The effect of “anticipatory behaviour”, generated by Pavlovian conditioning, on the development of play in early and normally weaned rats.

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Abstract

Since the classic studies of Pavlov, a wide variety of researchers have investigated the process of classical conditioning and used it to investigate animal behaviour. Recently, researchers in animal behaviour have discovered important new benefits associated with classical conditioning, now being applied as a way of enhancing “well-being”. Pavlovian conditioning occurs when an association between two stimuli, a neutral stimulus and a biologically relevant stimulus, is created by repeated paired presentations of both stimuli. When the presentation of the neutral stimulus is followed by a delay, before the biological stimulus is presented, “expectation” or “anticipatory behaviour” is generated. Anticipatory behaviour is known to elicit the release of dopamine. As play behaviour is also known to result in the release of dopamine, and correlates with positive welfare state, studies of the relationship between anticipatory behaviour and play may generate important new insights for animal welfare.

In this study I assessed if (i) early weaning influenced the development of playful attacks and “pinning” behaviour in rats and (ii) the effects of a Pavlovian conditioning paradigm on the occurrence of play in both early – and normally – weaned rats. Male pups were assigned to one of the following treatment groups: (1) an early weaned

(EW) group without exposure to conditioning, (2) a normally weaned (NW) group without exposure to conditioning, (3) an EW group with a conditional stimulus (CS) paired to an unconditional stimulus (US) to generate anticipatory behaviour (CS-US), (4) an EW group with CS-US unpaired, (5) an EW group with US only and (6) a NW group with US only. Animals in each group were observed for one hour each week from 4 to 7 weeks of life to record the frequency of playful attacks and pinning behaviour. EW pups that were not exposed to any form of conditioning had a significant reduction in the frequency of playful attacks relative to the NW pups without conditioning; pinning frequencies were low, but the effect was not statistically significant. The difference in frequencies of playful attacks between the NW group without exposure to conditioning and all the EW groups was statistically significant, with the NW groups displaying the highest frequencies of playful behaviour; pinning changes across those same treatment groups were not significant. Interestingly, the EW group under Pavlovian conditioning had a higher mean frequency of playful attacks and pinning behaviour relative to the EW control groups (i.e. EW with CS-US unpaired and EW with US only). Eliciting anticipatory behaviour may explain the increased levels of play behaviour in the EW conditioned group. Weight increases were similar across all EW and NW groups.

The results of this study are discussed in relation to the effects that early weaning has on behavioural development, the potential benefits of using anticipatory behaviour to reduce the impacts of stressful events, and its positive effects in the development of play behaviour in rats. The findings support the idea that a Pavlovian conditioning paradigm can be used to enhance the welfare of animals and that the expectation for the reward has a greater effect than the reward itself.

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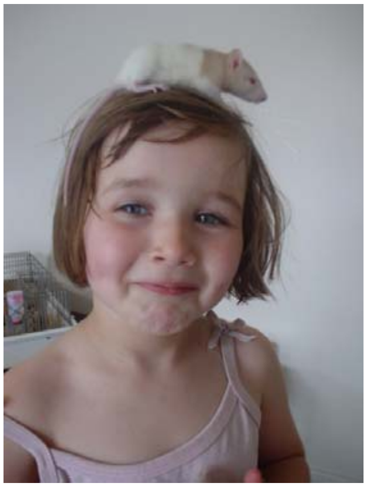

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Chapter 1

Literature review

1.1 Introduction

Pavlov discovered that the presentation of a relevant neutral stimulus (e.g. the sound of a bell; neutral stimulus or NS), when repeatedly paired with a relevant biological stimulus (e.g. food; unconditional stimulus or US), could eventually elicit physiological responses (e.g. salivation; unconditional response or UR; see Figure 1.1) in the absence of the US. Classical conditioning links the stimuli together and therefore the NS changes into a conditional stimulus (CS) capable of eliciting a conditional response (e.g. salivation).

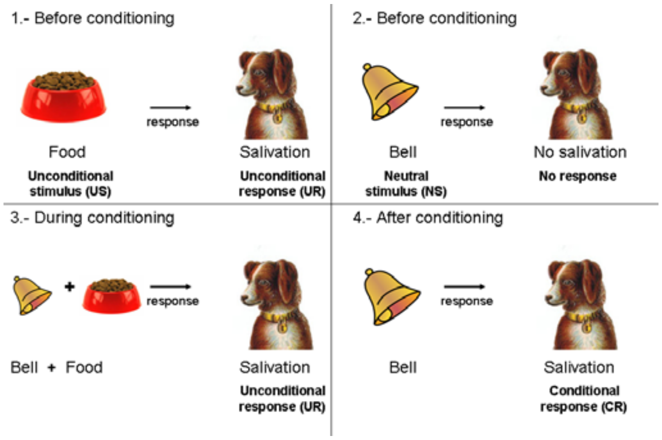

Figure 1.1: The process of classical conditioning. Pavlov conditioned salivation in dogs by pairing the sound of a bell with meat presentation. Repeated paired presentations of both stimuli created an association between the sound and the reward (the meat) so that the sound itself produced salivation.

When the presentation of the unconditional stimulus is delayed, the motivation to obtain the reward (or escape negative stimuli) creates “anticipatory behaviour”. When the unconditional stimulus is positive, anticipatory behaviour is correlated with dopamine release (O'Doherty *et al.*, 2002), just as play behaviour is also know to result in the release of dopamine. Welfare studies associate a positive welfare state with higher frequencies of play. Therefore, studies of the relationship between anticipatory behaviour generate important insights for animal welfare, allowing to assess the potential use of the Pavlovian conditioning paradigm for reducing the effects of stressful events (like early weaning (Dudink *et al.,* 2006), or being housed in environments that do not allow the expression of a wide range of behaviour patterns (Van der Harst *et al.,* 2003a)).

My research explores the effects of early weaning in play-fighting behaviour in rats and if anticipatory behaviour for a positive reward, created during the delay interval between the CS-US, can influence the development of play-fighting in early weaned rats (as expectation elicits dopamine release). Stressful events in early life, as well as chronic stress, can generate disorders such as depression and anhedonia (Bevins & Besheer, 2005). In the majority of cases of anhedonia, which is characterized by a lack of motivation for normally motivating events, dopamine release levels into the neurocircuitry of the brain are lower (Rygula *et al.*, 2005). The increase of dopamine associated with anticipatory behaviour can influence behaviour. Therefore, by identifying how a Pavlovian conditioning paradigm influences behaviour, we may be able to gain an understanding of the potential value of conditioning in the field of animal welfare (e.g. to reduce stereotypical behaviour and stress associated with captivity) and/or its possible uses to decrease mild depression in humans (without the use of antidepressants).

This research involves integrating the findings of several research areas: classical conditioning, the reward system and dopamine, and anticipatory behaviour. This first chapter of my thesis provides a literature review of each of the main areas presenting the mechanisms involved, hypotheses, results and findings from previous research. This review will familiarize the reader with the role of dopamine in reward, before explaining the use of reward in conditioning and the creation of anticipatory behaviour under the Pavlovian conditioning paradigm. The importance of play as a measure for welfare and the effects of weaning on play will be explained, followed by background information on the study species. This background information is necessary to explain the development of the research question. The chapter ends with an outline of the aims, hypotheses and format of this thesis.

1.2 What is a reward?

A reward as described by Shultz *et al*. (1997) is “an operational concept for describing the positive value that a creature ascribes to an object, a behavioral act, or an internal physical state”. However, Wise (2002) provides a very simple and more comprehensible definition by stating that rewards are “the environmental incentives we tend to return to after having previously contacted them”.

Behaviours related to rewards have been classified into two broad categories (Ikemoto & Panksepp, 1996). The first one is the appetitive or preparatory behaviour which includes behaviours performed before the consummatory phase (see below), when the reward is still not present. This phase includes behaviours such as exploration, increased activity, approach and instrumental responses (Barbano & Cador, 2005). During this phase, anticipatory behaviour can be observed as part of appetitive behaviour, in anticipation for the rewarding stimulus. The second category of activities related to rewards is the consummatory phase which is related to the terminal phase, when the rewarding stimulus is presented and obtained by the subject (Von Frijtag *et al.*, 2002a). With the use of intra-cranial stimulation, Olds and Milner (1954) obtained the necessary evidence for the existence of a "reward system" in the brain. The authors identified the regions of the brain where direct electrical stimulation was reinforcing by providing rats with access to a lever that would produce brief stimulation pulses when pressed. Higher rates of lever pressing (more than 6000 per hour) were found when stimulation occurred in these regions. Since Olds and Milner's (1954) discovery, numerous studies have examined the role of this system and its relation to dopamine (Bozarth, 1991; Spanagel &Weiss, 1999; Stairsa & Bardob, 2009).

1.3 The role of dopamine (DA) in rewards

In the literature, an incredible amount of data has been gathered linking and correlating the activation of the dopaminergic pathway with rewarding stimuli (e.g. food, sexual interactions, play, drugs, money, music and more; Cannon & Bseikri, 2004). Additionally, an extensive literature has been published on the important role that dopamine has in relation to reward systems (Wise & Rompre, 1989; Salamone *et al*., 1997; Spanagel & Weiss, 1999; Hyman & Malenka, 2001; Kakade & Dayan, 2002). Dopamine is one of the most studied neurotransmitter of the brain (Berridge & Robinson, 1998), with strong evidence indicating that DA plays an important role in mediating the ‘reward value’ (or the incentive properties) of a reinforcer. Evidence for the activation of the DA system by different types of rewards has been obtained by several methodologies (i.e. electrophysical, microdialysis and voltametric measures; Berridge & Robinson, 1998).

Furthermore, pharmacological studies provide information on the role of DA in the reward system. Studies using DA antagonistics show how both instrumental and consummatory behaviour decrease towards the reward. Similarly, studies in which ascending DA neurons were destroyed show how rats became indifferent and unmotivated even to food (i.e. starving to death if not fed artificially; Ungerstedt, 1971). Information has also been gathered to show how DA is released in response to a conditioned stimulus (Schultz *et al.,* 1997) with evidence suggesting that release of DA happens with the expectation of the reward, rather than by its distribution (de la Fuente-Fernández *et al*., 2002). Once there is a reliable link between a neutral stimulus and reward delivery, DA release happens at the stimulus onset rather than at the time of delivery or when the reward is obtained by the animal (Schultz *et al.,* 1997). This data suggests that the dopaminergic system encodes information about what to expect from the external stimuli and rewards.

1.4 Manipulations revealing impaired manifestations of reward value

Research on the effects of chronic stress with rats exposed to a social stress paradigm (i.e. being introduced into the territory of an aggressive male and housed individually after defeat) reveals a lack of appetitive behaviour towards a reward-related stimulus (Von Frijtag *et al.,* 2000). Chronically stressed rats were unable to anticipate a sucrose reward, had lower levels of social memory and long lasting effects of stress for up to three months. With this type of research, it has been shown that over-activation of the stress response mechanisms proved to be deleterious in the long term (Von Frijtag *et al.*, 2000).

Anhedonia has been suggested to result from the breakdown of the pathways associated with the brain's reward system involved in DA release (Rygula *et al.*, 2005). Anhedonia, a central part of depression symptoms in humans, and an important diagnostic feature of major depressive disorders (Keedwell *et al.*, 2005), indicates a desensitisation of the brain reward mechanisms (Rygula *et al.*, 2005). The decreased responsiveness of rats to rewards (like the reduction of appetitive behaviour found by Von Frijtag *et al.*, 2000) can be equated to the symptoms shown by humans in anhedonic states, serving as an animal model of depression. Human depressive patients show, just like other animals, signs of reduced motivation for pleasurable life events (Hughes *et al.*, 1985; Auriacombe *et al.*, 1997); additionally, they have difficulties in generating “happy thoughts” being biased towards negative stimuli (Surguladze *et al.*, 2005). A non-human animal model of anhedonia allows therefore to research plausible treatments (Papp *et al*., 1991) applicable to both humans and other animals.

Anhedonia and depression are generally treated in humans with the use of antidepressants. A study done with rats exposed to mild chronic stress, and treated with the antidepressants fluoxetine and maprotiline, supports the existence of a dopaminergic mechanism of antidepressant action to reverse the signs of anhedonia (Muscat & Papp, 1992). Treatments involving DA receptor agonists (like pramipexole; Willner *et al.,* 1994), or antagonists increasing the sensitivity to DA transmission (Muscat *et al.*, 1990), allows abnormalities of dopamine neurotransmission to be regulated and demonstrates the important and functional role of DA in reward behaviour (Papp *et al.,* 1991). Von Frijtag *et al.,* (2002b), in a study using imipramine as an antidepressant, provided further evidence that the inability to anticipate a reward is part of a depression-like state in animals that can be counteracted by chronic administration of antidepressants.

1.5 Anticipatory behaviour and the use of Pavlovian conditioning

It has been suggested that animals exposed to a Pavlovian conditioning paradigm, where the US is positive, alter the response of neurons by activating the mesolimbic dopaminergic and opiod systems, related to the reward system feedback mechanisms (Spruijt *et al*., 2001). Expectation for positive stimuli enhances appetitive behavioural patterns, while aversive stimuli suppress it (Spruijt *et al*., 2001). As mentioned above, it has been revealed that expectation of a reward, rather than its acquisition, causes the release of dopamine associated with the reward mechanisms in the brain (Schultz *et al*., 1997).

During a Pavlovian conditioning paradigm, the CS is paired with the delivery of a biologically relevant stimulus: the US. Once an association between a CS and a positive US is established, anticipation (or anticipatory behaviour) can be extended and exaggerated by gradually increasing the interval between the onset of the CS and the access to the US, creating an expectation for the specific reward (Van der Harst *et al.*, 2003).

In the field of applied animal welfare, anticipatory behaviour through a Pavlovian conditioning paradigm has been shown to counteract the impairment of anticipatory behaviour in rats exposed to a social stress paradigm (de la Fuente-Fernandez *et al*., 2002; Wightman & Robinson, 2002; Van der Harst *et al*., 2005). Additionally, anticipation of a reward can increase play behaviour and reduce stress-induced behaviours in piglets directly after weaning (Dudink *et al*., 2006). Dudink (2006) observed differences between three treatment groups of early weaned piglets: (1) sound cue (doorbell) paired with environmental enrichment (access to a hallway with straw and mixed seeds) with US delivery delayed by 30s to generate anticipatory behaviour (CS-US paired), (2) sound cue and environmental enrichment provided on a random schedule (CS-US unpaired) and (3) a no sound cue, no environmental enrichment group (no CS-US). Piglets under the CS-US paired treatment group showed more play behaviour and less aggression in comparison to the other treatment groups. These studies demonstrate potential for the use of Pavlovian conditioning in applied animal welfare.

Anticipatory behaviour is not expressed in the same way across species. Anticipatory behaviour towards positive rewards is expressed as an increase in behavioral transitions in rats (van der Harst *et al.*, 2003) and mink (Vinke *et al*., 2004), while in cats it is shown as a decrease in activity (van den Bos *et al*., 2003); anticipatory behaviour towards negative stimulus in rats is expressed as a decrease in activity (van den Harst *et al*., 2003). Quantitative changes in behavioural expression occur depending on the "subjective state" of an animal. Increased levels of deprivation or stress result in increased sensitivity for rewards (van der Harst et al., 2003); thus, anticipatory behaviour increases as the need for the reward increases (Spruijt *et al*., 2001). It is therefore inferred that anticipatory behaviour denotes "a general expression of the animals' need" (Spruijt *et al*., 2001) suggesting the use of anticipatory behaviour created via a Pavlovian conditioning paradigm as a tool for measuring welfare and assessing the need for a reward.

1.6 Play behaviour

1.6.1 Function and adaptive value

Play has generally been perceived as a good indicator of welfare for wild and captive animals and is generally related to somewhat stress-free contexts (Fagen & Fagen, 2004). Play is predominant in young animals, decreasing but still observed in adult mammals and birds (Power, 2000). Even though its function is still unclear (Caro, 1988; Pellis & Iwaniuk, 1999), it is a complex and costly behaviour normally interpreted as conveying benefits in the long term rather than the short term (Martin & Caro, 1985; Spinka *et al*., 2001; Sharpe *et al.*, 2002). Play behaviour is highly variable depending on nutritional, physiological and environmental factors (Burghardt, 1984; Caro, 1988), but it is known to be a key factor for the development of normal adult behaviour in mammals and some birds (Smith, 1982). Several experiments show play behaviour helps develop coping strategies for stressful and conflict situations, with play deprivation leading to abnormal social behavioural patterns in adulthood (Gerall *et al*., 1967; Hol *et al*., 1994; Hol *et al*., 1999; van der Berg *et al*., 1999; Von Frijtag *et al*., 2002a). Play is also known to facilitate forebrain dopaminergic neurotransmission (Vanderschuren *et al*., 1997); thus, a reduction or blockage of dopaminergic activity correlates with a reduction of play (Panksepp *et al*., 1987).

1.6.2 Play behaviour in rats

During the juvenile period, rats develop their play behaviour. Play in rats develops at around the age of 18 days (Panksepp, 1981) and is sexually dimorphic. The juvenile pattern of play behaviour is characterised by episodes of rough-and-tumble play or play-fighting behaviour which peaks at around 30 to 40 days old. Play behaviour then decreases after the onset of puberty (Loranca *et al*., 1999) turning into "partial rotations" (where the rat only rotates the forequarters allowing the hindquarters to remain stable while counterattacking; Pellis & Pellis 1987), and an increased frequency of defensive maneuvers (sidling, running away) and upright positions (boxing, pushing or kicking). Females play less than males and retain the juvenile pattern of play-fighting (Pellis & Pellis, 1987). The behaviour patterns discerned in play fighting and real fights are very similar. However, the activities can be differentiated as play behaviour has the attack or soft playful “bites” directed to the nape area while real fighting is generally directed toward the rump (Pellis & Pellis, 1987). Similarly, the consummatory behaviour for play fighting is ‘pinning’ the opponent; the result is one rat lying on top of the other with the pinned animal lying on its back in a belly-up position (Pellis & Pellis, 1987). In contrast, the consummatory behaviour during real fighting is serious injury (Blanchard & Blanchard 1977; Pellis & Pellis 1987).

1.7 The effects of weaning on behaviour

1.7.1 Effects of early weaning

Studies of several species have demonstrated that early weaning has dramatic effects on behaviour. For example, early weaning can result in a reduced frequency of play-fighting during developmental stages in the post-weaning period (e.g. rats (Shimozuru *et al*., 2007; Terranova & Laviola, 1995), pigs (Dudink *et al*, 2006; Donaldson *et al*., 2002)). Similarly, precocious weaning increases anxiety levels and aggressiveness in mice and rats (Ito *et al*., 2006). Play-fighting is the main social play behaviour exhibited by juvenile rats. Shimozuru *et al*. (2007) followed the development of play-fighting in juvenile rats until maturity and compared the development between normally weaned and early weaned males and females. Males and normally weaned rats showed a higher frequency of playful behaviour compared to females and early weaned groups respectively. However, the study did not rule out the possibility that the differences in behaviour across groups could be due to a reduction in the level of interactions between juvenile rats - the early weaned groups were housed in same sex pairs whereas the normally weaned group remained in larger mixed sex groups with the dam (2 female and 2 male pups with the dam). The study therefore not only had different levels of maternal separation but social contact differed across treatment groups. This was an important flaw in their experimental design.

1.8 Study species – Rattus norvegicus

Other than humans, rats are the most successful mammal on the planet. They were the first domesticated mammal used for scientific research, becoming since then a model organism in many areas of research (especially in medical research; Gibbs *et al.*, 2004). They are animals that grow and mature quickly, and are easy to care for, allowing research outcomes to be obtained rapidly.

The Sprague-Dawley, a commonly used laboratory-bred strain of albino rats originating from the Wistar Institute in Philadelphia, is a rodent descendent of the Norway rat (Calhoun, 1962; Pass & Freeth, 1993). Also known as the brown rat, it is thought to have its origins in Northern China (Robinson, 1965). This species is now present on all continents of the world, having displaced the black rat with its aggressive behaviour (Calhoun, 1962; Pass & Freeth, 1993). It has well developed senses, including ultrasonic hearing and a well developed olfactory sense. However, albino strains like the Sprague-Dawley, have poor vision due to the lack of melanin in the eyes (Pass & Freeth, 1993). Rats are predominantly nocturnal animals with good swimming abilities (Hall, 1981). Their reproduction rate is high as the polyoestrus females breed all year round producing several litters (up to 5) in a year with an average of 6 to 10 young per litter (Pass & Freeth, 1993). The female rats show strong maternal behaviour and the care of several litters is sometimes shared (Grota & Ader, 1969). The gestation time is very short, lasting an average of 21 to 25 days (Hall, 1981). However, the young are blind at birth and do not open their eyes until past the second week of life. They are born without fur but patterns of colour are distinguishable on their pink skin from birth. The young are normally weaned at around 21-28 days of age, although, they are able to feed by themselves as soon as the eyes are open and they become mobile (Hall, 1981). Post-weaning, this omnivorous species is able to eat almost anything (Nowak, 1999), eating a third of its body weight per day (Hall, 1981).

These rodents create social dominance structures (Cordero & Sandi, 2007) sharing colonial burrows where they groom and sleep together (McClintock, 1983). They have a life span of approximately two years (Woodall et al., 1996). Their body grows up to 25 cm long with a long tail of similar length. Males are generally bigger than females weighing around 350 g and 250 g on average respectively (Hall, 1981).

1.9. Research context

In this thesis I build on the findings of two previous research articles to examine how anticipatory behaviour towards a positive stimulus affects the development of play-fighting behaviour in early weaned rats. A reduction of play, related to early weaning, has been documented by Shimozuru *et al.* (2007). However, this study did not rule out the possibility that the behavioural differences detected between the groups were due to a reduction of interactions between juvenile rats with social contact acting as a confounding variable; that is, early weaned rats were housed in same pairs whereas the normally weaned groups remained in larger mixed sex groups (2 females and 2 males with the dam). In the second study of interest, Dudink *et al*. (2006) found that piglets exposed to a Pavlovian conditioning paradigm play more after early weaning than animals not exposed to a conditioning process; the results suggest that the conditioning process may be an effective strategy to reduce pre-weaning stress.

Amalgamating the methodologies of the two pieces of research identified above allows me to more accurately investigate: (i) how early weaning affects the development of rat behaviour and (ii) how the Pavlovian conditioning paradigm influences the development of play-fighting behaviour in juvenile rats. It is expected that by replicating the Shimozuru *et al.*'s (2007) weaning methodology and incorporating the Pavlovian conditioning paradigm to generate anticipatory behaviour via the expectation of a reward (as applied by Dudink *et al.,* 2006), the impacts of early weaning should be reduced. In my research, rather than focusing on the anticipatory behaviour (as research using anticipatory behaviour generated with a Pavlovian conditioning paradigm has created good background knowledge) I focus on its effects and possible applications.

Therefore, for this thesis research, Shimozuru *et al.*'s (2007) experiment provided the methodology for housing conditions, weaning procedure, play-fighting observations and data analysis. However, the living conditions of early and normally weaned pups were standardized to maintain the same levels of social interaction across all groups. Dudink *et al.*'s (2006) study was used as a model for creating the different experimental groups and applying the Pavlovian conditioning paradigm to elicit anticipatory behaviour and dopamine release (outlined in detail within Chapter 2). Expectation of a reward was generated by creating an association between a sound (bell) and the delivery of a reward (1 gram of cheese) delayed for 3 minutes. The behaviour of the rats was observed for an hour per week from their fourth to seventh week of life. Based on the frequency of pinning and playful attacks recorded, I determined if the Pavlovian conditioning paradigm (therefore the anticipatory behaviour, associated with a positive reward) influenced the development of play-fighting behaviour across normally – and early – weaned groups.

1.10 Hypotheses and expected outcomes

As outlined above, early weaned rats play less than normally weaned rats (Shimozuru *et al*., 2007); therefore, I hypothesised that the behaviour of the rats used in my study would conform. Furthermore, as early weaned piglets exposed to a conditioning paradigm with a positive US increased frequencies of play behaviour (Dudink *et al*., 2006), I hypothesised that the use of a Pavlovian conditioning paradigm should exaggerate levels of play behaviour in groups of rats exposed to a standard conditioning treatment (CS-US paired) in comparison to control groups. This may occur as a consequence of the activation of the reward system in the brain.

If anticipatory behaviour activates the reward system and influences DA release, it is expected that normally weaned groups will have the highest play frequencies, followed by early weaned groups exposed to the Pavlovian conditioning paradigm (CS-US paired). Early weaned control groups (i.e. those that have not been exposed to temporally predictable CS-US pairings) should display the lowest frequency of play behaviour.

If the procedure is successful, it would provide further support for the use of the Pavlovian conditioning paradigm as a method to improve animal welfare. Additionally, its applications could range from improving the welfare of captive animals (e.g. in zoos and laboratories), to reducing stereotypies, to enhancing the lives of depressive patients (i.e. by reducing the signs of anhedonia).

1.11 Format

This thesis has been formatted into three chapters. The first chapter includes the literature review that provides the necessary background information in support of the research question. The second chapter presents the research itself, formatted as a paper for publication. In the third and last chapter I present the general findings, logical areas for further research and the main implications of the findings for the field of animal welfare and human health. As chapter two has been formatted as an article for publication there is inevitable overlap of information across the chapters resulting in some degree of repetition.

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Chapter 2

The effect of “anticipatory behaviour”, generated by Pavlovian conditioning, on the development of play in early and normally weaned rats.

2.1 Abstract

Early weaning and abrupt separation from the mother is common practice in commercial farming. Maternal separation has long lasting behavioural and physiological effects, and has served as a method of investigating depression. New procedures for measuring and enhancing the welfare of animals exposed to stressful events (such as early weaning) that can be used across a range of situations and husbandry alternatives are urgently required. The use of anticipatory behaviour (i.e. exposing animals to a classical conditioning procedure in which the positive unconditional stimulus is progressively delayed from the onset of the conditional stimulus) has been suggested as a tool to enhance welfare of animals. In this study I examined (1) the effects of early weaning on the development of play fighting behaviour and (2) the role of anticipatory behaviour on the ontogeny of play fighting behaviour in early and normally weaned rats to determine if the Pavlovian conditioning paradigm could be used to improve animal welfare and reduce the effects of stressful events. Behavioural observations from week 4 to week 7 of age were carried out to detect differences in (a) playful attacks (i.e. one animal bringing its snout in contact with the nape area of the social partner in a fast movement) and (b) “pinning” frequency (i.e. one animal rolling and stradding the other in a belly-up position). Early weaned rats showed lower levels of pinning behaviour than normally weaned rats and a significant reduction in the frequency of playful attacks. Normally weaned rats had the highest frequencies of playful behaviour. Further, early weaned rats exposed to a Pavlovian conditioning paradigm had higher frequencies of playful attacks and pinning behaviour in comparison to early weaned rats that were not exposed to conditioning; conditioning increased frequency of both playful behaviours, however, levels did not reached those of normally weaned rats. The data suggest that without the need of food deprivation, food rewards can elicit anticipation effecting behavioural development. This research supports the idea that anticipatory behaviour generated by Pavlovian conditioning is a useful tool to counteract stress and an effective way to improve the welfare of animals.

Keywords: *Rattus norvegicus*, anticipatory behaviour, early weaning, play-fighting, Pavlovian conditioning, welfare, enrichment.

2.2 Introduction

In the domain of applied animal welfare many studies have focused on problems associated with weaning procedures. However, most of the weaning literature has focused on one particular physiological or behavioural process, or problems associated with a particular species (e.g. progressive versus sudden weaning in sheep (Orgeur *et al*., 1998), effects of weaning at different ages on piglet behaviour (Worobec *et al*., 1999), effects of communal areas pre- and post-weaning in pigs (Weary *et al*., 2002), physiological and behavioural influences of different housing systems on weanling horses (Heleski *et al.*, 2002)). Precocious or abrupt weaning, common in commercial farming, contributes to physiological consequences, such as reduced growth performance (Carroll et al., 1998) and behavioural disturbances, such as the instigation of negative social interactions (Terranova & Laviola, 1995) and increased aggression and anxiety (Kanari *et al*., 2005). These physiological and behavioural costs exacerbate the autonomic responses to stressful situations (Ito *et al*., 2006).

Early experiences in life have tremendous effects on the development of subsequent behaviour. Previous studies demonstrate that precocious weaning has an important impact on the development of play behaviour (commonly used as a measure of welfare), reducing its frequency. A reduction in the frequency of play behaviour is recorded in early weaned piglets (Dudink, *et al.*, 2006). Similarly, a reduction in the frequency of play behaviour is observed in early weaned (EW) rats when compared to normally weaned (NW) ones (Shimozuru *et al*., 2007). However, in Shimozuru *et al*.'s (2007) study, the authors did not rule out the possibility that the differences in behaviour across groups could have been due to a reduction in the level of interactions between juvenile rats - the early weaned groups were housed in same sex pairs whereas the normally weaned group remained in larger mixed sex groups (2 female and 2 male pups with the nursing dam). The level of social contact happened to be a confounding variable in this research, becoming an important flaw in their experimental design.

Behavioural economic principles have been used in research to assess animals' preferences since the late 1970's with the goal of enhancing animal welfare, and to understand to which degree a reward is rewarding for an animal (such preference and demand questions have been successfully investigated in a wide variety of species including: cows (Matthews & Temple, 1979); rats (Holm & Ladewig, 2007); and dogs, (Kos, 2005)). However, there is still debate on the proper interpretation of preference and demand results (Sumpter *et al*., 2002). Therefore, new procedures for measuring and enhancing the welfare of animals exposed to events like abrupt weaning that can be used across a range of situations and husbandry alternatives, are still greatly needed.

Recent research using anticipatory behaviour elicited via a Pavlovian conditioning paradigm showed that, anticipatory behaviour for a positive reward can increase the frequency of play behaviour in early weaned piglets exposed to conditioning prior to weaning (Dudink *et al*., 2006). In a Pavlovian conditioning paradigm, an initially neutral cue (such as a sound) is transformed into a conditional stimulus (CS) by repeatedly pairing it with an unconditional stimulus (US, such as food). Once the association is firmly established, increasing the time between the onset of the CS and the delivery of the US creates an expectation or an "anticipatory response". Anticipatory behaviour has been used in research as a measure to estimate the welfare status of animals, as deprivation of essential stimuli results in stronger anticipatory responses (e.g. farmed silver foxes (*Vulpes vulpes*) become more active in anticipation for a positive reward like a piece of salmon than for an aversive stimuli like being captured with a neck tong; Moe *et al*., 2006). This is linked to the fact that sensitivity to a reward is affected by stress (in humans and other animals) altering the threshold for appetitive behaviours (Spruijt *et al*., 2001). Therefore, it has been suggested that reward sensitivity could serve as a tool to investigate the "welfare balance" of animals (see Van der Harst & Spruijt, 2007, for a review of applications). However, it is important to be aware that the behavioural expression of anticipation for a positive stimulus is species specific (e.g. anticipatory behaviour in rats is expressed by hyperactivity while a reduction in behavioural transitions occurs in the domestic cat; van den Bos *et al.*, 2003).

Literature in neuroscience reveals that the neural mechanisms involved in the reward and pain feedback systems are both very dependent on dopamine release (Leknes & Tracey, 2008); dopamine release is associated with pain relief and increased feelings of pleasure (Leknes & Tracey, 2008). Furthermore, there is strong evidence of dopaminergic activity during anticipatory behaviour for a positive reward (Blackburn & Phillips, 1989; de la Fuente-Fernandez *et al*., 2002). Similarly, play behaviour is dependent on the mesolimbic dopaminergic system and is known to result in dopamine release (Dudink *et al*., 2006). Based on that knowledge, anticipatory behaviour, created through a Pavlovian conditioning paradigm, was used by Dudink *et al*. (2006) to counteract weaning stress in piglets resulting in an increase in the levels of play behaviour recorded post-weaning, enhancing as well the properties of the enrichment itself. Additionally, the Pavlovian conditioning methodology had positive effects on a variety of other measured behaviours (e.g. a reduction in aggression and the frequency of injuries; Dudink *et al*., 2006) proving to be an efficient methodology to enhance the welfare of the early weaned piglets.

A logical step forward, with respect to the research outlined above, would be to determine if the reductions in play observed in EW rats by Shimozuru *et al.* (2007) could be counteracted by the procedure applied on early weaned piglets (Dudink *et al*., 2006). Dudink *et al.*'s (2006) procedure should allow eliciting dopamine release in rats through anticipatory behaviour to reduce weaning stress. Therefore, there were three main goals in the present study: 1) to replicate Shimozuru *et al.* 's (2007) experiment to verify that EW causes a reduction in play; 2) to control for the level of social contact across all groups by standardizing the level of social contact across all treatment groups (therefore eliminating the level of social contact as a confounding variable in the development of play behaviour; and 3) to assess if anticipatory behaviour can counteract the effects of stress and increase the levels of play-fighting behaviour in EW rats.

I hypothesized that exposure to a Pavlovian conditioning paradigm, using a positive US, would create anticipatory behaviour and that the associated release of dopamine would effect play frequencies. If a conditioning process can be used to enhance the expression of play in rats and alleviate the impacts of EW (as it did in the early weaned piglets in Dudink *et al*., 2006), the use of anticipatory behaviour could have countless uses across commercial farming practices; the procedure is flexible enough to allow its use across a wide variety of situations to enhance the welfare of animals (e.g. as environmental enrichment for captive animals, as a method to reduce stereotypical behaviour, to counteract for past and future stressful events such as weaning or transportation, and to reduce anhedonic symptoms).

2.3 General methods

2.3.1 Subjects, housing and husbandry

Breeding for experiment one and two was done simultaneously. Eleven Sprague-Dawley females (source: Hercus Taieri Resource Unit, Dunedin, New Zealand) were bred to obtain the subjects for both experiments. Female rats were paired with one of three domestic bred black hooded male rats (source: Animates, Tauranga, New Zealand; Figure 2.1). Animals were housed within a University of Waikato animal facility (Hamilton, New Zealand). The colony rooms were maintained at 22^{o}C, under a reversed light-dark cycle (dim light: 0700-1900 h at 2.0 lux; white light: 1900-0700 h at 139.6 lux), with music from a radio serving as background noise (45 dB at 1m).

Figure 2.1: One of the male hooded rats used to fertilize female subjects.

Over the pregnancy period, females were weighed daily using a large transparent plastic cylinder over a standard kitchen scale (Figure 2.2; raw data for weight is available in Appendix 1). A female begins to gain weight within 24 hours after successful mating, with daily increases giving the assurance of a healthy and constant increase in the weight of the pups). Females were housed in all-female trios with water and pelleted food freely available (diet 86: lucerne, pollard/wheat by products, barley, meatmeal, wheat, fishmeal, Soya bean extract, rabbit premix supplement, milk powder and salt) in wire cages with a solid bottom (41 x 72 x 46 cm high); laboratory-grade pine and shredded paper were provided as bedding material, with a PVC box (see Figure 2.3) or cardboard box serving as shelter. Females were housed in these conditions (with cages cleaned every second day) until a few days before birth (detected by an increase in weight for 21 consecutive days and by signs of the belly becoming pinker and hairless); at that point, they were housed in the same conditions as above, but individually in a smaller cage (41 x 36 x 46cm high). Each cage contained shredded paper as nesting material and a clean carton or PVC box that was used by all females as a nest.

Figure 2.2: A rat being weighed.

Figure 2.3: Sprague-Dawley female rats housed in trios during early pregnancy, with nesting box (PVC box) and food containers.

Pairs of pups from each litter were allocated to the different treatment groups using a matrix (see appendix 2) so that a similar number of pups from each male were assigned across the different treatment groups (to control for genetic differences). For the same reasons, the four pairs of pups from each dam were divided up across the different treatment groups. The females remained housed individually until their last pup was weaned. From then on, the females were housed again in trios or pairs until re-housed (see appendix 3 for more information on re-housing).

All experiments complied with the New Zealand Guide to the Animal Welfare Act 1999 for the use of laboratory animals (Biosecurity New Zealand, 2000), and were undertaken with the approval from the University of Waikato Animal Ethics Committee.

2.4 Experiment 1

2.4.1 Methods

The procedures for this experiment closely followed those described in Shimozuru *et al*. (2007). The procedure was first used by Kanari *et al.* (2005) and Ito *et al.* (2006). The only change made to the original procedures was the standardization of the litters, done in two stages to control for the amount of social contact experienced across treatment groups (see below).

Weaning procedure

The birth of a litter was considered postnatal day 0 (PD0). From PD0 the dams remained undisturbed until PD3 when litters were standardized to 8 males per dam. As play behaviour in rats is sexually dimorphic, only male rats were used for the experiment (Beatty et al., 1981; Flynn et al., 2000). Transfers of pups across dams to standardize the litters were done using the closest litter in age, and by rubbing the unrelated pups in the bedding material of the adopting mother before introduction (Francis *et al.*, 2002). From then until PD16 only brief watering, feeding and cleaning sessions were undertaken, even though brief handling and separation from the mother does not affect the development of playful behaviour in juvenile rats (Arnold & Siviy, 2002). Pups become fully independent from their mother at around 4-5 weeks, being able to feed by themselves, maintain their body temperature and evacuate from 13 days old (Plaut & Davis, 1972).

On PD16, the early weaned pups (n = 12) were separated from the dam and housed in fours in standard laboratory rat cages with a solid bottom and wire top (26 x 48 x 21 cm high). These juvenile rats had free access to food and water and laboratory-grade pine for bedding material. The normally weaned pups remained in groups of four with their nursing dam (n = 12) until their natural weaning time at PD30 (Hahn & Kirby 1973). From PD30 all animals were housed in pairs under the same conditions as described above for the EW group. By following this procedure and standardizing the number of juveniles interacting with one another at two points in time (i.e. at PD16 and PD30), it was possible to control for the differences in group size experienced by subjects in the Shimozuru *et al.*'s (2007) experiment. The social pairs were maintained until the end of the experiment with their cages cleaned once a week on the corresponding observation day (see below).

Observation procedure

One rat from each pair was marked with an indelible pen (at least 24 hours before any observation). Observations were conducted in a dark room to which the cages with the pairs of rats were transported (using a wheeled trolley; Figure 2.4). The rats from each pair were individually weighed using the same procedure as for the pregnant rats (Figure 2.2); then they were transferred to a previously cleaned cage (using 95% ethanol) filled with new bedding material. Interactions between the two rats in the clean cages were videotaped for 1 hr for subsequent behavioural analysis (Figure 2.5). The tests were all run at the same time of the day (1000 h), to minimize any circadian influence.

Figure 2.4: Transport of cages to the observation room on wheeled trolley.

Figure 2.5: A pair of cages in the observation room being set up under the camera for the weekly observation period.

Behavioural analyses

Changes in the development of play-fighting behaviour in juvenile rats were monitored from week 4 (PD30) to week 7 (PD51) as in Shimozuru *et al.* (2007). The following behaviours were measured to quantify play-fighting: the frequency of pinning (one animal lying on its back while the other animal is on top; Panksepp, 1981) measured per pair of animals; and the frequency of playful attacks (one animal bringing its snout, in a fast movement, into close proximity with the nape area of the partner; Pellis & Pellis, 1990) measured per individual.

Statistical Analysis

Data are expressed as mean ± maximum-minimum value over 60 minutes (with the significance level set at $p = 0.05$). As the data were normally distributed and variances were equal, parametric statistics were used. Data were analyzed using Statistica release 8 (from

Statsoft Inc.). The frequency of playful attacks per individual and pinning frequency per pair of animals were analyzed using a repeated measures ANOVA followed by post hoc tests (Fisher's LSD) as in Shimozuru *et al.* 's (2007) analysis.

2.5 Results and discussion

2.5.1 Effects of early weaning on play development

In keeping with previous findings, play-fighting behaviour increased from week 4 to 5, reaching its maximum in week 6 and decreasing from week 7 (when rats approach sexual maturation; Janus, 1987; Panksepp, 1981; Pellis & Pellis, 1990; Shimozuru *et al.*, 2007; see Figure 2.6). Also, in agreement with earlier literature, the EW rats showed a lower frequency of play behaviour than normally weaned rats (Panksepp, 1981; Pellis & Pellis, 1990; Shimozuru *et al.*, 2007. Changes in playful attacks were signigicant (Figure 2.6a; F(4,7)=7.785; p=0.01); however, pinning frequency did not differ significantly between the EW and NW groups (F(4,1)=1.503; p =0.539), although values were consistently lower in the EW group across weeks (see Figure 2.6b). My results contrast those of Shimozuru *et al.* (2007) who found a significant difference in the frequency of pinning behaviour but not in playful attacks. Play-fighting behaviour was highly variable with some pairs playing constantly while other pairs showed low levels of play fighting frequencies (Pellis & McKenna, 1992).

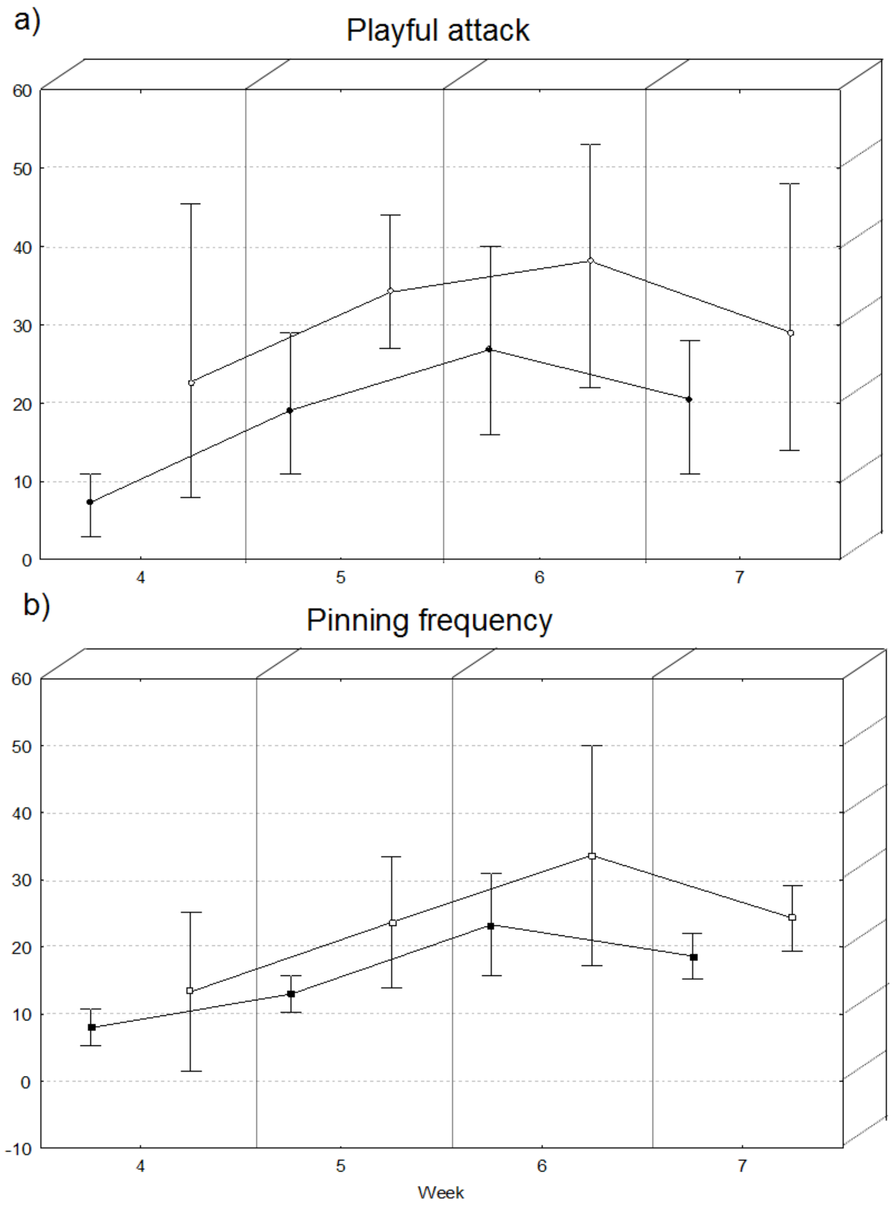

Figure 2.6: Changes in play behaviour for early weaned (closed) and normally weaned (open) rats from week 4 to 7. a) Playful attack frequencies per individual (n = 12) over 60 minutes weekly observation period; b) pinning frequency per pair (n = 6) over 60 minutes (data expressed as mean ± maximum and minimum values). For clarity, and to avoid overlap in error bars, the alignment of the curves have been manipulated but actual measurement times were identical across groups (at 4, 5, 6 and 7 weeks of age). Raw data available in appendices 4 and 5.

2.5.2 Weight gain

Weight gain from week 4 to 7 for the early weaned and normally weaned male rats is presented in Figure 2.7. Weight gain was very similar across early weaned and normally weaned rats. Both treatment groups had a constant and steady increase in body weight independently of being weaned at PD16 or PD30, consistent with previous literature (Shimozuru *et al.*, 2007).

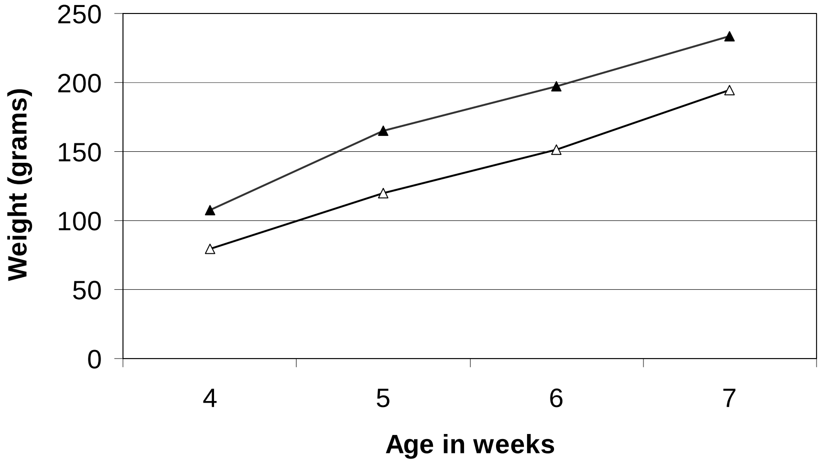

Figure 2.7: Patterns of increase in weight of early weaned (closed; week 4-7 SEs: ± 2.7, ± 4.5, ± 3.4, ± 3.8) and normally weaned (open; week 4-7 SEs: ± 2.5, ± 1.7, ± 4.1, ± 5.6) male rats over four weeks; data is expressed as weekly mean for the group. Raw data available in appendix 6.

2.6 Experiment 2

2.6.1 Methods

A Pavlovian conditioning paradigm was used for experiment two. Protocols for this experiment followed those described for experiment one. However, at weaning time (PD16 or PD30) the pairs of pups were separated to create four treatment groups and housed in one of two separate (and isolated) rooms (see description of experimental groups below and Figure 2.8): one room for the two groups exposed to a bell sound (Colony Room 1) and one room for the two groups without exposure to a bell sound (Colony Room 2, Figure 2.9).

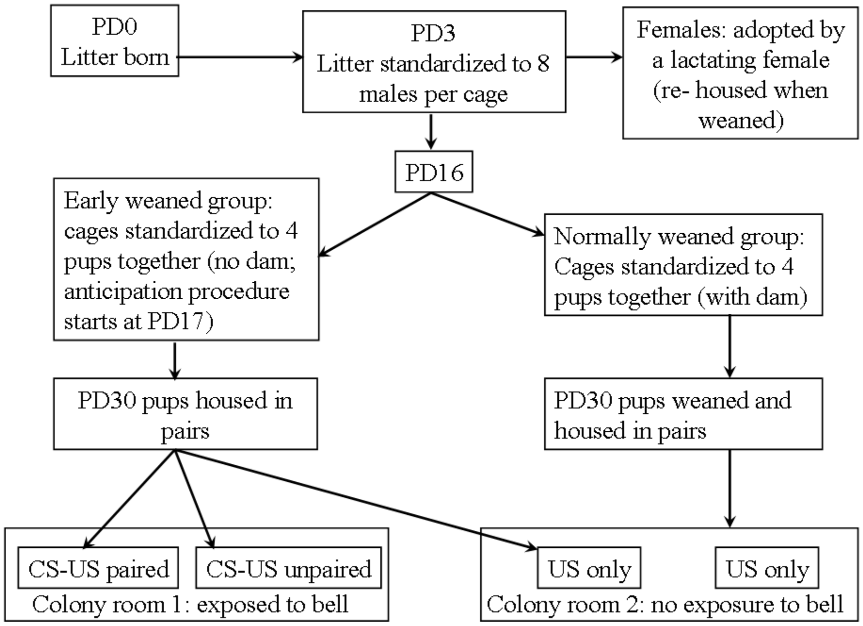

Figure 2.8: Experimental procedure for weaning time, group standardization, treatment and colony room allocation of the juvenile male rats included in the research.

Figure 2.9: A colony room (no bell). The small cages contain social pairs for the early weaned groups, while larger cages contain nursing mothers and pups for the normally weaned treatment groups.

2.6.2. Experimental groups

At PD30, the rats were assigned to one of four groups: three early weaned groups and one normally weaned group (Figure 2.8 and appendix 2).

1) EW group with classical conditioning and CS-US paired (EW/CS-USp; n = 12). The anticipation procedure started on PD17 (the day following early weaning). Training sessions were conducted in the housing room. This experimental condition involved exposing animals to the sound of a bell (3s at 78 dB at 1m) followed by the presentation of a food reward (pre-prepared portions of cheese 1 gram of cheese/individual/day; Edam from Pam's, New Zealand) in accordance with the times and intervals outlined in Table 2.1. Initially, training sessions involved the CS and the US presented with an overlap to generate an association between the two stimuli (see Table 2.1). Afterwards, the interval between the CS and the US were slowly increased within training sessions up to 180s to generate anticipatory behaviour (see Table 2.1). From the age of 17 to 28 days, the juveniles were exposed to four training sessions per day. From 29 days old, only one session was delivered daily (except on observation days in which the enrichment was not provided). To avoid the acquisition of an association between the time of day and training sessions and following Dudink *et al*. (2006), the training sessions were randomly assigned different times across the different days to fit the required number of training sessions. The bell was triggered from outside the room; therefore anticipatory behaviour occurred without disturbance or human association. The anticipation procedure and provision of the US was applied every day to the group except on observation days.

2) EW with random presentation of the CS and US stimulus (EW/CS-USr; n = 12). The only difference between this treatment condition and the group above relates to the presentation of the US (cheese). This group always received a random presentation of the US around the CS, with the US being delivered 0-30 min before or after the CS.

3) EW with US only (EW/US; n = 12). From PD17, the EW rats under this condition received their portion of cheese mixed with their normal food.

4) NW with US only (NW/US; n = 12). From PD17, the NW rats under this condition received the same treatment as the EW rats with US only.

Table 2.1: Training schedule for the presentation of the CS-US paired during the training days for the early weaned rats exposed to a Pavlovian conditioning paradigm (CS: bell sound; US: 1 gram cheese/individual/day). During the training/conditioning days PD17-PD27, 4 training sessions were done per day (the cheese portion was divided across sessions to provide only 1 gram of cheese/day). From PD28, only one training session per day was done (except on the observation day).

Post-natal day (PD)	Total number of days under this schedule	Interval time between CS-US	Total number of sessions	Total enrichment obtained per day/per individual
PD17-PD20	4	Overlap CS-US	16 trials	1 gram
PD21- PD22	2	30s interval	8 trials	1 gram
PD23	1	60s interval	4 trials	1 gram
PD24	1	120s interval	4 trials	1 gram
PD25- PD27	3	180s interval	12 trials	1 gram
PD28-PD57	30	180s interval	30 trials	1 gram

Note: inter-trial interval: minimum 9 min – maximum 30 min

Observation procedures, behavioural analyses and statistical analyses were as detailed for experiment one, following Shimozuru *et al.* (2007).

2.7 Results and discussion

2.7.1. Effect of a Pavlovian conditioning paradigm on play-fighting behaviour

Interestingly, the frequency of play-fighting behaviour of the EW/CS-USp group was positioned between the high frequency of play-fighting behaviour observed in the NW/US and the low frequencies observed in the remaining EW groups (EW/USr and EW/US; Figure 2.10 a and b). The results illustrate a positive beneficial effect from the paired Pavlovian conditioning paradigm (EW/CS-USp), as the rats in this group, despite being early weaned, showed a higher frequency of playful behaviour when compared to the EW/CS-USr and EW/US.

The repeated measures ANOVA showed significant variation across treatment groups in the frequency of playful attack (F(20, 90.499)=2.8173, p<0.001). Post-hoc tests revealed: significant differences across: (a) the EW/CS-USp and the NW/US animals (F(4,7) = 23.85; p<0.001); (b) the EW/CS-USp and the EW/US animals (F(4,7)=6.49; p=0.016); (c) the EW/CS-USr and the NW/US animals (F(4,7)=8.69, p=0.007); and (d) the EW/US and the NW/US animals (F(4,7)=18.43. p=0.0008). No significant differences were found between (e) the EW/CS-USp and the EW/CS-USr animals (F(4,7)=1.85, p=0.22) or (f) the EW/CS-USr and the EW/US animals (F(4,7)=0.31, p=0.85) in the frequency of playful attacks.

Pinning changes across the treatment groups were not significant (F(20, 30.799)=0.50249, p=.94464; Figure 2.10). However, there was an obvious trend seen with the EW groups, on average, having consistently lower frequencies of playful attacks and pinning frequencies when compared to the NW group (Figure 2.10 a and b).

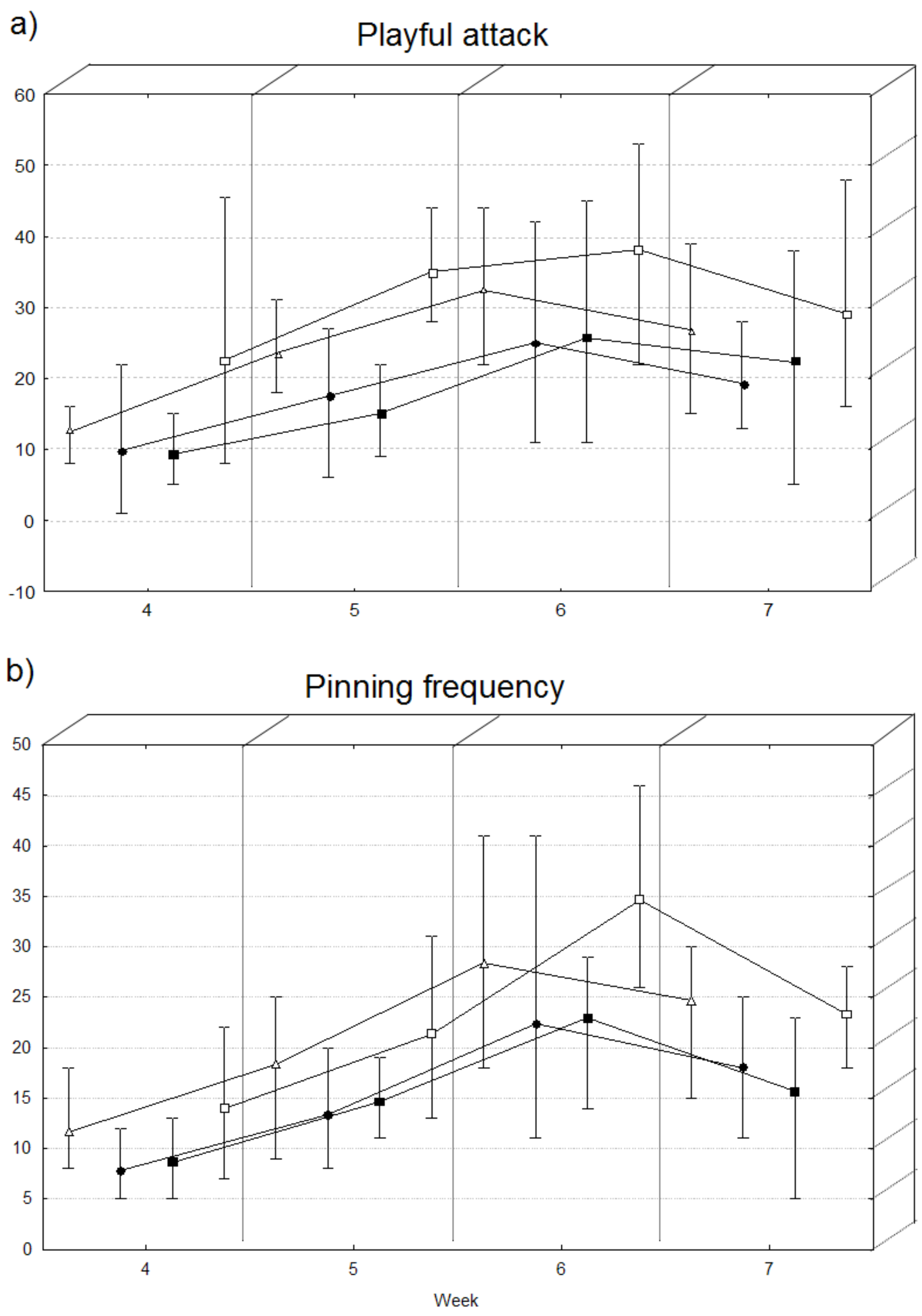

Figure 2.10: Development of play behaviour from week 4 to 7 in EW/US (closed square), NW/US (open square), EW/CS-USp (open triangle) and EW/CS-USr (closed circle). a) Playful attack frequencies measured per individual (n = 12); b) Pinning frequency measured per pair (n = 6). Data is expressed as mean ± maximum and minimum. For clarity, and to avoid overlap in the error bars, the alignment of the curves have been manipulated but actual measurement times were identical across groups (at 4, 5, 6 and 7 weeks of age). Raw data available in appendices 4 and 5.

The significant difference between the EW/CS-USp and the NW/US group reveals that the anticipatory behaviour created by the paradigm did not allowed the two groups to reach similar frequencies. However, the effect from the conditioning was sufficient as to create a significant difference between the EW/CS-USp and the EW/US. The significant difference between the EW/US and the NW/US groups is consistent with the literature characterized by higher frequencies of playful behaviour on NW animals when compared to EW ones (Dudink *et al.,* 2006 ; Shimozuru *et al.,* 2007).

2.7.2. Weight gain

The four treatment groups revealed no difference in the pattern of body-weight changes showing that weight gain was similar across groups (Figure 2.11). Rats are normally weaned by the dam at around 4 weeks of age (Rosenblatt, 1965) but pups are able to feed by themselves by the end of the second week of life (Plaut & Davis, 1972). This ability to feed independently from week two resulted in all experimental groups following a very similar linear pattern of weight gain, showing an even and constant increase in weight from week 4 to week 7.

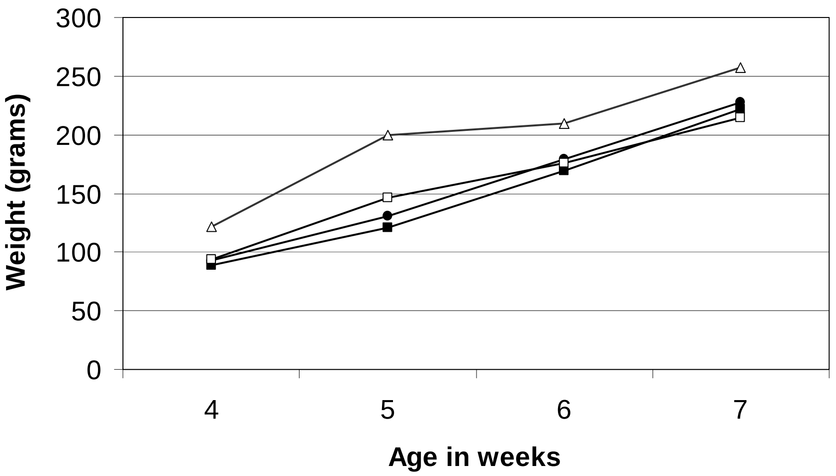

Figure 2.11. Patterns of increase in weight of early weaned and normally weaned male rats over four weeks for the four different treatment groups: early weaned with CS-US paired (open triangle; week 4-7 SEs: ± 3.0, ± 3.7, ± 4.9, ± 3.5), early weaned with CS-US unpaired (closed circle; week 4-7 SEs: ± 3.1, ± 3.6, ± 6.4, ± 5.1), early weaned with US only (closed square; week 4-7 SEs: ± 2.7, ± 3.4, ± 7.3, ± 4.1) and normally weaned with US only (open square; week 4-7 SEs: ± 5.3, ± 6.5, ± 4.0, ± 4.1). Raw data available in appendix 6.

2.8 General discussion

This study investigated whether early weaned male rats show lower frequencies of play-fighting behaviour when compared to normally weaned rats during the juvenile phase when play behaviour develops (from week 4 to week 7). In addition, I investigated whether announcing the arrival of a positive reward, by means of a Pavlovian conditioning paradigm, in order to generate anticipatory behaviour could demonstrably reduce the effects of early weaning. For that purpose, the pinning frequency and playful attacks of early weaned, normally weaned and early weaned rats exposed to a Pavlovian conditioning paradigm were observed over four weeks.

As found in previous research, the data confirms that early weaning effects the development of play in rats, leading to a marked but not significant reduction of pinning behaviour and a statistically significant reduction of playful attacks in juvenile rats observed from weeks 4 to 7 of age (Janus, 1987; Shimozuru *et al.*, 2007). During the behavioural observations, great variability was encountered in the amount of play behaviour recorded per social pair, including the NW groups (Figure 2.10; see also Pellis & McKenna, 1992).

Maternal separation and early weaning are known as stressors that effect behaviour (Ito *et al.*, 2006); deprivation of tactile and olfactory stimulation from the mother, more than milk deprivation, are the main factors affecting the pups (Hofer, 1975) as maternal care in rats tends to be quite intense prior to weaning (Kikusui *et al.*, 2005). Additionally, early weaning increases the levels of anxiety and reduces the levels of interactions between juvenile rats (Shimozuru *et al.*, 2007).

Early weaning was the stressor to which the rats were exposed during this experiment. All the EW groups showed a reduction in play frequencies, reflecting a decreased level of animal welfare. However, the level of play observed in the EW/CS-USp group was higher when compared to all the EW groups (EW/CS-USr, EW/US). This result is consistent with those recorded by Dudink *et al.*, (2006) who found increased levels of play in an "enrichment group" of early weaned pigs (which obtained access, for 15 minutes, to a hallway covered with 20g of mixed seeds provided daily in 500 g of straw changed weekly) when compared to the group that had the enrichment mixed with the normal meal. The EW/CS-USr group did not show a marked increase in the levels of play when compared to the conditioning group with paired stimulus (the same pattern was found in Dudink, *et al's.* (2006) research). This supports the postulate that expectation for the reward had a greater effect than the reward itself (Dudink *et al.*, 2006).

Anticipatory behaviour elicits dopamine release (Spruijt *et al.*, 2001). Therefore, expectation for the reward and the activation of the dopaminergic system aided in counteracting the effects of early weaning and allowed increasing the levels of play, a behaviour dependent on DA levels (Panksepp *et al.*, 1987). The higher levels of play behaviour instigated by creating anticipatory behaviour revealed that EW rats under a Pavlovian conditioning paradigm were less stressed than EW rats without conditioning. In previous research, anticipatory behaviour helped counteracting stress associated with early weaning in piglets in Dudink *et al.*'s (2006) research; similarly, anticipatory behaviour proved to be useful in reducing the effect of chronic stress in rats (van der Harst *et al.*, 2003, 2005). In my research, the Pavlovian conditioning paradigm used allowed to partially counteract weaning stress increasing the welfare of the EW rats under conditioning as reflected by the higher levels of play. The effects of anticipatory behaviour can be revealed in the frequency of play, suggesting that play remains a valuable welfare measure as proposed by Lawrence and Appleby (1996).

There is research that states that a degree of deprivation is generally required to create anticipatory behaviour (Barbano & Cador, 2005). Barbano and Cador (2005) found that without a certain degree of deprivation, anticipatory behaviour towards food could not be generated in rats exposed to a variety of foods differing in palatability. However, in my research, without the need for food deprivation, the anticipation for the reward was successfully elicited; similarly, in Dudink *et al.* (2006) expectation for 20g of mixed seeds and access to straw created anticipatory behaviour in EW piglets with water and food freely available (Dudink *et al.*, 2006). The results suggest that a Pavlovian conditioning paradigm can be used as an enrichment tool, creating anticipatory behaviour without the need for deprivation.

The body weight gain of rats in experiment one had a similar pattern compared to experiment two. This similarity in weight gain across all group confirms that weight gain followed a trend that was not affected by the US itself (i.e. rats in experiment 1 (EW) did not had access to any US and had a similar trend of weight gain when compared to the EW/US groups from experiment 2). Access to mother's milk in the NW groups did not lead to faster body weight gain either, as the weight gain was comparable to the one seen in the EW groups being fed with pelleted food. Then, it can be inferred that the nutritional properties of the enrichment had no effect on behavioural development (Dudink *et al.,* 2006; Shimozuru *et al.,* 2007).

Not being able to deliver the food reward without entering the room was a major limitation during this research. Providing the reward without human contact, as in Dudink *et al.* (2006), would have been ideal. There is the possibility that an association could have been formed, with a person entering the room creating anticipatory behaviour to some degree. Further research should aim to reduce the level of human disturbances when delivering the US (therefore being automatically or externally delivered as in Dudink *et al*., 2006).

This research only observed the short term effects of anticipatory behaviour. Further research could investigate the long term effects of anticipatory behaviour, or if the effects are still valid in mature animals. Anhedonic subjects have reduced levels of DA release, therefore loosing motivation and the feeling of pleasure for normally pleasurable events. Anticipatory behaviour increases DA release and restores the impairment to anticipate for positive stimulus (Von Frijtag *et al*., 2000). Therefore, it would be interesting to investigate the longevity of the effect of Pavlovian conditioning.

In summary this thesis allowed me to replicate Shimozuru *et al*. (2007), and to apply Dudink *et al*.'s (2006) procedure in a new species, the rat. A Pavlovian conditioning paradigm was used to create anticipatory behaviour to reduce the levels of stress caused by early weaning, in order to increase the frequency of play fighting behaviours in juvenile rats and increase their welfare. The main conclusions revealed by this study were that: (1) EW effects play fighting behaviour: pinning behaviour decreases markedly while the frequency of playful attacks is significantly reduced in juvenile rats observed over weeks 4 to 7. (2) The use of a Pavlovian conditioning paradigm demonstrably increased the frequency of play behaviour observed in EW rats when compared to NW ones. However, the level of play of the EW group under conditioning did not reach the level of the NW group. (3) The results indicate that announcement of enrichment (EW/CS-USp) and not the enrichment itself (EW/US) is the main factor allowing to increased play behaviour in early weaned rats, potentially counteracting the stress related to early weaning. (4) The results suggest that the Pavlovian conditioning paradigm is a useful enrichment tool to increase the welfare of animals by eliciting DA release.

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Chapter 3

Conclusions

The aim of this study was to examine (i) if early weaning influenced the development of playful attacks and pinning frequency in rats and (ii) the effects of anticipatory behaviour generated by a Pavlovian conditioning paradigm on early – and normally – weaned rats. This last chapter summarizes the implications of the study and highlights areas of future research.

3.1 Early weaning

Early weaning involves separating juveniles from their mother before their normal weaning time. Weaning is an important event in the life of animals, influencing their behavioural and physiological development as an outcome of having to become nutritionally and behaviourally independent (Kanari *et al.*, 2005). Additionally, early abrupt weaning, as commonly used in conventional husbandry systems, results in a stressful experience for animals instead of a gradual transition to independence (Dudink et al. 2006). My research adds to the literature revealing that early weaning effects behavioural development and decreases welfare as revealed by the decreased frequency of play-related behaviour in rats.

Many studies focus on only one aspect of problems associated with early weaning on a particular species (e.g. progressive versus sudden weaning in sheep (Orgeur *et al.*, 1998), effects of weaning at different ages on piglet behaviour (Worobec *et al.*, 1999), effects of communal areas pre and post weaning in pigs (Weary *et al.*, 2002), physiological and behavioural influences of different housing systems on weanling horses (Heleski *et al.*, 2002)). Early weaning generates a difference in diet, maternal separation, reduction in contact between juveniles and adults, and many other changes. It is therefore difficult to recognize the exact factor affecting behavioural development; however, maternal separation seems to be a major factor (Francis *et al*., 2002). In this research, a double standardization approach was taken when separating the pups from the dam to create a constant level of interaction between pups (first creating groups of four pups and then social pairs). However, even with the double standardization process, there were differences in levels of social interaction as the pups housed with their nursing mother would have been influenced by maternal contact received prior to weaning. Providing early weaned pups with a surrogate for adult interaction may not have solved that issue, as allowing contact with an unfamiliar dam can not equate contact with a nursing mother. Holloway and Suter (2003) evaluated the effect of play deprivation without social isolation by housing pairs of rats in cages divided by wire mesh and therefore allowing a certain degree of contact (olfactory, visual, auditory and to a certain degree tactile). A similar setup could have been used to potentially control for maternal contact across my normally and early weaned groups. Hofer (1975) found that the presence of the mother, even if available only behind a wire mesh, could significantly reduce the effects of maternal separation by providing tactile and olfactory stimulation. The use of the wire mesh technique opens new possibilities in future research to partially control the confounding variable of social isolation.

3.2 Pavlovian conditioning

I used previous studies to develop my hypothesis that generating anticipatory behaviour using Pavlovian conditioning reduces the effects of early weaning, as revealed by higher levels of play-related behaviours in the conditioned group in comparison to control groups. The difficulty of using the Pavlovian conditioning paradigm is that other unwanted associations can be created without the researcher being aware. All that is needed for an association to be formed is for two stimuli to have some degree of proximity in time; associations can even be formed without a predictive relationship between the stimuli (Savastano & Miller, 1998).

A major flaw in my research was that the reward was delivered to the conditioned groups manually (a person had to enter the colony room to deliver the reward). Dudink *et al.* (2006) devised a protocol that allowed for the reward to be delivered from outside the housing room. The constant repetition of procedures, to standardize the treatments across all the groups in my study, may have created some degree of anticipation in the non-conditioned groups. Even though care was taken to avoid creating an association between cleaning, feeding time and conditioning, there is the possibility that a human entering the room may have created enough expectation in the rats for something to happen, independently of the treatment group. However, as all the rats received the same amount of human contact, the results are still valid, supporting the effectiveness of Pavlovian conditioning to reduce the effects of stress. The increased levels of play, in the conditioned early weaned group appear to be a true result of anticipatory behaviour which is known to elicit the release of dopamine through the delayed presentation of the reward. Van der Harst *et al*. (2005) showed that the increased levels of dopamine associated with anticipatory behaviour were effective for reducing signs of anhedonia in rats. Similarly, in my research, anticipatory behaviour has revealed its effectiveness in reducing the effects of early weaning in rats.

3.3 Future research

Whereas some studies have found evidence to suggest that anticipatory behaviour can be elicited in appetitive tasks (Dudink *et al*., 2006), others have found that deprivation is required to elicit anticipatory behaviour (Barbano & Cador, 2005). Further studies exploring the magnitude and potential reinforcers that can be used in several species to create motivation and expectation are still required, as the magnitudes and rewards are species specific. In general, current research on magnitude of the reinforcers is based on differential response rates across groups (Gallistel & Gibbon, 2000). However, further studies should also investigate the effects of varied magnitude of the US as the magnitude determines the asymptote (Morris & Bouton, 2006). Anticipatory behaviour seems to follows Weber’s Law, therefore resulting in decreased appetitive behaviour with decreasing reward magnitudes (Pellegrini & Papini, 2007).

Even with the extensive research done so far in neurosciences, the release of dopamine and all its biological functions are still being assessed (Wise and Rompre, 1989). It would be unreasonable to think that dopaminergic neurons are the only and final path for all rewards. Other reward transmitter systems have been suggested to work independently and in parallel (Phillips, 1984). Studies elucidating the reward mechanism involved in expectation for rewards may provide further information on the possibilities and potential for the use of Pavlovian conditioning in animal welfare.

At present there is little research investigating and correlating behaviour and physiological measures. Faecal cortisol in rats is easy to sample using non invasive techniques. Due to the complexity of separating physiological parameters from other biological functions, studies including physiological and behavioural measures may provide further insight into the biological effects of subjects under a Pavlovian conditioning paradigm. Further research, based on the present findings, will investigate the effect of anticipatory behaviour on stress hormones, looking for correlations between stress hormone release and behaviour.

3.4 Conclusions

Previous studies have instigated and evaluated the use of anticipatory behaviour generated by a Pavlovian conditioning paradigm to enhance welfare in animals (e.g. Spruijt et al., 2001; van der Harst et al. 2003a; van der Harst et al., 2003b; Vinke et al., 2004; van der Harst et al., 2005; Dudink et al., 2006). This study adds to the list of studies demonstrating that a Pavlovian conditioning paradigm can be used effectively to decrease to some degree the effects of stressful events such as early weaning and that it can effect positively behaviour by increasing the levels of play behaviour in rats. Therefore, this study supports the findings of the two articles that inspired the research question: (1) early weaning does decrease playful behaviour and (2) anticipatory behaviour can at least partially counteract the effects of early weaning. The use of Pavlovian conditioning enhanced the welfare of early weaned piglets in Dudink *et al*.'s (2006) research. In my research, the welfare of early weaned rats was enhanced using a similar procedure. Pavlovian conditioning is an easy, non invasive and inexpensive procedure that can potentially be used to enhance the welfare of captive animals across a range of situations and species, having the advantage of being easily adapted to the specific needs of different species. Pavlovian conditioning can be used as a way of providing enrichment in a variety of contexts to enhance the welfare state of animals.

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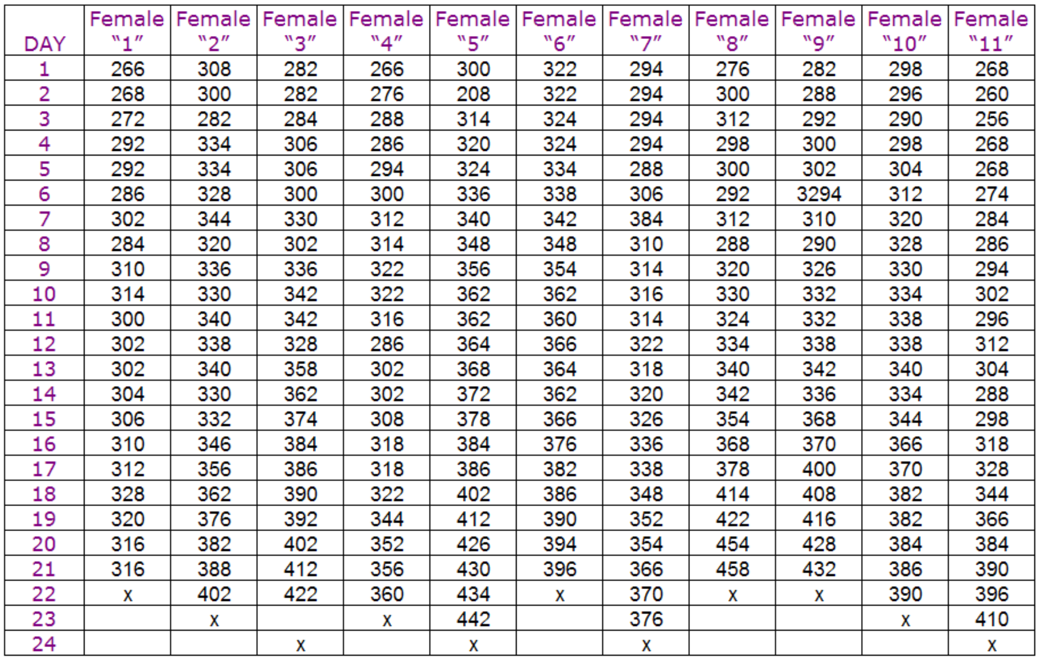
Appendices

Appendix 1

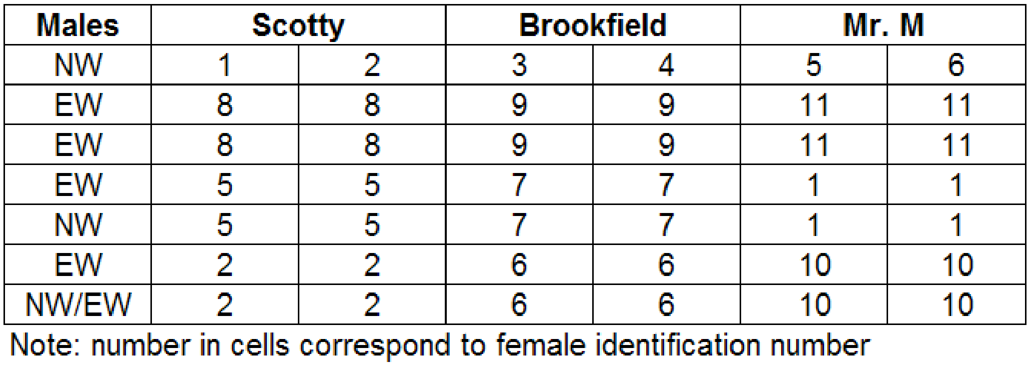
Pregnancy Weight Chart (in grams)



DAY	Female “1”	Female “2”	Female “3”	Female “4”	Female “5”	Female “6”	Female “7”	Female “8”	Female “9”	Female “10”	Female “11”
1	266	308	282	266	300	322	294	276	282	298	268
2	268	300	282	276	208	322	294	300	288	296	260
3	272	282	284	288	314	324	294	312	292	290	256
4	292	334	306	286	320	324	294	298	300	298	268
5	292	334	306	294	324	334	288	300	302	304	268
6	286	328	300	300	336	338	306	292	3294	312	274
7	302	344	330	312	340	342	384	312	310	320	284
8	284	320	302	314	348	348	310	288	290	328	286
9	310	336	336	322	356	354	314	320	326	330	294
10	314	330	342	322	362	362	316	330	332	334	302
11	300	340	342	316	362	360	314	324	332	338	296
12	302	338	328	286	364	366	322	334	338	338	312
13	302	340	358	302	368	364	318	340	342	340	304
14	304	330	362	302	372	362	320	342	336	334	288
15	306	332	374	308	378	366	326	354	368	344	298
16	310	346	384	318	384	376	336	368	370	366	318
17	312	356	386	318	386	382	338	378	400	370	328
18	328	362	390	322	402	386	348	414	408	382	344
19	320	376	392	344	412	390	352	422	416	382	366
20	316	382	402	352	426	394	354	454	428	384	384
21	316	388	412	356	430	396	366	458	432	386	390
22	x	402	422	360	434	x	370	x	x	390	396
23		x		x	442		376			x	410
24			x		x		x				x

Appendix 2

Males' allocation across breeding females and allocation of social pairs to treatment groups.



Males	Scotty		Brookfield		Mr. M	
NW	1	2	3	4	5	6
EW	8	8	9	9	11	11
EW	8	8	9	9	11	11
EW	5	5	7	7	1	1
NW	5	5	7	7	1	1
EW	2	2	6	6	10	10
NW/EW	2	2	6	6	10	10

Note: number in cells correspond to female identification number

Three male rats were allocated randomly across the different available females. Weaned pairs of pups from those females were then allocated to the different treatment groups according to the table above.

Appendix 3

Fate of the animals used for this experiment

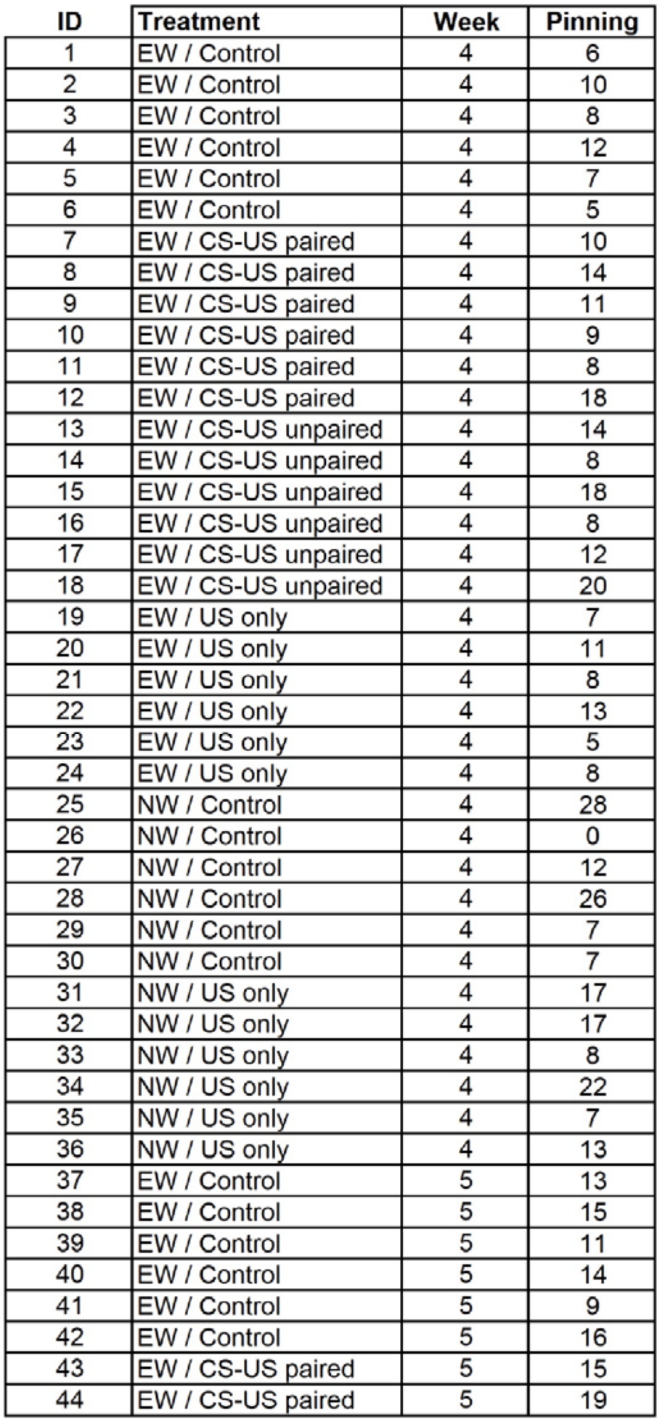
The protocol for this experiment allowed me to re-house all the animals at the end of the experiment. Only four extra males were born during the breeding period. Extra males and all female juveniles were successfully adopted by dams that had her pups removed to standardize the litters. For the dams to adopt the new pups, these were rubbed in the dams' bedding material. Pups remained with the dam until their normal weaning time, plus one week, before being put up for adoption (Figure A.1). To ensure the wellbeing of the pups, the animals were re-housed through several pet shops from the wider Waikato area and Bay of Plenty.

All the adults rats used for breeding (males and females), all the female pups born and 7 male pups were successfully re-housed. Sixty nine remaining males (approximately 60 days old) remained in the animal facilities and were used in further behavioural research.

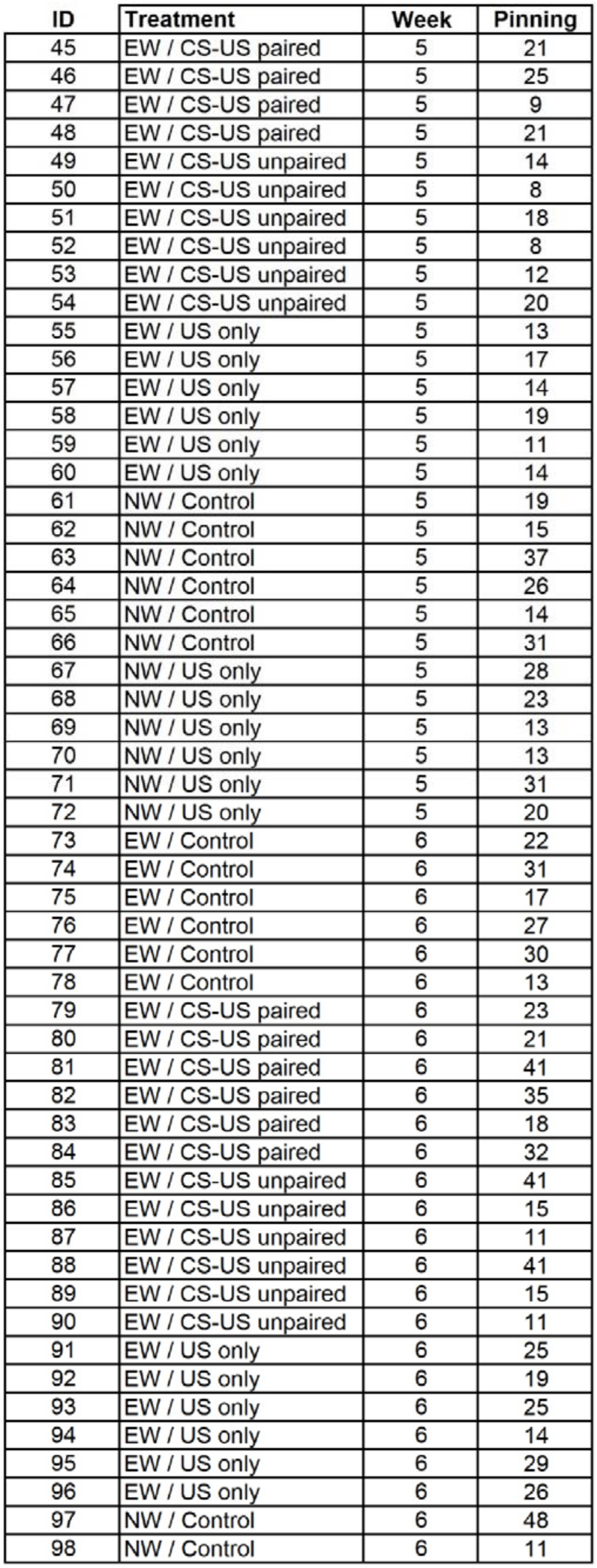
Figure A.1: Weaned female rats born during the course of this experiment ready to be re-housed as pets.

Appendix 4

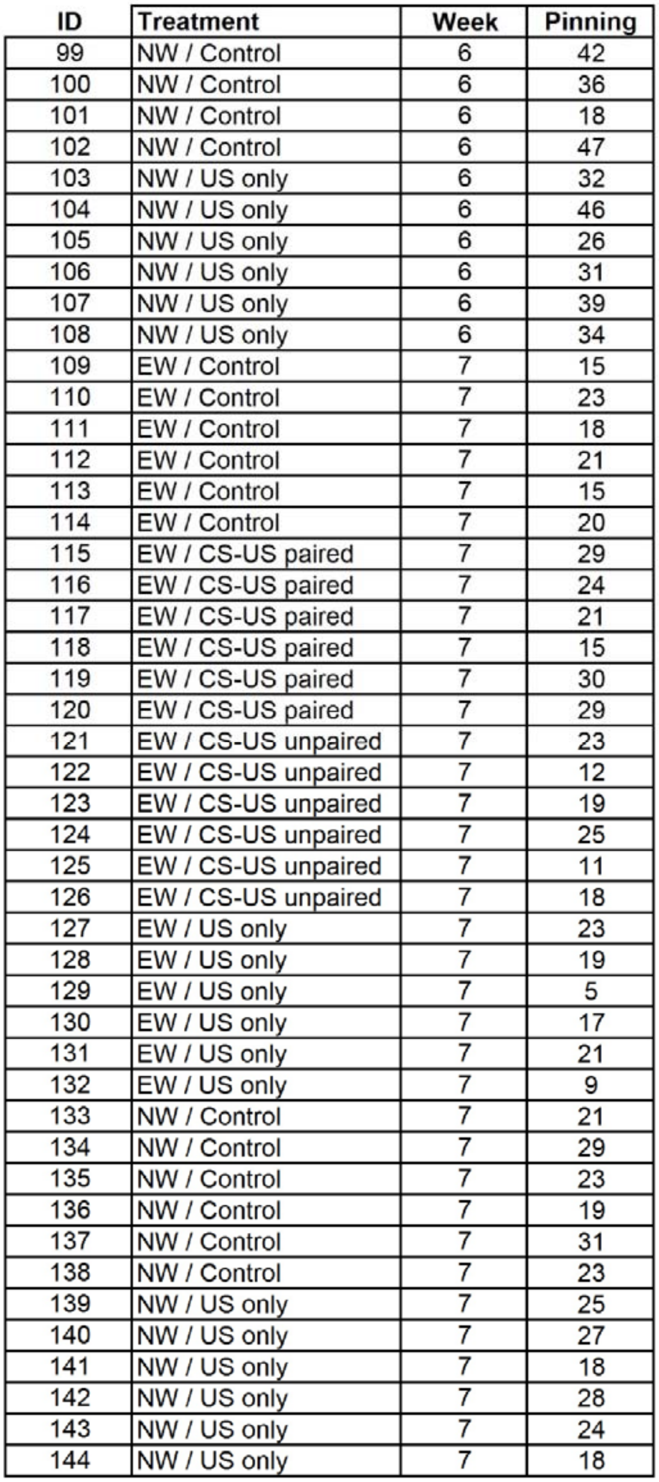
Weekly pinning frequency



ID	Treatment	Week	Pinning
1	EW / Control	4	6
2	EW / Control	4	10
3	EW / Control	4	8
4	EW / Control	4	12
5	EW / Control	4	7
6	EW / Control	4	5
7	EW / CS-US paired	4	10
8	EW / CS-US paired	4	14
9	EW / CS-US paired	4	11
10	EW / CS-US paired	4	9
11	EW / CS-US paired	4	8
12	EW / CS-US paired	4	18
13	EW / CS-US unpaired	4	14
14	EW / CS-US unpaired	4	8
15	EW / CS-US unpaired	4	18
16	EW / CS-US unpaired	4	8
17	EW / CS-US unpaired	4	12
18	EW / CS-US unpaired	4	20
19	EW / US only	4	7
20	EW / US only	4	11
21	EW / US only	4	8
22	EW / US only	4	13
23	EW / US only	4	5
24	EW / US only	4	8
25	NW / Control	4	28
26	NW / Control	4	0
27	NW / Control	4	12
28	NW / Control	4	26
29	NW / Control	4	7
30	NW / Control	4	7
31	NW / US only	4	17
32	NW / US only	4	17
33	NW / US only	4	8
34	NW / US only	4	22
35	NW / US only	4	7
36	NW / US only	4	13
37	EW / Control	5	13
38	EW / Control	5	15
39	EW / Control	5	11
40	EW / Control	5	14
41	EW / Control	5	9
42	EW / Control	5	16
43	EW / CS-US paired	5	15
44	EW / CS-US paired	5	19



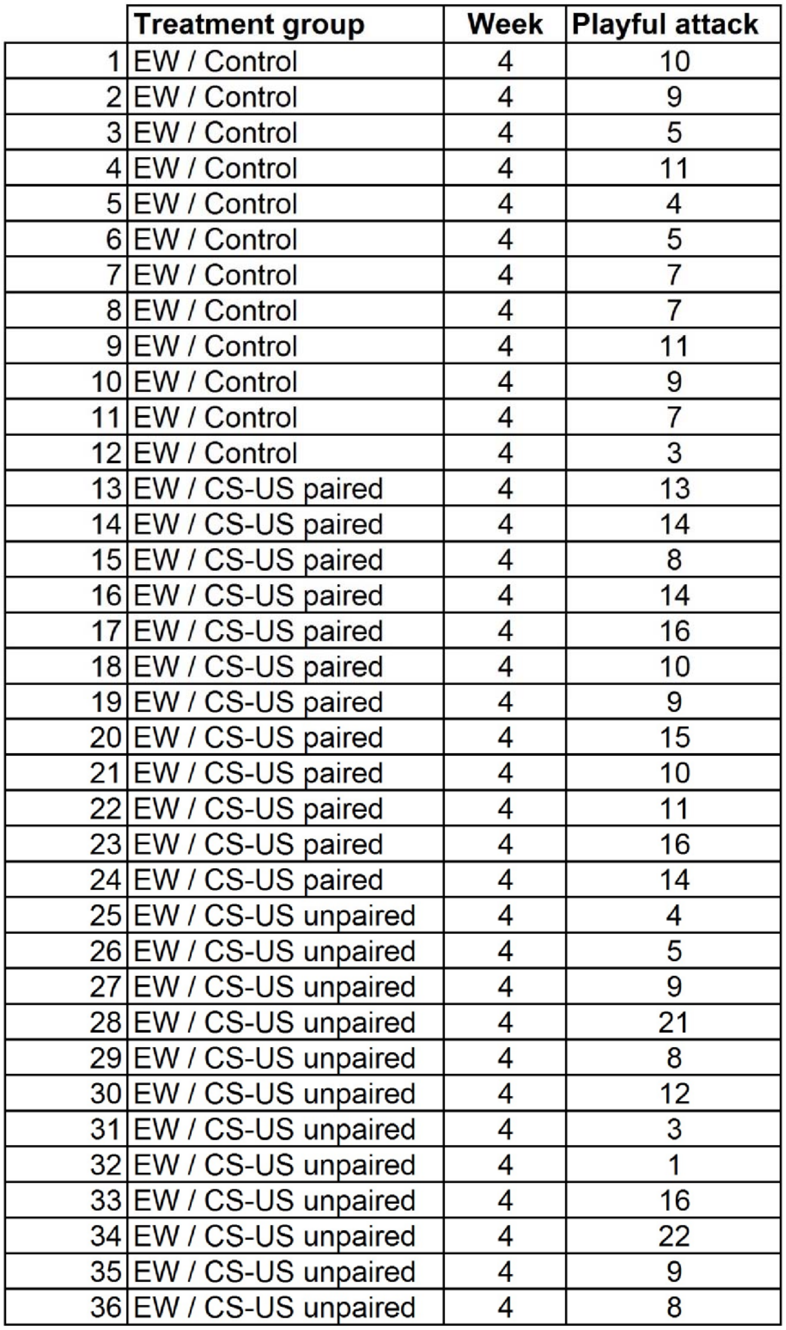
ID	Treatment	Week	Pinning
45	EW / CS-US paired	5	21
46	EW / CS-US paired	5	25
47	EW / CS-US paired	5	9
48	EW / CS-US paired	5	21
49	EW / CS-US unpaired	5	14
50	EW / CS-US unpaired	5	8
51	EW / CS-US unpaired	5	18
52	EW / CS-US unpaired	5	8
53	EW / CS-US unpaired	5	12
54	EW / CS-US unpaired	5	20
55	EW / US only	5	13
56	EW / US only	5	17
57	EW / US only	5	14
58	EW / US only	5	19
59	EW / US only	5	11
60	EW / US only	5	14
61	NW / Control	5	19
62	NW / Control	5	15
63	NW / Control	5	37
64	NW / Control	5	26
65	NW / Control	5	14
66	NW / Control	5	31
67	NW / US only	5	28
68	NW / US only	5	23
69	NW / US only	5	13
70	NW / US only	5	13
71	NW / US only	5	31
72	NW / US only	5	20
73	EW / Control	6	22
74	EW / Control	6	31
75	EW / Control	6	17
76	EW / Control	6	27
77	EW / Control	6	30
78	EW / Control	6	13
79	EW / CS-US paired	6	23
80	EW / CS-US paired	6	21
81	EW / CS-US paired	6	41
82	EW / CS-US paired	6	35
83	EW / CS-US paired	6	18
84	EW / CS-US paired	6	32
85	EW / CS-US unpaired	6	41
86	EW / CS-US unpaired	6	15
87	EW / CS-US unpaired	6	11
88	EW / CS-US unpaired	6	41
89	EW / CS-US unpaired	6	15
90	EW / CS-US unpaired	6	11
91	EW / US only	6	25
92	EW / US only	6	19
93	EW / US only	6	25
94	EW / US only	6	14
95	EW / US only	6	29
96	EW / US only	6	26
97	NW / Control	6	48
98	NW / Control	6	11



ID	Treatment	Week	Pinning
99	NW / Control	6	42
100	NW / Control	6	36
101	NW / Control	6	18
102	NW / Control	6	47
103	NW / US only	6	32
104	NW / US only	6	46
105	NW / US only	6	26
106	NW / US only	6	31
107	NW / US only	6	39
108	NW / US only	6	34
109	EW / Control	7	15
110	EW / Control	7	23
111	EW / Control	7	18
112	EW / Control	7	21
113	EW / Control	7	15
114	EW / Control	7	20
115	EW / CS-US paired	7	29
116	EW / CS-US paired	7	24
117	EW / CS-US paired	7	21
118	EW / CS-US paired	7	15
119	EW / CS-US paired	7	30
120	EW / CS-US paired	7	29
121	EW / CS-US unpaired	7	23
122	EW / CS-US unpaired	7	12
123	EW / CS-US unpaired	7	19
124	EW / CS-US unpaired	7	25
125	EW / CS-US unpaired	7	11
126	EW / CS-US unpaired	7	18
127	EW / US only	7	23
128	EW / US only	7	19
129	EW / US only	7	5
130	EW / US only	7	17
131	EW / US only	7	21
132	EW / US only	7	9
133	NW / Control	7	21
134	NW / Control	7	29
135	NW / Control	7	23
136	NW / Control	7	19
137	NW / Control	7	31
138	NW / Control	7	23
139	NW / US only	7	25
140	NW / US only	7	27
141	NW / US only	7	18
142	NW / US only	7	28
143	NW / US only	7	24
144	NW / US only	7	18

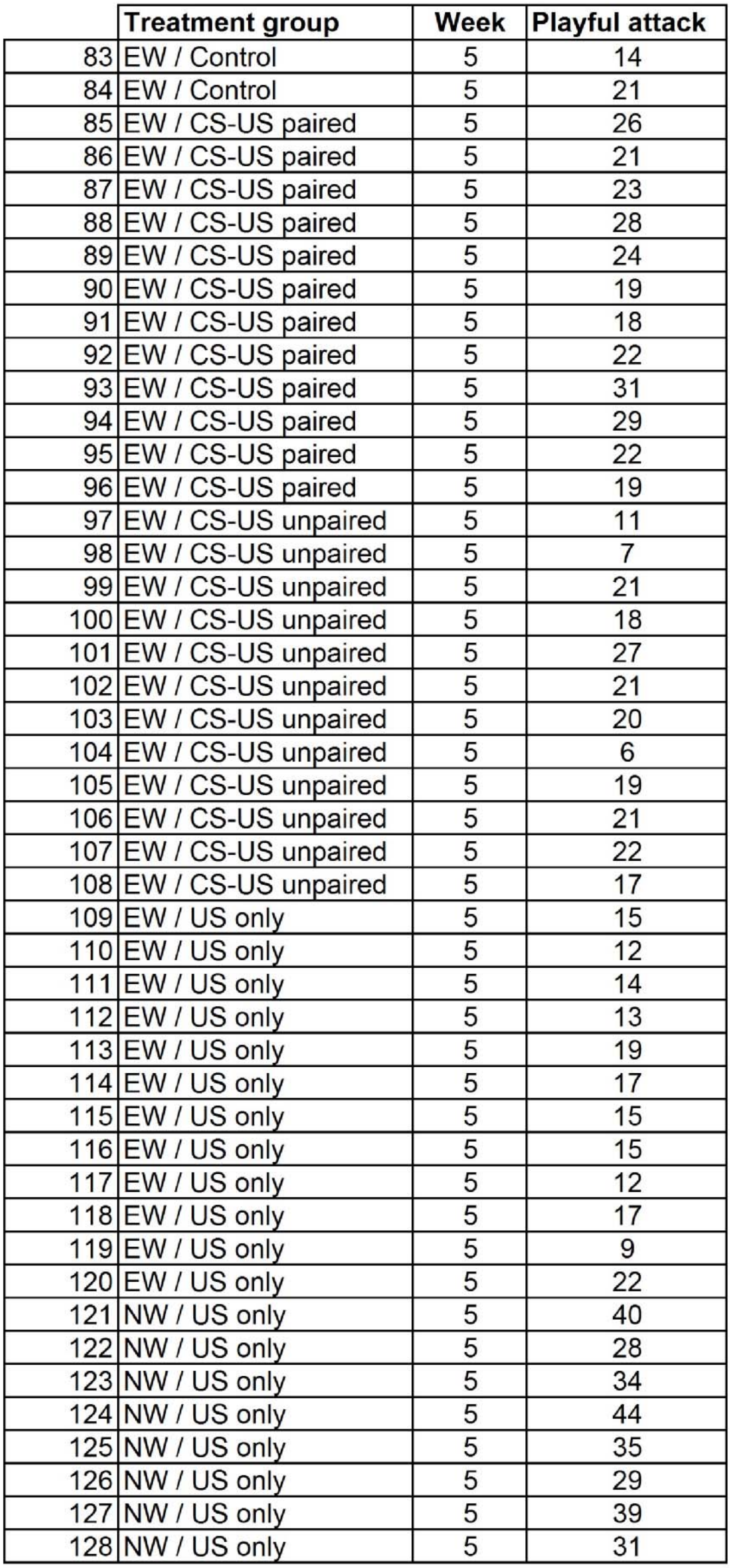
Appendix 5

Weekly playful attack frequency

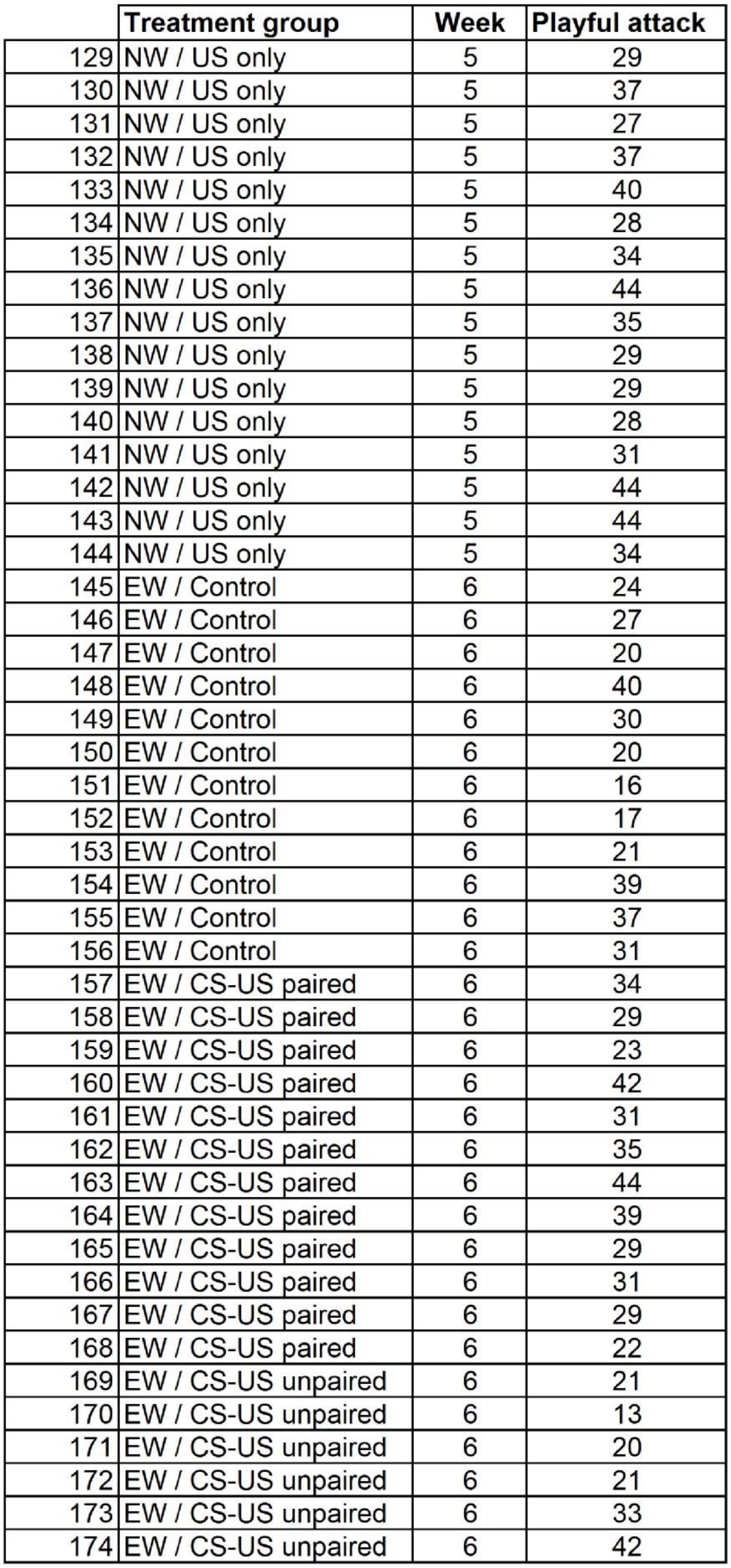


	Treatment group	Week	Playful attack
1	EW / Control	4	10
2	EW / Control	4	9
3	EW / Control	4	5
4	EW / Control	4	11
5	EW / Control	4	4
6	EW / Control	4	5
7	EW / Control	4	7
8	EW / Control	4	7
9	EW / Control	4	11
10	EW / Control	4	9
11	EW / Control	4	7
12	EW / Control	4	3
13	EW / CS-US paired	4	13
14	EW / CS-US paired	4	14
15	EW / CS-US paired	4	8
16	EW / CS-US paired	4	14
17	EW / CS-US paired	4	16
18	EW / CS-US paired	4	10
19	EW / CS-US paired	4	9
20	EW / CS-US paired	4	15
21	EW / CS-US paired	4	10
22	EW / CS-US paired	4	11
23	EW / CS-US paired	4	16
24	EW / CS-US paired	4	14
25	EW / CS-US unpaired	4	4
26	EW / CS-US unpaired	4	5
27	EW / CS-US unpaired	4	9
28	EW / CS-US unpaired	4	21
29	EW / CS-US unpaired	4	8
30	EW / CS-US unpaired	4	12
31	EW / CS-US unpaired	4	3
32	EW / CS-US unpaired	4	1
33	EW / CS-US unpaired	4	16
34	EW / CS-US unpaired	4	22
35	EW / CS-US unpaired	4	9
36	EW / CS-US unpaired	4	8

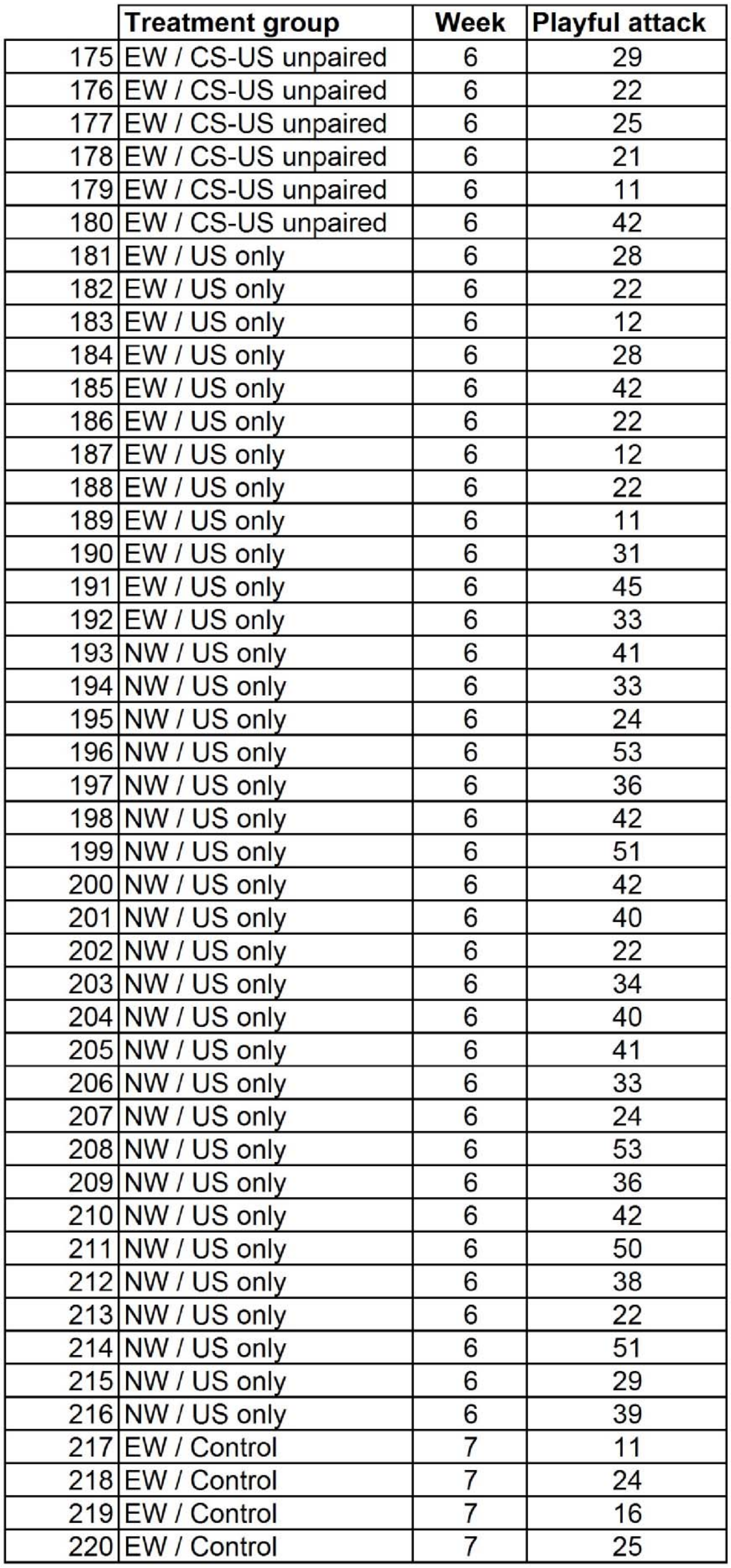
	Treatment group	Week	Playful attack
37	EW / US only	4	6
38	EW / US only	4	9
39	EW / US only	4	8
40	EW / US only	4	5
41	EW / US only	4	13
42	EW / US only	4	15
43	EW / US only	4	6
44	EW / US only	4	11
45	EW / US only	4	7
46	EW / US only	4	8
47	EW / US only	4	11
48	EW / US only	4	13
49	NW / US only	4	20
50	NW / US only	4	22
51	NW / US only	4	8
52	NW / US only	4	45
53	NW / US only	4	27
54	NW / US only	4	13
55	NW / US only	4	19
56	NW / US only	4	17
57	NW / US only	4	9
58	NW / US only	4	35
59	NW / US only	4	33
60	NW / US only	4	24
61	NW / US only	4	20
62	NW / US only	4	22
63	NW / US only	4	8
64	NW / US only	4	45
65	NW / US only	4	27
66	NW / US only	4	13
67	NW / US only	4	19
68	NW / US only	4	28
69	NW / US only	4	14
70	NW / US only	4	37
71	NW / US only	4	20
72	NW / US only	4	18
73	EW / Control	5	16
74	EW / Control	5	18
75	EW / Control	5	29
76	EW / Control	5	11
77	EW / Control	5	24
78	EW / Control	5	16
79	EW / Control	5	25
80	EW / Control	5	15
81	EW / Control	5	19
82	EW / Control	5	21



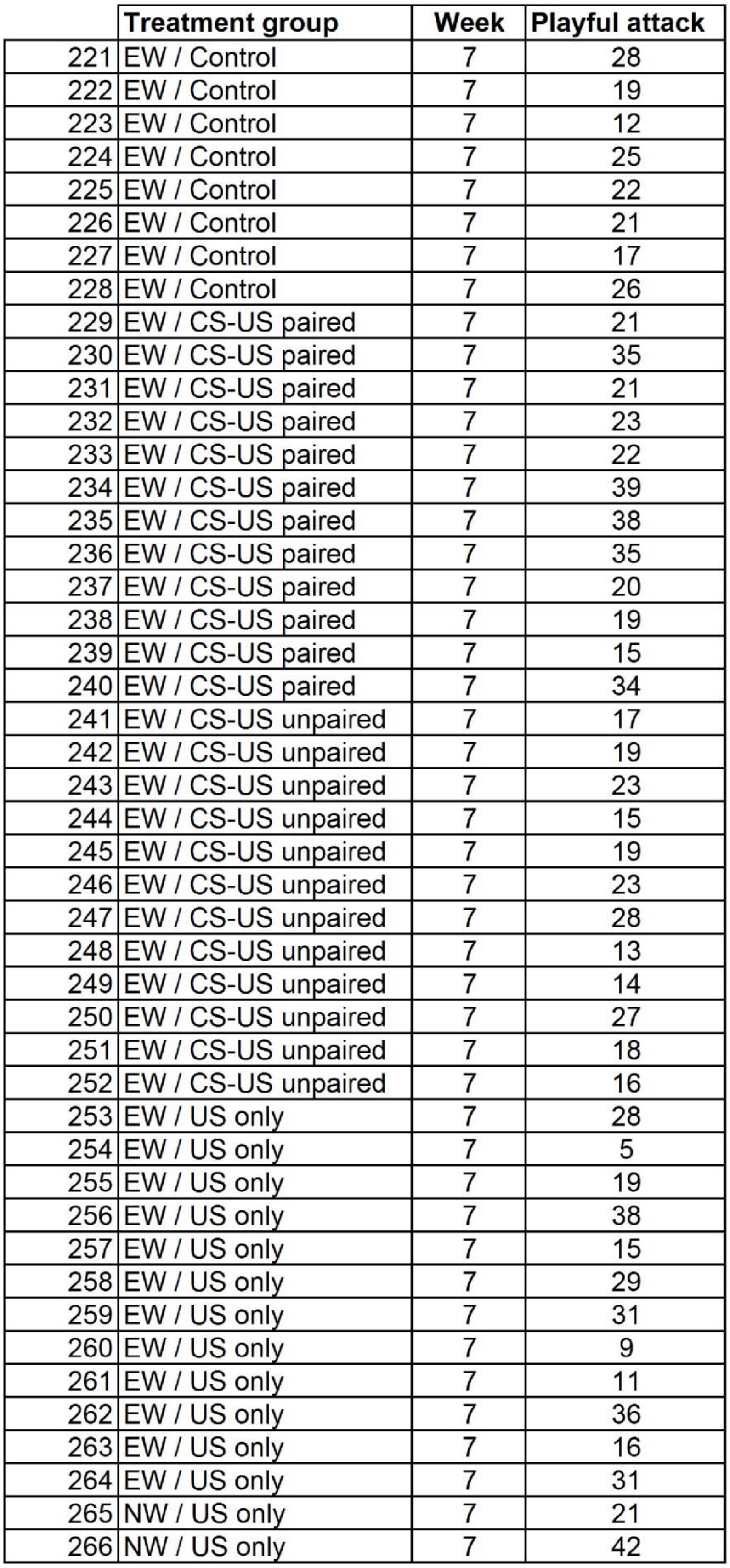
	Treatment group	Week	Playful attack
83	EW / Control	5	14
84	EW / Control	5	21
85	EW / CS-US paired	5	26
86	EW / CS-US paired	5	21
87	EW / CS-US paired	5	23
88	EW / CS-US paired	5	28
89	EW / CS-US paired	5	24
90	EW / CS-US paired	5	19
91	EW / CS-US paired	5	18
92	EW / CS-US paired	5	22
93	EW / CS-US paired	5	31
94	EW / CS-US paired	5	29
95	EW / CS-US paired	5	22
96	EW / CS-US paired	5	19
97	EW / CS-US unpaired	5	11
98	EW / CS-US unpaired	5	7
99	EW / CS-US unpaired	5	21
100	EW / CS-US unpaired	5	18
101	EW / CS-US unpaired	5	27
102	EW / CS-US unpaired	5	21
103	EW / CS-US unpaired	5	20
104	EW / CS-US unpaired	5	6
105	EW / CS-US unpaired	5	19
106	EW / CS-US unpaired	5	21
107	EW / CS-US unpaired	5	22
108	EW / CS-US unpaired	5	17
109	EW / US only	5	15
110	EW / US only	5	12
111	EW / US only	5	14
112	EW / US only	5	13
113	EW / US only	5	19
114	EW / US only	5	17
115	EW / US only	5	15
116	EW / US only	5	15
117	EW / US only	5	12
118	EW / US only	5	17
119	EW / US only	5	9
120	EW / US only	5	22
121	NW / US only	5	40
122	NW / US only	5	28
123	NW / US only	5	34
124	NW / US only	5	44
125	NW / US only	5	35
126	NW / US only	5	29
127	NW / US only	5	39
128	NW / US only	5	31



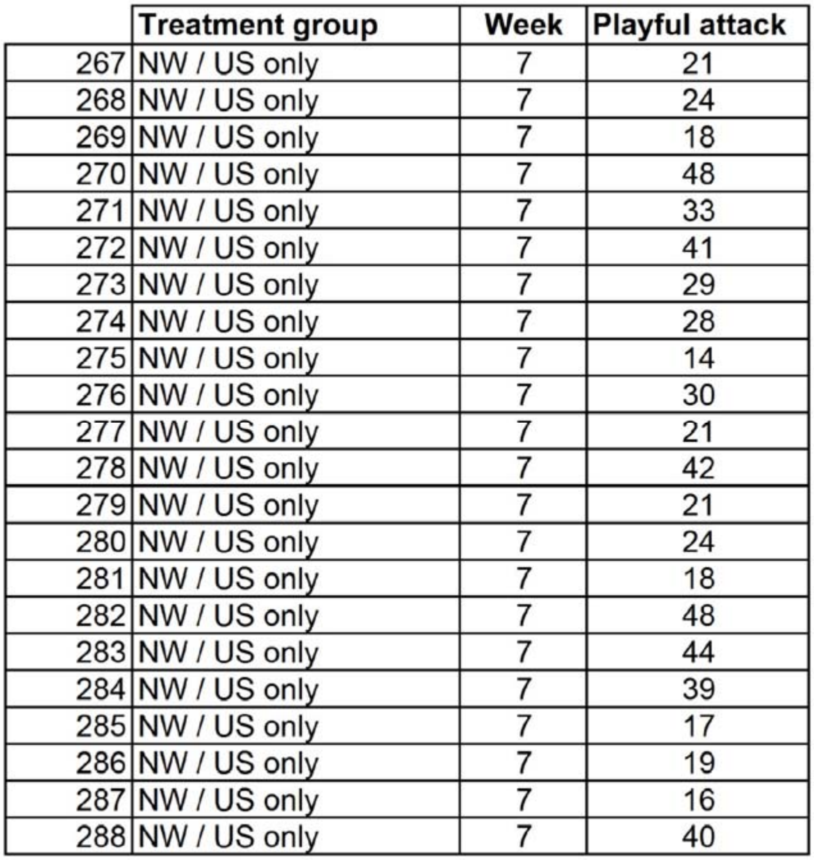
	Treatment group	Week	Playful attack
129	NW / US only	5	29
130	NW / US only	5	37
131	NW / US only	5	27
132	NW / US only	5	37
133	NW / US only	5	40
134	NW / US only	5	28
135	NW / US only	5	34
136	NW / US only	5	44
137	NW / US only	5	35
138	NW / US only	5	29
139	NW / US only	5	29
140	NW / US only	5	28
141	NW / US only	5	31
142	NW / US only	5	44
143	NW / US only	5	44
144	NW / US only	5	34
145	EW / Control	6	24
146	EW / Control	6	27
147	EW / Control	6	20
148	EW / Control	6	40
149	EW / Control	6	30
150	EW / Control	6	20
151	EW / Control	6	16
152	EW / Control	6	17
153	EW / Control	6	21
154	EW / Control	6	39
155	EW / Control	6	37
156	EW / Control	6	31
157	EW / CS-US paired	6	34
158	EW / CS-US paired	6	29
159	EW / CS-US paired	6	23
160	EW / CS-US paired	6	42
161	EW / CS-US paired	6	31
162	EW / CS-US paired	6	35
163	EW / CS-US paired	6	44
164	EW / CS-US paired	6	39
165	EW / CS-US paired	6	29
166	EW / CS-US paired	6	31
167	EW / CS-US paired	6	29
168	EW / CS-US paired	6	22
169	EW / CS-US unpaired	6	21
170	EW / CS-US unpaired	6	13
171	EW / CS-US unpaired	6	20
172	EW / CS-US unpaired	6	21
173	EW / CS-US unpaired	6	33
174	EW / CS-US unpaired	6	42



	Treatment group	Week	Playful attack
175	EW / CS-US unpaired	6	29
176	EW / CS-US unpaired	6	22
177	EW / CS-US unpaired	6	25
178	EW / CS-US unpaired	6	21
179	EW / CS-US unpaired	6	11
180	EW / CS-US unpaired	6	42
181	EW / US only	6	28
182	EW / US only	6	22
183	EW / US only	6	12
184	EW / US only	6	28
185	EW / US only	6	42
186	EW / US only	6	22
187	EW / US only	6	12
188	EW / US only	6	22
189	EW / US only	6	11
190	EW / US only	6	31
191	EW / US only	6	45
192	EW / US only	6	33
193	NW / US only	6	41
194	NW / US only	6	33
195	NW / US only	6	24
196	NW / US only	6	53
197	NW / US only	6	36
198	NW / US only	6	42
199	NW / US only	6	51
200	NW / US only	6	42
201	NW / US only	6	40
202	NW / US only	6	22
203	NW / US only	6	34
204	NW / US only	6	40
205	NW / US only	6	41
206	NW / US only	6	33
207	NW / US only	6	24
208	NW / US only	6	53
209	NW / US only	6	36
210	NW / US only	6	42
211	NW / US only	6	50
212	NW / US only	6	38
213	NW / US only	6	22
214	NW / US only	6	51
215	NW / US only	6	29
216	NW / US only	6	39
217	EW / Control	7	11
218	EW / Control	7	24
219	EW / Control	7	16
220	EW / Control	7	25



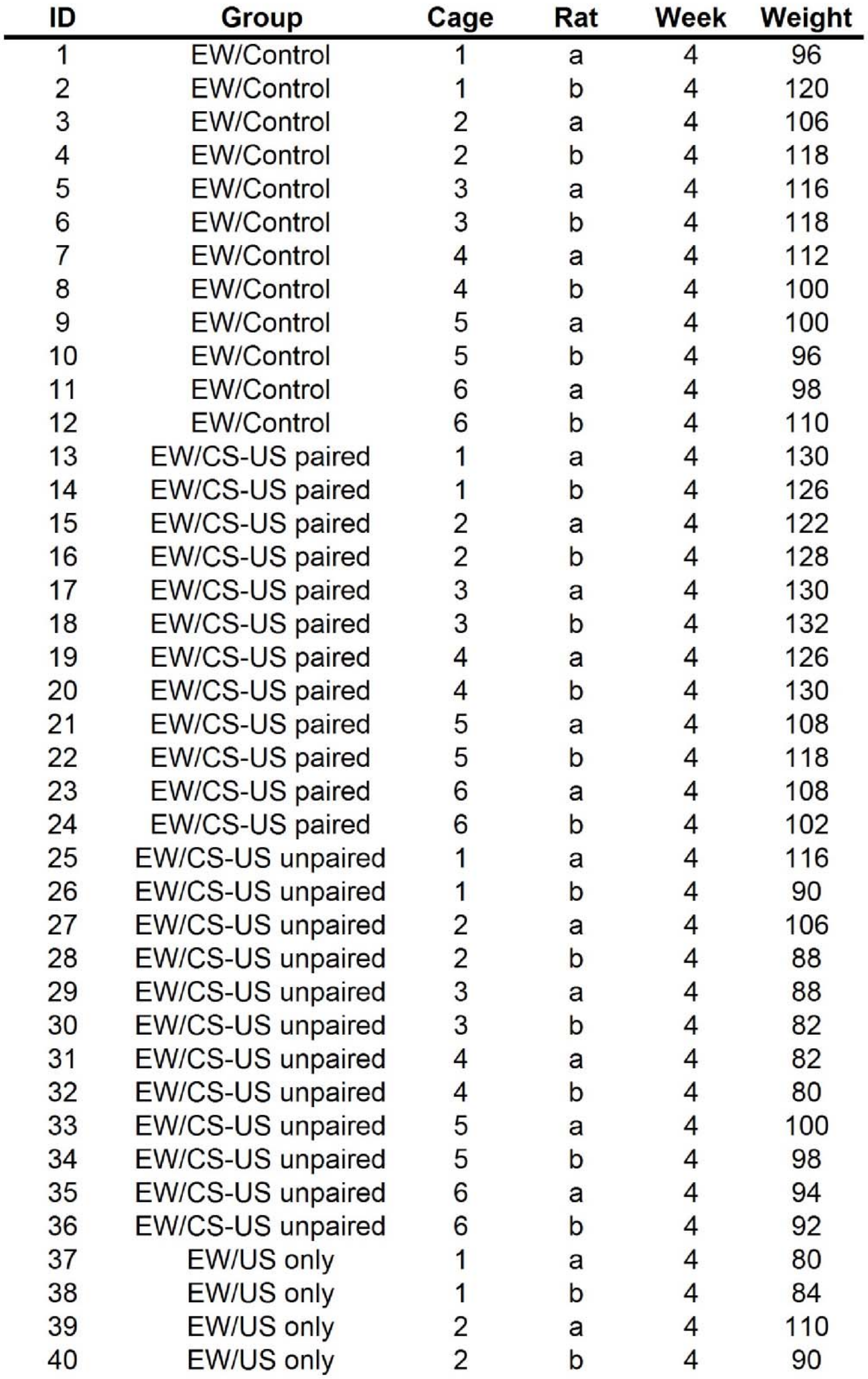
	Treatment group	Week	Playful attack
221	EW / Control	7	28
222	EW / Control	7	19
223	EW / Control	7	12
224	EW / Control	7	25
225	EW / Control	7	22
226	EW / Control	7	21
227	EW / Control	7	17
228	EW / Control	7	26
229	EW / CS-US paired	7	21
230	EW / CS-US paired	7	35
231	EW / CS-US paired	7	21
232	EW / CS-US paired	7	23
233	EW / CS-US paired	7	22
234	EW / CS-US paired	7	39
235	EW / CS-US paired	7	38
236	EW / CS-US paired	7	35
237	EW / CS-US paired	7	20
238	EW / CS-US paired	7	19
239	EW / CS-US paired	7	15
240	EW / CS-US paired	7	34
241	EW / CS-US unpaired	7	17
242	EW / CS-US unpaired	7	19
243	EW / CS-US unpaired	7	23
244	EW / CS-US unpaired	7	15
245	EW / CS-US unpaired	7	19
246	EW / CS-US unpaired	7	23
247	EW / CS-US unpaired	7	28
248	EW / CS-US unpaired	7	13
249	EW / CS-US unpaired	7	14
250	EW / CS-US unpaired	7	27
251	EW / CS-US unpaired	7	18
252	EW / CS-US unpaired	7	16
253	EW / US only	7	28
254	EW / US only	7	5
255	EW / US only	7	19
256	EW / US only	7	38
257	EW / US only	7	15
258	EW / US only	7	29
259	EW / US only	7	31
260	EW / US only	7	9
261	EW / US only	7	11
262	EW / US only	7	36
263	EW / US only	7	16
264	EW / US only	7	31
265	NW / US only	7	21
266	NW / US only	7	42



	Treatment group	Week	Playful attack
267	NW / US only	7	21
268	NW / US only	7	24
269	NW / US only	7	18
270	NW / US only	7	48
271	NW / US only	7	33
272	NW / US only	7	41
273	NW / US only	7	29
274	NW / US only	7	28
275	NW / US only	7	14
276	NW / US only	7	30
277	NW / US only	7	21
278	NW / US only	7	42
279	NW / US only	7	21
280	NW / US only	7	24
281	NW / US only	7	18
282	NW / US only	7	48
283	NW / US only	7	44
284	NW / US only	7	39
285	NW / US only	7	17
286	NW / US only	7	19
287	NW / US only	7	16
288	NW / US only	7	40

Appendix 6

Weekly weight data (in grams)



ID	Group	Cage	Rat	Week	Weight
1	EW/Control	1	a	4	96
2	EW/Control	1	b	4	120
3	EW/Control	2	a	4	106
4	EW/Control	2	b	4	118
5	EW/Control	3	a	4	116
6	EW/Control	3	b	4	118
7	EW/Control	4	a	4	112
8	EW/Control	4	b	4	100
9	EW/Control	5	a	4	100
10	EW/Control	5	b	4	96
11	EW/Control	6	a	4	98
12	EW/Control	6	b	4	110
13	EW/CS-US paired	1	a	4	130
14	EW/CS-US paired	1	b	4	126
15	EW/CS-US paired	2	a	4	122
16	EW/CS-US paired	2	b	4	128
17	EW/CS-US paired	3	a	4	130
18	EW/CS-US paired	3	b	4	132
19	EW/CS-US paired	4	a	4	126
20	EW/CS-US paired	4	b	4	130
21	EW/CS-US paired	5	a	4	108
22	EW/CS-US paired	5	b	4	118
23	EW/CS-US paired	6	a	4	108
24	EW/CS-US paired	6	b	4	102
25	EW/CS-US unpaired	1	a	4	116
26	EW/CS-US unpaired	1	b	4	90
27	EW/CS-US unpaired	2	a	4	106
28	EW/CS-US unpaired	2	b	4	88
29	EW/CS-US unpaired	3	a	4	88
30	EW/CS-US unpaired	3	b	4	82
31	EW/CS-US unpaired	4	a	4	82
32	EW/CS-US unpaired	4	b	4	80
33	EW/CS-US unpaired	5	a	4	100
34	EW/CS-US unpaired	5	b	4	98
35	EW/CS-US unpaired	6	a	4	94
36	EW/CS-US unpaired	6	b	4	92
37	EW/US only	1	a	4	80
38	EW/US only	1	b	4	84
39	EW/US only	2	a	4	110
40	EW/US only	2	b	4	90

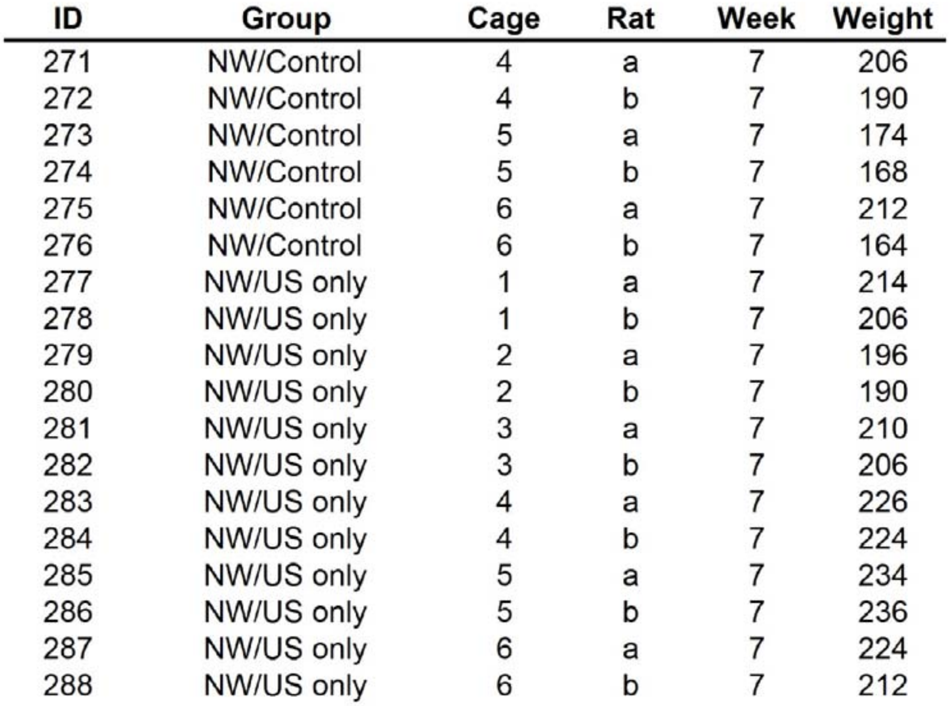
ID	Group	Cage	Rat	Week	Weight
41	EW/US only	3	a	4	78
42	EW/US only	3	b	4	86
43	EW/US only	4	a	4	92
44	EW/US only	4	b	4	96
45	EW/US only	5	a	4	80
46	EW/US only	5	b	4	86
47	EW/US only	6	a	4	100
48	EW/US only	6	b	4	88
49	NW/Control	1	a	4	80
50	NW/Control	1	b	4	74
51	NW/Control	2	a	4	74
52	NW/Control	2	b	4	76
53	NW/Control	3	a	4	78
54	NW/Control	3	b	4	70
55	NW/Control	4	a	4	80
56	NW/Control	4	b	4	64
57	NW/Control	5	a	4	96
58	NW/Control	5	b	4	86
59	NW/Control	6	a	4	88
60	NW/Control	6	b	4	86
61	NW/US only	1	a	4	84
62	NW/US only	1	b	4	82
63	NW/US only	2	a	4	72
64	NW/US only	2	b	4	82
65	NW/US only	3	a	4	84
66	NW/US only	3	b	4	78
67	NW/US only	4	a	4	86
68	NW/US only	4	b	4	86
69	NW/US only	5	a	4	122
70	NW/US only	5	b	4	122
71	NW/US only	6	a	4	116
72	NW/US only	6	b	4	110
73	EW/Control	1	a	5	176
74	EW/Control	1	b	5	176
75	EW/Control	2	a	5	162
76	EW/Control	2	b	5	184
77	EW/Control	3	a	5	176
78	EW/Control	3	b	5	178
79	EW/Control	4	a	5	174
80	EW/Control	4	b	5	164
81	EW/Control	5	a	5	158
82	EW/Control	5	b	5	154
83	EW/Control	6	a	5	134
84	EW/Control	6	b	5	142
85	EW/CS-US paired	1	a	5	204
86	EW/CS-US paired	1	b	5	210

ID	Group	Cage	Rat	Week	Weight
87	EW/CS-US paired	2	a	5	190
88	EW/CS-US paired	2	b	5	212
89	EW/CS-US paired	3	a	5	214
90	EW/CS-US paired	3	b	5	212
91	EW/CS-US paired	4	a	5	206
92	EW/CS-US paired	4	b	5	204
93	EW/CS-US paired	5	a	5	180
94	EW/CS-US paired	5	b	5	198
95	EW/CS-US paired	6	a	5	194
96	EW/CS-US paired	6	b	5	176
97	EW/CS-US unpaired	1	a	5	160
98	EW/CS-US unpaired	1	b	5	132
99	EW/CS-US unpaired	2	a	5	144
100	EW/CS-US unpaired	2	b	5	123
101	EW/CS-US unpaired	3	a	5	130
102	EW/CS-US unpaired	3	b	5	122
103	EW/CS-US unpaired	4	a	5	124
104	EW/CS-US unpaired	4	b	5	126
105	EW/CS-US unpaired	5	a	5	120
106	EW/CS-US unpaired	5	b	5	116
107	EW/CS-US unpaired	6	a	5	134
108	EW/CS-US unpaired	6	b	5	142
109	EW/US only	1	a	5	118
110	EW/US only	1	b	5	122
111	EW/US only	2	a	5	150
112	EW/US only	2	b	5	130
113	EW/US only	3	a	5	120
114	EW/US only	3	b	5	112
115	EW/US only	4	a	5	124
116	EW/US only	4	b	5	124
117	EW/US only	5	a	5	108
118	EW/US only	5	b	5	106
119	EW/US only	6	a	5	122
120	EW/US only	6	b	5	110
121	NW/Control	1	a	5	118
122	NW/Control	1	b	5	128
123	NW/Control	2	a	5	122
124	NW/Control	2	b	5	120
125	NW/Control	3	a	5	114
126	NW/Control	3	b	5	128
127	NW/Control	4	a	5	114
128	NW/Control	4	b	5	120
129	NW/Control	5	a	5	112
130	NW/Control	5	b	5	120
131	NW/Control	6	a	5	128
132	NW/Control	6	b	5	112

ID	Group	Cage	Rat	Week	Weight
133	NW/US only	1	a	5	130
134	NW/US only	1	b	5	118
135	NW/US only	2	a	5	112
136	NW/US only	2	b	5	126
137	NW/US only	3	a	5	158
138	NW/US only	3	b	5	154
139	NW/US only	4	a	5	134
140	NW/US only	4	b	5	142
141	NW/US only	5	a	5	166
142	NW/US only	5	b	5	176
143	NW/US only	6	a	5	174
144	NW/US only	6	b	5	168
145	EW/Control	1	a	6	210
146	EW/Control	1	b	6	208
147	EW/Control	2	a	6	186
148	EW/Control	2	b	6	214
149	EW/Control	3	a	6	202
150	EW/Control	3	b	6	214
151	EW/Control	4	a	6	194
152	EW/Control	4	b	6	186
153	EW/Control	5	a	6	188
154	EW/Control	5	b	6	180
155	EW/Control	6	a	6	192
156	EW/Control	6	b	6	192
157	EW/CS-US paired	1	a	6	222
158	EW/CS-US paired	1	b	6	224
159	EW/CS-US paired	2	a	6	210
160	EW/CS-US paired	2	b	6	228
161	EW/CS-US paired	3	a	6	180
162	EW/CS-US paired	3	b	6	182
163	EW/CS-US paired	4	a	6	220
164	EW/CS-US paired	4	b	6	226
165	EW/CS-US paired	5	a	6	194
166	EW/CS-US paired	5	b	6	216
167	EW/CS-US paired	6	a	6	218
168	EW/CS-US paired	6	b	6	200
169	EW/CS-US unpaired	1	a	6	228
170	EW/CS-US unpaired	1	b	6	192
171	EW/CS-US unpaired	2	a	6	192
172	EW/CS-US unpaired	2	b	6	200
173	EW/CS-US unpaired	3	a	6	188
174	EW/CS-US unpaired	3	b	6	176
175	EW/CS-US unpaired	4	a	6	172
176	EW/CS-US unpaired	4	b	6	174
177	EW/CS-US unpaired	5	a	6	162
178	EW/CS-US unpaired	5	b	6	158

ID	Group	Cage	Rat	Week	Weight
179	EW/CS-US unpaired	6	a	6	154
180	EW/CS-US unpaired	6	b	6	154
181	EW/US only	1	a	6	164
182	EW/US only	1	b	6	168
183	EW/US only	2	a	6	228
184	EW/US only	2	b	6	198
185	EW/US only	3	a	6	166
186	EW/US only	3	b	6	164
187	EW/US only	4	a	6	170
188	EW/US only	4	b	6	178
189	EW/US only	5	a	6	132
190	EW/US only	5	b	6	140
191	EW/US only	6	a	6	176
192	EW/US only	6	b	6	152
193	NW/Control	1	a	6	150
194	NW/Control	1	b	6	178
195	NW/Control	2	a	6	166
196	NW/Control	2	b	6	156
197	NW/Control	3	a	6	144
198	NW/Control	3	b	6	132
199	NW/Control	4	a	6	158
200	NW/Control	4	b	6	136
201	NW/Control	5	a	6	148
202	NW/Control	5	b	6	144
203	NW/Control	6	a	6	168
204	NW/Control	6	b	6	138
205	NW/US only	1	a	6	166
206	NW/US only	1	b	6	172
207	NW/US only	2	a	6	160
208	NW/US only	2	b	6	152
209	NW/US only	3	a	6	168
210	NW/US only	3	b	6	166
211	NW/US only	4	a	6	186
212	NW/US only	4	b	6	180
213	NW/US only	5	a	6	184
214	NW/US only	5	b	6	192
215	NW/US only	6	a	6	198
216	NW/US only	6	b	6	184
217	EW/Control	1	a	7	228
218	EW/Control	1	b	7	214
219	EW/Control	2	a	7	248
220	EW/Control	2	b	7	232
221	EW/Control	3	a	7	214
222	EW/Control	3	b	7	256
223	EW/Control	4	a	7	238
224	EW/Control	4	b	7	246

ID	Group	Cage	Rat	Week	Weight
225	EW/Control	5	a	7	242
226	EW/Control	5	b	7	230
227	EW/Control	6	a	7	228
228	EW/Control	6	b	7	226
229	EW/CS-US paired	1	a	7	262
230	EW/CS-US paired	1	b	7	260
231	EW/CS-US paired	2	a	7	234
232	EW/CS-US paired	2	b	7	262
233	EW/CS-US paired	3	a	7	250
234	EW/CS-US paired	3	b	7	254
235	EW/CS-US paired	4	a	7	260
236	EW/CS-US paired	4	b	7	276
237	EW/CS-US paired	5	a	7	240
238	EW/CS-US paired	5	b	7	258
239	EW/CS-US paired	6	a	7	274
240	EW/CS-US paired	6	b	7	254
241	EW/CS-US unpaired	1	a	7	274
242	EW/CS-US unpaired	1	b	7	238
243	EW/CS-US unpaired	2	a	7	236
244	EW/CS-US unpaired	2	b	7	242
245	EW/CS-US unpaired	3	a	7	224
246	EW/CS-US unpaired	3	b	7	212
247	EW/CS-US unpaired	4	a	7	220
248	EW/CS-US unpaired	4	b	7	218
249	EW/CS-US unpaired	5	a	7	218
250	EW/CS-US unpaired	5	b	7	222
251	EW/CS-US unpaired	6	a	7	212
252	EW/CS-US unpaired	6	b	7	216
253	EW/US only	1	a	7	258
254	EW/US only	1	b	7	194
255	EW/US only	2	a	7	230
256	EW/US only	2	b	7	220
257	EW/US only	3	a	7	224
258	EW/US only	3	b	7	208
259	EW/US only	4	a	7	266
260	EW/US only	4	b	7	258
261	EW/US only	5	a	7	190
262	EW/US only	5	b	7	186
263	EW/US only	6	a	7	224
264	EW/US only	6	b	7	204
265	NW/Control	1	a	7	188
266	NW/Control	1	b	7	208
267	NW/Control	2	a	7	204
268	NW/Control	2	b	7	194
269	NW/Control	3	a	7	230
270	NW/Control	3	b	7	198



ID	Group	Cage	Rat	Week	Weight
271	NW/Control	4	a	7	206
272	NW/Control	4	b	7	190
273	NW/Control	5	a	7	174
274	NW/Control	5	b	7	168
275	NW/Control	6	a	7	212
276	NW/Control	6	b	7	164
277	NW/US only	1	a	7	214
278	NW/US only	1	b	7	206
279	NW/US only	2	a	7	196
280	NW/US only	2	b	7	190
281	NW/US only	3	a	7	210
282	NW/US only	3	b	7	206
283	NW/US only	4	a	7	226
284	NW/US only	4	b	7	224
285	NW/US only	5	a	7	234
286	NW/US only	5	b	7	236
287	NW/US only	6	a	7	224
288	NW/US only	6	b	7	212