The effect of housing conditions on sex differences in spatial cognition in rats

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Declaration

The work contained within this thesis is my own and has not been done in collaboration, except where otherwise stated. The text does not exceed 70,000 words, and no part of this thesis has been submitted to any other university in application for a higher degree.

Anjanette Harris

Ethical note

All animal treatment, husbandry and experimental procedures were carried out in accordance with the Animals Scientific Procedures Act 1986, U.K. and the associated Guidelines under Home Office Project licence number 60/3531 and Personal licence number 60/9363. At the end of each experiment, all rats were humanely euthanased by exposure to a gently rising concentration of carbon dioxide. When possible, organs were harvested from the rats for use by other researchers in the department in an attempt to reduce the numbers of animals used.

Abstract

Male mammals typically outperform females in tests of spatial ability. However, in laboratory rats *(Rattus norvegicus),* from which the majority of data in support of this difference come, sex differences are not consistently found. Since stress affects cognition in males and females differently, I investigated possible sources of stress (e.g. housing conditions, spatial tasks) and the impact they have on cognitive performance in male and female rats.

Firstly, I investigated whether isolation housing, purported to be chronically stressful, affected the presence of sex differences in a dark-eyed and an albino strain of rat. Irrespective of sex and strain, I found that young or old rats isolated for long or short periods were not behaviourally or cognitively impaired relative to pair-housed conspecifics. I found, however, that behaviour caused by the acute stress of the task impeded performance. Furthermore, sex differences in performance were found only when the females were more stressed than the males during testing. Additionally, the degree to which the rats found the task stressful depended upon the age at which they travelled from the breeding establishment. In the dark-eyed strain, males were always less stressed than the females, but also out performed the females only if they travelled while young (4-5 weeks old). Both sexes seemed to be less stressed by the task if the rats travelled as adults. Conversely, in the albino strain, males outperformed females only if the rats travelled as adults, because in the young travellers both sexes were equally and highly stressed during testing. Therefore, the acute stress response, which seems to underlie sex differences in cognitive performance, was influenced by the age at which the rats travelled in a sex and strain dependent manner.

Next, I considered the impact of the physical attributes of the home cage on a rat's welfare and performance in a cognitive task. I found that, male and female rats housed with a barrier that reduced visual contact from their cage showed higher levels of behavioural stress in their home cage than did rats housed without a barrier between the cages. Rats housed with the barrier were also more stressed during spatial testing and had poorer cognitive performance relative to rats housed without the barrier. Pair housing did not ameliorate the effect of the barrier. Based on these data, although a rather unorthodox suggestion, I propose that single housing with a

view may be preferable to pair housing without a view. One implication of this finding is that the number of animals used in an experiment could be significantly reduced if the home cages allow sufficient visual interactions.

Lastly, I investigated the impact of environmental enrichment on spatial cognition and behavioural stress responses. I found, contrary to current opinion, that enriched rats outperformed non-enriched animals not because they had superior cognitive ability but because their behavioural stress response was reduced significantly during testing. Furthermore, withdrawing enrichment from rats for at least one week did not increase stress responses during testing or impair cognitive performance. Therefore, exposure to enrichment, even if later withdrawn, improves welfare by reducing stress during cognitive testing.

In conclusion, a differential behavioural stress response during cognitive testing may explain why males outperform females and why enriched animals do better than non-enriched animals in tests of spatial cognition. Furthermore, variation in this behavioural stress response may in part explain why sex differences in performance are not consistently found in laboratory rats.

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CHAPTER ONE: Introduction

Male mammals generally outperform females in tests of spatial cognition. However, the majority of data in support of this difference come from studies with laboratory rats *(Rattus norvegicus)* and sex differences are not consistently found in this species. In this thesis I examine potential explanations for the discrepancy in the literature regarding cognitive sex differences in rats. Stress affects cognition differently in males and females, therefore, I have focused on how stress, which is experienced either as a consequence of laboratory housing or imposed inadvertently during cognitive testing, affects male and female cognitive performance.

1.1. Introduction

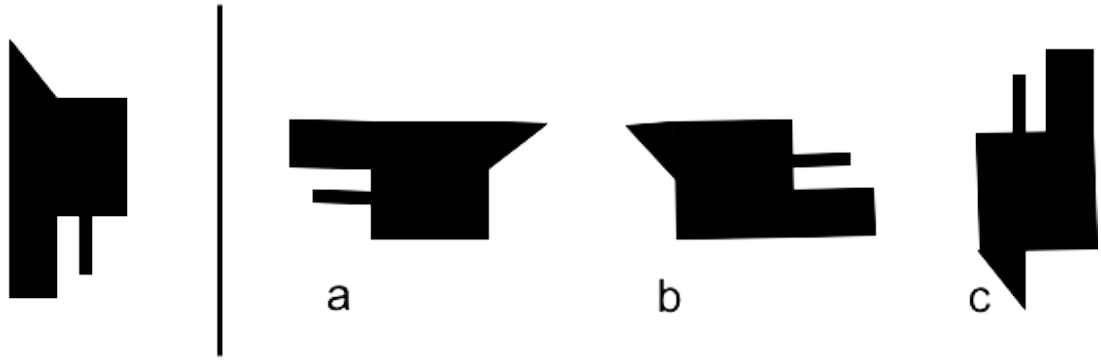
What is spatial cognition?

Spatial cognition encompasses a collection of cognitive abilities that are involved in the detection, processing, storage and retrieval of information relating to navigation and the perception of three-dimensional objects in space. Good spatial ability facilitates successful navigation around an environment and is an essential life skill for most animals (Shettleworth 1998). The majority of our understanding about how animals navigate (e.g. the types of cues that they use and what causes variation in spatial ability) comes from studying spatial learning and memory in the laboratory, where cues and subjects can be readily controlled or manipulated.

How is spatial cognition studied?

Spatial cognition in humans is often tested using mental rotation tests (Figure 1.1), map reading or computer-generated mazes. However, psychological and social variables (e.g. life experience) cannot be controlled in human studies. For example, some humans (boys, perhaps) may have more experience playing computer games and this may influence performance in computer-based tasks. There are also, for ethical reasons, limitations to the manipulations that can be applied to human subjects (there is particular opposition to performing brain lesions and hormonal manipulations on healthy human subjects). Consequently, the majority of what we know about spatial cognition comes from studying rodents, and the easiest way to test a rodent's spatial ability in the laboratory is in a maze.

Figure 1.1: An example of a typical mental rotation test for humans. Subjects have to identify which of the three choices on the right (a to c) is the same as the shape on the left, except for rotation. The answer is a.

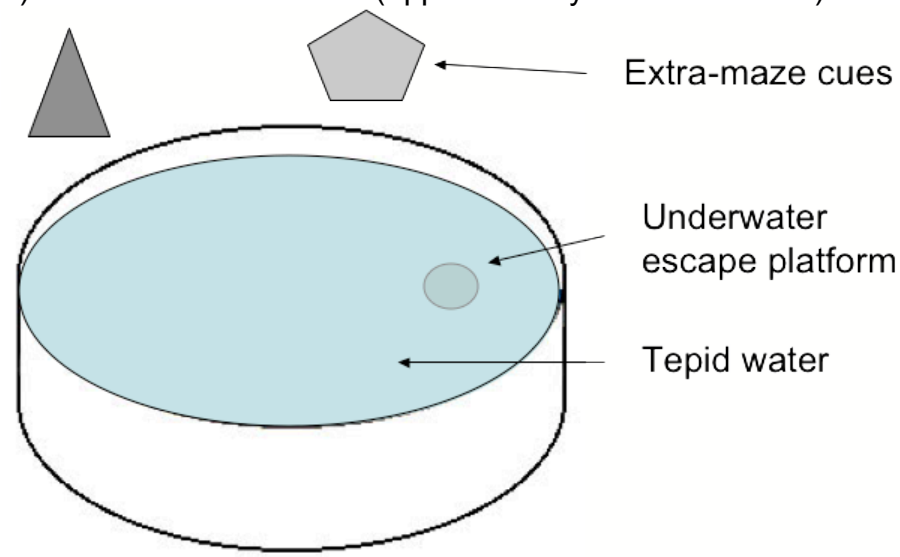


The most universally used mazes are the Morris water maze (MWM) and the radial arm maze (RAM; Levin 2001; Terry 2001). The MWM consists of a large circular pool (approximately 2m in diameter) filled with opaque tepid water in which a platform (approximately 15cm in diameter) submerged under the surface of the water is the only escape, see Figure 1.2.a (Morris et al. 1982; Morris 1984). With successive swims a rat learns to locate this platform remarkably quickly using extra-maze cues such as posters on the walls, the shape of the room and so on. The most common measure of performance in an MWM is the time taken to find the platform (Hodges 1996). Conversely, in a RAM, a rodent forages for food rewards at the ends of arms that radiate from a central chamber, see Figure 1.2.b (Olton and Samuelson 1976). To solve this maze, a rat can use either extra-maze cues, or, if all of the arms contain a reward, non-spatial strategies such as sequentially entering adjacent arms. The choice of the arms that the rodent enters provides a measure of spatial ability.

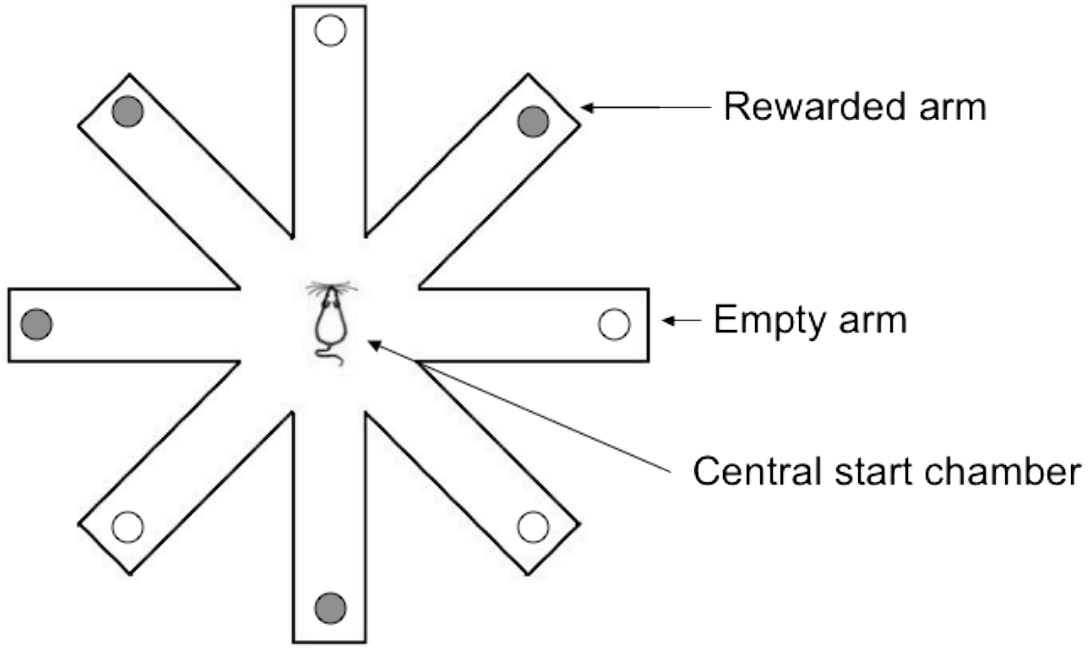
Both the RAM and the MWM can be used to assess the two major types of memory: working and reference memory. Working memory is defined as memory for information that is trial specific and so generally useful for a short period of time. Memory for information that is relevant from trial to trial, and so useful for longer periods of time, is referred to as reference memory.

Figure 1.2: The two most common mazes used to study spatial ability in rodents.

a) The Morris water maze (approximately 2 m in diameter).



b) The radial arm maze (approximately 2 m across).



Applications of studying spatial cognition

As well as shedding light on how animals navigate, assessing rodent cognition in a RAM or an MWM has numerous pharmaceutical and medical applications (reviewed in D'Hooge and De Deyn 2001). For example, the MWM can be used to investigate the effects of brain trauma (rodents are given brain lesions) on cognition (e.g. Hamm et al. 1992), the effects of aging and hormone replacement therapies on cognitive processes (e.g. Markowska 1999), and to develop drug therapies for neurodegenerative diseases (there are rodent models of Alzheimer's, Huntington's and Parkinson's Disease). Toxicological applications include testing the effects of drugs on a rat's performance in an MWM to ensure that various chemicals are safe and have no aversive side-effects on cognition in healthy animals (e.g. Mendez et al. 2008). In pharmaceutical and toxicological testing, typically, male rats are preferentially used over females because, for example, fluctuating hormone levels across the oestrous cycle in females may interact with drug effects (e.g. reviewed in Hughes 2007). However, testing only males raises welfare concerns, because large numbers of unwanted female rats are culled soon after birth. Even more problematic, is that potentially very important data are being ignored because females are not being tested (Hughes 2007). After all, half of most animal populations are female. Furthermore, there is evidence that the sexes differ in performance in the MWM (reviewed in Jonasson 2005).

1.2. Sex differences in spatial cognition

One of the best-studied causes of variation in spatial ability is sex. Males tend to outperform females in spatial tasks in a number of mammalian species (e.g. *Peromyscus maniculatus* (deer mice), Galea et al. 1996; *Macaca mulatta* (rhesus macaques), Lacreuse et al. 1999; *Mus musculus* (lab mice), Gresack and Frick 2003; *Rattus norvegicus* (lab rats), Isgor and Sengelaub 2003; *Homo sapiens* (humans), Rahman and Koerting 2007). Consequently, at least seven evolutionary hypotheses have been proposed to explain the existence of a sex difference in spatial cognition (reviewed in Jones et al. 2003). However, a significant proportion of the data for sex differences come from studies with laboratory rats and sex differences are not consistently found in this species. Although a large number of authors report that

male rats out-perform females in spatial tasks, almost as many authors report no difference in performance between male and female rats (see Table 1.1). While the type of task, strain of rat, and type of housing may explain some variation in the data, none of these variables appear to explain fully why sex differences are not consistently found (Table 1.1). Even within the same laboratory, under apparently identical conditions, sex differences are not reliably reproduced (e.g. Roof 1993a; Roof and Stein 1999).

The inconsistency with which sex differences are found in rats raises the possibility that sex differences in laboratory rats exist outside of the laboratory, but that some feature of the laboratory (e.g. the test situation or housing conditions) obscures the sex difference. Alternatively, sex differences in rats may be an artefact that is produced as a consequence of the animals' being in, or tested in, the laboratory.

1.3. Mechanisms for sex differences in spatial cognition

Although the functional explanations for sex differences in spatial cognition are currently under fervent debate (e.g. Jones et al. 2003; Ecuyer-Dab and Robert 2004; Silverman et al. 2007; Saucier et al. 2008), the proximate causes of sex differences in spatial ability have been relatively well characterized, at least, in rodents. Both sex and stress hormones pass through the blood-brain barrier, bind with their receptors in the hippocampus (the area of the brain implicated in processing spatial information in mammals: O'Keefe and Nadel 1978; Morris et al. 1982; Kessels et al. 2001) and influence spatial cognition. However, these explanations for sex differences in spatial ability have opposing implications: if sex hormones are the main mechanism then this implies that sex differences in spatial cognition exist outside the laboratory. On the other hand, if stress hormones are responsible it is possible that sex differences in spatial cognition in rats are an artefact due to the laboratory situation. Of course, it is also possible that both sex and stress hormones, and the interplay between them, shape an animal's cognitive ability. In this thesis I have investigated whether stress is a plausible explanation for the lack of consistency in, perhaps even the existence of, sex differences in spatial cognition in rats.

Table 1.1: Some of the most regularly cited studies that have investigated sex differences in spatial ability using unmanipulated rats. All sex differences in performance were due to males outperforming females. MWM = Morris water maze; RAM = radial arm maze; WM = working memory (memory for information within a trial); RM = reference memory (memory for information across trials). W = Wistar rats; SD = Sprague-Dawley rats; F = Fischer 344 rats (all albino strains) and LE = Long Evans rats; LH = Lister Hooded rats (all dark-eyed strains).

Study	Task	Memory	Strain	Rats/cage	Sex diff?
(Blokland et al. 2006)	MWM	RM	W	2	**Yes**
(Bucci et al. 1995)	MWM	RM	LE	1	No
(Cimadevilla et al. 1999)	MWM	RM	W	5	**Yes**
(Conejo et al. 2004) exp. b	MWM	RM	W	?	**Yes**
(Isgor and Sengelaub 1998)	MWM	RM	SD	?	**Yes**
(Isgor and Sengelaub 2003)	MWM	RM	SD	1	**Yes**
(Jonasson et al. 2004)	MWM	RM	LE	1	**Yes**
(Kanit et al. 1998)	MWM	RM	SD	3-4	No
(Kanit et al. 2000)	MWM	RM	SD	3-4	No
(Lukoyanov et al. 1999)	MWM	RM	W	?	No
(Perrot-Sinal et al. 1996) exp. a	MWM	RM	LE	1	**Yes**
(Perrot-Sinal et al. 1996) exp. b	MWM	RM	LE	1	No
(Snihur et al. 2008)	MWM	RM	LE	2	**Yes**

Study	Task	Memory	Strain	Rats/cage	Sex diff?
(Conejo et al. 2004) exp. a	MWM	WM	W	?	No
(Healy et al. 1999)	MWM	WM	LH	2	No
(Nuñez et al. 2000)	MWM	WM	LE	2	No
(Roof and Stein 1999) exp. a	MWM	WM	SD	group	**Yes**
(Roof and Stein 1999) exp. b	MWM	WM	SD	group	No
(Saucier et al. 2008)	MWM	WM	LE	5	**Yes**
(Warren and Juraska 1997)	MWM	WM	LE	2	No
(Markowska 1999)	MWM	WM and RM	F	3-4	No
(McFadden and Matuszewich 2007)	MWM	WM and RM	SD	2	**Yes**
(Einon 1980) exp. a	RAM	WM	W	1	No
(Einon 1980) exp. b	RAM	WM	W	5	**Yes**
(Endo et al. 1994) exp. a	RAM	WM	W	1	**Yes**
(Endo et al. 1994) exp. b	RAM	WM	W	1	No
(Juraska et al. 1984)	RAM	WM	LE	12 or 1	No
(Levin et al. 2005)	RAM	WM	SD	?	**Yes**
(Takase et al. 2008) exp. a	RAM	WM	W	2	**Yes**
(Takase et al. 2008) exp. b	RAM	WM	W	2	No

Study	Task	Memory	Strain	Rats/cage	Sex diff?
(Boakes et al. 2000)	RAM	WM and RM	W	8	No
(Gibbs and Johnson 2008)	RAM	WM and RM	SD	1	**Yes**
(Lund and Lephart 2001)	RAM	WM and RM	?	1	**Yes**
(Seymoure et al. 1996)	RAM	WM and RM	LE	13 or 1	**Yes**
(Roof 1993a) exp. a	RAM/ MWM	RM	SD	3-5	**Yes**
(Kolb and Cioe 1996)	RAM/ MWM	WM and RM	?	1	No

1.3.1. Sex steroids

When looking for mechanistic explanations for sex differences in spatial cognition, the most obvious place to start is with the sex hormones. Indeed, experimental manipulation of gonadal hormone titres confirms that sex steroids influence spatial learning and memory. Sex hormones that are experienced *in utero* and/or during early post-natal development can have an effect on an animal's spatial ability during adulthood (organisational effects). Additionally, sex hormones that are experienced during adulthood can also influence an adult animal's spatial cognition (activational effects). The greatest impact of gonadal hormones on spatial cognition appears to be during early development (i.e. organisational effects) because sex differences are found between male and female rats that have been gonadectomised at puberty (which removes all endogenous sources of sex steroids Williams et al. 1990) and castration in adulthood does not seem to impact on spatial memory in male rats (Isgor and Sengelaub 1998; Sandstrom et al. 2006; Gibbs and Johnson 2008). Whereas, neonatally castrated males, which have very low levels of testosterone during development, have poorer spatial ability than unmanipulated males (Joseph et al. 1978; Williams et al. 1990; Isgor and Sengelaub 2003). Additionally, female rats treated within 24 hours after birth with estradiol benzoate (a synthetic oestrogen that

has masculinising effects) outperform unmanipulated females in spatial tasks (Williams et al. 1990). Similarly, administration of testosterone to neonatal females improves spatial ability relative to control females (Roof and Havens 1992; Roof 1993a; Isgor and Sengelaub 1998). However, giving testosterone to neonatal gonadally intact males impairs adult cognition (Roof and Havens 1992; Roof 1993a). Taken together these data demonstrate that there is an inverse U-shaped relationship between testosterone and spatial cognition, and that the optimal level of testosterone exposure during early development, which engenders good spatial ability, lies in the low male range (Gouchie and Kimura 1991; Kimura 1999).

Insight into how sex hormones may influence (and so lead to sex differences in) spatial ability is provided by studies that demonstrate that sex hormones affect the hippocampus in rodents. The hippocampus is sexually dimorphic in adult and prepubescent rats. Specifically, the granular cell layer of the dentate gyrus (DG-GCL-) in the hippocampus in gonadally intact males is laterally asymmetrical (thicker on the right side than on the left side; Roof and Havens 1992; Roof 1993b). Additionally, the DG-GCL is approximately 9% thicker in right hemisphere in males than in females (Roof 1993b). And as with spatial ability, early hormone levels organise the development of the hippocampus (Roof 1993b; Nuñez et al. 2000). For example, females that are neonatally treated with testosterone develop a male-like hippocampus (Roof and Havens 1992; Roof 1993b). And males that are neonatally treated with flutamide (an anti-androgen) develop a female-like hippocampus (e.g. Isgor and Sengelaub 1998).

As well as exerting long-lasting effects during development, fluctuations in circulating levels of sex steroids at the time of testing can have transitory effects on spatial ability and hippocampal morphology. For example, in two species of cyclically breeding deer mice (*Peromyscus mainculatus* and *P. m. aretmisae),* there are no sex differences outside the breeding season when males are assumed to have low levels of testosterone levels (hormone levels were not explicitly measured: Galea et al. 1995). In a later study the neural and hormonal data were found to concur with the observed behavioural changes: the generation of new neurons in the hippocampus was highest in the breeding season when there was an approximate eight-fold increase in testosterone levels and spatial ability was enhanced (hormone

levels were measured in this study: Galea and McEwen 1999). Activational levels of hormones can, therefore, still influence cognition and the brain despite the organisational effects that have occurred during development.

Activational levels of sex hormones at the time of testing also influence spatial cognition in females. However, the relationship between circulating levels of oestrogen across the oestrous cycle (which lasts approximately five days in rats) and spatial cognition seems to be a rather obscure one. While there is some evidence that high levels of oestrogen (e.g. experienced during the pro-oestrous phase of the oestrous cycle) enhance working memory (Daniel et al. 1997; Healy et al. 1999), there are mounting data to the contrary (impaired working memory: Warren and Juraska 1997; Markowska 1999; Holmes et al. 2002). Additionally, high oestrogen appears to impair reference memory (Frye 1995; Galea et al. 1995; Warren and Juraska 2000). Conversely, there are a number of studies in which there is no effect of oestrogen, natural or exogenously administered, on spatial cognitive performance (e.g. Berry et al. 1997; Stackman et al. 1997; Varga et al. 2002). It is possible that the inconsistent reports are due to a dose dependent effect of oestrogen on cognition (i.e. in a non-linear relationship as is seen with testosterone). Additionally, other hormones may act in synergy with oestrogen. For instance, progesterone also fluctuates in harmony with oestrogen across the oestrous cycle. However, attempts to separate the effects of oestrogen and progesterone have produced opposing findings. For example, ovariectomised females given either hormone alone were impaired relative to control rats, but rats given both hormones together had impaired performance in the MWM (Chelser and Juraska 2000). Conversely, in another study, either hormone alone had no effect but both hormones together enhanced MWM performance (Sandstrom and Williams 2001). Nevertheless, while there is little consensus on whether oestrogen impairs or enhances cognition, it is entirely plausible that females under-perform relative to males only at certain points in the oestrous cycle. If performance in females fluctuates, and is then averaged across the cycle, then males will appear to do better.

While sex hormones experienced early in life affect both spatial cognition and the area of the brain that is involved in processing spatial information, there are few data from studies in which both organisational and activational levels have been

manipulated at the same time. Thus, while organisational effects of sex steroids are sufficient to cause sex differences in cognition (i.e. activational effects are not required) it is possible that circulating levels of hormones at the time of spatial testing swamp or alter these effects.

In sum, while sex hormones provide a potential proximate mechanism for sex differences in spatial cognition, the relationship between sex steroids and cognition is not straightforward and our understanding of this relationship is far from complete. Furthermore, sex steroids are not the only plausible mechanistic explanation for sex differences because stress steroids also influence spatial cognition.

1.3.2. Stress hormones

In addition to the sex hormone data, there are a plethora of data showing that stress hormones influence spatial cognition in rats. Stress causes the release of many hormones, peptides and neurotransmitters in to the blood, the exact profile depending, at least in part, upon the type of stressor (e.g. Van de Kar and Blair 1999). For example, when an animal perceives a stressor (real or imagined) stimulation of the adrenal glands via the sympathetic division of the autonomic nervous system results in the release of (nor) adrenaline and dopamine into the blood within 2-3 seconds. Additionally, endorphins (which have pain relieving properties) and vasopressin (which constricts blood vessels) may also be released by the pituitary gland (Olson et al. 1997; Mendl 1999). Stress also triggers the hypothalamic-pituitary-adrenal axis, which results in the release of glucocorticoids (e.g. corticosterone) from the adrenals within approximately 2-3 minutes (Joëls et al. 2006). The function of the physiological stress response is to enable the animal to cope with the source of stress either by fleeing or fighting. For example, glucose availability, heart rate, breathing, and blood pressure increase, and blood vessels and pupils dilate, as a result the animal enters a heightened state of arousal and its body is prepared for violent muscular activity (Joëls et al. 2006). Nevertheless, how an animal actually responds, in terms of behaviour, to a stressor is very complex and can depend on numerous factors e.g. the context of the stress, the source of stress, the availability of escape etc. Throughout this thesis I will use the blanket term ‘stress’

to refer to a short or long term event or condition that is perceived as threatening or unpleasant by an animal.

Stress can enhance or disrupt learning and memory depending, for example, upon the type of task, type of stress, training conditions and the sex of the animal. The simplistic view is that the relationship between stress and spatial cognition appears to be non-linear, with the best performance occurring when stress hormone levels are in the mid range (e.g. reviewed in Mendl 1999; Joëls et al 2006). In the hippocampus, the highest levels of long-term potentiation (a mechanism thought to underlie memory formation) also occur when stress levels are in the mid range (e.g. Pavlides et al. 1995).

The reason for suspecting that stress may underlie sex differences in cognition is that stress affects cognitive performance in males and females differently: females often respond more poorly to acute stress (short-term stress) than do males, while males respond more poorly to chronic stress (long-term stress) than do females. In the following section I shall outline: 1) some likely sources of stress that a laboratory rat may encounter during a cognitive experiment, and 2) how different kinds of stress (acute vs. chronic) impact on male and female spatial cognitive performance.

1) Sources of stress

A laboratory rat may face at least two possible sources of stress during an experiment. Firstly, housing conditions may be a source of chronic stress (e.g. grid flooring, isolation, crowding, barren cages). Typically, rats are housed in small barren cages to reduce environmentally-induced variation across and within laboratories. Rats are also often housed in isolation (see Table 1.1) and this can lead to behavioural and physiological changes in rats (reviewed in Krohn et al. 2006). Specifically, isolated rats may eat more (e.g. Fiala et al. 1977), gain more weight (Morgan and Einon 1975), and perform 'odd' behaviours in their home cage (bar biting, tail chasing, pawing at the air; Baenninger 1967; Hurst et al. 1997, 1998) relative to socially-housed conspecifics. Additionally, isolated rats can be aggressive when handled (Hatch et al. 1963), may have higher resting levels of stress hormones and show increased cardiovascular responses (heart rate and blood pressure) to saline

injection relative to socially-housed rats (Brown and Grunberg 1996; Sharp et al. 2002a, 2003). Since rats are a naturally gregarious species the behavioural and physiological changes that occur in response to isolation are believed to be indicative of increased stress levels. As a consequence of the negative impact of stress on welfare, the Home Office (the major regulatory body in the U.K. for experiments that use animals) strongly recommends that rats

"… should be socially housed wherever possible with compatible individuals, and only single housed if there is good justification on veterinary, husbandry or welfare grounds…" (Home Office 1995).

Nevertheless, isolation housing is still used in the U.K. and other parts of the world (e.g. Canada, USA) for scientific, animal welfare or logistical reasons. For example, rats may be housed alone after surgery, to determine food intake for one rat, to remove social stress associated with group housing or if cage mates die.

As well as impairing welfare, isolation can lead to spatial cognitive deficits. Isolation of neonatal rats (15-21 days old) for six hours per day for six days resulted in increased basal levels of CORT and significantly impaired performance in the RAM in adult males (Sandstrom 2005; Sandstrom and Hart 2005). Isolation during adulthood also seems to impair performance in the MWM and the RAM in males and females (e.g. Moser et al. 1994; Nilsson et al. 1999; Pham et al. 1999). However, the effect of isolation in these studies is confounded with the effects of increased environmental complexity, because comparison animals are often housed in larger cages that are supplemented with social and physical enrichment (>5 cage mates, nest boxes, bedding material, climbing ladders and so on). Additionally, it is currently unclear if isolation housing has a greater impact on spatial cognition in younger (i.e. post weaning) animals than it does in adult animals. Since 'play behaviour' in rats peaks at approximately 32-40 days of age and then declines into adulthood, if play is important during juvenile development it is possible that social isolation is particularly stressful for a young rat (e.g. Panksepp 1981).

A second major source of stress for a rodent in an experiment is the cognitive test itself (Sandi 1998). Cognitive tests may be stressful because animals are removed from their home cages, handled, and placed in unfamiliar environments that

may be unpleasant (e.g. brightly lit, water, open spaces etc). Additionally, experimental manipulations, for example, food restriction for appetitive tasks such as the RAM, may cause stress. Similarly, the MWM (the most commonly used spatial task) is considered to be an acutely stressful task because the animal is placed in an open-field like situation and immersed in lukewarm water in a brightly lit unfamiliar room. Immediately after the first swim in an MWM rats show an approximately three-to-four fold increase in corticosterone levels, and the colder the water the greater the stress response (Sandi et al. 1997; Beiko et al. 2004; Aguilar-Valles et al. 2005). Consequently, the Home Office deem the MWM a licensable procedure in some parts of the U.K. Additionally, rats initially swim thigmotactically (wall-hugging) when they are put into an MWM (e.g. Beiko et al, 2004). Since avoiding open spaces reduces a rodent's exposure to (aerial) predators, thigmotaxis is believed to have evolved as a natural anti-predator response (Treit and Fundytus 1989; Bonsignore et al. 2008). Because an increase in thigmotaxis indicates that a rodent is fearful of predation, this behaviour is considered a marker for anxiety in rodents in open-field like situations (Perrot-Sinal et al. 1996; Beiko et al. 2004; Herrero et al. 2006; Wilcoxon et al. 2007; Bonsignore et al. 2008). Furthermore, administration of anxiolytics significantly reduces thigmotaxis during open-field testing (e.g. Treit and Fundytus 1989) and administration of corticosterone significantly increases thigmotaxis in male and female rats during MWM testing (Snihur et al 2008).

Thus, rodents can face chronic stress from unfavourable housing/husbandry conditions and/or acute stress that is experienced inadvertently in the test situation. Crucially, both acute and chronic stress influence spatial cognition and the different types of stress seem to affect males and females differently.

2) Sex differences in response to stress

Acute and chronic stress affect the sexes differently. Spatial ability seems to be impaired to a greater degree in males than it is in females following exposure to chronic stress. For example, restraint for six hours per day for 21 days impairs male performance in the RAM, MWM and the Y-maze (a three armed version of the RAM) but enhances, or does not affect, female performance (Bowman et al. 2001; Bowman et al. 2003; Conrad et al. 2003; Kitraki et al. 2004; Bowman 2005). A

potential mechanism that may underlie this stress-induced cognitive deficit is that chronically high levels of corticosterone damage hippocampal neurons in male rats. For example, chronically elevated levels of corticosterone can lead to reversible atrophy of dendrites of hippocampal CA3 neurons (these neurones are important for long term potentiation; i.e. memory formation) and this is coupled with reversible learning and memory deficits in the RAM (Watanabe et al. 1992; Luine et al. 1994; Galea et al. 1997). It is possible then, that a sex difference in cognitive performance may not be found if rats are chronically stressed (e.g. housed in isolation) because males may underperform relative to if they were not stressed. This demonstrates how the effects of housing conditions could obscure sex differences in cognition.

Acute stress also appears to influence spatial cognition, and females respond with greater stress responses to acute stress. For example, female rats display higher resting levels of corticosterone and show faster and larger corticosterone responses for longer following exposure to acute stress (e.g. restraint, electric shock or forced running Kant et al. 1983; Handa et al. 1994). Additionally, females display more stress related behaviour, such as thigmotaxis, than do males during MWM testing (e.g. Beiko et al, 2004; Perrot-Sinal et al 1996). Typically stress related behaviours impede performance in cognitive tasks. For example, in an MWM a thigmotactic rat will take longer to find the platform than will a rat which ventures into the centre of the tank because the platform is never located in the outer edge.

In addition to behavioural impairments, females also display a greater disruption in learning than do males following exposure to acute stress. For example, hippocampal dependent learning is enhanced in male rats following exposure to acute stress: classical eye blink conditioning (which, similar to spatial learning, requires an intact hippocampus) is learned faster after exposure to restraint coupled with intermittent tail shocks (Shors et al. 1992). The opposite was found in females: eye blink conditioning was impaired following exposures to an acute stressor (Wood and Shors 1998). There is also evidence that spatial learning in the MWM is enhanced in male rats under acutely stressful conditions. If the water is cold (19°C) the rats have higher corticosterone responses and find the platform sooner than if the water is warmer (25°C). Females, however, were not tested in this study (Sandi et al. 1997).

There are at least three lines of evidence that support the hypothesis that sex differences in spatial cognition arise because females deal less well with acute stress during cognitive testing: 1) sex differences in both the MWM and the RAM are typically found early in testing, when tasks are unfamiliar and so potentially more stressful (e.g. Juraska et al. 1984; Williams and Meck 1991; Seymoure et al. 1996; Snihur et al. 2008); 2) if rats are pre-trained (which habituates the rats to the task) males and females perform equally well (Bucci et al. 1995; Perrot-Sinal et al. 1996; Healy et al. 1999; Nuñez et al. 2000; Beiko et al. 2004) and 3) adrenalectomy, which removes the endogenous source of stress hormones, removes sex differences in performance in the MWM (Beiko et al. 2004; Snihur et al. 2008). Under this final line of evidence, further support that stress causes apparent sex differences in cognition comes from experiments that involve the manipulation of endogenous opiate activity (which is associated with stress). In deer mice and meadow voles, administration of opiate antagonists (which inhibit opioid function) or exposure to an extremely low frequency magnetic field (which also inhibits opioid activity) improves MWM performance in females and minimises apparent sex differences in performance (Galea et al. 1994b; Kavaliers et al. 1996). These data illustrate that opiates, released in response to stress, impair female spatial cognition but have no impact on male cognition.

1.4. Summary and thesis questions

To summarise, sex differences in spatial cognition are not consistently found in laboratory rats. Although, sex hormones have both organisational and activational effects on spatial cognition, stress hormones also influence cognition differently in male and female rats. Since housing and cognitive tests provide potential sources of stress, it is entirely plausible that stress is the ultimate explanation for sex differences in spatial cognition in laboratory rats. Therefore, in this thesis I focussed on the impact of stress on spatial cognition in male and female rats. I manipulated chronic stress by using isolation housing (believed to be chronically stressful) and social housing (thought to alleviate stress and frustration; Patterson-Kane et al. 2002) and I tested cognition in an MWM because this is one of the most frequently used tests and it is acutely stressful. I did not manipulate acute stress levels but I could determine whether chronic isolation stress acted in concert with acute stress and whether males

and females were affected to the same degree. I measured thigmotaxis to assess acute stress during testing and bar biting in the home cage to assess stress associated with different housing conditions. Body weight and food intake were also monitored as additional measures of welfare (both typically increase in isolated rats e.g. Fiala et al. 1977).

The following questions were addressed in this thesis:

1) **Does isolation housing cause sufficient stress to impact on spatial ability and does this stress affect the sexes differently?**

I predicted that isolated rats would under perform relative to socially housed conspecifics (e.g. Lu et al. 2003). Additionally, since chronic stress impairs males to a greater degree (e.g. Luine 2002) I predicted that males would be cognitively impaired to a greater degree than would females, therefore, if sex differences exist (and are not an artefact due to laboratory testing), I predicted I would observe a sex difference in favour of males only in socially-housed rats.

2) **Does acute stress associated with the test situation (i.e. the MWM) impact on performance and does it affect males and females differently?**

I predicted that the MWM would be stressful (e.g. Aguilar-Valles et al. 2005) and that high levels of thigmotaxis would be correlated with impaired performance. Additionally, if sex differences are an artefact of stress that is caused by the test situation (and not by housing), then females would be more thigmotactic than would males and sex differences would be present irrespective of housing conditions.

3) **Does isolation housing have the same impact on young and adult rats? And does a longer period of isolation have greater impact on performance?**

I predicted that isolation would have a greater impact on younger animals because younger animals may value social contact more than do adult animals (e.g. adult rats play significantly less than do juvenile rats Panksepp 1981). It was unclear if longer periods of isolation would be more stressful than shorter periods of isolation.

4) **Do different strains of rat respond differently to stress caused by isolation housing or imposed by the test situation?**

Since reports of isolation-stress-syndrome are prevalent in albino strains of rat (e.g. Hatch al. 1963) and albino rats are reported to be more fearful in tests of anxiety and the MWM (e.g. Ennaceur et al. 2005; Schmidtt and Hiemke 1998) I predicted that an albino strain would respond more poorly than would a dark-eyed strain to stress associated with both isolation housing and cognitive testing in an MWM.

CHAPTER TWO: Does isolation housing cause sex differences in spatial cognition in Lister Hooded rats?

This chapter appears as the following publication: Harris, A. P., D'Eath, R. B. & Healy, S. D. 2008. Sex differences in spatial cognition are not caused by isolation housing. Behaviour, 145: 757-778.

The home cage behavioural data were collected with the assistance of Elizabeth Law and Marion Albinet. I analysed the data myself and produced the manuscript in collaboration with the authors listed.

Summary

In mammals, males typically have better spatial ability than do females. However, most of the data come from laboratory tests and it is possible that factors impacting on the captive animal cause the observed sex differences in spatial cognition. A common influence on cognitive ability is stress, which may have its effect acutely, in the testing situation, or chronically, due to the housing conditions. We used a spatial working and reference memory task (the Morris water maze) to investigate if isolation housing had a differential impact on spatial cognition in male and female rats. Either as juveniles or as adults, rats were housed in pairs or in isolation. We also manipulated the duration of isolation housing. Regardless of housing condition, we found a sex difference in spatial ability only in the youngest rats. However, we found no evidence that isolated rats were spatially impaired relative to pair-housed rats. We also found no difference in body weight, food intake or bar biting behaviour (indicators of welfare in rodents) between pair and isolated rats. We conclude that isolation housing causes insufficient stress to cause sex differences in spatial cognition.

2.1. Introduction

Males and females differ in morphology, physiology and, seemingly, in cognitive ability. The most consistent demonstration of a cognitive difference between the sexes is that males have better spatial skills than do females (Gaulin and Fitzgerald 1986; Williams et al. 1990; Lacreuse et al. 1999; Astur et al. 2004; Jones and Healy 2006). A number of evolutionary hypotheses (at least seven) have been proposed to explain this sexual dimorphism, the most strongly supported of which is the 'range size hypothesis', which proposes that relatively larger territory size selects for superior spatial ability in males (Jones et al. 2003). There are experimental data that conform to this prediction. For example, male meadow voles (*Microtus pennsylvanicus*) have larger home ranges than do conspecific females, superior spatial ability and a larger hippocampus (the area of the brain associated with processing spatial information) (Gaulin and Fitzgerald 1986, 1989). Conversely, the closely related pine vole (*Microtus pinetorum*) has no sexual dimorphism in home range size, spatial ability or size of the hippocampus (Jacobs et al. 1990).

Although there is debate as to the ultimate causes for a sexual dimorphism in spatial cognition, the proximate causes are well characterised. In mammals, at least, both organisational (before or soon after birth) and activational (circulating) levels of sex hormones impact on spatial ability throughout life. For example, girls exposed to high prenatal levels of testosterone have better spatial abilities than girls exposed to normal levels and administration of testosterone to newborn female rats results in the development of a male-like hippocampus and spatial ability akin to that of untreated males (Roof and Havens 1992; Grimshaw et al. 1995). Conversely, administration of testosterone to newborn male rats impairs adult spatial ability and neonatal castration of males (removal of a major source of testosterone) reduces adult spatial ability, indicating a possible U-shaped response curve between testosterone and spatial ability, with optimal levels in the low male range (Williams et al. 1990; Roof 1993a). Stress hormones also influence spatial ability. For example, social isolation of pre-weanling aged rats increases levels of the stress hormone corticosterone and results in poor spatial ability in adulthood (Frisone et al. 2002; Sandstrom and Hart 2005).

The majority of data for a sex difference in spatial ability come from laboratory experiments with rodents, largely rats. Male rats typically outperform females in the Morris Water Maze (MWM) and in a number of other spatial tests in the laboratory (Williams et al. 1990; Roof 1993a, b; Perrot-Sinal et al. 1996; Seymoure et al. 1996; Markowska 1999; Beiko et al. 2004). Although a male advantage is not always found, females rarely outperform males (Juraska et al. 1984; Bucci et al. 1995; Warren and Juraska 1997; Healy et al. 1999; Lukoyanov et al. 1999; Roof and Stein 1999). However, one potential problem with testing spatial cognition in the laboratory is that laboratory conditions may themselves affect the motivational and physiological state of the animal. Perhaps the most obvious example is that stress responses of males and females to their environment may differ and this may differentially affect cognitive performance between the sexes (Beck and Luine 2002).

Laboratory rats tested in spatial tasks may be exposed to stress from at least two sources. Firstly, the cognitive test itself may be a major source of stress for a laboratory rat. Spatial tasks such as the MWM are considered to cause stress because the animals are required to swim, usually in brightly lit conditions, to a platform hidden below the surface of the water at some distance from the side of the pool. Indeed, the UK Home Office classes the MWM as a licensable procedure due to the stress that it causes. Secondly, housing conditions of laboratory rats could be a potential source of stress. For example, the practice of housing rats alone may cause significant increases in stress for this naturally gregarious species (e.g. Patterson-Kane et al. 2004). Hatch et al. (1963) reported that after just four weeks of isolation, rats may be more aggressive, difficult to handle and show varying physiological impairments.

Concern about the effects of stress arises because the sexes appear to differ in their response to stress: females seem to cope less well than do males (e.g. Shors 2002). Certainly there is evidence that a sexually dimorphic response to stress during MWM testing can cause a sex difference in performance in rodents (Galea et al. 1994b; Perrot-Sinal et al. 1996; Beiko et al. 2004). For example, thigmotaxis (swimming close to the sides of the pool) is considered a marker of stress shown by rodents in open-field situations and females tend to engage in more thigmotactic

swimming in the MWM than do males. This will tend to result in impaired performance because the platform is never located at the edge of the pool (Treit and Fundytus 1989; Perrot-Sinal et al. 1996; Beiko et al. 2004; Herrero et al. 2006). Furthermore, there is also evidence that females respond less well to isolation housing than do males. For example, females show more escape-oriented behaviours (and more 'odd' behaviours such as tail chasing and bar chewing) when isolated (Hurst et al. 1998).

It is not yet clear whether the stress caused by isolation housing, either alone or in concert with the stress of swimming in an MWM task, can bring about the observed sex difference in spatial cognition. To address this question we housed male and female Lister Hooded rats alone or in pairs and tested their spatial cognition in an MWM. We sought to test whether isolation housing, purported to be stressful, could cause sufficient stress to impact on spatial cognition (possibly to a greater degree in females), and, thus result in a sex difference in spatial ability. During MWM testing we measured thigmotaxis as an indicator of stress. The MWM protocol we used allowed us to measure two different aspects of spatial memory: working memory (memory for within-trial specific information) and reference memory (memory for between trials). Additionally, we manipulated the timing and duration of isolation housing (Figure 2.1). We also measured body weight and food intake, and observed bar biting behaviour, all thought to be indicators of welfare in laboratory rodents (Würbel and Stauffacher 1996; Hurst et al. 1998).

We predicted 1) that the stress brought about by isolation housing would impair performance relative to pair housed controls; and 2) isolated females would perform more poorly than the other groups, such that a sex difference would only be seen in the isolated rats.

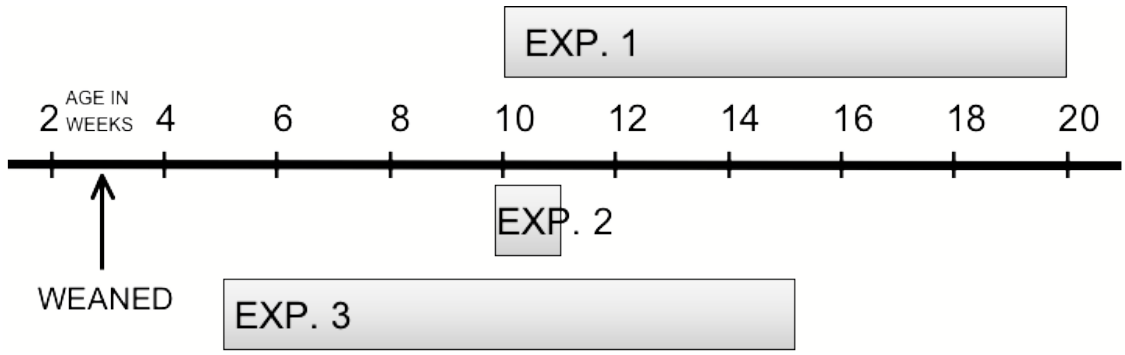


Figure 2.1: Schematic experimental timeline. We investigated the effect of isolation housing on spatial cognition in male and female Lister Hooded rats: in Experiment One, rats, aged 10 weeks at onset of the experiment, were tested after 10 weeks of isolation/pair housing; in Experiment Two rats 10 week old rats were tested after one week of the housing treatment; in Experiment Three, rats, aged four-five weeks at the onset of the experiment, were tested after 10 weeks of isolation/pair housing.

2.2. Experiment one: 10 weeks of isolation at 10 weeks of age

2.2.1. Materials and methods

Subjects and housing

The subjects were eighteen male and eighteen female Lister hooded rats, age 8-10 weeks obtained from Harlan U.K. Ltd. At time of arrival males weighed between 250-270 g and females 170-190 g. Six rats of each sex, were chosen at random and housed in isolation, the remaining 12 were housed in same sex pairs (N = 6 for each treatment group). Rats remained in their respective housing condition throughout the experiment. Paired rats were marked with hair dye (Schwarzkopf, R43) to enable identification. One rat from each pair was picked at random (dominance hierarchies, as determined by pinning rate 1, 7, 8 and 9 weeks post arrival, were unstable) to be the focal animal and this rat remained the only source of data from the pair for the duration of the experiment.

All rats were housed in standard plastic bottomed cages, dimensions 45x28x20 cm (North Kent Plastic cages Ltd., Kent, England). Due to the nature of the cages, visual, olfactory and auditory communication between neighbouring rats was not prevented. Rats were provided with *ad libitum* pellet food (RM3 diet, Special Diet Services, Ltd., Witham, Essex, U.K.) and tap water, and maintained under a 12L: 12D cycle (lights on at 0600 hours) at 21-24ºC. Each rat was handled (picked up using both hands clasped firmly and gently around the rat's body) at least twice weekly (e.g. during routine cage cleaning and weighing) for ten weeks prior to cognitive testing. At the start of MWM testing the majority of the rats appeared 'tame' (i.e. they did not struggle, squeak or attempt to bite during handling). During MWM testing each rat was handled daily for approximately 2-3 minutes in total (struggling and squeaking during placement in the MWM was frequently observed).

Rats experienced their respective housing condition for 10 weeks before spatial ability was assessed using the MWM. Each isolated and focal rat was tested in the MWM. Rats were euthanased in a carbon dioxide chamber at the end of the experiment.

Morris water maze apparatus

The MWM consisted of a circular tank made out of glass-fibre approximately 2 m in diameter, 65 cm high, with the bottom of the MWM raised 50 cm above floor level on a custom built platform. The MWM was situated in an experimental room (dimensions 4.25 m by 2.9 m) such that geometric cues (maze was not in the middle of the room) and landmark cues (e.g. light fittings, posters and shelving on walls) were available. The tank was filled to a depth of 32 cm with tap water, which was made opaque by the addition of 500 ml flooring latex, and maintained at 24±1ºC. An escape platform (white PVC of diameter 11 cm) was located 2 cm below the surface of the water and 30 cm from the edge of the pool in one of four possible locations (the four main compass points N, E, S or W). For each of the platform locations there were four possible release points into the pool: NE, SE, SW and NW. All trials were videotaped from above using a camera with a 4 mm wide-angle lens. To reduce both stress and distraction to the rats, all trials were observed via a video monitor.

Working memory procedure

Testing occurred during the light phase, to be consistent with the majority of published MWM research, which also uses daytime testing. Each rat received two days of training before testing began. During training each rat was given two swims to the hidden platform each day. The platform's location was the same within a day, but changed position each day and was pseudo-randomly determined so that it was never in the same place two days in a row.

For each swim the rat was gently lowered into the water at its predetermined release point and released facing the side of the tank. A swim started when the rat was released and finished when the rat found and subsequently climbed onto the platform. The time taken by the rat to find the platform was recorded to the nearest second. If the rat failed to find the platform within 120 seconds (s) it was gently guided to, and allowed to climb onto, the platform. Once on the platform the rat was left for 20 s before being picked up and released from one of the other three possible release points. After the final swim the rat was left on the platform for 20 s and then gently removed from the platform, towel dried, put back in its home cage and placed under a heat lamp for approximately 10 minutes to dry.

Testing started the day following the two days of training and was exactly as for training with the exception that each rat received four swims each day for 16 consecutive days in total.

Reference memory procedure

Reference memory was assessed from Day Two of testing to Day Five. The percentage of time that a rat spent swimming in each of the four quadrants of the maze in the first swim of each day (Days Two-Five only) was recorded. The quadrant that contained the platform was discounted and the proportion of time spent in the remaining three quadrants was calculated to establish if a rat spent more than 33.3% (chance) of its time searching in the target quadrant (the quadrant that contained the platform on the previous day).

Thigmotaxis

The percentage of time that a rat spent swimming within 15 cm of the wall of the maze was recorded for Swims One and Two on all test days.

Monitoring body weight and food intake

Body weight was measured at least once per week throughout the entire experiment for each isolated rat and each focal rat from each pair. Food intake was measured at least once per week from the second week post arrival to the week prior to MWM testing. To measure food intake, the entire contents of a food hopper (one per cage) were weighed before the food was topped up and re-weighed. Food intake per rat per day was estimated by dividing amount eaten by number of days since last weighed. Where rats were pair housed an average intake was calculated per cage.

Behaviour in home cage

The behaviour of the rats in their home cage was recorded by video camera (Sony mini DV digital handicam). Each cage was filmed for 10 minutes during the morning in the first and second months post arrival at the animal unit. This footage was then scored for the presence (1) or absence (0) of bar biting during 30 s intervals across the 10 minutes of footage. Every isolated rat and the focal rat from each pair were observed.

Data analysis

All data were analysed using the statistical software package JMP (version 5.1 for Apple MAC). Repeated measures data were analysed using a Repeated Measures Analysis of Variance (RM ANOVA); *between-subject* factors were sex (male and female) and housing condition (pair and isolated), and *within-subject* factors were Swim Number (One to Four) and Day (One to 16). All of these factors were included in the analyses, while interactions between main effects that were not significant were removed. The Mauchly-Criterion test was used to test for sphericity (the assumption that repeated measures have equal variances and that the correlations between any two measures are the same). When the assumption of sphericity was

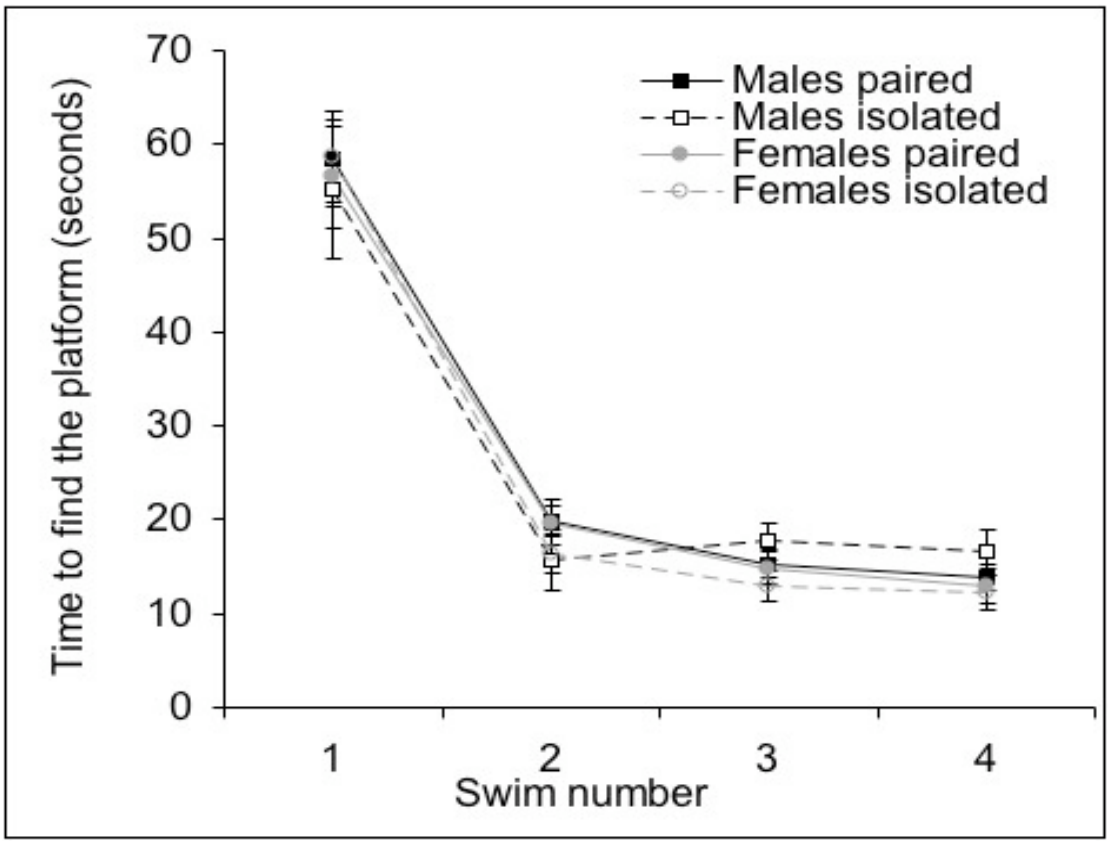
not met, the Greenhouse-Geisser adjusted degrees of freedom and the associated P-values were used, which is why the degrees of freedom reported are not always whole numbers (Quinn and Keough 2002). Bar biting data were analysed using Fishers Exact test. The assumptions of normality of residuals and homogeneity of variance were tested and appropriate transformations applied to the data, where necessary. Tukey's Honestly Significant Difference test ($P < 0.05$) was used to perform *post hoc* comparisons.

2.2.2. Results

Working memory

There was no significant effect of sex ($F_{1,21} = 0.80$, $P = 0.38$) or housing ($F_{1,21} = 0.25$, $P = 0.62$) on the time taken to find the platform (Figure 2.2). The sex by housing interaction was not significant. There was a highly significant effect of swim number on the time taken to find the platform: rats learnt the location of the platform during Swim One and swam almost directly to it in all three subsequent swims ($F_{1.3,27.4} = 154.20$, $P < 0.0001$; Figure 2.2). There was a significant effect of day on the time taken to reach the platform: as the days progressed the rats took less time to locate the platform ($F_{5.3,110.9} = 9.34$, $P < 0.0001$). No other interactions were significant.

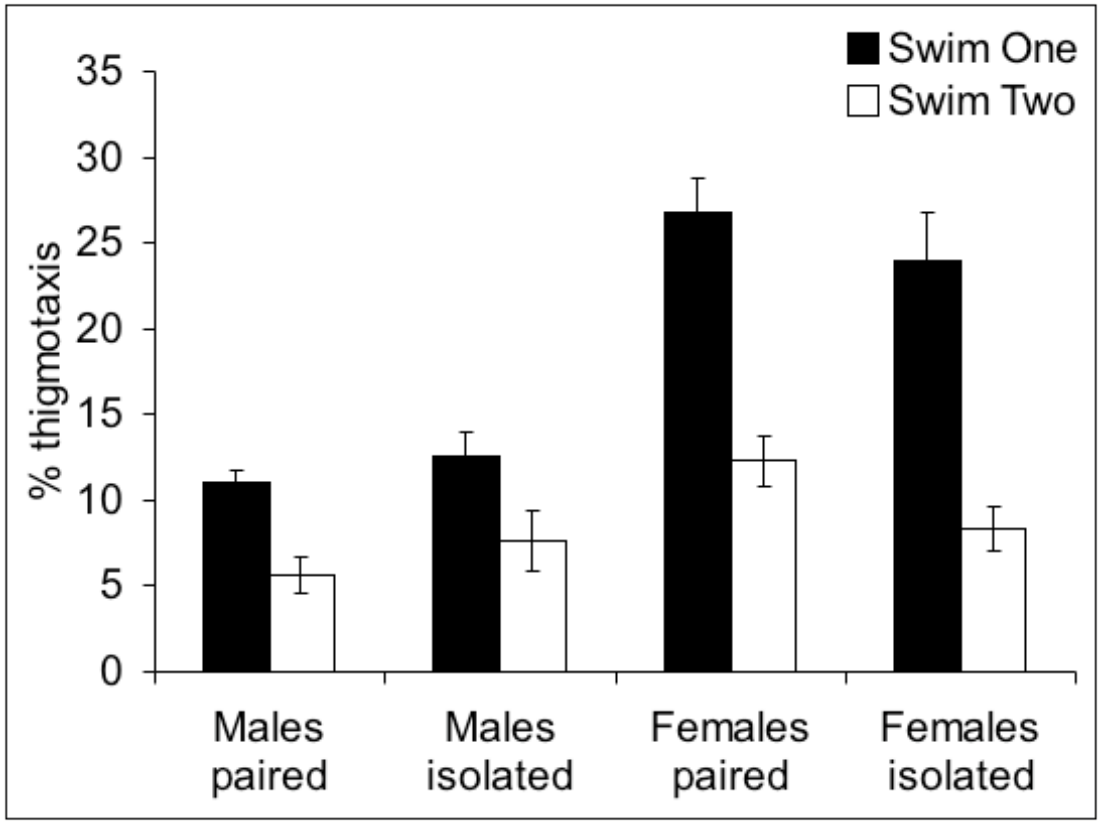
Figure 2.2: Average time taken to find the platform (mean ± SE) for male and female rats that were either pair or isolate housed, N = 6 per treatment group. Data are averaged across the 16 days of testing, analyses were conducted on daily swim data.



Thigmotaxis

There was a significant effect of sex ($F_{1,20}$= 55.54, P < 0.0001), which was dependent on housing condition ($F_{1,20}$= 4.99, P = 0.034). Paired males were significantly less thigmotactic than paired females (Tukey HSD, P < 0.05), however, isolated males and females were equally thigmotactic (Figure 2.3). The effect of sex was also dependent on swim number ($F_{1,20}$= 17.13, P = 0.0005). Males were less thigmotactic than females in Swim One (Tukey HSD, P < 0.05), but not in Swim Two. There was no effect of housing on thigmotaxis ($F_{1,20}$= 0.47, P = 0.50). There was a significant effect of Swim Number on thigmotaxis: thigmotaxis was significantly lower in Swim Two than in Swim One ($F_{1,20}$= 71.63, P < 0.0001; Figure 2.3).

Figure 2.3: Mean percentage (±SE) of Swim One (black bar) and Two (white bar) spent swimming thigmotactically. Data are averaged across the 16 days of testing, N = 6 per group.

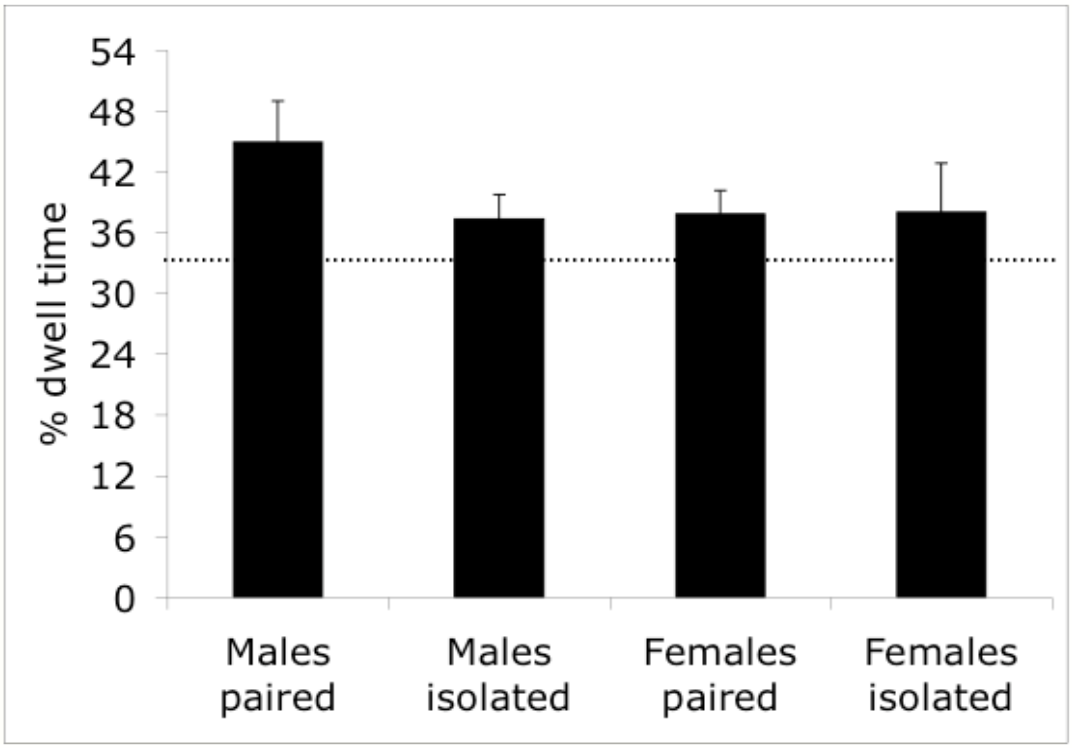


Reference memory

There was no effect of sex or housing condition on the proportion of time spent in the target quadrant (sex: $F_{1,21}$= 0.21, P = 0.65; housing: $F_{1,21}$=1.94, P = 0.18; see Figure 2.4). The sex by housing interaction was not significant. There was no effect of day ($F_{1.5,32.8}$=1.87, P = 0.18) and all interactions with day were not significant.

The proportion of time spent in the target quadrant by each rat was averaged across Days Two to Five, and pooled across sex and housing condition (since there was no significant effect of these factors) and tested against chance 33.3% (the quadrant that contained the platform was ignored) using a two tailed one sample t-test. Rats spent an average of 40% of Swim One in the target quadrant which was significantly longer than expected by chance (two-tailed t-test: t_{23} = 2.15, P = 0.042).

Figure 2.4: Reference memory expressed as the % (mean ± SE) dwell time in Swim One in the quadrant that contained the platform on the previous day. Male and female rats were either pair or isolate housed, N = 6 per treatment group. Data are averaged across Days Two to Five and the dotted line represents chance (33.3%).



Body weight and food intake

Males weighed more than the females ($F_{1,21}$= 513.50, P < 0.0001). Housing condition had no impact on body weight ($F_{1,21}$= 2.22, P = 0.15). All rats gained weight as the weeks progressed and males appeared to gain weight at a faster rate than females (week post arrival: $F_{2.9,55.6}$= 621.04, P < 0.0001; See Figure 2.5.a); sex by week interaction: $F_{2.9,55.6}$= 133.02, P < 0.0001). The housing by week interaction was not significant.

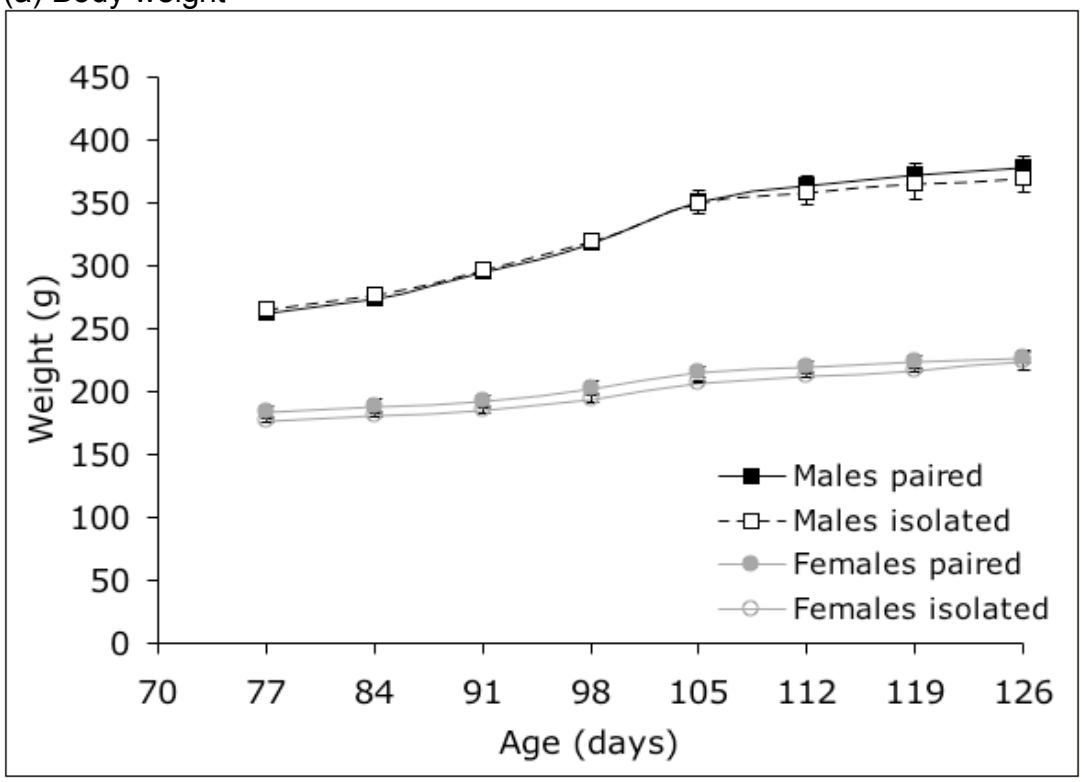
Males ate more than the females at all time points ($F_{1,21}$= 387.93, P < 0.0001; Figure 2.5.b). On average, males ate 24 ± 2 g and females ate 16 ± 1g (N = 12). Housing condition had no impact on food intake ($F_{1,21}$= 2.41, P = 0.14). There was a highly significant effect of week on food intake ($F_{6.0,126.3}$= 7.97, P < 0.0001). This effect appears to be due to natural fluctuations in the amount of food eaten as the weeks progress, and not a directional trend e.g. for the rats to eat more as they age. No other interactions were significant.

Behaviour in home cage

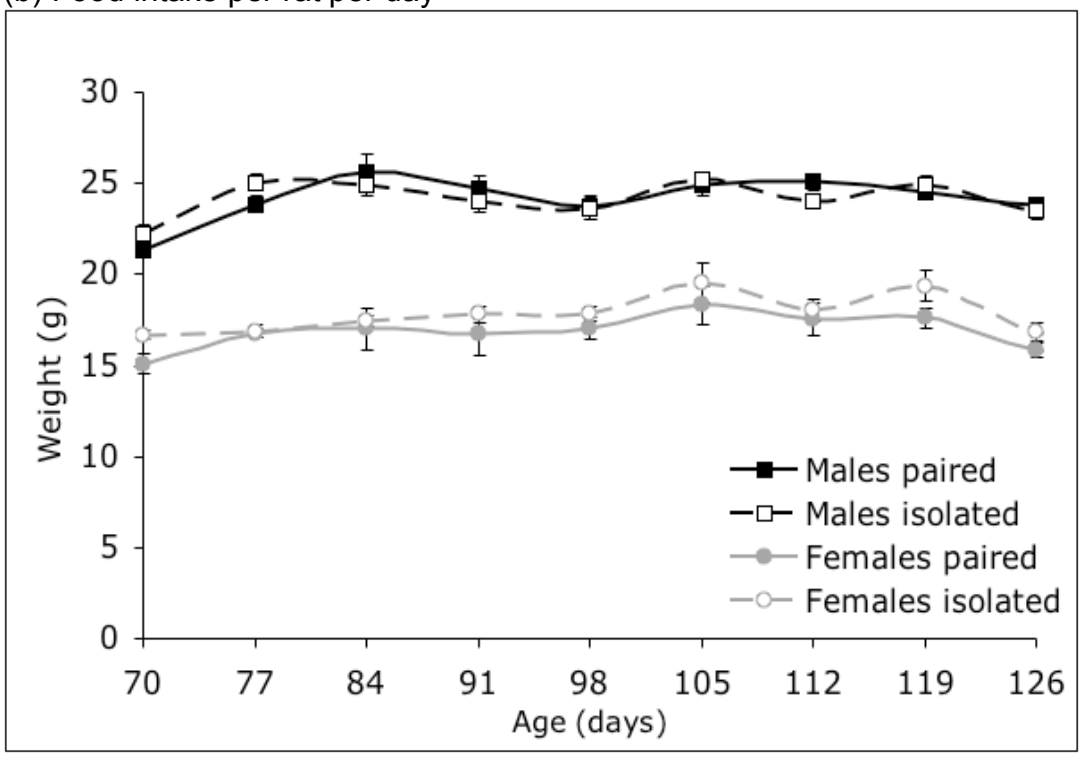
Males and females, irrespective of housing condition, were equally likely to bite the cage bars (P > 0.05, Fisher's exact test). During the observation period in the first month post arrival, four males and seven females spent approximately 5-25% of the time bar biting. During the observation period in the second month post arrival, only one paired female did any bar biting for 5% of the 10 minute observation period, suggesting a decrease in this behaviour that was independent of sex and housing condition. Overall, regardless of sex or housing condition, the occurrence of bar biting was extremely low, with the majority of bar biting occupying not more than 5-10% of the observation period.

Figure 2.5: Body weight and food intake (g) (mean ± SE) for male and female rats that were either pair or isolate housed, N = 6 per treatment group. Analyses were conducted on data up to when swimming started.

(a) Body weight



(b) Food intake per rat per day



2.2.3. Discussion

We found no significant effect of 10 weeks of isolation housing on working or reference memory in either male or female rats, nor did we find a sex difference in spatial ability. Similarly, body weight and food intake were not significantly affected by housing condition. Males were less thigmotactic in the MWM than females, but only if pair housed, and only in Swim One. Thigmotaxis decreased significantly from Swim One to Swim Two in all rats. Despite finding no evidence that the amount of time spent bar biting (a behavioural indicator of poor welfare) significantly differed between pair and isolate housed rats, it is possible that longer observation periods (e.g. > 2 hours per rat) and greater number of animals per housing treatment group would have yielded a significant difference in bar biting behaviour between paired and isolated rats (e.g. Hurst et al. 1997, 1998).

Male and female rats performed equally well in the MWM task finding the platform equally quickly across the four daily swims. These data are consistent with the results from a number of studies in which sex differences have not been reported in working memory MWM tasks (Bucci et al. 1995; Healy et al. 1999; Roof and Stein 1999). Although in our experiment working memory could only be assessed in Swims Two to Four (because the platform was moved each day) we included Swims One to Four in the analysis because differences in performance in Swim One may have indicated differences in searching strategy (e.g. thigmotaxis).

Males and females also appeared to retain information about the platform's location some 24 hours later spending significantly longer than expected by chance in the previous day's quadrant during Swim One. However, our method of measuring reference memory meant that a rat that was released in the quadrant that contained the platform on the previous day may have spent longer in this quadrant (i.e. by chance) or that a rat that was released near to the platform (and so found the platform quickly) may have spent less time in the previous day's quadrant. However, it is unlikely that this will have had a great impact on our data, because we ensured that rats within each treatment group were released from different starting points within a day. Furthermore, this method of measuring reference memory was preferable to placing the rats in the pool without a platform present (i.e. a probe trial) since this may have affected learning in subsequent trials. The ideal solution would have been

to use an Atlantis rising platform, since this platform rises from the bottom of the pool at the end of the probe trial.

We did find one sex difference: our pair-housed females were more thigmotactic than were the pair-housed males during Swim One in the MWM. Although this finding is consistent with other reports of higher thigmotaxis in females (Perrot-Sinal et al. 1996; Beiko et al. 2004), we did not find the cognitive performance of our females impaired. However, impairments in MWM performance due to thigmotaxis are generally reported when there are much higher levels of thigmotaxis than in our experiment (e.g. > 60% Perrot-Sinal et al. 1996; Beiko et al. 2004; Herrero et al. 2006). It is not clear why there should be such a disparity in levels of thigmotaxis as most of the methodological features that might lead to differences are similar in all these experiments, but it appears from our data that animals can spend up to 30% of the swim being thigmotactic without this behaviour affecting performance. While this might seem surprising, it is possible that the less thigmotactic males search rather inefficiently for the platform (e.g. zig-zagging across the pool). Certainly by Swim Two there is no sex difference in thigmotaxis so it appears that despite the higher initial stress in the females, one swim is sufficient to reduce stress levels significantly. This might be because they know where the platform is located or the stress in Swim One is largely due to getting wet (as is typical, rats were not dried between swims).

Isolation housing did not affect MWM performance. It may be that stress caused by isolation housing was insufficient to impact on cognitive performance, or that stress levels between isolated and paired rats did not differ. We propose the latter as an explanation for our results, since body weight, food intake and bar biting levels did not differ between the differentially housed rats.

We chose not to measure corticosterone levels because our animals showed no behavioural signs of stress and thus it is very unclear what corticosterone elevation, if found, would mean. There are, also, inconsistencies in the literature with respect to the relationship between corticosterone levels and behavioural measures of stress. In spite of this, corticosterone is often considered to be an appropriate physiological confirmation of behavioural indicators of stress, and, indeed, sometimes is used entirely alone to demonstrate stress. With respect to

isolation housing, there is no agreement that an elevation in corticosterone is an accurate indicator of stress, in either male or female rats. For example, isolated rats have been shown to have elevated (Hatch et al. 1963; Gamallo et al. 1986; Perelló et al. 2006), depressed (Hurst et al. 1997) or unaffected corticosterone levels relative to socially housed conspecifics (Morinan and Leonard 1980; Brown and Grunberg 1995; Scaccianoce et al. 2006).

There are several possible explanations for why isolation housing was not stressful: 1) isolation stress has its most significant effect on young animals and these rats were too old to be affected; 2) visual, olfactory and auditory communication between neighbouring cages may have mitigated the effects of physical isolation; 3) the handling and experimental conditions we imposed were sufficient to offset ill-effects of isolation; 4) although stressful at first, the rats habituate to isolation with time. The last of these hypotheses was tested in our next experiment.

2.3. Experiment two: 1 week of isolation at 10 weeks of age

In Experiment One, 10 weeks of isolation did not have a significant impact on cognitive ability. It is possible that housing exerts stressful effects early in the manipulation but that animals habituate to their conditions as time goes on. In Experiment Two, therefore, we investigated whether stress experienced during the initial stages of isolation housing affected performance in the MWM. We housed adult male and female Lister Hooded rats in isolation or in pairs and assessed spatial ability in the MWM after one week of exposure to their respective housing conditions.

2.3.1. Materials and methods

Subjects and housing

In this experiment, six rats of each sex were housed in isolation and 12 rats of each sex were housed in same sex pairs from 10 weeks of age (Figure 2.1). Rats remained in their respective housing conditions throughout the entire experiment. One week

after the beginning of the housing manipulation spatial ability was assessed using the MWM. Body weight and food intake, handling procedures and the MWM apparatus and testing procedure were identical to Experiment One (see section 2.2.1).

Behaviour in home cage

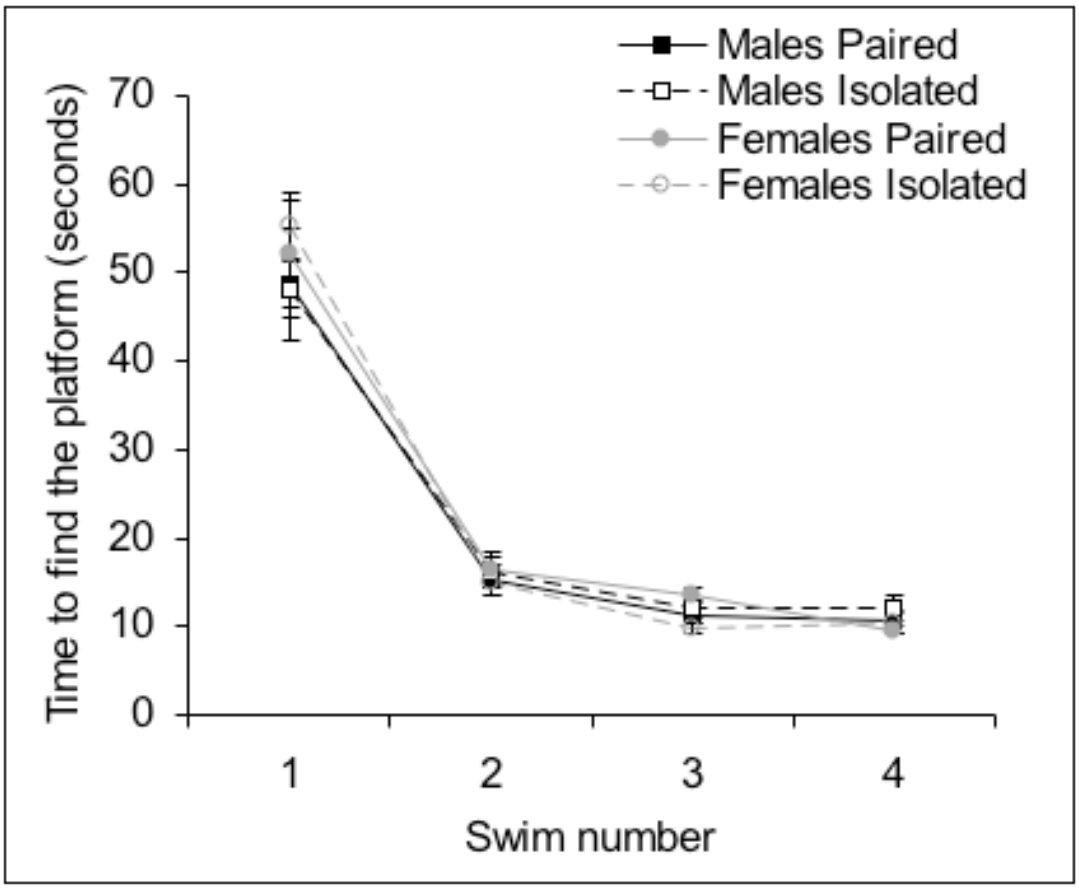
Rats are nocturnal, therefore a black and white wide-angle lens camera was set up to record home cage behaviour for six hours during the dark phase. A 40W red light bulb provided illumination. Film footage was scored for the absence (0) or presence (1) of bar biting behaviour every 30 seconds across 10 consecutive minutes, starting approximately at 2000 hours.

2.3.2. Results

Working memory

Males and females did not differ in the time they took to find the platform ($F_{1,21}$= 0.61, P = 0.44; Figure 2.6). One week of isolation housing also did not affect the time taken to find the platform ($F_{1,21}$= 0.02, P = 0.90). The sex by housing interaction was not significant. Rats learnt the location of the platform within the four daily swims; the largest decrease in swim time occurred between Swims One and Two ($F_{1.2,24.4}$= 200.40, P < 0.0001; Figure 2.6). There was a significant effect of day on performance ($F_{6.1,127.8}$ = 11.01, P < 0.0001) and a significant interaction between swim number and day ($F_{10.0,210.2}$ = 3.60, P = 0.0002). The time taken in Swim One decreased the most across the experiment (Tukey HSD, P < 0.05). No other interactions were significant.

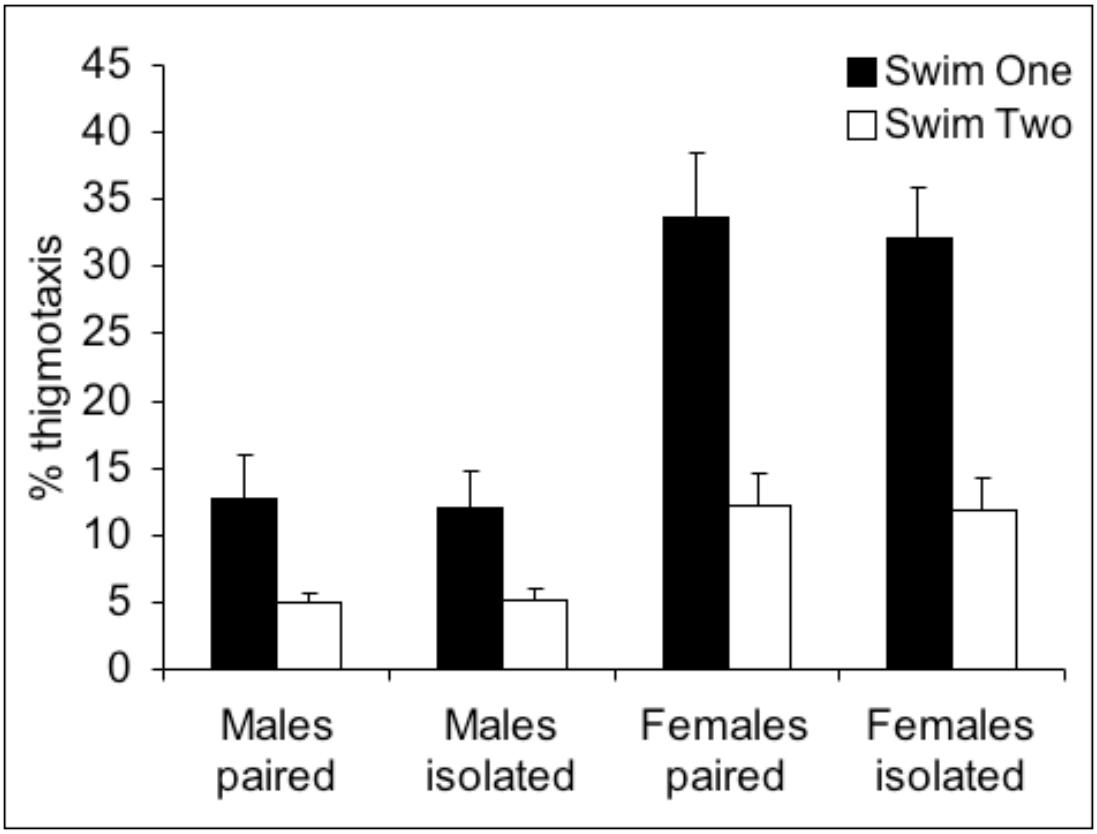
Figure 2.6: Average time (±SE) across the 16 days of testing to find the platform for male and female rats that were either pair or isolate housed, N = 6 rats per treatment group. Analyses were conducted on daily swim data.



Thigmotaxis

The data were log transformed to meet the assumptions of normality and homogeneity of variance. Thigmotaxis levels decreased significantly by Swim Two in males and females ($F_{1,21}$= 10.67, P = 0.0039). Females had higher levels of thigmotaxis than did males ($F_{1,20}$= 51.22, P < 0.0001; Figure 2.7). The effect of housing and all other interactions was not significant (P's > 0.05).

Figure 2.7: Mean percentage (±SE) of Swim One and Two spent swimming thigmotactically. Means were calculated for each rat on each day and then averaged over the 16 days of testing.

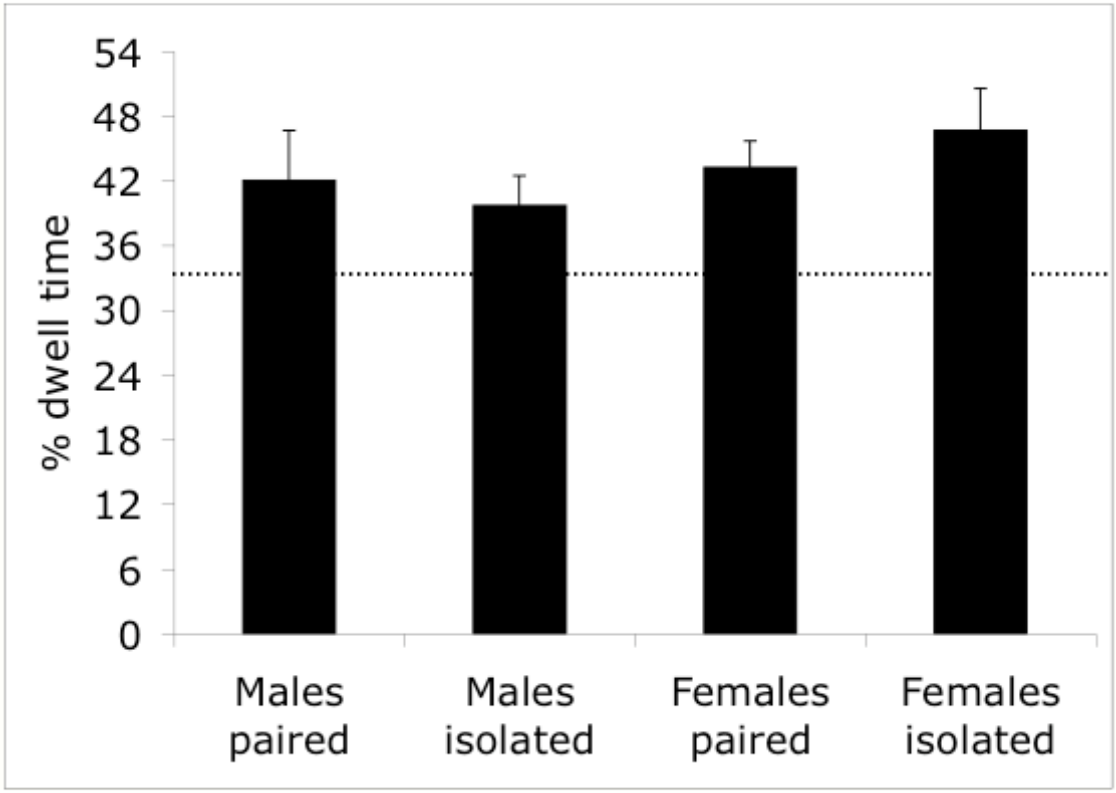


Reference memory

The data were arcsine square-root transformed before analysis. Neither sex nor housing condition had a significant impact on reference memory (sex: $F_{1,21}$= 1.76, P = 0.20; housing: $F_{1,21}$= 0.04, P = 0.85; see Figure 2.8).

The data were averaged across Days Two to Five and pooled across sex and housing condition then tested against chance (33.3%) using a one-sample t-test. The rats spent significantly longer than expected by chance in the target quadrant in Swim One (43% ±1.7%; two-tailed t-test: t_{23}= 3.65, P = 0.013).

Figure 2.8: Reference memory expressed as the % (mean ± SE) dwell time in Swim One in the quadrant that contained the platform on the previous day. Male and female rats were either pair or isolate housed, N = 6 per treatment group. Data are averaged across Days Two to Five. The dotted line represents chance (33.3%).



Body weight and food intake

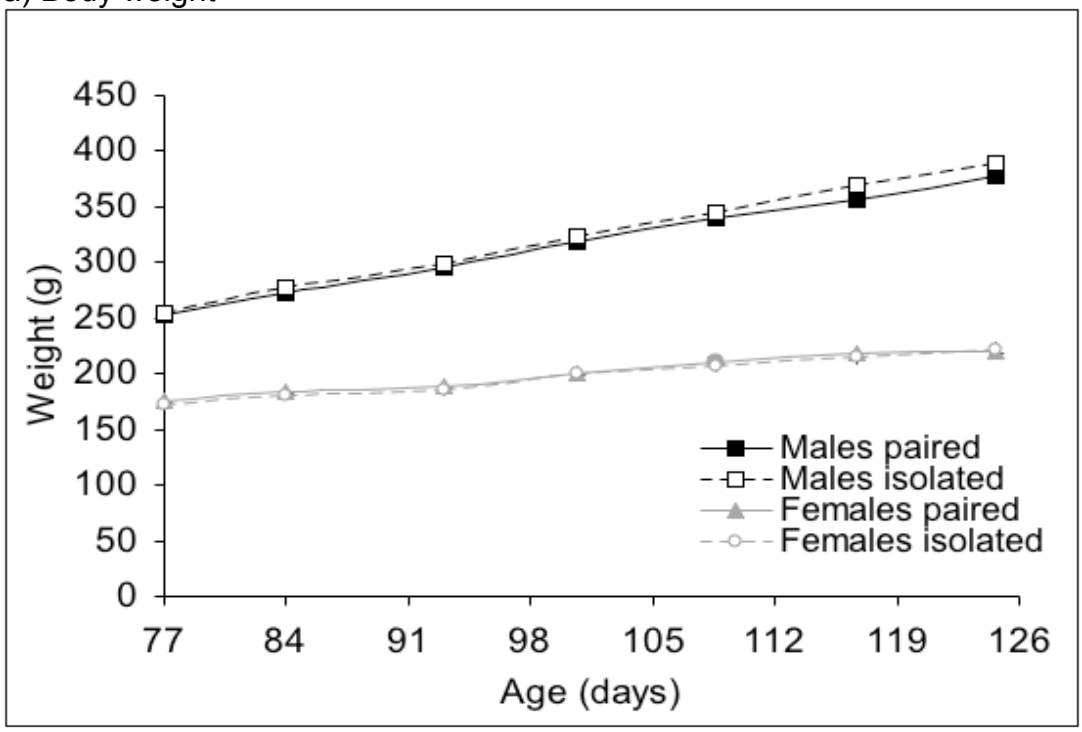
Males weighed significantly more than females ($F_{1,20}$= 774.73, P < 0.0001; Figure 2.9.a) and also gained weight at a faster rate (sex-by-week interaction: $F_{2.3,45.1}$= 199.18, P < 0.0001). This was, at least in part, due to males eating more than females ($F_{1,20}$= 332.73, P < 0.001). Housing condition had no effect on food intake or on body weight (food intake: $F_{1,20}$= 1.53, P = 0.24; body weight: $F_{1,20}$= 0.14, P = 0.75; Figure 2.9.b). No other interactions were significant.

Behaviour in home cage

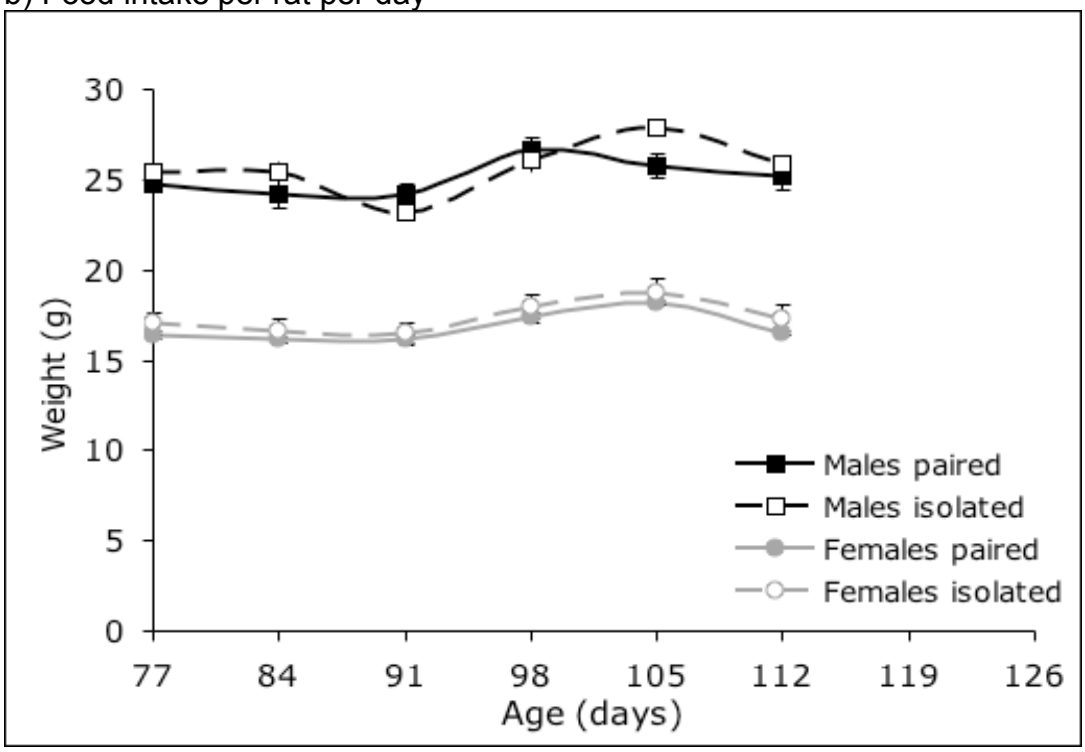
Bar biting behaviour did not differ between the sexes (P = 1, Fisher's Exact test). However, isolated rats were more likely to bar bite than pair-housed rats (P = 0.04, Fisher's Exact test). Bar biting behaviour was not seen for more than approximately 10% of the observation period in any rat. Bar biting was seen in three isolated males and two isolated females and was completely absent in all pair-housed rats.

Figure 2.9: Body weight and food intake (g) (mean ± SE) for male and female rats that were either pair or isolate housed, N = 6 per treatment group.

a) Body weight



b) Food intake per rat per day



2.3.3. Discussion

We found no effect of sex or of one week of isolation on working or reference memory in the MWM. Additionally, all females spent more time engaged in thigmotaxis in the MWM than did the males. Again, we found no effect of housing condition on body weight or food intake. However, in Experiment Two isolated rats (regardless of sex) were more likely to bite the cage bars than were pair-housed rats. Since bar biting was seen most in the first month post arrival in all rats regardless of housing condition in Experiment One, it seemed possible that stress levels were greatest during that period and would result in impaired performance in a cognitive task. This prediction was not met. Despite the higher incidence of bar biting in isolated rats (possibly indicating greater levels of anxiety) this stress was not sufficient to impact on cognitive ability since performance levels in the MWM were not affected by housing condition, and, were comparable between Experiments One and Two. Similarly, levels of thigmotaxis were not affected by housing condition. In this experiment, although females had higher levels of thigmotaxis in Swims One and Two, MWM performance did not differ between males and females. It appears that thigmotaxis needs to be greater than approximately 33% to impact on performance.

Coupled with the results from Experiment One, it appears that isolation for neither short nor long periods has much impact on cognitive ability in adult rats. We suggest four possible explanations: 1) the major impact of isolation occurs in young, rather than adult, rats; 2) the strain we used is one that is less susceptible to the stress of isolation; 3) that pair and isolation housing cause equal levels of stress; 4) isolation housing does not cause sufficient stress to impair performance in cognitive tests. We tested the first of these hypotheses in the next experiment.

2.4. Experiment three: 10 weeks of isolation at 4-5 weeks of age

To investigate if isolation housing has a greater impact on juvenile rats than it does on adults, male and female Lister Hooded rats were housed in isolation or pairs from four-to-five weeks of age, and spatial cognition was assessed, after 10 weeks, in the MWM (Figure 2.1).

2.4.1. Materials and methods

Subjects and housing

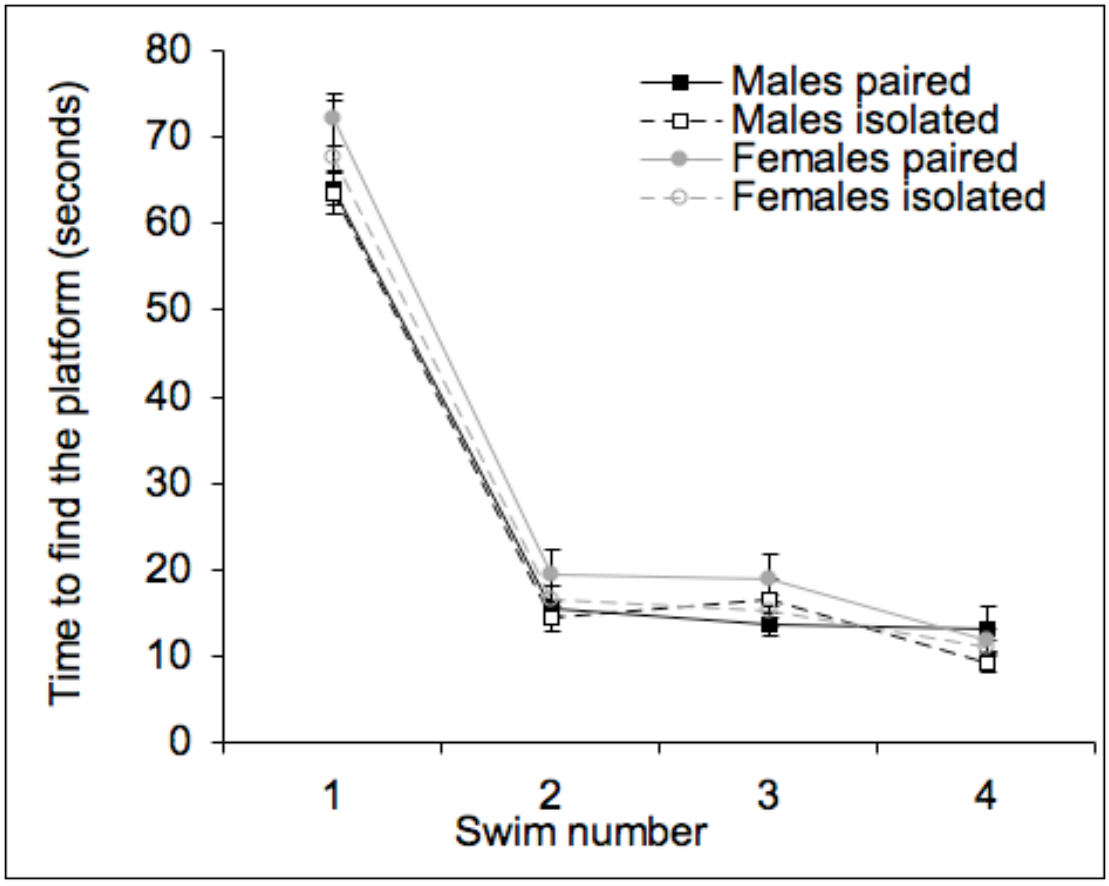
Six Lister Hooded rats of each sex were housed in isolation and 12 rats of each sex were housed in same sex pairs from four-to-five weeks of age for 10 weeks before spatial ability was assessed using the MWM. Housing, husbandry conditions and handling protocols and the MWM apparatus and testing procedure were identical to Experiment One (section 2.2.1). Body weight and food intake were measured as in Experiment One (section 2.2.1), and filming of in-cage behaviour as for Experiment Two (section 2.3.1).

2.4.2. Results

Working memory

There was a significant effect of sex on performance: males took less time to find the platform than did females ($F_{1,21}$= 5.21, P = 0.033; Figure 2.10). There was, however, no effect of housing on performance ($F_{1,21}$= 2.11, P = 0.16). As in Experiments One and Two, there was a significant effect of swim number on time taken to find the platform: the largest decline in swim time occurred between Swim One and two ($F_{1.9,39.0}$= 409.25, P < 0.0001; Figure 2.10). No other interactions were significant. The time taken to find the platform decreased across the experiment ($F_{15,315}$= 7.13, P < 0.0001). However, this effect was dependent on the sex of the rats: males improved their overall performance faster than females as testing progressed (sex by day interaction: $F_{15,315}$= 2.03, P = 0.013). No other interactions were significant.

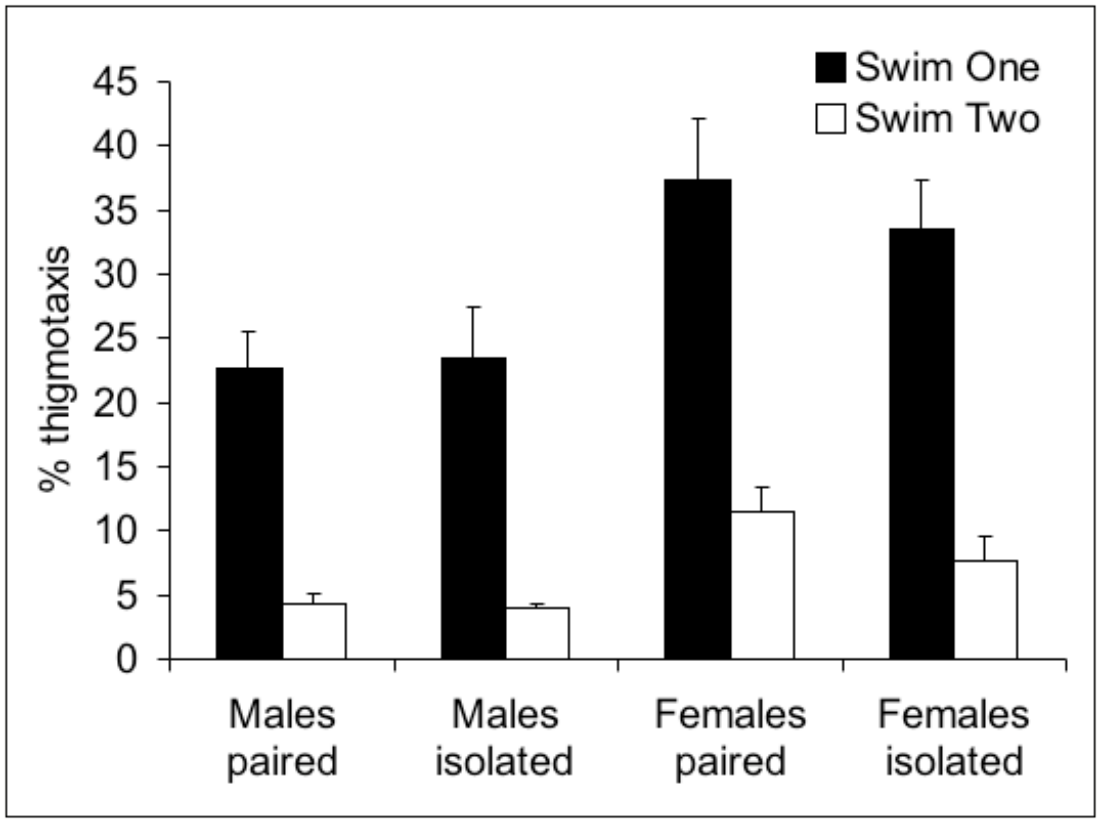
Figure 2.10: Mean time (±SE) taken to find the platform for male and female rats that were either pair or isolate housed, N = 6. Data are averaged across the 16 days of testing, analyses were conducted on daily swim data.



Thigmotaxis

The data were log transformed before analysis. Females were more thigmotactic than were males in Swim One and Swim Two ($F_{1,21}$= 21.07, P = 0.0002; Figure 2.11). There was no effect of housing and the sex by housing interaction was not significant (P's > 0.05). Thigmotaxis decreased significantly in Swim Two in males and females (swim: $F_{1,21}$= 264.75, P < 0.0001). No other interactions were significant (P's > 0.05).

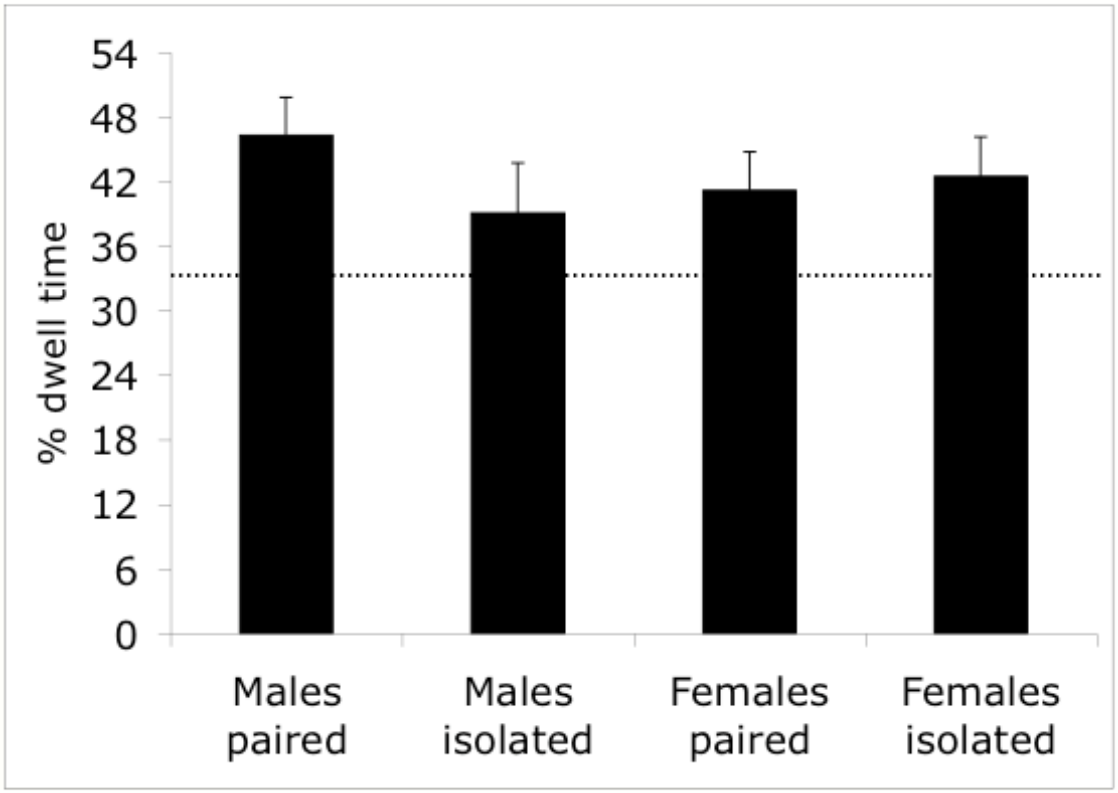
Figure 2.11: Mean (±SE) percentage of Swim One and Two spent swimming thigmotactically. Data are averaged across the 16 days of testing, N= 6 per treatment.



Reference memory

The data were arcsine transformed before analysis. There were no main effects of sex or housing on the amount of time spent searching in the target quadrant (sex: $F_{1,21}$= 0.04, P = 0.85; housing: $F_{1,21}$= 0.75, P = 0.40; Figure 2.12). Although there was a significant effect of day on the amount of time spent in the target quadrant ($F_{3,63}$= 3.95, P = 0.012), this appeared to be due to rats spending longer in the target quadrant on day four, rather than a progressive change across the days. The data were averaged across Days Two to Five and pooled across sex and housing condition and tested against chance (33.3%). Rats spent a mean of 42% ± 1.9% in the target quadrant: significantly longer than expected by chance (two-tailed t-test: t_{23}= 3.41, P = 0.0023).

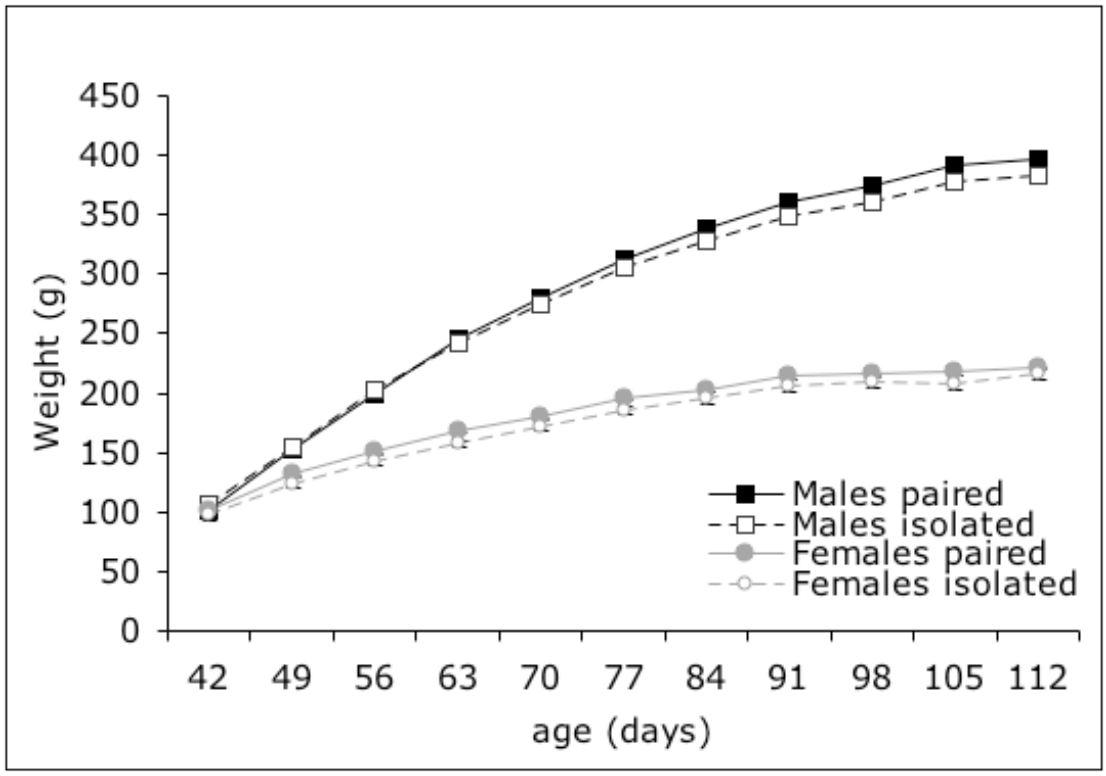
Figure 2.12: Reference memory expressed as the % (mean ± SE) dwell time in Swim One in the quadrant that contained the platform on the previous day. Male and female rats were either pair or isolate housed, N = 6 per treatment group. Data are averaged across Days Two to Five. Dotted line represents chance (33.3%).



Body weight and food intake

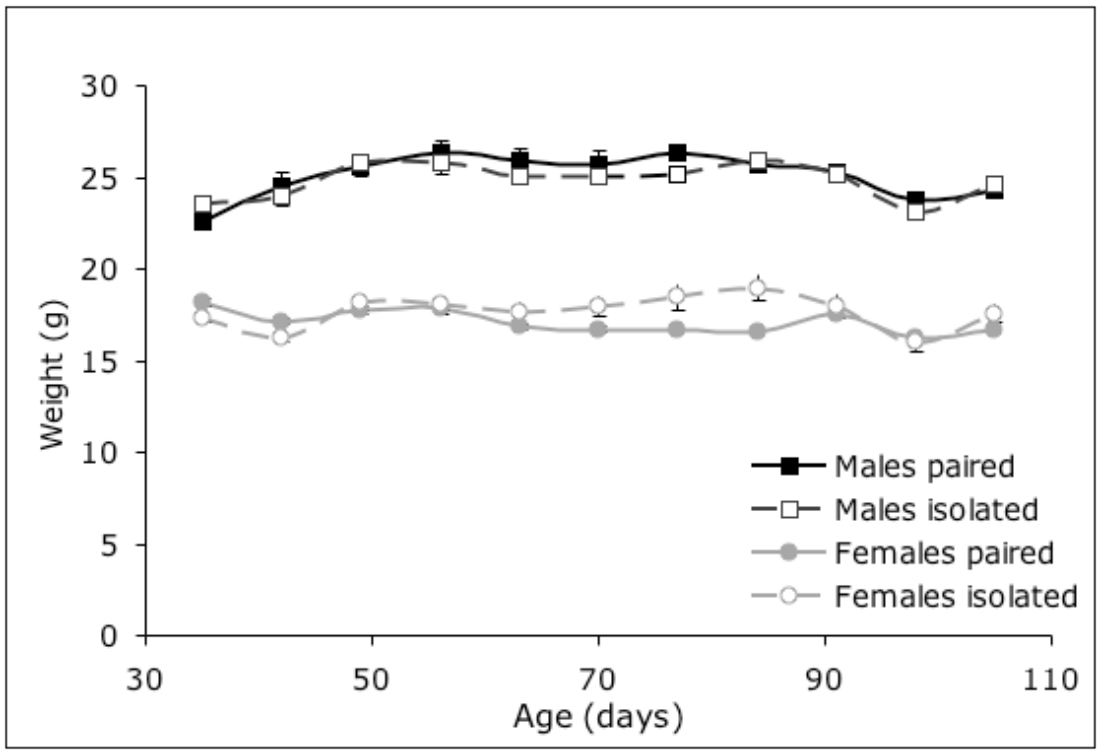
The males weighed significantly more than females ($F_{1,21}$ = 655.66, P < 0.0001; Figure 2.13) and also gained weight at a faster rate (sex-by-week interaction: $F_{1.8,38.3}$= 2617.57, P < 0.0001). Housing condition had no impact on body weight ($F_{1,21}$= 2.37, P = 0.14).

Figure 2.13: Body weight (g) (mean ± SE) for male and female rats that were either pair or isolate housed, N = 6 per treatment group. Analyses were conducted on data up to when swimming started.



Males ate more than females ($F_{1,21}$= 329.57, P < 0.0001) irrespective of housing condition ($F_{1,21}$= 0.03 P = 0.86; Figure 2.14). There was a non directional effect of week post arrival on food intake: this was at least in part due to natural daily fluctuations in intake, rather than any directional increase or decrease ($F_{4.0,83.8}$= 11.6, P < 0.0001).

Figure 2.14: Food intake per rat per day (g) (mean ± SE) for male and female rats that were either pair or isolate housed, N = 6 per treatment group. Analyses were conducted on data up to when swimming started.



Behaviour in the home cage

Male and female, pair and isolate housed rats were all equally likely to engage in bar biting behaviour (P's > 0.05, Fisher's Exact test). During the observation period bar biting was not seen for more than 10% of the observation period in any rat. This behaviour was absent in isolated males but present in one paired male, three paired females and two isolated females.

2.4.3. Discussion

Isolation housing did not affect working or reference memory in young male or female rats. However, males outperformed females in the working memory component of the MWM task. While this result is consistent with the common finding of superior male performance in the MWM (reviewed in Jonasson 2005) it is at odds with our hypothesis that isolation housing is a sufficient stressor to induce sex differences in cognition. As seen in older rats, none of the other behavioural indicators of stress were affected by isolation housing in these younger animals. However, as with Experiments One and Two, it is possible that small sample sizes and short observation periods reduced the likelihood of detecting a significant difference in bar biting behaviour between paired and isolated rats. These results are also not consistent with the widespread belief that isolation housing should have a negative impact on physiology and behaviour in rats, which are a naturally gregarious species.

2.5. General discussion

It is commonly considered that isolation housing is stressful and as females respond more poorly in cognitive testing to stress it seemed plausible that isolation housing might cause a sex difference in spatial cognition. Although we found a sex difference in the predicted direction in Experiment Three, it was not due to any of the housing manipulations we made. Rather, it occurred only in the rats that had travelled from the breeding establishment to our laboratory when the rats were aged four-to-five weeks. It is possible that travelling is stressful and this stress had a greater and longer lasting impact on females such that their performance in the MWM was impaired relative to males. This possibility seems plausible as housing manipulations of younger animals typically have a greater impact on behaviour than those imposed on older animals (e.g. Einon et al. 1981). Additionally, performance levels of the younger rats were slightly poorer than those of the older rats. It is possible, that travelling to or being in an unfamiliar setting immediately post weaning affects performance in both sexes, but to a greater extent in females.

Age at testing seems unlikely to be the explanation. We tested rats at several ages (77, 105 and 130 days) and as rats age although differences in spatial cognition

between the sexes may diminish or disappear completely (Bucci et al. 1995; Lukoyanov et al. 1999; Markowska 1999), we found a sex difference only in the 'middle-aged' rats. As none of the other proposed causes of sex difference via stress (e.g. water temperature, handling or training) differed among the experiments they cannot be the explanation for the sex difference.

Finally, the acute stress of swimming in the MWM does not appear to be the explanation for the occurrence of a sex difference only in Experiment Three. Although females were more thigmotactic than males in all three experiments, higher thigmotaxis in females did not lead to poorer performance. The only explanation we have for these data is that thigmotaxis acted as an alternate searching strategy in the female rats (e.g. see McCarthy and Konkle 2005). For example, it is possible that the females spiralled gradually out from the edge until encountering the platform, although a more plausible explanation is that the females went back and forth from the edge until they encountered the platform.

In summary, although we found a sex difference in spatial cognition it cannot be explained by stress imposed by isolation housing. We conclude that isolation housing is not sufficiently stressful to cause the observed sex differences in spatial cognition found in the literature. Rather, it appears that travel or introduction to a novel environment (i.e. our laboratory) when very young (i.e. four to five weeks old) has a much more significant impact on cognitive ability. Experimental manipulations are required to determine which of these two is the more important.

The following appendix does not appear in the previous publication.

2.6. Appendix: The effect of the oestrous cycle on spatial cognition

2.6.1. Introduction

Gonadal hormones can have both a positive and negative impact on female spatial cognition and hippocampal morphology. Effects can be seen both during development and later in life, and even over a single oestrus cycle, which typically lasts for five days in rats (Gould et al. 1990; Williams et al. 1990; Williams and Meck 1991; Isgor and Sengelaub 1998; Healy et al. 1999). Specifically, there is a wealth of literature reporting both enhancement and impairment of spatial ability

(across a variety of tasks) due to varying levels of oestrogen. Increased levels of oestrogen (either due to fluctuations across the oestrous cycle or administered exogenously following ovariectomy) improve spatial ability (Daniel et al. 1997; Healy et al. 1999) or impair spatial ability (Frye 1995; Warren and Juraska 1997). Conversely, other authors report that spatial ability does not vary significantly with changes in oestrogen levels (Berry et al. 1997; Stackman et al. 1997).

It is plausible, then, that the variation in reports as to whether there are sex differences in spatial ability could be due to fluctuations in gonadal hormone levels in cycling females, which impacts on their spatial ability. For example, when tested across the oestrous cycle, if females perform more poorly than males on some days but as well as males on other days, the average performance of females (over the oestrous cycle) will be poorer than that of males.

The purpose of this experiment was to determine if spatial cognition in females varies with oestrus cycle phase. During MWM testing in Experiment One, I examined spatial ability across the oestrus cycle. I compared female performance during oestrus, pro-oestrus and meta/di-oestrus (there appears to be no difference in performance between meta and di-oestrus phase females; Jones 2003). Given the similarity between the MWM protocol that I used and the protocol that Healy et al. (1999) used (a working memory task with four daily swims), I predicted, consistent with Healy et al.'s data, that females would perform better in the working memory task during the pro-oestrus phase (high oestrogen levels) and poorer during the oestrus phase (low oestrogen levels) i.e. oestrus females will need one extra swim to be sure of platform's location phase (e.g. Healy et al., 1999).

2.6.2. Materials and methods

Subjects and housing

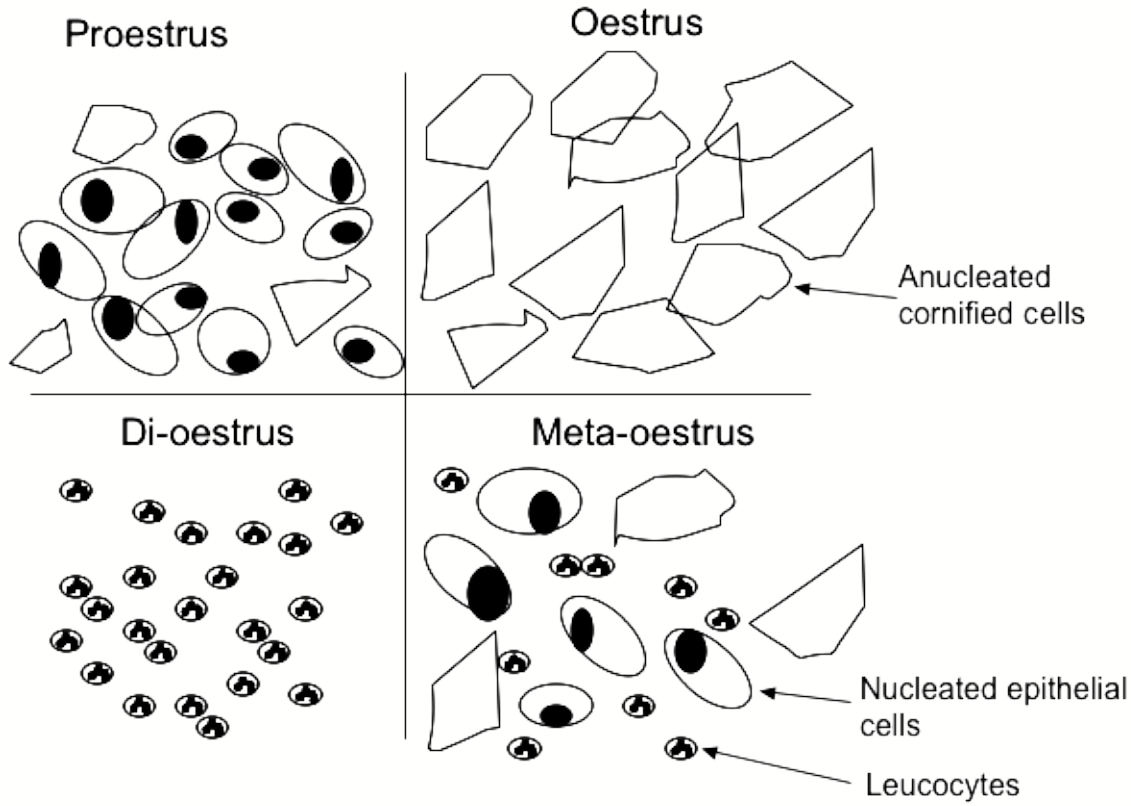
As for Experiment One (see section 2.2.1)

Smearing protocol

Day of oestrus cycle was determined daily for each female by taking a vaginal smear at 0900 hours and assessing the proportions of different cell types present in the smear (Figure 2.15; e.g. Marcondes et al. 2002). Taking a vaginal smear consisted

of using a plastic pastette to gently flush out the vagina with approx 200 ml of 1x phosphate buffered saline (PBS). The PBS was then dropped onto a glass cover slide, air dried, fixed with 100% methanol for one minute and then stained with Geimsa (Sigma, GS-1L) for 20 minutes. Slides were examined using a light microscope under a x40 objective lens to determine proportion of different cell types present. The expected cell types and naming of the different stages of oestrous differ slightly among sources, but the general consensus is that there are four stages in the rat oestrous cycle: oestrus, meta-oestrus, di-oestrus and pro-oestrus. The associated predominant cell types for each stage are as follows: a pro-oestrus smear consists mainly of nucleated epithelial cells, an oestrus stage smear consists of masses of aneucleated cornified cells, a meta-oestrus stage smear consists of the same proportion of polymorphonueclear leucocytes, cornified cells and nucleated epithelial cells and a di-oestrus stage smear consists of leucocytes (see Figure 2.15).

Figure 2.15: A schematic of the cell types and proportions present in a smear from the four stages of the oestrous cycle (after Jones 2003).



Data analysis

The average performance (time in seconds to find the platform) for each rat in each of the four oestrous cycle phases was calculated for the four daily swims. Repeated measures data were analysed using a Repeated-Measures-Analysis-Of-Variance (RM ANOVA). Within-subject factors were swim number (One to Four) and oestrous cycle phase (oestrus; pro-oestrus and meta/di-oestrus). The *between-subjects* factor was housing and was not significant ($F_{1,9}$= 0.33, P = 0.58) and nor was the interaction between housing and oestrous cycle phase (housing by oestrous interaction; $F_{2,18}$=1.5, P = 0.25), therefore, the data were pooled across housing conditions (thus, N = 12).

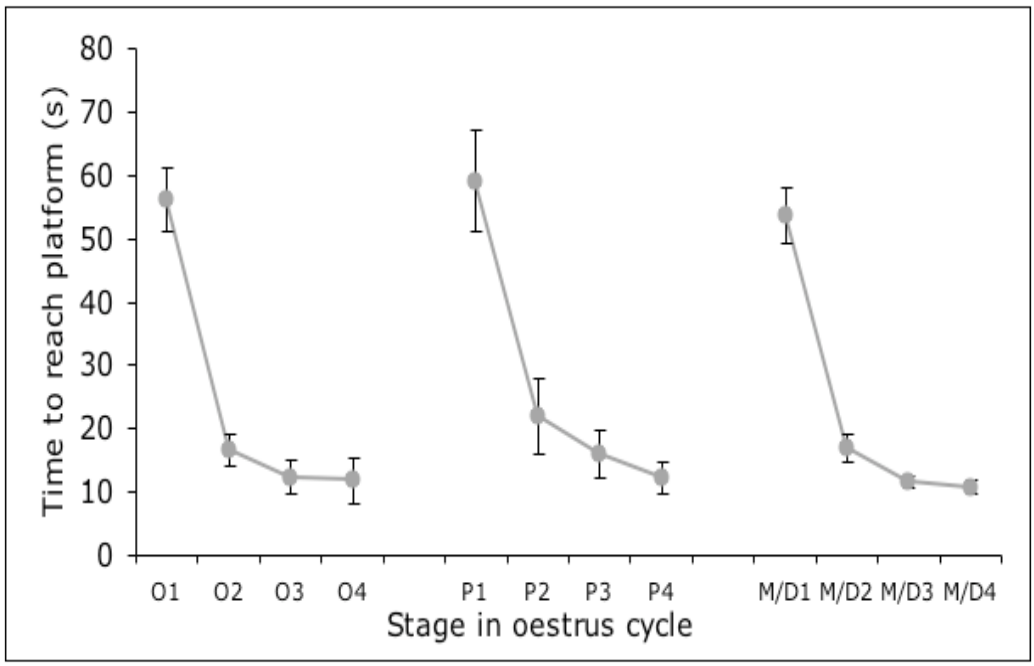
2.6.3. Results

Oestrus cycle phase did not explain any significant variation in performance in the working memory task ($F_{2,22}$= 1.2, P = 0.30; Figure 2.16.a). No other interactions with oestrous cycle phase were significant.

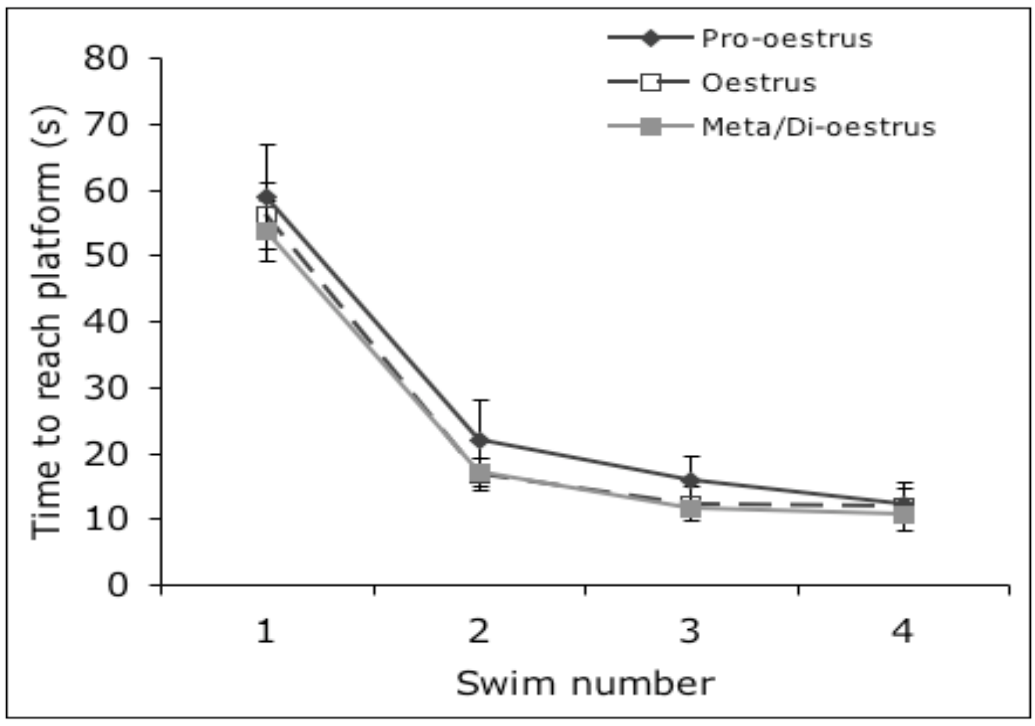
The *a priori* prediction was that oestrus females would take longer to find the platform in Swim Two (i.e. when the rats use memory to locate the platform) than would pro-oestrus females (as in Healy et al. 1999). Therefore, performance in Swim Two during the three oestrous phases was analysed separately. Oestrous cycle phase did not impact significantly on time to find the platform in Swim Two (Kruskal-Wallis: K_2= 0.2, P = 0.89; Figure 2.16.b).

Figure 2.16 Mean time (±SE) taken to find the platform in seconds for females for the four daily swims. Females were either in oestrus (O); pro-oestrus (P) or meta/di-oestrus (M/D), N = 12 rats.

(a) Data separated out by oestrous phase.



(b) Data separated out by Swim Number.



2.6.4. Discussion

I predicted that the performance of female rats in a working memory task would vary across the oestrous cycle and specifically, that females would perform better during pro-oestrus than during oestrus. This prediction was not met: females performed equally across all stages of the oestrous cycle. This result, however, is in agreement with that of Berry et al. (1997), who also found that performance in an MWM did not vary across the oestrous cycle, and Stackman et al. (1997), who found that performance in a radial arm maze was not affected by the oestrous cycle. Additionally, in the MWM, ovariectomised rats treated with oestrogen perform as well as rats that receive no oestrogen (Varga et al. 2002). However, there are an equal number of authors who report that (normal to high) physiological levels of oestrogen are associated with both a positive and negative impact on spatial ability (Frye 1995; Daniel et al. 1997; Warren and Juraska 1997; Healy et al. 1999; Sandstrom and Williams 2001; Holmes et al. 2002; Daniel and Lee 2004).

Some possible reasons why I (and other authors) do not find an effect of oestrous cycle phase on spatial cognition are discussed below. Firstly, it is possible that my smearing technique is poor, which lead me to wrongly identify which stage of oestrous the females were in. However, this does not seem a plausible explanation for my data, since I was confident in my determination of the cycle phase and all of the rats were definitely cycling through the four phases of the cycle. More importantly, consistent but incorrect determination of oestrous phase would be likely to result in an effect in the wrong direction, not the absence of an effect (because if the effect is real, mixing up oestrus with pro-oestrous would not cancel out the effect).

A second possible explanation for finding no effect of oestrous cycle is that there is a time-lag between cell changes in the uterus (as detected by smearing) and changes in spatial behaviour. For example, it could be that when a female presents a pro-oestrus smear, the point at which oestrogen levels peak has past (or is still to come), and as a result, the effect is missed. Nevertheless, there are data that show pro-oestrus females, as determined by smearing immediately prior to decapitation, have a greater synapse and dendritic spine density (a 30% increase) and greater long-term potentiation in the CA1 pyramidal cells in the hippocampus than do oestrus

females (Woolley et al. 1990; Woolley and McEwen 1992; Warren et al. 1995). These data would suggest that the smear corresponds closely with brain changes. But it is not clear when (if at all) these neuro-anatomical increases have a beneficial impact on behaviour. For example, it is unclear if it is the generation or the existence of the extra synapses and dendrites that imparts a beneficial effect on spatial ability. Furthermore, it is not clear if the generation of more synapses and so on, actually improves spatial ability, since superior spatial ability is also found in the phase of the cycle when the brain apparently has fewer dendrites (e.g. Warren and Juraska 1997). Thus, while it is generally considered that more neurones and synapses in the brain result in better cognition (Moser et al. 1994), it is possible that the rapid addition of extra spines and synapses adds 'noise' to the system, which impedes spatial learning and memory retrieval (e.g. Warren and Juraska 1997).

Even if the smearing technique accurately determines cycle phase, and the smear corresponds with behavioural and brain changes, the impact of oestrogen on spatial cognition still remains unclear. Because, authors who have carried out experimental manipulations of oestrogen (which removes phase determination errors and time-lags issues) after ovariectomy (to remove endogenous sources of oestrogen) still report conflicting effects of oestrogen on spatial ability. For example, oestrogen levels as experienced during pro-oestrus, enhances (Frye 1995; Bimonte and Denenberg 1999; Sandstrom and Williams 2001) impairs (Chelser and Juraska 2000; Holmes et al. 2002) or has no effect (Varga et al. 2002) on spatial ability. It is unclear why there are so many conflicting data; possibly age, strain, the level of hormone administered, or chronic versus acute administration may all play a role. It is also plausible that an inverse U-shaped relationship between oestrogen and spatial cognition exists, as it does with testosterone (Gouchie and Kimura 1991; Holmes et al. 2002).

The reason for investigating the effect of oestrous cycle on cognition stems from the belief that if females under-perform at certain points in the cycle, the average performance of females will be lower than that of males. However, sex differences in spatial ability are present in gonadectomised rats, which removes all sources of endogenous sex steroids, thus, the oestrous cycle cannot fully explain sex differences in cognition (Williams et al. 1990). Furthermore, authors who do report

significant effects of oestrous (regardless of the direction of the effect) do not report a sex difference in spatial ability when average male and female performance levels are compared across several cycles (Frye 1995; Warren and Juraska 1997; Healy et al. 1999). Therefore, fluctuation in oestrogen levels across the oestrous cycle may cause some variation in spatial cognition, but it does not appear to do so in a consistent manner. And more importantly, this variation does not seem to be enough to cause females to under-perform relative to males.

Further support that the oestrous cycle is unlikely to be responsible for the sex difference in spatial ability that I found (Experiment Three), comes from the finding that this sex difference was correlated with sex differences in stress: the females were more thigmotactic than were the males. And while there is some evidence that oestrous cycle phase can affect the stress response of females, the behavioural and hormonal data are in conflict with each other. For example, females in pro-oestrous (or administered with oestrogen to mimic pro-oestrus) have higher resting CORT levels and greater CORT responses to 20 minutes restraint than do females in oestrus/ dioestrus (Viau and Meaney 1991). Conversely, behavioural tests of anxiety (e.g. elevated plus maze; emergence test) show that females are less stressed during pro-oestrous than during oestrus (Frye et al. 2000). However, there was no effect of oestrus cycle phase on the cardiovascular response (heart rate, blood pressure) of rats subjected to restraint, injection or cage changing (Sharp et al. 2002b). Moreover, regardless of the direction of the impact it seems unlikely that fluctuations in stress response over the oestrous cycle explains why my females were more stressed than the males since I found the females in all three experiments were more stressed (thigmotaxic) than the males on every day, i.e. there was no conspicuous cycling in stress levels in any of the female rats across the days of testing.

In conclusion, despite the wealth neuro-anatomical and behavioural data, the impact of oestrogen on spatial cognition (and stress responses) remains unclear. And while oestrogen levels may cause some variation in female spatial ability, the data (from the current experiment and in the literature) do not consistently show that oestrous cycle phase sufficiently impacts on female cognition and, moreover, the oestrous cycle seems unlikely to contribute to sex differences in spatial ability (in my

data and in the literature). Furthermore, smearing females is potentially stressful (a differential stress response may underlie sex differences in cognition) and costly in terms of reagents and labour, therefore, I felt there was no significant justification for further collection of oestrous data in the following experiments.

CHAPTER THREE: The effect of isolation housing on performance in the radial arm maze

The data in this chapter were collected with the assistance of Marion Albinet.

Summary

Male rats typically, but not always, outperform females in radial arm maze tasks (RAM). However, it is possible that sex differences in this task are an artefact of stress, experienced either in the home-cage (e.g. chronic stress; such as isolation housing and/or food deprivation) or during testing (acute stress). The RAM is an appetitive task in which rodents have to remember the locations of several food rewards. Since stress can reduce appetite, a stressed rat may ignore food rewards, which will impair apparent cognitive performance. Here I tested if isolation housing was sufficiently stressful to impact on performance in the RAM in male and female rats. I measured how many rewards a rat ignored as a measure of acute stress during testing. I found that isolated and pair housed rats performed equally well and ignored a similar number of rewards. Additionally, males and females performed equally well and ignored a similar numbers of rewards. I conclude that 1) isolation housing is not sufficiently stressful to cause sex differences in an appetitive spatial task, and 2) further evidence is needed to confirm that ignored food rewards positively correlate with acute stress in the RAM.

3.1. Introduction

Male rats often outperform female rats in spatial tasks other than the MWM, such as the radial arm maze (Williams and Meck 1991; Roof 1993a; Seymoure et al. 1996; Lund and Lephart 2001; Takase et al. 2008) but not always (Einon 1980; Juraska et al. 1984). After the MWM, the RAM is the second-most commonly used spatial task in academic and pharmaceutical research (Buccafusco 2001). Although both of these tests enable the assessment of spatial ability, they differ in at least two ways. Firstly, the RAM is an appetitive (rather than aversive) spatial task in which rodents run along arms (usually eight, but can be as many as 17 arms) that radiate from a central arena to locate food rewards (Olton and Samuelson 1976). By rewarding only a subset of arms, both working (within-trial memory) and reference (between-trial memory) memory can be assessed simultaneously: entries into arms that never contain rewards are recorded as reference-memory errors and re-entries into arms that have been visited within the same trial are recorded as working-memory errors (e.g. Lund and Lephart 2001). Secondly, unlike the MWM in which rodents have to remember only one location per day, in a partially-rewarded RAM task rodents are required to remember several different locations each day, which may make the task more difficult (Hodges 1996). Increased task difficulty may lead to increased susceptibility of the task to stress impairments, so the RAM could be useful as a more sensitive method of assessing the effects of stress on spatial cognition.

During RAM testing there are at least three potential sources of stress that a rodent may face. Firstly, food deprivation, which is used to motivate foraging during testing, may be chronically stressful (e.g. Kant et al. 1988). Secondly, traversing down brightly-lit arms in a novel room, as is typical of RAM testing, may be acutely stressful. And lastly, housing conditions (e.g. isolation) provide another potential source of stress for a rodent during an experiment, albeit not a direct impact of the testing situation itself (see earlier sections). Despite finding no evidence that isolation housing impaired performance in an MWM, given that the RAM may be a more difficult task, it is possible that this test will be more sensitive to chronic stress than the MWM. Further, single housing is common during RAM testing so that rats can be accurately food deprived to (typically) 85-90% of their free-feeding body weight (e.g. Endo et al. 1994; Kolb and Cioe 1996; Lund and Lephart 2001; Gibbs

and Johnson 2008), and isolation coupled with this level of food deprivation may be especially stressful. Indeed, isolated rats often typically under-perform in the RAM, relative to socially-housed conspecifics (Einon 1980; Juraska et al. 1984; Seymoure et al. 1996).

Stress may directly affect cognitive processing, e.g. neurone functioning, consolidation of memories and so on (de Kloet et al. 1999). In the context of the RAM, stress might also indirectly impair performance because stress reduces appetite in rodents. For example, stress (e.g. 60 minutes of restraint or 10 minutes forced swim) immediately reduces feeding in rats (Mattioli and Perfumi 2007; Liu et al. 2008). Furthermore, males and females seem to differ in the magnitude of stress required to induce a response in feeding. For example, immediately following one hour of being able to see, hear and smell other rats that were receiving electric shocks, females ate significantly less than did males (Kuriyama and Shibasaki 2004). Thus, stressed rats may not eat the rewards in a RAM, which would lead to re-entries into arms previously visited, choices that are then (typically) recorded as working memory errors. Therefore, if females find testing more stressful than males (Handa et al. 1994; Beiko et al. 2004), they will ignore more food rewards and consequently apparently make more working memory errors. Consistent with this prediction, Jones (2003) found that after accounting for uneaten reward errors, the apparent trend for male superiority in performance disappeared. Also, isolated rats tend to be fearful of eating familiar food in a novel environment, which may also lead to isolated animals ignoring food rewards rather than making more incorrect choices (Holson 1986). Therefore, the number of rewards that are ignored during RAM testing could be recorded separately from working memory errors, in order to determine whether it is due to making this kind of error that females and isolated animals seem to perform so poorly.

As with the MWM, sex differences in performance in the RAM, then, may be an artefact of stress, since females seem to respond less well to stress associated with spatial testing (see earlier sections; Beiko et al. 2004; Perrot-Sinal et al. 1996). To determine whether performance in a potentially stressful appetitive task can be explained by variation in stress, rats were housed in isolation or in pairs for seven weeks and then tested in a RAM. To determine if stress was directly or indirectly

affecting performance, the number of rewards that were ignored was recorded during testing. I predicted that 1) RAM performance would be poorer in isolated animals than in pair-housed animals, specifically due to an increase in uneaten reward errors, and 2) females would make more uneaten reward errors than males and that this sex difference would be greater in the isolated animals.

3.2. Materials and methods

Subjects and housing

The rats from Chapter Two, Experiment Two (section 2.3) were used in this experiment. Three weeks after completion of MWM testing, each rat that swam in the MWM was tested in a RAM. The rats had therefore experienced either isolation or pair housing for approximately seven weeks prior to RAM testing. Consequently, the handling that these rats experienced was as for Chapter Two. Similarly, housing was as for Experiment Two (section 2.3.1) with the exception that during RAM testing the rats were food deprived for 17 hours prior to testing to ensure that the rats were motivated to forage. Food was taken away at 19:00 hours and replaced at 12:00 hours the following day after RAM testing. Body weight was monitored weekly and no rat lost more than 10% of its total body weight during RAM testing.

Food preferences

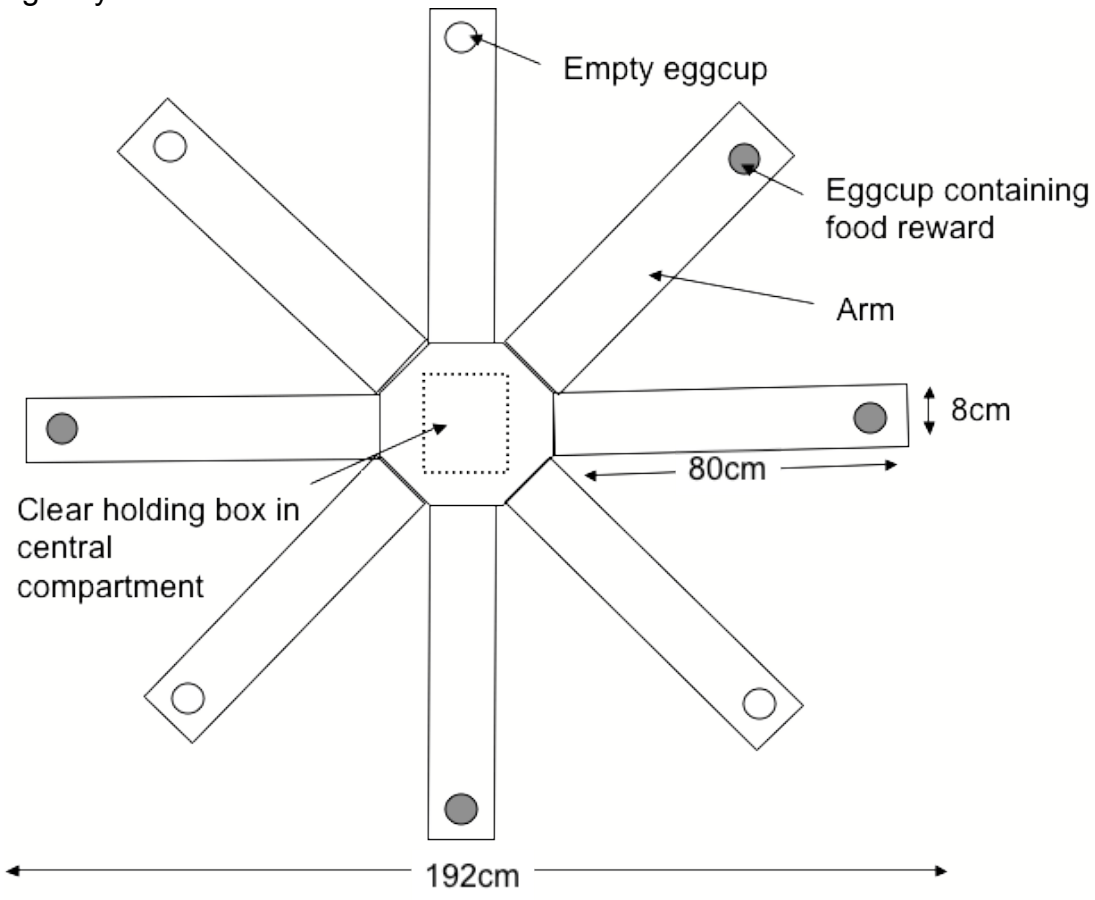
Before any RAM procedures took place I determined the food preferences of each rat. Two metal eggcups containing either 10 pieces of Nestlé chocolate cherio cereal or 10 pine nuts were placed in the home cage of each rat that was to be tested in the RAM (cage mates were temporarily removed). After three minutes the number of food items that had been removed (eaten or cached) was recorded. This enabled the food reward to be tailored to suit each rat (19 out of 24 rats preferred the chocolate cereal) and also exposed each rat to the metal eggcups and the association with food reward prior to RAM testing.

RAM testing: apparatus

The RAM consisted of a central octagonal compartment (diameter 32 cm) from which eight arms radiated at equal angles (see Figure 3.1). Each arm was made out

of clear Perspex and measured 80 cm in length, 8 cm in width with walls that were 20 cm high. The overall diameter of the RAM was 1.92 m. The RAM was positioned inside the empty MWM described in Experiment One (section 2.2.1). Metal eggcups were positioned 2 cm from the end of each arm, and could be filled with a food reward. In the rewarded arms one piece of Nestlé chocolate cherio cereal or one pine nut (depending on the results of the preference test) was used as a food reward. All trials were videotaped from directly above and observed via a monitor. To reduce the use of olfactory clues, cereal and pine nuts were scattered around the outside of the RAM, but out of view from the rats.

Figure 3.1: Schematic of the radial arm maze as viewed from directly above. Four arms contain eggcups with a food reward (grey circles) and four arms contain empty eggcups (white circles). Half of the rats received this pattern of rewarded arms and the remaining rats received this pattern rotated to the right by 90°.



Training

During training all eight arms contained a food reward, resulting in a working memory task designed to familiarise the rats with the procedure and the maze itself. Each rat was given one trial per day. For each trial the rat was carried by hand and placed in a clear holding box in the central compartment (20 cm by 20 cm: Figure 3.1) which was raised, by means of a pulley system after five seconds. The rat was then free to explore the maze for ten minutes or until it had eaten all eight rewards. The rats were tested in a different, predetermined random order each day. When a rat reached the criterion of entering all eight arms and eating at least four rewards within ten minutes, testing began. Rats took an average of four (±1) days of training to reach this criterion. The RAM was cleaned between rats: all eight arms, the eggcups and the central compartment were washed in soapy water and then sprayed with 70% ethanol. The RAM was dry before the next rat was tested.

Testing

During testing, four out of eight arms were rewarded. Each rat was given one trial a day for ten days and the same sub-set of arms were rewarded each day resulting in a working and a reference memory task. Half of the rats were assigned a pattern of rewarded versus non-rewarded arms, the other half of the rats were assigned this pattern rotated by 90º. Patterns with more than two adjacent rewarded arms and patterns with alternatively rewarded arms were avoided to reduce the likelihood that rats would develop turning strategies for visiting every other arm or every adjacent arm (Juraska et al. 1984; Levin 2001). The rats were placed in the maze as for training and left to explore until all four rewards were eaten or until 10 minutes had elapsed. An arm was recorded as being entered if a rat's front paws went more than 200 mm down the arm from the central compartment (i.e. 25% of the way down the arm). The following parameters were recorded during each trial: 1) the number of reference memory errors (entry into arms that never contained a reward; the maximum is four and re-entry was then counted as a working error) 2) the number of working memory errors (re-entries into an arm that had already been visited and did not contain a reward, either because it never did or because the rat had eaten it) 3) the

number of times a food reward was ignored, and 4) the total number of arms entered either before completion or in 10 minutes.

Data analysis

RAM testing took place over 10 consecutive days. The data were blocked into two-day averages (day 1-2, 3-4, 5-6, 7-8 and 9-10). Even after transformation, the data did not fit a normal distribution and the groups had unequal variances, therefore, analyses were conducted on the difference between Block One and five using the non-parametric Kruskal-Wallis test. This allowed comparison amongst the four groups (males paired; males isolated; females paired; females isolated) on the improvement in, for example, the number of reference memory errors made at the end of testing relative to those made at the beginning of testing. The interaction between sex and housing could not be investigated using this method of analysis. To assess the change in performance (e.g. improvement) over the course of testing, the difference between Block One and Five was tested with a two-tailed one-sample t-test against the null hypothesis that no change would occur, i.e. the difference in performance would be zero.

3.3. Results

Reference memory

The groups did not differ in the reduction of reference-memory errors from Blocks One to Five (Kruskal-Wallis: $K_3 = 1.35$, $P = 0.72$; median number of reference memory errors in Block One = 4 and in Block Five = 3). As testing progressed from Blocks One to Five the rats entered significantly fewer arms that never contained rewards (one-sample two-tailed t-test: $t_{23}=4.3$, $P < 0.0002$; the mean difference was 0.75 arms; Figure 3.2.a).

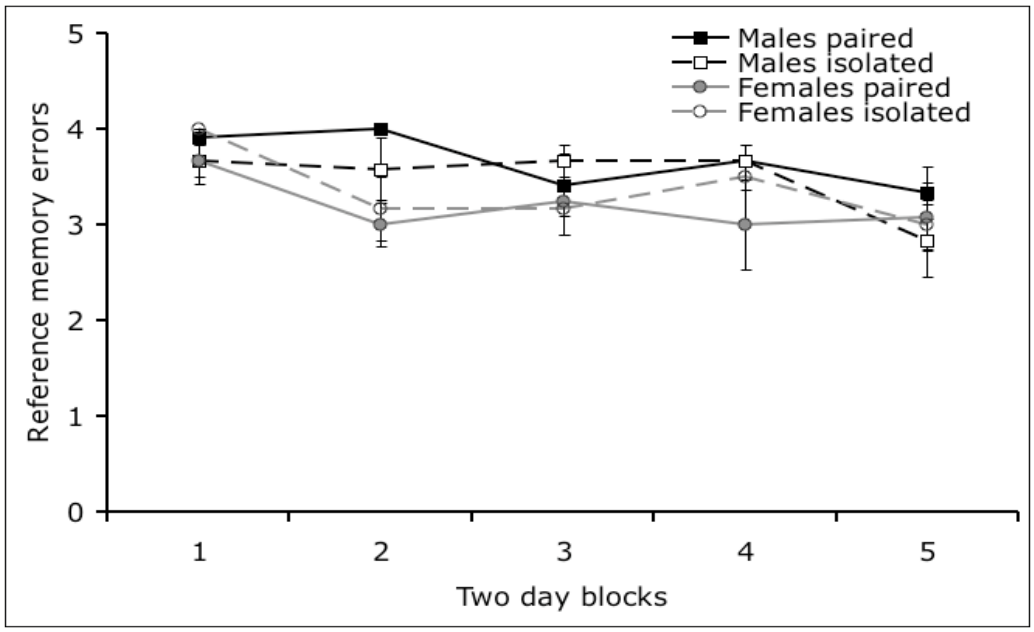
Working Memory

When re-entry into an arm containing an ignored reward was classed as an error, the groups did not differ in the decline in working memory errors across testing

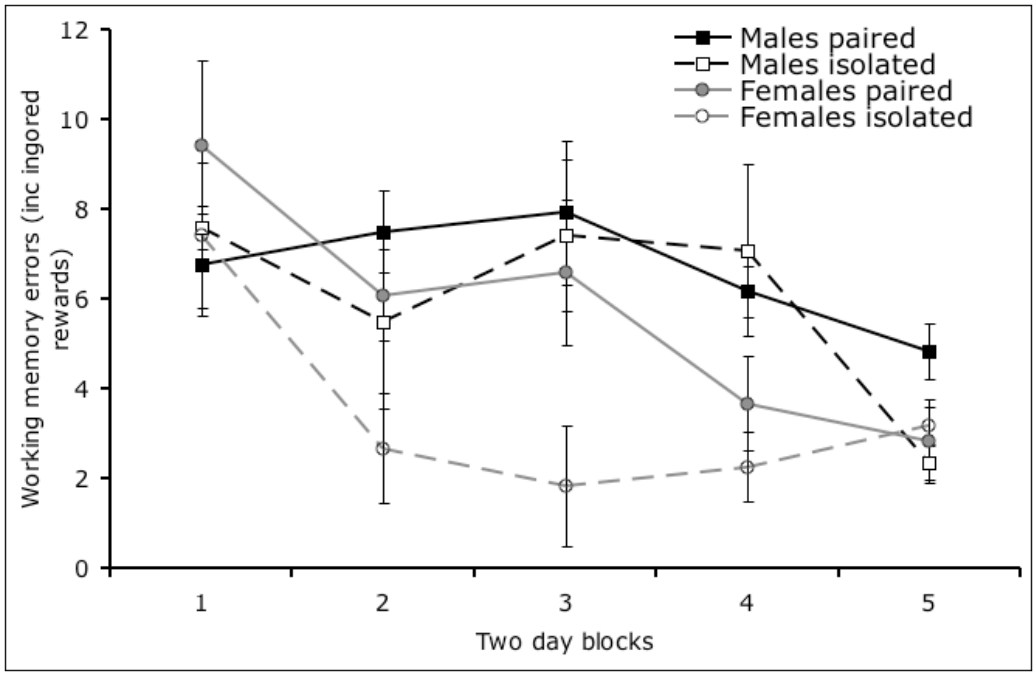
(Kruskal-Wallis: K_3 = 3.79, P = 0.28; median number of working memory errors (including entry to ignored rewards) in Block One = 8 and in Block Five = 4). There was a significant decrease in working memory errors of 4.9 from Block One to five (one-sample t-test: t_{23}=6.07, P < 0.0001; Figure 3.2.b). When ignored rewards were discounted, the groups still did not differ in the decline in working memory errors across testing (Kruskal-Wallis: K_3 = 3.25, P = 0.35; median number of working memory errors (minus ignored rewards) in Block One = 5 and in Block Five = 2; Figure 3.2.c). There was a significant decrease of 2.8 errors from Block One to Five (one-sample t-test: t_{23}= 4.66, P < 0.0001).

Figure 3.2: Performance in the RAM (mean ± SE) for male and female rats that were either pair or isolate-housed. N = 6 per treatment group. Each block represents the average over two days. Analyses were conducted on the difference between Block One and Five.

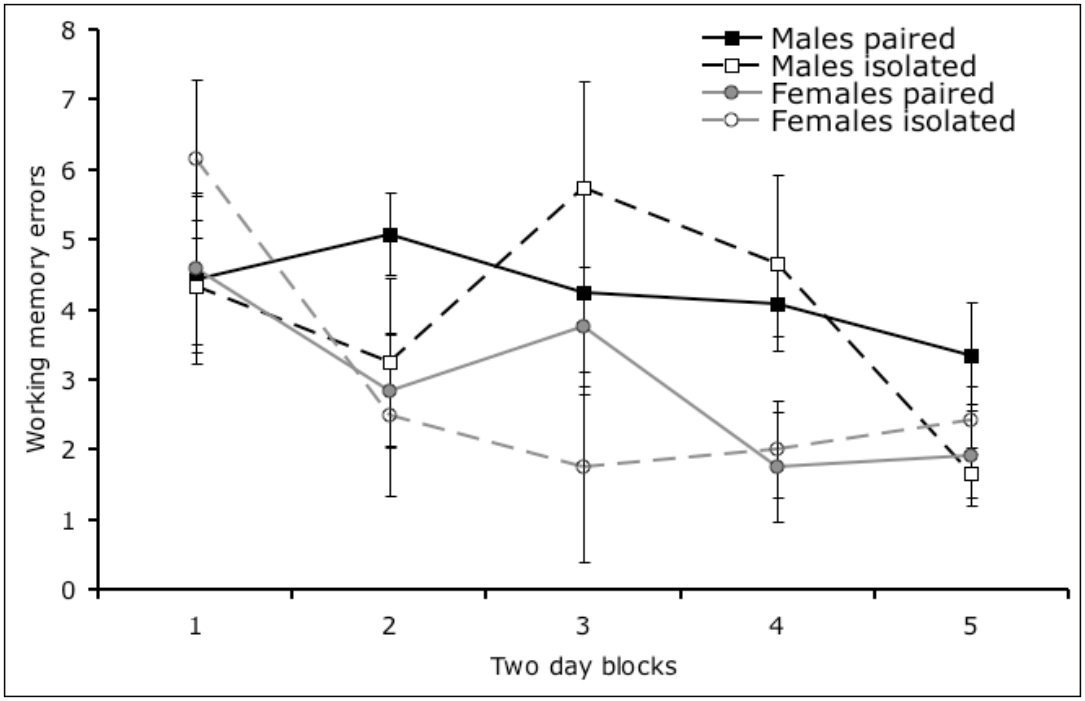
(a) Number of reference memory errors across the two-day blocks. (Note maximum number of errors is four).



(b) Number of working memory (including ignored rewards) errors across the two-day blocks.



(c) Number of working memory errors (excluding ignored rewards) across the two day blocks.



Number of rewards 'ignored'

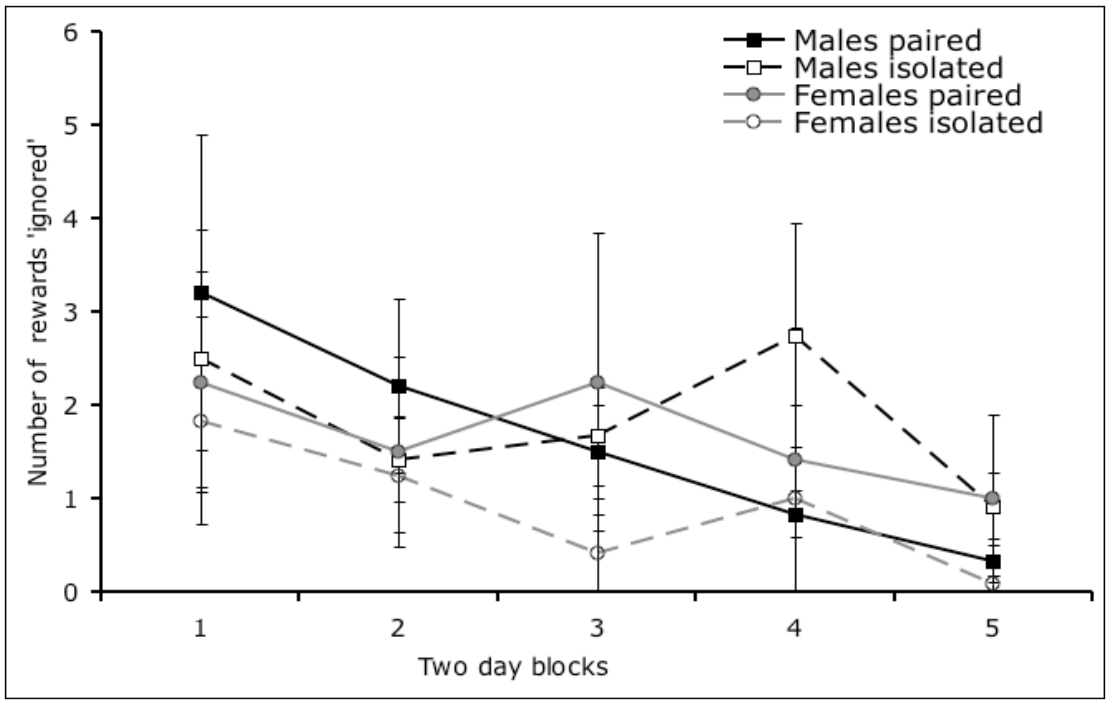
The groups did not differ in the decline in rewards that were ignored over testing (Kruskal-Wallis: $K_3 = 0.78$, $P = 0.85$; median number of rewards ignored in Block One = 2 and in Block Five = 0). There was a significant decrease in the number of rewards that were ignored as testing progressed: the difference between Block One and Five was 1.9 (one-sample t-test: $t_{23} = 3.21$, $P < 0.0038$; Figure 3.3.a).

Total number of arms visited

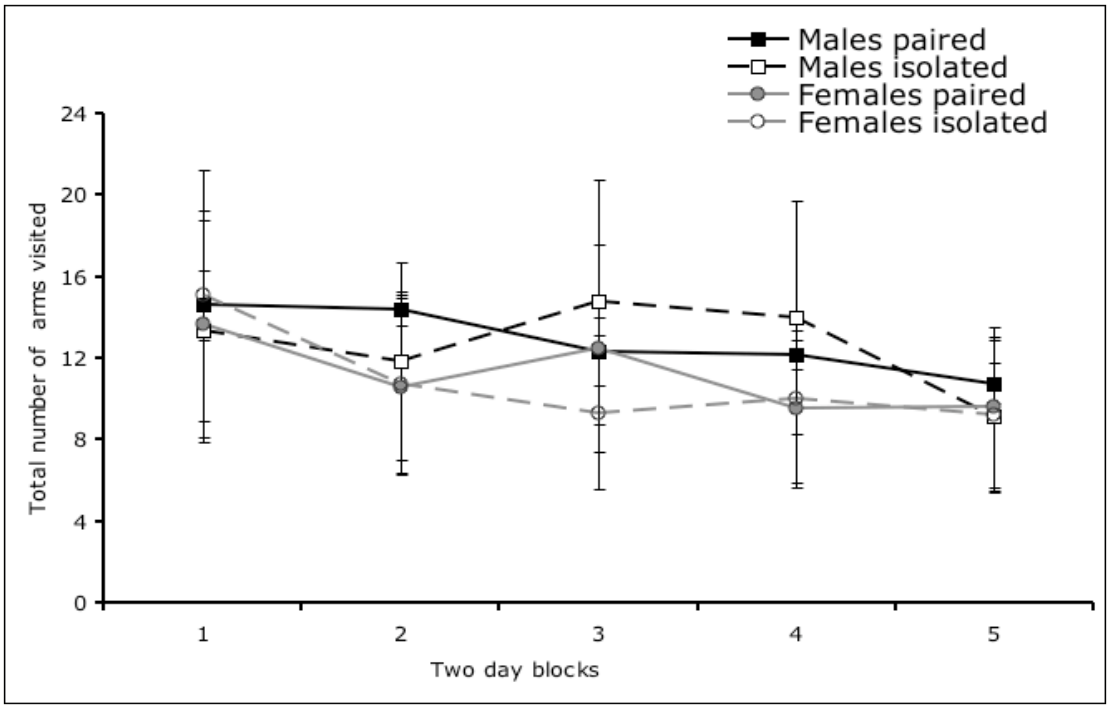
The groups did not differ in the reduction of the total number of arms they took to complete the task over the course of testing (Kruskal-Wallis: $K_3 = 1.59$, $P = 0.66$; median number of total arms to complete task (or before 10 mins elapsed) in Block One = 14 and in Block Five = 9; Figure 3.3.b). The number of arms entered before completing the task decreased significantly as testing progressed: the difference between Block One and Five in the number of arms visited was 4.5 (one-sample t-test: $t_{23} = 5.35$, $P < 0.0001$).

Figure 3.3: Performance in the RAM (mean ± SE) for male and female rats that were either pair or isolate-housed. N = 6 per treatment group. Each block represents the average over two days. Analyses were conducted on the difference between Block One and Five.

(a) Number of rewards that were ignored across the two-day blocks.



(b) Total number of arms entered across the two-day blocks.



3.4. Discussion

I predicted that stress brought about by isolation housing would impair performance in the RAM and that females would make more errors than males, specifically because isolated rats and female rats would ignore more rewards. Neither prediction was met: first, isolation did not impact on the change in performance from Blocks One to Five in either sex and second, males and females did not differ significantly in the number of rewards that they ignored, nor did they differ in the number of errors of any kind.

I can only find two other studies in which ignored food rewards are mentioned in sufficient detail that I can compare the performance of my rats with. In one of these studies, females ignore more rewards than males (1 vs 0.2) at the beginning of testing (Jones 2003). Jones suggested that this difference in ignored rewards explained the trend for males to make fewer working memory errors than the females at the beginning of testing. However, Boakes et al. (2000) found that more rewards were ignored at the beginning of testing (three) and there was no sex difference. I found, irrespective of sex, all of my rats ignored on average two rewards at the beginning of testing. Thus, my rats ignored slightly fewer than Boakes et al.'s rats and slightly more than Jones' rats, and I found no sex difference. It is possible that the different numbers of ignored rewards and the existence of sex differences in ignored rewards found across these studies can be explained in terms of different motivation levels in the rats due to the different food deprivation regimes that were used. For example, the rats in Boakes et al's study were not food deprived which may have lowered motivation to forage in all the rats. On the other hand, Jones maintained her rats at 90% of their free feeding body weight. It is possible that this chronic food deprivation was stressful for the females, making them ignore more rewards in the maze than did the males. I removed food from the rats for 17 hours prior to testing. Unlike Jones' animals, which were food-deprived relative to their own weight, my animals were all deprived for an equal length of time, which may or may not affect animals of different body weights to a different degree. I chose to food deprive in this (rather unorthodox) method because I had pair housed animals, and providing a ration of food may have lead to one of the pair monopolising the food and/or fighting. Future experiments are needed to determine if chronic food

deprivation is equally stressful for males and females and establish how (if at all) different deprivation regimes affect performance or ignored reward levels in the RAM in males and females. Ideally, a reward that the rats really covet should be used, so that food deprivation is not necessary.

Female rodents are typically more stressed (in terms of behaviour and stress hormones) than are males during MWM testing (Galea et al. 1994b; Perrot-Sinal et al. 1996; Beiko et al. 2004; Harris et al. 2008b) and in response to forced activity e.g. running (Kant et al. 1983). Therefore, I predicted that the females would be more stressed than were the males in the RAM, and so ignore more rewards. However, since ignored rewards did not significantly differ amongst the groups, it is possible that, unlike the MWM, the RAM is not a stressful task. Indeed, unlike the MWM, the RAM is not considered by the U.K. Home Office to be a procedure requiring a licence, implying that they do not consider the RAM to result in 'pain, suffering, distress or lasting harm' (Home Office 2000). Thus, the RAM may be the best task to use if a non-stressful spatial task is required.

As well as the RAM and/or food deprivation not being stressful, it is possible that ignored food rewards are a poor proxy for stress. Firstly, a rat may ignore a reward because it is not motivated to forage rather than because it is stressed. For example a rat may not be sufficiently food deprived and/or more motivated to explore than to feed. On the other hand, food deprivation, which should increase motivation to feed, may be stressful which may, paradoxically, cause a rat to ignore more rewards. Secondly, since a rat that ignores two rewards may not be twice as stressed as a rat that ignores one, stress is not as quantifiable during testing as it is during MWM testing (e.g. thigmotaxis correlates positively with stress/anxiety: Herrero et al. 2006). Thus, a non-appetitive and non-invasive measure of acute stress during RAM testing is needed to fully establish 1) if the RAM is stressful 2) if stress levels differ between treatment groups during testing, and 3) if food deprivation causes stress during testing.

I found no evidence that males and females differed in cognitive performance or stress levels in the RAM. This is at odds with authors who report that males demonstrate superior spatial abilities than do females in RAM tasks (Roof 1993a; Lund and Lephart 2001; Gibbs and Johnson 2008; Takase et al. 2008) and that

females are more stressed by cognitive testing (e.g. Beiko et al. 2004). This lack of sex difference in cognitive ability seems not to be because my RAM was too easy or hard, since my rats performed at similar levels to other researchers' rats in which a male advantage is seen, which implies that my females were performing well. For example, Sandstrom & Hart (2005) and Sandstrom (2005) found their rats make approximately 8 working memory errors in a 12-arm maze, the first block (an average over three trials) and after ten trials approximately 4 errors: the number of working memory errors (including ignored rewards) my rats made decreased from 8 in Block One to 4 in Block Five (other studies in which the number of working memory errors halves over ten trials: Williams et al. 1990; Seymoure et al. 1996). Also, in the same experiment, Sandstrom & Hart's rats make 6.5 reference memory errors in the first block and approx 5.5 after ten trials, which is a similar reduction to the number of reference memory errors made by my rats from Block One (four errors) to Block Five (three errors).

Isolated rats have been found to under-perform in the RAM relative to socially housed conspecifics (Einon 1980; Juraska et al. 1984; Seymoure et al. 1996). It is difficult to directly compare absolute levels of performance between these studies and my experiment (since my maze had fewer arms and these authors used a fully baited maze). For example, Seymoure et al. (1996) found that in a 17-arm maze their rats made approximately 8 working memory errors in Block One and after ten trials isolated rats made eight errors while socially-housed rats made half as many. Irrespective of housing, in the 8-arm maze my rats made five working errors in Block One and two errors after ten trials. Thus, my rats seem to be performing better than the isolated rats in Seymoure et al's study, but not as well as the socially housed animals (in spite of the difference in the number of arms in the maze). One explanation is that the socially housed rats in Seymoure et al's study also had physical enrichments in their cages (toys, nest boxes and so on), which may have improved spatial cognition independently of the social component (e.g. Kempermann et al 1997).

In conclusion, I found no difference in any measure of RAM performance between males and females, or between isolate and pair-housed animals. This latter result, coupled with those from my previous MWM experiments, suggest that

isolation housing, at least in the form practised in our animal house, is not sufficiently stressful to cause the observed sex differences in spatial cognition found in the literature, irrespective of whether the task is appetitive or aversive. Alternatively, it is possible that high levels of variation coupled with small sample sizes are obscuring potential differences in RAM performance between males and females and paired and isolated rats. It is unclear why performance levels were so variable, both across sex and housing treatments and within individuals. Additionally, since there is no measure of acute stress during testing, the MWM continues to be the most useful tool with which to examine both spatial cognition and stress simultaneously (since thigmotaxis in the MWM positively correlates with stress e.g. Beiko et al. 2004; Snihur et al. 2008; Herrero et al. 2006). Therefore, my further investigations into the impact of stress on spatial cognition will be carried out using an MWM.

In the next chapter I investigate the effect of *visual* isolation on cognitive performance in the MWM and stress levels in the home cage in male and female rats.

CHAPTER FOUR: Does visual isolation impair spatial cognition?

This chapter has been submitted as the following publication: Harris, A. P., D'Eath, R. B. & Healy, S. D. 2008. A cage without a view increases stress and impairs cognitive performance in rats. *Animal Welfare*

The home cage behavioural data and the thigmotaxis data in this chapter were collected with the assistance of Alex Brudenell and Emily Hope. I analysed all of these data myself and produced the manuscript in collaboration with the authors listed.

Summary

Isolation housing is believed to be chronically stressful and have a negative impact on welfare and cognition in rats. However, isolation does not consistently evoke stress-like responses nor does it consistently impair cognition. One possible explanation is that isolation effects vary depending on the severity of the isolation and that a significant contributor to that variation is the level of visual contact rats have with both neighbours and the rest of the holding room. In an experiment in which all cages were separated by a visual barrier, housing with a conspecific did not ameliorate the affects the barrier imposed on cognitive performance and stress levels during testing. Isolate-housed pigmented (dark-eyed) rats performed as well as pair-housed rats. Additionally, bar biting in the home cage did not differ between the two groups. However, rats housed without a barrier, whether housed with a cage-mate or alone, performed better in a spatial cognition task and were less stressed both in their home cages and during cognitive testing than were rats housed with a visual barrier between the cages. I conclude that interactions between the cages are of sufficient significance to rats such that single housing in a cage with a view to neighbouring rats and the rest of the laboratory holding room may be preferable to pair-housing in a cage without this view.

4.1. Introduction

Isolated rats develop 'odd' behaviours (tail chasing, 'pawing' at the air), eat more, put on more weight, are more aggressive, have heavier adrenal glands and under-perform in cognitive tests relative to socially-housed conspecifics (Hatch et al. 1963; Baenninger 1967; Hurst et al. 1998; Patterson-Kane et al. 1999, 2002; Sandstrom and Hart 2005). As a consequence of these findings, in the U.K. isolation housing is strongly discouraged by the Home Office (the major animal science regulatory body: Home Office 1989, 1995). Nevertheless, isolation housing is still widely used for logistical and ethical reasons, for example, to reduce the number of animals used, to avoid pseudoreplication, following surgery, or paradoxically to remove social stress (e.g. Nyska et al. 2002; Verwer et al. 2007).

However, despite the widespread belief that isolation housing impairs welfare, isolation does not consistently evoke greater stress hormone responses (Morinan and Leonard 1980) or result in heavier adrenal gland weights (adrenals secret the stress hormone corticosterone and enlarge with prolonged activity) than does social housing (Baldwin et al. 1995). Furthermore, isolated rats are not always cognitively impaired (Wongwitdecha and Marsden 1996) nor do they always eat and weigh more than socially-housed conspecifics (e.g. Baldwin et al. 1995). Additionally, in Chapters Two and Three I could find no compelling evidence that isolation housing had a detrimental impact on a range of typical welfare measures (body weight, food intake, bar biting behaviour) or on cognition in either male or female rats.

One explanation for these conflicting findings is that isolation effects vary depending on the severity of the isolation (Krohn et al. 2006). For example, if visual, olfactory and auditory communication between neighbouring rats is allowed, it is possible that isolation is less stressful than previously considered. Male rats housed alone but with visual, olfactory and auditory contact with neighbours are less aggressive when reintroduced to group housing than are rats without contact with neighbours (Hurst et al. 1997). Additionally, single-housed rats spend more time investigating a barrier between neighbours the more that barrier allows social contact (Hurst et al. 1997, 1998). These findings suggest that the degree of social contact among isolated rats may significantly affect the degree to which isolation is stressful.

Social contact can vary significantly with the type of cage in which the rodents are housed (often laboratory specific). In the U.K., 'standard' rat cages are opaque, plastic-bottomed cages with stainless steel wire mesh lids (see Figure 4.1 for an example of two cages supplied by North Kent Plastic Cages LTD, Kent, England). The wire mesh lids differ in size depending on the type of cage that is used (Figure 4.1a and b) leading to considerable variation in the degree of social contact a rat has with its neighbours and the laboratory holding room.

The following experiment had two aims: 1) to determine whether preventing visual contact (a barrier was placed between the cages) with neighbours was more stressful for rats housed alone than for pair-housed rats; and, 2) to determine if isolation without visual contact with neighbouring rats is sufficiently stressful to impair spatial cognition in a Morris water maze (MWM). I then used the data collected in this experiment to determine whether removal of visual contact has a significant impact on welfare and cognition in isolate-housed rats by comparing data from this experiment with those from animals in Chapter Two (Experiment Three), a similar experiment in which there were no visual barriers between the cages.

To measure stress I recorded bar biting in the home cage and I monitored thigmotaxis (swimming in the periphery of the pool) during the cognitive testing in the MWM (Hurst et al. 1998; Wilcoxon et al. 2007). Body weight and food intake were also monitored as basic indicators of welfare. If visual isolation does induce stress, I would expect 1) isolated rats to perform more poorly in a spatial cognition task and be more stressed due to the imposition of the barrier than would pair-housed rats; and, 2) isolated rats with a barrier between the cages to perform more poorly and be more stressed than isolated rats without a barrier (i.e. rats from Chapter Two).

Figure 4.1: Examples of two rat cages which present different levels of visual contact between neighbouring rats and the rest of the laboratory holding room.

(a) Dimensions: 38x25x20 cm high has a 2 cm strip of wire mesh at the top of the cage. Rats in these cages have no visual contact with neighbours when the cage is in the holding rack.

(b) Dimensions: 45x28x20 cm high has an 8 cm strip of wire mesh at the top of the cage. In this type of cage rats can see their neighbours and out into the rest of the room when the cage is in the holding rack.

4.2. Materials and methods

Subjects and housing

Eighteen male and eighteen female Lister Hooded rats, aged four-to-five weeks and obtained from Harlan U.K. Ltd were the subjects tested in this experiment. At the time of arrival, the males weighed 160g (± 11g) and the females weighed 120g (± 7g). Six rats of each sex, were chosen at random and housed in isolation, the remaining 12 were housed in same sex pairs (N = 6 per treatment group). Rats remained in their housing conditions throughout the experiment. Where pair-housed, one rat was marked with hair dye (Schwarzkopf, R43) to enable identification. To avoid pseudoreplication, one rat from each pair was picked at random to be the focal animal and this rat remained the only source of data from the pair for the duration of the experiment.

All rats were housed in standard plastic bottomed cages, dimensions 45x28x20 cm (RB3 cages; North Kent Plastic cages Ltd., Kent, England). A barrier made from white plastic and covered with white paper was slotted between each neighbouring cage within the holding rack. This barrier prevented visual contact between neighbouring rats and reduced visual contact with the rest of the holding room while not impeding olfactory and auditory communication. Rats were fed *ad libitum* pelleted food (RM3 diet, Special Diet Services, Ltd., Witham, Essex, UK) and tap water and maintained under a 12L: 12D cycle (lights on at 0600hours) at 21-24ºC. All of the rats were handled (picked up using both hands clasped firmly and gently around the rat's body) at least twice weekly (e.g. during cage cleaning and weighing) for ten weeks prior to cognitive testing. By the time cognitive testing began the majority of the rats were 'tame' (did not struggle, squeak or attempt to bite during handling). During MWM testing each rat was handled daily for approximately 2-3 minutes in total (struggling and squeaking during placement in the MWM was frequently observed).

Rats experienced their respective housing condition for ten weeks before spatial ability was assessed in an MWM. Each isolated and focal rat was tested in the MWM. At the end of the experiment all rats were humanely euthanased in a carbon dioxide chamber.

MWM apparatus

The MWM apparatus and procedure is described in detail in section 2.2.1. Briefly, the MWM consisted of a circular tank with its bottom raised 50 cm above floor level on a platform. The MWM was positioned in an experimental room with multiple visual spatial cues visible from the inside of the tank. The tank was filled with tap water (24±1ºC) and made opaque with non-toxic white paint. An escape platform was located 2 cm below the surface of the water and 30 cm from the edge of the tank in the centre of one of four imaginary quadrants (the four main compass points N, E, S or W). All trials were videoed from above and to prevent myself from distracting the rats during testing, all trials were observed via a video monitor once the rat was placed in the water.

MWM procedure

Each rat received two days of training before testing began. On a training day each rat received two consecutive swims to the hidden platform. The platform location was the same within each day but its position was changed from day to day. Platform location was pseudo-randomly determined so that the platform was never in the same place on two consecutive days. A swim started after the rat was gently lowered into the water by hand and released facing the side of the tank and ended when the rat found and subsequently climbed onto the platform. The time taken by the rat to find the platform was recorded to the nearest second using a stopwatch. If a rat failed to find the platform within 120 s it was gently guided to, and allowed to climb onto, the platform. Once on the platform a rat was left for 20 s before being picked up and released from one of the other three possible release points. After the final swim a rat was left on the platform for 20 s and then gently removed from the platform, towel dried, put back in its home cage and placed under a heat lamp for approximately 10 minutes to dry.

Testing proper started the day following the last day of training and the procedure was exactly as for training with the exception that each rat received four swims each day for 16 consecutive days. All trials were conducted between 1100 and 1500 hours.

Working and reference memory assessment

The time taken to reach the platform across the four daily swims provided the measure of working memory. To measure reference memory the percentage of time that a rat spent swimming in each of the four quadrants in Swim One of each day was recorded. The quadrant that contained the platform was discounted and the proportion of time spent in the remaining three quadrants was calculated to establish if a rat spent more than 33.3% (chance) of its time searching in the quadrant that contained the platform on the previous day. Reference memory was assessed from Day Two of testing to Day Five for two reasons: (1) because reference memory cannot be measured on Day One, and (2) moving the platform every day over 16 days of testing may have led to the rats learning to avoid the previous day's platform location.

Thigmotaxis measurement

The percentage of time that a rat spent swimming within 150 mm of the wall of the maze was recorded for Swims One and Two across the 16 days of MWM testing. The footage of each swim was watched on a TV monitor. An acetate sheet, placed over the TV, displayed the outer 150mm periphery and the time that a rat's head and shoulders spent in this area was recorded with a stopwatch.

Monitoring body weight and food intake

Body weight was measured once a week until the week that MWM testing began. Food intake was also measured once a week from the second week post arrival to the week prior to MWM testing. To measure food intake, the entire contents of a food hopper (one per cage) were weighed before the food was topped up and re-weighed. Food intake per rat per day was estimated by dividing the amount eaten by the number of days since the food was last weighed. Where rats were pair-housed an average intake was calculated for both of the rats.

Behaviour in home cage

The rats were filmed in their home cages for two hours during the dark phase (0300-0500hours) using a black and white wide-angle (4 mm) lens camera. During filming a 40W red light bulb was used for illumination. Each cage was filmed prior to MWM testing (approximately five-eight weeks post arrival). Bar biting was scored for each focal animal in the 1st, 12th, 24th, 36th, 48th, 60th, 72nd, 84th, 96th, 108th and 120th minute of the footage providing an observation period of 11 minutes per rat. Every five seconds for each of these minutes, we noted the presence or absence of bar biting. The total number of occurrences of bar biting were totalled and multiplied by five (duration of the 'sample observation') to equal total time spent bar biting, which was then converted to a percentage of the observation period. The total number of rats that showed at least one occurrence of bar biting behaviour was also noted.

Data analysis

Data that included repeated measures on the same subject were analysed using a repeated-measures-analysis of variance. The *between-subject* factors were sex (male/female) and housing condition (pair/isolated) and the *within-subject* factors were swim number (One to Four) and day (One to 16).

To investigate the impact of the visual barrier on behaviour, data from the current experiment were compared with data from Chapter Two Experiment Three. In Chapter Two six rats of each sex were isolated and 12 of each sex were pair housed (thus, as in this experiment, N = 6 per treatment group). The rats were of the same strain, age and were housed in pairs or isolation without barriers between the cages for 10 weeks before cognitive testing. Bar biting was recorded during the night approximately four to eight weeks post arrival in both experiments. Using RM ANOVA, the between-subject factors were barrier (present/absent), housing (pair/isolated) and sex (male/female); the within-subject factors were as above. All interactions were tested and, if not significant, were removed from the final model. The assumptions of sphericity were tested using the Mauchly-criterion test. If the assumption of sphericity was not met, we used Greenhouse-Geisser adjusted degrees

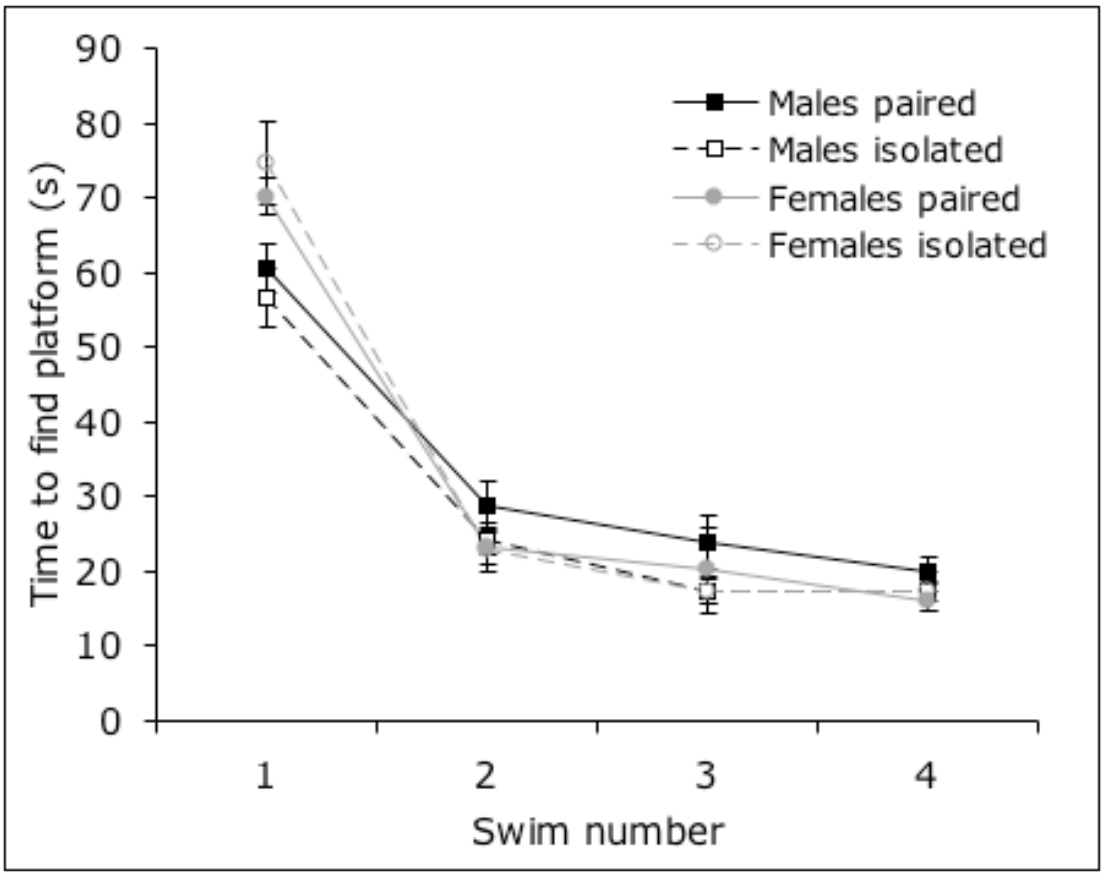
of freedom and the associated P-values, which is why the degrees of freedom we report are not always whole numbers (Quinn and Keough 2002). The assumptions of normality of residuals and homogeneity of variance were tested and appropriate transformations applied to the data, where necessary.

4.3. Results

Working memory

Isolated and pair-housed rats with a barrier between their cages performed equally well in the MWM ($F_{1,21}$= 0.82, P = 0.37; Figure 4.2). Although the average performance of males and females did not differ significantly (i.e. averaged over the four swims and across the 16 days of testing; $F_{1,21}$= 0.56, P = 0.46), females took significantly longer to find the platform in Swim One than did the males (swim-by-sex interaction: $F_{2.1,44.0}$= 9.47, P = 0.0003; Tukey HSD, P < 0.05; females took a mean of 72 ± 4s and males took 59 ± 4s). All of the rats took less time to reach the platform with increasing swim number within a day ($F_{2.1,44.0}$ = 292.98, P < 0.0001; Figure 4.2) and across days ($F_{7.1,149.1}$ = 8.76, P < 0.0001). No other interactions were significant.

Figure 4.2: Mean time (±SE) to find the platform (s) across the 16 days of testing for male and female rats that were housed in pairs or isolation with a barrier between the cages for ten weeks, N = 6 per group.



Irrespective of whether rats were housed alone or with a cage-mate, rats housed without a barrier found the platform significantly faster than rats housed with a barrier between the cages, although only in Swims Two and Four (barrier: $F_{1,43}$= 12.97, P = 0.0008; Swim-by-barrier interaction: $F_{2.0,86.52}$ = 5.27, P = 0.068; Tukey HSD, P < 0.05; Figure 4.3). The barrier had a similar effect on males and females ($F_{1,43}$= 0.30, P = 0.59) but males tended to outperform females in Swim One if housed with a barrier (swim-by-sex-by-barrier interaction: $F_{2.0,86.52}$ = 2.60, P = 0.080; Tukey HSD, P < 0.05; Figure 4.4). No other interactions were significant.

Figure 4.3: Comparison of the mean time (±SE) to find the platform across the 16 days of testing for rats that were housed in pairs or isolation with or without a barrier between the cages, data are pooled across sex, N = 12 per group.

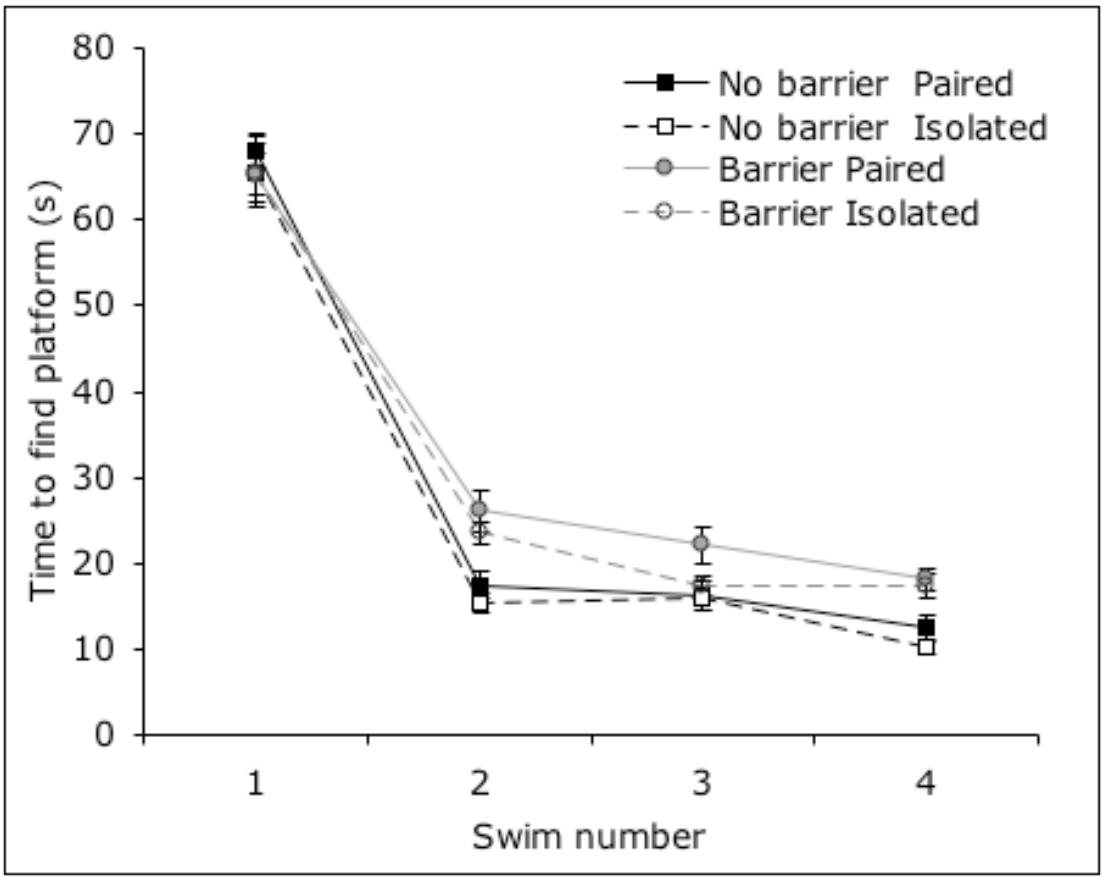
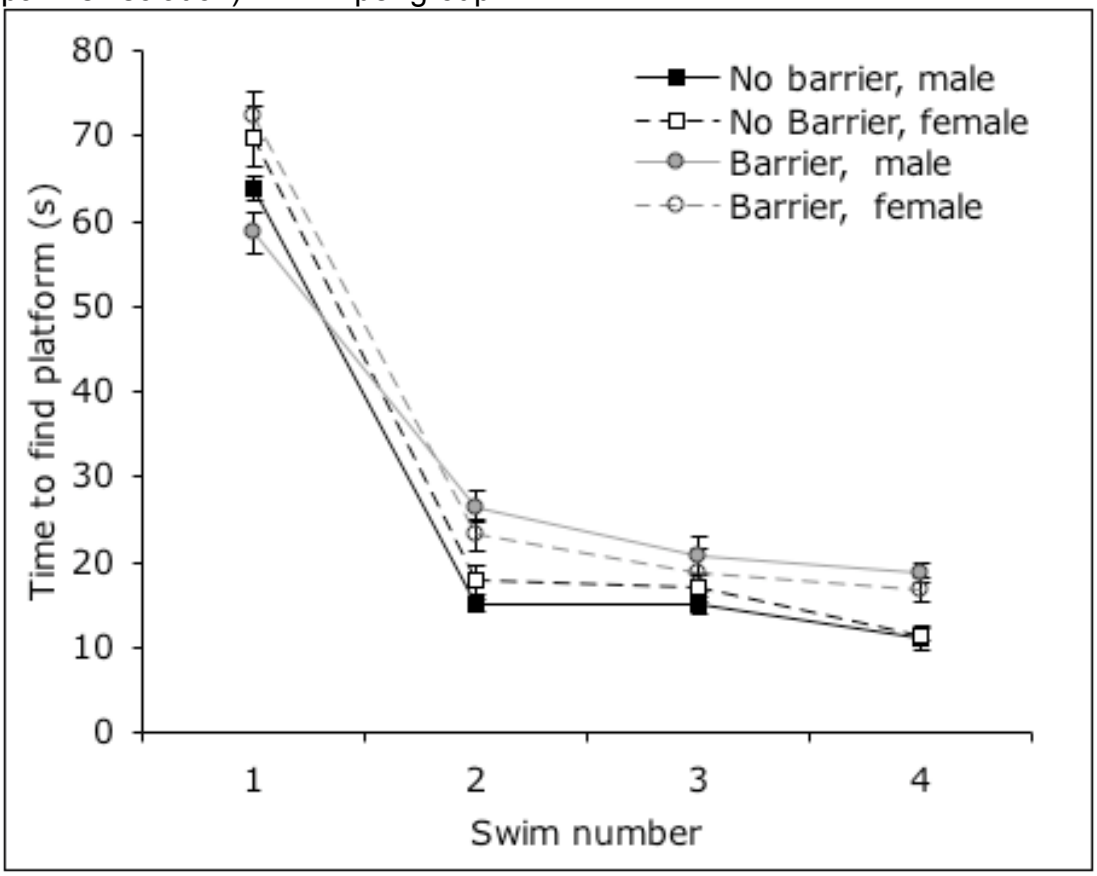


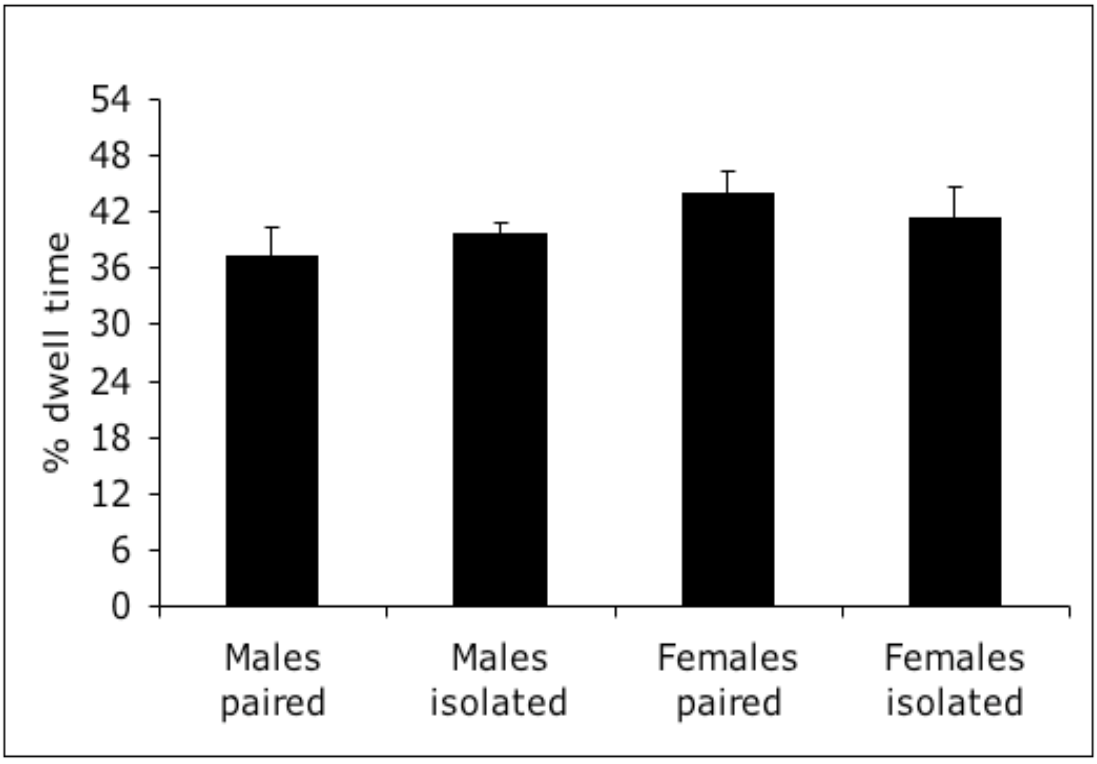
Figure 4.4: Comparison of the mean time (±SE) to find the platform across the 16 days of testing for male and female rats that were housed with or without a barrier between the cages, data are pooled across housing (i.e. pair vs. isolation) N = 12 per group.



Reference memory

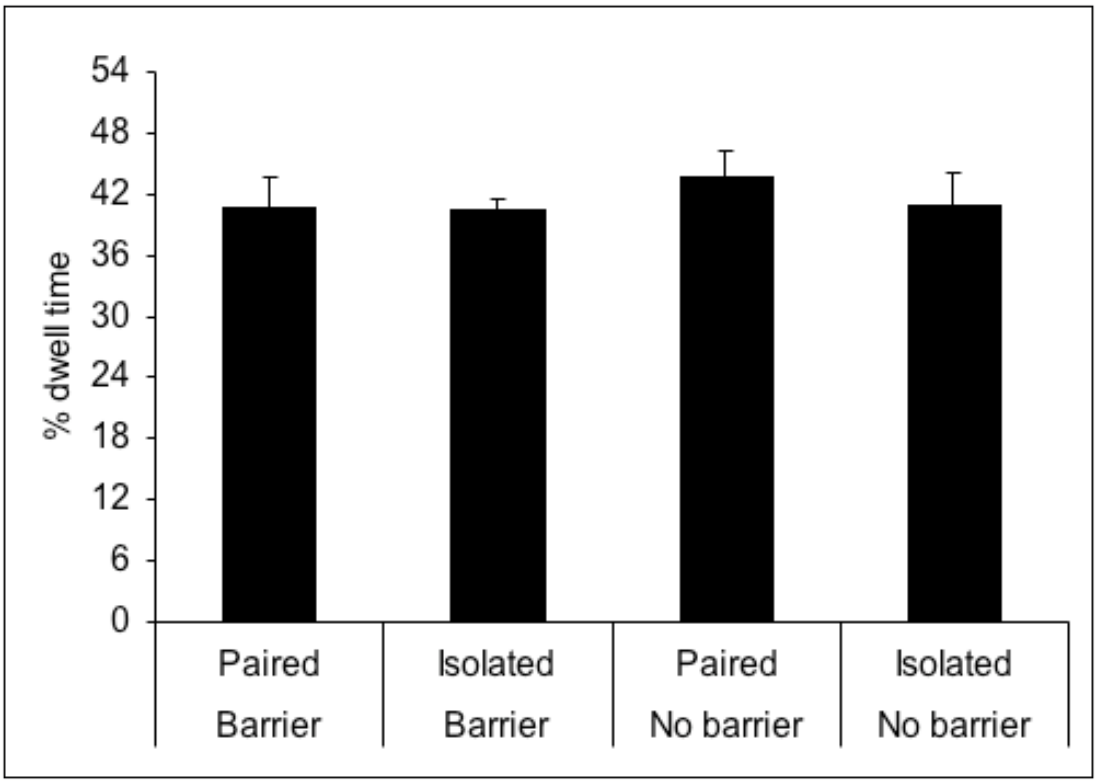
In Swim One, the isolated and pair-housed rats did not differ significantly in the amount of time they spent searching in the quadrant that contained the platform on the previous day ($F_{1,21} < 0.01$, P = 0.95; mean ±SE for pair-housed rats 40.7 ± 2.1% and for isolated rats 40.6 ± 1.7; Figure 4.5). Additionally, males and females did not significantly differ in the time they spent in the target quadrant ($F_{1,21}= 2.43$, P = 0.13; for males 38.5 ± 1.6% and females 42.6 ± 2.0%). The amount of time that the rats spent in the target quadrant varied significantly over Days Two to Five ($F_{2.2,47.0}= 4.0$, P = 0.02), but this seems to be due to non-directional fluctuations rather than to an increase or decrease across the days (time spent in Day Four is longer than on the other days). No other interactions were significant.

Figure 4.5: Mean time (±SE) spent in the target quadrant in Swim One across days Two to Five for male and female rats housed in pairs or isolation, all cages had a barrier between them, N= 6 per treatment group.



The average time spent in the target quadrant over the four days was calculated for each rat and pooled across sex for comparison with the comparable data from Chapter Two. Irrespective of whether rats were pair housed or isolated, the presence of the barrier had no impact on their reference memory performance (ANOVA; barrier: $F_{1,46}$= 1.30, P = 0.26; mean (±SE) for barrier-housed rats: 40.5 s ± 1.3%; without barrier: 37.9 s ± 1.9%; Figure 4.6).

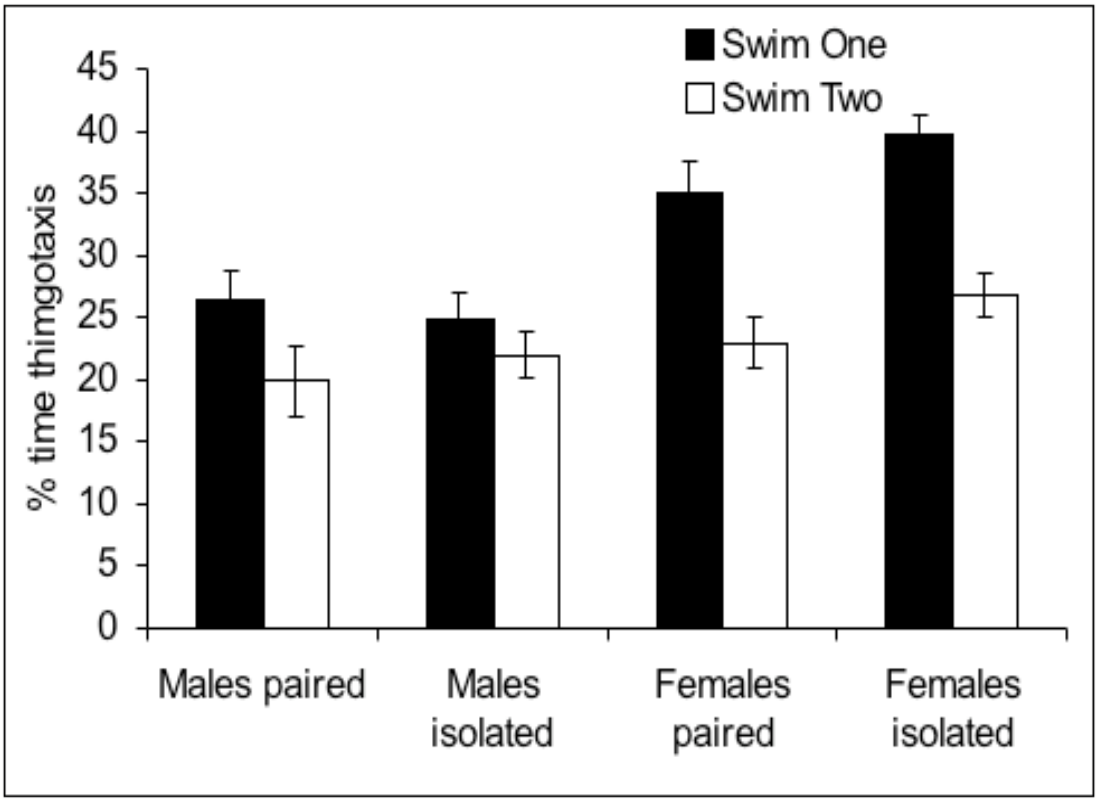
Figure 4.6: Comparison of the mean (±SE) time in the target quadrant in Swim One across days Two to Five for rats that were pair or isolated with or without a barrier between the cages, N= 12 per treatment.



Thigmotaxis

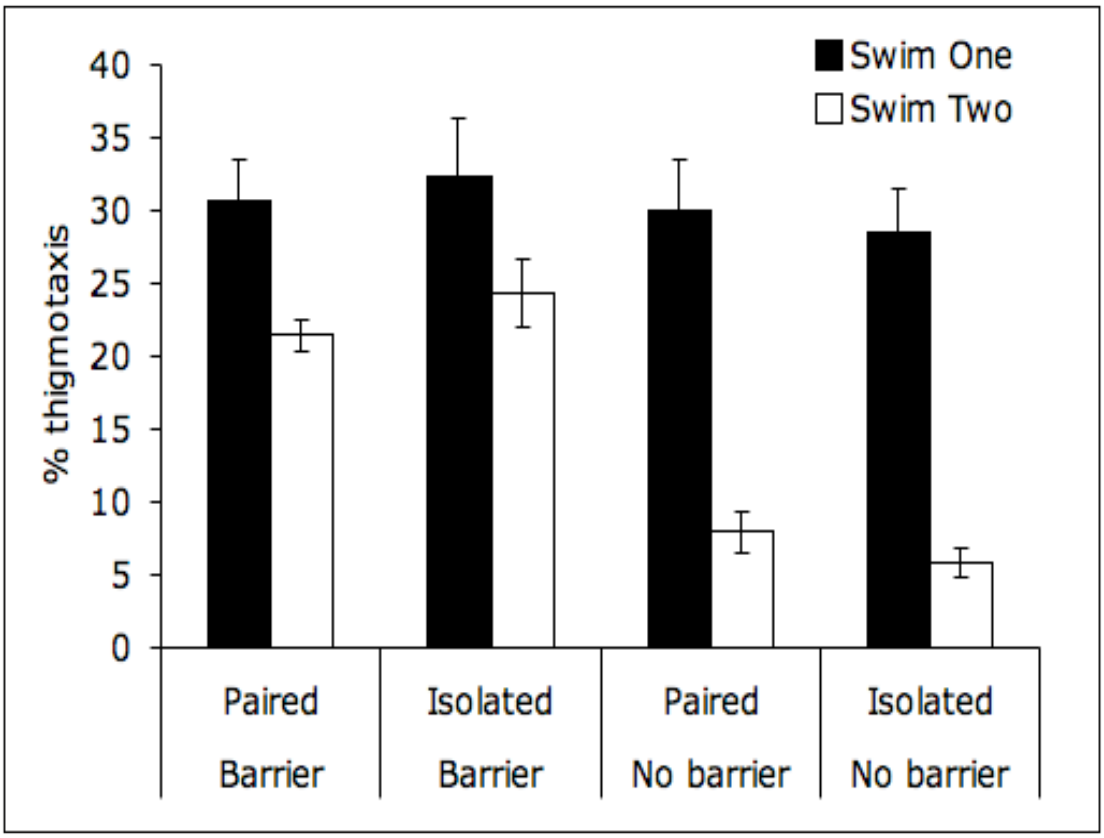
Isolated and pair-housed rats were equally thigmotactic during testing ($F_{1,21}$= 0.50, P = 0.49; Figure 4.7). Also, irrespective of housing conditions, the females were more thigmotactic than were the males ($F_{1,21}$= 6.03, P = 0.02) but only in Swim One ($F_{1,21}$= 8.57, P = 0.008; Figure 4.6). All of the rats were less thigmotactic in Swim Two than they were in Swim One ($F_{1,21}$= 41.6, P < 0.0001; Figure 4.7). Thigmotaxis levels changed significantly across testing ($F_{7.3,153.0}$= 4.12, P = 0.0003), but there was no directional trend (e.g. thigmotaxis on Day One is significantly higher than on Day eight but not Day 16; Tukey HSD, P < 0.05). No other interactions were significant.

Figure 4.7: Mean (±SE) percentage of Swim One and Two spent in thigmotaxis for male and female rats that were housed in pairs or isolation for ten weeks, N = 6 per group.



To determine the impact of the barrier on thigmotaxis the average thigmotaxis level for each rat across the 16 days of testing was calculated for Swims One and Two. These data were then compared with the corresponding data from Chapter Two. Rats housed with a barrier were significantly more thigmotactic than were rats housed without a barrier between the cages, irrespective of whether the rats were housed in pairs or in isolation (barrier: $F_{1,43}$= 21.45, P < 0.0001; housing-by-barrier interaction: P > 0.05; Figure 4.8). However, this difference was only significant in Swim Two ($F_{1,43}$= 39.37, P < 0.0001; Tukey HSD, P < 0.05; Figure 4.8). The presence of the barrier did not affect sex differences in thigmotaxis: males were more thigmotactic than the females in Swim One only (sex: $F_{1,43}$= 18.12, P < 0.0001; sex-by-swim interaction: $F_{1,43}$= 11.29, P = 0.0016; sex-by-barrier interaction: $F_{1,43}$= 0.08, P = 0.78).

Figure 4.8: Comparison of mean percentage of Swim One and Two (±SE) spent in thigmotaxis for rats were housed in pairs or isolation with or without a barrier between the cages, data are pooled across sex, N = 12 per group.



Body weight and food intake

Regardless of sex, isolated barrier rats tended to weigh more than the pair-housed barrier rats: average body weight (±SE) for females 186 ± 3g vs. 179± 3g; and males 310 ± 7g vs. 296 ± 8g, for isolated and pair-housed rats respectively ($F_{1,21}$= 4.28, P = 0.051). Males weighed more than females throughout the experiment ($F_{1,21}$= 478.8, P < 0.0001) and the males gained weight at a faster rate than did the females, as the experiment progressed ($F_{1.5,32.0}$= 372.33, P < 0.0001). No other interactions were significant.

Body weight in the 10th week post arrival was compared with that of the rats in Chapter Two using a three-way ANOVA (housing; sex; barrier). When the barrier was present the isolated animals weighed more than the paired animals and the opposite tended to be true when the barrier was absent ($F_{1,43}$= 2.07, P = 0.04; Tukey, HSD P < 0.05). The barrier had a similar effect on male and female body weights (P > 0.05).

In the current experiment, irrespective of sex, the isolated barrier rats ate significantly more than did the pair-housed barrier rats: mean daily intakes for females 19 ± 0.4g vs. 17 ± 0.4g; and males 25 ± 0.4g vs. 23 ± 0.3g, for isolate and pair-housed rats respectively ($F_{1,21}$= 418.55, P < 0.0001). Males ate significantly more than females throughout the experiment ($F_{1,21}$= 25.52, P < 0.0001) and there were significant changes in food intake during the course of the experiment, but neither an increase nor a decrease across the experiment ($F_{1.4,29.3}$= 8.39, P = 0.0034; Figure 4b).

The average food intake per cage in week ten post arrival was calculated and compared with that from Chapter Two (Exp. 3). The presence of the barrier and whether the rats were isolated or paired had a significant impact on food intake (barrier: $F_{1.43}$= 3.56, P = 0.0009; housing: $F_{1,43}$= 3.33, P = 0.0018). However, these main effects were mediated by a significant interaction: only when the barrier was present did the isolated animals eat significantly more than the paired animals ($F_{1, 43}$= 2.67, P = 0.011; Tukey, HSD P < 0.05).

Bar biting

Isolated barrier rats did not bite the bars more than did pair-housed barrier rats during the observation period ($F_{1,19}$= 1.83, P = 0.19; mean proportion of time bar biting for pair-housed males: 5 ± 1.6%; isolated males 9 ± 3.7%; paired females 2 ± 1%; isolated females 6 ± 3.2%) but not all rats were observed to bite the bars. Analysing only the data from the seven pair-housed and eight isolated rats that were observed to bite the bars, there was still no significant difference in bar biting levels between the two groups ($F_{1,13}$= 2.96, P = 0.11). Male and female rats also did not differ in the amount of time they spent bar biting ($F_{1,19}$= 2.04, P = 0.17; N = 22 because the data from two cages were lost due to human error). In comparison, four pair-housed and two isolated rats in Chapter Two were observed bar biting, half the number as when a barrier was placed between the cages.

4.4. Discussion

Reducing visual contact between rats by the insertion of an opaque barrier between neighbouring cages was expected to have a bigger effect on the behaviour of animals housed alone than those housed in pairs (prediction one). This prediction was not met: although I found that isolated animals ate more (by approx. 10%) and tended to be heavier (by approx. 5%) than pair-housed animals, bar biting did not differ between the groups. Additionally, there were no group differences in cognitive performance in the MWM nor in the amount of time spent in thigmotaxis (our behavioural measure of stress during testing). However, I found considerable evidence for my second prediction: in comparison with rats in a previous experiment housed without a barrier (Chapter Two), the presence of the barrier led to an increase in the number of rats performing bar biting, increased thigmotaxis in the MWM and consequently impaired cognitive performance. This was true for both isolated and pair-housed rats, implying that a cage-mate did not ameliorate any stress caused by the barrier.

Isolated rats tend to bar bite more than socially-housed rats apparently in frustrated attempts to leave the cage and seek social contact (e.g. Hurst et al. 1997, 1998; Nevison et al. 1999). Although I did not observe a difference in bar biting between the isolated and pair-housed animals in this experiment, it is possible that

differences in this behaviour existed but were not detected because of the small sample sizes (i.e. small number of rats and short observation period). Nevertheless, the presence of the barrier did lead to a greater incidence of bar biting than was observed in Chapter Two. An increase in this 'escape-related' behaviour suggests that reduced visual contact with neighbouring rats/the holding room potentially impairs welfare through an increase in frustration levels, which rise even when the rat has a cage-mate. Furthermore, isolated rats ate significantly more and tended to weigh more than pair-housed conspecifics only when the barrier was present (true for both males and females). It is unclear why isolated animals typically eat more than socially housed conspecifics (e.g. Morgan and Einon 1975; Fiala et al. 1977; Brown and Grunberg 1996).

The presence of the barrier also increased stress levels during cognitive testing: thigmotaxis levels were almost four times higher in the barrier rats than in the non-barrier animals in Swim Two (Figure 4.8). As thigmotaxis correlates positively with anxiety/stress levels (Treit and Fundytus 1989; Snihur et al. 2008) increases in this behaviour suggest impaired welfare during testing. Thigmotaxis also impairs performance in the MWM because the platform is never located in the outer periphery of the tank. Correspondingly, because thigmotaxis was so much higher in the barrier rats, the level of cognitive performance of the rats housed with a visual barrier never reached that of the rats housed without barriers. Although rats did not differ in the time they took to reach the platform on Swim One (all rats spent considerable periods of time in thigmotaxis), the barrier rats took significantly longer than the non-barrier rats to reach the platform on Swim Two, an effect still noticeable by the fourth daily swim and across the 16 days of testing (Figure 4.3). It seems likely that ceiling levels of thigmotaxis/stress were reached in Swim One by all of the rats, even those without the barrier. Visual isolation did not appear to be more stressful for isolated rats than pair housed rats because the presence of a cage mate did not appear to ameliorate the effect of the barrier on thigmotaxis or performance in the MWM. This suggests that the barrier increased stress levels not simply because it removed social contact, but perhaps because it reduced how much the rats could see out of the home cage and into the holding room. For example, it is possible that the barrier rats were less aware of people in the holding room and

therefore had less opportunity to habituate to people. Data regarding the ease of handling of the differentially housed rats may determine if this is a plausible hypothesis. An alternative explanation for higher stress levels in the barrier rats is that the noises of unidentified animals from behind the barrier may have been stressful for these rats. For example, unidentified noises may have induced fear of predation in the barrier-housed rats. To determine if this is a plausible explanation, the cognitive performance and stress related behaviour of rats housed with barriers and empty neighbouring cages could be investigated.

Conclusions and animal welfare implications

I found that the loss of visual interactions between caged rats and the holding room led to a significant increase in stress, both in terms of behaviour within the cage and the stress response during MWM testing. This affect was seen in males and females and did not seem to be ameliorated by the presence of a cage mate. This effect of the physical, rather than the social, attributes of housing is currently relatively unappreciated, with far more emphasis on within-cage enrichment (Home Office 1995; Patterson-Kane 2004; Balcombe 2006).

The conclusions that I present here are based on the comparison of data from two experiments that were carried out at different times (approximately four months apart). Although the rats were the same strain and age, kept in the same caging (but without a barrier between the cages) and tested in the same cognitive task after the same experience, it is possible that the presence of the barrier is not the only difference between the two experiments. To strengthen the conclusion that the barrier is responsible for the differences in the animals' behaviour and cognitive performance across the two experiments, this experiment needs to be repeated with all housing conditions applied within the same experiment.

Based on these data that I present here I cautiously conclude that the welfare of rats housed alone but with visual contact with neighbours may be better than that of pair-housed rats in cages that prevent visual contact with neighbours. Although barriers are not a feature of rat housing, many of the cages deemed suitable for housing rats preclude visual contact to a significant degree. A corollary of demonstrating that visual contact may lead to better welfare than does pair housing is

that the welfare costs of housing rats alone may be traded off against the reduction in numbers of animals required to deal with pseudoreplication (although group-housed, only one animal per group or an average per cage can be used as an independent source of data for analysis: Hurlbert 1984; Festing et al. 2002). Reducing the numbers of animals used in scientific research is one of the aims of the 3Rs (reduction, replacement and refinement) which form the basic principles of humane research (Russell and Burch 1959). Although single housing rats is a rather heretical suggestion, based on the results I present here, I would encourage further investigation (using additional measures of welfare to those employed here) into the role of visual contact allowed by different cages on the welfare of laboratory rats.

In the next chapter I investigate if isolation housing causes sufficient stress to impact on cognition in an albino strain of rat. I also examine if acute stress during testing affects performance, and if the sexes are affected differently by each of these sources of stress.

CHAPTER FIVE: Acute stress explains sex differences in cognitive performance in albino rats

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I collected and analysed all of the data myself and wrote the manuscript in collaboration with the authors listed.

Summary

Male rats typically outperform females in tests of spatial cognition. However, as stress affects cognition differently in the two sexes, performance differences may be an artefact of stress. Rats face at least two sources of stress during an experiment: the test situation (acute) and housing conditions (chronic, e.g. isolation). We used a task (the Morris water maze, MWM) that allowed testing of both spatial working and reference memory to investigate whether chronic stress (isolation housing) and/or acute stress (the task) has a differential impact on spatial cognition in male and female albino rats. Irrespective of age at onset of isolation housing, isolated rats were not spatially impaired relative to pair-housed rats. However, the acute stress of the task led to adult males apparently outperforming adult females: adult females took longer to reach the platform than did males because they spent more time in thigmotaxis (swimming close to the wall) during testing. In juvenile rats, the stress caused by swimming in the MWM resulted in both males and females being highly thigmotactic and no sex difference in performance. We conclude that stress can lead to apparent differences between the sexes in performance on a spatial cognition task.

5.1. Introduction

Male mammals typically outperform females in tests of spatial cognition (e.g. Galea et al. 1996; Astur et al. 2004; Jonasson 2005) and at least seven evolutionary hypotheses have been proposed to explain the existence of this sex difference (reviewed in Jones and Healy 2006). One reason for the number of hypotheses is that sex differences in spatial cognition have been observed in various experimental paradigms, each of which appears to differ in some important way. However, it is also the case that sex differences are not always observed and it is difficult to compare the predictions of these various evolutionary hypotheses if the supposed difference cannot be reliably produced even when looked for under apparently the same test conditions (e.g. Bucci et al. 1995; Healy et al. 1999).

This lack of reliable replication has at least two possible hormonal explanations: (1) variation in the sex hormones (known to affect spatial cognition in mammals e.g. reviewed in Williams and Meck 1991), as a result of either fluctuations in testosterone, causing male performance to go up or down, or, variation in oestrogen levels, causing changes in female performance; (2) variation in the stress levels of the animals under test (e.g. Bowman 2005). It is only the second of these that we consider here. The reason for suspecting that stress may explain the lack of replicability in, perhaps even the existence of, sex differences in spatial cognition, is that there are a plethora of data to show not only that stress affects spatial cognition, but also that it does so differently in females than in males: females tend to respond more poorly to acute stress, such as is imposed by a test situation, and yet their spatial performance may be unchanged or enhanced by chronic stress. Male cognitive abilities, on the other hand, may be adversely affected under conditions of chronic stress (Luine 2002; Conrad et al. 2003; Beiko et al. 2004; but see Conrad et al. 2004).

Compounded by the fact that most of the sex difference literature comes from laboratory tests on rodents (often rats), it is conceivable that many of these data are, actually, an artefact of stress caused by one or more laboratory variables. A further significant component is the strain of rat: sex differences in cognition have been more often found using albino rather than pigmented strains (Markowska 1999; Warren and Juraska 2000; Blokland et al. 2006). This apparent strain effect may be

because albino strains are more 'anxious' and 'emotional' than pigmented strains (e.g. more likely to freeze) in behavioural tests of anxiety (e.g. light/dark box, open field Schmitt and Hiemke 1998).

There are at least two potential sources of anxiety or stress that a laboratory rodent may face during an experiment: the testing situation and the housing conditions themselves (e.g. isolation housing, reviewed in Patterson-Kane 2001; Krohn et al. 2006). In cognitive tests a rat often has to venture out into an exposed, brightly lit area to locate a goal or an escape option. For example, during Morris water maze (MWM) testing, rats are required to swim in tepid water to locate a hidden escape platform. Albino strains perform less well than pigmented strains in MWM tasks (Tonkiss et al. 1992; Harker and Whishaw 2002) and while it is possible that poorer vision in albino rats may mean they find it more difficult to see extra-maze cues needed to solve the task than do pigmented rats, an alternative explanation is that albino rats find bright open-field tasks, such as the MWM, more aversive than do pigmented rats. If so, they are more likely to spend time being thigmotactic (Andrews 1996; Prusky et al. 2002). In the context of the MWM, high levels of thigmotaxis will lead to longer escape latencies since the platform is usually located at least 30 cm away from the edge of the tank (Treit and Fundytus 1989; Saucier and Cain 1995; Herrero et al. 2006).

Thigmotaxis (wall-hugging) is considered a marker of stress shown by rodents in open-field situations and provides a noninvasive measure of stress during MWM, which is readily quantified. Confirmation that this behaviour is an indicator of stress comes from the fact that both administration of anxiolytics and pretraining in the MWM reduce thigmotaxis (Galea et al. 1994b; Beiko et al. 2004). Additionally, thigmotaxis levels are positively correlated with both endogenous and exogenous corticosterone levels during MWM testing (Herrero et al. 2006; Snihur et al. 2008). Furthermore, hippocampal lesions, which impair MWM performance, do not affect thigmotaxis; thus a rodent does not swim near the pool wall simply because it does not know the location of the platform (Hostetter and Thomas 1967; Morris et al. 1982).

Housing conditions constitute a second potential source of stress for laboratory rodents. For example, isolation housing is reported to be stressful for rats,

and 'isolation-stress syndrome' (e.g. increased aggression and body weight, hyperactivity and impaired spatial cognition) is often reported in albino strains of rat (Hatch et al. 1963; Holson et al. 1991; Heidbreder et al. 2000; Lu et al. 2003; Shabanov et al. 2004).

If stress plays a significant role in the production of sex differences in spatial cognition in laboratory rats, a large proportion of the data used to support evolutionary hypotheses for those sex differences may be questionable. To determine the degree to which stress affects spatial cognition, we manipulated stress chronically (isolation housing) and tested rats under an acutely stressful situation (MWM testing). We examined the effects of these stressors on the animals' performance in both a working and a reference memory task. We measured thigmotaxis during MWM testing to determine stress behaviourally as well as measuring body weight and food intake as physiological markers of chronic stress, since these are reported to increase in isolated rats (Würbel and Stauffacher 1996; Hurst et al. 1998).

If chronic stress has an impact on performance in the MWM, we would predict that isolated males would respond more poorly than females and there would be no sex difference between these animals i.e. apparently removing a sex difference. If, on the other hand, acute stress impacts on MWM performance in a sex-dependent manner, all females, irrespective of housing condition, will have a greater stress response (higher thigmotaxis) during MWM testing and perform more poorly than males. If stress plays no role in producing sex differences in spatial ability, then we predict a sex difference in performance, regardless of housing, and no differences in stress levels during MWM testing. The prediction that isolation housing would impair spatial ability in males to a greater degree than in females, contrasts with the prediction in Chapter Two. This is because there is evidence that in albino rats, chronic stress has a greater impact on spatial cognition in males than in females (e.g. Bowman et al. 2002; Luine 2002; Conrad et al. 2003).

5.2. Experiment one: 10 weeks of isolation at 10 weeks of age

5.2.1. Materials and methods

Subjects and housing

We used 18 male and 18 female Wistar rats, aged 8-10 weeks obtained from Harlan U.K. Ltd were the subjects tested in this experiment. At the time of arrival males weighed 280g (±11g) and females 185g (±5g). Six rats of each sex, were chosen at random and housed in isolation, the remaining 12 were housed in same-sex pairs (N = 6 per housing and sex treatment group). One rat from each pair was chosen at random and marked with hair dye (Schwarzkopf, R43) for identification. To prevent hair dye odour or the marking procedure affecting behaviour, all of the rats were handled in similar manner and all of the marking was done one week prior to any data collection (e.g. Hurst et al. 1997). To avoid pseudoreplication, and since dominance hierarchies are unstable at this age (Adams and Boice 1989a), one rat from each pair was picked at random to be the focal animal and this rat remained the only source of data from the pair for the duration of the experiment. Rats remained in their respective housing condition throughout the entire experiment. The handling protocol followed that of Chapter 2. Briefly, the rats were gently handled at least twice weekly (e.g. during cage cleaning and weighing) for ten weeks prior to cognitive testing. The majority of the rats quickly 'tamed' (i.e. did not struggle, squeak or attempt to bite during handling). However, during MWM testing struggling and squeaking during placement in the MWM was frequently observed.

All rats were housed in plastic bottomed cages (45 x 28 cm and 20cm high; North Kent Plastic Cages Ltd., Kent, England). Visual, olfactory and auditory communication between neighbouring rats was not prevented. Rats were provided with ad libitum pellet food (RM3 diet, Special Diet Services, Ltd., Witham, Essex, U.K.) and tap water and maintained under a 12:12h light:dark cycle (lights on at 0600 hours) at 21-24ºC. After 10 weeks of the housing treatment, each isolated and focal rat was tested in the MWM.

Morris water maze apparatus

The MWM consisted of a circular tank made of glassfibre, approximately 2 m in diameter, 65 cm high, with the bottom of the MWM was raised 50 cm above floor level on a custom-built platform. The MWM was positioned in an experimental room (dimensions 4.25 m x 2.9 m) with geometric and landmark cues (e.g. room corners, posters and shelving on walls) visible from the inside of the tank. The tank was filled to a depth of 32 cm with tap water (24±1ºC) and made opaque with approximately 500 ml nontoxic white paint (Dulux). An escape platform (white PVC of diameter 11cm) was located 2cm below the surface of the water and 30 cm from the edge of the tank in the centre of one of four imaginary quadrants (the four main compass points: N, E, S or W). For each of the platform locations there were four possible release points into the pool: NE, SE, SW and NW. We videotaped all trials from above using a camera with a 4 mm wide-angle lens, and all trials were observed via a video monitor once the rat was placed in the water; this was to reduce both stress and distraction to the rats during testing.

Working memory

Each rat received two days of training before testing began. To reduce stress in the MWM to a degree sufficient to remove sex differences, training typically occurs for at least 10 days (e.g. Healy et al. 1999; Beiko et al. 2004). Two days of training is not considered sufficient to reduce stress; it merely provides the animal with knowledge of the platform's existence and as an escape possibility (indeed, the only one). On a training day each rat received two consecutive swims to the hidden platform. The platform location was the same within each day, but its position was changed from day to day. Platform location was pseudo-randomly determined so that the platform was never in the same place on two consecutive days.

Each swim began after the rat was gently lowered into the water and released facing the side of the tank, and ended when the rat found and subsequently climbed onto the platform. The time taken by the rat to find the platform was recorded (±1 s) using a stopwatch. Rats that failed to find the platform within 120 s were gently guided to, and allowed to climb onto, the platform. Once on the platform a rat was left for 20 s before being picked up and released from one of the other three possible

release points. After the final swim a rat was left on the platform for 20s and then gently removed from the platform and returned to its home cage.

Testing started the day following the last day of training and the procedure was exactly as for training with the exception that each rat received four swims (referred to as Swim One, Two, Three and Four) each day for 16 consecutive days in total. All trials were conducted between 1100 and 1500 hours.

Reference memory

Reference memory was assessed from Day Two (because memory cannot be assessed on Day One) of testing to Day Five (because moving the platform every day may, over the course of 16 days, lead to the rats learning to specifically avoid the location occupied by the platform on the previous day). We recorded the percentage of time that a rat spent swimming in each of the four quadrants of the maze in Swim One on Days Two to Five. The proportion of time spent in the three quadrants other than the quadrant containing the platform was calculated to establish whether a rat spent more than 33.3% (chance) of its time searching in the target quadrant (the quadrant that contained the platform on the previous day). The chance level was set at 33.3% because the quadrant that contained the platform on that day was discounted, because the presence of the platform may increase search time in this quadrant, for example, if a rat was to brush against but not climb onto the platform.

Thigmotaxis

The percentage of time that a rat spent swimming within 15 cm of the wall of the maze was recorded for Swim One and Two on all test days. The videotapes were watched on a TV monitor, over which an acetate sheet was attached. Marked on the acetate sheet were the circumference of the MWM and 15 cm from the edge of the MWM. All the time the rat spent in this outer perimeter was recorded.

Body weight and food intake

Body weight was measured once per week from week one post-arrival until the week of MWM testing. Food intake was measured once per week from week two post-

arrival until one week prior to MWM testing. To measure food intake, the entire contents of a food hopper (one per cage) were weighed before the food was topped up and re-weighed. Food intake was estimated per rat per day by dividing the amount eaten by the number of days since the food was last weighed. Where rats were pair-housed an average intake was calculated per cage.

Data analysis

Repeated-measures data were analysed with a Repeated-Measures-Analysis-of-Variance (RM ANOVA): *between-subject* factors were sex (male and female) and housing condition (pair and isolated), and *within-subject* factors were Swim (One to Four) and Day (One to 16). We included all of these factors in the analyses, and interactions between main effects that were not significant were removed. For within-subject statistics the assumptions of sphericity (that repeated measures have equal variances and that the correlations between any two measures are the same) were tested using the Mauchly-Criterion test. Greenhouse-Geisser corrections were used to account for violations of sphericity (resulting in adjustment of degrees freedom to non-whole integers). The assumptions of normality of residuals and homogeneity of variance were tested and appropriate transformations applied to the data, where necessary. Tukey's honestly significant difference test (HSD, $P < 0.05$) was used for the *post-hoc* comparisons of parametric data.

5.2.2. Results

Working memory

Males took less time than did females to reach the platform but only in Swim One (sex: $F_{1,21}= 4.35$, $P = 0.049$; sex-by-swim-number interaction: $F_{1.4,29.6} = 5.52$, $P = 0.017$; Tukey HSD: $P < 0.05$; Figure 5.1.a). Housing condition had no impact on performance in the MWM ($F_{1,21}= 1.76$, $P = 0.20$). The sex by housing interaction was not significant.

All rats learnt the location of the platform in Swim One and swam almost directly to it in all three subsequent swims ($F_{1.4,29.6} = 315.59$, $P < 0.0001$; Figure 5.1.a). There was a significant effect of day on the time taken to reach the platform:

as the experiment progressed the rats took less time to locate it ($F_{6.3,107.5} = 10.20$, $P < 0.0001$). There was a non-significant tendency for greater improvement in performance in Swim One than in the other swims, suggesting a change in level of thigmotaxis or in search strategy during the experiment (swim-number-by-day interaction: $F_{10.7,181.7} = 1.78$, $P = 0.062$). No other interactions were significant.

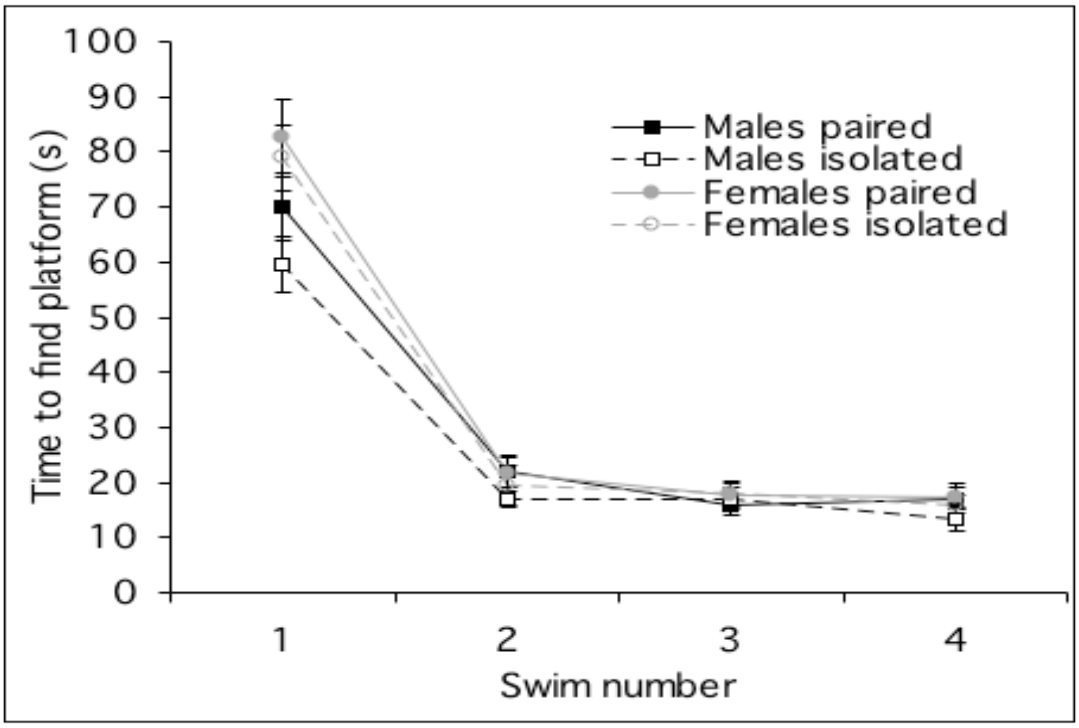
Reference memory

Across Days Two to Five, males spent longer in Swim One searching in the quadrant that had contained the platform in the previous day than did females ($F_{1,21} = 4.48$, $P = 0.047$; Figure 5.1.b). There was, however, no impact of housing condition on reference memory and the sex-by-housing interaction was also not significant (housing: $F_{1,21} = 0.07$, $P = 0.79$). There was non-directional day to day variation in the amount of time spent searching in the previous day's target quadrant (day: $F_{1.8,37.8} = 3.85$, $P = 0.034$). Days Two and Four differed from Days Three and Five (Tukey HSD: $P < 0.05$). No other interactions were significant.

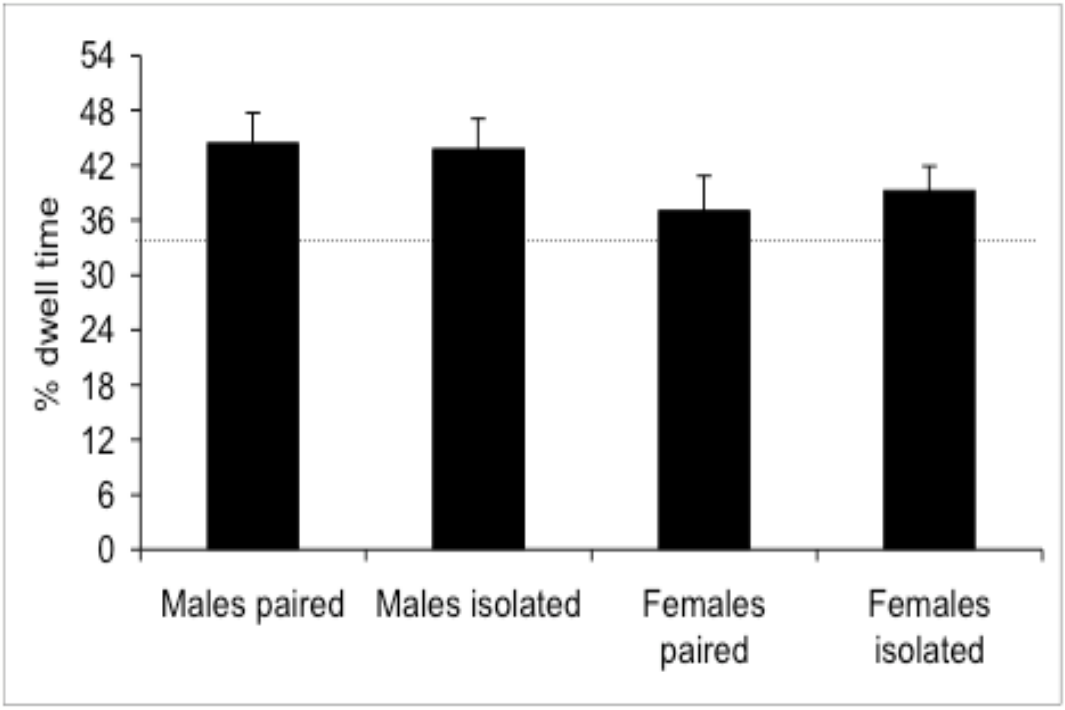
To compare reference memory performance with that expected by chance, the proportion of time spent in the target quadrant by each rat was averaged across Days Two to Five, and pooled across housing condition and tested against chance (33.3%; the quadrant that contained the platform was ignored) using a two-tailed one-sample t-test. Both males and females spent significantly longer than expected due to chance in the target quadrant (males: $t_{11} = 5.29$, $P = 0.0003$; females: $t_{11} = 2.59$, $P = 0.025$).

Figure 5.1: Experiment One, performance in the MWM (mean ± SE) for male and female rats that were either pair or isolate housed N = 6 per treatment

(a) Time taken to find the platform in Swims One to Four. Swim times are averages across the 16 days of testing, analyses were conducted on daily swim data.



(b) Percentage of Swim One in the target quadrant. The dotted line represents chance (33.3%).



Thigmotaxis

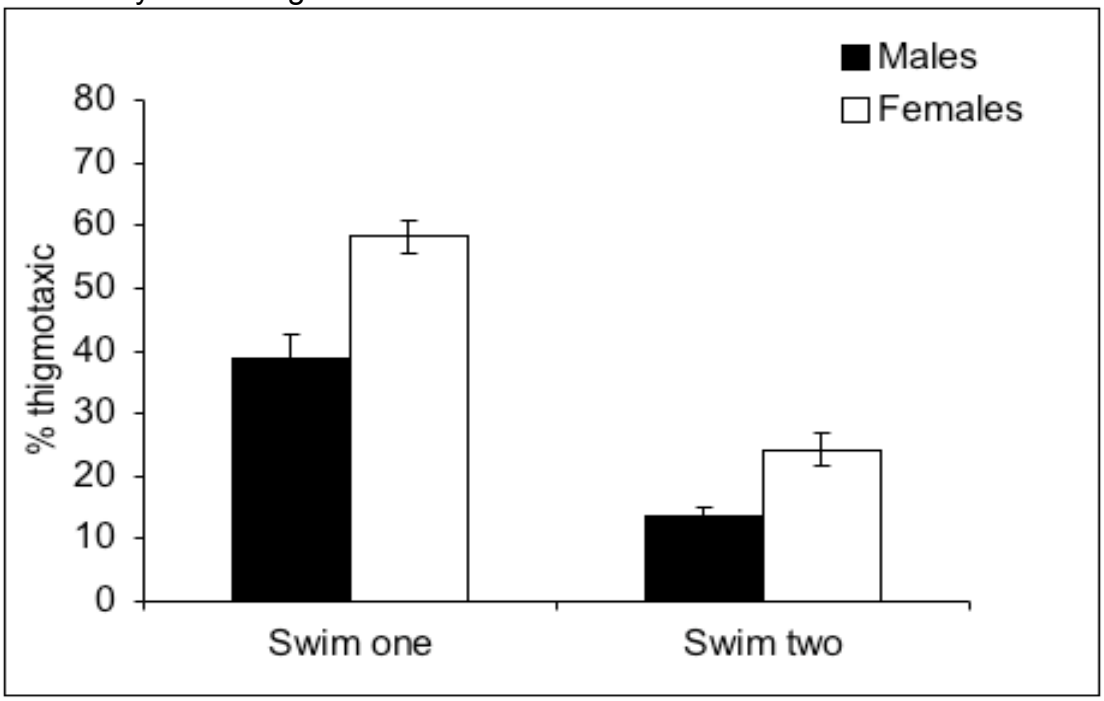
Males were significantly less thigmotactic than females in Swims One and Two ($F_{1,21}$= 19.58, P = 0.0002; Tukey HSD: Ps < 0.05; Figure 5.2.a and 5.2.b), and both males and females were less thigmotactic in Swim Two than they had been in Swim One ($F_{1,21}$= 268.19, P < 0.0001; Tukey HSD: Ps < 0.05; Figure 5.2.a). There was a significant interaction between these two factors: females had a greater decrease in thigmotaxis in Swim Two (sex-by-swim-number interaction: $F_{1,21}$= 6.36, P = 0.020). Housing condition was not correlated with variation in thigmotaxis ($F_{1,21}$= 2.69, P = 0.12) nor were any other interactions with housing significant. Thigmotaxis in Swim One declined significantly across the days of the experiment ($F_{15,330}$ = 4.94, P < 0.0001), but only for males (sex-by-day interaction: $F_{15,330}$ = 1.92, P = 0.020; Figure 5.2.b). No other interactions were significant.

Body weight and food intake

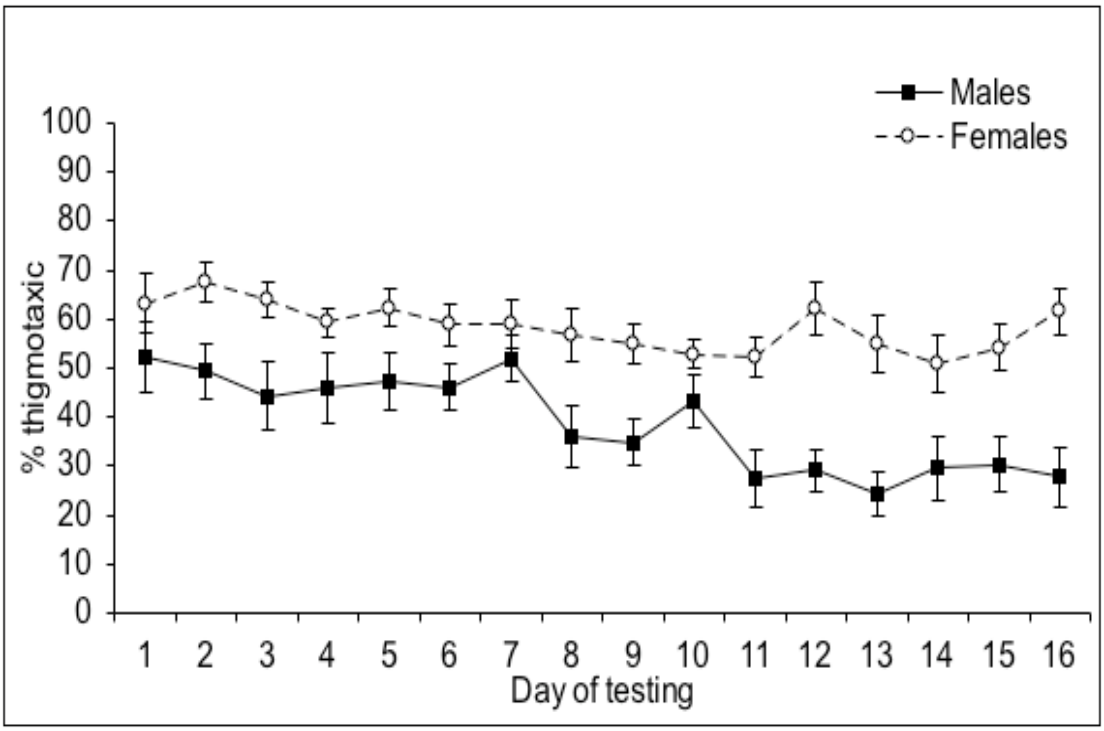
Males weighed more than females (as measured each week from 77 to 133 days old; sex: $F_{1,21}$ = 555.41, P < 0.0001) and gained weight at a faster rate (sex-by-week interaction: $F_{2.5,52.5}$ = 30.3, P < 0.0001). Housing condition had no impact on body weight and the housing-by-sex interaction was not significant (housing condition: $F_{1,21}$ = 0.11, P = 0.74). Males ate more than females ($F_{1,20}$ = 60.6, P < 0.0001; on average, males ate 22 ± 0.7g and females ate 18 ± 0.5g per day).

Figure 5.2: Mean percent of time swimming thigmotactically (±SE) (within 15 cm of the edge of the MWM). Data are pooled across housing condition (N = 12 per sex).

(a) Thigmotaxis in Swim One and Two. For each rat data are averaged over the 16 days of testing.



(b) Thigmotaxis in Swim One over the 16 days of testing.



5.2.3. Discussion

Females appeared to perform more poorly than did males in the MWM: males were both quicker to find the platform each day and spent more time in the first swim of each day in the quadrant that had contained the platform on the previous day. We did not measure path length as latency is the most common measure of performance in the MWM (e.g. Mendez et al. 2008; Saucier et al. 2008) and correlates so closely with path length that authors who do measure both typically report one in detail and mention only that the other measure of performance followed the same pattern (e.g. Roof 1993a; Kempermann et al. 1997; Nilsson et al. 1999). Importantly, there is no evidence that male and female swim speeds differ, the reason for wishing to consider distance in addition to latency (Jonasson et al. 2004; Snihur et al. 2008). Therefore, sex differences, both in our work and in the literature, are not explained by differences between latency and distance (as there are none).

Any stress caused by isolation housing had no discernible impact on MWM performance by either sex. However, the sex difference in performance in the MWM was explained by the difference in the proportion of time spent in thigmotaxis. It is possible that thigmotaxis reflects impaired allocentric learning (e.g. search 15 cm away from the edge of the tank) or it may be easier to view landmarks from this area of the maze. However, not only should easier viewing lead to better performance, but there is an overwhelming body of literature that demonstrates that thigmotaxis correlates positively with anxiety (e.g. Treit & Fundytus 1989; Beiko et al. 2004). Thus, the apparent sex difference in memory in this experiment, can be ascribed to the greater stress response of females swimming in the MWM and thus appearing to have a poorer memory for the platform's location.

We assessed the impact of stress on two measures of cognitive performance: reference memory and working memory. Working memory can be investigated by the time taken to find the platform in Swims Two to Four, when the animals use information acquired in the swims of that day to locate the platform. As the sexes did not differ on this measure, we interpret the sex difference in performance in Swim One to be a result of a difference in the stress response to swimming in an MWM, rather than to a cognitive impairment. Our finding that males and females

performed equally well in the working memory component of the task is consistent with other studies that find no sex difference in working memory in the MWM (Healy et al. 1999; Conejo et al. 2004).

We also attribute the apparently superior reference memory in our male rats to the higher levels of thigmotaxis in the females, since swimming around the edge necessarily precludes searching in the quadrant that contained the platform on the previous day. Furthermore, these data are consistent with the finding that stress can impair retrieval of long-term spatial memory in rats (de Quervain et al. 1998). Our findings strongly suggest that investigations into sex differences in reference memory in the MWM should include the consideration of thigmotactic behaviour. Since reference memory is typically measured either by giving a single swim per day or by averaging latencies over several swims per day, if females find the first swim of the day more stressful (Figure 5.2.a) it is possible that this methodology serves to bias the results in favour of the males (e.g. Roof and Havens 1992; Blokland et al. 2006).

Although in our experiment females were significantly more thigmotactic than males in Swim Two, they did not differ in working memory performance from the males. One explanation is that, to detect a performance difference, the time spent in thigmotaxis relative to the time taken to reach the platform must exceed some threshold. In Swim One females spent 60% of their time in thigmotaxis (49 s in real time), whereas in Swim Two they spent only 24% of their time in thigmotaxis relative to the males' 13%, which was a difference in real time of only 2 s. In a series of previous experiments (Chapter Two), we found that the sexes' performance differed significantly only when at least one of the sexes (always females in our experiments) spent at least 35% of the time swimming thigmotactically and the difference was at least 13% between the sexes, a difference in real time of approximately 11 s (Harris et al., 2008a). The results of the current experiment coupled with our previous work, in which adult Lister Hooded rats differed in thigmotaxis but not in cognitive performance (Harris et al., 2008a), as well as that of others (e.g. Beiko et al. 2004; Perrot-Sinal et al. 1996), suggest that thigmotaxis must reach a threshold level (>35%) before the performance of either sex is impaired. Furthermore, a sex difference (in contrast to simple performance impairment) is seen

when the difference between the sexes in thigmotaxis is greater than 13% (or 11s in absolute time).

Acute stress, then, may be the explanation for at least some of the sex differences in cognitive performance reported for adult laboratory rats. However, a considerable proportion of the data come from rats that were obtained from breeding establishments as juveniles (i.e. exposed to isolation rearing) so it is important to determine whether the effects seen in adults (i.e. only acute stress affecting performance, if at all) are also seen in juveniles. For this reason we carried out Experiment Two, in which all manipulations were as in Experiment One, but those manipulations began when the rats were only four-to-five weeks old.

5.3. Experiment two: 10 weeks of isolation at 4-5 weeks of age

Play behaviour in rats increases from 18 days of age, peaks at around 32-40 days of age and then gradually decreases into adulthood (Panksepp 1981). Thus, young rats play more than old rats and since social isolation removes the opportunity for play, it is plausible that social deprivation (i.e. isolation housing) of juvenile rats may cause greater chronic stress than it does in adults. For example, rats isolated from approximately 21 days of age show a variety of behavioural and cognitive changes, such as hyperactivity in an open field (Einon and Morgan 1977; Einon et al. 1978; Parker and Morinan 1986), impaired reversal learning (Schrijver et al. 2004), and impaired spatial learning (Lu et al. 2003; Hellemans et al. 2004). Greater chronic stress in juveniles might lead to differences in cognitive performance compared with that of adults, given that males appear to be more susceptible to chronic stress than do females (e.g. Bowman et al. 2003; Sandstrom and Hart 2005).

We housed rats aged four-to-five weeks in isolation or in pairs for 10 weeks before testing their spatial ability in an MWM. We made the same predictions as for Experiment One: that 1) if chronic stress impacts specifically on males, and if sex differences exist, isolated males should perform more poorly than pair-housed males but there should be no sex differences between isolated males and females; 2) if acute stress impacts specifically on females, females should perform more poorly

than males; 3) if stress is not a significant contributory factor to performance, we would predict a sex difference irrespective of housing condition.

5.3.1. Methods

Subjects and housing

We used 18 male and 18 female Wistar rats, aged four-to-five weeks, obtained from Harlan U.K. Ltd. At the time of arrival the males weighed 146± 4 g and the females weighed 121±2 g. Six rats of each sex were chosen at random to be housed alone; the remaining 12 were housed in same-sex pairs (N = 6 per sex and housing treatment group). Housing, handling and husbandry were the same as for Experiment One (Section 5.2.1)

Rats experienced their respective housing condition for 10 weeks before spatial ability was assessed using the MWM. MWM testing and apparatus were as for Experiment One. The measurement of thigmotaxis, body weight and food intake, data analyses and euthanasia were also as for Experiment One, Section 5.2.1.

5.3.2. Results

Working memory

The sexes did not differ in their working memory and the performance of neither sex was affected by housing condition (sex: $F_{1,21} = 0.43$, $P = 0.52$; housing condition: $F_{1,21} = 0.45$, $P = 0.51$; Figure 5.3.a). Additionally, the sex-by-housing interaction was not significant.

There was a highly significant effect of swim number on performance ($F_{1.7,36.2} = 177.94$, $P < 0.0001$; Figure 5.3.a): all rats took significantly longer to find the platform in Swim One than in all other swims (Tukey HSD: $P < 0.05$). There was also an effect of day on performance: as testing progressed, performance improved ($F_{7.2,151.3} = 16.85$, $P < 0.0001$). The day-by-swim interaction was not significant, indicating that the improvement across the days was seen in all four swims. No other interactions were significant.

Reference memory

The sexes did not differ in the amount of time spent in the target quadrant during Swim One across Days Two to Five ($F_{1,21}$ = 0.47, P = 0.50; see Figure 5.3.b) and there was no effect of housing ($F_{1,21}$ = 0.21, P = 0.65). The sex-by-housing interaction was not significant. There was, however, a significant effect of day on time spent in the target quadrant: as the experiment progressed rats spent less time in this quadrant ($F_{3,60}$ = 5.10, P = 0.003).

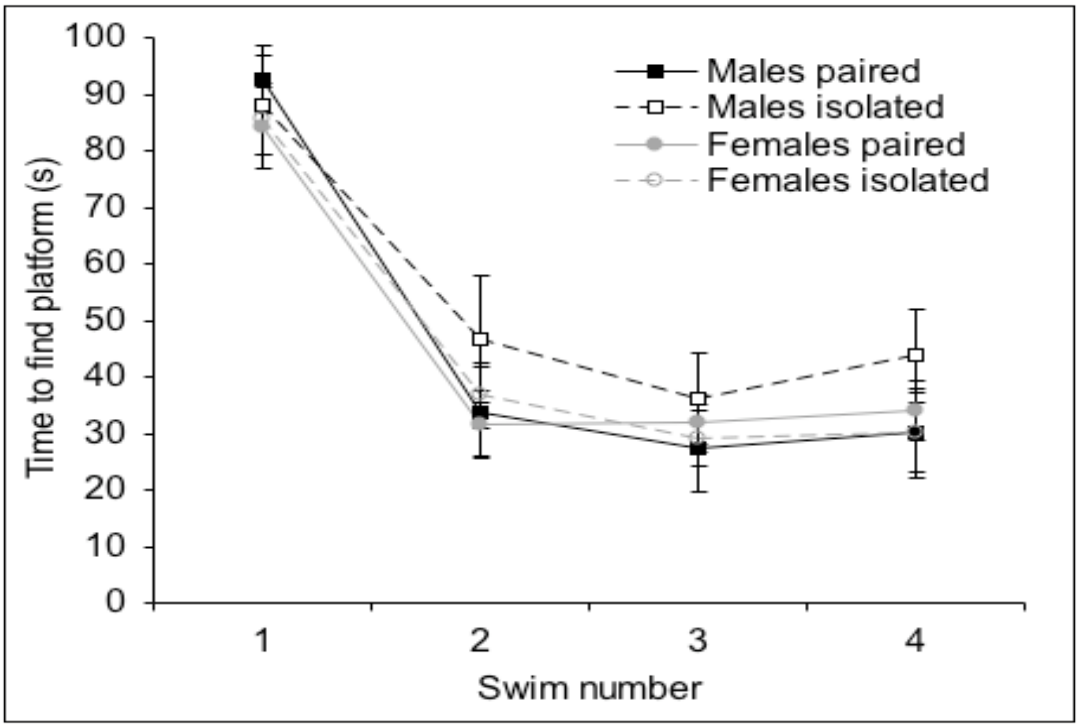
To compare performance with that expected by chance, the proportion of time spent in the target quadrant by each rat was averaged across Days Two to Five, pooled across sex and housing condition (thus N = 24) and tested against chance (33.3%; data from the quadrant that contained the platform were ignored) using a two-tailed one-sample t-test. Rats tended to bias their searching in Swim One, spending longer than expected by chance in the target quadrant (mean % of time: 37.4%; t_{23} = 2.07, P = 0.05).

Thigmotaxis

The sexes did not differ in the amount of time spent in thigmotaxis ($F_{1,21}$ = 0.002, P = 0.90; Figure 5.4.a and 5.4.b). There was no effect of housing condition on thigmotaxis and the sex-by-housing interaction was also not significant (housing: F1,21 = 0.003, P = 0.95; sex-by-housing interaction: P > 0.1). However, thigmotaxis decreased significantly between Swims One and Two ($F_{1,21}$ = 149.39, P < 0.0001; Figure 5.4.a). Thigmotaxis in Swim One changed significantly across the days of the experiment, but there was no directional trend ($F_{15,330}$ = 4.94, P < 0.0001; Figure 5b), and males and females did not differ significantly over the days (sex-by-day interaction: $F_{15,330}$ = 0.99, P = 0.46; Figure 5.4.b). No other interactions were significant.

Figure 5.3: Experiment Two: performance in the MWM (mean ± SE) for male and female rats that were either pair or isolate housed, N = 6 per treatment.

(a) Time taken to find the platform in Swims One to Four. Swim times are averaged across the 16 days of testing, analyses were conducted on daily swim data.



(b) Percentage of Swim One in the target quadrant averaged across Days Two to Five. The dotted line represents chance (33.3%).

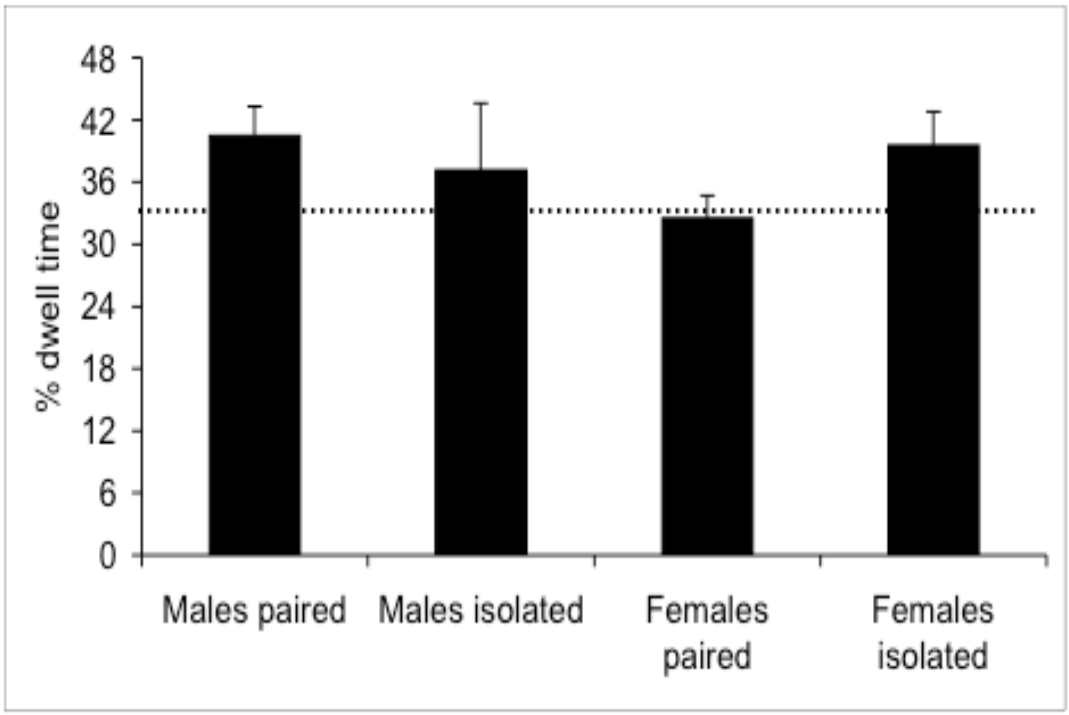
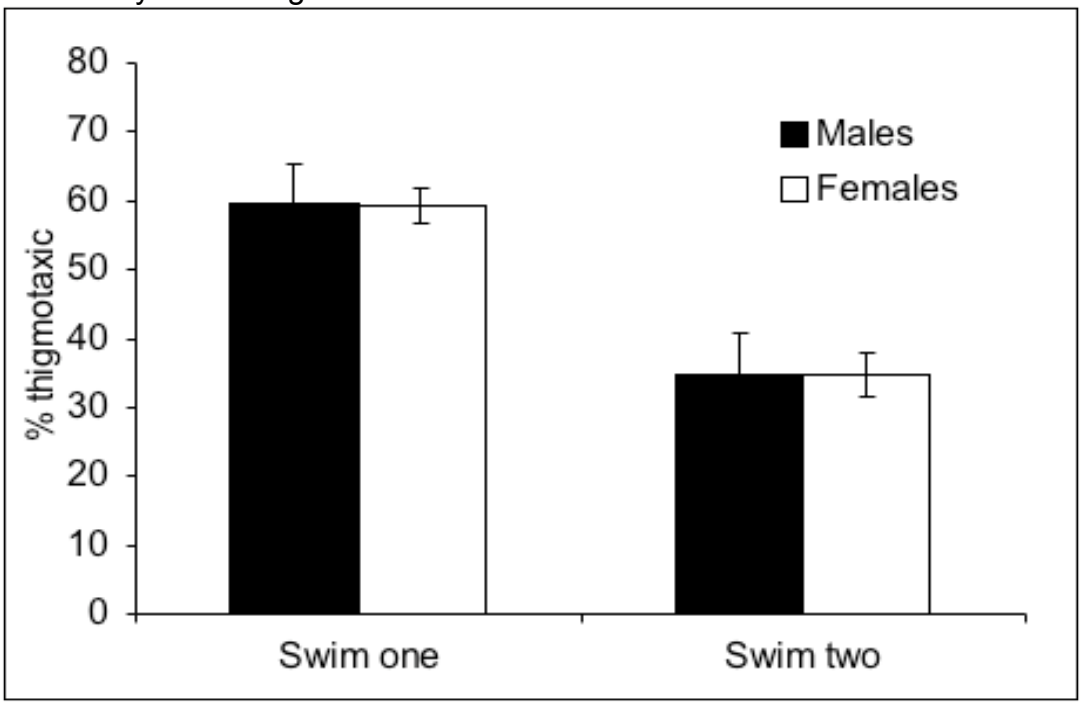
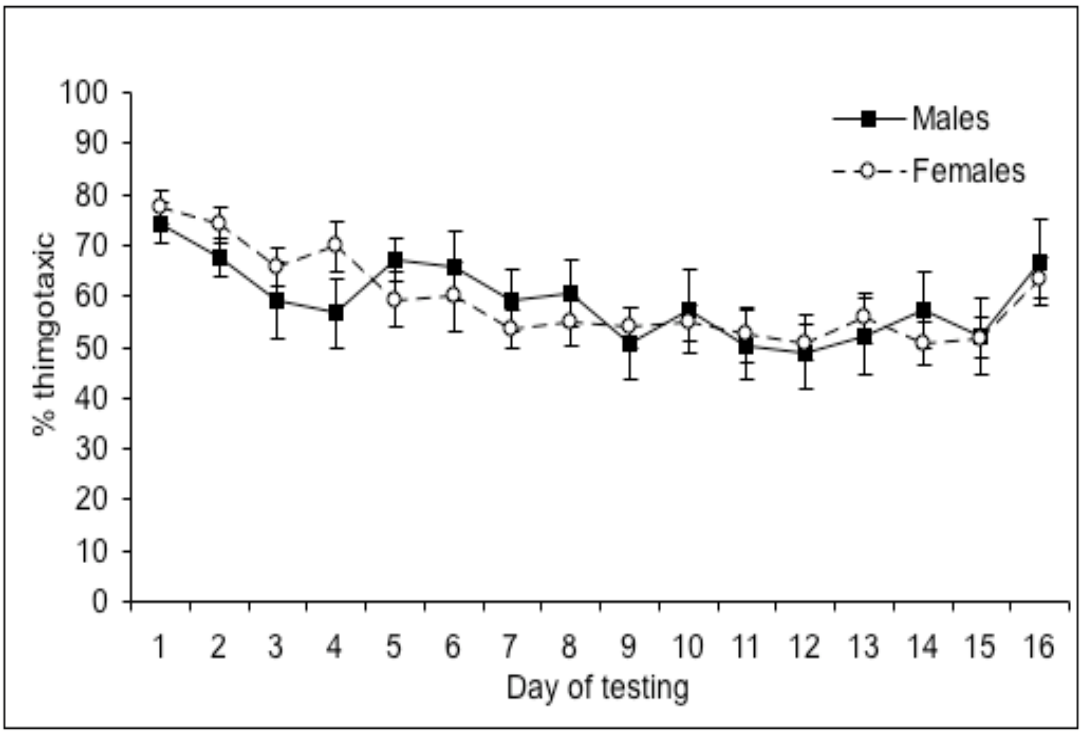


Figure 5.4: Experiment Two: mean percent of time swimming thigmotactically (±SE) (within 15 cm of the edge of the MWM). Data are pooled across housing condition (N = 12 per sex).

(a) Thigmotaxis in Swim One and Two. For each rat data are averaged over the 16 days of testing.



(b) Thigmotaxis in Swim One across the 16 days of testing.



Body weight and food intake

Males weighed more than females ($F_{1,21}$ = 200.84, P < 0.0001) and gained weight at a faster rate (sex-by-week interaction: $F_{2.3,49.5}$ = 235.87, P < 0.0001). Housing condition had no impact on body weight and the housing-by-sex interaction was not significant ($F_{1,21}$ = 1.5, P = 0.23). Males ate more per day (23 ± 0.4g) than females (18 ± 0.8g; $F_{1,21}$ = 89.94 P < 0.0001). Housing condition had no impact on food intake and the housing-by-sex interaction was not significant (housing condition: $F_{1,21}$ = 1.81, P = 0.19).

5.3.3. Discussion

We did not find a sex difference in either working or reference memory in the MWM in these juvenile rats. Isolation housing did not impact on cognitive performance or on food intake or body weight. We did, however, see a significant impact of acute stress on performance in the MWM: all of the rats spent about 60% of their first swim in thigmotaxis and correspondingly performance was poorer in Experiment Two than in Experiment One. Although the juvenile females spent a similar proportion of Swim One in thigmotaxis as did adult females in Experiment One, the difference between the two experiments is due to the much higher proportion of thigmotaxis observed in the juvenile males. Reference memory performance, then, was equally obscured in both sexes in the juveniles. Working memory, too, was equally impacted in both sexes: although thigmotaxis dropped in Swim Two, it remained at approximately 40% for both, a level similar to that of the adult females in Experiment One (and thus much higher than that of the adult males).

Isolation housing imposed at three weeks of age can have an impact on spatial cognition in males after as little as four to eight weeks (e.g. Wongwitdecha and Marsden 1996; Lu et al. 2003). However, in those studies, the 'control' groups were either housed with enrichments (e.g. cage furniture, toys) or in social groups of four to five rats, which confounds social housing with larger home cages and physical complexity. Additionally, the degree of isolation (auditory, olfactory and visual) was not made explicit in these studies. Despite our rats being young when the housing manipulation was imposed (4-5 weeks) and this exposure lasting 10+

weeks, no discernible impact on cognition or stress (thigmotaxis) was detected in either sex. To our knowledge, previous studies have not investigated thigmotaxis during MWM testing in juvenile rats following isolation housing. However, rats reared in isolation from 35 days of age show more 'stress-related' behaviours, such as bar biting and tail manipulation in their home cages, than group-reared conspecifics (Baenninger 1967; Hurst et al. 1997, 1998).

As in Experiment One, we attribute the outcome of this experiment to the effects of acute stress, the difference being that in this case, juvenile males were also affected to a degree similar to that of females.

5.4. General discussion

We proposed that variation in stress might lead to differences between the sexes in performance in spatial cognition, especially as chronic and acute stress seem to impact differentially on male and female spatial cognition. We manipulated chronic stress by housing animals alone or in pairs and acute stress by using the MWM as our memory task. As we could find no effect of isolation housing on performance in the spatial cognition test, we conclude that chronic stress, as incurred by isolation housing, is an unlikely explanation for sex differences in spatial ability in albino rats.

Chronic stress (e.g. six hours of daily restraint for 21 days) impairs male spatial ability but enhances or has no affect on female spatial ability (e.g. Bowman et al. 2001; Bowman 2005). We did not find that isolation housing impaired spatial ability, in either sex. However, as we saw no conspicuous signs of stress in the isolated rats, since food intake and body weight were indistinguishable between pair-housed and isolated rats, and none of the isolated rats had scaly tails (e.g. Hatch et al., 1963), it is possible that isolation housing did not impact on performance because it was not sufficient to cause chronic stress.

Our finding that isolation had little discernible impact on our rats conflicts with the widespread belief that housing rats alone is detrimental to their wellbeing because it is chronically stressful (Home Office 1995; Patterson-Kane et al. 2004). However, at least two reviews suggest that there is a distinct lack of in-depth, well-controlled studies in this area and more data are needed before concluding that isolation is stressful (Brain and Benton 1979; Krohn et al. 2006). Additionally, it is possible that the routine handling (detailed in section 5.2.1) our rats received was

sufficiently stimulating to mitigate any deleterious effects of isolation (e.g. see Holson et al. 1991; Heidbreder et al. 2000). Alternatively, isolation stress may become increasingly significant as visual, auditory and olfactory communication between neighbouring cages, none of which were prevented in our housing conditions, are reduced. Nevertheless, if rats can be singly housed without detrimental impact on either their welfare or the outcome of the experiment, data could be collected from all of the animals that are used (testing animals from one cage and then treating these data as independent data points in the analysis is pseudoreplication), which would ultimately reduce the number of rats used to study this specific question (as is encouraged by the U.K. Home Office).

Our data are, however, consistent with the hypothesis that performance by females in hippocampal-dependent tasks is affected to a greater degree by acute stress than is that of males (Shors and Miesegaes 2002; but see Conrad et al. 2004). Our data also support the hypothesis that acute stress, associated with the test situation, can explain the presence and absence of sex differences in cognitive tasks (e.g. Perrot-Sinal et al. 1996; Beiko et al. 2004; Harris et al. 2008a). A sex difference is caused when the sexes respond to a similar stressor to a different degree. In this case, females in Experiment One were more stressed than were males, leading them to be more thigmotactic and thus perform more poorly in the cognitive task as a result. However, when the sexes are equally stressed, there was no sex difference in performance (Experiment Two).

Importantly, the MWM is a task in which the effects of stress can be seen as variation in thigmotaxis, which provides a quantitative (non invasive) measure of stress while the animal is completing the task. More time spent in thigmotaxis in the first swim of the day will result in apparently poorer reference memory performance. Differences in thigmotaxis may also lead to apparent sex differences in cognition in the working memory version of this task: latency to reach the platform will inevitably be longer, the more time is spent in thigmotaxis. If thigmotaxis continues to be high in Swim Two, working memory in such thigmotactic animals will appear to be poorer, than in animals spending less time in thigmotaxis in Swim Two (Experiment One). In neither instance was there evidence for cognitive differences.

Another potential source of variation in spatial ability is hormonal fluctuations caused by the oestrous cycle. However, while fluctuations in hormone levels across the oestrous cycle may influence spatial ability in female rats, the findings are inconsistent. For example, performance in spatial cognition tasks may be enhanced during the pro-oestrus (high oestrogen) phase of the cycle (Healy et al. 1999), impaired during the pro-oestrus phase (e.g. Warren and Juraska 1997) or remain stable across the oestrous cycle (Stackman et al. 1997). Similarly, stress effects in females may also depend on the oestrous cycle phase; for example, greater stress responses are found during the pro-oestrus phase of the cycle (Viau and Meaney 1991; but see Frye et al. 2000; Sharp et al. 2002b). However, we tested our females over several oestrous cycles (there were 16 days of testing) and found no conspicuous cycling in performance or thigmotaxis levels across testing: the females were always more stressed (i.e. thigmotaxic) and underperformed, relative to the males in Experiment One, and equally stressed and performed equally in Experiment Two.

It is not clear what caused the increase in stress for the juvenile males in Experiment Two (nor, indeed, why the females in both experiments were more susceptible to acute stress). It is possible that travel to or the change in housing conditions, as occur between the producer (Harlan Ltd.) and our animal unit, affect females irrespective of age but males are susceptible only when young and there is some evidence that transportation of rodents is stressful (reviewed in Swallow et al. 2005) . Whatever the cause, it has a long-term affect on the rats' ability to deal with acute stress.

The effects of acute stress on performance have rarely been considered in typical MWM tasks, that is, reference memory tests. In at least two studies, however, in which the effects of acute stress levels (i.e. thigmotaxis) were explicitly investigated, sex differences in MWM performance are also accounted for by thigmotaxis (Perrot-Sinal et al., 1996; Beiko et al., 2004). Additionally, a role for acute stress is implicated in a number of studies in which rats received extensive pretraining leading to no differences in performance between the sexes (Bucci et al. 1995; Warren and Juraska 1997; Nunez et al 2000; Blokland et al. 2006). Similarly, in working memory tests, there was no sex difference after extensive pretraining in

the MWM nor when comparisons of performance were made for Swim Two only (Healy et al. 1999; Conejo et al. 2004).

As a final note, we did not measure levels of the stress hormone CORT in our rats because, in general, levels of CORT return to baseline levels after a period of chronic stress, which jeopardizes the value of CORT as a measure of chronic stress caused by isolation housing (Jensen et al. 1996). Correspondingly then, there is little consistency in the literature as to how CORT levels change in response to isolation housing. For example, there are studies that report that CORT levels in isolated rats are elevated (e.g. Perelló et al. 2006), depressed (e.g. Hurst et al. 1998) or unaffected relative to socially housed rats (e.g. Scaccianoce et al. 2006). It would also have been inappropriate to use CORT levels as an indicator of acute stress following MWM testing, since the blood sampling required may itself affect subsequent MWM performance (e.g. the next day).

In conclusion, we found that acute but not chronic stress had sufficient impact on the rats to cause apparent sex differences in cognitive performance in the MWM. However, when equally stressed, the sexes did not differ in performance. We suggest that a significant proportion of the sex difference literature that comes from testing laboratory rats may result from an artefact (stress) of the test situation rather than selection for better spatial cognition in males than in females. However, sex differences in spatial cognition have been demonstrated in a number of mammalian species using a range of tasks (Gaulin and Fitzgerald 1986; Galea et al. 1996; Lacreuse et al. 1999; Gresack and Frick 2003; Jones and Healy 2006). Our data do not, then, speak to all previous work demonstrating sex differences in spatial cognition but they may go some way to explain the inconsistencies in the contributions from testing laboratory rats. Our data also raise two concerns: (1) that it is possible during cognitive tests to bias tasks or data analyses inadvertently in such a way as to produce or to exaggerate sex differences in performance; (2) laboratory rats may not be ideal subjects for investigations into sex differences in spatial cognition. For understanding the causes of sex differences (i.e. hormonal), laboratory rats remain very useful. However, if the question of interest concerns the evolution of sex differences in spatial cognition, perhaps species recently taken from the wild would better suit the purpose (e.g. Healy et al. in press). Since males and

females taken from the wild may also differ in their response to stress, it may be more appropriate to test these animals in the wild (rather than in the lab) under non-stressful conditions, for example the animals could choose to participate in spatial tasks for food rewards (e.g. Bateson et al. 2002; Bateson et al. 2003; Healy et al. in press) or tests in which stress responses can be distinguished from cognitive performance should be used (e.g. the MWM).

CHAPTER SIX: Group housing rodents: the trade-off between good welfare and poor science

A version of this chapter will be submitted as the following publication:
Benefits of enrichment for laboratory rodents: a trade-off between good welfare and poor science. Harris, A. P., Colegrave, N. & Healy, S. D.

I wrote the manuscript in collaboration with the authors listed.

Summary

Environmental enrichment comes in two forms, often combined. The first is group housing of same-sex conspecifics and the second is provision of a variety of objects such as nest boxes, tubes to hide in and novel objects e.g. to chew and climb on. It is widely accepted that provision of such enrichment leads to beneficial effects on the neuroanatomy and cognitive abilities of these animals (e.g. Rosenzweig et al. 1978; Falkenberg et al. 1992; Kempermann et al. 1997; Kempermann et al. 1998). As it is also widely accepted that such enrichment, especially group housing, leads to good welfare in these animals, group housing of laboratory rodents has now become the norm, at least in the U.K (Home Office 1995, 2000).

Here we examine some potential downsides of group housing rodents, in particular the, apparently frequently unappreciated, effect that group housing can have on the statistical power of a study. We will focus on experiments that have investigated the impact of 'environmental enrichment' on a rodent's brain and behaviour, although the issues raised apply much more widely. We will then examine critically some of the proposed benefits to using group-housed rodents, and suggest that for rats at least, some of these benefits may have been over emphasised. We will finish with our recommendations for best practise.

6.1. Group housing can reduce independent replication and lead to pseudoreplication

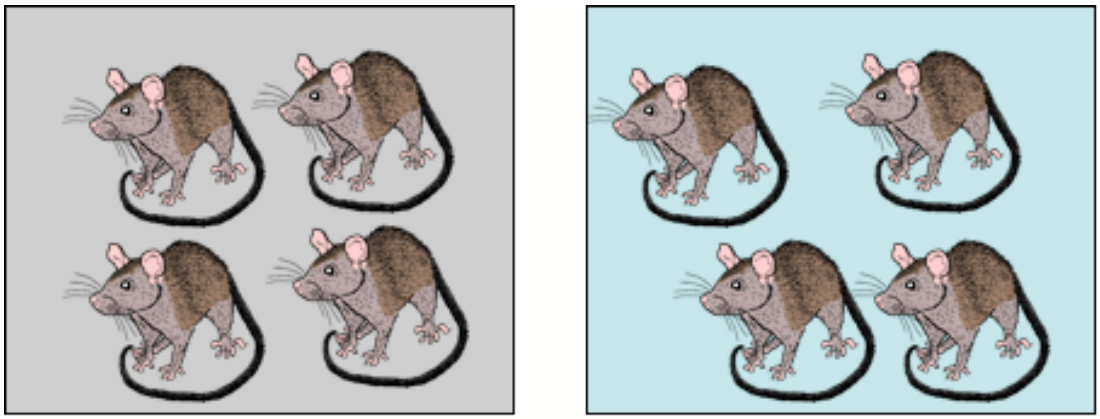
Good experimental design requires independent replication of the 'experimental unit' (Ruxton and Colegrave 2006). The 'experimental unit' can be defined as the smallest unit to which an experimental treatment can be independently applied. As such it is an independent source of data; it can be an animal, a liver, a brain and so on, but it should only contribute one data point to the data set. The statistical power of a study (the probability of correctly rejecting the null hypothesis) will depend, in part, on the number of experimental units in the experiment (typically, more units equals more power).

Group-housed laboratory rodents, however, share a common micro-environment i.e. the cage. If different experimental treatments can be applied independently to rodents within a single cage, for example a drug treatment and its placebo that can be administered intravenously, then individual rodents can still be regarded as independent units providing that an appropriate statistical test that allows cage effects to be considered and controlled for is used. However, if all of the animals within a cage receive the same treatment (e.g. enrichments, food, cleaning regime) the measures from the individual animals become pseudoreplicates rather than true replicates (see Figure 6.1; Hurlbert 1984; Festing et al. 2002). The independent unit of replication is the cage and not the animal. This can lead to two potential problems. First, if the stats are handled correctly (see below), the number of independent units is fewer than the number of rodents chosen for the study. This is very likely to lead to a reduction in the power of the experiment. For example, an experiment in which twenty rats, housed in groups of five, all receive the same treatment has four independent units (i.e. the number of cages) rather than twenty as intended. In practice, this means that an experiment with group housed rodents may require a greater total number of rodents than if the rodents were housed individually. Second, and more serious, if the statistics are handled incorrectly, and rats are treated as independent replicates rather than pseudoreplicates, we increase our chance of erroneously concluding that our treatment is having an effect (the type 1 error rate). Thus, imagine again our experiment above with twenty rats in four cages (two treatment and two control). All it requires in this imaginary experiment is

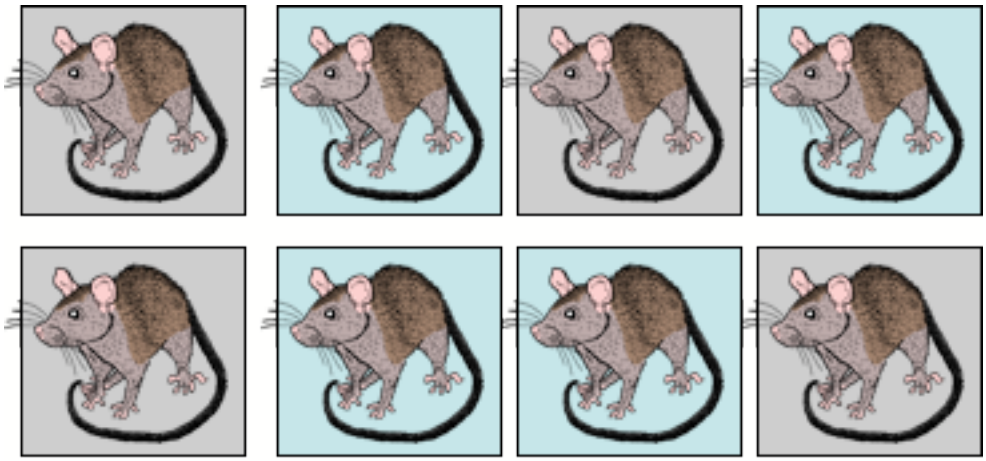
for a chance event such as differences in handling, an unwanted infection or even water bottle leakage to affect the parameter of interest (i.e. the body weight of the animals) in one of the cages for there to be a difference among the cages (see Figure 6.1.a for a scaled down example). This may then be interpreted as an effect of the treatment, since it affects half of the animals in one treatment and none in the other. Additionally, sequentially testing all of the animals from one cage may mean that the animal that gets tested last from each cage is extra stressed, and this may cause order effects in the data. There may also be variation in social interactions among the animals within and among the cages, which can lead to incorrect rejection of the null hypothesis and the conclusion that treatment was responsible for the significant outcome. Thus, in spite of the care to standardise the cage itself, there are many differences among real cages that may affect the individuals in those cages and lead to an increase in the type 1 error rate to an unknown degree if individual animals are treated as independent replicates.

Figure 6.1: A schematic representation of a) pseudoreplication and b) how to avoid it. The boxes represent laboratory cages and the different colours represent different treatments (could be diet, infection).

a) In this experiment because the rats in each treatment share a common microenvironment the rats are pseudoreplicates and so the sample size in this experiment is one, not four. Depending on the specificity of the response (e.g. behaviour, food intake etc) it is possible that a water bottle leak in e.g. the grey box, will increase the likelihood of wrongly rejecting the null hypothesis.



b) In this experimental design because the rats are in different cages each rat can be treated as a true replicate. And since treatment is applied to multiple cages the sample size in this experiment is four.



6.1.1. Pseudoreplication in animal experiments

Despite the ease with which laboratory cages (and their inmates) can be replicated many investigators appear either unaware of, or to ignore, the problems of pseudoreplication. It may even be that the enthusiasm for group housing has encouraged pseudoreplication as the lesser of two evils i.e. good welfare but a reluctance to increase the number of animals tested. Whichever the explanation, the way in which group housing is currently used has, in fact, led to an unwitting trade off between good welfare and good science. In the six months between April and August 2008 in Nature alone there are 31 studies (19 issues) using laboratory rodents. In two of these studies the rats were single housed because they had cannulae fitted. In the remaining 29 studies since no comment is made on cage numbers we are left to assume that group housing was used and that all rodents from a cage were treated as independent data points for the subsequent analysis (since degrees of freedom seem to match the number of rodents used). However, unless this is made explicit in the Methods section, referees, editors and readers cannot assess the value of the study.

It is ironic that that the problem of pseudoreplication is not less common for work on the effects of 'environmental enrichment' as it is for others using laboratory rodents in very different contexts. It may seem obvious that when the factor of interest is environmental enrichment i.e. the home cage that a rodent experiences, that this factor needs to be replicated. However, several highly influential papers from the last two decades (Falkenberg et al. 1992 cited > 140 times; Kempermann et al. 1997 cited > 1000 times; Kempermann et al. 1998 cited > 360 times) purport to show significant effects of enrichment on neuroanatomy and cognitive ability, and yet the experiments in these papers do not satisfactorily replicate the housing environment. This seems to be a rather common occurrence, as shown in Table 6.1. It is typical in these experiments for control animals to have been housed in separate cages while the enriched animals come from no more than one or two enclosures.

Table 6.1: Studies in which the true level of replication is much lower than reported. The 'Reported N' is the number of animals that were enriched and the 'Actual n' is the number of times the enriched environment was replicated. In eight studies it is not clear how many rats were housed per cage, how many enriched cages were used or what the total sample size was. All authors, we assume, collected data from all of the animals in each cage. All authors report significant effects of enrichment on brain or behaviour.

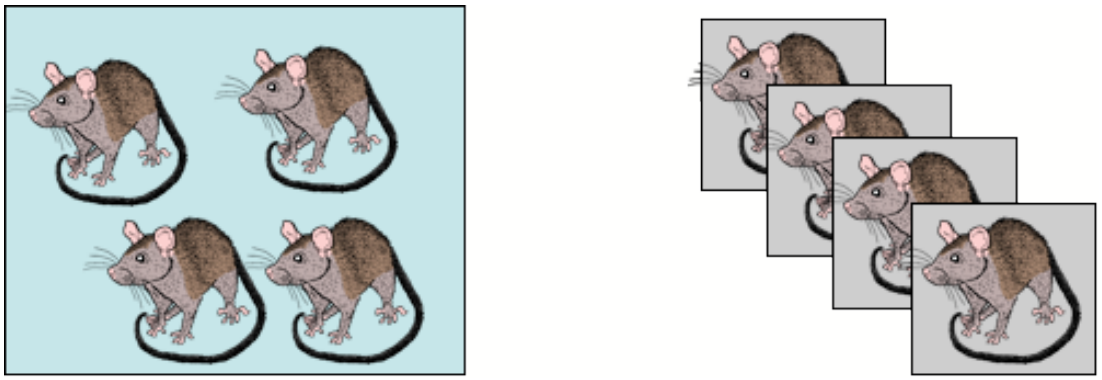
Study:	Effect of enrichment on:	Reported N =	Actual N =
(Altman and Das 1964)	neuroanatomy	unclear	1
(Amaral et al. 2008)	open field habituation	unclear	unclear
(Cummins et al. 1973)	neuroanatomy	10	2
(Frick and Fernandez 2003)	cognition, neuroanatomy	8	2
(Harburger et al. 2007)	cognition	16	2
(Hellemans et al. 2004)	cognition, stress response	12	1
(Juraska and Kopcik 1988)	neuroanatomy	10	1
(Kempermann and Gage 1999)	cognition, neuroanatomy	12	1
(Larsson et al. 2002)	stress responses	32 or 16	4 or 2
(Leggio et al. 2005)	cognition, neuroanatomy	17	2 (unclear)
(Meshi et al. 2006)	cognition, neuroanatomy	unclear	unclear
(Mohammed et al. 1990)	cognition, neuroanatomy	12	2
(Mohammed et al. 1993)	neuroanatomy	8	1
(Moser et al. 1994)	cognition, neuroanatomy	7 or 13	1
(Nilsson et al. 1999)	cognition, neuroanatomy	12	2
(Pham et al. 1999)	cognition, neuroanatomy	8	1
(Puurunen et al. 2001)	cognition, neuroanatomy	7 or 5	2 (unclear)
(Rosenzweig et al. 1978)	neuroanatomy	12	1

Study:	Effect of enrichment on:	Reported N =	Actual N =
(Roy et al. 2001)	stress responses	30	unclear
(van Dellen et al. 2000)	progression of disease	unclear	unclear
(Wright and Conrad 2008)	cognition and stress responses	6	1 (unclear)

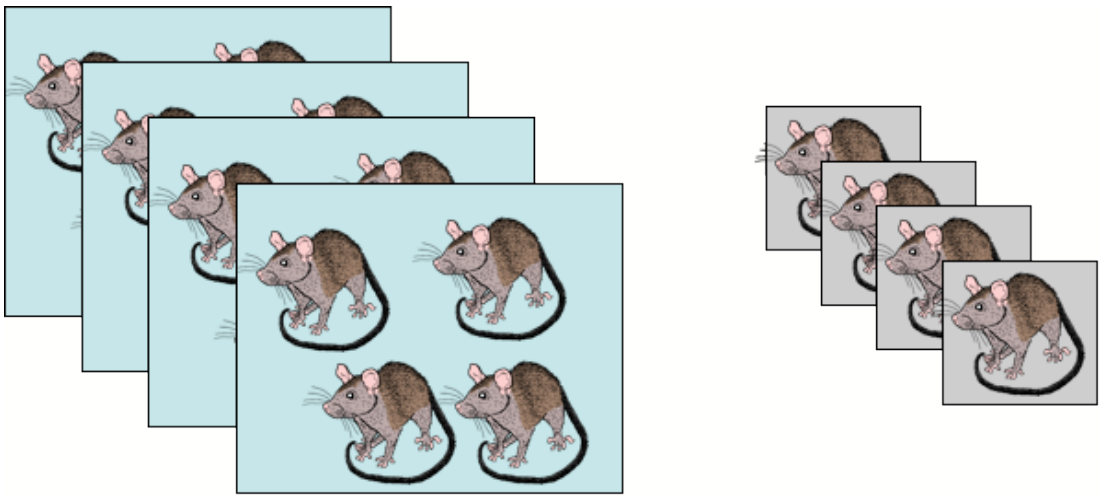
Testing multiple animals from one cage and replicating cage very few times, if at all, results in exceptionally small sample sizes no matter how many rodents are actually used (as depicted in Figure 6.2.a). In fact, the majority of enrichment studies are so heavily pseudoreplicated that the true level of replication is practically zero. At best, the interpretation that can be reached from these data is that living in that particular cage (with particular conspecifics) caused the effects seen (not enrichment *per se)*, a finding that is of limited interest. There are so few studies on enrichment that have not committed the sin of pseudoreplication that we conclude there are no useful data, yet, on the impact of enrichment on the brain and behaviour.

Figure 6.2: A schematic of how pseudoreplication occurs in enrichment experiments. The blue boxes represent enriched cages and the grey boxes are non-enriched cages

a) In this experiment the enriched animals all come from one enclosure and are therefore pseudoreplicates. The true sample size is one, despite using four animals per treatment.



b) In this experiment only one animal per blue box is sampled and so these represent true replicates of the enrichment treatment. The sample size in this experiment is four, despite using 16 animals. Group housing significantly increases the number of animals that are used, but does not necessarily increase the sample size.



The problem of pseudoreplication has long been appreciated in other areas of biological research such as ecology (Hurlbert 1984; Heffner et al. 1996; Hurlbert 2004) behaviour (e.g. Lombardi and Hurlbert 1996; Kroodsma et al. 2001; Ruxton and Colegrave 2006) and welfare (e.g. Lewis and Hurst 2004; Mason et al. 2004) even though true replication in these fields can be difficult. However, pseudoreplication has been much less well appreciated in the biomedical sciences where true replication is much more straightforward. Part of the problem may stem from lack of methodological detail provided (or expected) in papers in this field. Housing details, especially with regard to single or group housing must be made explicit. We also suspect that there is a general, but unfounded, belief that because the cages used for housing laboratory rodents appear identical, cage effects can be ignored.

6.1.2. Dealing with pseudoreplication

The best line of attack on pseudoreplication is to avoid it at the experimental design stage. For example, animals receiving the same treatment can be mixed up so that subjects belonging to one treatment group are not all housed together. However, mixed housing of subjects across treatments may not always be possible. For example, infected and non-infected, or male and female animals cannot always be housed together without unwanted consequences.

In situations where housing individuals from the same treatment group in the same cage is unavoidable (such as in enrichment studies), it is critical that experimental data are analysed in such a way that pseudoreplication is avoided. The simplest approach is to use a single measurement from each cage in the analysis. Practically this might be achieved by collecting data from one animal per cage (e.g. chosen at random, or the dominant animal in the cage, it depends on the biological question being asked), or, using the mean value across all the animals in a cage, see Figure 6.1 (Festing et al. 2002). If this had been done for the studies described in the previous section then the lack of true replication would become immediately apparent. Alternatively, the design of the experiment can be explicitly included in the statistical model, for example, by carrying out a Nested Analysis of Variance (Sokal and Rohlf 1995) or a General Linear Model with cage included as a random

factor (Crawley 2007). Whilst such analyses use the data from the individual rodents, the actual tests of the experimental hypotheses are based on the number of cages, not the number of rodents and pseudoreplication is avoided. Thus, whilst it is simply not possible to 'get around' the problem that the true level of independent replication is reduced, whatever statistical model is used, the appropriate model can be chosen so as to avoid pseudoreplication misleading us.

Finally, it is possible to formally test for variation between cages. If there is good evidence that such variation is very small and so likely to have limited effects on the type 1 error rate, then it may be possible to use the data points from the individual rodents as if they were true replicates in subsequent hypothesis tests, even though they are formally pseudoreplicates. Such "sacrificial pseudoreplication or pooling", as it has been called (Hurlbert 1984), can increase the power of the study by increasing the level of replication. However, there is considerable debate about this approach with some authors (e.g. Hurlbert 1984) arguing that it is never appropriate since there will always be some variation between cages and others taking a more pragmatic approach (Sokal and Rohlf 1995; Quinn and Keough 2002) but in practice the increased power obtained is often small (Quinn and Keough 2002). We also note that even if there is good evidence from previous studies that there are no measurable cage differences this does not guarantee that the same will apply in all future studies, even in the same laboratory. In sum, it is necessary to have multiple cages, not just multiple animals, in each treatment group if any inference about treatment effects is to be drawn, irrespective of which of the above procedures is used.

6.1.3. Group housing vs. single housing

An entirely different and somewhat unorthodox solution to the problem of pseudoreplication is to avoid group housing altogether. The major rationale for group housing is that providing opportunities for rodents to carry out species-specific behaviours leads to a significant reduction in stress and frustration to those animals in comparison to animals housed alone (e.g. Hatch et al. 1963; Dawkins 1988; e.g. Patterson-Kane et al. 1999; Würbel 2001). However, the effects of single housing are not straightforward to assess and the notion of isolation stress, in rats at least, has been challenged. At least two comprehensive surveys of the relevant data have

resulted in the conclusion that there is a distinct lack of well-controlled studies (Brain and Benton 1979; Krohn et al. 2006). Moreover, data that do come from well controlled studies do not equivocally demonstrate that isolation is stressful. For example, changes that occur in singly housed rats, such as increased food intake and decreased concentrations of brain-derived neurotrophic factor in the brain (a protein that is implicated in learning and memory), are not necessarily correlated with increases in stress, as measured by behavioural or physiological means (e.g. corticosterone titres, stereotyping levels: Morinan and Leonard 1980; Holson et al. 1991; Hunt and Hambly 2006; Scaccianoce et al. 2006). Also, rather paradoxically, there is good evidence that social housing leads to significant levels of stress for rodents, to the degree that in toxicological work, it is customary for rodents to be housed alone so as to remove the effects of social stress, thought to impact on the animal's response to the experimental chemicals (Hurst et al. 1999; Nyska et al. 2002; Fitchett et al. 2005). Indeed, it is possible that the benefits of group housing may have been, for rats at least, overstated (e.g. Wolfensen 1994).

A further problem is that group housing coupled with the necessity to replicate treatments over cages leads to an increase in the number of animals that are used (illustrated in Figure 6.2.b). This can lead to confrontation with the recommendations that stem from Russell and Birch's 3Rs: Reduction, Replacement and Refinement, to which all U.K. based scientists are expected to do their best to conform, since these form the basic principles for humane experimentation (Russell and Burch 1959). The use of multiple cages containing group-housed animals within each treatment group to provide adequate replication and statistical power leads to an increase in the number of animals involved, not Reduction as desired.

One advantage with group-housing rodents, however, which concerns the quality of the data collected, is that it is possible to increase the power of a study by using the mean from each cage, even though the number of experimental units drops (because fewer cages are used). In general, taking the mean of individuals in a cage as the data point, rather than the individuals themselves, will reduce the statistical power of an experiment (because there are fewer experimental units). However, *if* the behaviour of rodents housed individually is much more variable than when they are housed in groups, then an experiment based on isolated individuals may be less

powerful, due to the additional noise, than an experiment done with pairs in a cage. This might be so, even if we take an average measure for each pair (and so halve our number of independent units). However, while it may be presumed that isolated animals are more variable than socially housed animals (or vice versa) we are not aware of any experimental data to support that this is the case (e.g. Verwer et al. 2007).

Thus, it appears that both isolation and group housing have apparent benefits: avoidance of pseudoreplication and fewer animals used when animals are isolated, and, a better estimate of the mean and better welfare when animals are group housed. However, since a comprehensive and robust cost/benefit analysis of single versus group housing has not, in fact, been carried out, it is not clear which housing type should be recommended. Importantly, inadequate replication of an experimental treatment leads to poor science, limited inference and a waste of animals, just the opposite of the tenets of the 3Rs (perhaps, with the inclusion of Replication, the 3Rs could become the 4Rs).

6.2. Suggestions for best practise

The Home Office and Scientists alike want good science and to reduce the number of animals used. In order to achieve both of these goals we recommend that a thorough assessment of the costs and benefits of single versus group housing be carried out. This may well be required for each strain, age and sex of rodent, as social needs may vary in different ways for each. Before we can impose good practise we need the robust science to demonstrate what good practice should be. In the mean time there are some changes that could be implemented that will lead to the production of better science, though possibly at the cost of using more animals in the short term. Among these are: 1) researchers should avoid applying treatments at the cage level unless absolutely necessary. The fact that it is logistically easier to have cages containing only individuals of a single treatment is not adequate justification for doing so. 2) All journals that publish work in which laboratory rodents have been used should request explicit details for the Methods sections on how animals from different treatment groups were allocated across cages, and in situations where treatments are applied to all individuals within a cage, how the problem of pseudoreplication was

avoided. 3) In situations where grouping causes a significant reduction in the power of the experiments, researchers should consider carefully whether individual housing is appropriate for their study system. The answer to this question will be strongly system (and question) dependent. For example, if single housing affects an animal's behaviour causing them to behave atypically, then group housing is necessary. Similarly, if group housing reduces the variation between individuals an experiment based on groups may actually have more power than one based on individuals. Only with all of these in place can we begin to be sure that work using laboratory rodents is maximising the quality of the science while minimising animal suffering.

Since there are so few studies in which enriched cages are adequately replicated, in the next chapter I investigate the effect of environmental enrichment on cognitive performance in male and female rats. I also examine if enrichment influences behavioural stress responses during Morris water maze testing.

CHAPTER SEVEN: The effect of enrichment on spatial cognition and stress

A version of this chapter has been accepted for publication and appears as the following: Harris, A. P., D'Eath, R. B. & Healy, S. D. Environmental enrichment enhances spatial cognition in rats by reducing thigmotaxis (wall-hugging) during testing. Animal Behaviour.

I collected and analysed the data myself and wrote the manuscript in collaboration with the authors listed.

Summary

Rats housed with 'environmental enrichment' do better in tests of spatial cognition than do rats housed in barren cages. The leading hypothesis is that exposure to 'social and inanimate complexity' leads to better cognitive processing abilities, which directly enhances performance in a spatial task. However, enrichment is associated with reduced stress responses in novel or acutely stressful situations (cognitive tasks are typically both). Therefore, a plausible alternate hypothesis is that experience of enrichment indirectly enhances performance by reducing a rat's stress response during cognitive testing. We found that, irrespective of sex, enriched rats outperformed barren-housed rats in the Morris Water Maze. However, after accounting for the effects of thigmotaxis (a behavioural stress measure), there was no significant difference in performance between enriched and barren housed rats. Enriched rats were simply less thigmotactic and this indirectly improved their performance. This was true for both males and females. We conclude that enrichment reduces stress outside the home cage, in a testing situation, and subsequently, the cognitive benefits of enrichment occur because enriched animals are less stressed.

7.1. Introduction

The standard definition of enrichment for laboratory-housed animals is "a combination of complex inanimate and social stimulation" (Rosenzweig et al. 1978). Early investigations into the impact of enrichment found that rodents living in a complex environment have heavier brains than do conspecifics reared in a barren environment (e.g. Bennett et al. 1964; Diamond et al. 1965). More recently, however, the addition of environmental enrichment to the home cages of laboratory rodents has been recommended as a useful way to improve rodent welfare (Sørensen et al. 2004).

Typically, laboratory rodents are housed in barren cages and often in isolation. However, despite the intrinsic benefits associated with standardised housing (e.g. minimal environmentally induced variation, cheap, easy maintenance), living in barren conditions has been associated with the development of abnormal behaviours, high stress levels and impaired welfare in rodents (Würbel et al. 1998; Callard et al. 2000; Würbel 2001). Enrichment of home cages (both socially and physically) is thought to improve the welfare of caged animals by providing the animal with the opportunity to carry out species-specific behaviours that the animal is highly motivated to perform (Patterson-Kane 2004), leading to a reduction in stress and frustration and, to some extent, possibly 'boredom' (e.g. Würbel et al. 1998). As such, the U.K. Home Office encourages the addition of environmental enrichment to the home cages of laboratory rats and mice (Home Office 1995).

As well as improving welfare, enrichment also appears to improve cognitive ability in rodents. Rats that have experienced enrichment (social and physical) tend to outperform single or barren-housed (non-enriched) conspecifics, especially in tests of spatial cognition (Falkenberg et al. 1992; Nilsson et al. 1999; Pham et al. 1999; Larsson et al. 2002; Leggio et al. 2005). The currently favoured hypothesis to explain these data is that interacting with a socially and physically complex environment (informal learning) directly enhances cognitive ability (for reviews see: Rosenzweig and Bennett 1996; van Praag et al. 2000). However, rodents that have had exposure to enrichment also tend to have reduced stress responses to acutely stressful (e.g. novel) situations. For example, enriched rodents habituate to novel objects in a familiar test arena faster and enter an unforced open field test

significantly sooner (Chapillon et al. 1999; Zimmermann et al. 2001; Scrijver et al. 2002). They also spend longer in the open arms of the elevated plus maze (a classic test of anxiety), than do non-enriched animals (Roy et al. 2001). Socially and physically enriched rats explore more initially and habituate sooner to 'open-field'/novel arenas than do non-enriched rats (Falkenberg et al. 1992; Larsson et al. 2002). Enriched rats also have reduced corticosterone, adrenocorticotropin and adrenalin responses to repeated handling compared to non-enriched rats (Moncek et al. 2004). Thus, an alternative explanation for the superior performance of enriched rodents during cognitive testing is that exposure to enrichment reduces the stress response of these animals to the stresses associated with various cognitive tasks.

A reduced stress response during cognitive testing is likely to improve performance. For example, in the Morris water maze (MWM), a commonly used spatial task, the rodent swims in a brightly lit pool to a hidden escape platform, and, due to the nature of the MWM (a wet, brightly-lit, open-field task), it is widely accepted that this task is acutely stressful (e.g. D'Hooge and De Deyn 2001; Beiko et al. 2004). High levels of acute stress during MWM testing in rodents are manifested behaviourally as thigmotaxis (wall-hugging). Thigmotaxis may indirectly impair performance in the MWM because the platform is never located in the outer edge of the tank (Herrero et al. 2006).

There are several lines of evidence that support that thigmotaxis is a reliable non-invasive indicator of stress during MWM testing. Firstly, thigmotaxis is suppressed by anxiolytics and increases with corticosterone administration (Treit and Fundytus 1989; Snihur et al. 2008). Secondly, thigmotaxis levels are positively correlated with corticosterone levels after MWM testing and pre-training reduces thigmotaxis (Beiko et al. 2004; Herrero et al. 2006). And thirdly, hippocampal lesions, which impair MWM performance, do not affect thigmotaxis, suggesting that a rat does not swim near the pool wall simply because it does not know the location of the platform (Morris et al. 1982).

High acute stress levels during MWM testing often affect females to a greater degree and lead to apparent sex differences in performance (Perrot-Sinal et al. 1996; Beiko et al. 2004; Harris et al. 2008a), differences that can be reduced/eliminated by pre-training of animals prior to testing in a MWM or administration of anxiolytics

(Galea et al. 1994b; Perrot-Sinal et al. 1996). If enrichment enhances performance by reducing stress, it might be that sex differences in performance diminish as a result of this kind of housing.

Enrichment may directly enhance MWM performance independently of stress, or it may enhance MWM performance indirectly by reducing behavioural stress levels during testing. To determine whether variation in stress levels could help to explain the cognitive benefits of enrichment, we tested the impact of housing enrichment on spatial cognition and acute behavioural stress levels in the MWM in male and female rats. We also tested stress levels in a light/dark box. The light/dark box consists of two compartments, one of which is dark and the other brightly lit from above, joined by a wall containing a small opening. The rodent is placed in the dark compartment and can pass freely into the light compartment. The time the animal takes to fully enter the light side positively correlates with the animal's anxiety level, as demonstrated by dose-dependent effects of anxiolytics (Crawley and Goodwin 1980; Augustsson et al. 2003). If enrichment *directly* enhances cognition, then we predicted that: 1) enriched rats would out-perform the non-enriched animals in the MWM; 2) MWM performance would not be correlated with variation in stress between housing conditions; 3) enriched and non-enriched rats would not differ in time to enter the light compartment in the light/dark box. However, if enrichment *indirectly* enhances cognition by reducing stress levels during testing, we predicted that: 1) enriched rats would out-perform non-enriched rats; 2) males would out-perform females but only under non-enriched conditions; 3) enhanced MWM performance in enriched rats would be accompanied by a decrease in acute behavioural stress levels in these rats; 4) enriched rats would be faster to enter the light compartment in the light/dark box than non-enriched rats.

7.1.1. Materials and methods

Subjects and housing

The subjects were 30 male and 30 female Wistar rats, aged between four and five weeks, obtained from Harlan U.K. Ltd. On arrival, males weighed 84g (±7g) and females 81g (±5g). Six rats of each sex, were chosen at random and housed in

isolation in 'non-enriched' (NE) conditions, while the remaining 24 rats were housed in six same-sex groups of four in 'environmentally-enriched' (EE) conditions (N = 6 per housing and sex treatment). The NE rats were housed in plastic-bottomed cages (45x28x20 cm; NKP cages Ltd.), the EE rats in larger plastic-bottomed cages (56x38x20 cm; NKP cages Ltd.) and both groups were provided with a 2 cm layer of woodchip bedding. Additionally, each EE cage was provided with tissue-paper nesting material (Paper Wool, DBM Scotland), a transparent red tunnel (Rat retreat tunnel, size 89x153mm, DBM Scotland) on the floor of the cage and an opaque plastic tube (290x105mm) suspended from the cage top with chains. The EE rats were also given various novel objects (newspaper strips, small cardboard tubes and boxes, wood blocks, empty yogurt pots etc.) that were changed every three to four days. All rats had access to *ad libitum* pelleted food (RM3 diet, Special Diet Services, Ltd., Witham, Essex, UK) and tap water and were maintained under a 12L: 12D cycle (lights on at 0700 hours) at 21-24ºC. All cages were cleaned out and each rat was weighed once per week. Therefore, all of the rats were handled at least twice weekly for ten weeks before cognitive testing began. The majority of the rats appeared 'tame' at the start of cognitive testing (they were easy to catch and did not struggle, squeak or attempt to bite during handling).

To determine dominance hierarchies within a cage, we measured the amount of pinning (when a rat uses its forelimbs to pin another rat on its back) by each rat eight-nine weeks post arrival. The animals in each cage were observed for 10 mins between 1400 and 1700hours and which animal doing the pinning, the recipient and the number of pinning incidences was recorded; data are not shown. After finding no evidence of stable dominance hierarchies, as is consistent for rats at this age (e.g. Adams and Boice 1989b), so as to avoid pseudoreplication one rat was picked at random from each EE cage to be tested in the MWM (all NE rats were the comparison test group). At 10 weeks post-arrival these EE rats and all the NE rats were then tested for 22 days in a reference memory (memory across days for the same location) MWM task in addition to the two probe trials, followed by light/dark box testing 13 days later.

Figure 7.1: Photograph of an enriched cage on the left and a non-enriched on the right. Food and water bottles have been removed from the cages to give a clearer view.

Morris water maze apparatus

The MWM apparatus is described in detail in section 2.2.1.

Morris water maze procedure

Probe One: To assess how much time each rat spent in the four quadrants prior to training, the very first swim each rat received was a Probe Trial, during which the platform was removed from the MWM. This also allowed observation of thigmotaxis levels prior to knowing the platform's location. Each rat was gently released from a predetermined release point into the MWM close to, and facing the side of the tank and allowed to swim for 60 seconds (s). After 60 s the rat was lifted out of the tank, towel dried and placed into its home cage under a heat lamp for approximately 10 mins. All probe trials were video-taped so that the percentage of

time spent in each of the pre-determined quadrants (north, east, south and west) could be determined.

Training: Training proper began the day after Probe One. During spatial training the platform was either located in the east or the west quadrant but for each rat the platform remained in the same location across the days. Each rat received one swim per day and was released from a different release point each day (NE, NW, SE, SW). All swims took place between 1100hours and 1500hours. The rat was gently lowered into the water close to and facing the wall of the tank. The time taken for the rat to find and subsequently climb onto the platform was recorded. If a rat failed to find the platform within 120 s it was gently guided to, and allowed to climb onto, the platform. Rats were left on the platform for 20 s before being removed from the platform, towel dried and returned to its home cage under a heat lamp for approximately 10 mins. Rats had one swim per day for 22 consecutive days.

Probe Two: On the day after the last day of spatial training, each rat received a second Probe Trial, for which the platform was removed. The procedure for Probe Two followed that for Probe One.

Thigmotaxis

The percentage of time that a rat spent swimming within 15 cm of the wall of the maze was recorded manually during both probes and throughout training. The videotapes were watched on a TV monitor, over which an acetate sheet was attached. Marked on the acetate sheet were the circumference of the MWM and 15 cm from the edge of the MWM. All the time the rats head and shoulders spent in this outer perimeter was recorded.

Light/dark box

To determine stress levels in an alternative novel situation, eleven days after Probe Two each rat was tested once in a light/dark box. The light/dark box consisted of an open top rectangular cardboard box divided into two equal sized compartments using

a cardboard divider. A 10 x 10 cm hole in the divider provided free access between the compartments. Black card lined the dark compartment and white card lined the light compartment, both compartments measured 40 x 25 x 25 cm high. A 40 Watt light positioned directly over the centre of the light compartment provided the only illumination in the testing room. Testing took place between 1100 and 1600 hours. Rats were tested in a pre-determined random order. Each rat was placed in the middle of the dark compartment facing away from the opening to the light side and its behaviour was monitored for five minutes. The time taken for the rat to cross over into the light compartment completely (latency in seconds) was recorded. The box was cleaned between rats (faeces removed and box surfaces wiped with soapy water then sprayed with 70% ethanol).

Data analyses

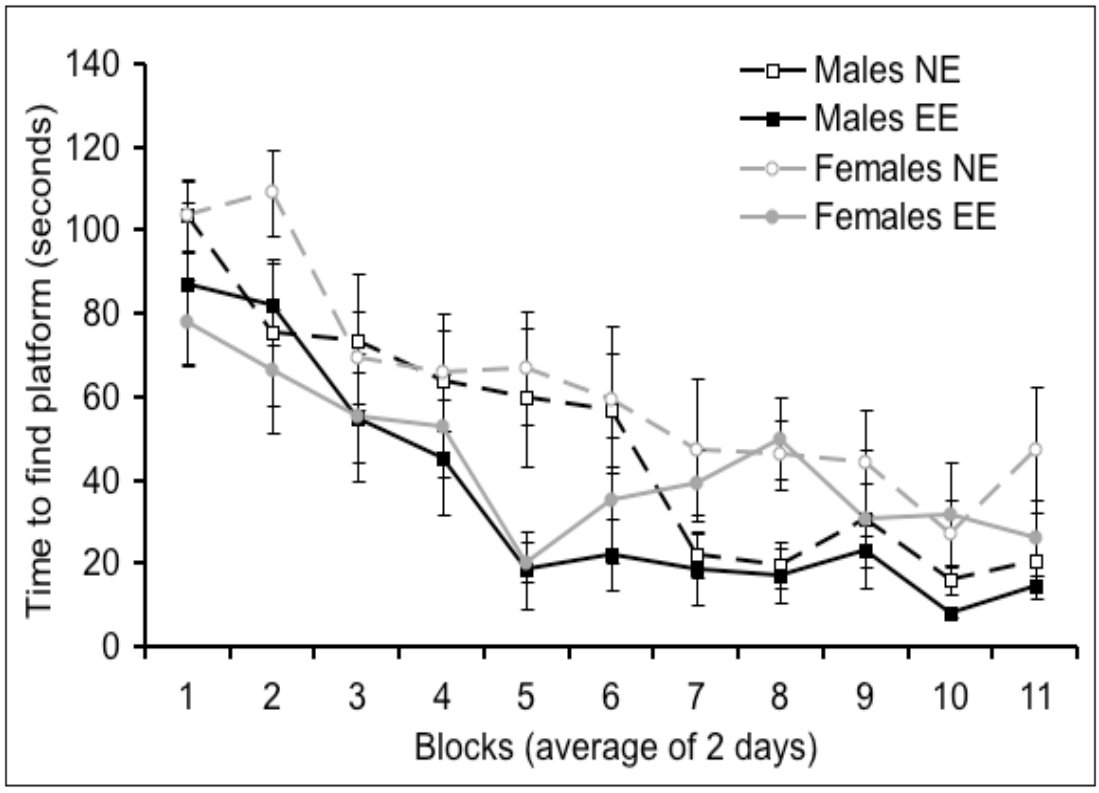
Repeated-measures data were analysed using a Repeated-Measures-Analysis-of-Variance (RM ANOVA): *between-subject* factors were Sex (male and female) and Housing Condition (EE and NE), *within-subject* factors were Block (block one to eleven; each block being an average of two days' trials). All of these factors were included in the analyses and interactions between main effects that were not significant were removed. The assumptions of sphericity (that repeated measures have equal variances and that the correlations between any two measures are the same) were tested using the Mauchly-Criterion test. Corrections were made using the Greenhouse-Geisser terms where appropriate. The assumptions of normality and homogeneity of variance were tested and transformations applied to the data where appropriate. The light/dark box data were normally distributed but did not have equal variances and so were analysed using a Welch ANOVA test. To determine if thigmotaxis explained any differences in performance between EE and NE rats, the mean time spent in thigmotaxis across testing was calculated and co-varied using an Analysis of Co-Variance (ANCOVA) with the mean time to find the platform. Housing and sex were included in this analysis.

7.1.2. Results

Reference memory

Males reached the platform significantly sooner than did females and NE rats took significantly longer to find the platform than did EE rats (sex: $F_{1,21}$= 4.39, P = 0.0485; housing: $F_{1,21}$= 9.45, P = 0.0058; Figure 7.2). All rats found the platform faster as the experiment progressed (block: $F_{10,210}$= 19.02, P < 0.0001). No other interactions were significant.

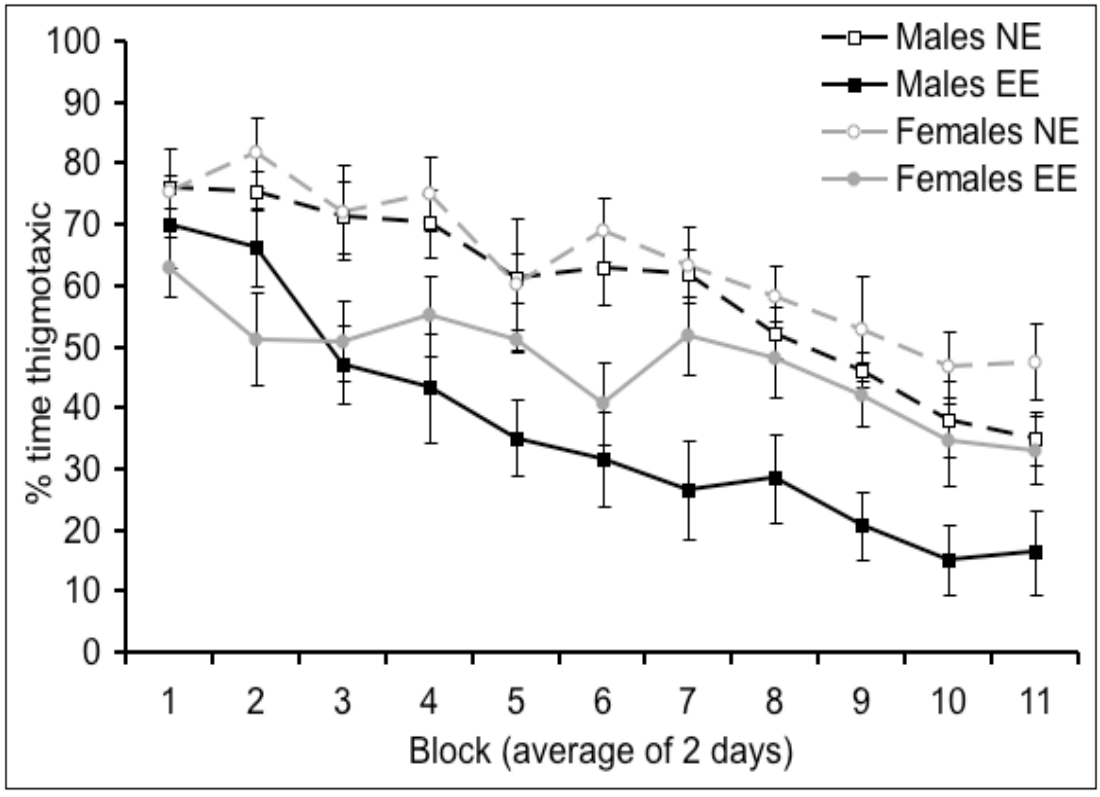
Figure 7.2: Time taken to reach the platform (mean ± SE) for males (black) and females (grey) that were either enriched (EE; solid lines) or non-enriched (NE; dotted lines). Data are blocked over two days and N = 6 per treatment group.



Thigmotaxis

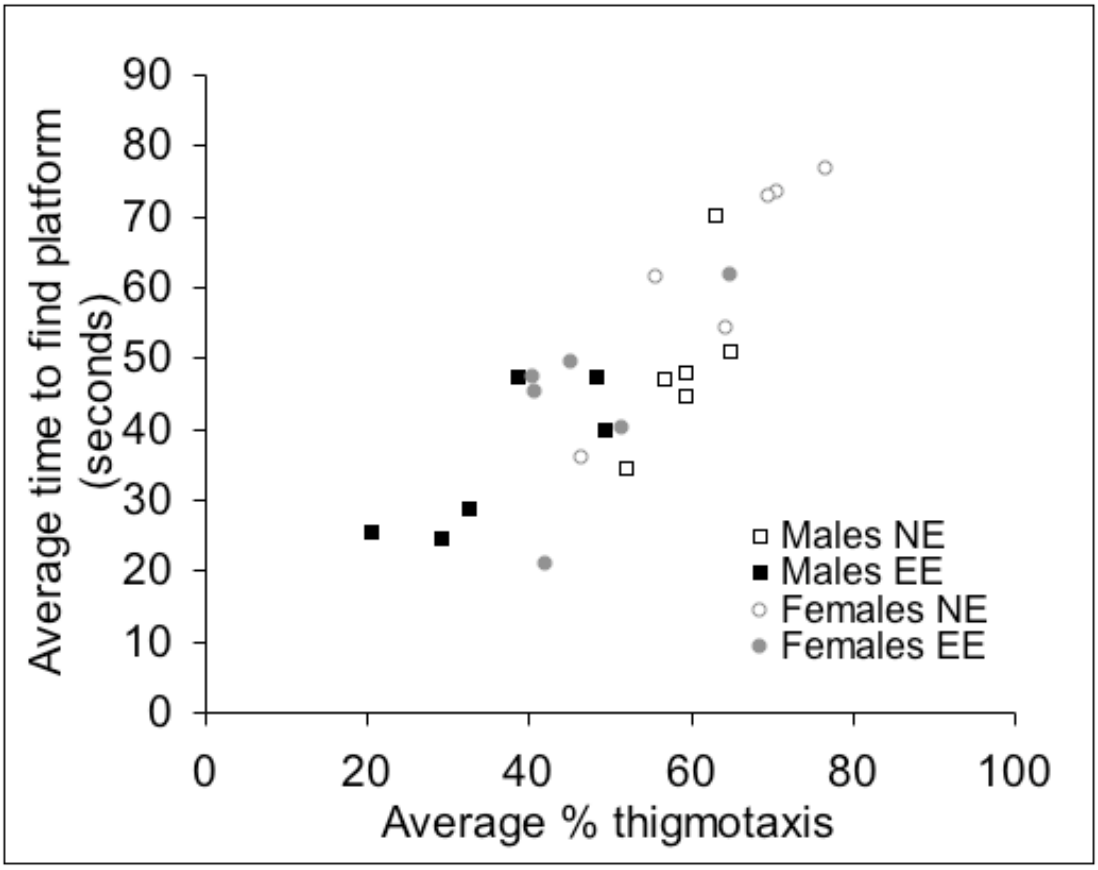
Females were more thigmotactic than were males and NE rats were significantly more thigmotactic than were EE rats (sex: $F_{1,21}$= 4.17, P = 0.05; housing: $F_{1,21}$= 25.93, P < 0.0001; see Figure 7.3). Thigmotaxis decreased as testing progressed ($F_{10,210}$= 24.64, P < 0.0001; Figure 7.3) and thigmotaxis in males tended to decrease faster than in females (sex-by-block interaction: $F_{10,210}$= 1.84, P = 0.056). No other interactions were significant.

Figure 7.3: Time spent swimming thigmotactically (mean ± SE) for males (black) and females (grey) that were either enriched (EE; solid lines) or non-enriched (NE; dotted lines). Data are blocked over two days and N = 6 per treatment group



The average time to find the platform across the 22 days of training was co-varied with the average time spent in thigmotaxis. Mean thigmotaxis levels positively correlated with the mean time the rats took to find the platform over the 22 days of testing (Pearson's correlation: $r^2 = 0.69$, N = 24, $P < 0.0001$). Moreover, once thigmotaxis was accounted for, the sex and housing effects were not significant (ANCOVA, thigmotaxis: $F_{1,20} = 19.64$, $P = 0.0003$; sex: $F_{1,20} = 0.65$, $P = 0.43$; housing: $F_{1,20} = 0.30$, $P = 0.59$; Figure 7.4)

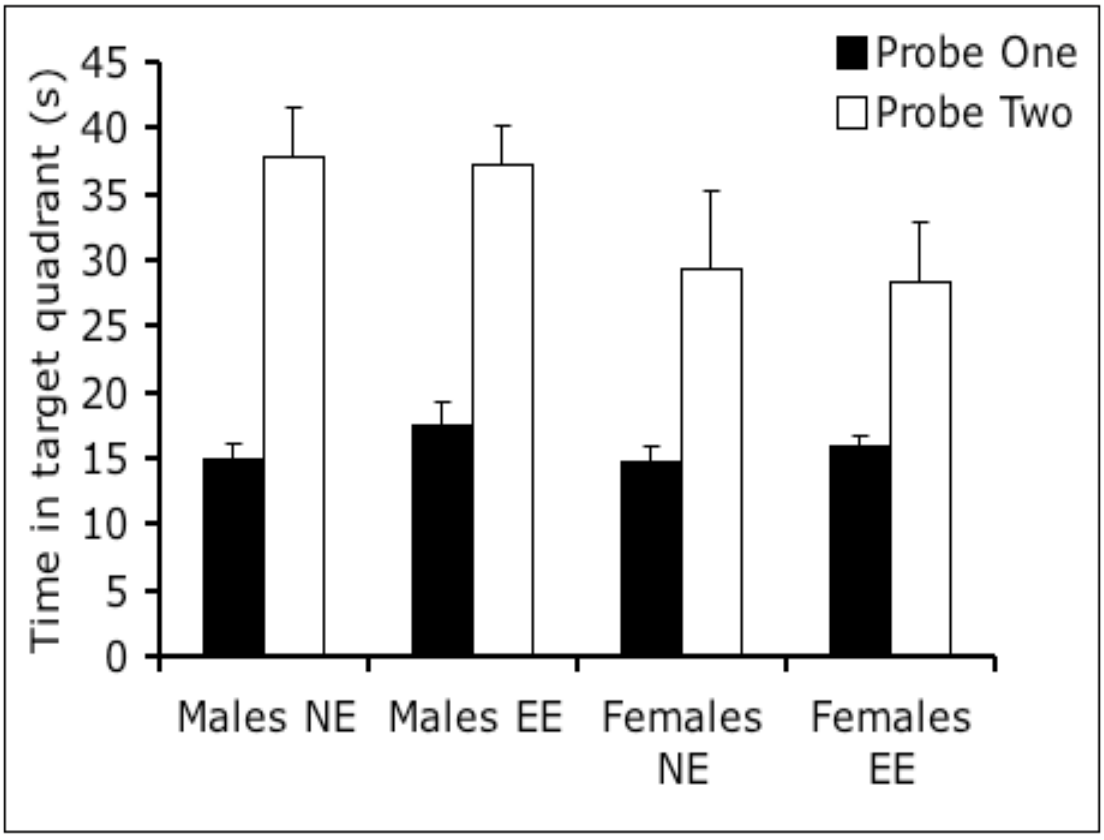
Figure 7.4: Mean time (s) taken to find the platform across the 16 test days co-varied with average thigmotaxis; EE enriched, NE non-enriched (%; across the 16 test days), N = 6 per group.



Probes One and Two

None of the rats showed a preference for the target quadrant in Probe One (one sample t-test, Probe One: $t_{23} = 1.32$; $P = 0.20$; Figure 7.5.). However, in Probe Two, after 22 days of spatial training, all rats searched for significantly longer in the target quadrant than expected by chance, indicating that the rats had learnt the platform's location (one sample t-test, Probe Two: $t_{23} = 8.23$, $P < 0.0001$). Similarly, all the rats spent significantly longer in the target quadrant in Probe Two than they had in Probe One (RM ANOVA: $F_{1,21} = 65.66$, $P < 0.0001$; Figure 7.5.). Males tended to spend longer than the females in the target quadrant in Probe Two (probe trial-by-sex interaction: $F_{1,21} = 3.23$, $P = 0.087$). Housing had no effect on time in the target quadrant in the probe trials and no other interactions were significant.

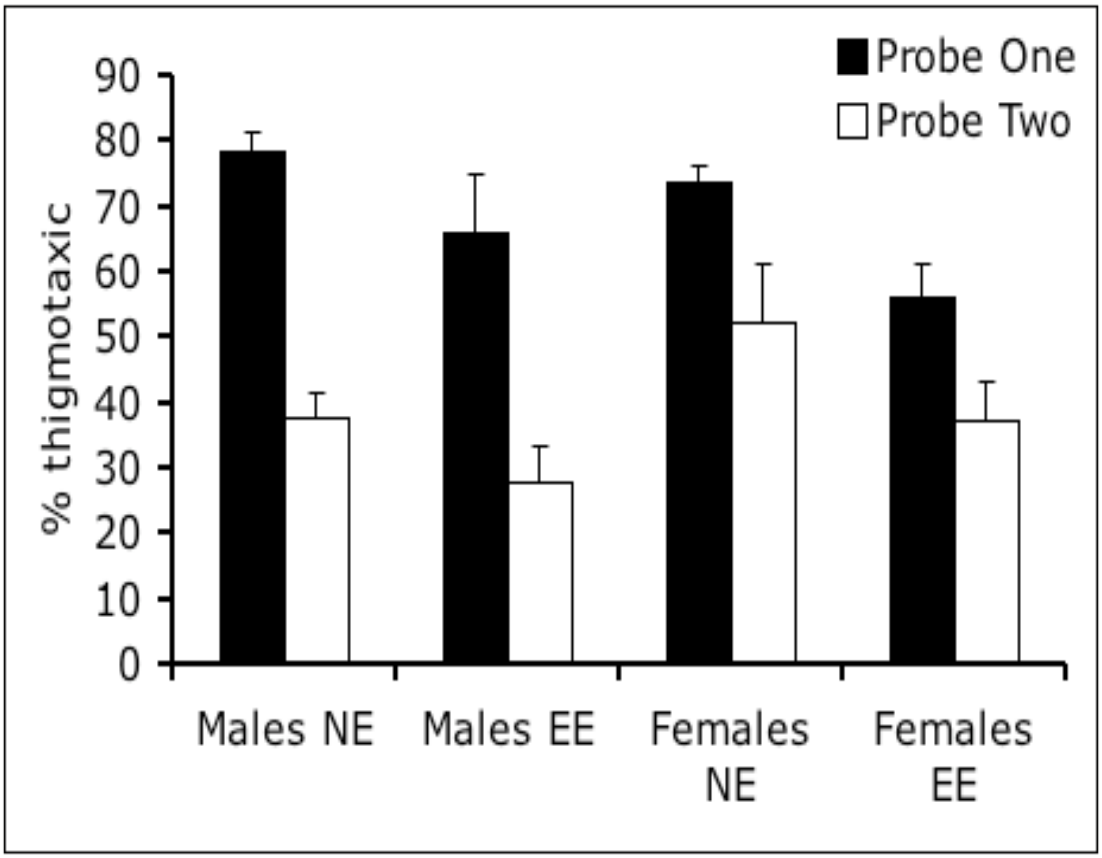
Figure 7.5: Mean dwell time (±SE) in the target quadrant during Probes One (black) and Two (white) for males and females that were either enriched (EE) or non-enriched (NE), N = 6 per group.



Thigmotaxis during Probes One and Two

The EE rats were significantly less thigmotactic than were NE rats during the probe trials (RM ANOVA: $F_{1,21}$= 13.72, P = 0.0013). All rats were significantly less thigmotactic in Probe Two than in Probe One ($F_{1,21}$= 43.94, P < 0.0001; Figure 7.6). Overall, males and females spent a similar proportion of time engaged in thigmotaxis ($F_{1,21}$= 0.43, P = 0.517). However, in Probe Two, males were significantly less thigmotactic than were females (sex-by-probe interaction: $F_{1,21}$= 4.61, P = 0.0435).

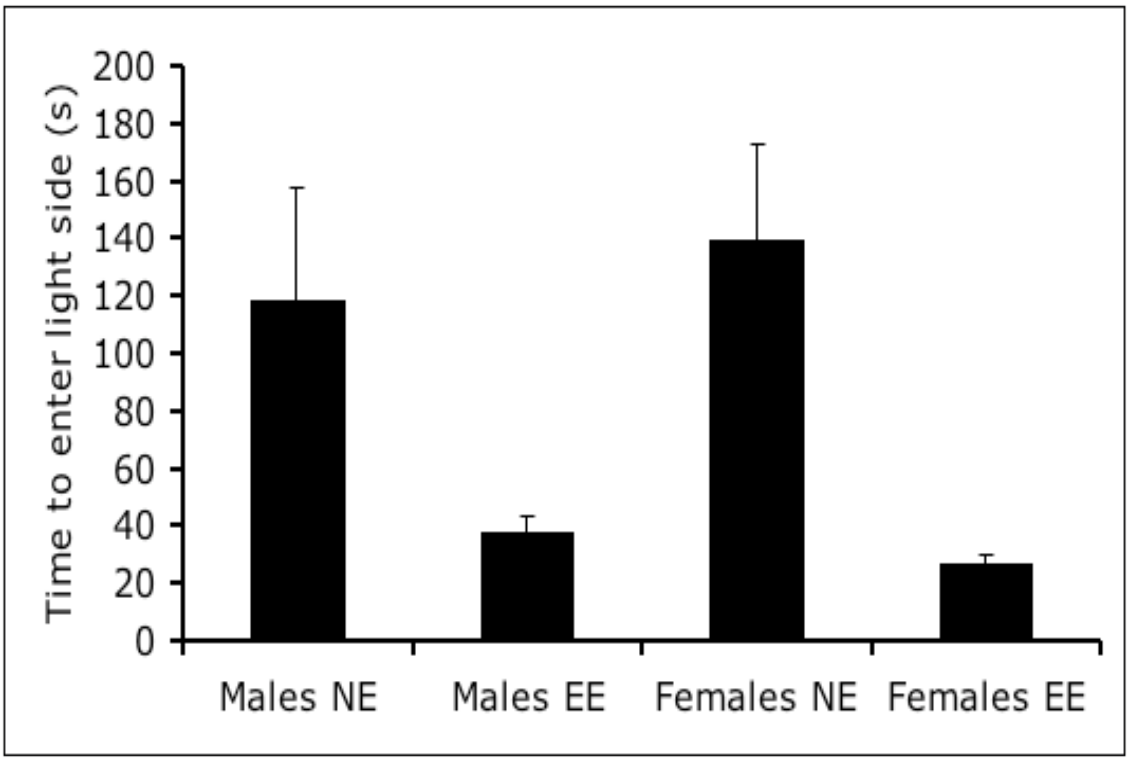
Figure 7.6: Mean thigmotaxis (±SE) during Probes One (black) and Two (white) for males and females that were either enriched (EE) or non-enriched (NE), N = 6 per group.



Light/dark box

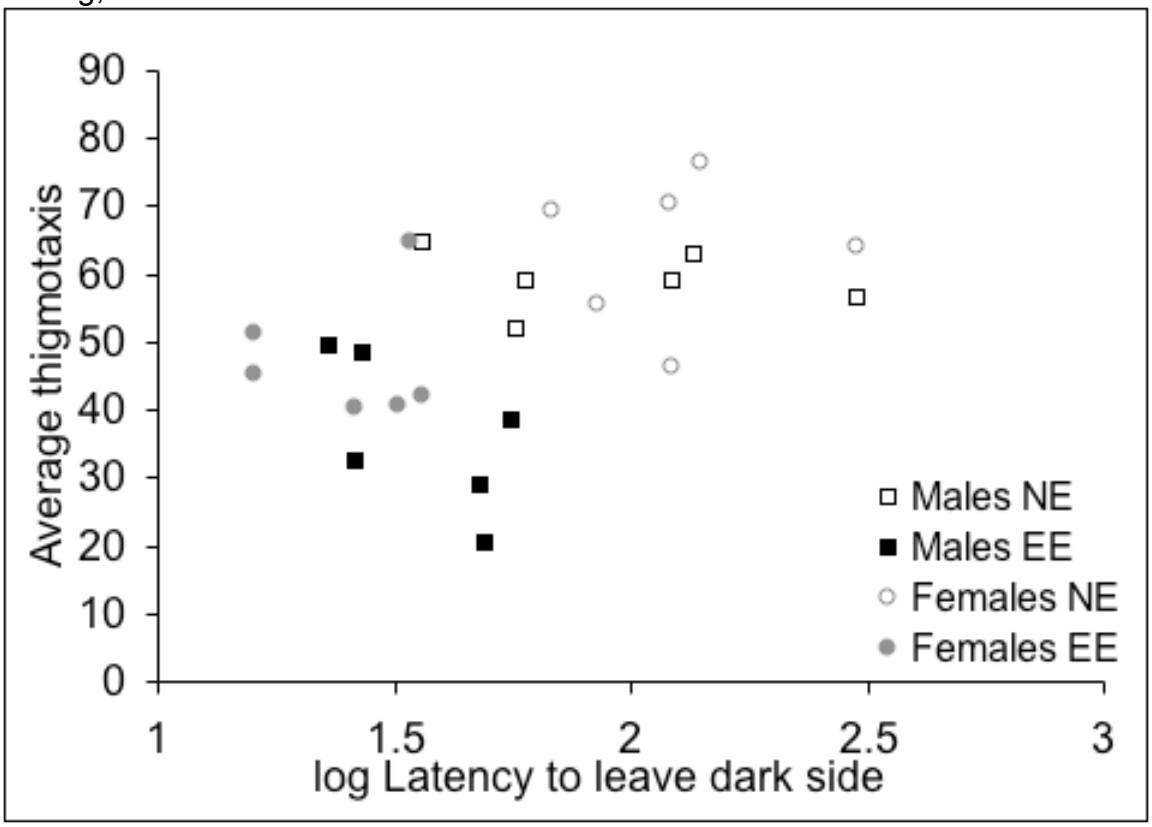
There was a significant difference amongst the groups with respect to the time taken to leave the dark side: irrespective of sex, EE rats were quicker to venture into the light compartment (Welch one-way ANOVA assuming unequal variances $F_{3,9.7}$ = 5.3, P = 0.0205; see Figure 7.7). The EE rats took a mean of 32 s (± 3 s) and the NE took a mean of 128 s (± 25 s) to leave the dark side and enter the light compartment.

Figure 7.7: Mean time taken (± SE) to leave the dark side in the light/dark box for male and female rats that were enriched (EE) or non-enriched (NE), N = 6 per treatment group.



To determine if the animals that took the longest to leave the dark side during light/dark testing were the most thigmotactic during MWM testing, the average percentage of time that each rat spent swimming thigmotactically across the 22 days of training was correlated with the latency that each rat took to leave the light side during light/dark box testing (light/dark box data were log transformed for this analysis). Mean thigmotaxis levels positively correlated with the time the rats took to leave the dark side: the most thigmotactic rats were the least willing to enter the light side during light/dark box testing (Pearson's correlation: $r^2 = 0.22$, N = 24, P = 0.02).

Figure 7.8: Correlation between thigmotaxis (% averaged over the 22 days of training) and the time taken to leave the dark side during light/dark box testing, N = 24.



7.1.3. Discussion

As predicted, the EE rats reached the platform sooner than did the NE rats and males were faster to do so than were females. However, both of these effects were entirely explained by the time spent in thigmotaxis (our proxy for stress).

Living in enriched conditions is associated with enhanced spatial cognition in rodents (e.g. Nilsson et al. 1999; Leggio et al. 2005) and the favoured interpretation is that it is through interactions with a (socially and physically) complex environment that cognitive abilities are enhanced via informal learning (e.g. Rosenzweig and Bennett 1996). However, as we found in our experiment that the time rats spent in thigmotaxis completely explained both sex and housing differences in performance, we propose that enhanced cognition in enriched rodents is a consequence of a reduced stress response during cognitive testing.

Our finding that, relative to impoverished animals, EE animals are less fearful/stressed/emotional when challenged with a stressful situation is consistent with other research (Chapillon et al. 2002). However, we are the first to show that this decrease in stress response can cause an apparent improvement in performance in the MWM, a cognitive task in which stress-related behaviour (expressed as thigmotaxis) impedes performance. Indeed, we believe the NE rats learnt the platform location as accurately as did the EE rats since in Probe Two the NE and EE rats spent similar amounts of time in the training quadrant. However, in Probe Two, the NE rats were significantly more thigmotactic than were the EE rats, which suggests that, despite knowing the platform's location, the NE rats were more reluctant to leave the maze wall than were the EE rats, which explains why the NE rats took longer to find the platform during training. Additionally, the NE animals were slower to emerge from the dark side of the light/dark box than were the EE animals. That thigmotaxis is a useful measure of anxiety is supported by our finding that the most thigmotactic animals were also the slowest to leave the dark side during light/dark box testing (Figure 7.8).

It is possible that the EE rats had a greater knowledge of the platform's location, which caused them to be bolder and to swim away from the edge of the tank. However, the EE rats were significantly less thigmotactic than were the NE rats in Probe One, during which no platform was present, which demonstrates that

the EE rats were bolder even when they did not know of the platform's location or existence.

In rodents, experience of enrichment is correlated with changes in areas of the brain associated with processing spatial information, including increased number and lifespan of neurones in the dentate gyrus (Kempermann et al. 1997; Nilsson et al. 1999), increased density of dendritic spines in the CA1 region of the hippocampus (Moser et al. 1994; Leggio et al. 2005), and increased cortical thickness (Bennett et al. 1964; Cummins et al. 1973). Although these brain changes provide a potential mechanism for enhanced cognition, there are only correlation data to support that these brain changes result in better cognition (e.g. Kempermann et al. 1997; Pham et al. 1999). In fact there are data that suggest newborn cells in the hippocampus do not mediate the changes in behaviour that occur after enrichment, since improved spatial ability was seen in mice after exposure to enrichment even when neurogenesis in the hippocampus was blocked using X-irradiation (Meshi et al. 2006). There are also no data, to date, confirming that these brain changes are directly caused by enrichment. These changes in the brain might be caused by stress reductions from enriched housing, not least because the hippocampus is a major target for corticosterone (de Kloet et al. 1999). And it is possible that these brain changes cause, or are coupled with changes that cause, the animal to cope better with stress. For example, in comparison to impoverished rats, enriched rats have a higher expression of the gene that encodes the glucocorticoid receptor (GR) in the hippocampus (e.g. Olsson et al. 1994). Since increases in this receptor enhance feedback efficacy of the hypothalamic-pituitary adrenal axis (responsible for turning stress responses on and off), it is hypothesised that increased GR expression is responsible for enriched rats coping better (cognitively and behaviourally) when challenged with a stressful situation (Meaney et al. 1991; Mohammed et al. 1993; Herrero et al. 2006).

The 'reduced stress via enrichment' hypothesis may also explain the results from other cognitive tasks. For example, EE rats make fewer reference and working memory (within trial memory) errors than do NE rats in the radial arm maze (RAM), an appetitive task in which rodents search at the ends of arms that radiate from a central arena to locate food rewards (Einon 1980; Juraska et al. 1984; Seymoure et al. 1996; Daniel et al. 1999). It is possible that food deprivation (used as motivation)

is chronically stressful and that being in a RAM is also acutely stressful, since rats are typically required to traverse along brightly lit arms in a novel room. Therefore stress, which can reduce appetite (e.g. Liu et al. 2008), may result in 1) a rat not eating food rewards and thus re-entering previously visited arms i.e. working memory errors; 2) a reduction in motivation to forage at all, or 3) a shift in motivation from foraging to exploring i.e. reference and working memory errors.

It may seem counterintuitive that a 'stress-free life', apparently experienced if housed with enrichment, should equip a rodent to deal well with acute stress or novelty. Instead, we propose that the addition of novel objects into the home cage of a rodent may actually be a mildly stressful experience. However, since no aversive outcome is experienced from interacting with the enrichment objects, the animal learns that novel objects are not coupled with negative outcomes and as a result becomes habituated to novelty. Our light/dark box data would suggest that EE rats may even seek novelty, since they were four times faster to enter the light side than were the NE rats. Similarly, social housing, through the development of social hierarchies or inability to escape from cage-mates (for whatever reason), results in a changing or novel environment, which may be mildly stressful. As enriched rats housed in large groups (10 per cage) have higher corticosterone levels and heavier adrenals than rats housed in groups of 3-4 in smaller barren cages (Moncek et al. 2004), it is possible that habituation to novelty explains why living in enriched housing is associated with a reduced stress response in acutely stressful situations.

Regardless of housing conditions, the males outperformed the females: males found the platform faster during spatial training and, during the final probe trial when the platform was absent, spent longer in the platform quadrant. This finding is consistent with other studies that also find a male advantage in reference memory tasks (Cimadevilla et al. 1999; Isgor and Sengelaub 2003; Saucier et al. 2008). However, females were more stressed than were the males by being tested in the MWM as they were always more thigmotactic than were the males. The sex difference in reference memory performance in both isolated and enriched rats may, then, be attributable to a differential acute stress response in the males and females during testing (Harris et al. 2008a, 2008b; Beiko et al. 2004; Perrot-Sinal et al. 1996). Reduction of the acute stress response, either through adrenalectomy,

anxiolytic drugs, opioid inhibitors or pre-training, leads to equivalent male and female reference memory performance (Galea et al. 1994b; Kavaliers et al. 1996; Perrot-Sinal et al. 1996; Beiko et al. 2004). There are no data on the effects of anxiolytics or pre-training with regard to cognitive differences between NE and EE rats but our data lead us to predict that either would remove, or substantially diminish, enrichment benefits on cognitive performance.

Surprisingly, given the sex difference in thigmotaxis in the MWM, we found no differences between males and females in the light/dark box. However, as behavioural, pharmacological and physiological validation of the light/dark box test (and, indeed, many other anxiety tests e.g. the elevated plus maze) has been carried out almost entirely in males, it is possible that this test is not an appropriate test of anxiety in females (e.g. Johnston and File 1991). Alternatively, the light/dark box is either not a very sensitive measure of acute stress or it is not as stressful as the MWM.

In summary, both better male performance and an apparent benefit of housing enrichment on MWM performance in our experiment can be ascribed to stress in the MWM. Females were always more stressed in the MWM than males (irrespective of housing) and although enrichment reduces stress during MWM testing in both sexes, the reduction was not enough to remove sex differences in performance. Therefore, enrichment indirectly enhanced performance in a cognitive task by reducing stress during testing. These data raise two issues. Firstly, these data cast doubt on the leading hypothesis that enrichment directly improves cognitive ability by increasing 'brain power' (e.g. Kempermann et al. 1997; Pham et al. 1999). Secondly, while it is widely accepted that enrichment reduces both stress in the home cage (e.g. Sørensen et al. 2004) and anxiety during exposure to threatening stimuli (e.g. handling, injection; Sharp et al. 2002a; Moncek et al. 2004), our data demonstrate that enrichment also reduces anxiety during cognitive testing in an MWM.

In the following appendix (which does not appear in the previous manuscript) I investigate the impact of environmental enrichment on performance in a working memory task in the MWM.

7.2. Appendix: The impact of enrichment on working memory

Introduction

Most evidence for enrichment leading to better cognitive performance in animals housed with social and/or physical enrichment is produced from reference memory testing using the MWM (e.g. Mohammed et al. 1990; Falkenberg et al. 1992; Leggio et al. 2005; Harburger et al. 2007). The hidden platform stays in the same place across the days and rats typically receive one or multiple swims per day. Performance is averaged across the swims to generate the daily performance level. In Chapter 7, I argue that enriched animals outperform impoverished animals in the MWM because they cope better with the stress of testing. As the first swim of each day appears to be the most stressful (thigmotaxis levels are highest in Swim One; Chapter 2 and 5) and in reference tasks the measure of cognition includes performance in Swim One, reference memory protocols may introduce a bias favouring animals that cope better with stress.

The full extent of the impact of stress on performance in a working memory MWM task is less clear because of differences in both protocol (e.g. duration between swims) and the data that are actually reported. In a working memory task a rat receives multiple swims per day and the platform is moved each day. There are few, if any, data concerning the working memory ability of enriched rats in the MWM. However, working memory of enriched rats in the RAM is typically better than that of non-enriched animals so it seems unlikely that enrichment only enhances reference memory (e.g. Juraska et al. 1984; Seymoure et al. 1996).

The aim of this experiment was to determine whether enrichment enhances cognitive performance in a working memory MWM task, or, whether differences are apparent only in the non-cognitive component of the task (Swim One). In a test comparing performance of enriched and non-enriched rats in the MWM using a working memory protocol I predicted that, if enrichment *directly* enhances cognition, the enriched rats would outperform non-enriched rats in the second swim when memory is used to locate the platform. Additionally, enriched animals would be as thigmotactic as non-enriched rats in Swim Two. However, if enrichment *indirectly*

enhances performance by lowering the enriched rats' response to stress (as found in Chapter 7), differences should be apparent only in the non-cognitive component of the task i.e. in Swim One the enriched rats would be significantly less thigmotactic and, therefore, find the platform sooner than would the NE rats. In Swim Two, the groups should not differ. Additionally, to determine whether prior MWM experience decreased stress levels during testing, I compared data from the non-enriched rats in this experiment with those from non-enriched rats in a similar experiment (Chapter 5, Experiment 2). The difference between these experiments was whether the rats had completed a reference task before the working memory task. I expected that previous experience of the MWM would reduce thigmotaxis.

7.2.1. Materials and methods

Subjects and housing

Environmentally enriched conditions (EE) and non-enriched conditions and handling protocols were as described for the experiment in Chapter 7 (section 7.1.1). Two days after light/dark box testing, each rat that had been tested in the reference memory task was then tested in a working memory task in the MWM; see Figure 7.6 for a schematic experimental timeline.

The MWM apparatus

As described in Chapter 2 (section 2.2.1).

Figure 7.6: A schematic of the experimental timeline. On day one (d1) the rats received their first probe trial (P1) and then were tested in a reference memory task (RM task; grey shaded box) for 22 days before receiving probe two (P2). Light/dark box testing (L/D) took place 11 days after Probe Two, and two days later the rats were re-tested in the MWM in a working memory task for 16 consecutive days (WM task; white box).

Working memory protocol

The platform location was pseudo-randomly determined so that it was never located in the same place for two consecutive days (four possible locations).

For each swim the rat was gently lowered into the water and released facing the side of the tank. A swim started when the rat was released and finished when the rat found and subsequently climbed onto the platform. The time taken by the rat to find the platform was recorded to the nearest second. If a rat failed to find the platform within 120 s it was gently guided to, and allowed to climb onto, the platform. Once on the platform the rat was left for 20 s before being picked up and released from one of the other three possible release points. Each rat received four swims per day, after the final swim the rat was left on the platform for 20 s and then gently removed, towel dried, put back in its home cage and placed under a heat lamp for approximately 10 minutes to dry. Testing occurred for 16 consecutive days. Thigmotaxis was measured in Swims One to Four; the protocol was as described in Chapter 7.

Data analyses

Repeated-measures data were analysed using a Repeated-Measures-Analysis-of-Variance (RM ANOVA): *between-subject* factors were sex (male and female) and

housing condition (enriched, EE and non-enriched, NE) and *within-subject* factors were swim number (One to Four) and day (One to 16). All of these factors were included in the analyses, and interactions between main effects that were not significant were removed. The assumptions of sphericity were tested using the Mauchly-Criterion test and where appropriate the Greenhouse-Geisser correction terms were used. Additionally, the assumptions of normality of residuals and homogeneity of variance were tested and appropriate transformations applied to the data, where necessary. Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$) was used to perform post-hoc comparisons.

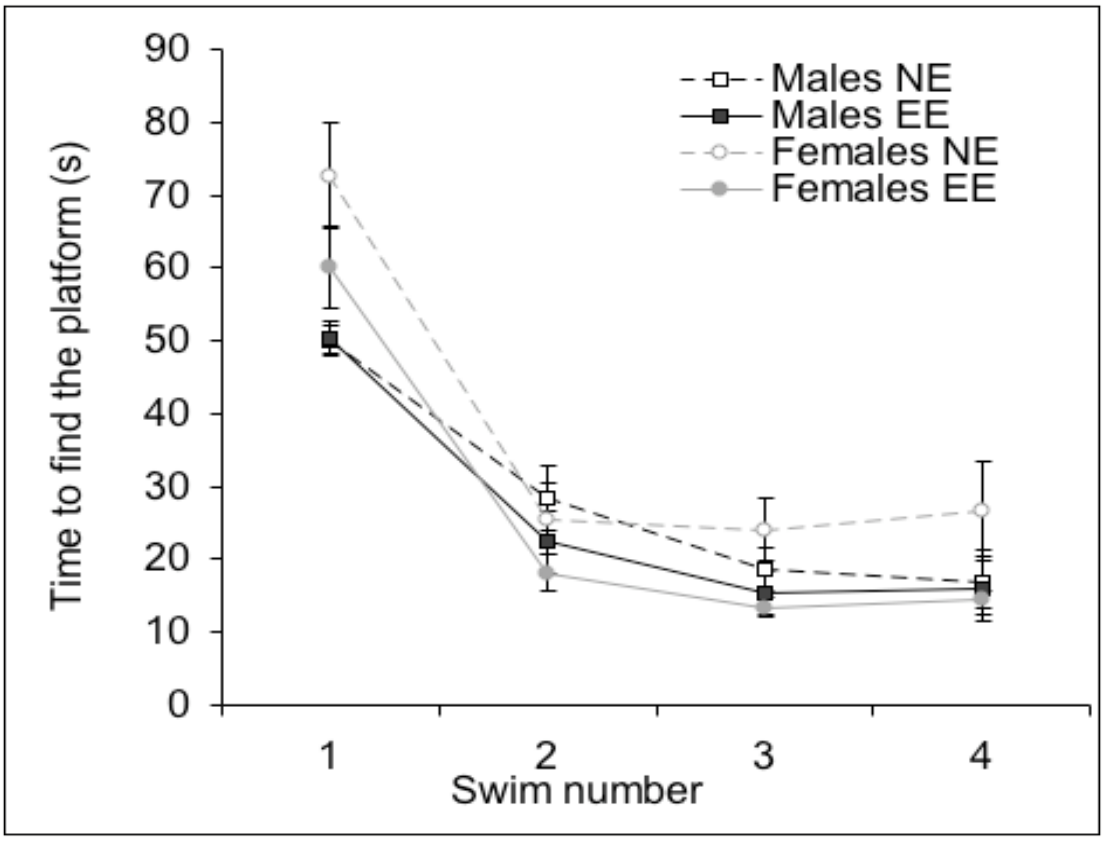
To determine if prior MWM experience affected stress levels, thigmotaxis in Swim One was averaged across the 16 days of testing for the NE rats (the EE rats did not have an appropriate comparison group and were not compared across the experiments) and compared with the corresponding data from rats that had not completed a reference memory task i.e. NE rats in Chapter 5 (Experiment two). In the experiment in Chapter 5, six rats of each sex were housed in isolation (in similar cages dimensions: 45x28x20 cm high) for ten weeks before being tested in a working memory task. The data were compared using a two-way ANOVA: factors were sex (male and female) and prior experience of the MWM ('prior experience' and 'no prior experience').

7.2.2. Results

Working memory

NE rats tended to take longer than EE rats to find the platform ($F_{1,21}$= 4.21, P = 0.059; Figure 7.7) but this difference was not dependent on the swim number ($F_{1.7,35.1}$= 0.04, P = 0.93). All of the rats found the platform sooner across the four daily swims and there was an improvement in performance (i.e. a decrease in latency), across the 16 days of testing (swim number: $F_{1.7,35.1}$= 171.62, P < 0.0001; day: $F_{15,315}$= 4.79, P < 0.0001). No other interactions with housing were significant.

Figure 7.7: Mean time taken to find the platform in Swims One to Four (± SE) for male and female rats that were either enriched (EE) or non-enriched (NE). Swim times are averages across the 16 days of testing. Analyses were conducted on daily swim data, N = 6 per treatment group.



The sexes did not differ in the time they took to find the platform ($F_{1,21}$= 2.03, P = 0.168; Figure 7.7). However, the males found the platform sooner than the females in Swim One (sex-by-swim number interaction: $F_{1.7,35.1}$= 7.74, P = 0.0027; Tukey

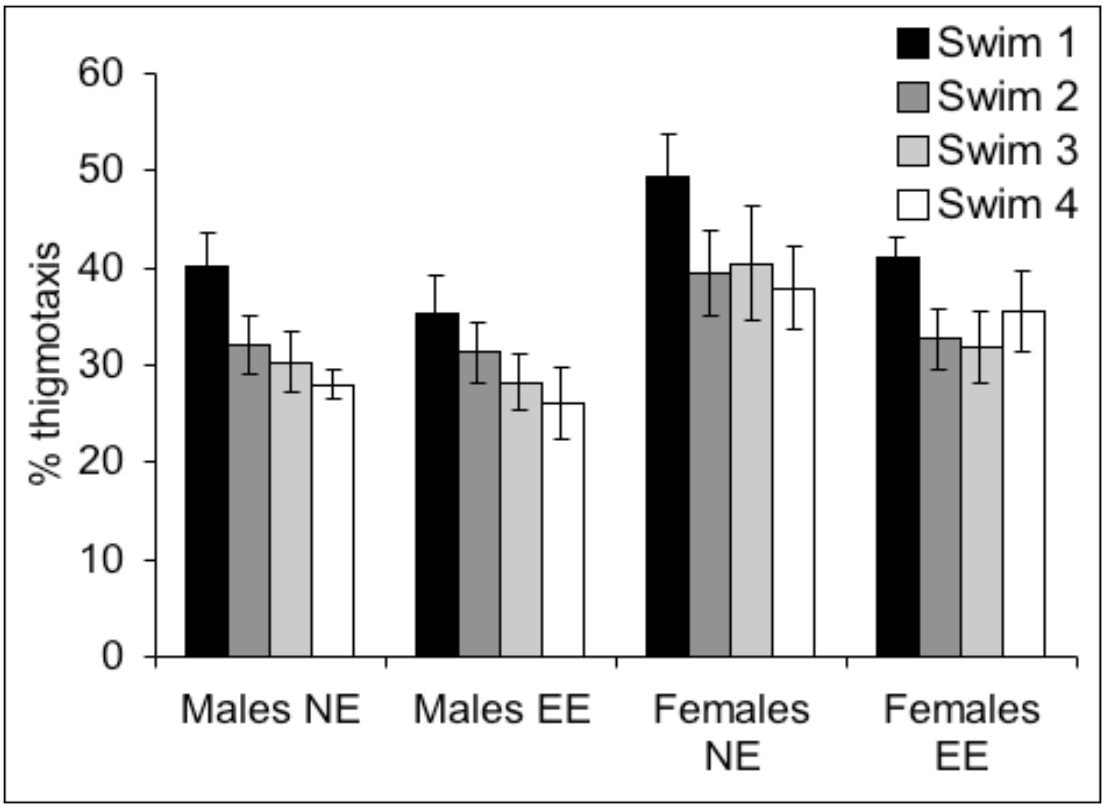
HSD, P < 0.05). The sex-by-housing interaction was not significant and was removed from the final model. No other interactions with sex were significant.

Thigmotaxis

The housing conditions had no impact on thigmotaxis: the NE and EE rats were equally thigmotactic in all four swims (housing: $F_{1,21}$= 1.97, P = 0.17; swim-by-housing interaction: $F_{3,63}$= 0.98, P = 0.41; the sex-by-housing interaction was removed from the final model).

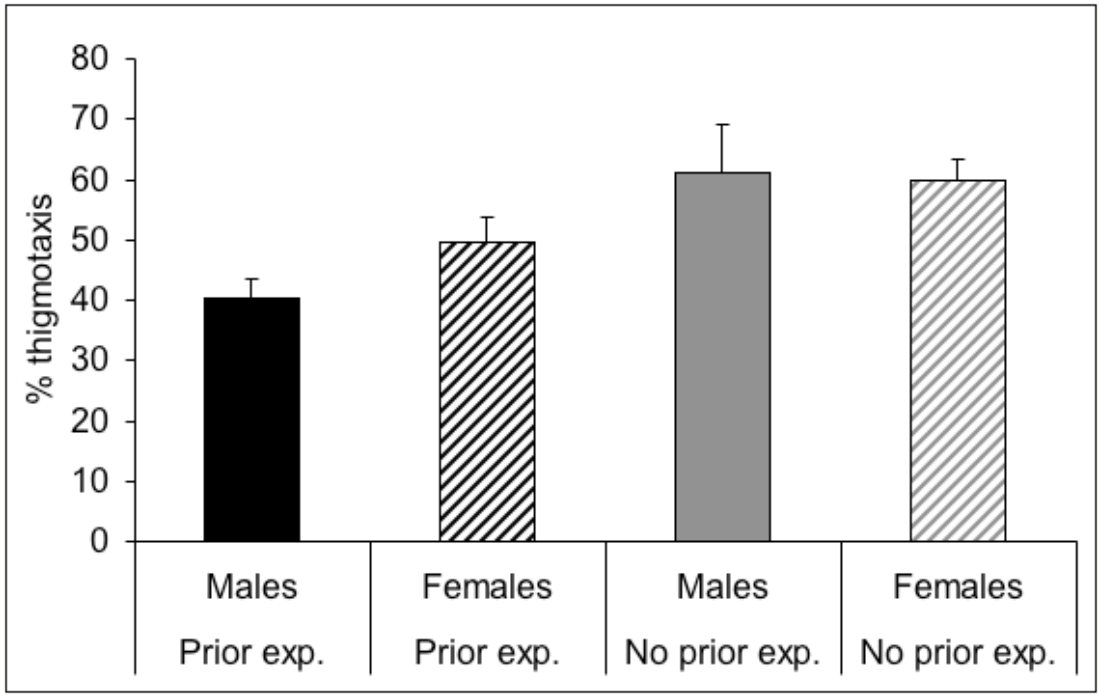
Females were significantly more thigmotactic than the males in all four swims (sex: $F_{1,21}$= 5.05, P = 0.035; sex-by-swim interaction: $F_{3,63}$= 1.26, P = 0.30). Thigmotaxis decreased significantly across the swims (swim: $F_{3,63}$= 19.98, P < 0.0001; Figure 7.8). Thigmotaxis did not change significantly across the days (day: $F_{6.4,134.1}$= 1.59, P = 0.15). No other interactions were significant.

Figure 7.8: Mean proportion of Swim One to Four (±SE) spent in thigmotaxis across the 16 days of testing for enriched (EE) and non-enriched (NE) rats. Analyses were conducted on daily data, N = 6 per treatment.



The rats without prior experience of the MWM (NE rats in Chapter 5, and NE rats in this experiment isolated in barren cages) were significantly more thigmotactic in Swim One than the rats in this experiment which were also isolated in barren cages but had completed a referenced memory task before testing (ANOVA: $F_{1,21} = 9.06$, P = 0.0067; mean (±SE) for prior experience rats 44.8 ±2.9% and for no prior MWM experience rats 60.4 ±4.2%; Figure 7.9). The sex-by-prior experience interaction term was not significant, thus, the effect of prior experience was the same for males and females.

Figure 7.9: Mean percentage thigmotaxis (±SE) across the 16 days of testing for NE males and females with or without prior MWM experience before testing, N = 6 per group.



7.2.3. Discussion

Enriched rats tended to find the platform sooner than did the NE rats although this effect was not significant. However, thigmotaxis levels did not differ between the two housing groups in any of the four swims. Additionally, the males took less time to reach the platform and were less thigmotactic than the females in Swim One only. I predicted that if the benefit of enrichment on cognitive performance was an indirect

one via the reduction of stress, MWM performance would differ between the groups only in Swim One and that thigmotaxis would explain that difference. If, on the other hand, the benefits were not related to stress, performance was expected to be better across all four swims. This latter prediction meets the data better than the former prediction.

Although this result is apparently consistent with evidence that EE rodents have superior spatial abilities relative to NE rodents in reference memory versions of the MWM (e.g. Mohammed et al. 1990; Falkenberg et al. 1992; Kempermann et al. 1997; Nilsson et al. 1999; Leggio et al. 2005), differences in performance of these same individuals in a reference memory task in Chapter 7 was, in fact, accounted for by variation in thigmotaxis. In the light of those reference memory data, the tendency for EE rats in this working memory experiment to outperform NE irrespective of thigmotaxis was, therefore, surprising. The EE rats in this working memory experiment seem to show better cognitive ability, which is consistent with the hypothesis that enrichment increases 'brain-power' (e.g. Rosenzweig and Bennett 1996; van Praag et al. 2000).

I would prefer to be cautious over the interpretation of these data, not least because the significance level is not compelling. Additionally, the rats tested in this experiment had prior experience of the MWM because they completed a reference memory task two weeks earlier. It is possible that prior experience of the MWM (procedure and the surrounding room) led to a similar degree of habituation in all animals to the test conditions, which resulted in similar levels of thigmotaxis across the housing groups. This seems likely as the NE rats from this experiment were significantly less thigmotactic than were the NE rats described in Chapter 5 (Experiment 2), which were of the same strain, age at purchase and housed under similar conditions to the rats in the current experiment. While NE rats in this experiment were, admittedly, five weeks older than those tested in Chapter 5, age at testing does not seem have a major impact on thigmotaxis in the MWM (Chapter 2).

Further experiments are needed to determine if 1) EE rats really do have better working memory abilities in the MWM relative to NE rats regardless of prior experience, 2) if prior MWM experience has a greater effect on NE rats and therefore removes differences in stress levels between NE and EE rats 3) if the type of prior

experience is important, for example, if rats only received experience of getting wet and being in a bright room rather than actually completing a spatial task, it is possible that no cognitive differences will be seen, if for example, differences are a result of performing in a spatial task.

Although prior MWM experience seems to have removed differences in thigmotaxis that were present between the housing groups in Chapter 7, it was not sufficient to remove sex differences in stress in Swim One. Prior MWM experience tends to remove sex differences in reference memory tests (Bucci et al. 1995; Perrot-Sinal et al. 1996; Beiko et al. 2004; Blokland et al. 2006), however, all of these authors tested Long-Evans or Lister Hooded rats (dark-eyed strains) and I used an albino strain. In the experiments described in Chapter 5, I found that on the last day of testing, Wistar (albino) females remain thigmotactic at end of testing despite having completed 15 days of testing (Figure 5.2.b). Therefore, data in this working memory experiment concurs with the data in Chapter 5, and shows prior experience does not necessarily reduce stress levels during testing in Wistar females. It is possible that this is because albino strains are more 'emotional/anxious', as demonstrated in anxiety tests (Schmitt and Hiemke 1998; Ennaceur et al. 2005). Further experiments are needed to ascertain if sex differences are present in stress responses after prior experience in other commonly used strains of albino rat (e.g. Sprague Dawley).

In summary, there was a tendency for EE rats to outperform NE rats during Swims Two to Four irrespective of similar thigmotaxis levels. Although this supports the suggestion that the groups differ in cognitive ability, I do not find this result compelling largely because of the unconvincing significance level, but also in part, because superior performance in these same rats in Chapter 7 was explained by thigmotaxis. Additionally, since prior MWM experience confounds this result, it is unclear if the same result would be found in rats with no prior MWM experience.

In the next Chapter I investigate if animals exposed to enriched environments continue to have reduced stress responses and improved cognition after a period of withdrawal from the enriched environment.

CHAPTER EIGHT: Does withdrawal of enrichment impair spatial cognition in rats?

The home cage behavioural data in this chapter were collected with the assistance of Andy Ladle.

Summary

Environmental enrichment reduces stress and frustration levels in laboratory rodents both in the home cage and in unfamiliar and stressful situations. However, provision of enrichment is sometimes followed by withdrawal of that provision, which may increase stress both in the home cage and in acutely stressful situations. As cognitive tests are typically stressful, an increased stress response would not only impair welfare during testing but may also impair cognitive performance. In this experiment I tested whether removing an animal from an enriched environment was sufficiently stressful to impact on cognitive performance. I found that, in comparison with rats that remained housed in social groups, rats that were removed from social housing and into isolation housing (and housed with or without physical enrichment) performed equally well in a Morris water maze (MWM) test and had similar levels of thigmotaxis (stress-related wall-hugging) during testing. Additionally, behaviour in the home cage and in the light/dark box (a standard anxiety test) did not differ significantly amongst the groups. In conclusion, removal of social and physical enrichment for one week does not necessarily cause sufficient stress to impact on cognitive performance in male rats.

8.1. Introduction

'Environmental enrichment', in the form of physical objects and cage mates, provides caged laboratory rodents with the opportunity to carry out species-specific behaviours that rodents are highly motivated to perform (Patterson-Kane 2001, 2004; Sørensen et al. 2004; Balcombe 2006). On the supposition that freedom to perform these behaviours reduces stress and improves welfare in caged rodents, the U.K. Home Office recommends that, where possible, social and physical enrichment is added to the home cages of rodents used in scientific research (Dawkins 1988; Home Office 1989, 1995; Würbel 2001; Olsson and Dahlborn 2002; Smith and Corrow 2005).

In addition to improving welfare by reducing stress in the home cage, environmental enrichment may also improve welfare by reducing the acute stress responses of enriched animals to novelty and acutely stressful situations outside the home cage. For example, in comparison with 'impoverished' rodents (typically isolated in barren cages), enriched rodents have reduced hormonal (corticosterone, CORT) and physiological (heart rate, blood pressure) stress responses to handling and saline injection, are more active at first but habituate sooner in unfamiliar arenas/'open-fields' and have reduced CORT responses to cat-odour exposure (Roy et al. 2001; Zimmermann et al. 2001; Larsson et al. 2002; Sharp et al. 2002a; Moncek et al. 2004; Meijer et al. 2006). Enriched animals also show reduced stress behaviour in standard anxiety tests. For example, they spend more time in the open arms of an elevated-plus-maze and feed sooner in a novel arena after food deprivation (Chapillon et al. 1999; Hellemans et al. 2004; Meshi et al. 2006). As most cognitive and behavioural tests as well as routine experimental procedures (e.g. weighing, taking blood) are stressful, novel or involve handling, enrichment has the potential, therefore, to improve welfare during all of these procedures by reducing the stress response of the animal.

The benefits to enrichment provision seem clear but there are few data, if any, on the relevant welfare benefits of the provision of physical enrichment versus social enrichment. Provision of physical enrichment (e.g. nest boxes and chewable items) to isolated rats can reduce both basal stress hormone levels (CORT and adrenocorticotrophic hormone) and stress hormone responses to saline injection

relative to isolated rats in barren cages (Belz et al. 2003). Thus, it is possible that provision of physical enrichment may be sufficient for good welfare and unimpaired cognitive ability and that isolation itself need not be the primary concern when considering housing conditions of laboratory rats.

Despite the benefits of enrichment, provision of enrichment may be coupled with removal of the animal from the enriched environment on occasion, sometimes permanently. For example, isolation housing with or without cage-furniture may occur post-surgery, if cage mates die (either through old age or because of experimental procedures) or after transition from a breeding facility to an experimental facility where housing conditions differ as a requirement of the experiment (examples of studies in which isolation housing was essential are Belz et al. 2003; Brillaud et al. 2005). Removal from an enriched environment is potentially stressful, and indeed there are data that correlate removal of enrichment with increases in stress/anxiety both in the home cage and in acutely stressful situations. For example, compared to rats that remain enriched, rats that have physical enrichment removed from their home cage respond with 'less optimistic-like judgement' to ambiguous stimuli (Burman et al. 2008b). Rats that have had physical and social enrichment removed also spend less time in the open-arms and make fewer open arm entries in an elevated plus maze than do permanently enriched rats (Hellemans et al. 2004). Additionally, insertion of a barrier between paired cage mates led to a significant increase in faecal corticosterone levels even though the barrier allowed some degree of tactile communication between the rats (Boggiano et al. (2008).

The magnitude of an animal's stress response not only impacts on its welfare but may also impact on the animal's performance in cognitive testing. This is because stress can directly affect performance by impairing cognitive functioning (e.g. consolidation or retrieval of memories) or indirectly by increasing behavioural responses that impede performance in tasks (e.g. thigmotaxis impairs Morris water maze performance Snihur et al. 2008). Consequently, it is plausible that removal from an enriched environment causes sufficient stress to impact negatively on performance in cognitive testing.

The following experiment had two aims: 1) to determine whether removal of social and physical enrichment, after an extended period of exposure to both, would have a significant impact on cognitive performance in an MWM test, and 2) to determine if removal of social enrichment (i.e. rats remain physically enriched) would have less impact on cognition than removal of both physical and social enrichment. I predicted that control animals, continually housed with both social and physical enrichment, would perform the best and animals from whose housing all enrichment was removed would perform the most poorly in the MWM. The group left with only physical enrichment was expected to perform at intermediate levels. I measured thigmotaxis levels during MWM testing to determine if any apparent cognitive differences amongst the groups were due to stress. I predicted that changes in cognitive performance would be explained by thigmotaxis (stress response). Stress levels in a non-cognitive context were assessed using a light/dark box and behaviour in the home cage was monitored for signs of stress/distress in the home cage (e.g. bar biting). These behaviours were expected to increase after removal from enriched housing.

8.2. Materials and methods

Subjects and housing

The subjects tested in this experiment were 30 male Wistar rats, aged between four and five weeks, obtained from Harlan U.K. Ltd. On arrival, the rats weighed 113±5 g. The rats were housed in groups of five in plastic-bottomed cages (56x38 and 20 cm high; North Kent Plastic cages Ltd.). Each cage was provided with a 2 cm layer of woodchip bedding, tissue-paper nesting material (Paper Wool, DBM Scotland), a red plastic tunnel (Rat retreat tunnel, size 9 x15.3 cm, DBM Scotland) on the floor of the cage and an opaque plastic tube (29 x 10.5 cm) suspended from the cage top with chains. Various novel objects (newspaper strips, small cardboard tubes and boxes, wood blocks, empty yogurt pots and so on) were provided and were changed every three to four days, or as needed. All rats had access to *ad libitum* pelleted food (RM3 diet, Special Diet Services, Ltd., Witham, Essex, U.K.) and tap water and were maintained under a 12:12 hour light: dark cycle (lights on at 0700 hours) at 21-24ºC.

All the cages were cleaned out and each rat was weighed once per week. For identification purposes all of the rats were marked with hair dye (Schwarzkopf, R43). Marking took place at least one week before any data were collected (in case the dye or marking procedure affected behaviour).

Dominance hierarchies were observed three and four weeks post arrival. To do this, each cage of five rats was observed for 15 minutes between 1400 and 1700 hours and the number of pinning incidences was recorded along with which animal did the pinning, and who the recipient was. Consistent with previous research (e.g. Adams et al. 1989a), a stable hierarchy was not apparent in any of the cages (all rats seemed to pin or be pinned in equal proportions across the observation periods). Thus, rats were allocated at random to the different housing conditions in the next stage of the experiment.

The next stage of the experiment began at six weeks post arrival (when the rats were 77 days of age; see Figure 8.1): one rat from each cage was re-housed alone in a cage (all of the cages were 56x38x20 cm; North Kent Plastic cages Ltd.) provided with a 2 cm layer of woodchip bedding but without any environmental enrichment (barren/isolated; N = 6 cage replicates); a second rat from each cage was re-housed alone in a cage with the same physical enrichments as the enriched group (enriched/isolated; N = 6). The remaining three rats were placed together in a clean cage with physical enrichments (enriched; N = 6). The rats remained in these new housing conditions for the rest of the experiment (Figure 8.1). One week after the change in housing conditions, each of the re-housed rats and one rat from each cage of the socially housed rats (chosen at random) was then tested in the MWM (see Figure 8.1).

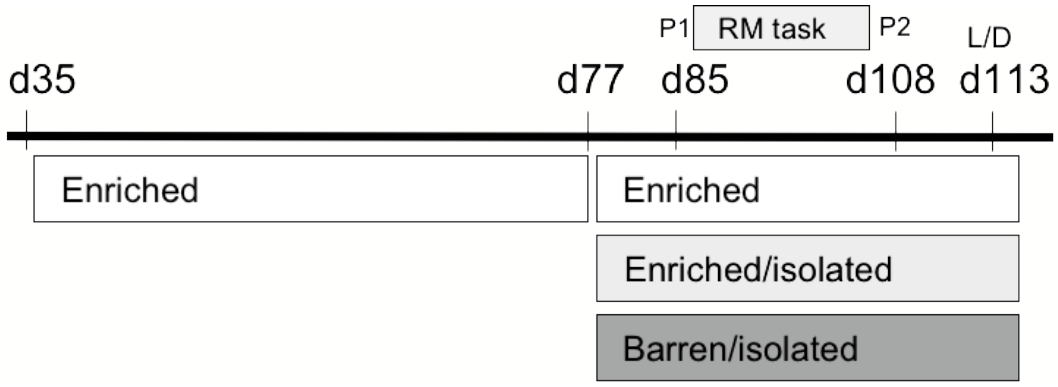


Figure 8.1: Schematic of experimental time line. The rats were housed with physical and social enrichment (five rats per cage) from 35 days of age for 42 days. At 77 days of age (77d) one rat from each of the socially and physically enriched cages (N = 6) was re-housed with physical enrichment in isolation, and one rat (N = 6) was re-housed in a barren cage in isolation. The remaining rats from the socially and physically enriched cages remained with their cage mates but were re-housed in a clean cage with physical enrichment (three rats per cage; N = 6). After one week of these new housing conditions one rat from each cage was tested in Probe One (P1) and then had 21 days of reference memory testing (RM task) followed by Probe Two (P2). All of the rats that were tested in the MWM were then tested in a light/dark box (L/D) four days after Probe Two.

Morris water maze apparatus

The MWM is described in detail in Chapter 2, section 2.2.1. Briefly, the MWM was a circular pool (2 cm diameter, 65 cm deep) filled with tap water (24±1ºC). Non-toxic paint was added to make the water opaque. An escape platform (white PVC of diameter 11 cm) was located in the centre of one of four imaginary quadrants (the four main compass points N, E, S or W) approximately 2 cm below the surface of the water and 30 cm from the edge of the tank. All trials were videotaped from above using a camera with a 4 mm wide-angle lens, which allowed observation via a video monitor once the rat was placed in the water.

Morris water maze procedure

The MWM procedure is described in detail in Chapter 7, section 7.1.1. Briefly, the first swim trial each rat received was a probe trial (Probe One), during which the platform was removed, to assess how much time the rats spent in the each quadrant prior to training. Each rat was gently placed in the water at the edge of the tank (in a predetermined release point) facing the side of the tank and allowed to swim for 60 seconds (s). After 60 s the rat was lifted out of the tank, towel dried and placed into its home cage under a heat lamp to dry. The percentage of time spent that each rat spent in each of the pre-determined quadrants (north, east, south and west) was determined from the video footage. A rat swimming at random should spend approximately 25% of the probe trial (i.e.15 s) in each quadrant. All swims took place between 1100 hours and 1500 hours.

Testing began the day after Probe One was carried out. The procedure was similar to that for Probe One, with two exceptions. First, an escape platform was located in the north or the west quadrant (but for each rat the platform remained in the same location across the days). Second, the time taken for the rat to find and subsequently climb onto the platform was recorded as the measure of performance. Rats that failed to find the platform within 120 s were gently guided to, and allowed to climb onto, the platform where they were left for 20 s before being removed. Rats had one swim per day for 21 consecutive days.

Probe Two: On Day 23, each rat received a second probe trial (Probe Two), during which the platform was removed. The procedure for Probe Two followed that for Probe One. Rats that had learnt the location of the platform were expected to spend more than 25% of the probe trial (i.e. 15 s) in the quadrant where the platform had been during training.

Thigmotaxis

The procedure followed that described in Chapter 7, section 7.1.1. Briefly, the amount of time a rat spent swimming within 15 cm of the wall of the maze was

recorded manually using an acetate sheet attached to a TV monitor. Thigmotaxis was recorded during both probes and throughout training.

Behaviour in the home-cage

Every night from days 77 to 95 (see Figure 8.1) animals in different cages were filmed between the hours of 1900-2000 hours and 0500-0600 hours (this is when rats are most active; e.g. Hurst et al. 1997). A 4 mm-wide angle lens black and white camera was used and a 40W red light bulb provided illumination. The cages were filmed in a predetermined order, blocking for housing condition. Behaviour was analysed from six clips, each two minutes long (randomly selected from the footage for each cage; three minutes from the first hour, and three from the second hour of footage) totalling 12 minutes per cage. For analysis, a rat was randomly pre-selected from each cage and was the only source of data from each cage. The percentage of the 12 minutes that the rats spent performing various behaviours (see Table 8.1) was calculated using computer software (The Observer, version 5, Noldus Information Technology, The Netherlands). Biting and investigating the bars, and digging were analysed because increases in these behaviours are associated with impaired welfare (e.g. Hurst et al. 1997). The amount of time spent interacting with the cage furniture and grooming were analysed to determine if removal of social enrichment increased the proportion of time that a rat spent with the enrichment objects or performing 'self maintenance' behaviours.

Light/dark box

To determine stress levels in a novel, non-cognitive situation, four days after Probe Two, rats that had swum in the MWM were tested once in a light/dark box. The light/dark box is described in Chapter 7, section 7.7.1. Briefly, each rat was placed in the middle of a dark compartment facing away from an opening to a light compartment. The rat's behaviour was monitored for five minutes. The latency for each rat to fully cross over from the dark side and into the light compartment was recorded to the nearest second. Rats were tested in a pre-determined random order. The box was cleaned between rats (faeces removed; box surfaces wiped with warm

soapy water and sprayed with 70% ethanol). Testing took place between 1000 and 1700hours.

Table 8.1: Ethogram of the behaviours that were analysed using the Noldus Observer behavioural coding computer program.

Behaviour	Description
Grooming	Scratching, licking, nosing with paw or mouth own or cage-mate's coat
Interacting with 'cage furniture'	In, on, chewing or sniffing physical enrichment objects
Digging	Moving sawdust (forwards or backwards) with forepaws
Consuming	Eating or drinking
Investigating the bars	Sniffing, hanging from, or touching the bars at the side or top of the cage with forepaws or nose
Bar-biting	Biting the cage bars

Data analyses

Repeated-measures data were analysed using a One-Way-Repeated-Measures-Analysis-of-Variance (RM ANOVA): the between-subject factor was Housing Condition (with three levels), the within-subject factor was Day (One to 21; Figures show performance averaged over two day blocks, except for block 11, which is performance on day 21) or, in the case of probe trials, Probes One and Two. All of these factors were included in the analyses and interactions between main effects that were not significant were removed. The assumptions of sphericity (that differences between all repeated measures have equal variances and that the correlations

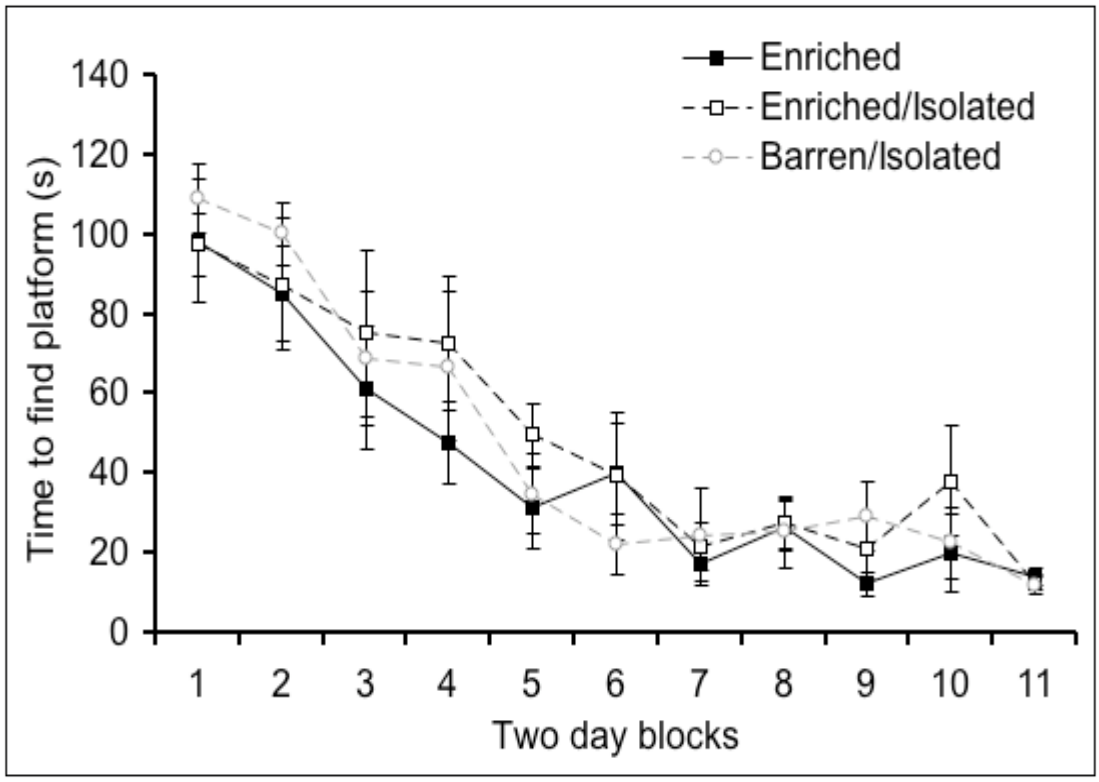
between any two measures are the same) were tested using the Mauchly-Criterion test, and where necessary, corrections were made using the Greenhouse-Geisser terms. Transformations were carried out where appropriate (i.e. if data violated the assumptions of normality and homogeneity of variance). The light/dark box data were normally distributed, but had unequal variances and so were analysed using the Welch ANOVA. Even after transformation, home cage behavioural data did not meet the assumptions of parametric testing and so were analysed using a non-parametric Kruskal-Wallis test.

8.3. Results

Reference memory and thigmotaxis

Housing conditions did not have a significant impact on the time taken to find the platform ($F_{2,15}$= 0.39, P = 0.68; Figure 8.2). Irrespective of housing conditions, the rats took less time to reach the platform as testing progressed (day: $F_{7.1,106.5}$= 17.27, P < 0.0001; housing-by-day interaction: $F_{14.2,106.5}$= 0.54, P = 0.83).

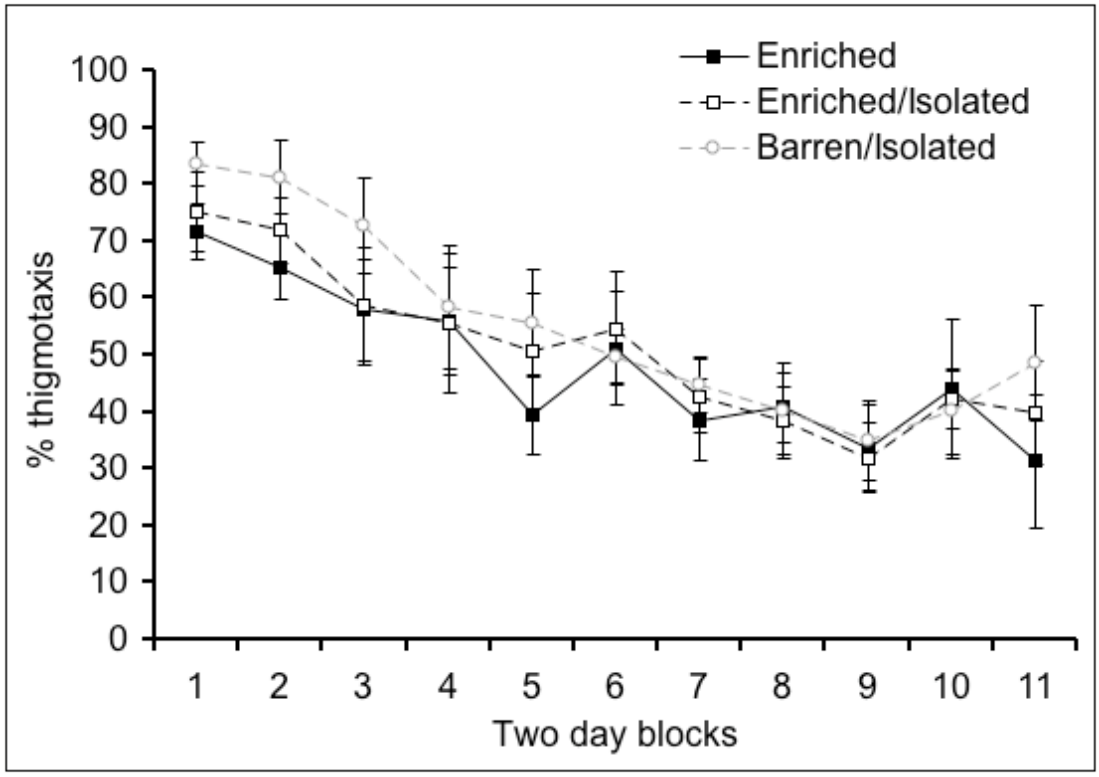
Figure 8.2: Mean time (s) to find the platform (±SE) for rats that were enriched, enriched and isolated or barren housed and isolated for one week. Prior to these housing conditions, all rats were socially housed with physical enrichment for six weeks. Each block is an average of two daily trials, analyses were conducted on daily data, N = 6 per group.



Thigmotaxis

Thigmotaxis levels did not differ significantly amongst the housing treatment groups ($F_{2,15}$= 0.29, P = 0.76; Figure 8.3) and, irrespective of housing conditions, thigmotaxis declined over the 21 days of testing (day: $F_{7.3,109.8}$= 13.3, P < 0.0001; housing-by-day interaction: $F_{14.6,109.8}$= 0.59, P = 0.88).

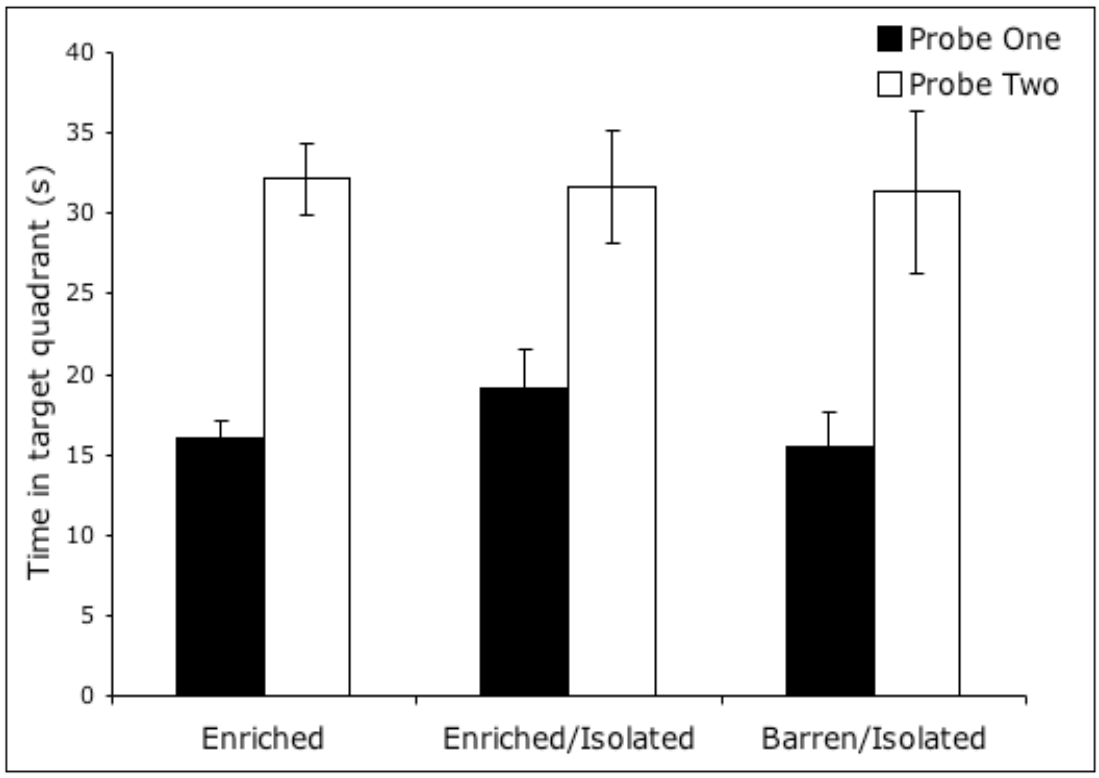
Figure 8.3: Mean percentage of each swim spent in thigmotaxis (±SE) for rats that were enriched, enriched and isolated or barren-housed and isolated for one week. Prior to these housing conditions, all rats were socially housed with physical enrichment for six weeks. Each block is an average of two daily trials, analyses were conducted on daily data, N = 6 per group.



Probes One and Two

In Probe One none of the rats spent a significantly different amount of time than would be expected by chance (two-tailed one-sample t-test against the expectation that a rat swimming at random would spend 15/60 s in the target quadrant: t_{17}= 1.66, P = 0.12; Figure 8.4). However, in Probe Two after 21 days of testing the rats showed a significant preference for the target quadrant (two-tailed one-sample t-test: t_{17}= 8.19, P < 0.0001; Figure 8.4). The housing conditions of the rats did not vary significantly in their impact on the amount of time spent in the target quadrant in either probe trial (RM ANOVA; $F_{2,15}$= 0.21, P = 0.82). All of the rats, irrespective of housing conditions, spent significantly longer in the target quadrant in Probe Two than in Probe One (probe: $F_{1,15}$= 41.28, P < 0.0001; probe-by-housing interaction: $F_{2,15}$= 0.26, P = 0.77; Figure 8.4).

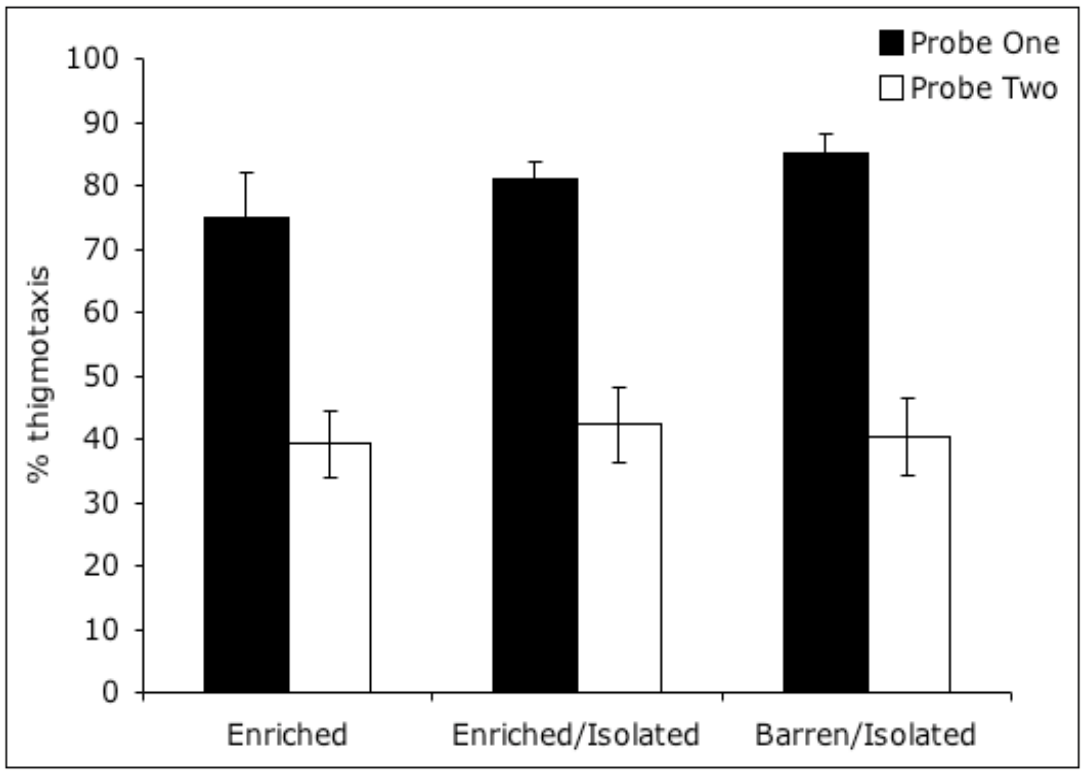
Figure 8.4: Mean time (s) in target quadrant (±SE) in Probe One (black) and Probe Two (white) for rats that were enriched, enriched and isolated or barren-housed and isolated, N= 6 per group.



Thigmotaxis in Probe One and Two

Thigmotaxis levels did not differ significantly amongst the housing treatment groups in either of the probe trials ($F_{2,15}$= 0.52, P = 0.60; Figure 8.5). Regardless of housing conditions, the duration of thigmotaxis was significantly longer in Probe One than in Probe Two ($F_{1,15}$= 104.97, P < 0.0001; housing-by probe interaction: $F_{2,15}$= 0.46, P = 0.64). The rats swam thigmotactically for almost twice as long in Probe One (mean ± SE: Probe One 80.3 ± 4%; Probe Two 40.6 ± 6%.).

Figure 8.5: Mean Percentage of time spent in thigmotaxis (±SE) in Probe One (black) and Probe Two (white) for rats that were enriched, enriched and isolated or barren-housed and isolated, N= 6 per group.



Behaviour in the home-cage

There were no significant differences amongst animals from the three different housing treatments in the percentage of time spent performing various behaviours; see Table 8.2. Most notable is the complete absence of bar-biting behaviour within the given observation period in all rats.

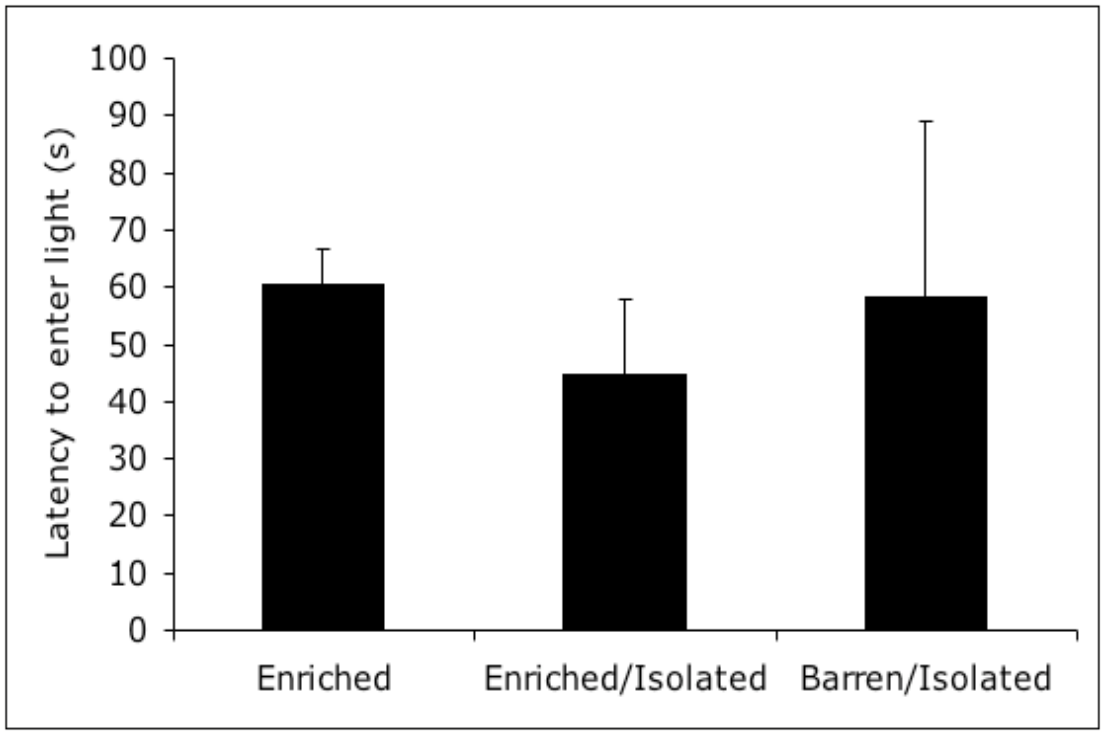
Table 8.2: The mean percentage (±SE) of time spent performing certain behaviours in the home cage for male rats that were enriched, enriched and isolated or barren-housed and isolated, N = 6 per group.

Behaviour	**Enriched**	**Enriched/isolated**	**Barren/isolated**	**Kruskall-Wallis test:**
Grooming	5.0 ±1.7	5.9 ±3.4	17.4 ±4.7	K = 4.15, df2, P > 0.05
Investigating objects	62.0 ±12	76.4 ±7	N/A	K = 0.77, df1, P > 0.05
Digging	3.6 ±3	1.6 ±1	0.7 ±0.5	K = 0.41, df2, P > 0.05
Consuming food/water	5.5 ±2	2.6 ±2	4.6 ±2	K = 1.9, df2, P > 0.05
Investigating bars	7.8 ±3	4.7 ±2	5.3 ±2	K = 0.37, df2, P > 0.05
Biting bars	0	0	0	N/A

Light/dark box

Housing conditions did not significantly influence the mean time that the rats took to leave the dark side and enter the light compartment during light/dark box testing (Welch ANOVA assuming unequal variances: $F_{2,8} = 0.54$, $P = 0.60$; Figure 8.6). The enriched rats took a mean of 60 ± 6 s, the enriched/isolated rats took 45 ± 13 s and the barren/isolated rats took 58 ± 30 s to enter the light compartment.

Figure 8.6: Mean (±SE) time (s) to enter the light compartment during light/dark box testing for male rats that were enriched, enriched and isolated or barren-housed and isolated, N= 6 per group.



8.4. Discussion

My first prediction was that removing a rat from social housing into isolation would lead to higher levels of thigmotaxis and, subsequently, to impaired performance in the MWM. This prediction was not met. Rats removed from social housing were as thigmotactic during testing and performed equally in the MWM as rats that remained socially housed.

My second prediction was that rats that had social enrichment removed while remaining physically enriched would perform better than rats that had all enrichment removed. This prediction was also not met: rats with all enrichment removed performed as well as did rats that had only social enrichment removed.

One possible explanation for these results is that animals in all treatment groups were stressed. For the control rats (the permanently enriched rats) this would be because they had some of their cage mates removed, which may have impaired the welfare of the rats that remained, even though they remained socially housed (e.g. Burman et al. 2008a). Burman et al. (2008a) investigated the impact of removing cage-mates on the welfare of the rats that remained behind. Thirteen rats were housed in a cage (six replicates), after one week, two rats were removed and after another week two more rats were removed. Burman et al. found that two hours after each of the removals the remaining rats (the 6 focal rats which were housed with at least nine other rats) spent significantly longer bar biting and aggressively grooming cage mates (i.e. pinning a cage mate on its back with forepaws while grooming it vigorously; Burman et al. 2008a). Additionally, audible vocalisations significantly increased and faecal corticosterone metabolite levels increased threefold in the rats that had had cage mates removed. One contrast between my experiment and the Burman et al. experiment, however, is that the effects of removal of cage mates in the Burman et al. experiment were recorded only two hours after removal when it is possible that the rats were still responding to the human intervention. I did not begin to record in-cage behaviour until at least 24 hours after I removed cage mates. This was specifically because I was concerned that there may be immediate but short-lived responses, not least because I also moved all animals into clean cages, a potentially disruptive and stressful procedure. By 24 hours, bar biting was not observed in any cages in my experiment. Additionally, comparison with previous

results (Chapter 7) does not reveal conspicuous differences in thigmotaxis or performance levels in the MWM. In the context of my experiment, at least, removal of social and/or physical enrichment had little discernible impact on behaviour in the home cage or in the MWM. However, it is possible that differences in behaviour existed amongst the differentially housed rats, but insufficient power (due to small sample sizes and short observation periods) reduced the likelihood of detecting significant differences in levels of behaviour in the home cage.

The lack of effect of removal in my experiment may be because the removal of enrichment itself was not stressful. Given that there are several studies that have shown that removal of enrichment is stressful (Hellemans et al. 2004; Burman et al. 2008b), this seems unlikely. However, there are details in both of these studies that leave that conclusion open. For example, removal of both social and physical enrichment in the Hellemans et al. experiment was coupled with re-housing the barren rats in much smaller cages. In the Burman et al. study, physical enrichment was removed from socially-housed rats, which might be stressful because the socially-housed rats were left with nowhere to hide.

A second possible explanation is that six weeks of enrichment (social and physical) is sufficient to provide a long-lasting beneficial impact on welfare and cognitive performance. However, this seems unlikely as Hellemans et al. (2004) enriched their animals for nine weeks and Burman et al. (2008b) enriched their animals for seven weeks and both subsequently found a negative impact on the rats after withdrawal. In contrast, in my experiment, I looked for effects of withdrawal after one week and they both waited at least 2.5 weeks before assessing the impact of withdrawal. Therefore, it may be that the effects of withdrawal depend on what is taken away (social and/or physical) and how long the withdrawal period is. If social or both physical and social enrichment are taken away and the cage size remains constant, then the benefits of enrichment appear to remain for at least one week. It is possible that removal of just physical enrichment, or both for longer, may have been more stressful for the rats. A more comprehensive experiment testing all these possibilities is required.

Finally, despite the vast body of literature regarding the neuroanatomical consequences of exposure to enrichment (e.g. Kempermann et al. 1997; Nilsson et al.

1999; Leggio et al. 2005) there are few data on the impact of enrichment withdrawal on the brain. Brain changes occur rapidly following exposure to enrichment, and are not dependent on continuous exposure. For example, exposure to enrichment for only two hours per day for 30 consecutive days is sufficient to result in significant increases in cerebral cortex weight and increases in the total activity of acetylcholinesterase and cholinesterase in the cortex of male rats (enzymes necessary for efficient neuron functioning because they enable neurones to return to resting states after firing; Rosenzweig et al. 1968). Upon withdrawal of enrichment there is some evidence that enrichment induced changes in overall brain weight and acetylcholinesterase and cholinesterase activity levels persist for longer after 80 days of exposure versus 30 days of exposure (Bennett et al. 1974). Additionally, Bennett et al. (1974) demonstrated that significant persistence of brain weight differences (between rats that were withdrawn from enrichment and rats that remained impoverished) were found 21 days after removal from the enriched environment and that significant persistence of differences in acetylcholinesterase and cholinesterase activity levels were found 47 days after withdrawal of enrichment. It is unclear, however, how long other changes in the brain that are specific to the hippocampus, (e.g. increased numbers of neurones, dendritic spine density etc) remain after withdrawal from the enriched environment. Nor is it clear if withdrawal of social or physical or both types of enrichment would have different effects on the brain. Based on my data, I would predict that brain changes in response to enrichment would persist for at least one week post removal if both or just social enrichment are withdrawn. To determine any longer lasting impact of enrichment on brain or behaviour, further studies are needed. For example, the impact of removing both or one type of enrichment on cognition, stress and the brain could be assessed after varying periods of withdrawal (different animals would be needed). Additionally, future studies are needed to determine whether cognitive and neural changes persist for longer after exposure to social and physical enrichment or whether physical enrichment is sufficient. There is some evidence that continuous provision of physical and social enrichment correlates with bigger effects (in terms of acetylcholinesterase and cholinesterase activities) than just social enrichment (Rosenzweig et al. 1978; Renner and Rosenzweig 1986). Additionally, there is some

evidence that, irrespective of social housing, provision of physical enrichment to male rats improves spatial learning and memory in an MWM (Scrijver et al. 2002, 2004).

In conclusion, my data have shed some light on the impact of removing social and physical enrichment on cognition: the benefits of social and physical enrichment appear to last at least one week after withdrawal in male albino rats.

CHAPTER NINE: General Discussion

Male rats typically outperform females in tests of spatial ability (Krasnoff and Weston 1976; Seymoure et al. 1996; Roof and Stein 1999; Lund and Lephart 2001; Beiko et al. 2004; Jones and Watson 2005; Levin et al. 2005; Gibbs and Johnson 2008; Saucier et al. 2008; Takase et al. 2008) but not always (Juraska et al. 1984; Bucci et al. 1995; Kolb and Cioe 1996; Perrot-Sinal et al. 1996; Healy et al. 1999; Lukoyanov et al. 1999; Blokland et al. 2006). The primary goal of the research described in this thesis was to investigate if stress could explain this inconsistency in the literature. To this end, I examined whether stress, which can be experienced inadvertently during testing or imposed in the home cage as a result of isolation housing, affected the spatial cognitive performance of male and female rats. I tested spatial cognition using a working memory protocol in an MWM, which is acutely stressful. And I attempted to manipulate chronic stress in the home cage by housing rats in isolation and control rats were housed in same-sex pairs. I measured stress levels behaviourally in the home cage (bar biting) and during cognitive testing (thigmotaxis). In the following discussion I will summarise my main findings, discuss their wider implications and highlight some suggestions for future work.

9.1. Summary of findings

- *Does isolation housing cause sufficient stress to cause sex differences in spatial cognition?* Irrespective of housing conditions, males outperformed females in three out of a total of six working memory MWM experiments (Chapters 2; 4 and 5) and in one reference memory MWM experiment (Chapter 7). Moreover, isolation housing did not impact on performance in either sex in all six MWM experiments or in the RAM experiment (Chapter 3).
- *Does isolation housing have a greater impact on young rats? And do longer periods of isolation have greater effects on performance than do shorter periods?* I found no discernible impact of age during exposure or duration of isolation on body weight, food intake or bar biting (measures of welfare) or spatial cognitive performance in an MWM (Chapters 2 and 5). However, stress levels (thigmotaxis) during MWM testing were increased if the rats were purchased from the commercial supplier at a younger age (4-5 weeks vs. 10 weeks; Chapters 2 and 5).
- *Does isolation or test stress have different effects on different strains of rat?* Isolation had no significant impact on MWM performance or bar biting, food intake and body weight in either Lister Hooded (a dark-eyed strain; Chapter 2) or Wistar rats (an albino strain; Chapter 5). However, the albino strain was more thigmotactic than the dark-eyed strain during MWM testing.
- *Does acute stress associated with the MWM impact on performance and are males and females affected differently?* Females were significantly more thigmotactic than were the males in five out of six MWM working memory experiments (Chapters 2; 4 and 5). In three of these five experiments the males outperformed the females. In the reference memory MWM task the males were significantly less thigmotactic than, and outperformed, the females (Chapter 7).

I also addressed the following questions, which were raised as my work progressed:

- *Is isolation more stressful if the rats cannot see each other/out into the room?* Rats housed with a barrier, which reduced the view from their home cage and removed visual contact with neighbouring rats, showed increased bar biting and food intake in the home cage, increased thigmotactic swimming during MWM testing and impaired cognitive performance relative to rats housed without a barrier between the cages. Paired housing, however, did not ameliorate the increases in bar biting or thigmotaxis that were seen in rats housed with a barrier between their cages. This was true for males and females (Chapter 4).
- *Does environmental enrichment enhance cognition or reduce stress during testing?* Environmental enrichment appeared to enhance reference memory MWM performance because it led to decreased thigmotaxis, rather than to increased spatial cognitive ability. This was true for males and females (Chapter 7).
- *Is it stressful to remove social enrichment? Can physical enrichment alone ameliorate stress caused by the removal of social enrichment?* Withdrawing physical and/or social enrichment for one week did not seem to cause sufficient stress to impact on reference memory MWM performance or behaviour in the home cage in male rats (Chapter 8).

9.2. The effect of isolation housing on spatial cognition and stress-related behaviour

The U.K. Home Office discourages isolation housing because it is reported to be chronically stressful for rats. It was, therefore, surprising to find little discernible impact of isolation on cognitive performance (as measured by the MWM), or for that matter, on any of the measures of welfare that I looked at in any of the six experiments. Strain, age and duration of exposure to isolation made no difference (Chapters 2 and 5). I conclude that isolation housing is not sufficiently stressful to contribute significantly to the presence or absence of sex differences in spatial ability in the literature.

One possible reason for finding that isolation was not a significant stressor for my rats is that the rats could see out of their home cages and into the cages of their neighbours when the cages were in the holding rack (i.e. the rats were not visually isolated). Indeed, removing visual contact, by means of a barrier between the cages, did lead to higher levels of bar biting in the home cage (a proxy for stress/frustration), higher thigmotaxis levels and impaired performance during MWM testing (Chapter 4). However, singly housed rats were not more stressed than were pair housed rats. It appears, then, that the presence of the barrier may have restricted the amount of visual contact the rats had with both their neighbours and the holding room in which they were housed which increased stress in the home cage and during testing. It is also possible that visual exposure to humans (which open-topped cages provide) habituates the rats to humans. A consequence of this would be that the MWM procedure is less stressful leading to better performance. Thus, paradoxically, in terms of welfare (i.e. reducing stress and the numbers of rats used) single housing in a cage with a view may be preferential to social housing in a cage with no view. However, the data that lead me to this suggestion come from one investigation with male and female Lister Hooded rats aged four-to-five weeks (Chapter 4). It is currently unclear if the same effect would be found in males and females of different strains or ages. For example, albino rats may find that open-topped cages do not provide enough shelter from laboratory holding room lights, which may be stressful or unpleasant for their light-sensitive eyes (Spencer et al. 1995), irrespective of whether they are housed with a cage mate or not ('closed-topped' cages have a much darker interior than 'open-topped' cages when the cage is in the holding rack). Additionally, it is not yet apparent what kind of view is necessary from an open-topped cage to reduce stress levels in rats. For example, it is unclear if a view of only one or two other cages would still reduce stress. Nor is it currently clear what is actually causing the greater levels of stress in animals housed with a barrier between the cages. Answers to these questions need to be in place before single housing in cages with a view is routinely recommended as 'best practice'

Although my data show that reduced visual input from outside the cage did not cause isolation to be stressful, it is possible that other housing and/or husbandry

protocols lead authors to conclude that isolation is stressful for a rat. For example, in some studies the effect of isolation on a rat's welfare is confounded with the use of cages with grid floors and/or minimal handling (e.g. cages cleaned less than once per week, if at all). Both of which are reported to increase stress, aggression and timidity during handling in rats (Holson et al. 1991; Manser et al. 1995; Sharp et al. 2003). Thus, husbandry protocols may interact with isolation to make it more stressful, or simply confound with the effects of isolation (making it difficult to determine what the main source of stress is). For example, authors who report significant differences between isolated and socially housed rats typically use grid flooring and/or minimal handling (Hatch et al. 1963; Baenninger 1967; Fiala et al. 1977; Holson et al. 1991; Hall et al. 1997; Hurst et al. 1997; Hurst et al. 1998; Heidbreder et al. 2000). Whereas authors who report no differences typically use smooth flooring and at least twice weekly handling (Morinan and Leonard 1980; Holson et al. 1988; Holson et al. 1991; Verwer et al. 2007; Harris et al. 2008a, 2008b). A comprehensive experiment that includes different types of flooring and handling regimes would help to determine if these husbandry protocols act in concert (or simply confound) with isolation to cause stress to laboratory rats.

There is one benefit to single housing that has not yet received much consideration in the literature. This is that it may lead, counterintuitively, to a significant reduction in the number of rats used in scientific research, in line with one of Russell and Burch's 3Rs (Reduction; Chapter 6). This is because only one animal per cage should be tested, or an average per cage generated so as to avoid pseudoreplication (Russell and Burch 1959; Hurlbert 1984; Festing et al. 2002). For example, if rats are pair housed, 12 rats are needed to make up a sample size of six (which means six rats may be 'wasted' for the sake of company). Furthermore, with group housing, rather than pair housing, the number of 'wasted' rats increases dramatically. Thus, experiments are needed to show whether single housing can be used, without detriment to the animals or the science, in other areas of research (i.e. not just in cognitive sex differences). If social housing is necessary in an experiment, then conclusive evidence is needed to show that group housing provides significantly greater welfare benefits than does pair housing.

Finally, isolated rats may retain social contact with neighbouring rats in ways that we are unaware of. For example, rats can emit both audible 'squeaks' that we can hear and vocalisations in the ultrasonic range that we cannot hear; these different vocalisations communicate both aversive and pleasurable experiences to conspecifics (Latham and Mason 2004; Burn 2008). Rats also rely heavily on olfaction for information about the environment and for communication with other rats (Otto et al. 2002). Additionally, my data show that rats value visual input from outside their home cage (Chapter 4). Thus, to assume that solitary housing deprives rats of social contact may be too anthropocentric. It is possible that the many noises, visions and scents that a rat encounters in its home cage (most of which we are unaware of) provide ample social contact.

Measuring stress behaviourally

Throughout my thesis I measured stress using behavioural non-invasive techniques. Stress can also be assessed physiologically. For example, telemetry can be used to monitor cardiovascular responses (heart rate, blood pressure) or basal levels of stress hormones can be determined from blood, urine/faeces or salivary samples. Some of these techniques, however, may require invasive sampling, which may alter the stress levels and/or the behaviour of the animals under test. Additionally, there may be time lag issues or time of day effects that cause variation in stress hormone levels (irrespective of housing conditions). Moreover, not enough is known about how chronic stress affects cardiovascular responses or the hypothalamic-pituitary-adrenal (HPA; this coordinates the stress response) axis e.g. prolonged stress exposure may lead to adaptation of the HPA axis. Additionally, it is unclear whether changes in daily means or secretory episodes are the important factor that determines how stressful an experience is. Thus, it can be misleading to use hormonal data alone to make claims about stress levels (and subsequently welfare). Alternative physiological measures of stress include organ weights (e.g. adrenal glands). Although organ weights are unlikely to be affected by stress associated with sampling, because the animals are dead, this also means that organ weights provide little insight into changes in stress levels over time e.g. over the course of MWM testing. Adrenal gland weight may also be an ineffective measure of chronic stress

because although adrenal glands were initially thought to increase in isolated animals (the adrenals secrete stress hormones and enlarge under conditions of stress; Hatch et al. 1963) there is mounting evidence that isolation does not affect adrenal gland weights (Stern et al. 1960; Morinan and Leonard 1980; Gamallo et al. 1986; Giralt and Amario 1989; Baldwin et al. 1995). Finally, it is unclear what differences in physiological measures of stress (e.g. hormone or organ weights) between isolated and pair-housed rats would mean in the absence of any behavioural differences.

9.3. Sex differences in spatial cognition and stress

I carried out a total of six MWM experiments using a working memory protocol and found in none of them that isolation housing caused or removed sex differences in cognitive performance. In three of the experiments females under performed relative to the males in the MWM. In these three experiments, however, the females were significantly more thigmotactic than were the males. Additionally, in a reference memory MWM task, the females were significantly more thigmotactic and under performed relative to the males, irrespective of housing conditions. Since a wealth of literature supports that thigmotaxis correlates positively with anxiety/stress in open-field like situations and in the MWM, I, like others, propose that females find the MWM more stressful than the males (Treit and Fundytus 1989; Simon et al. 1994; Perrot-Sinal et al.; Beiko et al. 2004; Herrero et al. 2006; Wilcoxon et al. 2007). It is simply because the platform is never located by the edge of the tank that the females take longer to locate it. Due to the effects of thigmotaxis on the time taken to reach the platform, performance in the MWM does not necessarily amount to cognitive ability.

It is also possible that stress-related behaviours impede performance, and that performance does not equal cognitive ability *per se,* in other tasks. For example, the RAM may be a stressful task (discussed in Chapter 3) and since stress can reduce appetite in rodents it is possible that during RAM testing a stressed rat will ignore food rewards or return to arms that have been emptied but are considered to be 'safe' (e.g. Shors and Dryver 1992). This is problematic if entries into these arms are recorded as working memory errors (discussed in Chapter 3). Additionally, it is unclear if stress affects appetite in males and in the same way or if feeding regimes

that restrict food intake (e.g. to 80% of free-feeding intake) have the same effect on males and females.

In sum, my data lead me to propose that stress is the causal factor for sex differences in spatial ability in laboratory rats. This has several implications. Firstly, the laboratory rat may not be an appropriate model with which to study evolutionary explanations for sex differences in spatial cognition because stress levels are relatively easy to manipulate or can inadvertently be affected relatively easily in a laboratory situation. Although I only tested two strains of rat, I deem this sufficient evidence to conclude that the rat is an inappropriate model for cognitive sex differences. Because even if sex differences in cognition are not caused by stress in other strains of rat, this implies that artificial selection (e.g. for different strain characteristics) has affected spatial cognition in ways that are unclear. Secondly, it is possible that stress is the causal factor for sex differences in spatial cognition in other mammals. This shows shortcomings in the various evolutionary hypotheses, which seek to explain the existence of a sex difference in cognition, because they lead only to predictions that males differ from females or not. Sex differences are found in other species of rodent (such as voles, deer mice and mice). Importantly all of these data come from testing in the laboratory, using a range of different mazes (e.g. MWM, water RAM, and the sunburst maze: Gaulin and Fitzgerald 1986; Galea et al. 1994a; Gresack and Frick 2003). The stress response of the rodents under test in these experiments has not yet been considered and so it is entirely plausible that a differential stress response could explain these data. Finally, another consequence of the finding that male and female rats differ in their stress responses (rather than cognition) is that the pharmaceutical and toxicology industry should be testing both sexes (rather than just males) because the data for males may not be representative of those for females.

Measuring stress in the water maze

During MWM testing I monitored thigmotaxis as a measure of acute stress. There is substantial evidence that thigmotaxis correlates positively with anxiety (e.g. Treit and Fundytus 1989; Beiko et al. 2004; Herrero et al. 2006). Additionally, measuring

thigmotaxis is non invasive and relatively easy. Indeed, there is mounting support that this behaviour is a useful indicator of stress during MWM testing (Wilcoxon et al. 2007; Bonsignore et al. 2008; Mendez et al. 2008; Santucci et al. 2008; Snihur et al. 2008). Since thigmotaxis confounds performance I propose that MWM data should not be presented/published without consideration of thigmotaxis. In addition to thigmotaxis anxiety levels of the animals under test could also be assessed using other tests (the light/dark box, elevated plus maze).

Evolutionary explanations for sex differences in stress

The 'range size hypothesis' is the currently favoured evolutionary explanation for why males have better spatial ability than do conspecific females (Jones et al. 2003). This hypothesis postulates that relatively larger territory size selects for superior spatial ability in males. The behavioural and neuroanatomical data that support this hypothesis come from experiments with voles. For example, male meadow voles (*Microtus pennsylvanicus*) have a larger home range, superior spatial ability and a larger hippocampus than do conspecific females (Gaulin and Fitzgerald 1986, 1989). Whereas the closely related pine vole (*Microtus pinetorum*) is not sexually dimorphic in home range size, spatial ability or size of the hippocampus (Jacobs et al. 1990). Although this all fits together, the range-size hypothesis is based on a two-sample comparison and the home-range of the rodents is confounded with breeding system. Pine voles are monogamous therefore males and females share a common territory with their mates, whereas meadow voles are polygynous and so males have a large territory that encompasses multiple female territories. Moreover, if sex differences in spatial cognition are due to differences in stress, the current focus on the evolution of variation in spatial cognition may be misdirected.

An alternative way to look at these sex differences is to consider whether or not there is a selection pressure for sex differences in stress response. One could speculate (as has been the case with most of these evolutionary hypotheses) that males have more to gain from risky behaviour than do females, and so natural selection has selected for males that cope well with stress. Ranging over a large home territory to mate with multiple females is risky because a male may encounter predators, aggressive conspecifics or get lost (mating with multiple females may also

be stressful). To determine if this is a plausible explanation for sex differences in stress and performance in cognitive tests, the stress responses of polygynous and monogamous rodents during cognitive testing or in anxiety tests are needed. In monogamous rodents I would predict there would be no sexual dimorphism in stress responsiveness, and in polygynous rodents I predict males would show reduced stress (or better learning under conditions of stress) relative to conspecific females.

Changing facility at a young age increases stress during testing

In my experiments, time spent in thigmotaxis during testing in the MWM was increased if the rats had travelled from the commercial breeder at a young age (four-to-five weeks). This effect was dependent on both the sex and the strain of the rats, such that male Lister Hooded rats that travelled as juveniles and male Wistar rats that travelled as adults outperformed the females that had travelled with them (Chapters 2 and 5). Thus, it would seem that age at travel is potentially a huge source of unwanted variation in the stress response of a rat that can affect cognitive performance. This may explain why sex differences in cognition are not consistently found in the literature.

Travelling while young may increase the stress response of a rat because travelling is a stressful experience (e.g. Tuli et al. 1995; Capdevila et al. 2007). It is unclear why travel should be more stressful for a young rat. Alternatively, changes in husbandry at a young age may be stressful. One example of a change that occurs between the commercial breeding establishment and a research facility is the type of food that rats receive. For example, Harlan U.K. Ltd provide a soft pellet food (name unknown), whereas the university of Edinburgh animal house (and most other institutes across the U.K.) provide their rats with the standard laboratory diet, which is a hard pellet food (RM3 diet, Special Diet Services, Ltd., Witham, Essex, U.K.). There is some evidence that females fed hard pellets have heavier adrenal glands than do females fed powdered pellets, so changing to hard pellets may be stressful (Endo et al. 1994). Furthermore, there is evidence that soft-diet feeding (i.e. powdered pellets) from three weeks until 16 weeks of age enhances female spatial cognition and that sex differences are not present if rats are fed a powdered diet from

six weeks of age (Endo et al. 1994; Takase et al. 2008). Thus, leaving Harlan early (i.e. 'travelling young') may impair female cognition because of changes in diet. Manipulation of travel experience independently of housing changes (and vice versa) would help to determine whether it is travel at a young age or changes in husbandry that cause stress for young laboratory rats.

Routine husbandry during early development at the commercial supplier (Harlan Ltd) may affect stress responsiveness and cognition in adulthood

I visited Harlan Ltd U.K. (Station Road, Bicester, Oxon, OX6 0TP; one of the main commercial suppliers of rodents for experimental research in the U.K.) to gain insight into the housing conditions that rodents experience before they reach a research laboratory. During my visit it became clear that Harlan's typical husbandry protocols include the cross-fostering of pups, housing on grid flooring and the use of a soft pellet diet. These husbandry protocols were of particular interest to me because they could be potential sources of stress leading to unwanted variation in a cognition experiment.

Cross-fostering of pups occurs because the consumer demand for male rats means that many females are destroyed soon after birth (< seven days old). Extensive cross-fostering of male pups is then employed to maintain the pups in same-sized litter groups of 12 (Gary Chambers, operations manager of Harlan Ltd. pers. comm.). This early manipulation of the litter means that: 1) the sex ratio of a litter is altered to become male biased, 2) mothers receive pups other than her own to nurse, and 3) pups are handled at a young age, albeit briefly. It is currently unclear whether replacing a mother's young with unfamiliar animals alters a mother's response to these new and her own offspring. Similarly, relatively little is known about how changing the sex ratio of a litter or handling the pups during early development affects mother-offspring interactions. These are important questions for cognitive researchers who purchase animals from commercial breeders, because there is considerable evidence that the amount of maternal care (licking and grooming) that a mother provides to a pup has long-term effects on the brain and

HPA axis of the pup during adulthood. For example, male rat pups that receive a high level of maternal licking and grooming during the first ten days of life show reduced corticosterone responses to acute stress, increased glucocorticoid receptor levels in the brain, increased synaptogenesis in the hippocampus (which underlies learning and memory) and enhanced spatial learning relative to pups that receive low levels of maternal attention (Liu et al. 1997; Liu et al. 2000). Furthermore, the sexes may be affected differently: unlike males, females that receive less attention from the mother have better spatial ability than females that receive more attention (Barha et al. 2007). Thus, if cross fostering alters mother-offspring interactions, this routine husbandry protocol may be a huge source of unwanted variation in data on sex differences in stress and cognition.

9.4. The effects of enrichment on cognition and stress

The stress response of rats housed with environmental enrichment was significantly reduced during MWM testing in my experiments relative to non-enriched rats (single housed in barren cages). This reduction in stress led the enriched animals to outperform the non-enriched animals during testing in the MWM. There is substantial evidence that enriched animals are cognitively superior to non-enriched animals (Cummins et al. 1973; Mohammed et al. 1990; Falkenberg et al. 1992; Moser et al. 1994; Kempermann et al. 1997; Kempermann et al. 1998; Harburger et al. 2007). However, in none of these experiments was the stress response of the animals under test taken into consideration. If enrichment induces superior performance solely because it reduces stress, I would predict that administration of anxiolytics, which reduce anxiety, would improve the performance of non-enriched rats but not the performance of enriched rats. Also, given the hypothesis that environmental enrichment habituates rats to novelty because it exposes rats to novelty, I would predict that enrichment would have a reduced effect on stress responses in the MWM if the enrichment objects are not renewed or changed frequently. Additionally, it is unclear what component of enrichment is beneficial: social, physical or a mixture of both. Similarly, more research is needed to establish if the neuroanatomical and behavioural changes that are correlated with 'experience of enrichment' result from informal learning, or repeated exposure to novelty. It is

also unclear if these brain changes directly cause enhanced spatial ability, or, simply provide the animal with better stress coping ability. Crucially, all future enrichment studies should have adequate independent replication of the enrichment enclosures to avoid the pseudoreplication issues that currently plague the enrichment literature (discussed in Chapter 6).

As a final note on the use of enrichment in research, a reduction in stress during MWM testing could be classed as a refinement of procedures (one of Russell and Burch's 3Rs). Based on my data, then, it would seem that a combination of social and physical enrichment (Chapter 7) are a refinement of procedures. However, it is currently unclear which kind of enrichment (social or physical) has the greatest beneficial impact on welfare or if the effects of enrichment are the same for different strains, ages and sex of rat. These questions need to be answered before encouraging widespread use of enrichment as a tool to improve welfare out side of the home cage.

9.5. Summary and conclusions

I could find no significant effects of isolation housing on behaviour in the home cage or on spatial cognitive performance in six experiments. It is not clear to me, then, that isolation is always necessarily stressful. Furthermore, isolation housing does not explain the inconsistencies in reports of sex differences in spatial ability in the literature. However, stress due to the test situation does seem to explain why males perform better than do females in the MWM in my experiments. Stress during cognitive testing also seems to explain why enriched animals outperform non-enriched conspecifics in my MWM experiments. In sum, I think my data help to explain the variation in observation of sex differences in spatial cognition.

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