THE ROLE OF PLAY-DERIVED EXPERIENCES ON THE DEVELOPMENT OF THE MEDIAL PREFRONTAL CORTEX AND ADULT SOCIAL BEHAVIOR

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DEDICATION

To my parents, for always encouraging me to play.

ABSTRACT

For many mammals, play with peers is a hallmark of the juvenile period. While these animals may play because it is fun, research has also shown that play during the juvenile period helps develop critical skills that allow animals to better adapt to unpredictable situations later in life. Therefore, play is hypothesized to build skills, especially executive functions that, in many mammals, depend on neural systems that incorporate the prefrontal cortex. This thesis uses novel approaches to explore the role of play in the development of social skills and the underlying brain mechanisms in rats. For the first time, the same rearing paradigm is used to compare the effects play deprivation has in both sexes. Not only do males and females exhibit the same social deficits and altered brain development, but this thesis also shows that both sexes depend on the same play-derived experiences.

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6-OHDA	6-hydroxydopamine
F344	Fischer 344 rats
FM	frequency modulated
kHz	kilohertz
LE	Long Evans rats
MC	motor cortex
mPFC	medial prefrontal cortex
OFC	orbitofrontal cortex
PFC	prefrontal cortex
SEM	standard error of the mean
USV	ultrasonic vocalizations

CHAPTER 1: GENERAL INTRODUCTION

Social play, specifically play fighting with peers, is the most common form of play reported in young mammals. Indeed, in the wild, animals will go to great lengths to engage in play, only decreasing the amount of play, or ceasing play altogether, when resources are scarce (Baldwin & Baldwin, 1974, 1976; Berger, 1980). As in many species, play in rats peaks during the juvenile period before declining after puberty. However, rats maintain a low level of play into adulthood (Panksepp, 1981; Pellis & Pellis, 1990). Rats are highly social creatures and have a large behavioral repertoire to support the formation of complex relationships (Baenninger, 1966). Because of their complex social behavior, and the ease with which their experiences can be manipulated within the laboratory environment, rats are a model organism for studying the neurobiology of social play (Pellis & Pellis, 2009; Siviy & Panksepp, 2011; Vanderschuren et al., 2016; Vanderschuren & Trezza, 2014). The goal of this thesis is to understand how play-derived experiences during the juvenile period affects both the development of adult social skills and the organization of the medial prefrontal cortex (mPFC).

Play fighting in rats involves competition for access to the nape of the neck, which is nuzzled with the tip of the snout when contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). To protect the nape from such contact, the defending rat can use a variety of tactics (Pellis & Pellis, 1987, 1990). Juvenile rats of both sexes use the same defensive tactics to defend from attack, the main difference in play at this age is quantitative – male rats play more than females (Meaney & Stewart, 1981; Pellis & Pellis, 1990). However, for both sexes, play peaks in frequency between 30 – 40 days of life (Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1990; Thor & Holloway, 1984). When attacked, a rat can either continue its current behavior or use various tactics to create space between the attacker's snout and its nape (see Figure 1.1). Approximately

90% of attacks are actively defended and can take the form of either an evasion or facing defense (Pellis & Pellis, 1990). Evasions involve the defending rat running, leaping, or swerving away to create distance between the attacker and its own nape, while facing defenses involve the rat turning its face toward its attacker, thereby blocking access to its nape. There are two main types of rotation rats can make: 1) they can rotate around the vertical axis, typically around the pelvis, to face their attacker, while remaining standing on, at least, their hind paws, or 2) they can rotate around the longitudinal axis, turning to face their partner with the ventral surface of the head. In turn, there are two versions of the longitudinal rotation tactic. The defending rats can: (i) partially rotate, turning the anterior of their torso toward their partner while maintaining contact with the floor with at least one hind paw, or (ii) completely rotate along their whole body until they lie supine on their backs (B. Himmler, Pellis, & Pellis, 2013). From both the vertical axis rotation and partial rotation tactic, the animals can rear onto their hind legs, termed ‘mutual upright’, and engage in a boxing contest (Pellis & Pellis, 1987). Complete rotations often lead to prolonged contact between the ventral surfaces of the rats, as the attacker remains standing on top, and both continue to lunge at each other’s napes.

The complete rotation tactic is the maneuver most likely to end in a ‘pin’ configuration, where one rat stands on top of the other as both try to access the others nape (Panksepp, 1981). Pins can also be achieved by the attacker pushing its partner onto its back from a mutual upright or partial rotation defensive tactic (S. Himmler, Himmler, Stryjek et al., 2016). Once the recipient of a nape attack defends itself successfully, it can launch a counterattack, which if successful can lead to a role reversal, where the original attacker becomes the defender (Pellis & Pellis, 1987). Counterattacks can be launched following both evasion and facing defense (S. Himmler, Himmler, Pellis & Pellis, 2016; Pellis et al., 1989); however, it is challenging to decide whether a counterattack from an evasion is truly a counterattack or a new attack. Therefore, in my work, I have opted for scoring counterattacks, ones that are successful and lead to a role reversal, only from facing defenses. As will be seen in the empirical chapters, this criterion leads to scoring a lower rate of counterattacks compared to previous studies (Pellis & Pellis, 1990), but it is still sufficient to detect experimental effects.

Playful attacks begin around the 17th day of life (Baenninger, 1967; Bolles & Woods, 1964) while defensive tactics develop in a piecemeal fashion. Evasions and partial rotations emerge first, followed by mutual uprights and complete rotations (Pellis & Pellis, 1997). During the pre-juvenile phase, which is between weaning and 30 days of age, the most common defensive tactics used by rats, regardless of sex, are evasions and partial rotations (Pellis & Pellis, 1997). It is not until rats enter the peak period of play that a switch in defensive tactics is seen, in which juvenile rats engage in proportionately more complete rotations. The increase in complete rotations, in turn, lead to more wrestling and more counterattacks (Pellis & Pellis, 1990). Once sexual maturity is reached, male rats again switch tactics, performing more evasions and partial rotations while females retain the juvenile typical play style of mostly performing complete rotations (Pellis & Pellis, 1990).

Even though play fighting is most frequent in the juvenile period and wanes with the onset of sexual maturity, it continues at a low rate well into adulthood (Pellis & Pellis, 1990; Thor & Holloway, 1984), but its expression becomes more sensitive to dominance relationships (Pellis, Pellis, & McKenna, 1993). Rats live in multi-male and multi-female colonies, with one adult male typically adopting the dominant role (Barnett, 1975; Calhoun, 1962; Lore & Flannelly, 1977). The dominant male receives an increased rate of affinitive contact, including grooming and play, from the other colony members (Adams & Boice, 1983, 1989; Pellis & Pellis, 1991). Additionally, while dominant males respond to playful attacks in the manner typical of adult males, when

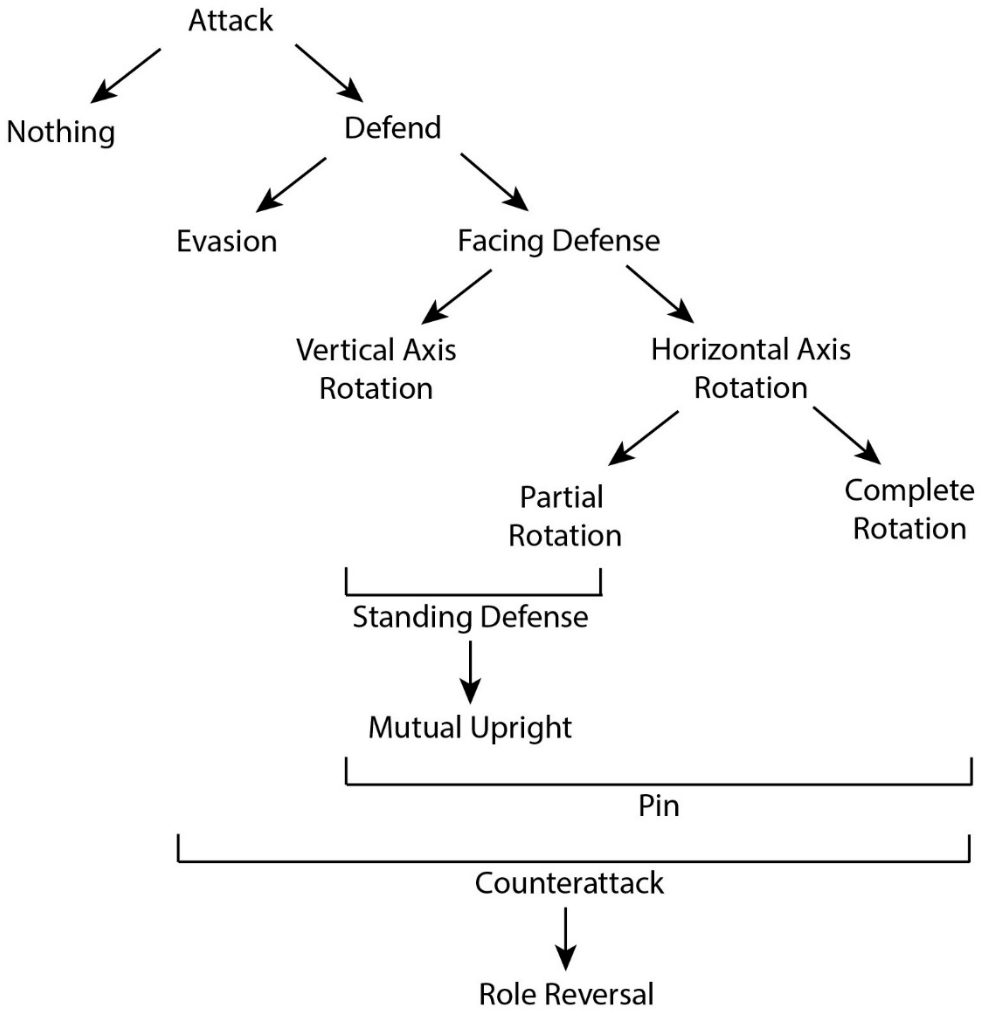

Figure 1.1 *Schematic used to assess play behavior in rats.*

attacked by the dominant rat, subordinate males respond in a more juvenile typical manner (Pellis, Pellis, et al., 1993; Pellis & Pellis, 1992). Adult females respond to playful attacks by either dominant or subordinate males in the same way, in the juvenile typical manner (Pellis et al., 2006; Smith et al., 1998a). The adoption of juvenile typical defensive tactics by the subordinates is thought to signal submission to the dominant and so function as means of appeasement (Pellis, Pellis, et al., 1993; Pellis & Pellis, 1992). Indeed, in semi-natural conditions, the subordinate males that keep well away from the dominant and thus are not maintaining their bond with the dominant, are more likely to be attacked and bitten when encountered (D. Blanchard & Blanchard, 1990). However, when unfamiliar adult males encounter one another in a neutral arena they engage in play, but both adopt the adult-typical patterns of defense, which results in a rougher form of play. Such rough play can lead to the establishment of a dominance relationship, with the nascent subordinate adopting the gentler form of play, or it can lead to an escalation to serious fighting before dominance is established (D. Blanchard et al., 1984; Smith et al., 1999). While dominance relationships guide the pattern of play among adults, a hallmark of play in juveniles is fairness and reciprocity.

To remain playful, play fighting must have some degree of reciprocity, sometimes referred to as the 50:50 rule (Altmann, 1962). While the methods of achieving reciprocity and how much turn-taking an animal engages in can vary with species, age, and sex, some degree of reciprocity is needed for play fighting to continue (Palagi, Cordoni, et al., 2016; Pellis & Pellis, 2017). In rats, one way reciprocity is achieved is through self-handicapping behaviors. Self-handicapping is achieved by performing behaviors that make it easier for the play partner to gain the advantage and reverse roles (Pellis & Pellis, 1998). Typically, when in the pin configuration, the attacking rat that is standing on top of its supine partner will anchor its hind paws on the floor. By anchoring its hind paws, the rat on top is creating stability while holding down its partner. However, there are instances in which the rat on top will adjust its positioning so as to stand on top of its supine partner, thus making its position more unstable (Foroud & Pellis, 2003). Self-handicapping makes it easier for the supine partner to launch a counterattack, and pinned rats are two to three times more likely to succeed when the on top rat is adopting the handicapping posture (Pellis et al., 2005). Additionally, the period of peak juvenile play – 30-40 days of age (Panksepp, 1981; Pellis & Pellis, 1990; Thor & Holloway, 1984) – is when self-handicapping behavior is most performed (Foroud & Pellis, 2002). Counterattacks can lead to role reversals, in which the defending rat is successful in its counterattack and switches positions, becoming the attacker. Reversing roles is a key component to play and maintaining reciprocity, requiring the rats to keep track of how many attacks each animal makes and then make decisions that lead to appropriate responses. Thus, reversing roles requires working memory, impulse control, and decision making, all of which are key executive functions that are supported by the medial prefrontal cortex (mPFC) and are impaired when rats are deprived of play (Baarendse et al., 2013; Burleson et al., 2016; Schneider et al., 2014; Schneider, Bindila, et al., 2016).

1.1 Play and the brain

In order to develop, the brain requires proper external inputs which fine-tune the activity and connectivity of the brain's neural circuits – the term 'use it or lose it' comes to mind. Some of the best examples of the importance of external inputs come from depriving animals of sensory information (for reviews, see Gainey & Feldman, 2017; Hensch, 2005). However, when uncovering the experiences required for the development of brain areas recruited for more generalized functions, such as executive functions related to the prefrontal cortex (PFC) (Bicks et al., 2020; Larsen & Luna, 2018), the deprivation of a single relevant experience is more difficult. Since animals that are deprived of play have impairments in executive functions (Pellis & Pellis, 2009; Špinka et al., 2001; Vanderschuren & Trezza, 2014), it would seem likely that the brain is primed and motivated to engage in play in order gain the experiences needed to adequately develop these functions (Siviy, 2016).

Play fighting in rats is a highly rewarding behavior (Panksepp et al., 1984; Pellis & Pellis, 2009; Trezza et al., 2011; Vanderschuren et al., 2016). For example, rats that are habituated in a place preference paradigm to a playful partner will prefer to spend more time in the context in which they experienced play (Calcagnetti & Schechter, 1992; Trezza et al., 2009). Additionally, the reward of playing with a partner has been used in operant conditioning paradigms. Rats are trained to perform an arbitrary task, such as a lever press, to gain access to a reward. In many cases, the reward is food, sucrose water, or an infusion of drugs of abuse. However, play has been shown to successfully elicit behaviors in the conditioned rat to gain access to a play partner (Achterberg et al., 2016). These studies provide evidence for the highly rewarding nature of play.

The role of the cortex

Animals that have more complex forms of play tend to have proportionally larger brains (Fagen, 1981; Iwaniuk et al., 2001; Kaplan, 2020). However, early studies that removed the entire neocortex shortly after birth found that the decorticate animals still engaged in play fighting during the juvenile period (Murphy et al., 1981; Panksepp et al., 1994; Pellis et al., 1992; Whishaw, 1990). Further analysis of play in decorticate rats found that, while there were no quantitative differences in the amount of play engaged in, there were qualitative differences in how the decorticates play when compared to intact controls (Panksepp et al., 1994; Pellis et al., 1992). Specifically, decorticate rats are less likely to perform defensive maneuvers that promote close-quarter wrestling, such as complete rotations. Decorticate rats will also engage in fewer instances of pins during the peak play period, and are more likely to evade an attack (Pellis et al., 1992). As decorticate male rats age, they show the age-typical decline in amount of play, but do not show the post-pubertal shift in defensive strategies that is seen in intact controls, nor do they modify the use of their defensive tactics when playing with partners of different status – dominant versus subordinate (Pellis et al., 1992). These data show that the neocortex is not needed to regulate the waxing and waning of the motivation to play but is needed to regulate some of the age-related changes in play and how to contextually modify play. As these changes could be the result of damage to any number of regions of the cortex, a closer look was needed.

Follow up research looked to uncover the roles of more specific subregions of the cortex, which led to some interesting results. As the PFC is important in regulating social behavior (Kolb, 1990), the role of two important sub-regions (Euston et al., 2012; Kolb, 1984), the orbital frontal cortex (OFC) and the medial prefrontal cortex (mPFC) was examined. When, as neonates, rats had their OFC ablated, they later showed juvenile typical levels of play and playful defenses and showed the adult typical switch in play tactics (Pellis et al., 2006). However, as adults, the OFC ablated rats failed to adjust their style of play when interacting with more dominant rats (Pellis et al., 2006). When, as neonates, rats had their mPFC ablated, they later showed juvenile typical levels of play and playful defenses, showed the adult typical switch in play tactics and were able to detect and alter their play based on the identity of their partner (Bell et al., 2009). However, rats with mPFC damage tended to preferentially use simpler tactics, such as evasion, rather than the more complex, facing defense tactics (Bell et al., 2009). A possible reason for the greater use of simpler defensive tactics is that the more complex tactics require greater inter-animal coordination, a skill that is eroded with damage to the mPFC. Using a different test paradigm, in which one rat protects a piece of food from being stolen by another rat (Whishaw, 1988), it was confirmed that, while rats with mPFC damage are still able to dodge laterally away from the robber, their ability to coordinate and match the robber's movement is impaired (B. Himmler, Bell, et al., 2014). The reduced ability to match the opponent's movements leads to a greater likelihood of the robber successfully stealing the food from the dodger's mouth (B. Himmler, Bell, et al., 2014; Whishaw

& Oddie, 1989). Using the same food protection paradigm with OFC damaged rats revealed that the rats could coordinate their movements with those of their partner, but failed to modify their dodges with the identity of their partner (Pellis et al., 2006). Therefore, it seems that the OFC is important for modulating behavior depending on the identity of the partner, and the mPFC is important for modulating behavior based on the actions performed by the partner (Pellis & Pellis, 2009).

A third cortical area has been shown to modulate another feature of play. When, as neonates, rats had their motor cortex (MC) ablated, they later showed juvenile typical levels of play, a waning of play with the onset of puberty and were able to modulate their playful defense with the identity of the partner, but did not show age related changes in defensive tactics seen in intact controls. Rats with an ablated MC played in the adult-typical manner at all ages (Kamitakahara et al., 2007). Therefore, the cortex can modify play in three distinct ways: it modulates the age-related changes of defensive tactics used (MC), it modulates play based on the identity of one's partner (OFC), and it modulates actions during play to coordinate with the actions of one's partner (mPFC). It should be noted that the modulatory roles of the OFC and the mPFC are not limited to play, but also to other forms of social interaction. Additionally, it is important to note that none of these studies that damaged all or part of the cortex, changed the rat's motivation to play or how that motivation changed with age. The motivation to play is regulated by subcortical regions of the brain.

The role of subcortical regions

As for measurements involving the size of the whole brain or the cortex (Iwaniuk et al., 2001; Kaplan, 2020; Lewis, 2000), the amount of social play in which a species engages is predictive of the size of several subcortical regions, at least in nonhuman primates – including the striatum, hypothalamus, amygdala, and cerebellum (Graham, 2011; Lewis & Barton, 2004, 2006; Pellis & Iwaniuk, 2002). That is, there are multiple subcortical areas involved in regulating play, the role of which has been detailed in several recent reviews (Siviy, 2016, 2019; Vanderschuren et al., 2016). In what follows, I will broadly describe how subcortical regions regulate play, and in so doing, indicate how cortical systems become involved. Rats with 6-OHDA lesions to the striatum, an area of the brain important for voluntary motor control (Báez-Mendoza & Schultz, 2013; Cools, 1980), show a decrease in nape attacks, tend to favor defensive tactics that limit play contact (i.e., evasions) and readily switch to other social behaviors, including non-playful ones, such as grooming or sexual mounting (Pellis, Castañeda, et al., 1993). These findings suggest that dopamine circuits within the striatum not only contribute to the maintenance of sequential organization and execution of actions during play but on the amount of play engaged in.

One area of the striatum that seems to be important in modulating play is the ventral striatum. This area undergoes dynamic changes in dopamine response during the juvenile period. Both juvenile and adult rats increase the release of dopamine into the nucleus accumbens – a subregion of the ventral striatum – in response to social interactions (Robinson et al., 2011). Upon subsequent introductions to social partners, adult rats showed a decrease in dopamine release and habituated to the social encounter, while the juveniles, upon subsequent introductions, maintained a consistent dopamine response (Robinson et al., 2011). Consistent with these findings are studies in which dopamine is increased in the nucleus accumbens by either the use of dopamine reuptake inhibitors or dopamine agonists – both of which increase the amount of play (Manduca et al., 2016). Amphetamines are powerful dopamine agonists, yet inactivation of dopamine receptors in the nucleus accumbens blocks the effects of amphetamine, resulting in a decrease of play behaviors (Manduca et al., 2016). These studies point to the importance of dopamine in the ventral striatum and the role the striatum plays in modulating, motivating, and priming the brain for social behavior, especially in juvenile rats.

Additional work examined the impact of play on the activation of substructures of the striatum and their connections within the cortex. Temporary inactivation of cortical structures, including the mPFC, reduces both nape attacks and pins without affecting locomotion, while inactivation of the dorsomedial striatum increases attacks and pinning (van Kerkhof et al., 2013). The dorsomedial striatum receives most of its projections from the somatosensory cortex (Voorn et al., 2004) and is thought to be implicated in motor planning and execution of behavior. Thus, this area of the striatum may be important for inhibiting social behavior while the mPFC prolongs social interactions, specifically play (van Kerkhof et al., 2013). This might explain why rats with striatal lesions have difficulty in regulating social behaviors, such as grooming, sexual mounting and play (Pellis, Castañeda, et al., 1993). These studies implicate dopamine as an important regulator of play, with both the dorsal and ventral striatum modulating play in different ways.

Many of the studies described above involved lesions that are permanent. However, the use of drug infusions to temporarily inactivate specific brain regions has greatly improved with advances of technology and has become more mainstream. All of this work assesses the role the brain has in modulating various aspects of play, but the converse is also true; experience modulates the brain. Therefore, we can assess the role that behaviors have on shaping and developing the brain by depriving rats of play.

1.2 Play deprivation paradigms

In the mid to late 70s and beyond, a research boom emerged on the effects of rearing rats in complete isolation between weaning and sexual maturity; this research showed that isolation leads to a host of sociocognitive and behavioral impairments (Arakawa, 2018; Baenninger, 1967; Byrd & Briner, 1999; da Silva et al., 1996; Einon et al., 1975; Einon & Morgan, 1976; Morgan, 1973), as well as causing a wide range of changes in various neural systems (e.g., Fone & Porkess, 2008; Hall, 1998). It was found that there is a sensitive period for when the effects of isolation are most pronounced – between 25 and 45 days of life (Einon & Morgan, 1977) – the same age at which social play peaks (Thor & Holloway, 1984). These studies suggest that it is the absence of play that may be critical for the development of sociocognitive skills. This conclusion was reinforced by the finding that brief periods of intermittent social contact can alleviate some of the negative effects of being reared in isolation (Einon et al., 1978). As has been pointed out by others, this latter finding was important since complete isolation deprives developing rats of more than just play (Bekoff, 1976). However, rearing paradigms that allow some social contact, but limit play during the juvenile period, still result in a host of impairments, implicating the importance of play.

Partition rearing paradigm

In the partition rearing paradigm, rats are reared with a partner, typically of the same strain and sex, but are separated with a perforated Plexiglas® or wire mesh partition (See Figure 1.2a). Being divided by a partition allows the animals to see, smell, and hear one another, as well as some limited tactile contact, but precludes the animals from playing. When rats are deprived of play using a partition during the juvenile period, they are impoverished as adults in their ability to coordinate their movements with those of a competitor in the food protection paradigm (Pellis et al., 1999).

Male rats reared in the partition paradigm have impoverished impulse control, cognitive function, and reduced synaptic inhibition in the PFC (Baarendse et al., 2013; Omrani et al., 2020). In a study by Omrani and colleagues (2020), it was found that while play deprived rats were able to learn an operant task as well as control animals, they had impairments in the reversal learning paradigm. That is, rats that had experienced play were better able to solve the reversal learning paradigm and adopted a more complex cognitive strategy. The deprived rats not only took longer to learn the reversal paradigm but also used a simplified cognitive strategy (Omrani et al., 2020). Additionally, voltage-clamp recordings of pyramidal cells of the mPFC showed that there was a reduction in GABAergic synaptic inputs in this area (Omrani et al., 2020). This lack of inhibitory input into the mPFC may be responsible for some of the behavioral deficits found in rats reared in the partition paradigm (Baarendse et al., 2013; Bell, 2014).

A limitation of this paradigm in assessing the role of play in development, that I believe makes it less than ideal, is that social contact of all kinds is severely curtailed. The rats may be able to touch and smell each other through the partition and, depending on the size of the perforations, may even be able to lick one another, but they cannot huddle together or groom one another. That is, they are deprived of a variety of complex social interactions, not just play. Also, as when completely isolated, the level of corticosterone, a stress-related hormone, remains high in rats housed in a partitioned cage (Boggiano et al., 2008). Chronic social isolation alters emotional reactivity to stress and causes the system that regulates stress to become hyperactive (Weiss et al., 2004). Additionally, in chronic stress paradigms, pair-housing the stressed rats can have a stress-reducing effect (Westenbroek et al., 2005). The findings that being housed in a partitioned cage does not reduce hormonal stress levels and that pair-housing can reduce the effects of chronic stress, points to there being a stress-regulation role for social contact. Consequently, partition rearing may not be a benign method for depriving rats of social play in the juvenile period, as prolonged lack of social contact could have a negative impact on stress and so produce many of the deficits attributed to the lack of play.

Adult rearing paradigm

In the adult rearing paradigm (see Figure 1.2b), juvenile rats are reared with an adult, until the juveniles are 60 days of age or more. This allows for the adult and juvenile to engage in affiliative behaviors, such as grooming and huddling, but limits the amount of play provided to the juvenile (Einon et al., 1978; Pellis & Pellis, 1997). A detailed analysis of the play between adult-juvenile pairs found that adults drastically limit the number of nape attacks they make toward their juvenile partner; around an average of six attacks during a 10-minute play session versus over 40 attacks for a matched juvenile playing with a juvenile (Pellis et al., 2017). However, the juvenile partner of the adult compensates for the lack of play by increasing the number of attacks it makes, on average, making 80 attacks per 10-minutes versus 51 for a matched juvenile playing with a juvenile (Pellis et al., 2017). When attacked, adults are just as likely to respond with an active defense as a juvenile being attacked; however, adults are more likely to evade an attack (Pellis et al., 2017). When the adult does respond with a facing defense, they are more likely to use partial rotations, often using a hind foot to kick the juvenile (Pellis et al., 2017). Most striking is the asymmetry and lack of reciprocity present in the adult-juvenile pair. The adult provides few opportunities for the juvenile to reverse roles. When the adult pins the juvenile, the adult holds the juvenile firmly to the ground, providing little opportunity for the juvenile to compete for access to the adult's nape, a necessary precursor for role reversals. On the other hand, when the juvenile is able to pin the adult, the adult never fully rotates onto its back and uses its superior size to dislodge the juvenile and move away (Pellis et al., 2017). Thus, when reared with an adult, juveniles have close contact when huddling and grooming together, and even experience some play, but the play they experience is atypical.

Juveniles reared with adults during the juvenile period, and thus over the peak play period (Meaney & Stewart, 1981; Thor & Holloway, 1984), have atypical development of the neurons of the prefrontal cortex. In an experiment by Bell and colleagues (2010), juvenile rats were either pair housed with an adult or same-sex peer, or were housed in quads, either with three adults or three peers. The idea was that juveniles housed with one or three adults would experience limited play, whereas juveniles reared with peers would experience more play, especially the juveniles reared with three peers. The latter was assumed to be the case because having more playful partners available makes individuals engage in more play (Pellis & McKenna, 1992; Reinhart et al., 2006). The dendritic arbor of pyramidal neurons of the OFC and the mPFC was used to detect the effects of the different rearing conditions (Bell et al., 2010). Juveniles reared with one or more peers had more pruned neurons in the mPFC. In contrast, juveniles reared with three partners, regardless of whether they were adults or peers, had more complex neurons in the OFC (Bell et al., 2010). Therefore, it was concluded that the neurons of the mPFC respond to play in a binary fashion and as long as a threshold is reached, appropriate pruning happens. In contrast, the OFC is sensitive to the number of partners the animal experiences, regardless of the types of social interactions in which they engage (Bell et al., 2010). These findings have been replicated (B. Himmler, Pellis & Kolb, 2013; B. Himmler et al., 2018), suggesting these are robust experiential effects on the development of the prefrontal cortex.

I also wish to point to another study supporting the findings that the mPFC is modulated by the amount of play received during the juvenile period. Burleson and colleagues (2016) reared Syrian hamsters through the juvenile period with either an adult or peer and looked at the pyramidal neurons in the mPFC and OFC. Juvenile males were reared with their mothers from weaning until 42 days of age (Burleson et al., 2016), which corresponds to the peak play period in hamsters

(Pellis & Pellis, 1988). After play deprivation, the hamsters were rehoused with their littermates until adulthood, at which time their brains were collected for histological analysis. Hamsters that were play deprived had increases in the length and branch number of dendrites in the mPFC, but no differences in the dendrites of the OFC (Burleson et al., 2016), similar to that which was found for rats (Bell et al., 2010). While no direct analysis of play between the adults and juveniles was provided, the authors did state that they monitored cages and did not witness any play between the adults and juveniles (Burleson et al., 2016), a finding that is consistent with other studies on this species (Guerra et al., 1999). In addition, the play deprived hamsters also overreacted to defeat stress as adults, suggesting that the altered development of the mPFC is associated with sociocognitive deficits (Burleson et al., 2016).

In conclusion, the studies using the adult rearing paradigm have shown that it is sufficient to deprive animals of play, resulting in changes to the neurons of the mPFC and the OFC in both rats (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013) and hamsters (Burleson et al., 2016). However, in rats, this paradigm can only be used with females, as males housed with an adult male will suffer stress from social defeat (A. Burke et al., 2017), or will begin exhibiting sexual behaviors during the onset of puberty if housed with an adult female (Pellis & Pellis, 1990). Consequently, for rats, only female juveniles reared with female adults have been used (Bell et al., 2010; B. Himmler, Pellis & Kolb, 2013; B. Himmler et al., 2018). In the hamster study, males were reared with their mothers, but this rearing set-up was terminated before they gained sexual maturity (Burleson et al., 2016). To uncover any sex differences resulting from play deprivation, deprivation models that can be used on both sexes are needed. In addition to the inability to assess both sexes is that stress from social defeat may not just be suffered by male juveniles (Martinez et al., 1998). Therefore, the Fischer 344 (F344) rearing paradigm may avoid the stressors present in both the isolation and partition rearing paradigms can be employed to deprive both sexes.

The Fischer 344 rearing paradigm

The F344 rat is an inbred strain that originated from Columbia University in the 1920s and has been extensively used for cancer research ever since (for a review see Foster & Frost, 2018). This strain gained the interest of play researchers when it was discovered that F344 rats had greater sensitivity to dopamine agonists (Helmeste, 1983), thought to be the result of an abundance of D2-dopamine receptors (Helmeste et al., 1981). A systematic dive into the play of the F344 rats, and comparing their relative playfulness with other strains, revealed that F344 rats do not engage in as much play (Siviy et al., 1997, 2003). Specifically, when playing with a partner from another strain, the F344 rats reduce the number of attacks they make. Additionally, when attacked, they are less likely to defend nape attacks actively and when they do, are less likely to use defensive tactics that promote playful wrestling – that is, complete rotations (Siviy et al., 1997, 2003). This work has shown that there are genetic differences accounting for the play differences in the F344 rat compared to other strains, which influence behaviors beyond play. For example, F344 mothers spend less time actively engaging in affiliative behaviors toward their pups (Moore et al., 1997), and this could indirectly influence subsequent play as the amount of maternal care a pup receives in infancy has been shown to impact the amount of play rats engage in as juveniles (Parent & Meaney, 2008). However, cross fostering F344 pups with mothers from more attentive strains showed no impact on the amount of play they engaged in during the juvenile period (Siviy et al., 2017), lending credence to the hypothesis that the low levels of play in F344 rats are likely due to genetic factors. Armed with this knowledge, researchers have capitalized on using the F344 strain to deprive rats from playful strains of play experiences (see Figure 1.2c).

A series of experiments by Schneider and colleagues (2014; 2016) found that rearing female juvenile Wistar rats, a playful strain (S. Himmler, Modlinska et al., 2014), with juvenile F344 peers, produces adult Wistar rats with sociocognitive impairments. In addition to sociocognitive impairments, the play deprived Wistar rats have increased pain sensitivity and alterations in cannabinoid receptors (Schneider et al., 2014; Schneider, Pätz, et al., 2016). The play between Wistar and F344 rats are similar to other reports of F344 rats playing with partners of other strains (Siviy et al., 1997, 2003). F344 rats initiate fewer playful contacts and when attacked by their Wistar partners, show a reduction in playful responses (Schneider et al., 2014; Schneider, Pätz, et al., 2016). When the F344 rat does respond to a playful attack, it shows a decrease in the number of pins, despite responding to the nape more often with a complete rotation during the peak play period (Schneider, Bindila, et al., 2016). As adults, Wistar rats reared with F344s show impairments in social interactions, with less instances of anogenital sniffing and follow/approach behaviors, as well as impairments in the social transmission of food preferences and in social recognition (Schneider, Bindila, et al., 2016).

The altered play of F344 rats thus appears to be insufficient to provide the appropriate play experiences for rats from more playful strains to develop normal sociocognitive skills. The advantages to using the F344 rats to deprive other rats of play are that, by being reared with a peer,

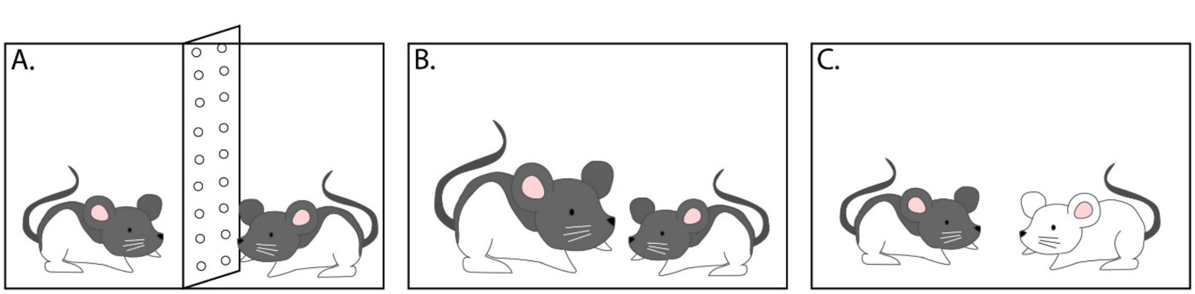

Figure 1.2 *Diagram of current methods for play deprivation.*

any potential stresses imposed by sharing an enclosure with an adult is eliminated and the same rearing paradigm can be used to test the effects of play deprivation on both sexes.

1.3 A new frontier for play deprivation studies

To date, no direct comparison between the sexes has been conducted on the long-term effects of play deprivation. While sex comparisons in the adult rearing paradigm are not feasible, they are for the other two paradigms described. For example, male and female rats were socially isolated using the partition rearing paradigm and then tested in the food protection paradigm (Pellis et al., 1999). Both sexes were found to have reduced ability to coordinate their movements if reared without play experience, but the measures available at the time were not sensitive enough to assess, quantitatively, if there was a difference in the magnitude of this impairment between the sexes. Moreover, the major criticism of the partition rearing paradigm is that it limits all close contact social behavior, not just play, so it cannot be assumed that it is play that provides the critical experiences (Bekoff, 1976). The F344 deprivation paradigm shows a lot of promise in this regard. I capitalized on the demonstrated value of this paradigm (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016) for my thesis research, by rearing male and female Long Evans (LE) rats, another highly playful strain (S. Himmler, Modlinska et al., 2014), with same-sex F344 peers. For female LE rats reared over the juvenile period with adult female partners, there is reduced pruning of the dendritic arbor of the pyramidal neurons of the mPFC (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013). Rearing LE males and LE females with F344 partners enabled me to compare the effects of play deprivation on the development of the mPFC between the sexes. Moreover, it has yet to be determined if the reduced mPFC pruning in the LE females reared with adults is correlated with impoverished social skills in adulthood. In the following chapters, male and female LE rats reared with F344 peers are tested in a version of the social interaction test (File, 2003; Smith et al., 1999) to assess whether, as adults, they exhibit sociocognitive deficits.

1.4 Thesis Objectives

The goal of this thesis is to explore the role of play experience during the juvenile period on the development of social competence and the development of the dendritic morphology of pyramidal neurons from the mPFC in male and female LE rats. To accomplish this, rats were purchased, and pair housed at PND28 in same-sex dyads composed of either same-strain or mixed-strain pairs. That is, a LE rat with another LE rat, or an LE rat with a F344 partner (Figure 1.3). The thesis is broken into four empirical chapters, each teasing apart different aspects of the larger question at hand.

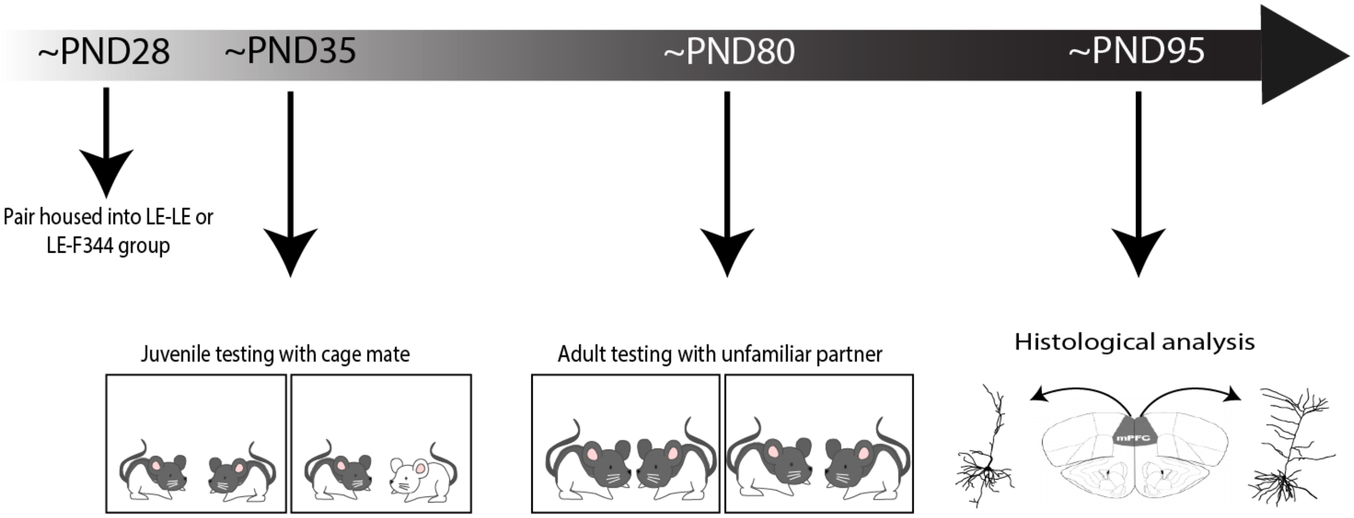

Figure 1.3 *Timeline of the cohort of rats used in this thesis. Rats were pair housed and tested during the juvenile period (Chapter 4), in adulthood males and females were separately assess (Chapters 2 and 3, respectively), after adult testing their brains were collected and assessed (Chapter 4).*

Adult behavior

Chapters 2 and 3 assess, respectively, the effects of play deprivation on adult social competence in male and female rats. As already mentioned, male and female rats play differently as adults. This difference in play is thought to be due to the function that play has at this stage of life. Adult males use play to assess and assert dominance with other males, whereas females use play, as do subordinate males within the colony, to maintain close, affiliative contact with dominant males (Pellis & Pellis, 1990; Pellis, Pellis, & McKenna, 1993; Smith et al., 1999). Among females, dominance hierarchies are more subtle and are less stable (Schweinfurth et al., 2017; Ziporyn & McClintock, 1991), and play does seem to be involved in negotiating these relationships (Pellis & Pellis, 1990; Smith et al., 1998a).

For these chapters of the thesis, I used the 'stranger paradigm' to assess the rats' social competence. When an intruder enters the territory of a rat colony, the dominant male, and to some extent, the dominant female, attacks the intruder, directing bites to its rump and face (D. Blanchard et al., 1984; D. Blanchard & Blanchard, 1990). In contrast, when two, unfamiliar, adult male rats meet in a neutral area, after a brief period of social investigation (Barnett, 1975), they begin to engage in a rough version of play, which appears to be used to determine which animal is dominant. Typically, this rough play is sufficient to assess dominance but can, on rare occasions, escalate to serious aggression – directing bites to the rump (Smith et al., 1999). I predicted that, for males, if the play deprivation led to deficits in social skills, these could be readily detected in the stranger paradigm by assessing aggression. Because females do not form strong dominance hierarchies within colonies and are not known to compete for dominance with unfamiliar females encountered in neutral arenas, I predicted that, if female-female interactions in the stranger paradigm were capable of revealing deficits in social skills, they would be more subtle than is the case for males. Even with this caveat, the stranger paradigm was used as a way of assessing social competence because it relies on naturally occurring behaviors between rats. After all, the ability of rats and other animals to navigate social interactions successfully requires that they develop a repertoire of behaviors and learn how to deploy them effectively (Barnett, 1975; Lore & Flannelly, 1977; Meaney & Stewart, 1979; Palagi, Burghardt, et al., 2016).

For these two chapters, I hypothesized that play experience during the juvenile period is essential for the development of appropriate social skills in adulthood. Based on previous work showing the sociocognitive impairments in female Wistar rats deprived of play (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016), I predicted that, if LE rats reared with a F344 partner do indeed have sociocognitive impairments, they should be less successful in navigating this novel social situation. In the stranger paradigm, the rats gradually establish a relationship, and to do so, they engage in play. This requires that they coordinate movements between each other, respond to playful attacks appropriately, inhibit overly assertive behaviors and monitor wins and losses. All of these skills have been linked to the mPFC (Baarendse et al., 2013; Chan et al., 2011; Omrani et al., 2020; van Kerkhof et al., 2014), which is altered when play experiences are limited (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013). Since there are differences in how males and females use play, along with sex-general predictions, I also made some sex-specific predictions about how the social incompetence would be detected in the stranger paradigm.

Juvenile behavior

Chapter 4 explores the aspects of play that the LE rat that is reared with a F344 partner is being deprived of during the juvenile period. Juvenile rats show a play rebound effect; that is, the longer they are deprived of the opportunity to play, the more they play once given the opportunity

(Panksepp & Beatty, 1980). Play behavior between LE and F344 cage mates were compared to the play between LE-LE cage mates after 24 hours of social isolation.

As mentioned above, F344 rats provide female Wistar rats with impoverished play experiences (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Based on this previous work, I hypothesize that the F344 rats would similarly provide deficient play experiences for LE cage mates. The play of LE male and female rats does not differentiate until adulthood (Pellis & Pellis, 1990), therefore I predict that both male and female LE would experience a similar play environment from their F344 partners. Additionally, based on the work by Schneider and colleagues, I was able to make specific predictions about the play experienced by the LE rats. Critically, these pertain to the asymmetry between play mates, since highly asymmetrical play experiences seem to be what is most damaging to the development of social skills (Pellis et al., 2017; Pellis, Pellis, Himmler et al., 2019).

Neuronal morphology

Chapter 5 explores the effects of play deprivation on the pruning of pyramidal neurons in the mPFC. Juvenile rats that are deprived of play have alterations in the neurons of their mPFC, leading to the hypothesis that play experience is important for pruning these neurons (Bell et al., 2010). Indeed, various experiences have been shown to induce changes to the neuronal morphology of the mPFC including social experience (Bock et al., 2008), environmental enrichment (Kolb et al., 2003), maternal separation (Muhammad & Kolb, 2011), chronic stress (Radley et al., 2009) and drugs of abuse (Robinson & Kolb, 2004). However, play seems to be an important modulator of experience. Juvenile rats that experience play in the juvenile period have been shown to be more sensitive to new experiences later in life (B. Himmler, Pellis, & Kolb, 2013).

Based on previous research, I made one prediction regarding the role of play during the juvenile period: LE rats reared with a F344 will have larger and more complex neurons indicative of a reduction in pruning, as has previously been shown for LE rats reared in the adult paradigm (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013). Neurons of male and female rats show sexual dimorphism, with the neurons in the mPFC of males typically being larger and more complex, suggesting that testosterone may modulate development of this area (Kolb & Stewart, 1991). However, other studies have found no sex differences (e.g., Kolb & Gibb, 2010; Muhammad & Kolb, 2011; Mychasiuk et al., 2012). Nonetheless, given that males tend to engage in more play fighting as juveniles (Meaney & Stewart, 1981; Pellis & Pellis, 1990; Thor et al., 1983), it is possible that play has a bigger effect on the pruning of mPFC neurons in males than females. It should be noted, however, that the presence and magnitude of the sex difference in play can vary with strain, rearing condition and the test paradigm that is used to assess play (Argue & McCarthy, 2015; S. Himmler, Himmler, Pellis, & Pellis, 2016; Thor & Holloway, 1984), Still, since the rats were reared and tested in the same paradigms, the magnitude of the difference in play between the sexes, whether large or small, should be consistent. Therefore, I was able to compare the effects of the same rearing paradigm on both sexes to test whether there was a sex difference on the magnitude of dendritic pruning in the mPFC.

Summary of objectives

There are four main objectives of this thesis:

1. To investigate the role of play deprivation on adult social skills in both male and female LE rats.
2. To investigate the key experiences missing in the play between LE and F344 rats that contribute to impairments in adulthood.

3. To investigate the role of play in the development of the mPFC in male and female LE rats.
4. To investigate if there are any sex differences in the effects of play on the development of social skills and the mPFC.

CHAPTER 2: MALE LONG EVANS RATS REARED WITH A FISCHER-344 PEER DURING THE JUVENILE PERIOD SHOW DEFICITS IN SOCIAL COMPETENCY: A ROLE FOR PLAY*

2.1 Abstract

Rats that are deprived of play during the juvenile period develop into adults that have a host of sociocognitive impairments. Thus, it has been hypothesized that peer play fighting during the juvenile period creates experiences that refine executive functions. The present study assesses the social ability of adult, male Long Evans hooded (LE) rats that have been reared with either a same age and sex LE rat or a Fischer 344 (F344), a low playing strain. As adults, their social skills were assessed. This was done by introducing each rat into a neutral arena with an unfamiliar partner. As predicted, rats from both conditions engaged the stranger in play, but the LE rats that had been reared with a F344 partner were more likely to escalate these playful interactions into aggressive ones. These findings support the hypothesis that play experience during the juvenile period is critical for the development of some executive functions.

Keywords: play fighting; aggression; play deprivation; Fischer-344; play signals; executive functions

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2.2 Introduction

Rough-and-tumble play or play fighting with peers is one of the most common forms of play in young mammals (Burghardt, 2005; Pellis & Pellis, 2009). The pioneering work by Jaak Panksepp with laboratory rats stimulated research into identifying the neural circuits that underlie such play (Panksepp, 1998; Panksepp et al., 1984). The impact that this work has had on furthering our understanding of the neural circuitry that controls play can be found in recent reviews (Achterberg & Vanderschuren, 2020; Siviy, 2016; Vanderschuren et al., 2016). In the present paper, we focus on the role that play in the juvenile period has on the development of social skills.

In rats, play fighting involves competition for access to the nape of the neck, which if contacted is gently nuzzled with the snout (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). Such play peaks in the juvenile period (30-40 days after birth) before declining, but continues, albeit at a lower frequency, well into adulthood (Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1990, 1991, 1997; Thor & Holloway, 1984). Rearing rats in isolation over the juvenile period leads to many behavioral and neural abnormalities (e.g., Arakawa, 2018; da Silva et al., 1996; Einon & Morgan, 1977; Fone & Porkess, 2008; Hall, 1998; Hol et al., 1999; Von Frijtag et al., 2002). However, given that all social contact is abolished by such isolation, it cannot be concluded that it is play that is the critical source of the experiences needed for normal development (Bekoff, 1976).

Rearing paradigms in which some contact, but not playful contact is allowed, such as intermittent contact with a non-playful peer is provided, or when juveniles are housed with social partners that provide reduced opportunities for play, still result in adult rats that have a variety of short-term memory, social and emotional deficits (e.g., Einon et al., 1978; Pellis et al., 1999; Potegal & Einon, 1989; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Moreover, these changes are accompanied by functional and anatomical changes in the brain (Siviy, 2019), especially in areas of the prefrontal cortex (e.g., Baarendse et al., 2013; Bell et al., 2010; B. Himmler, Pellis, & Pellis, 2013; van Kerkhof et al., 2014). These findings indicate that play fighting between peers in the juvenile period generates experiences that are important for refining the development of sociocognitive skills and the neural mechanisms that support those skills (Pellis et al., 2014, 2017; Vanderschuren & Trezza, 2014). More recently, these effects of juvenile peer-peer play have been extended to other rodents (Burleson et al., 2016; Marks et al., 2017) and evidence is mounting that the same is also true for many primates (Palagi, 2018), including humans (Nijhof et al., 2018).

To remain playful, play fighting must have a degree of reciprocity (Pellis & Pellis, 2017). This is achieved by the animals engaging in actions that, while competitive, also add a degree of cooperation, giving the partner a chance to reverse roles (S. Himmler, Himmler, Pellis, & Pellis, 2016). Recent studies have linked these role-reversal experiences as critical for the beneficial functions of play fighting in promoting the development of sociocognitive skills (Marks et al., 2017; Pellis et al., 2017, 2019; Schneider, Bindila, et al., 2016). For example, adults with diminished opportunity to engage in play fighting with peers during the juvenile period have reduced impulse control (Baarendse et al., 2013), impoverished social memory (Burleson et al., 2016; Schneider, Bindila et al., 2016) and impaired coordination of movements with a social partner (Pellis et al., 1999). When confronted by highly stressful situations, such as when attacked by a conspecific, these deficiencies become glaringly obvious as such animals react in ways that promote, rather than diminish, further attack (van den Berg et al., 1999). But even more benign forms of social contact can elicit responses that can quickly degenerate into aggression (Byrd & Briner, 1999). In the present paper, we used a rearing method that involved living with a social partner, but one that provided diminished opportunities for peer-peer play. The animals were then tested as adults in a situation that was socially challenging, but not overtly aggressive. This testing paradigm, the stranger paradigm, involves the introduction of two unfamiliar animals in a neutral arena. By combining these approaches, the specific effects of play in the rats' development of social skills could be tested.

To produce adult rats that experienced diminished opportunities for play, especially play involving role reversals, we capitalized on recent studies involving the rearing of rats from different strains. In studies by Schneider and colleagues (Schneider, Bindila et al., 2016; Schneider, Pätz et al., 2016), rats from a low playing strain, F344 rats (Siviy et al., 1997, 2003), were reared during the juvenile period with Wistar rats, a high playing strain (S. Himmler, Lewis, & Pellis, 2014). Analysis of the play during the juvenile period revealed that the F344 rats launch fewer attacks, and, when attacked, the F344 rats had a decreased likelihood to defend themselves, and most critically, had far fewer reciprocal exchanges (Schneider, Bindila et al., 2016; Schneider, Pätz et al., 2016). As adults, the Wistar rats had deficiencies in social memory (Schneider, Bindila et al., 2016). In a study with hamsters, in which juveniles were each reared with an adult, both sociocognitive deficits and reduced pruning of the dendritic arbor in the neurons of the medial prefrontal cortex (mPFC) were found (Burleson et al., 2016). Rearing juvenile LE rats with an adult also led to similarly altered development of the mPFC neurons (Bell et al., 2010; B. Himmler, Pellis & Kolb, 2013). Just like the Wistar rats reared with F344 peers, the juvenile LE rats reared with an adult experienced less play, with much less of that play involving role reversals (Pellis et al., 2017).

A limitation in the studies in rearing juveniles rats with adults is that they have only used females – if young males are reared with a female, with the onset of puberty, it leads to sexual behavior (Pellis & Pellis, 1990), and, if reared with an adult male, they can suffer stress from social defeat (A. Burke et al., 2017). Consequently, the neural and behavioral effects that have been found due to the impoverished play resulting from being reared with an adult has been for females (Bell et al., 2010; Burleson et al., 2016; B. Himmler, Pellis, & Kolb 2013). Similarly, the diminished sociocognitive skills reported for Wistar rats reared with F344 peers, has also been with females (Schneider, Bindila, et al., 2016). In the present study, juvenile male LE rats were either reared with a same age male LE rat or with a same age male F344 rat. In adulthood, the LE rats from both conditions were tested for their social skills, thus permitting the assessment of the impact of impoverished play in the juvenile period on males.

While social memory is important for social behavior and is a good reflection of underlying changes in the mPFC (Burleson et al., 2016), it does not assess whether affected subjects have impoverished social skills when interacting with another animal. Even complete strangers can successfully navigate social and sexual encounters (Smith et al., 1999; Whishaw & Kolb, 1985). Therefore, to test for effects on the social skills needed during social interactions, we used a test paradigm that capitalizes on a particular feature of the behavioral ecology of this species. Rats live in colonies with multiple adult males and adult females (Barnett, 1975). All adult males are dominant over females, but males form dominance relationships among themselves, with one becoming dominant over the others (Adams & Boice, 1983, 1989). And it is the dominant colony male that most vigorously attacks unfamiliar intruding males (D. Blanchard & Blanchard, 1990). Within a colony, adult males can use play fighting either to reinforce or reverse dominance status with peers (Pellis, Pellis, & McKenna, 1993).

While some subordinate males appear to adapt to their subordinate status, others try to reverse their position or leave the natal colony (R. Blanchard et al., 1988). Rats that leave their natal colony are likely to encounter similar adult males from other colonies. These encounters can be simulated in the laboratory. When unfamiliar adult males are introduced to one another in a neutral enclosure, they initially sniff one another as a means of identifying the other (Barnett, 1975), and then begin to engage in play, which can become very rough. In most cases, the rough play suffices to determine which partner becomes dominant, but occasionally, the encounters escalate to serious fighting, as is evidenced by the rats switching from attacking and nuzzling the nape to biting the rump (Smith et al., 1999). Social skills are important in negotiating such rough play and so avoiding escalation to aggression (Kisko, Euston, & Pellis, 2015). This 'stranger meets stranger' paradigm was used in the present study.

If deficient juvenile play experiences affect the development of social skills, we predicted that LE rats that were reared with a F344 cage mate should be less successful in navigating such a tense social situation. As the rats were placed in a neutral test enclosure, we predicted that they would not initiate agonistic attacks, as do males protecting their territory against an intruder (D. Blanchard & Blanchard, 1990). Rather, consistent with studies showing that play-deprived rats are highly motivated to engage partners in play, even later in life (Holloway & Suter, 2004; Varlinskaya & Spear, 2008), we predicted that play-deprived LE rats should initiate playful attacks and respond playfully to their partners. However, we also predicted that the play will be less stable and more likely to escalate to aggression as the play-deprived rats would be more likely to misread social cues (Byrd & Briner, 1999; Einon & Potegal, 1991). We also assessed the behavior of the partners of the subjects, as normally reared partners of experimentally treated rats may alter their behavior to adjust to the atypical behavior of their treated partners (Bell et al., 2009; Holloway & Suter, 2004; Pellis et al., 2006). Such adjustments can provide clues as to the deficiencies present in the experimental animals.

2.3 Methods

2.3.1 Subjects

Twenty-six weanling LE hooded male rats and ten weanling F344 rats were purchased from Charles River Laboratories (Kingston, New York) and arrived at the Canadian Centre for Behavioural Neuroscience at 24 days of age. At 26 days of age, the animals were housed in dyads consisting of either two LE rats (8 pairs) or a LE and a F344 (10 pairs). Another ten LE males were purchased from the same source at 75 days of age, housed in pairs and then used for the experiment when all the rats were 80 days of age. All animals were housed in polyethylene cages (46cm X 25cm X 20cm) with corncob bedding. Food and water were available *ad libitum.* Animals were maintained at a constant temperature of 21-23°C and were housed on a 12-hour light-dark cycle, lights off at 1900. All care and testing procedures were approved by the University of Lethbridge Animal Welfare Committee in compliance with guidelines from the Canadian Council for Animal Care.

2.3.2 Apparatus

Testing took place in a sound-attenuating chamber with a clear Plexiglas® enclosure (50cm X 48cm X 48cm) placed inside. The Plexiglas® box was filled with approximately 2-3cm of CareFresh® bedding. The chamber was lined with sound-attenuating foam (Primeacoustic, Port Coquitlam, British Columbia). An ExmourRS 4K Sony Handycam with night-shot capability was used for filming the interactions and was placed in a small window of the chamber at a 45° angle. Simultaneously, vocalizations during the encounter were recorded using an ultrasonic microphone (Model 4939, Brüel & Kjaer, Denmark) placed approximately 32-38 cm above the center of the Plexiglas® box and set to record sounds ranging between 4- and 100 kHz. Lastly, to synchronize the audio and video for post analysis, a device emitting a simultaneous red-light flash and audible sound was used. During all habituation and testing trials, three consecutive flash/beep signals were given to signify the start of the trial.

2.3.3 Procedure

At 80 days of age, the LE rats were ready for testing with an unfamiliar adult male LE rat in a neutral arena (Smith et al., 1999). One partner from each LE-LE pair was selected as the control animal to compare with the LE rats from the LE-F344 pairs. These rats had the base of their tails colored with a permanent marker pen (Sharpie®), to make them readily distinguishable from their play partner. An unfamiliar partner pool was created using the additionally purchased ten male LE rats and the second LE rat from the LE-LE pairs. Prior to play testing, cage mates were habituated to the testing enclosure for 15-minutes a day for four days. Fresh bedding was replaced and the Plexiglas® box was cleaned with Virkon® after each trial to reduce any odors left from the previous rats.

Prior to testing, the animals were socially isolated for 24-hours to increase playfulness when introduced to a play partner in the experimental enclosure (Panksepp & Beatty, 1980; Pellis & Pellis, 1990). Following social isolation, pairs of unfamiliar rats were introduced into the test enclosure for 10-minutes with video and audio recordings taken for the entire trial. To avoid the risk of loss of data due to equipment or other failure, each experimental animal was tested twice. After the first testing session, animals were rehoused with their cage mate for 24 hours before being socially isolated for the second test trial. In both trials, partners were assigned randomly so that they were unfamiliar to the experimental subjects (i.e., were not cage mates or in the case of the second trial, had not previously interacted in the first trial). At the end of the experiment, when the rats were around 90 days of age, they were sacrificed, and their brains removed for later histological analysis.

2.3.4 Behavioral analysis

For the present study, only the video files were analyzed as they provided the data suitable for testing the predictions. Video sequences from the first play trial only were evaluated using a combination of normal speed and frame-by-frame analysis to score various aspects of playful attack and defense (B. Himmler, Pellis & Pellis, 2013) and agonistic behavior (Smith et al., 1999). The first play trail was chosen for analysis, as none of the data from the pairings were lost. Also, as this was the first encounter with an unfamiliar partner, there was no risk of habituation to interacting with a new partner, as could be the case for the second session. Each video was scored in terms of the actions performed by the focal animal (the animal with the marked tail) and the actions directed toward the focal animal by its partner. A playful attack was scored when the snout of one rat was either in contact with or moved towards the nape of the other rat. The latency from the start of the trial to the first nape attack was also recorded. Once an attack was made, the recipient of the attack could either continue with its ongoing behavior or defend against the attack. Therefore, the probability of defense was calculated as the percentage of attacks that were defended. Defense involved either an evasion, in which the rat turned away from its attacker by running, leaping, or swerving away, or a facing defense, in which the rat turned to face its attacker. There are several types of facing defense and these are likely to lead to particular configurations in the play of adult male LE rats (Pellis & Pellis, 1987). When wrestling, the rats can end up with one lying on its back and its partner standing over it (i.e., a pin) (Panksepp, 1981), or they can remain standing, grappling with their forepaws in a mutually upright position. The former posture tends to reflect a more amicable state (Panksepp, 1998) than the latter, which tends to reflect a more agonistic state (R. Blanchard et al., 1977; Grant, 1963). Mutual upright measures can be useful in conveying overall patterns emerging from the dynamic wrestling that can follow facing defense (C. Burke, Kisko, Pellis, & Euston, 2017; B. Himmler et al., 2015) and can be scored either as the absolute number occurring per trial or as the percentage of encounters that end in those configurations. A key feature of play fighting is that the animals engage in actions that allow the partners to reciprocate roles of attack and defense (Palagi, Burghardt, et al., 2016; Pellis & Pellis, 2017). Therefore, to compare this cooperative aspect of play, the likelihood of play fights having a role reversal was also scored (S. Himmler, Himmler, Stryjek, et al, 2016).

As play between unfamiliar adult male rats is rougher than the play in the juvenile period or among familiar adult males (Pellis & Pellis, 1987, 1992), it has a higher risk of escalation to serious aggression (Smith et al., 1999). Gestures such as piloerection, lateral displays, and tail rattling are useful markers of aggression, with bites directed at the rump and lower flanks being a clear sign that the encounter has escalated into overt aggression (R. Blanchard et al., 1977; Kisko, Euston, et al., 2015; Smith et al., 1999; Takahashi & Lore, 1983). The absolute occurrence of each of these behaviors was scored during the 10-minute trials. For analysis, all these aggressive behaviors were combined into a total score, but, in addition, a separate score was kept for the number of bites for further comparison across the two conditions.

2.3.5 Statistical analysis

Two types of analysis were conducted: between pairs and between individuals. For the pairs, the total number of nape attacks, pins, mutual uprights, aggressive gestures, bites and the latency to make first contact on the nape, were scored. Numerical comparisons were made between the control pairs and the pairs containing a play-deprived partner. For individuals, the total number of nape attacks, aggressive gestures and bites were scored. In addition, the percentage of nape attacks defended and the percentage of defended attacks involving evasion were scored individually. To assess changes in the play-deprived rats, these values were compared between the play-deprived rats from the mixed pairs and a randomly selected partner (focal subject) from the control pairs. To assess the effects of playing with a play-deprived partner, the scores from the partners of the play-deprived rats were compared to the scores from the partners of the focal animals from the control pairs.

As all comparisons were pair wise and the data were normally distributed, unpaired Student's t-tests were used (R Core Team, 2018). One-tailed tests were used for comparisons with a directional prediction, otherwise, two-tailed tests were used. Differences were considered significant if *p*-values were ≤ 0.05. For tabular and graphical representation of the data, values were reported as group means with standard error of the mean (±SEM).

One observer scored all measures (RAS) and scores were compared to those from previous studies from the same laboratory that used the same experimental procedures and scoring techniques (S. Himmler, Stryjek, et al., 2013; Kisko, Euston, & Pellis, 2015; Pellis & Pellis, 1990). The values scored in the present study fell within the range of previously published values.

2.4 Results

As predicted, not a single interaction began with a partner lunging to bite the rump of an opponent. Also, as predicted, irrespective of rearing condition, the rats did engage in play. Pairs from both conditions initiated a similar number of nape attacks (t (16) = -1.455, p = 0.17; Figure 2.1a) and engaged in a similar number of pins (t (16) = -0.50483, p = 0.31; Figure 1b). Similarly, there were no significant differences in the percentage of attacks defended, the percentage of defended attacks involving evasion, or in the total number of mutual uprights or role reversals per trial (Table 2.1).

What did differ significantly was that the latency to the first nape attack, which was significantly longer for pairs with a play-deprived partner (27.0 ± 5.93s for control pair *versus* 52.0 ± 4.02s for the pair containing a play-deprived partner: t (16) = -3.606, p = 0.0024).

The pairs containing a play-deprived rat were significantly more likely to show signs of aggression (t (16) = 2.2833, p = 0.018), and also significantly more likely to escalate to biting (t (16) = 2.2331, p = 0.020) (Figure 2.2a). Closer inspection of the data showed that, when compared to the behavior of the focal rat in the control pairs, it was the play-deprived rat that was significantly more likely to show signs of aggression (t (16) = -2.6845, p = 0.008) and deliver more bites (t (16) = -2.343, p = 0.027) (Figure 2.2b). Consistent with the findings that it was the play-deprived rats that were more agonistic was that, even though they were just as likely to evade when playfully attacked as were the control subjects, a greater percentage of evasions by the play-deprived rats involved them kicking their partner with a hind leg (33.6 ± 7.16 by the matched control subjects *versus* 49.1 ± 5.76 by the play-deprived rats: t (16) = -2.6995, p = 0.016).

The partners of the pairs from the two groups differed significantly in the number of playful nape attacks launched with the partner of the play-deprived rat launching more nape attacks, while the percentage of attacks that were defended or the percentage of defended attacks that involved evasion were not significantly different (Table 2.2). Furthermore, the partners from the two groups did not differ significantly either in the overall expression of aggressive gestures (t (16) = -1.0932, p = 0.29) or in the number of bites (t (16) = -1.2494, p = 0.23).

Table 2.1

Various measures of play are compared between the focal rat from the control pairs and the play-deprived rat from the mixed pairs.

	Play-deprived rat	Control rat	t-test
Percentage of attacks defended	96.8 ± 1.02	91.2 ± 2.73	ns
Percentage of defense involving evasion	36.9 ± 4.19	32.0 ± + 6.67	ns
Number of mutual uprights per trial	11.6 ± 2.56	7.25 ± 2.07	ns
Percentage of defended attacks leading to a role reversal	5.6 ± 1.01	3.13 ± 0.69	ns

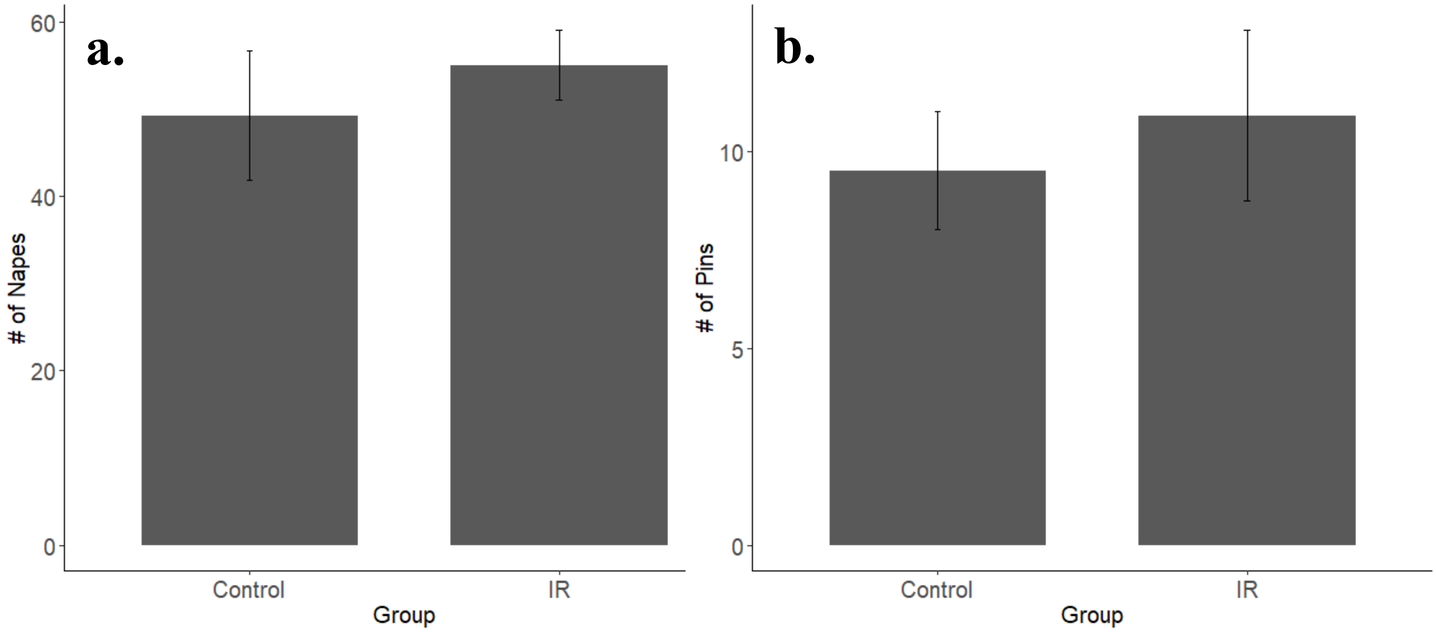

Figure 2.1 *The total number of playful attacks (a) and pins (b) per trial are compared between the control pair and the pair containing the play-deprived rats.*

Table 2.2

Various measures of play are compared between the partner of the control rat and the partner of the play-deprived rat.

	Partner of the play- derived rat	Partner of the control rat	Significance
Number of nape attacks	32.5 ± 2.97	20.6 ± 3.75	t = -2.5206 p = 0.022
Percentage of attacks defended	92.4 ± 1.61	90.5 ± 3.0	ns
Percentage of defense involving evasion	29.3 ±4.01	25.4 ± 4.84	ns

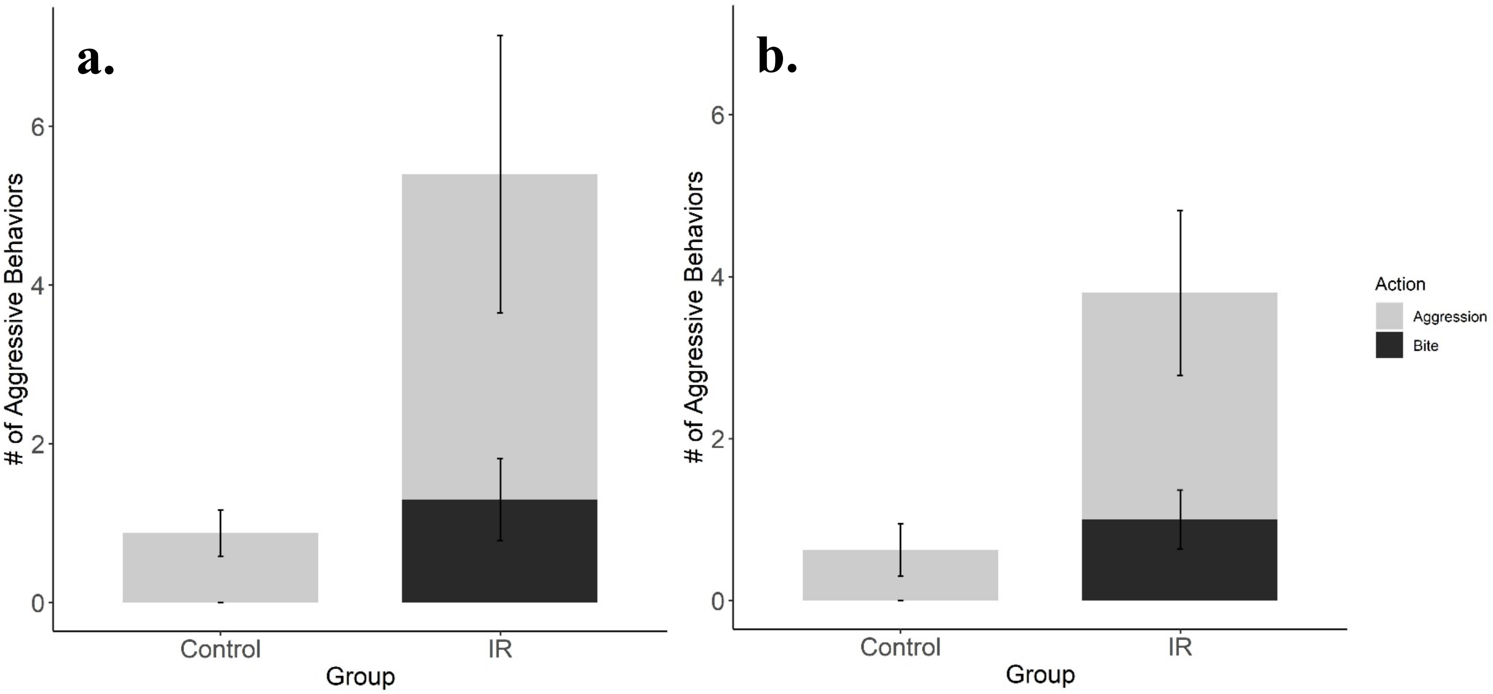

Figure 2.2 *The total number of aggressive actions per trial compared between control pairs and pairs containing a play-deprived rat (a) and between the focal rat from the control pairs and the play-deprived rat from the mixed pairs (b).*

2.5 Discussion

Being reared alone with a play-deprived partner leads to altered development of the mPFC and impoverished sociocognitive skills (Baarendse et al., 2013; Bell et al., 2010; Burleson et al., 2016; B. Himmler, Pellis, & Kolb, 2013; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). In our laboratory, we have used LE rats to evaluate these changes and have done so by rearing a juvenile female with an adult female partner. The adult infrequently initiates playful attacks and rarely provides opportunities for the young rat to negotiate the maneuvers needed to produce role reversals (Pellis et al., 2017). In a strain of rat in which the adult provides more such opportunities, the brain associated deficiencies are not present (Pellis, Pellis, Himmler, et al., 2019). To test whether being reared with a play-deprived partner affects sociocognitive skill development in male LE rats, in the present study, we used the Schneider rearing paradigm (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Juvenile male LE rats were reared with juvenile male F344 rats and then tested as adults in a socially challenging situation that is ethologically valid for males.

Unfamiliar male adults were introduced together in a neutral enclosure (Kisko, Euston, et al., 2015; Smith et al., 1999). If an unfamiliar adult male is introduced into the home cage of another adult male, the resident will attack aggressively and direct bites at the rump and lower flanks of the intruder (D. Blanchard & Blanchard, 1990). When the enclosure is not the territory of either rat, and so neutral, the strangers engage in a rough form of play fighting that can be sufficient to lead to one rat becoming the dominant animal (Smith et al., 1999). However, sometimes these encounters can escalate to aggression, especially if their ability to communicate is impaired (Kisko, Euston, et al., 2015). If LE rats reared with F344 partners are socially deficient, then we predicted that this would be a more difficult encounter to negotiate effectively.

Our data supported the prediction that the play-deprived LE rats would engage their partner in play (Figure 2.1) but that such play would also involve more instances of aggression (Figure 2.2). Based on the measures we used to score play (B. Himmler, Pellis, & Pellis, 2013; B. Himmler et al., 2015; S. Himmler, Himmler, Stryjek, et al., 2016), there were no obvious differences compared to the control rats that were reared with LE partners (Figure 2.1, Table 2.1). As is typical of this paradigm – strangers meeting in a neutral arena – aggression only emerged from encounters that began playfully (Kisko, Euston, et al., 2015; Smith et al., 1999). When the play involved a normally reared rat and a F344 reared LE rat, encounters were more likely to escalate to aggression (Figure 2.2a) and the aggression was most likely to be initiated by the play-deprived rat (Figure 2.2b). Clearly, during the playful interaction, the play-deprived rats were not assessing the context appropriately, suggesting that they are less socially competent than rats reared with more typically playing partners. Therefore, like females, males reared with an inadequately playing peer results in reduced social skills as adults (Schneider, Bindila, et al., 2016). We are currently beginning to compare the mPFC of male and female LE rats that were reared with either another LE or a F344 partner to assess whether these sociocognitive deficiencies are also reflected in comparable anatomical changes in the mPFC between the sexes. Before exploring the possible deficiencies in the LE rats reared with F344 peers, a potentially confounding factor needs to be considered.

Even though the F344 partner is a peer, it is different to a LE peer, not only in its play, but in its body size, locomotor activity (Siviy et al., 2003) and susceptibility to stress (e.g., Brodkin et al., 1998; Dhabhar et al., 1993), and, although the reports differ with test, age and sex, F344 rats may be more anxious in some situations (Bert et al., 2002; Chaouloff et al., 1995). Moreover, LE rats raised with Sprague-Dawley peers, which differ in some aspects of their play (S. Himmler, Modlisnka, et al., 2014), themselves modify their style of play (S. Himmler, Lewis, & Pellis, 2014). Consequently, the LE rats may not just be gaining atypical play experiences, but a variety of other experiences that could affect the development of sociocognitive skills.

While the influence of such factors cannot be discounted, there are several reasons that suggest it is the atypical play experiences of the LE rats reared with the F344 rats that are most crucial. First, Lewis rats cross-fostered with F344 mothers from birth, from whom they receive reduced maternal licking among other things, robustly retain most features of their strain-typical play as juveniles (Siviy et al., 2017). Second, unlike Wistar rats reared with F344 peers, Wistar rats reared with Sprague Dawley peers do not exhibit diminished social memory as adults (Schneider, Bindila, et al., 2016), suggesting that, despite some differences in their play (S. Himmler, Modlinska, et al., 2014), the Sprague Dawley rats must be providing the crucial play-derived experiences. Third, even though discordant in body size, playfulness and overall activity, adult female wild rats provide sufficient play-derived experiences to their juvenile partners to ensure the normal development for the mPFC (Pellis, Pellis, Himmler, et al., 2019). Together, these point to the lack of typical play experiences provided by F344 peers in the reduced socio-cogntive skills of their LE partners.

With regard to what the sociocognitive deficits may be, three further behavioral differences that were found provide possible clues. First, pairs in which one partner was play-deprived took almost twice as long to make the first nape attack compared to control pairs. That the play-deprived pair take longer to assess each other may be the result of deficits in processing social information. However, since the measurement was latency to the first nape attack regardless of who made the first nape attack, it remains to be determined as to why the intact animal is also apparently hesitant to initiate the first encounter. This hesitation raises the possibility that the intact animal is detecting some deficiency/abnormality in the play-deprived rat. Second, the partners of play-deprived rats initiated significantly more playful attacks than the controls (Table 2.2). Such an increased level of initiating play is typical of rats encountering unresponsive partners (Pellis et al., 2017; Reinhart et al., 2006; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Third, when evading their partners, play-deprived rats were more likely to kick at the pursuing animal with their ipsilateral hind leg than were control rats. Such kicking is often seen in agonistic encounters (R. Blanchard et al., 1977; Pellis & Pellis, 1987) and when a partner is avoiding the playful overtures of a partner (Holloway & Suter, 2004; Pellis et al., 2017). We hypothesize several processes by which impoverished juvenile play experiences could lead to these kinds of alterations in adult interactions.

As in other studies producing play-deprived rats (Varlinskaya & Spear, 2008), these rats were motivated to play (Figure 2.1, Table 2.1), but then found the actual contact initiated by their partner aversive, as indicated by increased kicking and escalation to aggression. One possible reason for this aversion to playful contact could be that their pain thresholds have been altered (Schneider et al., 2014). If this is so, the vigorous jumping on and pushing one another that are typical of play, may be perceived by them as unpleasant and so to be avoided. A second possibility is derived from the finding that there are separate neural mechanisms regulating the motivation to play and the pleasure derived from engaging in play (Vanderschuren et al., 2016). The failure to gain appropriate play experience in the juvenile period may affect the development of the pleasure mechanisms while leaving the motivational mechanisms largely intact (Kisko et al., 2016). If this is so, this would result in rats that 'want' to play, but do not 'like' playing.

A third possibility is that their ability to communicate may have been compromised. When one member in the stranger paradigm is unable to vocalize, it is much more likely that the pair will escalate from play to aggression (Kisko, Euston, et al., 2015). Rats emit many different kinds of ultrasonic calls (USVs) in a variety of social contexts (e.g., Bialy et al., 2000; 2016; Burgdorf et al., 2008; C. Burke et al., 2018; B. Himmler, Kisko, et al., 2014; Knutson et al., 1998; Seffer et al., 2014). During play, USVs are more likely to be emitted immediately before playful contact is made (B. Himmler, Kisko et al., 2014; Kisko, B. Himmler, et al., 2015). More specifically, particular types of calls are emitted in association with particular behavioral actions (C. Burke et al., 2018). Such coordination of calls and actions is disrupted when one partner is devocalized. In the stranger paradigm, it appears that failing to emit the appropriate call, when the pair begins to escalate the aggressiveness of the interaction, leads to biting (C. Burke, Kisko, Pellis, & Euston, 2017). Recently, it has been reported that increasing the amount of play experience gained during the juvenile period leads to an increased emission of USVs when the rats are socially isolated as adults (Emmerson et al., 2019). It is possible that the LE rats reared with F344 peers may have failed to learn when and how to insert particular calls, and this social inadequacy could account for their longer latency in commencing to play and their inability to coordinate encounters to prevent them from escalating to aggression.

Any of these factors could lead the play-deprived rat to give mixed messages to the normally-reared stranger – ‘come play with me, but don’t touch me, it hurts’, ‘come play with me, but don’t touch me, I don’t like it’, ‘my body says I am ready to play, but my calls say I am not’. The increased play initiation attempts by the partner of these disturbed rats may be an attempt to assess what is wrong with them. All these possibilities need to be explored experimentally, but what is clear is that the LE rats reared with F344 partners are deficient in their ability to use play as a social tool to negotiate a relationship with an unfamiliar partner.

CHAPTER 3: USING THE 'STRANGER PARADIGM' TO ASSESS SOCIAL COMPETENCY IN ADULT FEMALE LONG EVANS RATS REARED WITH A FISCHER 344 PARTNER*

3.1 Abstract

Rats reared with limited access to a play partner during the juvenile period develop into adults with impairments in various cognitive, emotional, and social skills. However, the majority of our understanding of the many deficits as a result of play deprivation comes from studying male rats. The present study assesses the consequences of play deprivation on adult social skills in female Long Evans (LE) rats that were reared with a low-playing Fischer 344 (F344) rat over the juvenile period. As adults, their social skills were assessed using the stranger paradigm, by pairing the deprived LE rats with a novel, typically reared LE partner in a neutral arena. While the deprived rat engages its partner in play, as expected, there were alterations in key aspects of play, such as reduced pinning and a longer latency to begin playing that suggest there are impairments in the social ability of the deprived rat. Most notable were the changes in the behavior of the typically reared partner, a reduction in the amount of play it initiated and fewer actions that produced reciprocal and prolonged interactions. The changes in the behavior of the normally reared partner suggest that it detected subtle changes in the play deprived LE rats. These findings support the ever-growing body of literature that provides evidence for the hypothesis that peer-peer play experiences during the juvenile period are important for the development of sociocognitive skills.

Keywords: social interaction; play deprivation; play fighting; executive functions, passing behavior

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* Manuscript submitted to Behavioural Processes. Under review.

3.2 Introduction

Being reared in social isolation during the juvenile period leads to adult rats that have a wide range of behavioral, emotional, cognitive, neural and physiological abnormalities (Arakawa, 2018; Einon & Morgan, 1977; Fone & Porkess, 2008; Hall, 1998; Potegal & Einon, 1989; van den Berg et al., 1999). Using rearing methods that prevent or limit peer-peer play, but not other forms of social contact, still lead to deficits in the development of some neural systems and various cognitive, emotional and social skills (Baarendse et al., 2013; Bell et al., 2010; Einon et al., 1978; B. Himmler, Pellis, & Kolb, 2013; Pellis et al., 1999; Potegal & Einon, 1989; Schneider etal., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016; Van Kerkhof et al., 2013), suggesting that play with peers is an important contributor to refining such skills, and the underlying neural mechanisms that support them (Pellis et al., 2014, 2017; Vanderschuren & Trezza, 2013).

The effects of absent or reduced experience with peer-peer play has been assessed in rats and other rodents by identifying significant changes in social memory, resilience to defeat stress, impulse control, and boldness in coping with unfamiliar non-social and social environments (e.g., Baarendse et al., 2013; Burleson et al., 2016; Hol et al., 1999; Marks et al., 2017; Schneider, Bindila, et al., 2016). While such deficits likely impact the animal's ability to interact with a partner, direct tests involving actual social behavior are rare. The older literature involving rearing rats in complete social isolation has provided abundant evidence of incompetent sexual behavior in adulthood (see review in Moore, 1985). But there are two problems with these studies. First, as noted above, complete social isolation deprives the rats of more than just the opportunity to play (Bekoff, 1976), and second, it is unclear what behavior are altered that impairs sexual encounters

(Pellis & Pellis, 2009). Using a food protection paradigm (Whishaw, 1988) to assess social responsiveness, a study preventing peer-peer play, but not restricting all social contact during the juvenile period, showed that while as adults they could perform species-typical defensive actions, the rats were less skilled in coordinating their movements with those of their partners (Pellis et al., 1999b). Importantly, similar deficits in inter-animal coordination during food protection has been shown in socially reared adult rats with damage to the mPFC (B. Himmler, Bell, et al., 2014), an area of the brain that is activated during social play (van Kerkhof et al., 2013), and is modified anatomically and physiologically by peer-peer play experience (Baarendse et al., 2013; Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013). These findings suggest that the effects of diminished peer-peer play experience in the juvenile period can be assessed directly in the actual social behavior performed by adults.

A commonly used paradigm to assess social behavior is the 'social interaction test', in which animals are placed together in a neutral arena with the latency to first contact and total amount of time spent in social contact during the test providing indices of social behavior (File & Seth, 2003). As originally designed, the test is used to assess anxiety and the value of various therapeutic drugs to alleviate stress. Prior to testing, rats are kept in social isolation for five to seven days, habituated individually to the test arena and then tested in pairs (File & Hyde, 1978, 1979). There are two limitations with this test to assess the negative effects of limited play experience. First, the extended period of social isolation prior to testing can have negative, acute effects, on social behavior, potentially masking the chronic deficits arising from the rearing manipulation (Einon & Potegal, 1991; Potegal & Einon, 1989). Second, likely due to the need to process large samples of rats tested with different drugs, the behavioral measures used are relatively simple, and do not assess the actual dynamics of the social interactions involved. Capitalizing on some characteristics of play in rats, the stranger paradigm, a different version of the social interaction test was developed to evaluate the way animals interact with one another and how this may be altered by differential experiences during the juvenile period.

Play fighting in rats involves competition for access to the nape of the neck, which is nuzzled with the snout if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). Play peaks during the juvenile period (from 30-40 days after birth) and while rats play less after sexual maturity, play continues well into adulthood (Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1990, 1991, 1997; Thor & Holloway, 1984). In natural settings, rats live in colonies containing multiple adult males and females, with all males dominant over all females and some males being dominant over the other males (Barnett, 1975; R. Blanchard et al., 1988; Calhoun, 1962). Within colonies, subordinate males and females preferentially initiate grooming and play interactions directed to the dominant males, seemingly to maintain affinitive bonds (Adams & Boice, 1983, 1989; Pellis, Pellis, & McKenna, 1993; Pellis & Pellis, 1991; Smith et al., 1998b). Given the evidence for a lack of stable long-term social bonds among rats (Schweinfurth et al., 2017), this could explain why punitive and affinitive interactions are initiated periodically, thus supporting some degree of temporal stability of relationships within groups (R. Blanchard et al., 1988; Pellis, Pellis, & McKenna, 1993).

The play directed to dominant males by subordinate males and females is gentler, resembling the play typical of juveniles. Whereas the play directed by dominant males to subordinate males and females is rougher, more typical of sexually mature males (Pellis & Pellis, 1991, 1992; Smith et al., 1998a). Sometimes, a subordinate male may engage in rougher play with the dominant, perhaps to test the relationship (Pellis, Pellis, & McKenna, 1993). Adult males thus use play to reinforce, assess and assert dominance in a colony setting (Pellis, Pellis, & McKenna, 1993). When a non-colony adult male intrudes into the territory, the dominant male attacks the unfamiliar male, by directing bites at their rump area (D. Blanchard & Blanchard, 1990). However, when two males who are unfamiliar with one another encounter each other in a neutral arena they engage in a rough form of play fighting (Smith et al., 1999).

In most cases, play between unfamiliar males in this neutral arena is sufficient to establish dominance, but the encounters can sometimes escalate to serious fighting, thereby switching from attempts to nuzzle the partner's nape to directing bites at the rump (Smith et al., 1999). Unlike the social interaction test, in the 'stranger paradigm', rats are housed with a partner and habituated to the neutral test enclosure with its cage mate and are only socially isolated for one day prior to being tested with the stranger, so reducing the acute effects of social isolation. Moreover, by videotaping the encounters (unlike the real time scoring of the social interaction test), the dynamics of the interactions can be analyzed to identify crucial moments in the playful encounters when escalation to aggression is most likely. Thus, post analysis is carefully carried out to characterize play tactics and communicative signals that may be used to mitigate such escalation (C. Burke Kisko, Pellis, & Euston, 2017; Kisko, Euston, et al., 2015). Consequently, the stranger paradigm can be used to assess if a rearing regime affects sociality, as is typically measured with the social interaction test, as well as to identify particular social skills that may be compromised.

Female Wistar rats, a highly playful strain (S. Himmler, Lewis, & Pellis, 2014), reared with age and sex matched F344 rat partners, a low playing strain (Siviy, 2020), develop into adults that have impairments in social memory, and in the social transmission of information (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Male LE rats, another high playing strain (S. Himmler, Modlinska, et al., 2014), reared with F344 rats, tested as adults in the stranger paradigm, show a deficit in social skills as their playful encounters are more likely to escalate to serious fighting (Stark & Pellis, 2020). In the present paper, we assess the social skills of adult LE female rats reared with age and sex matched F344 partners using the stranger paradigm. Some idiosyncrasies in the social relationships of females and their play may dampen the effectiveness of the stranger paradigm to identify social deficiencies.

In many group living species, females form dominance hierarchies for differential access to resources, such as food, water, space, or breeding opportunities (Hodge et al., 2008; Parish, 1994; Pusey et al., 1997). Adult female rats are subordinate to the adult males in a colony (Adams & Boice, 1983, 1989; R. Blanchard et al., 1988), and are rarely observed fighting other females in the colony, although resident females may attack unfamiliar females that intrude into the colony (DeBold & Miczek, 1984; Hood, 1981). If with a litter, the resident female may also attack intruding males, particularly if the female is lactating (Erskine et al., 1978). However, because of the low levels of aggression by adult females, dominance hierarchies among female rats were thought to be absent or play a minor role in colony dynamics (Barnett, 1975; Grant & Chance, 1958). Even so, some females within colonies are more likely to attack intruders to the colony, suggesting that these females may be more dominant animals (Adams & Boice, 1983; D. Blanchard et al., 1984). However, applying the same criteria used for identifying dominance among males, the dominance order identified among females is not stable (Grant & Chance, 1958; Takahashi & Lore, 1983). At best, the dominance relationships among females are subtle, but they can still influence social life. For example, females living in groups synchronize their oestrous cycles with those of the dominant female (McClintock, 1978, 1984). Moreover, having a higher dominance rank is correlated with greater reproductive success (Parent et al., 2013).

Both male and female rats continue to play into adulthood, but while adult females continue to use the same defensive tactics as when juvenile, adult males shift to using rougher tactics in play than when juvenile (Pellis & Pellis, 1990). This shift in defensive tactics used by male rats at sexual maturity (Smith et al., 1996, 1998a) makes it easier to use playful interactions to identify dominant versus subordinate males (Pellis et al., 2006). Whether dominance differences among females can be detected in their play remains to be determined. One indicator of possible dominance status is ‘passing’ (Ziporyn & McClintock, 1991), in which one rat moves along the flank of another. This can occur in face-to-face or face-to-tail orientations. In either case, the passing rat is asserting their dominance over the passed rat and it has been suggested to be a useful measure of dominance among females (Ziporyn & McClintock, 1991). Therefore, passing was measured in the present study to assess whether female rats attempt to assert dominance in the stranger paradigm.

If the impoverished play experienced by female LE rats reared with female F344 peers alters the development of social skills, then several features of their behavior in the stranger paradigm should be affected. Given the low levels of fighting and overt striving for dominance, it was predicted that, unlike males (Stark & Pellis, 2020), playful interactions would rarely, if ever, escalate to serious fighting. However, it was predicted that, like the males, if the females were socially impaired, there would be a greater latency for play to begin during the test trials. Also, it was predicted that the play deprived rats would be less likely to use tactics that promote continued wrestling and role reversals. That is, given that sustaining play fighting requires some degree of reciprocity (Pellis & Pellis, 2017), it is those actions that make playful interactions more symmetrical that are likely the ones that require greater social and communication skills to be able to coordinate actions with a partner (Kisko, Himmler, et al., 2015; Pellis, Pellis, Pelletier, & Leca, 2019). As this is the first attempt to measure passing in a playful context, I did not know what to expect.

Sometimes the measures used to assess changes in play in experimentally manipulated rats are not sufficiently sensitive to detect the effects of the treatment. However, subtle effects of the treatment may be revealed by alterations in the behavior of the untreated partner (Bell et al., 2009; Pellis et al., 2006). That is, if the behavior of the subject with impoverished play experience is atypical, then the intact partner should indicate this by being less likely to initiate play and more likely to avoid situations and actions that facilitate continued playful interaction.

3.3 Methods

3.3.1 Subjects

Thirty-six weanling female rats were purchased from Charles River Laboratories (Kingston, NY, USA), 26 were of the LE strain and ten were of the F344 strain and arrived at the Canadian Centre for Behavioural Neuroscience at 24 days of age. After two days of acclimatization, at 26 days of age, animals were housed in dyads consisting of eight same strain LE dyads and ten mixed strain LE-F344 dyads. Another ten LE females were purchased from Charles Rivers Laboratories (Kingston, NY, USA) at 75 days of age. After arrival, the rats were housed in dyads and used for the experiment when all rats were 80 days of age. Rats were housed in polyethylene cages (46 cm x 25 cm x 20 cm) with corncob bedding and food and water were provided *ad libitum*. The colony room was maintained at a constant temperature of 21°C-23°C and a 12-hour light-dark cycle (lights on at 0700). All care and testing procedures were approved by the University of Lethbridge Animal Welfare Committee in compliance with guidelines from the Canadian Council for Animal Care.

3.3.2 Apparatus

Testing took place in a sound-attenuating chamber lined with sound-attenuating foam (Primeacoustic, Port Coquitlam, BC, Canada). A clear Plexiglas® enclosure (50 cm x 48 cm x 48 cm) was placed inside and filled with 2-3 cm of CareFresh® pet bedding. Interactions were filmed with an ExmourRS 4K Sony Handycam with night shot capabilities at a 45° angle through a small window in the side of the chamber. Vocalizations were recorded during interaction with an ultrasonic microphone (Model 4939, Brüel & Kjaer, Denmark) mounted to the ceiling of the chamber, approximately 32-38 cm above the center of the Plexiglas® box and set to record sounds ranging between 4 and 100 kHz. At the start of every session in the chamber, a device is used that emits a synchronized light and audible sound, this is used for post analysis to synchronize the audio file with the video (B. Himmler, Kisko, et al., 2014).

3.3.3 Procedure

Animals were reared in same strain – LE-LE – or cross strain – LE-F344 – pairs from the juvenile period until adulthood. During the juvenile period, the play between pair mates in the dyads was tested (Stark et al, submitted). As adults, at 80 days of age, the LE rats were once again tested for their play, but this time in the stranger paradigm, with the findings for the males reported elsewhere (Stark & Pellis, 2020). One partner from each of the same strain dyads was selected as the control animals with which to compare with the LE rats from the cross-strain dyads. These rats had the base of their tails colored with a permanent marker pen to distinguish them from their play partner to facilitate video analysis. The remaining LE rats, the ones not chosen from the same strain dyads, and the additionally purchased LE rats, were used as the unfamiliar partners. Prior to the test with a stranger, cage mates were habituated to the testing enclosure for 15-minutes a day for four days. The Plexiglas® box was decontaminated with Virkon® between testing and habituation trial and the bedding was replaced.

Before the stranger paradigm trials, the rats were socially isolated for 24 hours to increase playfulness when introduced to a play partner in the experimental enclosure (Panksepp & Beatty, 1980; Pellis & Pellis, 1990). Following social isolation, pairs of unfamiliar rats were introduced in the test enclosure; audio and video of the ensuing interaction was recorded for ten minutes. To avoid any potential data loss due to equipment or other failure, each animal was retested two days later, following the same procedure. At the end of the behavioral trials, around 90 days of age, all animals were sacrificed, and their brains were collected for subsequent histological analysis (Stark et al., in progress).

3.3.4 Behavioral analysis

As all the trials from the first test were successfully recorded, those video files were used for the present study.

Play behavior

For each pair, the behavior of the focal animal and that of its partner was scored. The focal experimental animal was the LE rat reared with a F344 partner and the focal control animal was one of the LE rats from the LE-LE pairs. Video sequences were evaluated using a combination of normal speed and frame-by-frame analysis to score aspects of playful attack and defense (B. Himmler, Pellis, & Pellis, 2013). A playful attack was scored when the snout of one partner came in contact with, or moved towards, the nape of the neck of its partner. The total number of nape attacks each pair mate launched per trial was scored. In addition, the latency from when introduced into the test enclosure to the first nape attack was scored. The recipient of an attack could either continue its current behavior or defend itself, allowing the percentage of attacks defended to be calculated. Defended attacks can involve either an evasion, in which the defender runs, leaps, or swerves away to create distance between its nape and the attacker's snout, or a facing defense, in which the defender turns towards the attacker, placing its face between the attacking rat and its nape. As evasions and facing defense together comprise all defended attacks, for group comparisons, the percentage of defended attacks involving facing defense was calculated. Further, facing defenses can involve either 1) rotating around a vertical axis, usually around the pelvis, and so pivoting horizontally toward the attacker, or, 2) rotating cephalocaudally around the longitudinal axis. The second rotation tactic can take one of two forms: i) partial rotation, in which the defending rat rotates its forequarters, but maintains contact with the floor with at least one of its hind paws, and ii) complete rotation, in which the defending rat rotates until it lays fully supine on its back. As the complete rotation tactic is the modal form of facing defense in both juvenile and adult females (Pellis & Pellis, 1990; Smith et al., 1998a), the percentage of facing defense involving complete rotation was compared between the experimental and control rats.

The pin configuration, in which one partner lays supine on its back while the other rat stands over it (Panksepp, 1981), was also scored. In LE rats, most pins arise from the defender performing a complete rotation, but can also arise from the partner pushing the defender over when adopting any facing defense (S. Himmler, Himmler, Stryjek, et al., 2016). When using the complete rotation, the defending rat is actively selecting to adopt the supine defense position, but when the defending rat is pushed onto its back by its partner, a discrepancy between complete rotations and pins can reveal whether an attacker is more forceful when initiating play (B. Himmler, Pellis, & Pellis, 2013; Pellis & Pellis, 1987). Therefore, the percentage of facing defenses that ended with a pin was compared between the experimental and control animals. In addition, as longer lasting pins reflect continued wrestling between the partners as they compete to attack and defend their napes (C. Burke et al., 2018; Panksepp et al., 1994), the duration each partner of the dyad was pinned was scored and compared between the experimental and control rats. Since a certain number of role reversals, in which the attacker becomes the defender, are needed for playful interactions to remain playful (Pellis & Pellis, 2017), deviant rates of such reciprocity can indicate atypical play behavior (Pellis et al., 2017; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Therefore, counterattacks launched by one rat when engaging in facing defense that led to its partner adopting a defensive position, were scored (S. Himmler, Himmler, Pellis, & Pellis, 2016; Stark et al., submitted). The percentage of successful counterattacks launched during facing defenses was calculated to compare between the experimental and control rats.

Even though aggression between females is rare, it can happen. Therefore, encounters were also scored for agonistic behaviors. Threat signals such as piloerection, lateral displays and tail rattling are all used in aggressive encounters, usually leading up to serious aggression, which involves bites directed at the rump (R. Blanchard et al., 1977; Kisko et al., 2015; Smith et al., 1999; Takahashi & Lore, 1983).

Passing

Passes can be categorized according to two criteria, see Figure 3.1 (Ziporyn & McClintock, 1991). First, passing can vary in the orientation between the animals' bodies: head-to-head or head-to-tail. From the head-to-tail configuration, the passing rat is designated as the "winner". Second, the movement can be continuous or discontinuous. In the latter, the passing rat briefly stops before finishing the pass. Passes were only scored as 'passes' if the sides of the rats touched or were at least closely oriented toward the flank of the other. For example, if one rat passed the other's rump area, a pass was not recorded. Similarly, for discontinuous passes, the stop needed to be brief, if long enough for the rat to engage in another behavior, such as inspecting the bedding or sniffing its partner, a pass was not scored. All passes that occurred in the ten-minute trials were scored for each pair.

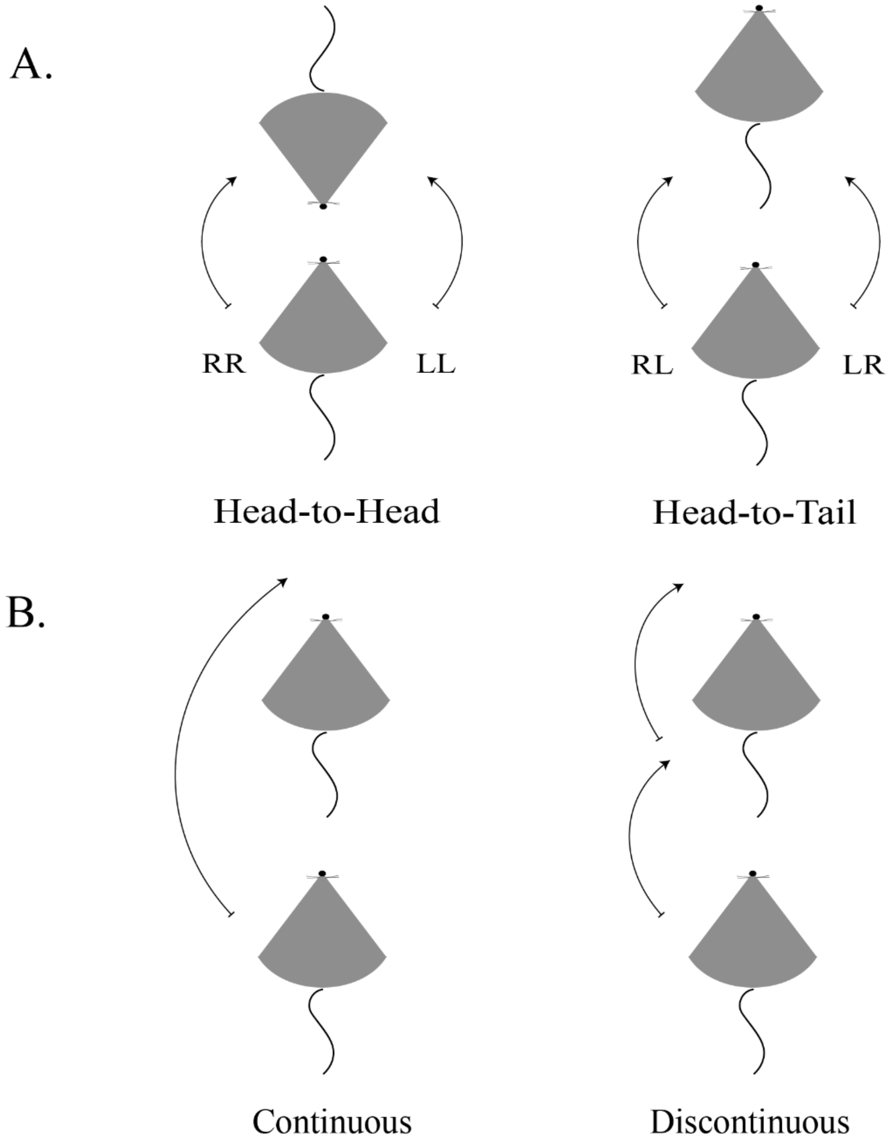

Figure 3.1 *Variation in passing behaviors in female rats. A) shows the two main orientations for passing, while B) shows the two main movement types.*

3.3.5 Statistical analysis

The data from the dyads were compared in two ways. The first was between the focal rats – the LE rats reared with a F344 peer (experimental) and one of the rats from the LE-LE pairs (control). The second was between the partners of the focal rats. These comparisons were analyzed with unpaired Student's *t*-tests (R Core Team, 2018). One-tailed tests were used for comparisons with a directional prediction, otherwise, a two-tailed test was used. Differences were considered significant if *p*-values were ≤ 0.05. All data are represented as group means and the standard error of the mean (± SEM).

3.4 Results

3.4.1 Play

In neither the pairs containing the experimental animals nor in the control pairs did any playful interactions escalate to serious fighting, involving biting. Indeed, none of the interactions even contained any agonistic posturing or signaling. There was a significant difference in the latency to the first nape attack ($t(16) = -2.68$, $p = 0.009$), with pairs containing an experimental rat taking almost triple the amount of time to begin playing (control pairs: 33.58 ± 12.33 s; experimental pairs: 96.23 ± 31.03 s).

Focal rats

There appeared to be a trend for the focal rats in the experimental pairs to initiate more nape attacks than the focal rats in the control pairs (Figure 3.2, dark grey bars), but given the large variance in both groups, this was not significant ($t(16) = 0.6307$, $p = 0.731$). There were no significant differences in the likelihood of a nape attack leading to a defense or in the type of defenses most likely to be used (Table 3.1). There were also no significant differences in either the likelihood that a facing defense led to a pin or to counterattacks. However, once pinned, there was a significant difference in the duration of time in remaining pinned (*t*(16) = -2.415, p = 0.014), with the experimental animal being much less likely to remain in the supine position (Figure 3.3).

Partners of the focal rats

There was a significant decrease in the number of nape attacks made by the partners of the experimental rats compared to the matched controls (*t*(16) = -3.178, p = 0.0029) (Figure 3.2). Whether attacked by the experimental or the control rats, the recipients were just as likely to defend against attacks and that defense was just as likely to be a facing defense (Table 3.2). However, when performing a facing defense, the partners of the experimental rats were significantly less likely to use a complete rotation and significantly less likely to launch counterattacks (Table 3.2). When they were pinned, the duration of time in remaining supine did not significantly differ whether attacked by an experimental or control rat (Figure 3.3).

3.4.2 Passing

Only five out of eight control pairs and six out of ten experimental pairs performed passes, with a total of 34 passes for the control pairs, and 35 for the experimental pairs. Of these, only 16 met the criteria for identifying a winner (N = 9 for control pairs, N = 7 for experimental pairs). A power analysis with a power of .8, a medium effect size (Cohen's d = .5) and an alpha level of 0.05, indicated that we would need more than double the passes available to conduct the kinds of targeted pairwise comparisons used with the play data. Therefore, no conclusions can be drawn as to whether particular types of passes were more likely between the two groups or if there were any differences in winning. Nonetheless, the distribution of passes appeared to be more asymmetrical in the pairs containing an experimental rat (Figure 3.4). A non-parametric test, Mood's Median Test, was used to assess this difference. The median number of passes made by the focal experimental rats was significantly different than the median number of passes made by their partners (z = 2.59, p = 0.01), while no difference was seen in the median number of passes made by the partners in the pairs from the control group (z = -0.85, p = 0.4).

Table 3.1

Various measures of playful defense are compared between the focal rats from the experimental pairs with that of the focal rat from the control pairs.

Behavior	Experimental rats	Matched control rats	t-tests
Percent of attacks defended	87.5 ± 5.28	86.7 ± 3.60	ns
Percent of defended attacks involving facing defense	67.7 ± 10.28	66.0 ± 6.04	ns
Percent of facing defenses involving complete rotation	26.5 ± 7.42	38.8 ± 10.7	ns
Percent of facing defenses ending in a pin	31.2 ± 7.34	47.4 ± 8.89	ns
Percent of facing defenses involving a counterattack	12.6 ± 3.98	12.6 ± 3.41	ns

Table 3.2

Various measures of playful defense are compared between the partners of the focal experimental and the focal control rats.

Behavior	Partner of the experimental rats	Partner of the matched control rats	t-tests
Percent of attacks defended	90.0 ± 4.06	86.8 ± 2.28	ns
Percent of defended attacks involving facing defense	62.2 ± 11.47	79.0 ± 3.89	ns
Percent of facing defenses involving complete rotation	25.2 ± 5.70	43.2 ± 6.79	t = -2.05, p = 0.029
Percent of facing defenses ending in a pin	41.4 ± 7.93	48.6 ± 10.39	ns
Percent of facing defenses involving a counterattack	6.4 ± 2.42	16.2 ± 4.88	t = -1.78, p = 0.047

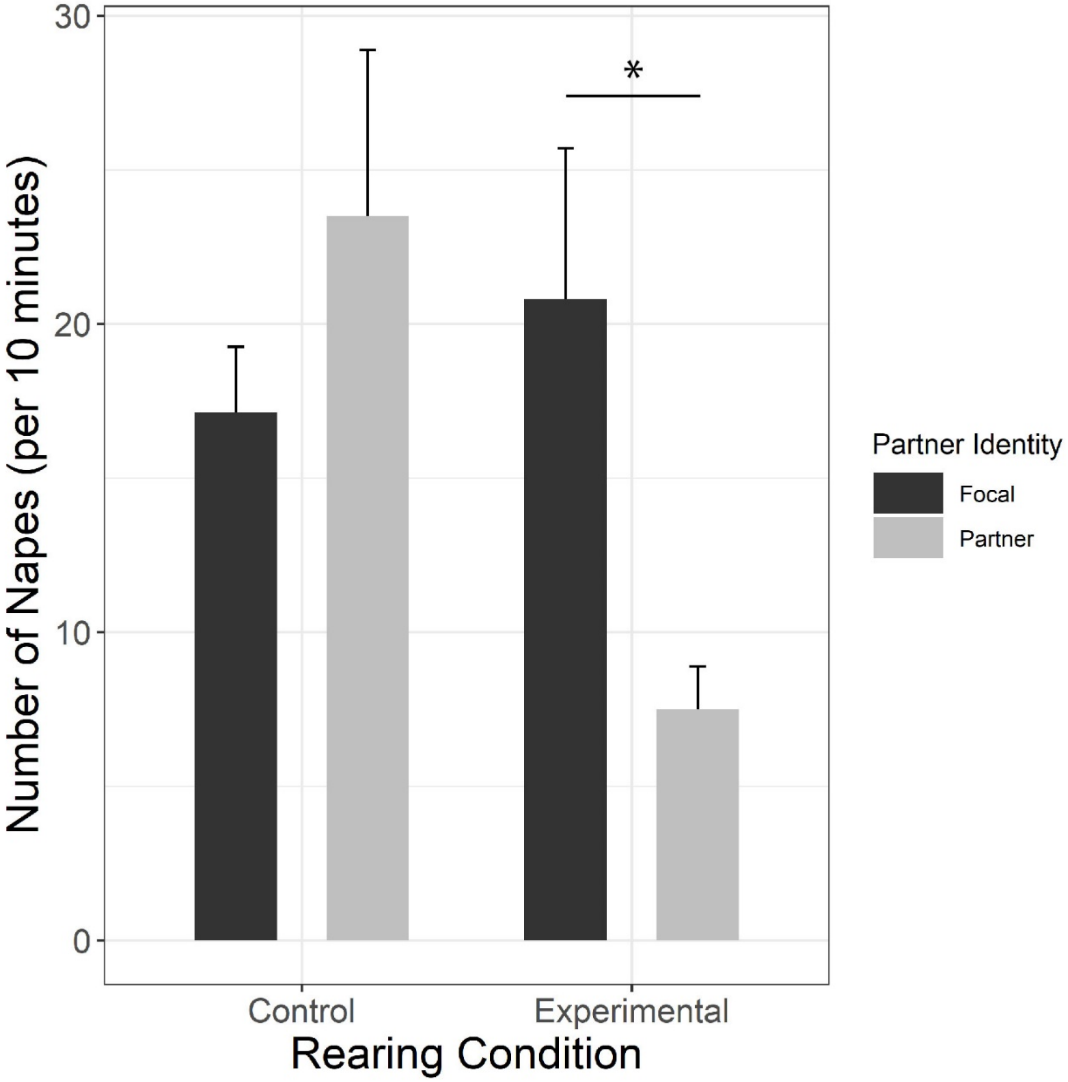

Figure 4. 2 *Shows the average ± the SEM number of nape attacks made during the ten-minute interaction of female Long Evans rats during the stranger paradigm, (*) denotes significant p ≤ 0.05.*

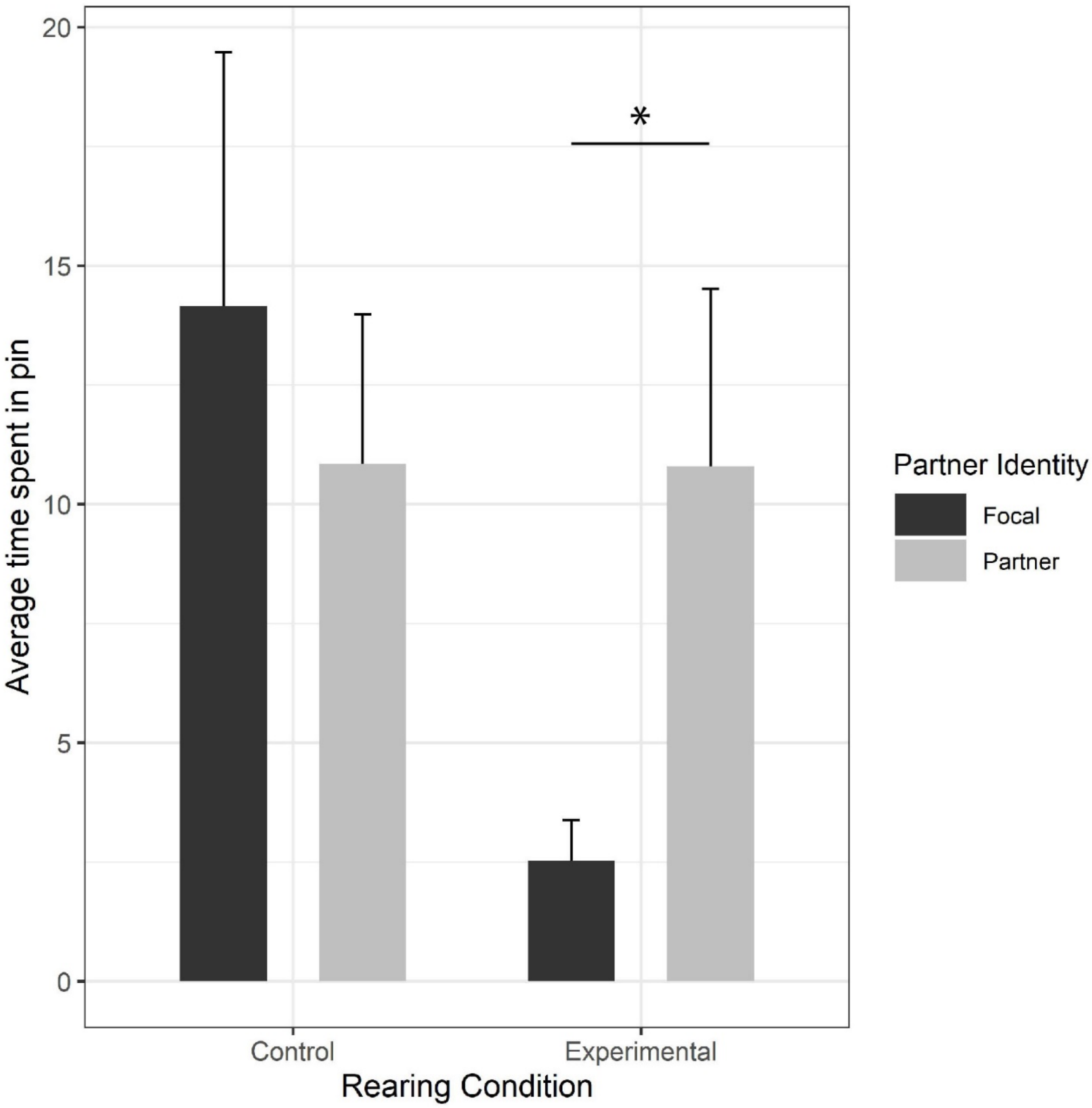

***Figure 3.3** Shows the average ± the SEM for the duration each rat in the dyad spent pinned by its partner during the stranger paradigm, (*) denotes significant p ≤ 0.05.*

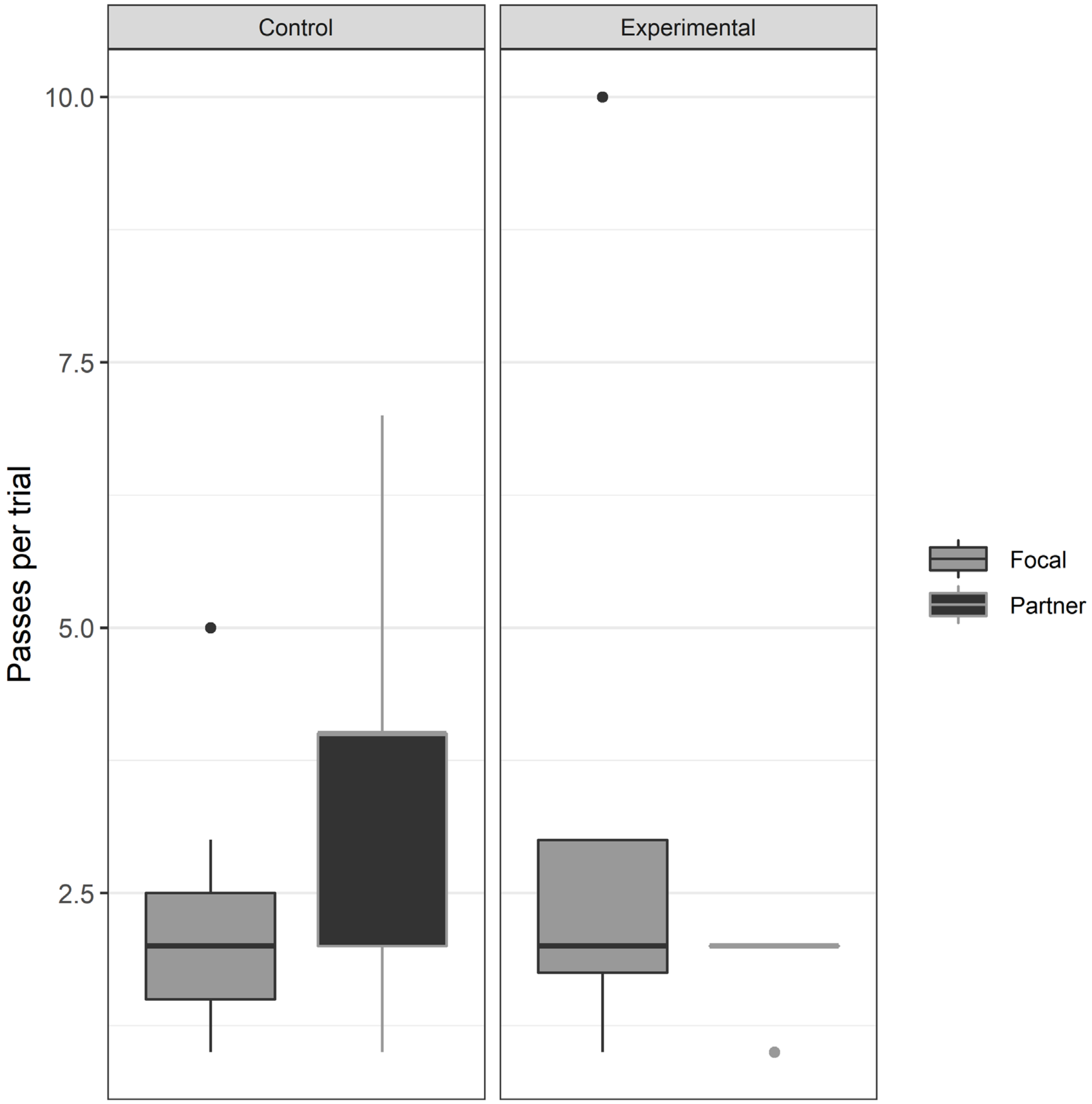

Figure 3.4 *Boxplot for the average number of passes during the ten-minute interaction between female Long Evans rats.*

3.5 Discussion

None of the female pairs in the present study escalated from playful interactions to serious fights, as can happen when adult males are tested in the stranger paradigm (Kisko, Euston, et al., 2015; Smith et al., 1999). Indeed, when we tested adult male LE rats that had been reared with F344 peers during the juvenile period in the stranger paradigm, escalation to serious fighting was significantly more likely than for LE males reared with LE peers (Stark & Pellis, 2020). One reason no aggression was seen in the current study is that, in this novel situation, female rats were not motivated to assert dominance over the stranger, unlike the case with males, in which the play is rough and only becomes gentler once one partner starts behaving in a more submissive manner (Smith et al., 1999). Dominant females may attack strangers, especially other females that intrude into the colony (D. Blanchard et al., 1984; DeBold & Miczek, 1984), but to our knowledge, no one has reported females fighting in neutral arenas. Consistent with this, passing was rare, suggesting that this subtle form of dominance assertion is more likely to occur among females within a colony (Ziporyn & McClintock, 1991), not against strangers in a neutral arena. Nonetheless, the passing which did occur was more asymmetrically distributed in the experimental pairs, with the LE rat reared with a F344 peer performing more passes than its partner (Figure 3.4). This may reflect the experimental animal's failure to assess the situation properly, and so may indicate impoverished social skills. However, given the small sample size, the potential disproportionate effect of outliers (Figure 3.4) and the low power of the statistical test used (Mood's Median Test has a high likelihood of making type 1 errors), whether the difference in passing is real remains to be determined. Future studies should assess such rats in competitive paradigms, such as gaining access to a water nozzle (Scott & Fredericson, 1951; Syme et al., 1974), to determine whether females are more likely to escalate these interactions to fighting.

In the absence of attempts to assert dominance during the playful encounters, the females are less likely to put themselves in situations in which social skills are needed to avoid escalating to serious fighting as is the case with males (C. Burke, Kisko, Pellis, & Euston, 2017). Consequently, using the stranger paradigm with females does not create a context in which social failure is readily identifiable – such as when biting one's partner. However, some of the features of play measured do suggest that the LE females reared with F344 peers are less attractive partners and less skilled at engaging in protracted and reciprocal interactions. Compared to the LE rats reared with an LE peer, the LE rats reared with a F344 peer initiated a similar number of playful attacks (Figure 3.2), were just as likely to defend themselves when attacked, and were just as likely to use the same tactics of defense (Table 3.1). Thus, the experimental LE rats appeared to engage in play typical of females of this strain at this age: they launched around 20-30 playful attacks, actively defended around 90% of the attacks received and did so in the majority of cases by turning to face their attacker, with rolling over to supine being one of the most commonly used facing defense tactics (Pellis & Pellis, 1990; Smith et al., 1998a). The partners of the experimental rats showed two differences in their play behavior that suggests that the experimental rats were not viewed as attractive play partners – they launched fewer playful attacks (Figure 3.2) and were significantly less likely to use the supine defense (Table 3.2). Several studies have shown that rats confronted with less attractive play partners initiate less play and avoid using tactics that promote close-quarter contact (Bell et al., 2009; Holloway & Suter, 2004; Pellis et al., 2006, 2017; Varlinskaya et al., 1999; Varlinskaya & Spear, 2008). Configurations and actions that promote continued playful contact, such as pinning, further indicate that there was something amiss with the experimental rats.

Adopting a position in which one rat lays on its back with its partner standing on top, the pin configuration (Panksepp, 1981) is characteristic of play fighting of many strains of rats (S. Himmler, Modlinska, et al., 2014). As such, the pin is a frequently used behavioral marker for scoring play in rats (e.g., Aguilar et al., 2009; Siviy et al., 2011; Trezza & Vanderschuren, 2008; Varlinskaya & Spear, 2009). From the pin configuration, rats continue to compete for access to each other's napes and this can lead to prolonged playful contact (Pellis & Pellis, 1987). Also, from this configuration, and to a lesser extent from other defensive positions, counterattacks can lead to role reversals (S. Himmler, Himmler, Pellis, & Pellis, 2016). Together, prolonged pinning and successful counterattacks are indicative of highly reciprocal interactions (Pellis et al., 2017, 2019). Reciprocity is not only essential for sustaining play fighting (Palagi, Cordoni, et al., 2016; Pellis & Pellis, 2017), but it also challenges the social skills of the participants (Palagi, Burghardt, et al., 2016; Pellis et al., 2014). Although being pinned is just as frequent in the experimental LE rats (Table 3.1), the time they remained in the supine position was significantly less (Figure 3.3). Short pin durations indicate that the partner on its back is remaining passive, rather than continuing the competition (Pellis & Pellis, 1987, 1990). It is during active pins that rats signal to one another the most (C. Burke et al., 2018, 2020). Moreover, the partners of the experimental rats were less likely to adopt the supine defense (Table 3.2), but remained in the supine position for as long a period as did the control rats (Figure 3.3). Given that most pins in LE rats arise from the defender using the complete rotation tactic (S. Himmler, Himmler, Stryjek, et al., 2016), the fact that the experimental partners are pinned at the same rate as controls but are less likely to adopt the supine defensive posture upon being attacked indicates that the experimental rats were more likely to push their partners over. Being repeatedly pushed over may have reduced the attractiveness of the experimental rats as play partners is further supported by the significantly reduced likelihood of their partners performing successful counterattacks (Table 3.2). That is, while the experimental rats counterattacked just as often as the controls (Table 3.1), their actions must have somehow been aversive to their partners, making them less inclined to reciprocate and so cutting short the interaction.

The features of play that differed from those of the controls (both focals and their partners) suggest that the experimental rats were not providing the appropriate signals or feedback to induce their partners to have prolonged, reciprocal interactions. A delay in beginning to interact with one's partner is a marker used in the social interaction test to identify abnormalities in the test subject (File & Seth, 2003), and such a delay was found in the stranger paradigm for both male LE rats reared with F344 peers (Stark & Pellis, 2020) and for female LE rats reared with F344 peers (present study). Therefore, taken together, the present study is consistent with the hypothesis that being reared with a partner that provides insufficient play experiences leads to defiencies in sociocognitive skills which is most likely related to atypical development of the supporting neural circuits of the mPFC (Baarendse et al., 2013; Bell et al., 2010; Burleson et al., 2016; Pellis et al., 2017; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016).

3.5.1 Females compared to males

Studies in which juveniles are reared with an adult play partner to restrict play experiences (Pellis et al., 2017) have only used female subjects (Bell et al., 2010; Burleson et al., 2016; B. Himmler, Pellis, & Kolb, 2013). Similarly, restricting play experience by rearing playful strains with F344 peers have also used females (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Studies using partitions to limit play but allow for visual, olfactory and auditory exchange and some physical contact, have mostly been conducted on males (Baarendse et al., 2013; Baarendse & Vanderschuren, 2012; Omrani et al., 2020). While in all cases, limiting play-derived experiences during the juvenile period has led to reduced sociocognitive skills and altered neural development, especially of the mPFC in both sexes (Pellis et al., 2014; Schneider, Bindila, et al., 2016; Van Kerkhof et al., 2014), it has not been possible to assess whether the magnitude of the effects is comparable between the sexes. One study used both sexes, limiting juvenile play experience using the partition approach, and found that, when tested as adults in a food protection paradigm, both males and females that had been deprived of play were less skilled in coordinating their movements with those of their partners (Pellis et al., 1999). However, the measures were not adequate to assess quantitatively whether there was a difference in the magnitude of these play deprivation effects between the sexes. In a study in which the amount of play experienced in the juvenile period was found to be positively correlated with being better at dealing with novel stressful situations (Marks et al., 2017), it was not possible to assess whether the magnitude of the effect differed between the sexes. The sexes need to be subjected to the same play limiting rearing condition and tested in a way that can assess potential sex differences in outcome.

To this end, we adopted the rearing of a playful strain with F344 partners in the manner after Schneider and colleagues, who housed Wistar rats in triads with either two same strain peers or two F344 peers (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). However, we modified the paradigm to conform with the juvenile-adult rearing paradigm (Bell et al., 2010). Being housed with multiple partners, irrespective of their playfulness, influences the development of the orbitofrontal cortex, whereas the mPFC is influenced by being reared with a playful partner, with the number of partners making no difference (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013; B. Himmler et al., 2018). Since these subareas of the prefrontal cortex influence different skills associated with social interactions (Bell et al., 2009; B. Himmler, Bell et al., 2014; Pellis et al., 2006), it is hard to know what is compromising sociocognitive skills when both are disrupted. Therefore, both male and female LE rats were reared as pairs with a same sex partner, either a F344 peer or a LE peer. As previously found for Wistar females being reared with same sex F344 peers (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016), as juveniles, the LE rats experienced less play and less reciprocity in their play when housed with F344 peers (Stark et al., submitted). To test the consequences of that juvenile experience the adults were tested in our version of the social interaction test, the stranger paradigm (Kisko, Euston, et al., 2015; Smith et al., 1999).

As already noted, LE males reared with F344 peers performed poorly – they were more likely to escalate the playful encounters into serious fighting (Stark & Pellis, 2020). LE females reared with F344 peers also seemed to perform less well than LE females reared with LE peers, with aspects of their encounters suggesting that they were less attractive partners, which led to altered play, especially those aspects related to reciprocity (present study). While these findings are consistent with the hypothesis that juvenile play experience affects the development of sociocognitive skills in both males and females, whether there is a difference in magnitude remains unknown. One approach to answer this question is to examine the anatomical changes in the pyramidal neurons of the mPFC, which have previously been shown to be altered in juvenile females reared with an adult (Bell et al., 2010; B. Himmler, Pellis & Kolb, 2013). We are currently analyzing the neurons from the mPFC of LE rats of both sexes reared in the F344 pairing paradigm. Preliminary data suggest that the neurons are changed in both sexes, but it is too early to conclude whether there is a magnitude difference between the sexes (work in progress). The other approach is to find a sex-neutral test paradigm with which to compare the rearing effects on socio-cogntive skills in the two sexes.

The kinds of tests that have been used to assess the effects of absent or reduced experience with peer-peer play are ones that evaluate cognitive and/or emotional responses and decision making, with some of these being appropriately sex-neutral potentially to test for sex differences in the effect size of the rearing manipulation (e.g., Baarendse et al., 2013; Burleson et al., 2016; Marks et al., 2017; Schneider, Bindila et al., 2016; van den Berg et al., 1999). However, what attracted us to the stranger paradigm was that it tested the animal's performance during an actual social interaction, in which moment-to-moment actions can derail the encounter. That is, it evaluates sociocognitive skills in the 'heat of battle'. Unfortunately, such encounters present different challenges to the two sexes, making this an unsuitable paradigm to determine if there are differences of magnitude between the sexes. The food protection paradigm (Whishaw, 1988) can be applied to both sexes (Field et al., 1997), with both sexes facing the same challenge, that of preventing a rat from robbing the food being held in the mouth of another rat. Since using the food protection paradigm to compare the effects of rearing condition on males and females (Pellis et al., 1999), a more sophisticated analytical framework has been developed to provide finer-grain measurements of inter-animal coordination (Bell, 2014; Bell & Pellis, 2011). This framework has been successfully applied to detect subtle changes in inter-animal coordination skills following damage to the mPFC (B. Himmler, Bell, et al., 2014). Such a paradigm may provide the framework needed to compare the effects of play experience in the juvenile period with the development of sociocognitive skills – a test that (1) can assess actual behavior during an interaction, (2) is sex-neutral, and (3) provides measurements that are sufficiently fine-grained to detect subtle, but potentially significant, sex differences.

3.6 Conclusion

The present study adds to the growing literature showing that limited or deficient peer-peer play experiences in the juvenile period results in impaired sociocognitive skills in adulthood (e.g., Baarendse et al., 2013; Burleson et al., 2016; Marks et al., 2017; Pellis et al., 1999; Schneider, Bindila et al., 2016). The stranger paradigm was found to be sufficient to detect sociocognitive impairment in both males (Stark & Pellis, 2020) and females (present study), but insufficient to assess whether the magnitude of impairment is the same between the sexes. Also, it remains to be determined what skills are deficient that lead to less sustainable playful encounters in the stranger paradigm.

One possible reason is that communication has been altered. Ultrasonic vocalizations (USVs) have been shown to be used to facilitate play (B. Himmler, Kisko et al., 2014; Knutson et al., 1998) and coordinate the actions of the partners during play (C. Burke et al., 2018, 2020). Play between devocalized juveniles is halved compared to intact peers and role reversals are reduced (Kisko, Himmler et al., 2015). Moreover, in the absence of hearing, pins are reduced (Siviy & Panksepp, 1987). If in the stranger paradigm, one of the adult males is devocalized, the risk of escalation to serious aggression is much greater (Kisko, Euston, & Pellis, 2015); this may arise because of the failure of that individual to emit particular vocalizations that are seemingly needed to deescalate the situation (C. Burke, Kisko, Pellis, & Euston, 2017). Given that, if reared with a F344 partner, the vocal milieu is different to that of LE rats reared with LE rats (Stark et al., submitted), it may be that they have not learned how to use USVs appropriately. Another possibility is suggested by the findings from Wistar rats reared with F344 peers, which have increased pain sensitivity (Schneider et al., 2014; Schneider, Pätz, et al., 2016). This could explain the reduced time spent in pins, as close-quarter wrestling could be painful to the LE rats reared with F344 peers. These, and other, possible mechanisms remain to be explored.

CHAPTER 4: DEFICIENT PLAY-DERIVED EXPERIENCES IN JUVENILE LONG EVANS RATS REARED WITH A FISCHER 344 PARTNER: A DEFICIENCY SHARED BY BOTH SEXES*

4.1 Abstract

Play fighting during the juvenile period has been shown to be an important experience for the development of sociocognitive skills and the underlying neural mechanisms that support them. Various paradigms have been used to deprive rats of play while still providing social contact. We used the paradigm of rearing a playful rat with a low-playing Fischer 344 (F344) partner to limit the play experienced by Long Evans (LE) rats during the juvenile period. This rearing paradigm has previously been shown to cause sociocognitive impairments in adulthood. In the present paper, we examined the play of same sex LE rats with LE or F344 partners at the peak juvenile period (around 35 days of age). F344 rats launched fewer playful attacks and when attacked, defended atypically compared to how LE do in LE-LE pairs. Playing with a F344 partner afforded LE rats fewer opportunities to engage in prolonged wrestling and fewer opportunities to ward off counterattacks (in which the defending rat becomes the attacker). In addition, there are fewer vocalizations emitted during the encounters in LE-F344 pairs and the types of calls most often emitted differed to those between LE-LE pairs. The altered play and communication experiences were equally present in male and female pairs. These findings are consistent with the hypothesis that, in such rearing paradigms, it is impoverished play experiences in the juvenile period that lead to impaired sociocognitive skills in adulthood.

Keywords: play deprivation, Fischer 344 rats, play fighting, executive functions, reciprocity, sex differences

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4.2 Introduction

Rats live in large social groups, with the males forming complex dominance hierarchies that are reinforced by overt aggression as well as by affiliative interactions involving play and grooming (Adams & Boice, 1983; Blanchard, Flannelly, & Blanchard, 1988; Pellis, Pellis, & McKenna, 1993). While females do not form strict hierarchies, there is evidence for subtle, dominance relationships (Ziporyn & McClintock, 1991). Therefore, functioning within the group requires a repertoire of social behaviors that can be used in contextually appropriate ways (Barnett, 1975; Lore & Flannelly, 1977; Meaney & Stewart, 1979). The development of these social skills, which are mediated by the executive functions dependent on neural circuits in the mPFC, has been linked to social play experienced during the juvenile period (Omrani et al., 2020; Pellis, Pellis, & Himmler, 2014; Schneider, Pätz, et al, 2016; Stark & Pellis, 2020; van Den Berg, et al., 1999; Vanderschuren & Trezza, 2014). For example, rats socially isolated during the juvenile period (postnatal day 21 – 42) have altered dopamine sensitivity of mPFC neurons and impoverished impulse control as adults (Baarendse et al., 2013). Similarly, rearing Wistar rats, a highly playful strain (S. Himmler, Himmler, Pellis, & Pellis, 2016), during the juvenile period with a F344 partner, a low playing strain (Siviy, 2020; Siviy et al., 1997, 2003), leads to impairments in social recognition in the Wistar as adults (Schneider, Bindila, et al., 2016). Moreover, when juvenile Long Evans rats, another highly playful strain (S. Himmler, Modlinska, et al., 2014), are only reared with adults, they experience impoverished play (Pellis et al., 2017) that appears to lead to atypical development in the dendritic morphology of their neurons in the mPFC (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013; B. Himmler et al., 2018). Changes in the development of the mPFC, and social skills, resulting from inadequate play experiences have also been shown in other rodent species that have complex patterns of social play (Burleson et al., 2016; Marks et al., 2017).

In rats, as in the young of many other mammal species, play fighting is the most common form of social play (Burghardt, 2005; Pellis & Pellis, 2009). Play fighting in juvenile rats involves competition for, and defense of the nape of the neck, which if contacted is gently nuzzled with the tip of the snout (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). Such play is highly rewarding (Trezza et al., 2010, 2011; Vanderschuren, 2010). When defending the nape, rats use several tactics. They can evade by turning away from their attacker, and by swerving laterally, running or jumping, or they can turn to face their attacker, thereby using their body to block access to the nape. Facing defense can involve partial rotation, in which the rat rotates its upper torso while leaving at least one of its hind paws in contact with the floor, a complete rotation to supine, or a standing defense, in which the animal rears onto their its hind legs and engages in a pushing or 'boxing' contest (Pellis & Pellis, 1987). Development of playful attacks directed at the nape begin at around 17 days after birth (Baenninger, 1967; Bolles & Woods, 1964), but defensive tactics, especially those involving complete rotation to supine, do not achieve their juvenile-typical form until around 28 days after birth (Pellis & Pellis, 1997). Thus, when play reaches its peak frequency between 30 and 40 days after birth, rats have a fully mature repertoire of playful actions (Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1990; Thor & Holloway, 1984). This is the age at which the effects of play are most pronounced on the development of sociocognitive skills and the development of the neural circuitry of the mPFC (Pellis et al., 2014; Vanderschuren & Trezza, 2014).

There are several components of the play dynamic that influence the development of social competency and the mPFC. When a juvenile rat is reared with a partner that affords an impoverished play experience - such as an adult, a drugged peer or a peer from a less playful strain, such as a F344 - one compensatory tactic employed by the impoverished rat is to increase the overall number of attacks it launches, thus increasing the overall number of play fights it experiences; however, despite such compensation, deficits still persist (Einon et al., 1978; Pellis et al., 2017; Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Therefore, increasing playful nape attacks is not sufficient to overcome the sociocognitive deficits that result from play deprivation. Rather, it is the defensive tactics of one's partner that appear critical, especially as these affect the reciprocity of the interactions (Pellis, Pellis, Pelletier, & Leca, 2019; Pellis & Pellis, 2017; Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016).

For play fighting to remain playful, some degree of reciprocity or turn-taking is needed (Altmann, 1962). Although the relative degree in the occurrence of turn-taking varies with the species, age, dominance relationship between the animals and their sex, a degree of reciprocity is needed for play fighting to continue (Pellis & Pellis, 2017). In rats, as with many other species, this is achieved by showing restraint in the execution of the tactics of attack and defense in a way that makes it easier for one's partner to gain the advantage (Pellis & Pellis, 1998). For example, in rats, a common configuration adopted during play fighting is the pin, in which one animal stands over its supine partner (Panksepp, 1981). The rat standing on top typically holds its supine partner down with its forepaws while at the same time anchoring its body weight by standing on the ground with its hind paws. Sometimes, the rat on top will stand on its supine partner with all four of its paws, reducing its own stability as the supine animal squirms (Foroud & Pellis, 2003). Counterattacks by the supine partner are two to three times more likely to lead to a role reversal when the on top rat does not have its hind paws anchored on the ground (Pellis et al., 2005). The unanchored position is most common between 30-40 days of age (Foroud & Pellis, 2002), the age at which play is most frequent (Thor & Holloway, 1984) and most likely to influence brain development (Vanderschuren & Trezza, 2014). The opportunity to reverse roles is a key component of play fighting (Pellis & Pellis, 2017), requiring the animals to track exchanges, so ensuring that they are reciprocal, and making decisions to respond to an attack appropriately; all of which engages executive functions related to the mPFC. It is likely that using these executive functions during play in the juvenile period fine-tunes the underlying mechanisms (Pellis et al., 2014; Pellis, Pellis, Himmler, et al., 2019). Reduced opportunity for reciprocity is not only present in LE juveniles playing with adults (Pellis et al., 2017), but also occurs in Wistar juveniles when playing with F344 rats of the same age and sex (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016).

Male Long Evans rats that are reared with a F344 during the juvenile period have reduced social competence as adults (Stark & Pellis, 2020), as do female Wistar rats reared with Fischer 344 rats (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). In the present paper, we test the hypothesis that the social deficits in adult Long Evans rats reared with Fischer 344 partners arises from their play experiences in the juvenile period. If this is true, the play of juvenile LE rats with F344 peers should be impoverished relative to that of LE juveniles playing with same strain partners. Based on previous research (Pellis et al., 2017; Pellis, Pellis, Himmler, et al., 2019; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016), this impoverishement should be reflected in several ways.

We predict (1) that LE rats reared with a F344 peer will experience less play and compensate for the reduced frequency of attacks by their F344 partner by increasing their own frequency of attacks, and (2) that less of the play between the LE and F344 rats will lead to wrestling and so result in fewer opportunities for them to gain experience with role reversals. Ultrasonic vocalizations (USV) seem to be important for coordinating behaviors during play, facilitating reciprocity, and avoiding escalation to aggression (C. Burke, Kisko, Pellis, & Euston, 2017; Kisko, Euston, et al., 2015; Kisko, Himmler, et al., 2015). Given that Wistar-F344 pairs have reduced rates of emission of USV (Schneider, Pätz, et al., 2016), we predict (3) that pairs of rats involving a LE and a F344 will emit fewer USV, especially the types of calls most associated with coordinating play (C. Burke et al., 2018, 2020).

Since previous research using both the adult rearing paradigm (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013; Pellis et al., 2017) and the F344 rearing paradigm (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016) only used females, it was not known whether both sexes have comparable experiences during play in the juvenile period when reared with a partner that plays atypically. Therefore, in the present study, a second goal was to compare the play experienced by pairs of females with that of pairs of males. Based on previous findings (B. Himmler, Bell, et al., 2014; Pellis et al., 2017; Schneider, Bindila, et al., 2016), we predict (4) that playing with an atypical play mate will effect the play experienced in both sexes, as in both cases, sociocognitive skills are compromised (Schneider, Bindila, et al., 2016; Stark & Pellis, 2020). However, what cannot be predicted *a priori* is whether the magnitude of changed experiences is similar in both sexes.

4.3 Methods

4.3.1 Subjects

A total of 72 weanling rats were purchased from Charles River Laboratories (Kingston, New York) and arrived at the Canadian Centre for Behavioural Neuroscience at 24 days of age. Fifty-two (26 males, 26 females) were LE rats and 20 (10 males, 10 females) were F344 rats. At 26 days of age, rats were housed in same sex pairs, of either two LE rats (8 male pairs and 8 female pairs; LE-LE), or as pairs composed of a LE and a F344 (10 male pairs and 10 female pairs; LE-F344). All animals were housed in polyethylene cages (46 cm x 25 cm x 20 cm) with corncob bedding, in the vivarium colony room maintained at a constant temperature (21 – 23°C), on a 12-hour light-dark cycle (lights off at 1900). Food and water were provided *ad libitum.* All care and procedures were approved by the University of Lethbridge Animal Welfare Committee in compliance with guidelines from the Canadian Council for Animal Care.

4.3.2 Procedure

Play was tested twice between 33 and 38 days of age that is within the peak period of play (Thor & Holloway, 1984). All pairs were habituated to the testing enclosure for 15-minutes each day for three days prior to testing. All habituation and testing trials occurred in complete darkness, which has been shown to increase play (Pellis & Pellis, 1997). After the third habituation session, pairs were isolated in separate cages for 24 hours before testing, a method that increases playfulness (Niesink & van Ree, 1989; Panksepp & Beatty, 1980; Pellis & Pellis, 1990). Test sessions were 10-minutes in length, an amount of time found to be sufficient to capture most aspects of play (Pellis & Pellis, 1990). After the first test session, animals were pair housed again for 24 hours before the isolation and subsequent testing for trial two was performed. The second trial was to ensure adequate data for analysis in case of data loss. The rats were tested again as adults (Stark & Pellis, 2020), and were then sacrificed, with their brains removed for histological analysis (work in progress).

4.3.3 Apparatus

Play trials were conducted in a 50 cm x 50 cm x 50cm Plexiglas® box with 1-2 cm of CareFresh® bedding. The Plexiglas® box was housed in a sound-attenuating chamber lined with sound-attenuating foam (Primeacoustic, Port Coquitlam, British Columbia). Video was recorded through a small window of the chamber from a 45° angle with an ExmourRS 4K Sony Handycam. The camera was set to record in night-shot to film in the dark. Vocalizations were also recorded simultaneously using a microphone capable of recording in the ultrasonic range (Model 4939, Brüel & Kjaer, Denmark) that was 32-38 cm above the center of the Plexiglas® box, and a red-light flash and audible sound was used to synchronize audio and video post filming. Sounds between 4-100 kHz were recorded. At the beginning of each habituation and test trial, three consecutive flash/beep signals were given to synchronize video and audio recordings.

4.3.4 Behavioral analysis

Only video files from the first test session were used for analysis, as there was no data loss during acquisition. Video was analyzed using a combination of normal speed and frame-by-frame inspection to score various aspects of playful attack and defense (B. Himmler, Pellis, & Pellis, 2013). The behavior of both partners in the dyad was scored. The play of the LE rat from the LE-F344 pairs was compared to the play of one of the rats from the LE-LE pairs (focal rats). The LE rat treated as the focal rat from the LE-LE pair was selected at random. Comparison of the focal rats provided a means of assessing the altered play experience of the LE rats reared with F344 partners. Comparison of the partners of the focal rats (the F344 partner and the other LE rat) provided a means of assessing the partners' contribution to the play experiences of the focal animals. Videos were scored in terms of the actions made by the focal animal toward their partner and those actions directed toward the focal animal by the partner. The pelage of the LE rats is white on the body, black on the head and has a black pattern extending down the back. During the trials, individual LE rats, especially those in the LE-LE pairs, were tracked by using their back patterns as well as by distinctive tail markings made with a Sharpie® before filming. The F344 rats have a fully white pelage and so could be easily tracked.

Playful attacks were scored when the snout of one rat either moved toward the nape or contacted the nape of the other rat. The recipient of an attack could either continue with its ongoing behavior or defend itself against the attack. The number of attacks defended divided by the total number of attacks gave the probability of defense. Defense involved two main types of tactics: evasion or a facing defense (B. Himmler, Pellis, & Pellis, 2013). When evading, the defending rat creates distance between the attacker's snout and its own nape by running, leaping, or swerving away. Facing defense involves the defending rat rotating towards its attacker, juxtaposing its face between its nape and the snout of its partner. The probability of a rat defending using a facing defense was calculated as the number of facing defenses divided by the total number of defenses. Three tactics of facing defense are used during play (B. Himmler, Pellis, & Pellis, 2013; Pellis & Pellis, 1990). The first facing defense is partial rotation, whereupon being attacked, the defending rat rotates its upper torso around its longitudinal axis to face its attacker, while keeping at least one of its hind paws on the ground. The second is complete rotation, whereupon being attacked, the defending rat rotates completely around its longitudinal axis to supine. The third facing defense is when, on being attacked, the defending rat rotates around its vertical axis at the level of its pelvis so that it remains prone when facing its attacker (Pellis et al., 1994). The vertical rotation tactic can lead to both rats rearing into a mutual upright position from which they push and box one another (Pellis & Pellis, 1987), although this is rarer in the juvenile period than it is later in development (Pellis & Pellis, 1990). The probability of each type of facing defense was calculated by dividing a particular type of defense (e.g., complete rotation) with the total number of facing defenses performed.

A common consequence of a facing defense is for one partner to end up lying on its back with its partner standing over it – referred to as a 'pin' (Panksepp, 1981). Although for some strains, most pins arise from the defender performing a complete rotation, the partner can also be forced over onto its back (S. Himmler, Himmler, Stryjek, et al., 2016; Pellis et al., 2017; Pellis & Pellis, 1997). Irrespective of how the pin configuration arises, it is thought to be important in providing some of the rewards derived from play (Panksepp, 1998), so the frequency of pins was scored. However, given the predicted differences across groups in the total number of play fights (Schneider et al., 2014), the probability of pins was calculated by dividing the number of pins by the total number of facing defenses performed, to standardize the comparison. Finally, because reciprocity is important for sustaining play fighting (Pellis & Pellis, 2017) and likely for the benefits derived from play in promoting the development of sociocognitive skills (Pellis et al., 2017; Pellis, Pellis, Himmler, et al., 2019; Schneider, Bindila, et al., 2016), a measure that assessed reciprocity was also scored. Playful encounters involving facing defense in which the defending animal was successful in launching a counterattack towards the nape of its attacker, thereby becoming the attacker, were scored. Again, because of the predicted group differences in the total amount of play fighting (Schneider et al., 2014), the probability of role reversals was used for comparison (S. Himmler, Himmler, Pellis, & Pellis, 2016). This was calculated by dividing the number of role reversals with the number of facing defenses performed.

In part, reduced reciprocity also leads to asymmetry in many aspects of the play between partners in a pair (Pellis et al., 2017). Therefore, to assess the asymmetry between the playmates in the different groups, the absolute difference between the scores of partners was calculated. By dividing this value with the total score of the pair, a score between 0 and 1 was produced. Values close to zero indicate little asymmetry in the behavior between partners and values close 1 indicate a high degree of asymmetry between partners.

4.3.5 Vocalization analysis

Acoustic data were analyzed using Raven Pro 1.4 software (Bioacoustics Research Program, Cornell Lab of Ornithology), which generates spectrograms with a 256-sample Hann window. A trained experimenter manually selects vocalizations for analysis. Spectral analysis was performed for all vocalizations emitted during the 10-minute play session. As well as scoring the number of calls emitted in the pairs of rats from the different groups, the differential use of some of the types of calls was also scored. Although there are many different types of 50 kHz ultrasonic vocalizations (Wright et al., 2010), for the present purposes, only four types were scored – flat calls, audible calls and frequency modulated (FM) calls, which were broken into two categories seen below (Burgdorf et al., 2008; Burgdorf & Panksepp, 2006; Wöhr et al., 2008). Altogether, vocalizations were categorized into four types based on visual identification, (a) trill calls, a form of FM ultrasonic call that are particularly common during play (B. Himmler, Kisko, et al., 2014), were identified based on their rapid frequency oscillations, (b) audible calls were identified based on the frequency at which they occur and were confirmed via audio playback detectable by the experimenter, (c) flat calls have a near constant frequency and occur above 30 kHz, and (d) FM vocalizations, include all non-trill ultrasonic vocalizations that involve frequency modulations. The total number of vocalizations included in the analysis is shown in Table 4.1. Trills are not only commonly emitted during play (B. Himmler, Kisko, et al., 2014), but are also highly correlated with nape attacks (C. Burke, Kisko, Swiftwolfe, et al., 2017; C. Burke et al., 2018). Similarly, some of the other FM calls are highly correlated with defensive actions that are likely to lead to role reversals (C. Burke et al., 2018, 2020).

Table 4.1

Total number of vocalizations included in the analyses of rates of vocalizations and types of calls emitted.

	LE-LE Pairs		LE-F344 Pairs	
	Male	Female	Male	Female
Number of vocalizations	7855	6087	5736	6817

4.3.6 Statistical analysis

All statistics were performed using RStudio (R Core Team, 2018) and the packages car (Fox & Weisberg, 2019) and lsmeans (Length, 2016), as well as the package ggplot2 (Wickham, 2016) to create the graphs. Comparisons of the proportion of playful attacks made by the focal and partner along with the probability of defense, facing defense, complete rotation, pin and successful counterattacks (i.e., role reversals), were tested using a two-way ANOVA, with rearing condition and sex as the factors. Comparisons of the average number of vocalizations emitted during the 10-minute play session, the average number of vocalizations emitted per play bout, as well as the proportion of call types emitted, were tested using a two-way ANOVA with the same parameters as above. As no significant interactions between sex and condition were found, *post hoc* analyses were not conducted. Differences were considered significant if the p-value was ≤0.05. All graphs and tabular data are presented as the group means and standard error of the mean.

4.4 Results

4.4.1 Play fighting: Focal subjects

Compared to the matched juveniles from the same strain dyads, there was no difference in the number of nape attacks launched by the LE paired with the F344 [F(1,32) = 0.11, p = 0.75] regardless of sex [F(1,32) = 3.39, p = 0.075] (Figure 4.1A). While there was no significant difference in the likelihood of defense [F(1,32) = 0.13, p = 0.73] (Figure 4.1B) or in the likelihood of using facing defense [F(1,32) = 0.75, p = 0.39] (Figure 4.1C), there was a significant difference in the likelihood of making a complete rotation [F(1,32) = 67.68, p < 0.0001] with the LE reared with a F344 less likely to rotate to supine (Figure 4.1D). There was a significant reduction in the likelihood of a facing defense resulting in the LE being pinned by the F344 [F(1,32) = 17.71, p = 0.00019] (Figure 4.1E), and there was a significant increase in the probability of an LE reared with a F344 in launching successful counterattacks [F(1,32) = 24.50, p < 0.0001] (Figure 4.1F). There were no significant sex differences for any of the measures.

4.4.2 Play fighting: Partners of the focal subjects

As seen in Figure 4.2A, compared to the matched juveniles from the same strain dyads, Fischer 344 rats launched fewer nape attacks [F(1,32) = 44.47, p < 0.0001] regardless of sex [F(1,32) = 0.44, p = 0.51]. While there were no differences found in the likelihood of defense between the F344 and the matched control LE [F(1,32) = 1.00, p = 0.32], there was a sex difference with females more likely to defend themselves [F(1,32) = 12.46, p = 0.0013] (Figure 4.2B). The Fischer 344 partners were more likely to respond with facing defense [F(1,32) = 32.02, p < 0.0001] (Figure 4.2C), but not in the likelihood of using complete rotation [F(1,32) = 0.91, p = 0.35] (Figure 4.2D) or in the likelihood of a facing defense resulting in a pin [F(1,32) = 0.53, p = 0.47]. There was a significant difference in the probability of launching successful counterattacks [F(1,32) = 54.93, p < 0.0001], with F344 partners being less likely to produce role reversals (Figure 4.1F). Except for the sex difference in the probability of defense, no other measures differed significantly between the sexes.

4.4.3 Asymmetry between play partners

The mixed strain pairs exhibited more asymmetry than the same strain control pairs in the number of nape attacks launched [F(1,32) = 34.84, $p < 0.0001$] (Figure 4.3A), the likelihood of facing defense [F(1,32) = 7.15, p = 0.012] (Figure 3C), the likelihood of complete rotations [F(1,32) = 15.07, p = 0.0004] (Figure 4.3D), the likelihood of pins [F(1,32) = 4.17, p = 0.049] (Figure 4.3E) and in the likelihood of launching a successful counterattack [F(1,32) = 39.37, $p < 0.0001$]. There was a trend for defending against nape attacks being more variable in the mixed strain pairs (Figure 4.3B), but this was not significant [F(1,32) = 2.79, p = 0.10]. There were no significant sex differences for any of the measures.

4.4.4 Vocalizations

There was a significant difference in the average number of vocalizations emitted during the 10-minute play session [F(1,32) = 6.91, p = 0.013], with mixed strain pairs emitting fewer calls (Figure 4.4A). However, there was no significant difference in the rate of calling per play bout [F(1,32) = 0.0019, p = 0.97] (Figure 4.4B). There were also significant differences in the call types that were emitted. The LE-F344 dyads were less likely to emit trills [F(1,32) = 29.19, $p < 0.0001$] (Figure 4.4C) and other FM calls [F(1,32) = 6.77, p = 0.014] (Figure 4.4F), as well as being more likely to emit audible calls [F(1,32) = 50.27, $p < 0.0001$] (Figure 4.4D), but there were no significant differences in flat calls [F(1,32) = 0.90, p = 0.35] (Figure 4.4E). There were no significant sex differences for any of the measures.

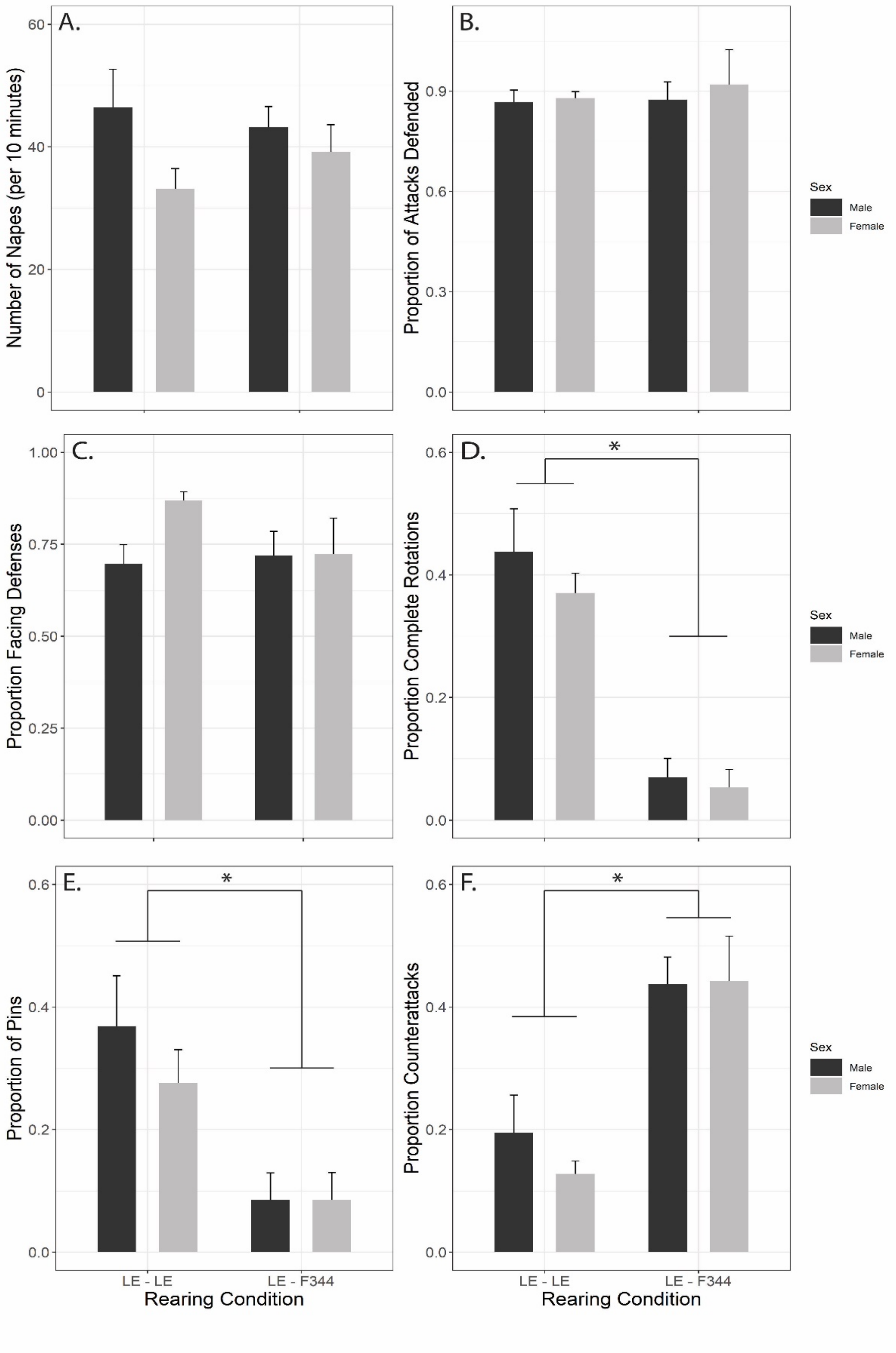

Figure 4.1 *Shows the play behaviors of the male and female focal rats, the LE paired with a F344 as the experimental condition and its matched control. A) shows the number of nape attacks made by the focal animal, while B - F) shows the reactions made by the focal rat in response to being attack by its play partner. All measures are represented as the average ± the SEM, (*) denotes significant p ≤ 0.05.*

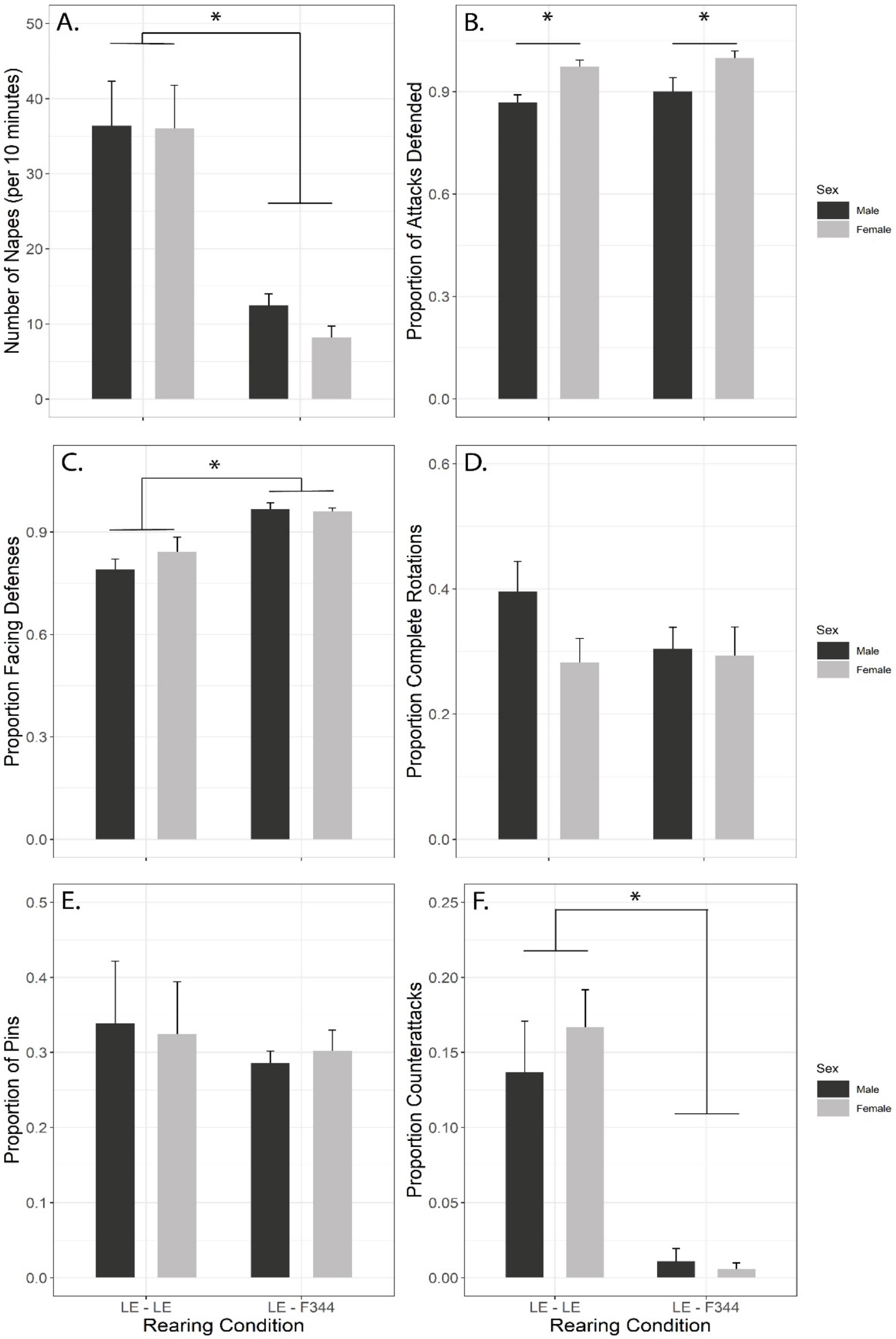

Figure 4.2 *Shows the play behaviors of the male and female partners of the focal rats, the F344 as the experimental condition and its matched control. A) shows the number of nape attacks made by the partner, while B - F) shows the reactions made by the partner in response to being attack by the focal rat. All measures are represented as the average ± the SEM, (*) denotes significant p ≤ 0.05.*

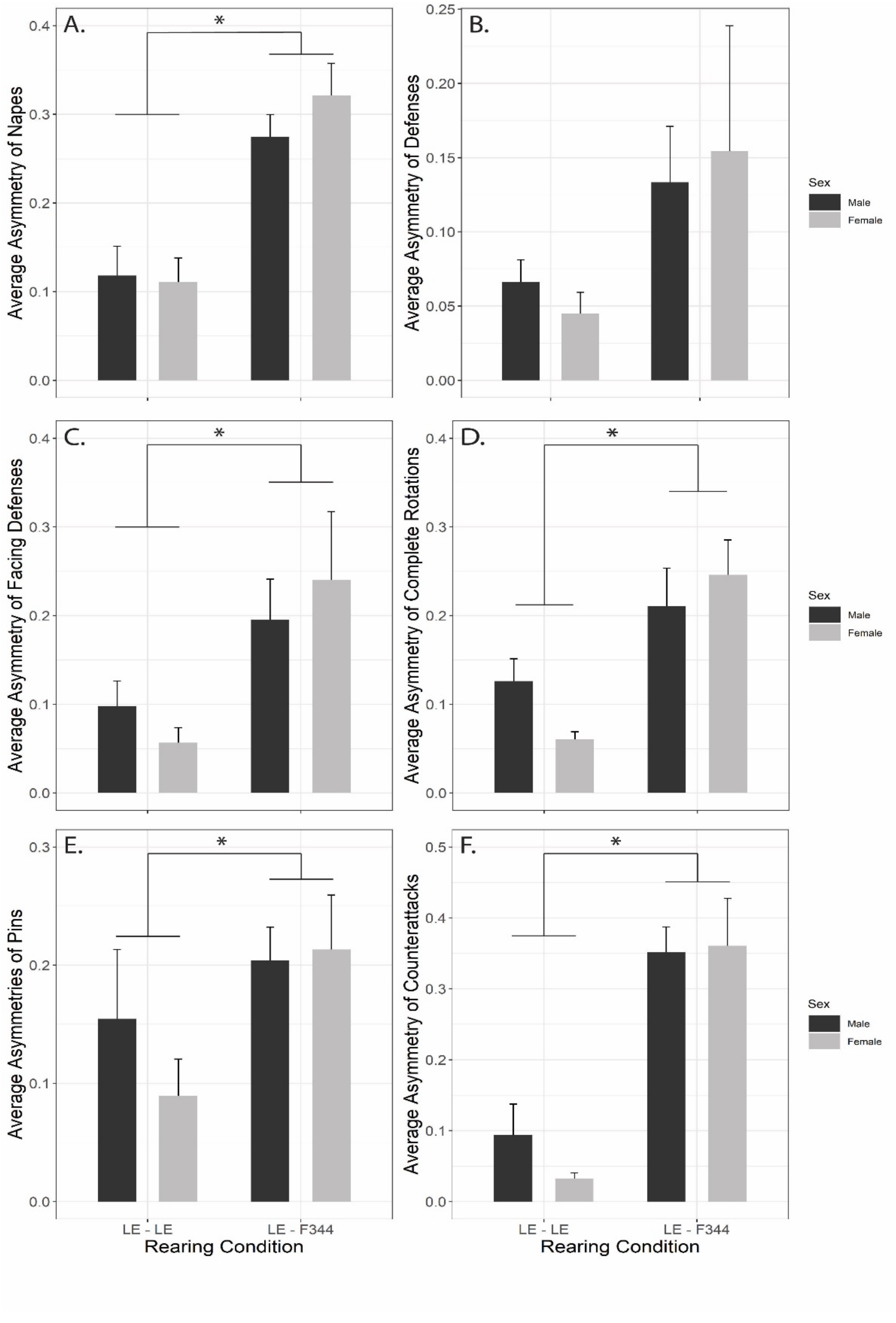

Figure 4.3 *Shows the asymmetry between the experimental and control partners in both male and female rats. A) shows the asymmetry of nape attacks made, B) shows the asymmetry in defending an attack, C) shows the asymmetry in the use of facing defenses, D) shows the asymmetry in a facing defense leading to a complete rotation, E) shows the asymmetry in a facing defense leading to a pin, and F) shows the asymmetry in a facing defense leading to a counterattack. All measures are represented as the average ± the SEM, (*) denotes significant $p \leq 0.05$.*

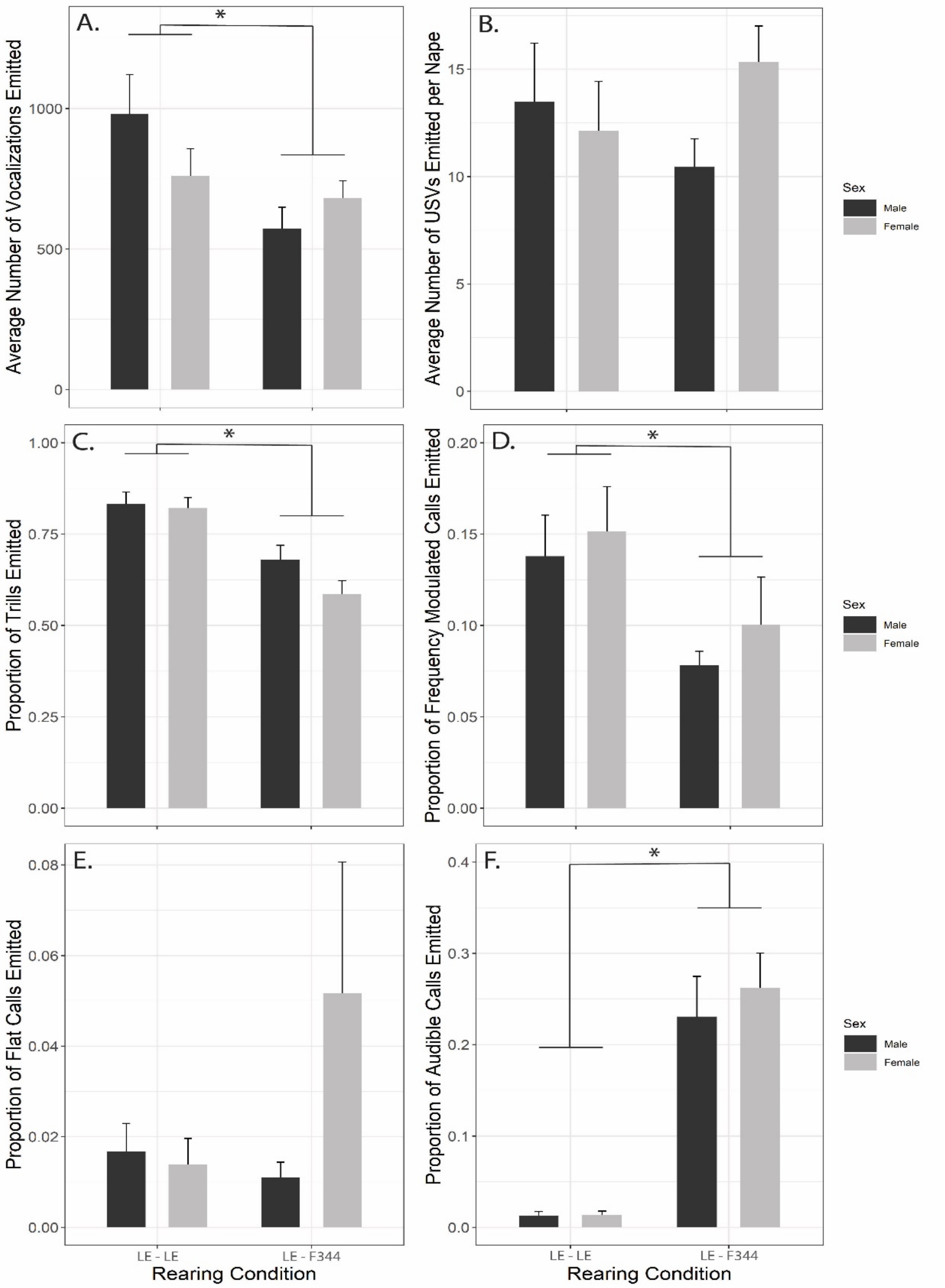

Figure 4.4 *Shows the average number and proportion of vocalizations emitted by experimental and control female and male rats. A) shows the average number of vocalizations emitted per 10-minute play session, B) shows the average number of vocalizations emitted per play bout, C) shows the proportion of trill call types emitted, D) shows the proportion of audible calls emitted, E) shows the proportion of flat calls emitted, and F) shows the proportion of frequency modulate calls emitted, on average per play session. All measures are represented as the average ± the SEM, (*) denotes significant p ≤ 0.05.*

4.5 Discussion

A LE rat reared with a F344 partner experiences a different play environment during the juvenile period than if it were reared with a LE partner. LE rats receive fewer attacks from their F344 partners (Figure 4.2A), and when the LE rats were attacked by their F344 partners, they were less likely to respond to a nape attack with a complete rotation (Figure 4.1D), leading to fewer instances of pins (Figure 4.1E). Even so, when attacked, the LE rats were more likely to launch successful counterattacks toward their F344 partners (Figure 4.1F). LE rats did not compensate for the lack of nape attacks from their F344 partners, as seen in LE juveniles playing with adults (Pellis et. Al., 2017), launching as many attacks as LE rats do with a same strain partner (Figure 4.1A). When attacked, F344s were more likely to defend with a facing defense (Figure 4.2C). Despite a comparable likelihood of defending an attack with a complete rotation (Figure 4.2D) and a similar frequency of pins (Figure 4.2E), F344s were less likely to launch successful counterattacks (Figure 4.2F).

There was greater asymmetry in the play between LE-F344 partners on virtually all measures of play, especially those reflecting reciprocity, such as pins and counterattacks. Given the importance of reciprocity in sustaining playful encounters (Palagi, Cordoni, et al., 2016; Pellis & Pellis, 2017), the disparity in the contributions to the play fights between LE and F344 partners was most striking. Even though playfulness varies across individuals within strains of rats (Lampe et al., 2017; Pellis & McKenna, 1992), the variation in asymmetry we found in this study for LE-LE partners was between 5-15% for all play measures, while the variation in asymmetry between LE-F344 partners was between 20-30% for the same measures (Figure 4.3). This clearly shows that an LE rat being reared with a F344 peer experiences a different pattern of play compared to an LE reared with an LE peer. Similarly, Wistar rats reared with F344 partners experience more asymmetry in their play compared to when reared with a Wistar partner (Schneider, Pätz, et al., 2016). Additionally, LE rats reared with F344 partners experience a different vocal environment during play (Figure 4.4), as was the case of Wistar rats reared with F344 rats (Schneider, Pätz, et al., 2016).

Juvenile and adult rats emit USVs that can be grouped into two broad categories based on the frequency at which the calls are emitted – 50 kHz and 22 kHz. Whereas the latter are produced during aversive contexts (Portfors, 2007), the 50 kHz calls are associated with positive contexts, such as when the rat is anticipating the arrival of play partner and during play (Burgdorf et al., 2008; Knutson et al., 1998). During play, various 50 kHz call types appear to promote play, which have been shown to facilitate reciprocity and deescalate aggressive situations (C. Burke, Kisko, Pellis, & Euston, 2017; Kisko, Euston, et al., 2015; Kisko, Himmler, et al., 2015). As is the case of Wistar rats playing with F344 rats (Schneider, Pätz, et al., 2016), when the total number of calls per trial was compared, the LE-F344 pairs emitted fewer calls (Figure 4.4A), but when adjusted for the number of play fights, the rate of calling per play fight did not differ with LE-LE pairs (Figure 4.4B). This suggests that the lower level of USV production in LE-F344 pairs arose from an overall reduction in the amount of play (Figures 4.1A, 4.2A). However, despite a similar number of vocalizations per nape attack, the types of calls used overall are different in their proportions in the LE-F344 pairs, which suggests that the calls most associated with promoting play and facilitating reciprocity, such as trills (Figure 4.4C) and other FM calls (Figure 4.4F) (C. Burke et al., 2018, 2020), are used more often in LE-LE pairs and could be influencing the increased symmetry in their play (Figure 4.3). Also, potentially important, is that audible calls are more commonly emitted in the LE-F344 pairs (Figure 4.4D); calls that are often used in threatening situations (Barnett, 1975). This may reflect a greater aversion in the play between LE and F344 rats. Together with not detecting any 22 kHz calls in these encounters, and an absence of any studies on how audible calls are used in play, caution should be exercised in interpreting this finding. Nonetheless, the large difference in the audible calls present in LE-F344 pairs, accompanied with the differences in play behaviors, does suggest that differences in play tactics may differentially require the use of audible calls to communicate some aspects of the interaction. Although, since which animal is emitting each vocalization cannot be distinguished, it is possible that excessive calling in the audible range is a peculiarity of F344 rats. Given that there are strain differences in the profile of vocalizations used (Schwarting, 2018b, 2018a), a detailed study of calling, both with regard to USVs and audible calls, in F344 rats is needed to understand the altered pattern of calling in LE-F344 pairs fully. However, the evidence available (Figure 4.4) does suggest that, when reared with a F344 peer, LE rats experience a different acoustic environment to that when reared with other LE rats.

4.5.1 Potential influences on the development of sociocognitive skills

The altered play experienced by LE rats reared with F344 peers may account for the sociocognitive deficits present in adult LE rats reared with F344 partners (Stark & Pellis, 2020). This is consistent with the findings on Wistar rats reared with F344 peers (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016) and LE rats reared with adults (Bell et al., 2009; B. Himmler, Pellis, & Kolb, 2013). Finding that impoverished play experience alters both the anatomy and physiology of the mPFC and the development of sociocognitive skills has been shown in Lister hooded rats reared with social exposure, but no opportunity for play (Baarendse et al., 2013; Baarendse & Vanderschuren, 2012; Omrani et al., 2020), and in hamsters, where juveniles were reared with adults (Burleson et al., 2016). Furthermore, the finding that ground squirrels that engaged in more social play as juveniles have greater sociocognitive skills (Marks et al., 2017), adds to the growing body of evidence that play fighting in the juvenile period refines sociocognitive skills and does so by modifying the underlying neural mechanisms involved (Pellis & Pellis, 2017). The present study also sheds light on the experiences derived from play that may be important for this function.

The play of LE-F344 pairs is highly asymmetrical (Figure 4.3), as has also been found for Wistar-F344 pairs (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). Similarly, the play of pairs of LE rats comprising a juvenile and an adult is also highly asymmetrical (Pellis et al., 2017). This common feature across strains and the type of play-deficient partner suggests that it is reciprocity that may be the experience derived from play that is critical. Three lines of evidence support this possibility. First, Wistar rats playing with F344 partners (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016), and LE rats playing with adult partners (Pellis et al., 2017), compensate for the lack of playfulness in their partners by increasing the number of playful attacks that they launch. While in the present study our LE did not increase the number of playful attacks when interacting F344 partners (Figure 4.1A), there was a significant asymmetry with the LE launching more attacks than their F344 partners (Figure 4.3A). Even in the absence of an overt compensatory increase in launching attacks, these rats engage in about the same amount of play as they would with same strain peers (Figure 4.1A), so it cannot be the physical actions performed during play that is crucial for training the mPFC and sociocognitive skills. Rather, it is likely that it is the engagement of executive functions, that occur during play, such as short-term memory, impulse control and decision making, that allow the animals to be reciprocal in their interactions that train these skills (Pellis et al., 2014). Second, unlike LE rats reared with an adult (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013), wild juvenile rats reared with an adult do not exhibit atypical development of the mPFC (Pellis et al., 2018), and unlike their LE counterparts, the play of juvenile-adult pairs of wild rats are more symmetrical (Pellis, Pellis, Himmler, et al., 2019). In particular, it is the opportunity for engaging in actions that lead to role reversals that are more symmetrical in the wild rats.

Third, while in juvenile-adult pairs, most of the counterattacks are by the adult (Pellis et al., 2017), in LE-F344 pairs, most of the counterattacks are by the LE partner (compare Figure 4.1E and Figure 4.2E). That is, in one context, the subject rat has too few opportunities to counterattack successfully and in the other, too many. So, it cannot be just performing the physical acts needed to counterattack that provides the essential experiences, but rather, it must be the engagement of the psychological processes of tracking encounters and deciding when to self-handicap to allow the partner to reverse roles that provides the training of sociocognitive skills and their underlying neural circuitry. There must be a level of symmetry and when the asymmetry becomes too great these experiences are insufficient (Baarendse et al., 2013; Bell et al., 2010; Burleson et al., 2016; B. Himmler, Pellis, & Kolb, 2013; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016; Stark & Pellis, 2020). Indeed, the asymmetry can be much less than 50:50 for play to still have this protective effect on development (Pellis, Pellis, Himmler, et al., 2019). While highly asymmetrical play is detrimental, there is the possibility that a certain level of asymmetry can produce additive improvements (Marks et al., 2017). Identifying the minimal level necessary and characterizing the magnitude of the gains made by playing beyond that minimal level has important practical implications for both animal welfare (Ahloy-Dallaire et al., 2018) and for developing policies for human development (Yogman et al., 2018). The identification of asymmetry as a suitable index for characterizing adequate play experience (Figure 4.3) provides a tool for further research.

4.5.2 Play and sex

Studies using both the adult rearing paradigm and the F344 partner paradigm have only used females (Bell et al., 2010; Burleson et al., 2016; B. Himmler, Pellis, & Kolb, 2013; Pellis et al., 2017; Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). These studies revealed the importance of social play during the juvenile period in facilitating the development of the neuronal morphology of the mPFC and sociocognitive skills. Other studies, using different paradigms to deprive juveniles of play, have primarily used males, and these have shown impairments in impulse control, altered cognitive strategies and altered neurotransmitter signaling in the mPFC and some connected neural systems (Baarendse et al., 2013; Baarendse & Vanderschuren, 2012; Omrani et al., 2020). One study used the paradigm in which pairs of rats were separated by a mesh barrier to prevent playing and compared the effects on both sexes. These play-deprived rats were then subjected to a food protection paradigm where a food item is given to one rat and protects it from being stolen by another rat by dodging laterally away (Whishaw, 1988). This paradigm revealed that, while species- and sex-typical food protection tactics emerged regardless of play experience, the coordination of the movements between partners, in adulthood, was reduced in both sexes in play deprived rats (Pellis et al., 1999). Furthermore, damage to the mPFC in adult males showed that the rats could still execute the typical protective tactics to defend a food item, but were less successful in doing so (Whishaw, 1988; Whishaw & Oddie, 1989) and this appears to be due to reduced ability to coordinate their movements with those of the rat trying to steal the food (B. Himmler, Bell, et al., 2014). These findings suggest that, in both sexes, juvenile play experiences affect the development of the mPFC and the sociocognitive skills dependent on this neural system. However, the stress of being fully or partially isolated from a social partner or being subjected to co-habitation with an adult may not be similar in both sexes

(Boggiano et al., 2008; Brown & Grunberg, 1996; A. Burke et al., 2017). It is thus difficult to use these paradigms to compare the effects of play deprivation between the sexes without the confounding of differential stress effects. The rearing of a playful strain, such as the LE or Wistar rats, with a play-deficient strain, such as the F344 strain, eliminates some of these confounds – the subject is reared in the physical presence of another rat with whom it can interact and that partner is an age- and sex-matched peer (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). The present study shows that, LE rats, whether male or female, experience the same distorted asymmetry in play when reared and tested with a familiar F344 peer. Therefore, in this rearing paradigm, the similar deficits in play experience in the juvenile period can be used to test for the effects of play in the development of the mPFC and sociocognitive skills.

4.6 Conclusion

Male LE rats that were reared with a F344 over the juvenile period were tested in the 'stranger paradigm', in which adults unfamiliar with one another were introduced in a neutral arena. In this context, the animals engage in a rough form of play that typically leads to one partner assuming the dominant status, but occasionally can escalate to serious aggression, especially if communication skills are impaired (Kisko, Euston, et al., 2015; Smith et al., 1999). In the stranger paradigm, the LE rats reared with a F344 partner were more likely to escalate these encounters to serious aggression (Stark & Pellis, 2020), indicating reduced sociocognitive skills. Females tested with other females in the stranger paradigm have subtle differences, but the absence of overt dominance relationships in females as opposed to males (Adams & Boice, 1983; R. Blanchard et al., 1988; Ziporyn & McClintock, 1991), makes this test less useful for assessing social interaction skills in females (Stark, unpublished results). Nonetheless, the finding that female Wistar rats reared with F344 partners have impaired social memory skills as adults (Schneider, Bindila, et al., 2016) suggests that the impoverished play experienced by females in the juvenile period impairs the development of sociocognitive skills as it does in males. Whether the magnitude of these play-induced effects are similar in both sexes will require testing them in the same, sex-neutral, sociocognitive tests and evaluating the size of the changes in the anatomy and physiology of the mPFC. As an important starting point, however, the present paper shows that the altered play experience of being reared with a F344 peer is comparable between the sexes.

CHAPTER 5: ATYPICAL PLAY EXPERIENCES IN THE JUVENILE PERIOD IMPACTS THE DEVELOPMENT OF THE MEDIAL PREFRONTAL CORTEX IN MALE AND FEMALE RATS

5.1 Abstract

In rats reared without play, or with limited access to play during the juvenile period, the pyramidal neurons of the medial prefrontal cortex (mPFC) have dendrites that are over exuberant. This suggests that play is critical for pruning the dendritic arbor of these neurons. However, the rearing paradigms that have been used so far to limit play have involved physical separation from a peer or sharing a cage with an adult, so the stressors produced could be the cause of the disrupted pruning. In this study, we used a known low playing strain of rats, the Fischer 344 rat (F344), to deprive playful Long Evans rats (LE) of play during the juvenile period. Housing same-sex peers together result in a less stressful rearing paradigm. We found that LE rats reared with an F344 partner had the reduced pruning of the pyramidal neurons of the mPFC compared to LE rats reared with an LE partner. This was the case for both males and females. The neurons of the play deficient LE rats not only took up more space, as determined by convex hull analyses, but were also longer than in typically reared rats. Unlike studies using more stressful rearing paradigms, the present effects were limited to the apical dendritic projections, suggesting that the previously reported effects on the basilar dendrites may have resulted from developmental disruptions caused by stress. If this is so, the present findings indicate that play-derived experiences over the juvenile period have a specific developmental effect that influences how the mPFC functions.

Keywords: Golgi-cox, medial prefrontal cortex, play deprivation, plasticity, pyramidal neurons, sex differences

5.2 Introduction

Being reared in social isolation during the juvenile period leads to a wide range of behavioral, emotional, cognitive, neural and physiological abnormalities in adult rats (Arakawa, 2018; Byrd & Briner, 1999; da Silva et al., 1996; Einon & Morgan, 1977; Fone & Porkess, 2008; Hall, 1998; Lukkes et al., 2009; Potegal & Einon, 1989; van den Berg et al., 1999; Von Frijtag et al., 2002). Given that social play peaks during the juvenile period, reaching its height between 30-40 days after birth (Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1990; Thor & Holloway, 1984), therefore it is thought that such play is important for the development of the aformentioned functions. However, because rearing rats in complete isolation limits more than just play, it cannot be concluded that play is the key experience that is leading to the deficits (Bekoff, 1976). Even so, rearing paradigms that prevent or limit peer-peer play during the juvenile period, but not other forms of social contact, still result in adults that have many of the deficits present in rats reared in complete social isolation (Baarendse et al., 2013; Bell et al., 2010; Einon et al., 1978; B. Himmler, Pellis, & Kolb, 2013; Omrani et al., 2020; Pellis et al., 1999; Potegal & Einon, 1989; Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016; van Kerkhof et al., 2013). Therefore, it does seem that play with peers is an important contributor to refining such skills, and the underlying neural mechanisms that support them (Pellis et al., 2014, 2017; Vanderschuren & Trezza, 2013).

One paradigm used to deprive rats of play is to place a perforated partition in the cage to separate the animals, allowing them to see, smell, hear and to a limited degree, touch one another, but prevents them from engaging in social grooming and play (Baarendse et al., 2013; Omrani et al., 2020; Pellis et al., 1999). Another paradigm involves rearing a juvenile rat with an adult, which allows a wide range of social behaviors, including some limited play (Pellis et al., 2017). Both of these rearing paradigms have shown that, with absent or reduced play, rats have reduced ability for impulse control and altered neural physiology of the mPFC (Baarendse et al., 2013) and reduced pruning of the dendritic arbor of the pyramidal neurons of the mPFC (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013). In a study with hamsters using the adult rearing paradigm, the same reduced pruning of the mPFC neurons was found along with reduced ability to tolerate social stress (Burleson et al., 2016). These studies show that in the absence of typical juvenile play experiences, both the development of the mPFC is altered and the animals have diminished executive functions that can influence effective deployment of social skills (Pellis et al., 2014; Vanderschuren & Trezza, 2013).

There are potential stress effects from the partition rearing paradigm arising from the inability to engage in close bodily contact, such as social grooming, huddling, and sleeping together. Such deprivation could differentially affect males and females, making this a suboptimal paradigm for comparing the sexes. Similarly, although the effect of the adult housing paradigm has been replicated multiple times with rats (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013, 2018) and hamsters (Burleson et al., 2016), this rearing paradigm is also not useful for comparing between the sexes. For rats, the adult rearing paradigm only works for females, as a male rat reared with an adult male could become stressed due to social defeat (A. Burke et al., 2017), and if reared with an adult female, would begin to show sexual behavior at the onset of puberty (Pellis & Pellis, 1990). In the hamster study, males were housed singly with their mothers, but the rearing manipulation had to be terminated prior to sexual maturity (Burleson et al., 2016), so before the age at which social play fully wanes in this species (Goldman & Swanson, 1975; Pellis & Pellis, 1988). To encompass the entire juvenile and adolescent period in the more sociable rat, the adult-rearing period needs to continue to around 60 days after birth (Bell et al., 2010), past the age at which sexual maturity begins (Clark & Price, 1981). Adult female rats tolerate juveniles and will engage in various social behaviors, such as grooming, huddling and sleeping together, but will avoid engaging in play with them (Cramer et al., 1990; B. Himmler et al., 2015; Pellis et al., 2017; Thiels et al., 1990). So, while adult rearing is a good paradigm to limit the experience of play during the juvenile period in females, it is not useful for comparing the effects of limiting play between the sexes. An alternative paradigm can potentially overcome this limitation.

The F344 paradigm involves rearing rats from highly playful strains with peers from a less playful strain, F344 rats (Siviy et al., 1997, 2003). For example, when Wistar rats, a highly playful strain (S. Himmler, Modlinska, et al., 2014), are reared with a low playing F344 peer, they experience an impoverished form of play and develop into adults with reduced pain thresholds, impaired social memory and diminished ability for the social transfer of information (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016). While these studies have only used females, we have shown that rearing LE rats, another high playing strain (S. Himmler, Modlinska, et al., 2014), with a same-sex F344 peer over the juvenile period leads to adult social impairments in both sexes (Stark & Pellis, 2020, Stark & Pellis, 2021, in review).

Not only can this cross-strain rearing paradigm allow males and females to be compared, it may also overcome a potential confounding factor that is present in the adult rearing paradigm. The adult is larger than the juvenile and actively avoids or even forcefully prevents the juvenile from initiating play (Pellis et al., 2017). Consequently, living with an adult may not be a completely benign experience even for juvenile females, and may cause some stress effects that could influence normal development. It is possible that the adult-induced stress effects could be the factor that is altering mPFC development. Since in the F344 rearing paradigm juveniles are reared with same-age peers, the likelihood of these potential stress effects are reduced, allowing the effects of impoverished play experience to be more readily assessed. Using the F344 rearing paradigm, we have two goals in this paper.

First, we wish to determine whether the negative effects of being reared with a low playing peer results in diminished pruning of the dendritic arbor of the pyramidal neurons of the mPFC as is the case for juveniles reared with adults (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013). Second, we wish to determine if the magnitude of the effect on the mPFC neurons is similar between the sexes. Sex differences in play have been long acknowledged, with males typically engaging in more play fighting than females (Ellis et al., 2008). However, whether this sex difference is present or not depends on the rearing condition (e.g., in pairs or in larger groups), the testing partner (e.g., same sex or opposite sex peer), the time sampled (e.g., five minutes versus longer periods), whether the play is naturally occurring or induced by prior social isolation, and the strain of rat being used (for a review of the literature see S. Himmler, Himmler, Pellis, & Pellis, 2016). What is clear is that, when tested under more natural group living conditions, males consistently engage in more play than females (e.g., Meaney & Stewart, 1981; Thor & Holloway, 1984). This greater amount of naturally occurring play in males would suggest that the experiences derived may be more critical to males than females (Fagen, 1981; Meaney et al., 1985), making it likely that the impact of play on the brain would be greater in males. Also, irrespective of rearing condition, females exhibit a greater degree of pruning that is due to intrinsic hormone-induced effects (Kolb & Stewart, 1991). Given that maternal behavior is critical among mammals, as only females can lactate, and more widespread than paternal behavior (Eisenberg, 1981; Panksepp, 1998), it is likely that females require a higher baseline level of sociocognitive skills than males (Pellis & Pellis, 2009). In females reared in the adult rearing paradigm, there is about an 18%

difference in pruning (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013), so if there is a sex difference, it would be predicted that the effect due to play would be greater in males.

5.3 Methods

5.3.1 Subjects

Fifty-two weanling LE rats (26 males, 26 females) and 20 weanling F344 rats (10 male, 10 female) were purchased from Charles River Laboratories (Kingston, New York). After 2 days of acclimation to the colony room, at 26 days of age, rats were pair housed into one of two same sex conditions: 1) the experimental condition was a mixed strain pairing of a LE and a F344 (10 pairs of each sex), and 2) the control condition was a same strain pairing of two LE rats (8 pairs of each sex). Animals were maintained in these pairs for the duration of the experiment. All animals were housed in polyethylene cages (46cm x 25cm x 20cm) with corncob as the bedding substrate, food and water were provided *ad libitum*. The colony room was maintained at a constant temperature (21 – 23°C), on a 12-hour light-dark cycle (lights off at 1900). All care and procedures were approved by the University of Lethbridge Animal Welfare Committee in compliance with guidelines from the Canadian Council for Animal Care.

5.3.2 Procedure

Once the weanling rats reached the juvenile period, the play between cage mates was assessed. Twice between the ages of 33 and 38, pairs were socially isolated for 24 hours before being reunited, in a habituated arena, where the subsequent play was recorded for 10-minutes. Play behaviors were analyzed to uncover the experiences that were provided to the LE rat by its F344 partner and compared to the experiences of a LE rat reared with another LE rat (Stark, Rakumar, & Pellis, 2021 in review). Rats were maintained in their pairs until adulthood. At 80 days of age, the animals underwent testing in the stranger paradigm. The LE rat reared with a F344 rat and a matched control for the same strain pairing were tested in a neutral arena, to which the animals were habituated, with an unfamiliar same-age, same-sex partner. Twenty (10 male, 10 female) LE rats were purchased from Charles Rivers Laboratories to serve as the unfamiliar partner pool. Cage mates were separated for 24 hours before the unfamiliar rats were introduced in the test enclosure for 10-minutes. Two trials were performed, each with an unfamiliar partner, to ensure no data loss. The behavioral results are reported elsewhere (Stark & Pellis, 2020; Stark & Pellis 2021, in review).

5.3.3 Histology

At 90 days of age, after the behavioral data were collected for the stranger paradigm, animals were anesthetized with 300mg/kg of sodium pentobarbital and perfused, intracardially, with 0.9% saline. The brains from 16 control (8 male, 8 female) and 20 experimental (10 male, 10 females) LE rats were removed, weighed, and placed into Golgi-Cox solution (Glaser & Van der Loos, 1981). The brains remained in the solution, in the dark, for 14 days after which the Golgi-Cox solution was replaced with a 30% sucrose solution. The brains were left in the sucrose solution for a minimum of 5 days before sectioning. Sectioning and staining was carried out following established procedures (Gibb & Kolb, 1998). Brains were sectioned with a vibratome at a thickness of 200µm and mounted onto 2% gelatin coated slides. Once mounted, slides were stored in a lightproof box, with a damp paper towel to prevent drying, for a maximum of 4 days. The brain sections were stained according to the procedure outlined by Gibb & Kolb (1998), cover slipped, and allowed to dry for at least 2 weeks before anatomical analysis.

Anatomical analysis

Neuronal morphology was quantified using established methodology (Brinkman, 2019). Area CG3 (mPFC) was selected for virtual slide imaging as defined by Zilles (1985). Virtual slides were created using an Olympus VS120 digital slide scanner with a 40x oil objective (UPlanFL N, 40x/1.30 oil, ∞/0.17/FN26.5) and Olympus VS-ASE FL software. Virtual slides consisted of a z-stack of 147 images spaced 0.68µm apart throughout the section, yielding 99.96µm of working distance. Once digital images were created, they were uploaded into Neurolucida 360® (MicroBrightField, Williston, VT, USA) for reconstruction of the neuron. Apical and basilar dendrites from pyramidal neurons from layer III were traced, but only if the cells were fully impregnated, free from large truncations, and centered within the z-stack. A total of five cells, with a minimum of three if five were not possible, were traced from each hemisphere. A total of 301 neurons were traced and included in the analysis (See Table 5.1). Due to technical errors during the histological preparation, one male and one female control brain was unable to be processed.

The traced neurons were analyzed using Neurolucida Explorer®. The following measurements were extracted for analysis: 1) convex hull volume for both apical and basal projections, as well as 2) the total convex hull surface area for both apical and basal projections, as well as 3) the total length of the apical and basal projections, which, including the length of the cell body, were summed to obtain the total cell length, 4) the volume of apical and basal projections, which, including the volume of the cell body, were summed for the total cell volume, 5) the branch number for both apical and basal projections, which was summed for a total branch number, 6) the branch order for both apical and basal projections, which was summed for a total branch order, and 7) the number of terminal nodes, which is the total number of endings on either apical or basilar projections, which, when summed, gives the total number of terminal nodes for each neuron.

Some of these measurements are similar to the ones typically extracted by hand using a Camera Lucida to trace dendrites (e.g., branch number, length, etc.) that our laboratory has used previously (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013). However, NeuroLucida® offers some novel measurements (e.g., convex hull measures) that provide more global analyses of cell size and shape, and so more refined measurements of potential functional changes to the cell. Such refinement may be critical to detect subtle differences, such as those predicted for males versus females (see Introduction). Neurolucida 360® traces in three dimensions, allowing more complex measures to be derived, such as convex hull measures of dendritic surface area and volume: measures that are not feasible using hand drawn methods of tracings. Convex hull measures have been used to uncover the overall territory occupied by the dendritic arbor (Rojo et al., 2016), and, by assessing the apical and basilar projections separately, may uncover patterns of information processing by the cell (Spratling, 2002). Additionally, changes in convex hull that are accompanied by changes in cell body size may reveal whether cells are generally increasing or decreasing in size, or if it is the projection of the dendrites that is increasing or decreasing in size. The volume of the projections is calculated by assuming that the projections are cylindrical, but then assuming that they are conical when they change in diameter. The last novel measure used scores the terminal nodes; these are the endpoints of the arbor and have been used as a measure of overall connectivity of the cell (Lui et al., 2021).

Intra-observer reliability

Since virtual slides can be assessed multiple times, intra-observer errors were assessed through the retracing of a single neuron in an image file. A single trained experimenter was responsible for the neuroanatomical analysis (RAS) and so assessing the reliability of the work is important. The same experimenter reconstructed the same neuron five times over the months that the cells were traced to ensure that, with the growing expertise of the experimenter, there were no changes or biases in the way cells were traced. The coefficient of variation was calculated for every measure and averaged to determine the variation between the tracings of the same cells. The coefficient of variation was 6.57%, which is similar to other studies (Brinkman, 2019).

Table 5.1

Number of neurons included in the analyses.

	Experimental	Control
Male	82	70
Female	85	64

5.3.4 Statistical analysis

All statistics were conducted in RStudio (R Core Team, 2018) and used the packages lme4 (Bates et al, 2015) and ggplot2 (Wickham, 2016) to create the graphical representations. Neuronal morphology measures were assessed with a linear mixed model to compare the effects of rearing condition and sex, and the interaction between the two. Condition and sex were set as the independent fixed effects and a random error term was assigned to account for the repeated measure of individual animals with hemisphere nested within each individual. Differences were considered significant if $p \leq 0.05$ using the Satterthwaite's approximation ANOV, this method approximates the degrees of freedom based on the data presented, which is why there may be differences in the results (Luke, 2017).

5.4 Results

Table 5.2 shows the average values (± standard error of the mean) for all measures analyzed. Percent differences are reported for all significant measures. Table 5.3 shows the results of the linear mixed model.

5.4.1 Convex hull

Overall, there was a significant main effect of rearing condition on convex hull volume ($F(1,24) = 8.69$, $p = 0.007$), with the apical projections significantly different ($F(1,22) = 5.40$, $p = 0.03$), but not the basilar projections ($F(1,22) = 3.09$, $p = 0.09$). Similarly, there was an overall significant main effect of rearing condition on convex hull surface area ($F(1,24) = 9.47$, $p = 0.005$), with the apical projections being significantly different ($F(1,22) = 9.21$, $p = 0.006$), but not the basilar projections ($F(1,22) = 0.45$, $p = 0.51$). There were no sex differences or interactions for any of the convex hull measures (Figure 5.1).

5.4.2 Length

There was a significant main effect of rearing condition on the total length of the neurons ($F(1,24) = 4.71$, $p = 0.04$), with a significant difference in the length of the apical projections ($F(1,51) = 0.009$), but not in either the length of the basilar projections ($F(1,24) = 1.967$, $p = 0.17$) or in the length of the cell bodies ($F(1,24) = 0.33$, $p = 0.6$). No sex differences or interaction were uncovered in the analysis (Figure 5.2).

5.4.3 Volume

There were no significant rearing condition, sex effect, or interaction effects for the measures of volume measures: total cell volume, apical projections, basilar projections, or cell body (Figure 5.2).

5.4.4 Branch number and order

There were no significant rearing condition, sex effect, or interaction effects for branch number for the total. There was a significant sex effect on total branch order (F(1,51) = 4.57, p = 0.04), but no rearing condition effect or interaction effects. Given the significant sex difference branch order, it is worth noting that there was a trend towards a significant sex difference in branch number (F(1,24) = 3.61, p = 0.069).

5.4.5 Terminal nodes

There were no significant differences in the number of terminal nodes based on rearing condition, sex, or their interaction.

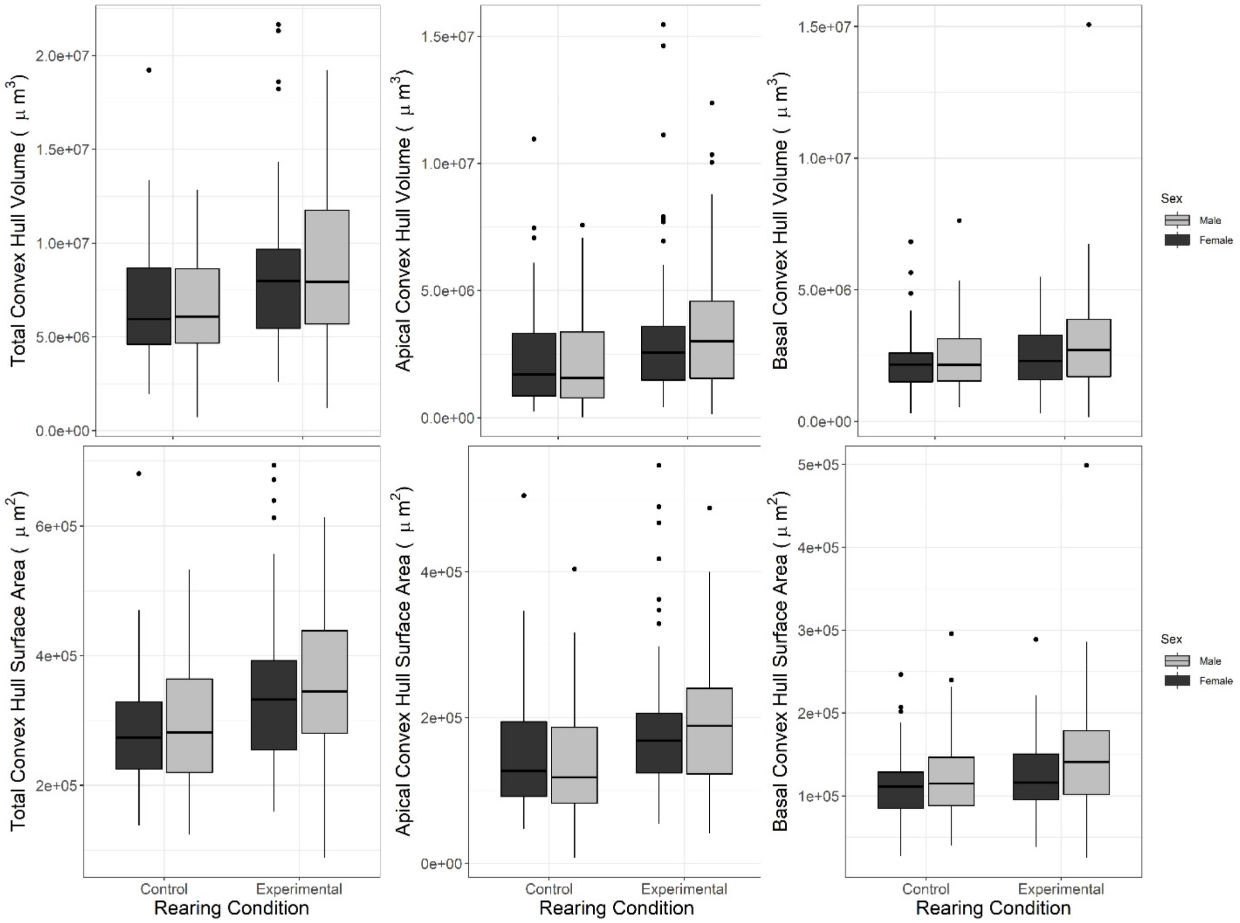

Figure 5.1 The boxplots show the results for the convex hull analyses of pyramidal neurons from layer III of area CG3, comparing both rearing condition and sex. The top panel shows the findings for volume and the bottom panel shows the findings for surface area. From left to right are shown the findings for the whole cell, apical and basilar projections, respectively. (*) denotes $p < 0.05$.

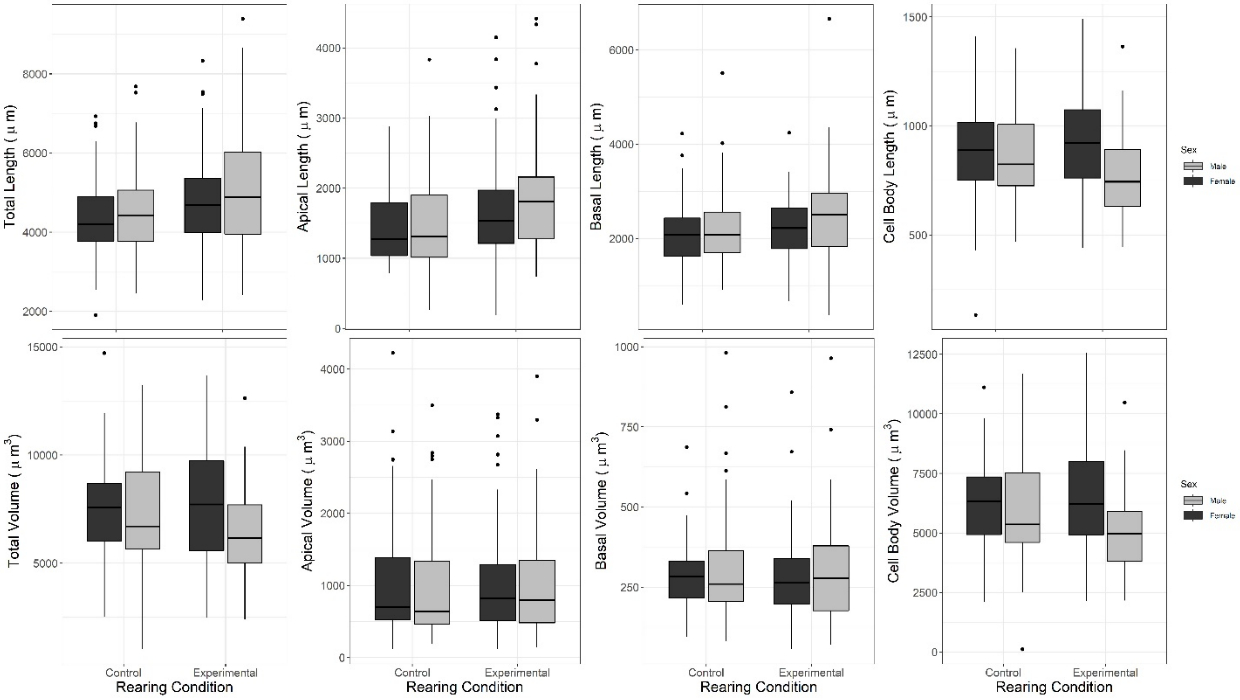

Figure 5.2 The boxplots show the results for the total length (top panel) and volume (bottom panel) of pyramidal neurons from layer III of area CG3, comparing both rearing condition and sex. The panels from left to right represent the total, apical, basilar, and cell body measures. (*) denotes $p < 0.05$.

Table 5.2

The average measurements (± standard error of the mean) of CG3 layer III pyramidal neurons of male and female rats that were either reared with a F344 partner or an age, sex, and strain matched partner are shown. The percent difference was only calculated for measures that were significantly different or trending significance.

** denotes significant rearing condition effect, # denotes significant sex difference, and ## denotes trending sex difference.*

Measurement	Control males	Control females	Experimental males	Experimental females	% difference
Convex hull volume (μm^3)	6695980 ± 343177	679923 ± 424144	8901396 ± 495374	8324340 ± 431508	21.5*
Apical convex hull volume (μm^3)	2215429 ± 216246	2424667 ± 281898	3440787 ± 301553	3160177 ± 311595	29.8*
Basilar convex hull volume (μm^3)	2467495 ± 150070	2216288 ± 152506	3012286 ± 250299	2448296 ± 138041	ns
Convex hull surface area (μm^2)	2941540 ± 11565	290418 ± 12556	361164 ± 14534	339568 ± 12722	16.4*
Apical convex hull surface area (μm^2)	139655 ± 9577	148614 ± 11113	193312 ± 88448	182621 ± 10564	9.6*
Basilar convex hull surface area (μm^2)	125122 ± 5858	111988 ± 5224	145753 ± 8237	123174 ± 5102	ns
Length (μm)	4549 ± 134	4446 ± 1079	5058 ± 174	4799 ± 131	8.5*
Apical length (μm)	1481 ± 78	1472 ± 74	1846 ± 92	1679 ± 79	15.9*
Basilar length (μm)	2198 ± 94	2099 ± 89	2435 ± 116	2203 ± 74	ns
Cell body length (μm)	869 ±26	875 ± 27	777 ± 24	916 ± 24	ns
Volume (μm^3)	7281 ± 303	7519 ± 304	6384 ± 260	7726 ± 285	ns
Apical volume (μm^3)	981 ± 89	1082 ± 110	1013 ± 90	1035 ± 84	ns
Basilar volume (μm^3)	305 ± 18	284 ± 14	290 ± 19	280 ± 14	ns
Cell body volume (μm^3)	5995 ± 265	6153 ± 230	5080 ± 215	6410 ± 242	ns
Branch number	65 ± 2	59 ± 2	69 ± 3	64 ± 2	7.8##
Apical branch number	29 ± 1	30 ± 2	31 ± 1	30 ± 1	ns
Basilar branch number	36 ± 2	29 ± 2	37 ± 2	33 ± 2	ns
Branch order	15 ± 0.5	14 ± 0.5	16 ± 0.6	14 ± 0.4	8.7#
Apical branch order	8 ± 0.5	7 ± 0.4	10 ± 0.5	8 ± 0.4	ns
Basilar branch order	6 ± 0.3	7 ± 0.4	6 ± 0.3	6 ± 0.3	ns
Terminal node	37 ± 1	35 ± 1	39 ± 1	36 ± 1	ns
Apical terminal node	13 ± 0.8	13 ± 0.7	15 ± 0.7	13 ± 0.6	ns
Basilar terminal node	24 ± 0.7	22 ± 0.8	25 ± 0.9	23 ± 0.7	ns

Table 5.3

Results of the linear mixed model analysis of condition, sex, and the interaction on 23 measures of CG3 layer II pyramidal neuron morphology. Results are reported with the fixed effects estimate, standard error, and p-values.

	Condition (Experimental)			Sex (Male)			Interaction			Intercept	
	Estimate	SE	p-value	Estimate	SE	p-value	Estimate	SE	p-value	Estimate	SE
Convex hull volume (μm^3)	1525098	903427	0.1	-109987	930771	0.9	696355	1271249	0.6	6799242	682926
Apical convex hull volume (μm^3)	735510	525349	0.2	-215574	541468	0.7	504687	739266	0.5	2424667	397127
Basilar convex hull volume (μm^3)	232009	438690	0.6	251208	451920	0.5	312782	617228	0.6	2216288	331619
Convex hull surface area (μm^2)	49150	26960	0.08	3531	27777	0.9	18445	37938	0.6	290418	20380
Apical convex hull surface area (μm^2)	34007	18906	0.08	-9267	19480	0.6	20445	26606	0.4	148615	14292
Basilar convex hull surface area (μm^2)	11186	15173	0.5	13134	15631	0.4	9445	21349	0.6	111988	11470
Length (μm)	353	285	0.2	98	294	0.7	165	401	0.7	4446	215
Apical length (μm)	207	152	0.2	5	156	0.9	168	214	0.4	1472	115
Basilar length (μm)	105	173	0.6	99	179	0.6	132	244	0.6	2099	131
Cell body length (μm)	40	65	0.5	-5	67	0.9	-134	91	0.2	875	49
Volume (μm^3)	207	699	0.8	-238	720	0.7	-1105	983	0.3	7519	528
Apical volume (μm^3)	-46	146	0.8	-102	150	0.5	83	205	0.7	1082	110
Basilar volume (μm^3)	-4	27	0.9	21	29	0.4	-11	39	0.8	284	21
Cell body volume (μm^3)	257	625	0.7	-158	644	0.8	-1172	880	0.2	6153	473
Branch number	4.3	4.2	0.3	5.9	4.3	0.2	-0.7	5.8	0.9	59.4	3.1
Apical branch number	-0.08	3.3	0.9	-0.9	3.4	0.8	2.0	4.6	0.7	30.4	2.5
Basilar branch number	4.4	4.9	0.4	6.9	5.0	0.2	-2.7	6.8	0.7	29.0	3.7
Branch order	0.07	0.9	0.9	0.6	0.9	0.5	1.4	1.2	0.3	13.9	0.7
Apical branch order	0.91	1.4	0.5	1.5	1.5	0.3	0.4	2.0	0.8	6.9	1.1
Basilar branch order	-0.9	1.0	0.4	-0.8	1.0	0.4	1.0	1.4	0.5	7.1	0.8
Terminal node	1.3	2.2	0.6	2.1	2.2	0.3	1.1	3.1	0.7	34.9	1.6
Apical terminal node	0.3	1.4	0.8	0.3	1.4	0.8	1.7	1.9	0.4	12.7	1.0
Basilar terminal node	1.0	1.4	0.5	1.9	1.4	0.2	-0.5	1.9	0.8	22.2	1.0

5.5 Discussion

Over the juvenile period, a high playing strain of rats, the LE strain, reared with low playing F344 peers, experience impoverished peer-peer play and have sociocognitive deficits as adults (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016; Stark & Pellis, 2020; Stark et al., 2021 in review). In the present paper, we show that, as is the case of LE rats reared with same strain adults (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013), LE rats reared with F344 peers have reduced pruning of the pyramidal neurons in the mPFC. The LE rats reared with F344 peers have larger and more complex pyramidal cells than LE rats reared with LE peers (Figure 5.1 and 5.2). This study adds support to the growing evidence that play-derived experiences in the juvenile period are important for the normal development of the mPFC (Baarendse et al., 2013; Bell et al., 2010; Burleson et al., 2016; B. Himmler, Pellis, & Kolb, 2013). It should be noted that even though these studies have involved different experimental manipulations to prevent or limit play in the juvenile period, the outcome is similar. Therefore, the first goal of our study was successfully achieved – LE rats reared with F344 partners had changes in the mPFC that were detectable at the anatomical level.

The second goal of this paper was to determine, if present, whether the magnitude of these effects was comparable between males and females. Both males and females were affected by the rearing condition, with no discernible sex difference in magnitude. The one sex difference that was found was that males had more complexity in branch structure, which was independent of rearing condition (Table 5.2). That is, as is the case for reduced pruning of the dendritic arbor of mPFC pyramidal cells in female rats (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013) and male hamsters reared with adults (Burleson et al., 2016), male and female rats reared in the same play-limiting condition showed comparable effects (Figures 5.1 and 5.2). Consequently, it can be concluded that both sexes gain the same developmental benefits from peer-peer play experiences in the juvenile period. Of course, it is possible that there may be differences at the physiological level that are not detectable in the anatomy of the cells. To conclude that the effects of juvenile play are the same in males and females, methods that have been shown to detect physiological changes in mPFC neurons following juvenile play deprivation (Baarendse et al., 2013) would need to be used. What can be unequivocally concluded is that peer-peer play in the juvenile period affects the development of the mPFC of both sexes and this has repercussions on the development of their sociocognitive skills (Burleson et al., 2016; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016; Stark & Pellis, 2020; Stark & Pellis, 2021, in review).

Convex hull analysis measures the volume or surface area contained within the dendritic arbor's most outward lying ends, and so provides a global measure of cell size. Figure 5.3 provides a visual recreation of these measures, clearly illustrating the smaller, more pruned cells of the rats reared with more playful partners. Our analyses show that the convex hull volume is 21.5% larger and convex hull surface area is 16.4 % larger in rats that had been reared in the play-impoverished condition. Consistent with these measures is an associated modest, but significant increase in dendritic length (8.5%). Together, these measures point to more expansive projections that can potentially make connections with a wider range of cells. Additionally, we found no significant difference in the size of the cell body – length or volume – suggesting that it is the dendritic projections becoming more expansive, rather than dendritic arbor expanding as a byproduct of increased cell size.

Cajal (1899) proposed the wiring economy principle, which states that neurons are arranged and connected in such a way as to minimize cost. In this way, the projections of the neurons, the axon and dendrites, are organized so as to save space and energy. Advances in

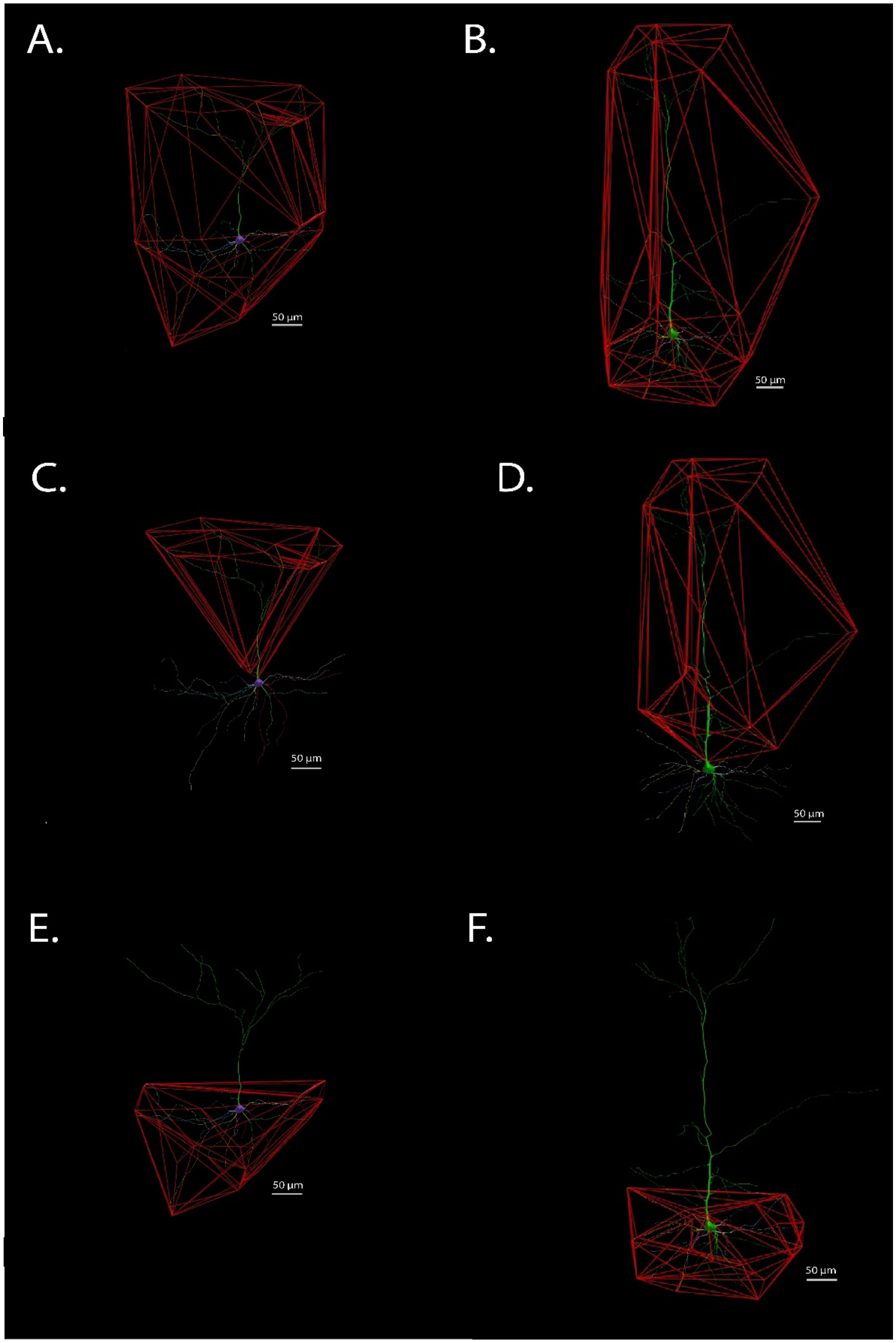

Figure 5.3 The convex hull analysis is represented for layer III pyramidal neurons from area CG3. The outward most lying ends are connected to create a geometric shape that encompasses the neuron, from which total volume and surface area can be calculated. The left panel is a neuron from a female rat that experienced play (control), while the right panel is a neuron from a female rat that had limited play experience (experimental). The whole neuron and the apical and basilar subfields are shown for each neuron.

technology, modeling and statistical methods, have revealed that neurons have optimal wiring patterns (Cuntz et al., 2010; Spruston, 2008). For example, pyramidal neurons in layers V and VI of the somatosensory cortex tend to be longer and have more branches than those in the layers closer to the surface (Anton-Sanchez et al., 2016). The greater surface area and volume of the pyramidal neurons in the mPFC of our play-impoverished rats could mean they are making more connections, and so decreasing efficiency. Indeed, even the modest increase in length could lead to suboptimal function, as even modest changes in branching angles can change the signaling patterns of the cell (Ferrante et al., 2013). While overall length and cell size play an important role in the functioning of neurons, we found most of the changes to be in the apical projections, not the basilar projections.

The neocortex has a laminar structure and pyramidal cells are found in all but the first layer. Pyramidal neurons have a distinctive morphology, comprising of an axon that can project to other cortical and subcortical structures, and a dendritic arbor divided into two components. The apical arbor ascends into more superficial layers while the basal arbor occupies the layer within which the cell resides (Crick & Asanuma, 1986). Thus, apical and basilar dendrites can detect signals from axons originating in different cortical areas. With a larger and more diffuse apical arbor, the control over action potential propagation becomes less precise. The point of pruning these neurons seems to function around the ability to create concise circuits that allow for executive control over behaviors implicated in executive functions. Indeed, even small changes in neurochemical signaling can cause a wide range of behavioral changes (Achterberg et al., 2016; Baarendse & Vanderschuren, 2012; Vanderschuren et al., 2016), which is altered when rats are deprived of play (Baarendse et al., 2013; Omrani et al., 2020). Thus, the lack of pruning seen in rats deprived of play (Bell et al. 2010; Himmler, Pellis, & Kolb, 2013; current paper), along with alterations in neurochemical signaling (Baarendse et al., 2013; Omrani et al., 2020), coupled with the alterations in social skills as adults (Stark & Pellis, 2020; Stark & Pellis, in review), seems to point to the lack of control the mPFC is able to exert over the behaviors performed. By fine tuning the connections of the apical arbor during the juvenile period, play would improve sociocognitive skills.

Stress during the preweaning period alters the development of neurons in the nucleus accumbens, the orbitofrontal cortex and mPFC, changes that are seen in adult rats (Muhammad et al., 2012). Importantly, it is the dendritic branching of the basilar arbor of the pyramidal neurons of the mPFC that were the most affected, while changes to the apical arbor were smaller and inconsistent (Muhammad et al., 2012). Previous findings using the adult rearing paradigm found increased complexity of both apical and basilar arbors of pyramidal neurons of the mPFC (Bell et al., 2010; B. Himmler, Pellis & Kolb, 2013). It could be that the changes to the basilar arbor resulted from the stress of being housed with an adult, while the changes to the apical arbor resulted from the impoverished play afforded by the adult. While additional research is needed to confirm this hypothesis, the absence of pruning of the apical dendrites, but not of the basilar dendrites in the LE rats reared with F344 peers, strongly suggests that play has a specific effect on the development of the apical dendrites and so the connections that they can make.

5.6 Conclusion

Consistent with previous work, we found that deprivation of play during the juvenile period results in reduced pruning of the mPFC. Moreover, by using a rearing paradigm that involves housing a rat from a high playing strain with a peer from a low playing strain (the F344 rearing paradigm), the specific contribution made by play experience in the juvenile period can be precisely assessed. Impoverished play experience reduced the pruning of the apical dendrites of the pyramidal neurons. Similarly, using the F344 rearing paradigm allowed for a sex neutral way to manipulate the play experiences, and this showed that play affects the development of mPFC neurons in the same way and to the same magnitude in both sexes. The F344 rearing paradigm has great potential for future studies to differentiate the effects of multiple partners on the development of other areas of the prefrontal cortex (Bell et al., 2010; B. Himmler et al., 2018) from the effects of play on the mPFC (Baarendse et al., 2013; Bell et al., 2010; Burleson et al., 2016; B. Himmler, Pellis, & Kolb, 2013).

CHAPTER 6: GENERAL DISCUSSION

Play with peers during the juvenile period has been hypothesized to be important for facilitating the development of executive functions and the underlying neural mechanisms that support these functions (Pellis et al., 2014; Vanderschuren & Trezza, 2014). In turn, improved executive functions results in more refined sociocognitive skills and so more effective social behavior (Pellis & Pellis, 2017). In this thesis, I used a peer-peer rearing paradigm involving housing LE rats with F344 partners, which reduces the play experienced, to investigate the role of play on the development of social competence, identify the key experiences that are important for this function, and replicate and extend previous investigations (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013) that show that play experience in the juvenile period has a role in the pruning of pyramidal neurons of the medial prefrontal cortex (mPFC). This is the first study to compare males and females directly, using the same rearing paradigm, and it has revealed that play has similar effects on both sexes.

Chapter 5 uncovered the changes to the neurons of pyramidal cells of the mPFC of rats reared with limited play experiences. I found that, as in previous studies, rats reared without adequate play experiences failed to prune the neurons of the mPFC but my work extended previous findings by showing that the neurons from play-deprived rats occupied more space. This was shown by increases in both convex hull measures (i.e., volume and surface area), and in the dendritic length of these neurons. These changes occurred mainly on the apical dendrites, which may have overarching functional implications as this part of the neuron receives inputs from layer I of the cortex. Layer I of the neocortex, especially the frontal lobe, receives many projections from within the cortex and from subcortical areas (thalamic nuclei) (Mitchell & Cauller, 2001). Thus, changes in the connectivity of the apical dendrites could have major implications in the functioning of the neurons, the control they exert over other areas and the behaviors implicated in the functioning of the circuitry. Furthermore, unlike previous experiments, in my study, there was no condition-induced reduction in pruning of the basilar dendrites. Being reared with a peer, even one that provides limited play (this study) may be less stressful than being reared with an adult (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013). The reduced pruning of the basilar dendrites in the adult-rearing paradigm could be due to the stress inherent in the adult rearing paradigm. If so, this would suggest that play has a specific effect on the development of the apical dendrites of the pyramidal neurons of the mPFC, and so modifies the behavioral functions dependent on those neural connections.

Chapters 2 and 3 assessed the role of play deprivation on the development of adult social competence. The 'stranger paradigm' was used to assess behaviors in a naturally occurring context. Rats are highly social, living in colonies with diverse social networks (Barnett, 1975). Therefore, it is imperative that rats develop their social repertoire and are able to react appropriately to different contexts and situations (Lore & Flannelly, 1977; Meaney & Stewart, 1979). The lack of play during the juvenile period has been shown to negatively impact the development of a wide range of social and cognitive functions (Baarendse et al., 2013; Omrani et al., 2020; Schneider et al., 2014; Schneider, Bindila, et al., 2016). I proposed that, if play deprivation causes atypical development of the mPFC, then the stranger paradigm would strain the limited social skills of these rats. I found that LE rats reared with a F344 partner were less successful in navigating this novel social situation (Chapter 2; Chapter 3). Both male and female experimental pairs took significantly longer to engage in play. Male LE rats reared with F344 partners were significantly more likely to escalate encounters to serious aggression. Because females do not compete for dominance in such contexts, in the female LE rats reared with F344 partners the signs of incompetence were subtler, mostly revealed by changes in the behavior of the normally reared unfamiliar partner. The typically reared partner showed avoidance behaviors and was more likely to adopt tactics that did not prolong encounters. These findings provide evidence that play experiences during the juvenile period are important for the development of sociocognitive skills as adults.

Chapter 4 evaluated the play between LE and F344 partners during the juvenile period to identify the experiences that were missing, compared to when two juvenile LE rats play together. The rearing paradigm used, like the adult rearing paradigm (Pellis et al., 2017), does not completely abolish play as does the partition rearing paradigm (see Figure 1.2). Therefore, because others have found that both the adult and F344 rearing paradigms result in atypical brain development and impaired sociocognitive skills (Bell et al., 2010; Burleson et al., 2016; B. Himmler, Pellis, & Kolb, 2013; Schneider, Bindila, et al., 2016), it is important to assess what aspects of play are missing. I found that F344 rats launch fewer attacks, and when attacked, respond in ways that limit close-quarter wrestling. Both aspects have previously been shown to be altered in LE juveniles reared with adults (Pellis et al., 2017) and juvenile Wistar rats reared with F344s (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016).

As one of my key goals was to compare the effects of reduced play on both sexes, I included males and females in all phases of my experiment. I hypothesized that both male and female juveniles would experience a similar play style when reared with a F344. This was based on previous work showing that male and female juveniles do not differ qualitatively in the way they play during the juvenile period (Pellis & Pellis, 1990). As expected, I found no differences in juvenile play. Adulthood is when sexual differentiation becomes more pronounced. Overall, as expected, males interacted in a quasi-aggressive manner in the stranger paradigm, playing in a rougher style in order to establish dominance. Since, in home colonies, females do not form overt or stable dominance relationships among themselves, as expected, there was no overt fighting in the stranger paradigm. Still, I looked at less overt measures that could reveal attempts at asserting dominance. Among familiar groups of females, passing – in which one animal overtakes the other in a leap frog fashion – has been used as an indicator of dominance (Ziporyn & McClintock, 1991a). In the stranger paradigm, passing was rare, and the versions of passing that are strongly indicative of assertion of dominance (head-to-tail passes) were even rarer, supporting the view that adult females that meet one another in a neutral arena do not compete for dominance. However, there was more asymmetry among the experimental pairs, with the F344 reared LE rat more likely to engage in passing. This asymmetry may be indicative of reduced social skills in the play deprived LE rats as it is consistent with the subtle changes in the play of the experimental pairs during the stranger paradigm, with the F344 reared LE rat being less attractive as a playmate when compared to its normally reared partner. Thus, while the stranger paradigm indicates that the play-deprived rats of both sexes are socially deficient, the test was not sufficiently sensitive to detect whether social skills are reduced by the same magnitude in both sexes. At the level of brain anatomy, both male and female LE rats deprived of play had more complex cytoarchitecture of the apical dendrites of the pyramidal neurons of the mPFC, and this was of the same order of magnitude. These findings suggest that both sexes may be equally affected by experiencing play in the juvenile period. There were sex differences: I found around an 8% sex difference between males and females in branch order (statistically significant) and branch number (trending significance), with males having more branching than females. Importantly, these sex differences were not dependent on play experience for their emergence. This is consistent with previous work suggesting that, compared to females, males have more dendritic branching in the mPFC, a difference likely arising from hormonal differences between the sexes (Bryan Kolb & Stewart, 1991).

6.1 Play experiences during the juvenile period

Previous research has shown that juvenile female rats reared with adults fail to prune the pyramidal neurons of the mPFC (Bell et al., 2010; B. Himmler, Pellis, & Kolb, 2013). A detailed analysis of the play between adults and juveniles found a drastic reduction in the amount of play received by the juvenile. Play between juvenile rats is relatively symmetrical, with both partners initiating close to 50% of the attacks, whereas in adult-juvenile pairs, only 7% of attacks are by the adult (Pellis et al., 2017). In the case of juvenile LE rats playing with a F344 partner, approximately 20% of attacks were by the F344 partner (Chapter 4). In the case of juveniles playing with adults, the juveniles seemed to compensate by launching more attacks than they would with another juvenile (Pellis et al., 2017). Unlike that found for Wistar juveniles playing with F344 peers (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016) or juveniles playing with adults (Pellis er al., 2017), I did not find such a compensatory increase for the LE rats playing with F344 partners (Chapter 4), However, F344 rats initiated fewer attacks toward Wistar rats than they did toward LE juveniles, so it is possible that, for the LE rats, a sufficiently rewarding number of play fights occurred (Vanderschuren et al., 2016), reducing the need for them to increase their attack rate. Regardless of whether the rats compensated for the lower levels of play or not, the level of play reached in all the experiments was insufficient to either prune the neurons of the mPFC or to improve sociocognitive skills (Bell et al., 2010; Burleson et al., 2016; B. Himmler, Pellis, & Kolb, 2013; Schneider, Bindila et al., 2016). While the quantity of play may remain stable because of compensatory changes, the quality of the play experienced by juveniles interacting with adults or

F344 partners was different. It is these qualitative differences that may be more important in the developmental effects of play.

While both adult and F344 rats are just as likely to respond to a nape attack, how they respond is markedly different to how a same strain peer would respond (Pellis et al., 2017; Chapter 4). Both adult and F344 rats respond to nape attacks with defensive tactics that limit close-quarter wrestling, favoring evasions and partial rotations, respectively (Pellis et al., 2017; Chapter 4). Adult rats limit the amount of wrestling engaged in with a juvenile by overpowering it, so thwarting its capacity to launch counterattacks that successfully result in role reversals. Indeed, in the rare event that a juvenile was able to launch a counterattack, the adult used its superior size to move away or kick the juvenile, as was similarly the case when an adult was pinned by a juvenile (Pellis et al., 2017). However, when a F344 partner provided an opportunity for close-quarter wrestling, the LE rat capitalized on this by increasing its counterattacks. Indeed, there is an interesting juxtaposition, while with an adult the opportunity for role reversals by LE juveniles is reduced (Pellis et al., 2017), in the case of an LE juvenile playing with a F344 peer, its opportunities for counterattacks is greater (Figure 4.1).

Not only is gaining some level of play, by compensatory increases in attacks (Pellis et al., 2017; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016) not sufficient to produce the beneficial effects of play, neither is compensating reciprocal exchanges by increasing the number of counterattacks performed (chapter 4). This leads me to suspect that it is not the performance of playful actions that is necessary, but the symmetry between the exchange itself between pair mates that is most important. Typically, no more than 30% of play fights involve a role reversal (Pellis & Pellis, 2017); however, it seems that the role reversals that do occur, need to be close to parity.

Indeed, on most measures of play, the LE-LE pairs were significantly more symmetrical than the LE-F344 pairs (see Figure 4.3). Work done on wild rats lends evidence to this hypothesis.

On average, juvenile wild rats launch fewer nape attacks and are less likely to adopt defensive tactics that promote close-quarter wrestling when compared to domestic juvenile LE rats (B. Himmler, Stryjek, et al., 2013). Despite the overall reduced play in wild juvenile rats, juvenile wild rats reared with an adult during the peak play period show no differences in the pruning of the pyramidal neurons of the mPFC compared to peer-reared wild juveniles (B. Himmler, 2015). Analysis of the play between wild juveniles and adults found that wild adults play in a more reciprocal manner (Pellis, Pellis, Himmler, et al., 2019). While wild adult rats launch fewer nape attacks, as is the case with domesticated adults, they are more likely to respond to an attack with playful wrestling and, most notably, participate in and receive counterattacks (Pellis, Pellis, Himmler, et al., 2019). That is, the wild adults create greater opportunity for parity in role reversals than do domesticated adults and these experiences of playful symmetry provide enough of the key experiences derived from play to develop the mPFC normally (Pellis, Pellis, Himmler, et al., 2019). These findings support the hypothesis that it is symmetry of action not the absolute number of playful actions that are performed that constitutes the important experience derived from play.

However, it should be noted that while wild adults are able to provide enough experiences for the juvenile to develop the mPFC, it is still unclear what the threshold is for adequate development. How many experiences of a certain type are needed and how often are they needed, are questions that still remain. For example, work done by Einon and colleagues in the 70s showed that complete social isolation globally impairs the development of social behavior and produces rats that are more anxious (Einon et al., 1975, 1978; Einon & Morgan, 1976). These behavioral deficits were most pronounced when juvenile rats were reared in isolation between the 25th and

45th days of life (Einon & Morgan, 1977). However, brief contact for one hour per day with a peer largely alleviated the deficits found (Einon & Morgan, 1977).

Strain differences in play may also shed light on what aspects of play are important for the development of the mPFC. Detailed analyses of strain differences have been conducted for a wide range of strains including those mentioned here – LE, F344s, and wild rats – but also Sprague-Dawley, Brown Norway, and Wistar rats (S. Himmler, Lewis, & Pellis, 2014; S. Himmler, Modlinska, et al., 2014; Northcutt, 2018; Siviy, 2020). These strains each have unique ways of playing that may offer insight into what aspects of play are important for the development of social skills and the brain. When rats of two strains with vastly different play styles are reared together over the juvenile period – LE and Sprague-Dawley rats – there is a convergence in the play style used during play. That is, each strain plays more like the other, so meeting in the middle (S. Himmler, Lewis, & Pellis, 2014). Not only does this convergence support the hypothesis that it is reciprocity that is important, and by playing more like your partner you are abiding by the 50:50 rule, but overall similarities in the play could uncover what aspects are important. For example, if counterattacks are important and the experience of performing and receiving counterattacks is crucial for development of the mPFC, as highlighted above, I would predict that, despite the alteration in play styles between LE and Sprague-Dawley rats, the number of counterattacks performed by each strain would be relatively equal despite other differences in play. Thus, although there is some level of plasticity in the style of play, such cross-rearing allows for the opportunity to identify the key elements that remain unchanged. As a control condition to rearing Wistar rats with F344 rats, Schneider, Bindila et al. (2016) also reared Wistar rats with either other Wistar rats or with Sprague Dawley peers. The play fighting style of Wistar rats is more similar to that of LE rats, and both are equally dissimilar to Sprague Dawley rats (S. Himmler, Modinska, et al., 2014). Yet while Wistar rats reared with F344 peers had sociocognitive deficits, those reared with Sprague Dawley peers did not, suggesting that the Sprague Dawley share elements of play critical to the development of the Wistar rats. Some strains, such as the F344, seem less plastic in their play style, retaining the core strain-typical style even after more extensive cross-fostering with a mother and litter from another strain of rats (Siviy et al., 2017). Future studies should capitalize on these strain differences to identify more precisely the key play experiences that are important, and the threshold level needed to influence the development of the mPFC and sociocognitive skills.

6.2 Anatomical considerations

While I showed that the mPFC is impacted by play deprivation in both male and females, future studies should also consider other cortical areas. Previous work, using lesions and multi-animal rearing conditions, have dissociated the influence of juvenile experiences on and the functions of the mPFC and OFC (Bell et al., 2010; Bell et al., 2009; Pellis et al., 2006). These studies found that the OFC is important for modulating behavior based on the identity of the partner (Pellis et al., 2006), and that the pyramidal neurons of the OFC have greater dendritic complexity if rats are reared with multiple partners during the juvenile period (Bell et al., 2010). Adult LE rats that were reared with a F344 had deficiencies in their ability to modulate behavior with another rat in the stranger paradigm (Chapters 2 and 3). While I believe these impairments are mainly due to the role that the mPFC plays in impulse control, short-term memory, and decision making (Baarendse et al., 2013; Omrani et al., 2020; Schneider, Bindila, et al., 2016), the paradigm I used involved pairs of rats reared together, so some of the disability could have resulted from underdevelopment of the OFC. Although the control rats fared better in the stranger paradigm than did LE rats reared with a F344 partner, clearly implicating the mPFC, it is possible that a LE rat reared with three same strain peers could do even better than the LE rat reared with one peer.

Since the stranger paradigm requires the appropriate coordination of behaviors between partners – which is dependent on the mPFC (Bell et al., 2009; B. Himmler, Bell, et al., 2014) – and uses an unfamiliar partner, the ability to detect and modify behaviors based on the partner's identity must also play a role – which is dependent on the OFC (Pellis et al., 2006). To dissociate the roles that these brain regions may have in the stranger paradigm, a rearing scheme similar to that of Bell et al. (2010) would be useful. By rearing LE rats with either play experience or deprivation and in groups or pairs and later testing them in the stranger paradigm with different types of unfamiliar partners (e.g., adult male, adult female, juvenile), it might be possible to discern how each brain area is contributing to the deficits seen in the Chapters 2 and 3. I would predict that LE rats reared with three F344 cage mates would fail to have properly pruned apical dendrites of the mPFC neurons, as would LE rats reared with one F344 peer (chapter 5), and LE rats from both conditions would perform poorly in the stranger paradigm (chapters 2 and 3). However, if the OFC is playing a role in modulating the interactions in the stranger paradigm, then LE rats reared with three F344s may be more successful in navigating the stranger paradigm if confronted by different types of strangers. Conversely, LE rats reared with three other LE rats would likely deal better with all situations, in that they would be able to modulate what actions to perform with different partners (OFC) and execute those actions more effectively (mPFC).

6.3 Ultrasonic vocalizations

USVs emitted by rats are broadly grouped into two categories based on the frequency range of the calls and emotional state of the animal when emitted. Fifty kHz calls are emitted in appetitive situations such as in anticipation of psychoactive drugs, during sexual encounters, in social play and when tickled by an experimenter (Bialy et al., 2000; B. Himmler, Kisko, et al., 2014; Knutson et al., 1999; Panksepp & Burgdorf, 2000). Twenty-two kHz calls are associated with negative situations such as social defeat, foot shock, drug withdrawal, and the presence of predator odor (R. Blanchard et al., 1991; Kaltwasser, 1990; Thomas et al., 1983; Tonoue et al., 1986). Researchers have further categorized 50 kHz calls based on their spectrographic characteristics, with some researchers proposing as little as two (Burgdorf & Panksepp, 2006; Wöhr et al., 2008) and as many as 15 different categories (C. Burke, Kisko, Swiftwolfe, et al., 2017; Wright et al., 2010).

During play, 50 kHz calls are more likely to be emitted prior to playful contact (B. Himmler, Kisko, et al., 2014), suggesting that vocalizations may be used as play signals to alert the partner of imminent contact (Bekoff, 1975, 1995). Additionally, using the larger range of proposed 50 kHz call categories (Wright et al., 2010), specific calls are correlated with specific behaviors both when anticipating play (C. Burke, Kisko, Swiftwolfe, et al., 2017) and when engaging in play (C. Burke et al., 2018), with at least some of those calls seemingly able to influence the subsequent behavior of the partner (C. Burke et al., 2020). Therefore, the appropriate use of vocalizations at particular times may be important in coordinating interactions with partners. Indeed, in the stranger paradigm, in which one partner was devocalized, adult male rats were more likely to escalate the play encounters to serious aggression than when both partners could vocalize (Kisko et al., 2015). Further analysis revealed that specific calls may be emitted at key moments to de-escalate the situation, leading to escalation when one animal was unable to vocalize (C. Burke, Kisko, Pellis, & Euston, 2017).

In chapter 4, I assessed four broad categories of vocalizations emitted when juvenile rats were playing. There were significant differences in the number of vocalizations emitted and in the proportion of specific call types emitted. As adults, male rats in the stranger paradigm emitted fewer vocalizations per play bout (see Appendix A). As indicated by the large standard errors, there was considerable variation across pairs in the frequency of emitting calls, and this likely precluded detecting significant patterns, but there were some interesting trends that deserve further study (Figure A.1). For example, 22 kHz calls tend to be emitted more frequently by the experimental pairs, the same pairs that are more likely to escalate to serious aggression (Chapter 2). As the majority of the aggression was initiated by the play-deprived rat, it would be interesting to know which partner was responsible for the increased emission of 22 kHz calls. If the 22 kHz calls were emitted by the partner, then this would suggest that the play-deprived rat may be misinterpreting the signal and continuing to contact the partner even though it is emitting a distress or warning call. The impoverished play environment afforded by being reared with a F344 peer was also associated with an atypical signaling context (see Figure 4.4), and so the impaired sociocognitive skills present in the play deprived LE rats could partly be due to a dysfunctional signaling ability. Future studies could be designed to determine whether it is the production of calls, their correct contextual use or their interpretation that is impaired due to being reared with a F344 partner.

A limitation in understanding how USVs are used in communication is in the inability to identify which animal is emitting which vocalization. The inability to distinguish signaler and receiver diminishes the conclusions that can be drawn from the data. For example, are the alterations seen in the number of vocalizations per play bout (Figure A.1 panel B) the result of the inadequately reared rat not using vocalizations at all, or is there a depression in the number of vocalizations emitted by both parties? Are the greater number of 22 kHz calls coming from the partner of the play deprived rat an attempt to mitigate aggression or from the play-deprived rat to signal its general agitation? There are some promising advances in using arrays of microphones and tracking software to try and pinpoint the animal emitting the vocalizations (Sangiamo et al., 2020). However, based on the close-quarter nature of play, I am unsure of how successful the technology will be for play. Once signaler and receiver are identifiable, however, these questions could become more easily answered.

6.4 The F344 paradigm

F344 rats have been systematically studied and shown to be less playful than other rat strains (Siviy et al., 1997a, 2003). Further, their style of play does not afford proper experiences, when paired with Wistar (Schneider et al., 2014; Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016) or LE rats (current thesis), to develop normal sociocognitive skills. As previously mentioned, when two playful strains of rats are housed together during the peak play period, there is a convergence of play styles (S. Himmler, Lewis, & Pellis, 2014). There is little evidence that play styles are converging when LE rats are reared with a F344; this is confirmed with the measures of asymmetry (Figure 4.3). However, the view that F344 rats are a low playing strain has been mainly derived from studies in which they were interacting with rats from other strains. F344 rats playing with F344 partners may convey a different impression.

Part of this project involved recording play interactions between F344 rats. While not directly pertaining to my thesis, another question arose: if F344s deprive LE rats of play, what happens to the F344s that are enriched? I automatically assumed that being reared with a more playful strain would be enriching. To answer this question, I also recorded the play between pairs of F344 rats to compare with that of F344 rats paired with LE partners. Are F344 rats reared with LE partners better able to cope in the stranger paradigm and would their mPFC neurons be different to those of F344 rats reared with F344 partners? An error made by the supply company from which I obtained the F344 rats, moving our laboratories from one building to another and a pandemic, did not fully permit exploring the data collected on the F344 rats. However, preliminary findings show that pairs of F344 rats launch nearly double the number of nape attacks compared to pairs of LE rats. Consistent with previous research (Siviy et al., 1997a, 2003), this analysis shows that F344 rats favor evasions and partial rotations as their defensive tactics of choice, evading from almost 60% of nape attacks as compared to 20% in LE rats. What is most surprising is that, despite the reduction in facing defenses in general, and the large reduction in complete rotations – with only 15% of facing defenses involving a complete rotation, compared to nearly 40% in LE rats – F344s are more likely to launch a successful counterattack than are LE rats (see Figure B.1).

These data reframe Bekoff's original concern about isolation rearing: "who's being deprived of what?" Clearly, further work is needed to elucidate why the above measures of play are so markedly different when a F344 rat plays with another F344 as compared to when it plays with a rat from another strain (see Table 6.1). Whatever the reason, F344 rats provide an impoverished environment for other playful strains, one sufficient to impair the development of adult social skills and the circuitry of the mPFC (Schneider, Bindila, et al., 2016; Schneider, Pätz, et al., 2016; present thesis).

Table 6.1

Comparison of playful attacks (per minute) launched by F344 rats towards other F344 rats versus rats from other strains.

	Napes per minute by F344	citation
F344 – F344	6.8	Appendix B
F344 – LE	1.3	Chapter 4
F344 – Sprague Dawley	2.6	Siviy et al., 2003
F344 – Buffalo	2.6	Siviy et al., 1997
F344 – Wistar	0.9	Schneider, Bindila, et al., 2016

CHAPTER 7: REFERENCES

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APPENDIX A: VOCALIZATIONS EMITTED DURING THE STRANGER PARADIGM BY ADULT MALES

A.1 Methods

A.1.1 Subjects

Rats were purchased from Charles River Laboratories (Kingston, New York) and reared at the Canadian Centre for Behavioural Neuroscience. Twenty-six male LE hooded rats and ten weanling F344 rats were purchased, arriving at 24 days of age. After a two-day acclimation period, animals were housed in pairs of either two LE rats, for a total of 8 pairs, or of one LE and one F344, for a total of 10 pairs. In addition, ten LE males were purchased at 75 days of age, were housed in pairs and used for experimentation when all rats were 80 days of age. All animals were pair-housed in standard polyethylene cages (46cm x 25cm x 20xm) with corncob bedding, food and water provided *ad libitum*. The colony room was maintained on a 12-hour light dark cycle, with lights on at 0700, and maintained at 21-23°C. All care and experimental procedures were approved by the University of Lethbridge Animal Welfare Committee in compliance with guidelines set in place by the Canadian Council for Animal Care.

A.1.2 Apparatus

Synchronous audio and video were taken in a sound-attenuating chamber with a removeable clear Plexiglas® enclosure (50cm x 48cm x 48xcm) inside. The chamber itself was lined with sound-attenuating foam (Primeacoustic, Port Coquitlam, British Columbia). For the recordings, CareFresh® bedding was provide as a soft substrate for the animals and also reduce the amount of ultrasonic noise (B. Himmler, Kisko et al., 2014). Video was recorded with an ExmourRS 4K Sony Handycam with night-shot capability, and the camera was placed in a small opening in the chamber at a 45° angle. Using a device that simultaneously emits an audible beep and a flash of light was used to synchronize the video and the audio. The audio was recorded using an ultrasonic microphone (Model 4939, Brüel & Kjaer, Denmark). This was placed ~35cm above the center of the Plexiglas® box. The audio was set to record sounds with a frequency range between 4 – 100 kHz. The microphone was connected to a Soundconnect™ amplifier (Listen, Inc., Boston, MA) and recorded at 195,313 Hz using a 32-bit resolution via a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, FL) using an in-house developed MATLAB acquisition program.

A.1.3 Procedure

Beginning at 80 days of age, before experimentation commenced, cage mates were habituated to the testing enclosure for 15-minutes each day for four consecutive days. After the fourth habituation session, cage mates were socially isolated for 24 hours; this increases the playfulness when introduced to a new partner (Panksepp & Beatty, 1980a; Pellis & Pellis, 1990). One partner from each LE-LE pair was selected as the control animal to be compared to a LE rat from the mixed pair and had the base of their tails colored with permanent marker (Sharpie®) for easy discrimination during video analysis. To run the stranger paradigm, a pool of unfamiliar partners was created with the ten additionally purchased males and the second LE rat from the LE-LE control pairs. After 24 hours of isolation, pairs of unfamiliar male rats were introduced into the experimental enclosure. Audio and video were recorded for 10-minutes before the animals were returned to their home cage. Twenty-four hours after the first test session, pairs underwent the same paradigm where they were, once again, socially isolated for 24 hours before being introduced to an unfamiliar partner in another trial. This was done to avoid any data loss due to equipment or other unforeseen experimental failure. In both trials, unfamiliar partners were assigned to the experimental animal, either the LE that had been reared with the F344 or the LE reared with another LE, and had not had any previous contact (therefore, neither the cage mate nor the partner from the first trial). After every habituation session or play trial, fresh bedding was replaced and the Plexiglas® box was cleaned with Virkon® to eliminate any odors from the previous trial.

A.1.4 Vocalization analysis

All vocalizations emitted during the 10-minute period were scored using Raven Pro 1.6.1 software (Bioacoustics Research Program, Cornell Lab of Ornithology). Spectrograms were generated with a 256-sample Hann window. Once generated, a trained scorer manually selected each vocalization for analysis, assigning it one of the 15 call-types described by Wright et al. (2010). One additional call-type was added: audible calls. Since these calls are emitted in an audible range they were first identified by visual inspection, then confirmed via audio inspection. In addition to call type, the number of USVs emitted per nape attack was calculated as seen in Chapter 4.

For the purpose of statistical analysis, call categories were grouped into those used in Chapter 4: trill type calls, audible calls, 50 kHz flat, and frequency modulated. Since play between adult males can escalate to aggression (Lori K. Smith et al., 1999), and 22 kHz calls are emitted during aggressive encounters (Takeuchi & Kawashima, 1986), calls with the lowest frequency component ranging between 20 - 30 kHz, were also assessed, as calls with flat components are emitted in this range while the frequency modulated component is above 30 kHz.

A.1.5 Statistical analysis

All statistics were performed using RStudio (R Core Team, 2018) and the packages car (Fox & Weisberg, 2019) and lsmeans (Length, 2016), as well as the package ggplot2 (Wickham, 2016) to create the graphs. Comparisons of the average number of vocalizations emitted during the 10-

minute play session, the average number of vocalizations emitted per play bout, as well as the proportion of call types emitted, were tested using a one-way ANOVA. Differences were considered significant if the p-value was ≤0.05. All graphs and tabular data are presented as the group means and standard error of the mean.

A.2.1 Results

Figure A.1 shows the differences between the vocal analysis based on rearing condition. The only significant difference was for the number vocalizations emitted per nape attack (F(1,16) = 4.51, p = 0.05), experimental pairs emitted fewer vocalizations per nape attack (panel B). No other significant differences were found. However, some interesting trends appeared. On average, experimental pairs emitted more frequency modulated, 22 kHz flat, and audible calls but emitted fewer 50 kHz flat calls. Additionally, experimental pairs had more calls with components lower that 30 kHz, which includes 22 kHz flat and audible calls.

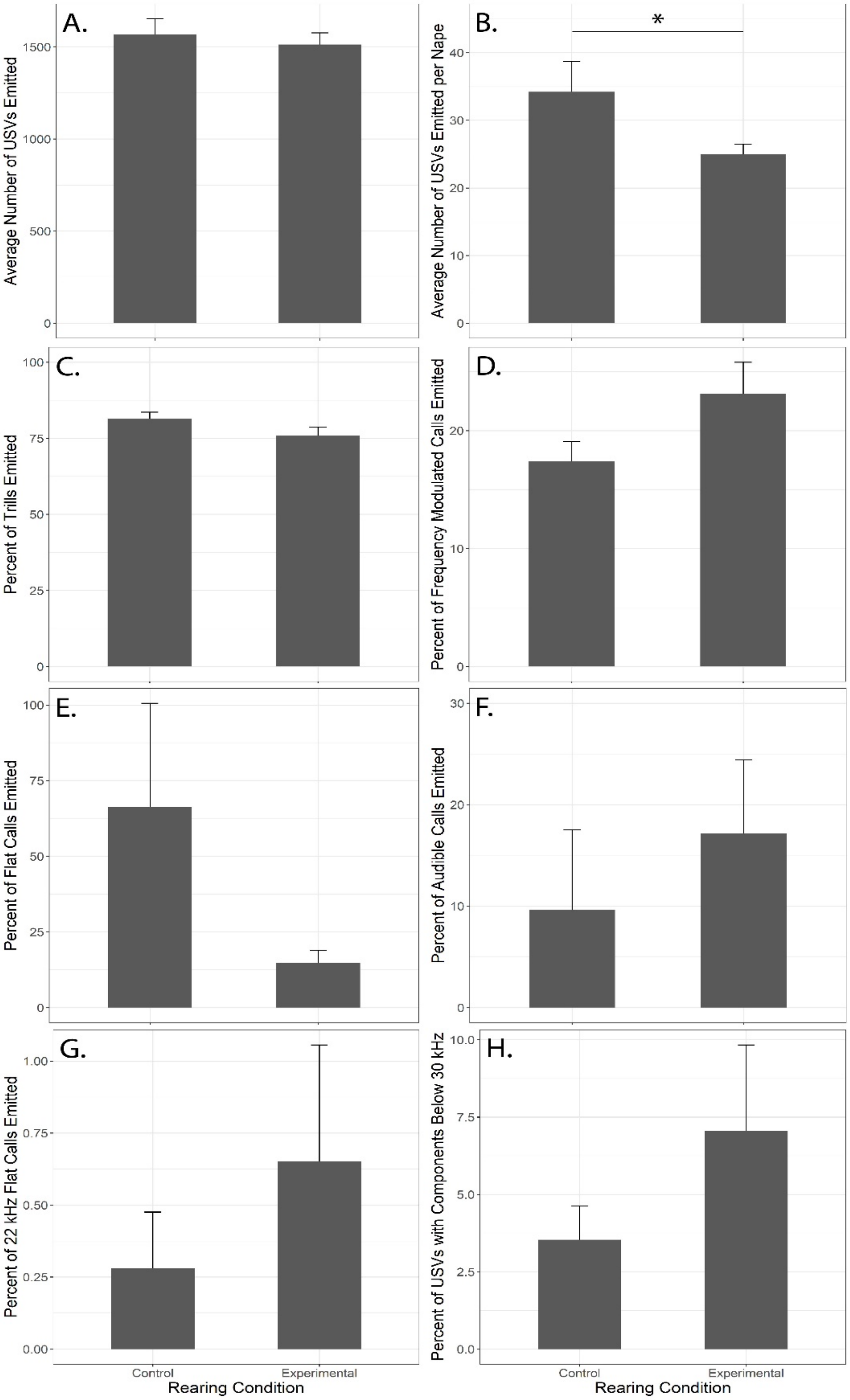

Figure A.1 *Vocalizations emitted by pairs of adult male rats in the stranger paradigm. Shown are A) the average number of vocalizations emitted during the 10-minute play session, B) the average number of vocalizations emitted per play bout, C) the percentage of trills that are emitted, D) the percent of frequency modulated calls emitted, E) the percentage of flat calls emitted, F) the percentage of audible calls emitted, G) the percentage of 22 kHz calls emitted, and H) the percentage of vocalizations that have a component below 30 kHz.*

APPENDIX B: COMPARING THE PLAY FIGHTING OF FISCHER 344 WITH THAT OF LONG EVANS RATS

A comparison was conducted between the number of nape attacks and the likelihood of using different types of defense tactics by the pairs of F344 rats and pairs of LE rats.

B.1 Methods

B.1.1 Subjects

A total of 32 weanling F344 rats were purchased, half of which were male, from Charles River Laboratories (Kingston, NY, USA). The sample of LE rats was the one used in Chapter 4, and it should be noted that the rats from the two strains were obtained and tested concurrently. All the animals arrived at the Canadian Centre for Behavioural Neuroscience at 24 days of age. At 26 days of age, the weanling rats were pair-housed into same sex pairs (8 male pairs; 8 female pairs), housed in polyethylene cages (46cm x 25cm x 20cm) in the colony room maintained at a constant temperature (21 – 23^{o}C), on a 12-hour light-dark cycle (lights off at 1900). Food and water were provided to the animals *ad libitum*. All care and procedures were approved by the University of Lethbridge Animal Welfare Committee in compliance with guidelines from the Canadian Council for Animal Care.

B.1.2 Procedure

Play between cage mates was tested twice during the peak juvenile period (Thor & Holloway, 1984), when the rats were between 33 and 38 days of age, in a neural arena. Cage mates were habituated to the testing enclosure, in complete darkness, for 15-minutes each day for three days leading up to testing. After the third habituation session, cage mates were socially isolated for 24 hours to increase playfulness on reunion (Niesink & van Ree, 1989; Panksepp & Beatty, 1980;

Pellis & Pellis, 1990). After isolation, cage mates were reunited in the test enclosure and their subsequent interaction was recorded 10-minutes. Once the test session was finished, pairs were housed together for 24 hours before undergoing the second testing session. The second test session was done to ensure enough data for analysis in the case of data loss. No data was lost during the process, thus the first play session will be used for analysis. Adult testing was performed when the F344 rats reached 80 days of age and followed a protocol described elsewhere (Stark & Pellis, 2020). Following adult testing, the rats were sacrificed, and their brains were removed for histological analysis.

B.1.3 Apparatus

The test enclosure was a box constructed of Plexiglas® measuring 50cm x 50cm x 50cm. Play trials were conducted in the box with 1-2 cm of CareFresh® bedding. The enclosure was housed in a sound-attenuating chamber lined with sound attenuating foam (Primeacoustic, BC, Canada). The video was recorded with an ExmourRS 4K Sony Handycam camcorder, set to record in night-shot, through a small window in the chamber allowing for a 45° angle of the interactions. Vocalizations were also recorded simultaneously using a microphone set to record sounds between 4-100 kHz (Model 4939, Brüel & Kjaer, Denmark). The microphone is situated above the center of the Plexiglas® box, 32-38 cm from the floor. At the beginning of each habituation or test trial, a series of three consecutive flash/beep signals were given to synchronize video and audio recordings post filming.

B.1.4 Behavioral analysis

As described in Chapter 4, behavioral analysis was carried out for both male and female F344 pairs of rats. As there are only two alternatives for defense, evasion and facing defense, they must sum to 100% (see Figure 1.1). As it has previously been reported that F344 rats are more likely to evade (Siviy et al., 1997. 2003), for comparative purposes, I contrasted the probability of evasion between the two strains and sexes. Similarly, of the facing defense tactics, complete rotation is reported to be less frequently used by F344 rats (Siviy et al., 1997. 2003); therefore, this was the tactic of facing defense compared between strains. Finally, prolonged wrestling can arise because rats adopt a pin configuration, which in LE rats most often arises from adopting the complete rotation tactic, but in other strains can frequently arise from being pushed over (S. Himmler, Himmler, Stryjek et al., 2016), and lead to role reversals resulting from counterattacks (S. Himmler, Himmler, Pellis& Pellis, 2016). Again, because of the reduced reciprocity, and so less prolonged wrestling reported in F344 rats (Schneider, Bindila et al., 2016; Schneider, Pätz et al., 2016; Siviy et al., 1997. 2003), the likelihood of a pin or a counterattack occurring in a bout of play fighting was compared between the two strains.

B.1.5 Statistical analysis

The number of nape attacks per pair per 10-minute trial, the likelihood of defense, use of different defensive tactics, and the likelihood that facing defenses led to counterattacks and pins were tested with a two-way ANOVA, with strain and sex as the factors. Differences were considered significant for *p* values of ≤ 0.05. The data are represented as the means and standard error of the mean.

B.2 Results

Overall, there was a significant main effect of strain on the number of nape attacks made ($F(1,28) = 39.4$, $p < 0.0001$), with F344 rats making more attacks than LE rats (Figure B.1a), and similarly, there was a significant main effect of strain on the probability of a nape attacks being defended (F(1,28) = 15.3, p = 0.0005), with F344 rats being more defensive (Figure B.1b). Of the number of nape attacks actively defended, there was a significant main effect of strain on the probability of using evasions (F(1,28) = 284, p < 0.0001), with F344 rats being more likely to use this tactic (Figure B.1c). When the rats defended with a facing defense, there was a significant main effect of strain on the probability of using a complete rotation (F(1,28) = 107.3, p < 0.0001), with LE rats being more likely to use this tactic (Figure B.1d).

For tactics and events leading to prolonged wrestling, there was a significant main effect of strain on the probability of facing defenses resulting in counterattacks (F(1,28) = 18.7, p = 0.0002), with F344 rats being more likely to launch counterattacks (Figure B.1e), and there was a significant main effect of strain on the probability of facing defenses resulting in pins (F(1,28) = 39.7, p < 0.0001), with LE rats being more likely to do so (Figure B.1f).

There was a significant main effect sex on the likelihood of defended attacks resulting in evasions (F(1, 28) = 6.1, p = 0.02), with males more likely to evade, and in the likelihood of facing defenses resulting in a complete rotations (F(1,28) = 6.1, p = 0.02), again with males more likely to do so. There were no significant interactions or other sex differences. Irrespective of the sex differences, inspection of the graphs (Figures B.1 c and d) clearly shows that the strain differences in these measures are of greater magnitude.

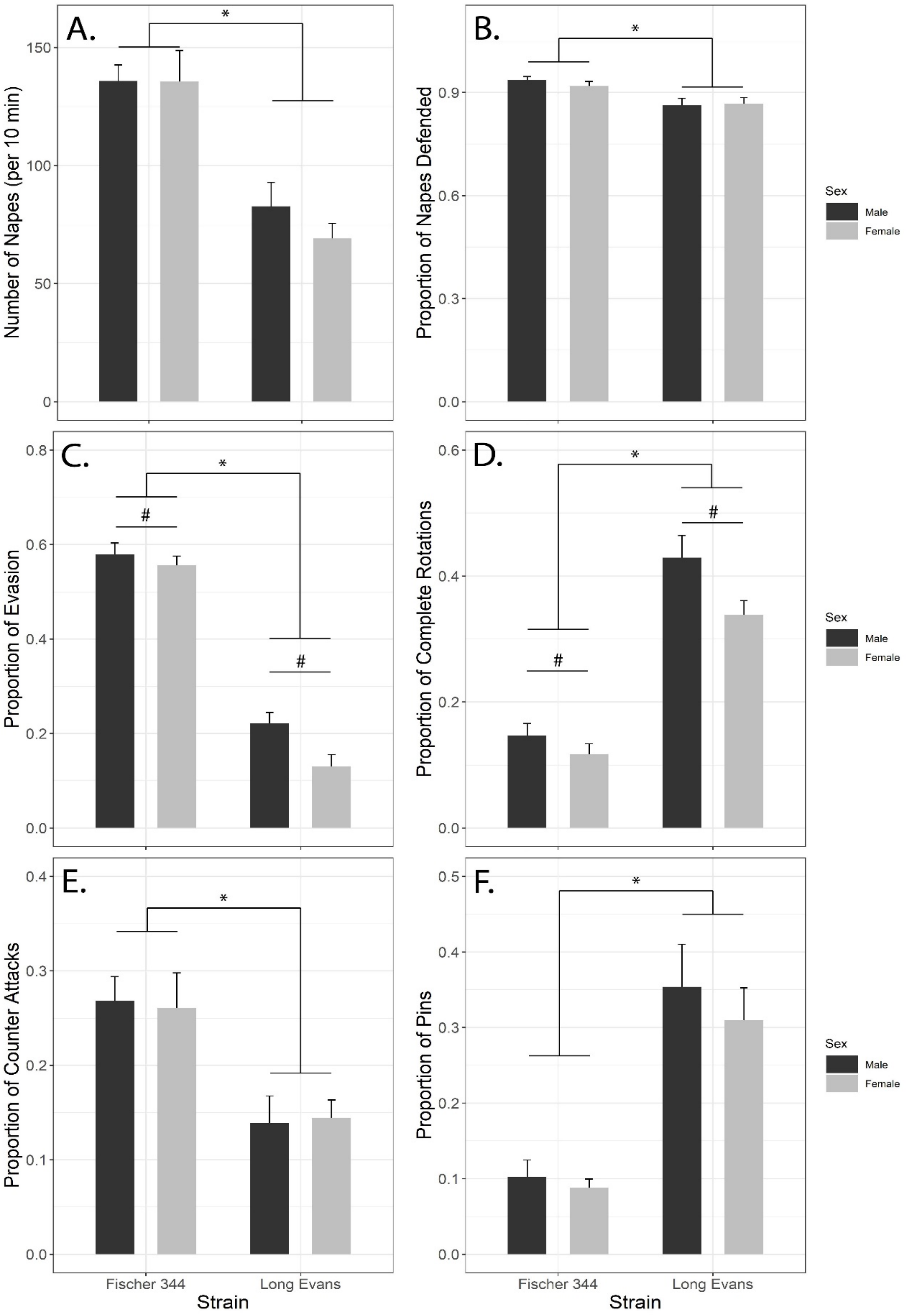

Figure B.1 *Strain and sex differences are shown for several measures of the microstructure of play fighting. (*) denotes significant strain differences, and (#) denotes significant sex differences. All data are represented as a mean ± standard error of the mean.*