects were reared in communal or single nests. AvpR1a expression in the BNST was log transformed. Non-significant interactions and covariates were removed from all models (A: body mass, B, C and D: body mass and number of offspring in the post-natal nest). A: subjects = 32, family ID = 8, breeding block = 2. B, C and D: subjects = 34, family ID = 8, breeding blocks = 2.*

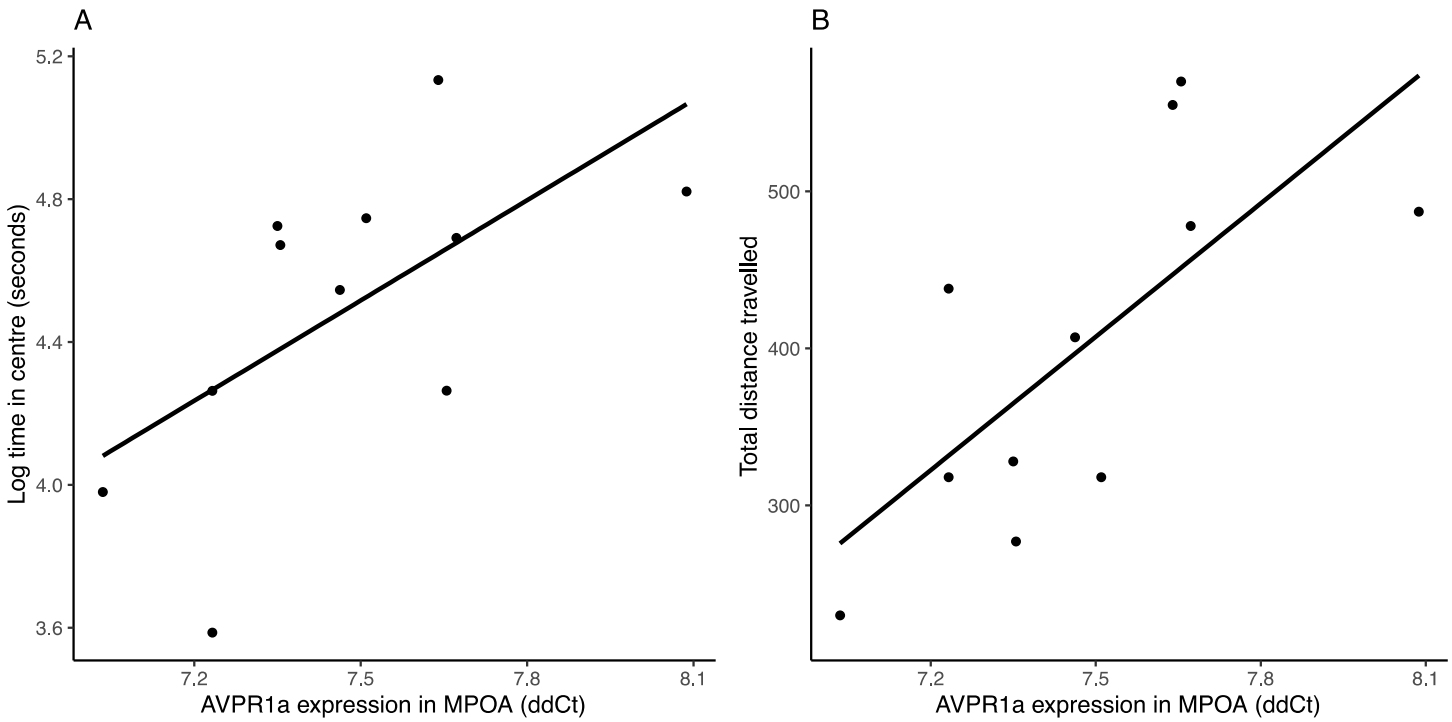
| Fixed effect | Estimate (SE) | t-value | p-value | Random effect | Variance (SD) |
|---|---|---|---|---|---|
| A: BNST OtR | | | | | |
| Intercept | 1.663 (0.119) | 13.978 | <0.001 *** | Family ID | 0.0003 (0.019) |
| Rearing environment | 0.205 (0.068) | 3.031 | 0.010 * | Block | 0 (0) |
| Offspring in nest | 0.025 (0.010) | 2.565 | 0.032 * | | |
| B: BNST AvpR1a | | | | | |
| Intercept | 2.123 (0.055) | 38.789 | 0.009 ** | Family ID | <0.001 (<0.001) |
| Rearing environment | -0.075 (0.043) | -1.732 | 0.093 . | Block | <0.001 (<0.001) |
| C: MPOA OtR | | | | | |
| Intercept | 8.203 (0.230) | 35.600 | 0.004 ** | Family ID | 0 (0) |
| Rearing environment | 0.048 (0.282) | 0.170 | 0.866 | Block | 0.050 (0.224) |
| D: MPOA AvpR1a | | | | | |
| Intercept | 7.506 (0.146) | 51.547 | <0.001 *** | Family ID | 0.062 (0.249) |
| Rearing environment | -0.292 (0.312) | -2.218 | 0.034 * | Block | 0.015 (0.021) |

*Table 4.3: Linear mixed effect models to investigate if the early rearing environment influences urinary corticosterone concentration of adult female house mice. Non-significant interactions and covariates were removed (subject mass, age and number of offspring in the post-natal nest). Urinary corticosterone was log transformed. Subjects = 21, family ID = 8, breeding block = 3.*

| Fixed effect | Estimate (SE) | t-value | p-value | Random effect | Variance (SD) |
|---|---|---|---|---|---|
| Urinary corticosterone | | | | | |
| Intercept | -0.173 (0.160) | -1.082 | 0.330 | Family ID | 0.06 (0.245) |
| Rearing environment | 0.023 (0.154) | 0.151 | 0.882 | Block | 0.013 (0.113) |

### *4.3.3. Anxiety-like behaviour and central receptor expression*

As we found a difference in OtR expression in the BNST and AvpR1a expression in the MPOA linked to communal rearing, we conducted additional analyses to investigate if this variation influences anxiety-related behaviour. A significant positive relationship was found between AvpR1a expression in the MPOA and time spent in the centre of the test arena (Figure 4.4A, Table 4.4A, $p = 0.030$, $n = 11$) and total distance travelled (Figure 4.4B, Table 4.4C, $p<0.001$, $n = 11$). No relationship was found between OtR expression in the BNST and time in the centre of the test arena (Table 4.4B, $p = 0.963$, $n =11$). However, a significant positive relationship was found between OtR expression in the BNST and total distance travelled (Table 4.4D, Figure 4.4C, $p<0.001$, $n = 10$).


A
5.2
4.8
4.4
4.0
3.6
Log time in centre (seconds)
7.2
7.5
7.8
8.1
AVPR1a expression in MPOA (ddCt)
B
500
400
300
Total distance travelled
7.2
7.5
7.8
8.1
AVPR1a expression in MPOA (ddCt)

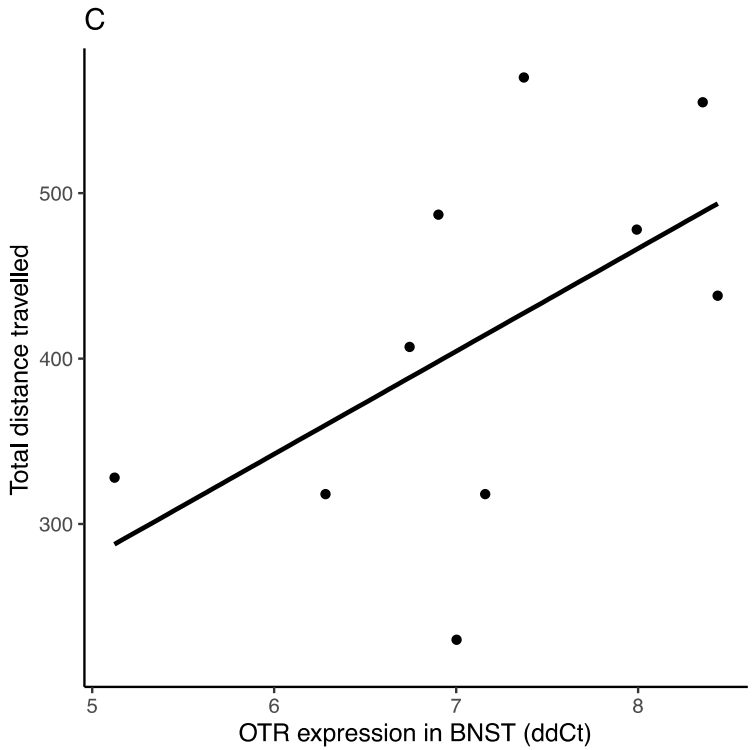



*Figure 4.4: Relationship between arginine vasopressin receptor 1a (AvpR1a) and oxytocin receptor (OtR) expression in the medial pre-optic area (MPOA) and bed nucleus of the stria terminalis (BNST) on anxiety like behaviour of adult female house mice, specifically time spent in the centre of a test arena and total distance travelled during a novel open field test. A significant positive relationship was found between AvpR1a expression in the MPOA and time in the centre of the test arena and (A: $p = 0.030$, $n = 11$) and total distance travelled (B: $p<0.001$, $n = 11$). A significant positive relationship was found between total distance travelled and OtR expression in the BNST (C: $p<0.001$, $n = 10$). Time in centre was log transformed.*

*Table 4.4: Linear mixed effect models to investigate whether central arginine vasopressin receptor 1A (AvpR1a) and the oxytocin receptor (OtR) in the medial pre-optic area (MPOA) and oxytocin receptor in the bed nucleus of the stria terminarlis (BNST) influences anxiety-related behaviour in adult female house mice in terms of time spent in the centre of the test arena (A and B) and total distance travelled (C and D). Time in the centre of the test arena was analysed using the lmer() function and was log transformed, total distance travelled was analysed using the glmer() function with a Poisson distribution. Family ID and breeding block were included as random factors. Non-significant interactions and covariates were excluded from all models (subject age, subject body mass and number of pups in the post-natal nest). A, B, C: subjects = 11, family ID = 7, breeding blocks =2. C and D: subjects = 10, family ID = 7, breeding blocks =2.*

| Fixed effect | Estimate (SE) | t-value | p-value | Random effect | Variance (SD) |
|---|---|---|---|---|---|
| A: Time in centre | | | | | |
| Intercept | -2.582 (2.603) | -0.991 | 0.354 | Family ID | 0 (0) |
| Rearing environment | 0.254 (0.203) | 1.251 | 0.250 | Block | 0.074 (0.272) |
| AvpR1a MPOA | 0.936 (0.346) | 2.708 | 0.030 * | | |
| B: Time in centre | | | | | |
| Intercept | 4.581 (1.026) | 4.466 | 0.007 ** | Family ID | 0.128 (0.358) |
| Rearing environment | -0.017 (0.349) | -0.050 | 0.963 | Block | 0.057 (0.239) |
| OtR BNST | -0.007 (0.141) | -0.049 | 0.963 | | |
| Fixed effect | Estimate (SE) | z-value | p-value | Random effect | Variance (SD) |
| C: Total distance travelled | | | | | |
| Intercept | -5.475 (1.649) | -3.321 | <0.001 *** | Family ID | 0.171 (0.134) |
| Rearing environment | -0.240 (0.057) | -4.177 | <0.001 *** | Block | <0.001 (0.023) |
| AvpR1a MPOA | 1.537 (0.219) | 7.014 | <0.001 *** | | |
| D: Total distance travelled | | | | | |
| Intercept | 4.596 (0.223) | 20.618 | <0.001 *** | Family ID | 0.042 (0.204) |
| Rearing environment | -0.073 (0.059) | -1.221 | 0.222 | Block | 0 (0) |
| OtR BNST | 0.199 (0.028) | 6.996 | <0.001 *** | | |

## 4.4. Discussion

Here we demonstrate that natural variation in early life experience, specifically being reared in a communal or single nest environment, may influence anxiety-related behaviours and stress physiology in adult female house mice. Our findings suggest that increased levels of maternal care and peer to peer interactions within communal nests may alter the neuropeptide system, which regulates the stress response and corresponding behaviour. The quality and quantity of social interactions during the early post-natal period has previously been shown to decrease stress-related behaviours (Meaney, 2001, Curley et al., 2009). Indeed, for BALB/C laboratory mice, Curley et al. (2009) concluded that a communal nest may be seen as a socially enriched environment. However, this may not be the case for house mice that naturally use both single and communal nest reproductive strategies, as it is often the smaller and less competitive females that nest communally, and rearing offspring in single nests results in overall greater lifetime reproductive success (Ferrari et al., 2019). For BALB/c mice, communally reared F1 subjects have been found to display significantly less anxiety-like behaviours, in terms of fewer faecal boli produced and less time spent immobile (Curley et al., 2009). In line with this, communally reared females in the current study may have shown significantly more exploratory behaviour (as highlighted by an increased distance travelled) and therefore less anxiety-related behaviour, suggesting increased maternal and sibling interaction may result in less anxious offspring. However, this was not replicated for time spent in the centre of the test arena and therefore caution should be used when interpreting this result. Furthermore, an open field test may not be the best behavioural assay to investigate anxiety-related behaviour in house mice (Jane L. Hurst, personal communications) highlighted by the stereotypic mice that were removed from analyses. It is important to note that we are likely to have removed the most anxious mice from the analysis (those showing stereotypic behaviours being the most anxious mice (Mason, 1991)). However, retaining these five mice would have prevented rigorous statistical analyses, and also the use of stereotypic mice in studies can lead to erroneous results (Jane L. Hurst, personal communications) (Novak et al., 2015).

Curley et al. (2009) found no influence of communal rearing on distance travelled or time spent in the inner area of an open field test for BALB/c laboratory mice. Additionally, Branchi et al. (2006b) found no effect of communal rearing on locomotor activities, and single and communally reared male F1 house mice did not differ in their response to a novel open field

test (Fischer et al., 2018). Studies using a variant of the communal rearing model (different aged pups reared by multiple females) suggest communal rearing may increase anxiety-related behaviour (Branchi and Alleva, 2006, Branchi et al., 2006b). The direction of the effect of communal rearing on anxiety-related behaviour may therefore be strain and context dependent.

Two brain regions that were selected for analysis are two that play key roles in the female stress response via the neuropeptide and HPA networks: the bed nucleus of the stria terminalis (BNST) and medial pre-optic area (MPOA) which are highly linked structures (Daniel and Rainnie, 2016, Dong and Swanson, 2006, Lebow and Chen, 2016, McDonald et al., 1994). These two areas were selected due to their importance in the stress response. The BNST is important in regulating anxiety and its plasticity is known to be sensitive to stress, mediating long term responses to anxiety (Daniel and Rainnie, 2016). The BNST mediates the stress response in two main ways. It contains corticotropin-releasing hormone (CRH) expressing long-range projecting neurons (Dedic et al., 2018), releasing CRH to activate the HPA axis (Davis et al., 2010). The BNST also projects AVP neurons to oxytocin neuronal cell bodies in the paraventricular nucleus (PVN) of the hypothalamus (Dong and Swanson, 2006, Dong et al., 2001) and activates both parvocellular and magnocellular neurons within the PVN (Lebow and Chen, 2016) which synthesise both oxytocin and AVP (Ross and Young, 2009). The BNST receives inputs from nuclei including the MPOA (Dong and Swanson, 2006), which also contains CRF-producing neurones (McDonald et al., 1994) with the potential to activate the HPA axis (Lebow and Chen, 2016). The MPOA is sexually dimorphic (McDonald et al., 1994) and is more widely known in the regulation of maternal behaviour (Klampfl et al., 2018) and maternal stress where it is vital for dampening down the post-partum stress response (Bosch and Neumann, 2012). Alternative receptor sites of interest that are key in the stress response that could have been included are the medial prefrontal cortex; however, this has previously been investigated mainly in male animals (Sabihi et al., 2017), the amygdala (Dong et al., 2017) and lateral septum (Zoicas et al., 2014).

The quantitation of hormone receptors in tissues can be achieved by two principal routes. Firstly, receptor protein can be estimated, for example by measuring receptor protein-specific gene expression by qPCR or by using a receptor-specific immunohistochemical method. Secondly, receptors can be quantified by ligand-specific binding, for example the use of a radioactive ligand followed by autoradiography (Curley et al., 2009), or using a ligand-specific

antibody to detect receptor-bound ligand. Each approach has advantages and disadvantages. Although binding assay approaches have the theoretical advantage of measuring biologically relevant receptor, there are drawbacks. For example the use of radioactive compounds requires a specialist laboratory which was not available for the current study, and quantifying receptor-bound ligand using antibodies, particularly for relatively small molecules such as oxytocin, can pose a problem of steric hindrance (Borst et al., 2017). The use of immunohistochemical methods to quantify specific proteins including receptors in tissues often suffer from a lack of reproducibility and standardisation in the many stages of the process including fixation conditions, specimen pre-treatment, reagents, detection methods, and interpretation of results (Rojo et al., 2009). Therefore, in the current study, the gene expression of oxytocin and AVP1a receptors were quantified using qPCR. qPCR is a widely used and accepted method which has the great advantage of being an objective quantitative methodology.

In the current study we demonstrated an effect of communal rearing on oxytocin receptor and AvpR1a levels in specific brain regions. Indeed, female anxiety-related behaviour is partially mediated by the BNST and MPOA of the hypothalamus (Klampfl et al., 2018). Here we have shown that within the BNST, OtR expression was significantly lower for communal nest reared females. However, the early rearing environment did not influence OtR expression in the MPOA. Contrasting this, Curley et al. (2009) found significantly greater oxytocin binding within the BNST of communally reared laboratory mice reflecting reduced anxiety-like behaviour. Indeed, there is evidence to suggest that increased levels of maternal grooming and peer to peer interactions from siblings within the nest are associated with an increase in OtR in offspring (Champagne et al., 2001, Champagne and Meaney, 2007, Francis et al., 2000). In contrast, maternal licking and grooming have also found to be positively correlated with AvpR1a and negatively with OtR expression in specific brain regions (Schmidt et al., 2018). Furthermore, pre-pubertal stress decreased the expression of AvpR1a in the pre-frontal cortex of female rats when adult (Brydges et al., 2020) and social instability stress resulted in decreased expression of both the AvpR1a and OtR in the pre-frontal cortex (Nowacka-Chmielewska et al., 2017), suggesting that there is no simple correlation between stress/anxiety-like behaviours and expression of OtR or AvpR1a. This is in line with the current study where communally reared females were found to have significantly lower OtR expression in the BNST and higher AvpR1a in the MPOA which may highlight species and strain-specific differences in responses to communal rearing. In the current study, there will

also be much broader genetic variability in randomly outbred wild stock house mice compared to the laboratory inbred stains of rodents used in other studies.

The BNST is crucial in regulating anxiety and its plasticity is known to mediate long term responses to anxiety (Daniel and Rainnie, 2016, Lebow and Chen, 2016). In the current study, OtR expression within the BNST was found to be positively correlated with total distance travelled, suggesting that higher oxytocin receptor expression was found in less anxious mice. Indeed, increases in OtR densities have previously been linked to decreased anxiety-like behaviour in rodents (Champagne and Meaney, 2007). This contradicts the possibility that the communally reared mice in the current study showed less anxiety-related behaviour and further highlights the need to use caution when interpreting the total distance travelled data for communally reared females. Indeed, following a study of the expression of AvpR1a and OtR in areas of the brain including the MPOA and BNST, related to behavioural changes associated with reproductive experience, Naik and de Jong (2017) concluded that their results emphasise the transient nature of these behavioural and molecular adaptations. Even though the neuropeptide system was found to be sensitive to the early rearing environment, which is known to modulate the HPA axis, no difference in urinary corticosterone response was detected between communally and singly reared females in the current study.

This study also found that communal reared females exhibited significantly higher AvpR1a expression within the MPOA. AvpR1a within the BNST however was not influenced by the early rearing environment. Curley et al. (2009) found decreased AvpR1a binding in one location, the lateral septum, for communally reared offspring, but no other brain region. We also found that AvpR1a expression in the MPOA was positively correlated with both total distance travelled and time spent in the centre of the test arena. This may be surprising as increased exploratory behaviour (increased distance and time in the centre of the test arena) are higher in mice displaying reduced anxiety-related behaviour and AVP is thought to be anxiogenic (Neumann and Landgraf, 2012, Amico et al., 2004, Windle et al., 2004, Neumann et al., 2000a, Neumann and Slattery, 2016, Mak et al., 2012). Indeed, AvpR1a knockout mice show decreased anxiety-like behaviour, specifically increased open-arm entries on an elevated plus maze (Egashira et al., 2007), and over expression of AVP receptor 1a within the lateral septum of wild-type mice results in mild increased anxiety-related behaviour, specifically decreased time spent in the light region of a light-dark box (Bielsky et al., 2005a). However, these studies investigating reduced AvpR activity are primarily focused on specific

brain regions of male rats. By contrast, AvpR1a knock-out female mice have unaltered anxiety-related behaviour (Bielsky et al., 2005b), and studies have also shown that AvpR antagonists can be associated with decreased anxiety-related behaviour (Everts and Koolhaas, 1999, Liebsch et al., 1996). This suggests that AVP mediated stress responses could be species, sex and receptor location dependent. Indeed, in the current study, the relationship between AvpR1a and reduced anxiety-related behaviour was only found in the MPOA. The MPOA is key in maternal behaviour, and motherhood is a known time of muted anxiety. It is widely reported in rodents that post-natal dams have a reduced state of anxiety to enable them to appropriately care for their offspring, for example, if an intruder was to appear (Bosch, 2011). Furthermore, the oxytocin system is up regulated during this post-partum period resulting in anxiolytic effects. As the MPOA is vital for maternal behaviour (Klampfl et al., 2018), and successful maternal behaviour is reliant on a lower innate level of anxiety (Bosch, 2011), one reason for this contradictory data within the MPOA could be because this region is critical for producing a lower innate anxiety level in adult females to prepare for motherhood.

In conclusion, the current study suggests that the stress response elicited in house mice to a novel open field test may partially be modulated by the central AVP and oxytocin systems within the BNST and MPOA of the hypothalamus, and that these systems are sensitive to the early rearing environment. Indeed, being reared in a communal nest resulted in female mice showing increased exploratory behaviour, significantly lower OtR expression in the BNST and significantly higher AvpR1a in the MPOA. Furthermore, significant relationships were found between receptor expression and behavioural responses to the open field test. These novel findings in female house mice offer new understanding of how natural variation in reproductive tactics may influence the response of offspring to novel situations.

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Ethical note

Animal use and care was in accordance with the EU directive 2010/63/EU and UK Home Office code of practice for the housing and care of animals bred, supplied or used for scientific purposes.

Declaration of interest

None.

4.5. References


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## 4.6. Supplementary Material

*Table S4.1: Summary of litters produced during the experiment across three breeding rounds and two treatment groups, and the number of female subjects from which physiological measures were taken and subject numbers used in the novel open field test. Each breeding trio consisting of 2 related females and 1 unrelated male and had the opportunity to breed 3 times, however not all females bred in each round. Abbreviations: Tx: breeding treatment, CN: communal and SN: single nest rearing environment (number of pups in each of the single nests are separated by |), uCC: urinary corticosterone.*

| Breeding trio | Round 1 | | Round 2 | | Round 3 | | uCC | Round 1 receptors | Round 2 receptors | Round 1 OFT | Round 2 OFT | Round 3 OFT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tx | # pups | Tx | # pups | Tx | # pups | | Subject # | Subject # | Subject # | Subject # | Subject # |
| 1 | CN | 14 | SN | 8\|6 | SN[2] | 6\|0 | 4 | 4 | 2 | 2 CN | 2 SN | 0 |
| 2 | CN | 13 | SN | 6\|8 | | 0 | 4 | 4 | 2 | 1 CN | 1 SN | 0 |
| 3 | CN | 8 | | 0 | | 0 | 2 | 2 | 2 | 2 CN | 0 | 0 |
| 4 | | 0 | SN | 7\|0 | CN | 9 | 2 | 0 | 2 | 0 | 2 SN | 0 |
| 5 | CN[1] | 11 | CN | 15 | | 0 | 1 | 2 | 2 | 1 CN | 0 | 0 |
| 6 | SN | 7\|4 | CN | 12 | SN | 7\|1 | 5 | 2 | 4 | 2 SN | 2 CN | 0 |
| 7 | SN | 3\|0 | SN[2] | 5\|0 | | 0 | 3 | 2 | 0 | 0 | 2 SN | 0 |
| 8 | SN | 4\|8 | CN | 13 | SN | 5\|0 | 5 | 2 | 4 | 1 SN | 2 CN | 1 SN |

[1] Initially a single nest treatment, females chewed through the central divide creating a communal nest at PND 6

[2] Designated into the communal nest treatment group but only one female bred resulting in a single nest

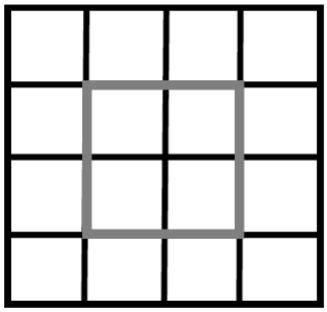

*Figure S4.1: For the purpose of analysis, the novel open field test arena (70 x 60 x 55cm MDF arena) was split into 16 equal squares and the distance travelled calculated by a count of the total number of lines crossed. Total time spent in the centre of the test arena (grey square) was also recorded.*

## Chapter 5: African wild dog (*Lycaon pictus*) faecal steroid hormone metabolite concentrations degrade over 4 hours in UK *ex situ* environments

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Abstract

The measurement of hormone metabolites in excreta has facilitated non-invasive physiological monitoring of both *in situ* and *ex situ* endangered species. Within *ex situ* populations, this enables evidenced-based management decisions to improve welfare and reproductive success. This is particularly important for species on the brink of extinction, including the African wild dog (*Lycaon pictus*). However, hormone metabolites within excreta are sensitive to environmental conditions and degrade, resulting in erroneous results and conclusions. Here we used controlled degradation studies (summer and winter) to investigate the impact of environmental conditions (sun / shade and wet / dry) on the rate of faecal hormone degradation for an *ex situ* wild dog population. The stability of faecal hormone metabolites of progesterone, glucocorticoids and testosterone was investigated. The stability varied between the different hormones of interest, and all were less stable during the summer. The concentration of the least stable hormone metabolite, glucocorticoid, varied significantly within 4 hours of the start of both the summer and winter trials. For all hormones of interest, concentrations were significantly higher in sunny compared to shaded locations during the summer, and significantly lower in wet locations compared to dry in the winter. Hence, we recommend faecal samples be collected promptly, not during wet weather, and that the condition of the sample site (sunny versus shaded location) be recorded for use as a covariate in analysis. Our study highlights the importance of robust sample collection protocols and the use of degradation studies prior to conducting a non-invasive faecal hormone metabolite study.

Keywords

*Lycaon pictus*, faecal steroid hormones, environmental sensitivity, degradation.

## 5.1. Introduction

African wild dogs (*Lycaon pictus*) are classed as "one of the world's most threatened carnivores", with fewer than 1500 mature adults left in the wild (Woodroffe and Sillero-Zubiri, 2020). Those that inhabit non-protected areas are subjected to human-related mortality, domestic dog diseases and habitat fragmentation (IUCN, 2017, Woodroffe et al., 2007, Woodroffe and Sillero-Zubiri, 2020). At the current rate of decline, captive breeding is vital to conserve this species. Unfortunately, captive groups of wild dogs suffer from high levels of aggression and poor reproductive success (Van der Weyde et al., 2015), including high pup mortality due to infanticide by conspecifics (Yordy and Mossotti, 2016, Van Heerden et al., 1996). To enable greater understanding of this endangered species and to improve conservation efforts, it is important to investigate factors perturbing captive wild dog populations.

Methods for measuring stress and reproductive hormones are vital to monitoring captive endangered species, allowing for informed management decisions to improve welfare and captive breeding programmes. Indeed, endocrinological analyses facilitate greater understanding of the physiology underpinning behaviour, reproduction and response to stressors. Obtaining blood samples for longitudinal hormone monitoring of captive populations is neither ethical nor desirable. The capture and handling of individual animals is highly stressful and is therefore likely to result in measured hormone levels that do not reflect normal physiology (de Villiers et al., 1997). Instead, longitudinal hormone analysis for captive endangered species requires non-invasive measurement in faeces, a widely successful application (Brown, 2006). Unlike the diurnal pulsatile variations in blood hormone concentrations, faecal hormone analysis provides an estimate of hormone levels over a number of hours (Touma and Palme, 2005). Before the hormone of interest reaches the urine or faeces, it is metabolised, resulting in high concentration of hormone metabolites, and these are measured rather than the original hormone (Macdonald et al., 1983). Steroid hormones undergo a two-phase metabolism, firstly via a reduction reaction, followed by conjugation to increase their water solubility (Schiffer et al., 2019).

Climatic variation has been shown to alter steroid hormone metabolites in excreta post deposition in a wide range of species, from wild bears (grizzly bears (*Ursus arctos horribilis*) and American black bears (*U. americanus*)) and captive jaguars (*Panthera onca*) (Stetz et al.,

2013, Mesa-Cruz et al., 2014) to semi-captive Asian elephants (*Elephas maximus*) (Wong et al., 2016). Indeed, hormone metabolites in faeces are known to be affected by temperature and ultraviolet light, all of which can influence the microbes within the excreted bolus, both directly and indirectly altering hormone metabolite concentrations via biotransformation (Macdonald et al., 1983, Touma and Palme, 2005, Schwartz and Monfort, 2008, Millspaugh and Washburn, 2004). Many studies have also shown that concentrations of hormone metabolites significantly vary over time from when defecation occurred, highlighting the requirement for rapid collection protocols. This is the case across multiple taxa including orangutans (*Pongo pygmaues*) (Muehlenbein et al., 2012) and Asian elephants (*Elephas maximus*) (Wong et al., 2016). Where non-invasive physiological monitoring is important, a degradation study is therefore vital to ensure accurate hormone monitoring in the captive species of interest.

Faecal hormone metabolites have been measured in wild dogs in multiple studies (Monfort et al., 1997, Creel, 2001, Van der Weyde et al., 2016, Santymire and Armstrong, 2010). The gut transit times for wild dogs is approximately 22 hours with peak metabolites reaching the faeces 18 hours following hormone infusion (Monfort et al., 1997, Van der Weyde et al., 2015). Due to management practices of dangerous animals, samples may not be collected for days following defecation, or maybe collected opportunistically (Van der Weyde et al., 2015). However, to date, no degradation study has been conducted for captive wild dogs to investigate the stability of faecal hormone metabolites between defecation and the time that samples are collected and stored for hormone analysis.

Prior to measuring hormone metabolites using an enzyme-immunoassay, the first step is to ascertain that the assay is capable of measuring the metabolites of interest without interference from other constituents within the sample. Firstly, a parallelism study needs to be conducted to ensure the assay is indeed measuring hormone metabolites that are structurally similar to the synthetic hormone to which the antibody within the assay was raised. Secondly, a background matrix interference assessment needs to be conducted to ensure there is no significant interference from other chemicals within the sample. Furthermore, the concentration of hormone metabolites in faecal samples can vary over time between the time of defecation and sample collection, and are dependent on environmental conditions including temperature and precipitation (Millspaugh and Washburn, 2004).

Experimental assessment of the influence of environmental conditions on faecal hormone metabolites is a crucial step to ensure methodological validity. Following this, long-term hormone assessments can be conducted on captive wild dog populations to decipher stressors or to perform reproductive assessments as required. Therefore, the aims of this project were to: 1) biochemically validate enzyme immunoassays for male, female and unknown/mixed sex faecal samples for progesterone, testosterone and corticosterone faecal metabolites, 2) determine the stability of faecal hormone metabolite from wild dogs over time in a captive setting, and 3) determine how environmental parameters including sun and rainfall influences faecal hormone metabolite concentration within defecated faecal boli. To achieve these aims, winter and summer faecal hormone metabolite degradation studies were conducted at Chester Zoo, UK.

## 5.2. Methods

To investigate if levels of faecal hormone metabolites vary over time, a degradation experiment was conducted at Chester Zoo, UK, with winter and summer trials. Faecal samples were collected from two adult male and one adult female captive wild dogs over two three-month periods (winter trial: January to March 2017 (faecal samples n = 10) and summer trial: June to August 2017 (faecal samples n = 10)) during routine enclosure management. Samples were collected by Keeping Staff at approximately 0900h and stored at -20$^{o}$C up to 18 months prior to analysis. Only samples of fresh appearance were collected (up to about 4-5 hours old). Because of the animal husbandry policies at Chester Zoo, it was not possible to identify the source or sex of individual faeces. Samples were homogenised to create a winter trial pool and a summer trial pool. The pack was fed a consistent diet of a full calf carcass every 48 hours (2.5kg of meat per dog, including hair and bones, but offal removed, with the addition of a carnivore vitamin and mineral supplement) throughout both study periods.

Each pooled faecal sample (winter and summer) was split into four experimental treatment groups: sun-wet, sun-dry, shade-wet and shade-dry. Duplicate faecal aliquots were placed in each treatment group as shown in Figure 5.1. The faecal aliquots were placed in real life conditions at Chester Zoo for three days and subsamples collected every two hours between 07:30 and 19:30 during days one and two, and every four hours between 07.30 and 19.30 on day three (for sample numbers see Supplementary Material, Table S5.1). Due to access limitations, subsamples could not be collected throughout a full 24-hour period. Dry faecal

aliquots were covered with a clear canopy to prevent rainfall, whereas wet faecal aliquots were left open to the rain (weather dependent). Sun faecal aliquots were placed in a sunny aspect that received sun from 09:00 until sundown (cloud dependent) whereas the shade faecal aliquots were placed in a 100% shaded location. During each collection period, humidity and temperature was recorded. Additionally, rainfall was measured. During collection, an area of the faecal aliquot was homogenised to include external and internal areas of the sample, placed into a labelled collection tube and stored at -20$^{o}$C until hormone extraction (up to n = 144 subsamples for each trial, Table S5.1).

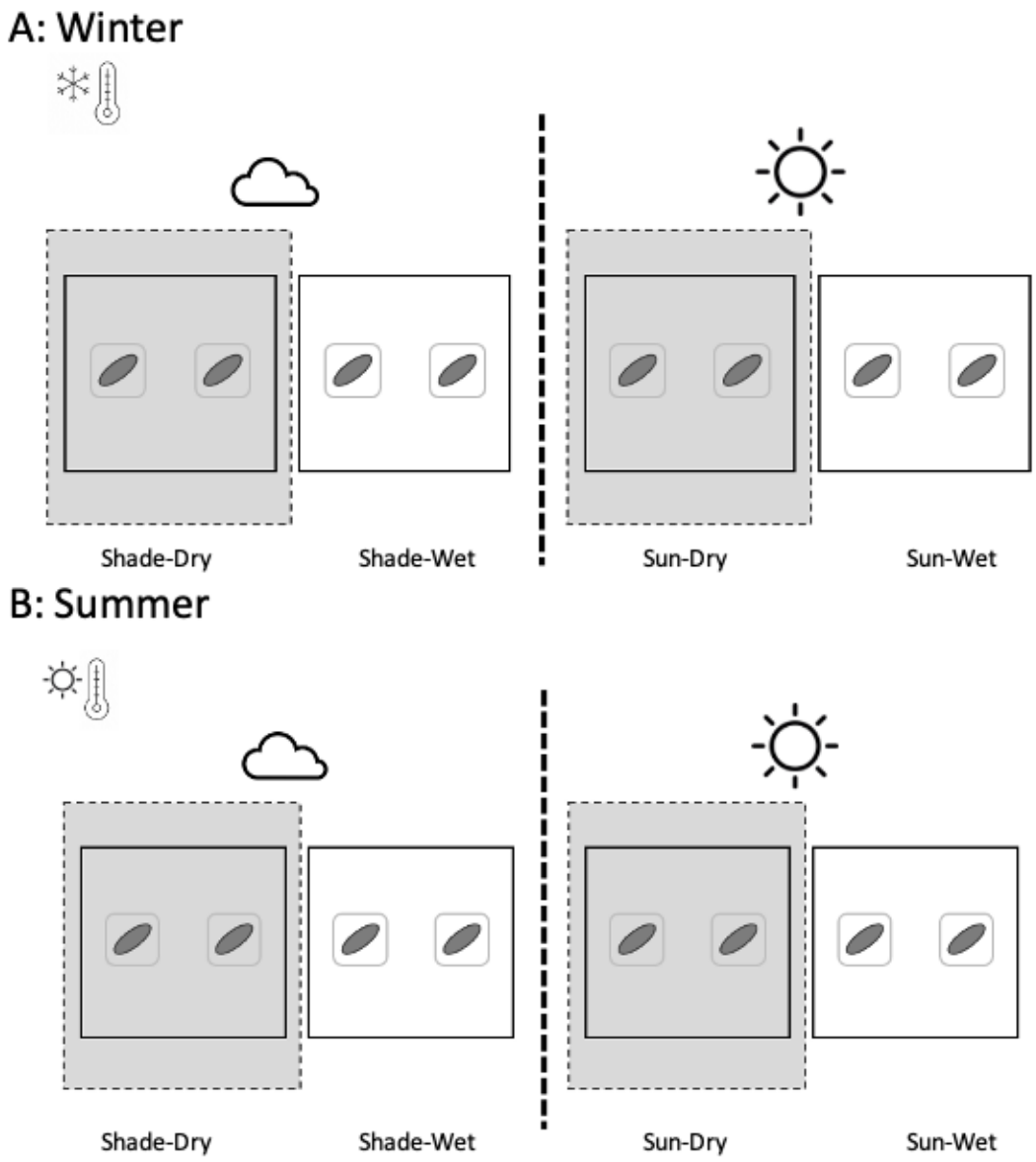


*Figure 5.1: Experimental set up. In both the winter (A) and summer (B) trials. Duplicate faecal aliquots (produced from pooled samples consisting of 10 homogenised faecal piles from 3 African wild dogs) were placed in weigh-boats and allocated into one of four treatment groups: shade-dry, shade-wet and sun-dry, sun-wet. Dry samples were placed under a clear canopy (filled grey rectangle) and shaded samples (cloud) were placed in a 100% shaded aspect. Sun samples (sun) were placed in a sunny aspect (cloud dependent).*

### *5.2.1. Hormone analysis*

#### Wet-weight extraction protocol

Hormone metabolites were extracted from each subsample using a wet-weight extraction technique as described previously (Walker et al., 2002, Edwards et al., 2016). Briefly, 0.5g of faecal material was mixed with 5ml 90% methanol, agitated overnight, dried and reconstituted in 1ml 100% methanol before being stored at -20°C until analysis.

### Enzyme immuno-assay and validation

Subsamples were biochemically validated (parallelism and matrix interference assessment) and analysed using enzyme immunoassays (EIAs) following methods previously published to measure progestogens (monoclonal antiserum progesterone metabolite CL425 (Walker et al., 2008)), glucocorticoids (corticosterone antiserum CJM006 (Watson et al., 2013, Munro and Stabenfeldt, 1984)) and testosterone (testosterone polyclonal antiserum R156/7 (Edwards et al., 2015)). CL425, CJM006 and R156/7 were provided by C. Munro, University of California Davis, CA, USA. For each hormone of interest, a parallelism study was conducted: faecal extract was serial diluted in duplicate alongside a serial dilution of synthetic hormone standard (progesterone standard range 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 pg/ml): corticosterone standard range: 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg/ml, testosterone standard range: 2.3, 4.7, 9.4, 18.8, 37.5, 75, 150, 300 and 600 pg/ml). Results were plotted and parallelism of the displacement curve and standard curve was visually inspected and confirmed by linear regression analysis. A matrix interference assessment was conducted to determine whether any other constituent of the sample matrix significantly interfered with the hormone measurement. A serial dilution of synthetic standard (corticosterone standard range: 7.8, 15.6, 31.2, 62.5, 125, 250 and 500 pg/ml; progesterone standard range: 3.12, 6.25, 12.5, 25, 50, 100 and 200 pg/ml and testosterone standard range: 9.4, 18.8, 37.5, 75, 150, 300 and 600 pg/ml) was spiked with an equal volume of faecal extract (faecal extract at a working dilution to achieve 50% binding: female samples: progesterone 1:1500, corticosterone 1:50 and testosterone 1:300; males samples: corticosterone 1:50 and testosterone 1:300; and group samples: corticosterone 1:50, progesterone 1:1024 and testosterone 1:120). The background interference was then deleted from the observed concentration and compared with synthetic standards, confirmed by linear regression analysis.

The antiserum cross-reactivities can be found in Tables S5.3 to S5.5 and are published elsewhere: CJM006 (Watson et al., 2013), R156/7 (Santymire and Armstrong, 2010) and CL425 (Graham et al., 2001). Intra-assay CVs for each sample was less than 10% and between plate CVs were as follows: testosterone: high quality control (26.79 %b/Bo)11.76% and low quality control (67.45%b/Bo) 13.53%; corticosterone: high quality control (22.66%b/Bo) 9.00% and low quality control (61.8%b/Bo) 5.47%; and progesterone: high quality control (16.48%b/Bo) 9.97% and low quality control (53.21%b/Bo) 6.23%.

*5.2.2. Statistical analysis*

Faecal hormone metabolite concentrations were analysed with linear mixed models using the lmer() function in the lme4 package in R (Bates et al., 2015). Hormone metabolite concentration was included as the response variable with time (2h, 4h, 6h, 8h, 10h, 12h, 24-30h, 32-36h and >48), location (shade versus sun) and condition (wet versus dry), maximum temperature, precipitation and humidity as covariates in the winter models. Summer models were identical other than excluding precipitation as it only rained twice during the summer test period. Both models included variation between faecal pile as a random factor (n = 8). Time was investigated as a categorical variable to best determine variation from time 0. Contrasts were set to compare each time point against time 0. The minimal model was determined using the drop1() and update() functions and non-significant interaction and covariates were dropped from the final model. Location and condition were retained in the models due to experimental design. P-values were calculated using the summary() function in the lmerTest package with Satterwaite approximation and P values $<0.05$ were deemed significant. Data were transformed where required (log) and outliers, identified on box and whisker plots, removed to achieve homogeneity and normality of residuals. Normality was confirmed by visual inspection of residuals in addition to Shapiro-Wilks and Lillie normality tests. Outliers were removed from the progesterone winter (n = 5) and both corticosterone models (winter outliers n = 3, summer outliers n = 1) to achieve homogeneity. Progesterone summer, and both testosterone models were log transformed to achieve homogeneity.

## 5.3. Results

*5.3.1. Biochemical validations*

Successful parallelism was achieved: the displacement curves generated by serial dilutions of extracted faecal metabolite were significantly parallel to the standard curve created by the standard synthetic hormone preparation to which the antibody was raised (Table S5.2, $p<0.001$) for the three EIAs of interest (corticosterone, progesterone and testosterone). No matrix interference was detected: the addition of diluted extracted faecal metabolite to the synthetic corticosterone, progesterone or testosterone antigen did not significantly alter the observed concentration (Table S5.2, $p<0.001$). Percentage recoveries ± SEM can be found in Table S5.2. Furthermore, the regression coefficient that represents the gradient was close to 1 in all validations, highlighting that the faecal extract matrix had no influence on the concentration of synthetic standard within the assay.

*5.3.2. Degradation study*

### Progesterone

During the winter trial, the mean concentration of faecal progesterone metabolite (fPGM) varied significantly over time (Figure 5.2A: Table 5.1A, $p<0.001$, $n = 139$), with metabolite concentration only remaining stable for up to 8 hours following the initiation of the trial. At the beginning of the degradation study (time 0, baseline), the mean (±SD) fPGM concentration was 421.2 ± 131.2 ng/g. After 10 hours, a significant difference was found in fPGM concentration from baseline ($p = 0.005$). During this time, the average measured fPGM concentration increased to 654.5 ± 161.3 ng/g faeces, a 55.5% increase in concentration. Within the winter model, location (sun versus shade) and condition (wet versus dry) both had a significant influence on fPGM concentration (location: $p = 0.007$, condition: $p = 0.013$, Table 5.1A), with concentrations being higher in the sunny location and dry condition. Significant positive relationships were also found between fPGM concentration and max temperature ($p = 0.003$) and humidity ($p <0.001$) (Table 5.1A).

The concentration of fPGM was less stable over time during the summer trial (Figure 5.2B: Table 5.1B, $p<0.001$, $n = 144$). At the beginning of the degradation study (time 0, baseline), the mean (±SD) faecal progesterone metabolite (fPGM) concentration was 302.0 ± 61.3ng/g. The metabolite concentration remained unchanged up to 4 hours following time 0 ($p = 0.070$). After 6 hours, a significant difference in concentration from baseline was found ($p = 0.009$). By 6 hours into the trial, fPGM concentration had more than doubled to 621.1 ± 260.6 ng/g faeces, an increase of 105.7%. Neither location nor condition were found to influence fPGC concentration (Table 5.1B).

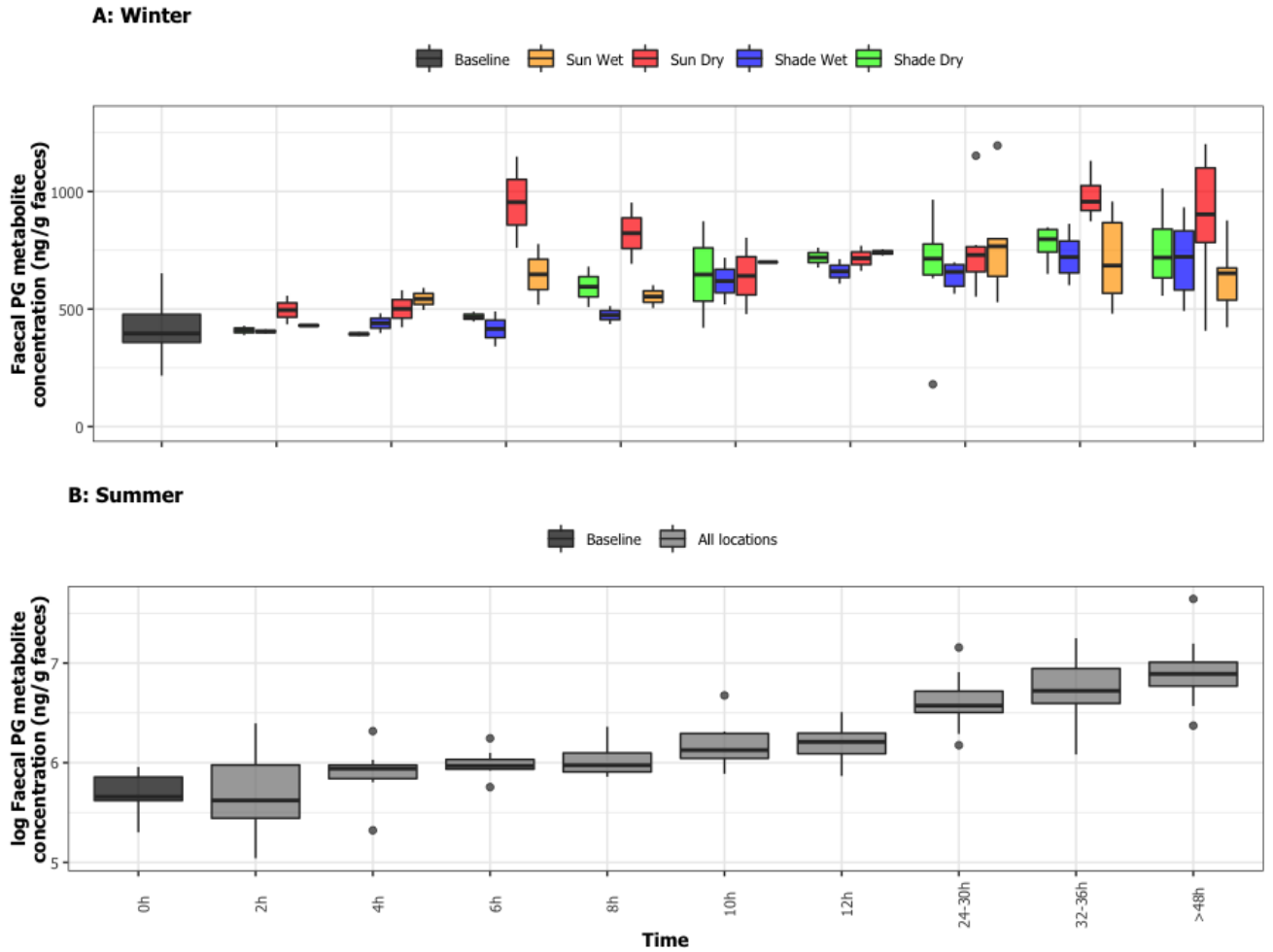


*Figure 5.2: The concentration of faecal progesterone metabolite (fPGM) varied significantly over time in both (A) the winter degradation study ($p<0.001$, $n = 139$) and (B) the summer degradation study ($p<0.001$, $n = 144$). In the winter trial (A), fPGM varied significantly between the sunny and dry locations ($p = 0.007$) and wet and dry conditions ($p = 0.013$). No effect of location or condition on fPGM concentration was found during the summer (B) study.*

*Table 5.1: Linear mixed models investigating factors that influenced faecal progesterone metabolite (fPGM) concentration during the (A) winter, n = 139, and (B) summer, n = 144, degradation trials. Outliers were removed from the winter model and fPGM concentration was log transformed to achieve homogeneity of residuals. Time point was included as a categorical variable and contrasts were set to compare each time point to baseline (time 0). Identification of the faecal pile "sample" was included as a random factor (n = 8) in both models. The non-significant covariates precipitation, temperature and humidity were removed from the summer model.*

| Fixed effect | Estimate (SE) | t-value | p-value | Random effect | Variance (SD) |
|---|---|---|---|---|---|
| A: Winter progesterone concentration | | | | | |
| Intercept | -212.666 (226.122) | -0.940 | 0.349 | Sample | 1024.000 (32.010) |
| Location (shade v sun) | 378.748(137.969) | 2.745 | 0.007 ** | | |
| Condition (wet v dry) | -125.160 (36.635) | -3.416 | 0.013 * | | |
| Baseline – 2 hours | 50.755 (74.006) | 0.686 | 0.494 | | |
| Baseline – 4 hours | -15.600 (81.081) | -0.192 | 0.848 | | |
| Baseline – 6 hours | 83.303 (81.204) | 1.026 | 0.307 | | |
| Baseline – 8 hours | 110.705 (80.573) | 1.374 | 0.172 | | |
| Baseline – 10 hours | 210.599 (73.890) | 2.850 | 0.005** | | |
| Baseline – 12 hours | 299.638 (72.207) | 4.150 | <0.001 *** | | |
| Baseline – 24-30 hours | 305.726 (98.878) | 3.092 | 0.002 ** | | |
| Baseline – 32-36 hours | 373.134 (105.608) | 3.533 | <0.001 *** | | |
| Baseline - ≥48 hours | 291.499 (85.472) | 3.410 | <0.001 *** | | |
| Max temp | 60.041 (19.706) | 3.047 | 0.003 ** | | |
| Humidity | 8.021 (2.229) | 3.598 | <0.001 *** | | |
| Precipitation (mm) | 40.998 (28.736) | 1.427 | 0.156 | | |
| Location : max temp | -30.520 (14.382) | -2.122 | 0.036 * | | |

| | | | | | |
|---|---|---|---|---|---|
| Max temp : humidity | -0.646 (0.151) | -4.275 | <0.001 *** | | |
| Condition: precipitation | 15.775 (12.414) | 1.271 | 0.206 | | |
| Humidity: Precipitation | -1.158 (0.384) | -3.020 | 0.003 ** | | |
| | | | | | |
| B: Summer progesterone concentration | | | | | |
| Intercept | 5.663 (0.094) | 60.392 | <0.001 *** | | |
| Location (shade v sun) | 0.143 (0.063) | 2.286 | 0.071 | Sample | 0.005 (0.071) |
| Condition (wet v dry) | -0.086 (0. 063) | -1.374 | 0.228 | | |
| Baseline – 2 hours | 0.003 (0.111) | 0.023 | 0.982 | | |
| Baseline – 4 hours | 0.204 (0.111) | 1.836 | 0.06873 | | |
| Baseline – 6 hours | 0.297107 (0.111) | 2.669 | 0.009 ** | | |
| Baseline – 8 hours | 0.344802 (0.111) | 3.097 | 0.002 ** | | |
| Baseline – 10 hours | 0.490521 (0.111) | 4.406 | <0.001** | | |
| Baseline – 12 hours | 0.515364 (0.111) | 4.629 | <0.001*** | | |
| Baseline – 24-30 hours | 0.915807 (0.091) | 10.075 | <0.001 *** | | |
| Baseline – 32-36 hours | 1.075367 (0.090) | 11.830 | <0.001 *** | | |
| Baseline - ≥48 hours | 1.206040 (0.086) | 13.986 | <0.001 *** | | |

*Corticosterone*

During the winter trial, fGCM concentration changed significantly over time (Figure 5.3A, Table 5.2A, $p<0.0001$, $n = 141$). The overall mean (±SD) faecal corticosterone metabolite (fGCM) concentration at time 0 was 94.6 ± 18.3 ng/g faeces, with a significant difference between baseline time 0 and 6 hours following the initiation of the study ($p = 0.039$). A significant difference was also found between time 0 and 2 hours after the start of the study; however, this difference disappeared at time point 4h so this appears to be an artefact. By 6 hours into the study, the average fGCM concentration had increased to 134.2ng/g faeces, an increase of 41.9%. Location (shade v sun) did not influence fGCM concentration ($p = 0.520$), however, fGCM was higher in dry conditions ($p = 0.050$). A positive relationship was found between temperature and fGCM concentration ($p = 0.001$; Table 5.2A). Humidity and precipitation had no influence on fGCM concentration during the winter trial.

In the summer trial, fGCM concentration again significantly varied over time (Figure 5.3B, Table 5.2B, $p<0.001$, $n = 142$). The mean faecal corticosterone metabolite (fGCM) concentration at time 0 was 79.2 ± 13.5 ng/g faeces (mean±SD) and only remained similar to baseline for up to 4 hours. By hour 6, fGCM concentration had significantly increased ($p<0.001$) to 854.5ng/g faeces, a staggering increase of 978.9%. fGCM concentration was found to be significantly higher in the sunny location ($p<0.001$) compared to the shaded location; however, no difference was found between wet and dry conditions ($p = 0.181$). A positive relationship was also found between increasing temperature and fGCM concentration ($p = 0.010$; Table 5.2B).

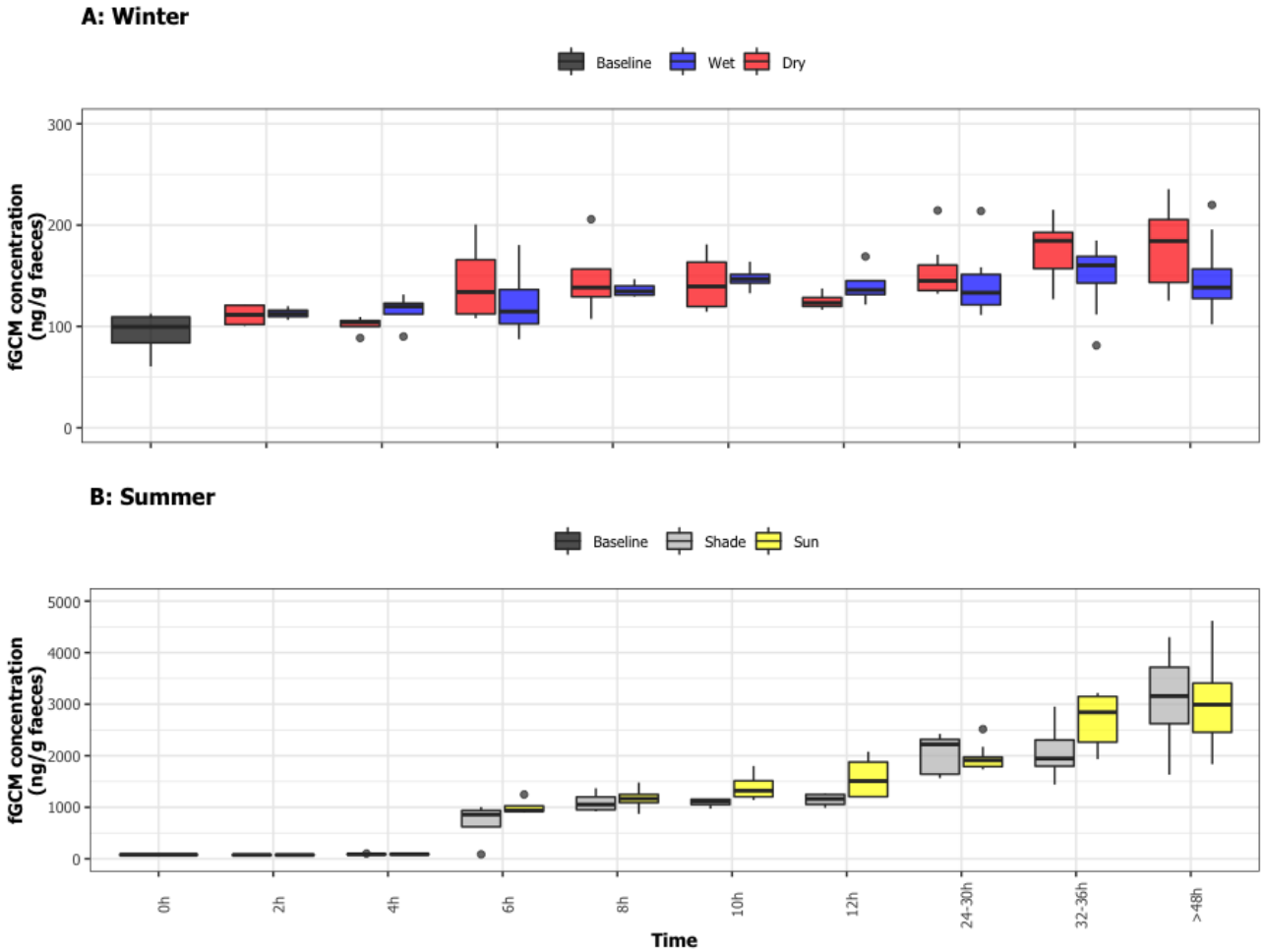


*Figure 5.3: The concentration of faecal corticosterone metabolite (fGCM) varied significantly over time during both the (A) winter ($p<0.001$, $n = 141$) and (B) summer ($p<0.001$, $n = 142$) degradation studies. The concentration of fGCM varied significantly between wet and dry conditions during the winter trial (A, $p = 0.05$) and between shaded and sunny locations in the summer trial (B, $p = 0.01$).*

*Table 5.2: Linear mixed models investigating factors that influenced faecal glucocorticoid metabolite (fGCM) concentration during the (A) winter, n = 141, and (B) summer, n = 142, faecal degradation trials. Outliers were removed from both models to achieve normality of residuals. Time point was included as a categorical variable and contrasts were set to compare time points to baseline time 0. Identification of the faecal pile "sample" was included as a random factor in both models (n = 8). Precipitation was excluded from the summer model as it only rained twice during the study period.*

| Fixed effect | Estimate (SD) | t-value | p-vaue | Random effect | Variance (SD) |
|---|---|---|---|---|---|
| A: Winter cort concentration | | | | | |
| Intercept | 4.322 (0.132) | 32.762 | < 0.001 *** | | |
| Location (shade v sun) | 0.028 (0.042) | 0.673 | 0.520 | Sample | 0. 001 (0.036) |
| Condition (wet v dry) | -0.097 (0.040) | -2.421 | 0.050 * | | |
| Baseline – 2 hours | 0.198 (0.082) | 2.412 | 0.017 * | | |
| Baseline – 4 hours | 0.039 (0.091) | 0.428 | 0.670 | | |
| Baseline – 6 hours | 0.188 (0.090) | 2.087 | 0.039 * | | |
| Baseline – 8 hours | 0.324 (0.088) | 3. 683 | <0.001 *** | | |
| Baseline – 10 hours | 0.433 (0.084) | 5.171 | <0.001 *** | | |
| Baseline – 12 hours | 0.366 (0.082) | 4.476 | <0.001 *** | | |
| Baseline – 24-30 hours | 0.606 (0.110) | 5.514 | <0.001 *** | | |
| Baseline – 32-36 hours | 0.638 (0.115) | 5.534 | <0.001 *** | | |
| Baseline - ≥48 hours | 0.529 (0.093) | 5.677 | <0.001 *** | | |
| Max temp | 0.0262 (0.008) | 3.320 | 0.001** | | |
| Humidity | 0.003 (0.002) | 1.578 | 0.117 | | |
| Precipitation | 0.025 (0.032) | 0.782 | 0.436 | | |
| Temperature : humidity | -0.0004 (0.0001) | -2.635 | 0.010 ** | | |

| | | | | | |
|---|---|---|---|---|---|
| Condition: precipitation | -0.012 (0.014) | -0.877 | 0.382 | | |
| Precipitation : humidity | -0.0009 (0.0004) | -2.200 | 0.030 * | | |
| | | | | | |
| B: Summer cort concentration | | | | | |
| Intercept | -189.345 (739.424) | -0.256 | 0.798 | | |
| Location (shade v sun) | 2042.025 (363.238) | 5.622 | <0.001 *** | Sample | 181030 (105.0) |
| Condition (wet v dry) | -161.257 (103.900) | -1.552 | 0.181 | | |
| Baseline – 2 hours | 20.582 (219.500) | 0.094 | 0.925 | | |
| Baseline – 4 hours | 180.596 (239.025) | 0.756 | 0.451 | | |
| Baseline – 6 hours | 925.905 (222.421) | 4.163 | <0.001 *** | | |
| Baseline – 8 hours | 1357.690 (236.845) | 5.732 | <0.001 *** | | |
| Baseline – 10 hours | 1379.671 (231.211) | 5.967 | <0.001 *** | | |
| Baseline – 12 hours | 1543.170 (236.320) | 6.530 | <0.001 *** | | |
| Baseline – 24-30 hours | 2119.517 (237.565) | 8.922 | <0.001 *** | | |
| Baseline – 32-36 hours | 2653.868 (229.592) | 11.559 | <0.001 *** | | |
| Baseline - ≥48 hours | 3152.666 (179.094) | 17.603 | <0.001 *** | | |
| Max temp | -70.912 (28.280) | -2.507 | 0.013 * | | |
| Humidity | -9.5939 (11.782) | -0.814 | 0.417 | | |
| Location : humidity | -32.013 (6.077) | -5.268 | <0.001 *** | | |
| Temperature : humidity | 1.691 (0.542) | 3.118 | 0.002 ** | | |

*Testosterone*

The concentration of faecal testosterone metabolite (fTM) varied significantly over time during the winter trial (Figure 5.4A, Table 5.3A, $p<0.001$, $n = 144$). The baseline mean (±SD) fTM concentration at time 0 was 195.0 ± 26.1 ng/g faeces. Within 8 hours of the trial, a significant increase in fTM was found ($p = 0.003$) to 291.1 ± 65.8 ng/g faeces, an increase of 49.3%. fTM concentration was found to be significantly higher in the sunny location compared to the shaded location ($p = 0.026$, Table 5.3A). The condition in terms of wet and dry was also found to influence fTM concentration, with significantly greater concentrations in dry conditions ($p = 0.046$; Table 5.3A). Maximum temperature, humidity and precipitation did not significantly influence fTM concentration in the winter trial.

In the summer, the concentration of fTM also significantly varied over time (Figure 5.4B, Table 5.3B, $p<0.001$, $n = 140$). The baseline mean (±SD) fTM concentration at time 0 was 171.6 ± 42.2 ng/g faeces. Six hours into the experiment, fTM concentration had significantly increased ($p = 0.005$) to 249.8 ± 62.1 ng/g faeces, an increase of 45.6%. Location was also found to significantly influence the concentration of fTM ($p = 0.007$; Table 5.3B), with a greater concentration of fTM found in the sunny condition. Wet versus dry conditions were found not to influence fTM concentration. Precipitation, maximum temperature and humidity did not influence fTM concentration in the summer trial.

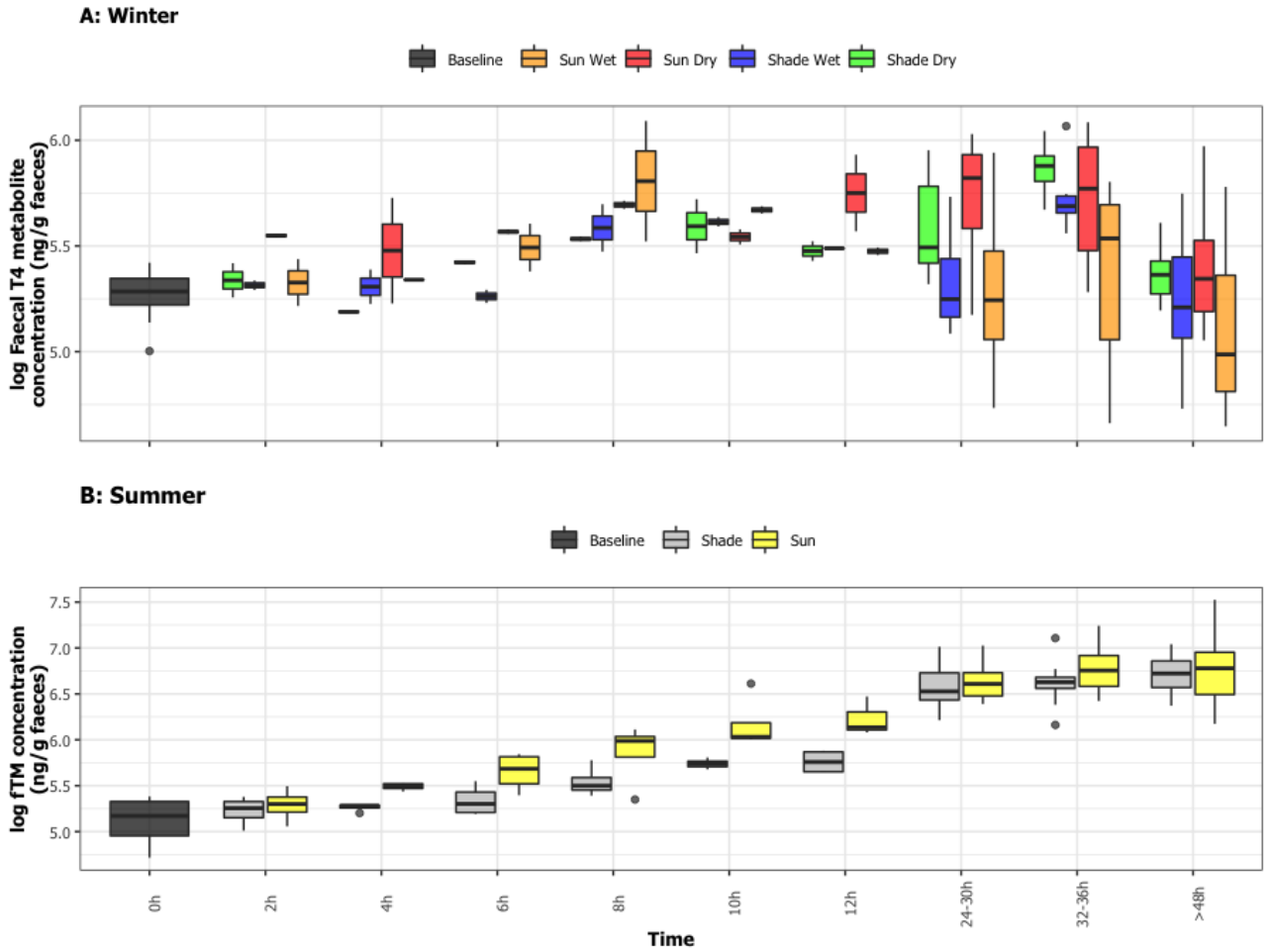


*Figure 5.4: The concentration of fTM varied significantly over time in both the (A) winter ($p<0.001$, $n = 144$) and (B) summer ($p<0.001$, $n = 140$) degradation studies. During the winter trial, fTM varied significantly between the wet and dry conditions (A, $p = 0.05$) and between the sunny and shaded locations (A, $p = 0.03$). In the summer trial, fTM concentration varied significantly between sunny and shaded locations (B, $p = 0.007$).*

*Table 5.3: Linear mixed models investigating factors that influence faecal testosterone metabolite (fTM, T) concentration during (A) the winter, n = 144, and (B) the summer, n = 140, faecal degradation trials. fTM concentration was log transformed for both models and outliers were removed from the summer trial to achieve homogeneity. Time point was included as a categorical variable and each were compared to baseline time 0. Identification of the faecal pile "sample" was included as a random factor (n = 8) in both modes. Non-significant covariates were removed from the models: maximum temperature from the winter model and precipitation and maximum temperature from the summer model.*

| Fixed effect | Estimate (SD) | t-value | p-vaue | Random effect | Variance (SD) |
|---|---|---|---|---|---|
| A: Winter log T concentration | | | | | |
| Intercept | 5.166 (0.130) | 39. 706 | < 2e-16 *** | | |
| Location (shade v sun) | 0. 265 (0.116) | 2. 273 | 0. 026 * | Sample | 0.059 (0.242) |
| Condition (wet v dry) | -0. 144 (0.058) | -2.491 | 0.04570 * | | |
| Baseline – 2 hours | 0.107 (0.122) | 0.882 | 0.37952 | | |
| Baseline – 4 hours | 0.052 (0.121) | 0.431 | 0.667 | | |
| Baseline – 6 hours | 0.163(0.122) | 1.333 | 0.185 | | |
| Baseline – 8 hours | 0.370 (0.121) | 3.044 | 0.003 ** | | |
| Baseline – 10 hours | 0.315 (0.122278) | 2.580 | 0.011 * | | |
| Baseline – 12 hours | 0.262 (0.121751) | 2.154 | 0.033 * | | |
| Baseline – 24-30 hours | 0.314 (0.134) | 2.335 | 0.021 * | | |
| Baseline – 32-36 hours | 0.379 (0.130) | 2.906 | 0.004 ** | | |
| Baseline - ≥48 hours | -0.005 (0.106) | -0.045 | 0.964 | | |
| Humidity | 0.003 (0.002) | 1.759 | 0.08109 | | |
| Precipitation | -0.024 (0.018) | -1.333 | 0.185 | | |
| Location : humidity | -0.004 (0.002) | -2.679 | 0.008 ** | | |
| Condition : precipitation | -0.037 (0.021) | -1.786 | 0.077 . | | |

| | | | | | |
|---|---|---|---|---|---|
| B: Summer log T concentration | | | | | |
| Intercept | 4.982 (0.189) | 26.388 | < 2e-16 *** | | |
| Location (shade v sun) | 0.458 (0.164) | 2.791 | 0.007 ** | Sample | 0.008 (0.918) |
| Condition (wet v dry) | -0.132 (0.075) | -1.762 | 0.138 | | |
| Baseline – 2 hours | 0.125 (0.118) | 1.060 | 0.291 | | |
| Baseline – 4 hours | 0.198 (0.125) | 1.584 | 0.116 | | |
| Baseline – 6 hours | 0.345 (0.121) | 2.844 | 0.005 ** | | |
| Baseline – 8 hours | 0.563 (0.124) | 4.558 | <0.001 *** | | |
| Baseline – 10 hours | 0.805 (0.123) | 6.563 | <0.001 *** | | |
| Baseline – 12 hours | 0.819 (0.127) | 6.461 | <0.001 *** | | |
| Baseline – 24-30 hours | 1.474 (0.101) | 14.574 | <0.001 *** | | |
| Baseline – 32-36 hours | 1.556 (0.120) | 14.165 | <0.001 *** | | |
| Baseline - ≥48 hours | 1.611 (0.090) | 16.530 | <0.001 *** | | |
| Humidity | 0.002 (0.002) | 1.070 | 0.287 | | |
| Location: humidity | -0.006 (0.003) | -2.222 | 0.028 * | | |

## 5.4. Discussion

Our findings demonstrate that for captive African wild dogs from faecal samples that were likely to be a maximum of 4-5 hours old at collection, no significant changes in hormone levels of progesterone, corticosterone and testosterone were measurable for at least 4 hours after collection, suggesting that the levels measured initially in this study reflect the true levels of hormones in these samples at the time of excretion. This held true regardless of condition, location, or time of year that the faecal samples were exposed to after collection. This information will help in the design of reliable studies to investigate and accurately monitor reproduction and adrenal activity in this species. Such studies will be valuable to enable evidence-based management decisions to improve welfare, for example by removing stressors, and to maximise reproductive output, for example by confirming an individual is in oestrous before introducing a mate.

We have successfully biochemically validated enzyme immunoassays for faecal hormone metabolites of progesterone (fPGM), corticosterone (fGCM) and testosterone (fTM) for captive wild dogs. Starting from a baseline of freshly collected samples, we have also shown that various hormone metabolites present in faeces degrade differently over time and under different weather conditions in an *ex situ* environment. The glucocorticoid, corticosterone was measured as an indication of adrenal activity as this has previously been evaluated to reflect adrenal activity in the African wild dog and is therefore thought to be useful for evaluating stress (Monfort et al., 1998). Indeed, faecal corticosterone has been measured in relation to dominance and the effect of the use of radio collars on wild African wild dogs (Creel et al., 1997, Monfort et al., 1997). Furthermore, in line with the current study, previous studies investigating glucocorticoid metabolites in wild dogs have also quantified corticosterone using EIAs (Santymire and Armstrong, 2010). An alternative glucocorticoid to measure is cortisol, which has been measured using radioimmunoassay in captive and free-ranging wild dogs to monitor stress (Van der Weyde et al., 2016).

Measured fPGM concentrations varied significantly from baseline after 10 hours in the cooler temperatures of winter but were found to be significantly higher from baseline after 6 hours in the summer, with a near significant difference after 4 hours. fGCM levels were found to degrade at a different rate to fPGM: concentrations were significantly higher after 6 hours in both the summer and winter trials. fTM levels were stable for up to 8 hours in the winter, but

only up to 6 hours in the summer trial. Additionally, in some treatments, weather conditions were also found to influence the degradation of hormone metabolites to varying degrees, with the sunny and dry samples having greater concentrations of metabolite than the shaded and wet samples. Although this could in part be caused by some drying out and therefore reducing the water content of the faecal samples in the sun (results were expressed per wet weight of faeces), the very differing changes observed between hormones in the summer samples (978.9% increase for fGCM; 49.3% increase for fTM) suggests that this is unlikely to be a major factor. These results provide evidence that sampling protocols must remain strict in *ex situ* environments to enable reliable hormone analysis. Samples need to be collected as quickly as possible. Millspaugh and Washburn (2004) state it is important to collect samples within at least two hours of defecation. However, for captive animals, carnivores especially, it is often difficult to collect samples within a short time frame due to management restrictions, and this could result in erroneous data indicating excessively high hormone concentrations. It is important to conduct degradation studies covering the time frame available for sample collection when conducting studies and including varying weather conditions, and to maintain careful records of such conditions.

Biological validation of the EIAs were not performed in the current study as we were interested in the degradation of faecal hormone metabolites over time. The corticosterone antibody (CJM0006) has previously been opportunistically biologically validated in black rhino (*Diceros bicornis*) (n = 1), blue-headed macaw (*Primolius couloni*) (n = 1) and Asian elephants (*Elephas maximus*) (n = 4), where fGCM concentrations were found to be significantly higher following a stressful event (Watson et al., 2013). The testosterone (R156/7) and corticosterone (CJM0006) antisera have previously been used to measure faecal testosterone metabolites in wild dogs but no biological validations were reported (Santymire and Armstrong, 2010).

Results from the current study are consistent with previously published results showing significant changes in hormone metabolite concentrations over time in Asian elephants (*Elephas maximus*) (Wong et al., 2016), wild jaguars (*Panthera onca*) (Mesa-Cruz et al., 2014), white-tailed deer (*Odocoileus virginianus*) (Washburn and Millspaugh, 2002) and African wild dogs (Crossey et al., 2018). Crossey et al. (2018) demonstrated that faecal glucocorticoid metabolite concentrations in wild dogs remained reasonably stable for up to 24 hours post-defecation in South African zoological collections. However, unlike the current study,

subsamples examined by Crossey et al. (2018) were left at room temperature (12-20$^{o}$C) in laboratory conditions without the inclusion of environmental variables, for example, rainfall, sunlight or humidity which will affect microbial activity. In addition, unlike the current study which used a wet-weight extraction method, (Crossey et al., 2018) used a dry-weight extraction method. Furthermore, the starting samples could also have been up to 24 hours old.

Over time, the hormone metabolites are likely to undergo molecular degradation which may result in an altered affinity to the antibody within the different assays used (Palme et al., 2005). Microbial action in the soil onto which a faecal bolus has been deposited has been shown to influence the breakdown of steroid hormones. A greater proportion of unsterilised soil, containing a greater number of microorganisms, has been shown to degrade the steroid hormone 17β-estradiol faster than partially sterile soil (Xuan et al., 2008). In the current study, samples were placed in weigh boats, removing the continual influence of soil; however, soil particles will be present from initial faecal boli collection. In a more real-life situation, the degradation rate found in this study could therefore be even greater if animals are housed on a soil substrate.

In line Yarnell and Walker (2017) and Wong et al. (2016), degradation rates in the current study were generally found to be greater, resulting in increased metabolite concentration, in sunny aspects compared to the shade, resulting in an increase in measured metabolite concentration. However, the effect of photo degradation and UV intensity on steroid hormones is not thought to be significant (Fan et al., 2008, Mesa-Cruz et al., 2014). In the current study, faecal hormone metabolites were also found to be significantly higher in the dry compared to wet conditions. In contrast, Washburn and Millspaugh (2002) found significantly greater concentrations of steroid metabolites in simulated rainfall treatments on fGCM in white-tailed deer faeces. Similarly, Mesa-Cruz et al. (2014) also found rain to increase faecal steroid hormone metabolite concentration in wild jaguars. However, both these studies measured faecal hormone metabolite in dry-weight faecal samples, compared to the wet-weight faecal extraction method used here. Hence, in the dry conditions in the current study, it is likely that the samples simply contained less water and therefore a somewhat higher concentration of faecal metabolite at the time of extraction. Using a dry-weight extraction protocol may remove this variability (Palme et al., 2005). However, dry-weight extraction methods often include many hours of heating at temperatures of 55$^{o}$C (Price et al., 2019),

which could erroneously influence the resulting hormone metabolite concentration by breaking down the hormones of interest. An alternative option would be to use a wet-weight extraction process followed by EIA analysis then correcting the result to dry matter weight measured in a second aliquot of sample collected at the same time. Wong et al. (2016), used a similar wet-weight extraction protocol to the current study and found no significant influence of water treatment on fGCM concentration in semi-wild Asian elephant (*Elephas maximus*) populations. This could be due to the voluminous and fibrous nature of elephant dung compared to small pellets (deer) or carnivorous boli (jaguars or wild dogs), or water may alter the immunoreactivity of different metabolites, which can be detected by some, but not all immunoassays (Wong et al., 2016, Mesa-Cruz et al., 2014). Abáigar et al. (2010) found rainfall to dilute hormone metabolite concentration in wet-weight extracted faeces in a hormone specific manner. In agreement with these authors, we therefore recommend not to collect faecal boli after rain.

In conclusion, we have shown that for hormone monitoring of captive wild dogs in the UK, faecal metabolites are stable from fresh samples (those collected about 4-5 hours after defecation) for no more than 4 hours. Samples for hormone monitoring should preferably be collected from a shaded location and not during or following rain. Furthermore, faecal boli are likely to be more sensitive to degradation in the higher temperatures of the summer months. Overall, to ensure accurate non-invasive hormone data, this study highlights the requirement for robust sample collection protocols and degradation studies for different hormones of interest for all species prior to conducting a non-invasive faecal hormone metabolite study.

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Declaration of interest

None.

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## 5.6. Supplementary Material

*Table S5.1: 144 subsamples were collected for hormone analysis from the winter and summer degradation trials. Samples were split into four treatment groups, and within each treatment group there were two faecal pools from which subsamples were collected over a 36-hour period.*

| Treatment | Winter | | Summer | |
|---|---|---|---|---|
| | Faecal samples | Subsamples | Faecal samples | Subsamples |
| *Sun-wet* | 2 | 36 | 2 | 36 |
| *Sun-dry* | 2 | 36 | 2 | 36 |
| *Shade-wet* | 2 | 36 | 2 | 36 |
| *Shade-dry* | 2 | 36 | 2 | 36 |

*Table S5.2: Validation of L. pictus faecal extracts on corticosterone, testosterone and progesterone EIAs through the parallel displacement of extract and standard curve and matrix interference assessment. Parallelism was successfully achieved for all EIAs as confirmed by linear regression analysis (p<0.001). Non-significant background interference was determined for all EIAs as confirmed by linear regression analysis in the matrix interference assessment (p<0.001).*

| EIA | Sample | Parallelism | Matrix interference assessment | % Recovery ± s.e.m |
|---|---|---|---|---|
| Corticosterone CJM006 | Female African wild dog (faecal extract) | Sample % binding = 0.991 (standard % binding) – 2.278, $R^2$=0.991, $F_{1,7}$ = 736.500, p <0.001 | Observed = 1.146 (expected) – 4.391 $R^2$= 0.997, $F_{1,7}$ = 2206.825, p<0.001 | 108.05 ± 4.07 % |
| | Male African wild dog (faecal extract) | Sample % binding = 0.860 (standard % binding) + 20.1792 $R^2$=0.961, $F_{1,7}$ = 170.450, p <0.001 | Observed = 1.222 (expected) – 0.325 $R^2$= 0.999, $F_{1,7}$ = 10845.970, p<0.001 | 125.73 ± 6.73 % |
| | Mixed African wild dog (faecal extract) | Sample % binding = 0.937 (standard % binding) + 9.625, $R^2$=0.986, $F_{1,7}$ = 170.450, p <0.001 | Observed = 1.257 (expected) – 2.303 $R^2$= 0.996, $F_{1,7}$ = 1591.313, p<0.001 | 122.36 ± 6.36 % |
| Testosterone R156/7 | Female African wild dog (faecal extract) | Sample % binding = 0.860 (standard % binding) + 0.896, $R^2$=0.995, $F_{1,7}$ = 1343.812, p <0.001 | Observed = 1.093 (expected) – 1.615 $R^2$= 0.997, $F_{1,7}$ = 2050.298, p<0.001 | 101.77 ± 3.73 % |
| | Male African wild dog (faecal extract) | Sample % binding = 0.860 (standard % binding) – 0.212, $R^2$=0.995, $F_{1,7}$ = 1357.970, p <0.001 | Observed = 1.169 (expected) – 0.230 $R^2$= 0.997, $F_{1,7}$ = 2355.237, p<0.001 | 123.58 ± 5.58 % |

| | | | | |
|---|---|---|---|---|
| | Mixed African wild dog (faecal extract) | Sample % binding = 0.915 (standard % binding) + 1.841, $R^2$=0.996, $F_{1,7}$ = 1653.829, p <0.001 | Observed = 1.049 (expected) + 1.349 $R^2$= 0.999, $F_{1,7}$ = 19803.750, p<0.001 | 103.04 ± 10.37 % |
| Progesterone CL425 | Female African wild dog (faecal extract) | Sample % binding = 0.900 (standard % binding) – 2.878, $R^2$=0.988, $F_{1,7}$ = 563.673, p <0.001 | Observed = 0.862 (extracted) – 0.709 $R^2$=0.999, $F_{1,7}$= 5679.573, p <0.001 | 72.71 ± 8.08 % |
| | Mixed African wild dog (faecal extract) | Sample % binding = 0.858 (standard % binding) + 1.584, $R^2$=0.9798, $F_{1,7}$ = 339.631, p <0.001 | Observed = 0.881 (extracted) + 0.643 $R^2$=0.998, $F_{1,7}$= 3188.998, p <0.001 | 98.00 ± 6.58 % |

*Table S5.3: Cross-reactivities of antibody CJM006 to different steroids relative to the binding of corticosterone*

| Steroid | Cross-reactivity (%) |
|---|---|
| Corticosterone | 100 |
| Desoxycorticosterone | 14.25 |
| Progesterone | 2.65 |
| Tetrahydrocorticosterone | 0.90 |
| Testosterone | 0.64 |
| Cortisol | 0.23 |
| Prednisolone | 0.07 |
| 11-Desoxycortisol | 0.03 |
| Prednisone | <0.01 |
| Cortisone | <0.01 |
| Estradiol-17β | <0.01 |
| | <0.01 |

*Table S5.4: Cross-reactivities of antibody R516/7 to different steroids relative to the binding of testosterone*

| Steroid | Cross-reactivity (%) |
|---|---|
| Testosterone | 100 |
| 5α-Dihydrotestosterone | 57.37 |
| Androstenedione | 0.27 |
| Androsterone | 0.04 |
| DHEA | 0.04 |
| Cholesterol | 0.03 |
| β-Oestradiol | 0.02 |
| Progesterone | <0.02 |
| Pregnenolone | <0.02 |
| Hydrocortisone | <0.02 |
| Cholic acid | <0.02 |
| Chenodeoxycholic acid | <0.02 |
| Cholic acid methyl ester | <0.02 |
| Dehydrocholic acid | <0.02 |
| Deoxycholic acid | <0.02 |
| Lithocholic acid | <0.02 |
| Glycholic acid | <0.02 |
| Taurodeoxycholic acid | <0.02 |
| Taurocholic acid | <0.02 |
| Taurochenodeoxycholic acid | <0.02 |
| Glycochenodeoxycholic acid | <0.02 |

*Table S5.5: Cross-reactivities of antibody CL425 to different progesterone metabolites relative to the binding of progesterone*

| Progesterone metabolite | Common name | Cross-reactivity (%) |
|---|---|---|
| 4-Pregnen-3,20-dione | Progesterone | 100 |
| 4-Pregnen-3α-o1-20-one | | 188 |
| 4-Pregnen-3β-o1-20-one | | 172 |
| 4-Pregnen-11α-o1-3,20-dione | | 147 |
| 5α-Pregnan-3β-o1-20-one | | 94 |
| 5α-Pregnan-3α-o1-20-one | | 64 |
| 5α-Pregnan-3,20-dione | | 55 |
| 5β-Pregnan-3β-o1-20-one | | 12.5 |
| 5β-Pregnan-3,20-dione | | 8 |
| 4-Pregnen-11β-o1-3,20-dione | | 2.7 |
| 5β-Pregnan-3α-o1-20-one | | 2.5 |
| 5β-Pregnan-3α,20α-diol | Pregnanediol | <0.1 |
| 5α-Pregnan-3α,20β-diol | | <0.1 |
| 5β-Pregnan-3,17-dione | Androstenedione | <0.1 |
| 5β-Pregnan-11β,21-diol-3,20-dione | Corticosterone | <0.1 |

# Chapter 6: Factors affecting survival, aggression and reproductive success in captive African wild dogs (*Lycaon pictus*) in European zoological collections.

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Abstract

With natural populations of the endangered African wild dog (*Lycaon pictus*) in decline, captive breeding programmes are a proposed method to assist in preventing their extinction. However, since wild dogs have complex social and reproductive behaviours linked to obligate cooperative breeding, this can present challenges for their successful management in captivity. Here we investigate factors influencing the success of the European wild dog breeding programme, with the aim of assisting in the development of evidence-based husbandry guidelines. We utilised stud book data over a 20-year period to explore factors influencing survival of wild dogs in the European captive population. Offspring survival was significantly greater for younger dams (for both first time dams and all dams combined) and for dams that had previous success of rearing offspring to post-natal day (PND) 30. Offspring survival was significantly lower for dams that had previously tried, but unsuccessfully raised offspring to PND 30. The juvenile mortality rate was found to be 44.7%, but due to recording difficulties, this is likely to be an underestimate. Loss of the entire litter occurred for 40% of litters born during the study period. Trauma caused by conspecifics was the most commonly reported cause of juvenile mortality. We also present results of a husbandry questionnaire sent to European institutions housing groups of wild dogs. Among responding institutions, 53% reported adverse breeding experiences, 88% reported aggression, and nearly one third (30%) experienced severe aggression resulting in death or euthanasia. Higher reported aggression could be linked to having more than one pack within an institution, more adults within a pack and possibly smaller enclosure sizes. These findings could be used to inform husbandry guidelines therefore potentially improving offspring survival and reducing adult aggression in the European wild dog population.

Keywords

African wild dog; *ex situ* breeding; cooperative breeding; juvenile mortality; survival.

## 6.1. Introduction

The African wild dog (*Lycaon pictus*) is an obligate cooperatively breeding canid, with a monogamous mating system and flexible social organisation (Kleiman, 1977, Frame et al., 1979, Malcolm and Ken, 1982). Wild dogs are in decline and are classed as endangered on the International Union for the Conservation of Nature (IUCN) red list (Woodroffe and Sillero-Zubiri, 2020). Indeed, they are thought to be "one of the world's most threatened carnivores" (Woodroffe, 2004, Woodroffe et al., 2007, Woodroffe and Sillero-Zubiri, 2020, McNutt and Silk, 2008), with a recent estimate of 1409 mature breeding individuals left in the wild (Woodroffe and Sillero-Zubiri, 2020). In addition to habitat protection and restoration, human education and awareness, and increasing legislation, reintroductions are needed for this species to survive (Woodroffe and Sillero-Zubiri, 2020). One way to facilitate this is through *ex situ* breeding programmes, which are also vitally important for maintaining the genetic variability required for endangered species survival (Hoffmann et al., 2010).

In the wild, female wild dog's dominance increases with age, resulting in a relationship between litter size and maternal age (Creel and Creel, 2002). Overall, however, middle-aged females (three to six years old) successfully raise more pups than older or younger females (Creel and Creel, 2002). Reported juvenile mortality in the wild is variable across packs (Van Heerden et al., 1995); it has been reported to be as low as 30% in the Selous Game Reserve (Creel and Creel, 2002) and as high as 76% in the Serengeti (Malcolm, 1979, unpublished dissertation in (Yordy and Mossotti, 2016)), with an average of 56% (Creel and Creel, 2002). Juvenile mortality may be variable due to differences in pack size, as pack size is positively correlated with pup survival (Courchamp et al., 2002). Indeed, larger packs are able to predate larger prey, resulting in higher calorie intakes enhancing pup survival (Creel and Creel, 1995), and are better able to defend their pups from predating carnivores (Creel and Creel, 2002, Courchamp et al., 2002).

With the absence of inter-species competition, diseases and human-related dangers, it might be expected that *ex situ* wild dog populations should achieve relatively high reproductive success and survival rates. However, to date, *ex situ* breeding programmes for wild dogs have had varying success (Frantzen et al., 2001, Woodroffe, 2004, Yordy and Mossotti, 2016). Captive juvenile mortality rates still remain high, both in South Africa (63%) (Frantzen et al., 2001) and in North America (53%) (Yordy and Mossotti, 2016). This is despite intensive

breeding efforts for this species and goes against the general 'rule' for canines that offspring mortality decreases with an increased number of litters bred in captivity (Ginsberg and Macdonald, 1990). Furthermore, causes of juvenile mortality in *ex situ* wild dog populations are often associated with aggression / cannibalism performed by the dam, and loss of the an entire litter of pups, which is rare in the wild (Burrows et al., 1995), is unfortunately common in captivity (Yordy and Mossotti, 2016).

The poor reproductive performance of *ex situ* wild dogs may have a number of causes. The confinement of captivity creates a number of possible stressors that can influence reproductive success, including unnatural social groups (of particular importance for cooperatively breeding species), space constraints, unnatural noises and an unnatural diet and food presentation (Morgan and Tromborg, 2007). Requirements for species with complex social and environmental needs can be more difficult to recreate in a captive setting, resulting in higher than expected infant mortality rates (Schmalz-Peixoto, 2003). Furthermore, captive female wild dogs may have higher levels of faecal glucocorticoid metabolites than their wild counterparts, suggesting captivity could influence adrenal activity in this species (Van der Weyde et al., 2016). High-levels of circulating glucocorticoids are associated with heightened aggression (Creel, 2001), a problem faced by captive wild dog populations, and an obvious welfare concern. Chronic stress, resulting in long-term raised glucocorticoid levels, is also known to reduce reproductive output and alter reproductive behaviour (Creel, 2001). Additionally, the inability of wild dogs to express their naturally complex social behaviours in captivity could lead to abnormal behaviours including stereotypies (Hohmann et al., 2013). An additional potential issue for captive wild dog populations is inbreeding, resulting in reduced genetic variability (Frantzen et al., 2001). For example, it has been shown that pups born to unrelated parents had a significantly greater survival probability than those born to related parents (Yordy and Mossotti, 2016). In the North American captive wild dog population, offspring survival was greater for pups born to younger dams. Indeed, for primiparous dams, the youngest females achieved the lowest juvenile mortality (Yordy and Mossotti, 2016).

The aim of this study was to investigate the factors that influence reproductive success and aggression in the European captive population of wild dogs. Data were collected from the European stud book, Zoological Information Management Systems database, and a questionnaire sent to European wild dog Keeping Staff. Our specific objectives were to

investigate 1) how maternal age and experience influence offspring mortality, and 2) how different management practices affect aggressive behaviour and reproductive success.

## 6.2. Methods

### *6.2.1. Maternal effects on offspring mortality*

Data on the European population of captive African wild dogs were collected from the Zoological Information Management Systems (ZIMS) and studbooks ranging from 1996 until 2016. Data were limited to the past 20 years as veterinary and husbandry practices have changed dramatically since the first wild dog was managed in captivity (Marsden et al., 2013).

### *6.2.2. Survival analysis*

Data were used to investigate whether survival and juvenile mortality were influenced by maternal age at parturition or previous maternal reproductive experience and success. Following Yordy and Mossotti (2016), survival analysis was conducted using Kaplan-Meir survival curves which were compared using the survdiff() function via Mantel-Haenszel log-rank tests in the survival package in R (Dardis, 2015). This analysis enabled individuals that were lost to follow up (unknown outcome), or still alive at the end of the study period, to be censored in the analysis.

#### Maternal age at parturition

Offspring were grouped depending on the age of their dam at parturition: offspring born to dams <2.5 years old (yo), 2.5-4.5yo, 4.5-6.5yo, 6.5-8.5yo and >8.5yo. Two year age gaps were used in accordance with previous studies (Yordy and Mossotti, 2016), however, due to larger sample sizes in the current study, an additional dam age group was included (>8.5yo). To investigate if pup survivorship varied in relation to dam age, a log-rank test was conducted using the comp() function in the survMisc package (Yordy and Mossotti, 2016). Median and mean survival rates are reported. Pup mortality, defined as pup death up to and including post-natal day (PND) 30, was investigated between the maternal age groups using the chisq.test() function in R (Yordy and Mossotti, 2016).

#### Maternal experience

To investigate whether prior maternal experience influenced offspring survival, offspring were split into three groups: pups born into a dam's first litter, pups born into a litter following

previous reproductive success (a dam had previously successfully raised at least one pup to PND 30) and pups born into a litter following attempted but no previous reproductive success (a dam had previously reproduced but failed to raise at least one pup to PND 30). Juvenile mortality was investigated between dams of varying reproductive experience using the chisq.test() function in R (Yordy and Mossotti, 2016).

Although not always the case, it is likely that dams that have previously reproduced are older than first time dams (mean age for first time dams: 3.5yo, mean age for experienced dams: 5.5yo), therefore further analysis was conducted. Kaplan-Meir survival curves were compared using the survdiff() function, and survival trends due to increasing maternal age were investigated using comp() for dams of varying experience.

*6.2.3. Effects of husbandry on aggressive behaviour and reproductive success*

A survey was conducted to investigate the effects of husbandry, pack composition, enrichment, enclosure size and neighbouring species on reproductive success and aggression in captive wild dogs. A questionnaire (see Supplementary Material) was sent to 45 EAZA collections that house wild dogs, details of which were collected from the Taxon Advisory Group. There were 26 respondents to the questionnaire.

### Reproduction

Only 46% (n = 12) of responding collections housed breeding groups of African wild dogs and only 2 had offspring. Given the limited sample size, we are therefore only able to present descriptive results for this part of the study.

### Aggression

Causes of aggression were analysed in collections that (1) reported any level of aggression, (2) housed more than one individual and (3) where dogs were housed in a single pack (n = 20). To investigate the level of aggression, each collection was given a total aggression score which depended on (1) the severity and (2) the frequency of the aggression (Table 6.1). These two scores were multiplied together to give a total aggression score. To analyse which factors influenced aggression in captive wild dogs, results were analysed using generalised linear models using the glm() function in the lme4 package in R (Bates et al., 2015). Total aggression score was transformed using the square root function to result in normal distribution of residuals. A non-transformed model was also investigated, and model best fit was decided by

the lowest AIC. Normality was checked by visual inspection of Q-Q plots, and Kolmogorov-Smirnov and Shapiro-Wilk normality tests. Explanatory variables were removed where non-significant. Explanatory variables included pack composition, breeding group status, pup presence, enclosure size, neighbouring species and level of enrichment. Carcass feeding was excluded from analysis as it reduced the n value. Four collections reported housing more than one wild dog pack, and aggression scores of single and multi-pack collections were analysed using a non-parametric one-way ANOVA (Kruskal-Wallis). Further analysis into which factors result in greater aggression within multiple pack collections was not possible due to the limited sample size.

*Table 6.1: Total aggression scores were created for each institution by multiplying the frequency of aggression by the severity of the aggression score.*

| Frequency of aggression | Score | Severity of aggression | Score | Severity of aggression description |
|---|---|---|---|---|
| Daily | 4 | Very mild | 1 | Fighting seen but no obvious sign of injury |
| Weekly | 3 | Mild | 2 | Fighting seen with evidence of superficial wounds, or observation of superficial wounds that required no further interventions |
| Monthly | 2 | Moderate | 3 | Wounds required veterinary treatment |
| Other (annually or less often) | 1 | Moderate severe | 4 | Wounds required veterinary treatment and staff had to intervene and separate group members |
| Never | 0 | Severe | 5 | Wounds resulted in death or required euthanasia |

The goal of the survey and questionnaire was to acquire evidence-based management decisions to improve reproductive success and reduce aggression to aid in the production of Best Practice Guidelines for European captive wild dogs.

## 6.3. Results

During the study period, 1342 individual African wild dogs were reported to be born into 287 litters in European *ex situ* collections. Of these, across all ages, 1056 died and 37 were lost to

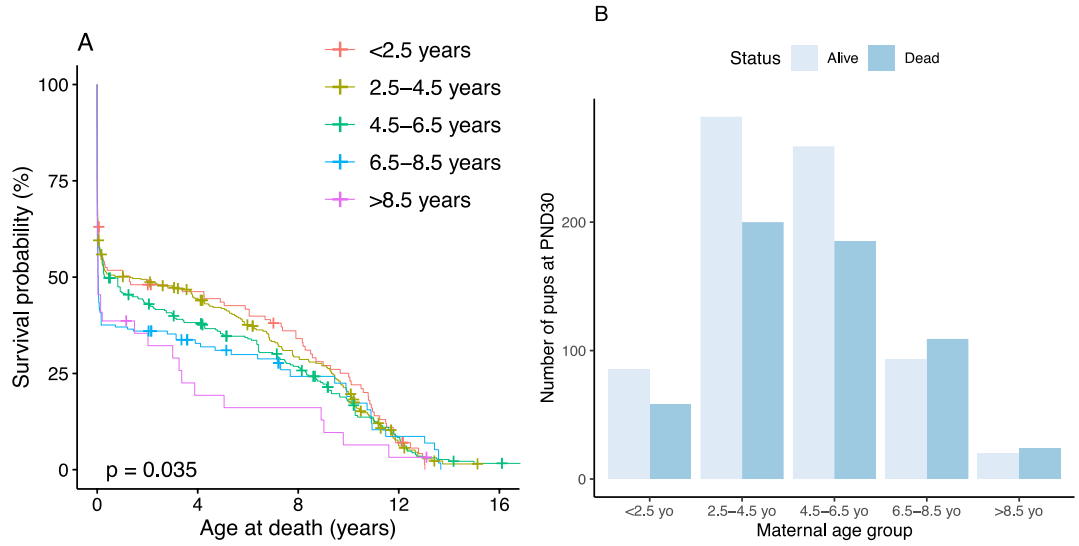
follow up (unknown outcome on records). At parturition, the mean dam and sire ages were 4.8 and 6.3 years respectively. The mean number of pups per litter was 5 (min: 1, max: 18). The mean age of offspring at death was 2.6 years, and the median age at death was 79 days (Table 6.2). Where sex was reported, males had a significantly greater survival probability than females ($X^2$=4.700, df=1, p=0.030, n=834). Fifty-four per cent of offspring were reported to be male.

*Table 6.2: Summary of age of death of offspring and maternal age extracted from ZIMS and studbooks of all wild dogs born in European zoos from 1996 until 2016.*

| | Offspring age at death (days) | Maternal age at parturition (days) |
|---|---|---|
| n | 1058 | 117 |
| Mean | 947 | 1747 |
| Median | 79 | 1801 |
| Minimum | 0 | 349 |
| Maximum | 6276 | 4030 |

### *6.3.1. Maternal influences on offspring mortality*

The youngest female to reproduce during the study period was aged 11.5 months, however, this litter was not successful with no pups surviving past PND 0. Survival was significantly different for offspring born to dams of varying age groups (Table 6.3A, Figure 6.1A, p = 0.040) with a significant log-rank trend in decreasing survival for increasing maternal age at parturition (Z = -2.941, p=0.003). Juvenile mortality rates up to and including PND30 varied significantly across maternal age groups ($X^2$ = 13.008, df = 4, p=0.010) and were below 50% for offspring born to dams in age classes <2.5 years, 2.5-4.5 years and 4.5-6.5 years. Offspring born to dams >6.5 years at parturition had a juvenile mortality rate of greater than 50% (Figure 6.1B).




| Maternal age | <2.5 | 2.5-4.5 | 4.5-6.5 | 6.5-8.5 | >8.5 |
|---|---|---|---|---|---|
| Median | 468 | 481 | 103 | 17 | 10 |
| Mean | 1349 | 1065 | 956 | 558 | 596 |

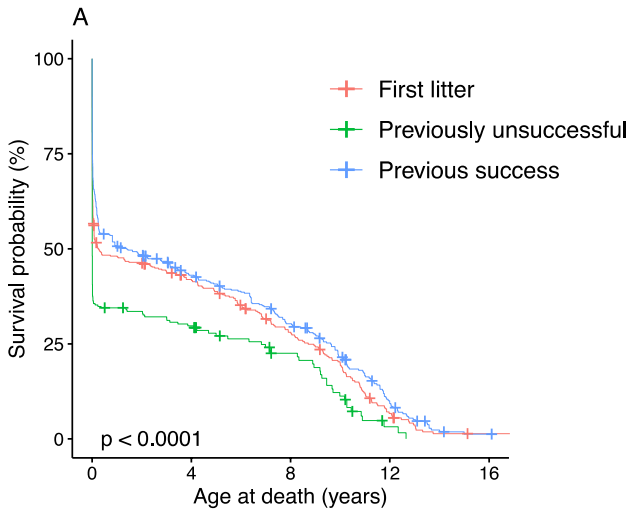
$X^2 = 10.30$, df = 4, p = 0.040, n = 1278

*Figure 6.1: Effect of maternal age group on (A) offspring survival ($X^2 = 10.30$, df = 4, p = 0.040) and (B) juvenile mortality ($X^2 = 13.008$, df = 4, p = 0.010).*

Survival differed significantly for offspring born to dams with contrasting maternal experience (Table 6.3B, Figure 6.2A, $p<0.001$) with experienced dams having significantly greater offspring survival. Further analysis found no difference in offspring survival between first time dams and dams that had successfully raised at least one litter to PND30 (Table 6.3C, $p = 0.06$). Combining these dams and comparing their offspring survival rates to those of previously unsuccessful dams, we found evidence of significantly greater survival for first time and successful dams compared to those with previous unsuccessful experience of litter rearing (Table 6.3D, $p<0.001$). Offspring survival rates differed significantly for first time dams of varying age class (Table 6.2E, Figure 6.3B, $p<0.001$), with a significant trend found for decreasing survival in relation to increasing maternal age at first litter ($Z = -4.037$, $p<0.001$). Maternal age had no significant influence on offspring mortality for previously successful dams (Table 6.3F, Figure 6.2C, $p =0.20$), however, offspring survival again varied significantly for unsuccessful dams of differing age class (Table 6.2G, $p<0.001$). A log rank test for trend was not significant; there was no change in survival with increased maternal age for unsuccessful dams ($Z = -1.60$, $p = 0.10$). Juvenile mortality to PND30 varied significantly between dams of varying experience ($X^2 = 70.282$, $df = 2$, $p<0.001$). Juvenile mortality was 33% for previously successful mothers but was nearly double for previously unsuccessful dams at 64%.

*Table 6.3: Summary of Kaplan-Meir survival curve log rank tests of maternal influences on offspring survival analysed using the survdiff() function of the survival package in R. A: maternal age group consisted of <2.5 years, 2.5-4.5 yo, 4.5-6.5yo, 6.5-8.5yo and > 8.5yo. B: Maternal experience consisted of first-time dams, unsuccessful and successful dams. C: First time versus previously successful dams. D: first time plue successful versus unsuccessful dams. E: Maternal age group (as described for A) for first time dams. F: Maternal age group for successful dams (as described for A) and G: maternal age group for unsuccessful dams (as described for A).*

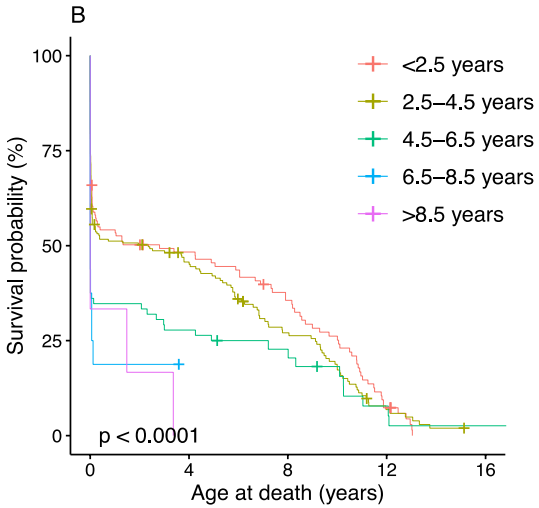
| | $X^2$ | df | p | n |
|---|---|---|---|---|
| A: Maternal age group | 10.3 | 4 | 0.040* | 1278 |
| B: Maternal experience | 41.6 | 2 | <0.001*** | 1272 |
| C: First time vs successful dams | 3.5 | 1 | 0.060 | 1272 |
| D: First time and successful vs unsuccessful dams | 38.6 | 1 | <0.001 *** | 1272 |
| E: Maternal age group for first time dams | 24.8 | 4 | <0.001 *** | 454 |
| F: Maternal age group for successful dams | 4.9 | 4 | 0.200 | 553 |
| G: Maternal age group for unsuccessful dams | 18.8 | 4 | <0.001*** | 265 |




A: Varying maternal experience

| Experience | Median | Mean |
|---|---|---|
| First | 87 | 1070 |
| Successful | 510 | 1093 |
| Unsuccessful | 2 | 501 |

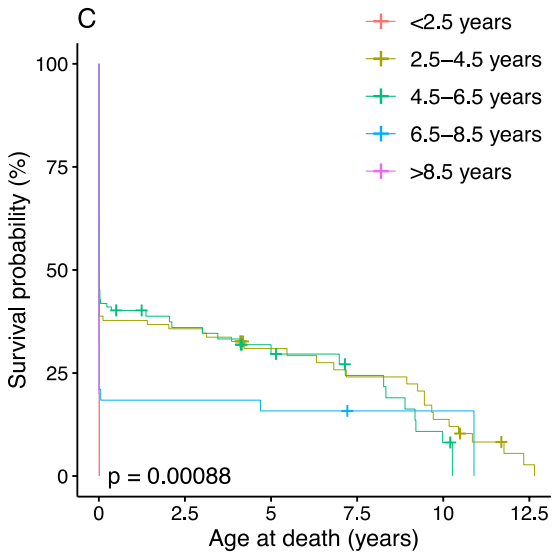
$X^2 = 41.60$, df = 2, $p<0.001$, n = 1272




B: First time dams

| Dam age | Median | Mean |
|---|---|---|
| <2.5 | 1024 | 1421 |
| 2.5-4.5 | 858 | 1073 |
| 4.5-6.5 | 2 | 736 |
| 6.5-8.5 | 2 | 7 |
| >8.75 | 0 | 295 |

$X^2 = 24.80$, df = 4, $p<0.001$, n = 454




C: Previously unsuccessful dams

| Dam age | Median | Mean |
|---|---|---|
| <2.5 | 0 | 0.8 |
| 2.5-4.5 | 2 | 741 |
| 4.5-6.5 | 4 | 459 |
| 6.5-8.5 | 2 | 174 |
| >8.75 | 1 | 1.6 |

$X^2$ = 18.80, df = 4, p<0.001, n = 265

*Figure 6.2: Offspring survival varies significantly between (A) dams of varying reproductive experience ($X^2$ = 41.60, df = 2, p<0.001, n = 1272), (B) between first time dams of varying age class ($X^2$ = 24.80, df = 4, p<0.001, n = 454) and (C) between dams with no previous breeding success ($X^2$ = 18.8, df = 4, p<0.001, n = 265).*

*6.3.2. Juvenile mortality*

One hundred and seventy-seven pups died on PND zero (16.8% of all deaths) and 600 pups died by PND 30 (56.8% of all deaths occurred by PND 30) or juvenile mortality rate of 44.7% of all births during the 20-year period). Loss of entire litters was found to be common with 40% of litters produced being lost before PND 30, which is 77% of all litters where juvenile mortality occurred in at least one pup. The cause of juvenile mortality was reported in 55% of cases. The most common reported cause of juvenile mortality was trauma caused by conspecifics (45%). Pack behaviour was responsible for 16% of juvenile mortality and 13% of pups were reported to be stillborn.

*6.3.3. Effects of husbandry on aggressive behaviour and reproductive success: results from the questionnaire*

*Husbandry*

The average number of wild dogs per institution was 6.5 individuals (min: 1, max: 22, median: 4). Thirty-eight per cent of the collections housed single sex groups and only two housed pups (<1 year old). Four of the 26 collections split their wild dogs into more than one pack and half of these were in close enough proximity to communicate. The average on-show enclosure size was 3716$m^2$ (min: 55$m^2$, max: 30,000$m^2$). The average off-show area was 309$m^2$ (min: 9.4$m^2$, max: 4000$m^2$).

Feeding regimes were similar across institutions, with wild dogs most commonly being fed small (85%) and large carcasses (79%). Wild dogs were also fed large (85%) and small (62%) joints of meat, offal (19%), and there were two reports of kibble being used. Food was presented on the ground/ scattered on the ground, by zip-line use and tethering. Eighty-one per cent of feeding occurred at group level and 8% at an individual level. Respondents commented that individual feeding was used to ensure adequate nutrition was provided to subordinate individuals or to maintain the best body condition score. Enrichment was used in all but one collection and 90% of this was conducted at group level. Enrichment included moveable objects (e.g. balls), sensory (e.g. herbs), training (e.g. target training) and food. Training was used in 54% of collections.

*Reproduction*

Forty-six per cent of collections housed breeding groups of wild dogs. Where respondents included their perception of breeding success, 50% reported that their breeding programme was moderately successful, with one litter produced every two years, and 50% reported their programme as successful with a litter produced annually. However, only six respondents completed this question. There were no reports of an unsuccessful breeding programme with less than one litter produced every two years. However, comments provided by the respondents could highlight a more suboptimal picture. Eleven respondents provided additional comments about reproduction. If we include the negative comments as poor breeding success, 53% of breeding groups have had adverse breeding experiences. Insufficient numbers prevented further analysis of the factors that could be influencing poor reproductive performance.

Twenty-seven per cent of collections use contraception in their dogs, with five female and two male hormonal contraception uses, and two female and one male surgically sterilised. All reported that chemical contraception was achieved using Desorelin acetate (Suprelorin®) subcutaneous implants. Three collections believed that contraception use influenced social hierarchy, one negatively, resulting in death of an implanted female, and one positively influencing social ranking. There were also three reports of contraception reducing aggression and one where contraception increased aggression. No institutions separated the dam from the pack at any time during the breeding season but five (42%) reported having a separate breeding enclosure.

*Aggression*

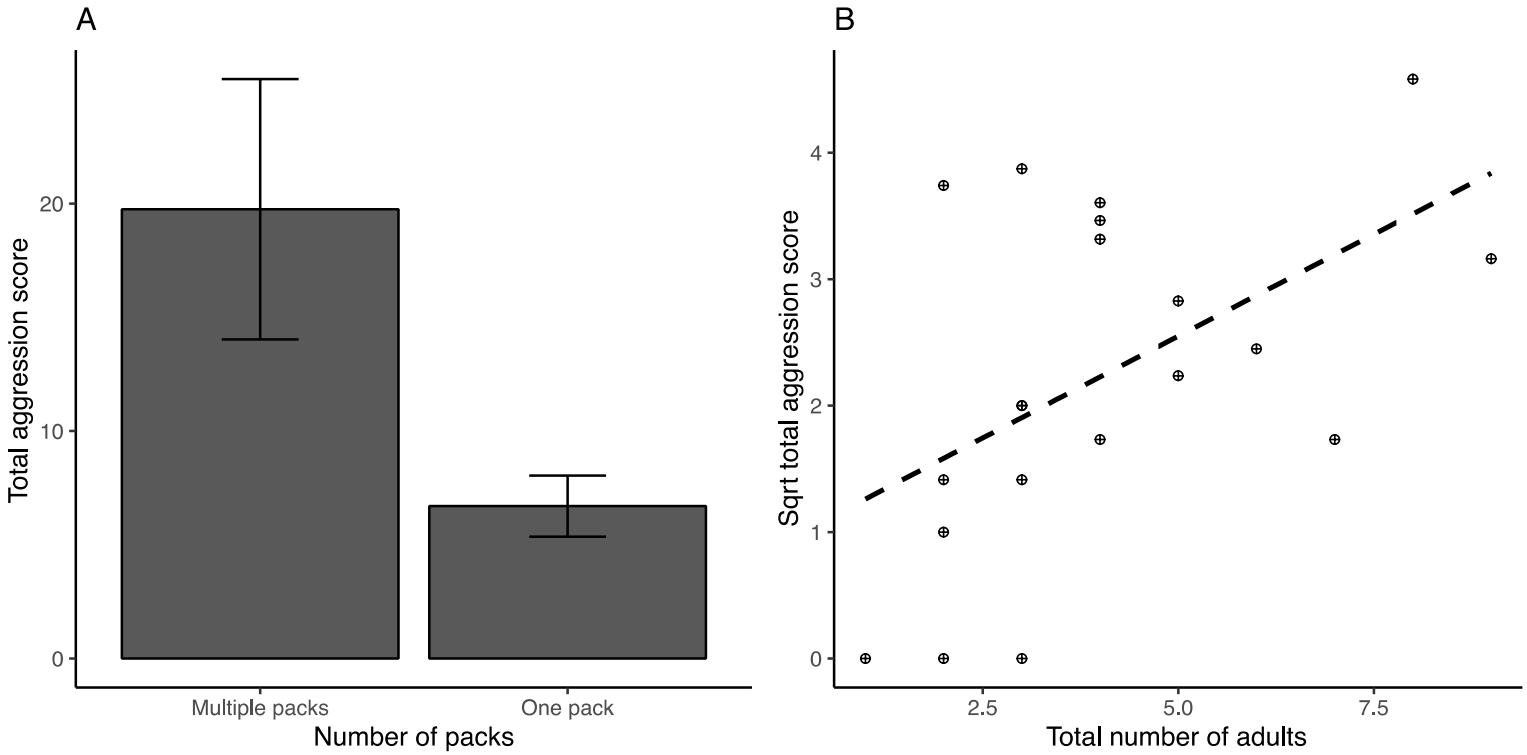
Eighty-eight per cent of institutions that housed more than one wild dog reported aggression to some degree. Of these, 91% reported very mild aggression and 30% reported severe aggression. Severe aggression, where wounds resulted in death or euthanasia, was connected to either “nothing specific”, “pack introduction” or “breeding”. Aggression was reported to occur for no specific reason in 43% of cases. Pack formation was responsible for 18% of reported aggression. Breeding was only found to be responsible for 8% of aggression.

Four of the 26 respondents split their population of wild dogs into multiple packs and half of these could visually communicate. Collections that contained more than one pack reported significantly more aggression than single pack institutions (Figure 6.3A: $X^2 = 5.070$, df = 1, p =

0.024). For single pack institutions that reported aggression, a significant positive relationship was found between the total number of adults and total aggression score (Figure 6.3B, Table 6.4, p = 0.017). A positive trend was found between the number of adults per square metre of enclosure and aggression (Figure 6.3C, Table 6.4, p = 0.083). However, this was heavily influenced by one small enclosure, and the trend disappears when this influential point is removed from analysis. A near significant difference in aggression level was found between wild dogs housed in close proximity to predators (lions) and those housed near to non-predator species (Figure 6.3D, Table 6.4, p = 0.073).

*Table 6.4: Generalised linear model investigating which factors influence aggression score. Total aggression score was square root transformed. Neighbours: neighbouring predators) Non-significant covariates were excluded from the model (pack composition, breeding group status, pup presence and enclosure size). n = 20.*

| | Estimate (SE) | t-value | p-value |
|---|---|---|---|
| (Intercept) | 1.0574 (0.5571) | 1.898 | 0.076 . |
| Total number of adults | 0.3176 (0.1193) | 2.663 | 0.017 * |
| Adults per $m^2$ of enclosure | 37.6790 (20.3712) | 1.850 | 0.083 . |
| Neighbours | 1.1914 (0.6198) | -1.922 | 0.073 . |


A
Total aggression score
20
10
0
Multiple packs
One pack
Number of packs
B
Sqrt total aggression score
4
3
2
1
0
2.5
5.0
7.5
Total number of adults

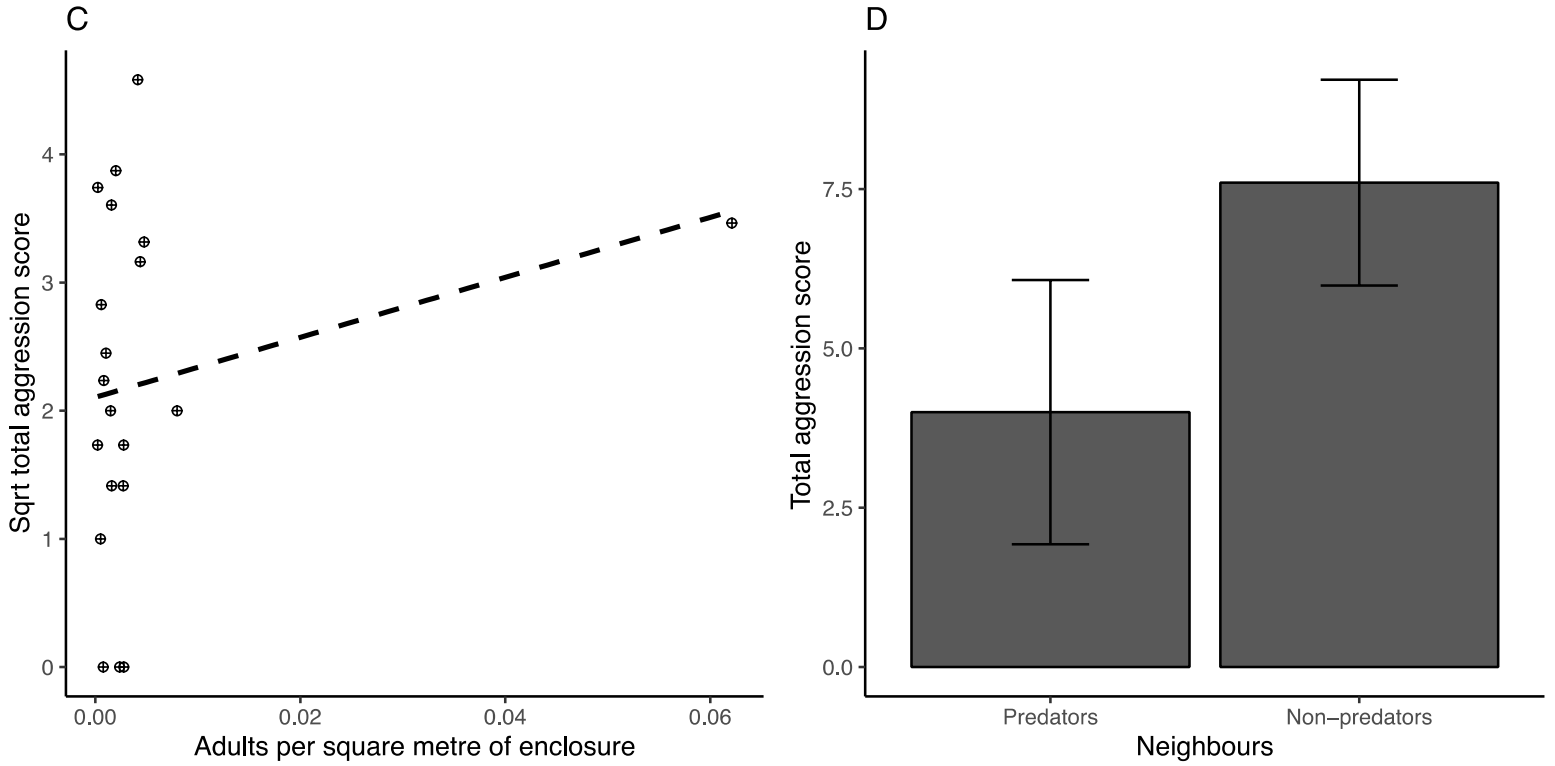



*Figure 6.3: The effect of (A) number of packs in one institution (mean ± SE), (B) the total number of adult wild dogs in one pack, (C) number of adults per square metre of enclosure and (D) neighbour type, on total aggression score in captive wild dogs (mean ± SE). A. Significantly more aggression was reported in institutions that housed more than one pack (p = 0.024, n = 24). B. A significant positive relationship was found between the total aggression score and total number of adults in the pack (p = 0.017, n = 20). C. A positive trend was found between aggression score and number of adults per square metre of enclosure (p = 0.086, n = 20), however, this was caused by one influential data point and no such trend was found on removal of this point. D. Wild dogs that were housed next to non-predators displayed near significantly more aggression than those housed next to predators (p = 0.073, n = 20).*

## 6.4. Discussion

We found that younger dams have the best offspring survival and if a dam is unsuccessful at rearing offspring to PMD 0, they have a significantly reduced offspring survival for in future pregnancies compared to first time and previously successful dams. Between 1996 and 2016, juvenile mortality was 44.7% and the most common cause of juvenile death was trauma by conspecifics. The questionnaire found 53% of institutions that housed breeding groups of wild dogs had experienced adverse breeding experiences and 88% of institutions reported aggression. Higher aggression was possibly linked to numbers of adults in a pack and possibly smaller enclosure sizes per dog.

In wild populations, survival to independence is higher for male than female wild dogs (Creel and Creel, 2002), resulting in a male bias within the population (McNutt and Silk, 2008). Within a pack, males are significantly less likely to disperse than females (Creel and Creel, 2002), and therefore greater survival probability by males could be due to greater parental investment in the sex that is likely to remain in the natal group and serve as helpers (Malcolm and Marten, 1982, Creel and Creel, 2002). Consistent with these findings for wild populations, we also found a male bias in the captive European wild dog population, and a significantly higher survival probability for male offspring, which might also be explained by greater parental investment.

In the current study, offspring survival was found to be greater for younger dams. Juvenile survival has been reported to be lower for offspring born to older dams in a number of species including chimpanzees (*Pan troglodytes*) (Pusey et al., 1997) and Japanese macaques (*Macaca fuscata*) (Sugiyama and Ohsawa, 1982). Indeed, Yordy and Mossotti (2016) found lower pup mortality for younger mothers in captive wild dogs. In line with this, data from the current study found breeding success to be greater for younger dams with a significant trend in decreasing survival with increasing dam age. Age at parturition for first time dams follow the same trend, but age had no influence on pup survival for previously successful dams. Pup mortality was greater than 50% for dams over 6.5yo, suggesting a reproductive decline (physiologically or behaviourally) may begin at around this age. This is in accordance with Yordy and Mossotti (2016), who similarly found female reproductive decline to be around 6.5yo for captive female wild dogs in North America. In the wild, female age has a significant effect on the fecundity of wild dogs; however, in a different way to the current results. Only

the dominant female is guaranteed to breed, and social dominance increases with female age; therefore the number of pups successfully raised increases with dam age until approximately 7yo (Creel and Creel, 2002), suggesting group composition differs in captivity. Indeed, carnivores have been found to have greater reproductive success later on in life; however, this could be due to behavioural effects (e.g. increased experience) rather than having a physiological basis (Sharp and Clutton-Brock, 2010, Durant, 2000). Furthermore, constraints of captivity may also apply, as females are only able to breed once they reach a certain age due to current breeding recommendations or space within collections. As juvenile mortality exceeded 50% for females >6.5yo in the current study, reproductive decline can be assumed to be reached at approximately 6.5-7yo in both wild (Creel and Creel, 2002) and captive female wild dogs. This is higher than for another cooperative canid, the grey wolf (*Canis lupus*), where reproductive decline is reported to start at 4-5yo in the wild (Stahler et al., 2013). The current study also found a juvenile mortality rate of 64-75% for primiparous dams aged over 4.5yo at parturition. This suggests it may not be beneficial for institutions to try to breed females for the first time once they reach 4.5yo.

Offspring born to dams that had previously failed to raise offspring to PND30 were found to have a significantly lower survival than those born to first time dams or females with previous experience of successfully rearing a litter. These data, along with the finding that increasing maternal age at first litter also has a significant negative influence on offspring survival, suggests breeding institutions should focus on breeding young captive female wild dogs. Moreover, if the first litter is not successful, it may not be worth trying to breed from that female a second time with the same partner. This however does not take into account the requirement to breed from genetically valuable animals or the difficulties of moving animals to create new breeding packs. This highlights the difficulties faced by managers of captive populations of wild dogs, who need to weigh up the requirements to breed specific individuals to maintain genetic diversity (Marsden et al., 2013), and the reproductive success and welfare of animals in their care.

Between 1954 and 1977, Frantzen et al. (2001) found juvenile mortality to be 63% in Southern African wild dog *ex situ* populations. However, veterinary care and husbandry practices have since improved. More recently, Yordy and Mossotti (2016) found a 53% mortality rate in North American captive wild dog populations. In the current study, juvenile mortality was found to be 45%, with loss of entire litters being common. However, this may be an underestimate due

to collections recording entire deceased litters as one individual. Yordy and Mossotti (2016) hypothesised that institutions may not record the exact number of individuals born when a litter is lost early (recording the litter as one individual), and this hypothesis was supported in the current questionnaire data, which concluded only 10% of collections reported the actual number of pups lost. This is likely due to the inability to count newborn pups in a den that cannot be disturbed, and sometimes there is very little evidence to suggest parturition had even occurred. If these numbers are adjusted to include the average number of pups in a wild dog litter (eight; Frame et al., 1979, McNutt and Silk, 2008, Gusset and Macdonald, 2010, Comizzoli et al., 2009) rather than one recorded individual, juvenile mortality could be 57%; similar to those reported for other captive populations. These reported juvenile mortality rates within captivity are high, considering wild populations have been reported to have juvenile mortality rates as low as 30% in one population (Creel and Creel, 2002).

Based on responses to our questionnaire, around half of the European institutions attempting to breed African wild dogs have had adverse breeding experiences. However, sub-fertility or infertility does not seem to be the main issue influencing poor reproductive performance in captive wild dogs. Juvenile mortality due to trauma caused by conspecifics was the most commonly recorded cause of death. Infanticide is more likely in species where maternal energetic investment is higher (Lukas and Huchard, 2018), and wild dogs invest in large litter sizes (Geffen et al., 1996). Indeed, Yordy and Mossotti (2016) often found pups to be killed directly by the dam. Proposed reasons for high maternal infanticide in captive wild dogs include a post-copulatory mechanism for mate choice, and inbreeding avoidance for breeding pairs with high levels of relatedness (Yordy and Mossotti, 2016). High parental kinship was found to have a significant negative impact on offspring survival in North American captive wild dog populations (Yordy and Mossotti, 2016). Kinship data was not included in the current study, although Marsden et al. (2013) found genetic diversity to be reasonably conserved in the European wild dog population. However, inbreeding was also found to be common, with a large reproductive skew resulting from a few individuals producing many offspring, and many individuals producing no offspring (Marsden et al., 2013). Indeed, within captive settings, there are restraints on space and movement resulting in little or no opportunity for mate choice.

Only two institutions reported housing pups (less than 1yo) at the time of the survey; one litter was born to a pack consisting of multiple adults and sub-adults, and one litter was born

to a lone pair with no other individuals present, making it impossible to decipher the group size effect. In the wild, there is a non-significant but positive correlation between number of helpers and number of surviving pups (Malcolm and Ken, 1982) and there is a significant correlation between reproductive success and pack size (Courchamp and Macdonald, 2001, Creel et al., 2004). An alternative hypothesis for high juvenile mortality in captivity could therefore be infanticide due to a lack of helpers; obligate cooperative breeders require helpers to survive in the wild (Lukas and Clutton-Brock, 2013) and dams might kill their offspring due to a lack of helpers in captive settings. Conversely, this explanation may be less likely as there is less energetic demand on captive packs of wild dogs due to provisions of food.

In the current questionnaire, some degree of aggression was reported in 88% of the collections that housed more than one wild dog, and a significant positive relationship was found between total numbers of adult wild dogs per collection and levels of reported aggression. In cooperatively breeding mammals, fitness benefits have been found to directly increase with the size of group, termed the "group size effect" (Clutton-Brock, 2002). The increase in aggression seen with increasing numbers of adults therefore appears to contradict this requirement for complex social groupings to successfully reproduce. However, in a confined space, there is likely to be more aggression due to a greater number of interactions between a greater number of individuals (Boutelle and Bertschinger, 2010). Indeed, in the current study, a near significant trend was found between aggression and square metre of enclosure per dog; however, this was skewed by one small enclosure. This is a similar finding to Yordy and Mossotti (2016) who also found a possible association between enclosure size and juvenile mortality. Even though insufficient numbers enabled further investigation into additional husbandry factors that may influence aggression levels, factors including food presentation may also contribute. For example, if a carcass is presented tethered to the ground and lower ranking individuals try to feed first, more dominant dogs could aggressively remove them from the carcass. This could be a reason for some institutions reporting that they feed dogs individually to ensure adequate body condition score is maintained. Furthermore, contraception use may cause heightened aggression. Indeed, results from the questionnaire found one example of contraception use potentially resulted in the death of the implanted female, and there were three further reports of contraception use increasing aggression.

High levels of aggression reported in *ex situ* wild dog populations could be associated with high levels of circulating glucocorticoids. Glucocorticoids are known mediators of aggression, including within wild dog populations (Creel, 2001), and captive female wild dogs may have higher levels of faecal glucocorticoid metabolites than their wild counter-parts (Van der Weyde et al., 2016), suggesting heightened stress of captivity. Worryingly, fighting within captive groups can be extreme enough to result in mortality (Van Heerden et al., 1996) and this level of aggression was reported in 30% of the responding institutions. Interestingly, aggression was found to be greater in those institutions that house more than one pack. If possible, housing only one pack could negate this aggression, however, if this is not possible, housing them as far away as physically possible (Van Heerden et al., 1996) may help, but due to small sample numbers, caution must be taken with this interpretation. In the current study, it was found that neighbouring lions may reduce aggression, however, this result should also be taken with caution as there could be many other factors at play. However, if this is the case, it could be due to increased cooperation within the pack due to a perceived threat of kleptoparasitism and parasitism (Ritchie and Johnson, 2009).

In conclusion, since age of female wild dogs at first litter significantly influences offspring survival, we advise to initiate breeding at as young an age as possible following sexual maturity. However, if a female has previously attempted to breed but has been unsuccessful at raising offspring to PND 30, our findings suggest that the chances of success for a second breeding attempt are also reduced. Finding an alternative mate may be beneficial, although more research is needed to assess breeding success under such conditions. Our study also provides limited evidence to suggest that relatively large enclosures may help to minimise aggression; however, further investigation is required. Using genetic analysis to ascertain the relatedness of possible breeding partners may be highly beneficial to reduce juvenile mortality rates and should be investigated for European collections of wild dogs.

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Contributions

All authors contributed to the design of the study; P.S. R.L.B. and E.H. compiled and conducted the questionnaire; E.H. completed preliminary analyses, R.L.B analysed the data. R.L.B, P.S and L.H drafted manuscript.

Conflict of Interest

The authors have no conflict of interest to declare.

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## 6.6. Supplementary Material

### Questionnaire

#### PAGE 1: INSITUTE INFORMATION

1. Name of institution
2. Name of lead contact
3. Email address of lead contact

#### PAGE 2: CONSENT

4. I have read and understood the Participant Information Sheet, I understand this questionnaire is voluntary and I agree to take part.
Tick box "I Agree"
*Participants will only be able to go on to complete the questionnaire if this box is ticked.*

## PAGE 3: DOG INFORMATION

5. How many painted dogs do you currently have in your collection?
Adult female (>2 years)
Adult male (>2 years)
Sub-adult female (1-2 years)
Sub-adult male (1-2 years)
Pups (<1 year) -

6. Are your painted dogs separated into more than one pack in different enclosures?
Yes / No

7. If you answered yes to question 6, can the different packs see each other through the barriers?
Yes/No

## PAGE 4: FEEDING INFORMATION

8. What are the painted dogs in your collection usually fed?

| | Frequency<br><br>→ drop down options | How<br><br>→ drop down options | Presentation<br><br>→ tick box |
|---|---|---|---|
| Small raw meat joints eg. End of horse limb | Twice daily; Once daily; Once a week; Once a fortnight; Once a month; Other | Individually; As a group; Both; Other | Scattered on the floor; Buried; Zipline; Tethered; Left on ground; In a bowl; Other |
| Large raw meat joints eg. Side of ribs | Twice daily; Once daily; Every second day; More than twice a week; Once a week; Once a fortnight; Once a month; Other | Individually; As a group; Both; Other | Scattered on the floor; Buried; Zipline; Tethered; Left on ground; In a bowl; Other |
| Small raw whole carcass eg. Rabbit | Twice daily; Once daily; Every second day; More than twice a week; Once a week; Once a fortnight; Once a month; Other | Individually; As a group; Both; Other | Scattered on the floor; Buried; Zipline; Tethered; Left on ground; In a bowl; Other |
| Large raw whole carcass eg. Calf | Twice daily; Once daily; Every second day; More than twice a week; Once a week; Once a fortnight; Once a month; Other | Individually; As a group; Both; Other | Scattered on the floor; Buried; Zipline; Tethered; Left on ground; In a bowl; Other |
| Offal | Twice daily; Once daily; Every second day; More than twice a week; | Individually; As a group; Both; Other | Scattered on the floor; Buried; Zipline; Tethered; Left on |

| | | | |
|---|---|---|---|
| | Once a week; Once a fortnight; Once a month; Other | | ground; In a bowl; Other |
| Kibble | Twice daily; Once daily; Every second day; More than twice a week; Once a week; Once a fortnight; Once a month; Other | Individually; As a group; Both; Other | Scattered on the floor; Buried; Zipline; Tethered; Left on ground; In a bowl; Other |

9. If you answered "Other" to the above question, please provide details below.

10. Are your painted dogs usually physically separated to be fed?
Yes
No
Details

11. Do you have any other comments on your feeding regime?

PAGE 5: ENCLOSURE INFORMATION

12. What is the approximate size of the enclosure in $m^2$?
Number box (on- /off-show)

13. How many natural dens are present within the enclosure?

| | On show<br><br>→ drop down options | Off show<br><br>→ drop down options |
|---|---|---|
| Natural dens | 1-10 | 1-10 |
| Artificial dens | 1-10 | 1-10 |

14. From how many sides of the on-show enclosure are the dogs directly visible to the public?
1
2
3
4

15. Please list which species are housed around your painted dog enclosure.
Comments box

16. Do you have any other comments on your enclosure?

PAGE 6: ENRICHMENT INFORMATION

17. Please provide details on the enrichment you provide for your painted dogs.

| | Frequency<br><br>→ drop down options | Presentation<br><br>→ drop down options |
|---|---|---|
| Moveable objects eg. Ball | Never; Twice daily; Once daily; Every two days; More than twice a week; Less than twice a week; Once a week; Once every | Individually; As a group; Both; Other |

| | | |
|---|---|---|
| | two weeks; Once a month; Less frequently; Other | |
| Sensory eg. Prey species dung sack, scent trails | Never; Twice daily; Once daily; Every two days; More than twice a week; Less than twice a week; Once a week; Once every two weeks; Once a month; Less frequently; Other | Individually; As a group; Both; Other |
| Training eg. Target training | Never; Twice daily; Once daily; Every two days; More than twice a week; Less than twice a week; Once a week; Once every two weeks; Once a month; Less frequently; Other | Individually; As a group; Both; Other |
| Food eg. Blood icicles, small food items in a sack | Never; Twice daily; Once daily; Every two days; More than twice a week; Less than twice a week; Once a week; Once every two weeks; Once a month; Less frequently; Other | Individually; As a group; Both; Other |
| Other | Never; Twice daily; Once daily; Every two days; More than twice a week; Less than twice a week; Once a week; Once every two weeks; Once a month; Less frequently; Other | Individually; As a group; Both; Other |

18. If you answered "Other" for the above question, please provide details.

19. Do you separate your painted dogs for training?
Yes, we separate individuals then train them individually
No, we train them without separation from the group
No training given

20. Do you have other comments on your enrichment programme?

## PAGE 7: REPRODUCTION INFORMATION

*If you have a non-breeding group, please skip to question 26.*

21. When you are attempting to breed, how successful is your breeding programme?
Tick options
Successful – one litter per year
Moderate – one litter every two years
Unsuccessful – less than one litter every two years
Breeding not attempted / non-breeding group

22. Do you have a separate breeding enclosure?
Tick options
Yes
No
If yes, is this on show to the public?

23. Is the dam separated from the rest of the pack at any point during or after pregnancy?

Tick options
Not separated
From first signs of pregnancy
During whelping
For 2 weeks following whelping
Other, please specify

24. If a litter is unsuccessful before a head count is completed, how do you record this on ZIMS / stud book?
Tick options
Not aware of any pregnancy loss
Lost litter recorded as 1 individual
Lost litter recorded as 6 individuals (average litter size)
Not recorded
Other, please specify

25. Do you use any of the following reproduction management options?
Male hormonal contraception
Female hormonal contraception
Male surgical contraception
Female surgical contraception
None
Other, provide details

*If you do not use any reproductive management options, please skip to question 32.*

26. Which hormonal contraception methods do you use to manage reproduction in your painted dogs?
Tick options for males and females.
Desorelin acetate (Suprelorin): subcutaneous implant
Leuprolide acetate (Lupron®): subcutaneous injection
Leuprolide acetate (Lupron®): intramuscular injection
Other

27. If you answered "Other" for the above question, please provide details.

28. If you have used hormonal contraception in your painted dogs, was a change in social hierarchy observed?
Yes
No
Please provide details

29. If you have used hormonal contraception in your dogs, have you observed a change in aggression levels?
Increased aggression
Decreased aggression
Unknown – no monitoring conducted
Please provide details

30. Do you have any other comments on factors that may be influencing reproductive success?

PAGE 8: AGGRESSION INFORMATION

31. How often do you observe aggression between your painted dogs?

| | Frequency<br><br>→ drop down options | Activity association<br><br>→ drop down options |
|---|---|---|
| Very mild – fighting seen but no obvious sign of injury | Never; Daily; Weekly; Monthly; Other | No particular activity; Feeding; Breeding; Enrichment; Pack introduction |
| Mild – fighting seen with evidence of superficial wounds, or observation of superficial wounds that required no further interventions | Never; Daily; Weekly; Monthly; Other | No particular activity; Feeding; Breeding; Enrichment; Pack introduction |
| Moderate – wounds required veterinary treatment | Never; Daily; Weekly; Monthly; Other | No particular activity; Feeding; Breeding; Enrichment; Pack introduction |
| Moderate severe – wounds required veterinary treatment and staff had to intervene and separate group members | Never; Daily; Weekly; Monthly; Other | No particular activity; Feeding; Breeding; Enrichment; Pack introduction |
| Severe – wounds resulted in death or required euthanasia | Never; Daily; Weekly; Monthly; Other | No particular activity; Feeding; Breeding; Enrichment; Pack introduction |

32. If you answered “Other” for the above question, please provide details.

33. Do you have any further comments on aggression within your painted dog pack?

PAGE 9: THANK YOU

34. Thank you for taking the time to complete our survey. Do you have any further comments?

# Chapter 7: General Discussion

## 7.1. Chapter Overview

In this chapter I discuss the results of this thesis within a broader context. Human activity is causing a catastrophic decline in biodiversity, and we have a duty to act before it is too late. In addition to protecting wild spaces and species habitats, it is also important to ensure endangered species do not go extinct. Zoos act as an insurance policy by housing breeding groups of endangered species. Not only does this mean there are continuing genetic lines of rare species, but also enables *ex situ* conservation research to take place. Unfortunately, many cooperatively breeding species are threatened with extinction and so *ex situ* breeding programmes are important to prevent the loss of these species. One example of an endangered cooperatively breeding mammal is the African wild dog and this species struggles with poor reproductive performance and high levels of aggression in captivity. Using a model species, the house mouse, I wanted to decipher whether variations to the early rearing environment could alter adult phenotypes, specifically social and anxiety-related behaviour. Could unnatural groupings for endangered cooperatively breeding mammals be resulting in increased aggression and heightened anxiety? The broad aim of this thesis was to add to evidence-based management practices for African wild dogs to add to best practice guidelines for this endangered species.

I begin with discussing the validation required for the methodology used in this thesis including enzyme immunoassays and the importance of a faecal hormone metabolite degradation study for different species. I then discuss how variation in the early rearing environment has the potential to alter the neuropeptide system, specifically oxytocin and vasopressin (AVP), and the hypothalamic pituitary adrenal (HPA) axis and corresponding social and anxiety-related behaviour using house mice (*Mus musculus domesticus*) as a model cooperatively breeding mammal. I then consider the wider implications of this work in relation to captive endangered cooperatively breeding species with a focus on the African wild dog (*Lycaon pictus*) with an aim to improve evidence-based management practices for this endangered species.

## 7.2. Methodological validation

Investigating physiological mediators of behaviour can often require invasive sample collection, which is stressful and can result in erroneous data. However, for longitudinal behavioural studies or studies on endangered *ex situ* populations, invasive sample collection is not possible or ethical and non-invasive samples are often collected instead. Non-invasive samples, for example excreta, are reasonably easy to collect, however, it is vital that rigorous assay validations are conducted. In Chapter 2, I successfully biochemically validated a commercially available oxytocin EIA for mouse urine, as well as for PNV homogenate and serum. Mice produce small volumes of urine in a single void making it impossible to extract oxytocin from urine within the sensitivity range of the assay. However, a significant correlation between extracted and non-extracted oxytocin was found. Correlations between extracted and non-extracted oxytocin have previously been reported for different matrices (Michopoulos et al., 2011, Leeds et al., 2018). Using wild house mice (*Mus musculus domesticus*), I was able to investigate the relationships between central, peripheral and non-invasive measures of oxytocin. No relationship was found between serum and PVN homogenate oxytocin, and no relationship was found between urinary oxytocin and serum or PVN homogenate oxytocin. This may not be surprising as central and peripheral release of oxytocin is thought to be differentially regulated with both the parallel and independent release of oxytocin (Engelmann et al., 1999, Insel, 2010). Indeed, the lack of correlations found in the current study are likely to be due to the fact they were collected during a baseline period rather than following a specific stimulus (Valstad et al., 2017).

In addition to urine, faeces provide an alternative non-invasive matrix for monitoring aspects of the endocrinology of species in captivity. Unlike the ease of collecting samples from known individuals in laboratory bred wild house mice where, for this thesis, mice were placed in recovery cages for short periods of time, it is often difficult to obtain fresh faecal samples and to identify from which individual animal any faecal sample was derived due to management practices in *ex situ* situations. This is particularly the case for dangerous carnivores where keeping staff are unable to enter enclosures without first securing the animals elsewhere, which in itself can affect behaviour. The lag time between defecation and faecal collection (and therefore cold storage) can be variable and long, and these factors may erroneously alter results. For example, this could provide results indicating high levels of adrenal hormones suggesting an animal is under stress, when in fact, this could purely be due to collection

techniques. In agreement with current literature (Yarnell and Walker, 2017, Wong et al., 2016, Mesa-Cruz et al., 2014, Washburn and Millspaugh, 2002), in Chapter 5 I found all faecal hormone metabolite concentrations investigated varied over time during both winter and summer months. Furthermore, hormone concentrations were found to be higher in the sunny aspect and dry conditions. In addition to the obvious fact these samples will simply contain less water and therefore hormone metabolites are likely to be more concentrated, conjugated side-group cleavage from hormone metabolites, or the release of steroid hormones from lipid micelles within the bolus may also cause the increased concentrations found (Washburn and Millspaugh, 2002). Furthermore, higher temperatures may cause an increase in microbial growth which can cause an increase in hormone metabolite concentration (Yarnell and Walker, 2017). Indeed, these data suggest that it is vitally important to ensure collection times are short and within a known time frame to ensure accuracy of results. For wild dogs, Chapter 5 shows faecal hormone metabolite concentrations were stable for four hours following the initiation of the degradation study. Environmental conditions should also be taken into consideration when investigating hormone correlates of behaviour for this species. Due to more controlled conditions within the laboratory, there was little to no lag time between defecation and cold storage for house mouse samples. Additionally, the conditions within the animal rooms are constant, removing variations in environmental conditions. However, if there were to be a time lag between excretion and cold storage, or variations in environmental conditions, a degradation study to cover this time and environmental variables would be an important consideration.

A broad understanding of the physiology of a species is vital to ensure evidence-based management practices provide optimal welfare standards and high levels of reproductive success. Furthermore, greater understanding of how intrinsic and extrinsic factors influence individuals and populations is important. This understanding should facilitate the removal of factors that negatively affect welfare or reproduction, may reduce abnormal behaviours, and may provide evidence for reasons behind mate choice. Hormone analysis has the potential to provide this greater understanding and is a tool often used successfully in *ex situ* breeding programmes (Brown, 2006). For *ex situ* programmes, the development of non-invasive hormone measures is key, and strict validations are required to ensure accurate measures of the hormone of interest are being achieved. Gaps in knowledge hinder a zoo's ability to provide optimal animal welfare. In a number of zoos, housing and husbandry conditions are based on tradition and word of mouth rather than evidence of what is best (Melfi, 2009).

Guidelines may adhere to the Farm Animal Welfare Committee 1979 five freedoms of animal welfare: (1) freedom from hunger and thirst; (2) discomfort; (3) pain, injury, or disease; (4) fear and distress; and (5) freedom to express normal behaviour. However, even though this may be satisfactory, it may not be optimal, and using a species-specific evidence-based method of management practices could allow a species to thrive rather than just survive. Any change in husbandry or housing practice due to an empirical study should then be followed up with "post occupancy evaluations" to ensure any change indeed has been beneficial (Melfi, 2009). However, evidence-based management practices are increasing with EAZA and other governing bodies adopting this approach. Indeed, journals are now publishing special issues dedicated to evidence-based zoo animal management to create a forward-thinking approach to management.

### 7.3. Early rearing environment

Using house mice as a model of a cooperatively breeding mammal, I investigated whether the early rearing environment influenced social behaviour (Chapter 3) and anxiety-related behaviour (Chapter 4). In Chapter 3 we found that early life experience in terms of being reared in a communal or single nest, had no influence on their social behaviour or urinary oxytocin concentration when meeting unfamiliar conspecifics. Female mice displayed an ability to recognise unfamiliar kin, consistent with previous studies (Ferrari et al., 2019, Green et al., 2015). Indeed, more affiliative and less aggressive behaviours were directed towards related than unrelated conspecifics. Affiliative behaviour directed towards close kin may be mediated by the oxytocin system, and affiliative behaviour directed towards unfamiliar close relatives was significantly corelated with urinary oxytocin concentration. Furthermore, any effect of the early rearing environment on anxiety-related behaviour was inconclusive. Both communal and single nest environments produced female mice that showed stereotypic behaviours which skewed the data and so these mice were removed from the final analysis. Results suggested that communal nest reared females travelled significantly greater distances within the novel field test than single nest reared females, indicating such communal reared females showed lower anxiety levels than those reared in a single nest. However, time in the centre of the test arena was not affected by early rearing experience. Chapter 4 provides evidence that the early rearing environment does, indeed, influence oxytocin receptor and AVP receptor 1a expression in specific brain regions. Furthermore, anxiety-related behaviour may be mediated by central oxytocin receptor and AVP receptor expression.

For communal rearing species like cooperatively breeding mammals, dams make a decision to rear their offspring either in a communal or single nest environment (Weidt et al., 2014). This decision is likely to have lasting impacts on offspring brain development and therefore behaviour following a period of plasticity in very young animals (Taborsky, 2017). The neuropeptide network and HPA axis are both sensitive to the early rearing environment (Bosch et al., 2007, Dantzer et al., 2013, Champagne and Meaney, 2006, Provencal and Binder, 2015, Sakhai et al., 2016, Curley et al., 2009) . Natural variations in nesting (communal versus single nest environment) may therefore affect these networks, as can unnatural nesting arrangements that occur within captivity. The neuropeptide system is responsible for a plethora of behavioural responses including affiliative behaviour, kin recognition, aggression and anxiety-related behaviour (Veenema and Neumann, 2008, Young and Wang, 2004, Bosch, 2013, Anacker and Beery, 2013, Beery and Zucker, 2010, Choleris et al., 2009, Oettl et al., 2016, Winter and Jurek, 2019). Changes to the neuropeptide network due to variation in early rearing environment may therefore result in differing social and anxiety-related behaviours of offspring. This has wide ranging implications, especially for captive breeding of endangered cooperatively breeding mammals. Artificial constraints of captivity may prevent natural breeding groups from forming, causing epigenetic changes in offspring reared in unnatural nests. Via the neuropeptide system, this may have permanent consequences on adult behaviour. Unfortunately, many cooperatively breeding species are threatened with extinction therefore requiring *ex situ* breeding programmes for long term survival. However, these species also suffer from high levels of aggression and poor reproductive performance in captivity. The unnatural rearing environment could therefore be enhancing the aggressive social behaviour and heightened anxiety seen in captive African wild dogs.

## 7.4. Broader implications

It would be interesting to investigate variations in oxytocin in captive endangered cooperatively breeding mammals, however, there are limitations in numbers and ability to receive samples. Often few individuals of each species are found across various zoological collections. Data values of such studies will therefore often be low, and so such an approach would benefit from multi-institutional collaborations (Leeds et al., 2018).

As shown in Chapter 3, oxytocin physiology mediates social behaviour directed towards unfamiliar relatives, but not unfamiliar unrelated individuals. Within captive settings, specific unnatural groups are formed to ensure genetic diversity is maintained, for example for the captive wild dog population. For cooperative breeders, groups of unfamiliar and or unrelated individuals placed together to form a pack could produce lower affiliative behaviour levels partially mediated by the oxytocin system. Furthermore, as shown in Chapter 4, variation in the early rearing environment influences both anxiety-related behaviour and central oxytocin receptor concentrations in laboratory-manipulated female house mice. Indeed, single nest reared female house mice exhibited less exploratory behaviour in terms of total distance travelled, suggesting single nest reared females may show increased anxiety-related behaviour. For captive wild dogs therefore, at a central level, smaller pack sizes and less interaction within the den, could be causing variations in neuropeptide receptor concentrations, as seen in the house mice in Chapter 4. Furthermore, the pack composition of wild dogs assessed in Chapter 6 was not known, and pack composition could be a factor that may be influencing the poor reproductive success in captive wild dogs. Indeed, within a communal nest, the relatedness of the females that form a communal nest affects its success (König and Lindholm, 2012).

In the work described in this thesis, the relatedness of conspecifics within captive wild dogs are unknown, and there is the possibility that unrelated females may be placed within the same zoological collections. Unrelated females within the confines of captivity are likely to display increased aggression, especially during the breeding season. For example, there would be increased aggression to secure alpha female status because following breeding, an unrelated helper will not be indirectly passing on any genes to the future generation (Hamilton, 1964a, Hamilton, 1964b). Additionally, the high level of infant mortality caused by conspecifics found in this thesis, could again be due to unrelated helpers not being willing to share parental responsibilities. However, even if females within a nest were related, competition will still arise, especially during periods of reproduction. The constraints of captivity are likely to enhance this due to space restrictions, and, for subordinate individuals, a complete lack of breeding opportunities without fighting for them. In Chapter 6, it was found that 88% of the zoological collections that responded to the wild dog husbandry survey reported aggression. Unfortunately, it is not known whether the aggression was between males, females, both or across the different sexes, however, personal observation of one captive wild dog pack, found the highest level of aggression to be between two sisters at the

start of the breeding season. An additional possible factor that may be contributing to increased aggression could be that humans have removed all reasons for wild dogs to cooperate. In captivity, wild dogs do not need to cooperate for food or for protection; there are no reasons to cooperate.

Within zoological institutions, enclosure size limitations may result in smaller social groups. Indeed, the median wild dog pack size in Europe (Chapter 6) was found to be four, below the described minimum threshold for successful breeding for this species of five (Courchamp et al., 2002). Naturally, obligate cooperative breeders, including African wild dogs, require the addition of helpers to successfully rear offspring, and these may be lacking in artificially managed groups. Reduced physical interactions resulting from smaller pack sizes may be influencing anxiety-related behaviours similar to that seen in house mice. Furthermore, the early rearing environment is known to also influence the HPA axis. Even though no difference was found in the corticosterone response between communal and single nest reared female house mice in Chapter 4, this is not the case in other studies. Smaller pack sizes in captivity may be influencing the developing HPA axis of pups due to less licking and grooming within the nest, resulting in an altered HPA development and heightened HPA responses. Indeed, captive female wild dogs are known to have higher faecal glucocorticoid metabolite concentrations than their wild counterpart (Van der Weyde et al., 2016) reflecting increased stress levels, and the early rearing environment could partially be influencing this. As increased glucocorticoids is a known mediator of aggression, this could be one factor contributing to high levels of aggression experienced by captive wild dogs evidenced in Chapter 6.

Physiological parameters including faecal hormone metabolites were not available for the wild dogs discussed in Chapter 6; however, captive female wild dogs have previously been found to have higher faecal glucocorticoid metabolite concentrations than their wild counterparts (Van der Weyde et al., 2016). It is currently unknown whether concentrations of reproductive hormones are influenced in a captive setting for this species; however, increased glucocorticoids are known to supress reproduction (Creel and Creel, 2002). Could the stress of captivity be supressing reproduction, adding to the poor reproductive performance seen in the European captive population of wild dogs? Indeed, in the Chapter 6 questionnaire, over a half of participating institutions reported adverse breeding experiences within their pack of wild dogs. Additionally, juvenile mortality was found to be high in captive European collections

of wild dogs over the past 20 years. It was impossible to deduce whether or not pack size influenced reproductive success, as this information was not available in the stud book data and only two participating zoological collections had pups (<1yo) when the questionnaire was completed. In fact, one of these packs contained helpers, whereas the other was a lone breeding pair, which goes against the rule of requiring a minimum of five dogs to successfully reproduce (Courchamp et al., 2002). In Chapter 6, survival analysis of wild dogs born between 1996 and 2016 found greatest offspring survival for the youngest dams and those that had previously successfully reproduced (reared at least one pup to PND 30). Breeding efforts may therefore be best placed in the younger females.

## 7.5. Impact

We are undergoing the sixth mass extinction, and unlike previous extinction events, this one is anthropogenic (Ceballos et al., 2015) and ultimately threatening our own survival. The protection of natural spaces can only be sustainable with the maintenance of a diverse range of flora and fauna which have evolved within those spaces. In a world of ever decreasing biodiversity, *ex situ* breeding programmes are vital for the maintenance of threated animal species. Not only do zoological institutions provide a 'reservoir' of endangered species, but they also provide education and public awareness of conservation related issues including the extinction crisis. *Ex situ* breeding programmes aim to be self-sustaining (Leus et al., 2011) and follow evidence based management practices (Lacy, 2013). However, for an obligate cooperatively breeding mammal, the confines of captivity may be causing perturbations during the early stage of epigenetic plasticity that occurs to enable individuals to adapt to a changing environment (Taborsky, 2017). Unnatural breeding groups, resulting in unnatural early rearing environments may be influencing social and anxiety-related behaviours, mediated by variations in the HPA axis and neuropeptide systems. These variations could result in aggressive or anxious phenotypes which are not suited to captivity. Furthermore, smaller nests within the confines of captivity may be altering maternal behaviour. Indeed, for laboratory mice, larger communal nests result in female offspring expressing increased maternal care when they reached adulthood (Curley et al., 2009). Smaller nests in the confines of captivity could result in reduced maternal care, which may be evidenced as reduced maternal protection. This could be one reason for the high level of juvenile mortality in captive wild dogs being caused by trauma inflicted by conspecifics (Chapter 6). Additionally, the high level of infanticide found in wild dogs in the current thesis could be due to the complete

removal of mate choice in captivity and potentially high parental kinship (Yordy and Mossotti, 2016) that was not investigated in the current study. High parental kinship was found to have a significant negative impact on offspring survival in North American captive wild dog populations (Yordy and Mossotti, 2016).

The additional stressors of captivity from human interference, unnatural groups, limited space, no mate choice and so on make it hard to decide upon the winning combination for reduced aggression and increased reproductive performance for the African wild dog. In this thesis I have found that the early rearing environment of a cooperatively breeding mammal (the house mouse) may influence anxiety-related behaviour of future offspring and applying this to a captive endangered cooperatively breeding mammal could suggest that the unnatural constraints of captivity could be altering physiology via epigenetic changes occurring in the nest. In addition, cooperative behaviour directed towards kin may partially be mediated by the oxytocin system. The future of African wild dogs as a species is in the hands of a currently far from perfect *ex situ* breeding programme. Here we can direct best practice guidelines to breed females at a young age, not to put resources into breeding previously unsuccessful pairs and increasing enclosure sizes. But maybe we should also be trying to make captive environments more natural by increasing pack sizes. Within European zoological institutions this will always be constrained by enclosure size. Even the largest enclosure in a zoo cannot come close to the vast lands these animals have evolved to roam. Logistically it is impossible to allow for mate choice, however, genetic screening to reveal relatedness of potential breeding partners prior to introduction may be massively beneficial (Yordy and Mossotti, 2016). But with so few individuals left, and with numbers in the wild decreasing, habitats decreasing and fragmented, maybe an alternative tool is required in addition to captive breeding for example the use of cryogenic biobanking and artificial reproductive technology is an emerging tool in the conservation toolbox.

## 7.6. References

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