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Exploring Social Interactions and Olfactory Communication in The Common Brushtail Possum: Implications for Management

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Abstract

The common brushtail possum (*Trichosurus vulpecula*) was introduced to New Zealand in the mid-1800's and has since become highly invasive in this country. Causing considerable damage to both native flora and fauna and being a wildlife reservoir of bovine tuberculosis, this species requires intensive management and control. Management will be improved by having a more complete understanding of possum social interactions and communication. The focus of this thesis is to, therefore, improve our understanding in these areas of possum biology.

The first part of this thesis focuses on possum social interactions. Of specific interest is whether or not differences exist in the duration and frequency of interactions between possums during the breeding and non-breeding seasons. Furthermore, the possibility of a relationship between genetic relatedness and tolerance levels between female possums and mate choice between males and females is investigated. The results of this study suggest that male and female possums interact far more than females do with females and than males do with males during both the breeding and non-breeding season. Furthermore, genetic relatedness does not appear to have a strong effect on mate choice or on the amount of time that females spend in close contact with other females during the non-breeding season.

The second part of this study focuses on determining how possums use various scents to communicate with each other. Their responses to sternal gland scent (from both genders and from two different populations) are investigated with the intention of discerning whether or not they have any preferences based on gender and/or familiarity. Also researched is whether or not they show a preference when presented with possum sternal gland scent and a popular food lure to see if possum produced scents may function as a lure which can be used in their management. Due to the low level of response during these trials, it is not possible to come to a conclusion on possum preferences for different possum scents. The trends suggest, however, that they take more interest in the scents of familiar females over familiar males and in foreign possums over familiar possums. What is evident, however, is that they take more interest in the scent of cinnamon apples over that of foreign female sternal gland scent.

The final part of this thesis is a pilot study which investigates using a molecular technique, denaturing gradient gel electrophoresis (DGGE), to examine the composition and dynamics of the bacterial communities that inhabit the cutaneous surface of the possum sternal gland. Unique bacterial profiles during the same season as well as changes in individual profiles between the breeding and non-breeding seasons are investigated. It was determined that new technologies, such as amplicon sequencing, should be utilized to conduct such evaluations due to problems encountered with DGGE. Although conclusions could not be made due to a small sample size, the trends suggest that these bacterial communities do not change based on season (i.e. physiological state of the possum) and that possums do not have unique bacterial profiles in this specific anatomical region. The issues associated with this technique as well as the need for further research are discussed.

Due to the adverse effects that this species has on New Zealand's economy and ecology, it is imperative that their numbers are reduced, hopefully to the point that they are

completely removed. Hopefully with the understanding gained by this study, our ability to control possums will improve, further enabling us to reach this goal.

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Photo by Olivia Hamilton

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Chapter One: Introduction

The social lives of animals are complex, dynamic, and correlated with other aspects of animal behaviour such as cognition and communication (Byrne & Whitten, 1988), making sociality a fascinating field to study. The degree of socialization varies from species to species and sometimes from one population to the other within the same species based on factors such as resource and mortality patterns (e.g. Macdonald & Bacon, 1982). As sociality is a broad field, including interactions such as cooperation, competition, the establishment and maintenance of dominance (Whitehead, 1997), allogrooming, food sharing, mating, and denning (Houseknecht, 1969; Day *et al.*, 2000), understanding sociality has the potential to enhance many areas of applied biology.

Understanding the social lives of animals can potentially have profound benefits to wildlife management and conservation. The spread of disease and the use of self-disseminating forms of biological control for invasive species, for example, are highly dependent upon interactions such as mating (e.g. Barlow, 1994), denning (e.g. Cowan *et al.*, 2006), fighting (e.g. Holtcamp, 2007), and food sharing (e.g. Wright & Gompper, 2005). Social interactions are thus a focus of this study as understanding how and why animals interact may help us to better manage them and, in the case of invasive species and disease vectors, may also help us to devise more productive methods of control.

Communication plays an important role in social behaviour of animals. Communication, an action of one individual which leads to a change in the probability of behavioural occurrences in another individual (Wilson, 1975), can take place using different modes. Animals may send messages to each other via touch, sounds, the visual mode (e.g. facial expressions, displays), and via olfaction (Feldhamer *et al.*, 2004). These different modes of communication vary with regards to the duration of the message's effectiveness, how far it is carried, the energetic cost to the producer of the message, and the effectiveness of the message in the absence of light, amongst other properties (Feldhamer *et al.*, 2004).

Animals communicate with each other for multiple reasons such as for group coordination (e.g. hunting and foraging), for recognition (e.g. kin recognition to avoid inbreeding or for kin selection), for reproductive purposes (e.g. advertising reproductive

status, harem protection), to advertise social status, to alarm others of a threat, for mother-offspring interactions, and to solicit play (reviewed in Feldhamer *et al.*, 2004). All of these are important for resource defence, for the maintenance of groups, and for the cooperative behaviours upon which many animals function. Animal communication is of interest in this study as an understanding of communication also has the potential to facilitate the management of wildlife, which can have significant implications for conservation. The type of communication that is of interest in this study is olfactory communication.

1.1 Social Interactions

There is considerable variation in the degree of sociality of mammals. Some animals, such as naked mole-rats (*Heterocephalus glaber*), are social to the point of being eusocial (Burda *et al.*, 2000) while others, such as the Siberian chipmunk (*Eutamias sibiricus lineatus*), may come together only to breed (Kawamichi *et al.*, 1987). In between these two extremes are many species that live and move in cohesive groups (reviewed in Feldhamer *et al.*, 2004) and also some, such as the striped skunk (*Mephitis mephitis*), that are mainly solitary but that will den together on occasion (e.g. Shirer & Fitch, 1970; Larivière & Messier, 1998).

Whether gregarious or solitary, non-sexual interactions occur between individuals and these interactions can be divided into two types: agonistic and affiliative. Agonistic interactions serve multiple purposes such as offspring protection (Wolff & Peterson, 1998), territory protection (e.g. Palombit, 1993), access to mates (e.g. Cant *et al.*, 2002), and to release frustration (Sapolsky, 2004). Similarly, there are multiple purposes of affiliative behaviour such as forming and maintaining bonds/coalitionary relationships (Henzi & Barrett, 1999) and post-fight reconciliation (e.g. Schino, 1998).

Gregarious and solitary mammals also interact for sexual purposes as mammals require internal fertilization (Feldhamer *et al.*, 2004) and thus need to physically be together to successfully mate. Many mammals mate seasonally, with females coming into oestrous only during certain times of the year, and majority of female mammals are only receptive to copulation during this time of oestrous (Feldhamer *et al.*, 2004). There are only a few exceptions to this, for example humans (*Homo sapiens*) and bonobos (*Pan paniscus*) (Blount, 1990). When considering animal interactions, therefore, the breeding

season is an important time of the year as individuals of both gregarious and solitary animals will be seeking out and interacting with conspecifics.

1.1.2 Management Implications of Social Interactions

Understanding animal interactions can greatly benefit conservation efforts as well as economic and health issues. With regards to conservation, efforts are being made to find more humane methods of dealing with the issue of invasive species. One such method is the development of a self-disseminating vector to spread a bio-control agent (such as an immunocontraception agent) among the target animals. Multiple studies have been undertaken to find such a system that will work with various invasive animals (e.g. Barlow, 1994; Saunders *et al.*, 2010). Such a system, however, depends on the ability of the vector to spread itself through a population which ultimately depends on animals coming into contact with one another. It is thus necessary to discover and understand patterns in the frequency and duration of animal interactions so that we know if such a method is feasible with the species of interest, and to be able to efficiently execute a biological control program using a self-disseminating form of control.

Social interactions are also extremely important when trying to control disease transmission in animals which often have serious health and economic implications. For example, bovine tuberculosis, a common wildlife disease, has impacts on humans as it has the potential to affect livestock and thus may have serious implications for livestock trade (Coleman & Livingstone, 2000). Being that this and other diseases are spread via close contact, it is important to understand the social interactions of its carriers both when considering the rate and patterns of disease spread and when considering control options.

1.2 Olfactory Communication

Olfactory communication is a complex behaviour that allows organisms to communicate with conspecifics as well as with other species and to relay a wide variety of messages using different substances (Wyatt, 2003). This type of communication has advantages as it is not very costly, the message stays around for a long time after the messenger has left the area, it often does not necessitate direct contact between the releaser

and the receiver, and the messages can be carried over long distances and do not require visual contact (i.e. it can be used at night) (Mykytowycz, 1972; Wilson, 1975, Wyatt, 2003; Knapp *et al.*, 2006).

Olfactory communication is accomplished by the release of chemical signals referred to as semiochemicals (Law & Regnier, 1971). Semiochemicals can then be classified into different types based on how they are used. Semiochemicals that are used between different species are referred to as allelochemicals while those that are used to send messages to conspecifics are referred to as pheromones (Wyatt, 2003). There are differences in the chemical structure and make-up of pheromones depending on the type of message (Brennan & Zufall, 2006). For example, pheromones used to attract or alarm others, are typically volatile and small in size to allow the fast spread of the message. Pheromones that are used to provide personal information about the producer, however, are typically larger molecules (peptides or proteins) and thus less volatile, probably to ensure that the message is associated with that individual (Brennan & Zufall, 2006), and maybe also to allow for a wide range of molecular combinations which are needed for individual recognition.

Pheromones are classified based on the effect that they have on the receiver. Those which immediately cause a change in the receiver's behaviour via a central nervous system response are referred to as releaser pheromones while those which affect the receiver's endocrine system and therefore physiology (having longer lasting effects) are called primer pheromones (Wilson & Bossert, 1963). There is not a clear distinction between these two types of pheromones, however, as some pheromones may act as both a primer and a releaser (Wilson & Bossert, 1963; Wyatt, 2003).

1.2.1 Mammalian Scent Production and Use

Scents are produced in different regions of the body with many being produced by glands (Quay, 1976; Shanas & Terkel, 1997) and many being found in urine, faeces, saliva, and vaginal secretions (Mykytowycz, 1972; Lombardi *et al.*, 1976; Massey & Vandenbergh, 1980; Koyama & Kamimura, 2000; Wyatt 2003). These scents are used for purposes such as resource defence (Roper *et al.*, 1986), imprinting between mother and young (Macfarlane, 1975; Russell, 1976; Kaplan *et al.*, 1977; Stoddart, 1980; Holmes,

1992), advertising reproductive receptiveness (Michael *et al.*, 1972; Stoddart, 1980; Rasmussen *et al.*, 1997; Smith & Abbott, 1998; Swaisgood *et al.*, 2000), marking one's territory (Rich & Hurst, 1998; Summarized in Wyatt, 2003), and for defence (Cuyler, 1924; Wood, 1999).

1.2.2 Variation in Scent

Within species, there can be considerable variation in the final scent products that are emitted. Variations could be a result of internal sources such as genetics, specifically the major histocompatibility complex (MHC) which plays a role in immunity (Yamazaki *et al.*, 1979; Brown, 1995), as well as the specific chemistry of the animal's scent glands and the diet of the animal (Brown, 1979; Brown, 1995; Salamon, 1995). Furthermore, hormonal fluctuations, either natural or induced by hormonal contraception, can also lead to a change in scent production in animals (Dryden & Conaway, 1967; Brown, 1979; Zhang *et al.*, 2001; Crawford *et al.*, 2010).

In addition, bacterial activity can have an effect on the scent of secretions (Brown, 1979; Albone, 1984; Studier, 1984; Nordstrom *et al.*, 1989; Woolhouse *et al.* 1994; Salamon, 1994; Lanyon *et al.*, 2007) as they breakdown the chemicals secreted by animals (e.g. Gorman, 1976). Studies which investigated differences in the olfactory cues given off by animals raised in sterile *versus* conventional housing support the role that bacteria play in the scent production of animals (Singh *et al.*, 1990; Schellinck *et al.*, 1995). As bacteria play such a role, it is important to consider the dynamics of the bacterial communities that are symbiotic with animals. The physiological state of an animal can determine the composition of the bacterial communities living within or on the surface of the animal through the variable proteins, metabolites, and other products that are made available for consumption by bacteria (Nordstrom *et al.*, 1989; Wintzingerode *et al.*, 1997; Penn & Potts 1998, cited in Lanyon *et al.*, 2007). Furthermore, Albone *et al.* (1977) suggest that bacteria can facilitate communication types which modulate through time (e.g. reproductive status advertisement). Changes in substrate availability for commensal microbial species allows for changes in the individual's scent (into scents that attract conspecifics). For example, the microbially derived vaginal secretion volatiles of female rhesus monkeys (*Macaca mulatta*) are produced during oestrous to attract male conspecifics (Michael *et al.*, 1972).

1.2.3 Management Implications of Olfactory Communication

Wildlife management can benefit from studies on olfactory communication as scents can be used as both lures and deterrents. Multiple studies have been conducted on such a practical use of scents (Melchior & Leslie, 1985; Epple *et al.*, 1993; Clapperton *et al.*, 1999; Bramley & Waas, 2001; Russell & Banks, 2005; Borgo *et al.*, 2006; Russell & Banks, 2007). Such research demonstrates the possibility of using scents for purposes ranging from attracting animals to monitoring devices, such as tracking tunnels or hair snares, to keeping a certain species from using roosting cavities. While some studies have not yielded expected or desired results (e.g. Russell & Banks, 2007), and further research is needed, many studies demonstrate the potential that lies in utilizing olfactory communication in wildlife management.

1.3 The Common Brushtail Possum

The common brushtail possum (*Trichosurus vulpecula*) is a marsupial mammal averaging three kilograms and is indigenous to Australia (Clout & Ericksen, 2000) (Figure 1.1). These nocturnal animals are mainly arboreal (Cowan & Clout, 2000), consuming a wide variety of food types (Tyndale-Biscoe, 2005). Belonging to the *Phalangeridae* family, common brushtail possums have a relatively large range in Australia including both the mainland and Tasmania (Tyndale-Biscoe, 2005).

1.3.1 Possums as Pests in New Zealand

The possum was introduced into New Zealand from mainland Australia and Tasmania due to its value in the fur trade industry. The first successful introduction occurred in 1858 and was followed by further introductions and liberations by acclimatization societies and the New Zealand government into the 1900's (L.T. Pracy, 1974; reviewed in Clout & Ericksen, 2000). It was not until 1947 that the government recognized the invasiveness of this species and began to take action to limit its numbers (Clout & Ericksen, 2000). This species has been highly successful in New Zealand,



Figure 1.1 The common brushtail possum (*Trichosurus vulpecula*). Photo by Idan Shapira.

especially when compared with its density in Australia. In its homeland, they are found at densities from 0.5 ha^{-1} to 8 ha^{-1} but can be found at densities up to 25 ha^{-1} in New Zealand (Efford, 2000; Tyndale-Biscoe, 2005). This success is at least partly attributable to the fact that they have far fewer predators (two compared to five) and parasites (fourteen compared to sixty-six) in New Zealand (Clout & Ericksen, 2000).

Possums are opportunists, eating a wide variety of food depending on what is available. They are known to eat plants such as introduced grasses and clover, indigenous woody plants, ferns, mistletoe, pine pollen and pine cones, and the fruits and flowers of both native and introduced plants (Dunnet *et al.*, 1973; Warburton, 1978; Nugent *et al.*, 2000). Even though they are flexible with their diet, they do have preferences. Comparing the data of multiple dietary studies, Nugent *et al.* (2000) found that in twelve of twenty studies, Kāmahī (*Weinmannia racemosa*) and Tōwai (*Weinmannia silvicola*) were in the top five favoured plants of *T. vulpecula*. Their ability to eat so many different types of plants despite having preferences is most likely another factor that has allowed them to successfully invade New Zealand enabling them to make a living in a diverse range of environments.

T. vulpecula feeding habits have caused damage to forests throughout New Zealand and, interestingly, the responses of forests to their removal have been variable (Rose *et al.*, 1992; Allen *et al.*, 1997; Payton *et al.*, 1997; Norton, 2000; Nugent *et al.*, 2001). Some studies did not find any significant recovery in forests following possum removal operations and even reported a continued decline in the condition of some plant species (Tyndale-Biscoe, 2005; Cowan *et al.*, 1997; Payton *et al.*, 1997; Smale, M.C. *et al.*, cited in Norton, 2000; Tubbs, M., cited in Norton, 2000). Studies in other areas, however, have found significant recovery amongst forest plants (Atkinson, I.A.E., cited in Norton, 2000; de Lange, P.J. & Norton, D.A., cited in Norton 2000, Tyndale-Biscoe, 2005). It is likely that other factors are involved in this complex relationship between possums and forests. Such factors may include the timing of the removal operation with respect to possum density, a time lag in the plant response, the number of possum survivors remaining (and if these individuals continue to consume the same individual plants), and reduction in seed dispersal by native birds due to possum (or other invasive species) predation/competition (Pekelharing & Batcheler, 1990; Ladley & Kelly, 1996; Payton *et al.*, 1997; Norton, 2000).

In addition to eating plants, brushtail possums also eat fungi (Tyndale-Biscoe, 2005) and animals such as insects, gastropods, and birds (Warburton, 1978; Sadleir, 2000; Cowan, 2001). Their consumption of birds has received the most attention as some of these species are rare or endangered. They are known to eat the eggs, juveniles, and/or adults of wood pigeons (*Hemiphaga novaeseelandiae*), kōkako (*Callaeas cinerea*), kiwi (*Apteryx* spp), and fantails (*Rhipidura fuliginosa*) amongst others (Brown *et al.*, 1993; James & Clout, 1996; McLennan *et al.*, 1996; Innes *et al.*, 1996; Nugent *et al.*, 2000). Although they are not the only introduced species to prey on these animals (cats, dogs, rodents, and mustelids can also take these birds), their involvement in the decline of these species is important and in need of regulation. This is especially the case with species that are endangered or who are suffering population declines as is the case with kōkako and kiwi (Leathwick *et al.*, 1983; Innes *et al.*, 1996; McLennan *et al.*, 1996).

Possums also affect birds in a more indirect way. As possums consume large amounts of plant material, they can compete with some native birds for food. Owen and Norton (1995) found that some food items that are important to birds such as kaka, bellbirds, tui, and wood pigeons are also favoured by possums. There is also an overlap in

the diets of possums (and other introduced mammals such as goats (*Capra hircus*) and red deer (*Cervus elaphus*)) and kōkako (*C. cinerea*) (Leathwick *et al.*, 1983). Possums feed on the leaves of plants, such as five finger (*Pseudopanax arboreus*) and whitey wood (*Melicactus ramiflorus*), just as kōkako do and both species also consume the fruits of some of the same plants to supplement their diets (Fitzgerald, 1984). Thus, possums have the ability to significantly impact New Zealand's wildlife not only through predation, but also through competition.

1.3.2 Possums as Bovine Tuberculosis Reservoirs

Just as significant as their dietary habits is the role that possums play in maintaining bovine tuberculosis in New Zealand. Considered to be the main wildlife reservoir of Tb in this country, possums are the main reason that this disease has not been eradicated from livestock herds (Coleman & Caley, 2000). This disease, caused by the bacterium *Mycobacterium bovis*, is important because it is transmissible between humans and other warm-blooded animals and has the potential to cause significant economic damage. For example, in the early 19th century, *M. bovis* alone caused more livestock deaths across the US than all other pathogens combined (Vantiem, 2002). Bovine Tb is of major economic concern in New Zealand as a high level of incidence of Tb in cattle and deer can harm trade relations with other countries (Coleman & Livingstone, 2000).

Possums spread this disease to each other via pseudo vertical transmission, such as when a mother passes it to her offspring through her milk, or through direct horizontal transmission which occurs during close interactions such as mating, fighting, denning, and grooming (Fairweather *et al.*, 1987; Morris & Pfeiffer, 1995; Coleman & Caley, 2000). Although not confirmed, it seems likely that the disease is spread to cows and deer due to the inquisitive nature of these animals. Studies revealed that cows and deer approach and investigate possums that were drugged to simulate the behaviour of terminally ill possums and that some cows sniff and lick the possums (Paterson & Morris, 1995; Sauter & Morris, 1995). The transmission of bovine Tb from possums to farmed animals is clearly a concern and is yet another reason why possums need to be controlled in New Zealand. Due to their involvement in the decline of New Zealand flora and fauna and because of the role they play in the spread of bovine Tb, possums are an ideal animal for this study on olfactory

communication as management and control efforts may benefit from a better understanding of their communication.

1.3.3 Possum Scent Glands

Possoms have many of the scent glands that are found in marsupials. Studies have noted that they have odourous glands in the labial region, the ears, eyes, chin, sternal region, in the pouch, in between the digits, in the ventral (hairless) surface of the tail, as well as paracloacal and circumanal glands (Green, 1963; summarized in Russell, 1985). Secretions from these various glands are suspected to provide information such as identity, status, and age, to communicate fear and submission, to reinforce the bond between mothers and their young, to familiarize females with potential mates, to establish and maintain hierarchies, and possibly to protect resources (summarized in Russell, 1985; Hynes, 1999).

Of interest to this study is the sternal gland. Located on the chest, this gland causes staining of the hair covering the glandular area resulting in a hair patch that is reddish brown (Green, 1963; Russell, 1985) (Figure 1.2). The amount of staining, the size of the stained area, and the development of both the associated sebaceous and sudoriferous glands are greater in mature males than in females or juveniles (Green, 1963; Russell, 1985; Hynes, 1999; personal observation) (Figure 1.3).

Findings from previous studies suggest that sex hormones, particularly testosterone, play a significant role in sternal gland development and secretion (A. Bolliger, cited in Hynes, 1999). The Bolliger study found that castration in young males led to the lack of stained hairs covering the sternal gland while this effect was reversed upon treatment with testosterone propionate. Additionally, adult males who were castrated and treated with oestrogen experienced a loss in size of the stained sternal region as well as lightening of the sternal hairs to a yellow brown. The sternal hairs of ovariectomized young females failed to become stained (even with oestradiol dipropionate treatment) but did become stained to a degree comparable to that of males following testosterone propionate treatment. There were no significant changes in the sternal hairs, besides from turning slightly more yellow, in adult females who had been ovariectomized.

Another study by Biggins (cited in Hynes, 1999) suggests a relation between testosterone and sternal marking behaviour in possums. While sternal marking behaviour ceased in castrated males, this behaviour returned following testosterone treatment. It would be curious to see if there are any effects of ovariectomy and testosterone treatment on female marking behaviour. The sternal gland is used to mark various structures in the home range of possums. Hynes (1999) found that structures such as tree trunks (mostly close to the ground), fallen logs, rocks, traps, etc. are marked by possums as are the areas around den entrances. She proposes that possums may mark their dens as a way to inform others that it is occupied and/or it may be a way for the resident of the den to boost his or her confidence by covering the area in his or her scent. Possums do not, however, mark the boundaries of their home ranges.

Hynes (1999) also found that there are differences in the marking behaviour and sternal gland morphology of males and females at different times of the year. Adult males experience the highest degree of sternal staining and the most marking behaviour in the first three months of the year (pre-breeding season). Although there is some overlap in staining with females as mature females experience the highest degree of staining when they are in oestrous (around March), females mainly mark when they have offspring. She thus suggests that sternal marking may be used by males to inform females as to whom their potential mates are and may be used by females to protect resources required for her and her young. It is also possible that both genders use this scent to establish and maintain dominance hierarchies between adults and juveniles.

1.4 Previous Research on Possum Social and Olfactory Biology

Previous studies have looked into the social interactions of possums. These studies investigated factors such as the frequency and duration of interactions during the breeding and non-breeding seasons, interactions between males and females who are either in oestrous or anoestrous, and the relationship between contact rates and population density (Day *et al.*, 2000; Ramsey *et al.*, 2002; Ji *et al.*, 2005). There were limitations with these studies as the only interactions that were observed were between males and females (Ji *et al.*, 2005), or interactions were observed in captivity (Day *et al.*, 2000), or the locations of



Figure 1.2 The sternal stain of an adult female possum. Photo by Ateret Shabtay.



Figure 1.3 The much larger area of sternal staining of an adult male possum. Photo by Phil Riley.

the possums were only recorded once every forty-five to sixty minutes (Ramsey *et al.*, 2002). Thus further studies on possum sociality are required. Previous research has also looked at different aspects of olfactory communication in *T. vulpecula* such as the chemical composition of their secretions, their response to predator scents, and their preferences for different scents used in poisoned baits (Innes, J. & Frampton, C., cited in Woolhouse *et al.* 1994; Salamon, 1994; Woolhouse *et al.* 1994; Morgan *et al.*, 1995; Todd, 1995; Bramley & Waas, 2001; Russell & Banks, 2005). However, the possibility of using possum scents to attract conspecifics has not yet been tested. Moreover, studies on the bacterial species that are symbiotic with possums, and thus may affect their odour, are lacking with only one being found in the literature. This study researched the bacterial species that inhabit the possum pouch (Deakin & Cooper, 2004).

1.5 Study Objectives

The first objective of this study (Chapter 3) is to gain a better understanding of the social interactions that occur between wild possums. Of specific interest is whether or not there are differences in the frequency and duration of interactions between female-female, female-male, and male-male pairs both during and outside of the breeding season. Furthermore, it is of interest to see if female possums show more tolerance to other female possums based on relatedness and if mates are chosen based on relatedness. Such analyses will hopefully allow us to better understand patterns of social behaviour that will ultimately have implications for possum control and management in New Zealand.

The second objective of this thesis (Chapter 4) is to 1) gain a better understanding of how wild possums respond to conspecific sternal gland scents, and to test the luring ability of these scents for management purposes. Hynes (1999) studied sternal gland marking behaviour in brushtail possums in Tasmania and speculated as to what kinds of messages were being communicated based on where and when marking occurred. She suggests that sternal marking may be used to establish and maintain intrasexual dominance hierarchies, to familiarize females with potential mates, and/or to protect resources (see section 1.3.3). Thus, it is not known if possums use their sternal gland scent to attract conspecifics, or to deter them, or both (perhaps depending on gender and/or season). The other option is that the scent neither attracts nor deters possums. Testing the responses of

possums to various scents is, therefore, necessary as it will help us to understand how they use these sternal gland scents to communicate. Furthermore, the attracting abilities of possum scents are of interest as such scents may be able to be used as lures in possum management, facilitating the control of this highly invasive marsupial.

The third objective (Chapter 5) is to investigate a technique that can be used to explore the composition of the bacterial communities inhabiting the cutaneous surface of the brushtail possum sternal gland and to gather preliminary results on this topic. As mentioned in section 1.2.2, it is possible that the physiological state of an animal influences the community composition of the animal's bacteria by determining which proteins, metabolites, etc. are made available to the bacteria (Nordstrom *et al.*, 1989; Wintzingerode *et al.*, 1997; Penn & Potts 1998, cited in Lanyon *et al.*, 2007). It would be interesting to investigate this in the brushtail possum by determining whether compositional changes occur in their bacterial communities based on season (breeding or non-breeding) and thus on their hormonal status. Furthermore, it would also be of interest to see if individual possums have unique bacterial profiles when compared with others during the same season, which might provide them with a unique odour. This study, therefore, includes a pilot study into such an investigation.

In summary, I would like to test the following hypotheses:

1. Possums have higher rates of interactions (frequency and duration) during the breeding season with there being higher rates between males and females compared with female-female interactions.

A previous study on wild possums found significantly more interactions during the breeding season than the non-breeding season (Ji *et al.*, 2005). This is expected as individuals seek each other out or are sought out during the breeding season. Furthermore, female possums are noted to be more tolerant of other possums during the breeding season (J.N. Jolly 1981, cited in Day *et al.*, 2000). It is therefore expected that contact rates increase during this time of the year.

2. Male-female interactions of a longer duration during the breeding season, which suggests sexual interactions, mainly occur between individuals with less degree of genetic relatedness to avoid inbreeding.

Inbreeding avoidance is common in the animal kingdom with various tactics being used by different species. Some are able to recognize and avoid close relatives when it comes to mating, some have extra-pair relations, and some species employ mechanisms of delayed maturation or reproductive suppression (reviewed in Pusey & Wolf, 1996). It is thus expected that possums too are capable of avoiding close relatives when it comes to reproduction.

3. There is a positive correlation between the frequency of long-lasting (and therefore assumed to be affiliative) interactions and degree of relatedness between female possums.

It is common for mammals to be more tolerant of and to have long-lasting relationships that are not of a sexual nature with conspecifics that are more closely related (Poole, 1985). Many examples of these types of relationships are found in the mammalian class (e.g. spotted hyenas (*Crocuta crocuta*) (Wahaj *et al.*, 2004); multiple species of primates (reviewed in Smith *et al.*, 2003); California sea lions (*Zalophus californianus*) (Hanggi & Schusterman, 1990). Furthermore, female possums are more tolerant of their female offspring than of their male offspring as male offspring are chased off while female offspring typically remain in the area and have overlapping territories with their mother (Clout & Efford, 1984). It is thus predicted that females will be more tolerant of each other based on their relatedness to each other.

4. The scents of both male and female possums from different populations will attract more interest than scents collected from individuals in the same population.

This hypothesis follows from the MHC-dependent mating preferences seen in studies conducted on humans and rodents (reviewed in Penn & Potts, 1999; Penn, 2002) as well the study done on the agile antechinus which showed that females are more attracted to the

scents of males who are more distantly related (Parrott *et al.*, 2007). Although previous MHC research has mainly focused on urinary scents and has covered a limited number of taxa, it is a worthwhile area to pursue as it may enhance our ability to attract possums with scents, improving the management of the species.

5. Possum scents are more effective at attracting possums than wheat bran and cinnamon coated apples, which is the lure that was used to live trap possums during this study.

Although food lures clearly work to some degree with brushtail possums, there is some evidence which suggests that food is not extremely efficient (Morgan *et al.*, 1995; Todd, 1995). Due to the lack of enthusiasm that possums have shown for food lures in these studies, it would be beneficial to find a substance(s) that act as a stronger attractant. Morgan *et al.* (1995) discuss how effective lures that have been found for other mammalian species typically contain a scent that the animals recognize, thus it is possible that the scent of another possum (especially a scent that is used to advertise mates and reproductive status during the breeding season, the time when tests were conducted) may prove to be a more efficient lure.

Chapter Two: General Methods

2.1 Study Sites

Samples for both the scent testing (Chapter 4) and sternal bacterial flora (Chapter 5) aspects of this study were collected from a wild possum population inhabiting a 26ha forest remnant in Coatesville (36°42'58.71"S, 174°38'33.41"E) which is located approximately 30 km north of Auckland, New Zealand. This is also where the experiments on possum responses to possum scent were carried out (Chapter 4). This site, which is a fenced, mixed broadleaf-podocarp forest remnant, is surrounded by pasture and private residences. The average of possum density estimates at this site estimated during a study from 1999 to 2001 was around 4.3 possums ha⁻¹ (Ji *et al.*, 2005). The northern twelve hectare section of this bush patch was used for this study.

Additional sternal bacterial and scent samples were collected from a second wild possum population on the North Island, located in Mercer (37° 16'41.22"S, 175° 02'41.22"E). The habitat in Mercer is also a mix broadleaf podocarp forest remnant similar to the Coatesville site but has less undergrowth, likely to be due to ungulate grazing. This site is also lined with pasture and private residences, although far fewer than in Coatesville.

Scent samples were also collected from Landcare Research's captive facility in Lincoln, on New Zealand's South Island for use in the scent test trials. The possums housed at this facility were originally trapped in the Lewis Pass region of New Zealand's South Island and had been in captivity for two to five months (J. Duckworth, Landcare Research, Lincoln, pers. comm.). The locations of the three sites can be seen in Figure 2.1.

2.2 Trapping of Possums

Possums were caught in live cage traps baited with cinnamon and wheat bran dusted apples. A trapping grid consisting of 32 traps, with 50 metres in between each trap, was used in Coatesville. The Mercer site consisted of three trap lines with each line consisting of five traps separated by 50 metres.

2.3 Determination of Reproductive Status

The reproductive status of females and males were determined using guidelines outlined by Ji *et al.* (2000). Females were considered sexually mature by the presence of an

invaginated pouch. Males with a testes width of 16.94 ± 1.24 mm are known to have successfully reproduced (Ji *et al.*, 2000), this same standard was thus used in this study and males with testes width of 15.7 mm or over were regarded as sexually mature.

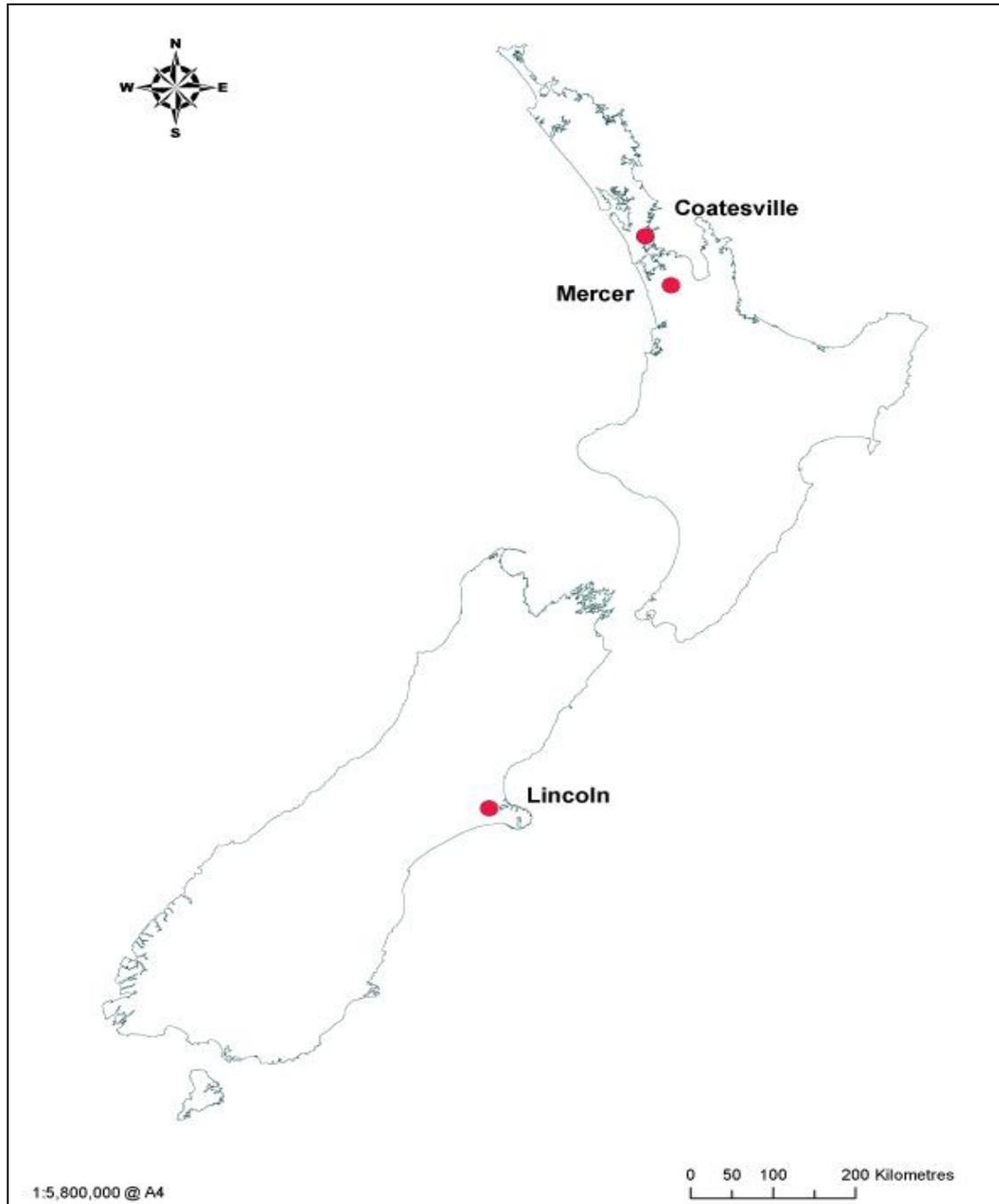


Figure 2.1 The locations of the two field sites, Coatesville and Mercer, and the captive site, Lincoln, where possums were sampled for this study. All sites are located in New Zealand. Map data source from Land Information New Zealand (LINZ 2008).

2.4 Further Methodology Details

Detailed methods for the study on possum sociality, possum responses to possum scents, and on possum sternal gland bacterial composition and dynamics are given in chapters 3, 4, and 5, respectively.

Chapter Three: Possum Social Interactions

3.1 Introduction

Understanding the social interactions of animals has the potential to greatly benefit the fields of conservation biology and wildlife management as social interactions are highly important for multiple reasons. For example, understanding aspects such as the mating system of a species and the dominance relationships of a group of animals has the potential to benefit captive breeding efforts. Furthermore, as discussed in chapter one, understanding interactions and the patterns of interactions is necessary for studies concerned with the spread and control of diseases and the use of self-disseminating forms of biological control. These last two reasons are important for the management of brushtail possums in New Zealand.

Previous studies on possum social interactions found that the contact rate of possums is not related to population density in a linear fashion (Ramsey *et al.*, 2002; Ji *et al.*, 2005). These studies also looked at differences in the frequency of interactions between the breeding and non-breeding seasons. Two studies that looked at frequencies (and one also looked at duration) found conflicting results yet this is likely due to the fact that one of the studies looked at captive possums (Day *et al.*, 2000) while the other looked at wild possums (Ji *et al.*, 2005). The captive study found a higher frequency of interactions during the non-breeding season with threats being the most frequent type of encounter recorded (Day *et al.*, 2000). However, as discussed by the authors, this is likely the result of the possums being in confined areas. The study on wild possums found that there were more interactions and that interactions lasted much longer in the breeding season (Ji *et al.*, 2005). Due to the limitations of the method, this previous study on interactions between wild possums only revealed the close contacts between females and males. Information on social interactions that occur between same sex dyads is also important for understanding the rate of disease transmission in possum populations. Such information is crucial for evaluating the effectiveness of any potential biological control methods that rely on possum contacts for the dissemination of control agents.

Another interesting result from another study on wild possums is that there is not a significant difference between the contact rates that males have with oestrous *versus* non-oestrous females (Ramsey *et al.*, 2002). This is important as it suggests that possums may

regularly come into contact with each other for reasons other than to breed. This can have implications for both disease transmission and for a self-disseminating form of biological control. Unfortunately, however, the locations of the possums were only recorded once an hour or once every forty-five minutes in this study which is likely to have left many interactions undetected. It is thus important to conduct further studies in this area to gain a more complete picture of possum social interactions.

It is also important to investigate how social interactions within a population are affected by genetic relatedness. The degree of importance of genetic relatedness on social interactions can vary from species to species. For example, relatedness impacts the social structure of groups of animals such as coatis (*Nasua narica*) (Gompper *et al.*, 1997), Tammar wallabies (*Macropus eugenii*) (Blumstein *et al.*, 2002), and African elephants (*Loxodonta Africana*) (Archie *et al.*, 2006). A situation found in other species, however, suggests that familiarity, not genetic relatedness, has more of an impact on social interactions (e.g. Kareem & Barbard, 1982; Fredrickson & Sackett, 1984). It would thus be interesting to investigate the possibility of a relationship between relatedness and sociality in the common brushtail possum to determine whether relatedness is important to these mammals with regards to mate choice and to the tolerance of conspecifics.

The aim of this study is to further investigate the possibility of patterns in possum social interactions and to determine whether or not their interactions are correlated with degree of relatedness. Three hypotheses have been tested in this section: 1. The frequency and duration of interactions are greater during the breeding season, with there being more interactions between males and females than between female dyads. This is expected as female possums are more tolerant of other possums during this season (J.N. Jolly 1981, cited in Day *et al.*, 2000), because a previous study on wild possums found such a pattern (Ji *et al.*, 2005), and because the breeding season is the time of year that mammals seek each other out for reproductive purposes. 2. Genetic relatedness is negatively correlated with the frequency and duration of interactions between males and females during the breeding season as inbreeding avoidance is a common mechanism used for mate choice in the animal kingdom. 3. The degree of genetic relatedness is positively correlated with the frequency and duration of interactions between females during the non-breeding season.

This pattern is predicted to occur in brushtail possums as higher levels of tolerance for closely related conspecifics is observed in many mammals (Poole, 1985).

3.2 Methodology

3.2.1 Proximity Data Loggers

Data in this chapter were previously collected during 2006 and 2007. The proximity data loggers used were a relatively new product developed by Sirtrack Ltd, New Zealand, which can be attached to the possums via a neck collar. The weight of each logger is 40g, which is 1.3% to 2.4% of a possum body weight (using the weight of the lightest and heaviest possums trapped in this study).

Each logger contains a UHF transceiver that broadcasts a unique ID code which can be detected by another logger within a user defined range, while it simultaneously detects the ID code of the other logger. Once detected, the receiving unit queries an onboard real-time clock (RTC) and begins counting. Once the contact is broken for a user definable period of time (15 seconds in this study), the ID code, date, time of contact and the duration of contact of the transmitting unit are stored into a non-volatile memory. Each unit also contains a VHF transmitter. The logger unit can be connected to a PC via a USB port through an interface to download data and set user parameters.

The receiving range of all data loggers purchased from SirTrack was tested before being deployed in the field. The minimum recording range varies among loggers from 0.5m to 4m. However, the loggers which had a minimum range over 0.5m recorded the presence of another logger as a string of 1 second events from a distance of up to 4m until within a range of 0.8 to 1.2m when the presence of a logger were recorded as a true interval. Therefore, instead of using the same record settings for all loggers, the recording settings were adjusted according to the sensitivity of each logger and loggers were standardised with a maximum recording range of 1.2m.

3.2.2 Fitting of Collars

Possums were live trapped in the Coatesville study site and, once transferred from the live trap into a secure box, were anesthetized with gaseous isoflurane and then fitted with a collar. A total of 11 females and 5 males had collars during the non-breeding

season. During the breeding season, 11 females and 4 males were fitted with collars. These numbers represent the number of males and females that were trapped. Data for the 'non-breeding' season were collected from November to February, a time of the year that breeding does not occur, while 'breeding season' data were collected between the months of August and October following removal of pouch young (to induce oestrous in the females).

3.2.3 Determination of Genetic Relatedness

A small ear tissue sample was taken from each possum captured. Genomic DNA was extracted using proteinase K digestion and a Bio-Rad AquaPure Genomic DNA isolation kit. Individuals were genotyped at 7 polymorphic microsatellite markers (TV12, Tv16, Tv19, Tv27, TV53, Tv54, and TV58) previously isolated from possums (Taylor, 1998). Microsatellite DNA amplification was performed using Polymerase Chain Reaction (PCR) with either the forward or the reverse of each microsatellite primer pair end-labelled with fluorescent dye (Applied Biosystems, Lincoln, USA). A combination of different dyes, the size of amplified DNA, and the optimum annealing temperature made it possible to combine all 7 primers in a single multiplex for PCR. Multiplex PCR amplifications were carried out in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Lincoln, USA) (Table 3.1) using a touch-down cycle: 4 min at 95°C, 10x(30sec at 94°C, 45sec at 70-60°C (-0.1°C each time), 45sec at 72°C), 35x(30sec at 94°C, 45sec at 60°C, 45sec at 72°C), 40min at 72°C and reduce to 10°C. Amplifications were performed in 10 µl reactions with FastStart Taq DNA Polymerase PCR buffer (Roche PCR Kit), 10 nmol of each primer, 2 mM dNTPs, 10 nmol BSA, 0.8 U FastStart Taq polymerase (Roche PCR Kit), and approximately 20 ng of gDNA. To verify amplifications, PCR products were run on a 2% agarose gel along with a 1kb DNA ladder as a molecular weight marker. Gels were stained with Ethidium bromide and exposed to UV light for visual checking.

PCR products were separated using capillary electrophoresis on an Applied Biosystems Genetic Analyser 3130xl (Applied Biosystems, Lincoln, USA) according to the manufacturer's instructions. Allele sizes were scored using Genemapper v4.0 software (Applied Biosystems, Lincoln, USA).

The maximum-likelihood method (Konovalov & Heg, 2008) was used to calculate genetic relatedness between each pair of possums using allele size data. The analysis was carried out using the Kingroup software (Konovalov *et al.*, 2004).

Locus name	TV12	TV16	TV19	TV27	TV53	TV54	TV58
Allele size	232-234	132-146	261-277	174-190	240-268	91-121	137-159
Fluorescent colour label	NED	VIC	VIC	VIC	6FAM	NED	6FAM

Table 3.1 Microsatellite loci used for genotyping possum DNA samples, recorded allele size, and fluorescent label used for multiplex PCR.

3.2.4 Data Analyses

To investigate the duration and number of interactions between the three different dyads (female-male, female-female, and male-male) for the breeding and non-breeding seasons, the duration and frequency of interactions were pooled according to dyad and season and then compared. For those pairs who had multiple interactions, the mean of their interactions was used. Statistical analyses were not conducted on these data as the non-independent nature of the data would make such analyses difficult. Furthermore, the patterns in the data are explicit in the graphs (A. Smith, pers. comm.). However, box plots were constructed to show the range of durations and frequencies for the three dyads during the two seasons (PASW Statistics v18) (Figures 3.1-3.6).

In order to investigate whether a correlation exists between relatedness and mating in the breeding season, interactions between males and females that lasted longer than two minutes, suggesting sexual interactions, were isolated. A minimum of two minutes was used for these analyses as mating typically last for two to four minutes in this species (Winter, 1976). The frequencies of these longer lasting interactions were then plotted against relatedness (Microsoft Office Excel 2007). Furthermore, genetic relatedness was plotted against the duration (for all durations lasting longer than two minutes and for those who did not interact at all) to test for a relationship between duration and relatedness.

To determine whether females are more tolerant of other females based on relatedness during the non-breeding season, interactions lasting longer than one minute

were isolated and their frequencies plotted against relatedness in a scatter plot (Microsoft Office Excel 2007). As with the female-male interactions, relatedness was also plotted against duration (over a minute). One minute was chosen as such a duration suggests that they are tolerable of one another if they stay within close contact for that long. Also, fighting would not be expected to last a minute so agnostic interactions can be ruled out. For both male-female and female-female interactions, the relatedness data for pairs that did not interact at all were also included on plots as a lack of interaction may be as important as the occurrence of interactions.

3.3 Results

3.3.1 Contact Rates During the Breeding Season and Non-Breeding Season

Female-Male dyads

During the non-breeding season, over 1,829 close interactions, ranging from 2.38 to 642.58 minutes, were recorded between 7 of the possible 55 pairs of females and males. During the breeding season, we recorded 2,461 interactions ranging from 0.28 to 756.15 minutes between 15 of the possible 44 pairs (Figures 3.1 and 3.2).

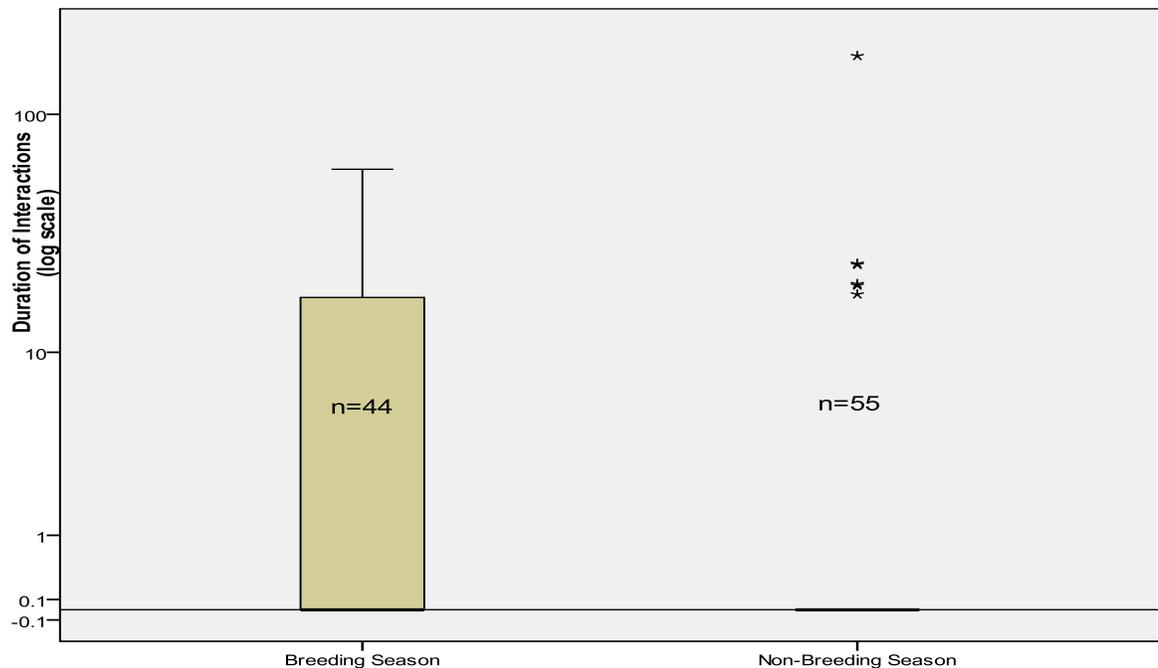


Figure 3.1 The amount of time (minutes) that male and female pairs spent interacting during the breeding and non-breeding seasons, with time on a log scale. For pairs who had multiple encounters, the mean duration of their visits was used. The horizontal line shows the location of zero seconds. The asterisks are outlier data points.

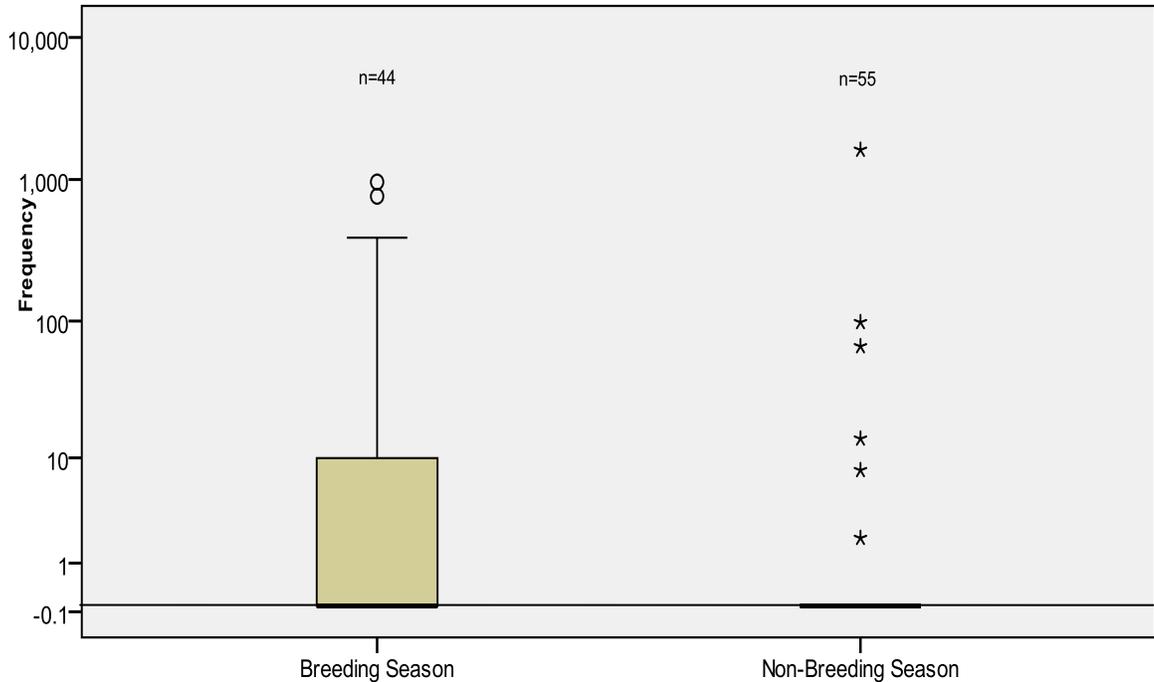


Figure 3.2 The frequency of interactions between male and female possums during the breeding and non-breeding seasons with frequency on a log scale. The horizontal line shows the location of zero seconds. The asterisks are outlier data points.

Female-Female dyads

During the non-breeding season, 247 interactions were recorded between 12 of the possible 55 female-female pairs. The total duration of interactions ranged from 0.17 to 31.13 minutes for the different dyads. During the breeding season, 58 interactions (Figures 3.3 and 3.4) were recorded between 7 of the possible 56 pairs of collared females totalling 0.42 to 11.5 minutes for different pairs.

Male-Male dyads

Close interactions between males did not occur in the non-breeding season. During the breeding season, 1 of the possible 6 male-male dyads was in close contact for a total of 0.58 minutes over 6 interactions (Figures 3.5 and 3.6).

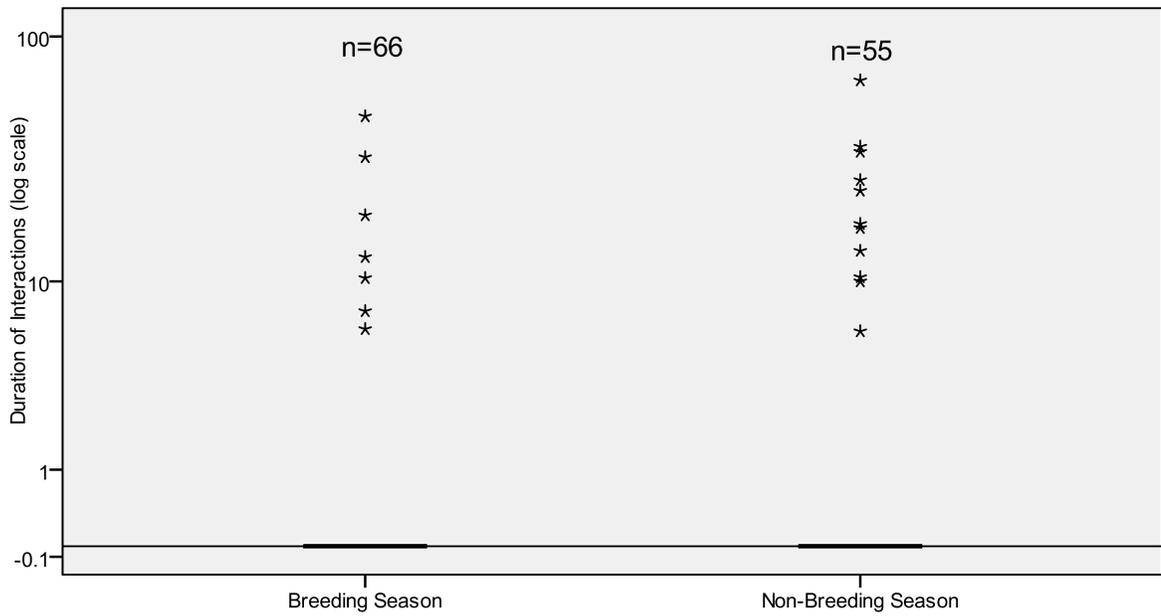


Figure 3.3 The amount of time in minutes that female pairs spent interacting during the breeding and non-breeding seasons, with time on a log scale. For pairs who had multiple encounters, the mean duration of their visits was used. The horizontal line shows the location of zero seconds. The asteriks are outlier data points.

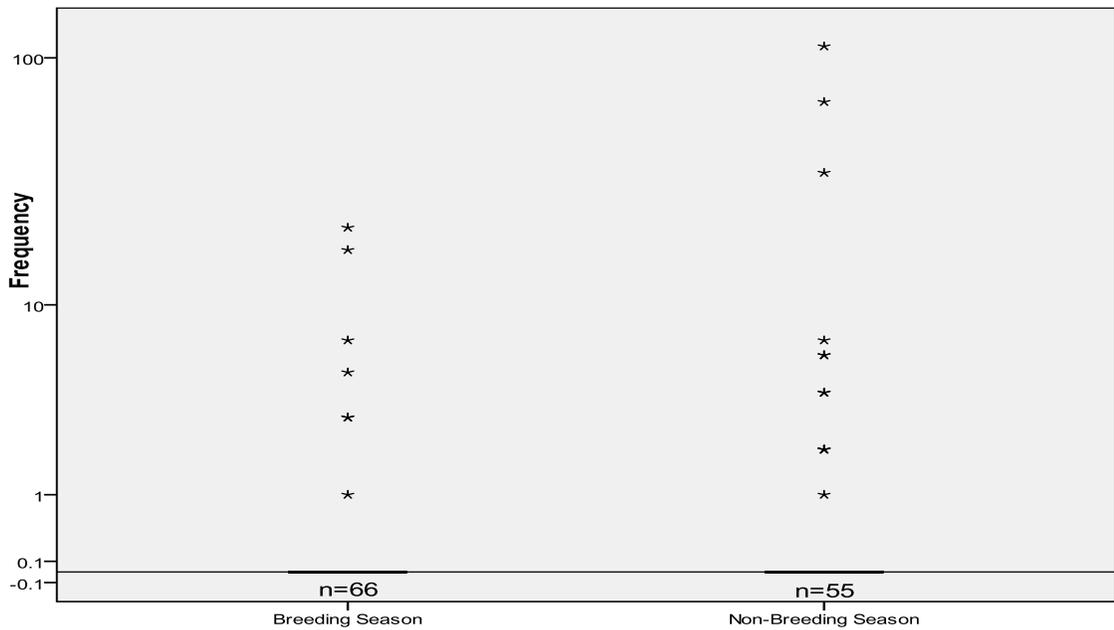


Figure 3.4 The frequency of interactions between female possums during the breeding and non-breeding seasons with frequency on a log scale. The horizontal line shows the location of zero seconds. The asteriks are outlier data points.

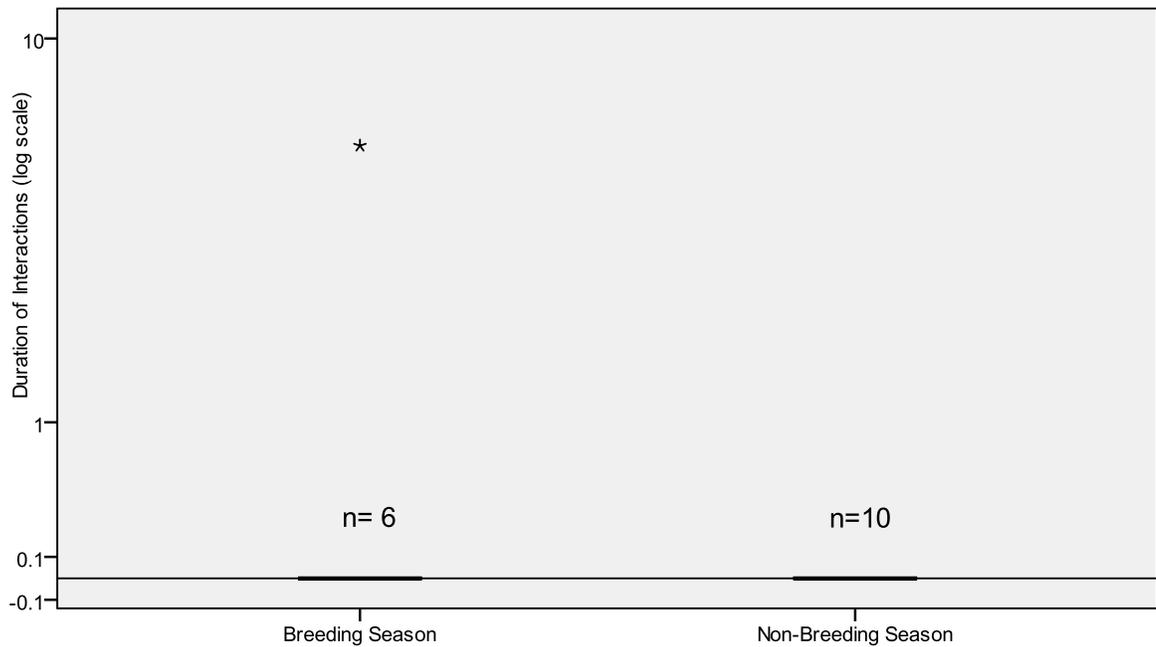


Figure 3.5 The amount of time in minutes that male pairs spent interacting during the breeding and non-breeding seasons, with time on a log scale. For pairs who had multiple encounters, the mean duration of their visits was used. The horizontal line shows the location of zero seconds.

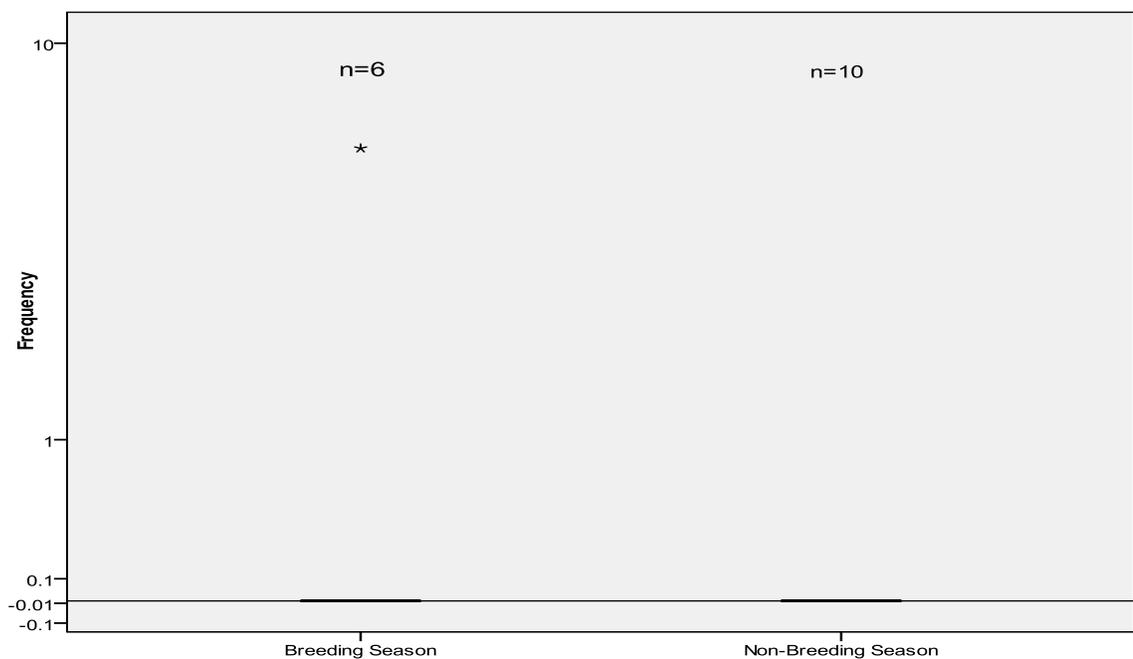


Figure 3.6 The frequency of interactions between male possums during the breeding and non-breeding seasons with frequency on a log scale. The horizontal line shows the location of zero seconds. The asteriks are outlier data points.

3.3.2 Correlation Between Frequency and Duration of Interactions and Relatedness Between Male-Female Dyads

There is no significant correlation between relatedness and the frequency of interactions between males and females that lasted longer than 2 minutes ($R^2=0.0058$; $n=35$, Figure 3.7). Relatedness does not have a significant effect on the duration of interactions either ($R^2=0.0087$; $n=35$, figure 3.8). Eight pairs, five of whom did not interact at all, were not included in the analyses as genetic data were unavailable for at least one the possums.

3.3.3 Correlation Between Frequency of Long Lasting Interactions and Genetic Relatedness Between Female-Female Dyads

Among females that interacted with each other, genetic relatedness is not correlated with the frequency of interactions lasting over 1 minute ($R^2=0.0007$; $n=39$, figure 3.9) or with the duration of interactions ($R^2=0.0029$; $n=39$, figure 3.10). As with the previous section, genetic data were not available for all females so the data for ten pairs (none of them interacting) were not used in these analyses.

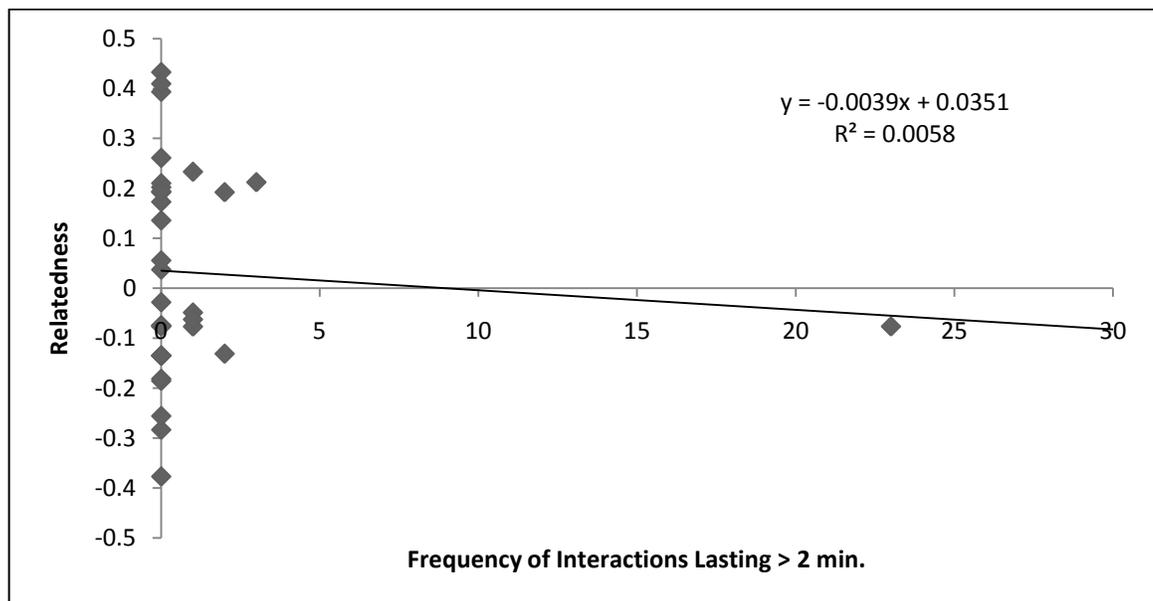


Figure 3.7 The relatedness between different male-female dyads plotted against the frequency of interactions that lasted for more than two minutes, suggesting mating, during the breeding season. Male-female pairs that never interacted (i.e. frequency equals zero) are included.

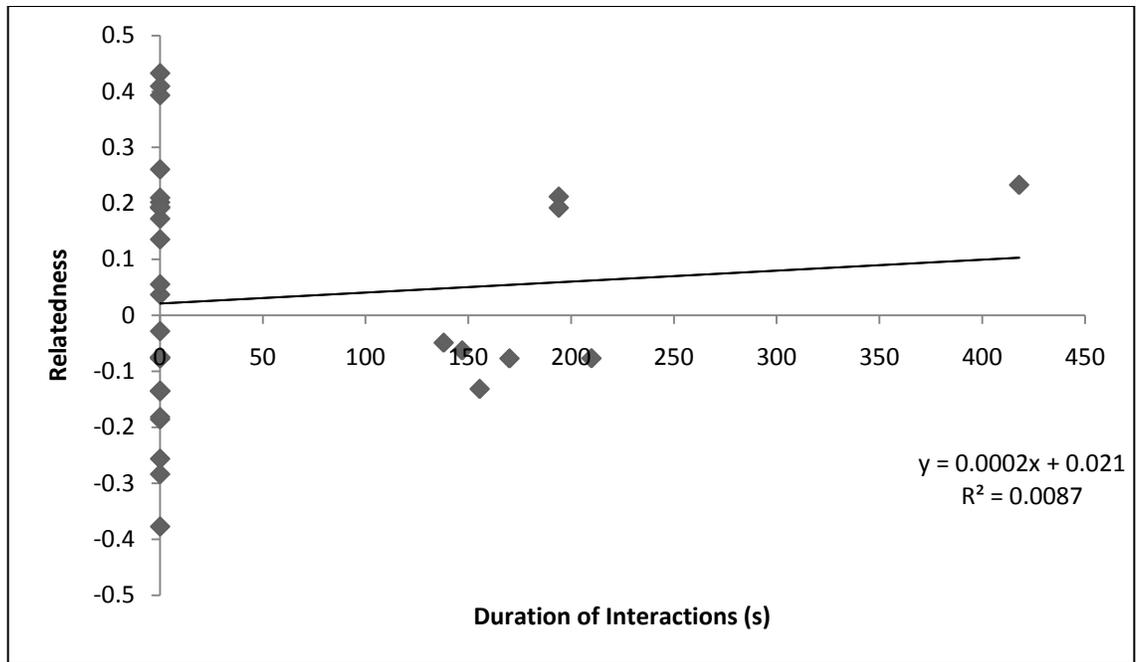


Figure 3.8 The relatedness between male and female pairs plotted against the duration of their interactions. For pairs who interacted more than once, the mean duration was used.

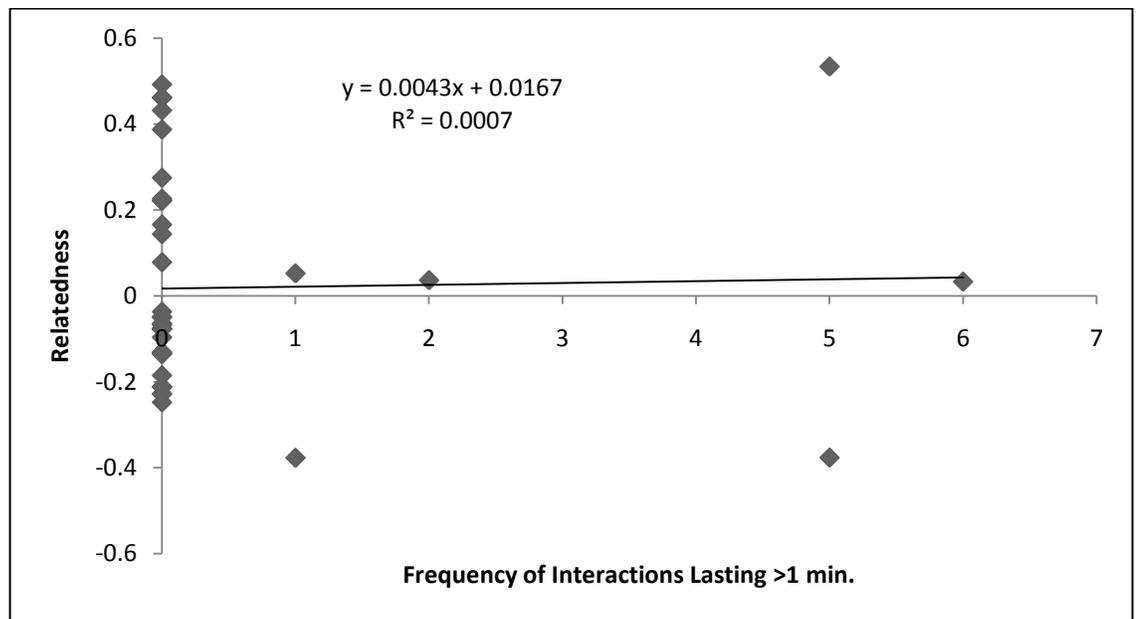


Figure 3.9 The relatedness between different female-female dyads plotted against the frequency of interactions that lasted for more than one minute, suggesting a high level of tolerance, during the non-breeding season. Female-female pairs that never interacted (i.e. frequency equals zero) are included.

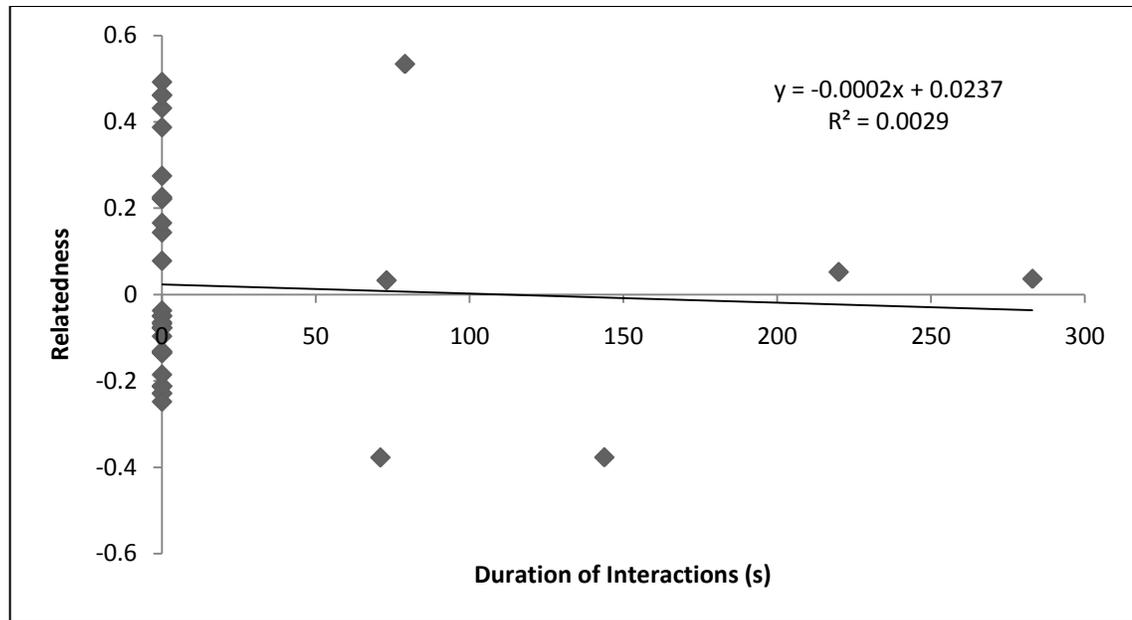


Figure 3.10 The relatedness between female pairs plotted against the duration of their interactions. For pairs who interacted more than once, the mean duration was used.

3.4 Discussion

3.4.1 Contact Rates During the Breeding Season and Non-Breeding Season

Close interactions have been recorded between females and between males and females in both the breeding season and the non-breeding season. There are significantly more interactions between females and males than between the same sex, reflected in both duration and frequency of contacts. Close interactions between males are rare, brief and are only recorded in the breeding season. There is a clear difference between the time that females spend together *versus* the time that they spend with males during both the breeding and non-breeding seasons with female-male dyads spending significantly more time together in both seasons. Despite the occurrence of more interactions between females in the non-breeding season (12 *versus* 7 male-female pairings), males and females, on average, spent 12.3 times as much time together. Although the difference in time spent is even more significantly different between these two types of dyads in the breeding season with males and females spending, on average, 48.1 times as many seconds together, it must be taken into account that there were more male-female pairings (15 *versus* 7 for female-female pairings). The patterns for number of interactions are the same in the non-breeding season with males and females engaging in 7.40 times as many interactions as female-

female pairs. Similarly, in the breeding season there were 42.4 times as many male-female interactions as there were female-female interactions.

Contrary to the results for these two types of dyads, interacting dyads composed of two males were rare in this study with only one dyad interacting on six occasions during the breeding season (Figures 3.5 and 3.6). While it is possible that the results of this study at least partially reflect the small number of males that were present to be fitted with collars (5 males versus 11 females in the non-breeding season; 4 males *versus* 11 females in the breeding season), it should be noted that having such a small number of collared males did not result in a rare number of interactions between males and females. Avoidance between males has been noted in another study which was conducted in captivity (Day *et al.*, 2000).

These data suggest that whilst both female-female and female-male interactions occur during both the breeding and non-breeding seasons, that it is mixed-gender interactions that are much more common during both of these seasons and that interactions between males are quite rare. Such a pattern between frequency of interactions and the gender of participants has also been noted in another marsupial species, the Virginia opossum (*Didelphis virginiana*). Across both the breeding and non-breeding seasons, male-female opossum pairs had the highest frequency of interactions followed by female-female pairs and then by male-male pairs (Holmes, 1991).

The findings in this study regarding interactions between males and females during the different seasons are similar to the results of another study on possum interactions which did not find a significant difference in the amount of time that males spend with oestrous and anoestrous females (Ramsey *et al.*, 2002). On average, the amount of time that collared males and females interacted between the breeding and non-breeding seasons in our study is not very different (6,901 seconds on average in the breeding season *versus* 6,255 on average seconds in the non-breeding season). This shows that there are indeed many mixed-sex interactions occurring when females are not in oestrous.

These data are in contrast to the study conducted by Day *et al.* (2000) as they found that interactions were more frequent during the non-breeding season. However, as previously mentioned, the authors propose that the results reflect the fact that the study was conducted in captivity. Furthermore, interactions were more frequent between female-female pairs than between female-male pairs which is in contrast with the results presented

herein. The results of my study are more in line with the results of the previous study that was conducted on wild possums (Ji *et al.*, 2005) as both studies found, as to be expected, that there are more interactions during the breeding season.

Perhaps as technology improves and with the necessary funding, such a study can be repeated using data loggers that can be set to log interactions that occur at a much closer distance. The distance that the loggers were triggered at in this study, 1.2 metres, is quite a large distance and can include interactions such as displays which are not necessarily important when considering issues such as the spread of bovine tuberculosis or a self-disseminating vector for possum control. These concerns would be better served by gaining an understanding of the frequency and duration of much closer interactions such as when they are within less than half a metre of each other. This would allow us to be more confident that they are actually engaged in physical contact whether it be mating, denning, grooming, or fighting.

3.4.2 Correlation Between Duration of Interactions for Male-Female Dyads and Relatedness

Although the trend shows a higher frequency of long interactions between males and females as relatedness decreases, relatedness does not have a significant effect on mate choice in brushtail possums. The null hypothesis that relatedness does not influence the frequency of sexual interactions thus cannot be rejected. These data suggest that possums are either unable to detect degree of relatedness or that there are other factors that are more influential when it comes to mate choice, at least in these particular possums. It is unfortunate, however, that genetic data were not available for possums involved in the longest interactions (36,570 and 45,369 seconds) as these two interactions were distinctly longer than any others and may have an impact on the trend of the data.

As majority of the interactions in this study occurred between females and only two males, these data may not accurately reflect the true nature of possum mate choice. Perhaps possums will mate with another individual who is relatively closely related but focus more of their attention on those who are less related. Other possibilities are that these data actually reflect the mating system of possums and that relatedness is not important either due to detection issues or due to lack of importance. Another possibility is that they

do have preferences for distantly related individuals but are limited to those mates with whom their territory overlaps. This is supported by a study which found that matings mainly only occurred between possums who had adjacent ranges (Clinchy *et al.*, 2004). A captive study may help resolve this as one male or female possum can be in an enclosure with multiple individuals on the opposite sex and of varying degrees of relatedness. This may allow us to see, if given a choice, if they have a preference for individuals who are more or less related. It is thus necessary to conduct further studies with a larger number of animals so that a more general pattern can be detected. Captive studies may help reveal whether relatedness affects mate choice while studies in different wild populations will allow for other ecological factors, such as population density, to be considered.

3.4.3 Correlation Between Duration of Interactions for Female-Female Dyads and Relatedness

Similarly, relatedness does not have a statistically significant effect on the frequency of long lasting interactions between female-female pairs during the non-breeding season although the trend shows a slight increase in the frequency of such interactions with an increase in relatedness. Thus, the null hypothesis that relatedness does not have an effect on the tolerance levels that female possums display towards one another cannot be rejected.

As mentioned previously, captive studies may help us to further understand any patterns related to whom possums choose to spend time with. Animals may be more tolerant of and less agonistic towards those who are genetically related (e.g. Holmes, 1986; Blumstein *et al.*, 2002; Archie *et al.*, 2006) or to those with whom they are more familiar (e.g. Kareem & Barbard, 1982; Fredrickson & Sackett, 1984). It would thus be interesting to see how much time female possums spend grooming and nesting with other females of varying relatedness and familiarity. In a captive setting, relatedness and familiarity can both be controlled and may thus prove to be an effective way to test how important these two factors are to female possums.

Chapter Four: Possum Sternal Gland Communication and the Use of Scents as Lures

4.1 Introduction

How animals use various scents to communicate with one another is an aspect of animal behaviour that has the potential to be very applicable in the field of conservation biology. Understanding what kinds of messages are left behind and how animals respond to the messages can assist in the management of wildlife. Determining how animals respond to olfactory messages can then be taken a step further and tested for practical purposes. Specifically, we can ask questions such as can various scents be used to either attract animals to a certain location or to deter them from a location? And if so, is there variation in the effectiveness of different scents with regards to the gender or physiological status of the scent producer, for example? This is where olfactory communication can be particularly useful for wildlife managers.

Numerous studies have been conducted on possible applications of olfactory communication such as using scents as attractants or repellents. Clapperton *et al.* (1999) demonstrated that scents from the anal glands of stoats (*Mustela erminea*) were successful at attracting conspecifics to tracking tunnels. This can have significant management applications as stoat scent has the potential to provide a more species specific lure and may be stronger than food lures. Furthermore, the use of scented lures (beaver castoreum and catnip oil) successfully attracted a higher number of lynx (*Lynx canadensis*) to hair snares, and induced rubbing behaviour at the snares, which can assist in lynx detection studies (McDaniel *et al.*, 2000). Thus scents have the potential to assist in wildlife management as they may be used to attract animals to specific locations.

Other studies have focused on using the scents of predators (from urine, faeces, anal gland secretions, and hair) to deter prey animals. For example, Borgo *et al.* (2006) demonstrated that flying squirrels (*Glaucomys volans*), who compete with the endangered red-cockaded woodpecker (*Picoides borealis*) for roosting cavities, were less likely to use roosting sites that contained the scents of the following predators: fox squirrel (*Sciurus niger*), bobcat (*Lynx rufus*), red fox (*Vulpes vulpes*), raccoon (*Procyon lotor*), king snake (*Lampropeltis getula*), and corn snake (*Elaphe guttata*). Additionally, predator scents

successfully reduced the amount of browsing done by penned black-tailed deer (*Odocoileus hemionus columbianus*) (Melchior & Leslie, 1985) and penned brushtail possums (Woolhouse & Morgan, 1995). Other studies, however, did not find the subject animals to be deterred and a few animals were actually attracted to traps containing predator scents (Bramley & Waas, 2001; Russell & Banks, 2005; Russell & Banks, 2007). These studies demonstrate the potential that scent applications can have and also show that more research is needed as some studies have yielded unexpected results or were not done in a natural setting which could have altered the behaviour of the animals.

Previous studies on the luring ability of food have not been promising with brushtail possums. When tested as lures, scents such as cherry, orange, plum, and nuts either did not show a significant increase in possum visitation or, as was the case with cinnamon, only attracted individuals if they were within two meters of the station (Morgan *et al.*, 1995). Todd (1995) found similar results in her testing of possum odour preferences. She found that possums were not attracted to cinnamon, almond, peanut, cloves, orange, or the odour of woodrose (*Dactyloctenium aegyptium*) when compared with water. These studies demonstrate a need for research into the development of a more attractive and effective lure.

This study investigates how possums respond to the scent of other possums including possums of different genders and possums from a different population. Specifically, I tested two hypotheses: 1. Possums will be more attracted to the scent of unfamiliar possums. This is the predicted outcome as studies on other species show that individuals are more attracted to individuals who are less related (reviewed in Penn & Potts, 1999; Penn, 2002; Parrott *et al.*, 2007). 2. Possums are more interested in possum scent than in the scent of a food item. This is predicted as effective lures for other mammals contain scents with which the animals are familiar (Morgan *et al.*, 1995), because possums have not displayed a great interest in food lures that have been tested in other studies (Morgan *et al.*, 1995; Todd, 1995), and because tests were conducted during the breeding season, a time when possums are searching for conspecifics.

4.2 Methodology

4.2.1 Scent Sample Collection

Scent samples were collected from the sternal area of live trapped possums at the Coatesville site and from captive possums inhabiting Landcare Research's facility in Lincoln, New Zealand. For details of live trapping, handling, and anaesthetising, see chapter 2. Once the possum was anaesthetized, a piece of sterile gauze was rubbed multiple times on the sternal gland area in order to collect the animal's sternal gland scent. The gauze was immediately placed into a small vial and stored in a chilly bin for no more than three to four hours until being stored at -80 °C.

4.2.2 Experimental Design for Testing the Response of Possums to Scent Samples

Test sites selection:

Experiments were carried out in 5 locations at the Coatesville study site during March of 2010 as this is the time of the year that most breeding occurs (Fletcher & Selwood, 2000). These locations were selected based on the information of capture rate obtained from the live trapping sessions in other parts of this study. The areas with highest capture rate, indicating higher possum traffic relative to other areas, were chosen as test sites.

Scent presentation

In total, five different experiments were conducted over the study period (Table 4.1) aiming to test different responses between genders, foreign and familiar possums, and between possum scent and food. The scents of mature possums were utilized in these trials as only a small number of juvenile samples were collected from the wild and captive possums (majority of trapped possums were adults). For each experiment, three different pieces of gauze were presented to the possums, two being treatments and one being a control (a plain piece of sterile gauze). All gauze pieces utilized were pre-sterilized (by the manufacturer) and individually packaged when purchased. Each combination of scents was presented four times, with the order of presentation changing each time. Latex gloves were worn while handling the pieces of gauze to avoid the transfer of other biological scents.

Documentation of possum responses to scent samples

Individual ID by tail fur clipping

Before the testing period, possums were live trapped and marked for identification purposes. While under anaesthesia, each possum had his/her tail hair trimmed in a distinctive pattern (figure 4.1). Possums were trapped throughout the entire study area in Coatesville regardless of how close the traps were to the experimental sites to ensure a high level of success at identifying possums in the video footage. A total of eighteen possums were marked.

Test	Sample 1	Sample 2	Sample 3
1	Mature Coatesville Female	Mature Coatesville Male	Control
2	Mature Lincoln Female	Mature Lincoln Male	Control
3	Mature Coatesville Male	Mature Lincoln Male	Control
4	Mature Coatesville Female	Mature Lincoln Female	Control
5	Mature Coatesville Female	Wheat-bran/Cinnamon Apple	Control

Table 4.1 The five different tests that were conducted to test the response of possums to the scents of males *vs* females, to the scents of familiar *vs* foreign possums, and to the scents of possums *vs* a popular food item.

Equipment set up

Pilot study on possum nocturnal activity

A pilot study was conducted in order to test the equipment, the set up (scent presentation, see above), and to determine what hours of the night receive the most possum activity. For the pilot study, recording occurred over four nights in February, 2010. Sessions commenced in the hour of sunset (2000 hrs and 1900 hours) and continued until 0500 hours when power was usually lost. In these early studies, it was evident that majority of possum activity occurred between the hour of sunset and 0100 hrs (Figure 4.2).



Figure 4.1 The distinctive tail hair trimming given to adult male, Neewa.

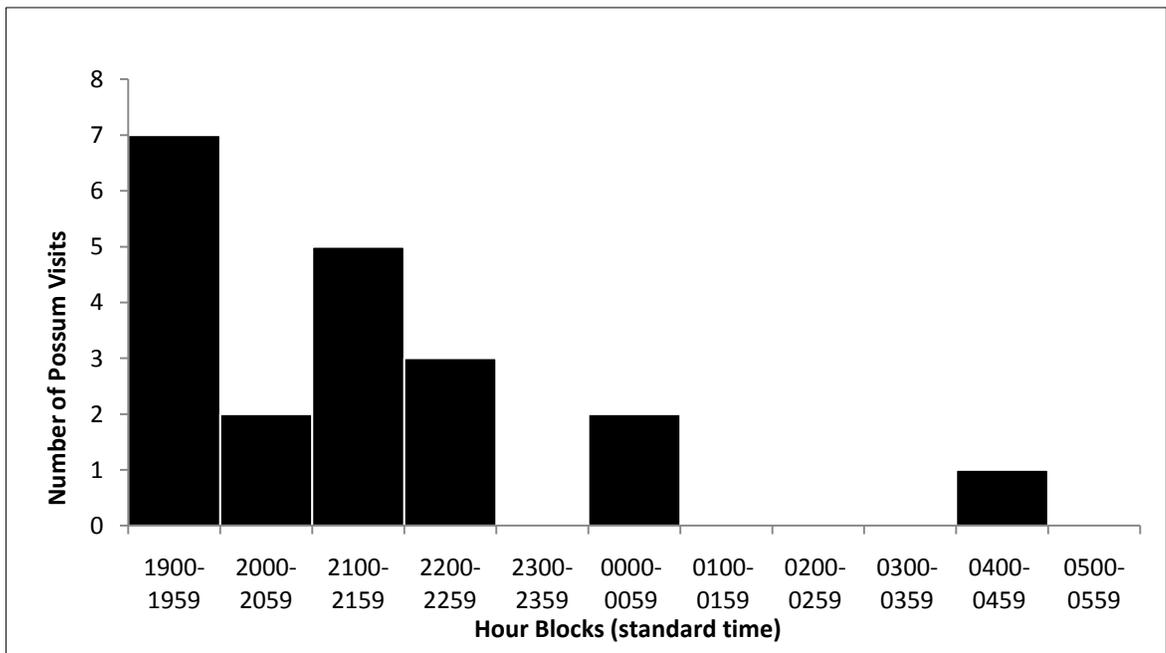


Figure 4.2 The number of possum visits that occurred during different hour blocks when DVR recorders recorded activity until 0500 hours. The data is the sum of activity from five different nights (twenty visits in total).

The responses of the possums to the various scents presented were recorded using a DVR recorder (Avermedia EB1304MOB) and infra-red, waterproof cameras (Sony IP67). These were powered by deep cycle or car batteries. Gauze pieces were taped to a tree at a height of approximately 25 cm above ground level (approximately the height of a possum's head) and approximately 25 cm apart (figure 4.3). Due to low levels of visitation during the pilot study, a few pieces of wheat-bran/cinnamon apples were left at the test station and in the surrounding area in an attempt to attract more individuals to the location.

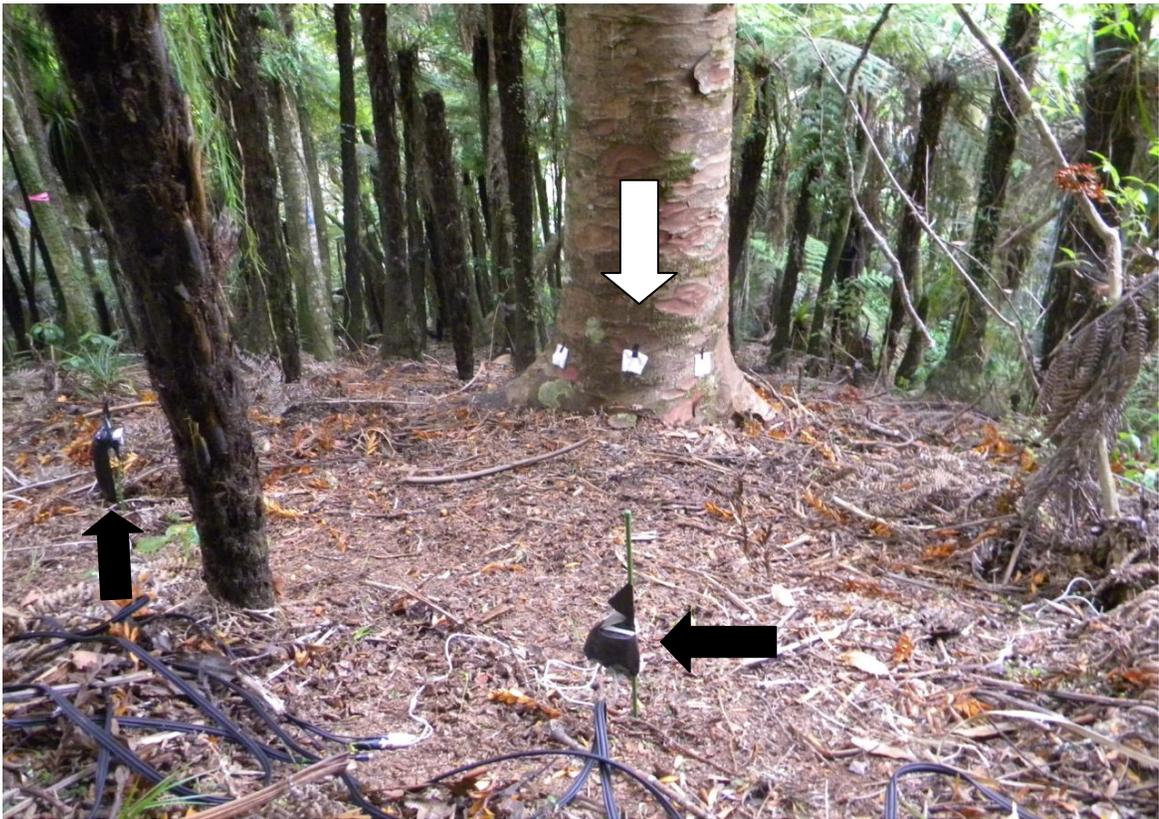


Figure 4.3 Photo of the experimental set-up at one of the test sites in Coatesville showing the placement of the three pieces of gauze (white arrow) and two of the three cameras (black arrows).

Habituation period

Tests were conducted at each site for three continuous nights and then the equipment was moved to the next location. The move was done 4-5 days before commencement of the next experimental session to allow the possums in the area to become acclimated to the presence of the equipment.

Recording intervals during the trial

Testing commenced in the hour of sunset (originally 1900 hrs and then 1800 hrs) and continued until battery power was lost which was typically between 0200 and 0400. Using lighter and more manageable batteries that did not run as long was justified by analysing the data from the pilot study and initial parts of the formal study when a larger battery (hence running all night) was used. Trials took place between 8 March and 29 March, 2010.

4.2.3 Analyses

Data were downloaded from the DVR's and analyzed using Avermedia USB Playback Console. The amount of time spent by each possum investigating each of the three pieces of gauze in a test was calculated as well as the identity of the possum investigating the scents (the duration of investigation was timed three times from the video footage, and then averaged). If a possum could not be identified, the data was only used in the analysis of the "total" response and in the analysis of behavioural responses (see below). In a small number of instances, a possum investigated a piece of gauze after a different possum had over-marked that piece of gauze at an earlier time. Such data were excluded from the 'investigation duration' and 'behavioural response' analyses (4.3.1, 4.3.2) as it could not be certain if the possum was responding to the original scent or to that of the previous investigator. These data were used, however, for the 'order and presence of conspecifics' section (4.3.3).

Duration of Investigation

To determine which scents attract more investigation time, a relative interest, the amount of time that each possum spent at a particular scent minus the amount of time that the same possum spent investigating the control gauze, was used and these were compared across all individuals (if the same individual made multiple visits, an average of the relative interest for that individual was used in the analysis). This was done separately for all five tests and the differences in their responses towards the scents were compared using a Wilcoxin matched-pair signed rank test. The null hypothesis was rejected for one of the five tests so a Fischer's exact test was utilized to determine whether the differences seen in

the responses were due to the gender of the visiting possum. SPSS version 18 was used for all of these analyses.

Behavioural Response

The observed responses of possums to scent samples ranged from lack of investigation to over-marking the gauze and marking the surrounding area following scent investigation (Table 4.2). The different responses were assigned ranks accordingly (Table 4.2). Level of behavioural response was averaged for males, females, and juveniles for

Behaviour	Description	Rank
Ignore Scent	The possum walks past the scent without any investigation	0
Sniff Scent	The possum places his/her nose on the gauze and displays active investigation	1
Rip gauze off tree to investigate	Following investigation, the possum removes the gauze from the tree for further investigation	2
Mark area following investigation	The possum marks (sternal, chin, anal, etc.) the tree and/or the ground in the test area after investigation	3
Over-mark gauze 1 time	The possum over-marks the gauze one time with any gland (sternal or chin)	4
Over-mark gauze >1 time	The possum over-marks the gauze multiple times	5
Over-mark gauze and the surrounding area	Following investigation, the possum over-marks the gauze and surrounding area (tree, ground, etc.)	6

Table 4.2 An ethogram of the different behavioural responses recorded as well as the rank given to the different responses.

each trial to visualize any patterns that may be present. Due to such a small sample size, statistical analyses were not utilized for this section.

Additionally, multi-dimensional scaling (MDS) plots were constructed (using Primer 5) for each of the five tests to see if behavioural responses were similar based on gender or maturity. MDS plots were used as they provide a visual representation of relationships with the distance between variables on the plot reflecting the degree of similarity between them. This method therefore allows for the visualization of patterns (or lack thereof). For animals that made multiple visits during a test, the mode of their responses was used. In addition to MDS plots, the data for each test were also analyzed for patterns using an analysis of similarity (ANOSIM) (Primer 5). ANOSIM, which is essentially a non-parametric version of analysis of variance (ANOVA), was used to analyse these data as it allows for the behavioural responses of the possums to different treatments to be compared against one another.

Order of Visitation and Presence of Conspecifics

The data collected were also analyzed to consider the possible effect of the experimental design on the results. In order to analyse this, I looked at how frequently the first investigated scent was the first scent encountered by the possum (based on the direction that possum entered from). Additionally, I looked at what proportion of possums investigated only that scent which was investigated first and also what percentage of investigators were alone and how many had conspecifics present at the test site. To look at the possible effect that conspecifics may have on the behaviour of the investigator, a Fischer's exact test (GraphPad Software) was used to analyze the differences between the number of possums who had a conspecific present versus those who did not for the following groups: those who only investigated one scent versus those who investigated more than one and then for those who investigated any scent versus those who investigated none at all. For these analyses, tests one through four were combined and test five was analyzed separately as this test was different from the others because a non-possum scent was used (table 4.1).

4.3 Results

4.3.1 Investigation Duration

There is a trend of possums spending more time investigating the scent of Coatesville females (relative interest average 7.09 seconds) than Coatesville male scent (average 0.47 seconds). However, the difference is not statistically significant ($p=0.075$; $n=6$) (Figure 4.4).

No difference was detected between the time possums spent investigating Lincoln male scents (average 2.12 seconds) and the time spent investigating Lincoln female scents (average 1.85 seconds) ($p= 0.715$; $n=4$) (Figure 4.4).

The average time that possums spent investigating the Lincoln male scent seem to be longer (average relative interest 7.85 seconds) than the time investigating Coatesville male scents (average 4.13 seconds). However, the difference is not statistically significant ($p=0.398$; $n=7$) (Figure 4.2).

Similarly, the possums showed more interest in the scent of unfamiliar females when Lincoln female and Coatesville female scents were compared, although the difference is not significant. The average relative interest was -0.41 seconds for the Coatesville female scent and 7.51 seconds for the scents of Lincoln females ($p=0.180$; $n=5$) (Figure 4.4).

The possums showed a clear preference for the food scent over Lincoln female scents ($p=0.033$; $n=13$), with an average relative interest of 13.36 seconds at the food scent and only 3.22 seconds at the Lincoln female possum scent (Figure 4.4). The difference seen in the responses of the possums to these two scents is not dependant on the gender of the investigators ($p=0.692$; $n=10$).

4.3.2 Behavioural Responses

For the trial comparing Coatesville female and male scents, both males and females exhibited the highest level of response to the scent of males (average rank = 2.5 and 2, respectively). Male possums then responded to the control gauze (2) with the next highest level of intensity followed by the scent of females (1). Female possums, on the other hand, respond to the scent of females with a greater intensity (1.67) than to the control gauze (1) (all in Figure 4.5; refer to Table 4.2 for description of ranks).

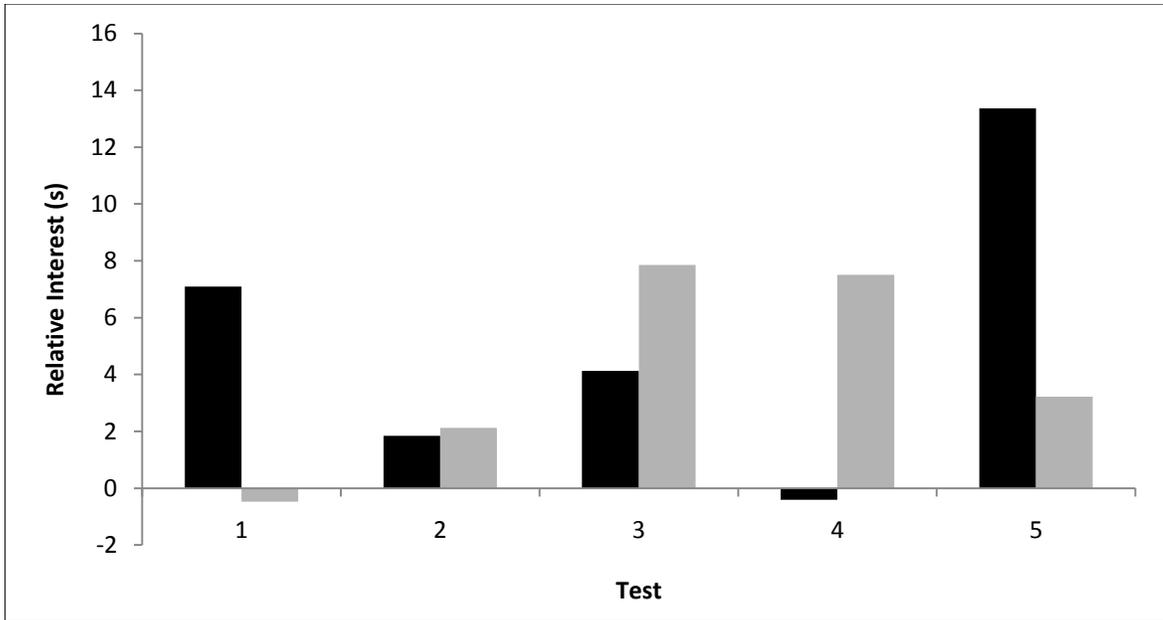


Figure 4.4 The relative interest (duration in seconds spent investigating the scents minus the duration in seconds investigating the control) of all possums to various scents during the five trials. Test 1: Coatesville female (black); Coatesville male (grey). Test 2: Lincoln female (black); Lincoln male (grey). Test 3: Coatesville male (black); Lincoln male (grey). Test 4: Coatesville female (black); Lincoln female (grey). Test 5: cinnamon/wheat-bran apple (black); Lincoln female (grey).

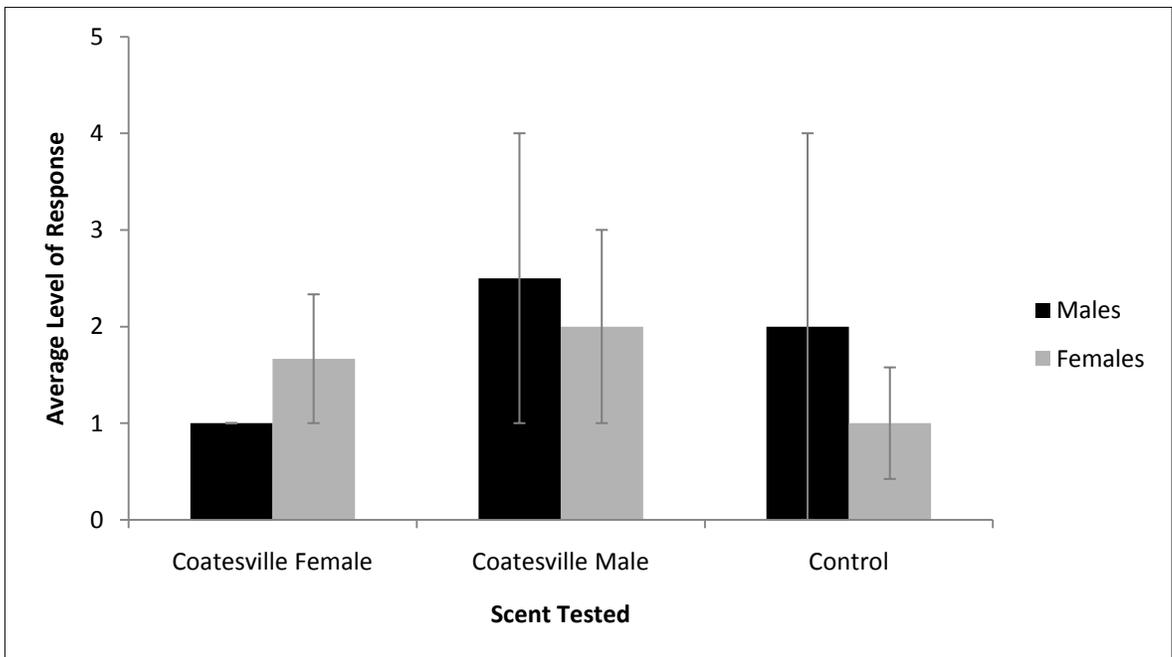


Figure 4.5 The mean level of behavioural response displayed by possums during the trials comparing Coatesville female and male scents. Error bars represent the standard error. Refer to table 4.2 for description and rank of responses.

The Coatesville male possum had a stronger response to the Lincoln male scent (3) than to the Lincoln female scent and the control (0 and 0, respectively). Coatesville female possums displayed a slightly higher level of response to Lincoln female scents (2.5) *versus* Lincoln male (2) and control (2). The one juvenile visitor had a higher level of response to the Lincoln female scent (1) compared with the response to Lincoln male scent and control (0 and 0, respectively) (Figure 4.6).

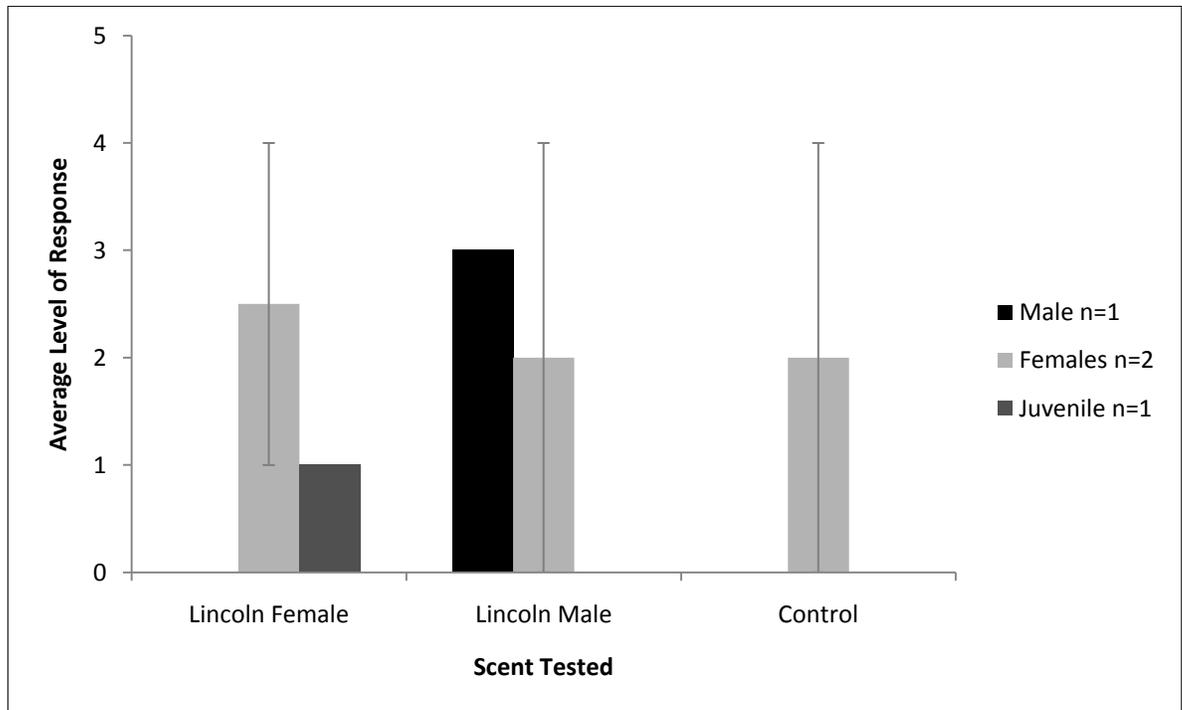


Figure 4.6 The mean level of behavioural response displayed by possums during the trials comparing Lincoln female and male scents. Error bars represent the standard error. Refer to table 4.2 for description and rank of responses.

There was a large difference in the level of response by the one male visitor to scents of Lincoln males (6) over that of Coatesville males and the control (0 and 0, respectively). Females showed a slightly higher level of response to the Lincoln male scents (2.25) *versus* the scents of Coatesville males (2) and to the control (1.25). The response levels of the two juveniles in this test were the same for the two treatments and control (all = 0.5) (Figure 4.7).

Male visitors responded with equal intensity to the scents of Coatesville and Lincoln females (3) which were greater than their response to the control (0.5). The one

female visitor responded equally to all three presentations (6). The unmarked adult displayed a response of level 2 to the control while the two treatments were ignored (0). The one juvenile investigator also responded more to the gauze (1) than to the two treatments (both = 0) (Figure 4.8).

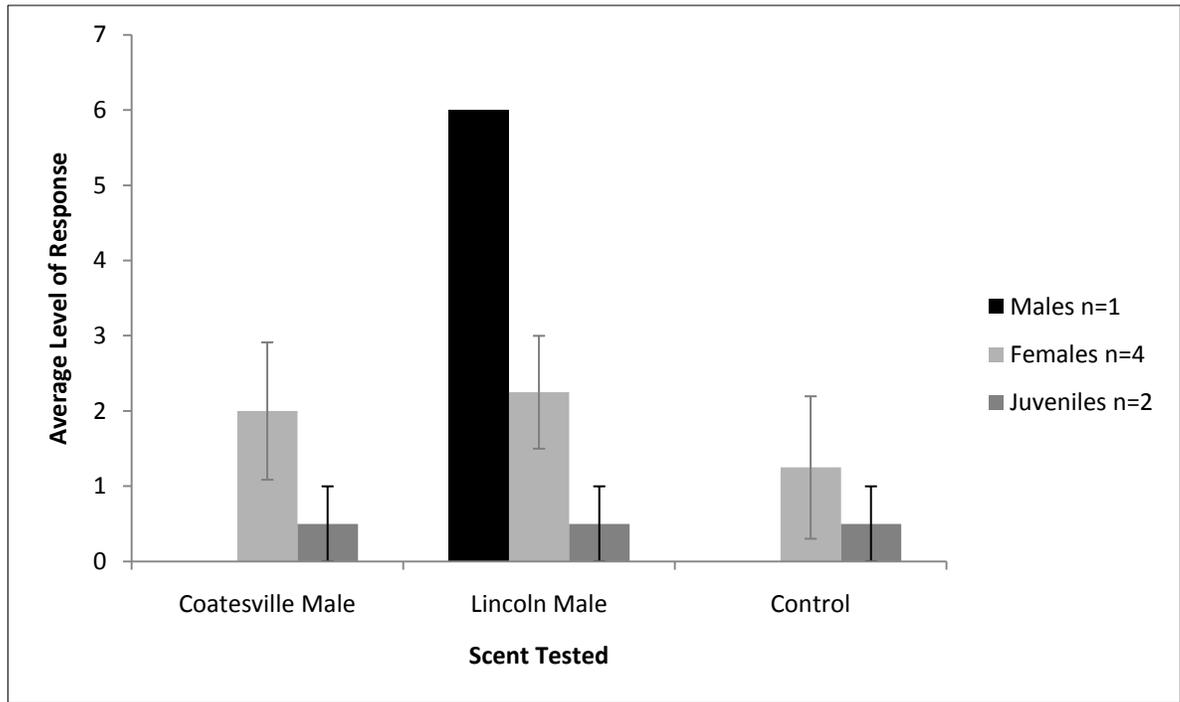


Figure 4.7 The mean level of behavioural response displayed by possums during the trials comparing Coatesville and Lincoln male scents. Error bars represent the standard error. Refer to table 4.2 for description and rank of responses.

In the test comparing the responses of the possums to Lincoln female scents and the scent of cinnamon/wheat-bran apple (Figures 4.9 and 4.10), male visitors had a greater response to the apple scent (3.5) than to that of Lincoln females (1.5) and control (0.5). Females showed a greater level of response to the Lincoln female scent (3) than they did to the control (1.6) and apple (1.4). The unmarked adult’s levels of response were greatest for the apple (4) than for Lincoln females (1) and control (1). The two juveniles showed more of a response to the scent of the Lincoln females (2) than to the apple (1.5) and control (1.5).

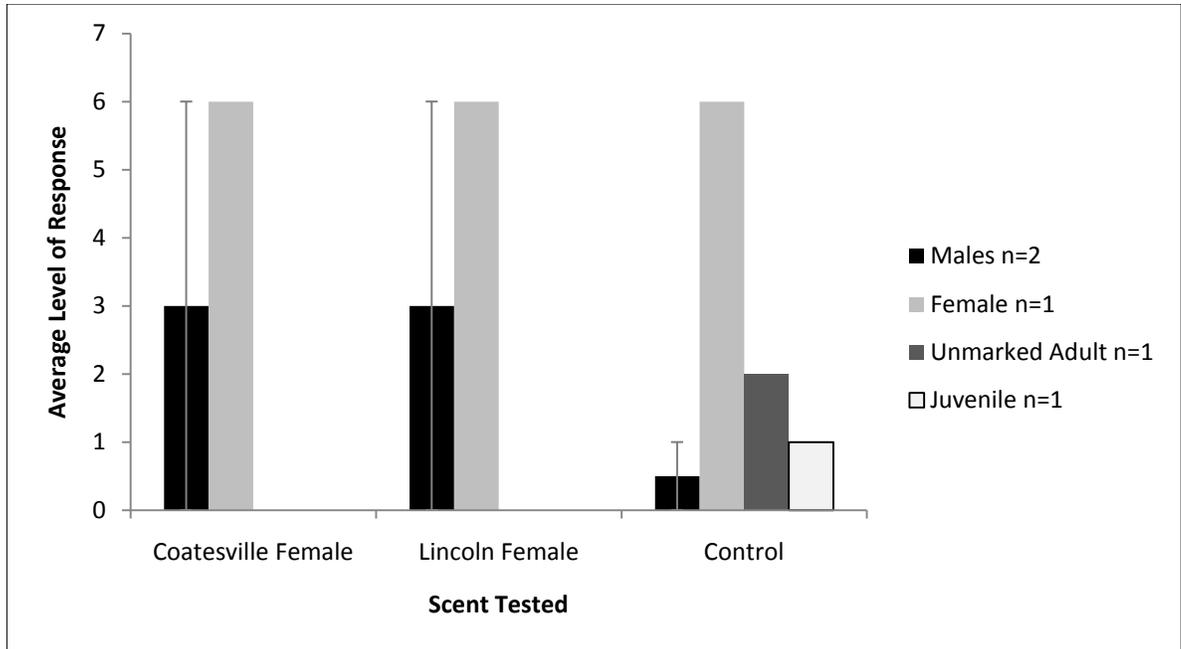


Figure 4.8 The mean level of behavioural response displayed by possums during the trials comparing Coatesville and Lincoln female scents. Error bars represent the standard error. Refer to table 4.2 for description and rank of responses.

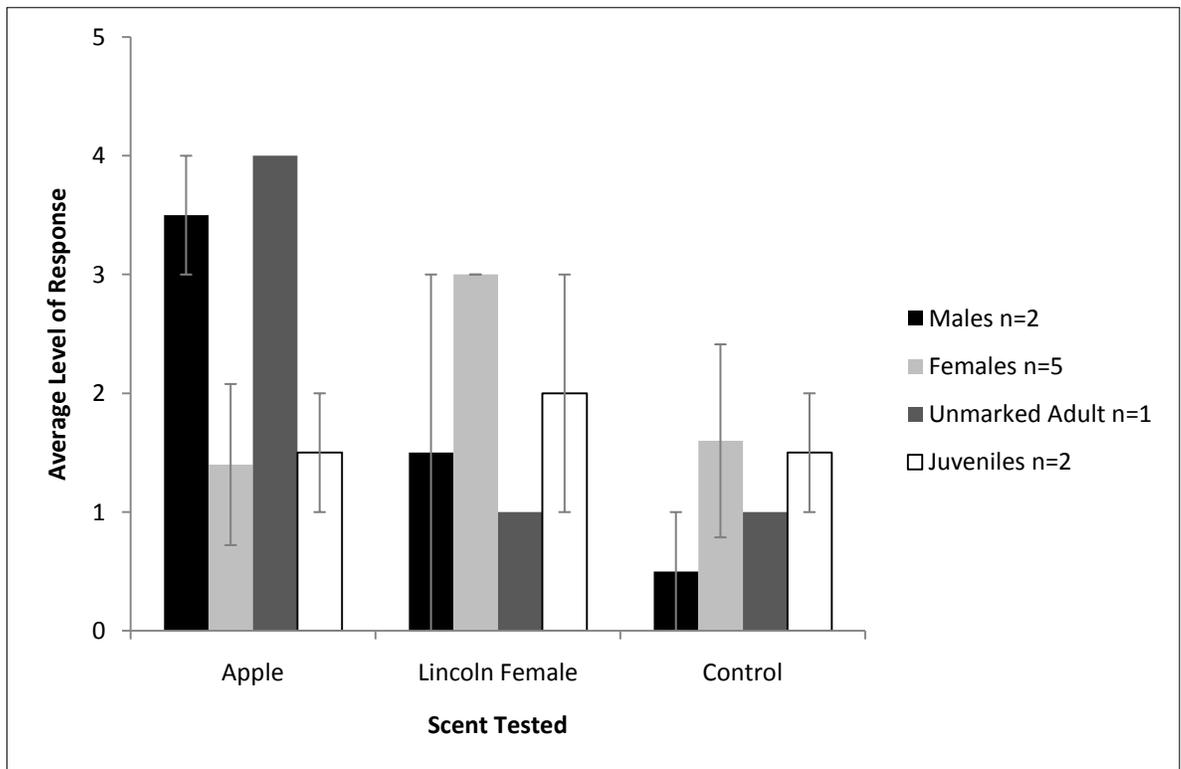


Figure 4.9. The mean level of behavioural response displayed by possums during the trials comparing cinnamon wheat-bran apple and Lincoln female scents. Error bars represent the standard error. Refer to table 4.2 for description and rank of responses.



Figure 4.10 An adult female possum sternal marking the control gauze (black arrow) while her offspring investigates the gauze scented with cinnamon/wheat-bran apple scent (white arrow).

The MDS plots for all five tests did not show any pattern in behavioural response according to gender or age group although in some tests there were not multiple individuals of a gender or age-class so it was not possible to detect a pattern (Figures 4.11-4.15). ANOSIM tests confirmed the lack of pattern in these trials: Coatesville Female vs. Coatesville Male ($R=-0.125$; $n=5$); Lincoln Female vs. Lincoln Male ($R=-1.0$; $n=4$); Coatesville Male vs. Lincoln Male ($R=0.417$; $n=6$); Coatesville Female vs. Lincoln Female ($R=0$; $n=5$); Lincoln Female vs. Apple ($R=0.273$; $n=9$). The test statistic 'R' ranges from -1 to 1 with -1 meaning that more similarity is found between groups than within and 1 meaning that there is more similarity found within than between groups.

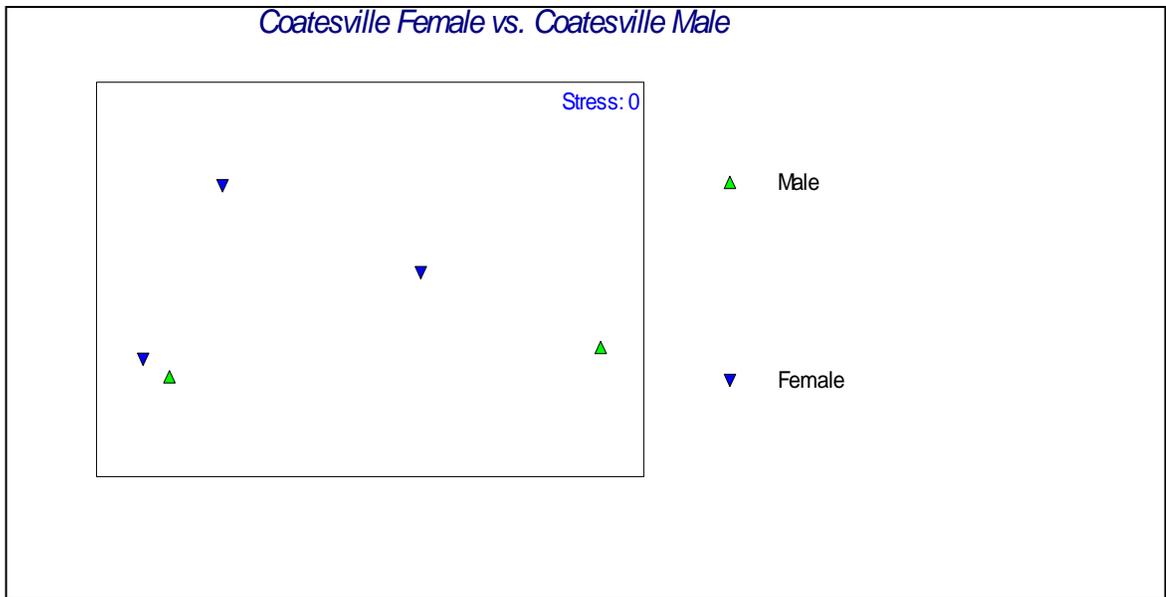


Figure 4.11 MDS plot showing the behavioural response levels of different possums, labelled by gender, to Coatesville females and Coatesville males. The distances between the different symbols represent the degree of similarity between the ranks of the respective individual's behavioural responses.

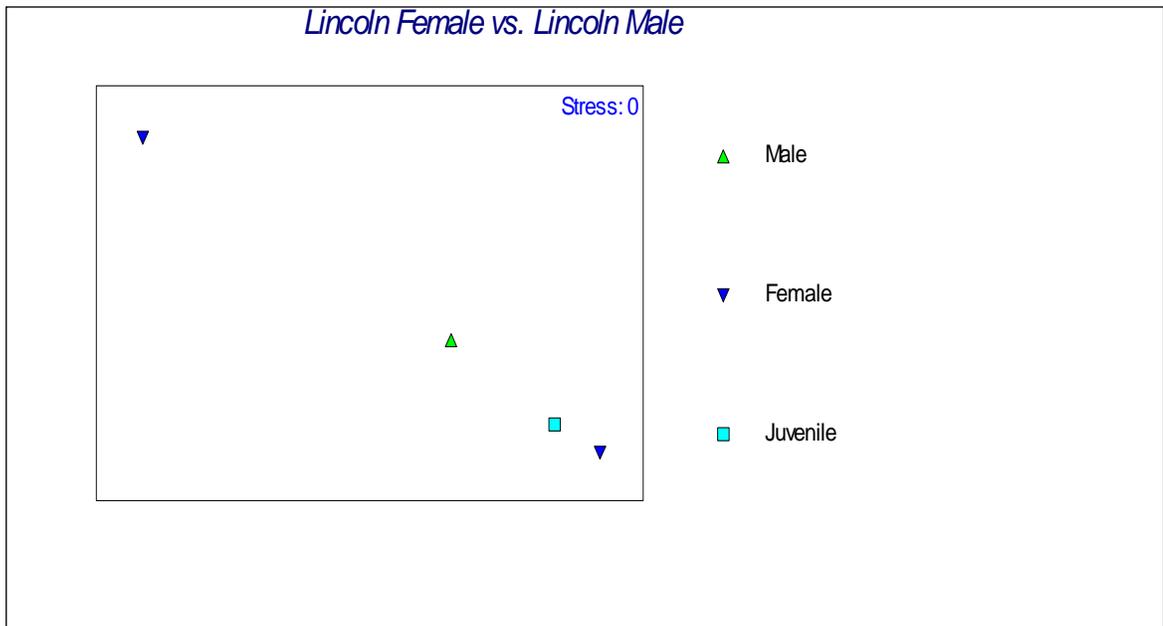


Figure 4.12 MDS plot showing the behavioural responses of different possums, labelled by gender and age group, to Lincoln females and Lincoln males. The distances between the different symbols represent the degree of similarity between the ranks of the respective individual's behavioural responses. Possums of unknown gender are only listed by their age-class.

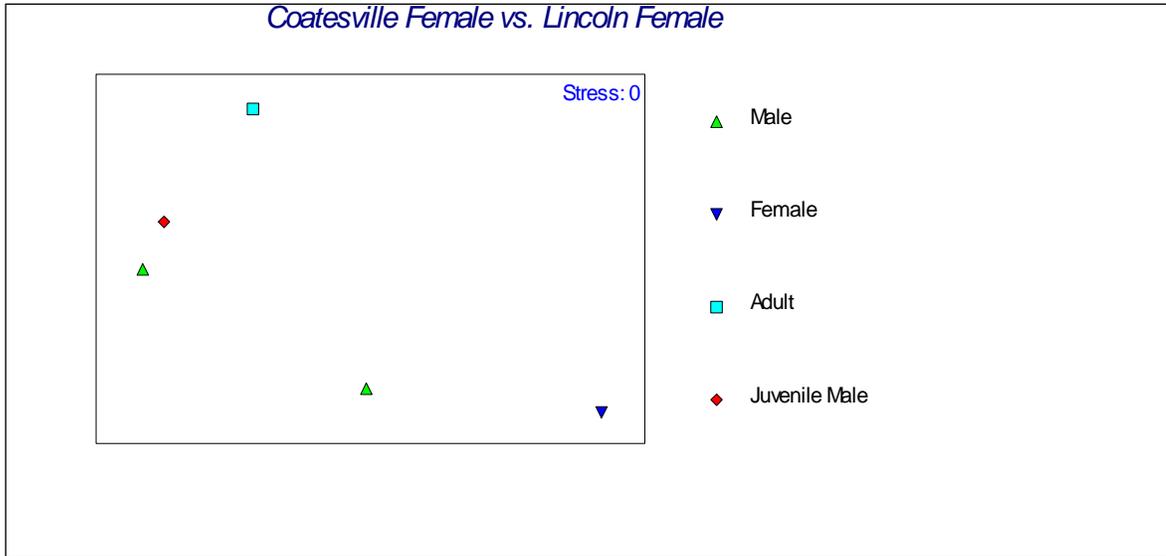


Figure 4.13 MDS plot showing the behavioural responses of different possums, labelled by gender and age group, to Coatesville females and Lincoln females. The distances between the different symbols represent the degree of similarity between the ranks of the respective individual's behavioural responses. Possums of unknown gender are only listed by their age-class.

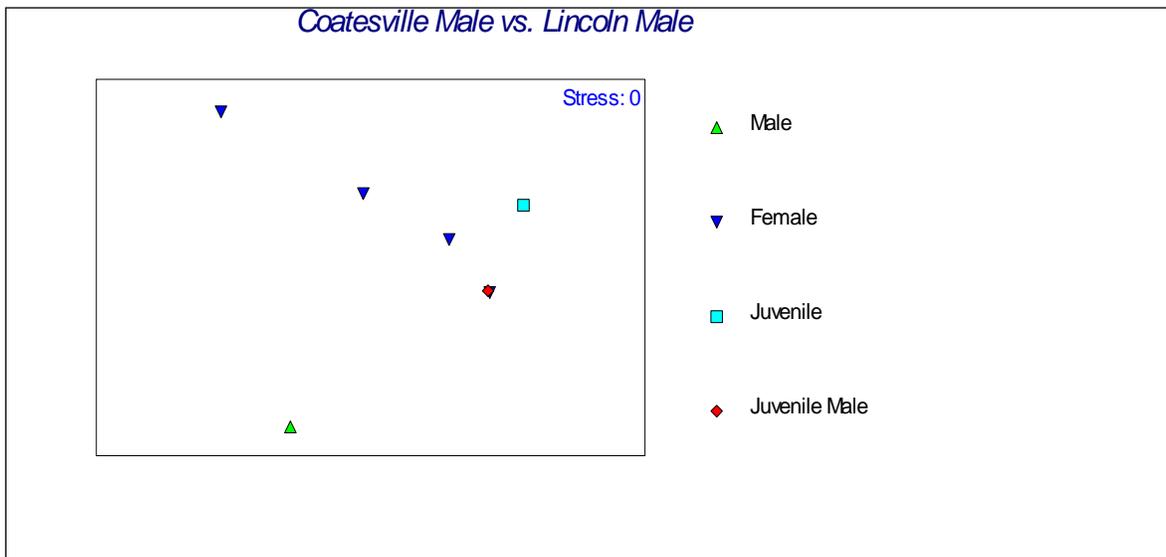


Figure 4.14 MDS plot showing the behavioural responses of different possums, labelled by gender and age group, to Coatesville males and Lincoln males. The distances between the different symbols represent the degree of similarity between the ranks of the respective individual's behavioural responses. Possums of unknown gender are only listed by their age-class.

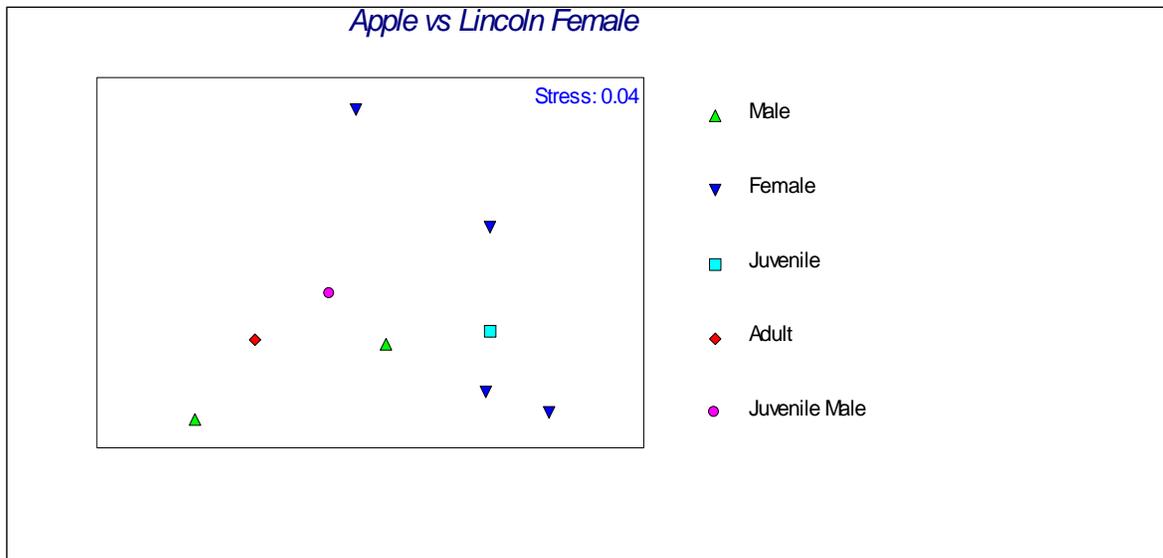


Figure 4.15 MDS plot showing the behavioural responses of different possums, labelled by gender and age group, to Lincoln female and cinnamon/wheat-bran apple scent. The distances between the different symbols represent the degree of similarity between the ranks of the respective individual's behavioural responses. Possums of unknown gender are only listed by their age-class.

4.3.3 Order of Visitation and the Effect of Conspecific Presence

Of the sixty-three possum visits recorded during all trials, 52.4% of the possums investigated the first scent that they came across while 22.2% passed up one or two scents on their way to investigate the first scent (the remaining 25.4% did not investigate any scents). Of the possums that took interest in the first scent encountered, 45.5% went on to investigate the other scents. Of those who did not investigate the first scent encountered, 71.4% went on to investigate at least one other scent sample (Figure 4.16).

When comparing the group who investigated only one scent against those who investigated more than one, the presence of a conspecific does not have had a significant effect on the behaviour of possums in either tests one through four ($p=0.4401$; $n=27$) or test five ($p=0.1577$; $n=20$) (Figures 4.17 and 4.18). When combining the two investigator groups and comparing them against those who did not investigate any scent all, the presence of a conspecific did not have a significant effect on the behaviour of possums in tests one through four ($p=0.2553$; $n=36$) but it did in test five ($p=0.0329$; $n=27$) (Figures 4.19 and 4.20).

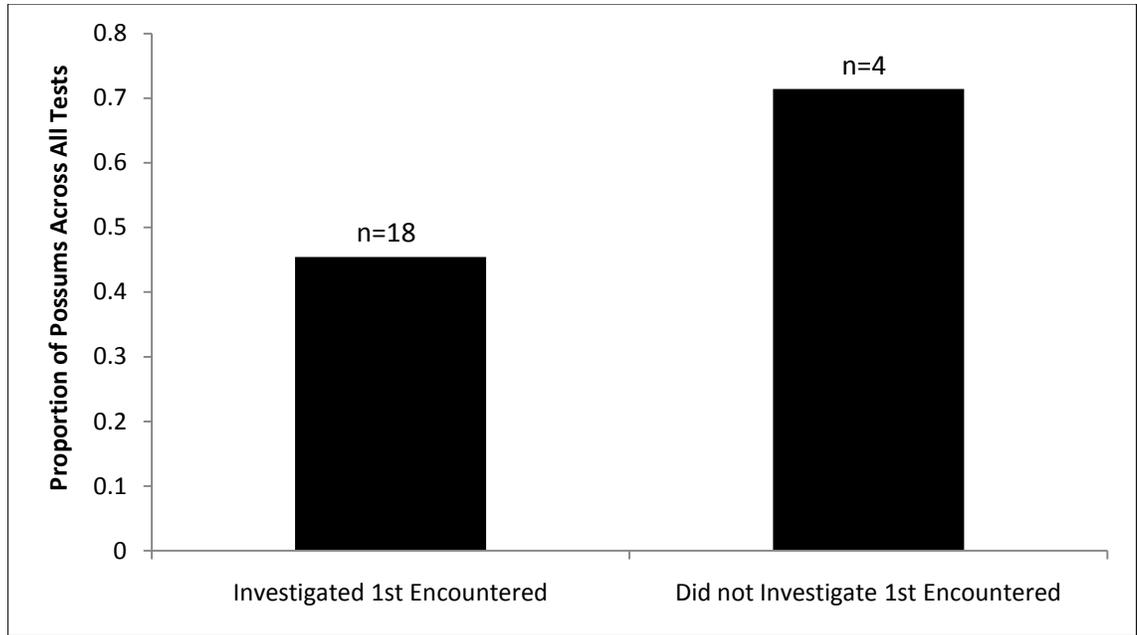


Figure 4.16 The proportion of possums that investigated more than one scent (as opposed to those who only investigated one) for two groups: those that investigated the first scent encountered and those who did not investigate the first scent encountered.

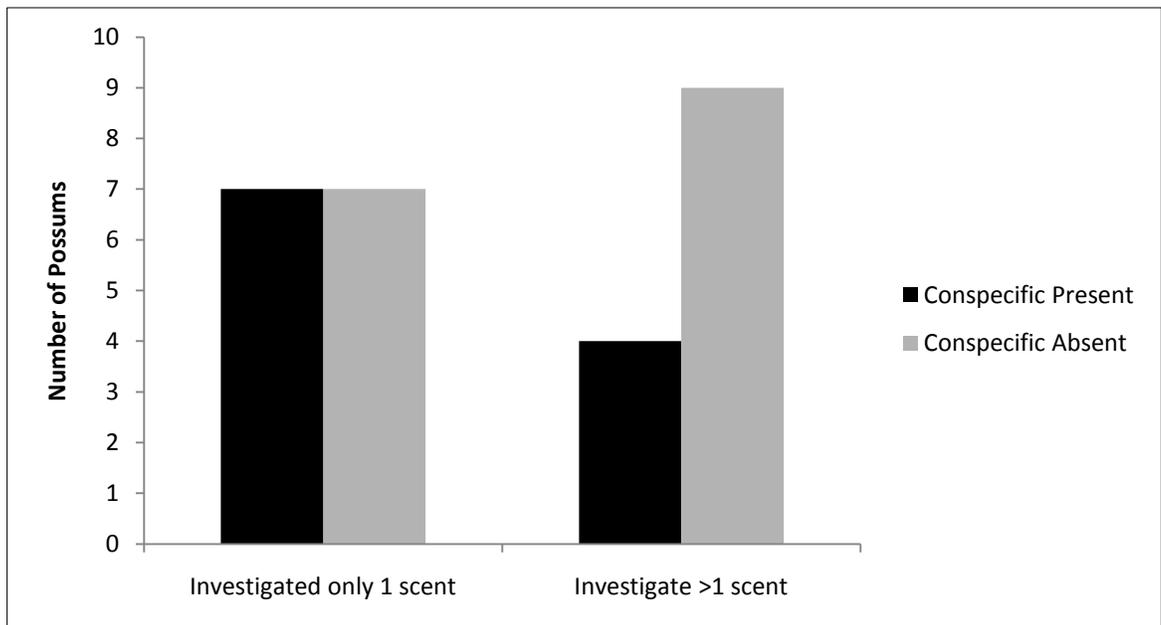


Figure 4.17 The effect of conspecific presence on the number of different scent samples investigated for trials on different possum scents (Trials 1-4).

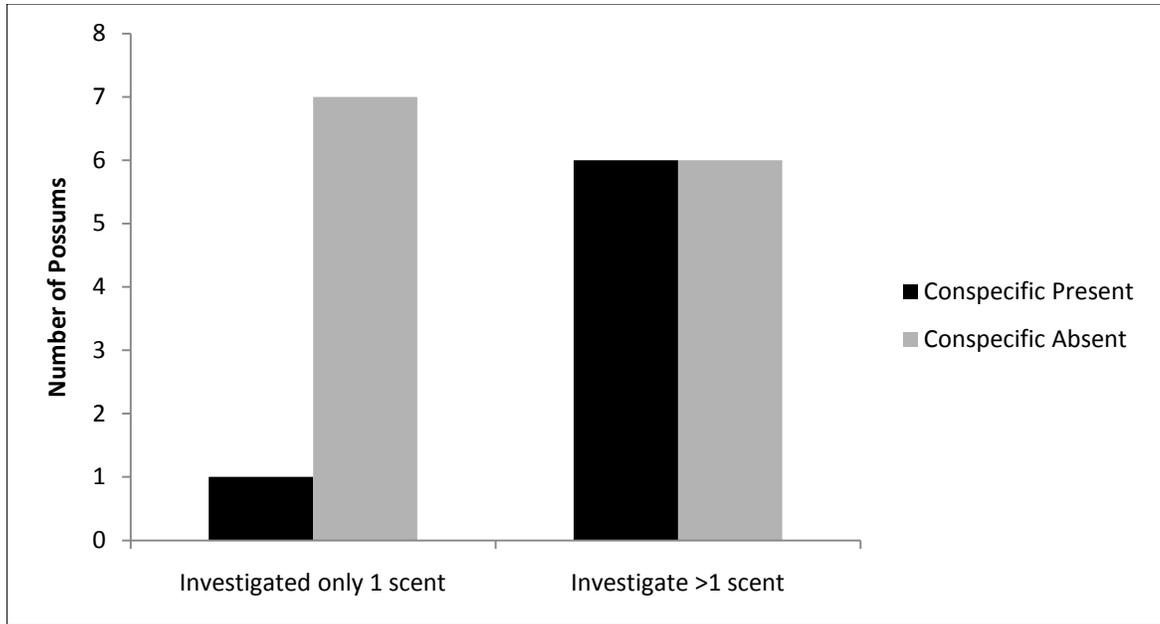


Figure 4.18 The effect of conspecific presence on the number of different scent samples investigated for the trials on possum scent vs. apple scent (Trial 5).

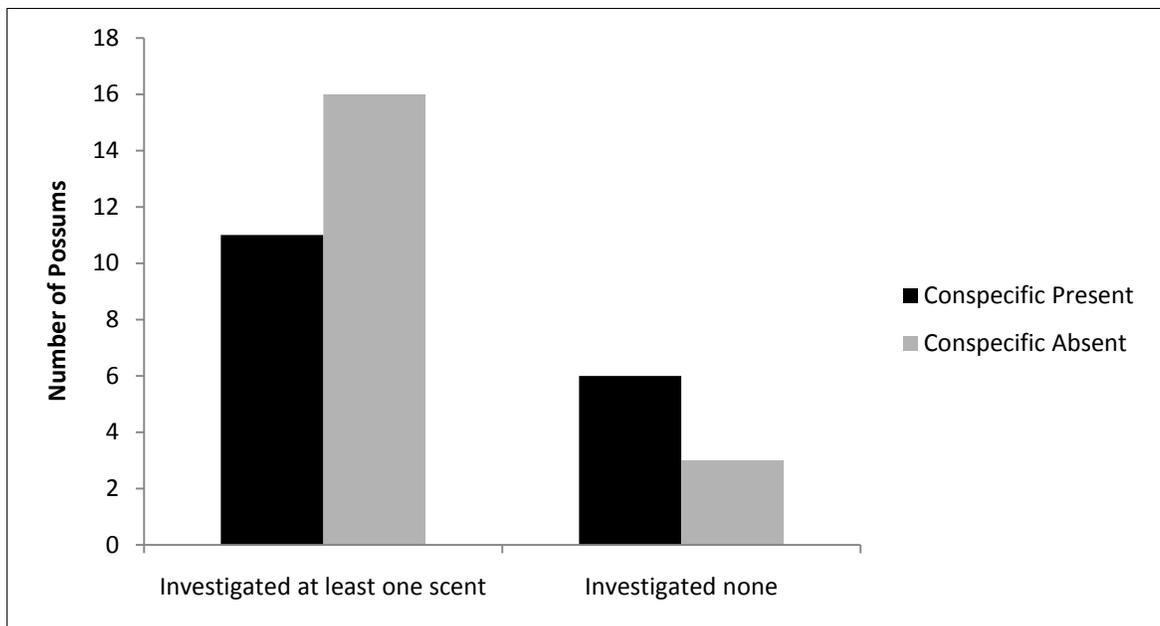


Figure 4.19 Number of possums from tests one through four who had a conspecific present for two groups: those who investigated at least one scent and those who investigated none at all.

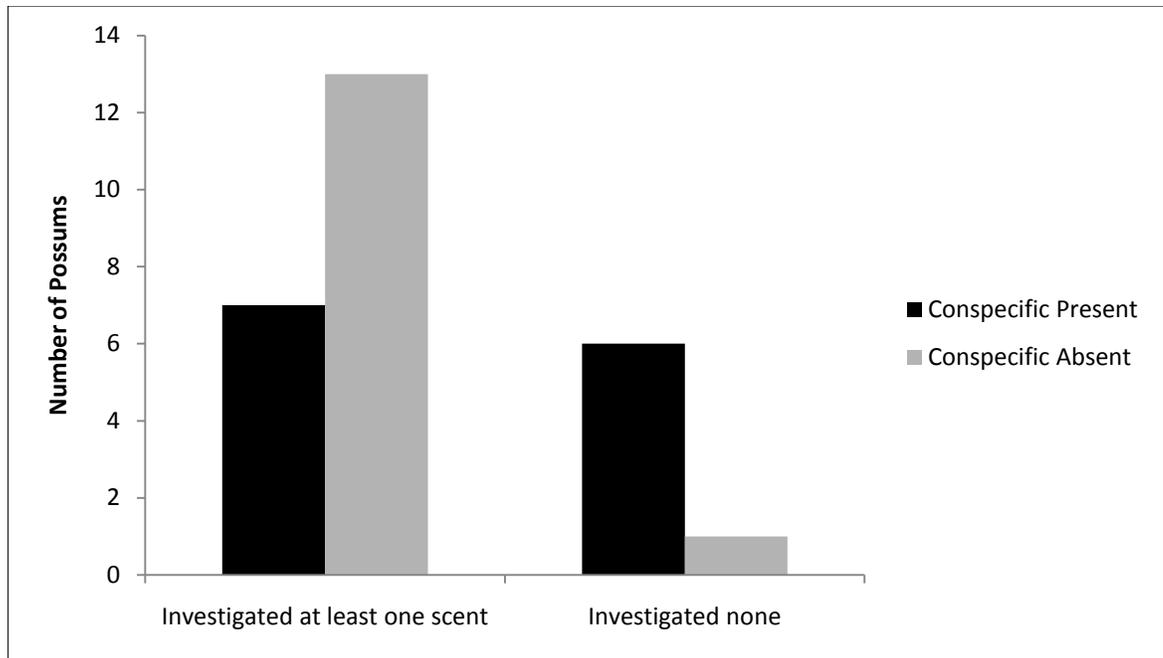


Figure 4.20 Number of possums from test five who had a conspecific present for two groups: those who investigated at least one scent and those who investigated none at all.

4.4 Discussion

4.4.1 Investigation Duration

While there are some trends in the possums' responses to the different scents, most tests did not yield statistically significant results. The trends suggest that the possums in this study are more interested in the scents of familiar females over familiar males and that they are more interested in unfamiliar scents over familiar scents (both male and female scents) (Figure 4.2). Despite this trend, the null hypothesis that no difference exists between the responses of Coatesville possums to the scents of familiar and foreign possum scents cannot be rejected as results were not statistically significant. Despite the lack of significance, these data suggest that the scents of unfamiliar possums elicit more investigatory behaviour and when it comes to familiar possums, the scents of females seemed to be more interesting.

Although further tests and repeated tests with larger sample sizes need to be conducted before this can be confirmed, the trends do make sense. Considering that these trials occurred during the breeding season, it is expected that possums would find the scents of foreign possums of opposite sex to be more interesting as foreign possums are more

distantly related. As reviewed by Penn and Potts (1999), MHC-dependant mating preferences are likely to occur as they are hypothesized to benefit the immunocompetence of offspring, benefit hosts in an evolutionary arm's race with parasites, and to help with inbreeding avoidance. Thus, in evolutionary terms, the trends seen in this study are sound.

This study shows that possums are statistically more interested in the scent of cinnamon/wheat-bran apples than in the scent of unfamiliar females. Due to the small sample size in this study, however, the null hypothesis that no difference exists in the interest of possums to the scent of foreign females or the scent of a popular food item cannot be rejected. Despite previous studies showing that food lures are not significantly more attractive than water (Todd, 1995) and that cinnamon only attracted possums if they were within two metres (Morgan *et al.*, 1995), the food lure used in this study was still far more appealing to these possums than the scent of unfamiliar female possums. These data suggest that food, specifically cinnamon/wheat-bran coated apples, is an efficient lure to use at this time. This is unfortunate as a more species specific lure would be desirable to avoid trapping or poisoning non-target species (for example, black birds and quail were both caught in the live traps multiple times during this study and traps were regularly tripped by rats, leaving them unavailable for possums). Perhaps trying other possum scents, such as one that is likely to convey messages such as reproductive receptiveness, will lead to the discovery of a more efficient lure. As discussed in chapter one, such messages have been noted in the female urine of Asian elephants (*Elephas maximus*), common marmosets (*Callithrix jacchus*), and panda bears (*Ailuropoda melanoleuca*) (Rasmussen *et al.*, 1997; Smith & Abbott, 1998; Swaisgood *et al.*, 2000). It would be interesting to see if possum urine also contains messages relating to reproduction, perhaps this would be a more promising lure.

4.4.2 Behavioural Responses

With regards to the behavioural responses of the possums to the various scents in this study, the sample size was too small to be able to test for statistically significant differences in the levels of their responses. Nevertheless, trends and patterns were investigated. For the test in which possums were presented with the scents of Coatesville females and Coatesville males, the possums displayed on average more of a behavioural

response to the scents of Coatesville males yet displayed more investigatory behaviour towards the scents of Coatesville females (see section 4.2.1). This could suggest that female scents are more interesting and appealing while male scents elicit more of a competitive response, encouraging them to over mark the scent or to mark other items in the area. The sample size for this test is too small to indicate whether a more competitive response (e.g. over marking) is dependent upon the gender of the visiting possum, however.

During the trial comparing Lincoln female and male scents, the one male that visited these scents had a more intense response to the male scent than to the others as was the case with the males in the first test (see above). While it was only one male that visited during this test, his response may be some sort of an intra-sexual challenge or display, as discussed in the paragraph above. Hynes (1999) suggested that males may use their sternal gland to inform the females of their presence. Thus, it is possible that this particular male (and the males from the first test discussed above) over-marked the scents of other males in order to make his scent known.

During the tests trialling the scents of Coatesville and Lincoln males, there was a conspecific present while the one male investigator was in the testing area which, as discussed in section 4.4.3, could have affected his behaviour. The females did not display much difference in their levels of response to the two possum scents but three of the four females also had a conspecifics present. Thus, it is not possible to speculate as to the meaning of the differences in levels of response exhibited by the male or the females as they may have been distracted.

For the test comparing Coatesville and Lincoln female scents, both males had a conspecifics present as did the one visiting juvenile, again making it difficult to speculate as to the meaning behind their responses. The unmarked adult was alone, however, but was one of the possums who only investigated the first piece of gauze that was encountered making it difficult to speculate as to why he/she responded in such a way. Perhaps, as discussed in section 4.3.3, this possum was not even aware of the other scents. The female that visited the test station did not show a difference in response to the possum scents or the control (she over-marked all three pieces of gauze), suggesting that the familiarity of the scent (or lack of scent) did not affect her impulse to over-mark the gauze pieces.

When a food scent was tested against the scent of a foreign female possum, males may have displayed more of a behavioural response to the food scent as they just want their own scent to cover any conspicuous scents they may come across in their range to ensure that females will be aware of their presence. Potentially females showed the lowest level of behavioural response, but more investigation, towards the food scent because it was during the breeding season, when they were likely to be pregnant and therefore more interested in food than in mating.

No specific pattern of behavioural response could be related to gender or age-class mostly due to a low number of replicates. For example, when comparing Coatesville and Lincoln females, only one female approached the testing area. It is therefore not possible to come to a conclusion on the possibility of behavioural patterns in brushtail possums due to the small sample size and lack of replicates in this study.

As with other parts of this study, a captive setting may prove beneficial to gaining an understanding of how the genders respond to possum scents based on the gender and degree of familiarity of the scent donator. In order to come to a conclusion, a much larger sample size is needed. Furthermore, repeated visits by the same individuals are necessary to test whether or not their responses are consistent. Finally, conducting such tests with the same individuals during both the breeding and non-breeding seasons is necessary as their responses are likely to differ depending on their reproductive status. This is especially likely to be the case if Hynes (1999) was correct in her speculation that females mainly sternal mark to protect resources while with young and that males mainly sternal mark to inform the females of their presence. If this is the case, both genders may respond differently to the scents of other possums depending on the season.

Furthermore, evaluating the responses of possums within the same gender group requires further investigation. There were a couple of occurrences during this study where there was a high degree of variability in the level of response for two possums of the same gender. For example, in the test comparing Lincoln female and male scents, one female over-marked all pieces of gauze while the other female sniffed one scent and ignored the others. It would be interesting to see if the dominance position of the individual has an effect on how likely they are to over-mark the scents of other possums. A positive correlation between dominance status and frequency of scent marking occurs with other

mammals such as the golden hamster (*Mesocricetus auratus*) (Drickamer *et al.*, 1973) and stoats (*Mustela erminea*) (Erlinge *et al.*, 1982). This would be another interesting aspect of possum olfactory communication that is worth researching.

4.4.3 Order of Visitation and Presence of Conspecifics

The data on order of visitation and on the presence of conspecifics suggest that the results obtained in this study are unlikely to truly reflect the interests of these possums due to the experimental design. In many scenes a possum approached the testing area from the side, investigated the one piece of gauze that was on the same side of the tree, then turned around and left the scene. The type of scent does not appear to have had an effect on this behaviour as there is no pattern in the type of scent that was investigated during these occurrences (e.g. it was not always the scent of a possum which, if this had been the case, may have suggested that it was perhaps the scent of a more dominant individual and that the scent may have deterred the investigator). It is therefore possible and highly likely that the possum was not aware of the presence of the other pieces of gauze in many of these cases. Thus, we cannot say for certain that these possums “preferred” the investigated scent over the others as the possum did not even encounter the other scents in many cases. A captive study may be more successful at discerning their preferences as it may be easier to control the direction of their movements as well as the distance that they are allowed to travel from the scents. Furthermore, in a captive setting, some sort of a structure may be able to be built where the possums could be funnelled into the presentation area and made aware of all scents being presented. Such a structure may be necessary, especially if, as is suspected in this study, the scents being tested are not highly volatile and therefore not strong enough to draw possums into the area.

Another factor which may have had an impact on the accuracy of these results is the effect that conspecifics appear to have on the behaviour of the subjects. The presence of conspecifics did not have a significant effect on whether or not possums investigated only one scent or more than one scent for either the trials comparing possum scents or for the ones comparing possum scent and food. However, when comparing possums who investigated any number of scents at all with those did not, there does appear to be an affect. For the trials comparing possum scents, the result was not statistically significant

yet the trends suggest that there is an effect. But the effect of conspecific presence was statistically significant when comparing these two groups for the trials comparing possum scent and food scent. There were some video scenes which showed obvious effects of conspecifics which support these results. For example, in one scene, an adult male was investigating a scent and then stopped investigating and ran away when an adult female and her offspring approached the area. In another scene, an adult male was chased away from the testing area by a mature female. The level of distraction in all experiments is unknown, however, as the presence of conspecifics was only known when they appeared in view of the cameras. It is possible that there were other times that another possum was present but was just not detected.

As conspecific distraction does seem to have an effect on the behaviour of possums during such trials, it is recommended that such tests are carried out in a captive, and therefore controlled, setting in the future. This will allow possums to spend as much time as they want at the test stations, improving the accuracy of results. Furthermore, more accurate results will be obtained after conducting such a study with a larger sample size. Such studies will allow us to better understand how possums respond to the sternal gland scent of others as well as any patterns of preference that may exist.

Chapter Five: Possum Cutaneous Bacteria Composition and Dynamics

5.1 Introduction

Symbiotic relations between microbes and animals are important to consider as the interactions between these different organisms are often significant. For example, some bacterial-animal relations are significant to the point of being necessary for survival of the animal (Gil-Turnes & Fenical, 1992), some lead to physiological and behavioural phenomena such as bioluminescence (McFall-Ngai, 1994), and many relations involve processes such as digestion and host protection (reviewed in Guarner & Malagelada, 2003). Another outcome of bacterial-animal relationships is scent production (Quay, 1976; Studier, 1984; Singh *et al.*, 1990; Schellinck *et al.*, 1995), which is the relationship that is of interest in this study.

Bacteria play a role in converting some otherwise inactive or scentless secretions into pheromones (Quay, 1976). For example, mice cannot distinguish between the urinary odours of mice who are housed in sterile conditions *versus* the urinary odours of those who are housed in conventional housing (Schellinck *et al.*, 1995). Rats raised in a sterile environment also do not produce distinguishable urine until they are placed in conventional housing (Singh *et al.*, 1990). In another study, the odours given off by greater bulldog bats (*Noctilio leporinus*) were compared with the odours produced by cultures of bacteria that were sampled from these bats. The peaks on the gas chromatograph from both the bat and the bacterial culture odour were similar, suggesting that the bacteria are at least partially involved in the production of the bats' scent (Studier, 1984).

The composition and dynamics of bacterial communities that have symbiotic relations with brushtail possums is not known. Only one study concerning bacterial-possum relations has been found in the literature. This particular study investigated the bacteria living in the pouch of possums and that in females who were in oestrous, not in oestrous, with a pouch young, and with a back rider (Deakin & Cooper, 2004). While this study revealed the bacterial species that pouch young are exposed to as well as their immune competency, we continue to lack data regarding the bacterial species that inhabit, and thus

are likely to metabolize the products of, other glandular areas, specifically those glands involved with communication between adult possums.

This chapter presents the results of a pilot study on using denaturing gradient gel electrophoresis (DGGE) as a technique for investigating the composition of the bacterial communities inhabiting the cutaneous surface of the brushtail possum sternal gland. The sternal gland, and the possible involvement of bacteria in the production of the final scent product emitted by the gland, is of interest as it is a gland that possums utilise to communicate with conspecifics (see 1.3.3). It is likely that this gland is used by males to advertise their presence to females, used by both genders to establish and maintain hierarchies, and used by females to protect resources when with young (Hynes, 1999). The possibility of bacterial composition changes between the breeding and non-breeding seasons was of interest as a correlation between bacterial community composition and hormonal status has been suggested for other animals (Nordstrom *et al.*, 1989; Wintzingerode *et al.*, 1997; Penn & Potts 1998, cited in Lanyon *et al.*, 2007). Also of interest is whether there are differences in the bacterial profiles of different individuals during the same season to investigate the possibility of unique bacterial profiles for individual possums.

5.2 Methodology

5.2.1 Bacterial Sampling

Once unconscious following anaesthesia, the possum was carefully laid on his or her back. The skin covering the area of the sternal gland, which is easily located due to the presence of red, sebum-stained hairs, was rubbed several times with a sterile cotton swab which was then placed into a sterile Eppendorf tube. Swabs were moistened with sterile physiological saline solution prior to sampling to enhance the efficiency of sampling. All samples were kept on ice in a chilly bin until being placed at -80°C for storage. Latex gloves were worn by the handler for the entire process and care was taken to not allow anything to come into contact with the sternal region of the possums (e.g. their underside was not allowed to come into contact with the ground while they were extracted from the box).

5.2.2 Microbial Community Analysis

DNA Extraction and Amplification

DNA was extracted from the collected bacteria using either the Sigma REDExtract-N-Amp Plant PCR Kit or the X-Tractor Gene robotic unit (Corbett Robotics). The DNA obtained from the extraction process was then amplified using the polymerase chain reaction (PCR) with primers that are specific to part of the bacterial genome that codes for 16s ribosomal RNA. The ribosomal RNA gene was utilized as this gene is found in all known bacteria and is a large enough molecule with enough regions of variability that it can be used to distinguish between different bacteria (Clarridge III, 2004). The optimal mixture consisted of the following: 13.8 μ l water, 2.5 μ l 10x buffer, 2.5 μ l dNTP's, 0.5 μ l of each primer, 1.0 μ l BSA, 0.2 μ l Taq polymerase, and 4 μ l DNA template. The universal bacterial primers used were 357f-GC and 907r (Table 5.1). The PCR conditions used in this study were 94°C for 3 minutes, 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute, and then 72°C for 10 minutes, with 35 cycles. Agarose gel electrophoresis was then used to analyze the PCR products.

Primer	Sequence (5' to 3')
357f-GC	CCTACGGGAGGCAGCAGCGCCCGCCGCGCGGGCGGGCGGG GCGGGGGCACGGGGGG
907r	CCGTCAATTCMTTGTGAGTTT

Table 5.1 The sequence of the two primers used in this study. Sequences obtained from Kaksonen *et al.*, 2004.

Separation of Bacterial DNA

Denaturing gradient gel electrophoresis (DGGE) was then used to separate the DNA of the various bacterial cells that were amplified from the samples using the CBS Scientific DGGE system. During DGGE, DNA fragments are exposed to an increasing level of denaturant (urea and formamide) as they travel through a polyacrylamide gel. The DNA of each operational taxonomic unit (OTU), depending on its nucleotide sequence, melts at a different concentration of denaturant and therefore stops at a certain location in the gel (Sheffield, 1989) (Figure 5.1). Theoretically, nearly all single base differences (between

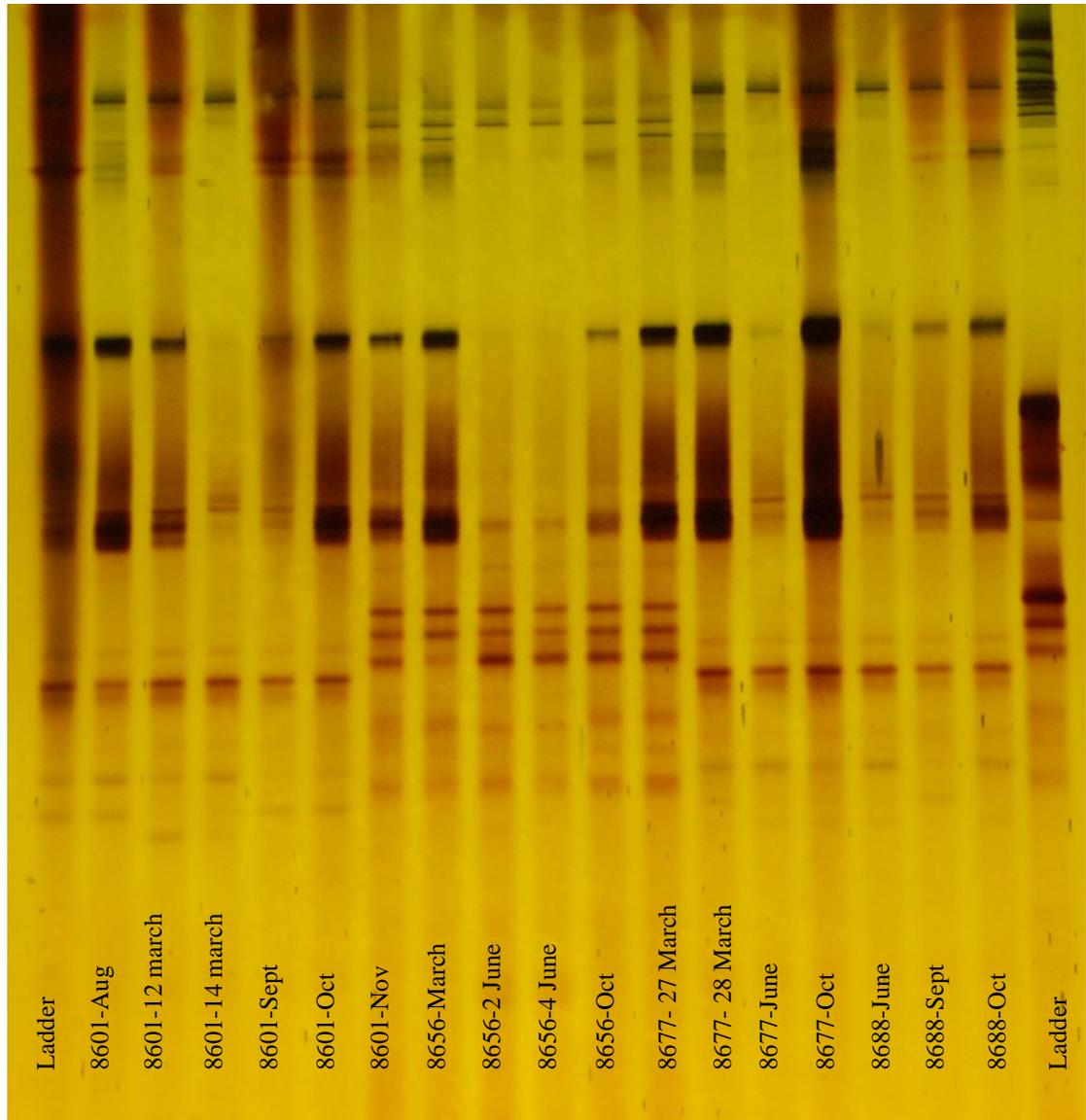


Figure 5.1 A DGGE gel containing bacterial samples that were collected during different times of the year from four male possums. Each column is a separate sample and each band is a different bacterial OTU.

different DNA molecules) should be detectable by this method (Myers *et al.*, 1985; Sheffield *et al.*, 1989). Furthermore, this technique is highly sensitive and thus has the ability to detect the presence of organisms that make up a small percent of a population (Muyzer *et al.*, 1993). Thus, changes in the composition of a microbial community can be detected as the arrival of or disappearance of new OTU's will be represented as new or lost bands on a DGGE gel. For the samples obtained in this study, a gradient of 60% - 62% was the most efficient as bands were the most separated at this gradient. Gels ran for

approximately ten minutes at 200 V (until samples entered the gel) and then for at least sixteen hours at 90V at 60° C. A TGGE/DGGE 2401 system (CBS Scientific) unit was used to run samples in this study.

5.2.3 Analyses

DGGE gels were analyzed using the computer program PFQuest (version 5.10) (Bio-Rad). Binary tables were constructed which show whether or not a band (and therefore an OTU) is present in the samples tested. Tables were constructed in such a way that allowed for the testing between various samples of the same individual (taken at different times of the year) and for testing between different individuals during the same time of year. The binary data were then analyzed using multi-dimensional scaling (MDS) plots to see how similar (or dissimilar) the different bacterial profiles are relative to each other (Primer 5). MDS plots display the relative similarity between different data points by spacing data points on the graph according to how similar the samples are to one another. For example, if possum A has a more similar bacterial composition to possum B than she has to possum C, then possum A will be placed closer to possum B on the plot than she is to possum C. When MDS plots are produced, x and y coordinates are given for each sample. These coordinates were used to obtain numerical values for the distances between the samples. Thus, a larger distance suggests a larger degree of dissimilarity between samples whereas a smaller distance suggests that the samples are more similar in composition. To analyze the distance between replicate samples and between samples from different animals, a Wilcoxin matched-pair signed rank test was utilized (SPSS version 18).

5.3 Results

5.3.1 Number of Bacterial Operational Taxonomic Units

On average, more bacterial OTU's were found on female possums (49 +/- 5.9 OTU's) than on male possums (24 +/- 3.4 OTU's) ($p=0.006$) (Figure 5.2). For individual females, the number of detected OTU's ranged from 31 to 80 while the number of OTU's detected on males ranged from 10 to 36. As the collected samples were not sequenced, the identity of these different OTU's is not known. Furthermore, it cannot be said for certain that these OTU's all represent unique species as some of the bands on the gel may represent different copies of the 16s gene from the same species (see 4.4.4).

5.3.2 Degree of Similarity Between Replicate Samples Versus Those Between Different Possums

The average distance between replicate samples (n=9), 1.263, and between samples of different animals (n=106), 1.347, are similar with replicate samples only being slightly more similar than those between different possums (Figure 5.3). The difference between these two groups of samples is not significant (p=0.066).

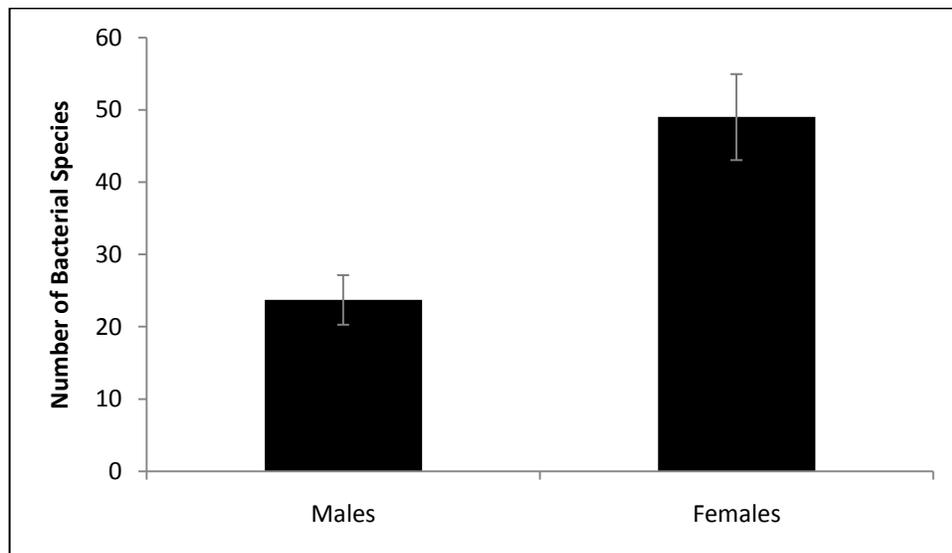


Figure 5.2 The average number of bacterial OTU's found on male and female possums. The error bars represent the standard error.

5.3.3 Individual Bacterial Profiles During Different Seasons

Males

Male possum, 8601, had replicate samples taken in March and June (two samples in each month). The distances between the replicate pairs are at the median of the data (June) or higher than the median (March). There is a relatively tight cluster of similar samples: September, the second June sample, and August. The sample taken in October is the most unique in composition (Figure 5.4).

For possum 8656, the two replicate samples from June are the most similar and both June samples also share the greatest amount of dissimilarity with the sample collected in March (Figure 5.5).

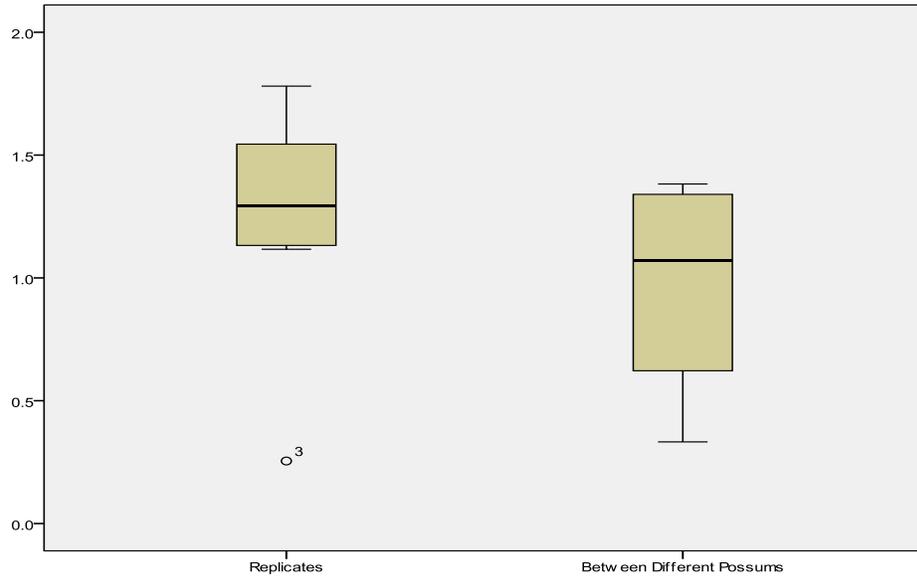


Figure 5.3 Box plots of the distance between replicate samples of all possums in this study (n=9) and the distance between the samples of different individuals (n=106). Distances were calculated using the numeric distances between the sample points produced on the MDS plots.

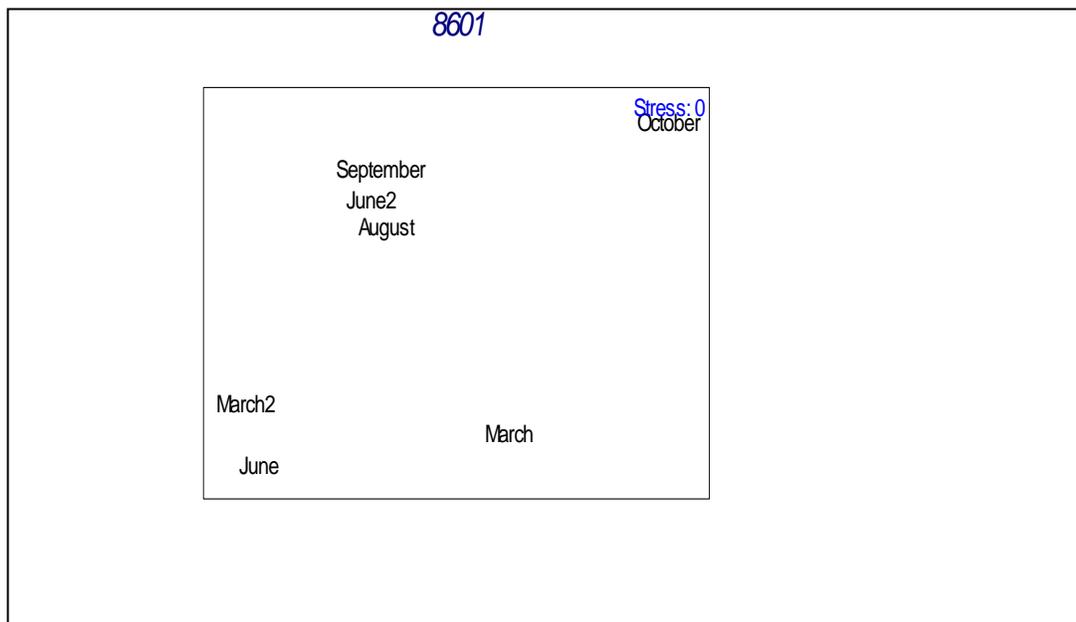


Figure 5.4 MDS plot showing samples collected from male possum 8601 during different times of the year.

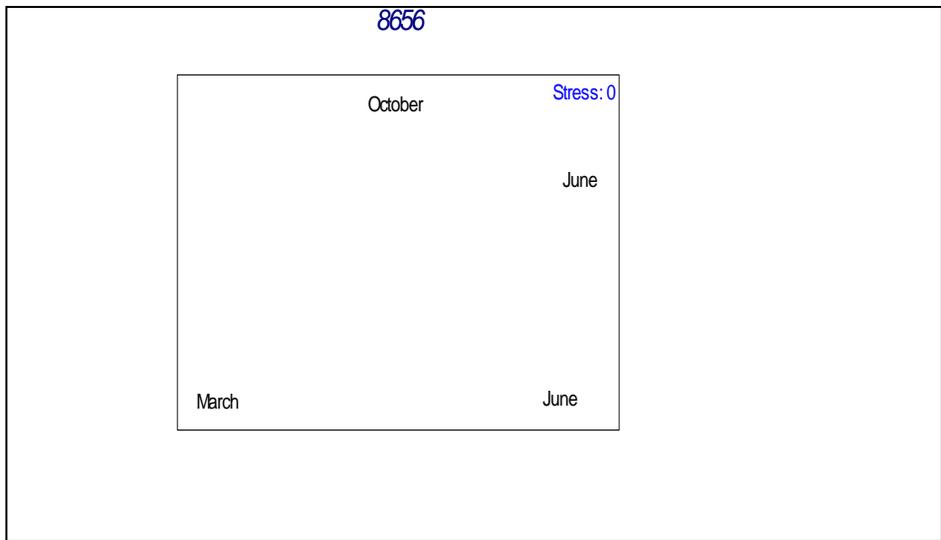


Figure 5.5 MDS plot showing samples collected from male possum 8656 during different times of the year.

The two replicates collected from possum 8677 in March are fairly similar with only one other comparison being more similar (March 2 and October). The second March sample is also quite similar to the June sample yet the first March sample shares the least amount of similarity with the October and June sample (Figure 5.6).

For possum 8688, the samples from September and October are the most similar, the samples from June are September are the next closest followed by samples from June and October (Figure 5.6).

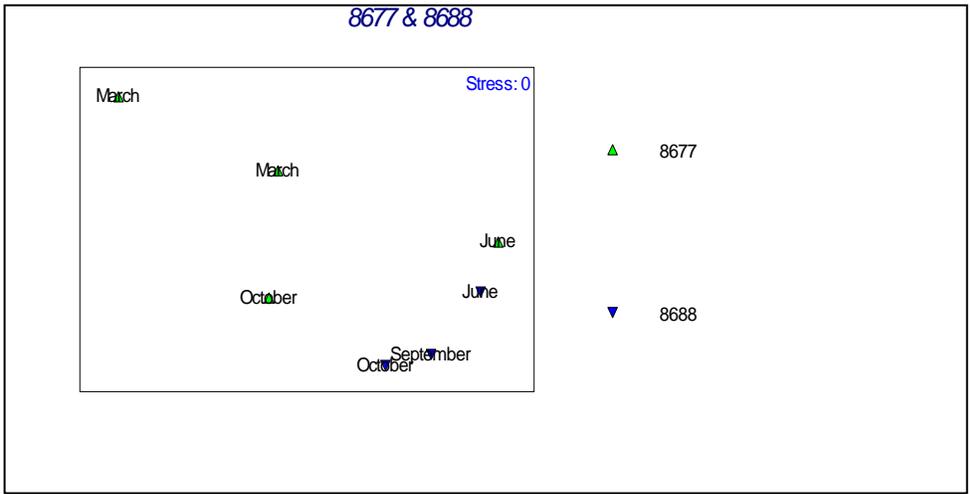


Figure 5.6 MDS plot showing samples collected from two different male possums during different times of the year.

Females

Female 4132's sample from March was most similar with her September sample and was the least similar with the sample collected in October (Figure 5.7). For possum 8636, her two samples collected in March are less similar than either is to the sample collected in June (Figure 5.7).

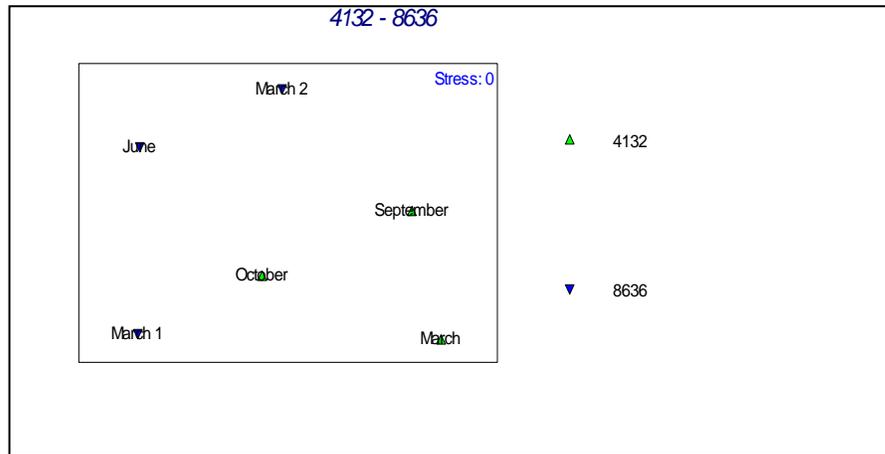


Figure 5.7 MDS plot showing samples collected from two different female possums, 4132 and 8636, during different times of the year.

For female 2907, her two samples collected in March are not the most similar (the most similar are October and September, October and the second March sample, and September and second March sample) but are more similar than both the mean and median of the data. January is the month that the most unique sample was collected from this individual (Figure 5.8).

5.3.4 Bacterial Profiles between Different Individuals

Males

The results for the samples collected from males in March (n=6) show both similarities and differences between the different samples with the two samples taken from the same individual (8677) being more different than some of the samples between different individuals (Figure 5.9; Table 5.2). The results from the samples taken in October (n=7) show three very distinct groups of possums with similar bacterial profiles (Figure 5.10; Table 5.3).

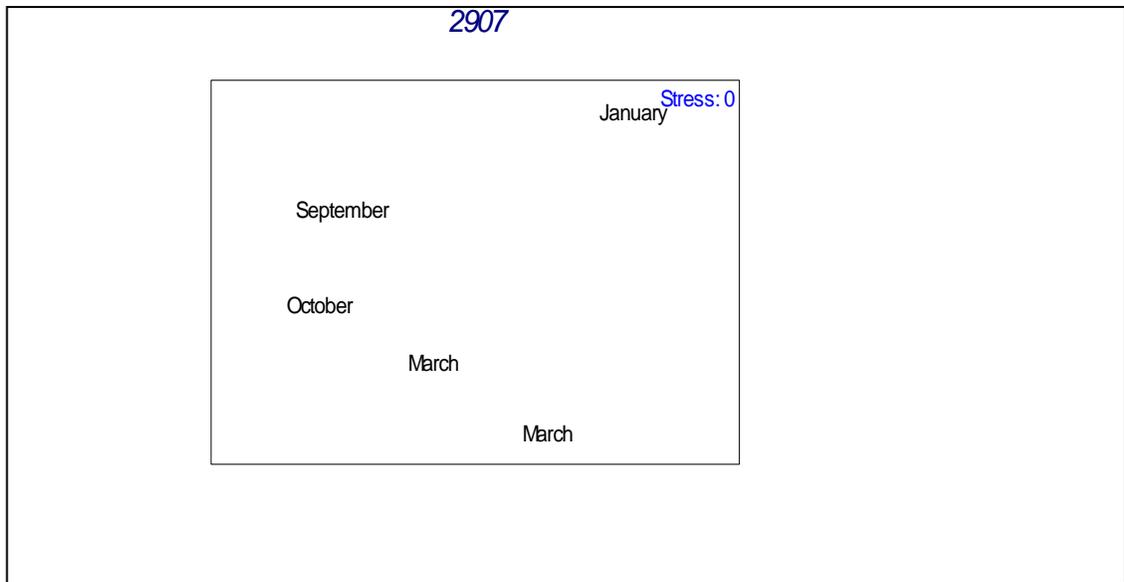


Figure 5.8 MDS plot showing samples collected from female possum, 2907, during different times of the year.

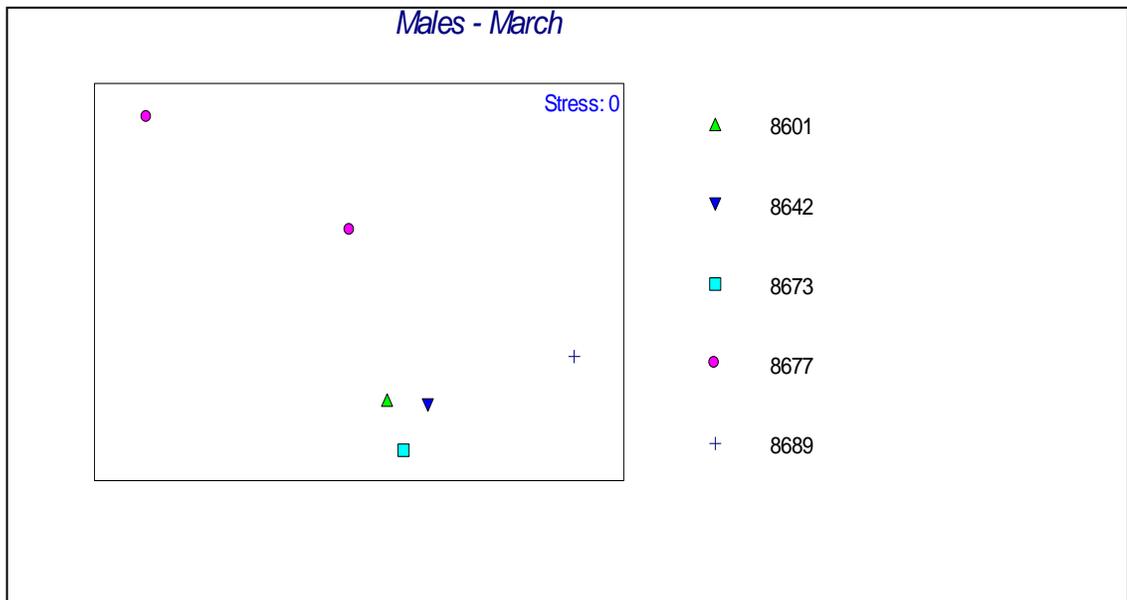


Figure 5.9 A MDS plot of the samples taken from different male possums during the breeding season (March) of 2009. Possum 8601 is the only individual from Coatesville, all others are from Mercer.

Possum Pair	Distance Between Possum Pair
8642-8673	0.332
8601-8642	0.413
8601-8673	0.622
8642-8689	1.022
8601-8677	1.071
8601-8689	1.082
8673-8689	1.340
8673-8677	1.351
8642-8677	1.382
8677-8677-2	1.545
8601-8677-2	1.842
8677-8689	2.110
8642-8677-2	2.251
8677-2-8689	2.297
8673-8677-2	2.435

Table 5.2 The distance between male possum bacterial profiles in March. The median for these data is 1.351 and the average distance is 1.406.

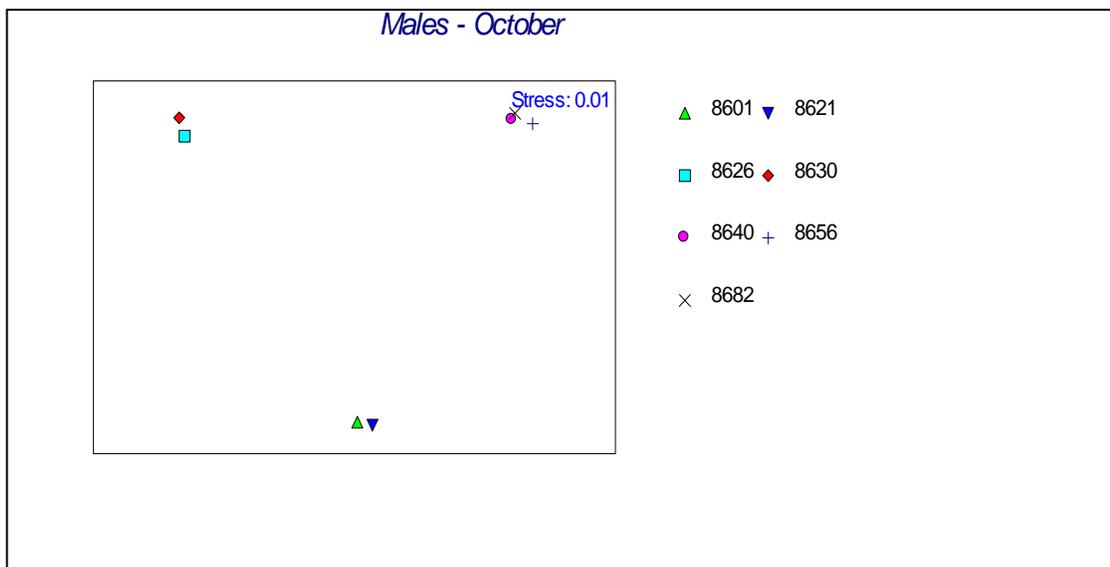


Figure 5.10 A MDS plot of the samples taken from different males during October of 2009. Possum 8630 is from Mercer while all others are from Coatesville.

Possum Pair	Distance Between Possum Pair
8640-8682	0.058
8601-8621	0.094
8640-8656	0.108
8656-8682	0.125
8636-8630	0.126
8601-8636	1.649
8621-8640	1.673
8601-8640	1.702
8621-8656	1.704
8621-8636	1.715
8621-8682	1.728
8601-8656	1.739
8636-8640	1.746
8630-8640	1.752
8601-8682	1.757
8636-8682	1.759
8630-8682	1.760
8601-8630	1.761
8621-8630	1.824
8636-8656	1.851
8630-8656	1.858

Table 5.3 The distance between male possum bacterial profiles in October. The median for these data is 1.728 and the average distance is 1.357.

Females

The MDS plot for the samples collected in March (n=9) shows a similar pattern to that of the March male samples with there, in some cases, being more similarity between the profiles of different individuals than between the replicate samples of the same individuals (Figure 5.11; Table 5.4). The plots of the samples taken in both June (n=4) and September (n=5) do not show any type of clustering (Figures 5.12 and 5.13; Tables 5.5 and 5.6, respectively). The results from the October (n=7) samples show a high degree of clustering with only two of the seven samples not being in the cluster (Figure 5.14; Table 5.7).

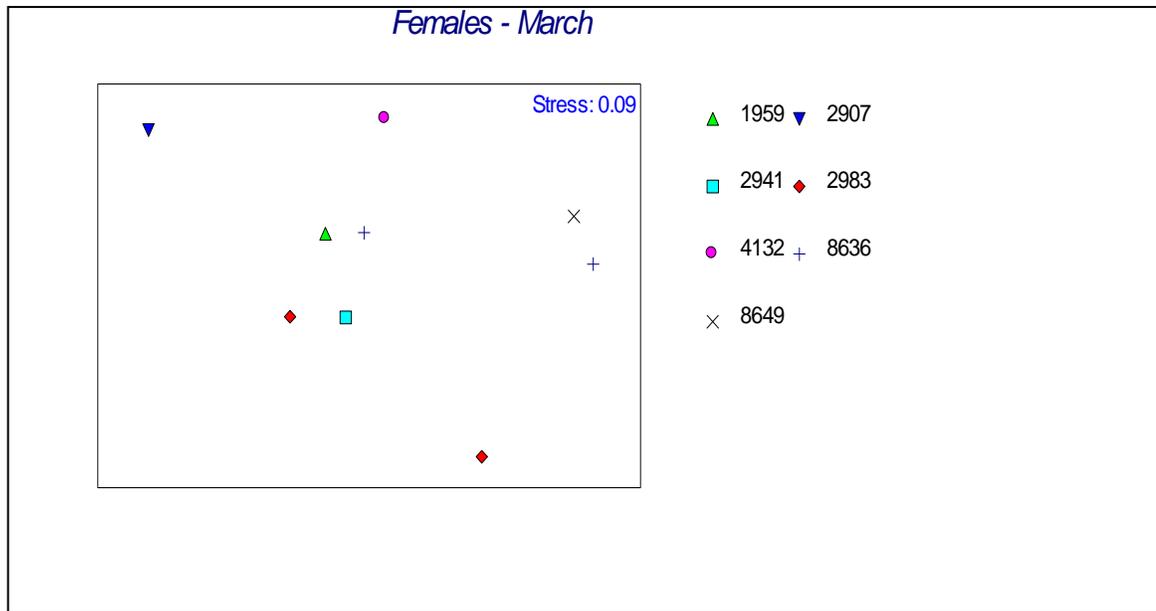


Figure 5.11 A MDS plot of the samples taken from different female possums during the breeding season (March) of 2009. All females in this group are from Coatesville.

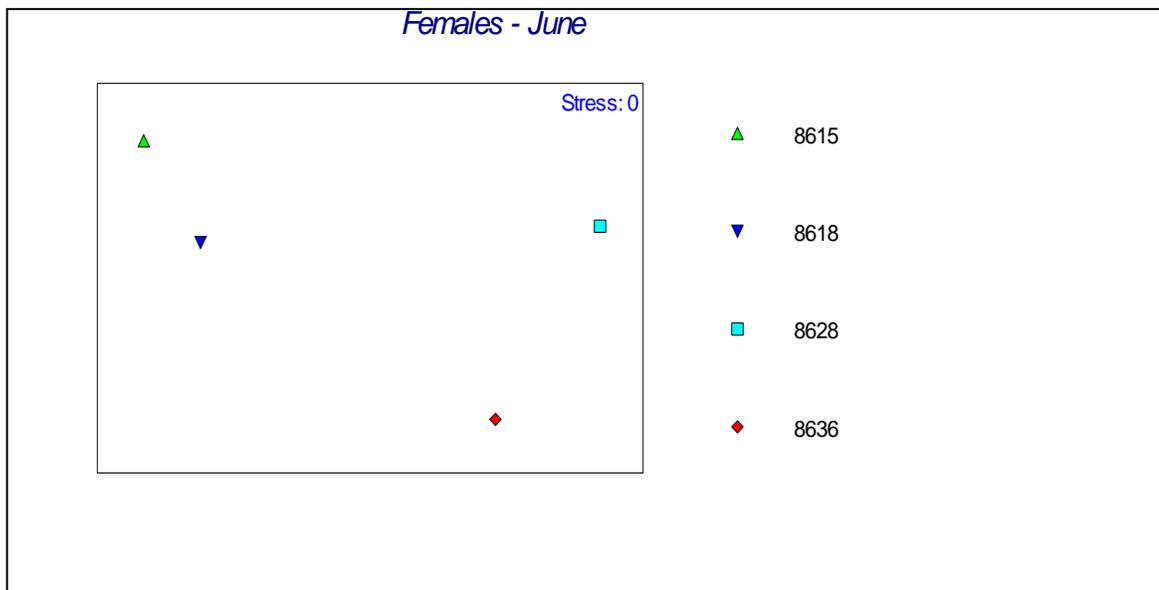


Figure 5.12 A MDS plot of the samples taken from different female possums during June of 2009. Female 8628 is from Mercer while all others are from Coatesville.

Possum Pair	Distance Between Possum Pair
1959-2983	0.447
1959-8636	0.563
2941-8636	0.622
2983-8636	0.680
2941-4132	0.766
8649-8636-2	0.769
4132-8636	0.794
1959-2983-2	0.908
2941-8649	0.985
1959-2941	1.019
2983-2-8649	1.057
4132-2907	1.073
2941-8636-2	1.082
2907-8636	1.250
2941-2983	1.281
2983-2983-2	1.293
2907-2983	1.294
8649-1959	1.315
8636-8649	1.341
1959-4132	1.357
2983-2-8636	1.358
2983-4132	1.370
2941-2983-2	1.488
1959-2907	1.600
8636-8636-2	1.665
2907-2941	1.669
4132-8636-2	1.721
2983-8649	1.749
4132-8649	1.750
2983-2-8636-2	1.817
1959-8636-2	1.867
4132-2983-2	2.098
2983-8636-2	2.246
2907-2983-2	2.507
2907-8649	2.570
2907-8636-2	2.732

Table 5.4 The distance between female possum bacterial profiles in March. The median for these data is 1.315 and the average distance is 1.392.

Possum Pair	Distance Between Possum Pair
8628-8636	1.068
8615-8618	1.091
8618-8628	1.203
8618-8636	1.321
8615-8628	2.246
8615-8636	2.350

Table 5.5 The distance between female possum bacterial profiles in June. The median for these data is 1.262 and the average distance is 1.546.

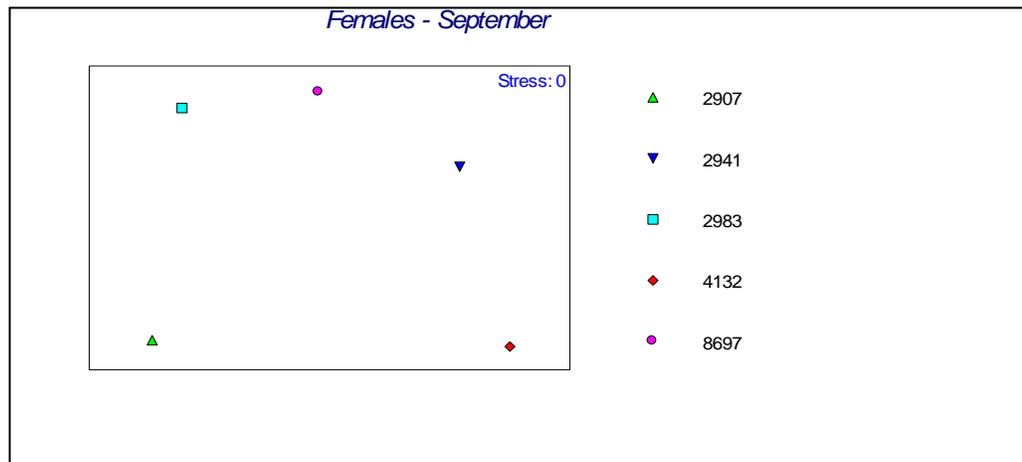


Figure 5.13 A MDS plot of the samples taken from different female possums during September of 2009, which is a time of year that some females undergo a second oestrous. All females in this group are from Coatesville.

Possum Pair	Distance Between Possum Pair
2983-8697	0.684
2941-8697	0.850
2941-4132	0.980
2907-2983	1.310
2907-8697	1.424
2941-2983	1.533
4132-8697	1.643
2907-2941	1.973
2907-4132	2.173
2983-4132	2.286

Table 5.6 The distance between female possum bacterial profiles in September. The median for these data is 1.478 and the average distance is 1.486.

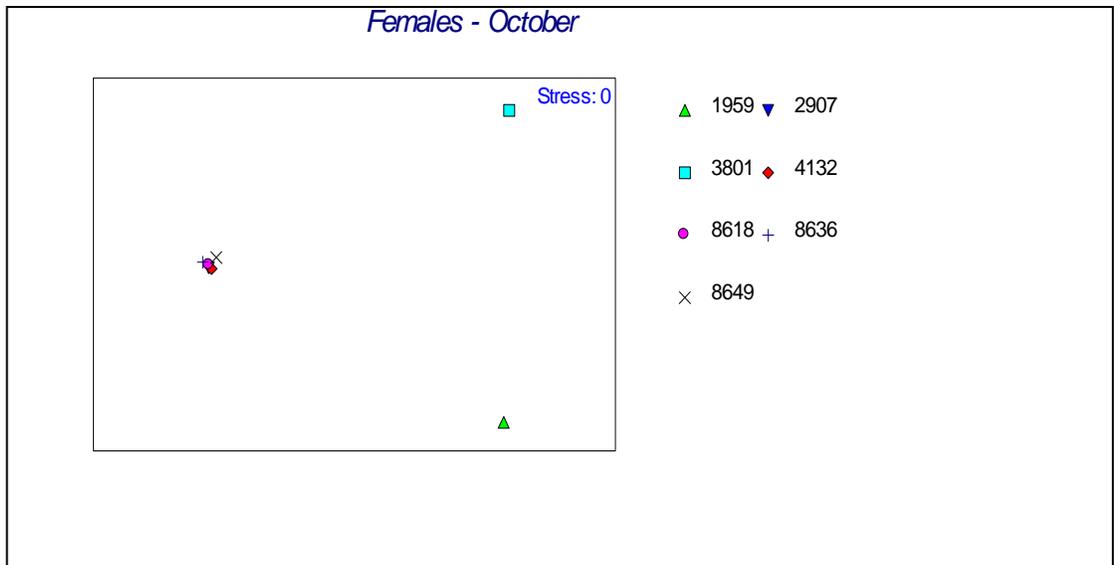


Figure 5.14 A MDS plot of the samples taken from different female possums during October of 2009. All individuals are from Coatesville.

Possum Pair	Distance Between Possum Pair
4132-8618	0.014
8618-8636	0.020
2907-8618	0.022
2907-8636	0.022
2907-4132	0.022
8636-8649	0.022
4132-8636	0.032
8618-8649	0.041
2907-8649	0.042
4132-8649	0.054
3801-1959	1.810
1959-8618	2.120
1959-4132	2.125
3801-8649	2.126
1959-8649	2.129
1959-8636	2.129
2907-1959	2.143
3801-8636	2.144
3801-8618	2.152
3801-4132	2.165
2907-3801	2.166

Table 5.7 The distance between female possum bacterial profiles in October. The median for these data is 1.810 and the average distance is 1.119.

5.4 Discussion

5.4.1 Number of Bacterial Operational Taxonomic Units

A much larger number of bacterial OTU's were found on the cutaneous surface of the female possum sternal gland than on that of male possums. As there is only a slight overlap between the lowest number of OTU's found on a female and the highest number found on a male, it is unlikely that these results strongly reflect sampling error. If errors had occurred which would have led to such results, a more random pattern in sampling efficiency, and hence more overlap in numbers, would be expected.

It is therefore likely that females do, in general, carry more bacteria in the sternal region. Intersexual differences in microbial (bacterial and fungal) load have also been detected in different regions of the human body (Noble *et al.*, 1976; Marples, 1982; Leeming *et al.*, 1989). However, these studies all found there to be more bacterial species on males than on females. Marples (1982) suggests that the obvious differences in cutaneous physiology and anatomy between the genders are likely to effect the composition of cutaneous flora, leading to such results. It is thus possible that the same holds true for other species such as possums.

5.4.2 Degree of Similarity Between Replicate Samples Versus Those Between Different Possums

There is not a significant difference between the degree of similarity between replicate samples from the possums in this study and the degree of similarity between the samples of different possums. These data suggest that community structure of an individual's cutaneous bacteria is highly variable and is nearly as variable as that between different individuals. There were, however, considerably fewer replicate samples than samples between different animals so a definite conclusion cannot be made. Further studies, with more replicates from more individuals are required.

There are many possible explanations for the high degree of variation found between replicate samples in this study. Contamination can lead to extra OTU's being present in some samples, for example. However, extra care was taken in both the field and laboratory to reduce the chance for contamination and negative controls contained no DNA indicating that swabs, saline, lab reagents, etc. were not contaminated.

Another possibility is that there was variation in the efficiency of PCR. OTU's that occurs at low levels in a sample may not be replicated during PCR as the few strands of its DNA molecules may recombine with other strands that are at least somewhat complementary and end up being part of a chimeric molecule (Wang & Wang, 1996). For example, when Wang and Wang (1996) replicated different DNA templates at a 1:10 ratio, almost all of the strands of the less occurring DNA template recombined. A similar occurrence was noted in another study by Wang and Wang (1997). Thus, if either fewer cells of a species happened to occur on an animal during a certain sampling session or if fewer cells were collected due to inefficiencies in the sampling procedure, the DNA of these cells may have not been replicated during PCR. Furthermore, due to the logarithmic nature of PCR, DNA from cells that occur at a relatively low quantity in a sample may not amplify as much as DNA from cells that occur at a higher quantity (B. Weir, pers. comm.).

This leads into another possibility concerning repeated sampling within one to two days of each other. If sampling had removed majority of bacterial cells on one day, these cells may not have recovered back to a large enough number by the next day or two which may lead to fewer cells and then less efficient amplification. Studies which have investigated the doubling time of bacterial cells in nature (e.g. in rabbits (Latin name not given) and in pond water) found that the doubling time varies from one to eight hours (Bott & Brock, 1970; Small *et al.*, 1986). The doubling time for different species and in different environments is likely to be highly variable, thus specific studies on possums and their symbiotic bacteria are required to see how long it takes their bacteria to repopulate following a disturbance. Furthermore, if a large number of bacterial cells are in fact being removed during sampling, a founder effect may occur which may result in decreased genetic variation which has the potential to have impact the pattern of recolonization.

Furthermore, many bacterial species compete with each other, inhibiting colonization. For example, *Cornebacterium* species and non-*aureus* staphylococci inhibit the colonization of *Staphylococcus aureus* in the human nose, leaving *S. aureus* colonization dependent upon the bacterial community that is present in one's nasal cavity (Lina *et al.*, 2003). Thus, if many cells of dominant species were removed from the possums during sampling, this may have allowed other species to colonize the area due to a decrease in competition. Conducting studies on how long it takes for their bacterial cells to

recover as well as competitive relationships between the species found on these animals may help determine whether or not this is an issue. To resolve this, the same individuals should be sampled on a regular basis, perhaps ranging from every few hours to every few days or so, in order to see how much variation exists over a short time span, to see the point at which the populations re-establish themselves, and to study species turn-over following disruption. It would be best to conduct such a study on captive animals as re-trapping the same animals in the wild over short time periods is difficult and therefore a rare occurrence.

One other possibility is that the bacterial profile on the possum sternal gland actually does change on a semi-regular basis, thus leading to the results found in this study. Possums use this gland to mark various items in their habitat and are likely to pick up microbes from these different items as they mark them. It is not unreasonable to propose, therefore, that the bacterial OTU's found on this glandular area may change depending on what bacteria they encountered which depends on what items they marked before being caught for sampling. Perhaps the possums whose replicate samples did not differ greatly had not marked vastly different items on the two nights before being trapped while those with highly dissimilar samples marked items that contain a different array of bacterial species. Further studies into the possibility of variation in microbial community composition based on having contact with different items would prove beneficial. A captive situation, but one that is large and diverse with multiple surfaces to mark, may suit such a study.

5.4.3 Individual Bacterial Profiles During Different Seasons

Although a conclusion cannot be made regarding a correlation between hormonal status and cutaneous bacteria community composition in brushtail possums due to a small sample size in this study, the trends seen in these data suggest that a correlation does not exist. Although few replicates were collected from possums in the same month, majority of the replicates gathered were less similar than samples collected from the same possum from different months (or from different possums). If a correlation exists between bacterial composition and hormonal status, a higher degree of similarity would be expected to be found between replicate samples taken within a day or two of each other.

It is possible, however, that there is a correlation between hormonal status and bacterial community composition and that the results of this study failed to expose this relation due to the possible errors and inefficiencies discussed above (see 5.4.2). For the same reasons discussed above, conducting further tests on a larger number of possums are necessary to better understand the nature of their cutaneous bacterial communities and the variations that exist in these communities across time. Again, captive studies may be beneficial for sample size considerations, for the number and frequency of replicate sampling sessions, and for the ability to control more variables (e.g. what structures are available for the possums to mark).

5.4.4 Bacterial Profiles between Different Individuals

Similarly, a conclusion cannot be made regarding the possibility of unique microbial signatures in this species due to the small sample size in this study. As with the results of section 5.4.1, replicate samples of individual possums that were collected within a day or two of each other were less similar than those samples collected between different individuals. The trend thus suggests that possums do not have cutaneous bacteria communities that are unique but can also be the result of the issues discussed in 5.4.2.

Furthermore, some individuals had matching, or close to matching, bacterial profiles. Such a result can be seen in the samples collected from males in October and from females in October (Figures 5.10 and 5.14). If one's symbiotic microflora is at least partially dependent upon one's physiology as has been hypothesized, we would expect these matching profiles to occur between replicates of an individual (which has not occurred in this study, these matching profiles are from different animals) and we would also expect to see individuals from the same populations grouped together due to a higher degree of relatedness, which we do not see either. For example, during June, female 8628 is the sole female from Mercer and yet the dyad with the greatest amount of difference in this group consists of two females from Coatesville (Figure 5.12).

There are other limitations associated with the techniques used in this study. For example, the sampling technique used in this study may have affected the results of this study in general, leading to an underrepresentation of OTU's in the samples. Morgan *et al.* (1985) tested the sampling efficiency of two techniques, swabbing and excision. Both

techniques detected bacterial species in majority of trials but both also failed to detect organisms in some trials when the organisms were known to be present. Furthermore, both the study by Morgan *et al.* and another study which also compared different sampling techniques on carcasses (Dorsa *et al.*, 1996) found that sampling with cotton swabs, which were recommended for use in this study, is not the most efficient method to use. It is therefore likely that variation in sampling occurred and also that many OTU's may have been missed in some or all samples.

Furthermore, the full diversity of bacterial OTU's inhabiting the sternal surface of the possums may not be represented in the results due to the primers that were used. As exceptions to the universality of some primers are being found as more environmental sequence studies are conducted, Hugenholtz and Goebel (2001) suggest using multiple primer sets in order to obtain a more complete picture of the OTU's present. Due to funding limitations, multiple primer sets were not used in this study.

There are other issues associated with these techniques that can suggest that there are actually more OTU's present than there are in reality. As mentioned above, chimeras can be formed during PCR which can show up as a distinct band on the DGGE gel, suggesting another OTU when it is, in fact, not an OTU at all. Chimeras can be formed when two templates, similar in nucleotide sequence, recombine. Recombination sites typically have 20-30 identical bases (Hugenholtz & Goebel, 2001). Chimeras can be formed between DNA strands of closely related species or between two of the multiple rRNA genes that most bacterial species possess (Wang *et al.*, 1997). Intensive cell lysis procedures which damage DNA molecules, such as those sometimes used for Gram positive bacteria (Wintzingerode *et al.*, 1997), as well as a high number of PCR cycles, a short elongation period, and a high diversity of DNA templates (Qiu *et al.* 2001) can all lead to a higher probability of chimeric formations.

It is also possible to obtain results which suggest that there are actually fewer OTU's than there are in reality. Due to issues with the apparatus, the current dropped while one of the gels was running causing the DNA to not travel as far down the gel as it had in other gels. Due to this, the DNA molecules may not have separated as much on this gel as they did on other gels leading to skewed results as a collection of multiple small bands may just appear as one large band. This may lead to some variation being undetected on the gels

that did not separate as well. Similarly, the resolution differed between the gels with some being relatively clear while others being smeared which made analyses difficult. This is another way that variations (or lack thereof) in the samples ran on certain gels may have gone undetected.

Furthermore, it is not possible to come to conclusions regarding the relative abundances of various bacterial OTU's as PCR does not necessarily allow for communities to be analyzed in a quantitative manner. Suzuki and Giovannoni (1996) found that one pair of primers yielded products in a 1:1 ratio regardless of the initial proportion of the DNA templates while the products of a different set of primers represented the initial ratio. As experiments were not conducted to determine whether the primers used in this study yield products that represent the initial proportions of templates, it is not possible to come to a quantitative conclusion on the bacterial community composition. Additionally, DNA strands that contain many guanine (G) and cytosine (C) nucleotides do not disassemble into separate strands as easily as these two nucleotides are bound by three hydrogen bonds (compared to two bonds as is the case with adenine and thymine). This can lead to biases during PCR as DNA containing less G and C will be separated easier and may therefore amplified more (Suzuki & Giovannoni, 1996). This could potentially lead to some species not being represented, leading to results which suggest less variability than there actually is, and/or others being overrepresented, which is another reason why we cannot come to a quantitative conclusion on these data.

Further studies are required to gain an understanding of the bacterial profiles of the sternal gland surface of brushtail possums as well as the dynamics of these communities. If the methods used in this study are to be utilized in further investigations, the sampling technique should be revised (perhaps to the sterile virgin sponge used by Dorsa *et al.* (1996)) and disadvantages and possible biases (discussed above) need to be considered and discussed. This technique has the advantage of allowing for the different OTU's in a sample to be separated on a gel and, when feasible, for the bands to be literally excised from the gel and sequenced. Additionally, MDS plots can be constructed using the gels to compare the composition of different samples with one another and the x and y coordinates provided (during MDS plot construction) can be used to numerically analyse relationships. When samples need to be compared to one another, however, they need to be ran on the same

gel. The program used to analyze the gels (PFQuest (version 5.10) (Bio-Rad)) was unable to make comparisons between different gels. This needs to be considered when planning future studies. It would ultimately be better, however, to analyze samples for such a study via amplicon sequencing using the Roche 454 sequencer (B. Weir, pers. comm.) to avoid the issues discussed above. Unfortunately, this technique was not available when this project was undertaken, but it would be useful in future studies.

In addition to further analyzing the composition of the bacterial communities inhabiting possums scent glands, it would be interesting to identify (via sequencing) the OTU's found on these animals and to see how many of the bacterial OTU's are shared between individuals and shared between males and females. Looking at different possum populations would also be interesting to determine the degree of bacterial variation found on animals inhabiting different regions. Sequencing the DNA of the OTU's found will also ensure that all bands on the DGGE gels truly represent unique OTU's, and are not just different copies of the 16s rRNA gene that can be found in the same OTU. Variation between the different copies of the 16s rRNA gene can differ by up to 5% in some species (Clayton *et al.*, 1995). Sequencing should thus be conducted as it will enable us to confirm the actual number of unique species in a sample and to evaluate the degree of species sharing between different animals.

It would also be interesting to look at what factors affect the dynamics of their bacterial communities to see if hormonal status truly does have an effect. If a correlation is found between season and bacterial profile, however, this will not necessarily support the hypothesis as other factors, such as seasonal changes in possum diet (if this occurs) and what items they mark, may also have an effect on bacterial community dynamics. Changes in the chemical composition of the sternal gland secretion is impacted by the diet of possums (Salamon, 1994) and this may very well have an effect on the bacterial species present due to changes in nutrient availability. Further studies, such as treating possums with hormones while monitoring their bacterial composition, as well as studies on their diet and on the relations between their diet and bacterial composition, will thus need to be conducted.

Additionally, studies involving the relationship between bacterial profile and scent production should be conducted to understand how important bacteria are in the production

of possum scents. This may be accomplished by altering the bacterial composition (perhaps via antibiotic treatment) on the surface of their glands while monitoring the chemical composition of their scents at the same time. It would also be interesting to see if behavioural responses to the scents change based on which bacterial species are present to metabolize their secretions. Changes in the behavioural responses of male lemurs (*Lemur catta*) to female lemurs who are treated with hormonal contraception have been noted (Crawford *et al.* 2010). It would be interesting to see how bacteria are involved in the relationship between the hormonal status and odour cues of the scent producer and to determine the extent to which they are involved in animal communication.

Chapter Six: Conclusions and Implications

6.1 Possum Social Interactions

Studying the social interactions of animals, particularly the interactions of animals that are ecologically significant (e.g. invasive or endangered), is important as understandings in this area have the potential to facilitate their management. Understanding the social interactions of brushtail possums in New Zealand is important as they are invasive in this country and they are also bovine tuberculosis reservoirs. It is therefore necessary to have an insight into how frequently they come together and whether or not they interact during different seasons. Such information may allow researchers to devise an efficient and appropriate type of self-disseminating biological control for this species and may also assist in the control of bovine tuberculosis.

The findings of this study suggest that female possums in this area engage in close interactions with other females and with males during both the breeding and non-breeding seasons. Males, however, seem to avoid one another. These findings have implications for both the issue of bovine tuberculosis in New Zealand and for the possible use of a self-disseminating vector in the control of possums. As bovine tuberculosis is spread via close contact (e.g. mating, fighting, denning, and grooming) (Fairweather *et al.*, 1987; Morris & Pfeiffer, 1995; Coleman & Caley, 2000) and close contact would also be required for certain types of biological control, these patterns of close interactions are highly important.

For bovine tuberculosis and its control, it is important to know such patterns of interaction so that control methods can be implicated at the correct time. If, for example, social interactions dropped during the non-breeding season, it would be beneficial to focus control efforts during the breeding season. But with the patterns found in this population, the data suggest that the spread of this disease is possible and likely even outside the breeding season. Thus, if control of this disease were to be attempted, it would be important to control and monitor it throughout the year.

With regards to a self-disseminating form of biological control for brushtail possums, these results are promising. If males and females are coming into close contact even during the non-breeding season as are females and females, a form of control requiring some sort of close contact may be feasible. Even if males do not interact with other males, they are apparently likely to interact with females who can then pass it vector

off to other males. Before such a type of biological control can be implemented, however, further tests are required. First of all, it is necessary to determine how close they have to be for the vector to be passed on and if the possums are coming into this range. As discussed in chapter three, it would be beneficial and perhaps necessary to repeat such studies with data loggers that only record interactions at much closer ranges. For example, if the possums are mainly spending time only within a metre of each other, for example, this may not be close enough for the use of a biological control agent that is self-disseminating. Secondly, it would be necessary to ensure that the patterns seen in this population are also found in other populations as it would be most efficient to devise a control method that works in majority if not all populations.

This study did not find a significant relationship between genetic relatedness and interactions between males and females or between female-female pairs. Determining why a significant relationship is lacking should be pursued in future studies. With regards to sexual relations, it would be interesting to see if possums of other populations are restricted to mating with possums with whom their ranges neighbour as suggested by Clinchy *et al.* (2004). If this is the case, additional studies looking at the genetic relatedness and frequency of matings between possums and their immediate neighbours would be a promising study in determining whether or not relatedness is important in possum mate choice. Furthermore, as previously suggested, captive studies may also be beneficial as relatedness can be controlled for in such studies allowing us to gain better insight into whether or not possums use degree of genetic relatedness to choose mates. It would be important to properly design such experiments and to conduct them in proper enclosures (i.e. areas that are large enough to allow each possum enough personal space) to avoid the problems of aggressive interactions encountered by Day *et al.* (2000).

Similarly, captive studies conducted in an appropriate enclosure may be a beneficial way to study if possums are more tolerant of conspecifics based on relatedness or familiarity as both of these variables can be controlled for in captive situation. Furthermore, as suggested for studies on mate choice and relatedness, studies in the wild looking at the genetic relatedness of neighbouring possums and levels of longer lasting interactions may be beneficial as the studies will focus on those possums who are more likely to cross paths with each other.

6.2 Behavioural Response to the Scent of the Possum Sternal Gland

Possum management may also benefit from insights into how possums respond to the scents of other possums. Understanding this aspect of their olfactory communication may be beneficial as possums scents, if they prove to be attractive enough, may be used as species specific lures in their management. It is therefore necessary to study how they respond to different scents and if their responses vary according the gender of the scent donator as well as the degree of familiarity that they have with the donors. Other factors, such as reproductive state of the donors and the season of testing, should be studied as well and would be interesting areas of research in the future. This will allow us to understand patterns of interest in, if any exist, as well as the luring abilities of possum produced scents.

As the sample size in this part of the study was low, it is not possible to come to conclusions on how possums respond to the sternal gland scent of other possums. The trends suggest that they are more interested in the scents of familiar females over familiar males and in the scents of foreign possums over familiar possums. Further studies are required, however, before a definite conclusion can be made. What can be concluded, however, is that the possum sternal gland scent would not function as a proper lure. It is clear that they are far more interested in the scent of cinnamon apples, making this a more efficient lure to use with this species. Further studies should be conducted on the luring ability of other possum scents, however, as a species specific lure would reduce the number of catches of non-target species.

When designing future experiments of this nature, it is important to consider the likely effect that experimental design had in this study. Some sort of a structure should be used to ensure that the possums are exposed to all scents. Furthermore, the presence of conspecifics should be controlled as conspecifics seemed to have had an effect on the number of scents investigated by visiting possums. For these reasons, a captive environment may be a more appropriate setting to begin investigating such matters. With a more controlled environment, a larger sample size, and a higher number of replicate visits from the same possums, we are more likely to be able to come to a conclusion of their responses and level of interest for various scents.

6.3 Denaturing Gradient Gel Electrophoresis (DGGE) as a Technique for Investigating Bacterial Community Composition and Dynamics on the Possum Sternal Gland

Studies investigating the composition and dynamics of bacterial communities inhabiting the skin of the glandular areas of animals are of interest as bacteria play a role in scent production. It would, therefore, be interesting to gain an understanding of how much of a role bacterial play, how bacterial communities change through time, and how such changes are affected by factors such as physiology and the diet of the animal on which they live.

The pilot study investigating DGGE as a technique to investigate the compositional dynamics of the bacterial communities inhabiting the cutaneous surface of the possum sternal gland suggests that other techniques may be more appropriate for such a study. While DGGE has advantages such as allowing for the visualization of the number and pattern of operational taxonomic units for different samples, there are technical difficulties associated with the technique as well as difficulties in interpreting the results. More advanced technology, such as amplicon sequencing using the Roche 454 sequencer, are now available and should be pursued for future studies in this area.

The results obtained during this pilot study are inconclusive due to the small sample size in this study. The trends seen, however, suggest that the composition of bacterial communities on the skin surface of possum sternal gland is highly variable as the compositions of replicate samples from the same individuals (within a day or two of each other) were less similar than that of the samples collected from different animals. In this pilot study, a correlation was not found between hormonal status (i.e. breeding and non-breeding season) and the composition of the bacterial community on the possum sternal region. Unique bacterial profiles have also not been found. Profiles of some individuals were nearly identical and yet large variations in the profiles were recorded for the same individuals in the same season.

Further studies are undoubtedly required before conclusions can be made. Such studies require a much larger sample size and a high number of replicate samples for all individuals. This will allow us to see how regularly the composition changes. It is also necessary to investigate the potential effect that sampling has on their bacterial

communities. More advanced technologies (as opposed to DGGE) should be utilized as this will hopefully lead to more accurate and interpretable results, allowing for a better understanding of possum sternal gland bacterial community composition and dynamics.

Chapter Seven: References

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