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The behavioural and neuroendocrinological mechanisms affecting group formation in the African striped mouse, *Rhabdomys*

Kirsty-Jane Hartman

This thesis is submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

February 2018

DECLARATION

I hereby declare that this thesis is my own unaided work and that recognition has been given to the references used. It is being submitted for the Degree of Doctor of Philosophy in the Faculty of Science at the University of the Witwatersrand.



Kirsty-Jane Hartman

16 February 2018

ABSTRACT

Social systems vary within and between species, resulting in some populations living in complex social groups while others adopt a solitary lifestyle. Social systems are ultimately emergent properties of the interactions and relationships between individuals within a population. Social flexibility (a form of phenotypic plasticity) describes hormonally mediated reversible switches in social tactic in response to prevailing environmental conditions. I investigated the mechanisms underlying social interactions, and ultimately social structure in the striped mouse genus, *Rhabdomys*. It is widespread in southern Africa, occurring along an aridity gradient, which influences the social structure of constituent species. A desert species, *R. pumilio*, switches from group to solitary-living, and I asked whether a solitary-living grassland species, *R. dilectus*, could form stable groups. Four experiments were undertaken. 1) To examine the behaviours underlying group formation initiation, age-matched triads of unrelated, sub-adult conspecific female grassland and desert striped mice were formed in captivity. Nest-cohabitation occurred in all desert and 90% of grassland triads, yet desert triads were more aggressive than grassland triads on initial meeting. Both species spent more time interacting amicably as time in triads progressed. 2) Measurements were taken of circulating corticosterone and testosterone concentrations, and 3) neuropeptide arginine vasopressin (AVP) and oxytocin concentrations in the brain of triads and singly-housed controls. Grassland females in triads had greater corticosterone and AVP concentrations than desert female triads, but levels did not differ between triads and solitary-housed controls of both species; testosterone and oxytocin were unaffected. 4) I experimentally injected females of both species sequentially with a saline control, a low and a high dose of AVP, and measured their social motivation (i.e. propensity to spend time with a conspecific) and recognition of familiar unrelated conspecific females. Exogenously administered AVP enhanced the motivation to be social, by increasing the approaches toward the conspecific by focal females in the high AVP treatment in grassland mice and the low AVP treatment in desert mice. Neither species displayed a preference for an unfamiliar conspecific, but amicability toward familiar females was greater in grassland mice, but decreased in desert mice, under high AVP. Solitary grassland striped mice displayed tolerance of conspecifics, underpinned by different inherent hormonal control. My study has demonstrated that the naturally solitary *R. dilectus* can form similar social groupings to that of the facultatively social *R. pumilio*. Switching between social tactics

might have potential survival benefits in unpredictably changing environments predicted for southern Africa.

DEDICATION

For my family, and friends who have become family

- a reminder that dreams do come true.

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CHAPTER 1
General Introduction

Social systems

The social system of a population refers to the social interactions and subsequent relationships between the individuals (social structure) of that population which influences the composition of groups (social organisation; Lott, 1991; Kappeler and van Schaik, 2002). Social systems vary within and between species, resulting in some leading a solitary life, whereas others live in social groups (Lott, 1991; Kappeler et al, 2013). Social systems are the outcome of a trade-off between costs and benefits associated with resource availability, predation risk, reproductive competition and thermoregulatory advantages (Randall et al, 2005; Schradin et al, 2010). Ecologically, group living occurs, for example, when resources are temporally and spatially unpredictable, there is a short growing season of vegetation, limited nesting sites or cover, and high population densities (Poole, 1985; Schradin, 2005). In contrast, solitary living occurs when, for example, resources are widely distributed, food quality is poor, the growing season of vegetation is longer (which results in a longer breeding season), an abundance of cover is present (allowing for more available nest sites), and population density is relatively low (Poole, 1985; Schradin, 2005). The social system adopted by a population would be the one where the benefits are greater than the costs to an individual's fitness (Silk, 2007). These functional reasons for variation in social systems have been investigated extensively, whereas investigation into the proximate mechanisms underlying the development of social systems has been relatively neglected in comparison (Choleris et al, 2004; Hofmann et al, 2014).

THE NEUROENDOCRINE MECHANISMS OF SOCIALITY

Sociality is the tendency of an animal to live in a social group, which may vary in size and composition, and not merely the formation of aggregations (Alexander, 1974; Lacey and Sherman, 2007). Sociability describes an animal's propensity to spend time with conspecifics rather than remaining alone (Moy et al, 2004; Lim and Young, 2006). In order for an animal to live in a group, it must be motivated to interact with conspecifics and demonstrate a level of tolerance of conspecifics, as well as be able to discriminate between familiar and unfamiliar conspecifics (Engelmann et al, 1995; Choleris et al, 2009). Tolerance requires reduced or no aggression and the expression of affiliative (amicable) behaviours, allowing for bonding between conspecifics to occur and the potential for group formation (Young, 2002; Choleris et

al, 2004; Storm and Tecott, 2005). A direct relationship between the androgenic steroid hormone testosterone and aggression is well established (Nelson, 2005). In the case of amicable behaviour, many rodent species exhibit reduced circulating testosterone concentrations during winter to facilitate huddling, which leads to a reduction in energy demands of thermoregulation (Nelson, 2005). Progesterone is another steroid hormone that has been linked to aggressive behaviour in females in several species (e.g. golden hamster, *Mesocricetus auratus* (Payne and Swanson, 1971), and Galapagos marine iguanas, *Amblyrhynchus cristatus* (Rubenstein and Wikelski, 2005)). The glucocorticoid hormone, corticosterone, is released in response to stressors via stimulation of the hypothalamus-pituitary-adrenal (HPA) axis, and through mobilisation of glucose, allows animals to react to energetically demanding contexts (Sapolsky et al, 2000; Rubenstein, 2007). Agonistic social interactions can elicit corticosterone release, and high levels of circulating corticosterone have been associated with dispersal in group-living animals (Creel, 2001; Schoepf and Schradin, 2013).

Social recognition enables group living animals to express the appropriate affiliative behaviours towards group members, and to react to intruders entering the group's territory (Young, 2002). Arginine vasopressin (AVP) and oxytocin are two neuropeptides that have been implicated in mediating social recognition and social behaviour (Neumann, 2008; Albers, 2012). In the peripheral circulation, both neuropeptides function as hormones. AVP is important in water balance by regulating the reabsorption of water in the kidney as a response to changes in plasma osmotic potential and plasma volume, and in the cardiovascular system for stabilising blood pressure via vasoconstriction (Nelson, 2005; Curtis et al, 2007; Leng et al, 2012; Carter et al, 2008). Oxytocin is important for its stimulation of the contraction of smooth muscle in several organs and glands, including uterine contractions during parturition and milk-ejection in lactation in females, and enabling penile erection and stimulating ejaculation during copulation in males (Nelson, 2005; Curtis et al, 2007; Leng et al, 2012; Carter et al, 2008).

The central actions and concentrations of AVP and oxytocin originate from extra-hypothalamic sites and fibres that project from the paraventricular nucleus (PVN) into the medial amygdala, bed nucleus of the stria terminalis (BNST), lateral septum, anterior hypothalamus, ventromedial hypothalamus, and the periaqueductal grey (area of the midbrain involved in autonomic responses) of the brain (collectively referred to as the "social behaviour network" (SBN)), where they mediate social behaviours by acting on specific receptors in these regions (Hawthorn et al, 1980; Bradford, 1986; Bielsky and Young, 2004; Veenema and Neumann, 2008; Leng et al, 2012). Oxytocin has only one receptor type, while AVP has three

different types, but of the three, the V1a receptor is primarily implicated in its behavioural actions (Carter, 2003; Albers, 2012; Leng et al, 2012).

AVP is important in social recognition and social communication, and is usually closely associated with male-typical social behaviours, such as paternal care, mate guarding and courtship (Young and Wang, 2004). Oxytocin is involved in mediating several affiliative behaviours, such as grooming, sexual behaviour, pair bonding, and social communication (Nelson, 2005; Nair and Young, 2006; Neumann, 2008). The majority of studies on the role of oxytocin in sociality have focused on pair bonding, mainly in the monogamous prairie vole, *Microtus ochrogaster* (e.g. Young and Wang, 2004). Oxytocin has also been confirmed to be important in tolerance and establishing social attachments with conspecifics in laboratory mice and rats (Carter, 2003). Knock-out of oxytocin expressing genes have demonstrated that AVP has the ability to assume some of the functions of oxytocin, indicating that both neuropeptides regulate social behaviour in both sexes (Carter, 2003; Carter et al, 2008; Donaldson and Young, 2008).

VARIATION IN SOCIALITY

Sociality is influenced by an interaction between the external environment and the internal (neuroendocrine and genetic) interactions, and thus variation in sociality occurs both between and within species (Lott, 1991; Kappeler et al, 2013; Hofmann et al, 2014). An integrated understanding of the internal mechanisms that prime animals to exhibit a particular suite of social behaviours, and which result in animals responding to various environmental conditions, is essential to our understanding of social evolution (Hofmann et al, 2014). Interspecific variation in sociality seen in closely related species of voles, *Microtus* genus, has been partly attributed to differences in the environment in which each species occurs, and to differences in intrinsic inclination for social contact or avoidance (Getz et al, 1993; Insel and Shapiro, 1992). The communal group-living monogamous prairie vole, *M. ochrogaster*, experiences higher population densities than the solitary-living promiscuous montane vole, *M. montanus* (Getz et al, 1993; Insel and Shapiro, 1992). These two species also differ in terms of behavioural responses to artificially administered increases of AVP and oxytocin, which elicits mate guarding aggression towards males and promotes bond formation with females in male prairie voles, *M. ochrogaster*, but does not affect these behaviours in the montane vole, *M. montanus* (reviewed in Carter et al, 2008; and Veenema and Neumann, 2008).

Intraspecific variation in sociality can occur because of one or more of three processes: genetic differences between populations; stochastic environmental processes; or phenotypic

plasticity (Lott, 1991; Schradin, 2013). 1) Genetic reasons for differences in sociality are especially noticeable between individuals of the same species from different localities which display local adaptations to different environmental conditions (Schradin, 2013). An example of such genetic variation is seen in salt marsh wolf spiders, *Pardosa purbeckensis*, where individuals born in a laboratory setting but originating from salt marshes of various sizes, differed in their propensity to disperse (and utilisation of wind for dispersal) in direct correlation with the size of the marsh from which they originated: individuals from large salt marshes had a greater propensity for dispersal than spiders from small salt marshes (Bonte et al, 2007). 2) Stochastic events are extrinsic factors that cause variation in social organisation within a species, such as the death or removal of a dominant breeder (Schradin, 2013). Stochastic processes influencing sociality can be non-adaptive or might not have an ultimate or proximate explanation (Schradin, 2013). For example, common marmosets, *Callithrix jacchus*, typically live as nuclear family groups, consisting of a dominant monogamous breeding pair and their non-reproducing offspring, but following the death of the breeding male and introduction of a new male, a polygynous mating system developed, with mother and daughters in sexual competition with one another (reviewed in Anzenberger and Falk, 2012). This kind of disruption carries significant fitness costs, such as infanticide after changing from social monogamy to a new group composition (as in the case of the common marmosets above), which often leads to the eventual restoration of the original social organisation (Anzenberger and Falk, 2012; Schradin, 2013).

3) Phenotypic plasticity describes phenotypic change without alteration in genetic makeup in response to environmental conditions. Here, individuals possess genotypes capable of producing different phenotypes to improve individual fitness as a function of prevailing environmental conditions (i.e. reaction norms; Van Buskirk, 2012; Dingemanse et al, 2009). Two forms of phenotypic plasticity exist: i) developmental plasticity and ii) phenotypic flexibility (Schradin, 2013). Developmental plasticity, describes the process when an individual adopts a tactic, which is maintained throughout its adult life, based on its experiences and environment during development (Sol et al, 2002; Randall et al, 2005; Schradin et al, 2012). Red foxes, *Vulpes vulpes*, display developmental plasticity in sociality (living solitarily or in monogamous family groups) depending on resource distribution and the occurrence of larger canids in their natal home range (Cavallini, 1996). Phenotypic flexibility describes a situation where changes in phenotype are reversible and can occur in adults (Schradin, 2013). For example, giant kangaroo rats, *Dipodomys ingens*, show seasonal changes in home range

size, where males permit females to share their territories, resulting in more individuals exploiting limited resources in the winter months of low food availability, and increased breeding opportunities for both sexes in the breeding season (Cooper and Randall, 2007). Pumas, *Puma concolor*, are typically territorial, solitary-living carnivores, but display instances of tolerance of conspecifics and co-feeding at large kills (Elbroch et al, 2017). Phenotypic plasticity could provide a potential advantage in cases of disruption and times of rapid environmental change (Dingemanse et al, 2009; Rymer et al, 2013).

Social flexibility, a form of phenotypic plasticity, is defined as reversible changes in the social organisation in adult animals which alternate between solitary and group living strategies within their lifetime, due to changes in environmental conditions (Schradin et al, 2012). Social flexibility considers the way individuals interact with conspecifics, such as developing a tolerance for other individuals, resulting in the formation of a social group, and a change in reproductive tactic, such as a female changing from breeding solitarily with a single male to forming a group with other females where all females assist in raising offspring (Randall, 2007). In the white-fronted bee-eater, *Merops bullockoides*, individuals often switch between being cooperative helpers at the nest to independent breeders in response to variable environmental conditions (Koenig et al, 1992). Eastern grey kangaroos, *Macropus giganteus*, also display such flexibility in response to extrinsic factors such as food availability and body condition, with females in good body condition feeding alone rather than in groups (Menz et al, 2017).

Social flexibility is predicted to be common in short lived species (Schradin et al, 2012), and has been described in two desert rodent species, the great gerbil, *Rhombomys opimus*, and the African striped mouse, *Rhabdomys pumilio*. In *R. opimus*, the traditional social system is made up of social groups, consisting of one male with up to six females, which defend a common territory against non-members while behaving amicably toward group members (Randall et al, 2005). A two-year drought resulted in low population density due to high mortality, and thus, group sizes were greatly reduced, with the majority of females changing to a solitary lifestyle during this time. Male great gerbils adjusted their reproductive tactic to suit the change in the female distribution, resulting in the usual social organisation consisting of group-living, social *R. opimus* changing to a system of solitary individuals, each defending its own exclusive territory (Randall et al, 2005). Similarly, in the usually group-living *R. pumilio*, a year of drought resulted in a low population density, leading to a total change in the social

organisation of both sexes changing from group living to living solitarily (Schradin et al, 2012); further details are provided below.

Study species

THE AFRICAN STRIPED MOUSE, *RHABDOMYS*

The African striped mouse, genus *Rhabdomys*, is a diurnal, murid rodent, widely distributed throughout southern Africa, occupying a variety of biomes from moist grasslands to semi-desert (Skinner and Chimimba, 2005). *Rhabdomys* is aptly named the striped mouse due to four distinguishing dark longitudinal stripes running dorsally from the base of its neck to the base of its tail (Skinner and Chimimba, 2005). No distinct sexual dimorphism occurs in *Rhabdomys*, with adult body mass of both sexes being between 32-55g (Schradin, 2005; Skinner and Chimimba, 2005). The pelage is reddish-brown to grey-brown dorsally, with a light off-white to pale grey-brown underbelly, and the ears are yellowish- to reddish-brown (Skinner and Chimimba, 2005; Stuart and Stuart, 2007). This colouration tends to vary with geographical location, as does tail length (8-11cm) and body size (Coetzee, 1970; Skinner and Chimimba, 2005; Stuart and Stuart, 2007). In South Africa, striped mice from the xeric western regions tend to have a lighter body mass, paler pelage and longer tails than striped mice occupying the mesic eastern regions (Pillay, 2000a; Pillay, 2000b; Skinner and Chimimba, 2005).

Historically, *Rhabdomys* was considered to be monospecific (i.e. *R. pumilio*), despite its vast geographic distribution (de Graaff, 1981) and evidence of reproductive isolation between geographically distant populations (Pillay, 2000a; Pillay, 2000b). In 2003, Rambau and colleagues proposed, through mitochondrial DNA phylogeography and karyotypic analysis, that these different populations comprised of two separate (yet closely related) species of *Rhabdomys*, with *R. pumilio* occurring in xeric environments and *R. dilectus* in mesic areas, which includes two subspecies, *R. d. dilectus* and *R. d. chakae*. *R. pumilio* is the putative ancestral form of the genus *Rhabdomys* and *R. dilectus* is a derived form (Rambau et al, 2003; Pillay et al, 2006; Meynard et al, 2012). Subsequent research by du Toit et al (2012) recognised four clades within *Rhabdomys*, identifying three distinct mtDNA lineages within *R. pumilio* (namely *R. pumilio, R. bechuanae,* and *R. intermedius*, and *R. dilectus* being the fourth clade).

I considered two clades of striped mouse, *R. pumilio* and *R. d. dilectus* (confirmed genetically prior to study). Distinct differences in sociality exist between these two clades, which occupy localities with contrasting environmental conditions (Schradin and Pillay, 2005). The arid Succulent Karoo of South Africa occupied by *R. pumilio* experiences very low rainfall

(160mm per annum) falling primarily during the winter months (Schradin, 2005), which results in a shorter growing season for the vegetation as well as a shorter breeding season for *R. pumilio*. Here, striped mice feed on the protein rich, patchily distributed wildflowers, young plants, and insects (Schradin, 2005). From long term studies on one population in Goegap Nature Reserve (Northern Cape Province), this species is facultatively group-living, with groups consisting of one breeding male, which displays paternal care, and up to four breeding females and their philopatric offspring of different ages (Schradin and Pillay, 2003; Schradin and Pillay, 2004). Group members share a nest, display amicable behaviour toward group members, defend a common territory, but forage solitarily during the day (Schradin, 2005).

The grassland species used here, *R. dilectus*, has a solitary social structure, with individual territories that overlap exclusively with territories of the opposite sex (Brooks, 1974; Schradin and Pillay, 2005). Females raise offspring alone and only interact with males during the breeding season, although laboratory studies have revealed paternal care exists in this species (Schradin and Pillay, 2003). Paternal care occurring in captivity but not in nature suggests this behaviour is plesiomorphic in *R. dilectus* (Pillay et al, 2006) but this assumption requires testing. The grassland areas of central South Africa experience summer rainfall of between 700-1000mm per annum. *R. dilectus* does not eat the grasses themselves, but the seeds of various grasses as well as berries and insects (Schradin, 2005). It breeds for 7 months (October to April) in the grasslands (Skinner and Chimimba, 2005).

SOCIAL FLEXIBILITY IN *R. PUMILIO*

As mentioned above, the group-living *R. pumilio* was first observed displaying social flexibility when a drought brought about a drop in the population density in the Succulent Karoo (Schradin et al, 2012). This resulted in a change to solitary-living in both sexes (Schradin et al, 2012; Hill et al, 2015). In winter, individuals formed unrelated groups for huddling, but changed to a solitary structure at the start of the breeding season, only returning to a social group structure after the breeding season (Schradin et al, 2012). This switch in social tactic has since been observed regularly during the breeding season under low population densities (Schradin et al, 2009; Schoepf and Schradin, 2012). This change in social tactic during a low population density is attributed to fierce reproductive competition experienced in groups in the form of reproductive suppression in males and infanticide in females (Schradin et al, 2009; Schoepf and Schradin, 2012).

In addition to switching social tactics, *R. pumilio* also displays alternative reproductive tactics (Schradin et al, 2009; Schradin et al, 2013), another form of phenotypic plasticity. An

alternative reproductive tactic is where an individual can select one of several reproductive strategies to maximise individual fitness (Gross, 1996), which can be fixed if an individual assumes a strategy and maintains it throughout its life, or flexible if an individual can change between tactics reversibly within its lifetime (Taborsky et al, 2008). In *R. pumilio*, individuals of both sexes can assume one of three reproductive tactics: i) group-living dominant breeder, ii) group-living non-breeding philopatric or, iii) live solitarily (Schradin et al, 2009; Hill et al, 2015). Schradin et al (2013) investigated the role of AVP in male *R. pumilio* that adopt three social tactics: i) breeders, ii) philopatric males, and iii) solitary roamers. They found that the solitary roamers had higher levels of AVP than the two group-living classes (dominant breeders and philopatric), and hypothesised that this difference may be a mechanism to allow the solitary male the opportunity to quickly switch to living in a group, should the opportunity arise to become a breeder with a group of females. Within this free-living population of *R. pumilio*, hormonal differences have been identified between group-living males and solitary roamers, with the latter having higher concentrations of testosterone and lower corticosterone than group-living breeder males (Schoepf and Schradin, 2013). Solitary breeding females had lower corticosterone concentrations than group-living females, but did not differ in testosterone, progesterone or oestrogen concentrations (Hill et al, 2015). AVP concentrations have not been measured in female *R. pumilio* adopting different tactics.

The switch of sociality and reproductive tactic has never been observed in *R. dilectus*. Social flexibility is expected to occur in animals in an arid environment in response to unpredictable environmental changes (Cooper and Randall, 2007). Desert animals have a higher circulating level of AVP due to increased necessity to reduce water loss in their hot and dry environments (Carter, 2003; Nelson, 2005). It could be possible that these higher levels of AVP prime individuals for group-living, which may explain why the solitary *R. dilectus*, living in a predictable, moist environment, does not form social groups. Since female *R. pumilio* typically form groups which are later joined by a male (Schradin and Pillay, 2003), a closer examination and comparison of the proximate factors affecting sociality (behaviour, hormones and neuropeptides) between females of these two socially different species of *Rhabdomys* should help tease apart the role of these proximate factors in social evolution.

Motivation for the study

Anthropogenic induced environmental change, including climate change, poses a threat to biodiversity. The rate of change is potentially unsustainable and some believe will contribute to the next mass extinction (McLaughlin et al, 2002; Friedlingstein, 2008; Rymer et al, 2013).

Genetic change and evolutionary adaptation might not offer a viable response to these environmental changes because of the slow speed of change (reviewed in Rymer et al, 2013; Rymer et al, 2016). Our lab at the University of the Witwatersrand has been studying whether phenotypic plasticity could provide a potential advantage in times of rapid environmental change. Behavioural plasticity is a subset of phenotypic plasticity and describes changes in the behavioural phenotype in response to prevailing environmental conditions (Dingemanse et al, 2009). One form of behavioural plasticity that has been the focus of research in our group is social flexibility. Rymer et al (2013, 2016) and others (Sol et al, 2002; Randall et al, 2005; Cooper and Randall, 2007) postulate that social flexibility, and indeed behavioural flexibility generally, offers the potential for species to respond to unpredictable environmental changes. To understand whether behavioural flexibility can provide such an opportunity, the underlying causal mechanisms must be identified since these form the basis of such responses.

This thesis is concerned with understanding the mechanisms underlying social interactions, and ultimately the formation of social systems. While many studies of sociality and social tactics exist, the underlying basis of how a group is formed, taking into account the behavioural and physiological differences that may exist between species, is rarely explored. By comparing the behaviour and physiology of two species that have different social systems, we are afforded the opportunity to compare the hormonal and behavioural mechanisms underpinning group formation between species. I examined some of these mechanisms here. First, I aimed to determine whether *R. dilectus* is capable of tolerating conspecifics and displaying behaviours to promote group formation, and compared these to the behaviour of *R. pumilio* (Chapter 2). Second, I investigated the physiological markers of sociality in each species, including the circulating corticosterone and testosterone profiles (Chapter 3), and central AVP and oxytocin concentrations (Chapter 4) in response to simulated solitary and social contexts. Third, I investigated the role of AVP in sociality and social recognition in each species (Chapter 5), to establish whether they responded differently to this neuropeptide to better understand its role in social behaviour and group formation in *Rhabdomys*. Such information should provide insight into whether the genus *Rhabdomys* and its constituent species can demonstrate social flexibility as a response to environmental change, or whether the grassland species, in particular, will succumb to the changes and be replaced by the semi-arid species, as postulated by Rymer et al (2013). Our understanding of the evolution of the social systems that currently exist may allow us to better understand whether, and how, animals

can respond flexibly to the rapid changes occurring due to anthropogenic induced environmental change.

Format of the thesis

This thesis is comprised of a general introductory chapter (Chapter 1), four experimental chapters (Chapter 2 to 5) and a general discussion (Chapter 6). The experimental design included creating triads to conduct the behavioural (Chapter 2), hormonal (Chapter 3), and neuropeptide (Chapter 4) comparisons between the two species to an imposed social context. A separate experimental design tested the behavioural response to artificially increased AVP in both species in the form of a sociability and social novelty preference test (Chapter 5). An appendix chapter is included, which provides a detailed comparison of the protocols for neuropeptide extraction from the brain used in Chapter 4 of the thesis. All experimental chapters have been written as manuscripts for publication, and thus, each chapter has its own reference list and subsequently an overlap exists of references between chapters. There is inevitably an overlap of information in the introduction and discussion sections across the thesis chapters. The intended journal for submission for each chapter is suggested below the chapter title of each chapter, but the format of each experimental chapter follows the thesis editing style. Figures and tables are numbered sequentially in each chapter, and pages are numbered sequentially throughout the entire thesis.

All experimental work was approved by the animal ethics committee of the University of the Witwatersrand (clearance numbers: 2012/28/2A; 2014/34/B).

Glossary of terms

Mating system. Representation of the outcome of a combination of reproductive strategies adopted by each sex. It is a distinct subset of social interactions identifying who mates with whom within the population and how often mating occurs. Mating systems can be broadly described as monogamous, polygamous (polygyny or polyandry), or promiscuous. A component of a social system (Kappeler and van Schaik, 2002; Kappeler et al, 2013).

Phenotypic plasticity. The ability of one genotype to produce different phenotypes (behavioural, physiological, or morphological) in response to different environmental conditions. The set of phenotypes a single genotype can produce in particular environmental conditions is referred to as a reaction norm. It can occur in response to the early environment of an individual which leads to permanent changes (termed developmental plasticity), or in

adults where the phenotypic changes are reversible (termed phenotypic flexibility; Dingemanse et al, 2009; Schradin, 2013).

Sociability. The motivation or propensity for an animal to seek out contact with a conspecific rather than remaining alone (same as social motivation; Moy et al, 2004; Lim and Young, 2006).

Sociality. Describes the gregariousness or tendency of individuals in a population to live in social groups, and their relationships with other individuals. This varies within and between species, often in response to environmental conditions (Alexander, 1974; Lacey and Sherman, 2007; Silk, 2007; Menz et al, 2017).

Social flexibility. Reversible changes in the social organisation of a population due to individual adult animals of both sexes alternating their social and reproductive tactics within their lifetime, in response to changes in environmental conditions (Schradin et al, 2012).

Social organisation. The composition of a group of animals: size, sexual composition, genetic composition, and spatiotemporal cohesion of a social system. Includes categorisations such as "solitary" or "group-living." A component of a social system (Kappeler and van Schaik, 2002; Schradin et al, 2012; Kappeler et al, 2013).

Social recognition. The ability to distinguish between individuals (e.g. known and unknown individuals, or between group members and non-group members). This allows for the appropriate behavioural response to be displayed when encountering a conspecific, based on the stored information about the identity of that individual (social memory) from previous interactions (Curtis et al, 2007).

Social structure. The pattern of interactions (e.g. affiliative or agonistic) between individuals and the resulting relationships between members of a population, either promoting group-living or solitary-living. The most basic foundation of a social system (Kappeler and van Schaik, 2002).

Social system. A set of conspecifics that interact regularly and tend to interact with each other more often than any other conspecifics (same as society). This is the umbrella concept incorporating social organisation, social structure and mating system of the population (Kappeler and van Schaik, 2002).

Strategy. A particular life-history pattern or genetically based set of decision rules of an individual, determining which tactic it will follow i.e. which phenotypes it will express (Gross, 1996; Taborsky et al, 2008).

Tactic. A trait (phenotype) or set of traits (phenotypes) that result from a strategy (decision rule) to serve a particular function (Gross 1996; Taborsky et al, 2008).

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CHAPTER 2

Initiation of group formation in the African striped mouse, *Rhabdomys:* a behavioural comparison between group-living and solitary-living females

(To be submitted to Ethology. Editing follows the style of the thesis)

Abstract

The variation of animal social systems (solitary to complex societies) has long been attributed to environmental drivers, such as predation pressure and resource distribution. An important predictor of a social system is how individuals within populations interact with one another, as certain behaviours promote group-living (attraction, tolerance), whereas others promote solitary-living (avoidance, aggression). Individuals of some species are able to switch between social tactics by adjusting their behaviour flexibly towards conspecifics. Comparisons of interactions with conspecifics between a socially flexible group-living species and a solitary species, provides an opportunity to examine the proximate mechanisms of group formation in both social systems. In this study, I compared the behaviour of female conspecifics in a socially flexible, naturally group-living species, *Rhabdomys pumilio*, to its closely related solitary-living congener, *R. dilectus*, to assess how group formation is initiated and whether it occurs in *R. dilectus*. By forming triads of age-matched, unfamiliar females from each species, I compared how they interacted on Day 1 of triad formation. Thereafter, I assessed whether they could form groups and share a nest, and the duration they engaged in amicable and aggressive behaviour periodically over three weeks after triad formation. Triads of both species predominantly displayed amicable behaviour on Day 1, with *R. pumilio* approaching triad members aggressively more often compared to *R. dilectus*. Using sociograms, comparisons of interactions on Day 1 revealed that 67% of *R. dilectus* triads had all three females interacting compared to 100% of *R. pumilio* triads. Triads in both species shared a nest with conspecifics from Day 2 onwards. Over the 3 weeks of study, both species displayed amicable behaviour predominantly, which continued to increase significantly over time. This study confirms that *R. dilectus* has the ability to form social groups like *R. pumilio*, and potentially also to be socially flexible.

Introduction

Why certain animal populations consist of solitary individuals while others are made up of complex social groups has been of interest to animal behaviourists for decades (Alexander, 1974; Crook et al, 1976). Lukas and Clutton-Brock (2013) suggest that solitary-living is the ancestral form of sociality for all mammalian species, but today mammalian populations exist in a variety of social systems, ranging from solitary-living to complex societies. Many explanations exist for the ultimate reasons why animals would live in groups, such as the effect of ecological adaptations, physiological demands, and reproductive competition (Banks et al, 2007; Silk, 2007; Schradin et al, 2012). Ecological adaptations promoting group-living include the benefits of improved predator avoidance (e.g. the dilution effect), improved foraging-vigilance trade-off, sharing in limited high-quality food sites with group members, and/or improved ability to hunt prey for carnivores (Bacon et al, 1991; Krebs et al, 1993; Tchabovsky et al, 2001; Kerth, 2008).

The distribution of individuals within a habitat also affects the social system adopted. For example, high population density is expected to promote philopatry and group-living (habitat saturation hypothesis), when offspring can share in limited resources (e.g. nest/den sites) with their parents (Pitelka et al, 1974; Schradin et al, 2012; Hofmann et al, 2014), as occurs in red fox, *Vulpes vulpes* (Lindström, 1986). The primary physiological demand making group-living advantageous in mammals is the benefit of less energy expenditure on thermoregulation gained through huddling in winter (Kerth, 2008). Low reproductive competition might favour group-living, but if it is high, dispersal of offspring and solitary-living are more favourable (Emlen, 1982; Schradin et al, 2012; Schoepf and Schradin, 2012a). In this case, dispersal avoids the occurrence of reproductive suppression (Schradin et al, 2012) or the risk of infanticide, two disadvantages of group-living (Krebs et al, 1993; Kappeler and van Schaik, 2002). Other costs of group-living include increased risk of disease or parasite transmission (Krebs et al, 1993; Côté and Poulinb, 1995; Kappeler and van Schaik, 2002). The social system adopted by a population would be the one where the benefits are greater than the costs to an individual's fitness (Silk, 2007).

The proximate reasons for differences in sociality and group formation are of interest in this study. The most basic component of all forms of sociality is the nature of the interactions between individuals (Hinde, 1976). Experiences with conspecifics can lead to individuals either being attracted to each other (positive experiences) or avoiding each other (negative experiences; Hinde, 1976; Kappeler and van Schaik, 2002). These experiences and interactions

between individuals determine the types of relationships or associations that exist in a population (Hinde, 1976). In solitary species, individuals show low tolerance for conspecifics and aggressively defend exclusive territories (e.g. the fat sand rat, *Psammomys obesus*), whereas social species tolerate conspecifics (e.g. the great gerbil, *Rhombomys opimus*; Tchabovsky et al, 2001; Szenczi et al, 2012). Amicable behaviour, which describes affiliative behaviours between individuals (e.g. allo-grooming), is emergent of tolerance and is an important aspect of group-living (Young, 2002; Choleris et al, 2004; Storm and Tecott, 2005). Amicable behaviours potentially facilitate social recognition by allowing group members to gain information from group constituents, such as nose rubbing in Bechstein's bats, *Myotis bechsteinii* (Kerth et al, 2003; Safi and Kerth, 2003), and recognition of members in turn promotes amicable behaviours between members to promote group stability (Kerth, 2008; Manno, 2008). Exceptions to these behavioural trends of solitary and group-living exist, such as increased affiliative behaviour displayed postpartum in solitary-living montane vole females, *Microtus montanus* (Insel and Shapiro, 1992), sharing of large kills by typically-solitary pumas, *Puma concolor* (Elbroch et al, 2017), and aggressive behaviour seen in group-living dwarf mongoose, *Helogale parvula*, and coypu, *Myocaster coypus*, to establish a social hierarchy within the group (Creel, 2001; Guichón et al, 2003).

Social systems are not necessarily static. There is a constant feedback interaction between how constituent individuals behave towards each other, which leads to a particular social organisation and demography of a population, and in turn affects the behaviour of individuals in that population (Kappeler and van Schaik, 2002). Social behaviour involves both positive (affiliative, cooperative) and negative (aggressive, aversive) interactions (Hofmann et al, 2014). High occurrences of aggressive behaviour can lead to mutual avoidance and potentially promote the dispersal of individuals, affecting different social ranks and sexes differently (Hofmann et al, 2014). In cases where the interactions influence the distribution and behaviour of one sex (usually females), this affects the behaviour of the opposite sex (usually males; Hofmann et al, 2014). The resulting sex ratio of a population can be skewed in favour of either sex, and the opposite sex would need to respond behaviourally to adjust to the sex ratio, such as forming cooperative breeding groups or remaining philopatric after sexual maturity, as underpinned by prevailing environmental conditions (Emlen, 1982). For example, some primate groups have multiple males (e.g. olive baboons, *Papio anubis*), while others have a single male (e.g. geladas, *Theropithecus gelada*) with multiple females (Shultz et al, 2011),

depending on whether or not females display synchronised receptivity, limiting the ability of males to monopolise females (Kappeler and van Schaik, 2002).

Variation in social systems occurs both between and within species, mediated by the environmental conditions in the area each species inhabits, as well as the physiology of the species (Lott, 1991; Kappeler et al, 2013). Interspecific variation is seen in closely related species of voles, which adopt different social systems: the monogamous prairie vole, *Microtus ochrogaster*, lives in communal groups (Getz et al, 1993), whereas, the solitary, promiscuous montane vole, *M. montanus*, lives in isolated burrows and avoids contact with conspecifics (Jannett, 1982; Insel and Shapiro, 1992). It has been suggested that these differences in social systems are due to differences in the environments in which each species occurs (higher population densities experienced by prairie voles, *M. ochrogaster*) and to differences in inherent propensity for social contact or affiliation in each species (Getz et al, 1993; Insel and Shapiro, 1992).

Variation in sociality can occur with populations of a species. Such variation can occur because of genetic differences between populations, stochastic environmental processes, or phenotypic plasticity (Schradin, 2013; C. Schradin et al, in prep). Developmental plasticity, describes the process when an individual adopts a tactic which is maintained throughout its adult life based on its experiences and environment during development (Sol et al, 2002; Randall et al, 2005; Schradin et al, 2012). For example, red foxes, *V. vulpes*, will either adopt a solitary roaming strategy or live in monogamous family groups, depending on the distribution of resources available and presence or absence of larger canids in their natal home range (Cavallini, 1996). On the other hand, social flexibility is a form of phenotypic plasticity, which describes circumstances where an adult individual is able to change, reversibly, between different social tactics in response to prevailing environmental conditions (Schradin et al, 2012). This predominantly occurs in species living in unpredictably changing environments, such as in the desert dwelling great gerbil, *R. opimus* (Randall et al, 2005). This flexibility provides the benefit of being able to exploit the best aspects of both group-living and solitary-living in response to the prevailing environmental conditions, enhancing an animal's lifetime reproductive success (Rymer et al, 2013).

Investigating how social flexibility is achieved may provide clues to understanding social evolution (Schradin et al, 2012). Individuals would need to have the genetic ability to display the variety of behaviours required for the different social tactics, with their ability to show behavioural plasticity depending on how broad the behavioural reaction norm (allowing

for phenotypic flexibility) is for that individual in response to its environment (Dingemanse et al, 2009). Thus, an individual should be able to tolerate conspecifics and have the motivation to seek out (attraction) company of conspecifics to allow for group-living, as well as have the ability to switch to avoiding conspecifics and defending a territory alone to live solitarily (Schradin et al, 2012). If a species does not display this flexibility, it does not necessarily imply that they lack the ability to be flexible; they may have simply never experienced the conditions that require them to do so. Comparisons of a socially flexible group-living species to a solitary species in how they interact with conspecifics might add to our understanding of how group formation is initiated, affording the opportunity to identify the behaviours required to allow for the successful formation of social groups.

The African striped mouse, genus *Rhabdomys*, is a small (adult body mass 32-55g) diurnal, murid rodent, widely distributed throughout southern Africa, occurring in a variety of biomes from arid semi-desert (160mm rainfall per annum) to moist grasslands (700-1000mm rainfall per annum; Skinner and Chimimba, 2005). My study considers two closely related species of *Rhabdomys*: *R. pumilio* occurring in xeric environments and *R. dilectus* in mesic areas of South Africa (Rambau et al, 2003). Distinct differences in sociality exist between these species in nature. *R. pumilio* in the arid Succulent Karoo is facultatively group-living, usually forming social groups consisting of one breeding male, up to four closely related breeding females and their philopatric offspring, with males displaying paternal care (Schradin and Pillay, 2003; Schradin and Pillay, 2004). Group members interact amicably and bask together at dawn and dusk, share a nest, and defend a common territory, but forage solitarily during the day (Schradin, 2005). These striped mice have a widespread year-round abundant food source, feeding on shrubs and succulents (Schradin, 2005). *R. pumilio* is socially flexible, switching to solitary-living under low population densities, associated with solitary breeding females (Hill et al, 2015) and males (Schradin and Pillay, 2006; Schradin et al, 2010a). This occurs primarily during the breeding season during low population density, when *R. pumilio* can experience reproductive competition in the form of reproductive suppression in males and infanticide in females (Schradin et al, 2009; Schoepf and Schradin, 2012a).

The grassland species, *R. dilectus*, is solitary-living, with individual territories overlapping exclusively with territories of the opposite sex (Schradin and Pillay, 2005). Females raise offspring alone and interact with males only during the breeding season, yet males display paternal care in captivity (Schradin and Pillay, 2003). Food in the grasslands are scarce and patchily distributed, so *R. dilectus* has a larger home range size (6 to 10 times larger)

than *R. pumilio* (Schradin, 2005; Schradin and Pillay, 2005). *R. dilectus* also has a lower population density than *R. pumilio*, possibly indicating that lower population density promotes solitary-living (Schradin and Pillay, 2005). The breeding season (7 months) of *R. dilectus* is more than double that of *R. pumilio* (3 months; Schradin and Pillay 2005; Skinner and Chimimba, 2005). Reproductive competition has been identified as a major driver for solitary living in *R. pumilio* (Schradin et al, 2010b; Schoepf and Schradin, 2012a). The potential for *R. dilectus* to be socially flexible, like *R. pumilio,* would need to be tested in the absence of reproductive competition. This provides the opportunity to investigate, firstly, whether this naturally solitary-living species has the potential to be group-living, and secondly, whether they differ in how they interact with conspecifics on initial meeting that facilitates or hinders group formation.

How group formation is initiated is surprisingly underrepresented in the literature. Here, I investigated whether or not three unrelated, unfamiliar conspecific female striped mice display the social tolerance necessary to facilitate and initiate social group formation, in the naturally solitary-living *R. dilectus* and the naturally group-living *R. pumilio*; and whether tolerance (nest sharing and amicability) of conspecifics is maintained over time in each species (referred to as stable groups in this study). Females were tested in this study because groups in *R. pumilio* are usually made up of multiple females with one breeding male, and males follow the distribution of females when selecting a social/reproductive tactic (Schradin et al, 2012). Female free-living *R. pumilio* form groups with closely related females (Schradin and Pillay, 2004). However, in this study**,** I used unrelated females to test whether *Rhabdomys* has the ability to form social groups with non-kin. Sisters of both species have been recorded to always cohabit in captivity (N. Pillay pers com), thus using sisters in this study would not afford the opportunity to investigate the motivation to form groups (D.L. Hill unpublished data). I predicted that, because unrelated female triads were established, social interactions were unlikely to be amicable immediately after establishing triads, so aggressive interactions and avoidance of triad members was expected on first encounter between triad members in both species. Nonetheless, I expected that aggression would be reduced in *R. pumilio* triads compared to *R. dilectus* triads. I predicted that the naturally group-living *R. pumilio* would initiate groups (i.e. greater amicability and sharing nests) sooner than *R. dilectus* and maintain these relationships over the next 3 weeks of study. In contrast, I predicted that the naturally solitary-living *R. dilectus* would be unlikely to form groups, or would take longer to do so. Amicable behaviour is important for maintaining groups, and thus I expected amicability to

occur more often than aggressive behaviour once the triads formed groups (i.e. increased affiliative behaviour after nest cohabitation occurs).

Materials and methods

STUDY SPECIES

All experiments took place in the Milner Park Animal Unit at the University of the Witwatersrand, Johannesburg, South Africa. The striped mice used in this study were from the captive colony at the Milner Park Animal Unit. The naturally group-living species, *Rhabdomys pumilio*, originated from the Goegap Nature Reserve in the semi-arid Nama Karoo of South Africa (average rainfall of 160 mm per annum, winter rainfall). The naturally solitary-living species, *Rhabdomys dilectus dilectus* (referred to as *Rhabdomys dilectus* here), originated from the moist grassland area in the Highveld region of central South Africa (average rainfall of 700 mm per annum, summer rainfall). All individuals used in the study were bred in captivity and were either F2 or F3 captive born. This study was approved by the animal ethics screening committee at the University of the Witwatersrand (AESC number: 2012/28/2A).

HUSBANDRY

This study was conducted under partially controlled environmental conditions: 14:10 light: dark cycle (lights on at 05h00); 30-60% rH; and a room temperature of 24-28°C. Approximately 40-45 breeding pairs per species were randomly formed simultaneously before experiments began. Breeding pairs were housed in clear Labotec™ cages (L x H x W: 425mm x 150mm x 240mm), containing a PVC nest-box (L x H x W: 98mm × 64mm × 77mm), ±3cm wood shavings, a handful of *Eragrostis* grass (± 20g) and ± 5g of paper tissue provided for nesting material. Enrichment was provided in the form of a cardboard tube (L x D: 99mm x 38mm) and a small wooden block for chewing. The daily diet included 5g Epol® mouse cubes, approximately 5g fresh fruit or vegetables and approximately 2g millet per mouse (millet was gradually increased to 4g for pregnant and lactating females); water was provided *ad libitum* for the entire period of the study.

At 20 days of age, pups produced from the breeding pairs were weaned and housed alone for a further 30 days in Labotec™ cages, furnished and enriched as described above for the breeding pairs. *R. pumilio* can disperse and occur alone in nature, and thus solitary housing

post-weaning is unlikely to be stressful (Schoepf and Schradin, 2013). From these juveniles, a total of 30 *R. pumilio* and 30 *R. dilectus* females were used in this study.

EXPERIMENTAL DESIGN

At approximately 43 days of age (one week prior to forming triads), all striped mice were uniquely marked using commercial hair dye (Innecto™, Pinetown, South Africa). Three unrelated age-matched conspecific females were randomly assigned to a triad. A total of 10 triads were formed per species. A triad was housed together from approximately 50 days old to approximately 85 days old in an aluminium tank with a clear Perspex front (L x H x W: 460mm x 350mm x 300mm) and a wire-mesh lid. These tanks contained wood shavings for bedding, and grass and nesting material, as described above. Tanks contained three separate nest boxes of equal size (L x H x W: 100mm x 77mm x 63.5mm) to give the mice a choice to not co-habit within triads. The feeding regime per animal followed the diet described above.

The interactions immediately after introducing the three mice into experimental tanks (approximately 20 minutes) on Day 1 of the triad were observed through direct observation to assess the levels of aggression, so that triads could be terminated if excessive or damaging fights occur (no behaviour was formally recorded in this period as the purpose was to separate the mice should fighting occur). No fights were observed in any of the triads during this period. Following this 20-minute direct observation period, the triads were filmed using Sony Handycam® video cameras from 09h00-12h00, when striped mice are most active (Pillay, 2000). Triads were filmed on Day 1, Day 3, Day 6, Day 10, Day 15, and Day 21 of triad formation. Video footage was used to collect data of the behaviour of the females in each triad (Table 2.1).

To investigate the initiation of group formation in the two species, the three hours (09h00-12h00) of footage from Day 1 of triad formation was scored continuously, recording the frequency of all interactions between all members of the triad. This was done by recording dyadic interactions between members of each triad, including which animal initiated an interaction, which mouse they initiated the interaction toward, the manner of the approach (amicable or aggressive), and the response (amicable, aggressive, or avoidance) of the approached mouse (Table 2.1). I calculated the frequency of aggressive approaches, amicable approaches, avoidance, and aggressive responses to an approach from these data from Day 1 (behaviours defined in Table 2.1). Sociograms were used to provide an indication of how the behaviours expressed were distributed between triad members on initial encounter, to establish whether all three mice interacted on the first day, or whether there was one key player in

initiating interactions and group formation. Sociograms were plotted using the frequency of sniffing of an individual by another. Sniffing was used as the metric of the initiation of an interaction between individuals because rodents use sniffing as a means to initiate interactions and identify conspecifics (Engelmann et al, 1995; Wesson, 2013).

Table 2.1. Behaviours scored in female triads in 2 species of *Rhabdomys*. The duration of the defined behaviours was scored for interactions between dyads over the 21-day study period, and the frequency of the behaviours occurring when one mouse was moving toward another mouse (approach) or in response to another mouse approaching (response) was scored on Day 1 of triad formation.

Behaviour categories and components	Definition
Aggression	
- Chasing	Running after another mouse, usually resulting in the other mouse fleeing
- Fighting	Attacking, biting, or wrestling with another individual, lunging toward or jumping on another individual.
- Kleptoparatising food	Pulling food away from another individual, from paws or mouth of the individual
- Pushing	Pushing or shoving another individual with the forelimbs
Amicable behaviour	
- Allogrooming	Grooming another individual or being groomed by another individual
- Sitting in body contact	Sitting next to another individual with body contact, huddling or staying in the same nest as another individual
- Sniffing	Sniffing another individual or being sniffed by another individual
Avoidance behaviour	Moving away as soon as another individual approached, includes walking away and running away
Nest co-habitation	All members of the triad sleeping in the same nest at the same time; confirms group formation

To confirm whether triads maintained groups over time (i.e. whether stable groups formed), the duration of amicable and aggressive interactions (defined in Table 2.1) between dyads within each triad was scored from the video footage recorded on Day 1, Day 3, Day 6, Day 10, Day 15, and Day 21 of experimental triad formation, and the presence of nest co-habitation was recorded from direct observation. Behaviours were scored continuously for 15-minutes during the period of most activity of the three hours of video footage recorded for all

triads on the 6 non-consecutive days over the 21-days of sampling. This 15-minute period was determined by a pre-screening of the footage from Days 1 and 3 of the first triads. Nest cohabitation was recorded using direct observation at times when the mice were resting or asleep, usually between 14h00-15h00. This was done every day (excluding weekends when not filming) following formation of triads and was used to confirm whether group formation had occurred in a triad (see Schradin et al, 2010b; Schoepf and Schradin, 2012b).

DATA ANALYSIS

Nest co-habitation was recorded from live observations outside of the filming times, and was used as an indication of group formation (see Schradin et al, 2010b; Schoepf and Schradin, 2012b). The number of the 10 triads of each species that formed groups, as indicated by nest co-habitation of all 3 mice maintained over the study period, was recorded as a percentage.

One *R. dilectus* triad was terminated on Day 4 of triad formation due to excessive fighting. Video footage of one *R. pumilio* triad was corrupted and was not available for any analysis of behavioural interactions. Both of these triads are not considered in the data analysis of the Day 1 interactions (sociograms and the frequency of approach and response type) or behaviour during 6 non-consecutive days over 21-day period of sampling, resulting in data set of n = 9 triads for both species for these analysis, but these triads are included in the nest co-habitation percentage calculation.

Day 1 interactions

Sociograms were plotted using the library "qgraph" (Epskamp et al, 2012) in R© version 3.2.4 (2016, The R Foundation for Statistical Computing, Vienna, Austria) software, run through RStudio© Version 0.99.892 (2016, RStudio, Inc.), based on the data of initiated interactions (sniffing) between dyads as a proportion of all interactions initiated within the triad (i.e. the total interactions initiated between all females in a total of 3 dyad combinations) on Day 1 (first 3 hours) of each triad. To establish whether dyadic interactions occurred above or below chance within the triads, I ran a cell-wise χ^2 analysis comparison using adjusted residuals of the total interactions initiated (sniffs) between dyads in MatMan™ version 1.1.4 (Ballintijn et al, 2003; Noldus Information Technology, Netherlands). The *p* values were calculated from the *z* values generated, using GraphPad QuickCalcs (GraphPad Software Inc, USA) to establish which dyads had significant associations within each triad.

The frequency of the type of approaches (amicable or aggressive) and responses to an approach (aggressive, or avoidance) on Day 1 were analysed using a multivariate analysis of

variance (MANOVA) in Dell Statistica version 12 (Dell Inc., USA). Data of the various approach and response behaviours were first transformed to a normal distribution using the appropriate transformation as indicated by the lambda value generated by a Box-Cox power analysis. Species was included as a categorical predictor and the frequency of aggressive approaches, amicable approaches, avoidance of approaches, and aggressive responses to approaches were set as multiple response variables.

Behaviour during 6 non-consecutive days of sampling over 21 days

The duration of amicable and aggressive behaviour displayed during the 6 days of observation during 3 weeks of study were analysed in R using a generalised linear model (GLZ), using the car (Fox and Weisberg, 2011), lme4 (Bates et al, 2015), and lmerTest (Kuznetsova et al, 2016) libraries in R. A separate GLZ was done for each behaviour, with either aggression or amicable behaviour listed as the response variable, and species and Day, and the interaction between species and Day as fixed factors in the model. Triad was included as a random effect. To correct for the multiple comparisons of amicable and aggressive behaviour, I adjusted *p* values using the Benjamini and Hochberg (1995) method. Models were validated by comparing the residuals to the fitted values (Crawley, 2009). Day was releveled in the model to determine where significant differences occurred in the six days sampled by comparing the least square means as a post hoc. Data are presented as mean ± standard error throughout.

Results

From direct observations of nest cohabitation occurrence, I found that females of both species had formed stable groups by Day 3 of triad formation, with all three females sharing one nest from Day 2 for 100% of *R. pumilio* triads (10 of 10 triads) and 90% of *R. dilectus* triads (9 of 10 triads; the 1 triad terminated early due to damaging fights is included here in nest cohabitation counts).

INTERACTIONS ON DAY 1 OF TRIAD FORMATION

On Day 1 of triad formation, sociograms revealed that all individuals interacted by sniffing in all triads of *R. pumilio* (Figure 2.1), but only for 67% of *R. dilectus* triads (Figure 2.2). Analysis of the individual initiations of interactions (sniffs) between triad members indicated that only one *R. pumilio* triad (number 6) had interactions that were more (indicating attraction), or less (indicating avoidance) likely to occur than chance (Figure 2.1). In *R. pumilio* Triad 6,

individual 1 and 2 were attracted to and initiated interactions with individual 3 (*a* ($p = 0.035$) and *b* ($p = 0.007$) respectively on sociogram), while individual 3 avoided initiating interactions with individual 2 (*c* on sociogram, $p = 0.042$), and individual 2 avoided initiating interactions with individual 1 (*d* on sociogram, $p = 0.032$; Figure 2.1). In 3 of the 9 *R. dilectus* triads (Triad 3, 5 and 6; Figure 2.2), one female interacted with both the other females, but neither of those mice interacted with each other. In a fourth *R. dilectus* triad (Triad 4; Figure 2.2), one female received interactions from the two females, but never initiated interactions with either of the other mice. None of the *R. dilectus* triads had interactions occurring more frequently above or below chance (Figure 2.2).

Species was a significant predictor ($F_{1,16} = 3.32$, $p = 0.044$) of the type of approaches and responses to an approach on Day 1 of triad formation (Figure 2.3). *R. pumilio* displayed more aggressive approaches ($F_{1,16} = 9.75$, $p = 0.007$), aggressive responses ($F_{1,16} = 13.77$, $p = 0.002$), and avoidance ($F_{1,16} = 9.41$, $p = 0.007$) than *R. dilectus*, but there was no significant difference in the frequency of amicable approaches ($F_{1,16} = 1.04$, $p = 0.323$) between the species (Figure 2.3), which occurred most frequently of all the behaviours recorded.

BEHAVIOUR DURING 6 NON-CONSECUTIVE DAYS OF SAMPLING OVER 21 DAYS

Day was a significant predictor of duration of amicable behaviour ($\chi^2_5 = 60.74$, adjusted $p = 0.041$ Benjamini and Hochberg method; Figure 2.4). The post hoc tests revealed that amicable behaviour duration was significantly lower ($p < 0.001$) on Day 1 than the subsequent days, and the duration of amicable behaviour on Day 21 was significantly greater than on Day 3 ($p = 0.01$) and Day 6 ($p = 0.03$; Figure 2.4). Species was not a significant predictor of amicable duration ($\chi^2_1 = 0.78$, adjusted $p = 0.167$), nor was there a species x Day interaction ($\chi^2_5 = 4.39$, adjusted $p = 0.250$). In both species, amicable behaviour occurred more frequently than aggressive behaviour during the study (Figure 2.4).

Species ($\chi^2_1 = 1.16$, adjusted $p = 0.083$), Day ($\chi^2_5 = 5.16$, adjusted $p = 0.208$) and their interaction ($\chi^2_5 = 5.42$, adjusted $p = 0.125$; Figure 2.4) did not affect aggressive behaviour.

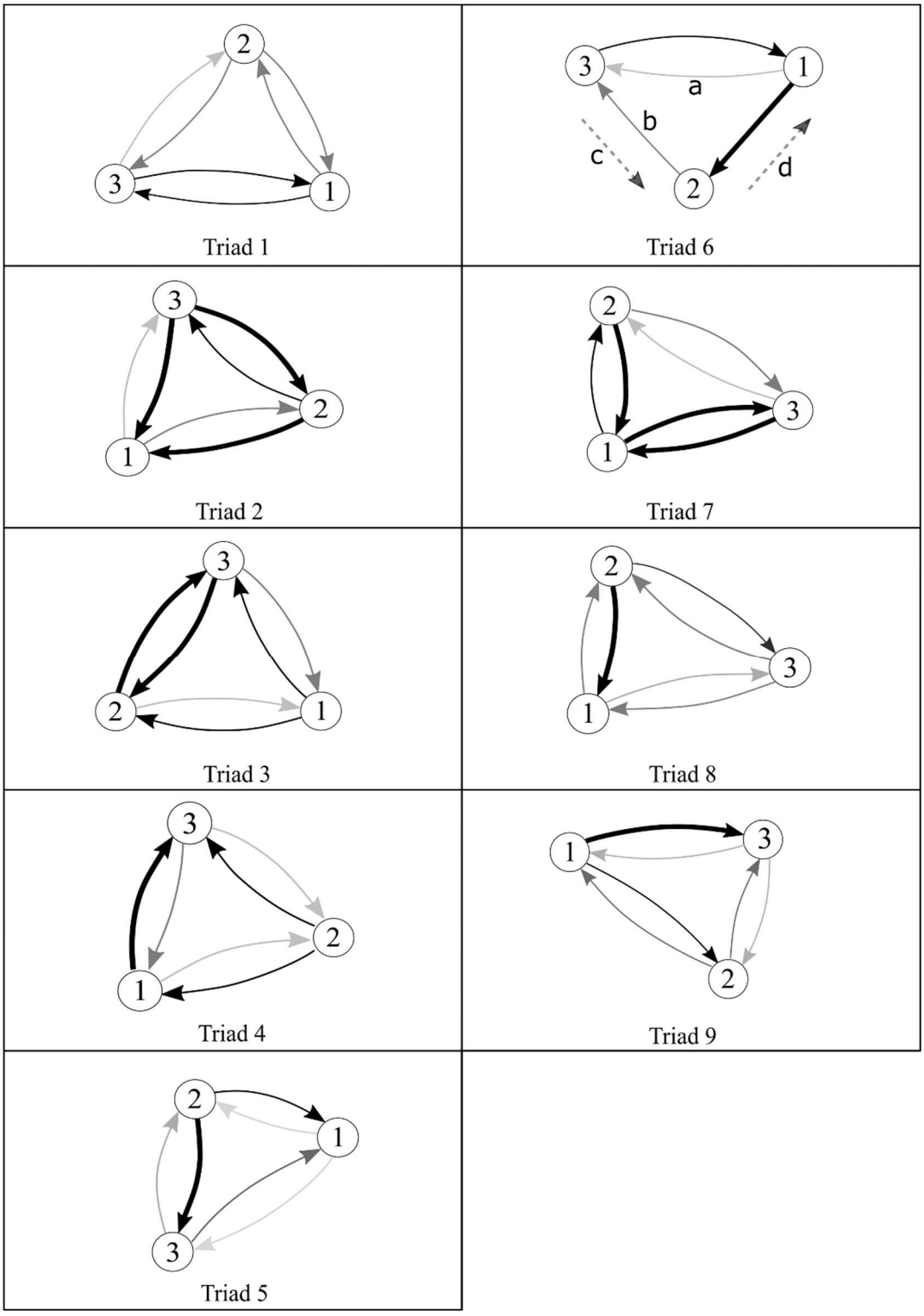


Figure 2.1. Sociograms of each of 9 *R. pumilio* triads. Line thickness and depth of colour indicates frequency of interactions initiated (sniffs) between individuals, as a proportion of all interactions initiated and received by each female within the triad (thicker and darker lines indicate more interactions). Each node (numbered 1, 2, or 3) denotes a different female within each triad, and arrows indicate the direction of the interaction. Only Triad 6 had interactions occurring above (indicating attraction: interaction arrows labelled as a ($p = 0.035$) and b ($p = 0.007$)) or below (indicating avoidance: dashed arrows where no interaction occurred labelled c ($p = 0.042$) and d ($p = 0.032$)) expected by chance, all other triads had no significant associations ($p > 0.05$).

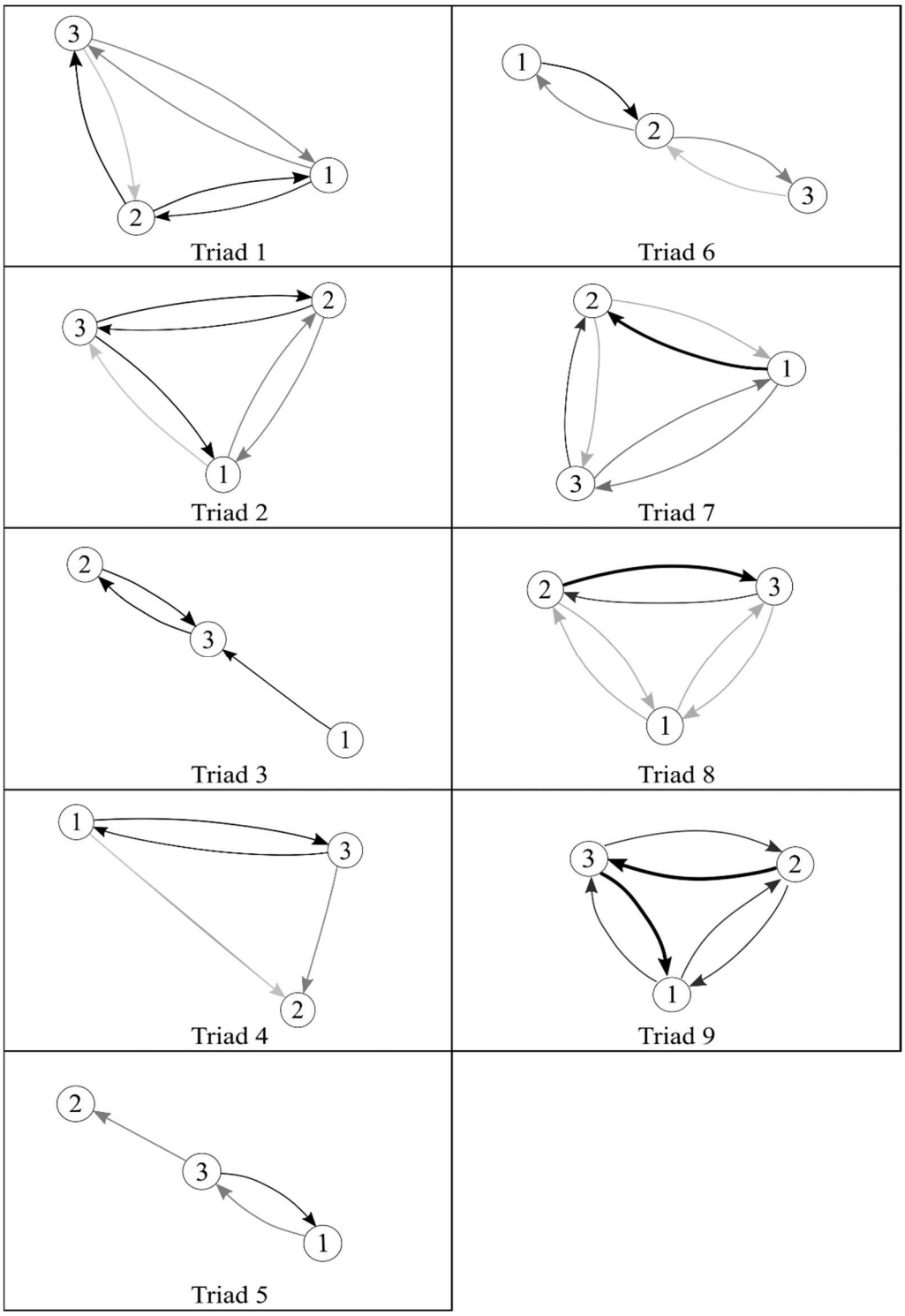


Figure 2.2. Sociograms of each of the 9 *R. dilectus* triads analysed. Line thickness and depth of colour indicates frequency of interactions initiated (sniffs) between individuals, as a proportion of all interactions initiated and received by each female within the triad (thicker and darker lines indicate more interactions). Each node (numbered 1, 2, or 3) denotes a different female within each triad, and arrows indicate the direction of the interaction. None of the triads had significant associations ($p>0.05$) occurring above or below chance.

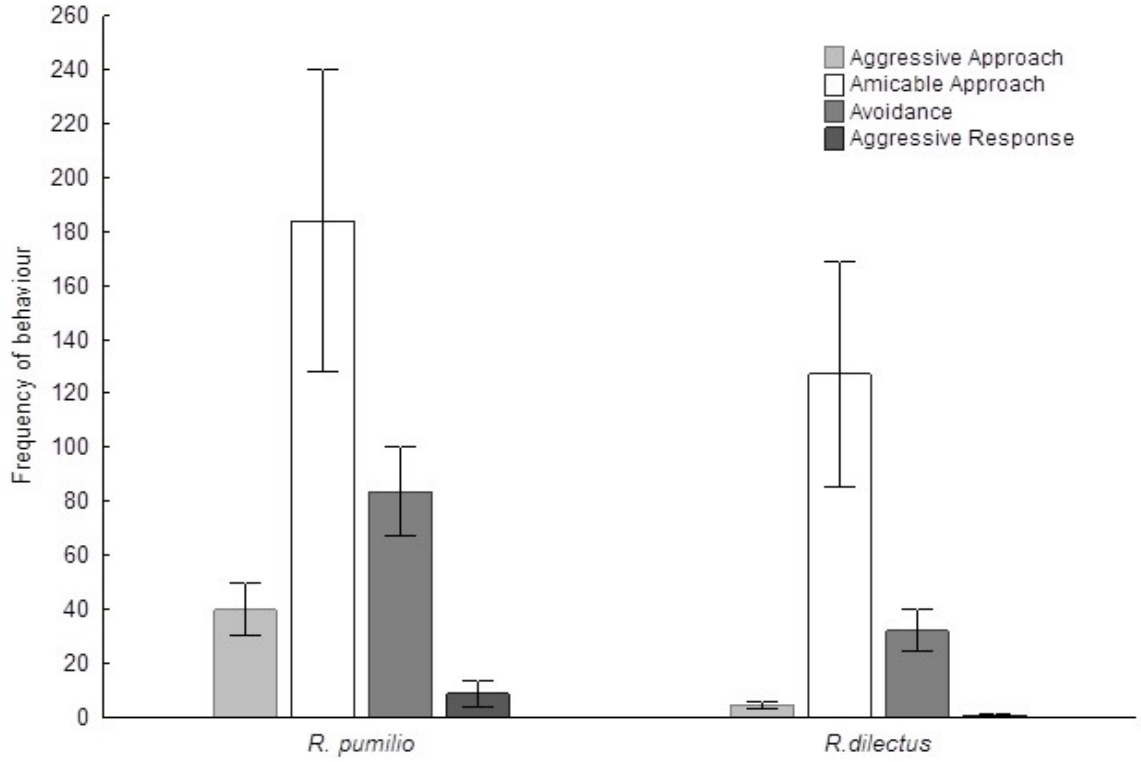


Figure 2.3. Frequencies of aggressive and amicable approaches, aggressive responses, and avoidance between triad members on Day 1 of triad formation in two species of *Rhabdomys*. Columns indicate mean frequency and error bars show the standard error.

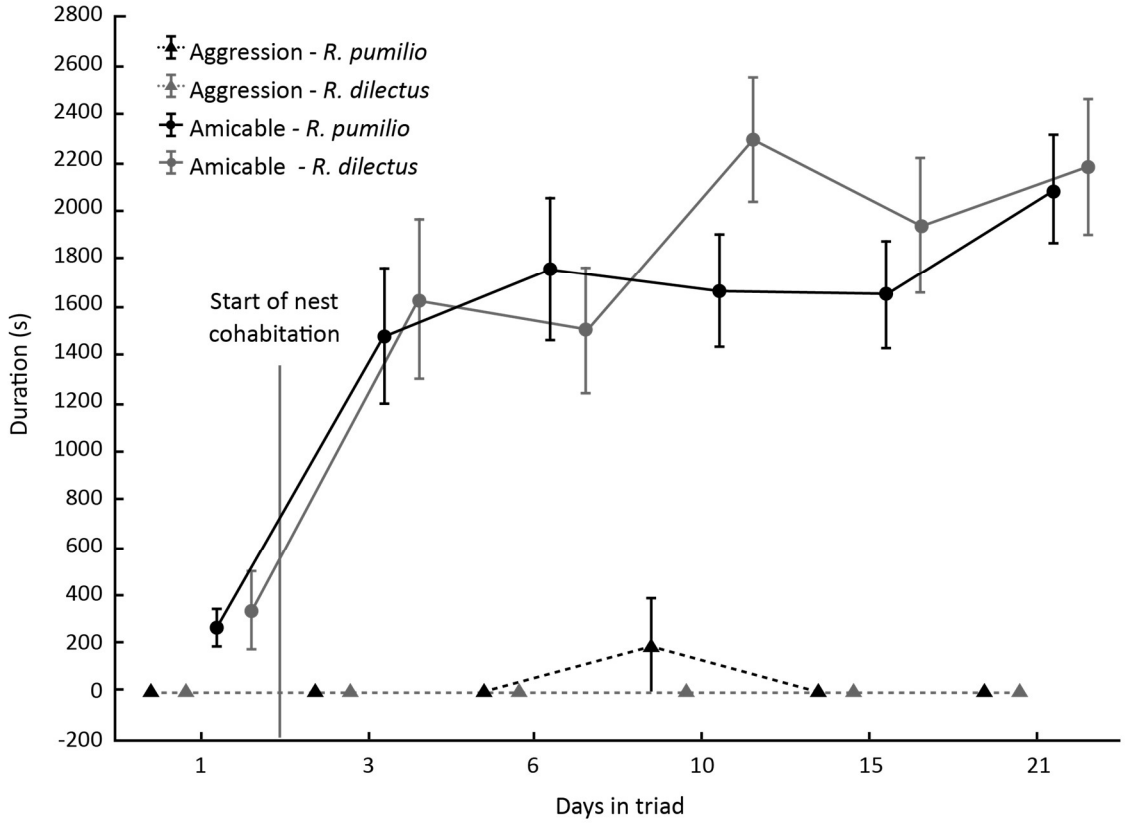


Figure 2.4. Duration of amicable (circular points on the solid line) and aggressive (triangular points on the dashed line) behaviour of two species of *Rhabdomys* housed in triads over 21 days. *R. pumilio* data displayed with black lines, *R. dilectus* data displayed with grey lines. Data displayed as mean and standard error. Vertical grey line indicates Day 2, when nest cohabitation first occurred.

Discussion

This study aimed to investigate whether females of two sister species with different social systems could tolerate unrelated conspecifics and promote social group formation, and to examine how group formation is initiated as a result of this tolerance. I expected that *R pumilio* would be more tolerant of conspecifics than *R. dilectus*, and thus start sharing nests sooner than *R. dilectus* (indicative of group formation). I found that both *R. pumilio* and *R. dilectus* can form social groups involving unfamiliar, unrelated females, and maintain these groups over 21 days. Interestingly, triads of both species were sharing nests by the second day of the experiment. I expected the naturally group-living *R. pumilio* to display greater amicability and less aggression than *R. dilectus*, with more avoidance behaviour expected in *R. dilectus* on Day 1. The majority of approaches on Day 1 in triads were amicable for both species, and the species did not differ in the frequency of amicable behaviour displayed on Day 1. Interestingly, *R. pumilio* displayed more aggressive approaches and responses to conspecifics, and more avoidance of conspecifics than was observed in *R. dilectus*. Sociograms revealed that the species differed in how they interacted on initial meeting: all three females interacted in all *R. pumilio* triads on the first day, but not in all *R. dilectus* triads. In addition, one (*R. pumilio*) triad had interactions occurring more and less often than expected by chance, indicative of attraction and avoidance between certain members of this triad. Amicable behaviour was expected to occur more often than aggressive behaviour once the triads started to cohabit in nests (formed groups). This was observed in both species, with amicable behaviour increasing gradually with time during the study.

While the two species appear to differ slightly in how they initiated group formation, my study demonstrates that both species do have the ability to tolerate unfamiliar conspecifics, and the potential to form social groups. I expected *R. pumilio* to be more tolerant of conspecifics than *R. dilectus* (measured as less aggression), but *R. pumilio* was more aggressive toward conspecifics than *R. dilectus* on initial meeting. Naturally occurring groups of *R. pumilio* typically consist of extended family members (Schradin and Pillay, 2004), so using unrelated females here may be an explanation for why *R. pumilio* was more aggressive than was expected on Day 1. Kin often constitute communal groups, or are the closest neighbours for solitary animals (reviewed in Kawata, 1990; Silk, 2007). In nature, *R. pumilio* displays high levels of aggression toward non-group members and are amicable toward group members (Schradin, 2004). However, amicable behaviour, not aggression, was the predominant behaviour displayed between triad members here, so one could infer that the higher occurrence of

aggression in *R. pumilio* triads, than that observed in *R. dilectus*, may be due to *R. pumilio* females establishing a hierarchy amongst themselves, which has been observed between females in breeding groups (D.L. Hill, unpublished data). Schoepf and Schradin (2012b) found dispersing *R. pumilio* of both sexes to be highly aggressive toward same-sex individuals and have high frequencies of social investigation. Similarly, *R. pumilio* triads showed higher levels of aggression and rates of social investigation compared to *R. dilectus* in the present study.

R. pumilio may have had more aggressive approaches and responses, and avoidance simply as a result of more individuals interacting than in *R. dilectus*. The sociograms revealed that all members of all *R. pumilio* triads interacted with one another, whereas 33% of *R. dilectus* triads had two triad members that did not interact on Day 1. This absence of interactions may indicate that *R. dilectus* is not as motivated as *R. pumilio* is to interact with conspecifics. One *R. pumilio* triad showed attraction and avoidance, but it was not apparent whether any one female initiated interactions in this triad. Furthermore, no clear hierarchical pattern or dominant individual could be identified from the sociograms for any of the triads, potentially indicating an egalitarian structure in the triads. D.L. Hill (unpublished data) observed a dominance hierarchy between *R. pumilio* sisters breeding communally in the laboratory, with one sister consistently producing litters before her siblings. The absence of a hierarchy in my triads may possibly result from 1) a social hierarchy not being established on initial encounter, or 2) the absence of reproductive competition. The dominance hierarchy observed by D.L. Hill (unpublished data) may be an emergent property of reproductive competition between females, even though they later display alloparental care in breeding groups (Schubert et al, 2009).

Nest cohabitation occurred in all *R. pumilio* triads and 90% of *R. dilectus* triads by the second day of triad formation, and was maintained for the remaining duration of the study. *R. dilectus* was not expected to form groups as readily as *R. pumilio*. Sharing a nest with conspecifics requires tolerance and is often used as an indication of groups being formed (Andrews and Belknap, 1993; Rusu and Krackow, 2004; Schradin et al, 2010b; Schoepf and Schradin, 2012b). Three nest boxes were provided for each triad to preclude forced nest cohabitation. Nest sharing under optimal conditions in captivity might not derive the benefits as in nature (thermoregulatory benefits, infant care, and vigilance; Schradin and Pillay, 2004; Schradin, 2005). Similarly, prairie voles, *M. ochrogaster*, share nests with individuals of the opposite sex with which they have pair bonds, being amicable toward each other without mating opportunities, limited nest sites, or any other functional benefits driving cohabitation (Hofmann et al, 1984). The occurrence of amicable behaviour was more frequent after the mice

started cohabiting, and consistently increased with time of study. The increasing duration of amicable behaviour between group members over time may be the result of increased familiarity between group members, especially through behaviours such as allogrooming, which also facilitates social recognition of group members (Kerth, 2008). Female free-tailed bats, *Tadarida teniotis*, exhibit more amicable behaviour toward familiar conspecifics (Ancillotto and Russo, 2014). Similarly, meadow voles, *M. pennsylvanicus*, that form unrelated communal nesting groups in winter, reduce agonistic and increase amicable interactions toward group-members, but display heightened aggression toward unfamiliar non-group members (Kawata, 1990).

Positive (affiliative) interactions are important for collecting information for social recognition, to form social memories, and to form and maintain social bonds between group-living animals (Carter, 2003; Kerth, 2008; Manno, 2008). Female vervet monkeys, *Chlorocebus aethiops pygerythrus*, have preferred grooming partners rather than simply grooming whichever females happen to be in the closest proximity to them (Henzi et al, 2013). This example shows how social recognition enables an animal to discriminate between conspecifics, adjusting how the individual interacts with and responds to conspecifics (Choleris et al, 2004). My results show that both species of striped mouse were more amicable toward triad members when they were more familiar with one another (from Day 2), which may potentially be for the formation and maintenance of social bonds between group members in this genus.

Differences in physiological states or processes alter the motivation to interact with conspecifics and the manner in which animals interact with conspecifics (aggressive or tolerant/amicable), subsequently shape variation in social systems (Lott, 1991; Porges, 1998). The neuropeptides arginine vasopressin (AVP) and oxytocin have been implicated in mediating social recognition and bonding, with social and solitary species showing differences in social behaviours associated with differences in these peptides (reviewed in Carter et al, 2008; Veenema and Neumann, 2008). For example, the socially monogamous prairie vole, *M. ochrogaster* increases selective aggression associated with mate guarding and pair bonding with females when AVP and oxytocin are experimentally administered, but these have no effect in the promiscuous solitary montane vole, *M. montanus* (reviewed in Carter et al, 2008; and Veenema and Neumann, 2008). A comparison of these neuropeptides in *R. pumilio* males adopting either a solitary or group-living tactic revealed that solitary males have more AVP-immunoreactive cells than group-living males, but did not differ in AVP1a receptor distribution

in the brain, nor in oxytocin-immunoreactive cells (Schradin et al, 2013; Schradin et al, 2014; Schoepf et al, 2015). Neuropeptide changes, thus, could modulate social behaviour in this socially flexible species, but it has not yet been tested whether differences exist in the neuropeptergic systems in females or between the two socially differing species.

Certain solitary species may have the ability to live in social groups if that tactic were to become most beneficial under new environmental conditions (e.g. habitat fragmentation leading to patchy food distribution or mate availability) but the prevailing environmental conditions have always favoured maintaining solitary living (low population density, widely distributed resources; Banks et al, 2007). This variation in sociality is seen seasonally in meadow voles, *M. pennsylvanicus*, which are typically solitary but will form communal nesting groups to exploit limited resources in the winter months (Kawata, 1990), and in the solitary grizzly bear, *Ursus arctos*, which will form aggregates at river mouths to exploit the spawning salmon (Lott, 1991). Tolerance of conspecifics at large kills also occurs in pumas, *Puma concolor*, a solitary-living carnivore (Elbroch et al, 2017). My results confirm that *R. dilectus* has the ability to tolerate conspecifics like *R. pumilio*, suggesting a potential for group formation should this become more advantageous than solitary-living. However, whether these groups could be maintained into the breeding season under the pressure of reproductive competition is unknown as this was not tested in this study. Similarly, it is uncertain if these groups would be maintained should competition arise for other contested resources, such as limited food availability.

R. dilectus appears to have a latent ability to display behaviours necessary to initiate the formation of social groups, although social groups have never been observed in nature. Similarly, this species does not display paternal care in the field, but does so in captivity (Schradin and Pillay, 2003). *R. pumilio* is the putative ancestral form of the genus *Rhabdomys* and *R. dilectus* is a derived form (Rambau et al, 2003; Pillay et al, 2006; Meynard et al, 2012). Thus, it is possible that these behaviours (paternal care and group formation) arose in a desert-living ancestor and are plesiomorphic in *R. dilectus* (Pillay et al, 2006). This raises the question of whether *R. dilectus* might have retained the ability to be socially flexible, like *R. pumilio*. This may be an indicator of *Rhabdomys* having a broad reaction norm for the genotype regulating social behaviour in this genus, with *R. dilectus* not being observed to display these behaviours in nature as the environmental conditions that elicit these behaviours in *R. pumilio* are absent for *R. dilectus* because the two species occupy very different environments (dry semi-desert versus moist grassland; Dingemanse et al, 2009; Meynard et al, 2012). Ongoing

studies in some parts of its range provides anecdotal information of *R. dilectus* sometimes forming small social groups, but the reasons for, and the demographic composition of, such group-living are unknown (C.M.S. Dufour et al, in prep).

In conclusion, this is one of few studies to consider how group formation is initiated. Here, I have demonstrated similarity in the response to conspecifics of a naturally solitary-living and naturally group-living species. This indicates 1) that the behaviours of initiation of groups amongst unfamiliar females is similar in species that differ in their natural social systems, and 2) a possible latent potential of the solitary species to form groups, at least over a short period in captivity. Thus, *Rhabdomys* appears to have an inherent motivation (internally driven) to form groups, regardless of kinship, in captivity. Whether *R. dilectus* has the potential to be socially flexible is not known, but it would be advantageous in future unpredictable environmental conditions (as seen in *R. pumilio*; Rymer et al, 2016). Future studies should consider whether limited resources (such as food or nests) influence group formation directionally. Furthermore, future studies are needed into the endocrinological profiles of these animals, to investigate whether females are physiologically motivated to live in groups or are in fact stressed in social groups. Such information on the proximate mechanisms could address the question of whether species are physiologically primed to be social, solitary (Lott, 1991), or socially flexible (Schradin et al, 2012).

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CHAPTER 3

Hormonal correlates of group formation in females of solitary-living and group-living African striped mice, *Rhabdomys*

(To be submitted to General and Comparative Endocrinology. Editing follows the style of the thesis)

Abstract

Social systems vary within and between species, ranging along a continuum between solitary-living to living in complex societies. Environmental factors influence the type of social system adopted, but not how they are maintained over time, which is likely to be under hormonal influence. In this study, I investigated the hormonal correlates of experimentally imposed group-living in a naturally solitary striped mouse species, *Rhabdomys dilectus* and a closely related sister species *Rhabdomys pumilio*, which is naturally group-living. I measured corticosterone and testosterone from blood serum of unrelated females housed in triads for 35 days; age matched solitarily housed females of each species served as controls. Females in triads of the naturally solitary *R. dilectus* had significantly higher (1.3x) concentrations of corticosterone than *R. pumilio*. Concentrations of corticosterone did not differ between treatments within each species. The solitary controls of *R. dilectus* did not differ in their corticosterone concentration to *R. pumilio* triads and controls. Testosterone concentration did not vary between species and treatments. In a concurrent behavioural study, triads of *R. dilectus* co-habited and formed social groups, but my findings here indicate that these females were physiologically stressed in a group (indicated by higher corticosterone concentrations). However, females of both species were less likely to engage in damaging fights, which was also apparent in the similar levels of testosterone in treatments. In conclusion, although *R. dilectus* has the potential to live in experimentally manipulated groups, females in such groups displayed greater stress than the naturally group-living *R. pumilio*.

Introduction

Animal populations adopt a variety of social systems, varying from solitary-living to living in complex societies, which are shaped by the interplay of intrinsic (within the individual) and extrinsic (environmental) factors (Hofmann et al, 2014). The social system adopted usually reflects the net fitness benefits of individuals (Silk, 2007). For example, group-living offers benefits such as reduced predation risk through increased vigilance (Blumstein and Fernandez-Juricic, 2010), but has costs including increased risk of disease and parasites (Ebensperger, 2001; Blumstein and Fernandez-Juricic, 2010), and reproductive competition (Schradin et al, 2010a). Solitary-living can avoid these costs, but this carries the cost of increased energy expenditure defending monopolisable resources, and potentially increased predation risk (Blumstein and Fernandez-Juricic, 2010).

Social systems can also be said to be emergent properties of the types of interactions between individuals within the population (Hinde, 1976; Kappeler and van Schaik, 2002). For example, a solitary social system emerges when animals exhibit heightened aggression toward conspecifics, increasing social aversion between individuals (e.g. golden hamster, *Mesocricetus auratus*; Wommack et al, 2003). In contrast, individuals that display more tolerance of conspecifics (less aggression and more amicability) encourage positive relationships and group-living to occur (Hinde, 1976; Engelmann et al, 1995; Young, 2002; Choleris et al, 2004; Storm and Tecott, 2005; Choleris et al, 2009). The manner in which animals interact with each other is driven by various internal factors, such as hormones and neuropeptides (McCall and Singer, 2012; Hofmann et al, 2014). The hormonal underpinnings of these interactions are of interest in this study, particularly the steroid hormones testosterone and corticosterone.

Testosterone was once thought to only be important in male reproductive behaviour, but has been found to be an important modulator of behaviour in females too (Staub and de Beer, 1997). Testosterone is important in social interactions, influencing aggression, territory defence, body size, sexual behaviour, and potentially social status (Staub and de Beer, 1997; Soto-Gamboa et al, 2005). Testosterone has also been associated with inhibition of the hypothalamus-pituitary-adrenal (HPA) axis (the pathway that meditates stress responses) and reducing anxiety (McCall and Singer, 2012). This reduction in anxiety by testosterone is proposed to increase risk-taking behaviour (Aikey et al, 2002; Schradin et al, 2009). Some species increase testosterone secretion in response to social interactions, and high

concentrations of testosterone have been linked to social aggression, such as territorial aggression (Wingfield, 2005).

The glucocorticoid hormone corticosterone is released via stimulation of the HPA axis in response to stressors (e.g. a sudden attack by a predator, or aggression from a conspecific; Sapolsky et al, 2000). Corticosterone mobilises glucose to provide energy in response to stress, and increased circulating corticosterone is considered as a stress response (Rubenstein, 2007), although a predictable response to chronic stress does not exist across all species in all contexts perceived as stressful (Dickens and Romero, 2013). An increase in corticosterone in the short term allows for efficient immune response, but over the long term can reduce immunity, increasing the animal's susceptibility to pathogens (Gust et al, 1991; Bartolomucci, 2007; Ebensperger et al, 2011).

Social stress can lead to increased circulating corticosterone concentrations (Zayan, 1991). Dickens and Romero (2013) propose that while a generalised endocrine profile of chronic stress does not exist, social stress is associated with a glucocorticoid increase in many species. Solitary and social species experience social stress differently (Blanchard et al, 2001). For example, social species mount a stress response when separated from conspecifics, as seen in several primate species (Zayan, 1991), and weanling African striped mice, *Rhabdomys pumilio* (Mackay et al, 2014). In contrast, solitary species can experience stress in response to social interaction with conspecifics, such as in the golden hamster, *M. auratus*, which experiences immune-system decline in groups, with a shorter life span as group size increases (Germann et al, 1989; Wommack et al, 2003). Moreover, group-living animals can experience social buffering where they display a reduced corticosterone response to a stressful situation due to the presence of conspecifics to which they are socially bonded, potentially via inhibition of the HPA axis (Hennessy et al, 2009).

Typically, corticosterone suppresses testosterone secretion, with high concentrations of corticosterone usually being accompanied by low testosterone (Soto-Gamboa et al, 2005; Deviche et al, 2014). This relationship is not evident in all species, however (Boonstra et al, 2001), as is seen in territorial male Rufous-winged sparrows, *Peucaea carpalis,* which have high concentrations of both hormones during the breeding season (Deviche et al, 2014). Similarly, female Galapagos marine iguanas, *Amblyrinchus cristatus,* have high concentrations of testosterone and corticosterone during the breeding season, when aggression is high, and testosterone then decreases after mating during nesting and egg laying, while corticosterone concentrations remain high (Rubenstein and Wikelski, 2005). Different social tactics also vary

in hormonal profile, such as in the seasonally social degu, *Octodon degus*, where resident males with females and high rates of social interactions, have higher corticosterone and testosterone concentrations than transient males which have lower rates of social interaction (Soto-Gamboa et al, 2005). The vast variation of hormonal patterns across social systems raises the question of how a normally solitary animal responds hormonally when forced to cohabit with conspecifics. Although it is difficult to conclude whether a hormonal change is responsible for a displayed behaviour, or the behaviour responsible for the hormonal response (Rubenstein and Wikelski, 2005), a comparison of the profile of corticosterone and testosterone could provide information on how species respond to different social scenarios.

The African striped mouse, genus *Rhabdomys*, is a small (adult body mass 32-55g) diurnal, murid rodent, widely distributed throughout southern Africa, occurring in a variety of biomes from moist grasslands (700-1000mm rainfall per annum) to arid semi-desert (160mm rainfall per annum; Skinner and Chimimba, 2005). I considered two closely related species of *Rhabdomys*: *R. pumilio* occurring in xeric environments and *R. dilectus* in mesic areas of South Africa (Rambau et al, 2003). Distinct differences in sociality exist between these species. *R. pumilio* in the arid Succulent Karoo is facultatively group-living, usually forming social groups consisting of one breeding male with up to four breeding females and their philopatric offspring, with males displaying paternal care (Schradin and Pillay, 2003; Schradin and Pillay, 2004). Group members share a nest and defend a common territory, but forage solitarily (Schradin, 2005). *R. pumilio* is highly socially flexible, switching to solitary-living under low population densities, associated with solitary breeding males and females occurring in the same area (Schradin and Pillay, 2006; Schradin et al, 2010b; Hill et al, 2015). *R. dilectus* is solitary-living and territorial, with individual territories overlapping exclusively with territories of the opposite sex (Schradin and Pillay, 2005). Females raise offspring alone and only interact with males during the breeding season (Schradin and Pillay, 2003).

In free-living *R. pumilio*, individuals of both sexes adopting different tactics (i.e. group-living, solitary living, or individuals "switching" from group-living to solitary) have lower corticosterone concentrations when solitary than in a group, and solitary males have higher testosterone concentrations than group-living males (Schoepf and Schradin, 2013). Hill et al (2015) found that female *R. pumilio* which breed solitarily had lower baseline corticosterone concentrations when compared to their group-living counterparts. These solitary breeders did not differ from the group-living females in testosterone, progesterone or oestrogen concentrations (Hill et al, 2015). Schoepf and Schradin (2013) found that although females that

switched from group-living to solitary-living showed an elevated testosterone concentration, these concentrations were not significantly different to group-living females. Hormone concentrations vary seasonally between sexes and breeding vs. non-breeding group members within social groups of *R. pumilio*: females (breeders and non-breeders) had lower concentrations of corticosterone and testosterone in the non-breeding season than the breeding season, while breeding males had the lowest corticosterone concentrations and highest testosterone concentrations during the breeding season (Schradin, 2008). While variation in hormonal response in *R. pumilio* has been well investigated, a comparison of the hormonal profile with the closely related *R. dilectus* has not been investigated. These closely related, yet socially different, species offer a unique opportunity to compare the endocrine patterns of different social systems.

Ten triads of each species were used and the behaviour of individuals in the triads was recorded (K-J. Hartman unpublished data, see Chapter 2). While the majority of initial interactions were amicable in triads of both species, *R. pumilio* displayed more aggression toward conspecifics than *R. dilectus* triads did on Day 1 of triad formation. All *R. pumilio* triads shared a nest from Day 2 of triad formation and 9 of the ten *R. dilectus* triads cohabited from Day 2, with one triad being terminated early due to damaging fights. The behavioural ability of *R. dilectus* to form social groups under a simulated high population density, like *R. pumilio* does, raises the question of whether these two species differ hormonally in their response to social grouping or not.

I investigated the serum corticosterone and testosterone concentration patterns in *R. pumilio* and *R. dilectus* housed under experimentally imposed group-living conditions in captivity. Progesterone was not considered here as it is primarily involved in female reproductive behaviour, which is not the focus of this study, and Hill et al (2015) indicated that it does not correlate with reproductive tactics in free-living female *R. pumilio*. Groups of free-living *R. pumilio* comprise of one breeding male with up to four closely related communally-breeding females (Schradin and Pillay, 2004). Therefore, in this study**,** I used unrelated females to test whether *Rhabdomys* has the ability to form social groups with unrelated individuals. Sisters would not afford the opportunity to investigate the motivation to form groups because sisters of both species naturally cohabit in captivity (D.L. Hill unpublished data). I hypothesised that associating with conspecifics would be more stressful for *R. dilectus* than *R. pumilio*. I predicted that 1) *R. dilectus* would have higher concentrations of corticosterone than *R. pumilio* in social groups; 2) *R. pumilio* in groups would have higher concentrations of

testosterone than *R. dilectus* groups, based on *R. pumilio* being more aggressive in triads (K-J. Hartman unpublished data, Chapter 2); and 3) social groups of both species would have greater concentrations of corticosterone and testosterone than solitary-housed controls of both species, due to social stress and competition experienced in groups.

Materials and methods

STUDY SPECIES

The striped mice used in this study were from the captive colony at the Milner Park Animal Unit at the University of the Witwatersrand, Johannesburg, South Africa. The naturally group-living species, *Rhabdomys pumilio*, originated from the semi-arid Nama Karoo of South Africa (average rainfall of 160 mm per annum, winter rainfall). The naturally solitary-living species, *Rhabdomys dilectus dilectus (*referred to as *Rhabdomys dilectus* here), originated from the moist grassland area in the highveld of central South Africa (average rainfall of 700 mm per annum, summer rainfall). The individuals used were bred in captivity and were either F2 or F3 captive born.

HUSBANDRY

This study was conducted in the Milner Park Animal Unit under partially controlled environmental conditions: 14:10 light:dark cycle (lights on at 5:00); 30-60% rH; and a room temperature of 24-28°C. Breeding pairs were housed in clear Labotec™ cages (L x H x W: 425mm x 150mm x 240mm) containing a PVC nest-box (L x H x W: 98mm × 64mm × 77mm), ±3cm wood shavings, a handful of *Eragrostis* grass (± 20g) and ± 5g of paper tissue provided as nesting material. Environmental enrichment was provided in the form of a cardboard tube (L x D: 99mm x 38mm) and a small wooden block. The diet of the striped mice throughout this study included 5g Epol® mouse cubes, approximately 5g fresh fruit or vegetables and approximately 2g millet and sunflower seeds daily per mouse; water was provided *ad libitum* for the entire period of the study.

Approximately 40 to 45 breeding pairs of both species were established before experiments began. At 20 days of age pups were weaned and housed alone for 30 days in Labotec™ cages (L x H x W: 425mm x 150mm x 240mm), furnished and enriched as described above for the breeding pairs. From these pairs, a total of 40 *R. pumilio* and 40 *R. dilectus* female offspring were used in this study.

EXPERIMENTAL DESIGN

At approximately 43 days of age (one week prior to forming the experimental groups), all females used in this study were uniquely marked using commercial hair dye (Innecto™, Pinetown, South Africa) to allow for individual identification. Triads were formed when mice were approximately 50 days of age (adult). Triads consisted of three unrelated, age-matched females which were randomly assigned to a triad. Triads comprised females of the same species, a total 10 triads per species were created (K-J. Hartman unpublished data; Chapter 2).

Triads were housed together for 35 days. The initial interactions (approximately 20 minutes) of the triad were observed using direct observation to assess the levels of aggression, so that triads could be terminated immediately should excessive or damaging fights occur (which was not the case in all triads). Each triad was housed in a large aluminium tank with a clear Perspex front (L x H x W: 460mm x 350mm x 300mm). These tanks contained wood shavings for bedding, grass and nesting material as described above. The tanks contained three separate nest boxes of equal size (L x H x W: 100mm x 77mm x 63.5mm) in order to prevent forced co-habituation of the triad. All *R. pumilio* triads, and 9 *R. dilectus* triads, shared a single nest box from the second day in the triad (i.e. from age 51 days to 85 days; K-J. Hartman unpublished data, see Chapter 2), so triads were assumed to have formed stable social groups (as described in Schradin et al, 2010a, Schoepf and Schradin, 2012a). A control treatment (10 per species) in which females were maintained in solitarily housing from 50 to 85 days of age to provide an age-matched comparison to triads. These individuals were also marked and placed in new cages on the same days as experimental triads. Control animals were housed in Labotec™ cages prepared as described above. Since *R. pumilio* can occur alone in nature, the solitary housing post-weaning and of controls is not stressful (Schoepf and Schradin, 2013).

At approximately 85 days of age, all subjects (triads and controls) were euthanased through isoflurane inhalation, as approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number: 2012/28/A). Triads were maintained for 35 days before euthanasia and blood collection to measure serum corticosterone and testosterone. Mice were euthanised and blood collected within 2-3 minutes of first contact to minimise the effect of the stress of handling on the circulating corticosterone concentrations. Mice were confirmed dead by testing for a pulse, then immediately decapitated and approximately 500µL of blood was collected in an Eppendorf™ tube from the trunk of the animal. Following the methods described by Schradin (2008), blood samples were kept at room temperature for 1.5 hours to allow for coagulation before being centrifuged at 8000 rpm for 10 minutes. The serum

was then pipetted into another Eppendorf™ tube and centrifuged for a further 10 minutes at 8000 rpm. The serum was then pipetted into two different aliquots (20µL of serum for corticosterone, and 50µL for testosterone) which were stored at -20°C. Hormone concentrations were measured using enzyme-immunoassays, performed at the University of Strasbourg, France, using commercial kits from IBL Hamburg specific to each hormone, following the procedures validated for striped mice by Schradin (2008). For both hormones, serial dilution of striped mouse sample pools (2 for each hormone) paralleled the standard curve and the slopes were not different (Schradin, 2008). Intra- and inter-assay variability was determined with pools from wild striped mice. For testosterone, inter-assay variability for one pool with 6 samples was 15.6%. For corticosterone, inter-assay variability (5 samples) for two pools were 11.5% and 15.6%. Intra-assay variability was only determined for corticosterone for both pools and was 10.2% (5 samples) and 4.6% (8 samples). Hormone concentrations are expressed as nano-gram of hormone measured per millilitre of serum (ng/ml).

DATA ANALYSIS

One *R. dilectus* triad was terminated early due to damaging fights, thus data from 9 *R. dilectus* triads (n = 27), 10 *R. pumilio* triads (n = 30), and 10 controls per species were analysed. All data analysis was done in R version 3.2.4 (The R Foundation for Statistical Computing, Vienna, Austria) software. Corticosterone concentration data were normally distributed, confirmed by a Shapiro-Wilk normality test. Testosterone concentration data were square root transformed to achieve a normal distribution; the appropriate transformation was identified using a Box-Cox power transformation in the "car" library (Fox and Weisberg, 2011). All data are presented as mean and 95% confidence interval. The influence of species and treatment on corticosterone and testosterone concentrations were analysed using a general linear model (GLM), using the lme4 (Bates et al, 2015), lmerTest (Kuznetsova et al, 2016) and car (Fox and Weisberg, 2011) libraries in R. Each hormone was listed as the response variable in a separate GLM. Species and treatment, and the interaction between treatment and species, were fixed factors in the model. Treatment group (i.e. triads and each control), and litter ID were included as random effects. Litter ID was included to account for the litter from which females were derived having possible influences on group dynamics, as litter mates were assigned to different triads or as controls. Likelihood ratios tests were used to generate χ^2 statistics and p values to indicate significance of factors (Bates et al, 2015). Models were validated by comparing the residuals to the fitted values (Crawley, 2009).

To establish whether a correlation between the hormonal profiles and behaviours exists, Spearman's Rank Order correlation between the behaviour displayed on Day 1 of triad formation (Chapter 2) and the hormone concentrations (measured here) was run in Dell Statistica version 12 (Dell Inc., USA). Corticosterone and testosterone were each compared to the frequency of amicable behaviour, aggressive behaviour, and avoidance behaviour displayed by each individual in the triads (n = 54). Since corticosterone suppresses testosterone secretion in some species (Soto-Gamboa et al, 2005; Deviche et al, 2014), I also used a Spearman rank correlation to establish whether these hormones were correlated (n = 77, as the *R. dilectus* triad terminated early was excluded).

Results

Species (χ^2_1 = 3.80; p = 0.049) was a significant predictor of circulating corticosterone concentrations. *R. dilectus* had higher corticosterone concentrations (mean: 2480.79 ng/ml) than *R. pumilio* (mean: 1852.75 ng/ml). Treatment (χ^2_1 = 1.141; p = 0.285) was not a predictor of corticosterone concentrations, nor was there a species x treatment interaction (χ^2_1 = 2.22; p = 0.137) effect. Concentrations of corticosterone did not differ between treatments for both species, but were highest in *R. dilectus* triads compared to *R. pumilio* in triads and singly housed controls of *R. pumilio*. (Figure 3.1).

A significant, weak, negative correlation existed between corticosterone concentration and the frequency of amicable behaviour (Spearman R = -0.41, p = 0.002) and avoidance (Spearman R = -0.39, p = 0.004) displayed on initial encounter, but there was no correlation with the frequency of aggressive behaviour (Spearman R = -0.22, p = 0.115).

Testosterone concentration was not significantly predicted by species (χ^2_1 = 1.73; p = 0.188), treatment (χ^2_1 = 0.11; p = 0.738), or species x treatment interaction (χ^2_1 = 2.22; p = 0.136; Figure 3.2).

Serum testosterone concentration was not correlated to the frequency of amicable (Spearman R = 0.12, p = 0.4), aggressive (Spearman R = 0.15, p = 0.27), or avoidance behaviour (Spearman R = 0.05, p = 0.73) displayed on Day 1 of triad formation. Testosterone and corticosterone were not correlated (Spearman R = -0.16, p = 0.16).

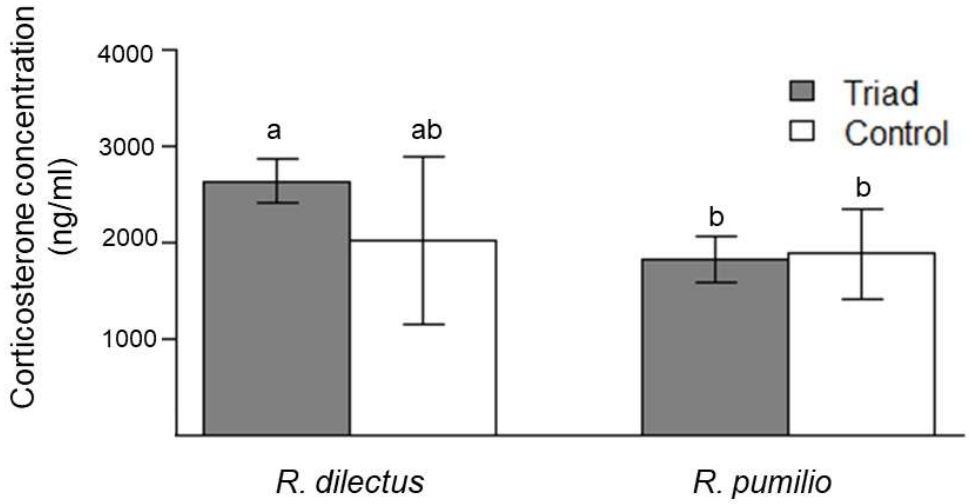


Figure 3.1. Serum corticosterone concentration (ng/ml) of two species of *Rhabdomys*, comparing two treatments: triads (in grey) and solitarily (in white) housed females (controls). Data presented as mean ± 95% CI. n = 27 for *R. dilectus* triads, n = 30 for *R. pumilio* triads, n = 10 for controls of each species. Different letters indicate significant differences ($p < 0.05$).

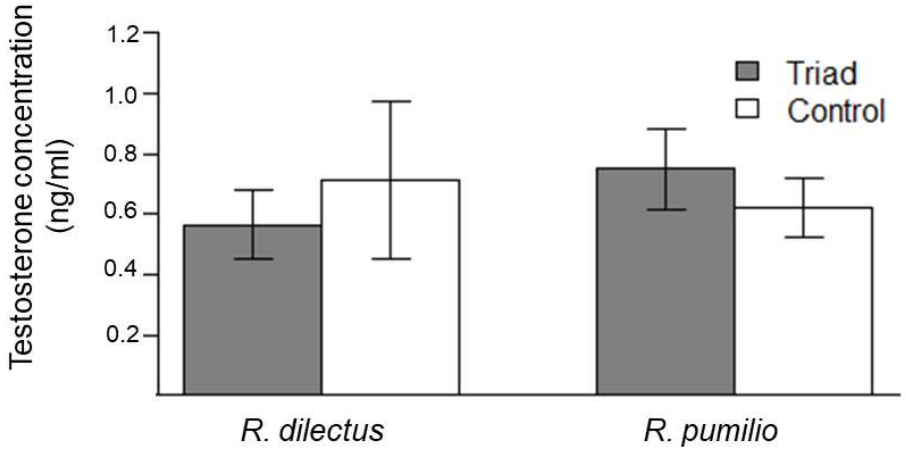


Figure 3.2. Serum testosterone concentration (ng/ml) of two species of *Rhabdomys*, comparing two treatments: triads (in grey) and solitarily housed females (controls, in white). Data presented as mean ± 95% CI. n = 27 for *R. dilectus* triads, n = 30 for *R. pumilio* triads, n = 10 for controls of each species. There were no significant differences between species, treatment and their interactions.

Discussion

I investigated whether experimentally housed unrelated female triads of a naturally solitary-living species differed hormonally to female triads of a naturally group-living species. I expected *R. dilectus* to be more stressed during associations with conspecifics than *R. pumilio*, expressed as higher corticosterone concentrations. This prediction was supported, as the naturally solitary-living *R. dilectus* showed a significantly higher concentration of circulating

corticosterone in triads than *R. pumilio* triads and controls. I expected both species to have higher corticosterone in groups than in the solitary controls, but this was not the case, as neither species differed in corticosterone concentration between treatments. Corticosterone concentration had a weak negative relationship with the frequency of amicable and avoidance behaviour displayed when triads were initially formed. *R. pumilio* was more aggressive than *R. dilectus* when triads were initially formed (35 days prior to the hormone measurements in the current study; K-J. Hartman unpublished data, see Chapter 2), and thus I expected *R. pumilio* in groups would have higher concentrations of testosterone than *R. dilectus* groups. I also expected the triads to have greater concentrations of testosterone than solitary-housed controls of both species, due to competition within groups. However, there was no difference in circulating testosterone concentrations between the two species, regardless of social setting (triad vs. control). Testosterone was not correlated with any of the behaviours displayed when triads were formed, nor was testosterone correlated with corticosterone.

In a previous study, unrelated female *R. dilectus* shared a single nest (K-J. Hartman unpublished data, see Chapter 2), a behavioural indication of social tolerance (Andrews and Belknap, 1993; Rusu and Krackow, 2004; Schradin et al, 2010a), but they appear to experience stress, as indicated by elevated corticosterone, in this group setting. It is typical for individuals to experience increased corticosterone concentrations during the initial interactions with unfamiliar individuals (Mendoza et al, 1978), and the stress response should dissipate once the social group is stable (maintaining a consistent composition over time; DeVries et al, 2003). This may indicate that while the socially flexible *R. pumilio* is able to settle into a stable social group, *R. dilectus* could share a nest, but these triads may not have been stable. A stress response induced by social interactions usually stems from increased aggression or harassment from conspecifics (Creel, 2001; DeVries et al, 2003), which is usually observed during group instability (Rubenstein, 2007). For example, in the superb starling, *Lamprotornis superbus*, helpers have higher corticosterone concentrations because they are harassed by the more dominant breeders when establishing rank (Rubenstein, 2007). However, *R. dilectus* interacted amicably in triads and aggression was never observed after the first day of triad formation (K-J. Hartman unpublished data, see Chapter 2).

R. pumilio triads had lower corticosterone concentrations than *R. dilectus* triads, indicating that the naturally group-living *R. pumilio* is less stressed in a group than *R. dilectus*. Species with differing social systems differ in what is perceived as socially stressful, with either social isolation or social grouping having the potential to be stressful (Blanchard et al, 2001).

Social buffering is experienced by social animals that form social bonds with conspecifics, where the presence of those conspecifics can reduce corticosterone response to a stressful situation (Hennessy et al, 2009). One suggestion for this effect is the inhibition of the HPA axis by the neuropeptide oxytocin, responsible for stimulating affiliative behaviour between individuals that encourages social bonding and reduces corticosterone production (DeVries et al, 2003). Since social bonds cannot be measured directly, they are usually inferred from the response of an individual to separation from a group or partner (DeVries et al, 2003), as seen in the monogamous prairie vole, *Microtus ochrogaster*, which increases corticosterone concentrations when separated from their pair-bonded partner (Carter, 1998). A previous study showed that *R. pumilio* weanlings were more behaviourally (anxiety) and hormonally (corticosterone) stressed than *R. dilectus* weanlings when separated from their family groups (Mackay et al, 2014). This suggests that *R. pumilio* forms more lasting social bonds and that *R. dilectus* copes better with isolation, even at a young age. This finding and the results of the current study indicate that *R. dilectus* may be under physiological stress in a social group, even though this might not manifest in a behavioural stress response.

Corticosterone concentrations in the naturally group-living *R. pumilio* did not differ between triads and controls, even though females of this species in communally breeding groups in field studies have higher corticosterone than solitary individuals (Schoepf and Schradin, 2013; Hill et al, 2015). These results likely differ from these field studies for two reasons: 1) the field studies were conducted on naturally occurring groups, which usually consist of closely related females (Schradin and Pillay, 2004), whereas my experimental groups consisted of unrelated females; and 2) field studies were conducted during the breeding season, when reproductive competition is high, and has been confirmed as a driver of striped mice dispersing to live solitarily (Schoepf and Schradin, 2012b). The current study was conducted under optimal conditions in captivity, free from reproductive competition (absence of a breeding male). My results also differ to those of Mackay et al (2014), who found weanling *R. pumilio* to be stressed when housed alone, alluding to an age and timing-effect of isolation. Thus, adult *R. pumilio* are not stressed when solitary, complementing the field data showing that *R. pumilio* switch readily to a solitary social tactic from group-living under low population densities (Schradin et al, 2010a; Schoepf and Schradin, 2013).

Testosterone concentrations did not vary by species or treatment. Hill et al (2015) also found no difference in testosterone concentration between solitary and group living female *R. pumilio* employing different reproductive tactics. Moreover, Schoepf and Schradin (2013)

reported no differences in testosterone concentration in solitary and group-living female *R. pumilio* across seasons. My study is the first to measure testosterone concentration in *R. dilectus*, which again showed that testosterone may not have an important role in female social behaviour in *Rhabdomys*. The role of testosterone in female behaviour still requires much investigation. Female Galapagos marine iguanas, *Amblyrinchus cristatus*, have lower concentrations of testosterone when fighting than when they are not fighting (Rubenstein and Wikelski, 2005), contesting the idea of a direct relationship between testosterone and aggression. In contrast, Woodley and Moore (1999) found that increases in testosterone concentrations were associated with periods of heightened aggression in female mountain spiny lizards, *Sceloporus jarrovi*, but determining a causal link between hormonal concentration and a behaviour is nonetheless difficult without consideration of environmental and neurological effects.

Several environmental factors affect sociality, these include food availability, population density, access to mates and predation pressure (Maher and Lott, 2000; Silk, 2007). While these factors can explain why animals may aggregate initially, they do not explain why animals would form and maintain social groups (Jones, 2014). The triads in my study simulated a situation of high population density. All female *R. dilectus* used a single nest box (i.e. co-habited). Even though the females had access to sufficient food, water and independent nest boxes, such housing was stressful. Higher corticosterone concentrations impact on the immune function and reproductive output, leading to reduced fitness (Gust et al, 1991; Bartolomucci, 2007; Ebensperger et al, 2011). Therefore, group-living in female *R. dilectus,* as imposed here, is unlikely to be stable, even if groups were to form under high population density in nature; striped mouse population density can exceed 200 individuals per ha in grassland localities, leading to overlapping home ranges among females (N. Pillay unpublished data). In *R. pumilio*, groups form when reproductive competition is absent (non-breeding season), or population density is high with limited nest sites and food availability (Schradin et al, 2010a; Schoepf and Schradin, 2012b).

The hormonal regulation of, and response to, social interactions is important for understanding how animals form stable groups and social bonds. My results show that two sister species that differ in their social systems display different patterns of corticosterone concentrations in groups, but no differences to solitary controls. Testosterone concentrations were not associated with living in triads in both species. Group-living in *R. pumilio* occurs because of ecological constraints, such as limited nest sites and food (Schradin et al, 2010a;

Schradin et al, 2010b). Here, I also demonstrated that unrelated female *R. pumilio* can form social groups, and they do not mount a stress response (raised corticosterone) in these groups. *R. dilectus* may have the latent ability to cohabit under conditions where resources are not contested, but the elevated corticosterone concentrations indicate that, while females are tolerant of group members, they are stressed, so the stability of the group may fail, which might explain the lack of group-living in nature under high population density. Future studies are needed in other closely related species with differing social systems to show the generalizability of my findings. In addition, examining the neuro-endocrinological correlates of group-living might show whether and how other hormones are associated with group formation and stability.

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CHAPTER 4

Neuropeptide mediation of group-formation: a comparison of a group-living and solitary-living species of the striped mouse, *Rhabdomys*

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Abstract

The neuropeptides arginine vasopressin (AVP) and oxytocin are well-known for their role in mediating social behaviour in mammalian species, and are part of the proximate drivers of social systems. The social tactic of females dictates the tactic adopted by males in most species. Thus, it is important to investigate the central AVP and oxytocin concentrations in females of socially different species to understand the proximate mechanisms of group formation. I investigated the concentration of these two neuropeptides in the whole brain from females of two species of *Rhabdomys* with contrasting social systems, namely the facultatively group-living *R. pumilio* and the solitary-living *R. dilectus*. I imposed group-living conditions in captivity by creating triads of unfamiliar females for each species and compared the concentrations of AVP and oxytocin between the two species after housing them in triads for 35 days, as well as made comparisons with age-matched solitary controls of each species. I used two peptide extraction protocols, utilising either an acidified methanol buffer or a half RIPA buffer, and found half RIPA buffer protocol to be more suitable. I predicted there would be no difference in AVP between species in triads, but if *R. pumilio* is primed to be social, this species should have higher AVP than *R. dilectus* in the solitary controls. Triads of both species were expected to have higher oxytocin than solitary controls. *R. pumilio* controls were expected to have higher oxytocin than *R. dilectus* controls. *R. dilectus* in triads had significantly higher concentrations of AVP than *R. pumilio* in triads, but neither differed from their solitary controls. Surprisingly, solitary controls of the two species did not differ in AVP concentrations, potentially indicating that AVP is released in response to the social environment and not a primer of how each species will behave socially. Interestingly, there were no significant differences in oxytocin concentration across the two species or between treatments. I demonstrated that two socially different species of *Rhabdomys* do not differ in neuropeptide concentration when socially isolated, but do differ in AVP concentration when in a group, and oxytocin concentration does not differ with social context or species. It appears that AVP is more important than oxytocin as a proximate driver of social behaviour in *Rhabdomys*.

Introduction

Social systems vary on a continuum between solitary-living and living in complex societies, depending on what is most advantageous to individuals in a population in its specific environmental conditions (Silk, 2007). While different social systems offer different costs and benefits based on the environment in which a population occurs (Poole, 1985; Jones, 2014), the social system itself can be an emergent property of the interactions that occur between the individuals of that population (Hinde, 1976). In social groups, individuals recognise and tolerate group members, leading to more sociable behaviour towards them and repulsive behaviours (e.g. aggression) towards non-group members (Carter, 2003; Choleris et al, 2004; Albers, 2012). In contrast, solitary living is characterised by more agonistic (aggression and avoidance) behaviour toward conspecifics, except for particular events such as mating and the rearing of offspring (Blanchard et al, 2001). These differences in social behaviour are likely to be driven by neuroendocrine mechanisms (Carter et al, 2008). My study considers the proximate mechanisms mediating social behaviours in species that differ in their sociality.

Two important neuroendocrine mediators of social behaviour in mammals are arginine vasopressin (AVP) and oxytocin (reviewed in Carter et al, 2008). These nonapeptides differ in structure by only two amino acids and have similar roles in social behaviour, even influencing each other's receptors in the brain (Carter et al, 2008; Leng et al, 2012). Both are synthesised in magnocellular neurosecretory neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and are stored in the posterior pituitary gland for secretion into the systemic circulation as hormones performing various physiological functions (Leng et al, 2012). As circulating hormones, AVP regulates water balance and blood pressure, and oxytocin stimulates contraction of smooth muscle, such as uterine contractions during parturition (Nelson, 2005; Curtis et al, 2007; Carter et al, 2008; Leng et al, 2012). Travelling through the brain via projections from the PVN and via diffusion, AVP and oxytocin act centrally to mediate social behaviour by acting on specialised receptors within various regions of the brain, collectively referred to as the "social behaviour network" (SBN; Hawthorn et al, 1980; Bradford, 1986; Bielsky and Young, 2004; Veenema and Neumann, 2008; Leng et al, 2012). Oxytocin has only one receptor type, while AVP has three different types, but of these, the V1a receptor is mainly implicated in behaviour (Carter, 2003; Albers, 2012; Leng et al, 2012).

AVP mediates social recognition, social communication and social memory, as well as sex typical behaviours such as maternal aggression and paternal care, mate guarding, and

courtship (Young and Wang, 2004; Carter, 2007; Curtis et al, 2007; Albers, 2012; Anacker and Beery, 2012; McCall and Singer, 2012). Oxytocin is implicated in several affiliative behaviours, including social bond formation, pair bonding, sexual behaviour, grooming, social recognition and communication, and maternal behaviour (Nelson, 2005; Nair and Young, 2006; Neumann, 2008). Sex differences in social behaviour are usually ascribed to the synthesis of AVP being androgen dependent while oxytocin synthesis is oestrogen dependent, but both neuropeptides influence the behaviour of both sexes (Carter et al, 2008).

Veenema and Neumann (2008) proposed that the diversity of social systems observed in nature may be partly due to difference in neuropeptide synthesis, neuropeptide release, and their receptor distribution in the SBN across vertebrate species and sexes. Comparisons of a social and solitary species of tuco-tuco, genus *Ctenomys*, revealed vast differences in the distribution of AVP and oxytocin receptors in regions of the SBN between the species (Beery et al, 2008). Similarly, social bonds, following centrally administered oxytocin and AVP, was observed in the monogamous social prairie vole, *Microtus ochrogaster*, but not in the promiscuous solitary montane vole, *M. montanus* (Choleris et al, 2004). These neuroanatomical differences, and different behavioural responses to administered AVP and oxytocin, suggest that social and solitary species may be differently primed physiologically to facilitate or prevent social approach or avoidance, shaping their social systems (Carter et al, 2008; Veenema and Neumann, 2008).

The African striped mouse, genus *Rhabdomys*, includes species that have been recorded displaying social flexibility, a phenomenon where individuals in a population reversibly switches between social tactics in response to prevailing environmental conditions (Schradin et al, 2012), as well as a species that has only ever been observed living solitarily in nature (Schradin and Pillay, 2005). This provides us with the opportunity to compare neuropeptide influences on social behaviour. While such comparisons have been made between group-living and solitary species, socially-flexible species have not been considered in the literature.

Rhabdomys, a diurnal, murid rodent, is commonly found in a variety of biomes in southern Africa, including arid semi-deserts (160mm rainfall per annum) and moist grasslands (700-1000mm rainfall per annum; Skinner and Chimimba, 2005). In this study, I consider two closely related species of *Rhabdomys* with distinctly different social systems: the facultatively group-living *R. pumilio*, which occurs in the xeric Nama-Karoo, and the naturally solitary-living *R. dilectus* from the mesic grassland areas of South Africa (Rambau et al, 2003). Social groups in *R. pumilio* typically consist of one breeding male with 2-4 breeding females and their

philopatric offspring, with males exhibiting paternal care for offspring (Schradin and Pillay, 2003; Schradin and Pillay, 2004). Group members forage solitarily during the day, but interact amicably and bask together at dawn and dusk, share a nest, and defend a shared territory (Schradin, 2005). This species is socially flexible, switching to solitary-living during the breeding season under low population densities, adopting a social system of solitary breeding females and males occurring in the same area, potentially as a means to avoid reproductive competition in the form of reproductive suppression in males and infanticide in females (Schradin and Pillay, 2006; Schradin et al, 2009; Schradin et al, 2010; Schoepf and Schradin, 2012a; Hill et al, 2015). The grassland species, *R. dilectus*, is solitary-living, and defends individual territories that overlap exclusively with individuals of the opposite sex (Schradin and Pillay, 2005). Females interact with males during the breeding season and raise offspring alone, yet male *R. dilectus* display paternal care in captivity (Schradin and Pillay, 2003).

A comparison of AVP-immunoreactive (ir) and oxytocin-ir cells in the brain of free-living *R. pumilio* males adopting either a group-living (philopatric or breeder) or solitary tactic, in captivity and nature, found that the solitary roamer males have more AVP-ir cells in their PVN than the group-living males (Schradin et al, 2013; Schoepf et al, 2015). Schradin et al (2013) proposed that the higher densities measured in the solitary males were indicative of the stored AVP which could be released when required to initiate social bond formation to switch tactics. This was confirmed in a separate captive study of *R. pumilio* in which the occurrence of AVP V1a receptors in various regions of the brain did not differ between the male social tactics, so males of each tactic can respond in the same way to AVP secretion (Schradin et al, 2014). The oxytocin-ir cells did not differ between the tactics in males studied in captivity, which may be due to oxytocin not having as much functional importance in males as it would in females (Schradin et al, 2013), but field studies found philopatric group-living males had lower oxytocin-ir cells than solitary roamers and breeding males, indicating that oxytocin may be more relevant to sexual experience than social behaviours in male *R. pumilio* (Schoepf et al, 2015). The concentration of AVP and oxytocin in the brain of *R. dilectus* has never been studied, nor have the brain concentrations of these neuropeptides been investigated in female *R. pumilio*. Since female *R. pumilio* form social groups which are later joined by a breeding male (Schradin and Pillay, 2003), it is important to know whether the females of these two socially different species differ in AVP and oxytocin concentrations to understand the proximate mechanisms of group formation and, possibly, social flexibility.

I investigated AVP and oxytocin concentrations in the whole brain of females of *R. pumilio* and *R. dilectus*. This was done by comparing these concentrations between the two species under experimentally imposed group-living conditions (i.e. female triads), and against solitarily-housed controls of each species. In these forced group-living conditions, both species formed stable social groups (maintained over 33 days), with all group members sharing a single nest and displaying predominantly amicable behaviour, indicating that *R. dilectus* can form social groups like *R. pumilio* (K-J. Hartman unpublished data, see Chapter 2), in captivity. To extract the neuropeptides from the whole brains, I developed two protocols (using either acidified methanol buffer, or half strength radioimmunoprecipitation assay (commonly known as half RIPA) buffer) to determine which is the most suitable for extracting AVP and oxytocin from whole mouse brains to measure concentration with commercial enzyme-immunoassay (ELISA) kits.

A previous study by Schoepf et al (2015) found the social behaviours displayed were not correlated to the density of AVP-ir cells and oxytocin-ir cells in the brain of male *R. pumilio*. Thus, the tolerance and predominantly amicable behaviour seen in female triads of both species (K-J. Hartman unpublished data, see Chapter 2) does not necessarily mean that social behaviour of the two species is similarly associated to central AVP and oxytocin concentration. I predicted that *R. dilectus* would not differ in terms of brain AVP concentration to *R. pumilio* in a group-housing context, but triads of both species would have lower AVP than solitary controls, based on the suggestion by Schradin et al (2013) that solitary individuals may have higher AVP to facilitate the induction of social bonds and group formation when the opportunity arises. *R. pumilio* naturally forms social groups, thus I expected *R. pumilio* housed solitarily to have higher AVP concentrations than *R. dilectus* controls, because *R. dilectus* does not naturally form groups (Schradin and Pillay, 2004; Schradin and Pillay, 2005). I predicted that oxytocin would be higher in females housed in groups than those housed alone for both species, due to the bond forming action of affiliative behaviours such as allogrooming (Carter, 2003; Neumann, 2008). If the naturally social *R. pumilio* is primed to be social (or socially flexible; Schradin et al, 2012; Schradin et al, 2013), I expected that solitary *R. pumilio* would have higher concentrations of oxytocin than *R. dilectus* in isolation.

Materials and methods

Study species

All striped mice used in this study were obtained from the captive colony at the Milner Park Animal Unit at the University of the Witwatersrand, Johannesburg, South Africa. The naturally group-living species, *Rhabdomys pumilio*, originated from the semi-arid Nama Karoo region of South Africa. The naturally solitary-living species, *Rhabdomys dilectus dilectus* (referred to as *R. dilectus* in this paper), originated from the moist grassland Highveld region of central South Africa. All individuals used in this study were bred in captivity and were either F2 or F3 captive born.

Husbandry

This study was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number: 2012/28/A). All experiments took place at the Milner Park Animal Unit under partially controlled environmental conditions: daily light: dark cycle of 14:10 (lights on at 5:00); 30-60% rH; and a room temperature of 24-28°C. Breeding pairs were housed in clear Labotec™ cages (L x H x W: 425mm x 150mm x 240mm) provided with a PVC nest-box (L x H x W: 98mm × 64mm × 77mm), ± 3cm wood shavings, and a handful of *Eragrostis* grass (± 20g) and ± 5g of tissue paper for nesting material. Each cage included a cardboard tube (L x D: 99mm x 38mm) and a small wooden block to serve as environmental enrichment. Throughout this study, each mouse was given a daily diet of 5g Epol® mouse cubes, approximately 5g fresh fruit or vegetables and approximately 2g millet and sunflower seeds; water was provided *ad libitum*.

Before experiments began, approximately 40 to 45 breeding pairs of each species were established, the offspring produced by these pairs were used as study subjects in this study. At 20 days of age, pups were weaned and these juveniles were housed alone for 30 days in Labotec™ cages (described above). These cages were furnished and enriched as described above for the breeding pairs. A total of 40 *R. pumilio* and 40 *R. dilectus* females from these pairs were used in this study.

Experimental design

One week prior to forming the experimental groups, all females used in this study were uniquely marked using commercial hair dye (Innecto™, Pinetown, South Africa). At approximately 50 days of age (adults) individuals were randomly assigned to triads, which

consisted of three unrelated, age matched, females from the same species. In total, 10 triads per species were formed.

Triads were housed together for a total of 35 days. The initial interactions (approximately 20 minutes) of the triad were observed using direct observation to assess the levels of aggression, so that triads could be terminated immediately should excessive or damaging fights occur (which did not occur in all 20 triads). Each triad was housed in a large aluminium tank with a clear Perspex front (L x H x W: 460mm x 350mm x 300mm). These tanks contained wood shavings for bedding, grass and nesting material as described above. The tanks contained three separate nest boxes of equal size (L x H x W: 100mm x 77mm x 63.5mm) in order to prevent forced co-habituation of the triad. Triads were assumed to have formed stable social groups if they shared a nest box (as described in Schradin et al, 2010 and Schoepf and Schradin, 2012b). All 10 *R. pumilio* triads, and 9 *R. dilectus* triads (one triad was terminated at 54 days of age due to damaging fights), shared a single nest box from the second day in the triad (i.e. from age 51 days to 85 days; K-J. Hartman unpublished data, see Chapter 2).

To provide an age-matched comparison, 10 control animals per species were solitarily housed for the same duration (from 50 to 85 days of age). Control animals were housed in Labotec™ cages prepared as described above. These individuals were marked, placed in new cages, and moved to the same room as the experimental triads in synchrony with the experimental triads' protocol. Since *R. pumilio* can occur alone in nature, the solitary housing post-weaning and of controls is unlikely to be stressful (Schoepf and Schradin, 2013).

BRAIN AND BLOOD SAMPLE COLLECTION

After triads were maintained for 35 days (approximately 85 days of age) all subjects (triads and controls) were euthanased via isoflurane inhalation. Mice were confirmed to be dead by testing for a pulse, then immediately decapitated and the brain was dissected out. Once extracted, the brain was immediately weighed using a 3-place precision balance (Mettler AE160). The brains were stored at -80°C within 2-3 minutes of euthanasia to reduce the effects of protease on the neuropeptide concentrations post-mortem (Strum et al, 2010).

Blood osmolality was also measured because of its association with AVP concentration in the brain, due to the role of AVP in water regulation once in the blood (systemic circulation; Carter, 2003; Nelson, 2005). At the point of decapitation, approximately 500µL of blood was immediately collected in an Eppendorf™ tube from the trunk of the animal. Following the methods described by Schradin (2008), blood samples were kept at room temperature for 1.5 hours to allow for coagulation before being centrifuged at 8000 rpm for 10 minutes. The serum

was then pipetted into another Eppendorf™ tube and centrifuged for a further 10 minutes at 8000 rpm. Following this, the serum was then pipetted into two aliquots that were stored at -20°C. 30µL of serum was used to measure the osmolality of each animal, using an osmometer (VAPRO 5600, Wescor, USA).

NEUROPEPTIDE EXTRACTION AND ANALYSIS

No clear technique for extracting the two neuropeptides from the same sample appeared in the literature. Therefore, I developed two protocols to extract the total protein smaller than 10 kDa in size from the entire brain. Both protein extraction protocols are loosely based on the method described by Strum et al (2010), with modifications being made to better suit the resulting extract for use in a commercial enzyme-immunoassay (ELISA) kit. The first protocol used acidified methanol as the extraction buffer, as described in Strum et al (2010), while the second used a combination of half strength radioimmunoprecipitation assay (commonly known as half RIPA) buffer to lyse the cells and extract the total protein and a protease inhibitor cocktail (Sigma-Aldrich®, St. Louis, MO, USA) to ensure no degradation of the proteins occurred after the brain had thawed. Full details on the protocols used for peptide extraction are reported in Appendix 1.

The sample set, including experimental triads and control individuals, was divided evenly with half being assigned to the acidified methanol protocol's sample set and the other half to the half RIPA protocol's sample set. The brains were individually homogenised using a micro-pestle in an Eppendorf™ tube containing their respective buffer, and the resulting supernatant was filtered through a 10 kDa cut off filter and the resulting filtrate was separated into two aliquots before being lyophilised to concentrate the extract. Two separate ELISA kits were used, one specific to mouse AVP (CUSABIO Biotech Co., Ltd; CSB-E09272m) and one specific to oxytocin (Enzo Life Sciences, NY, USA; ADI-900-153), and the extract was reconstituted using the buffer supplied by the respective kits and both ELISAs were performed following the protocol described in the kit manuals. For AVP, the kit specificity was for mouse AVP from supernatant, and had no significant cross reactivity or interference, according to manual specifications. Kit sensitivity was less than 0.31 pg/ml and inter-assay precision CV was <10%, and intra-assay precision CV was <8%. Recovery was between 4.5-903%, the linearity produced a slope r-squared of 0.37. The oxytocin kit sensitivity was 11.7 pg/ml, and inter-assay CV was between 5.2-14.5% and intra-assay CV was 9.1-12.4%, according to kit specifications. Recovery was between 63-134%. The linearity slope had an r-squared value of

0.96. For both AVP and oxytocin, the low volume of samples and limited sample size prevented measures of parallelism, intra- and inter-assay CV specific to the samples was not possible. Neuropeptide concentrations are expressed as pico-gram of neuropeptide per millilitre of extract per gram of brain mass (pg/ml/g), determined by dividing the neuropeptide concentration measured by the ELISA (pg/ml) by the mass of the brain (grams).

DATA ANALYSIS

The extraction protocol using acidified methanol as the buffer failed to yield measurable results for the concentration of both AVP and oxytocin. Whether this was due to the chemical properties of how the buffer interacts with the proteins or its interaction with the ELISA kit was unclear. Thus, the data available for analysis was half that of the number of animals used in the experiments, because only the data from the protocol using the half RIPA buffer could be used (n = 5 controls per species and n = 15 experimental animals per species). From these data, outliers were identified and removed from the analysis, as their presence in the data set was preventing model convergence. Removal of outliers improved model strength and did not change the overall trends of the data. AVP and oxytocin outliers were from different individuals. Thus, AVP concentration was analysed from n = 5 controls per species, n = 14 experimental animals per species, and oxytocin concentration was analysed from n = 5 controls per species, n = 14 experimental animals for *R. pumilio*, and n=15 experimental animals for *R. dilectus*.

All data analyses were done using R© version 3.2.4 (2016, The R Foundation for Statistical Computing, Vienna, Austria) software, run through RStudio© Version 0.99.892 (2016, RStudio, Inc.). I used generalised linear mixed effect models with penalised quasilikelihood (glmmPQL; from the "MASS" library, Venables and Ripley, 2002) with Poisson error distribution to analyse the effect of species and treatment (categorical predictors), and the interaction of the two, on each neuropeptide concentration. Each neuropeptide was set as the response variable in its own model, with triad included as a random effect for both neuropeptides. Osmolality (covariate) and Litter ID (random factor) were initially included in the model but were subsequently omitted as their variance component was close to zero in the analysis for both neuropeptides (i.e. they did not contribute to the variance of both neuropeptides; osmolality is reported in appendix Table A1). Where significance was indicated by the glmmPQL, I used the general linear hypothesis and multiple comparisons (glht) function of the "Multcomp" package to run a Tukey's HSD post hoc test to identify where the difference occurred (Hothorn et al, 2008). Model fit was assessed by plotting the model residuals against

fitted values and by comparing if there was residual deviation from the best fit line (Crawley, 2009). Tests were two-tailed and alpha set at 0.05. Data are presented as mean and 95% confidence intervals.

A Spearman's Rank Order correlation was run in Dell Statistica version 12 (Dell Inc., USA) to establish whether the behaviour displayed on Day 1 of triad formation (amicable, aggressive, avoidance; see Chapter 2) and the concentrations of AVP and oxytocin (measured here) were correlated (n = 25 for AVP; n = 26 for oxytocin). The correlation between serum corticosterone (see Chapter 3) and brain AVP were also performed as these sometimes interact at the hypothalamus-pituitary-adrenal (HPA) axis (n = 38).

Results

Neither species (glmmPQL, t = -0.190, p = 0.852) nor treatment (glmmPQL, t = 1.45, p = 0.168) were significant predictors of AVP concentration. However, species x treatment was a significant predictor of AVP concentration (glmmPQL, t = -2.19, p = 0.044). *R. dilectus* in triads had higher concentrations of AVP than *R. pumilio* in triads (Tukey's HSD post hoc: $p < 0.005$; Figure 4.1). There was no significant difference between the species in the solitary controls (Tukey's HSD post hoc: p = 0.99; Figure 4.1). Neither species differed between treatments, with both triads and controls having the same concentration in *R. pumilio* (Tukey's HSD post hoc: p = 0.870) and *R. dilectus* (Tukey HSD post hoc: p = 0.388; Figure 4.1).

AVP concentration in the brain was not correlated with the frequency of amicable (Spearman R = 0.03, p = 0.9), aggressive (Spearman R = -0.04, p = 0.9), or avoidance behaviour (Spearman R = -0.3, p = 0.1) displayed on Day 1 of triad formation. AVP was not correlated with the concentration of serum corticosterone (Spearman R = 0.31, p = 0.056).

Brain oxytocin concentration was the same in both species in treatments and controls (Figure 4.2), and was not predicted by species (glmmPQL, t = -0.02, p = 0.982), treatment (glmmPQL, t = -0.45, p = 0.658), or the interaction between species x treatment (glmmPQL, t = 0.62, p = 0.544). Oxytocin concentration was not correlated with the frequency of amicable (Spearman R = 0.14, p = 0.51), aggressive (Spearman R = -0.03, p = 0.9), or avoidance behaviour (Spearman R = 0.2, p = 0.33) displayed on Day 1 of triad formation.

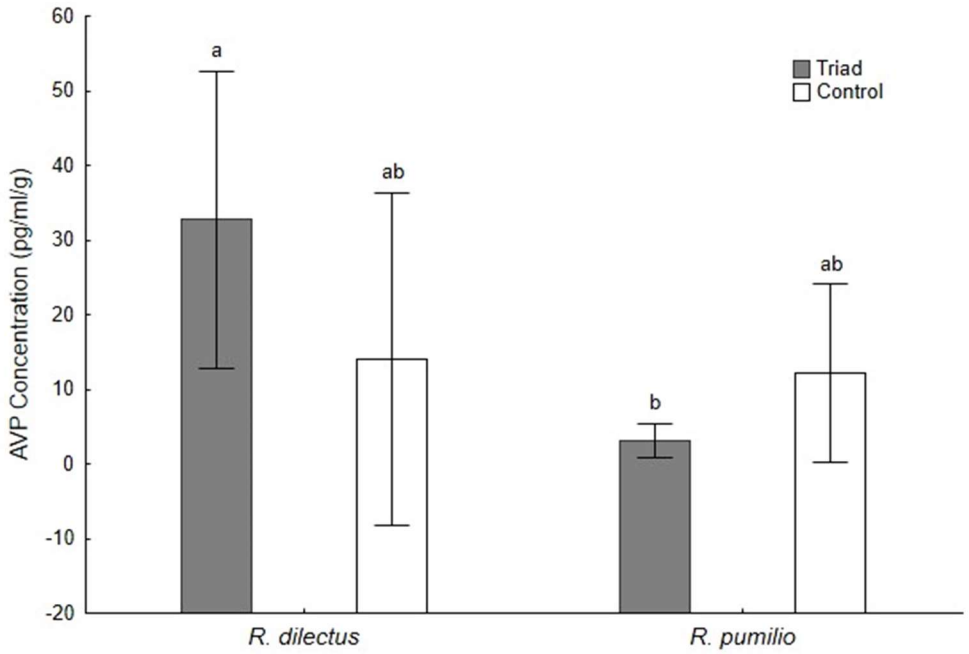


Figure 4.1. Mean and 95% confidence intervals of the concentration of AVP (pg/ml), measured per gram of brain mass, from two species of *Rhabdomys* after being housed as either experimental triads (grey columns) or solitary controls (white columns). Different letters indicate significant differences of the species x treatment interaction effect as indicated by the Tukey's HSD post hoc test (glmmPQL, t = -2.19, df = 16, p = 0.044).

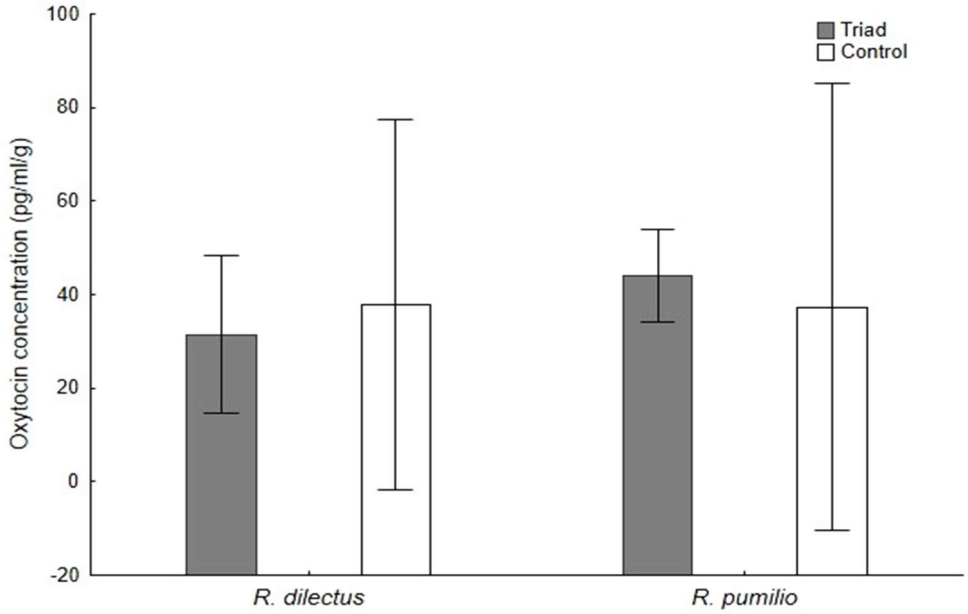


Figure 4.2. Mean and 95% confidence intervals of the concentration of oxytocin (pg/ml/g) from the whole brain in two species of *Rhabdomys*, housed as either experimental triads (grey columns) or solitary controls (white columns). There were no differences between species and treatments.

Discussion

In this study, I compared the brain concentrations of AVP and oxytocin in females of a naturally group-living species and a naturally solitary-living species of *Rhabdomys* to investigate whether these species differ in their central concentrations of these peptides in a social and isolated context. I used two novel peptide extraction techniques (acidified buffer or half RIPA buffer) to extract AVP and oxytocin from the whole brain. The half RIPA technique produced measurable data. I expected no difference in AVP concentration between the two species in a group setting. Interestingly, females of the naturally solitary-living *R. dilectus* triads had significantly higher AVP concentrations than females of *R. pumilio* triads. *R. pumilio* housed solitarily was predicted to have higher AVP than *R. dilectus* controls. Surprisingly, there was no species difference in brain AVP concentration when housed alone. I predicted higher oxytocin concentrations in triads than controls housed alone for both species. I also expected that the naturally group-living, and socially flexible, *R. pumilio* would have higher concentrations of oxytocin when housed alone than *R. dilectus* in isolation. There was no significant difference in the concentration of oxytocin in a group setting or in solitary controls between the two species, and between the triads and controls of each species. This indicates that the two species are not physiologically different, in terms of central AVP and oxytocin, outside of a social context, but do differ in how their central AVP concentrations respond to social grouping. *R. dilectus* exhibit increased AVP concentrations in the brain when in a social group while *R. pumilio* central concentrations remain unchanged whether isolated or in a group. Neither AVP nor oxytocin were correlated with the behaviours displayed on initial encounter with triad members (see Chapter 2), and AVP was not correlated to blood osmolality.

Previous studies on male *R. pumilio* demonstrated that solitary roamers had more AVP-ir cells in their PVN than group-living males (but did not differ in the density of AVP V1a receptors), indicating that solitary males have the potential to secrete more AVP (Schradin et al, 2013; Schradin et al, 2014; Schoepf et al, 2015). While direct comparisons between the studies are not possible because of the differences in techniques and quantification of AVP, it is nonetheless of interest that the sexes differed in AVP. These results potentially indicate that AVP has a different role in females compared to males of this species as there was no difference in AVP concentration when individuals were kept solitary or in a group, but further research would be required to confirm this sex differences using similar techniques used here.

This is the first time AVP concentration has been investigated in *R. dilectus*. I found that this naturally solitary species exhibited elevated AVP when in a forced social group. While

AVP is an important mediator of social memory and social recognition, it is also linked to anxiety and territoriality in mammals (including rodents and humans; Blanchard et al, 2001; Caldwell et al, 2008; McCall and Singer, 2012). The same individuals studied here had elevated corticosterone in groups (K-J. Hartman unpublished data, see Chapter 3), which potentially indicates that the elevated AVP could be stress related. A study comparing AVP V1a receptor knockout (KO) mice to normal (wild-type) mice revealed lower anxiety-related behaviour in the AVP V1a KO mice when compared with normal mice that were receptive to AVP (i.e. with functional AVP V1a receptors; Egashira et al, 2007), suggesting an important role of AVP in anxiogenesis. This elevated AVP combined with previous findings of elevated corticosterone suggests that *R. dilectus*, while able to be tolerant of group members, appears to experience physiological stress and anxiety in groups.

Group-living animals are expected to have higher oxytocin concentrations due to the frequent display of affiliative behaviours, such as allogrooming and body contact observed in grouped individuals (Carter, 2003; Lim and Young, 2006), including the striped mice in my study (K-J. Hartman, unpublished data, see Chapter 2). I found that the female *R. pumilio* and *R. dilectus* had no significant differences in oxytocin concentration, whether in groups or housed solitarily. Schoepf et al (2015) suggested oxytocin is related to sexual experience more than social behaviour in *R. pumilio*, since they found no differences in oxytocin-ir cells in breeding males (whether solitary or group-living), but these males differed to sexually inexperienced philopatric males. This could potentially explain why I found no differences in oxytocin concentration across social contexts in previously unmated females. Oxytocin also enables individuals to adapt to (or cope in) a socially novel situation (Tops et al, 2013), and thus the lack of a difference between grouped and solitarily-housed individuals may be a consequence of measuring the neuropeptide concentrations after all individuals had habituated to the group-living context after being housed in groups for 35 days. Similarly, the observed lack of a difference in oxytocin concentration could be because it is important for the initial formation of social bonds, but not necessarily for the maintenance of those bonds (Carter, 1998). Another possibility for the lack of difference is that using the whole brain rather than selected parts could have diluted differences that may have existed. This study involved comparisons of how the two species were similar or different across social contexts, and thus it is not possible to comment on whether the observed concentrations of oxytocin were notably high or low across both contexts. Nonetheless, my results add support to the idea of oxytocin

having less functional importance than AVP in regulating social behaviour in *Rhabdomys* in the long term (Schradin et al, 2013; Schoepf et al, 2015).

My results suggest that central AVP concentrations in *Rhabdomys* respond to the social environment of the individual, though it is difficult to establish whether a physiological factor is mediating observed behaviours or whether the observed behaviours regulated the release of a physiological factor (Rubenstein and Wikelski, 2005). The two species did not differ in AVP and oxytocin concentration in solitary living conditions, suggesting that neither one is physiologically primed to be either group-living or solitary, but rather respond differently to social grouping. The differences in the social systems of these two species may potentially be shaped more strongly by the extrinsic cues, such as prevailing environmental conditions, than intrinsic cues priming them to adopt the observed social tactics (Silk, 2007; Schradin et al, 2013).

In conclusion, my study is the first to measure the AVP and oxytocin concentrations in the whole brain of striped mice and to demonstrate that two socially different species of *Rhabdomys* do not differ in terms of basal brain concentrations of these neuropeptides when in isolation, but they do differ in how they respond to social grouping in terms of AVP concentration. The mediation of social behaviour by these neuropeptides is seen in many different species (reviewed in Carter et al, 2008). It is possible that the role of AVP is more important than oxytocin in regulating sociality in *Rhabdomys*. The elevated concentrations of AVP in the naturally solitary-living *R. dilectus* in imposed groups may be a response to allow for recognition of group members, but may also indicate anxiety. To better assess the role of AVP, future studies should investigate experimental manipulation of these neuropeptides (specifically AVP) and the consequent social behaviour, to clarify the role of these peptides in group formation. Future studies quantifying the density of oxytocin-ir cells and receptor distribution in females of both species would also be of value, as rodent social behaviour is a product of complex interactions of various brain regions and neurotransmitter systems within the brain allowing the appropriate response to external stimuli (Curtis et al, 2007).

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Appendices

Table A1. Osmolality values for experimental (triad) and control striped mice.

Species	Treatment	Identification	Osmolality (mmol/kg)
R. dilectus	Triad	D1-a	331.86
	Triad	D1-b	341.40
	Triad	D1-c	384.03
	Triad	D4-a	332.42
	Triad	D4-b	399.55
	Triad	D4-c	336.05
	Triad	D6-a	365.10
	Triad	D6-b	321.51
	Triad	D6-c	344.96
	Triad	D7-a	367.87
	Triad	D7-b	332.48
	Triad	D7-c	321.37
	Triad	D8-a	400.28
	Triad	D8-b	391.91
	Triad	D8-c	395.11
	Control	c11	318.96
	Control	c12	318.56
	Control	c14	318.97
	Control	c17	328.77
	Control	c18	339.03
R. pumilio	Triad	P1-a	327.83
	Triad	P1-b	349.12
	Triad	P1-c	341.15
	Triad	P7-a	329.02
	Triad	P7-b	354.80
	Triad	P7-c	331.03
	Triad	P8-a	388.32
	Triad	P8-b	399.21

	Triad	P8-c	349.91
	Triad	P9-a	384.47
	Triad	P9-b	360.58
	Triad	P9-c	398.14
	Triad	P10-a	389.64
	Triad	P10-b	348.58
	Triad	P10-c	360.63
	Control	cp21	334.06
	Control	cp23	380.12
	Control	cp24	359.01
	Control	cp28	356.67
	Control	cp30	390.84

CHAPTER 5

The association between arginine vasopressin and sociality in the African striped mouse, *Rhabdomys*

(To be submitted to Hormones and Behaviour. Editing follows the style of the thesis)

Abstract

In order for animals to be able to live in social groups, they need to be motivated to interact with conspecifics and be able to distinguish between group members and intruders. Arginine vasopressin (AVP) is a known modulator of several social interactions, including social recognition, affiliative behaviour, and social memory. In this study, I investigated the role of AVP in mediating the motivation to be social (sociability) and social recognition in two socially different species of *Rhabdomys*. This was done with the established three-chamber apparatus of testing sociability and social recognition using the social discrimination paradigm, which uses the preference for social novelty as a measure of social recognition. Ten females each of the naturally group-living *R. pumilio* and naturally solitary-living *R. dilectus* were used as the focal animals in the tests. Focal mice were sequentially injected with 20 µl of saline, 1 µg/g AVP (low AVP), or 1.5 µg/g AVP (high AVP) on separate experimental days, before being subjected to the sociability and social recognition tests. In each test, I recorded the frequency of entries to each chamber, the duration in each chamber, and the frequency of approaches toward the stimulus animals in each chamber. I also classified the type of interaction initiated by the focal animal as either amicable or aggressive. Both species displayed a propensity to be social in the sociability test, with high AVP significantly enhancing the frequency of approach toward a conspecific in both species. AVP did not affect the preference for social novelty in either species, with neither species displaying preference for social novelty or familiarity in all three treatments. Closer examination of the type of interactions elicited by the focal mice when in contact with the stimulus animals revealed opposing responses to AVP in the two species. High AVP enhanced the display of amicable behaviour toward the familiar mouse in *R. dilectus*, and enhanced amicable behaviour displayed toward the novel mouse in *R. pumilio*. My findings suggest that AVP facilitates social approach in *Rhabdomys*, but does not affect the preference for familiar or novel conspecifics. AVP also affects affiliative behaviour toward known and novel individuals in the two species differently, potentially indicating a mechanism behind the differences in sociality in these species.

Introduction

All social systems are emergent properties of the interactions between individuals within the population (Hinde, 1976). The ability to distinguish between individuals is an important determinant of how individuals respond to and interact with conspecifics (approach or avoid, display affiliative or aggressive behaviour), and the recall of previous social interactions with an individual is important for the development of social familiarity (Winslow and Insel, 2004; Choleris et al, 2009). Social recognition describes an animal's ability to distinguish between a known and unknown conspecific (familiarity) and also more complex aspects such as distinguishing between sexes, social rank in dominance hierarchies, kin, and oestrous stage to determine sexual reciprocity of females (Bluthé and Dantzer, 1990; Song et al, 2016). Social recognition and discrimination are modulated by neuroendocrine and hormonal factors. My study is concerned with the role of the nonapeptide arginine vasopressin (AVP) on social behaviour. I investigated the role of AVP rather than oxytocin, the well-known social bonding peptide, because central AVP concentrations and immunoreactive cells differ in different social contexts but this was not seen for oxytocin, and it has been proposed that oxytocin is more relevant to sexual experience than social behaviour in *R. pumilio* (Schradin et al, 2013; Schoepf et al, 2015; K-J. Hartman, unpublished data, see Chapter 4).

Synthesised in the hypothalamus, AVP is stored in the posterior pituitary gland for secretion into the blood as a hormone where it performs several physiological functions, including the regulation of water balance and blood pressure (Nelson, 2005; Leng et al, 2012). AVP also acts as a neuromodulator, traveling from the hypothalamus to a collection of brain regions referred to as the social behaviour network (SBN) that contain specialised receptors (V1a) which bind with AVP to regulate social and cognitive behaviours (Hawthorn et al, 1980; Bradford, 1986; Bielsky et al, 2004; Veenema and Neumann, 2008; Leng et al, 2012; Dumais and Veenema, 2016). AVP is known to promote affiliative behaviour, regulating many social behaviours, including social recognition and social bonding (Young, 2002; Winslow and Insel, 2004; Lim and Young, 2006; Carter et al, 2008; Albers, 2012). For example, a naturally vasopressin-deficient strain of Long-Evans rat, Brattleboro rat, lacks the ability to exhibit social recognition, but AVP administered to the brain restored social recognition (van Wimersma Greidanus, 1982; Engelmann and Landgraf, 1994).

Regulation of social behaviour by AVP is not always predictable and varies with the social organisation of a species (Insel and Young, 2000). For example, artificial increases of the AVP-homologue vasotocin (AVT) in males of the territorial field sparrow, *Spizella pusilla,*

was correlated with an increase in vocalisations and aggression, but had no effect on agonistic song frequency and a weak effect on aggression in male colonial zebra finch, *Taeniopygia guttate* (Goodson, 1998, Goodson and Adkins-Regan, 1999). Artificial increase of AVP in the monogamous male prairie vole, *Microtus ochrogaster*, which typically displays mate guarding and territorial defence, increases affiliative behaviour toward females and increases aggression toward other males, but this effect is absent in the promiscuous montane vole, *M. montanus* (Winslow et al, 1993; Stribley and Carter, 1999; Choleris et al, 2004). AVP has been demonstrated to facilitate social approach, with administration of AVP, both centrally to specific brain regions and peripherally into systemic circulation, increasing the duration of social engagement, olfactory investigation and huddling with conspecifics in prairie voles, *M. ochrogaster* (Cho et al, 1999; Young et al, 1999), and laboratory rats (Dantzer et al, 1987; Bluthé and Dantzer, 1990; Winslow and Insel, 2004; Albers, 2012).

Species with different social organisations differ in neuropeptide synthesis, release, and receptor distribution in the SBN (Insel and Young, 2000; Veenema and Neumann, 2008). These differences and the variation in behavioural response to AVP suggest that social organisation may have an underlying physiological mechanism priming species to be either social or solitary, promoting or preventing social approach or avoidance (Carter et al, 2008; Veenema and Neumann, 2008). An animal must be socially motivated to approach a conspecific before a social bond can form (Lim and Young, 2006). This motivation is mediated by AVP and its sister nonapeptide oxytocin (reviewed by Lim and Young, 2006; and Carter et al, 2008). Here, I test the role of AVP in the sociability (i.e. animal's propensity to spend time with an unfamiliar conspecific rather than remaining alone; Moy et al, 2004; Lim and Young, 2006) and the social recognition (i.e. distinguishing between familiar and unfamiliar conspecifics) ability of two species of the African striped mouse, *Rhabdomys*, to test whether a facultatively group-living species, *R. pumilio*, is primed to be social when compared to a naturally solitary-living sister species, *R. dilectus* (Rambau et al, 2003).

In the xeric Nama-Karoo of Southern Africa, *R. pumilio* typically occurs in social groups consisting of one breeding male, with two to four breeding females and their philopatric offspring (Schradin and Pillay, 2003; Schradin and Pillay, 2004). Group members share a nest and defend a shared territory, interact amicably and bask together at dawn and dusk, but forage solitarily during the day (Schradin, 2005). This population of *R. pumilio* switches from group-living to solitary-living during the breeding season under low population densities, assuming a social system of solitary breeding females and males occurring in the same area (Schradin and

Pillay, 2006; Schradin et al, 2009; Schradin et al, 2010; Hill et al, 2015). Captive and field studies revealed that solitary males have higher densities of AVP immunoreactive (ir) cells in the brain than males adopting a group-living (philopatric or breeder) tactic (Schradin et al, 2013; Schoepf et al, 2015), but males adopting these different tactics had the same central expression and distribution of AVP V1a receptors, indicating that they would have a similar response to AVP secretion (Schradin et al, 2014). Groups are initially formed by females which are later joined by males to create a breeding group (Schradin and Pillay, 2003), and thus the neuroendocrine correlates of female social behaviour needs investigation. In captivity, female *R. pumilio* do not differ in central concentration of AVP in both imposed group-living and solitary-living (K-J. Hartman, unpublished data; see Chapter 4).

R. dilectus occurs in the mesic grassland areas of South Africa, is solitary-living, and defends intersexually overlapping individual territories (Rambau et al, 2003; Schradin and Pillay, 2005). Females interact with males only during the breeding season, but raise offspring alone (Schradin and Pillay, 2003). This species has not been studied as extensively as *R. pumilio* in terms of brain physiology and its behavioural correlates. A previous study demonstrated that *R. dilectus* has the ability to form social groups when group-living is imposed, but group housed females had significantly higher concentrations of AVP in the brain than that of *R. pumilio* females (K-J. Hartman, unpublished data, see Chapter 4). This implies a potential difference exists in how AVP modulates social behaviour and sociability between the species.

I aimed to investigate the role of experimentally administered AVP on sociability and social recognition in females of two *Rhabdomys* species, using the sociability test and the social discrimination paradigm, respectively (Engelmann et al, 1995; Ferguson et al, 2002; Choleris et al, 2009). In these tests, a focal female was presented initially with one conspecific stimulus female (sociability) test and next with another conspecific stimulus female (i.e. the first familiar female and second unfamiliar female). Focal females were subjected to three treatments on different days, and were injected sequentially with saline (control), a low dose of AVP, or a high dose of AVP to examine whether AVP affects the behavioural response in both species. Synthetic AVP has not been administered to *Rhabdomys* previously, and thus the two doses were administered in sequence of low to high dose as a precaution against possible over-dose, and to allow for identification of a possible dose response. From results of earlier experiments (K-J. Hartman, unpublished data, see Chapter 4), I predicted that AVP would increase the social motivation in *R. dilectus*, expressed as more time spent with (i.e. sociability), and more

approaches to, an unfamiliar mouse compared to an empty chamber, but no difference was predicted for *R. pumilio* across treatments. I further expected that AVP would not affect the preference of social novelty in *R. pumilio*, but AVP would reduce the preference for social novelty in *R. dilectus* (higher affinity for familiarity).

Pearson et al (2010) recommended the inclusion of measures of aggression and amicability in the sociability and social recognition protocols to better assess the motivation of the focal animal to investigate the stimulus animals. I predicted that *R. pumilio* would be more aggressive toward the stimulus animals than *R. dilectus* in both tests, as they were more aggressive in a previous study (K-J. Hartman, unpublished data, see Chapter 2). Amicable behaviour increased with duration of group formation (Chapter 2), which was accompanied by higher brain AVP concentrations in *R. dilectus* (K-J. Hartman, unpublished data, see Chapter 4). Thus, AVP was not expected to affect aggression or amicability in *R. pumilio*, but I predicted AVP to reduce aggressive behaviour and increase amicable behaviour in *R. dilectus*.

Materials and methods

STUDY SPECIES

Rhabdomys is a genus of diurnal, murid rodents. Two closely related species of *Rhabdomys* were considered in this study: the facultatively group-living *R. pumilio*, and solitary-living *R. dilectus dilectus* (referred to as *R. dilectus*; Rambau et al, 2003; Schradin and Pillay, 2005). Individuals of each species were either F2 or F3 captive born from a captive colony maintained at the Milner Park Animal Unit at the University of the Witwatersrand, Johannesburg, South Africa. The *R. pumilio* mice originated from the semi-arid Nama Karoo (160mm rainfall per annum), and *R. dilectus* in the colony originated from the moist grassland region of South Africa (receives 700-1000mm of rainfall per annum; Skinner and Chimimba, 2005).

HUSBANDRY

This study was conducted in the Milner Park Animal Unit at the University of the Witwatersrand. Subjects were housed and reared under partially controlled environmental conditions, with a light:dark cycle of 14:10. A total of 60 virgin adult (older than 75 days of age) female striped mice from the captive colony were used for this study, comprising of 30 *R. pumilio* and 30 *R. dilectus*. All mice were housed solitarily in Lab-o-tec home cages (L x H x W: 425mm x 150mm x 240mm) from 25 days of age and throughout the study. Home cages

included ±3cm wood shavings, a handful of *Eragrostis* grass (± 20g) and ± 5g of tissue paper, provided as nesting material, a cardboard tube (L x Diameter: 99mm x 38mm), and a small piece of wood for gnawing as enrichment. All home cages were cleaned weekly with F10®SC disinfectant soap (Health and Hygiene (Pty) Ltd) and water, and air dried. Mice were provided with 5g Epol mouse cubes, 5g fresh vegetables and 2g millet daily, with water being available *ad libitum*.

Ten females of each species were randomly selected to be the focal individuals. The remaining 20 individuals per species were used as stimuli, and all were unrelated and unfamiliar to the focal individuals. Stimulus animals were categorised into weight matched pairs for use in the experiments, to avoid variation in the size of a stimulus animal affecting the responses of the focal animal. Focal animals were housed in a separate room to the stimulus animals throughout the study period. This study was approved by the animal ethics screening committee of the University of the Witwatersrand, AESC number: 2014/34/B.

EXPERIMENTAL DESIGN

Apparatus

The social behaviour testing apparatus used was adapted from Moy et al (2004) and Crawley et al (2007), consisting of a glass aquarium/tank (L x H x W: 600mm x 400mm x 300mm) divided into three zones/chambers by two clear flexiglass barriers (Figure 5.1), with wood shavings on the floor. The middle area (referred to as "neutral zone") was the largest (260mm wide), while the two outer "unfamiliar zones" measured 170mm wide each. A 60mm x 60mm archway allowed the focal animal to move freely between the three areas of the experimental cage; archways (chamber entrances) were positioned in the front of the tank and could be opened or closed by the experimenter. The two test areas (unfamiliar zones) contained a mesh holding cage (L x H x W: 130mm x 105mm x 95mm) which was covered on top by a small white tile to block interaction between the mice from above the cage, and to weigh the cage down. The holding cages were positioned in the section of the chamber furthest from the chamber entrance (Figure 5.1). This mesh holding cage was used to house the unfamiliar/stimulus individuals during trials to avoid the risk of damaging fights between individuals and to control for the focal animal being the only animal able to initiate interactions.

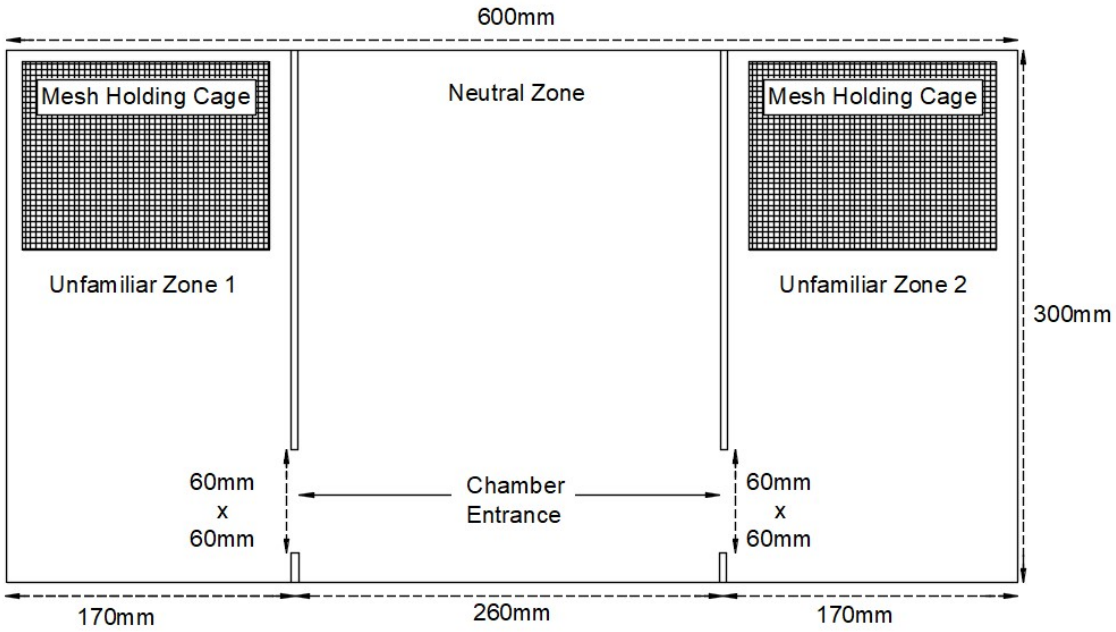


Figure 5.1. The three-chambered test apparatus as viewed from above, with dimensions. The unfamiliar zones are the test area/chambers that contained the stimulus mice within mesh holding cages. White tiles above holding cages are omitted from this image. Zones were divided by 3mm thick clear flexiglass barriers with breathing holes.

The focal animal was placed in the experimental tank between 13h00 and 15h00 the day before experiments to allow it to habituate to the tank, and was provided with 5g mouse cubes, 2g sunflower seeds, water, and a nest box. During the habituation period, the two empty mesh holding cages were present in their respective zones. This habituation period was included to ensure that the social stimulus was the only novel stimulus in the set up during the experimental trial, avoiding the potential for the measures of sociability and social recognition being confounded by the focal mouse's investigation of a novel set-up. All experimental trials were done between 09h00 – 12h00 when striped mice are most active (Pillay, 2000). The behaviour of the focal mouse was filmed from above the tank through a clear flexiglass lid using Sony Handycam® video cameras. No human observers were in the room during recording.

Each focal animal underwent 3 treatments over a 5-day period, being injected subcutaneously, into the "scruff" of the neck, on Day 1, Day 3, and Day 5 sequentially with 20µl saline, 1 µg/g AVP (referred to as low AVP), or 1.5 µg/g AVP (referred to as high AVP) respectively. The treatments were administered in this sequence as a precaution of exposing individuals too soon to high dose AVP, particularly since AVP had never been administered to *Rhabdomys* previously. This allowed me to establish whether any dose responses or threshold concentration of AVP exist without jeopardising the data collection for this study. These

dosages were selected based on research done by Schaller et al (2010) on murine mice and the naturally occurring plasma AVP levels recorded in field studies of *Rhabdomys* (Schoepf and Schradin, 2014). Each focal female had a minimum of one day rest period between each treatment. AVP injected subcutaneously has a half-life of 2 to 8 hours and was assumed to be fully eliminated from the mice before the subsequent experimental day. Animals were restrained and not anesthetised before injections were administered, as this type of injection is not painful for the animal and a small dose volume was used (as per guidelines of Boston University IACUC, 2014). Subcutaneous injection of AVP has elicited behavioural responses in previous studies (reviewed by Ferguson et al, 2002), and thus was expected to cross the blood brain barrier in sufficient amounts to act on central V1a receptors.

Sociability and social recognition

The three-chambered testing arena allowed me to measure sociability and social novelty. Sociability is used to quantify social motivation by comparing the duration the focal animal spends with a stimulus animal rather than in an empty chamber (Engelmann et al, 1995; Young, 2002; Moy et al, 2004; Choleris et al, 2009). Social recognition is most sensitively measured using the social discrimination paradigm (Choleris et al, 2009), where the focal animal's ability to recognise conspecifics is tested by introducing a novel stimulus animal into the once empty chamber (in the prior sociability test) simultaneously with the previously unfamiliar (now known) stimulus animal, giving the focal animal the choice between the two (Engelmann et al, 1995; Ferguson et al, 2002; Bielsky and Young, 2004; Moy et al, 2004). Because the focal animal is the only animal in the apparatus able to initiate interactions, this type of sociability and social recognition test has been used in several studies on rodent species to test the effect of administered neuropeptides and certain hormones on sociability and social recognition (reviewed by Bielsky and Young, 2004; Choleris et al, 2009).

Sociability test

On each experimental day, the subjects were tested as follows: following a 5 minute habituation to the lighting and video camera in the filming room, the subject was injected with either saline or synthetic AVP, as described above. The focal female was returned to the experimental tank, where the food, water bottle, and nest box had been removed, and was allowed 10 minutes to settle down after the injection and handling. The focal female was then contained in the middle neutral zone by blocking the chamber entrances with the holding cage cover tiles and a stimulus

animal (referred to as unfamiliar 1) added to a holding cage in one of the two outer zones, which was randomly selected for each trial. The entrances were opened and exploration and behavioural interactions of the focal female with the stimulus female was video-recorded for 10 minutes. The frequency of entries to each chamber, duration in each chamber, frequency of approaches to the stimulus animal or empty cage, duration in amicable contact with the stimulus mouse, and duration of aggression toward the stimulus animal (definitions of all behaviours are in Table 5.1) were later scored from the video footage using The Observer (version 9.0, Noldus, Netherlands) software.

Table 5.1. The behaviours scored from video footage in the sociability and social novelty preference tests

Behaviour Scored	Definition
Enter chamber	Focal female's head and forelimbs crossed the chamber entrance leading into the chamber, with the head facing the chamber. The duration in chamber was recorded from the instant the focal mouse entered the chamber and was terminated when the focal mouse entered an adjoining chamber.
Approach	Focal female moved toward the holding cage, within less than half a body length of the holding cage. Focal female had to be moving in and facing the holding cage's direction.
Amicable contact	Focal female in body contact with the holding cage, with no aggression displayed. Included resting against the cage, sniffing the stimulus mouse, any amicable interactions with the stimulus mouse through the cage.
Aggressive contact	Focal female lunged toward, lifted, bit or pulled on the holding cage containing the stimulus mouse.

Social novelty preference test

Directly after the 10-minute sociability test, each focal mouse was tested for social novelty preference, or the preference to interact with a new unfamiliar, as a measure of social recognition (i.e. social discrimination; Engelmann et al, 1995). The focal female was coaxed into the central neutral zone and the entrances blocked off. A second, novel, stimulus animal,

(referred to as unfamiliar 2) was added to the other holding cage in the previously empty chamber. The doors were opened simultaneously, allowing the focal female access to both stimulus mice, and the exploration and social interactions were recoded for 10 minutes. The frequency of entries to each chamber, duration in each chamber, the frequency of approaches toward each stimulus animal, duration in amicable contact with each stimulus animal, and duration of aggression displayed toward each stimulus mouse were scored from the video footage as described above (Table 5.1). Following this 10-minute trial all three females were then returned to their respective home cages. Between trials, the experimental equipment was cleaned with F10®SC disinfectant soap (Health and Hygiene (Pty) Ltd) and water, then air dried, to remove the scent of all other mice from the apparatus.

Identification of oestrous stage

To account for potential oestrous stage effects on behavioural responses (Mora et al, 1996), vaginal swabs were taken from the focal and stimulus individuals after experiments were complete on each day of testing. Swabs were always collected between 11h30 and 12h30 on experimental days. Swabs were done by restraining the mouse by hand while gently inserting a cotton swab into the vagina using a rolling motion. The swab was then rolled on a clean glass slide and the smear allowed to dry before viewing under a compound light microscope. The smears were examined at 100x magnification, where the oestrous cycle phase was determined by identifying the proportions of leukocytes, nucleated epithelial cells, and anucleated cornified epithelial cells present in the dried, unstained smear. Four stages were identified: 1) oestrous (predominantly cornified epithelial cells), 2) met-oestrous (equal proportions of leukocytes, cornified epithelial cells, and nucleated epithelial cells), 3) di-oestrous (predominantly leukocytes), and 4) pro-oestrous (predominantly nucleated epithelial cells). The identification of oestrous cycle phases from cytology was based on Bennett (1999), Marcondes et al (2002), and Byers et al (2012).

DATA ANALYSIS

The variables of interest (response variables) were the frequency of entries to each chamber, duration spent in each chamber, frequency of approaches to the stimulus animal's holding cage, and duration of amicable or aggressive contact with the stimulus animals. Each response variable was analysed using a separate mixed model repeated measures analysis of variance (ANOVA), considering variance between and within species and treatments, for both the

sociability and social novelty preference tests. Data were analysed in R© version 3.2.4 (2016, The R Foundation for Statistical Computing, Vienna, Austria) software, using the car (Fox and Weisberg, 2011) and lmerTest (Kuznetsova et al, 2016) libraries. Since the duration in the three chambers summed to 10 minutes, the data collected for the behaviours of the focal mouse in each chamber (unfamiliar 1 chamber, neutral central zone, or unfamiliar 2/empty chamber) were mutually exclusive measures, and thus statistical analyses include data collected in the side chambers only (the neutral central zone was excluded). In the sociability test, duration in each chamber and frequency of entries and approaches to the holding cage were set as response variables in three separate models, chamber (unfamiliar mouse or empty), treatment (saline, low AVP, or high AVP), and species were set as categorical predictors, treatment was set as the within-subjects factor, and focal mouse ID was a random effect. The analysis of duration of amicable and aggressive contact in the sociability test considered only the chamber containing the stimulus mouse, and thus treatment (saline, low AVP, or high AVP), and species were set as categorical predictors with treatment set as the within-subjects factor, and focal mouse's ID was set as a random effect. In the social novelty preference test, the total duration in each chamber, frequency of entries to a chamber, frequency of approaches to holding cage, duration in amicable contact with the cage of the stimulus mice, and duration in aggressive contact with the cage of the stimulus mouse were the response variables in five separate models, with chamber (familiar or novel mouse), treatment (saline, low AVP, or high AVP), and species set as categorical predictors, treatment was set as the within-subjects factor, and focal mouse ID was set as a random effect.

The behaviours displayed and duration in the chambers were not affected by the oestrous stage of the focal or stimulus mice, the body mass of the focal or stimulus mice, and the side of the chamber (i.e. randomly assigned chamber) containing the stimulus mice for both the sociability and social novelty preference test, as revealed by a Spearman's Rank Order correlation, run in Dell Statistica version 12 (Dell Inc., USA; 2.5% of weakly correlated variables were recorded and were omitted from the analysis, see tables A1, A2 and A3 in chapter appendices). Therefore, none of these random effects were considered in the models described above.

Models were validated by comparing the residuals to the fitted values and qqnorm plots (Crawley, 2009). Model significance was set at $p > 0.05$ for all tests. Where significant predictors were indicated, post hoc tests were run by comparing the difference of least square means. All data are presented as mean ± 95 % confidence intervals.

Results

SOCIABILITY TEST

Duration in chamber, frequency of entries to chambers and approaches to stimulus animal

Females of both species showed a significant proclivity for social contact with a conspecific rather than being in the empty chamber (Table 5.2). The frequency of entries to a chamber was significantly predicted by treatment, but not predicted by species, chamber, or any interactions of the three categorical predictors (Table 5.2). Focal animals entered the chambers more frequently when given the high AVP treatment than the saline treatment (Figure 5.2).

Chamber (containing unfamiliar female or empty) was a significant predictor of the duration spent in each chamber in the sociability test, but there were no species, treatment, or interaction effects (Table 5.2). Overall, the focal animals spent more time in the chamber containing the stimulus animal than in the empty chamber (Figure 5.3).

Treatment and chamber both significantly predicted the frequency of approaches of the focal mouse to the stimulus mouse or empty cage (Table 5.2). Neither the species, nor any interaction of the three categorical predictors were significant predictors. Overall, the focal mouse approached the holding cage containing the stimulus mouse more frequently than the empty holding cage, and the saline treatment produced lower frequencies of approach than both AVP treatments (Figure 5.4).

Behaviour toward unfamiliar stimulus mouse in the sociability test

Neither the species nor treatment predicted the duration of aggressive or amicable behaviour of the focal mouse toward the unfamiliar stimulus mouse (Table 5.2, see Figure 5.5 A and B). The interaction of species x treatment was also not a significant predictor of aggressive or amicable behaviour.

Table 5.2. The output of the mixed model repeated measures ANOVA analysis for the sociability test, comparing the behaviour of females from two species of *Rhabdomys* after being treated with saline, 1 µg/g AVP, and 1.5 µg/g AVP injected subcutaneously. Predictors and test statistics in bold indicate significant predictors of the respective response variable. Statistics = Type II Wald χ^2 Test

Response variable	Predictors	Statistics
Frequency of entries to chamber	Species	$\chi^2_1 = 0.12$; p = 0.731
	Treatment	**$\chi^2_2 = 6.18$; p = 0.046**
	Chamber	$\chi^2_1 = 0.31$; p = 0.580
	Species*Treatment	$\chi^2_2 = 2.64$; p = 0.267
	Species*Chamber	$\chi^2_1 = 0.03$; p = 0.867
	Treatment*Chamber	$\chi^2_2 = 3.90$; p = 0.142
	Species*Treatment*Chamber	$\chi^2_2 = 1.22$; p = 0.544
Total duration in chamber	Species	$\chi^2_1 = 0.18$; p = 0.671
	Treatment	$\chi^2_2 = 0.25$; p = 0.884
	Chamber	**$\chi^2_1 = 7.64$; p = 0.006**
	Species*Treatment	$\chi^2_2 = 0.41$; p = 0.815
	Species*Chamber	$\chi^2_1 = 0.86$; p = 0.354
	Treatment*Chamber	$\chi^2_2 = 3.66$; p = 0.161
	Species*Treatment*Chamber	$\chi^2_2 = 1.86$; p = 0.394
Frequency of approaches	Species	$\chi^2_1 = 0.22$; p = 0.642
	Treatment	**$\chi^2_2 = 13.72$; p = 0.001**
	Chamber	**$\chi^2_1 = 14.09$; p < 0.001**
	Species*Treatment	$\chi^2_2 = 1.31$; p = 0.518
	Species*Chamber	$\chi^2_1 = 0.29$; p = 0.589
	Treatment*Chamber	$\chi^2_2 = 2.44$; p = 0.296
	Species*Treatment*Chamber	$\chi^2_2 = 1.38$; p = 0.501
Duration in aggressive contact	Species	$\chi^2_1 = 0.82$; p = 0.365
	Treatment	$\chi^2_2 = 2.83$; p = 0.243
	Species*Treatment	$\chi^2_2 = 3.74$; p = 0.154
Duration in amicable contact	Species	$\chi^2_1 = 1.29$; p = 0.255
	Treatment	$\chi^2_2 = 1.55$; p = 0.460
	Species*Treatment	$\chi^2_2 = 0.26$; p = 0.879

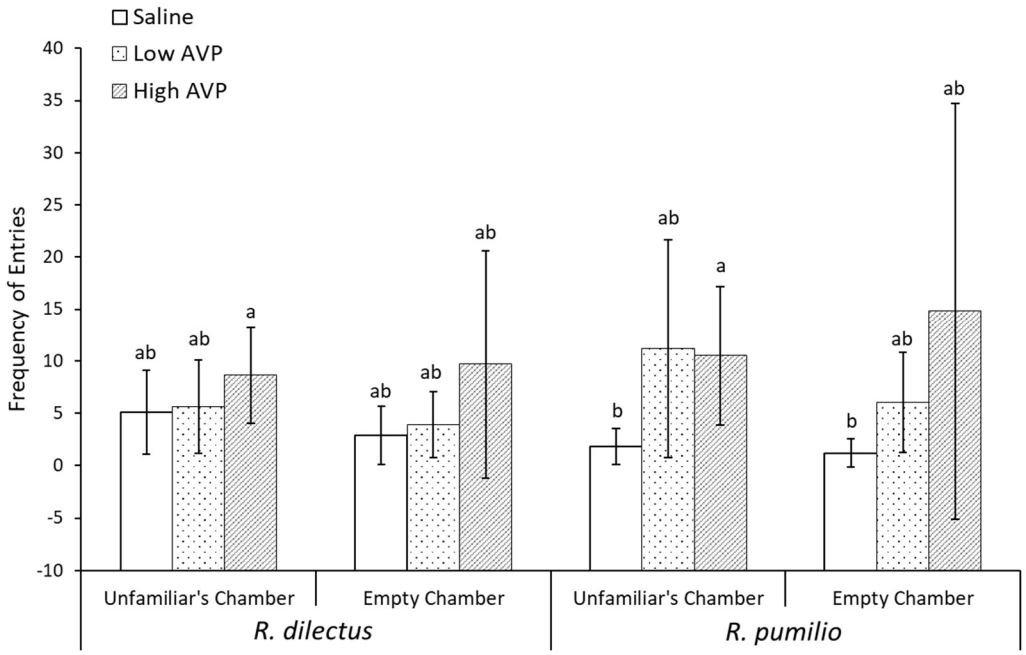


Figure 5.2. Mean frequency of entries into each chamber in three treatments (saline (white bars), low dose of AVP (dotted bars), and high dose of AVP (bar with diagonal lines) injected subcutaneously) in the sociability test, comparing females of two species of *Rhabdomys.* "Unfamiliar's chamber" was the chamber containing the stimulus mouse in a holding cage, and "empty chamber" contained an empty holding cage. Error bars represent the 95% confidence intervals. Treatment was a significant predictor of the frequency of entries. Different letters denote where significant differences were found ($p < 0.05$).

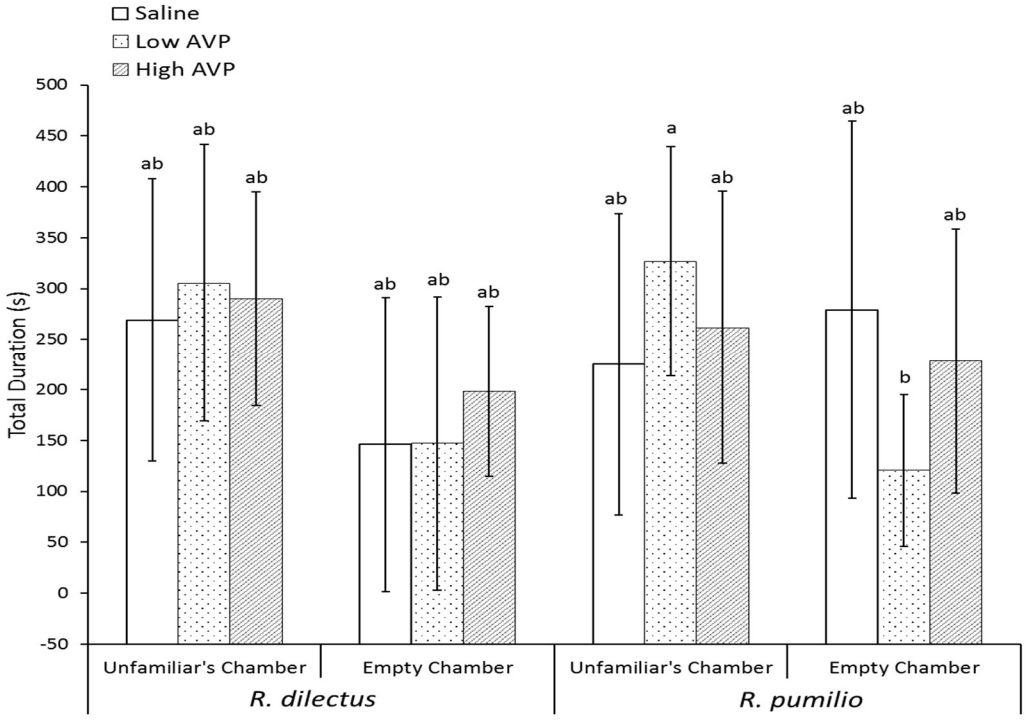


Figure 5.3. Mean total duration in each chamber in the sociability test, compared between two species of *Rhabdomys* after being treated with saline (white bars), low dose of AVP (dotted bars), and high dose of AVP (bar with diagonal lines) injected subcutaneously. "Unfamiliar's chamber" was the chamber containing the stimulus mouse in a holding cage, and "empty chamber" contained an empty holding cage. Error bars represent 95% confidence intervals. Chamber was a significant predictor of duration. Different letters denote significant differences as indicated in post hoc test ($p < 0.05$).

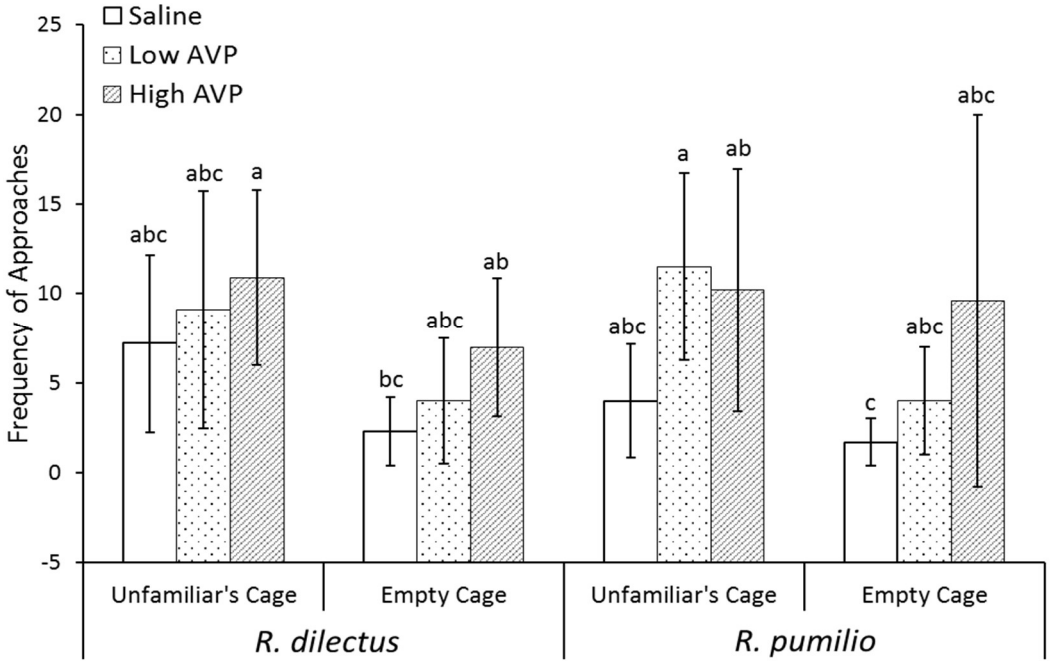


Figure 5.4. Mean frequency of approaches to stimulus mouse or empty holding cage across three treatments (saline (white bars), a low dose of AVP (dotted bars), or a high dose of AVP (bar with diagonal lines) injected subcutaneously) in the sociability test, comparing females of two species of *Rhabdomys*. Error bars represent the 95% confidence intervals. Treatment and chamber were significant predictors of approach. Different letters denote where significant differences were found ($p < 0.05$).

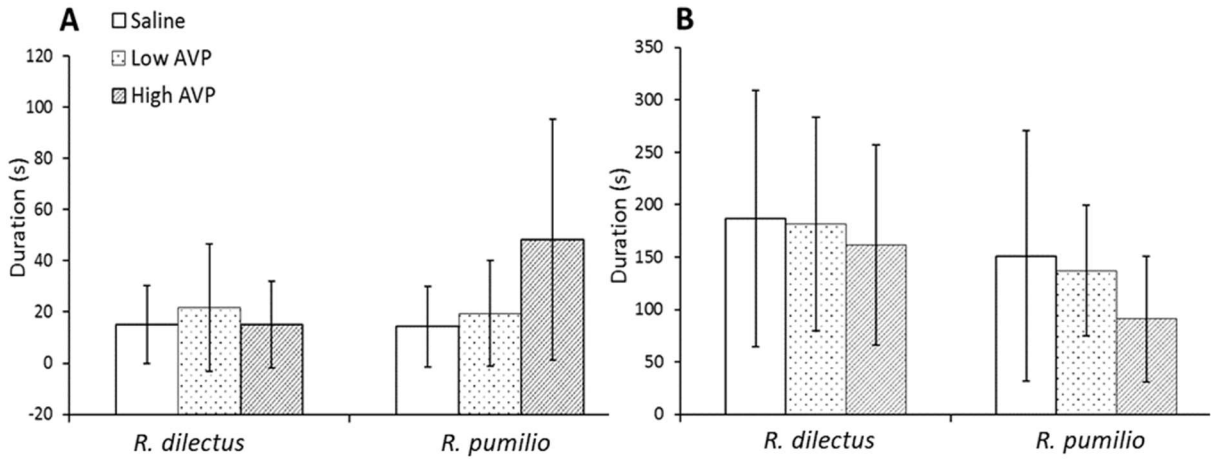


Figure 5.5. Behavioural responses to an unfamiliar stimulus mouse in the sociability test by focal females of two species of *Rhabdomys* after being treated with either saline (white bars), a low dose of AVP (dotted bars), and a high dose of AVP (bar with diagonal lines) injected subcutaneously. Mean duration of the duration of aggressive behaviour (A) and amicable behaviour (B) are indicated by the columns. The error bars represent 95% confidence intervals. No significant differences were found for species, treatment, and the interaction of species x treatment.

SOCIAL NOVELTY PREFERENCE TEST

Duration in chamber, frequency of entries into each chamber and approaches to stimulus mice

Both species did not show a preference for social novelty. The frequency of entries to the chambers was not significantly predicted by species, treatment, the chamber itself, or any interaction of the three categorical predictors (Table 5.3). Focal mice entered both the novel and familiar mouse's chambers the same number of times for all treatments (Figure 5.6). Likewise, the duration spent in each chamber was not significantly predicted by species, treatment, or chamber (familiar or novel mouse's chamber; Table 5.3), nor by any interaction of the three categorical predictors. The duration in each stimulus mouse's chamber was similar in all treatments for both species (Figure 5.7).

Species, treatment, and chamber were not significant predictors of the frequency of approaches toward either the familiar stimulus mouse or the novel stimulus mouse, although treatment did tend towards significance (Table 5.3). No interactions of the three categorical predictors were significant. Focal animals approached both stimulus animals (familiar and novel) the same number of times, regardless of treatment or species (Figure 5.8).

Behaviour toward the two stimulus mice in the social novelty preference test

The behaviour toward the stimulus animal differed between the familiar and novel mouse. Aggressive behaviour toward the stimulus mice was significantly predicted by the chamber, but not by the species or treatment or any interaction of the fixed terms (Table 5.3.). Focal mice displayed aggression for a longer duration toward the novel mouse than the familiar mouse (Figure 5.9).

The duration of amicable contact with the stimulus mouse was significantly predicted by the interaction of species x treatment x chamber, but not by any of the categorical predictors at the first level (see Table 5.3). It is interesting to note that the two species respond in an opposite manner to each other in the treatments in expressing amicability (Figure 5.10). *R. dilectus* spent more time in amicable contact with the familiar stimulus mouse than *R. pumilio* did with the familiar stimulus mouse under the high AVP treatment. In contrast, *R. pumilio* expressed more amicable behaviour toward the novel mouse in this treatment (Figure 5.10).

Table 5.3. The output of the mixed model repeated measures ANOVA analysis for the social novelty preference test, comparing the behaviour of females from two species of *Rhabdomys* after being treated with saline, 1 µg/g AVP, and 1.5 µg/g AVP injected subcutaneously. Predictors and test statistics in bold indicate significant predictors of the respective response variable. Statistics = Type II Wald χ^2 Test

Response variable	Predictors	Statistics
Frequency of entries to chamber	Species	$\chi^2_1 = 0.06$; p = 0.803
	Treatment	$\chi^2_2 = 0.94$; p = 0.624
	Chamber	$\chi^2_1 = 0.68$; p = 0.410
	Species*Treatment	$\chi^2_2 = 3.10$; p = 0.212
	Species*Chamber	$\chi^2_1 = 0.68$; p = 0.410
	Treatment*Chamber	$\chi^2_2 = 2.05$; p = 0.359
	Species*Treatment*Chamber	$\chi^2_2 = 2.28$; p = 0.321
Total duration in chamber	Species	$\chi^2_1 = 0.04$; p = 0.838
	Treatment	$\chi^2_2 = 0.33$; p = 0.846
	Chamber	$\chi^2_1 = 0.04$; p = 0.837
	Species*Treatment	$\chi^2_2 = 0.33$; p = 0.847
	Species*Chamber	$\chi^2_1 = 2.27$; p = 0.132
	Treatment*Chamber	$\chi^2_2 = 1.00$; p = 0.605
	Species*Treatment*Chamber	$\chi^2_2 = 4.96$; p = 0.084
Frequency of approaches	Species	$\chi^2_1 = 0.68$; p = 0.408
	Treatment	$\chi^2_2 = 5.21$; p = 0.074*
	Chamber	$\chi^2_1 = 1.37$; p = 0.242
	Species*Treatment	$\chi^2_2 = 4.73$; p = 0.094
	Species*Chamber	$\chi^2_1 = 0.03$; p = 0.867
	Treatment*Chamber	$\chi^2_2 = 3.11$; p = 0.211
	Species*Treatment*Chamber	$\chi^2_2 = 0.86$; p = 0.649
Duration in aggressive contact	Species	$\chi^2_1 = 0.24$; p = 0.624
	Treatment	$\chi^2_2 = 1.02$; p = 0.601
	Chamber	**$\chi^2_1 = 4.07$; p = 0.044**
	Species*Treatment	$\chi^2_2 = 0.93$; p = 0.629
	Species*Chamber	$\chi^2_1 = 0.72$; p = 0.398
	Treatment*Chamber	$\chi^2_2 = 0.80$; p = 0.669
	Species*Treatment*Chamber	$\chi^2_2 = 2.70$; p = 0.259
Duration in amicable contact	Species	$\chi^2_1 = 2.26$; p = 0.133
	Treatment	$\chi^2_2 = 0.46$; p = 0.793
	Chamber	$\chi^2_1 = 1.10$; p = 0.294
	Species*Treatment	$\chi^2_2 = 1.00$; p = 0.606
	Species*Chamber	$\chi^2_1 = 0.46$; p = 0.498
	Treatment*Chamber	$\chi^2_2 = 0.21$; p = 0.899
	Species*Treatment*Chamber	**$\chi^2_2 = 8.89$; p = 0.012**

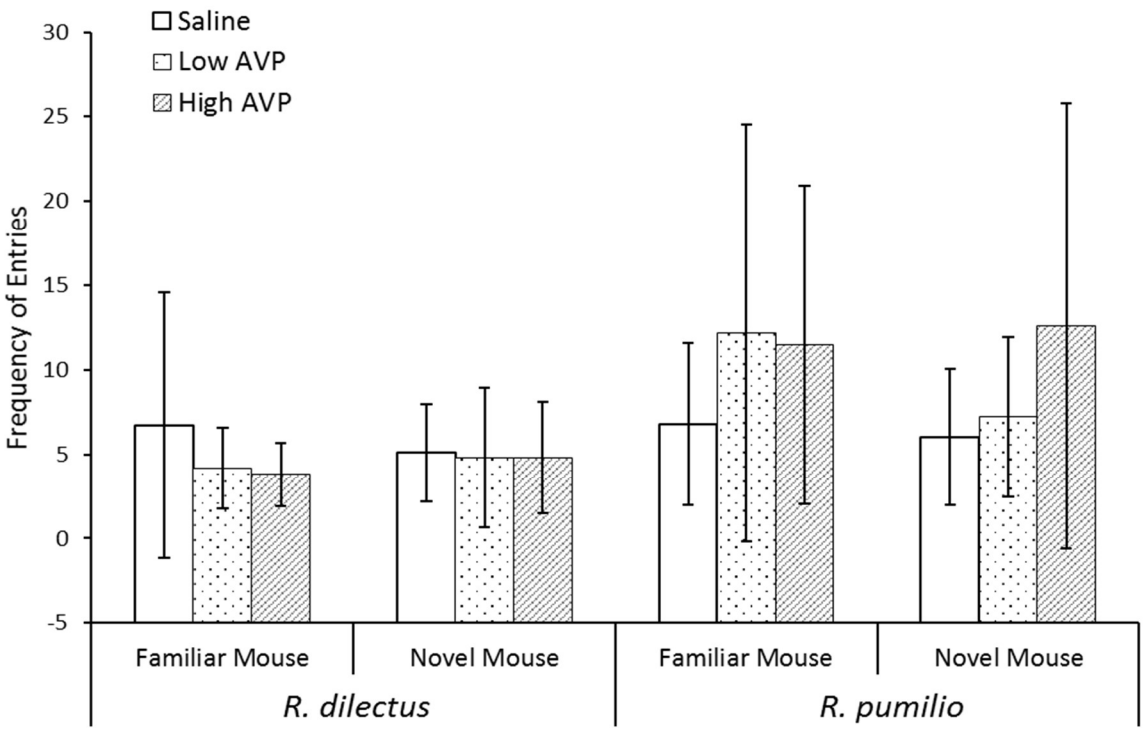


Figure 5.6. Mean frequency of entries into chambers containing a familiar and novel stimulus mouse in the social novelty preference test by focal females of two species of *Rhabdomys* in three treatments (saline (white bars), low dose of AVP (dotted bars), and a high dose of AVP (bar with diagonal lines) injected subcutaneously). Error bars represent 95% confidence intervals. No significant effects were found for species, treatments, chambers, or any interaction of the three.

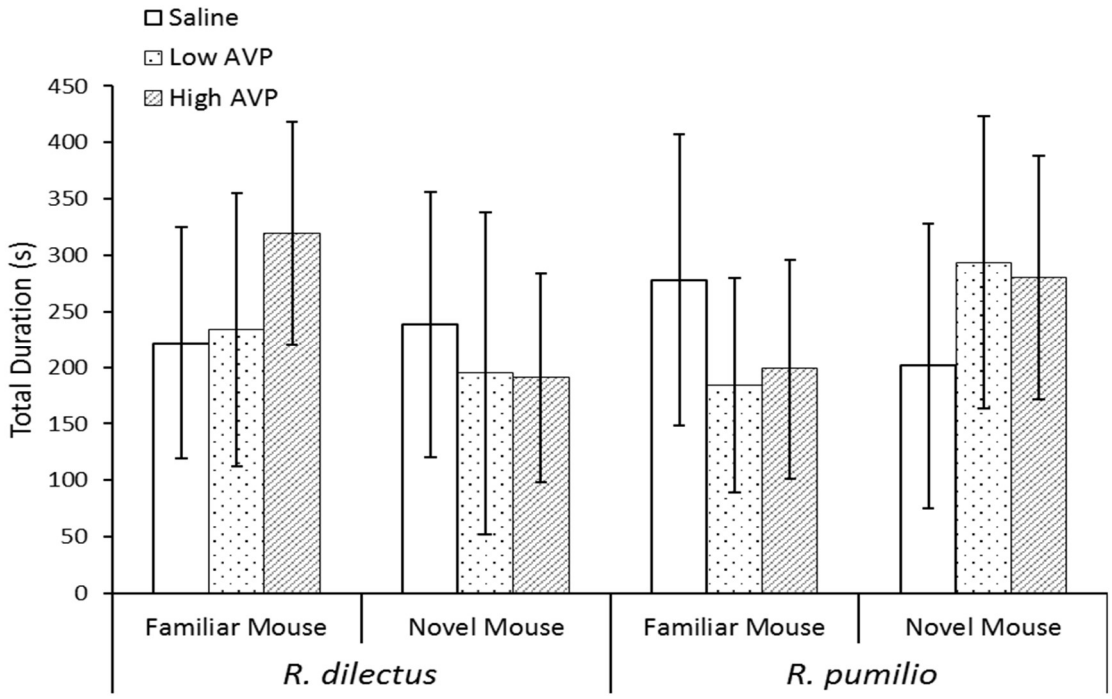


Figure 5.7. Mean total duration in chambers containing a familiar and novel stimulus mouse in the social novelty preference test, compared between two species of *Rhabdomys* in three treatments (saline (white bars), low dose of AVP (dotted bars), and a high dose of AVP (bar with diagonal lines) injected subcutaneously). Error bars represent 95% confidence intervals. No significant effects were found for species, treatments, chambers, or any interaction of the three.

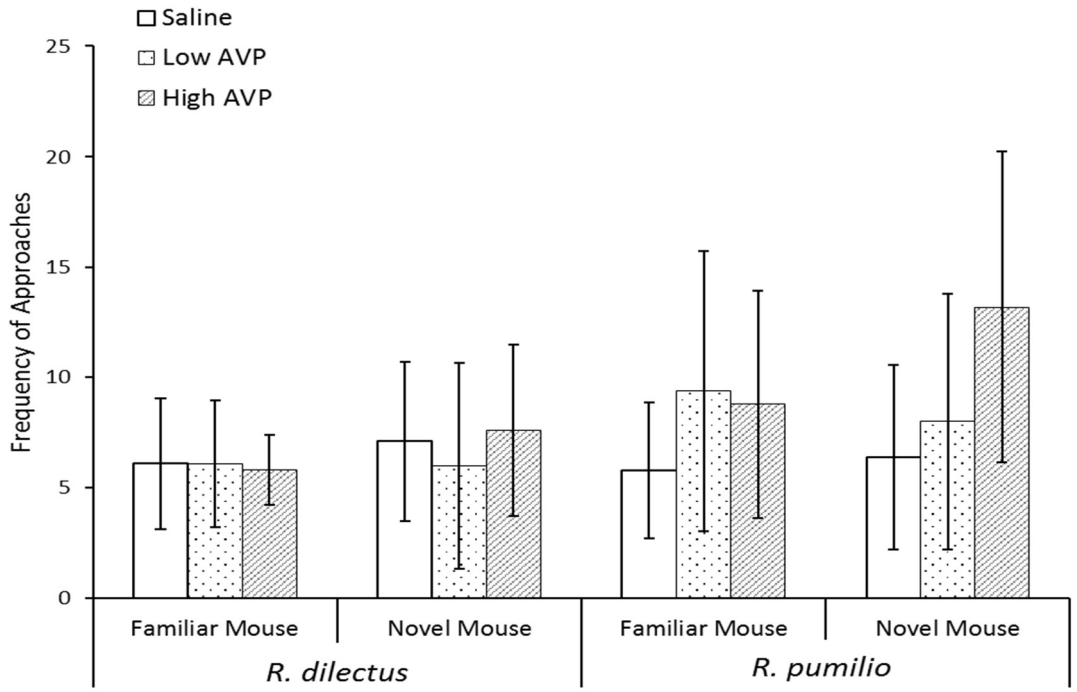


Figure 5.8. Mean frequency of approaches toward either the familiar or novel stimulus mouse in the social novelty preference test by females of two species of *Rhabdomys* after being injected subcutaneously with saline (white bars), low dose of AVP (dotted bars), and high dose of AVP (bar with diagonal lines). Error bars represent 95% confidence intervals. No significant differences were found in species, treatments or chambers, or in any interaction of the three.

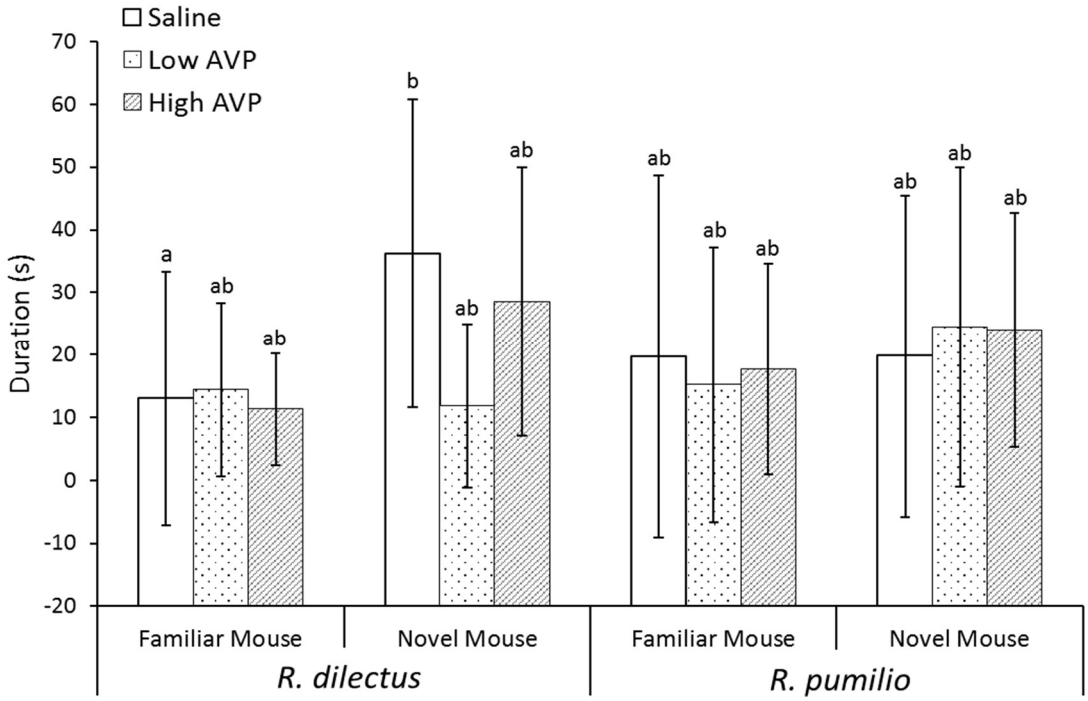


Figure 5.9. Mean duration of aggressive behaviour displayed toward a familiar and novel stimulus mouse in the social novelty preference test by focal females of two species of *Rhabdomys* after being treated with saline (white bars), low dose of AVP (dotted bars), and high dose of AVP (bar with diagonal lines) injected subcutaneously. Chamber was a significant predictor of aggression. Different letters indicate a significant difference, as identified in the post hoc test ($p > 0.05$). The error bars represent 95% confidence intervals.

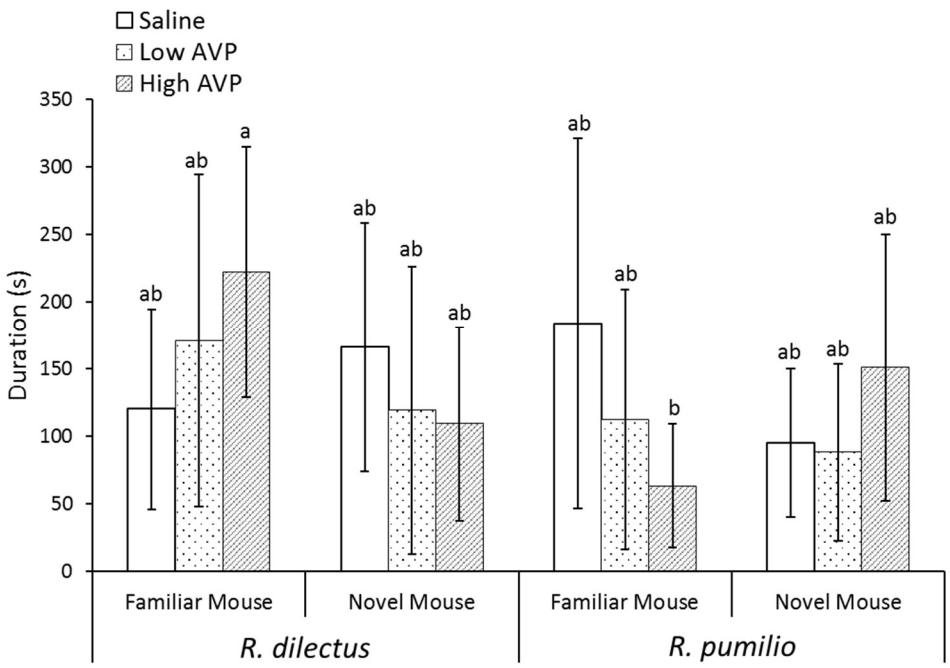


Figure 5.10. Mean duration of amicable behaviour displayed toward a familiar and novel stimulus mouse in the social novelty preference test by focal females of two species of *Rhabdomys* in three treatments (saline (white bars), low dose of AVP (dotted bars), and high dose of AVP (bar with diagonal lines) injected subcutaneously). The interaction of species x treatment x chamber was a significant predictor of amicable duration. Different letters denote significant differences ($p < 0.05$). The error bars represent 95% confidence intervals.

Discussion

This study aimed to investigate the role of AVP in promoting sociability and the ability to distinguish between familiar and unfamiliar conspecifics in two species of *Rhabdomys* that differ in their degree of sociality. AVP was predicted to increase the motivation to be social in the naturally solitary-living *R. dilectus*, but was not predicted to affect sociability in the naturally group-living *R. pumilio*. Interestingly, *R. dilectus* and *R. pumilio* did not differ in their social motivation, with both species spending significantly more time in the chamber with the stimulus mouse, and approached the unfamiliar mouse more than they did an empty holding cage, indicating a motivation to interact with the conspecific. AVP increased how often they approached the conspecific, indicating a potential of AVP to motivate *Rhabdomys* to be social, as approach and contact with conspecifics are accepted measures of social motivation (reviewed in Lim and Young, 2006). AVP was associated with an overall increased frequency to enter chambers, potentially demonstrating an overall increase in activity when striped mice were injected with the highest dose of AVP. The preference for social novelty was predicted to not be affected by AVP in *R. pumilio*, with this naturally social species being expected to seek contact with both stimulus animals equally, but AVP was predicted to reduce social

novelty preference in *R. dilectus* (increasing the affinity for familiarity). Neither species displayed a preference for social novelty, spending an equal duration in each chamber, and this was not affected by AVP. These results indicate that striped mice either lack the ability to discriminate between the novel and familiar stimuli, or they simply do not have a preference for either stimulus mouse.

Examination of the behaviour displayed toward the stimulus animals assists in understanding the motivation of the focal animal to approach the stimulus animals (Pearson et al, 2010). *R. pumilio* was predicted to show more aggression toward the stimulus animal than *R. dilectus*, since a previous study recorded more aggression toward an unfamiliar conspecific on initial meeting in this species (K-J. Hartman, unpublished data; Chapter 2). AVP was not expected to affect aggression or amicability in *R. pumilio*, but I expected AVP to reduce aggressive behaviour and increase amicable behaviour in *R. dilectus*. Interestingly, the two species did not differ in the levels of amicable or aggressive behaviour they displayed toward the unfamiliar conspecific in all three treatments in the sociability test. This indicates that AVP did not appear to mediate the aggressive or amicable responses to a conspecific on the first encounter in striped mice. Both species displayed more aggression toward the novel mouse than the familiar mouse. *R. dilectus* was significantly more amicable toward the familiar mouse than was *R. pumilio* in the high AVP treatment. Interestingly, in *R. dilectus*, AVP was associated with an increase in the duration of amicable behaviour towards the familiar mouse and reduced amicability toward the novel mouse. In contrast, in *R. pumilio*, females on the saline treatment showed longer durations of amicable behaviour toward the familiar than novel mouse, but *R. pumilio* displayed reduced levels of amicable behaviour to the familiar mouse and was more amicable toward the novel mouse in the high AVP treatment. Thus, AVP seems to increase amicable behaviour toward familiar mice in *R. dilectus*, but increases amicable behaviour toward novel mice in *R. pumilio*.

AVP is known to increase the motivation to seek contact with a conspecific and thus increase approaches in several species, such as laboratory rats and prairie voles, *M. ochrogaster* (reviewed by Lim and Young, 2006), and my results suggest the same for *Rhabdomys.* AVP was associated with an increase in the approaches toward a conspecific in both *R. pumilio* and *R. dilectus* when provided with the choice between interacting with an unfamiliar conspecific or remaining alone in a separate (empty) chamber. Once an animal has decided to socially engage, or approach, a conspecific, it can collect information about the identity of that conspecific for social recognition and form a social memory of that individual, allowing for

potential bonds to form or avoidance at a future interaction with that individual (Lim and Young, 2006). The increased social approaches observed here suggest an increased potential to initiate social contact, and thus facilitate the initial phase of group formation in both species.

When presented with a choice between a familiar and novel conspecific, both species displayed an absence of a preference for social novelty, interacting equally with both individuals, with the duration of contact and frequency of approaches to each stimulus mouse not being associated with AVP. Examining the manner of interactions between the focal mouse and stimulus mice provides additional indicators of the focal mouse's motivational state when interacting with a conspecific, and whether the approach is of an affiliative or agonistic nature (Pearson et al, 2010). The observed absence of social novelty preference, in terms of duration in chamber and frequency of approaches, does not appear to indicate a lack of social recognition as both species appeared to differentiate between the familiar and novel stimulus mouse because they interacted differently toward each mouse in terms of amicable contact. *R. pumilio* was more amicable toward the novel female, whereas *R. dilectus* was more amicable toward the familiar female. Thus, *R. dilectus* displayed a preference for familiarity when subjected to high AVP concentrations, whereas *R. pumilio* displayed a heightened preference for novel conspecifics at higher AVP concentrations.

Field studies of *R. pumilio* have shown that solitary males had higher densities of AVP-ir cells in the paraventricular nucleus (PVN) and medial bed nucleus of the strata terminalis (BNST) of the brain than their group-living counterparts; such difference potentially facilitates solitary males to transition to group-living more efficiently should the opportunity present itself (Schradin et al, 2013; Schoepf et al, 2015). My results suggest that AVP is associated with social approach, and facilitates amicable behaviour toward novel conspecifics. While AVP has been found to modulate social behaviour differently in males and females of some species, as has been demonstrated in laboratory rats and mice (reviewed by Winslow and Insel, 2004, and Dumais and Veenema, 2016), my results complement the idea that high AVP facilitates the transition to group-living in female *R. pumilio*. Similarly, social approach behaviour in the naturally solitary-living *R. dilectus* is also associated with high AVP. *R. pumilio* forms social groups in nature in the hot and dry non-breeding season (Schoepf and Schradin, 2014). AVP plays an important physiological role in the systemic circulation as a hormone mediating the maintenance of water balance, increasing in times of low water availability to reduce water loss via the kidneys (Nelson, 2005; Leng et al, 2012). The increased affiliative behaviour toward novel mice, observed here in response to artificially increased AVP, could potentially mirror

the increase in circulating AVP due to the environmental scarcity of water during the non-breeding season facilitating the initiation of group formation in free-living *R. pumilio*, but confirmation of this would require further investigation.

In conclusion, this is the first study demonstrating that both *R. dilectus* and *R. pumilio* are motivated to be social, and the potential role of AVP in enhancing social approach in both species. The suggested contrast in how these socially different species react to AVP may explain in part why the two species differ socially, but social behaviour is a complex behavioural construct with multiple systems including sensory, autonomic, motor, emotional and neural networks involved (Carter et al, 2008). AVP did not have a role in mediating a preference for social novelty or enhancing social recognition in these species, but it did promote affiliative behaviours toward conspecifics in both species, either toward familiar individuals in *R. dilectus*, or novel individuals in *R. pumilio*. The complexity of social behaviour needs to consider the environmental factors that could drive differences in sociality in species, and I have demonstrated here that proximate factors, such as AVP can affect how striped mice respond to conspecifics. AVP being associated with more affiliative behaviour towards a familiar individual in *R. dilectus*, but *R. pumilio* being more amicable toward a novel individual, could be explained by the location of the V1a receptors in the brain. Future studies should consider this comparison of V1a receptor location between the two species to illuminate the way in which AVP mediates social behaviours displayed in response to social cues in these socially different species. AVP is known to be involved in the processing of socially relevant cues in lab mice, actively mediating social recognition by acting on V1a receptors (Bielsky et al, 2004). Subcutaneous injections of AVP enhanced social recognition in both male and female rats (Dantzer et al, 1987; Bluthé and Dantzer, 1990), and transgenic V1a receptor knockout mice failed to display social recognition (Bielsky et al, 2004), demonstrating the importance of AVP and its receptors in social recognition in other rodent species. Knowledge of the distribution of receptors in the brain for this neuropeptide will also be valuable to further explain the development of social behaviour in these two species of *Rhabdomys*, and is currently being investigated by this research group.

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Appendices

"Unfamiliar 1" is the first unfamiliar stimulus mouse introduced in the sociability test, which becomes the "known" mouse in the social novelty test. "Unfamiliar 2" refers to the novel mouse introduced in the social novelty test.

Table A1. Spearman's Rank Order correlation results of random effects/continuous predictors and dependent variables for the sociability test. N=60 for all interactions considered (20 focal animals across 3 treatments).

Random effect	Dependent variable	Chamber	Spearman's R	t(N-1)	p-value
Baseline osmolality of focal animal	Total duration in chamber	Unfamiliar	0.064	0.489	0.627
		Empty chamber	-0.187	-1.452	0.152
	Frequency of entries	Unfamiliar	0.034	0.259	0.797
		Empty chamber	0.054	0.413	0.681
	Frequency of approaches	To unfamiliar	0.019	0.141	0.888
		To empty cage	0.090	0.687	0.495
Focal animal's body mass	Total duration in chamber	Unfamiliar	-0.049	-0.375	0.709
		Empty chamber	0.081	0.619	0.538
	Frequency of entries	Unfamiliar	-0.066	-0.505	0.615
		Empty chamber	-0.052	-0.393	0.696
	Frequency of approaches	To unfamiliar	-0.079	-0.606	0.547
		To empty cage	-0.049	-0.373	0.711
Focal animal's oestrous stage	Total duration in chamber	Unfamiliar	-0.107	-0.822	0.414
		Empty chamber	0.021	0.157	0.876
	Frequency of entries	Unfamiliar	**0.344**	**2.790**	**0.007**
		Empty chamber	**0.298**	**2.378**	**0.021**
	Frequency of approaches	To unfamiliar	0.183	1.417	0.162
		To empty cage	0.250	1.966	0.054
Unfamiliar animal's oestrous stage	Total duration in chamber	Unfamiliar	-0.033	-0.253	0.801
		Empty chamber	-0.024	-0.180	0.858
	Frequency of entries	Unfamiliar	-0.067	-0.512	0.611
		Empty chamber	-0.050	-0.380	0.705
	Frequency of approaches	To unfamiliar	-0.085	-0.653	0.516
		To empty cage	-0.069	-0.528	0.599
Unfamiliar animal's body mass	Total duration in chamber	Unfamiliar	-0.075	-0.576	0.567
		Empty chamber	0.185	1.435	0.157
	Frequency of entries	Unfamiliar	0.148	1.136	0.261
		Empty chamber	0.146	1.121	0.267
	Frequency of approaches	To unfamiliar	0.091	0.699	0.487
		To empty cage	0.142	1.095	0.278

Side of unfamiliar animal's chamber	Total duration in chamber	Unfamiliar	0.075	0.575	0.567
		Empty chamber	-0.176	-1.358	0.180
	Frequency of entries	Unfamiliar	0.132	1.013	0.315
		Empty chamber	0.101	0.776	0.441
	Frequency of approaches	To unfamiliar	0.133	1.025	0.310
		To empty cage	0.162	1.254	0.215
Side of empty chamber	Total duration in chamber	Unfamiliar	-0.075	-0.575	0.567
		Empty chamber	0.176	1.358	0.180
	Frequency of entries	Unfamiliar	-0.132	-1.013	0.315
		Empty chamber	-0.101	-0.776	0.441
	Frequency of approaches	To unfamiliar	-0.133	-1.025	0.310
		To empty cage	-0.162	-1.254	0.215

Table A2. Spearman's Rank Order correlation results of random effects/continuous predictors and dependent variables for the social novelty test. N=60 for all interactions considered (20 focal animals across 3 treatments).

Random effect	**Dependent variable**	**Chamber**	**Spearman's R**	**t(N-1)**	**p-value**
Baseline osmolality of focal animal	Total duration in chamber	Known animal	0.058	0.442	0.660
		Novel animal	-0.105	-0.804	0.425
	Frequency of entries	Known animal	-0.131	-1.010	0.317
		Novel animal	-0.059	-0.449	0.655
	Frequency of approaches	Known animal	-0.092	-0.703	0.485
		Novel animal	-0.037	-0.279	0.782
Focal animal's body mass	Total duration in chamber	Known animal	-0.145	-1.115	0.269
		Novel animal	0.159	1.227	0.225
	Frequency of entries	Known animal	0.164	1.266	0.211
		Novel animal	0.132	1.016	0.314
	Frequency of approaches	Known animal	-0.003	-0.025	0.981
		Novel animal	0.081	0.617	0.540
Focal animal's oestrous stage	Total duration in chamber	Known animal	-0.079	-0.604	0.548
		Novel animal	0.103	0.786	0.435
	Frequency of entries	Known animal	**0.426**	**3.587**	**0.001**
		Novel animal	**0.411**	**3.431**	**0.001**
	Frequency of approaches	Known animal	0.236	1.849	0.070
		Novel animal	**0.311**	**2.495**	**0.015**
Unfamiliar 1's oestrous stage	Total duration in chamber	Known animal	0.073	0.557	0.580
		Novel animal	**-0.280**	**-2.218**	**0.030**
	Frequency of entries	Known animal	-0.093	-0.714	0.478
		Novel animal	-0.062	-0.469	0.641
	Frequency of approaches	Known animal	-0.144	-1.110	0.271
		Novel animal	-0.248	-1.948	0.056

Unfamiliar 2's oestrous stage	Total duration in chamber	Known animal	0.042	0.322	0.749
		Novel animal	-0.063	-0.477	0.635
	Frequency of entries	Known animal	0.020	0.154	0.878
		Novel animal	-0.185	-1.433	0.157
	Frequency of approaches	Known animal	0.116	0.888	0.378
		Novel animal	-0.239	-1.873	0.066
Unfamiliar 1's body mass	Total duration in chamber	Known animal	-0.163	-1.258	0.213
		Novel animal	0.233	1.826	0.073
	Frequency of entries	Known animal	**0.295**	**2.349**	**0.022**
		Novel animal	**0.296**	**2.360**	**0.022**
	Frequency of approaches	Known animal	0.100	0.768	0.445
		Novel animal	**0.314**	**2.519**	**0.015**
Unfamiliar 2's body mass	Total duration in chamber	Known animal	-0.157	-1.214	0.230
		Novel animal	0.193	1.501	0.139
	Frequency of entries	Known animal	**0.275**	**2.178**	**0.033**
		Novel animal	**0.268**	**2.121**	**0.038**
	Frequency of approaches	Known animal	0.061	0.468	0.642
		Novel animal	**0.284**	**2.257**	**0.028**
Side of unfamiliar 1's chamber	Total duration in chamber	Known animal	0.066	0.501	0.618
		Novel animal	-0.135	-1.039	0.303
	Frequency of entries	Known animal	0.136	1.042	0.302
		Novel animal	0.122	0.937	0.353
	Frequency of approaches	Known animal	0.088	0.673	0.504
		Novel animal	0.130	0.996	0.324
Side of unfamiliar 2's chamber	Total duration in chamber	Known animal	-0.066	-0.501	0.618
		Novel animal	0.135	1.039	0.303
	Frequency of entries	Known animal	-0.136	-1.042	0.302
		Novel animal	-0.122	-0.937	0.353
	Frequency of approaches	Known animal	-0.088	-0.673	0.504
		Novel animal	-0.130	-0.996	0.324

Table A3. Spearman's Rank Order correlation results of random effects/continuous predictors and the duration of behaviours when in contact with stimulus mice in the sociability and social novelty tests. N=60 for all interactions considered (20 focal animals across 3 treatments).

Random effect	Stimulus & Test	Behaviour	Spearman's R	t(N-1)	p-value
Baseline osmolality of focal animal	Unfamiliar in sociability test	Amicable	0.210	1.635	0.107
		Aggressive	-0.087	-0.668	0.507
	Known mouse in social novelty test	Amicable	0.202	1.573	0.121
		Aggressive	-0.087	-0.662	0.511
	Novel mouse in social novelty test	Amicable	0.019	0.142	0.888
		Aggressive	0.102	0.780	0.439

Focal animal's body mass	Unfamiliar in sociability test	Amicable	-0.205	-1.597	0.116
		Aggressive	-0.029	-0.223	0.825
	Known mouse in social novelty test	Amicable	**-0.255**	**-2.005**	**0.050**
		Aggressive	-0.142	-1.096	0.278
	Novel mouse in social novelty test	Amicable	0.021	0.157	0.876
		Aggressive	-0.091	-0.694	0.490
Focal animal's oestrous stage	Unfamiliar in sociability test	Amicable	-0.146	-1.126	0.265
		Aggressive	0.128	0.982	0.330
	Known mouse in social novelty test	Amicable	-0.084	-0.644	0.522
		Aggressive	0.075	0.576	0.567
	Novel mouse in social novelty test	Amicable	0.066	0.501	0.618
		Aggressive	0.146	1.128	0.264
Unfamiliar 1's oestrous stage	Unfamiliar in sociability test	Amicable	-0.118	-0.901	0.371
		Aggressive	**-0.377**	**-3.100**	**0.003**
	Known mouse in social novelty test	Amicable	0.039	0.294	0.770
		Aggressive	-0.235	-1.840	0.071
	Novel mouse in social novelty test	Amicable	**-0.341**	**-2.762**	**0.008**
		Aggressive	**-0.343**	**-2.777**	**0.007**
Unfamiliar 2's (novel mouse) oestrous stage	Known mouse in social novelty test	Amicable	-0.008	-0.057	0.955
		Aggressive	0.012	0.088	0.930
	Novel mouse in social novelty test	Amicable	**-0.268**	**-2.115**	**0.039**
		Aggressive	-0.224	-1.752	0.085
Unfamiliar 1's body mass	Unfamiliar in sociability test	Amicable	-0.178	-1.377	0.174
		Aggressive	0.236	1.854	0.069
	Known mouse in social novelty test	Amicable	-0.239	-1.873	0.066
		Aggressive	-0.009	-0.071	0.944
	Novel mouse in social novelty test	Amicable	0.107	0.818	0.417
		Aggressive	0.049	0.375	0.709
Unfamiliar 2's (novel mouse) body mass	Known mouse in social novelty test	Amicable	-0.250	-1.970	0.054
		Aggressive	-0.010	-0.077	0.939
	Novel mouse in social novelty test	Amicable	0.026	0.195	0.846
		Aggressive	0.048	0.368	0.714

Side of unfamiliar 1's chamber	Unfamiliar in sociability test	Amicable	0.039	0.294	0.770
		Aggressive	0.203	1.583	0.119
	Known mouse in social novelty test	Amicable	0.031	0.235	0.815
		Aggressive	0.138	1.060	0.294
	Novel mouse in social novelty test	Amicable	-0.041	-0.309	0.758
		Aggressive	0.158	1.217	0.229
Side of empty/unfamiliar 2 (novel mouse) chamber	Unfamiliar in sociability test	Amicable	-0.039	-0.294	0.770
		Aggressive	-0.203	-1.583	0.119
	Known mouse in social novelty test	Amicable	-0.031	-0.235	0.815
		Aggressive	-0.138	-1.060	0.294
	Novel mouse in social novelty test	Amicable	0.041	0.309	0.758
		Aggressive	-0.158	-1.217	0.229

CHAPTER 6
General Discussion

Phenotypic plasticity provides a plausible solution for animals to respond swiftly to unpredictable and rapid changes in their environments resulting from rapid environmental changes. Social flexibility is one example of phenotypic plasticity which has been observed in several species living in unpredictable environments (Randall et al, 2005; Cooper and Randall, 2007; Schradin et al, 2012). The ability to change between group-living and solitary-living might be advantageous for coping with changing environmental conditions (Rymer et al, 2013). This phenomenon also provides an opportunity to study the foundational mechanisms of variation in social systems.

In group-living species, individuals must be capable of recognising and sequentially tolerating conspecifics. Lott (1991) proposed that animals may be physiologically primed to be either solitary-living or group-living. Two questions arise. 1) How would a socially flexible species be primed physiologically? 2) Are there physiological markers to indicate this ability? Several studies have investigated the variation in the social systems of animal populations (reviewed in Alexander, 1974; Crook et al, 1976; Bacon et al, 1991; Krebs et al, 1993; Kappeler and van Schaik, 2002; Banks et al, 2007; Silk, 2007; Schradin et al, 2012; Lukas and Clutton-Brock, 2013; Hofmann et al, 2014), with several ecological drivers and constraints being identified as reasons for animals forming aggregations and benefiting from social groups, but more investigation into the behavioural and physiological mechanisms underlying the initiation of group formation is required.

I aimed to investigate the mechanisms underlying social interactions, and ultimately social group formation in two species of the African striped mice, *Rhabdomys*. I compared two species with differing social systems: *R. pumilio* from the xeric western regions of South Africa, which is facultatively group-living and highly socially flexible; and the solitary-living *R. dilectus* which occurs in the moist grassland regions of South Africa, and is solitary-living. I studied females of each species because social groupings are typically formed by females and later joined by males in *R. pumilio* (Schradin et al, 2012). I compared the behaviour of females of these two species in their initial interactions with unfamiliar conspecifics, their behaviour over 21-days of imposed group-living, their base-concentrations of corticosterone and testosterone, and the response of these two hormones to the social-living context. I also examined the brain concentrations of the socially relevant neuropeptides AVP and oxytocin

across social contexts, and the role of AVP in social motivation and social novelty preference in each species. My results suggest a latent ability of *R. dilectus* to form social groups, as is observed in *R. pumilio*, but also revealed interesting differences in the physiological and behavioural responses that exist between these two species. In this chapter, I review the key findings from my study, compare these results to other species, discuss their implications to the broader understanding of social evolution, and suggest avenues for future studies.

Key findings

My first objective was to identify the behaviours of initiating and allowing for group formation, by investigating the behavioural response to unfamiliar, unrelated conspecifics in the naturally solitary *R. dilectus* females and naturally group-living *R. pumilio* females. I confirmed that both species are capable of being socially tolerant of unfamiliar conspecifics, but differ slightly in how they initiate group formation (Chapter 2). Females of both species were tolerant of female conspecifics, displaying predominantly amicable behaviour toward unfamiliar triad members on initial meeting and sharing nests by the second day in triads, but female *R. pumilio* was more aggressive toward conspecifics on initial meeting than *R. dilectus*. Both species maintained nest sharing over 21-days in triads, with the duration of amicable behaviour displayed between triad members increasing the longer the females were in the triads.

My second objective was to investigate the physiological markers of sociality in each species by comparing their neuroendocrine profiles in an imposed social grouping or in isolation for 35-days. I investigated the concentration of circulating corticosterone and testosterone to identify whether any physiological stress response occurs in the different social contexts and whether there is any hormonal priming for aggression (Chapter 3). Social and solitary-living species experience social stress differently – for a social animal, isolation from conspecifics can elicit a physiological stress response, whereas interactions with conspecifics can be physiologically stressful for a solitary animal (Zayan, 1991; Blanchard et al, 2001; Wommack et al, 2003). Examination of corticosterone revealed that *R. dilectus* is physiologically stressed in a social group compared to *R. pumilio*. Interestingly, the two species did not differ in terms of corticosterone concentration when in isolation, indicating that adult female *R. pumilio* are not stressed when housed alone. This is different to juveniles of this species, which display physiological and behavioural evidence of separation anxiety and stress when in isolation (Mackay et al, 2014). The two species did not differ in their concentrations of testosterone in the treatments, which complements the similarity in amicable behaviour

displayed by both species in the triads after 3-weeks, as well as field studies of free-living *R. pumilio* (Schoepf and Schradin, 2013; Hill et al, 2015).

I measured the concentration of the neuropeptides AVP and oxytocin in the brain after 35-days in each social context (Chapter 4). These neuropeptides are important in modulating social recognition, social memory, and social bonding in several mammalian species (Young, 2002; Winslow and Insel, 2004; Lim and Young, 2006). I demonstrated that these two socially different species differ in their response to social grouping, with the naturally solitary *R. dilectus* having higher AVP in groups than *R. pumilio* in groups. The two species did not differ in terms of base neuropeptide concentrations when housed in isolation, nor did they differ in oxytocin concentration in groups. This difference in AVP concentration between the two species in imposed groups raised the question of whether AVP affects social behaviour in different ways in each species. Both species displayed the behaviours expected to promote group stability and bonding (such as tolerance and allo-grooming; Chapter 2), but experienced opposing concentrations of a neuropeptide known to be instrumental in mediating social behaviour (Chapter 4). As with all hormonal patterns, neuroendocrine changes could happen as a result of the social context, or the social behaviour expressed could be as a result of the effect of neuropeptide mediation (Rubenstein and Wikelski, 2005). Thus, I endeavoured to identify the role AVP plays in the motivation to be social in *Rhabdomys.*

My third objective was to investigate the role of AVP in sociability (social motivation) and social recognition in these two species (Chapter 5). Species with differing social structures respond differently to AVP (Insel and Young, 2000), including avian species where artificially increased AVP-homologue vasotocin elicits increased aggression in territorial male field sparrows, *Spizella pusilla*, but not in male colonial zebra finch, *Taeniopygia guttate* (Goodson, 1998; Goodson and Adkins-Regan, 1999). My experiments were designed to allow a single focal animal the opportunity to interact with unfamiliar conspecifics or remain alone, testing their motivation to be social (Engelmann et al, 1995). The focal female was administered a saline control and two dosages of AVP (low and high) sequentially. I demonstrated that females of both species are motivated to be social since both species displayed a preference for social contact, electing to spend more time with a conspecific than in isolation, independently of the treatment. I found that AVP increases the frequency of approaches toward the unfamiliar mouse in both species, indicating a potential for AVP to be associated with the propensity to be social in *Rhabdomys*. Social recognition was tested using the social discrimination paradigm of the social novelty preference test (Choleris et al, 2009). Here, the same focal animal as in

the sociability test was given the choice between interacting with a familiar conspecific or a novel conspecific, under the same three treatments of saline, low and high AVP dose. When examining the duration of contact with each stimulus mouse and frequency of approaches, AVP appeared to have no role in mediating social novelty preference in *Rhabdomys*. However, closer examination of the behaviours expressed toward the two stimulus females revealed that both species were more amicable toward a familiar mouse than the novel mouse. The two species had contrasting responses to AVP in terms of amicable behaviour toward the stimulus mice, where *R. dilectus* increased the duration of amicable behaviour toward the familiar mouse as AVP increased, whereas *R. pumilio* reduced the duration of amicable behaviour toward the known mouse as AVP increased, having the highest duration of amicable behaviour toward the novel mouse in the highest AVP treatment. This indicates that proximate mechanisms, such as AVP, have the ability to affect how striped mice respond to conspecifics, but different species respond differently to these mediators.

Implications of key findings

Hofmann et al (2014) highlighted the importance of utilising an integrative approach to studying and understanding social behaviour in vertebrates. Social interactions are affected by a combination of attributes, ranging from proximate mechanisms such as genetic and neuroendocrine modulators, to functional drivers such as predation pressure and resource distribution (Hofmann et al, 2014). My study examined the proximate mechanisms underlying sociality in *Rhabdomys*. My findings contribute to a broader understanding of social evolution in *Rhabdomys* and are not merely laboratory artefacts, firstly, because behavioural and physiological measures were associated in my study, and secondly, because consistently similar behavioural and physiological findings have been generated in both the field and laboratory contexts for several behavioural parameters in this genus (Kinahan and Pillay, 2008; Yuen et al, 2016).

My work complements ongoing research within our research group at the University of the Witwatersrand, which has already extensively examined the environmental pressures driving and affecting social behaviour in this genus. Social flexibility observed in *R. pumilio* has provided another means of studying and understanding social evolution in mammals (Schradin et al, 2012). It is known that food quality and distribution, vegetation cover, and population density drive social tactics in *Rhabdomys* (Schradin, 2005; Schradin and Pillay, 2005; Schradin and Pillay, 2006), with fierce reproductive competition being a strong driver

for solitary-living during the breeding season for *R. pumilio* (Schradin et al, 2010; Schoepf and Schradin, 2012). The physiological correlates of social flexibility have also been investigated in *R. pumilio* in an attempt to identify endocrine underpinnings of this phenomenon. The primary focus has been on males, with investigations of the oxytocin and AVP immunoreactive(ir) cells (Schoepf et al, 2015) and receptors (Schradin et al, 2014) in the brains of male *R. pumilio* adopting either a solitary roamer social tactic or a group-living tactic of either breeder or philopatric males. Hormonal analyses of free-ranging *R. pumilio* have found that solitary individuals have lower corticosterone concentrations than group-living mice, and solitary males have higher testosterone than group living males (Schoepf and Schradin, 2013), but females do not differ in terms of testosterone concentration in their tactics (Hill et al, 2015).

In the pursuit of understanding social evolution in this genus, I continued the investigation into the proximate mechanisms mediating social behaviour in the genus *Rhabdomys* by comparing the solitary sister species, *R. dilectus,* of *R. pumilio*. Firstly, I investigated whether *R. dilectus* could live in groups, indicating a potential to be socially flexible, like *R. pumilio*. Secondly, I built on the current knowledge of mammalian sociality because behavioural, ecological, and neurobiological data are essential should one hope to gain an integrated understanding of social behaviour in a species (Hofmann et al, 2014). Jones (2014) maintained that while environmental conditions can explain why aggregations would form initially, they cannot explain why that aggregation would be maintained and why stable groups form. I believe that social bonds are what maintain groups over time, and for an animal to be social it needs both the motivation to initiate group formation and the capacity to maintain social relationships.

In order for any animal to form and live in a social group, it must first be motivated to approach and interact with conspecifics (Lim and Young, 2006). I found that AVP increases the propensity of female *Rhabdomys* to approach a conspecific, and thus, AVP appears to enhance the motivation to be social in this genus (Figure 6.1; Chapter 5). Once an animal has approached and initiated interactions with a conspecific, the manner in which it interacts with that individual will affect the type of relationship (affiliative or agonistic) that exists between the two, and the nature of future interactions with that individual (social memory of that conspecific), which is also affected by AVP (Lim and Young, 2006; Hofmann et al, 2014; Chapter 5 of this thesis). The type of interaction also depends on whether the individual is socially stressed (elevated corticosterone), in which case they may be likely to display more avoidance or aggressive behaviour than an animal that is not stressed in groups (Blanchard et

al, 2001). This hormonal stress response could occur from a social memory of a past negative interaction (e.g. aggression; Creel, 2001) which could lead to future aggressive interactions or avoidance of conspecifics (Figure 6.1). Similarly, amicable behaviour reduces stress or anxiety in other rodent species (Bielsky et al, 2004; Egashira et al, 2007), and thus amicable interactions could minimise this response and enhance tolerance of conspecifics and promote nest sharing. Nest cohabitation and social bond formation could in turn increase amicable behaviour. This subsequently would create a positive association with that conspecific (Figure 6.1). This is in agreement with and adds to the hypotheses described by Hinde (1976) and Kappeler and van Schaik (2002) that the most basic foundation of any social system is the nature of interactions and relationships between individuals in a population, and that these interactions are a result of a constant feedback and integration of neuroendocrine mediation of behaviours and responses to behaviours displayed between individuals (Figure 6.1). The pattern of behaviour displayed promotes a particular social tactic which in turn shapes the social structure of a population (Figure 6.1). Plasticity in social tactics would then be possible in animals which have the capability of displaying the behaviours that promote each social tactic (Figure 6.1).

A switch in tactic could be triggered by a change in environmental conditions that in turn affects the physiology of the animal, which facilitates a change in behaviour. Animals living in arid and unpredictable environments, and which display social flexibility, have high circulating levels of AVP due to limited water availability (Carter, 2003; Nelson, 2005; Cooper and Randall, 2007; Schradin et al, 2012). While behavioural and physiological sex differences may exist in *R. pumilio*, my results support the idea that higher AVP facilitates the transition to group-living in this species (Schradin et al, 2013; Schradin et al, 2014), because female *R. pumilio* injected with AVP are more amicable toward novel females. This species forms social groups in nature during the dry season, which correlates with elevated plasma AVP concentrations (Schoepf and Schradin, 2014), and thus it is possible to speculate that AVP does facilitate this switch in social tactic by promoting social motivation. High AVP dosages enhanced preference for known individuals in *R. dilectus*, and triads of this species had higher central concentrations of AVP that was associated to high durations of amicable behaviour, indicating potential for AVP to facilitate sociality in this species too.

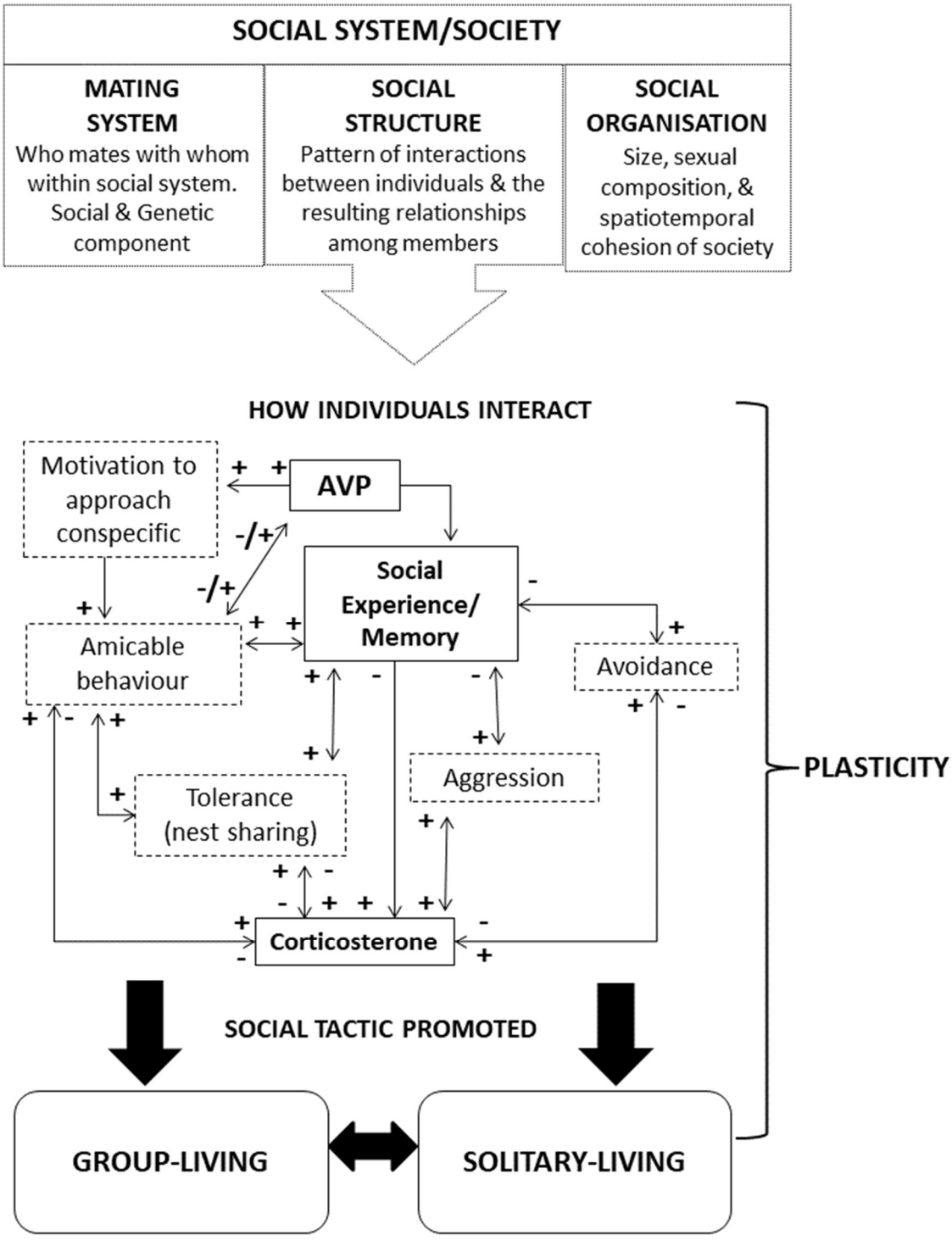


Figure 6.1. An integrative model of the proximate factors affecting social behaviour in female *Rhabdomys*, facilitating either group-living or solitary-living. Definitions taken from Kappeler and van Schaik (2002). The manner in which individuals interact in female *Rhabdomys* is affected by arginine vasopressin (AVP) via its role in promoting social approach and social memory. Memory of previous interactions with conspecifics shapes how an individual will respond to conspecifics in future. Whether these experiences were positive (+) or negative (-) will affect whether the interaction will elicit a stress response of raised corticosterone concentrations. Constant feedback occurs between social experiences, behaviours expressed, and neuroendocrine responses and mediation. Annotations of "+"and "-" signs indicate an increase or decrease in level, respectively. Signs closest to the box of a component indicates the change in the level of that component in response to the change on the same side of the arrow as the connected component.

Generally, the temporal and spatial distribution of females and social tactic employed by females of a population dictates the distribution and tactic adopted by males (Kappeler and van Schaik, 2002; Jones, 2014), and thus understanding female behaviour is important for understanding the variation of social systems observed in nature. Because environmental change can result in changes in population density and resource availability, changes in phenotypic plastic traits (e.g. sociality) might mitigate against changes by presenting rapid responses to environmental change (Banks et al, 2007; Rymer et al, 2013). The requirement for an animal which is usually solitary to tolerate conspecifics has been displayed in meadow voles, *Microtus pennsylvanicus*, which form communal nesting groups in winter to exploit limited resources (Kawata, 1990). *R. dilectus* may also be capable of such a short-term change in social tactic because it is motivated to interact socially and display amicable behaviour. Familiarity is an important aspect of the amount of amicable behaviour an animal will display, as is seen in free-tailed bats, *Tadarida teniotis*, which are more amicable toward familiar conspecifics within a communal nest (Ancillotto and Russo, 2014). Moreover, who an animal interacts with, and the type of interaction, is not merely a case of who is located nearby, but the identity of that individual (i.e. whether there is a social bond or the nature of a past interactions), which relies on the ability to recognise specific individuals (Choleris et al, 2004). For example, female vervet monkeys, *Chlorocebus aethiops pygerythrus*, groom and receive grooming from preferred partners (Henzi et al, 2013). While social behaviour is a complex construct affected by a vast number of factors (reviewed in Carter et al, 2008), the physiological mechanisms driving the ability to recognise individuals and facilitate affiliative behaviours may provide a means of allowing animals to transition from solitary-living to group-living, and vice versa, should it become more advantageous.

Future studies

I have demonstrated that a species known to be solitary in free-living populations has the ability to display the behaviours appropriate for encouraging social group formation (amicability, tolerance, nest sharing), and can maintain these groups for weeks in captivity. This was also the first study to confirm that *R. pumilio* can form groups of unrelated females. The similarities in social behaviour in *R. pumilio* and *R. dilectus* over the three weeks in triads indicate a latent ability of *R. dilectus* to be group-living, which could potentially be a plesiomorphic trait in this species (*R. pumilio* being the putative ancestral form of *Rhabdomys*; Rambau et al, 2003; Pillay et al, 2006; Meynard et al, 2012). However, whether these groups can be maintained over a

longer time under reproductive competition should be investigated. I hypothesise that these groups will break down easily, as the elevated corticosterone (measured in my study; Chapter 3) is not sustainable since it could reduce resistance to pathogens (Gust et al, 1991; Bartolomucci, 2007; Ebensperger et al, 2011; Hayes et al, 2011). Extrinsic factors are known to affect sociality in animals, such as in free-living female eastern grey kangaroos, *Macropus giganteus*, where body condition, food availability and reproductive state predictably affect sociality (Menz et al, 2017). Concurrent field research by C.M.S. Dufour et al (in prep) has identified cases of tolerance and nest sharing in free-living *R. dilectus* during the breeding season, but these groups are not stable and their demographic composition is unknown. Further studies of free-living populations of *R. dilectus* pin-pointing the extent to which sociality is shaped by environmental drivers, and identification of these drivers, as have been done for *R. pumilio*, will add valuable contributions to our understanding of social behaviour in this genus.

I have demonstrated that two species of a single genus that differ socially also differ in how AVP affects their social behaviour. I assumed that this difference could be attributed to differences in the distribution of AVP-V1a receptors in the brain of these species. This was seen in a social, *Ctenomys sociabilis*, and solitary, *C. haigi*, species of tuco-tuco, which have prominent differences between the species in the distribution of AVP-V1a receptors in regions of the social behaviour network of the brain (Beery et al, 2008). A comparison of receptor distribution between species will be valuable to understand why this difference in response to AVP occurs in the two species of *Rhabdomys*, and potentially point to the regions in the brain responsible for facilitating social behaviour in this genus.

Conclusions

I used a multipronged approach to understanding social behaviour in the African striped mouse, highlighting two findings. Firstly, I demonstrated similarity in the response to conspecifics of a naturally solitary-living and naturally group-living species, indicating that some commonality exists in the behaviours required for the initiation of group formation. Secondly, I demonstrated that, while the two species studied displayed similar social behaviours, they differed in how they responded to social groupings in terms of their neuroendocrine profiles. This indicates that while the solitary species is capable of tolerating conspecifics, the social context is stressful for them. Oxytocin did not vary with social context, adding support to the idea that it does not have an important role in sociality in *Rhabdomys* compared to AVP. Contrasting AVP

concentrations in the brains of the two species following imposed social groupings prompted further investigation into its role in sociality in this genus. This is the first study to demonstrate that both species are motivated to be social, and that AVP enhances social approach in both species, potentially indicating that this neuropeptide is key to modulating social behaviour. The ability for a solitary species to form groups in captivity hints toward the potential for this species to also be socially flexible, which would allow them to respond to changing environmental conditions, an ability which is expected to be particularly advantageous in the face of anthropogenic environmental change (Rymer et al, 2013; Rymer et al, 2016). My work here has added to our understanding of the foundations of social behaviour in mammals generally, but further studies are needed in more species and in other taxonomic groups to assess the generalisability of my findings.

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APPENDIX 1

Extraction of arginine vasopressin and oxytocin from whole mouse brain for ELISA analysis

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i. Summary/Abstract

The role of the neurohypophyseal nonapeptide hormones oxytocin and arginine-vasopressin in many physiological and behavioural processes has been of interest for many biological and pharmaceutical studies. The complex nature of the mammalian central nervous system and the small size of these neuropeptides have posed many challenges for quantifying the

concentrations of central oxytocin and arginine-vasopressin. Extraction from a tissue sample needs to safeguard against post-mortem protease activity as well as ensure optimum extraction of the peptides from cells without affecting the integrity of the peptides. We endeavoured to identify buffer conditions for optimal extraction of the neuropeptides such that they are readily available and suitable for use in an ELISA. This would allow for analysis of concentrations during fieldwork and situations where access to mass spectrometry or HPLC equipment is restricted.

ii. Keywords

Arginine vasopressin, Oxytocin, Neuropeptide, Extraction, ELISA

1. Introduction

The role of oxytocin (OT) and arginine vasopressin (AVP) in mammalian nervous and systemic systems has been of interest in many pharmacological and behavioural studies ***(1, 2, 3, 4, 5)***. These cyclic nonapeptides are synthesised in the magnocellular neurons of the paraventricular nuclei (PVN) and supraoptic nuclei (SON) of the hypothalamus, are present in the amygdala, hippocampus and neocortex of the brain, and secreted into the blood by the posterior pituitary gland ***(6, 7, 8, 9).*** The small size of these peptides (OT 1007 g/mol, AVP 1084 g/mol) ***(10, 11)*** and short chain structure (OT: Cys-Try-Ile-Gln-Asn-Cys-Pro-Leu-Gly; AVP: Cys-Try-Phe-Gln-Asn-Cys-Pro-Arg-Gly) ***(12)*** makes quantifying the amount present in the brain challenging.

While mass spectrometry and high-performance liquid chromatography (HPLC) offer means of identifying neuropeptides in a tissue sample ***(13)***, we were interested in optimising a protocol specifically for quantifying OT and AVP concentrations in the mouse brain with an ELISA. ELISA is more readily available, lends itself to quantification, and does not require equipment with the level of sophistication of mass spectrometry and HPLC. This was achieved by first producing protein lysates, followed by the isolation of polypeptides that are smaller than 10kDa from the whole brain using two readily available buffers. The resulting extract was perfectly suited to ELISA analyses.

The two buffers used were acidified methanol, based on its use in previous studies ***(13, 14)***, and a modified radioimmunoprecipitation assay buffer (RIPA) ***(15, 16).*** Alcohol-based buffers have a propensity to induce protein precipitation ***(17)*** which is not desirable for ELISA analysis as it may prevent access to epitopes, thus prompting the use of a modified RIPA buffer as an alternative. The actions of this low salt buffer are based on the mild detergent properties of Triton X-100, a non-ionic detergent ***(18)***, together with low concentrations of deoxycholate and sodium dodecyl sulfate (SDS) to aid in dissociating weak and non-specific protein interactions that may arise after cellular lysis ***(15, 19, 20)***.

Presented here is a relatively easy to follow protocol for the quantification of the concentration of OT and AVP from the mouse brain, including dissection of the brain and extraction of the neuropeptides from the mouse brain with two buffers, for analysis with neuropeptide-specific ELISA kits.

2. Materials

2.1. Mouse Dissection

1. Isoflurane (Abbott Laboratories S.A. (Pty) Ltd, FORANE® liquid) (see **Note 1**)

2. Self-contained plastic box, prepared by rinsing in 70% ethanol
3. Three place precision balance (Mettler AE160)

2.2. Neuropeptide Extraction

2.2.1. Acidified methanol as extraction buffer

1. Ice (protocol performed on ice)
2. Refrigerated Centrifuge set at 4 ºC (PrismR, Labnet International, Inc.)
3. Cold acidified methanol (90% methanol 9% acetic acid 1% distilled water), prepared fresh and kept at 4 ºC
4. Microtube pestles (compatible with 1.5 ml microcentrifuge tubes), prepared by rinsing in 70% Ethanol and then distilled water
5. 10 kDa centrifugal filter units (Merck Millipore Ltd., Tullagreen, Co. Cork, IRL, Ultracel®, Amicon® Ultra) (see **Note 2**)
6. 1.5ml Microcentrifuge tubes, rinsed with acidified methanol before use

2.2.2. Modified RIPA buffer as extraction buffer

1. Ice (protocol below to be performed on ice)
2. Modified-RIPA lysis and extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.5% Triton X-100, 0.05% sodium dodecyl sulphate (SDS), made up to final volume with distilled water) stored at 4°C
3. Refrigerated Centrifuge, set at 4°C (PrismR, Labnet International, Inc.)
4. Modified-RIPA mixed with protease inhibitor cocktail (Sigma - Aldrich®; St Louis, MO) (see **Note 3**)
5. Microtube pestles (compatible with 1.5 ml microcentrifuge tubes), clean by rinsing in 70% ethanol and then distilled water

6. 10 kDa centrifugal filter units (Merck Millipore Ltd., Tullagreen, Co. Cork, IR, Ultracel®, Amicon® Ultra) (see **Note 2**)
7. 1.5ml Reaction/Eppendorf tubes and "Flow-through collection tubes" to be rinsed with the RIPA buffer (prepared above) before use.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

1. ELISA kits specific to oxytocin (Enzo Life Sciences Inc., ADI-900-153) and arginine vasopressin (CUSABIO®, CSB-E09272m)
2. Microplate reader (ASYS Expert Plus, v. 1.4)
3. Precision pipettes and tips for volumes between 5µl and 1000 µl (see **Note 4**)
4. Incubator capable of providing stable incubation conditions up to 37℃ (see **Note 5**)

3. Methods

3.1. Mouse Dissection

1. Mice are placed in a glass jar saturated with isoflurane, and are decapitated once breathing has stopped. (see **Note 6**)
2. The brain is immediately removed from the skull and weighed.
3. Once weighed, the brain is placed in a plastic container and frozen at -80℃, where it remains until neuropeptide extraction. (see **Note 7**)

3.2 Neuropeptide Extraction

3.2.1 Acidified methanol buffer

1. Cut the frozen brain into quarters and place in prepared Eppendorf tube.

2. Add 300µl of cold acidified methanol to frozen sample and allow to thaw on ice for 20 minutes
3. Homogenise on ice using a microtube pestle for 120 seconds
4. Centrifuge at 14 000 RCF at 4℃ for 15 minutes
5. Transfer the supernatant to a prepared filter fitted over a flow through collection tube and centrifuge at 14 000 RCF at 4℃ for 15 minutes. (see **Note 2**)
6. Vortex the filtrate, briefly, in the collection tube to mix and aliquot into two prepared 1.5ml microcentrifuge tubes (see **Note 8**)
7. Lyophilise the sample to dryness. (see **Note 9**)

3.2.2 Modified-RIPA buffer

1. Cut frozen brain into quarters and place in 1.5 ml Eppendorf tube.
2. Add 300 µl of modified-RIPA-protease-inhibitor combination to frozen sample and allow thawing on ice for 20 minutes. (see **Note 3**)
3. Homogenise on ice using a microtube pestle for 120 seconds
4. Add another 300µl modified-RIPA-protease-inhibitor combination and return to the ice for 30 minutes
5. Centrifuge at 14 000 RCF at 4 ºC for 15 minutes
6. Transfer the supernatant to the prepared filter, fitted over the flow-through collection tubes and centrifuge at 14 000 RCF at 4 ºC for 15 minutes. (see **Note 2**)
7. Add another 300µl modified-RIPA-protease-inhibitor combination to the pellet and homogenise for 120 seconds on ice, stand on ice for 30 minutes
8. Pellet again as in step 5 above, and then transfer the supernatant to a new prepared filter; and then centrifuge again for 15 minutes at 14 000 RCF at 4ºC (see **Note 2**, **10**)

9. Vortex the filtrate in the collection tube to mix and aliquot into two prepared 1.5ml microcentrifuge tubes (see **Note 8**)
10. Lyophilise samples to dryness. (see **Note 9**)

3.3 Enzyme Immunoassay Analysis

The concentration was determined using ELISA kits specific to oxytocin (Enzo Life Sciences Inc., ADI-900-153) and arginine vasopressin (CUSABIO®, CSB-E09272m), following the method described in the respective kit manuals. The ASYS Expert Plus (v. 1.4) microplate reader was used to measure the optical density of the wells at 405nm for oxytocin and 450nm for arginine vasopressin, as advised in the kit manuals.

3.4 Data Analysis

The data were reduced using Master Plex 2010 (Hitachi Software Engineering America, Ltd.) software to generate a four parameter logistic curve-fit. This software was chosen due to its ease of accessibility online and ease of data input, as no file conversions are necessary for both input of raw data and export of the analysed concentrations and graphs. (see **Note 11**)

4. Notes

1. Isoflurane was selected for euthanasia as it does not affect the chemistry of the brain ***(21)***.
2. Filters and flow-through collection tubes pre-cleaned by rinsing in distilled water and then cold acidified methanol, at 14000 RCF, at 4 ºC for 3min.
3. The ratio of half RIPA to protease inhibitor cocktail used was 200:1

4. A 20-200µl pipette is used most frequently during the ELISA.
5. Specific temperature required is determined by the ELISA specifications.
6. The African striped mouse, *Rhabdomys* species, was used in this study.
7. To reduce the effects of protease degradation of proteins post-mortem, the brain should be frozen within 120 seconds after death.
8. Filtrate collected in the collection tube contains the neuropeptides.
9. Store dried sample at -70°C until reconstitution for ELISA.
10. The pellet is homogenised again to ensure the maximum yield of peptides from the brain as possible. This is only included for the RIPA buffer protocol as we observed an increase in the volume of supernatant collected than the initial volume of buffer added. In the acidified methanol buffer protocol, no increase was observed and the concern of a second addition of buffer diluting the final concentration of neuropeptides in the filtrate.
11. Often the plate reader itself will be able to reduce the data using the built-in equations available. Raw data of the optical density measures (including blank wells) from the plate reader were saved in Microsoft Excel and imported to Master Plex's virtual plate for reduction.

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