ADOLESCENT ENVIRONMENTAL CHALLENGES AFFECT ADULT FUNCTION IN MALE AND FEMALE LONG EVANS RATS

by

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DEDICATION

This thesis is dedicated to my wonderful parents, Mr. M.C. Joshi and Mrs. Neena Joshi, who kindled my interest in analyzing the world around me and pushing the limits of my knowledge.

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Abstract

Stress in adolescence is a putative risk factor for developing mental illnesses such as schizophrenia and mood disorders. Symptoms for these illnesses first emerge in late adolescence and early adulthood, with both incidence and severity being sexually dimorphic. Animal models can shed light on the neurobiological underpinnings of these disorders by allowing one to explore the relationship between a risk factor such as stress, and development of symptoms. In the current work the role of adolescent stress is explored in the development of biomarkers that are associated with adolescent-onset illnesses using Long Evans rats. Repeated exposure to predator odour was combined with social isolation during adolescence to create a novel stressor model. The specific objectives of this study were to determine (i) if repeated predator odour exposure altered measures related to sensorimotor gating (measured as prepulse inhibition, PPI), startle, and emotionality, and (ii) whether social support affected the outcome of predator odour stress. Predator odour elicited immediate avoidance, which did not habituate with repeated exposures, suggesting a strong behavioural stress response. In contrast to past work, few significant long-term effects were observed in animals exposed to predator odour compared with ones exposed to a non-threatening odour. Unexpectedly, animals exposed to a no odour (control) condition displayed altered PPI, startle response, anxiety-related behaviour, and memory, compared to rats exposed to a non-threatening, control odour or a predator odour. Moreover, the no odour animals showed altered expression of dopamine D2R receptor protein in the medial prefrontal cortex. The outcomes for this group were remarkably similar to those seen in animals raised in social isolation, suggesting an underlying similarity in the neurobiological mechanisms associated with these experiences that likely can be traced to being raised in environments lacking adequate social and physical complexity. Sex differences were noted in PPI, startle response, tests of anxiety- and depression-like behaviour, memory, and levels of dopamine D2R receptors, although the sex of the animal did not interact with stressor treatment to affect these measures. In conclusion, results of the current work provide further evidence for the importance of the social and physical environment to normal development during adolescence, as well as the importance of being male versus female.
(Words = 356)

List of Abbreviations Used

ACTH: Adrenocorticotrophic Hormone
AVP: Arginine Vasopressin
ASR: Acoustic Startle Response
CA1: *Cornu Ammonis1*
CCR-1: Cholecystokinin receptor-1
CRH: Corticotrophin Releasing Hormone
CORT: Corticosterone
CSS: Central Sympathetic System
D1R: Dopamine D1 Receptor
D2R: Dopamine D2 Receptor
DAT: Dopamine Transporter
dB: Decibel
DHEA: Dehydroepiandrosterone
DNA: Deoxyribonucleic Acid
DOPAC: 3,4-dihydroxyphenylacetic acid
DTI: Diffusion Tensor Imaging
EPM: Elevated Plus Maze
fMRI: Functional Magnetic Resonance Imaging
FSH: Follicle Stimulating Hormone
GABA: Gamma-Amino Butyric Acid
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GnRH: Gonadotropin Releasing Hormone
HPA axis: Hypothalamus-Pituitary-Adrenal axis
HPG axis: Hypothalamus-Pituitary-Gonadal axis
HC: Hippocampus
IL: Inter Leukin
LH: Lutenizing Hormone
mPFC: medial Prefrontal Cortex
MRI: Magnetic Resonance Imaging
mRNA: Messenger Ribonucleic Acid
ms: Millisec
MTL: Medial Temporal Lobe
mV: Millivolts
NMDA: N-methyl-D-aspartate
NOR: Novel Object Recognition
OFC: Orbitofrontal Cortex
OFT: Open Field Test
PET: Positron Emission Tomography
PFC: Prefrontal Cortex
PTSD: Post Traumatic Stress Disorder
PnC: Caudal Pontine Reticular Nucleus
PND: Post Natal Day
PVN: Paraventricular Nucleus
V_{Max}: Amplitude of maximum response to the acoustic stimulus

SAM: Sympathetic Adrenal Medullary
SPT: Sucrose Preference Test
T_{Max}: Latency to reach maximum response (i.e. V_{Max})
TNF: Tumor Necrosis Factor

Abbreviations for group names:
PH: Pair Housed
SH: Single Housed
NO: No Odour
NPO: No Predator Odour
PO: Predator Odour

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Chapter 1: Introduction

1.1. Adolescence: A period of development:

Adolescence is considered a pivotal developmental period in many species, including humans, because it transforms a juvenile to an independent adult (Schneider, 2008, Sturman et al., 2011, and Sisk and Foster, 2004) . The terms adolescence and puberty are often used interchangeably in common parlance. This is not surprising considering the similarities in the meaning of their Latin roots: adolescence is derived from "adolescere" which means to grow up, while the term puberty is derived from "pubertas" which means adulthood (Blondell et al., 1999). But the biological definitions of these terms are different. Puberty most often refers to the attainment of sexual maturation by activation of the hypothalamic-pituitary-gonadal (HPG) axis. On the other hand, adolescence often refers to the sum total of all biological changes and social and environmental experiences involved in the transition from childhood to adulthood. To cite a review, " Puberty differs from adolescence in that it is just one change (maturation of the reproductive system) that occurs during adolescence" (Pinyerd et al., 2005). In general, adolescence is said to begin around puberty and end with the assuming of adult social roles and responsibilities (reviewed in Spear, 2000). While it is difficult to demarcate this period exactly in every species, there are certain commonly-used age ranges within which most of the physiological, behavioural and social changes associated with adolescence are known to take place. In humans, this period is often thought to occur from 10-19 years (approximately) (e.g. Petersen et al., 1996). In non-human primates, considerable debate exists on the exact period of adolescence although the latter half of

the juvenile phase and early sub-adult phases are considered comparable to human adolescence (reviewed in Spear, 2000). In rats the period from post natal day (PND) 28-60 encompasses the timing of most of the adolescent-specific biological changes (reviewed in Spear, 2000), and can therefore, be considered the adolescent period.

Together, the biological changes occurring during puberty and adolescence result in the attainment of fertility followed by the ability to reproduce successfully (reviewed in Pinyerd and Zipf, 2005). The main changes that occur during this period are: body growth, development of sexual organs or genitals, and the appearance of secondary sexual characteristics. By the end of adolescence, both sexes have undergone a growth spurt as a result of which there is an increase in height and weight, with boys emerging taller and heavier than girls on average (Abbassi, 1998). Additionally, this period is accompanied by the development of body image or the individual's inner conception of his/her appearance (reviewed in Pinyerd and Zipf, 2005). Adolescents can be overly critical of their body image, particularly girls, which in severe cases can bring about mental illnesses like anorexia or bulimia, or depression (Weinshenker, 2002). Acne vulgaris is another physical change that occurs in many adolescents and contributes towards a negative body image; it is one of the most prevalent skin disorders in adolescence and begins around the age of 12.2 years (Lucky et al., 1991 and 1994).

A seminal event that triggers these pubertal and adolescent changes is the increased activation of the HPG axis, which is a major regulator of the levels of sex hormones (or gonadal hormones) released in the body. The information summarized in this paragraph is reviewed in Sisk and Foster (2004). Most mammals show a brief activation of the HPG axis in the late prenatal and early postnatal period resulting in an increase in gonadal hormones that contribute to sexual differentiation and developmental

programming. Thereafter, the release of gonadal hormones slows down dramatically until puberty, during which a dramatic increase is seen in the production of gonadotropin releasing hormone (GnRH) in the median eminence of the basal hypothalamus. Unfortunately, the exact mechanism that triggers increased release of GnRH during puberty is not yet clear though recent studies are starting to shed light on this matter (reviewed in Moenter et al., 2003 and Kelly and Wagner, 2002). GnRH is a decapeptide that is released in pulses by specialized neurons though the exact mechanism of episodic release also remains a mystery (reviewed in Sisk and Foster, 2004). GnRH stimulates the pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH)- two gonadotropins that are vital to gonadal function and reproductive behaviour. LH stimulates testosterone production while FSH triggers sperm production in boys. In girls, FSH stimulates development and maturation of a single follicle in one of the two ovaries present, while LH and FSH together result in ovulation or the release of a fertile gamete (egg) from one of the ovaries. These gonadotropins also regulate the activity of the HPG axis by controlling the release of GnRH via negative feedback. These processes are driven by androgens such as testosterone in males, and estrogens in females though both types of gonadotropins are present in both sexes (albeit in different amounts).

It is of interest that the period of adolescence is accompanied by an increase in the incidence of a number of mental illnesses including major depressive disorder, anxiety-disorders, eating disorders and schizophrenia (Sturman and Moghaddam, 2011). The National Comorbidity Survey Replication study revealed that the peak age of onset for any mental disorder is 14 years (Kesslet et al., 2005). The combined influence of ongoing developmental changes and environmental elements, such as psychosocial factors, is thought to trigger an onset of symptoms of different mental disorders in vulnerable

individuals. For example, in the case of schizophrenia, it has been proposed that a difference in the cortical synaptic density due to a difference in the rate of selective elimination of cortical synapses in adolescence is linked to psychosis proness (Saugstad, 1994). Selective elimination of cortical synapses is a routine occurrence during adolescence. According to Saugstad's theory, completing this maturational process very early and very late compared to normal adolescents would result in an increased risk for psychosis due to an excessively large or small number of synapses in adulthood, respectively (Saugstad, 1989 and 1994) Experimental studies have offered support to this theory (e.g. Kaiser and Gruzelier, 1999). Therefore, in order to understand the etiology of any of these adolescent-onset illnesses like schizophrenia (one of the aims of the current thesis), it is important to first understand the normal developmental processes taking place in adolescence. These are discussed in the following sections.

1.1(i) Neurobiology of adolescent development in humans:

Behavioural changes in adolescence include an increase in risk-taking and impulsivity (Spear, 2000). This is reflected in the greater number of suicides, traffic accidents, unsafe sexual practices, excessive intake of alcohol and other drugs of abuse (Scott, 1992). In fact, approximately 70% of the total deaths of adolescents recorded annually in the United States occur due to motor accidents, unintentional injuries, homicides and suicide (Eaton et al., 2006). It has been suggested that adolescent-typic behaviours such as increased impulsivity, novelty-seeking and risk-taking, which help most mammalian adolescents (including humans) learn the skills necessary to become independent of their parents, can, ironically, also place adolescents in dangerous situations and increase their probability of injury and harm relative to children or adults

(Kelley et al., 2004). In general, adolescents show heightened responsiveness to incentives and peer-context while retaining little ability to control impulses (reviewed in Kelley et al., 2004).

One of the possible factors underlying these insalubrious behavioural choices are ongoing structural changes occurring in different brain regions in human adolescents, as revealed by modern imaging studies. Before discussing the results of these studies, a brief summary of the main imaging techniques used to study adolescent brain development is provided. These techniques are: magnetic resonance imaging (MRI), diffusion tensor imaging (DTI), and functional magnetic resonance imaging (fMRI).

In a nutshell, the source of the signal for MRI studies is the water present in the cell bodies of gray matter and the fat present in the white matter (Henderson, 1983, and Bradley, 1984). Changes in the relative amount of water and fat in different regions provides an indication of possible changes in the relative amount of gray and white matter (Henderson, 1983, and Bradley, 1984). The methods used to analyze MRI data have changed with time; while volumetric changes were primarily used in the past, newer studies also make use of techniques such as voxel-based morphometry and Cortical Pattern Matching which are not biased by the need to make anatomical delineations between regions (unlike the more traditional volumetric-based analysis) (Sowell et al., 2004).

DTI is used to measure the degree of myelination and the directionality of fiber tracts between different regions of the brain (Pierpaoli et al., 1996), whereas fMRI techniques are used to study patterns of brain activity while the subject performs a specific task (e.g. Casey et al., 1997). DTI is sensitive to morphological features of

neurons such as axon size, density, organization and degree of myelination (Tamnes et al., 2010).

fMRI studies of human adolescents allow researchers to measure the degree of activation of different regions of the brain in response to performance on behavioural tasks designed to measure risk-taking, impulse-control or other adolescent-typical behaviours (Henderson, 1983). fMRI measures the level of oxygen in the blood being circulated in different regions of the brain; increased level of oxygen being sent to a certain region is thought to indicate increased level of neural activation in that region (Henderson, 1983). Tasks such as the Go/No Go task measure impulse control in the subjects (Donders, 1868), and thus the regions of the brain that show increased level of activation in a task such as the Go/No Go task are assumed to be important for impulse control. In general it is observed that adolescents show greater limbic system activation during such tasks than adults; moreover, performance and degree of activation of PFC increase with age (reviewed in Casey et al., 2008 and 2011).

Post-mortem analysis of the adolescent brain is a non-imaging technique used to study its development; this was the main technique used to study brain development before the emergence of imaging techniques such as MRI, fMRI and DTI. An obvious limitation of this technique is the normalcy of the brains being studied - unlike most of the normal population that survive adolescence unharmed, these subjects died in adolescence. Thus, it is difficult to distinguish effects of normal development from the changes brought on as a result of death. Sample sizes of such studies are also very restricted, limiting the power to detect effects and the conclusions one can draw from the results (these limitations are summarized in Sowell et al., 2004).

Two key changes revealed by post-mortem studies of adolescence are the continued myelination (Benes et al., 1994), and the decrease in synaptic density (Huttenlocher, 1979, and Huttenlocher and de Courten, 1987) brought on by pruning in the cortical regions (Figure 1.2). Using MRI, it has been shown that although the total volume of the brain doesn't change much between adolescence and adulthood (the brain reaches 90% of its adult volume by age 6, the relative volume of white matter increases and that of gray matter decreases during this period (Tamnes et al., 2010). In fact, the volume of gray matter and cortical thickness follow an inverted U-shaped curve with maximum volume reached in late childhood and decline beginning in early adolescence until adult-volume is reached (Tamnes et al., 2010). This development is regionally specific with regions with a simple laminar architecture (3-layered allocortex) showing a linear developmental trajectory while regions with 6-layered isocortex showing complex trajectories. Moreover, within the isocortex, the primary sensory and motor areas attain adult thickness before secondary areas and association areas (Tamnes et al., 2010). Loss of gray matter in parts of the temporal lobe and dorsolateral PFC occurs mainly in late adolescence (Giedd et al., 1999, Shaw et al., 2008, and Sowell et al., 2003), and is disrupted in schizophrenia as evidenced by an analysis of post mortem brain tissue (Mauney et al., 2013). In conclusion, gray matter development progresses along a posterior-to-anterior direction and along a lateral to medial direction (Tamnes et al., 2010). By contrast, the developmental trajectory of white matter follows a roughly linear path, with levels peaking around the fourth decade of life, and showing little regional variation (Tamnes et al., 2010). Sub-cortical regions also show such changes in volume of gray and white matter, particularly the basal ganglia (Giedd et al., 1996, Sowell et al., 1999).

Taken together, these results suggest that throughout adolescence, in various parts of the brain, synapses are first over-produced and then reduced to adult levels, consistent with the inverted U-shaped curve found for gray matter volume in MRI studies (reviewed in Casey et al., 2008). However, these synaptic changes have only been demonstrated to exist in post-mortem tissue and in animal models; imaging techniques used at present cannot provide information about such microscopic changes in structure (limitations reviewed in Paus et al., 2010, and Paus, 2010). At the level of the cortex, there is a reduction in the depth of sulci and an increase in their width leading to an overall thinning of gray matter (Aleman-Gomez et al., 2013). On the other hand, the increase in white matter is thought to occur due to myelination of tracts connecting the frontal regions with sub-cortical areas such as basal ganglia and amygdala. This explanation of the change in white matter volume could also explain the increased impulse control and reduced risk-taking in young adults compared to adolescents. Adolescents are presumably lacking in risk-taking and impulse-control because of reduced control of sub-cortical regions by PFC due to a lack of appropriate myelination of the white matter tracts.

To conclude, while imaging studies can provide information about macroscopic brain development in adolescence, they cannot reveal the microscopic mechanisms that underlie these macroscopic changes (Bradley, 1984, Henderson, 1983). Such questions can only be addressed using animal models as discussed below.

1.1(ii) Neurobiology of adolescent development in animal models:

In addition to studies on humans, adolescent development has been studied in different primate and non-primate species. Animal models, particularly rodents, provide a relatively inexpensive way to study the phenomena of adolescence, and allow researchers

the opportunity to study cause and effect, which cannot be done in humans. The validity of such animal models is assessed using three criteria borrowed from psychological testing literature (reviewed in Razafsha et al., 2013, and Spear, 2000). Face validity refers to apparent similarities between the animal model and the phenomenon being studied while construct validity refers to homology between the underlying physiological changes that result in the apparent similarities between the animal model and the human phenomenon being modeled (Albelda and Joel, 2012). The processes associated with adolescence in humans and animals like rats show certain similarities that lend some face and construct validity to the latter but it is important to bear in mind that assessment of validity of a model is an ongoing process. Moreover, validity of animal models is ultimately determined by "their usefulness in expanding understanding of the phenomena under investigation, propagating further testable hypotheses and generating data to refine the model and further assess validity" (Spear, 2000). Discussion of the issue of validity is continued in a later section of this chapter.

Adolescence is clearly not unique to humans; primates and several non-primates also display such a phase in development when the young prepare themselves for an independent existence. In general, during this transition, individuals of all these species show an increase in peer-directed social interactions (Steinberg, 1989), novelty seeking and risk-taking behaviours (Steinberg, 2008). The goal of all these changes is to prepare the individuals to eke out an adult existence in the real world, outside of the protective fold of their parents and siblings. When viewed through this prism, the increase in risk-taking and novelty seeking appear to be adaptations to allow the animals to acquire new skills, and disperse away from their family unit in order to live as independent adults. Additionally, like humans, other species like monkeys and rats show changes in brain

regions like the PFC in adolescence, along with developmental hyperphagia and accelerated growth rates. Therefore, these animals are often used to study aspects of human adolescence, although certain experiences are unique to humans and can not be modeled in animals, such as peer-pressure, self-esteem, impact of parenting on parent-offspring conflict, obsession with aspects of physical beauty such as thinness among females, and cultural differences in the experience and importance of adolescence (reviewed in Spear, 2000).

Much like humans, monkeys also show a reduction in density of synapses and dendritic spines over the course of adolescence (Zecevic and Rakic, 1991). Additionally, in the PFC region, neurons in layer III show pruning of axonal arbors (Zecevic and Rakic, 1991). Furthermore, in monkeys, synapse number was reduced in the visual cortex and other cortical areas from 2 to 5 years of age (a period roughly considered the adolescence phase of these animals) (Bourgeois et al., 1993). The authors of that study concluded that pruning of synapses could not account for the bulk of volume reduction in the cortical gray matter because synaptic boutons formed a very small fraction of the total volume of cortical gray matter (Bourgeois et al., 1993). It has been suggested that due to synaptic pruning, the metabolic and energy requirements of these neurons would be reduced resulting in a reduction in the surrounding glial cells, which could contribute towards the reduction in the volume of gray matter (Huttenlocher, 1979). Additionally, it is proposed that increased myelination occurs within the cortex (besides occurring in the sub-cortical regions) resulting in a relative reduction in the volume of cortical gray matter as measured in MRI (Giedd, 2004). These alternate explanations for the reduction in gray matter volume are less accepted and discussed in the literature as most investigators consider

pruning of synapses in the cortex to be the primary reason behind the reduction in the volume of gray matter in the cortex in adolescence.

As previously mentioned, human adolescents show a reduction in the volume of gray matter in cortical regions like the frontal cortex between adolescence and early adulthood, leading researchers to suggest a possible reduction in the number of neurons due to apoptosis, as well as synaptic, dendritic and axonal pruning (Markham et al., 2007). Such processes can be directly studied in rats but in consideration of such results, an important question arises: is the cortical region researchers accept to be PFC in rats homologous to the PFC (or specifically the dorsolateral PFC) in humans and other primates? This region is responsible for higher-order thinking such as abstract thought, attention, and the ability to adapt to the shifting demands of the environment in order to attain a goal (called cognitive flexibility) (Gilbert and Burgess, 2008, and Klanker et al., 2013). Moreover, it is undergoing important developmental activity throughout adolescence and early adulthood and is found affected in schizophrenics (Sun et al., 2009). Researchers believe rats to have a prefrontal cortex that has functionally dissociated regions (much like primates); one of these regions (medial PFC) is considered homologous to the primate PFC (or the dorsolateral region of the primate frontal cortex) and is responsible for higher-order cognitive functions such as attention, and cognitive flexibility (Uylings et al., 2003). Lesions in this region of the cortex produce a deficit in a test of cognitive flexibility called the Attentional Set Shifting Task (Ng et al., 2007). This is essentially the rat version of the Wisconsin Card Sorting Task (Ng et al., 2007, and Birrell and Brown, 2000) and the deficits observed in rats on the Attentional Set Shifting Task are similar to that seen in patients with lesions in the dorsolateral PFC on the Wisconsin Card Sorting Task (Klanker et al., 2013).

Evidence of loss of gray matter in the medial PFC throughout adolescence in rats has been revealed (Markham et al., 2007). Neuron number, glial cell number, and volumes were estimated for the dorsal (including anterior cingulate region) and ventral (including prelimbic and infralimbic delineations) sub regions of the medial PFC in male and female Long Evans rats in adolescence (PND35) and adulthood (PND90) using stereological techniques (Markham et al., 2007). The number of neurons was significantly reduced in adulthood in the ventral medial PFC; while in layers II/III this effect was seen across both sexes, in layer V/VI, this reduction in neuronal number in adulthood was only seen in females (Markham et al., 2007). Furthermore, females, but not males, displayed a reduction in the volume of the ventral medial PFC from adolescence to adulthood. Neuron number and volume were unchanged in the dorsal part of medial PFC between adolescence and adulthood (Markham et al., 2007). Additionally, the volume of white matter in the frontal cortex increased in both sexes by adulthood (Markham et al., 2007). Thus, this study was able to establish adolescence in rats as a period of ongoing developmental activity in the PFC, much like in humans. The length, complexity and density of dendritic spines in the medial PFC and basolateral amygdala also show similar changes from the late juvenile to the pubertal period with all measures showing an increase (Koss et al., 2011 and 2014). But from adolescence to adulthood dendritic density decreased in the medial PFC, while being unchanged in the basolateral amydala (Koss et al., 2011). This study lends further support to the view that dendritic pruning occurs during much of adolescence in specific regions of the brain, particularly the PFC.

Over-production and subsequent reduction is not only limited to dendritic arbors or synapses; this pattern of change is also found to occur at the level of dopamine D1-like and D2-like receptors in adolescence (Andersen et al., 1997, 2000, and 2002).

Autoradiography and homogenate binding were used in these studies; both involve binding of radiolabelled ligand to the receptors of interest. These studies demonstrate that D1R and D2R levels in the rat striatum increase in number until PND40 (mid-adolescence); thereafter they continue to decline until they reach a stable value in early adulthood. This pattern is also observed for PFC D1R and D2R, although pruning is more protracted in the PFC, lasting until middle adulthood; this process is much less dramatic in the nucleus accumbens (Andersen et al., 2000 and 2002). This process is sexually dimorphic in the striatum and nucleus accumbens but it is not known whether sex differences exist in the over-production and elimination of dopamine receptors in the PFC. Within the striatum, male rats display greater variation in the density of D1R and D2R throughout adolescence even though there is no difference in the adult levels of these receptors (Andersen et al., 1997). Additionally, in the nucleus accumbens, D1R receptors show a higher level in adolescence and adulthood than females (Andersen et al., 1997). Gonadal hormones do not underlie these changes (Andersen et al., 2000).

In the next section, certain psychiatric disorders are discussed that first emerge during adolescence or early adulthood (e.g. schizophrenia). In addition, the role of a potential risk factor- repeated stress (particularly when experienced in adolescence)- in the etiology of such disorders is discussed.

1.2. Stress and adolescent mood disorders and psychoses:

1.2(i) Stress: a risk factor in schizophrenia:

Exposure to stressors in adolescence is considered a putative risk factor for schizophrenia (Brown et al., 1972); in fact, exposure to stress in adolescence precipitates

and exacerbates symptoms of schizophrenia in adolescence and early adulthood (reviewed in Arnsten, 2011, and Dinan, 2005). Note that schizophrenia is a multi-symptom disorder. This will be discussed briefly in the next section, detailing one of the measures related to schizophrenia, and relevant to this current thesis, - sensorimotor gating. Major depressive disorder will also be introduced as another stress-related disorder that emerges in adolescence and involves stress as a risk factor. The results of the current work have implications for both schizophrenia, and major depressive disorder, although categorizing depressive symptoms, such as anhedonia, as being indicative of a separate disorder is not necessary, as depressive symptoms like anhedonia are often present in schizophrenia.

1.2(i)(a) Schizophrenia: A stress-related, adolescent-onset disorder:

Schizophrenia is accompanied by multiple, disparate symptoms that can be broadly classified into three categories: positive symptoms, negative symptoms and cognitive symptoms. Some researchers also recognize a fourth category, that of mood symptoms (reviewed in Guillin et al., 2007). While positive symptoms include hallucinations, delusions, paranoia and thought disorganization, negative symptoms include affective flattening, anhedonia, impoverished speech and ambivalence. Cognitive symptoms include distractibility, learning deficits, memory deficits, and impairment in abstract thought whereas mood symptoms include dysphoria, anxiety, agitation and suicidality (Figure 1.5).

Exposure to stressors, as well as genetic liability, are together thought to result in schizophrenia. Specifically, the stress-vulnerability model considers exposure to internal or external stressors along with an inherent genetic predisposition vital for the onset of

psychosis. Not surprisingly then, stressful life-events such as migration and combat experience are associated with an increased onset of psychosis, as are minor everyday-hassles (Bebbington et al., 1993, and Beighley et al., 1992). Factors such as urbanity are also associated with an increased risk of emergence and development of psychoses, and social adversity, isolation and the stress associated with urban life are considered the key elements of urban life that are responsible for this outcome (Bebbington et al., 1993, Janssen et al., 2004).

Studies examining the mechanism linking such environmental adversity in adolescence to psychosis suggest a role for monoamines, the hypothalamus-adrenal-pituitary (HPA) axis, hippocampus, and altered activity of the central dopamine system as mediators between stress and the onset of psychosis (Meyer-Lindenberg and Tost, 2012, and Kirkbirde et al., 2007, and reviewed in Heinz, 2002, and Heinz et al., 2013). Altered hippocampal structure and function are consistently found in studies of schizophrenic patients (imaging and post-mortem tissue analysis) and in preclinical models of schizophrenia and is suggested as a target for therapeutic intervention (reviewed in Lodge and Grace, 2011). A key target of heavy research into the neurobiology of schizophrenia is the central dopamine system and this is discussed in the next sub-section.

1.2(i)(b) Dopamine hypothesis of schizophrenia:

The classical dopamine hypothesis of schizophrenia states that the positive symptoms of schizophrenia are likely caused by excessive activation of the D2R receptors (reviewed in Guillin et al., 2007). This was based on the observation that antipsychotics like reserprine that successfully reduce psychotic symptoms, bind to, and block D2R in the striatum, and their clinical potency is correlated with their ability to block D2R

(Carlsson and Lindqvist, 1963, Creese et al., 1976, and Seeman and Lee, 1975). This scenario continues to be supported by recent work. For example, unmedicated patients newly diagnosed with schizophrenia show higher D2R availability in the striatum compared to unaffected controls, and an increased release of endogenous striatum in response to an amphetamine challenge (Abi-Dargham et al., 2000, and 2012; Corripio et al., 2011). An amphetamine challenge involves administration of amphetamine (a synthetic analog of dopamine) resulting in release of endogenous dopamine (and other monoamines) (sample protocol in Pogarell et al., 2012).

It is important to note that the initial iteration of the dopamine hypothesis of schizophrenia attempted to explain *all* categories of symptoms of schizophrenia and thought of dopamine as working in isolation (Matthysse, 1973, Snyder, 1976). Post-mortem analysis of schizophrenics' brains revealed a finding that led to a reworking of the classical dopamine hypothesis. Contrary to expectations, no dopamine increase was seen in the cerebrospinal fluid indicating that dopaminergic levels were probably elevated only in specific regions of the brain (Davis et al., 1991). Furthermore, clozapine, an antipsychotic, was found to have a low affinity to D2R even though it had a high clinical potency. Additionally, with the emergence of positron emission tomography (PET), researchers were able to establish the reduced cerebral blood flow to the frontal cortex (PFC) in schizophrenics suggesting a deficit in this region as well. Animal studies too showed that lesion of the PFC resulted in an elevation in dopamine levels in the striatum and other sub-cortical areas (reviewed in Eyles et al., 2012).

As a result, a new iteration of the dopamine hypothesis was proposed which stated that schizophrenia was accompanied by a hypo-dopaminergic tone in the frontal cortex (resulting in the negative symptoms) and a consequent hyper-dopaminergic tone in the

striatum (resulting in the positive symptoms) (Abi-Dargham et al., 1998, Davis et al., 1991). The most recent iteration of this hypothesis focuses on the importance of presynaptic dopamine dysregulation (possibly due to D2R levels) in the psychotic symptoms of schizophrenia while also emphasizing the importance of multiple adverse events or "hits" in triggering the initial onset of symptoms (Howes and Kapur, 2009). This hypothesis is still relatively new and evidence to support or refute it is still being gathered through myriad studies.

1.2(i)(c) The Glutamate hypothesis of schizophrenia:

Apart from the dopamine hypothesis of schizophrenia, the glutamate hypothesis of schizophrenia is one that has been actively researched, and which is as prominent as the dopamine hypothesis. The origins of this hypothesis are in the PCP model of schizophrenia, which was proposed by Luby et al. in 1959 (reviewed in Javitt, 2010). This model was based on the similarities between the effects of the drugs phenylcyclidine (PCP) and ketamine in healthy individuals and the symptoms seen in schizophrenics such as apathy, thought disorder and psychosis, and neurochemical changes that accompany it. Thereafter it was revealed that both PCP and ketamine block N-methyl-D-aspartate (NMDA) type glutamate receptors non-competitively (Carlsson et al., 1999). This paved the way for a glutamate hypothesis of schizophrenia that was first proposed in the 1990s and which states that glutamatergic hypofunction could be a neural basis for many of the symptoms seen in schizophrenia. Due to the widespread distribution of the NMDA receptors throughout the brain, this model can better predict and explain a number of symptoms seen in schizophrenia including cognitive dysfunction. Moreover, unlike the dopaminergic model of schizophrenia (which attributes the disorder to circumscribed

central dopamine dysfunction), the glutamate model emphasizes widespread glutamatergic dysfunction as the reason behind this disorder. Moreover, due to the regulatory role played by these receptors in the release of dopamine, it has been suggested that dopaminergic dysfunction seen in schizophrenia may be a result of underlying glutamatergic dysfunction (reviewed in Javitt 2010). However, at the moment, the subjects of glutamate and dopamine interactions and which might possibly be the site of primary dysfunction in schizophrenia, are still being investigated.

1.2(i)(d) Biology of sensorimotor gating (prepulse inhibition):

Sensorimotor gating is a "pre-attentive filtering process" by which certain incoming sensory information alters (reduces) the organism's motor responses to other sensory stimuli (reviewed in Swerdlow et al., 1994, and Geyer, 2006). It is measured in the form of PPI, defined as a reduction in the startle response to an acoustic stimulus if the startling stimulus is preceded by a sub-threshold stimulus (called the prepulse). This sub-threshold stimulus can be of any modality (acoustic, olfactory, visual, or tactile) although most experiments including the one used in the current study use an acoustic stimulus for a prepulse. In humans, the startle response measured is an eye-blink assessed using electromyography (e.g. Braff et al., 1978). In animals such as rats and mice, the movement produced within the entire body in response to the startle stimulus is measured (e.g. Geyer et al., 1993), although recent publications have suggested a similar approach to testing PPI in humans (reviewed in Geyer, 2006). Although PPI itself is not a cognitive process, deficits in PPI (such as those seen in schizophrenia) are considered predictive, or reflective, of the existence of cognitive deficits.

While a circuit operating at the level of the brainstem mediates the acoustic startle response, higher-order structures are involved in mediating and regulating PPI (Swerdlow et al., 1994). As shown in Figure 1.8, the circuit involved in mediating the acoustic startle response includes the dorsal and ventral cochlear nuclei, cochlear root nucleus, ventrolateral tegmental nucleus, and the caudal pontine reticular nucleus (PnC) (reviewed in Kohl et al., 2013). PnC sends direct projections to the motor neurons. The dorsal and ventral cochlear nuclei as well as the cochlear root nucleus stimulate the midbrain inferior colliculus that activates the superior colliculus. The superior colliculus projects onto the pedunculopontine tegmental nucleus that inhibits the PnC, reducing the startle response. This circuit mediates PPI, but PPI is regulated by higher order structures including the hippocampus, medial PFC and orbitofrontal cortex, basolateral amygdala and the nucleus accumbens (Figure 1.8) (reviewed in Fendt and Koch, 2013). PPI, startle and their underlying circuits are conserved across different species making this a useful measure to assess and compare across animal and human studies.

1.2(i)(e) Role of dopamine system (and glutamate) in regulating PPI:

Evidence from work on rats and mice suggests that the neurotransmitters modulating PPI are dopamine (e.g. Davis, 1988, Mansbach et al., 1989, Swerdlow et al., 1990, and Peng et al., 1990), glutamate (e.g. Wan and Swerdlow, 1996), GABA, and serotonin (reviewed in Swerdlow et al., 2001). The main evidence for the role of dopamine in PPI is from animal studies (rats and mice) that tried to model PPI deficits seen in schizophrenics. Administration of direct or indirect dopamine receptor agonists such as apomorphine (binds to both D1-like and D2-like dopamine receptors) and amphetamine (dopaminergic psychostimulant) to rats results in a reduction in PPI

comparable to that seen in schizophrenics (Varty and Higgins, 1994, Swerdlow and Geyer, 1993). These effects are partly attributable to D2R in the striatum and nucleus accumbens (Swerdlow et al., 1994). PPI disruption is also produced in rats by experimental treatments that reduce the dopaminergic tone of the medial PFC such as infusion of D1R and/or D2R receptor antagonists, suggesting an important role for the mesolimbic dopamine system (including D2R and possibly, D1R though the strongest evidence exists for D2-like receptors including D2R) in regulating PPI (Ellenbroek et al., 1996).

Most studies of the role of dopamine in regulating PPI have looked at dopamine systems in the sub-cortical regions, primarily the nucleus accumbens and striatum. They suggest that at least part of the effect of dopamine agonists on PPI is mediated by dopamine receptors in the nucleus accumbens although other factors such as gamma-aminobutyric acid (or GABAergic) projections from the nucleus accumbens to the ventral pallidum also contribute to this effect of dopamine agonists (Geyer and Braff, 1987, Varty and Higgins, 1994, Swerdlow and Geyer, 1993).

Glutamatergic NMDA receptors are also implicated in modulating PPI; for example, mutant mice strains lacking the NMDA receptor in the PFC showed reductions in PPI even though startle response remained unchanged (Rompala et al., 2013). Atypical antipsychotics too block NMDA receptors in addition to blocking dopamine receptors (Swerdlow, Platten, et al., 2001). The NMDA receptors in the medial PFC, amygdala and dorsal hippocampus but not the nucleus accumbens or ventral hippocampus appear to be important in regulating PPI as evidenced by a study in which an NMDA receptor antagonist, dizocilpine, was injected in specific regions of the rat brain to disrupt glutamatergic transmission (Bakshi and Geyer, 1998).

1.2(ii) Stress: a risk factor in major depressive disorder:

As mentioned earlier, much like schizophrenia and other psychoses, major depressive disorder also shows a greater likelihood of emergence in adolescence. Environmental factors, particularly daily hassles and chronic stressors, have long been considered important to the development of this disease (reviewed in Nestler et al., 2002). Of particular note are adolescent social stresses such as negative family relationships, peer victimization and bullying (Lund et al., 2008, Jordanova et al., 2007, McCabe et al., 2010), stresses associated with interactions with peers as well as adults (including altered parent-child dynamics), and the challenges of creating and sustaining romantic relationships (Soller, 2014, La Greca and Harrison, 2010). Moreover, the adolescent-onset major depressive disorder is associated with a lack of adequate social support, which often protects against the damaging effects of different social stressors.

1.2(ii) (a) HPA axis and major depressive disorder:

The HPA axis is dysregulated in major depressive disorder resulting in increased cortisol in patients and this dysfunction is believed to precede the onset of symptoms, suggesting a role in the etiology of the disorder (evidence reviewed in Guerry and Hastings, 2011). In fact, aberrant HPA activity is one of the most consistently reported results in the study of major depressive disorder. Furthermore, increased basal levels of cortisol, higher cortisol levels in response to stressors, and higher response to the dexamethasone suppression test suggest a deficit in the feedback regulation of the HPA axis and in CRH production by the hypothalamus (Beaton et al., 2006, Birmaher et al., 1992 and 1996, Goodyer et al., 2001, Lopez-Duran et al., 2009). Dexamathasone is a synthetic analog of cortisol and it is administered to test subjects in an effort to activate

the HPA axis above basal levels and test the efficacy of feedback regulation (Lopez-Duran et al., 2009).

The circuit connecting the amygdala to the hippocampus and the PFC (which regulates the activity of the HPA axis) and the one connecting the striatum and PFC as well as the dopamine system are also likely mediators of this link between adversity in adolescence and emergence of depression (reviewed in Schwab et al., 1968, Thapar et al., 2012, and Seeman, 2013). To conclude, the role of developmental (i.e. adolescent) stress in the etiology of stress-related, adolescent-or-early-adulthood onset mental disorders like schizophrenia, is being investigated at present using numerous techniques and model systems. The current work is one such attempt focusing on the disruption of normal adolescent development by the experience of adolescent stress.

The next section is will briefly address the role of dopamine in depression, particularly psychotic depression.

1.2(ii) (b) Dopamine and depression:

Depression is often thought of as a single, homogeneous disorder. Moreover, most research into the neurobiology of depression has long focused on serotonin and norepinephrine alone. However, recent research suggests that depression may not be a homogeneous disorder after all, and there may be variants to it, each with its own distinct etiological origins. This is best illustrated in a recent publication which considers depression to consist of three principle sub-types, each of which is brought on by deficits in a certain neurotransmitter system (Mahli et al., 2005). These sub-types are psychotic melancholia, non-psychotic melancholia, and non-melancholic depression. Of these, deficits in dopamine transmission are considered most relevant to the etiology of

psychotic melancholia, whereas norepinephrine and serotonin, respectively, are considered important for the other two sub-types of depression (reviewed in Mahli et al., 2005). Of all these sub-types, clearly psychotic melancholia is most relevant to the current work because it involves psychosis as a symptom (which is a symptom of schizophrenia as well) and dopamine as a possible neural substrate.

Briefly, two main lines of evidence connect dopamine dysfunction with depression. The first evidence is of the reduced presence of dopamine metabolites such as homovanillic acid in the cerebrospinal fluid of depressed patients (Pepsechi et al., 1971, and Goodwin et al., 1973). Furthermore, dopamine metabolites, such as 3,4-dihydroxyphenylacetic acid (DOPAC), are reduced in the urine of depressed patients compared to controls (Roy et al., 1986). The second line of evidence that links dopamine dysfunction to psychotic depression is the altered activity of the mesolimbic dopamine system. In fact, increased levels of dopamine are found in the serum of patients with psychotic depression compared to patients with non-psychotic depression (Devanand et al., 1985).

In the next section, the concepts of stress, and animal models of adolescent stress are discussed in greater detail. This will be followed by a discussion of the design and rationale of the current study.

1.3 Stress: Historical details, biology, and animal models

1.3(i) Historical origins of stress research:

The story of the origin of modern stress research is discussed briefly in this paragraph; for more details readers are advised to consult Goldstein and Kopin (2007),

Weissman (2007), Cooper (2008), and Szabo et al. (2012). Briefly, the origins of modern stress research can be traced to three researchers: Claude Bernard, Walter Cannon and Hans Selye. Bernard was the first scientist to introduce the idea of an internal environment within the body apart from the external environment. His major discovery was the fact that the liver converted glucose to glycogen. Prior to this discovery, it was thought that only plants made sugar, and animals digested sugar by combustion in the lungs or capillaries. While discovering the glycogenic function of the liver, he also discovered the regulation of blood supply to this organ by sympathetic nerves; this led him to generalize that all cells were surrounded by an internal environment which needed to be maintained in a constant state for optimal health and well-being. Cannon extended this concept further and used the term "homeostasis" to describe the process of maintaining various physiological variables within an optimum range. Disparate threats to homeostasis such as exposure to cold, traumatic pain or emotional distress activate the adrenal medulla and the sympathetic nervous system (referred to as the sympathoadrenal or sympathico-adrenal-medullary, SAM, system) that maintain homeostasis. Thus, Cannon is credited with the discovery of the role of catecholamines (from the adrenal medulla) in the response to non-specific injury. The concept of negative feedback was introduced by Norbert Weiner, an engineering mathematician, and is used in the stress literature to describe self-regulatory processes carried out in order to maintain homeostasis.

The role of adrenal cortical "corticoids" in the stress response was discovered by Selye (among other discoveries), and described in his landmark paper published in the journal "Nature". On injecting mice with an ovarian extract, he recorded an expression of alarm in the mice, and he referred to this alarm as being the beginning of the general

adaptation syndrome (Selye, 1946). In his words, "the general adaptation syndrome is the sum of all non-specific, systemic reactions of the body which ensue upon long continued exposure to stress" (Selye, 1946). It is important to note that Selye did not coin the word stress; it was Walter Cannon who used the term in his paper "Stresses and strains of homeostasis" published shortly before Seyle's paper. Selye however, did draw a distinction between stressor and stress (Figure 1.3). The former refers to the alarming or threatening stimulus while the latter refers to the body's response to the threat (Selye, 1976). He also coined the terms distress and eustress in the early 1970s based on whether the response was triggers by unpleasant or pleasant stressors. Additionally, he also discovered and coined the terms adrenal glucocorticoids and mineralcorticoids. Finally, he insisted that variable stressors elicit the same non-specific activation of the corticoid and catecholamine response.

In common parlance, stress refers to any event that causes or is likely to cause distress and discomfort. Given such a generic definition, it is not surprising that this term "has been applied to almost every sling and arrow endured by sentient creatures" (Weissmann, 2007). In biological terms, stress is defined as "an adverse circumstance that disturbs or is likely to disturb, the normal physiological or psychological functioning of an individual" (Weissmann, 2007). Furthermore, as suggested by Selye, a distinction is now drawn between the words stressor and stress (Figure 1.3). While the word stressor refers to a potentially threatening stimulus, the word stress refers to the experience of stress in the animal in response to a stressor (Figure 1.3). The current work also follows this definition of stressor and stress. Additionally, there is a distinction between the terms homeostasis, allostasis and allostatic load (McEwen, 2007). Homeostasis is a state of equilibrium, which is threatened by the presence of stressors; on the other hand, allostasis

refers to the "process of responding to a challenge to the body by triggering chemical mediators of adaptation (HPA, autonomic, metabolic, immune) that operate in a nonlinear network. Allostasis is essential to maintaining homeostasis in the face of challenges" (McEwen and Gianaros, 2011). Allostatic load refers to the wear and tear that can result from "chronic dysregulation of mediators of allostasis" (McEwen and Gianaros, 2011).

1.3(ii) Biology of the stress response:

1.3(ii) (a) Stress and the hypothalamus-pituitary-adrenal axis:

The brain is the main organ involved in the stress response mounted to maintain homoeostasis and improve survival of the individual. Physiological changes brought on to withstand stress include increased cardiovascular tone, respiratory rate and decreased feeding, digestion, growth, reproduction and immunity (reviewed in Smith and Vale, 2006). The main structures involved in initiating this response are the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland and the adrenal gland (together called the HPA axis). In addition, the locus coeruleus-noradrenergic system (central sympathetic system, CSS) in the brainstem, the sympathetic circuits in the adrenal medulla and the parasympathetic system also play a role in mounting the stress response (Swanson and Hartman, 1975, Stone, 1975).

A stressful experience results in activation of the PVN of the hypothalamus to release corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP). These hormones act synergistically to stimulate the anterior pituitary to release adrenocorticotropic hormone (ACTH) into the systemic circulation. This hormone acts on the adrenal cortex to stimulate the release of glucocorticoids such as cortisol in humans (its equivalent in animals is CORT). The diverse adaptive and maladaptive responses to

stress are triggered by the binding of glucocorticoids to their receptors on different organs of the body.

Glucocorticoids also regulate their own release through a mechanism of negative feedback. These effects are mediated by two sub-types of receptors: Type I or mineralcorticoid receptors and Type II or glucocorticoid receptors (Reul and de Kloet, 1985). The former have a greater affinity for glucocorticoids and are localized in the hippocampus and septum; the latter sub-type of receptors are localized in the brainstem, limbic system, hypothalamus, pituitary and cerebral cortex, and have a higher affinity for synthetic steroids like dexamethasone but a lower affinity for corticoids (reviewed in de Kloet et al., 1998). Mineralcorticoid receptors are responsible for maintaining basal HPA activity while glucocorticoid receptors play a part when the levels of corticoids rise above basal levels, often in response to a stressor. In such cases, glucocorticoid receptors are also responsible for negative feedback driven inhibition of the HPA axis at the level of the hypothalamus and pituitary (reviewed in Jacobson and Sapolsky, 1991).

Glucocorticoid receptors are present in different regions of the brain and peripheral tissues. Under normal conditions, glucocorticoid receptors are present in an inactive state in the cytosol of cells bound to heat shock proteins. Upon binding of glucocorticoid molecules, these receptors undergo a conformational change, separate from the heat shock proteins and enter the nucleus where they bind to specific parts of the promoter region of glucocorticoid responsive genes in the DNA and regulate their transcription (reviewed in Smith and Vale, 2006). Negative regulation of the HPA axis is also provided by the hippocampus and medial PFC while the amygdala provides positive regulation of PVN of the hypothalamus (reviewed in Tsigos and Chrousos, 2002). Figure 1.4 shows a diagrammatic representation of the activation of the HPA axis and related

structures in response to exposure to a stressor while Table 1.1 lists some of the primary mediators involved in maintaining homeostasis such as glucocorticoids and adrenaline (adapted from McEwen, 2002). These act on different organs and tissues to change structure and function resulting in different secondary outcomes. For example, primary mediators like cortisol can result in secondary outcomes such as an increase in systolic and diastolic blood pressure as well as an increase in waist-hip ratio (secondary outcomes of cortisol are reviewed in McEwen, 2002).

It may be argued that the most important biological changes that occur in response to a stressor are behavioural. Presumably, the above changes in HPA activity and SAM activity trigger biological cascades that ultimately result in altered behaviour to help the organism adapt to the stressor (e.g. Berridge and Dunn, 1989). These behavioural changes form as much a part of the stress response as the neural, endocrinological and other physiological changes. Some of the behavioural changes seen in response to stressor exposure include a decrease in food intake (Pare, 1964), reduction in appetitively-motivated behaviours (Annau and Kamin, 1961), reduction in sexual activity, and an increase in defensive and certain-learning behaviours (Overmier and Seligman, 1967). In other words, behaviours that help an individual combat the stressor are encouraged while maintenance behaviours are reduced. These behavioural changes can be used as biomarkers of the animals' stress response, similar to hormonal changes. For example, stress-induced increase in arousal is one such behavioural response that allows animals to cope with the stressful situation. Increased arousal may be defined as an increase in the awareness of and sensitivity to environmental stimuli, and is modulated by noradrenergic projections to various brain areas such as the hippocampus, cortical areas, and the basal forebrain (Dahlstrom and Fuxe, 1964). Such behavioural changes in response to stress are

referred to as the psychological features of stress in contrast to the physiological indices such as HPA and SAM activity. Measurement of the former provides important information on the animal's response to the stressor experience much like assays of CORT/cortisol or adrenaline do.

1.3(ii) (b) Central dopamine system and adolescent stress:

An observation that can be made based on the dopamine hypothesis and its continual reworking over the last few decades is that the central dopamine system, particularly dopamine receptors, continues to be considered important to the etiology of schizophrenia (particularly psychosis), and therefore, is investigated in various studies. Consequently, in the current work, the levels of some dopamine receptors were measured in animals exposed to various aversive and stressful environmental experiences. Specifically, the levels of dopamine D1R and D2R receptors were measured in the striatum (caudate-putamen region), and medial PFC. Even though these receptors are only one cog in a very convoluted system (as illustrated in figure 1.7), their study can still provide useful clues with respect to the effect of environmental stressors during adolescence, particularly since these receptors are still undergoing development (over-production followed by selective reduction) throughout adolescence and early adulthood as shown in rat models (Andersen et al., 2000).

Some studies using animal models of social stress in adolescence have revealed perturbations in the dopamine system in response to stressor treatment, although others have reported no changes. For example, social stress administered over a few days during adolescence was accompanied by an increase in dopamine release in the medial PFC in adolescence, though dopamine activity decreased below baseline by adulthood. Blocking

D2R pharmacologically prevented this reduction in dopamine activity in medial PFC of animals exposed to adolescent social stress (Watt et al., 2013). The authors proposed that the increase in dopamine in adolescence in response to social stress might result in activation of D2 autoreceptors, leading to inhibition of dopamine activity over the long-term (Watt et al., 2013). Adolescent social stress also reduced the level of dopamine transporter (DAT) in select regions of the medial PFC in adulthood (Novick et al., 2011). By contrast, some other studies using similar models of adolescent stress have shown changes in the dopamine system, particularly in the medial PFC (e.g. Vidal et al., 2007). This would include repeated exposure to a psychological stressor across the peripubertal period resulting in lower expression of D2R in medial PFC in adulthood (Wright et al., 2008).

1.3(iii) Biomarkers of stress:

Biomarkers may be defined as an objective measurement of normal processes; they can be used to define pathological states or the response to a pharmacological or other experimental treatment (the concept of biomarkers is reviewed in Sokolowska et al., 2013 with a few key points from the review presented in this paragraph). The important requirement for a biomarker is that it should be easily measured, and should unambiguously differentiate between normal versus experimental/pathological states. Genes, proteins, and RNA molecules can all serve as biomarkers. Based on this definition, the products of the activation of the SAM axis or the HPA axis could also be considered as biomarkers for stress. Both these axes are the first response systems engaged by the presence of a threatening or aversive stimulus (Pacák et al., 1995). Their activation results in the coordination and synchronization of the different physiological

systems of the body to help the organism cope with the stimulus and for the body to return back to its homeostatic state following cessation of the stressful experience (reviewed in Koolhaas et al., 2011). Levels of the main hormone produced by the activation of the HPA axis, CORT (in rats) or cortisol (in primates), are often used as biomarkers for stress in many studies. In addition to these, behaviour is also an effective biomarker as mentioned in the previous section. Behaviours such as avoidance of the stressor can provide an indication of the animal's response to the stressor. In rats, for example, in the predator odour model, physical avoidance of the stressor stimulus is used as an indicator of stress in the animals (predator odour model reviewed in Dielenberg et al., 2001, and Staples and McGregor, 2006). This stimulus also results in other behaviours (such as head-out response, in which the rat sticks its neck out of an enclosed, protective box in order to scan the environment for potential threats) (e.g. Wright et al., 2008).

In the current study, such behavioural changes, and not levels of CORT were used as biomarkers of stress. An advantage to using behaviour as a biomarker is that it does not focus on individual regions or hormones. Behaviour is the final product of the action of numerous regions of the central nervous system, and of different neurotransmitters and hormones. Stress-induced changes in HPA and SAM activity as well as other changes in the central nervous system ultimately result in changes in learning and memory (Overmier and Seligman, 1967, Annau and Kamin, 1961), arousal, defensive behaviours and non-defensive behaviours such as sexual activity and foraging (Pare, 1964), that allows adaptive behaviours (e.g. Berridge and Dunn, 1989). It therefore, provides a holistic measure of the animal's response to the stressor. Behavioural measures are considered valid in such situations because aberrant behaviour is often the starting point for diagnosing many stress-based psychiatric illnesses (Myrbakk and Tetzchner, 2008).

Moreover, the measurement of CORT is fraught with numerous challenges. For example, levels of CORT are extremely sensitive to numerous factors such as the time of day (Hiroshinge et al., 1969), sex of the animal (Askari, 1970), loud noises (Arts et al., 2012), transportation (Arts et al., 2012) and this makes it likely that the HPA axis's response to predator odour (one of the two stressors used in the current study) could be confounded. Additionally, blood extraction can be a stressful procedure as well.

Thus, immediate behavioural changes in response to control and predator odour exposure were used as biomarkers of stress in the current study. In general, predator odour exposure results in an increase in defensive behaviours and a decrease in non-defensive behaviours (Staples, 2010). Physical avoidance of the odour source is one such behaviour, which was used as a biomarker in the current study. It is hypothesized to be greater in animals exposed to predator odour than those exposed to control odour as has been previously demonstrated (e.g. Mashoodh et al., 2008 and Staples and McGregor, 2006). The following table summarizes the behavioural markers of the predator odour experience used in this study (also consult figure 2.3 showing a schematic of the odour exposure arena).

Signs of behavioural avoidance of odour source (Biomarkers of predator odour experience):	
(i). Percent duration in odour region	Predator odour exposed animals spend *less* time in the odour region compared to animals exposed to control odour.
(ii). Percent duration in third region (most distant from the odour source)	Predator odour exposed animals spend *more* time in the third region compared to animals exposed to control odour.
(iii). Number of entries into odour region	Predator odour exposed animals make *lower* number of entries to odour region than animals exposed to control odour.

(iv). Number of entries into third region	Predator odour exposed animals make *greater* number of entries into the third region than animals exposed to control odour

(Note 1: An increase in other behaviours like investigation of the cat collar, and a reduction in behaviours like rearing and grooming can also be considered indicators of the predator odour experience.)

(Note 2: Additionally, levels of D1R and D2R were assessed in the medial PFC and caudate-putamen to assess activity of the dopamine system, which can be affected by predator odour experience.)

Additionally, the stress of single housing was assessed using two different biomarkers. Rearing post-weaning rats in single housing condition is one of the most commonly used and accepted models for inducing changes in sensorimotor gating and startle abnormalities in rats (e.g. Stevens et al., 1997 and Varty et al., 1999). In the current study, the author took advantage of this outcome by using these two measures (PPI, and startle) as biomarkers of the single housing experience. This information is summarized in the table below.

Biomarkers of the single housing experience

(i). Prepulse inhibition (PPI) changes	Single housing of rats results in changes in PPI (often a reduction though instances of increases in PPI also exist)
(ii). Startle amplitude changes	Single housing of rats results in increased startle amplitude.

In addition, levels of dopamine D1R and D2R receptors in the medial PFC were also measured to gauge activity of the dopamine system. Exposure to cat odour results in an increase in dopamine levels in the medial PFC in Long Evans rats (the strain of rats

used in the current study) and post-stress dopamine and CORT release are positively correlated (Sullivan and Gratton, 1998). Increased dopamine can result in reduced expression of dopamine receptors in order to regulate the level of signaling. Moreover, predator odour stress has been shown to result in reduced dopamine D2R levels in the medial PFC (Wright et al., 2008). The medial frontal cortical dopamine system regulates both the SAM axis as well as the HPA axis (reviewed in Walker et al., 2008). An increased release of dopamine in the left and right medial PFC results from exposure to cat odour (Sullivan and Gratton, 1998).

1.3(iv) Modeling repeated stress in adolescent rats:

The goal of chronic stress models in rats is to model certain aspects of sustained stress as experienced by humans. The term model refers to "any experimental preparation developed for the purpose of studying a condition" in the same or different species (Markou et al., 2009). It is advisable to specify the purpose for which the model is to be used in order to determine the criteria it must satisfy to establish its validity (Hitzemann, 2000). It is also advisable to limit the purpose of a single model so that it offers more cross-species validity. For example, instead of modeling all symptoms of a complex illness such as schizophrenia, it is advisable to model only one aspect of its signs and symptoms such as the deficit seen in sensorimotor gating (Markou et al., 2009).

The next section contains an overview of the criteria used in evaluating animal models (reviewed in Markou et al., 2009, Chadman et al., 2009, and Razafsha et al., 2013). Thereafter, specific models of stress in rats will be discussed.

1.3(iv) (a) Validity of animal models:

In general, animal models of human phenomena much have face, construct, and predictive validity so that the information gathered from them can be extrapolated to human conditions (reviewed in Meyer and Feldon, 2010). Moreover, both the independent variable (i.e. the manipulation performed by the experimenter) and the dependent variable (i.e. the outcome measured by the experimenter in response to the manipulation) should be validated. Types of validity relevant for the independent variable include etiological validity, construct validity, and face validity; it is proposed that etiological validity is the more important of the three (reviewed in Markou et al., 2009, Chadman et al., 2009, and Razafsha et al., 2013). Construct, convergent, discriminant and face validity are most relevant for a dependent variable. Furthermore, not all types of validity are equally important in assessing the animal model; predictive validity and reliability are considered the most important by several researchers (Markou et al., 2009, Chadman et al., 2009, Razafsha et al., 2013). In the following paragraphs are discussed some of the main validity criteria needed to assess animal models of chronic stress (for more in-depth analysis, the reader is directed towards the reviews by Markou et al., 2009, Chadman et al., 2009, and Razafsha et al., 2013).

Face validity refers to the apparent similarity between the model and the phenomenon being modeled (in other words, a similarity in phenotype). In the context of animal models of stress, an appropriate model of stress would be one that is visibly similar to the experience of stress faced by humans. For example, social stress such as bullying faced by adolescents is often modeled by the resident-intruder paradigm that involves exposure of the test rat to a larger, more aggressive conspecific (reviewed in Meyer and Feldon, 2010). Clearly, in both cases, the individual is faced with interaction with an aggressive conspecific. It is important to note that judging a model based on face

validity alone can be misleading and therefore, other types of validity must also be established.

Reliability refers to the stability and reproducibility of the phenotype (reviewed in Hitzemann, 2000); in other words, it addresses how reliably manipulation of the independent variable will produce the expected pattern of change in the dependent variables. This implies that the experimenter must be able to manipulate the independent variable precisely, to measure the dependent variable objectively and unambiguously, and exactly reproduce the phenomenon and its effects on the dependent variable. Additionally, reliability also requires minimal within-subject and between-subject variability of the dependent variable.

In addition to apparent similarity in phenotype, the model system must also be similar in the biological mechanisms that drive the phenotype; this is referred to as construct validity (Meyer and Feldon, 2010). Establishing construct validity can be tricky. Construct validity is said to have two sub-types: convergent validity and discriminant validity. Convergent validity refers to the degree of correlation between the model in question and other tests that attempt to measure the same phenomenon. Divergent validity refers to the degree to which the model in question measures aspects of a phenomenon that are distinct from those measured by other tests (which assess different aspects of the same phenomenon). Establishing construct validity in animal models of stress can be tricky because the observable behaviour seen in response to stressor exposure is the result of multiple biological pathways and systems interacting with each other, and therefore, establishing construct validity would require the researcher to assess the working of each of those systems. A common technique for establishing construct validity in an animal model of stress is to measure behavioural and physiological indices of stress;

physiological indices include the level of CORT and cortisol (products of the activation of the HPA axis) in the blood or feces, whereas behavioural indices include measurement of defensive and non-defensive behaviours (such as done in the current study).

The final type of validity that will be discussed in this section is etiological validity that is said to be present if the model and the phenomenon being modeled have similar etiology. But establishing etiological validity for models of mental disorders is challenging because the etiology of the disorders themselves is often unknown (reviewed in Markou et al., 2009, Chadman et al., 2009, Razafsha et al., 2013). In the next section, some of the important models of adolescent stress used in rats will be discussed.

1.3(iv) (b) Different rat models of adolescent stress:

The following paragraphs contain a discussion of some of the more commonly encountered models of adolescent stress in rats. These include repeated restraint, social instability model, the isolation-rearing model and its variants, the chronic unpredictable stress model, the resident-intruder model, and the predator and predator odour models.

Repeated restraint has been used by various researchers on animals of all ages including adolescent rats. In this paragraph, the author will discuss some such studies done in adolescent rats, whose outcomes are of some relevance to the current work. For example, Negron-Oyarzo et al. (2014) used 7-days of restraint (30-min. per session, PND42-48) to compare responses of rats immediately after the final stressor exposure in adolescence, and in adulthood on tests of anxiety-related behaviour and fear conditioning. Rats that were stressed in adolescence showed higher anxiety-related behaviour in adolescence as well as adulthood. Additionally, in adolescence, stressed rats showed reduced extinction of learned fear and excitatory transmission in the prelimbic region of

the medial PFC although these changes did not endure in adulthood. Suo et al. (2013) used another model of restraint wherein rats were restrained for 28-days in adolescence (PND28-55) although each session lasted a mere 5-min. Rats that were repeatedly restrained showed *reduced* anxiety-related and depressive-related behaviour in early adulthood along with an increase in signaling of mammalian target of Rapamycin (mTOR) in the PFC. Romeo et al. (2006) used a 7-day period of restraint stress (each session of restraint lasting 30-min) administered to male Sprague Dawley rats in adolescence; the stressor period lasted from PND22-28 in rats stressed in adolescence and PND70-77 in rats stressed in adulthood. They also compared effects of acute stress in adolescent (PND28) and adult (PND77) rats in this study. Adolescent males released greater ACTH and CORT immediately following the final session of restraint compared to adult rats (Romeo et al., 2006). However, adolescent rats exposed to repeated restraint showed *lower* ACTH and CORT levels than adult rats 45-min after termination of the final stressor session (Romeo et al., 2006). Neuronal activation was gauged by counting the number of fos-immunoreactive cells in various regions of the brain. In addition, the number of CRH and AVP immunolabelled cells were also measured in this study. No changes were seen in the fos immunoreactivity in the medial PFC in response to repeated stress at either age, although in the nucleus accumbens (shell region), stressed adolescent males showed greater fos expression than stressed adult rats (Romeo et al., 2006). Additionally, adolescent males that were stressed repeatedly showed greater number of CRH and fos double-immunolabelled cells in the paraventricular nucleus of the hypothalamus immediately after cessation of the final stressor session and 45-min after termination of stressor compared to adult males that were repeatedly stressed (Romeo et al., 2006). These findings suggest that significant differences exist in the stress reactivity

of adolescent and adult male rats, and point towards the CRH-containing neurons in the PVN of the hypothalamus as being one of the mediators of these changes.

A final variant of the repeated restraint stress model will be discussed here before discussing other stressor models. Gomez et al. (2002) stressed male Sprague Dawley rats by restraining them on three consecutive days in adolescence, each session lasting 3-hrs (PND40-42), or on three consecutive days in adulthood (PND60-62). Testosterone, ACTH and CORT levels were gauged from blood samples taken each day following the restraint session. Basal ACTH levels were unchanged between adolescent and adult rats. However, repeated restraint had a different effect on ACTH levels at either age even though ACTH levels were significantly elevated from basal levels in response to restraint at both ages. Rats that were restrained repeatedly in adolescence showed higher ACTH levels compared to those stressed in adulthood. This ACTH response habituated in adolescent rats but not the adult ones (Gomez et al., 2002). CORT levels were no different on the first and final days of stressor exposure (Gomez et al., 2002). Lastly, testosterone levels were also differently affected by repeated restraint at both ages. Adolescent rats showed reduced testosterone levels following repeated restraint whereas adult rats showed an elevation in testosterone levels. Together, these results suggest a rapid change in the functioning and regulation of the HPA axis throughout adolescence compared to that in early adulthood.

However, an important limitation of the restraint model is the degree of physical discomfort and distress it causes the experimental animals. This is particularly important in studies that aim to model human psychological stress in rats (just as the current study does). Therefore, a more useful alternative to the restraint stress model is the social instability model of repeated stress that is used almost exclusively in adolescent animals.

It involves a 15-day period in early adolescence (PND30-45) of housing the rats in an aversive environment. The animals are housed in isolation for 1-hr followed by housing with a novel cagemate that is also part of the experiment and undergoing the same stress regime (called "SS" group) (McCormick et al., 2007). The control group (called "CTL") consisted of pair-housed animals that had been left undisturbed except for weekly cage changes (McCormick et al., 2007). In addition, another group of rats were exposed to isolation for 1-hr followed by rehousing with the original cagemate (called "ISO") Males from both ISO and SS groups showed a significant reduction in body weight compared to CTL group (on PND45) while females showed no such change. Baseline blood CORT levels were unchanged on PND45 in the all three groups of either sex. However, on PND45, among males, CORT levels (measured after 1-hr of isolation) were significantly lowered in both ISO and SS groups compared to the CTL group. Among females, on PND45, post-isolation CORT levels were significantly lowered in the ISO group compared to SS group. Additionally, baseline CRH mRNA in the PVN was increased on PND45 in the SS and ISO groups compared to CTL group. CRH mRNA levels in PVN measured an hour after isolation on PND45 showed an increase (compared to baseline) in the CTL group only (McCormick et al., 2007). CRH mRNA levels in the central amygdala in showed a main effect of stress treatment with lower levels for ISO than SS group (McCormick et al., 2007). In short, this study was able to establish the social instability model of stress as a viable alternative to more traditional models such as long periods of single housing (also called isolation housing or simply isolation in the literature).

A more traditional variant of single housing begins immediately after weaning and continues for 6-8 weeks until early adulthood. For example, Hall et al. (1997 and 1998)

isolated male Lister Hooded rats from weaning (PND21) for a period of 8 weeks before behavioural and physiological tests were conducted. Similarly, Ferdman et al. (2007) housed male and female Wistar rats in isolation from weaning for 14 weeks. Isolation housing was accompanied by an increase in social behaviour in male rats during the social interaction test (a test of anxiety-related behaviour in rats) indicating a reduction in anxiety-related behaviour. A similar model of isolation housing used male Wistar rats housed in isolation from weaning at PND21 to adulthood (PND82) (Toth et al., 2011). Isolated rats showed no changes in blood CORT levels or diurnal oscillations in the heart rate (Toth et al., 2011) although they showed abnormally aggressive responses during social interactions with conspecifics (including an increase in defensive behaviours, deficient social communication and increased attacks on vulnerable conspecifics) (Toth et al., 2011). A similar isolation protocol has also been shown to disrupt PPI and startle response, as well as behaviours in tests of emotionality in various studies (discussed later in this chapter). In the current work, this protocol was modified to exclude the period from weaning to PND28 because this period is not traditionally included in adolescence (reviewed in Lupien et al., 2009), and the author wanted to focus on the impact of isolation on adolescence alone, excluding the juvenile period.

Another model of adolescent stress using social stress is the chronic variable stress model by (Kabbaj et al., 2002). It involves exposing rats to a series of social stressors throughout adolescence from PND28 for up to 28-days. Rats are exposed to one of several social stressors for 2-hr each day; these stressors are isolation, introduction of cagemates to a novel environment, crowding, housing with animals from a different litter, as well as housing with older and bigger resident rats. Another group of rats was exposed to a similar stressor model that relied on physical instead of social stressors (chronic

unpredictable physical stress) which involved exposure to one of the following stressors for 2-hrs a day: cold exposure, ether exposure, forced swim, restraint, and loud noise. Of the two protocols, the protocol prevented behavioural sensitization to amphetamine (Kabbaj et al., 2002). Chronic physical stress altered peripheral indices of HPA activity but these changes were reversed by a 3-week period of recovery in the form of stressor-free, standard housing. Specifically, animals exposed to physical stress showed increased adrenal weight and reduced thymus and body weight compared to the control and chronic variable stress groups (Isgor et al., 2004). Additionally, physical stress alone inhibited growth in regions of the hippocampus that became apparent after the 3-week recovery period. These structural deficits were accompanied by deficits in spatial navigation as assessed by the Morris Water Maze task (Isgor et al., 2004).

An additional type of the social stress model is the resident-intruder paradigm that is administered to male rats during mid-adolescence. As part of this model rats were individually exposed to a larger, more aggressive male rat (called the resident) for 60-min five times during adolescence (on PND45, 48, 51, 54 and 57) (Vidal et al., 2007) and control rats were isolated throughout the 60-min duration. Following a period of rest, rats from both groups were tested for anxiety-like behaviour; animals exposed to the resident-intruder protocol showed increased social anxiety-related behaviour although the level of monoamines in different regions of the brain including the medial PFC and hypothalamus were not different from controls (Vidal et al., 2007). In spite of its usefulness, there exist certain limitations of the social defeat model, which make it less appealing and practical for stress research. The first limitation of this model is the fact that in such a model, animals show few signs of distress until they are physically attacked by the more-aggressive conspecific. Thus, any behavioural or other biological markers of stress

become most apparent after the experimental animal has received some degree of physical discomfort and pain, which can lead to possible complications that may confound the outcome of the experiment (Blanchard and Blanchard, 1977). Moreover, this model is more usable in males than females because male rats, like most mammals, are more physically aggressive than their female counterparts. Furthermore, dominance hierarchies are more easily made and maintained in males (Blanchard and Blanchard, 1977).

The final category of naturalistic models is therefore, more preferable for stress studies as it circumvents most of the above-mentioned limitations of the resident-intruder and other models. This model and its variants involve exposure to a predator or its odour. These models trace their origin to experiments conducted a few decades ago when rats were placed in an enclosed arena similar to their natural habitat in the wild, called the visible burrow system (Blanchard and Blanchard, 1972). Rats were placed in the visible burrow system and were periodically exposed to a live cat, which was placed at the entrance of the visible burrow system (Blanchard and Blanchard, 1972). Such an exposure to a live cat resulted in an increase in the following behaviours (viewed as a sign of defensiveness in the rat): avoidance of the cat, freezing behaviour or immobility, and ultrasonic vocalizations that signal distress in the animals (Blanchard and Blanchard, 1972). Cat exposure also resulted in reduced rearing and grooming, and an increase in crouching behaviour (Blanchard et al., 1998). These behavioural changes usually last for 24-hr or longer (Blanchard et al., 1998).

Cat exposure can be performed in one of two ways. The first way is to expose rats to a non-attacking predator, which is often achieved by physically confining the cat or

shrouding it from view (Blanchard et al., 1998). Such single exposures to cats can also result in increased anxiety-like behaviours as assessed by the EPM task, which last for up to 21-days after the exposure (Williams and Barber, 1990). The second type of cat exposure involves exposure to a live, moving cat, and such an exposure results in a much greater degree of freezing (Blanchard et al., 1998). Exposure to a live cat is associated with increased activation of the amygdala, a region traditionally associated with fear (although this is true of exposure to cat odour as well) (Martinez et al., 2011).

A variant of this model involves exposure to odour related to the cat instead of the cat itself; this is likely a more practical model of use because of the possibility of rats being harmed when exposed to live cats. Moreover, the logistics of performing an experiment with a cat odour stimulus may be easier to manage than that involving a live cat. Besides, these two experiences (i.e. cat odour exposure and exposure to a live cat) share a fair degree of similarity in the responses evoked in the rat and the neural regions activated (Blanchard et al., 1990).

Cat odour can be presented in various forms. A common technique is to use soiled cat litter (that primarily contains cat urine because fecal boli are removed before use); such a protocol results in an increase in startle amplitude, and anxiety-related behaviour in the EPM task (e.g. Cohen et al., 2008). An alternative to this model uses cat fur instead of urine to stress the animals (Munoz-Abellan et al., 2008). A comparison of cat urine and fur/skin as stressors revealed that both elicited similar ACTH and CORT responses but plasma glucose levels were higher in response to the fur/skin stimulus than the urine odour stimulus (Munoz-Abellan et al., 2008). Both odour sources had little effect on the locomotor activity of the rats whereas anxiety-related behaviour in the Elevated Plus

Maze or EPM task measured 7-days after an acute exposure was higher in response to the fur/skin stimulus than the urine odour stimulus although the ACTH response to the EPM was comparable in both cases (Munoz-Abellan et al., 2008).

Predator odour can also be administered in the form of a chemical that predominantly contributes to the predator scent (e.g. trimethylthiazoline, or TMT, which is considered a key component of fox feces, and used as a predator odour model) (Staples et al., 2008). TMT elicits avoidance and freezing behaviour in rats although no risk assessment or fear conditioning behaviours are seen in response to its presence (Wallace and Rosen, 2000, Fendt et al., 2005, and McGregor et al., 2002). In a comparison of cat odour stimulus and TMT, it was found that a single exposure to cat odour, not TMT, resulted in reduced grooming and increased escape attempts (Staples et al., 2008). Moreover, only cat odour (administered in the form of a piece of collar previously worn by a cat) resulted in increased activation of fos in the accessory olfactory bulb, anterior olfactory nucleus, the medial PFC, medial hypothalamus, striatum and medial hypothalamic circuit associated with defensive behaviour (Staples et al., 2008). TMT activated internal granular layer of the olfactory bulb and the central amygdala whereas both stimuli activated the glomeruli of the olfactory bulb, the piriform cortex, ventral orbital cortex, and anterior cortical amygdala (Staples et al., 2008). It was concluded that TMT lacked the "pheromone-like quality" associated with the cat stimulus (Staples et al., 2008). A stimulus similar to TMT is 2-propylthietane, the main component of the weasel anal gland (Perrot-Sinal et al., 1999). A brief exposure to it resulted in increased production of ACTH and CORT while locomotor activity remained unchanged (Perrot-Sinal et al., 1999).

A variant of the cat fur/skin predator odour model has also been used in the Perrot Lab and involves use of a J-cloth that has been rubbed against the body of a cat as a stressor (e.g. Wright et al., 2008). This model has resulted in increased HPA activity as well as altered dopamine D2R receptor expression (refer to tables 1.2 and 1.4 for more details of the design and outcomes of this study along with a couple of others from the Perrot Lab).

In the current study, a variant of this model was used involving use of a piece of cat collar as a stressor instead of a J-cloth (both J-cloth and collar piece contain cat fur and skin as the major sources of cat odour). This model (used in the current study) involves repeated exposure to predator odour administered using a piece of a collar worn by a domestic cat for at least a fortnight (Dielenberg et al., 2001, Masoodh et al., 2008, Wright et al., 2012, 2013). It allows one to study both sexes of rats unlike some other models, which can only be used on one sex (such as the resident-intruder paradigm). Additionally, it is an ethologically relevant model (unlike some other models such as chronic restraint) that makes use of the rats' natural aversion to cats. It is therefore, more likely to make use of the rats' natural defence mechanisms. Moreover, such a predator odour model allows us to analyze the rats' behaviour *during* odour exposure unlike a lot of other models. It allows the use of behavioural biomarkers of stress to gauge the rats' response to stress as it is being experienced. In cases when multiple rats are being exposed to predator odour at the same time, this also provides a useful opportunity to study the interaction of these rats as they face the same stressor as was done in Wright et al. (2008, 2012 and 2013). The design of these studies (i.e. Wright et al., 2008, 2012, and 2013) and their key findings are outlined in tables 1.2 and 1.4, respectively. Table 1.3 outlines the design of the current study.

The model used in the current study combined the predator odour stress model described previously in this section (using a piece of collar that has been worn by a domestic cat) with the more commonly used isolation-housing model; this provides the opportunity to determine the outcome of an etiologically relevant model of stress as well as determine the role of a cagemate in ameliorating the impact of such a stress (if at all).

1.4. Rational for the different behaviours measured in current study:

The battery of tests used in this study was meant to gauge the impact of experimental treatments (namely, repeated predator odour exposure and single housing) on specific endophenotypes associated with schizophrenia (sensorimotor gating that is assessed by prepulse inhibition (PPI). In addition, the author wanted to assess changes in depression-related (anhedonia, measured using the Sucrose Preference Test, SPT) and anxiety-related behaviour (using the EPM task, and Open Field Test or OFT), as well as cognition (memory deficit, measured using the Novel Object Recognition or NOR test). This is illustrated in figure 1.6. Startle amplitude can also be interpreted in terms of anxiety-related behaviour. It can also be considered a potential endophenotype of schizophrenia because increased anxiety is often a symptom of schizophrenia, or a co-morbid condition (reviewed in Phillips et al., 2006). In short, the measures used in this study can be applied to illnesses such as schizophrenia and major depressive disorder, which are associated with childhood adversity and are likely to emerge in adolescence and early adulthood (Zahn-Waxler et al., 2008). This is summarized in the table below:

Behavioural construct	Test used to assess that behavioural construct in the current study

1. Pre-attentive filtering of information	1. Prepulse inhibition of startle
2. Anxiety-related behaviour	1. Startle amplitude 2. Open field test 3. Elevated plus maze test
3. Depression-related behaviour (specifically, anhedonia)	1. Sucrose preference test
4. Object recognition memory	1. Novel object recognition test

(Note: PPI is an endophenotype of schizophrenia. Startle amplitude is also considered a potential endophenotype of schizophrenia and anxiety-related disorders.)

Stress and its associated dysregulation of the HPA axis are considered important in the origin of schizophrenia (reviewed in Yeap and Thakore, 2005). Cortisol levels are elevated in patients at the onset of schizophrenia, and atypical antipsychotics reduce cortisol levels in both patient and control populations (Walker and Diforio, 1997). Studies in rats or mice that aim to establish a cause-and-effect relationship between stress and the onset of schizophrenia and psychoses often measure sensorimotor gating changes. Changes in startle, which is also measured as part of measuring PPI, also accompany certain psychiatric conditions such as PTSD and generalized anxiety disorders (Bakker et al., 2009) and are studied in animal models due to their perceived link to the origins of those conditions and their cross-species validity.

1.4(i) Rationale for measuring prepulse inhibition:

Prepulse inhibition is considered a potential endophenotype of schizophrenia. In order to be considered an endophenotype, a measure has to fulfill some of the following criteria such as specificity (i.e. it should be strongly linked to one or more psychiatric

conditions), heritability, state independence (i.e. it should be stable over time and independent of the course of the illness), it should occur more frequently in affected than control populations, and it should occur more frequently in relatives of patients than the control population (reviewed in Pizzagalli, 2014, and Gottesman and Gould, 2003). PPI fulfills most of these criteria, and is therefore, a likely endophenotype for schizophrenia. Changes in sensorimotor gating ability (PPI) were measured in the current study in response to adolescent stress because exposure to stressors is considered a risk factor for developing schizophrenia (reviewed in Walker et al., 2008) and other mental illnesses such as anxiety disorders and PTSD (reviewed in Swerdlow et al., 1994). Briefly, schizophrenics and patients suffering from affective psychosis show elevated basal cortisol and ACTH levels in comparison to unaffected controls (reviewed in Walker et al., 2008). Additionally, atypical antipsychotics reduce cortisol and ACTH secretion in schizophrenics and unaffected controls (reviewed in Walker et al., 2008). A negative correlation exists between elevation in cortisol levels in patients once they stop taking atypical antipsychotics and the rise in negative symptoms. These results suggest that HPA axis dysregulation may be an important etiological factor in schizophrenia, and atypical antipsychotics may be effective due to their suppression of HPA activity. Moreover, PPI is also reduced in prodromal populations (i.e. adolescents with a genetic susceptibility to schizophrenia) suggesting an etiological link between stress-related changes in PPI, and schizophrenia (e.g. Ziermans et al., 2012). In fact a reduction in PPI in such individuals with a genetic predisposition towards developing schizophrenia has been proposed as an early vulnerability marker by Ziermans et al. (2012). Compared to other potential endophenotypes like P50 suppression and Smooth Pursuit Eye Movements, only PPI was reduced in adolescents that had a familial history of schizophrenia compared to age-

matched controls (Ziermans et al., 2012). In another similar study, PPI was compared between first-episode schizophrenics, prodromals (i.e. individuals with a genetic liability to develop schizophrenia) and age-matched controls (Quednow et al., 2008). Expectedly, PPI was significantly reduced in prodromals and unmedicated first-episode schizophrenics compared to controls and medicated schizophrenics (Quednow et al., 2008). In other words, a reduction in PPI in schizophrenics compared to controls is thought to be an outcome of neural changes that are linked to the etiology of this disorder, and that eventually result in symptoms such as cognitive deficits (e.g. an inability to attend to relevant stimuli), sensory flooding and cognitive fragmentation. In fact, the change in PPI in a population of male schizophrenics was correlated with the negative and positive symptoms exhibited by the patients (Braff et al., 1999).

Additionally, PPI has cross-species validity and is, therefore, measured routinely in studies on animal models investigating the origins of schizophrenia and psychosis (reviewed in Swerdlow et al., 2001). Such studies also support the view that stress in adolescence (e.g. post-weaning isolation) causes changes in startle and PPI. Sustained exposure to stress, while resulting in beneficial long-term adaptations, can also render an individual more susceptible to developing illnesses; stress is therefore cited as a risk factor in illnesses such as major depressive disorder and PTSD (reviewed in Thapar et al., 2012).

1.4(ii) Rationale for measuring startle amplitude:

Changes in startle are seen in various mental disorders such as generalized anxiety disorder and PTSD (reviewed in Grillon et al., 1998, Grillon, 2002, Bakker et al., 2006). In fact, increased startle is listed as one of the symptoms of PTSD in the Diagnostic and

Statistical Manual of Mental Disorders IV (reviewed in Grillon et al., 1998). Early life stress such as trauma or abuse is proposed as a risk factor and an important mediator in developing these illnesses. Furthermore, it is proposed that only individuals in whom stressor exposure results in changes in startle go on to develop stress-related psychiatric illnesses such as PTSD and anxiety disorders; startle increase therefore, appears to be a biomarker for increased vulnerability to stress (Nalloor et al., 2011). Evidence from animal models supports the view that early life stress or trauma can cause changes in startle as well as an increase in anxiety-related behaviour in the EPM task (Nemeroff, 2007). Therefore, in the current study, changes in startle and PPI are used as biomarkers for aversive environmental experiences such as single housing.

1.4(iii) Need for multiple tests for measuring anxiety-related behaviour:

Animal models probing the link between stressor exposure and the emergence of increased anxiety-related behaviours also make use of classic behavioural tests of anxiety such as the EPM and OFT in addition to measuring startle amplitude. Although these tests do not assess exactly the same behaviour or underlying neural structures, there is reason to believe that there exists considerable overlap between the behaviours and neural structures involved in the OFT and EPM (reviewed in Ramos, 2008). Both the EPM and OFT tests are based on the natural conflict within rodents between the urge to explore a novel environment and the urge to avoid it for fear of predation (reviewed in Ramos, 2008). It has been suggested that these tests assess similar but distinct aspects of the anxiety construct of rats (Ramos et al., 1997). For example, while both tests are sensitive to the effects of drugs that reduce anxiety in humans (such as benzodiazepines like diazepam and chlordiazepoxide) (Ramos et al., 1997), there still exist multiple instances

of inter-test differences. For example, an anxiolytic called NKP608 resulted in reduced anxiety-related behaviour in the OFT but not in EPM in one strain of rats called the spontaneously hypertensive rats (only in males, not in females) (Vendruscolo et al., 2003). Similarly, another study revealed anxiolytic properties of the neurohoromone melatonin and the drug UCM765 in the EPM but not in the OFT (Ochoa-Sanchez et al., 2012). These results suggest the possibility that the different tests of anxiety do not assess the same phenomenon but measure different aspects of anxiety-related behaviour. On the other hand, when multiple genetic strains of mice or rats are compared for anxiety-related behaviour in the EPM task and OFT, the results of the two tests generally point in the same direction (Ramos et al., 1997). For example, both Lewis rats and spontaneously hypertensive rats show increased anxiety-related behaviour in both OFT and EPM (Ramos et al., 2008). This suggests that there is a similarity in the aspects being measured by these tests (Ramos et al., 2008). To reconcile these observations, it has been proposed that emotionality is a multi-dimensional construct and that each of these tests assesses one or more of these dimensions (Ramos et al., 1997 and 2008). Furthermore, there is reason to predict a certain degree of overlap between the dimensions assessed by these two tests (Ramos et al., 2008). It is recommended that multiple tests of emotionality be conducted in order to measure as many different dimensions of emotionality as possible. Therefore, in the current study, EPM and OFT were both used to assess the animals' anxiety levels.

1.4(iv) Rationale for using the novel object recognition test to assess recognition memory:

In the present study, the NOR test of recognition memory was also added to the battery of tests conducted on the animals because memory impairment is one of the

cognitive deficits commonly recorded in both schizophrenia and anxiety-related disorders (Andreason, 1995). Recognition memory is a type of conscious memory (declarative memory) that is defined as the ability to distinguish a novel stimulus from a previously encountered one (reviewed in Squire et al., 2007). It involves two processes: recollection and familiarity. Recollection implies remembering "specific contextual details about a prior learning episode" whereas familiarity refers to "simply knowing that an item was presented without having available any additional information about the learning episode" (Squire et al., 2007).

NOR is one of the more commonly used tests of recognition memory in rats and mice. An advantage of this task is the fact that it does not involve extensive prior training or aversive reinforcement (Warburton and Brown, 2010). It is therefore, less stressful to the animals (Warburton and Brown, 2010). Performance in this task is disrupted by lesions of the perirhinal cortex. The protocol used in the current work is a rat-version of a memory task used in humans and monkey (called the visual paired comparison task or the NOR test) that tests recognition memory. All versions of this task are based on the same principle: organisms are drawn towards a novel object compared to a familiar one if they retain the memory of the previously encountered object (reviewed in Squire et al., 2007).

Parts of the brain considered important for performance on any test of recognition memory such as the NOR, include the medial temporal lobe and medial PFC including the infralimbic and prelimbic regions (Warburton and Brown, 2010). The medial temporal lobe consists of the hippocampus and adjacent entorhinal, perirhinal, parahippocampal cortices (Holdstock, 2005). Its different regions are thought to be involved co-operatively in recognition memory (Warburton and Brown, 2010, and Squire et al., 2007) though the exact role played by individual parts in different aspects of

recognition is being currently investigated (Warburton and Brown, 2010). The involvement of the hippocampus in NOR test is somewhat controversial (Warburton and Brown, 2010) though Clark et al. (2000) showed an impairment in performance following hippocampal lesions (Note: The current study uses the same protocol as Clark et al., 2000). Ibotenic acid lesions as well as radio-frequency lesions of the hippocampus were accompanied by a significant reduction in percent preference for the novel object, although this result was true only for a 1-hr delay period (Clark et al., 2000). Performance was unaffected during delay periods shorter or longer than 1-hr (Clark et al., 2000).

1.4(iv) Rationale for using sucrose preference test for assessing depressive behaviour (anhedonia):

Finally, the SPT was used to measure anhedonia in the rats. Anhedonia refers to the inability to experience pleasure and is seen in patients suffering from major depressive disorder and also represents one of the negative symptoms of schizophrenia (Paus et al., 2008, Blakemore and Mills, 2014, Walker et al., 2008 and 2009, Braff et al., 1999 and 2005, and Zahn-Waxlet er al., 2008). Major depressive disorder and its symptoms seen in schizophrenics place a crippling burden on healthcare systems in North America (Pizzagalli, 2014). These symptoms are classified based on clinical course instead of etiology, as is the case for other mental illnesses. Research into the etiology of this disorder, however, focuses on one or few symptoms that appear to share a common biological origin (Vialou et al., 2013, and Paus et al., 2008). One such symptom is anhedonia, which is proposed as a promising endophenotype of major depressive disorder (Pizzagalli, 2014). Anhedonia fulfills many of the requirements of a potential endophenotype such as familial association, state independence, heritability, although it

does not show much specificity as it occurs in other psychiatric disorders as well such as schizophrenia (Pizzagalli, 2014). Regardless, anhedonia has now emerged as a candidate endophenotype of major depressive disorder and is used in animal models attempting to study the etiology of this disorder.

One of the most popular models of anhedonia in rats is the chronic variable stress (also referred to as chronic mild stress) model discussed earlier in this chapter (Pizzagalli, 2014). It was developed in the 1980s as an animal model of major depressive disorder with the focus being on anhedonia (Willner, 2005). It was based on an observation made by Katz about the reduction in the intake of sugary fluids by rats that had been exposed to a series of severe stressors (Willner, 2005). Chronic mild stress results in reduced preference for the sucrose solution compared to control rats (Mao et al., 2014).

A major criticism of SPT was that reduced consumption of sucrose solution in the SPT of rats exposed to chronic mild stress was likely a result of reduced body weight, and not a reflection of an inability to experience pleasure (Hill et al., 2012). This criticism has been successfully addressed in studies that calculated sucrose preference per body weight (Hill et al., 2012). In the current study, too, sucrose consumption was normalized against the body weight of the animals measured the night before the test.

Apart from chronic mild stress, other models of stress also used SPT to assess anhedonia. In this paragraph, a few studies will be discussed that have explored this question in adolescent rats. Predictable repeated stress in the form of 5-min of restraint (from PND28-56) did not alter preference in the SPT though it did reduce anxiety-related behaviour in the EPM (Suo et al., 2013). A more unpredictable stressor protocol, when administered from PND30-50 also failed to change performance in the SPT or OFT (Chaby et al., 2013). The stressor protocol used in this study was essentially similar to the

chronic variable stress protocol and involved exposure to cramped, tilted cages with soiled, damp bedding, as well as exposure to social stressors such as isolation, and crowding (Chaby et al., 2013). It appears that such a mild stressor protocol, whether predictable (as in Suo et al., 2013) or unpredictable (as in Chaby et al., 2013) does not produce anhedonia. In adult rats, on the other hand, a chronic variable stress protocol produces anhedonia (e.g. Kompagne et al., 2008) similar to a chronic unpredictable regime of restraint (Tynan et al., 2010). Brief social defeat (7-days of defeat) in adulthood too reduces sucrose preference, suggesting an increase in anhedonia (Patki et al., 2013). It can therefore, be concluded that in adult rats, both chronic variable stress as well as repeated social defeat can result in anhedonia. In adolescence, on the other hand, even profound social stressors such as isolation seem incapable of producing anhedonia, at least in males. For example, a prolonged period of social isolation (PND30-50), followed by resocialization (PND50-70) did not change sucrose preference in Sprague Dawley males (Hong et al., 2012). Females, on the other hand, showed an increase in sucrose preference indicating *decreased* anhedonia (Hong et al., 2012). Therefore, in the current study, it was hypothesized that no change would occur in sucrose preference of male rats that had been housed in isolation in adolescence, although females reared in isolation could likely show an increase in sucrose preference.

1.5. Study design and hypotheses:

The current project was conceived to determine if repeated stress in adolescence and early adulthood results in changes in measures of sensorimotor gating, emotionality, and cognition. Specifically, the project addressed the following questions:

1. Does repeated stressor exposure in adolescence affect behaviour in adulthood?

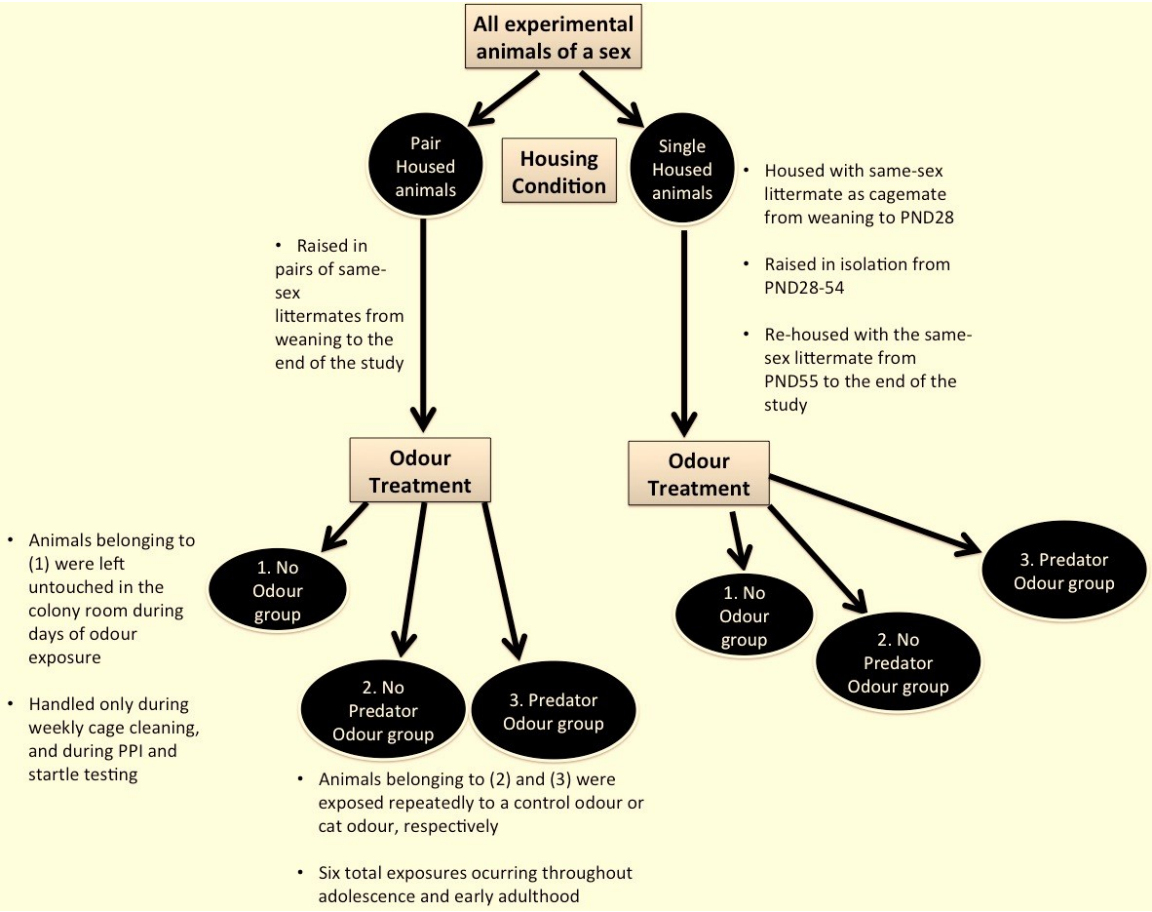
2. Does the support offered by a cagemate influence the outcome of stressor exposure?
3. Does the sex of the rat affect the outcome of (1) and (2).

The outcomes being examined here are biomarkers that have been associated with the etiology of mental illnesses such as schizophrenia, major depression and anxiety disorders. These disorders emerge in adolescence or early adulthood, have stress and a lack of social support as putative risk factors, and show sex differences in incidence and severity (as discussed earlier in this chapter). The following measures were assessed in rats in this study: measures of sensorimotor gating (PPI), anxiety-related behaviour (using EPM, OFT, startle amplitude), depression-related behaviour (SPT) and memory (NOR test). These measures are similar to certain symptoms or endophenotypic changes seen in schizophrenia (PPI, NOR), anxiety disorders (startle amplitude, EPM, OFT), and depression (SPT).

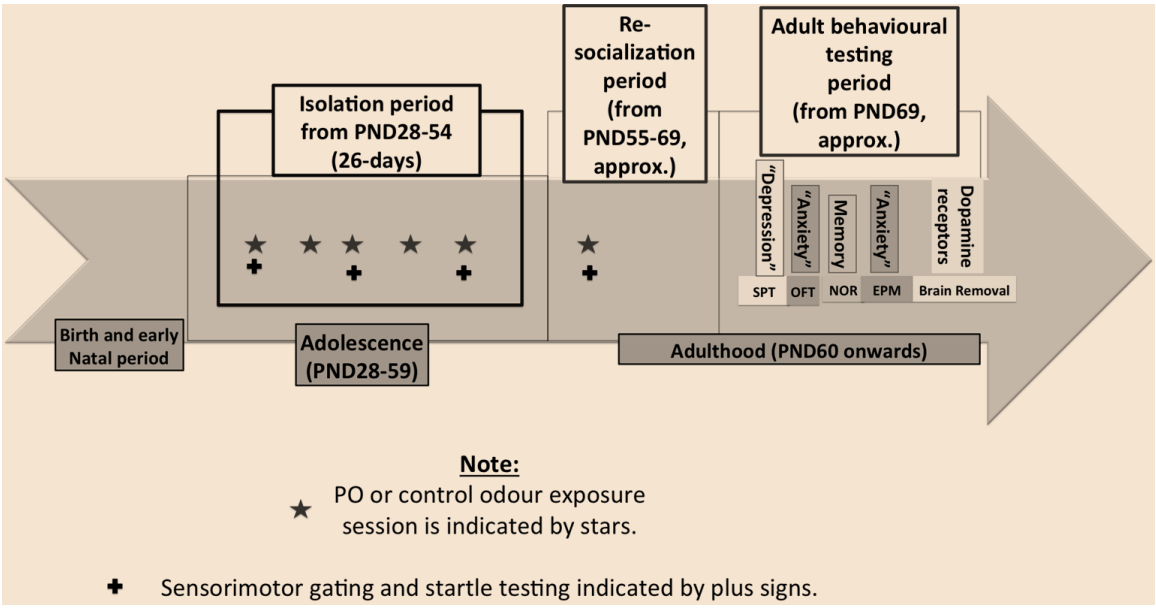
All of the complex symptoms of these disorders cannot be assessed in rats as such. Instead, some symptoms and/or neural changes common to a few illnesses are modeled in rats (Figures 1.6 and 1.7). For example, the memory dysfunction seen in schizophrenia can be assessed using any of the various memory tests available for rats such as the Morris Water Maze or the NOR test. The latter was used in the current study because it is relatively less stressful to the rats compared to a test such as Morris Water Maze task, which involves a stressful immersion of the rats in a large pool of water (Morris, 1984). Additionally, the NOR protocol uses the same arena as the OFT, and allowed us to shorten the habituation period in the NOR protocol by a day because the rats were already exposed once to the arena during the OFT (which preceded the NOR test).

In the current study, repeated exposure to cat odour was combined with isolation housing to provide an aversive environment to the animals. Both of these manipulations are ethologically relevant as they make use of the animals' innate fear of predators (cats) and need for social support. They are therefore, more likely to invoke the animals' natural response and serve as better models of the experience of adverse environments among human adolescents.

The following figure shows the different experimental groups for each sex, and explains the various housing and odour conditions used in this study. Also consult figure 2.2 and table 2.1 for details of the different experimental groups.



The following figure shows a simplified timeline of the experiments (for detailed timeline, consult figure 2.1).



It was hypothesized that animals exposed to either or both stressors (i.e. cat odour and single housing) would reveal deficits in one or more of the parameters measures. In particular, single housing was expected to cause an increase in startle amplitude and a change in PPI (either an increase or a decrease because both have been shown to occur in previous research). This is because such deficits have been consistently reported in the literature. Additionally, it was hypothesized that both single housing and predator odour exposure would result in a deficit in dopamine receptor levels in the medial PFC because repeated exposure to predator alone has been shown to alter D2R levels in the medial PFC of the adult animals (Wright et al., 2008). The addition of single housing is aimed at reducing any buffering effects that the presence of a cagemate might produce. Finally, based on previous work, it was hypothesized that single housing would cause an increased in anxiety-related behaviour (as assessed by the EPM task and OFT) whereas no change would occur in SPT performance of single housed males compared to pair housed controls, although single housed females could show an increase in sucrose preference, as discussed in the previous section. Additionally, it was hypothesized that

there would be an increase in anxiety-related behaviour in rats exposed to repeated predator odour (based on the results of Wright et al. (2012, 2013), which are summarized in tables 1.2 and 1.4.

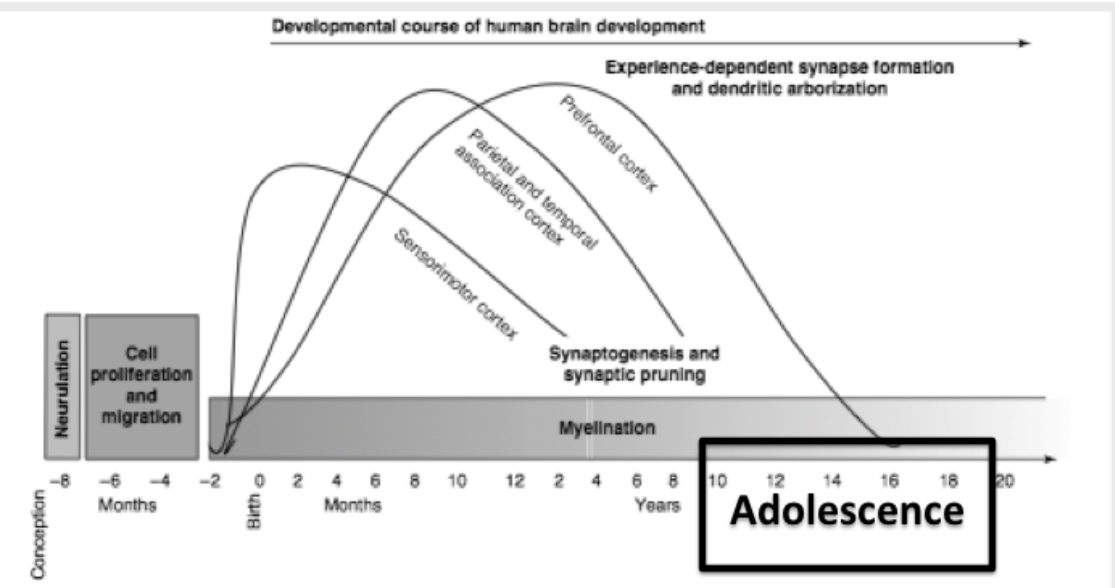


Figure 1.1. A schematic of the microscopic changes occurring in the brain throughout development (adapted from Casey et al., 2005).

A schematic of some of the key changes that occur during different stages of human brain development. These include the earliest changes that occur during pre-natal and early natal stages (such as neurulation, cell proliferation and migration), as well as ones that occur during adolescence (such as myelination and synaptic pruning). As seen in the figure, the prefrontal cortex is the site where most of the changes occurring in adolescence take place.

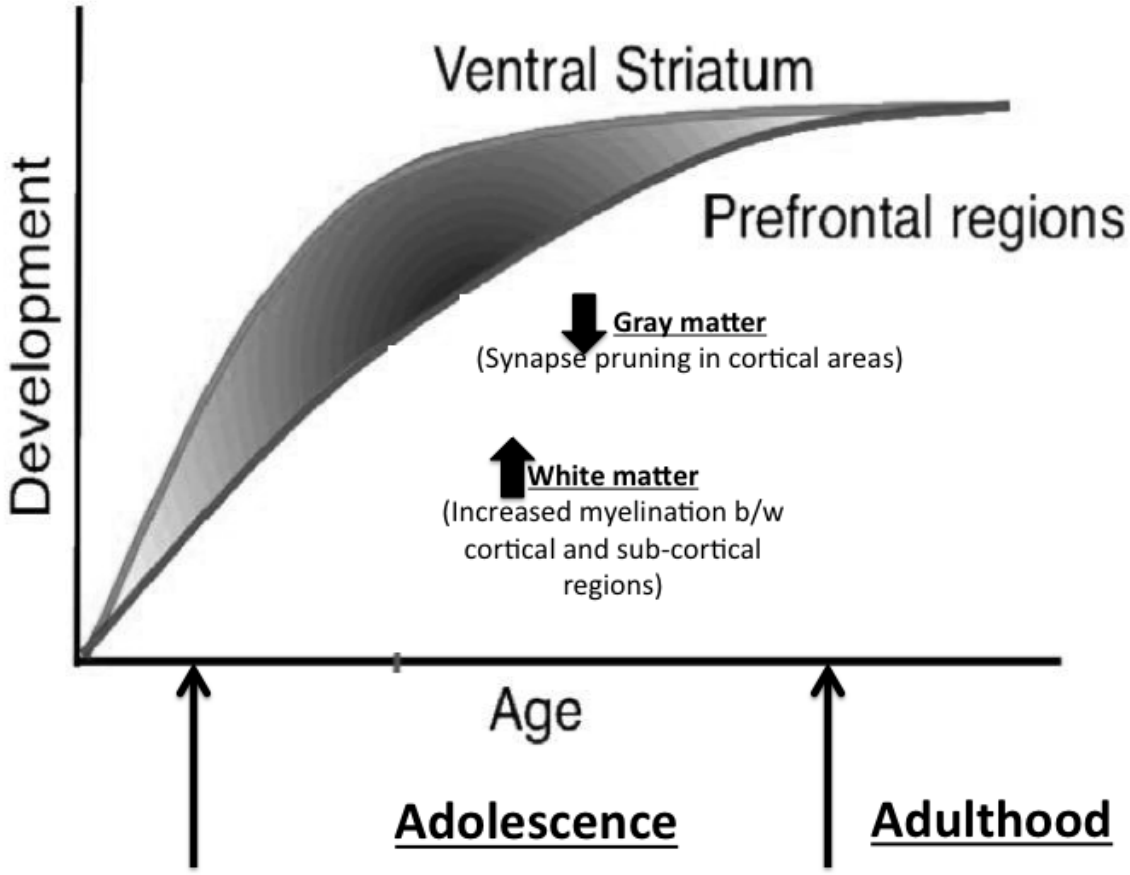


Figure 1.2. A schematic depicting the different rates of development of prefrontal cortical (PFC) and sub-cortical regions (such as the ventral striatum) in adolescence (adapted from Casey et al., 2011).

The above schematic shows the differential pace of developmental activity occurring during adolescence in the ventral striatum and the PFC. Two of the main developmental processes occurring in these regions are a reduction in gray matter volume, presumably due to pruning of synapses, and an increase in white matter volume, which is likely due to increased myelination of axons connecting the PFC and sub-cortical regions. This schematic is based on human and rodent studies of adolescent brain development. It suggests that a difference in the pace of development of the PFC and sub-cortical regions like the ventral striatum may explain the increased impulsive and risk-taking behaviour seen in adolescence.

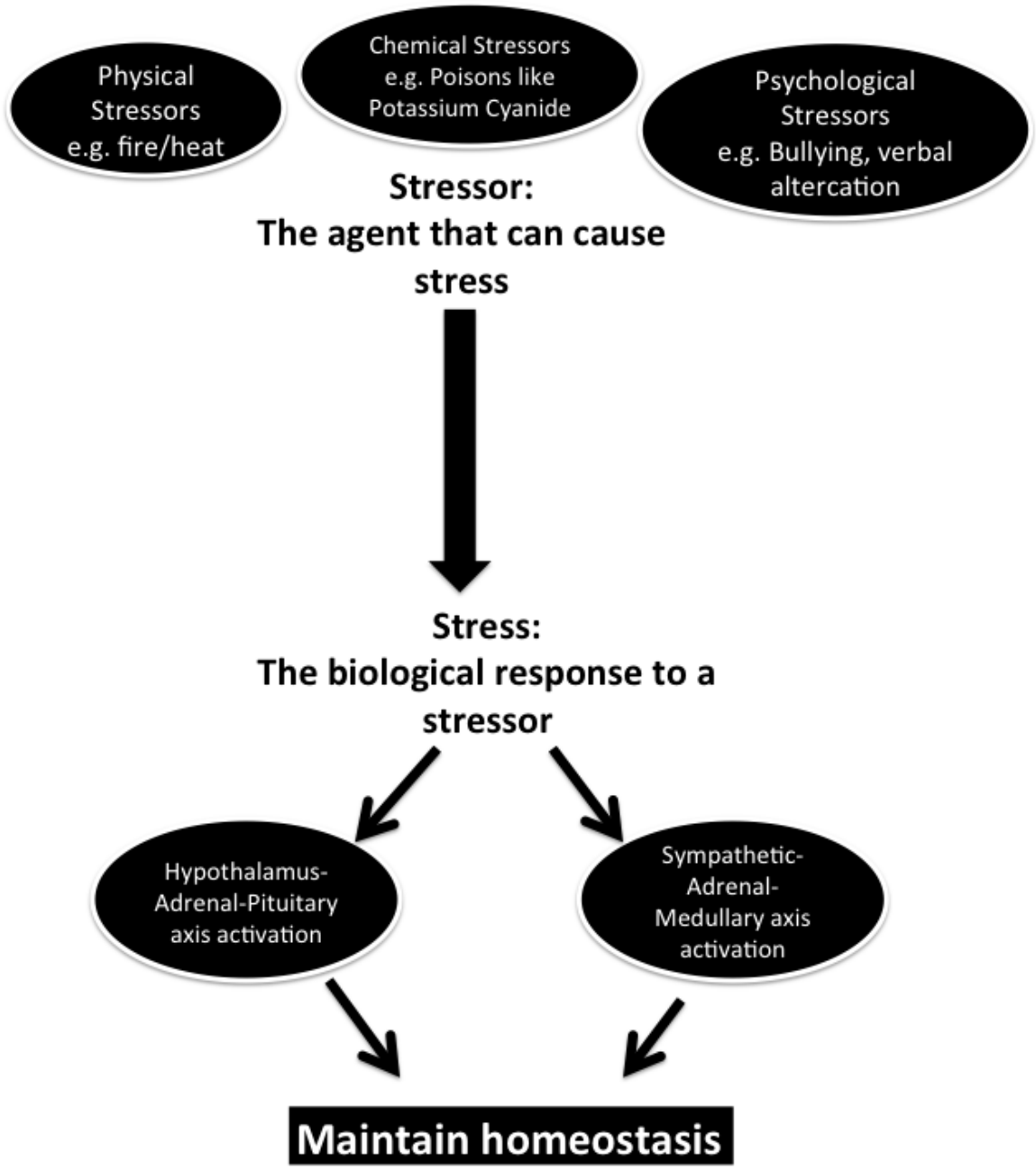


Figure 1.3. A schematic illustrating the difference between the terms "stressor" and "stress" as explained by Hans Selye (adapted from Szabo, 2012).

This figure is adapted from a schematic published in a much-lauded paper by Hans Selye on the concept of stress and the neuroendocrine system. It defines the term "stressor" as the "agent which causes stress", and "stress" as the body's physiological and behavioural response to the stressor. Stress usually entails activation of the sympathetic-adrenal-medullary axis (or the central sympathetic system), and the hypothalamic-adrenal-pituitary axis to meet the short-term and long-term demands of the body to maintain homeostasis, respectively.

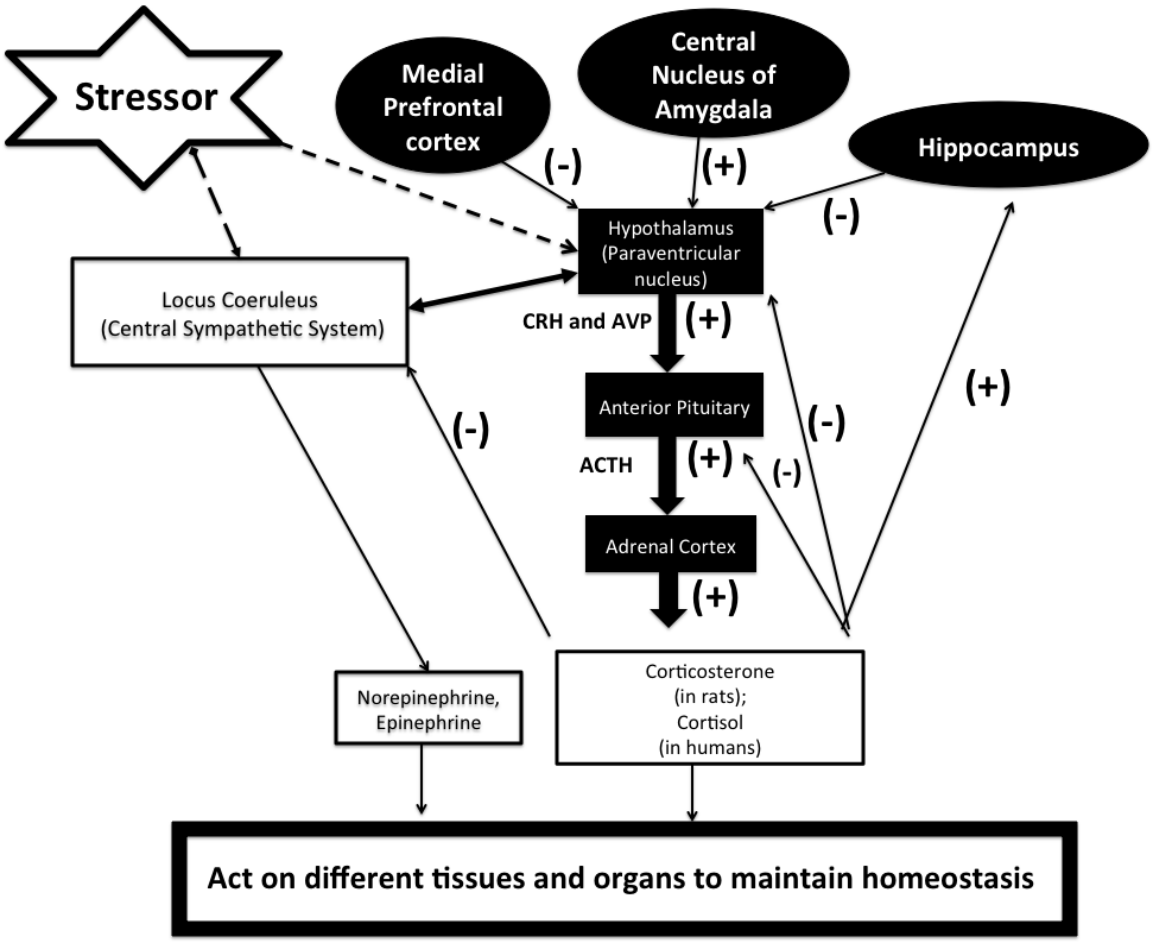


Figure 1.4. A simplified diagram of the various biological systems activated by any stressor.

A stressful experience activates the autonomic nervous systems (called Central Sympathetic System, CSS, in the figure), and the hypothalamic pituitary adrenal (HPA) axis. Both these systems attempt to counter the stressful experience as well as maintain homeostasis (adapted from Romeo and McEwen, 2006, and Tsigos and Chrousos, 2002). The HPA axis is responsible for the long-term effects of stressor exposure. The CSS is responsible for the immediate response to stressor exposure. CSS releases catecholamines like norepinephrine and epinephrine. The HPA axis comprises of the hypothalamus, anterior pituitary, and the adrenal cortex. The hypothalamus, on being stimulated by a stressor, releases corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). Both these stimulate the pituitary to release adrenocorticotropin (ACTH), which stimulates the adrenal cortex to release glucocorticoids. Corticosterone is the main glucocorticoid in rats whereas cortisol is the main glucocorticoid in humans. These act on a wide range of organs and tissues to bring about changes that help maintain homeostasis. The prefrontal cortex, amygdala, and hippocampus regulate the HPA axis, which also regulates itself through negative feedback. *(Note: "+" and "-" indicate positive and negative regulation, respectively).*

Table 1.1. A list of the primary mediators of the physiological response to stress (adapted from McEwen, 2002)

Primary mediators of the physiological response to stress:	
I. Systemic mediators:	1. Glucocorticoids (The primary glucocorticoid in humans is cortisol and in rats, corticosterone.) 2. Dehydroepiandrosterone (DHEA) 3. Catecholamines (Norepinephrine, epinephrine) 4. Cytokines (e.g. Inter Leukin (IL)-1, IL-2, Tumor Necrosis Factor-alpha (TNF- α)) 5. Systemic hormones (e.g. thyroid hormone, insulin, leptin) 6. Pituitary hormones (e.g. prolactin, adrenocorticotropic hormone or ACTH)
II. Tissue mediators:	1. Corticotrophin releasing hormone (CRH) 2. Excitatory amino acids 3. Monoamines (e.g. serotonin, dopamine, histamine) 4. Other neurotransmitters (e.g. Gamma-aminobutyric acid or GABA, glycine)
III. Other neuropeptides	1. Neuropeptide Y 2. Cholecystokinin 3. Enkephalin 4. Many cytokines (e.g. TNF- α, IL-1, IL-4, IL-6 etc.) 5. Some pituitary hormones (e.g. Prolactin)

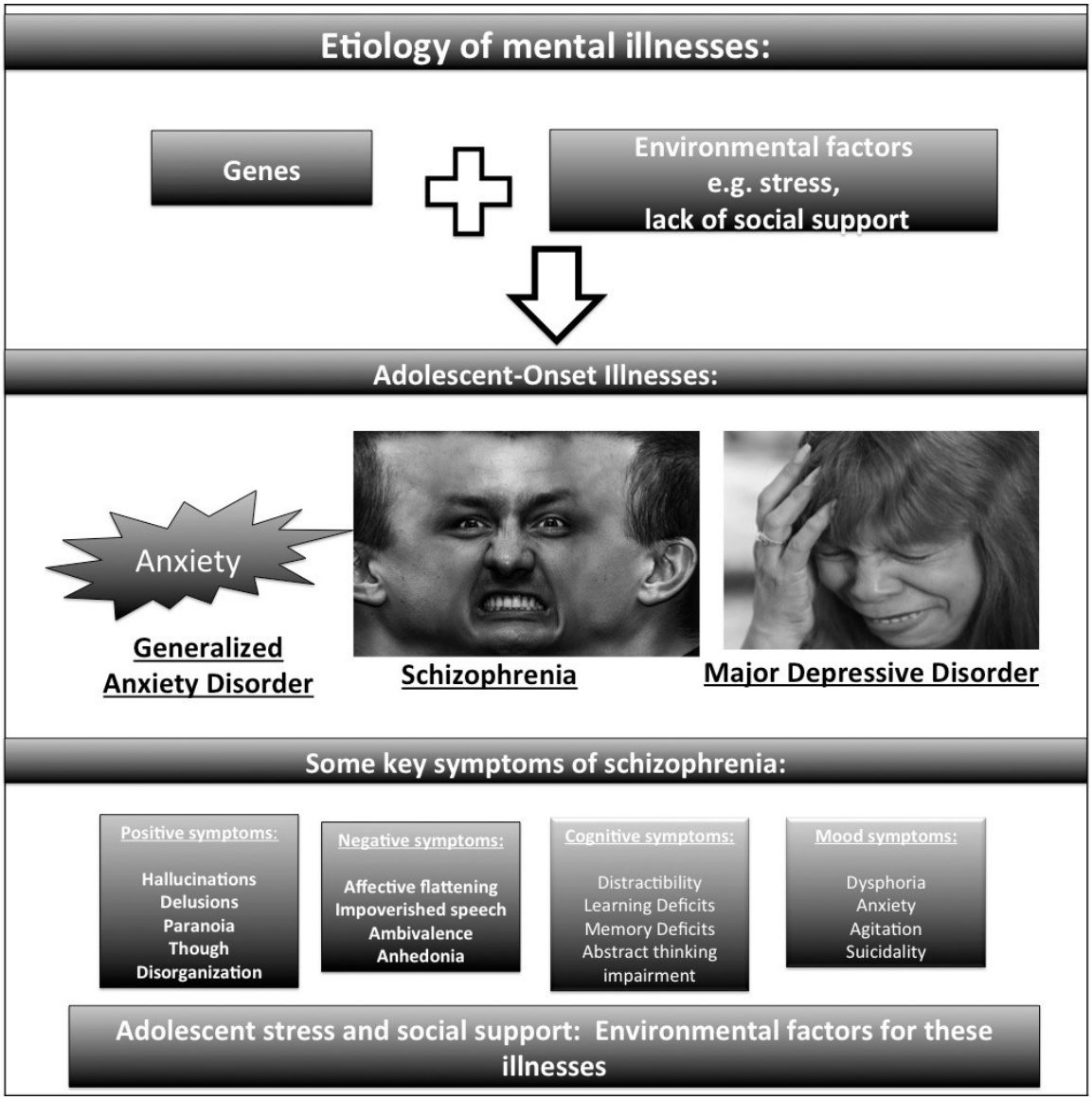


Figure 1.5. A conceptual diagram illustrating the role of both genetic and environmental factors in the etiology of adolescent-onset disorders like schizophrenia.

The purpose of this diagram is to underscore the fact that the current study is one of several attempting to understand the etiology of adolescent-onset illnesses such as schizophrenia, generalized anxiety disorder and major depressive disorder. These illnesses are a result of genetic and environmental factors, and have several disparate symptoms. Therefore, animal models are unable to model *all* these complexities. Most animal models of these illnesses are designed to model the effect of a particular genetic or environmental element on a few, often related symptoms.
(Images are from www.medicinenet.com/schizophrenia_pictures_slideshow/article.htm.)

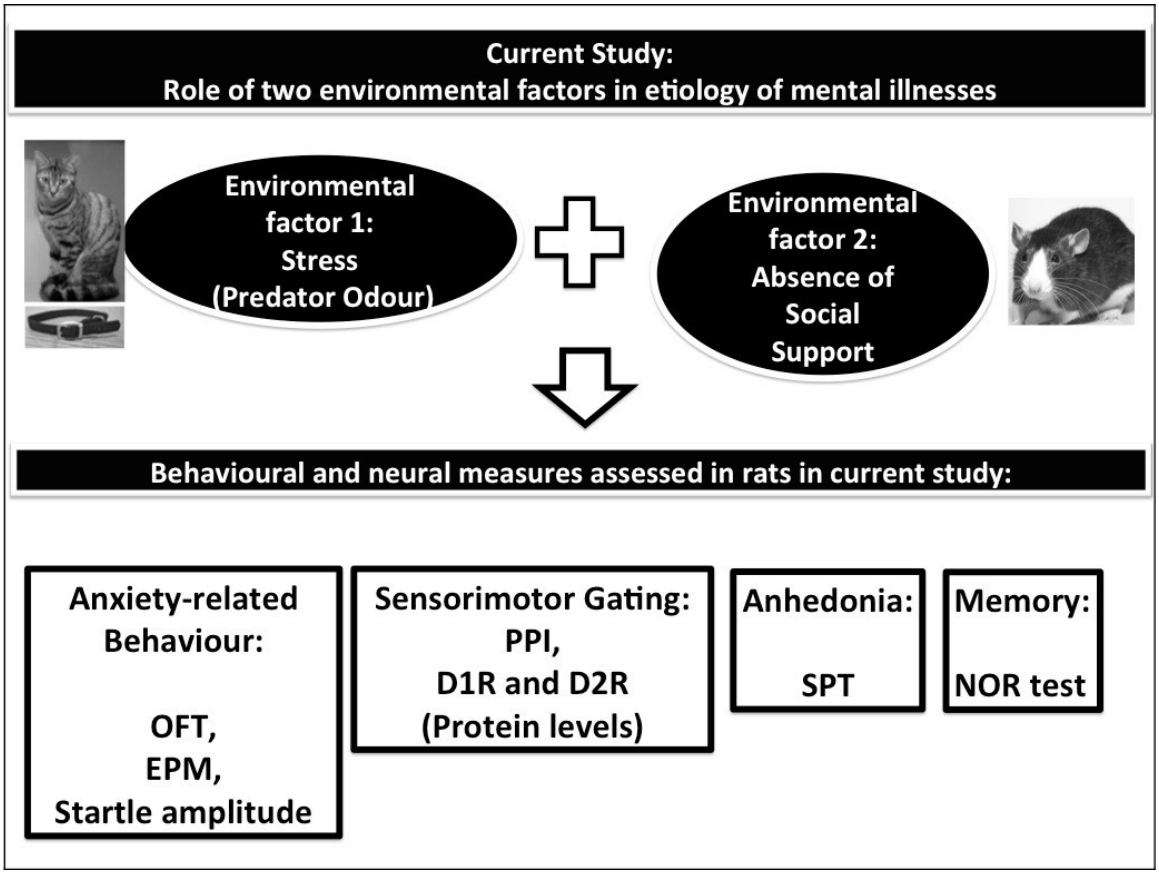


Figure 1.6. A conceptual diagram outlining the current study

The aim of the current work was to study the impact of two environmental factors- repeated stress (administered through repeated exposure to cat odour), and the lack of social support (administered through housing rats in isolation) in adolescence- on various behaviours and physiological parameters in adulthood, which are relevant to stress-related, adolescent-onset illnesses such as generalized anxiety disorder, schizophrenia and major depressive disorder. Anxiety-related behaviour was assessed by measuring startle amplitude, and behaviour in the open field test (OFT) and the elevated plus maze (EPM) task. Deficits in sensorimotor gating were assessed using prepulse inhibition (PPI) and dopamine D1R and D2R receptor protein levels. Anhedonia was assessed using the sucrose preference test (SPT) whereas recognition memory was assessed using the novel object (NOR) test.

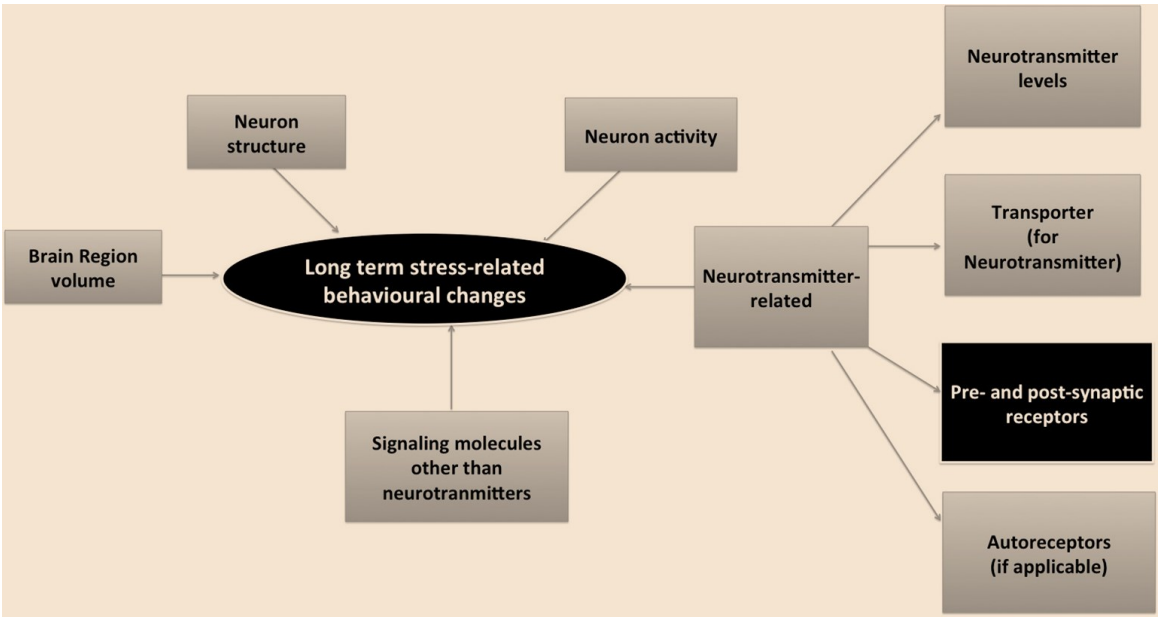


Figure 1.7. A diagram illustrating some of the potential mediators of the behavioural changes to stressor exposure.

Behavioural changes in response to long-term exposure to stressors can be the result of one or more changes, some of which are: changes in the volume of specific brain regions, or the structure or activity of neurons (and other cells like glia) in that region, the level of neurotransmitters, or receptors or transporters of these neurotransmitters, or other signaling molecules in that region. In the current work, the influence of one small cog in this complex wheel was assessed by examining the levels of dopamine D1R and D2R receptors in the medial prefrontal cortex and caudate-putamen.

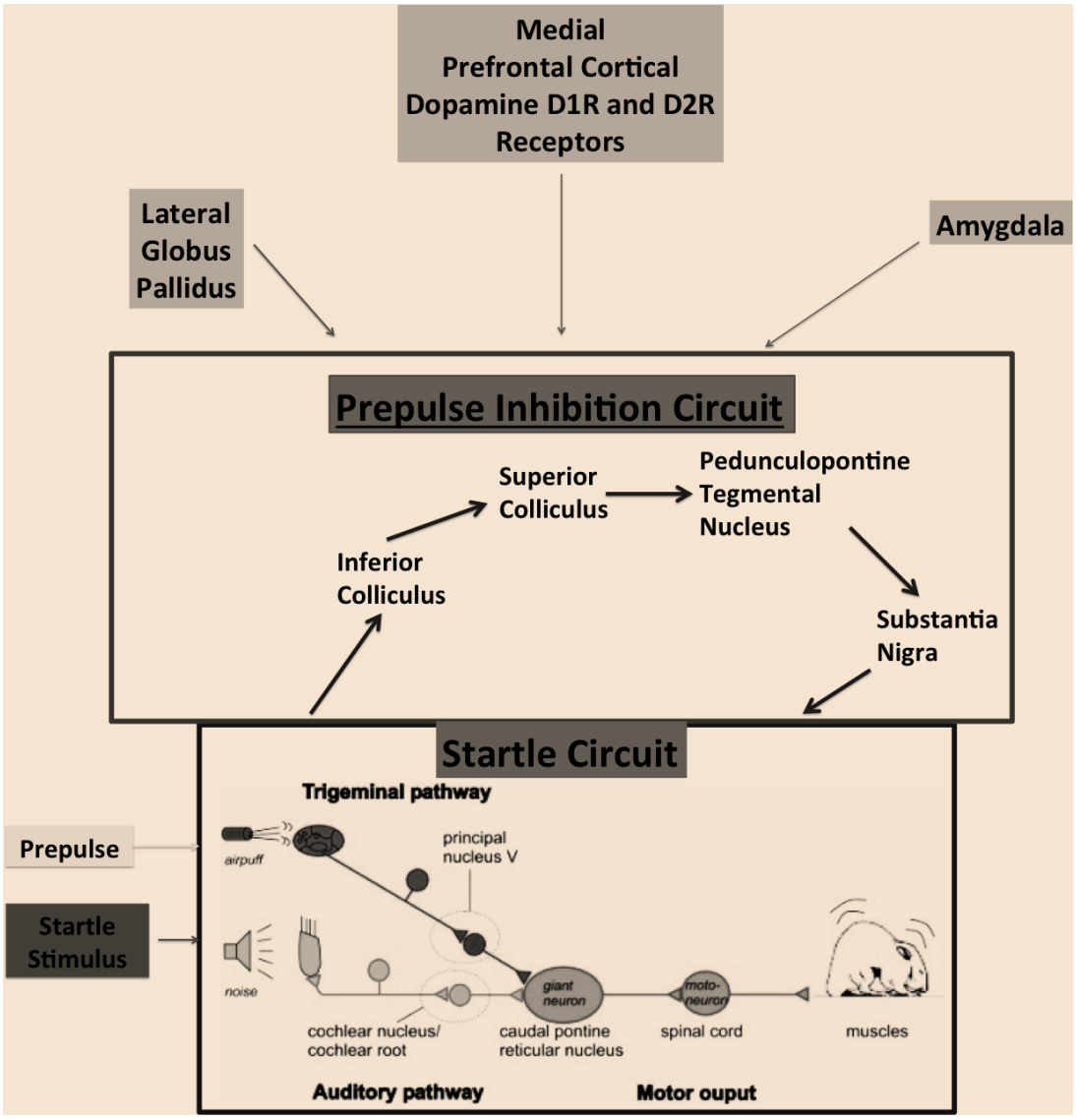


Figure 1.8. A diagram showing a simplified version of the startle and prepulse inhibition circuit in order to reveal the key neural structures involved.

Lateral glubus pallidus, medial prefrontal cortex (including its dopamine receptors), and the amygdala are believed to be key regulators of the prepulse inhibition circuit. Part of this figure is adapted from Simons-Weidenmaier et al. (2006).

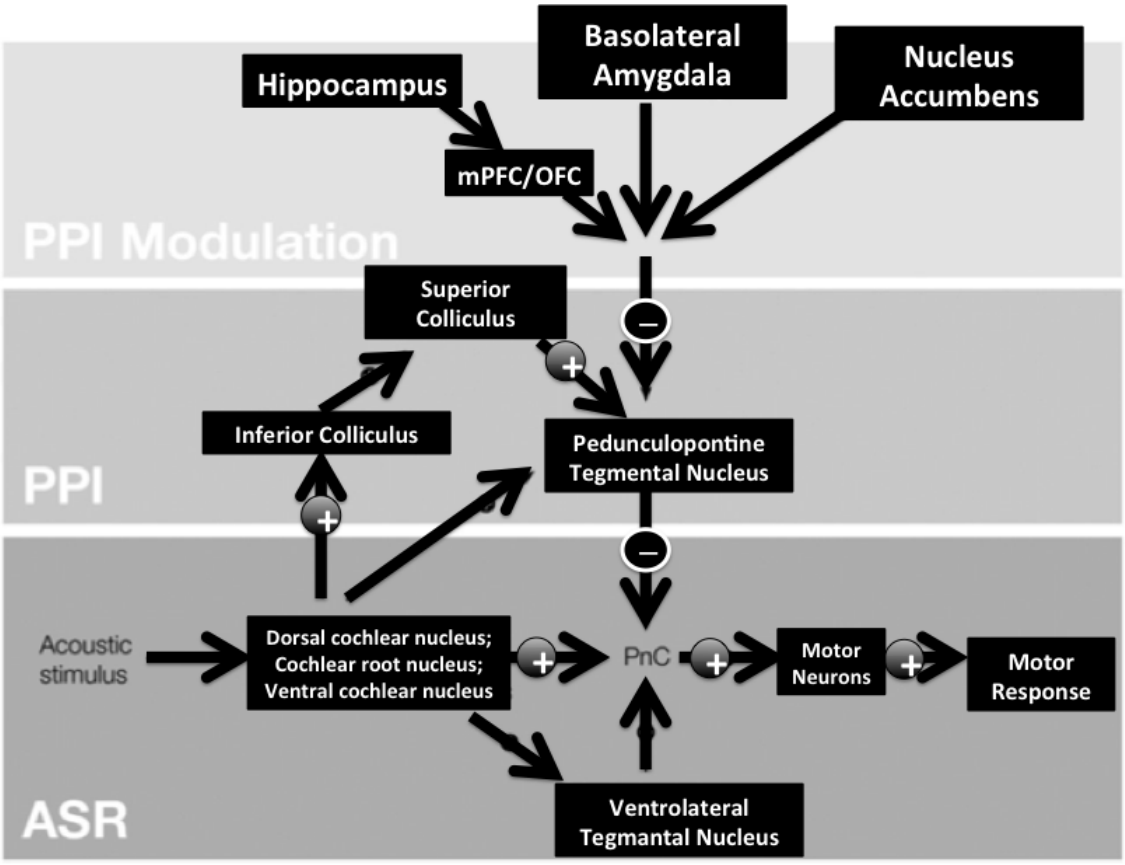


Figure 1.9. The structures involved in mediating the acoustic startle response (ASR), prepulse inhibition (PPI), and in modulation of PPI.

The circuit mediating the acoustic startle response (ASR) includes the dorsal and ventral cochlear nuclei, cochlear root nucleus, ventrolateral tegmental nucleus, and the caudal pontine reticular nucleus (PnC). The circuit mediating prepulse inhibition (PPI) includes the superior colliculus, inferior colliculus and pedunculopontine tegmental nucleus. PPI, in turn, is modulated by the hippocampus through the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC), the basolateral amygdala and the nucleus accumbens. Note: "+" and "-" refer to positive and negative regulation, respectively (Adapted from Kohl et al., 2013).

Table 1.2. A summary of the design of various studies done in the Perrot Lab using a predator odour model of repeated stress (using Long Evans rats of both sexes).

	Wright et al., 2008	Wright et al., 2013	Wright et al., 2012
Purpose of the study	Effect of repeated adolescent stress on adolescent and adult stress responding	Comparing effects of two models of repeated cat odour stress in adolescence	Comparing effects of repeated adult and adolescent stress
Odour stimulus	J-cloth	J-cloth, Collar	Collar
Age at weaning	PND22	PND21	PND21
Age at 1st, and 5th (last) exposures	PND40, and 48	PND38, and 46	PND38, and 46
Behaviours measured during first 7-min of 30-min odour exposure sessions in adolescence	• Movement b/w three regions of arena (*), • Duration and rate of entry into each region of arena (*), • Duration and rate of contact with cagemate		
Open Field Test (OFT) arena design	• Hide-box (HB) present at one end, odour stimulus at the other end (Odour Region, OR), and center (Center Region, CR) • Test was conducted over two days (20-min/day/animals) in a dimly-lit room illuminated by red light		
Behaviours measured during first 10-min of 20-min/day OFT (conducted on two days)	Duration and rate of: • Locomotion within entire open field (*), • Rearing within entire open field (*), • Grooming within entire open field, • Head-out of HB, • Odour stimulus contact, • Duration and rate within OR, CR, and HB,		
Behaviours measured during first 10-min of 25-min Predator Odour Test (PT) (conducted on 2-	Duration and rate of: • Rearing, • Grooming, • Head-out, • Odour stimulus contact, • Space use (OR, CR, HB),		

days)	• Locomotion within entire arena.		
Blood CORT levels	Blood CORT levels in response to repeated stress in adolescence (on PND48), and after PT in adulthood.		
Dopamine receptor levels	• D1R, D2R in striatum and medial PFC in adulthood • Infralimbic and dorsopeduncular regions of medial PFC used to assess dopamine receptor levels	N/A	N/A
Other behaviours assessed	N/A	N/A	N/A

(Note:"" refers to behaviours assessed in the current study as well.)*

Table 1.3. A summary of the design of the current study using a predator odour model and Long Evans rats of both sexes.

	Current study
Purpose of the study	Characterizing effects of repeated predator odour (PO) and of single housing (SH) across adolescence in rats
Odour stimulus	Collar piece
Age at weaning	PND22
Average age at 1st, and last exposures	PND32, and 62/69
Behaviours during first 7-min of 30-min/session odour exposures	Duration and rate of: • rearing, • grooming, and • collar investigation
Open Field Test (OFT) arena	• No hide box (HB), or odour stimulus in arena. Arena divided into center (C) and periphery (P) • Test conducted on a single day (5-min/animal) in a brightly lit room
Behaviours during entire duration (5-min) of OFT	Duration and rate in • C and in P • Rearing in C and in P, • Thigmotaxis
Behaviours during Predator Odour Test (PT)	N/A
Blood CORT levels	N/A
Dopamine receptor levels	• D1R, D2R in medial PFC, and caudate-putamen (striatum) in adulthood • Prelimbic, infralimbic, and dorsopeduncular sub-regions of medial PFC used to assess dopamine receptor levels
Other behaviours assessed	1. Prepulse Inhibition 2. Startle amplitude and habituation

	3. Sucrose Preference Test 4. Novel Object Recognition Test 5. Elevated Plus Maze test

Table 1.4. Key outcomes of various studies conducted in the Perrot Lab using a predator odour model.

Study authors	Behavioural test and measures used	Effect of repeated predator odour (PO) exposure
Wright et al., 2008	1. Behaviour assessed during first 7-min of 30-min odour exposure	During 1st odour exposure, the following changes were seen: 1. inhibition of locomotor activity within the odour exposure arena, 2. physical avoidance of the region of the arena containing the odour source (spending more time in part of arena furthest from odour source), and 3. increased social contact with cagemate in animals exposed to predator odour over controls Most of these were reversed or no longer significant by the final odour exposure, indicating habituation to repeated odour exposure.
	2. Behaviours during first 10-min of 20-min (per day) Open Field Test (OFT)	On the 1st day of the OFT, compared to controls, PO exposed animals: 1. spent significantly less time in center, 2. spent significantly more time in the hide box, and 3. showed a lower rate of rearing. These changes were not seen on the 2nd day.
	3. Behaviours during 25-min Predator Odour test (PT)	Animals exposed to PO in adolescence showed: 1. significantly greater rate of rearing, 2. significantly reduced duration of grooming, 3. significantly increased rate of entries to center, and to hide box, and 4. significantly increased locomotor activity. Increased locomotor activity and reduced grooming suggest that the effect of repeated adolescent stress endured into adulthood.
	4. Blood CORT levels (expressed as percent of pre-odour baseline)	1. Unchanged in males during 1st and final exposures 2. By final exposures, CORT was significantly lowered in PO-exposed females compared to controls

	5. Dopamine D1R and D2R receptor levels in medial PFC and striatum	1. Striatum: No change in D1R or D2R in response to PO exposure 2. Medial PFC: D2R was lowered in PO-exposed rats compared to controls (no change in D1R in medial PFC)
Wright et al., 2013	1. Behaviour assessed during first 7-min of 30-min odour exposure	Compared to controls, both sources showed: 1. reduced locomotor activity, 2. reduced rate of rearing, 3. reduced rate of entries to odour region (among females only), 4. reduced rate of entries to middle region of arena, and 5. rate of contact with cagemate. The two PO sources elicited some differences in responding that are listed below: 1. Collar-exposed animals spent less time in contact with the odour stimulus than controls. 2. Among males, only collar-exposed rats showed reduced rate of entries to odour region.
	2. Behaviours during first 10-min of 20-min (per day) Open Field Test (OFT)	Animals exposed to PO using a collar stimulus showed significantly reduced rate of entries to the center.
	3. Behaviours during 25-min Predator Odour test (PT)	1. Females exposed to PO using either stimulus spent significantly less time investigating the odour stimulus during the PT. 2. Females exposed to PO using the collar stimulus made fewer rate of entries to the hide box compared to controls.
	4a. Blood CORT levels in adolescence	1. Baseline CORT levels were no different between the various groups. 2. After final exposure, CORT increased only in animals exposed to PO using a J-cloth stimulus compared to controls.
	4b. Blood CORT levels in adulthood (before and after	No effect of adolescent PO treatment on baseline CORT or post-PT CORT levels in adulthood.

	acute PO exposure in PT)	
Wright et al., 2012	1. Behaviour assessed during first 7-min of 30-min odour exposure	1. Compared to age-matched controls, adolescent stressed rats show significantly lower rate of locomotor activity (not so with adult PO animals) 2. Adolescent PO-exposed animals showed significantly reduced rearing duration after the 1st exposure compared to controls. By the 5th exposure, the converse was true (no such difference in control and PO-exposed adult rats). 3. Adolescent PO-exposed rats spent significantly greater duration immobile than age-matched controls after the 1st exposure (not true of rats exposed to PO in adulthood). 4. Adolescent PO-exposed rats spent significantly less time in the odour region compared to controls after the 1st exposure (not true of rats exposed to PO in adulthood).
	2. Behaviours during first 10-min of 20-min long (per day) Open Field Test (OFT)	1. Females exposed to PO in adolescence showed reduced locomotor activity in OFT compared to controls. This effect was not seen in rats exposed to PO in adulthood. 2. Females exposed to PO in adolescence spent less time in the hide box than age-matched controls (on second day of OFT).
	3. Behaviours during 25-min Predator Odour test (PT)	1. Adolescent PO-exposed rats showed reduced duration and rate of investigating the odour stimulus compared to age-matched controls. This was not seen in animals exposed to PO in adulthood. 2. Animals exposed to PO in adolescence spent less time investigating the odour source than those exposed to PO in adulthood. 3. Adolescent odour exposure (control or PO) resulted in significantly reduced rate of entry to region containing the odour source, and increased duration spent in hide box compared to animals exposed to either odour in adulthood. 4. Adolescent odour exposure (control or PO) also resulted in reduced grooming duration compared to animals exposed to either odour in adulthood.
	4a. Blood CORT levels	1. Baseline CORT levels were not different between control and PO groups in adolescent and adult rats.

	immediately before 1st exposure and after last odour exposure	2. PO exposure elevated CORT levels significantly compared to controls (regardless of age at exposure or sex of the animals). 3. Among males, repeated exposure in adolescent resulted in significantly higher CORT than exposure in adulthood regardless of the nature of the odour.
	4b. Blood CORT levels in adulthood (before and after PT)	1. Baseline CORT levels measured a night before PT were significantly elevated in animals exposed repeatedly to PO (regardless of age at exposure) compared to baseline CORT measured immediately before 1st odour exposure. This effect was not seen in control odour-exposed rats. 2. The PT did not affect CORT levels in animals exposed to either odour in adolescence or in adulthood.
	5. Testosterone levels	Testosterone levels were unaffected by odour exposure at either age.

Chapter 2. Materials and Methods

2.1 Subjects:

2.1(i) Animal husbandry details:

Male and female Long Evans rats (approximately 55 days old, to be used as breeders to generate experimental animals) were purchased from Charles River (Quebec, Canada), and acclimated to the colony room for at least 7 days prior to any handling. They were housed in same-sex pairs (except during breeding and gestation) in 22 X 24 X 48 cm polypropylene cages with a wire lid, containing wood-chip bedding and a 5 inch black, polyvinyl-carbonate tube for enrichment. The cages were kept in a colony room with a temperature of 20 ± 1 ° C, under a reversed 12:12 light: dark cycle (lights off at 0930 h). Food (Purina Lab Chow, Canada) and tap water were available *ad libitum.* All experimental procedures followed the guidelines laid out by the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

2.1(ii) Breeding:

A sexually experienced male rat was placed into a clean standard home cage (described above) with one or two females for 7-9 days. Thereafter, males were removed and females were housed singly. Starting from the first possible day for parturition, females were checked daily for litters and the day of birth was designated postnatal day 0 (PND0). All litters generated 8-12 pups and none were culled at birth. Animals of each litter were housed in same-sex pairs from weaning (PND22) to PND28. All animals

belonging to the pair housed sub-groups continued with this arrangement while animals from the single housed sub-groups were housed singly from PND28-PND54; thereafter, these rats were re-paired with their previous same-sex cagemates.

2.1(iii) Experimental group designation:

On PND22, weanlings of each sex were randomly assigned to one of six experimental groups. Details of these experimental groups along with the abbreviations used for them throughout this thesis are provided in Figure 2.2 and Table 2.1. For each experimental sub-group, sample size is given below:

Group Name	Sample size
Pair Housed and No Odour group (Males)	15
Pair Housed and No Predator Odour group (Males)	10
Pair Housed and Predator Odour group (Males)	10
Single Housed and No Odour group (Males)	10
Single Housed and No Predator Odour group (Males)	7
Single Housed and Predator Odour group (Males)	6
Pair Housed and No Odour group (Females)	6
Pair Housed and No Predator odour group (Females)	12
Pair Housed and Predator Odour group (Females)	14
Single Housed and No Odour group (Females)	7
Single Housed and No Predator Odour group (Females)	9
Single Housed and Predator Odour group (Females)	11

Twelve litters were generated for the experiments in this thesis; every effort was made to ensure that each litter contributed equally to all the experimental groups. Individual members of a cage were identified using markings made on the base of their tails using non-toxic ink; these were reapplied weekly. *(Note: In the current study, for the most part, dependent variables for the different behavioural tests (odour exposure behaviours, PPI, startle amplitude, startle habituation, OFT, EPM, NOR, and SPT) that did not show any significant effects of the various factors are displayed in the form of graphs or tables and presented in the appendix (Appendix B) in this document.)*

2.2 Measuring biomarkers of predator odour and single housing experiences:

Behavioural responses to odour exposure (referred to as Odour Exposure Behaviours in several places in the current document) elicited by the no predator odour and predator odour conditions were measured during the 1st, 3rd, 5th, and 6th exposures, and sensorimotor gating and startle-related variables were measured immediately after these same exposures. These measures were also tested a day before the odour exposures began and a day before the 6th odour exposure.

Figure 2.1 shows a detailed timeline of these procedures. Odour exposure occurred on PND32-34 (1st), PND36-38 (2nd), PND43-48 (3rd), PND46-51 (4th), PND49-53 (5th), and PND63-69 (6th). Odour exposures, and all behavioural testing (except startle and sensorimotor gating) were done in the same room. The animals were brought to this testing room in their home cages at least 15-min to 30-min before testing began.

Table 2.2 lists the various behaviours assessed in rats during the 1st, 3rd, 5th and 6th odour exposures. For each variable mentioned on that list, a mixed-design (repeated measures) 2x2x2 ANOVA was performed for effects of Sex (male, female), Housing (pair housing, single housing), and Odour Treatment (no predator odour, predator odour). Exposure Period was the repeated measure with 4 levels because behaviours were analyzed during the 1st, 3rd, 5th, and 6th exposures. As mentioned earlier, relative changes in some of these behaviours are used as biomarkers for the predator odour experience.

Behaviours measured during odour exposure were used as biomarkers of the predator odour experience. In particular, the animal's propensity to stay as far away from the collar stimulus as possible (can be called behavioural avoidance) was used as a biomarker for predator odour exposure (reviewed in Apfelbach et al., 2005). Animals

exposed to predator odour spend less time in the odour region and more time in the third region of the arena (which is furthest away from the odour source) when compared to animals exposed to the control odour. Similarly, animals exposed to predator odour make the fewest rate of entries into the odour region and greater rate of entries into the other regions (particularly the third region) when compared to animals exposed to the control odour (reviewed in Apfelbach et al., 2005). This propensity to avoid being near the source of predator odour can be called behavioural avoidance, and is used here as a biomarker for the predator odour experience. This has been described in detail in a table in the first chapter (under the section on biomarkers of stress), and in table 2.2.

While the propensity to avoid being near the odour source can be considered a defensive behaviour, rearing and total horizontal locomotor activity could either be considered defensive behaviours (both can help the animal accumulate information about the potential threat) or exploratory (and therefore, non-defensive) behaviours (Blanchard and Blanchard, 1989, Blanchard et al., 1991). In this study, these behaviours were categorized as exploratory. Additionally, grooming can also be considered a defensive behaviour. This is usually done when the different sub-stages involved in grooming are also being measured (for more details consult Kalueff and Tuohimaa, 2004). Any stress can result in a disruption in the multi-stage grooming process, and an analysis of the various stages of grooming can therefore, be used as a biomarker of the aversive/stressful experience. In the current study, however, the different sub-stages of grooming were not investigated. This was due to limitations of the scoring program in use, which could only score whether the animal performed grooming or not.

Change in PPI and in startle amplitude were used as biomarkers for the single housing experience. This has already been described in a table in the first chapter (in the section on biomarkers of stress).

2.2(i) Behaviours measured during odour exposures:

2.2(i) (a) Odour exposure protocol:

During each odour exposure, animals in the no predator odour and predator odour groups were taken to the testing room while the ones in the no odour groups were left undisturbed in the colony rooms (except on the days of sensorimotor gating and startle testing). During an odour exposure session, animals belonging to no predator odour groups were exposed to a 2-inch piece of a brand new Petmate® cat collar handled briefly by the experimenter using bare hands (source of non-threatening odour). Animals in the predator odour group were exposed to a piece of collar that had been worn by a domestic, reproductively active female cat for at least a week. Once removed from the cats, the collars were cut up into approximately 2-inch pieces and placed in double Ziploc bags in a -20^0 C freezer until use. Collars worn by multiple cats were cut and put in a bag together to reduce the probability of the rats being exposed to the scent of the same cat during subsequent exposures; this prevents the animals from habituating (Staples et al., 2008). Collar pieces used for no predator odour groups were stored in a similar way albeit in a separate part of the freezer to avoid contamination. All collar pieces were brought to room temperature before being used on the day of odour exposure. All predator odour exposures occurred after the no predator odour exposures and in a separate arena. Following each exposure, the arenas were washed thoroughly with non-scented soap and rinsed with water.

Each exposure session lasted 30-min and occurred during the dark (active) phase of the light: dark cycle (approximately 1300h) and was carried out in a transparent Plexiglas arena measuring 35.5cm X 27cm X 60cm. The arena was placed on top of a wooden desk in the testing room (see figure 2.3 for a picture of the odour exposure arena). Before placing the animals into the arena, the appropriate odour source was placed into an alligator clip attached to one of the end walls (centered 6.5cm from the top of the arena). Pair Housed animals were exposed to the relevant odour along with their cagemate because even brief periods of isolation can cause substantial stress to the animals.

For two consecutive days before odour exposures began (approximately PND30 and 31) animals from the no predator odour and predator odour groups were habituated to the testing room and the odour arena for 30-min in order to reduce the stress generated by these two parameters.

2.2(i) (b) Quantifying behaviours during odour exposure:

The entire 30-min exposure session was recorded for the 1st, 3rd, 5th, and 6th exposures using two Sony 8mm digital cameras (CCD0TRV65 or CCD0TRV108) placed on either side of the arena. The first 7min of these videos were scored for various behaviours similar to a previous study (Wright et al., 2008). *(Note: While scoring the videos, the experimenter was blind to the experimental treatments that the animal had received. Each video was tagged by a unique identification number that was assigned to the animal featured in the video. After scoring was completed, information about the experimental treatment meted out to the animals was revealed to the scorer. This is true of the behaviours scored in all other behavioural tests as well.)*

The odour arena was divided into three virtual regions of approximately equal area using two virtual lines for the purpose of scoring certain behaviours (Figure 2.3). The region containing the odour stimulus was referred to as the Odour Region. The region adjacent to the odour region was called the Middle Region, and the region furthest from the odour region was called Third Region.

Horizontal locomotor activity within the *entire* arena was assessed using the number of times the rats moved into a new sub-region of the arena (odour region, middle region or third region) by crossing one of the two virtual lines (Figure 2.3). This serves as a measure of movement displayed by the rat during odour exposure.

In addition, the time spent and total number of entries into each of the three regions were also assessed. An animal was said to occupy a region of the arena when its forelimbs *and* hindlimbs were within the boundaries of that region. Duration spent in, and the number of entries into the odour region vs the third region was used to gauge behavioural avoidance. In general, animals exposed to cat odour spend less time in the odour region alongside an increase in blood CORT levels (Wright et al., 2008, 2012, and 2013). This behaviour is, therefore, considered a biomarker of predator odour exposure.

In addition to the above, another defensive behaviour- collar investigation- was measured. Collar investigation behaviour is defined as sniffing, biting, touching or licking of the collar piece.

Grooming and rearing were also measured. Grooming is defined as a series of uninterrupted movements using the mouth and paws to clean the body (involving licking, biting, and/or rubbing of any part of the animal's body using its mouth or paws) (Wright et al., 2008).

Rearing was said to occur when the animal stood upright on its hind limbs (it could rest its forelimbs on one of the walls of the arena). Instances when the animal was upright but grooming were excluded.

For all the above behaviours, total duration and number of events were measured. For collar investigation and grooming, additional measures were assessed: latency to initiate the behaviour, duration of the shortest and longest bouts, and duration of an average bout. This information is summarized in table 2.2.

All behavioural scoring was performed using the Observer XT 10.1.548 software (2010) (Noldus, Wageningen, the Netherlands).

2.2(ii) Measuring sensorimotor gating (prepulse inhibition), startle amplitude, and startle habituation:

As previously mentioned, startle and PPI were used as biomarkers for the single housing experience. In general, single housing results in a change in startle amplitude and/ PPI (reviewed in Swerdlow et al., 2001). The following sub-sections will provide details of how this was achieved.

2.2(ii) (a) Startle and prepulse inhibition testing apparatus:

Startle and PPI testing were performed in commercially available startle chambers (San Diego Instruments, San Diego, CA) (Figure 2.4). Prior to testing, an animal was placed inside a clear Plexiglas cylinder, which was mounted on a white Plexiglas platform kept inside a wooden, soundproof chamber (Figure 2.4). A high-frequency loudspeaker located directly above the Plexiglas cylinder was used to deliver the various sound stimuli. Any body movement produced by the animal in response to a sound stimulus was measured using a piezoelectric unit mounted at the bottom of the Plexiglas platform that

sent an analog signal to the computer. This was then digitized and stored in the computer (expressed in milliVolts or mV).

2.2(ii) (b) Startle and prepulse inhibition testing procedure:

Startle and PPI testing was performed immediately after 1st, 3rd, 5th, and 6th odour exposures for all experimental groups. For no predator odour and predator odour sub-groups, this was also done on the second day of habituation to the odour arena, as well as a day or so before the final odour exposure. *(Note: The PPI measured a day before the 1st exposure and a day before the 6th exposure is to as "baseline" PPI because during the pilot study for this project, this "baseline" PPI was used to normalize PPI measured immediately after the odour exposure sessions. This was done to minimize variability in the data, though it was discontinued.)*

Appendix 1 lists the trial-wise details of each testing session (also consult Figure 2.5). Each testing session consisted of a 5-min period of acclimation during which only background stimulus was present (65dB sound pulses), followed by 52 acoustic trials, which were presented in pseudo-random order. These trials included 22 Startle Alone trials in which the 120dB startle stimulus was presented for 40ms. Of these, 5 startle trials were presented at the beginning of the testing session (called Block 1 trials, i.e. trials# 1 to 5 in Appendix 1) and 5 at the very end (called Block 3 trials, i.e. trials#58 to 62 in Appendix 1) while the remaining 12 were presented randomly interspersed between the 30 Prepulse and 10 No Stimulus trials which comprised the remaining test session (all these trials formed the Block 2 trials, i.e. trials# 6 to 57 in Appendix 1).

Of the 30 Prepulse trials, 10 were devoted to each of the three prepulses used- 68dB, 71dB and 77dB. During a prepulse trial, the specific prepulse was presented for

20ms; 100ms afterwards, the 120dB startle stimulus was presented for 40ms and the animal's response measured (protocol from Powell et al., 2002). Since the three prepulse (68dB, 72dB and 77dB) trials were respectively 3dB, 6dB and 12dB above the background, they are referred to as 3dB, 6dB and 12dB Prepulse Trials in this thesis. The machine was calibrated regularly to maintain accuracy (protocol adapted from Powell et al., 2002).

2.2(ii) (c) Data analysis:

For each trial in each testing session, two measures were produced: a V_{max} and a T_{max} (Figure 2.5). V_{max} refers to the maximal response produced by the animal to the stimulus in a particular trial, while T_{max} indicates the latency to achieve that maximum response. The table below lists the PPI-related dependent measures assessed in this study. Details of the formulae used in calculating them and statistical analyses performed on them are provided in table 2.3.

I. PPI (Measured immediately after the 1st, 3rd, 5th, and 6th exposures)
1. PPI for 3dB prepulse 2. PPI for 6dB prepulse 3. PPI for 12dB prepulse 4. PPI (Average of all prepulses)
II. "Baseline" PPI *(Measured a day before 1st exposure and 6th exposure)*
1. "Baseline" PPI (3dB prepulse) 2. "Baseline" PPI (6dB prepulse) 3. "Baseline" PPI (12dB prepulse) 4. "Baseline" PPI (Average of all prepulses

The table below lists the startle-related dependent measures assessed in this study. The details of the formula used to calculate them and the statistical analysis performed on each are listed in table 2.4.

Startle amplitude-related:
1. Startle amplitude (After consecutive startle-alone trials) 2. Startle amplitude (After non-consecutive startle-alone trials) 3. Startle amplitude (After *all* startle trials, that is both consecutive and non-consecutive startle-alone trials) 4. Startle Habituation (SH) 5. No stimulus response
(Note 1: Consecutive and non-consecutive startle-alone trials are part of the same testing protocol. Consecutive startle-alone trials are part of Block 1 and Block 3 in the protocol whereas non-consecutive startle-alone trials are parts of Block 2 trials.) *(Note 2: No Stimulus response refers to the average response to the No Stimulus trials in the prepulse inhibiton testing protocol.)*

The baseline movement of the animal in response to the background noise alone was calculated by averaging the animal's response to the No Stimulus trails. In addition, T_{max} data were also analyzed to determine the effect of experimental treatments on the latency to produce the maximal response during various prepulse trials. The following table summarizes these dependent measures. Details of the formula used and statistical analyses are given in table 2.5.

Latency to reach maximum response during prepulse trials (Calculated using T_{max} data for each prepulse trial)
1. T_{max} at 3dB prepulse 2. T_{max} at 6dB prepulse 3. T_{max} at 12dB prepulse 4. Average T_{max} at all prepulses

2.3 Dopamine D1R and D2R receptor analysis in the medial prefrontal cortex and caudate-putamen:

2.3(i) Collection of brain tissue:

The day following the last round of behavioural testing, animals were placed into a CO_2 chamber until unresponsive to a toe pinch, after which they were decapitated, and brains quickly removed. Animals used in experiments performed during the period of 2011-2013 were rendered unconscious and unresponsive to toe pinch by an overdose of Euthanyl (sodium pentobarbital) prior to decapitation. This was done due to a change in the guidelines set out by the Canadian Council on Animal Care.

Whole brains were flash-frozen and stored at -80^0 C until microdissection. Samples were thawed to -15^0 C in a temperature controlled cryostat chamber and microdissected using razor blades and a Kopf® Rat Brain Blocker. Prefrontal cortical (including the prelimbic, infralimbic, and dorsopeduncular regions) sections were microdissected between +2.2 and +3.2 relative to Bregma (Paxinos and Watson, 1998). The striatum (caudate-putamen) was sectioned as well from sections dissected between +1.7 and -0.40 Bregma. Appendix C shows relevant images from Paxinos and Watson (1998).

2.3(ii) Western blot analysis of the brain dopamine D1R and D2R receptors:

Samples were homogenized in a fixed amount of lysis buffer (40µl for PFC samples and 100µl for caudate putamen samples). The lysis buffer consisted of 50 mM Tris-HCl, 0.25% Na-deoxycholate, 1% w/v Triton X-100, 150 mM NaCl, 1 mM EDTA, 1mM activated Na-orthovanadate, and a protease inhibitior cocktail (1 µg/ml aprotinin, leupeptin and pepstatin; 1mM phenylsulfonyl fluoride). Homogenizing involved

manually disrupting the tissue using Teflon-pestles after lysis buffer was added to each tube. Samples were then centrifuged at 3000g at 4^0 C for 30-min and the supernatant was removed and analyzed for total protein content using a Bradford Assay.

Western blotting protocol was adopted from Wright et al. (2008). A fixed amount of each protein from each sample (30µg) was loaded into individual wells of a 17% SDS-polyacrylamine gel. This fixed amount of protein was determined by performing a series of Western blots using a control sample at different primary antibody dilutions. Such optimization was performed for each of the primary antibodies used in this study.

After electrophoresis was complete, the separated proteins were transferred to a 0.2µm polyvinyl difluoride membrane (Trans-Blot Turbo Transfer Pack from Biorad). Once the transfer was complete, membranes were washed in 0.1 M Tris-buffered saline containing 0.05% Triton X-100 (TTBS) and then blocked for 60-min at room temperature using a 5% solution of non-fat milk. Membranes were incubated over-night at 4^0 C in a 1% milk solution made in TTBS-containing anti-D2R (Santa Cruz; 1µg/ml) and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; Chemicon; 25ng/ml). The next day, the membranes were washed 3X in TTBS and then incubated in the secondary antibodies (goat anti-rabbit, 1:1,000; goat anti-mouse horseradish peroxidase-conjugated antibodies, 1:10,000) for 30-min. This was followed by three rinses in TTBS, two in TBS, and then membranes were imaged using a Chemidoc XRS+ system (Biorad) using enhanced chemiluminescence. Dopamine receptor D1R was detected as a band at 74kDa (another band was detected in samples at around 65kDa but was not considered in the analysis because of the absence of information in the literature about such a band. Moreover, the 74kDa band was darker and more consistently visible). Dopamine D2R

was seen as a band of about 50kDa while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected as a 36kDa band.

2.4. Measuring anxiety-related behaviours:

For this section and the subsequent behavioural sections, all behavioural scoring was done using the Observer XT 10.1.548 software (2010) (Noldus, Wageningen, the Netherlands). While scoring videos, the experimenter was kept blinded to the experimental treatments used on the animals. This was accomplished by tagging each video using a unique animal identification number. After scoring was completed, information about the experimental treatment meted out on the animals was revealed to the scorer.

2.4(i) Open field test protocol:

The open field test (OFT) was conducted using a black Plexiglas arena with a grid of white lines drawn on the floor (79 cm x 79 cm x 35.5 cm; Figure 2.6) (protocol adapted from Perrot-Sinal et al., 2004). Two such arenas were placed in the same room to allow us to test two rats at the same time. The test room was lit with two bright, fluorescent overhead lights (one above each arena) and the entire session was recorded using Sony 8mm digital cameras (CCD0TRV65 or CCD0TRV108). Each animal was placed into the arena for 5-min.

Four virtual lines (shown in white (thick font) in Figure 2.6) were used to divide the arena into a center and a periphery for the purpose of quantifying behaviour. Total duration spent in, and number of entries into, each region was measured; an animal was

considered to occupy a region when its forelimbs and hindlimbs were within the boundaries of that region.

Both the center and the periphery of the open field were further divided into sub-regions using the grid of white lines that were painted on the floor of the arena (Figure 2.6). Movement within a region was gauged by the frequency and rate of crossing these lines. Movement within the *entire* open field was assessed by summing the movement within the center, and the periphery.

Thigmotaxis is defined as the propensity to remain in close contact with the walls of an arena; in the wild, this would minimize the chance for predation (Simon et al., 1994). The duration, number and latency to display thigmotaxis were measured when at least some part of the animal's body (except the tail and vibrissae) was in contact with one of the four walls of the arena (as observed by the experimenter while scoring the videos); instances when the animal's forelimbs are rested on the walls of the arena are excluded as they form part of other behaviours such as rearing and escape attempts. Additionally, rearing was measured to assess exploration in the open field. As previously mentioned in this chapter, rearing occurred when the animal stood upright on its hind limbs (it could rest its forelimbs on one of the walls of the arena). Instances when the animal was upright but grooming were excluded.

All the behaviours mentioned above are summarized in table 2.6. For each of these measures, a 2x2x3 ANOVA was performed to determine the effect of Sex, Housing, and Odour Treatment.

All behavioural scoring was done using the Observer XT 10.1.548 software (2010) (Noldus, Wageningen, the Netherlands).

2.4(ii) Elevated plus maze test protocol:

A wooden elevated plus maze (EPM) with 50cm long and 10cm wide arms joined by a central platform (10cm x 10cm) was used in this study (adapted from Mitra et al., 2009). Forty cm high walls protected the two closed arms whereas the remaining two open arms were unprotected with only a 3cm high edge around them. The test was conducted in a well-lit room and the entire test session was recorded using two Sony 8mm digital cameras (CCD0TRV65 or CCD0TRV108) Each animal was placed in the central platform while facing one of the open arms at the beginning of the test and returned to its home-cage 5min later. The videos recorded were used to analyze the time spent and the amount of locomotor activity and exploratory behaviours displayed in the different regions of the arena. All behavioural scoring was done using the Observer XT 10.1.548 software (2010) (Noldus, Wageningen, the Netherlands).

The percent duration spent in and rate of entries into each of the three regions of the EPM (center, open arms, and closed arms) was calculated. An animal was said to be in a region of the EPM (open arms, closed arms or center) if both its forelimbs and hindlimbs were within the boundaries of that region. These measures were shown for closed arms and center whereas the measures for open arms were not graphed as such. This was done because it seemed more prudent and conventional to express the duration and rate of entries into the open arms as a fraction of the time and rate of entries into the rest of the EPM. Therefore, the relative duration spent in the open arms, and the relative entries made into the open arms was calculated using the following formulae:

Relative duration in open arms= Percent duration in open arms/ (Percent duration in center + Percent duration in closed arms + percent duration in open arms)

Relative rate of entry into open arms= Rate of entry into open arms/ (Rate of entry into center + Rate of entry into closed arms + Rate of entry into open arms)

These two measures were graphed and analyzed statistically for the effect of different factors. The following formula was used to calculate an Anxiety Index (AI) that integrates the above two measures into a single measure (adapted from Cohen et al., 2012):

Anxiety Index (AI)= 1- [{(Relative duration in open arms) + (Relative rate of entries into open arms)}/2]

AI was also graphed and analyzed statistically for the effect of different factors. Additionally, percent duration and rate of three exploratory behaviours was also measured. These are defined below.

Rearing occurred when the animal stood upright on its hind limbs (it could rest its forelimbs on one of the walls of the arena). Instances when the animal was upright but engaged in grooming were excluded.

Head Dipping occurred when the animal was sitting in the Center or an Open Arm with its snout peering over the edge of the region.

Risk Assessment, another exploratory behaviour, involved the animal sitting within or close to the Center while stretching the front half of its body to investigate the region ahead.

These dependent measures are summarized in the table below.

Type of behaviour	Specific dependent measures

(a) Anxiety-related measures	1(i). Relative duration in open arms *(Calculated using percent duration in open arms, as well as 2(i) and 3(i))* 1(ii). Relative rate in open arms *(Calculated using rate of entry into open arms, as well as 2(ii) and 3(ii))* 1(iii). Anxiety Index *(Calculated using 1(i) and 1(ii))*
(b) Locomotor activity-related measures	2(i). Percent duration in closed arms 2(ii). Rate of entry into closed arms 3(i). Percent duration in center 3(ii). Rate of entry into center
(c) Exploratory activity-related measures	4(i). Percent duration of rearing 4(ii). Rate of rearing 5(i). Percent duration of head dipping 5(ii). Rate of head dipping 6(i). Percent duration of risk assessment 6(ii). Rate of risk assessment

For each dependent measure in the EPM test, a 2x2x3 ANOVA was performed to assess the effect of Sex, Housing, and Odour Treatment. The only exceptions were percent duration spent in the open arms and rate of entries into the open arms. As mentioned earlier in this section, these measures were not analyzed statistically (or shown in this thesis) because they were used to calculate the relative duration and relative rate of entries into the open arms.

2.5 Measure of depression-related behaviour:

2.5(i) Sucrose preference test protocol:

The animals were first acclimated for a week to a 1% sucrose solution made using tap water (w/v) (Kompagne et al., 2008). During this time, two bottles were placed on each cage - one contained tap water and the other contained the 1% sucrose solution. The

position of the bottles was switched daily to prevent the rats from making an association between the nature of the solution in the bottle and its position.

Animals were weighed the night before the test; this data is presented in the next chapter. On the day of the test, the rats were deprived of food and water for 5-hrs. Thereafter, they were placed individually into new cages and given access to the two bottles: one containing sucrose solution and the other containing tap water. The animals were left undisturbed for 60-min after which they were returned to their home cages. Both bottles were measured before and after the test to calculate the weight of sucrose solution, and tap water consumed. Sucrose preference was calculated according to the following equation:

Percent sucrose preference = (Total sucrose soln. consumed (g))/ (Total sucrose soln. consumed (g) + Total tap water consumed (g)) × 100

2.6 Measuring object recognition memory:

2.6(i) Novel object recognition test protocol:

Object recognition memory was assessed using a NOR test adapted from Clark et al. (2000) (original protocol from Wood and Phillips, 1991, and adapted by Clark et al., 2000). The arena used in the OFT was used here as well (measuring 79cm x 79cm x 35.5cm) (Figure 2.7). Table 2.7 illustrates the protocol for each stage of the task.

Briefly, the animals were first habituated to the arena for 5-min a day for 5 consecutive days. This constitutes the habituation phase. On the next two days, the NOR test was conducted (one trial on each day). Each trial comprised of a familiarization phase and a test phase, conducted 60-min apart. During the familiarization phase of each trial,

the animal was put in the arena with two identical copies of an object (positions of the objected are indicated in figure 2.7). After being allowed to explore the objects for 5-min, the animal was put back in its homecage and taken to the colony room. An hour later, the animal was brought back into the test room and put in the arena. The arena contained another copy of the previously encountered object, and a novel object. The animal was allowed to investigate both objects for 5-min. This constituted the test phase of the task. These stages of the task were repeated the next day (for trial 2) using new pairs of novel and familiar objects. These stages are listed in the table below (and mentioned in greater detail in table 2.7).

	Phase of the NOR test
Day 0-5	Habituation Phase
Day 1	Trial 1: Test Day (Familiarization Phase)
Day 1 (60-min. later)	Trial 1: Test Day (Test Phase)
Day 2	Trial 2: Test Day (Familiarization Phase)
Day 2 (60-min later)	Trial 2: Test Day (Test Phase)

The first trial used Ziploc containers and crystal tumblers as familiar and novel objects. For approximately half of the animals in each group, the Ziploc containers were used as the familiar object while for the remaining animals the crystal tumblers were used as the familiar object. For trial 2, metal flower pots and used beer bottles (cleaned with soap, water and 70% ethanol prior to use) were used. For half the animals tested, the metal flower pots were used as the familiar object, while for the remaining, the used beer bottles were the familiar object (Figure 2.8).

These objects were chosen because they appeared to be sufficiently different in shape, and feel from each other to be distinguishable by the rats (reviewed in Ennaceur, 2010). Most published papers using the NOR test (including Clark et al., 2000, from which the current protocol was adapted) do not mention the exact pairs of objects used in

each trial. However, objects were chosen that were likely to be differentiated by the rats based on touch, i.e. they were made of materials that were likely to feel different when touched (e.g. Ziploc containers and crystal tumblers), and the shapes of the objects were quite different from each other. These objects also allowed the rats to climb on them, an important criterion to sustain rats' interest in exploring them (Ennaceur, 2010). They were also *not* smaller than the rat, or larger than 2.5 times the rat's size (Clark et al., 2000). Most objects were of such a weight that they could not be displaced easily by the rat while exploration (though they were taped to the floor of the arena using masking tape and Scotch tape) (Clark et al., 2000). Moreover, based on personal communication with a fellow experimenter (Mrs. Rhiann Gunn), objects that had distracting elements like looped openings (e.g. in cups with handles) were avoided. Additionally, ceramic flower pots were used in earlier rounds of the experiment (used along with metal flower pots for trial 2). However, these were replaced with used beer bottles to provide greater visual differences between the objects. The beer bottles used were more dramatically different in shape, size and colour from the metal flower pots and therefore, were preferred over the ceramic flower pots. The arena and objects were cleaned thoroughly with non-scented soap, water and 70% ethanol after use.

Both the familiarization and test phases were recorded using Sony 8mm digital cameras (CCD0TRV65 or CCD0TRV108), and the videos analyzed to determine relative amount of time spent in investigating the various objects in each of the phases of the task. An animal was identified as investigating an object when its snout was directed towards the object and it was within 3-5cm of the object. Instances when the animal was merely using the object to prop itself up and not actively investigating it were not included for

obvious reasons. Data from Trial 1 and Trial 2 were used to calculate the following measures. Formulae used to calculate each is listed in table 2.8.

I. Familiarization Phase
1. Total object interaction duration (sec) 2. Percent duration spent in total object interaction
II. Test Phase:
1. Percent preference for novel object 2. Total object interaction duration (sec) 3. Percent duration spent in total object interaction 4. Percent duration spent with *novel* object 5. Percent duration spent with *familiar* object

All these variables were analyzed statistically using a 2x2x3 ANOVA with Sex, Housing, and Odour Treatment as the between subject factors (alpha= 0.05) (as discussed in the next section of this chapter). However, only (1) and (2) from test phase and (1) from familiarization phase are included in this thesis for the sake of brevity, and because they are most affected by the various independent measures.

In addition, for most of the dependent measures listed in the above table, data from both trials were also averaged, and analyzed using a 2x2x3 ANOVA for effects of Sex, Housing, and Odour Treatment. However, due to the absence of any major effect of one or more of the three factors, these data (obtained by averaging both trials) were not included in this thesis. An exception to this is the effect of Sex on percent preference for the novel object during the test phase, which is mentioned in the appropriate section of the next chapter.

2.7 Statistical analysis of the data:

2.7(i) Startle, prepulse inhibition, and odour exposure behaviours:

2.7(i) (a) Generating group averages:

T_{max}, V_{max}, startle amplitude, and habituation values obtained for each experimental group for each round of testing (i.e. for each exposure period) were averaged and standard error of the mean (SEM) calculated. The tables and figures in the next chapter show these results (average +/- SEM).

For each behaviour measured during odour exposure, group averages and SEM were calculated, and some of these are presented as tables or graphs in the next chapter. In addition, some variables for some behaviours were also collapsed across one or more independent variables and the resulting averages (+/- SEM) graphed to illustrate the Main Effect or an Interaction Effect of variables.

2.7(i) (b) Statistical analysis:

All odour exposure behaviour data, and all startle and PPI data were analyzed using mixed-design 2x2x3x4 ANOVAs with Sex, Housing, and Odour Treatment as the between-subject variables and Exposure Period as the within-subject factor; significance (alpha) level was set at 0.05. Where necessary to resolve interactions, simple effect analyses were performed. For these simple effects analyses, in order to reduce probability of Type I errors, a new significance level was obtained by dividing 0.05 by the total number of simple effects analysis done to resolve the interaction in question.

While performing the mixed-design ANOVA on SPSS, the option to perform Mauchly's test of sphericity was chosen, and when needed, the Greenhouse-Geisser correction was employed.

2.7(ii) Adult behavioural and dopamine D1R and D2R receptor data:

2.7(ii) (a) Statistical Analysis:

Striatal tissue data were collapsed across sex because sample sizes were not sufficient to analyze each sex separately. Medial PFC dopamine D1R and D2R receptor intensity was analyzed separately for each sex using an independent t-test. Levels of D1R and D2R in striatal (caudate-putamen) tissue each were collapsed across sex and analyzed for effect of Odour Treatment using a t-test. Adult behavioural data (for OFT, EPM, SPT, and NOR tests) was analyzed using separate 2x2x3 ANOVAs with Sex, Housing, and Odour Treatment as the between subject factors (alpha= 0.05). Where necessary to resolve interactions, simple effect analyses were performed. For simple effects analysis, in order to reduce probability of Type I errors, p-value was obtained by dividing 0.05 by the total number of simple effects analysis done to resolve the interaction in question (i.e. Bonferroni correction).

2.7(iii) Correlations:

Correlational analyses using Pearson's product-moment test were performed between relative dopamine D1R, and D2R receptor densities in different regions, and startle and PPI data. Similar analyses were also performed between relative dopamine D1R and D2R receptor densities in different regions, and various dependent measures on

the OFT, and EPM. Significant results that enlighten our interpretation of the data will be presented in the next chapter.

2.7(iv) Interpreting Effect Size:

The following table (adapted from Morris and Fritz, 2013) can be used to interpret effect sizes reported in the next chapter.

Effect size	**Partial Eta Squared ($\eta^2_{Partial}$)**
Small effect	$0.01 \leq \eta^2_{Partial} \geq .06$
Medium effect	$0.06 \leq \eta^2_{Partial} \geq 0.14$
Large effect	$\eta^2_{Partial} \geq 0.14$

Recent scientific literature has emphasized the reporting of effect sizes along with significance values (Morris and Fritz, 2013). The significance value informs the reader about the probability that the significant difference observed between two groups occurred due to a random chance. Effect sizes, on the other hand, are supposed to inform the reader about the "practical significance" of the experimental results (Schuele and Justice, 2006). In the words of a researcher, "Effect-size estimates are metrics designed specifically to characterize results in more functional and meaningful ways by discussing the *magnitude* of an effect in addition to estimates of probability" (Schuele and Justice, 2006). This is particularly important because significance values are heavily dependent on the sample sizes, and large samples can reveal significant effects even though those effects may have little clinical or practical relevance. Likewise, smaller sample sizes can "hide" effects of potential clinical relevance.

Interpreting effect sizes can be done in two ways. Either the effect sizes obtained in a study can be compared to a pre-defined benchmarks as those stated in the table in the previous page. However, this is not considered a very sophisticated way of interpreting one's data because these benchmarks (based on Cohen's interpretations) are somewhat arbitrary (Thompson, 2007).

A better way to interpret effect sizes would be to take into account the values that have been obtained in previous experiments performed under similar circumstances, which explore similar questions (Morris and Fritz, 2013). In other words, exploring and comparing one's effect sizes with those found in previous work in one's field. This is, however, extremely hard to do in the current work because most papers discussed here do not discuss or report effect sizes. Therefore, in the current work, the author has merely documented the effect sizes obtained for each measure that was statistically significant without interpreting them.

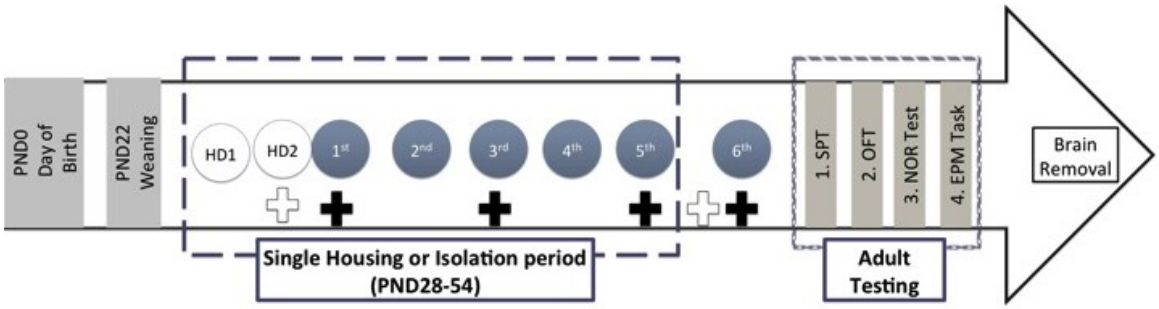


Figure 2.1. Schematic of the timeline of the experimental procedures.

The day of birth was designated as post natal day (PND) 0. Animals were weaned on PND22 and housed in same-sex pairs from PND22-27. From PND28-54, a sub-group of the animals was housed singly; thereafter, they were re-paired with their former same-sex cagemates until the end of the experiment. Odour Treatment was administered to select groups on 6 occasions marked by black circles in the schematic. Two days prior to the 1st exposure, these animals were habituated to the arena used in odour exposures (marked by white circles in the schematic; "HD" refers to Habituation Day). A startle and PPI measurement was taken on HD2, and a day before the 6th odour exposure in adulthood (this is marked by white plus signs). Startle and PPI measurements taken immediately after the 1st, 3rd, 5th and 6th exposures are marked by black plus signs. Adult testing began with the Sucrose Preference Test (SPT), followed by the Open Field Test (OFT), the Novel Object Recognition test (NOR test), and the Elevated Plus Maze (EPM) task/test. Following these tests, the animals were sacrificed and their brains removed for analysis of dopamine D1R and D2R receptors.

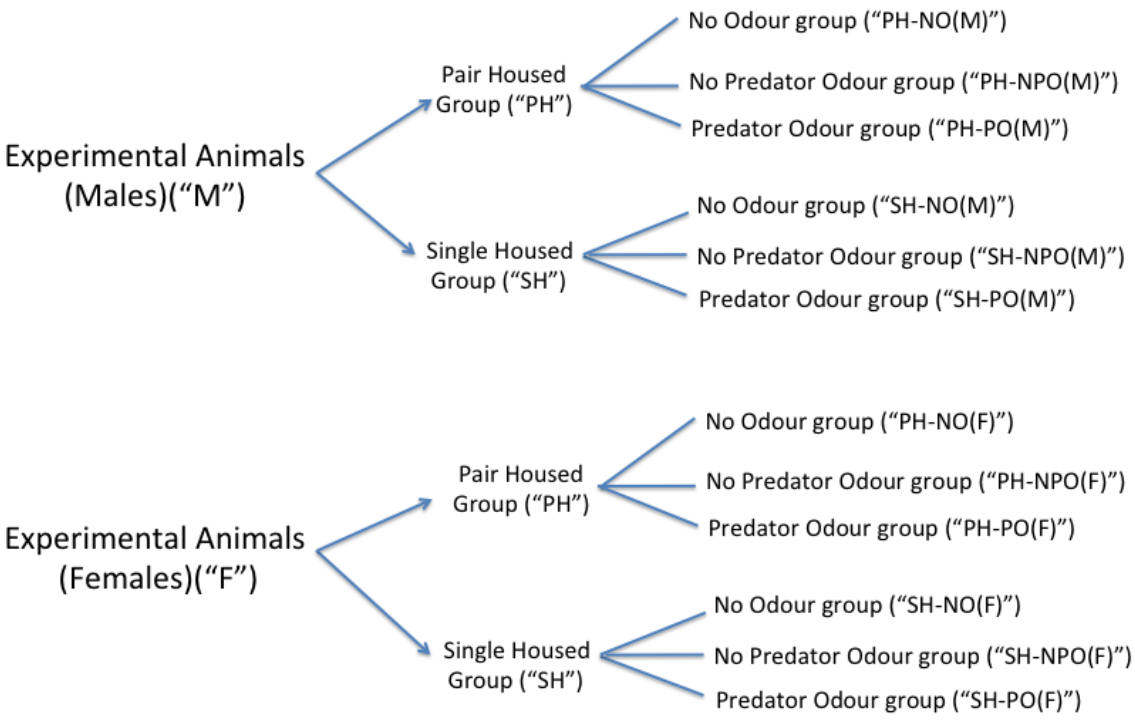


Figure 2.2. Schematic of the different experimental groups.

All experimental animals were first divided by sex. Animals of each sex were then divided into two groups based on the housing condition: Pair Housed (PH), and Single Housed (SH) groups. Animals from each of these groups were then divided into three groups based on the odour treatment they would receive in adolescence: No Odour (NO), No Predator Odour (NPO), and Predator Odour (PO) groups.

(Note: During various points in the text, terms like "pair housed sub-groups" and "predator odour sub-groups" are used for brevity. Essentially, the term "pair-housed sub-group" refers to animals that have received pair-housed treatment (regardless of their sex or odour treatment). Therefore, it refers to "PH-NO(M)", "PH-NPO(M)", "PH-PO(M)", "PH-NO(F)", "PH-NPO(F)", and "PH-PO(F)" shown above. Similarly, the term "predator odour sub-groups" refers to all animals that have received predator odour treatment regardless of sex or housing condition. Therefore, it refers to "PH-PO(M)", "SH-PO(M)", "PH-PO(F)", and "SH-PO(F)" in the above figure.)

Table 2.1. Details of the housing and odour treatments received by each sub-group of animals.

1. Pair-Housed (PH) male groups		
1(a). No Odour (NO) group	Same sex pairs per cage	No exposure to any odours *(Animals left undistrubed in colony room except during days of sensorimotor gating and startle testing)*
1(b). No Predator Odour (NPO) group	Same sex pairs per cage	Six 30-min exposures to control odour
1(c). Predator Odour (PO) group	Same sex pairs per cage	Six 30-min exposures to predator odour
2. Single Housed (SH) male groups		
2(a). No Odour (NO) group	Single animal per cage	No exposure to any odours *(Animals left undistrubed in colony room except during days of sensorimotor gating and startle testing)*
2(b). No Predator Odour (NPO) group	Single animal per cage	Six 30-min exposures to control odour
2(c). Predator Odour (PO) group	Single animal per cage	Six 30-min exposures to predator odour
3. Pair-Housed (PH) female groups		
3(a). No Odour (NO) group	Same sex pairs per cage	No exposure to any odours *(Animals left undistrubed in colony room except during days of sensorimotor gating and startle testing)*
3(b). No Predator Odour (NPO) group	Same sex pairs per cage	Six 30-min exposures to control odour
3(c). Predator Odour (PO) group	Same sex pairs per cage	Six 30-min exposures to predator odour
4. Single Housed (SH) female groups		
4(a). No Odour (NO) group	Single animal per cage	No exposure to any odours *(Animals left undistrubed in colony room except during days of sensorimotor gating and startle testing)*
4(b). No Predator Odour (NPO) group	Single animal per cage	Six 30-min exposures to control odour
4(c). Predator Odour (PO) group	Single animal per cage	Six 30-min exposures to predator odour

a. Photograph of the arena used for odour exposure

b. Schematic of the arena:

Third Region (TR)	Middle Region (MR)	Odour Region (OR)

Figure 2.3. A photograph (a) and schematic (b) of one of two identical arenas used for exposing select groups of animals to a control or threatening (predator) odour.

Each arena was made of transparent Plexiglas walls (and lid), with a white Plexiglas floor, measuring 35.5 cm x 27 cm x 60 cm. Before placing the animals into the arena, the appropriate odour source (a piece of brand-new, unused cat collar, or a collar previously worn by a cat) was placed into an alligator clip attached to one of the end walls approximately 6.5 cm from the top of the arena. (b) A schematic of the arena is displayed depicting the division of the arena into three virtual regions of approximately equal size; these were used for the purpose of measuring various behaviours exhibited by the animals while in the arena. The collar piece is placed in the Odour Region (refer to Chapter 2, section 2.2 for details).

Table 2.2. Details of the various odour exposure behaviours assessed in this study.

These behaviours were assessed during the 1st, 3rd, 5th and 6th odour exposures for the No Predator Odour and Predator Odour sub-groups.

1. Activity in different regions of arena:	Time spent in and number of entries into each region of the arena was measured. An animal was said to occupy a region of the arena when its forelimbs and hindlimbs were within the boundaries of that region. The total duration (sec) spent in, and number of entries made to each of the three regions of the arena was assessed.
2. Horizontal locomotor activity within entire arena	Horizontal locomotor activity within the arena was judged from the total number of times the rat crossed the two virtual lines that separate the arena into three regions. An animal was said to have crossed a line when its forelimbs and hindlimbs have crossed it.
3. Collar investigation behaviour	Collar investigation involved biting, sniffing, touching or licking the piece of collar present in the arena.
4. Grooming	Grooming was defined as a series of uninterrupted movements using the mouth and paws to clean the body. This could involve licking, biting, or rubbing of any part of the animal's body using its mouth and paws.

Note 1: For both Collar Investigation and Grooming, the following measures were assessed: Total duration spent (sec); Total number of bouts; Duration of shortest, continuous bout (sec); Duration of longest, continuous bout (sec); Duration of an average bout (sec); and Latency to initiate the behaviour (sec).

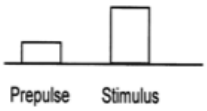
5. Rearing	Rearing occurred when the animal stood upright on its hind limbs. The forelimbs could be resting on one of the walls of the arena. Instances when the animal was upright but grooming were excluded. The following measures were assessed: the total duration spent rearing (sec), and total number of rears.

(Note 2: For each variable mentioned here, a mixed-design 2x2x2 ANOVA was performed for effects of Sex (male, female), Housing (pair housing, single housing), and Odour Treatment (no predator odour, predator odour.). Exposure Period was the repeated measure with 4 levels (because odour exposure behaviours were analyzed during the 1st, 3rd, 5th, and 6th exposure sessions.))

Figure 2.4. Photograph of the apparatus used for measuring sensorimotor gating (PPI) and startle-related variables.

The black star indicates the clear Plexiglas cylinder within which the animal was placed during testing.

a.

	Background noise used	Prepulse stimulus used	Startle stimulus used	Illustration of the trial
No Stimulus Trial	65dB	n/a	n/a	
Startle Alone Trial	65dB (present throughout the trial)	n/a	120dB	
Prepulse Trials: **i. 3dB Prepulse Trial** **ii. 6dB Prepulse Trial** **iii. 12dB Prepulse Trials**	65dB (present throughout the trial)	i. 68dB ii. 71dB iii. 77dB	120dB (presented 100ms after prepulse stimulus)	

b.

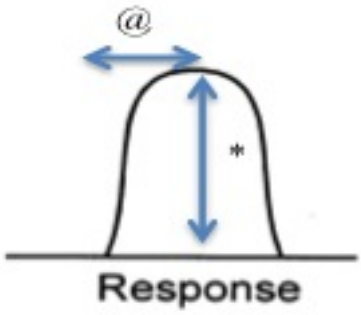


Figure 2.5. A tabular and pictorial representation of protocol used for startle and PPI measurement.

(a) Description of the different stimuli present during each type of trial in a sensorimotor gating and startle testing session. An illustration is also drawn for each type of trial. (b) The bottom part of the figure illustrates a recording of the animal's response and corresponding dependent measures - "*" refers to the maximum startle response displayed by the animals (V_{max}), while "@" refers to the time taken to reach that response (T_{max}).

Table 2.3. Details of formulae used to calculate, and statistical analysis used for the dependent measures related to prepulse inhibition (PPI), including "baseline" PPI.

I. PPI *(Measured immediately after the 1st, 3rd, 5th, and 6th exposures)*	
1. PPI for 3dB prepulse	= 100-[100*(Startle response for 3dB prepulse)/(Average response to all non-consecutive startle trials)]
2. PPI for 6dB prepulse	= 100-[100*(Startle response for 6dB prepulse)/(Average response to all non-consecutive startle trials)]
3. PPI for 12dB prepulse	= 100-[100*(Startle response for 12dB prepulse)/(Average response to all non-consecutive startle trials)]
3. PPI (Average of all prepulses)	=Average PPI values obtained for 3, 6, and 12dB (i.e. average (1),(2) and (3))

Note: For (1), (2), and (3), a mixed-design (repeated measures)2X2X3X4 ANOVA was performed with Sex (male, female), Housing (Pair housing, Single housing), and Odour Treatment (No Odour, No Predator Odour, and Predator Odour) as the independent variables and Exposure period as the repeated measure (4 levels).

II. "Baseline" PPI *(Measured a day before 1st exposure and 6th exposure)*	
4. "Baseline" PPI (3dB prepulse)	= 100-[100*(Startle response for 3dB prepulse)/(Average response to all non-consecutive startle trials)]
5. "Baseline" PPI (6dB prepulse)	= 100-[100*(Startle response for 6dB prepulse)/(Average response to all non-consecutive startle trials)]
6. "Baseline" PPI (12dB prepulse)	= 100-[100*(Startle response for 12dB prepulse)/(Average response to all non-consecutive startle trials)]
7. "Baseline" PPI (Average of all prepulses)	=Average "baseline" PPI values obtained for 3, 6, and 12dB (i.e. average (4), (5) and (6))

Note: For (4)-(7), a mixed-design (repeated measures) ANOVA was performed with Exposure period as repeated measure (2 levels), and Sex (male, female), Housing (Pair housing, Single housing), and Odour Treatment (No Predator Odour, and Predator Odour) as the independent variables (2X2X2X2 ANOVA)

Table 2.4. Details of formula and statistical analysis for the various startle-related depedent measures (i.e, startle amplitude, startle habituation, and response to no-stimulus trials).

Dependent measure and formula used to calculate it	
1. Startle amplitude (After consecutive startle-alone trials)	=Average response to *consecutive* startle-alone trials in a testing session
2. Startle amplitude (After non-consecutive startle-alone trials)	=Average response to *non-consecutive* startle-alone trials in a testing session
3. Startle amplitude (After *all* startle trials)	=Average response to *consecutive and non-consecutive* startle-alone trials in a testing session
4. No stimulus response	=Average response to all No Stimulus trials in a testing session
5. Startle habituation	= 100-[100* (average response to Block 1 consecutive startle-alone trials)/average response to Block 3 consecutive startle-alone trials)]

Note 1: For each of the variables above, a mixed-design 2X2X3X4 ANOVA was performed with Sex (male, female), Housing (Pair housing, Single housing), and Odour Treatment (No Odour, No Predator Odour, and Predator Odour) as the independent variables, and Exposure period as the repeated measure (4 levels).

(Note 2: Consecutive and non-consecutive startle-alone trials are part of the same testing protocol. Consecutive startle-alone trials are part of Block 1 and Block 3 in the protocol whereas non-consecutive startle-alone trials are parts of Block 2 trials. For more detail, consult section 2.2 of chapter 2.)

Table 2.5. Details of formula and statistical analysis for the latency to reach the maximum response at each prepulse trial (i.e. T_{max}).

Dependent measure	Formula used
1. T_{max} at 3dB prepulse	=Average T_{max} of the animal for all 3dB prepulse trials in a testing session
2. T_{max} at 6dB prepulse	=Average T_{max} of the animal for all 6dB prepulse trials in a testing session
3. T_{max} at 12dB prepulse	=Average T_{max} of the animal for all 12dB prepulse trials in a testing session
4. Average T_{max} at all prepulses	=Average T_{max} for 3dB, 6dB, and 12dB prepulse trials

Note: For each of the variables above, a mixed-design 2X2X3X4 ANOVA was performed with Sex (male, female), Housing (Pair housing, Single housing), and Odour Treatment (No Odour, No Predator Odour, and Predator Odour) as the independent variables, and Exposure period as the repeated measure (4 levels).

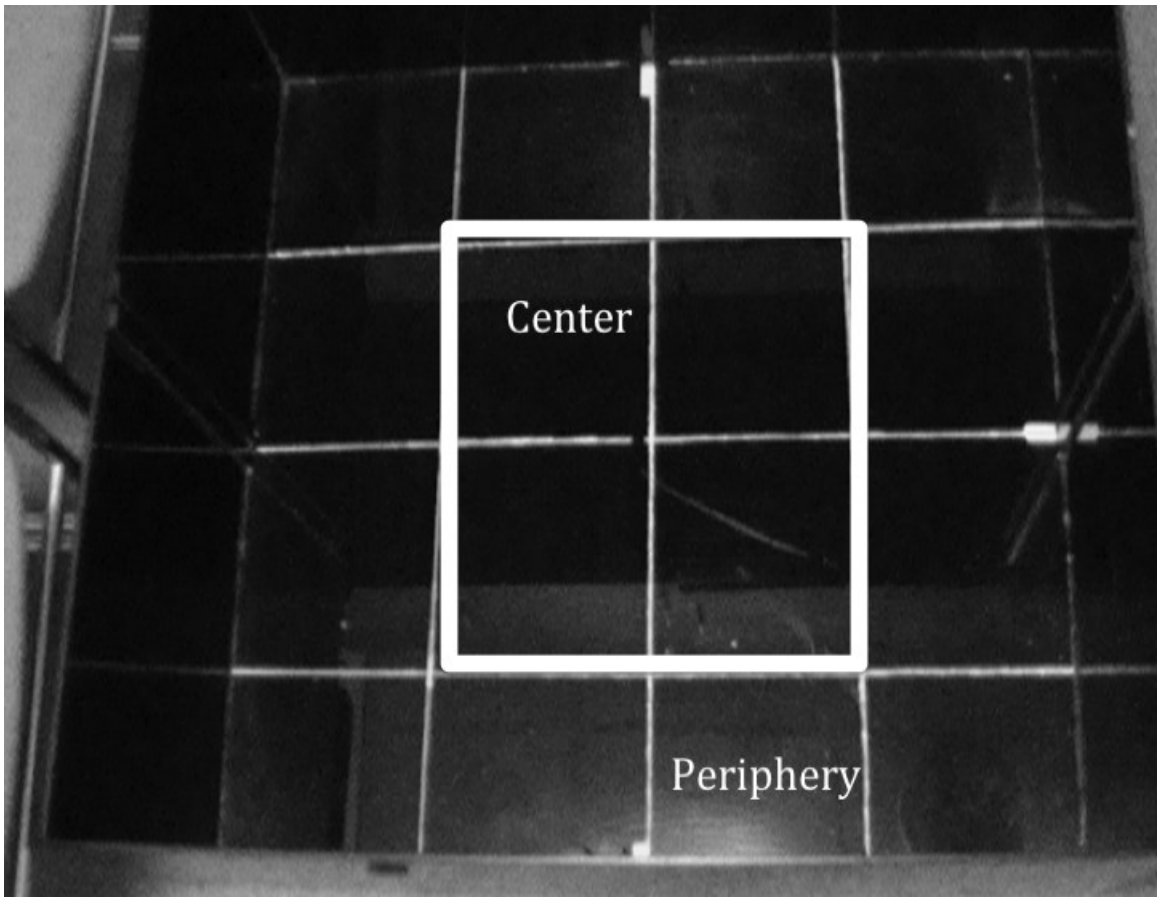


Figure 2.6. An annotated photograph of the arena used for Open Field Test (OFT).

The black Plexiglas arena measured 79 cm x 79 cm x 35.5 cm and its floor was divided into 16 equal sized squares using white paint for behavioural scoring. The white box (in thick lines) drawn over the center of the photograph divides the arena virtually into a central region (Center) and a peripheral region (Periphery). Movement into each region was quantified during the OFT; an animal entered the Center or Periphery when all forelimbs were within that region. Thigmotaxic behaviour was measured as the duration and total number of instances when any part of the animal (except the tail and vibrissae) was in contact with at least one of the four walls.

Table. 2.6. Behaviours and dependent measures assessed in the open field test.

Type of behaviour	Specific depedent measures
Anxiety-related measures	1(i). Percent duration in center 1(ii). Rate of entry into center 2(i). Percent duration in thigmotaxis 2(ii). Rate of thogmotaxis 2(iii). Latency to thigmotaxis
Locomotor activity-related measures	2(i). Percent duration in periphery 2(ii). Rate of entry into periphery
Exploratory activity-related measures	4(i). Percent duration of rearing in entire open field 4(ii). Rate of rearing in entire open field 5(i). Percent duration of rearing in center 5(ii). Rate of rearing in center 6(i). Percent duration of rearing in periphery 6(ii). Rate of rearing in periphery

(Note: For each measure, a 2X2X3 ANOVA was performed for determining effects of Sex, Housing, and Odour Treatment.)

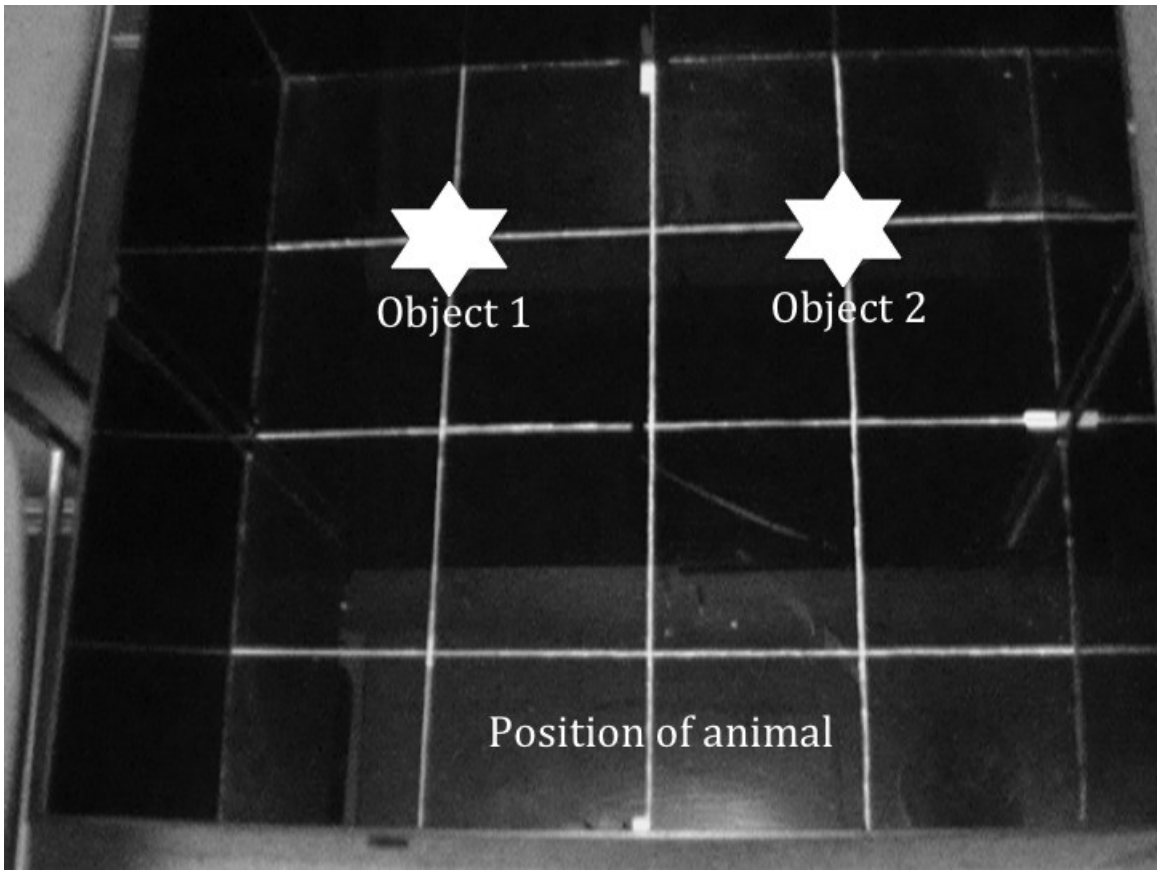


Figure 2.7. An annotated photograph of the arena used for Novel Object Recognition test (NOR).

The arena measured 79 cm x 79 cm x 35.5 cm and was used in the Open Field Test as well. The stars indicate the positions of the two objects during the familiarization or test phases of the task while the position of the animal at the beginning of each phase of the task is also shown.

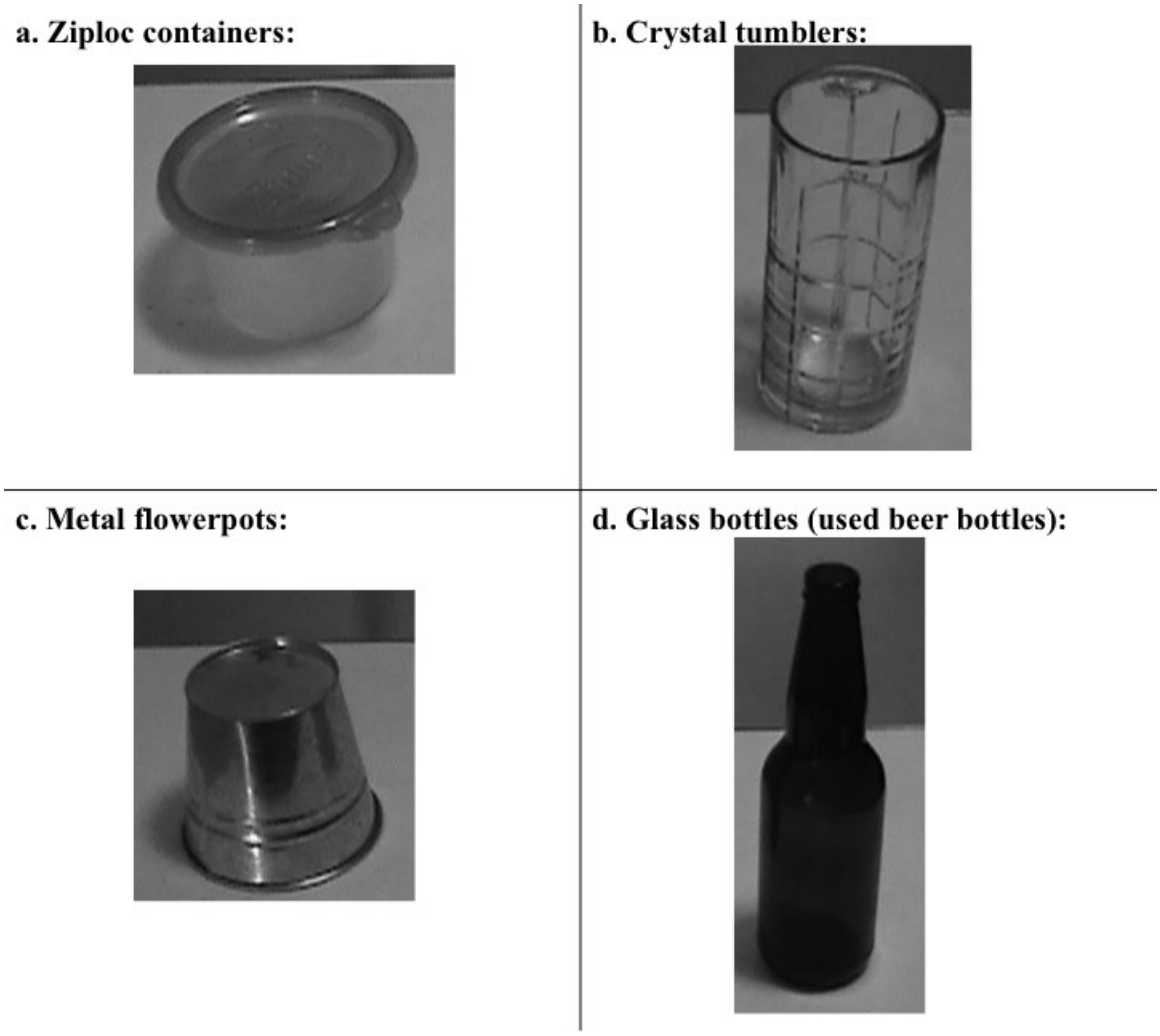


Figure 2.8. Photographs of objects used in each of the two trials of the Novel Object Recognition (NOR) test.

The first trial used Ziploc containers (a) and crystal tumblers (b). For approximately half of the animals in each group, Ziploc containers were used as the familiar object, while for the remaining animals, crystal tumblers were used as the familiar object. For the second trial, metal flower pots (c) and glass bottles (clean used beer bottles) (d) were used. For half of the animals tested, the metal flower pots were used as the familiar object, while for the remaining, the used beer bottles were the familiar object.

Table 2.7. A summary of the protocol for each stage of the novel object recognition test.

	Phase of the NOR test	Details
Day 0-5	Habituation Phase	Animals were habituated to the arena by placing them individually in the arena for 5-min each day.
Day 1	Trial 1: Test Day (Familiarization Phase)	Each animal was re-habituated to the arena for 1-min following which two identical objects (familiar object) were placed in the arena. The animal was allowed to explore these objects for 5-min following which it was taken back to the colony room.
Day 1 (60 min. later)	Trial 1: Test Day (Test Phase)	An hour later, the animal was placed back in the arena for 5-min with a third copy of the Familiar Object and a different object that the animal had not previously encountered (novel object). Thereafter the animal was returned to the colony room. The arena and objects were cleaned thoroughly with non-scented soap, water and 70% ethanol between animals.
Day 2	Trial 2: Test Day (Familiarization Phase)	Protocol is same as for Trial 1 Familiarization phase. The objects used on Day 2 (both familiar and novel objects) were different from those used on Day 1.
Day 2 (60 min later)	Trial 2: Test Day (Test Phase)	Protocol is same as for Trial 1 Test phase. The objects used on Day 2 (both familiar and novel objects) were different from those used on Day 1.

Table 2.8. The dependent measures used in the familiarization and test phases of of the novel object recognition test.

I. Familiarization Phase	
1. Total object interaction duration (sec)	= [(Total duration spent with left object)+ (Total duration spent with right object)]
2. Percent duration spent in total object interaction	=100* [((Total duration spent with left object)+(Total duration spent with right object))/(Total analyzed duration)] *(Note: Total analyzed duration= 5min)*
II. Test Phase:	
1. Percent preference for novel object	=100*[(Total duration spent with novel object)/((Total duration spent with novel object)+(Total duration spent with familiar object))]
2. Total object interaction duration (sec)	= (Total duration spent with novel object) + (Total duration spent with familiar object)
3. Percent duration spent in total object interaction	= 100* [(Total duration spent with novel object)+ (Total duration spent with familiar object)/ Total analyzed duration] *(Note: Total analyzed duration= 5min)*
4. Percent duration spent with *novel* object	= 100* [(Total duration spent with *novel* object)/((Total duration spent with novel object)+ (Total duration spent with familiar object))]
5. Percent duration spent with *familiar* object	=100* [(Total duration spent with *familiar* object)/((Total duration spent with novel object)+ (Total duration spent with familiar object))]

(Note: For each measure, a 2X2X3 ANOVA was performed for determining effects of Sex, Housing, and Odour Treatment.)

Chapter 3: Results

3.1. Biomarkers of predator odour experience:

3.1(i) Behaviours during odour exposures:

3.1(i) (a) Horizontal movement within entire arena increased over time, and in pair housed males:

Horizontal movement within the entire arena was gauged by the number of times the animal crossed either of the two virtual lines that divided the arena into the odour, middle and third regions. Total number of line crosses increased with exposure periods (main effect of Exposure Period)(1^{st} <5^{th}, 1^{st} <6^{th}, 3^{rd} <5^{th}, 3^{rd} <6^{th}; $F(2.71, 195.40)= 20.25$, $p= 0.001$, Effect Size= 0.22).

Moreover, single housed males showed lower activity within the arena than pair housed males (Housing X Sex interaction; $F(1,72)= 4.64$, $p= 0.03$, Effect Size= 0.06) (Figures 3.1, 3.2) and this effect was not seen in females.

3.1(i) (b) Time spent in, and number of entries into individual regions of the arena increased among no predator odour exposed animals, and among pair housed animals:

Time spent in, and the number of entries into each of the three virtual regions of the arena was assessed in this study. A reduction in either time spent or number of entries to the odour region (which houses the cat collar piece) in predator odour exposed rats compared to control odour (that is, no predator odour) exposed rats was taken to imply behavioural avoidance of the odour source. Alternately, an increase in the time spent in or number of entries to the third region (which is furthest away from the odour source) in predator odour exposed rats compared to rats exposed to no predator odour also indicated behavioural avoidance of the odour source (Figures 3.3 to 3.5, and AB.1 to AB.5). Each

of these variables was analyzed separately using a 2x2x2 mixed-design (repeated measures) ANOVA with Exposure Period as the repeated measure, and Sex, Housing and Odour Treatment as the independent variables.

The effect of Exposure Period are discussed in this paragraph. Essentially, the duration in the middle region of the arena increased with each subsequent exposure (main effect of Exposure Period)(1^{st} <3^{rd}, 1^{st} <5^{th}, 1^{st} <6^{th}, 3^{rd} <6^{th}) (Figure AB.2). The number of entries into all three regions of the arena also increased with subsequent exposures, as indicated by a main effect of Exposure Period (1^{st}<5^{th}, 1^{st}<6^{th}, 3^{rd}<5^{th}, 3^{rd}<6^{th}) (Figures AB.3-AB.5) (see table below).

Dependent Variable(s) displaying effect of Exposure Period	**F**	**p**	**Effect Size**
Duration in Middle Region	7.65(3, 216)	0.001	0.096
Number of entries to Odour Region	19.73(2.49, 179.52)	0.001	0.215
Number of entries to Middle Region	21.34(2.68, 193.17)	0.001	0.230
Number of entries to Third Region	18.95(3, 216)	0.001	0.210

In this paragraph, the effects of odour treatment will be discussed. Firstly, odour treatment had a main effect of duration spent in the odour region ($F(1, 191.57)= 57.93$, $p= 0.001$, Effect size= 0.45) (Figure 3.3, top panel, Figure AB.1). Simple effects analyses revealed that animals exposed to predator odour spent significantly *less* time in the odour region than those exposed to predator odour. In addition, this variable showed a Odour Treatment X Sex X Exposure Period interaction ($F(2.66, 191.57)= 3.81$, $p= 0.01$, Effect Size= 0.05; Figure 3.3, bottom panel, and Figure AB.1). Simple effects analyses revealed that females exposed to predator odour spent *less* time in the odour region near the odour stimulus compared to females exposed to no predator odour at each exposure period. However, the same effect was seen in predator odour exposed males compared to ones

exposed to no predator odour only during the 5th exposure period (Figure 3.3, Figure AB.1).

Conversely, time spent in the third region revealed an effect of Odour Treatment in males after the 3rd and 5th exposure periods (no predator odour < predator odour), and in females after each exposure period (no predator odour < predator odour) (Sex X Odour Treatment X Exposure Period interaction; F(2.70, 194.44)= 4.53, p= 0.001, Effect Size= 0.06) (Figure 3.4). Thus, predator odour exposed females spent more time in the third region during each exposure whereas predator odour exposed males did the same only during the 3rd and 5th exposures.

Next, the effect of housing on these measures will be discussed. Specifically, single housed animals made fewer entries into the odour region than pair housed ones (main effect of Housing; F(1.00, 72.00)= 9.27, p= 0.001, Effect Size= 0.11) (Figure AB.3). Moreover, single housed males made fewer entries into both the middle region and the third region compared to pair housed males (Sex X Housing interaction) (Number of entries to third region: F(1.00, 72.00)= 5.09, p= 0.02, Effect Size= 0.07; Number of entries to middle region: F(1.00, 72.00)= 4.35, p= 0.04, Effect Size= 0.06) (Figures 3.5, AB.3-AB.5). In other words, isolated animals avoided the odour region whereas isolated males showed lesser movement between regions of the arena than males raised in pairs of same sex littermates.

3.1(i) (c) Collar investigation was reduced among predator odour exposed animals and increased among single housed animals:

Collar investigation was another behaviour assessed in this study. It involved sniffing, biting, or touching the odour stimulus. The number of collar investigations increased with each subsequent exposure (Main effect of Exposure Period; 1st <5th, 3rd<

5th) (F(3.00, 219.00)= 4.67, p= 0.001, Effect Size= 0.06, Figure 3.7). Latency to begin collar investigations also revealed such an effect (Main effect of Exposure Period; 1st <5th, 3rd< 5th) (F(1.95, 140.18)= 5.47, p= 0.001, Effect Size= 0.07) (Figure 3.8). Thus, as the exposures progressed and the animals grew older, they showed greater exploration of the collar stimulus although they took longer to begin the investigation of the stimulus.

Next the interaction effect between Sex and Housing on the duration and number of collar investigations will be discussed; simple effects analyses revealed an effect of Sex in single housed animals (Male > Female), but not pair housed ones (For duration of investigation: F(1.00, 73.00)= 6.40, p= 0.01, Effect Size= 0.08; For number of investigations: F(1.00, 73.00)= 9.44, p= 0.001, Effect Size= 0.11) (Figures 3.6 to 3.7).

Expectedly, no predator odour exposed animals spent a greater total duration investigating the collar than the predator odour group (F(1.00, 73.00)= 7.73, p= 0.001, Effect Size= 0.09). This was accompanied by an Odour Treatment X Housing interaction for the number of collar investigations (F(1.00, 73.00)= 4.81, p= 0.03, Effect Size= 0.06). Among single housed animals, those exposed to no predator odour showed a higher number of collar investigations relative to those exposed to predator odour. Pair housed animals did not show such an effect.

The number of collar investigations (F(1.00, 73.00)= 9.44, p= 0.001, Effect Size= 0.11, Figure 3.7), and the latency to investigate the collar (F(1.00, 73.00)= 4.75, p= 0.03, Effect Size= 0.06, Figure 3.8), was also significantly affected by an interaction between Housing and Sex. Among females, single housed animals showed more collar investigations and a shorter latency to begin the investigations than pair housed rats. No other effects were revealed while performing simple effects analyses to tease apart the Housing and Sex interaction (Figures 3.6 to 3.8, Table AB.1).

However, relative to pair housed animals, single housed animals showed a shorter value for each of the dependent measures listed in the table below (Main Effect of Housing; also see Table AB.1).

Dependent Variable(s) displaying effect of Housing	F	p	Effect Size
Duration of the shortest investigation bout	4.45(1.00, 73.00)	0.030	0.06
Duration of the longest investigation bout	6.01(1.00, 73.00)	0.010	0.07
Mean duration of an investigation bout	14.88(1.00, 73.00)	0.001	0.17

3.1 (ii) Grooming and rearing behaviours:

3.1(ii) (a) Grooming was reduced in predator odour exposed animals, and in single housed animals:

Overall, grooming increased with subsequent exposures. Specifically, Exposure Period had a Main Effect on the duration of grooming, and number of grooming bouts; in each case, the value of the variable increased with subsequent exposures compared to the first odour exposure ($1^{st}<3^{rd}$, $1^{st}<5^{th}$, $1^{st}<6^{th}$) (Figure 3.9). Duration of longest grooming bout, also showed a similar result ($1^{st}<3^{rd}$, $1^{st}<5^{th}$; Tables AB.2 and AB.3).

Dependent Variable(s) displaying effect of Exposure Period	F	p	Effect Size
Duration spent grooming	7.15 (3.00, 213.00)	0.001	0.09
Number of grooming bouts	13.84 (3.00, 213.00)	0.001	0.16
Duration of longest grooming bout	3.13 (3.00, 213.00)	0.020	0.04
Duration spent rearing	25.59 (3.00, 213.00)	0.001	0.26
Number of rears	29.49 (3.00, 213.00)	0.001	0.29

In addition, latency to groom showed an Exposure Period X Housing X Odour Treatment interaction ($F(3.00, 213.00)= 7.59$, $p= 0.001$, Effect Size= 0.09.). Simple effects revealed an effect of Exposure Period on animals exposed to both single housing

and predator odour ($1^{st} < 3^{rd}$, $1^{st} < 5^{th}$, $1^{st} < 6^{th}$). Simple effects also revealed an effect of Housing on the predator odour group during the 1^{st} exposure (pair housed >single housed) and an effect of Odour Treatment on single housed animals after the 1^{st} exposure (no predator odour > predator odour); this is discussed again a few paragraphs below with the other effects of Housing)(Figures 3.10, Tables AB.3).

A Main Effect of Odour Treatment was observed for total grooming duration, number of grooming bouts, and the duration of the longest grooming bout; in each case, no predator odour groups had a higher average than predator odour groups (see table beneath for F, p-value and effect size). Thus, animals exposed to cat odour showed reduced grooming behaviour indicating behavioural inhibition.

Dependent Variable(s) displaying effect of Odour Treatment	**F**	**p**	**Effect Size**
Duration spent grooming	14.15(1.00, 71.00)	0.001	0.17
Number of grooming bouts	10.43(1.00, 71.00)	0.001	0.13
Duration of longest grooming bout	11.90(1.00, 71.00)	0.001	0.14

An effect of Housing was also observed for the latency to groom for the predator odour group during the 1^{st} odour exposure (pair housed > single housed) (Exposure Period X Housing X Odour Treatment interaction: F(3.00, 213.00)= 7.59, p= 0.001, Effect Size= 0.09)(Figure 3,10, Table AB.3).

(Note: In addition, the Exposure Period X Housing X Odour Treatment interaction on latency to groom also revealed an effect of Exposure Period on animals exposed to both single housing and predator odour ($1^{st} < 3^{rd}$, $1^{st} < 5^{th}$, $1^{st} < 6^{th}$), and an effect of odour treatment on single housed animals during the 1^{st} exposure (no predator odour> predator odour). These have been mentioned on the previous page when the effect of Exposure Period and Odour Treatment, respectively.)

3.1(ii) (b) Rearing increased among females, with subsequent exposures, and among no predator odour exposed animals:

Rearing was another behaviour that was assessed in this study. It involves standing on the hind limbs and exploring the surrounding area. Among the no predator odour group, females spent longer rearing than males (Sex X Odour Treatment interaction: $F(1.00, 73.00)= 4.26$, $p= 0.04$, Effect Size= 0.05); no other significant effects were revealed while resolving this interaction (Figures 3.11).

Overall, the number of rears increased with subsequent exposures ($1^{st} <3^{rd}$, $1^{st} <5^{th}$, $1^{st} <6^{th}$, $3^{rd} <5^{th}$, $3^{rd} <6^{th}$) (main effect of Exposure Period: $F(3.00, 219.00)= 29.49$, $p= 0.00$, Effect Size= 0.29, data not shown). Among the predator odour exposed animals only, the duration spent rearing increased with subsequent exposures ($1^{st} <3^{rd}$, $1^{st} <5^{th}$, $1^{st} <6^{th}$, $3^{rd} <5^{th}$) (Exposure Period X Odour Treatment interaction: $F(3.00, 219.00)= 3.53$, $p= 0.01$, Effect Size= 0.05, data not shown).

After the 1^{st} exposure, no predator odour exposed animals spent a greater duration rearing compared to the predator odour group (Exposure Period X Odour Treatment interaction: $F(3.00, 219.00)= 3.53$, $p= 0.01$, Effect Size= 0.05, Figure 3.11). No predator odour exposed animals also reared significantly more times than the predator odour exposed animals (main effect of Odour Treatment: $F(1.00, 73.00)= 14.61$, $p= 0.001$, Effect Size= 0.17) (Figures 3.12).

3.2. Biomarkers of single housing experience:

3.2(i) Measures of startle (particularly startle amplitude and prepulse inhibition), and dopamine activity:

3.2(i) (a) Prepulse inhibition increased with exposure period, and in single housed animals but decreased in no odour animals:

As indicated earlier, changes in PPI and startle amplitude were used as biomarkers of the single housing experience in this study. In particular, single housed is associated with a change in startle amplitude to startle alone trials and a change in PPI.

PPI (calculated from V_{max} response to the various prepulse trials) measured after the 1^{st}, 3^{rd}, 5^{th}, and 6^{th} exposure periods will be discussed first. At 3dB prepulse, a Main Effect of Sex was seen i.e. females showed a greater PPI than males ($F(1.00, 106.00)= 5.63$, $p= 0.01$, Effect Size= 0.05; Figure 3.13).

Moreover, Exposure Period exerted an effect on PPI calculated using V_{max} response to 6dB prepulse ($1^{st}<6^{th}$, $5^{th}<6^{th}$) ($F(2.70, 286.44)= 3.07$, $p= 0.03$, Effect Size= 0.03), and to 12dB prepulse ($1^{st}<5^{th}$, $1^{st}<6^{th}$, $3^{rd}<6^{th}$, $5^{th}<6^{th}$) ($F(2.69, 286.11)= 11.84$, $p= 0.001$, Effect Size= 0.10) (Figures AB.6, AB.8 and AB.10)

Resolving an Exposure Period X Odour Treatment interaction ($F(5.04, 269.70)= 2.27$, $p= 0.04$, Effect Size= 0.04) showed that this effect of exposure period on average PPI in the no predator odour group ($1^{st} <6^{th}$, $3^{rd} <6^{th}$, $5^{th} <6^{th}$)and in the predator odour group ($1^{st} <6^{th}$)(Figure 3.14 and 3.15). PPI measured immediately after the 6^{th} exposure revealed an effect of Odour Treatment (no odour< no predator odour, no odour< predator odour) (Figure 3.14 and 3.15).

Now the latency to reach the maximum response (T_{max}) for various prepulse trials will be discussed (measured immediately after 1^{st}, 3^{rd}, 5^{th} and 6^{th} exposure periods).

Exposure Period and Housing had an interaction effect on T_{max} at 6dB ($F(3.00, 309.00)= 2.95$, $p= 0.03$, Effect Size= 0.03, Figure AB.9), 12dB ($F(2.75, 284.12)= 3.07$, $p= 0.03$, Effect Size= 0.03, Figure AB.11), and average of all prepulses ($F(2.73, 295.27)= 4.99$, $p= 0.001$, Effect Size= 0.04, Figure 3.16). At 6dB, this effect was resolved into an effect of Exposure Period in pair housed animals ($1^{st} <6^{th}$) (Figure AB.9. At 12dB and for the average of all prepulses, this effect was resolved into an effect of Exposure Period in pair housed ($1^{st} <6^{th}$) and single housed animals ($1^{st} <3^{rd}$) (Figure 3.16 and AB.11). This implies that by the final odour exposure, animals took longer to reach the maximum startle response. (*Note: Resolving the Exposure Period X Housing interaction also revealed an effect of Housing at each exposure period (pair housed< single housed animals) for 12dB and average of all PPI datasets, as mentioned below while discussing effects of Housing of different PPI measures.)*

As mentioned in the previous section, average PPI (calculated using V_{max} response to all three prepulse trial types) showed an Exposure Period X Odour Treatment interaction ($F(5.04, 269.70)= 2.27$, $p= 0.04$, Effect Size= 0.04). Simple effects analyses revealed as effect of Odour Treatment after the 6^{th} exposure (no odour< no predator odour, no odour< predator odour), and an effect of Exposure Period on the no predator odour ($1^{st}<6^{th}$, $3^{rd}<6^{th}$, $5^{th}<6^{th}$), and predator odour ($1^{st}<6^{th}$) groups (Figures 3.14 and 3.15).

PPI measured a day before the first, and a day before the final odour exposures ("Baseline" PPI) showed an effect of Housing (Pair Housed < Single Housed) at 6dB ($F(1.00, 70.00)= 22.80$, $p= 0.001$, Effect Size= 0.25, Figure 3.17), 12dB ($F(1.00, 70.00)= 11.09$, $p= 0.001$, Effect Size= 0.14, Figure 3.18), and for the average of all prepulses ($F(1.00, 70.00)= 32.16$, $p= 0.001$, Effect Size= 0.31, Figures 3.18). In addition, at 3dB,

there was an Exposure Period X Housing interaction effect for this variable (F(1.00, 70.00)= 4.09, p= 0.04, Effect Size= 0.05) and simple effects analyses revealed an effect of Housing for both exposure periods (pair housed< single housed) (Figure 3.17).

At 3dB, Housing had a significant effect on the latency to reach the maximum response or T_{max} (Pair Housed < Single Housed; F(1.00, 103) = 7.45, df= 1.00, p= 0.007, Effect Size= 0.07, Figure AB.7). A similar effect was also seen while teasing apart the interaction between Exposure Period and Housing at 12dB (F(2.76, 284.12)= 3.07, p= 0.03, Effect Size= 0.03), and for the average of all prepulses (F(2.73, 295.27)= 4.99, p= 0.001, Effect Size= 0.04). In both cases, simple effects analyses revealed an effect of Housing at each exposure period (pair housed < single housed) (Figure 3.16 and AB.11). Thus, single housing resulted in increased PPI as well as increased latency to achieve this maximum response. (*Note: Resolving the Exposure Period X Housing interaction for 12 dB and average PPI, an effect of Exposure Period on pair housed animals (1^{st}<6^{th}), and on single housed animals (1^{st} <3^{rd}) was also noted, and has been mentioned in an earlier paragraph listing effects of Exposure Period.)*

3.2(i) (b) Startle amplitude increased with repeat testing, and in single housed animals, and in no odour exposed animals:

An interaction between Sex and Exposure Period for the latency to reach the maximum response (T_{max}) during the consecutive startle trials (F(3.00, 324)= 3.60, p= 0.01, Effect Size= 0.03) was noted, which was resolved into an effect of Sex at each Exposure Period (males<females, data not shown). Data obtained by combining latency to respond to consecutive and non-consecutive startle trials (T_{max}) revealed a main effect of Sex (F(1.00, 104.00)=5.60, p= 0.02, Effect Size=0.051)(males< females), and Housing

(F(1.00, 104.00)=7.31, p= 0.008, Effect Size= 0.066) (pair housed animals<single housed animals, data not shown).

There was a significant interaction of Exposure Period and Odour Treatment on the amplitude (V_{max}) of non-consecutive startle (F(3.64, 180.52)= 2.96, p= 0.02, Effect Size= 0.06); simple effects analyses revealed an effect of Exposure Period on different Odour Treatment sub-groups (no odour group: 1^{st}<3^{rd}, 1^{st}<5^{th}, 1^{st}<6^{th}, 3^{rd}<6^{th}, 5^{th}<6^{th}; no predator odour group: 1^{st}<3^{rd}, 1^{st}<5^{th}, 1^{st}<6^{th}, 3^{rd}<6^{th}, 5^{th}<6^{th}; predator odour group: 1^{st}<3^{rd}<5^{th}<6^{th}), and an effect of Odour Treatment after the 1^{st}, 3^{rd}, and 6^{th} exposure periods (no odour > no predator odour, no odour > predator odour groups) (Figure 3.19 and AB.12). The amplitude of the response to consecutive startle trials also showed an interaction effect of Exposure Period and Odour Treatment (F(2.91, 147.03)= 8.02, p= 0.001, Effect Size= 0.14). Simple effects analyses revealed an effect of Exposure Period in each Odour Treatment group (no odour group: 1^{st} <3^{rd} <5^{th} <6^{th}; no predator odour group: 1^{st} <3^{rd}, 1^{st} <5^{th}, 1^{st}<6^{th}, 5^{th}<6^{th}; and predator odour group: 1^{st} <3^{rd}, 1^{st}<5^{th}, 1^{st}<6^{th}, 3^{rd} <6^{th}), and an effect of Odour Treatment (no odour group >no predator odour group, no odour group> predator odour group) after each exposure (Figure AB.13). Data obtained by averaging the response to non-consecutive and consecutive startle trials revealed an interaction effect of Exposure Period and Odour Treatment (F(2.91,147.03)=5.22, p= 0.001, Effect Size= 0.09) (Figure AB.14). Simple effects analyses revealed an effect of Odour Treatment at each exposure period (no odour group>no predator odour group, no odour group>predator odour group), and an effect of Exposure Period on the no odour group (1^{st} <3^{rd} <5^{th} <6^{th}), no predator odour group, and predator odour group (in each case, all exposure periods are significantly different from each other except the 3^{rd} and 5^{th}).

Exposure Period and Housing had a significant interaction effect on the amplitude of the response to consecutive startle trials ($F(1.45, 147.03)= 3.48$, $p= 0.04$, Effect Size= 0.03)(Figure AB.13), and non-consecutive startle trials ($F(1.82, 180.52)= 5.29$, $p= 0.001$, Effect Size= 0.05)(Figure AB.12). In each case, simple effects analyses revealed an effect of Exposure Period on pair housed ($1^{st} <3^{rd}$, $1^{st}<5^{th}$, $1^{st}<6^{th}$, $3^{rd}<6^{th}$, $5^{th}<6^{th}$) and on single housed animals ($1^{st} <3^{rd} <5^{th} <6^{th}$).

A significant interaction between Housing and Odour Treatment was found for amplitude of the response to non-consecutive startle trials ($F(2.00, 99.00)= 4.35$, $p= 0.01$, Effect Size= 0.08, Figure) and simple effects analyses revealed an effect of Odour Treatment on pair housed animals only (no odour group > no predator odour group, no odour group > predator odour group) (Figure AB.12). A similar interaction was observed between Housing and Odour Treatment for the amplitude of the response to consecutive startle trials ($F(2.00, 101.00)= 5.00$, $p= 0.001$, Effect Size= 0.09); simple effects analyses revealed an effect of Odour Treatment on pair housed animals (no odour group > no predator odour group, no odour group > predator odour group), and an effect of Housing for the no predator odour group and the predator odour group (in each case, pair housed < single housed) (Figure AB.13). Data obtained by averaging consecutive and non-consecutive startle response data also showed an interaction effect of Housing and Odour Treatment interaction: ($F(2.00, 101.00)= 6.62$, $p= 0.001$, Effect Size= 0.11)(Figure AB.14). Simple effects analyses revealed an effect of Odour Treatment on pair housed animals (no odour group> no predator odour group, no odour group > predator odour group), and an effect of Housing on the no predator odour group and the predator odour group (in each case, pair housed animals <single housed animals).

For consecutive startle data, an interaction between Housing and Odour Treatment was observed for T_{max} ($F(2.00, 108.00)= 5.00$, $p= 0.001$, Effect Size= 0.09), and simple effects analyses revealed an effect of Housing for both no predator odour and predator odour groups (in each case, pair housed< single housed), and an effect of Odour Treatment in pair housed animals (no odour group > no predator odour group, no odour group > predator odour group) (Figure AB.13).

Lastly, there was a significant interaction between Housing and Exposure Period for the magnitude of the No Stimulus trials (V_{max}) ($F(1.50, 148.56)= 4.62$, $p= 0.01$, Effect Size= 0.04); simple effects analyses revealed an effect of Housing after the 5^{th} and 6^{th} exposure periods (in each case, pair housed groups> single housed groups), and an effect of Exposure Period on pair housed animals ($1^{st}<3^{rd}$, $1^{st}<5^{th}$, $1^{st}<6^{th}$, $3^{rd}<5^{th}$, $3^{rd}<6^{th}$), and single housed animals ($1^{st}<5^{th}$). In addition, the latency to respond to the No Stimulus (T_{max}) trials showed a Main Effect of Exposure Period ($1^{st}<3^{rd}$, $1^{st}<5^{th}$, $1^{st}<6^{th}$, $3^{rd}<5^{th}$, $3^{rd}<6^{t}$; $F(3.00, 309)= 18.38$, $p= 0.001$, Effect Size= 0.15) (Figure AB.17 and AB.18).

Startle Habituation was unaffected by any of the factors (Figure AB.15 and AB.16).

3.2(i) (c) Dopamine D1R receptor levels increased in single housed animals whereas D2R levels decreased in single housed animals and in animals exposed to no odour:

Sex had a Main Effect on the levels of dopamine D2R in the medial PFC (Males< Females) ($F(1.00, 50.00)= 5.437$, $p= 0.024$, Effect Size= 0.098) (Figure 3.21).

An interaction between Sex, Housing and Odour Treatment ($F(2.00, 50.00)= 4.997$, $p= 0.011$, Effect Size= 0.185) was found for the dopamine D1R levels in medial PFC. Simple effects analyses revealed an effect of Housing in no predator odour males (pair housed animals< single housed animals) (Figure 3.20). However, single housed

animals showed *fewer* D2R in the caudate-putamen than pair housed ones (Main Effect of Housing; F(1.00, 74.00)= 4.195, p= 0.044, Effect Size= 0.054) (Figure 3.22).

Animals from the no odour group showed significantly lower D2R levels in the medial PFC compared to the two odour treatments (Main Effect of Odour Treatment; F(2.00, 50.00)= 4.505, p= 0.016, Effect Size= 0.153) (Figures 3.21). Figure 3.23 displays GAPDH, D1R and D2R bands from some of the Western Blots run for this study.

3.3. Measure of anxiety-related behaviours:

3.3(i) Open field test:

3.3 (i) (a) Single housed animals and animals exposed to no odour revealed an increase in anxiety-related behaviour:

Anxiety-related behaviour in the OFT was assessed using conventional measures such as the duration and rate of entry into the center, and the periphery. In addition, locomotor activity exhibited while in the center and in the periphery of the open field was also measured. Finally, total locomotion displayed by the rats in the *entire* open field was also gauged by summing the locomotor activity in the center and in the periphery. Table 2.6 in the previous chapter summarizes these variables.

Specifically, there was a significant interaction of Sex, Housing, and Odour Treatment on rate of movement within the center of the open field (F(2.00, 106.00)= 3.26, p= 0.04, Effect Size= 0.06) and simple effects analyses revealed an effect of Housing on the no predator odour exposed females (pair housed< single housed), as well as an effect of Sex for animals exposed to both single housing and no predator odour (males< females)(Figure AB.21). Latency to move while in the periphery was higher in pair

housed animals relative to single housed ones (Main Effect of Housing; F(1.00, 106.00)= 4.663, p= 0.033, Effect Size= 0.042) (Figure 3.27).

Similarly, percent duration spent in the center showed a main effect of Housing i.e. pair housed animals >single housed animals (F(1.00, 104.00)= 5.07, p= 0.02, Effect Size= 0.04)(Figure 3.24). Rate of thigmotaxis too showed an effect of Housing i.e. pair housed animals < single housed animals (F(1.00, 107.00)= 9.88, p= 0.001, Effect Size= 0.08)(Figure 3.28). Thus, single housed animals spent less time in the center, and showed a greater frequency of thigmotaxic behaviour suggesting an increase in anxiety-related behaviour.

The latency to move when in the center of the open field was affected by an interaction between Sex and Odour Treatment (F(2.00, 106.00)= 3.297, p= 0.041, Effect Size= 0.059). Simple effects analyses revealed an effect of Odour Treatment on females (no odour animals> no predator odour animals, no odour animals > predator odour animals) (Figure 3.26). The latency to initiate thigmotaxis was lower in animals exposed to no odour relative to predator odour (Main Effect of Odour Treatment; F(2.00, 106.00)= 3.72, p= 0.02, Effect Size= 0.06) (Figure 3.29). Thus, animals exposed to no odour in adolescence initiated thigmotaxis earlier than the other groups suggesting increased anxiety-related behaviour.

In addition to the above, exploratory activity in the OFT was assessed. To do so, rearing behaviour was measured while the rat was in the center, and in the periphery of the open field. Rate of rearing in the center of the open field was unaffected by any of the factors (Figure 3.31). Rate of rearing in the periphery of the open field showed a Main Effect of Odour Treatment (F(2.00, 104.00)= 5.120, p= 0.008, Effect size= 0.090; no odour group > no predator odour group, no odour group > predator odour group). It also

showed a Main Effect of Housing (F= 4.445, df= 1, p= 0.037, Effect size= 0.041; Pair Housed< Single Housed groups) (Figure 3.32). Percentage duration spent in rearing in the center or in the periphery showed no effect of any of the factors. Percentage duration spent in rearing in the *entire* open field showed an interaction effect of Housing and Odour Treatment (F(2.00, 104.00)= 3.129, p= 0.048, Effect size= 0.057); simple effects analyses revealed an effect of Odour Treatment on pair housed animals (no odour groups >no predator odour groups) (Figure 3.30). Rate of rearing in the *entire* open field (obtained by analyzing rearing in the center *and* the periphery) showed a Main Effect of Odour Treatment i.e. no odour group >no predator odour group, and no odour group >predator odour group (F(2.00, 104.00)= 5.122, p= 0.008, Effect size= 0.090) (Figure. 3.33).

3.3(ii) Elevated plus maze test:

3.3(ii) (a) Females, single housed animals, and animals exposed to no odour showed reduced anxiety-related behaviour:

The EPM was also used to assess anxiety-related behaviour. To do so, a number of dependent measures were used. Some measures were used to gauge anxiety-related behaviours (e.g. relative duration in the open arms and relative rate of entries into the open arms), whereas others were used to assess locomotor and exploratory activity. A table in the previous chapter summarizes the various dependent measures used. A 2x2x3 ANOVA was performed on each of these measures to determine the effect of Sex, Housing, and Odour Treatment. In the following paragraphs, anxiety- and locomotor activity-related measures will be discussed whereas exploratory activity-related measures will be discussed in the next sub-section.

This paragraph contains information about the effect of Sex on various measures. Females spent a significantly greater relative duration in the open arms than males (Main Effect of Sex; F= 6.299, df= 1, p= 0.014, Effect Size= 0.056; Figure 3.34). Females also had a higher rate of entry into the center relative to males (Main Effect of Sex; F= 5.719, df= 1, p= 0.019, Effect Size= 0.051) (Figure 3.36). Consequently, the Anxiety Index was significantly *lower* for females than males (Main Effect of Sex; F(1.00, 106.00)=4.234, df= 1, p= 0.042, Effect Size= 0.038) (Figure 3.38). In other words, females showed *reduced* anxiety-related behaviour than males.

There was also a Main Effect of Odour Treatment on the Anxiety Index such that it was significantly greater in no predator odour exposed animals relative to the no odour exposed animals (F(2.00, 106.00)= 5.140, p= 0.007, Effect Size= 0.088) (Figures 3.38). This implies that animals exposed repeatedly to the unthreatening odour showed *higher* anxiety-related behaviour than those exposed to none of the odours.

An interaction between Housing and Odour Treatment was found for the rate of entries made into the center of the EPM (F(2.00, 106.00)= 9.144, p= 0.0001, Effect Size= 0.147, Figure 3.36). Simple effects analyses revealed an effect of Odour Treatment among pair housed animals (no odour group >no predator odour group; no predator odour group <predator odour group), and an effect of Housing among the no predator odour animals (pair housed animals <single housed animals) (Figure 3.36).

An interaction between Housing and Odour Treatment was also found for the rate of entries made into the closed arms (F(2.00, 106.00)= 7.207, p= 0.001, Effect Size= 0.120). Simple effects analyses revealed an effect of Housing among no predator odour animals (pair housed animals >single housed animals), and an effect of Odour Treatment among pair housed animals (no predator odour animals <predator odour animals) (Figure

3.35). To conclude, among the animals exposed repeatedly to the unthreatening odour, the ones housed in pairs made more frequent entries to the closed arms and less frequent entiries to the center than those raised in isolation.

3.3(ii) (b) Single housed animals, and animals exposed to neither odour displayed greater exploration in the elevated plus maze test:

Certain exploratory behaviours revealed an effect of Sex. For example, the percent duration spent in head dipping behaviour ($F(1.00, 103.00)= 6.938$, $p= 0.010$, Effect Size= 0.063, Figure 3.40), and the rate of head dipping behaviour ($F(1.00, 103.00)= 5.176$, $p= 0.025$, Effect Size= 0.048, Figure AB.29) were higher in females than males. In conclusion, females showed reduced anxiety-related and increased exploration-related behaviours relative to males in the EPM.

A significant Interaction Effect of Housing and Odour Treatment was found for the percent duration spent in risk assessment ($F(2.00, 103.00)= 7.972$, $p= 0.001$, Effect Size= 0.134) and simple effects analyses revealed an effect of Odour Treatment in pair housed animals (no odour group< no predator odour group, no odour group< predator odour group), and an effect of Housing among no predator odour animals and among predator odour animals (in each case, pair housed animals > single housed animals) (Figure 3.39).

A significant interaction of Housing and Odour Treatment was noted for the percent duration spent in head dipping behaviour ($F(2.00, 103.00)= 8.121$, $p= 0.001$, Effect Size= 0.136), and simple effects analyses revealed an effect of Odour Treatment among pair housed animals (no odour group> no predator odour group, no odour group> predator odour group), and an effect of Housing among the no predator odour group and among the predator odour group (in each case, pair housed animals< single housed

animals) (Figure 3.40). This means that animals exposed to both pair housing and either control or predator odour exposure show reduced exploration in the EPM. On the other hand, isolated rats that are exposed to either type of odour show *increased* exploration in the EPM.

Similarly, rate of head dipping too showed an interaction effect of Housing and Odour Treatment ($F(2.00, 103.00)= 8.872$, $p= 0.0001$, Effect Size= 0.147), which resolved into an effect of Odour Treatment in pair housed animals (no odour group> no predator odour group, no odour group > predator odour group), and an effect of Housing in no predator odour group and the predator odour group (pair housed animals < single housed animals) (Figure AB.29). An interaction between Housing and Odour Treatment for the rate of rearing ($F(2.00, 103.00)= 4.187$, $p= 0.018$, Effect Size= 0.073) was resolved into an effect of Housing in no predator odour exposed animals (pair housed animals< single housed animals) (Figure 3.41). In summary, isolated rats that were exposed to an unthreatening odour repeatedly in adolescence showed more frequent exploratory activity than those that were housed with a cagemate and exposed to an unthreatening odour.

3.4. Measure of depression-related behaviour (anhedonia):

3.4(i) Sucrose preference test:

3.4(i) (a) Preference for sucrose solution was unaffected by housing condition or odour treatment, although females showed greater preference than males:

Andehonia, i.e. the inability to feel pleasure, was assessed in the experimental animals using the SPT. For each animal, the percent sucrose preference was calculated from the total tap water and total sucrose solution consumed during the 60-min test. This

was normalized against the weight of the animals, measured the night before the test. A 2x2x3 ANOVA was run for each of these dependent measures (including the body weight) to assess the effect of Sex, Housing, and Odour Treatment. A reduction in the percent sucrose preference indicates anhedonia.

The impact of Sex on these dependent measures will be discussed in this paragraph. As expected, males weighed more than females (Main Effect of Sex; F(1.00, 106.00)=168.80, p=0.001, Effect Size=0.61) (Figure 3.43). *(Note: In addition, in each of the three odour groups, males were heavier than females. This was revealed when analyzing the interaction effect between Sex and Odour Treatment, and is discussed in more detail in the next paragraph).* In order to control for this difference in weight between the two sexes, percent sucrose preference was calculated per 100gm body weight. However, despite controlling for body weight, Sex had a significant effect on percent sucrose preference with females showing greater percent sucrose preference than males (F(1.00, 106.00)=13.29, p=0.001, effect size = 0.11) (Figure 3.42, Table 3.4). To conclude, females, although lighter than males, showed a greater percent preference for sucrose solution. In other words, females showed *less* anhedonia than males.

The Interaction Effect of Sex and Odour Treatment on various dependent measures will be discussed next. Body weight showed a significant interaction between Sex and Odour Treatment (F(2.00, 106.00)=6.57, p=0.001, Effect Size = 0.11) (Figure 3.43). Simple effects analyses revealed that predator odour exposed males weighed significantly *less* than the no predator odour males, and males were significantly heavier than females in each of the three Odour Treatments (as mentioned in the previous paragraph). In other words, males that were exposed to predator odour repeatedly in

adolescence weighed less than those exposed to the control odour. No other significant effects were revealed during simple effects analyses.

A significant Interaction Effect of Sex and Odour Treatment was also noted on the total water consumed ($F(2.00, 106.00)=4.75$, $p=0.01$, Effect Size = 0.82) (Table 3.4). Simple effects analyses revealed an effect of Odour Treatment among females (no odour exposed females >predator odour exposed females), and an effect of Sex among the no odour group (male<female), and among the predator odour group (male >female). Total fluid consumed (obtained by adding the weight of total water and total sucrose consumed) however, was unaffected by any of the variables (Table 3.4). In short, females exposed repeatedly to predator odour consumed *less* tap water than those exposed to neither odour.

(Note: An important caveat to bear in mind about the sucrose preference test protocol is that it assumes a difference in the weight of the bottle of fluid over the 60-min test period to arise solely due to consumption of that fluid by the animal and not due to spillage or some other reason.)

3.5. Measures of object recognition memory:

3.5(i) Novel object recognition test:

3.5(i) (a) Females, and animals exposed to no odour showed greater preference for the novel object and higher levels of object interaction:

The NOR test was used to assess recognition memory in the rats. This test was conducted twice on each test animal (trial 1, and trial 2). The two trials were a day apart and involved the use of different novel and familiar object pairs. Videos of the animals' performance during the familiarization phase of each trial were scored to assess the total duration (in sec) spent investigating the two objects. This variable was also expressed as a

percentage of the total analyzed duration (i.e. 5-min). Similarly, videos from the test phase of each trial were used to score total duration spent investigating *both* objects (also expressed as a percentage of the total analyzed duration, i.e. 5-min), as well as duration spent investigating the familiar object, and the novel object (each of these was also expressed as a percentage of total duration spent investigating both objects). During the test phase of each trial, the relative duration spent with the novel object was calculated and this measure was called the percent preference for the novel object. It is the main measure that is often used to assess recognition memory (e.g. Jurdak et al., 2009). Each of these dependent measures were subject to a 2x2x3 ANOVA to assess effects of Sex, Housing, and Odour Treatment. The following paragraphs contain information on the outcomes of these analyses.

Sex had a Main Effect on the animals' responses during the test phase of trial 1 and the familiarization phase of trial 2. Specifically, females showed greater percent preference for the novel object in the test phase of trial 1 ($F(1.00, 115.00)= 6.979$, $p= 0.009$, Effect Size= 0.057) (Figure 3.44). This same Main Effect of Sex was also seen in data obtained by averaging the percent preference for the novel object across both trials (i.e. trials 1 and 2) ($F(1.00, 104.00)= 8.782$, $p= 0.004$, Effect size= 0.078) (data not shown). Moreover, during test phase of trial 1, Sex showed a significant Main Effect on the percent duration spent with the familiar object ($F(1,115)= 5.64$, $p=0.019$, Effect size=0.047; Male>Female) (data not shown), and on duration spent with the novel object ($F(1,115)= 6.97$, $p= 0.009$, Effect size= 0.057; Male<Female)(data not shown). However, such an effect was not seen on the total duration spent (sec) with both (familiar and novel) objects (Figure 3.45). No such effects of Sex were seen on any of the variables from the familiarization phase of trial 1 (Figures AB.30 and AB>31). However, during trial 2, Sex

had a Main Effect on the total object interaction duration during the familiarization phase ($F(1.00, 82.00)= 9.917$, $p= 0.002$, Effect Size= 0.108, males > females) (Figures AB.30-AB.33). To conclude, females showed a better recognition memory than males in trial 1 (increased percent preference for the novel object among females). During trial 2, on the other hand, females showed *less* interaction with the objects during the familiarization phase though no difference was seen in the extent of object interaction or recognition memory performance during the test phase.

Certain variables during the NOR test showed an effect of Housing. For example, during the test phase of trial 1, among the no predator odour exposed animals, the ones that were single housed showed greater percent preference for the novel object than the ones that were pair housed (test phase of trial 1) (Interaction Effect of Odour Treatment and Housing; $F(2.00, 115.00)= 4.379$, $p= 0.015$, Effect Size= 0.071) (Figure 3.44). In general, single housed animals also showed greater total object interaction during the familiarization phase of trial 1 than pair housed ones (Main Effect of Housing; $F(1.00, 92.00)= 4.076$, $p= 0.046$, Effect Size= 0.042) (Figure AB.30). Additionally, during the test phase of trial 1, single housed animals showed greater total object interaction (sec) than pair housed ones (Main Effect of Housing; $F(1.00, 115.00)= 6.436$, $p= 0.013$, Effect Size= 0.053) (Figure 3.45). Single housed animals also spent greater percent duration with both objects during the test phase of trial 1($F(1,115)= 7.334$, $p=0.008$, Effect size= 0.060, data not shown). They also spent a greater percent duration with the novel object ($F(1,115)= 7.719$, $p= 0.006$, Effect size= 0.063, data not shown) during the test phase of trial 1.

However, during the test phase of trial 1, single housed animals spent *less* percent duration with the familiar object than pair housed ones ($F(1,115)= 6.423$, $p=0.013$, Effect

size=0.053, data not shown). To summarize, single housed animals displayed a better recognition memory and greater interaction with the novel object and the familiar one than pair housed animals. However, during the familiarization phase of trial 2, single housed animals displayed *lower* total object interaction relative to the pair housed ones (F(1.00, 82.00)= 6.263, p= 0.014, Effect Size= 0.071) (AB.31), although no differences were seen in recognition memory (i.e. percent preference for the novel object).

Finally, certain dependent measures that showed a Main Effect of Odour Treatment. The percent duration spent with both objects (F(2,115)= 6.476, p=0.002, Effect size= 0.101, data not shown), and total object interaction (F(2.00, 115.00)= 5.965, p= 0.003, Effect Size= 0.094, Figure 3.45) during trial 1 both showed a Main Effect of Odour Treatment (in each case, no odour animals > no predator odour animals, no odour animals > predator odour animals). Thus, animals exposed to neither odour (i.e. the no odour group) showed the greatest level of interaction with both objects albeit during the test phase of trial only. Remaining dependent measures from trials 1, 2, and average of both trials showed no effect of any of the factors.

3.6. Correlations:

As mentioned in the previous chapter, a number of dependent measures were correlated with one another. Select correlations that are relevant to the rest of the results are depicted in Figures 3.46 to 3.49.

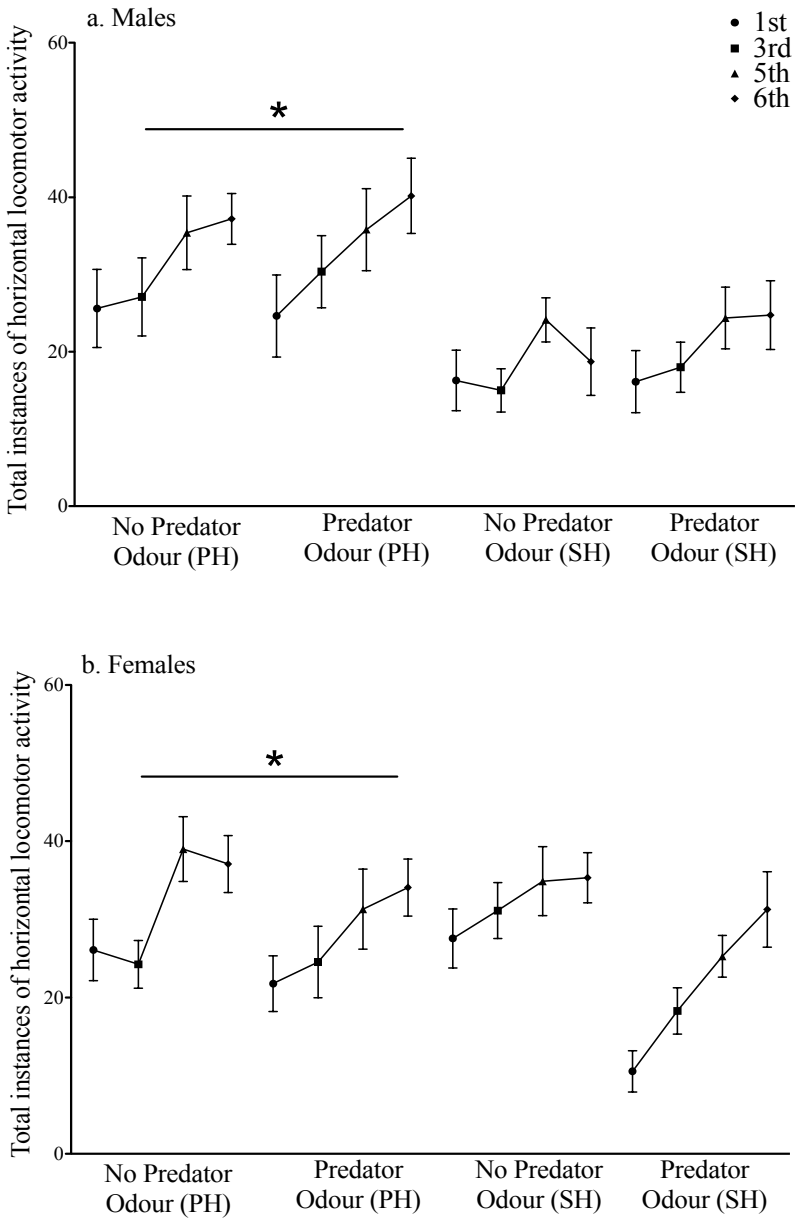


Figure 3.1. <u>Odour exposure behaviour</u>: Horizontal locomotor activity between the different regions of the arena during individual odour exposure periods (Mean +/- SEM).

The graphs above show the horizontal locomotor activity displayed by male (a) and female (b) rats during odour exposure sessions. It was assessed as the number of times the rats crossed one of the two virtual lines that divide the arena into three regions. This dependent measure increased with subsequent exposures ($1^{st} < 5^{th}$, $1^{st} < 6^{th}$, $3^{rd} < 5^{th}$, $3^{rd} < 6^{th}$). Single Housed (SH) males showed lower activity than Pair Housed (PH) males (Housing X Sex interaction). *(* significantly different from SH)*

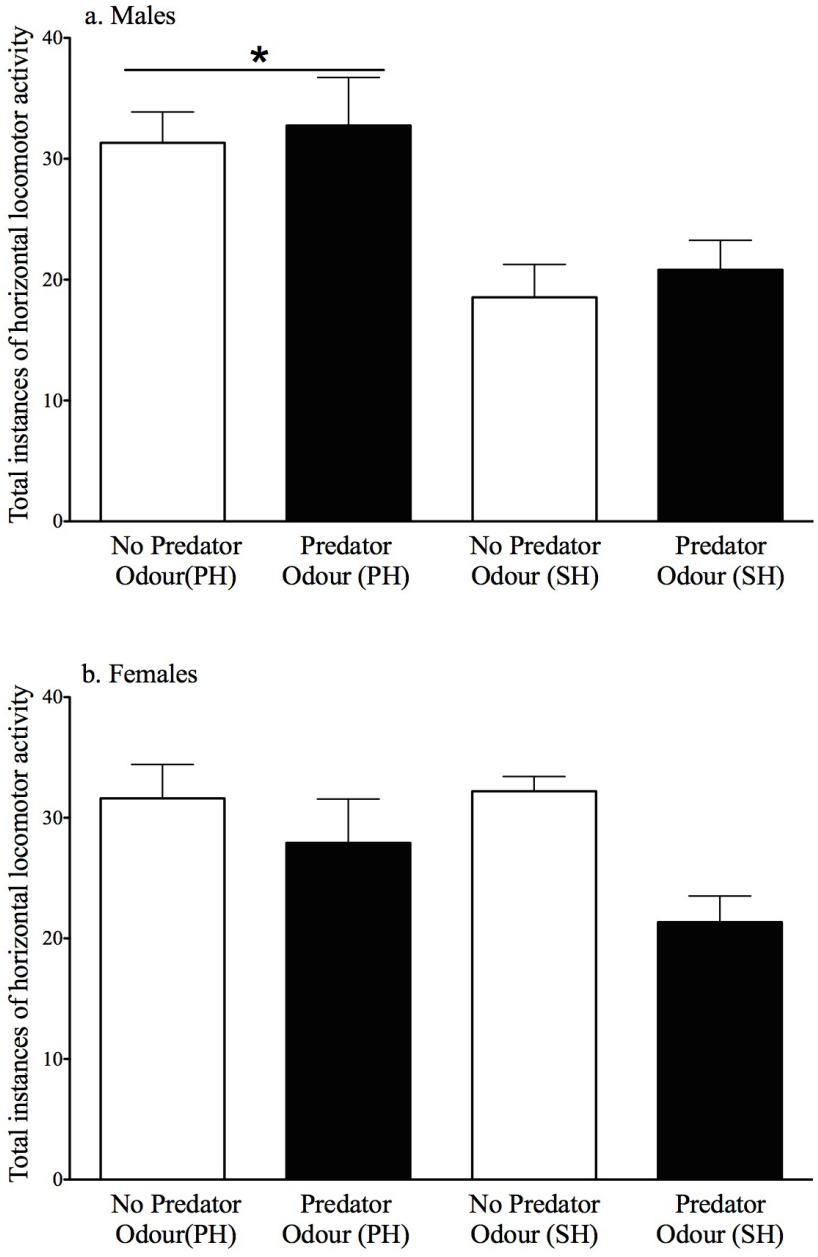


Figure 3.2. <u>Odour exposure behaviour</u>: Horizontal locomotor activity between different regions of the arena during odour exposure (data collapsed across Exposure Period) (Mean +/- SEM).

The graph above shows the horizontal locomotor activity during odour exposure displayed by males (a) and females (b) (data are collapsed across Exposure Period). Horizontal locomotor activity was assessed as the number of times the rats crossed one of the two virtual lines that divide the arena into three regions. Single Housed (SH) males showed lower activity than Pair Housed (PH) males (Housing X Sex interaction). *(* significantly different from SH of same sex)*

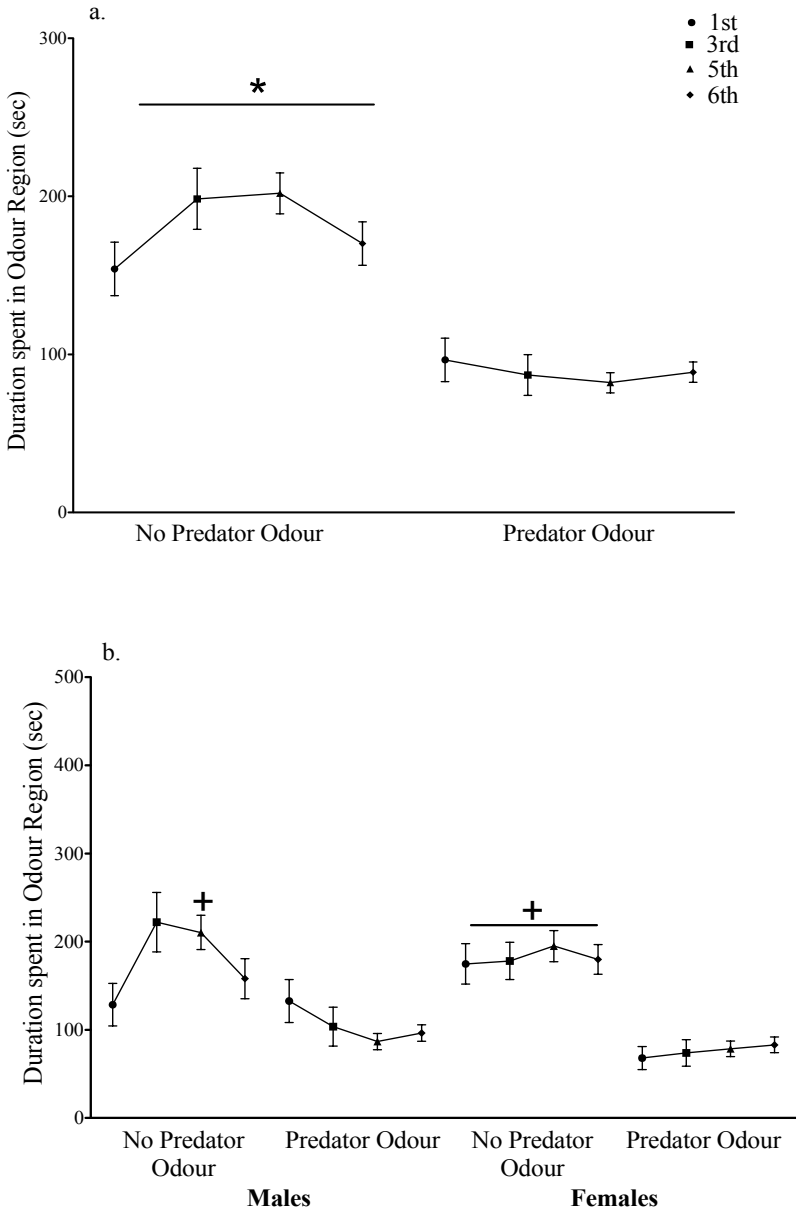


Figure 3.3. <u>Odour exposure behaviour</u>: Mean (+/- SEM) duration spent in the Odour Region (OR) of the arena by rats, collapsed across Sex and Housing (a), and Housing (b).

(a) In general, predator odour exposed animals spent *less* time in the odour region than those exposed to no predator odour (main effect of Odour Treatment). *(* is significantly different from predator odour group.)* (b) Additionally, females exposed to predator odour spent less time in the odour region compared to females exposed to no predator odour, during *each* exposure period. Predator odour exposed males, on the other hand, spent less time in the odour region compared to no predator odour exposed males only during the 5th exposure (Odour Treatment X Sex X Exposure Period interaction). *("+" is significantly different from predator odour group of the same sex and exposure period.)*

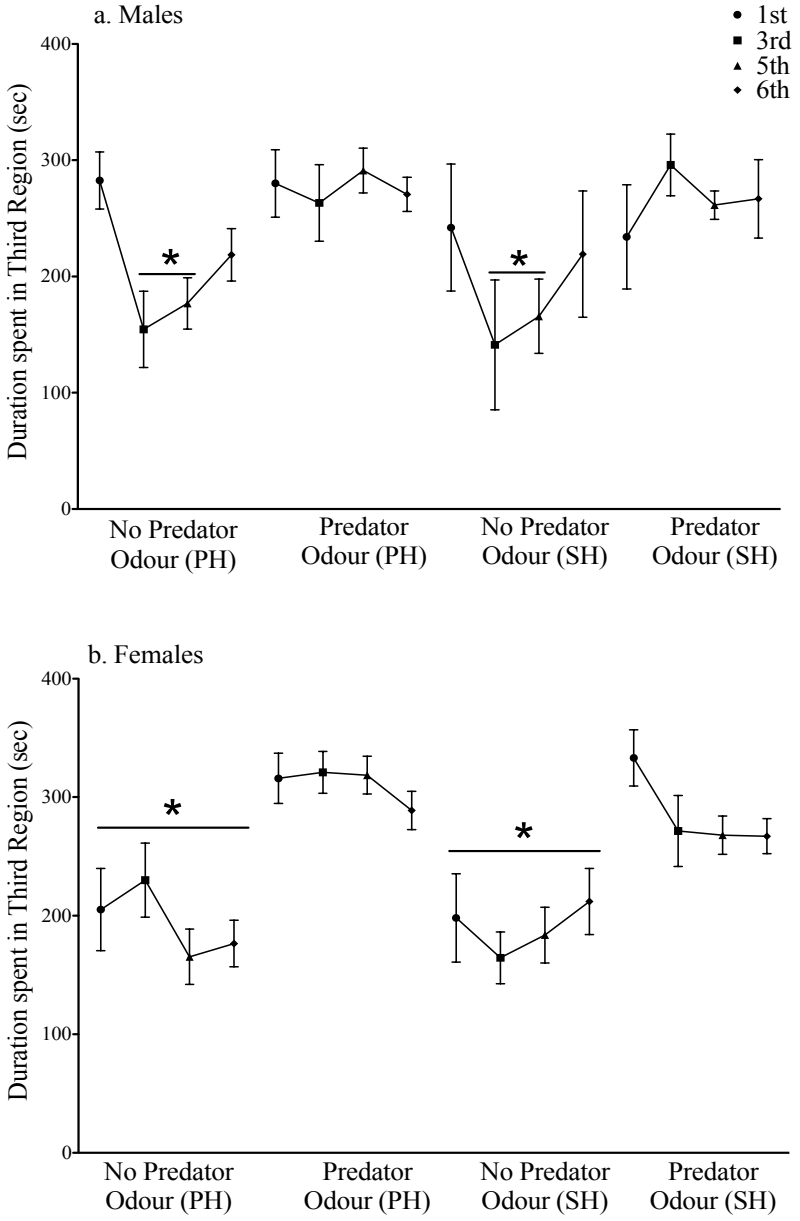


Figure 3.4. <u>Odour exposure behaviour</u>: Mean duration (+/- SEM) spent in the Third Region (TR) of the arena for males (a) and females (b).

This measure revealed an effect of Odour Treatment in males after the 3rd and 5th exposure periods (No Predator Odour < Predator Odour), and in females after each exposure period (No Predator Odour < Predator Odour) (Sex X Odour Treatment X Exposure Period interaction). *("*" is significantly different from PO group of the same sex and exposure period.)*
(Note: "PH" and "SH" refer to "Pair Housed" and "Single Housed" respectively.)

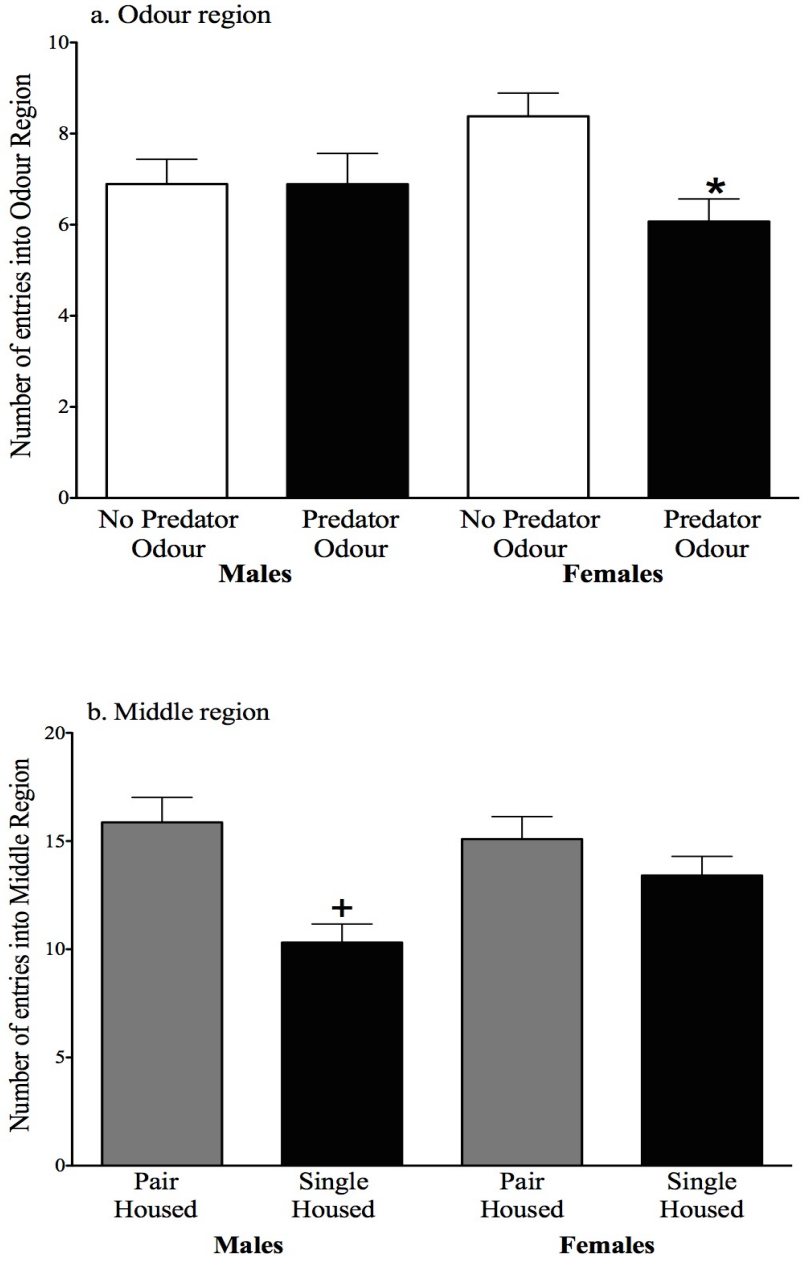


Figure 3.5. <u>Odour exposure behaviour</u>: Mean (+/- SEM) number of entries made into the Odour Region (OR) and the Middle Region (MR) by rats during odour exposure (data collapsed across one or more factors).

The data are collapsed across Housing and Exposure Period in the top figure (a), and across Odour Treatment and Exposure Period in the bottom figure (b). Females exposed to Predator Odour made fewer entries into the OR region compared to No Predator Odour (NPO) females. Single-Housed males made fewer entries into the MR than their Pair-Housed (PH) equivalents. *("*" is significantly different from NPO group of same sex, and "+" is significantly different from PH of same sex.)*

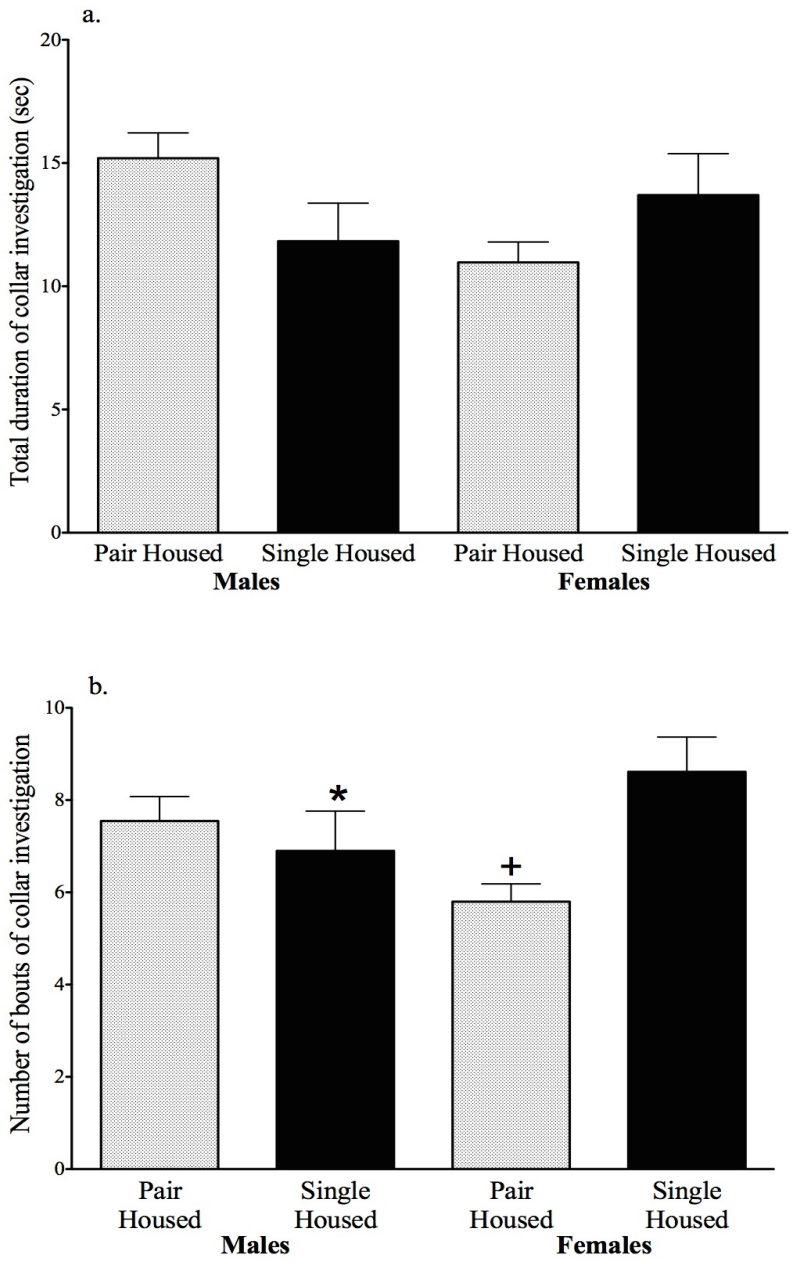


Figure 3.6. <u>Odour exposure behaviour</u>: Mean (+/- SEM) duration and number of collar investigations.

(a) Data are collapsed across Exposure Period and Odour Treatment. None of the factors had an effect on this dependent measure. (b) Date are collapsed across Exposure Period and Odour Treatment. Sex and Housing had an interaction effect on this dependent variable. Simple effects analyses revealed an effect of sex among Single Housed animals (Males<Females; "*"), and an effect of Housing among females (Pair Housed < Single Housed, "+").

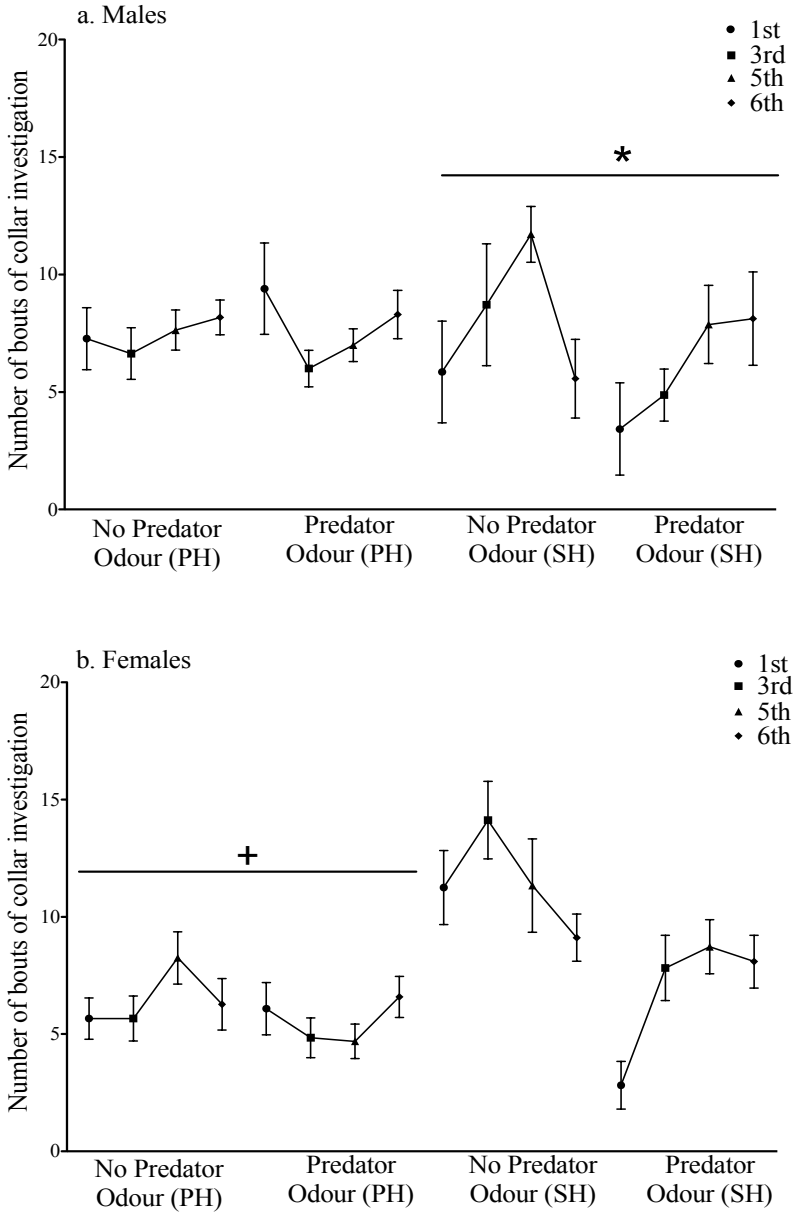


Figure 3.7. <u>Odour exposure behaviour</u>: Mean (+/- SEM) number of collar investigations across for males (a) and females (b).

The number of investigations of the collar increased with each subsequent exposure (1^{st} S<5^{th}, 3^{rd}< 5^{th}). Sex and Housing had an interaction effect on number of collar investigations; simple effects analyses revealed an effect of Sex in SH animals (Male> Female) and of Housing among females (PH<SH). *("*" is significantly different from females of SH group, and "+" is significantly different from SH of same sex.) (Note: "PH" and "SH" refer to "Pair Housed" and "Single Housed" respectively.)*

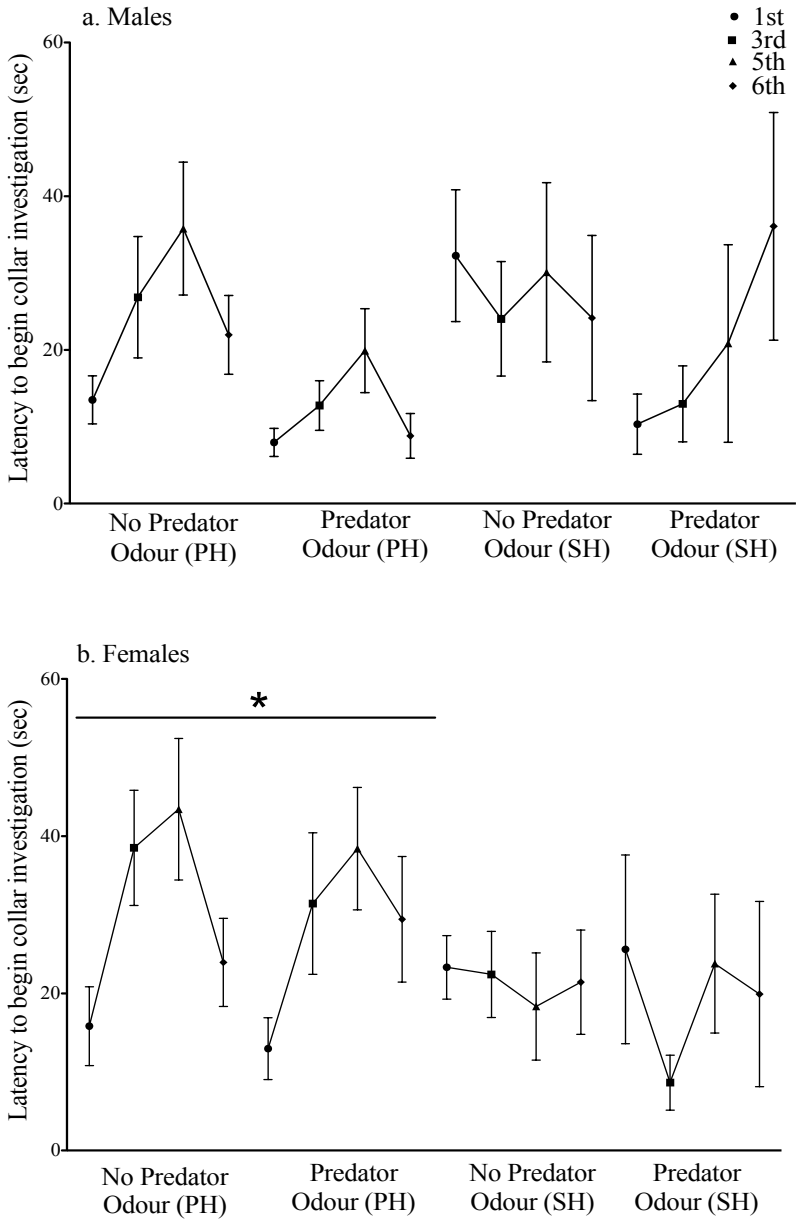


Figure 3.8. <u>Odour exposure behaviour</u>: Mean (+/- SEM) latency to initiate collar investigations for males (a) and females (b).

Exposure Period affected this measure ($1^{st} < 5^{th}$, $3^{rd} < 5^{th}$). Sex, and Housing had an interaction effect on this measure; simple effects analyses revealed an effect of Housing on females (PH>SH). *("*" is significantly different from SH group of the same sex.) (Note: "PH" and "SH" refer to "Pair Housed" and "Single Housed" respectively.)*

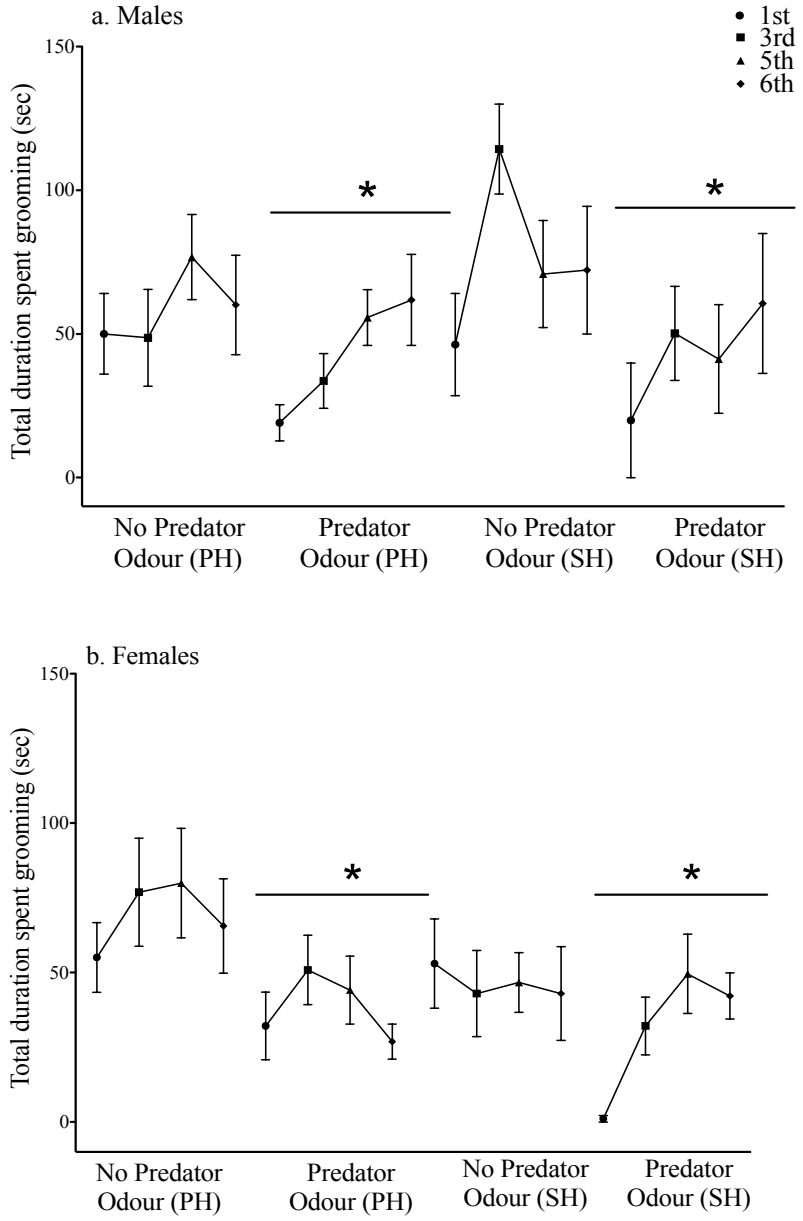


Figure 3.9. <u>Odour exposure behaviour</u>: Mean (+/- SEM) duration spent grooming for males (a) and females (b) during odour exposure.

Predator Odour animals spent less time grooming compared to No Predator Odour ones. Exposure Period also had a Main effect on this dependent measure ($1^{st}<3^{rd}$, $1^{st}<5^{th}$, $1^{st}<6^{th}$). *("*" is significantly different from the no predator odour exposed animals.) (Note: "PH" and "SH" refer to "Pair Housed" and "Single Housed", respectively.)*

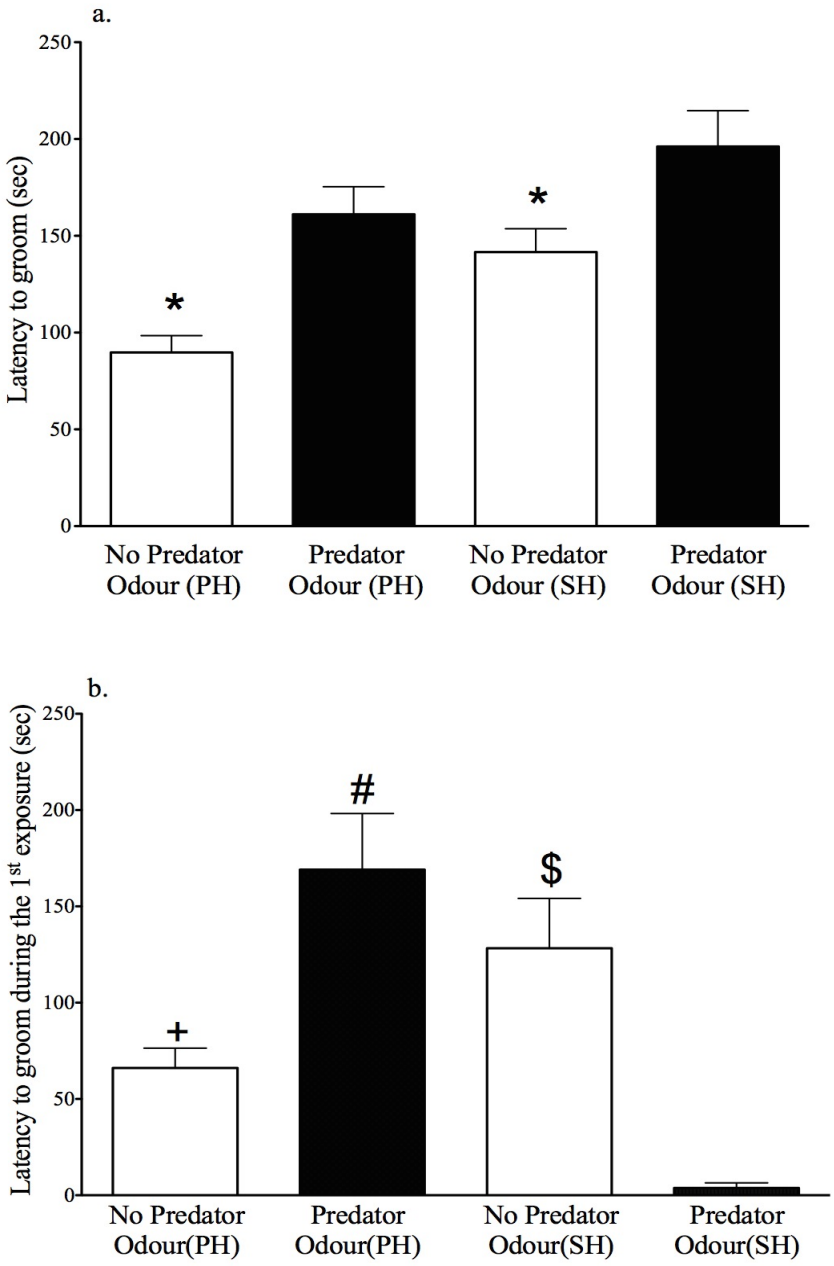


Figure 3.10. <u>Odour exposure behaviour</u>: Mean (+/- SEM) latency to groom (sec) collapsed across Exposure Period and Sex (a), and the latency to groom during the 1st exposure collapsed across Sex (b).

Latency to groom showed a main effect of Odour Treatment (NPO >PO). Also, an effect of Housing was seen for the PO group during the 1st odour exposure (PH > SH), and an effect of Odour Treatment on the SH group during the 1st exposure (NPO>PO) (Exposure Period X Housing X Odour Treatment interaction).
("" is significantly different from predator odour group. ""+" and "$" are significantly different from the predator odour group of the same housing condition, and "#" is significantly different from the SH animals of the same odour treatment.)*

(Note: "PH" and "SH" refer to "Pair Housed" and "Single Housed", respectively. And "NPO" and "PO" refer to "No Predator Odour" and "Predator Odour", respectively.)

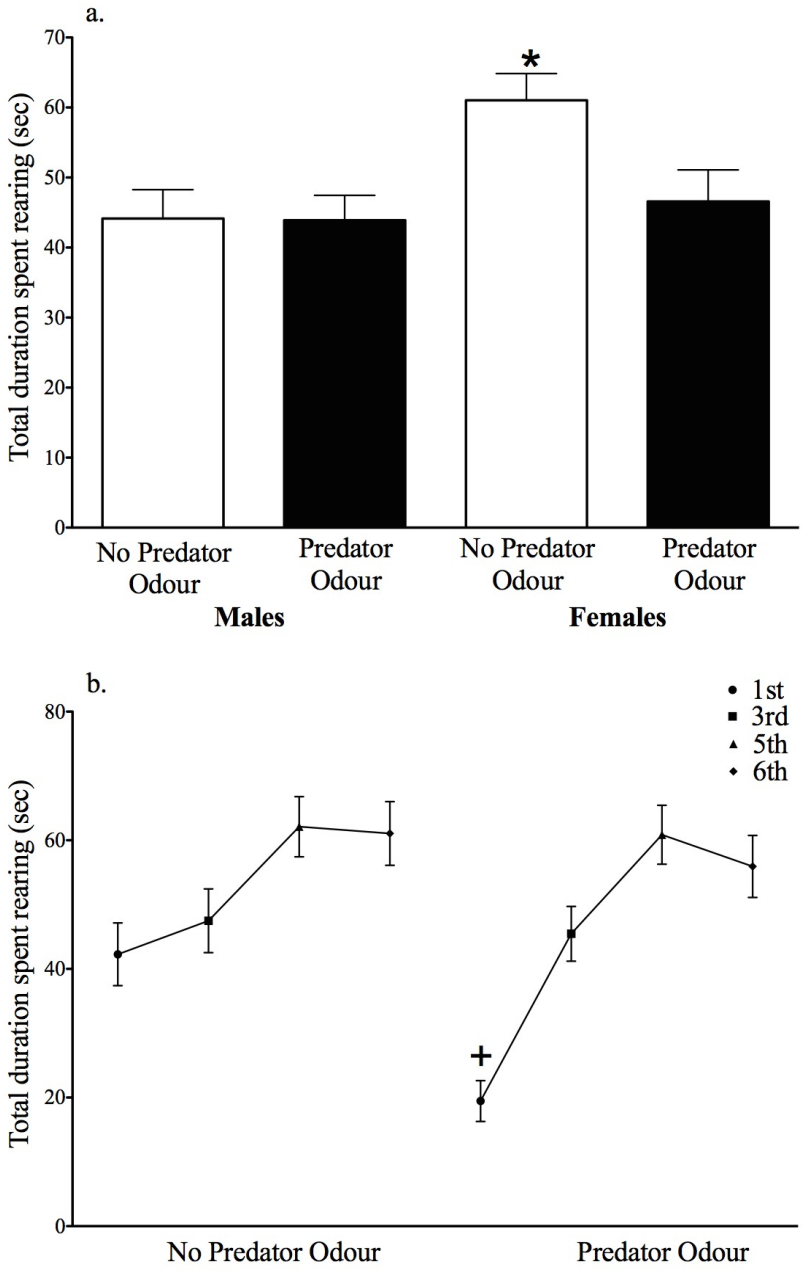


Figure 3.11. <u>Odour exposure behaviour</u>: Mean (+/- SEM) duration spent rearing during odour exposure.

Panel (a) shows data collapsed across Housing, and Exposure Period, and (b) shows data collapsed Sex and Housing. Among the No Predator Odour group, females spent longer rearing than males (Sex X Odour Treatment interaction). During the 1st exposure, No Predator Odour animals spent more duration rearing compared to the Predator Odour group (Exposure Period X Odour Treatment interaction). *("*" is significantly different from males of the same odour treatment, whereas "+" is different from the no predator odour group during the same exposure period.)*

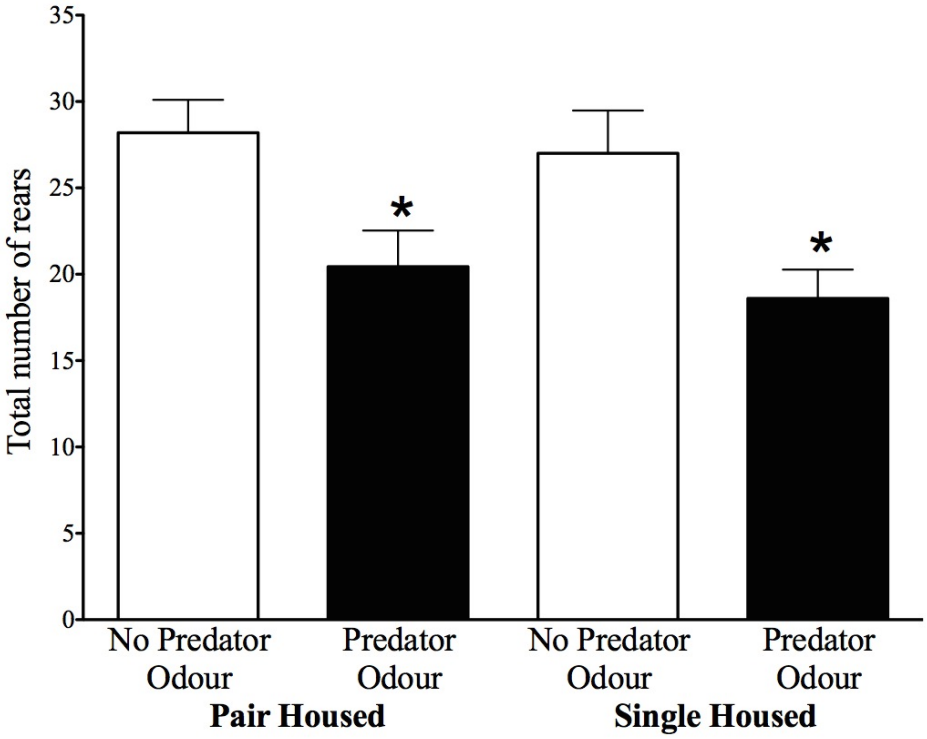


Figure 3.12. <u>Odour exposure behaviour</u>: Mean (+/- SEM) number of rears shown by the animals (data collapsed across Sex, and Exposure Period).

No Predator Odour animals reared significantly more times than the Predator Odour animals (main effect of Odour Treatment) *("*" is significantly different from no predator odour group, collapsed across sex, exposure period and housing condition*.)

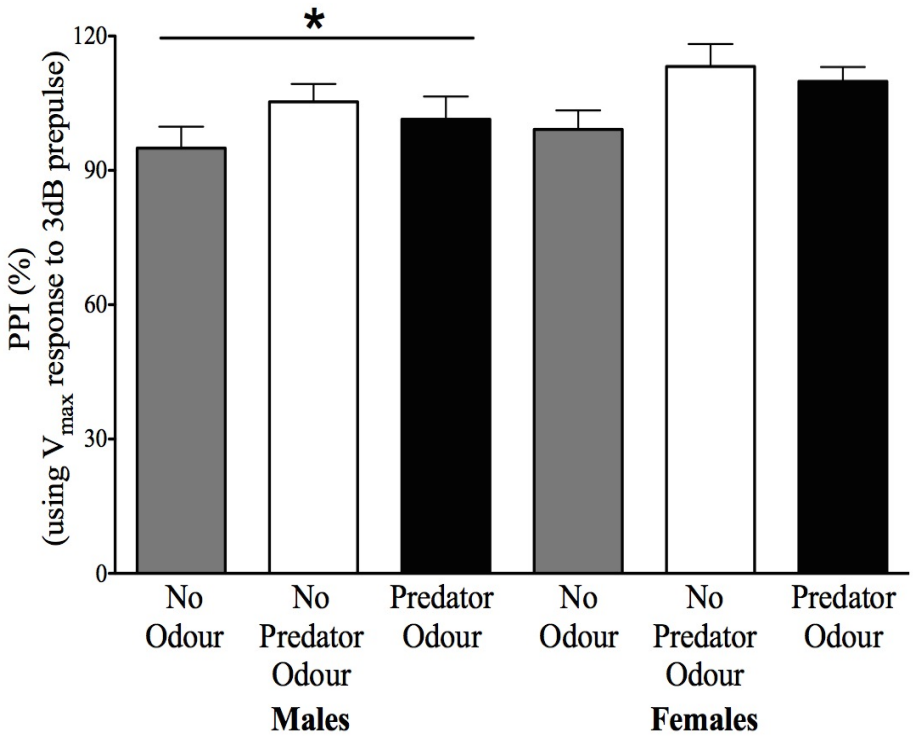


Figure 3.13. Prepulse Inhibition of startle: Mean (+/- SEM) percent prepulse inhibition (PPI) at 3dB collapsed across Exposure Period and Housing.

Females showed a greater PPI than males (main effect of Sex, "*").

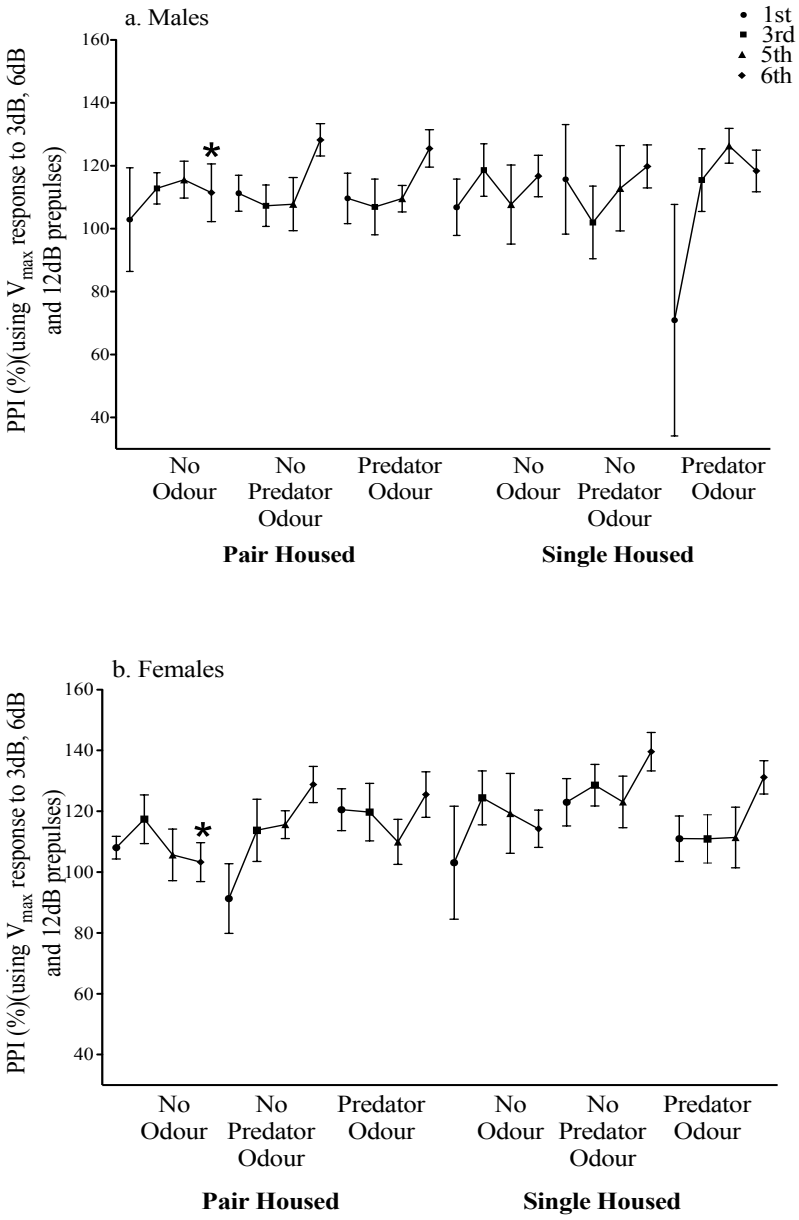


Figure 3.14. <u>Prepulse Inhibition of startle</u>: Mean (+/- SEM) percent prepulse inhibition (PPI) obtained by averaging the PPI response to each of the three prepulses (3dB, 6dB, and 12dB) for males (a) and females (b).

Exposure Period and Odour Treatment had an interaction effect; simple effects revealed an effect of Odour Treatment after the 6th exposure (No Odour< No Predator Odour, No Odour < Predator Odour), and an effect of Exposure Period on the No Predator Odour (1st<6th, 3rd<6th, 5th<6th), and Predator Odour (1st<6th) groups.
("" is significantly different from no predator odour and predator odour groups during the same exposure period.)*

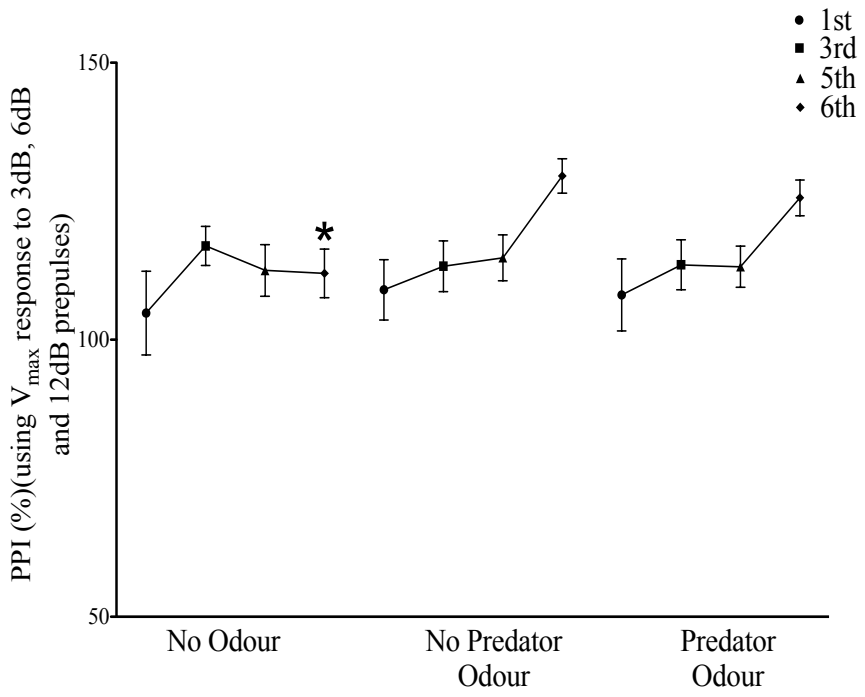


Figure 3.15. <u>Prepulse Inhibition of startle</u>: Mean (+/- SEM) percent prepulse inhibition (%PPI) collapsed across Sex and Housing.

Exposure Period and Odour Treatment had an interaction effect; simple effects revealed an effect of Odour Treatment after the 6th exposure (No Odour< No Predator Odour, No Odour < Predator Odour), and an effect of Exposure Period on the No Predator Odour (1st<6th, 3rd<6th, 5th<6th), and Predator Odour (1st<6th) groups. *("*" is significantly different from no predator odour and predator odour groups during the same exposure period.)*

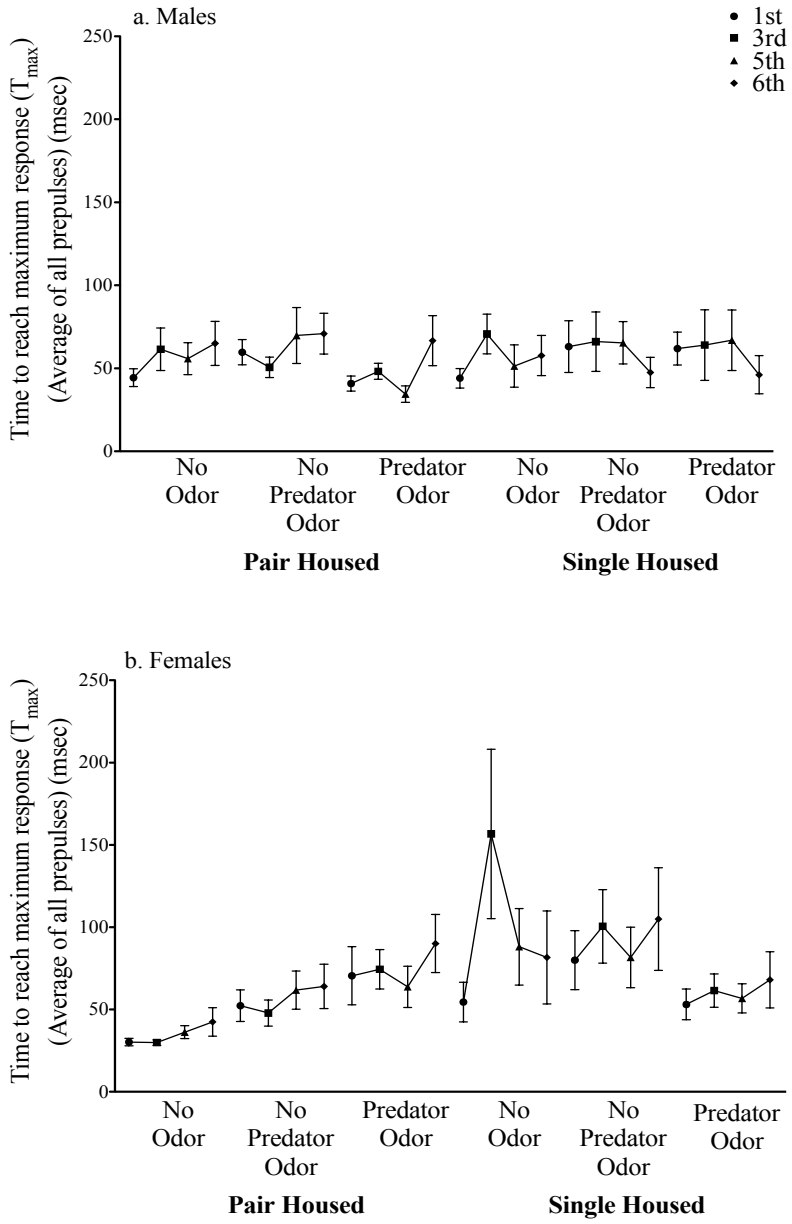


Figure 3.16. <u>Prepulse Inhibition of startle</u>: Mean (+/- SEM) time to reach maximum response obtained by averaging the time to reach maximum response for each of the three prepulses used (3dB, 6dB, 12dB) for males (a) and females (b).

An interaction between Exposure Period and Housing was found; simple effects analyses revealed an effect of Exposure Period on Pair Housed ($1^{st} < 6^{th}$) and Single Housed animals ($1^{st} < 3^{rd}$), and an effect of Housing at each exposure period (Pair Housed< Single Housed).

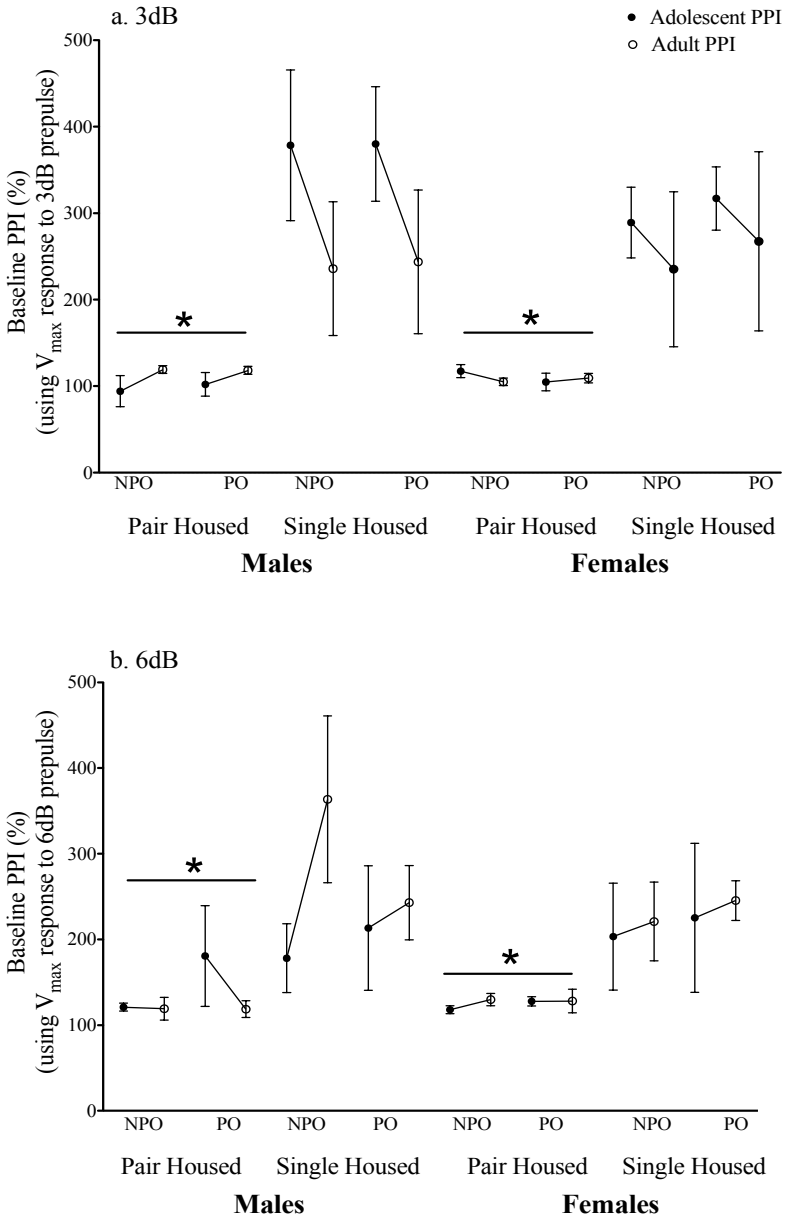


Figure 3.17. <u>Prepulse Inhibition of startle ("Baseline" PPI)</u>: Mean (+/- SEM) "baseline" PPI response to (a) 3dB, and (b) 6dB prepulse trials for the various experimental groups.

At 3dB and 6dB prepulses, Housing has a main effect on the "baseline" PPI (pair housed< single housed). Additionally, at 3dB prepulse, Housing has an interaction effect with Exposure Period. Simple effects analyses reveal an effect of Housing at both Exposure Periods (in each case, Pair Housed< Single Housed). *("*" is significantly different from the single housed group, collapsed across Sex, Odour Treatment and Exposure Period).*

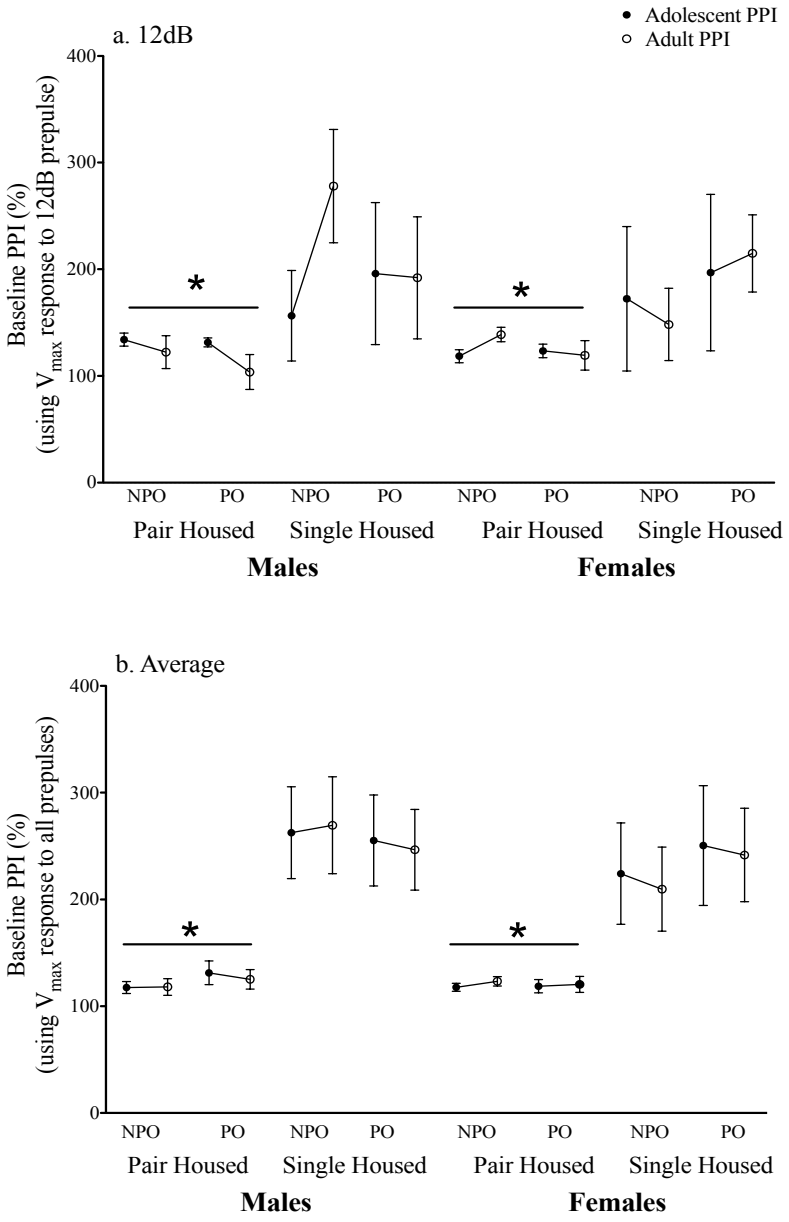


Figure 3.18. <u>Prepulse Inhibition of startle ("Baseline" PPI)</u>: Mean (+/- SEM) "baseline" PPI response to (a) 12dB, and (b) average of all three prepulse trials for the various experimental groups.

In each case, Housing has a main effect (Pair Housed < Single Housed). *("*" is significantly different from the single housed group.)*

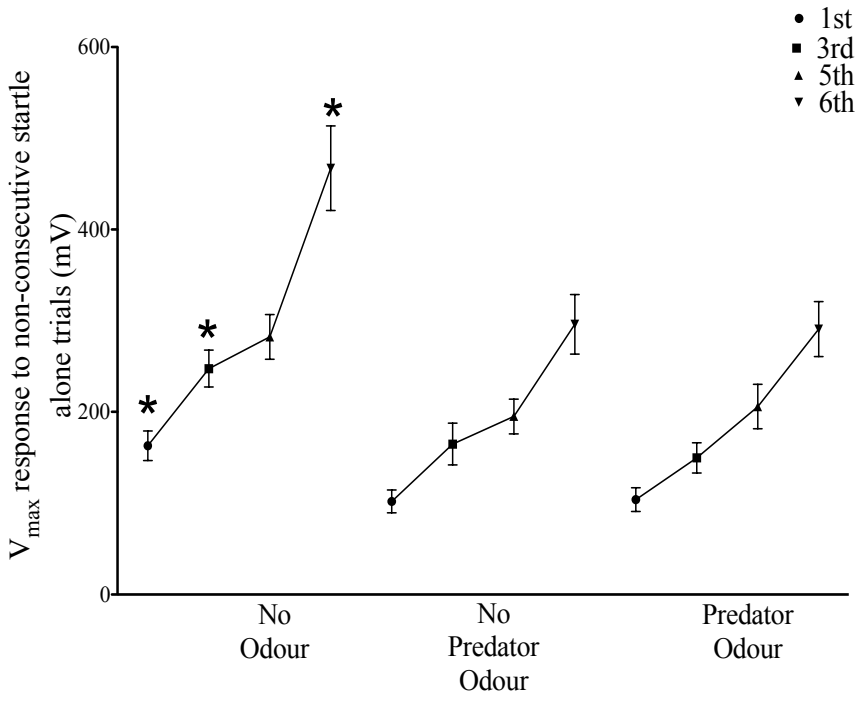


Figure 3.19. <u>Acoustic startle response</u>: Mean (+/- SEM) to startle alone trials collapsed across Sex and Housing.

Exposure Period and Odour Treatment had an interaction effect; simple effects analyses revealed an effect of Exposure Period on No Odour (NO), No Predator Odour (NPO), and Predator Odour (PO) groups (NO: $1^{st}<3^{rd}$, $1^{st}< 5^{th}$, $1^{st}< 6^{th}$, $3^{rd}<6^{th}$, $5^{th}< 6^{th}$; NPO: $1^{st}<3^{rd}$, $1^{st}<5^{th}$, $1^{st}<6^{th}$, $3^{rd}<6^{th}$, $5^{th}<6^{th}$; PO: $1^{st}<3^{rd}<5^{th}<6^{th}$), and an effect of Odour Treatment after the 1^{st}, 3^{rd}, and 6^{th} exposure period (NO> NPO, NO> PO). *("*" is significantly different from NPO and PO groups during the same exposure period.)*

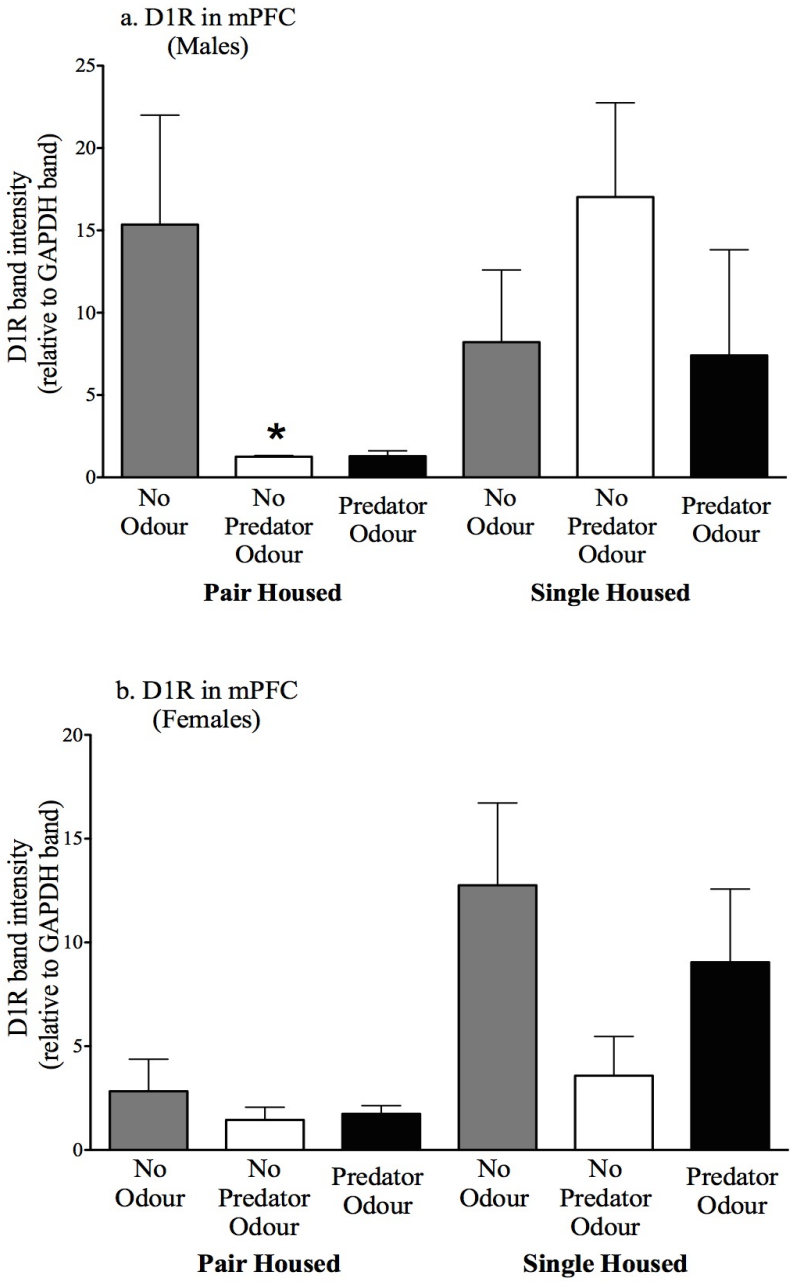


Figure 3.20. <u>Dopamine receptor levels</u>: Mean (+/- SEM) intensity of the dopamine receptor D1R in the medial prefrontal cortex of males (a) and females (b).

An Interaction Effect of Sex, Housing and Odour Treatment was seen; simple effects analyses revealed an effect of Housing on No Predator Odour males (Pair Housed < Single Housed)(represented by "*" in the figure).

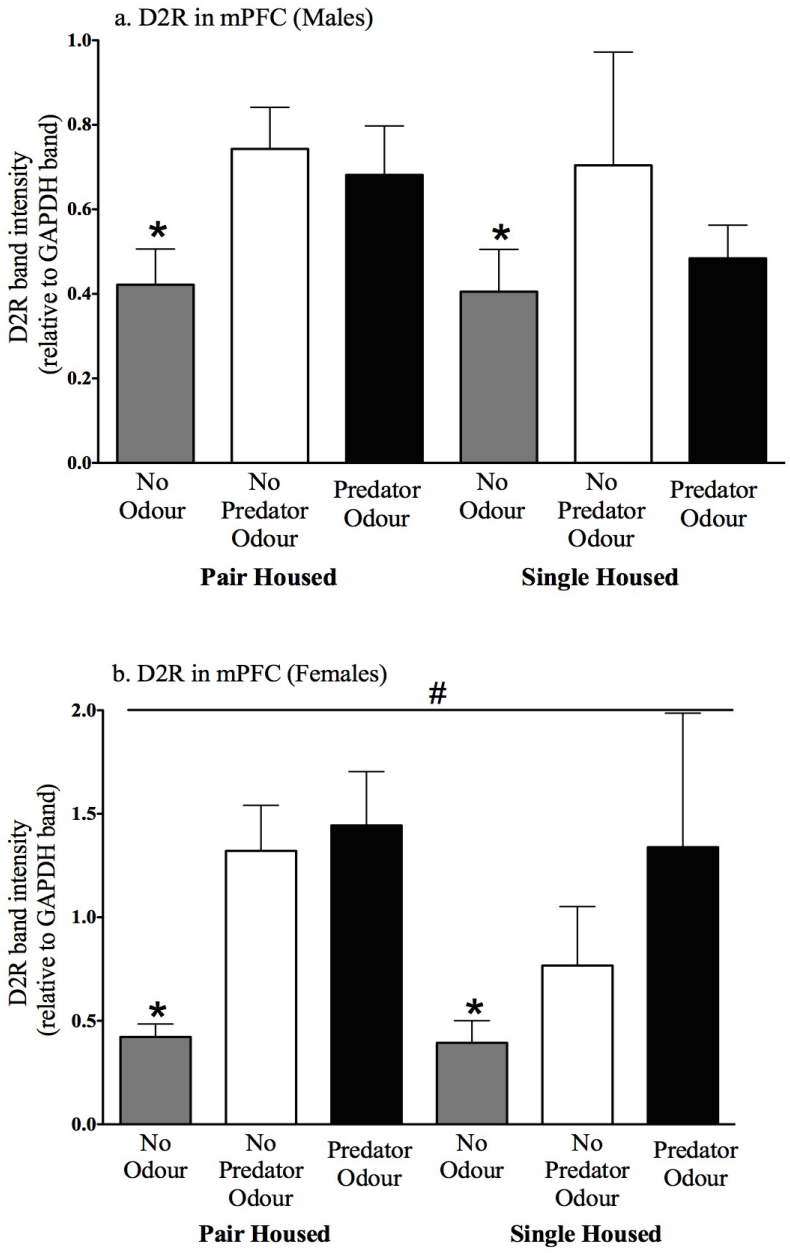


Figure 3.21. <u>Dopamine receptor levels</u>: Mean (+/- SEM) intensity of the dopamine receptor D2R in the medial prefrontal cortex of males (a) and females (b).

Females showed greater levels of D2R than males (represented by "#" in the figure). Animals from the No Odour group showed significantly lower D2R levels compared to the other two odour treatments (represented by "*" in the figure).

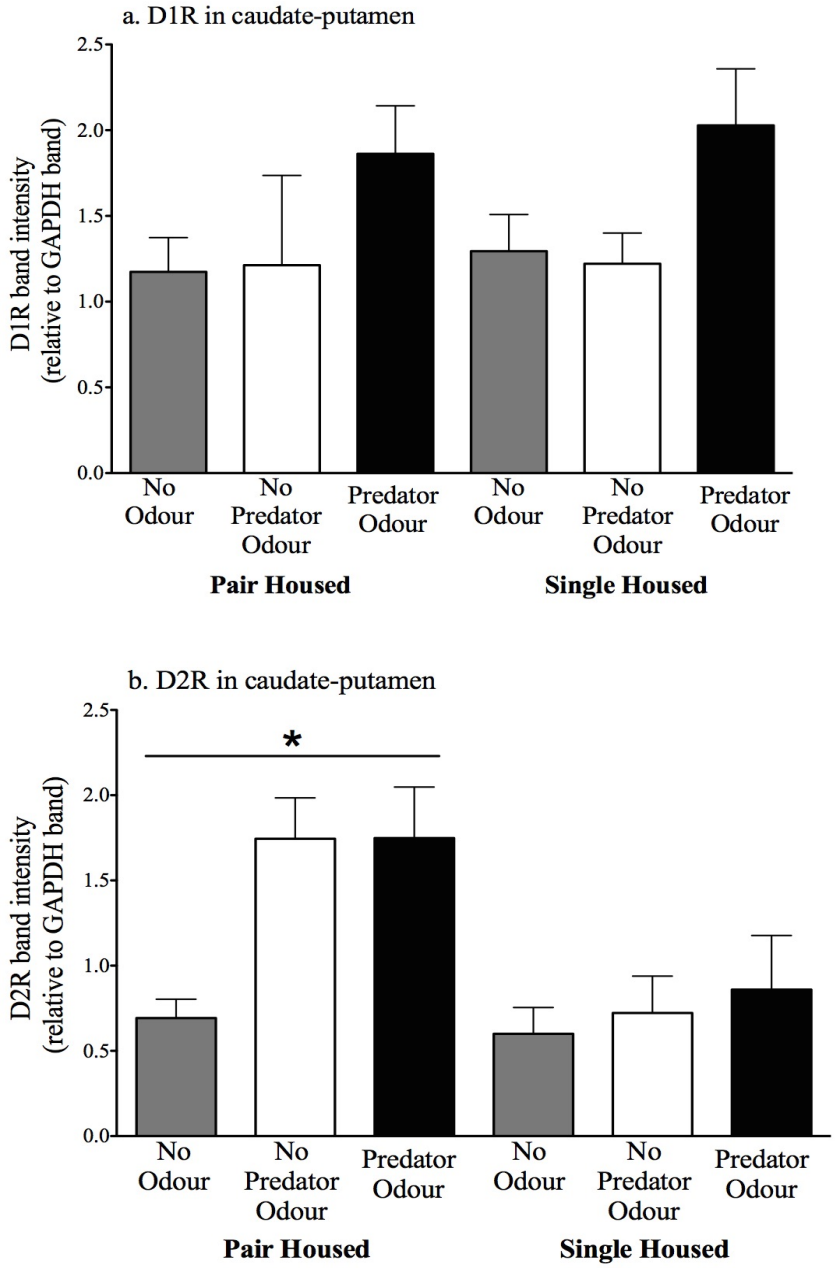


Figure 3.22. <u>Dopamine receptor levels</u>: Mean (+/- SEM) intensity of the dopamine receptor D1R (a) and D2R (b) in the caudate-putamen.

Data are collapsed across sex due to insufficient number of animals. Single Housed animals showed fewer D2R in the caudate-putamen than Pair Housed animals (represented by "*" in the figure).

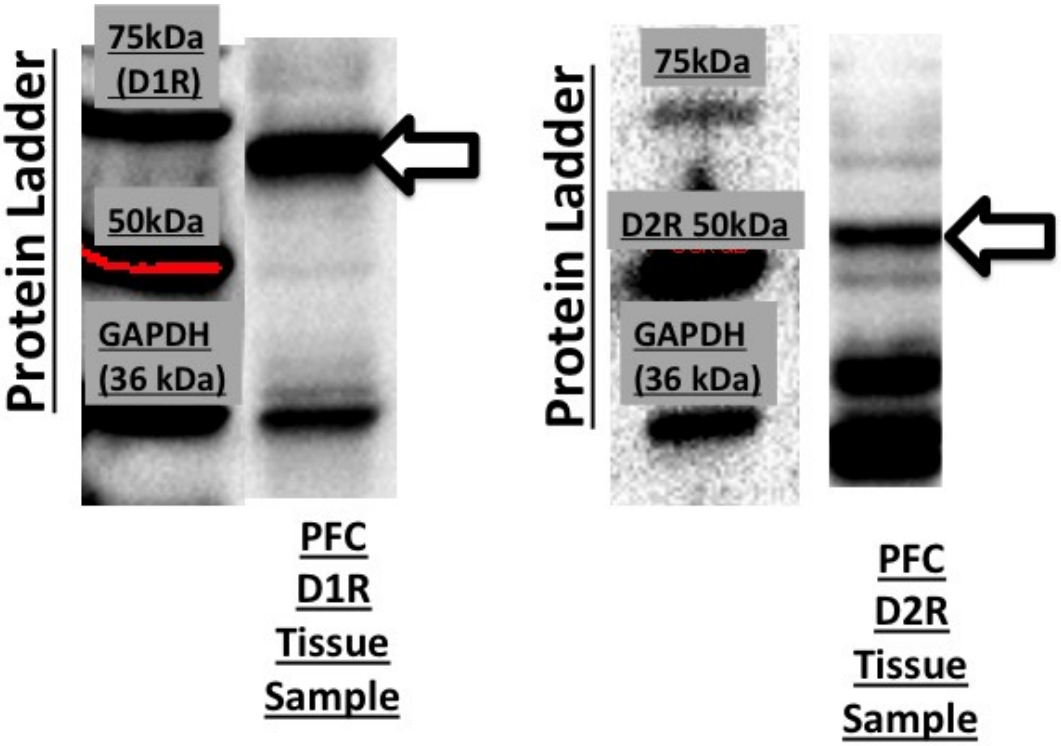


Figure 3.23. Dopamine receptor levels: Images of representative D1R and D2R bands from Western Blots run on rat tissue samples.

Note that the arrow on the left points towards the D1R protein band in a prefrontal cortex (PFC) sample whereas the arrow on the right points towards the D2R protein band in another PFC sample.

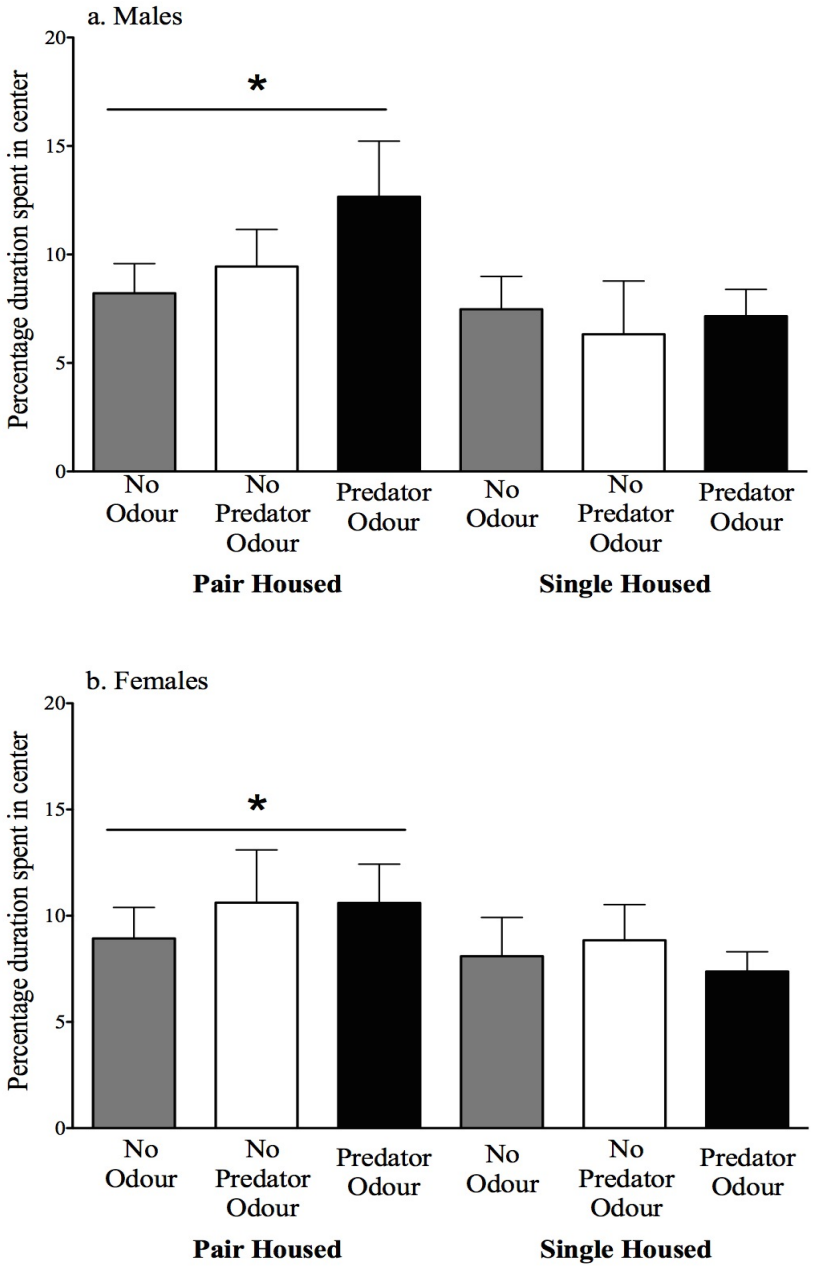


Figure 3.24. Open field test: Mean (+/- SEM) duration spent in the center during the Open Field Test by males (a) and females (b).

Pair Housed animals showed a higher percent duration spent in the center relative to Single Housed ones ("*").

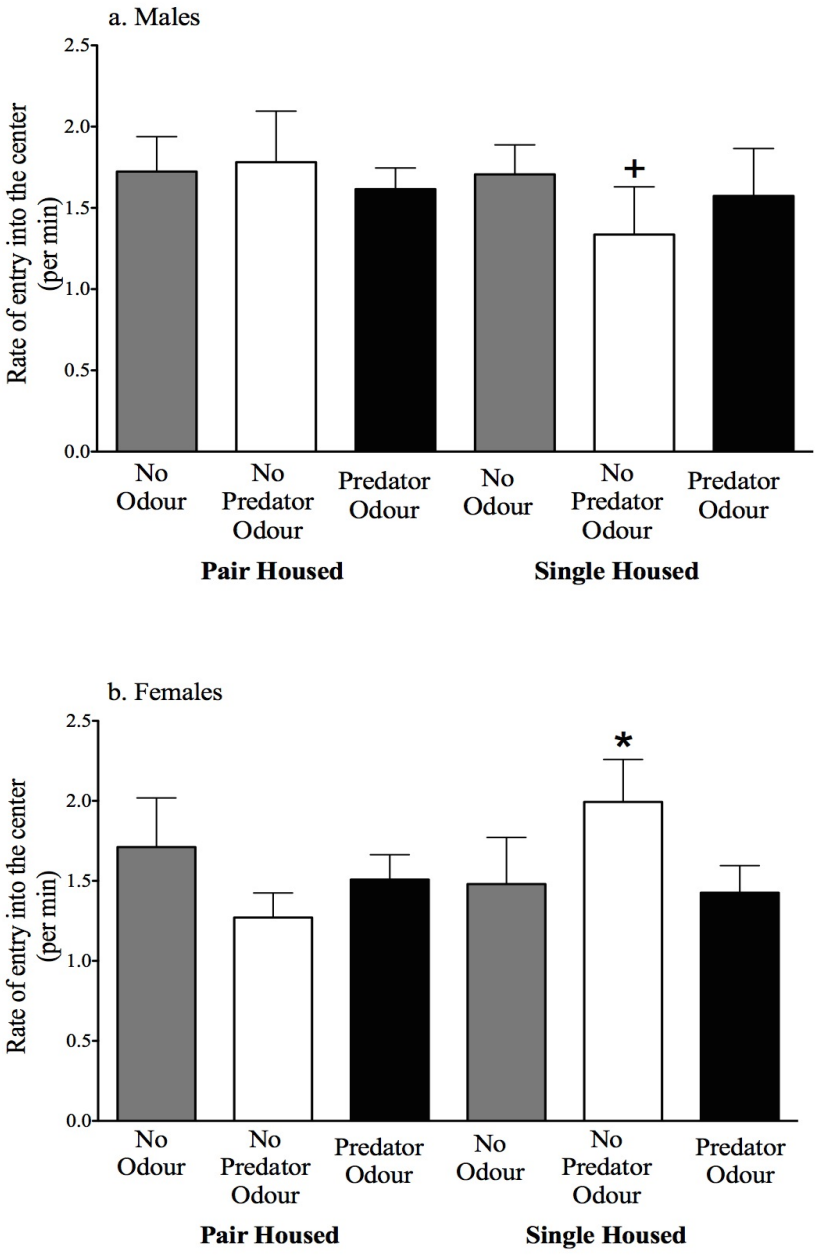


Figure 3.25. <u>Open field test</u>: Mean (+/- SEM) rate of entries made into the center by males (a) and females (b) during the Open Field Test.

Sex, Housing, and Odour Treatment had an interaction effect on this variable; simple effects analyses revealed an effect of Housing on No Predator Odour exposed females (Pair Housed < Single Housed), as well as an effect of Sex for animals exposed to Single Housing and No Predator Odour (Male< Female). *("+" is significantly different from females of the same housing condition and odour treatment whereas "*" is significantly different from pair housed animals of the same odour treatment and sex.)*

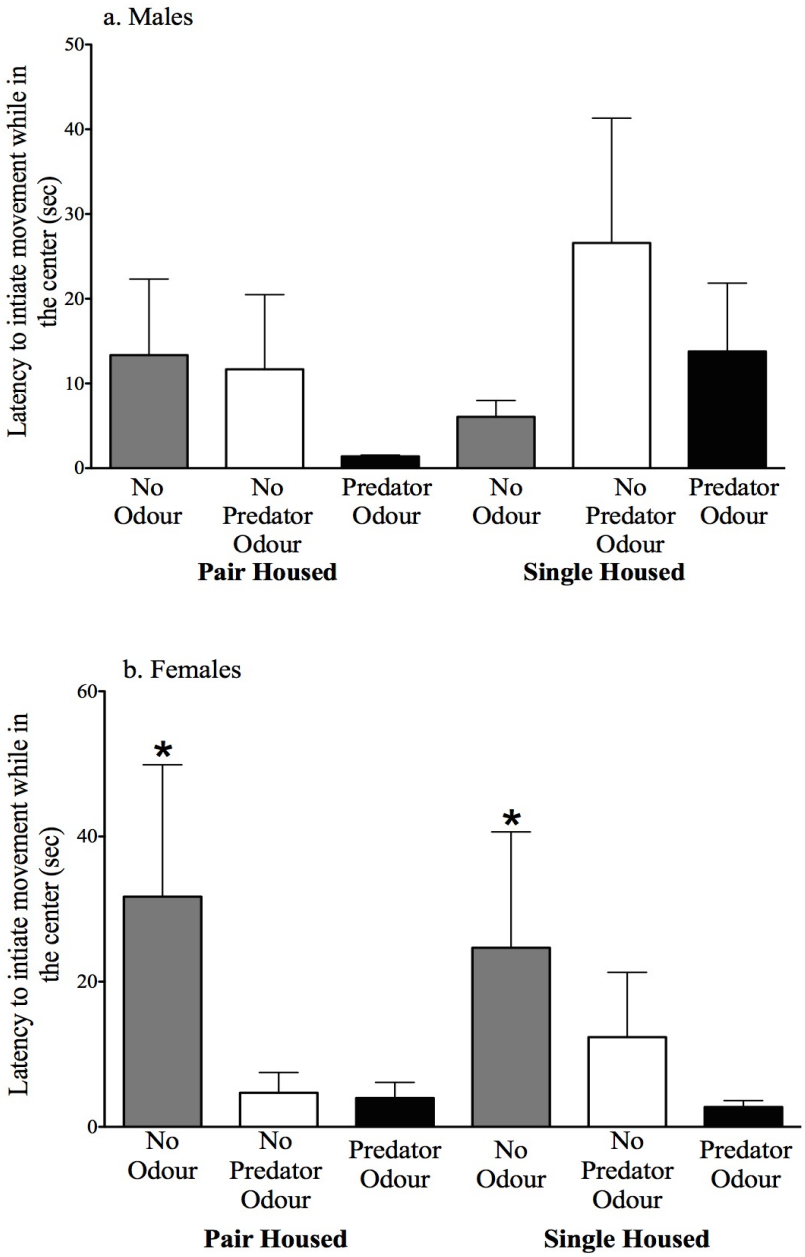


Figure 3.26. <u>Open field test</u>: Mean (+/- SEM) latency to move while in the center by males (a) and females (b) during the Open Field Test.

Sex and Odour Treatment had an interaction effect on this dependent measure. Simple effects analysis revealed an effect of Odour Treatment on females (No Odour> No Predator Odour, No Odour> Predator Odour). *("*" is significantly different from the no predator odour and predator odour treatments of the same sex.)*

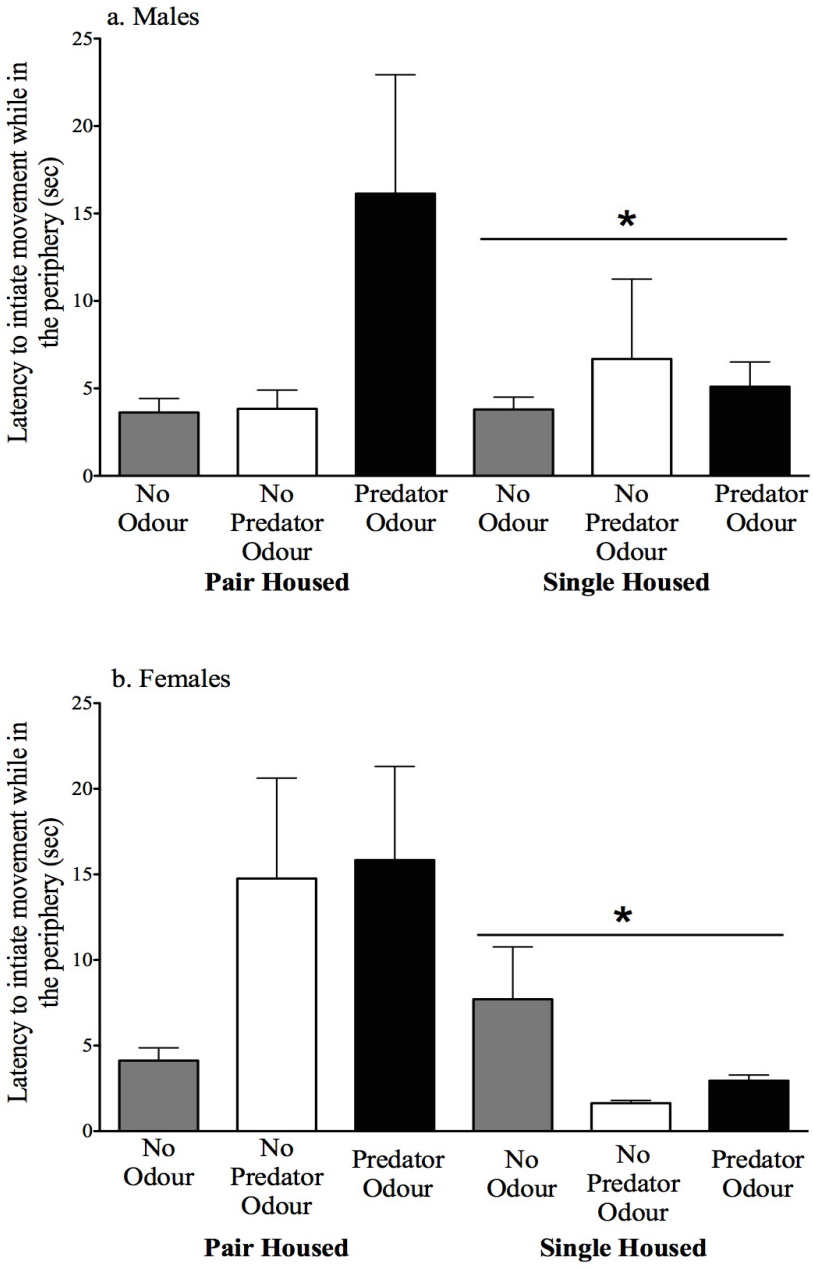


Figure 3.27. <u>Open field test</u>: Mean (+/- SEM) latency to move while in the periphery by males (a) and females (b) during the Open Field Test.

This measure was greater in Pair Housed animals than Single Housed ones ("*").

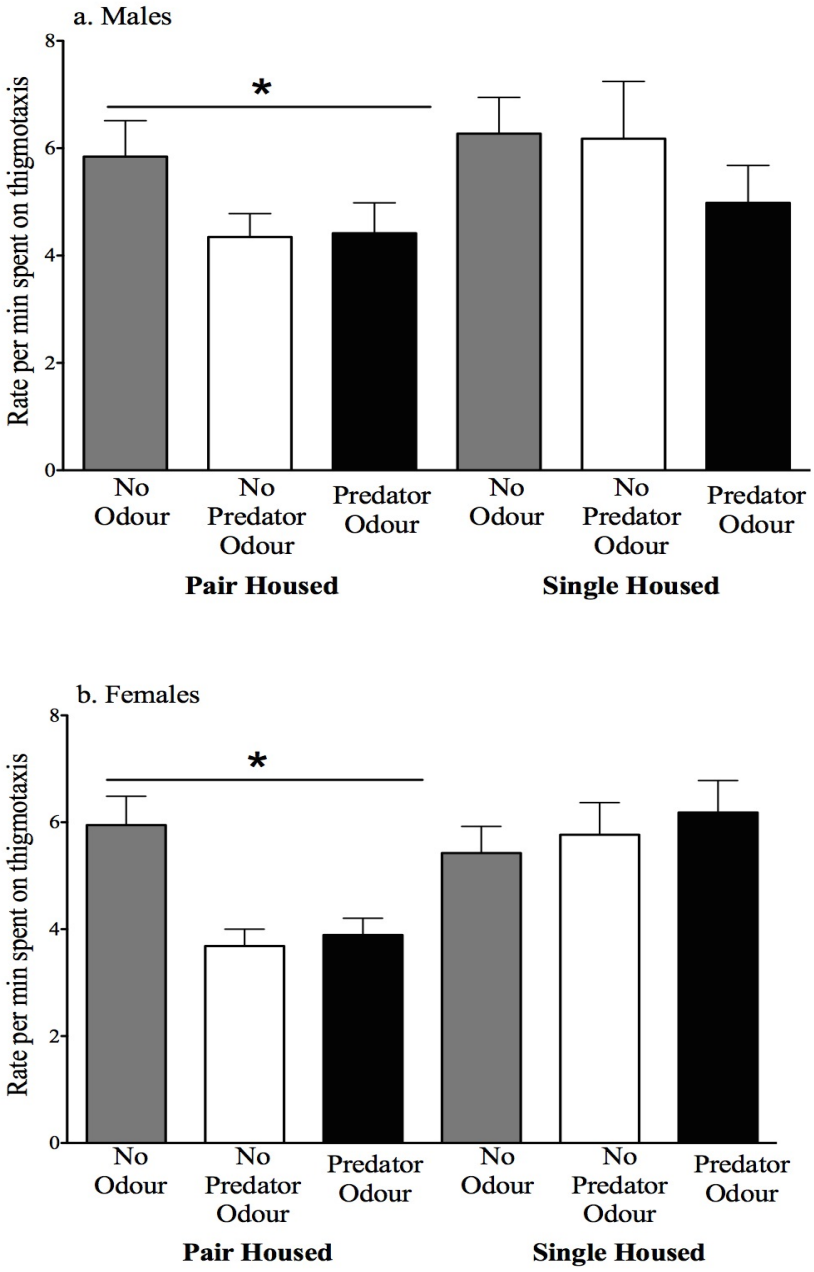


Figure 3.28. <u>Open field test</u>: Mean (+/- SEM) rate of thigmotaxic behaviour in the Open Field Test exhibited by males (a) and females (b).

Pair Housed animals showed a lower rate of thigmotaxis than Single Housed ones ("*").

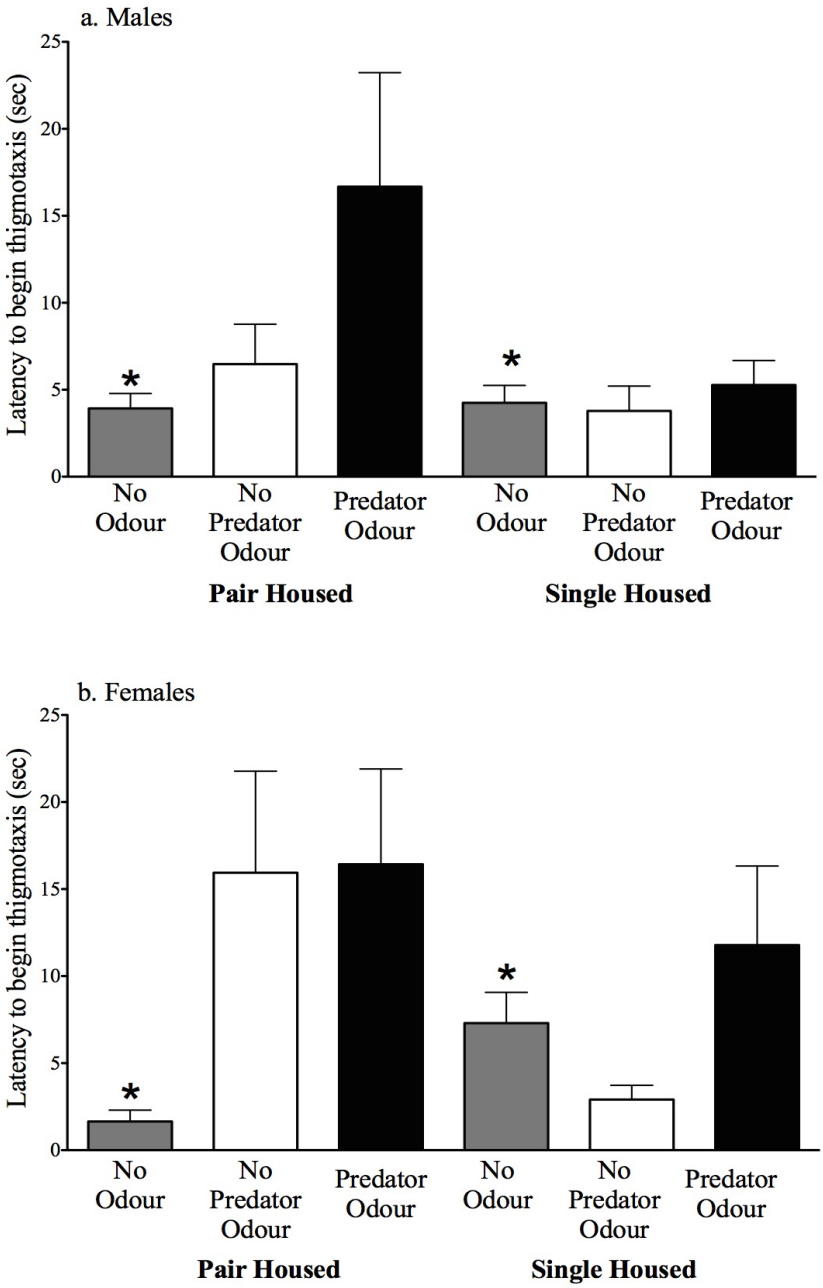


Figure 3.29. <u>Open field test</u>: Mean (+/- SEM) latency to initiate thigmotaxic behaviour in the Open Field Test exhibited by males (a) and females (b).

Odour Treatment had a main effect on this dependent measure (No Odour<Predator Odour). *("*" is significantly different from the predator odour group.)*

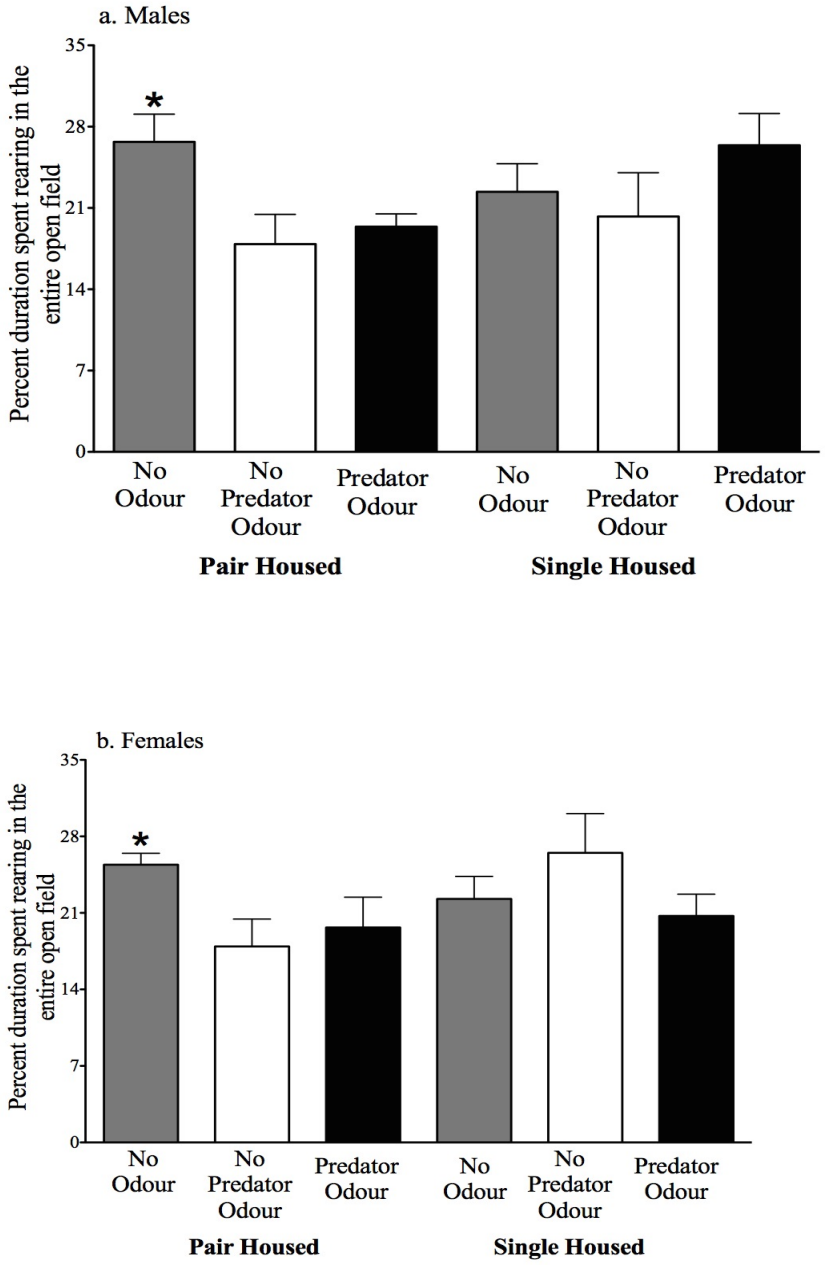


Figure 3.30. <u>Open field test</u>: Mean (+/- SEM) duration spent in rearing in the entire open field by males (a) and females (b) during the Open Field Test.

Percentage duration spent rearing in the entire open field showed an interaction effect of Housing and Odour Treatment; simple effects analyses revealed an effect of Odour Treatment on Pair Housed animals (No Odour>No Predator Odour). *("*" is significantly different from the no predator odour group, collapsed across sex.)*

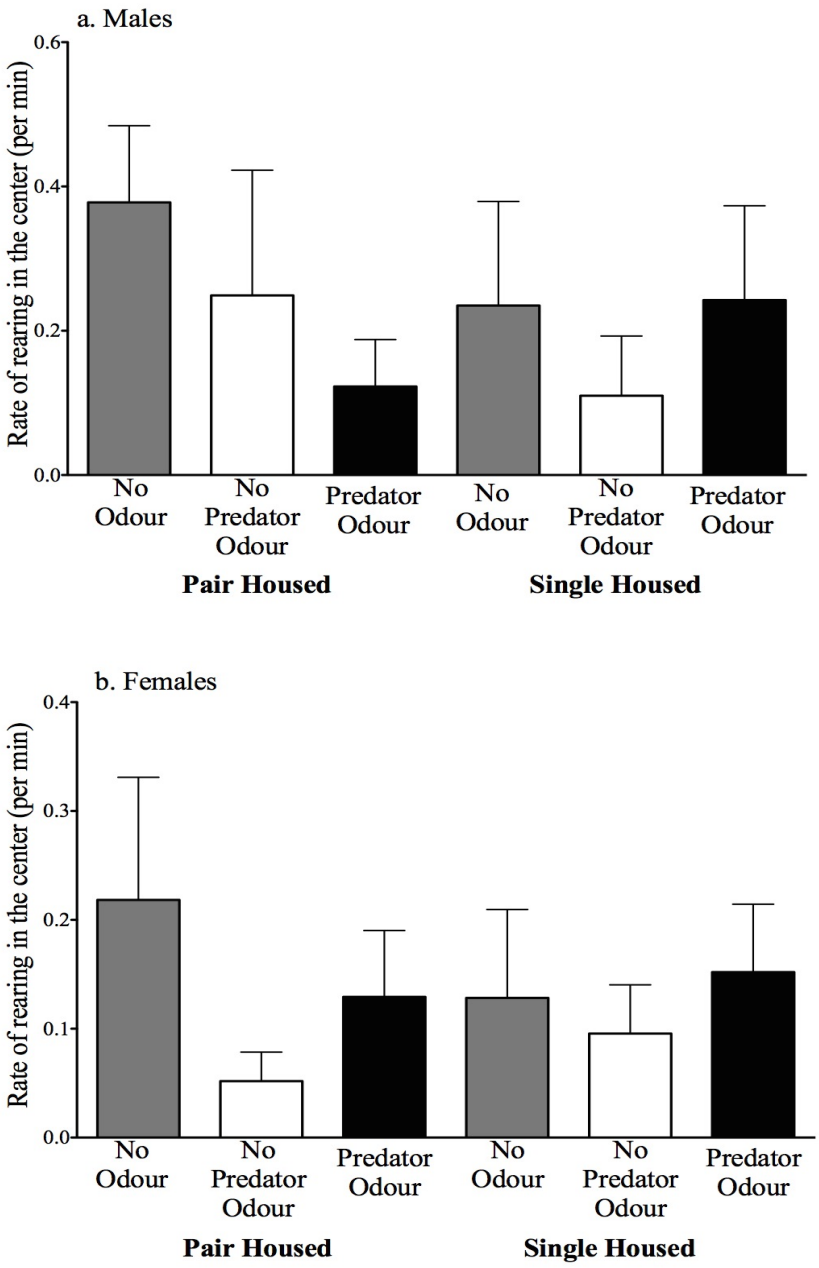


Figure 3.31. <u>Open field test</u>: Mean (+/- SEM) rate of rearing in the center of the open field by males (a) and females (b) during the Open Field Test.

Rate of rearing in the center of the open field was unaffected by any of the factors.

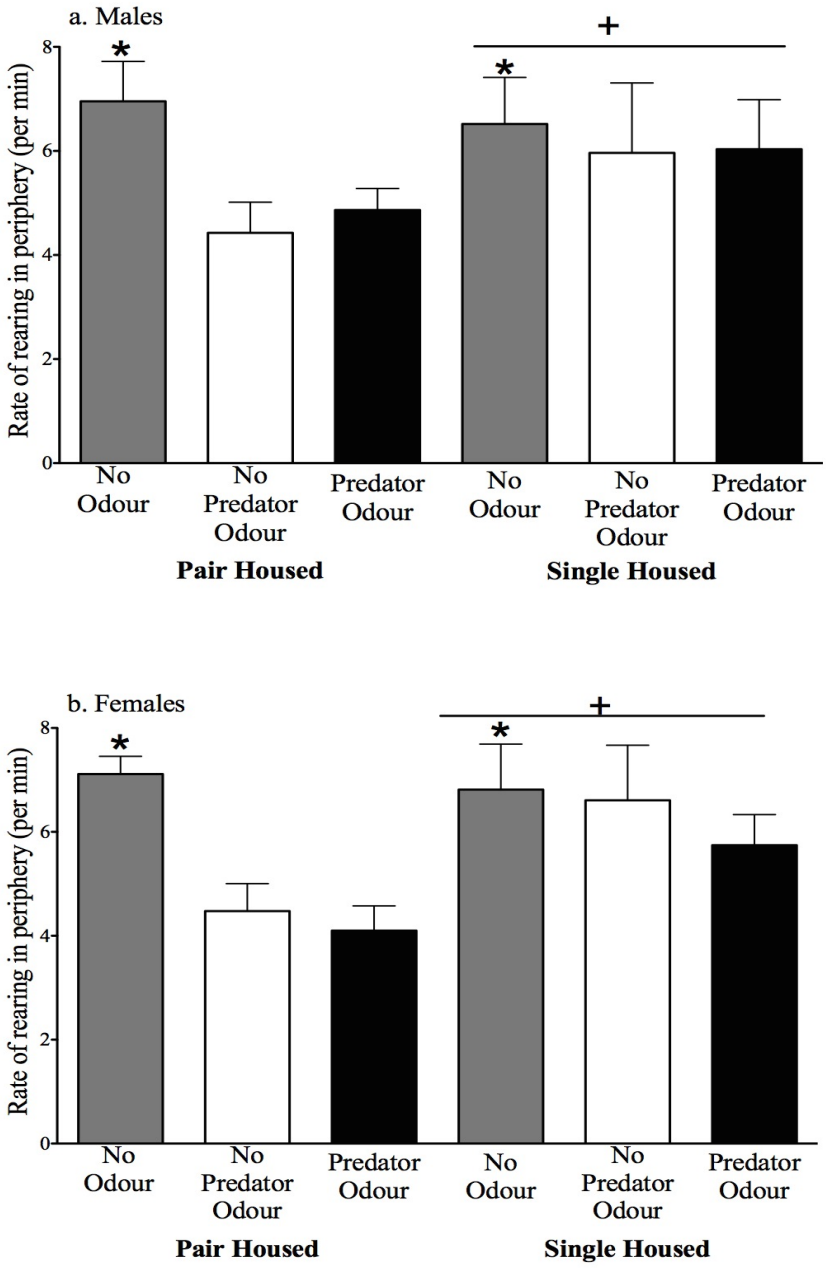


Figure 3.32. <u>Open field test</u>: Mean (+/- SEM) rate of rearing in the periphery of the open field by males (a) and females (b) during the Open Field Test.

Rate of rearing in the periphery of the open field showed a main effect of Odour Treatment (No Odour>No Predator Odour, No Odour>Predator Odour), and of Housing (Pair Housed< Single Housed). *("*" is significantly different from no predator odour and predator odour groups whereas "+" is significantly different from pair housed animals.)*

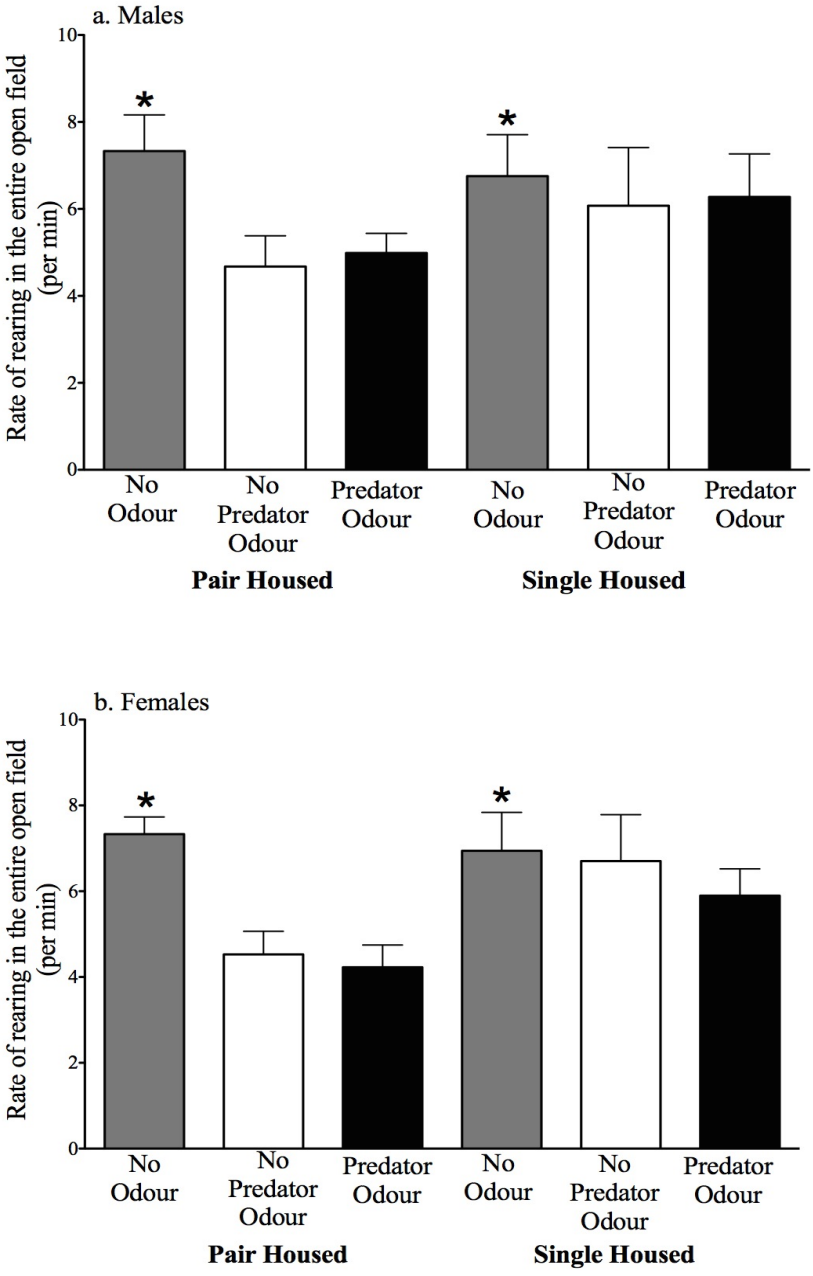


Figure 3.33. <u>Open field test</u>: Mean (+/- SEM) rate of rearing in the entire open field by males (a) and females (b) during the Open Field Test.

Rate of rearing in the entire open field showed a main effect of Odour Treatment (N0 Odour>No Predator Odour, No Odour>Predator Odour). *("*" is significantly different from no predator odour and predator odour groups.)*

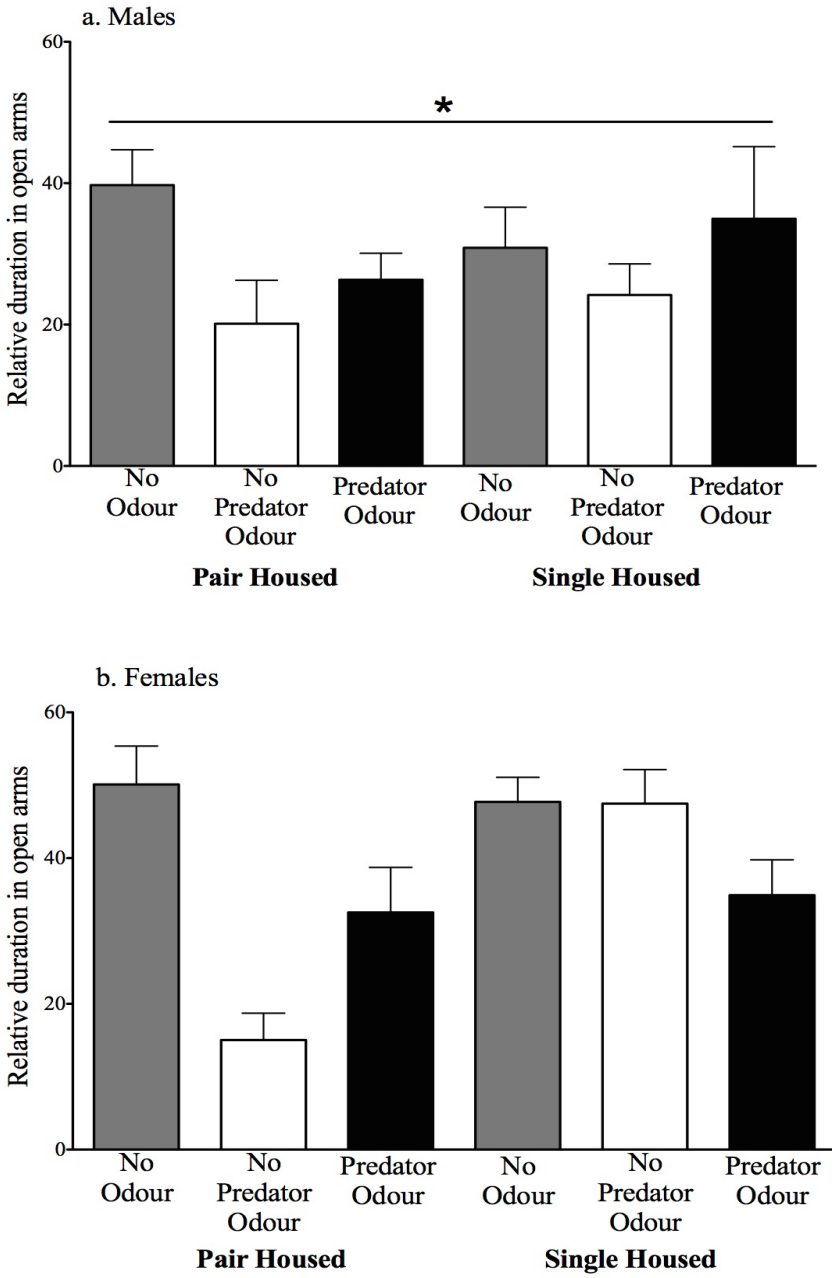


Figure 3.34. Elevated plus maze test: Mean (+/- SEM) duration spent in the open arms of the Elevated Plus Maze by males (a) and females (b).

Females spent a significantly greater relative duration in the Open Arms than males.*("*" is significantly different from females.)*

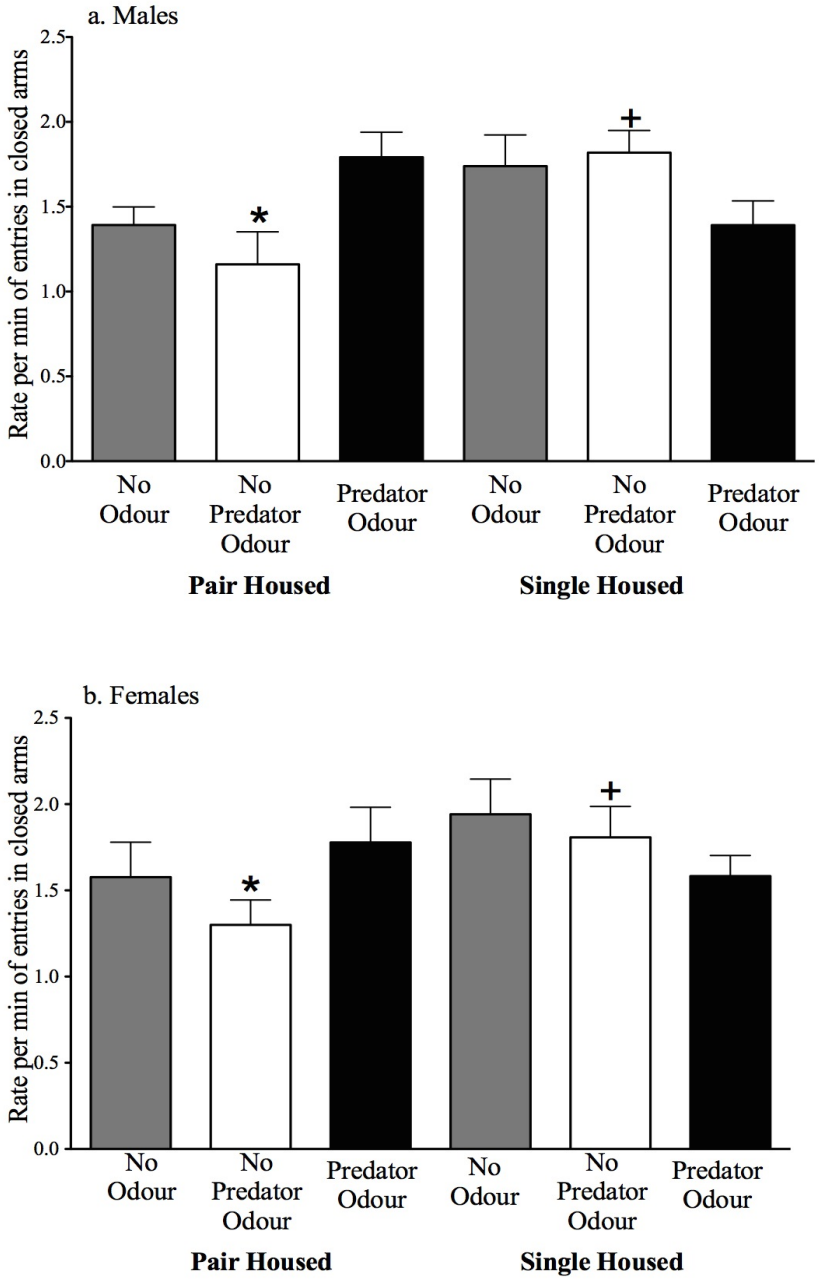


Figure 3.35. <u>Elevated plus maze test</u>: Mean (+/- SEM) rate of entries into the closed arms of the Elevated Plus Maze for males (a) and females (b).

An interaction between Housing and Odour Treatment was found on this measure; simple effects analyses on the rate of entries into the closed arms revealed an effect of Housing on No Predator Odour animals (Pair Housed >Single Housed), and an effect of Odour Treatment on Pair Housed animals (No Predator Odour <Predator Odour). *("*" is significantly different from the predator odour group of the same housing treatment whereas "+" is significantly different from pair housed animals of the same odour treatment.)*

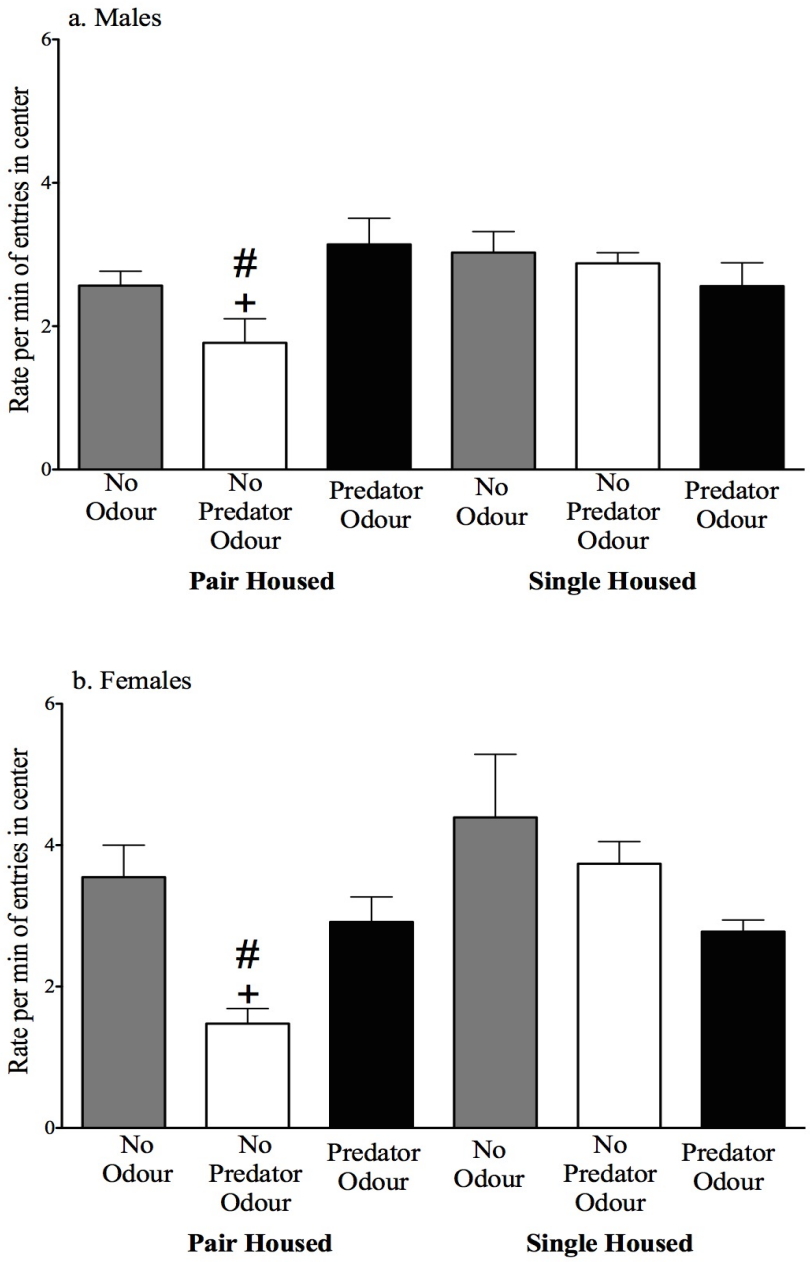


Figure 3.36. <u>Elevated plus maze test</u>: Mean (+/- SEM) rate of entries into the center of the Elevated Plus Maze for males (a) and females (b).

An interaction effect of Housing and Odour Treatment was found on this measure; simple effects analyses revealed an effect of Odour Treatment among Pair Housed animals (No Odour>No Predator Odour; No Predator Odour <Predator Odour), and an effect of Housing among No Predator Odour animals (Pair Housed <Single Housed). *("#" is significantly different from no odour and predator odour groups of the same housing condition. "+" is significantly different from the single housed animals of the same odour treatment.)*

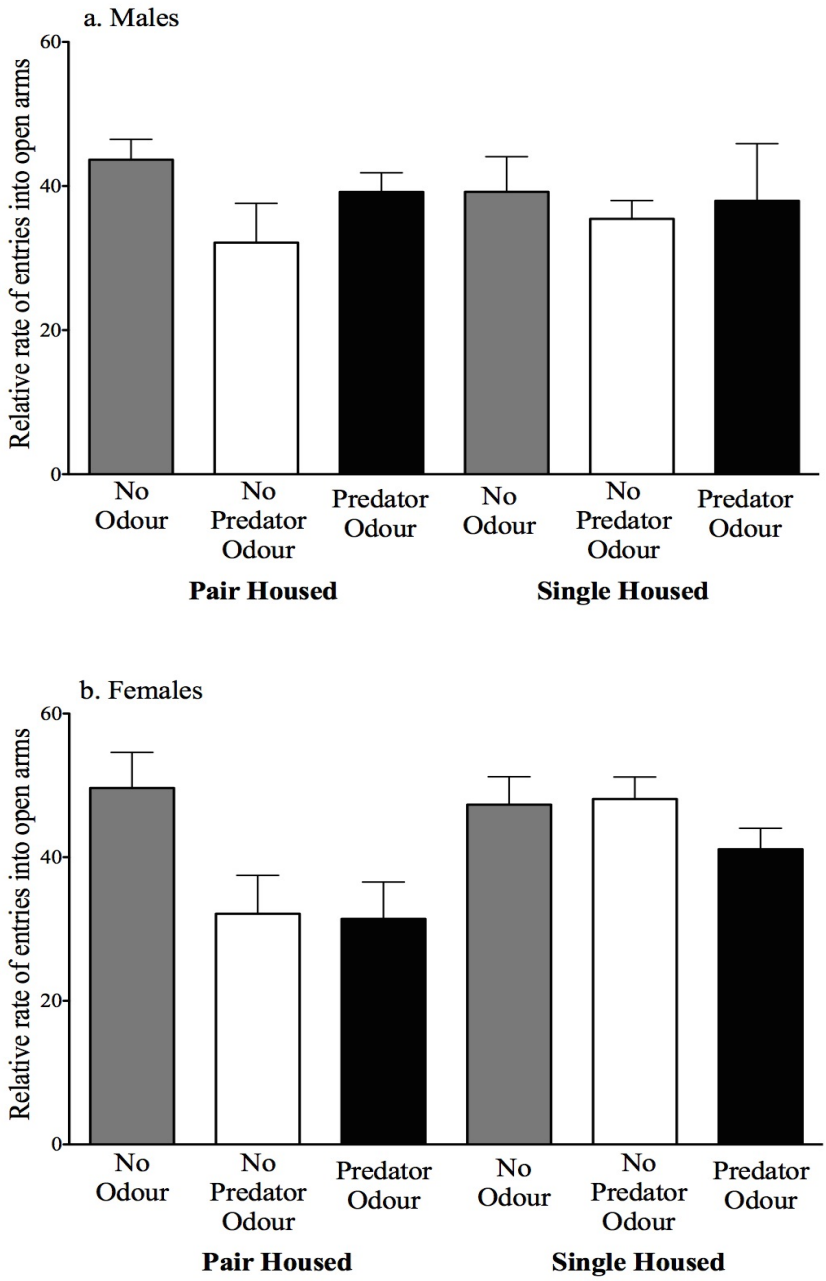


Figure 3.37. Elevated plus maze test: Mean (+/- SEM) relative rate of entries made into the open arms by males (a) and females (b) in the Elevated Plus Maze test.

None of the factors had any effect on this dependent measure.

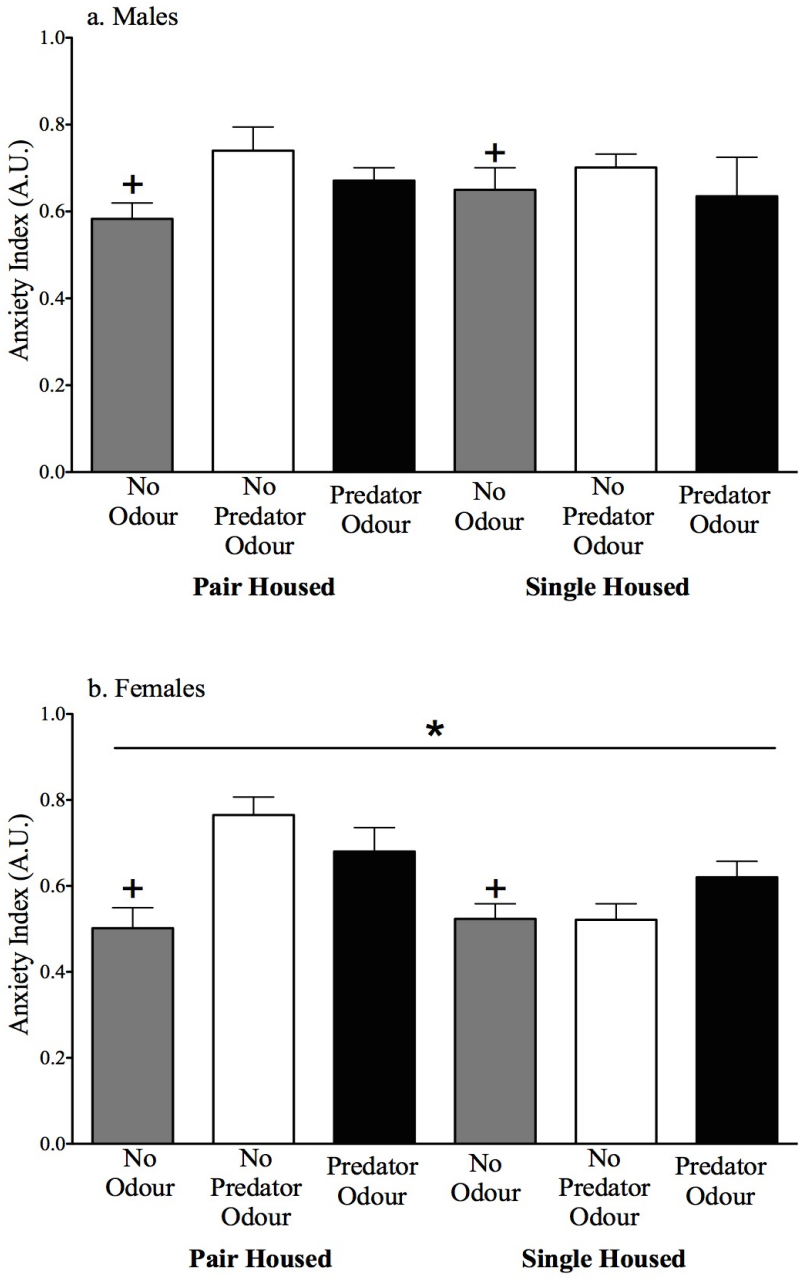


Figure 3.38. <u>Elevated plus maze test</u>: Mean (+/- SEM) Anxiety Index (AI) for males (a) and females (b) calculated from measures obtained in the Elevated Plus Maze test.

No Odour animals showed significantly lower AI than the No Predator Odour group. In addition, AI was significantly lower for females than males. *("*" is significantly different from males, whereas "+" is significantly different from no predator odour animals.)*

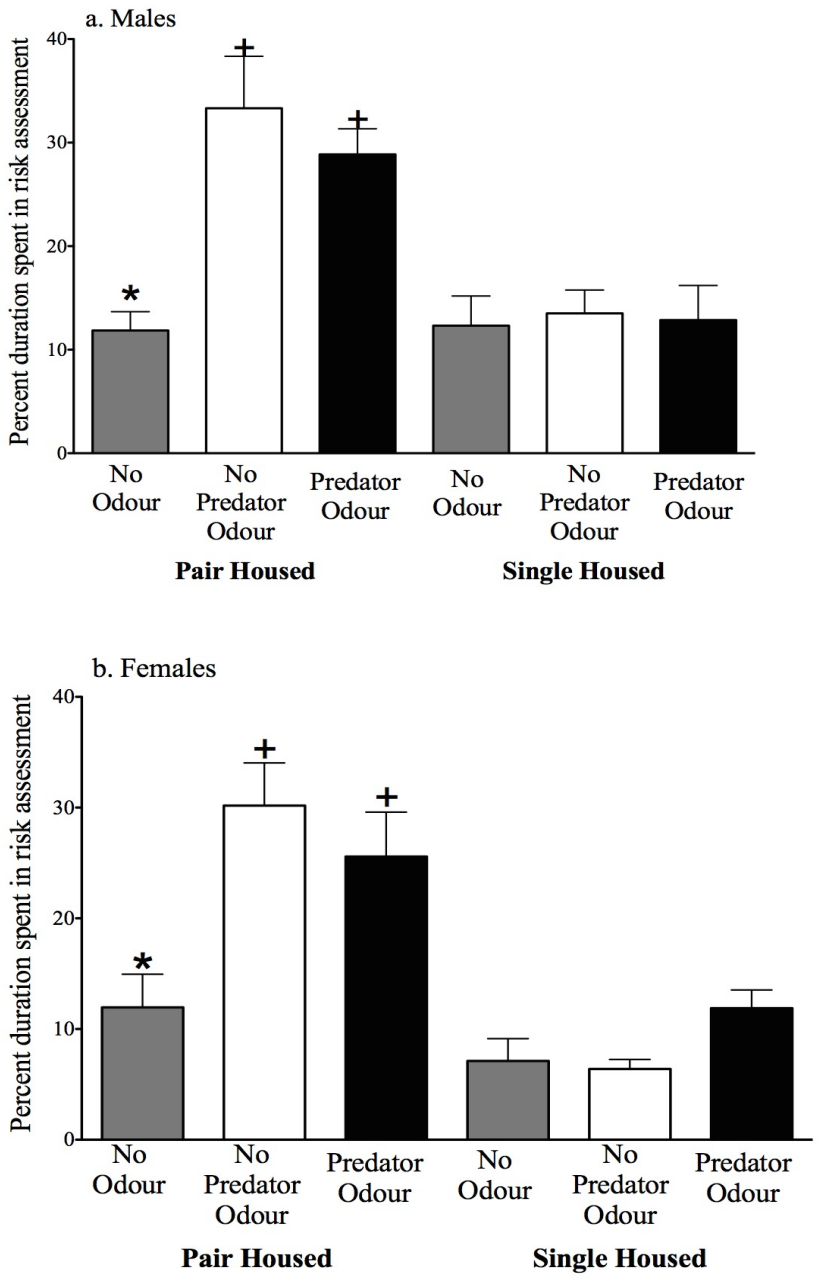


Figure 3.39. <u>Elevated plus maze test</u>: Mean (+/- SEM) duration spent in risk assessment during the Elevated Plus Maze test by males (a) and females (b).

A significant interaction of Housing and Odour Treatment was found on this measure; simple effects analyses revealed an effect of Odour Treatment on Pair Housed animals (No Odour < No Predator Odour, No Odour< Predator Odour), and an effect of Housing on No Predator Odour and Predator Odour animals (Pair Housed > Single Housed). *("*" is significantly different from no predator odour and predator odour groups of the same housing condition whereas "+" is significantly different from single housed animals of the same odour condition.)*

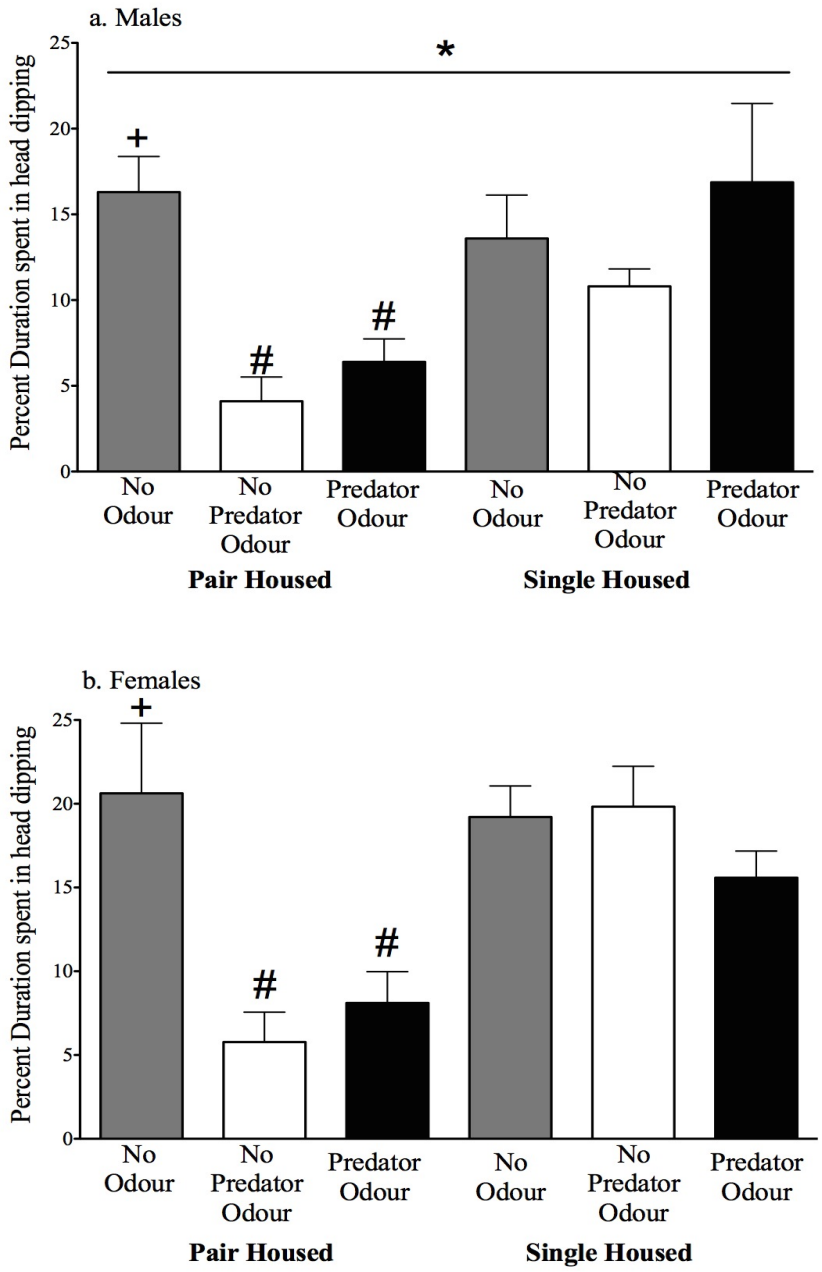


Figure 3.40. <u>Elevated plus maze test</u>: Mean (+/- SEM) duration spent head dipping during the Elevated Plus Maze test by males (a) and females (b).

This dependent measure was higher in females than males. A significant interaction of Housing and Odour Treatment was noted; simple effects analyses revealed an effect of Odour Treatment on Pair Housed animals (No Odour> No Predator Odour, No Odour> Predator Odour), and an effect of Housing on No Predator Odour and Predator Odour groups (Pair Housed< Single Housed). *("+" is significantly different from no predator odour and predator odour groups of the same housing condition whereas "#" is significantly different from single housed animals of the same odour condition. Additionally, "*" is different from females.)*

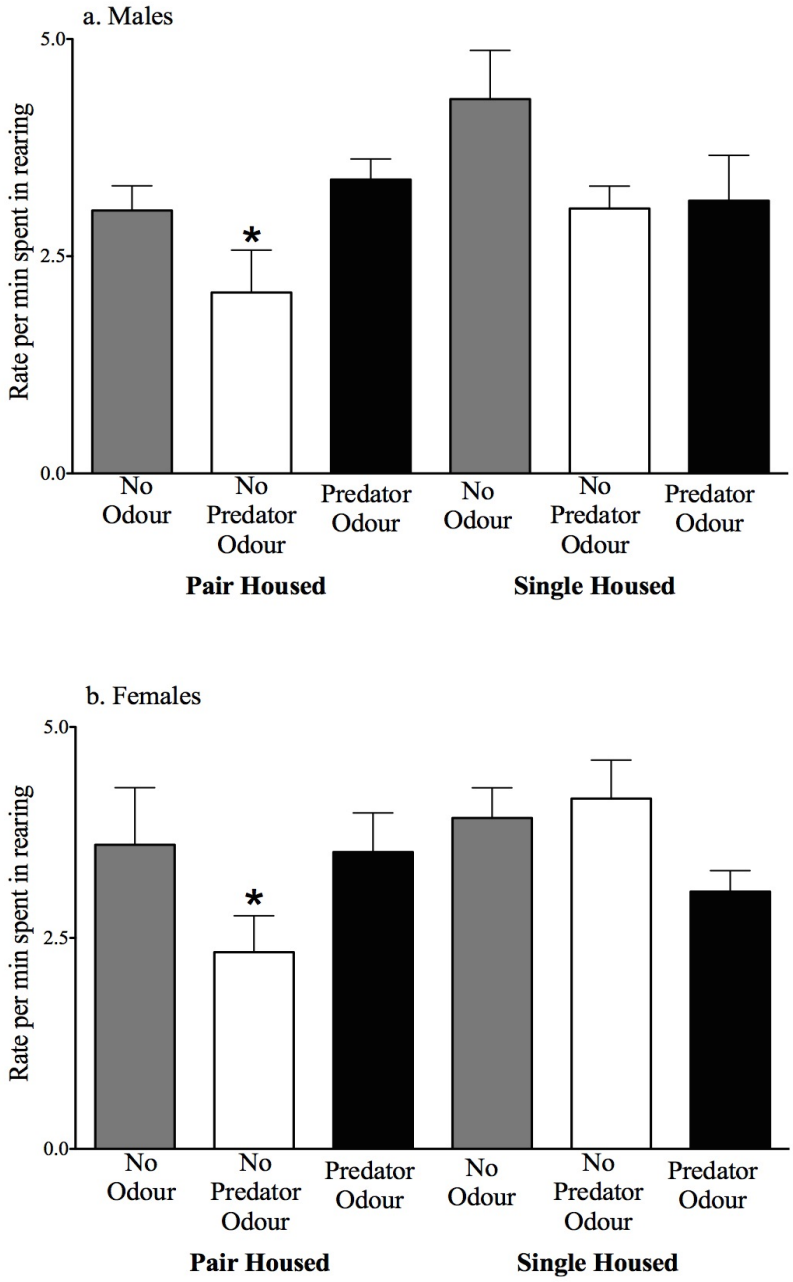


Figure 3.41. <u>Elevated plus maze test</u>: Mean (+/- SEM) rate spent rearing during the Elevated Plus Maze test by males (a) and females (b).

An interaction Effect between Housing and Odour Treatment was seen; simple effects revealed an effect of Housing on No Predator Odour animals (Pair Housed< Single Housed). *("*" is significantly different from single housed animals of the same odour condition.)*

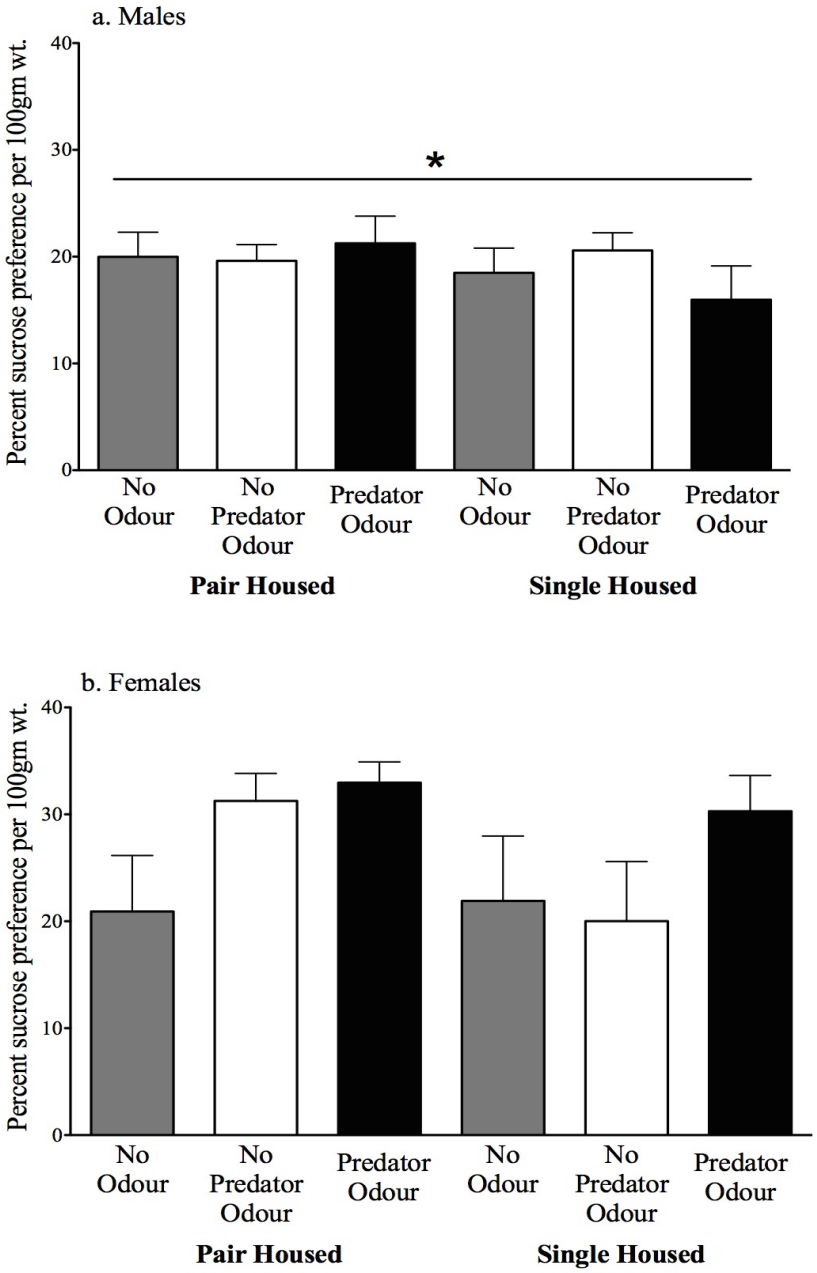


Figure 3.42. <u>Sucrose preference test</u>: Mean (+/- SEM) preference for the sucrose solution among males (a) and females (b) during the Sucrose Preference Test.

Sex had a significant effect on this dependent measure with females showing greater percent sucrose preference than males. *("*" is significantly different from females.)*

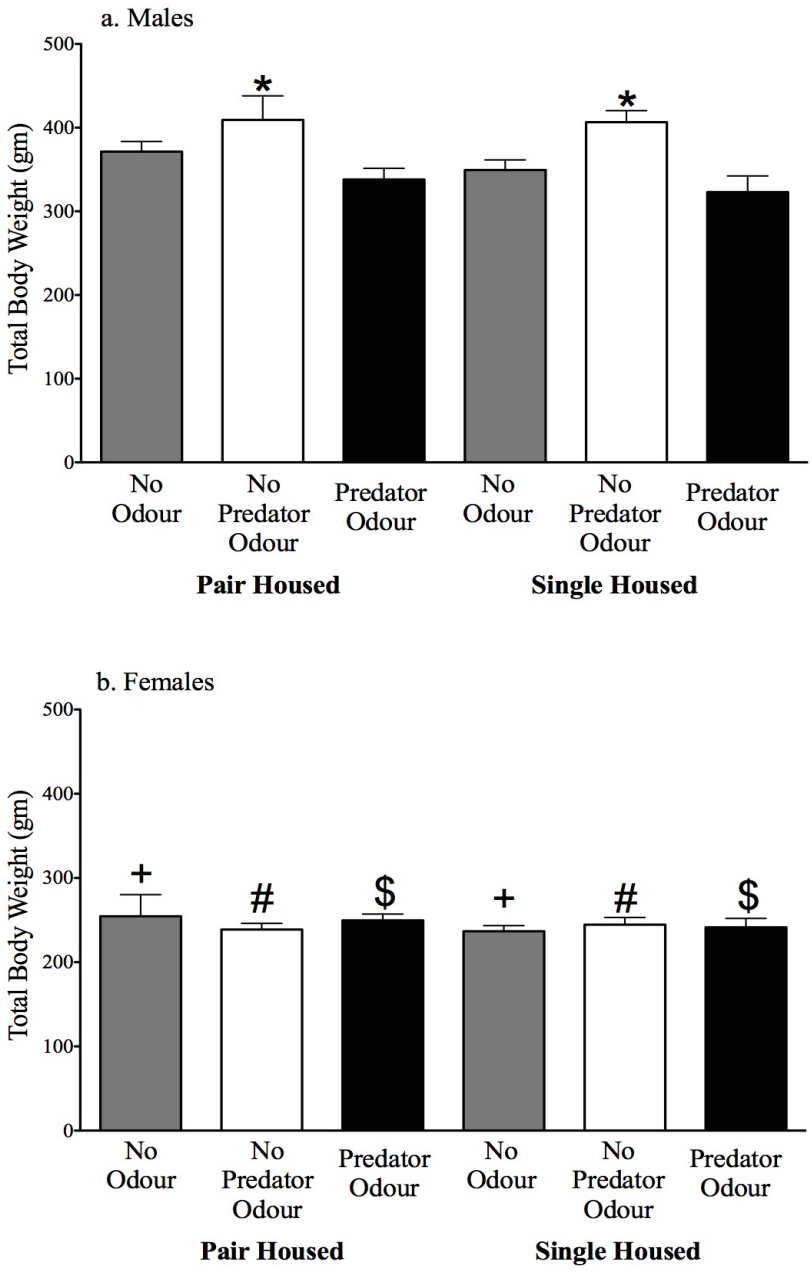


Figure 3.43. <u>Sucrose preference test</u>: Mean (+/- SEM) body weight of adult males (a) and females (b) taken prior to the Sucrose Preference Test.

Total body weight in adulthood showed a significant interaction between Sex and Odour Treatment; simple effects analyses revealed that Predator Odour males weighed significantly less than the No Predator Odour males, and males were significantly heavier than females in each of the three Odour Treatments. *("*" is significantly different than predator odour animals of the same sex, collapsed across housing condition. Additionally, "+", "#', and "$" are significantly different from males of the same odour treatment, collapsed across housing condition.)*

Table 3.1. Sucrose preference test: Mean (SEM) fluid and water consumed by different experimental sub-groups during the Sucrose Preference Test.

A significant interaction of Sex and Odour Treatment was found on total water consumed and simple effects analyses revealed an effect of Odour Treatment on females (NO> PO), and an effect of Sex on the NO (Male <Female), and PO (Male >Female) groups.
(Note: "PH, "SH", "NO", "NPO", and "PO" refer to "Pair Housed", "Single Housed", "No Odour", "No Predator Odour", and "Predator Odour" respectively.)

Experimental sub-groups			Total fluid consumed (g) (=Total water consumed + Total sucrose solution consumed)	Total water consumed (g)
Males	PH	NO	13.00 (2.70)	1.94 (0.44)
		NPO	12.00 (1.00)	2.51 (0.57)
		PO	9.30 (0.94)	2.64 (0.71)
Males	SH	NO	11.00 (0.85)	2.69 (0.53)
		NPO	12.00 (1.30)	1.78 (0.23)
		PO	11.00 (1.50)	2.94 (0.36)
Females	PH	NO	11.00 (0.88)	4.12 (0.52)
		NPO	8.80 (1.10)	2.03 (0.39)
		PO	10.00 (1.40)	1.75 (0.34)
Females	SH	NO	9.40 (2.90)	3.96 (1.30)
		NPO	12.00 (1.50)	3.62 (1.30)
		PO	6.90(1.00)	1.85 (0.36)

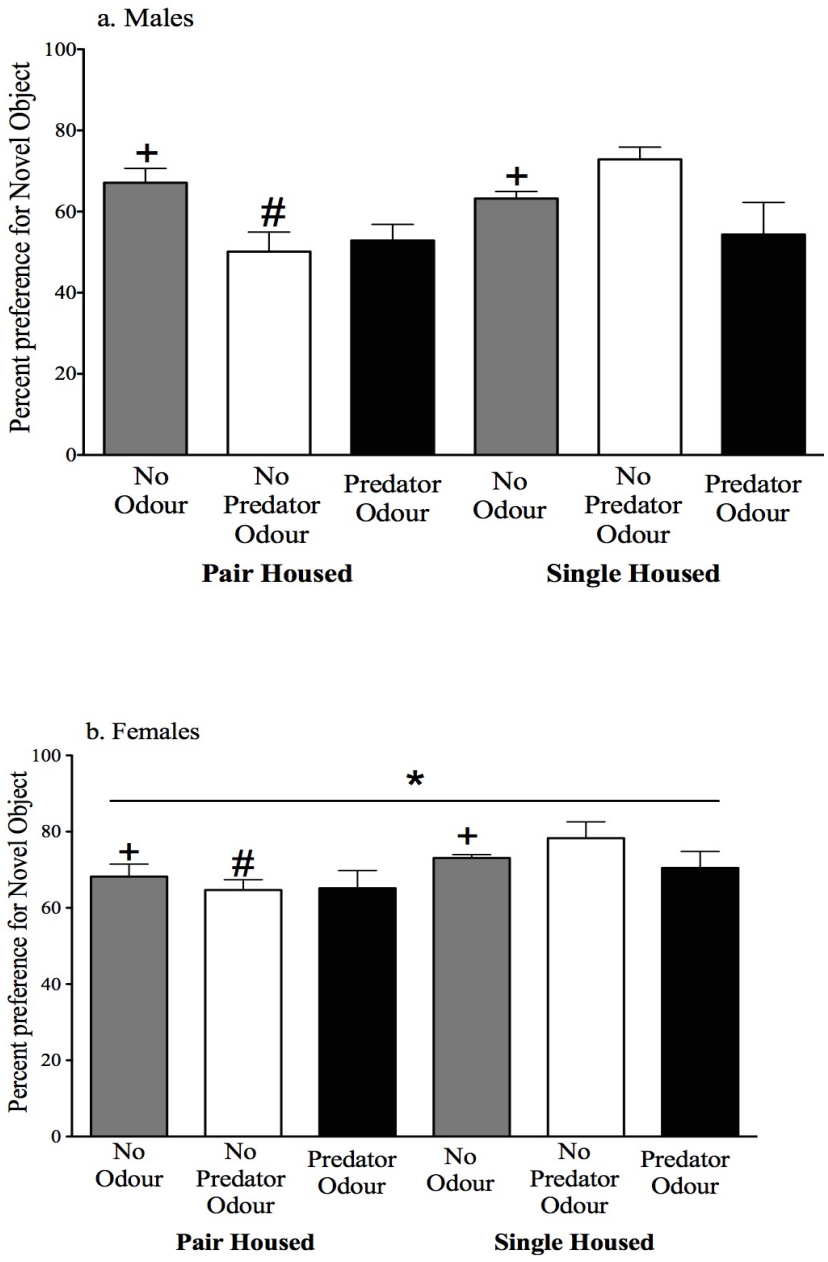


Figure 3.44. <u>Novel object recognition test:</u> Mean (+/- SEM) preference for the novel object during Novel Object Recognition test (Trial 1, Test Phase) is displayed for males (a) and females (b).

Females showed greater percent preference than males. An interaction was seen between Odour Treatment and Housing; simple effects revealed an effect of Housing on No Predator Odour group (Pair Housed <Single Housed). *("+" is significantly different from both no predator odour and predator odour groups, collapsed across sex and housing condition, whereas "#" is significantly different from single housed animals of the same odour condition, collapsed across sex. Finally, "*" is significantly different from males.)*

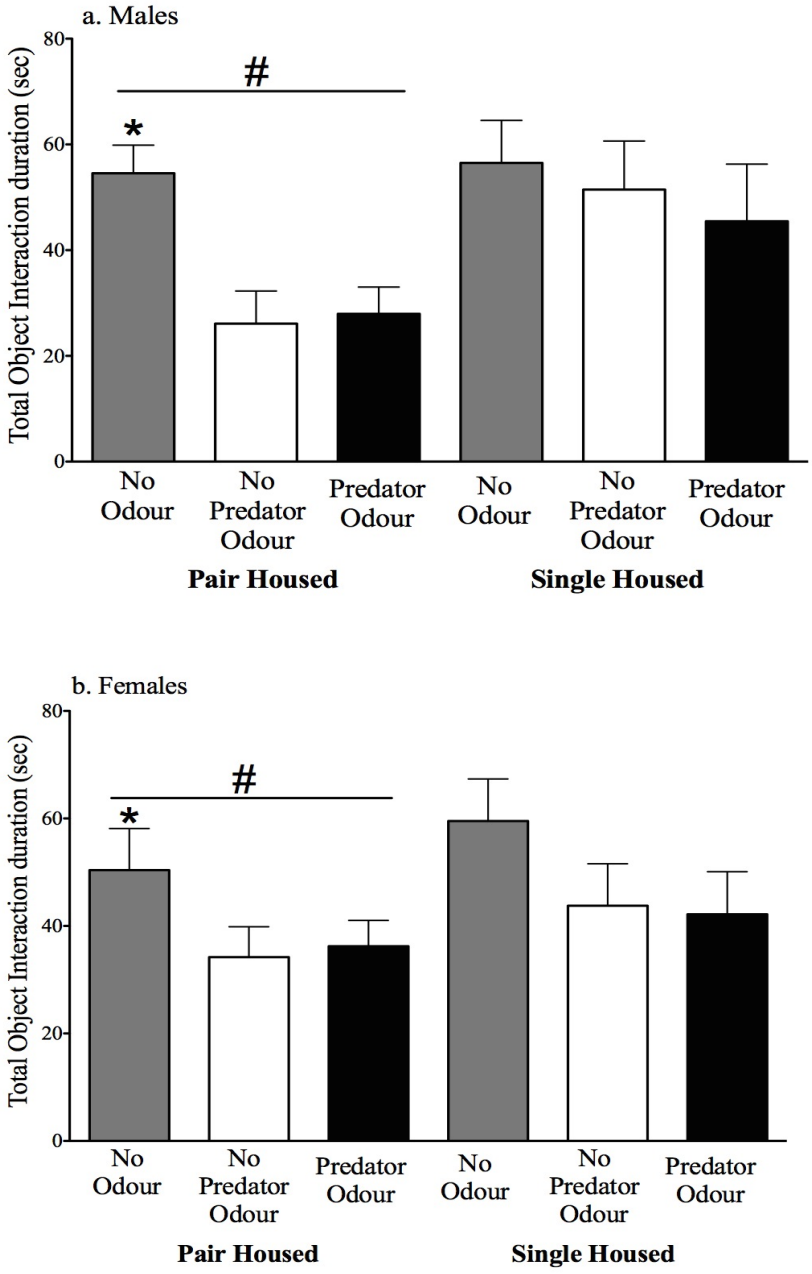


Figure 3.45. <u>Novel object recognition test:</u> Mean (+/- SEM) duration spent interacting with both the familiar and the novel object during the Novel Object Recognition test (Trial 1, Test Phase) is displayed for males (a) and females (b).

Odour Treatment had a main effect on this measure (No Odour> No Predator Odour, No Odour> Predator Odour). Housing too had a main effect on this measure (Pair Housed< Single Housed). *("#" is significantly different from single housed animals, collapsed across sex and odour treatment, whereas "*" is significantly different from no predator odour and predator odour groups, collapsed across sex and housing condition.)*

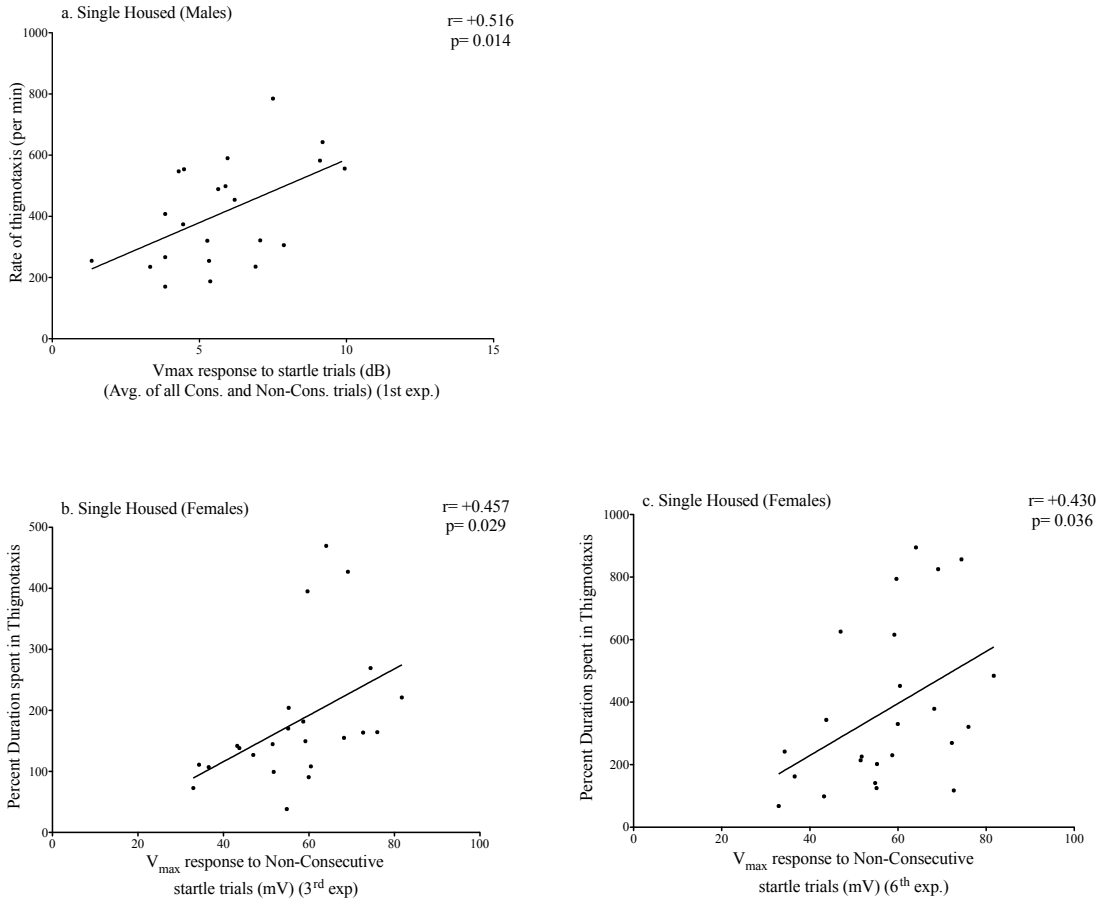


Figure 3.46. Correlations between the various measures of the Open Field Test (OFT) and response to startle trials (data collapsed across Sex, Odour Treatment and Housing).

Panel (a) shows the correlation between rate of thigmotaxis and maximum response to all non-consecutive and consecutive startle trials (after the 1st odour exposure). These measures show a significant positive correlation. Panels (b) and (c) show the correlation between percent duration spent in thigmotaxis and maximum response to non-consecutive startle trials after the 3rd and 6th odour exposures, respectively. In both cases, a significant positive correlation exists.

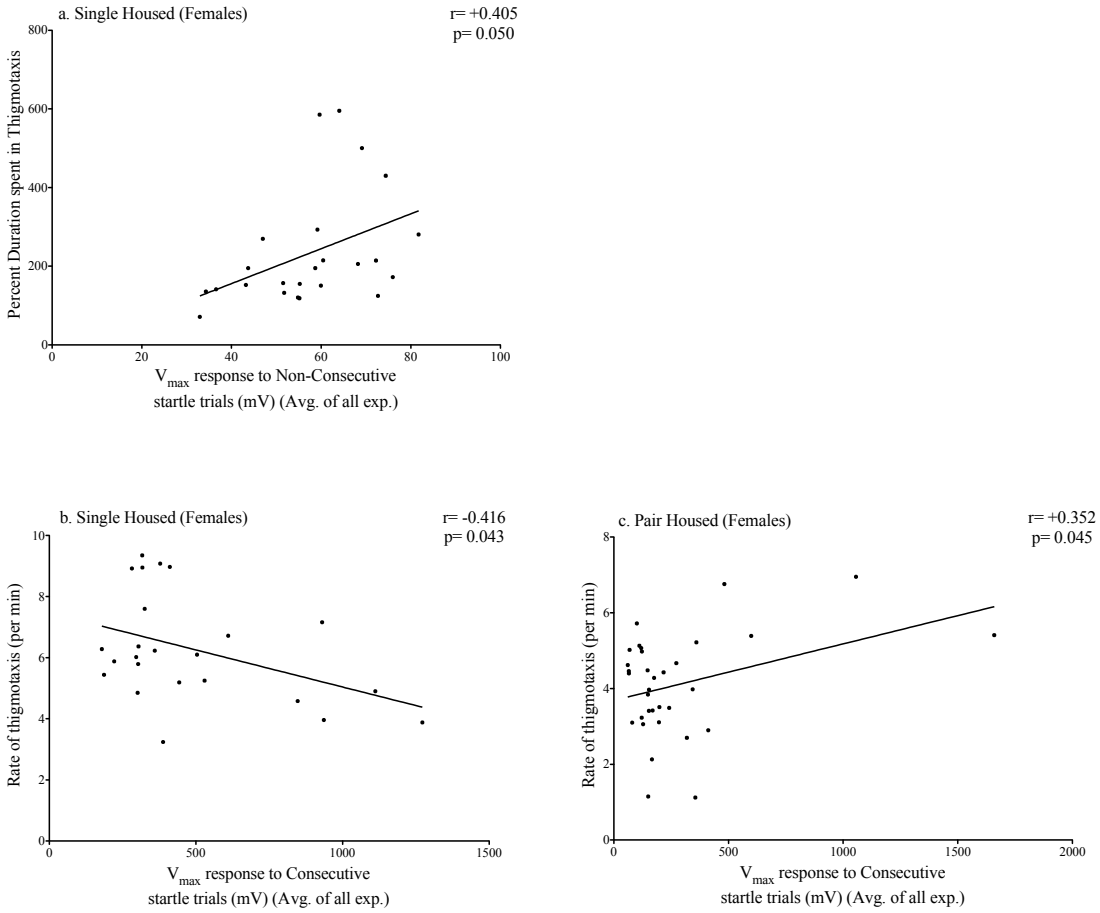


Figure 3.47. Correlations between the various measures of thigmotaxis behaviour in the Open Field Test (OFT) and response to startle trials for female rats of various groups (data collapsed across Odour Treatment and Housing).

Panel (a) shows the correlation between the percent duration spent in thigmotaxic behaviour in the OFT and maximum response to non-consecutive startle trials (averaged across all exposure periods) for single housed female rats. These show a significant positive correlation. Panels (b) and (c) show the correlation between rate of thigmotaxis and maximum response to all consecutive startle trials (averaged across all exposure periods) for single housed females and pair housed females, respectively. These show a significant negative and positive correlation, respectively.

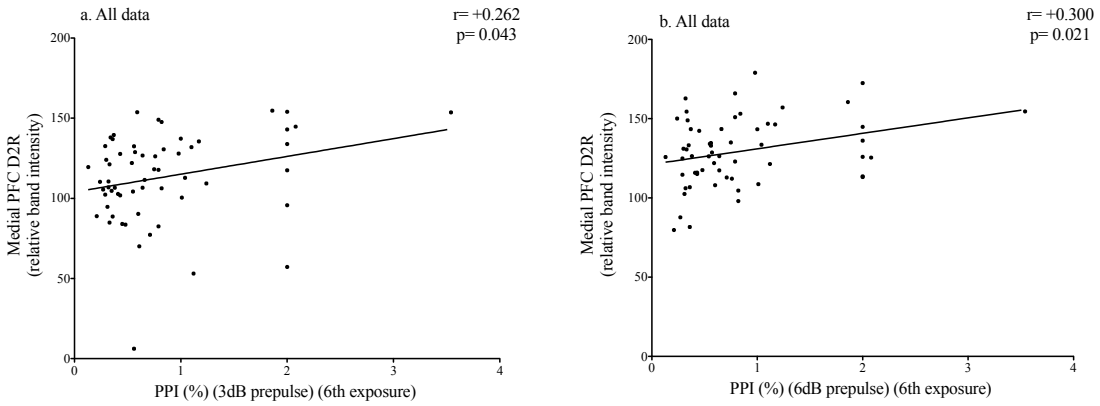


Figure 3.48. Correlation between dopamine receptor levels in medial prefrontal cortex (PFC) and percent prepulse inhibition (PPI) (data collapsed across Sex, Odour Treatment and Housing).

Panels (a) and (b) display the correlation between the dopamine D2R receptor in the medial PFC and PPI measured after the 6th odour exposure for 3dB and 6dB prepulse data, respectively. In both cases, the correlations are significant.

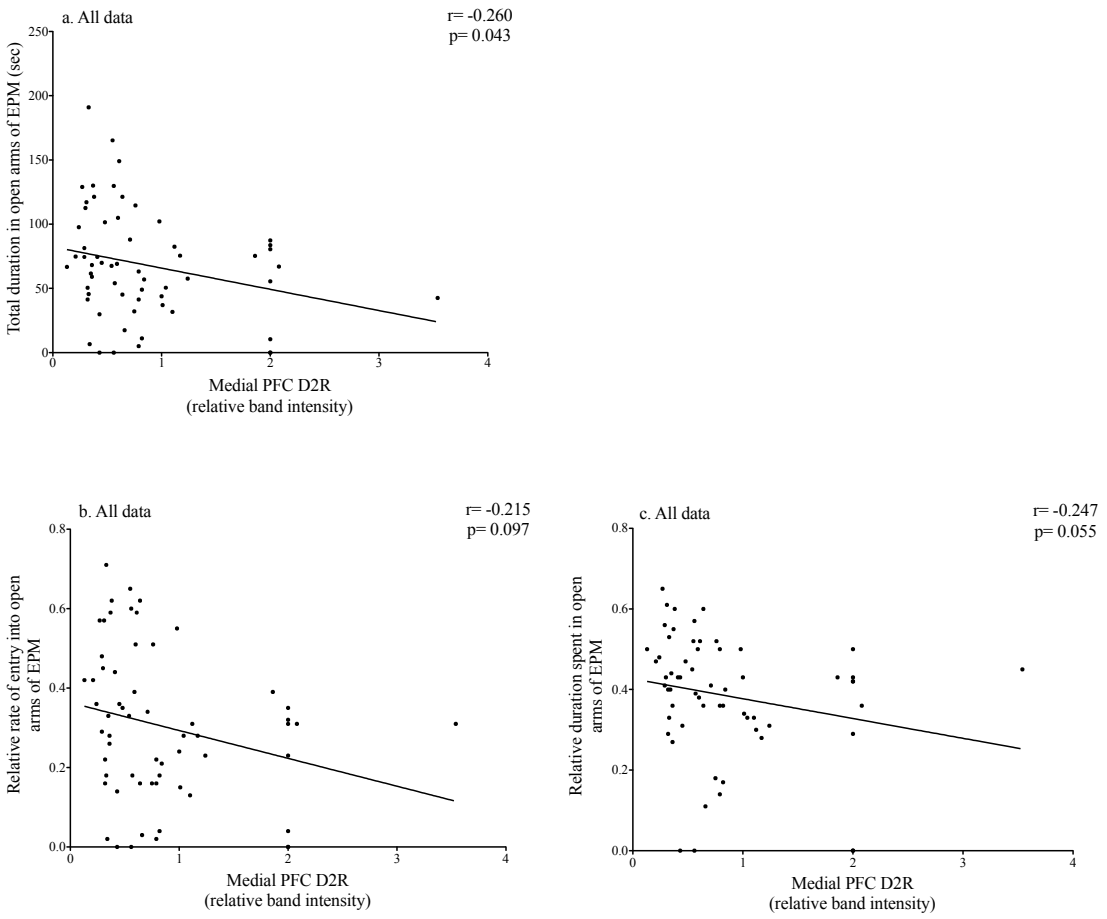


Figure 3.49. Correlations between dopamine D2R levels in the medial prefrontal cortex (PFC) and various dependent measures of the elevated plus maze (EPM).

Panel (a) shows the correlation between dopamine D2R levels in the medial PFC and total duration spent in the open arms of the EPM. Both measures show a significant negative correlation (p= 0.04). Panels (b) and (c) show the correlation between dopamine D2R levels and relative rate and relative duration spent in the open arms of the EPM, respectively. Both measures show a trend towards significance.

Chapter 4: Discussion

The main purpose of this work was to determine if repeated stress (predator odour) experienced during adolescence caused enduring, sex-specific changes in startle, sensorimotor gating ability, emotionality and memory in rats, and whether social support played any part in combating the impact of this ethologically relevant stressor. Previous work suggests that social support may act to buffer against stressful experiences (e.g. Kiyokawa et al., 2004, Wright et al., 2012, 2013, Hennessy et al., 2009, and DeVries et al., 2003). To achieve this, a novel model of stress was employed that combines single housing in adolescence with repeated exposure to predator odour occurring throughout adolescence and early adulthood. The effects of this model on development of basic stress-related behaviours as well as functional integrity of the PFC was examined in adulthood. Behavioural tests that measure sensorimotor gating ability (PPI), startle, anxiety-related behaviours (EPM, OFT), memory (NOR), and depression-related behaviour (SPT) were therefore, chosen to assess the effect of the predator odour experience and the single housing experience, as outlined in detail in the first chapter. These tests approximate certain negative symptoms (anhedonia, measured using SPT), and cognitive symptoms (memory deficit, measured using NOR test), associated with schizophrenia as well as an endophenotypic change seen in prodromal populations (sensorimotorgating dysfunction, measured using PPI). Additionally, some of these measures also apply to illnesses such as generalized anxiety disorder and major depressive disorder, which are associated with childhood adversity and are likely to emerge in adolescence and early adulthood (Zahn-Waxler et al., 2008). Of all the manipulations performed in this study, single housing was accompanied by the most

consistent and notable effects; such animals displayed increased sensorimotor gating, higher startle amplitude, greater anxiety as measured in the OFT, and increased D1R in medial PFC. Taken together, these results suggest that single housing induces an anxiety-related phenotype. Repeated exposure to predator odour did not have lasting effects on these measures, but animals that were not exposed to any odour (that is, the no odour group, conceived initially as a control against the odour exposure experience) showed similar behavioural changes to the single housed animals, suggesting an underlying similarity in the single housing and no odour experiences. It is proposed that a dramatic reduction in complexity of the physical and social environment of these animals might be a unifying experiential factor driving this outcome. Recent work examining effects of the rearing environment of rats supports this view (e.g. Varty et al., 2000, Zeeb et al., 2013, and Kirkpatrick et al., 2013).

4.1 Does predator odour result in stressing the rats?

Behaviours such as collar contact, grooming and rearing were analyzed during exposure to predator odour in order to determine if the predator odour experience was aversive and stressful to the rats (refer to section 3.1 of chapter 3 for details). Under these conditions, an increase in certain behaviours such as avoidance of the odour source, is considered a biomarker of the aversive or "stressful" experience, much like HPA activation, increased blood pressure and heart rate, and increased release of epinephrine. Measuring blood CORT and ACTH levels was avoided in this study because the stress involved in handling and extracting blood from the animals could potentially confound the outcome of the odour treatment. Collection of fecal samples for analysis of the surge in CORT following predator odour exposure is a challenging and expensive alternative to

collecting blood samples. While it is less likely to stress the rats (unlike blood collection), it is harder to determine the exact time when the increased CORT will be metabolized and the metabolites appear in the fecal samples. Moreover, changes in ACTH and CORT levels are not necessarily predictive of changes in other physiological parameters associated with stress. For example, disassociations between the effect of acute predator odour exposure and immobilization stress on the HPA axis activity (ACTH and CORT levels) and measures of anxiety-like behaviour have been noted (Munoz-Abellan et al., 2008). This suggests that while HPA activation is an important biological change in response to a variety of stressors, it is not the mediator of *all* biological effects associated with stressor exposure, and its connection to behavioural outcomes is unclear in some cases. Moreover, some changes may be brought about without any contribution of HPA hormones. Considering these factors, and the costs associated with the analysis of HPA hormones, it is reasonable for experimenters to use alternative biomarkers of stress such as behavioural changes associated with stressor exposure. This is easily achieved with the predator odour model because, as mentioned earlier, it allows researchers to record and assess the rats' behaviour *during* the predator odour exposure.

In the current study, the predator odour experience proved to be more aversive than the exposure to the unthreatening odour. Specifically, while total locomotor activity levels were not different between animals exposed to either odour, the number of entries into the odour region was higher among animals exposed to the unthreatening odour compared to those exposed to the predator odour. Furthermore, this effect was seen consistently (i.e. at each exposure) only among females. As indicated earlier, the number of entries made by the animal into the odour region represents the animal's behavioural avoidance of the odour stimulus, and therefore, informs the researcher about whether the

animal is responding to the stressful stimulus or not. Similarly, the time spent in the region furthest from the stimulus also provides an indication of this. In this study, animals exposed to predator odour also spent significantly greater time in the region of the arena furthest from the cat collar stimulus (i.e. the Third Region), showing clear avoidance behaviour. Yet again, females avoided the odour stimulus during each exposure while males first displayed avoidance only during the third odour exposure. This sex-difference in avoidance behaviour is not entirely without precedent as female rats have displayed greater defensive behaviour in response to a predator threat in the past (Blanchard et al., 1991 and 1992; Blanchard and Blanchard, 1989). This may be particularly beneficial during the time that females are raising their young and may increase the likelihood of dams protecting most of their young in an attack from a predator.

There were modest effects of odour exposure on grooming and rearing with both behaviours decreasing in response to predator odour exposure, although these effects were not always seen during the first exposure. Animals exposed to predator odour showed reduced bouts of grooming compared to ones exposed to no predator odour (collapsed across exposure periods). Similarly, animals exposed to predator odour showed reduced rearing compared to animals exposed to the unthreatening odour. The latter spent longer rearing during the acute exposure (first odour exposure), and displayed a greater number of rears than the former. This increase in what can essentially be considered non-defensive behaviours (e.g. Blanchard et al., 1991) in animals exposed to a non-threatening odour compared to a threatening odour lends further support to the assertion that the predator odour experience was perceived to be more aversive to the rats than the non-threatening odour. In conclusion, rats exposed to predator odour displayed behavioural biomarkers of stress, compared to rats exposed to a non-threatening odour; thus, the

predator odour model was effective in stressing rats. The pattern of behavioural response to cat odour is consistent with what has been previously shown using predator odour models (e.g. Wright et al., 2008, 2012, and 2013, Mashoodh et al., 2008, Apfelbach et al., 2005, McGregor, 2004).

Continued avoidance of the odour stimulus despite repeated exposures to it can be considered one of the strengths of our protocol as it implies that the animals are receiving the maximum aversive experience even after multiple exposures to the same type of stimulus. This lack of habituation of avoidance behaviour can be partly attributed to our use of odour from different cats for the different odour exposures (Staples, 2010, Wright et al., 2012, 2013). Behaviours that have been shown to habituate to repeated odour of the *same* cat include investigation of the odour source, behaviours such as risk assessment, head-out behaviour in exposure arenas containing a hide-box, and duration spent hiding in the said hide-box as well as grooming, and foraging (Mashoodh et al., 2008, and Staples et al., 2008). Reduction in these behaviours is also accompanied by reduction in c-fos activation that is normally observed in response to cat odour (fur/skin) exposure in brain regions such as accessory olfactory regions (mitral and granular layers of the posterior accessory olfactory bulb and posteroventral medial amygdala), the ventromedial and dorsal pre-mammillary hypothalamic nuclei, basolateral amygdala and periaqueductal grey (Staples et al., 2008). Subsequent exposure to the odour of a different cat, however, results in dis-habituation of both behaviour and brain activation (Staples et al., 2008). Therefore, in the current study, odour from multiple cats was used as the stressor.

Another behaviour measured in the current study was investigation of the odour stimulus, which did not differ between rats exposed to predator odour or the unthreatening odour. Previous work on the effect of predator odour on this measure has

yielded mixed results. For example, while May et al. (2012) found a decline in stimulus investigation in response to repeated exposure to cat body rubbings, Hubbard et al. (2004) found no change in this measure. On the other hand, Staples et al. (2008) found rats exposed to cat odour to display a greater degree of contact with the stimulus than ones exposed to a control odour. In the current work, too, no difference was found in collar investigation between the animals exposed to either odour. Clearly, unlike physical avoidance of the odour region, contact with the odour source is one of the behaviours that is not consistently affected by cat odour. Differences in outcome between the studies mentioned here are likely due to methodological variations such as choice of rat strain, source of cat odour used, and number of odour exposures administered.

One potentially important methodological issue is the fact that cat odour administered through a collar or cloth piece is not particularly volatile (Blanchard et al., 2013). It is therefore, reasonable to expect the rats to investigate the collar piece repeatedly, particularly in the first few odour exposures. This further suggests cat odour stimulus may elicit some, but not all, behavioural responses ordinarily associated with the presence of a predator. This is hardly surprising because a predator odour is considered a partial predator stimulus. While it mimics certain aspects of a predator's presence, it cannot replicate the entire experience associated with encountering a predator in the wild (Hubbard et al., 2004).

The current paragraph will summarize the changes in HPA activity in response to cat odour as revealed by previous studies. This is done in an effort to understand the physiological basis of the sex difference in avoidance behavior seen in this study. As discussed previously, exposure to cat odour results in HPA axis activation as indicated by elevated CORT levels in rats. In the Perrot lab (where the experiments mentioned in this

thesis were carried out), previous studies have investigated the effect of two versions of the predator odour model on HPA activity. The design of all these previous studies and the current one, as well as the main outcomes are summarized in tables 1.2 to 1.4. CORT levels immediately following the final predator odour exposure in adolescence did not show any effect of odour treatment (Wright et al., 2012, 2013) even though the animals showed signs of behavioural avoidance. Baseline CORT levels in adulthood, however, revealed an effect of adolescent odour treatment with predator odour exposed rats showing great CORT levels in the blood (Wright et al., 2012, 2013). HPA reactivity to the same stressor (cat odour) in adulthood also revealed effects of adolescent predator odour treatment. Specifically male rats exposed to predator odour in adolescence showed reduced CORT release during the predator odour test in adulthood compared to rats exposed to control odour in adolescence (Wright et al., 2012, 2013). To conclude, repeated predator odour in adolescence increased baseline HPA activity in adulthood whereas HPA reactivity to a homotypic stressor was reduced (in males only). This sex difference in reactivity is very similar to the sex difference seen in avoidance behaviour of rats in the current study. Specifically, in the current study, predator odour exposure reduced the duration spent in the vicinity of the odour source in females during each exposure period. In males, on the other hand, such avoidance emerged only during the final odour exposure.

The above discussion of the effect of cat odour stimulus on HPA activity also supports the view that CORT changes do not always provide a consistent measure of the effect of a stressor. Moreover, changes in CORT can take longer to emerge whereas the behavioural changes in response to predator odour emerge immediately during each exposure. For example, animals exposed to cat odour spend more time avoiding the

region of the exposure arena in close proximity to the odour source, and spend more time in the region furthest from the odour source, and in the hide-box (Wright et al., 2008). Cat odour exposure has also been shown to reduce the time spent approaching the odour source while increasing the number of head-outs, implying increased vigilance (Staples and McGregor, 2006). Cat odour also resulted in increased escape attempts and inhibition of behaviours like grooming (Staples et al., 2008). Therefore, we believe that immediate behavioural responses such as avoidance of the odour source, head-out behaviour, and activity levels may be better measures of recording the effect of cat odour because they show an effect of the stress that is consistent across odour sources (e.g. collar, J-cloth, fur) and different laboratories and studies.

In the current study, although the predator odour model was effective in stressing rats (as indicated by behavioural biomarkers), it did not exert any long-term effects. Unlike the present study, however, previous work using a similar model of stress has shown changes in OFT performance and dopamine D1R and D2R receptor levels following repeated predator odour exposure in adolescence (e.g. Wright et al., 2008, 2012, and 2013). (Note: These will be discussed in greater detail in the upcoming paragraphs). In order to investigate the possible reasons behind this difference in outcome, a comparison was made between the current work and these previous studies. However, because of the differences in methodology, only some behaviours measured during the first odour exposure could be directly compared between the present study and past ones by Wright et al. (2008, 2012, and 2013). For example, horizontal locomotor activity levels as well as the rate of entries and duration spent in each part of the arena during odour exposure for pair housed animals that were exposed to the unthreatening odour and those exposed to the cat odour, were directly compared to these previous

studies. Locomotor activity levels during the first odour exposure (collapsed across sex) were comparable in cat odour exposed animals, but animals in Wright et al. (2008, 2012, and 2013) had a noticeably higher activity level in response to control odour than animals in the current study. Also, while the number of entries made into the odour region of the exposure arena for females from pair housed-no predator odour group and the pair housed-predator odour group were nearly identical in the two studies, stressed males displayed fewer entries into the odour region in Wright et al. (2013) compared to the current study. Moreover the duration spent in the third region (called Safe Region in Wright et al., 2012, 2013) was higher for pair housed-no predator odour males, pair housed-no predator odour females, and pair housed-predator odour females in the current study when compared to similar groups in Wright et al. (2012, 2013). Duration in the third region for pair housed-predator odour males was comparable in the two studies (referred to as Safe Area in Wright et al. (2008, 2012, and 2013). These differences are clearly not limited to one experimental group or sex alone. This suggests that they are likely due to the combined effect of various methodological differences between the two studies, such as age at first exposure, age of weaning, culling of litters, extent and frequency of interaction with the experimenter, and cohort differences.

4.2 Repeated odour stress did not produce any long-term changes in behaviour or dopamine receptor levels:

In the present study, there was no effect of repeated cat odour exposure on PPI, startle, dopamine receptor expression, performance in tests of anxiety-related behaviours (e.g., OF and EPM), of anhedonia (e.g., SPT) or of recognition memory (e.g., NOR) in adulthood (refer to sections 3.1 to 3.5 of chapter 3 for details). This was unexpected

because there were immediate behavioural changes in response to repeated predator odour (e.g. physical avoidance of the odour stimulus) indicative of increased stress much like in previous studies by Wright et al. (2008, 2012, and 2013). This section will attempt to understand potential reasons behind this unexpected finding, beginning with a comparison between the present study and previous ones done by Wright et al. (2008, 2012, and 2013).

As indicated earlier, both the current study and the ones by Wright et al. (2008, 2012, and 2013) investigated the effects of repeated predator odour exposure in adolescence on adult behaviour and dopamine receptor levels. While most dependent measures used in the present study are unique, the OFT and dopamine D1R and D2R receptor levels are two measures common to the present study and the ones by Wright et al. (2008, 2012, and 2013). Wright et al. (2008) reported the effect of repeated adolescent stress on the dopamine D1R and D2R receptor levels in the medial PFC and striatum. Cat odour in that study was administered using a piece of J-cloth that had been rubbed against the body of an adult cat, and was therefore, a potent source of cat odour. All exposures occurred in mid-adolescence (between PND40-48), with no more than a 48-hr period between successive exposures (Wright et al., 2008). In response to such a stressor protocol, a reduction in D2R in the medial PFC was revealed in adult rats, while D1R levels in the same region remain unchanged. Furthermore, the level of dopamine D1R and D2R receptors remained similar in caudate-putamen (striatum) between the two groups in Wright et al. (2008). In the current study too, the level of D1R and D2R in caudate-putamen was not affected by prior exposure to either the control odour or the predator odour (in both studies, data were collapsed across sex). Additionally, no difference was seen in either group in the level of D1R and D2R in the medial PFC. Unlike Wright et al.

(2008), in the current study, the author analyzed the level of these dopamine receptors in the medial PFC separately for the two sexes. Furthermore, unlike the current study, Wright et al. (2008) revealed a reduction in D2R in the medial PFC in response to repeated PO exposure.

Methodological differences between the current study and the previous ones are likely to explain at least some of these disparate outcomes. The first difference between some of these studies (namely Wright et al., 2008, and a sub-section of animals used in Wright et al., 2012, 2013) is in the nature of the cat odour stimulus used. In the current study, pieces of a collar previously worn by an adult cat were used as stressor stimuli whereas in some previous studies mentioned here (e.g. Wright et al., 2008, 2012, 2013), a piece of J-cloth that had been rubbed against the body of an adult cat was used as stressor. Previous work has shown the two odour sources to result in some similar effects as well as some profoundly different effects on adolescent rats (Wright et al., 2012, 2013). Most notably, repeated exposure to cat odour in the form of a J-cloth reduced blood CORT levels by the final exposure in adolescence, while no such change was seen in response to exposure to a collar stimulus (Wright et al., 2012, 2013). The collar stimulus, on the other hand, reduced the rate of entry into the odour region for both sexes, while the cloth stimulus only affected females (males exposed to either odour showed no difference in the rate of entry to the odour region) (Wright et al., 2012, 2013). Both types of stimuli, however, produced comparable reduction in locomotor activity during odour exposure, and a comparable reduction in the duration spent in the middle region of the arena (Wright et al., 2012, 2013). Similarly, certain measures only showed an effect of the collar stimulus while rats exposed to predator odour using a J-cloth behaved no different from the controls (e.g. rate of rearing, and duration and rate of cagemate contact during

odour exposure) (Wright et al., 2012, 2013). Behaviors of the two groups in the OFT also showed mixed results. For example, while exposure to both types of odour stimuli reduced locomotor activity in the OFT compared to controls, exposure to the collar stimulus alone caused a reduction in the rate of center entry (a conventionally-used measure of assessing "anxiety" in the OFT) (Wright et al., 2013).

Another notable difference between the current study and Wright et al. (2008, 2012, and 2013) is in the total number of odour exposures included in the stressor protocol as well as the age of the rats at each odour exposure during adolescence. Both these differences could have, conceivably, contributed to the differences in outcomes. In the current study, rats were exposed to predator or control odour on six occasions whereas Wright et al. exposed rats to predator odour on five occasions (Wright et al., 2008, 2012, and 2013). Additionally, the age of exposure and the inter-stressor intervals were different between these studies. While previous work involved exposing rats to predator odour in early adolescence with no more than 2 days between successive exposures (Wright et al., 2008, 2012, and 2013), the current work used longer inter-stressor intervals such that predator odour exposures covered the entire period of adolescence and early adulthood. This potentially allowed the animal enough time to recover from each individual exposure as longer inter-stressor intervals would be more likely to produce lasting behavioural and endocrinological habituation.

In spite of these profound differences in methodology, certain results were comparable between the current study and the previous ones completed in our lab (Wright et al., 2008, 2012, and 2013). The similarities in odour exposure behaviours have already been mentioned in an earlier paragraph. Additionally, both in the previous study (Wright et al., 2008) and the current one, no changes were seen in adult D1R and D2R receptor

levels in the caudate-putamen/striatum in response to repeated predator odour exposure. However, unlike the current study, Wright et al. (2008) reported a decrease in D2R in the medial PFC of rats exposed to adolescent stress. As adolescence is a period of rapid developmental changes in different parts of the brain including the PFC and caudate-putamen (reviewed in Spear, 2000) , it is hardly surprising that the same stressor protocol can have different effects based on when it is applied during adolescence.

Additionally, these results also suggest that the dopamine receptor levels in the caudate-putamen/striatum may be resilient to the impact of repeated predator odour (though they do change following single housing in the current study as will be discussed later in this chapter). This is surprising considering the fact that D1R and D2R levels in the caudate-putamen/striatum also undergo rapid developmental changes, much like the D1R and D2R levels in the PFC (Andersen et al., 2000, Andersen and Teicher, 2000, and Teicher et al., 2003). But it is important to note that in both Wright et al. (2008) and the current study, data were collapsed across sex due to a lack of adequate samples. This may be important given that there are sex differences in the pubertal development of D1R and D2R in the caudate-putamen/striatum (Andersen et al., 1997 and 2000). In general, males show a much more dramatic increase and decrease in dopamine D1R and D2R levels between PND25 and puberty compared with females particularly with respect to D1R and D2R levels in the striatum (Andersen et al., 2000). Therefore, in order to unequivocally answer the question of whether predator odour affects dopamine receptor levels in the striatum/caudate-putamen, tissue from both sexes may be needed to be analyzed separately.

Additionally, much like the present study, Wright et al. (2012, 2013) used the OFT to measure anxiety-related behaviour in adulthood. However, unlike the protocol

used in the current study, Wright et al. (2012, 2013) tested animals in the OFT over two days with each session lasting 20-min. Moreover, the open field used in Wright et al. (2012, 2013) contained a hide-box, and rate and duration of entry into the opaque hide-box (that contained a single entrance for the rat) were calculated. The hide-box was placed along the middle of one of the four walls of the arena and during the predator odour test, the odour stimulus was placed across from it. In addition to the other behaviours, the duration and rate of head-out behaviour was also measured. A head-out occurs when the front half of the animal's body including the head is sticking out of the hide-box. It is considered to be a risk-assessment behaviour that allows the animal to gingerly explore the threatening environment (Wright et al., 2008). In the present study, on the other hand, in the OFT, the rate and duration of entry into the periphery were scored along with thigmotaxis behaviour. At a conceptual level, these behaviours could be considered comparable to the hide-box behaviour as in both cases the rat is trying to shield itself from any potential risks by avoiding the open part of the arena.

Rate of entrance to the center was influenced by adolescent odour treatment (though data from the first and second days had to be collapsed to arrive at this result) (Wright et al., 2012, 2013). On the first day of the OFT, no effect of odour treatment was found on behaviours such as duration and rate of entrance into the hide-box, or duration spent in head out for either sex (Wright et al., 2012, 2013). However, locomotor activity was reduced in rats exposed to cat odour (either in the form of collar or cloth stimulus) (Wright et al., 2012). Collar-exposed rats also showed a reduced rate of entrance into the center (Wright et al., 2012, 2013). These results suggest a reduction in anxiety-related behaviours in response to adolescent predator odour treatment (in the form of a collar stimulus). In the current study, on the other hand, there were no differences between the

control or predator odour exposed groups with respect to any of the behaviours measured in the OFT. In conclusion, previous work using the predator odour model has shown changes in dopamine receptor levels, anxiety-related behaviour and HPA activity indicating enduring changes in structure and function; this result is in opposition to the results observed following predator odour exposure in the present study wherein very few long-term effects of predator stress were seen.

As mentioned earlier, part of the reason for the absence of any effects of repeated predator stress might be the fact that the inter-stressor intervals in the current study were long compared to previous studies. Changing inter-stressor interval can affect the behavioural and endocrinological response to the stressor. For example, Masini et al. (2008) found that six acoustic stress exposures, each separated by 24-hr, produced habituation of the behavioural and HPA axis response to a subsequent homotypic stressor administered 48-hr later. But when the same six stressor sessions were administered in a single day, the resulting behavioural and endocrinological habituation did not last 48-hr after the last stressor session. From this study, it can be concluded that stressor protocols with *shorter* inter-stressor intervals are *unlikely* to produce lasting habituation of HPA activity.

In a similar study, the effects of two protocols were compared that differed in the duration of each session, and number of sessions administered although the time lag between consecutive sessions was identical (24-hr) (Gray et al., 2010). Specifically, the authors compared the effects of restraint stress when administered for 10 sessions (30-min/session) and when administered for 5 sessions (3-hr/session) (Gray et al., 2010). Both protocols produced the same level of habituation of the ACTH and CORT responses. They did, however, differ in the levels of CRH mRNA and AVP released in various

regions of the brain following stressor exposure. CRH mRNA levels were only increased in the 3-hr protocol (in central amygdala and bed nucleus of stria terminalis (BNST), whereas AVP levels were increased in both albeit in different regions. While 30-min restraint increased AVP levels in medial amygdala, the 3-hr restraint stress increased AVP in the PVN of hypothalamus and BNST (Gray et al., 2010). Together, these results imply that both protocols engaged the HPA axis and its components to different degrees. It is, therefore, conceivable that that these two stress protocols may be recruiting different pathways downstream of the HPA axis for stress adaptation despite involving identical stressors.

These studies also highlight the importance of factors other than the nature of stressor used (such as the duration of inter-stressor interval and individual stressor sessions) in affecting the outcome of a stressor protocol. Furthermore, the outcomes of these studies also present a strong case for investigating the effects of different types of stressor protocols on other components of the animal's stress response system (i.e. components other than the HPA axis). This will likely provide a more complete picture of the biological effect of different stressor protocols.

To conclude, the lack of effect of repeated cat odour exposure on most measures assessed in the current study may not be particularly surprising considering the fact that the inter-stressor interval was longer than is commonly used in studies of this kind.

4.3 Isolation was accompanied by increased sensorimotor gating, anxiety-related measures, and D1R expression in the medial prefrontal cortex:

4.3(i) Summary of effects of single housing in the current study:

In the present study, rats that were housed alone showed increased "baseline" PPI indicating an increase in sensorimotor gating, as well as the latency to reach that response (refer to sections 3.2(i) and 3.3(i) for details). Isolated rats also showed reduced D2R levels in the caudate-putamen and increased D1R levels in the medial PFC (though this latter effect was only seen in predator odour exposed males). The first "baseline" PPI was measured 5 days after single housing began (on PND33); thus, a deficit in PPI first emerged following only a very brief period of isolation and endured for approximately 20-days of isolation followed by 15-days of re-housing the animals with their former cagemates.

In addition, isolated rats showed signs of increased anxiety-related behaviour. Specifically, isolated animals showed increased startle, spent less time in the center and more time in the periphery of the OFT, and displayed an increased rate of thigmotaxis in the OFT. These changes in startle and OFT behaviour suggest an increase in anxiety-related behaviour in animals housed singly for a little over three weeks in adolescence, followed by a little over two weeks of housing with a cagemate. Behaviour in the EPM also hinted at this result. Though the EPM test did not reveal an effect of single housing on any of the conventional measures of anxiety-related behaviour such as relative duration in the open arms and relative number of entries to the open arms, isolated animals did, however, show certain behaviours that could be interpreted as increased "anxiety". For example, isolated animals showed greater rate of entry into the closed arms compared to animals housed in pairs (a statistical trend). Isolated rats also spent a significantly greater duration in the center of the EPM compared to animals raised in pairs.

Elevated startle amplitude is considered a reliable indicator of anxiety-related behaviour (Walker et al., 2003). In this study too, this link between startle amplitude and anxiety-related behaviour is supported by the results of correlational analyses between OFT and startle data. A negative correlation was found between the percent duration spent in the center of the open field and the amplitude of the startle response (data not shown). This means that animals with greater startle amplitude show increased anxiety-like behaviour (i.e. spent less time in the center of an open field). Additionally, the rate of thigmotaxis was positively correlated with certain startle measures, namely, the average of all startle trials after the 1st exposure, the average of non-consecutive startle trials after the 3rd exposure, and the 6th exposure, and the average of all consecutive startle trials (averaged across all exposure periods, Figure 3.46 and 3.47). This means that animals that showed greater startle amplitude also preferred being in a protected position by the walls of the open field. These results support the idea that both startle amplitude and the OFT are measuring related, over-lapping aspects of anxiety-related behaviour.

Taken together, these results suggest that the isolated rats preferred being in the center and closed arms instead of the more-exposed open arms. In short, it can be concluded that rats reared in isolation showed greater anxiety-related behaviour than those reared in pairs. The following paragraphs will discuss these results in the context of other such work carried out on isolated animals.

4.3(ii) Prior studies have established single housing during the post-weaning period as detrimental:

Isolation was hypothesized to be particularly challenging for adolescent rats because social support is an important factor throughout development, and the lack of

social support brought about by social isolation is a known risk factor for many diseases (Karelina and DeVries, 2011) . This is especially true in adolescence during which the process of becoming independent from parents, and learning to eke out an independent existence becomes underway in most species (Blakemore, 2012), and the opinion of, and interaction with, peers becomes increasingly important. For example, compared to young children and adults, the mood of adolescents was most affected by social exclusion from their peers during an online ball game (Sebastian et al., 2010). In addition, adolescents that live in socially unpredictable environments experience short-term and long-term reductions in mental and physical health (Brumbach et al., 2009). Such increased sensitivity to social influences and interest in social interactions is accompanied by development of abilities that assist in these interactions such as social emotional processing, mentalizing, and face processing, as well as brain regions that assist in these functions such as the dorsomedial PFC and posterior superior temporal sulcus (Blakemore, 2012). Additionally, social isolation among adolescents is associated with an increased risk for depressive symptoms, suicide attempts, and low self-esteem (Hall-Lande et al., 2007).

Social interactions are also pivotal to development in adolescence (Vanderschuren et al., 1997); social play is possibly the most important and intense peer-directed social interaction exhibited during this time by mammals including rats, which is comparable (up to a point) to the peer-directed social interactions of human adolescents, though in rats this occurs mostly during the weaning to early adolescent phase before sexual maturity is attained (reviewed in Spear, 2000). Structures such as the PFC, which is developing during adolescence, and glutamatergic inputs into the striatum are considered important for the emergence of play behaviour. Furthermore, neurotransmitter systems in

the brain, which regulate social behaviour are also undergoing extensive development such as changes in the levels of monoamines, density of serotonin and dopamine transporters, as well as dopaminergic receptor levels, in various parts of the brain including the medial PFC and striatum (Han et al., 2011). Consequently, a disruption in social interactions, including but not limited to, play behaviour brought on by single housing in adolescence has the potential to result in irrevocable changes in brain structure and function.

Isolation rearing of rats from weaning to adulthood changes social behaviour as well as the underlying monoamine levels. For example, isolation resulted in increased playful fighting and social contact behaviours (but not serious aggressive behaviour) compared to controls in the social interaction test conducted in adulthood as well as an accompanying increase in dopamine and serotonin levels in the medial PFC and nucleus accumbens, and an increase in serotonin turnover in nucleus accumbens (Han et al., 2011). A 12-week isolation rearing protocol starting from PND25 revealed an increase in the number of spontaneously firing dopaminergic neurons and in the proportion of dopaminergic neurons expressing irregular and bursting activity in the ventral tegmental area; these changes in activity are believed to underlie the behavioural phenotype associated with such prolonged isolation including increased locomotor hyperactivity in a novel environment and a deficit (decrease) in PPI and startle amplitude (Fabricius et al., 2010).

Considering these changes in dopamine transmission in the medial PFC, it was hypothesized that the protocol used in the present study would result in altered dopamine signaling in the medial PFC. As expected, isolation housing increased PPI and changed

dopamine receptor levels. Specifically, isolated rats had increased D1R in medial PFC and decreased D2R in caudate-putamen.

Furthermore, previous work has shown isolation housing to result in increased anxiety-related behaviour. For example, a 4-week isolation-housing regime starting from PND21-48 resulted in increased anxiety-like behaviour in the OFT and increased level of social interaction and aggression in the Social Interaction test; these changes were reversed by housing the animals in groups for 4-weeks from PND49-77 (Meng et al., 2010). Even though isolation in the current study began almost a week after weaning (i.e. in early adolescence), a similar increase in anxiety-related behaviour was hypothesized in the isolated animals considering the importance of social interactions in adolescence in rats (as explained above). Thus, it was predicted that an increase in anxiety-related behaviour would be seen in one or more of the following tests which are thought to reflect anxiety: startle amplitude, OFT, and EPM. The outcome of the current study supported this hypothesis as startle amplitude and OFT behaviour of isolated rats showed increased anxiety-related behaviour. In the following paragraphs, each of these results will be discussed.

4.3(iii) Isolated animals showed increased sensorimotor gating, and changed dopamine receptor levels:

As mentioned earlier, the current study revealed a change in sensorimotor gating and dopamine receptor levels in response to isolation housing. Previous research too has shown similar changes in response to isolation housing of rats. In general, rearing rats in isolation (particularly from weaning day onwards, up to adulthood or longer) results in the so-called isolation syndrome, characterized by a reduction in PPI, increase in startle,

increased behavioural perseveration, increased locomotor activity in a novel environment, as well as increased responsiveness to dopaminergic agonists, possibly due to an increase in the number of dopamine receptors (e.g. Day-Wilson et al., 2006, Cilia et al., 2005, Powell et al., 2002, Möller et al., 2012, and Fitzgerald et al., 2013).

Studies on the effects of isolation rearing on PPI and startle have, however, produced some conflicting results in the past that newer research is attempting to reconcile. For example, Varty et al. (1999) tested effects of varying periods of isolation occurring at different time-points during adolescence on PPI, startle, and habituation in male Sprague Dawley rats. Only a continuous isolation beginning on PND21 and lasting for about 4-weeks resulted in a deficit in PPI, with startle and habituation being unaffected by such a treatment (Varty et al., 1999). In line with this observation, a shorter (2-week) period of isolation commencing from weaning did not produce this deficit in male Sprague Dawley (Liu et al., 2011) while 6 to 9-weeks of continuous post-weaning isolation reduced PPI in male Sprague Dawley (Roncada et al., 2009). In Long Evans rats, however, it is found that 3-weeks of post-weaning isolation is sufficient to reduce PPI in both sexes (Powell et al., 2002).

It was therefore, decided to use an approximately 3-week isolation period in the current study, although it began a week after weaning compared to earlier ones. In this way, confining isolation housing to adolescence, instead of including the pre-adolescent period, allowed me to delineate the outcomes specific to isolation housing in adolescence. This is important because disorders such as schizophrenia (whose sensorimotor gating deficits the current work is trying to model) emerge mostly in adolescence, not childhood (pre-adolescence) (Fendt and Koch, 2013). Moreover, this protocol allowed me to

investigate whether re-socialization can rescue some of the effects caused by isolation housing during a developmentally sensitive time like adolescence.

The current results, however, contradict earlier findings (e.g. Powell et al., 2002) as isolation increased PPI instead of reducing it. Specifically, approximately 3-weeks of isolation housing initiated a week from weaning, followed by about 2-weeks of re-socialization produced an increase in PPI. Previous research, on the other hand, has shown isolation to reduce PPI (as discussed in the earlier paragraphs). Two possible, somewhat-related reasons could explain this contradiction. Firstly, the current study followed the 3-week isolation housing with an approximately 2-week re-socialization period. In adult Wistar males, a similar protocol of brief isolation followed by re-socialization resulted in an *increase* in PPI (Rosa et al., 2005).

Secondly, isolation in the present study began a week after weaning while most protocols that produce a reduction in PPI begin isolation immediately after weaning. Developmental changes occurring around, or a little before, puberty might be important in mediating in isolation-induced reduction in PPI. For example, the increased level of testosterone available at puberty might be important for isolation housing to reduce PPI. To this effect, it has been shown that isolation from PND21 up to 6-weeks reduces testosterone release in response to sexual arousal in male Wistar rats (Amstislavskaya et al., 2013), and evidence exists that testosterone levels affect PPI. For example, castration of adult rats reduces the disruption of PPI by a serotonin 5HT1A receptor agonist; this effect is reversed by administration of synthetic testosterone (Gogos and van den Buuse, 2003). These results support the view that the pubertal changes in gonadal hormone levels might be important mediators of the reduction in PPI following post-weaning isolation.

However, further work needs to be done in this area before making any definitive statements.

It is relevant to mention that not all PPI measures recorded in this study revealed an effect of single housing. PPI measured immediately after the 1st, 3rd, 5th, and 6th odour exposures were not significantly affected by housing. But analysis of PPI measured a day before the odour exposures began (i.e. a measure of sensorimotor gating about 4-days after single housing began), and that measured 1-day before the final odour exposure, revealed an effect of single housing. All these PPI measurements could not be analyzed together because there was a subtle difference in the experimental condition in the two cases ("baseline" vs post-odour PPI measurement). "Baseline" PPI was recorded on days when there was no odour exposure, and animals were taken to the PPI testing room directly from the colony room. On the other hand, post-odour PPI measurement was carried out after the animals were taken from the colony room to the testing room where they were exposed to a control or predator odour. Following odour exposure, they were taken to the PPI testing room for PPI measurement.

The fact that "baseline" PPI but not "post-odour" PPI showed an effect of isolation suggests that isolation may affect PPI differently based on the age of the animal and/or the period of isolation it has undergone. The PPI measurement recorded after the 1st, 3rd, 5th, and 6th odour exposures were taken at approximately PND33, 37, 45, 48, 50, and 66. "Baseline" PPI, on the other hand, was measured at approximately PND32 and PND64 (after re-socialization). The emergence of PPI deficits in response to isolation housing towards the end of adolescence and beginning of adulthood has been shown previously in Lister Hooded and Sprague Dawley rats (Varty et al., 1999). This finding suggests that

the changes that occur during adolescence, such as reduction in the number of dopamine receptors in the medial PFC and striatum, may be important for PPI deficits to emerge.

Additionally, isolated animals showed a greater T_{max} (i.e. they required more time to achieve the maximum startle response in response to prepulse trials). This could mean that the underlying neural structures are slower in processing the prepulse stimulus (because T_{max} for startle-alone trials remained unchanged suggesting that the animals showed no change in the time they took to reach the maximum startle response to the startle stimulus).

To summarize, these results suggest a complicated picture of the effect of single housing on PPI and likely the neural structures mediating it. They also underscore the need to measure PPI at multiple time points throughout the experiment as well as a need to look at T_{max} as well while measuring PPI. Animal studies, for the most part, do not measure T_{max}. Our results, on the other hand, indicate the possibility that single housing might influence this measure as much as it influences V_{max}. Additionally, there is no reason to believe that changes in T_{max} might not reflect changes in the neural substrates mediating PPI much as changes in V_{max} are believed to do.

Furthermore, in the current study, isolated rats showed increased D1R in medial PFC and decreased D2R in caudate-putamen. In other words, it appears that isolated rats showed reduce dopaminergic tone in the cortical regions and an increased dopaminergic tone in the sub-cortical regions. This is similar to the changes predicted to occur in schizophrenia i.e. a hypo-dopaminergic tone in the cortical regions and a hyper-dopaminergic tone in the sub-cortical regions (Howes and Kapur, 2009).

Some of the changes in the dopamine system found using different isolation-rearing protocols are as follows. Isolation rearing from weaning to adulthood increases

levels of dopamine in the medial PFC and nucleus accumbens (Han et al., 2011). Isolation housing from PND21-85 also increased dopaminergic D2R levels in the medial PFC and nucleus accumbens of Sprague Dawley rats (Han et al., 2012). In line with this result, isolation housing also alters the activity of VTA dopaminergic neurons (that project to the PFC and nucleus accumbens) to irregular and bursting (Fabricius et al., 2010). Comparing results of the present study to these earlier studies suggests that isolation, if begun at weaning, and carried on until adulthood, will reduce dopamine transmission in the medial PFC (by reducing D1R) much like that suspected in schizophrenia. On the other hand, if isolation is begun later in adolescence (around puberty or later), it will likely not produce this result. In the earlier discussion of the PPI results too the conclusion was reached that if the peripubertal/weaning period is missed, isolation will likely not reduce PPI. The same holds true for D1R levels in medial PFC. On the other hand, if isolation is begun later in adolescence and followed by a period of re-socialization, it can increase PPI as seen in the current study. The same appears likely for D1R levels in the medial PFC as well. Moreover, correlational analyses suggest an association between dopamine D1R and D2R receptor levels and PPI. Specifically, a significant positive correlation was found between D2R levels in medial PFC and PPI after the 6^{th} odour exposure (only at 3dB and 6dB, Figure 3.48). A correlation between D1R in medial PFC and PPI after the 1^{st} odour exposure (at 6dB) showed a trend towards significance ($p= 0.065$, data not shown). These results, together, suggest the possibility that changes in the level of the dopamine receptors in response to isolation are related to the changes in PPI.

At this point, it is important to point out that by measuring D1R and D2R in two regions of the brain, the author of the current study was only able to get a look at one of the multiple components of the central dopamine machinery, and this image of

dopaminergic transmission is incomplete. A more comprehensive, but still incomplete, picture of dopaminergic function in response to isolation housing was demonstrated in a recent study wherein male Long Evans rats were housed in isolation from PND28-77 (Gill et al., 2013). Following isolation, dopamine release and uptake in the nucleus accumbens core were studied using fast-scan voltammetry (Gill et al., 2013). Isolation housing was accompanied by increased release and uptake of dopamine in the nucleus accumbens core; this change persisted into later adulthood (Gill et al., 2013). Isolated rats also showed increased anxiety-related behaviour in the EPM as evidenced by a reduction in the time spent in the open arms. Not surprisingly, a negative correlation was found between the time spent in the open arms and the amount of dopamine released and taken up during fast-scan voltammetry (Gill et al., 2013). Furthermore, D2-autoreceptor activity in the nucleus accumbens was unaffected by the housing condition (Gill et al., 2013). This study underscores the need for a multi-pronged approach to studying the effect of isolation housing (or *any* experimental treatment, for that matter) on the sub-cortical and/or cortical dopamine system. Apart from looking at the levels of receptors, autoreceptors and the dopamine transporter, researchers should also attempt to look at the amount of dopamine released and taken up in response to electrical stimulation as is done in fast-scan voltammetry studies such as the one mentioned earlier in the paragraph. Unfortunately, it is challenging to perform all these analyses along with behavioural studies within the same study due to time, and budgetary constraints.

The current work estimated levels of dopamine D1R and D2R receptor proteins using the Western Blotting technique. As mentioned earlier, this technique provides only a brief glimpse into the workings of a multi-faceted system, and conclusions must therefore, be drawn from it with caution. A related point of concern is that dopamine

receptor levels in different sub-cellular regions of a neuron may be differently affected by isolation housing. Moreover, the effect of single housing may be evident in one sub-region of the PFC alone (the current work looked at dopamine receptor levels in three sub-regions of the PFC together- the prelimbic, infralimbic, and dorsopeduncular regions). This is best exemplified by the findings of a recent study that looked at the change in the density of D2R in PFC in male Sprague Dawley rats. Using dual electron microscopic immunolabelling, the authors studied the sub-cellular distribution of pre-synaptic and post-synaptic D2R receptors in the prelimbic region of the PFC (Fitzgerald et al., 2013). Isolation did not affect the global density of pre-synaptic or post-synaptic D2R in the prelimbic cortex. It did, however, reduce the density of post-synaptic D2R in the dendritic region in the prelimbic cortex (Fitzgerald et al., 2013). Thus, the current work suggests an alteration in the central dopamine system in response to single housing but the exact nature of the changes seen here (increase in D1R in medial PFC and decrease in D2R in caudate-putamen) are different from those seen previously. Part of the reason behind this outcome is the difference in the single housing protocol and duration used in these studies. Furthermore, these studies differ in the exact part of the dopamine system being investigated.

4.3(iv) Isolation was accompanied by increased anxiety-related behaviours:

Isolation housing increased anxiety-related behaviour in rats (refer to sections 3.3(i) and 3.3(ii) for details). Specifically, isolated rats displayed

(1) an increase in startle amplitude, and

(2) an increase in percent duration spent in the center of the open field during the OFT compared to their pair housed counterparts.

There was no effect of isolation on any of the conventional measures of anxiety-related behaviour in the EPM such as relative duration in the open arms and relative number of entries to the open arms. Isolating animals did, however, show certain behaviours that could be interpreted as increased "anxiety". For example, isolated animals showed greater rate of entry into the closed arms compared to pair housed animals (a statistical trend). Isolated rats also spent a significantly greater duration in the center of the EPM compared to pair housed ones. Taken together, these results suggest that the isolated rats preferred being in the center and the closed arms instead of the more-exposed open arms. In short, it can be concluded that rats reared in isolation showed greater anxiety-related behaviour in both OFT and EPM task. Thus, in all three tests of anxiety-related behaviours, isolated animals displayed increased "anxiety".

Previous work on isolation housing in adolescent Long Evans rats showed a similar increase in anxiety-related behaviour. Long Evans rats isolated from PND28-77 revealed increases in anxiety-related behaviour in the EPM (Chappell et al., 2013, and Yorgason et al., 2013). Isolation housing was also accompanied by an increase in dopamine release, and an increase in dopamine transporter activity in the nucleus accumbens though D2 autoreceptor activity in nucleus accumbens was unchanged (Yorgason et al., 2013). Furthermore, these changes were negatively correlated with a measure of anxiety in the EPM (i.e. with duration spent in the open arms). In the current work, however, the main effects of isolation housing on anxiety-related behaviour were seen in the startle measure and OFT, though certain behaviours in EPM did suggest increased anxiety-related behaviour (such as the preference to stay in the closed arms and center over the open arms). Furthermore, like Yorgason et al. (2013), the current work

also revealed a correlation between various measures of EPM and dopamine D1R and D2R levels in the medial PFC. These correlations are listed below:

1. Levels of D1R in PFC were *negatively* correlated with the percent duration spent in the closed arms of the EPM (data not shown).
2. Levels of D1R in PFC also showed a trend towards a significant *positive* correlation with the percent duration spent in the center of the EPM ($p= 0.067$, data not shown).
3. Levels of D2R in PFC were *negatively* correlated with the total duration spent in the open arms of the EPM (Figure 3.49).
4. Levels of D2R in PFC also showed a trend towards a significant *negative* correlation with relative rate of entry into the open arms ($p= 0.097$, Figure 3,49).
5. Levels D2R in PFC also showed a trend towards a significant *negative* correlation with the relative percent duration spent in the open arms ($p= 0.055$, Figure 3.49)

These correlations suggest the possibility that increased "anxiety" in the EPM is associated with an increase in D2R in medial PFC. It is also likely, that increased "anxiety" in EPM is associated with changes in D1R levels in the medial PFC though the current study offers limited evidence for this view. Even though the three tests of "anxiety" in this study yielded similar effects of isolation housing, OFT and startle amplitude data did not show similar correlational relationships with dopamine receptor data. None of the OFT measures showed any correlation with D1R or D2R receptor levels in the medial PFC. Startle amplitude data, on the other hand, presented a slightly more mixed picture. A trend towards a positive correlation between the startle amplitude obtained by averaging all consecutive and non-consecutive startle trials (averaged across all Exposure Periods as well), and PFC D1R levels suggested that an increase in

dopaminergic activity may be linked to increased "anxiety" in the rats ($p= 0.060$, data not shown). This is similar to the result obtained for EPM and dopamine data, and that obtained by an earlier study (Yorgason et al., 2013). However, startle amplitude obtained from consecutive trials, and that obtained by average of consecutive and non-consecutive startle trials revealed a significant negative correlation with the PFC D2R levels at all Exposure Periods. This correlation suggests that increased "anxiety" as evidenced by an increase in startle is associated with a *reduction* in PFC D2R levels. Given the limited information known at the moment, all that can be concluded is that even though isolation in adolescence increased "anxiety" in EPM, OFT, and measures of startle, dopamine D1R and D2R in medial PFC likely mediate only the changes in behaviour exhibited in the EPM.

Isolation housing in adult rats is also associated with an increase in anxiety-related behaviour. Adult male Sprague Dawley rats and male wild type rats showed increased "anxiety" in the EPM (Carnevali et al., 2012) and the Light/Dark Box test of anxiety-related behaviour following 3 to 4-weeks of isolation (Carrier and Kabbaj, 2012). However, when the behaviour of animals isolated in adolescence is compared to those isolated in adulthood, it was found that the former (i.e. those isolated in adolescence) showed greater anxiety-like behaviour in the EPM (Yorgason et al., 2013). Thus, it appears that, although isolation can produce an increase in "anxiety" at any age, its effects are more profound when it begins in early adolescence. The current results suggest that even if isolation in adolescence is followed by a period of re-socialization, the formerly isolated rats continue to show increased anxiety-related behaviours. Therefore, it can be concluded that these deficits cannot be rescued by re-socialization. It is unclear is the same holds true for adult isolation effects such as those seen in a couple of earlier studies

(Carnevali et al., 2012, and Carrier and Kabbaj, 2012). This needs to be investigated in the future to determine if isolation housing in adolescence is more detrimental than isolation in adulthood. With certain behaviours such as PPI, it has been shown that isolation rearing needs to be carried out for at least a few weeks, preferably encompassing the earliest weeks of adolescence/puberty for a PPI deficit to manifest itself (as mentioned earlier in the chapter).

To summarize, in the current study, rats isolated throughout most of adolescence revealed an increase in anxiety-related behaviours as gauged by the startle amplitude and performance in the OFT and EPM task. Additionally, correlational analyses suggest that changes in dopamine D1R and D2R receptor levels in the medial PFC likely mediate only the behaviour seen in the EPM task. Further work needs to be carried out in the area before any further, definitive conclusions can be drawn.

4.3 (iv) Isolation was not accompanied by depression-like behaviour such as anhedonia in rats:

Isolated rats showed no changes in behaviour in the SPT. Specifically, there was no change in the animals' preference for the sucrose solution relative to tap water, implying no change in anhedonic behaviour in response to isolation (refer to section 3.4(i) of chapter 3 for details). As mentioned in the first chapter, mild stressors in adolescence do not produce anhedonia in male rats although females did show increased preference for the sucrose solution in SPT (Hong et al., 2012). In the present study, there did exist a sex difference in percent sucrose preference with females showing increased percent preference for sucrose than males although there was no difference in the effect of a stressor such as isolation on either sex. This result appears inconsistent with the findings

relating to major depressive disorder in humans where a more definitive link appears to have been established between stressor exposure and the onset of depressive symptoms (e.g. Slattery et al., 2012). It is said that chronic stressors that are uncontrollable and/or involve a loss of status are particularly associated with an increased risk of major depressive disorder (Slattery et al., 2012). Furthermore, stressors appear to have a greater role to play in triggering the first episode of major depressive disorder than later episodes (Slattery et al., 2012). In fact, facing severe adversity in childhood/early adolescence is associated with an increase in anhedonic symptoms associated with major depressive disorder (Slattery et al., 2012). It is therefore unexpected to see that a severe adolescent stressor in rats such as isolation does not affect anhedonia or depressive-behaviour in general. However, as mentioned in chapter 1, this result in not entirely without precedent in the adolescent rat literature. Several studies have found no change in the SPT in response to adolescent stressors such as repeated restraint (Suo et al., 2013) or repeated social stressors such as isolation and crowing (Chaby et al., 2013).

The first possible reason for the absence of an effect of isolation stress could be the fact that stressors such as isolation failed to measure the reward sensitivity of these rats in addition to measuring anhedonia. In fact it has been suggested that the apparent anhedonic tendencies seen in major depressive disorder are not due to an inability to experience pleasurable rewards, but due to a lack of incentive salience (i.e. a lack of wanting rather than liking the rewarding stimulus) (Slattery et al., 2012). The SPT, unfortunately, is not designed to measure incentive salience in rats. It merely measures the propensity of rats to choose a sucrose solution over tap water under normal housing conditions. Performing a test of incentive salience in rats such as the conditioned place

preference test along with the SPT could help further clarify if isolation does indeed bring about depressive behaviours in rats.

Another useful addition to these potential experiments would be to include some measurement of the activity of the mesolimbic dopamine system, particularly at the level of the nucleus accumbens, a region traditionally associated with reward sensitivity. It has been proposed that one reason behind anhedonic symptoms seen in major depressive disorder could be an under-performing mesolimbic dopamine system (Slattery et al., 2012). In fact, the chronic mild stress model of major depressive disorder has been shown to cause both a decrease in percent sucrose preference in adult rats as well as a reduction in conditioned place preference (i.e. a reduced ability to revisit a place that was previously paired with a reward) (Willner, 2005). This is also associated with reduced dopamine transmission in the striatum and increased dopamine transmission in the PFC (Willner, 2005). In the current study, on the other hand, isolation was associated with an increase in dopamine D1R receptor levels in the medial PFC and a decrease in the D2R receptor levels in the caudate-putamen. This suggests a reduction in dopamine levels in the medial PFC as well as an increase in the dopamine levels in the caudate-putamen (and possibly, in other sub-cortical regions as well). In another study, isolation housing was accompanied by an increase in dopamine D2R levels in the nucleus accumbens, a region related to sensitivity to rewards (Han et al., 2011).

To summarize, it appears that social isolation is capable of changing cortical and sub-cortical dopamine transmission (at least at the level of D1R and D2R). However, the few differences in the outcome of chronic stressor exposure in the current study and others mentioned here suggest that the role of social stress and dopamine in altering

adolescent sensitivity to rewards, and anhedonia needs to be studies a bit further before definite conclusions can be drawn.

4.3 (v) Isolation alone did not affect memory although it improved memory when combined with repeated exposure to a control odour:

In the current study, approximately 3.5-weeks of isolation in adolescence resulted in no change in memory as assessed using the NOR test. However, a combination of isolation and repeated exposure to a control odour did improve memory. Specifically, it increased percent preference for the novel object over the familiar one. Animals raised in pairs and exposed to a control odour repeatedly, on the other hand, did not show this effect (refer to section 3.5(i) of chapter 3 for details). As mentioned earlier, performance in the NOR reflects the recognition memory of rats. Greater preference for the novel object means that the rat is able to remember the previously encountered (familiar) object and able to distinguish it from the novel one. In other words, the rat's recognition memory is in working condition.

These results contradict the initial hypothesis and are somewhat surprising because aversive events or stressors are known to reduce performance in the NOR test (e.g. (Eagle et al., 2013). In fact, in the present study, a sub-section of isolated animals that were exposed to the control odour did show increased preference for the novel object compared to the their pair-housed equivalents suggesting an improvement in performance in response to isolation (and repeated exposure to a non-threatening odour). This result hints at the possibility of long-term stress in adolescence having some beneficial effects, although it is uncertain why this effect is only seen in the isolated animals that were also exposed repeatedly to a control odour.

Previous research offers some support for this result as isolation has been shown previously to alter NOR performance. For example, 6-weeks of isolation beginning from the day of weaning (PND21) altered the ability of Lister Hooded rats to differentiate novel objects from familiar ones (Zamberletti et al., 2010, Watson et al., 2012). This change in recognition memory seen in Lister Hooded rats raised in isolation can be reversed by D3R competitive antagonists, which also have some affinity for D2R such as S33084 and S33138 (Watson et al., 2012). This suggests that the change in NOR performance may have arisen due to excessive activity of the D2R and D3R receptors. In the current work, a change in NOR performance was accompanied by a reduction in D2R in the caudate-putamen and an increase in D1R in the medial PFC. This, however, was not exclusive to the isolated animals exposed repeatedly to a control odour. Therefore, at this point, it cannot be unequivocally concluded that dopamine receptors in the caudate-putamen and medial PFC mediate the change in NOR performance in these animals without further experimentation.

Other models of adolescent stress have also investigated effects on recognition memory. Much like the effect of isolation housing, effects of adolescent stress vary across the board. For instance, chronic unpredictable stress administered from PND30-70 that included social stressors like isolation and crowding, reduced the latency to approach a novel object, suggesting boldness, though object recognition memory was not measured (Chaby et al., 2013). Long Evans rats that were exposed to 3-days of stress in adolescence (PND27-29) showed no difference in their ability to explore novel objects in an open arena though recognition memory per se was not assessed (Saul et al., 2012). Prolonged stressor experiences in adolescence have been shown to alter structures that contribute towards performance in tests of recognition memory (Squire et al., 2007). For example,

increased activation of the stress axes such as the HPA axis possibly affects the functioning of regions such as the medial PFC, HC and amygdala, all of which are involved in regulating memory (Squire et al., 2007). Expectedly, a 72-hr period of isolation housing resulted in an increase in CORT along with a reduction in neuronal activity (long term potentiation) in *Cornu Ammonis* 1 or CA1 region of the HC in two strains of mice which is thought to mediate performance in various tests of memory like the NOR test (Kamal et al., 2014). This change was reversed by treatment with a glucocorticoid receptor blocker suggesting a key role for CORT and hippocampal glucocorticoid receptor in reducing long term potentiation. Therefore, it is likely that isolation could cause profound alterations in the NOR performance of rats were the current study replicated with a larger sample size. The author's own experience with the NOR test suggests a noticeable degree of variability in the performance of rats on this task, indicating a need to use greater sample sizes for differences to achieve statistical significance.

To conclude, the current study revealed no changes in recognition memory of isolated rats although the combination of isolation and repeated exposure to a control odour improved recognition memory.

4.4 The unique behaviour of the rats exposed to neither odour:

4.4(i) Are isolation and no odour exposure conditions two sides of the same coin, i.e. sub-optimal environmental stimulation?

Much like isolation housing, animals exposed to neither odour type showed altered PPI, D2R in medial PFC, and startle amplitude. Specifically, these rats showed reduced PPI and D2R in medial PFC compared to animals in the other two odour

conditions as well as an increase in startle. These results suggest an underlying similarity between the experiences of isolation and no odour exposure, which is discussed in greater detail in the following paragraphs.

When the experiments discussed in this document were conceived, the no odour condition was devised as a control for the experience of repeated exposure to the arena (that was used in odour exposure), and the testing room, just as the no predator odour condition was a control for the experience of repeated encounter with a distinct, threatening odour. The animals belonging to the no odour group were left undisturbed in the colony room except on days involving startle and PPI testing, unlike animals from the other odour sub-groups who were repeatedly taken to the testing room and placed inside an arena for exposure to a control or threatening odour. It was therefore, hypothesized that these animals would show minimum changes/disruption in different behaviours and dopamine receptor levels compared to the other odour sub-groups. Clearly, this hypothesis was unsupported because these animals showed increased startle amplitude, decreased PPI, and a decrease in anxiety-related behaviour in the EPM. Most of these changes (except for the reduced anxiety in the EPM task) were seen in response to isolation as well. This led to the suggestion that both treatments may have an underlying commonality, namely a sub-optimal level of environmental stimulation. While isolated animals experienced reduced social interaction during adolescence, animals exposed to neither odour missed out on the experience of repeatedly exploring a novel arena along with a novel (control) or threatening odour stimulus.

The animals exposed to no odour behaved in a unique manner compared to the ones exposed to a control or predator odour. As previously mentioned, these animals were not moved to the testing room until the adult behavioural testing began (starting with the

OFT), unlike animals from the other odour sub-groups who were put in an odour arena for 30-min, 6 times during adolescence and early adulthood. Animal from the no odour condition were, however, handled briefly (approximately 5-sec) twice a week for cage-changes similar to the rats in the other two odour groups. Additionally, the tails of animals that were housed in pairs were marked on a weekly basis by the experimenter, a procedure that involved physical contact between the animals and experimenter that lasted about 5-sec. This contact was a little different from that involved in cage cleaning because cage changes were mostly performed by the animal care personnel and involved about 2-sec of physical contact with the base of the animal's tail. Marking tails, on the other hand, was done by the experimenter; it involved picking up the animal and placing it close to the body of the experimenter for about 3 to 5-sec. It is important to note that animals belonging to the no odour condition were either housed singly or in pairs; of these two sub-groups, the ones that were isolated till re-socialization received the least amount of environmental stimulation. Regardless, both isolated and pair housed no odour sub-groups may have received less than optimal environmental stimulation, potentially influencing their behaviour and dopamine receptor levels in adulthood.

Studies of isolation housing as well as of environmental enrichment can also be viewed as investigating the role of experiential complexity in guiding development and function. While isolation is at one end of this spectrum of experiential complexity, at the other end are different types of environmental enrichment protocols, which result in increased social and physical stimulation for the animals. The current work provided the opportunity to examine the effects of two types of environmental deprivation on the experimental animals: a lack of social contact, and a lack of physical complexity in the animals' environment. Comparing this work with previous studies presents a challenge

because although isolation housing is a routinely employed protocol, no studies could be found which had a treatment condition comparable to the no odour condition of the present study. Nonetheless, there are studies that investigated the role of physical and social complexity on the animal and these provide some insight, as discussed below.

Varty et al. (2000) found startle amplitude in isolated and enriched animals to be the same; startle was significantly higher in both groups than in standard, pair housed controls suggesting an increase in anxiety-like phenotype with isolation and enrichment. Similarly, both these groups showed increased rate of locomotor activity in an open arena as measured using behavioural pattern monitor (BPM) (Varty et al., 2000). Isolated rats, however, did not habituate in the 10-min period. PPI, on the other hand, showed a reduction in isolated animals compared to both enriched and pair housed controls; isolated rats also showed more rearing than the other two groups in the behavioural pattern monitor (Varty et al., 2000). Amphetamine-induced locomotor stereotypy showed a similar difference between isolation- and enrichment-housed animals (Pritchard et al., 2013). These similarities in the effects of isolation and enrichment support the view that a certain optimal level of environmental stimulation is needed for ideal function. This idea is also supported by the observed similarities between enriched and pair housed control groups. This also suggests the possibility of pair housed control housing conditions being adequate to achieve that optimal level of environmental stimulation. These results also suggest that the effect of environmental parameters is dependent on the behaviours or structures being studied.

Lending support to this view are the following studies of isolated and enriched rats in various types of maze tests to assess anxiety-related behaviour. For example, isolated rats have been shown to spend less time in open arms of the EPM than enriched

rats, indicating increased anxiety-related behaviour though this difference was obliterated by routine handling (Pritchard et al., 2013, Ravenelle et al., 2013). Interestingly, a study using adult rats that were exposed to enriched conditions for a mere 3-hr a day revealed the opposite effect on anxiety-related behaviour; specifically, rats exposed to enriched conditions revealed *reduced* anxiety-related behaviour in the elevated zero maze as well as the radial arm water maze (Sampedro-Piquero et al., 2013). Comparing the results of these studies suggests the possibility of the effect of enrichment (and isolation) on anxiety-related behaviour to be dependent on the duration for which these conditions are experienced. While sustained exposure to enriched environment is useful in reducing anxiety, brief bouts of exposure may serve to increase anxiety possibly because of reduced habituation to the novel environment. Further work needs to be carried out to investigate these possibilities**.**

Apart from behaviour, effects of isolation and environmental enrichment have also been tested on physiological measures such as HPA activity and dopamine receptor levels. Baseline CORT levels were comparable between pair housed controls and enriched rats though it is uncertain how they compare against isolated rats; following an acute stressor, CORT levels returned to baseline sooner in enriched rats (Konkle et al., 2003) . These results suggest that isolation as well as enriched conditions can alter physiological HPA activity though it remains to be seen how other HPA axis hormones and regulatory receptor levels change in different parts of the brain such as the PFC, HC, and amygdala. Levels of dopamine receptors, a key player in the stress response as well as regulation of HPA activity, also show effects of enrichment; specifically, enriched rats show D2R levels comparable to isolated ones in the caudate-putamen although in the nucleus accumbens, D2R levels are higher in the enriched animals than isolated or social

controls (Ravenelle et al., 2013). Future work needs to be done to investigate the effects of isolation and of enrichment on other regulators of HPA activity.

4.5 The unique behaviour of females compared to males:

In the current study, females showed distinctly different behaviour from males in a number of measures. They displayed increased PPI, startle latency, and D2R in medial PFC. Additionally, in the EPM, they showed greater activity in the open arms than males while in the SPT, they showed a greater preference for the sucrose solution than males. These results imply less anxiety-like and depression-like behavioural tendencies in female Long Evans rats than males in adulthood. This outcome is consistent with the existence of profound differences in the neurobiology of male and female brains in rats in adulthood and earlier, and the need to take these differences into account while conducting research on animal models of human disorders with sex differences in prevalence, onset and other parameters. To begin with, female (Long Evans) rats show higher basal levels of CORT than males (Wright et al., 2013); furthermore, females have a profoundly larger HPA axis response to stress compared to males as well as higher diurnal changes in ACTH and CORT levels (Handa et al., 1994). Additionally, while males gain weight in response to adolescent stress, females show an increase in CORT levels (McCormick et al., 2005). Adolescent stress increases behavioural sensitization to nicotine in females, not males (McCormick et al., 2005), while repeated restraint impairs Radial Arm Maze performance of males, but not females (Bowman et al., 2002).

Future researchers need to, therefore, prioritize a detailed investigation of the sex differences between control populations of model animals such as different strains of rats. As things currently stand, quite a few studies use male rats/mice only as their test subjects (Short et al., 2013); the ones that do use female subjects often consider sex merely as a possible confounding factor that might influence the outcome without investigating the possible mechanistic factors behind the influence of sex (Short et al., 2013). Additionally, the ontogeny of behavioural and structural changes in both sexes of model organisms like Long Evans rats (and other strains) needs to be established; this is particularly important in modeling symptoms of diseases which show sex differences in incidence and disease progression. For example, schizophrenia, anxiety and depressive disorders show pronounced sex differences in their incidence such as the age of onset, and rapidity of progression (Handa et al., 1994). In the current work, sex was not treated as a peripheral, confounding factor to be controlled for. Instead, it was ones of the three main independent variables used to determine the existence of sex differences in the behaviours being assayed. The author wanted to contribute towards chronicling and understanding sex differences in control rats and rats exposed to adverse developmental environments. However, an amendment to the design of the current work that has the potential to make it more informative would be to record the estrous stage of the female subjects at different points in the experiment. This would have allowed the author to hypothesize if any of the sex differences observed in these behaviours could be attributed to the routine cycling of estrogen and progesterone in female rats.

In the current work, females revealed a higher PPI, latency to startle and D2R levels in the medial PFC than males. Unaffected human females, on the other hand, show a *lower* PPI than males; moreover, PPI varies with the cyclical fluctuation of hormones

during the estrous cycle (PPI is lowest in the luteal phase) (Kumari, 2011) though no consistent differences have been recorded in startle or startle latency. Most studies attempting to model sensorimotor gating changes in humans use male animals (rats or mice), an understandable choice considering the higher incidence of disorders involving sensorimotor gating deficits (e.g. schizophrenia) in males (Swerdlow et al., 2000). But the results of the current work emphasize the need to reconsider this choice; moreover, additional work needs to be done to systematically investigate the temporal changes in PPI and related measures in both sexes of various strains of rats and mice. Though labor-intensive and time-consuming, such as investigation can help understand how this function varies with sex and time in natural populations; since PPI changes in schizophrenia and other deficits are consistently recorded in multiple populations spread across different geographical regions (e.g. Csomor et al., 2009, Braff et al., 2005, and Ziermans et al., 2012) and PPI is one of the few measures with cross-species validity (Li et al., 2009). Furthermore PPI, startle amplitude and even habituation show a significant level of heritability in both control and schizophrenic populations (Hasenkamp et al., 2010) making it a potentially invaluable tool in investigating the genetic basis of these disorders.

In the current study, females showed a greater preference for the sucrose solution than males. In other words, females showed *reduced* anhedonia insofar as SPT reflects a rat's ability to experience pleasure. Furthermore females spent more time in and made more entries to the open arms than males suggesting an inherently lower anxiety-related behaviour than males. Curiously, these differences are contrary to the differences seen in humans; as mentioned earlier, both major depressive disorder and its comorbid anxiety occurring more frequently in females than males (Mehta et al., 2013). A caveat to the

results of the current study is the fact that statistical significance was reached only after pooling data from all experimental treatments of each sex together. A more convincing display of sex differences in EPM and SPT would be the emergence of a statistically significant effect of sex in a study in which only control (untreated) male and female rats were studied.

While not many studies in rats, particularly Long Evans rats, have been undertaken with the express intent of chronicling sex differences, a few have recorded sex differences between control populations. For example, Huynh et al. (2011) found higher open arm activity among untreated females than males as well as an increase in the time spent in the center in the OFT. In other words, females showed lower anxiety-like behaviour in two tests of "anxiety" whereas in the current study, no effects of sex were seen on performance in the OFT or on startle amplitude (another measure believed to be linked to trait anxiety (Koch and Schnitzler, 1997). This could imply a task-specific increase in anxiety-related behaviour. It is also possible that, because the tests were performed on different days, sex differences in emotionality depend on the age at testing. Nonetheless, that does suggest a more-labile, fluctuating difference between the two sexes in these measures. Clearly, future work needs to be undertaken to clarify these issues.

It is likely that gonadal hormones may mediate some of these changes. Previous work suggests an effect of the estrous cycle on certain measures such as performance in cognitive tests in female rats. For example, estrous cycle of rats affects performance in the NOR test. Females showed better performance in the proestrous and estrous phases compared to metestrous and diestrous phases (van Goethem et al., 2012). Unfortunately, in the current study, levels of gonadal hormones were not assessed. Additionally, no

record was kept of the stage of the estrus cycle of the female rats during different stages of the experiment. Follow up studies could investigate these questions to better understand the mechanism behind these differences.

4.6 Caveats associated with the current work:

4.6(i) Caveats associated with choice of husbandry details:

The first set of caveats discussed here relate to details of rat strain, supplier and animal husbandry. The choice of rat strain can influence the outcome of a study such as the current one. In particular, the effects of early-life isolation on PPI and startle depend on the rat strain used (Bakshi et al., 1998, Bakshi and Geyer, 1999). While Sprague Dawley rats require 4-weeks of continuous isolation for effect to emerge, Long Evans rats require a longer duration (about 6-weeks). In the current study, it is possible that isolation did not produce pronounced deficits in PPI (only affecting "baseline" PPI) because the isolation period lasted less than 6-weeks. Furthermore, choice of rat strain can also affect other parameters related to PPI and startle, such as disruption of these measures by dopamine agonists such as apomorphine. Apomorphine is a non-selective agonist for D1-like and D2-like receptors, and its disruption of PPI is believed to occur by binding of these receptors, particularly the latter (Millan et al., 2002). While apomorphine disrupted PPI without affecting startle amplitude in Wistars, the converse was true for CD rats (a strain derived from Sprague Dawley rats)(Rigdon, 1990). Even though the effects of apomorphine were not investigated in the current study, this result lends support to the assertion that choice of rat strain can have a pronounced effect on the outcome of studies measuring PPI, startle, and related functions.

Choice of rat strain can also affect recognition memory as gauged by performance in the NOR test. Wistars, Long Evans and Sprague Dawley rats show an effect of strain on NOR performance; though "baseline" performance is comparable between the three, they differ in the amount of muscarinic acetylcholine receptor blocker scopolamine needed to disrupt NOR performance (van Goethem et al., 2012). Mice strains also differ in the "baseline" ability to discriminate novel objects from familiar ones during the NOR test. Only two strains (namely NMR1 and OF1 strains) were able to distinguish between novel and familiar stimuli (van Goethem et al., 2012); one strain called SJL strain, showed lower object exploration and no retention following a 60-min interval between the familiarization and test trials (van Goethem et al., 2012). Because baseline performance is comparable between Long Evans, Wistars, Sprague Dawley rats, one can be a little more confident when comparing the results of the current study to those involving Wistar and Sprague Dawley rats (as done in the section discussing NOR tests).

It is useful to speculate why the choice of rat strain has such a pronounced effect on the outcome of experiments. Part of the reason for this is likely genetic (Ellenbroek et al., 2005); unfortunately, the knowledge of the rat genome and of techniques to manipulate elements in the genome is currently very limited. It is therefore, not possible to directly compare genomes across various rat strains to find out sequences that are different between strains. This also makes it difficult to generate rat strains with targeted mutations in order to investigate the role of a specific gene or protein in a specific context. Therefore, standard rat strains that are not bred for investigating specific research questions are used in most rat studies of stress.

But differences in physiology and behaviour of the various rat strains have been documented. Of these, differences in the stress response systems are particularly relevant

to the current work. Knowledge of these differences can help researchers make more judicious choice of rat strain in studies of stress. Most studies compare only two strains with each other, which is why pairs of rat strains will be discussed here. The first pair of rat strains to show difference in HPA activity is Fischer 344 and Lewis rat strains (Grota et al., 1997, Armario et al., 1995, and Chaouloff et al., 1995). Both are types of inbred strains; while Fischer 344 was developed in 1920 by Curtiss and Donning, Margaret Lewis developed Lewis rat strain by repeatedly inbreeding Wistar rats. Lewis rats have a blunted response to difference stressors such as forced swimming and restraint compared to F344 though baseline levels of HPA activity is similar in both (Grota et al., 1997, Armario et al., 1995, and Chaouloff et al., 1995). The source of this difference is likely a reduced amount of AVP released by the hypothalamus in Lewis rats; AVP, much like CORT, stimulates the pituitary to release ACTH (Grota et al., 1997, Armario et al., 1995, and Chaouloff et al., 1995). This explanation has been proposed because other parameters of the HPA axis function such as levels of various HPA hormones released in response to stress, the extent of negative feedback, levels of corticosteroid receptors etc. are comparable for the two strains (Grota et al., 1997, Armario et al., 1995, and Chaouloff et al., 1995).

To further the point about the effect of rat strains on stress studies, a few studies will be discussed in which effects of a chronic mild stress protocol were compared on adult Long Evans and Sprague Dawley rats (Bielajew et al., 2002, 2003, Konkle et al., 2003). Chronic mild stress, as mentioned in Chapter 1, is a stressor model that uses a series of "everyday" experiences such as overnight illumination, overnight food and water deprivation, and brief confinement in order to stress rats (Bielajew et al., 2002). Three weeks of chronic mild stress did not alter fecal output per week or the weight of the

spleen though body weight was reduced and CORT levels increased in both Sprague Dawley and Long Evans strains (Bielajew et al., 2002). Chronic mild stress resulted in a heavier adrenal gland in Long Evans rats only (Konkle et al., 2003). Also, the two strains differed in the blood CORT response to a brief stressor (Bielajew et al., 2002). Sprague Dawley rats exposed to chronic mild stress showed an identical response to the acute stressor as ones from the control group (Bielajew et al., 2002). But Long Evans rats exposed to chronic mild stress showed a blunted response to the acute stressor compared to control rats (Bielajew et al., 2002). This suggests that the HPA axis in Long Evans rats is more susceptible than Sprague Dawley rats to being molded by previous heterotypic stressor experiences. Not surprisingly, adult Long Evans rats exposed to chronic mild stress show greater changes in their response to an acute stressor (forced swim session) than Sprague Dawley rats (Bielajew et al., 2003). Long Evans rats may therefore, be more useful to study long-term effects of chronic stressor protocols than Sprague Dawley rats, particularly with respect to effects mediated by CORT. This finding offers support to our decision of using Long Evans rat strains in the current study.

The author would like to add a final word about her choice of rat strain for the current study. Long Evans strain was chosen in order to enable a comparison of the current work with work previously performed in the Perrot Lab using the predator odour model in order to establish the reliability of the model, as well as to add to the body of findings that has already been produced. Furthermore, using Long Evans rat strain also allowed one to compare the current work with that of other researchers that study adolescent stress in Long Evans rats such as Green et al. (2013), Green and McCormick (2013), Wilkin et al. (2012), and Chappell et al. (2013).

Along with rat strain, the supplier of rats used can also affect the outcome. For example, a comparison of Wistar rats from two different suppliers (American Supplier: Harlan, USA; English Supplier: Bantin-Kingman, UK) revealed that rats from the American supplier showed a higher baseline PPI than those from the English supplier (Swerdlow et al., 2000 and 2001). These differences were also reflected in PPI disruption by apomorphine. Sprague Dawley rats, on the other hand, showed no effect of supplier in baseline PPI or PPI at various doses of apomorphine except at 0.25mg/kg; at this latter concentration, Sprague Dawleys from the American supplier showed significantly lower PPI than Sprague Dawleys from the English supplier. Clearly, not all rat strains are equally susceptible to the effect of supplier on measures such as PPI; Wistar rats seem more susceptible than Sprague Dawley rats. This could be the reason behind the popularity of this strain in stress research.

Because the genetic background of same strains of rats from different suppliers is identical, the difference in their response to various experimental conditions is probably due to environmental factors. These factors include the level of background noise, cleanliness, adeptness of the animal care staff in handling the animals, the temperature and humidity of the colony room, as well as the stress associated with transport of animals from the supplier to the receiver. In order to minimize the effect of at least one of these factors (namely transport stress), it was decided to bring in breeders from a commercial supplier, and generate the experimental animals. This does, however, make it difficult to make a direct comparison of the present results against those from many other studies because the latter used experimental animals purchased from a commercial supplier. Another way to address supplier issues is to use cohorts from multiple suppliers for the same experiment. This may help establish reliable outcomes of experimental

treatments and has, to our knowledge, been attempted in at least one recent study of stress in rats that used Lister Hooded rats from two different suppliers to assess the effect of isolation housing on NOR test performance and related hippocampal protein levels (Bianchi et al., 2006). Rats from both suppliers showed similar effects of isolation housing as discussed previously in this chapter (Bianchi et al., 2006).

Such husbandry-related choices are particularly important to take into account and possibly, standardize across laboratories because certain measures like PPI are particularly susceptible. For example, repeated handling of animals (occurring biweekly in the colony rooms used in the current study) involving approximately 2 to 5-sec of touching by the base of the tail to move the animal from one cage to another) has been shown to prevent development of PPI deficits in isolation-housed animals (Rosa et al., 2005).

Along the same lines, details related to animal housing, such as the type of cage bottoms used, affect HPA axis activity and other parameters like locomotor activity in a novel open field (Heidbreder et al., 2000). PPI deficits in response to isolation housing appeared only in animals raised in sawdust cages and not in grid-floor cages (Heidbreder et al., 2000). Such a conclusion was also reached by an earlier study wherein the effects of isolation housing were inextricably dependent on husbandry practices such as the nature of cages used (plastic or hanging metal), amount of handling of the animals, and test conditions such as the order of testing the animals and whether other animals were present in the testing room during behavioural testing (Holson et al., 1991). Subsequent work over many years has established the importance of such details in influencing the outcome of experimental treatments. These factors are important to bear in mind while comparing different studies of adolescent stress in rats to the current one. Some of the

differences in outcome could be partly explained by differences in husbandry-related choices.

4.6(ii) Caveats associated with data interpretation:

The next set of caveats pertain to the challenges faced in interpreting data such as that produced in the current study. The first such challenge is in interpreting the meaning of the changes seen in response to our experimental treatment, and extrapolating these findings to human subjects. For example changes in PPI are believed to reflect changes in attention (Fendt and Koch, 2013). In rats too, animals bred for high PPI show correlations with performance in some complex behavioural assays such as the radial arm maze (Fendt and Koch, 2013). But this link between the PPI and higher cognitive functions is still not irrevocably established in rat or human research. At best, it can be said that PPI is an endophenotype which *may* be linked (conceptually and otherwise) to certain cognitive deficits like inattention seen in schizophrenia and some other mental illnesses, and deficits in PPI *may* be the result of the same neural processes that could eventually result in the full-blown symptoms (Fendt and Koch, 2013).

Another challenge is posed in interpreting startle data. On the one hand, it provides information about a reflex response regulated by a circuit within the brainstem (as discussed in Chapter 1). On the other hand, changes in startle amplitude are also considered suggestive of changes in anxiety-related behaviour (Dreissen et al., 2012). In human subject, for example, higher startle amplitude is associated with increased trait anxiety (De Pascalis et al., 2013). Moreover, increased startle amplitude is a proposed endophenotype of certain anxiety-related disorders such as post traumatic stress disorder even though evidence about whether startle is increased in PTSD and other anxiety

disorders is mixed (Dreissen et al., 2012). Correspondingly, in various rat studies, changes in startle amplitude are often interpreted in terms of changes in anxiety-related behaviour. But we must consider startle amplitude and anxiety-related behaviour (measured in rats using EPM or OFT) as overlapping but distinct phenomena. This is because startle amplitude is a reflex response mediated by a circuit operating at the level of the brainstem, whereas the anxiety-like phenotype measured in animal tests of anxiety likely involve higher-order brain structures as well such as the medial PFC (Lacroix et al., 2000). It is therefore, important to be cautious in interpreting changes in PPI and startle in response to experimental treatments.

Another challenge in data interpretation lies in making sense of the changes the levels of dopamine receptors, and whether these changes imply a role for this dopamine system in mediating changes in startle and PPI. In the current study, startle and PPI increased in response to isolation. Animals exposed to neither odour, on the other hand, showed decreased PPI but increased startle. Some of these changes, particularly in PPI, could be mediated by the changes in the dopamine system in the medial PFC and caudate-putamen (reflected in changed dopamine receptor levels in this study). These results are of consequence because similar changes have been reported in other animal studies as well as studies of human prodromal and schizophrenic populations. But these results do not imply a deficit in cognitive function in isolated rats and in rats exposed to neither odour. To establish such an outcome, the present study will need to be repeated to test rats for effects of isolation and of no odour exposure on PPI, startle, as well as higher cognitive function assessed by tests such as the attentional set shifting task. Similar effects of isolation and no odour exposure on all these measures as well as a correlation

between PPI or startle *and* performance in attentional set shifting task could suggest a deeper link between these two types of measures.

Moreover, it is important to point out that most studies that provide information about the role of specific neurotransmitters or regions in mediating or in regulating PPI or startle (including the current one) need to be interpreted cautiously. They do not provide any information on whether a certain neurotransmitter or region mediates or regulates PPI during normal, physiological conditions (Swerdlow et al., 2001). At best, they can provide information on whether a certain manipulation/dysfunction in a region can alter PPI (Swerdlow et al., 2001). For instance, a study involving measuring PPI following lesion of the medial PFC in rats can tell one whether a certain structural deficit in the medial PFC (aka lesion) can result in altered PPI (e.g. Lacroix et al., 2000). This information can help understand the neurobiology of gating deficits in disorders such as schizophrenia and PTSD. It does not, however, provide any information on whether medial PFC has a role to play in mediating or regulating PPI in normal rats under standard conditions (Swerdlow et al., 2001). This is also true of the other dependent measures tested in this study, such as NOR, EPM, OFT or SPT performance. The current study sheds some light on the potential effects of exposure to a chronic stressor such as isolation, and the mediating role (if any) played by dopamine receptors. It does not, however, expressly address the question of whether chronic stress experienced in the wild would result in such deficits. Furthermore, it does not address the equally important question of whether stresses (such as the feeling of social exclusion and loneliness) encountered in routine life by humans would result in deficits similar to the ones seen in the current study with single housed animals.

Another caveat associated with the current study pertains to interpreting changes in anxiety-related behaviour and other behavioural tests. It is important to recognize that anxiety-related tests such as EPM and OFT were first devised to compare the effects of therapeutic drugs used to treat humans with that of potential drugs in order to discover novel treatments. Nowadays these tests are used routinely to explore effects of other types of experimental treatments in animal models. Even though these tests have some validity (as discussed earlier), questions persist about the interpretation of changes in the performance of rats and how they relate to humans. In the current study, rats housed in isolation showed reduced anxiety-like behaviour in the OFT, much like those exposed to no odour treatment. The latter showed reduced anxiety-like behaviour in the EPM. These changes indicate a change in the behaviour of rats in response to either treatment. Furthermore, these behaviours appear to have more than a passing connection to human anxiety construct. But the most that can be said about human stress and anxiety based on the current work is that a lack of social and physical stimulation in adolescence could result in an increase in certain types of anxiety behaviours. These issues regarding the construct validity of the behaviours measured in the rodent OFT and EPM tests need to be resolved in order to ascertain the implications of studies such as the current one for human disorders.

Another point to bear in mind while comparing the current study to earlier work is that not all studies of stress use the same control. For example, in certain studies of stress, isolated animals are used as a control (Vidal et al., 2007). This is problematic because isolation is itself a stressful and aversive experience for rats as has been shown in a number of studies including the current one. It is more appropriate to compare rats exposed to a stressor to ones that have experienced minimal stress such as socially housed

rats that receive minimal handling (e.g. Wright et al., 2008). Additionally, not all studies define "standard housed" condition in the same way. For example, in Gill et al. (2013), the standard housed control group consisted of animals housed in groups of 4 instead of 2 as seen in the current study (and others such as Wright et al., 2012, 2013). These were compared with the isolated group to assess the impact of isolation rearing (Gill et al., 2013). Similarly, in (Fitzgerald et al., 2013), isolated animals were compared to standard housed controls, which were housed in groups of three. In Fabricius et al. (2010), the authors compared isolated animals with ones housed in groups of 5 (the latter forming the control group). Such comparisons can, understandably, result in different outcomes of the same experimental treatment. In other words, the outcome of a study depends on the nature of the experimental treatment as well as the control group. In the current study, the outcome would likely be different if single housed-no predator odour exposed animals and pair housed-no predator odour exposed animals were considered controls and no no odour-exposed sub-groups were included. Since both under- and over-crowding is a potent stressor in rats (Botelho et al., 2007), it might behoove one to establish the number of cagemates that should be housed together to constitute the standard housed, control condition.

4.6(iii) Additional caveats relating to prepulse inhibition and startle measurement:

The final set of caveats relates to PPI and startle data only. These are:

1. The effect of consumption of sucrose solution (1% w/v) on PPI and startle of rats
2. The effect of ambient illumination on startle and PPI of rats

3. The effect of testing rats in groups or individually, and of repeat testing on startle and PPI

The first of these caveats pertains to the possible effect of consumption of sucrose solution on PPI and startle. This is relevant because the final set of PPI and startle recordings occurred when rats were being habituated to the sucrose solution ahead of the SPT and were therefore, consuming sucrose regularly. Specifically, PPI and startle measured a day before (adult "baseline" PPI and startle), and immediately after the 6th odour exposure were measured while rats had been consuming 1% sucrose solution as part of their regular diet (in addition to tap water and standard rodent chow). Little research has been done into this question though a few research articles lend support to the possibility that sucrose consumption can affect PPI and startle. To begin with, rats that had been selectively bred to ingest less saccharin (Occidental LoS rats) show increased PPI and startle, and anxiety-related behaviours compared to rats that had been bred for high saccharin consumption (HiS rats) suggesting a link between saccharin consumption and PPI and startle, and emotionality (Gonzales et al., 2008). Along the same lines, daily sucrose consumption was associated with increased startle in rats that were genetically mutated for a cholecystokinin receptor (CCK-1 deficient rats) (DeJonghe et al., 2005). CCK-1 regulates mesolimbic dopamine, and therefore, can conceivably play a part in regulating PPI and startle (DeJonghe et al., 2005). Moreover, consumption of sucrose solution increased dopamine release in the nucleus accumbens of adult Sprague Dawley rats (Hajnal and Norgren, 2001), a result which strengthens the view that sucrose consumption could affect PPI and startle because mesolimbic dopamine is known to affect both PPI and startle (Braff, 2010).

The next issue of possible relevance to the PPI and startle data measured in this study and others is the effect of changes in ambient lighting. A transition from dark to light conditions reduced startle and PPI although repeated changes in illumination rescued this effect (Schmajuk et al., 2009). This outcome suggests a potential role of diurnal cycle on startle and PPI. This needs to be borne in mind when comparing the current study to ones where rats were tested during the light phase of the diurnal cycle.

In addition to ambient lighting, the presence of a cagemate in the testing room could also alter the animal's PPI and startle responses as can repeated testing using the same equipment and protocol (Faraday and Grunberg, 2000). In the current study, pair housed rats were taken to the startle testing room together though they were measured individually. The presence of a cagemate in the testing room could affect the response of pair housed rats compared to single housed ones, which were taken to the testing room individually. To support this view, it has been shown that male rats tested individually exhibited greater startle amplitude and PPI than males tested in groups (Faraday and Grunberg, 2000). Moreover, in males, these effects of a cagemate's presence were only felt on the first round of PPI and startle testing (Faraday and Grunberg, 2000). On the other hand, female rats showed an effect of the presence of a cagemate on PPI alone. This effect also depended on the testing period: 1st and 3rd round of testing revealed an increase in PPI of females tested individually whereas during the 2nd round of testing, females tested individually showed *reduced* PPI (Faraday and Grunberg, 2000). By the 4th round of testing, females no longer showed an effect of social or individual testing on PPI (Faraday and Grunberg, 2000). These results also highlight the importance of repeated testing to determine the effect of an experimental treatment on PPI and startle, particularly of female rats.

4.7 Conclusions and summary:

The main purpose of the current study was to assess the impact of two environmental risk factors associated with several adolescent and adult-onset psychiatric illnesses such as schizophrenia. These are repeated exposure to an aversive environment, as well as the absence of social support. In short, rats were exposed to predator odour repeatedly in adolescence in order to assess the impact on behaviours such as PPI, startle, anxiety-related and depressive behaviours as well as on recognition memory. By raising half of these rats in isolation, the role of the absence of a cagemate was assessed on adolescent development in general, and on the effect of repeated predator exposure in particular.

In general, repeated exposure to predator odour produced behavioural avoidance in the rats without causing any long-term changes. Isolation, on the other hand, caused pronounced changes in behaviours such as increased PPI, startle, anxiety-related behaviour in the OFT and D1R in the medial PFC. Additionally, the no odour exposure group, originally intended as a control against the experience of repeated exposure to a novel, non-threatening odour, also displayed a change in PPI, startle and D2R expression in the medial PFC. The similarities in the outcomes associated with isolation and no odour exposure led us to propose that the two represent a sub-optimal environment for the adolescent rats. While isolation is associated with less-than-optimal social experiences, no odour exposure is associated with less-than-optimal experiences exploring novel physical environments (such as the room and the arena used for odour exposure). Therefore, both seemingly disparate manipulations result in similar effects. The current study also found

evidence for the existence of pronounced differences between male and female Long Evans rats.

To conclude, the current study established the importance of social support during adolescence. It also recorded extensive sex differences between male and female Long Evans rats, supporting the use of both sexes in studies of stress in adolescence. Finally, it was able to establish the importance of a certain optimal amount of social and physical stimulation for proper adolescent development.

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Appendices:

Appendix A: Sensorimotor gating and startle protocol

Table AA.1.Trialwise details of the protocol used for measuring sensorimotorgating and related measures.

Trial #	Type	Trial #	Type	Trial #	Type	Trial #	Type
1.	Startle	18.	PP71	35.	PP71	55.	Startle
2.	Startle	19.	NS	36.	PP71	56.	NS
3.	Startle	20.	PP77	40.	PP68	57.	PP77
4.	Startle	21.	PP68	41.	Startle	58.	Startle
5.	Startle	22.	PP68	42.	PP71	59.	Startle
6.	NS	23.	Startle	43.	PP77	60.	Startle
7.	PP68	24.	PP77	44.	PP68	61.	Startle
8.	NS	25.	NS	45.	Startle	62.	Startle
9.	PP77	26.	Startle	46.	PP68		
10.	NS	27.	PP71	47.	PP68		
11.	Startle	28.	NS	48.	Startle		
12.	PP68	29.	PP77	49.	PP71		
13.	PP77	30.	PP71	50.	Startle		
14.	PP77	31.	Startle	51.	Startle		
15.	PP71	32.	Startle	52.	NS		
16.	PP68	33.	NS	53.	PP77		
17.	NS	34.	Startle	54.	PP71		

(Note 1: Block 1 refers to trials 1-5 in this table, Block 2 refers to trials 6-57, and Block 3 refers to trials 58-62. While Block 1 and 3 consist entirely of startle-alone trials (called consecutive startle trials), Block 2 consists of startle-alone trials (called non-consecutive startle trials), no stimulus trials, and three different types of prepulse trials.)

(Note 2: NS refers to no stimulus trials.)

Appendix B. Supplementary Figures and Tables

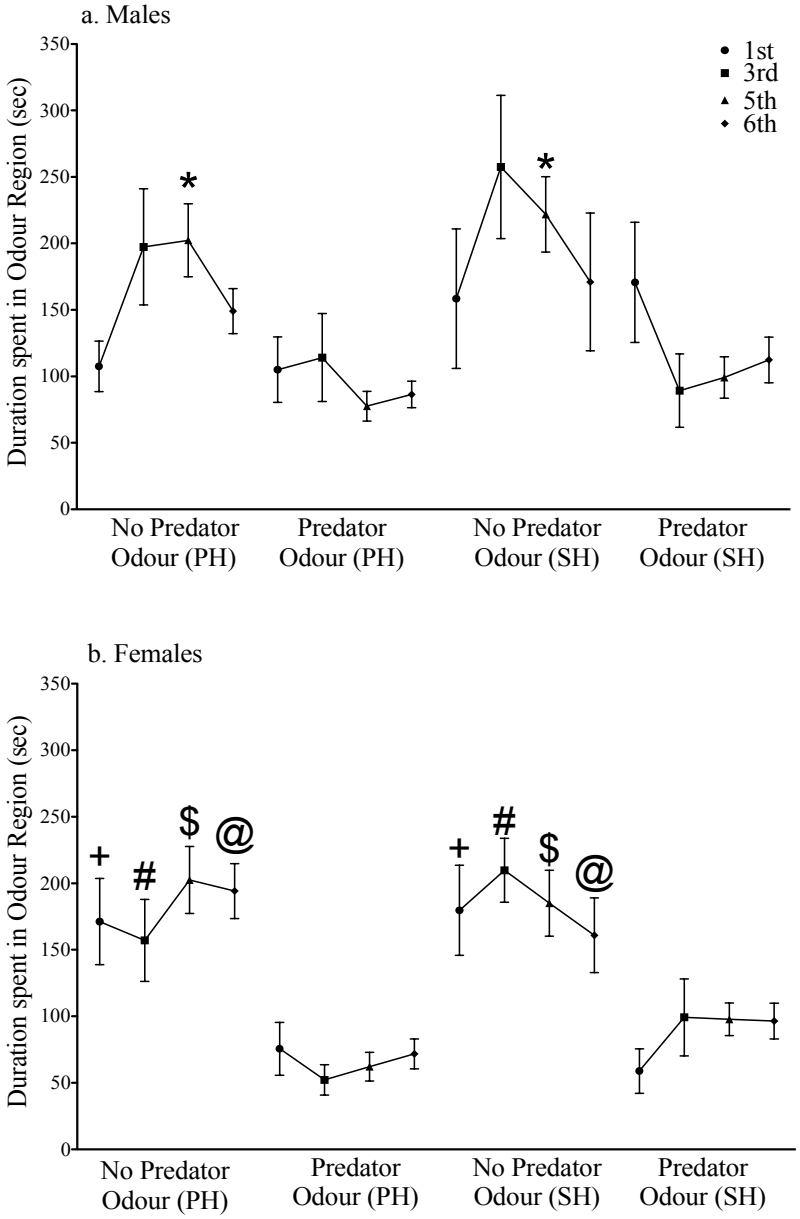


Figure AB.1. <u>Odour exposure behaviour:</u> Mean (+/- SEM) duration spent in the Odour Region (OR) of the arena by males (a) and females (b).

In general, predator odour exposed animals spent *less* time in the odour region than those exposed to no predator odour (main effect of Odour Treatment). Additionally predator odour exposed females spent less time in the odour region at *each* exposure period compared to no predator odour exposed females. Meanwhile, predator odour exposed males spent less time in the odour region only during the 5th exposure period (Odour Treatment X Sex X Exposure Period interaction). *("*", "+", "#", "$", and "@" are significantly different from the predator odour treatment for the same sex and exposure period, collapsed across housing.)*
(*Note: "PH" and "SH" refer to "Pair Housed" and "Single Housed", respectively.)*

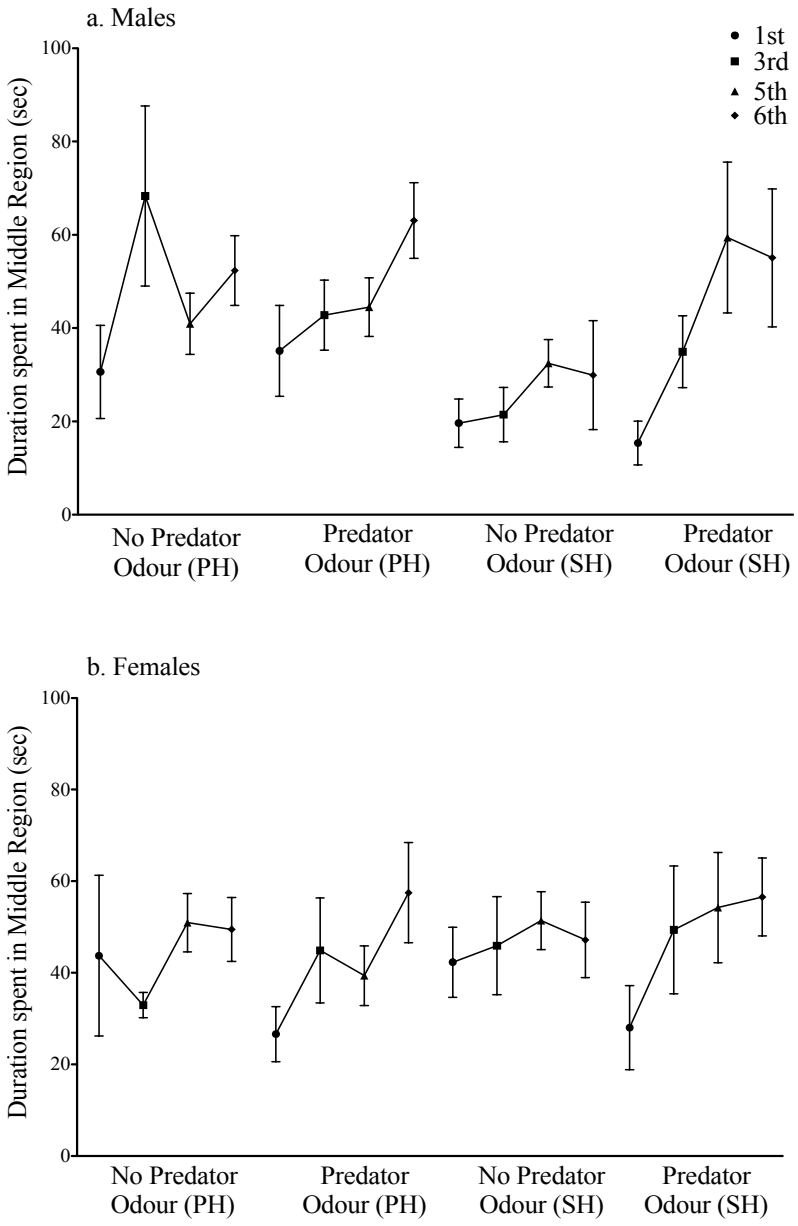


Figure AB.2. <u>Odour exposure behaviour:</u> Mean duration (+/- SEM) spent in the Middle Region (MR) of the arena for males (a) and females (b).

This measure increased with each subsequent exposure ($1^{st} < 3^{rd}$, $1^{st} < 5^{th}$, $1^{st} < 6^{th}$, $3^{rd} < 6^{th}$). (*Note: "PH" and "SH" refer to "Pair Housed" and "Single Housed", respectively.)*

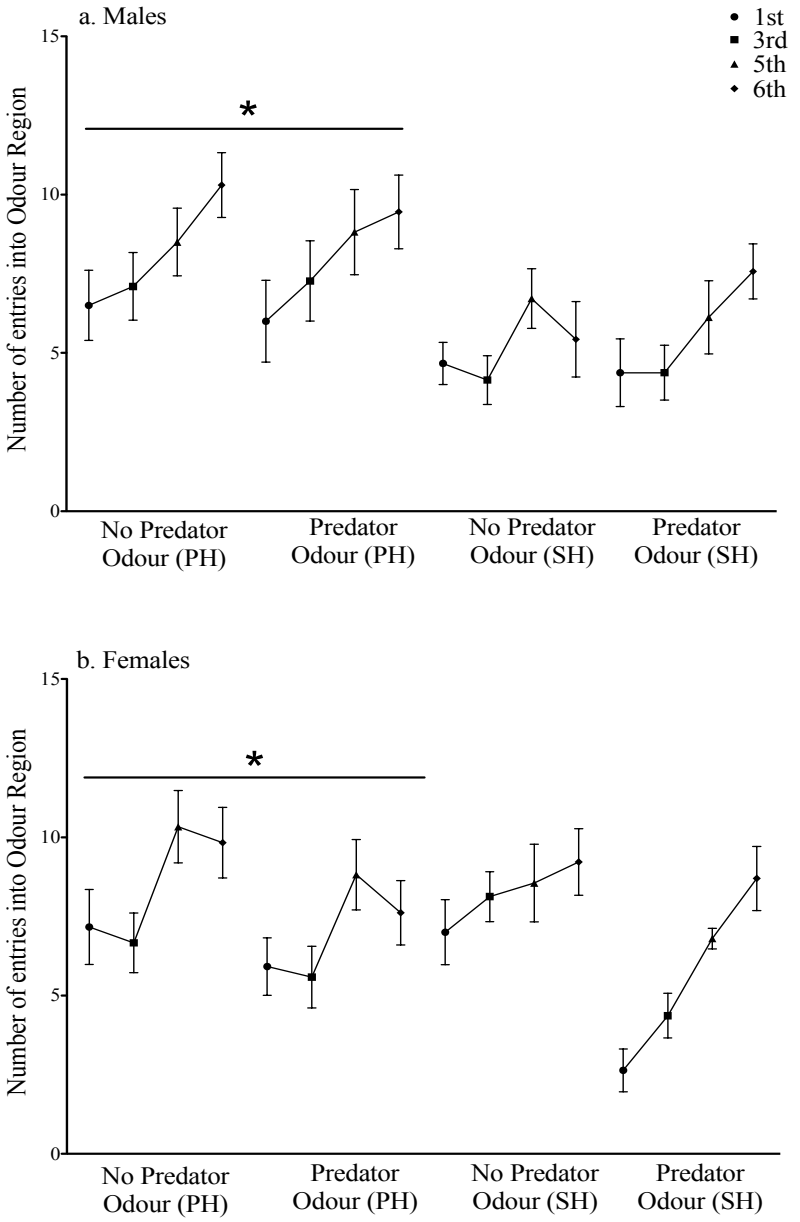


Figure AB.3. <u>Odour exposure behaviour:</u> Mean (+/- SEM) number of entries into the Odour Region (OR) of the arena for male (a) and female (b) rats.

Single housed (SH) animals made fewer entries into the odour region than pair housed (PH) ones (main effect of Housing). The number of entries into OR also increased with subsequent exposures, indicating a main effect of Exposure Period ($1^{st} < 5^{th}$, $1^{st} < 6^{th}$, $3^{rd} < 5^{th}$, $3^{rd} < 6^{th}$). *("*" is significantly different from single housed animals, collapsed across sex, odour treatment and exposure period.)*

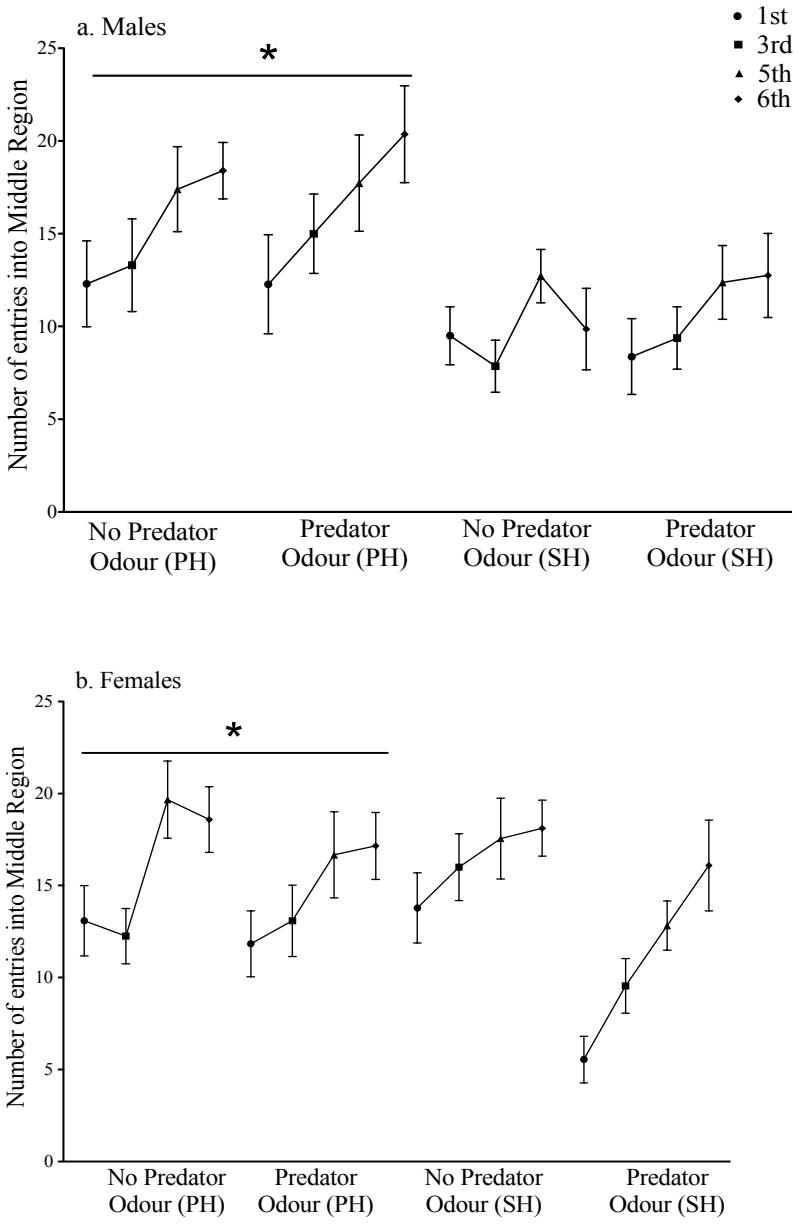


Figure AB.4. <u>Odour exposure behaviour:</u> Mean (+/- SEM) number of entries into the Middle Region (MR) of the arena for male (a) and female (b) rats.

Single housed (SH) males made fewer entries into the MR compared to pair housed (PH) males (Sex X Housing interaction). The number of entries also increased with subsequent exposures (main effect of Exposure Period: $1^{st} < 5^{th}$, $1^{st} < 6^{th}$, $3^{rd} < 5^{th}$, $3^{rd} < 6^{th}$). *("*" is significantly different from single housed animals, collapsed across sex, odour treatment and exposure period.)*

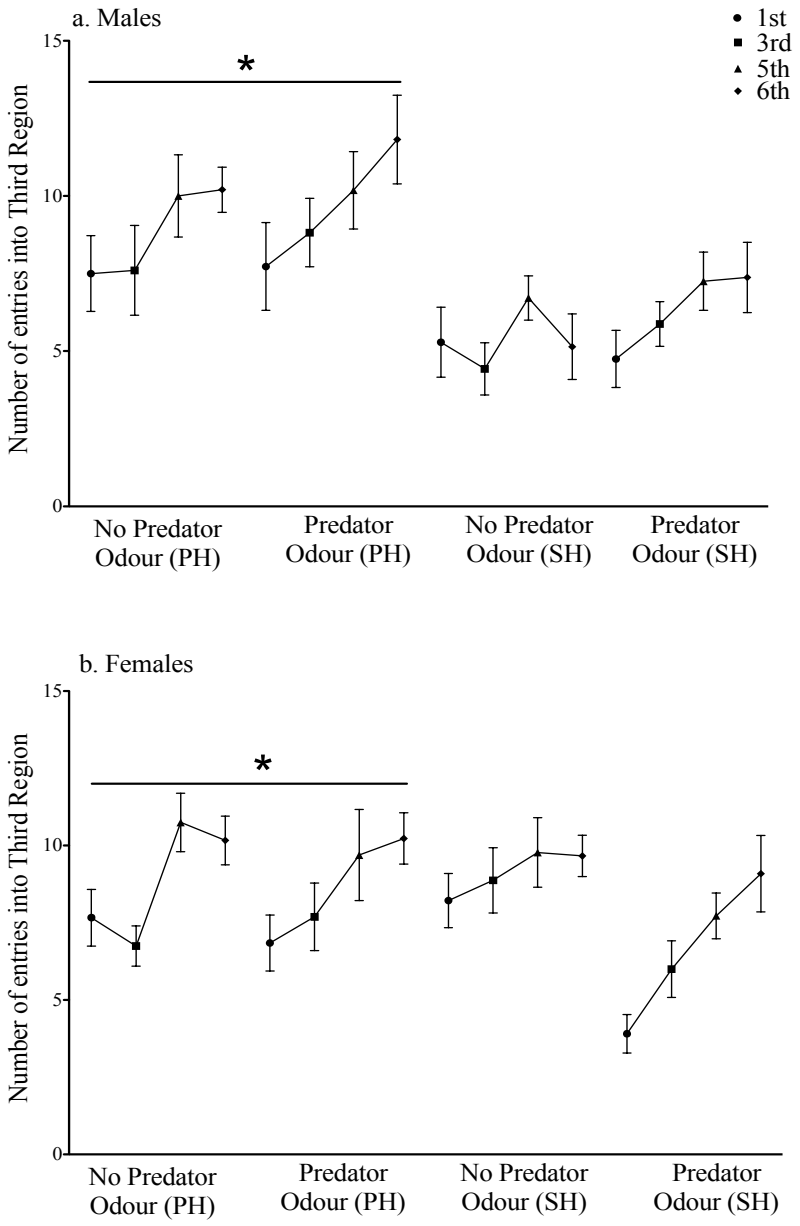


Figure AB.5. <u>Odour exposure behaviour:</u> Mean (+/- SEM) number of entries into the Third Region (TR) of the arena for male (a) and female (b) rats.

Single housed (SH) males made fewer entries into the TR compared to pair housed (PH) males (Sex X Housing interaction). The number of entries also increased with subsequent exposures (1^{st}<5^{th}, 1^{st}<6^{th}, 3^{rd}<5^{th}, 3^{rd}<6^{th}). *("*" is significantly different from single housed animals, collapsed across sex, odour treatment and exposure period.)*

Table AB.1. Odour exposure behaviour: Mean (SEM) durations (sec) of collar investigations for different experimental treatments.

For each dependent measure, PH>SH. (*Note: "PH", "SH", "NO", "NPO" and "PO" refer to "Pair Housed", "Single Housed", "No Odour", "No Predator Odour", and "Predator Odour", respectively.)*

		Collar Investigation			
I. Duration of shortest bout of collar investigation					
Males		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	1.07 (0.56)	0.85 (0.27)	0.31 (0.02)	0.31 (0.03)
PH	**PO**	0.49 (0.16)	0.45 (0.09)	0.40 (0.04)	0.54 (0.22)
SH	**NPO**	0.16 (0.06)	0.34 (0.06)	0.29 (0.04)	0.24 (0.07)
SH	**PO**	0.41 (0.27)	0.53 (0.19)	0.37 (0.06)	0.25 (0.05)
Females		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	0.46 (0.06)	0.75 (0.29)	0.28 (0.03)	0.47 (0.12)
PH	**PO**	0.55 (0.21)	1.72 (1.21)	2.09 (1.74)	0.51 (0.10)
SH	**NPO**	0.36 (0.11)	0.21 (0.03)	0.27 (0.04)	0.27 (0.01)
SH	**PO**	0.50 (0.29)	0.32 (0.09)	0.34 (0.03)	0.34 (0.03)
II. Duration of longest bout of collar investigation					
Males		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	7.37 (1.98)	9.79 (1.92)	5.24 (1.34)	8.31 (1.65)
PH	**PO**	7.40 (2.39)	4.05 (1.20)	4.46 (0.92)	4.78 (0.69)
SH	**NPO**	4.52 (2.08)	3.23 (1.43)	7.05 (1.52)	2.93 (1.11)
SH	**PO**	3.83 (2.25)	4.61 (1.54)	3.78 (0.68)	3.30 (0.87)
Females		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	7.52 (1.38)	3.97 (0.50)	4.06 (0.69)	6.78 (1.71)
PH	**PO**	4.37 (1.18)	3.91 (1.10)	6.01 (1.67)	5.18 (0.83)
SH	**NPO**	7.93 (1.93)	5.20 (1.52)	4.10 (0.90)	4.88 (2.40)
SH	**PO**	3.64 (1.72)	4.35 (1.23)	3.51 (0.63)	4.29 (1.07)
III. Duration of an average bout of collar investigation					
Males		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	3.12 (1.21)	3.42 (0.72)	1.66 (0.32)	2.27 (0.32)
PH	**PO**	2.52 (0.62)	1.41 (0.19)	1.48 (0.15)	2.13 (0.39)
SH	**NPO**	1.08 (0.42)	1.15 (0.25)	1.86 (0.29)	1.10 (0.36)
SH	**PO**	1.27 (0.64)	1.93 (0.46)	1.56(0.27)	1.01 (0.17)
Females		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	2.71 (0.55)	1.93 (0.28)	1.49 (0.25)	2.07 (0.46)
PH	**PO**	2.21 (0.83)	2.44 (1.16)	3.27 (1.66)	1.71 (0.22)
SH	**NPO**	1.95 (0.43)	1.25 (0.25)	1.26 (0.22)	1.28 (0.35)
SH	**PO**	1.30 (0.43)	1.36 (0.27)	1.30 (0.16)	1.28 (0.21)

Table AB.2. Odour exposure behaviour: Mean (+/- SEM) duration (sec) spent grooming for different experimental treatments.

A Main Effect of Odour Treatment was observed for the duration of the longest grooming bout (NPO > PO). *(Note: "PH" and "SH" refer to "Pair Housed" and "Single Housed" respectively. Also, "NPO", "PO" refer to "No Predator Odour" and "Predator Odour" respectively).*

I. Duration of shortest bout of grooming

Males		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	1.69 (0.82)	0.86 (0.26)	3.21 (1.04)	2.27 (0.62)
PH	**PO**	4.26 (2.10)	2.71 (0.85)	7.53 (6.49)	1.60 (0.37)
SH	**NPO**	0.67 (0.25)	0.98 (0.23)	2.04 (0.89)	1.13 (0.35)
SH	**PO**	0.17 (0.17)	2.14 (0.70)	1.34 (0.38)	1.06 (0.28)
Females		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	1.03 (0.17)	1.75 (0.33)	1.23 (0.38)	1.26 (0.46)
PH	**PO**	7.67 (4.37)	1.43 (0.41)	1.78 (0.72)	1.79 (0.48)
SH	**NPO**	1.67 (0.50)	0.86 (0.32)	2.19 (0.96)	0.88 (0.30)
SH	**PO**	0.07 (0.07)	8.99 (7.29)	3.42 (1.27)	2.49 (0.97)

II. Duration of longest bout of grooming

Males		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	16.40 (3.47)	18.97 (6.23)	22.12 (2.90)	34.01 (11.27)
PH	**PO**	13.26 (5.14)	17.33 (5.81)	23.40 (5.91)	19.98 (5.91)
SH	**NPO**	20.93 (7.80)	39.11 (6.07)	37.92 (11.22)	25.33 (5.51)
SH	**PO**	5.73 (5.73)	19.55 (6.58)	17.53 (6.11)	12.75 (3.33)
Females		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	25.96 (5.73)	23.00 (5.23)	27.60 (7.18)	27.73 (5.51)
PH	**PO**	18.06 (5.21)	23.20 (5.50)	19.70 (4.64)	10.43 (1.91)
SH	**NPO**	23.94 (6.18)	19.31 (5.16)	21.41 (4.66)	23.33 (10.05)
SH	**PO**	0.80 (0.80)	19.24 (7.44)	18.72 (4.24)	21.40 (4.31)

Table AB.3. Odour exposure behaviour: Mean (+/- SEM) duration spent (sec) and latency (sec) to begin grooming for different experimental treatments.
A Main Effect of Exposure Period (1^{st}<3^{rd}, 1^{st}<5^{th}), and of Odour Treatment (NPO<PO) was observed for the latency to begin grooming. Additionally, during the 1^{st} exposure, latency to groom showed an effect of Odour Treatment on SH animals (NPO>PO), and an effect of Housing on PO animals (PH>SH). Finally, an effect to Exposure Period was seen in the animals exposed to both SH and PO (1^{st}< 3^{rd}, 1^{st}< 5^{th}, 1^{st}< 6^{th}) (Exposure Period X Housing X Odour Treatment interaction).
(Note: "PH" and "SH" refer to "Pair Housed" and "Single Housed" respectively. Also, "NPO", "PO" refer to "No Predator Odour" and "Predator Odour" respectively).

I. Duration of an average bout of grooming

Males		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	8.28 (1.86)	6.26 (1.71)	9.69 (1.14)	10.12 (1.62)
PH	**PO**	8.46 (2.97)	8.45 (2.33)	13.56 (6.03)	8.34 (1.79)
SH	**NPO**	6.41 (2.31)	12.23 (2.10)	12.92 (2.65)	10.21 (2.22)
SH	**PO**	2.22 (2.22)	8.80 (2.56)	6.90 (1.82)	5.31 (1.04)
Females		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	8.42 (1.48)	8.74 (1.87)	8.80 (1.63)	8.36 (1.54)
PH	**PO**	11.04 (4.18)	7.62 (1.77)	7.97 (1.88)	5.13 (0.90)
SH	**NPO**	9.11 (2.21)	6.50 (1.76)	8.44 (1.44)	6.65 (1.92)
SH	**PO**	0.37 (0.37)	12.96 (7.06)	9.61 (1.64)	9.70 (2.43)

I1. Latency to begin grooming

Males		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	65.57 (14.85)	80.90 (34.97)	97.51 (20.65)	75.87 (13.62)
PH	**PO**	153.64 (47.25)	116.30 (30.80)	121.75 (27.59)	97.54 (15.42)
SH	**NPO**	88.19 (36.85)	120.72 (23.69)	130.76 (30.39)	129.03 (23.68)
SH	**PO**	5.26 (5.26)	129.86 (46.83)	232.36 (52.86)	132.81 (40.76)
Females		**1st**	**3rd**	**5th**	**6th**
PH	**NPO**	66.53 (14.87)	60.56 (13.95)	90.22 (19.88)	109.29 (20.86)
PH	**PO**	167.97 (37.38)	116.60 (23.35)	125.07 (26.11)	148.22 (31.76)
SH	**NPO**	145.16 (34.63)	128.70 (42.19)	166.47 (22.36)	100.34 (29.12)
SH	**PO**	2.69 (2.69)	124.83 (33.22)	187.41 (24.83)	151.28 (26.21)

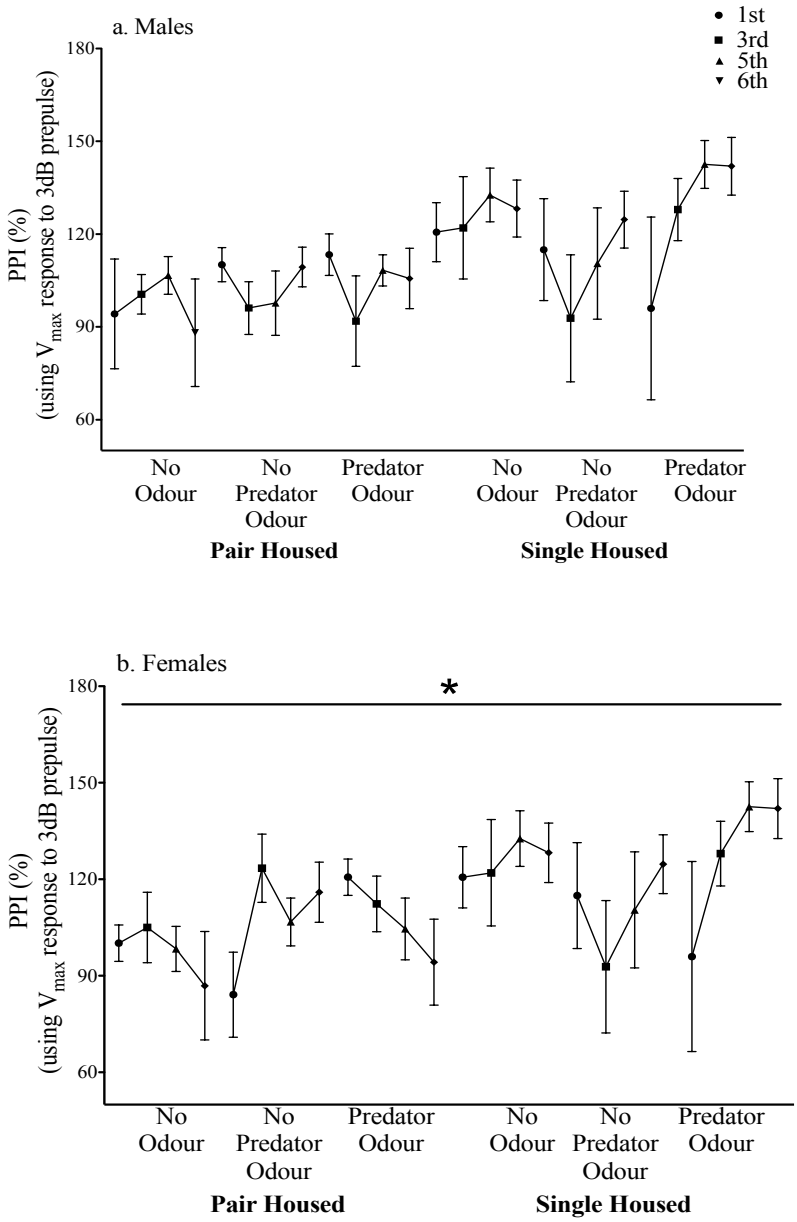


Figure AB.6. <u>Prepulse inhibition of startle:</u> Mean (+/- SEM) percent prepulse inhibition (PPI) at 3dB for males (a) and females (b).

Females showed a greater PPI than males (main effect of Sex). *("*" is significantly different from males.)*

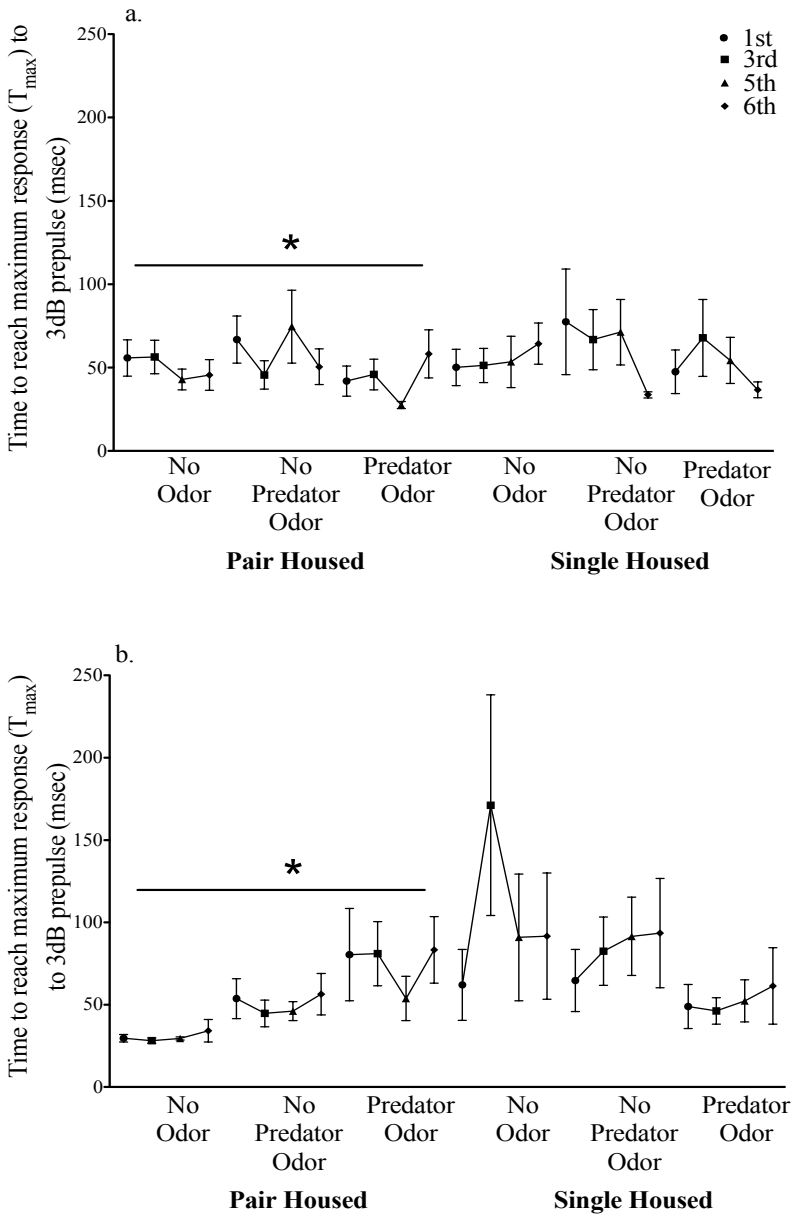


Figure AB.7. <u>Prepulse inhibition of startle:</u> Mean (+/- SEM) time to reach maximum response to 3dB prepulse trials for males (a) and females (b).

Housing had a significant effect on this dependent variable (Pair Housed< Single Housed). *("*" is significantly different from single housed animals, collapsed across sex, odour treatment and exposure period.)*

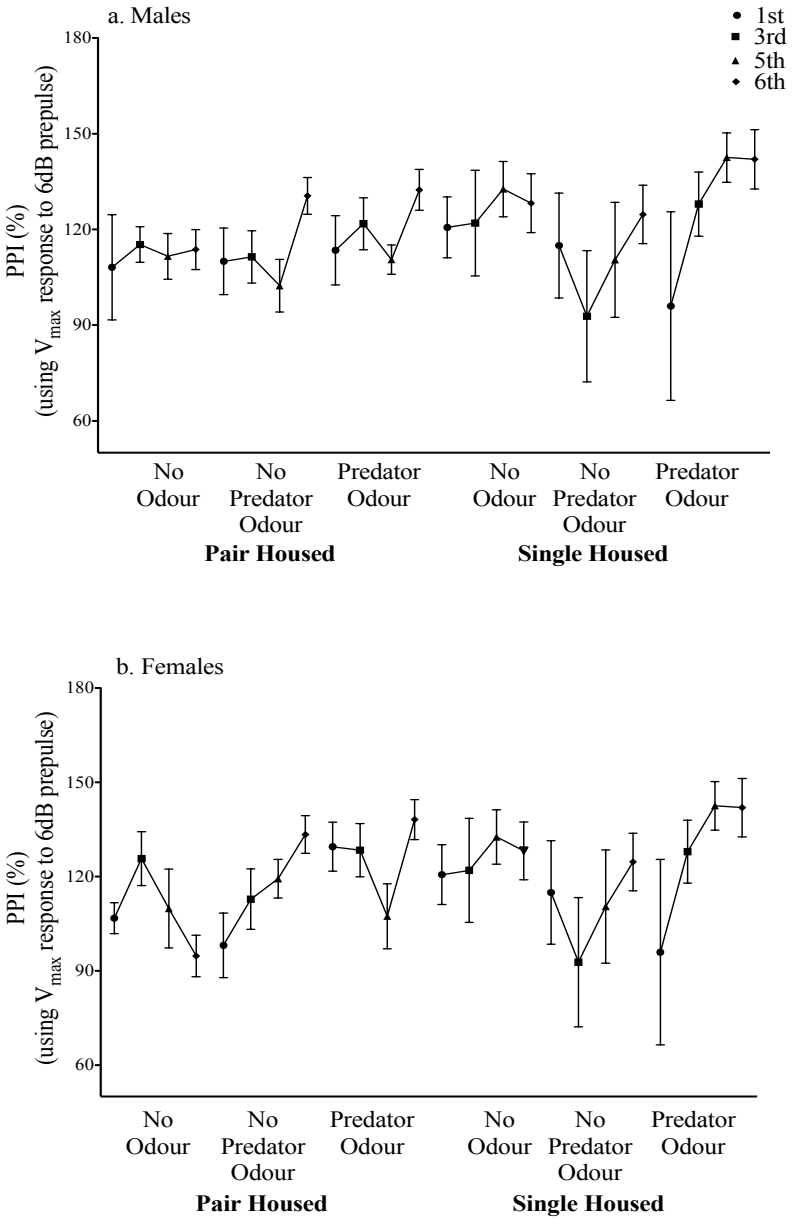


Figure AB.8. <u>Prepulse inhibition of startle:</u> Mean (+/- SEM) percent prepulse inhibition (PPI) at 6dB for males (a) and females.

Exposure Period had a main effect on this measure ($1^{st} < 6^{th}$ $5^{th} < 6^{th}$).

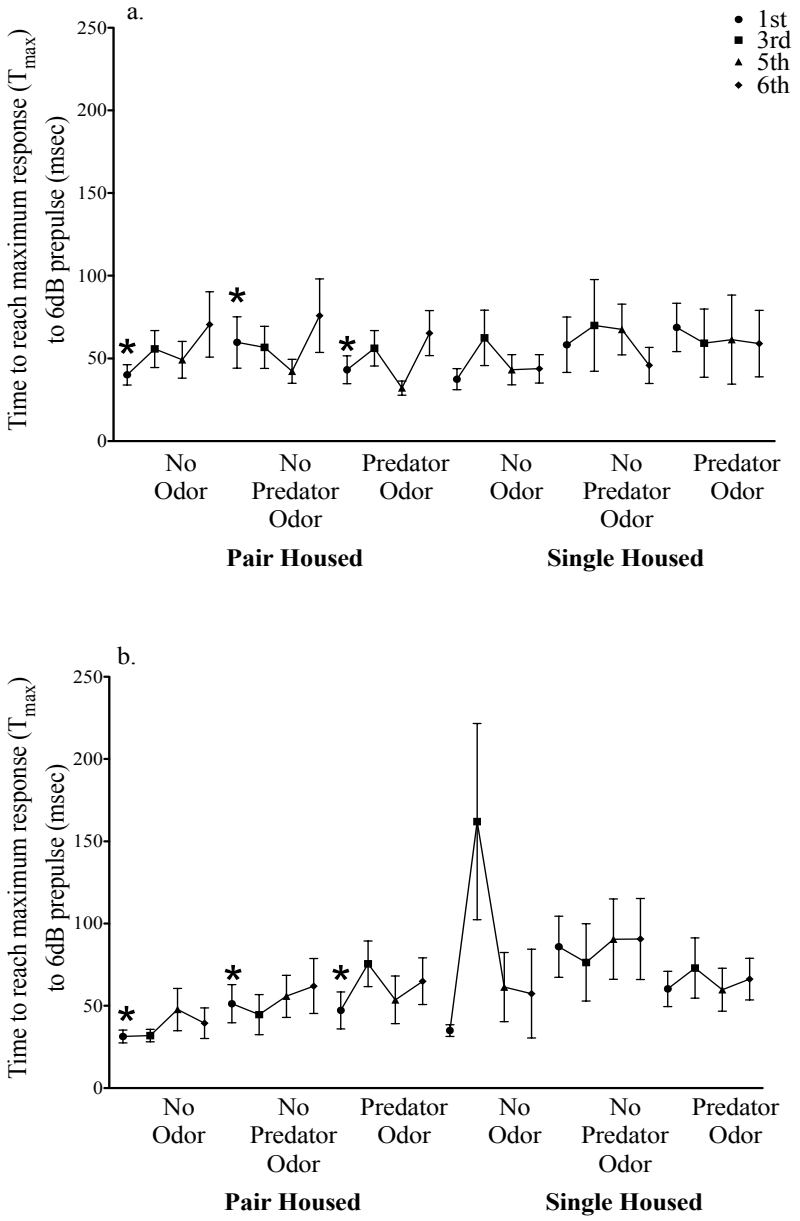


Figure AB.9. <u>Prepulse inhibition of startle:</u> Mean (+/- SEM) time to reach maximum response to 6dB prepulse trials for males (a) and females (b).

An interaction between Exposure Period and Housing was found; simple effects analyses revealed an effect of Exposure Period on Pair Housed animals (1^{st} <6^{th}). *("*" is significantly different from the 6^{th} exposure period animals of the same housing condition, collapsed across sex and odour treatment.)*

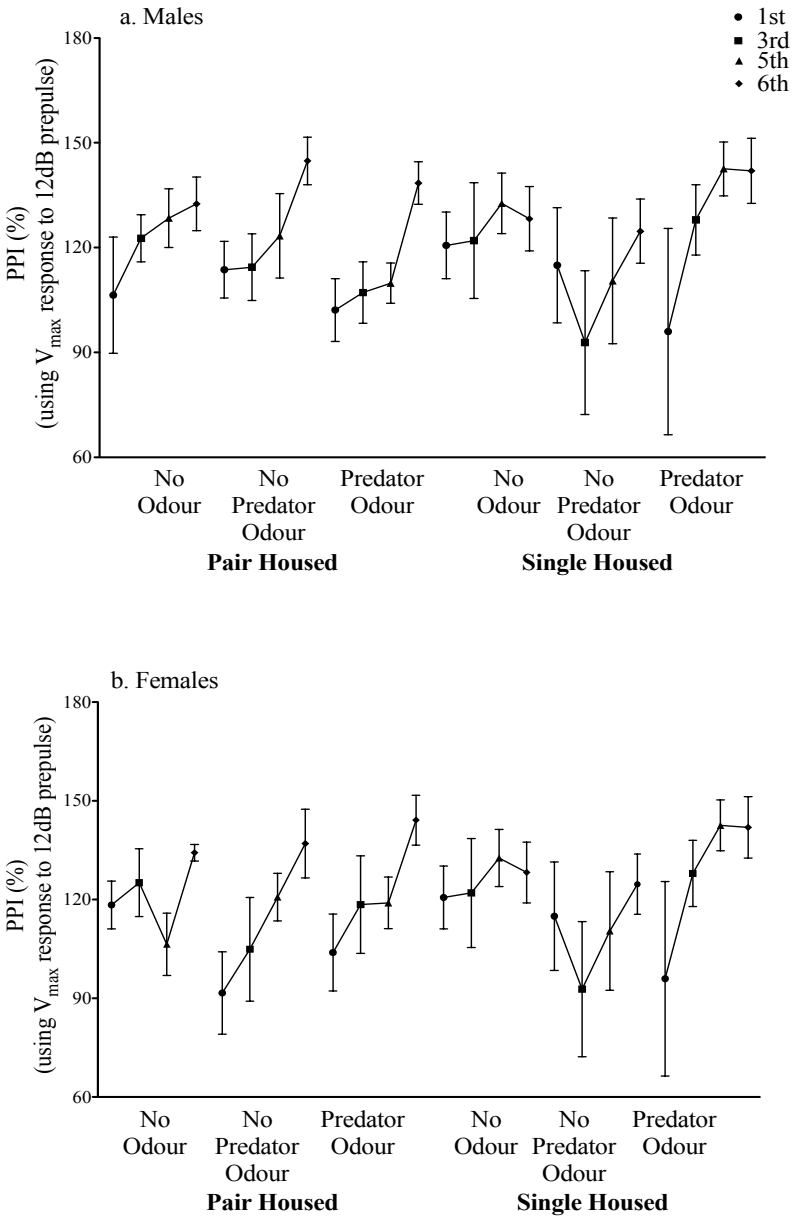


Figure AB.10. <u>Prepulse inhibition of startle:</u> Mean (+/- SEM) percent prepulse inhibition (PPI) at12dB for males (a) and females (b).

Exposure Period had a main effect on this measure (1^{st} <5^{th}, 1^{st}<6^{th}, 3^{rd}<6^{th}, 5^{th}<6^{th}).

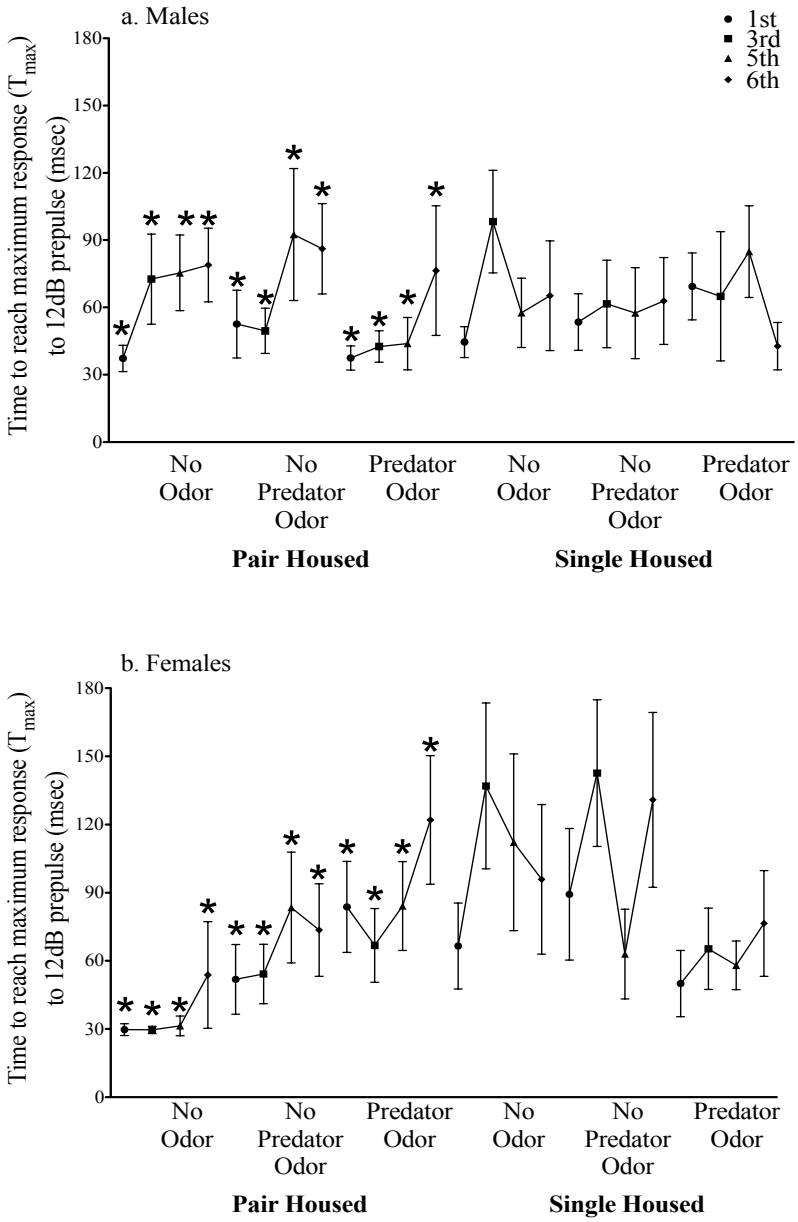


Figure AB.11. <u>Prepulse inhibition of startle:</u> Mean (+/- SEM) time to reach maximum response to 12dB prepulse trials for males (a) and females (b).

An interaction between Exposure Period and Housing was found on this measure; simple effects analyses revealed an effect of Exposure Period on Pair Housed ($1^{st} < 6^{th}$) and Single Housed animals ($1^{st} < 3^{rd}$), and an effect of Housing at each exposure period (PH< SH). *("*" is significantly different from single housed animals at the same exposure period, collapsed across sex, and odour treatment.)*

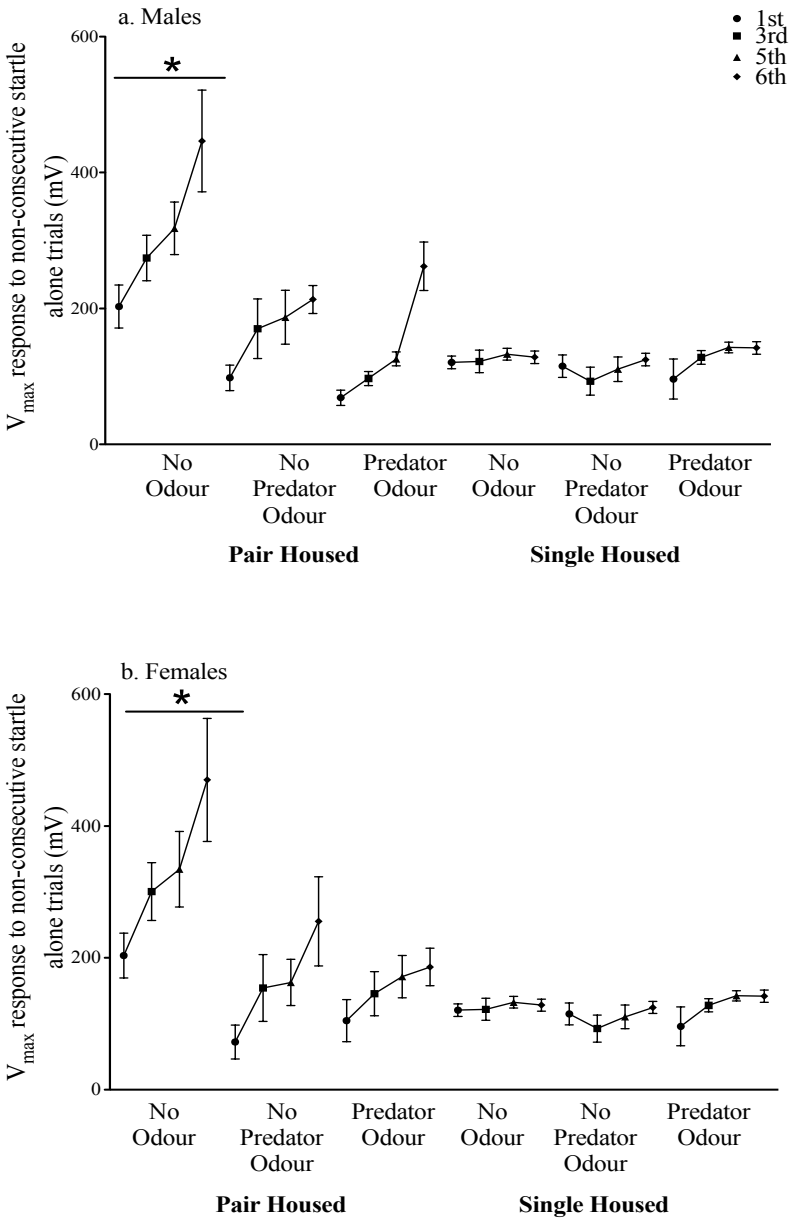


Figure AB.12. <u>Acoustic startle response</u>: Mean (+/- SEM) response to the non-consecutive startle alone trials for males (a) and females (b).

A significant interaction between Housing and Odour Treatment was found; simple effects analyses revealed an effect of Odour Treatment on PH animals (NO > NPO, NO > PO) only. Additionally, an effect of Exposure Period was seen at each Odour Treatment, and an effect of Odour Treatment after the 1st, 3rd, and 6th exposure periods (in each case, NO > NPO, NO > PO) (Exposure Period X Odour Treatment interaction). Finally, an effect of Exposure Period was seen on PH and SH animals (Exposure X Housing interaction) (both described in detail in chapter 3). *("*" is significantly different from no*

predator odour and predator odour groups of the same housing condition, collapsed across sex.)
(*Note: "PH", "SH", "NO", "NPO" and "PO" refer to "Pair Housed", "Single Housed", "No Odour", "No Predator Odour", and "Predator Odour", respectively.)*

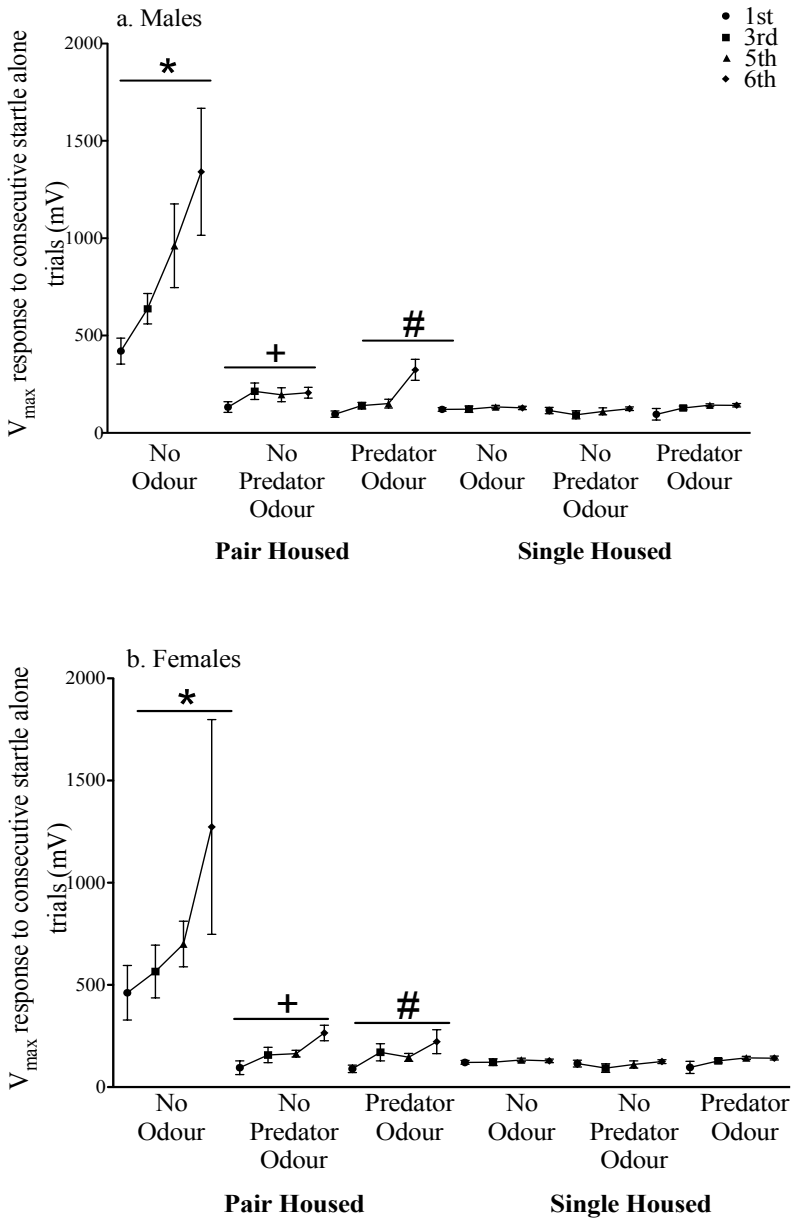


Figure AB.13. <u>Acoustic startle response</u>: Mean (+/- SEM) response to the consecutive startle alone trials for males (a) and females (b).

This figure shows some key effects of the various factors on the startle response. An effect of Odour Treatment was seen on pair housed animals (NO> NPO, NO> PO), and an effect of Housing on NPO and PO groups (in each case, pair housed < single housed) (Housing X Odour Treatment interaction). Additionally, interaction effects were seen between Exposure Period and Odour Treatment, and Exposure Period and Housing (both described in detail in chapter 3). *("*" is significantly different from no predator odour and predator odour groups of the same housing condition, collapsed across sex, whereas "+" and "#" are significantly different from single housed animals of the same odour treatment, collapsed across sex and exposure period.)*

(*Note: "NO", "NPO" and "PO" refer to "No Odour", "No Predator Odour", and "Predator Odour", respectively.)*

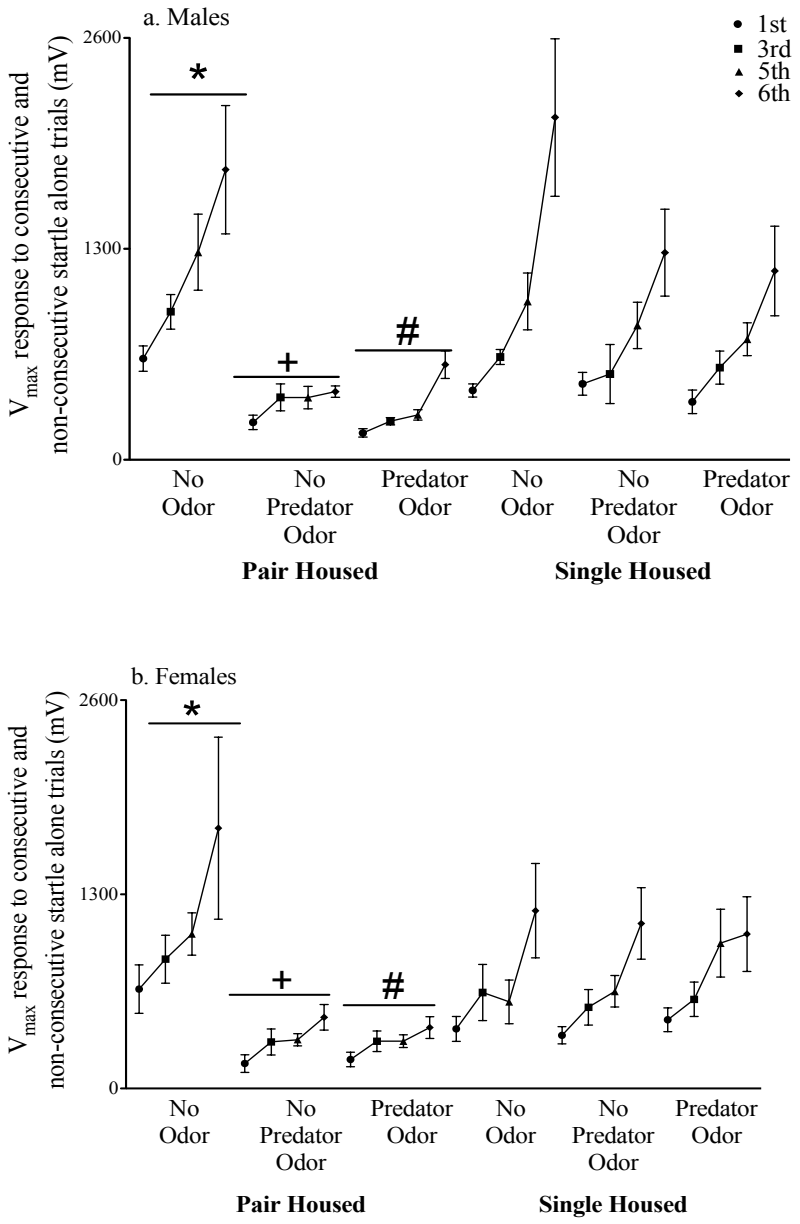


Figure AB.14. <u>Acoustic startle response</u>: Mean (+/- SEM) response to the non-consecutive and consecutive startle alone trials for males (a) and females (b).

This figure shows some key effects of the various factors on the startle response. An effect of Odour Treatment was seen on pair housed animals (NO> NPO, NO> PO), and an effect of Housing on NPO and PO groups (in each case, pair housed < single housed) (Housing X Odour Treatment interaction). Additionally, an interaction effect was seen between Exposure Period and Odour Treatment (described in chapter 3). *("*" is significantly different from no predator odour and predator odour groups of the same housing condition, collapsed across sex, whereas "+" and "#" are significantly different from single housed animals of the same odour treatment, collapsed across sex and exposure period.)*

(*Note: "PH", "SH", "NO", "NPO" and "PO" refer to "Pair Housed", "Single Housed", "No Odour", "No Predator Odour", and "Predator Odour", respectively.)*

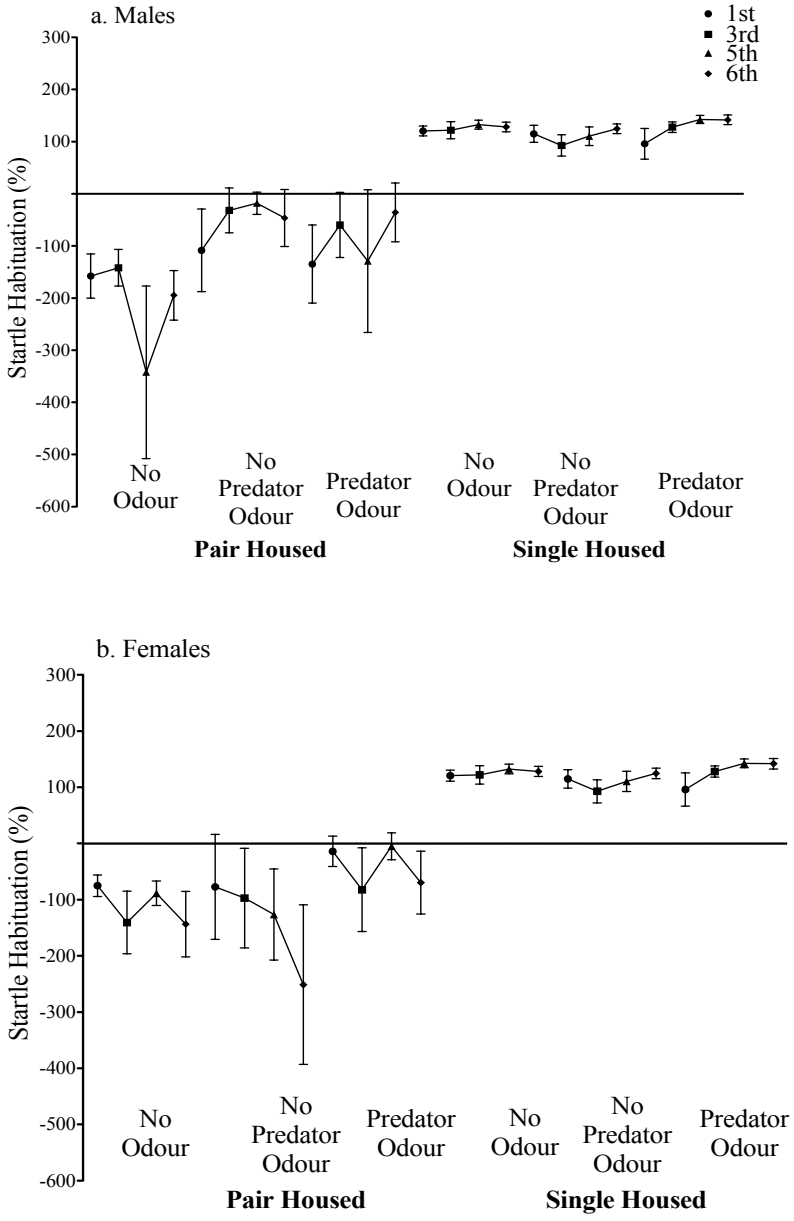


Figure. AB.15. <u>Acoustic startle habituation</u>: Mean (+/- SEM) startle habituation in males (a) and females (b).

None of the factors had any effect on this dependent measure.

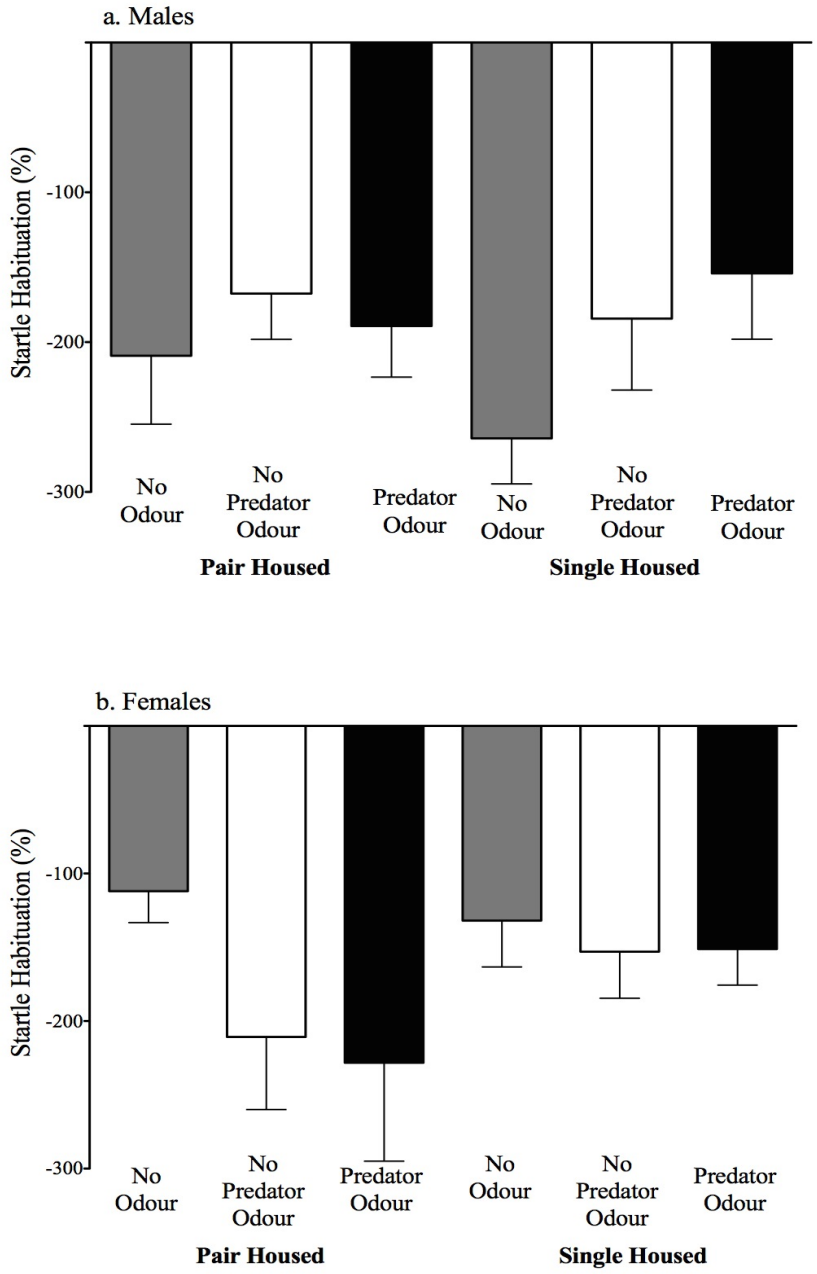


Figure AB.16. <u>Acoustic startle habituation</u>: Mean (+/- SEM) startle habituation (data collapsed across Exposure Period) in males (a) and females (b).

None of the factors had any effect on this dependent measure.

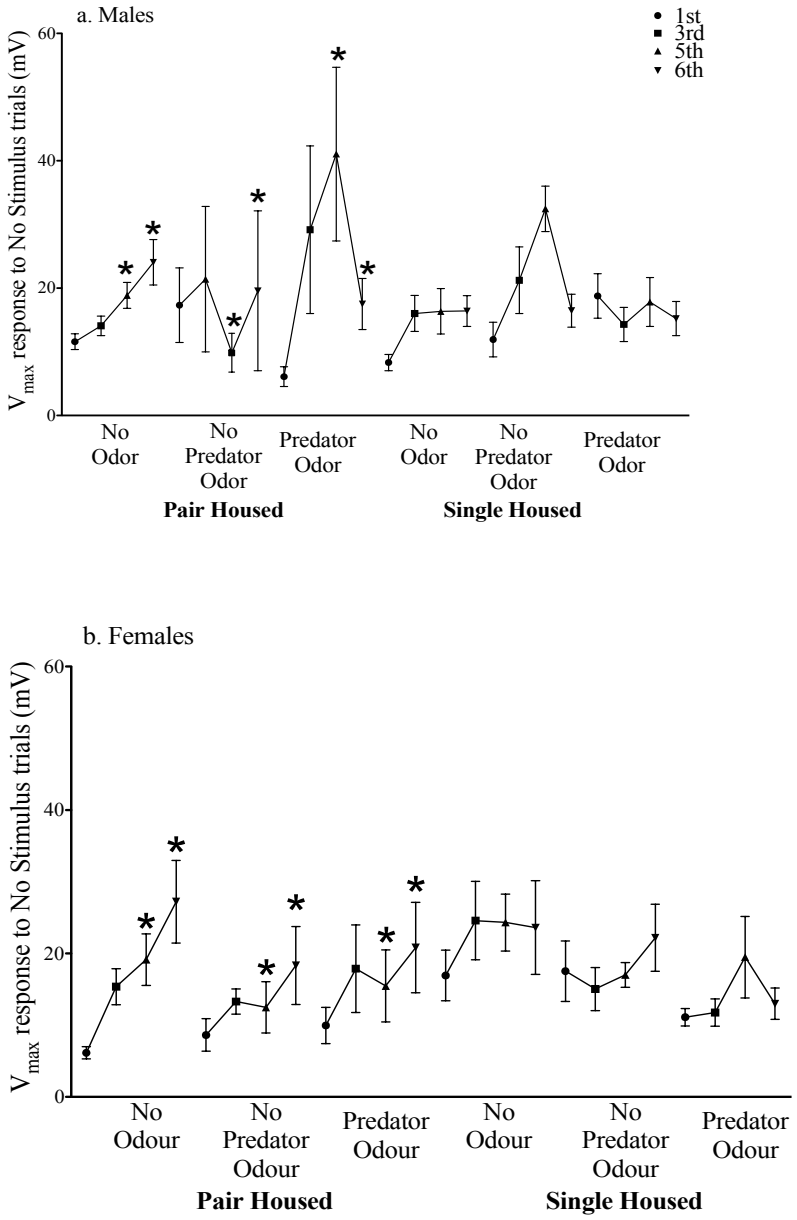


Figure AB.17. <u>No stimulus response</u>: Mean (+/- SEM) response to No Stimulus trials in males (a) and females (b).

Housing and Exposure Period had an Interaction Effect on this dependent variable. Simple effects analyses revealed that single housed animals had a significantly lower response to no stimulus trials than pair housed ones after the 5^{th} and 6^{th} exposure periods. Additionally, Exposure Period had a significant effect on pair housed animals (1^{st}<3^{rd}, 1^{st}<5^{th}, 1^{st}<6^{th}, 3^{rd}<5^{th}, 3^{rd}<6^{th}), and on single housed (1^{st}<5^{th}) ones. *("*" is significantly different from the single housed group at the same exposure period, collapsed across sec and odour treatment.)*

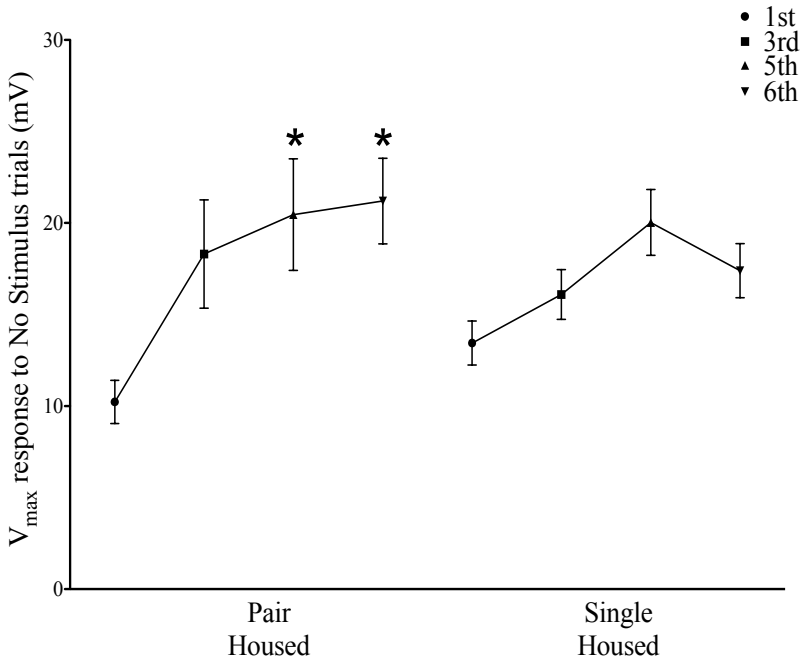


Figure AB.18. <u>No stimulus response</u>: Mean (+/- SEM) response to No Stimulus trials collapsed across Sex and Odour Treatment.

Housing and Exposure Period had an Interaction Effect. Simple effects analyses revealed that Pair Housed (PH) had a significantly higher response than Single Housed (SH) after the 5^{th} and 6^{th} exposure periods, and that Exposure Period had a significant effect on PH (($1^{st}<3^{rd}$, $1^{st}<5^{th}$, $1^{st}<6^{th}$), ($3^{rd}<5^{th}$, $3^{rd}<6^{th}$)), and SH ($1^{st}<5^{th}$) animals. *("*" is significantly different from the single housed group at the same exposure period, collapsed across sec and odour treatment.)*

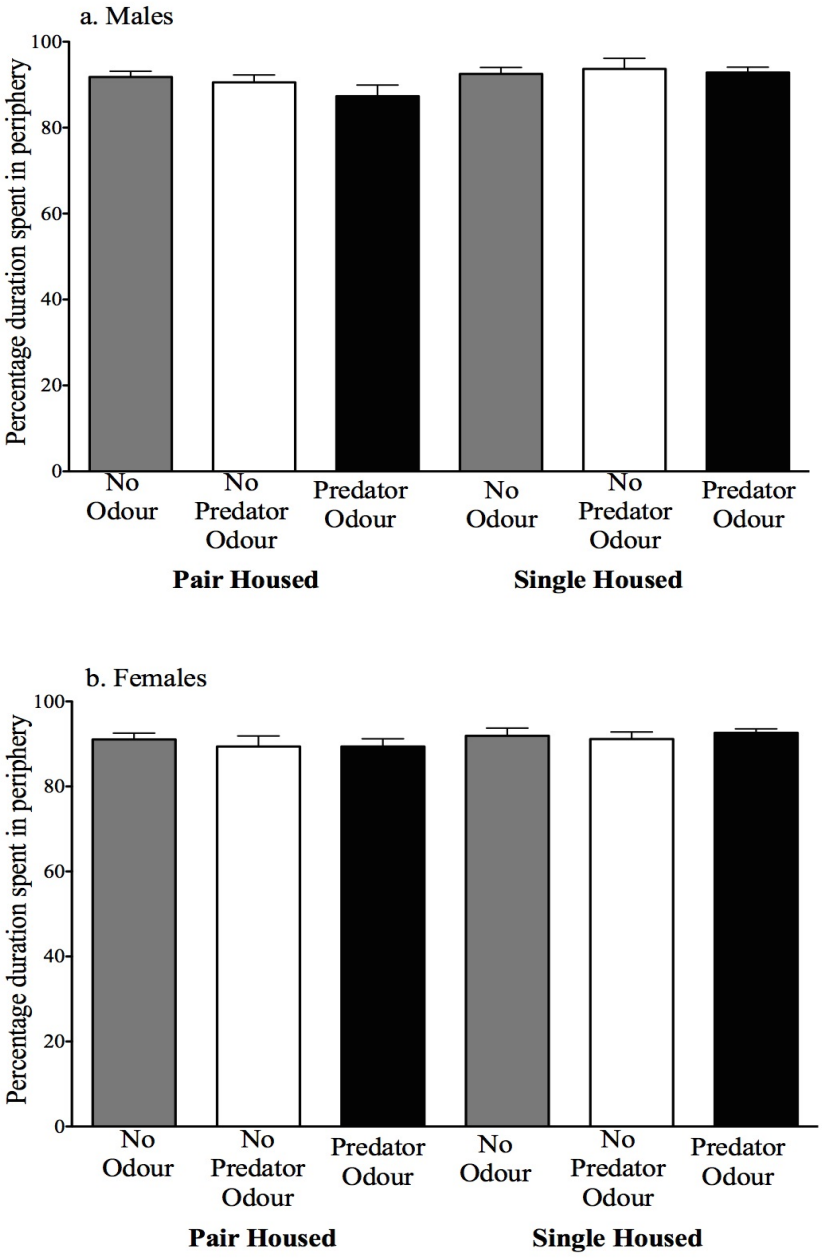


Figure AB.19. <u>Open field test:</u> Mean (+/- SEM) duration spent in the periphery during the Open Field Test in males (a) and females (b).

None of the factors had any effect on this dependent measure.

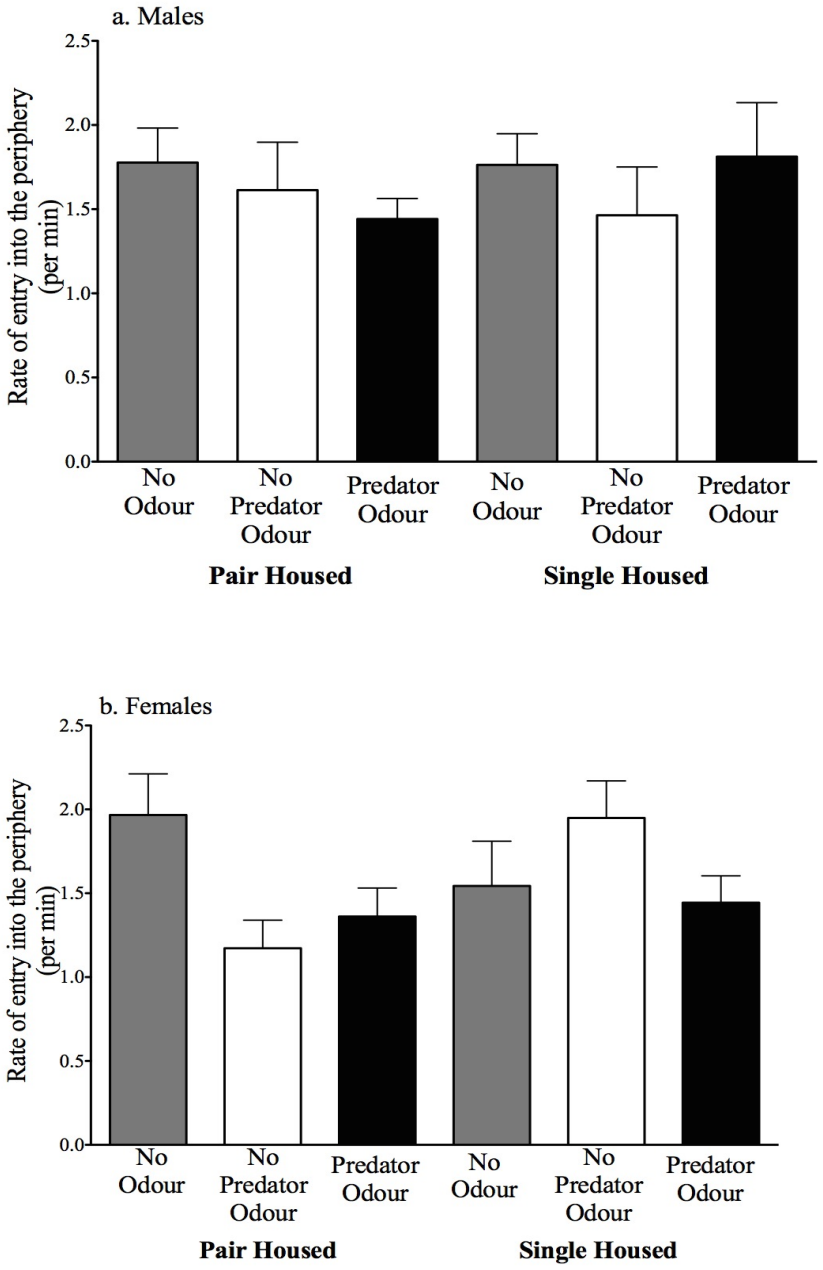


Figure AB.20. <u>Open field test:</u> Mean (+/- SEM) rate of entry into the periphery during the Open Field Test in males (a) and females (b).

None of the factors had any effect on this dependent measure.

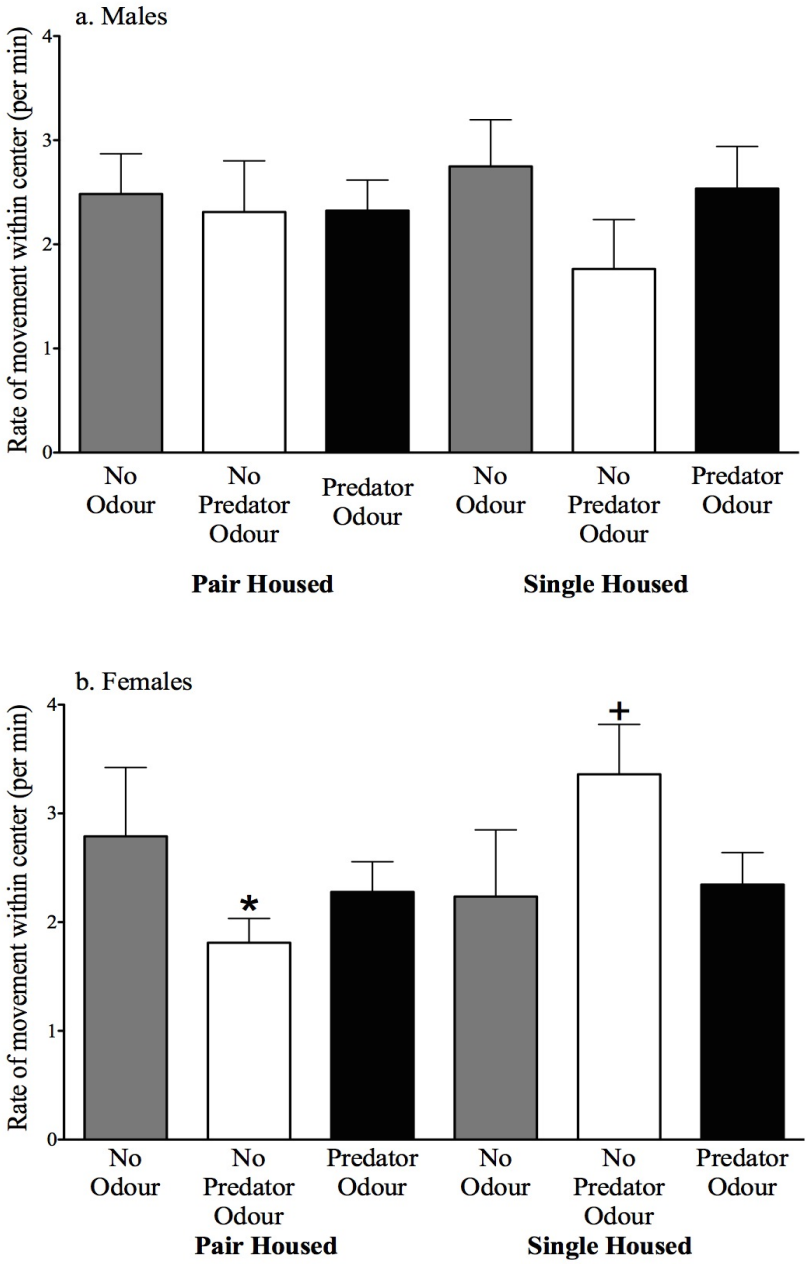


Figure AB.21. <u>Open field test:</u> Mean (+/- SEM) rate of movement within the center during the Open Field Test in males (a) and females (b).

There was a significant interaction of Sex, Housing, and Odour Treatment on rate of movement within the center of the open field. Simple effects analyses revealed an effect of Housing on NPO exposed females (PH< SH), as well as an effect of Sex for animals exposed to both SH and NPO (Male< Female). *("*" is significantly different from single housed animals of the same sex and odour treatment, whereas "+" is significantly different from males of the same housing and odour treatment.)*

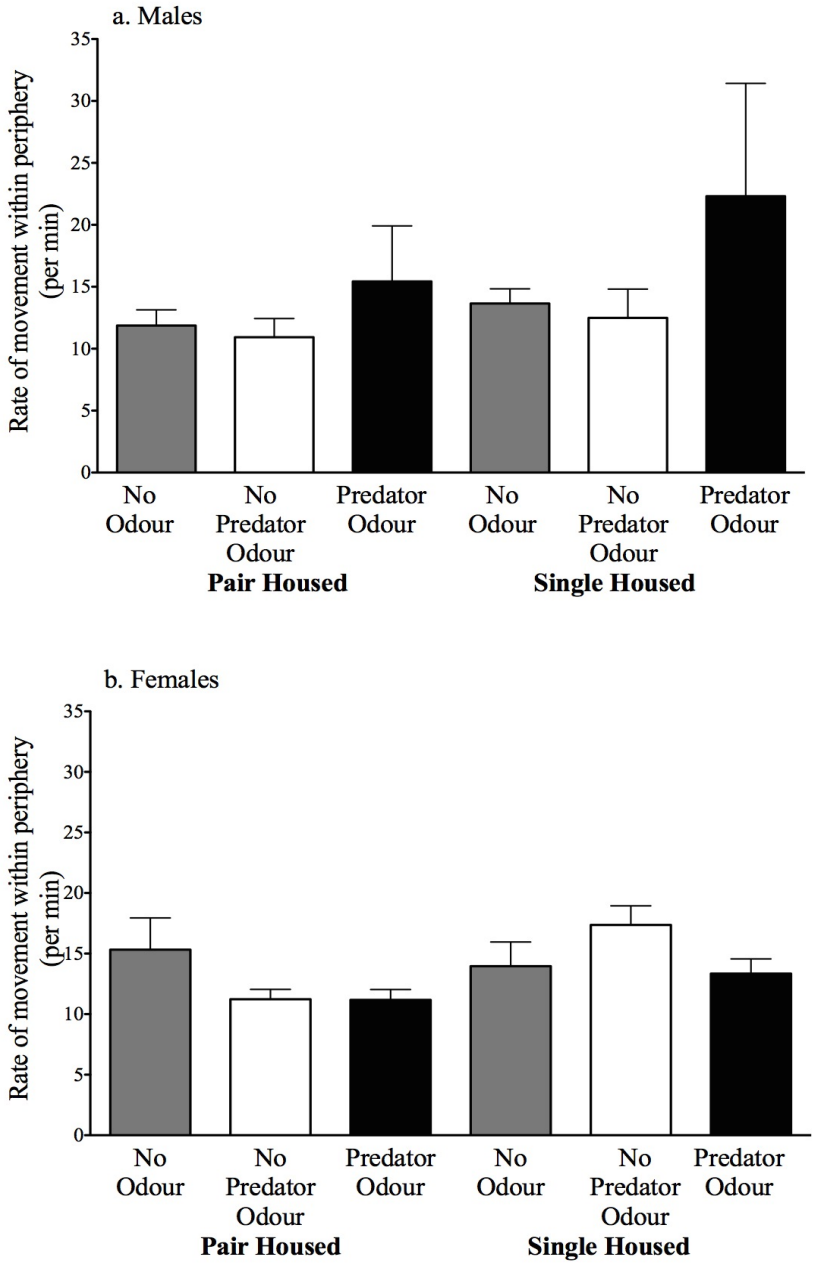


Figure AB.22. <u>Open field test:</u> Mean (+/- SEM) rate of movement within the periphery during the Open Field Test in males (a) and females (b).

None of the factors had any effect on this dependent measure.

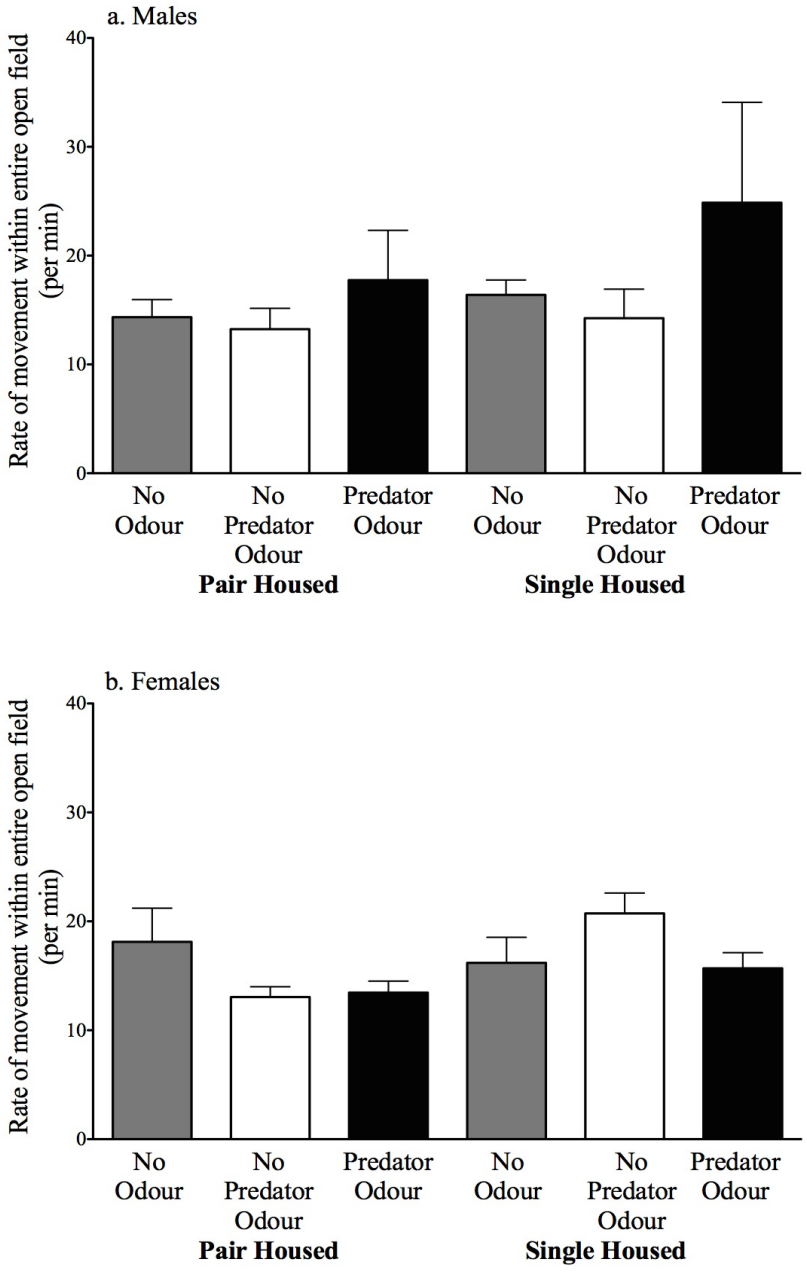


Figure AB.23. <u>Open field test:</u> Mean (+/- SEM) rate of movement within the entire open field during the Open Field Test in males (a) and females (b).

None of the factors had any effect on this dependent measure.

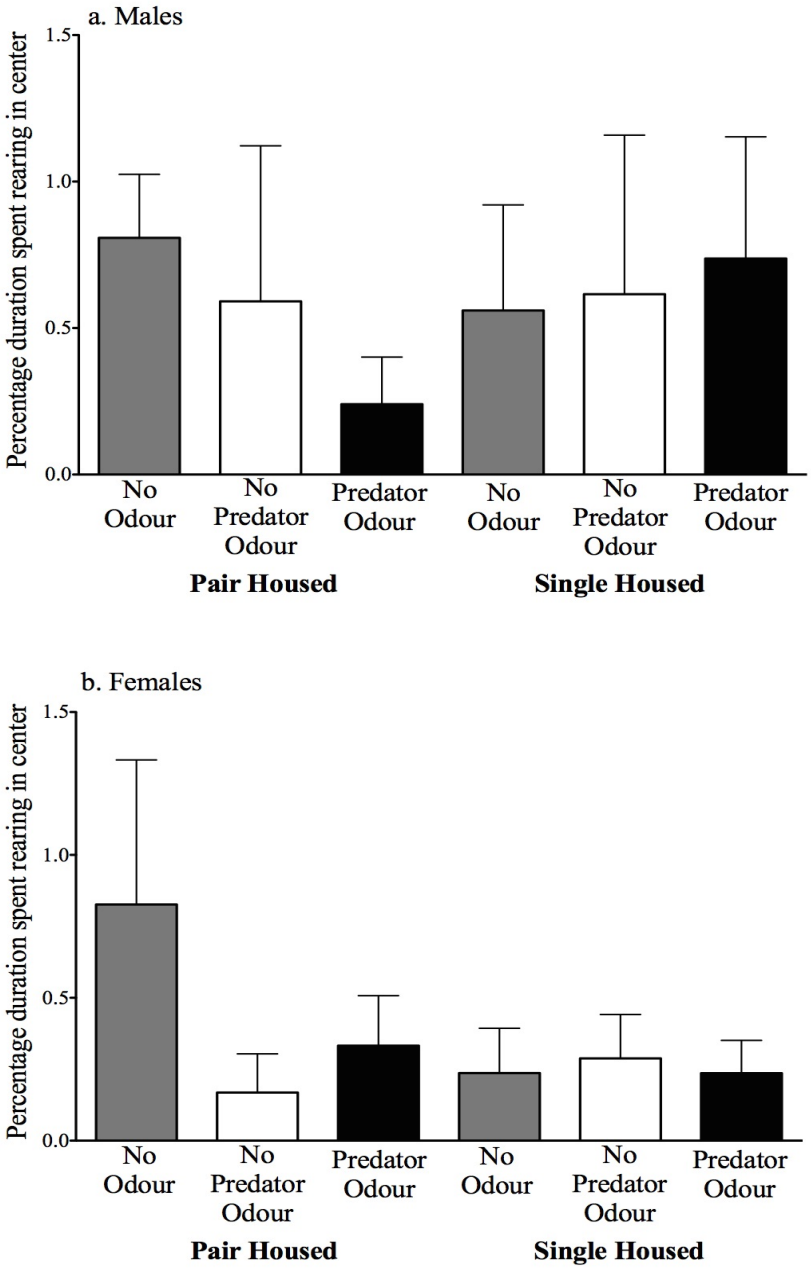


Figure AB.24. <u>Open field test:</u> Mean (+/- SEM) duration spent in rearing in the center of the open field by males (a) and females (b) during the Open Field Test.

None of the factors had any effect on this dependent measure.

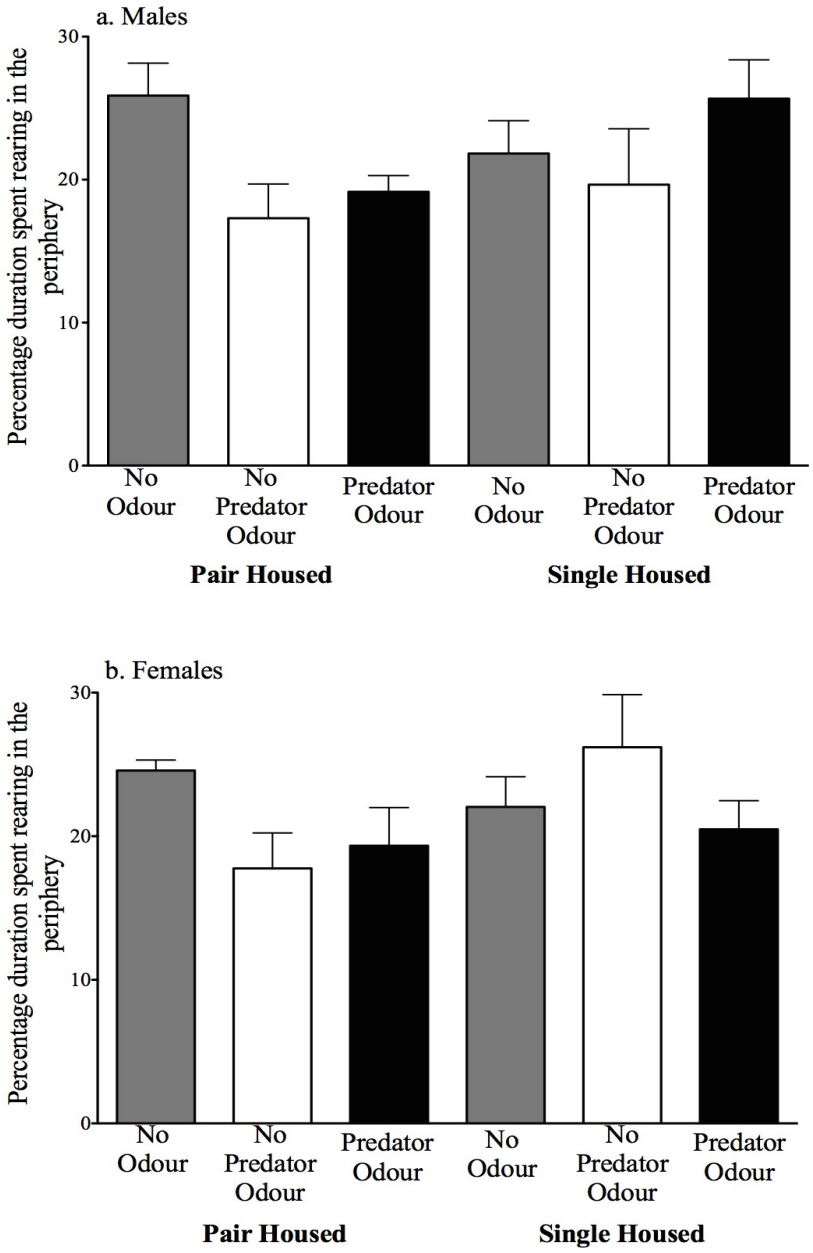


Figure AB.25. <u>Open field test:</u> Mean (+/- SEM) duration spent in rearing in the periphery of the open field by males (a) and females (b) during the Open Field Test.

None of the factors had any effect on this dependent variable.

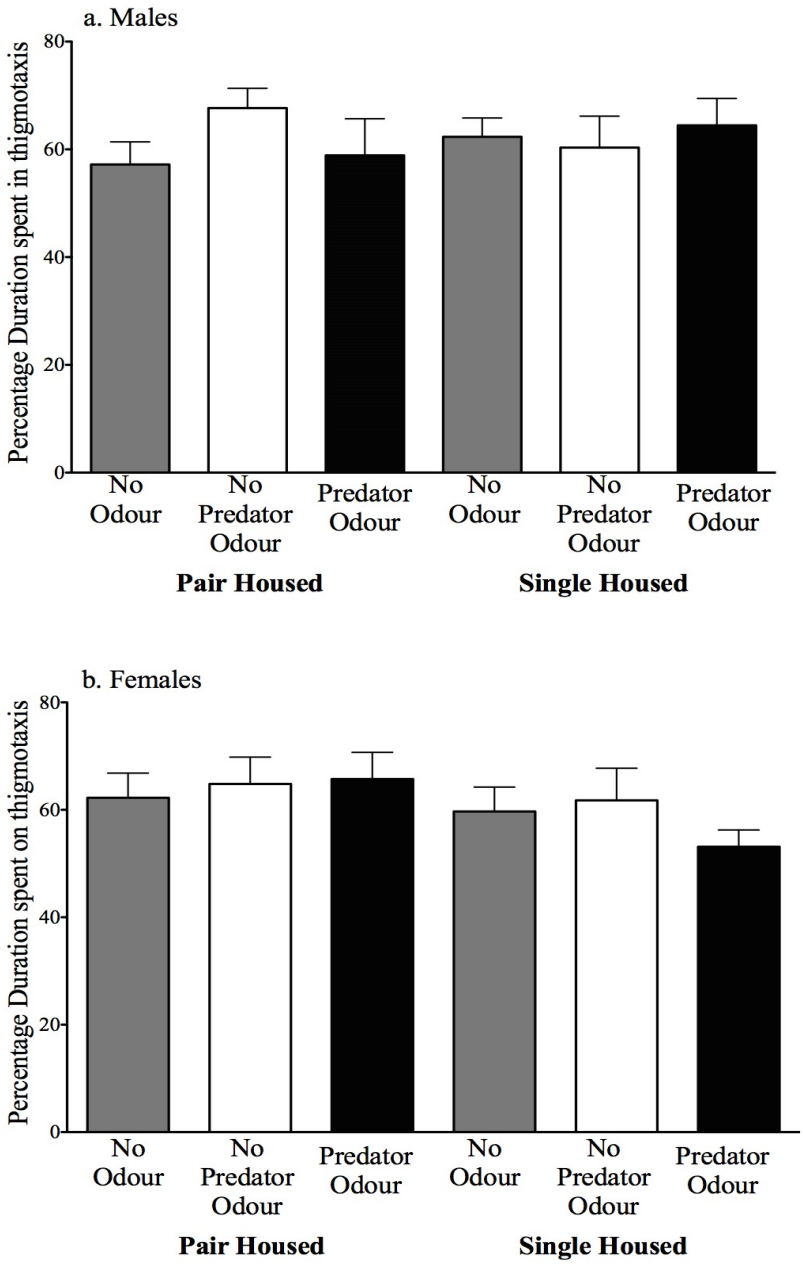


Figure AB.26. <u>Open field test:</u> Mean (+/- SEM) duration spent on thigmotaxis during the Open Field Test in males (a) and females (b).

None of the factors had any effect on this dependent measure.

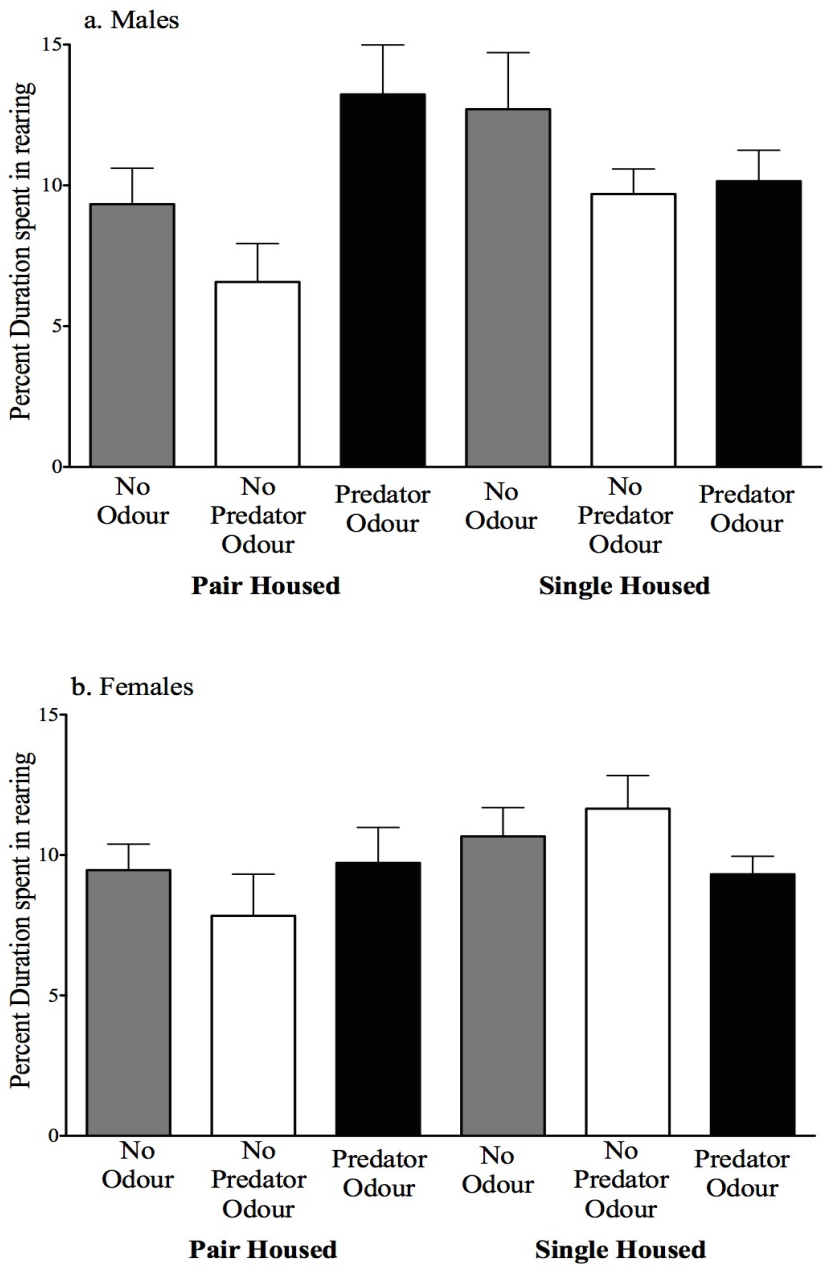


Figure AB.27. <u>Elevated plus maze:</u> Mean (+/- SEM) percent duration spent in rearing during the Elevated Plus Maze test by males (a) and females (b).

None of the factors had any effect on this dependent measure.

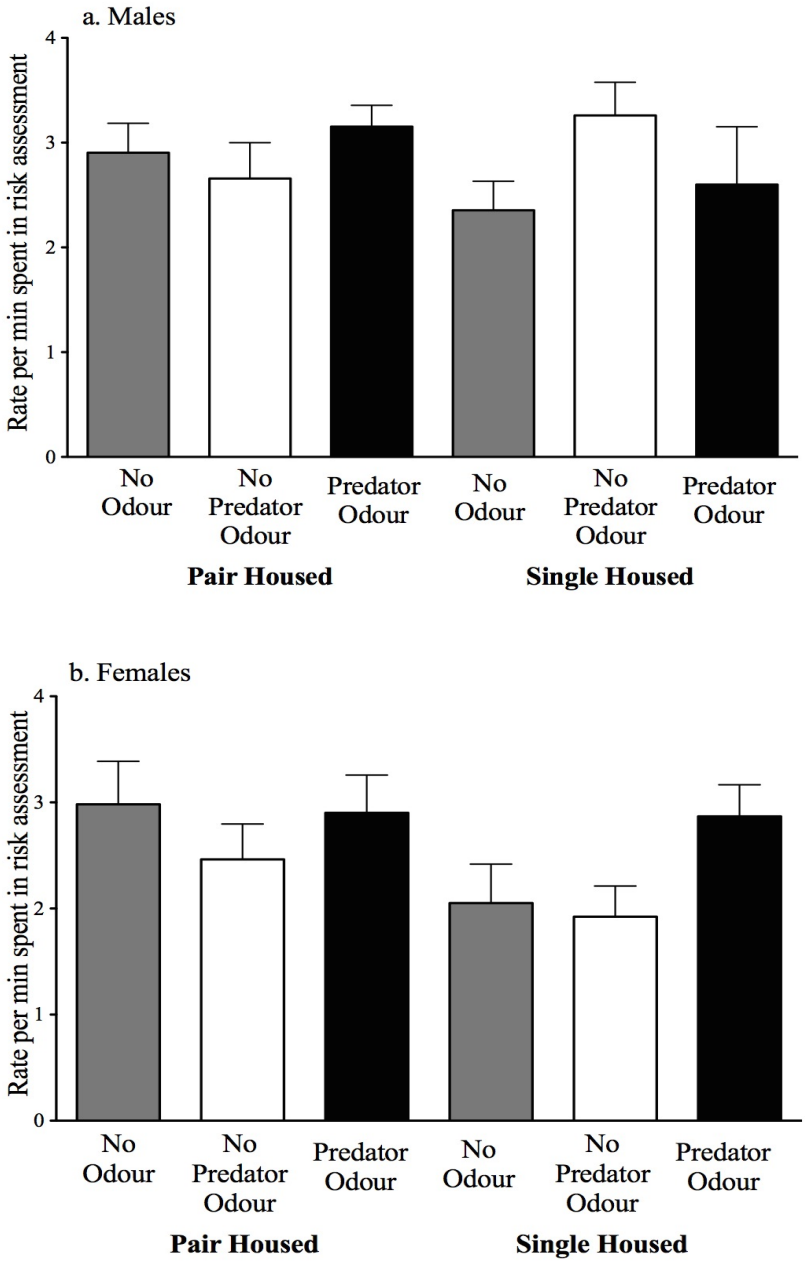


Figure AB.28. <u>Elevated plus maze:</u> Mean (+/- SEM) rate of risk assessment during the Elevated Plus Maze test by males (a) and females (b).

None of the factors had any effect on this dependent measure.

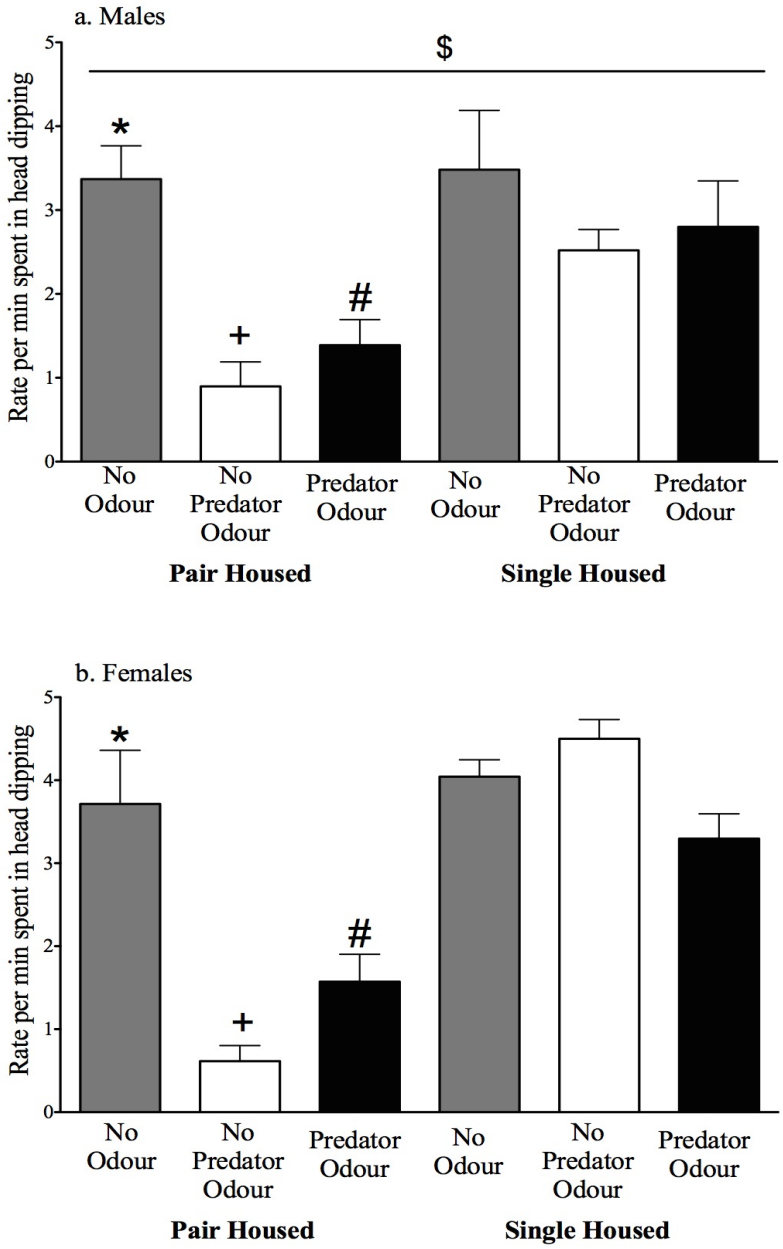


Figure AB.29. <u>Elevated plus maze:</u> Mean (+/- SEM) rate of head dipping during the Elevated Plus Maze test by males (a) and females (b).

An interaction effect of Housing and Odour Treatment was seen; simple effects revealed an effect of Odour Treatment on Pair Housed animals (No Odour> No Predator Odour, No Odour> Predator Odour), and an effect of Housing on No Predator Odour and Predator Odour groups (Pair Housed < Single Housed). This dependent measure was also higher in females than males. *("*" is significantly different from the no predator odour and predator odour treatments of the same housing condition, collapsed across sex. "+" and "#" are significantly different from the single housed groups of the same odour condition, collapsed across sex. "$" is significantly different from males.)*

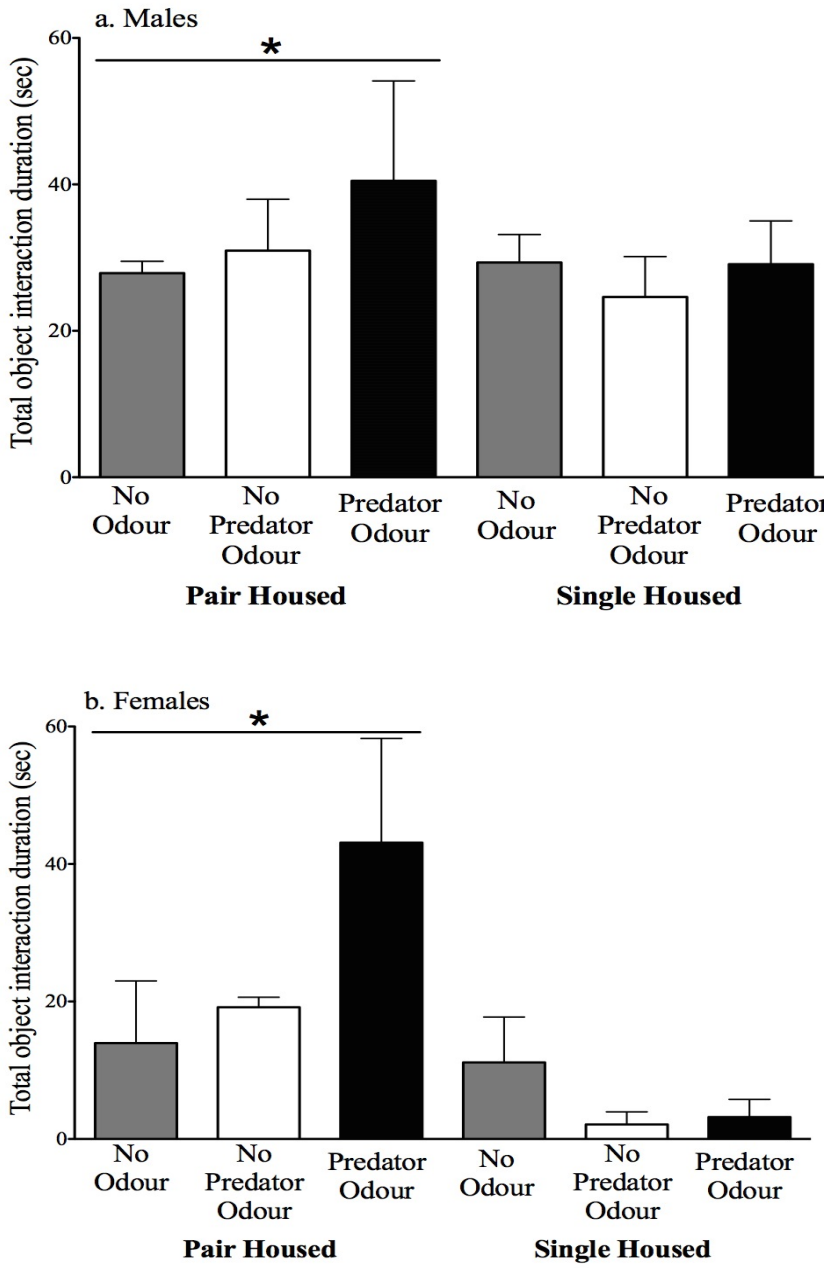
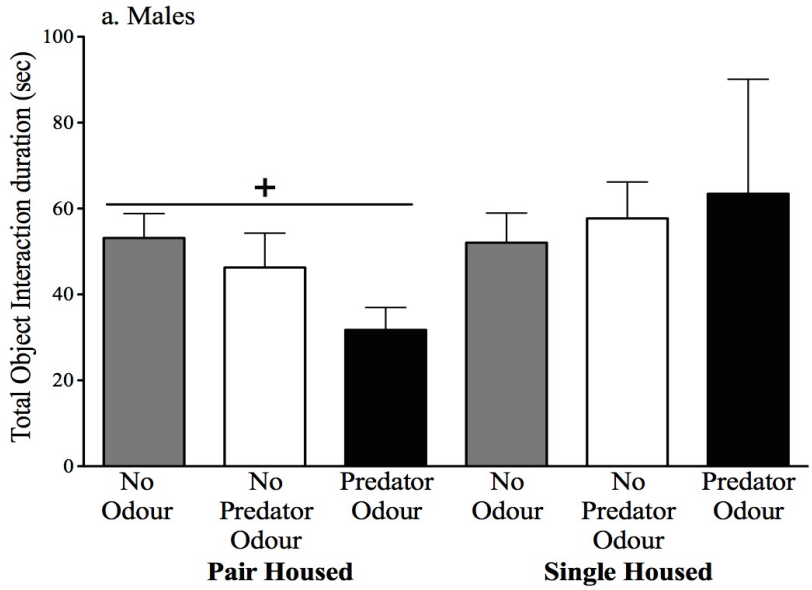


Figure AB.30. <u>Novel object recognition test:</u> Mean (+/- SEM) duration spent interacting with both identical objects during the Novel Object Recognition test (Trial 1, Familiarization Phase) is displayed for males (a) and females (b).

Housing had a main effect on this dependent measure (Pair Housed < Single Housed). *("*" is significantly different from single housed animals, collapsed across sex, and odour treatment.)*



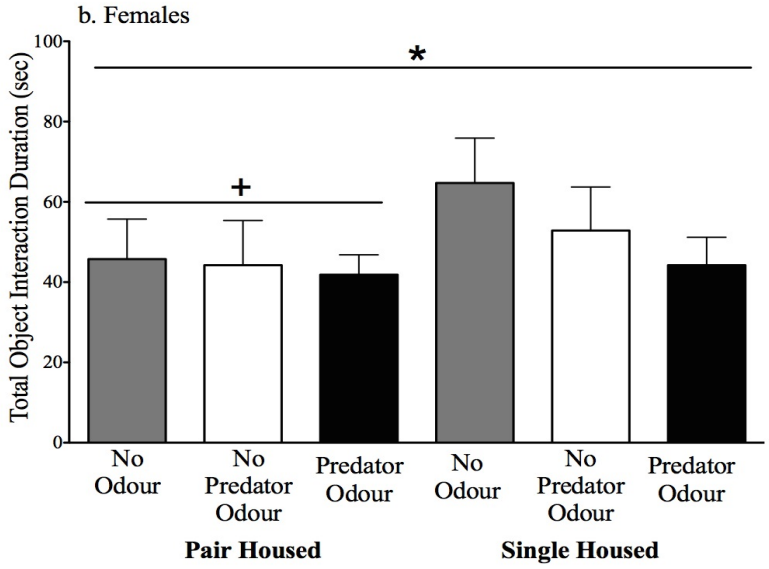


Figure AB.31. <u>Novel object recognition test:</u> Mean (+/- SEM) duration spent interacting with both identical objects during the Novel Object Recognition test (Trial 2, Familiarization Phase) is displayed for males (a) and females (b).

Sex (Male> Female), and Housing (Pair Housed> Single Housed) had a main effect on this dependent measure. *("*" is significantly different from males, whereas "+" is significantly different from single housed animals, collapsed across sex, and odour treatment.)*

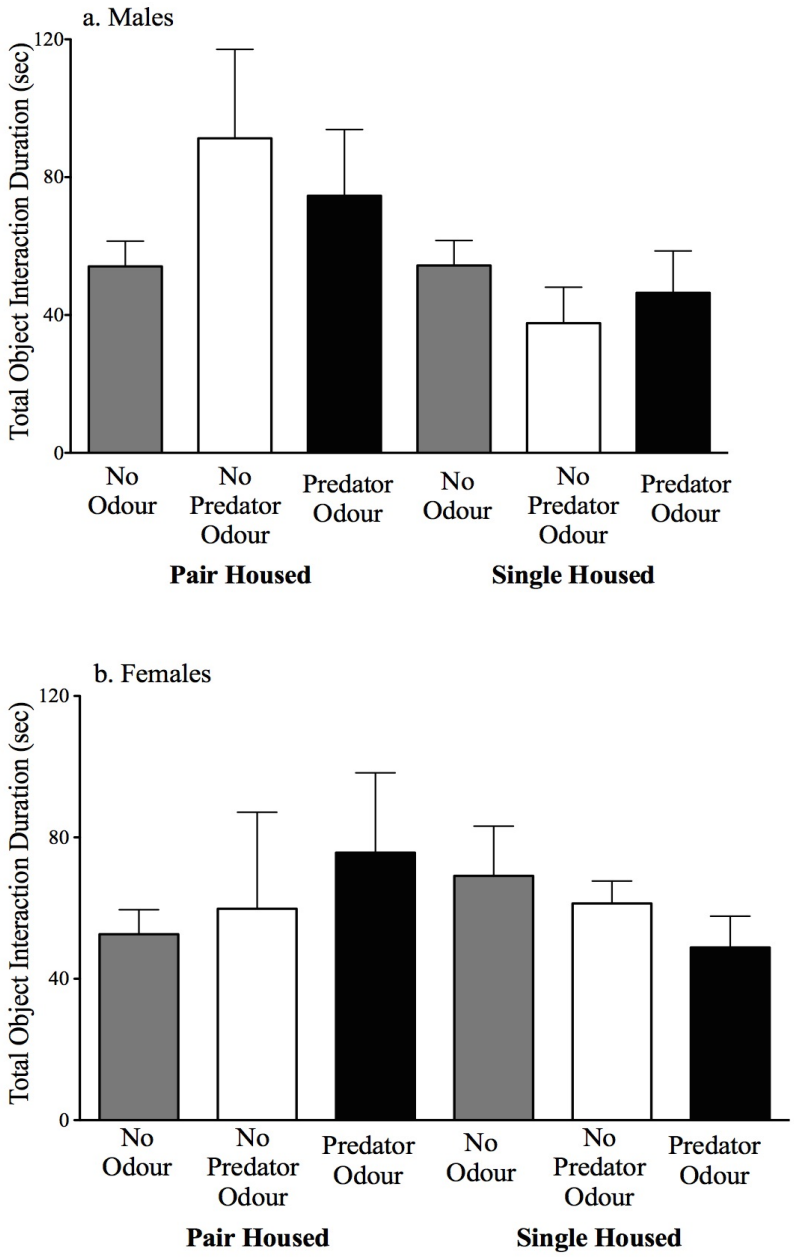


Figure AB.32. <u>Novel object recognition test:</u> Mean (+/- SEM) duration spent interacting with both the novel and the familiar object during the Novel Object Recognition test (Trial 2, Test Phase) is displayed for males (a) and females (b).

None of the factors had any effect on this dependent measure.

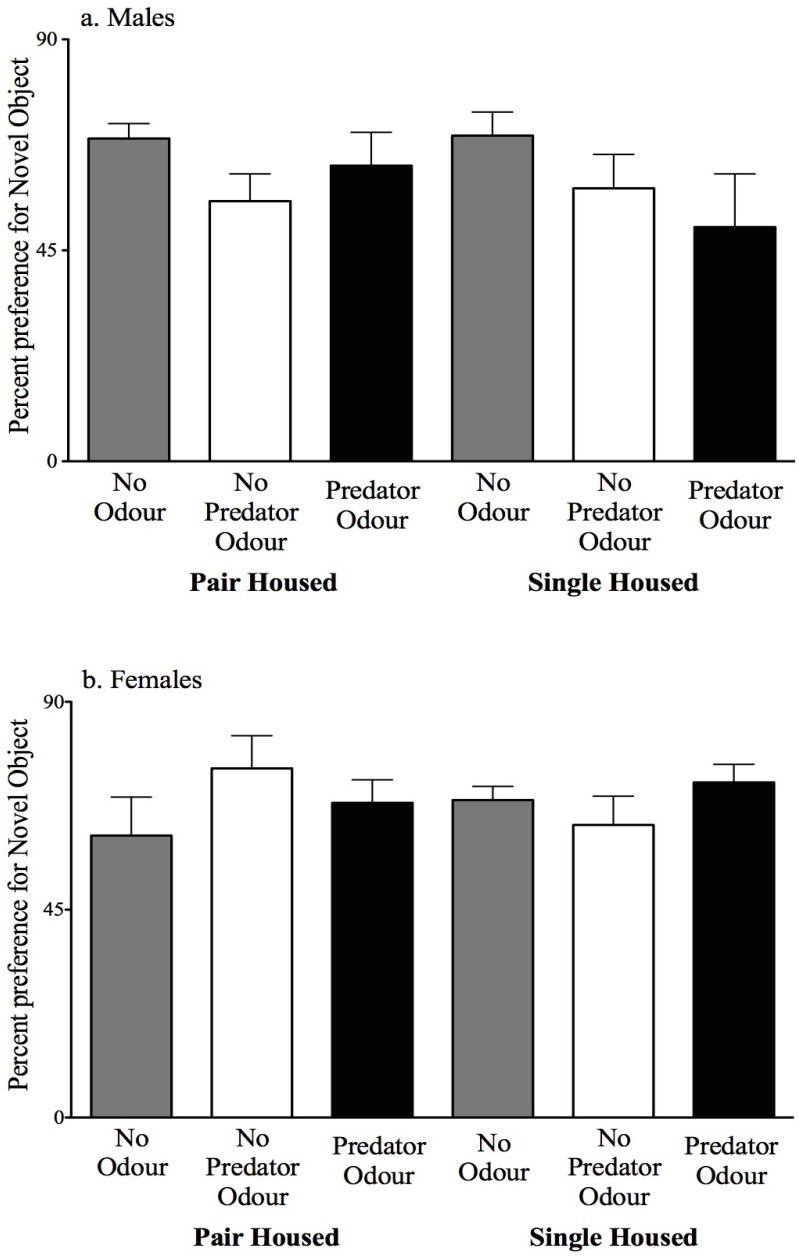


Figure AB.33. <u>Novel object recognition test:</u> Mean (+/- SEM) percent preference for the novel object during the Novel Object Recognition test (Trial 2, Test Phase) is displayed for males (a) and females (b).

None of the factors had any effect on this dependent measure.

Appendix C. Images from Rat Atlas (Paxinos and Watson, 1998) for microdissecting tissue for analysis.

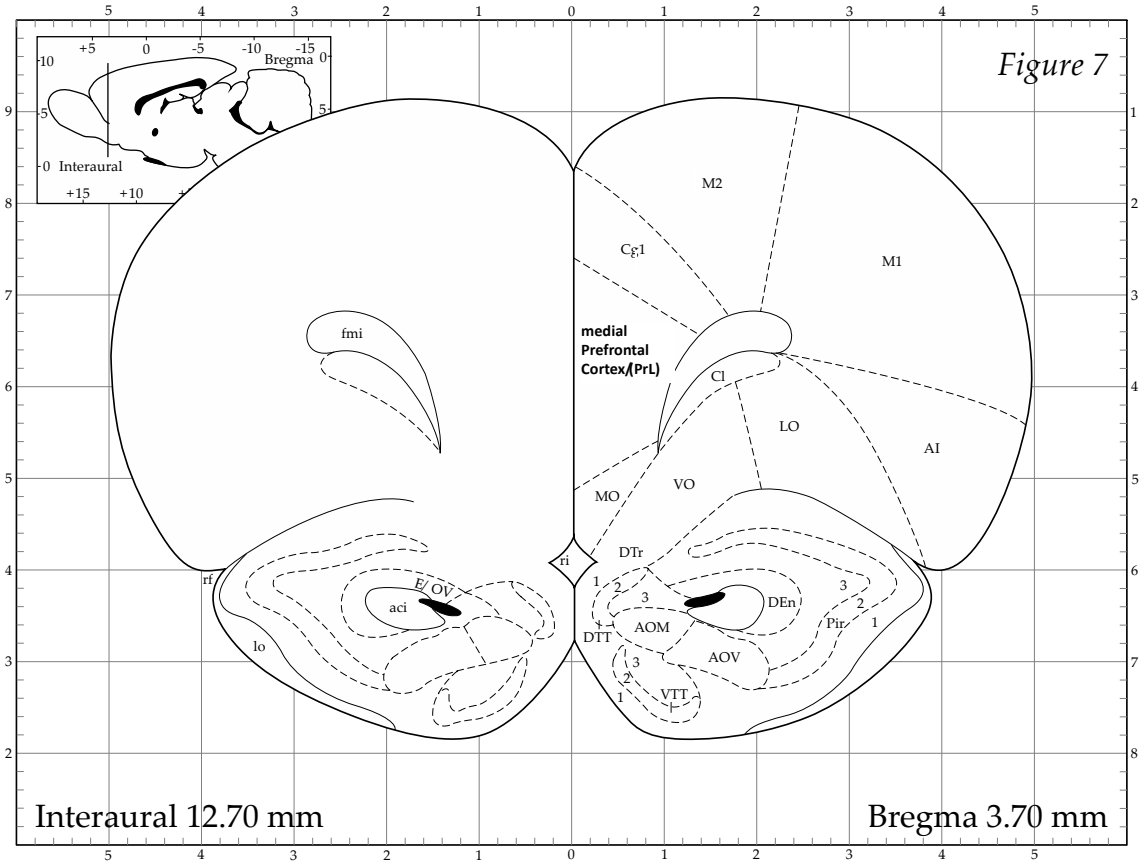


Figure AC.1. Image from rat atlas: A diagram of a cross-section of the rat brains displaying the medial prefrontal cortex from Paxinos and Watson (1998) atlas. *(Note: "PrL" refers to prelimbic sub-region of the medial prefrontal cortex.)*

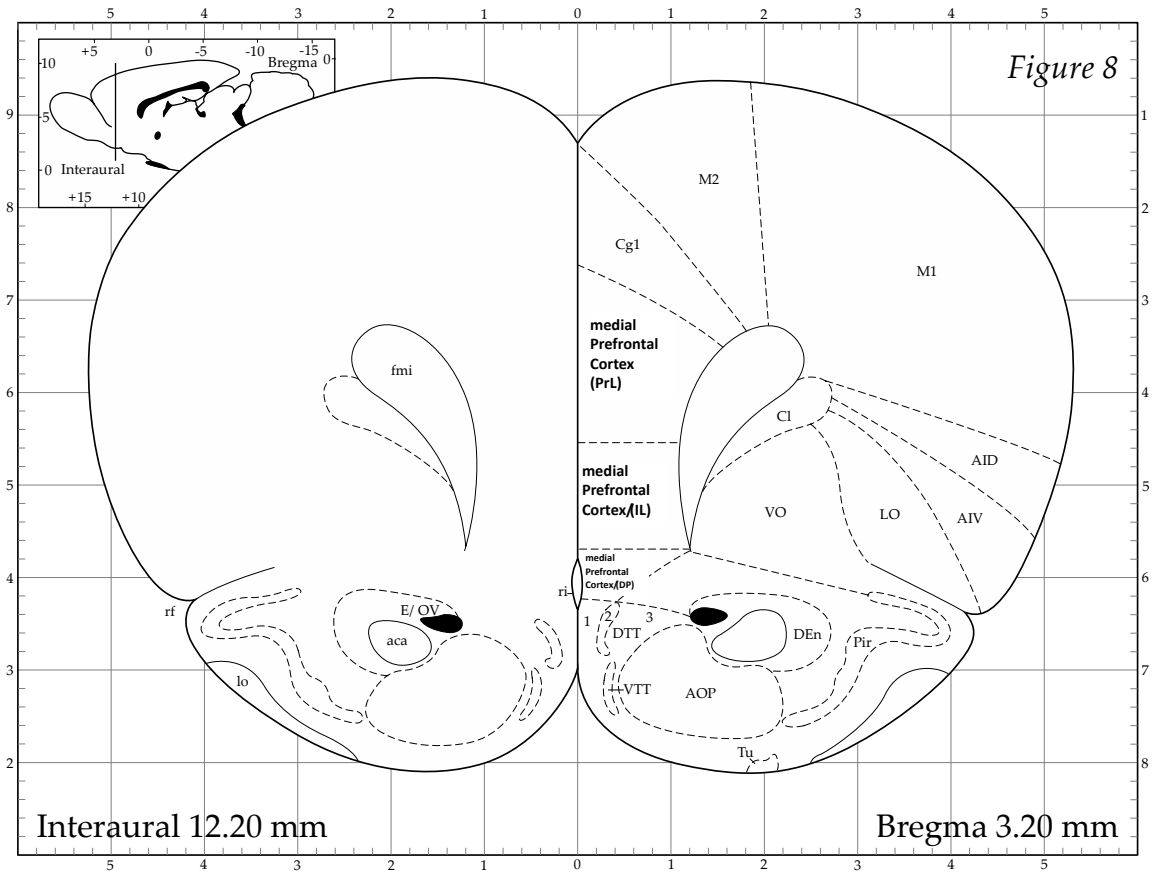


Figure AC.2. <u>Image from rat atlas</u>: A diagram of a cross-section of the rat brains displaying the medial prefrontal cortex from Paxinos and Watson (1998) atlas. *(Note: "PrL", "IL" and "DP" refer to the prelimbic, infralimbic, and dorsopeduncular sub-regions of the medial prefrontal cortex.)*

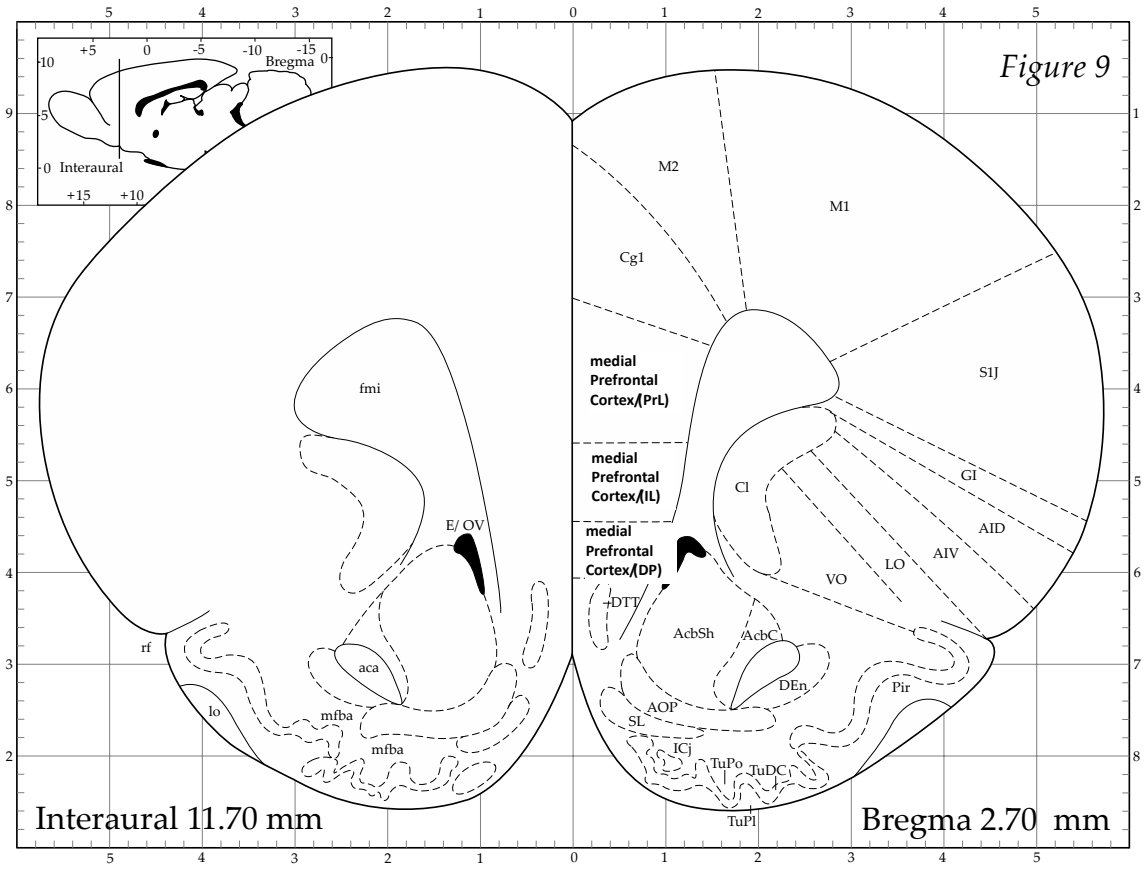


Figure AC.3. <u>Image from rat atlas</u>: A diagram of a cross-section of the rat brains displaying the medial prefrontal cortex from Paxinos and Watson (1998) atlas. *(Note: "PrL", "IL" and "DP" refer to the prelimbic, infralimbic, and dorsopeduncular sub-regions of the medial prefrontal cortex.)*

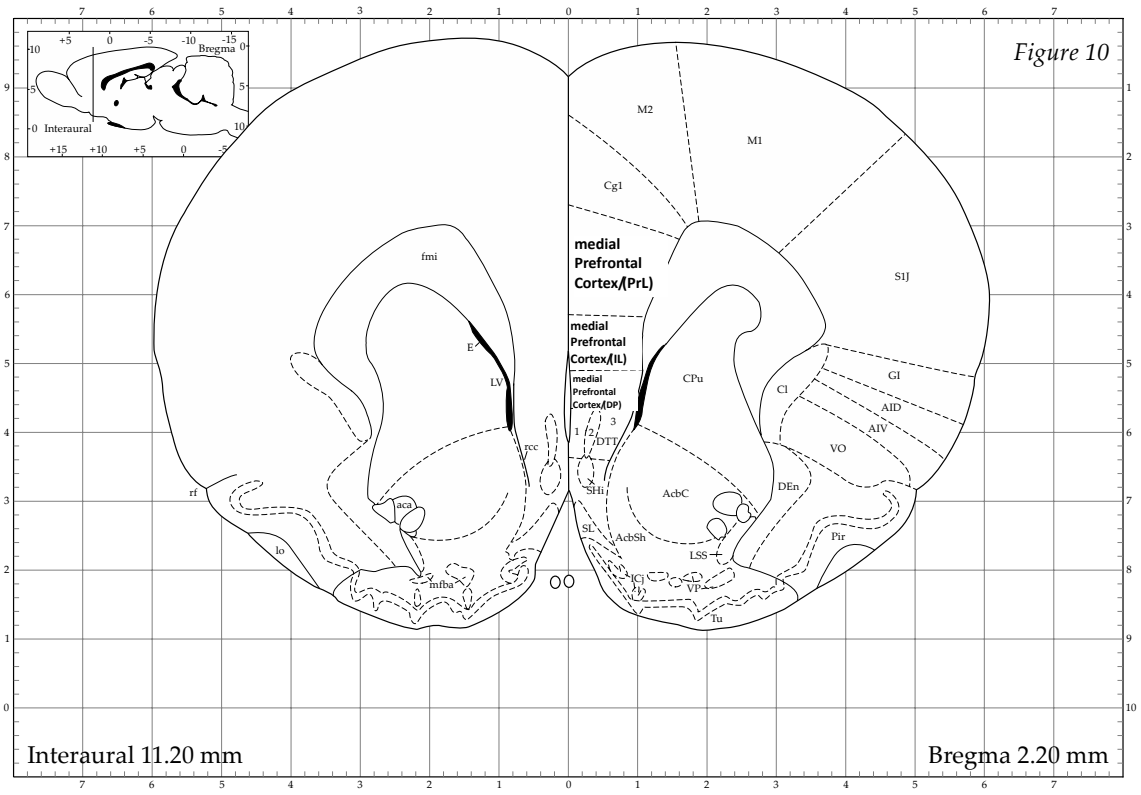


Figure AC.4. <u>Image from rat atlas</u>: A diagram of a cross-section of the rat brains displaying the medial prefrontal cortex from Paxinos and Watson (1998) atlas. *(Note: "PrL", "IL" and "DP" refer to the prelimbic, infralimbic, and dorsopeduncular sub-regions of the medial prefrontal cortex.)*

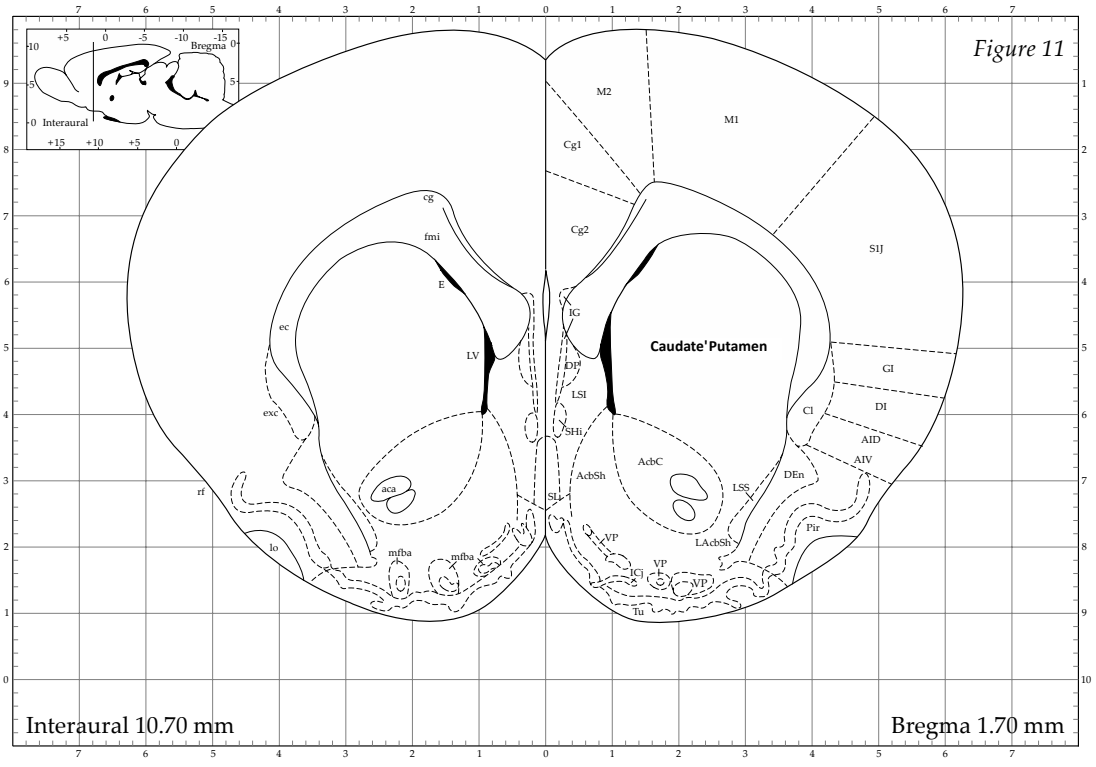


Figure AC.5. Image from rat atlas: A diagram of a cross-section of the rat brains displaying the caudate-putamen from Paxinos and Watson (1998) atlas.

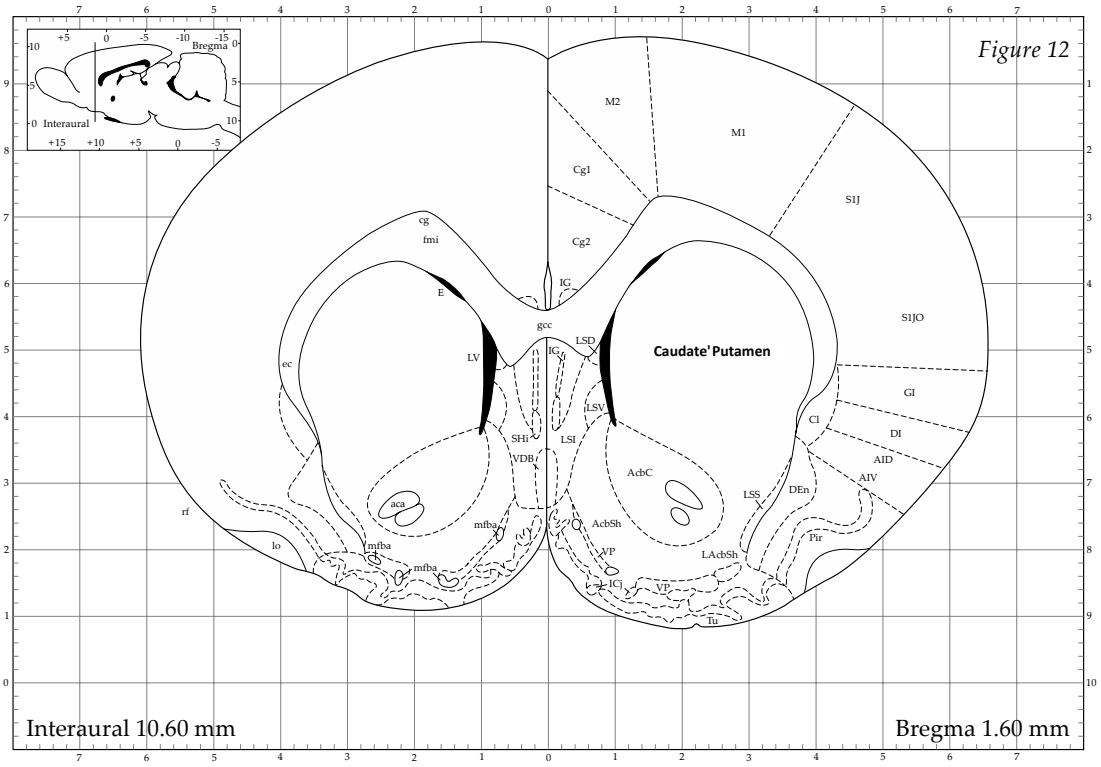


Figure AC.6. <u>Image from rat atlas</u>: A diagram of a cross-section of the rat brains displaying the caudate-putamen from Paxinos and Watson (1998) atlas.

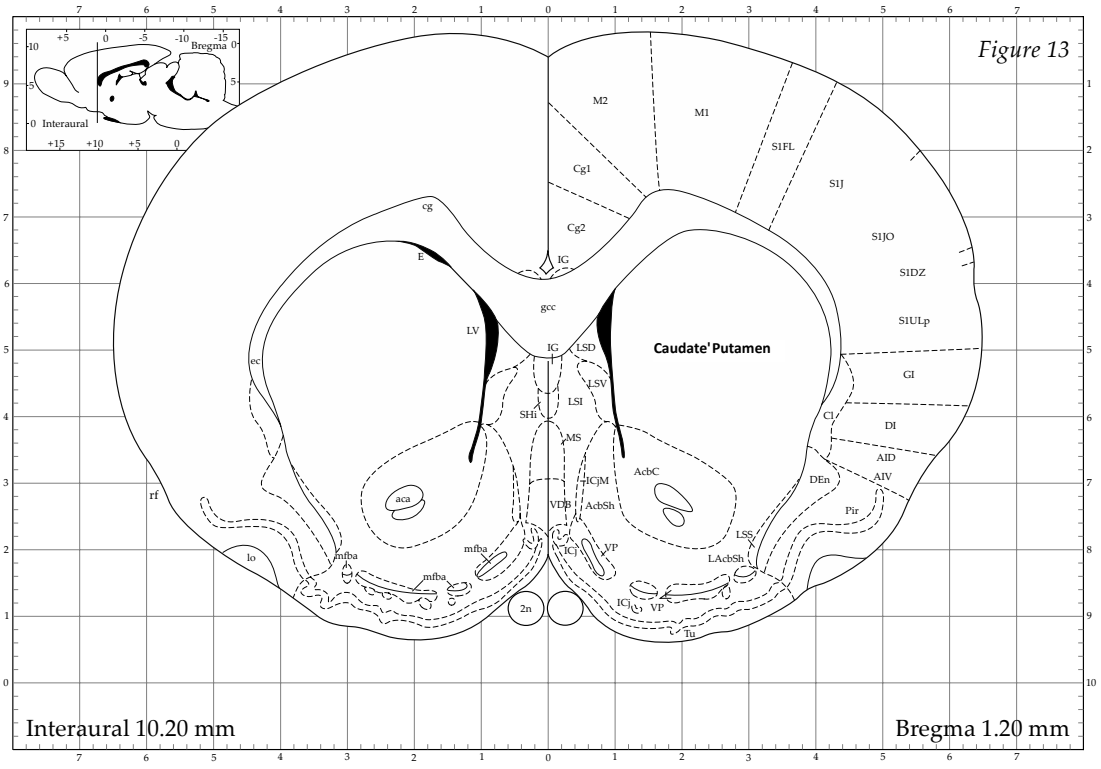


Figure AC.7. <u>Image from rat atlas</u>: A diagram of a cross-section of the rat brains displaying the caudate-putamen from Paxinos and Watson (1998) atlas.