Reducing the Impact of Rodenticides on Non-Target Wildlife

Thesis submitted in accordance with the requirements of the

University of Liverpool for the degree of Doctor in Philosophy

by

Nicola Beth Davidson

May 2018

Table of Contents

List of Figures

Supplementary figures

List of Tables

Supplementary tables

Abstract

Reducing the impact of rodenticides on non-target wildlife

Nicola Davidson

There is a global reliance on pesticides to control animal pests. Often, pesticides are not species specific, leading to poisoning of non-target species. This is a particular problem for pest control aimed at rats, primarily affecting small rodents including wood mice (*Apodemus sylvaticus*), bank voles (*Myodes glareolus*) and field voles (*Microtus agrestis*). Secondary poisoning of small rodent predators is widespread, affecting birds of prey, foxes and mustelids. The aim of this thesis is to identify methods to reduce the poisoning of non-target rodents by rat poisons.

One method investigated was the use of rat odour as a species specific repellent to repel non-target rodents, whilst not repelling target rats. Odour from rats of both sexes was repellent to non-target rodents in laboratory arenas and reduced non-toxic bait take in semi-natural enclosures. However, concern over possible repellent effects of male rat odour on male rats led to a focus on female rat odour and non-sex specific rat odour cues as non-target rodent repellents. Analysis of female and male rat odour identified 4-ethyl phenol as the most abundant volatile compound in both sexes. 4-ethyl phenol was tested with bank voles and wood mice and found to be repellent to the former, but not the latter. Under natural conditions, female rat odour reduced non-toxic bait take by non-target rodents, but repellency was context dependent and not as strong as found in more controlled conditions. Thus, there is good potential for rat odour to be used as a non-target repellent. However, further development will be needed before it can be used during pest control operations.

A second method of reducing the exposure of non-target rodents to rat poisons is to use species specific attractants to attract rats to alternative control measures, without attracting non-target rodents. A candidate species specific rat attractant is 50 kHz calls, produced by rats in prosocial situations and found to attract other rats. When tested in laboratory arenas, rats were attracted to 50 kHz rat calls and sounds of rat movement. There was no sex specificity in this attraction and calls from unfamiliar rats were attractive to conspecifics. In addition, bank voles were not attracted to, nor repelled by, 50 kHz calls. These calls have the potential to be used as a species specific rat attractant, but field testing will be required to determine their efficacy on wild rats.

A requirement for reducing the impact of rodenticides on non-target rodents is the need to monitor populations of these animals, especially during rat control campaigns. Current monitoring methods are labour intensive and time consuming. An emerging technology that has the potential to be used to monitor rodents, without these problems, is Rapid Evaporative Ionisation Mass Spectrometry (REIMS). In a series of tests, REIMS was capable of identifying rodent species to a high level of accuracy with minimal effects of sample storage and subject diet. REIMS has good potential to be used as a monitoring tool for use in pest control, as well as research and ecology applications.

In conclusion the use of natural social cues, including rat odour and 50 kHz rat calls, has the potential to reduce the exposure of non-target rodents to rodenticides. In addition, application of REIMS to rodent faecal pellets has the potential to improve monitoring of target and non-target rodents.

Acknowledgements

This thesis would not have been possible without the help and support of a number of people.

I would particularly like to thank my supervisors Professor Jane Hurst and Professor Rob Beynon for their help and support throughout my studies. Thanks especially to Jane for being an amazingly supportive supervisor. I have really enjoyed our discussions on experimental design and hypothesis testing, and I have learned so much from your example of how to be a professional, respected researcher. Thanks to Rob for your unceasing enthusiasm and excellent advice, and for teaching me to love proteins and mass spectrometers. Specific thanks also to Rob for originating the idea of using REIMS as a method of identifying rodent species based on faecal samples.

Thank you to the Biotechnology and Biological Sciences Research Council and the University of Liverpool for funding and supporting my work.

Thank you to all the members of the Mammalian Behaviour and Evolution group at the University of Liverpool for their support, advice and friendship. Particular thanks to Amanda Davidson for teaching me the laboratory techniques necessary to complete my work and for not getting too angry when I put things in the wrong bins. I would like to thank all the animal technicians I have worked with for the high levels of care they gave to my experimental animals, particularly John Waters. Thank you to Rick Humphries for your help with temperature controllers and for keeping the buildings running. Thank you to Mark Prescott for running my samples on the GC-MS and helping me to understand the results. Thank you to Joscelyn Harris for all her help with running and using the REIMS and for writing the matlab code used to analyse the results.

Thank you to the staff at Ness gardens, Wood Park Farm and Ness Heath Farm, in particular Jessica Mehers, Timothy Baxter, John Cameron and Nigel Jones, for allowing me to trap rodents and collect rodent droppings from their facilities. Thanks also to Mike Flynn from Alpha Pest Control for allowing me to accompany him on his rounds to collect rat faeces samples.

Thank you to Emrys Jones and all the team at Waters in Wilmslow for providing the REIMS source, and for the helpful technical support and feedback required when working on such a new piece of equipment.

Thank you to my amazing husband, Phil, for giving me so much support, particularly during the last few months of writing up. I couldn't have done it without you. Thank you to my gorgeous daughter, Isabel, for making me smile and giving me someone to work hard for. I hope you are proud of me when you are old enough to understand this achievement. Thank you to my sister, dad and parents-in-law for being great listeners and supporting me in whatever I do. Thank you to my amazing friends, especially Hanna and Nicola, for putting up with my grumblings and keeping me going through thick and thin.

Chapter 1

1. Introduction

This thesis aims to examine ways to reduce the impact of rodenticides on non-target wildlife. To introduce this topic, I will provide a background on what causes an animal to be considered as a pest, as well as roles of non-pests in ecosystems, with a particular focus on rodents. I will then move on to outline the ways in which pest animals are controlled, again with a focus on rodents. In discussing methods of pest control I will describe non-target effects of pesticides, highlighting the problem of non-target poisoning in pest control aimed at rats. This will lead on to an introduction of the potential options to mitigate non-target poisoning in rat control, including the use of scents and sounds, as well as the role of pest monitoring. Finally, I will outline the objectives and issues addressed in each chapter of this thesis.

1.1. What makes a pest?

1.1.1. The role of non-pests

Throughout the world, animals may be categorised by their relationship to humans and human activity, depending on whether this relationship is viewed as positive or negative (Arluke & Sanders, 1996). Many animals are seen as having a positive association with humans. These animals may form close relationships with humans, such as pets or farmed animals (Arluke & Sanders, 1996). In addition, animals may be viewed positively simply by playing their part in normal ecosystem function (Arluke & Sanders, 1996). Within ecosystems, the roles of animals are complexly interlinked, with the activities of many species causing ecosystem changes that affect the availability of resources for other species (Moore, 2006; Naiman, 1988). Animals may function as part of food webs, both as consumers of lower trophic levels or as providers of food for higher trophic levels (Williams & Martinez, 2000). In addition, many species serve to pollinate plants, and cause landscape changes that help other animals survive (Dickman, 1999; Moore, 2006; Naiman, 1988). For example, bush elephants (*Loxodonta africana*) modify vegetation structure affecting food availability to other herbivores (Naiman, 1988). More subtly, the use of silk by caddisfly larvae to build cases and filtration nets alters the structure of stream beds, providing new habitats and preventing erosion (Moore, 2006). Crucially, these ecosystem roles do not interfere with human activity, or damage things that are valuable to humans and so are viewed positively by humans (Arluke & Sanders, 1996).

1.1.2. Characteristics of pests

At the other end of the scale to animals that are viewed positively by humans, are those animals that are viewed negatively and are labelled as pests (Arluke & Sanders, 1996). Pests interfere with human activity or damage things that are valuable to humans, including effects on ecosystem function (Arluke & Sanders, 1996; Buckle & Fenn, 1992; Harris, 1989; Oerke, 2006; Oliveira *et al.*, 2014). Effects on ecosystem function can be a particular problem where pests are introduced to new habitats, either intentionally or otherwise, by human activity (Pimentel *et al.*, 2005; Westphal *et al.*, 2008). Pest animals tend to share certain characteristics which make them successful as pests. They often have a fast reproductive cycle, with a high capacity for fast population growth (Nair, 2007). In addition, pest animals are often highly adaptable both in terms of diet preferences and habitat utilisation (Nair, 2007). These features allow pests to colonise new habitats with ease and rapidly expand their populations to take advantage of those habitats. For example, *Hyblaea puera*, a moth pest of teak plantations, exhibits rapid population growth in the presence of new teak growth (Nair, 2007). However, some pest animals are less adaptable and have slow population growth. These animals are often considered pests on a local scale where humans consider any level of damage as a problem or where human activity interferes with natural population regulation (Nair, 2007). In addition, some species may be considered pests in some ecosystems, but not in others, depending on their role within that individual ecosystem and how that role impacts human activity. For example, rabbits are considered as pests in agricultural fields but are an important maintainer of flora on chalk downs (Putman, 1989). Fundamentally, when a species interferes with human activity it is labelled as a pest.

1.1.3. Damage caused by pests

Pest animals cause damage by a variety of means, and the scale of this damage can be considerable. Of particular concern are pests that damage human food supplies, threatening global food security. Pests may be primary consumers of growing crops, spread crop and livestock diseases and eat or contaminate stores of food stuffs (Oerke, 2006; Oliveira *et al.*, 2014; Pimentel *et al.*, 2005). Plagues of pest animals can wipe out huge amounts of food causing starvation in local human populations (Bebber *et al.*, 2013; Oerke, 2006; Oliveira *et al.*, 2014). Pests not only spread diseases to crops and livestock, but also carry and transmit many human pathogens (Meerburg *et al.*, 2009; Pimentel *et al.*, 2005). Pests cause physical damage to human infrastructure by damaging buildings and electrical cables (Ghaly & Edwards, 2011). For example, termites consume dry wood, reducing the structural integrity

of buildings (Ghaly & Edwards, 2011). In addition, invasive pest species can heavily impact ecosystems by damaging populations of indigenous species (Pimentel *et al.*, 2005). This is a particular concern in island habitats where invasive species can cause local or global extinction of rare and endangered species (Buckle & Fenn, 1992; Hilton & Cuthbert, 2010). The cost of the losses and damages associated with pest animals is vast, with estimates of $67 billion annually in the USA alone (Pimentel *et al.*, 2005).

1.1.4. Rodents as pests

Rodents are responsible for a high proportion of the overall costs associated with pests. Of the $67 billion cost of pests in the USA, $19 billion is attributable to rodents, in particular rats (*Rattus spp.;* (Pimentel *et al.*, 2005). Rodents are a particular threat to global food security (Singleton *et al.*, 1999). They consume or destroy food crops and food stores throughout the food chain (Lund, 1994). In Asia rodents consume 5-10% of the annual rice crop, enough to feed 180 million people (Singleton, 2003). In Bangladesh 12% of the annual wheat crop may be eaten (Lund, 1994). Up to 15% of stored crops in Turkey are destroyed by rodents and similar figures can be found for stores of maize, barley, rice and legumes throughout the world (Lund, 1994). As well as consuming food supplies, rodents also carry or transmit over 50 infectious diseases, including several that are a severe public health risk, such as bubonic plague, leptospirosis, hanta viruses and leishmania (Meerburg *et al.*, 2009). In addition, rodents are adept chewers, damaging human infrastructure by chewing through electrical cables, walls and doors (Pimentel *et al.*, 2005). Rodents have a high capacity for invasion into new habitats, often being transported by human activity (Clapperton, 2006). Once in these new habitats rodents often pose a significant threat to endemic species and have been associated with local extinctions, particularly in island habitats (Hilton & Cuthbert, 2010; Pimentel *et al.*, 2005). The level of rodent impacts on human activity and ecosystems has led to them being viewed as significant pests worldwide (Capizzi *et al.*, 2014).

Not all rodent species are considered as pests and some can be considered as pests in some environments but not in others. There are over 2000 known rodent species, but only 7% of these are counted as pests (Capizzi *et al.*, 2014). Globally, rodent species of most concern are the Black rat (*Rattus rattus*), the brown or Norway rat (*Rattus norvegicus*) and the house mouse (*Mus musculus*; (Capizzi *et al.*, 2014). Among the other important rodent pests are several additional species of rat, as well as voles and squirrels, some of which are highly important on a local scale (Capizzi *et al.*, 2014). For example, field voles (*Microtus agrestis*)

are considered as pests in forests in mainland Europe, but do not cause a problem in the UK (Capizzi *et al.*, 2014; Richards, 1989). Rodents that are not classed as pests play important roles in many ecosystems. Rodents act as seed dispersers for many plant species and some rodent species assist with pollination (Dickman, 1999; Xiao *et al.*, 2006). Many species prey on rodents and rely on them for a large proportion of their diet (Mukherjee *et al.*, 2004). In addition, many rodent species burrow and chew, altering the structure of the local environment and affecting habitat structure and plant diversity (Dickman, 1999). It is important that these roles are not forgotten, as consideration of the negative impacts of pest rodents has the potential to override the positive role of non-pest species.

Of the species of rodents that are considered as pests, many have adaptations that make them particularly likely to become pests and these have contributed to their global importance. As mentioned above, many rodent species have a high capacity to invade new habitats. Once in those habitats they easily become established due to their high adaptability and high reproductive capacity (Clapperton, 2006). Rodent adaptability applies to several aspects of their life history. Most species of rodents are omnivorous and can adapt to a range of diets depending on what is currently available (Buckle & Fenn, 1992). A high level of behavioural flexibility allows rodents to readily adapt to new ecosystems and cope with environmental change (Clapperton, 2006; Macdonald & Fenn, 1994). Many rodent species have a short gestation length, sizable litters and reach sexual maturity at a relatively young age, leading to a fast generation turnover that allows fast population growth and quick adaptation to environmental pressures (Macdonald & Fenn, 1994). This combination of traits has made rodents some of the most successful and devastating pest species worldwide (Capizzi *et al.*, 2014; Singleton, 2010).

1.2. Controlling pest animals

1.2.1. General pest control principles

There are a variety of options available to reduce the damage done by pest animals, including rodents. The two main options are to target the pest or to protect what the pest damages. Protecting vulnerable items from pest damage, such as food crops and infrastructure, can be effective in reducing pest damage (Linnie, 1994; Mason & Littin, 2003; Vincent *et al.*, 2003; Witmer, 2007). For example, fencing may be used to exclude flying insects, such as cabbage flies (*Delia radicum*), from vulnerable crops (Vincent *et al.*, 2003). However, this option is underutilised, often due to costs of implementation and maintenance (Linnie, 1994; Mason

& Littin, 2003; Vincent *et al.*, 2003). Targeting a pest can involve killing it, altering its behaviour or controlling its reproduction. Reproductive control has found some success in insect pests using techniques such as sterile male release (Alphey *et al.*, 2010; Vreysen *et al.*, 2000). Behavioural modifications have also been successful for insect pests, including repellents, attractants and other compounds that exploit intra-specific communication and can directly impact reproduction, such as oviposition inhibitors (Wallingford *et al.*, 2017; Witzgall *et al.*, 2010).

The option to kill pests can take several forms. The most commonly used option is to use a pesticide (Capizzi *et al.*, 2014; Williamson *et al.*, 2008; Wilson & Tisdell, 2001). Kill traps have been used for a variety of species, including insects and mammals, with varying degrees of success (Capizzi *et al.*, 2014; Cook *et al.*, 2007; Roomaney *et al.*, 2012). Release of biological predators and diseases has been attempted for many species, particularly insect pests, although there is concern that these released species can cause pest problems of their own (Bellows, 2001; Lacey *et al.*, 2001; Louda *et al.*, 2003). For example, parasites of the winter moth (*Operophtera brumata*) were introduced to Canada and effectively reduced the population of this hardwood forest pest (Bellows, 2001). However, the introduction of European ladybirds (*Coccinella Septempunctata*) to control wheat aphids in North America led to the decline of native ladybird species (Louda *et al.*, 2003).

New control strategies have found favour recently that combine many of the above types of control to improve efficacy and reduce some of the issues associated with individual control methods. For instance, integrated pest management attempts to combine pest control strategies in a manner specific to each individual pest control situation (Fitt, 2000; Singleton *et al.*, 2005). For example, to control cotton pests, pesticides have been integrated with intercropping to increase habitat diversity and transgenic cotton to resist pest damage (Fitt, 2000). Another option is push-pull control where repellents are combined with attractants to push pests away from vulnerable items, such as crops, and pull them towards control measures, such as traps (Cook *et al.*, 2007). This control method has been trialled for many insect pests, including the Colorado potato beetle (*Leptinotarsa decemlineata*) that can be repelled from potato plants using an antifeedant, whilst simultaneously attracting it towards a trap crop using host plant volatile compounds (Cook *et al.*, 2007). Due to the variety of pest control options available, there are many options for combining measures to improve efficacy, many of which are yet to be explored.

1.2.2. Rodent pest control

Pest control aimed at rodents has followed many of the trends of pest control aimed at other species. As with other pest control, rodent pest control is heavily reliant on poisons as the mainstay of control (Capizzi *et al.*, 2014). Several other options are available. Traps have been trialled in a variety of settings to control rodents, but they are labour intensive to implement and are susceptible to the development of behavioural resistance (Barbosa *et al.*, 2013; Clapperton, 2006; Wang *et al.*, 2017). Behavioural resistance is a particular problem for rodents, due to their intelligence, behavioural flexibility and, especially in rats, a high level of neophobia in relation to control devices and poisons (Clapperton, 2006; Macdonald & Fenn, 1994). Exclusion of rodents by blocking off entry points in stores and housing can be very effective against rodents, but is often underutilised (Mason & Littin, 2003). Integrated pest management, combining a variety of control techniques, has been shown to be cheaper and more effective than using one method alone (Singleton *et al.*, 2005; Witmer, 2007). However, poisons remain the main control method for most rodent pest species (Capizzi *et al.*, 2014).

There are several types of poison available to control rodents, but the most commonly used are the anticoagulant rodenticides (Capizzi *et al.*, 2014; Memmott *et al.*, 2017). The anticoagulant rodenticides were developed in the 1940s, following observations that warfarin was toxic to rodents, and proved to be highly successful (Hadler & Buckle, 1992). However, over the next few decades both behavioural and physiological resistance led to modification of these poisons to create the second generation anticoagulant rodenticides (SGARs; Hadler & Shadbolt, 1975). These poisons were more potent and bioaccumulative than the original anticoagulants, meaning that rodents only had to consume a small amount of them to cause fatality (Erickson & Urban, 2004; Hadler & Buckle, 1992; Vandenbroucke *et al.*, 2008). All anticoagulant rodenticides kill by interfering with blood clotting, causing death from uncontrolled bleeding (Hadler & Buckle, 1992). Other rodent poisons interfere with calcium metabolism, cause cytotoxicity or lead to an anaesthetic overdose (Mason & Littin, 2003). These poisons have been used in a variety of different settings and can be highly effective (Mason & Littin, 2003). However, anticoagulant rodenticides are the most popular rodent poison (Capizzi *et al.*, 2014; Dawson *et al.*, 2001). A global study of scientific literature and case studies found that 61% of poison control involved anticoagulant use (Capizzi *et al.*, 2014). A report on rodenticide use in UK farms found that SGARs accounted for 91% of poison use (Dawson *et al.*, 2001). In addition, 80% of pest controllers in Massachusetts, USA

used SGARs rather than other rodenticides (Memmott *et al.*, 2017). Thus, there are a variety of poisons available for the control of rodents, but there is heavy reliance on the anticoagulant rodenticides.

1.2.3. Problems with pesticides

The global reliance on pesticides to control pest animals, including rodents, has led to several problems. Several studies have shown that pesticides are the mainstay of control for most pest animal groups, including insects and rodents (Capizzi *et al.*, 2014; Williamson *et al.*, 2008; Wilson & Tisdell, 2001). There are many reasons for the reliance on pesticides, including lack of available alternatives and human behaviour in relation to their application (Health and Safety Executive, 2012; Wilson & Tisdell, 2001). This reliance is despite the many problems associated with pesticide use (Wilson & Tisdell, 2001). One of the main problems with pesticides is that many animals are becoming resistant to them. This resistance can be physiological or behavioural (Clapperton, 2006; Macdonald & Fenn, 1994). Behavioural resistance causes pests to avoid ingestion of poisons, whilst physiological resistance prevents poisons working once they are ingested. Both types of resistance can render a pesticide effectively useless and are very costly. For example, physiological resistance to pesticides in budworms led to the loss of over a quarter of a million hectares of cotton in Mexico (Pimentel, 2005). The total cost of pesticide resistance has been estimated at $1.5 billion annually in the USA (Pimentel, 2005). Another serious problem with pesticides is their effects on non-target species. Most pesticides are not species specific and can kill or harm large numbers of species that are not the target of pest control (Devine & Furlong, 2007). Non-target poisoning can have devastating impacts on ecosystems, including damaging species that are important to humans, such as pollinators (Pimentel, 2005). As pesticides are used so extensively to control pests, these negative effects are a serious concern.

Rodent poisons carry similar problems to other animal pesticides, which has led to wide ranging impacts. As described above, rodents have a high degree of behavioural flexibility and can often be highly neophobic, especially in relation to ingestion of poisons and approaching traps (Clapperton, 2006). This has led to the widespread development of behavioural resistance to poisons. As well as avoiding consumption of novel food items, rodents, especially rats, will often taste small amounts of a novel food and not consume that food again if they become unwell after eating it (Gentle *et al.*, 2006). This has obvious implications for poison use, where rodents may suffer negative effects relatively quickly after

consumption of small amounts of bait (Gentle *et al.*, 2006). In addition, poison baits are often placed in environments where there are ample alternative food sources, such as grain stores. In these situations, the incentive for rodents to consume poisons can be very low indeed (Witmer, 2007). Of grave concern for the use of rodent poisons is their effect on non-target species. No rodent poisons are species specific (Mason & Littin, 2003). In fact, the most commonly used poisons, the anticoagulant rodenticides, are toxic to all mammals and birds (Hadler & Buckle, 1992). The levels of exposure to rodent poisons in non-target species can be extremely high and are a major cause for concern (Health and Safety Executive, 2012; Walker *et al.*, 2013).

1.2.4. Non-target poisoning

In general, non-target poisoning by pesticides can affect many species and cause a wide range of issues. Non-target species may be poisoned by direct consumption of poisons, termed primary poisoning. Alternatively, predators may be poisoned when they consume animals that contain poison residues, termed secondary poisoning (Buckle & Fenn, 1992; Colvin *et al.*, 1988). As stated above, most species are not pests and have highly beneficial roles to play. Non-target poisoning can damage populations of many of these beneficial species (Pimentel, 2005). This can include species essential for human food production, such as pollinating bees and other insects (Pimentel, 2005). Fish stocks can be damaged when pesticides run-off into water courses (Devine & Furlong, 2007; Pimentel, 2005). In addition, predators of pests are often killed by pesticides, leading to an increase in the need for pesticide use (Devine & Furlong, 2007). In island habitats, where invasive pests are often targeted by intensive control operations, non-target poisoning can be a major risk for endangered species (Hoare & Hare, 2006; Holmes *et al.*, 2015). In less precarious habitats, non-target poisoning of wildlife can be found widely, particularly in predators who are vulnerable to pesticides that accumulate as they pass up the food chain, such as birds of prey (Walker *et al.*, 2013). The combined annual cost of non-target poisoning has been estimated at $3 billion in the USA alone, calculated as a combination of losses due to poisoning of birds, bees, fish, livestock and natural pest predators (Pimentel, 2005). Consequently, the non-target effects of pesticides are a serious cause for concern.

1.2.5. Non-target poisoning in rat control

Non-target poisoning is a particular problem for rodent control, especially control aimed at rats. The global reliance on poisons to control rats has led to non-target poisoning of many species. In European countries, the most commonly poisoned non-target species are non-target rodents, such as wood mice (*Apodemus sylvaticus*), bank voles (*Myodes glareolus*) and field voles (*Microtus agrestis*; (Brakes & Smith, 2005; Geduhn *et al.*, 2014). In addition, insects, molluscs and birds will consume rat poisons (Brooke *et al.*, 2013; Elliott *et al.*, 2013; Hernandez-Moreno *et al.*, 2013; Hoare & Hare, 2006). Accumulation of poisons in the tissues of these primary consumers is particularly a problem for the most commonly used rat poisons, the SGARs. As stated above, to overcome resistance problems these poisons were particularly designed to be highly toxic to be effective as single feedants (Erickson & Urban, 2004; Hadler & Buckle, 1992). They are also bioaccumulative, leading to high levels of exposure in species that predate on rodents, including birds of prey, mustelids and foxes (Elmeros *et al.*, 2011; Geduhn *et al.*, 2015; McDonald *et al.*, 1998; Thomas *et al.*, 2011; Walker *et al.*, 2013). In addition, insectivores, such as hedgehogs, and avivores, such as sparrow hawks, have been found to contain rodenticide residues (Dowding *et al.*, 2010; Hughes *et al.*, 2013). The scale of non-target exposure due to rat poisons is huge. Residues of rat poisons have been found in over 75% of red kites, barn owls and red foxes in the UK (Shore *et al.*, 2016; Tosh *et al.*, 2011a; Walker *et al.*, 2016). Other countries, including Denmark, Germany and the USA, have also recorded high rates of rat poison residues in these species, as well as stoats, weasels, and bobcats (Elmeros *et al.*, 2011; Geduhn *et al.*, 2015; Geduhn *et al.*, 2016; Serieys *et al.*, 2015). The high percentages of exposure rates in these cases have led to non-target poisoning by rat poisons being considered a major issue globally and has led to their continued use being questioned in the UK and across Europe (Colvin *et al.*, 1988; Health and Safety Executive, 2012; Hoare & Hare, 2006; Walker *et al.*, 2013).

1.2.6. Rat poison effects on non-target species

Non-target species can be affected by rat poisons in a variety of ways. Of primary concern are fatalities associated with primary or secondary exposure to rat poisons. These fatalities can affect population sizes in affected species (Brakes & Smith, 2005; Eason *et al.*, 2002; Nogeire *et al.*, 2015). However, sublethal doses can also cause serious effects. Residues of rat poisons have been associated with poor body condition, particularly in young animals, including kestrel fledglings and mustelids (Elmeros *et al.*, 2011; Martinez-Padilla *et al.*, 2017). An increase in disease susceptibility has been correlated with rat poison exposure in some

species. For example, voles exposed to rat poisons in Spain had a high incidence of the pathogen *Francisella tularensis* (Vidal *et al.*, 2009). In addition, parasite burdens of bobcats have been associated with residues of rat poisons (Serieys *et al.*, 2015). Another concern is that rat poisons could affect the reproductive capacity of non-target rodents. There was some suggestion that the breeding output of New Zealand Moreporks was affected by exposure to rat poisons (Stephenson *et al.*, 1999). This issue may be compounded by the potential for some rat poisons to transfer from mother to foetus during pregnancy, as was found in a case involving bobcats (Serieys *et al.*, 2015). For many species, the dose of rat poison required to cause pathology is unknown (Eason *et al.*, 2002; Giraudoux *et al.*, 2006). Therefore, it is not currently possible to determine the extent of damage done by rat poisons to non-target wildlife, but it is likely to be extensive due to the potential for both fatal and non-fatal effects.

1.3. How to reduce the impact of rodenticides on non-target wildlife

1.3.1. Current options

To date, several options have been suggested to reduce the exposure of non-target wildlife to rodenticides. The most complete solution would be to ban the use of rat poisons, particularly the anticoagulant rodenticides and other bioaccumulative poisons, as these are responsible for the majority of non-target poisoning (Erickson & Urban, 2004; Walker *et al.*, 2013). Unfortunately, the efficacy of currently available alternative control methods is not high enough to replace poisons, so banning them is not considered to be a viable option at present (Health and Safety Executive, 2012). In addition, the pest control industry is heavily reliant on the use of poisons and has not invested in the development of new, alternative options (Capizzi *et al.*, 2014; Jackson, 2001). Therefore, poison use has been allowed to continue (Health and Safety Executive, 2012). Species specific control measures, such as reproductive control and sterilisation have been suggested as tools to reduce non-target exposure (Liu *et al.*, 2012; Prowse *et al.*, 2017; Tran & Hinds, 2013). However, there have been difficulties in developing these tools for use in rodents due to a lack of long lasting effects (Tran & Hinds, 2013). In addition, there is controversy over the use of mechanisms, such as gene drives, to spread lethal or infertility genes through a pest population, with concerns that these genes could be transferred to non-pest populations (Prowse *et al.*, 2017; Webber *et al.*, 2015).

Another way to reduce the exposure of non-target wildlife to rat poisons is to restrict the use of these compounds, particularly in outdoor and rural environments, where non-target poisoning is more common (Health and Safety Executive, 2012). These restrictions can include banning placement of poisons away from buildings, restrictions on the time poisons can be left in the environment, and regular inspections to remove poisoned carcasses (Campaign for Responsible Rodenticide Use UK, 2015; Health and Safety Executive, 2015). Such restrictions have been implemented in some countries, such as Denmark, however there are concerns about their effectiveness due to a lack of compliance and some emerging evidence that restrictions are not as effective as hoped at reducing non-target exposure (Campaign for Responsible Rodenticide Use UK, 2015; Elmeros *et al.*, 2018; Memmott *et al.*, 2017; Tosh *et al.*, 2011b).

Further options to reduce non-target poisoning have focused on restricting access of non-target animals to rat poisons. A standard method of reducing non-target exposure to rodenticides is to confine rat poisons within secure bait boxes (Campaign for Responsible Rodenticide Use UK, 2015; Clapperton, 2006). Bait boxes are effective at reducing access to rodenticides by large non-target species, such as domestic pets, but they cannot prevent access by small non-target rodents, as entrance holes have to be big enough to allow access by rats (Campaign for Responsible Rodenticide Use UK, 2015; Hadler & Buckle, 1992). In island habitats, where poisoning of non-target species can be particularly devastating, there have been attempts to protect particularly vulnerable species by either taking them into captivity during rodent control operations, or by providing vitamin K supplements to non-target species to prevent the effects of anticoagulant rodenticides (Campbell *et al.*, 2015; Olivera *et al.*, 2010). Whilst these methods have found some success, they are not appropriate for use in general pest control settings. Therefore, new methods are needed to reduce the problem of non-target exposure to rodenticides.

1.3.2. Using repellents and attractants

A novel method to reduce exposure of non-target rodents to rodenticides is to use cues that repel non-target rodents from poisons whilst attracting, or at least not repelling, target rats. The idea is to increase the species specificity, and potentially the efficacy, of current rat poisons without the need to modify existing control methods. There are three main ways in which cues could work to achieve this. An ideal cue would repel non-target species from rat poisons, whilst attracting target rats. This would allow continued use of current rat poisons,

by increasing their attractive potential to rats and reducing their consumption by non-target species. A less ideal, but still effective cue would repel non-target rodents and neither attract, nor repel rats. Such a cue would increase the species specificity of rat poisons, reducing non-target exposure, but would not have the added benefit of increasing attraction to rats. Another option is a cue that does not repel non-target species, but does attract rats. Such a cue could be used to increase the attractiveness of control methods, such as traps, that do not rely on poisons. This would reduce exposure of non-target species to rat poisons by removing the need for use of poisons altogether, provided such a cue did not attract non-target species. A cue, or combination of cues, that fulfil any of these options, has the potential to reduce the exposure of non-target rodents to rodenticides.

1.3.3. The nature of repulsion and attraction

In order to find a cue that fits one of the three options outlined above, an understanding is needed of the nature of attraction and repulsion and how that may differ between rodent species. Animals rely on many senses to determine which objects in their environment are desirable, and which pose a threat (Burn, 2008). Desirable objects are often attractive to animals, but the nature of attraction can vary with the location of the object and how desirable it is. For example, a valuable food source may be highly attractive, but this attractive potential may be decreased if the food is located in a difficult to reach place, or if it is located in close vicinity to a predator (Sulikowski, 2017). There is a constant trade off of benefits gained from obtaining a desirable item, with the costs associated with obtaining it (Sulikowski, 2017; Zylberberg & DeWeese, 2011). Of particular interest for developing attractants and repellents is how this cost/benefit evaluation affects an animal's willingness to perform certain behaviours, especially those relating to movement and food consumption. Movement behaviours of interest include willingness to enter a particular area, willingness to enter a confined space and willingness to spend time in a particular location once it has been entered. Food related behaviours relate to a willingness to consume a food item, and how much to consume. A combination of these effects may also be desirable. For instance, to encourage a rat to consume more poison, an ideal attractant would attract a rat to enter an area where the poison is present, spend time in that location and encourage the rat to consume sufficient poison. The distance over which attractive and repellent cues are required to be effective may vary depending on the intended use of the cue. For instance, it may be desirable for an attractant to work over large distances to draw pests towards control measures, for example, to attract insect pests towards traps, away

from vulnerable food crops (Eigenbrode *et al.*, 2016). However, this may not always be desirable, as repellents may only need to work over short distances to push animals away from a poison source without affecting their normal behaviour. Distance of effectiveness, and the nature of attraction and repulsion produced by a cue, should all be considered when assessing the potential of a cue as a non-target repellent or target attractant.

1.3.4. Differences between target and non-target rodents

The starting point in the search for cues that could reduce the exposure of non-target rodents to rat poisons is to identify behavioural and ecological differences between target and non-target rodents. Cues can then be identified that exploit these differences, particularly cues that differ in their attractiveness or repulsiveness between species. The most obvious difference between rats and non-target rodents is size. Rats are larger than non-target rodents (Hansson, 1971; Yin *et al.*, 2011). To use this difference to reduce exposure of non-target rodents to rat poison, it would be necessary to build a poison box that only allowed entry of larger, heavy rats. An elevated bait station was developed for this purpose on Anacapa Island, which allowed access of invasive roof rats, but excluded native deer mice (Erickson *et al.*, 1990). The bait station succeeded in only allowing roof rats to access poisoned bait, but it was not known if effective rat control was possible using such a bait station. Given the highly neophobic nature of rats in relation to existing poison boxes (Clapperton, 2006), further complications of box design may increase bait avoidance in rats. Other differences between rats and non-target rodents include differences in ecology, including diet and habitat preferences. Such differences have been used to deter birds from eating rat poisons, such as taste and colour preferences for food (Clapperton *et al.*, 2012; Day *et al.*, 2003). However, the differences observed between rats and non-target rodents are more subtle and they tend to be attracted and repelled by similar compounds (Hansen *et al.*, 2015; Hansen *et al.*, 2016). Thus, size, diet and habitat differences between rat and non-target rodents may not be good options when looking for cues that may induce differential behavioural responses.

1.3.5. Predator prey relationships

A difference between rats and non-target rodents that may induce differential responses is the predator prey relationship between these species. Rats are known predators of house mice, particularly when they are food deprived (Bridgman *et al.*, 2013; O'Boyle, 1974; Paul *et al.*, 1971). The potential for a predator prey relationship between rats and non-target

rodents has not been studied. However, it may be expected that as non-target rodents, such as wood mice, bank voles and field voles, have similar vulnerabilities to house mice, they may be preyed upon by rats. Even if rats do not commonly prey on non-target rodents, they are likely to be potentially dangerous heterospecific competitors. In order to avoid predation, prey animals will avoid cues that indicate the presence of a predator (Apfelbach *et al.*, 2005; Hettena *et al.*, 2014). As rats are potential predators or heterospecific competitors of non-target rodents, it would be expected that non-target rodents may avoid cues that indicate rat presence.

1.3.6. Touch and sight

Cues that indicate predator presence can include touch and visual cues. Rodents are highly sensitive to touch, using their whiskers to sense vibrations in the environment (Burn, 2008; Diamond & Arabzadeh, 2013). These vibrations may be used to detect predator presence (Diamond & Arabzadeh, 2013). Other specialist touch sensors, such as the antennae of crickets, are capable of sensing differences in predatory species (Okada & Akamine, 2012). However, it is difficult to imagine that the difference between the vibrations generated by a rat and another predator would be significant enough to repel non-target rodents without repelling rats. Visual cues are used by some rodents to indicate predator presence, such as looming visual stimuli that replicate the threat of an aerial predator (Yilmaz & Meister, 2013). As rats are ground based predators, non-target rodents may not use visual cues as an indication of rat presence. In addition, it would be difficult to create a visual stimulus that was repellent to non-target rodents, but not to rats. Therefore, touch and visual cues are unlikely to be useful as non-target repellents.

1.3.7. Rat odour as a non-target repellent

A cue that indicates predator presence that is distinct to rats, and thus may cause differential responses between rats and non-target rodents, is scent. Both predator and prey species deposit scent cues in the environment, either deliberately to communicate with conspecifics or as a by-product of metabolism (Parsons *et al.*, 2018). Heterospecifics may then use these scents as a warning that a predator is present (Apfelbach *et al.*, 2005; Ferrero & Liberles, 2010). Responses to predator odours include avoidance, changes in activity patterns, habitat shifts and reductions in non-defensive behaviours, such as foraging (Apfelbach *et al.*, 2005; Parsons *et al.*, 2018). All of these responses could be useful for a non-target repellent. There is evidence that wood mice and water voles (*Arvicola amphibious*) avoid feeding from

stations scented with rat odour (Barreto & Macdonald, 1999; Richards, 2012). In addition, rats are attracted to the odour of other rats, although there may be sex differences in responses (Christiansen, 1976; Selvaraj & Archunan, 2006; Takács et al., 2016a). Therefore, rat odour has the potential to function as a non-target repellent, and possible rat attractant.

To assess the potential of rat odour as a non-target repellent, an understanding of odour biology and predator odour responses is needed. In rodents, odour cues are mainly sensed by the main olfactory epithelium and the vomeronasal organ (Ferrero & Liberles, 2010; Li & Liberles, 2015). From these organs, signals are projected to the brain, triggering behavioural responses (Buck, 2000; Li & Liberles, 2015). These responses can be innate, automatic responses, or they may be learned responses associated with past experiences of predatory threat (Ferrari *et al.*, 2007; Karlson & Luscher, 1959; Wyatt, 2010). Odour cues that generate specific, innate responses during intra-specific communication are termed pheromones (Karlson & Luscher, 1959; Wyatt, 2010). Specific innate responses may also be generated during inter-specific communication (Ferrero & Liberles, 2010). Where these responses are to the benefit of the recipient, these odour cues are termed kairomones (Ferrero & Liberles, 2010). Kairomones include predator odours that cause innate avoidance of predators, to the benefit of prey species (Ferrero & Liberles, 2010). The responses of prey species to predator odours have been found in diverse taxa, including invertebrates, birds and reptiles and fish, but have been most extensively studied in mammals (Amo *et al.*, 2008; Armsworth *et al.*, 2005; Barnes *et al.*, 2002; Kelley & Magurran, 2003; Lloyd *et al.*, 2009). Thus, predator odours are capable of triggering innate responses in prey species and these responses have been widely studied.

Predators produce multiple odour cues that can be used by prey animals to indicate predator presence (Apfelbach *et al.*, 2005; Parsons *et al.*, 2018). The origin of an odour cue can affect the response of a prey animal to that cue (Apfelbach *et al.*, 2005). This may be due to what that odour indicates about how likely a predator is to be present. For example, urine and faeces may have been deposited by a predator sometime in the past, but body odour may indicate more likely recent predator presence, due to differences in persistence of odour cues (Blanchard *et al.*, 2003). Prey animals are more likely to respond to cues that indicate recent predator presence, as this indicates a higher potential for contact with the predator (Blanchard *et al.*, 2003; Lima & Bednekoff, 1999). This is illustrated well in the response of rats to cat odour. Rats consistently avoid cat body odour, which indicates recent predator presence, but often fail to avoid cat urine, which may have been deposited sometime in the

past (Bramley & Waas, 2001; Fendt, 2006; McGregor *et al.*, 2002; Wright *et al.*, 2013). Therefore, odour source may be important in the response of non-target rodents to rat odour.

Other considerations for use of predator odours as repellents concern habituation and response variability. Habituation is a decrease in response to a stimulus occurring after repeated exposure to that stimulus (Harris, 1943). Habituation to predator odour has been shown in some studies (Dielenberg & McGregor, 1999; Hegab *et al.*, 2014) and could reduce the usefulness of predator odours as non-target repellents. Repeated testing is needed to ensure that habituation is not likely to occur. Responses to predatory odours by prey species have varied between experiments (Apfelbach *et al.*, 2005; Parsons *et al.*, 2018). This may be due to a variation in experimental design, strength of odour cue used, or the measures assessed (Apfelbach *et al.*, 2005; Parsons *et al.*, 2018). Therefore, repeated testing using a variety of experimental designs will be needed to fully assess the potential of rat odour as a non-target repellent.

1.3.8. Use of scents in pest control

There is evidence to indicate that scent cues exploiting intra and inter-specific communication could be used as effective repellents for use in pest control settings. Most of the work using scents for the purposes of pest control has focused on insects. There have been several successful examples of use of both repellents and attractants to control insect pests (Cook *et al.*, 2007; Witzgall *et al.*, 2010). There is research evidence to suggest that scent cues, including predator odours and pheromones, could be exploited for use in mammalian pest control (Apfelbach *et al.*, 2005; Calder & Gorman, 1991; Sullivan *et al.*, 1988; Takács *et al.*, 2016a; Woolhouse & Morgan, 1995). However, very few of these trials has led to commercialised pest control products (Clapperton *et al.*, 2017). From the work done with insect pests, several benefits have emerged from using scent cues as repellents. First, scent cues often have species specific effects, reducing the effect of control measures on non-target species (Witzgall *et al.*, 2010). For example, a pheromone specific to palm weevils has been used to attract these pests to traps (Witzgall *et al.*, 2010). Second, small quantities of scent cues are often effective, reducing costs of application, although overall discovery and production costs can be high (Clapperton *et al.*, 2017; Cook *et al.*, 2007; Witzgall *et al.*, 2010; Wyatt, 2014). Finally, as scents used for pest control exploit species communication channels, pest species need to evolve alternative communication channels to develop

resistance to a scent cue (Witzgall *et al.*, 2010). Therefore, resistance may be slow to develop to scent cues, particularly if multiple cues are used synergistically (Cook *et al.*, 2007; Witzgall *et al.*, 2010). These factors make scents highly appealing as potential non-target repellents. This, combined with existing evidence of non-target repulsion, indicates that there is good reason to investigate the non-target repellent potential of rat odour.

1.3.9. Rat calls as a non-target repellent

Another cue that may indicate rat presence, and so may cause differential responses between rats and non-target rodents, is rat calls. Rodents use a wide variety of calls for conspecific communication (Sales, 2010). The frequency of these calls extends from human hearing range into the ultrasonic range (Sales, 2010). Wood mice, bank voles and field voles all produce ultrasonic calls, although the role of these calls in conspecific communication has not been fully established in these species (Kapusta & Kruczek, 2016; Sales, 2010). These calls are produced in the same ultrasonic range as rats, indicating that it is likely they will be able to hear rat calls (Sales, 2010).

Rats produce a variety of calls in the ultrasonic range and these calls have been characterised into three subtypes (Panksepp & Burgdorf, 2000). The first subtype is produced by infant rats when separated from their mother and litter mates (Zippelius & Schleidt, 1956). These calls are produced at a range of frequencies, particularly when body temperature decreases, and direct maternal behaviour to return pups to the nest (Brunelli *et al.*, 1994; Takács *et al.*, 2016b; White *et al.*, 1992). The second subtype is an alarm call, produced at around 22 kHz (Blanchard *et al.*, 1991; Litvin *et al.*, 2007). The third subtype are prosocial calls. These calls are produced at around 50 kHz and are thought to have a variety of functions, including social cohesion, de-escalation of aggressive encounters and as a signal of positive emotions (affect) (Brudzynski, 2013; Burke *et al.*, 2017; Panksepp & Burgdorf, 2000).

Prosocial, 50 kHz calls attract rats (Sadananda *et al.*, 2008; Seffer *et al.*, 2014; Wöhr & Schwarting, 2007), making them potentially useful as an attractant for the purposes of pest control, although they have not been tested for this purpose. Again, exploiting the predator prey (or dominant competitor) relationship between rats and non-target rodents, 50 kHz rat calls may repel non-target rodents as a signal of a predator. Prey species are known to avoid the calls of their predators (Hettena *et al.*, 2014). However, there are no published tests of the response of non-target rodents to 50 kHz rat calls. The attractive potential of these calls

to other rats and the concept that they could be repellent to non-target rodents warrants further testing.

1.3.10. Monitoring rodents

As well as developing non-target repellents, reducing the exposure of non-target rodents to rodenticides requires rodents to be monitored. Monitoring of pest populations is a key part of control strategies for all pest animals. Before the start of pest control operations, monitoring allows an assessment of population size and distribution to gauge the level of control needed and where it should be targeted (Campbell *et al.*, 2002). During pest control operations, monitoring allows progress to be tracked to ensure selected control measures are effective (Campbell *et al.*, 2002). After pest control has ceased, monitoring ensures control has been successful and provides an early warning of reinvasion (Campbell *et al.*, 2002). Monitoring technologies are also vital in other areas, including tracking populations of endangered and cryptic species for conservation and for ecological and behavioural research of free living animals (Barbosa *et al.*, 2013; Galan *et al.*, 2012; Witmer, 2007). To reduce the exposure of non-target rodents to rodenticides, monitoring is needed to assess if non-target rodents are present in an area prior to the start of rat control. This would allow targeted use of any non-target repellent, or other approaches to reduce non-target exposure where this is required (as these approaches may increase costs). In addition, in conditions where non-target species are causing a pest issue, for example bank voles in forestry (Capizzi *et al.*, 2014), identification of species involved would ensure that non-target repellents are not used inappropriately. Another role for monitoring is to assess population sizes of non-target rodents at the end of rat control campaigns. This would allow the magnitude of effect that rat control has on non-target rodents to be calculated.

1.3.11. Current monitoring options

A good monitoring technique for rodents should encompass several factors. Ideal methods should be cheap, provide rapid identification, accurately identify species and utilise samples that are easy and non-invasive to obtain (Barbosa *et al.*, 2013; Engeman & Witmer, 2000). Many current monitoring techniques require identification of live animals, but these have many drawbacks. Identification of live animals has been attempted by live trapping, photography and tracking boards (Galan *et al.*, 2012; Glen & Dickman, 2003; Nelson *et al.*, 2002; Whisson *et al.*, 2005; Yu *et al.*, 2013). These methods are very labour intensive and require expert identification to distinguish species (Barbosa *et al.*, 2013; Galan *et al.*, 2012).

In addition, trapping will only sample the subset of the population that are willing to enter traps (Stoddart, 1982). Another live tracking method is non-toxic bait blocks. These blocks are often used by pest controllers to locate populations of pest rodents (Nelson *et al.*, 2002; Whisson *et al.*, 2005). Non-toxic bait blocks are simple to use, but do not provide any information on which species have consumed them (Whisson *et al.*, 2005). A viable alternative to live animal identification is to use faecal samples. Collection of faeces is less labour intensive than methods to detect live animals (Barbosa *et al.*, 2013). In addition, faecal samples may be the only material available to identify cryptic species (Barbosa *et al.*, 2013; Witmer, 2005). Current species identification from faecal samples relies on analysis of macroscopic remains or DNA analysis (Taberlet & Fumagalli, 1996; Waits & Paetkau, 2005). These methods can be protracted and time consuming and require fresh faecal samples for accurate identification (Barbosa *et al.*, 2013; Reed *et al.*, 1997; Taberlet & Fumagalli, 1996; Waits & Paetkau, 2005). A method that utilised faecal samples, but was able to rapidly analyse both fresh and aged samples would have good potential to be used as a monitoring method for rodents during pest control.

1.3.12. REIMS as a monitoring method

One potential method to rapidly identify rodent species from faecal samples is rapid evaporative ionisation mass spectrometry (REIMS). REIMS is a new technique that originated as a tool to identify cancerous tissue during surgery (Schaefer *et al.*, 2009). To obtain information on samples by REIMS, samples are heated using a high-frequency electrical current (Balog *et al.*, 2010). The heat generated effectively burns the material, creating an aerosol of molecules, which are drawn in to a mass spectrometer by an air pump (Balog *et al.*, 2010). The spectra produced by this process are characteristic of the original biological tissue (Balog *et al.*, 2010; Balog *et al.*, 2013). Further development of REIMS has led to its use in food security to identify meat and fish samples and in microbiology for the rapid identification of fungal and bacterial colonies (Balog *et al.*, 2016; Black *et al.*, 2017; Bolt *et al.*, 2016; Cameron *et al.*, 2016; Strittmatter *et al.*, 2013). So far, REIMS has not been tested on faeces, despite the potential for rapid identification of samples. Another benefit of REIMS relevant to faecal identification is that little or no sample preparation is required prior to testing, increasing the speed of analysis (Balog *et al.*, 2010; Cameron *et al.*, 2016). Therefore, there are sufficient indications that REIMS has the potential to identify rodent species from faecal samples to support further investigation.

1.4. Thesis objectives

There are three main objectives to this thesis.

1. To assess the potential of rat odour to reduce the exposure of non-target rodents to rat poisons, and identify effective odour components that could be used as non-target repellents.
2. To assess the potential of 50 kHz rat calls to reduce the exposure of non-target rodents to rat poisons.
3. To assess if REIMS has the potential to be used as a monitoring method for rodents, with the purpose of assessing what species of non-target rodent are present during rat control campaigns.

1.5. Thesis structure and chapter outlines

The format of this thesis is as a series of independent papers, followed by an overall discussion. The work documented in this thesis is my own, except where clearly attributed otherwise.

Chapter 2: Male rat odour repels non-target rodents

As stated above, previous studies have indicated that rat odour may be repellent to non-target rodents. In Chapter 2 I examined the response of three non-target rodent species, wood mice, bank voles and field voles, to male rat odour by testing them in laboratory arenas and semi natural enclosures. These experiments combined multiple sources of rat odour, including body odour and faeces. I next addressed if the response of wood mice to rat body odour was the same as to rat faeces. In addition, I assessed the spatial extent of responses to rat odour. Finally, to ensure that male rat odour is not repellent to target rats, I tested the response of rats of both sexes to male rat odour. This Chapter also establishes laboratory and semi-natural enclosure bioassays that are used in subsequent chapters.

Chapter 3: The response of non-target rodents to female rat odour and 4-ethyl phenol

Following on from the results of Chapter 2, in Chapter 3 I examined the response of bank voles and wood mice to female rat odour with the aim of establishing if non-target rodents are repelled by sex-specific rat odour cues or generic species cues. For these experiments I used bioassays developed in Chapter 2. To identify compounds that may be responsible for

the behavioural responses I observed in non-target rodents, I then began analysis of the volatile compounds in rat odour. This analysis identified the most abundant volatile odour compound in rat odour. This compound was tested with bank voles and wood mice to determine if it produced the same response as whole rat odour.

Chapter 4: Field trials of rat odour as a non-target repellent

Having established that rat odour is repellent to non-target rodents in laboratory arenas and semi-natural enclosures, I next tested the response of non-target rodents to rat odour in natural conditions. In a series of field trials, I assessed the response of non-target rodents to rat odour in a variety of settings. To assess if rat odour affected willingness of non-target rodents to enter a confined space, I examined the number of non-target rodents caught in live, rat odoured traps. To assess if rat odour affected the willingness of non-target rodents to feed from bait boxes, I examined the weight of non-toxic bait consumed by non-target rodents from rat odoured bait boxes. In addition, I examined the response of non-target rodents to two different sizes of rat odoured bait boxes, to determine if context of odour presentation affects behavioural responses to that odour.

Chapter 5: Testing the potential of 50 kHz rat calls as a species specific rat attractant

As stated above, previous research has indicated the attractive potential of 50 kHz rat vocalisations to rats. However, several aspects of this attraction have not been established, nor has the effect of 50 kHz rat calls on non-target rodents been tested. In this chapter I tested the response of rats and bank voles to 50 kHz rat calls. I particular I assessed if the response of rats to 50 kHz calls is sex specific and if these calls were able to attract rats inside bait boxes. In addition, I tested if prior familiarity with calling rats is required for listening rats to respond to 50 kHz calls. Finally, I assessed the response of bank voles to 50 kHz rat calls.

Chapter 6: Species identification by novel mass spectrometric analysis of rodent faeces

Monitoring of rodent species is essential to assess the impact of rat poisons on their abundance. It could also play a vital role in reducing non-target poisoning by allowing targeted use of non-target repellents and indicating what restrictions should be used prior to placement of rat poisons. I assessed the potential of REIMS to identify rodent species using faecal samples as a non-invasive monitoring method. This technology was tested with the aim of establishing a rapid throughput monitoring method that could be used for rodents and other species. I tested the classification accuracy of REIMS to identify wild and laboratory

housed rodent species. In addition, I examined the effects of subject diet and sample storage on REIMS classification accuracy.

Chapter 7: Discussion

In the overall discussion I summarise the results of Chapters 2 to 6. I then discuss the benefits and issues associated with the use of rat odour and 50 kHz rat calls as methods to reduce the exposure of non-target rodents to rat poisons and the use of REIMS to identify rodent species. I then discuss when and how each of these measures could be implemented, including legislative and cost considerations. I finish with a discussion of future work required to further develop these measures for use in a commercial setting.

1.6. References

Alphey, L., Benedict, M., Bellini, R., Clark, G. G., Dame, D. A., Service, M. W. & Dobson, S. L. (2010). Sterile-Insect Methods for Control of Mosquito-Borne Diseases: An Analysis. *Vector-Borne and Zoonotic Diseases.* **10**, 295-311.

Amo, L., Galván, I., Tomás, G. & Sanz, J. (2008). Predator odour recognition and avoidance in a songbird. *Functional Ecology.* **22**, 289-293.

Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A. & McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neuroscience & Biobehavioral Reviews.* **29**, 1123-1144.

Arluke, A. & Sanders, C. R. (1996). The Sociozoologic Scale. In *Regarding Animals.* pp. 167-186. Temple University Press, Philadelphia.

Armsworth, C., Bohan, D., Powers, S., Glen, D. & Symondson, W. (2005). Behavioural responses by slugs to chemicals from a generalist predator. *Animal Behaviour.* **69**, 805-811.

Balog, J., Szaniszlo, T., Schaefer, K.-C., Denes, J., Lopata, A., Godorhazy, L., Szalay, D., Balogh, L., Sasi-Szabo, L., Toth, M. & Takats, Z. (2010). Identification of Biological Tissues by Rapid Evaporative Ionization Mass Spectrometry. *Analytical Chemistry.* **82**, 7343-7350.

Balog, J., Sasi-Szabo, L., Kinross, J., Lewis, M. R., Muirhead, L. J., Veselkov, K., Mirnezami, R., Dezso, B., Damjanovich, L., Darzi, A., Nicholson, J. K. & Takats, Z. (2013). Intraoperative Tissue Identification Using Rapid Evaporative Ionization Mass Spectrometry. *Science Translational Medicine.* **5**, 1-11.

Balog, J., Perenyi, D., Guallar-Hoyas, C., Egri, A., Pringle, S. D., Stead, S., Chevallier, O. P., Elliott, C. T. & Takats, Z. (2016). Identification of the Species of Origin for Meat Products by Rapid Evaporative Ionization Mass Spectrometry. *Journal of Agricultural and Food Chemistry.* **64**, 4793-4800.

Barbosa, S., Pauperio, J., Searle, J. B. & Alves, P. C. (2013). Genetic identification of Iberian rodent species using both mitochondrial and nuclear loci: application to noninvasive sampling. *Molecular Ecology Resources.* **13**, 43-56.

Barnes, M. C., Persons, M. H. & Rypstra, A. L. (2002). The Effect of Predator Chemical Cue Age on Antipredator Behavior in the Wolf Spider *Pardosa milvina* (Araneae: Lycosidae). *Journal of Insect Behavior.* **15**, 269-281.

Barreto, G. R. & Macdonald, D. W. (1999). The response of water voles, *Arvicola terrestris*, to the odours of predators. *Animal Behaviour.* **57**, 1107-1112.

Bebber, D. P., Ramotowski, M. A. & Gurr, S. J. (2013). Crop pests and pathogens move polewards in a warming world. *Nature Climate Change.* **3**, 985–988.

Bellows, T. S. (2001). Restoring Population Balance through Natural Enemy Introductions. *Biological Control.* **21**, 199-205.

Black, C., Chevallier, O. P., Haughey, S. A., Balog, J., Stead, S., Pringle, S. D., Riina, M. V., Martucci, F., Acutis, P. L., Morris, M., Nikolopoulos, D. S., Takats, Z. & Elliott, C. T. (2017). A

real time metabolomic profiling approach to detecting fish fraud using rapid evaporative ionisation mass spectrometry. *Metabolomics.* **13**, 1-13.

Blanchard, D. C., Markham, C., Yang, M., Hubbard, D., Madarang, E. & Blanchard, R. J. (2003). Failure to produce conditioning with low-dose trimethylthiazoline or cat feces as unconditioned stimuli. *Behavioral Neuroscience.* **117**, 360-368.

Blanchard, R. J., Blanchard, D. C., Agullana, R. & Weiss, S. M. (1991). 22 kHz alarm cries to presentation of a predator, by laboratory rats living in visible burrow systems. *Physiology & Behavior.* **50**, 967-972.

Bolt, F., Cameron, S. J. S., Karancsi, T., Simon, D., Schaffer, R., Rickards, T., Hardiman, K., Burke, A., Bodai, Z., Perdones-Montero, A., Rebec, M., Balog, J. & Takats, Z. (2016). Automated High-Throughput Identification and Characterization of Clinically Important Bacteria and Fungi using Rapid Evaporative Ionization Mass Spectrometry. *Analytical Chemistry.* **88**, 9419-9426.

Brakes, C. R. & Smith, R. H. (2005). Exposure of non-target small mammals to rodenticides: short-term effects, recovery and implications for secondary poisoning. *Journal of Applied Ecology.* **42**, 118-128.

Bramley, G. & Waas, J. (2001). Laboratory and Field Evaluation of Predator Odors as Repellents for Kiore (*Rattus exulans*) and Ship Rats (*Rattus rattus*). *Journal of Chemical Ecology.* **27**, 1029-1047.

Bridgman, L. J., Innes, J., Gillies, C., Fitzgerald, N. B., Miller, S. & King, C. M. (2013). Do ship rats display predatory behaviour towards house mice? *Animal Behaviour.* **86**, 257-268.

Brooke, M. d. L., Cuthbert, R. J., Harrison, G., Gordon, C. & Taggart, M. A. (2013). Persistence of brodifacoum in cockroach and woodlice: Implications for secondary poisoning during rodent eradications. *Ecotoxicology and Environmental Safety.* **97**, 183-188.

Brudzynski, S. M. (2013). Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. *Current Opinion in Neurobiology.* **23**, 310-317.

Brunelli, S. A., Shair, H. N. & Hofer, M. A. (1994). Hypothermic vocalizations of rat pups (rattus-norvegicus) elicit and direct maternal search behavior. *Journal of Comparative Psychology.* **108**, 298-303.

Buck, L. B. (2000). The molecular architecture of odor and pheromone sensing in mammals. *Cell.* **100**, 611-618.

Buckle, A. & Fenn, M. (1992) Rodent control in the conservation of endangered species. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 36-41.

Burke, C. J., Kisko, T. M., Pellis, S. M. & Euston, D. R. (2017). Avoiding escalation from play to aggression in adult male rats: The role of ultrasonic calls. *Behavioural Processes.* **144**, 72-81.

Burn, C. C. (2008). What is it like to be a rat? Rat sensory perception and its implications for experimental design and rat welfare. *Applied Animal Behaviour Science.* **112**, 1-32.

Calder, C. & Gorman, M. (1991). The effects of red fox *Vulpes vulpes* faecal odours on the feeding behaviour of Orkney voles *Microtus arvalis*. *Journal of Zoology.* **224**, 599-606.

Cameron, S. J. S., Bolt, F., Perdones-Montero, A., Rickards, T., Hardiman, K., Abdolrasouli, A., Burke, A., Bodai, Z., Karancsi, T., Simon, D., Schaffer, R., Rebec, M., Balog, J. & Takáts, Z. (2016). Rapid Evaporative Ionisation Mass Spectrometry (REIMS) Provides Accurate Direct from Culture Species Identification within the Genus Candida. **6**, 1-10.

Campaign for Responsible Rodenticide Use UK (2015). *CRRU UK code of best practice.* pp. 1-24. CRRU UK, Ossett.

Campbell, J., Mullen, M. & Dowdy, A. (2002). Monitoring stored-product pests in food processing plants with pheromone trapping, contour mapping, and mark-recapture. *Journal of Economic Entomology.* **95**, 1089-1101.

Campbell, K. J., Beek, J., Eason, C. T., Glen, A. S., Godwin, J., Gould, F., Holmes, N. D., Howald, G. R., Madden, F. M., Ponder, J. B., Threadgill, D. W., Wegmann, A. S. & Baxter, G. S. (2015). The next generation of rodent eradications: Innovative technologies and tools to improve species specificity and increase their feasibility on islands. *Biological Conservation.* **185**, 47-58.

Capizzi, D., Bertolino, S. & Mortelliti, A. (2014). Rating the rat: global patterns and research priorities in impacts and management of rodent pests. *Mammal Review.* **44**, 148-162.

Christiansen, E. (1976) Pheromones in small rodents and their potential use in pest control. In Siebe, C. C., Ed. *Proceedings of the Seventh Vertebrate Pest Conference, Monterey*. University of California. pp. 185-195.

Clapperton, B. K. (2006). *A review of the current knowledge of rodent behaviour in relation to control devices.* Department of Conservation, New Zealand.

Clapperton, B. K., Porter, R. E. R., Day, T. D., Rwaas, J. & Matthews, L. R. (2012). Designer repellents: combining olfactory, visual or taste cues with a secondary repellent to deter free-ranging house sparrows from feeding. *Pest Management Science.* **68**, 870-877.

Clapperton, B. K., Murphy, E. C. & Razzaq, H. A. A. (2017). *Mammalian Pheromones: New Opportunities for Improved Predator Control in New Zealand.* Science for Conservation, pp. 1-53. **330**. Department of Conservation, New Zealand.

Colvin, B. A., Hegdal, P. L. & Jackson, W. B. (1988). Review of non-target hazards associated with rodenticide use in the USA. *EPPO Bulletin.* **18**, 301-308.

Cook, S. M., Khan, Z. R. & Pickett, J. A. (2007). The use of push-pull strategies in integrated pest management. *Annual Review of Entomology.* **52**, 375-400.

Dawson, A., Bankes, J. & Garthwaite, D. (2001). *Rodenticide use on farms in Great Britain growing arable crops 2000.* pp. 1-22. DEFRA, York.

Day, T. D., Matthews, L. R. & Waas, J. R. (2003). Repellents to deter New Zealand's North Island robin *Petroica australis longipes* from pest control baits. *Biological Conservation.* **114**, 309-316.

Devine, G. J. & Furlong, M. J. (2007). Insecticide use: Contexts and ecological consequences. *Agriculture and Human Values.* **24**, 281-306.

Diamond, M. E. & Arabzadeh, E. (2013). Whisker sensory system–from receptor to decision. *Progress in Neurobiology.* **103**, 28-40.

Dickman, C. R. (1999). Rodent-ecosystem relationships: a review. *Ecologically-based Management of Rodent Pests. ACIAR Monograph.* 113-133.

Dielenberg, R. A. & McGregor, I. S. (1999). Habituation of the hiding response to cat odor in rats (*Rattus norvegicus*). *Journal of Comparative Psychology.* **113**, 376-87.

Dowding, C. V., Shore, R. F., Worgan, A., Baker, P. J. & Harris, S. (2010). Accumulation of anticoagulant rodenticides in a non-target insectivore, the European hedgehog (*Erinaceus europaeus*). *Environmental Pollution.* **158**, 161-6.

Eason, C. T., Murphy, E. C., Wright, G. R. & Spurr, E. B. (2002). Assessment of risks of brodifacoum to non-target birds and mammals in New Zealand. *Ecotoxicology.* **11**, 35-48.

Eigenbrode, S. D., Birch, A. N. E., Lindzey, S., Meadow, R. & Snyder, W. E. (2016). Review: A mechanistic framework to improve understanding and applications of push-pull systems in pest management. *Journal of Applied Ecology.* **53**, 202-212.

Elliott, J., Hindmarch, S., Albert, C., Emery, J., Mineau, P. & Maisonneuve, F. (2013). Exposure pathways of anticoagulant rodenticides to nontarget wildlife. *Environmental Monitoring and Assessment.* **186**, 895-906.

Elmeros, M., Christensen, T. K. & Lassen, P. (2011). Concentrations of anticoagulant rodenticides in stoats (*Mustela erminea*) and weasels (*Mustela nivalis*) from Denmark. *Science of the Total Environment.* **409**, 2373-2378.

Elmeros, M., Lassen, P., Bossi, R. & Topping, C. J. (2018). Exposure of stone marten (*Martes foina*) and polecat (*Mustela putorius*) to anticoagulant rodenticides: Effects of regulatory restrictions of rodenticide use. *Science of the Total Environment.* **612**, 1358-1364.

Engeman, R. M. & Witmer, G. W. (2000) IPM strategies: indexing difficult to monitor populations of pest species. In T.P. Salmon & A.C. Crabb, Eds. *Proceedings of the Nineteenth Vertebrate Pest Conference, San Diego*. University of California Davis. pp. 183-189.

Erickson, W. A., Marsh, R. E. & Halvorson, W. L. (1990). A Roof Rat Bait Station That Excludes Deer Mice. *Wildlife Society Bulletin.* **18**, 319-325.

Erickson, W. A. & Urban, D. J. (2004). *Potential risks of nine rodenticides to birds and nontarget mammals: a comparative approach.* pp. 1-225. U.S. Environmental Protection Agency, Washinton D.C.

Fendt, M. (2006). Exposure to urine of canids and felids, but not of herbivores, induces defensive behavior in laboratory rats. *Journal of Chemical Ecology.* **32**, 2617-2627.

Ferrari, M. C. O., Gonzalo, A., Messier, F. & Chivers, D. P. (2007). Generalization of learned predator recognition: an experimental test and framework for future studies. *Proceedings of the Royal Society B-Biological Sciences.* **274**, 1853-1859.

Ferrero, D. M. & Liberles, S. D. (2010). The secret codes of mammalian scents. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine.* **2**, 23-33.

Fitt, G. P. (2000). An Australian approach to IPM in cotton: integrating new technologies to minimise insecticide dependence. *Crop Protection.* **19**, 793-800.

Galan, M., Pages, M. & Cosson, J. F. (2012). Next-Generation Sequencing for Rodent Barcoding: Species Identification from Fresh, Degraded and Environmental Samples. *PLOS ONE.* **7**, 1-13.

Geduhn, A., Esther, A., Schenke, D., Mattes, H. & Jacob, J. (2014). Spatial and temporal exposure patterns in non-target small mammals during brodifacoum rat control. *Science of the Total Environment.* **496**, 328-338.

Geduhn, A., Jacob, J., Schenke, D., Keller, B., Kleinschmidt, S. & Esther, A. (2015). Relation between Intensity of Biocide Practice and Residues of Anticoagulant Rodenticides in Red Foxes (*Vulpes vulpes*). *PLOS ONE.* **10**, 1-15.

Geduhn, A., Esther, A., Schenke, D., Gabriel, D. & Jacob, J. (2016). Prey composition modulates exposure risk to anticoagulant rodenticides in a sentinel predator, the barn owl. *Science of the Total Environment.* **544**, 150-157.

Gentle, M., Massei, G. & Quy, R. (2006). Diversity of diet influences the persistence of conditioned taste aversion in rats. *Applied Animal Behaviour Science.* **97**, 303-311.

Ghaly, A. & Edwards, S. (2011). Termite damage to buildings: Nature of attacks and preventive construction methods. *American Journal of Engineering and Applied Sciences.* **4**, 187-200.

Giraudoux, P., Tremollières, C., Barbier, B., Defaut, R., Rieffel, D., Bernard, N., Lucot, É. & Berny, P. (2006). Persistence of bromadiolone anticoagulant rodenticide in *Arvicola terrestris* populations after field control. *Environmental Research.* **102**, 291-298.

Glen, A. S. & Dickman, C. R. (2003). Monitoring bait removal in vertebrate pest control: a comparison using track identification and remote photography. *Wildlife Research.* **30**, 29-33.

Hadler, M. R. & Shadbolt, R. S. (1975). Novel 4-hydroxycoumarin anticoagulants active against resistant rats. *Nature.* **253**, 275-277.

Hadler, M. R. & Buckle, A. P. (1992) Forty five years of anticoagulant rodenticides - past, present and future trends. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 149-156.

Hansen, S. C., Stolter, C. & Jacob, J. (2015). The smell to repel: The effect of odors on the feeding behavior of female rodents. *Crop Protection.* **78**, 270-276.

Hansen, S. C., Stolter, C., Imholt, C. & Jacob, J. (2016). Plant Secondary Metabolites as Rodent Repellents: a Systematic Review. *Journal of Chemical Ecology.* **42**, 970-983.

Hansson, L. (1971). Small Rodent Food, Feeding and Population Dynamics - Comparison between Granivorous and Herbivorous Species in Scandinavia. *Oikos.* **22**, 183-198.

Harris, J. D. (1943). Habituatory response decrement in the intact organism. *Psychological Bulletin.* **40**, 385-422.

Harris, S. (1989). When Is a Pest a Pest. *New Scientist.* **121**, 49-51.

Health and Safety Executive (2012). *Environmental risk mitigation measures for second generation anticoagulant rodenticides proposed by the UK* pp. 1-30. Health and Safety Executive, UK.

Health and Safety Executive (2015). *UK anticoagulant rodenticide product authorisation and the CRRU stewardship scheme.* pp. 1-12. Health and Safety Executive, UK.

Hegab, I. M., Shang, G., Ye, M., Jin, Y., Wang, A., Yin, B., Yang, S. & Wei, W. (2014). Defensive responses of Brandt's voles (*Lasiopodomys brandtii*) to chronic predatory stress. *Physiology & Behavior.* **126**, 1-7.

Hernandez-Moreno, D., de la Casa-Resino, I., Lopez-Beceiro, A., Fidalgo, L. E., Soler, F. & Perez-Lopez, M. (2013). Secondary poisoning of non-target animals in an Ornithological Zoo in Galicia (NW Spain) with anticoagulant rodenticides: a case report. *Veterinarni Medicina.* **58**, 553-559.

Hettena, A. M., Munoz, N. & Blumstein, D. T. (2014). Prey Responses to Predator's Sounds: A Review and Empirical Study. *Ethology.* **120**, 427-452.

Hilton, G. M. & Cuthbert, R. J. (2010). Review article: The catastrophic impact of invasive mammalian predators on birds of the UK Overseas Territories: a review and synthesis. *Ibis.* **152**, 443-458.

Hoare, J. M. & Hare, K. M. (2006). The impact of brodifacoum on non-target wildlife: gaps in knowledge. *New Zealand Journal of Ecology.* **30**, 157-167.

Holmes, N. D., Griffiths, R., Pott, M., Alifano, A., Will, D., Wegmann, A. S. & Russell, J. C. (2015). Factors associated with rodent eradication failure. *Biological Conservation.* **185**, 8-16.

Hughes, J., Sharp, E., Taylor, M. J., Melton, L. & Hartley, G. (2013). Monitoring agricultural rodenticide use and secondary exposure of raptors in Scotland. *Ecotoxicology.* **22**, 974-984.

Jackson, W. B. (2001). Current rodenticide strategies. *International Biodeterioration & Biodegradation.* **48**, 127-136.

Kapusta, J. & Kruczek, M. (2016). Ultrasonic Reaction of Bank Vole Males to the Presence of Females Varying in Hormonal Activity. *Ethology.* **122**, 468-480.

Karlson, P. & Luscher, M. (1959). Pheromones - New Term for a Class of Biologically Active Substances. *Nature.* **183**, 55-56.

Kelley, J. L. & Magurran, A. E. (2003). Learned predator recognition and antipredator responses in fishes. *Fish and Fisheries.* **4**, 216-226.

Lacey, L. A., Frutos, R., Kaya, H. & Vail, P. (2001). Insect pathogens as biological control agents: do they have a future? *Biological Control.* **21**, 230-248.

Li, Q. & Liberles, Stephen D. (2015). Aversion and Attraction through Olfaction. *Current Biology.* **25**, 120-129.

Lima, S. L. & Bednekoff, P. A. (1999). Temporal Variation in Danger Drives Antipredator Behavior: The Predation Risk Allocation Hypothesis. *The American Naturalist.* **153**, 649-659.

Linnie, M. J. (1994). Pest control in natural history museums: A world survey. *Journal of Biological Curation.* **1**, 43-58.

Litvin, Y., Blanchard, D. C. & Blanchard, R. J. (2007). Rat 22kHz ultrasonic vocalizations as alarm cries. *Behavioural Brain Research.* **182**, 166-172.

Liu, Q., Qin, J., Chen, Q., Wang, D. & Shi, D. (2012). Fertility control of *Rattus nitidus* using quinestrol: effects on reproductive organs and social behavior. *Integrative Zoology.* **8**, 9-17.

Lloyd, R., Alford, R. A. & Schwarzkopf, L. (2009). Chemical discrimination among predators by lizards: Responses of three skink species to the odours of high- and low-threat varanid predators. *Austral Ecology.* **34**, 50-54.

Louda, S. M., Pemberton, R. W., Johnson, M. T. & Follett, P. A. (2003). Nontarget effects - The Achilles' heel of biological control? Retrospective Analyses to Reduce Risk Associated with Biocontrol Introductions. *Annual Review of Entomology.* **48**, 365-396.

Lund, M. (1994). Commensal Rodents. In *Rodent Pests and their Control.* Buckle, A. P. & Smith, R. H. (Eds.), pp. 23-44. CAB International, Oxon.

Macdonald, D. W. & Fenn, M. G. P. (1994). The natural history of rodents: preadaptations to pestilence. In *Rodent Pests and their Control.* Buckle, A. P. & Smith, R. H. (Eds.), pp. 1-22. CAB International, Oxon.

Martinez-Padilla, J., Lopez-Idiaquez, D., Lopez-Perea, J. J., Mateo, R., Paz, A. & Vinuela, J. (2017). A negative association between bromadiolone exposure and nestling body condition in common kestrels: management implications for vole outbreaks. *Pest Management Science.* **73**, 364-370.

Mason, G. & Littin, K. E. (2003). The humaneness of rodent pest control. *Animal Welfare.* **12**, 1-37.

McDonald, R. A., Harris, S., Turnbull, G., Brown, P. & Fletcher, M. (1998). Anticoagulant rodenticides in stoats (*Mustela erminea*) and weasels (*Mustela nivalis*) in England. *Environmental Pollution.* **103**, 17-23.

McGregor, I. S., Schrama, L., Ambermoon, P. & Dielenberg, R. A. (2002). Not all 'predator odours' are equal: cat odour but not 2, 4, 5 trimethylthiazoline (TMT; fox odour) elicits specific defensive behaviours in rats. *Behavioural Brain Research.* **129**, 1-16.

Meerburg, B. G., Singleton, G. R. & Kijlstra, A. (2009). Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology.* **35**, 221-270.

Memmott, K., Murray, M. & Rutberg, A. (2017). Use of anticoagulant rodenticides by pest management professionals in Massachusetts, USA. *Ecotoxicology.* **26**, 90-96.

Moore, J. W. (2006). Animal Ecosystem Engineers in Streams. *BioScience.* **56**, 237-246.

Mukherjee, S., Goyal, S. P., Johnsingh, A. J. T. & Leite Pitman, M. R. P. (2004). The importance of rodents in the diet of jungle cat (*Felis chaus*), caracal (*Caracal caracal*) and golden jackal (*Canis aureus*) in Sariska Tiger Reserve, Rajasthan, India. *Journal of Zoology.* **262**, 405-411.

Naiman, R. J. (1988). Animal Influences on Ecosystem Dynamics. *BioScience.* **38**, 750-752.

Nair, K. S. S. (2007). Population dynamics: What makes an insect a pest? In *Tropical Forest Insect Pests: Ecology, Impact, and Management.* pp. 119-133. Cambridge University Press, Cambridge.

Nelson, J. T., Woodworth, B. L., Fancy, S. G., Lindsey, G. D. & Tweed, E. J. (2002). Effectiveness of Rodent Control and Monitoring Techniques for a Montane Rainforest. *Wildlife Society Bulletin.* **30**, 82-92.

Nogeire, T. M., Lawler, J. J., Schumaker, N. H., Cypher, B. L. & Phillips, S. E. (2015). Land Use as a Driver of Patterns of Rodenticide Exposure in Modeled Kit Fox Populations. *PLOS ONE.* **10**, 1-15.

O'Boyle, M. (1974). Rats and mice together: The predatory nature of the rat's mouse-killing response. *Psychological Bulletin.* **81**, 261-269.

Oerke, E. C. (2006). Crop losses to pests. *Journal of Agricultural Science.* **144**, 31-43.

Okada, J. & Akamine, S. (2012). Behavioral response to antennal tactile stimulation in the field cricket *Gryllus bimaculatus*. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology.* **198**, 557-565.

Oliveira, C. M., Auad, A. M., Mendes, S. M. & Frizzas, M. R. (2014). Crop losses and the economic impact of insect pests on Brazilian agriculture. *Crop Protection.* **56**, 50-54.

Olivera, P., Menezes, D., Trout, R., Buckle, A., Geraldes, P. & Jesus, J. (2010). Successful eradication of the European rabbit (*Oryctolagus cuniculus*) and house mouse (*Mus musculus*) from the island of Selvagem Grande (Macaronesian archipelago), in the Eastern Atlantic. *Integrative Zoology.* **5**, 70-83.

Panksepp, J. & Burgdorf, J. (2000). 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables. *Behavioural Brain Research.* **115**, 25-38.

Parsons, M. H., Apfelbach, R., Banks, P. B., Cameron, E. Z., Dickman, C. R., Frank, A. S., Jones, M. E., McGregor, I. S., McLean, S. & Müller-Schwarze, D. (2018). Biologically meaningful scents: a framework for understanding predator–prey research across disciplines. *Biological Reviews.* **93**, 98-114.

Paul, L., Miley, W. M. & Baenninger, R. (1971). Mouse killing by rats: Roles of hunger and thirst in its initiation and maintenance. *Journal of Comparative and Physiological Psychology.* **76**, 242-249.

Pimentel, D. (2005). Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. *Environment, Development and Sustainability.* **7**, 229-252.

Pimentel, D., Zuniga, R. & Morrison, D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics.* **52**, 273-288.

Prowse, T. A. A., Cassey, P., Ross, J. V., Pfitzner, C., Wittmann, T. A. & Thomas, P. (2017). Dodging silver bullets: good CRISPR gene-drive design is critical for eradicating exotic vertebrates. *Proceedings of the Royal Society B-Biological Sciences.* **284**, 1860-1869.

Putman, R. J. (1989). Introduction: Mammals as Pests. In *Mammals as Pests.* Putman, R. (Ed.) pp. 1-20. Chapman and Hall Ltd, London.

Reed, J. Z., Tollit, D. J., Thompson, P. M. & Amos, W. (1997). Molecular scatology: The use of molecular genetic analysis to assign species, sex and individual identity to seal faeces. *Molecular Ecology.* **6**, 225-234.

Richards, C. G. J. (1989). The pest status of rodents in the United Kingdom. In *Mammals as Pests.* Putman, R. (Ed.) pp. 21-33. Chapman and Hall Ltd, London.

Richards, S. (2012). Behavioural responses of wood mice (*Apodemus sylvaticus*) and bank voles (*Myodes glareolus*) to rat odours. University of Liverpool [Unpublished thesis].

Roomaney, R., Ehrlich, R. & Rother, H. A. (2012). The acceptability of rat trap use over pesticides for rodent control in two poor urban communities in South Africa. *Environmental Health.* **11**, 1-6.

Sadananda, M., Wöhr, M. & Schwarting, R. K. W. (2008). Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. *Neuroscience Letters.* **435**, 17-23.

Sales, G. D. (2010). Chapter 3.4 - Ultrasonic calls of wild and wild-type rodents. In *Handbook of Mammalian Vocalization : an Integrative Neuroscience Approach.* Brudzynski, S. M. (Ed.) pp. 77-88. Academic Press, Boston.

Schaefer, K.-C., Denes, J., Albrecht, K., Szaniszlo, T., Balog, J., Skoumal, R., Katona, M., Toth, M., Balogh, L. & Takats, Z. (2009). In Vivo, In Situ Tissue Analysis Using Rapid Evaporative Ionization Mass Spectrometry. *Angewandte Chemie-International Edition.* **48**, 8240-8242.

Seffer, D., Schwarting, R. K. W. & Wöhr, M. (2014). Pro-social ultrasonic communication in rats: Insights from playback studies. *Journal of Neuroscience Methods.* **234**, 73-81.

Selvaraj, R. & Archunan, G. (2006). Efficacy of male scent glands and urine in masking poison bait odour in female house rats, *Rattus rattus*. *Journal of Pest Science.* **79**, 255-258.

Serieys, L. E. K., Armenta, T. C., Moriarty, J. G., Boydston, E. E., Lyren, L. M., Poppenga, R. H., Crooks, K. R., Wayne, R. K. & Riley, S. P. D. (2015). Anticoagulant rodenticides in urban bobcats: exposure, risk factors and potential effects based on a 16-year study. *Ecotoxicology.* **24**, 844-862.

Shore, R. F., Walker, L. A., Potter, E. D., Pereira, M. G., Sleep, D. & Thompson, N. J. (2016). *Second generation anticoagulant rodenticide residues in barn owls 2016.* pp. 1-21. Centre for Ecology & Hydrology, Lancaster.

Singleton, G. R., Leirs, H., Hinds, L. A. & Zhang, Z. (1999). *Ecologically-based management of rodent pests – re-evaluating our approach to an old problem.* Ecologically-based Management of Rodent Pests, pp. 17-29. Australian Centre for International Agricultural Research (ACIAR), Canberra.

Singleton, G. R. (2003). *Impacts of rodents on rice production in Asia.* IRRI Discussion Paper Series, No. 45, pp. 1-30. International Rice Research Institute, Philippines.

Singleton, G. R., Jacob, J. & Krebs, C. (2005). Integrated management to reduce rodent damage to lowland rice crops in Indonesia. *Agriculture, Ecosystems & Environment.* **107**, 75-82.

Singleton, G. R. (2010). *Rodent outbreaks: ecology and impacts.* International Rice Research Institute, Philippines.

Stephenson, B. M., Minot, E. O. & Armstrong, D. P. (1999). Fate of moreporks (*Ninox novaeseelandiae*) during a pest control operation on Mokoia Island, Lake Rotorua, North Island, New Zealand. *New Zealand Journal of Ecology.* **23**, 233-240.

Stoddart, D. M. (1982). Does trap odour influence estimation of population size of the short-tailed vole, *Microtus agrestis*? *Journal of Animal Ecology.* **51**, 375-386.

Strittmatter, N., Jones, E. A., Veselkov, K. A., Rebec, M., Bundy, J. G. & Takats, Z. (2013). Analysis of intact bacteria using rapid evaporative ionisation mass spectrometry. *Chemical Communications.* **49**, 6188-6190.

Sulikowski, D. (2017). From sensory to social: the information that impacts animal foraging decisions. *Current Opinion in Behavioral Sciences.* **16**, 93-99.

Sullivan, T. P., Sullivan, D. S., Crump, D. R., Weiser, H. & Dixon, E. A. (1988) Predator odors and their potential role in managing pest rodents and rabbits. In Crabb, A. C. & Marsh, R. E., Eds. *Proceedings of the Thirteenth Vertebrate Pest Conference, Monterey*. University of California. pp. 145-150.

Taberlet, P. & Fumagalli, L. (1996). Owl pellets as a source of DNA for genetic studies of small mammals. *Molecular Ecology.* **5**, 301-305.

Takács, S., Gries, R., Zhai, H. & Gries, G. (2016a). The Sex Attractant Pheromone of Male Brown Rats: Identification and Field Experiment. *Angewandte Chemie-International Edition.* **55**, 6062-6066.

Takács, S., Kowalski, P. & Gries, G. (2016b). Natural and synthetic vocalizations of brown rat pups, *Rattus norvegicus*, enhance attractiveness of bait boxes in laboratory and field experiments. *Pest Management Science.* **72**, 1873-1882.

Thomas, P. J., Mineau, P., Shore, R. F., Champoux, L., Martin, P. A., Wilson, L. K., Fitzgerald, G. & Elliott, J. E. (2011). Second generation anticoagulant rodenticides in predatory birds: Probabilistic characterisation of toxic liver concentrations and implications for predatory bird populations in Canada. *Environment International.* **37**, 914-920.

Tosh, D. G., McDonald, R. A., Bearhop, S., Lllewellyn, N. R., Fee, S., Sharp, E. A., Barnett, E. A. & Shore, R. F. (2011a). Does small mammal prey guild affect the exposure of predators to anticoagulant rodenticides? *Environmental Pollution.* **159**, 3106-3112.

Tosh, D. G., Shore, R. F., Jess, S., Withers, A., Bearhop, S., Ian Montgomery, W. & McDonald, R. A. (2011b). User behaviour, best practice and the risks of non-target exposure associated with anticoagulant rodenticide use. *Journal of Environmental Management.* **92**, 1503-1508.

Tran, T. T. & Hinds, L. A. (2013). Fertility control of rodent pests: a review of the inhibitory effects of plant extracts on ovarian function. *Pest Management Science.* **69**, 342-354.

Vandenbroucke, V., Bousquet-Melou, A., De Backer, P. & Croubels, S. (2008). Pharmacokinetics of eight anticoagulant rodenticides in mice after single oral administration. *Journal of Veterinary Pharmacology and Therapeutics.* **31**, 437-445.

Vidal, D., Alzaga, V., Luque-Larena, J. J., Mateo, R., Arroyo, L. & Viñuela, J. (2009). Possible interaction between a rodenticide treatment and a pathogen in common vole (*Microtus arvalis*) during a population peak. *Science of the Total Environment.* **408**, 267-271.

Vincent, C., Hallman, G., Panneton, B. & Fleurat-Lessard, F. (2003). Management of agricultural insects with physical control methods. *Annual Review of Entomology.* **48**, 261-281.

Vreysen, M. J. B., Saleh, K. M., Ali, M. Y., Abdulla, A. M., Zhu, Z. R., Juma, K. G., Dyck, V. A., Msangi, A. R., Mkonyi, P. A. & Feldmann, H. U. (2000). *Glossina austeni* (Diptera : Glossinidae) eradicated on the Island of Unguja, Zanzibar, using the sterile insect technique. *Journal of Economic Entomology.* **93**, 123-135.

Waits, L. P. & Paetkau, D. (2005). Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *Journal of Wildlife Management.* **69**, 1419-1433.

Walker, L. A., Chaplow, J. S., Llewellyn, N. R., Pereira, M. G., Potter, E. D., Sainsbury, A. W. & Shore, R. F. (2013). *Anticoagulant rodenticides in predatory birds 2011: a Predatory Bird Monitoring Scheme (PBMS) report.* pp. 1-29. Centre for Ecology & Hydrology, Lancaster.

Walker, L. A., Chaplow, J. S., Moeckel, C., Pereira, M. G., Potter, E. D., Sainsbury, A. W. & Shore, R. F. (2016). *Anticoagulant rodenticides in red kites (Milvus milvus) in Britain 2010 to 2015: a Predatory Bird Monitoring Scheme (PBMS) report.* pp. 1-14. Centre for Ecology & Hydrology, Lancaster.

Wallingford, A. K., Cha, D. H., Linn, C. E., Wolfin, M. S. & Loeb, G. M. (2017). Robust Manipulations of Pest Insect Behavior Using Repellents and Practical Application for Integrated Pest Management. *Environmental Entomology.* **46**, 1041-1050.

Wang, D., Li, Q. X., Li, K. & Guo, Y. W. (2017). Modified trap barrier system for the management of rodents in maize fields in Jilin Province, China. *Crop Protection.* **98**, 172-178.

Webber, B. L., Raghu, S. & Edwards, O. R. (2015). Opinion: Is CRISPR-based gene drive a biocontrol silver bullet or global conservation threat? *Proceedings of the National Academy of Sciences.* **112**, 10565-10567.

Westphal, M. I., Browne, M., MacKinnon, K. & Noble, I. (2008). The link between international trade and the global distribution of invasive alien species. *Biological Invasions.* **10**, 391-398.

Whisson, D. A., Engeman, R. M. & Collins, K. (2005). Developing relative abundance techniques (RATs) for monitoring rodent populations. *Wildlife Research.* **32**, 239-244.

White, N. R., Adox, R., Reddy, A. & Barfield, R. J. (1992). Regulation of rat maternal behavior by broadband pup vocalizations. *Behavioral and Neural Biology.* **58**, 131-137.

Williams, R. J. & Martinez, N. D. (2000). Simple rules yield complex food webs. *Nature.* **404**, 180-3.

Williamson, S., Ball, A. & Pretty, J. (2008). Trends in pesticide use and drivers for safer pest management in four African countries. *Crop Protection.* **27**, 1327-1334.

Wilson, C. & Tisdell, C. (2001). Why farmers continue to use pesticides despite environmental, health and sustainability costs. *Ecological Economics.* **39**, 449-462.

Witmer, G. W. (2005). Wildlife population monitoring: some practical considerations. *Wildlife Research.* **32**, 259-263.

Witmer, G. W. (2007). *The ecology of vertebrate pests and integrated pest management (IPM).* USDA National Wildlife Research Center-Staff Publications, pp. 393-410. University of Nebraska, U.S.A.

Witzgall, P., Kirsch, P. & Cork, A. (2010). Sex Pheromones and Their Impact on Pest Management. *Journal of Chemical Ecology.* **36**, 80-100.

Wöhr, M. & Schwarting, R. K. (2007). Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior? *PLOS ONE.* **2**, 1-12.

Woolhouse, A. D. & Morgan, D. R. (1995). An evaluation of repellents to suppress browsing by possums. *Journal of Chemical Ecology.* **21**, 1571-1583.

Wright, L. D., Muir, K. E. & Perrot, T. S. (2013). Stress responses of adolescent male and female rats exposed repeatedly to cat odor stimuli, and long-term enhancement of adult defensive behaviors. *Developmental Psychobiology.* **55**, 551-567.

Wyatt, T. D. (2010). Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology.* **196**, 685-700.

Wyatt, T. D. (2014). *Pheromones and animal behavior : chemical signals and signatures.* 2nd ed. Cambridge University Press, New York.

Xiao, Z., Jansen, P. A. & Zhang, Z. (2006). Using seed-tagging methods for assessing post-dispersal seed fate in rodent-dispersed trees. *Forest Ecology and Management.* **223**, 18-23.

Yilmaz, M. & Meister, M. (2013). Rapid Innate Defensive Responses of Mice to Looming Visual Stimuli. *Current Biology.* **23**, 2011-2015.

Yin, B. F., Fan, H. M., Li, S. P., Hegab, I., Lu, G. Y. & Wei, W. H. (2011). Behavioral response of Norway rats (*Rattus norvegicus*) to odors of different mammalian species. *Journal of Pest Science.* **84**, 265-272.

Yu, X., Wang, J., Kays, R., Jansen, P. A., Wang, T. & Huang, T. (2013). Automated identification of animal species in camera trap images. *Eurasip Journal on Image and Video Processing.* **52**, 1-10.

Zippelius, H. M. & Schleidt, W. M. (1956). Ultraschall-Laute Bei Jungen Mausen. *Naturwissenschaften.* **43**, 502-502.

Zylberberg, J. & DeWeese, M. R. (2011). How should prey animals respond to uncertain threats? *Frontiers in Computational Neuroscience.* **5**, 1-7.

Chapter 2

2. Male rat odour repels non-target rodents

2.1. Abstract

The majority of rodent species are not considered as pests and play an important role in many ecosystems. However, these species are often poisoned during efforts to control pest rodents. This non-target exposure to poisons is widespread, affecting not only non-target rodents but also the predators that prey on them. To reduce non-target rodent exposure to rat poisons, we tested if male rat odour could be used to repel non-target rodents from rat bait boxes.

To test if rat odour is repellent to non-target rodents under controlled conditions, the response of wood mice (*Apodemus sylvaticus*; n = 12), bank voles (*Myodes glareolus*; n = 12) and field voles (*Microtus agrestis*; n = 12) to male rat odour was tested in laboratory arenas. In these conditions, bank voles and field voles avoided spending time in boxes containing rat odour (body odour, faeces and soiled nest material), but wood mice did not. To test if rat odour is repellent to non-target rodents in natural conditions, the response of wood mice (n = 40), bank voles (n = 60) and field voles (n = 60) to male rat odour was tested in outdoor, semi-natural enclosures. In these conditions, all three species avoided eating non-toxic bait from boxes scented with rat odour (body odour and faeces). Further testing of wood mice in outdoor semi-natural enclosures (n = 20) revealed that wood mice avoid rat body odour more than rat faeces and the spatial extent of rat odour avoidance is limited to the odoured box.

An important caveat of any non-target repellent is that it does not repel target rats. To assess this, the response of male (n = 8) and female rats (n = 11) to rat odour was tested in a two compartment laboratory arena. Overall, rats were neither significantly attracted to, nor repelled by, male rat odour. However, the response of male rats was highly variable and one male rat was observed to be particularly cautious when close to the odour source.

Thus, male rat odour, particularly body odour, is repellent to three non-target rodent species. This repellence is sufficient to significantly reduce intake of attractive food baits. However, there is some concern about the effect of male rat odour on male rats. Therefore, whilst male rat odour has the potential to reduce the exposure of non-target rodents to rat poisons, other cues, such as female rat odour, should be trialled to reduce the risk of repelling target rats.

2.2. General Introduction

Rodents play a vital role in many ecosystems. They contribute to environmental structure by burrowing and building nests (Dickman, 1999). Herbivorous rodents alter the abundance and variety of plants in the local ecosystem (Dickman, 1999). In addition, rodents are important in the reproductive cycle of many plant species as seed dispersers and, to a lesser extent, as pollinators (Dickman, 1999; Xiao *et al.*, 2006). Rodents are an important part of food webs, with many species relying on them as prey (Jaksić *et al.*, 1980; Mukherjee *et al.*, 2004).

However, not all rodent species are beneficial to ecosystems. These species are classified as pests and comprise approximately 7% of known rodent species (Capizzi *et al.*, 2014). These pest species often have a high reproductive potential. Populations can go from a few individuals to many thousands in a single breeding season (Singleton *et al.*, 2001). In addition, many pest species are highly adaptable in both the food they eat and how they live, meaning they can survive in a wide range of habitats, often alongside humans (Berry, 1981; Buckle & Fenn, 1992; Major *et al.*, 2007; Triggs, 1991). This capacity for fast population growth, coupled with adaptability to novel food sources makes pest rodents a major threat to global food security (Capizzi *et al.*, 2014; Singleton, 2010). In addition, pest rodents are reservoirs for over 50 zoonotic diseases and cause vast damage to human infrastructure (Meerburg *et al.*, 2009; Pimentel *et al.*, 2005). Several pest species have invaded new habitats, where they threaten populations of endemic species (Buckle & Fenn, 1992; Pimentel *et al.*, 2005). These factors have contributed to rodents being considered as one of the most important groups of pests worldwide.

Controlling pest rodents follows general pest control principles. The main ways to prevent damage by a pest are to kill the pest, repel it, protect what it damages or remove sources of food and shelter (Cook *et al.*, 2007; Davis, 1953; Meerburg *et al.*, 2008; Witmer, 2007). Pests are killed using pesticides, traps, biological predators or targeted pathogen release (Bellows, 2001; Capizzi *et al.*, 2014; Meerburg *et al.*, 2008). Often, the ideal solution is to combine methods in an integrated pest management strategy (Fitt, 2000; Singleton *et al.*, 2005). However, pest control relies heavily on the use of pesticides as a single main control technique (Capizzi *et al.*, 2014; Fitt, 2000; Simon *et al.*, 2011). Rodent control is no exception to this rule, with pesticides being the number one control method in all continents (Capizzi *et al.*, 2014).

Unfortunately, pesticides have many disadvantages, especially their effects on non-target species. Non-target species may be poisoned directly by pesticides (primary poisoning), or they may be poisoned when they consume other species that have been exposed (secondary poisoning; Buckle & Fenn, 1992; Colvin *et al.*, 1988). In addition to killing non-target animals, non-lethal doses of pesticides can reduce fitness and increase disease susceptibility (Elmeros *et al.*, 2011; Martinez-Padilla *et al.*, 2017; Serieys *et al.*, 2015). Non-target exposure to pesticides is of major global concern due to the scale of the problem. In the USA alone, non-target poisoning by pesticides has been estimated to cost $3 billion per year (Pimentel, 2005).

Non-target poisoning is a particular concern for pest control aimed at rats (*Rattus spp.*). Among rodents, rats are the most important pest species (Capizzi *et al.*, 2014). They threaten global food security, consuming 5-10% of rice yields in Asia every year, and are an important reservoir for many diseases including bubonic plague, listeriosis and typhus (Meerburg *et al.*, 2009; Singleton, 2003). Control of rats is heavily reliant on the use of pesticides, particularly the anticoagulant rat poisons (Capizzi *et al.*, 2014). These poisons are potentially toxic to all species of mammals and birds (Hadler & Buckle, 1992). Second generation anticoagulant rat poisons are highly potent and bioaccumulative and so pass up food chains, causing secondary poisoning (Hadler & Buckle, 1992; Vandenbroucke *et al.*, 2008). Most primary non-target poisoning by anticoagulant rat poisons affects small rodents, such as wood mice (*Apodemus sylvaticus*), bank voles (*Myodes glareolus*) and field voles (*Microtus agrestis*; (Brakes & Smith, 2005; Geduhn *et al.*, 2014; Tosh *et al.*, 2012). As these rodents are a major food source for many avian and mammalian predators, secondary poisoning by rat poisons is widespread. For example, in the UK over 75% of barn owls, red kites and foxes have residues of anticoagulant rat poisons, often at toxic levels (Shore *et al.*, 2016; Tosh *et al.*, 2011a; Walker *et al.*, 2016).

Several solutions have been suggested to solve the problem of non-target poisoning by anticoagulant rat poisons. The most obvious solution would be to ban their use. Unfortunately, this is not an option due to a lack of viable alternatives to control pest rats (Health and Safety Executive, 2012). As an alternative, many countries have introduced restrictions on the use of these poisons, particularly reducing their use in outdoor habitats where non-target species are particularly susceptible (Campaign for Responsible Rodenticide Use UK, 2015; Elmeros *et al.*, 2018; Health and Safety Executive, 2015). However, these restrictions are often not adhered to and there is evidence that they do not fully mitigate the

risks of non-target poisoning (Elmeros *et al.*, 2018; Tosh *et al.*, 2011b). Another solution would be to increase the species specificity of rat poisons by developing repellents that selectively repel non-target rodent species, but not target rats. Such repellents have been developed in insects, but not in mammals (Witzgall *et al.*, 2010). Species-specific repellents may offer a viable means to reduce the exposure of non-target species to rat poisons, without the need to ban anticoagulant poisons or further restrict their use.

To develop a species specific repellent for use in rat control, an understanding is needed of how that repellent would work. Repellents can take the form of odours, sounds and visual cues (Apfelbach *et al.*, 2005; Clapperton *et al.*, 2012; Hansen *et al.*, 2016; Hettena *et al.*, 2014; Wallingford *et al.*, 2017). Some species specific repellents that have been developed include insect pheromones to prevent oviposition on food crops and coloured baits to deter non-target birds from eating rodent poisons (Clapperton *et al.*, 2012; Wallingford *et al.*, 2017). Good knowledge of the biology of the pest and non-target species is needed to exploit differences in their ecology that can then be used as a species specific repellent.

One candidate odour cue that might be a suitable species specific repellent for use in rat control is rat odour. To find a suitable non-target repellent, we need to identify differences in ecology or behaviour between target rats and the non-target rodents that are most commonly poisoned by rat control. One difference in the ecology of these species is their relationship as predator and prey. Rats are potential predators of small rodents (Bridgman *et al.*, 2013; O'Boyle, 1974; Paul *et al.*, 1971). Therefore, small rodents may avoid contact with rats. Many species will avoid the odour of their predators as an indication of recent presence of the predator (Apfelbach *et al.*, 2005). Therefore, we might expect that small rodents would avoid rat odour. There is some evidence for this. Papes *et al.* (2010) found that house mice (*Mus musculus*) avoid rat odour in laboratory arenas and Richards (2012) found that wood mice avoid male rat odour in outdoor enclosures. To be effective as a non-target repellent, rat odour should not repel target rats. There is some evidence that rats are not repelled, and may even be attracted by the odour of other rats, although there are sex differences in responses (Christiansen, 1976; Selvaraj & Archunan, 2006; Takács *et al.*, 2016). Therefore, rat odour has the potential to function as a species specific non-target repellent.

In a series of experiments we tested the effects of male rat odour on the behaviour of three commonly poisoned non-target species from the UK, wood mice, bank voles and field voles. When tested in laboratory arenas, male rat odour was repellent to bank voles and field voles, but not wood mice. When tested in outdoor, semi-natural enclosures, male rat odour was

repellent to all three species. In addition, rat body odour had a stronger repellent effect than rat faeces, and this repellent effect was confined to the box in which the odour was located. In addition, when tested in laboratory arenas male rat odour was not attractive or repellent to rats, but the response of male rats was highly variable, with one male appearing highly cautious in the presence of male scent. The results of this study suggest that rat odour is a candidate non-target repellent worth pursuing, but further work is needed to reduce the risk of repelling target rats.

2.3. Experiment 1. Bank voles and field voles, but not wood mice, avoid male rat odour in laboratory arenas

2.3.1. Introduction

To assess the potential of rat odour as a non-target rodent repellent, we tested wild caught wood mice, bank voles and field voles with rat odour in a laboratory arena. The use of laboratory arenas allowed close monitoring of test subject behaviour in a controlled environment. Previous data, recorded in outdoor semi-natural enclosures, indicated that wood mice avoid male rat odour (Richards, 2012). However, wood mice, bank voles and field voles show differing antipredator responses. Wood mice respond to a predatory threat by running away, whereas voles hide or freeze (Hansson, 1987; Jędrzejewski *et al.*, 1992). We hypothesised that wood mice would avoid rat odour by moving away from the odour source, but would not necessarily hide, whereas bank voles and field voles would hide in a location separate from the odour source.

2.3.2. Methods

2.3.2.1. Ethics statement

During this experiment test subjects were exposed to the odour of a predator in an enclosed arena, with the potential to cause transient stress responses. The study was carried out under UK Home Office licence (PPL40/3492) in accordance with international best practice guidelines (Animals (Scientific Procedures) Act, 1986; Directive 2010/63/EU, 2010; Association for the Study of Animal Behaviour, 2018; NC3Rs *et al.*, 2017). The study was approved by the University of Liverpool Animal Welfare Committee.

2.3.2.2. Animal subjects

Behavioural test subjects were 6 male and 6 female bank voles, 6 male and 6 female field voles and 6 male and 6 female wood mice. All subjects were wild caught in Northwest England 1 to 6 months prior to testing. Odour donors were 6 adult male Wistar rats (HsdHan®:WIST, InVivo, Bicester, UK), aged 11 months. Male rats were used as odour donors as there is some evidence that male rats are more likely than female rats to predate small rodents and male rat odour is repellent to wood mice in semi-natural enclosures (Paul *et al.*, 1971; Richards, 2012).

All test subjects were housed in the same room. Bank voles and field voles were housed singly in 48 x 15 x 13 cm cages (M3, North Kent Plastics, Coalville, UK). Wood mice were

housed singly in 38 x 25 x 18 cm cages (RM2, North Kent Plastics, UK). Odour donor rats were housed in a separate room to test subjects, in same sex pairs, in 56 x 38 x 22 cm cages (RC2R, North Kent Plastics, UK), in a room containing both male and female rats. Separate protective clothing was worn when interacting with rats to ensure no transfer of scent to test subjects.

All animals were fed 5002 certified rodent diet (LabDiet, St Louis, USA) *ad libitum* and had access to *ad libitum* water. The diets of wood mice, bank voles and field voles were supplemented with Harry Hamster complete muesli (Supreme Petfoods Ltd., Ipswich, UK) and hay. Field voles were also given cut grass. All cages had Corn Cob Absorb 10/14 substrate (IPS Product Supplies Limited, London, UK) lining the base. Cardboard tubes and paper wool nest material were provided to all animals for enrichment. In addition, rats were given 15 x 8 cm plastic tubes for enrichment.

All housing and test rooms were maintained at 21 °C, 55% humidity and 20 air changes per hour. Throughout the test period all animals were housed on a reversed 12:12 hour light-dark cycle with lights off at 0800 h. All behavioural experiments were conducted in the dark phase of the light cycle under dim red lights.

2.3.2.3. Laboratory arena design

The laboratory arena measured 1.2 x 1.2 m, with solid laminated chipboard walls and base. Two 28 x 25 x 13 cm Roguard® Extra bait boxes (BASF, Cheadle Hulme, UK) were placed on opposing sides of the arena (Figure 2.1). Bait boxes were used in all experiments to demonstrate avoidance from an object relevant to pest control.

2.3.2.4. Laboratory arena test procedure

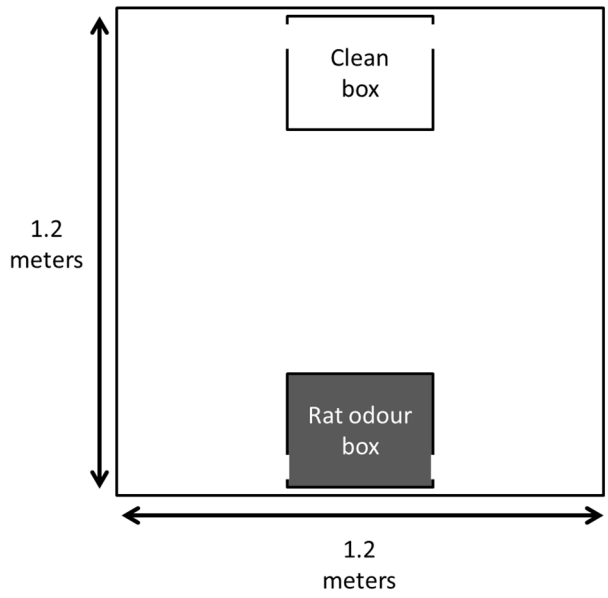
Test subjects were given a choice between a rat odoured box and a clean box. Twenty four to 48 hours prior to testing, subjects were habituated to the test arena for 30 minutes, with no odour stimulus in either box. Following habituation, one of the boxes was scented with rat odour by confining two adult male Wistar rats in it for 10 minutes, following which 10 faecal pellets and approximately 5 g of soiled nest material was added to the box. The other box contained approximately 5 g of clean nest material. Subjects were placed into the centre of the test arena and their behaviour was recorded in an adjacent room on DVD for 30 minutes. The order of habituation, testing and side of arena containing the odoured box were randomised across all species in a balanced design. Habituation and testing were conducted in the dark phase of the light cycle under dim red lights. Between each subject,

the arena was cleaned with Teepol Multipurpose detergent (Teepol, Orpington, UK) and 70% ethanol. Analysis of DVD recordings was performed blind to the arena side containing the odour stimulus. The duration inside each box, number of entries to each box and duration in each half of the arena was recorded.

2.3.2.5. Data analysis

To assess if each species avoided rat odour, Wilcoxon signed-rank tests compared within subject differences between arena sides. To assess if there was a difference in strength of avoidance between species, bias scores were calculated by subtracting values for the odoured side of the arena from the clean side of the arena. Bias scores were compared between species by Kruskal-Wallis tests. To compare overall hiding time between species, total time spent inside both boxes was compared between species by Kruskal-Wallis test. All statistical tests were bidirectional and a p-value of less than 0.05 was taken as significant. All statistical analyses were carried out using R 3.4.0. (R Core Team, 2016) with packages 'exactRankTests' (Hothorn & Hornik, 2015) and 'ggplot2' (Wickham, 2009).

A)



B)

C)

Figure 2.1. Design of laboratory test arena. A) Overall arena design. **B)** Closed bait box. **C)** Open bait box.

2.3.3. Results

Bank voles, field voles and wood mice (n = 12 for each species) were given a choice between a box that was scented with rat odour and a clean box in a laboratory arena.

Bank voles (Wilcoxon signed rank test, V = 73, p = 0.005) and field voles (V = 68, p = 0.02) strongly avoided spending time inside boxes containing rat odour (Figure 2.2A). However, wood mice did not avoid spending time inside rat odoured boxes (V = 44, p = 0.73; Kruskal-Wallis test for difference between species, $\chi^2 = 10.41$, p = 0.005).

Bank voles entered boxes containing rat odour fewer times than clean boxes, but the difference was minimal (Wilcoxon signed rank test, V = 59.5, p = 0.02; Figure 2.2B). Field voles (V = 57.5, p = 0.16) and wood mice (V = 45.5, p = 0.64) entered rat odoured and control boxes with equal frequency (Kruskal-Wallis test, $\chi^2 = 3.44$, p = 0.18).

While bank voles and field voles avoided boxes containing rat odour, they did not avoid spending time in the general vicinity of rat odoured boxes (duration in arena side, outside boxes; Wilcoxon signed rank test, V = 52, p = 0.34; V = 51, p = 0.38; Figure 2.2C). Wood mice also did not avoid spending time in the general vicinity of rat odoured boxes (V = 58, p = 0.15; Kruskal-Wallis test for difference between species $\chi^2 = 0.02$, p = 0.99)

A lack of avoidance of rat odoured boxes by wood mice could be explained by their reduced use of hiding as an antipredator response compared to voles. To test this we compared the total time all three species spent inside boxes. Overall, wood mice tended to spend slightly less time inside bait boxes compared to bank voles and field voles, although this did not reach significance (Kruskal-Wallis test, $\chi^2 = 5.07$, p = 0.08; Figure 2.2D).

2.3.4. Discussion

In this experiment, bank voles and field voles avoided spending time in a box scented with rat odour, but wood mice did not. The lack of avoidance of rat odour by wood mice in our experiment is contrary to expectations, as Richards (2012) found that wood mice avoid rat odour when tested in outdoor semi-natural enclosures. There are multiple possible explanations for this discrepancy. First, the discrepancy may be due to the difference in antipredator responses of wood mice, bank voles and field voles. While bank voles and field voles tend to hide in dense undergrowth, wood mice will more often flee (Jędrzejewski *et al.*, 1992). In our laboratory arenas, we were able to measure the hiding response of voles by time spent inside the clean box. However, it was more difficult to measure a fleeing

response of wood mice using this experimental design. We observed wood mice repeatedly trying to jump out of the test arena and frequently running in and out of bait boxes. It may be that wood mice were trying to escape from the arena due to the presence of rat odour, but a lack of side bias in their escape response prevented us from detecting it. To assess if rat odour causes a general effect on wood mouse, bank vole and field vole behaviour an additional test could be run with two clean boxes in the arena. Test subject behaviour could then be compared between a clean arena and one containing a rat odoured box.

A further explanation is that, in our test arena, wood mice were too stressed to respond to rat odour. We have observed that wood mice do not adapt to captivity as well as voles and tend to be highly stressed when placed into open arenas, such as the one used in this experiment. This stress is displayed by repeated escape attempts and extreme restlessness when in open arenas. The stress of being in open arenas may have overridden any behavioural response to the presence of rat odour.

Another explanation is that the low light levels in our test arena reduced the likelihood that wood mice would respond to predator odour. Our test arenas were lit by low intensity red lighting, making them very dark. However, Richards (2012) outdoor experiment, whilst conducted overnight, would have had higher light levels due to moon illumination and ambient light from surrounding buildings. Increased light levels (due to moon illumination) led to reduced trapping of wood mice when fox odour was present at trap entrances (Navarro-Castilla & Barja, 2014). Thus, we might expect that in the low light level conditions of our test arena, wood mice would show reduced avoidance of predator odour.

Overall, it would appear that open laboratory arenas may not be the most appropriate environment to test the anti-predatory responses of wood mice. Further testing in outdoor enclosures is needed to test if rat odour is avoided under more naturalistic conditions, where wood mice have more chance to display their natural antipredator responses.

It was interesting to observe that even though bank voles and field voles spent less time in rat odoured bait boxes, they did not avoid the side of the arena containing these bait boxes. This may indicate that rat odour is only detectable by these animals when they are very close to its source. Alternatively, avoidance of rat odour may be restricted to a small area, close to the odour source.

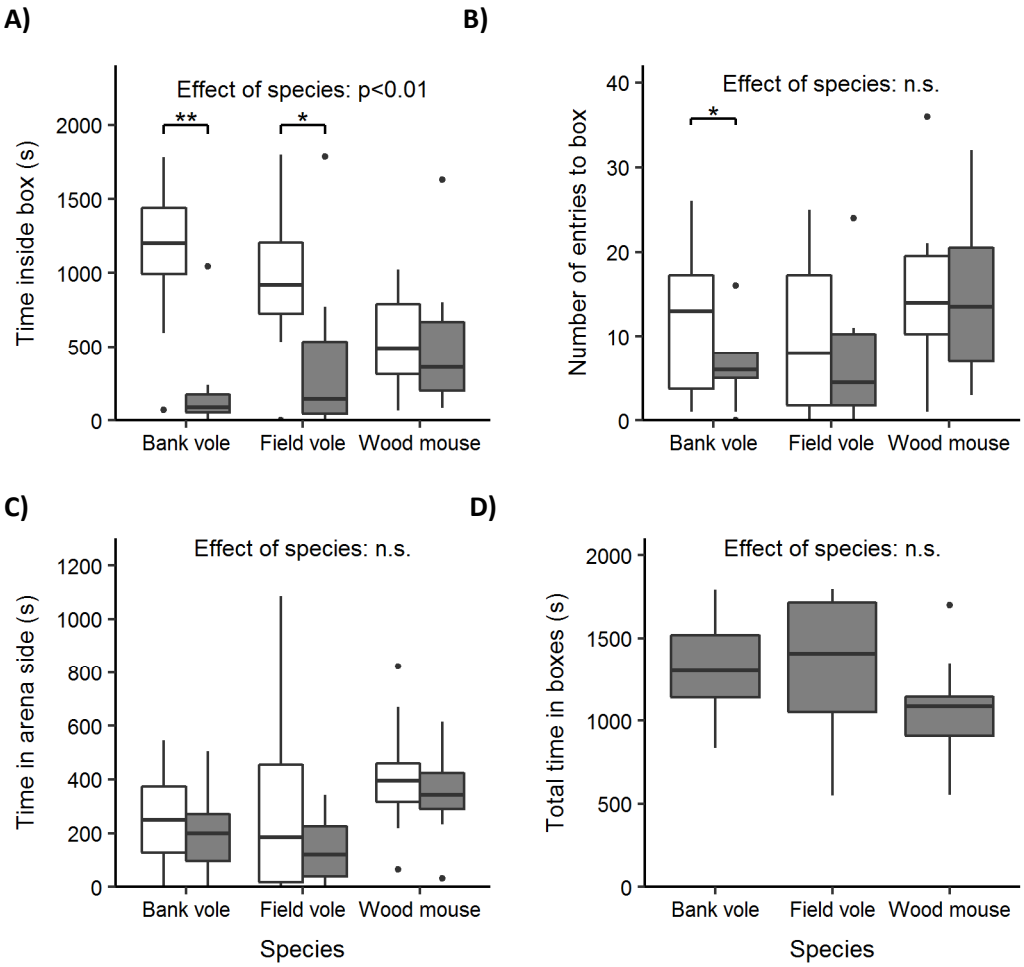


Figure 2.2. Response of three rodent species to rat odour in a laboratory arena. Animals were presented with a box scented with rat body odour, faeces and nest material (dark grey bars) versus a clean box with clean nest material (white bars). **A)** Duration inside boxes. **B)** Number of entries to boxes. **C)** Duration in arena side, outside boxes. **D)** Total duration inside both bait boxes. Box plots show medians (black bar), interquartile range (IQR; boxes), 1.5 IQR (whiskers) and outliers (dots). Effect of species was compared with Kruskal-Wallis tests. Odour versus control were compared with Wilcoxon Signed Rank tests (* $p < 0.05$, ** $p < 0.01$).

2.4. Experiment 2. Wood mice, bank voles and field voles avoid male rat odour in outdoor semi-natural enclosures.

2.4.1. Introduction

To test if male rat odour is repellent to non-target rodents in a more naturalistic environment than laboratory arenas, we tested the response of wood mice, bank voles and field voles to male rat odour in outdoor semi-natural enclosures. The experimental design was similar to Richards (2012), who compared the response of wood mice to boxes scented with rat odour with their response to clean boxes in large, outdoor enclosures. In a control test, Richards (2012) compared the response of wood mice to house mouse odoured boxes with their response to clean boxes, to test if avoidance of rat odour was due to rat odour itself, or the presence of a novel odour. Richards (2012) found that wood mice avoided rat odour, but not house mouse odour. In this experiment house mouse odour was included with rat odour and clean boxes in one test. We hypothesised that wood mice, bank voles and field voles would consume less food from boxes scented with rat odour, compared to clean boxes and boxes scented with house mouse odour.

2.4.2. Methods

2.4.2.1. Ethics statement

All outdoor enclosure experiments were non-invasive behavioural tests that did not involve pain, suffering or lasting harm and were carried out in accordance with international best practice guidelines (Animals (Scientific Procedures) Act, 1986; Directive 2010/63/EU, 2010; Association for the Study of Animal Behaviour, 2018; NC3Rs *et al.*, 2017). The study was approved by the University of Liverpool Animal Welfare Committee. No specific licences were required to carry out the work, as animals were free to approach or avoid the test stimuli.

2.4.2.2. Animal subjects

Subjects were adult male and female field voles and bank voles (n = 30 each species and sex) and adult male and female wood mice (n = 20 each sex). Subjects were wild caught from North West England 1 to 17 months prior to the start of the experiment. Following capture, subjects were maintained indoors until transfer to an outdoor enclosure, except male wood mice, which were placed into an outdoor enclosure on the day they were captured. Indoors, subjects were maintained under conditions as described for Experiment 1. Animals captured before March 2015 were fed 5002 certified rodent diet (LabDiet, USA). Animals captured

after March 2015 were fed 5FL2 EURodent Diet (LabDiet, USA). Odour donors were 7 adult male Wistar rats (HsdHan®:WIST, InVivo, UK), aged 6 to 12 months, and 7 adult male wild derived house mice (bred for 5 - 10 generations from populations captured in Northwest England), aged 12 to 22 months. Rats were housed and maintained as described for Experiment 1. House mice were housed singly in 48 x 15 x 13 cm cages (M3, North Kent Plastics, UK), in a separate room to test subjects and rats, and maintained as described for Experiment 1.

2.4.2.3. Outdoor enclosure design

Outdoor enclosures were 25 x 40 m, enclosed by 2 m high encircling walls, the bottom half of which was solid metal sheeting and the top half wire mesh. The ceiling of the enclosure was covered with wire mesh. Each enclosure contained grassland habitat, surrounded by a 0.5 m flagstone border. Laboratory animal food (5002 certified rodent diet or 5FL2 EURodent Diet (LabDiet, USA) mixed with Harry Hamster complete muesli (Supreme Petfoods Ltd., UK)) was provided to subjects in 5 covered plastic boxes, 4 of which were placed halfway along each side of the enclosure, within the vegetation, and the final one placed in the centre of the enclosure. Water was provided beside food boxes in plastic dispensers. Additional shelter was provided by placing the bottom part of 4 cages (RM2, North Kent Plastics, UK), filled with hay, upside down in each quadrant of the enclosure.

Test subjects were transferred to outdoor enclosures as shown in Table 2.1. Each species was housed separately so we could assign bait consumption to a particular species. Each sex was housed separately to ensure animal numbers remained constant for the duration of the study. No testing was carried out for the first 14 days following transfer to allow subjects to habituate to test enclosures.

Table 2.1. Timing of transfer of test subjects to outdoor semi-natural enclosures.

Species	Sex	Date of transfer
Wood mouse	Female	November 2014
	Male	April to May 2017
Field vole	Female	February to April 2015
	Male	September 2015
Bank vole	Female	October 2015
	Male	August 2015

2.4.2.4. Outdoor enclosure test procedure

To test the feeding rate of rodents from scented bait boxes, wood mice, bank voles and field voles were given the choice to feed from bait boxes containing rat odour, house mouse odour or clean boxes. House mouse odour was used as a control odour to confirm that avoidance is specific to rat odour, and not a response to the presence of a novel odour. Bait boxes were placed into an enclosure each evening and collected the following morning.

In each enclosure, 21 Roguard® Extra bait boxes (BASF, UK) were placed at 5 m intervals. For wood mice bait boxes were arranged around the circumference of enclosures (Figure 2.3A). When bait boxes were arranged around the circumference of enclosures containing voles, they did not feed regularly from them. Therefore, for voles, bait boxes were arranged throughout the enclosure (Figure 2.3B).

Each evening, a 20 g Detex® non-toxic bait block (Bell laboratories, Sudbury, UK) was placed in each bait box. Non-toxic bait blocks contain the same ingredients as bait normally used in pest control, but do not contain any poison. Use of non-toxic bait blocks allows a better assessment of the feeding behaviour of non-target rodents in real-world rodent control conditions than if normal laboratory food was used. Food boxes were removed prior to placing bait boxes to ensure that bait boxes were the only major source of food during testing. Food boxes were replaced during the day, when bait boxes were not present in the test enclosure. Subjects were habituated to feeding from bait boxes for at least 4 nights prior to testing.

For testing, 7 bait boxes were scented with rat odour by confining one adult male Wistar rat in each box for 10 minutes, and adding 10 faecal pellets from the rat's cage. Seven bait boxes were scented by confining one adult male wild derived house mouse in each box for 10 minutes, and adding 10 faecal pellets from the mouse's cage. The final 7 boxes were clean. The order of bait box scent was randomised within trios of bait boxes each night (Figure 2.3). Each morning the weight of bait eaten from each bait box was recorded and all bait boxes were washed with dilute Teepol Multipurpose detergent (Teepol, UK). Between each night of testing bait boxes were moved 2.5m clockwise or anticlockwise to prevent subjects associating an odour with a particular location. Wood mice were tested for 4 nights. Bank voles and field voles were tested for 8 nights as they fed from bait boxes less frequently than wood mice.

2.4.2.5. Data analysis

To assess if box odour affected the likelihood of any bait being consumed, Pearson's Chi-squared test compared the frequency of any bait being consumed between box odours. Planned 2x2 Chi-squared tests then compared differences between rat odour and control odours (clean and house mouse). As activity varied across enclosures, no bait was eaten from many bait boxes, leading to an excess of zero values in our data. To overcome this we first divided boxes into trios based on location within an enclosure, and assigned each trio a location number (Figure 2.3). If no bait was consumed from any of the boxes in a trio location during a test night we removed all boxes in that trio from the analysis.

To assess if box odour affected the weight of bait eaten, where bait was consumed, boxes with no bait take were removed from the analysis. Weight of bait eaten from bait boxes was then log transformed and analysed using a linear mixed effects model with box odour as a fixed effect. As each sex of each species was housed in separate enclosures at different time points we could not make direct comparisons for differences in responses between species and sexes. Therefore, each sex of each species was assigned a group number (1 to 6) and group was added as a random effect to the model. Day of testing (1 to 4 for wood mice and 1 to 8 for voles) was nested within group number to account for repeated measures. Box position (1 to 21) was nested within group number to account for variation in bait take due to microhabitat differences. Model significance was calculated by ANOVA comparison to a null model (see supplementary information). The model was validated using quantile-quantile (Q-Q) plots and residual distributions (Figure S2.1). P-values of model fixed effects were calculated using likelihood ratio tests (Crawley, 2007). Rat odour was set as the reference to allow comparison with control odours (clean and house mouse).

For all analyses, a p-value of less than 0.05 was taken as significant. Statistical analysis was carried out using R 3.4.0. (R Core Team, 2016) with packages 'lme4' (Bates *et al.*, 2015), 'lsmeans' (Lenth, 2016) 'afex' (Singmann *et al.*, 2017) and 'ggplot2' (Wickham, 2009).

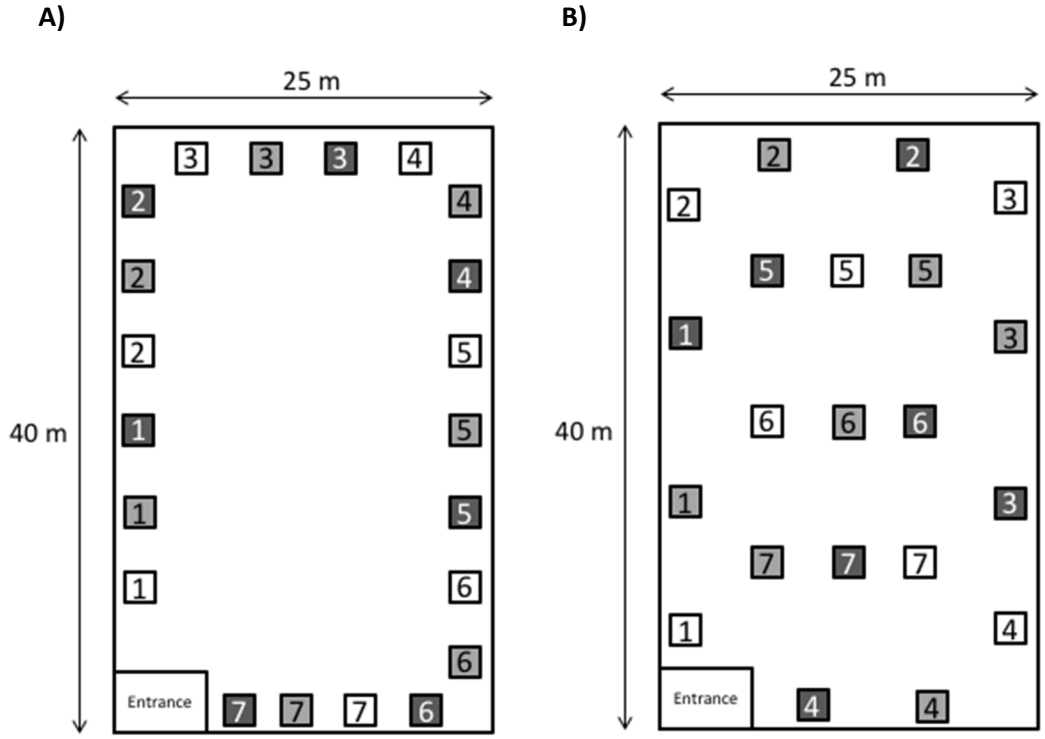


Figure 2.3. Outdoor enclosure layout. Clean bait boxes (clear squares), house mouse odoured bait boxes (light grey squares) and rat odoured bait boxes (dark grey squares) were arranged in an outdoor enclosure. All boxes contained a 20 g non-toxic bait block. **A)** Wood mouse layout. **B)** Bank vole and field vole layout. Numbers denote locations defined for statistical analysis.

2.4.3. Results

Wood mice, bank voles and field voles were given a choice to feed from bait boxes scented with rat odour, bait boxes scented with house mouse odour and clean bait boxes in outdoor semi-natural enclosures. Testing was carried out over multiple nights (4 nights for wood mice, 8 nights for bank voles and field voles).

Wood mice, bank voles and field voles were more likely to feed from clean bait boxes than from rat odoured bait boxes ($\chi^2_1 = 13.77$, $p < 0.001$; Table 2.2). There was a non-significant trend for non-target rodents to take bait more frequently from house mouse odoured bait boxes compared to rat odoured bait boxes ($\chi^2_1 = 3.54$, $p = 0.06$).

Where bait was consumed, non-target rodents ate less bait from bait boxes scented with rat odour compared to bait boxes scented with house mouse odour and clean bait boxes (linear mixed effects model, overall model, $F_{2, 445} = 50.5$, $p < 0.001$, rat versus mouse, $t_{438} = 5.89$, $p < 0.001$; rat versus clean $t_{441} = 10.05$, $p < 0.001$). Where bait was consumed, median bait take was 4.85 g from clean boxes, 3.3 g from house mouse odoured boxes and 2.05 g from rat odoured boxes. Due to the experimental design it is not appropriate to compare effect sizes directly, but there was a consistent trend for each species to consume less from rat odoured boxes compared to clean boxes and house mouse odoured boxes (Figure 2.4).

Overall (including boxes where no bait was consumed), median bait take was 3.5 g from clean boxes, 2.2 g from house mouse odoured boxes and 0.9 g from rat odoured boxes. This represents a substantial decrease in consumption (74%) from rat odoured boxes compared to control boxes.

Table 2.2. Number of bait boxes eaten from by non-target rodents.

		Box odour			
		Clean	**House mouse**	**Rat**	**Total**
Wood mouse	Bait eaten	55 (34.6%)	54 (34.0%)	50 (31.4%)	159
	Bait not eaten	1 (11.1%)	2 (22.2%)	6 (66.7%)	9
Bank vole	Bait eaten	90 (36.4%)	84 (34.0%)	73 (29.6%)	247
	Bait not eaten	21 (24.4%)	27 (31.4%)	38 (44.2%)	86
Field vole	Bait eaten	59 (38.6%)	49 (32.0%)	45 (29.4%)	153
	Bait not eaten	23 (24.7%)	33 (35.5%)	37 (39.8%)	93
Total (All species)	Bait eaten	204 (36.5%)	187 (33.5%)	168 (30.1%)	559
	Bait not eaten	45 (23.9%)	62 (33.0%)	81 (43.1%)	188

Bait eaten refers to number of boxes where any bait was consumed.

Bait not eaten refers to number of boxes where no bait was consumed.

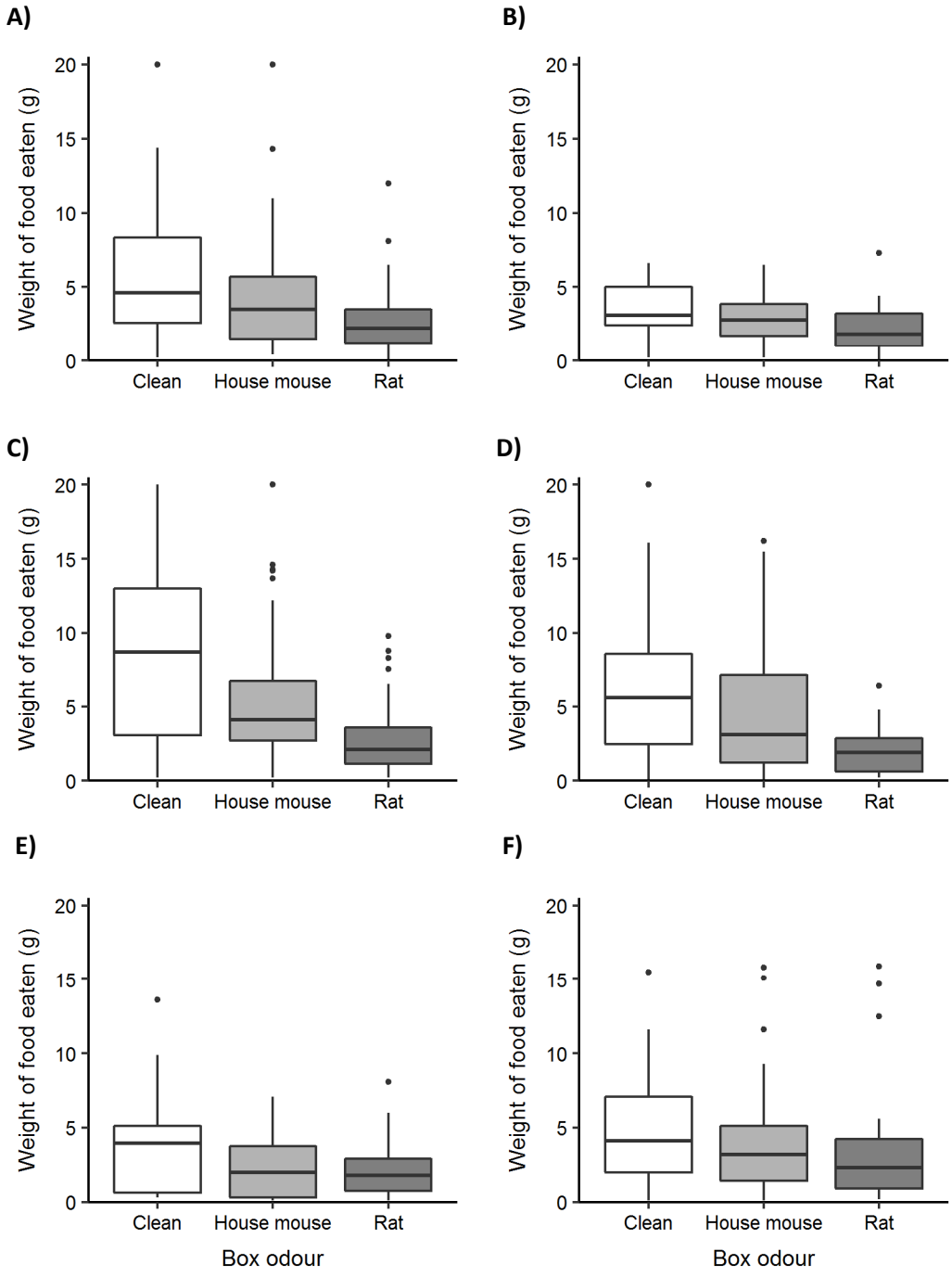


Figure 2.4. Weight of bait eaten per box per night by three rodent species from bait boxes scented with rodent odour. Animals were presented with bait boxes scented with rat body odour and faeces (dark grey bars), house mouse body odour and faeces (light grey bars) and or clean bait boxes (white bars). Bait boxes where no bait was consumed were removed from the analysis. All boxes contained a 20 g non-toxic bait block. Weight of food eaten by **A)** female wood mice, **B)** male wood mice, **C)** female bank voles, **D)** male bank voles, **E)** female field voles, **F)** male field voles. Box plots show medians (black bar), IQR (boxes), 1.5 IQR (whiskers) and outliers (dots).

2.4.4. Discussion

In outdoor semi-natural enclosures, wood mice, bank voles and field voles ate less non-toxic bait from bait boxes scented with rat odour compared to clean bait boxes and bait boxes scented with house mouse odour. The reduction in food take from rat odoured boxes compared to clean boxes indicates that non-target rodents avoided rat odour. However, from this result it is not possible to say if non-target rodents are avoiding rat odour specifically, or simply the presence of a novel odour. The finding that non-target rodents ate less from rat odoured boxes compared to house mouse boxes indicates that non-target rodents are specifically repelled by rat odour and not by any heterospecific odour cue.

These results are consistent with previous findings that male wood mice avoid eating from bait boxes scented with rat odour (Richards, 2012). However, these results are inconsistent with the lack of response of wood mice to rat odour in our laboratory test arena (Experiment 1). As discussed above, there are several possibilities why wood mice may not avoid rat odour in our laboratory arenas, but may avoid rat odour in a more natural environment.

2.5. Experiment 3. Rat body odour is more effective than rat faeces at repelling wood mice in outdoor semi-natural enclosures.

2.5.1. Introduction

Having established in Experiment 2 that non-target rodents ate less food from boxes scented with rat odour, we wanted to identify which source of rat odour caused the greatest reduction in bait take. We decided to use wood mice for this part of the experiment, as they are the most likely of our three species to be involved in non-target poisoning (Brakes & Smith, 2005; Geduhn *et al.*, 2014). We compared avoidance of an odour that rats would deposit in the environment (faeces) to the body odour of the animal itself. We hypothesised that wood mice would avoid both rat body odour and rat faeces, as both are indicators of potential presence of a rat. However, as rat body odour is likely to be a more reliable indicator of recent rat presence, we hypothesised that avoidance of rat body odour would be stronger than avoidance of rat faeces.

2.5.2. Methods

2.5.2.1. Animal subjects

Subjects were 20 female wood mice housed as for Experiment 2.

2.5.2.2. Outdoor enclosure design

The test enclosure was the same as for Experiment 2. The same female wood mice were tested in this experiment as in Experiment 2, with testing for this experiment beginning the day after the conclusion of Experiment 2.

2.5.2.3. Outdoor enclosure test procedure

Testing was carried out in a similar manner to Experiment 2. For this experiment bait boxes contained rat body odour, rat faeces, or were clean. On each night of testing 21 bait boxes were placed into the enclosure. Seven boxes were scented by confining one adult male Wistar rat in each bait box for 10 minutes, 7 boxes were scented by adding 10 faecal pellets from an adult male Wistar rat's cage and 7 boxes were clean. Any faeces deposited by rats in boxes during body odour collection were removed before boxes were placed into the enclosure. Each morning the weight of non-toxic bait eaten was measured. This experiment was repeated for 4 nights. The order of scent within each trio of boxes was randomised each night and the boxes were moved 2.5 m clockwise or anticlockwise each night.

2.5.2.4. Data analysis

Weight of bait eaten was log transformed and compared by a linear mixed effects model with box odour as a fixed effect. Date and position of box (1-21) were included as random effects. Model significance was calculated by ANOVA comparison to a null model (see supplementary information). P-values of model fixed effect were calculated using likelihood ratio tests (Crawley, 2007). Planned comparisons were calculated between all pairs of treatment groups using Tukey contrasts. Models were validated using Q-Q plots and residual distributions (Figure S2.2). A p-value of less than 0.05 was taken as significant. All statistical analyses were carried out using R 3.4.0. (R Core Team, 2016) with packages 'lme4' (Bates *et al.*, 2015), 'lsmeans' (Lenth, 2016) 'afex' (Singmann *et al.*, 2017) and 'ggplot2' (Wickham, 2009).

2.5.3. Results

A population of 20 female wood mice were given a choice to feed from bait boxes scented with rat body odour, bait boxes scented with rat faeces and clean bait boxes in outdoor semi-natural enclosures. Testing was carried out over four nights.

Female wood mice ate less from bait boxes scented with rat faeces (linear mixed effects model, $F_{2, 60} = 19.0$, $p < 0.001$; Tukey contrast, $t_{60} = 2.75$, $p = 0.02$) and rat body odour ($t_{60} = 6.17$, $p < 0.001$) compared to clean bait boxes. Female wood mice ate less from bait boxes scented with rat body odour compared to rat faeces ($t_{60} = 3.42$, $p = 0.003$; Figure 2.5).

Median bait take was 21% less from rat faeces boxes, and 62% less from rat body odoured boxes, compared to clean boxes.

2.5.4. Discussion

In outdoor semi-natural enclosures, wood mice avoided eating from boxes scented with rat body odour and boxes scented with rat faeces. Avoidance of rat body odour was greater than avoidance of rat faeces. These findings are consistent with Blanchard *et al.* (2003), who found that rats avoided both cat faeces and body odour, but only body odour was able to produce aversive conditioning. In addition, Masini *et al.* (2005) found that exposure to ferret body odour, but not faeces, increased levels of corticosterone in rats. When a prey animal encounters the odour of a predator they can ignore it and continue with their normal behaviour, or they can initiate defensive behaviours (Zylberberg & DeWeese, 2011). Defensive behaviours can include fleeing, hiding, a reduction in time spent foraging and

movement to different habitats (Apfelbach *et al.*, 2005). However, there are costs to defensive behaviours, including depletion of energy due to fleeing and loss of foraging time (Hegab *et al.*, 2015; Zylberberg & DeWeese, 2011). Computer modelling has indicated that the decision to initiate defensive behaviours depends on the certainty with which a predator cue indicates the presence of a predator (Zylberberg & DeWeese, 2011). Body odour is a more reliable indicator than faeces of recent predator presence (Blanchard *et al.*, 2003). Therefore, prey species should show defensive behaviours more readily in the presence of predator body odour than predator faeces and this is consistent with our findings.

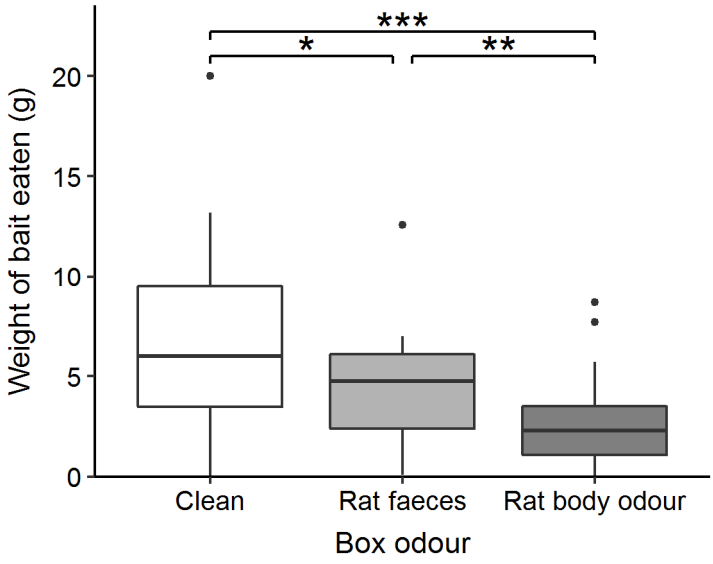


Figure 2.5. Weight of bait eaten per box per night by female wood mice from bait boxes scented with rat odour. Animals were presented with bait boxes scented with rat body odour (dark grey bars), bait boxes scented with rat faeces (light grey bars) and bait boxes that were clean (white bars). All boxes contained a 20 g non-toxic bait block. Box plots show medians (black bar), IQR (boxes), 1.5 IQR (whiskers) and outliers (dots). Weight of food eaten from bait boxes was compared using a linear mixed effects model, followed by Tukey post-hoc comparisons (* $p < 0.05$, ** $p < 0.01$), *** $p < 0.001$).

2.6. Experiment 4. The repellent effects of rat odour do not extend beyond the scented bait box.

2.6.1. Introduction

An ideal non-target repellent would not affect the behaviour of non-target rodents or their use of the environment, unless they were close to pest control devices. In Experiments 2 and 3 wood mice consumed more bait from clean bait boxes compared to rat odoured boxes placed 5 m away. In Experiment 1 bank voles and field voles avoided boxes containing rat odour, but did not avoid the area surrounding those boxes. To investigate further if rat odour repulsion extends beyond the odoured box we tested wood mice in semi-natural enclosures with clean bait boxes placed directly next to rat odoured bait boxes. We hypothesised that avoidance of a bait box containing rat odour would be confined to the odoured box, and not extend to adjacent boxes.

2.6.2. Methods

2.6.2.1. Animal subjects

Subjects were 20 female wood mice housed as for Experiment 2.

2.6.2.2. Outdoor enclosure design

The test enclosure was the same as for Experiment 2. Female wood mice were tested for this experiment one month after the conclusion of Experiment 3.

2.6.2.3. Outdoor enclosure test procedure

For this experiment 40 bait boxes were placed into the enclosure in 20 pairs of 2 boxes. Within each pair, bait boxes were placed so that they touched, but entrances were on outside edges (Figure 2.6B). Boxes were organised by alternating pairs of boxes comprising a box scented with rat body odour and a clean box, with pairs comprising two clean boxes (Figure 2.6). This gave a total of 10 rat odoured boxes and 30 clean boxes in the enclosure. Rat odoured boxes were scented by confining one adult male Wistar rat in each box for 10 minutes. Boxes were placed into the enclosure each evening and the weight of non-toxic bait eaten was measured each morning. This experiment was repeated for 4 nights. The location of scent within and between box pairs was randomised each night and the boxes were moved 2.5 m clockwise or anticlockwise each night.

2.6.2.4. Data analysis

Weight of bait eaten was log transformed and compared by a linear mixed effects model with box odour as a fixed effect. Date and position of box (1-40) were included as random effects (see supplementary information). Orthogonal contrasts were used to compare weight of bait eaten within and between box pairs (Crawley, 2007). First, we assessed if presence of rat odour caused a reduction in weight of bait eaten within a box pair. We compared weight of bait eaten within box pairs comprising one rat odoured box and one clean box ('Rat' versus 'Clean by rat'). As a control, we compared weight of bait eaten within box pairs comprising two clean boxes ('Clean 1' versus 'Clean 2'). Next, we assessed if presence of rat odour within a box pair caused a reduction in overall bait take in that pair compared to clean box pairs placed at a distance. We compared weight of bait eaten from two clean boxes with weight of bait eaten from one clean box and one rat odoured box ('Clean 1' and 'Clean 2' versus 'Rat' and 'Clean by rat'; Table S2.1). These contrasts were defined *a priori* based on the experimental design.

Model significance was calculated by ANOVA comparison to a null model (see supplementary information). P-values of model fixed effect were calculated using likelihood ratio tests (Crawley, 2007). Models were validated using Q-Q plots and residual distributions (Figure S2.3). A p-value of less than 0.05 was taken as significant. All statistical analyses were carried out using R 3.4.0. (R Core Team, 2016) with packages 'lme4' (Bates *et al.*, 2015), 'lsmeans' (Lenth, 2016) 'afex' (Singmann *et al.*, 2017) and 'ggplot2' (Wickham, 2009).

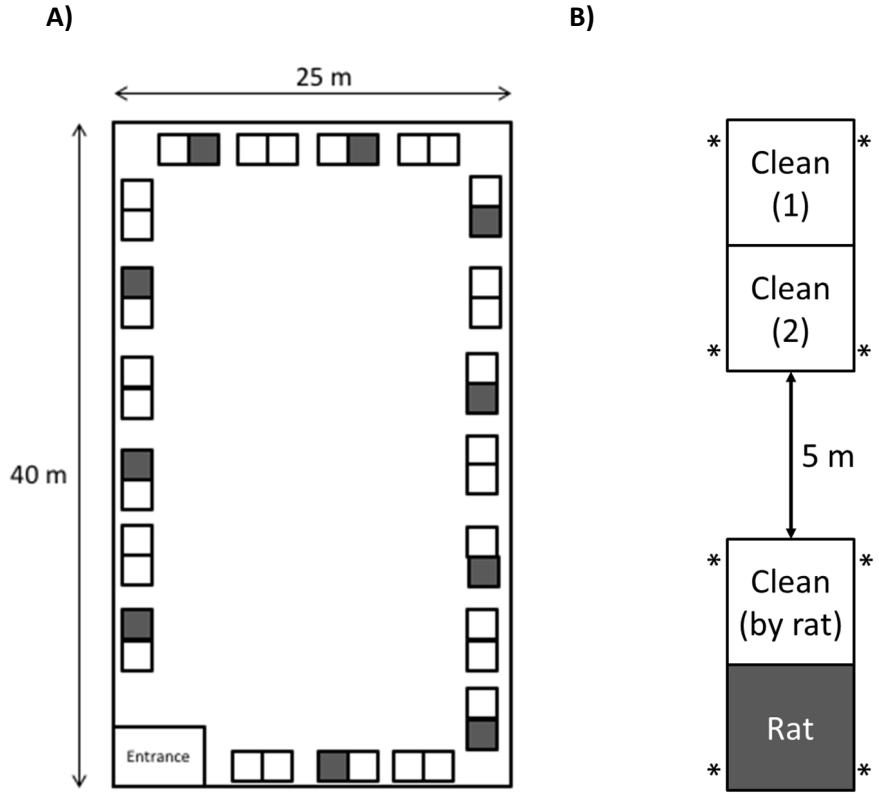


Figure 2.6. Arrangement of box pairs within enclosure. Clean bait boxes (clear squares), and rat odoured bait boxes (dark grey squares) were arranged in pairs in an outdoor enclosure. **A)** Overall enclosure plan. **B)** Close up of bait box pairs (* indicates location of bait box entrances).

2.6.3. Results

A population of 20 female wood mice were given a choice to feed from pairs of bait boxes containing one rat scented bait box and one clean box, or two clean boxes, in outdoor semi-natural enclosures. Testing was carried out over four nights.

Within box pairs, wood mice ate less from boxes scented with rat odour compared to adjoining clean boxes (linear mixed effects model, $F_{3, 114} = 10.1$, $p < 0.001$; $t_{114} = 5.11$, $p < 0.001$; Figure 2.7). Median bait take was 87% less from rat odoured boxes compared to clean boxes adjoining rat odoured boxes. There was no difference in the weight of bait eaten by wood mice within pairs of clean boxes ($t_{114} = 0.43$, $p = 0.67$).

Wood mice ate slightly less from pairs of boxes containing one rat odoured box compared to pairs of clean boxes ($t_{114} = 5.11$, $p = 0.05$; Figure 2.7). Median bait take was 32% less from pairs of pairs of boxes containing one rat odoured box, compared to clean boxes.

2.6.4. Discussion

Wood mice did not avoid eating from clean bait boxes when rat odoured bait boxes were placed next to them. This response indicates that the effect of rat body odour is confined to the box in which it is present, rather than to the area surrounding the box. The lack of effect of rat odour on an adjacent clean bait box could indicate that rat odour was not strong enough to be detectable outside of bait boxes. Alternatively, it could be that wood mice only perceive rat odour as threatening in close proximity to the odour. Wood mice ate slightly less non-toxic bait from pairs of clean and rat odoured boxes compared to pairs of clean boxes. However, median bait take was higher from clean boxes adjoining rat odoured boxes (3.0 g) compared to clean boxes 5 m away from rat odoured boxes (2.4 g/2.1g). This suggests that wood mice may compensate for not feeding from a high-risk site by increasing their food intake from a neighbouring low-risk site.

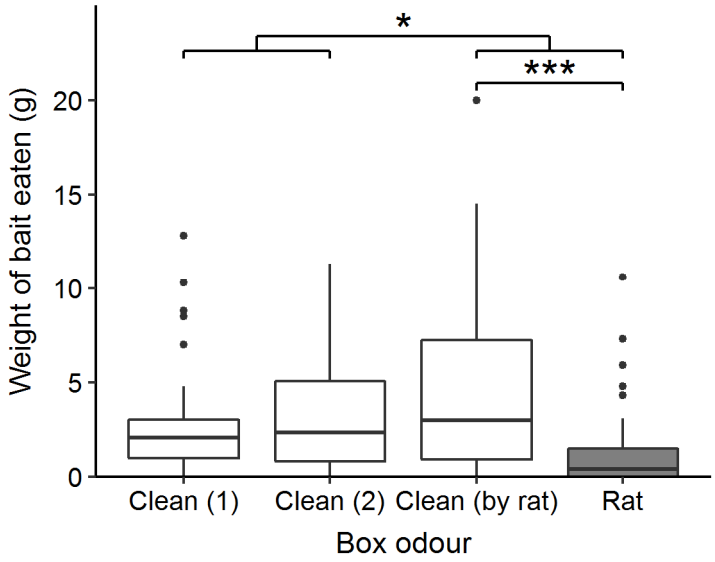


Figure 2.7. Weight of bait eaten per box per night by female wood mice from bait boxes scented with rat odour. Animals were presented with bait boxes scented with rat body odour (grey bars) and bait boxes that were clean (white bars). All boxes contained a 20 g non-toxic bait block. Box plots show medians (black bar), IQR (boxes), 1.5 IQR (whiskers) and outliers (dots). Weight of food eaten from bait boxes was compared using a linear mixed effects model, with planned orthogonal contrasts (* $p < 0.05$, *** $p < 0.001$).

2.7. Experiment 5: The response of male and female rats to male rat odour.

2.7.1. Introduction

The potential for male rat odour to be used to reduce exposure of non-target rodents to rat poisons relies on male rat odour not repelling target rats. Current data indicates that the response of rats to male rat odour is complicated by sex differences in responses. Female rats are attracted to a variety of male odour cues, including volatile compounds specific to male rats (Selvaraj & Archunan, 2006; Takács *et al.*, 2016; Zhang *et al.*, 2008). However, the response of male rats to male rat odour is more variable (Takács *et al.*, 2016). We tested the response of rats of both sexes to male rat odour in a compartmentalised laboratory arena. We hypothesised that rats would not be repelled by male rat odour.

2.7.2. Methods

2.7.2.1. Animal subjects

Subjects were 11 male and 11 female, 3 month old, first generation crosses between Brown Norway (BN/SsNOlaHsd, InVivo, UK) and Wistar rats (HsdHan®:WIST, InVivo, UK). Odour donors were 6 male, 14 month old Wistar rats (HsdHan®:WIST, InVivo, UK).

Rats were housed in same sex pairs in 56 x 38 x 22 cm cages (RC2R, North Kent Plastics, UK), in rooms containing both male and female rats. Subject rats were housed in a separate room to odour donor rats. Rats were maintained as described for Experiment 1.

2.7.2.2. Laboratory arena design

The laboratory test arena consisted of two 1.2 x 1.2 m arenas, with laminated chipboard walls and bases, connected by a 6 cm wide by 5 cm long tunnel. In each arena an empty 28 x 25 x 13 cm Roguard® Extra bait box (BASF, UK) was placed against the wall opposite the connecting tunnel (Figure 2.8). Bait boxes were used in this experiment as we wanted to check the response of rats to an odour cue presented in a confined space, typical of that used during rat control.

2.7.2.3. Laboratory arena test procedure

Test subjects were given a choice between a rat odoured box and a clean box. Subjects were habituated to the test arena, for 30 minutes one day prior to testing, with no odour stimulus in either box. During habituation, subjects were placed individually into one side of the test arena. The side of subject placement was randomised. Following habituation, one box was scented with rat odour by confining an adult male Wistar rat in it for 10 minutes, following

which 10 faecal pellets were added to the box. The other box was clean. Subject rats were introduced into the arena on one side of the tunnel. Behaviour of subjects was recorded for 30 minutes using remote video monitoring. The order of rat subjects, side of subject placement and side of arena containing the odoured box were randomised in a balanced design. Habituation and testing were conducted in the dark phase of the light cycle under dim red lights. Between each subject, the arena was cleaned with Teepol Multipurpose detergent (Teepol, UK) and 70% ethanol. Analysis of DVD recordings was performed blind to the arena side containing the odour stimulus. The duration inside each box, number of entries to each box and duration in each half of the arena was recorded.

2.7.2.4. Data analysis

Bias scores were calculated by subtracting values for the odoured side of the arena from the clean side of the arena. Bias scores were calculated for time spent in boxes, number of entries to boxes and time spent in arena sides (outside boxes). To assess differences in responses between sexes, bias scores were compared between males and females using t-tests where data approximated normality and Mann-Whitney U tests where data was not normal. To test for attraction or avoidance bias scores were compared to zero using one-sample t-tests where data approximated normality and Wilcoxon signed-rank tests where data was not normal. Three male rats were discounted from the analysis because they did not enter both sides of the arena during the 30 minute test period. Two of these rats were placed into the arena side containing the rat odoured box. The third rat was placed into the arena side containing the clean box. All statistical tests were bidirectional. A p-value of less than 0.05 was taken as significant. All statistical analyses were carried out using R (R Core Team, 2016) with packages ‘exactRankTests’ (Hothorn & Hornik, 2015) and ‘ggplot2’ (Wickham, 2009).

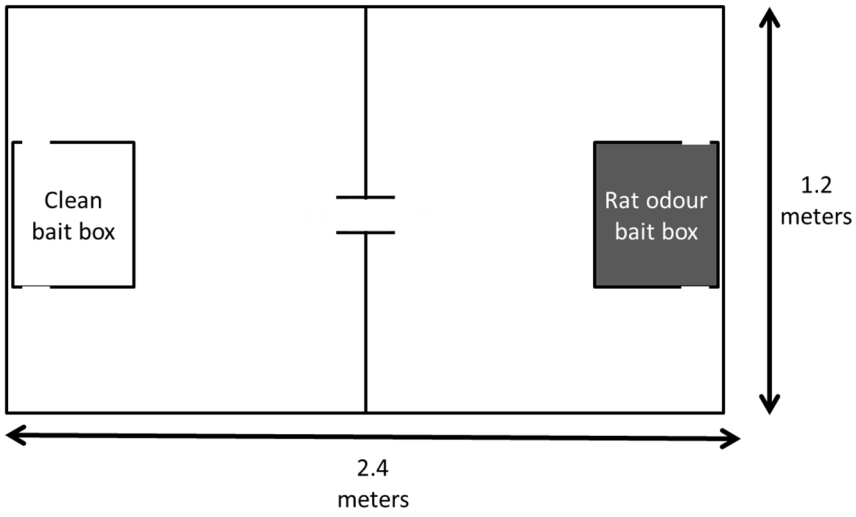


Figure 2.8. Design of laboratory test arena.

2.7.3. Results

Rats (n = 11 females, n = 8 males) were given a choice between a box that was scented with rat odour and a clean box in a compartmentalised laboratory arena.

Rats of both sexes showed no preference for, nor avoidance of, spending time inside a box scented with rat odour (arena side, Wilcoxon signed rank test, V = 106, p = 0.68; sex, *t*-test, t_8 = 0.37, p = 0.72; Figure 2.9A). In addition, rats of both sexes did not enter boxes more often when they were scented with rat odour or when they were clean (arena side, Wilcoxon signed rank test, V = 122, p = 0.12; sex, Wilcoxon rank sum test, W = 55, p = 0.38; Figure 2.9B). Furthermore, rats of both sexes showed no preference, nor avoidance of, spending time in an arena side containing a box scented with rat odour (arena side, *t*-test, t_{18} = 0.86, p = 0.40; sex, *t*-test, t_8 = 1.15, p = 0.28; Figure 2.9C).

There was greater variability in the willingness of male rats to enter, and spend time inside, boxes scented with rat odour, compared to female rats (Figure 2.9A-B). This variability was also seen for time spent in an arena side containing a rat odoured box (Figure 2.9C). One male rat was observed to behave very cautiously when near the entrances of the rat odoured bait box. He frequently approached the box, sniffed the entrance, and then quickly moved away.

2.7.4. Discussion

Overall, male and female rats were neither attracted to, nor repelled by, male rat odour presented in boxes in a laboratory arena. However, there was more variation in the response of male rats compared to female rats and one male rat appeared to be actively repelled by the presence of odour from another unfamiliar male.

High variability in the response of male rats to male rat odour could be due to the design of the test arena. The tunnel that connected the two halves of the test arena was relatively narrow compared to an adult male rat, meaning that we had to exclude three male rats from our analysis as they did not visit both sides of the arena during the test period. Female rats were smaller than male rats and so were able to pass between arena sides without impairment. This problem may have reduced the inclination of all male rats to pass between arena sides, increasing the variability of our results.

Another explanation for high variability in male rat response is the effects of dominance. Responses of males to odour cues deposited by other males can be highly variable and can

be influenced by a variety of factors, including dominant/subordinate relationships (Brown, 1992; Roberts, 2007). Responses can include investigation and avoidance of scent marked objects (Roberts, 2007). In our experiment, if a test subject rat perceived itself to be subordinate to an odour donor rat, and potentially threatened by a possible encounter, then it may have preferred to avoid the odour donor. This would particularly be the case if an odour indicated that the donor was present in a threatening situation, such as the small confined space of the boxes used in this experiment. However, a test subject rat that perceived itself to be dominant to an odour donor rat, may not avoid the odour donor's scent. As we randomised which rat was presented with which odour donor's scent and did not test for dominance in our rats, it was not possible to test for this effect directly. However, the high variability in the response of male rats to male rat odour, is in line with this explanation. The individual cautious behaviour we observed in one male rat may be due to this effect. However, cautious behaviour was only observed in one male. Further testing is needed to confirm if other male rats show a similar response.

Our results differ to those of Takács *et al.* (2016), who found that laboratory male and female rats were attracted to male rat specific odour compounds. In that study, rats were presented with boxes scented with a blend of male specific volatile odour compounds and clean boxes in laboratory arenas. All boxes were baited with non-toxic food lures. There are two main differences between that study and our experiment that could account for the differences observed. First, Takács *et al.* (2016) used Brown Norway laboratory rats as test subjects, whereas we used Brown Norway crossed Wistar laboratory rats. Wistar rats spend more time hiding and greater reluctance to approach a water stimulus, compared to Brown Norway rats (Yin *et al.*, 2011). It may be that Brown Norway crossed Wistar rats are more cautious than pure Brown Norway rats, leading to different behaviour in the presence of odour cues. An alternative explanation is that we used a different odour cue to Takács *et al.* (2016), which may have affected it's attractive potential. We used whole body odour and faeces, whereas Takács *et al.* (2016) used a combination of male specific volatile compounds. These compounds may not have been present in our odour cue, reducing its attractive potential. Alternatively, compounds may have been present in our odour cue, which were not present in Takács *et al.* (2016) compound blend that cancelled out the attractive potential of our odour cue.

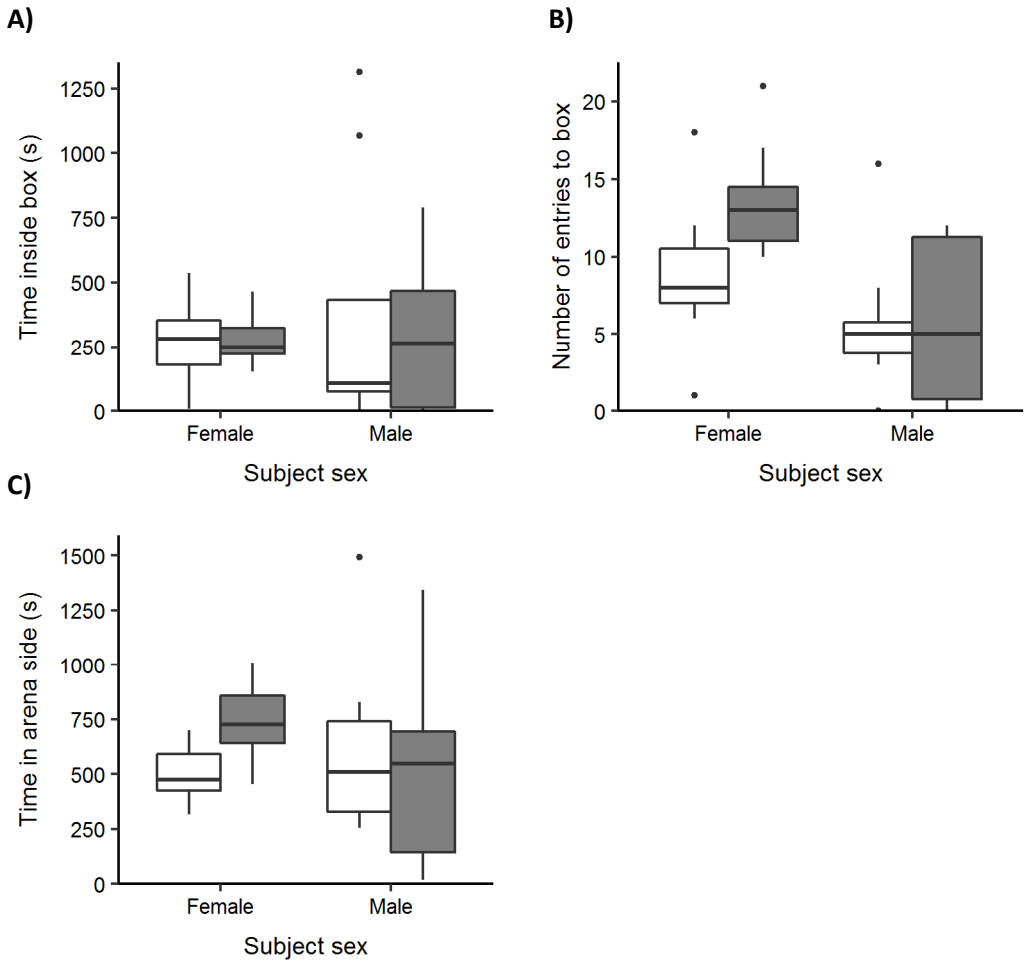


Figure 2.9. Response of rats to rat odour in a laboratory arena. Animals were presented with a side containing a box scented with rat body odour and faeces (dark grey bars) versus a side containing a clean box (white bars). **A)** Duration inside boxes. **B)** Number of entries to boxes. **C)** Duration in arena side, outside boxes. Box plots show medians (black bar), IQR (boxes), 1.5 IQR (whiskers) and outliers (dots). Durations on either side of the arena were compared with *t*-tests were data was normal and Wilcoxon Signed Rank tests where data was not normal.

2.8. General discussion

Our results demonstrate substantial avoidance of rat odour by wood mice, bank voles and field voles in outdoor semi-natural enclosures. Bank voles and field voles, but not wood mice, strongly avoided rat odour in laboratory arenas. Wood mice avoided rat body odour more than rat faeces in outdoor semi-natural enclosures. Avoidance of rat body odour appeared to be specific to the location of the odour and did not extend outside the scented box. Rats of both sexes did not avoid male rat odour. However, the response of male rats to male rat odour was highly variable.

Overall, we found a clear and consistent avoidance of rat odour by bank voles, field voles and wood mice. In laboratory arenas, the presence of rat odour substantially reduced time bank voles and field voles spent in bait boxes. Rat odour reduced the willingness of bank voles, field voles and wood mice to feed from bait boxes in outdoor semi-natural enclosures, with an overall reduction of 74% when all species are considered together. These results are consistent with findings that wood mice, house mice and water voles avoid rat odour in laboratory arena and outdoor enclosure trials (Barreto & Macdonald, 1999; Papes *et al.*, 2010; Richards, 2012). Rats have been observed killing house mice (Lidicker, 1976; Paul *et al.*, 1971). O'Boyle (1974) and Bridgman *et al.* (2013) examined the nature of mouse killing by rats and determined that it is a predatory response. So, it is likely that small rodents would avoid rats as a potential predator. In order to avoid rats, small rodents need to detect their presence. Prey species often use odour cues to detect and avoid predator contact (Apfelbach *et al.*, 2005; Hegab *et al.*, 2015). Therefore, it seems likely that the non-target rodents in this study avoided rat odour as it indicated of the presence of a predator.

The spatial scale of avoidance of rat odour appears to be limited to the bait box in which odour is present. We found this to be the case in laboratory arenas and outdoor semi-natural enclosures. In laboratory arenas, bank voles and field voles avoided spending time in rat odoured bait boxes, but did not avoid spending time in the side of an arena containing a rat odoured bait box. In outdoor semi-natural enclosures, female wood mice ate less non-toxic bait from boxes scented with rat odour, but did not avoid feeding from a clean bait box placed along side. Rats displayed defensive behaviours when a cat collar was placed 5 cm behind a mesh barrier, but not when it was placed 20 cm behind the barrier (Dielenberg & McGregor, 1999), indicating that other predator odours may only induce defensive behaviours over short distances. It may be that some predator odours are not detectable

over large distances and prey animals can only respond to odours once they have sensed them (Dielenberg & McGregor, 1999). This may be true of protein based odour cues, including some predator odours (Papes *et al.*, 2010). Alternatively, prey animals may only consider predator odours to indicate a threat when they are very close to the source. As stated previously, there is a cost to defensive behaviours, so prey animals should only respond to predator odours when they indicate a definite threat (Helfman, 1989; Zylberberg & DeWeese, 2011). Sensing an odour cue at a distance may not induce a defensive response if it is not a reliable threat indicator (Zylberberg & DeWeese, 2011).

Avoidance of rat odour by non-target rodents has the potential to reduce non-target exposure to rat poisons by repelling non-target rodents from rat bait boxes. Support for this conclusion comes from our finding of a clear and consistent avoidance of rat odour by all three species of non-target rodents when tested in a semi-natural environment. Both likelihood to consume bait and weight of bait consumed were reduced. In addition to this finding, two observations from our experiments increase the potential value of rat odour as a non-target repellent. First, in the context of developing a non-target repellent for use in rodent control programmes, the repellent should cause substantial reduction in bait take. In Experiment 2 all three non-target species consumed less than half the quantity of bait from rat odoured bait boxes compared to clean bait boxes. However, complete avoidance of rat odour was not observed in any of our experiments. Thus, in its current form, rat odour has the potential to substantially reduce bait consumption by non-target rodents, but cannot completely eliminate it. Second, during rat control campaigns rat bait boxes are often left out for long periods of time (Tosh *et al.*, 2011b), so non-target rodents could habituate to rat odour, reducing its effectiveness as a non-target repellent. Habituation to predator odour cues has been observed previously (Dielenberg & McGregor, 1999; Hegab *et al.*, 2014). However, despite repeated testing of female wood mice in outdoor enclosures, we observed no reduction in avoidance of rat odour. Overall, the response of non-target rodents to rat odour in these experiments indicates that rat odour has good potential as a non-target repellent.

A concern for the use of male rat odour as a non-target repellent is the potential for male rat odour to repel some male rats. The results of Experiment 5 indicate that rat odour may have some repellent effects on some male rats. Experiment 5 was conducted with laboratory rats and had a small sample size. However, the results are in line with another study that found that wild male rats are repelled by male specific compounds (Takács *et al.*, 2016). In

that study free-living wild rats were exposed to traps scented with male specific volatile compounds and clean traps. All traps were baited with food. Traps scented will male specific compounds caught more female rats than clean traps, but the opposite was true for male rats. This result contrasted with a laboratory experiment conducted in the same study where male and female rats were attracted to male specific compounds. The same compounds were used in both experiments (Takács *et al.*, 2016). This indicates that wild and laboratory rats may differ in their response to intraspecific odour cues. Inconsistencies between laboratory and field studies have been seen previously (Banks, 1998; Fendt & Endres, 2008; Wallace & Rosen, 2000). Therefore, we cannot extrapolate our results to pest control settings without testing the response of wild rats to male rat odour. Despite this limitation, our results combined with Takács *et al.* (2016) provide evidence that we need to carefully consider the use of male rat odour as a non-target repellent for use in rat control campaigns. There is potential for male rat odour to reduce visits of male rats to bait boxes, reducing the likelihood that they will consume poison. This could reduce the efficacy of rat control. Whilst reduction of the non-target effects of rat poisons is important, this cannot be at the cost of reducing the efficacy of those poisons against rats.

An alternative to using male rat odour as a non-target repellent is to trial female rat odour or non sex-specific odour compounds as non-target repellents. Field and laboratory trials have shown compounds found in female rat odour to be attractive to female rats and non-repellent to male rats (Takács *et al.*, 2016). Theoretically, the use of female rat odour as a non-target repellent is likely to be less problematic than male rat odour, as encountering a female is less likely to lead to an aggressive interaction than encountering a male (Stockley & Campbell, 2013). Female rat odour may, therefore, be used to repel non-target rodents with less risk of repelling target rats. In addition, non sex-specific compounds could be trialled. Repulsion of individual male rats in this study may be due to dominance effects, likely communicated in odour cues by sex specific compounds such as those found by Takács *et al.* (2016). However, there are several odour compounds that are common to both male and female rats (Takács *et al.*, 2016; Zhang *et al.*, 2008). These compounds may be repellent to non-target rodents, but not cause the sex-specific repulsion observed in rats.

Further studies will be needed to develop rat odour further as a non-target rodent repellent. Due to the concern that male rat odour may be repellent to some male rats, female rat odour should be tested for its repellent effects on non-target rodents. If female rat odour is repellent to non-target rodents, rat odour should be analysed to identify chemical

components common to both sexes. These components can then be tested for their repellent effects on non-target rodents. The response of rats to any identified compounds should also be tested. In addition, whilst the outdoor semi-natural enclosures used in this study give a good approximation for natural conditions, animals may behave differently when in the wild. The presence of predator odours other than rat odour in natural conditions and the increased energy demands of free-living animals may reduce the efficacy of rat odour as a repellent. Therefore, field experiments testing rat odour with non-target rodents will be essential. For all subsequent studies the use of rat body odour is recommended in the first instance, as it induced the greatest avoidance response in our experiments.

In conclusion, male rat odour, in particular body odour, has the potential to reduce the ingestion of rat poisons by non-target rodents. However, there is some concern over the potential effect of male rat odour on target rats. Therefore, further work should prioritise testing female rat odour, or non sex-specific rat odour compounds, to repel non-target rodents.

2.9. Appendix

2.9.1. Supplementary information

2.9.1.1. Linear mixed effects model and null model for Experiment 2 (rat and house mouse odour outdoor enclosure experiment).

Model: Log (Eaten+1) ~ Odour + (1 | Group / Test day) + (1 | Group / Box position)

Null model: Log (Eaten+1) ~ 1 + (1 | Group / Test day) + (1 | Group / Box position)

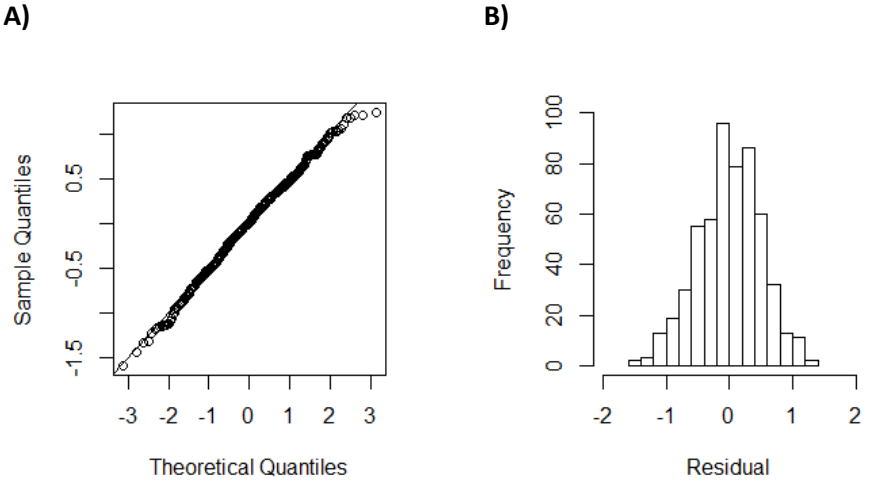


Figure S2.1. Experiment 2 residual plots. A) Normal Q-Q Plot. **B)** Histogram.

2.9.1.2. Linear mixed effects model and null model for Experiment 3 (rat body odour and faeces outdoor enclosure experiment).

Model: Log (Eaten+1) ~ Odour + (1 | Test day) + (1 | Box position)

Null model: Log (Eaten+1) ~ 1 + (1 | Test day) + (1 | Box position)

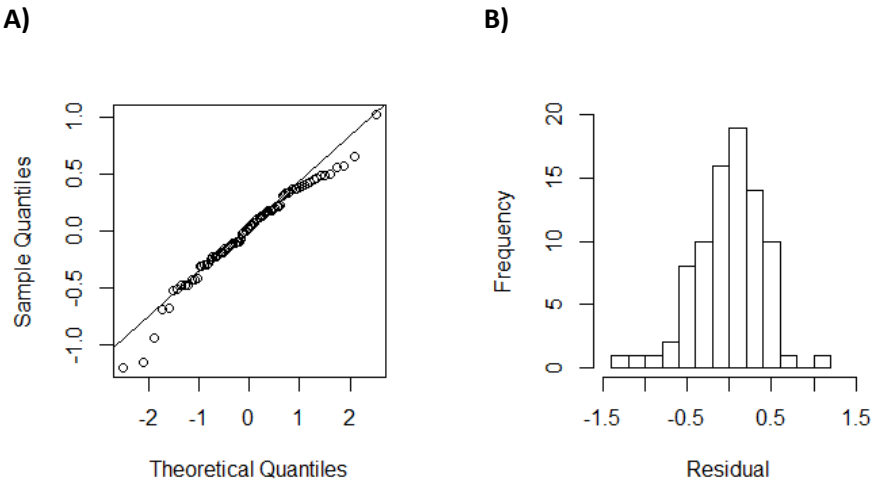


Figure S2.2. Experiment 3 residual plots. A) Normal Q-Q Plot. **B)** Histogram.

2.9.1.3. Linear mixed effects model and null model for Experiment 4 (rat odour spatial effects enclosure experiment).

Model: Log (Eaten+1) ~ Odour + (1 | Test day) + (1 | Box position)

Null model: Log (Eaten+1) ~ 1 + (1 | Test day) + (1 | Box position)

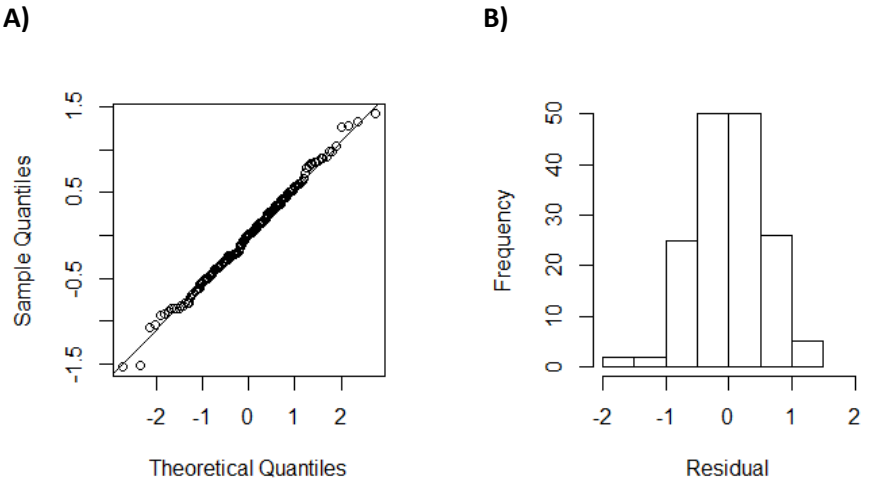


Figure S2.3. Experiment 4 residual plots. A) Normal Q-Q Plot. **B)** Histogram.

Table S2.1. Orthogonal contrasts for comparing within and between box pairs for Experiment 4.

Box odour	Rat versus Clean (by rat)	Clean (1) versus Clean (2)	Clean (1) and Clean (2) versus Rat and Clean (by rat)
Clean (1)	0	-1	-1
Clean (2)	0	1	-1
Clean (by rat)	-1	0	1
Rat	1	0	1

2.10. References

Animals (Scientific Procedures) Act. (1986). UK.

Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A. & McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neuroscience & Biobehavioral Reviews.* **29**, 1123-1144.

Association for the Study of Animal Behaviour (2018). Guidelines for the treatment of animals in behavioural research and teaching. *Animal Behaviour.* **135**, I-X.

Banks, P. B. (1998). Responses of Australian Bush Rats, *Rattus fuscipes*, to the Odor of Introduced *Vulpes vulpes*. *Journal of Mammalogy.* **79**, 1260-1264.

Barreto, G. R. & Macdonald, D. W. (1999). The response of water voles, *Arvicola terrestris*, to the odours of predators. *Animal Behaviour.* **57**, 1107-1112.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software.* **67**, 1-48.

Bellows, T. S. (2001). Restoring Population Balance through Natural Enemy Introductions. *Biological Control.* **21**, 199-205.

Berry, R. (1981). Town mouse, country mouse: adaptation and adaptability in *Mus domesticus* (*M. musculus domesticus*). *Mammal Review.* **11**, 91-136.

Blanchard, D. C., Markham, C., Yang, M., Hubbard, D., Madarang, E. & Blanchard, R. J. (2003). Failure to produce conditioning with low-dose trimethylthiazoline or cat feces as unconditioned stimuli. *Behavioral Neuroscience.* **117**, 360-368.

Brakes, C. R. & Smith, R. H. (2005). Exposure of non-target small mammals to rodenticides: short-term effects, recovery and implications for secondary poisoning. *Journal of Applied Ecology.* **42**, 118-128.

Bridgman, L. J., Innes, J., Gillies, C., Fitzgerald, N. B., Miller, S. & King, C. M. (2013). Do ship rats display predatory behaviour towards house mice? *Animal Behaviour.* **86**, 257-268.

Brown, R. E. (1992). Responses of dominant and subordinate male-rats to the odors of male and female conspecifics. *Aggressive Behavior.* **18**, 129-138.

Buckle, A. & Fenn, M. (1992) Rodent control in the conservation of endangered species. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 36-41.

Campaign for Responsible Rodenticide Use UK (2015). *CRRU UK code of best practice.* pp. 1-24. CRRU UK, Ossett.

Capizzi, D., Bertolino, S. & Mortelliti, A. (2014). Rating the rat: global patterns and research priorities in impacts and management of rodent pests. *Mammal Review.* **44**, 148-162.

Christiansen, E. (1976) Pheromones in small rodents and their potential use in pest control. In Siebe, C. C., Ed. *Proceedings of the Seventh Vertebrate Pest Conference, Monterey*. University of California. pp. 185-195.

Clapperton, B. K., Porter, R. E. R., Day, T. D., Rwaas, J. & Matthews, L. R. (2012). Designer repellents: combining olfactory, visual or taste cues with a secondary repellent to deter free-ranging house sparrows from feeding. *Pest Management Science.* **68**, 870-877.

Colvin, B. A., Hegdal, P. L. & Jackson, W. B. (1988). Review of non-target hazards associated with rodenticide use in the USA. *EPPO Bulletin.* **18**, 301-308.

Cook, S. M., Khan, Z. R. & Pickett, J. A. (2007). The use of push-pull strategies in integrated pest management. *Annual Review of Entomology.* **52**, 375-400.

Crawley, M. J. (2007). *The R book.* Wiley, Chichester.

Davis, D. E. (1953). The characteristics of rat populations. *The Quarterly Review of Biology.* **28**, 373-401.

Dickman, C. R. (1999). Rodent-ecosystem relationships: a review. *Ecologically-based Management of Rodent Pests. ACIAR Monograph.* 113-133.

Dielenberg, R. A. & McGregor, I. S. (1999). Habituation of the hiding response to cat odor in rats (*Rattus norvegicus*). *Journal of Comparative Psychology.* **113**, 376-87.

Directive 2010/63/EU. (2010). EU.

Elmeros, M., Christensen, T. K. & Lassen, P. (2011). Concentrations of anticoagulant rodenticides in stoats (*Mustela erminea*) and weasels (*Mustela nivalis*) from Denmark. *Science of the Total Environment.* **409**, 2373-2378.

Elmeros, M., Lassen, P., Bossi, R. & Topping, C. J. (2018). Exposure of stone marten (*Martes foina*) and polecat (*Mustela putorius*) to anticoagulant rodenticides: Effects of regulatory restrictions of rodenticide use. *Science of the Total Environment.* **612**, 1358-1364.

Fendt, M. & Endres, T. (2008). 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor - Just repugnant or really fear-inducing? *Neuroscience and Biobehavioral Reviews.* **32**, 1259-1266.

Fitt, G. P. (2000). An Australian approach to IPM in cotton: integrating new technologies to minimise insecticide dependence. *Crop Protection.* **19**, 793-800.

Geduhn, A., Esther, A., Schenke, D., Mattes, H. & Jacob, J. (2014). Spatial and temporal exposure patterns in non-target small mammals during brodifacoum rat control. *Science of the Total Environment.* **496**, 328-338.

Hadler, M. R. & Buckle, A. P. (1992) Forty five years of anticoagulant rodenticides - past, present and future trends. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 149-156.

Hansen, S. C., Stolter, C., Imholt, C. & Jacob, J. (2016). Plant Secondary Metabolites as Rodent Repellents: a Systematic Review. *Journal of Chemical Ecology.* **42**, 970-983.

Hansson, L. (1987). An Interpretation of Rodent Dynamics as Due to Trophic Interactions. *Oikos.* **50**, 308-318.

Health and Safety Executive (2012). *Environmental risk mitigation measures for second generation anticoagulant rodenticides proposed by the UK* pp. 1-30. Health and Safety Executive, UK.

Health and Safety Executive (2015). *UK anticoagulant rodenticide product authorisation and the CRRU stewardship scheme.* pp. 1-12. Health and Safety Executive, UK.

Hegab, I. M., Shang, G., Ye, M., Jin, Y., Wang, A., Yin, B., Yang, S. & Wei, W. (2014). Defensive responses of Brandt's voles (*Lasiopodomys brandtii*) to chronic predatory stress. *Physiology & Behavior.* **126**, 1-7.

Hegab, I. M., Kong, S. S., Yang, S. M., Mohamaden, W. I. & Wei, W. H. (2015). The ethological relevance of predator odors to induce changes in prey species. *Acta Ethologica.* **18**, 1-9.

Helfman, G. (1989). Threat-sensitive predator avoidance in damselfish-trumpetfish interactions. *Behavioral Ecology and Sociobiology.* **24**, 47-58.

Hettena, A. M., Munoz, N. & Blumstein, D. T. (2014). Prey Responses to Predator's Sounds: A Review and Empirical Study. *Ethology.* **120**, 427-452.

Hothorn, T. & Hornik, K. (2015). *exactRankTests: Exact Distributions for Rank and Permutation Tests*. https://CRAN.R-project.org/package=exactRankTests

Jaksić, F. M., Schlatter, R. P. & Yáñez, J. L. (1980). Feeding Ecology of Central Chilean Foxes, *Dusicyon culpaeus* and *Dusicyon griseus*. *Journal of Mammalogy.* **61**, 254-260.

Jędrzejewski, W., Jędrzejewska, B. & McNeish, E. (1992). Hunting success of the weasel *Mustela nivalis* and escape tactics of forest rodents in Białowieża National Park. *Acta Theriologica.* **37**, 319-328.

Lenth, R. V. (2016). Least-Squares Means: The R Package lsmeans. *Journal of Statistical Software.* **69**, 1-33.

Lidicker, W. Z. (1976). Social-Behavior and Density Regulation in House Mice Living in Large Enclosures. *Journal of Animal Ecology.* **45**, 677-697.

Major, H. L., Jones, I. L., Charette, M. R. & Diamond, A. W. (2007). Variations in the diet of introduced Norway rats (*Rattus norvegicus*) inferred using stable isotope analysis. *Journal of Zoology.* **271**, 463-468.

Martinez-Padilla, J., Lopez-Idiaquez, D., Lopez-Perea, J. J., Mateo, R., Paz, A. & Vinuela, J. (2017). A negative association between bromadiolone exposure and nestling body condition in common kestrels: management implications for vole outbreaks. *Pest Management Science.* **73**, 364-370.

Masini, C., Sauer, S. & Campeau, S. (2005). Ferret odor as a processive stress model in rats: neurochemical, behavioral, and endocrine evidence. *Behavioral Neuroscience.* **119**, 280-292.

Meerburg, B. G., Brom, F. W. & Kijlstra, A. (2008). The ethics of rodent control. *Pest Management Science.* **64**, 1205-1211.

Meerburg, B. G., Singleton, G. R. & Kijlstra, A. (2009). Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology.* **35**, 221-270.

Mukherjee, S., Goyal, S. P., Johnsingh, A. J. T. & Leite Pitman, M. R. P. (2004). The importance of rodents in the diet of jungle cat (*Felis chaus*), caracal (*Caracal caracal*) and golden jackal (*Canis aureus*) in Sariska Tiger Reserve, Rajasthan, India. *Journal of Zoology.* **262**, 405-411.

Navarro-Castilla, A. & Barja, I. (2014). Does predation risk, through moon phase and predator cues, modulate food intake, antipredatory and physiological responses in wood mice (*Apodemus sylvaticus*)? *Behavioral Ecology and Sociobiology.* **68**, 1505-1512.

NC3Rs, BBSRC, DEFRA, MRC, NERC & Wellcome Trust. (2017). Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. NC3Rs, London.

O'Boyle, M. (1974). Rats and mice together: The predatory nature of the rat's mouse-killing response. *Psychological Bulletin.* **81**, 261-269.

Papes, F., Logan, D. W. & Stowers, L. (2010). The Vomeronasal Organ Mediates Interspecies Defensive Behaviors through Detection of Protein Pheromone Homologs. *Cell.* **141**, 692-703.

Paul, L., Miley, W. M. & Baenninger, R. (1971). Mouse killing by rats: Roles of hunger and thirst in its initiation and maintenance. *Journal of Comparative and Physiological Psychology.* **76**, 242-249.

Pimentel, D. (2005). Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. *Environment, Development and Sustainability.* **7**, 229-252.

Pimentel, D., Zuniga, R. & Morrison, D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics.* **52**, 273-288.

R Core Team. (2016). *R: A language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Richards, S. (2012). Behavioural responses of wood mice (*Apodemus sylvaticus*) and bank voles (*Myodes glareolus*) to rat odours. University of Liverpool [Unpublished thesis].

Roberts, S. C. (2007). Scent marking. In *Rodent Societies: an Ecological and Evolutionary Perspective.* Wolff. J.O. & Sherman, P. W. (Eds.), pp. 255-266. The University of Chicago Press, London.

Selvaraj, R. & Archunan, G. (2006). Efficacy of male scent glands and urine in masking poison bait odour in female house rats, *Rattus rattus*. *Journal of Pest Science.* **79**, 255-258.

Serieys, L. E. K., Armenta, T. C., Moriarty, J. G., Boydston, E. E., Lyren, L. M., Poppenga, R. H., Crooks, K. R., Wayne, R. K. & Riley, S. P. D. (2015). Anticoagulant rodenticides in urban bobcats: exposure, risk factors and potential effects based on a 16-year study. *Ecotoxicology.* **24**, 844-862.

Shore, R. F., Walker, L. A., Potter, E. D., Pereira, M. G., Sleep, D. & Thompson, N. J. (2016). *Second generation anticoagulant rodenticide residues in barn owls 2016.* pp. 1-21. Centre for Ecology & Hydrology, Lancaster.

Simon, S., Brun, L., Guinaudeau, J. & Sauphanor, B. (2011). Pesticide use in current and innovative apple orchard systems. *Agronomy for Sustainable Development.* **31**, 541-555.

Singleton, G. R., Krebs, C. J., Davis, S., Chambers, L. & Brown, P. (2001). Reproductive changes in fluctuating house mouse populations in southeastern Australia. *Proceedings of the Royal Society of London. Series B: Biological Sciences.* **268**, 1741-1748.

Singleton, G. R. (2003). *Impacts of rodents on rice production in Asia.* IRRI Discussion Paper Series, No. 45, pp. 1-30. International Rice Research Institute, Philippines.

Singleton, G. R., Jacob, J. & Krebs, C. (2005). Integrated management to reduce rodent damage to lowland rice crops in Indonesia. *Agriculture, Ecosystems & Environment.* **107**, 75-82.

Singleton, G. R. (2010). *Rodent outbreaks: ecology and impacts.* International Rice Research Institute, Philippines.

Singmann, H., Bolker, B., Westfall, J. & Aust, F. (2017). *afex: Analysis of Factorial Experiments*. https://CRAN.R-project.org/package=afex

Stockley, P. & Campbell, A. (2013). Female competition and aggression: interdisciplinary perspectives. *Philosophical Transactions of the Royal Society B: Biological Sciences.* **368**, 1-11.

Takács, S., Gries, R., Zhai, H. & Gries, G. (2016). The Sex Attractant Pheromone of Male Brown Rats: Identification and Field Experiment. *Angewandte Chemie-International Edition.* **55**, 6062-6066.

Tosh, D. G., McDonald, R. A., Bearhop, S., Lllewellyn, N. R., Fee, S., Sharp, E. A., Barnett, E. A. & Shore, R. F. (2011a). Does small mammal prey guild affect the exposure of predators to anticoagulant rodenticides? *Environmental Pollution.* **159**, 3106-3112.

Tosh, D. G., Shore, R. F., Jess, S., Withers, A., Bearhop, S., Ian Montgomery, W. & McDonald, R. A. (2011b). User behaviour, best practice and the risks of non-target exposure associated with anticoagulant rodenticide use. *Journal of Environmental Management.* **92**, 1503-1508.

Tosh, D. G., McDonald, R. A., Bearhop, S., Llewellyn, N. R., Montgomery, W. I. & Shore, R. F. (2012). Rodenticide exposure in wood mouse and house mouse populations on farms and potential secondary risk to predators. *Ecotoxicology.* **21**, 1325-1332.

Triggs, G. S. (1991). The population ecology of house mice (*Mus domesticus*) on the Isle-of-May, Scotland. *Journal of Zoology.* **225**, 449-468.

Vandenbroucke, V., Bousquet-Melou, A., De Backer, P. & Croubels, S. (2008). Pharmacokinetics of eight anticoagulant rodenticides in mice after single oral administration. *Journal of Veterinary Pharmacology and Therapeutics.* **31**, 437-445.

Walker, L. A., Chaplow, J. S., Moeckel, C., Pereira, M. G., Potter, E. D., Sainsbury, A. W. & Shore, R. F. (2016). *Anticoagulant rodenticides in red kites (Milvus milvus) in Britain 2010 to 2015: a Predatory Bird Monitoring Scheme (PBMS) report.* pp. 1-14. Centre for Ecology & Hydrology, Lancaster.

Wallace, K. J. & Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behavioral Neuroscience.* **114**, 912-22.

Wallingford, A. K., Cha, D. H., Linn, C. E., Wolfin, M. S. & Loeb, G. M. (2017). Robust Manipulations of Pest Insect Behavior Using Repellents and Practical Application for Integrated Pest Management. *Environmental Entomology.* **46**, 1041-1050.

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, New York. http://ggplot2.org

Witmer, G. W. (2007). *The ecology of vertebrate pests and integrated pest management (IPM).* USDA National Wildlife Research Center-Staff Publications, pp. 393-410. University of Nebraska, U.S.A.

Witzgall, P., Kirsch, P. & Cork, A. (2010). Sex Pheromones and Their Impact on Pest Management. *Journal of Chemical Ecology.* **36**, 80-100.

Xiao, Z., Jansen, P. A. & Zhang, Z. (2006). Using seed-tagging methods for assessing post-dispersal seed fate in rodent-dispersed trees. *Forest Ecology and Management.* **223**, 18-23.

Yin, B. F., Fan, H. M., Li, S. P., Hegab, I., Lu, G. Y. & Wei, W. H. (2011). Behavioral response of Norway rats (*Rattus norvegicus*) to odors of different mammalian species. *Journal of Pest Science.* **84**, 265-272.

Zhang, J.-X., Sun, L., Zhang, J.-H. & Feng, Z.-Y. (2008). Sex-and gonad-affecting scent compounds and 3 male pheromones in the rat. *Chemical Senses.* **33**, 611-621.

Zylberberg, J. & DeWeese, M. R. (2011). How should prey animals respond to uncertain threats? *Frontiers in Computational Neuroscience.* **5**, 1-7.

Chapter 3

3. The response of non-target rodents to female rat odour and 4-ethyl phenol

3.1. Abstract

The majority of animal pests are controlled using pesticides, most of which are not species specific. This lack of specificity has led to large scale exposure of non-target species to pesticides, and is a particular problem for pest control aimed at rats. One suggested method of reducing this issue is to repel non-target rodents from rat poison stations using rat odour. Male rat odour repels non-target rodents, but there is some concern that it may be repellent to some male rats. Female rat odour, or generic rat odour components have been suggested as alternatives, but their efficacy as non-target rodent repellents has not been tested. To determine the repellent potential of female rat odour, bank voles and wood mice were tested in laboratory arenas and semi-natural enclosures respectively. Female rat odour was repellent to bank voles and wood mice. The volatile odour components of male and female rat nest material water elutions were analysed and 4-ethyl phenol was the most abundant compound in all samples. Bank voles were repelled by 4-ethyl phenol presented at the highest amount found in nest material elutions, but wood mice were not repelled by 4-ethyl phenol presented at either the highest or lowest amounts found in nest material elutions. Rat odour appears to have good potential as a non-target rodent repellent, but further work is needed to determine which volatile compounds are actively repellent to wood mice.

3.2. General Introduction

There is a global reliance on pesticides to control pest animals (Capizzi *et al.*, 2014; Devine & Furlong, 2007; Williamson *et al.*, 2008). Unfortunately, many pesticides are not species specific and numerous non-target animals are killed as a by-product of pest control (Devine & Furlong, 2007; Pimentel, 2005). Non-target species can be poisoned by consuming pesticides directly, or by ingesting prey containing residues of poisons (Buckle & Fenn, 1992). The costs of non-target poisoning are huge, both in terms of damage to ecosystems and damage to resources, such as waterways and pollinators (Devine & Furlong, 2007; Pimentel, 2005). In the USA alone, the annual costs of non-target poisoning have been estimated at $3 billion (Pimentel, 2005).

Non-target poisoning is of particular concern for pest control aimed at rats. As with control of other pest animals, rat control relies heavily on poisons (Capizzi *et al.*, 2014). Most rat poisons are not species specific and can kill non-target species (Mason & Littin, 2003). This includes the most widely used rat poisons, the anticoagulant rodenticides (Capizzi *et al.*, 2014; Mason & Littin, 2003). These poisons are toxic to all mammals and birds (Hadler & Buckle, 1992). They interfere with blood clotting, causing animals to bleed to death (Hadler & Buckle, 1992). The most commonly used anticoagulant rodenticides are the second generation anticoagulants, which are highly potent and bioaccumulative, passing up the food chain to affect predatory species such as birds of prey, foxes and mustelids (Capizzi *et al.*, 2014; Dawson *et al.*, 2001; Elmeros *et al.*, 2011; Hadler & Buckle, 1992; Tosh *et al.*, 2011a; Walker *et al.*, 2013). The scale of the problem is such that over 75% of red kites, barn owls and red foxes in the UK have residues of these poisons, often at toxic levels (Shore *et al.*, 2016; Tosh *et al.*, 2011a; Walker *et al.*, 2016). There are also concerns that non-toxic levels of these poisons could be causing poor body condition and increasing disease susceptibility (Martinez-Padilla *et al.*, 2017; Serieys *et al.*, 2015).

Several solutions have been suggested to reduce the problem of non-target poisoning by rat poisons. The most obvious solution is to ban the use of these poisons all together. Unfortunately, there are few viable alternatives to control rats, and so rat poisons remain in use (Health and Safety Executive, 2012). There have been attempts to restrict use of rat poisons, particularly in areas where non-target poisoning is likely, such as away from buildings (Elmeros *et al.*, 2018; Health and Safety Executive, 2015). However, these restrictions are often not adhered to, and even when they are, they may not be effective at

reducing levels of non-target poisoning (Elmeros *et al.*, 2018; Memmott *et al.*, 2017; Tosh *et al.*, 2011b). Another option to reduce non-target exposure to rat poisons is to use scent cues that selectively repel non-target animals, but not target rats.

One cue that has the potential to reduce non-target exposure to rat poisons is rat odour. The first step in identifying a scent cue that could be used as a non-target repellent is to identify differences in ecology or behaviour between rats and non-target animals. The most common species poisoned during rat control are small rodents such as wood mice (*Apodemus sylvaticus*), bank voles (*Myodes glareolus*) and field voles (*Microtus agrestis*; (Brakes & Smith, 2005; Geduhn *et al.*, 2014). These small rodents are potential prey for rats (O'Boyle, 1974; Paul *et al.*, 1971). Therefore, there is incentive for them to avoid rat scent cues and there is evidence that they do so. Wood mice, bank voles and field voles all avoid male rat odour in semi-natural enclosures (Chapter 2), indicating that rat odour has the potential to be used as a non-target repellent. However, there is concern that male rat odour may be repellent to some male rats (Takács *et al.*, 2016; Chapter 2). Repulsion of target rats would make rat odour unsuitable for use as a non-target repellent. However, it is not known what components of male rat odour non-target rodents respond to. They may respond to male-specific compounds, or to generic rat odour compounds, produced regardless of sex or status. Generic rat odour compounds may not cause the sex-specific repulsion observed for male rat odour in rats and could be of use as non-target repellents if non-target rodents are repelled by them.

To assess if non-target rodents respond to generic rat odour compounds or sex-specific compounds, an understanding is needed of how animals respond to scent cues both within, and between species. Much of what is known about intraspecific scent communication is focused on sex-specific cues. These cues are deliberately deposited in the environment, often for breeding purposes (Johnson, 1973). Sex-specific cues are often deposited by males to attract females (Hurst, 1990). These cues are frequently deposited over a wide area (Hurst, 1987), so they are not a good indication of recent animal presence. Non-target animals are likely to avoid scent cues if they perceive the animal that deposited them as a threat, either as a predator or competitor. Cues that indicate recent animal presence are much more likely to be of use in indicating recent predator presence, and sex-specific cues that are deposited all over the environment are not likely to indicate recent animal presence.

As well as sex-specific odour cues, animals deposit generic species cues, and these may be repellent to prey species. Generic species cues include scents such as body odour and some of the components of faeces and urine that are found in all members of a species. Little is known about the use of these cues in intraspecific communication. However, generic species cues have been studied extensively for their effects on other species, particularly prey species. For example, TMT is a generic fox faecal compound that is repellent to several rodent species (Fendt & Endres, 2008; Sullivan *et al.*, 1988). Predator odours are often sensed through the vomeronasal organ, an accessory scent gland that requires an animal to make close contact with a scent (Papes *et al.*, 2010; Parsons *et al.*, 2018). Therefore, predator odours often only work when a prey animal is close to them. This makes sense as it is beneficial for a prey species to avoid meeting a predator, but it would not necessarily be beneficial to avoid the predator's entire habitat (Lima & Bednekoff, 1999). An example of this is the effect of cat odour on rats. Rats avoid cues that indicate potential close contact with a cat, such as a collar that contains body odour (McGregor *et al.*, 2002; Wright *et al.*, 2013). The effect of this is more pronounced the closer a rat is to the scent (Dielenberg & McGregor, 1999). However, the response to cat urine, a cue deposited widely in the environment often in a sex-specific manner, is much more variable, and rats will often not avoid this cue (Bramley & Waas, 2001; Fendt, 2006). As generic species cues can indicate recent presence of any member of a potentially predatory species, they may be more likely to elicit response from a prey species than sex-specific compounds. This suggests that non-target rodents may be more likely to be responding to generic species cues than sex-specific cues when they avoid rat odour, but this needs to be tested.

Current knowledge of rat scents and the compounds that make them up is mostly confined to sex-specific cues, with little known about generic rat odour cues. Female specific cues, such as urine from females in oestrus, are attractive to male rats (Christiansen, 1976). Male specific cues are attractive to females, but can repel males (Selvaraj & Archunan, 2006; Takács *et al.*, 2016). None of these studies assessed the response of rats to generic species cues. There have been some attempts to isolate compounds involved in rat scent communication, but again this has focused on sex-specific cues. For instance, six male specific compounds have been isolated, which, when blended together attract wild female rats, but repel wild male rats (Takács *et al.*, 2016). In addition, 4-ethyl phenol and 2-heptanone from urine and squalene from preputial glands were found to be higher in males than females and attracted female rats when added to castrated male urine (Zhang *et al.*,

2008). These three compounds have not been tested on male rats. Compounds found in both male and female rat urine, preputial glands and soiled bedding have been identified (Takács *et al.*, 2016; Zhang *et al.*, 2008), but very little is known about how rats respond to these generic species cues. Further analysis of rat scent cues is needed to help identify what components of rat odour repel non-target rodents.

To test if non-target rodents are repelled by female rat odour, we tested bank voles in laboratory arenas and wood mice in semi-natural enclosures with female rat nest material. Both species were repelled by female rat odour. As a first step in identifying the volatile components of rat odour that non-target rodents are responding to, water elutions of male and female rat nest material were examined by gas chromatography mass spectrometry (GC-MS). The major volatile compound was 4-ethyl phenol at a calculated range of 0.006 mg to 0.3 mg. When tested in laboratory arenas, the higher level repelled bank voles to the same extent as female rat odour. However, wood mice were not repelled by this higher level, nor by 0.006 mg 4-ethyl phenol when tested in semi-natural enclosures. We conclude that non-target rodents are repelled by generic rat odour compounds, but species may respond to different compounds and further work is needed to confirm the identity of the compound/s responsible for repulsion.

3.3. Experiment 1. Bank voles avoid female rat odour in laboratory arenas

3.3.1. Introduction

Bank voles avoid male rat odour when tested in laboratory arenas (Chapter 2). To assess if avoidance of rat odour generalises to odours from both sexes, we tested bank voles with female rat odour in laboratory arenas, using a previously established testing procedure (Chapter 2). Bank voles were given a choice between boxes scented with female rat nest material and clean boxes. In Chapter 2 rat body odour was collected by confining a single rat, or pair of rats, inside a bait box for 10 to 20 minutes. For this study, soiled nest material was used as a source of body odour, instead of confining rats inside boxes. Soiled nest material was chosen as it is more amenable to chemical analysis than odoured boxes. We hypothesised that bank voles would avoid female rat odour.

3.3.2. Methods

3.3.2.1. Ethics statement

All procedures were non-invasive behavioural tests that did not involve pain, suffering or lasting harm and were carried out in accordance with international best practice guidelines (Animals (Scientific Procedures) Act, 1986; Directive 2010/63/EU, 2010; Association for the Study of Animal Behaviour, 2018; NC3Rs *et al.*, 2017). The study was approved by the University of Liverpool Animal Welfare Committee. No specific licences were required to carry out the work, as animals were free to approach or avoid the test stimuli and showed no signs of distress when exposed to the odour in an arena.

3.3.2.2. Animal subjects

Subjects were 6 male and 6 female bank voles. All subjects were wild caught in Northwest England 1 to 2 months prior to testing. Odour donors were 51 adult female, 3rd to 5th generation crosses between Brown Norway (BN/SsNOlaHsd, InVivo, Bicester, UK) and Wistar rats (HsdHan®:WIST, InVivo, UK), aged 3 to 13 months. Female bank voles were housed singly in 48 x 15 x 13 cm cages (M3, North Kent Plastics, Coalville, UK). Male bank voles were housed in 1.2 m square arenas. Odour donor rats were housed in a separate room to test subjects, in same sex pairs or trios in 56 x 38 x 22 cm cages (RC2R, North Kent Plastics, UK), in a room containing both male and female rats. Separate protective clothing was worn when interacting with rats to ensure no transfer of scent to test subjects.

All animals were fed 5FL2 EURodent Diet (LabDiet, St Louis, USA) *ad libitum* and had access to *ad libitum* water. Bank vole diet was supplemented with parakeet seed mix (Rob Harvey, Tongham, UK), sunflower seeds, a variety of chopped fruit and vegetables and hay. All cages had Corn Cob Absorb 10/14 substrate (IPS Product Supplies Limited, London, UK) lining the base. Cardboard tubes and paper wool nest material (IPS Product Supplies Limited, UK) were provided to all animals for enrichment. In addition, rats were given 15 x 8 cm plastic tubes for enrichment.

All housing and test rooms were maintained at 21 °C, 55% humidity and 20 air changes per hour. Throughout the test period bank voles were housed on a reversed 16:8 hour light-dark cycle with lights off at 0900 h. Rats were housed on a reversed 12:12 hour light-dark cycle with lights off at 0800 h. All behavioural experiments were conducted in the dark phase of the light cycle under dim red lights.

3.3.2.3. Laboratory arena design

The laboratory arena measured 1.2 x 1.2 m, with solid laminated chipboard walls and base. Two 28 x 25 x 13 cm Roguard® Extra bait boxes (BASF, Cheadle Hulme, UK) were placed on opposing sides of the arena (Figure 3.1). Bait boxes were used in this experiment as we wanted to demonstrate avoidance from an object relevant to pest control.

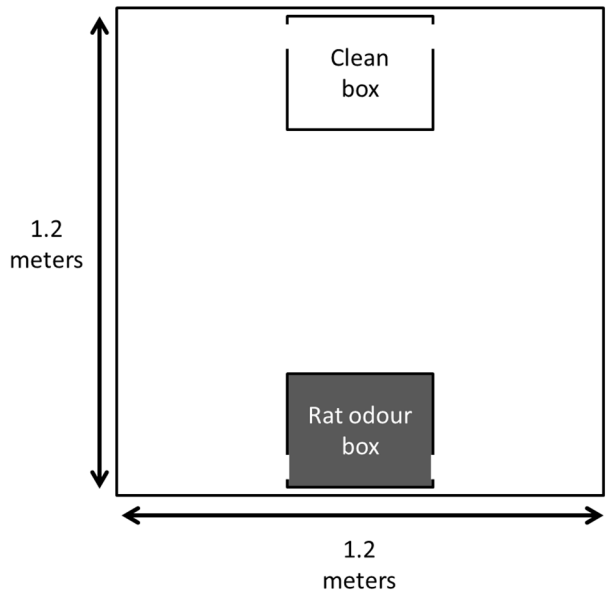
3.3.2.4. Laboratory arena procedure

Test subjects were given a choice between a rat odoured box and a clean box. Twenty four hours prior to testing, subjects were habituated to the test arena for 30 minutes, with no odour stimulus in either box. Following habituation, one of the boxes was scented with female rat odour by adding 5 g of nest material that had been in female rat cages for 7 days. Nest material was pooled from multiple cages, and mixed, before being placed in bait boxes. The other bait box was clean and contained 5g of clean nest material. Subjects were placed into the centre of the test arena and their behaviour was recorded in an adjacent room on DVD for 30 minutes. The order of habituation, testing and side of arena containing the odoured box were randomised in a balanced design. Habituation and testing were conducted in the dark phase of the light cycle under dim red lights. Between each subject, the arena was cleaned with Teepol Multipurpose detergent (Teepol, Orpington, UK) and 70% ethanol. Analysis of DVD recordings was performed blind to the arena side containing the odour stimulus. The duration inside each box, number of entries to each box and duration in each half of the arena was recorded.

3.3.2.5. Data analysis

To assess if bank voles avoided female rat odour, Wilcoxon signed-rank tests compared within subject differences between arena sides. All statistical tests were bidirectional and a p-value of less than 0.05 was taken as significant. All statistical analyses were carried out using R 3.4.0. (R Core Team, 2016) with packages 'exactRankTests' (Hothorn & Hornik, 2015) and 'ggplot2' (Wickham, 2009).

A)



B)

C)

Figure 3.1. Design of laboratory test arena. A) Overall arena design. **B)** Closed bait box. **C)** Open bait box containing 5 g nest material.

3.3.3. Results

Bank voles (n = 12) were given a choice between a box that was scented with female rat nest material and a clean box in a laboratory arena.

Bank voles strongly avoided spending time in boxes containing rat odour (Wilcoxon signed rank test, V = 72, p = 0.006; Figure 3.2A), but did not avoid spending time in the general vicinity of rat odoured boxes (duration in arena side, outside boxes; V = 38, p = 0.97; Figure 3.2C). Bank voles entered rat odoured boxes more often than control boxes, but this difference was small (V = 9, p = 0.04; Figure 3.2B).

3.3.4. Discussion

Bank voles avoided female rat odour when tested in laboratory arenas. This avoidance was confined to the odoured box and did not extend to the surrounding area. These results are similar to those obtained previously, where bank voles avoided male rat odoured boxes, but not the surrounding area (Chapter 2). In Chapter 2, bank voles spent 86.9 ± 122.5 s (median ± IQR) in rat odoured boxes, a very similar length of time compared to this experiment where bank voles spent 114.9 ± 99.1 s in rat odoured boxes. Avoidance of male and female rat odour by bank voles supports the hypothesis that non-target rodents are avoiding generic rat odour cues, rather than sex-specific cues.

There was a slight tendency for bank voles to enter female rat odoured boxes more frequently. This may be due to bank voles investigating the rat odoured box more than the control box. The concept of 'predator inspection' has been observed previously when prey encounter a predatory odour (Parsons *et al.*, 2018), and may account for the behaviour observed here. However, in Chapter 2 bank voles were observed to enter male rat odoured boxes less often than control boxes, although again the difference was small. It may be that number of box entries is not a reliable measure of avoidance behaviour, especially if differences are small.

Avoidance of male rat odour by bank voles in laboratory arenas correlates with avoidance in semi-natural enclosures (Chapter 2). It is likely that this would also be the case for female rat odour, so bank voles were not tested with female rat odour in semi-natural enclosures.

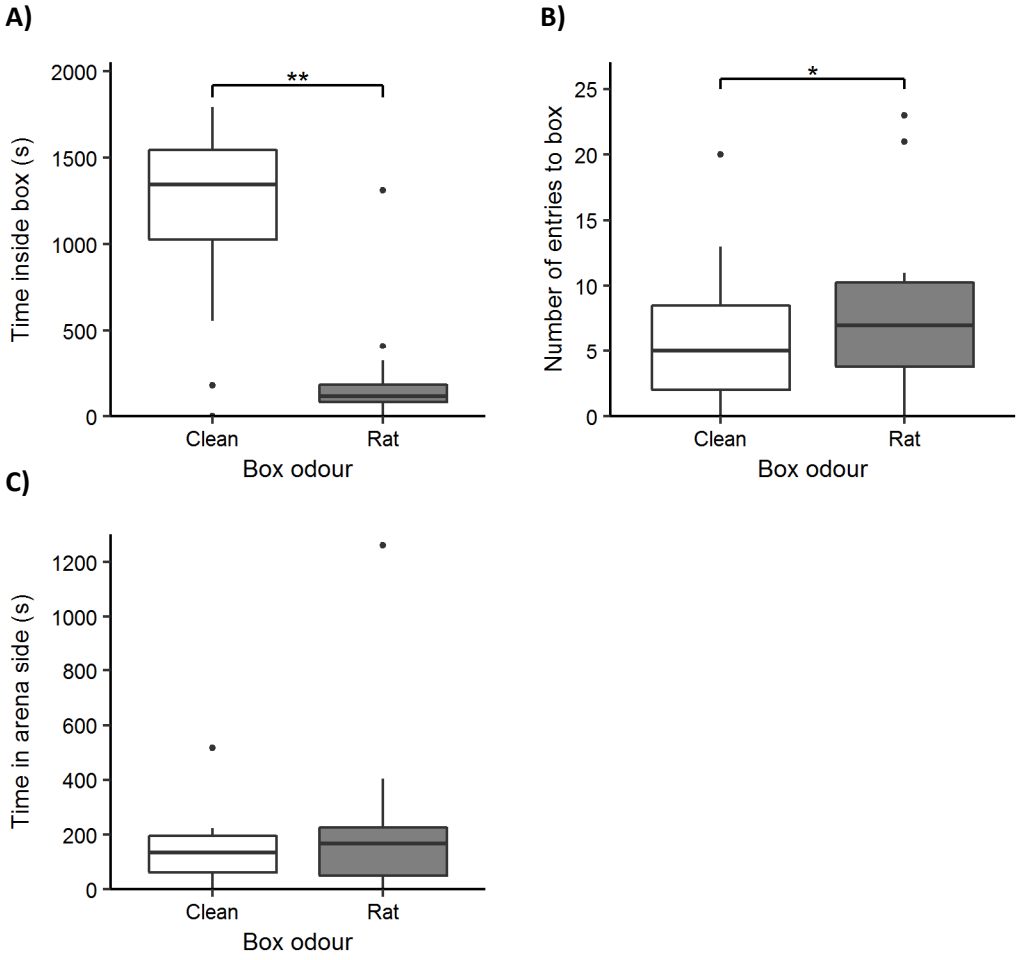


Figure 3.2. Response of bank voles to rat odour in a laboratory arena. Animals were presented with a box scented with rat nest material (dark grey bars) versus a clean box scented with clean nest material (white bars). **A)** Duration inside boxes. **B)** Number of entries to boxes. **C)** Duration in arena side, outside boxes. Box plots show medians (black bar), interquartile range (IQR; boxes), 1.5 IQR (whiskers) and outliers (dots). Odour versus control was compared with Wilcoxon Signed Rank tests (** p<0.01, * p<0.05).

3.4. Experiment 2. Wood mice avoid female rat odour in outdoor semi-natural enclosures.

3.4.1. Introduction

Wood mice show a consistent avoidance of bait boxes containing male rat odour, but only when tested in semi-natural enclosures (Chapter 2). When tested in laboratory arenas, wood mice do not avoid rat odour, likely due to the stress of being in open arenas (Chapter 2). Therefore, to assess if wood mice are repelled by female rat odour we tested wood mice with female rat odour in outdoor semi-natural enclosures. In this experiment wood mice were given the choice to feed from boxes scented with female rat nest material, boxes scented with a water elution of female rat nest material or clean boxes. We included a water elution of female rat nest material as a first step in analysing which components of rat odour non-target rodents are avoiding. We hypothesised that wood mice would avoid female rat odour. We hypothesised that wood mice would avoid female rat nest material elution.

3.4.2. Methods

3.4.2.1. Animal subjects

Subjects were 20 adult male wood mice. Subjects were wild caught from North West England 1 to 2 months prior to the start of the experiment and placed into an outdoor enclosure on the day they were captured. Odour donors were 51 adult female, 3rd to 5th generation crosses between Brown Norway (BN/SsNOlaHsd, InVivo, UK) and Wistar rats (HsdHan®:WIST, InVivo, UK), aged 3 to 13 months. Rats were housed as described for Experiment 1.

3.4.2.2. Preparation of scent cues

Female rat nest material: We collected nest material that had been in female rat cages for 7 days. Nest material was pooled from multiple cages, mixed, and frozen at -18°C for a maximum of 7 days and defrosted 1-2 hours before use.

Female rat nest material elution: We collected female rat nest material as described above. We placed 5 g of nest material inside a 20 ml syringe (Beton, Dickinson and Company, Franklin Lakes, USA) with the plunger removed. 2.5 ml distilled water was added to the top of the nest material. The syringe containing the wet nest material was placed into a 50 ml centrifuge tube (Fisher Scientific, Loughborough, UK) and spun at 2000 rpm for 10 minutes in an Eppendorf 5702 centrifuge (Eppendorf UK Ltd., Stevenage, UK). This gave approximately 1.5 ml elution.

Clean nest material elution was prepared in a similar method to soiled rat nest material elution, except 5 g clean nest material was used instead of rat scented nest material and 5 ml distilled water was added to give approximately 1.5 ml elution. A larger volume of water was added to clean nest material compared to soiled nest material as the water absorbing properties of clean nest material are greater.

Rat and clean nest material elutions were freshly prepared on each day of testing.

3.4.2.3. Outdoor enclosure design

Outdoor enclosures were 25 x 40 m, enclosed by 2 m high encircling walls, the bottom half of which was solid metal sheeting and the top half wire mesh. The ceiling of the enclosure was covered with wire mesh. Each enclosure contained grassland habitat, surrounded by a 0.5 m flagstone border. Laboratory animal food (5FL2 EURodent Diet, LabDiet, USA) mixed with Harry Hamster complete muesli (Supreme Petfoods Ltd., Ipswich, UK) was provided to subjects in 5 covered plastic boxes, 4 of which were placed halfway along each side of the enclosure, within the vegetation, and the final one placed in the centre of the enclosure. Water was provided beside food boxes in plastic dispensers. Additional shelter was provided by placing the bottom part of 4 cages (RM2, North Kent Plastics, UK), filled with hay, upside down in each quadrant of the enclosure.

Male wood mice were transferred to the outdoor enclosure in April and May 2017. No testing was carried out for the first 14 days to allow subjects to habituate to test enclosures.

3.4.2.4. Outdoor enclosure testing procedure

To test the feeding rate of wood mice from odoured bait boxes, wood mice were given a choice between bait boxes scented with female rat nest material, female rat nest material elution or clean boxes. 21 Roguard® Extra bait boxes (BASF, UK) were placed around the enclosure circumference at 5 m intervals (Figure 3.3).

Each evening, a 20 g Detex® non-toxic bait block (Bell laboratories, Sudbury, UK) was placed in each bait box. Non-toxic bait blocks contain the same ingredients as bait normally used in pest control, but do not contain any poison. Use of non-toxic bait blocks allows a better assessment of the feeding behaviour of non-target rodents in real-world rodent control conditions than if normal laboratory food was used. Food boxes were removed prior to placing bait boxes to ensure that bait boxes were the only major source of food during testing. Food boxes were replaced during the day, when bait boxes were not present in the

test enclosure. Subjects were habituated to feeding from bait boxes for 4 nights prior to testing.

For testing, 7 bait boxes were scented by adding 5 g of soiled female rat nest material. Seven boxes were scented by adding 5 g of clean nest material, onto which was pipetted 1.5 ml of female rat nest material elution. The final 7 boxes contained 5 g of clean nest material onto which was pipetted 1.5 ml clean nest material elution. Each morning the weight of bait eaten from each bait box was recorded and bait boxes were washed with dilute Teepol multipurpose detergent (Teepol, UK). To control for any differences in spatial distribution of wood mice in the enclosure, bait boxes were divided into trios. The order of scent within trios was randomised each night in a repeating pattern (Figure 3.3). Between each night of testing bait boxes were moved 2.5m to prevent subjects associating a particular location with one of the odours. The experiment was repeated for 4 nights.

3.4.2.5. Data analysis

Weight of bait eaten was log transformed and compared by a linear mixed effects model with box odour as fixed effect. Date and position of box (1-21) were included as random effects to control for repeated measures and variation in bait take due to microhabitat differences in the enclosure. Model significance was calculated by ANOVA comparison to a null model (see supplementary information). P-values of model fixed effect were calculated using likelihood ratio tests (Crawley, 2007). Planned comparisons were calculated between all pairs of treatment groups using Tukey contrasts. Models were validated using quantile-quantile (Q-Q) plots and residual distributions (Figure S3.1). A p-value of less than 0.05 was taken as significant. All statistical analyses were carried out using R 3.2.4 (R Core Team, 2016) with packages 'lme4' (Bates *et al.*, 2015), 'lsmeans' (Lenth, 2016) 'afex' (Singmann *et al.*, 2017) and 'ggplot2' (Wickham, 2009).

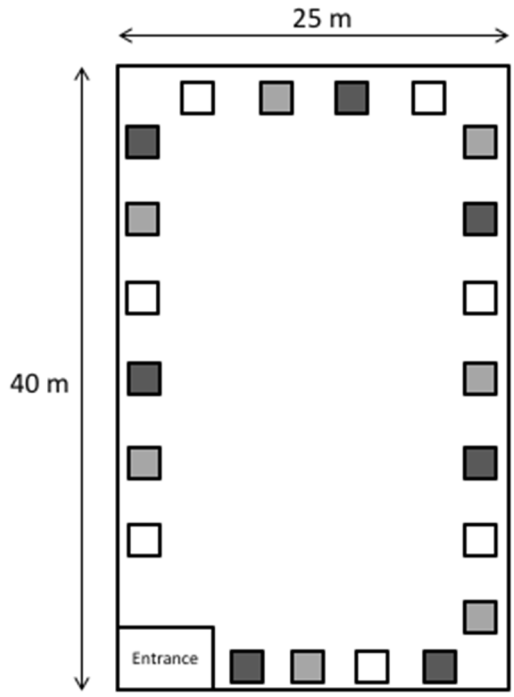


Figure 3.3. Outdoor enclosure layout. Bait boxes containing clean nest material (clear squares), nest material with female rat nest material elution (light grey squares) and female rat nest material (dark grey squares) were arranged in an outdoor enclosure. All boxes contained a 20 g non-toxic bait block.

3.4.3. Results

A population of 20 male wood mice were given a choice to feed from bait boxes scented with female rat nest material, bait boxes scented with an aqueous elution of female rat nest material on clean nest material or bait boxes containing clean nest material in outdoor semi-natural enclosures.

Wood mice ate considerably less from boxes containing female rat nest material compared to clean boxes (linear mixed effects model, $F_{2, 61.4} = 33.8$, $p < 0.001$; Tukey contrast, $t_{64.5} = 8.02$, $p < 0.001$, Figure 3.4). Wood mice tended to feed less from boxes containing an aqueous elution of this female rat odour applied to clean nest material compared to clean boxes, although this did not reach significance ($t_{64.5} = 2.34$, $p = 0.06$). The amount of food eaten in the presence of the original scented nest material was significantly less than that in response to the aqueous elution from the same amount of material ($t_{64.5} = 5.68$, $p < 0.001$).

Median bait take was 21% less from nest material elution odoured boxes, and 65% less from nest material odoured boxes, compared to clean boxes.

3.4.4. Discussion

Bait take was substantially reduced by female rat nest material, in outdoor, semi-natural enclosures. These results are similar to those obtained previously, where wood mice avoided consuming bait from male rat odoured bait boxes in outdoor enclosures (Chapter 2). This reinforces the findings of Experiment 1, and further supports the hypothesis that non-target rodents are avoiding generic rat odour cues, not sex-specific cues. This is the most likely interpretation as all rats are potentially dangerous to non-target rodents, whether sexually mature or not. However, it is possible that non-target rodents are repelled by male specific and female specific cues.

Wood mice did not avoid female rat nest material elution in this experiment, although there was a trend towards slight avoidance. It may be that some of the components of rat odour that are repellent to wood mice are not extracted during the process of aqueous elution. Alternatively, the amount of odour components in nest material elutions may be reduced, compared to the amount in soiled nest material, reducing the repellent effect of nest material elutions.

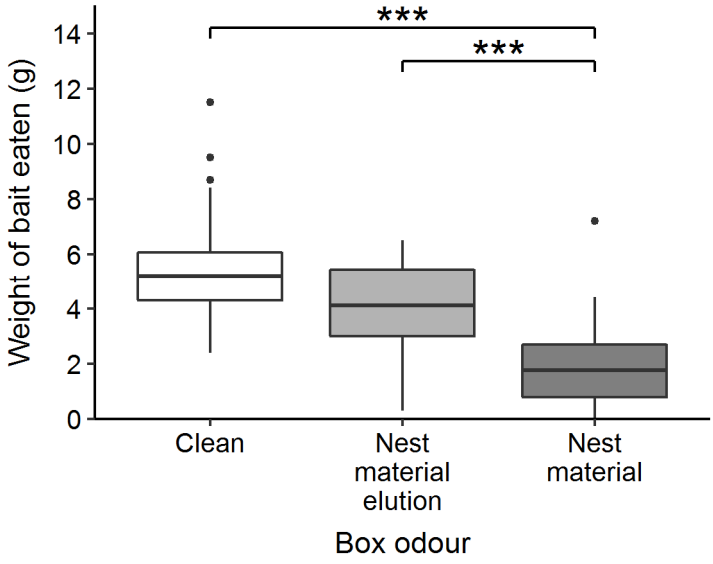


Figure 3.4. Weight of bait eaten per box per night by male wood mice from bait boxes scented with rat odour. Animals were presented with bait boxes scented with female rat nest material (dark grey bars), bait boxes scented with female rat nest material elution (light grey bars) and bait boxes scented with clean nest material elution (white bars). Box plots show medians (black bar), IQR (boxes), 1.5 IQR (whiskers) and outliers (dots). Weight of food eaten from bait boxes was compared using a linear mixed effects model, followed by Tukey comparisons (*** $p<0.001$).

3.5. Experiment 3. The main volatile components of female and male rat nest material

3.5.1. Introduction

Having established that wood mice and bank voles avoid male and female rat odour, and are likely avoiding rat odour cues generic to both of these sources, our next step was to analyse soiled nest material to identify compounds common to both sexes. We analysed nest material elutions from rats of both sexes by GC-MS to identify the most abundant volatile components present. Aqueous elutions of nest material were chosen for analysis due to this work already being in progress as part of a study looking at attraction of rats to rat odour.

3.5.2. Methods

3.5.2.1. Odour donors

Odour donors were 7 male and 9 female adult 3rd to 5th generation, Brown Norway (BN/SsNOlaHsd, InVivo, UK) crossed Wistar rats (HsdHan®:WIST, InVivo, UK), aged 4 to 10 months. Odour donors were housed as for Experiment 1 in single sex pairs, trios or quads.

3.5.2.2. Preparation of nest material elution

We collected nest material that had been in rat cages for 7 days. Two samples were collected from each of six cages (3 male and 3 female cages). Each sample of nest material was eluted as described for Experiment 2. Nest material elutions were stored at -18°C prior to mass spectrometric analysis. Clean nest material was also analysed as a control.

3.5.2.3. GC-MS method

For overview of workflow see Figure 3.5. Aqueous elutions of rat odoured and clean nest material were extracted using hexane. A sub-sample of 50 µl of each nest material elution was added to 400 µl of hexane. Samples were left to stand for 2 hours at room temperature (approximately 20°C).

1 µl of the organic layer from each hexane extraction was removed using a 50 µl glass syringe and then injected into a GCT GC-MS (Waters, Elstree, UK). The GC column was a 30 m x 0.25 mm diameter x 0.25 mm film thickness DB-WAX (Agilent J & W, Santa Clara, USA). Helium was used as a carrier gas at a rate of 0.7 ml/min. The injector was set to splitless mode at a temperature of 250°C. The temperature programme was 40°C to 240°C, at an increase rate

of 10°C per minute, with a 2 minute initial hold. A mass range of 45 to 500 Dalton (Da) was measured with a scan time of 0.9 s.

3.5.2.4. Data analysis

Peaks with the greatest intensity on the GC-MS trace were selected to identify specific compounds. Identification of peaks was carried out by comparison of retention times and mass spectra of authentic compounds. For identified peaks, total ion chromatogram (TIC) peak areas were calculated for all samples. To calculate the highest concentration of identified compounds in male and female nest material elutions, male and female samples with the largest peaks on the GC trace were selected. TIC peak area was calculated for 1 µl authentic compound at a concentration of 10 µg/µl, and for 1 µl of nest material hexane extract. It was assumed that most of the volatile compounds present in the nest material elution were recovered during hexane extraction. TIC peak area for nest material hexane extract was divided by TIC peak area for authentic compounds to give the amount of volatile compound present in 1 µl of extract. This was multiplied by 400 (to correct for the total volume of hexane, 400 µl) to give the amount of volatile compound in 50 µl of nest material elution. The concentration (in mg/ml) and total amount (in mg) of volatile compound in the original nest material elution was then calculated (assuming a 1.5 ml mean starting elution volume; Figure 3.5).

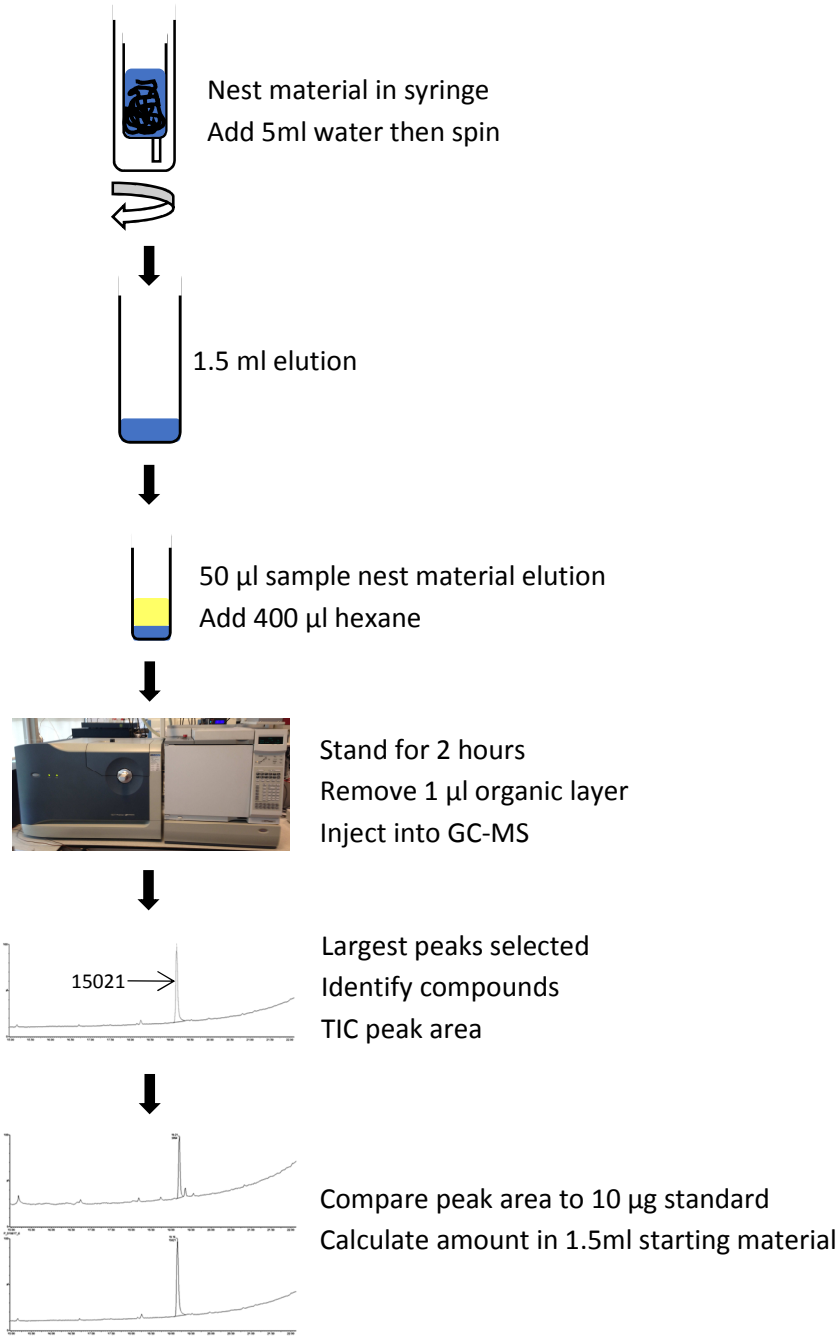


Figure 3.5. Workflow for calculation of amount of volatile in nest material elution. GC-MS work, hexane extractions and peak area calculations carried out by M. Prescott, University of Liverpool.

3.5.3. Results

Water elutions of soiled nest material from rats of both sexes were analysed by GC-MS, following hexane extraction.

By far the most abundant volatile compound in both male and female rat nest material was 4-ethyl phenol (Figures 3.6 and 3.7). 4-ethyl phenol was present in all male and female samples, the only volatile compound that appeared to be. A clear peak of 4-methyl phenol was present in two male and one female samples, at a lower concentration than 4-ethyl phenol. Neither 4-ethyl phenol, or 4-methyl phenol were present in elutions of clean nest material.

The range of 4-ethyl phenol abundance was very wide, especially for female samples (Table 3.1). The maximum amount of 4-ethyl phenol was calculated to be 0.3 mg in female rat nest material elutions and 0.09 mg in male rat nest material elutions (Table 3.2).

Table 3.1. Peak areas of 4-ethyl phenol from male and female rat nest material elutions.

Sex	Number of rats in cage	Sample	4-ethyl phenol peak area
Female	3	1	3179
		2	7568
	3	1	14567
		2	15021
	4	1	1850
		2	5860
Male	2	1	2032
		2	2695
	3	1	2326
		2	2795
	3	1	4791
		2	9557

GC-MS work, hexane extractions and peak area calculations carried out by M. Prescott, University of Liverpool.

Table 3.2. Maximum concentration and amount of 4-ethyl phenol from male and female rat nest material elutions.

Sex	4-ethyl phenol peak area	4-ethyl phenol concentration (mg/ml)	Amount of 4-ethyl phenol in 1.5 ml elution (mg)
Female	15021	0.2	0.3
Male	9557	0.06	0.09

GC-MS work, hexane extractions and peak area calculations carried out by M. Prescott, University of Liverpool.

3.5.4. Discussion

The most abundant volatile compound in both male and female rat nest material elution was 4-ethyl phenol. This compound has been identified previously as being present in male and female rat nest material and urine (Takács *et al.*, 2016; Zhang *et al.*, 2008). 4-ethyl phenol has also been identified in male mouse urine (Achiraman & Archunan, 2002). One study on rat urine found levels of 4-ethyl phenol to be higher in males than in females (Zhang *et al.*, 2008). This contrasts with the present study where the concentration of 4-ethyl phenol was higher in females than in males. However, as nest material may be soiled by faeces and body odour as well as urine, the two measures cannot be compared directly.

Although Zhang *et al.* (2008) found that 4-ethyl phenol was present in male rat urine at a concentration of 0.04 mg/ml, female rats were not attracted to this concentration in a sniffing test but were attracted to a concentration of 0.2 mg/ml. 0.2 mg/ml was the highest concentration of 4-ethyl phenol in female rat nest material elutions in this study. However, in laboratory arena tests, this concentration of 4-ethyl phenol was neither attractive nor repellent to male and female rats, when measured as total time spent near the odour (K. Pounder, personal communication).

A wide range of concentrations of 4-ethyl phenol was present in female rat nest material elutions. This may account for the lack of significant avoidance of female rat nest material elutions by wood mice observed in Experiment 2. It may be that wood mice only avoided

boxes odoured with nest material elution where the concentration of 4-ethyl phenol was at its highest. Therefore, testing of non-target rodents with 4-ethyl phenol at the top concentration found in nest material elutions is merited.

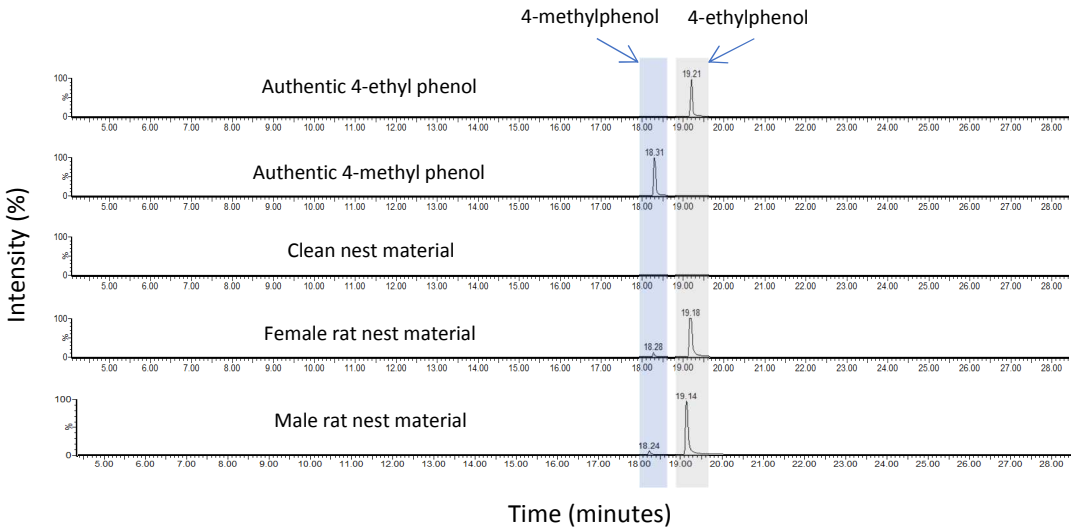


Figure 3.6. Representative extracted ion chromatogram of male and female rat nest material extracts and authentic compounds. Extracted ion chromatogram of the most abundant 4-ethyl phenol mass (107 Da), scaled to 5.35 x 10^4 for all samples except male rat nest material, which is scaled to 5.37 x 10^4. 10 ng of each authentic compound was injected into the GC-MS. GC-MS work and spectral image creation carried out by M. Prescott, University of Liverpool.

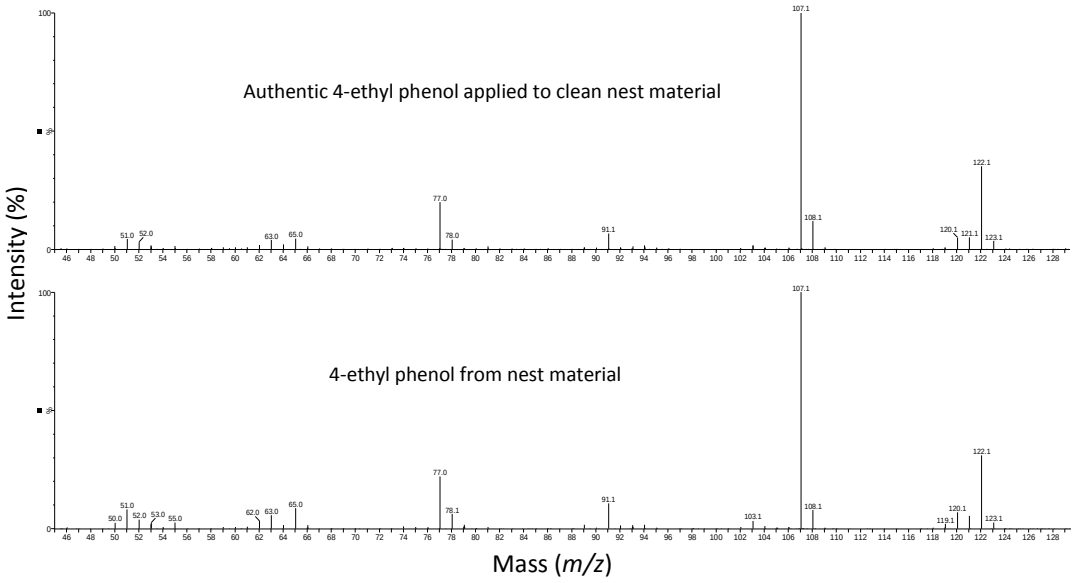


Figure 3.7. Representative mass spectra of 4-ethyl phenol from rat nest material and authentic 4-ethyl phenol. GC-MS work and spectral image creation carried out by M. Prescott, University of Liverpool.

3.6. Experiment 4. Bank voles avoid 4-ethyl phenol in laboratory arenas.

3.6.1. Introduction

Having identified, in Experiment 3, that 4-ethyl phenol is the most abundant volatile component of male and female rat nest material, we next assessed the response of bank voles to 4-ethyl phenol in laboratory arenas. Bank voles were given the choice between a box scented with 4-ethyl phenol and a clean box. In a separate test, bank voles were given the choice between a box scented with female rat nest material and clean box as a positive control. Each bank vole was presented with 0.3 mg 4-ethyl phenol, the highest amount found in rat nest material elutions. We hypothesised that bank voles would avoid 4-ethyl phenol approximately to the same extent as they avoid female rat odour.

3.6.2. Methods

3.6.2.1. Animal subjects

Test subjects and odour donors were the same as used in Experiment 1. Testing for this experiment was carried out 7 weeks after the end of Experiment 1.

3.6.2.2. Laboratory arena testing procedure

Test subjects were given a choice between an odoured box and a clean box in a series of two tests, using the arena design described for Experiment 1. In one test the odoured box was scented with 4-ethyl phenol. In the other test the odoured box was scented with female rat nest material as a positive control to ensure bank voles had not habituated to rat odour volatiles. A different arena was used for each test to prevent bank voles learning the location of the odour cue in the first test and avoiding this location, rather than the cue itself. One week prior to testing, subjects were habituated to one of the test arenas for 30 minutes, with no odour stimulus in either box. Following habituation, bank voles were tested twice, with a different arena used for each test, with two days between tests. During one test, one bait box was scented by adding 5 g of clean nest material, onto which was pipetted 0.3 mg 4-ethyl phenol (Sigma-Aldrich, Gillingham, UK) dissolved in 1.5 ml distilled water. The other bait box contained 5 g clean nest material, onto which was pipetted 1.5 ml of distilled water. During the other test, one bait box was scented by adding 5 g of nest material that had been in female rat cages for 7 days. Nest material was pooled from multiple cages, and mixed, before being placed in bait boxes. The other bait box was clean and contained 5 g of clean

nest material. The order of subjects, order of tests, arena used for each test and the side of the arena in which the odoured boxes were placed were randomised in a balanced design. Subjects were placed into the centre of the test arena and their behaviour was recorded in an adjacent room on DVD for 30 minutes. Habituation and testing were conducted in the dark phase of the light cycle under dim red lights. Between each subject, the arena was cleaned with Teepol Multipurpose detergent (Teepol, UK) and 70% ethanol. Analysis of DVD recordings was performed blind to odour treatment and arena side containing the odour stimulus. The duration inside each box, number of entries to each box and duration in each half of the arena was recorded.

3.6.2.3. Data analysis

Bias scores were calculated by subtracting durations and frequencies in the odoured side of the test arena from the control side of the test arena. To test if treatment odour affected the response of bank voles, bias scores were compared between odour cues using Wilcoxon Signed Rank tests. To test if bank voles avoided odour cues, bias scores were compared to zero using Wilcoxon Signed Rank tests. All statistical tests were bidirectional and a p-value of less than 0.05 was taken as significant. All statistical analyses were carried out using R 3.4.0. (R Core Team, 2016) with packages 'exactRankTests' (Hothorn & Hornik, 2015) and 'ggplot2' (Wickham, 2009).

3.6.3. Results

To test if bank voles avoid 4-ethyl phenol, bank voles (n = 12) were given a choice to spend time in an odoured box or a clean box. Bank voles were tested twice with two odour treatments: 1) 0.3 mg 4-ethyl phenol and 2) female rat nest material.

Bank voles spent considerably less time in odoured boxes compared to clean boxes, a bias that did not differ between 4-ethyl phenol and rat odour (Wilcoxon signed rank test, side, V = 241, p = 0.008; odour, V = 24, p = 0.27; Figure 3.8A). However, bank voles did not avoid spending time in the general vicinity of either 4-ethyl phenol or rat odoured boxes, compared to clean boxes (Wilcoxon signed rank test, side, V = 157, p = 0.86; odour, V = 62, p = 0.08; Figure 3.8C). In addition, there was no difference in the number of times bank voles entered odoured boxes compared to clean boxes for either odour treatment (Wilcoxon signed rank test, side, V = 176.5, p = 0.24; odour V = 29, p = 0.90; Figure 3.8B).

3.6.4. Discussion

Bank voles avoided 4-ethyl phenol to the same extent as female rat nest material. The lack of avoidance of the area surrounding the odoured box for both 4-ethyl phenol and female rat nest material indicates that the spatial extent of avoidance is similar for both odour cues. Bank voles may have avoided rat odour as they use this volatile compound to indicate the presence of a rat. Alternatively, this amount of 4-ethyl phenol may be aversive as an offensive stimulus. However, rats were not repelled by this amount of 4-ethyl phenol presented in laboratory arenas (K. Pounder, personal communication). This indicates that rats did not find this amount of 4-ethyl phenol offensive. Bank voles may have a different olfactory sensitivity to rats, but this seems unlikely. Therefore, it unlikely that bank voles found 4-ethyl phenol aversive as an offensive stimulus.

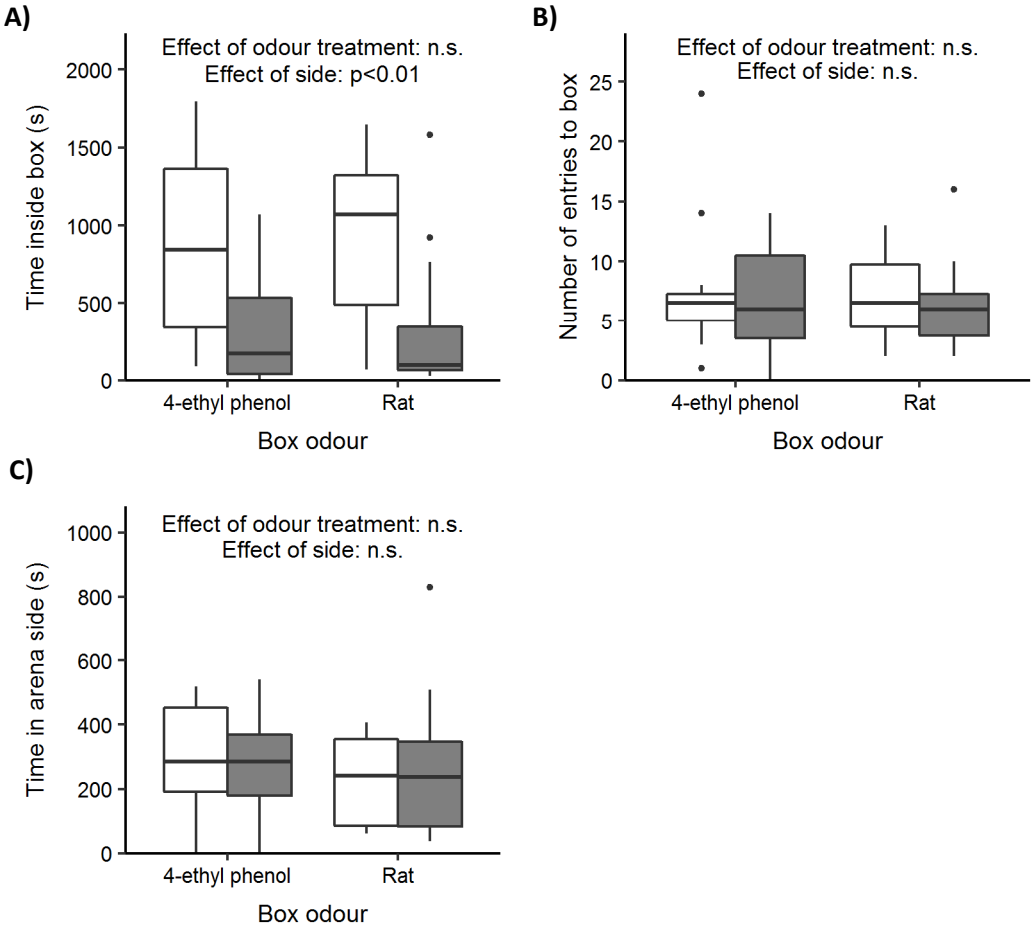


Figure 3.8. Response of bank voles to odour stimuli in laboratory arenas. Animals were presented with a side containing a box with scented with female rat nest material or 0.3 mg 4-ethyl phenol (dark grey bars) versus a side containing a clean box with clean nest material (white bars). **A)** Duration inside boxes. **B)** Number of entries to boxes. **C)** Duration in arena side, outside boxes. Arena sides and odour treatments were compared with Wilcoxon Signed Rank tests. Box plots show medians (black bar), IQR (boxes), 1.5 IQR (whiskers) and outliers (dots).

3.7. Experiment 5. Wood mice do not avoid 4-ethyl phenol in outdoor semi-natural enclosures.

3.7.1. Introduction

Having established that bank voles avoid 0.3 mg 4-ethyl phenol applied to nest material in laboratory arenas, we next assessed the response of wood mice to the same amount of 4-ethyl phenol in outdoor semi-natural enclosures. We gave wood mice the choice between boxes scented with 4-ethyl phenol, boxes scented with female rat nest material and clean boxes. We hypothesised that wood mice would avoid 4-ethyl phenol to the same extent as female rat odour, as was the case for bank voles.

3.7.2. Methods

3.7.2.1. Animal subjects

Subjects were 20 male wood mice housed as for Experiment 2. Odour donors were 51 adult female, 3rd to 5th generation crosses between Brown Norway (BN/SsNOlaHsd, InVivo, UK) and Wistar rats (HsdHan®:WIST, InVivo, UK), aged 3 to 13 months. Rats were housed as described for Experiment 1.

3.7.2.2. Outdoor enclosure design

The test enclosure was the same as for Experiment 2. Male wood mice were tested in this experiment 4 months after the conclusion of Experiment 2.

3.7.2.3. Outdoor enclosure testing procedure

Testing was carried out in a similar manner to Experiment 2. For this experiment bait boxes were scented with female rat nest material, 4-ethyl phenol, or contained 5 g of clean nest material. On each night of testing 21 bait boxes were placed into the enclosure. Seven bait boxes were scented by adding 5 g of nest material that had been in female rat cages for 7 days. Nest material was pooled from multiple cages, and mixed, before being placed in bait boxes. Seven boxes were scented by adding 5 g of clean nest material, onto which was pipetted 0.3 mg 4-ethyl phenol (Sigma-Aldrich, UK) in 1.5 ml distilled water. The final 7 boxes contained 5 g clean nest material, onto which was pipetted 1.5 ml of distilled water. Each morning the weight of non-toxic bait eaten was measured. This experiment was repeated for 4 nights. The order of scent within each trio of boxes was randomised each night and the boxes were moved 2.5 m clockwise or anticlockwise each night.

3.7.2.4. Data analysis

Weight of bait eaten was log transformed and compared by a linear mixed effects model with box odour as fixed effect. Date and position of box (1-21) were included as random effects. Model significance was calculated by ANOVA comparison to a null model (see supplementary information). P-values of model fixed effect were calculated using likelihood ratio tests (Crawley, 2007). Planned comparisons were calculated between all pairs of treatment groups using Tukey contrasts. Models were validated using Q-Q plots and residual distributions (Figure S3.2). A p-value of less than 0.05 was taken as significant. All statistical analyses were carried out using R 3.2.4 (R Core Team, 2016) with packages 'lme4' (Bates *et al.*, 2015), 'lsmeans' (Lenth, 2016) 'afex' (Singmann *et al.*, 2017) and 'ggplot2' (Wickham, 2009).

3.7.3. Results

A population of 20 wood mice were given a choice to feed from bait boxes scented with 0.3 mg 4-ethyl phenol, bait boxes scented with female rat nest material and clean bait boxes in outdoor semi-natural enclosures. Testing was carried out over four nights.

Wood mice did not avoid feeding from 4-ethyl phenol odoured boxes compared to clean boxes (linear mixed effects model, $F_{2, 59.8} = 33.7$, $p < 0.001$; Tukey contrast, $t_{63} = 1.24$, $p = 0.44$; Figure 3.9). Wood mice did avoid feeding from female rat nest material boxes, eating considerably less non-toxic bait from them compared to clean boxes ($t_{63} = 7.67$, $p < 0.001$). In addition, wood mice ate considerably less from boxes containing female rat nest material compared to 4-ethyl phenol odoured boxes ($t_{63} = 6.43$, $p < 0.001$).

Median bait take was equal for 4-ethyl phenol boxes compared to clean boxes. However, wood mice ate 87% less from nest material odoured boxes compared to clean boxes.

3.7.4. Discussion

Wood mice avoided female rat nest material, but did not avoid 4-ethyl phenol in outdoor semi natural enclosures. This could be due to a loss of 4-ethyl phenol overnight. Rat odoured nest material will likely contain traces of urine, which contain major urinary proteins (MUPs) that could capture 4-ethyl phenol and slow its release (Gomez-Baena *et al.*, 2014; Hurst & Beynon, 2013). Nest material treated with 4-ethyl phenol would not contain these MUPs and so the 4-ethyl phenol may be released much faster. This could mean that wood mice were exposed to lower levels of 4-ethyl phenol in treated nest material, compared to rat odoured

nest material, especially later in the night. An alternative explanation is that while bank voles appear to be using 4-ethyl phenol to indicate the presence of rats, wood mice may be using other cues or may need a combination of compounds to indicate rat presence.

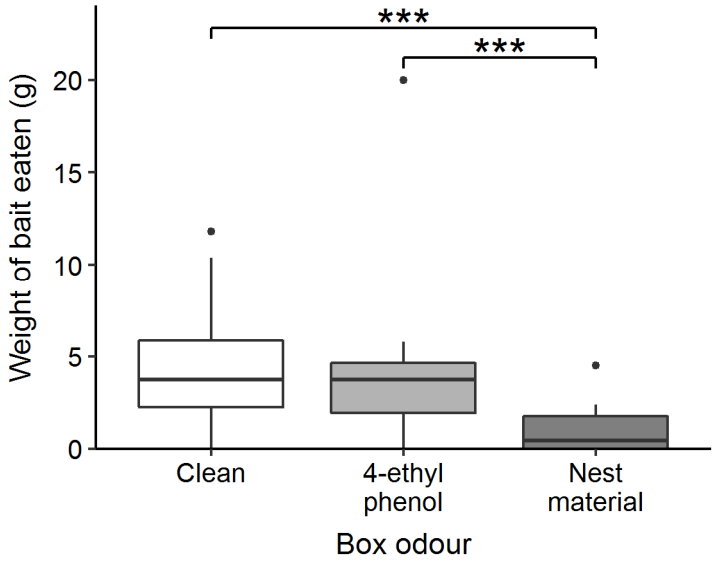


Figure 3.9. Weight of bait eaten per box per night by female wood mice from bait boxes scented with rat odour. Animals were presented with bait boxes scented with female rat nest material (dark grey bars), bait boxes containing nest material scented with 0.3 mg 4-ethyl phenol (light grey bars) and bait boxes containing clean nest material (white bars). Box plots show medians (black bar), IQR (boxes), 1.5 IQR (whiskers) and outliers (dots). Weight of food eaten from bait boxes was compared using a linear mixed effects model, followed by Tukey comparisons (*** $p<0.001$).

3.8. Experiment 6. Changes in concentration of 4-ethyl phenol overnight

3.8.1. Introduction

In Experiment 5, wood mice did not avoid 0.3 mg 4-ethyl phenol when presented on clean nest material in outdoor enclosures. This amount of 4-ethyl phenol was originally selected as it was the highest amount present in rat nest material elutions. One explanation for the lack of avoidance of 0.3 mg 4-ethyl phenol by wood mice is that 4-ethyl phenol evaporates faster from treated nest material, compared to that found on female rat nest material. This would lead to wood mice being exposed to a lower amount of 4-ethyl phenol on treated nest material, compared to female rat nest material. To test this, we compared the levels of 4-ethyl phenol on treated nest material and on female rat nest material when fresh, and after being left in open vials overnight. We hypothesised that the level of 4-ethyl phenol would be lower after being left overnight in treated nest material compared to female rat nest material.

3.8.2. Methods

3.8.2.1. Odour donors

Odour donors were 19 adult female, 3rd to 5th generation, Brown Norway (BN/SsNOlaHsd, InVivo, UK) crossed Wistar rats (HsdHan®:WIST, InVivo, UK), aged 7 to 12 months. Odour donors were housed as for Experiment 1 in single sex pairs, trios or quads.

3.8.2.2. Preparation of nest material elution

Nest material was collected that had been in rat cages for 7 days. Nest material was pooled from multiple cages, and mixed, before being stored at -18°C prior to mass spectral analysis.

To assess the total amount of 4-ethyl phenol present within each sample, nest material volatile compounds were analysed by hexane extraction. In addition, to assess the amount of 4-ethyl phenol released from each sample, nest material was sampled using headspace solid phase micro-extraction (SPME) and GC-MS. For overview of workflow see Figure 3.10.

3.8.2.3. GC-MS hexane extraction method

The amount of 4-ethyl phenol present in soiled nest material was compared to the amount present when 4-ethyl phenol was added to clean nest material using GC-MS of hexane extractions. For soiled nest material samples, 0.25 g soiled nest material was placed in a 15 ml vial. For 4-ethyl phenol samples, 15 µg authentic 4-ethyl phenol (Sigma-Aldrich, UK) in 75 µl distilled water was applied to 0.25g of clean nest material in 15 ml vials. Three replicates

were analysed for soiled nest material and 4-ethyl phenol added to clean nest material. 10 ml hexane was added to all samples, and samples were left to stand for 1 hour at room temperature (approximately 20°C).

1 µl of the organic layer from each hexane extraction was removed using a 50 µl glass syringe and then injected into a GCT GC-MS (Waters, UK). The GC column was a 30 m x 0.25 mm diameter x 0.25 mm film thickness DB-WAX (Agilent J & W, USA). Helium was used as a carrier gas at a rate of 0.8 ml/min. The injector was set to splitless mode at a temperature of 220°C. The temperature programme was 40°C to 240°C, at an increase rate of 6°C per minute, with a 2 minute initial hold. A mass range of 45 to 200 Da was measured with a scan time of 0.9 s.

A second set of samples was treated exactly the same as the first set, but these samples were left to stand unsealed, overnight at ambient temperature (approximately 20°C). The following day these samples were extracted using hexane and analysed by GC-MS using the same method as on day 1.

3.8.2.4. GC-MS headspace method

The amount of 4-ethyl phenol released from soiled nest material was compared to the amount released from 4-ethyl phenol added to clean nest material using GC-MS of SPME fibres. Samples were tested immediately and then after being left open to the atmosphere overnight. For soiled nest material samples, 0.25 g soiled nest material was sealed in 4 ml headspace vials. For 4-ethyl phenol samples, 15 µg authentic 4-ethyl phenol (Sigma-Aldrich, UK) in 75 µl distilled water was applied to 0.25g of clean nest material in 4ml vials then sealed. Three replicates were analysed each for soiled nest material and 4-ethyl phenol added to clean nest material. After sealing, a 1 cm SPME fibre DVB/CAR/PDMS (Sigma-Aldrich, UK) was inserted into each vial. SPME fibres were left in situ for 1 hour at ambient temperature (approximately 20°C). SPME fibres were then analysed on a GCT GC-MS (Waters, UK). The GC column was a 30 m x 0.25 mm diameter x 0.25 mm film thickness DB-WAX (Agilent J & W, USA). Helium was used as a carrier gas at a rate of 0.8 ml/min. The injector was set to splitless mode at a temperature of 220°C. The temperature programme was 40°C to 240°C, at an increase rate of 6°C per minute, with a 2 minute initial hold. A mass range of 45 to 200 Da was measured with a scan time of 0.9 s. Following SPME analysis sample vials were unsealed and left overnight at ambient temperature (approximately 20°C). The following day SPME analysis was repeated using the same method as on day 1.

3.8.2.5. Data analysis

To assess the relative amount of 4-ethyl phenol in samples, peak areas of the three most abundant 4-ethyl phenol masses (77, 107 and 122) were measured and summed. Mean TIC peak area of 4-ethyl phenol was compared before and after overnight evaporation for SPME and hexane extraction.

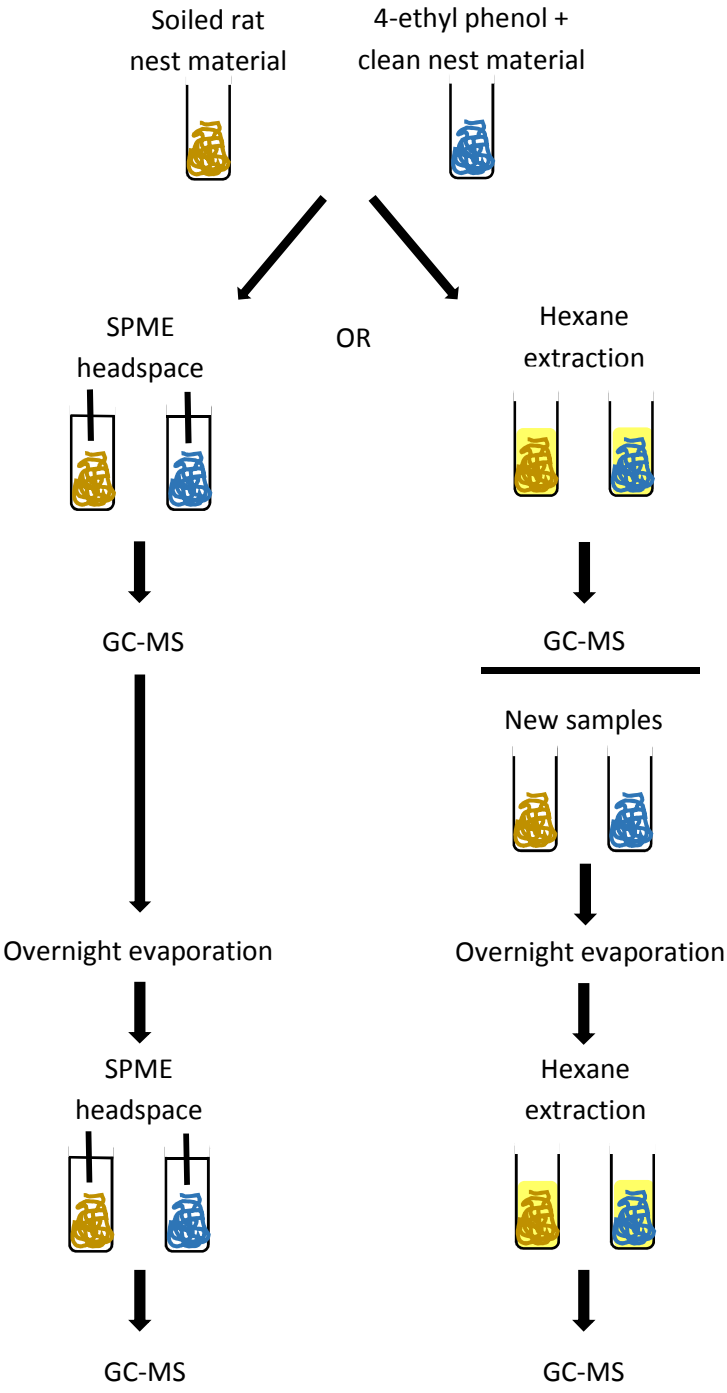


Figure 3.10. Workflow for comparison of 4-ethyl phenol in nest material before and after overnight evaporation. GC-MS work and hexane extractions carried out by M. Prescott, University of Liverpool.

3.8.3. Results

The relative abundance of 4-ethyl phenol in soiled rat nest material and 4-ethyl phenol applied to clean nest material was compared by headspace GC-MS and hexane extraction. Abundances were compared before and after samples were left open to evaporation overnight.

For sampling by hexane extraction and headspace GC-MS, 4-ethyl phenol was the most abundant volatile compound in soiled rat nest material for all samples (Figures 3.11, 3.12).

Hexane extractions indicate the amount of volatile compound present in a sample. The amount of 4-ethyl phenol present in soiled rat nest material was 50 fold lower than in samples where 0.3 mg 4-ethyl phenol was added to clean nest material, immediately and after overnight evaporation (Table 3.3). Looking at the amount of 4-ethyl phenol lost from nest material overnight, the amount of 4-ethyl phenol decreased at a similar rate from soiled nest material compared to clean nest material.

Headspace analysis indicates the amount of volatile compound released from a sample. This gives an indication of how much volatile compound is present in the atmosphere to be detected by an animal.

The amount of 4-ethyl phenol released from soiled nest material was 100 fold lower than was released from 0.3 mg 4-ethyl phenol added to clean nest material, when measured immediately (Table 3.3). 4-ethyl phenol was released faster from soiled nest material than from clean nest material.

Table 3.3. 4-ethyl phenol SPME peak areas before and after overnight evaporation.

Sample type	Processing method	Mean initial SPME peak area	Mean SPME peak area after overnight evaporation	Reduction in peak area following overnight evaporation (%)
4 Ethyl phenol + clean nest material	Hexane extraction	660.7 (601 – 692)	282.7 (110 – 487)	57.2
Soiled nest material		12.0 (8 – 18)	5.3 (2 – 8)	55.6
4 Ethyl phenol + clean nest material	Headspace	15342.3 (13096 – 17450)	9862.0 (8514 – 11628)	35.7
Soiled nest material		141.3 (105 – 194)	46.3 (42 – 53)	67.2

Parentheses under mean peak areas indicate range.

GC-MS work, hexane extractions and peak area calculations carried out by M. Prescott, University of Liverpool.

3.8.4. Discussion

Overall, a much greater quantity of 4-ethyl phenol was present in, and released from, 0.3 mg 4-ethyl phenol applied to clean nest material, compared to soiled rat nest material. The amount of 0.3 mg 4-ethyl phenol was originally obtained from hexane extractions of aqueous elutions of soiled nest material (Experiment 3). It may be that during the aqueous elution process 4-ethyl phenol is concentrated, leading to higher levels than are present in the starting material.

These results indicate that 4-ethyl phenol does not evaporate more quickly from clean nest material compared to soiled nest material. This suggests that the lack of avoidance of 0.3 mg 4-ethyl phenol by wood mice is not due to it being lost rapidly overnight. In fact, it would appear that wood mice were exposed to a much higher amount of 4-ethyl phenol when 0.3 mg was applied to clean nest material, than was released from soiled nest material. This indicates that wood mice are either not repelled by 4-ethyl phenol, or they are repelled by an amount of 4-ethyl phenol much lower than 0.3 mg, that reflects that in soiled rat bedding.

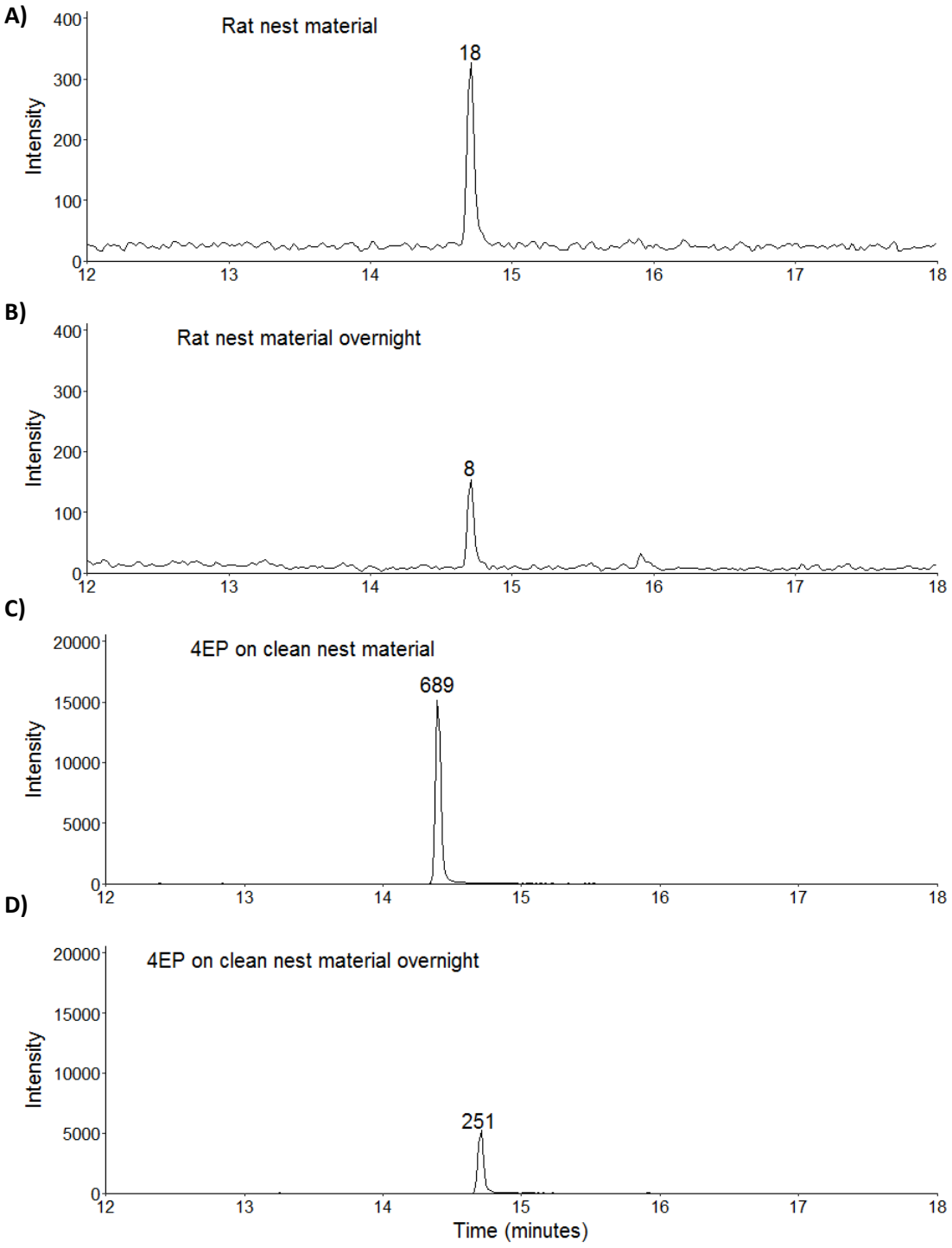


Figure 3.11. Representative hexane extraction total ion chromatograms. A) Soiled rat nest material. **B)** Soiled rat nest material after overnight evaporation. **C)** 0.3 mg 4-ethyl phenol added to clean nest material. **D)** 0.3 mg 4-ethyl phenol added to clean nest material after overnight evaporation. Numbers indicate 4-ethyl phenol peak area. GC-MS work carried out by M. Prescott, University of Liverpool.

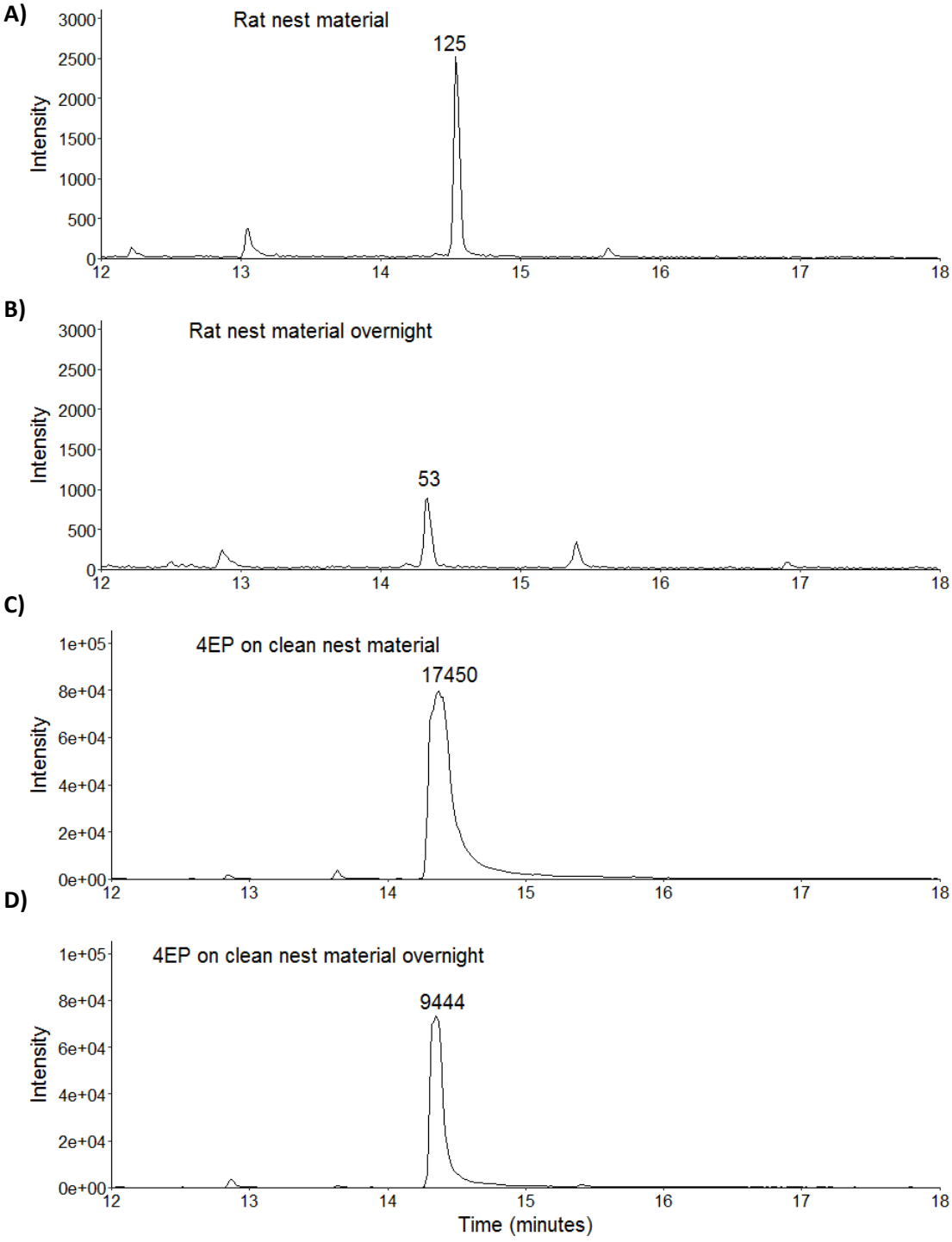


Figure 3.12. Representative SPME total ion chromatograms. A) Soiled rat nest material. **B)** Soiled rat nest material after overnight evaporation. **C)** 0.3 mg 4-ethyl phenol added to clean nest material. **D)** 0.3 mg 4-ethyl phenol added to clean nest material after overnight evaporation. Numbers indicate 4-ethyl phenol peak area. GC-MS work carried out by M. Prescott, University of Liverpool.

3.9. Experiment 7. Wood mice do not avoid 0.006 mg 4-ethyl phenol in outdoor semi-natural enclosures.

3.9.1. Introduction

The results of Experiment 6 indicated that the amount of 4-ethyl phenol present in 0.3 mg 4-ethyl phenol applied to clean nest material was much higher than that released from soiled rat nest material. Although it seems unlikely that wood mice are repelled by 4-ethyl phenol at a much lower amount than 0.3 mg, it is important to rule this out. Therefore, we tested wood mice with an amount of 4-ethyl phenol equivalent to that present in soiled rat nest material in Experiment 6. We hypothesised that wood mice would avoid 4-ethyl phenol at this level. However, we expected that wood mice would not avoid this amount of 4-ethyl phenol, as it is unlikely they would be repelled by such a low amount when they were not repelled by 0.3 mg.

3.9.2. Methods

3.9.2.1. Animal subjects

Subjects were 20 male wood mice housed as for Experiment 2. Odour donors were 33 adult female, 3rd to 5th generation crosses between Brown Norway (BN/SsNOlaHsd, InVivo, UK) and Wistar rats (HsdHan®:WIST, InVivo, UK), aged 5 to 13 months. Rats were housed as described for Experiment 1.

3.9.2.2. Outdoor enclosure design

The test enclosure was the same as for Experiment 2. Male wood mice were tested in this experiment 5 months after the conclusion of Experiment 5.

3.9.2.3. Outdoor enclosure testing procedure

In Experiment 6, the amount of 4-ethyl phenol present in soiled rat nest material was 50 fold less than 0.3 mg 4-ethyl phenol applied to rat nest material. Therefore, a 50 fold reduction in 4-ethyl phenol (0.006 mg) was tested with wood mice in outdoor semi-natural enclosures. Testing was carried out in a similar manner to Experiment 2. For this experiment bait boxes were scented with female rat nest material, 0.006 mg 4-ethyl phenol, or were clean. On each night of testing 21 bait boxes were placed into the enclosure. Seven bait boxes were scented by adding 5 g of nest material that had been in female rat cages for 7 days. Nest material was pooled from multiple cages before being placed in bait boxes. Seven boxes were scented by adding 5 g of clean nest material, onto which was pipetted 0.006 mg 4-ethyl phenol (Sigma-

Aldrich, UK) dissolved in 1.5 ml distilled water. The final 7 boxes contained 5 g clean nest material, onto which was pipetted 1.5 ml of distilled water. Each morning the weight of non-toxic bait eaten was measured. This experiment was repeated for 4 nights. The order of scent within each trio of boxes was randomised each night and the boxes were moved 2.5 m clockwise or anticlockwise each night.

3.9.2.4. Data analysis

Weight of bait eaten was square root transformed and compared by a linear mixed effects model with box odour as fixed effect. Date and position of box (1-21) were included as random effects. Model significance was calculated by ANOVA comparison to a null model (see supplementary information). P-values of model fixed effect were calculated using likelihood ratio tests (Crawley, 2007). Planned comparisons were calculated between all pairs of treatment groups using Tukey contrasts. Models were validated using Q-Q plots and residual distributions (Figure S3.3). A p-value of less than 0.05 was taken as significant. All statistical analyses were carried out using R 3.2.4 (R Core Team, 2016) with packages 'lme4' (Bates *et al.*, 2015), 'lsmeans' (Lenth, 2016) 'afex' (Singmann *et al.*, 2017) and 'ggplot2' (Wickham, 2009).

3.9.3. Results

A population of 20 wood mice were given a choice to feed from bait boxes scented with 0.006 mg 4-ethyl phenol, bait boxes scented with female rat nest material and clean bait boxes in outdoor semi-natural enclosures. Testing was carried out over four nights.

Wood mice did not avoid feeding from 4-ethyl phenol odoured boxes compared to clean boxes (linear mixed effects model, $F_{2, 59.6} = 12.5$, $p < 0.001$; Tukey contrast, $t_{60} = 0.93$, $p = 0.62$; Figure 3.13). Wood mice did avoid feeding from female rat nest material boxes, eating considerably less non-toxic bait from them compared to clean boxes ($t_{60} = 4.72$, $p < 0.001$). In addition, wood mice ate considerably less from boxes containing female rat nest material compared to 4-ethyl phenol odoured boxes ($t_{60} = 3.79$, $p = 0.001$).

Median bait take was 13% less from 4-ethyl phenol boxes, and 66% less from nest material odoured boxes, compared to clean boxes.

3.9.4. Discussion

Wood mice avoided female rat nest material, but did not avoid 0.006 mg 4-ethyl phenol in outdoor semi natural enclosures. Therefore, it is likely that 4-ethyl phenol on its own is not

responsible for the repellent effect of soiled nest material on wood mice. It may be that wood mice are repelled by a different compound, or a combination of compounds, present in rat nest material.

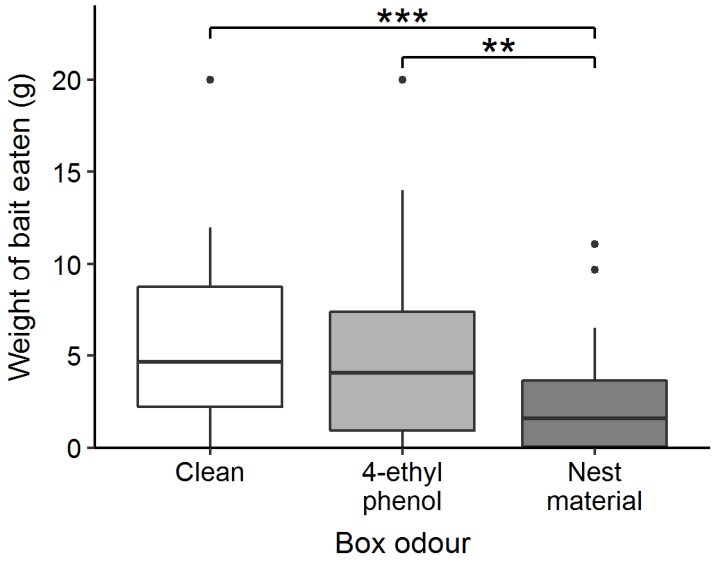


Figure 3.13. Weight of bait eaten per box per night by female wood mice from bait boxes scented with rat odour. Animals were presented with bait boxes containing female rat nest material (dark grey bars), bait boxes containing nest material scented with 0.006 mg 4-ethyl phenol (light grey bars) and bait boxes containing clean nest material (white bars). Box plots show medians (black bar), IQR (boxes), 1.5 IQR (whiskers) and outliers (dots). Weight of food eaten from bait boxes was compared using a linear mixed effects model, followed by Tukey comparisons (** $p<0.01$, *** $p<0.001$).

3.10. General discussion

Our results demonstrate avoidance of female rat odour by bank voles in laboratory arenas and wood mice in semi-natural enclosures. 4-ethyl phenol was the most abundant volatile compound in female rat nest material and aqueous elutions of male and female rat nest material. 4-ethyl phenol was avoided by bank voles in laboratory arenas, but not by wood mice in semi-natural enclosures.

Avoidance of male and female rat odour by bank voles and wood mice supports the hypothesis that non-target rodents are avoiding rat odour cues generally, rather than sex-specific cues. This is a logical conclusion. Whilst there is a small amount of evidence that male rats are more likely than female rats to predate small rodents (Paul *et al.*, 1971), it is likely that rats of both sexes would be a threat to small rodents. Therefore, it would be expected that non-target rodents would avoid all rats. The most efficient way for non-target rodents to avoid all rats is to use generic rat cues to indicate rat presence, although we cannot rule out that they use both male specific and female specific cues.

Whilst bank voles avoided 4-ethyl phenol, wood mice did not. Wood mice did not avoid 4-ethyl phenol at the upper, or lower end of the ranges identified in this study. The results of Experiment 6 suggest that 4-ethyl phenol is not lost rapidly overnight when applied to clean nest material. Therefore, it seems likely that 4-ethyl phenol is not repellent to wood mice when presented on its own. When wood mice are repelled by rat odour, they may be responding to a different volatile compound in rat odour than 4-ethyl phenol. On the other hand, they may respond to 4-ethyl phenol, but require the addition of other compounds to indicate rat presence. Wood mice showed a slight tendency to avoid rat nest material elutions, but this was not significant. This may have been due to the high variability in the concentration of volatile compounds in nest material elutions (Experiment 3). Alternatively, some components of rat odour may be lost during the elution process. In this study, only the volatile components of rat odour were examined. Other molecules present in predator odour cues include proteins, which cause repulsion in some species (Papes *et al.*, 2010). A more detailed study of the volatile and protein components of rat odour may indicate additional molecules that may be repellent to wood mice.

The results of this study indicate that rat odour has potential as a non-target repellent. There was a substantial reduction of 65-87% in non-toxic bait take for wood mice when comparing rat odoured boxes with control boxes. Both wood mice and bank voles continued to avoid

rat odour after repeated testing, indicating that habituation to rat odour does not develop quickly. We have established that non-target rodents are likely avoiding generic rat odour cues, rather than sex specific cues. This is advantageous due to the concern that sex-specific cues could repel some rats (Takács *et al.*, 2016). By using generic rat cues to repel non-target rodents, the effect on target rats may be minimised. This is backed up by rats not avoiding the generic rat odour cue 4-ethyl phenol (Zhang *et al.*, 2008), although further tests will be needed to confirm that identified compounds are not repellent to rats. 4-ethyl phenol has the potential to repel bank voles, but further work will be needed to find a volatile compound, or mixture of compounds, from rat odour that is capable of repelling wood mice.

In addition to establishing the volatile components of rat odour that wood mice are repelled by, further tests should also establish the effect of female rat nest material on other non-target rodents such as field voles. Given the results obtained for wood mice and bank voles, it is likely that field voles would be repelled by both male and female rat odour, especially as field voles will avoid male rat odour and the odours of other predators, such as weasels and foxes (Bolbroe *et al.*, 2000; Carlsen *et al.*, 1999; Chapter 2). However, this should be confirmed. Field trials are also needed to test if rat odour is repellent to non-target rodents in natural conditions.

In conclusion, we have shown that female rat odour is repellent to bank voles and wood mice, indicating that these species are likely to be avoiding generic rat odour cues. We have also shown the main volatile component of rat nest material, 4-ethyl phenol, is repellent to bank voles. With further refinement and testing, rat odour has the potential to be used as a non-target repellent.

3.11. Appendix

3.11.1. Supplementary information

3.11.1.1. Linear mixed effects model and null model for Experiment 2 (female rat nest material and nest material elution experiment)

Model: Log (Eaten+1) ~ Odour + (1 | Test day) + (1 | Box position)

Null model: Log (Eaten+1) ~ 1 + (1 | Test day) + (1 | Box position)

A) **B)**

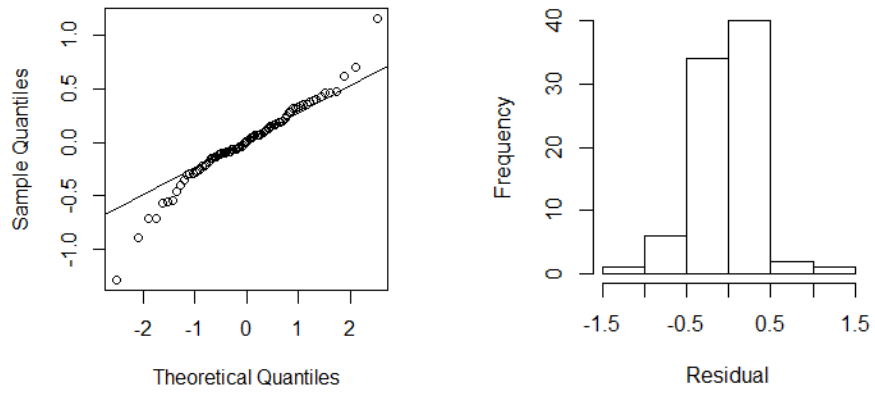


Figure S3.1. Female rat nest material residual plots. A) Normal Q-Q Plot. **B)** Histogram.

3.11.1.2. Linear mixed effects model and null model for Experiment 5 (0.3 mg 4-ethyl phenol and rat nest material experiment)

Model: Log (Eaten+1) ~ Odour + (1 | Test day) + (1 | Box position)

Null model: Log (Eaten+1) ~ 1 + (1 | Test day) + (1 | Box position)

A) **B)**

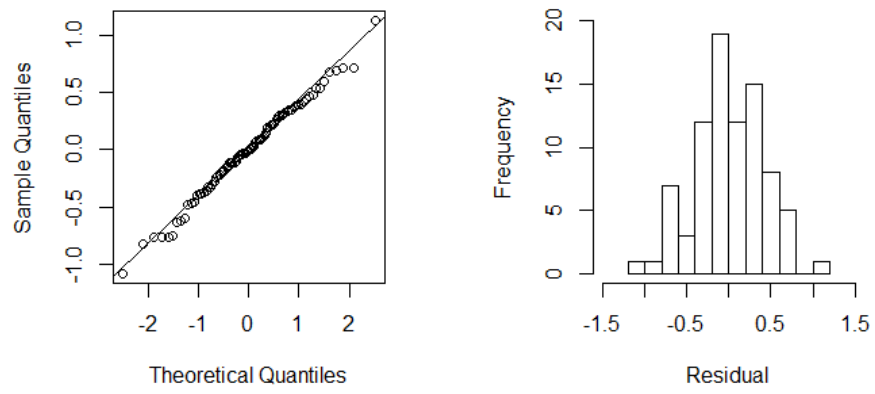


Figure S3.2. 0.3 mg 4-ethyl phenol residual plots. A) Normal Q-Q Plot. **B)** Histogram.

3.11.1.3. Linear mixed effects model and null model for Experiment 7 (0.006 mg 4-ethyl phenol and rat nest material experiment)

Model: Square root (Eaten+1) ~ Odour + (1 | Test day) + (1 | Box position)

Null model: Square root (Eaten+1) ~ 1 + (1 | Test day) + (1 | Box position)

A) **B)**

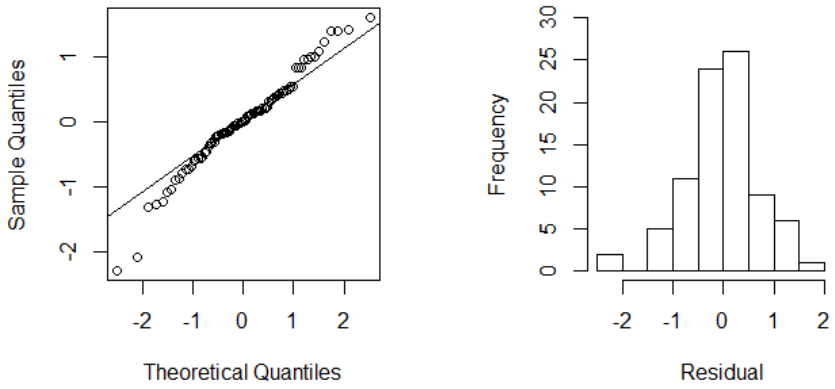


Figure S3.3. 0.006 mg 4-ethyl phenol residual plots. A) Normal Q-Q Plot. **B)** Histogram.

3.12. References

Achiraman, S. & Archunan, G. (2002). Characterization of urinary volatiles in Swiss male mice (*Mus musculus*): bioassay of identified compounds. *Journal of Biosciences.* **27**, 679-686.

Animals (Scientific Procedures) Act. (1986). UK.

Association for the Study of Animal Behaviour (2018). Guidelines for the treatment of animals in behavioural research and teaching. *Animal Behaviour.* **135**, I-X.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software.* **67**, 1-48.

Bolbroe, T., Jeppesen, L. L. & Leirs, H. (2000). Behavioural response of field voles under mustelid predation risk in the laboratory: more than neophobia. *Annales Zoologici Fennici.* **37**, 169-178.

Brakes, C. R. & Smith, R. H. (2005). Exposure of non-target small mammals to rodenticides: short-term effects, recovery and implications for secondary poisoning. *Journal of Applied Ecology.* **42**, 118-128.

Bramley, G. & Waas, J. (2001). Laboratory and Field Evaluation of Predator Odors as Repellents for Kiore (*Rattus exulans*) and Ship Rats (*Rattus rattus*). *Journal of Chemical Ecology.* **27**, 1029-1047.

Buckle, A. & Fenn, M. (1992) Rodent control in the conservation of endangered species. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 36-41.

Capizzi, D., Bertolino, S. & Mortelliti, A. (2014). Rating the rat: global patterns and research priorities in impacts and management of rodent pests. *Mammal Review.* **44**, 148-162.

Carlsen, M., Lodal, J., Leirs, H. & Jensen, T. S. (1999). The effect of predation risk on body weight in the field vole, *Microtus agrestis*. *Oikos.* **87**, 277-285.

Christiansen, E. (1976) Pheromones in small rodents and their potential use in pest control. In Siebe, C. C., Ed. *Proceedings of the Seventh Vertebrate Pest Conference, Monterey*. University of California. pp. 185-195.

Crawley, M. J. (2007). *The R book.* Wiley, Chichester.

Dawson, A., Bankes, J. & Garthwaite, D. (2001). *Rodenticide use on farms in Great Britain growing arable crops 2000.* pp. 1-22. DEFRA, York.

Devine, G. J. & Furlong, M. J. (2007). Insecticide use: Contexts and ecological consequences. *Agriculture and Human Values.* **24**, 281-306.

Dielenberg, R. A. & McGregor, I. S. (1999). Habituation of the hiding response to cat odor in rats (*Rattus norvegicus*). *Journal of Comparative Psychology.* **113**, 376-87.

Directive 2010/63/EU. (2010). EU.

Elmeros, M., Christensen, T. K. & Lassen, P. (2011). Concentrations of anticoagulant rodenticides in stoats (*Mustela erminea*) and weasels (*Mustela nivalis*) from Denmark. *Science of the Total Environment.* **409**, 2373-2378.

Elmeros, M., Lassen, P., Bossi, R. & Topping, C. J. (2018). Exposure of stone marten (*Martes foina*) and polecat (*Mustela putorius*) to anticoagulant rodenticides: Effects of regulatory restrictions of rodenticide use. *Science of the Total Environment.* **612**, 1358-1364.

Fendt, M. (2006). Exposure to urine of canids and felids, but not of herbivores, induces defensive behavior in laboratory rats. *Journal of Chemical Ecology.* **32**, 2617-2627.

Fendt, M. & Endres, T. (2008). 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor - Just repugnant or really fear-inducing? *Neuroscience and Biobehavioral Reviews.* **32**, 1259-1266.

Geduhn, A., Esther, A., Schenke, D., Mattes, H. & Jacob, J. (2014). Spatial and temporal exposure patterns in non-target small mammals during brodifacoum rat control. *Science of the Total Environment.* **496**, 328-338.

Gomez-Baena, G., Armstrong, S. D., Phelan, M. M., Hurst, J. L. & Beynon, R. J. (2014). The major urinary protein system in the rat. *Biochemical Society Transactions.* **42**, 886-92.

Hadler, M. R. & Buckle, A. P. (1992) Forty five years of anticoagulant rodenticides - past, present and future trends. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 149-156.

Health and Safety Executive (2012). *Environmental risk mitigation measures for second generation anticoagulant rodenticides proposed by the UK* pp. 1-30. Health and Safety Executive, UK.

Health and Safety Executive (2015). *UK anticoagulant rodenticide product authorisation and the CRRU stewardship scheme.* pp. 1-12. Health and Safety Executive, UK.

Hothorn, T. & Hornik, K. (2015). *exactRankTests: Exact Distributions for Rank and Permutation Tests*. https://CRAN.R-project.org/package=exactRankTests

Hurst, J. L. (1987). The Functions of Urine Marking in a Free-Living Population of House Mice, *Mus-Domesticus* Rutty. *Animal Behaviour.* **35**, 1433-1442.

Hurst, J. L. (1990). Urine Marking in Populations of Wild House Mice *Mus-Domesticus* Rutty .3. Communication between the Sexes. *Animal Behaviour.* **40**, 233-243.

Hurst, J. L. & Beynon, R. J. (2013). Rodent Urinary Proteins: Genetic Identity Signals and Pheromones. In *Chemical Signals in Vertebrates 12.* East, M. L. & Dehnhard, M. (Eds.), pp. 117-133. Springer, New York.

Johnson, R. P. (1973). Scent Marking in Mammals. *Animal Behaviour.* **21**, 521-535.

Lenth, R. V. (2016). Least-Squares Means: The R Package lsmeans. *Journal of Statistical Software.* **69**, 1-33.

Lima, S. L. & Bednekoff, P. A. (1999). Temporal Variation in Danger Drives Antipredator Behavior: The Predation Risk Allocation Hypothesis. *The American Naturalist.* **153**, 649-659.

Martinez-Padilla, J., Lopez-Idiaquez, D., Lopez-Perea, J. J., Mateo, R., Paz, A. & Vinuela, J. (2017). A negative association between bromadiolone exposure and nestling body condition in common kestrels: management implications for vole outbreaks. *Pest Management Science.* **73**, 364-370.

Mason, G. & Littin, K. E. (2003). The humaneness of rodent pest control. *Animal Welfare.* **12**, 1-37.

McGregor, I. S., Schrama, L., Ambermoon, P. & Dielenberg, R. A. (2002). Not all 'predator odours' are equal: cat odour but not 2, 4, 5 trimethylthiazoline (TMT; fox odour) elicits specific defensive behaviours in rats. *Behavioural Brain Research.* **129**, 1-16.

Memmott, K., Murray, M. & Rutberg, A. (2017). Use of anticoagulant rodenticides by pest management professionals in Massachusetts, USA. *Ecotoxicology.* **26**, 90-96.

NC3Rs, BBSRC, DEFRA, MRC, NERC & Wellcome Trust. (2017). Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. NC3Rs, London.

O'Boyle, M. (1974). Rats and mice together: The predatory nature of the rat's mouse-killing response. *Psychological Bulletin.* **81**, 261-269.

Papes, F., Logan, D. W. & Stowers, L. (2010). The Vomeronasal Organ Mediates Interspecies Defensive Behaviors through Detection of Protein Pheromone Homologs. *Cell.* **141**, 692-703.

Parsons, M. H., Apfelbach, R., Banks, P. B., Cameron, E. Z., Dickman, C. R., Frank, A. S., Jones, M. E., McGregor, I. S., McLean, S. & Müller-Schwarze, D. (2018). Biologically meaningful scents: a framework for understanding predator–prey research across disciplines. *Biological Reviews.* **93**, 98-114.

Paul, L., Miley, W. M. & Baenninger, R. (1971). Mouse killing by rats: Roles of hunger and thirst in its initiation and maintenance. *Journal of Comparative and Physiological Psychology.* **76**, 242-249.

Pimentel, D. (2005). Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. *Environment, Development and Sustainability.* **7**, 229-252.

R Core Team. (2016). *R: A language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Selvaraj, R. & Archunan, G. (2006). Efficacy of male scent glands and urine in masking poison bait odour in female house rats, *Rattus rattus*. *Journal of Pest Science.* **79**, 255-258.

Serieys, L. E. K., Armenta, T. C., Moriarty, J. G., Boydston, E. E., Lyren, L. M., Poppenga, R. H., Crooks, K. R., Wayne, R. K. & Riley, S. P. D. (2015). Anticoagulant rodenticides in urban bobcats: exposure, risk factors and potential effects based on a 16-year study. *Ecotoxicology.* **24**, 844-862.

Shore, R. F., Walker, L. A., Potter, E. D., Pereira, M. G., Sleep, D. & Thompson, N. J. (2016). *Second generation anticoagulant rodenticide residues in barn owls 2016.* pp. 1-21. Centre for Ecology & Hydrology, Lancaster.

Singmann, H., Bolker, B., Westfall, J. & Aust, F. (2017). *afex: Analysis of Factorial Experiments*. https://CRAN.R-project.org/package=afex

Sullivan, T. P., Crump, D. R. & Sullivan, D. S. (1988). Use of predator odors as repellents to reduce feeding damage by herbivores: III. Montane and meadow voles (*Microtus montanus* and *Microtus pennsylvanicus*). *Journal of Chemical Ecology.* **14**, 363-377.

Takács, S., Gries, R., Zhai, H. & Gries, G. (2016). The Sex Attractant Pheromone of Male Brown Rats: Identification and Field Experiment. *Angewandte Chemie-International Edition.* **55**, 6062-6066.

Tosh, D. G., McDonald, R. A., Bearhop, S., Lllewellyn, N. R., Fee, S., Sharp, E. A., Barnett, E. A. & Shore, R. F. (2011a). Does small mammal prey guild affect the exposure of predators to anticoagulant rodenticides? *Environmental Pollution.* **159**, 3106-3112.

Tosh, D. G., Shore, R. F., Jess, S., Withers, A., Bearhop, S., Ian Montgomery, W. & McDonald, R. A. (2011b). User behaviour, best practice and the risks of non-target exposure associated with anticoagulant rodenticide use. *Journal of Environmental Management.* **92**, 1503-1508.

Walker, L. A., Chaplow, J. S., Llewellyn, N. R., Pereira, M. G., Potter, E. D., Sainsbury, A. W. & Shore, R. F. (2013). *Anticoagulant rodenticides in predatory birds 2011: a Predatory Bird Monitoring Scheme (PBMS) report.* pp. 1-29. Centre for Ecology & Hydrology, Lancaster.

Walker, L. A., Chaplow, J. S., Moeckel, C., Pereira, M. G., Potter, E. D., Sainsbury, A. W. & Shore, R. F. (2016). *Anticoagulant rodenticides in red kites (Milvus milvus) in Britain 2010 to 2015: a Predatory Bird Monitoring Scheme (PBMS) report.* pp. 1-14. Centre for Ecology & Hydrology, Lancaster.

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, New York. http://ggplot2.org

Williamson, S., Ball, A. & Pretty, J. (2008). Trends in pesticide use and drivers for safer pest management in four African countries. *Crop Protection.* **27**, 1327-1334.

Wright, L. D., Muir, K. E. & Perrot, T. S. (2013). Stress responses of adolescent male and female rats exposed repeatedly to cat odor stimuli, and long-term enhancement of adult defensive behaviors. *Developmental Psychobiology.* **55**, 551-567.

Zhang, J.-X., Sun, L., Zhang, J.-H. & Feng, Z.-Y. (2008). Sex-and gonad-affecting scent compounds and 3 male pheromones in the rat. *Chemical Senses.* **33**, 611-621.

Chapter 4

4. Field trials of rat odour as a non-target repellent

4.1. Abstract

Pest rats (*Rattus spp.*) are a major problem globally and are commonly controlled using poisons. Unfortunately, these poisons are not species specific and kill large numbers of non-target animals, especially small rodents, such as wood mice (*Apodemus sylvaticus*) and bank voles (*Myodes glareolus*). Rat odour has been suggested as a repellent that could reduce the amount of poisoned baits consumed by non-target rodents, without repelling target rats. However, it is not known how non-target rodents respond to rat odour under natural conditions. In a series of field trials, we assessed the response of non-target rodents to rat odour. Rat odour did not repel non-target rodents when presented in live rodent traps. In addition, presence of rat odour did not reduce the weight of non-toxic bait eaten from mouse bait boxes. However, presentation of rat odour in rat bait boxes led to a highly significant, 26% reduction in bait take in rat odoured boxes compared to clean boxes. We conclude that rat odour has the potential to be used as a non-target repellent but further work is needed to fully optimise the odour cue and conditions required to ensure its effectiveness.

4.2. Introduction

Non-target poisoning is a major problem during animal pest control and is a particular problem for pest control aimed at rats (*Rattus spp.*; (Hoare & Hare, 2006; Pimentel, 2005; Walker *et al.*, 2013). Globally, rats are considered as some of the most important pest species (Capizzi *et al.*, 2014). They are a major threat to global food security, carry or transmit over 50 infectious diseases and cause large amounts of damage to human infrastructure (Meerburg *et al.*, 2009; Pimentel *et al.*, 2005; Singleton, 2003; Singleton, 2010). In addition, they are highly invasive and threaten endangered species, particularly in island habitats (Buckle & Fenn, 1992; Major *et al.*, 2007). The most common form of rat control is poisons, particularly the second generation anticoagulant rodenticides (SGARs; Capizzi *et al.*, 2014; Dawson *et al.*, 2001). SGARs are highly toxic to all mammals and birds (Hadler & Buckle, 1992). In addition, they are bioaccumulative, passing up the food chain to harm not only primary consumers of rat poison, but their predators as well (Erickson & Urban, 2004). The extent of this problem is such that over 75% of red kites, barn owls and red foxes in the UK have residues of SGARs in their internal organs (Shore *et al.*, 2016; Tosh *et al.*, 2011; Walker *et al.*, 2016).

One proposed method to reduce the exposure of non-target animals to rodenticides is to use odour cues that cause differential responses between rats and non-target species (Chapters 2, 3). The non-target species most commonly poisoned during rat control are smaller rodents such as wood mice (*Apodemus sylvaticus*), bank voles (*Myodes glareolus*) and field voles (*Microtus agrestis*; (Brakes & Smith, 2005; Geduhn *et al.*, 2014). These rodents are potential prey for rats (Bridgman *et al.*, 2013; Paul *et al.*, 1971) and thus would be expected to avoid the odour of rats. Experiments conducted in laboratory arenas and outdoor, semi-natural enclosures have confirmed that all three of these non-target rodent species are repelled by both male and female rat odour (Chapters 2, 3). While male rat odour may be repellent to some male rats, studies have shown that female rat odour does not appear to be repellent to rats of either sex (Takács *et al.*, 2016; K. Pounder, personal communication). Thus, female rat odour has potential to be used as a non-target repellent without reducing the efficacy of poisons against rats.

To further test the suitability of female rat odour as a non-target repellent field trials are needed, as avoidance of predator odour in laboratory studies may not be reproducible under natural conditions. For example, rats avoid fox odour in laboratory tests, but do not avoid

traps scented with this odour in the field (Banks, 1998; Fendt & Endres, 2008; Wallace & Rosen, 2000). The way in which animals respond to predator odours can be influenced by many factors that cannot be replicated in the controlled environment of the laboratory, or even the semi-natural environment of outdoor enclosures. Careful consideration of these factors is needed in order for a predator odour to be used effectively as a repellent in the field.

An important factor to consider in the application of predator odours in the field is where to apply the odour. When an animal detects a predator odour it can either continue with its normal behaviour or initiate defensive behaviours, including escape, habitat shifts and a reduction in foraging (Apfelbach *et al.*, 2005). Defensive behaviours may make the animal less likely to encounter the predator, however these behaviours are costly (Zylberberg & DeWeese, 2011). The predation risk allocation hypothesis indicates that a prey animal will respond to predator odour depending on how likely the odour indicates that the predator may be encountered, and the cost associated with avoiding that odour cue (Lima & Bednekoff, 1999). Even if an odour indicates a high risk of predator presence, a prey animal may not avoid that cue if its fear is overcome by a need to forage (Lima & Bednekoff, 1999). This effect is exacerbated if a prey animal is constantly exposed to a predator odour over large areas (Lima & Bednekoff, 1999). To overcome this problem predator odour should be applied to the environment in a targeted manner that indicates short term, high risk of recent predator presence (Lima & Bednekoff, 1999). In previous studies using rat odour as a predator odour, the odour has been presented in bait boxes, rather than spread over large areas (Chapters 2, 3). This limits non-target rodent exposure to rat odour to finite locations, replicating short term predator exposure that is more likely to lead to avoidance.

In addition to where predator odour is applied, it is important to consider which source of odour should be used. Field studies of predator odour have found avoidance of body odour, faeces and urine by a variety of rodent species (Bytheway *et al.*, 2013; Navarro-Castilla & Barja, 2014; Sullivan *et al.*, 1988). However, there are indications from other studies that body odour may cause a greater avoidance response than other bodily secretions (Blanchard *et al.*, 2003; Masini *et al.*, 2005). In addition, it has been shown that rat body odour is more repellent than rat faeces to wood mice (Chapter 2). Increased avoidance of body odour compared to urine or faeces is thought to be due to body odour being a more reliable indicator of recent predator presence than faeces or urine (Blanchard *et al.*, 2003). This is in line with the predation risk allocation hypothesis, which theorises that avoidance of predator

odour will increase with increasing likelihood of encountering a predator (Lima & Bednekoff, 1999). Therefore, field trials of rat odour should use an odour cue that incorporates body odour, such as nest material.

Another factor that could affect the likelihood of predator odour avoidance in the field is the presence of other predator odours in the environment. As stated above, prey animals should avoid predator odour only when the risks of meeting the predator outweigh the costs of avoiding the predator. In the field, prey animals must make these judgements within a landscape of multiple predatory scents. It has been suggested that prey will respond to the threat of a predator depending on the level of threat posed by that predator (Helfman, 1989). For example, damselfish are more likely to respond to large predators that appear to be about to attack (Helfman, 1989). For field studies trialling rat odour as a repellent, presence of other predators could be highly important as rats are likely to pose a lower threat than predators that commonly prey on small rodents, such as mustelids (Barreto & Macdonald, 1999; Ylönen *et al.*, 1992). Thus, small rodents may not avoid rat odour if odour cues are present from more threatening predators in the environment. Distribution of predators will vary over different locations (Harris *et al.*, 1995). Therefore, it is advisable to test rat odour over multiple locations in the field, to control for the possible presence of other predators.

Context of predator odour presentation may also have an effect on predator odour avoidance in the field. Behavioural responses to predator cues are often specific to individual predators. For example, voles will move into dense cover when exposed to a kestrel, but move out into the open when exposed to a weasel (Korpimaki *et al.*, 1996). Thus, avoidance of a predator odour may be dependent on how it is presented. This is likely to be tied to prey vulnerability (Parsons *et al.*, 2018). A predator odour that is presented in a context where a prey animal feels vulnerable to predation will be more likely to be avoided than a predator odour presented where a prey animal does not feel vulnerable (Parsons *et al.*, 2018). Extrapolating from the above example, presentation of weasel odour to voles in open cover may not lead to as strong an avoidance response as when it is presented in dense cover, where voles are more susceptible to weasel predation. For rat odour, the confined space of a trap or bait box, with only one or two escape holes, may be perceived as a threatening environment, compared to open habitat. Thus, presentation of rat odour inside traps or bait boxes, may be more likely to generate an avoidance response than presenting rat odour in the open. A further consideration of predator odour context is the distance between the

odour and the resource to be protected (Parsons *et al.*, 2018). Ideally, predator odours should be placed close to the resource to be protected (Parsons *et al.*, 2018). For rat odour this means placing the odour source close to poisoned baits, such as within a trap or bait box.

In a series of experiments, we assessed the response of non-target rodents to rat odour under natural conditions. First, we wanted to determine if wild non-target rodents were repelled from entering control devices by rat odour. To assess this, we gave wild non-target rodents the choice between live traps scented with rat odour and clean traps (all traps were baited with food). We hypothesised that the presence of rat odour would reduce the number of non-target rodents willing to enter live traps. However, we found that non-target rodents did not avoid entering live traps scented with female rat odour. Following on from this result, we wanted to determine if the presence of rat odour reduced the amount of non-toxic bait consumed by non-target rodents. To assess this, we gave wild non-target rodents a choice between mouse bait boxes scented with rat odour and clean mouse bait boxes (all bait boxes were baited with non-toxic bait). Mouse bait boxes were chosen as their entrances are too small for rats to enter. We hypothesised that the presence of rat odour would reduce the weight of bait eaten from mouse bait boxes. However, we found that non-target rodents did not reduce bait take from mouse bait boxes scented with rat odour. To further assess if context of odour presentation was important in avoidance of rat odour, we repeated the mouse bait box experiment, but substituted larger rat bait boxes for mouse bait boxes (entrances were partially covered to prevent access by rats). We hypothesised that the presence of rat odour would reduce the weight of bait eaten from rat bait boxes. In this experiment, non-target rodents ate significantly less non-toxic bait from rat bait boxes scented with rat odour. We conclude that rat odour is repellent to non-target rodents in the field, but context of presentation is highly important in this response.

4.3. Methods

4.3.1. Ethics statement

All procedures, including live trapping, were non-invasive behavioural tests that did not involve pain, suffering or lasting harm and were carried out in accordance with international best practice guidelines (Animals (Scientific Procedures) Act, 1986; Directive 2010/63/EU, 2010; Association for the Study of Animal Behaviour, 2018; NC3Rs *et al.*, 2017). The study was approved by the University of Liverpool Animal Welfare Committee. No specific licences were required to carry out the work.

4.3.2. Study areas

Field work was carried out at Ness Botanical Gardens (53.273° N, -3.043° E) and Wood Park farm (53.290°N, -3.033° E) in North West England. Ness Botanical Gardens are located close to the coast at 34 m above sea level. Habitat on site is a mixture of deciduous woodland, wild flower meadows, scrubland and intensively managed gardens. Wood Park farm is a dairy farm, located 2 miles inland from Ness Botanical Gardens at 50 m above sea level. Habitat on site is farmland and deciduous woodland. Brown rats (*Rattus norvegicus*) were known to be present at both Wood Park farm and Ness Gardens, but not in the locations used in this study. Least weasels (*Mustela nivalis*) were known to be present at Ness Gardens. House mice (*Mus musculus*) were not known to be present at either site.

4.3.3. Odour donors

Odour donors were 51 adult female, 3rd to 5th generation crosses between Brown Norway (BN/SsNOlaHsd, InVivo, Bicester, UK) and Wistar rats (HsdHan®:WIST, InVivo, UK), aged 3 to 13 months. Odour donors were housed in a room maintained at 21 °C, 55% humidity and 20 air changes per hour on a reversed 12:12 hour light-dark cycle with lights off at 0800 h. Female rats were housed in same sex pairs or trios in 56 x 38 x 22 cm cages (RC2R, North Kent Plastics, Coalville, UK), in a room containing both male and female rats.

Rats were fed 5FL2 EURodent Diet (LabDiet, St Louis, USA) *ad libitum* and had access to *ad libitum* water. All cages had Corn Cob Absorb 10/14 substrate lining the base. Paper wool nest material (IPS Product Supplies Limited, London, UK) and 15 x 8 cm plastic tubes were provided to all rats for enrichment.

4.3.4. Odour collection

Nest material, soiled by female rats was used as an odour source as we had previously shown that it is repellent to non-target rodents (Chapter 3). Paper wool nest material was collected after it had been in female rat cages for 7 days. Nest material was pooled from multiple cages and mixed before being frozen at -18°C for up to 21 days prior to use in field experiments.

4.3.5. Schedule of experiments

Three different experiments were carried out: live trapping, bait take from mouse bait boxes and bait take from rat bait boxes. Full details of timing and location of experiments is given in Table 4.1 and Figure 4.1.

Table 4.1. Details of experimental timing and locations.

Session	Dates	Sites	Method	Weight of nest material in each trap/box (g)
1	19/06/17-23/06/17	Ness Gardens a-e	Small mammal traps	2
2	14/06/17-18/08/17	Ness Gardens d-f Woodpark Farm g-h	Mouse bait boxes	2
3	18/09/17-22/09/17	Ness Gardens d-f Woodpark Farm g-h	Mouse bait boxes	2
4	23/10/17-27/10/17	Ness Gardens d-f Woodpark Farm g-h	Rat bait boxes	5

A) **B)**

Figure 4.1. Experimental sites. Satellite image of **A)** Ness Botanical Gardens and **B)** Wood Park farm with experimental sites highlighted.

4.3.5.1. Live trapping experiment

The response of non-target rodents to rat odour was first examined by live trapping. Non-target rodents were given a choice between traps scented with rat odour and clean traps. Trapping was carried out in June 2017 at 5 sites in Ness Gardens (Figure 4.1A, Table 4.1). Trap sites were selected that contained good habitat for small rodents, including deciduous woodland, long grass and scrubland. Twenty traps were placed at each trapping site, giving 100 traps in total. Of the 100 traps used, 40 were Longworth traps (Figure 4.2C), 30 were Mk1 TubeTraps (BioEcoSS Ltd, Shropshire, UK; Figure 4.2A) and 30 were Mk2 TubeTraps (BioEcoSS Ltd, UK; Figure 4.2B). Longworth traps are made of aluminium. Pressure on a metal wire located halfway along the trap triggers the trap, causing a door at the front to close, trapping an animal inside. TubeTraps work on the same principle as Longworth traps, but are made of plastic, with a plastic bar located halfway along the trap as the trigger mechanism. Mk1 traps have a solid door, whereas Mk2 traps have a small hole in the door to allow shrews to escape.

Traps were set for 4 consecutive nights giving 400 trapping nights (4 nights x 100 traps). Traps were set in pairs of the same trap type, with at least 30 cm between traps within a pair and at least 5 m between trap pairs. Within each trap pair one trap was scented by adding 2 g paper wool nest material soiled by female rats for 7 days. The other trap in each pair contained 2 g clean paper wool nest material. The location of odour placement within each trap pair was randomised. All traps were baited with parakeet seed mix (Rob Harvey, Tongham, UK) and a piece of apple. Traps were checked in the morning, locked closed during the day, and then reopened at night.

For each small mammal trapped we recorded trap site, species, sex (determined by anogenital distance, which is larger in males) and weight. Each animal was tagged with a Pet-ID 8 mm radio-frequency identification microchip (Pet-ID Microchips Ltd, West Sussex, UK) to record recaptures. All captured animals were scanned with a Pet-ID scanner (Pet-ID Microchips Ltd, UK) to detect recaptures. Any trap that was triggered was removed, washed with dilute Teepol Multipurpose detergent (Teepol, Orpington, UK) and replaced on the same day. Fresh clean or rat odoured nest material was added to cleaned traps as appropriate. Nest material was not replaced in non-triggered traps for the duration of data collection.

4.3.5.2. Bait box experiments

Following live trapping, the effect of rat odour on weight of bait consumed by non-target rodents was examined using bait boxes baited with non-toxic bait. Non-target rodents were given a choice between bait boxes scented with rat odour and clean bait boxes. Bait box experiments were carried out over 3 separate weeks. Mouse bait boxes were used during the first two weeks. Mouse bait boxes were used initially as they were small enough to prevent access by rats. Rat bait boxes were used during the final week. Rat bait boxes (with partially covered entrances to prevent rat entry) were used to determine if size of bait box affected the avoidance response of non-target rodents to rat odour.

Bait box experiments were carried out in August, September and October 2017 at 3 sites in Ness Gardens (Figure 4.1A, Table 4.1) and 2 sites in Wood Park farm (Figure 4.1B, Table 4.1). Three sites at Ness Gardens used for live trapping were not used for bait box trials due to low numbers of rodent captures, particularly wood mice, at those sites (sites a-c). The three new sites were selected where high numbers of wood mice and bank voles had been captured in previous years (sites f-h). Twenty bait boxes were placed at each trapping site, giving 100 bait boxes in total. Bait boxes were placed in pairs with at least 5 m between bait box pairs. Within each bait box pair one bait box was scented by adding paper wool nest material soiled by female rats for 7 days. The other bait box in each pair contained clean paper wool nest material. The order of odour placement within each bait box pair was randomised. Bait boxes were baited with a 20 g Detex® non-toxic bait block (Bell laboratories, Sudbury, UK). Non-toxic bait blocks contain the same ingredients as bait normally used in pest control, but do not contain any poison.

Bait boxes were placed out on day 1 of the study and then checked daily for 4 days. At each check rat odoured nest material and clean nest material were replaced with fresh material in all boxes. Bait blocks were replaced if there was any sign of chewing. Weight of bait eaten from chewed bait blocks was recorded. Bait boxes were cleaned at the end of each experimental week, but were not cleaned during each week.

For data collection in August and September, mouse bait boxes (11 x 8 x 4 cm AF Advance Mouse boxes (Killgerm, Ossett, UK)) were used, containing 2 g of clean or rat odoured nest material (Table 4.1). Mouse bait box entrances were too small for rats to enter (2.5 x 2.5 cm). Mouse bait boxes were placed with at least 30 cm between bait boxes (Figure 4.3A). For data collection in October, rat bait boxes (28 x 25 x 13 cm Roguard® Extra bait boxes (BASF,

Cheadle Hulme, UK)) were used, containing 5 g of clean or rat odoured nest material (Table 4.1). More nest material was used in rat bait boxes than mouse bait boxes as the air space inside rat bait boxes is much larger. The entrances to rat bait boxes were partially blocked to prevent access by rats, but allow access by small rodents. Entrances were blocked by taping wire grids over entrance holes, leaving a 2.5 x 3 cm gap (Figure 4.3C). Rat bait boxes were placed so that they touched with entrances on outside edges (Figure 4.3B).

To check numbers of non-target rodents present during each bait box experiment, live traps were placed at the study sites during the week following bait box data collection. This was done after all three data collection weeks. One trap was placed at each bait box pair site, giving 50 traps. Of the 50 traps used, 20 were Mk1 TubeTraps (BioEcoSS Ltd, UK; Figure 4.2A) and 30 were Mk2 TubeTraps (BioEcoSS Ltd, UK; Figure 4.2B). All traps were baited with parakeet seed mix (Rob Harvey, UK) and a piece of apple. Traps were set for 4 consecutive nights and checked twice daily. For each rodent trapped we recorded the site of the trap, species of rodent, and sex (determined by anogenital distance, which is larger in males). Fur from the rear end of each animal was clipped to record recaptures. Traps were not cleaned for the duration of each trapping session.

4.3.6. Data analysis

4.3.6.1. Live trapping experiment

To determine if rat odour affected the number of small mammals captured, Pearson's Chi squared tests compared number of captures between odoured and clean traps. Primary captures and recaptures were analysed separately.

4.3.6.2. Bait box experiments

Data from both mouse bait box weeks was combined prior to further analysis. Data from mouse and rat bait box experiments were analysed separately. For each night of testing, box pairs were removed from the analysis if there was no bait take from both boxes, or if all bait was eaten from both boxes, to ensure model assumptions were met. To assess if box odour affected the likelihood of taking any bait, Pearson's Chi-squared test compared the frequency of any bait being consumed between box odours. To assess if box odour affected the weight of bait eaten, where bait was consumed, bait boxes where no bait had been consumed were removed from the analysis. Weight of bait eaten from bait boxes was then log transformed and analysed using a linear mixed effects model with box odour as a fixed effect and data collection site and night of sampling as random effects. Model significance

was calculated by ANOVA comparison to a null model (see supplementary information). P-values of model fixed effect were calculated using likelihood ratio tests (Crawley, 2007). Models were validated using quantile-quantile (Q-Q) plots and residual distributions (Figures S4.1, S4.2). For all analyses, a p-value of less than 0.05 was taken as significant. Statistical analysis was carried out using R 3.4.0 (R Core Team, 2016) with packages 'lme4' (Bates *et al.*, 2015), 'lsmeans' (Lenth, 2016) 'afex' (Singmann *et al.*, 2017) and 'ggplot2' (Wickham, 2009).

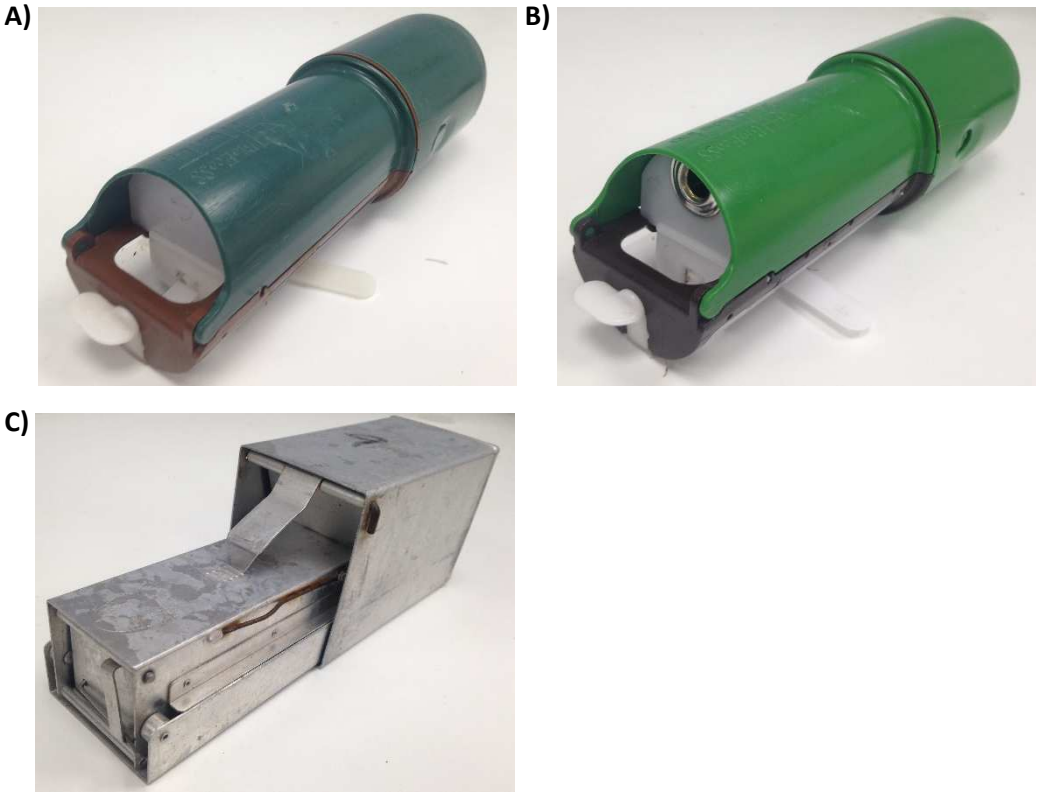
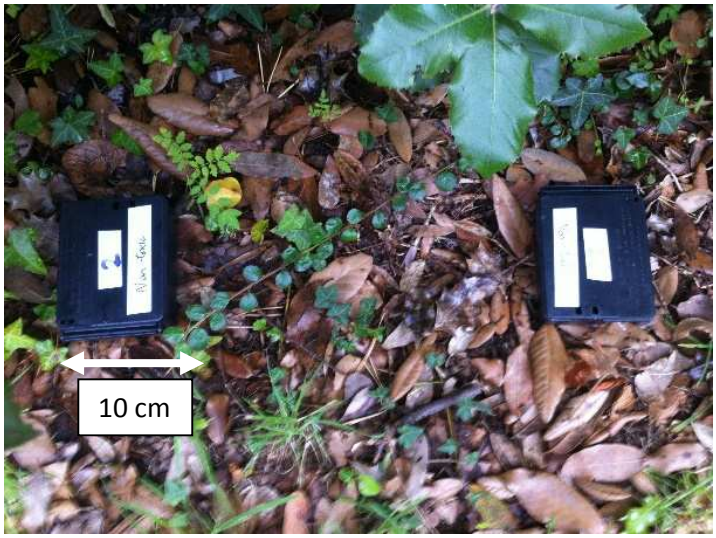


Figure 4.2. Traps used for live trapping. A) Mk1 TubeTrap. **B)** Mk2 TubeTrap. **C)** Longworth trap. All traps were approximately 30 cm in length.

A)



B)

C)

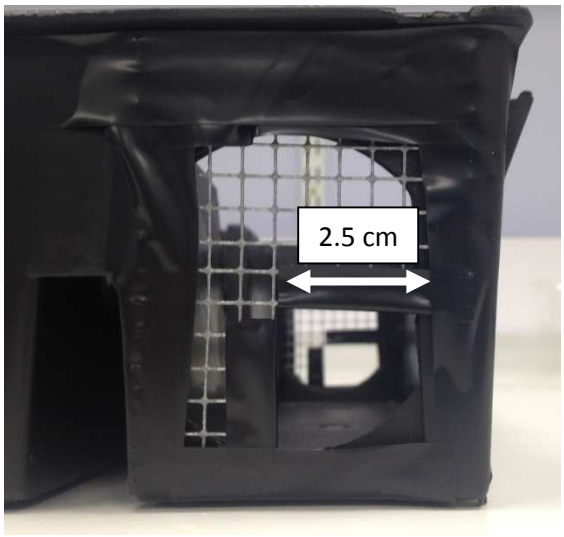


Figure 4.3. Bait boxes used for bait box experiments. A) Mouse bait boxes in situ. **B)** Rat bait boxes in situ. **C)** Close up of partially blocked rat bait box entrance.

4.4. Results

4.4.1. Live trapping experiment

To test if wild, non-target rodents avoid rat odour in the field we compared the number of small mammals caught in traps containing female rat nest material to the number caught in traps containing clean nest material. Trapping was carried out over four nights and 100 traps were set each night.

For primary captures, presence of rat odour did not deter rodents from entering traps (χ^2_1 = 0.26, p = 0.61; Figure 4.4A, Table 4.2). Primary captures included 29 bank voles, five wood mice and one common shrew, giving a total of 35 animals. Of these 35 captures, 19 (54%) were in rat odoured traps, indicating no evidence of any repellent effect of rat odour on the likelihood of non-target rodents to enter traps.

As rat nest material was not refreshed for the duration of this experiment, except where traps were cleaned, it is possible that the repellent effect of rat odour was lost from uncleaned traps over the 4 nights of testing. However, on the first night of testing, when all traps contained fresh nest material, more non-target rodents were caught in rat odoured traps compared to clean traps (Table 4.2).

As avoidance of rat odour might be a learned response, exposure to the presence of rat odour may lead to a reduction in the number of animals revisiting traps scented with rat odour. However, we found that number of recaptures was not affected by presence of rat odour (χ^2_1 = 0.05, p = 0.82; Figure 4.4B). All recaptured animals were bank voles. In total, 11 bank voles were recaptured, four were recaptured once, six were recaptured twice and one was recaptured three times, giving 19 recaptures in total.

Twenty two out of the 35 primary captures (63%) occurred at two of the five trapping sites (d and e, Figure 4.1A). Therefore, for subsequent experiments sites a, b and c were not used and three new sites (f, g and h, Figure 4.1) were used instead.

Table 4.2. Number of primary small mammal captures in traps scented with rat odour.

Odour	Date				
	20/06/2017	21/06/2017	22/06/2017	23/06/2017	Total
Rat	7	3	2	7	**19**
Clean	2	6	1	7	**16**
Total	**9**	**9**	**3**	**14**	**35**

4.4.2. Mouse bait box experiment

Having found that presence of female rat odour did not reduce the number of non-target rodents willing to enter live traps, we next assessed if presence of female rat odour reduced the amount of non-toxic bait consumed by non-target rodents. Non-toxic bait was presented in mouse bait boxes as these boxes prevent the entry of rats. Non-target rodents were given a choice to feed from mouse bait boxes scented with rat nest material or clean nest material over 8 nights.

Bait was consumed from an almost equal number of clean and rat odoured boxes ($\chi^2_1 = 0.23$, $p = 0.63$; Table 4.3). Where bait was consumed, presence of rat odour did not affect the weight of bait eaten by non-target rodents (linear mixed effects model, $t_{258.8} = 0.42$, $p = 0.68$; Figure 4.5). Focusing on boxes where bait was consumed, although not significant, median bait take was 18% lower for rat odoured boxes compared to clean boxes. For these boxes, median bait take was slightly lower from rat odoured boxes, compared to clean boxes, for all sites, except site g (Figure 4.5).

If bait take from all boxes is considered, median bait take was 27% lower for rat odoured boxes compared to clean boxes (Figure 4.6). Median bait take varied across the five sites, being lower for rat odoured boxes compared to clean boxes at sites e, f and h. The total weight of bait eaten across all nights of testing was 995 g for clean boxes and 927 g for rat odoured boxes, indicating that presence of rat odour had no effect on the amount of non-toxic bait entering the food chain.

Live trapping was carried out following each week of bait box experimentation to assess numbers of non-target species present at each experimental site. Following the mouse bait box experiment, in total 205 captures were made (primary and recaptures), of which 60%

were wood mice and 39.5% were bank voles. The most common primary captures were wood mice, followed by bank voles (Table 4.4). However, this differed between sites. Wood mice were more commonly captured than bank voles at sites g and h. Bank voles were more commonly captured than wood mice at sites d and e and f. Field voles were very rare, with only one captured at site e.

Table 4.3. Number of mouse bait boxes eaten from over 8 nights.

	Mouse Box		
	Clean	**Rat odour**	**Total**
Boxes with bait take	**137 (50.7%)**	**133 (49.3%)**	**270**
Boxes without bait take	**46 (47.9%)**	**50 (52.1%)**	**96**

Table 4.4. Number of rodents captured following mouse bait box experiment (primary captures).

		Site					Total
		d	**e**	**f**	**g**	**h**	
Week 1	Wood mouse	1	10	5	3	7	26
	Bank vole	3	9	9	1	3	25
	Field vole	0	1	0	0	0	1
	Total	**4**	**20**	**14**	**4**	**10**	**52**
Week 2	Wood mouse	2	8	12	13	10	45
	Bank vole	7	12	10	0	0	29
	Total	**9**	**20**	**22**	**13**	**10**	**74**

4.4.3. Rat bait box experiment

The lack of avoidance of rat odour observed in the mouse bait box experiment was surprising, given previous evidence that non-target rodents avoid consuming bait from boxes scented with female rat odour in semi-natural enclosures (Chapter 3). One difference between previous semi-natural enclosure experiments and the mouse bait box experiment is the type of bait box used. In semi-natural enclosure experiments rat bait boxes were used, which are much larger than mouse bait boxes. It is possible that non-target rodents do not feel as threatened in a small, confined mouse bait box that rats and other predators cannot enter, compared to the larger, open space of a rat bait box. To test if this was the case, we repeated the mouse bait box experiment, but we replaced mouse bait boxes with rat bait boxes. To prevent access by rats, the entrances of our rat bait boxes were partially blocked to be the same size as mouse bait box entrances (Figure 4.3C). Weight of nest material was increased in rat bait boxes to account for their larger air space compared to mouse bait boxes. Non-target rodents were given a choice to feed from rat bait boxes scented with rat nest material or clean nest material over 4 nights.

Bait was consumed from an almost equal number of clean and rat odoured boxes (proportion of boxes showing some bait take, $\chi^2_1 = 0.96$, $p = 0.33$; Table 4.5). However, where bait was consumed, non-target rodents ate less from boxes scented with rat odour compared to clean boxes (linear mixed effects model, $t_{207.3} = 4.19$, $p < 0.001$; Figure 4.7). Focusing on boxes where bait was consumed, median bait take was 49% less for rat odoured boxes compared to clean boxes. For these boxes, median bait take was lower from rat odoured boxes, compared to clean boxes, for all sites.

If bait take from all boxes is considered, median bait take was 31% less for rat odoured boxes compared to clean boxes (Figure 4.8), indicating that there is an overall repellent effect of rat odour on bait take. Median bait take was lower from rat odoured boxes, compared to clean boxes, for sites e, f and h, but higher for sites d and g. The total weight of bait eaten during the rat bait box experiment was 1198 g for clean boxes and 891 g for rat odoured boxes, a difference of 26%.

Live trapping was carried out following bait box removal to assess numbers of non-target species present at each experimental site. In total 96 captures were made (primary and recaptures), of which 71% were wood mice and 29% were bank voles. The proportion of wood mice captured was slightly higher for this experiment compared to the mouse bait box

experiment. The most common primary captures were wood mice, followed by bank voles (Table 4.6). However, this differed between sites. Bank voles were more commonly captured than wood mice at site e. At all other sites, wood mice were more commonly captured than bank voles. This differed from the mouse bait box experiment, where more bank voles were captured than wood mice at sites d and f.

Table 4.5. Number of rat bait boxes eaten from over 4 nights.

	Rat box		
	Clean	**Rat odour**	**Total**
Boxes with bait take	**104 (48.4%)**	**111 (51.6%)**	**215**
Boxes without bait take	**37 (55.2%)**	**30 (44.8%)**	**67**

Table 4.6. Number of rodents captured following rat bait box experiment (primary captures).

	Site					Total
	d	**e**	**f**	**g**	**h**	
Wood mouse	9	4	8	13	9	**43**
Bank vole	6	10	6	1	1	**24**
Total	**15**	**14**	**14**	**14**	**10**	**67**

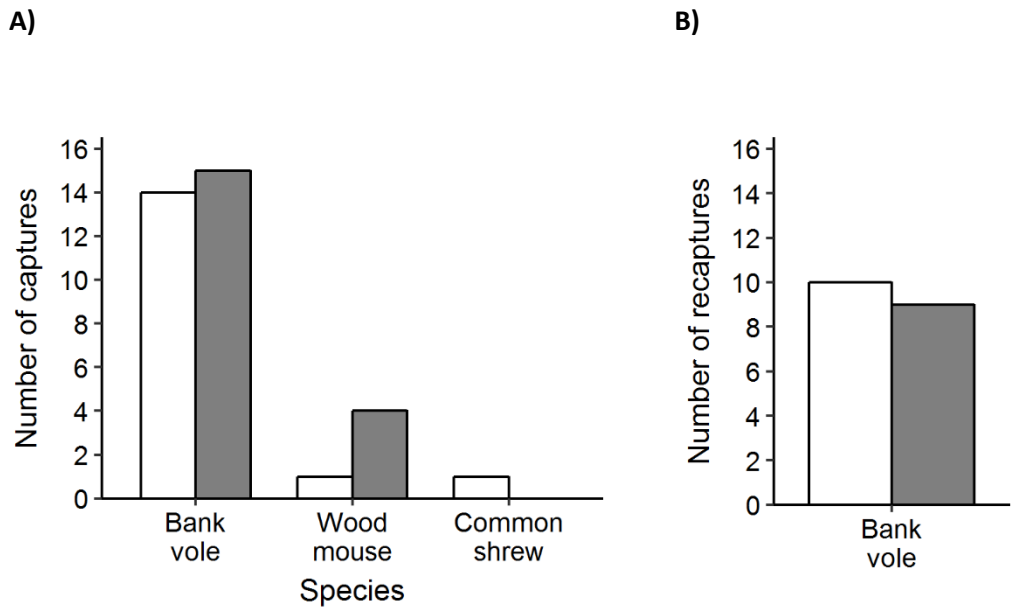


Figure 4.4. Number of small mammals captured in traps scented with rat odour. Traps were scented with female rat nest material (dark grey bars) or clean nest material (white bars). **A)** Primary captures. **B)** Recaptures.

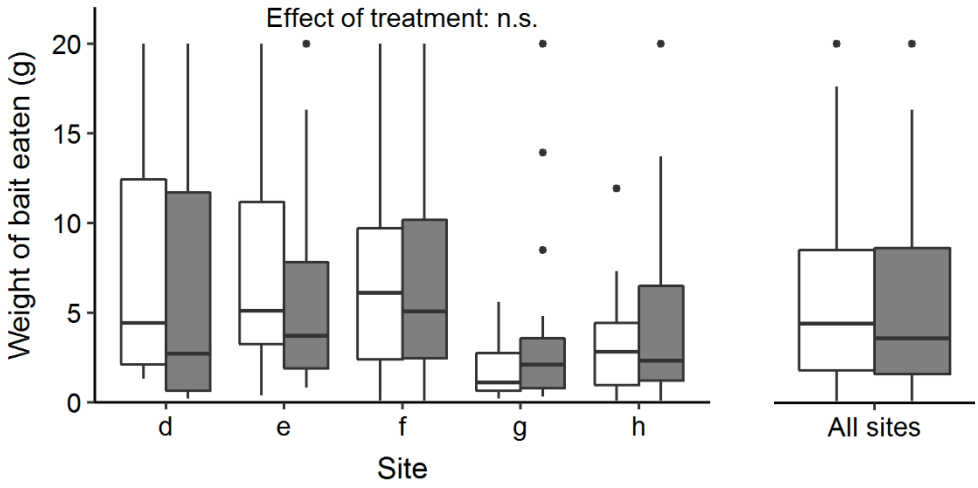


Figure 4.5. Weight of bait eaten per box per night from mouse bait boxes scented with rat odour. Bait boxes were scented with female rat nest material (dark grey bars) or clean nest material (white bars). Bait boxes where no bait was consumed were removed from the analysis. Box plots show medians (black bar), interquartile range (IQR; boxes), 1.5 IQR (whiskers) and outliers (dots). Weight of food eaten from bait boxes was compared using a linear mixed effects model.

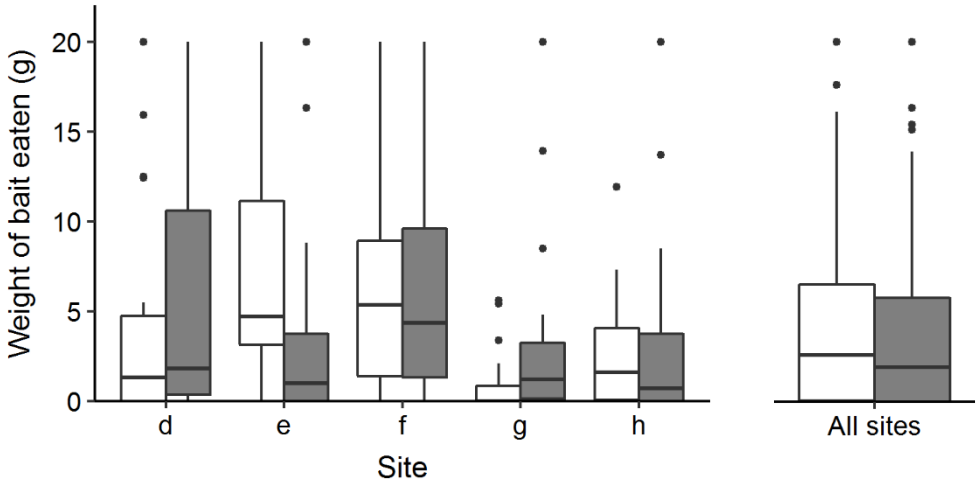


Figure 4.6. Weight of bait eaten per box per night from mouse bait boxes scented with rat odour. Bait boxes were scented with female rat nest material (dark grey bars) or clean nest material (white bars). Box plots show medians (black bar), interquartile range (IQR; boxes), 1.5 IQR (whiskers) and outliers (dots).

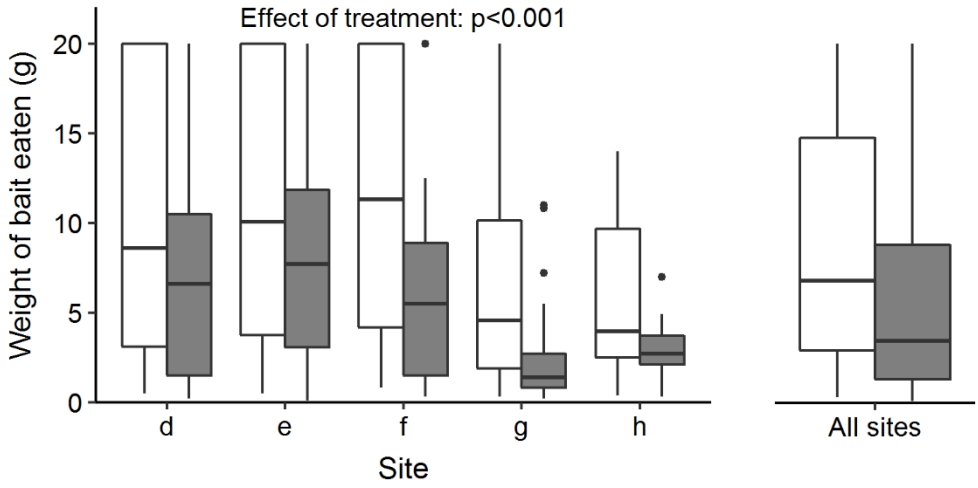


Figure 4.7. Weight of bait eaten per box per night from rat bait boxes scented with rat odour. Bait boxes were scented with female rat nest material (dark grey bars) or clean nest material (white bars). Bait boxes where no bait was consumed were removed from the analysis. Box plots show medians (black bar), IQR (boxes), 1.5 IQR (whiskers) and outliers (dots). Weight of food eaten from bait boxes was compared using a linear mixed effects model.

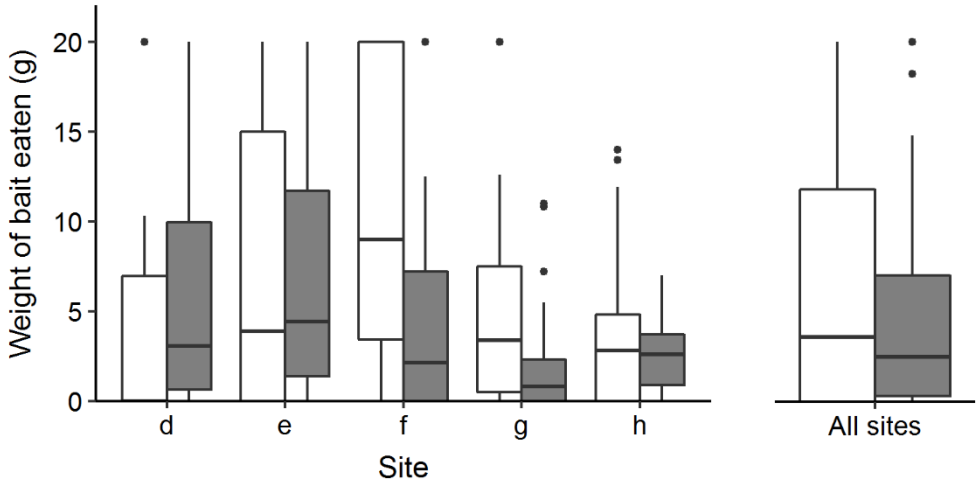


Figure 4.8. Weight of bait eaten per box per night from rat bait boxes scented with rat odour. Bait boxes were scented with female rat nest material (dark grey bars) or clean nest material (white bars). Box plots show medians (black bar), interquartile range (IQR; boxes), 1.5 IQR (whiskers) and outliers (dots).

4.5. Discussion

In field trials conducted at multiple sites, non-target rodents did not avoid entering small rodent traps scented with female rat nest material. In addition, non-target rodents did not avoid consuming non-toxic bait from mouse bait boxes scented with female rat nest material. However, non-target rodents did consume less bait from rat bait boxes scented with rat odour. This avoidance led to a 26% reduction in overall bait take during 4 nights of testing.

There were some limitations to this study that could have affected the validity of the results. First, during live trapping very few wood mice were captured, limiting the ability to determine if rat odour repels wood mice from entering small rodent traps. It is possible that wood mice avoided all traps as a rat odoured trap was present at all trapping sites, within 30 cm of clean traps. This would account for the higher numbers of wood mice captured during the trapping sessions following the mouse and rat bait box experiments, when all traps were clean. An alternative explanation is that numbers of wood mice were low during the live trapping experiment, but increased later in the year during the trapping sessions following the mouse and rat bait box experiments. The latter explanation seems more plausible, as 4 of the 5 wood mice captured in the live trapping experiment were found in rat odoured traps, indicating it is unlikely that wood mice avoided rat odoured traps.

A second limitation of the live trapping experiment is that rat nest material was not refreshed each night of the study in all traps, but was refreshed in traps that had been triggered. This meant there was a mixture of fresh and aged rat nest material present in traps across study sites. There is both theoretical and experimental evidence to indicate that ageing predator odour cues may be less effective repellents than fresh cues (Bytheway *et al.*, 2013; Parsons *et al.*, 2018). However, on the first night of testing, when all traps contained fresh nest material, more non-target rodents were captured in rat odoured traps compared to control traps. One explanation for this apparent discrepancy may be due to investigation of rat odour by non-target rodents. As defensive behaviours in response to predator odours are costly to prey species, they should only initiate them only if encountering a predator is likely (Zylberberg & DeWeese, 2011). In order to determine the likelihood of encountering a predator it may be necessary for a prey animal to investigate a predator odour before initiating defensive behaviours. This investigatory behaviour has been observed previously and has been termed ‘predator inspection’ (Parsons *et al.*, 2018). During predator inspection

prey animals will approach a predator scent and appear to investigate it (Parsons *et al.*, 2018). One of the possible causes for this behaviour is to determine the nature of risk posed by the presence of an odour cue, prior to initiating defensive behaviours, such as fleeing (Parsons *et al.*, 2018). In this study, if non-target rodents investigated rat odour, they would be trapped and unable to subsequently avoid rat odour. Therefore, predator investigation may explain the apparent attraction of non-target rodents to fresh rat odoured nest material on the first night of live trapping. However, it is not possible to draw conclusions about the effect of ageing of rat odour from this study, due to the mixture of fresh and aged material across study sites on subsequent nights. To assess if age of rat odour does affect repulsion it would be necessary to design an experiment specifically to address this question.

A limitation of the mouse and rat bait box experiments is that, during these experiments, there were several box pairs where all bait was consumed from both boxes. This meant we could not determine if box odour was having an effect on bait take for those box pairs. Increasing the total weight of non-toxic bait in each box may have allowed a better assessment of the effect of rat odour in these highly visited sites.

It was interesting that non-target rodents avoided rat odour when it was presented in rat bait boxes, but did not avoid rat odour when it was presented in mouse bait boxes. This may have been due to the increased amount of bedding in rat bait boxes, compared to mouse bait boxes. This may potentially have led to a greater level of odour stimulus in rat bait boxes compared to mouse bait boxes. However, the presence of a rat odour cue from the same source in both types of box should present an equally threatening environment, and may support the concept that context of odour presentation is important in predator odour responses. As stated above, predator inspection may be a means of assessing the necessity of initiating defensive behaviours (Parsons *et al.*, 2018). In this study, it may be that non-target rodents entered mouse and rat bait boxes, investigated rat odour where it was present, and only determined in the case of rat bait boxes that presence of rat odour indicated a viable threat.

There are two potential reasons for non-target rodents to find rat bait boxes more threatening than mouse bait boxes. First, as rat bait boxes are much larger they may be perceived as a more threatening environment by small non-target rodents. This could be because non-target rodents are unable to tell if another animal is present within the large bait box, whereas in a small bait box they are able to assess the full extent of the space easily

and determine that no other animal is present. Alternatively, small non-target rodents may perceive that a rat could not fit inside a mouse bait box, and so are not threatened by the presence of rat odour in such a box. In a rat bait box a rat could easily fit inside and so this space may be viewed as more threatening. Such predator specific behavioural responses have been identified in other studies. For example, bank voles will avoid entering narrow tunnels in the presence of weasel odour, but do not avoid the same tunnels in the presence of fox odour (Jędrzejewski *et al.*, 1993).

An alternative explanation for the difference in response to mouse and rat bait boxes is that there was more food available in the environment during the rat bait box experiment compared to the mouse bait box experiment due to seasonal variation. Seasonal variations in food availability can affect avoidance of predator odours. Whilst an animal may avoid a food source scented with predator odour when there is plenty of alternative food available, avoidance may be lessened when alternative food sources are scarce (Calder & Gorman, 1991), such as in winter. In addition, fluctuations in food requirements with breeding seasonality may affect predator odour avoidance. For example, bank voles are captured more often in weasel odoured traps in the breeding season compared to the non-breeding season (Borowski, 2002). Once again, there is a balance between cost of avoiding a food source and risk of encountering a predator (Zylberberg & DeWeese, 2011). When an animal has a high energy demand during breeding or lactation, or cannot find sufficient food in the environment, the risk of starvation may outweigh the risk of encountering the predator. In this study, if there was a scarcity of food during one of the experiments it could have led to non-target rodents being more willing to risk entering bait boxes to source high quality food than if there was an abundance of food. The mouse bait box experiment was carried out during late summer and early autumn. The rat bait box experiment was carried out in mid-autumn. There would have been differences in food availability between these times, with more nuts and berries being available in autumn and more foliage available in summer (personal observation at study sites), which may have influenced the likelihood of avoidance. The diet of bank voles and wood mice is similar, although bank voles tend to consume more foliage than wood mice (Hansson, 1971; Hansson, 1987), so seasonal differences in food availability for each species may have been present, although slight. Looking at the results of the mouse bait box experiment, which was carried out in summer and autumn, there was no indication of a difference in response between the two sessions. Overall, seasonality of food

availability may have had an impact on the results of this study, but it appears that this effect is likely to be small.

Non-target rodents ate less from rat odoured rat bait boxes compared to clean rat bait boxes. However, the magnitude of this response was not as large as when wood mice were tested in semi-natural enclosures. In semi-natural enclosures median bait take was reduced by 65%, when comparing clean and rat odoured boxes (Chapter 3), whereas in this field trial median bait take was reduced by 31%. There are several explanations that could account for this difference. First, between enclosure experiments, captive wood mice were provided with ample high quality food supplies, lessening the cost of avoiding rat odour in baited boxes, compared to wild rodents where alternative food sources may not have been so plentiful. Second, during enclosure experiments no other predator odours would have been present in the environment. During field experiments there could be many other predator odours present that may affect how rodents respond to rat odour. The presence of other predator odours could alter the perception of rat odoured boxes from a high risk environment when no other odours are present, to a lower risk environment when the odour of a more dangerous predator, such as a mustelid, is present. This could lead to less avoidance of rat odour in the field compared to semi-natural enclosures. This would be in accordance with the predator risk allocation hypothesis, which theorises that prey animals will respond to predator cues depending on the amount of risk these cues imply (Lima & Bednekoff, 1999). Third, all the animals in our enclosure experiment were adult animals with no opportunity to breed. Actively breeding animals and lactating females may have been present during field experiments. These animals will have a greater energy demand than non-breeding animals and so may be more willing to risk spending time in the locality of a predator odour, as has been seen previously with voles (Borowski, 2002). Finally, during enclosure experiments the entrances to rat bait boxes were not partially blocked, whereas they were during field experiments to ensure rats could not enter. This may have led to non-target rodents perceiving boxes with covered entrances as safer than non-covered boxes in a similar manner to the differences between mouse and rat bait boxes discussed above. This would have led to an increased bait consumption from rat bait boxes in the field, compared to enclosures.

Overall, the results of these field trials indicate that rat odour has the potential to significantly reduce the amount of bait being consumed by non-target rodents. However, context of odour presentation requires optimisation in order to achieve the best results.

Further testing is needed to achieve this optimisation. Field testing at other sites will be needed to confirm the results found in these experiments in a greater variety of habitats. Those field trials should use rat bait boxes, baited with greater than 20 g of non-toxic bait to allow the best assessment of the effectiveness of rat odour to repel non-target rodents. As a strategy to overcome the effects of food availability on avoidance of rat odour, a high quality food could be provided in a separate, safe compartment where no rat odour is present and with a small entrance hole that rats cannot get into. Provision of an alternative, high quality food proved successful in preventing the consumption of sugar-beet seeds by wood mice (Harris, 1989; Pelz, 1989) and so may be of use here. As stated above, it is possible that covering entrances to rat bait boxes affected the avoidance response of non-target rodents. Testing rat odoured rat bait boxes in the field without entrance covers would help to determine if entrance covers have an effect on rat odour avoidance. However, steps would need to be taken to ensure that rats were not entering bait boxes and consuming bait.

In conclusion, the use of rat odour as a non-target repellent looks promising, even in the field where other factors may be complicating the response. However, further work is need to fully optimise the repellent effects of rat odour for use in commercial settings.

4.6. Appendix

4.6.1. Supplementary information

4.6.1.1. Linear mixed effects model and null model for rat and mouse bait box experiments

Model: Log (Eaten+1) ~ Odour + (1 | Site) + (1 | Date)

Null model: Log (Eaten+1) ~ 1 + (1 | Site) + (1 | Date)

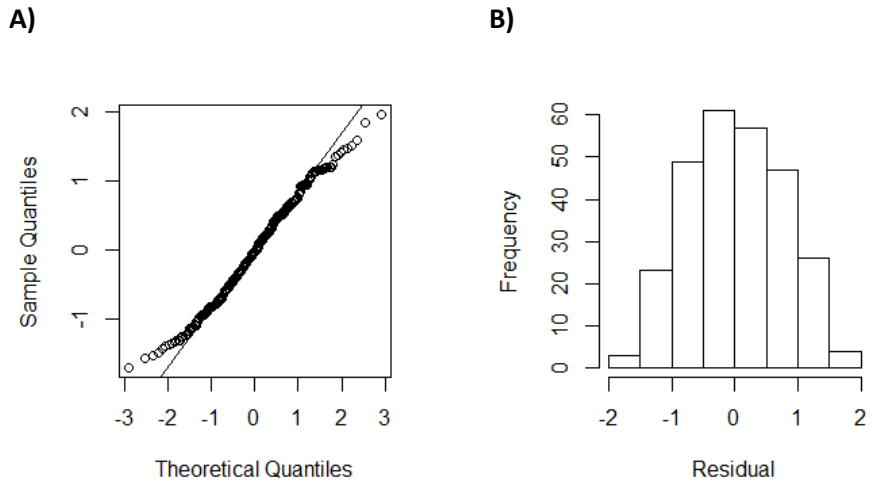


Figure S4.1. Mouse bait box experiment residual plots. A) Normal Q-Q Plot. **B)** Histogram.

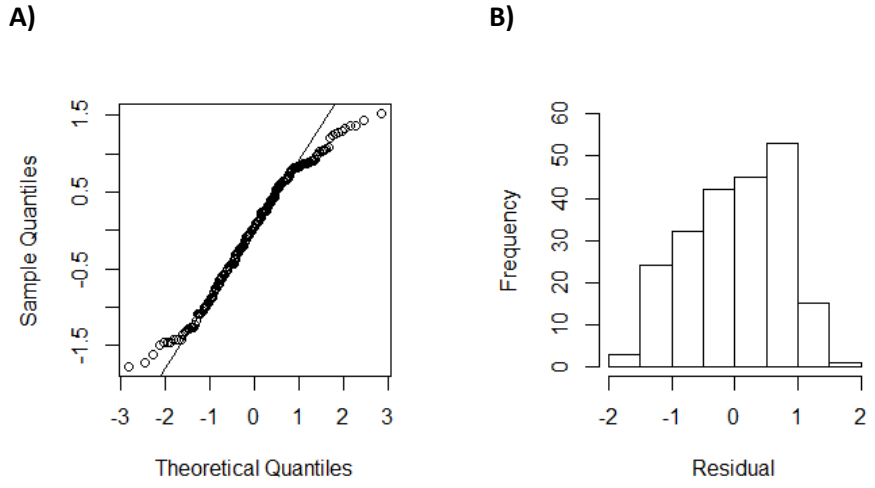


Figure S4.2. Rat bait box experiment residual plots. A) Normal Q-Q Plot. **B)** Histogram.

4.7. References

Animals (Scientific Procedures) Act. (1986). UK.

Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A. & McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neuroscience & Biobehavioral Reviews.* **29**, 1123-1144.

Association for the Study of Animal Behaviour (2018). Guidelines for the treatment of animals in behavioural research and teaching. *Animal Behaviour.* **135**, I-X.

Banks, P. B. (1998). Responses of Australian Bush Rats, *Rattus fuscipes*, to the Odor of Introduced *Vulpes vulpes*. *Journal of Mammalogy.* **79**, 1260-1264.

Barreto, G. R. & Macdonald, D. W. (1999). The response of water voles, *Arvicola terrestris*, to the odours of predators. *Animal Behaviour.* **57**, 1107-1112.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software.* **67**, 1-48.

Blanchard, D. C., Markham, C., Yang, M., Hubbard, D., Madarang, E. & Blanchard, R. J. (2003). Failure to produce conditioning with low-dose trimethylthiazoline or cat feces as unconditioned stimuli. *Behavioral Neuroscience.* **117**, 360-368.

Borowski, Z. (2002). Individual and seasonal differences in antipredatory behaviour of root voles a field experiment. *Canadian Journal of Zoology.* **80**, 1520-1525.

Brakes, C. R. & Smith, R. H. (2005). Exposure of non-target small mammals to rodenticides: short-term effects, recovery and implications for secondary poisoning. *Journal of Applied Ecology.* **42**, 118-128.

Bridgman, L. J., Innes, J., Gillies, C., Fitzgerald, N. B., Miller, S. & King, C. M. (2013). Do ship rats display predatory behaviour towards house mice? *Animal Behaviour.* **86**, 257-268.

Buckle, A. & Fenn, M. (1992) Rodent control in the conservation of endangered species. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 36-41.

Bytheway, J., Carthey, A. R. & Banks, P. (2013). Risk vs. reward: how predators and prey respond to aging olfactory cues. *Behavioral Ecology and Sociobiology.* **67**, 715-725.

Calder, C. & Gorman, M. (1991). The effects of red fox *Vulpes vulpes* faecal odours on the feeding behaviour of Orkney voles *Microtus arvalis*. *Journal of Zoology.* **224**, 599-606.

Capizzi, D., Bertolino, S. & Mortelliti, A. (2014). Rating the rat: global patterns and research priorities in impacts and management of rodent pests. *Mammal Review.* **44**, 148-162.

Crawley, M. J. (2007). *The R book.* Wiley, Chichester.

Dawson, A., Bankes, J. & Garthwaite, D. (2001). *Rodenticide use on farms in Great Britain growing arable crops 2000.* pp. 1-22. DEFRA, York.

Directive 2010/63/EU. (2010). EU.

Erickson, W. A. & Urban, D. J. (2004). *Potential risks of nine rodenticides to birds and nontarget mammals: a comparative approach.* pp. 1-225. U.S. Environmental Protection Agency, Washinton D.C.

Fendt, M. & Endres, T. (2008). 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor - Just repugnant or really fear-inducing? *Neuroscience and Biobehavioral Reviews.* **32**, 1259-1266.

Geduhn, A., Esther, A., Schenke, D., Mattes, H. & Jacob, J. (2014). Spatial and temporal exposure patterns in non-target small mammals during brodifacoum rat control. *Science of the Total Environment.* **496**, 328-338.

Hadler, M. R. & Buckle, A. P. (1992) Forty five years of anticoagulant rodenticides - past, present and future trends. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 149-156.

Hansson, L. (1971). Small Rodent Food, Feeding and Population Dynamics - Comparison between Granivorous and Herbivorous Species in Scandinavia. *Oikos.* **22**, 183-198.

Hansson, L. (1987). An Interpretation of Rodent Dynamics as Due to Trophic Interactions. *Oikos.* **50**, 308-318.

Harris, S. (1989). When Is a Pest a Pest. *New Scientist.* **121**, 49-51.

Harris, S., Morris, P., Wray, S. & Yalden, D. (1995). *A review of British mammals: population estimates and conservation status of British mammals other than cetaceans.* pp. 1-183. Joint Nature Conservation Committee, U.K.

Helfman, G. (1989). Threat-sensitive predator avoidance in damselfish-trumpetfish interactions. *Behavioral Ecology and Sociobiology.* **24**, 47-58.

Hoare, J. M. & Hare, K. M. (2006). The impact of brodifacoum on non-target wildlife: gaps in knowledge. *New Zealand Journal of Ecology.* **30**, 157-167.

Jędrzejewski, W., Rychlik, L. & Jędrzejewska, B. (1993). Responses of bank voles to odours of seven species of predators: experimental data and their relevance to natural predator-vole relationships. *Oikos.* **68**, 251-257.

Korpimaki, E., Koivunen, V. & Hakkarainen, H. (1996). Microhabitat use and behavior of voles under weasel and raptor predation risk: predator facilitation? *Behavioral Ecology.* **7**, 30-34.

Lenth, R. V. (2016). Least-Squares Means: The R Package lsmeans. *Journal of Statistical Software.* **69**, 1-33.

Lima, S. L. & Bednekoff, P. A. (1999). Temporal Variation in Danger Drives Antipredator Behavior: The Predation Risk Allocation Hypothesis. *The American Naturalist.* **153**, 649-659.

Major, H. L., Jones, I. L., Charette, M. R. & Diamond, A. W. (2007). Variations in the diet of introduced Norway rats (*Rattus norvegicus*) inferred using stable isotope analysis. *Journal of Zoology.* **271**, 463-468.

Masini, C., Sauer, S. & Campeau, S. (2005). Ferret odor as a processive stress model in rats: neurochemical, behavioral, and endocrine evidence. *Behavioral Neuroscience.* **119**, 280-292.

Meerburg, B. G., Singleton, G. R. & Kijlstra, A. (2009). Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology.* **35**, 221-270.

Navarro-Castilla, A. & Barja, I. (2014). Antipredatory Response and Food Intake in Wood Mice (*Apodemus sylvaticus*) under Simulated Predation Risk by Resident and Novel Carnivorous Predators. *Ethology.* **120**, 90-98.

NC3Rs, BBSRC, DEFRA, MRC, NERC & Wellcome Trust. (2017). Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. NC3Rs, London.

Parsons, M. H., Apfelbach, R., Banks, P. B., Cameron, E. Z., Dickman, C. R., Frank, A. S., Jones, M. E., McGregor, I. S., McLean, S. & Müller-Schwarze, D. (2018). Biologically meaningful scents: a framework for understanding predator–prey research across disciplines. *Biological Reviews.* **93**, 98-114.

Paul, L., Miley, W. M. & Baenninger, R. (1971). Mouse killing by rats: Roles of hunger and thirst in its initiation and maintenance. *Journal of Comparative and Physiological Psychology.* **76**, 242-249.

Pelz, H. J. (1989). Ecological aspects of damage to sugar beet seeds by *Apodemus sylvaticus*. In *Mammals as Pests.* Putman, R. (Ed.) pp. 34-48. Chapman and Hall Ltd, London.

Pimentel, D. (2005). Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. *Environment, Development and Sustainability.* **7**, 229-252.

Pimentel, D., Zuniga, R. & Morrison, D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics.* **52**, 273-288.

R Core Team. (2016). *R: A language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Shore, R. F., Walker, L. A., Potter, E. D., Pereira, M. G., Sleep, D. & Thompson, N. J. (2016). *Second generation anticoagulant rodenticide residues in barn owls 2016.* pp. 1-21. Centre for Ecology & Hydrology, Lancaster.

Singleton, G. R. (2003). *Impacts of rodents on rice production in Asia.* IRRI Discussion Paper Series, No. 45, pp. 1-30. International Rice Research Institute, Philippines.

Singleton, G. R. (2010). *Rodent outbreaks: ecology and impacts.* International Rice Research Institute, Philippines.

Singmann, H., Bolker, B., Westfall, J. & Aust, F. (2017). *afex: Analysis of Factorial Experiments*. https://CRAN.R-project.org/package=afex

Sullivan, T. P., Crump, D. R. & Sullivan, D. S. (1988). Use of predator odors as repellents to reduce feeding damage by herbivores: III. Montane and meadow voles (*Microtus montanus* and *Microtus pennsylvanicus*). *Journal of Chemical Ecology.* **14**, 363-377.

Takács, S., Gries, R., Zhai, H. & Gries, G. (2016). The Sex Attractant Pheromone of Male Brown Rats: Identification and Field Experiment. *Angewandte Chemie-International Edition.* **55**, 6062-6066.

Tosh, D. G., McDonald, R. A., Bearhop, S., Lllewellyn, N. R., Fee, S., Sharp, E. A., Barnett, E. A. & Shore, R. F. (2011). Does small mammal prey guild affect the exposure of predators to anticoagulant rodenticides? *Environmental Pollution.* **159**, 3106-3112.

Walker, L. A., Chaplow, J. S., Llewellyn, N. R., Pereira, M. G., Potter, E. D., Sainsbury, A. W. & Shore, R. F. (2013). *Anticoagulant rodenticides in predatory birds 2011: a Predatory Bird Monitoring Scheme (PBMS) report.* pp. 1-29. Centre for Ecology & Hydrology, Lancaster.

Walker, L. A., Chaplow, J. S., Moeckel, C., Pereira, M. G., Potter, E. D., Sainsbury, A. W. & Shore, R. F. (2016). *Anticoagulant rodenticides in red kites (Milvus milvus) in Britain 2010 to 2015: a Predatory Bird Monitoring Scheme (PBMS) report.* pp. 1-14. Centre for Ecology & Hydrology, Lancaster.

Wallace, K. J. & Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behavioral Neuroscience.* **114**, 912-22.

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, New York. http://ggplot2.org

Ylönen, H., Jedrzejewska, B., Jedrzejewski, W. & Heikkilä, J. (1992). Antipredatory behaviour of Clethrionomys voles - 'David and Goliath' arms race. *Annales Zoologici Fennici.* **29**, 207-216.

Zylberberg, J. & DeWeese, M. R. (2011). How should prey animals respond to uncertain threats? *Frontiers in Computational Neuroscience.* **5**, 1-7.

Chapter 5

5. Testing the potential of 50 kHz rat calls as a species specific rat attractant

5.1. Abstract

In general, control of animal pests relies heavily on the use of pesticides that are often avoided and are not species specific. These problems are particularly acute for pesticides used to control rats (*Rattus spp.*). Pest control aimed at rats could be improved by attracting rats to control measures using species specific cues. One cue that has the potential to attract rats is 50 kHz calls, emitted by rats in positive social situations. We tested the potential 50 kHz rat calls to act as a species specific rat attractant. We examined the response of rats (*Rattus norvegicus*; n = 48) and non-target bank voles (*Myodes glareolus*; n = 16), to male and female 50 kHz rat calls in a compartmentalised laboratory arena. Sounds of rat movement and white noise were used as control sound treatments and each sound treatment was tested against a silent control. When sound cues were played above an empty bait box, rats of both sexes were attracted to move close to 50 kHz rat calls. When sound cues were played inside an empty bait box, rats of both sexes were attracted to enter the box when 50 kHz rat calls and sounds of rat movement were playing, but were not attracted by regularly intermittent white noise. Bank voles showed no attraction or aversion to 50 kHz rat calls or to regularly intermittent white noise, playing from inside an empty bait box. These findings indicate that the attraction of rats to 50 kHz rat calls is not sex specific and that bank voles are neither attracted to, nor repelled by these calls. Using 50 kHz rat calls could help to improve the efficacy of pest control aimed at rats by providing a species specific lure that may be attractive even in the absence of food bait.

5.2. Introduction

Globally, many animal species are considered as pests. Those animals classified as pests typically interfere with human activity (Harris, 1989). This can be in the form of consumption of or damage to food supplies, spread of disease to humans or livestock and expensive damage to infrastructure (Capizzi *et al.*, 2014; Pimentel *et al.*, 2005; Singleton, 2010). In addition, invasive species may be classified as pests where they threaten populations of endemic species (Pimentel *et al.*, 2005). Pest species come from diverse taxa and are typified by being highly adaptable and having high reproductive potential (Capizzi *et al.*, 2014; Nair, 2007; Pimentel *et al.*, 2005). The cost of animal pests is huge, estimated at $67 billion a year in the USA alone (Pimentel *et al.*, 2005). This cost is due not only to the damage pests cause, but also to the cost of controlling them (Pimentel, 2005; Pimentel *et al.*, 2005).

Control of pest species relies heavily on use of chemical pesticides (Capizzi *et al.*, 2014; Pimentel, 2005), the use of which is associated with several problems. One of the big problems with the use of pesticides has been the development of resistance, particularly behavioural resistance, which leads to pests avoiding consumption of pesticides (Clapperton, 2006; Cook *et al.*, 2007; Hadler & Shadbolt, 1975). This can be a big problem, particularly where poisons are presented in secure stations, such as bait boxes, which pests may avoid (Clapperton, 2006). Another big issue with the use of pesticides is their lack of species specificity. This means that where pesticides are used they can kill or harm large numbers of non-target species (Brakes & Smith, 2005; Hoare & Hare, 2006; Pimentel, 2005; Walker *et al.*, 2013).

To reduce the problems associated with pesticides, we need to reduce avoidance of bait stations, and improve the species specificity of control measures. It may be possible to do this by using species specific attractants that overcome avoidance of control measures by pest species, but do not attract non-target species. In the past few decades, the search for species specific attractants for use in pest control has included the examination of pest species communication channels (Christiansen, 1976; Cook *et al.*, 2007). Communication within a species can take many forms, including sounds, odours and visual cues, which may attract or repel an animal from a location (Cook *et al.*, 2007). By replicating the cues involved, there is the potential to influence the movement of animals, with the view to attracting them towards a control measure, or repelling them away from a potential source of damage, e.g. a food crop (Cook *et al.*, 2007). So far, much of this manipulation has focused on scent cues,

particularly in insect pests, but other cues such as visual cues have found some success (Cook *et al.*, 2007). The advantages of using social cues for pest control are species specificity (which reduces effects on non-target species), strength of response and a degree of protection against the development of resistance, particularly where the chosen cues are necessary for survival (Cook *et al.*, 2007).

Improvements in the attractiveness and species specificity of control measures are particularly needed for pest control aimed at rats (*Rattus spp.*). Rats, especially black rats (*Rattus rattus*) and Norway rats (*Rattus norvegicus*), are some of the biggest pests worldwide (Capizzi *et al.*, 2014; Pimentel *et al.*, 2005). They are a major threat to global food security, carry and transmit over 50 zoonotic diseases and are a threat to endangered species, particularly in island habitats (Buckle & Fenn, 1992; Meerburg *et al.*, 2009; Singleton, 2003; Singleton, 2010). As with pest control aimed at other animals, control of rats relies mostly on the use of pesticides (Capizzi *et al.*, 2014). Some of these pesticides are not species specific and are bio-accumulative, passing up the food chain to affect higher trophic levels (Hadler & Buckle, 1992). As such, rat poisons are often presented within secure bait boxes to prevent access of pets and children (Hadler & Buckle, 1992). However, these bait boxes present two main problems. First, it is difficult to attract rats into the unfamiliar enclosed space of a bait box and so rats avoid entering them (Clapperton, 2006). Second, whilst the boxes prevent access by large non-target animals they do not prevent access by small non-target rodents. Due to this, high numbers of non-target rodents and their predators have been found with residues of rat poisons, often linked to poor body condition and fatalities (Brakes & Smith, 2005; Martinez-Padilla *et al.*, 2017; Serieys *et al.*, 2015; Tosh *et al.*, 2011; Tosh *et al.*, 2012; Walker *et al.*, 2013).

One method of increasing rat entry to bait boxes, but not attracting non-target species, is to use species specific rat attractants. As stated above, social cues have the potential to be species specific attractants. One social cue that has the potential to attract rats is prosocial 50 kHz calls. These calls were discovered by Panksepp & Burgdorf (2000) when tickling laboratory rats, and have since been linked to positive affect in this species (Brudzynski, 2013). 50 kHz calls are increased during play and sexual encounters, and their emission reduces aggression between conspecifics (Brudzynski & Pniak, 2002; Brudzynski, 2009; Burgdorf *et al.*, 2008; Burke *et al.*, 2017). Rats also appear to use these calls when searching for familiar conspecifics. 50 kHz calling increases when cage mates are separated, or when a lone rat is exposed to the scents of other rats from its colony (Brudzynski & Pniak, 2002;

Wöhr *et al.*, 2008). In open arenas rats prefer to spend time near to a sound source playing 50 kHz calls, but the sex specificity of this attraction has not been determined (Sadananda *et al.*, 2008; Seffer *et al.*, 2014; Wöhr & Schwarting, 2007). The attraction of rats to 50 kHz calls indicates they might be suitable as an attractant for use in pest control.

Key questions remain to assess the potential of 50 kHz calls as a rat attractant for the purposes of pest control. First, it is necessary to determine if these calls are sex specific, as ideally lures need to be attractive to both sexes of a pest species. Although some studies have looked at attraction of both sexes to 50 kHz calls (Seffer *et al.*, 2014; Snoeren & Ågmo, 2013; Snoeren & Ågmo, 2014), no study has directly compared the response of male and female rats to male and female calls. Second, it is necessary to determine if 50 kHz calls attract rats into unfamiliar, enclosed spaces, such as those used in current pest control. So far, the response of rats to 50 kHz calls has only been studied in open arenas. Third, it is necessary to determine if rats need to be familiar with the calling rat to be attracted to their calls. If 50 kHz calls require prior familiarity to be attractive, it would not be possible to generalise them to pest control, as recordings of local rats would be needed for every use. Finally, to reduce impact of rat control on non-target rodents, 50 kHz rat calls ideally need to be species specific, so they do not attract non-target species. As rats are potential predators of smaller rodents (Bridgman *et al.*, 2013; Paul *et al.*, 1971), it is possible that non-target rodents will avoid 50 kHz rat calls. Answering these questions would be a first step to determining the potential of 50 kHz calls as a species specific attractant for use in pest control.

In a series of experiments on rats, we determined that attraction to 50 kHz rat calls is not specific to the sex of the caller or listener. We found that rat sounds can attract rats to approach and enter a small enclosed space, and can attract individuals unfamiliar with the calling rat. In a final experiment, we found that bank voles, a common non-target rodent species in the UK (Brakes & Smith, 2005; Harris *et al.*, 1995), were not attracted or repelled by 50 kHz rat calls.

5.3. Methods

5.3.1. Ethics statement

All procedures were non-invasive behavioural tests that did not involve pain, suffering, distress or lasting harm and were carried out in accordance with international best practice guidelines (Animals (Scientific Procedures) Act, 1986; Directive 2010/63/EU, 2010; Association for the Study of Animal Behaviour, 2018; NC3Rs *et al.*, 2017). The study was approved by the University of Liverpool Animal Welfare Committee. No specific licences were required to carry out the work, as animals were free to approach or avoid the test stimuli.

5.3.2. Subjects

Behavioural test subjects were 16 male and 16 female Wistar rats (HsdHan®:WIST, InVivo, Bicester, UK) aged 12 to 15 months (Experiments 1 and 2), 8 male and 8 female Wistar rats aged 3 to 4 months (Experiment 3), and 8 male and 8 female bank voles aged 11 to 12 months (Experiment 4). Vocalisation donors were 3 male and 3 female Wistar rats, aged 12 months. Rats were housed in same sex pairs or trios in 56 x 38 x 22 cm cages (RC2R, North Kent Plastics, Coalville, UK), in a room containing both male and female rats. Rats were obtained from suppliers at 4 to 8 weeks old. Test subject rats were housed in the same room as vocalisation donor rats for Experiments 1 and 2, and in a different room from vocalisation donor rats for Experiment 3. Bank voles were housed singly in 48 x 15 x 13 cm cages (M3, North Kent Plastics, UK), in a separate room to all rats. Bank voles were first generation, captive bred from wild parents caught in Northwest England. Of the 16 bank voles, 13 (7 females and 6 males) had briefly been exposed to rat odour in a separate behavioural test. Neither bank voles nor rats had previous exposure to the test arena or bioassay used in these experiments.

All housing and test rooms were maintained at 21 ± 2°C, 55% humidity and 20 air changes per hour. Throughout the test period rats were housed on a reversed 12:12 hour light-dark cycle with lights off at 0800 h. Bank voles were housed on a reversed 16:8 hour light-dark cycle with lights off at 0900 h. All behavioural experiments were conducted in the dark phase of the light cycle under dim red lights.

All animals were fed 5FL2 EURodent Diet (LabDiet, St Louis, USA) *ad libitum* and had access to *ad libitum* water. All cages had Corn Cob Absorb 10/14 substrate lining the base.

Cardboard tubes and paper wool nest material were provided to all animals for enrichment. In addition, rats were given 15 x 8 cm plastic tubes for enrichment.

5.3.3. Composition of playbacks

Playbacks were recorded using a condenser ultrasound microphone CM16/CMPA (Avisoft-Bioacoustics, Glienicke, Germany) connected to an UltraSoundGate 116H (Avisoft-Bioacoustics, Germany). Sound files were recorded and edited to create 15 minute playbacks as follows.

Rat playback: 50 kHz rat calls were recorded individually from 3 male and 3 female Wistar rats in their own, soiled, home cage after removal of their cage mate, in accordance with Wöhr *et al.* (2008). From each recording, a 30 s section of 50 kHz calls was isolated. Each playback consisted of this 30 s section of 50 kHz calls on constant loop repeat. Individual playbacks were composed for each of the 6 rats recorded. Selection of playback during testing was randomised.

Sound of rat movement playback: We recorded one male Wistar rat during exploration of a home cage, with no vocalisations emitted. Each playback consisted of 30 s of the sound of rat movements on constant loop repeat.

Regularly intermittent white noise playback: We recorded background sound in the room in which rat sounds were recorded (with no animals present) for 15 s. We then generated 15 s of white noise using Avisoft SASLab Pro (Avisoft-Bioacoustics, Germany). White noise volume was matched to the lower end of volume for sound of rat movement playback. The two 15 s sound files were combined to give a 30 s composition. Each playback consisted of this 30 s composition on constant loop repeat.

The volume of playbacks was adjusted so that they matched the volume initially recorded. Maximum playback volume was approximately 61 dB for female rat playbacks, 56 dB for male rat playbacks, 48 dB for sound of rat movement playback and 40 dB for white noise playback (measured at 30 cm from sound source).

5.3.4. Bioassay of attraction or avoidance

The bioassay was conducted in a 120 x 120 x 80 cm laminated chipboard arena divided into three sections using polypropylene sheets, with a 45 x 120 cm compartment at each side and a 30 x 120 cm compartment in the middle. The compartments were connected through a 15

x 15 cm gap in the dividing walls towards one end of the arena. A 28 x 25 x 13 cm Roguard® Extra bait box (BASF, Cheadle Hulme, UK) was placed on the lateral wall of each side of the arena, with its entrances close to the lateral walls (Figure 5.1A and B). On one side of the arena a speaker was placed so that it either played above a bait box (Figure 5.1A) or through a bait box (Figure 5.1B). A matching dummy speaker (empty box of the same dimensions as the speaker) was placed on the other side of the arena. For the speaker (or dummy speaker) to play through a bait box, a 12 cm hole was cut into one side of each bait box, matching an 8 cm hole in the arena wall. Test subjects were prevented from escaping through this hole by a wire mesh barrier.

Three experiments used rats as test subjects (n = 16 for each experiment). The final experiment used bank voles as test subjects (n = 16). An overview of experiments is given in Table 5.1. For all experiments, each rat was tested with three different sound treatments and each bank vole was tested with two different sound treatments, with a 6-9 day gap between treatments. The order of test subjects, sound treatments, and side of speaker placement was randomised in a balanced design. To minimise stress induced by handling (Hurst & West, 2010), test subjects were moved into and out of the test arena using a clear plastic handling tunnel, capped with wire mesh (33 x 11 cm for rats and 18 x 5 cm for bank voles). Before each treatment, test subjects were familiarised with the arena for 15 minutes with the speaker turned off. Subjects were then confined to a handling tunnel, the speaker was turned on, and subjects were reintroduced to the arena for a 15 minute test period. Subject behaviour was recorded in an adjacent room by remote video monitoring. Between each subject the arena was cleaned with Teepol Multipurpose detergent (Teepol, Orpington, UK), followed by 70% ethanol. Transcription of video recordings was performed blind to sound treatment. We recorded the time spent in each side of the arena, time spent on top of each box and time spent inside each box. An animal was recorded as in a location when its whole body (not including the tail) was in that location.

5.3.5. Statistical analyses

Bias scores were calculated by subtracting time spent in the silent side of the arena from time spent in the side of the arena where sounds were played. Bias scores were calculated for time spent in four locations: total time spent in arena side, time spent inside box, time spent on top of box and time spent on arena floor (in arena side, not interacting with boxes).

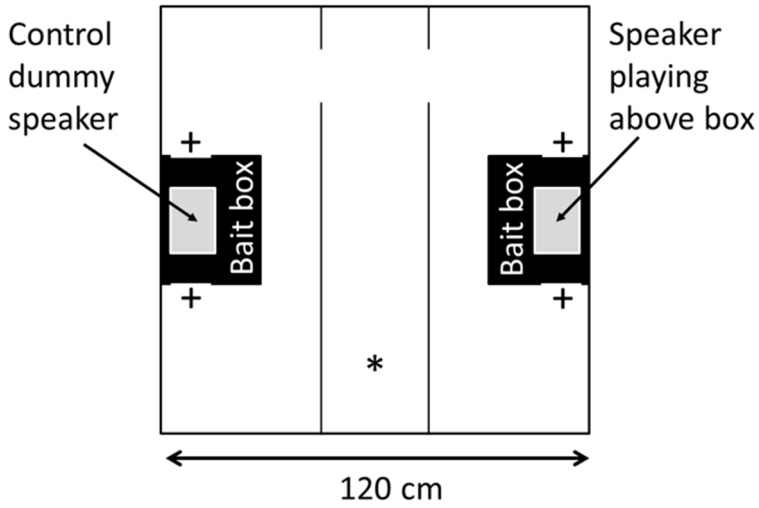
In addition, bias scores were calculated for number of entries to bait boxes for Experiments 2 and 3. Time spent in the central compartment of the arena was not included in the analysis.

We analysed the effect of sound treatment and test subject sex on bias scores for each of our four locations by two-way repeated measures ANOVA (where data approximated normality) or two-way non-parametric ANOVA (where data was not normally distributed). Where sound treatment had a significant effect, differences between each pair of groups were assessed using pairwise t-tests, with Holm-Bonferroni correction for multiple comparisons, (where data approximated normality) and Wilcoxon signed-rank tests, with Holm-Bonferroni correction (where data was not normally distributed).

To assess if rats were attracted to, or avoided, sound cues we analysed bias scores for each of our four locations using one-sample t-tests (where data was normally distributed) or Wilcoxon signed-rank tests (where data was not normally distributed), with Holm-Bonferroni correction where multiple comparisons were made. Where results of ANOVA indicated that sound treatment had a significant effect on bias between arena sides, bias observed in each sound treatment was analysed in a separate test. Where results of ANOVA indicated that sound treatment did not have a significant effect on bias between arena sides, all sound treatments were pooled and analysed in one test.

All statistical tests were bidirectional. A p-value of less than 0.05 was taken as significant. Two-way non-parametric ANOVAs were carried out using software provided by Barnard *et al.* (2007). All other statistical analyses were carried out using R 3.4.0 (R Core Team, 2016) with packages 'ez' (Lawrence, 2015), 'ggplot2' (Wickham, 2009), 'nortest' (Gross & Ligges, 2015), 'exactRankTests' (Hothorn & Hornik, 2015), 'extrafont' (Chang, 2014) and 'gridExtra' (Auguie, 2016).

A)



B)

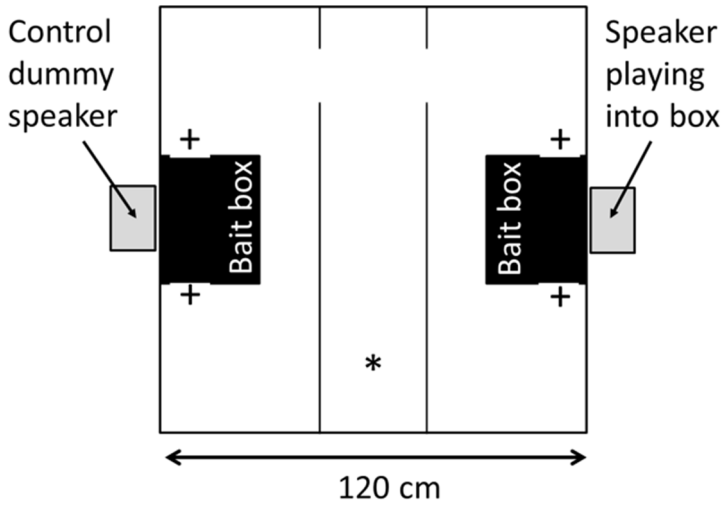


Figure 5.1. Design of laboratory test arena. A) Speaker placed to play above bait box. **B)** Speaker placed outside arena to play through bait box. (* = Position of subject placement into arena via handling tunnel, + = bait box entrances.)

Table 5.1. Overview of experiments.

Experiment number	Test subjects (n = 8 male, 8 female for all)	Test subject age (months)	Familiarity with call donors	Direction of sound	Test playbacks	Control playbacks
1	Wistar rats	12-15	Familiar (housed in same room)	From above box	Female 50 kHz calls Male 50 kHz calls	Sound of rat movement
2	Wistar rats	12-15	Familiar (housed in same room)	From within box	Female 50 kHz calls Male 50 kHz calls	Sound of rat movement
3	Wistar rats	3 – 4	Unfamiliar (housed in separate room)	From within box	Female 50 kHz calls	Sound of rat movement White noise
4	Bank voles	11 – 12	Unfamiliar (housed in separate room)	From within box	Female 50 kHz calls	White noise

5.4. Results

5.4.1. Experiment 1. Rats are attracted to 50 kHz calls, regardless of sex.

To test the sex specificity of 50 kHz rat calls in the attraction of conspecifics, we compared the response of eight male and eight female Wistar rats to three sound treatments, versus a silent control: 1) 50 kHz calls from an adult male Wistar rat, 2) 50 kHz calls from an adult female Wistar rat and 3) rat movement sounds, without any calls. To test if 50 kHz rat calls attracted rats to move close to a sound source, a speaker was placed so that it played above a box.

Overall, rats were attracted to spend more time in the sound side of the arena (one sample *t*-test, t_{47} = 5.05, p < 0.001), regardless of which sound was playing (repeated measures ANOVA, $F_{2, 28}$ = 1.62, p = 0.22) or test subject sex ($F_{2, 28}$ = 0.27, p = 0.76; Figure 5.2A). However, attraction to male and female rat calls appeared stronger than attraction to rat movement sounds.

Looking in more detail, close attraction to the sound source (rats moving on top of boxes) was seen when male (one sample *t*-test, t_{15} = 4.48, p < 0.001) and female rat calls were playing (t_{15} = 5.54, p < 0.001), but not when rat movement sounds were playing (t_{15} = 1.21, p = 0.24; Figure 5.2B). Rats spent substantially more time close to female rat calls (paired *t*-test, t_{15} = 6.02, p < 0.001) and male rat calls (t_{15} = 5.23, p < 0.001) compared to rat movement sounds. Rats spent slightly more time close to female rat calls compared to male rat calls (t_{15} = 2.13, p = 0.05), but attraction to move close to the speaker was not influenced by test subject sex (repeated measures ANOVA, $F_{2, 28}$ = 0.16, p = 0.86).

While rats spent more time close to the source of calls when rat calls were playing, this did not result in an attraction to spend more time inside boxes (one sample *t*-test, t_{47} = 0.29, p = 0.78; Figure 5.2C). This lack of response was not affected by sound treatment (repeated measures ANOVA, $F_{2, 28}$ = 0.25, p = 0.78) or test subject sex ($F_{2, 28}$ = 0.33, p = 0.72).

When rats were in the vicinity of the speaker, but not interacting with boxes, they were attracted by all three sound treatments (repeated measures ANOVA, $F_{2, 28}$ = 0.40, p = 0.67; Wilcoxon signed rank test, V = 950, p < 0.001; Figure 5.2D), a bias that did not differ according to test subject sex ($F_{2, 28}$ = 1.08, p = 0.35).

5.4.2. Experiment 2. Rat sounds attract rats into boxes.

To test if sound coming from within a box can attract rats to enter, we placed a speaker so that it played inside a box. Eight male and eight female Wistar rats were each tested with three sound treatments, versus a silent control: 1) 50 kHz calls from an adult male Wistar rat, 2) 50 kHz calls from an adult female Wistar rat and 3) rat movement sounds, without any calls.

Overall, rats were attracted to spend more time in the sound side of the arena (one sample *t*-test, $t_{47} = 5.97$, $p < 0.001$; Figure 5.3A), regardless of which sound was playing (repeated measures ANOVA, $F_{2, 28} = 0.91$, $p = 0.41$) or test subject sex ($F_{2, 28} = 0.08$, $p = 0.93$).

Looking at attraction to spend time close to the sound source, all three types of rat sound attracted rats to spend more time inside boxes (repeated measures ANOVA, $F_{2, 28} = 0.13$, $p = 0.88$; one sample *t*-test, $t_{47} = 5.12$, $p < 0.001$; Figure 5.3C). This attraction did not differ according to test subject sex (repeated measures ANOVA, $F_{2, 28} = 0.25$, $p = 0.78$).

All three types of rat sound attracted rats to enter boxes more often (median ± IQR, 3 ± 6.25; non-parametric two-way ANOVA, $H_{2, 28} = 3.8$, $p = 0.15$; Wilcoxon signed rank test, $V = 758.5$, $p < 0.001$). Female rats entered boxes where sounds were playing more often than male rats for all three treatment sounds (female, 6 ± 6.5; male, 2 ± 4.25; non-parametric two-way ANOVA, $H_{1, 14} = 5.59$, $p = 0.02$).

Rats spent no more time on top of the box in the sound side of arena compared to the silent side of the arena (Wilcoxon signed rank test, $V = 437$, $p = 0.10$; Figure 5.3B). This response was not influenced by sound treatment (non-parametric two-way ANOVA, $H_{2, 28} = 0.78$, $p = 0.68$) or test subject sex ($H_{2, 28} = 2.44$, $p = 0.30$).

When rats were in the vicinity of the speaker, but not interacting with boxes, they were attracted by all three sound treatments (non-parametric two-way ANOVA, $H_{2, 28} = 0.15$, $p = 0.93$, Wilcoxon signed rank test, $V = 194$, $p = 0.002$; Figure 5.3D), a bias that did not differ according to test subject sex (non-parametric two-way ANOVA, $H_{2, 28} = 1.01$, $p = 0.60$).

5.4.3. Experiment 3. Unfamiliar female 50 kHz calls attract rats into boxes.

The first aim of this experiment was to test whether prior familiarity is necessary for rats to be attracted to 50 kHz calls. In both Experiment 1 and Experiment 2, test rats were housed in the same room as vocalisation donor rats. Familiarity with vocalisation donor calls could be an explanation for the attraction we observed. To investigate this further we tested rats that had no prior exposure to vocalisation donor rats.

The second aim of Experiment 3 was to determine the specificity of attraction of rats to sound cues. The results of Experiment 2 showed attraction to both 50 kHz rat calls and sounds of a rat moving around. To confirm that rats are attracted to the sounds of rats (50 kHz calls and rat movement sounds) specifically, and not just the presence of any sound cue, rats were tested with regularly intermittent white noise as a second control.

An ultrasound speaker played sounds through a box, as for Experiment 2. Eight male and 8 female Wistar rats were each tested with 3 sound treatments, versus a silent control: 1) 50 kHz calls from an adult female Wistar rat, 2) rat movement sounds, without any calls and 3) regularly intermittent white noise. Female rat calls were chosen, as they were slightly more attractive than male rat calls in Experiment 1.

Overall, rats were attracted to spend more time in the sound side of the arena when female rat calls (one sample *t*-test, $t_{15} = 6.10$, $p < 0.001$), and rat movement sounds were played ($t_{15} = 3.11$, $p = 0.014$), but not when white noise was played ($t_{15} = -0.18$, $p = 0.86$; Figure 5.4A). Attraction to spend time in arena side was greater for female rat calls compared to white noise (paired *t*-test, $t_{15} = 3.12$, $p = 0.02$). Although rats showed a significant attraction to spend more time in the vicinity of rat movement sounds, the strength of this attraction was intermediate, not differing significantly from either female rat calls ($t_{15} = 1.77$, $p = 0.15$) or white noise ($t_{15} = 1.93$, $p = 0.15$). Attraction into arena side did not depend on test subject sex (repeated measures ANOVA, $F_{2, 28} = 0.22$, $p = 0.81$).

Attraction to spend time in arena side was explained by time spent inside boxes. Rats were attracted to spend more time inside bait boxes when female rat calls (one sample *t*-test, $t_{15} = 7.84$, $p < 0.001$), and rat movement sounds were played ($t_{15} = 2.94$, $p = 0.02$), but not when white noise was played ($t_{15} = -0.13$, $p = 0.90$; Figure 5.4C). Rats spent more time inside boxes when female rat calls were played compared to white noise (paired *t*-test, $t_{15} = 3.19$, $p = 0.018$). As with time spent in arena side, the strength of attraction to spend more time inside

a box when rat movement sounds were playing was intermediate, not differing significantly from either female rat calls (t_{15} = 1.09, p = 0.29) or white noise (t_{15} = 1.76, p = 0.20). Attraction to spend time in boxes did not depend on test subject sex (repeated measures ANOVA, $F_{2, 28}$ = 0.07, p = 0.93).

As well as attracting rats to spend more time inside boxes, female rat calls attracted rats to enter boxes more often (10 ± 6.75; Wilcoxon signed rank test, V = 136, p = 0.001). This attraction was not observed when white noise was played (1 ± 3; V = 65, p = 0.44). Comparing between sound treatments, rats were attracted to enter boxes more often when female rat calls were played compared to white noise (V = 1, p = 0.002). Rats entered boxes more often when rat movement sounds were played (2 ± 4.25; V = 91.5, p = 0.03), but the increase in number of entries was small, being much less compared to female rat calls (V = 1, p = 0.002) and not significantly different compared to white noise (V = 85.5, p = 0.15). Male rats entered boxes where sounds were playing slightly more often than female rats for all three treatment sounds (male, 3.5 ± 9; female, 2 ± 5; non-parametric two-way ANOVA, $H_{1, 14}$ = 25.32, p < 0.001).

Rats spent slightly more time on top of the box in the side of the arena where sounds were played compared to the silent control side (one sample *t*-test, t_{47} = 2.49, p < 0.016; Figure 5.4B), but the increase in time was very small. Sound treatment (repeated measures ANOVA, $F_{2, 28}$ = 0.68, p = 0.52) and test subject sex ($F_{2, 28}$ = 0.11, p = 0.90) had no influence on this response.

Attraction towards the sound source was not seen outside boxes. Rats spent no more time on the sound side of the arena floor (time not on top of, or inside boxes), compared to the silent side (one sample *t*-test, t_{47} = 1.56, p = 0.13; Figure 5.4D). Sound treatment (repeated measures ANOVA, $F_{2, 28}$ = 2.81, p = 0.08) and test subject sex ($F_{2, 28}$ = 0.67, p = 0.52) had no influence on this lack of response.

5.4.4. Experiment 4. Bank voles are neither attracted to, nor repelled by, 50 kHz rat calls.

As a first step in determining the species specificity of attraction to 50 kHz rat calls, we tested the response of heterospecific bank voles to these calls. An ultrasound speaker played sounds through a box, as for Experiment 2. Eight male and eight female bank voles were each

tested with two sound treatments, versus a silent control: 1) 50 kHz calls from an adult female Wistar rat and 2) regularly intermittent white noise.

Overall, bank voles showed no bias for either side of the test arena (one sample *t*-test, $t_{31} = -0.75$, p = 0.46; Figure 5.5A). Sound treatment and subject sex had no influence on this lack of response (repeated measures ANOVA, interaction $F_{1, 14} = 0.08$, p = 0.78; sound $F_{1, 14} = 1.96$, p = 0.18; sex $F_{1, 14} = 0.05$, p = 0.83).

When looking at this result in more detail, bank voles showed no bias to spend time close to the speaker, inside boxes (one sample *t*-test, $t_{31} = -1.22$, p = 0.27; Figure 5.5C). This lack of response was not influenced by sound treatment or test subject sex (repeated measures ANOVA, interaction $F_{1, 14} < 0.00$, p = 0.99; sound $F_{1, 14} = 1.74$, p = 0.21; sex $F_{1, 14} = 0.18$, p = 0.68).

Bank voles spent very little time on top of boxes (Figure 5.5B), a response that was not influenced by arena side, sound treatment or test subject sex (Wilcoxon signed rank test, side, V = 51, p = 0.74; non-parametric two-way ANOVA, interaction, $H_{1, 14} = 0.18$, p = 0.67; sound, $H_{1, 14} = 2.33$, p = 0.13; sex $H_{1, 14} = 0.58$, p = 0.45).

Bank voles showed no bias to spend time outside boxes on either side of the test arena (Wilcoxon signed rank test, V = 261, p = 0.96; Figure 5.5D). This lack of response was not influenced by sound treatment or test subject sex (repeated measures ANOVA, interaction, $F_{1, 14} = 0.45$, p = 0.52; sound, $F_{1, 14} = 0.96$, p = 0.34; sex, $F_{1, 14} = 0.57$, p = 0.46).

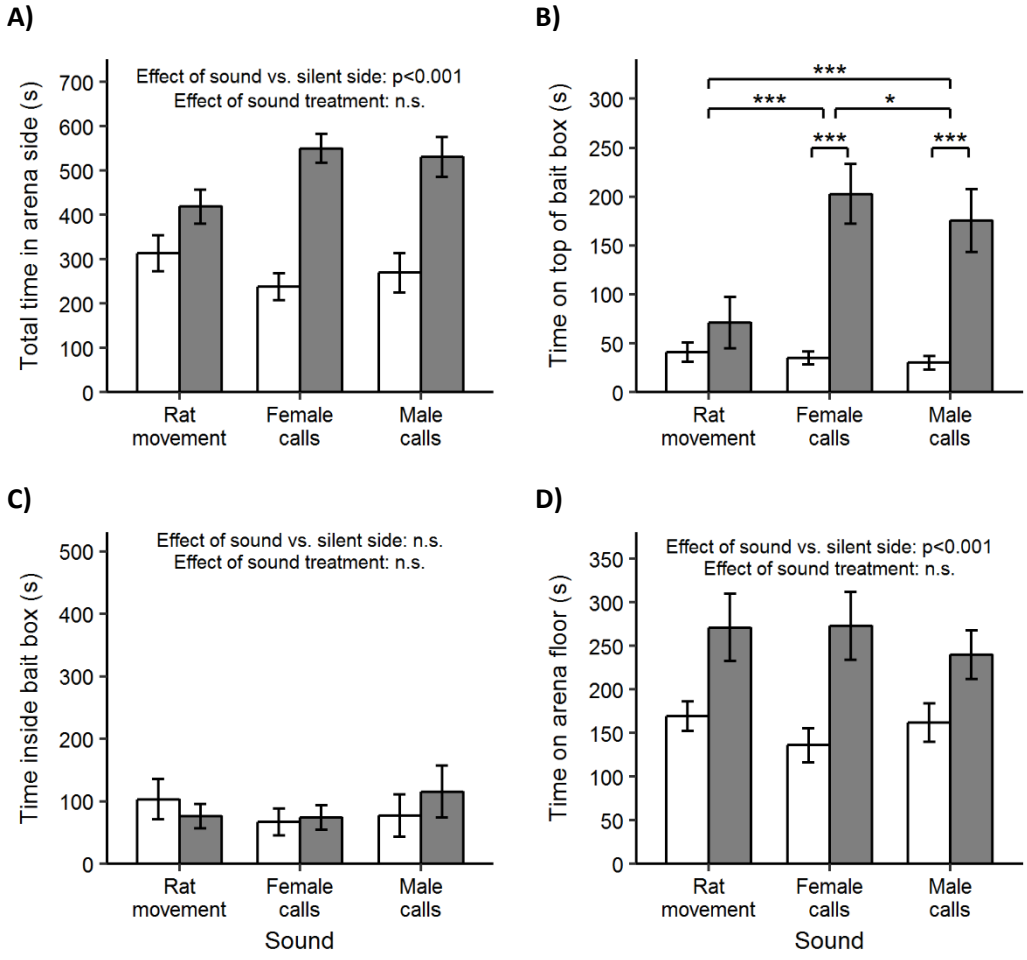


Figure 5.2. Response of Wistar rats to auditory stimuli (mean ± standard error of mean). Adult male and female Wistar rats were presented with sound playing above a box (grey bars) versus no sound (white bars, control) in opposite sides of a test arena. **A)** Total duration in each side of arena. **B)** Duration on top of box. **C)** Duration inside box. **D)** Duration on arena floor (in arena side, not interacting with box). Bias between sides was compared between treatments using repeated measures ANOVA, followed by pairwise t-tests (where data approximated normality) and non-parametric two-way ANOVA, followed by Wilcoxon signed-rank tests (where data was not normal). Within each treatment, attraction or avoidance was assessed using one-sample t-tests or Wilcoxon signed-rank tests. (*p<0.05, ***p < 0.001).

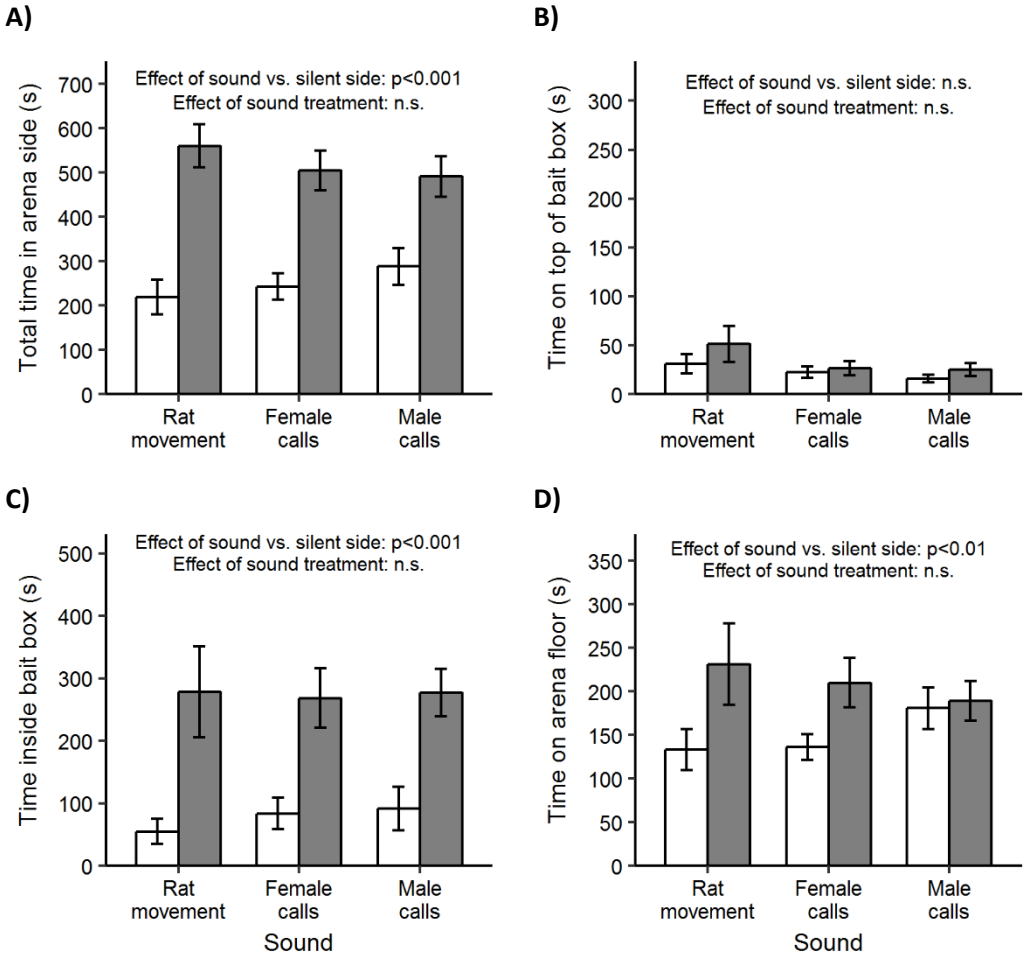


Figure 5.3. Response of Wistar rats to auditory stimuli (mean ± standard error of mean). Adult male and female Wistar rats were presented with sound playing through a box (grey bars) versus no sound (white bars, control) in opposite sides of a test arena. **A)** Total duration in each side of arena. **B)** Duration on top of box. **C)** Duration inside box. **D)** Duration on arena floor (in arena side, not interacting with boxes). Bias between sides was compared between treatments using repeated measures ANOVA, followed by pairwise t-tests (where data approximated normality) and non-parametric two-way ANOVA, followed by Wilcoxon signed-rank tests (where data was not normal). Within each treatment, attraction or avoidance was assessed using one-sample t-tests or Wilcoxon signed-rank tests.

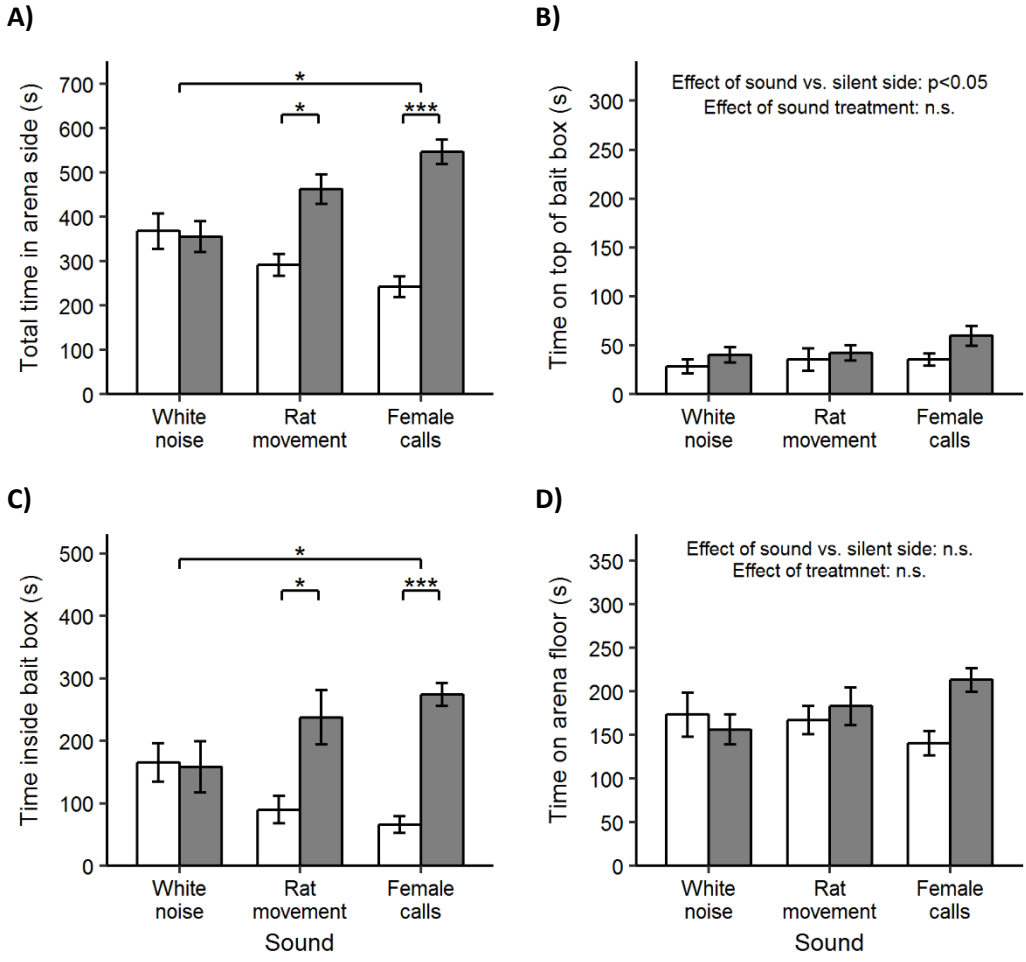


Figure 5.4. Response of Wistar rats to auditory stimuli (mean ± standard error of mean). Adult male and female Wistar rats were presented with sound playing through a box (grey bars) versus no sound (white bars, control) in opposite sides of a test arena. **A)** Total duration in each side of arena. **B)** Duration on top of box. **C)** Duration inside box. **D)** Duration on arena floor (in arena side, not interacting with boxes). Bias between sides was compared between treatments using repeated measures ANOVA, followed by pairwise t-tests (where data approximated normality) and non-parametric two-way ANOVA, followed by Wilcoxon signed-rank tests (where data was not normal). Within each treatment, attraction or avoidance was assessed using one-sample t-tests or Wilcoxon signed-rank tests. (*p<0.05, ***p < 0.001).

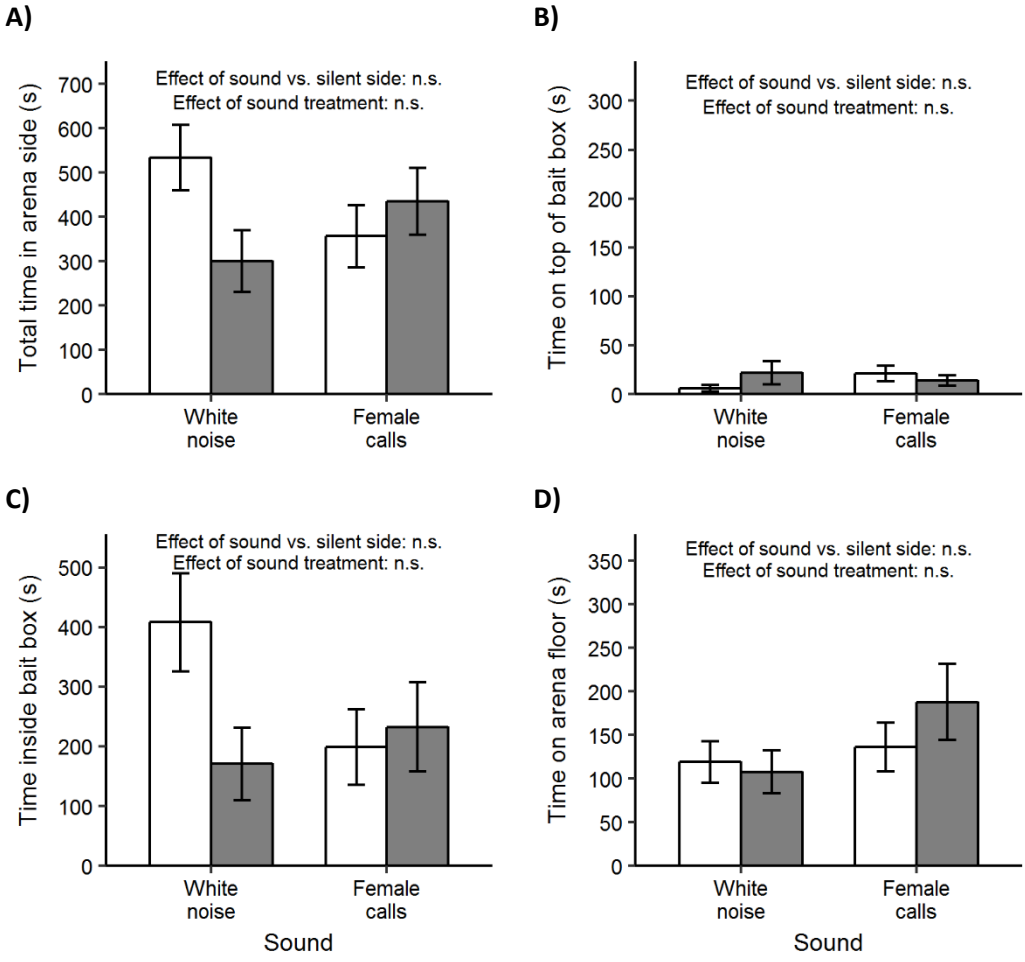


Figure 5.5. Response of bank voles to auditory stimuli (mean ± standard error of mean). Adult male and female bank voles were presented with sound playing through a box (grey bars) versus no sound (white bars, control) in opposite sides of a test arena. **A)** Total duration in each side of arena. **B)** Duration on top of box. **C)** Duration inside box. **D)** Duration on arena floor (in arena side, not interacting with boxes). Bias between sides was compared between treatments using repeated measures ANOVA, followed by pairwise t-tests (where data approximated normality) and non-parametric two-way ANOVA, followed by Wilcoxon signed-rank tests (where data was not normal). Within each treatment, attraction or avoidance was assessed using one-sample t-tests or Wilcoxon signed-rank tests.

5.5. Discussion

We found that rats of both sexes were attracted towards male and female 50 kHz rat calls and sounds of rat movement. However, attraction to male and female 50 kHz rat calls was more consistent than attraction to rat movement sounds. Whilst rat movement sounds attracted rats to spend time in the general vicinity of a sound source, only male and female 50 kHz calls attracted rats on top of boxes. In addition, when listening rats were unfamiliar with calling rats, they showed an intermediate level of attraction to rat movement sounds, compared to a strong attraction to female 50 kHz calls, and no attraction to white noise. We found no sex specificity in the response of rats to 50 kHz calls or rat movement sounds, although there was some evidence that female 50 kHz calls are slightly more attractive than male 50 kHz calls. Bank voles were neither attracted to, nor repelled by 50 kHz rat calls.

The attraction of rats to 50 kHz rat calls in this study is consistent with the findings of several previous studies. In a series of experiments using a radial arm maze, adults of both sexes and juvenile male rats were all attracted towards male rat 50 kHz calls (Seffer *et al.*, 2014; Willadsen *et al.*, 2014; Wöhr & Schwarting, 2007; Wöhr & Schwarting, 2012). In a compartmentalised arena, similar to the design used here, male rats were attracted to male 50 kHz calls (Sadananda *et al.*, 2008). All these studies used Wistar rats, recorded their 50 kHz vocalisations in a manner similar to that employed here and used sounds of rat movement as a control.

As rat movement sounds, and 50 kHz rat calls combined with rat movement sounds, attracted rats into boxes, we cannot conclude that the attraction into boxes in our experiments was attributable to 50 kHz rat calls alone. Attraction to rat movement sounds was seen in both Experiments 2 and 3, relative to a silent control, although the level of attraction was reduced in Experiment 3 compared to Experiment 2. This reduced attraction in Experiment 3 could be due to the reduced familiarity with sound donors, or the age difference in test subjects, between Experiments 2 and 3. Lack of attraction to white noise in Experiment 3 confirms that rats were attracted to specific sound cues, not just the presence of sound in our experiments. Rat movement sounds, recorded in the same manner as in this study, were used as a control in radial arm maze experiments (Seffer *et al.*, 2014) and no attraction was seen. One explanation for the attraction to rat movement sounds that we observed is that rats respond to sound cues depending on the context of their presentation. This could be particularly true for cues such as movement sounds that are not

specific to rats. When sounds are played in an open environment, as in Experiment 1, rats may only be interested in cues that are specific to another rat. However, when sounds are played in an enclosed environment, as in Experiments 2 and 3, rats may be interested in any sound that indicates the presence of another animal. Another explanation is that rats are willing to make more effort to get close to 50 kHz calls, but not rat movement sounds. We were able to observe this effect in Experiment 1, when rats had to move on top of a box to get close to the sound source. However, in Experiments 2 and 3, no extra energy was required to get close to the sound source, so rats may have been willing to move close to the sound source for all sound treatments. A final explanation involves the opacity of boxes used in these experiments. It could be that, in Experiments 2 and 3, there was a difference in response between 50 kHz rat calls and sounds of background movement, but we could not observe this difference as our boxes were opaque. Further experiments could begin to assess these explanations by increasing the effort rats have to go to in order to enter boxes and using clear boxes to observe test subject behaviour in more detail when close to the speaker.

Our results indicate that there is no sex specificity in the attraction of rats to 50 kHz rat calls or rat movement sounds. In contrast to our findings, Snoeren & Ågmo (2013) observed that female 50 kHz calls were not attractive to male rats. The experimental design used by Snoeren & Ågmo (2013) differed from our design in two important aspects. First, Snoeren & Ågmo (2013) used a single compartment arena, which was similar in size to one side of the test arena used in this experiment. This may have meant that, in this smaller arena, test subject rats were less able to determine the source of the 50 kHz calls. Second, Snoeren & Ågmo (2013) recorded 50 kHz calls when a female rat was sniffing a male rat, in an arena where she had previously copulated with a male. In our study 50 kHz calls were recorded on separation of a same sex cage mate. Wright *et al.* (2010) defined 14 different subtypes of call in the 50 kHz range for rats and assigned different call subtypes to sexual encounters versus cage exploration. It seems likely that if rats are producing different subtypes of call in different social contexts that these would elicit differing behavioural responses in listening rats. In a follow up study to Snoeren & Ågmo (2013), male 50 kHz calls were attractive to female rats using the same single compartment arena design (Snoeren & Ågmo, 2014). It may be that during sexual encounters male rats produce 50 kHz calls with the purpose of attracting females, but that the calls produced by females during these events are a response to the male, and do not function as an attractant. These sexual differences would not

necessarily apply to calls produced during separation of cage mates, which could be why we found attraction to both male and female non-sexual 50 kHz calls.

This is the first study to examine the effects of 50 kHz rat calls on heterospecifics. In this study bank voles were not attracted to, or repelled by, 50 kHz rat calls. As rats are potential predators of small rodents (Bridgman *et al.*, 2013; Paul *et al.*, 1971), such as bank voles, it may be expected that bank voles would avoid 50 kHz rat calls. The lack of response to 50 kHz rat calls that we observed could be due to several reasons. One possible explanation is that bank voles are not acoustically sensitive to sounds in the 50 kHz range. However, bank vole calls have been recorded between 20 and 50 kHz (Kapusta & Kruczek, 2016; Sales, 2010) and it is likely they would be able to hear conspecific calls, making this explanation unlikely. Another explanation is that avoidance of rat 50 kHz calls may be learned, rather than innate. The bank voles used in this experiment were first generation, captive bred animals and had no experience of rat vocalisations prior to this study. While testing wild caught bank voles would allow for more naturalistic responses, it would not be possible to control previous experience of rats or their vocalisations prior to capture. In addition, 50 kHz rat calls would not be useful as a non-target repellent if non-target rodents require prior exposure to rats before they are repelled by their calls. Finally, bank voles may use other sensory modalities, such as odour cues, alone or in combination, to alert them to the presence of rats, rather than using sound cues alone.

So, do 50 kHz calls have the potential to be used as a rat attractant for the purposes of pest control? Our results suggest that 50 kHz calls, combined with rat movement sounds, are attractive to rats and can attract them into bait boxes routinely used by pest controllers. The attraction of both male and female rats to male and female 50 kHz calls in Experiment 1, indicates that there is no sex specificity in attraction, which is highly beneficial for pest control. In Experiment 3 of our study, subject rats were unfamiliar with rats used to record sound files, indicating that these calls have the ability to attract unfamiliar individuals, increasing their potential for use in pest control settings. Calls from unfamiliar rats were also used in previous experiments where attraction to 50 kHz rat calls was seen (Sadananda *et al.*, 2008; Seffer *et al.*, 2014; Wöhr & Schwarting, 2007), supporting the results seen here. Overall, our results indicate that 50 kHz calls, combined with rat movement sounds, are candidate rat attractants that have the potential to be used for pest control.

However, some factors of this study need to be considered in translation of these findings to pest control settings. First, only laboratory rats were studied here, in laboratory arenas. Very little is known about the production and use of 50 kHz calls among wild rats. Ultrasonic calls produced by laboratory bred California mice (*Peromyscus californicus)* were recorded in wild California mice, but wild California mice had higher frequency and more variable calls (Kalcounis-Rueppell *et al.*, 2010). If such differences exist among wild rats, there may also be differences in behavioural responses to these calls that may affect their attractant potential. Second, a combination of cues may be required to attract wild rats. A recent field study played infant rat calls through bait boxes and found an increase in the number of female rats captured (Takács *et al.*, 2016). However, in that study infant calls were combined with a bait and female rat soiled bedding. Removal of either the infant calls or the odour cue dramatically reduced attraction. Neither cue was tested alone, making it difficult to judge the attractive potential of infant calls in the absence of bait (Takács *et al.*, 2016). Lastly, we tested responses under controlled laboratory conditions. In the field, ambient noise and other sensory distractions, such as odour cues, could reduce the attractive potential of 50 kHz rat calls. Field trials of 50 kHz rat calls, in pest control settings, would help to address the issues highlighted above. The finding here that female 50 kHz rat calls are slightly more attractive than male 50 kHz rat calls suggests that they should be trialled as a priority in field experiments.

The lack of repulsion of 50 kHz calls to bank voles means that these calls may not be of use in reducing the consumption of rat poisons by non-target rodents. Two solutions could alleviate this problem. First, 50 kHz rat calls could be used alongside control measures that do not rely on food lures, such as traps. Non-target rodents are unlikely to enter small confined spaces, such as bait boxes, unless there is something to attract them inside, such as a food lure. Removal of the food lure should reduce visitation by non-target rodents, but the use of 50 kHz rat calls would encourage visitation by rats. The rats could then be killed by alternative methods to consumable poisons. An alternative solution is to add another cue to 50 kHz calls, such as an odour, that repels non-target rodents, but not rats. By repelling non-target rodents from bait boxes, food lures could continue to be used to attract rats to consume poison. The addition of 50 kHz calls would then, hopefully, increase attraction of rats into bait boxes, without attracting non-target rodents. In our study, we tested only a single species of non-target rodent. Testing other non-target rodents, such as wood mice, is needed to confirm that 50 kHz rat calls are not attractive to them in the field.

In conclusion, our study has shown that 50 kHz rat calls, combined with rat movement sounds, are attractive to both male and female rats, with female 50 kHz calls being slightly more attractive than male 50 kHz calls. In addition, this study has shown that 50 kHz rat calls do not attract or repel bank voles. These findings hold promise to improve the efficacy and reduce the environmental impact of pest control aimed at rats by utilising 50 kHz rat calls as a species specific lure.

5.6. References

Animals (Scientific Procedures) Act. (1986). UK.

Association for the Study of Animal Behaviour (2018). Guidelines for the treatment of animals in behavioural research and teaching. *Animal Behaviour.* **135**, I-X.

Auguie, B. (2016). *gridExtra: Miscellaneous Functions for "Grid" Graphics*. https://CRAN.R-project.org/package=gridExtra

Barnard, C. J., Gilbert, F. S. & McGregor, P. K. (2007). *Asking Questions in Biology: A Guide to Hypothesis-testing, Analysis and Presentation in Practical Work and Research.* Pearson Education, Harlow.

Brakes, C. R. & Smith, R. H. (2005). Exposure of non-target small mammals to rodenticides: short-term effects, recovery and implications for secondary poisoning. *Journal of Applied Ecology.* **42**, 118-128.

Bridgman, L. J., Innes, J., Gillies, C., Fitzgerald, N. B., Miller, S. & King, C. M. (2013). Do ship rats display predatory behaviour towards house mice? *Animal Behaviour.* **86**, 257-268.

Brudzynski, S. M. & Pniak, A. (2002). Social contacts and production of 50-kHz short ultrasonic calls in adult rats. *Journal of Comparative Psychology.* **116**, 73-82.

Brudzynski, S. M. (2009). Communication of Adult Rats by Ultrasonic Vocalization: Biological, Sociobiological, and Neuroscience Approaches. *Ilar Journal.* **50**, 43-50.

Brudzynski, S. M. (2013). Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. *Current Opinion in Neurobiology.* **23**, 310-317.

Buckle, A. & Fenn, M. (1992) Rodent control in the conservation of endangered species. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 36-41.

Burgdorf, J., Kroes, R. A., Moskal, J. R., Pfaus, J. G., Brudzynski, S. M. & Panksepp, J. (2008). Ultrasonic vocalizations of rats (*Rattus norvegicus*) during mating, play, and aggression: Behavioral concomitants, relationship to reward, and self-administration of playback. *Journal of Comparative Psychology.* **122**, 357-367.

Burke, C. J., Kisko, T. M., Pellis, S. M. & Euston, D. R. (2017). Avoiding escalation from play to aggression in adult male rats: The role of ultrasonic calls. *Behavioural Processes.* **144**, 72-81.

Capizzi, D., Bertolino, S. & Mortelliti, A. (2014). Rating the rat: global patterns and research priorities in impacts and management of rodent pests. *Mammal Review.* **44**, 148-162.

Chang, W. (2014). *extrafont: Tools for using fonts*. https://CRAN.R-project.org/package=extrafont

Christiansen, E. (1976) Pheromones in small rodents and their potential use in pest control. In Siebe, C. C., Ed. *Proceedings of the Seventh Vertebrate Pest Conference, Monterey*. University of California. pp. 185-195.

Clapperton, B. K. (2006). *A review of the current knowledge of rodent behaviour in relation to control devices.* Department of Conservation, New Zealand.

Cook, S. M., Khan, Z. R. & Pickett, J. A. (2007). The use of push-pull strategies in integrated pest management. *Annual Review of Entomology.* **52**, 375-400.

Directive 2010/63/EU. (2010). EU.

Gross, J. & Ligges, U. (2015). *nortest: Tests for Normality*. https://CRAN.R-project.org/package=nortest

Hadler, M. R. & Shadbolt, R. S. (1975). Novel 4-hydroxycoumarin anticoagulants active against resistant rats. *Nature.* **253**, 275-277.

Hadler, M. R. & Buckle, A. P. (1992) Forty five years of anticoagulant rodenticides - past, present and future trends. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 149-156.

Harris, S. (1989). When Is a Pest a Pest. *New Scientist.* **121**, 49-51.

Harris, S., Morris, P., Wray, S. & Yalden, D. (1995). *A review of British mammals: population estimates and conservation status of British mammals other than cetaceans.* pp. 1-183. Joint Nature Conservation Committee, U.K.

Hoare, J. M. & Hare, K. M. (2006). The impact of brodifacoum on non-target wildlife: gaps in knowledge. *New Zealand Journal of Ecology.* **30**, 157-167.

Hothorn, T. & Hornik, K. (2015). *exactRankTests: Exact Distributions for Rank and Permutation Tests*. https://CRAN.R-project.org/package=exactRankTests

Hurst, J. L. & West, R. S. (2010). Taming anxiety in laboratory mice. *Nature Methods.* **7**, 825-826.

Kalcounis-Rueppell, M. C., Petric, R., Briggs, J. R., Carney, C., Marshall, M. M., Willse, J. T., Rueppell, O., Ribble, D. O. & Crossland, J. P. (2010). Differences in Ultrasonic Vocalizations between Wild and Laboratory California Mice (*Peromyscus californicus*). *PLOS ONE.* **5**, 1-10.

Kapusta, J. & Kruczek, M. (2016). Ultrasonic Reaction of Bank Vole Males to the Presence of Females Varying in Hormonal Activity. *Ethology.* **122**, 468-480.

Lawrence, M. A. (2015). *ez: Easy Analysis and Visualization of Factorial Experiments*. https://CRAN.R-project.org/package=ez

Martinez-Padilla, J., Lopez-Idiaquez, D., Lopez-Perea, J. J., Mateo, R., Paz, A. & Vinuela, J. (2017). A negative association between bromadiolone exposure and nestling body condition in common kestrels: management implications for vole outbreaks. *Pest Management Science.* **73**, 364-370.

Meerburg, B. G., Singleton, G. R. & Kijlstra, A. (2009). Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology.* **35**, 221-270.

Nair, K. S. S. (2007). Population dynamics: What makes an insect a pest? In *Tropical Forest Insect Pests: Ecology, Impact, and Management.* pp. 119-133. Cambridge University Press, Cambridge.

NC3Rs, BBSRC, DEFRA, MRC, NERC & Wellcome Trust. (2017). Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. NC3Rs, London.

Panksepp, J. & Burgdorf, J. (2000). 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables. *Behavioural Brain Research.* **115**, 25-38.

Paul, L., Miley, W. M. & Baenninger, R. (1971). Mouse killing by rats: Roles of hunger and thirst in its initiation and maintenance. *Journal of Comparative and Physiological Psychology.* **76**, 242-249.

Pimentel, D. (2005). Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. *Environment, Development and Sustainability.* **7**, 229-252.

Pimentel, D., Zuniga, R. & Morrison, D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics.* **52**, 273-288.

R Core Team. (2016). *R: A language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Sadananda, M., Wöhr, M. & Schwarting, R. K. W. (2008). Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. *Neuroscience Letters.* **435**, 17-23.

Sales, G. D. (2010). Chapter 3.4 - Ultrasonic calls of wild and wild-type rodents. In *Handbook of Mammalian Vocalization : an Integrative Neuroscience Approach.* Brudzynski, S. M. (Ed.) pp. 77-88. Academic Press, Boston.

Seffer, D., Schwarting, R. K. W. & Wöhr, M. (2014). Pro-social ultrasonic communication in rats: Insights from playback studies. *Journal of Neuroscience Methods.* **234**, 73-81.

Serieys, L. E. K., Armenta, T. C., Moriarty, J. G., Boydston, E. E., Lyren, L. M., Poppenga, R. H., Crooks, K. R., Wayne, R. K. & Riley, S. P. D. (2015). Anticoagulant rodenticides in urban bobcats: exposure, risk factors and potential effects based on a 16-year study. *Ecotoxicology.* **24**, 844-862.

Singleton, G. R. (2003). *Impacts of rodents on rice production in Asia.* IRRI Discussion Paper Series, No. 45, pp. 1-30. International Rice Research Institute, Philippines.

Singleton, G. R. (2010). *Rodent outbreaks: ecology and impacts.* International Rice Research Institute, Philippines.

Snoeren, E. & Ågmo, A. (2013). Female ultrasonic vocalizations have no incentive value for male rats. *Behavioral Neuroscience.* **127**, 439-450.

Snoeren, E. & Ågmo, A. (2014). The incentive value of males' 50-kHz ultrasonic vocalizations for female rats (*Rattus norvegicus*). *Journal of Comparative Psychology.* **128**, 40-55.

Takács, S., Kowalski, P. & Gries, G. (2016). Natural and synthetic vocalizations of brown rat pups, *Rattus norvegicus*, enhance attractiveness of bait boxes in laboratory and field experiments. *Pest Management Science.* **72**, 1873-1882.

Tosh, D. G., McDonald, R. A., Bearhop, S., Lllewellyn, N. R., Fee, S., Sharp, E. A., Barnett, E. A. & Shore, R. F. (2011). Does small mammal prey guild affect the exposure of predators to anticoagulant rodenticides? *Environmental Pollution.* **159**, 3106-3112.

Tosh, D. G., McDonald, R. A., Bearhop, S., Llewellyn, N. R., Montgomery, W. I. & Shore, R. F. (2012). Rodenticide exposure in wood mouse and house mouse populations on farms and potential secondary risk to predators. *Ecotoxicology.* **21**, 1325-1332.

Walker, L. A., Chaplow, J. S., Llewellyn, N. R., Pereira, M. G., Potter, E. D., Sainsbury, A. W. & Shore, R. F. (2013). *Anticoagulant rodenticides in predatory birds 2011: a Predatory Bird Monitoring Scheme (PBMS) report.* pp. 1-29. Centre for Ecology & Hydrology, Lancaster.

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, New York. http://ggplot2.org

Willadsen, M., Seffer, D., Schwarting, R. K. & Wöhr, M. (2014). Rodent ultrasonic communication: Male prosocial 50-kHz ultrasonic vocalizations elicit social approach behavior in female rats (*Rattus norvegicus*). *Journal of Comparative Psychology.* **128**, 56-64.

Wöhr, M. & Schwarting, R. K. (2007). Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior? *PLOS ONE.* **2**, 1-12.

Wöhr, M., Houx, B., Schwarting, R. K. & Spruijt, B. (2008). Effects of experience and context on 50-kHz vocalizations in rats. *Physiology & Behavior.* **93**, 766-776.

Wöhr, M. & Schwarting, R. K. (2012). Testing social acoustic memory in rats: effects of stimulus configuration and long-term memory on the induction of social approach behavior by appetitive 50-kHz ultrasonic vocalizations. *Neurobiology of Learning and Memory.* **98**, 154-164.

Wright, J. M., Gourdon, J. C. & Clarke, P. B. (2010). Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context. *Psychopharmacology.* **211**, 1-13.

Chapter 6

6. Species identification by novel mass spectrometric analysis of rodent faeces

6.1. Abstract

Accurate identification of species is required for many purposes, including pest control, conservation and scientific research. However, many species identification techniques are labour intensive, time consuming and costly. In this study, we describe a new approach to species identification from faecal samples, based on analysis of a molecular signature generated by rapid evaporative ionisation mass spectrometry (REIMS). Faecal pellets from five different rodent species were analysed by REIMS and complex mass spectra were rapidly acquired, typically a few seconds per sample. The uninterpreted mass spectra (signatures) were then analysed by discriminant function analysis and classification models based on random forests. For both laboratory housed and wild rodents, species identification returned high accuracy rates. The REIMS signatures were stable to prior storage of the faecal material under a range of different conditions and were not altered radically by changes in diet. REIMS offers a completely novel method for the rapid analysis of faecal samples and has considerable potential as a new tool as a species identification technique.

6.2. Introduction

Identification of species in the field is required for a variety of reasons, including pest control, conservation and scientific research (Barbosa *et al.*, 2013; Campbell *et al.*, 2002; Galan *et al.*, 2012). For pest control, species identification is required to monitor pest populations, to ensure that control measures are targeted to where they are required, and to ensure control measures have been successful (Campbell *et al.*, 2002; Witmer, 2007). In addition, due to the non species specific nature of many pesticides, particularly rodenticides, species identification may allow for the detection of non-target species prior to the start of control campaigns, allowing mitigation techniques to be deployed (Mason & Littin, 2003; Witmer, 2007). Species identification is also essential for conservation programmes to monitor populations and track the effectiveness of interventions (Barbosa *et al.*, 2013; Gibbs *et al.*, 1999). Furthermore, species identification is often needed during scientific studies that focus on the behaviour and ecology of free living animals (Galan *et al.*, 2012; Glennon *et al.*, 2002; Meek *et al.*, 2013). Thus, there is a wide applicability for effective species identification techniques.

Current species identification techniques have many drawbacks. To be effective, species identification techniques should be cost effective, rapid, accurate and utilise non-invasive samples (Barbosa *et al.*, 2013; Engeman & Witmer, 2000). Species identification is often accomplished by identification of live animals by trapping, photography or tracking plates (Galan *et al.*, 2012; Glen & Dickman, 2003; Nelson *et al.*, 2002; Whisson *et al.*, 2005; Yu *et al.*, 2013). However, live identification is often labour intensive, reducing cost effectiveness and is only accurate if carried out by trained experts (Barbosa *et al.*, 2013; Galan *et al.*, 2012). An alternative to identification of animals themselves is to use material deposited by animals in the field, such as faeces. Faeces are a common 'calling card' left by animals in the wild, and such deposits have proven to be a valuable source of information regarding species, sex, diet and physiological status, notably stress (Barbosa *et al.*, 2013; Eggert *et al.*, 2003; Farrell *et al.*, 2000; Hansen & Jacobsen, 1999; Mostl *et al.*, 2002). Collection of faeces is less labour intensive than methods to detect live animals; furthermore, faeces may be the only material available to identify cryptic species (Barbosa *et al.*, 2013; Whisson *et al.*, 2005). The predominant modes of faecal analysis are based on visual categorisation of macroscopic remains, such a bones or wing carapaces, or by analysis of DNA, both of which can be protracted and require expert skills (Taberlet & Fumagalli, 1996; Waits & Paetkau, 2005).

There is scope for novel approaches to analysis of faeces as an alternative to current faecal based identification techniques, especially if such new methods were rapid.

One of the major areas of development in biological mass spectrometry has been the development of new ambient ion sources that permit mass spectral data to be collected without prior sample preparation (Balog *et al.*, 2010; Cameron *et al.*, 2016; Cooks *et al.*, 2006). One potential method for analysis of faeces is by the relatively new technique of rapid evaporative ionisation mass spectrometry (REIMS). In REIMS acquisition, samples are subjected to a high frequency alternating current that generates heat in the sample that in turn creates an aerosol containing biological molecules. The molecules are then subjected to 'soft' ionisation that generates information-rich molecular ions (Schaefer *et al.*, 2009). To date, REIMS has found applications in the provision of new information during surgical diathermy 'electro surgery', in the examination of foodstuffs, primarily for analysis of species of origin and adulteration, and in microbial typing (Balog *et al.*, 2010; Balog *et al.*, 2013; Balog *et al.*, 2015; Balog *et al.*, 2016; Black *et al.*, 2017; Bolt *et al.*, 2016; Cameron *et al.*, 2016; Schaefer *et al.*, 2009; Strittmatter *et al.*, 2014; Verplanken *et al.*, 2017). REIMS can be used with solid or semi-solid samples and requires little or no sample preparation or prior separation before analysis (Balog *et al.*, 2010; Cameron *et al.*, 2016).

We were intrigued by the possibility of using REIMS to analyse faecal material for the purpose of species identification. Exploration of the use of REIMS to analyse rodent faeces samples was particularly interesting due to the difficulty in distinguishing faecal pellets from similarly sized rodent species. We analysed faecal samples from several rodent species, maintained under standard laboratory conditions and collected from the natural environment. We found that individual species are clearly resolvable with a high degree of confidence and that the signatures are robust to storage and diet. REIMS has the potential to be a powerful new tool, with applicability to commercial and research settings.

6.3. Methods

6.3.1. Ethics statement

Trapping and laboratory faecal collection were carried out in accordance with international best practice guidelines (Animals (Scientific Procedures) Act, 1986; Directive 2010/63/EU, 2010; Association for the Study of Animal Behaviour, 2018; NC3Rs *et al.*, 2017). Neither procedure involved pain, suffering or lasting harm. Traps were checked twice daily, there was minimal handling of subjects to determine species and sex, following which subjects were released. The study was approved by the University of Liverpool Animal Welfare Committee. No specific licences were required to carry out the work.

6.3.2. Laboratory housed rodent sample collection

Samples were collected from five laboratory housed rodent species, bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*), wood mice (*Apodemus sylvaticus*), house mice (*Mus musculus domesticus*) and rats (*Rattus norvegicus*) for analysis by REIMS. The faecal pellets of these species are visually indistinguishable (Figure 6.1A), except for rat faecal pellets, which are much larger than the others.

Test subjects were 10 male and 10 female bank voles, 8 male and 10 female field voles, 6 male and 10 female wood mice, 10 male and 10 female wild derived house mice and 10 male and 10 female crosses between Brown Norway and Wistar rats. Bank voles and field voles were a mixture of animals wild caught in Northwest England 9 to 15 months prior to the start of the study, and first generation offspring of individuals wild caught in Northwest England aged 5 to 18 months. Wood mice were all wild caught in Northwest England 22 to 27 months prior to the start of the study. Wild derived house mice were 9 to 17-month-old, bred for 5 - 10 generations from populations captured in Northwest England. Brown Norway Cross Wistar rats were 8 to 9 months old, bred for three generations from Wistar (HsdHan®:WIST, InVivo, Bicester, UK) and Brown Norway (BN/SsNOlaHsd, InVivo, UK) laboratory strains. Bank voles, field voles and male house mice were housed singly in 48 x 15 x 13 cm cages (M3, North Kent Plastics, Coalville, UK). Female house mice were housed in groups of between 2 and 4 full siblings in 45 x 28 x 13 cm cages (MB1, North Kent Plastics, UK). Wood mice were housed singly in 38 x 25 x 18 cm cages (RM2, North Kent Plastics). Rats were housed in same sex pairs in 56 x 38 x 22 cm cages (RC2R, North Kent Plastics, UK).

All animals were fed 5FL2 EURodent Diet (LabDiet, St Louis, USA) *ad libitum* and had access to water *ad libitum*. Wood mice, bank voles and field voles were supplemented with Harry Hamster complete muesli (Supreme Petfoods Ltd., Ipswich, UK) and hay. Field voles were also given cut grass. All cages had Corn Cob Absorb 10/14 substrate (IPS Product Supplies Limited, London, UK) lining the base. Cardboard tubes and paper wool nest material were provided to all animals for enrichment. In addition, rats were given 15 x 8 cm plastic tubes for enrichment.

Faeces were collected from laboratory housed rodents by placing them individually into a clean laboratory cage for 1 to 2 hours. Multiple faecal pellets were collected from each individual. The order of sample collection from each animal was randomised. Faecal pellets were stored in Eppendorf tubes overnight at 4°C before being processed by REIMS.

6.3.3. Storage conditions sample collection

Samples were collected from laboratory housed house mice and stored at a variety of temperatures and storage conditions prior to analysis by REIMS. Test subjects were twelve, 11 to 13 month old, male wild derived house mice (bred for 5 - 10 generations from populations captured in Northwest England). Multiple faecal samples were collected from each subject and stored in closed Eppendorf tubes at four temperatures: -18°C, -4°C, 21°C and ambient (mean 18.25°C, maximum 24°C, minimum 17°C). Ambient temperature samples were stored in open or closed Eppendorf tubes. Samples were stored for 1 day, 1 week or 4 weeks. Samples were randomly allocated to each temperature and time condition. For each test subject a sample was stored for each temperature and time condition, giving 15 samples per test subject.

6.3.4. Diet study sample collection

Samples were collected from laboratory housed house mice maintained on a variety of diets for analysis by REIMS. Test subjects were 48 male wild derived house mice (9 to 18 months old, bred for 5 - 10 generations from populations captured in Northwest England). Test subjects were fed 5FL2 EURodent Diet (LabDiet, USA) *ad libitum* prior to the start of the study. At the start of the study, house mice were assigned to four groups of 12. Each group of 12 house mice was fed a different diet for 5 weeks. During week 1, subjects were fed a mixture of 5FL2 EURodent Diet (LabDiet, USA) and their new diet. From week 2 house mice were fed only their new diet. Diets were Poultry Grower (SDS, Braintree, UK), Harry Hamster complete muesli (Supreme Petfoods Ltd., UK), Turbo 40 pig feed (Massey Bros Feeds Ltd,

Crewe, UK) and 5FL2 EURodent Diet (LabDiet, USA). On the first day of the study faecal samples was collected from each house mouse and were then collected from each subject at weekly intervals throughout the study.

6.3.5. Wild rodent sample collection

Samples were collected from four species of wild rodents and laboratory housed, wild derived house mice for analysis by REIMS. For wood mouse, bank vole and field vole samples, Longworth traps, Mk1 TubeTraps (BioEcoSS Ltd, Shropshire, UK), Mk2 TubeTraps (BioEcoSS Ltd, UK) and Ugglan traps were set in multiple locations. These locations were Kielder Forest (Northumberland, UK), Ness Botanic Gardens (Wirral, UK), Wood Park Farm (Wirral, UK), University of Liverpool Leahurst campus (Wirral, UK) and a private garden in Mouldsworth (Cheshire, UK). Traps were checked twice daily. Species and sex of all trapped animals was recorded and multiple faecal pellets were taken from each trap. Sex was determined using the anogenital distance (larger in males). When more than one animal was captured, no faecal samples were taken. Rat faecal samples were obtained as loose droppings from building floors at Wood Park Farm (Wirral, UK), Ness Heath Farm (Wirral, UK) and Shotton Industrial Estate (Wirral, UK). In total, samples were collected from 80 bank voles, 40 field voles, 74 wood mice and 29 rats (Table S6.1). Samples were stored at 4°C for up to 7 days or at -18°C for up to 15 days before analysis. Wild derived house mouse faecal samples were obtained from the 48 male wild derived house mice used for the diet study. Samples from these house mice were obtained at the end of the diet study (at the end of week 5).

6.3.6. REIMS processing of faecal samples

Faecal samples were burned using an electrosurgical pencil, producing an ionised aerosol, which was evacuated into a mass spectrometer, generating complex mass spectra (Figure 6.1B-D).

All sampling was conducted in a Ductless Fume box (Air Science, Lydiate, UK). REIMS requires that samples contain sufficient water to conduct an electric current to heat the sample and generate a vapour or aerosol. As faecal pellets collected in the field may have dried, we optimized a rehydration protocol. Faecal pellets were placed onto 25 mm glass microfiber filter paper disks (GE Healthcare, Chalfont St Giles, UK), moistened with MilliQ water. Pellets were then individually hydrated with 200 µL of MilliQ water for 1 to 2 minutes. From a small rodent, a typical faecal pellet (dry weight approximately 10 mg) was, after hydration, able to conduct electricity and burn rapidly during REIMS acquisition. An aerosol was generated

using a monopolar electrosurgical pencil with two buttons powered by a VIO 50 C electrosurgical generator on the dry cut setting at 35 W. Sampling comprised of three pellets from the same individual and/or condition that enabled data acquisition for 2-5 seconds per pellet. Faecal pellets were subjected to 1 to 5 burn events, each of which generated complex mass spectra, taking in a total analysis time of 30 to 120 seconds. Sample processing was conducted blind to treatment condition of the sample and the order of sample processing was randomised.

Aerosol particles were aspirated using a Venturi gas jet pump powered by nitrogen on the REIMS source via a 3 m evacuation tubing incorporated into the electrosurgical pencil. The aerosol was directed into the mass spectrometer using a whistle located in the Venturi valve. Propan-2-ol (Sigma-Aldrich, Gillingham, UK) was introduced directly into the REIMS source (Waters, Manchester, UK) via the whistle at 150 µL/minute. Laboratory animal and storage studies were conducted using the beta version of the impactor (ceramic cylinder) whereas the wild animal and diet study samples were analysed using the commercial version (metallic coil). Mass spectra were recorded on a Synapt G2-Si (Waters, UK) in full scan resolution, negative ion mode at a scan rate of 1 scan per second from 50 – 1200 *m/z*. The sample cone was set to 60 V and the heater bias was set to 60 V.

6.3.7. Data analysis

The data analysis protocol for all samples was optimised by comparison of analysis techniques and mass range selection (see supplementary material).

Individual burn spectra for each faecal pellet were aggregated to generate a single raw data file for each sample. Mass spectra were imported into Waters Offline Model Builder software (version 1.1.28; Waters, UK). Within the Offline Model Builder spectral data above the intensity threshold of 3 $x10^5$ were summed for each data point, this comprised of up to 3 faecal pellets from the same animal. Mass spectra were then lockmass corrected to an IPA background peak at 325.19 *m/z*. For analysis a mass range of 400 to 1100 *m/z* was used as limiting the mass range removed peaks from propan-2-ol and noise peaks. The resulting spectra were normalised, scaled and binned by the Offline Model Builder at 0.1 Da bin width. Binned data was exported from the Offline Model Builder as .csv data files that were analysed by principal component analysis (PCA), followed by discriminant function analysis (DFA) using SSPS version 24 (IBM, Portsmouth, UK) and random forest using R version 3.4.0.

(R Core Team, 2016) with packages 'randomForest' (Liaw & Wiener, 2002) and 'ggplot2' (Wickham, 2009).

6.3.7.1. Laboratory housed rodent analysis

Binned spectra from laboratory housed rodent samples were analysed by PCA, followed by DFA on the top 12 principle components. Spectra were also analysed by random forest using 1000 trees and 83 variables available for splitting at each tree node. A confusion matrix was generated to determine the accuracy of classification for each species. Overall classification accuracy was calculated as 100 minus the out of bag error rate.

6.3.7.2. Storage conditions analysis

To analyse the effect storage conditions would have on the accuracy of species classification, male house mouse samples from the laboratory housed rodent study were removed from that data set. Samples from male house mice in the storage study that had been stored at 4°C for one day (the same storage conditions as for laboratory housed rodent study) were added to the laboratory data set. Binned spectra from the resulting data set were analysed by random forest using 1000 trees and 83 variables available for splitting at each tree node. A confusion matrix was generated to determine the accuracy of classification for each species. Overall classification accuracy was calculated as 100 minus the out of bag error rate. We then used the predict function of the random forest package to determine the accuracy of our model to correctly predict species classification as house mouse for each of the 15 storage conditions.

6.3.7.3. Wild rodent analysis

Binned spectra from wild rodent samples were combined with spectra from week 5 samples from the house mouse diet study. As we were not able to capture wild house mice for inclusion in this analysis, we aimed to replicate the mixed diet conditions of wild rodents by including samples from house mice on multiple diets. Data from samples of house mice on day 35 of our diet study were added to the wild rodent sample dataset. The combined wild rodent and house mouse data set was analysed by PCA, followed by DFA on the top 12 principle components. Spectra were also analysed by random forest using 1000 trees and 83 variables available for splitting at each tree node. A confusion matrix was generated to determine the accuracy of classification for each species. Overall classification accuracy was calculated as 100 minus the out of bag error rate.

6.3.7.4. Diet study analysis

To analyse the effect diet conditions would have on the accuracy of species classification, we first constructed a random forest model as described for wild rodent analysis, but we substituted samples from house mice on day 35 for samples on day 0 (all house mice on lab diet). We then used the predict function of the random forest package to determine the accuracy of our wild rodent model to correctly predict species classification as house mouse for each of the 6 weekly time points of the house mouse diet study.

6.3.7.5. Separate location analysis

To assess if REIMS could accurately classify species from a location not included in the random forest model we first removed samples from Wood Park farm (n = 25 wood mice, 11 bank voles) from the dataset. The resulting dataset was run as for wild rodent analysis above to give a training model. We then used the predict function of the random forest package to determine the accuracy of classification for Wood Park farm samples.

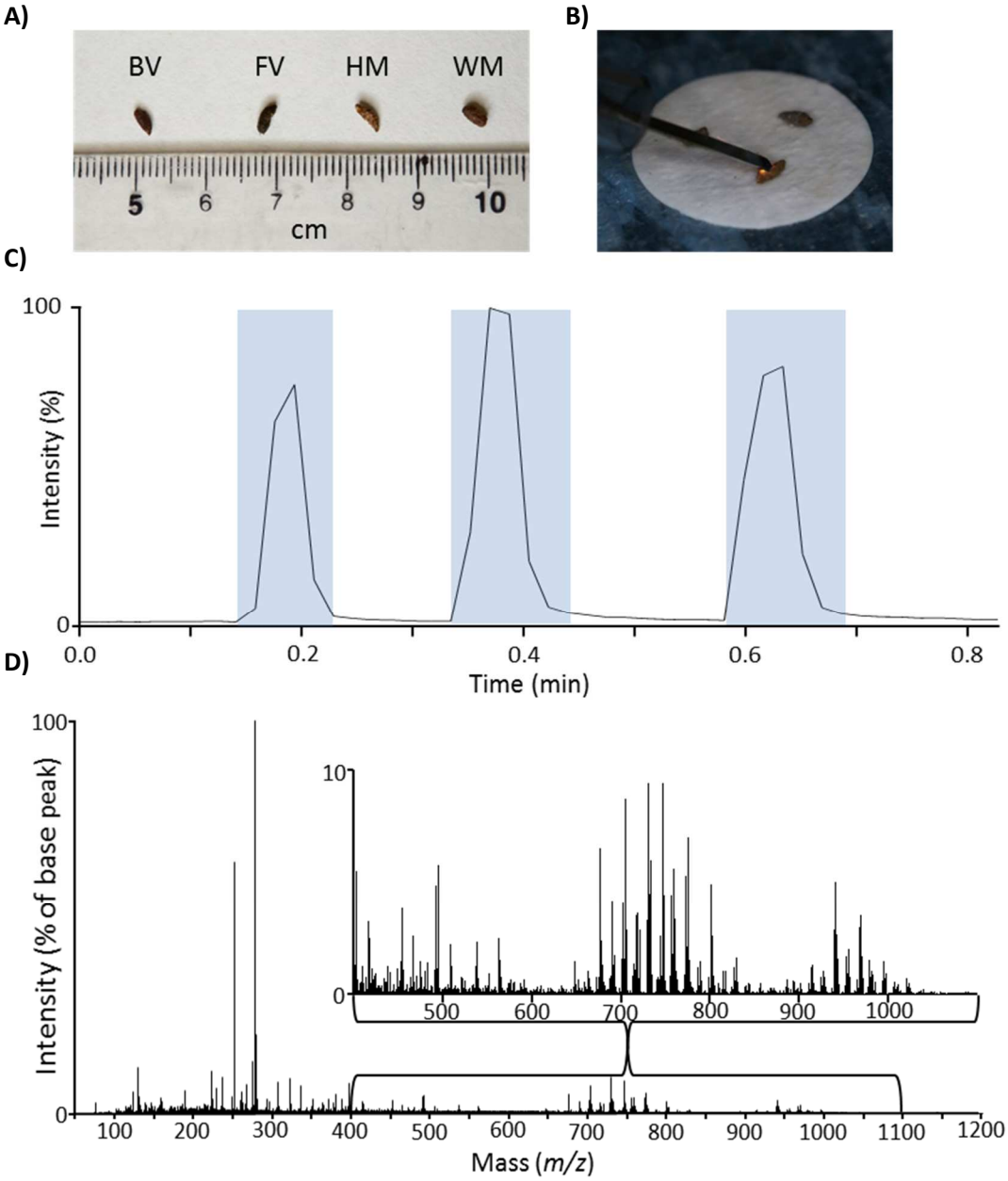


Figure 6.1. Overview of REIMS workflow. A) Faecal pellets from a bank vole (BV), field vole (FV), house mouse (HM) and wood mouse (WM) are visually indistinguishable (*Rattus norvegicus* pellet not included as it is easily distinguishable by size, being much larger than the others). **B)** Faecal pellets are hydrated and burned using a monopolar diathermy electrode. **C)** Each burn event (highlighted in blue) results in a burst of ion current at the mass spectrometer. **D)** Resulting complex mass spectrum from a single burn event (mass range selected for analysis is highlighted). Spectral images created by Dr J. Harris, University of Liverpool.

6.4. Results

6.4.1. Faecal REIMS can classify species of laboratory-housed rodents

We used laboratory-housed rodents for proof of principle that REIMS could classify rodent faecal samples based on species of origin. Samples from laboratory-housed rodents reduced variation in faecal composition attributable to environmental effects, such as diet, intestinal microbiome and housing. Faecal samples were collected from bank voles, field voles, wood mice, house mice and laboratory rats (Brown Norway crossed Wistar). Samples were stored overnight at 4°C before being analysed on the REIMS.

The five species generated complex mass spectra that were relatively similar within individuals from the same species, but consistently different between species. The mass spectra were binned using Offline Model Builder software to yield a 7001 point discretised spectrum for each sample prior to analysis by DFA. Using DFA, species were resolved with some clustering (Figure 6.2A). Using random forest classification, REIMS could resolve rodent species (housed under laboratory conditions) to an accuracy of 83% (random forest analysis, n = 94; Figure 6.2B). The highest classification accuracy was for rats (95%). Of the 20 rat samples, one was misclassified as wood mouse. The lowest classification accuracy occurred for house mice and wood mice (75% for both). Of the 20 house mouse samples, two were misclassified as wood mouse, two as rat and one as bank vole. Of the 16 wood mouse samples, two were misclassified as house mouse, one as rat and one as field vole.

6.4.2. Sample storage does not affect REIMS classification accuracy

To be of greatest value, the mass signature obtained through REIMS should be stable over time and under different environmental conditions. However, there is potential for time and changes in ambient temperature to cause variance in the mass spectra. This could be relevant to samples collected in the field of indeterminate history and subject to a broad range of environmental conditions (from -20°C to +21°C). We therefore explored the effect of sample history on classification accuracy.

We collected house mouse faeces and maintained them for extended periods under different conditions. Under all storage conditions, the pattern was remarkably invariant (Figure 6.3). The REIMS spectra that were obtained were classified using the random forest model established for laboratory housed rodents. No samples were misclassified for samples stored for 1 day. For samples stored for one week there was one misclassification out of 12

samples for samples stored in closed vials at each of three temperatures: ambient (mean 18°C), 21°C and -18°C. For samples stored for 4 weeks there was one misclassification out of 12 for samples stored in open vials at ambient temperature (mean 18°C). One mouse was misclassified twice, the other misclassifications occurred for separate individuals. The stability of the signature, and the classification, was surprising, even under conditions (ambient, open) where some of the faecal samples could be seen to have significant coatings of fungal hyphae.

6.4.3. Diet has limited impact on faecal REIMS classification accuracy

A potential source of variation in the faecal REIMS signature is the diet of donor animals, which would vary more in populations of wild rodents. To assess whether diet was a major influence on the REIMS signature, we took faeces from house mice maintained on four different commercial diets. We collected samples over several weeks as the mice were changed from a standard laboratory diet to their new diet (n = 12 for each diet).

Overall, the longer the house mice were maintained on their new diet, the more difficult it was for REIMS to classify them correctly (Figure 6.4). House mice maintained on the same laboratory rodent diet were classified to 100% accuracy for all time points measured. When house mice were transferred to a hamster diet, classification accuracy was reduced, with three misclassifications at 14 days, two at 21 days, three at 28 days and five at 35 days. House mice transferred to a pig diet had a higher classification accuracy, with one misclassification after 14 days and two at 35 days. House mice transferred to a poultry diet were slightly less accurately classified than those maintained on a pig diet, with one misclassification after 14 days and three misclassifications after 28 days. Of the 20 misclassifications, 15 house mice were misclassified as wood mice, three as field voles and two as bank voles. No individual mouse was misclassified more than two times. Overall, the accuracy of classification only decreased by 16% over the 5 week period, indicating that diet influences the signature, but is not the principal source of variation in the REIMS signature.

6.4.4. Faecal REIMS can classify wild rodents with a high degree of confidence

Having confirmed that REIMS could classify rodent faeces samples, and there was very limited effect of sample storage on classification ability, we next analysed field-collected samples from bank voles, field voles, wood mice, and brown Norway rats from several field sites. As we were not able to capture wild house mice for inclusion in this analysis, we used

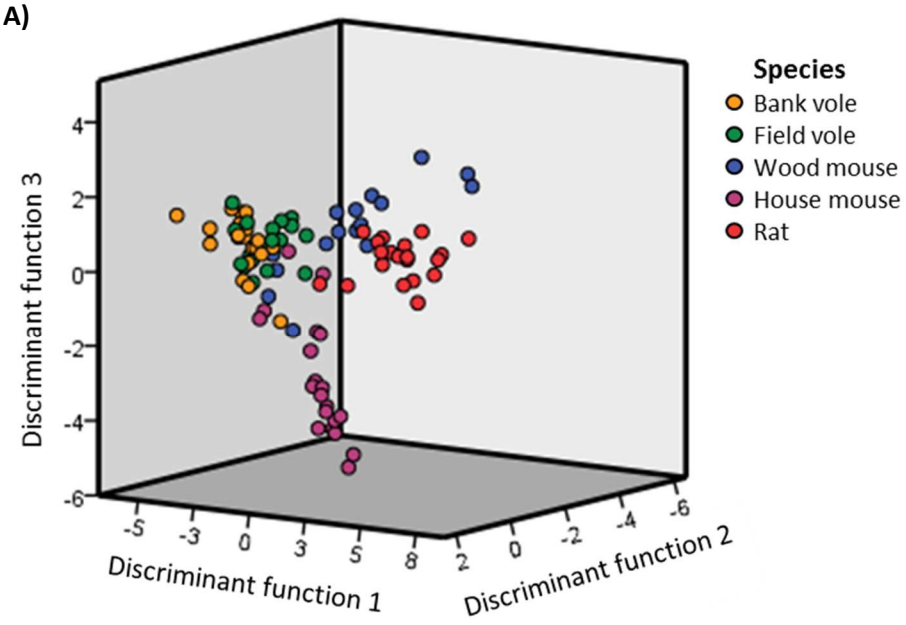
samples from week 5 of the house mouse diet study. We aimed to replicate the mixed diet conditions of wild rodents by including samples from house mice on multiple diets.

REIMS could classify rodent species to a high accuracy of 93.4% (Figure 6.5). The highest misclassification rate occurred for bank voles at 8.8%. Out of 80 bank vole samples, four were misclassified as wood mouse and three as field vole. Wood mice were misclassified at a rate of 8.1%; out of 74 wood mouse samples, four were misclassified as bank vole and two as house mouse. Out of 48 house mouse samples two were misclassified as bank vole and one as wood mouse, giving a misclassification rate of 6.3%. One rat sample was misclassified as a house mouse out of 29 samples, giving a misclassification rate of 3.4%. One field vole was misclassified as a bank vole out of 40 samples, giving a misclassification rate of 2.5%.

6.4.5. REIMS can classify species drawn from populations not included in the original data set

For REIMS to be of value in identification of species of rodents in new study sites, it should be possible to use pre-existing data as a learning set to extrapolate to new populations. To test this using our dataset, we selected one of our wild rodent trapping sites. We selected Wood Park Farm where we had obtained 25 wood mouse and 11 bank vole samples. We excluded these samples from our wild rodent data set and ran a Random Forest training model on the remaining data. This training model gave a classification accuracy of 93.6%. Subsequently, samples from Wood Park Farm were passed through the Random Forest model using the predict function to classify species. The model classified 25 out of 25 wood mouse samples correctly and 8 out of 11 bank vole samples correctly. Three bank vole samples were misclassified as wood mice.

A)



B)

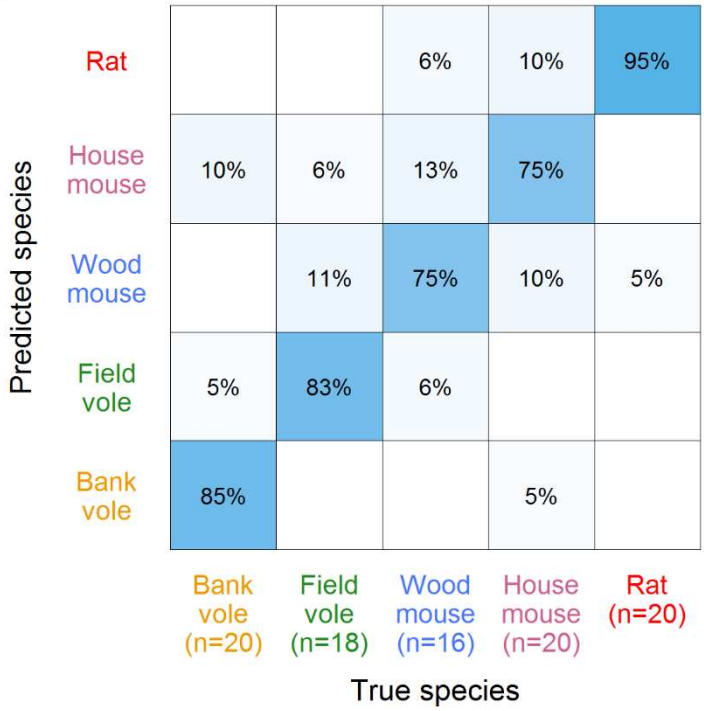


Figure 6.2. Classification of laboratory housed rodent species by REIMS. REIMS data were collected for faecal pellets between 16 and 20 individuals of both sexes of five different rodent species, all maintained under laboratory conditions and fed the same diet. **A)** Discriminant function analysis of the top 12 components from principle component analysis. **B)** Random forest confusion matrix for the classification of species, expressed as the percentage of each species classified as the correct or incorrect species.

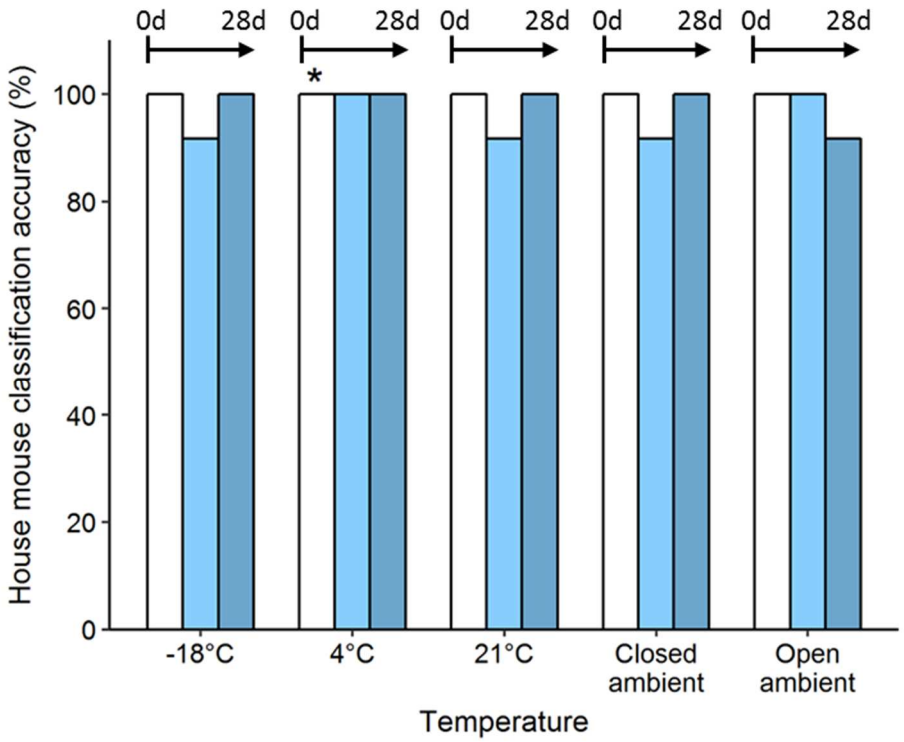


Figure 6.3. Stability of REIMS signature to storage. House mouse faecal pellets were stored in closed tubes for up to 28 days at -18°C, 4°C or 20°C. Further samples were stored for the same period under ambient temperature (average 18°C), either in closed tubes or open to the environment. All samples were then analysed by REIMS, and the classification accuracy under the different conditions was assessed by random forest analysis relative to baseline house mouse data taken from samples stored for 1 day at 4°C (marked with an asterisk).

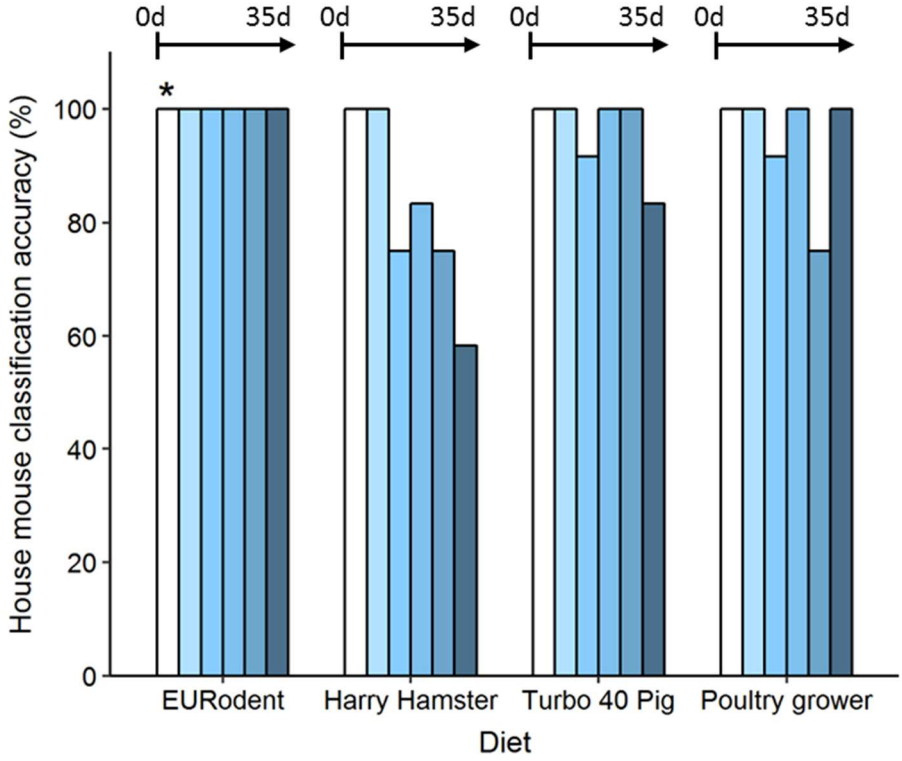


Figure 6.4. Stability of REIMS signature to dietary variation. House mice, initially fed a standard laboratory rodent diet, were acclimated to a new diet for a seven-day period, during which they were also given access to the new diet before being transferred solely to the new diet. At multiple time points, faecal pellets were collected from all animals up to four weeks after diet change, giving a 35 day experimental period. Classification accuracy, assessed by random forest analysis was relative to the baseline data from day 0 of the study (marked with an asterisk).

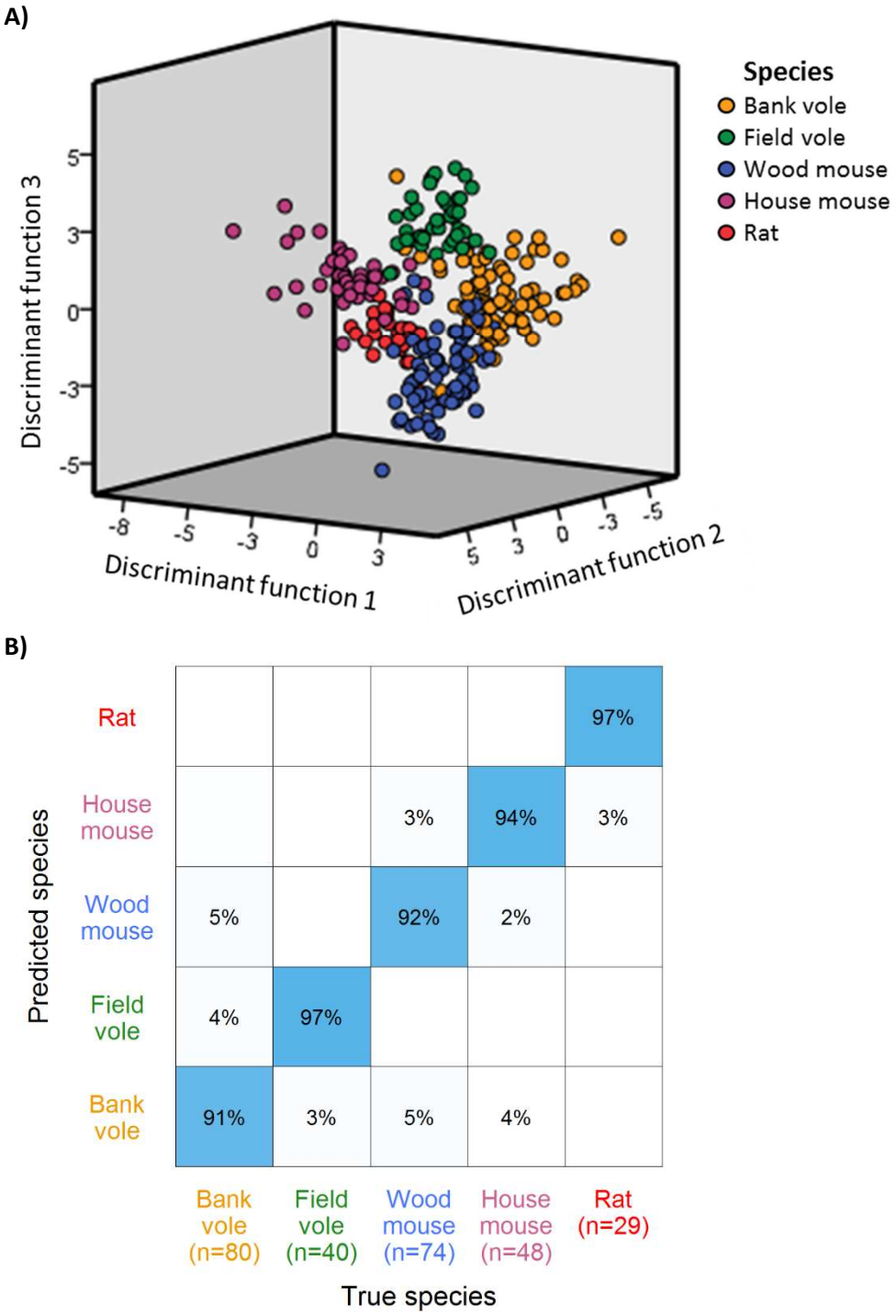


Figure 6.5. Classification of wild rodent species by REIMS. REIMS data were collected for faecal pellets between 29 and 80 wild caught individuals of both sexes. **A)** Discriminant function analysis of the top 12 components from principle component analysis. **B)** Random forest confusion matrix for the classification of species, expressed as the percentage of each species classified as the correct or incorrect species.

6.5. Discussion

This study demonstrated that REIMS can generate informative mass spectra from rodent faeces, and this can be used to classify species of origin with high performance (over 90% for wild rodents). This level of accuracy is comparable with previous studies that used REIMS to identify bacterial and fungal species (Cameron *et al.*, 2016; Strittmatter *et al.*, 2014).

An important part of this study was the examination of different data analysis methods for REIMS. The REIMS signature does not comprise a series of molecules, identified by their mass spectra. Rather the pattern of ions, uninterpreted in molecular terms, is the overall signature that is used for discrimination. Various analysis techniques have been used to analyse the ion pattern generated by REIMS, including PCA/DFA, random forest and univariate analysis (Bolt *et al.*, 2016; Cameron *et al.*, 2016). By examining multiple analysis techniques, we were able to increase the classification accuracy of our analysis. For this study, random forest gave a higher classification accuracy, compared to PCA/DFA. As improved classification accuracy of random forest compared to PCA/DFA has been noted before (Bolt *et al.*, 2016), random forest analysis is recommended for future REIMS studies.

REIMS classification of laboratory housed rodents was high, (between 75% and 95%). However, the performance when applied to faecal samples collected in traps from wild caught animals was very high (between 91% and 97%). The increased accuracy when applied to wild samples could indicate that diet has an effect on the ability of REIMS to distinguish species. As wild rodents will likely have a varied diet (Hansson, 1971), arguably, maintaining laboratory housed animals on a single diet could have impaired the distinction between the different species. During the diet study, there was a high level of classification accuracy following diet change in house mice. However, there was some loss of precision, especially when house mice were maintained on a hamster diet. The overall resilience of the REIMS signature to changes in diet implies that a major part of the REIMS signature is attributable to the animal, possibly due to species specific compounds excreted in the faeces. Such compounds may originate from anal gland secretions, and have been previously identified in faeces (Goodrich *et al.*, 1990a; Goodrich *et al.*, 1990b). The loss of classification accuracy observed when house mice were maintained on a hamster diet could be due to this being the most varied of the four diets used in that experiment, leading to a greater variation in dietary intake by individual mice. It may be possible that shifts in the gut microbiome elicited by dietary shifts could be manifest in the faecal molecular profile. Thus, the REIMS signature

may be composed of a combination of species specific elements, and elements influenced by other factors, such as diet. Overall, the high classification accuracy of wild rodent faecal samples, compared to those from laboratory housed rodents bodes well for future field studies.

Surprisingly, the REIMS ion pattern was robust to storage conditions, which provides reassurance that field-collected samples could be retained and analysed after the field study. This contrasts with instability of specific faecal metabolites (Hadinger *et al.*, 2015; Khan *et al.*, 2002). If controlled sample conditions were achievable, then the effects would be ameliorated further, but it is probable that in the absence of a freezer, the best storage solution would be to allow the faecal samples to dry completely, as they are fully rehydrated prior to REIMS.

There are many features of REIMS that indicate it has potential to be an effective monitoring tool for rodents, and other species. Species identification accuracy of REIMS is high, at a comparable level with other faecal based identification techniques (Alasaad *et al.*, 2011; Barbosa *et al.*, 2013; Galan *et al.*, 2012), and higher than other techniques, including photography and footprint identification (Meek *et al.*, 2013; Russell *et al.*, 2009; Yu *et al.*, 2013). Another benefit of REIMS is the high speed of sample processing, typically 5- 10 seconds per sample, with a maximum processing time of up to 2 minutes per sample. This is much faster than the standard protocol used to identify species from faeces, involving DNA extraction, PCR amplification and downstream analysis, which can take hours (Waits & Paetkau, 2005). In addition, although the core instrumentation necessary for REIMS is costly, it is inexpensive to build comprehensive profiles of large numbers of samples from a REIMS-based workflow, and the signatures incorporated into the learning set seem to permit classification for an extended period of future analysis.

The ease with which REIMS spectra are acquired, and indeed, the lack of any requirement for detailed molecular interpretation of the spectra, means that this method might be applicable to many ecological applicants, including conservation and biological research. Although samples must be brought to the instrument, the stability of the signature to storage makes this feasible. At hundreds of samples per day, it would be possible to develop a detailed profile over significant geographical or temporal scales. This could allow REIMS to be used as a central diagnostic service to which samples are sent for commercial pest control, conservation and research applications. It is possible that with suitable training data sets, the

information that might be gained could extend to other measures of, for example, sex, diet or even stress.

In conclusion, the classification accuracy of REIMS to identify rodent species is very high and is robust to variations in sample storage and diet. This accuracy, coupled with the rapidity of sample processing, suggests that REIMS has good potential to be applied to a variety of settings, including pest control, conservation and ecology studies.

6.6. Supplementary material

6.6.1. Wild rodent sample collection

Table S6.1. Faecal samples collected from different locations.

Species	Kielder Forest 55.234° N -2.579° E	Leahurst 53.290° N -3.027° E	Mouldsworth 53.230° N -2.732° E	Ness Gardens 53.273° N -3.043° E	Ness Heath Farm 53.286° N -3.029° E	Wood Park Farm 53.290°N -3.033° E	Shotton 53.236° N -3.043° E	**Grand Total**
Bank vole	3	17	3	46		11		**80**
Field vole	37			3				**40**
Rat					13	1	15	**29**
Wood mouse		7	20	22		25		**74**
Total	**40**	**24**	**23**	**71**	**13**	**37**	**15**	**223**

6.6.2. Data analysis

6.6.2.1. Selection of analysis technique

REIMS data has been analysed in multiple ways, including principal component analysis followed by linear discriminant analysis and random forest (Bolt *et al.*, 2016; Cameron *et al.*, 2016). To decide which approach was best to use for analysing our data we compared multiple analysis techniques. Laboratory animal data was used for this comparison. The workflow for data analysis comparison is shown in Figure S6.1.

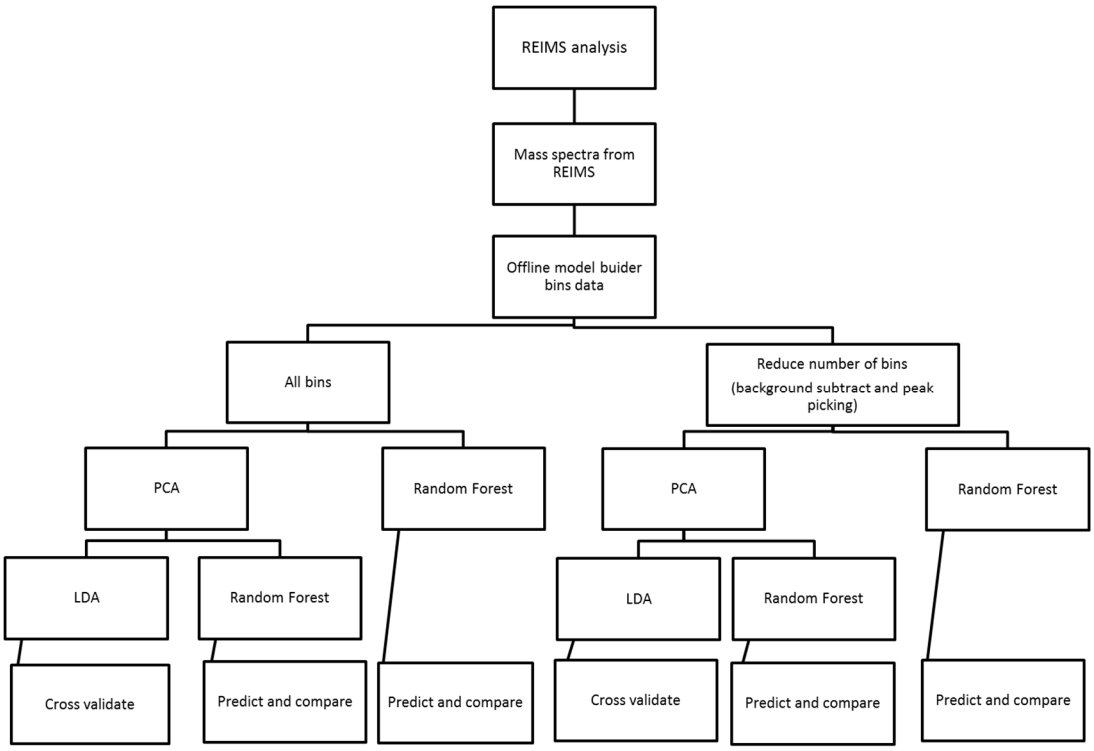


Figure S6.1. Comparison workflow for optimisation of data analysis methods using laboratory housed rodent data. MatLAB code for peak picking written by Dr J. Harris, University of Liverpool.

For all analyses, mass spectra were imported into Waters Offline Model Builder software (version 1.1.28; Waters, UK). Within the Offline Model Builder spectral data above the intensity threshold of 3 x10^5 were summed for each data point, this comprised of up to 3 faecal pellets from the same animal. Mass spectra were then lockmass corrected to an IPA

background peak at 325.19 *m/z*. The resulting spectra were limited to a range of 400 to 1100 *m/z*, normalised, scaled and binned by the Offline Model Builder at 0.1 Da bin width. Binned data was exported from the Offline Model Builder as .csv data files.

The first studies on REIMS used principal component analysis on all bins of the data, followed by linear discriminant analysis (LDA; Balog *et al.*, 2010; Schaefer *et al.*, 2009). Therefore, this was the first analysis tool we used. Principal component analysis was run with no rotation. All components with an Eigenvalue greater than 1 were extracted and saved as variables. All the resulting principal components were classified by stepwise DFA using the Wilks' lambda method with an F value entry of 3.84 and removal of 2.71. Prior probabilities were computed from group sizes and leave-one-out classification was performed to determine the accuracy of the model. The results of the stepwise DFA indicated that the top 12 principal components were providing the greatest contribution to the classification. Non-stepwise DFA was run on the top 12 principal components with leave-one out classification.

During examination of the data, we found that many bins that were important in PCA classification had a low intensity. In an attempt to analyse higher intensity peaks, data were subjected to further background subtraction and peak picking using in-house MatLAB code (version R2015a; The MathWorks, Inc., Massachusetts, USA). An average spectrum was produced from the total data matrix exported from OMB. From this average spectrum a background intensity threshold of a bin was calculated as twice the mean intensity of a 100 bin window surrounding the bin in question. The background intensity threshould was extrapolated for the first and last 50 bins. The in-built MatLAB function "findpeaks" was used and any peak below the threshold was discarded. This led to a reduced number of bins in the data (approximately 500). The resultant bins were analysed by PCA, followed by DFA on the top 12 principle components.

Bolt *et al.* (2016) found that Random Forest analysis produced greater classification accuracy than LDA for REIMS data. We used Random Forest to analyse laboratory animal data based on 1000 trees and the square root of the total number of variables available for splitting at each tree node. Accuracy was calculated as 100 minus the out of bag error rate. We also ran Random Forest on the top 12 principal components extracted from our original analysis to see if this improved classification accuracy. We then used Random Forest to analyse our MatLAB peak picked data and the top 12 principal components from the PCA analysis of the peak picked data.

We were also concerned that due to the high number of variables, there was a risk that the data analysis would be able to accurately categorise even a random variable. To test this hypothesis, we generated a random variable with five categories (as for the laboratory animal species variable) three times. We subjected the three random variables to the full data analysis run described above (Table S6.2).

As can be seen from Table S6.2, Random Forest on all binned data produced the greatest accuracy in classification of laboratory species. Therefore, random forest analysis on all bins of the data was used for analysis of all other data.

PCA and DFA analysis were conducted in SPSS version 24 (IBM, UK). Random Forest analysis was conducted in R version 3.4.0. (R Core Team, 2016) with packages 'openxlsx' (Walker, 2017), 'randomForest' (Liaw & Wiener, 2002) and 'ggplot2' (Wickham, 2009).

Table S6.2. Comparison of REIMS analysis methods. In variables included "All" refers to all binned data from the offline model builder (7001 variables), "Peak picking" refers to the reduced dataset processed in MatLAB (557 variables). Accuracy on species classification is ability to classify 5 species groups (house mouse, wood mouse, bank vole, field vole, Brown Norway x Wistar rat). Accuracy on random data is overall ability to classify a random set of 5 groups (the randomisation was performed 3 times and the table displays the mean of these 3 sets).

Variables included	Analysis method	Accuracy on species classification (%)	Accuracy on random data (%)
All	PCA/DFA	69.1	16.6
	Random Forest	**83.0**	**13.1**
	PCA/Random Forest	72.3	20.9
Peak picking	PCA/DFA	79.8	16.3
	Random Forest	80.9	16.7
	PCA/Random Forest	78.7	19.9

MatLAB code for peak picking written by Dr J. Harris, University of Liverpool.

6.6.2.2. Selection of mass range

For selection of analysis technique, a mass range of 400 to 1100 *m/z* was initially used as limiting the mass range removed peaks from propan-2-ol and noise peaks. To confirm that this mass range gave the highest classification accuracy, we exported the full mass range (50 to 1200 *m/z*) from the Offline Model Builder. We subjected the full mass range, and a selection of mass range subsets, to random forest analysis (Table S6.3). The highest classification accuracy was obtained for a mass range of 400-1100 *m/z*. Therefore, we used this mass range for all subsequent analyses.

As the Offline Model Builder scales spectra after mass range selection, classification accuracy varies depending on whether the mass range is limited before or after export from the Offline Model Builder. We obtained a higher classification accuracy if we limited the mass range within the Offline Model Builder (83.0%), compared to limiting the mass range following export of the full spectra (78.8%). Therefore, for all subsequent analyses a mass range of 400-1100 *m/z* was selected within the Offline Model Builder prior to exporting binned spectra.

Table S6.3. Random Forest classification accuracy at different mass ranges.

Mass range (*m/z*)	Classification accuracy (%)
50 - 1200	73.4
200-1200	76.6
300-1200	77.7
300-1100	73.4
400-1200	75.0
400-1100	**78.7**
400-1000	77.7
500-1100	76.6
600-1000	71.3
600-900	72.3

6.7. References

Alasaad, S., Sanchez, A., Marchal, J. A., Piriz, A., Garrido-Garcia, J. A., Carro, F., Romero, I. & Soriguer, R. C. (2011). Efficient identification of *Microtus cabrerae* excrements using noninvasive molecular analysis. *Conservation Genetics Resources.* **3**, 127-129.

Animals (Scientific Procedures) Act. (1986). UK.

Association for the Study of Animal Behaviour (2018). Guidelines for the treatment of animals in behavioural research and teaching. *Animal Behaviour.* **135**, I-X.

Balog, J., Szaniszlo, T., Schaefer, K.-C., Denes, J., Lopata, A., Godorhazy, L., Szalay, D., Balogh, L., Sasi-Szabo, L., Toth, M. & Takats, Z. (2010). Identification of Biological Tissues by Rapid Evaporative Ionization Mass Spectrometry. *Analytical Chemistry.* **82**, 7343-7350.

Balog, J., Sasi-Szabo, L., Kinross, J., Lewis, M. R., Muirhead, L. J., Veselkov, K., Mirnezami, R., Dezso, B., Damjanovich, L., Darzi, A., Nicholson, J. K. & Takats, Z. (2013). Intraoperative Tissue Identification Using Rapid Evaporative Ionization Mass Spectrometry. *Science Translational Medicine.* **5**, 1-11.

Balog, J., Kumar, S., Alexander, J., Golf, O., Huang, J., Wiggins, T., Abbassi-Ghadi, N., Enyedi, A., Kacska, S., Kinross, J., Hanna, G. B., Nicholson, J. K. & Takats, Z. (2015). In Vivo Endoscopic Tissue Identification by Rapid Evaporative Ionization Mass Spectrometry (REIMS). *Angewandte Chemie-International Edition.* **54**, 11059-11062.

Balog, J., Perenyi, D., Guallar-Hoyas, C., Egri, A., Pringle, S. D., Stead, S., Chevallier, O. P., Elliott, C. T. & Takats, Z. (2016). Identification of the Species of Origin for Meat Products by Rapid Evaporative Ionization Mass Spectrometry. *Journal of Agricultural and Food Chemistry.* **64**, 4793-4800.

Barbosa, S., Pauperio, J., Searle, J. B. & Alves, P. C. (2013). Genetic identification of Iberian rodent species using both mitochondrial and nuclear loci: application to noninvasive sampling. *Molecular Ecology Resources.* **13**, 43-56.

Black, C., Chevallier, O. P., Haughey, S. A., Balog, J., Stead, S., Pringle, S. D., Riina, M. V., Martucci, F., Acutis, P. L., Morris, M., Nikolopoulos, D. S., Takats, Z. & Elliott, C. T. (2017). A real time metabolomic profiling approach to detecting fish fraud using rapid evaporative ionisation mass spectrometry. *Metabolomics.* **13**, 1-13.

Bolt, F., Cameron, S. J. S., Karancsi, T., Simon, D., Schaffer, R., Rickards, T., Hardiman, K., Burke, A., Bodai, Z., Perdones-Montero, A., Rebec, M., Balog, J. & Takats, Z. (2016). Automated High-Throughput Identification and Characterization of Clinically Important Bacteria and Fungi using Rapid Evaporative Ionization Mass Spectrometry. *Analytical Chemistry.* **88**, 9419-9426.

Cameron, S. J. S., Bolt, F., Perdones-Montero, A., Rickards, T., Hardiman, K., Abdolrasouli, A., Burke, A., Bodai, Z., Karancsi, T., Simon, D., Schaffer, R., Rebec, M., Balog, J. & Takáts, Z. (2016). Rapid Evaporative Ionisation Mass Spectrometry (REIMS) Provides Accurate Direct from Culture Species Identification within the Genus Candida. **6**, 1-10.

Campbell, J., Mullen, M. & Dowdy, A. (2002). Monitoring stored-product pests in food processing plants with pheromone trapping, contour mapping, and mark-recapture. *Journal of Economic Entomology.* **95**, 1089-1101.

Cooks, R. G., Ouyang, Z., Takats, Z. & Wiseman, J. M. (2006). Ambient mass spectrometry. *Science.* **311**, 1566-1570.

Directive 2010/63/EU. (2010). EU.

Eggert, L. S., Eggert, J. A. & Woodruff, D. S. (2003). Estimating population sizes for elusive animals: the forest elephants of Kakum National Park, Ghana. *Molecular Ecology.* **12**, 1389-1402.

Engeman, R. M. & Witmer, G. W. (2000) IPM strategies: indexing difficult to monitor populations of pest species. In T.P. Salmon & A.C. Crabb, Eds. *Proceedings of the Nineteenth Vertebrate Pest Conference, San Diego*. University of California Davis. pp. 183-189.

Farrell, L. E., Romant, J. & Sunquist, M. E. (2000). Dietary separation of sympatric carnivores identified by molecular analysis of scats. *Molecular Ecology.* **9**, 1583-1590.

Galan, M., Pages, M. & Cosson, J. F. (2012). Next-Generation Sequencing for Rodent Barcoding: Species Identification from Fresh, Degraded and Environmental Samples. *PLOS ONE.* **7**, 1-13.

Gibbs, J. P., Snell, H. L. & Causton, C. E. (1999). Effective monitoring for adaptive wildlife management: lessons from the Galapagos Islands. *Journal of Wildlife Management.* **63**, 1055-1065.

Glen, A. S. & Dickman, C. R. (2003). Monitoring bait removal in vertebrate pest control: a comparison using track identification and remote photography. *Wildlife Research.* **30**, 29-33.

Glennon, M. J., Porter, W. F. & Demers, C. L. (2002). An alternative field technique for estimating diversity of small-mammal populations. *Journal of Mammalogy.* **83**, 734-742.

Goodrich, B. S., Gambale, S., Penncuik, P. & Redhead, T. D. (1990a). Volatile compounds from excreta of laboratory mice (*Mus musculus*). *Journal of Chemical Ecology.* **16**, 2107-2120.

Goodrich, B. S., Gambale, S., Pennycuik, P. & Redhead, T. D. (1990b). Volatiles from feces of wild male house mice. *Journal of Chemical Ecology.* **16**, 2091-2106.

Hadinger, U., Haymerle, A., Knauer, F., Schwarzenberger, F. & Walzer, C. (2015). Faecal cortisol metabolites to assess stress in wildlife: evaluation of a field method in free-ranging chamois. *Methods in Ecology and Evolution.* **6**, 1349-1357.

Hansen, M. M. & Jacobsen, L. (1999). Identification of mustelid species: otter (*Lutra lutra*), American mink (*Mustela vison*) and polecat (*Mustela putorius*), by analysis of DNA from faecal samples. *Journal of Zoology.* **247**, 177-181.

Hansson, L. (1971). Small Rodent Food, Feeding and Population Dynamics - Comparison between Granivorous and Herbivorous Species in Scandinavia. *Oikos.* **22**, 183-198.

Khan, M. Z., Altmann, J., Isani, S. S. & Yu, J. (2002). A matter of time: evaluating the storage of fecal samples for steroid analysis. *General and Comparative Endocrinology.* **128**, 57-64.

Liaw, A. & Wiener, M. (2002). *Classification and Regression by randomForest*. https://cran.r-project.org/package=randomForest

Mason, G. & Littin, K. E. (2003). The humaneness of rodent pest control. *Animal Welfare.* **12**, 1-37.

Meek, P. D., Vernes, K. & Falzon, G. (2013). On the reliability of expert identification of small-medium sized mammals from camera trap photos. *Wildlife Biology in Practice.* **9**, 7-19.

Mostl, E., Maggs, J. L., Schrotter, G., Besenfelder, U. & Palme, R. (2002). Measurement of cortisol metabolites in faeces of ruminants. *Veterinary Research Communications.* **26**, 127-139.

NC3Rs, BBSRC, DEFRA, MRC, NERC & Wellcome Trust. (2017). Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. NC3Rs, London.

Nelson, J. T., Woodworth, B. L., Fancy, S. G., Lindsey, G. D. & Tweed, E. J. (2002). Effectiveness of Rodent Control and Monitoring Techniques for a Montane Rainforest. *Wildlife Society Bulletin.* **30**, 82-92.

R Core Team. (2016). *R: A language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

Russell, J. C., Hasler, N., Klette, R. & Rosenhahn, B. (2009). Automatic track recognition of footprints for identifying cryptic species. *Ecology.* **90**, 2007-2013.

Schaefer, K.-C., Denes, J., Albrecht, K., Szaniszlo, T., Balog, J., Skoumal, R., Katona, M., Toth, M., Balogh, L. & Takats, Z. (2009). In Vivo, In Situ Tissue Analysis Using Rapid Evaporative Ionization Mass Spectrometry. *Angewandte Chemie-International Edition.* **48**, 8240-8242.

Strittmatter, N., Rebec, M., Jones, E. A., Golf, O., Abdolrasouli, A., Balog, J., Behrends, V., Veselkov, K. A. & Takats, Z. (2014). Characterization and Identification of Clinically Relevant Microorganisms Using Rapid Evaporative Ionization Mass Spectrometry. *Analytical Chemistry.* **86**, 6555-6562.

Taberlet, P. & Fumagalli, L. (1996). Owl pellets as a source of DNA for genetic studies of small mammals. *Molecular Ecology.* **5**, 301-305.

Verplanken, K., Stead, S., Jandova, R., Van Poucke, C., Claereboudt, J., Vanden Bussche, J., De Saeger, S., Takats, Z., Wauters, J. & Vanhaecke, L. (2017). Rapid evaporative ionization mass spectrometry for high-throughput screening in food analysis: The case of boar taint. *Talanta.* **169**, 30-36.

Waits, L. P. & Paetkau, D. (2005). Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *Journal of Wildlife Management.* **69**, 1419-1433.

Walker, A. (2017). *openxlsx: Read, Write and Edit XLSX Files*. https://CRAN.R-project.org/package=openxlsx

Whisson, D. A., Engeman, R. M. & Collins, K. (2005). Developing relative abundance techniques (RATs) for monitoring rodent populations. *Wildlife Research.* **32**, 239-244.

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, New York. http://ggplot2.org

Witmer, G. W. (2007). *The ecology of vertebrate pests and integrated pest management (IPM).* USDA National Wildlife Research Center-Staff Publications, pp. 393-410. University of Nebraska, U.S.A.

Yu, X., Wang, J., Kays, R., Jansen, P. A., Wang, T. & Huang, T. (2013). Automated identification of animal species in camera trap images. *Eurasip Journal on Image and Video Processing.* **52**, 1-10.

Chapter 7

7. Discussion

The aim of this thesis was to examine ways to reduce the exposure of non-target rodents to rodenticides. To accomplish this aim I tested rat odour as a non-target repellent and 50 kHz rat calls as a rat attractant. In addition, I tested rapid evaporative ionisation mass spectrometry (REIMS) as a monitoring method for target and non-target rodents. From the results of these investigations the following conclusions can be drawn. First, rat odour reduces the amount of non-toxic bait consumed by non-target rodents when tested in semi-natural enclosures and in natural conditions. In semi-natural enclosures a reduction in median bait take of over 60% was consistently achieved, whereas in the field a reduction of 31% was achieved. Some progress has been made towards isolating the chemical components responsible for this repellency. Further work is needed to confirm which components of rat odour cause repellency and to optimise the repellent effects of rat odour in the field. Second, rats are attracted to 50 kHz rat calls, when combined with rat movement sounds. There is no sex specificity in this response. There is no evidence, so far, that non-target rodents are attracted or repelled by 50 kHz rat calls. Third, REIMS is capable of identifying wild rodent species from faecal samples and has the potential to be applied to pest control settings, as well as to conservation and ecological research.

In this discussion I will consider the benefits of using rat odour and rat vocalisations to reduce the exposure of non-target rodents to rodenticides and the issues involved in this potential application, particularly focusing on the behavioural aspects of using these cues. I will then consider the benefits and issues of using REIMS as a monitoring technique for rodents. I will then discuss when and how rat odour, rat vocalisations and REIMS could be applied to pest control, including practical implementation, legislation and costs. Finally, I will suggest future work that will be required before these solutions can be applied in pest control settings.

7.1. Benefits and Issues

7.1.1. Rat odour

There are several potential benefits to the use of rat odour as a non-target repellent. One of the benefits is that, theoretically, selection pressure favours continued avoidance of rat odour. A non-target rodent that avoids rat odoured bait boxes will avoid consuming rat poison, and will avoid the fitness and survival costs consumption of rat poison entails. A non-target rodent that does not avoid rat odour and consumes rat poison will incur fitness costs from consumption of the poison, and may be killed. Therefore, there is a fitness benefit to

avoidance of rat odoured bait boxes containing poison baits. In a sense, the presence of rat odour may encourage non-target rodents to develop behavioural resistance to consumption of rat poisons, as has naturally developed in pest species (Clapperton, 2006; REX Consortium, 2013). However, there may be short term fitness benefits to consumption of a highly valuable food source, particularly during the breeding season or when food is scarce, that may give a slight fitness benefit to consuming small amounts of poisoned baits. Due to the high fitness costs of consuming poison, the former pressure may exceed the latter. However, the complexities of opposing selection pressures can make behavioural phenotypes difficult to predict (Schluter *et al.*, 1991), so it will be important to consider both possibilities.

Another benefit of using rat odour to repel non-target rodents is the lack of habituation observed across multiple experiments. In Chapters 2 and 3, I found that, despite repeated testing, non-target rodents did not habituate to the presence of rat odour and were still repelled after repeated exposure. This is in line with previous studies that found little evidence of habituation to predator odours after repeated exposure (Apfelbach *et al.*, 2005; Borowski, 1998; File *et al.*, 1993; Wallace & Rosen, 2000). However, the evidence is conflicting as some studies have found habituation to odour cues, whilst others have found habituation in some measured parameters, but not others (Dielenberg & McGregor, 1999; Hegab *et al.*, 2014; Mashoodh *et al.*, 2008; Takahashi *et al.*, 2005). For example, rats will consistently avoid cat body odour after repeated exposures, but freezing and grooming behaviours return to normal levels (File *et al.*, 1993; Mashoodh *et al.*, 2008; Takahashi *et al.*, 2005). In addition, exposure to rat odour during pest control may occur for longer periods than tested in Chapters 2 and 3, particularly where rat bait boxes are used permanently. Thus, it may be necessary to test for habituation to rat odour over extended periods and to examine behavioural responses of non-target rodents more closely after repeated exposure to rat odour.

Aside from the benefits of using rat odour as a non-target repellent there are some concerns with its use. First, the signalling complexity of mammals compared to other pests, such as invertebrates, make it difficult to create effective repellents and / or attractants (Christiansen, 1976). In invertebrates it is often possible to use single compounds that have a strong, innate effect on behaviour (Witzgall *et al.*, 2010). In mammals the search for such compounds has proven extremely difficult (Christiansen, 1976; Clapperton, 2006). Cues require to be presented at the correct concentration to be effective (Inagaki *et al.*, 2014; Zhang *et al.*, 2008). In addition, multiple compounds may be required (Inagaki *et al.*, 2014;

Osada *et al.*, 2013). This makes the synthesis of behaviour modifying mixtures extremely difficult. The finding that bank voles avoid 4-ethyl phenol, but wood mice do not, suggests a different mixture of compounds may be required for each non-target rodent species, further complicating this issue.

A second concern for the use of rat odour as a non-target repellent is that it does not fully eliminate consumption of bait by non-target rodents. In Chapters 2, 3 and 4 I found that whilst rat odour is repellent to non-target rodents, they are not completely repelled by it, and still consume bait from bait boxes. This was particularly the case for field experiments where the maximum reduction in overall bait consumption was 26%. Therefore, if the rat odour used in these studies was used as a non-target repellent, residues of rat poisons would still get into the environment, all be it at a reduced level. It may be possible to increase the effectiveness of rat odour as a non-target repellent by optimising the context of its presentation and isolating the most effective components of the odour cue, as discussed in Chapters 3 and 4. However, due to the issues discussed above, this may be difficult to achieve in mammals and it is unlikely that full repellency would be achieved, as predator odours are rarely completely repellent to prey species (Apfelbach *et al.*, 2005; Hegab *et al.*, 2015). Thus, whilst the use of rat odour may significantly reduce consumption of rat poison by non-target rodents, full repellency is unlikely to be achievable.

A third concern is that, whilst rat odour repels non-target rodents, its effects have not been tested on other types of primary non-target species, such as birds and invertebrates. Rats are potential predators of some bird and invertebrate species (Jones *et al.*, 2008; Major *et al.*, 2007; Miller & Miller, 1995; Woodward, 1972). In addition, birds and invertebrates have been shown to avoid predator odours (Amo *et al.*, 2008; Armsworth *et al.*, 2005; Barnes *et al.*, 2002). However, the response of these animals to rat odour is unknown. Therefore, further testing will be needed to determine if rat odour is repellent to birds and invertebrates that may consume rat poisons. If rat odour is not repellent to these species, another repellent or other means will need to be found to reduce their exposure to rodenticides.

A final concern with the use of rat odour as a non-target repellent is the potential for unintended effects on non-target rodents. Exposure to predator odour has been shown to affect habitat use and to supress reproduction in some species (Apfelbach *et al.*, 2005; Parsons *et al.*, 2018; Ylönen & Ronkainen, 1994). It may be necessary to test for these effects on non-target species if rat odour is used as a non-target repellent. I have shown that the

repellent effect of rat odour is confined to the box it is presented in. By confining the use of rat odour to bait boxes, unintended non-target effects may be minimised, but this should be tested.

Overall, rat odour has the potential to substantially reduce the consumption of bait by non-target rodents. Non-target rodents do not appear to habituate to rat odour and selection pressure favours continued avoidance of this cue. However, rat odour does not eliminate bait consumption by non-target rodents and its efficacy is unknown with regard to other non-target species, such as birds and invertebrates. In addition, manufacture of a synthetic cue is complicated by mammalian signalling complexity and rat odour may cause unintended side effects in non-target species. These concerns do not preclude the use of rat odour as a non-target repellent, but they require careful consideration and complicate its development and use.

7.1.2. Rat vocalisations

Moving on to consider 50 kHz rat calls as a species specific rat attractant, there are several potential benefits and issues associated with its application to influence the behaviour of rats. One of the main potential benefits is that 50 kHz rat calls may be able to attract rats to control measures that do not rely on the use of poison baits. If this was the case, it may be possible to remove or reduce the use of poisons, which would decrease the potential for non-target poisoning. However, due to natural variation and flexibility in rat behaviour (Macdonald & Fenn, 1994; Wöhr *et al.*, 2008), it is unlikely that all rats will be equally attracted to these calls. Therefore, 50 kHz rat calls may not provide an effective attractant on its own. A combination of control measures and attractants, including poisons and the use of 50 kHz calls to attract rats to alternative control devices, may have the potential to be effective at controlling rats, whilst reducing the use of poisons. Improvements in the efficacy of rat control by using 50 kHz calls may have the additional benefit of eliminating, rather than reducing the number of, rats from a locality. This would reduce the requirement for continued exposure to poisons and other lethal devices that endanger non-target species.

One of the concerns with using 50 kHz rat calls as an attractant is the potential for the development of resistance. As opposed to rat odour effects on non-target rodents, where repellent effects are beneficial to fitness and so are against resistance development, attraction to 50 kHz rat calls will lead rats to control measures. Therefore, rats that are not attracted to 50 kHz calls will have a fitness advantage, encouraging the development of

resistance (REX Consortium, 2013). The potential to develop resistance to approach of 50 kHz calls is further complicated in rats by their high innate levels of adaptability and flexibility (Macdonald & Fenn, 1994). This means that individual rats can quickly modify their behaviour to accommodate changes in the environment, allowing fast development of behavioural resistance (Macdonald & Fenn, 1994). Very little is currently known about the use of 50 kHz calls by wild rats and how important their use is to survival (Portfors, 2007). The more important a cue is for survival, the greater the fitness costs will be of developing resistance to normal behavioural responses to that cue (Cook *et al.*, 2007; Kliot & Ghanim, 2012). Therefore, understanding the importance of 50 kHz calls in wild rat behaviour may help to gauge how fast resistance is likely to develop.

An additional concern with the current knowledge of rat responses to 50 kHz rat calls is that the response of wild rats to these calls is unknown (Portfors, 2007). Wild rats are attracted to ultrasonic infant rat vocalisations in field conditions (Takács *et al.*, 2016), indicating that there is potential for them to be attracted to 50 kHz calls. However, this needs to be tested. Field tests of 50 kHz vocalisations and studies on the use of 50 kHz vocalisations by wild rats would help to assess the likely usefulness of 50 kHz calls as a species specific rat attractant.

7.1.3. REIMS

There are several potential benefits and issues of REIMS as a monitoring method for rodents, and other species. One of the main benefits of REIMS is rapidity of sample analysis (Verplanken *et al.*, 2017). Samples do not require any preparation prior to analysis, except the simple addition of water in the case of faecal samples (Balog *et al.*, 2010; Cameron *et al.*, 2016; Chapter 6). Mass spectral acquisition and processing can be done in under one minute for each sample (Verplanken *et al.*, 2017; Chapter 6). This rapid, high throughput data analysis is in sharp contrast to the time consuming faecal analysis techniques currently in use to identify species, such as DNA and diet composition analysis (Taberlet & Fumagalli, 1996; Waits & Paetkau, 2005).

A further benefit of REIMS is the use of faeces as the source of species identification. Faeces samples require relatively little effort to collect, compared to other identification methods (Barbosa *et al.*, 2013). In addition, they are not invasive to obtain, reducing welfare issues associated with monitoring wild animals (Waits & Paetkau, 2005). An extra benefit of REIMS is the potential to include large numbers of species as potential candidates in its database (Bolt *et al.*, 2016; Strittmatter *et al.*, 2014). This means that it may be possible to identify

non-target species from a diverse range of taxa before the instigation of rat control campaigns, including rodents, birds and invertebrates.

The main practical issue with REIMS is the requirement for a large database of samples to ensure high accuracy when classifying unknown samples (Chapter 6). However, when collecting samples for REIMS analysis, I was able to build up a large database relatively quickly. For use of REIMS in pest control, there is good potential to gather the data necessary to create a database for most species, particularly non-target rodents, which are often abundant in their natural habitats.

7.2. Implementation

7.2.1. Application of rat odour and rat vocalisations

With further research, rat odour and 50 kHz rat calls have the potential to be used to reduce the exposure of non-target rodents to rodenticides. However, careful consideration is needed to ensure they are used appropriately and effectively. One of the main considerations is when and where each cue should be applied in a pest control setting. As a non-target repellent, the use of rat odour will be best targeted to where non-target rodents are likely to be found. It is generally assumed that the majority of non-target poisoning takes place when rat poisons are placed away from buildings (Elmeros *et al.*, 2018; Health and Safety Executive, 2012). This is logical as wood mice, bank voles and field voles are most often found in farmland and woodland habitats and are not common in urban areas (Ashby, 1967; Gorman *et al.*, 1993; Green, 1979; Harris *et al.*, 1995; Ivanter *et al.*, 2013). However, poisoning of non-target rodents close to buildings may also be an important route of non-target exposure to rat poisons (Elmeros *et al.*, 2018; Tosh *et al.*, 2012). Therefore, it may be necessary to apply rat odour to all bait stations placed outside of buildings. To use rat odour more efficiently, surveys for the presence of non-target rodents could be undertaken prior to the start of rat control campaigns. Potentially, REIMS could be used for this purpose (see below), allowing targeted use of rat odour as a non-target repellent where it is most needed.

A further consideration for the use of rat odour during pest control, is how to optimise its effectiveness. It may be possible to increase the repellent effects of rat odour by adding an attractant in another location to pull non-target rodents away from poisons. This strategy is known as push-pull and has been used effectively to control insect pests (Cook *et al.*, 2007; Menger *et al.*, 2014). The concept involves the use of a repellent cue to push an insect away from a food crop, and an attractant to pull it towards a control measure (Cook *et al.*, 2007).

Combining cues in this way can increase the efficacy of weak attractants and repellents (Cook *et al.*, 2007). It is also possible to slow the development of resistance using this technique as resistance has to develop to multiple cues, rather than a single cue (Cook *et al.*, 2007). For non-target rodents one potential push-pull technique may be to add rat odour to bait boxes to push non-target rodents away from poisons and then provide an attractant to further discourage them from consuming poisoned bait. Previous studies have found that wood mice will avoid consuming sugar beet seeds if provided with an alternative food source (Harris, 1989; Pelz, 1989). The provision of a high quality food source in an adjacent compartment of a bait box, not scented with rat odour, may attract non-target rodents away from baits. If this compartment was inaccessible to rats (by using a small entrance) it would not deter rats from consuming bait. This strategy has the potential to increase the efficacy of rat odour in reducing the consumption of bait by non-target rodents.

As the purpose of 50 kHz rat calls during pest control would be to attract rats, rather than repel non-target rodents, they may be used in different situations. 50 kHz calls may be of use to attract rats wherever rat control is needed. However, due to the concerns over resistance development stated above, it may be wiser to use 50 kHz rat calls using a more targeted approach. There is often concern that rats avoid poisons and traps due to behavioural resistance (Campaign for Responsible Rodenticide Use UK, 2015; Clapperton, 2006). It may be possible to use 50 kHz rat calls in situations where behavioural resistance is suspected, as a way to encourage rats to enter bait boxes or traps. As 50 kHz calls are a sound cue, they may not be of use in noisy environments, such as industrial plants. However, in quieter conditions, such as domestic houses and farm buildings, they may be more efficacious. Further research will be needed to establish how much background noise influences the effectiveness of 50 kHz calls as a rat attractant. 50 kHz calls have the advantage of occurring outside the range of human hearing (Ashihara, 2007) and, therefore, may be used in close proximity to human activity without causing a disturbance. As there is no current evidence that 50 kHz rat calls are attractive to non-target rodents (Chapter 5), it may be possible to use them to attract rats to pest control devices that do not rely on poisons. This may be particularly useful where rat control is needed in areas of high non-target rodent density, such as farmland. Overall, targeted application of 50 kHz rat calls may have the benefit of improving rat control efficiency and reducing non-target exposure to poisons, whilst minimising the risk of resistance development.

As well as being used alone, rat odour and 50 kHz rat calls could be used in combination, either with each other or other control methods. The combination of rat odour and 50 kHz rat calls has good potential as a species specific cue, being attractive to rats and repellent to non-target rodents. The addition of other control techniques, such as trapping, may reduce the need for poisons considerably, or even completely. Combining pest control techniques, also called integrated pest management, has been found to be highly effective in controlling pests and is often more economical than using a single control method (Brenner *et al.*, 2003; Singleton *et al.*, 2005; Witmer, 2007). One of the facets of this type of control is tailoring control to the individual situation (Brenner *et al.*, 2003). Control of a small number of pest rats in a building, where it may be possible to prevent reinfestation by rodent-proofing, will require a different strategy to a heavy infestation in farmland where constant reinfestation is unavoidable (Mason & Littin, 2003; Singleton *et al.*, 2005). However, combining control techniques can be more complex and less practical than using single control measures, limiting usage (Cook *et al.*, 2007). Careful consideration of practicalities of using either cue will be needed to promote an integrated approach to control.

So far I have focussed on the use of rat odour and 50 kHz rat calls in pest control in developed countries, particularly the UK where wood mice, bank voles and field voles are not considered as pests (Harris, 1989; Richards, 1989). In other countries, it may not be feasible, or even desirable to use these cues as they would be used in the UK. For example, in some European countries voles are considered as pests (Capizzi *et al.*, 2014), in these areas it would not be desirable to use rat odour in bait boxes as it may deter target pests. Control of pests on island habitats is a major problem, particularly due to the risk pests pose to endemic species (Buckle & Fenn, 1992; Hilton & Cuthbert, 2010). Many islands have inaccessible areas, so poison bait is often distributed by aerial broadcast, without the use of bait boxes (Holmes *et al.*, 2015). This precludes the use of rat odour and 50 kHz rat calls in their current formats, as both require the use of enclosed bait boxes. In developing countries pest rats can cause devastating impacts on crops, particularly rice crops in Asia (Singleton *et al.*, 1999; Singleton, 2003; Singleton, 2010). Here, it may be possible to use 50 kHz rat calls to attract rats to control measures such as traps, although cost is likely to be an issue.

Although rat odour and 50 kHz rat calls may not be useful in all countries to reduce the exposure or non-target rodents to rat poisons, there is the potential to apply the general principles used to develop these cues internationally. The main principle used to identify rat odour and 50 kHz rat calls as species specific attractants or repellents was to identify

differences in the ecology and behaviour of target and non-target rodents. This required a good understanding of the basic ecology and behaviour of each species involved. This understanding is essential for many aspects of pest control, as well as non-target poisoning mitigation (Capizzi *et al.*, 2014; Singleton *et al.*, 1999). For non-target protection, knowledge of the ecology and behaviour of all species involved has the potential to lead to identification of communication channels that have the potential to be exploited either as attractants and / or as repellents. It may then be possible to find cues that cause differential behavioural responses between different species that could be used to reduce non-target poisoning.

7.2.2. Costs

An important consideration in the use of attractants and repellents in pest control is their cost. Costs must be weighed against the potential benefits derived from the use of attractants and repellents (Cook *et al.*, 2007). Benefits will relate to level of efficacy and consequences of not using the cue in question. Proven, quantifiable benefits will make this analysis much simpler. Costs must be considered, both in terms of how much these cues cost to manufacture, but also how much they cost to apply (Menger *et al.*, 2014). Cues that are cheap to produce, but require multiple applications, may be more expensive overall than cues that are expensive to manufacture, but only require one application. Manufacturing costs must take into account development, legalities and production (Cook *et al.*, 2007). Ideally, cues should require little development, be easy to protect legally and be simple and cheap to produce. The costlier a cue is, both in terms of manufacture and application, the less likely it is to be taken up by industry (Cook *et al.*, 2007).

Currently, it is difficult to calculate the costs of using rat odour as a non-target repellent. It appears that non-sex specific odour compounds are responsible for repelling non-target rodents, but the specific compounds involved have not been determined (Chapter 3). It may be that a combination of compounds is required. The costs required to identify these compounds may vary, depending on the complexity of the effective cue (Cook *et al.*, 2007). If these compounds can be identified, and it is possible to manufacture a synthetic cue that is as effective as rat odour, application of the cue must then be considered. Rat bait boxes are often checked at intervals of up to two weeks (Campaign for Responsible Rodenticide Use UK, 2015; Tosh *et al.*, 2011). Increasing the frequency of checks required will increase labour costs of rodent control, which is likely to be resisted by the pest control industry (Health and Safety Executive, 2012). Therefore, a non-target repellent will need to last at

least several days, and ideally up to two weeks, to be useful. As volatile odour compounds can evaporate relatively quickly (James, 2003), it will not be possible to apply the repellent on its own. To last in the environment, slow release technologies will be required (James, 2003). Such technologies do exist and include slow release gels and other materials that delay release of volatile compounds and can control release at a steady rate (Xu *et al.*, 2018). Provided a synthetic blend can be manufactured cheaply and the blend can be deployed in a slow release mechanism, rat odour has the potential to be relatively cost effective to use.

The costs of applying 50 kHz rat calls to pest control are likely to be more expensive than the application of rat odour. This is due to the technology required to broadcast these calls. 50 kHz calls need to be broadcast from an ultrasound speaker. This speaker would need to be small enough to be compatible with pest control devices. Such a speaker has been developed for broadcast of infant rat vocalisations (Takács *et al.*, 2016). It seems likely that this speaker may only require small modifications to allow it to transmit 50 kHz calls. To use 50 kHz calls combined with non-poison based control measures as the sole or main control method, it may be necessary to use large numbers of these speakers if one is required for each control device. If this is the case, the unit cost of the speakers may be prohibitive to their use. It may be more cost effective to deploy 50 kHz calls as attractants in situations where rats are present at low population densities, or to use them in conjunction with other control methods as described above.

7.2.3. Legislation

There are two main legislative considerations for the use of rat odour and 50 kHz rat calls in pest control. First, it may be possible to protect these cues using intellectual property (IP) law (Friedmann, 2015). Such protection has the benefit of encouraging investment in further development of these cues (Falvey *et al.*, 2006). However, it prevents wide use of these cues and has the potential to drive prices up (Boldrin & Levine, 2005). Volatile odour compounds, or blends of these compounds, can be protected by IP law (Mishra, 2008). This has been done for insect volatile pheromones that are now used in pest control (Sampson & Kirk, 2013). It is possible to protect sounds by IP law, potentially allowing protection of specific recordings of 50 kHz rat calls (Friedmann, 2015; Mishra, 2008).

A second legislative consideration is the use of legislation to ensure that these cues are used. This would not be necessary for 50 kHz rat calls. If they are efficacious and cost effective it is likely industry would adopt their use. However, the use of rat odour as a non-target repellent

is unlikely to increase the efficacy of rat control, unless rat attractive compounds can also be used to repel non-target species. If the main purpose of rat odour, in this context, is to mitigate the damage done by pest control on the environment, pest controllers may be unlikely to adopt its use, without a legislative incentive to make them bear the costs of rat odour application. Currently, the drive to reduce the exposure of non-target rodents to rat poison in the UK has focused on a voluntary stewardship scheme (Campaign for Responsible Rodenticide Use UK, 2015; Health and Safety Executive, 2015). The concept of this scheme is to encourage pest controllers to adopt measures to reduce non-target exposure, without committing these measures to law (Campaign for Responsible Rodenticide Use UK, 2015; Health and Safety Executive, 2015). The measures proposed include avoidance of permanent baiting, a focus on prevention of rodent pest problems before they become established and carrying out risk assessments prior to poison use (Campaign for Responsible Rodenticide Use UK, 2015). This scheme will be monitored by knowledge surveys and checking levels of rat poisons in predatory birds, such as barn owls (Health and Safety Executive, 2015). The incentive to industry is in the consequences of non-compliance with the scheme. If the scheme is not complied with, restrictions will be placed on rat poisons, limiting their use to industry (Health and Safety Executive, 2015). It is hoped that this scheme will reduce the exposure of non-target animals to rat poisons without the need for legislation and policing requirements (Health and Safety Executive, 2015). It may be possible to include the use of rat odour as a non-target repellent in this scheme. This would require proof of the efficacy of rat odour as a non-target repellent. Thus, if rat odour is to be used as a non-target repellent it will require policy backing and industry motivation to ensure it is used successfully. However, the use of 50 kHz rat calls as a rat attractant may only require proof of efficacy and cost effectiveness to encourage use by the pest control industry.

7.2.4. Application of REIMS

The use of REIMS as a tool to reduce the exposure of non-target rodents to rodenticides will require different practical considerations than the use of rat odour and 50 kHz rat calls. The main benefit of REIMS in the reduction of non-target poisoning is to identify the presence of non-target rodents, and other species, prior to the start of rat control campaigns. This may allow targeted use of rat odour and 50 kHz rat calls where they are most likely to be successful. It could also be used after rat control campaigns to check that non-target populations have not been damaged. However, the use of REIMS has the potential to be much broader than restricting it to non-target poisoning. As stated above, REIMS has the

potential to identify large numbers of species (Bolt *et al.*, 2016; Strittmatter *et al.*, 2014). Thus, it could be used to identify pest species, as well as non-target species, and may help to confirm pest populations have been completely removed following control campaigns. REIMS could be applied internationally to many pest control situations, including island habitats and developing countries. The only requirements are for a database of faecal samples of species likely to be found in a particular site, and the availability of necessary equipment. Equipment cost and maintenance is the main barrier to REIMS use. Mass spectrometers are expensive to buy and maintain (Verplanken *et al.*, 2017). However, there is the potential to use REIMS as a central diagnostic service where samples are sent. Such a service is made easier by the lack of effect of sample storage on REIMS classification accuracy (Chapter 6). Rapidity of sample processing means that large numbers of samples can be processed in a day (Verplanken *et al.*, 2017), allowing a diagnostic service to be set up that is capable of analysing large numbers of samples relatively quickly. This high turnover should keep costs down. A recent study suggested that pork samples could be tested by REIMS at less than 1 euro per sample in an abattoir production line (Verplanken *et al.*, 2017). Thus, it may be possible to offer REIMS as a faecal diagnostic service at relatively low cost, with sample results available very quickly. In addition, a REIMS test centre could apply REIMS to a variety of diagnostic requirements, so may not depend solely on requests for faecal diagnostics. Overall, there is good potential for REIMS to be of practical use in a wide variety of pest control settings, both in the UK and internationally.

7.3. Future work

Having established the benefits and issues of rat odour, 50 kHz rat calls and REIMS to reduce the exposure of non-target rodents to wildlife I will now consider what the next stages are in their development.

There is still much work to be done before rat odour can be used during rat control campaigns. In Chapter 4 I demonstrated that rat odour can be effective at reducing the amount of rat poison consumed by non-target rodents in the field. However, further work is needed to increase the level of reduction. This will include optimisation of the context of rat odour presentation, including bait box design, timing of bait placement to take into account seasonality and habitat structure, and length of time bait is left in the environment. The use of a high quality food supplement as part of a push-pull strategy should be trialled to see if this can improve rat odour repellence efficacy. Currently, the use of rat odour would require

a supply of soiled rat bedding. Therefore, further testing is required to identify which compounds in rat odour are responsible for its repellency. From this information, it may be possible to manufacture a synthetic rat odour blend to be used by industry. Legal protection in the form of intellectual property rights should be taken out for such a blend to ensure investment in its development. The pest control industry should be involved at the development stage to ensure any repellent is produced in a form that is practical for them to use. Early involvement of industry will, hopefully, encourage use of rat odour if it is available in a commercially practical format. Completion of this work will, hopefully, move rat odour from a concept to a useful commercial product.

A large amount of further work is needed to get 50 kHz rat calls into a commercial setting. Unlike rat odour, 50 kHz rat calls have not been tested with wild rodents under natural conditions. The first step in establishing if 50 kHz rat calls could be used is to test them with wild rats. This could be tested in semi-natural conditions with a wild population to get an initial indication of their attractive potential in the field. An understanding of how wild rats use these calls may allow an assessment of how and where they are likely to be of most use. This is especially needed as there is almost no knowledge of how wild rats use these calls. Additionally, it will be necessary to develop a speaker that can be used inside bait boxes, or other control measures, to transmit 50 kHz calls. Such speakers have been developed for other studies (Takács *et al.*, 2016), but these may be protected by patent laws. As for rat odour development, collaboration with industry will be necessary to ensure 50 kHz rat calls are practical to use. If the efficacy of 50 kHz calls on wild rodents can be proven, and deployment costs can be minimised, these calls have the potential to be successful rat attractants.

The use of REIMS as a faecal species identification tool should not require a great deal of further development. I have shown that species identification can be done to a high degree of accuracy and is robust to sample storage and test subject diet. However, further samples will be needed to build a more accurate classification model. This includes samples from more locations and additional species, whose faeces may be encountered during rodent control. These species could include invertebrates, birds and bats. Surveys of the pest control industry may provide an insight into what species should be focused on to build up the sample database. From this database, studies should be conducted to test classification accuracy of sites that are not present in the original model, as was done in Chapter 6, demonstrating REIMS effectiveness in novel locations. With this knowledge, it may be

possible to set up a diagnostic service where faecal samples can be sent for identification. There are already multiple examples of REIMS being applied to a commercial setting (Balog *et al.*, 2016; Black *et al.*, 2017; Verplanken *et al.*, 2017). Thus, little work may be required to move REIMS from research to industry use.

In this thesis I have investigated the use of rat odour, 50 kHz rat calls and REIMS to reduce the exposure of non-target animals to rat poisons. I have found that rat odour has good potential to be used as a non-target rodent repellent. I have found that 50 kHz rat calls have potential to be used as rat attractants. In addition, I have found that REIMS shows promise as a monitoring tool for non-target species, as well as other applications in pest control, research and conservation. Much work remains to be done to make these tools commercially viable. I sincerely hope that they can all be further developed to reduce the problems of non-target poisoning and increase the efficacy of rat control.

7.4. References

Amo, L., Galván, I., Tomás, G. & Sanz, J. (2008). Predator odour recognition and avoidance in a songbird. *Functional Ecology.* **22**, 289-293.

Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A. & McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neuroscience & Biobehavioral Reviews.* **29**, 1123-1144.

Armsworth, C., Bohan, D., Powers, S., Glen, D. & Symondson, W. (2005). Behavioural responses by slugs to chemicals from a generalist predator. *Animal Behaviour.* **69**, 805-811.

Ashby, K. R. (1967). Studies on ecology of field mice and voles (*Apodemus sylvaticus, Clethrionomys glareolus* and *Microtus agrestis*) in Houghall wood Durham. *Journal of Zoology.* **152**, 389-513.

Ashihara, K. (2007). Hearing thresholds for pure tones above 16kHz. *The Journal of the Acoustical Society of America.* **122**, EL52-EL57.

Balog, J., Szaniszlo, T., Schaefer, K.-C., Denes, J., Lopata, A., Godorhazy, L., Szalay, D., Balogh, L., Sasi-Szabo, L., Toth, M. & Takats, Z. (2010). Identification of Biological Tissues by Rapid Evaporative Ionization Mass Spectrometry. *Analytical Chemistry.* **82**, 7343-7350.

Balog, J., Perenyi, D., Guallar-Hoyas, C., Egri, A., Pringle, S. D., Stead, S., Chevallier, O. P., Elliott, C. T. & Takats, Z. (2016). Identification of the Species of Origin for Meat Products by Rapid Evaporative Ionization Mass Spectrometry. *Journal of Agricultural and Food Chemistry.* **64**, 4793-4800.

Barbosa, S., Pauperio, J., Searle, J. B. & Alves, P. C. (2013). Genetic identification of Iberian rodent species using both mitochondrial and nuclear loci: application to noninvasive sampling. *Molecular Ecology Resources.* **13**, 43-56.

Barnes, M. C., Persons, M. H. & Rypstra, A. L. (2002). The Effect of Predator Chemical Cue Age on Antipredator Behavior in the Wolf Spider *Pardosa milvina* (Araneae: Lycosidae). *Journal of Insect Behavior.* **15**, 269-281.

Black, C., Chevallier, O. P., Haughey, S. A., Balog, J., Stead, S., Pringle, S. D., Riina, M. V., Martucci, F., Acutis, P. L., Morris, M., Nikolopoulos, D. S., Takats, Z. & Elliott, C. T. (2017). A real time metabolomic profiling approach to detecting fish fraud using rapid evaporative ionisation mass spectrometry. *Metabolomics.* **13**, 1-13.

Boldrin, M. & Levine, D. K. (2005). The economics of ideas and intellectual property. *Proceedings of the National Academy of Sciences of the United States of America.* **102**, 1252-1256.

Bolt, F., Cameron, S. J. S., Karancsi, T., Simon, D., Schaffer, R., Rickards, T., Hardiman, K., Burke, A., Bodai, Z., Perdones-Montero, A., Rebec, M., Balog, J. & Takats, Z. (2016). Automated High-Throughput Identification and Characterization of Clinically Important Bacteria and Fungi using Rapid Evaporative Ionization Mass Spectrometry. *Analytical Chemistry.* **88**, 9419-9426.

Borowski, Z. (1998). Influence of predator odour on the feeding behaviour of the root vole (*Microtus oeconomus*). *Canadian Journal of Zoology.* **76**, 1791-1794.

Brenner, B. L., Markowitz, S., Rivera, M., Romero, H., Weeks, M., Sanchez, E., Deych, E., Garg, A., Godbold, J. & Wolff, M. S. (2003). Integrated pest management in an urban community: a successful partnership for prevention. *Environmental Health Perspectives.* **111**, 1649-1653.

Buckle, A. & Fenn, M. (1992) Rodent control in the conservation of endangered species. In Borrecco, J. E. & Marsh, R. E., Eds. *Proceedings of the Fifteenth Vertebrate Pest Conference, Newport Beach*. University of California. pp. 36-41.

Cameron, S. J. S., Bolt, F., Perdones-Montero, A., Rickards, T., Hardiman, K., Abdolrasouli, A., Burke, A., Bodai, Z., Karancsi, T., Simon, D., Schaffer, R., Rebec, M., Balog, J. & Takáts, Z. (2016). Rapid Evaporative Ionisation Mass Spectrometry (REIMS) Provides Accurate Direct from Culture Species Identification within the Genus Candida. **6**, 1-10.

Campaign for Responsible Rodenticide Use UK (2015). *CRRU UK code of best practice.* pp. 1-24. CRRU UK, Ossett.

Capizzi, D., Bertolino, S. & Mortelliti, A. (2014). Rating the rat: global patterns and research priorities in impacts and management of rodent pests. *Mammal Review.* **44**, 148-162.

Christiansen, E. (1976) Pheromones in small rodents and their potential use in pest control. In Siebe, C. C., Ed. *Proceedings of the Seventh Vertebrate Pest Conference, Monterey*. University of California. pp. 185-195.

Clapperton, B. K. (2006). *A review of the current knowledge of rodent behaviour in relation to control devices.* Department of Conservation, New Zealand.

Cook, S. M., Khan, Z. R. & Pickett, J. A. (2007). The use of push-pull strategies in integrated pest management. *Annual Review of Entomology.* **52**, 375-400.

Dielenberg, R. A. & McGregor, I. S. (1999). Habituation of the hiding response to cat odor in rats (*Rattus norvegicus*). *Journal of Comparative Psychology.* **113**, 376-87.

Elmeros, M., Lassen, P., Bossi, R. & Topping, C. J. (2018). Exposure of stone marten (*Martes foina*) and polecat (*Mustela putorius*) to anticoagulant rodenticides: Effects of regulatory restrictions of rodenticide use. *Science of the Total Environment.* **612**, 1358-1364.

Falvey, R., Foster, N. & Greenaway, D. (2006). Intellectual Property Rights and Economic Growth. *Review of Development Economics.* **10**, 700-719.

File, S. E., Zangrossi Jr, H., Sanders, F. L. & Mabbutt, P. S. (1993). Dissociation between behavioral and corticosterone responses on repeated exposures to cat odor. *Physiology & Behavior.* **54**, 1109-1111.

Friedmann, D. (2015). EU opens door for sound marks: will scent marks follow? *Journal of Intellectual Property Law & Practice.* **10**, 931-939.

Gorman, M. L., Akbarbin, Z. & Ahmad, M. (1993). A comparative study of the ecology of woodmice *Apodemus sylvaticus* in two contrasting habitats: deciduous woodland and maritime sand-dunes. *Journal of Zoology.* **229**, 385-396.

Green, R. (1979). Ecology of wood mice (*Apodemus-sylvaticus*) on arable farmland. *Journal of Zoology.* **188**, 357-377.

Harris, S. (1989). When Is a Pest a Pest. *New Scientist.* **121**, 49-51.

Harris, S., Morris, P., Wray, S. & Yalden, D. (1995). *A review of British mammals: population estimates and conservation status of British mammals other than cetaceans.* pp. 1-183. Joint Nature Conservation Committee, U.K.

Health and Safety Executive (2012). *Environmental risk mitigation measures for second generation anticoagulant rodenticides proposed by the UK* pp. 1-30. Health and Safety Executive, UK.

Health and Safety Executive (2015). *UK anticoagulant rodenticide product authorisation and the CRRU stewardship scheme.* pp. 1-12. Health and Safety Executive, UK.

Hegab, I. M., Shang, G., Ye, M., Jin, Y., Wang, A., Yin, B., Yang, S. & Wei, W. (2014). Defensive responses of Brandt's voles (*Lasiopodomys brandtii*) to chronic predatory stress. *Physiology & Behavior.* **126**, 1-7.

Hegab, I. M., Kong, S. S., Yang, S. M., Mohamaden, W. I. & Wei, W. H. (2015). The ethological relevance of predator odors to induce changes in prey species. *Acta Ethologica.* **18**, 1-9.

Hilton, G. M. & Cuthbert, R. J. (2010). Review article: The catastrophic impact of invasive mammalian predators on birds of the UK Overseas Territories: a review and synthesis. *Ibis.* **152**, 443-458.

Holmes, N. D., Griffiths, R., Pott, M., Alifano, A., Will, D., Wegmann, A. S. & Russell, J. C. (2015). Factors associated with rodent eradication failure. *Biological Conservation.* **185**, 8-16.

Inagaki, H., Kiyokawa, Y., Tamogami, S., Watanabe, H., Takeuchi, Y. & Mori, Y. (2014). Identification of a pheromone that increases anxiety in rats. *Proceedings of the National Academy of Sciences.* **111**, 18751-18756.

Ivanter, E. V., Kurkhinen, Y. P. & Sokolov, A. V. (2013). Ecology of the field vole (*Microtus agrestis L.*) in indigenous and anthropogenic landscapes of Eastern Fennoscandia. *Russian Journal of Ecology.* **44**, 213-220.

James, D. G. (2003). Synthetic herbivore-induced plant volatiles as field attractants for beneficial insects. *Environmental Entomology.* **32**, 977-982.

Jones, H. P., Tershy, B. R., Zavaleta, E. S., Croll, D. A., Keitt, B. S., Finkelstein, M. E. & Howald, G. R. (2008). Severity of the effects of invasive rats on seabirds: a global review. *Conservation Biology.* **22**, 16-26.

Kliot, A. & Ghanim, M. (2012). Fitness costs associated with insecticide resistance. *Pest Management Science.* **68**, 1431-1437.

Macdonald, D. W. & Fenn, M. G. P. (1994). The natural history of rodents: preadaptations to pestilence. In *Rodent Pests and their Control.* Buckle, A. P. & Smith, R. H. (Eds.), pp. 1-22. CAB International, Oxon.

Major, H. L., Jones, I. L., Charette, M. R. & Diamond, A. W. (2007). Variations in the diet of introduced Norway rats (*Rattus norvegicus*) inferred using stable isotope analysis. *Journal of Zoology.* **271**, 463-468.

Mashoodh, R., Wright, L. D., Hébert, K. & Perrot-Sinal, T. S. (2008). Investigation of sex differences in behavioural, endocrine, and neural measures following repeated psychological stressor exposure. *Behavioural Brain Research.* **188**, 368-379.

Mason, G. & Littin, K. E. (2003). The humaneness of rodent pest control. *Animal Welfare.* **12**, 1-37.

Menger, D. J., Otieno, B., de Rijk, M., Mukabana, W. R., van Loon, J. J. A. & Takken, W. (2014). A push-pull system to reduce house entry of malaria mosquitoes. *Malaria Journal.* **13**, 1-8.

Miller, C. & Miller, T. (1995). Population dynamics and diet of rodents on Rangitoto Island, New Zealand, including the effect of a 1080 poison operation. *New Zealand Journal of Ecology.* **19**, 19-27.

Mishra, N. (2008). Registration of non-traditional trademarks. *Journal of Intellectual Property Rights.* **13**, 43-50.

Osada, K., Kurihara, K., Izumi, H. & Kashiwayanagi, M. (2013). Pyrazine Analogues Are Active Components of Wolf Urine That Induce Avoidance and Freezing Behaviours in Mice. *PLOS ONE.* **8**, 1-9.

Parsons, M. H., Apfelbach, R., Banks, P. B., Cameron, E. Z., Dickman, C. R., Frank, A. S., Jones, M. E., McGregor, I. S., McLean, S. & Müller-Schwarze, D. (2018). Biologically meaningful scents: a framework for understanding predator–prey research across disciplines. *Biological Reviews.* **93**, 98-114.

Pelz, H. J. (1989). Ecological aspects of damage to sugar beet seeds by *Apodemus sylvaticus*. In *Mammals as Pests.* Putman, R. (Ed.) pp. 34-48. Chapman and Hall Ltd, London.

Portfors, C. V. (2007). Types and functions of ultrasonic vocalizations in laboratory rats and mice. *Journal of the American Association for Laboratory Animal Science.* **46**, 28-34.

REX Consortium (2013). Heterogeneity of selection and the evolution of resistance. *Trends in Ecology & Evolution.* **28**, 110-118.

Richards, C. G. J. (1989). The pest status of rodents in the United Kingdom. In *Mammals as Pests.* Putman, R. (Ed.) pp. 21-33. Chapman and Hall Ltd, London.

Sampson, C. & Kirk, W. D. J. (2013). Can Mass Trapping Reduce Thrips Damage and Is It Economically Viable? Management of the Western Flower Thrips in Strawberry. *PLOS ONE.* **8**, 1-8.

Schluter, D., Price, T. D. & Rowe, L. (1991). Conflicting selection pressures and life history trade-offs. *Proceedings of the Royal Society B: Biological Sciences.* **246**, 11-17.

Singleton, G. R., Leirs, H., Hinds, L. A. & Zhang, Z. (1999). *Ecologically-based management of rodent pests – re-evaluating our approach to an old problem.* Ecologically-based

Management of Rodent Pests, pp. 17-29. Australian Centre for International Agricultural Research (ACIAR), Canberra.

Singleton, G. R. (2003). *Impacts of rodents on rice production in Asia.* IRRI Discussion Paper Series, No. 45, pp. 1-30. International Rice Research Institute, Philippines.

Singleton, G. R., Jacob, J. & Krebs, C. (2005). Integrated management to reduce rodent damage to lowland rice crops in Indonesia. *Agriculture, Ecosystems & Environment.* **107**, 75-82.

Singleton, G. R. (2010). *Rodent outbreaks: ecology and impacts.* International Rice Research Institute, Philippines.

Strittmatter, N., Rebec, M., Jones, E. A., Golf, O., Abdolrasouli, A., Balog, J., Behrends, V., Veselkov, K. A. & Takats, Z. (2014). Characterization and Identification of Clinically Relevant Microorganisms Using Rapid Evaporative Ionization Mass Spectrometry. *Analytical Chemistry.* **86**, 6555-6562.

Taberlet, P. & Fumagalli, L. (1996). Owl pellets as a source of DNA for genetic studies of small mammals. *Molecular Ecology.* **5**, 301-305.

Takács, S., Kowalski, P. & Gries, G. (2016). Natural and synthetic vocalizations of brown rat pups, *Rattus norvegicus*, enhance attractiveness of bait boxes in laboratory and field experiments. *Pest Management Science.* **72**, 1873-1882.

Takahashi, L. K., Nakashima, B. R., Hong, H. & Watanabe, K. (2005). The smell of danger: A behavioral and neural analysis of predator odor-induced fear. *Neuroscience & Biobehavioral Reviews.* **29**, 1157-1167.

Tosh, D. G., Shore, R. F., Jess, S., Withers, A., Bearhop, S., Ian Montgomery, W. & McDonald, R. A. (2011). User behaviour, best practice and the risks of non-target exposure associated with anticoagulant rodenticide use. *Journal of Environmental Management.* **92**, 1503-1508.

Tosh, D. G., McDonald, R. A., Bearhop, S., Llewellyn, N. R., Montgomery, W. I. & Shore, R. F. (2012). Rodenticide exposure in wood mouse and house mouse populations on farms and potential secondary risk to predators. *Ecotoxicology.* **21**, 1325-1332.

Verplanken, K., Stead, S., Jandova, R., Van Poucke, C., Claereboudt, J., Vanden Bussche, J., De Saeger, S., Takats, Z., Wauters, J. & Vanhaecke, L. (2017). Rapid evaporative ionization mass spectrometry for high-throughput screening in food analysis: The case of boar taint. *Talanta.* **169**, 30-36.

Waits, L. P. & Paetkau, D. (2005). Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *Journal of Wildlife Management.* **69**, 1419-1433.

Wallace, K. J. & Rosen, J. B. (2000). Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behavioral Neuroscience.* **114**, 912-22.

Witmer, G. W. (2007). *The ecology of vertebrate pests and integrated pest management (IPM).* USDA National Wildlife Research Center-Staff Publications, pp. 393-410. University of Nebraska, U.S.A.

Witzgall, P., Kirsch, P. & Cork, A. (2010). Sex Pheromones and Their Impact on Pest Management. *Journal of Chemical Ecology.* **36**, 80-100.

Wöhr, M., Houx, B., Schwarting, R. K. & Spruijt, B. (2008). Effects of experience and context on 50-kHz vocalizations in rats. *Physiology & Behavior.* **93**, 766-776.

Woodward, P. W. (1972). *The Natural History of Kure Atoll, Northwestern Hawaiian Islands.* Atoll Research Bulletin, pp. 1-318. Smithsonian Institution Press, Washington D.C.

Xu, Q., Hatt, S., Lopes, T., Zhang, Y., Bodson, B., Chen, J. & Francis, F. (2018). A push–pull strategy to control aphids combines intercropping with semiochemical releases. *Journal of Pest Science.* **91**, 93-103.

Ylönen, H. & Ronkainen, H. (1994). Breeding suppression in the bank vole as antipredatory adaptation in a predictable environment. *Evolutionary Ecology.* **8**, 658-666.

Zhang, J.-X., Sun, L., Zhang, J.-H. & Feng, Z.-Y. (2008). Sex-and gonad-affecting scent compounds and 3 male pheromones in the rat. *Chemical Senses.* **33**, 611-621.