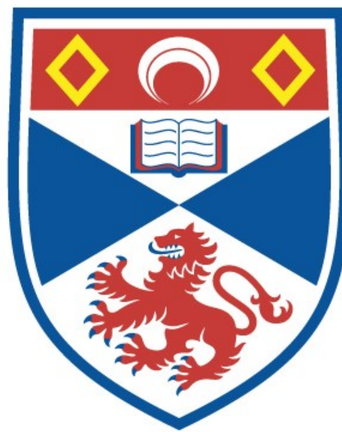
ADOLESCENT STRESS AND SOCIAL EXPERIENCES: DEVELOPMENTAL ANTECEDENTS OF ADULT BEHAVIOURAL RESPONSES TO UNFAMILIAR STIMULI AND THE UNDERLYING NEUROENDOCRINE MECHANISMS

Michael G. Emmerson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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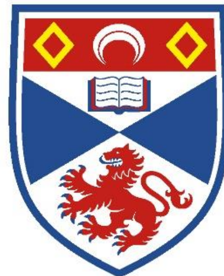
Adolescent stress and social experiences: developmental antecedents of adult behavioural responses to unfamiliar stimuli and the underlying neuroendocrine mechanisms

Michael G. Emmerson



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24th May, 2017

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Abstract

During adolescence, animals leave the natal home and interact with potentially threatening stimuli (i.e. stressors), e.g. unfamiliar environments and conspecifics. Adolescent stressors can result in fewer interactions with unfamiliar stimuli in adulthood, plausibly due to sustained effects of glucocorticoid exposure on stress physiology (e.g. glucocorticoid secretion and receptor expression). The current thesis tested the hypothesis that adolescent glucocorticoid exposure and social experiences act as stressors by quantifying the effects of the adolescent experiences on behavioural responses to unfamiliar stimuli and the underlying neuroendocrine mechanisms when in adulthood using two captive species, zebra finches and rats. In study one, adolescent zebra finches were dosed with the glucocorticoid corticosterone. In adulthood, birds dosed with corticosterone in early adolescence took longer to enter an unfamiliar environment when tested individually and had lower expression of the glucocorticoid receptor GR in the hippocampus and hypothalamus, brain regions that regulate stress responses. Glucocorticoids therefore appear to be an endocrine mechanism behind the long-term effects of adolescent stress. Subsequent studies explored whether higher social density and more unfamiliar social interactions during adolescence act as stressors. In study two, early adolescent zebra finches were housed in groups varying in conspecific number and density. In adulthood, females raised in larger groups secreted a higher stressor-induced corticosterone concentration and, if raised at lower density, spent more time in an unfamiliar environment when group housed. In study three, adolescent female rats were housed in familiar pairs or exposed to unfamiliar conspecifics. Unfamiliar adolescent interactions had no effects on responses to unfamiliar environments or stress physiology in adulthood, but heightened ultrasonic call rates. In this thesis, adolescent social experiences do not act like stressors, but modulate (especially female) social behaviour. Adolescent stressors and social experiences therefore have distinct effects on responses to unfamiliar stimuli and stress physiology that are maintained into adulthood.

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The behavioural responses to unfamiliar environments in adulthood and the corticosterone concentration secreted in response to capture and restraint during both adolescence and adulthood have been published in the journal *Hormones and Behavior*. The reference is provided below.

Emmerson, M.G., & Spencer, K.A. (2017). Long-term effects of adolescent stress on neophobic behaviors in zebra finches are modulated by social context when in adulthood. *Hormones and Behavior*, *90*, 48-55.

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The corticosterone concentration secreted in response to capture and restraint during adolescence and adulthood, along with the basal concentrations of adult male testosterone and female estradiol, are currently in press for publication in *General and Comparative Endocrinology*. The reference is provided below.

Emmerson, M.G., & Spencer, K.A. (In press). Group housing during adolescence has long-term effects on the adult stress response in female, but not male, zebra finches (*Taeniopygia guttata*). *General and Comparative Endocrinology*.

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Chapter 1

General Introduction

Adolescence is a stage of development which occurs across a range of taxa, during which individuals leave the natal home, begin interacting with unfamiliar conspecifics, and transition toward sexual maturity (Brown & Spencer, 2013; Nelson, Leibenluft, McClure, & Pine 2005; Schlegel & Barry, 1991; Spear, 2000). In humans, interacting with unfamiliar conspecifics allows adolescents to integrate into a social group that they may spend the rest of their life with (Blakemore & Mills, 2014; Choudhury, Blakemore, & Charman, 2006; Schlegel & Barry, 1991). Integrating into a peer group can provide benefits, with peers acting as 'social buffers' by providing social support in order to help cope with stress (Gunnar & Hostinar, 2015). However, adolescence is also the most common age for the onset of anxiety-related disorders (Kessler et al., 2005; McEvoy, Grove, & Slade, 2011), which may be caused by stress resulting from some adolescent social interactions, such as social instability and social rejection (Bakker, Ormel, Verhulst, & Oldehinkel, 2010; Sebastian, Viding, Williams, & Blakemore, 2015). Adolescence is therefore a stage of development in which individuals have the opportunity to establish a social network of peers that can be recruited in times of adversity, but adolescents are also vulnerable to the deleterious effects of stress and interpersonal relationships (Romeo, 2010; Romeo, 2015).

Adolescence is not limited to humans, with animals across a range of taxa leaving the natal home and interacting with unfamiliar conspecifics around the time of puberty, and these interactions can have long-term effects on adult social and stress-related phenotypes (Brown & Spencer, 2013; Hollis, Isgor, & Kabbaj, 2013; McCormick, Hodges, & Simone, 2015; Sachser, Hennessey, & Kaiser, 2011; Spear, 2000). Investigating adolescence in captive non-human animals can be advantageous, as the lab environment can provide the opportunity to make detailed observations of behavioural changes across adolescence. In addition, the

physiological mechanisms (e.g. neural receptor expression) that underpin any behavioural changes can be investigated in more detail than can be achieved in humans. The study of human adolescence also relies on the use of correlational designs that limit the extent to which the variation in adolescent experiences can be said to cause later-life variation in adult phenotypes. The use of lab animals permits better controlled designs that can infer causation. The current thesis aims to investigate the long-term consequences of adolescent stressors, social housing, and social interactions on adult stress-related and social phenotypes in non-human animals. Rodents and, to a lesser extent, birds have been studied during adolescence (Brown & Spencer, 2013) and the following introduction will therefore focus on outlining how these taxa contribute to an understanding of the short- and long-term effects of adolescent responses to stress, social housing, and social interactions. First, the events constituting adolescence will be described in more detail. Second, the responses of adolescents to stressors and social interaction will be outlined. Third, an explanation will be given for how adolescent stress and social interactions can have long-term effects on adult stress-related and social phenotypes. The structure of the remaining chapters of the thesis will then be outlined.

1.1. Adolescence

Adolescence is not a clearly demarcated stage of development, but a gradual transition toward living outside the natal home independent from parental influence within a species-typical social organisation as a sexually mature individual (Sachser, Hennessy, & Kaiser, 2011; Schlegel & Barry, 1991; Spear, 2000). This definition of adolescence will be elaborated in the following sections.

1.1.1. Parental independence

Nutritional independence facilitates dispersal from the natal home, as parental interactions become non-essential (Thiels, Alberts, & Cramer, 1990). Glucocorticoids (GCs), such as

corticosterone (CORT), are an endocrine mechanism facilitating dispersal across many taxa (Wada, 2008). Rats (*Rattus norvegicus*), for example, have a protracted stressor-induced secretion of CORT during days 28-30 (Klein & Romeo, 2013; Romeo, Patel, Pham, & So, 2016), i.e. prior to nutritional independence and dispersal around day 30-35 (Calhoun, 1963; Thiels et al., 1990). The protracted rise in GC may be attributable to maturation of the adrenal glands (i.e. adrenarche: Pignatelli, Xiao, Gouveia, Ferreira, & Vinson, 2006). The immediate effects of an acute secretion of GCs include enhancing learning capability, in particular of fear-evoking stimuli, and eliciting behaviours to avoid potential threats (Sapolsky, Romero, & Munck, 2000) which may be beneficial to adolescents entering unfamiliar environments and interacting with unfamiliar conspecifics (Wada, 2008).

1.1.2. Peer interactions

Social interactions with age-similar conspecifics supplant parental interactions during adolescence (Gunnar & Hostinar, 2015; Nelson et al., 2005; Nelson, Jarcho, & Guyer, 2016; Schlegel & Barry, 1991). Adolescents, compared to pre-adolescents, engage in more affiliative play with age-similar conspecifics across many taxa. Adolescent play is well documented in rodents, such as rats (Panksepp, Siviy, & Normansell, 1984; Vanderschuren, Niesink, & Ree, 1997; Varlinskaya & Spear, 2008), mice (*Mus musculus*; Terranova et al., 1993), and golden hamsters (*Mesocricetus auratus*; Taravosh-Lahn & Delville, 2004; Wommack, Taravosh-Lahn, David, & Delville, 2003). Rats are especially playful compared to other rodents (Pellis & Iwanuik, 2004). Adolescent birds also engage in playful interactions, such as play fighting in keas (*Nestor notabilis*) and play chasing in ravens (*Corvus corax*) (Diamond & Bond, 2003; Graham & Burghardt, 2010). However, some species (e.g. zebra finches, *Taeniopygia guttata*) are not observed engaging in adolescent play (Zann, 1996). Instead, zebra finches perch in closer proximity to unfamiliar conspecifics as adolescence progresses (Adkins-Regan & Leung, 2006), but more work is needed to determine if perching proximity is affiliative.

Affiliative behaviours are regulated by nonapeptides, such as oxytocin and vasopressin in mammals (OT and VP, respectively; Engelmann, Landgraf, & Wotjak, 2004; Neumann & Landgraf, 2012) and mesotocin and vasotocin in birds (MT and VT, respectively; Adkins-Regan, 2009). Affiliative social relationships between conspecifics can be broken down into multiple stages, as animals first perceive a conspecific as a stimulus to approach and then begin to engage in affiliative social interactions, such as huddling in small rodents (Lim & Young, 2006), and then need to remember the conspecific and interactions with the conspecific in order to maintain the affiliative relationship (Choleris, Clipperton-Allen, Phan, & Kavaliers, 2009). Nonapeptides, in particular OT/MT, regulate these aspects of affiliative social interactions, as inhibiting OT/MT functioning (e.g. blocking OT/MT receptors) results in animals that spend less time in proximity to one another and engage in fewer affiliative interactions (Lim & Young, 2006), and animals that are unable to discriminate between familiar and unfamiliar conspecifics indicating impaired social memory (Choleris et al., 2009). One example of this affiliation/nonapeptide interaction is pair bond formation in socially monogamous animals, such as prairie voles (*Microtus ochrogaster*) and zebra finches, in which two conspecifics establish and maintain a long-term socio-sexual relationship with one another; a relationship that can be formed or broken by the addition or inhibition of OT/MT, respectively (McGraw & Young, 2010; Prior & Soma, 2015).

Little is known about the function of nonapeptides during adolescence, but OT and VP expression in the rat hypothalamus increases with age during adolescent development (Miller, Ozimek, Milner, & Bloom, 1989; van Tol, van den Busse, de Jong, & Burbach, 1988) and administering a VP receptor antagonist can lower playful social interaction time in rats compared to administering a vehicle solution (Veenema, Bredewold, & De Vries, 2012; Veenema, Bredewold, & De Vries, 2013). Administering OT (which binds to MT receptors) in adult zebra finches results in birds that perch alongside larger groups (vs. smaller groups) for

a longer duration compared to birds dosed with a vehicle solution (Goodson, Schrock, Klatt, Kabelik, & Kingsbury, 2009), suggesting MT can modulate social approach and affiliative behaviour in birds. The role of MT in adolescent social behaviour, such as flocking, in zebra finches and other birds remains to be investigated.

GCs may also to play a role in adolescent affiliative interactions (Spencer, 2017). A positive correlations is present between an indirect measure of GC levels (faecal GC metabolites) and measures of affiliative behaviour (perching proximity, allopreening) in ravens (*Corvus corax*: Stöwe, Bugnyar, Schloegl, Heinrich, Kotrschal, & Möstl, 2008). A higher GC concentration (like higher OT/MT concentration) may cause more affiliative interactions, but because of the correlational design adolescent social interactions could also cause higher GC levels. In support of the former hypothesis, rats injected with GC during adolescence engage in a higher quantity of play compared than saline injected rats when the animals are each allowed to interact with an unfamiliar conspecific (Veenit, Cordero, Tzanoulinou, & Sandi, 2013). GCs therefore play a role in adolescent affiliative interactions, and data so far appear to show that higher GC concentration result in animals engaging in more affiliative interactions.

1.1.3. Sexual maturation

During adolescence, concentrations of gonadal hormones, such as testosterone and estradiol, become higher as age progresses (Delemarre-van de Waal, 2002; Sisk & Foster, 2004). Gonadal hormones facilitate the development of primary (e.g. gonads) and secondary (e.g. adult plumage in birds) sexual characteristics and elicit motivation for sexual behaviour during adolescence (Blakemore, Burnett, & Dall, 2010; Delemarre-van de Waal, 2002; Sisk & Foster, 2004). When gonadal hormone concentrations begin to rise varies between species. In adolescent rats (postnatal days 35-65), testosterone concentration rises between days 35-65 in males and estradiol concentration rises between days 32-50 in females (Pignatelli et al., 2006;

Vetter-O'Hagan & Spear, 2012). In adolescent zebra finches (postnatal days 30-100), testosterone concentration begins to rise during late adolescence in males (around day 70), but when estradiol concentration rises in female zebra finches is not known (Zann, 1996).

Adolescence ends when an individual is sexually mature, displaying adult-typical primary and secondary sexual characteristics (Brown & Spencer, 2013; McCutcheon & Marinelli, 2009; Schulz, Molenda-Figueira, & Sisk, 2009; Spear, 2000). For example, between days 30-100 zebra finches begin to develop adult plumage (black-barred chest, chestnut coloured flanks, orange beak and cheek patches) and learn to produce (males) or respond (females) to stereotyped songs used in adult mating (Zann, 1996). Sexual maturity is more than merely displaying adult typical morphology, and also reflects competent expression of socio-sexual behaviour within a species-typical social organisation. For example, male guinea pigs (*Cavia porcellus*) are sexually competent by day 90, but typically do not reproduce until after day 240 as males need to negotiate access to females by attaining a dominant status in a social hierarchy (Hennessey, Kaiser, & Sachser, 2009; Sachser, 1986). When adolescence actually ends is therefore, to some extent, dependent on social context.

1.2. Adolescent stress response

1.2.1. Stressors

Leaving the natal home exposes adolescents to a number of (potentially) life-threatening stimuli, including predation (Yoder, Marschall, & Swanson, 2004), group conflict (Calhoun, 1963), and unfamiliar environments and conspecifics (Spear, 2000). Any unpredictable, uncontrollable, and (potentially) life-threatening stimuli are termed stressors (Schulkin, 2003; Chrousos, 2009; McEwen & Wingfield, 2010; Koolhaas et al., 2011). Stressors can be immediately threatening, such as predation or conflict, but can also be potentially threatening, such as unfamiliar environments and conspecifics (Herman et al., 2003; Herman, Ostrander,

Mueller, & Figueiredo, 2005). Stressors can also be acute (e.g. capture by a predator; Romero & Reed, 2005) or chronic (e.g. separation from conspecifics; Remage-Healey, Adkins-Regan, & Romero, 2003). Evidence suggests that adolescents respond to stressors differently compared to pre-adolescent and adult animals in terms of both behaviour and physiology (Brown & Spencer, 2013; Estanislou & Morato, 2006; Klein & Romeo, 2013), and this will be outlined in more detail below. The adult response to stressors will first be outlined, then comparisons will be made between the adolescent and adult responses to stressors.

1.2.2 Adult responses to stressors

Encountering a stressor triggers the secretion of GCs, such as CORT in rats and passerine birds and cortisol in humans and guinea pigs, into the general circulation (Chrousos, 2009; McEwen, 1997; Romero & Butler, 2007; Sapolsky, Romero, & Munck, 2000). GCs are elevated above baseline within 2-3 minutes after a stressor is encountered, with CORT concentration typically peaking between 10-30 minutes after stressor onset, and returning to baseline approximately 90 minutes after stressor onset (Koolhaas, Meerlo, De Boer, Strubbe, & Bohus, 1997; Romero, 2004; Romero & Butler, 2007). An acute stressor-induced rise in circulating GC concentration raises blood glucose concentration and cardiovascular output, inhibits growth and reproduction, and alters affect, behaviour, and cognition in ways that facilitate the detection and termination of stressors (Chrousos & Gold, 1992; Chrousos, 2009; McEwen & Wingfield, 2003; Sapolsky et al., 2000). Although an acute stress response may be adaptive, a chronic stress response (i.e. prolonged GC secretion) may be maladaptive due to wear and tear of the physiological systems that regulate GC secretion (McEwen & Stellar, 1993; McEwen, 1998).

Behavioural responses to stressors in rats manifest as attempts to escape the stressor, with exposure to an unfamiliar environment triggering secretion of GCs that rise concomitantly

with locomotor activity, open space avoidance, rearing on the hind legs to assess risk by visually scanning the environment, and transient immobilisation (Haller, Halasz, Makara, & Kruk, 1998; Koolhaas, de Boer, Coppens, & Buwalda, 2010; Rodgers et al., 1999). Similarly, great tits (*Parus major*) selected for relatively low exploration of unfamiliar environments (vs. higher exploration) also secrete a higher concentration of CORT in response to a stressor (Baugh et al., 2012) or have higher faecal CORT metabolites (Stöwe, Rosivall, Drent, & Möstl, 2010). In contrast, a higher stressor-induced CORT concentration can result in some animals engaging in less avoidant behaviour compared to animals secreting less CORT. For example, adult zebra finches bred for higher stressor-induced CORT concentration are quicker to consume food from an unfamiliar feeder when placed in an unfamiliar environment compared to birds bred for relatively low CORT (Martins, Roberts, Giblin, Huxham, & Evans, 2007). However, being quicker to approach an unfamiliar feeder could plausibly still reflect greater stressor-avoidance in birds selected for relatively higher CORT concentration (e.g. more readily finding food could result in greater avoidance of under-nutrition and then more readily leaving and avoiding the potential threat of an unfamiliar environment). Social behaviour can also be affected during an acute stress response, with higher CORT concentration resulting in more antagonistic behaviour in adult male rats that may assist in coping with a social stressor, like a challenge by an unfamiliar conspecific over territory (Mikics, Kruk, & Haller, 2004). Stressors can also result in more affiliative behaviour (e.g. huddling in rats) that may result in animals seeking social support in order to cope with a stressor (Bowen et al., 2012), but whether an acute secretion of CORT is an endocrine mechanism resulting in more affiliative behaviour remains to be determined.

GC secretion in birds and mammals is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Boonstra, 2004; Romero & Butler, 2007; Tsigos & Chrousos, 2002). In response to a stressor, hypothalamic paraventricular (PVN) neurons secrete corticotrophin releasing

hormone (CRH) into the hypophyseal portal circulation connecting the hypothalamus and pituitary gland (Bale & Vale, 2004; Romero & Butler, 2007; Tsigos & Chrousos, 2002). CRH binds to receptors in corticotroph cells of the anterior lobe of the pituitary gland, with corticotrophs secreting adrenocorticotrophic hormone (ACTH) into the general circulation (Bale & Vale, 2004). CRH works in conjunction with the nonapeptide VP (in mammals) or VT (in birds) to further stimulate ACTH secretion (Aguilera & Rabadan-Diehl, 2000; Lightman, 2008; Scott & Dinan, 1998). ACTH binds to receptors in the adrenal cortex to initiate GC secretion into the general circulation (Elias & Clark, 2000) to initiate changes in behaviour (e.g. stressor avoidance) that have been identified above.

The HPA axis is regulated by brain structures, such as the amygdala, hippocampus, and medial prefrontal cortex (mPFC; Herman et al., 2003; Herman et al., 2005). GC secretion is raised by amygdala stimulation of the PVN, whereas GC secretion is lowered via the hippocampus stimulating inhibitory neurons of the peri-PVN regions of the hypothalamus and by the mPFC inhibiting the amygdala (Herman et al., 2005; McEwen & Gianaros, 2011; Smith & Vale, 2006). The avian hippocampus and amygdala can modulate the HPA axis in a similar fashion to mammals (Boonstra, 2004), but the role of an mPFC-like structure in avian stress regulation is not known. The cortex of mammalian brains are laminated, whereas the cortex of avian brains are nucleated; cross-taxa comparisons are therefore difficult (Dugas-Ford, Rowell, Ragsdale, 2012; Harris, 2015). However, the avian nidopallium caudolaterale has been indicated as a putative homologue of the mammalian PFC (Güntürkün, 2005; Rose & Colombo, 2005) and requires investigation as a possible site of avian HPA axis regulation.

Basal and stressor-induced CORT concentrations are regulated by receptors expressed in many tissues, such as the amygdala, hippocampus, and mPFC (Smith & Vale, 2006). Under basal conditions, the suprachiasmatic nucleus stimulates the PVN to maintain a basal GC concentration that fluctuates along a circadian rhythm; peaking around the time of waking and

then declining over time (Lightman et al., 2008; Sarabdjitsingh, Joels, & de Kloet, 2012). Mineralocorticoid receptors (MR), especially those in the hippocampus, regulate basal GC concentration (Joëls, Karst, DeRijk, & de Kloet, 2007; de Kloet et al., 2000). Stressor-induced CORT concentration is regulated by the glucocorticoid receptor (GR), with CORT binding to GR in the PVN, anterior pituitary gland, hippocampus, and mPFC to lower further secretion of CORT due to negative feedback effects on the HPA axis (Joëls et al., 2007; Kloet et al., 2000). However, CORT binding to GR in the amygdala can further stimulate the secretion of CORT due to positive feedback effects on the HPA axis (Herman, McKlveen, Solomon, Carvalho-Netto, & Myers, 2012; Shepard, Barron, & Myers, 2003). A stress response is therefore a trade-off between negative and positive feedback mechanisms regulating GC secretion.

1.2.3 Adolescent responses to stressors

Adolescent stress responses have been investigated in a small number of rodent species, most commonly rats. Compared to adult rats, adolescent rats have a prolonged secretion of CORT in response to a capture and restraint stressor (Klein & Romeo, 2013) and, when tested in unfamiliar environments, spend less time in open spaces (Arakawa, 2005; Estanislau & Morato, 2006; McCormick, Smith, & Mathews, 2008) and engage in more risk assessment (rearing) behaviour (Estanislau & Morato, 2005; Estanislau & Morato, 2006). A more prolonged secretion of CORT during adolescence compared to adults may therefore be responsible for more avoidant behavioural responses toward unfamiliar stimuli (i.e. neophobia) in adolescent rats compared to adult rats. However, when adolescents are exposed to an unfamiliar environment they have a lower stressor-induced CORT concentration compared to adults in a number of species, including rats (McCormick et al., 2008), mice (Adriani & Laviola, 2000), and guinea pigs (Hennessey, Hornschuh, Kaiser, & Sachser, 2006). Age-related differences in stressor-induced CORT secretion may therefore be dependent on stressor type and/or intensity,

but no study has yet compared CORT secretion in response to a number of different stressors across both adolescence and adulthood that would be necessary to test the hypothesis.

Despite investigation, no adolescent age-related changes in GR or MR expression have been found in the HPA axis or HPA axis regulators; no differences are found when comparing adolescents to adults on GR expression in PVN of rats (Dziedzic, Ho, Adabi, Foilb, & Romeo, 2014; Goel & Bale, 2007), GR expression in mPFC of rats (Dziedzic et al., 2014), and both GR and MR expression in the hippocampus of rats and mice (Dziedzic et al., 2014; Green, Nottrodt, Simone, & McCormick, 2016; Pryce, 2008). The absence of age-related differences in GR expression also appears across taxa, with the number and density of GR-immunoreactive neurons being no different between pre-adolescent and adult male zebra finches (Shahbazi, Schmidt, & Carruth, 2011). Whether neural GR expression in adolescent zebra finches differs from pre- and post-adolescent neural GR expression remains to be tested. Furthermore, age-related changes in GR and MR still need to be determined in all brain regions regulating stress responses (e.g. amygdala). However, current evidence suggests central receptor mechanisms are not responsible for age-related changes in stress responses. Peripheral mechanisms have recently been implicated, as pre-pubertal rats have a higher expression of the ACTH receptor in the adrenal gland compared to adult rats (Romeo et al., 2014). Whether adrenal sensitivity to stress is higher during adolescence compared to adults is not known. Furthermore, whether nonapeptide regulation of HPA axis functioning (e.g. VP/VT co-stimulation of ACTH in conjunction with CRH) is different between adolescents and adults remains to be investigated.

1.3. Adolescent social interactions

During adolescence, adolescents begin to interact more with unfamiliar conspecifics compared to when the animals were pre-adolescent (Blakemore & Mills, 2014; Nelson et al., 2005; Nelson et al., 2016; Sachser et al., 2011). Adolescent peer interactions facilitate learning of

species-typical social behaviour (Blakemore, 2008; Sachser et al., 2011), such as living within a social dominance hierarchy in rats (Pellis & Pellis, 2007; Pellis, Pellis, & Bell, 2010) and guinea pigs (Sachser, Lick, & Stanzel, 1993; Sachser, Kaiser, & Hennessy, 2013). In passerine songbirds, peer interactions can facilitate learning of nest building behaviour (Breen, Guillette, & Healey, 2016) and how to produce or respond to species-typical song in a social context (Beecher & Burt, 2004; King, West, & White, 2003; Mann & Slater, 1995). The following sections will outline the importance of adolescent peer interactions for two social behaviours, the ability to enter and maintain a position in a social group (i.e. social integration) and the ability to utilise a social group to lower responses to stressors (i.e. social buffering).

1.3.1. Social integration

Social integration refers to the ability to enter an unfamiliar group of conspecifics and then establish a position within the unfamiliar social network (Blakemore, 2008; Sachser et al., 2011). In birds, adolescent social interactions are important for learning how to integrate into, and then live in, a species-typical flock social structure (Ruploh, Bischof, & von Engelhardt, 2014; Templeton, Campbell, & Beecher, 2012; White, 2010). For example, learning to engage in affiliate interactions (e.g. clumping) that may result in more central positions in a flock social network (e.g. Ruploh et al., 2014). Social integration into a flock, as well as other social groups, is dependent on communicative competence, especially regarding mating interactions (Taborsky & Oliveira, 2012). Wild zebra finches live in groups of 150-350 birds (Griffiths & Buchanan, 2010; Zann, 1996) and during adolescence males learn to produce a stereotyped song from their father, whilst females learn to respond to a song (Holveck & Riebel, 2014; Nowicki, Peters, & Podos, 1998; Zann, 1996). Song is necessary to integrate into a group, as males emit song in order to attract a female and therefore pair bond with a group member (Hauber, Campbell, & Woolley, 2010; Zann, 1996). However, males who are already pair bonded may challenge unfamiliar males attempting to enter a social group due to potential mate

competition; learning when not to sing is therefore also important (Dunn & Zann, 1997; Ruploh, Bischof, & von Engelhardt, 2013; Ruploh et al., 2014). Adolescent social interactions are therefore important for learning how to integrate into a social group, but also for learning how to avoid group conflict that might threaten integration.

Social separation can affect stress physiology, with higher stressor-induced CORT concentration detected in single housed animals compared to pair tested animals in a number of species (Armario, Luna, & Balasch, 1983; Hennessy, 1997; Remage-Healey et al., 2003). CORT elicits behavioural patterns that can terminate stressors (Sapolsky et al., 2000), such as re-establishing social contact (Hawkley, Cole, Capitanio, Norman, & Cacioppo, 2012). Adult male rats attempt to solicit social contact during separation by emitting ultrasonic calls (Wöhr, Houx, Schwarting, & Spruijt, 2008). Rats typically emit 22 kHz calls in response to aversive stimuli, such as pain, and 50 kHz in response to appetitive stimuli, such as social interaction and mating; 50 kHz call rate is are also lowered in response to aversive stimuli (Brudzynski, 2009; Burgdorf et al., 2008; Portfors, 2007). 50 kHz calls are emitted in the frequency range of 30-80 kHz and can be classified based on structure as either frequency-modulated or constant (Brudzynski, 2009; Burgdorf et al., 2008; Portfors, 2007). Wöhr et al. (2008) report that during separation male rats emit both frequency-modulated and constant 50 kHz calls. 50 kHz calls in rats may therefore be emitted in response to social interaction in order to re-establish social contact and avoid separation. During adolescence, 50 kHz calls are emitted prior to and during play (Himmler Kisko, Euston, Kolb, & Pellus, 2014; Knutson, Burgdorf, & Panksepp, 1998) and absence of social contact during adolescence can lower overall 50 kHz call rate in adult male rats (Inagaki, Kuwahara, Tsubone, & Mori, 2013; Seffer, Rippberger, Schwarting, & Wöhr, 2015). Adolescence may therefore be a stage of development in which rats undergo vocal learning and develop communicative competencies, similarly to that seen in birds. Whether emitting 50 kHz calls in order to solicit social contact during separation, are

also affected by adolescent social conditions other than single housing remains to be investigated.

1.3.2. Social buffering

Integration into a social group can be advantageous when coping with stress, as behavioural and physiological responses to stressors are lower (‘buffered’) when familiar conspecifics are present compared to when alone in mammalian species (Beery & Kaufer, 2015; DeVries, Glasper, & Detillion, 2003; Gunnar & Hostinar, 2015; Hennessey et al., 2009; Kikusui, Winslow, & Mori, 2006; Sanchez, McCormack, & Howell, 2015). For example, adult rats exposed to an unfamiliar environment in familiar pairs, compared to individually tested, exhibit fewer immobilisations (Latané, 1969; Taylor, 1981; Kiyokawa, Hiroshima, Takeuchi, & Mori, 2014) and lower post-test CORT concentration (Armario et al., 1983). Birds may also be capable of social buffering, but the behavioural tasks used in the available data is open to other interpretations. Birds housed in familiar groups are quicker to contact a food-baited novel object compared to individually housed birds in zebra finches (Coleman & Mellgren, 1994) and budgerigars (*Melopsittacus undulatus*; Soma & Hasegawa, 2004). Being quicker to contact a food-baited novel object could be due to social buffering (i.e. lower neophobia in a group vs. individual context), but could also be due to social competition (e.g. competing over limited resources like food; Clayton, 1978; Webster & Ward, 2011). The current evidence for social buffering in birds therefore only reflects social facilitation, i.e. improved task performance in a group vs. individual context (Clayton, 1978; Nicol, 1995; Webster & Ward, 2011; Zajonc, 1965). Further measures of social behaviour during the tasks (e.g. affiliation during buffering, antagonism in competition) could to be recorded to aid in interpretation of any context-dependent effects on behavioural responses to unfamiliar stimuli.

Peer-directed social buffering emerges during adolescence as animals begin to spend more time interacting with unfamiliar conspecifics (Gunnar & Hostinar, 2015; Hennessey et al., 2009; Hostinar & Gunnar, 2013; Siviy, 2010). Most research on adolescent social buffering, however, has been conducted on rodents and other taxa (e.g. birds) have not been investigated. Pairs of adolescent rats have lower CORT concentration after exposure to an unfamiliar environment compared to same-aged rats tested alone (Terranova, Cirulli, & Laviola, 1999). In adult rats, physical contact (huddling) occurs after stressor exposure (Bowen et al., 2012) and physical contact with conspecifics is necessary for social buffering to occur (Nakayasu & Kato, 2008). Whether physical contact influences adolescent social buffering remains to be determined, but play has been suggested as a behavioural mechanism of adolescent social buffering (Siviy, 2010). However, no differences have been detected in post-interaction CORT concentration between familiar and unfamiliar pairs, despite unfamiliar pairs engaging in more play than familiar pairs (Cirulli, Terranova, & Laviola, 1996). In Cirulli et al. (1996), CORT concentration was only determined at one time point. Group differences may have emerged during a specific time in the physiological stress response, but went unmeasured. For example, in guinea pigs, unfamiliar females emerge as social buffers for adolescents by lowering CORT secretion in response to an unfamiliar environment compared to when single housed (Graves & Hennessey, 2000; Hennessey, Maken, & Graves, 2000); an effect that emerges at 30 minutes (but not 10 minutes) into the task (Graves & Hennessy., 2000). Peer-directed social buffering can therefore emerge during adolescence, but the behavioural mechanisms (e.g. play) have not yet been determined.

1.3.3. Social behaviour network

Social behaviour are regulated by hormones binding to receptors throughout the social behaviour network (SBN; Goodson, 2005; Newman, 1999). The SBN comprises six inter-connected regions of the brain: medial amygdala, lateral septum, preoptic area, anterior

hypothalamus, ventromedial hypothalamus, and midbrain (Goodson, 2005; Newman, 1999). Gonadal hormones were originally proposed as the endocrine mechanisms of social behaviour due to their effects on the SBN (Newman, 1999). Gonadal hormones, such as androgens (e.g. testosterone) and estrogens (e.g. estradiol), are primarily secreted from the gonads (Blakemore et al., 2010; Delemarre-van de Waal, 2002; Sisk & Foster, 2004). Estrogens act on estrogen receptors (ERα, ERβ) in order to stimulate the expression of female sexual behaviour (Wallen, 1990; Rissman, Early, Taylor, Korach, & Lubahn, 1997) and affiliative behavioural responses, such as maternal care (Champagne, Weaver, Diorio, Sharma, & Meaney, 2003). Androgens act on the androgen receptor (AR) to stimulate the expression of male sexual behaviour in response to mating opportunities (Rubinow & Schmidt, 1996; Wilson, 2001) and antagonistic behaviour in response to social status challenge (Eisenegger, Haushofer, & Fehr, 2011; Gleason, Fuxjager, Oyegbile, & Marler, 2009; Oliveira & Oliveira, 2014). Estradiol also stimulates the expression of male sexual behaviour due to the conversion of testosterone into estradiol by the enzyme aromatase (McCarthy, 2010).

Nonapeptides also facilitate the expression of affiliative and antagonistic behaviour (Goodson, 2008). Nonapeptides are synthesised by the hypothalamus (paraventricular and supraoptic nuclei) and secreted into the extracellular fluid surrounding the hypothalamus and, by the posterior pituitary gland, into the general circulation (Engelmann et al., 2004; Neumann, 2008; Neumann & Landgraf, 2012). The behavioural effects of nonapeptides are mediated by neural receptors, with one receptor for OT/MT (OT receptor in mammals; VT3 receptor in birds) and three receptors for VP/VT (V1a, V1b, V2 receptors in mammals; VT1, VT2, VT4 receptors in birds) having been identified (Baeyens & Cornett, 2006; Goodson, Kelly, & Kingsbury, 20012; Leung et al., 2011). Adults dosed with OT/MT, vs. saline, appear more affiliative, noted by OT-dosed rats spending a longer duration of time in contact with one another (Witt, Winslow, & Insel, 1992) and MT-dosed zebra finches perching for a longer

duration of time in proximity to larger (vs. smaller) groups (Goodson et al., 2009) compared to vehicle dosed animals. VP/VT are implicated in the expression of antagonistic behaviour; VP/VT mRNA expression in the hypothalamic supraoptic nucleus is positively correlated with antagonistic behaviour, whilst VP/VT binding in the medial bed nucleus of the stria terminalis can inhibit antagonistic behaviour (Kelly & Goodson, 2014). The supraoptic nucleus itself has not been shown to cause antagonistic behaviour, so VP/VT mRNA expression may become higher in response to antagonistic interactions (Kelly & Goodson, 2014). The bed nucleus of the stria terminalis stimulates the HPA axis and can result induce fearful behaviour (Lebow & Chen, 2016; Walker, Toufexis, & Davis, 2003), with higher VP/VT binding likely inhibiting antagonistic interactions due to a more fearful response to conspecifics (Kelly & Goodson, 2013; Kelly & Goodson, 2014).

1.3.4. Glucocorticoid interactions with gonadal hormones and nonapeptides

Stressors can affect circulating concentration of gonadal hormones and nonapeptides, with exposure to an acute stressor in mammals stimulating testosterone and estradiol secretion (Ortiz, Armario, & Castellanos, 1984; Romeo, Lee, Chuua, McPherson, & McEwen, 2004; Yilmaz, 2003) as well as OT and VP secretion (Jezova, Skultetyova, Tokarev, Bakos, & Vigas, 1995; Uvnäs-Moberg, Handlin, & Petersson, 2015). GC receptors are expressed through almost all cell types within an animal, including brain regions implicated in the expression of social behaviour like the SBN (Joëls et al., 2007; Morimoto, Morita, Ozawa, Yokoyama, & Kawata, 1996; Sarabdjitsingh et al., 2012). Stressors may therefore modulate circulating concentration of gonadal hormones and nonapeptides (and therefore social behaviour) due to the actions of GCs, but the hypothesis requires testing. The following paragraphs will outline what is known about the adolescent emergence of glucocorticoid interaction with gonadal hormones and nonapeptides.

Androgen and estrogen concentrations become higher with progressing age during adolescence, with males typically having higher androgen concentrations and females having a higher estrogen concentrations by adulthood (Blakemore et al., 2010; Delemarre-van de Waal, 2002; Sisk & Foster, 2004). During adolescence, gonadal hormones also begin to regulate the secretion of CORT (McCormick & Mathews, 2007; Green & McCormick, 2016). Stressor-induced CORT is not affected by castration in pre-pubertal males (Romeo et al., 2004; Foilb, Lui, & Romeo, 2011) or ovariectomy in pre-pubertal females (Romeo, Lee, & McEwen, 2004). However, adult stressor-induced CORT concentration is higher in castrated vs. intact males, and lower in ovariectomised, vs. intact, females (Handa & Weiser, 2014; McCormick & Mathews, 2007). Androgens therefore appear to inhibit stressor-induced CORT secretion, an effect that emerges during adolescence in male rats (Gomez, Manalo, & Dallman, 2004) and male guinea pigs (Hennessey et al., 2002). Whereas estrogens appear to stimulate stressor-induced CORT responses (McCormick & Mathews, 2007), but whether the effect emerges during adolescence remains to be determined. Social interactions stimulate secretion of androgens, such as testosterone (Lürzel, Kaiser, & Sachser, 2011). Androgens can in turn inhibit the HPA axis (Gomez et al., 2004; Hennessey et al., 2002), so androgens may be one mechanisms by which social buffering occurs (Lürzel et al., 2011). The interaction between the HPA axis and gonadal hormones is bidirectional, with chronic exposure to GCs resulting in an inhibition of gonadal hormone secretion (Toufexis, Rivarola, Lara, & Viau, 2014). Androgens may therefore be able to buffer acute stress responses, but chronic stress may inhibit androgen secretion and therefore lower social buffering responses.

During adolescent development OT and VP expression in the rat hypothalamus become higher with progressing age (Miller et al., 1989; van Tol et al., 1988), but little is known about the behavioural effects that emerge from rising nonapeptide concentrations during adolescence (e.g. Veenema et al., 2012). Whether nonapeptides are involved in the adolescent emergence

of social integration, social buffering, or HPA axis function have not been investigated. However, studies using adult animals clearly show OT/MT are implicated in these measures (DeVries et al., 2003; Hennessey et al., 2009). Higher OT/MT concentration results in behavioural responses that might be expected to improve social integration (e.g. more social contact: Witt et al., 1992; preference for larger flocks; Goodson et al., 2009). OT may also have social buffering effects (DeVries et al., 2003; Hennessey et al., 2009; Neumann, Krömer, Toschi, & Ebner, 2000). Social interactions also can cause a rise in OT concentration (Uvnäs-Moberg, 1998; Uvnäs-Moberg et al., 2015). OT, vs. vehicle, dosed rats also spend more time in open areas of unfamiliar environments that may be indicative of lower neophobia (Bale, Davis, Auger, Dorsa, McCarthy, 2001; Blume et al., 2008; Windle, Shanks, Lightman, & Ingram, 1997) and have lower stressor-induced CORT concentration (Legros, 2001; Windle, Shanks, Lightman, & Ingram, 1997). OT may therefore have social buffering effects due to inhibitory effects on the HPA axis (DeVries et al., 2003; Hennessey et al., 2009; Neumann, Krömer, Toschi, & Ebner, 2000). Sex differences are present in neural expression of OT, with female rodents having more OT-immunoreactive neurons than males in regions such as the PVN (Dumais & Veenema, 2016). OT dosing can also sometimes only affect female not male affiliative behaviour (e.g. gaze duration in dogs, *Canis familiaris*: Romero, Nagasawa, Mogi, Hasegawa, & Kikusui, 2014). Male and female animals therefore need to be included in further investigations of OT on social behaviour and HPA axis regulation. Whether MT in birds functions similarly to OT in mammals has only been indirectly investigated (e.g. Goodson et al., 2009), so further work in avian species is required.

As previously mentioned, VP/VT (by binding to V1b or VT2 receptors in mammals or birds, respectively, in the anterior pituitary gland) act in conjunction with CRH to further stimulate ACTH secretion and therefore further stimulate CORT secretion during a stress response (Aguilera & Rabadan-Diehl, 2000; Cornett, Kang, & Kuenzel, 2013). Unlike OT/MT,

VP/VT may therefore counter social buffering effects by stimulating the HPA axis. VP/VT may also impair social integration, as in most instances higher VP/VT concentration results in more antagonistic behaviour between conspecifics (Goodson & Thompson, 2010). However, VP/VT effects on social behaviour are far from clear and likely dependent on context (Albers, 2015; Caldwell, Lee, Macbeth, & Young, 2008). For example, male zebra finches dosed with a VT antagonist engage in fewer antagonistic interactions with unfamiliar male conspecifics when initially placed in a novel mixed-sex colony compared to males dosed with a vehicle solution (Kabelik, Klatt, Kingsbury, & Goodson, 2009). However, after being housed with the colony for one day, the males dosed with a VT antagonist engaged in more antagonistic interactions with unfamiliar male conspecifics compared to vehicle dosed males (Kabelik et al., 2009). VT therefore appears to regulate antagonistic behaviour, but whether VT results in more or less antagonistic behaviour depends on contextual factors like conspecific familiarity. In some instances VP/VT manipulations only modulate antagonistic behaviour in male animals and have no effect on females (e.g. Albers, 2015; de Vries & Panzica, 2006). Sex differences are present in neural expression of VP/VT, with males having higher neural expression of VP/VT mRNA in regions such as the lateral septum and bed nucleus of the stria terminalis compared to females (Albers, 2015; Caldwell et al., 2015; Goodson & Thompson, 2010). Any investigation of VP/VT therefore needs to include both male and female animals to elucidate any sex differences.

1.4. Adolescent stress: long-term effects

1.4.1. Developmental stress

The environmental conditions an individual experiences during development can have long-term effects on later-life phenotypes (Knudsen, 2004; Kuzawa, 2005; Wells, 2007). One hypothesis, the predictive-adaptive response, proposes that developmental conditions forecast

conditions that an individual will experience when in adulthood, and thereby modulate an individual's phenotype in order to better function in the anticipated future environment (Bateson, Gluckman, & Hanson, 2014; Fawcett & Frankenhuis, 2015; Fusco & Minelli, 2010; Groothius & Taborsky, 2015; Nettle, Frankenhuis, & Rickard, 2013; Sheriff & Love, 2013). For example, developmental exposure to stressors may indicate that the adult environment will be adverse (e.g. high predation risk or scare food availability) and thereby induce a more stressor-avoidant adult phenotype (e.g. more neophobic behavioural responses and greater stressor-induced CORT secretion) compared to animals that were exposed to fewer stressors during development (Bateson et al., 2014; Groothius & Taborsky, 2015; Nettle et al., 2013). Under adverse conditions, a more stressor-avoidant phenotype may result in improved survival compared to animals with a less avoidant phenotype, as the developmentally stressed animals may be better able to avoid life-threatening risks, such as predation (Ferrari, McCormick, Meekan, & Chivers, 2015; Ferrari, McCormick, Allan, et al., 2015).

Long-term effects of developmental stress exposure do not have to be (potentially) beneficial to an animal, as is assumed under the predictive-adaptive response hypothesis (e.g. Fawcett & Frankenhuis, 2015; Nettle et al., 2013). Chronic exposure to stress contributes to 'wear and tear' of physiological systems involved in regulating a stress response (McEwen & Stellar, 1993; McEwen, 1998). For example, chronic exposure to stress in rats results in lower hippocampal neurogenesis and lower hippocampal cell survival that lead to impaired hippocampal function (e.g. impaired spatial learning and memory) compared to controls that experienced no such stress (McEwen, Eiland, Hunter, & Miller, 2012; McEwen, Nasca, & Gray, 2016). In male songbirds, chronic stress can impair neural social nuclei and the social leaning of song, that may in turn impair reproductive success as song is used during courtship (Nowicki et al., 1998; Spencer & MacDougall-Shackleton, 2011). Chronic stress can also lower survival prospects perhaps due to higher disease susceptibility, as has been reported in lab rats

(Cavigelli & McClintock, 2003; Cavigelli, Ragan, Michael, Kovacsics, & Bruscke, 2009) and lab housed zebra finches (Monaghan, Heidinger, D'Alba, Evans, & Spencer, 2012). The effects of chronic stress during development can persist into adulthood (e.g. Isgor, Kabbaj, Akil, & Watson, 2004) and can therefore have clearly deleterious effects on animal fitness and welfare.

Hormones have organisational effects on the developing brain, i.e. re-structuring of the brain resulting in sustained changes in hormone secretion and behaviour patterns in later-life (Blakemore et al., 2010; Brown & Spencer, 2013; Kawata, 1995; Lupien, McEwen, Gunnar, & Heim, 2009; McCarthy, 2010; Romeo, 2003). Hormones can therefore act as a mechanism whereby developmental experiences can induce changes in later-life phenotypes (Dufty, Colbert, & Møller, 2002; Monaghan, 2008). One endocrine mechanism that may underlie the long-term effects of developmental stress are GCs (Khulan & Drake, 2012; Schoech, Rensel, & Wilcoxen, 2012; Seckl, 2004; Spencer, Evans, & Monaghan, 2009; Welberg & Seckl, 2001). For example, exposing foetal Japanese quail (*Coturnix coturnix*) to CORT by injecting CORT (vs. peanut oil) into fertile eggs results in adult birds with a higher expression of GR in the pituitary gland and hypothalamus (Zimmer & Spencer, 2014), lower restraint stressor-induced CORT secretion (Hayward, Richardson, Grogan, & Wingfield, 2006; Zimmer, Boogert, & Spencer, 2013; Zimmer, Larriva, Boogert, & Spencer, 2017), and lower latency to enter an unfamiliar environment when tested individually (Zimmer et al., 2013; Zimmer et al., 2017). Whether the long-term effects of foetal CORT dosing reflect a predictive-adaptive response (e.g. more exploratory CORT dosed birds perhaps better at locating food and avoiding under-nutrition in novel territory than oil dosed birds; Zimmer et al., 2013) or wear and tear (e.g. lower stressor-induced CORT secretion in CORT dosed birds may be an ineffective response to (potentially) threatening novelty compared to oil dosed birds) remains to be determined.

During prenatal development, a foetuses experience of the environment are indirect due to mediation by the mother (e.g. placental transfer of hormones in mammals and depositing

hormones into eggs in birds) whereas during postnatal development animals experience their own environment directly (Groothius & Taborsky, 2015; Macri & Würbel, 2006; Uller, 2008). GCs can be orally administered to passerine birds during postnatal development to mimic stressor exposure and explore GCs as an endocrine mechanism of the long-term effects of developmental stress (Pakkala, Norris, Sedinger, & Newman, 2016; Schmidt, MacDougall-Shackelton, Soma & MacDougall-Shackelton, 2014; Spencer, Buchanan, Goldsmith, & Catchpole, 2003). Zebra finches dosed with CORT, vs. vehicle, during nestling and fledgling development are quicker to approach an unfamiliar object in adolescence if male (Spencer & Verhulst, 2007) and in adulthood (Crino, Driscoll, Ton, & Breuner, 2014), and occupy more central foraging network positions in adolescence (Boogert, Farine, & Spencer, 2014). Birds dosed with CORT, vs. vehicle, during nestling and fledgling stages of development also have higher restraint stressor-induced CORT concentration in later-life (song sparrows, *Melospiza melodia*: Schmidt et al., 2014; zebra finches: Spencer et al., 2009). Whether a higher secretion of CORT and more readily contacting an unfamiliar object in CORT (vs. vehicle) dosed birds reflects more stressor-avoidance (e.g. quicker to locate food and potentially avoid under-nutrition; Martins et al., 2007), better stressor-coping (e.g. quicker to learn that the unfamiliar stimuli are non-threatening), or an impaired response to a stressor (i.e. under-estimation of potential threat from unfamiliarity) remains to be determined. Furthermore, whether the long-term effects of nestling and fledgling CORT dosing are due to developmental CORT exposure having organisational effects on the HPA axis remains to be investigated.

1.4.2 Adolescent stress

Exposure to stressors appears to have less pronounced effects on later-life phenotypes the later in postnatal development that the stressor occurs (e.g. Tsoory & Richter-Levin, 2006), but adolescence also appears to be a stage of development in which stressors can have long-term effects on adult phenotypes (Hollis, Isgor, & Kabbaj, 2013). Adolescent rats exposed to

chronic variable stressors, compared to rats that experienced no such stress, develop into adults that spend less time exploring open spaces in unfamiliar environments when individually tested (Eiland & Romeo, 2013; Hollis et al., 2013; Romeo, 2010) and less time interacting with an unfamiliar conspecific in a dyadic interaction (Marquez et al., 2013; Tzanoulinou et al., 2014); potentially neophobic effects that can be attributed to higher (restraint) stressor-induced CORT concentration (Isgor et al., 2004; Pohl, Olmstead, Wynne-Edwards, Harkness, & Menard, 2007) that in turn is attributable to down-regulation of GR expression in HPA axis inhibitors, such as the hippocampus (Isgor et al., 2004). Whether adolescent stress affects behavioural responses to unfamiliar environment in a group context and/or behavioural responses to unfamiliar conspecifics in a more ecologically realistic group context remains to be tested.

The strength of the organisational effects of steroid hormones reduces as an individual progresses through post-natal development, but adolescence has also been shown to be a stage of development in which steroid hormones can have-long term effects on later-life phenotypes (Schulz et al., 2009; Schulz & Sisk, 2016). CORT may therefore be the endocrine mechanism behind the long-term effects of adolescent stress, but empirical investigations of this hypothesis are limited. In one study, male rats injected with CORT during adolescence (7 doses between days 28-42) developed into adults that spent less time interacting with an unfamiliar same-sex rat, but no differences between CORT and saline injected rats were found on adult behavioural responses to an unfamiliar environment when in adulthood (Veenit et al., 2013). CORT may therefore be a mechanism behind the long-term effects of adolescent stress on social behaviour, but not behavioural responses to unfamiliar non-social stimuli, like novel environments. The responses to an unfamiliar environment in Veenit et al., (2013) are clearly counter to multiple studies showing adolescent stress typically results in rats spending less time in exposed areas in unfamiliar environments compared to controls (Hollis et al., 2013). However, Veenit et al. (2013) is only one study that used one unfamiliar environment and unfamiliar conspecific task

in order to reach these conclusions. Research is therefore needed to corroborate the unexpected findings and rule out limitations in the design of Veenit et al. (2013). For example, further research could use a dose that is physiologically relevant to the animals, remove injection stress from the dosing protocol that may mask any effects of CORT, and quantify behaviour across multiple tasks. Furthermore, the effects of adolescent CORT dosing on later-life social behaviour require elucidation. Spending less time with an unfamiliar conspecific can occur for several reasons (e.g. more neophobic, more antagonistic), so more detailed analyses of the long-term effects of adolescent stress and CORT dosing on social behaviour are needed to determine if the protocols result in less social interaction time with an unfamiliar conspecific, compared to controls, for the same reasons.

The long-term behavioural effects of developmental CORT dosing appear to differ depending on whether doses are administered prior to or during adolescence. For example, nestling/fledgling zebra finches dosed with CORT develop into interacting with more conspecifics than vehicle dosed birds in later-life (Boogert et al., 2014) whereas adolescent rats dosed with CORT spend less time interacting with conspecifics in later-life compared to vehicle dosed rats (Veenit et al., 2013). The effects on developmental CORT dosing on social behaviour could be due to species-typical social organisation, but little consideration has been given to the hypothesis. Zebra finches live in large flocks (150-350 birds) and spent a large proportion of time engaging in affiliative interactions with a pair-bonded partner (Griffith & Buchanan, 2010; Zann, 1996), whereas rats live largely solitary lives except for antagonistic and reproductive interactions (Calhoun, 1963). CORT dosing may therefore exaggerate a species-typical phenotype, with CORT-dosed rats more solitary than vehicle dosed rats (Veenit et al., 2013) and CORT dosed zebra finches more gregarious than vehicle dosed finches (e.g. Boogert et al., 2014; Spencer & Verhulst, 2008). Whether such differences in species-typical social organisation account for the species differences in behavioural responses have garnered

little consideration and require direct investigation. The different long-term effects of CORT dosing on adult social behaviour could also be due to the different ages of animals used. One way to test this would be to dose adolescent zebra finches with CORT and compare the long-term effects to those reported in other species (e.g. rats). In the current thesis, Chapter 2 presents a study in which adolescent zebra finches were orally dosed with CORT or saline during adolescence and behavioural responses to unfamiliar stimuli (environments and conspecifics), stress physiology, and social physiology were then quantified in later-life.

1.5. Adolescent social interactions: long-term effects

As has previously been discussed, peer-directed interactions become more common during adolescence compared to pre-adolescence (Nelson et al., 2005; Schlegel & Barry, 1991; Varlinskaya & Spear, 2008). Adolescent social interactions both trigger the secretion of hormones and are regulated by hormones, suggesting social interactions during adolescence can have organisational effects on the developing individual and thereby influence later-life phenotypes (Buwalda, Geerdink, Vidal, & Koolhaas, 2011; McCormick et al., 2015; Sachser et al., 2011). The following section will outline three lines of research that attempt to understand how adolescent social interactions can influence later-life behavioural responses to unfamiliar social and non-social stimuli and the endocrine mechanisms that underpin such differences in stress and social physiology, namely: social re-housing, social novelty, and social density. The majority of the research that examines these adolescent social conditions have been studied in laboratory rodents that typically live in small groups, especially rats (e.g. McCormick et al., 2015), mice (e.g. Van Loo, Mol, Koolhaas, Van Zutphen, & Baumans, 2001), and guinea pigs (e.g. Lürzel et al., 2011). As a general criticism, a more comparative approach is needed to determine if the reported effects of adolescent social conditions on later-life phenotypes is dependent on factors such as typical group size (e.g. small to large) and social organisation (e.g. solitary to gregarious).

1.5.1. Social re-housing

Adolescent rats play more if their play partner is unfamiliar compared to familiar (Cirulli et al., 1996; McCormick, Merrick, Secen, & Helmreich, 2006; Smith, Wilkins, Mogavero, & Veenema, 2015; Veenema et al., 2012). Taking advantage of this, a protocol has been developed to investigate the long-term effects of more adolescent social interactions by repeatedly pair housing unfamiliar rats together for twenty three hours after one hour of single housing over a period of fifteen days during adolescence (postnatal days 30-45: McCormick et al., 2015). In adulthood, male rats that underwent social re-housing, vs. rats left in familiar pairs, engage in more locomotor activity and spend less time in open spaces in an unfamiliar environment (McCormick et al., 2008), spend a shorter duration of time in contact with a novel object (Green, Barnes, & McCormick, 2013), and spend a shorter duration of time interacting with an unfamiliar male rat (Green et al., 2013). Male adolescent social interactions may therefore result in a more neophobic behavioural response when in adulthood, similar to the effects that emerge in response to rats that are exposed to more stressors during adolescence (Hollis et al., 2013). Adolescent social re-housing raises circulating CORT concentration during adolescence (Mathews et al., 2008) and may exert long-term effects on later-life responses to unfamiliar stimuli due to organisational effects on the HPA axis. Whether unfamiliar social interactions cause the long-term effects on behaviour is difficult to state, as exposing rats to an unfamiliar conspecific is conflated with single housing and re-housing without control groups for comparison. Single housing or re-housing stress, not unfamiliar social interactions, may therefore be causing the long-term effects of the protocol on behaviour.

Social re-housing in adolescent female rats results in animals that spend more time in open areas in an unfamiliar environments during proestrous when in adulthood compared to female rats that were raised in stable pairs during adolescence (McCormick et al., 2008). In contrast to male rats, adolescent social re-housing result in female rats that are less neophobic

when in adulthood compared to stable housed rats. However, during proestrous female rats attempt to solicit sexual interactions from males by engaging in more exploratory behaviour than when in other estrous cycle stages (Morgan, Schulkin, & Pfaff, 2004). Socially re-housed females may therefore not be less neophobic, but spending more time exploring an unfamiliar environment in order to find a mate compared to stable housed females. The long-term effects of social re-housing may therefore not be affecting behavioural responses to unfamiliar stimuli due to organisational effects on the HPA axis. Instead, adolescent social re-housing may be affecting social (e.g. reproductive) behaviour in later-life due to organisational effects on social physiology (e.g. HPG axis and/or SBN). No study has investigated the long-term effect of adolescent social re-housing on female rats socio-sexual behaviour. However, adolescent social re-housing in male rats results in delayed copulation time when in adulthood compared to stable housed males, suggesting social re-housing can affect adult reproductive behaviour (McCormick et al., 2013). In promiscuous rats, delayed copulation time would likely be a disadvantage to males due to lost mating opportunities (McCormick et al., 2013) whereas more exploration in proestrous may improve reproductive success in females due to more effective solicitation of a mate (Morgan et al., 2004). Further work is needed to explore the extent to which adolescent social re-housing may impair later-life sexual behaviour (i.e. wear and tear hypothesis) or improve reproductive success in certain contexts (i.e. predictive-adaptive response) in both males and females.

The long-term effects of adolescent social re-housing on behavioural responses to unfamiliar stimuli, a mild stressor, could be attributed to differences in HPA axis functioning. However, social re-housing in adolescent rats has no long-term effects on stressor-induced CORT concentration (McCormick, Robarts, Gleason, & Kelsey, 2004; McCormick, Robarts, Kopeikina, & Kelsey, 2005; Mathews, Wilton, Styles, & McCormick, 2008; McCormick et al., 2008). All stressors used were asocial (e.g. restraint, open platform, forced swim) and social

interactions may only modify CORT secretion in response to social stressors. Alternatively, adolescent social re-housing may affect later-life phenotypes via modulation of social hormones, such as gonadal hormones and nonapeptides. For example, social challenge raises circulating testosterone concentration when in adulthood (Wingfield, Hegner, Dufty, & Ball, 1990; Wingfield, Ball, Dufty, Hegner, & Ramenofsky, 2005) and adolescent testosterone can have long-term effects on adult phenotypes (Schulz et al., 2009; Schulz & Sisk, 2016). Adolescent male rats injected with testosterone spend less time in open areas of an unfamiliar environment when in adulthood (Olivares et al., 2014); similar to the effects of adolescent stress (Hollis et al., 2013) and social re-housing in males (McCormick et al., 2008). Social re-housing may raise testosterone during adolescence, and have long-term organisational effects on the HPG axis that may affect later-life responses to unfamiliar stimuli. Adolescent social re-housing in male rats results in lower basal testosterone concentration in adulthood compared to adolescent males from stable pairs (McCormick et al., 2013). A testosterone-mediated effect of social re-housing on adult behavioural responses to unfamiliar stimuli is plausible, but requires further testing to elucidate the mechanism (e.g. lower androgenic inhibition of the HPA axis). Whether social re-housing affects other social hormones (e.g. estradiol, OT, and VP) and what mechanisms may be affected in female's remains to be investigated.

The organisational effects of adolescent social re-housing on neural GC receptor distribution have not been investigated in rats, so inferring that any long-term effects of adolescent social re-housing on behavioural responses to unfamiliar stimuli are due to such changes is premature. However, adult GR and MR have been investigated in response to a re-housing protocol in mice (Sterlemann et al., 2008). Adolescent mice were re-housed in unfamiliar same-sex groups of four twice per week for seven weeks during adolescence (Schmidt et al., 2010). Social re-housing in male mice has no effect on hippocampal GR expression, but lowered hippocampal MR when compared to mice that had stable adolescent

housing (Sterlemann et al., 2008). The limited effects of adolescent social re-housing in mice on hippocampal GC receptor expression cannot explain the long-term behavioural effects of adolescent social re-housing in rats. However, further investigation is required to assess how adolescent social re-housing affects GC receptor expression in other regions regulating the HPA axis (e.g. hypothalamus and amygdala). Adolescent social re-housing may not affect later-life responses to stressors, like unfamiliar stimuli, via organisational effects on the HPA axis but may do so by affecting social physiology (e.g. HPG axis and/or SBN). However, to date no study has investigated the effect of adolescent social re-housing on later-life neural expression of gonadal hormone or nonapeptide receptors to test the hypothesis.

One caveat of the social rehousing in mice is that the protocol had no long-term effects on behavioural responses to unfamiliar environments (Sterlemann et al., 2008; Schmidt et al., 2007; Schmidt et al., 2010) or basal CORT concentration (Sterlemann et al., 2008); stressor-induced CORT concentration was not quantified. Social re-housing in mice would therefore be predicted not to have organisational effects on the HPA axis. The mouse social re-housing protocol, unlike the rat social re-housing protocol, does not conflate unfamiliar social interactions with single housing. Absence of effects of mouse social re-housing on responses to unfamiliar stimuli may therefore be further evidence that any effects of adolescent social re-housing in rats are not caused by unfamiliar social interactions. Instead, single housing either by itself or in interaction with unfamiliar conspecifics may be the cause of the long-term effects of adolescent social re-hosing in rats. Further work is needed to determine whether unfamiliar adolescent social interactions have long-term effects on behavioural responses to stressors and stress physiology using a design that excludes single housing effects to adequately test whether adolescent social interactions affect later-life phenotypes via organisational effects on the HPA axis.

1.5.2. Social novelty

Re-housing protocols have investigated the effects of prolonged exposure to unfamiliar conspecifics during adolescence, but animals become familiar to each other as time progresses. The initial interactions with an unfamiliar conspecific (i.e. social novelty) may be sufficient to affect later-life responses to stressors. One hypothesis has proposed that interactions with unfamiliar age-similar conspecifics prepares an animal to respond to unfamiliar stimuli in later-life, thereby lowering neophobic behavioural responses and CORT secretion in adulthood (Cooke & Shukla, 2011; Pellis & Pellis, 2007; Spinka, Newberry, & Bekoff, 2001). In line with the hypothesis, pair housed guinea pigs (one male, one female per pair) exposed to a different unfamiliar adult conspecific for 10 minutes on alternate days in late adolescence (20 sessions total) had a lower CORT concentration in response to a mild stressor (novel environment) when in adulthood compared to pairs of guinea pigs that did not experience such social novelty (Lürzel et al., 2011). Twice weekly novel social interactions (8 sessions in total) in late adolescent guinea pigs had no effect on adult CORT concentration (Lürzel, Kaiser, & Sachser, 2010), indicating that unfamiliar interactions need to be of sufficient quantity and/or duration to affect phenotypes in later-life. A sufficient number of novel social interactions with unfamiliar adult conspecifics appear to lower the acute physiological response to stress when in adulthood. Social re-housing studies do not find long-term effects of adolescent social interactions on later-life CORT secretion (e.g. Mathews et al., 2008), perhaps because the effects of social novelty were lessened by familiarisation with the novel conspecific during re-housing. Social re-housing studies also typically expose animals to unfamiliar adolescents (e.g. McCormick et al., 2015), whereas social novelty studies have exposed animals to unfamiliar adults (Lürzel et al., 2010). Whether brief interactions with novel age-similar conspecifics during adolescence affects later-life responses to unfamiliar stimuli, social behaviour, and the neuroendocrine mechanisms underpinning any differences in behaviour remains to be tested.

Whether the long-term effect of adolescent social novelty on CORT are present in females remains to be assessed as, despite opposite-sex adolescent pairs being exposed to novel conspecifics in Lürzel et al. (2011), only male guinea pigs were tested in adulthood. The immediate effects of adolescent social novelty have been well documented in male and female rats (e.g. Cirulli et al., 1996; Himmler et al., 2014; McCormick et al., 2006; Smith et al., 2015; Veenema et al., 2012). A clear sex difference in adolescent social novelty is present, with adolescent male rats engaging in a greater quantity of play and different style of play (e.g. greater use of counter-attacks) than adolescent females when paired with an unfamiliar age-similar rat when placed in both same- and opposite-sex pairings (Argue & McCarthy, 2015a; Argue & McCarthy, 2015b; Pellis, 2002; Pellis, Field, Smith, & Pellis, 1997; Smith et al., 2015; Smith, Forgie, & Pellis, 1998; Veenema et al, 2012). Adolescent male rats also emit more 50 kHz calls than females during adolescent play (Himmler et al., 2014). Whether the sex difference in adolescent social novelty affects responses to stressors in later-life in a similar manner to males has not been investigated. In the current thesis, Chapter 4 presents a study to discern whether adolescent social novelty in female rats has long-term effects on adult behavioural and endocrine responses to unfamiliar stimuli.

1.5.3. Social density

Adult animals in a number of species (e.g. rats, mice, meadow voles: *Microtus pennsylvanicus*, and chickens: *Gallus gallus domesticus*) housed in higher, vs. lower, densities engage in more antagonistic interactions and have a higher basal CORT concentration; perhaps as competition over limited resources becomes more common (e.g. territory, mates, and food: Boonstra & Boag, 1992; Christian, 1950; Craig & Swanson, 1994; Creel, Dantzer, Goymann, & Rubenstein, 2013; Kang, Park, Kim, & Kim, 2016; Van Loo et al., 2001). High density housing may therefore act as a stressor due to familiar conspecifics engaging in antagonistic interactions with one another than at lower density (Creel et al., 2013; Van Loo et al., 2001).

Higher social density housing during adolescence may therefore function as a stressor, resulting in animals that engage in more neophobic behaviour and secrete a higher concentration of stressor-induced CORT when in adulthood. In line with this hypothesis, mice raised at higher social density in adolescence spend less time in the exposed areas of an unfamiliar environment when in adulthood (Reiss, Wolter-Sutter, Krezel, & Ouagazzal, 2007) and secrete a higher concentration of CORT when single housed and exposed to a loud noise in adulthood (Ortiz, Armario, Castellanos, & Balasch, 1985) compared to conspecifics raised at lower densities. However, mice housed at higher density spend more time in the open spaces of an unfamiliar environment when still in adolescence compared to mice raised at lower density (Ago et al., 2014) and mice housed at different social densities have similar basal CORT concentrations (Ago et al., 2014; Laviola, Adriani, Morley-Fletcher, & Terranova, 2002). Adolescent social density may therefore not have long-term effects by acting as a stressor and modulating the developing stress physiology. However, the studies investigating the short-term effects of adolescent density on CORT concentration did not compare the behavioural responses to unfamiliar stimuli across ages and only basal CORT concentrations were quantified (e.g. Ago et al., 2014; Laviola et al., 2002). Further work is therefore needed to fully explore the effects of adolescent social density on behavioural responses to unfamiliar stimuli and the acute stress response, including basal and stressor-induced CORT concentrations, in both adolescent and adult animals to adequately assess the effects of adolescent social density on responses to stressors.

Two important caveats are present in social density studies: conflating group size with density, and only studying male animals. Social density studies achieve higher social density by placing more animals in a cage without changing cage size (e.g. Ago et al., 2014; Ruploh et al., 2014; Sachser et al., 1993) and therefore conflate social density and group size effects (Van Loo et al., 2001). Future work therefore needs to control for group size when investigating the

effects of social density to determine whether the absolute number of conspecifics and/or housing density of conspecifics during adolescence has long-term effects on responses to unfamiliar stimuli. Adolescent social density studies also mostly use male animals (e.g. Ortiz et al., 1985; Sachser et al., 1993). Adult females respond similarly to adult males when housed at higher social density (e.g. more antagonistic behaviour and higher basal CORT: Craig & Swanson, 1994; Kang et al., 2016) compared to lower social density. However, sex differences have occurred in response to social density in some species (e.g. rats) with higher density raising CORT concentration in male rats but lowering CORT concentration in female rats (Brown & Grunberg, 1995). Whether sex differences are present in response to adolescent social density variations is little investigated, but future research needs to include both male and female animals to detect any potential sex differences in response to adolescent social density. Chapter 3 presents a study in which adolescent zebra finches (both male and female) were housed in cages varying in conspecific number and density in order to identify the long-term effects of the variables on later-life behavioural responses to unfamiliar stimuli (e.g. environments, objects, and conspecifics) and the neuroendocrine mechanisms that underlie any behavioural differences (i.e. concentrations of CORT and gonadal hormones).

1.6. Thesis aims and structure

The introductory chapter has outlined adolescence as a transition from the natal home to the adult environment, during which individuals interact with stressors and conspecifics that have long-term effects on behavioural responses to stressors, such as unfamiliar stimuli, and the neuroendocrine mechanisms that may underpin differences in behaviour, e.g. HPA axis, HPG axis, and SBN (Hollis et al., 2013; McCormick et al. 2015). The data presented in Chapters 2, 3, and 4 outline attempts to further investigate the long-term effects of adolescent CORT exposure, adolescent social density, and adolescent social novelty. The results outlined in the experimental chapters are then discussed in Chapter 5; comparing the novel findings

with those previously described in the available literature, identifying any limitations of the research conducted for this thesis, and outlining directions for future work.

Chapter 2 will investigate the hypothesis that exposure to CORT is the mechanism behind the long-term effects of adolescent stress. Adolescent zebra finches were orally dosed with CORT and the behavioural responses to unfamiliar environments were quantified in adulthood in both an individual context and a group context. The contexts were chosen to quantify behaviour in and out of a group context. The behavioural responses to an unfamiliar group of birds was also undertaken to investigate how adolescent CORT affects later-life social behaviour. To determine the long-term neuroendocrine effects that may cause behavioural differences between adolescent CORT dosed and control animals, CORT concentration was quantified in response to a restraint stressor (in adolescence and adulthood) and neural expression of the GC receptors (GR, MR) were quantified in the HPA axis (in adulthood). To investigate the endocrine mechanism that may mediate the effects of adolescent CORT dosing on social behaviour, the basal concentrations of gonadal hormones (i.e. testosterone and estradiol) and the neural expression of nonapeptide receptors (VT1, VT3) were quantified in adulthood.

The remaining chapters focused on how adolescent social experiences could affect later-life responses to unfamiliar stimuli. Chapter 3 describes a study in which adolescent zebra finches were housed in cages varying in the number and density of conspecifics. When in adulthood, the same behavioural and plasma hormone measures as described in Chapter 2 were quantified in order to compare the extent to which adolescent social density has similar long-term effects to adolescent CORT dosing. Chapter 4 then goes on to describe a study that investigated the long-term effects of brief unfamiliar social interactions in adolescent female rats on adult responses to unfamiliar stimuli, such as unfamiliar environment. 50 kHz call rate was also quantified in response to social separation from and then reunion with a familiar

conspecific. CORT secretion in response to a familiar vs. unfamiliar conspecific were also quantified. The latter two tasks were included to examine the effects of adolescent social novelty on social measures in adulthood.

The thesis investigates two species that have previously been used to explore the long-term effects of adolescent stress and social experiences, rats (e.g. Veenit et al., 2013) and zebra finches (e.g. Ruploh et al., 2013). These two species vary in some aspects of their life histories, including species-typical social organisation and mating system. Rats spend most of their time alone, with the few social interactions engaged in typically involving antagonistic interactions in order to establish or maintain a positon in a social hierarchy, and reproductive encounters with several different conspecifics due to a promiscuous mating system (Calhoun, 1963). In contrast, zebra finches are affiliative animals that live in groups and establish socially monogamous pair bonds (Zann, 1996). Adolescent rats engage in play fighting with unfamiliar conspecifics in order to learn how to function in a social hierarchy in adulthood (Pellis & Pellis, 2007; Pellis et al., 2010), whilst adolescent zebra finches learn to maintain proximity to unfamiliar conspecifics (Adkins-Regan & Leung, 2006) possibly to better function in a social group in adulthood. Through the inclusion of these two species comparisons can be made between rats and zebra finches to explore the potential effects of species-typical differences in adolescent social behaviour (i.e. play fighting vs. proximity), adult social behaviour (i.e. antagonistic vs. affiliative interactions), social organisation (i.e. solitary vs. group-living), and mating system (i.e. promiscuous vs. pair bond) as variables that may mediate the long-term effects of adolescent stress and social experiences on adult behavioural responses to unfamiliar stimuli and stress physiology.

Chapter 2

Adolescent corticosterone exposure in zebra finches: long-term effects on behavioural responses to unfamiliar stimuli, stress physiology, and gonadal hormones

2.1. Introduction

Exposure to CORT during early-life can modulate later-life responses to unfamiliar stimuli, but most studies expose developing animals to stressors and assume CORT is causing the effects (Hollis et al., 2013; Sachser et al., 2011; McCormick et al., 2015). In contrast, studies using passerine birds have involved oral dosing with solutions containing CORT. CORT dosing during pre-adult development typically results in birds that more readily interact with unfamiliar environments and objects in later-life compared to vehicle dosed controls (Pakkala et al., 2016; Schmidt et al., 2014; Spencer et al., 2003). For example, male zebra finches dosed with CORT during the nestling and fledgling stages (twice per day during postnatal days 12-28) were quicker to contact an unfamiliar object than vehicle dosed birds when tested later in life (Crino, Driscoll, Ton et al., 2014; Spencer & Verhulst, 2007). Approaching unfamiliar objects may be a stressor-avoidant response in zebra finches, as CORT dosed birds were more likely to find food in unfamiliar foraging locations and avoid under-nutrition compared to controls (Crino, Driscoll, Ton, et al., 2014). The zebra finch studies tested the birds' responses to unfamiliar objects when the birds were single housed. In adulthood, familiar conspecifics can lower physiological responses to stressors (e.g. CORT secretion) and thereby lower stressor-induced behavioural responses compared to when single housed, potentially due to a social buffering effect (DeVries et al., 2003; Hennessy et al., 2009). Assuming unfamiliar stimuli are stressors (Haller et al., 1998; Rodgers, Cao, Dalvi, & Holmes, 1997), the presence of familiar conspecifics during adult testing may buffer (i.e. cancel out) the long-term effects of developmental CORT dosing on behavioural responses to unfamiliar stimuli. However, no study has yet investigated whether developmental CORT dosing affects behavioural responses to unfamiliar stimuli in both individual and familiar group contexts.

Behavioural effects of developmental CORT dosing in passerine birds appear to have similar effects on social behaviour. Zebra finches dosed with CORT prior to adolescence (twice

per day from postnatal days 12-28) developed into adolescents that entered a seed-filled foraging box with a greater number of unfamiliar conspecifics compared to vehicle dosed birds when the birds were housed in mixed-sex and mixed-age flocks of 29-34 birds (Boogert et al., 2014). Interacting with more unfamiliar birds could be due CORT dosed birds interacting with all unfamiliar stimuli more, but CORT could also have effects on behavioural interactions between conspecifics. For example, CORT dosed birds may engage in fewer antagonistic interactions (e.g. beak fencing, perch displacement) and/or more gregarious interactions (e.g. allopreening, clumping) resulting in the birds interacting with more unfamiliar conspecifics than control birds. However, pre-adolescent CORT dosing in zebra finches has no effect on antagonistic behaviour when in adolescence (Spencer & Verhulst, 2007), and the effects on gregarious behaviour have not yet been quantified. Interacting with more unfamiliar birds does not necessarily reflect social competence, but could reflect that a bird is unable to establish reliable foraging partners and must therefore interact more with unfamiliar birds. In zebra finches, pre-adolescent CORT dosing results in adult males with lower quality and less attractive songs than vehicle dosed controls that may be due to CORT impairing song learning (Spencer et al., 2003; Spencer et al., 2005). CORT dosing could therefore also impair other social behaviour not yet quantified, resulting in CORT dosed birds interacting with a greater number of birds than vehicle dosed birds. Whether CORT dosing results in more social interactions with unfamiliar conspecifics than controls due to more gregariousness, more social impairment, or less neophobia remains to be tested.

The long-term effects of pre-adolescent CORT exposure appear similar across species, as pre-adolescent rats dosed with CORT (transferred in milk from CORT-fed mothers) develop into adults that interact with unfamiliar stimuli more than rats fed by non-dosed mothers (Catalani, Alemà, Cinque, Zuena, & Casolini, 2011). Interacting more with unfamiliar stimuli in response to CORT exposure during pre-adolescent may therefore be a typical occurrence

across taxa, rather than a phenomenon specific to passerine birds. This comparative approach has begun to hint at a route via which developmental CORT exposure results in lasting effects on adult behaviour. CORT-dosed mother rats engage in more maternal care than control mothers (Catalani et al., 2011), and greater quantity of maternal care can result in offspring developing into adults that interact with unfamiliar stimuli more than control offspring (Diorio & Meaney, 2005; Francis & Meaney, 1999). The long-term effects of pre-adolescent CORT dosing may therefore not be indirect modulation of parental behaviour, rather than the direct effect of elevated CORT concentration. Whether the similar long-term effects of pre-adolescent CORT exposure found across species is similarly due to CORT modulating parental behaviour has not been studied.

One way to investigate the long-term effects of CORT exposure without the effect of parental behaviour would be to dose animals in adolescence, as animals can live independent of their parents during this time (Sachser et al., 2011; Spear, 2000). However, the organisational effects of exposure to steroid hormones declines as age progresses (Schulz & Sisk, 2009; Schulz & Sisk, 2016). Adolescent CORT dosing may therefore have diminished or even no effects on later-life phenotypes. The one study to investigate the long-term effects of adolescent CORT dosing on behavioural responses to unfamiliar stimuli (7 injections of CORT in male rats between postnatal days 28-42) found no difference between CORT and saline injected rats' responses to an unfamiliar environment (Veenit et al., 2013). The effects of CORT exposure on adult phenotypes may only be present in response to pre-adolescent CORT dosing, perhaps due to indirect effects on parental behaviour. However, the design used in Veenit et al. (2013) has several limitations. The study ended up comparing two stress groups; injection stress vs. injection stress and CORT. Animals in both groups would therefore be expected to interact less with unfamiliar stimuli compared to non-injected controls that were not included in the design. Any effect of CORT dosing on responses to unfamiliar stimuli in Veenit et al. (2013) may have

been masked by comparing two injection stress groups. Avoiding injections, for example by orally dosing with CORT, would therefore allow less ambiguous inferences to be made. Oral dosing with CORT is achievable in passerine birds (e.g. Spencer et al., 2003), so investigating the effects of adolescent CORT exposure may be better determined using such species.

Adolescent CORT exposure affects later-life behavioural responses to unfamiliar social stimuli, as adolescent male rats injected with CORT (vs. saline) develop into adults that spend less time interacting with an unfamiliar conspecific (Veenit et al., 2013). Adolescent CORT exposure may therefore influence the ontogeny of social behaviour. Male rats, in semi-natural enclosures, are observed to be mostly solitary except for occasional bouts of antagonistic interactions between males (Calhoun, 1963). A lower interaction time in CORT (vs. saline) dosed rats may reflect that the rats are more avoidant of potentially injurious encounters with unfamiliar conspecifics. However, spending less time with an unfamiliar conspecific could also reflect that CORT dosed rats are more socially impaired (i.e. don't know how to interact with an unfamiliar conspecific) than controls. The two dosing conditions could also be engaging in different types of social behaviour (e.g. less gregarious or more antagonistic interactions in CORT vs. saline dosed rats). Whether adolescent CORT dosing affects specific adult behavioural interactions with conspecifics (e.g. affiliative or antagonistic) have not been investigated, as Veenit et al. (2013) only reported social interaction time. Further work is therefore needed to determine how adolescent CORT affects later-life social behaviour, not just social interaction time.

To determine the effects expected to occur from adolescent glucocorticoid exposure, animals can be directly exposed to stressors during adolescence. Experiments in rats and mice show that exposure to stressors during adolescence results in adult animals that are less likely to explore unfamiliar environments and less likely to interact with unfamiliar objects when in adulthood compared to control animals that received no such stress (Eiland & Romeo, 2013;

Hollis et al., 2013). Like the effects seen for CORT dosing, adolescent rats exposed to stressors also spend less time with an unfamiliar conspecific than control animals when in adulthood (Marquez et al., 2013; Tzanoulinou et al., 2014). Adolescent stressor exposure can therefore result in animals that interact less with unfamiliar stimuli compared to controls, and research now needs to establish whether adolescent CORT has a similar effect. The long-term effects of adolescent stressor and CORT exposure (in rats; Hollis et al., 2013; Veenit et al., 2013) are the opposite of those reported in response to pre-adolescent CORT dosing (rats: Catalani et al., 2011; zebra finches: Spencer & Verhulst, 2007). Differences in developmental stage (i.e. preadolescent vs. adolescent) are the most likely explanation for the age-specific effects, with adolescent effects perhaps resulting from exposure to developmental stress without the compensatory effects of greater parental care. However, differences in testing context may also explain the reported effects. Responses to unfamiliar conspecifics, for example, were quantified in mixed-sex mixed-age flocks of 29-34 birds in zebra finches (Boogert et al., 2014), but in response to a single male conspecific in rats (Veenit et al., 2013). The social context during testing (e.g. number, density, age, and sex of conspecifics present during testing) may therefore mediate the long-term effects of CORT exposure on behavioural responses to unfamiliar conspecifics. Quantifying the behavioural effects of adolescent CORT exposure across different social testing contexts when in adulthood could help explore these putatively mediating variables.

Developmental stressor exposure has long-term effects on the secretion of CORT in response to an acute stressor when in adulthood (e.g. Isgor et al., 2004; Spencer et al., 2009). For example, adolescent stress in rats results in adult animals with a prolonged CORT secretion in response to restraint compared to rats that experienced no such stress (Isgor et al., 2004; Pohl et al., 2007). CORT dosing prior to adolescence has a similar effect, as zebra finches dosed with CORT during nestling and fledgling stages had a prolonged secretion of CORT in

response to restraint compared to vehicle dosed controls when the birds were adolescents (Spencer et al., 2009). Song sparrows dosed with CORT (twice per day from days 7-60) continuously through nestling, fledgling, and adolescent stages had a higher CORT concentration in response to an ACTH challenge in adulthood compared to control birds (Schmidt et al., 2014). Greater stressor-induced CORT concentration can result in more neophobic behavioural responses in adult rats (Haller et al., 1998), so developmental stressors may result in greater stressor-induced CORT secretion as a proximate mechanism underlying the long-term effects on behavioural responses to unfamiliar stimuli (Isgor et al., 2004; Spencer & Verhulst, 2007). However, recent research has not been able to corroborate the effects in Spencer et al. (2009) and found that CORT dosing zebra finches during nestling and fledgling stages had no effect on the acute stress response at either day 60 or day 90 (Crino, Driscoll, & Breuner, 2014). CORT dosed birds in Crino et al. (2014) did have a prolonged CORT secretion to restraint at day 30 compared to control birds, so CORT dosing may only have transient effects on the acute stress response. However, Crino et al. (2014) used the same stressor (i.e. restraint) at all three ages and the bird's absence of differences at later ages may reflect that the birds had habituated to the restraint stressor after the first exposure. If a different stressor was used at each sampling age then differences in stressor-induced CORT concentration between the conditions may have been present at later-ages.

The greater secretion of stressor-induced CORT in adulthood that occurs in response to adolescent stress (Isgor et al., 2004; Pohl et al., 2007) may be attributable to lower negative feedback and/or more positive feedback acting on the HPA axis (Isgor et al., 2004). Adolescent stress in male rats results in lower expression of the glucocorticoid receptor GR in the hippocampus, a brain region that can inhibit the HPA axis, when in adulthood compared to adult control rats that were not subject to such adolescent stress (Isgor et al., 2004). Adolescent stress can therefore result in lower negative feedback of the HPA axis compared to controls;

further work is necessary to test whether the effect is seen in the other brain regions that can inhibit the HPA axis (e.g. prefrontal cortex and hypothalamus: Herman et al., 2003; Herman et al., 2005). The nidopallium caudolaterale in avian species has similar functions to the prefrontal cortex in mammals (e.g. working memory: Güntürkün, 2005), but whether developmental stress has similar effects on these structures (e.g. lower GR expression) remains to be assessed. Whether adolescent stress also results in more positive feedback by up-regulating GR expression in HPA axis stimulators (e.g. amygdala: Herman et al., 2012; Shepard et al., 2003) compared to control animals also remains to be determined. Higher MR expression can cause higher basal activity of the HPA axis that can result in a quicker rise in CORT concentration during an acute stress response (de Kloet et al., 2000; Joëls et al., 2007; Oitzl, Champagne, van der Veen, & de Kloet, 2010). Adolescent stress does not appear to affect basal CORT concentration in rats (Isgor et al., 2004; Pohl et al., 2007), so adolescent stress would be expected to also have no effect on neural MR expression. However, the hypothesis needs testing as to date no study has quantified the long-term effects of adolescent stress or CORT dosing on MR expression when in adulthood. An additional measure, the GR/MR ratio has been linked to HPA axis functioning, with lower GR to MR ratio in brain regions that can inhibit the HPA axis (e.g. hippocampus) resulting in lower negative feedback efficiency compared to animals with more balanced GR to MR ratio (de Kloet, Vreugdenhil, Oitzl, & Joëls, 1998; Groneweg, Karst, de Kloet, & Joëls, 2011). Whether adolescent stress results in a lower ratio of GR to MR in order to lower HPA axis negative feedback remains to be tested.

The effects of developmental stress or CORT dosing could be attributed to differences in CORT secretion, but ‘social hormones’ (i.e. gonadal hormones and nonapeptides) are also a possible mechanism. Adolescent stress in rats has no long-term effects on basal testosterone concentration in males or basal estradiol concentration in females compared to control rats that received no such stress in adolescence (Bourke et al., 2013). Gonadal hormones may therefore

not be a mechanism by which adolescent stress has long-term effects on behavioural responses to unfamiliar stimuli. However, adolescent CORT dosing has inconsistent effects on later-life measures of gonadal hormones. Adolescent CORT dosing in rats resulted in lower basal testosterone concentration in adult males compared to control animals, but did not affect basal estradiol concentration in females (Kaplowitz et al., 2016). In song sparrows, CORT dosing during nestling, fledgling, and adolescent development resulted in higher basal testosterone than in adult males, and lower estradiol in females, compared to same-sex controls (Schmidt et al., 2014). The inconsistent effects of CORT dosing on later-life gonadal hormone concentration may reflect species-specific effects for reasons that are currently unclear. Any effects may also be confounded by the doses used, as neither study specified why the doses were selected, with administered CORT doses potentially lacking physiological relevance to the animals. Whether administering physiologically relevant CORT doses during adolescence affects basal concentration of gonadal hormones when in adulthood remains to be tested.

Nonapeptides also regulate social behaviour by acting on nonapeptide receptors in the social behaviour network (Goodson, 2005, Goodson, 2013). Oxytocin binds to the oxytocin receptor and mesotocin binds to the VT3 receptor in mammals and birds, respectively, resulting in more affiliative interactions with unfamiliar conspecifics (e.g. Witt et al., 1992; Goodson et al., 2009). Vasopressin binds to V1a/V1b receptors and vasotocin binds to VT1/VT4 receptors in mammals and birds, respectively, and can result in more antagonistic interactions with unfamiliar conspecifics (Goodson & Thompson, 2010); but fewer antagonistic interactions with familiar conspecifics (e.g. Kabelik et al., 2009). In mammals (mostly rats and mice), early postnatal stressors (e.g. parental absence) can result in adults with lower oxytocin receptor binding in the social behaviour network (Francis, Young, Meaney, & Insel, 2002; Lukas, Bredewold, Neumann, & Veenema, 2010) and higher vasopressin mRNA expression in the paraventricular nucleus of the hypothalamus (Pan, Liu, Young, Zhang, & Wang, 2009;

Veenema, Blume, Niederle, Buwalda, & Neumann, 2006) compared to control animals without such stress. However, non-social stressors (e.g. maternal restraint during pregnancy) in development have little effect on nonapeptide receptor expression in the offspring in later-life (Lee, Brady, Shapiro, Dorsa, & Koenig, 2007). Social experiences in development may therefore have long-term effects on the nonapeptide system, but developmental stressors may have no such long-term effects. Developmental CORT dosing affects behaviour that are regulated by nonapeptides (e.g. social interaction with unfamiliar conspecifics: Boogert et al., 2014; Veenit et al., 2013), but whether developmental CORT dosing (in order to mimic developmental stress) has any effects on nonapeptide functioning in later-life remains to be tested.

The current study aimed to investigate the hypothesis that adolescent exposure to glucocorticoids, such as CORT, are an endocrine mechanism that has long-term effects on behavioural responses to unfamiliar stimuli and the acute stress response when in adulthood due to organisational effects on glucocorticoid receptor expression in the regulatory mechanisms of the HPA axis. The study also investigated the hypothesis that the long-term effects of adolescent glucocorticoid exposure on later-life phenotypes is more pronounced the earlier in pre-adult development that the exposure occurs. Zebra finches were used as several studies have used the species to detail the long-term effects of CORT dosing during pre-adolescent development on behavioural responses to unfamiliar stimuli (Boogert et al., 2014; Crino, Driscoll, Ton, et al., 2014; Spencer & Verhuslt, 2007) and the acute stress response (Crino et al., 2014; Spencer et al., 2009). The previous work can therefore be used here in order to make informed predictions about the effects of adolescent CORT dosing.

Zebra finches in the current study were dosed with CORT or saline during early (day 40-60) or late (day 65-85) adolescence. The age ranges were selected as they are ten days either side of different stages of social maturation in adolescent zebra finches. Around day 50, zebra

finches begin to spend more time with unfamiliar conspecifics compared to the parents (Adkins-Regan & Leung, 2006). Around day 75, zebra finches begin to use courtship behaviour in mating contexts and begin to form pair bonds (Zann, 1996). The two age ranges were selected to assess the age-related effects of steroid hormone dosing. As the organisational effects of steroid hormones appear to decline with age, all effects throughout this chapter were predicted to be more pronounced in response to early adolescent CORT dosing compared to late adolescent CORT dosing (based on Schulz & Sisk, 2016). Birds were trained to enter a 'dosing box' attached to the home cage in order to consume cucumber cubes injected with the dose; a dosing method that avoids injection stress. Latency to enter the dosing box was recorded in order to determine whether the dosing was having any immediate effects on behaviour.

In adulthood (day 100+), birds were exposed to unfamiliar environments that contained spinach-baited unfamiliar objects in an individual context and a group context. In line with previous research (e.g. Pakkala et al., 2016; Schmidt et al., 2014; Spencer et al., 2003), CORT-dosed birds were predicted to interact more with unfamiliar stimuli than saline dosed birds when in an individual context (quicker to enter the unfamiliar environments, more entries into the unfamiliar environments, more time perching in the unfamiliar environments, more time foraging next to the unfamiliar objects, more hops between perches, and more head turns). Number of head turns increases when birds are exposed to predators (Jones, Krebs, & Whittingham 2007), and this measure was recorded here as a novel measure of risk assessment in zebra finches. In a group context, familiar conspecifics may cancel out any long-term effects of developmental CORT dosing (e.g. DeVries et al., 2003; Hennessey et al., 2009), so no differences were predicted to occur between adolescent conditions on behavioural responses to the environment (listed above) or social behaviour during the task (allopreening, beak fencing, clumping, perch displacement, and time alone).

Later in adulthood, behavioural responses to an unfamiliar mixed-sex group of birds were quantified. CORT-dosed birds were predicted to spend less time alone than saline-dosed birds, as CORT-dosed birds were expected to spend more time interacting with unfamiliar conspecifics compared to control birds (based on Boogert et al, 2014). CORT-dosed birds were predicted to engage in fewer courtship behaviour (fewer directed song bouts emitted by males or directed to females, fewer mounts) than saline-dosed birds (based on Spencer et al., 2003; Spencer et al., 2005). Gregarious behaviour (allopreens, clumps) and antagonistic behaviour (displacements, beak fencing) were not expected to differ between dosing conditions (based on Spencer & Verhulst, 2007).

CORT concentration was quantified in response to a standard capture and restraint stressor in adolescence and adulthood. Basal concentrations of male testosterone and female estradiol were also quantified in adulthood. Plasma was taken to determine basal mesotocin and vasotocin concentrations, but due to unavailability of equipment necessary for extraction the hormones were not quantified. Stressor-induced CORT concentration was predicted to be higher in CORT-dosed birds during both adolescence and adulthood (based on Spencer et al., 2009), but testosterone and estradiol concentrations were predicted not to differ between CORT and saline dosed birds (based on Bourke et al., 2013)

RNA expression of neural glucocorticoid and nonapeptide receptors were determined using quantitative polymerase chain reaction (qPCR). GR expression was predicted to be lower in brain regions that may inhibit the HPA axis (i.e. hypothalamus, hippocampus, nidopallium caudolaterale) and higher in brain regions that may stimulate the HPA axis (i.e. amygdala) in CORT-dosed compared to saline-dosed birds (based on Isgor et al., 2004; Herman et al., 2012). MR expression was predicted not to differ across dosing conditions (based on Isgor et al., 2004; Pohl et al., 2007). Expression of oxytocin-like VT3 receptor and the vasotocin receptor VT1 were not predicted to be different between adolescent conditions (based on Lee et al., 2007).

2.2. Methods

2.2.1. Ethical statement

All ethical guidelines and requirements, as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85–23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986, were adhered to under project licence 70/8159 and personal licences IDFA58352, IEBE43CFF, and 60/13261.

2.2.2. Pilot study

To determine the physiologically relevant dose of CORT that was to be administered to the adolescent zebra finches, a pilot study was undertaken. Previous research has dosed birds with a concentration at two standard deviations above the sample mean of CORT in response to a restraint stressor (Crino et al., 2014; Spencer et al., 2009). In order to determine the restraint stressor-induced CORT concentration of adolescent zebra finches ten birds (5M, 5F) aged 40-60 days (mean = 51.45) and ten birds (5M, 5F) aged 65-85 days (mean = 73.14), were obtained from local breeders. Birds were housed in same-sex age-similar groups of two or three in cages measuring 90 x 40 x 40 cm (length x height x depth) in a single holding room (lights on 07:00-19:00, temperature 22±2°, and relative humidity 55±5%). Birds had *ad libitum* access to seed in hoppers (Food for Finches, Johnson & Jeff, UK), water hoppers, grit tray, and a water bath. Diets were supplemented with spinach leaves once per week with spinach provided to the birds in black plastic dishes.

Birds were captured for blood sampling between 9-11 AM on one day. Cages containing the birds were arranged so the experimenter could capture a cage of birds without being seen by the other cages; a disturbance that might affect CORT concentration in birds yet to be sampled. Two blood samples were collected by puncturing a brachial vein with a 27-gauge needle. The first sample (basal) of approx. 40µl was collected within three minutes of

entering the holding room. Birds were then restrained in a black cloth bag for fifteen minutes to induce restraint stress. A second sample (stressor-induced) of approx. 40µl was collected following the same method. The second sample was collected at 10 minutes, as previous research has indicated this time point as the peak CORT concentration in response to a restraint stressor in zebra finches (Spencer et al., 2009). Blood samples were centrifuged (3500g for 10 minutes), and plasma was removed and stored at -20°C. CORT concentration was then determined using a radioimmunoassay (Zimmer et al., 2013), the procedural details of which can be found in the *2.2.8. Hormone assays* sub-section later in this chapter. All pilot study samples were run in a single assay with an intra-assay co-efficient of variation of 10.72% and with 50% binding of 0.65 ng/ml.

A linear mixed model with age group, sex, and sample time entered as fixed factors (main effects and interactions) and sample time entered as a repeated measure was used to assess whether any differences were present in the CORT concentration (ng/ml). CORT concentration was subject to a square root transform to achieve normality due to a positive skew in the raw residuals (Shapiro-Wilk, $p < 0.05$). No differences were found between ages ($F_{1,16} = 0.006$, $p = 0.939$), sexes ($F_{1,16} = 0.067$, $p = 0.799$), or an age and sex interaction ($F_{1,16} = 0.640$, $p = 0.435$). Sampling time was significant with a higher CORT concentration at 10 minutes than baseline ($F_{1,16} = 66.588$, $p < 0.001$, $d = 2.18$; Figure 2.1.). Sampling time did not interact with any variable (age: $F_{1,16} = 0.052$, $p = 0.822$; sex: $F_{1,16} = 0.022$, $p = 0.883$; age x sex: $F_{1,16} = 1.002$, $p = 0.332$). The mean stressor-induced CORT concentration across all birds was 12.14 ng/ml (SD = 6.34). It was therefore determined that the CORT concentration in the plasma should be raised to 25 ng/ml (i.e. two standard deviations above the sample mean) for all birds as no effects of age or sex emerged.

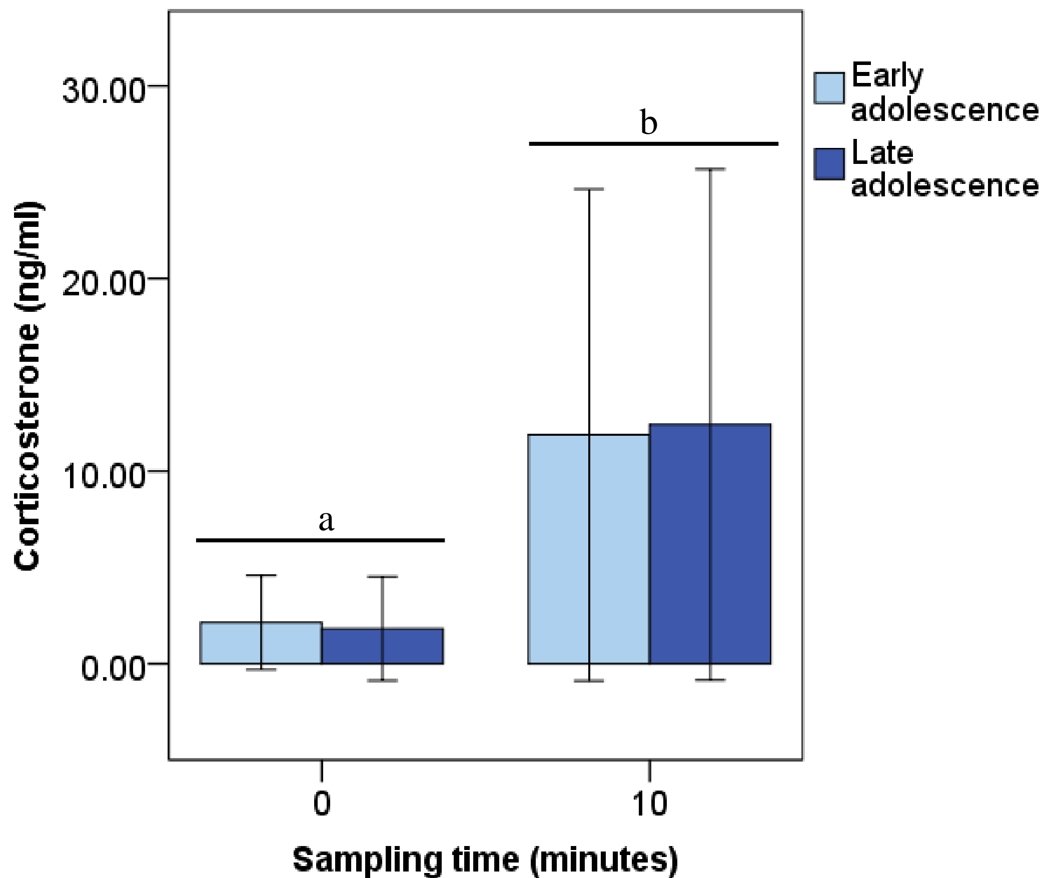

Figure 2.1. Corticosterone concentration (ng/ml) secreted by early and late adolescent zebra finches at baseline and 10 minutes after restraint. Values are mean ± two standard deviation of the mean. a vs. b = $p < 0.05$.

2.2.3. Establishing the experimental population

A total of 66 zebra finches were used in the current study. Birds were bred from twenty adult breeding pairs from the breeding colony based at the University of St Andrews. All birds (breeding pairs and offspring) were housed in a single colony room with environmental conditions and food / water availability the same as those described in the pilot study. Diets were supplemented with spinach once per week. Adult breeding birds (n = 20 male, 20 female) were selected from an in house breeding stock and placed in one of two white powder coated steel breeding cages (100 x 100 x 50 cm, length x height x depth) with 10 males and 10 female per cage. Birds were observed daily for two hours, one hour between 8:30-13:00 and one hour between 13:00-17:30, for fourteen days to identify birds that had, or were beginning to have, formed a pair bond. Pairs were identified by observing a male and female engaging in mating

behaviour on three consecutive observations. Mating behaviours were: following, directed song, mounting, and sharing a nest box (Zann, 1996). Birds were allowed to choose their partner as this results in a higher number of offspring than birds that are force paired (Griffith et al., 2017) and may therefore reduce the number of breeding animals needed for the study.

Once a pair was identified, the individuals were removed from the colony cage and housed in individual white powder coated steel breeding cages (60 x 50 x 50 cm, length x height x depth; MB 3612 Metal Double Breeding Cage, R.J. Leigh Ltd., UK). Breeding cages had a layer of wood pellets covering the base of the cage (Stovies Wood Pellets, Arbuthnott Wood Pellets Ltd, UK) and contained two 50 cm plastic perches. Each breeding cage also had access to a cardboard nest box (14 x 11 x 11 cm, height x length x depth) with a rectangular aperture of 7.5 x 11 cm (height x length). Nests were checked daily and each breeding cage was given new nesting materials (hay, jute fibre; Liverine Pet and Animal Health Care Ltd., UK) and fresh egg food (approx. 1.5g of CéDé Premium Egg Food, Belgium) after each nest check. New nesting materials were given until egg incubation began and fresh egg food was given until the offspring reached nutritional independence on post-hatch day (PHD) 32.

Upon laying, each egg was removed and replaced with a fake egg (Staedtler Fimo Soft Oven Hardened Modelling Clay (white), UK). Eggs were returned when females had stopped laying on two consecutive days. Eggs were returned on the same day in order to standardise hatch day and thereby control for any variation in hatch order, a variable that has been shown to influence adult exploratory behaviour and attractiveness (Mainwaring, Blount, & Hartley, 2012; Mainwaring & Hartley, 2013). On incubation day 7, clutches were candled and infertile clutches were removed to allow relaying. After hatching, chicks were given a temporary ID on PHD 5 (coloured nail polish applied to each leg, re-applied on PHD 8) and a permanent ID on PHD 10 (one uniquely numbered orange leg ring, one coloured leg ring: pink, yellow, light blue, or white). Leg ring colour can affect zebra finch behaviour (e.g. red bands are perceived

as more attractive and can result in males engaging in more courtship behaviour: Burley, Krantzberg, & Radman, 1982; Zann, 1994), so the colours used in the current study were selected as there is no evidence indicating they affect zebra finch behaviour. Offspring (n = 66) were re-housed in same-sex, non-sibling triplets in cages identical to the breeding cages at PHD 35 ($\pm$ 2 days). The three birds in each cage were of a similar mass (within 1g of one another).

2.2.4. Molecular sexing

To determine the sex of the birds prior to dosing, a blood sample was taken for molecular sexing. A 5µl sample was taken from each bird on PHD 12-15 after pricking a brachial vein with a 27-gauge needle. DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen Ltd) following the nucleated erythrocytes protocol. PCR was used to amplify CHD gene fragments with the primer pair P2 (5'-TCTGCATCGCTAAATCCTTT-3') / P17 (5'-GAAGAAAATAATTCCAGAAGTCCA-3') that has been developed for sexing zebra finches (Arnold et al., 2003). All reactions were run in a final volume of 10µL (0.8µL of P2 and P17 primers, 200µM of each dNTP, 0.8µL of 25mM MgCl2, 2µL (5x) of GoTAQ Flexi buffer (Promega, UK), 0.35 units of GoTaq polymerase (Promega, UK), and 100 µM of target DNA). All reactions were carried out on a TGradient 96 Biometra thermal cycler (Biometra GmBH, Goettingen, Germany) at: 94°C for 2 min, 29 cycles of 94°C for 30s, 49°C for 45s, 72°C for 40s, 49°C for 1 min, and 72°C for 5 min. PCR products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized using a Bio-Rad Gel Doc XR+ system (Bio-Rad Laboratories Ltd.). All molecular sexing was performed by Aileen Adams at Glasgow University.

2.2.5. Experimental design

Adolescent dosing. Each cage of three birds was allocated to one of four groups that varied in the age of dosing and type of dose: early adolescent saline dosing (E-SAL; n = 9M, 6F); early

adolescent CORT dosing (E-CORT; n = 9M, 9F), late adolescent saline dosing (L-SAL; n = 9M, 6F), and late adolescent CORT dosing (L-CORT; n = 9M, 9F). Early adolescent birds were dosed twice per day from PHD 40-60 and late adolescence birds were dosed twice per day from PHD 65-85. Ages the birds were dosed in relation to the rest of the experiment are presented in Figure 2.2. Saline dosed birds were given 10µl of autoclaved saline (PBS, Sigma Aldrich, UK; 0.01M, pH 7.4). CORT dosed birds were administered 10µl of a 0.36mg/ml CORT solution (Sigma Aldrich, UK) that was expected to raise the plasma concentration of CORT to 25ng/µl based on Spencer et al. (2009). The CORT solution was made from powdered CORT (18mg) that was initially dissolved in 300µl of 100% ethanol before being diluted in autoclaved saline to a final volume of 50ml. The saline solution also contained 300µl of 100% ethanol in a final volume of 50ml to control for ethanol content between dose types. Solutions were stored at 4°C prior to and between uses.

Doses of saline or CORT were administered by feeding the birds a cube of cucumber (approximately 0.5 cm^3) injected with the relevant substance twice per day. Doses were administered twice per day, with CORT dosed birds receiving a total daily CORT of 7.2µg. To avoid the effects of handling stress, birds were trained to enter a dosing box apparatus (a plastic mesh box affixed to the front of the home cage) in order to receive their cucumber. All birds therefore remained in the familiar triplets in the home cage during dosing. The rectangular boxes measured 13 x 13 x 23 cm (length x height x depth). Birds entered the dosing box and cucumber was placed next to them via a hole in the box wall and onto a small black dish. ID rings were used to ensure each bird received one dose per trial. Birds learned to enter box from their parents, who were trained to enter the box to obtain cucumber (not dosed) starting when their chicks were PHD 5. All of the fledglings were observed following their parents into the dosing box between PHD 19-23 and then independently entering the dosing box between PHD 28-33.

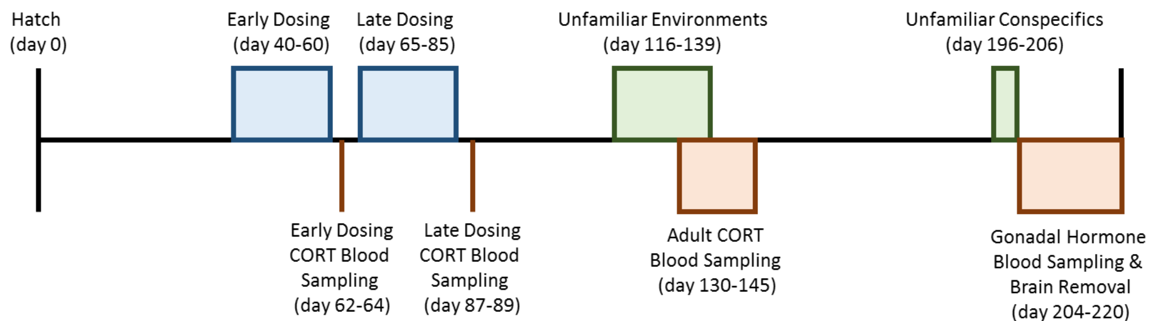

Figure 2.2. Timeline detailing the age at which the birds were dosed with corticosterone (blue), when behavioural testing occurred (green), and when blood sampling occurred and brains were removed (red).

2.2.6. Behavioural measures

Adolescent behaviour. Latency (seconds) to enter the dosing box was recorded for each bird. A bird was considered inside the box when both feet were on the floor of the dosing box.

Individual and group responses to unfamiliar environments. Adult behavioural responses to an unfamiliar environment where quantified when individually housed and when housed in familiar social groups during adulthood (PHD 116-139; see Figure 2.2.). Task order (individual or group context first) was counter-balanced across treatment groups and sexes. Tasks were carried out in white powder coated steel cages measuring 120 x 50 x 50 cm (length x height x depth) that were identical to the home cage, but twice the length. Cages were split into two zones each measuring 60 x 50 x 50 cm (length x height x depth) by placing an opaque white wrought iron divider down the middle of the cage. One zone was identical to the home cage (two 50 cm plastic perches and *ad libitum* access to seed, water, and grit) and was therefore considered a familiar environment. The other zone was largely identical to the home cage (two 50 cm perches, but no seed, water, or grit), but had the addition of three unfamiliar objects attached to the two perches. The side of the cage that was ‘familiar’ or contained the unfamiliar objects was counter-balanced across treatment groups and sexes. In the individual context the objects were a pink ball, a pyramid of three coloured blocks, and two intertwined dark blue pipe cleaner rings. In the group context the objects were a yellow tub, a green pipe cleaner

helix, and a 'U' of coloured blocks. A new set of unfamiliar objects was used in each context in order to prevent habituation to the unfamiliar objects across contexts. Unfamiliar objects had a black dish of shredded spinach (identical to those used for weekly spinach supplements) next to them to encourage birds to interact with the objects. A schematic diagram of each unfamiliar environment cage can be found in Figure 2.3. A pilot study was conducted on stock adult birds (n = 6M, 6F) to see whether the objects were sufficiently aversive. Object arrangements used resulted in a mean latency (minutes) to enter the unfamiliar environment of 21.09 mins (SD = 5.481) with individual context objects and 22.32 mins (SD = 4.42) with group context objects.

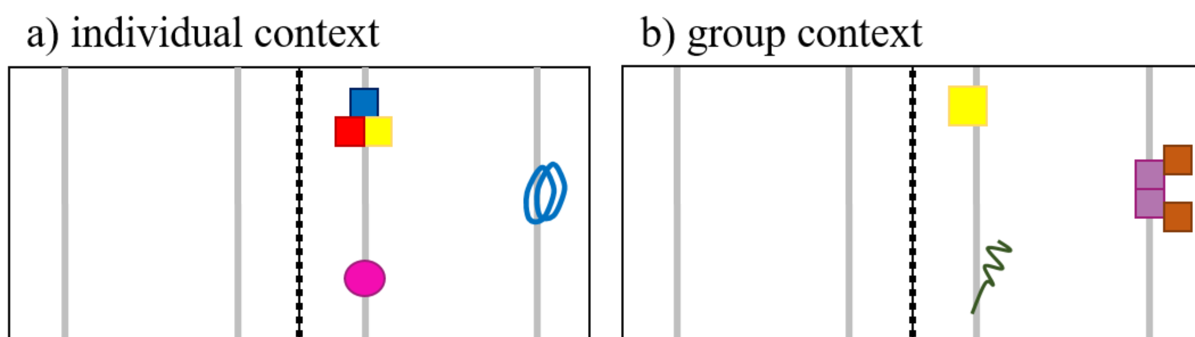

Figure 2.3. Schematic diagram of the unfamiliar environments used in an a) individual context and b) group context. Within each diagram, a familiar environment is shown on the left and the unfamiliar object filled environment on the right. A dashed line shows where a removable divider was placed to separate the familiar and unfamiliar environments.

In the individual context, birds were captured from their home cages and single housed in the 'familiar' environment in the unfamiliar environment task cages placed in a room separate from the colony room. All three birds from a single cage were captured at the same time and housed in the task cages whilst in acoustic, but not visual, contact with one another. One home cage of birds (i.e. three birds) was housed in the task room at any given time. Birds had *ad libitum* access to food and water, a grit tray, and a water bath as in the home cage. Birds were left 24 hours to habituate to the task cages before the task began and at no point could see the unfamiliar environment. In the group context, instead of capture and single housing, birds

were captured and immediately returned to their colony room home cage with their familiar cage mates in order to control for any handling effects across contexts.

A video camera (Sony Handycam, HDR-PJ24OE) was placed around 60 cm in front of each task cage. Prior to the task, birds were recorded for 30 minutes in order to quantify locomotor and risk assessment behaviour (i.e. pre-exposure). Locomotor behaviour recorded was *number of hops between perches* and risk assessment behaviour recorded was *number of ninety degree head turns*. After 30 mins pre-exposure recording had elapsed, the opaque white divider was removed from each of the three cages to expose the birds to the unfamiliar environment (i.e. exposure). Birds were free to explore the unfamiliar environment for sixty minutes during which *latency to enter the unfamiliar environment (seconds)*, *number of entries into the unfamiliar environment*, *duration of time spent perching in the unfamiliar environment (seconds)*, *number of object contacts*, *duration of time spent in contact with the objects (seconds)*, *total number of hops between perches*, and *total number of ninety degree head turns* were recorded. A bird was considered inside the unfamiliar environment if both feet were in the unfamiliar environment, and considered to be in contact with an object when physically touching any of the objects. In a group context, additional measures were taken of social behaviour throughout the task, namely: *number of allopreens*, *number of beak fence bouts*, *number of clumps*, and *duration of time perching alone (seconds)*. These behaviours are defined in Table 2.1. All behaviour were recorded manually, with a stopwatch used to record durations. After sixty minutes had elapsed the birds were ushered back into the familiar environment and the divider replaced. Birds were then captured and returned to their home cages in their familiar groups of three.

Table 2.1. Description of the social behaviour coded during the group context response to an unfamiliar environment and when housed with unfamiliar conspecifics based on the definitions in Alger, Juang, & Riters (2011), Goodson & Adkins-Regan (1999), Ruploh et al. (2014), and Zann (1996).

Behaviour	Definitions
Perching alone	A bird is on a perch with no other bird on the perch
Ground alone	A bird is on the ground with no other bird on the ground
Allopreen	A bird grooms the neck and/or head of another bird with its beak
Clumping bout	Two birds sit in contact with one another for at least 5 seconds
Displacement	A bird hops at another bird causing the receiving bird to hop away; can be given or received
Beak fence bout	Two birds jab their beaks together for at least 2 seconds
Directed song bout	A male looks at a female and emits a stereotyped series of calls for at least 2 seconds
Mount	A male hops on top of a female as an attempt to copulate

Unfamiliar conspecifics task. Behavioural responses of experimental birds to a mixed-sex group of unfamiliar conspecifics were quantified between PHD 196-206 (see Figure 2.2.). A total of 24 birds (12 male, 12 female) were used to create the mixed-sex groups, with each group consisting of two male and two females. Birds in the mixed-sex groups were selected from an in-house stock and had been housed together for at least thirty days before testing began and were therefore considered familiar to one another. Each mixed-sex group was housed in a white powder coated steel cage measuring 60 x 50 x 50 cm (length x height x depth) that was identical to the home cages, but in a room separate from the colony room. Each unfamiliar social group was in auditory, but not visual contact, with one another.

To begin the task, test birds (i.e. three home cage birds) were captured and placed in separate cages each containing an unfamiliar mixed-sex group. Each group was then video

recorded (Sony Handycam, HDR-PJ24OE) for sixty minutes starting when a test bird was placed in the cage from a distance of around 60 cm using a separate camera for each group. The social behaviour of the bird introduced into each group was then quantified (see Table 2.1. for definitions). Affiliative and gregarious behaviour patterns recorded were: *number of allopreens*, *number of clumps*, *duration of time perching alone (seconds)*, and *duration of time on ground alone (seconds)*. Antagonistic behaviour patterns recorded were: *number of beak fence bouts*, *duration of beak fence bouts (seconds)*, *number of displacements given*, and *number of displacements received*. Each antagonistic behaviour with an unfamiliar bird was individually recorded and then summed for each unfamiliar bird sex. Courtship behaviour recorded were: *number of directed song bouts emitted by males*, *number of directed song bouts received by females*, *total duration of song bouts emitted by males (seconds)*, *total duration of song bouts received by females (seconds)*, *number of mounts given by males*, and *number of mounts received by females*. After sixty minutes had elapsed, the three test birds were captured and returned to the home cages in their familiar triplets. Each unfamiliar social group was introduced to one unfamiliar test bird per day over eleven consecutive days.

2.2.7. Hormone sampling

Plasma corticosterone, testosterone, and estradiol concentrations were determined as described below. CORT concentration in response to a standard capture-restraint stressor (Wingfield et al., 1997) was quantified at two age points, first three days after each dosing period had ended (E-SAL and E-CORT birds at PHD 63, L-SAL and L-CORT birds at PHD 88) and then in adulthood between PHD 130-145 (see Figure 2.2.). Basal concentrations of testosterone in males and estradiol in females were determined once between PHD 204-220 (see Figure 2.2.).

Corticosterone. White curtains were hung in the holding room prior to blood sampling so the birds could not see the experimenter catching another cage of birds; a disturbance that might

affect CORT concentration in birds yet to be sampled. Curtains were hung 48 hours prior to blood sampling so the birds could habituate to the curtains. On the sampling day, all three birds in a home cage were captured and transferred in a carry cage to a separate room for blood sampling. A brachial vein of each bird was punctured with a 27-gauge needle to collect a basal blood sample (approximately 40µl). Basal samples were collected within three minutes of entering the colony room to ensure that a basal concentration was determined (Romero, 2004). Blood samples were collected into heparinised capillary tubes and then placed into an Eppendorf on wet ice. Birds were then placed into separate black cloth bags to induce restraint stress. At 10 and 30 minutes after entering the colony room, birds were removed from their black cloth bags and further blood samples (approximately 30µl) were taken as described above. The time points were chosen as previous work in zebra finches has revealed that 10 and 30 minutes reflect peak and post-peak CORT concentration, respectively, in response to a restraint stressor (Spencer et al., 2009). After the final sample was taken, birds were returned to their home cages in their familiar triplets. Blood samples were then centrifuged (3500g for 10 minutes) to separate the plasma and red blood cells. Plasma was then removed and placed into a separate Eppendorf tube that was then stored at -20°C.

Gonadal hormones. Curtains were hung in the holding room like that described for CORT sampling. On the sampling day, all three birds in a home cage were captured and transferred to a separate room for blood sampling. A brachial vein was punctured with a 27-gauge needle tip and 100µl of blood was collected into heparinised capillary tubes. All samples were collected within four minutes of entering the holding room to ensure basal testosterone concentration was collected (Wingfield & Wada, 1989). Blood samples were placed into an Eppendorf on wet ice until the samples could be centrifuged (3500g for 10 minutes) to separate the plasma and red blood cells. Plasma was then removed and placed into a separate Eppendorf tube that was then stored at -20°C.

2.2.8. Hormone assays

Corticosterone. Radioimmunoassay was used to quantify CORT in 10-30µl of plasma as described in Spencer et al. (2009). All samples were extracted with 1ml diethyl ether after being spiked with 25µl of [1,2,6,7-3H]-CORT label (Perkin Elmer Inc., UK). Extracted samples were evaporated at 42°C and reconstituted in 300µl of assay buffer (0.01M PBS, pH 7.4, 0.25% BSA). 50µl aliquots of the reconstituted samples were used to determine the extraction efficacy, which ranged between 71.24-100%. CORT concentration was then determined in two 100µl aliquots of the reconstituted samples using anti-CORT antiserum (Esoterix Endocrinology, USA, B3-163; 1:15000 dilution in assay buffer) and [1,2,6,7-3H]-CORT label (Perkin Elmer, UK). The reactions were incubated for 24 hours at 4°C, and the unbound antigens were then removed by the addition of 100µl of a dextran coated charcoal suspension (0.5% charcoal, 0.25% dextran in assay buffer) and centrifuging the samples at 2000g for 20 minutes. The supernatant was then removed and used to quantify the bound antigens using a radioactivity counter (Packard Tri-Carb 1600 TR Liquid Scintillation Analyser, Perkin Elmer Inc., UK). A total of six assays were performed, with all samples from a single individual run in duplicate in the same assay. Treatment groups and sexes were spread across the assays. Each assay included a ten point standard curve ranging from 0.04-20 ng/ml. 50% binding (ng/ml) were 0.68, 0.64, 0.64, 0.72, 0.65, and 0.73. Intra-assay coefficients of variation (%) were 9.13, 8.3, 11.34, 11.11, 10.07, and 12.42. Inter-assay coefficient of variation (%) was 14.64.

Testosterone. Radioimmunoassay was used to quantify testosterone in 20-30µl of plasma following the protocol described for CORT. However, anti-testosterone antiserum (MP Biomedicals, LLC., USA, 07-189016) and [1,2,6,7-3H]-testosterone label (Perkin Elmer, UK) were used in the testosterone assay. A ten point standard curve ranging from 0.04-20 ng/ml was included in the assay. Extraction efficiency was 75-100%. All samples were run in

duplicate in one assay. 50% binding was 0.38 ng/ml, intra-assay co-efficient of variation was 10.62%, and the detection limit was 0.04 ng/ml.

Estradiol. An enzyme immunoassay kit (Cayman Chemical Company, Estradiol EIA Kit, Ann Arbor, Michigan, USA) that has been previously used to quantify estradiol in zebra finches (Remage-Healey, Maidment, & Schlinger, 2008; Remage-Healey, Dong, Chao, & Schlinger, 2012) was used to determine estradiol concentration in the female plasma samples. Following the manufacturer's guidelines, 10-30µl samples of plasma were diluted in assay buffer to 105µl. 50 µl aliquots were run in duplicate on one plate with an eight point standard curve ranging from 6.6-4000 pg/ml. The plate was read on a Biochrom Anthos 2010 Microplate Reader, ADAP 2.0 (Biochrom Ltd., UK) at a wavelength of 405 nm. Intra-assay coefficient of variation was 12.73% and the detection limit was 6.6 pg/ml.

2.2.9. Neural receptor expression

All birds were injected with around 0.5 ml sodium pentobarbital (Dolethal: Vetoquinol, Buckingham, UK) immediately after the blood samples were taken for gonadal hormones between PHD 204-220 (see Figure 2.2.). The brain was quickly extracted from the skull of each bird (≤ 90 seconds), frozen on dry ice, and then stored at -80°C.

Micro-dissection. Each brain was placed ventral side up into a brain matrix (Roboz Surgical Instrument Co., Gaithersburg, MD, USA) with a 1mm graduated scale. The matrix was placed on top of a mixture of dry and wet ice to keep the brain frozen. A brain atlas for zebra finches (Nixdorf-Bergweiler & Bischof, 2007) and a detailed study of the blue tit brain (*Cyanistes coeruleus*: Montagnese, Szekely, Csillag, & Zchar, 2015) were then used as guides for micro-dissection. The blue tit atlas was used as blue tits have a similarly sized brain to that of zebra finches, and the blue tit guide was more detailed than that for zebra finches. Starting at the rostral orientation, a razor blade was used to make a coronal slice 1mm into the brain and a

second razor blade was used to make a coronal slice 2mm into the brain. The 1mm coronal section was then removed from the brain and two bilateral punches (each 1mm in diameter) were obtained from one area that included BNST, nucleus accumbens, and lateral septum (i.e. BNS punch; abbreviation of BNST, nucleus accumbens, and septum). The BNS punch contained all of these regions as the individual regions were too small to punch and would not have yielded enough RNA for qPCR. Starting from the rostral orientation, a razor blade was placed 4mm into the brain and a second razor blade was placed 6mm into the brain. The 2mm coronal section was then removed from the brain and two 1mm bilateral punches were obtained for each of three brain regions corresponding to the medial amygdala, hippocampus, and nidopallium caudolaterale. A single 1mm punch was taken from a posterior portion of the section corresponding to the medial hypothalamus. The five samples were immediately placed in separate Eppendorf's and kept on dry ice until they could be stored at -80°C.

qPCR. Total RNA was extracted from each sample using Absolutely RNA Miniprep kits (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's guidelines for extracting extremely small samples. Extracted samples were eluted in 60µl (30µl x2) of warmed (60°C) elution buffer. Quantity and integrity of RNA were determined using an RNA 6000 Pico assay kit with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) following the manufacturer's guidelines. Mean RIN was 8.50 (range 4.7–10). Some samples had low quality and/or insufficient RNA and therefore could not be kept in the experiment (23.1% of BNS samples, 21.5% of hypothalamus samples, 4.6% of hippocampus samples, and 0% of amygdala and nidopallium samples were excluded). Final sample sizes for each group and for each brain region used for qPCR are summarised in Table 2.2. After RNA measures were quantified, samples were stored at -80°C. RNA was reverse transcribed to produce cDNA using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, USA) following the manufacturers guidelines for the use of random primers. Extraction and reverse transcription

can contribute to inter-sample variation (Kozera & Rapacz, 2013; Vandesompele et al., 2002). A GeNorm kit (PrimerDesign, Southampton, UK) was therefore used to determine a reference gene that was stable in brain tissue across the adolescent conditions that could then be used to scale each sample to an endogenous control. Experimental conditions can affect reference gene expression (Kozera & Rapacz, 2013), but selecting a stable reference gene allows the detection of a reference gene that was likely unaffected by adolescent condition. The GeNorm kit was performed for six reference genes available for zebra finches (ATP5B, B2M, RPS13, RPL30, ACT5C, & GAPDH) on brain tissue from 16 birds (2 male, 2 female from each adolescent group). GAPDH was determined as the most stable reference gene (M value = 0.6).

Table 2.2. Final sample sizes used for qPCR split by each brain region and adolescent dosing condition.

Region	Adolescent Condition				Total
	E-SAL	E-CORT	L-SAL	L-CORT	
BNS	12	17	10	11	50
Hypothalamus	8	16	15	12	51
Hippocampus	14	17	16	15	62
Amygdala	14	18	18	15	65
Nidopallium	14	18	18	15	65

Specific hydrolysis probes were designed and validated by PrimerDesign for the genes of interest based on the published zebra finch genome. GenBank Accession No. for the genes of interest were: GR (XM_002192952), MR (NM_001076690), VT1 (XM_002195382), VT3 (XM_002188266), and GAPDH (NM_001198610). qPCR reactions for each reference gene within each brain region for each individual were run in duplicate on BrightWhite real-time PCR plastic plates (PrimerDesign, UK). Each well contained 10µl of x2 Brilliant III Ultra-Fast QPCR MasterMix (Agilent Technologies, USA), 1µl of reference gene specific hydrolysis primer, and 9µl of the appropriate cDNA. To determine the optimal concentration of cDNA, standard curves were performed on the same samples that were used for the GeNorm kit. qPCR output from a 10 point standard curve (range 25ng to 50pg total cDNA) revealed that the

quantity of cDNA necessary to detect genes of interest (Ct value below 30) was 300pg total cDNA. R^2 for each gene of interest were: GAPDH = 0.955, GR = 0.932, MR = 0.943, VT1 = 0.934, VT3 = 0.942. qPCR reactions were carried out on a Stratagene MX 3005P (Agilent Technologies) at 95°C for 3 minutes, then 40 cycles of 95°C for 20 seconds and 60°C for 20 seconds with fluorescence collected via the FAM channel at the end of each cycle. The delta Ct method was used to quantify expression of GR, MR, VT1, and VT3 relative to GAPDH expression with the formulae $2^{-(\text{Ct for gene of interest} - \text{Ct for GAPDH})}$ (Dorak, 2006).

2.2.10. Data Analysis

SPSS version 22 was used to conduct all analyses. After performing each test, residuals were checked for normality (Shapiro-Wilk, $p > 0.05$). Variables with residuals that were not normally distributed due to a positive skew in their distribution were either square root or log10 transformed to achieve normality where stated. Variables that could not be transformed to achieve a normal distribution were analysed using generalized linear models. Nest ID and brood size were entered as random factors in all mixed models to control for pre-adolescent variation. In adolescent dosing box latency model and behavioural response to an unfamiliar environment group context mixed models, home cage ID was entered as a random factor to control for the effects of cage mates on individual bird behaviour. In the behavioural response to unfamiliar conspecifics mixed models, test cage ID was entered as a random factor to control for the effect of being housed with different groups. Statistically significant effects were identified with $p < 0.05$. Sidak and Bonferroni post hoc tests were used to further investigate any significant effects for independent and repeated measures, respectively. Cohen's d was calculated as a measure of effect size for all significant post hoc pairwise comparisons. All data presented are mean $\pm$ one standard error of the mean.

Adolescent behaviour. Latency to enter the dosing box was calculated as the mean of the two dosing sessions on each day, with latency for each twenty dosing days reduced to five time

blocks by calculating the mean latency of trials for every consecutive four days, i.e. 1-4, 5-8, 9-12, 13-16, and 17-20. Latency to enter the dosing box for the five time blocks was entered as a dependent variable in a linear mixed model (LMM) with adolescent condition, sex, and time block entered as fixed factors (main effects and interactions). Time block (1-5) was also entered as a repeated measure to account for the non-independence of latencies across blocks.

Individual context unfamiliar environment. Square root latency to enter the unfamiliar environment, square root number of entries into unfamiliar environment, square root duration of time perching in unfamiliar environment, square root number of hops between perches during exposure, and number of ninety degree head turns during exposure were entered as dependent variables in separate LMMs. Adolescent condition and sex (main effects and interaction) were entered as fixed factors in all models. For hops and head turn exposure models, pre-exposure number of hops and number of head turns were entered as co-variates to control for any differences in behaviour prior to the task. No differences were found in pre-exposure measures (see Appendix A Table A.1. and Table A.2. for analysis and output). Number of object contacts, and duration of object contacts were not analysed as the behaviour occurred too infrequently (i.e. fewer than 1 in 10 birds displayed each behaviour).

Group context unfamiliar environment. Square root latency to enter unfamiliar environment, square root number of entries into unfamiliar environment, square root duration of time perching in unfamiliar environment, square root number of hops between perches, square root number of head ninety degrees turns, and duration of time perching alone were entered as dependent variables in separate LMMs. Adolescent condition and sex (main effects and interaction) were entered as fixed factors in all models. For hops and head turn exposure models, pre-exposure number of hops and number of head turns were entered as co-variates to control for any differences in behaviour prior to the task. No differences were found in pre-exposure measures (see Appendix A Table A.1. and Table A.2. for analysis and output).

Allopreening, beak fencing, clumping, and perch displacements, number of object contacts, and duration of object contacts were not analysed as the behaviour occurred too infrequently (i.e. fewer than 1 in 10 birds displayed each behaviour).

Unfamiliar conspecifics. Exploratory correlations (Spearman's rank) revealed some behaviour to be correlated. Number and duration of beak fence bouts were highly correlated ($r = 0.96$, $p < 0.001$) and therefore only one variable, duration of time beak fencing, was analysed. Song bout number, song bout duration, and mount number variables were correlated within each sex (Table 2.3.). As the two song variables were highly correlated, song bout duration was omitted from subsequent analyses. Principal component analyses revealed that number of song bouts and number of mounts could be reduced to a single variable for males (KMO = 0.500; Bartlett's Test, χ^2 (1) = 4.127, $p = 0.042$) and females (KMO = 0.500; Bartlett's Test, χ^2 (1) = 4.324, $p = 0.038$). The PCA model was used to create a new component (i.e. courtship behaviour) score between the variables. Allopreening and clumping occurred too infrequently (i.e. fewer than 1 in 10 birds displayed each behaviour) and were therefore not analysed.

Table 2.3. Correlations between courtship behaviour quantified during the unfamiliar conspecifics task split by sex.

Variables	Male		Female	
	r	p	r	p
Song bout number and song bout duration	0.928	<0.001	0.960	<0.001
song bout number and mount number	0.406	0.014	0.448	0.013
song bout duration and mount number	0.487	0.003	0.461	0.010

Duration of time alone was entered as a dependent variable in a LMM with location (perch, ground), adolescent condition, and sex entered as fixed factors (main effects and all interactions). Location was also entered as a repeated measure to control for non-independence of time alone between locations. Log10 duration of time spent beak fencing, number of times a test bird displaced an unfamiliar bird, and log10 number of times a test bird was displaced by

an unfamiliar bird were entered as dependent variables in separate LMMs with adolescent condition, test bird sex, and unfamiliar bird sex entered as a fixed factors (main effects and interactions). In beak fencing and displacement models, unfamiliar bird sex was also entered as a repeated measure to control for non-independence of each behaviour between unfamiliar males and females. Male courtship behaviour and female courtship factor scores were entered as dependent variables in separate LMMs with adolescent condition entered as a fixed factor. Variables excluded in response to PCA analysis, i.e. number of beak fence bouts and duration of song bouts, were analysed in an identical manner to their respective included variables as described above. Models for included and excluded variables within each behaviour (i.e. beak fencing and courtship) had similar outputs (data not shown), indicating that excluding these variables did not qualitatively affect the results.

Hormones. Log10 CORT concentration (ng/ml) was analysed in a LMM with adolescent condition, sampling time (0, 10, and 30 min), age (adolescent adult), and sex entered as fixed factors (main effects and interactions). Sampling time and age were also entered as repeated measures. Highest CORT concentration (i.e. highest concentration at either 10 or 30 mins) was analysed to further investigate the physiological stress response. Highest CORT concentration (ng/ml) was entered as a dependent variable in a LMM with adolescent condition, age, and sex entered as fixed factors (main effects and interactions). Age was also entered as a repeated measure (to control for the non-independence of CORT across ages) and basal CORT was entered as a co-variate (to control for initial CORT concentration). Log10 male testosterone concentration (ng/ml) and raw female estradiol concentration (pg/ml) were entered as dependent variables in separate LMMs with adolescent condition entered as a fixed factor.

qPCR. Each gene of interest (GR, MR, VT1, and VT3) within each brain (BNS, hypothalamus, hippocampus, amygdala, and nidopallium caudolaterale) region was analysed in separate LMMs that had adolescent condition and sex (main effects and interaction) entered as fixed

factors. For the BNS, all genes of interest were square root transformed; for the hippocampus, GR was log10 transformed and all other genes were square root transformed; for the hypothalamus and amygdala, only VT1 was square root transformed and raw data were used for the remaining variables; and for the nidopallium caudolaterale, GR and MR were square root transformed and raw data were used for the remaining variables.

GR/MR ratio was calculated by dividing GR by MR for each bird within each brain region. GR/MR ratio was entered as a dependent variable in separate models for each brain region. GR/MR ratio was square root transformed in BNS and hippocampus models and log10 transformed in the hypothalamus model. Amygdala and nidopallium caudolaterale GR/MR ratio were analysed using generalized linear mixed models with data fitted to a gamma distribution with a log link function as the residuals could not be transformed to normality.

Correlations. As hormones mediate the relationship between brain and behaviour, the correlations between hormone concentrations with both behavioural and neural receptor variables were assessed using Spearman's rank correlation. Models were conducted separately for adolescent and adult variables, with models then split by sex if sex-specific effects occurred. Adolescent CORT measurements were correlated with adolescent latency to enter the dosing box. Adult CORT and gonadal hormone variables were correlated with behaviour and neural receptor variables in which a main effect or interaction implicating adolescent condition were found to see if variation in hormone concentration was related to the observed effects. Bonferroni corrections were applied to account for multiple comparisons.

2.3. Results

2.3.1. Adolescent behaviour

E-CORT birds took longer to enter the dosing box with each subsequent dosing block. Latency to enter the dosing box differed between dosing conditions ($F_{3,14.650} = 6.261$, $p = 0.006$), time blocks ($F_{4,58} = 9.831$, $p < 0.001$), and an interaction was found between dosing condition and time block ($F_{12,58} = 11.578$, $p = < 0.001$; Figure 2.4.). Post hoc tests exploring the interaction revealed that E-CORT birds took longer to enter the dosing box than all other groups during time block 3 (E-SAL: $p = 0.032$, $d= 0.86$; L-SAL: $p = 0.029$, $d = 1.15$; L-CORT: $p = 0.035$, $d = 0.92$), time block 4 (E-SAL: $p = 0.001$, $d = 1.62$; L-SAL: $p = 0.002$, $d = 1.32$; L-CORT: $p < 0.001$, $d = 1.67$), and time block 5 (E-SAL: $p < 0.001$, $d = 2.30$; L-SAL: $p < 0.001$, $d = 2.06$; L-CORT: $p < 0.001$, $d = 2.63$). Sex did not influence any variable (sex: $F_{1,14.650} = 0.032$, $p = 0.860$; sex x condition: $F_{3,14.650} = 2.258$, $p = 0.125$; sex x session: $F(_{4,58}) = 0.341$, $p = 0.849$; sex x condition x session: $F_{12,58} = 0.822$, $p = 0.627$).

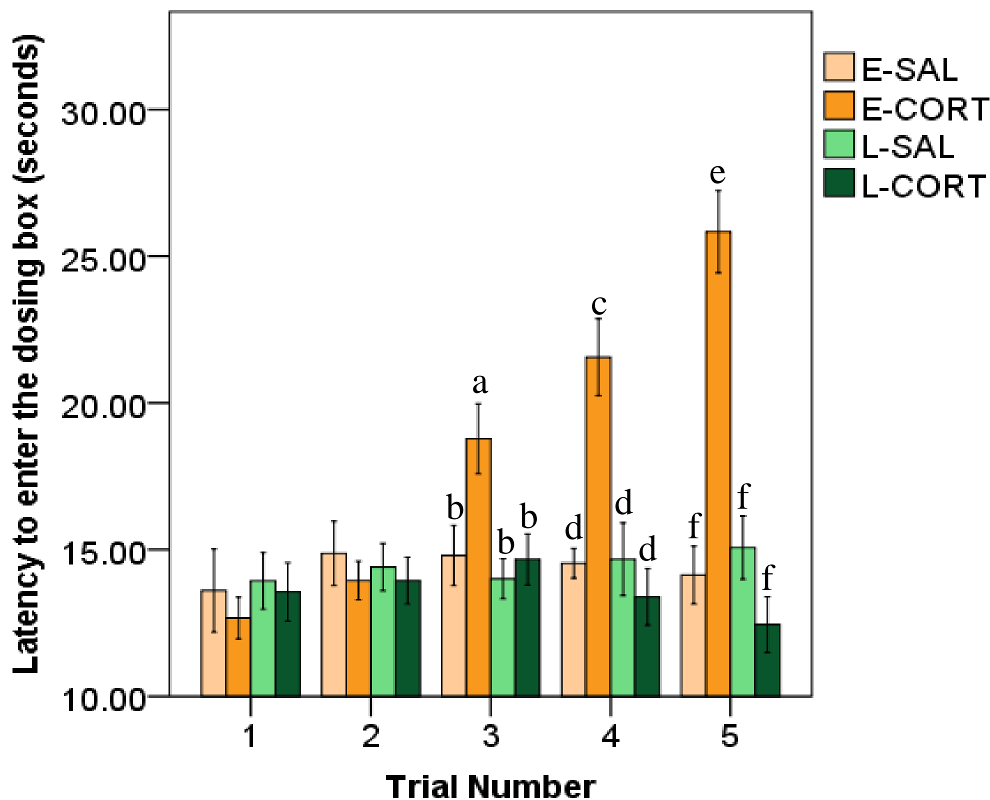

Figure 2.4. Latency to enter the dosing box (seconds) across the five time blocks split by adolescent dosing condition. Data presented are mean + one standard error of the mean. Letters denote significant differences ($p < 0.05$) within each time block, i.e. a vs. b, c vs. d, e vs. f.

2.3.2. Adult behavioural responses to unfamiliar environments

In the individual context unfamiliar environment task, E-CORT birds took longer to enter the unfamiliar environment than birds from other dosing conditions (dosing condition: $F_{3,58}$ = 4.095, p = 0.011; E-CORT vs. E-SAL: p = 0.048, d = 1.08; E-CORT vs. L-SAL; p = 0.030, d = 0.88; E-CORT vs. L-CORT: p = 0.047, d = 1.02; Figure 2.5.1.a). However, individual context latency was not affected by sex (sex: $F_{1,58}$ = 1.486, p = 0.228; dosing condition x sex: $F_{3,58}$ = 0.508, p = 0.678). No effects were found on latency to enter the unfamiliar environment in the group context (adolescent group: $F_{3,58}$ = 0.311, p = 0.817; sex: $F_{1,58}$ = 2.078, p = 0.155; adolescent group x sex: $F_{3,58}$ = 2.129, p = 0.106; Figure 2.5.1.b).

Birds entered the unfamiliar compartment a similar number of times regardless of dosing condition and sex in both an individual context (adolescent condition: $F_{3,58}$ = 1.540, p = 0.214; sex: $F_{1,58}$ = 0.009, p = 0.925; adolescent condition x sex: $F_{3,58}$ = 0.777, p = 0.511; Figure 2.5.2.a) and a group context (adolescent condition: $F_{3,58}$ = 0.367, p = 0.777; sex: $F_{1,58}$ = 0.164, p = 0.687; adolescent condition x sex: $F_{3,58}$ = 1.591, p = 0.201; Figure 2.5.2.b).

In the individual context, E-CORT birds spent more time perching in the unfamiliar environment than birds from other dosing conditions ($F_{3,58}$ = 13.056, p < 0.001; E-CORT vs. E-SAL: p < 0.001, d = 1.42; E-CORT vs. L-SAL: p < 0.001, d = 1.26; E-CORT vs. L-CORT: p < 0.001, d = 1.58 Figure 2.5.3.a). Individual context duration of time perching in the unfamiliar environment was no different between sexes (main effect, sex: $F_{1,58}$ = 1.569, p = 0.215; interaction, adolescent dosing and sex: $F_{3,58}$ = 1.014, p = 0.393). In contrast to the individual context, birds spent a similar duration of time spent perching in the unfamiliar environment in group context regardless of adolescent dosing and sex (adolescent condition: $F_{3,58}$ = 0.383, p = 0.766; sex: $F_{1,58}$ = 1.124, p = 0.294; adolescent condition x sex: $F_{3,58}$ = 1.345, p = 0.269; Figure 2.5.3.b).

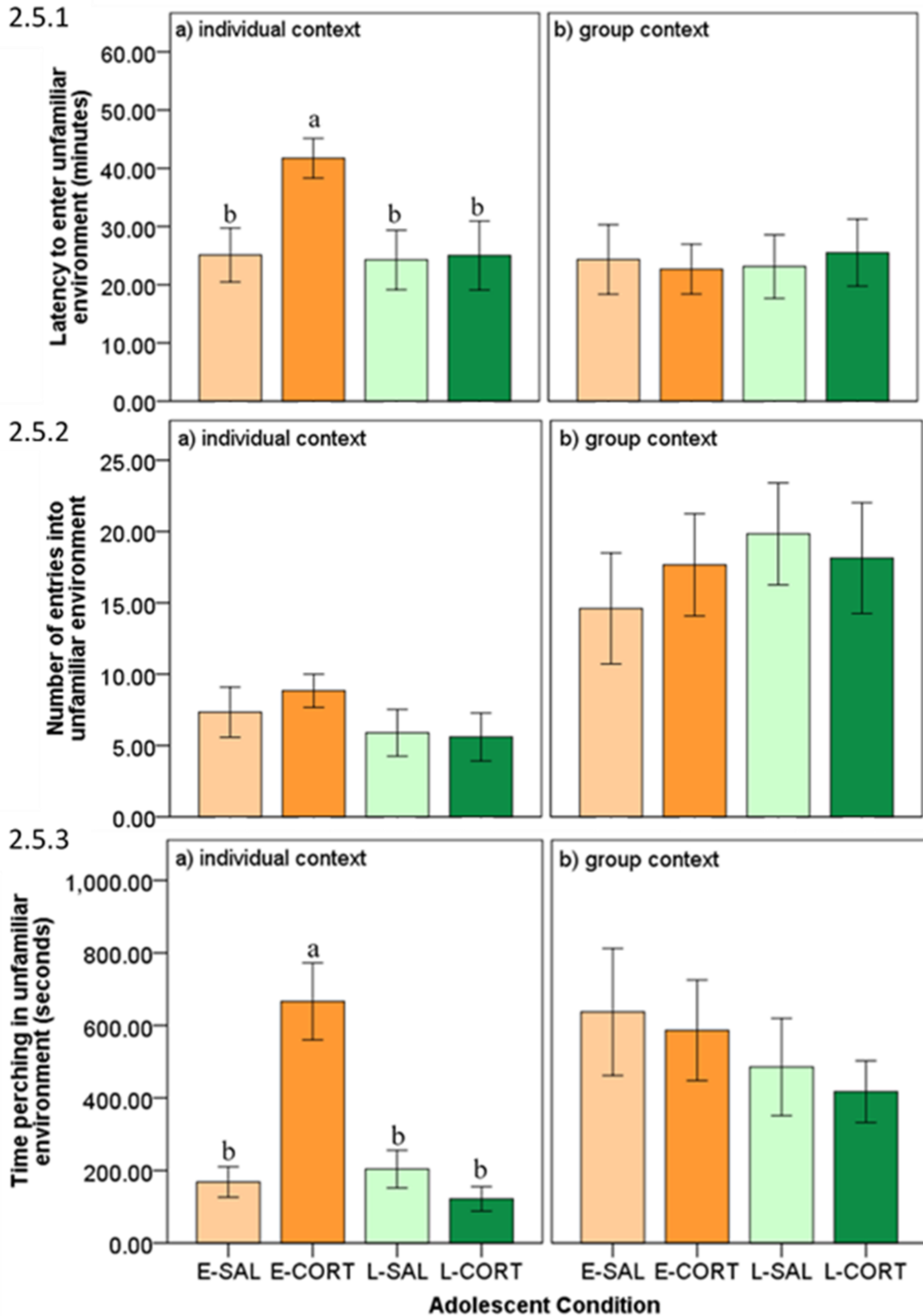

Figure 2.5. Latency (mins) to enter the unfamiliar environment (2.5.1.), number of entries into the unfamiliar environment (2.5.2.), and duration (secs) perching in unfamiliar environment (2.5.3.) split by dosing condition in a) individual context and b) group context. Data presented are mean $\pm$ one standard error of the mean. Significant differences ($p < 0.05$) between conditions within a single behavioural measure in a single context are shown by a vs. b.

Number of hops between perches during exposure did not differ between adolescent conditions in either an individual context ($F_{3,57}$ = 0.290, p = 0.832; Figure 2.6.a) or a group context ($F_{3,57}$ = 0.487, p = 0.693; Figure 2.6.b). In addition, no sex differences were found on number of hops in the individual context (sex: $F_{1,57}$ = 0.002, p = 0.968; sex x group: $F_{3,57}$ = 0.220, p = 0.882) or the group context task (sex: $F_{1,57}$ = 0.929, p = 0.339; sex x group: $F_{3,57}$ = 0.396, p = 0.757).

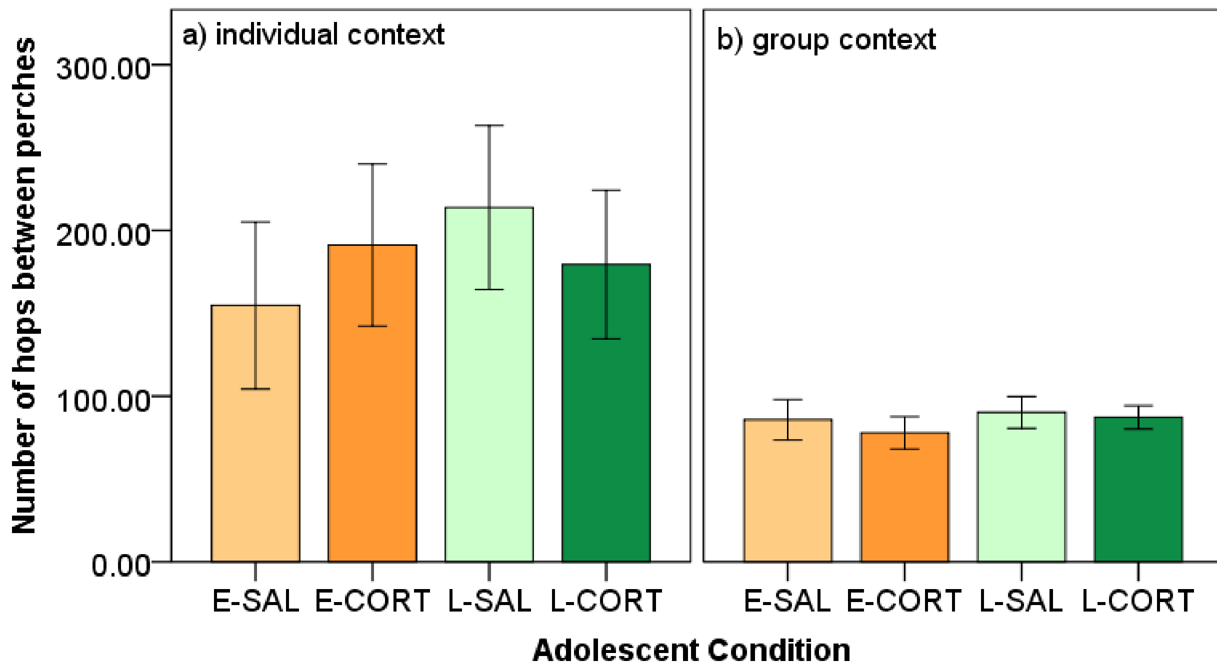

Figure 2.6. Number of hops when exposed to an unfamiliar environment split by adolescent dosing condition in an a) individual context and b) group context. Data presented are mean $\pm$ one standard error of the mean. All comparisons were not significant (p > 0.05).

In the individual context, number of head turns was affected by dosing condition ($F_{3,57}$ = 5.953, p = 0.001) but the effect was dependent on sex (sex x dosing condition: $F_{3,57}$ = 4.824, p = 0.005; Figure 2.7.a). E-CORT females engaged in more head turns when exposed to an unfamiliar environment compared to all other conditions (E-CORT vs. E-SAL, p < 0.001, d= 1.98; E-CORT vs. L-SAL, p < 0.001, d = 2.38; E-CORT vs. L-CORT, p = 0.002, d = 1.72). Males and females overall had a similar number of head turns in the individual context ($F_{1,58}$ = 2.119, p = 0.151). In a group context, number of head turns when exposed to an unfamiliar environment was similar for all birds regardless of dosing condition ($F_{3,57}$ = 0.038, p = 0.990) or sex (sex: $F_{1,57}$ = 0.013, p = 0.908; sex x group: $F_{3,57}$ = 1.188, p = 0.322; Figure 2.7.b).

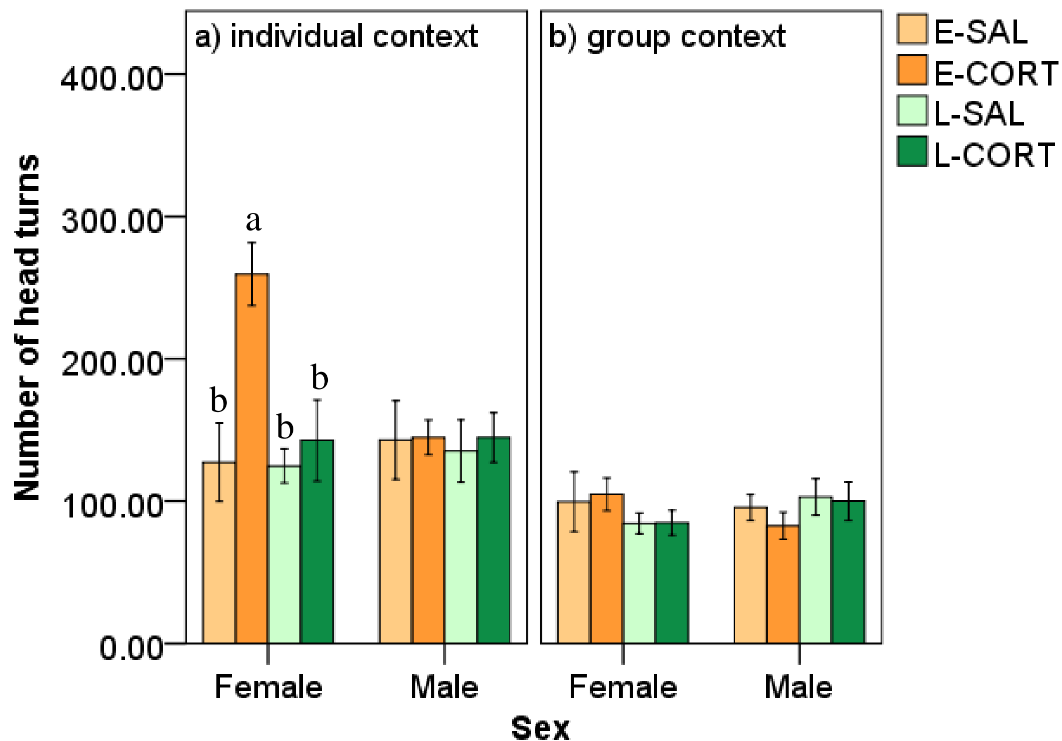

Figure 2.7. Number of head turns when exposed to an unfamiliar environment split by sex and adolescent dosing condition in an a) individual context and b) group context. Data presented are mean $\pm$ one standard error of the mean. Significant differences ($p < 0.05$) between female groups in an individual context are shown, a vs. b.

In the group context, all birds spend a similar number of seconds perching alone when exposed to an unfamiliar environment irrespective of adolescent dosing condition ($F_{3,57} = 1.306$, $p = 0.281$; E-SAL: M = 398.73, SEM = 33.25; E-CORT: 325.44, SEM = 31.04; L-SAL: 396.47, SEM = 40.84; L-CORT: 322.67, SEM = 34.85). Duration of time spent perching alone when exposed to an unfamiliar environment in a group context similarly did not differ according to a bird's sex (sex: $F_{1,57} = 0.557$, $p = 0.459$; sex x condition: $F_{3,57} = 0.340$, $p = 0.796$).

2.3.3. Adult behavioural responses to unfamiliar conspecifics

Time alone. Duration of time alone differed between adolescent dosing conditions at a specific location (dosing condition: $F_{3,57.987} = 3.179$, $p = 0.031$; location: $F_{1,58} = 55.240$, $p < 0.001$; dosing condition x location: $F_{3,58} = 4.781$, $p = 0.005$; Figure 2.8.a,b). Post hoc tests revealed that differences between adolescent conditions were only present when birds perched alone, with CORT dosed birds spending less time alone on the perches compared to birds dosed with saline (E-CORT vs. E-SAL, $p = 0.002$, $d = 1.73$; E-CORT vs. L-SAL, $p = 0.017$, $d = 1.43$; L-CORT vs. E-SAL, $p = 0.002$, $d = 1.42$; L-CORT vs. L-SAL, $p = 0.015$, $d = 1.17$). Duration of tie alone did not differ according to sex (test bird sex: $F_{1,57.987} = 0.039$, $p = 0.845$; test bird sex x dosing condition: $F_{3,57.987} = 0.268$, $p = 0.849$; test bird sex x location: $F_{1,58} = 0.594$, $p = 0.444$; test bird sex x dosing condition x location: $F_{3,58} = 0.361$, $p = 0.781$).

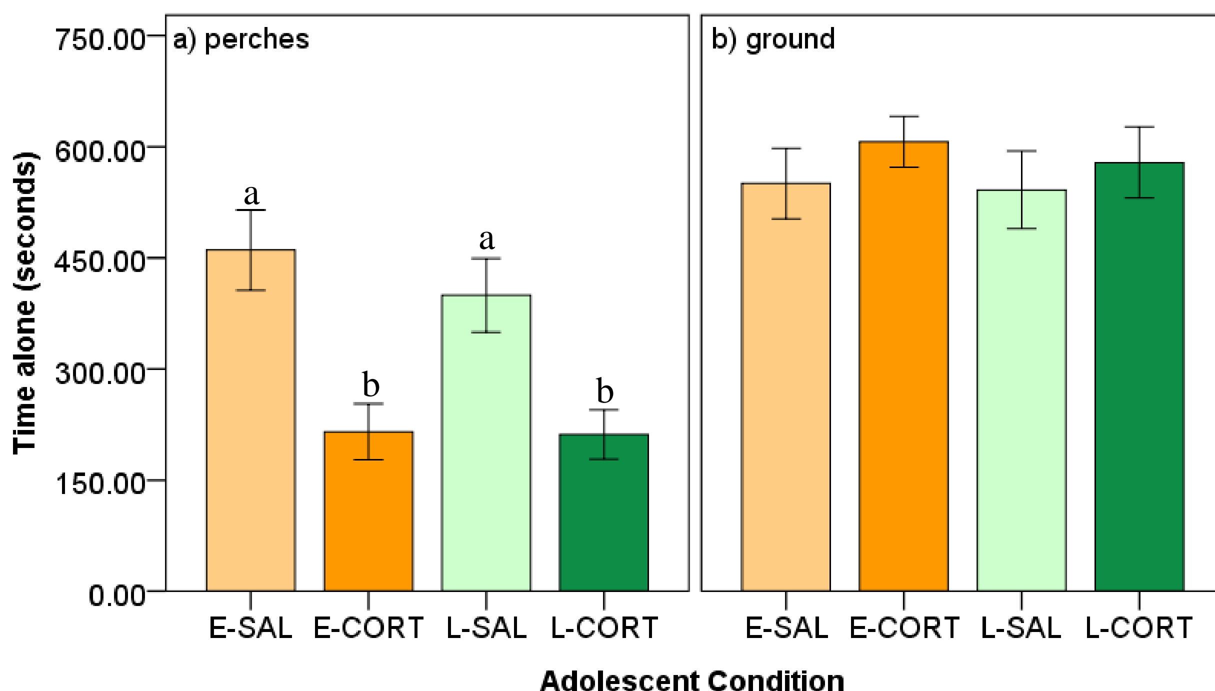

Figure 2.8. Duration of time (seconds) a) perching alone and b) on the ground alone when housed with an unfamiliar group of conspecifics split by dosing condition. Data presented are mean $\pm$ one standard error of the mean. Significant differences ($p < 0.05$) between conditions are shown by a vs. b.

Beak fencing. Male test birds spent more time beak fencing than female test birds ($F_{1,57.608}$ = 5.695, p = 0.020; male: M = 64.42, SEM = 6.25; female: M = 41.43, SEM = 5.41), and unfamiliar males engaged in a longer duration of beak fencing than unfamiliar females ($F_{1,50.417}$ = 30.354, p < 0.001; male: M = 73.44, SEM = 6.60; female: M = 34.50, SEM = 4.42). However, test bird and unfamiliar bird sex did not interact to affect duration of time beak fencing ($F_{1,50.417}$ = 2.114, p = 0.152). All main effects and interactions that investigated the effect adolescent CORT dosing on fencing factor score were not significant (dosing condition: $F_{3,62.161}$ = 0.576, p = 0.633; test bird sex x dosing condition: $F_{3,62.161}$ = 0.569, p = 0.637; unfamiliar bird sex x dosing condition: $F_{3,51.981}$ = 1.957, p = 0.132; test bird sex x unfamiliar bird sex x dosing condition: $F_{3,51.981}$ = 0.556, p = 0.647).

Displacements. Test birds dosed with CORT in late adolescence displaced unfamiliar birds less often than saline dosed birds (dosing condition: $F_{3,58.022}$ = 5.030, p = 0.004: L-CORT vs. E-SAL, p = 0.003, d = 1.22; L-CORT vs. L-SAL, p = 0.046, d = 0.95; Figure 2.9.a), but test birds dosed with CORT in early adolescence displaced unfamiliar birds to the same extent as all other groups (E-CORT vs. E-SAL, p = 0.356; E-CORT vs. L-SAL, p = 0.930; E-CORT vs. L-CORT, p = 0.298). Sex differences were also apparent as male test birds displaced unfamiliar birds significantly more than female test birds ($F_{1,57.996}$ = 5.471, p = 0.023; male: M = 23.50, SEM = 1.56; female: M = 17.38, SEM = 1.34), and test bird displaced unfamiliar males significantly more than unfamiliar females ($F_{1,58.022}$ = 37.346, p < 0.001; male: M = 24.44, SEM = 1.52; female: M = 17.00, SEM = 1.39). All interactions involving test bird sex and unfamiliar bird sex were not significant (test bird sex x unfamiliar bird sex: $F_{1,58.048}$ = 0.751, p = 0.390; test bird sex x dosing condition: $F_{3,57.996}$ = 0.537, p = 0.659; unfamiliar bird sex x dosing condition: $F_{3,57.996}$ = 0.847, p = 0.474; test bird sex x unfamiliar bird sex x dosing condition: $F_{3,58.022}$ = 0.406, p = 0.749).

Number of times a test bird was displaced by an unfamiliar bird was similar in all birds regardless of dosing condition ($F_{3,54.484} = 1.106$, $p = 0.355$; Figure 2.9.b). However, male test birds were displaced by unfamiliar birds more often than female test birds were displaced by unfamiliar birds ($F_{1,55.108} = 4.510$, $p = 0.038$; male: M = 50.88, SEM = 8.50; female: M = 27.18, SEM = 7.11). In addition, test birds were displaced by unfamiliar males more often than unfamiliar females ($F_{1,57.098} = 22.231$, $p < 0.001$; male: M = 62.74, SEM = 10.05; female: M = 17.47, SEM = 3.93). All interactions involving test bird and unfamiliar bird sex were not significant (test bird sex x unfamiliar bird sex: $F_{1,57.098} = 0.081$, $p = 0.776$; test bird sex x dosing condition: $F_{3,54.484} = 1.085$, $p = 0.306$; unfamiliar bird sex x dosing condition: $_{3,56.448} = 0.869$, $p = 0.463$; test bird sex x unfamiliar bird sex x dosing condition: $F_{3,56.448} = 1.102$, $p = 0.356$).

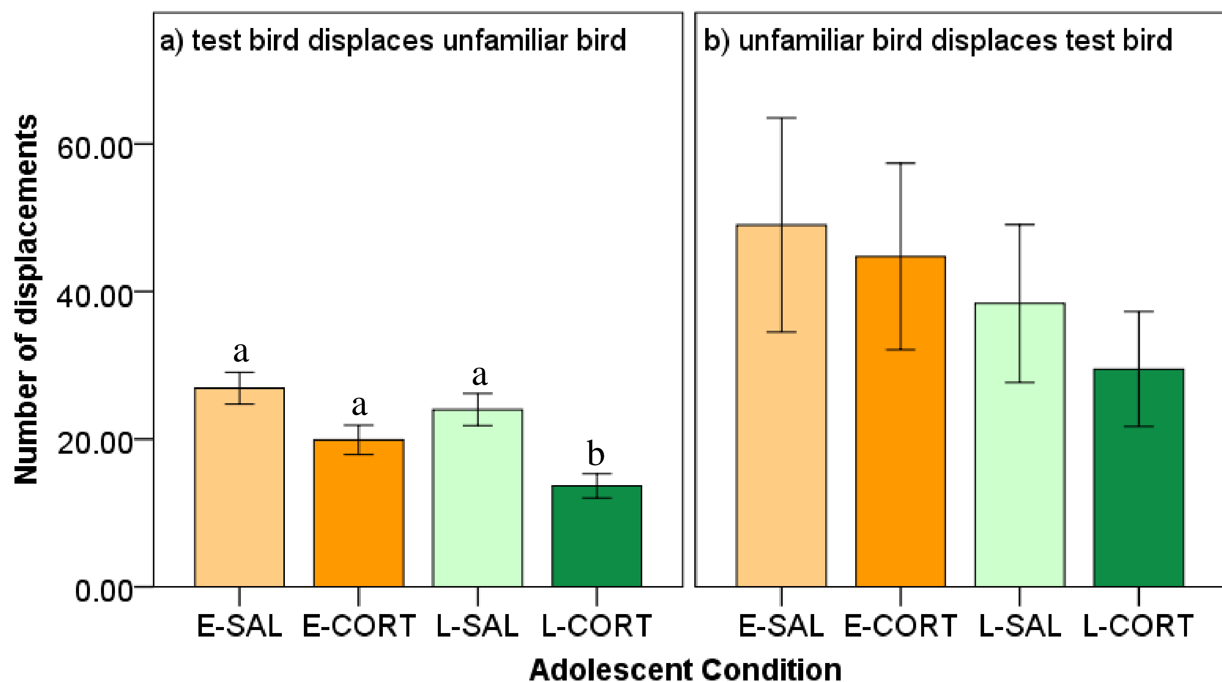

Figure 2.9. Number of times a) a test bird displaces and unfamiliar bird and b) a test bird is displaced by an unfamiliar bird split by adolescent dosing condition. Data presented are mean ± one standard error of the mean. Significant differences ($p < 0.05$) between conditions are shown by a vs. b.

Courtship. Females had similar courtship factor scores regardless of dosing condition ($F_{3,26}$ = 0.143, p = 0.933; Figure 2.10.a). In males, birds dosed with CORT had lower courtship factor scores than males dosed with saline ($F_{3,32}$ = 16.959, p < 0.001: E-CORT vs. E-SAL, p = 0.001, d = 2.51; E-CORT vs. L-SAL, p = 0.011, d = 2.03; L-CORT vs. E-SAL, p < 0.001, d = 2.85; L-CORT vs. L-SAL, p < 0.001, d = 2.22; Figure 2.10.b).

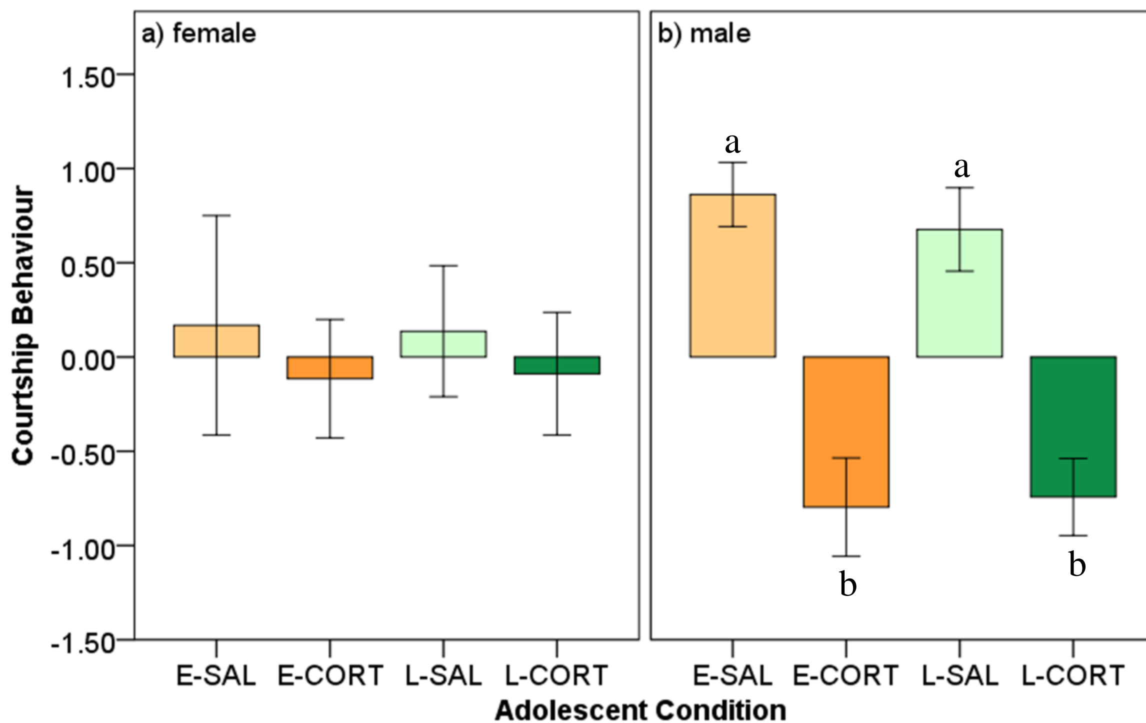

Figure 2.10. Courtship behaviour (number of song bouts and number of mounts) expressed as a component score calculated in a PCA model for a) females and b) males split by adolescent dosing condition. Data presented are mean ± one standard error of the mean. Significant differences (p < 0.05) between conditions are shown by a vs. b.

2.3.4. Hormones

Corticosterone time-response to capture and restraint. Birds had similar CORT concentrations in response to capture and restraint irrespective of a bird's dosing condition or sex (dosing condition: $F_{3,55.9}$ = 0.887, p = 0.454; sex: $F_{1,55.331}$ = 0.286, p = 0.595; dosing condition x sex: $F_{3,56.282}$ = 2.237, p = 0.094). However, CORT concentration was affected by restraint stress and changed across sampling times ($F_{2,58.034}$ = 220.357, p < 0.001; Figure 2.11.). Post hoc tests revealed that when samples were averaged across dosing conditions, ages, and sexes, CORT concentration was higher at 10 mins compared to baseline (p < 0.001, d = 1.21), 30 mins

compared to baseline ($p < 0.001$, $d = 1.78$), and 30 mins compared to 10 mins ($p < 0.001$, $d = 0.65$). CORT concentration was also affected by age ($F_{1,57.942} = 16.378$, p = < 0.001, $d = 0.15$) and the age difference was dependent on sampling time ($F_{2,57.934} = 20.718$, $p < 0.001$). Post hoc tests revealed that when samples were averaged across dosing conditions and sexes, CORT concentration was higher in adolescents compared to adults at baseline ($p < 0.001$, $d = 1.11$) and 10 mins ($p = 0.040$, $d = 0.46$) but no age difference was detected at 30 mins ($p = 0.244$). Age differences at specific sampling times did not further differ due to a bird's dosing condition or sex (dosing condition: $F_{6,57.928} = 0.603$, $p = 0.727$; sex: $F_{2,57.934} = 2.078$, $p = 0.134$; dosing condition x sex: $F_{6,57.928} = 0.377$, $p = 0.890$). Sampling time differences in CORT concentration did not differ according to a bird's dosing condition or sex (dosing condition: $F_{6,58.030} = 0.398$, $p = 0.877$; sex: $F_{2,58.034} = 1.065$, $p = 0.351$; dosing condition x sex: $F_{6,58.030} = 0.502$, $p = 0.804$) and neither did age differences in CORT concentration (dosing condition: $F_{3,57.934} = 0.536$, $p = 0.660$; sex: $F_{1,57.942} = 0.086$, $p = 0.771$; dosing condition x sex: $F_{3,57.938} = 1.238$, $p = 0.304$).

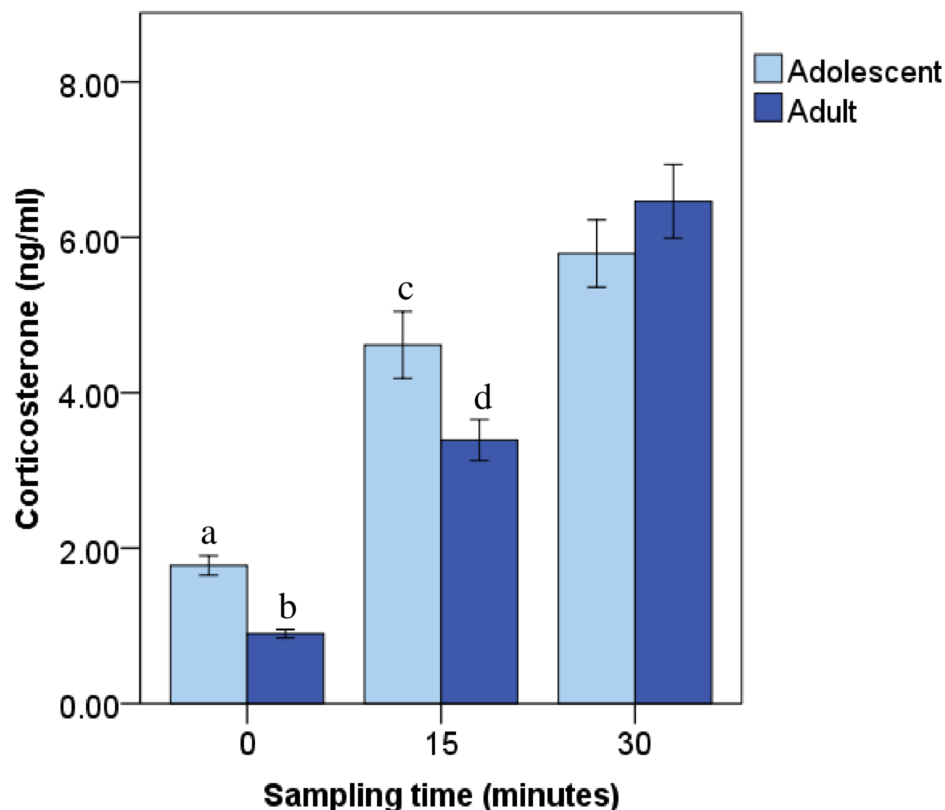

Figure 2.11. Corticosterone concentration (ng/ml) in response to capture and restraint in adolescent and adult birds. Data presented are mean ± one standard error of the mean. Significant differences ($p < 0.05$) within each sampling time are shown: a vs. b, c vs. d.

Highest corticosterone concentration in response to capture and restraint. Highest CORT concentration secreted in response to capture and restraint was not different between adolescent conditions ($F_{3,57.953} = 0.456$, $p = 0.714$; Figure 2.12.a,b) and no differences were found between adolescent conditions when split by age ($F_{3,57.424} = 0.506$, $p = 0.680$), sex ($F_{3,59.416} = 1.241$, $p = 0.303$), or age and sex ($F_{3,57.861} = 0.556$, $p = 0.646$). Similarly, highest CORT concentration was not different between birds according to sex ($F_{3,57.942} = 0.531$, $p = 0.469$), age ($F_{1,67.860} = 2.110$, $p = 0.151$), or an interaction between the two ($F_{1,57.475} = 0.939$, $p = 0.337$).

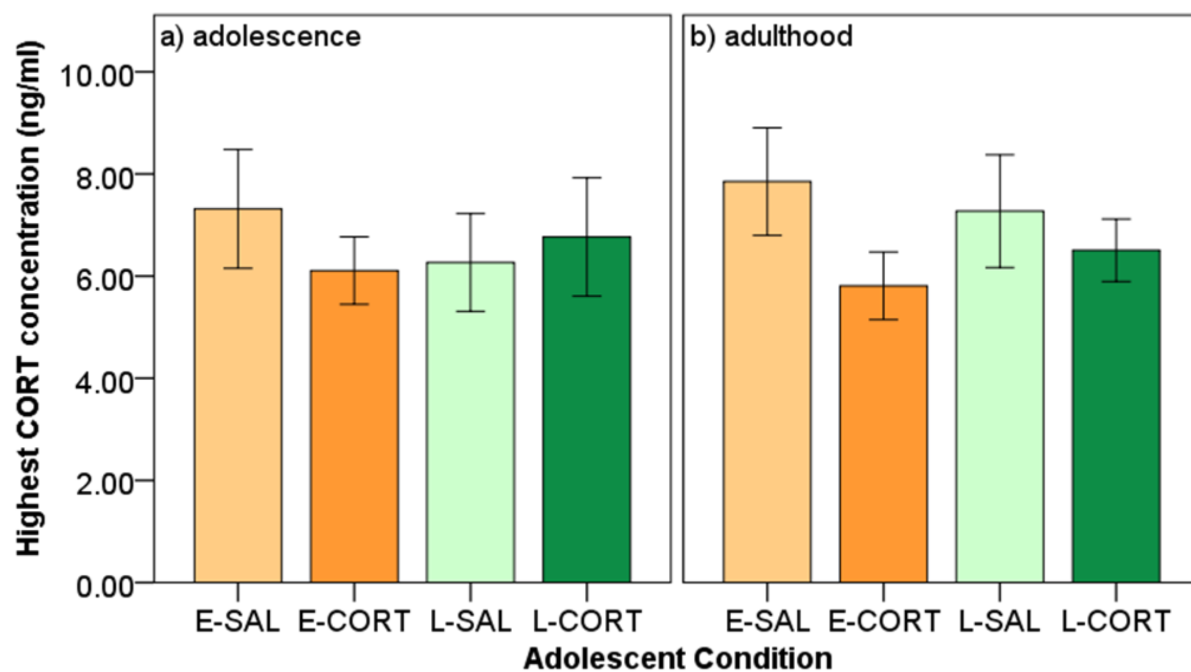

Figure 2.14. Highest corticosterone concentration (ng/ml) in response to capture and restraint split by dosing condition in a) adolescent and b) adult birds. Data presented are mean $\pm$ one standard error of the mean. All comparisons were not significant ($p > 0.05$).

Gonadal hormones. Male testosterone concentrations were similar regardless of adolescent dosing ($F_{3,29} = 0.591$, $p = 0.626$), as were female estradiol concentrations ($F_{3,26} = 0.211$, $p = 0.888$). The concentrations of each hormone are summarised in Table 2.4.

Table 2.4. Male testosterone concentration (ng/ml) and female estradiol concentration (pg/ml) split by dosing condition. Data are mean (standard error of the mean).

Hormone	Adolescent Condition			
	E-SAL	E-CORT	L-SAL	L-CORT
Testosterone (ng/ml)	1.23 (0.29)	1.04 (0.21)	1.54 (0.37)	1.29 (0.25)
Estradiol (pg/ml)	43.44 (17.92)	40.66 (6.78)	51.58 (10.22)	38.01 (9.57)

2.3.5. Neural receptor expression

Glucocorticoid receptors. In the hypothalamus, E-CORT birds had significant lower relative GR expression than saline dosed birds (dosing condition: $F_{3,43} = 3.891$, p = 0.015; E-CORT vs. E-SAL, p = 0.036, d = 1.62; E-CORT vs. L-SAL, p = 0.044, d = 1.15 Figure 2.13a), whereas L-CORT birds were no different from any other group (E-SAL, p = 0.578; E-CORT, p = 0.611; L-SAL, p = 0.824). MR expression was similar across birds regardless of dosing condition ($F_{3,43} = 0.742$, p = 0.533; Figure 2.13b). No sex differences were detected for hypothalamic relative GR expression (sex: $F_{1,43} = 1.957$, p = 0.169; sex x adolescent condition: $F_{3,43} = 0.499$, p = 0.685) or relative MR expression (sex: $F_{1,43} = 0.033$, p = 0.857sex x adolescent condition: $F_{3,43} = 0.223$, p = 0.880).

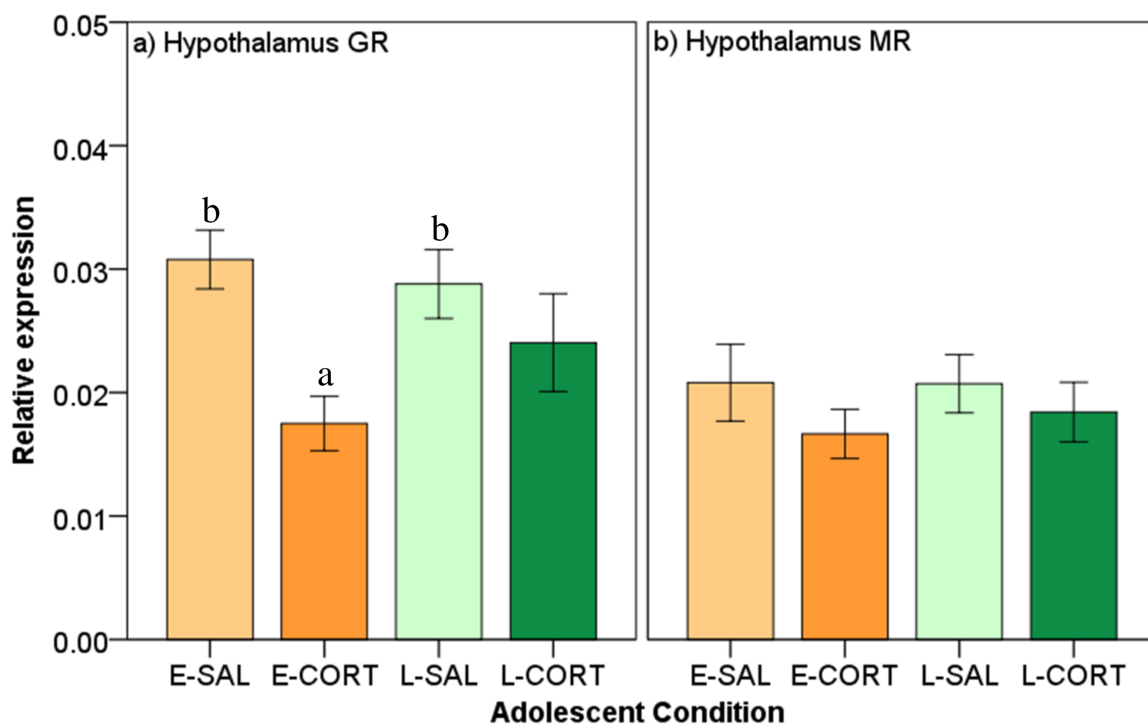

Figure 2.13. Relative expression of the glucocorticoid receptors (a) GR and (b) MR in the hypothalamus split by adolescent dosing condition. Data presented are mean ± one standard error of the mean. Significant differences (p < 0.05) between conditions shown by a vs. b.

Relative GR expression in the hippocampus was lower in E-CORT birds compared to saline dosed birds (dosing condition: $F_{3,54} = 4.160$, p = 0.010; E-CORT vs. E-SAL, p = 0.035, d = 1.13; E-CORT vs. L-SAL, p = 0.016, d = 1.10 Figure 2.14a), whereas L-CORT birds were no different from any group (E-SAL, p = 0.824; E-CORT, p = 0.462; L-SAL, p = 0.695). Relative expression of hippocampal MR was similar for all birds irrespective of dosing condition ($F_{3,54}$ = 0.221, p = 0.882; Figure 2.14b). No sex differences were found for hippocampal relative GR expression (sex: $F_{1,54} = 0.093$, p = 0.761; sex x adolescent condition: $F_{3,54} = 0.564$, p = 0.641) or relative MR expression (sex: $F_{1,54} = 0.153$, p = 0.697; sex x adolescent condition: $F_{3,54}$ = 0.425, p = 0.736).

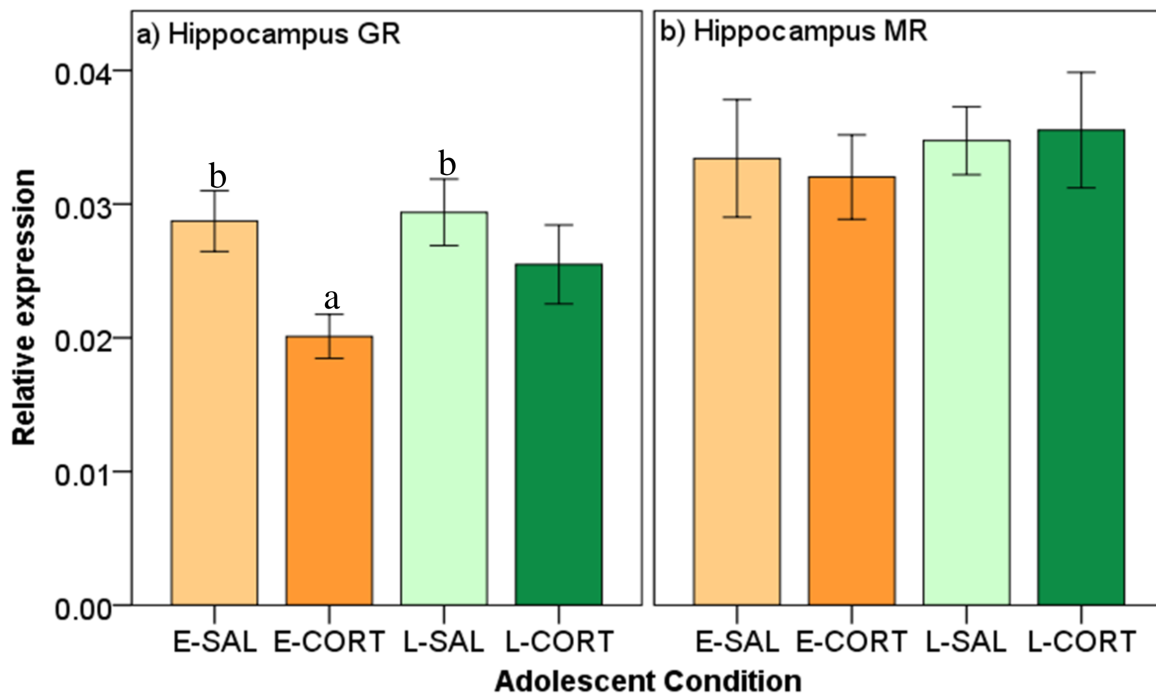

Figure 2.14. Relative expression of the glucocorticoid receptors (a) GR and (b) MR in the hippocampus split by adolescent dosing condition. Data presented are mean ± one standard error of the mean. Significant differences (p < 0.05) between conditions shown by a vs. b.

Adolescent condition had no effect on relative expression of either GC receptor in the BNS (GR: $F_{3,42} = 0.512$, $p = 0.676$; MR: $F_{3,42} = 0.114$, $p = 0.952$), amygdala (GR: $F_{3,57} = 0.902$, $p = 0.446$; MR: $F_{3,57} = 0.419$, $p = 0.740$), or nidopallium (GR: $F_{3,57} = 0.522$, $p = 0.669$; MR: $F_{3,57} = 0.051$, $p = 0.985$). Relative expression of GR and MR split by group are summarised for the BNS, amygdala, and nidopallium in Table 2.5.

Table 2.5. Relative expression of genes of interest (GOI) GR and MR in BNS, amygdala, and nidopallium caudolaterale punches split by adolescent dosing condition. Data presented are mean ± one standard error of the mean. All comparisons were not significant ($p > 0.05$).

GOI	Region	Adolescent Condition			
		E-SAL	E-CORT	L-SAL	L-CORT
GR	BNS	0.0165 (0.0024)	0.0204 (0.0028)	0.0171 (0.0017)	0.0164 (0.0015)
	Amygdala	0.0214 (0.0021)	0.0270 (0.0026)	0.0229 (0.0023)	0.0239 (0.0027)
	Nidopallium	0.0183 (0.0021)	0.0181 (0.0023)	0.0155 (0.0015)	0.0157 (0.0019)
MR	BNS	0.0183 (0.0029)	0.0189 (0.0024)	0.0169 (0.0029)	0.0176 (0.0027)
	Amygdala	0.0206 (0.0022)	0.0224 (0.0020)	0.0188 (0.0024)	0.0201 (0.0027)
	Nidopallium	0.0155 (0.0040)	0.0148 (0.0027)	0.0159 (0.0031)	0.0160 (0.0025)

Relative expression of GR did not differ according to a bird's sex in the BNS (sex: $F_{1,42} = 0.002$, $p = 0.961$; sex x dosing condition: $F_{3,42} = 0.437$, $p = 0.728$), amygdala (sex: $F_{1,57} = 0.034$, $p = 0.854$; sex x dosing condition: $F_{3,57} = 0.313$, $p = 0.816$), and nidopallium (sex: $F_{1,57} = 0.058$, $p = 0.810$; sex x dosing condition: $F_{3,57} = 1.946$, $p = 0.132$). An identical pattern was found for MR, as relative MR expression did not differ according to a bird's sex in the BNS (sex: $F_{1,42} = 0.219$, $p = 0.642$; sex x dosing condition: $F_{3,42} = 0.052$, $p = 0.984$), amygdala (sex: $F_{1,57} = 0.383$, $p = 0.538$; sex x dosing condition: $F_{3,57} = 0.211$, $p = 0.888$), and nidopallium (sex: $F_{1,57} = 0.198$, $p = 0.658$; sex x dosing condition: $F_{3,57} = 1.187$, $p = 0.323$).

The ratio between GR and MR was not different between adolescent dosing conditions (all $p > 0.229$) and/or sexes (sex, main effect: all $p > 0.437$; sex x dosing condition, interaction: all $p > 0.280$). Data are not shown, but outputs from GR/MR models are provided in Table 2.6.

Table 2.6. Outputs for models exploring the ratio between GR and MR in BNS, hypothalamus, hippocampus, amygdala, and nidopallium punches. All comparisons were not significant ($p > 0.05$).

Region	Factor	GR:MR		
		F or χ^2	df	p
BNS	Condition	F = 0.232	3,42	0.954
	Sex	F = 0.110	1,42	0.632
	Condition * Sex	F = 0.305	3,42	0.822
Hypothalamus	Condition	F = 0.472	3,43	0.703
	Sex	F = 0.567	1,43	0.456
	Condition * Sex	F = 0.413	3,43	0.744
Hippocampus	Condition	F = 1.486	3,54	0.229
	Sex	F = 0.002	1,54	0.966
	Condition * Sex	F = 0.675	3,54	0.571
Amygdala	Condition	χ2 = 0.304	3,57	0.823
	Sex	χ2 = 0.529	1,57	0.470
	Condition * Sex	χ2 = 0.186	3,57	0.905
Nidopallium	Condition	χ2 = 0.311	3,57	0.817
	Sex	χ2 = 0.002	1,57	0.969
	Condition * Sex	χ2 = 1.310	3,57	0.280

Nonapeptide receptors. Relative VT1 and VT3 receptor expression in the BNS, hypothalamus, hippocampus, amygdala, and nidopallium did not differ according to a bird's adolescent dosing condition, sex, or an interaction between the two (Table 2.7). Relative expression of VT1 and VT3 split by dosing condition are summarised in Table 2.8.

Table 2.7. Model outputs exploring VT1 and VT3 receptor expression in BNS, hypothalamus, hippocampus, amygdala, and nidopallium. All comparisons were not significant (p > 0.05).

Region	Factor	VT1			VT3		
		F	df	p	F	df	p
BNS	Condition	0.133	3,42	0.940	0.470	3,42	0.705
	Sex	0.331	1,42	0.568	0.006	1,42	0.938
	Condition * Sex	0.105	3,42	0.957	0.176	3,42	0.912
Hypothalamus	Condition	0.545	3,43	0.654	0.316	3,43	0.814
	Sex	0.021	1,43	0.885	0.030	1,43	0.863
	Condition * Sex	0.503	3,43	0.682	0.509	3,43	0.678
Hippocampus	Condition	0.283	3,54	0.837	0.212	3,54	0.888
	Sex	0.001	1,54	0.976	0.217	1,54	0.643
	Condition * Sex	1.070	3,54	0.370	0.680	3,54	0.568
Amygdala	Condition	0.283	3,57	0.837	1.072	3,57	0.368
	Sex	0.017	1,57	0.897	0.298	1,57	0.588
	Condition * Sex	0.873	3,57	0.461	2.713	3,57	0.053
Nidopallium	Condition	0.988	3,57	0.405	0.071	3,57	0.975
	Sex	0.101	1,57	0.752	0.227	1,57	0.636
	Condition * Sex	1.197	3,57	0.319	0.173	3,57	0.914

Table 2.8. Relative expression of genes of interest (GOI) VT1 and VT3 receptors split by brain region and adolescent dosing condition. Data presented are mean ± standard error of the mean.

GOI	Region	Adolescent Condition			
		E-SAL	E-CORT	L-SAL	L-CORT
VT1	BNS	0.0031 (0.0003)	0.0033 (0.0004)	0.0030 (0.0004)	0.0032 (0.0004)
	Hypothalamus	0.0033 (0.0005)	0.0038 (0.0004)	0.0032 (0.0004)	0.0036 (0.0004)
	Hippocampus	0.0019 (0.0003)	0.0018 (0.0003)	0.0022 (0.0004)	0.0016 (0.0003)
	Amygdala	0.0027 (0.0003)	0.0026 (0.0002)	0.0030 (0.0004)	0.0027 (0.0004)
	Nidopallium	0.0016 (0.0003)	0.0019 (0.0002)	0.0014 (0.0002)	0.0015 (0.0002)
VT3	BNS	0.0041 (0.0005)	0.0047 (0.0004)	0.0041 (0.0006)	0.0048 (0.0006)
	Hypothalamus	0.0052 (0.0003)	0.0050 (0.0003)	0.0050 (0.0004)	0.0046 (0.0004)
	Hippocampus	0.0030 (0.0005)	0.0026 (0.0005)	0.0028 (0.0005)	0.0024 (0.0004)
	Amygdala	0.0030 (0.0004)	0.0038 (0.0005)	0.0032 (0.0004)	0.0040 (0.0005)
	Nidopallium	0.0025 (0.0003)	0.0028 (0.0004)	0.0026 (0.0004)	0.0026 (0.0004)

2.3.6. Correlations

After correcting for multiple comparisons all adolescent correlations were not significant (all $p > 0.076$; Bonferroni corrected $\alpha = 0.0025$) and all adult correlations were not significant (all $p > 0.012$; Bonferroni corrected $\alpha = 0.0017$). Outputs from correlation analyses are presented in Appendix B (Table B.1., Table B.2., and Table B.3.).

2.4. Discussion

The findings presented in this chapter are the first to document that adolescent CORT exposure can modulate responses to both unfamiliar environments and unfamiliar conspecifics when in adulthood. However, the effects of CORT on responses to unfamiliar environments are dependent on adolescent age of dosing and adult social context. When individually housed, birds dosed with CORT in early adolescence took longer to enter an unfamiliar environment in adulthood, perched in the unfamiliar environment for longer, and, if female, engaged in more head turns when compared to all other birds. No group differences in response to an unfamiliar environment were found when the birds were housed in familiar groups. Late adolescent CORT dosing had no long-term effects on behavioural responses to an unfamiliar environment. In response to unfamiliar conspecifics, birds dosed with CORT during adolescence spent less time perching alone compared to birds dosed with saline which, in males, may be attributable to lower courtship behaviour from CORT dosed compared to saline dosed males. The behavioural effects do not appear to be attributable to differences in circulating CORT, T, or E2 concentrations. However, birds dosed with CORT in early adolescence had lower relative expression of GR in the hypothalamus and hippocampus that may reflect that the birds' HPA axis had lower negative feedback efficiency compared to all other dosing conditions. The findings presented partially support the hypothesis that adolescent CORT exposure would result in birds that interacted more with unfamiliar stimuli in adulthood. However, the data revealed more nuanced effects that will now be discussed.

2.4.1. Adolescent behaviour

During adolescent dosing, immediate behavioural effects were detected, with birds dosed with CORT during early adolescence gradually taking longer to enter the dosing box compared to all other conditions. The longer latency to enter the dosing box cannot be attributed to short-term changes in CORT concentration, as CORT concentration quantified shortly (three

days) after the dosing had ended were no different between conditions. However, quantifying CORT concentration three days after dosing had ended may have been too late to indicate the short-term effects of dosing. Future work may therefore wish to determine how adolescent CORT dosing influences the acute stress response during the early adolescent dosing window.

CORT dosing may have induced behavioural changes in early (but not late) adolescent birds that were causing the effect on dosing box latency. Dosing sessions were not recorded, so any behavioural changes that may have been present were not quantified. A longer latency to enter the dosing box in E-CORT birds could reflect that the birds are more avoidant of the dosing box, perhaps because the birds formed an association between the dosing box and an aversive experience that arises from consuming CORT. For example, CORT dosing may trigger a stress response that is perceived as aversive or CORT doses may taste more unpleasant than saline doses. Adult rats will self-administer a physiologically relevant dose of stressor-induced CORT (but not doses that are more/less than a physiologically relevant concentration) indicating that CORT dosing may not be experienced as aversive (Piazza et al., 1993). Lower aversive response to CORT and/or less unpleasant taste perception in late vs. early adolescent zebra finches would also be necessary in order for the age-specific effects to occur, but such ideas have not been investigated. Rather than associating the dosing box with aversive effects, delayed entry latency in E-CORT birds may be due to changes in stress-related behaviour, such as lower locomotor activity (Koolhaas et al., 1999), heightened risk assessment behaviour (Rodgers et al., 1999), or modulation of startle response (Glowa, Geyer, Gold, & Stemberg, 1993). Social behaviour could also be changed by dosing, with E-CORT birds possibly distracted from the dosing box by attempting to spend more or less time interacting with conspecifics than other dosing conditions (Boogert et al., 2014; Veenit et al., 2012). To determine if behavioural changes are causing the effects on dosing box latency further work is necessary to quantify stress-related and social behavioural responses to adolescent CORT

dosing. Whether adolescent age-related changes in stress-related and/or social behaviour occur in zebra finches has not been explored, so both early and late adolescent zebra finches should be included in any future work attempting to explore the short-term effects of adolescent CORT dosing in order to explore the age-specific effects on dosing box entry latency.

The absence of effects of late adolescent CORT dosing on dosing box latency may be consistent with the finding that animals become less responsive to steroid hormones as adolescence progresses, as indicated for gonadal steroids (e.g. Schulz & Sisk, 2016). However, CORT may have had short-term effects on unmeasured behavioural responses. Further research is needed to quantify the short-term effects of CORT vs. saline dosing in late adolescence on stress-related and social behaviour to determine whether CORT has any immediate effects on unmeasured late adolescent behaviour. An alternative explanation for the lack of effects of CORT on late adolescent behaviour is that the doses used may have been inadequate for effects to occur in late adolescence. A pilot study determined a physiologically relevant dose of stressor-induced CORT to administer to the birds. The model output suggested that there was no difference in stressor-induced CORT between early and late adolescence, so identical doses were used for early and late adolescent birds in the current study. However, model used to analyse the data may have been over-parametrised by including three fixed factors, two of which were repeated measures, with a sample size of only twenty birds. This may have resulted in a sample with high variability and lack of statistical power, so any small differences in stressor-induced CORT concentration between early and late adolescence may have been undetected. Further research is therefore needed to replicate the pilot study from the current thesis with a larger sample size to determine if the doses used in the current study were accurate.

2.4.2. Adult behavioural responses to unfamiliar environments

In adulthood, birds dosed with CORT during early adolescence took longer to enter the unfamiliar environment when individually housed compared to birds from all other conditions. Adolescent stress in rats results in a similar effect, with 'stressed' rats taking longer to contact an unfamiliar object when in adulthood when compared to rats that received no such stress (Eiland & Romeo, 2013; Hollis et al., 2013; McCormick et al., 2015). Stressor exposed and CORT dosed adolescents taking longer to interact with unfamiliar stimuli may be indicative of a more neophobic behavioural response when in adulthood compared to controls. Whether CORT is an endocrine mechanism underlying the long-term effects of adolescent stress on responses to unfamiliar stimuli has remained unclear (Veenit et al., 2013), but the data in the current chapter are the first to support the hypothesis.

Female zebra finches that received early adolescent CORT doses engaged in more head turns than birds from all other conditions. Only males have been studied to investigate the long-term effects of adolescent CORT dosing on responses to unfamiliar stimuli (Veenit et al., 2013). The current data are therefore the first to indicate that adolescent CORT exposure can affect behavioural responses to unfamiliar environments in a sex-dependent manner. Number of head turns has been proposed as a measure of risk assessment in birds (Fernandez-Juricic, 2012; Jones et al., 2007), but the current data are the first to quantify the behaviour in zebra finches. Female birds dosed with CORT in early adolescence may therefore be engaging in more risk assessment (potentially a more neophobic response) than birds in all other dosing conditions. As the measure is novel, head turns may not reflect risk assessment as is proposed here. Female birds may be more responsive to social separation, with more head turns reflecting an attempt to find conspecifics. However, no group differences in head turns were found during pre-exposure, indicating that more head turns in E-CORT birds than other dosing conditions is not due to absence of conspecifics, but is a response to an unfamiliar environment.

Although E-CORT birds took longer to enter the unfamiliar environment and, if female, engaged in more head turns in response to the environment, the same birds spent a longer duration of time perching in the unfamiliar environment. E-CORT birds did not spend more time in the unfamiliar environment because they entered the environment more often than birds from other conditions. Previous work has typically found that adolescent stress results in adult rats that spend less time in exposed areas of unfamiliar environments compared to animals that did not experience any such stress (Eiland & Romeo, 2013; Hollis et al., 2013; McCormick et al., 2015), but developmental stress can also result in animals spending more time in unfamiliar environments than control animals (e.g. Toledo-Rodriguez & Sandi, 2011; Zimmer et al., 2013). Why E-CORT birds spent more time in the unfamiliar environment is not clear, but stressor-induced immobilisation is a possible explanation (Koolhaas et al., 1999). E-CORT birds may have been more immobile in the unfamiliar environment than birds from other conditions; an effect that would be consistent with E-CORT birds having a more neophobic response to an unfamiliar environment than controls. If E-CORT birds were more immobile then the birds would be expected to engage in fewer hops, but no difference in number of hops was found between conditions. The birds rarely interacted with the novel objects, so were also unlikely to have been immobile whilst investigating and/or hiding amongst the objects. Stressor-induced immobility is therefore unlikely, with future work required to explore why E-CORT birds spent more time perching in the unfamiliar environment. For example, E-CORT birds may spend more time in the environment to forage more and avoid the stress of under-nutrition compared to other dosing conditions (e.g. Crino, Driscoll, Ton, et al., 2014). Foraging behaviour was not quantified in the current chapter, but future research could do so.

The effects of adolescent CORT on behavioural responses to an unfamiliar environment were entirely dependent on social context. In the presence of familiar birds, adolescent CORT dosing had no effects on any behavioural measure during the unfamiliar environment task.

Group housing with familiar conspecifics has been shown to act as a social buffer, with neophobic behavioural responses lower when animals are tested with familiar conspecifics compared to when single housed (Beery & Kaufer, 2015; Sanchez et al., 2015). The current findings may therefore suggest that presence of familiar conspecifics in adulthood can buffer the long-term effects of adolescent CORT on adult behavioural responses to an unfamiliar environment. Adolescent CORT exposure has no effect on adult behaviour in a social buffering context when in adulthood, either in terms of task performance (e.g. latency to enter unfamiliar environment) or social interaction (e.g. more affiliative or less antagonistic behaviour). Adolescent CORT dosing may therefore not improve or impair social behaviour related to social buffering when in adulthood, an effect that is in line with previous work investigating the developmental antecedents of social buffering (e.g. pre-adolescent social density: Branchi & Alleva, 2006). However, the birds may not interpret group exposure to an unfamiliar environment as a social buffering context. For example, the birds may interpret the test as competition over limited food resources as the unfamiliar objects in the unfamiliar environment were baited with spinach. Adolescent CORT dosing may therefore have no effects on foraging competition in the presence of familiar conspecifics, rather than social buffering. Further work could avoid different behavioural interpretations by investigating whether adolescent CORT dosing affects group vs. individual responses to stressor-induced CORT concentration to determine if adolescent CORT affects later-life social buffering of the acute stress response.

Although the group context may cancel out any effects of adolescent CORT dosing, an individual context may also have effects on animals that facilitate the emergence of behavioural differences in response to an unfamiliar environment. For example, zebra finches have a higher basal CORT concentration 24 hours after single housing compared to when housed with a pair mate (Remage-Healey et al., 2003). Consequently, social context may affect behaviour via changes in stress physiology with higher pre-test CORT concentration in an individual context

may prime birds to respond to a stressor. Birds that were dosed with CORT in early adolescence may have a higher CORT concentration than other conditions when individually housed in adulthood prior to any tasks. Future work could explore how adolescent CORT dosing affects pre-test CORT concentration across social contexts, e.g. individual vs. group, when in adulthood to test the hypothesis. Another difference between the contexts was the unfamiliar object arrangements. Adult birds responded similarly to both sets of objects during the pilot study, suggesting that object differences are not the cause of the context-dependent effects of early adolescent CORT dosing. However, further work is needed to repeat the current experiment but with the unfamiliar object arrangements counter-balanced across contexts to rule out any effects of the apparatus used.

2.4.3. Adult behavioural responses to unfamiliar conspecifics

As predicted, when separated from a familiar conspecific and housed with an unfamiliar group, CORT dosed birds spent less time alone on the perches compared to birds dosed with saline during adolescence. Spending less time alone in an unfamiliar group in adulthood could not be attributed to CORT dosed birds engaging in more gregarious interactions with unfamiliar birds as no differences could be detected for clumping and allopreening as the behaviour were too infrequent for analysis. Spending less time alone need not reflect gregariousness, but could also reflect that CORT dosed birds were just sitting in proximity to others with little interaction compared to saline dosed birds. Counter to the latter hypothesis, zebra finches dosed with CORT as nestlings and fledglings forage with more unfamiliar conspecifics than vehicle dosed birds when in adolescence (Boogert et al., 2014). Whether a similar effect occurs in response to adolescent CORT dosing remains to be tested, but the evidence intimates that CORT dosed birds may not spend less time alone due to more social inactivity than saline dosed birds.

Zebra finches have a higher CORT concentration when separated from a familiar pair mate compared to when with a pair mate, possibly indicating that separation from a familiar conspecific is a stressor in the species (Remage-Healey et al., 2003). Birds in the current study were separated from familiar conspecifics immediately prior to testing. CORT dosed birds may experience more separation stress than saline dosed birds, resulting in CORT dosed birds spending less time alone with unfamiliar birds to lower their greater separation stress compared to saline dosed birds. No differences between adolescent conditions were found in pre-exposure behaviour in the individual context unfamiliar environment tasks, indicating that the birds may have an equal response to social separation. However, pre-exposure behaviour was quantified 24 hours after single housing and may not reflect immediate behavioural differences in response to social separation. Further work needs to explore whether E-CORT birds display greater separation stress (e.g. more locomotor activity, more risk assessment, higher CORT concentration) immediately after being single housed compared to birds from other conditions.

Male birds dosed with CORT engaged in less courtship behaviour when exposed to an unfamiliar mixed-sex group in adulthood. A lower expression of courtship behaviour could reflect that CORT dosed males were less sexually competent than saline dosed males as has been reported in rats in response to adolescent stress (Almeida, Kempinas, & Carvalho, 2000; McCormick et al., 2013; Toth et al., 2008). A lower expression of courtship behaviour could also indicate that CORT dosed males are less attractive than saline dosed males, as has been reported in zebra finches that were dosed with CORT during nestling and fledgling development (Spencer et al., 2003; Spencer et al., 2005). Courtship competence and attractiveness were not quantified, so further work is needed to determine if males dosed with CORT are less sexually competent or less attractive than saline dosed males. If CORT dosing does not impair courtship behaviour or lower attractiveness compared to saline dosing, then the effects may be context-dependent. CORT dosed males may lower courtship behaviour to

better integrate into an unfamiliar group and better avoid social separation stress compared to saline dosed birds, but when already in a familiar group no group differences may be detected in courtship behaviour as there is no separation stress. Further work is therefore needed to determine the effects of adolescent CORT on courtship behaviour across social contexts (e.g. with and without familiar conspecifics).

Whether adolescent stress affects female courtship competence and/or attractiveness has been largely ignored by previous research (e.g. Almeida et al., 2000; Toth et al., 2008), but the current study found no effects of adolescent CORT on female courtship behaviour. Only male courtship behaviour may have been affected as, for the measures taken in the current study, males can inhibit their expression of courtship behaviour whereas females cannot (e.g. males can stop singing, but females cannot stop being sung at). Female courtship behaviour may have been affected by adolescent CORT, just not on the measures that were quantified in the current study. For example, female zebra finches may attract a mate by being active (e.g. hopping: Zann, 1996) which may have been lower in CORT dosed females compared to saline dosed females. Adolescent CORT has sex-dependent effect on adult courtship behaviour, emphasising that future work should include male and female animals to elucidate any further sex differences in adult courtship behaviour (e.g. activity).

L-CORT birds displaced unfamiliar birds less than birds from any other condition. Zebra finches dosed with CORT as nestlings and fledglings develop into adolescents that are supplanted from a perch more often than vehicle dosed birds when competing over access to a single perch, suggesting birds dosed with CORT during development may be more subordinate or less dominant than controls in later-life (Spencer & Verhulst, 2007). L-CORT birds in the current chapter may have engaged in fewer antagonistic interactions as the birds were more subordinate or less dominant than birds from other dosing conditions, but the hypothesis requires testing. Early adolescent CORT dosing had no effect on antagonistic behaviour when

in adulthood. Adolescent CORT exposure may therefore interact with age-specific expression of competitive behaviour that emerges in late adolescence. For example, zebra finches begin to compete over mates and establish pair bonds during late adolescence (around day 70; Zann, 1996) and CORT may affect antagonistic behaviour used during mate competition during this period and have persistent effects that result in more antagonistic behaviour when in adulthood. However, more work is necessary to document the ontogeny of adolescent zebra finch social behaviour (e.g. competitiveness, dominance) and then explore how exposure to stressors and CORT can shape the developmental trajectories of such behaviour.

2.4.4. Glucocorticoids and glucocorticoid receptors

No differences in either basal or stressor-induced CORT concentrations were found between dosing conditions. The current chapter therefore provides no support for the prediction that stressor-induced CORT concentration would be higher in birds dosed with CORT compared to birds dosed with saline. Previous work in rats has shown that adolescent stress results in a higher stressor-induced secretion of CORT compared to control rats that were not exposed to the adolescent stressors (Isgor et al., 2004; Pohl et al., 2007). The contradictory effects are unlikely due to a difference in stressor type (i.e. CORT dosing vs. external stressors) as nestling/fledgling zebra finches dosed with CORT have a higher stressor-induced CORT concentration when in adolescence compared to control birds (Spencer et al., 2009). Instead, absence of group differences in CORT concentration are likely due to an incomplete stress response being quantified. Birds in the current study were continuing to secrete a higher CORT concentration at each consecutive sampling point, with no post-peak concentration quantified. A similar effect has also been reported for zebra finches fed CORT during the nestling/fledgling period (Crino et al., 2014), suggesting that a time series from 0-30 mins is insufficient to quantify all aspects of a stress response in zebra finches. However, the models used to analyse CORT concentrations may have also been over-parameterised. The CORT

response across sampling times model included four fixed factors of which two were repeated measures, and the peak CORT concentration model included three fixed factors one of which was a repeated measure. Adolescent CORT dosing may have had an effect on stressor-induced CORT concentration, but the models used to analyse this data may have lacked statistical power due to the use of complex models and smaller than required sample sizes. The experiment needs to be replicated with a larger sample size in order to rule out that adolescent CORT dosing had no effect on stressor-induced CORT concentrations due to statistical limitations.

GR was significantly lower in the hippocampus and hypothalamus of E-CORT birds compared to saline dosed birds dosed. GR regulates stressor-induced CORT concentration, with higher hippocampal and hypothalamic GR acting to inhibit CORT secretion during a stress response (Herman et al., 2003; Herman et al., 2005). E-CORT birds may have lower negative feedback efficiency of the HPA axis compared to birds dosed with saline. Lower hippocampal GR has previously been found in adult rats exposed to stress during adolescence compared to rats that experienced no such stress (Isgor et al., 2004). The current results are the first to suggest that early adolescent exposure to CORT is a mechanism behind the long-term effects of adolescent stress on GR and that hypothalamic GR may also be affected by adolescent stress. Late adolescent CORT dosing effects on relative GR expression appear intermediate between saline and early CORT dosing, indicating that the extent to which CORT can affect neural GR expression may decline with age. Previous work that has shown similar effects for other steroid hormones, like testosterone (Schulz & Sisk, 2009; Schulz & Sisk, 2016), but more research is needed to determine why the age-related decline occurs.

GR expression in the amygdala and NPC were not affected by adolescent CORT. CORT can have a positive feedback effect on the HPA axis by binding to amygdala GR to further stimulate CORT secretion during a stress response (Herman et al., 2012; Shepard et al., 2003), but can also have a negative feedback effect on the HPA axis by binding to GR in the

PFC to inhibit further CORT secretion during a stress response (Diorio, Viau, & Meaney, 1993; Herman et al., 2012). The current study suggests that adolescent CORT dosing does not have long-term effects on an amygdala-mediated positive feedback mechanism or a NPC-mediated negative feedback mechanism that act on the HPA axis. The NPC is an avian homologue of the mammalian PFC (Güntürkün, 2005; Rose & Colombo, 2005), but future work should investigate adolescent CORT dosing across taxa to see if the dosing has long-term effects on GR expression in the mammalian PFC and amygdala.

No differences were found in the relative expression of MR or the ratio between GR and MR. In brain regions that can inhibit the HPA axis like the hippocampus and hypothalamus, a higher MR expression results in a faster rise in stressor-induced CORT concentration by lowering the threshold for triggering a stress response (de Kloet et al., 2000; Joëls et al., 2007; Oitzl et al., 2010). A higher expression of MR in brain regions that inhibit the HPA axis can also result in a lower GR/MR ratio in these regions, which in turn can result in lower negative feedback efficiency of the HPA axis compared to animals with a more balanced GR to MR ratio (de Kloet et al., 1998; Groneweg et al., 2011). Adolescent CORT having no influence on MR expression may be advantageous as a lower threshold for responding to stress can be costly, with higher sensitivity resulting in more frequent false positive responses to benign events (Bateson et al., 2011; Nettle & Bateson, 2012). Through a selective lowering of hippocampal and hypothalamic GR, adolescent CORT exposure may result in animals with a greater response to unambiguous stressors than control animals whilst being no more likely than controls to mount a stress response to benign events.

The samples collected for glucocorticoid receptor analysis were coarse, with RNA expression quantified from large samples in some areas (e.g. hypothalamus and amygdala) and from multiple regions in a single punch for others (e.g. BNS). Taking such coarse samples may have obscured some effects of adolescent CORT dosing, so future work is necessary to use

techniques other than qPCR that can provide a more detailed analysis of the long-term effects of adolescent CORT dosing on glucocorticoid receptor expression. For example, in situ hybridisation could be used to display the RNA distribution of glucocorticoid receptors on a finer scale. For example, adolescent CORT may have specific effects on GR or MR expression in the BNST that may be obscured by conflating multiple regions into a single punch. Immunohistochemistry could also be used to display the protein distribution of glucocorticoid receptors on a finer scale. Glucocorticoid receptor RNA and protein measures typically correlate (e.g. Han, Ding, & Shi, 2014; Sotnikov et al., 2014), but protein expression still needs to be quantified to determine if adolescent conditions have protein-specific effects. Adolescent stress can impair neurogenesis in the hippocampus, resulting in fewer hippocampal neurons compared to control animals (Isgor et al., 2004). Using techniques that are more detailed than qPCR could indicate whether adolescent CORT results in lower hippocampal GR due to fewer hippocampal neurons.

2.4.5. Gonadal hormones and nonapeptide receptors

Basal concentration of testosterone in males and estradiol in females were no different between CORT and saline dosed birds. A similar effect was found in rats, with rats subjected to chronic variable stress in adolescence no different from rats that experienced no such stress in basal testosterone or basal estradiol concentrations in males and females, respectively (Bourke et al., 2013). In contrast to basal measures, adolescent stress can blunt a stressor-induced rise in testosterone in male rats and blunt a stressor-induced fall in estradiol in female rats (Bourke et al., 2013). A lower testosterone concentration in male guinea pigs is associated with lower antagonistic and courtship behaviour when housed with an unfamiliar male and female (Sachser et al., 1993). In the current study, lower courtship behaviour in CORT dosed males compared to saline dosed males could be due to lower testosterone secretion in response to being housed in an unfamiliar mixed-sex group but the hypothesis requires testing. Further

work is needed to explore how adolescent CORT dosing influences time-dependent gonadal hormone responses to unfamiliar stimuli, like environments and conspecifics.

Nonapeptides are a plausible mechanism that contribute to the social behaviour effects found in the current chapter, but quantifying circulating nonapeptide concentrations was not possible as the equipment necessary for extraction was not available. However, nonapeptide receptors VT1 and VT3 were quantified in adulthood and CORT dosing had no effect on relative expression of either receptor. Adolescent CORT therefore has little influence on neural regulation of nonapeptide functioning, but VT2 receptor expression in the pituitary gland and VT4 receptor throughout the SBN still require quantification. In mammals, parental absence and low parental care typically result in adult animals with lower oxytocin receptor binding in the BNS (e.g. Francis et al., 2002; Lukas et al., 2010) and higher vasopressin RNA expression in the PVN of the hypothalamus (e.g. Pan et al., 2009; Veenema et al., 2006). Developmental stressors that are not social (e.g. maternal restraint during offspring prenatal development) have little effect on offspring nonapeptide receptor expression in later-life (Lee et al., 2007). Nonapeptide functioning may be affected by developmental social experiences, not stressors. Adolescent CORT dosing would not be expected to have any influence on nonapeptide receptor expression, but adolescent social experiences like social instability (McCormick et al., 2015) and social density (Sachser et al., 1993) may. However, the hypothesis that adolescent social experiences affect adult nonapeptide receptor expression requires testing.

2.4.6. Summary

In summary, the current chapter clearly show that adolescent CORT dosing can have long-term effects on later-life responses to unfamiliar stimuli (environments, objects, and conspecifics) and on stress physiology by affecting neural expression of GR. The long-term effects of adolescent CORT dosing on behavioural responses to an unfamiliar environment and

GR expression were similar to the effects seen in response to adolescent stress in rats. CORT may therefore be an endocrine mechanisms behind the long-term effects of adolescent stress. Encountering stress during adolescence is a common occurrence as animals leave the natal home and encounter unfamiliar environments and predators (Spear, 2000; Yoder et al., 2004). The findings presented in the current chapter suggest that, due to greater glucocorticoid exposure, exposure to stressors in adolescence can affect behavioural responses to unfamiliar stimuli and stress physiology when in adulthood.

Chapter 3

Early adolescent group size and social density in zebra finches: long-term effects on behavioural responses to unfamiliar stimuli, corticosterone, and gonadal hormones

3.1. Introduction

Living at higher social density during adulthood may be a stressor, as animals may engage in more antagonistic interactions as they competition over limited resources (e.g. territory, food) becomes more common (Craig & Swanson, 1994; Judge & deWaal, 1993; van Loo et al., 2001). Whether higher social density is a stressor in adolescents is unclear. Adolescent social density may modulate social behaviour rather than stress, with adolescents raised at higher social density developing into adult animals that engage in more affiliative behaviour in adulthood compared to conspecifics raised at a lower density. For example, male zebra finches housed six birds per cage in adolescence clump more with unfamiliar birds when housed in an unfamiliar social group in adulthood compared to birds raised two per cage in adolescence (Ruploh et al., 2014). However, the long-term effects of adolescent social density could be interpreted in terms of stress. In Ruploh et al. (2014), the birds were removed from their adolescent housing conditions (two or six birds per cage) in early adulthood (postnatal day 108-112) and then single housed for a mean duration of 245 days prior to testing. High density reared birds may therefore clump with unfamiliar birds more than low density reared birds to better avoid social separation stress. In line with the stress avoidance hypothesis, male mice raised seven per cage in adolescence develop into adults that spend less time in open spaces in an unfamiliar environment compared to mice raised one per cage (Reiss et al., 2007). Higher adolescent social density may therefore result in adults that are more stress avoidant than animals raised at lower density. However, adult social context may modulate the effect. Mice raised at higher density prior to adolescence (three nests per cage) developed into adults that spent less time in open areas of an unfamiliar environment than mice raised at lower density, but only when the animals were individually tested (Branchi & Alleva, 2006). When tested in familiar pairs, no group differences were present (Branchi & Alleva, 2006). Familiar conspecifics can lower stressor avoidant responses due to social buffering (DeVries et al., 2003;

Hennessy et al., 2009). Familiar conspecifics may therefore buffer the behavioural responses to unfamiliar stimuli in adulthood, thereby negating any long-term effects of adolescent social density. However, the hypothesis requires testing as all studies that have assessed the long-term effects of adolescent density on responses to unfamiliar environments and objects have done so in an individual context.

Adolescent social density can have different effects on responses to unfamiliar stimuli depending on an animal's developmental stage during testing. Male mice housed twenty per cage during adolescence spend more time interacting with an unfamiliar conspecific and more time in open spaces in an unfamiliar environment whilst in adolescence compared to male mice housed four per cage (Ago et al., 2014). The mice in Ago et al. (2014) were tested immediately after the housing variation, so the effects may reflect either developmental stage (i.e. adolescence vs. adulthood) or time between housing variation and testing (i.e. short-term vs. long-term). In contrast to Ago et al. (2014), adult male rats housed at higher density spend less time in open spaces in an unfamiliar environment compared to rats housed at lower density when tested immediately after the housing density variation (Botelho, Estanislou, & Morato, 2007; Daniels, Pietersen, Carstens, Daya, & Stein, 2000). Developmental stage, not duration of time until testing, therefore better explains the effect in Ago et al. (2014). Higher adolescent social density may result in animals that are more likely to approach unfamiliar stimuli in adolescence, but more likely to avoid unfamiliar stimuli in adulthood. The rise in gonadal hormones during adolescence results in more risk-taking behaviour within adolescence (Cyrenne & Brown, 2011; Steinberg, 2008), and social density may interact with such pubertal maturation in order to result in the adolescent-specific effects reported in Ago et al. (2014). However, no study has quantified the short- and long-term effects of adolescent social density on behavioural responses to unfamiliar stimuli within the same study to determine whether the

same animals display different responses across development and therefore determine if the effects of Ago et al. (2014) are age-specific.

Adolescent social experiences, such as social re-housing or single housing, have long-term effects on adult behavioural responses to unfamiliar stimuli, and these effects have been attributed to higher exposure to glucocorticoids during adolescence compared to control animals (Buwalda et al., 2011; McCormick et al., 2015; Sachser et al., 2011). Higher social density in adult animals results in a higher basal CORT concentration compared to animals raised at lower density of some species, suggesting that higher social density may act as a stressor (Christian, 1950; Creel, 2013). Higher adolescent social density could therefore also act as a developmental stressor, resulting in higher CORT concertation when in adulthood as has been shown to occur in response to other adolescent stressors (Hollis et al., 2013). Adolescent social density has no effect on basal CORT concentration in adolescent mice (Ago et al., 2014; Laviola et al., 2002) or adult mice (Ortiz et al., 1985), but male mice housed at higher density in adolescence secrete a higher concentration of CORT when exposed to a stressor (loud noise when single housed) in adulthood compared to male mice from a lower density housing condition (Ortiz et al., 1985). However, male guinea pigs housed at higher density throughout adolescence had lower cortisol concentrations in response to encountering two unfamiliar conspecifics (one male, one female) in adulthood compared to guinea pigs raised in pairs in adolescence (Sachser et al., 1993). Adolescent social density may therefore affect stressor-induced CORT secretion in a context- or species-dependent manner. Animals raised at higher social density in adolescence may secrete more CORT in an individual context (or if mice; Ortiz et al., 1985), but secrete less CORT in a group context (or if guinea pigs; Sachser et al., 1993), compared to animals reared at a lower social density However, further work is needed to determine the effects of adolescent social density on adult CORT secretion

in response to a stressor (e.g. unfamiliar environment) in individual and group contexts within the same animals to test these hypotheses.

Adult animals engage in more antagonistic interactions when at higher social density compared to lower social density across a range of taxa (e.g. birds: Craig & Swanson, 1994; primates: Judge & deWaal, 1993; rodents: van Loo et al., 2001), and more antagonistic interactions results in a higher testosterone concentration (Wingfield et al., 1990; Wingfield et al., 2005). Animals living at higher (vs. lower) social density may therefore have higher concentrations of testosterone, as has been shown in both males and females adults across taxa as social density varies over the breeding season (e.g. birds: Smith, Raouf, Brown, Wingfield, & Brown, 2005; mammals: Hirschenhauser & Oliveira, 2006; Schradin, 2008). Adolescent male guinea pigs raised at higher density throughout adolescence have higher basal testosterone concentration in adolescence compared to guinea pigs raised at lower density (Sachser & Pröve, 1988). Adolescent social density could therefore affect gonadal hormone functioning, with subsequent effects on adult social behaviour. However, adolescent social density has no long-term effects on basal testosterone concentration in adulthood for mice (Nicholson et al., 2009; Ortiz et al., 1984; Smith, Mabus, Stockwell, & Muir, 2004) or male guinea pigs (Lürzel et al., 2010). Whether adolescent social density affects estradiol concentration in male and/or female animal's remains to be investigated. Furthermore, whether adolescent social density modulates gonadal hormone concentration in avian species has not been investigated. Adolescent social density affects adult social behaviour, as animals raised at higher density in adolescence engage in less courtship and antagonistic behaviour when in adulthood compared to animals raised at lower density (e.g. zebra finches, Ruploh et al., 2014; guinea pigs, Sachser et al., 1993). The current evidence suggests that the effects of adolescent social density on adult social behaviour cannot be attributed to any long-term changes in differences in the circulating concentration of gonadal hormones.

Nonapeptides also regulate behavioural responses to conspecifics, but have not been investigated in the context of adolescent social density. Higher adolescent social density results in adult animals that engage in more affiliative behaviour, less antagonistic behaviour, and less courtship behaviour when in adulthood compared to animals raised at lower density (Ruploh et al., 2013; Ruploh et al., 2014; Sachser et al., 1993). Higher concentration of oxytocin (OT; in mammals) and mesotocin (MT; in birds) results in animals typically engaging in more affiliative behaviour with unfamiliar conspecifics, such as social contact in rats (Witt et al., 1992) and flocking in birds (Goodson et al., 2009). Higher concentration of vasopressin (VP; in mammals) and vasotocin (VT; in birds) results in animals typically engaging in more antagonistic behaviour with unfamiliar conspecifics (Goodson & Thompson, 2010), for example in a mate competition context in zebra finches (Kabelik et al., 2009). Higher adolescent social density could therefore result in animals with higher OT/MT concentration and lower VP/VT concentration when in adulthood compared to animals raised in lower density groups in order to explain the effects of adolescent social density on adult social behaviour, but these suggestions require testing.

An important caveat in the social density literature is that animals are often made to live at higher social density by housing animals in larger groups. For example, social density has been varied in zebra finches by housing birds in groups of either two or six in same sized enclosures (Ruploh et al., 2013). Any effects reported to arise from variation in social density could be attributed to either variation in the absolute number of animals an individual was housed with and/or the number of animals per square metre. Larger group size in adults, independent of social density, can either result in more antagonistic behaviour (Craig & Swanson, 1994; van Loo et al., 2001) or less antagonistic behaviour (D'Eath & Keeling, 2003; Hughes, Carmichael, Walker, & Grigor, 1997). A social hierarchy may become more difficult to maintain in larger groups, so animals may engage in more antagonistic behaviour to better

establish a position in the hierarchy (van Loo, van Zutphen, & Baumans, 2003) or less antagonistic behaviour as animals begin to use alternative methods (for example, body size and/or mass) for forming a hierarchy (D'Eath & Keeling, 2003; Pagel & Dawkins, 1997). During adolescence, animals enter larger social networks than before adolescence (Nelson et al., 2016) and learn how to function in social hierarchies (Pellis & Pellis, 2007). Adolescent group size itself, or in interaction with social density, could therefore influence responses to unfamiliar stimuli through changes in antagonistic behaviour but these variables are rarely tested concurrently.

The current study aimed to investigate the hypothesis that living at high social density during adolescence acts as a stressor, resulting in adult animals that spend less time interacting with unfamiliar stimuli and have a prolonged secretion of CORT during an acute stress response compared to animals raised at a lower density in adolescence. Zebra finches were used, as adolescent social density has previously been shown to have long-term effects on social behaviour in the birds (Ruploh et al., 2014) and Chapter 2 revealed when in adolescence zebra finches appear most sensitive to glucocorticoid exposure (postnatal days 40-60). During days 40-60 zebra finches begin to perch in closer proximity to unfamiliar conspecifics instead of the parents (Adkins-Regan & Leung, 2005; Zann, 1996), so the age range may be a time when adolescent zebra finches are most responsive to different social contexts. Zebra finches were therefore housed in groups varying in number and density of birds from days 40-60.

On day 60, the manipulation was ended by re-housing birds in same-sex pairs and the behavioural interactions between the re-housed birds were quantified to assess the short-term effects of the housing conditions. Birds raised at high density during days 40-60 were predicted to spend less time alone during the initial re-housing compared to all other groups (based on Ago et al., 2014). Adolescents raised at higher density were also expected to engage in more gregarious behaviour (allopreens, clumps), less antagonistic behaviour (beak fences,

displacements), and less neophobic behaviour (hops, head turns) compared to the other housing conditions (based on Branchi & Alleva, 2006; Pellis & Pellis, 2007).

In adulthood (day 100+), behavioural responses toward unfamiliar environments and objects were quantified in individual and group contexts. The unfamiliar environments were identical to those described in Chapter 2, but the unfamiliar object tasks were new and consisted of exposing the birds to a novel perch with coloured card on the end. In line with previous work investigating adolescent stressors (e.g. Chapter 2; Hollis et al., 2013), high density raised birds, compared to all other groups, were predicted to interact less with an unfamiliar environment when individually tested (slower to enter unfamiliar environment, fewer entries into unfamiliar environment, less time perching in unfamiliar environment, less time foraging next to the unfamiliar objects, more hops between perches, and more head turns) and to an unfamiliar object when individually tested (slower to perch on unfamiliar object, fewer number of times contacting unfamiliar object, shorter time perching on unfamiliar object, more hops between perches, and more head turns). In a group context, adolescent social density was not predicted to have any effects on behavioural responses to an unfamiliar environment or object and social behaviour during the task (time alone, allopreening, clumping, beak fencing, perch displacements) based on previous research (Chapter 2; Branchi & Alleva, 2006).

The birds' behavioural responses to an unfamiliar mixed-sex group of conspecifics were quantified later in adulthood in a task identical to that described in Chapter 2. Birds reared at higher adolescent social density were predicted to spend less time alone and, if male, engage in less courtship behaviour compared to all other groups (based on Chapter 2; Ruploh et al., 2014). Birds raised at higher density were also predicted to engage in more gregarious behaviour (allopreens, clumping) and less antagonistic behaviour (displacements, beak fences) compared to all other groups (based on Ruploh et al., 2014; Sachser et al., 1993).

CORT concentration was quantified in response to a standard capture and restraint stressor during the adolescence and in adulthood. Basal concentrations of male testosterone and female estradiol were quantified in adulthood. Blood samples were collected in adulthood for nonapeptide assays, but due to lack of equipment the assays were not performed. Birds reared at higher density were predicted to have a higher stressor-induced CORT concentration in adolescence and adulthood compared to all other groups (based on Ortiz et al., 1985). Basal testosterone and estradiol concentrations were predicted to be unaffected by group size or social density in line with previous research (e.g. Ortiz et al., 1984; Smith et al., 2004).

3.2. Methods

3.2.1. Ethical statement

All ethical guidelines and requirements, as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85–23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986, were adhered to under project licence 70/8159 and personal licences IDFA58352, IEBE43CFF, and 60/13261.

3.2.2. Establishing the experimental population

A total of 76 zebra finches were used in the current study. The zebra finches were offspring from 23 breeding pairs that were established as described in Chapter 2. 12 males and 12 females were taken from an in-house breeding stock and housed together in one of two 100 x 100 x 50 cm (length x height x depth) cages. Birds were observed to determine opposite-sex pairs that engaged in any sexual behaviour (e.g. following, directed song, mounting). Once a pair was identified they were removed from the colony cage and housed in individual breeding cages (60 x 50 x 50 cm, length x height x depth; MB 3612 Metal Double Breeding Cage, R.J. Leigh Ltd., UK) with access to a cardboard nest box (11 x 14 x 11 cm, length x height x depth) with a rectangular aperture of 11 x 7.5 cm (length x height). Birds had ad libitum access to seed

hoppers (Food for Finches, Johnson & Jeff, UK), water hoppers, a water bath, and a grit tray at all times. Diets were supplemented with spinach leaves once per week. Breeding cages were outfitted with two 50 cm perches, and the cage floor was covered with wood pellets (Stovies Wood Pellets, Arbuthnott Wood Pellets Ltd, UK). All breeding birds were housed in a single colony room with lights on at 07:00-19:00, temperature 22±2°, and relative humidity 55±5%.

Each breeding cage was given new nesting material (hay and jute fibre; Liverine Pet and Animal Health Care Ltd., UK) once per day until the birds had laid a clutch and egg food (approx. 1.5g of CéDé Premium Egg Food, Belgium) once per day until the chicks reached nutritional independence (30 days old). After laying each egg, the egg was removed and replaced with a fake egg (Staedtler Fimo Soft Oven Hardened Modelling Clay (white), UK). All of the real eggs were returned to the birds when the females had stopped laying for two consecutive days. Real eggs were returned at the same time in order to synchronise hatching and remove any effects that can arise from hatch order (e.g. exploration: Mainwaring et al., 2012; attractiveness: Mainwaring & Hartley, 2013). Clutches were candled on incubation day 7 to determine egg fertility, with any infertile clutches removed to allow relaying.

On the first day of hatching, brood sizes were standardised to four chicks per nest to control for variation in pre-adolescent group size and social density. Any nests with more than four chicks (2/24) were reduced to four by placing chicks in donor nests that were not used, whilst nests with fewer than four chicks (2/24) were not used in the study. Each chick was given a temporary ID by applying coloured nail polish to each leg when 5 and 8 days old. Chicks were given a permanent ID when 10 days old (one uniquely numbered orange leg ring, one coloured leg ring: pink, yellow, light blue, or white). The colours were selected as no evidence suggests that they affect behaviour (unlike for red bands, e.g. Burley et al., 1982).

3.2.3. Experimental design

At 40 days of age, adolescent birds were housed in one of four groups of same-sex and age-similar (±1 day) birds that varied in the number and density of birds per cage. Groups were termed low number (LN), low number/control (LN/C), high number/low density (HN/LD), and high number/high density (HN/HD; Figure 3.1). The birds were continuously housed in the four conditions between days 40 and 59. On day 60 (±1), group size and density was standardised by pair housing all birds in cages measuring 0.6 x 0.5 x 0.5 metres (length x height x depth). HN/LD, HN/HD, and LN birds were re-housed with a same-sex age-similar (±1 day) bird from a different replicate of the same group (birds were unfamiliar to one another), whereas LN/C birds were re-housed with their familiar cage mate. LN/C birds were included in the design to investigate the effects of re-housing a bird with an unfamiliar conspecific as part of the attempt to standardise housing. LN/C birds can be compared to LN birds in order to draw inferences regarding the effects of adolescent social novelty on later-life measures.

Group size was investigated by the inclusion of low number housing conditions (LN & LN/C) that could be compared with higher number housing conditions (HN/LD & HN/HD). LN and LN/C groups were housed two birds per cage (each condition had n = 18, 10 male and 8 female, split into 9 replicates) in cages measuring 0.6 x 0.5 x 0.5 metres (length x height x depth). To investigate social density, birds in HN/LD and HN/HD conditions were housed five birds per cage (each condition had n = 20, 10 male and 10 female, split into four replicates). Each HN/LD replicate was housed in cages measuring 1.2 x 0.5 x 0.5 metres (length x height x depth), whilst each HN/HD replicates were housed in cages measuring 0.6 x 0.5 x 0.5 m (length x height x depth). The HN/LD group had $0.06m^3$ per bird whereas the HN/HD group had $0.03m^3$ per bird, hence being termed high number/low density and high number/high density. To control for differences in foraging competition across group sizes, large group size replicates had access to two seed hoppers, two water hoppers, two water baths, and two grit

trays whereas smaller group replicates only had one of each item. Birds in HN/HD, LN, and LN/C replicates had access to two 50cm perches, whereas birds in the HN/LD replicates had access to four 50cm perches so they could occupy more space. The ages the birds underwent the housing manipulation in relation to the rest of the experiment is presented in Figure 3.2.

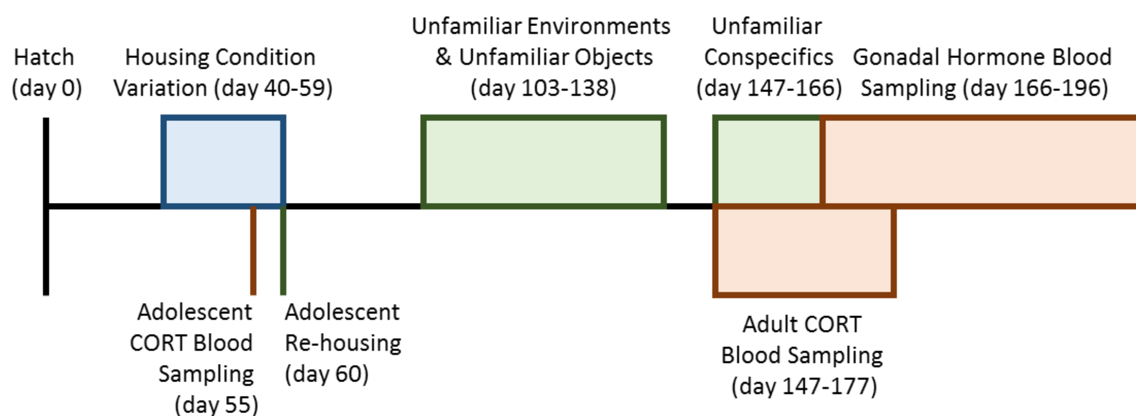

Figure 3.1. Summary diagram of the early adolescent housing condition variation. Birds were housed in one of four groups varying in group size and/or density between days 40-59. On day 60 the LN, HN/HD, and HN/LD were re-housed in unfamiliar pairs.

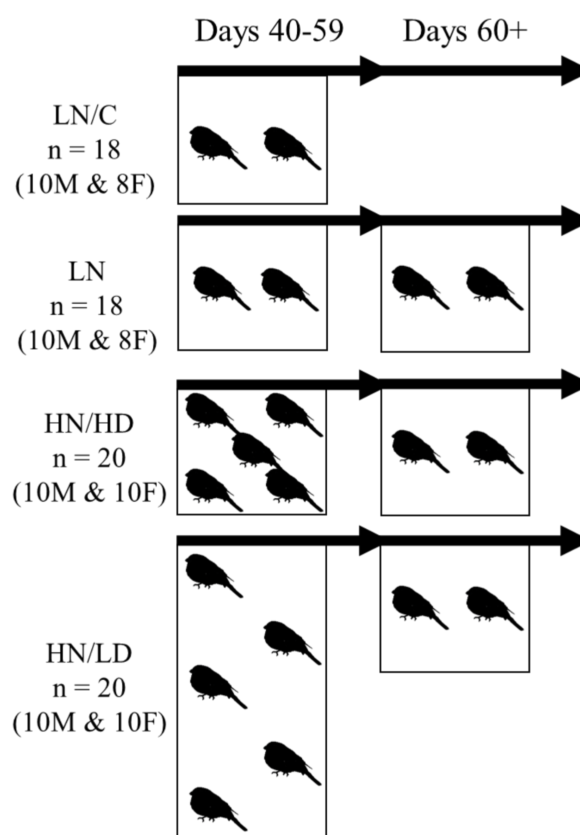

Figure 3.2. Timeline detailing the age at which the birds underwent the adolescent variation in housing conditions (blue), when behavioural testing occurred (green), and when blood sampling occurred (red).

3.2.4. Adolescent behavioural responses to conspecifics during re-housing

On day 60, birds were video recorded for the first thirty minutes after being re-housed. Digital cameras (Sony Handycam, HDR-PJ24OE) were positioned around 60 cm in front of the cages. Recordings were used to quantify affiliation-related behaviour (duration of time perching alone, number of allopreens, and number and duration of clumping), antagonistic behaviour (number of perch displacements, number and duration of beak fencing), courtship behaviour (number and duration of directed song, number of mounts), and neophobia-related behaviour (number of hops, number of head turns) that are described in Table 3.1.

Table 3.1. Descriptions of zebra finch behaviour that were quantified during the adolescent social interactions, adult group context unfamiliar stimuli tasks, and adult social integration task. Definitions are based on those in Alger et al. (2011), Goodson & Adkins-Regan (1999), Ruploh et al. (2014), and Zann (1996).

Behaviour	Description
Perching alone	A bird is on a perch with no other bird on the perch
Allopreen	A bird grooms the neck and/or head of another bird with its beak
Clumping bout	Two birds sit in contact with one another for at least 5 seconds
Displacement	A bird hops at another bird causing the receiving bird to hop away; can be given or received
Beak fence bout	Birds jab their beaks together for at least 2 seconds
Directed song bout	A male looks at a female and emits a stereotyped series of calls for at least 2 seconds
Mount	A male hops on top of a female as an attempt to copulate
Hop	A bird jumps from one location to another; hops can be perch-to-perch, perch-to-ground, or ground-to-perch.
Head turns	A bird turns its head ninety degrees, with zero degrees being head facing forward with beak aligned with the midline of the body

3.2.5. Adult behavioural testing

Four tasks were used to quantify behavioural responses to unfamiliar stimuli when in adulthood (PHD 103-138); two tasks exposed birds to an unfamiliar environment, two tasks exposed birds to an unfamiliar object. Each task occurred once when birds were individually housed (individual context) and once when birds were housed in familiar social groups (group context). Tasks within each context occurred on the same day for each bird, with each task separated by four hours (individual context start time 9 AM or 1 PM; group context start time 9.15 AM or 1.15 PM). Individual and group context tasks were separated by at least 7 days for each bird and counter-balanced across treatment groups and sexes. Unfamiliar environment and object task order were counter-balanced across treatment groups and sexes within each day. Later in adulthood (PHD 147-166) after behavioural responses to unfamiliar non-social stimuli were completed, birds were exposed to a fifth task used to quantify behavioural responses to an unfamiliar mixed-sex social group of birds.

Unfamiliar environment tasks. The tasks were identical to those described in Chapter 2, and only a brief summary will be presented here. Tasks were carried out in cages measuring 120 x 50 x 50 cm (length x height x depth) that were split into two zones each measuring 60 x 50 x 50 cm (length x height x depth) with a white wrought iron divider. One zone was identical to the home cage and was therefore considered a familiar environment, whereas the other identical to the home cage but with the addition of three unfamiliar objects attached to the perches. Individual context objects were a pink ball, a pyramid of three coloured blocks, and two intertwined dark blue pipe cleaner rings. Group context objects were a yellow tub, a green pipe cleaner helix, and a ‘U’ of coloured blocks. Unfamiliar objects were baited with a dish of spinach to encourage the birds to approach the objects. Familiar/unfamiliar cage side was counter-balanced across treatment groups and sexes.

In individual context tasks, birds were captured from their home cages and single housed in the familiar environment of the unfamiliar environment task cages that were in a room separate from the colony room. All birds from a single cage were captured at the same time and housed in the unfamiliar environment cages whilst in acoustic, but not visual, contact with one another. Birds were left twenty fours before the first task began. A video camera (Sony Handycam, HDR-PJ24OE) was placed around 60 cm in front of the task cages. For each task, birds were recorded for thirty minutes in order to quantify locomotor activity (*number of hops between perches*) and risk assessment behaviour (*number of ninety degree head turns*) prior to exposing the birds to the unfamiliar environment (i.e. pre-exposure). After thirty minutes had elapsed the divider separating the familiar/unfamiliar compartments was removed to expose birds to the unfamiliar environment (i.e. exposure) for sixty minutes. During exposure the following behaviour were recorded: *latency to enter unfamiliar environment (seconds)*, *number of entries into unfamiliar environment*, *duration of time spent perching in unfamiliar environment (seconds)*, *number of objects contacts*, *duration of time spent in contact with objects (seconds)*, *total number of hops between perches*, and *total number of ninety degree head turns*.

In group context tasks, birds were captured and immediately returned to their home cage in the colony room with their familiar cage mates to control for handling effects between individual and group conditions. After 24 hours had then elapsed, the task began and was identical to that described for the individual context unfamiliar environment task except two birds were tested at the same time. In the group context, the same behavioural measures were recorded as in the individual context task for each bird. Additionally, measures were taken of social behaviour, namely: *number of allopreens*, *number of beak fence bouts*, *duration of time beak fencing (seconds)*, *number of clump bouts*, *duration of time clumping (seconds)*, and *duration of time perching alone (seconds)*. Social behaviour are defined in Table 3.1. After

sixty minutes had elapsed birds were ushered back into the familiar environment and the divider replaced. If the individual context unfamiliar environment task was the last task of the day, birds were captured and returned to their home cages in their familiar pairs in the colony room.

Unfamiliar object tasks. The individual context task occurred in what was the familiar environment in the unfamiliar environment task cages described above, whereas the group context task occurred in the home cage. The task consisted of inserting a 15cm white perch into the centre of the front of the cage (level with the two 50cm perches already present). On the end of each perch was a coloured piece of card (Paper2Go, Rainbow Card Bright's) that the birds had never been exposed to, making the perch an unfamiliar object. The individual context object was a 7cm diameter orange circle (Figure 3.3. a), whereas the group context task object was a 6 x 6 cm pink square (Figures 3.3. b). A pilot study was conducted on stock adult birds (n = 5M, 5F) to determine which objects should be used. When individually housed, stock birds had a mean latency to perch on the novel objects described above of around 25 minutes (individual context object: M = 26.25, SD = 6.03; group context object: M = 24.73, SD = 5.86) in a 60 minute test. Objects were therefore considered sufficiently and similarly aversive.

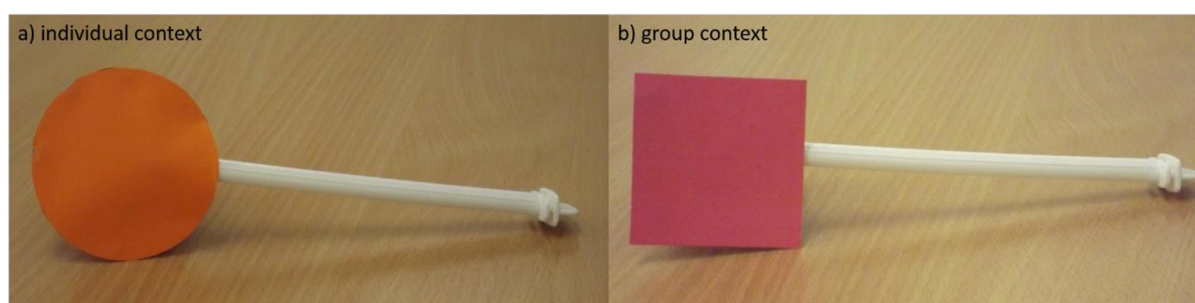

Figure 3.3. Objects used in unfamiliar object tasks, a) individual context and b) group context.

In order to quantify any behavioural differences prior to the tasks, locomotor behaviour (*number of hops between perches*) and risk assessment (*number of ninety degree head turns*) were recorded for thirty minutes prior to exposing the birds to the unfamiliar objects. After thirty minutes had elapsed the objects were inserted into the task cages. Objects remained in

the task cages for sixty minutes and the behavioural responses to the objects were recorded. Behaviour quantified were *duration of time flying in response to the object* (seconds), *latency to perch on unfamiliar object (seconds)*, *number of perches on unfamiliar object*, *duration of time spent perching on unfamiliar object (seconds)*, *total number of hops between perches*, and *total number of ninety degree head turns*. In the group context, social behaviour between the birds was also quantified. Social behaviour quantified were *number of allopreens*, *number of beak fence bouts, duration of beak fence bouts (seconds)*, *number of clumps*, *duration of time clumping (seconds)*, and *duration of time perching alone (seconds)*. After sixty minutes of exposure to the unfamiliar object the object was removed. If the unfamiliar object task was the last task of the day, birds were then captured and returned to their home cages in their familiar pairs in the colony room.

Unfamiliar conspecifics task. A test bird was captured from the home cage and housed in one of four unfamiliar social groups. Each unfamiliar social group consisted of two adult male and two adult female birds taken from a stock population. Each unfamiliar social group has been housed in cages measuring 60 x 50 x 50 cm (length x height x depth) for 30 days prior to the beginning of testing task and were therefore considered familiar to one another. All four unfamiliar social groups were housed in the same room and were in auditory, but not visual, contact with one another. The room in which the unfamiliar conspecifics task occurred was outside that of the holding room.

Two birds from the same home cage were captured and housed in separate unfamiliar social groups for 60 minutes and cages were video recorded (Sony Handycam, HDR-PJ24OE) from a distance of around 60 cm in front of the test cages. Video records were used to quantify social behaviour that the test bird engaged in with unfamiliar birds. Affiliation-related behaviour recorded were: *number of allopreens*, *number of clumps*, *duration of time perching alone (seconds)*, and *duration of time on ground alone (seconds)*. Antagonistic behaviour

recorded were: *beak fence bouts (number and duration)*, *number of times a test bird displaced an unfamiliar bird, number of times an unfamiliar bird displaced a test bird.* Courtship behaviour recorded were: *directed song bouts emitted by test males toward unfamiliar females (number and duration)*, *directed song bouts sang to test females by unfamiliar males (number and duration)*, *number of times a test male and an unfamiliar female engaged in mounting,* and *number of times a test female and unfamiliar male engaged in mounting*. Beak fencing and perch displacement behaviour were recorded separately for unfamiliar males and unfamiliar females. Social behaviour are defined in Table 3.1. After sixty minutes had elapsed both test birds were captured and returned to their home cage. Each unfamiliar social group was exposed to one test bird every day over a period of nineteen days.

3.2.6. Hormone sampling

Plasma CORT concentration in response to a standard capture-restraint stressor (Wingfield et al., 1997) was quantified during adolescence (PHD 54-56) and then in adulthood (PHD 147-177). Basal plasma concentration of testosterone in males and estradiol in females were determined between from blood samples taken between PHD 166-196.

Corticosterone. Three blood samples were collected from each bird in response to a standard capture and restraint stressor over a period of 45 minutes. The first sample (approx. 40µl) was collected within three minutes of entering the holding room to ensure that CORT was at basal concentration (Romero, 2004). Birds were then restrained in black cloth bags. A second sample (approx. 30µl) was taken 15 minutes into restraint, and a third sample (approx. 30µl) was taken 45 minutes into restraint. The time points were chosen to attempt to quantify basal, peak, and post-peak CORT concentration in response to a stressor. Chapter 2 revealed that 10 minutes was an inaccurate time to detect peak CORT concentration and 30 minutes was an insufficient duration of time to determine post-peak CORT. Consequently, the blood sampling times were

later compared to chapter 2. 15 minutes and 45 minutes into restraint were selected as later sampling times to attempt to quantify peak and post-peak CORT concentrations, respectively. Blood samples were collected by pricking a brachial vein of each bird with a 27-gauge needle tip. Samples were collected into heparinised capillary tubes, placed in an Eppendorf on wet ice, and then centrifuged at 3500g for 10 minutes to separate the plasma and red blood cells. Plasma was then removed and placed into a separate Eppendorf tube that was then stored at -20°C. All birds in a home cage were captured and sampled at the same time to control for any effects of cage disturbance. After the final blood sample was taken, the birds were returned to their home cages in their familiar pairs.

Gonadal hormones. Birds from a home cage were captured and transferred to a separate room for blood sampling. A brachial vein was punctured with a 27-gauge needle tip and 100µl of blood was collected into heparinised capillary tubes. All samples were collected within four minutes of entering the holding room to ensure basal testosterone concentration was collected (Wingfield & Wada, 1989). Blood samples were placed into an Eppendorf on wet ice before being centrifuged at 3500g for 10 minutes to separate the plasma and red blood cells. Plasma was then removed and placed into a separate Eppendorf tube that was then stored at -20°C.

3.2.7. Hormone assays

CORT. Radioimmunoassay (Spencer et al., 2009) was used to quantify CORT in 10-30µl of plasma. All samples were extracted with 1 ml diethyl ether after being spiked with 25µl of [1,2,6,7-3H]-CORT label (Perkin Elmer Inc., UK). Extracted samples were evaporated at 42°C and reconstituted in 300µl of assay buffer (0.01M PBS, pH 7.4, 0.25% BSA). 50µl aliquots of the reconstituted samples were used to determine the extraction efficacy, which ranged between 71.24-100%. The concentration of CORT was then determined in two 100µl aliquots of the reconstituted samples using anti-CORT antiserum (Esoterix Endocrinology, USA, B3-163;

1:15000 dilution in assay buffer) and [1,2,6,7-3H]-CORT label (Perkin Elmer, UK). The reactions were incubated for 24 hours at 4°C, and the antibody-bound antigens were then removed by the addition of 100µl of a dextran coated charcoal suspension (0.5% charcoal, 0.25% dextran in assay buffer) and centrifuging the samples at 2000g for 20 minutes. The supernatant was then removed and used and radioactivity quantified using a radioactivity counter (Packard Tri-Carb 1600 TR Liquid Scintillation Analyser, Perkin Elmer Inc., UK). A total of six assays were performed, with all samples from a single individual run in duplicate in the same assay. Treatment groups and sexes were spread across the assays. Each assay included a ten point standard curve ranging from 0.04-20 ng/ml. Intra-assay coefficients of variation (%) were 10.51, 9.74, 12.86, 10.12, 11.37, and 13.15. Inter-assay coefficient of variation (%) was 12.70. 50% binding (ng/ml) were 0.79, 0.74, 0.80, 0.76, 0.92, and 0.81.

Testosterone. Radioimmunoassay was used to quantify testosterone in 20-30µl of plasma following the protocol described for CORT. However, anti-testosterone antiserum (MP Biomedicals, LLC., USA, 07-189016) and [1,2,6,7-3H]-testosterone label (Perkin Elmer, UK) were used in the testosterone assay. A ten point standard curve ranging from 0.04-20 ng/ml was included in the assay. The extraction efficiency was 75-100%. All samples were run in duplicate in one assay. The intra-assay co-efficient of variation was 5.34%, 50% binding was 0.39 ng/ml, and the detection limit was 0.04 ng/ml.

Estradiol. An enzyme immunoassay kit (Cayman Chemical Company, Estradiol EIA Kit, Ann Arbor, Michigan, USA) was used to determine estradiol concentration in female plasma samples. The kit has previously been used to quantify estradiol in zebra finches (Remage-Healey et al., 2008; Remage-Healey et al., 2012). Following the manufacturer's guidelines 10-30µl samples of plasma were diluted in assay buffer to 105µl. 50 µl aliquots were run in duplicate on one plate with an eight point standard curve ranging from 6.6-4000 pg/ml. The plate was read on a Biochrom Anthos 2010 Microplate Reader, ADAP 2.0 (Biochrom Ltd.,

UK) at a wavelength of 405 nm. Intra-assay coefficient of variation was 9.74% and the detection limit was 6.6 pg/ml.

3.2.8. Data Analysis

SPSS version 22 was used to conduct all statistical tests. Residuals from each analysis were checked for normality (Shapiro-Wilk, $p > 0.05$). Variables that had positively skewed residuals were square root or log10 transformed to achieve normality where stated. Transformations are indicated in brackets after each dependent variable; no bracketed information indicates that raw data was used. If a variable could not be transformed to normality, a generalized linear model was used. Each model was conducted twice for all dependent variables. The first model included only LN and LN/C as a fixed factor to determine the effects of re-housing a bird with a familiar or unfamiliar conspecific in adolescence (referred to as the familiarity model). The second model included LN/C, HN/LD, and HN/HD data as a fixed factor to determine the effects of early adolescent group size and/or social density (referred to as the housing condition model). Nest ID was entered as a random factor in all mixed models to control for pre-adolescent variation. Home cage ID was added as a random factor in group context behavioural responses to unfamiliar environment and object mixed models to control for the effects of conspecifics on individual bird behaviour. Test cage ID was entered as a random factor in behavioural response to unfamiliar conspecific mixed models to control for effects of different groups on test bird behaviour. An alpha value of $p < 0.05$ was used as the threshold for statistical significance in all tests. Sidak and Bonferroni post hoc tests were used to investigate significant effects for independent and repeated measures in all models, respectively. Partial eta squared was calculated as a measure of effect size for significant main effects and interactions in general linear models, whilst Cohen's d was calculated as a measure of effect size for all significant post hoc comparisons. All data presented are mean $\pm$ one standard error of the mean.

Adolescent behaviour. Exploratory correlations (Spearman's rank) revealed significant relationships between clumping behaviour (number and duration of clumps: familiarity model, $r = 0.622$, $p < 0.001$; housing condition model: $r = 0.747$, $p < 0.001$) and between beak fencing behaviour (number and duration of beak fence bouts: familiarity model, $r = 0.867$, $p < 0.001$; housing condition model, $r = 0.854$, $p < 0.001$). Consequently, only one clumping variable (duration of time clumping) and one beak fencing variable (duration of time spent beak fencing) were used for analysis of these behaviour in each model. Durations were chosen in order to maintain a consistent scale of measurement between adolescent social behaviour (i.e. along with duration perching alone).

Duration of time perching alone and number of head turns were entered as dependent variables in separate linear mixed models (LMM). Duration of time spent clumping (square root in housing condition model) and time spent beak fencing (square root in housing condition model) were entered as a dependent variables in separate general linear models (GLM). For each behaviour, one model included familiarity conditions (LN, LN/C) as a fixed actor and one model included housing conditions (LN, HN/LD, HN/HD) as a fixed factor. Sex was entered as a fixed factor in all models. In time alone and head turn models, individual birds were the unit of analysis with cage number entered as a random factor to control for the dependency of birds behaviour on one another. In clumping and beak fencing models, cage number was the unit of analysis as each behaviour was not specific to a bird but emerged whilst two birds were interacting. Allopreening, perch displacements, song, and mounts occurred infrequently (i.e. fewer than 1 in 10 birds displayed each behaviour) and were therefore not analysed. Variables excluded in response to PCA analysis, i.e. number of beak fence bouts and duration of song bouts, were analysed in an identical manner to their respective included variables as described above. Models for included and excluded variables within each behaviour (i.e. clumping and

beak fencing) had similar outputs (data not shown), indicating that excluding these variables did not qualitatively affect the results.

Unfamiliar environment individual context. Latency to enter unfamiliar environment, number of entries into unfamiliar environment, and duration of time perching in unfamiliar were entered as dependent variables in a multivariate general linear model. Number of hops during exposure and number of head turns during exposure (square root in both familiarity condition and housing condition models) were entered as dependent variables in separate LMMs. For each behaviour, one model included familiarity conditions (LN, LN/C) as a fixed actor and one model included housing conditions (LN, HN/LD, HN/HD) as a fixed factor. Sex was entered as a fixed factor in all models. In hop and head turn models, the pre-exposure quantities of each behaviour were entered into corresponding models as co-variates to control for any behavioural differences prior to the task. No differences were found in pre-exposure measures (see Appendix A Table A.3., Table A.4, and Table A.5.). Number of object contacts and duration of object contact occurred infrequently (i.e. fewer than 1 in 10 birds displayed each behaviour) and were therefore not analysed.

Unfamiliar environment group context. Latency to enter unfamiliar environment, number of entries into unfamiliar environment, duration of time perching in unfamiliar environment, number of hops during exposure, number of head turns during exposure, and duration of time perching alone during exposure (square root in both familiarity and housing condition models) were entered as dependent variables in separate LMMs. For each behaviour, one model included familiarity conditions (LN, LN/C) as a fixed actor and one model included housing conditions (LN, HN/LD, HN/HD) as a fixed factor. Sex was entered as a fixed factor in all models. In hop, head turn, and time alone models, pre-exposure quantities of each behaviour were entered into corresponding models as co-variates to control for any differences prior to the task. No differences were found in pre-exposure measures (see Appendix A Table A.3.,

Table A.4., and Table A.5.). Allopreening, beak fencing, clumping, perch displacements, number of object contacts, and duration of object contact occurred infrequently (i.e. fewer than 1 in 10 birds displayed each behaviour) and were not analysed.

Unfamiliar object individual context. Latency to perch on unfamiliar object, duration of time perching on unfamiliar object, number of hops (square root in both familiarity and housing condition models), and number of ninety degree head turns were entered as dependent variables into separate LLMs. Number of perches on unfamiliar object were entered as a dependent variable in a generalized linear mixed model (GzLM) with data fitted to a gamma distribution with a log link function. For each behaviour, one model included familiarity conditions (LN, LN/C) as a fixed actor and one model included housing conditions (LN, HN/LD, HN/HD) as a fixed factor. Sex was entered as a fixed factor in all models. In hop and head turn models, pre-exposure quantities of each behaviour were entered into corresponding models as co-variates to control for any differences prior to the task. No differences were found in pre-exposure measures (see Appendix A Table A.6., Table A.7., and Table A.8.).

Unfamiliar object group context. Latency to perch on unfamiliar object, number of perches on unfamiliar object, duration of time perching on unfamiliar object, number of hops between perches (square root in housing condition model), number of ninety degree head turns, and duration of time perching alone during exposure were entered as dependent variables in separate LMMs. For each behaviour, one model included familiarity conditions (LN, LN/C) as a fixed actor and one model included housing conditions (LN, HN/LD, HN/HD) as a fixed factor. Sex was entered as a fixed factor in all models. In hop, head turn, and time alone models, the pre-exposure quantities of each behaviour were entered into corresponding models as co-variates to control for any behavioural differences prior to the task. No differences were found in pre-exposure measures (see Appendix A Table A.6., Table A.7., and Table A.8.).

Allopreening, beak fencing, clumping, and displacements were not analysed as the behaviour occurred infrequently (i.e. fewer than 1 in 10 birds displayed each behaviour).

Unfamiliar conspecifics task. Exploratory correlations (Spearman's rank) revealed a significant relationship between beak fencing behaviour (number and duration of beak fences: familiarity model, $r = 0.953$, $p < 0.001$; housing condition model, $r = 0.969$, $p < 0.001$). Only duration of time beak fencing was therefore used in further analyses. Correlations were also present between courtship behaviour in males and between courtship behaviour in females (Table 3.2.). Duration of song bouts was omitted from future analyses. A principal components analysis (PCA) revealed the number of song bouts and number of mounts could be reduced to single factors for males (familiarity condition: KMO = 0.682; Bartlett's Test, χ^2 (1) = 30.676, $p < 0.001$; housing condition: KMO = 0.749; Bartlett's Test, χ^2 (1) = 78.281, $p < 0.001$), and females (familiarity condition: KMO = 0.517; Bartlett's Test, χ^2 (1) = 25.874, $p < 0.001$; housing condition: KMO = 0.700; Bartlett's Test, χ^2 (1) = 52.491, $p < 0.001$). The PCA model was used to create a new component (i.e. courtship behaviour) score between the variables. Allopreening and clumping occurred infrequently (i.e. less than 1 in 10 birds engaged in the behaviour) and were therefore not analysed.

Table 3.2. Correlations between courtship behaviour quantified in the unfamiliar conspecifics task split by sex and adolescent condition model (familiarity, housing).

Variables	Male		Female	
	r	p	r	p
Familiarity conditions model				
Song bout number and song bout duration	0.825	<0.001	0.770	<0.001
Song bout number and mount number	0.475	0.034	0.778	<0.001
Song bout duration and mount number	0.513	0.021	0.512	0.013
Housing conditions model				
Song bout number and song bout duration	0.900	<0.001	0.807	<0.001
Song bout number and mount number	0.760	<0.001	0.763	<0.001
Song bout duration and mount number	0.716	<0.0001	0.611	0.001

Duration of time alone was entered as a dependent variables in LMMs. One model contained familiarity condition (LN, LN/C) as a fixed factor, whilst a second model contained housing condition (LN, HN/LD, HN/HD) as a fixed factor. Sex and location (perch, ground) were entered as fixed factors in both models, and location was also entered as a repeated measure. Duration of time spent beak fencing, number of times a test bird displaces an unfamiliar bird (square root transformed in housing condition model), and number of times an unfamiliar bird displaces a test bird (square root transformed in familiarity and housing condition models) were entered as dependent variables in separate LMMs. One model contained familiarity condition (LN, LN/C) as a fixed factor, whilst a second model contained housing condition (LN, HN/LD, HN/HD) as a fixed factor. Test bird sex and unfamiliar bird sex were also entered as fixed factors in beak fencing and displacement models, with unfamiliar bird sex also entered as a repeated measure to account for non-independence of the behaviour between unfamiliar bird sexes. Male and female courtship factors were entered as dependent variables in separate LMMs. Familiarity condition (LN, LN/C) and housing condition (LN, HN/LD, HN/HD) were entered as fixed factors in two separate models for each sex. Variables excluded in response to PCA analysis, i.e. number of beak fence bouts and duration of song bouts, were analysed in an identical manner to their respective included variables as described above. Models for included and excluded variables within each behaviour (i.e. beak fencing and courtship) had similar outputs (data not shown), indicating that excluding these variables did not qualitatively affect the results.

Hormones. To analyse CORT concentration over time in response to capture and restraint, log10 CORT concentration (ng/ml) was entered as a dependent variable in two separate LMMs (familiarity model and housing condition model). Adolescent familiarity conditions (LN, LN/C) or housing condition (LN, HN/LD, HN/HD) were entered as fixed factors in separate models alongside the fixed factors of sampling time, age group, and sex that were entered as

in both models. Sampling time and age were also entered as repeated measures in both models. To further analyse CORT, change in CORT concentration between 15 and 45 mins into restraint was calculated (i.e. 45 min sample minus 15 min sample for each bird). Change in CORT concentration (ng/ml) was entered as a dependent variable in two separate LMMs (square root transformed data was used for the familiarity model and raw data was used for the housing condition model). Adolescent familiarity (LN, LN/C) or housing condition (LN, HN/LD, HN/D) were entered as fixed factors alongside in separate models alongside the fixed factors of age group and sex entered in both models. Age group was also entered as a repeated measure, and basal CORT concentration was entered as a co-variate in both models. Log10 male testosterone concentration (ng/ml) and square root female estradiol concentration (pg/ml) were each entered as dependent variables in two separate LMMs. For each hormone, one model had familiarity condition (LN, LN/C) entered as a fixed factor and one model had housing condition (LN, HN/LD, HN/HD) entered as a fixed factor. Spearman's rank correlation were conducted to investigate the relationship between hormone concentrations and behavioural variables that were significantly affected by adolescent group size and/or density, with models conducted separately for each age group and sex. In adolescent models, beak fencing and clumping factors were omitted as the unit of analysis was cage and these variables could not be correlated with CORT concentrations quantified for individual birds.

Correlations. Correlations between hormone concentrations with behavioural variables were assessed using Spearman's rank correlation. Models were conducted separately for adolescent and adult variables and for each sex. Adolescent CORT measurements were correlated with adolescent behavioural after re-housing that were affected by adolescent condition. Adult CORT and gonadal hormone concentrations were correlated with behaviour in which a main effect or interaction implicating adolescent housing condition were found. Bonferroni corrections were applied to account for multiple comparisons.

3.3. Results

3.3.1. Adolescent behavioural responses to conspecifics after re-housing

Perching alone. Birds that were unfamiliar spent more time perching alone than birds that were familiar ($F_{1,15.813} = 14.479$, $p = 0.002$; Figure 3.4.); effects that were independent of sex (sex: $F_{1,15.321} = 0.018$, $p = 0.896$; sex x familiarity: $F_{1,14.956} = 0.088$, $p = 0.770$). When housed with an unfamiliar conspecific, time perching alone differed according to a bird's prior housing condition and sex (housing condition: $F_{2,28.704} = 3.929$, $p = 0.031$; sex: $F_{1,30.104} = 13.600$, $p = 0.001$; housing condition x sex: $F_{2,25.050} = 3.666$, $p = 0.040$; Figure 3.4.). Within females, HN/HD birds perched alone less than LN birds ($p = 0.002$, $d = 3.16$) but HN/LD birds perched alone to a similar extent as birds from other conditions (LN, $p = 0.210$; HN/HD, $p = 0.180$). Within males, all birds perched alone to a similar extent regardless of prior housing conditions (LN vs. HN/LD, $p = 0.998$; LN vs. HN/HD, $p = 0.995$; HN/LD vs. HN/HD, $p = 1.0$). HN/HD females spent less time perching alone than HN/HD males ($p < 0.001$, $d = 0.29$), but sex differences were not present between LN ($p = 0.062$) and HN/LD conditions ($p = 0.988$).

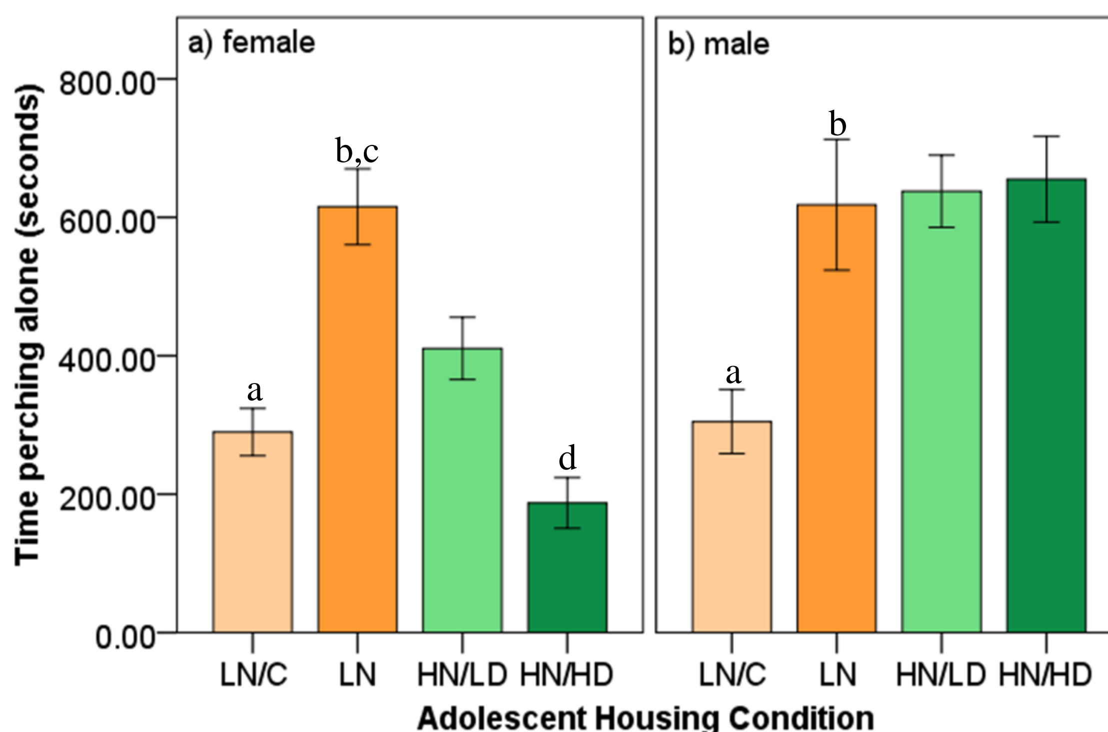

Figure 3.4. Duration of time birds spent perching alone during adolescent social interactions split by housing condition for a) female birds and b) male birds. Data are mean ± one standard error of the mean. Significant differences ($p < 0.05$) in each sex are shown: a vs. b, c vs. d.

Clumping. Unfamiliar pairs of bird's spent less time clumping together than familiar pairs of birds ($F_{1,14}$ = 8.444, p = 0.012, η_p^2 = 0.376; Figure 3.5.), and this effect was present regardless of a bird's sex (sex: $F_{1,14}$ = 0.205, p = 0.658; sex x familiarity: $F_{1,14}$ = 3.870, p = 0.069). When housed with an unfamiliar conspecific, clumping duration differed with a bird's prior housing condition and sex (housing condition: $F_{2,23}$ = 8.823, p = 0.001, η_p^2 = 0.595; sex: $F_{1,23}$ = 11.578, p = 0.002, η_p^2 = 0.335; housing condition x sex: $F_{2,23}$ = 16.885, p < 0.001, η_p^2 = 0.434; Figure 3.5.). Within females, HN/HD birds spent more time clumping than birds from other housing conditions (LN, p < 0.001, d = 6.03; HN/LD, p = 0.001, d = 5.21) and clumping duration was similar between LN and HN/LD birds (p = 0.934). Within males, LN birds clumped more than HN birds (HN/LD, p = 0.002, d = 1.69; HN/HD, p = 0.022, d = 1.55) and clumping duration was similar between HN birds (p = 0.715). LN males clumped more than LN females (p = 0.040, d = 1.27), but HN females clumped more than HN males (HN/LD, p = 0.040, d = 0.97; HN/HD, p < 0.001, d = 6.57).

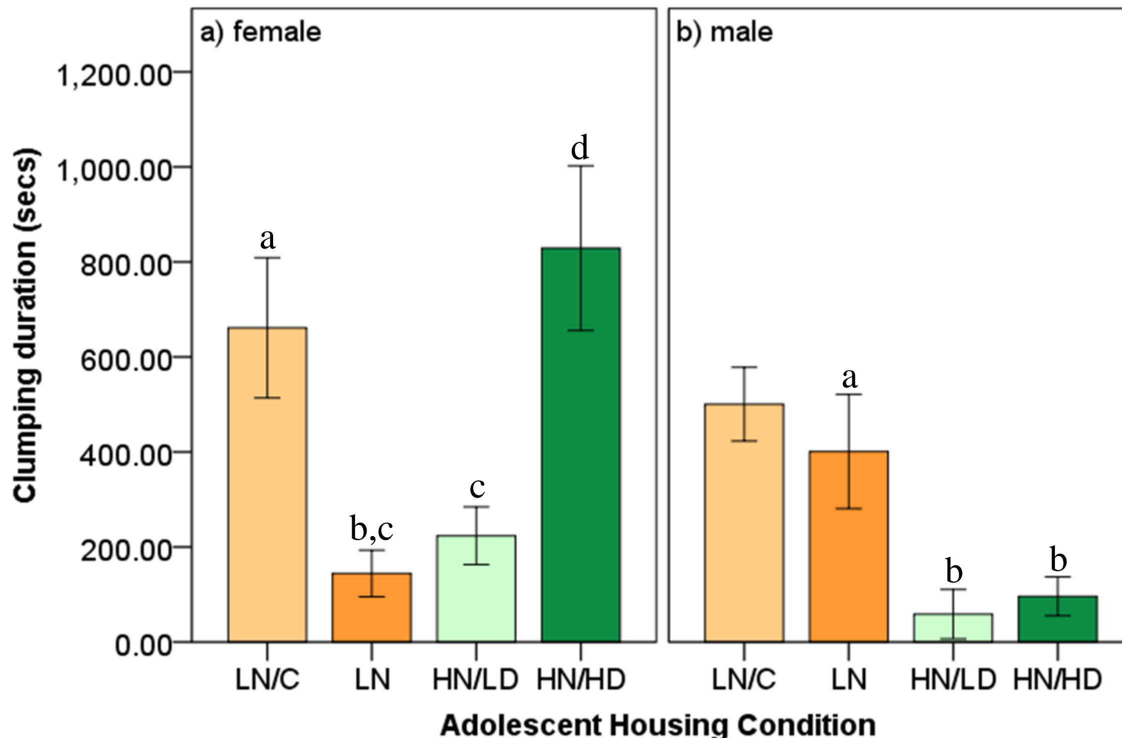

Figure 3.5. Clumping duration of birds during adolescent social interactions for a) female birds and b) male birds. Data are mean ± one standard error of the mean. Significant differences (p < 0.05) within each sex are shown: a vs. b and c vs. d.

Beak fencing. Unfamiliar pairs of birds spent more time beak fencing than familiar pairs ($F_{1,14} = 5.888$, $p = 0.029$, $\eta_p^2 = 0.296$; Figure 3.6.); an effect that was present regardless of a bird's sex (sex: $F_{1,14} < 0.001$, $p = 1.0$; sex x familiarity: $F_{1,14} = 0.073$, $p = 0.791$). When housed with an unfamiliar conspecific, beak fencing duration did not differ with housing condition itself ($F_{2,23} = 2.197$, $p = 0.134$) but did differ with a bird's prior housing condition and sex (sex: $F_{1,23} = 17.353$, $p < 0.001$, $\eta_p^2 = 0.430$; housing condition x sex: $F_{2,23} = 5.064$, $p = 0.015$, $\eta_p^2 = 0.306$; Figure 3.6.). In males, HN birds fenced more than LN birds (LN vs. HN/LD, $p = 0.044$, $d = 0.84$; LN vs. HN/HD, $p = 0.003$, $d = 2.18$). In females, all birds beak fenced for a similar duration regardless of prior housing condition (LN vs. HN/LD, $p = 0.867$; LN vs. HN/HD, $p = 0.867$; HN/LD vs. HN/HD, $p = 1.0$). Males from HN conditions fenced more than females from the respective HN conditions (HN/LD, $p = 0.005$, $d = 1.77$; HN/HD, $p < 0.001$, $d = 2.69$), but male and female LN birds fenced to a similar extent ($p = 0.905$).

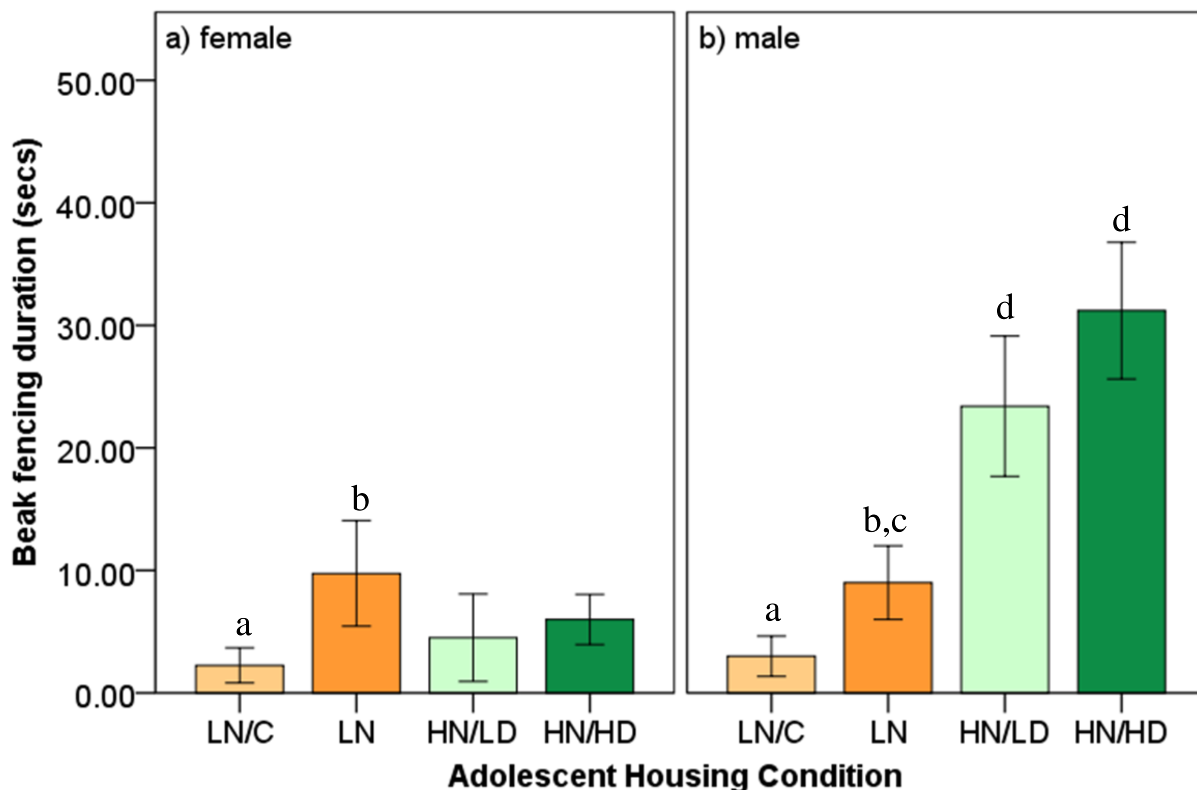

Figure 3.6. Duration of time spent beak fencing during adolescent social interactions split by housing condition for a) female birds and b) male birds. Data are mean $\pm$ one standard error of the mean. Significant differences ($p < 0.05$) within each sex are shown: a vs. b, c vs. d.

Head turns. Birds housed with an unfamiliar conspecific engaged in more head turns than birds housed with a familiar conspecific ($F_{1,50.320} = 15.018$, $p < 0.001$); effects that were independent of a bird's sex (sex: $F_{1,30.088} = 0.010$, $p = 0.921$; sex x familiarity: $F_{1,29.705} = 0.371$, $p = 0.547$). The number of head turns that occurred in response to an unfamiliar conspecific was similar irrespective of a bird's prior group size and social density ($F_{2,48.073} = 0.656$, $p = 0.523$) and irrespective of a bird's sex (sex: $F_{1,50.227} = 1.385$, $p = 0.245$; sex x adolescent condition: $F_{1,47.075} = 0.270$, $p = 0.764$).

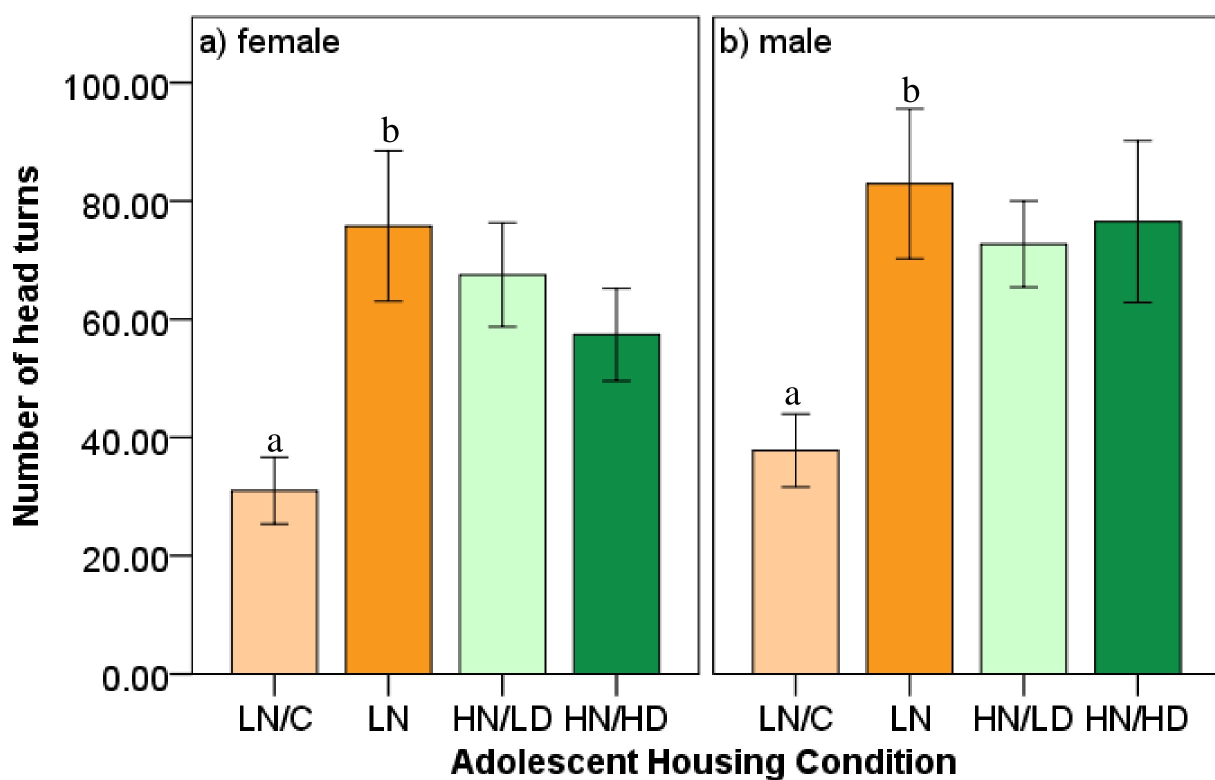

Figure 3.7. Number of head turns by birds during the adolescent social interactions split by adolescent housing condition and sex. Data are mean ± one standard error of the mean. Significant differences ($p < 0.05$) are shown by a vs. b.

3.3.2. Adult behavioural responses to unfamiliar environments

Adolescent re-housing with a familiar vs. unfamiliar conspecific had no effect on the latency to enter the unfamiliar environment, number of entries into the unfamiliar environment, or duration of time spent in the unfamiliar environment when in an individual context (familiarity: $F_{1,32} = 0.519$, $p = 0.673$; sex: $F_{1,32} = 0.594$, $p = 0.624$; familiarity x sex: $F_{1,32} = 0.236$, $p = 0.871$). Adolescent group size and/or social density prior to being re-housed with an unfamiliar conspecific in adolescence also had no effect on the same behavioural measures (housing condition: $F_{2,51} = 1.028$, $p = 0.388$; sex: $F_{1,51} = 1.828$, $p = 0.154$; housing condition x sex: $F_{2,51} = 0.298$, $p = 0.827$). Data for the latency to enter the unfamiliar environment (Table 3.3.), number of entries into the unfamiliar environment (Table 3.4.), and duration of time spent perching in the unfamiliar environment (Figure 3.8.) are presented below alongside group context data for comparison.

In a group context, all birds entered the unfamiliar environment at a similar time regardless of whether they were re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,14.334} = 2.356$, $p = 0.147$; sex: $F_{1,14.373} = 0.185$, $p = 0.674$; familiarity x sex: $F_{1,14.277} = 0.017$, $p = 0.898$; Table 3.3.) and regardless of early adolescent group size and and/or social density (housing condition: $F_{2,23} = 1.122$, $p = 0.372$; sex: $F_{1,23} = 0.437$, $p = 0.534$; housing condition x sex: $F_{2,23} = 0.850$, $p = 0.372$; Table 3.3.).

Table 3.3. Latency to enter the unfamiliar environment split by housing condition in individual and group contexts. Data are mean (one standard error of the mean). All comparisons were not significant ($p > 0.05$).

Behaviour	Context	Adolescent Housing Condition			
		LN/C	LN	HN/LD	HN/HD
Latency to enter unfamiliar environment	Individual	36.81 (2.91)	37.60 (3.15)	38.23 (2.84)	34.49 (2.62)
	Group	36.89 (3.13)	34.42 (2.91)	29.72 (3.93)	38.64 (3.09)

Birds entered the group context unfamiliar environment a similar number of times regardless of whether the birds were re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,14} = 0.028$, $p = 0.868$; sex: $F_{1,14} = 0.309$, $p = 0.587$; familiarity x sex: $F_{1,14} = 0.028$, $p = 0.868$; Table 3.4.) and regardless of early adolescent group size and/or social density (housing condition: $F_{2,23} = 0.313$, $p = 0.721$; sex: $F_{1,23} = 0.125$, $p = 0.731$; housing condition x sex: $F_{2,23} = 0.342$, $p = 0.812$; Table 3.4.).

Table 3.4. Number of entries into the unfamiliar environment split by housing condition in individual and group contexts. Data are mean (one standard error of the mean). All comparisons were not significant ($p > 0.05$).

Behaviour	Context	Adolescent Housing Condition			
		LN/C	LN	HN/LD	HN/HD
Number of entries into unfamiliar environment	Individual	6.67 (0.88)	8.28 (1.27)	8.16 (1.53)	7.10 (0.84)
	Group	15.28 (2.63)	14.33 (3.48)	17.95 (4.26)	15.05 (3.47)

In the group context, birds re-housed with a familiar or unfamiliar conspecific in adolescence spent similar lengths of time perching in the unfamiliar environment (familiarity: $F_{1,32} = 0.246$, $p = 0.623$; sex: $F_{1,32} = 0.224$, $p = 0.639$; familiarity x sex: $F_{1,32} = 1.428$, $p = 0.241$; Figure 3.8.). Duration of time perching in the unfamiliar environment was also similar between birds housed in different group sizes and densities in adolescence ($F_{1,49.478} = 2.768$, $p = 0.073$). However, adolescent group size and density had sex-specific effects on unfamiliar environment perching duration (sex: $F_{1,50.140} = 4.582$, $p = 0.037$; sex x housing condition: $F_{1,48.906} = 5.683$, $p = 0.006$; Figure 3.8.). In females, HN/LD birds perched in the unfamiliar environment longer than birds from other conditions (LN, $p = 0.006$, $d = 1.90$; HN/HD, $p = 0.007$, $d = 1.68$), but no difference was found between LN and HN/HD birds ($p = 0.992$). In males, birds perched in the unfamiliar environment for similar durations regardless of housing condition (LN vs. HN/LD, $p = 1.0$; LN vs. HN/HD, $p = 0.496$; HN/LD vs. HN/HD, $p = 0.459$). Female HN/LD birds perched in the unfamiliar environment longer than HN/LD males ($p < 0.001$, $d = 1.57$), but no sex differences were found for LN ($p = 0.691$) and HN/HD birds ($p = 0.554$).

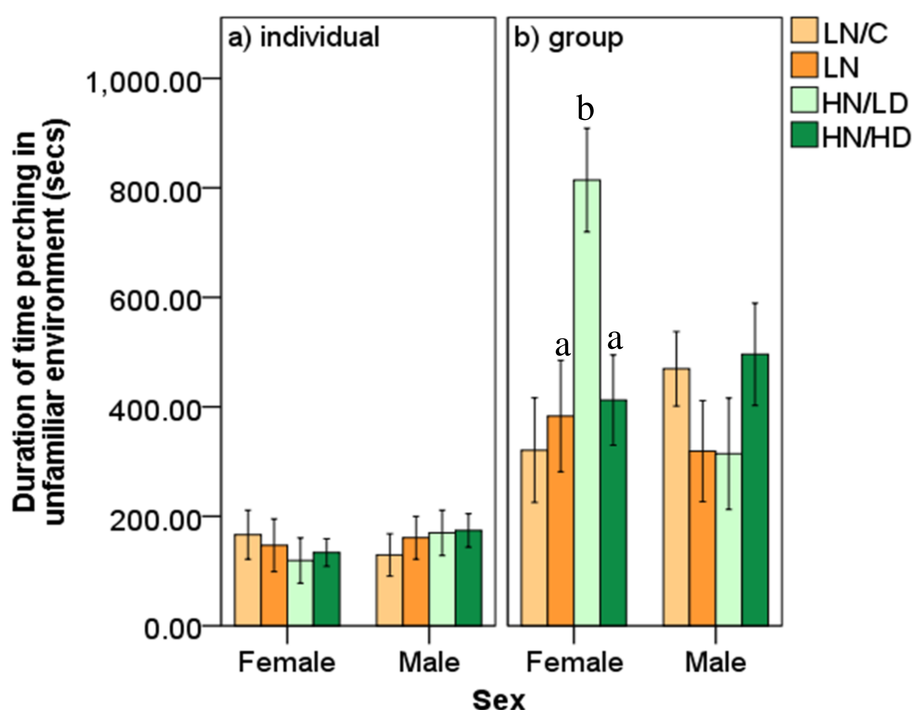

Figure 3.8. Duration of time perching in the unfamiliar environment split by sex and housing condition in a) individual context and b) group context task. Data are mean ± one standard error of the mean. Significant differences ($p < 0.05$) between female housing conditions in a group context are shown: a vs. b, $p < 0.05$.

When exposed to an unfamiliar environment in an individual context, the number of hops was not differ according to whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,18.042} = 0.356$, $p = 0.558$; sex: $F_{1,30.463} = 0.488$, $p = 0.490$; familiarity x sex: $F_{1,30.903} = 0.600$, $p = 0.444$) or what group size and/or social density a bird was raised in during early adolescence (housing condition: $F_{2,50} = 0.329$, $p = 0.721$; sex: $F_{1,50} = 0.670$, $p = 0.417$; housing condition x sex: $F_{2,50} = 2.032$, $p = 0.142$).

Identical effects were found for the number of hops that occurred in when exposed to an unfamiliar environment in a group context, with a similar number of hops occurring in birds re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,15.820} = 0.121$, $p = 0.733$; sex: $F_{1,16.493} = 2.005$, $p = 0.175$; familiarity x sex: $F_{1,15.372} = 0.307$, $p = 0.587$) and in birds housed in different group sizes and/or social densities in early adolescence (housing condition: $F_{2,21.622} = 0.440$, $p = 0.650$; sex: $F_{1,21.683} = 1.051$, $p = 0.317$; housing condition x sex: $F_{2,21.160} = 0.170$, $p = 0.844$). Hops data are summarised in Table 3.5.

Table 3.5. Number of hops by birds when exposed to an unfamiliar environment split by housing condition and social context. Data are mean (one standard error of the mean). No significant differences were found in each task ($p > 0.05$).

Behaviour	Context	Adolescent Housing Condition			
		LN/C	LN	HN/LD	HN/HD
Number of hops	Individual	20.83 (4.73)	24.22 (4.86)	23.16 (3.98)	28.35 (5.73)
	Group	258.33 (28.25)	269.11 (34.04)	244.80 (30.88)	290.25 (31.10)

In an individual context, the number of head turns a bird engaged in when exposed to an unfamiliar environment did not differ according to whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,31} = 1.359$, $p = 0.253$; sex: $F_{1,31} = 0.611$, $p = 0.440$; familiarity x sex: $F_{1,31} = 0.047$, $p = 0.830$) or what group size and/or social density a bird was raised in during early adolescence (housing condition: $F_{2,51} = 0.281$, $p = 0.756$; sex: $F_{1,51} = 1.126$, $p = 0.294$; housing condition x sex: $F_{2,51} = 1.378$, $p = 0.261$).

In a group context, the number of head turns a bird engaged in when exposed to an unfamiliar environment was similar regardless of whether birds were re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,31} = 0.002$, $p = 0.962$; sex: $F_{1,31} = 0.403$, $p = 0.530$; familiarity x sex: $F_{1,31} = 0.014$, $p = 0.905$) and what group size and/or social density the birds were housed in during early adolescence (housing condition: $F_{2,51} = 1.403$, $p = 0.255$; sex: $F_{1,51} = 0.049$, $p = 0.825$; housing condition x sex: $F_{2,51} = 0.228$, $p = 0.797$). Head turns data are summarised in Table 3.6.

Table 3.6. Number of head turns by birds when exposed to an unfamiliar environment split by housing condition and social context. Data are mean (one standard error of the mean). No significant differences were found ($p > 0.05$).

Behaviour	Context	Adolescent Housing Condition			
		LN/C	LN	HN/LD	HN/HD
Number of head turns	Individual	101.06 (17.43)	119.56 (15.33)	130.37 (18.86)	111.95 (14.27)
	Group	136.56 (11.95)	126.11 (14.30)	162.30 (17.42)	135.35 (9.41)

Birds re-housed with a familiar or unfamiliar conspecific in adolescence spent similar lengths of time perching alone when exposed to the unfamiliar environment (familiarity: $F_{1,12.963} = 0.108$, $p = 0.748$; sex: $F_{1,13.491} = 0.003$, $p = 0.961$; familiarity x sex: $F_{1,13.179} = 0.437$, $p = 0.520$; Figure 3.9.). Duration of time perching alone also differed between birds housed in different group sizes and/or densities in early adolescence in a sex-specific manner (housing condition: $F_{2,22.352} = 9.388$, $p = 0.001$; sex: $F_{1,22.291} = 7.614$, $p = 0.011$; sex x housing condition: $F_{2,22.289} = 9.314$, $p = 0.001$; Figure 3.9.). Within females, HN/LD birds spent more time alone than birds from other housing conditions (LN, $p < 0.001$, $d = 2.40$; HN/HD, $p < 0.001$, $d = 2.09$) and LN and HN/HD birds spent similar lengths of time perching alone ($p = 0.883$). Within males, birds spent a similar duration of time perching alone regardless of adolescent group size and/or density (LN vs. HN/LD, $p = 0.779$; LN vs. HN/HD, $p = 0.178$; HN/LD vs. HN/HD, $p = 0.641$). Female HN/LD birds also perched alone more than male HN/LD birds ($p < 0.001$, $d = 2.34$), but sex differences were not present for LN ($p = 0.628$) and HN/HD ($p = 0.476$) birds.

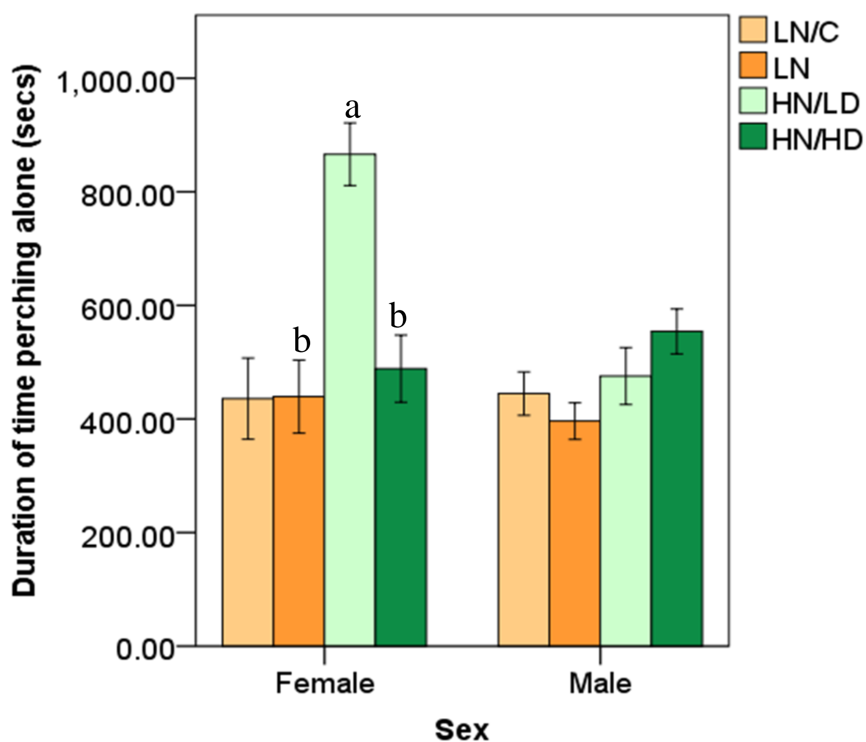

Figure 3.9. Duration of time perching alone in a group context unfamiliar environment split by sex and housing condition. Data are mean ± one standard error of the mean. Significant difference ($p < 0.05$) within females are shown by a vs. b.

3.3.3. Adult behavioural responses to unfamiliar objects

Birds re-housed with either a familiar or unfamiliar conspecific in adolescence had similar latencies to contact the unfamiliar object in both an individual context (familiarity: $F_{1,23.480} = 0.637$, $p = 0.433$; sex: $F_{1,31.243} = 0.066$, $p = 0.799$; familiarity x sex: $F_{1,31.929} = 0.098$, $p = 0.757$) or group context (familiarity: $F_{3,28.796} = 0.277$, $p = 0.841$; sex: $F_{1,29.940} = 0.158$, $p = 0.694$; familiarity x sex: $F_{3,29.435} = 0.199$, $p = 0.896$).

Latency to contact the unfamiliar object did not differ with early adolescent group size and/or density in an individual context (housing condition: $F_{2,43.757} = 0.433$, $p = 0.651$; sex: $F_{1,45.268} = 0.764$, $p = 0.387$; housing condition x sex: $F_{2,39.964} = 1.241$, $p = 0.300$) or group context (housing condition: $F_{2,22.608} = 0.178$, $p = 0.838$; sex: $F_{1,22.753} < 0.001$, $p = 0.999$; housing condition x sex: $F_{2,22.458} = 0.070$, $p = 0.933$). Latency data are summarised in Table 3.7.

Table 3.7. Latency to perch on the unfamiliar object in an individual and group context split by housing condition. Data are mean (one standard error of the mean). No significant differences were found ($p > 0.05$).

Behaviour	Context	Adolescent Housing Condition			
		LN/C	LN	HN/LD	HN/HD
Latency to perch on unfamiliar object (mins)	Individual	25.23 (1.82)	27.43 (1.80)	27.31 (1.47)	28.46 (1.78)
	Group	24.67 (2.03)	27.32 (1.95)	26.86 (1.88)	25.35 (2.03)

Birds re-housed with either a familiar or unfamiliar conspecific in adolescence had similar latencies to contact the unfamiliar object in both an individual context (familiarity: $\chi^2_{1,32} = 0.206$, $p = 0.653$; sex: $\chi^2_{1,32} = 0.376$, $p = 0.544$; familiarity x sex: $\chi^2_{1,32} = 0.876$, $p = 0.356$) or group context (familiarity: $F_{1,14.001} = 0.562$, $p = 0.466$; sex: $F_{1,14.001} = 0.587$, $p = 0.456$; familiarity x sex: $F_{1,14.001} = 0.307$, $p = 0.588$).

Latency to contact the unfamiliar object did not differ with early adolescent group size and/or density in an individual context (housing condition: $\chi^2_{2,51} = 0.101$, $p = 0.904$; sex: $\chi^2_{1,51} = 0.190$, $p = 0.665$; housing condition x sex: $\chi^2_{2,51} = 0.484$, $p = 0.619$) or group context (housing condition: $F_{2,22.514} = 0.802$, $p = 0.461$; sex: $F_{1,22.789} = 0.849$, $p = 0.367$; housing condition x sex: $F_{2,22.269} = 0.460$, $p = 0.637$). Latency data are summarised in Table 3.8.

Table 3.8. Number of perches on an unfamiliar object in an individual and group context split by housing condition. Data are mean (one standard error of the mean). No significant differences were found ($p > 0.05$).

Behaviour	Context	Adolescent Housing Context			
		LN/C	LN	HN/LD	HN/HD
Number of perches on unfamiliar object	Individual	2.56 (0.40)	2.22 (0.35)	2.47 (0.50)	2.20 (0.28)
	Group	6.00 (0.95)	4.94 (0.88)	6.50 (0.82)	7.20 (1.05)

Duration of time spent perching on the unfamiliar object did not differ between birds that were re-housed with a familiar or unfamiliar conspecific in adolescence when the birds were exposed to the unfamiliar object either individually (familiarity: $F_{1,32} < 0.001$, $p = 0.991$; sex: $F_{1,32} = 0.239$, $p = 0.629$; familiarity x sex: $F_{1,32} = 0.345$, $p = 0.561$) or when in groups (familiarity: $F_{1,32} = 0.042$, $p = 0.840$; sex: $F_{1,32} = 1.026$, $p = 0.319$; familiarity x sex: $F_{1,32} = 0.064$, $p = 0.802$).

Birds spent a similar length of time perching on an unfamiliar object when individually tested regardless of early adolescent group size and social density (housing condition: $F_{2,46.535} = 0.153$, $p = 0.859$; sex: $F_{1,47.676} = 2.959$, $p = 0.092$; housing condition x sex: $F_{2,43.778} = 0.873$,

p = 0.425). In the group context, duration of time perching on the unfamiliar object differed in response to adolescent housing in different group sizes and densities in a sex-specific manner (housing condition: $F_{2,19.968} = 12.086$, $p < 0.001$; sex: $F_{1,20.009} = 11.918$, $p = 0.003$; sex x housing condition: $F_{1,19.588} = 4.049$, $p = 0.034$; Figure 3.10.). Within females, HN/LD birds perched on the unfamiliar object longer than birds from other conditions (LN, p = 0.001, d = 2.49; HN/HD, p < 0.001, d = 2.23), but no difference was found between LN and HN/HD birds (p = 0.989). Within males, birds perched on the unfamiliar object for a similar lengths of time regardless of early adolescent group size and/or social density (LN vs. HN/LD, p = 0.709; LN vs. HN/HD, p = 0.949; HN/LD vs. HN/HD, p = 0.396). HN/LD females also perched on the unfamiliar object longer than HN/LD males (p < 0.001, d = 2.27), but no sex differences were found for LN (p = 0.474) and HN/HD birds (p = 0.337).

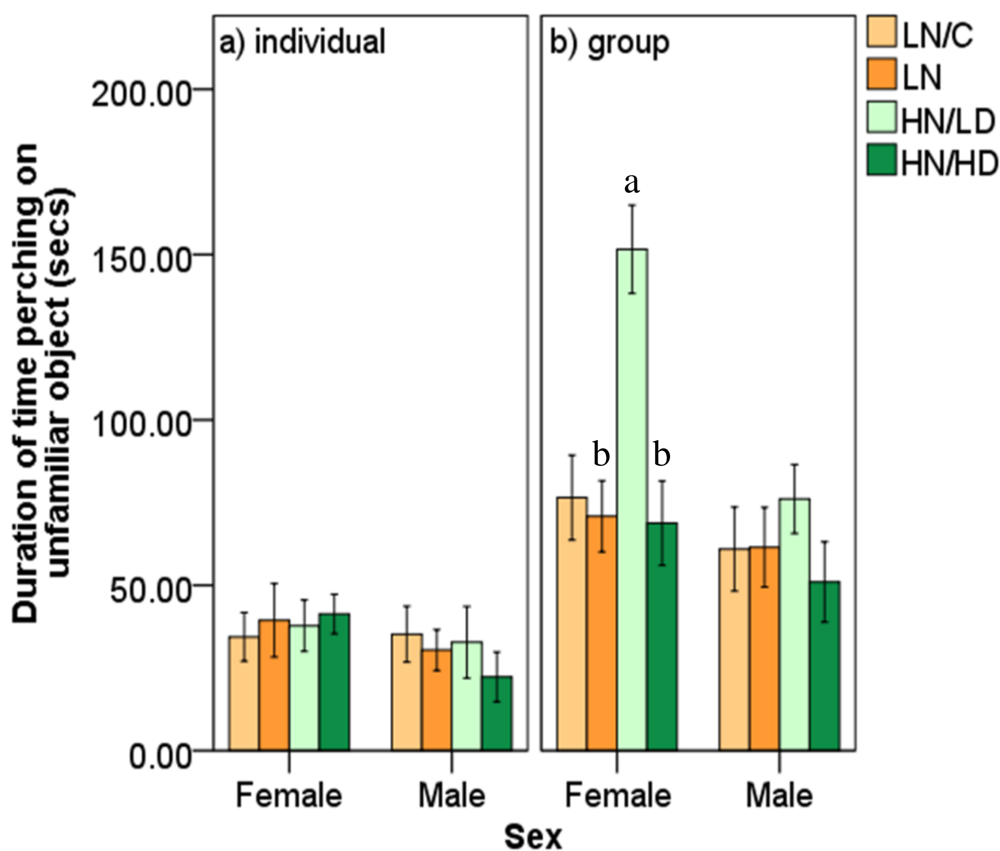

Figure 3.10. Duration of time perching on the unfamiliar object split by sex and housing condition in a) individual context and b) group context. Data are mean ± one standard error of the mean. Significant differences (p < 0.05) between female conditions in a group context are shown by a vs. b.

The number of hops that occurred in response to being individually exposed to an unfamiliar object did not differ according to whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,23.050} = 0.007$, $p = 0.933$; sex: $F_{1,30.871} = 0.015$, $p = 0.902$; familiarity x sex: $F_{1,30.807} = 0.039$, $p = 0.846$) or what group size and/or social density a bird was raised in during early adolescence (housing condition: $F_{2,44.977} = 0.803$, $p = 0.454$; sex: $F_{1,46.107} = 1.833$, $p = 0.182$; housing condition x sex: $F_{2,42.201} = 0.452$, $p = 0.639$).

Similarly, number of hops that occurred in response to being exposed to an unfamiliar object in pairs did not differ for birds re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,12.154} = 0.111$, $p = 0.744$; sex: $F_{1,12.558} = 0.226$, $p = 0.643$; familiarity x sex: $F_{1,12.594} = 0.012$, $p = 0.915$) and for birds housed in different group sizes and/or social densities in early adolescence (housing condition: $F_{2,23.022} = 0.470$, $p = 0.631$; sex: $F_{1,23.410} = 0.744$, $p = 0.397$; housing condition x sex: $F_{2,22.803} = 0.671$, $p = 0.521$). Hops data are summarised in Table 3.9.

Table 3.9. Number of hops when exposed to an unfamiliar object split by housing condition and social context. Data are mean (one standard error of the mean). No significant differences were found within each task ($p > 0.05$).

Behaviour	Context	Adolescent Housing Condition			
		LN/C	LN	HN/LD	HN/HD
Number of hops	Individual	17.39 (3.86)	17.50 (3.89)	16.58 (3.94)	12.15 (2.86)
	Group	22.28 (4.26)	24.78 (4.37)	32.35 (4.40)	28.00 (3.96)

Number of head turns in response to being exposed to an unfamiliar environment in an individual context did not differ depending on whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,31} = 2.658$, $p = 0.113$; sex: $F_{1,31} = 0.275$, $p = 0.604$; familiarity x sex: $F_{1,31} = 2.523$, $p = 0.122$) or what group size and/or social density a bird was raised in during early adolescence (housing condition: $F_{2,44.843} = 0.152$, $p = 0.860$; sex: $F_{1,46.075} = 1.691$, $p = 0.200$; housing condition x sex: $F_{2,42.117} = 2.807$, $p = 0.072$).

Number of head turns in response to being exposed to an unfamiliar environment in a group context also did not differ depending on whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,13.592} = 0.019$, $p = 0.893$; sex: $F_{1,13.473} = 0.019$, $p = 0.894$; familiarity x sex: $F_{1,13.396} = 0.512$, $p = 0.487$) or housed in a different group sizes and/or social densities in early adolescence (housing condition: $F_{2,21.451} = 2.611$, $p = 0.097$; sex: $F_{1,21.364} = 4.115$, $p = 0.055$; housing condition x sex: $F_{2,21.422} = 0.173$, $p = 0.842$). Head turns data are summarised in Table 3.10.

Table 3.10. Number of head turns when exposed to an unfamiliar object split by housing condition and social context. Data are mean (standard error of the mean). No significant differences were found within each task ($p > 0.05$).

Behaviour	Context	Adolescent Housing Condition			
		LN/C	LN	HN/LD	HN/HD
Number of head turns	Individual	59.33 (10.38)	79.17 (9.09)	90.26 (9.50)	84.25 (9.51)
	Group	61.78 (8.27)	59.94 (5.90)	76.10 (5.59)	79.20 (6.62)

Birds re-housed with a familiar or unfamiliar conspecific in adolescence did not differ in time spent perching alone when exposed to an unfamiliar object (familiarity: $F_{1,12.741} < 0.001$, $p = 0.992$ sex: $F_{1,13.078} = 0.328$, $p = 0.576$; familiarity x sex: $F_{1,13.233} = 0.568$, $p = 0.464$; Figure 3.11.). Duration of time perching alone also did not differ between males and females ($F_{1,21.157} = 0.243$, $p = 0.627$), but did differ between birds housed in different group sizes and/or densities in early adolescence in a sex-specific manner (housing condition: $F_{2,21.116} = 7.137$, $p = 0.004$; sex x housing condition: $F_{2,21.037} = 5.165$, $p = 0.015$; Figure 3.11.). In females, HN/LD birds perched alone more than birds from other conditions (LN, $p = 0.001$, $d = 2.69$; HN/HD, $p = 0.004$, $d = 2.16$) and LN and HN/HD birds were no different in duration of time perching alone ($p = 0.685$). In males, birds spent similar lengths of time perching alone irrespective of adolescent group size and/or density (LN vs. HN/LD, $p = 0.914$; LN vs. HN/HD, $p = 0.887$; HN/LD vs. HN/HD, $p = 1.0$). Female HN/LD birds perched alone more than male HN/LD birds ($p = 0.008$, $d = 1.52$), but similar durations were found for males and females from LN ($p = 0.256$) and HN/HD conditions ($p = 0.419$).

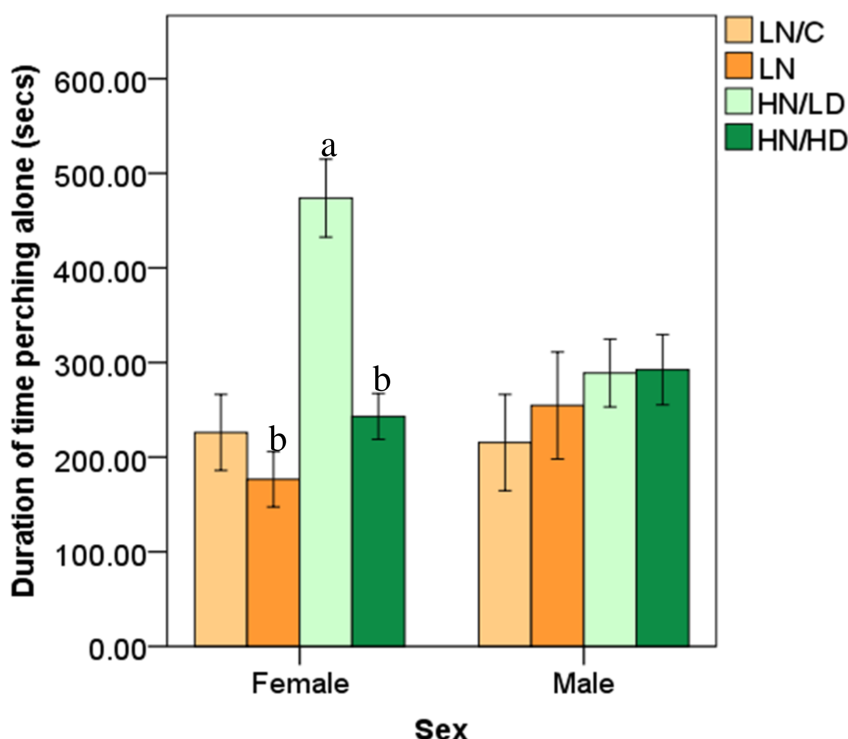

Figure 3.11. Duration of time perching alone when exposed to an unfamiliar object split by sex and adolescent housing condition. Data are mean $\pm$ standard error of the mean. Significant differences between female housing conditions are shown: a vs. b, $p < 0.05$.

3.3.4. Adult behavioural responses to unfamiliar conspecifics

Time Alone. Whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescence had no effect on the length of time a bird spent alone when housed with unfamiliar conspecifics in adulthood (Table 3.11.).

Table 3.11. Output for the model exploring the effects of re-housing with a familiar or unfamiliar conspecific in adolescence on duration of spent alone when housed with unfamiliar conspecifics in adulthood. No significant differences were found ($p > 0.05$).

Variable	Test spent alone		
	df	F	p
Familiarity	1,30.006	1.043	0.315
Location	1,32	0.062	0.805
Sex	1,29.968	1.043	0.315
Familiarity x location	1,32	0.152	0.700
Familiarity x sex	1,32.272	2.856	0.101
Location x sex	1,32	0.119	0.733
Familiarity x location x sex	1,32	0.742	0.395

Duration of time spent alone when housed with unfamiliar conspecifics differed with adolescent group size and/or social density in a location-specific manner (housing condition: $F_{2,40.491} = 3.619$, $p = 0.036$; location: $F_{1,52} = 4.424$, $p = 0.040$; housing condition x location: $F_{2,52} = 3.410$, $p = 0.041$; Figure 3.12.). HN/LD birds spent less time alone on the ground compared to birds from other housing conditions (LN, $p = 0.003$, $d = 0.35$; HN/HD, $p = 0.004$, $d = 0.45$), but LN and HN/HD birds spent similar lengths of time on the ground ($p = 0.988$). HN/LD birds also spent less time alone on the ground compared to the perches ($p = 0.002$, $d = 0.48$), but birds from all other housing conditions spent an equal duration of time on the ground and perches (LN, $p = 0.613$; HN/HD, $p = 0.310$). Male and female birds spent a similar length of time alone when housed with unfamiliar conspecifics (sex: $F_{1,42.684} = 0.442$, $p = 0.510$; sex x housing condition: $F_{2,41.640} = 0.852$, $p = 0.434$; sex x location: $F_{1,52} = 0.355$, $p = 0.554$; sex x housing condition x location: $F_{2,52} = 0.315$, $p = 0.731$).

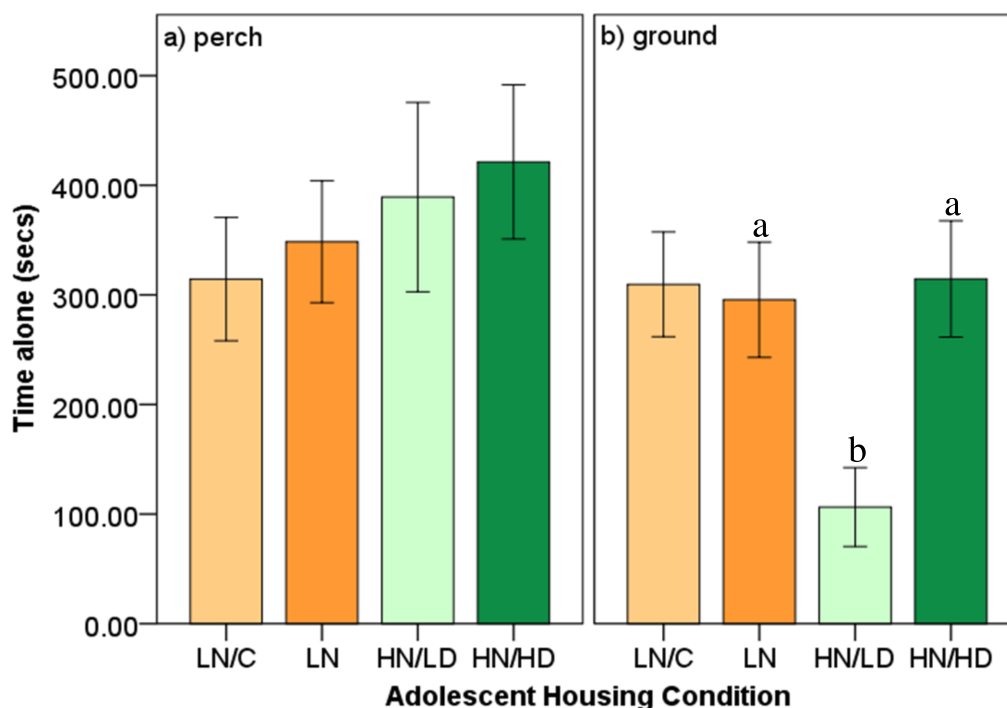

Figure 3.12. Duration of time alone on a) perches and b) ground when housed with an unfamiliar group of conspecifics split by housing condition. Data are mean ± one standard error of the mean. Significant differences ($p < 0.05$) are shown by a vs. b.

Beak fencing. Length of time spent beak fencing with unfamiliar conspecifics did not differ depending on whether a birds was re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,32.041} = 0.748$, $p = 0.394$; familiarity x test bird sex: $F_{1,18.649} = 0.140$, $p = 0.712$; familiarity x unfamiliar bird sex: $F_{1,32} = 0.058$, $p = 0.812$; familiarity x test bird sex x unfamiliar bird sex: $F_{1,32} = 0.393$, $p = 0.535$). Regardless of whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescences, test males spent more time beak fencing than test females ($F_{1,18.096} = 9.511$, $p = 0.006$; test male: $M = 139.1$, $SEM = 9.65$; test female: $M = 91$, $SEM = 6.52$) and birds spent more time beak fencing with unfamiliar males than unfamiliar females ($F_{1,32} = 7.880$, $p = 0.008$; unfamiliar male: $M = 132.5$, $SEM = 9.92$; unfamiliar female: $M = 102.94$, $SEM = 8.40$). These sex differences were dependent on one another ($F_{1,32} = 4.732$, $p = 0.037$) as male test birds spent more time beak fencing with unfamiliar males than unfamiliar females ($p < 0.001$, $d = 1.14$; unfamiliar male: $M = 163.25$, $SEM = 12.37$; unfamiliar female: $M = 114.95$, $SEM = 12.97$), but test birds spent an equal

duration of time beak fencing an unfamiliar male or female ($p = 0.675$; unfamiliar male, M = 94.06, SEM = 9.85; unfamiliar female, M = 87.94, SEM = 8.81).

Birds raised in different group sizes and/or social densities during early adolescence did not differ in duration of time spent beak fencing with unfamiliar conspecifics (housing condition: $F_{2,52} = 0.442$, $p = 0.658$; housing condition x test bird sex: $F_{2,52} = 0.107$, $p = 0.899$; housing condition x unfamiliar bird sex: $F_{2,52} = 0.669$, $p = 0.517$; housing condition x test bird sex x unfamiliar bird sex: $F_{2,52} = 0.091$, $p = 0.913$). Irrespective of whether early adolescent group size and/or social density, test males spent more time beak fencing than test females ($F_{1,152} = 22.227$, $p < 0.001$; test male: M = 134.12, SEM = 8.97; test female: M = 80.45, SEM = 5.39) and birds spent more time beak fencing with unfamiliar males than unfamiliar females ($F_{1,52} = 21.458$, $p < 0.001$; unfamiliar male: M = 128, SEM = 8.87; unfamiliar female: M = 88.41, SEM = 6.79). These sex differences were dependent on one another ($F_{1,52} = 13.623$, $p = 0.001$) as male test birds spent more time beak fencing with unfamiliar males than unfamiliar females ($p < 0.001$, $d = 1.16$; unfamiliar male: M = 168.57, SEM = 11.40; unfamiliar female: M = 99.67, SEM = 10.75), but test birds spent an equal duration of time beak fencing an unfamiliar male or female ($p = 0.675$; unfamiliar male: M = 84.54, SEM = 7.68; unfamiliar female: M = 76.36, SEM = 7.63).

Displacements. The number of times a test bird displaced an unfamiliar bird did not differ depending on whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescence (familiarity: $F_{1,21.590} = 0.226$, $p = 0.640$; familiarity x test bird sex: $F_{1,46.349} = 0.143$, $p = 0.708$; familiarity x unfamiliar bird sex: $F_{1,48.911} = 0.122$, $p = 0.729$; familiarity x test bird sex x unfamiliar bird sex: $F_{1,48.911} = 0.284$, $p = 0.597$). Regardless of whether birds were re-housed with a familiar or unfamiliar conspecific, male test birds displaced more unfamiliar birds than female test birds ($F_{1,37.165} = 17.677$, $p < 0.001$; test male: M = 17, SEM = 1.05; test female: M = 11.19, SEM = 0.91) and test birds displaced more unfamiliar males than unfamiliar

males ($F_{1,48.911}$ = 6.379, p = 0.015; unfamiliar male: M = 16.08, SEM = 1.20; unfamiliar female: M = 12.75, SEM = 0.94). These sex differences were not dependent on one another ($F_{1,49.911}$ = 0.474, p = 0.494).

Birds raised in larger groups in early adolescence displaced unfamiliar birds less often than birds reared in smaller groups, regardless of whether large group reared birds were raised at low density (housing condition: $F_{2,48.507}$ = 11.168, p < 0.001;LN vs. HN/LD, p < 0.001, d = 1.19) or high density (LN vs. HN/HD, p = 0.001, d = 0.92 Figure 3.13.a). Birds raised in large groups in early adolescence displaced unfamiliar birds to a similar extent (p = 0.941). Male test birds displaced unfamiliar birds more often than female test birds ($F_{1,50.528}$ = 7.397, p = 0.009; test male: M = 11.48, SEM = 0.84; test female: M = 8.27, SEM = 0.72) and unfamiliar male birds displaced test birds more often than unfamiliar female birds ($F_{1,52}$ = 4.6, p = 0.037; unfamiliar male: M = 10.72, SEM = 0.82; unfamiliar female: M = 9.12, SEM = 0.79); these effects did not depend on one another ($F_{1,52}$ = 0.106, p = 0.746). The effects of adolescent group size on the number of times a test bird displaced an unfamiliar bird did not differ with sex (housing condition x test bird sex: $F_{2,47.68}$ = 0.071, p = 0.931; housing condition x unfamiliar bird sex: $F_{2,52}$ = 0.091, p = 0.913; housing condition x test bird sex x unfamiliar bird sex: $F_{2,52}$ = 0.608, p = 0.548).

Birds re-housed with a familiar or unfamiliar conspecific in adolescence were displaced by an unfamiliar bird a similar number of times (familiarity: $F_{1,29.564}$ = 1.647, p = 0.209; familiarity x test bird sex: $F_{1,30.115}$ = 0.150, p = 0.701; familiarity x unfamiliar bird sex: $F_{1,32}$ = 0.092, p = 0.763; familiarity x test bird sex x unfamiliar bird sex: $F_{1,32}$ = 0.091, p = 0.765). Irrespective of whether the birds were re-housed with a familiar or unfamiliar conspecific in adolescence, male test birds were displaced by unfamiliar birds more than female test birds ($F_{1,29.478}$ = 7.659, p = 0.010; test male: M = 14, SEM = 0.92; test female: M = 10.59, SEM = 0.91) and test birds were displaced more by unfamiliar males than unfamiliar females ($F_{1,32}$ =

7.664, $p = 0.009$; unfamiliar male: M = 14.14, SEM = 0.99; unfamiliar female: M = 10.83, SEM = 0.85); effects that were not dependent on one another ($F_{1,32} = 0.025$, $p = 0.876$).

Birds were displaced by an unfamiliar conspecific a similar number of times regardless of early adolescent group size and/or social density (housing condition: $F_{2,45.019} = 1.837$, $p = 0.171$; housing condition x test bird sex: $F_{2,43.483} = 0.693$, $p = 0.506$; housing condition x unfamiliar bird sex: $F_{2,52} = 0.507$, $p = 0.605$; housing condition x test bird sex x unfamiliar bird sex: $F_{2,52} = 0.038$, $p = 0.962$; Figure 3.13.b). Irrespective of early adolescent group size and/or social density, male test birds were displaced by unfamiliar birds more than female test birds ($F_{1,47.842} = 7.875$, $p = 0.007$; test male: M = 14.20, SEM = 0.72; test female: M = 11.93, SEM = 0.66) and test birds were displaced more by unfamiliar males than unfamiliar females ($F_{1,52} = 16.441$, $p \leq 0.009$; unfamiliar male: M = 15.02, SEM = 0.72; unfamiliar female: M = 11.19, SEM = 0.60); effects that were not dependent on one another ($F_{1,52} = 0.041$, $p = 0.841$).

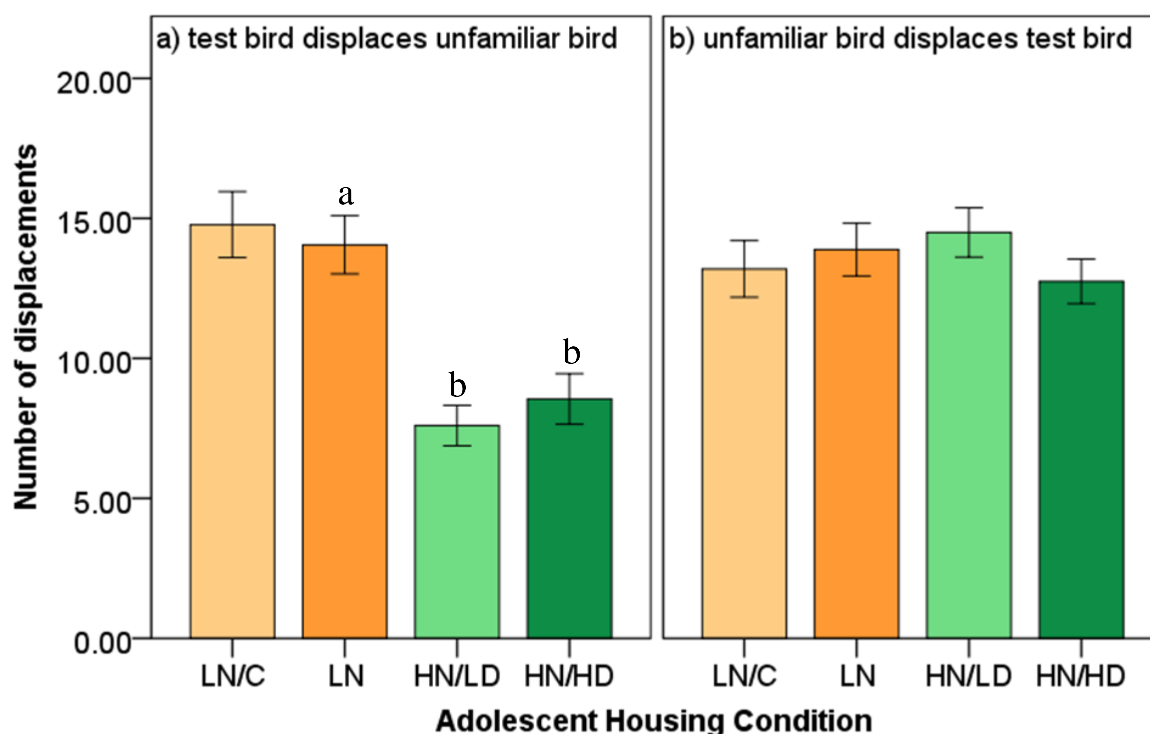

Figure 3.13. Number of times a) a test bird displaces an unfamiliar bird and b) an unfamiliar bird displaces a test bird when housed with an unfamiliar group of conspecifics split by adolescent housing condition. Data are mean $\pm$ one standard error of the mean. Significant differences ($p < 0.05$) shown by a vs. b.

Courtship factors. Female courtship behaviour was similar for all birds regardless of whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescence ($F_{1,7.811} = 0.340$, $p = 0.576$) or by being housed in a different group size and/or social density housing condition ($F_{1,20.096} = 2.803$, $p = 0.084$; Figure 3.14.a). In males, courtship behaviour was similar between birds re-housed with a familiar or unfamiliar conspecific in adolescence ($F_{1,18} = 0.003$, $p = 0.956$; Figure 3.14.b). However, males raised in larger groups engaged in less courtship behaviour than males reared in smaller groups (housing condition: $F_{1,18.176} = 11.177$, $p = 0.001$), regardless of whether large group reared males were raised at low density (LN vs. HN/LD, $p = 0.001$, $d = 1.46$) or high density (LN vs. HN/HD, $p = 0.005$, $d = 1.28$). Males raised in larger groups engaged in similar quantities of courtship behaviour irrespective of early adolescent social density (HN/LD vs. HN/HD, $p = 0.860$)

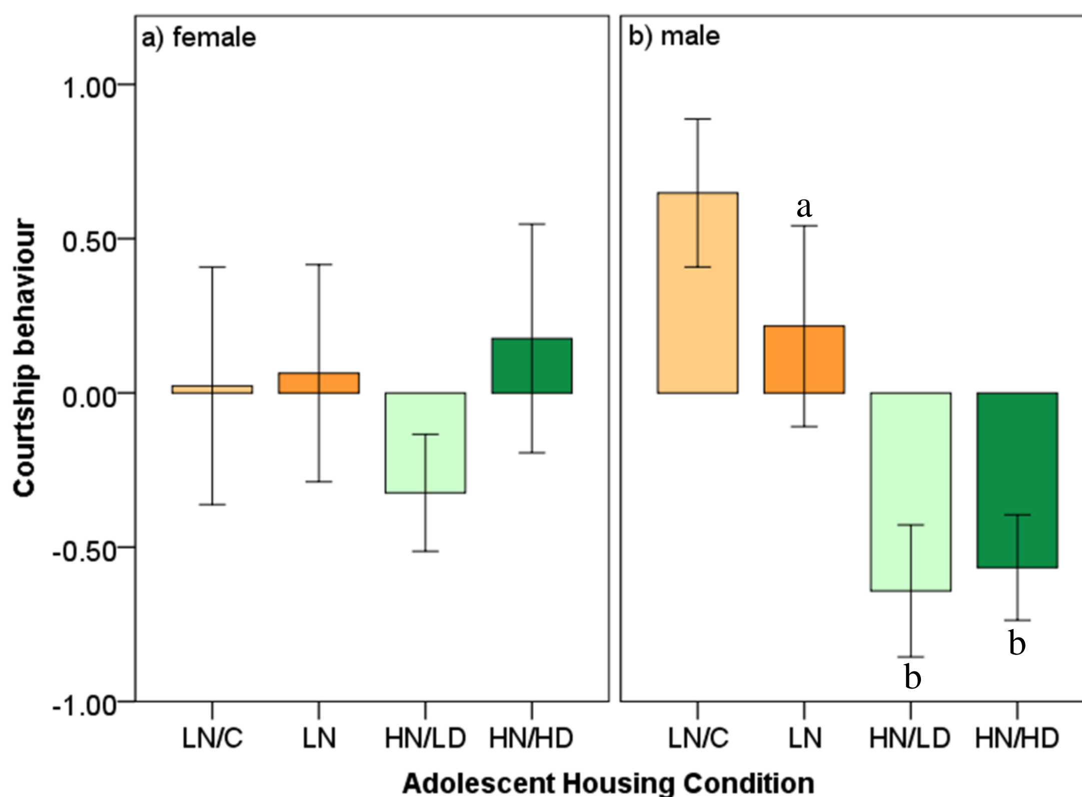

Figure 3.14. Courtship behaviour for a) females and b) males when housed with an unfamiliar group of conspecifics split by housing condition. Data are mean ± one standard error of the mean. Significant differences ($p < 0.05$) shown by a vs. b.

3.3.5. Hormones

Corticosterone time-response. Regardless of whether a bird was re-housed with a familiar or unfamiliar conspecific in adolescence, CORT concentration secreted in response to capture and restraint differed over time (Table 3.12.; Figure 3.15.). Birds had a lower CORT concentration at baseline compared to 15 min ($p < 0.001$, $d = 2.18$) and 45 min ($p < 0.001$, $d = 1.94$); no difference in CORT concentration was found between 15 and 45 mins ($p = 0.707$).

Table 3.12. Outputs from models exploring the effect of re-housing a bird with a familiar or unfamiliar conspecific in adolescence, and adolescent group size and density on corticosterone concentration in response to capture and restraint. Significant effects ($p < 0.005$) are in bold.

Variable	Familiarity			Group size and density		
	df	F	p	df	F	p
Adolescent condition	1,31.365	1.431	0.241	2,43.223	2.481	0.095
Sex	1,22.804	2.101	0.161	1,44.628	1.213	0.277
Age	1,32	0.477	0.495	1,52	3.855	0.055
Time	**2,32**	**201.788**	**<0.001**	**2,52**	**278.069**	**<0.001**
Adolescent condition x sex	1,22.379	0.019	0.891	2,39.306	0.145	0.865
Adolescent condition x age	1,32	0.007	0.935	2,52	2.188	0.122
Adolescent condition x time	2,32	0.005	0.995	**4,52**	**2.674**	**0.042**
Sex x age	1,32	0.418	0.522	1,52	0.144	0.706
Sex x time	2,32	1.371	0.268	2,52	5.118	0.059
Age x time	2,32	2.149	0.133	**2,52**	**13.787**	**<0.001**
Sex x age x time	2,32	0.529	0.594	**2,52**	**10.018**	**<0.001**
Adolescent condition x sex x age	1,32	0.619	0.437	2,52	1.349	0.268
Adolescent condition x sex x time	2,32	0.605	0.552	**4,52**	**7.621**	**<0.001**
Adolescent condition x age x time	2,32	0.318	0.730	4,52	0.791	0.536
Adolescent condition x sex x age x time	2,32	0.531	0.593	4,52	0.913	0.464

Irrespective of early adolescent group size and/or social density, CORT concentration changed across sampling times (Table 3.12.) with basal concentrations lower than later time points (15 min: $p < 0.001$, $d = 1.98$; 45 min: $p < 0.001$, $d = 1.87$) and CORT concentration at 45 mins higher than 15 mins ($p = 0.004$, $d = 0.55$). CORT concentration in response to restraint differed over time in response to early adolescent group size and did so in a sex-specific manner when birds CORT concentrations were averaged (mean) across ages (Table 3.12.; Figure

3.15.). In female conditions, basal CORT concentrations were similar (LN vs. HN/LD, p = 0.727; LN vs. HN/HD, p = 0.680; HN/LD vs. HN/HD, p = 1.0). 15 mins into restraint, CORT concentrations of HN/HD females were lower than those of female birds from other conditions (LN, p = 0.002, d = 0.78; HN/LD, p = 0.006, d = 0.90) whilst LN and HN/LD were not different (p = 0.944). 45 mins into restraint, females from larger groups had higher CORT concentration than females from smaller groups (LN vs. HN/LD, p = 0.009, d = 1.08; LN vs. HN/HD, p = 0.006, d = 1.12) and females from larger groups did not differ (HN/LD vs. HN/HD, p = 0.999). All comparisons between male housing conditions were not significant at 0 min (LN vs. HN/LD, p = 0.704; LN vs. HN/HD, p = 0.914; HN/LD vs. HN/HD, p = 0.323), 15 min (LN vs. HN/LD, p = 0.994; LN vs. HN/HD, p = 0.958; HN/LD vs. HN/HD, p = 0.995), and 45 min (LN vs. HN/LD, p = 0.926; LN vs. HN/HD, p = 0.416; HN/LD vs. HN/HD, p = 0.164).

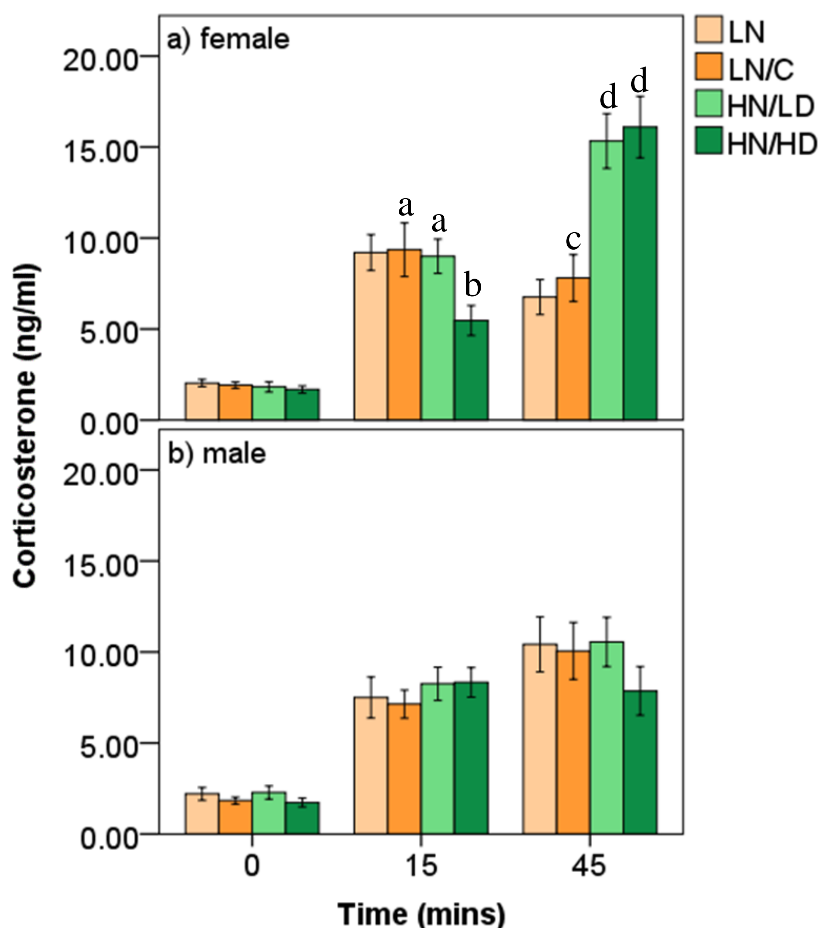

Figure 3.15. Corticosterone concentration (ng/ml) in response to capture and restraint averaged across ages for a) females and b) males split by sampling time and housing condition. Data are mean ± one standard error of the mean. Significant differences ($p < 0.05$) in each female sampling time are show by a vs. b, c vs. d.

CORT concentration secreted in response to capture and restraint differed over time in an age-specific manner, and these age-specific effects were in turn sex-specific when averaged (mean) across housing conditions (Table 3.12.; Figure 3.16). In adolescent females, birds had a CORT concentration that was higher than baseline at both 15 min ($p < 0.001$, d = 2.72) and 45 min ($p < 0.001$, d = 2.57), but 15 and 45 min concentrations were no different ($p = 0.997$). In adult females, birds had a higher CORT concentration at each subsequent sampling time (0 vs. 15 min, $p < 0.001$, d = 1.53; 0 vs. 45 min, $p < 0.001$, d = 2.28; 15 vs. 45 min, $p < 0.001$, d = 1.23). In adolescence and adult males, birds had a CORT concentration that was higher than baseline at 15 min (adolescent: $p < 0.001$, d = 2.42; adult: $p < 0.001$, d = 2.36) and 45 min (adolescent: $p < 0.001$, d = 1.77; adult: $p < 0.001$, d = 1.78), whilst no differences were found between 15 and 45 min concentrations (adolescent, $p = 0.948$; adult, p = 0874).

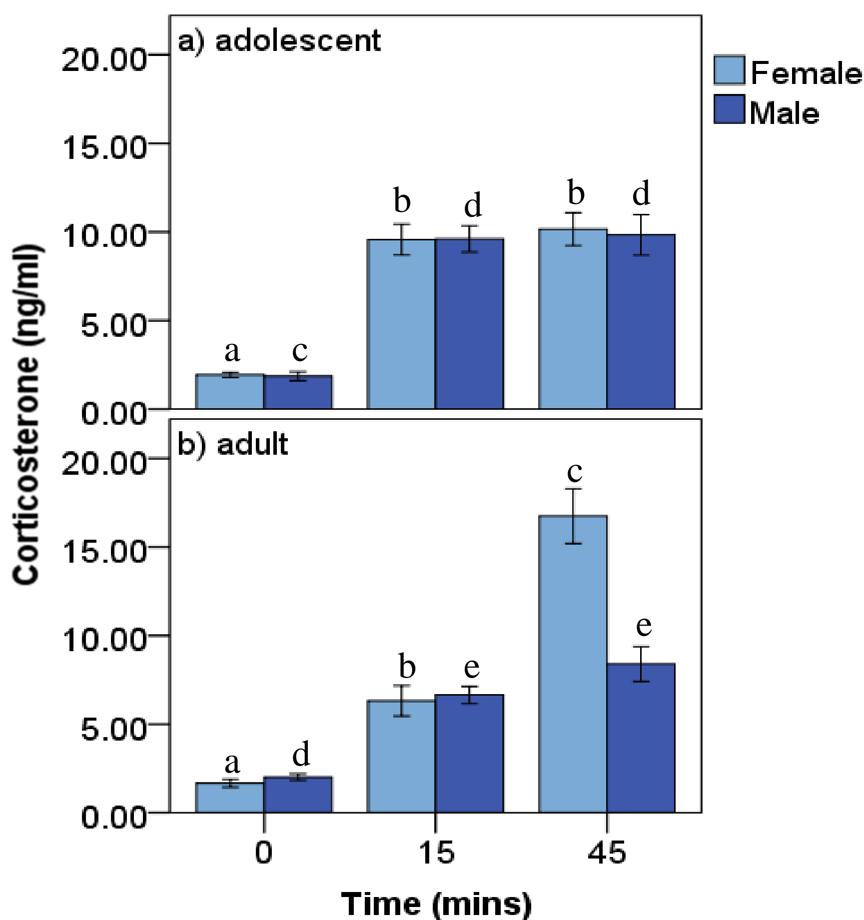

Figure 3.18. Corticosterone concentration (ng/ml) in response to capture and restraint averaged across housing conditions for a) adolescents and b) adults split by sampling time and sex. Data are mean $\pm$ one standard error of the mean. Significant differences ($p < 0.05$) within each sex in adolescence are a vs. b and c vs. d, within each sex in adults are a vs. b vs. c and d vs. e.

Stressor-induced corticosterone concentration change over time. Being re-housed with a familiar or unfamiliar conspecific in adolescence had no effect on the change in stressor-induced CORT concentration between 15 and 45 mins into restraint (Table 3.13.).

Table 3.13. Outputs from models exploring the effect of re-housing a bird with a familiar or unfamiliar conspecific in adolescence, and adolescent group size and density on change in corticosterone concentration between 15 and 45 mins into restraint. Significant effects ($p < 0.005$) are in bold.

Variable	Familiarity			Group size and social density		
	df	χ^2	p	df	χ^2	p
Housing condition	1,25	<0.001	0.998	2,43.863	2.455	0.098
Sex	1,25	2.763	0.109	**1,44.911**	**15.057**	**<0.001**
Age	1,25	1.172	0.289	**1,49.558**	**28.012**	**<0.001**
Housing condition x sex	1,25	0.493	0.489	**2,41.941**	**8.677**	**0.001**
Housing condition x age	1,25	2.602	0.199	**2,49.578**	**3.696**	**0.032**
Sex x age	1,25	0.061	0.807	**1,50.087**	**16.656**	**<0.001**
Housing condition x sex x age	1,25	1.072	0.31	**2,50.261**	**3.522**	**0.043**

Early adolescent housing condition itself did not affect the change in stressor-induced CORT concentration between 15 and 45 mins into restraint, but females had a higher change in CORT concentration than males when data were averaged (mean) across ages and housing conditions (female: M = 3.34, SEM = 0.85; male: M = 0.90, SEM = 0.70) and adults had a higher change in CORT concentration than adolescents when data were averaged (mean) across sexes and housing conditions (adolescent: M = 0.24, SEM = 0.62; adult: M = 3.87, SEM = 0.88) over the same period of time during restraint (Table 3.13.). The sex-specific and age-specific effects depended on one another (Table 3.13.), as adult females had a higher change in CORT between 15 and 45 mins than adolescent females ($p < 0.001$, d = 1.25; adolescent female: M = 0.20, SEM = 0.72; adult female: M = 6.47, SEM = 1.36) but no age differences were found in males ($p = 0.127$; adolescent male: M = 0.27, SEM = 0.99; adult male: M = 1.52, SEM = 1.01). The female- and adult-specific effects on the change in CORT concentration also depended on early adolescent group size (Table 3.13.; Figure 3.17.). Within adolescent females, change in CORT concentration between 15 and 45 min did not differ with early adolescent group size and/or

social density (LN vs. HN/LD, $p = 1.0$; LN vs. HN/HD, $p = 0.527$; HN/LD vs. HN/HD, $p = 0.384$). Within adult females, birds raised in larger group during early adolescence had a higher change in CORT concentration between 15 and 45 min relative to birds raised in smaller groups in early adolescence (LN vs. HN/LD, $p = 0.005$, $d = 1.31$; LN vs. HN/HD, $p < 0.001$, $d = 1.53$); no difference was detected between birds raised in larger groups at different densities (HN/LD vs, HN/HD, $p = 0.440$). Comparing across ages, females raised in larger groups in early adolescence had a higher change in CORT concentration between 15 and 45 min when in adulthood compared to when in adolescence (HN/LD, $p < 0.001$, $d = 1.29$; HN/HD, $p < 0.001$, $d = 1.42$). Females reared in smaller early adolescent groups had a similar change in CORT concentration across ages (LN, $p = 0.469$). Within males, birds from different early adolescent housing condition had similar changes in CORT concentration between 15 and 45 mins when in adolescence (LN vs. HN/LD, $p = 0.994$; LN vs. HN/HD, $p = 0.538$; HN/LD vs. HN/HD, $p = 0.724$) and in adulthood (LN vs. HN/LD, $p = 0.989$; LN vs. HN/HD, $p = 0.698$; HN/LD vs. HN/HD, $p = 0.513$). Males from different early adolescent housing conditions also had similar changes in CORT concentrations between 15 and 45 mins across adolescence and adulthood (LN, $p = 0.743$; HN/LD, $p = 0.397$; HN/HD, $p = 0.788$).

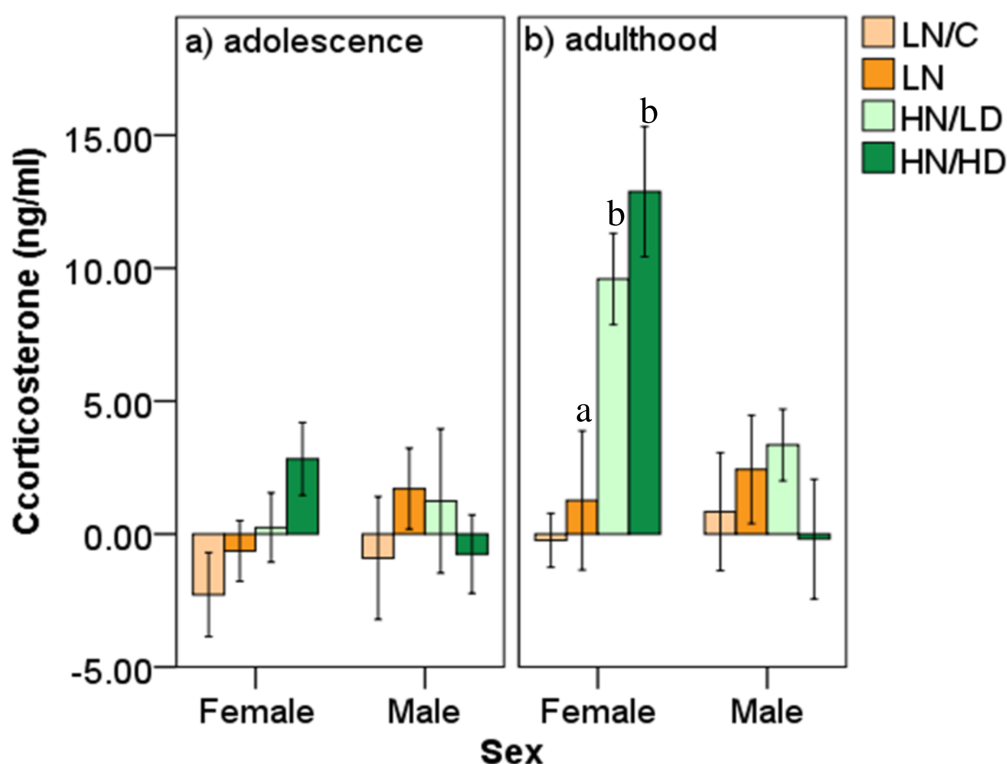

Figure 3.17. Change in corticosterone concentration (ng/ml) between 15 and 45 min in response to capture and restraint split by sex and housing conditions for a) adolescents and b) adults. Data are mean $\pm$ one standard error of the mean. Significant differences ($p < 0.05$) between housing conditions in adult females are show by a vs. b.

Gonadal hormones. Male testosterone concentrations were similar between all male birds regardless of whether a bird was re-housed with a familiar or unfamiliar bird in adolescence ($F_{1,12.605} = 0.469$, $p = 0.506$;) or early adolescent group size and/or social density ($F_{2,20.587} = 0.034$, $p = 0.967$). Estradiol concentration was also similar in all female birds irrespective of whether a bird was re-housed with a familiar or unfamiliar bird in adolescence ($F_{1,14} = 0.006$, $p = 0.942$) or early adolescent group size and/or social density ($F_{2,15.261} = 1.348$, $p = 0.289$). The gonadal hormone data are summarised in Table 3.14.

Table 3.14. Female estradiol concentration (pg/ml) and male testosterone concentration (ng/ml) split by housing condition. Data are mean (one standard error of the mean). Comparisons between conditions in each sex were not significant ($p > 0.05$).

Gonadal Hormone	Adolescent Housing Condition			
	LN	LN/C	HN/LD	HN/HD
Estradiol (pg/ml)	51.38 (11.08)	50.13 (12.68)	39.02 (6.00)	48.00 (12.13)
Testosterone (ng/ml)	1.36 (0.43)	1.58 (0.39)	1.42 (0.45)	1.15 (0.27)

3.3.6. Correlations

All correlations were not significant when alpha values were Bonferroni corrected for multiple comparisons in both adolescent models ($p > 0.036$; Bonferroni corrected $\alpha = 0.003$) and both adult models ($p > 0.031$; Bonferroni corrected $\alpha = 0.0014$). Outputs from correlation analyses are presented in Appendix B (Table B.4., Table B.5., and Table B.6.).

3.4. Discussion

The current chapter tested the hypothesis that higher social density during adolescence would act as a stressor, resulting in animals that spent less time with unfamiliar stimuli in adulthood and a greater stressor-induced CORT concentration compared to animals raised at lower density. However, the findings presented suggest that higher social density in adolescence is not a stressor. Instead, adolescent group size by itself and, in some instances, in interaction with low density rearing conditions, affect social behaviour. In the short-term, males raised in large groups were more antagonistic toward an unfamiliar conspecific and females raised at high density were more affiliative toward an unfamiliar conspecific compared to lower number and lower density conditions, respectively. These immediate effects of adolescent housing are not directly mirrored by behavioural measures taken in later-life. Female birds raised in larger groups at lower density during adolescence spent more time in an unfamiliar environment, more time on an unfamiliar object, and more time perching alone during adult testing compared to all other conditions. In response to unfamiliar conspecifics, birds raised in larger groups at lower density spent less time alone on the ground compared to all other conditions. However, birds raised in larger groups engaged in fewer perch displacements and males raised in larger groups engaged in less courtship behaviour when the birds were compared to birds raised in smaller groups. The effects of adolescent housing conditions on social behaviour are inconsistent, and emphasise that differences in age, sex, and social context can modulate the

effects of adolescent social housing. Hormonal effects were more consistent, with a longer stressor-induced secretion of CORT found in female birds from larger adolescent groups compared to smaller groups, an effect that emerges in adolescence and is sustained into adulthood. However, peak CORT was higher in females raised in larger groups compared to smaller groups only in adulthood. The nuanced effects of adolescent group size and density on behavioural responses to unfamiliar stimuli and the endocrine mechanisms underpinning the effects will now be discussed.

3.4.1. Adolescent behavioural responses to conspecifics after re-housing

Female zebra finches housed at higher density spent less time alone and engaged in more clumping behaviour with an unfamiliar same-sex conspecific during adolescence compared female zebra finches housed in smaller and lower density groups. A similar effect has been reported in male mice, with male mice raised in higher density groups during adolescence spending more time interacting with an unfamiliar conspecific compared to male mice raised in lower density groups during adolescence (Ago et al., 2014). However, the mouse findings created a higher density by housing more animals per cage without changing cage size thereby conflating group size with social density. Furthermore, the mouse findings did not quantify social behaviour to discern why higher density reared mice spent more time interacting with an unfamiliar conspecific than lower density reared mice (e.g. more affiliative or antagonistic interactions). These two limitations in Ago et al. (2014) were not present in the current study, with the present findings revealing that higher social density (not group size) results in more affiliative (not antagonistic) interactions between unfamiliar conspecifics compared to animals raised at lower social density for female zebra finches.

Male zebra finches raised in larger groups in early adolescence engaged in less clumping behaviour and more beak fencing behaviour compared to males raised in smaller

groups. This is in agreement with previous work in territorial adult animals (e.g. Craig & Swanson, 1994; Van Loo et al., 2001). As group size becomes larger, maintaining a position in a social hierarchy may become more difficult, with animals resorting to more antagonistic behaviour to maintain high status (van Loo et al., 2003). Although zebra finches rarely engage in antagonistic behaviour outside of a mating context (Zann, 1996), the birds do live in social hierarchies that can be maintained by use of antagonistic behaviour (Ardia, Broughton, & Gleicher, 2010; Bonoan et al., 2013; Zann, 1996). A larger adolescent group size may therefore affect dominance-related behavioural responses to unfamiliar conspecifics. However, the current study did not test birds in a context that would be required to uncover such dominance-related behaviour. Further work could therefore explore whether a larger adolescent group size results in birds that engage in more dominance-related behaviour in tasks such as competing over a single perch (Spencer & Verhulst, 2007) or over limited food (Bonoan et al., 2013).

Previous work investigating the effects of adolescent social density on social behaviour has predominantly used males (e.g. Ago et al., 2014; Ruploh et al., 2014; Sachser et al., 1993). Consequently, no sex-specific predictions regarding the effects of adolescent social density and group size on adolescent social behaviour could be made a priori. The clearly sex-dependent difference in adolescent behavioural responses to unfamiliar conspecifics presented in the current chapter show the need to include both male and female animals in future research investigating the effects of adolescent group size and social density. Why the sex differences occurred is not known. In adult zebra finches, males engage in more antagonistic behaviour than females (Ikebuchi & Okanaya, 2006; Zann, 1996), but no sex differences are found in affiliative behaviour (Goodson et al., 2009; Silcox & Evans, 1982; Svec, Licht, & Wade, 2009; Zann, 1996). In adolescence (days 50-60), no sex differences in antagonistic behavioural response to unfamiliar conspecifics are present (Spencer & Verhulst, 2007), and no attempt has been made to investigate sex differences in adolescent affiliative behaviour. Although data are

clearly limited, sex differences in behavioural responses to unfamiliar conspecifics appear to emerge during early adolescence and, as suggested in the current chapter, are modulated by social context. Further research is needed to document the behaviour of zebra finches throughout adolescence, with birds housed in groups varying in group size and density, to track the ontogeny of sex differences in antagonistic and affiliative behaviour of zebra finches across different social contexts. Male birds would be expected to engage in more antagonistic interactions with an unfamiliar conspecific compared to females during early adolescence, and a larger group size in males (but not females) may result in even more antagonistic interactions between unfamiliar conspecifics. A further concern regarding the nature of these sex-dependent effects is that they are based on small sample sizes (n = 4-5 per housing condition), as clumping and beak fencing were analysed with cage (not individual bird) as the unit of analysis. The models used to analyse these behaviour may therefore have been over-parameterised given the small samples, resulting in higher variability in the dataset and lower statistical power than would be achieved with larger sample sizes. The experiment therefore needs to be replicated with larger sample sizes in order to corroborate the novel sex-specific effects that have been reported here.

3.4.2. Adult behavioural responses to unfamiliar environments and objects

Adolescent group size and/or social density had no long-term effects on behavioural responses to an unfamiliar environment or an unfamiliar object when the birds were tested individually in adulthood. Adolescent mice housed at a higher social density spent less time in open spaces in an unfamiliar environment when individually tested in adulthood compared to adolescent mice raised at a lower social density (Reiss et al., 2007); an effect that also occurs in adolescent rodents exposed to developmental stressors (Buwalda et al., 2011; Hollis et al., 2013). Previous rodent work therefore suggests higher adolescent social density may be a stressor that results in animals engaging in more stressor avoidant behaviour in adulthood, but

no support for the hypothesis was found in the current chapter. A difference in species between previous research and the current chapter may account for the different findings. Mice engage in more antagonistic interactions at higher density (Van Loo et al., 2001), but the behavioural responses to social density variation in zebra finches has not been quantified. As zebra finches are affiliative birds (Zann, 1996), the birds may not engage in more antagonistic behaviour at higher social density. Social density may only be a stressor in species that respond to higher social density by engaging in more antagonistic interactions (e.g. mice; Reiss et al., 2007; Van Loo et al., 2001), and therefore account for the absence of effects in the current chapter.

Methodological differences were also present between previous research investigating the long-term effects of adolescent social density on later-life responses to unfamiliar stimuli and the current chapter. In Reiss et al. (2007), the variation in group size and social density were greater (1 vs. 7 mice per cage) and maintained for longer (13 weeks) compared to the parameters used in the current chapter. Higher adolescent social density may therefore act as a stressor, but only when animals are housed at a sufficiently high density for a sufficiently long duration, a hypothesis that now requires direct testing. Reiss et al. (2007) also compared mice raised at higher density with mice that were housed one per cage. Mice housed one per cage are not just living in a smaller group or less dense cage, but were also experiencing social deprivation and single housing stress (Fone & Porkess, 2008). Whether single housed animals are an appropriate comparison is debatable, given that density effects are conflated with social deprivation and stress effects. Zebra finches in the current study were always with other conspecifics when in different housing conditions, thereby excluding single housing effects. The absence of group size and/or social density on later-life behavioural responses to unfamiliar stimuli in an individual adult context in the current study may therefore be due to the more appropriate comparison of small vs. large groups and low vs. high density that have been omitted in previous work.

In the presence of a familiar conspecific, female zebra finches raised in a larger group at a lower density spent more time in an unfamiliar environment and more time perching on an unfamiliar object compared to all other groups. The study is the first to show that variation in adolescent group size and density can have long-term effects on behavioural responses to unfamiliar stimuli in a group context. The findings may indicate that rearing in a larger group at a sufficiently low density during adolescence may improve social buffering when in adulthood, as the birds were more likely to interact with unfamiliar stimuli when in groups, but not individually. However, no differences between adolescent conditions were found in other behavioural measures related to neophobia (e.g. locomotor activity or risk assessment). Female zebra finches reared in larger groups at lower density may therefore be more effective social buffers than birds from other housing conditions, but the data are not sufficiently consistent to exclude other hypotheses related to social facilitation (e.g. competition: Clayton, 1978; Webster & Ward, 2011). As the unfamiliar objects in both tasks were baited with spinach, the effects may have resulted from differences in foraging competition. For example, female birds raised in larger groups at a lower density may have spent more time in the unfamiliar environment and on the unfamiliar perch as the birds were attempting to consume more spinach than their cage mate compared to other housing conditions, but the hypothesis requires direct testing.

Why group context behavioural responses to unfamiliar stimuli were affected by an interaction between adolescent group size and social density in female zebra finches is not known. In male mice, communal rearing results in pups engaging in a higher number of affiliative interactions compared to mice raised in single nests (Branchi, 2009). A higher quantity of affiliative interactions during pre-adult development may therefore have long-term effects on adult behavioural responses to unfamiliar stimuli when in a group context. However, pre-adolescent communal (vs. single nest) rearing in mice had no effects on group context

responses to unfamiliar stimuli when in adulthood (Branchi & Alleva, 2006). Furthermore, female zebra finches reared in larger groups at lower density did not appear more affiliative than any other housing condition in terms of behavioural responses to an unfamiliar conspecific in adolescence. Quantifying interactions with an unfamiliar conspecific may not be an adequate way of assessing whether birds engaged in more affiliative interactions during the housing manipulation. The current experiment therefore needs to be repeated in order to quantify affiliative behaviour (e.g. allopreening, clumping, and perching proximity) during days 40-60, when the birds were housed in varied group sizes and densities. The effects cannot be attributed to differences in adult social behaviour during the group context tasks as all birds engaged in too few encounters for analysis. However, female birds raised in larger groups at lower density did spend more time perching alone in both the unfamiliar environment and object tasks compared to all other conditions. Adolescent group size and density may therefore have long-term effects on a bird's proximity to other birds. For example, female birds raised in large groups at low density in adolescence may develop into adults that have less interest in interacting with a familiar conspecific or be more able to tolerate lack of physical proximity to a familiar conspecific compared to other housing conditions, but the hypotheses require testing.

The findings in the current chapter suggest that adolescent social housing has long-term effects on behavioural responses to unfamiliar stimuli in a group context, but limitations in the design and statistical analyses need to be addressed before such a conclusion can be assured. For example, the birds raised in larger groups at lower density were housed in larger cages during adolescence compared to all other housing conditions. Prior experience with housing in a larger cage may therefore have resulted in female birds spending more time in a larger unfamiliar environment in adulthood. However, such an explanation cannot account for why female birds were no different across housing conditions in response to a larger environment in an individual context. Furthermore, female birds raised in larger groups at lower density also

spent more time on an unfamiliar object in the group context unfamiliar object task, when the birds were not housed in larger cages. The context-specific and cross-task effects suggest that adolescent social housing, not cage size, appear to have long-term effects on behavioural responses to unfamiliar stimuli. To investigate any potential effects of cage size, a further study could use a two-by-two design (crossing group size with density, with density varied by changing cage size) to investigate the effects of adolescent group size, social density, and cage size on later-life behavioural responses to unfamiliar stimuli. A second design limitation in the design of the current study is that two different sets of objects were used between individual and group context tasks. The effect of context may therefore actually be an effect of object set. Although pilot studies were conducted to ensure the objects elicited similar responses, further work could repeat the current experiment with the objects counter-balanced across contexts to rule out any effects of object type. A further limitation is that the statistical models used to analyse the behaviour may have been over-parameterised, especially given the smaller sample sizes used to explain the housing condition- and sex-specific effects that were found. The novel findings in the current study may have therefore been a product of potentially more variable data given the small sample sizes, so the experiment needs to be replicated with larger samples in order to corroborate the effects presented here.

3.4.3. Adult behavioural responses to unfamiliar conspecifics

Birds raised in larger groups spent less time alone on the ground compared to all other conditions when raised at a sufficiently low density, but no group differences were found for duration of time alone on the perches. Zebra finches spend most of their time perching in proximity to relatively familiar birds (Zann, 1996), so unfamiliar birds may be relegated to more distant locations from the group, such as the ground, before gradually integrating into a more central position in a group. Birds raised in larger groups at lower density may therefore have an accelerated social integration response intimated by spending less time alone the

ground. If testing had been longer, the large groups birds may have also gone on to spend less time alone on the perches. A larger adolescent group size has previously been indicated to have such an accelerating effect on adult social integration in male zebra finches (Ruploh et al. (2014), but the current data specify that a larger group size must be at a sufficiently low density for the effect to occur and that the effect occurs in both males and females.

Why birds raised in larger groups at lower density spent less time alone on the ground is not obvious from the current data. Birds raised in larger groups at lower density do not appear to spend less time alone because they are more sociable than any other condition, as no differences could be detected in affiliative behaviour (i.e. allopreening and clumping). However, the behaviour were rarely seen and could not be analysed. Greater sociability can therefore not be ruled out as an explanation until more data is available to show what effects adolescent social housing conditions have on adult affiliative behaviour in zebra finches. Instead of sociability, adolescent group size and social density may interact to affect later-life responses to unfamiliar stimuli when in a group context; like that identified in tasks measuring group context behavioural responses to an unfamiliar environment and object. Birds raised in larger groups at a sufficiently low density during adolescence may develop into adults that are more likely to approach or less likely avoid unfamiliar stimuli, but only when in a group context. As the effects are limited to a group context, social facilitation effects may be the cause of the adolescent group differences in duration of time spent on the ground alone when housed with unfamiliar conspecifics. Further work is now necessary to explore the long-term effects of adolescent group size and social density on later-life social facilitation related behaviour when with unfamiliar conspecifics. For example, testing whether birds raised in large groups at low density are more effective social buffers with unfamiliar conspecifics or engage in more social competition with unfamiliar conspecifics when in adulthood compared to other groups.

Birds reared in larger groups in early adolescence engaged in fewer displacements and, if male, engaged in less courtship behaviour directed toward unfamiliar females. Previous work has also shown that larger group size can result in lower antagonistic and courtship behaviour in male guinea pigs (Sachser et al., 1993) and male zebra finches (Ruploh et al., 2014). However, the previous work did not control for cage size and therefore conflated group size with social density. The work presented in this chapter controlled for social density effects and clearly showed that group size, not density, has long-term effects on adult antagonistic and courtship behaviour. Furthermore, previous work investigating the long-term effects of adolescent group size and social density on later-life social behaviour only used male animals. The current work indicates that adolescent group size affects antagonistic behaviour in both males and females, but group size only affects male courtship behaviour. Male and female animals therefore need to be included in future work to determine sex differences in the long-term effects of adolescent group size and density on adult social behaviour. Why only male courtship behaviour was affected is not known. For the current measures, males can inhibit their expression of courtship behaviour whereas females cannot (e.g. males can stop singing, but females cannot equally stop being sung at). Further work could therefore assess whether courtship-related behaviour that females can regulate (e.g. activity, such as hops: Zann, 1996) are also lower in adulthood in response to being reared in a larger adolescent group size.

During adolescence, animals enter into larger social networks (Nelson et al., 2016) and learn how to function in social hierarchies by, for example, avoiding competition with dominant conspecifics (Pellis & Pellis, 2007). Adolescent group size may therefore affect dominance-status when in adulthood. Animals raised in larger groups may adopt a more subordinate position amongst unfamiliar conspecifics compared to animals raised in smaller groups. Lower antagonistic behaviour and male courtship behaviour in birds raised in larger adolescent groups may therefore reflect that the birds are less likely to compete with unfamiliar

conspecifics over a mate compared to birds raised in smaller groups during adolescence. An alternative (but not mutually exclusive) explanation is that adult birds raised in larger adolescent groups are less attractive than birds raised in smaller groups, an effect that has been previously found in male zebra finches (Ruploh et al., 2013). However, Ruploh et al. (2013) conflated group size with social density, so no data are currently available to determine whether larger adolescent group size is the cause of lower attractiveness when in adulthood. Further work is necessary to elaborate on the hypotheses presented above, for example, by investigating the long-term effects of adolescent group size on adult competitive and dominance-related behaviour (e.g. competition over a single perch: Spencer & Verhulst, 2007) as well as measures of attractiveness (e.g. male song quality: Spencer et al., 2003; Spencer et al., 2005).

3.4.4. Corticosterone

Female birds housed in larger groups in early adolescence had a higher concentration of CORT 45 mins into restraint in both adolescence and adulthood compared to birds housed in smaller groups and males housed in larger groups. In addition, females that were housed in larger groups in early adolescence had a greater change in CORT concentration between 15 and 45 mins into restraint when in adulthood (but not in adolescence) compared to birds raised in smaller groups where no change in CORT concentration occurred between 15 and 45 mins into restraint. Female birds raised in larger groups in early adolescence therefore appear to have a longer stress response when in adulthood relative to females raised in smaller groups. The age-dependent effects may be attributable to the fact that different aspects of the stress response are quantified, i.e. concentrations at specific time-points during restraint in the first model and change in CORT concentration between 15 and 45 mins in the second model. However, the models also differed in complexity. The initial model analysing CORT concentration across sample times included four fixed factors of which two factors were repeated measures, perhaps resulting in a model that was over-parameterised, given the sample sizes and sex-specific

effects, resulting in a lack of statistical power. The CORT concentration change between 15 and 45 mins model removed the repeated measure of restraint duration from the analysis, likely improving the power of the model output and providing a more accurate summary of the effects of adolescent group size. Adolescent group size may therefore only affect, or have a stronger effect on, adult CORT secretion compared to adolescent CORT secretion. Previous work has found that variation in adolescent group size and social density has no short-term effects on basal glucocorticoid concentration (Ago et al., 2014; Laviola et al., 2002; Ortiz et al., 1985; Sachser et al., 1993). The current data may extend upon the inferences drawn from the previous work to suggest that both basal and stressor-induced secretion of glucocorticoids may not be affected by adolescent group size and/or density in the short-term, or at least less affected by adolescent group size and/or density than in the long-term. The absence (or lower magnitude) of short-term effects of adolescent social density on glucocorticoid secretion may be due to the age at which the neural expression of glucocorticoid receptors differs between low and high density reared birds. The difference in adolescent social density may set the birds on different developmental trajectories, with glucocorticoid receptor expression differences accumulating with age and only resulting in functional differences in the acute stress response in later-life (e.g. adulthood). To date, no study has determined when (and if) glucocorticoid receptor expression in the brain occurs in response to different adolescent social densities and therefore needs to be investigated to explore the hypothesis outlined above (i.e. that adolescent social density results in differences in glucocorticoid receptor expression in the brain in later-life, not immediately).

In the current thesis, glucocorticoids were quantified 15 days after the birds were placed into different housing conditions. Prior work investigating the short-term effects of adolescent group size and social density on glucocorticoid secretion has quantified the hormones between 9 and 29 days into the housing variation (e.g. Ago et al., 2014; Laviola et al., 2002; Ortiz et al.,

1985). Any immediate effects of adolescent group size and density on glucocorticoid concentration have therefore not been quantified. Initially, adult animals respond to housing in larger and denser groups by engaging in more antagonistic interactions that gradually decrease over time (deWaal, 1989; Judge & deWaal, 1993; Judge & deWaal, 1997). Whether adolescents also engage in more antagonistic interactions upon initially being housed in larger groups compared to smaller groups remains to be determined. If found to be the case, adolescents living in larger groups may secrete more CORT than adolescents living in smaller groups but only shortly after being placed in different group sizes due to the initially higher number of antagonistic interactions in larger groups compared to smaller groups. The absence (or lower magnitude) of short-term effects of group size and density on glucocorticoid secretion in the current thesis and previous work (e.g. Laviola et al., 2002; Ortiz et al., 1985) may therefore be due to quantifying the hormones too late into the variation in group size and/or social density. Hormone sampling therefore needs to occur at multiple time points after housing animals in different group sizes during adolescence to plot how glucocorticoid concentrations may change over time (for example, sampling animals at postnatal days 40, 50, and 60 in the design used in the current study).

Female birds raised in larger groups in early adolescence secreted a higher CORT concentration between 15 and 45 mins into restraint when in adulthood compared to all other conditions. This effect may reflect that female birds raised in larger groups in early adolescence have a longer stress response when in adulthood, resulting in more CORT being secreted in response to a stressor in larger group reared females. Previous work has shown that male mice raised in larger adolescent groups develop into adults with a higher CORT concentration in response to a stressor (loud noise when single housed) compared to male mice raised in smaller groups (Ortiz et al., 1985). However, Ortiz et al., (1985) only quantified CORT at one time point during stressor exposure. By measuring CORT more than once during stressor exposure

when zebra finches were sampled, the findings from the current thesis suggest that adolescent group size may result in a higher stressor-induced CORT concentration as the animals have a longer stress response than birds from smaller adolescent groups. Two stressor-induced samples were taken from zebra finches at two different times as an attempt to quantify peak and post-peak stressor-induced CORT concentration (e.g. Spencer et al., 2009). However, birds from all conditions did not show a decline in CORT concentration with progressing restraint time. Further experiments are therefore required to quantify aspects of the stress response that were not measured in the current experiment, i.e. peak and post-peak stressor-induced CORT concentrations, in order to obtain more holistic representations of how birds from different adolescent housing conditions respond to a stressor. As adult females from larger adolescent groups appear to have a longer stress response, stressor-induced CORT concentration in females from larger groups would be expected to take longer to return to baseline, indicated by higher post-peak CORT concentration, relative to females from smaller adolescent groups. This prediction now needs to be tested.

In contrast to the zebra finches in the current thesis and the mice in Ortiz et al. (1985), male guinea pigs raised in a larger group throughout adolescence secreted a lower concentration of cortisol in response to being housed with an unfamiliar male and female when in adulthood (mate competition context) compared to guinea pigs raised in smaller groups in adolescence (Sachser et al., 1993). Social context may therefore modulate the long-term effects of adolescent group size on CORT secretion. Stressor-induced CORT secretion affects behavioural responses to stressors, for example by eliciting avoidant behaviour to escape a stressor (Haller et al., 1998; Rodgers et al., 1999) or by eliciting antagonistic behaviour to compete against a social stressor (Mikics et al., 2004). Adolescent group size may modulate later-life CORT secretion, but in a direction that results in a greater avoidance of stress. Secreting more CORT when in an individual context may therefore result in better avoidance

of a (potentially) threatening stimuli, whereas secreting less CORT in a social context may result in less antagonistic interactions and lower injury risk. In order to test the hypothesis, research is now necessary to investigate the long-term effects of adolescent group size on adult CORT secretion across social contexts in the same animals, for example CORT secretion in response to an unfamiliar object vs. an unfamiliar conspecific.

3.4.5. Corticosterone: sex differences

Adolescent group size and density had no effects on secretion of CORT in male zebra finches. Why early adolescent group size only affected CORT secretion in females is not clear. The sex-dependent effects were not predicted, but most research investigating adolescent group size and social density have only used males (e.g. Ago et al., 2014; Lürzel et al., 2010; Lürzel et al., 2011; Ortiz et al., 1985; Sachser et al., 1993), and the one study to include both males and females found no sex-dependent effects of group size/density on CORT secretion in adolescent mice (Laviola et al., 2002). The current work is therefore the first to investigate the long-term effects of adolescent group size on later-life CORT secretion in females. Previous work has shown that adolescent female zebra finches (day 60) have a higher basal and peak CORT concentration in response to capture and restraint compared to age-similar males (Crino et al., 2014), but these effects may be limited to birds from smaller broods (i.e. three chicks per nest vs. more than three chicks per nest: Spencer et al., 2009). The standardised brood size in the current study rules out brood size effects, but the prior findings emphasise that social context during development can influence sex differences in CORT secretion. Sex-dependent effects of early-life social conditions (e.g. brood size, adolescent group size) on glucocorticoid secretion may be attributable to differences in nonapeptide functioning. Nonapeptides are regulated by social context, can either inhibit (e.g. mesotocin) or stimulate (e.g. vasotocin) CORT secretion, and have sex differences in functioning, e.g. female rats have a higher number of OT-immunoreactive neurons in the PVN compared to male rats (Dumais & Veenema, 2016;

Goodson et al, 2015; Neumann, 2008). Social conditions experienced during development may modulate sex differences in nonapeptide functioning to account for the effects of nestling brood size and adolescent group size on glucocorticoid secretion outlined above. For example, housing zebra finches with fewer familiar siblings in the nest and, separately, more unfamiliar conspecifics when in adolescence may result in lower mesotocin and/or higher vasotocin concentration in female birds that would be expected to result in greater glucocorticoid secretion. No research has yet investigated whether the social conditions birds experience during development affect the nonapeptide systems, but these could now be a focus of future investigation.

Sex differences in pubertal maturation are typically not present in zebra finches, as male and female birds develop over a similar time scale and reach sexual maturity at similar ages (Perfito, 2010; Zann, 1996). The adolescent sex differences in CORT secretion found in the current chapter and previous work (e.g. Spencer et al., 2009) are therefore not likely to be due to male and female birds being in different stages of maturation. As adolescence progresses, basal testosterone concentration becomes higher in male zebra finches compared to females (around day 75, i.e. late adolescence; Zann, 1996). Higher basal testosterone concentration may result in more antagonistic behaviour in males, as observed in adults (Ardia, Broughton, & Gleicher, 2010). Males raised in larger and/or denser groups during late adolescence may, due to the effects of testosterone, engage in more antagonistic interactions and have a higher CORT concentration than males reared in smaller and/or less dense groups during late adolescence, as has been reported in adult male rodents (e.g. Creel, 2013; Van Loo et al., 2001). CORT secretion in males may therefore have been affected by variation in group size and/or social density in late adolescence. The hypothesis now requires testing by repeating the current experiment in late adolescence, for example between days 65-85 as investigated in Chapter 2.

3.4.6. Gonadal hormones and nonapeptides

Basal gonadal hormone concentrations were no different between housing conditions in the current chapter, suggesting adolescent group size and social density do not have long-term effects on adult behaviour due to organisational effects on gonadal hormone secretion. Previous work has also shown that adolescent group size and/or social density do not affect basal testosterone concentration (Nicholson et al., 2009; Ortiz et al., 1984; Sachser et al., 1993; Smith et al., 2004), but the novel finding here is that estradiol in females is similarly unaffected. Estradiol stimulates the HPA axis in mammalian species, resulting in higher CORT concentration in female compared to male animals (McCormick & Mathews, 2007). As adult estradiol concentration was unaffected by adolescent housing conditions it is therefore unlikely to be a mechanism contributing to the effects of group size on stressor-induced CORT concentration. However, work is still needed to determine the long-term effects adolescent group size and density on the time-dependent responses of gonadal hormones to social challenge (Wingfield et al., 1990) and stressors (Ortiz et al., 1984; Romeo et al., 2004b; Yilmaz, 2003). In addition to, or instead of, affecting circulating gonadal hormone concentrations, adolescent housing conditions may affect an animal's neural sensitivity to gonadal hormones by regulating the neural expression of gonadal hormone receptors. For example, greater neural sensitivity to androgens due to higher androgen receptor expression in the PVN of the hypothalamus may result in lower stressor-induced CORT (Handa & Weiser, 2014). Neural expression of androgen and estrogen receptors in adulthood therefore still need to be quantified in animals raised in the different adolescent housing conditions used in the current chapter.

VP/VT regulate antagonistic interactions, with higher VP/VT resulting in more antagonistic interaction with unfamiliar conspecifics (e.g. Goodson & Thompson, 2010). A lower VT concentration could account for the lower antagonistic behaviour toward unfamiliar conspecifics that was observed in birds reared in larger groups compared to smaller groups, but

this suggestion requires testing. Oxytocin and possibly mesotocin inhibit CORT secretion (Neumann, 2008; Goodson, Schrock, & Kingsbury, 2015), whereas vasopressin and vasotocin further stimulate CORT secretion during a stress response (Aguilera & Rabadan-Diehl, 2000; Cornett et al., 2013). A lower mesotocin and/or higher vasotocin plasma concentration in female zebra finches raised in larger groups during adolescence compared to smaller groups could result in effects on CORT concentration found in the current study. Further work is needed to explore the effects of adolescent housing conditions on adult nonapeptide concentrations to test the above hypotheses.

3.4.7. Summary

In summary, the findings presented in the current chapter clearly show that adolescent group size and social density have long-term effects on adult behavioural responses to unfamiliar stimuli (environments, objects, and conspecifics) when in a group context. Birds raised in larger groups at a sufficiently low density in early adolescence interact more with unfamiliar objects and environments (if female) and spend less time alone amongst unfamiliar conspecifics (males and females) when in the presence of familiar conspecifics in adulthood. Birds raised in larger groups in early adolescence also engage in less antagonistic and courtship behaviour when amongst unfamiliar conspecifics in adulthood. The long-term behavioural effects of group size in females may be attributable to differences in stress physiology, with large group reared females secreting a greater concentration of CORT in response to an acute stressor when in adulthood relative to smaller group reared females. The mechanisms underpinning the effects of density and any effects in males still remain to be elucidated. Previous work has focused on male animals and conflated the effects of group size with social density. The current work is an improvement upon much prior work as the design could detect the separate effects of group size and social density, whilst both males and females were included in order to detect sex differences. Entering into unfamiliar social groups that vary in

both number and density of conspecifics is an inevitable event in many social species, especially in highly gregarious species like zebra finches (Griffith & Buchanan, 2010; Zann, 1996). The current findings indicate that adolescent group size and density can independently, and in interaction with one another, affect behavioural responses to unfamiliar stimuli and stress physiology in adulthood.

Chapter 4

Adolescent interactions with unfamiliar conspecifics: long-term effects on behavioural and endocrine responses to unfamiliar stimuli

4.1. Introduction

Upon dispersing from the natal home, adolescents begin to interact with unfamiliar conspecifics (Nelson et al., 2005; Nelson et al., 2016; Schlegel & Barry, 1991). Unfamiliar adolescent interactions have been proposed to affect responses to unfamiliar stimuli in later-life, with more unfamiliar social interactions during adolescence resulting in adult animals that interact more with unfamiliar stimuli (i.e. less neophobic) compared to animals that engaged in fewer unfamiliar social interactions during adolescence (Spinka et al., 2001). Late adolescent pairs of guinea pigs (days 80-120) briefly exposed to unfamiliar conspecifics for ten minutes per day every other day (20 interactions in total), vs. stable pair housing, had lower cortisol concentration in response to an unfamiliar environment later in adolescence (Lürzel et al., 2011); but ten interactions had no effect (Lürzel et al., 2010). A sufficient number of unfamiliar interactions can therefore lower the physiological response to unfamiliar stimuli, a mild stressor, in adolescent guinea pigs. The guinea pigs exposed to more unfamiliar social interactions (vs.no such interactions) would therefore be expected to engage in less neophobic behaviour when in adulthood. However, the long-term effects of unfamiliar adolescent social interactions on adult neophobic behaviour and glucocorticoid secretion or the adolescent behavioural cause of any long-term effects on such adult measures remain to be determined in the species.

Attempts have been made to determine the adult behavioural effects of unfamiliar adolescent social interactions in rats by repeatedly re-housing unfamiliar rats together for twenty three hours after one hour of single housing on successive days during early adolescence (days 30-45: McCormick et al., 2015). Re-housed male rats (domesticated, Long Evans), vs. familiar stable pair housing, developed into adults who moved more in an unfamiliar elevated-plus maze (McCormick et al., 2008) and spent less time in contact with an unfamiliar object (Green et al., 2013). In contrast to males, female rats (domesticated, Long Evans) subjected to

the adolescent re-housing protocol described above developed into adults who spent more time on the open arms of an elevated-plus maze during estrous (McCormick et al., 2008).Activity and avoidance are neophobic behavioural responses (Haller et al, 1998), suggesting more unfamiliar adolescent social interactions may result in a more neophobic phenotype in male rats and less neophobic phenotype in female rats (McCormick et al., 2015). However, the re-housing protocol conflates unfamiliar social interactions with single housing and absence of a familiar rat. Social separation is a stressor in a number of social species (Ferland & Schrader, 2011; Hennessey, 1997; Remage-Healey et al., 2003). Any effects of the re-housing protocol could therefore be attributed to unfamiliar social interactions, but could also reflect stress from single housing and/or absence of familiar conspecifics.

A rat's appraisal of unfamiliar stimuli can be interpreted in part by the type of ultrasonic call emitted during exposure to the stimuli (Portfors, 2007; Schwarting & Whör, 2012). In response to unfamiliar stimuli rats can emit 22 kHz calls to signal a stimulus is perceived as (potentially) threatening and should be avoided (e.g. predator, handling) and 50 kHz calls to signal a stimulus is (potentially) appetitive and can be approached (e.g. adolescent play, adult mating) (Brudzynski, 2009; Burgdorf et al., 2008; Portfors, 2007). During social separation male rats also emit 50 kHz calls, perhaps as an attempt to solicit social contact to terminate social separation (Whör et al., 2008). Adolescent social interactions affect the emission of ultrasonic calls in later-life, with male rats single housed during adolescence developing into adults that emit fewer 22 kHz calls in response to handling than male rats pair housed during adolescence (Inagaki et al., 2005). Adolescent social interactions also affect behavioural responses to ultrasonic calls in later-life, with male rats single housed during adolescence approaching playback of 50 kHz calls to a lesser extent than male rats pair housed during adolescence (Seffer et al., 2015). Single housing may therefore remove opportunities for vocal learning in adolescence, resulting in lower and/or impaired vocal production in adulthood. The

effect of more unfamiliar adolescent social interactions on vocal behaviour in later-life remains to be tested.

The long-term behavioural effects of unfamiliar adolescent social interactions on adult responses to unfamiliar stimuli may reflect changes in CORT secretion (McCormick et al., 2015; Sachser et al., 2011). However, the rat re-housing protocol described earlier has no long-term effects on CORT concentration (McCormick et al., 2004; McCormick et al., 2005; Mathews et al., 2008; McCormick et al., 2008). To date, all re-housing studies have only quantified CORT concentration in response to non-social stressors, such as restraint and confinement on an open platform. Adolescent social interactions may only have effects on adult social interactions, including the secretion of CORT in response to social interactions (van den Berg et al., 1999). When investigating the long-term endocrine effects of adolescent social interactions, the social context in which CORT concentration is quantified should be taken into consideration.

The reason why unfamiliar adolescent social interactions have long-term effects on adult responses to unfamiliarity is not clear. However, the adult differences may emerge during adolescence from behavioural differences in responses to familiar vs. unfamiliar conspecifics. There is no clear evidence of differences in non-social investigation (e.g. locomotor activity and rearing) occurring between familiar and unfamiliar rats (Cirulli et al., 1996; Terranova et al., 1999), but social behaviour is less ambiguous. Rodents, especially domesticated rats, engage in rough-and-tumble play and investigatory sniffing with one another in adolescence (Panksepp et al., 1984; Pellis & Iwanuik, 2004; Pellis & Pellis, 2007; Varlinskaya & Spear, 2008) and emit 50 kHz calls during play (Himmler et al., 2014). Play and sniffing is more common between unfamiliar rats compared to familiar rats during brief (10-30 minute) encounters (Barefoot et al., 1975; Cirulli et al., 1996; McCormick et al., 2006; Thor &

Holloway, 1984; Veenema et al., 2012), suggesting the quantity of social interaction may be the cause of the long-term effects on adult responses to unfamiliarity.

The current study investigated two hypotheses. First, interacting with unfamiliar conspecifics during adolescence results in adult animals that are less neophobic, indicated by the animals interacting more with unfamiliar non-social stimuli and secreting a lower glucocorticoid concertation in response to an unfamiliar conspecific (Spinka et al., 2001). Second, interacting with unfamiliar conspecifics in adolescence results in adult animals engage in more and/or improved vocal communication in adulthood, indicated by the animals emitting more 50 kHz calls at baseline and fewer 50 kHz calls in response to social separation (based on Seffer et al., 2015; Whör et al., 2008). Male rats have been the focus of most prior research investigating unfamiliar social interactions during adolescence on adult phenotypes (McCormick et al., 2015). The current study used only female rats as an attempt to provide a detailed investigation of how female rats specifically respond to unfamiliar adolescent social interactions. Previous research has shown that, in line with the hypothesis being tested in the current chapter, female (not male) rats interacted with unfamiliar stimuli more in adulthood after being re-housed with unfamiliar conspecifics compared to stable housing (McCormick et al., 2008). However, McCormick et al. (2008) cannot convincingly claim the effects were due to more unfamiliar social interactions due to the uncontrolled confounds of single housing and re-housing that were present in the design. These confounds will be controlled for in the current study in order to provide less ambiguous evidence of the long-term effects of unfamiliar adolescent social interactions on adult responses to unfamiliar stimuli. This study is also the first to investigate the long-term effects of adolescent social novelty on adult vocalisation rate in female rats.

During early adolescence (days 34-46) rats in the current study experienced one of three conditions: paired with an unfamiliar partner in a test arena for ten minutes per day (n = 12),

paired with a familiar cage mate in a test arena for ten minutes per day (n = 12), or left in the home cage (n = 12). Previous work had indicated that 10 minutes is sufficient for unfamiliar pairs of rats to have engaged in more play than familiar pairs of rats (Cirulli et al., 1996) and long-term effects to occur in guinea pigs (Lürzel et al., 2011). During the interactions in the test arena recordings were made of social behaviour (time in proximity, number of investigative sniffs, number of play encounters) and risk-related behaviour (rearing, freezing) as an indication of the proximate behavioural cause of any long-term effects. Unfamiliar, vs. familiar, pairs were predicted to be more affiliative, indicated by more time in proximity, engaging in more sniffing and play (based on, for example, McCormick et al., 2006; Thor & Holloway, 1984) but no differences were expected for rears and freezes (based on Cirulli et al., 1996; Terranova et al., 1999).

In adulthood (day 65+), behavioural responses to unfamiliar stimuli were quantified in three unfamiliar environments. The first environment was an elevated-plus maze, which permits rats to explore aversive open arms and less aversive closed arms on an elevated ‘plus’ shaped maze (Carobrez & Bertoglio, 2005). The second environment was a light-dark box, which permits rats to freely enter an aversive well-lit compartment from a less aversive dark compartment (Bourin & Hascoët, 2003). The third environment was an object/social novelty task, which permits rats to explore an unfamiliar arena containing a novel object and novel male rat (Brown, Kulbarsh, Spencer, & Duval, 2015). Rats exposed to unfamiliar adolescent interactions, vs. other conditions, were predicted to interact more with unfamiliar stimuli in the elevated-plus maze (enter the open arms more, spend more time on the open arms, an engage in more risk assessment on the open arms), light-dark box (entering the light compartment more quickly, entering the light compartment more often, spending more time in the light compartment, and rearing in the light compartment more), and object/social novelty task (more

time in proximity to the unfamiliar object and conspecific, more rears when in proximity to the unfamiliar object and conspecific) based on the findings of McCormick et al. (2008).

In adulthood, responses to unfamiliar social contexts were quantified in two separate tasks. First, the number of ultrasonic calls were quantified prior to, during, and immediately after single housing. 50 kHz call rate during social separation was predicted to be lower in rats that interacted with unfamiliar conspecifics during adolescence compared to control conditions that did not interact with unfamiliar conspecifics (based on Wöhr et al., 2008). Second, urinary CORT concentration was quantified both prior to and after housing with a familiar and unfamiliar conspecific. CORT concentration in response to an unfamiliar conspecific was predicted to be lower in rats that interacted with unfamiliar conspecifics during adolescence compared to control conditions that did not interact with unfamiliar conspecifics (based on Lürzel et al., 2011).

4.2. Methods

4.2.1. Ethical statement

Ethical guidelines as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85–23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986 were adhered to under Project Licence 60/4354 and Personal Licence IDFA58352.

4.2.2. Animals and housing

36 female Lister hooded rats derived from nine litters that were bred in-house from stock animals (Harlan, UK) were used as test subjects in the current study. Additionally, 12 females and 6 males from these litters were used as unfamiliar social interaction partners. Pups were reared by their mothers in plastic cages measuring 52 x 40 x 26 cm (length x depth x height) with a wire mesh top. Pups were weaned on postnatal day (PND) 26 and housed in same-sex sibling pairs in cages measuring 45 x 28 x 21 cm cages (length x depth breadth x height). Pups

were weighed upon weaning and given unique tail markings with semi-permanent marker. Rats continued to be weighed and marked weekly throughout the experiment. Rats had *ad libitum* access to soya-free pellet food (DBM Food Hygiene Supplies Ltd, Scotland) and water when in the home cage. All animals were held in a single holding room throughout the experiment and were kept on a 12:12 light-dark cycle (lights on at 07:00), with temperature and humidity maintained at 20±1° and 55±5%, respectively. All interactions with the rats occurred during lights-on. All cages received weekly husbandry.

4.2.3. Experimental Design

Adolescence. Three groups were established that differed in adolescent social experience: familiar social interactions (FS; n = 12 pair housed rats), unfamiliar social interactions (US; n = 12 pair housed rats), and unmanipulated controls (C; n = 12 pair housed rats). The litters were distributed across the different conditions to reduce any pre-weaning effects, and both rats in the same cage were in the same group. FS rats were housed in a test arena with their cage mate for 10 minutes per day, US rats were housed in a test arena with one unfamiliar same-sex age-similar (±1 day) play mate for 10 minutes per day, and C rats were left undistributed in their home cages except for regular husbandry in order to control for the effects of handling, transport, and exposure to the test arena. As all rats were handled regularly during weekly husbandry a separate handling control group was not needed. The unfamiliar playmates (n = 14) used were not from the same litter as the US rat undergoing testing.

The FS and US interactions occurred in one of two identical and adjoined arenas measuring 48 x 47 x 44 cm (length x height x depth) with sawdust covering the base and black card covering the exterior walls. The arenas were separated from one another with an opaque plastic barrier. The arena was surrounded by a black curtain to standardise the testing environment. US rats were carried in a test cage to the test arena and placed one rat per test

arena. Each test arena contained one unfamiliar play mate rat that was placed in the arena less than two minutes prior to placing the US rat in the arena. The unfamiliar rats were allowed to interact for 10 minutes before being placed back into a carry box and returned to the home cage with their familiar partner. FS rats were carried to the test arena in a carry box and both rats were placed in the same arena and allowed to interact for 10 minutes before being put back in a carry box and returned to the home cage with their familiar partner. FS and US rats underwent this process once per day from PND 34-45. Unfamiliar playmates were used once per day. The test arenas were wiped down with 70% ethanol between each interaction to remove odour cues.

A video camera mounted into the ceiling was used to video record the interactions. Six of the twelve daily interactions were video recorded (half of animals were recorded on odd days and half on even days) to provide a measure of behaviour change across adolescence. The six interactions are referred to as sessions. From the view of the camera, the test arena was divided into equally sized quadrants by lines of string that were fastened to the top of the test arena. The *duration of time both rats were in the same quadrant* (seconds) was recorded as a measure of social proximity. *Number of rough-and-tumble play behaviours*, *number of investigative sniffs*, *number of rears*, and *number of freezes* were also recorded. Table 4.1. contains definitions of the coded behaviour. Measured behaviour were not independent of each rat, so each behaviour was recorded per pair of rats.

Adulthood. In adulthood (PND 96-111), behavioural responses to unfamiliar stimuli were quantified by exposing animals to unfamiliar environments (see '*Unfamiliar environments*' below). Rats were tested over a period of three weeks with one task per week. The three environments used were an elevated-plus maze, a light-dark box, and an object/social novelty task. The first task occurred on PND 96 or 97, the second on PND 103 or 104, and the third on PND 110 or 111. All tasks were counter-balanced across testing ages, adolescent conditions, and time of day (8:30-13:00 vs.13:00-17:30). Later in adulthood, responses to changes in social

context were quantified (see '*CORT response to social interactions*' below). In one task, urine samples were collected before and after social interaction with a familiar or unfamiliar same-sex adult female rat (PND 122-132). A pre-interaction urine sample was taken after removing a rat from a home cage, and a post-interaction urine sample was taken after three hours of pair housing with a familiar or unfamiliar rat. Three hours has been indicated as a sufficient duration of time to determine stressor-induced CORT in urine (Bamberg, Palme, & Meingassner, 2001). Urine sampling for each rat occurred on two consecutive days, with partner familiarity and conditions counter-balanced across the two days. The chronological order of testing was also counter-balanced across conditions. Later in adulthood (PND 156-169), ultrasonic calls were recorded (see '*Ultrasonic call response to social separation and reunion'* below). The calls were recorded during three phases of a social separation task (pre-separation, separation, and reunion) during which a rats was separated from its familiar cage mate and single housed. A summary diagram of the design used in the current chapter is presented in Figure 4.1.

Table 4.1. Behaviours coded during adolescent observations which were based on: 1. Cirulli et al. (1996), 2. Klein, Padow, & Romeo, 2010, 3. Rodgers et al. (1999), 4. Blanchard, Griebel, Pobbe, & Blanchard (2011), 5. Kiyokawa et al. (2004).

Variable	Composite Behaviours	Description
Play[1,2]	Nape attack	A rat contacts the back of the neck of another rat
	Boxing	Rats stand on their hindpaws and push one another with their forepaws
	Pin	A rat holds another rat down on its back or side
	Evasion	A rat runs, leaps, or swerves from a rat during play
Investigation[1,2]	Facial sniffs	A rat contacts, with its snout, the head and nape of another rat
	Flank sniffs	A rat contacts, with its snout, either side of the body of another rat
	Anogenital sniffs	A rat contacts, with its snout, the ano-genital region of another rat
Rears[3,4]	-	A rat raises its forepaws off the ground and stands on the hind legs to horizontally investigate the environment
Freezes[4,5]	-	A rat suddenly stops moving for a minimum of two seconds

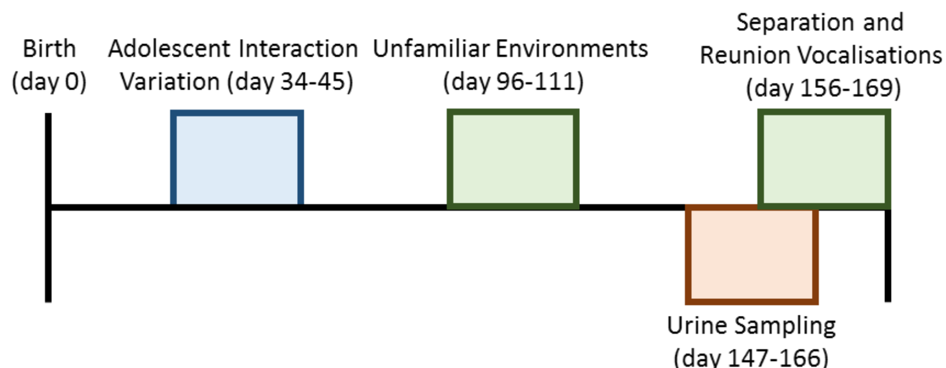

Figure 4.1. Timeline detailing the age at which the rats were underwent the familiar or unfamiliar social interaction manipulation (blue), when behavioural responses were recorded (green), and when urine sampling occurred (red).

4.2.4. Apparatus and measures

Unfamiliar environments. All unfamiliar environment testing occurred in the same room as the adolescent interaction sessions. Each unfamiliar environment apparatus was surrounded by a black curtain to standardise the testing environment. Each task was recorded using a camera mounted into the ceiling above the apparatus. All rats were tested individually and each apparatus was wiped down with 70% ethanol after each test to remove odour cues.

Elevated-plus maze (EPM). The EPM consisted of a wooden 'plus' shaped platform made up of four grey-painted wooden arms. Each arm was 51cm long and 11cm wide, extending out from a central square (11 x 11 cm) to form a 'plus' shape. Two opposing arms were open platforms, whilst the other two opposing arms had walls (back wall, two side walls). The central square was present to ensure behavioural recordings from each arm were independent from one another. A metal frame elevated the maze to a height of 56cm.

Recording began when a single rat was placed in the centre square facing an open arm. *Number of open and closed arm entries* (all four paws in an arm), *time spent in open and closed arms* (seconds), and *number of rears in open and closed arms* (rat stands on its hind legs within an arm) were recorded for five minutes before returning the rat to the home cage.

Light-dark box (LDB). An arena (119 x 47 x 44 cm, length x height x width) was split into two adjacent compartments with an opaque plastic divider An 11 x 11cm archway at the bottom of the divider allowed movement between compartments. A dark compartment (49 x 47 x 44 cm, length x height x width) was created by covering the exterior walls in black card and covering the compartment with a wooden lid. A light compartment (70 x 47 x 44 cm, length x height x width) was created by covering the exterior walls of the compartment with white card and exposing the compartment from above with artificial light (150 lux on compartment floor).

A single rat was placed in the dark compartment to begin the task. *Latency to enter the light compartment* (all four paws in light compartment), *number of head pokes into the light compartment* (only snout to ears in light compartment), *number of entries into the light compartment* (all four paws in light compartment), *duration of time spent in the light compartment* (seconds), and *number of rears in the light compartment* (rat stands on hind legs in light compartment) were recorded for five minutes before returning the rat to the home cage.

Object/social novelty (OSN) task. An arena (119 x 47 x 44 cm, length x height x width) was covered with black card on the exterior walls. A Perspex box (24 x 46 x 21 cm, length x height x width) was placed in the top right corner and the bottom left corner of the arena. During testing one box ('object box') contained one of six novel objects (glass jar filled with stones, blue plastic bottle filled with sand, orange plastic watering can, spiky yellow ball, and a multi-coloured tower of Lego® blocks) and the other box ('social box') contained one of six novel males (adult males from same litters as females in the current study, but not related to the test female). The arena floor was divided into three zones: left, right, and centre. The left and right zones were 48 x 47 cm (length x depth) and each included one of the Perspex boxes. The left and right zones are referred to as 'object area' or 'social area' depending on the contents of each Perspex box in that zone. The centre zone measured 24 x 47 cm (length x depth) and was included so behaviour in the left and right zones could be measured independent of one another.

The order of each object and male (1-6) and the location of the object box/area and social box/area (left vs. right) were counter-balanced across conditions.

A rat was placed in the centre zone to start testing. The *number of entries into the object area and social area* (four paws in the area), the *duration of time in the object area and social area* (seconds), the *number of rears in the object area and social area* (rat stands on hind legs), the *number of times in contact with the object and social box* (rat touches a box with head, body, or paws), and the *duration of time in contact with the object and social box* (seconds) were recorded for five minutes before returning the rat to the home cage.

CORT response to social interaction. A pair of rats were moved to a separate room for urine collection. Rats were individually housed in clean cages (39 x 26 x 57 cm, length x height x depth) with cling film covering the base of the cage. A pre-interaction urine sample (minimum 50 µl) was collected by holding a rat under the arms and lifting the rat off the ground; the rat responded by urinating on the cling film. Urine was collected in an Eppendorf, put on wet ice, and stored at -20°C until assayed. Rats were then pair housed in new clean cages identical to the urine collection cages, but with sawdust bedding and *ad libitum* access to soya-free pellets and water, in a separate room to the urine collection room. Rats were pair housed with either a familiar or unfamiliar rat for three hours. The familiar rats were cage mates. The unfamiliar rats were one of the subjects in the current study was not the cage mate. The condition (FS, US, C) that the unfamiliar rat belonged to was counter-balanced with the condition of the rat undergoing testing. After three hours had elapsed the rats were returned to the urine collection room and a second urine sample was collected like that described above. Rats were then returned to their home cages with their original familiar cage mate.

Ultrasonic call before, during, and after social separation. During pre-separation, two familiar rats were removed from their home cage and placed in separate but adjacent 32 x 47 x 44 cm

(length x height x depth) arenas for five minutes. Arenas were covered with black card on the exterior walls and a perforated clear Perspex divider separated one arena from the other. A rat was in visual, auditory, and olfactory contact with the other rat. Rats were then single housed in separate 39 x 26 x 57 cm cages for sixty minutes with one rat moved to a different room to ensure no contact could occur (separation). Rats had *ad libitum* soya-free pellets and water during separation. After sixty minutes elapsed, rats were returned to their original pre-separation compartments (reunion) for five minutes before being returned to their home cages in the holding room. To control for any relocation effects the task occurred twice (separated by seven days) with each rat experiencing relocation. Chronological order of testing was counter-balanced across conditions.

Ultrasonic calls were recorded during social separation task using UltraSoundGate Condenser Microphone CM16/CMPA (Avisoft-96 Bioacoustics, Germany; frequency range 10–200 kHz). During pre-separation and reunion a microphone was suspended above each arena to record calls from each rat for five minutes. During separation, a microphone was suspended above each cage and calls were recorded for the first five minutes of single housing. Audacity®, version 2.0.5 was used to visualise calls. 50 kHz calls (frequency range 30-80 kHz) were classified into one of fifteen call types based on call shape, duration, and bandwidth (Table 4.2.) based on the call types described in Wright, Gourdon, & Clarke (2010). 22 kHz calls (near constant structure, frequency range 20-25 kHz, duration $\geq$ 0.1 seconds) were also quantified, but so few rats emitted them (4/36) that the calls were not further considered.

Table 4.2. 50 kHz call types coded during social separation and reunion. Calls followed by a (FM) are frequency modulated (frequency range ≥5 kHz) and calls followed by a (C) are constant (frequency range <5 kHz).

Call	Definition
Complex (FM)	Call with at least three directional changes; frequency range > 8 kHz; duration ≥ 0.02 seconds
Sub-complex (FM)	Call with at least three directional changes; frequency range 5-8 kHz; duration ≥ 0.02 seconds
Upward ramp (FM)	Call gradually increases in frequency and may end in a plateau or slight dip; frequency range ≥ 5 kHz; duration > 0.02 seconds
Downward ramp (FM)	Call gradually decreases in frequency and may end in a plateau or slight rise; frequency range ≥ 5 kHz; duration ≥ 0.02 seconds
Step up (FM)	A constant call that terminates with an isolated call that is a higher frequency; frequency range ≥ 10 kHz; duration ≥ 0.02 seconds
Step down (FM)	A constant call that terminates with an isolated call that is a lower frequency; frequency range ≥ 10 kHz; duration ≥ 0.02 seconds
Multi-step (FM)	Central section of a call is separated from preceding/succeeding sections and falls to a lower frequency; sections can be any call, but not trills; frequency range ≥ 10 kHz; duration ≥ 0.05 seconds
Trill (FM)	Continuous and connected rapid frequency oscillations; frequency range of each trill component is > 8 kHz; duration ≥ 0.04 seconds
Trill plus (FM)	A trill call that contains any additional call type
Inverted U (FM)	A single call that contains a frequency increase then frequency decrease; frequency range ≥ 5 kHz; duration ≥ 0.005 seconds
Cluster inverted U (FM)	Two or more inverted U calls separated by no more than 0.005 seconds
Composite (FM)	Two or more different types of call as detailed above; must not contain trills
Short (C)	Rapid call isolated from other calls by ≥ 0.03 seconds; frequency range < 5kHz; duration ≤ 0.015 seconds
Flat (C)	A constant call with frequency range ≤ 3 kHz; duration ≥ 0.012 seconds; must be detected above 30 kHz
Near constant (C)	A mostly constant call with a frequency range 3-5 kHz; duration ≥ 0.015 seconds; must be detected above 30 kHz

4.2.5. Hormone assays

Creatinine. An enzyme immunoassay kit was used to determine the concentration of creatinine in each urine sample to control for urine concentration (Creatinine (urinary) Colorimetric Assay Kit, Cayman Chemical, USA). Following the manufacturers guidelines, 5µl urine samples were diluted with distilled water to a final concentration of 1:20 in 40µl. 15µl samples were run in duplicate across two plates, with all samples from a single animal on the same plate and condition distributed across the plates. Each plate contained a separate eight point standard curve ranging from 0-15 mg/dl. All plates were read on a Biochrom Anthos 2010 Microplate Reader, ADAP 2.0 (Biochrom Ltd., UK) at a wavelength of 492 nm. The intra-plate coefficients of variation (%) were 9.74% and 11.52. The inter-plate coefficient of variation (%) was 10.63.

CORT. Radioimmunoassay following a previous protocol (Spencer et al., 2009) was used to determine CORT concentration in 50µl urine samples. Prior to assay the samples were spiked with 25µl of [1,2,6,7-3H]-CORT label (Perkin Elmer Inc., UK) and extracted with 1ml diethyl ether. Extracted samples were evaporated at 42°C and reconstituted in 300µl of assay buffer (0.01M PBS, pH 7.4, 0.25% BSA). 50µl aliquots of the reconstituted samples were taken to determine the extraction efficacy, which ranged between 75-100%. The CORT concentration was then determined in 100µl aliquots of the reconstituted samples using anti-CORT antiserum (Esoterix Endocrinology, USA, B3-163; 1:15000 dilution in assay buffer) and [1,2,6,7-3H]-CORT label (Perkin Elmer, UK). After incubating the reactions for 24 hours at 4°C the unbound antigens were removed by adding 100µl of a charcoal-dextran suspension (0.5% charcoal, 0.25% dextran in assay buffer) to each sample and centrifuging the samples at 2000g for 20 minutes. The supernatant was collected in order to quantify the antibody-bound antigens using a radioactivity counter (Packard Tri-Carb 1600 TR Liquid Scintillation Analyser, Perkin Elmer Inc., UK). All samples were run in duplicate across two assays. Each assay contained

all samples from a single rat, and conditions were distributed across assays. Each assay contained a separate ten point standard curve ranging from 0.04-20 ng/ml. 50% binding (ng/ml) for each assay was 0.71 and 0.75. Intra-assay coefficients of variation (%) for each assay were 14.35 and 11.33. Inter-assay coefficient of variation (%) was 12.84.

4.2.6. Data Analysis

SPSS version 22 was used to conduct all analyses. After performing each test the residuals were checked for normality (Shapiro-Wilk, $p > 0.05$), with some variables found to be non-normal due to a positive skew in the distribution. Consequently, some variables were square root or log10 transformed to achieve normality in residuals. Those variables that could not be transformed to normality were analysed using generalized linear models. In any mixed models, litter ID and litter size was entered as random factors to control for pre-adolescent experiences. Statistically significant effects were classed as those with a $p < 0.05$. Significant main effects and interactions were further explored using Bonferroni post hoc tests. Cohen's d was calculated as a measure of effect size for all significant post hoc pairwise comparisons. All data presented are means $\pm$ standard error of the mean.

Adolescent interactions. Linear mixed models (LMM) were used to analyse the adolescent behaviour as the interaction sessions were a repeated measure. Duration of time in same quadrant, square root number of play behaviours, number of investigatory sniffs, square root number of rears, and number of immobilisations were entered as dependent variables in separate models. Fixed factors included adolescent condition, session, and the interaction between the two. Session was entered as repeated measure to account for the non-independence of observations across the adolescence recordings.

EPM. Exploratory correlations (Spearman's rank) revealed significant correlations between the variables (arm entries v. arm duration: $r = 0.832$, $p < 0.001$; arm entries vs. rears: $r = 0.445$, p

< 0.001; arm duration vs. rears: $r = 0.498$, $p < 0.001$). A multivariate general linear model would be inappropriate given the repeated nature of the data (i.e. open v. closed arm). One variable, duration of time in each arm, was therefore analysed. Square root duration of time in the arms was entered as a dependent variable in a LMM with arm type (open, closed) and adolescent condition entered as fixed factors. Arm type was also entered as a repeated measure to account for the non-independence between spending time in each arm. Variables excluded in response to the PCA analysis, i.e. number of arm entries and number of rears, were analysed in models identical to those for duration of time on the arms. Model outputs from the excluded variable models were identical to that of the included variable (data not shown), indicating that excluding these variables did not qualitatively affect the results.

LDB. A multivariate analysis of variance was used to analyse LDB variables in order to simultaneously analyse all variables whilst accounting for the co-variance between variables. Log10 light latency (seconds), number of head pokes, number of light compartment entries, duration of time in light compartment (seconds), and number of rears were entered as dependent variables in one model with adolescent condition entered as a fixed factor.

OSN. Exploratory correlations (Spearman's rank) found that the dependent variables recorded were positively correlated with one another (see Appendix B Table B.7.). A multivariate general linear model would be inappropriate given the repeated nature of the data (i.e. social v. object area/box). Only one behaviour, duration of time in contact with the stimulus box, was therefore analysed. Duration of time in contact with the stimulus box was entered as a dependent variable in a LMMs with adolescent condition and box type (social, object) entered as fixed factors. Box type was also entered as a repeated measure to account for the non-independence between times spent with either stimulus box. The variables excluded in response to the PCA analysis, i.e., were analysed in models identical to those for duration of time in contact with the stimulus box. Model outputs from the excluded variable models were identical

to that of the included variable (data not shown), indicating that excluding these variables did not qualitatively affect the results.

CORT response to social interactions. The creatinine concentration was calculated following the manufacturers guidelines for the creatinine EIA kit. CORT concentration was expressed as 10^{-5} CORT (ng/ml)/creatinine (mg/dl). Log10 pre-interaction CORT concentration was first analysed for differences between conditions using a univariate general linear model with adolescent condition entered as a fixed factor. No significant difference was detected ($F_{2,69}$ = 0.236, p = 0.791). A change in urinary CORT concentration variable was then created by subtracting pre-interaction CORT concentration from post-interaction CORT concentration. A LMM was then used to analyse change in 10^{-5} CORT (ng/ml)/creatinine (mg/dl) with adolescent condition and partner familiarity entered as fixed factors. Partner familiarity was also entered as a repeated measure to account for the non-independence of CORT across task contexts. Partner condition was entered as a random factor to control for any effects of partner adolescent experience. The pre-interaction CORT concentration was entered as a co-variate to control for individual differences in the pre-interaction CORT concentration.

Ultrasonic calls before, during, and after social separation. The mean call rate across the two recording days was calculated, and then split into two categories: FM calls ($\geq$5 kHz bandwidth), and constant calls (<5 kHz bandwidth). The total number of calls was entered as a dependent variable in a generalized linear mixed model with adolescent condition, call category, task phase, and the interaction between them entered as fixed factors. The call categories and task phases were also entered as repeated measures to account for the non-independence of calls across categories and task phases. The data were fitted to a gamma distribution (with log link) using a robust estimation in order handle possible violations of the model assumptions. A gamma distribution was chosen to account for a positive skew in the residuals of the raw data.

4.3. Results

4.3.1. Adolescent interactions

Unfamiliar pairs spent more time in the same quadrant than familiar pairs ($F_{1,17.356}$ = 20.965, p < 0.001; familiar: M = 268.47, SEM = 8.43; unfamiliar: M = 328.61, SEM = 5.45), but session had no effect on quadrant time (session: $F_{5,16.543}$ = 2.247, p = 0.098; session x condition: $F_{5,16.543}$ = 1.815, p = 0.165). Unfamiliar pairs also engaged in more investigatory sniffs than familiar pairs ($F_{1,17.992}$ = 40.063, p < 0.001; familiar: M = 10.11, SEM = 0.73; unfamiliar: M = 20.67, SEM = 0.72), but session had no effect on sniffing (session: $F_{5,16.985}$ = 1.503, p = 0.241; session x condition: $F_{5,16.985}$ = 1.503, p = 0.241). Unfamiliar pairs also played more than familiar pairs ($F_{1,17.973}$ = 18.549, p < 0.001), but this was dependent on session number ($F_{5,16.905}$ = 9.273, p < 0.001; Figure 4.2.). Unfamiliar pairs played more than familiar pairs in session 4 (p = 0.02, d = 1.15), 5 (p < 0.001, d = 2.14), and 6 (p < 0.001, d = 1.88). Session itself did not affect play ($F_{5,16.905}$ = 2.046, p = 0.124).

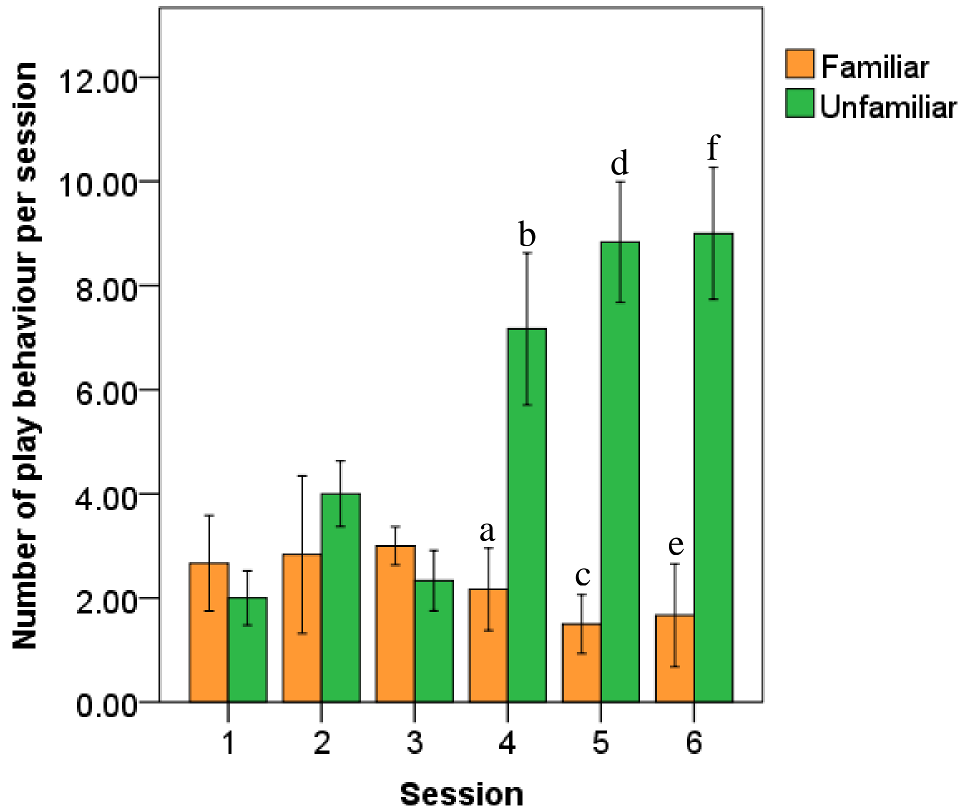

Figure 4.2. Number of play behaviour between familiar and unfamiliar rats during adolescent social interaction sessions. Data are mean $\pm$ one standard error of the mean. Letters denote significant difference (p < 0.05) between conditions in a session, i.e. a vs. b, c vs. d, e vs. f.

Rats engaged in fewer freezes in session 1 compared to all other sessions (session: $F_{5,15.966} = 18.396$, $p < 0.001$; 1 vs. 2: $p < 0.001$, $d = 1.49$; 1 vs. 3: $p < 0.001$, $d = 1.84$; 1 vs. 4: $p < 0.001$, $d = 1.86$: 1 vs 5: $p < 0.001$, $d = 2.13$; 1 vs. 6: $p < 0.001$, $d = 2.59$). Data are summarised in Table 4.3. Adolescent condition did not affect freezing (condition: $F_{1,16} = 0.437$, $p = 0.518$; session x condition: $F_{5,15.966} = 0.951$, $p = 0.476$).

Table 4.3. Number of freezes during each of the six sessions of adolescent social interaction split between familiar and unfamiliar pairs. Data presented are mean (one standard error of the mean). A main effect of session was found denoted by bold vs. not bold, with fewer freezes in session 1 vs. session 2-6 regardless of condition ($p < 0.05$).

Session	Freezes	
	Familiar (M±SEM)	Unfamiliar (M±SEM)
1	**2.67 (1.04)**	**2.04 (0.48)**
2	5.75 (1.54)	6.33 (0.84)
3	8.00 (0.55)	7.25 (1.23)
4	8.92 (0.98)	6.79 (1.11)
5	10.25 (2.25)	9.38 (1.22)
6	9.58 (1.21)	8.91 (1.07)

Rats engaged in a similar number of rears regardless of adolescent condition or test session (condition: $F_{1,15.931} = 2.031$, $p = 0.173$; session: $F_{5,15.769} = 1.848$, $p = 0.161$; condition x session: $F_{5,15.769} = 1.108$, $p = 0.39$). Data are summarised in Table 4.4.

Table 4.4. Number of rears during each of the six sessions of adolescent social interaction split between familiar and unfamiliar pairs. Data presented are mean (one standard error of the mean). No significant effects were detected ($p > 0.05$).

Session	Rears	
	Familiar (M±SEM)	Unfamiliar (M±SEM)
1	41.17 (2.35)	38.96 (1.69)
2	37.25 (1.36)	42.96 (2.38)
3	41.67 (3.08)	42.17 (2.65)
4	33.58 (4.34)	35.17 (1.99)
5	34.33 (2.31)	40.29 (2.14)
6	36.42 (1.39)	42.91 (3.33)

4.3.2. Adult behavioural responses to unfamiliar environments

In the EPM, rats did spend more time on the open arms compared to the closed arms regardless of adolescent condition ($F_{2,33}$ = 67.927, p < 0.001; Figure 4.3.). However, rats spend a similar total duration of time on the arms regardless of adolescent conditions (dosing condition: $F_{2,33}$ = 0.449, p = 0.642) and a similar duration of time on each specific arm (dosing condition x arm type: $F_{2,33}$ = 0.367, p = 0.695).

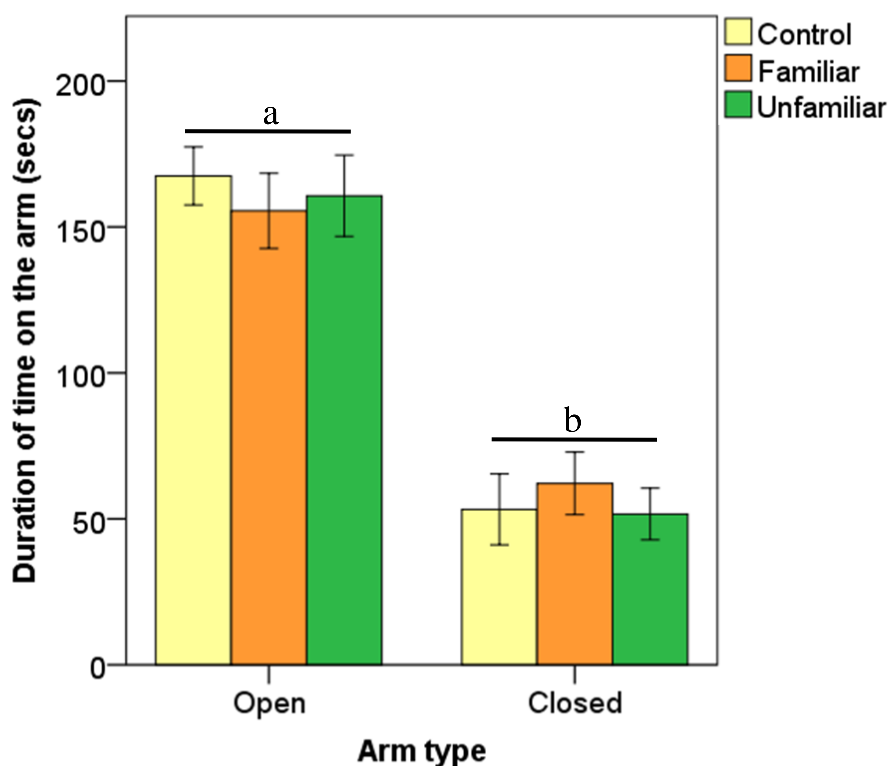

Figure 4.3. Duration of time spent on the arms of an elevated-plus maze split by arm type and adolescent condition. Data presented are mean $\pm$ one standard error of the mean. Letters denote a significant differences; a vs. b, p < 0.05.

In the LDB, all rats entered the light compartment at a similar time ($F_{2,32} = 0.050$, $p = 0.952$), entered the light compartment a similar number of times ($F_{2,32} = 0.463$, $p = 0.634$), and spent a similar duration of time in the light compartment ($F_{2,32} = 0.1$, $p = 0.905$). In addition, all rats engaged in a similar number of head pokes into the light compartment ($F_{2,32} = 3.0$, $p = 0.065$) and a similar number of rears in the light compartment ($F_{2,32} = 0.51$, $p = 0.951$). Data are summarised in Table 4.5.

Table 4.5. Behavioural measures in the LDB split by adolescent condition. Data presented are mean (one standard error of the mean). No significant effects were detected ($p > 0.05$).

Measure	Adolescent Condition		
	Control ($M \pm SEM$)	Familiar ($M \pm SEM$)	Unfamiliar ($M \pm SEM$)
Latency to enter light compartment (sec)	52.48 (24.42)	46.8 (20.58)	49.68 (28.58)
Number of head pokes into light compartment	2.09 (0.44)	2.64 (0.56)	3.9 (0.59)
Number of light compartment entries	3.91 (0.65)	4 (0.45)	4.7 (0.76)
Duration in light compartment (sec)	78.63 (15.55)	73.88 (12.61)	69.61 (14.07)
Number of rears in light Compartment	4.73 (0.54)	4.91 (0.72)	5.0 (0.56)

In the OSN task, rats spent more time in contact with the social box compared to the object box ($F_{2,32}$ = 128.212, $p < 0.001$; Figure 4.4.). However, rats spent a similar duration of time interacting with the stimulus boxes regardless of adolescent condition (condition: $F_{2,32}$ = 1.910, p = 0.165; condition x area: $F_{2,32}$ = 0.739, p = 0.485).

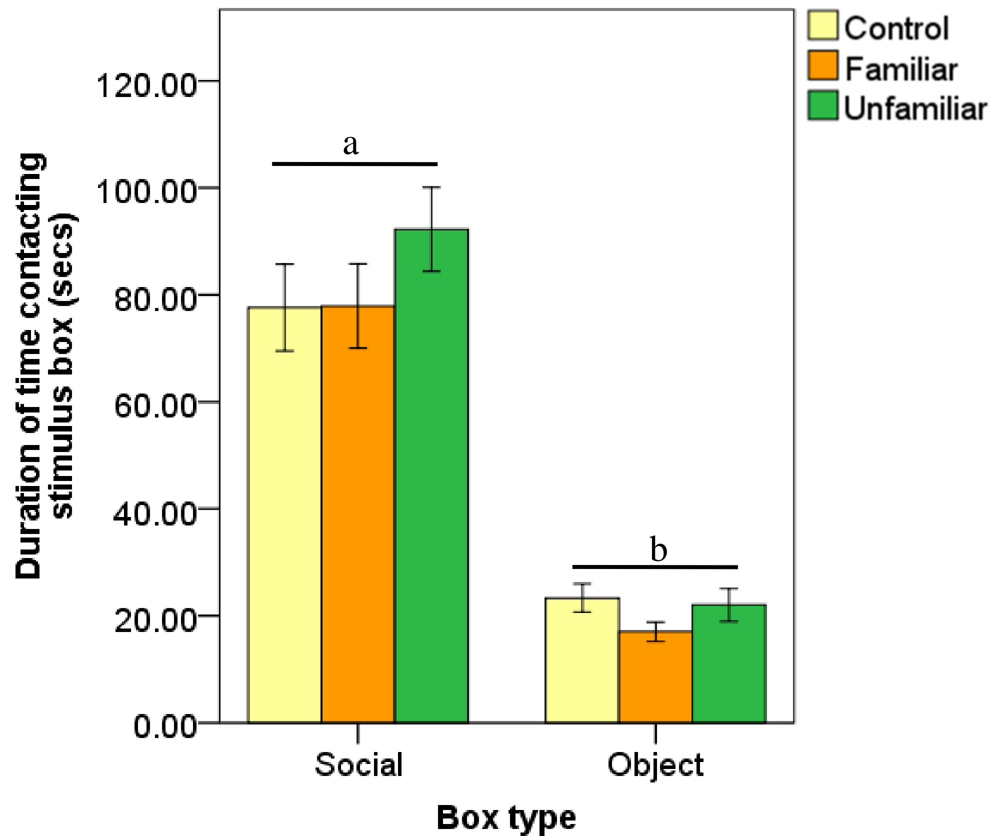

Figure 4.4. Duration of time spent in contact with the stimulus boxes in the OSN task split by box type and adolescent condition. Data presented are mean $\pm$ one standard error of the mean. Letters denote a significant differences; a vs. b, $p < 0.05$.

4.3.3. CORT response to social interactions

Change in urinary CORT/creatinine ratio was similar in all rats regardless of adolescent condition and partner familiarity (condition: $F_{2,15.220} = 0.459$, $p = 0.641$; partner familiarity: $F_{1,27.068} = 0.124$, $p = 0.727$; condition x partner familiarity: $F_{2,29.301} = 1.204$, $p = 0.314$; Figure 4.5.).

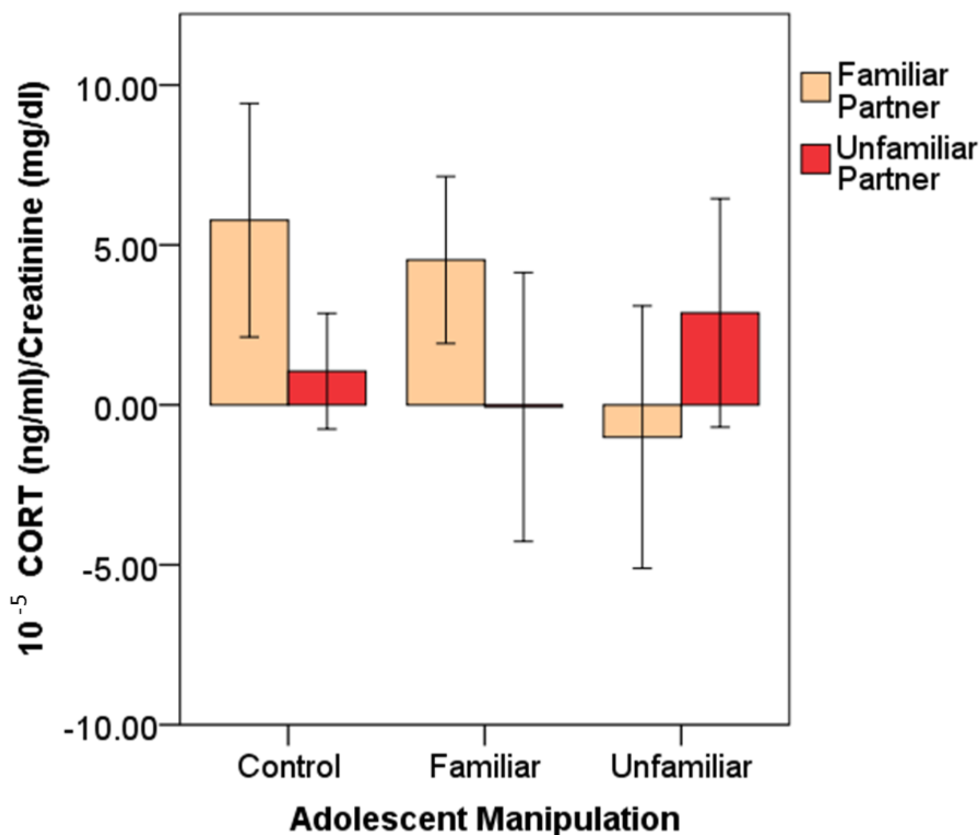

Figure 4.5. Change in CORT concentration in response to social interaction (post-interaction minus pre-interaction) scaled for creatinine concentration. CORT concentration is presented for each condition split by familiar vs. unfamiliar partner. Data presented are mean $\pm$ one standard error of the mean. No significant differences were detected ($p > 0.05$).

4.3.4. Ultrasonic calls before, during, and after social separation

In the social separation task, the number of 50 kHz calls emitted by rats depended on the task phase ($F_{2,172} = 54.656$, $p < 0.001$). Compared to pre-separation, more calls were emitted during separation ($p < 0.001$, $d = 1.16$) and reunion ($p < 0.001$, $d = 0.55$). In addition, more calls were emitted during separation compared to reunion ($p = 0.025$, $d = 0.55$). Comparison between call categories was also significant, with rats emitting more FM calls compared to constant calls ($F_{1,172} = 467.206$, $p < 0.001$). In all task phases rats emitted more FM calls compared to constant calls (task phase x call type: $F_{2,172} = 11.407$, $p < 0.001$: pre-separation: $p < 0.001$, $d = 0.60$; separation: $p < 0.001$, $d = 1.36$; reunion: $p < 0.001$, $d = 0.71$).

Total number of 50 kHz calls emitted by the rats differed with adolescent condition ($F_{2,172} = 7.109$, $p = 0.002$), but this was dependent on task phase ($F_{4,172} = 6.347$, $p < 0.001$) and call category ($F_{2,172} = 13.279$, $p < 0.001$). These separate interactions were overshadowed by a three way interaction between adolescent condition, task phase, and call category ($F_{4,172} = 4.034$, $p = 0.004$; Figure 4.6.). First, the adolescent social experience effects on FM calls were dependent on task phase. During pre-separation, US rats emitted more FM calls compared to FS ($p = 0.018$, $d = 0.92$) and C rats ($p = 0.014$, $d = 0.93$), but C and FS rats were no different ($p = 0.608$). There were no effects of adolescent condition on FM calls during separation (C vs. FS, $p = 0.456$; C vs. US, $p = 0.423$; FS vs. US, $p = 0.241$). During reunion, US rats emitted more FM calls than C rats ($p = 0.023$, $d = 0.90$) but not FS rats ($p = 0.302$) and no difference was found between C and FS ($p = 0.161$). Second, condition effects on constant calls were also dependent on task phase. During pre-separation, US rats emitted more constant calls than C ($p = 0.016$, $d = 0.81$) and FS rats ($p = 0.001$, $d = 0.98$) but no differences were found between C and FS rats ($p = 0.204$). No differences were found between adolescent conditions for constant calls during separation (C vs. FS, $p = 0.157$; C vs. US, $p = 0.287$; FS vs. US, $p = 0.062$) or reunion (C vs. FS, $p = 0.955$; C vs. US, $p = 0.333$; FS vs. US, $p = 0.333$).

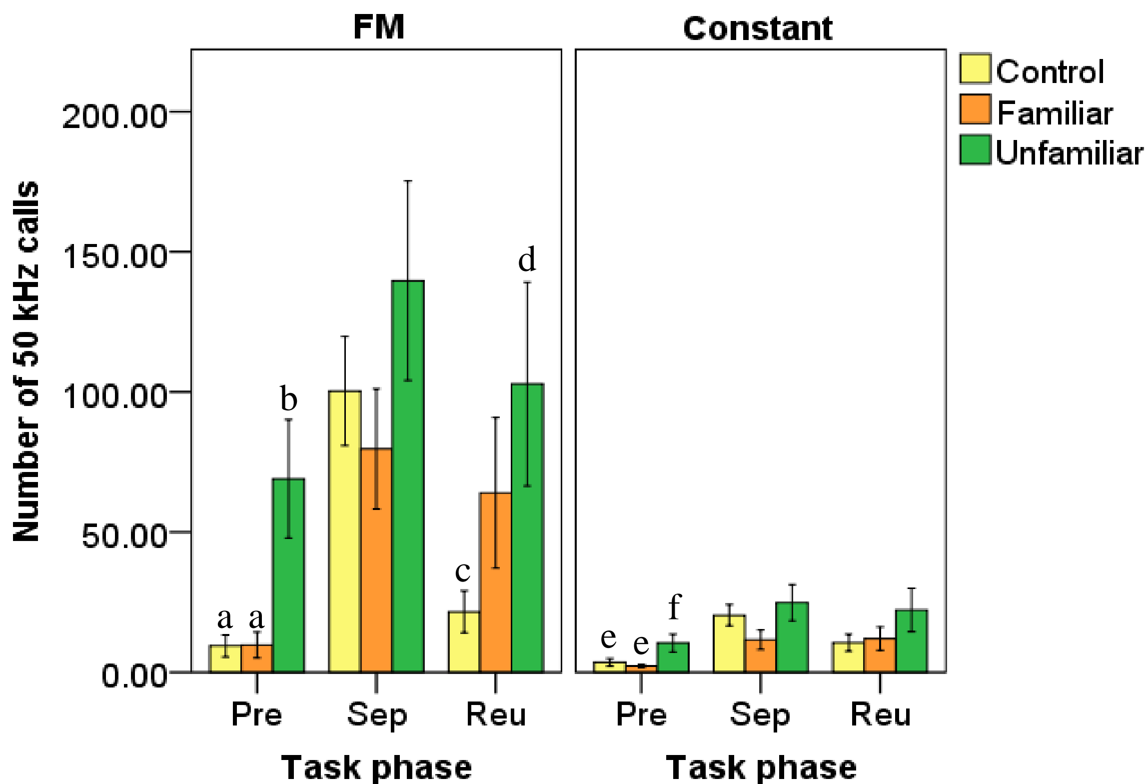

Figure 4.6. Number of 50 kHz calls emitted during social separation and reunion split by call categories, task phase, and adolescent condition. Data presented are mean ± one standard error of the mean. Significant differences ($p < 0.05$) between conditions within a task phase shown by a vs. b, c vs. d, e vs. f.

4.4. Discussion

The findings of the current study clearly show that brief unfamiliar social interactions during early adolescence have no effect on adult behavioural responses to unfamiliar stimuli or CORT secretion in domesticated female rats. Instead, unfamiliar adolescent interactions resulted in adult female rats that emitted more 50 kHz calls both before and after separation in adulthood. During adolescence, unfamiliar rats played and sniffed one another more than familiar rats. However, no difference were found in risk assessment or freezing behaviour. The hypothesis that unfamiliar social interactions during adolescence would result in adult animals that interact more with unfamiliar stimuli when in adulthood is therefore rejected. The results instead suggest that brief unfamiliar social interactions in female rats have immediate social effects and may modulate adult social behaviour through changes in auditory communication.

4.4.1. Adolescent interactions

During adolescent social interactions, unfamiliar rats were found to maintain closer proximity, engage in more play fighting, and engage in more investigative sniffing than familiar pairs of rats; effects that are corroborated by a plethora of previous work investigating how both male and female rats respond to unfamiliar conspecifics (e.g. Cirulli et al., 1996; McCormick et al., 2006; Thor & Holloway, 1984; Veenema et al., 2012). The current study focused on the behaviour in the test arena, but did not quantify the home cage behaviour after each treatment session. Unfamiliar interactions may have also resulted in rats playing more in the home cage thereby amplifying the effects of the unfamiliar interactions to permit any long-term effects, but this requires testing. In addition, only behaviour was documented during adolescence. Given that vasopressin is an endocrine factor that regulates play in rats (Smith et al., 2015; Veenema et al., 2012), it could be hypothesised that vasopressin secretion would differ between FS and US rats and could provide a mechanism through which adolescent social behaviour could affect adult phenotype. Future work is necessary to determine the relative contribution of each of these hypotheses on adult social behaviour. In addition, males typically play more than females during adolescence (Argue & McCarthy, 2015a; Pellis, 2002; Smith et al., 1998), play is more antagonistic in males than females (Pellis et al., 1997; Pellis, 2002), and males emit more 50 kHz calls during play compared to females (Himmler et al., 2014). If differences in play are the mechanism behind the long-term effects of unfamiliar social interactions then sex differences would be expected in the long-term effects of adolescent social interactions. Future work should therefore also investigate any potential sex differences in the short- and long-term effects of unfamiliar adolescent social interactions on behavioural responses to unfamiliar stimuli.

Prior research investigating non-social behaviour during adolescent play is limited, but those studies that report any effects are not consistent (Cirulli et al., 1996; Terranova et al.,

1999). In the current study, the familiarity of the interaction partner during adolescence had no effect on non-social activities (i.e. freezes or rears). However, the number of freezes became higher as sessions progressed. A sudden cessation of activity typically occurs when a rat is startled, possibly as a method of avoiding detection (Blanchard et al., 2011; Boissy, 1995). The unfamiliar test arena would have been expected to be a mild stressor, with the number of freezes declining across sessions as the rats habituate to the test arena due to repeated exposure (i.e. familiarisation) to the arena. However, the progressively higher number of freezes suggests rats may have become sensitised to the test arena over time. The test arena may therefore have not acted as a stressor. Freezing is a complete cessation of movement (Blanchard et al., 2011; Boissy, 1995), but the camera angle (birds-eye view) may not have been able to discern whether the rat had completely stopped moving. The rats may have therefore just stopped abruptly to investigate the ground, with more investigation occurring as the test sessions progressed.

4.4.2. Adult behavioural responses to unfamiliar stimuli

Unfamiliar adolescent social interactions do not appear to affect later-life responses to unfamiliar stimuli as no differences between conditions were detected on any behavioural measure in the EPM, LDB, or OSN tasks. Previous work has shown that unfamiliar re-housing during early adolescence in rats results in estrous females that may be less neophobic (e.g. spend more time on the open arms in the EPM) when tested individually compared to estrous females raised in stable housing (McCormick et al., 2008). Estrous cycle was not determined in the current study and effects dependent on estrous cycle stage may therefore have been masked by merging rats' behavioural responses across estrous cycle stages. Alternatively, as the short-term effects of the current study are purely social, unfamiliar adolescent social interactions may have only modulated adult behavioural responses to unfamiliar stimuli in a group context. Adolescent unfamiliar social interactions may have effected social behaviour that influences social buffering ability when in adulthood, but the effects may not have been

detected in the current chapter as rats were only tested in an individual context. Future work would benefit by investigating the effects of adolescent social interactions on behavioural responses to unfamiliar stimuli across social contexts.

The unfamiliar environments used during adult testing are standard tests used with small rodents. The EPM is one of the most common tests for quantifying behavioural responses to unfamiliarity (Carobrez & Bertoglio, 2005). Female rats typically spend a small proportion (20-40%) of test time on the open arms (Lynn & Brown, 2010; Marcondes, Miguel, Melo, & Spadari-Bratfisch, 2001; McCormick et al., 2008), but in the current study female rats spent most of the time on the open arms. Why the rats performed in such a manner is not obvious, but could be due to the testing environment. Rats spend more time on the open arm when light intensity is low (Garcia, Cardenas, & Morato, 2005), but rats have spent more time in the closed arms at light levels lower than the 40 lux used here (e.g. 25 lux: Lynn & Brown, 2010). Regardless of cause, the atypical performance of rats in the EPM questions the credibility of any inferences drawn from the task. However, the absence of findings in the EPM are corroborated by typical performance in the LDB and OSN tasks. Previous work with the LDB has indicated that female rats spend around 25% of test time in the light compartment (Brown et al., 2015; Ramos et al., 2002) and in the current study female rats spent a similar proportion of time in the light compartment. In the OSN task female rats spend around 25% of the test time in contact with the social stimulus and spend very little time with the object (Cavigelli et al., 2011), and both findings were found in the current study.

4.4.3. CORT response to social interactions

Across conditions, rats were no different from one another in urinary CORT concentration in response to a familiar or unfamiliar social interaction. Previous work has also shown that re-housing unfamiliar rats together during adolescence has no effect on adult plasma

CORT secretion in response to non-social stressors (McCormick et al., 2004; McCormick et al., 2005; Mathews et al., 2008; McCormick et al., 2008). Rats therefore provide no support for the hypothesis that CORT is an endocrine mechanism via which unfamiliar adolescent social interactions can modulate later-life behaviour (McCormick et al., 2015; Sachser et al., 2013). Although the effect was not significant, US rats appear to have secreted less CORT in response to handling, restraint, and re-housing with a familiar conspecific whereas controls secreted more CORT in response to the protocol. US rats' greater reduction in CORT concentration in a familiar social context may be a tentative indication that US rats are better social buffers than control rats. Little is known about the developmental antecedents of social buffering competency (Armario et al., 1983; Beery & Kaufer, 2015), but brief unfamiliar adolescent social interactions may have been in the process of shifting US rats toward improved social buffering. One reason the difference did not reach statistical significance may be that urinary CORT concentration was time-integrated and very variable. Plasma CORT is a more accurate, dynamic, and time-sensitive measure of stressor-induced CORT concentration (Koolhaas et al., 1997) and may have revealed an effect of condition at a specific time-point in the stress response. Future work would benefit by determining the plasma CORT response to social interactions and potential effects of social buffering to account for any inaccuracies in the urine sampling method.

4.4.4. Ultrasonic calls in response to social separation and reunion

Brief unfamiliar social interactions during adolescence had a long-term effect on communication, with US rats developing into adults that emitted more 50 kHz calls (FM and constant) compared to control rats. Adolescent social interactions can therefore influence adult rat communication as has been documented previously (Inagaki et al., 2005; Seffer et al., 2015), but the current study is the first to show that 50 kHz call rate can become higher in response to more adolescent interactions. Unfamiliar adolescent interactions may therefore be able to

modulate later-life social behaviour, given that ultrasonic communication is vital to functioning in a rat social organisation (Brudzynski, 2009; Burgdorf et al., 2008; Portfors, 2007). One function of 50 kHz calls (FM and constant) in male rats is to solicit social contact during social separation (Wöhr et al., 2008). The current study corroborates Wöhr et al. (2008), and further indicates that female rats may also solicit social contact through increased 50 kHz call rate during separation. In addition, unfamiliar adolescent social interactions may heighten the attempt to solicit social contact as US rats emitted more FM calls than C rats during reunion. Future work could investigate whether physiological responses to social separation, such as CORT secretion (Ferland & Schrader, 2011; Hennessey, 1997; Remage-Healey et al., 2003), are also modulated in the same direction. Call rate during reunion was also no different (i.e. intermediate) between FS rats and the other conditions, suggesting that handling and re-housing may have subtle effects on 50 kHz call rate during social reunion when in adulthood. Further research would therefore be useful to explore whether later-life call rates can be modulated by more or less handling and/or re-housing during adolescence. Future research may also wish to use larger sample sizes than those used here, as FM call rates during separation and reunion were noticeably variable. The variable nature of the separation and reunion FM call rate may have result in models with diminished statistical power, resulting in some effects not reaching statistical significance (e.g. comparison between FS and US rats FM calls during reunion). A larger sample size may also counter the complex nature of the model used to analyse the 50 kHz calls data, a model that included three fixed factors of which two were repeated measures. Replicating the current study with a larger sample size is now necessary to account for the statistical limitations outlined above and to corroborate the findings presented here.

50 kHz calls (both FM and constant) were already higher in US compared to control rats prior to separation, suggesting that the 50 kHz calls may have functions other than soliciting social contact upon separation. In the current study, US rats spent more time in

contact with the social box than the other conditions during the OSN task but the effect did not reach significance. A sufficient number of brief unfamiliar adolescent interactions may therefore influence gregariousness or mate attraction. Brief unfamiliar adolescent interactions with females could signal that the fore-coming adult social environment is populous and/or has a female-biased sex ratio. Adult US rats emitting more 50 kHz calls could reflect a more gregarious and/or reproductively competitive life history strategy. These hypotheses are yet to be investigated, but deprivation of adolescent social contact provides tentative support for both. Male rats single housed in adolescence develop into adults who lacked the typical approach to a playback of 50 kHz calls compared to pair housed rats (Seffer et al., 2015), suggesting rats have become less gregarious in response to fewer social interactions. Male rats singly housed during adolescence also develop into adults who emit fewer 50 kHz calls in response to an unfamiliar female compared to pair housed rats (Inagaki et al., 2013), possibly because the rats have become less reproductively-competitive in response to fewer interactions. Single housing is a stressor in rats (e.g. Lukkes, Mokin, Scholl, & Forster, 2009) and the effects of the social deprivation studies conflate social and stress effects. The specific effects of unfamiliar adolescent social interactions on later-life social behaviour requires direct investigation.

4.4.5. Summary

In summary, the findings from the current study show that female rats that engaged in more unfamiliar social interactions during adolescence developed into individuals that emitted more 50 kHz calls in adulthood. A higher number of playful and social investigatory behaviour between unfamiliar, compared to familiar, rats during adolescence is likely the behavioural mechanism behind the effect. The current study is the first to show that more unfamiliar adolescent interactions can result in a higher 50 kHz call rate. Further research is necessary to establish the context in which a higher 50 kHz call rate may be useful, such as attracting a mate or living in a larger group. In contrast, female rats that engaged in more unfamiliar social

interactions during adolescence were no different from control rats on measures of behavioural and endocrine responses to unfamiliar stimuli when in adulthood. The novel findings are at odds with similar work that has investigated the long-term effects of unfamiliar adolescent social interactions on adult behavioural responses to unfamiliar stimuli in rats (McCormick et al., 2015), plausibly because the unfamiliar interaction protocol used in the current study improved on previous studies designs by controlling for stress confounds.

Chapter 5: General Discussion

The current thesis aimed to investigate the long-term effects of adolescent stress, social housing, and social interactions on behavioural responses to unfamiliar stimuli (environments, objects, and conspecifics) when in adulthood and the physiological mechanisms that underpin these effects. Chapter 2 explored the long-term effects of mimicking adolescent stress via CORT dosing, whilst Chapters 3 and 4 investigated the long-term effects of adolescent social housing via manipulating group size and density (Chapter 3) and the quantity of unfamiliar interactions (Chapter 4). This discussion will outline and explain the key findings from the chapters, then address limitations in the current research and the direction for future research.

5.1. Hormonal sensitivity

Developmental exposure to steroid hormones has effects on later-life behaviour, but the later in development the hormone exposure occurs the lower the change in later-life behaviour (i.e. decline in hormone sensitivity: Schulz & Sisk, 2009; Schulz & Sisk, 2016). Previous work has found a decline in sensitivity to stressors from pre-pubertal to adolescent development in male rats (Tsoory & Richter-Levin, 2006), but the data presented in Chapter 2 are the first to show that sensitivity to CORT declines during adolescence, at least for some measures. For example, zebra finches dosed with CORT in early adolescence (E-CORT) developed into adults with lower hippocampal and hypothalamic GR compared to birds dosed with saline, but no effects were found in birds dosed with CORT in late adolescence. However, time perching alone and male courtship behaviour amongst unfamiliar conspecifics in adulthood was equally lowered by early and late adolescent CORT dosing compared to control birds. No clear explanation is available for why sensitivity to CORT declined for some measures and not others. In zebra finches, sensitive periods for both sexual imprinting and song learning end during adolescence; an effect that is attributed to a decline in neuronal spine density in regions

regulating these behaviour in response to relevant stimuli, e.g. opposite-sex conspecifics for sexual imprinting (Bischof & Rollenhagen, 1999; Bischof, 2007). The current thesis focused on glucocorticoid receptor expression, but further work could explore whether adolescent CORT exposure differently affects neuronal spine density in brain regions that regulate the HPA axis (e.g. hippocampus) and social behaviour (e.g. amygdala) during adolescence to explain how different sensitivities may occur across different measures.

5.2. Individual context behavioural responses to unfamiliar environments and objects

Animals that experience stress during adolescence are assumed to be more neophobic (i.e. interact less with unfamiliar stimuli) than control animals in adulthood (e.g. Hollis et al., 2013; McCormick & Green, 2013; Romeo, 2010). In Chapter 2, E-CORT birds took longer to enter an unfamiliar environment, spent longer perching in the unfamiliar environment, and, if female, engaged in more head turns compared to other adolescent conditions, supporting the hypothesis that CORT affects later-life responses to unfamiliar stimuli. E-CORT bird's behavioural responses to an unfamiliar environment are not consistent, and could be interpreted as more or less novelty-avoiding depending on the measure in question. A more parsimonious explanation may be that E-CORT birds navigate an unfamiliar environment differently compared to other adolescent conditions. Adolescent stress in male rats can result in poorer spatial learning when in adulthood compared to control rats that experienced no such stress (Isgor et al., 2004; McCormick et al., 2012). E-CORT birds may have slower spatial learning, with E-CORT birds needing more time (longer latency to enter unfamiliar environment), more exposure (more time perching in unfamiliar environment), and more environmental scanning (more head turns in females) to learn the layout of an unfamiliar environment compared to other conditions. Chapter 2 thus clearly revealed that early adolescent CORT exposure modulates later-life behavioural responses to unfamiliar environments, but more work is needed to explore why the effects occurred (e.g. responses to unfamiliar settings and/or spatial

learning) and why some effects were limited to females (i.e. head turns). The explanations may not be mutually exclusive, with greater neophobia possibly explaining the delayed entry latency and slower spatial learning perhaps explaining greater perching duration and head turn number. Spatial learning has been investigated in zebra finches by housing birds in an unfamiliar cage with access to empty and seed-filled opaque hoppers, then upon re-exposure a day later quantifying the bird's ability to find the seed-filled hoppers (Bailey, Wade, & Saldanha, 2009). Early adolescent nutritional stress (food restriction) has no long-term effect on such spatial learning in adult zebra finches (Kriengwatana, Farrell, Aitken, Garcia, & MacDougall-Shackleton, 2014). However, a future study could investigate whether mimicking stress by dosing early adolescent zebra finches with CORT leads to lower ability to find seed-filled hoppers in a spatial memory task when in adulthood compared to saline dosed control.

Adolescent social stressors, such as chronic variable social stress and social instability, can mimic the effects of non-social adolescent stressors and result in adult rats that interact less with unfamiliar stimuli than controls that did not experience social stress (Isgor et al., 2004; McCormick et al., 2015). Adolescent social experiences in the current thesis, such as variation in group size and density in zebra finches (Chapter 3) or exposure to unfamiliar conspecifics in rats (Chapter 4), had no effect on behavioural responses to unfamiliar environments and objects when in adulthood. In contrast to the current thesis, prior social stress research has exposed adolescent rats to social interactions that were structured to resemble stressors (i.e. unpredictable, uncontrollable, and potentially threatening) and then find long-term effects on responses to unfamiliar stimuli (Isgor et al., 2004; McCormick et al., 2015). Social interactions may therefore only affect later-life responses to unfamiliar objects and environments when structured to act as stressors. For example, in rats, repeated unfamiliar social interactions do not affect later-life behavioural responses to unfamiliar environments (Chapter 4) but when taking place in addition to repeated single housing and re-housing the interactions can result in

animals that interact less with unfamiliar stimuli when in adulthood compared to controls (e.g. McCormick et al., 2008). The absence of effects of adolescent social conditions on behavioural responses to unfamiliar stimuli in an individual context may reflect that the conditions were not sufficiently like stressors for long-term effects to emerge. However, it cannot be ruled out that the adolescent social conditions used here did sufficiently resemble stressors but were insufficiently intense for long-term effects to emerge. Repeating the experiments in the current thesis, but with larger and/or denser groups than those in Chapter 3 and more conspecifics than in Chapter 4, would be one way to test whether more intense versions of the social conditions manipulated in the current thesis can result in long-term effects on adult phenotypes.

The developmental age at which social conditions used in the current thesis were varied may also explain why the conditions had no long-term effects on adult behavioural responses to unfamiliar stimuli in an individual context. Adolescents engage in more antagonistic interactions in late adolescence compared to early adolescence (e.g. Delville et al., 2003; Terranova et al., 1993). Had the adolescent social conditions varied in the current thesis been varied later in adolescence then, due to higher rates of antagonistic interactions, the conditions may have acted as more intense stressors and long-term effects may have emerged. Late adolescent animals predominantly engage in antagonistic interactions with adult conspecifics (e.g. Sachser & Pröve, 1988; Templeton et al., 2012), as adolescents begin attempts to enter into social groups outside the natal home that contain likely more dominant unfamiliar adult conspecifics (Spear, 2000). Presence of more dominant conspecifics and/or a higher adult to adolescent ratio during adolescence may act as stressors, resulting in animals that are less likely to interact with unfamiliar stimuli in adulthood. Subjugation by more dominant conspecifics during adolescence can result in more neophobic behaviour when in adulthood in rats (Ver Hoeve et al., 2013; Vidal et al., 2011), but a higher adult to adolescent ratio in adolescent zebra finches had no effect on behavioural responses to unfamiliar conspecifics when in adulthood

(Bölting & von Engelhardt, 2017). Having more interactions with dominant conspecifics in adolescence, rather than having more interactions with older conspecifics, may result in animals that interact less with unfamiliar stimuli when in adulthood. However, research still needs to explore whether a higher adult to adolescent ratio results in less interaction with unfamiliar non-social stimuli (e.g. objects) in zebra finches and whether age ratio during adolescence has any long-term effects in different species. As zebra finches are particularly affiliative (Zann, 1996), absence of effects of adolescent age ratio in the species may be due to low levels of antagonistic interactions between adolescents and adults; but no study has yet quantified such interactions. Further research could therefore explore the effects of adolescent age ratio in more antagonistic species (e.g. rats) to explore whether such a social condition has long-term effects on adult behavioural responses to unfamiliar stimuli that are dependent on species-typical social behaviour.

5.3. Group context behavioural responses to unfamiliar environments and objects

Social facilitation refers to the higher expression of a given behaviour when in the presence of conspecifics compared to when alone, that is often attributed to either a social buffering or social competition effect (Clayton, 1978; Nicol, 1995; Webster & Ward, 2011; Zajonc, 1965). Adolescents learn to utilise conspecifics outside of the natal home as social buffers (Gunnar & Hostinar, 2015). In the current thesis, housing female (but not male) birds in larger groups at lower density during early adolescence resulted in adult birds that interacted more with unfamiliar stimuli when with familiar conspecifics compared to other housing conditions (Chapter 3) whereas exposure to CORT during adolescence had no effect on responses to an unfamiliar environment when in a group context (Chapter 2). Adolescent social interactions, not CORT exposure, may therefore affect later-life social buffering ability. In rodents, physical contact (huddling) occurs after stressor exposure (e.g. Bowen et al., 2012) and social buffering is dependent on physical contact with conspecifics (Nakayasu & Kato,

2008). In Chapter 2, adolescent dosing had no effect on group context responses to an unfamiliar environment and, as would expected, no differences were found on measures related to social proximity (e.g. time spent alone). However, female birds raised in larger groups at lower density spent more time interacting with unfamiliar stimuli than other housing conditions despite spending more time alone during testing than the other conditions (Chapter 3). Rodents and birds may differ in the behavioural basis of social buffering, with only rodents needing social contact for buffering to occur. However, exposure to a mild stressor like unfamiliar stimuli may not be an appropriate stimulus to measure social buffering effects. Further work is necessary to explore how adolescent experiences affect social buffering of adult fear-related responses to more threatening stimuli than unfamiliar stimuli. For example, adult rats can act as social buffers to one another in response to predation cues (Bowen et al., 2012) and foot shocks (Kiyokawa et al., 2014). No study has yet investigated whether adolescent experiences effect adult social buffering in response to similarly threatening stimuli, but future research could test whether female zebra finches raised in larger groups at lower density in early adolescence can buffer fear-related responses to exposure to predation cues when in adulthood.

In adulthood, birds more readily contact food dishes and consume more food when in groups compared to when alone regardless of whether feeders used were familiar (Dally, Clayton, & Emery, 2008) or unfamiliar to the birds (Coleman & Mellgren, 1994; Soma & Hassegawa, 2004). Foraging competition, rather than social buffering of responses to unfamiliarity, may therefore better explain the group context behavioural effects in Chapters 2 and 3. Unfamiliar stimuli used in unfamiliar environment and unfamiliar object tasks were baited with a food source (spinach) to encourage birds to engage with the task. Birds' diets were supplemented with a limited quantity (once per week) of spinach outside the behavioural testing period. Birds may have interpreted the group context unfamiliar environment and object tasks as competition over a limited quantity of appetitive food. In Chapter 3, spending more

time alone and more time amongst the spinach-baited stimuli may reflect more competitive behaviour in female birds raised in larger groups at lower density during early adolescence compared to other housing conditions. Nutritional independence occurs around the start of adolescence in some altricial species (e.g. rats: Thiels et al., 1990; zebra finches: Zann, 1996). Animals therefore learn foraging behaviour during adolescence, and further research could explore whether adolescent experiences can affect foraging behaviour. For example, one hypothesis that could be investigated is whether a larger adolescent group size at a sufficiently low density results in animals that forage in larger groups when in adulthood compared to animals raised in smaller groups, an effect that in turn could result in more foraging competition. Why female, but not male, zebra finches behaviour was affected also remains to be determined by including both sexes in future studies. For example, females reared in larger groups at sufficiently low density may be more competitive in a foraging context than males reared in similar conditions, but the hypothesis requires testing.

5.4. Behavioural responses to unfamiliar conspecifics

Adolescent experiences can have long-term effects on social behaviour related to social integration and the formation of social networks (Ruploh et al., 2014; Veenit et al., 2013). In the current thesis, adolescent CORT exposure (Chapter 2) and early adolescent housing in larger groups at lower density (Chapter 3) resulted in zebra finches that spent less time alone with unfamiliar conspecifics when in adulthood. Spending less time alone when housed with unfamiliar conspecifics could result in birds that are better able to integrate into an unfamiliar group (Ruploh et al., 2014). Previous work has already found that developmental CORT exposure in nestling and fledgling zebra finches resulted in adolescent birds with more central positions in foraging networks compared to control birds that were not CORT dosed (Boogert et al., 2014). However, social proximity networks may be better indicators of social networks than foraging networks (Kendal et al., 2010). For example, perching networks may be more

indicative of social networks than foraging networks in birds (Boogert, Nightingale, Hoppitt, & Laland, 2014). Further work is now necessary to explore whether adolescent experiences, such as CORT dosing and group size/density, have long-term effects on social network position. One hypothesis that requires testing is whether zebra finches dosed with CORT (vs. saline) or raised in larger groups at lower density (vs. smaller groups and higher density groups) in adolescence results in birds that are more socially integrated into adult flocks by occupying more central positions in adult perching networks.

Group living in many species provides access to potential mates (Evans, Votier, & Dall, 2012; Silk, 2007). Male zebra finches engaged in fewer courtship behaviour with unfamiliar female conspecifics when housed with an unfamiliar mixed-sex group in adulthood if dosed with CORT in adolescence compared to saline dosed controls (Chapter 2) or if the housed in larger groups in early adolescence compared to smaller groups (Chapter 3). Engaging in fewer courtship behaviour may indicate impaired socio-sexual behaviour (e.g. Almeida et al., 2000; Toth et al., 2008) or lower attractiveness (e.g. Spencer et al., 2003; Spencer et al., 2005). However, effects of adolescent experiences on socio-sexual behaviour are typically quantified in short interactions between small groups of unfamiliar conspecifics (e.g. Chapters 2 and 3; Ruploh et al., 2014; Toth et al., 2008). The testing context is not ecologically realistic for many species, like colonially breeding zebra finches (Griffith & Buchanan, 2010; Zann, 1996), and does not indicate reproductive success between familiar breeding pairs. Few studies have investigated the effects of developmental stress on reproductive success in a group breeding context (Crino & Breuner, 2015). One study has found that male zebra finches dosed with CORT during nestling and fledgling development raised more chicks to fledging when in a group context compared to vehicle dosed birds (Crino, Prather, Driscoll, Good, & Breuner, 2014). However, following the same dosing protocol, reproductive success is no different between CORT vs. saline dosed zebra finches that are forced and housed together in individual

breeding cages when in adulthood (Monaghan et al., 2012). Developmental CORT exposure may therefore be able to improve reproductive success in an ecologically relevant context.

The differences in reproductive success between CORT dosed and control birds when in a group context may be due to differences in socio-sexual interactions with non-pair bonded birds. In Crino et al. (2014), control males reared more non-genetic (but a similar number of genetic) offspring than CORT dosed males. CORT dosed males also interact with a greater number of unfamiliar conspecifics in later-life relative to control males (Boogert et al., 2014); an effect that may result in CORT dosed males interacting with more non-pair bonded females in adulthood. A greater number of interactions with non-pair bonded females may result in more reproductive encounters and higher reproductive success. However, more detailed analyses are needed of how developmental CORT exposure affects later-life socio-sexual interactions to explore this suggestion. Socio-sexual interactions (e.g. perching proximity, sharing nest box) could be quantified using social network analyses to explore the formation and maintenance of socio-sexual networks that may mediate the effects of adolescent conditions on reproductive success. Previous work investigating how adolescent experiences affect socio-sexual behaviour have used males (e.g. Almeida et al., 2000; Toth et al., 2008), but mixed-sex flocks could be used to explore male and female effects within the same context.

5.5. Stress physiology

The current thesis found that a larger early adolescent group size in female zebra finches resulted in a higher restraint stressor-induced CORT concentration compared to female birds raised in smaller groups (Chapter 3). However, adolescent CORT dosing (Chapter 2) or unfamiliar social interaction quantity (Chapter 4) had no effect on later-life measures of CORT. Glucocorticoid hormones (GCs) are secreted into the general circulation as part of an acute stress response and bind to glucocorticoid receptors to have effects (Sapolsky et al., 2000).

Plasma glucocorticoids are bound to binding globulins, such as corticosterone binding globulin (CBG), with only unbound GCs able to bind to glucocorticoid receptors (Breuner, Delehanty, & Boonstra, 2013; Malisch & Breuner, 2010). CBG bound GCs can be biologically active in some instances, with CBG/CORT complexes acting on membrane binding sites to facilitate intracellular translocation of CORT, e.g. at sites of inflammation (Breuner & Orchinik, 2002; Hammond, 1995). However, the effects of GCs are primarily mediated by the unbound GCs binding to glucocorticoid receptors (Breuner & Orchinik, 2002). Only total CORT (bound and unbound combined) was quantified in the current thesis and any inferences drawn may be misleading without accounting for what proportion of CORT is unbound. Further work is now necessary to explore the effects of adolescent experiences on CBG and unbound CORT concentrations. For example, a larger early adolescent group size in female zebra finches would be expected to result in higher unbound CORT concentration in response to a stressor compared to birds raised in smaller or denser groups.

An acute rise in glucocorticoids can aid in coping with a short-term stressor, as secreting more CORT in response to a stressor can elicit risk-avoidant behaviour (Haller et al., 1998) that may result in more successful avoidance of life-threatening stressors in harsh environments (e.g. high predation risk: Ferrari et al., 2015; Ferrari, McCormick, Allan, et al., 2015). However, glucocorticoids exposure can also have detrimental effects (McEwen, 1998; McEwen & Wingfield, 2003). For example, glucocorticoid exposure can result in exposure to oxidative stress (Constantini, Maraasco, & Møller, 2011) and greater disease susceptibility (Cavigelli et al., 2009) that would be expected to lower longevity (Cavigelli & McClintock, 2003; Cavigelli et al., 2009; Monaghan et al., 2012) and impair fitness (Bonier, Martin, Moore, & Wingfield, 2009; Breuner, Patterson, & Hahn, 2008). In Chapter 3, female zebra finches reared in larger groups during early adolescence secreted more CORT in response to a stressor compared to female birds reared in smaller groups. Secreting more CORT in response to a

stressor could impair fitness (more oxidative stress, shorter lifespan) or improve fitness (more threat-avoidance in harsh environments), but further work is needed to investigate these hypotheses and explore how adult context (e.g. high vs. low predation risk) may modulate the effects of adolescent experiences on fitness. As the group size effect on stressor-induced CORT concentration were female-specific, any future research investigating the fitness-related effects of adolescent group size needs to include both sexes to reveal any potentially sex-dependent effects.

Adolescent stressors result in lower hippocampal GR expression compared to control animals that experienced no such stress (Isgor et al., 2004), and comparable effects were found in Chapter 2, with birds exposed to CORT in early adolescence having lower hippocampal and hypothalamic GR expression compared to all other adolescent conditions. Adolescent stress, via greater exposure to CORT, may therefore lower negative feedback efficiency of the HPA axis. Glucocorticoid receptors are well documented regulators of HPA axis function (de Kloet et al., 1998; Oitzl et al., 2010), but how other stress-related mechanisms are affected by adolescent experiences have been little investigated. For example, corticotrophin releasing hormone (CRH) regulates behavioural responses to unfamiliar stimuli via CRH-1 and CRH-2 receptors (Bale & Vale, 2004). Mice exposed to adolescent stress have lower amygdala CRH-2 receptor expression and spend less time in exposed areas of an unfamiliar environment when in adulthood compared to control mice that had no such stress (Yohn & Blendy, 2017). CRH stimulates adrenocorticotrophic hormone (ACTH) secretion from the anterior pituitary gland that binds to melanocortin type 2 (MC2) receptors in the adrenal cortex to stimulate CORT secretion (Fridmanis, Roga, & Klovins, 2017; Gallo-Payet, 2016). Rats with a history of adolescent stress have lower ACTH secretion in adulthood compared to control rats (e.g. Goliszek et al., 1996), but no work has investigated whether adolescent stress affects adrenal sensitivity to ACTH via MC2 receptor expression. Stressor-induced ACTH secretion is co-

stimulated by vasopressin (via V1b receptors) and vasotocin (via VT2 receptors) in mammals and birds, respectively (Aguilera & Rabadan-Diehl, 2000; Cornett et al., 2013; Leung et al., 2011). However, the effects of adolescent stress on anterior pituitary V1b/VT2 receptor expression remains to be investigated. Future studies could examine whether adolescent CORT dosing affects CRH/CRH-R, ACTH/MC2R, and pituitary VP/VT function to provide a broader understanding of the mechanisms behind the long-term effects of adolescent stress. For example, two hypotheses that could now be addressed are whether adolescent stress results in higher stressor-induced CORT secretion in adulthood (Isgor et al., 2004; Pohl et al., 2007) due to higher adrenal gland MC2 receptor expression and/or higher pituitary gland V1b/VT2 expression compared to control animals.

5.6. Social physiology

Social behaviour patterns are regulated by both gonadal hormones (Eisenegger et al., 2011; Oliveira & Oliveira, 2014) and nonapeptides (Adkins-Regan, 2009; Kelly & Goodson, 2014; Goodson, 2005). Basal plasma concentrations of gonadal hormones rise during adolescence (Delemarre-van de Waal, 2002; Sisk & Foster, 2004), as do basal concentrations of nonapeptides (Miller et al., 1989; van Tol et al., 1988). In the current thesis, adolescent exposure to CORT (Chapter 2) or variation in early adolescent group size/density (Chapter 3) have no effect on adult basal concentrations of male testosterone and female estradiol. Whether adolescent conditions affected basal nonapeptide concentrations remains to be quantified. However, adolescent experiences may have long-term effects on the concentration of gonadal hormones and nonapeptides secreted in response to stressors rather than basal concentrations of the hormones. Acute stressors can stimulate testosterone and estradiol secretion (Ortiz et al., 1984; Romeo et al., 2004; Romeo, Lee, & McEwen, 2004; Yilmaz, 2003), as well as oxytocin and vasopressin secretion (Uvnäs-Moberg, 1998; Uvnäs-Moberg et al., 2015). In male rats, stressor exposure during adolescence results in a blunted stressor-induced rise in testosterone

when in adulthood compared to control rats (Bourke et al., 2013). As testosterone can inhibit CORT secretion (Gomez et al., 2004), a lower rise in testosterone concentration may result in a higher stressor-induced CORT concentration that has been reported to occur in response to adolescent stress (e.g. Pohl et al., 2007). One hypothesis that could be further investigated is whether adolescent stress results in higher secretion of stressor-induced CORT in adulthood due to effects on social hormones. For example, adolescent stress could result in a lower stressor-induced secretion of social hormones that inhibit the HPA axis, like testosterone (Gomez et al., 2004) and oxytocin/mesotocin (Windle et al., 1997), and/or higher stressor-induced secretion of social hormones that stimulate the HPA axis (e.g. estradiol, McCormick & Mathews, 2007: vasopressin/vasotocin: Cornett et al., 2013).

Plasma testosterone concentration rises during antagonistic interactions (Wingfield et al., 1990; Wingfield et al., 2005), and plasma oxytocin concentration rises during affiliative interactions (Uvnäs-Moberg, 1998; Uvnäs-Moberg et al., 2015). Testosterone secretion in response to antagonistic interactions emerges during adolescence in male guinea pigs (e.g. Lürzel et al., 2010; Sachser & Pröve, 1988), but emergence of an oxytocin response to affiliative interactions during adolescence has not been investigated. CORT does not appear to be the mechanism behind the effects of adolescent social experiences, as few similarities were present between the findings in Chapter 2 compared to Chapters 3 and 4. However, gonadal hormones and/or nonapeptides may be mechanisms behind long-term effects of adolescent social experiences. For example, similarly to the long-term effects of adolescent stress, male rats repeatedly injected with testosterone during adolescence developed into adults that engaged in more antagonistic interactions with an unfamiliar same-sex rat and spent less time in exposed areas of unfamiliar environments when in adulthood compared to male rats that were injected with vehicle (e.g. Olivares et al., 2014). Adolescent testosterone dosing studies typically administer supra-physiological doses to mimic anabolic androgen abuse

(Cunningham, Lumia, & McGinnis, 2013). Whether physiologically relevant testosterone doses affect later-life responses to unfamiliar stimuli remains to be investigated. Further work is needed to quantify gonadal hormone and/or nonapeptide concentrations in response to social interactions during adolescence that could be used to determine a physiologically relevant dose (like for CORT in Chapter 2). Dosing adolescent animals with gonadal hormones or nonapeptides, like for CORT in Chapter 2, could then reveal whether these hormones function as an endocrine basis of the long-term effects of adolescent social interactions on later-life responses to unfamiliar stimuli.

The behavioural results from the current thesis have indicated that the long-term effects of adolescent experiences depend on social context when in adulthood, but the extent to which the neural correlates of these effects are also context-dependent has only recently begun to be investigated (Ahern, Goodell, Adams, & Bland, 2016; Lukkes, Burke, Zelin, Hale, & Lowry, 2012; Wall, Fischer, & Bland, 2012). In one recent study, rats single housed during adolescence had fewer c-fos positive neurons (indicating lower neural activity) in the amygdala and lateral septum when exposed to an unfamiliar conspecific in later-life relative to rats that were group housed in adolescence (Ahern et al., 2016). No differences in c-fos expression between rats that were single housed and group housed during adolescence were found when the rats were placed in an asocial context (i.e. single housed: Ahern et al., 2016). The amygdala and lateral septum are regions of the SBN (Goodson, 2005; Newman, 1999), so the findings of Ahern et al. (2016) may indicate that context-dependent SBN activity may be a neural mechanism underpinning the context-dependent behaviour patterns that vary in response to adolescent experiences. Whether the context-dependent behavioural effects in this thesis can be attributed to context-dependent activity in the SBN now requires testing. Research could now begin to explore this hypothesis, but using a wider range of adolescent social conditions and adult social contexts. Ahern et al. (2016) conflated group size and density in the adolescent group housing

condition and only quantified c-fos expression in response to two social contexts (asocial and unfamiliar conspecific). One future study could raise adolescents in groups that vary in number and/or density, then quantify adult social behaviour and c-fos expression in the SBN in response to more varied social contexts (e.g. asocial, familiar conspecific, and unfamiliar conspecifics) and/or more varied characteristics than conspecific presence and familiarity (e.g. adolescent vs. adult, male vs. female, subordinate vs. dominant). This research could highlight that activity in the neural mechanisms underlying the effects of adolescent experiences on adult social behaviour is, like behaviour itself, flexible and dependent on adult social context.

5.7. Test vs. control comparisons

The current thesis explored the long-term effects of experiencing more glucocorticoid exposure in adolescence (Chapter 2), living with more conspecifics (Chapter 3), and more interactions with unfamiliar conspecifics (Chapter 4). To determine the effects of these experiences the control conditions included animals only exposed to stress (and presumably glucocorticoid exposure) during regular husbandry (Chapter 2) and animals that lived in pairs of familiar conspecifics (Chapters 3 and 4). These control comparisons may not be appropriate given the typical ecology of adolescent animals. Adolescents typically experience rises in glucocorticoid concentration as they encounter stressors outside of the natal home (e.g. Brown & Spencer, 2013; Wada, 2008), enter into social networks and live amongst age-similar conspecifics (e.g. Nelson et al., 2005; Nelson et al., 2016), and engage in interactions with unfamiliar conspecifics (e.g. Adkins-Regan & Leung, 2006; Pellis & Iwanuik, 2004). The test conditions may therefore be more realistic control conditions, and the control conditions perhaps better resemble test conditions by depriving adolescents of typical stress and social experiences.

The results from this thesis could be reinterpreted, with the test conditions assumed to be more realistic control conditions. For example, zebra finches that lived with no stress except

for regular husbandry during early adolescence developed into adults that may have been less neophobic than birds exposed to additional stress via CORT dosing (Chapter 2). Less exposure to glucocorticoids during adolescence may prevent animals from acquiring stressor avoidant behavioural responses, potentially a disadvantage given that animals typically live in ecological contexts that contain many stressors that will need to be avoided in order to survive (Boonstra, 2013). Zebra finches that lived in smaller groups in early adolescence engaged in more antagonistic and (if male) more courtship behaviour with unfamiliar conspecifics in adulthood compared to birds raised in larger groups (Chapter 3). Living in a smaller group during adolescence may therefore deprive animals of opportunities to learn how to live within a group, resulting in more behaviour that would likely impair social integration (as has been suggested to occur in guinea pigs, e.g. Sachser et al., 2011). Rats that were raised in familiar pairs throughout adolescence emitted fewer 50 kHz calls when in adulthood compared to rats that interacted with unfamiliar conspecifics (Chapter 4). Interacting with fewer conspecifics during adolescence may result in fewer opportunities for vocal learning (as has been suggested by adolescent single housing studies in rats, e.g. Seffer et al., 2015). The test conditions used in this thesis may therefore be more ecologically realistic controls, with the control conditions revealing effects due to deprivation from stress and social experiences that would typically experience by an animal.

The lab environment provides greater control over experimental variables than can be achieved in field studies, whilst field studies provide more ecological realism (e.g. Calisi & Bentley, 2009). The current thesis may indicate that simulating more ecologically realistic conditions during development may strengthen any inferences drawn by accounting for alternative explanations of the data (e.g. deprivation effects). One approach to gain more ecological realism whilst retaining control over independent variables would be to replicate the experiments in the current thesis in a semi-natural setting. For example, the effects of

adolescent glucocorticoid exposure could be investigated by dosing adolescent zebra finches with CORT whilst the birds are housed in outdoor aviaries to provide exposure to typical field stressors (e.g. weather) and living in flocks to provide typical social stress (e.g. social hierarchy formation). As all birds would have been exposed to stressors, any effects of CORT could not be due to comparison with a control condition that had been deprived of nearly all stress. Adolescent group size/density and number of unfamiliar conspecifics could also be experimentally manipulated in avian species whilst the birds live in flocks in aviaries. As all birds would have been living in groups, any effects could not be due comparing animals reared in larger/denser flocks or animals exposed to more unfamiliar conspecifics with control conditions that had been deprived of social interactions. Through the use of experiments in semi-natural settings more realistic experimental and control conditions can be created to provide more convincing inferences regarding the effects of adolescent experiences on adult phenotypes than those drawn from the data presented in the current thesis.

5.8. Comparative perspectives

Comparative psychology can provide a more holistic understanding of behaviour by comparing how behaviour are expressed across species that differ in certain species-typical characteristics, such as social organisations and mating systems (Adkins-Regan, 1990). The species studied in this thesis vary in terms of both species-typical social organisation and mating system. Rats are largely solitary animals, except for occasional bouts of antagonistic interactions to maintain a positon in a social hierarchy, and occasional reproductive encounters with different conspecifics as part of a promiscuous mating system (Calhoun, 1963). Contrastingly, zebra finches are group-living animals that typically engage in affiliative interactions with conspecifics and establish socially monogamous pair bonds (Zann, 1996). Comparisons between rats and zebra finches could therefore provide insights into whether species-typical

social structure and mating system mediate the long-term effects of adolescent stress and social experiences on adult phenotypes.

To a large extent the findings from the current thesis reveal similar effects occurring in response to adolescent stress and social experience between rats and zebra finches. For example, adolescent stress appears to impair spatial memory and/or result in more neophobic behaviour when individually exposed to an unfamiliar environment in rats (Hollis et al., 2013; Isgor et al., 2004) and zebra finches (Chapter 2). Adolescent social experiences also appear to have similar effects across species, as providing more opportunities to interact with age-similar conspecifics during adolescence may result in more affiliative adult behaviour in rats (Chapter 4) zebra finches (Chapter 3). Species-typical social organisation may, however, mediate the effects of adolescent stress on adult behavioural responses to unfamiliar conspecifics. In male rats, adolescent stress results in animals that, in adulthood, spend less time interacting with an unfamiliar conspecific (Tzanoulinou et al., 2014; Veenit et al., 2013) and engage in more antagonistic behaviour with an unfamiliar male (Marquez et al., 2013; Veenit et al., 2013) compared to control rats that experienced no such stress. Contrary effects were found in Chapter 2, as zebra finches dosed with CORT during adolescence spent less time alone when housed with unfamiliar conspecifics and, if dosed in late adolescence, engaged in less antagonistic behaviour compared to saline dosed birds. Adolescent CORT exposure may result in relatively solitary and/or antagonistic animals, like male rats (Calhoun, 1963), becoming more solitary and antagonistic, but relatively gregarious and/or affiliative animals, like zebra finches (Zann, 1996), may become more gregarious and affiliative. Future research could explore how adolescent stress affects later-life social behaviour in species varying along a solitary-gregarious continuum. For example, finch species vary from territorial pair-living Melba finches to more gregarious flock-living zebra finches (Goodson & Kingsbury, 2011; Goodson et al., 2012). A hypothesis that now requires testing is whether adolescent CORT

dosing results in more affiliative adult behaviour in zebra finches and more antagonistic adult behaviour in Melba finches compared to saline dosed control birds within each species.

Comparative psychology can also be used to provide insights into human behaviour, in particular to address research questions that may be difficult to explore using human subjects, such as the causes of mental illness and the underpinning physiological mechanisms (Palanza, 2001; Rodgers et al., 1997; Steimer, 2011). Adolescence in humans is the most common time of life for the diagnosis of anxiety-related disorders (Kessler et al., 2005; McEvoy et al., 2011), with such psychopathologies possibly caused by adolescent stress (Bakker et al., 2010; Sebastian et al., 2015). Adolescent rats and zebra finches have been used as model organisms in order to explore human development and psychopathologies (e.g. McCormick & Green, 2013; Mori & Wada, 2015). The findings from the current thesis may therefore provide insights into the effect of stress and social experiences on adolescent development and the emergence of anxiety-related disorders in humans. In the current thesis, zebra finches dosed with CORT in early adolescence may have engaged in more neophobic behaviour in response to an unfamiliar environment; an effect that may have been attributable to lower GR expression in the hypothalamus and hippocampus, regions that inhibit the HPA axis (Chapter 2). Greater neophobic behaviour is indicative of anxiety-related disorders in humans (Kagan & Snidman, 1999). Chapter 2 may therefore suggest that glucocorticoid exposure during adolescence is a contributing factor to the emergence of anxiety-related illnesses in humans, potentially due to a lowering of glucocorticoid receptor expression in brain regions that inhibit the HPA axis.

The findings from the current thesis also provide tentative support for methods that aid in the prevention or treatment of anxiety-related disorders. Social support can buffer responses to stressors in humans, and a greater availability of social support is associated with a lower incidence of anxiety-related disorders (Kawachi & Berkman, 2001; Smith & Christakis, 2008). In zebra finches, early adolescent CORT dosing had no effect on behavioural responses to an

unfamiliar environment in adulthood when the birds were housed with familiar conspecifics. Chapter 2 may therefore indicate that conspecifics in adulthood can potentially buffer the long-term effects of adolescent stress, further suggesting that social support in adulthood could be used to prevent or treat anxiety-related disorders. Social support within adolescence (i.e. greater number of friends) can also be used to lower the likelihood of diagnosis of an anxiety-related disorder (Frenkel et al., 2015; La Greca & Harrison, 2005). Friendships are established by individuals engaging in mutually reciprocated affiliative interactions (Hartup & Stevens, 1997). Adolescent social conditions that foster affiliative interactions between conspecifics may therefore result in individuals with greater access to social support, resulting in a lower likelihood of being diagnosed with an anxiety-related disorder (Frenkel et al., 2015; La Greca & Harrison, 2005). In the current thesis, a larger group size at a sufficiently low density in zebra finches (Chapter 3) and interacting with more unfamiliar conspecifics in rats (Chapter 4) appear to result in animals that engage in more affiliative behaviour in adulthood. In zebra finches, a larger adolescent group size at a sufficiently low density appeared to lower neophobic behaviour in response to an unfamiliar environment in adulthood when familiar conspecifics were present. The findings from Chapter 3 may therefore support the notion that affiliative adolescent interactions can result in adults that can better use social support and thereby buffer anxiety-like responses to stressors. One advantage of identifying this putative effect in a lab animal is that further investigations can now elucidate the underlying mechanism of the effect. Higher oxytocin concentration results in more affiliative behaviour and social buffering (Neumann, 2008; Witt et al., 1992), with mesotocin perhaps having a similar effect in birds (Goodson et al., 2009; Goodson et al., 2015). More affiliative interactions during adolescence may therefore result in adults with higher plasma oxytocin/mesotocin concentration and/or higher oxytocin/mesotocin receptor expression in the SBN compared to animals with fewer

affiliative interactions in adolescence; such predictions could not be adequately explored on human subjects due to the difficulty in collecting the necessary tissue.

5.9. Developmental perspectives

Developmental stages that occur prior to adulthood (i.e. foetal, early postnatal, and adolescent stages) are described as sensitive periods, and experiences that occur during these sensitive periods can have long-term effects on adult phenotypes (e.g. Kapoor et al., 2006; Levine, 2005; Romeo, 2010). The current thesis has explored the long-term effects of adolescent experiences, including adolescent exposure to glucocorticoids (Chapter 2) and different social conditions (Chapters 3 and 4), whilst leaving foetal and early postnatal development unmanipulated. This approach allowed for a direct exploration of adolescence as a sensitive period of development, but animals are unlikely to experience glucocorticoid exposure and different social conditions only during adolescence. During foetal development, for example, animals can be exposed to glucocorticoids from the mother via placental hormone transfer in mammals (Macri & Würbel, 2006) and depositing hormones into eggs in birds (Groothius & Taborsky, 2015). In early postnatal development, animals can also be exposed to different social conditions such as variation in parental care (e.g. Angelier & Chastel, 2009; Champagne et al., 2003; Levine, 2001). Animals are therefore likely to experience variations in glucocorticoid exposure and social conditions across multiple developmental stages, and such experiences likely interact in order to shape adult phenotypes (e.g. Gottlieb & Lickliter, 2004; Romeo & McEwen, 2006).

Adolescent stressor exposure can mediate the long-term effects of pre-adolescent stressor exposure (Ricon, Toth, Leshem, Braun, & Richter-Levin, 2012; Toth, Avital, Leshem, Richter-Levin, & Braun, 2008). Exposure to pre-adolescent maternal separation and adolescent chronic variable stress results in adult rats that potentially engage in more neophobic behaviour (i.e. more locomotor activity in an unfamiliar environment) than occurs in rats that experienced

either stressor independently (Toth, Avital, et al., 2008). However, exposure to pre-adolescent maternal separation and a milder adolescent chronic variable stress paradigm than that of Toth, Avital, et al. (2008) has been shown to result in animals that are no different from unmanipulated controls in terms of neophobic behaviour (Ricon et al., 2012). Pre-adolescent stress and higher intensity adolescent stressors may therefore have cumulative long-term effects, whilst the long-term effects of pre-adolescent stress and lower intensity stressors may negate one another. The mechanism explaining these different effects remains to be determined, but future work could explore the role of glucocorticoid exposure. An investigation could explore if glucocorticoid exposure prior to and during adolescence results in, a) more neophobic behaviour in adulthood than when the dosing periods occur independently due to a cumulative effect of developmental stress (Toth, Avital, et al., 2008), or b) neophobic behavior that is no different from non-dosed controls as glucocorticoid exposure during both developmental stages negate one another (Ricon et al., 2012). Physiologically-relevant glucocorticoid doses have been determined for pre-adolescent and adolescent zebra finches (Spencer & Verhulst, 2008; Chapter 2), so a future study could dose zebra finches with CORT during early postnatal development and/or during adolescence to explore how glucocorticoid exposure interacts across development stages in order to shape adult phenotypes. The findings could indicate whether adolescence is a period of vulnerability to the accumulating effects of glucocorticoid exposure (Toth, Avital, et al., 2008), or an opportunity to undo the potentially deleterious effects of pre-adolescent glucocorticoid exposure (Ricon et al., 2012).

Adolescent enrichment may attenuate the long-term effects of pre-adolescent stress, as rat pups exposed to maternal separation develop into adults that engage in more neophobic behaviour and have greater stressor-induced CORT concentration in later-life compared to rats not exposed to such separation, but no difference between the conditions is found if maternally separated rats are housed in enriched environments (e.g. larger cage size, access to toys, more

conspecifics) in adolescence (e.g. Berdaro, Fabio, & Pautassi, 2016; Francis, Diorio, Plotsky, & Meaney, 2002; Vivinetto, Suárez, & Rivarola, 2013). Interacting with more conspecifics during adolescence, one variable manipulated during environmental enrichment, itself may be able to lessen or negate the long-term effects of experiences prior to adolescence on adult stress-related phenotypes, but few investigations have directly explored such a suggestion (e.g. Gariépy, Rodriguiz, & Jones, 2002). The social conditions explored in the current thesis, namely larger and/or denser group housing (Chapter 2) and interacting with more unfamiliar conspecifics (Chapter 4), may lessen or negate the long-term effects of pre-adolescent stress on adult stress-related measures (e.g. neophobic behaviour, stressor-induced glucocorticoid secretion, glucocorticoid receptor expression). Zebra finches dosed with CORT during nestling and fledgling development are quicker to approach an unfamiliar object later in life (Spencer & Verhulst, 2008), but if raised in larger (vs. smaller) group size during adolescence the effects may not emerge. The results could demonstrate whether adolescence presents an opportunity to overturn the potentially deleterious long-term effects of pre-adolescent stress via appropriate social housing conditions, a suggestion that has received limited empirical exploration (e.g. Gariépy et al., 2002).

5.10. Conclusion

Adolescent exposure to stressors and social conditions can have long-term effects on later-life responses to potentially threatening stimuli and conspecifics (Brown & Spencer, 2013; Hollis et al., 2013; McCormick et al., 2015). The findings from the current thesis clearly show that CORT is a mechanism behind the long-term effects of adolescent stressor exposure (Chapter 2), but the long-term effects of adolescent group size/density (Chapters 3) and number of unfamiliar social interactions (Chapter 4) did not have similar effects to those reported in Chapter 2 or previous research exploring the long-term effects of adolescent stress. Adolescent social conditions in the current thesis may have therefore not been sufficiently intense stressors

for long-term effects to occur, or may have needed to occur in different periods of adolescent for long-term effects to occur (e.g. late adolescence). Alternatively to acting as stressors, adolescent social conditions may have instead affected social learning of a sex- and species-specific repertoire of social behaviour and thereby have long-term effects on later-life responses to social stimuli (Sachser et al., 2011). Future work should therefore take a more comparative approach that includes both male and female animals to explore how species-specific social organisation and sex mediate the long-term effects of adolescent stressors and social conditions. Through further exploration of endocrine mechanisms, such as gonadal hormones and nonapeptides, and a broader range of behavioural measures, such as socio-sexual and foraging behaviour, research can continue to elucidate the formative experiences in adolescence that affect phenotypic traits an individual may live with for the rest of their life.

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Appendix A: supplementary data analyses

The number of hops, number of head turns, and duration of time (second) spent perching alone prior to exposure to an unfamiliar environment were entered as dependent variables in separate LMMs. Any transformations required to achieve normality of the residuals (Shapiro-Wilk, $p > 0.05$) are stated below. Adolescent dosing condition and sex were entered as fixed factors (main effects and interaction). In Chapter 2 models, nest ID and brood size were entered as random factors in all models to control for pre-adolescent experiences. In Chapter 3 models, only nest ID was entered as a random factor as brood size was standardised. All models were conducted separately for each context (individual, group), with cage number entered as a random factor in group context models to account for group influence on individual behaviour. All comparisons were not significant and each section below provides a table of raw data split adolescent condition for each task and then two tables (one for familiar conditions model, one for housing conditions model) summarising the model output for each task.

Chapter 2: Behaviour prior to exposure to an unfamiliar environment

Prior to analysis, the number of hops in an individual context was square root transformed and the duration of time spent perching alone in a group context was log10 transformed. Raw data were used for all other variables. Raw data split by adolescent dosing condition are summarised in Table A.1. and the output from the models are summarised in Table A.2.

Table A.1. Behavioural measures taken prior to exposure to an unfamiliar environment in Chapter 2 split by adolescent dosing condition and social context of testing. Data presented are mean (standard error of the mean).

Measure	Adolescent dosing condition			
	E-SAL	E-CORT	L-SAL	L-CORT
Individual context				
Number of hops	39.07 (6.40)	48.56 (6.25)	50.44 (8.65)	53.69 (8.87)
Number of head turns	72.40 (6.17)	53.83 (6.32)	65.61 (7.74)	69.40 (7.89)
Group context				
Number of hops	45.21 (6.26)	47.06 (7.68)	52.67 (6.08)	48.88 (4.63)
Number of head turns	33.33 (4.84)	46.28 (6.66)	42.56 (3.22)	37.93 (4.52)
Time perching alone	159.27 (20.96)	153.83 (17.95)	169.33 (24.99)	153.56 (21.44)

Table A.2. Output from models analysing the effects of adolescent dosing condition in Chapter 2 on behavioural measures taken prior to exposure to an unfamiliar environment split by social context of testing. All comparisons were not significant ($p > 0.05$).

Dependent variable	Independent variable	Individual context			Group context		
		df	F	p	df	F	p
Number of hops	Sex	1,58	0.587	0.447	1,58	0.855	0.359
	Dosing condition	3,58	0.421	0.738	3,58	0.152	0.928
	Sex x dosing condition	3,58	0.661	0.579	3,58	1.605	0.198
Number of head turns	Sex	1,58	0.012	0.912	1,58	0.807	0.373
	Dosing condition	3,58	1.211	0.314	3,58	0.947	0.424
	Sex x dosing condition	3,58	0.251	0.860	3,58	0.343	0.794
Time perching alone	Sex	-	-	-	1,58	0.172	0.680
	Dosing condition	-	-	-	3,58	0.113	0.952
	Sex x dosing condition	-	-	-	3,58	0.116	0.950

Chapter 3: Behaviour prior to exposure to an unfamiliar environment

Prior to analysis, square root transformations were applied to the number of hops in a group context for the familiarity conditions model and number of hops in an individual context for the housing conditions model. A log10 transformation was applied to the number of turns in an individual context for the familiarity conditions model and the number of hops in a group context for the housing conditions model. Raw data split by adolescent housing condition are summarised in Table A.3. and the output from the models are summarised in Table A.4. (familiarity conditions model) and Table A.5. (housing conditions model).

Table A.3. Behavioural measures taken prior to exposure to an unfamiliar environment in Chapter 3 split by adolescent condition and social context of testing. Data presented are mean (standard error of the mean).

Measure	Adolescent condition			
	LN	LN/C	HN/LD	HN/HD
Individual context				
Number of hops	56.78 (7.67)	66.83 (10.89)	69.42 (10.16)	64.65 (10.49)
Number of head turns	36.17 (4.13)	45.39 (5.59)	38.26 (3.80)	35.50 (3.36)
Group context				
Number of hops	139.89 (29.70)	191.22 (35.96)	136.75 (18.61)	173.35 (28.23)
Number of head turns	33.28 (6.45)	45.00 (7.53)	50.50 (4.52)	55.55 (5.38)
Time perching alone	348.61 (34.83)	359.67 (30.32)	430.65 (55.14)	372.25 (52.14)

Table A.4. Output from models analysing the effects of adolescent familiarity condition in Chapter 3 on behavioural measures taken prior to exposure to an unfamiliar environment split by social context of testing. All comparisons were not significant (p > 0.05).

Dependent variable	Independent variable	Individual context			Group context		
		df	F	p	df	F	p
Number of hops	Sex	1,28.664	1.000	0.326	1,14	0.959	0.344
	Familiarity condition	1,27.468	1.420	0.243	1,14	0.792	0.388
	Sex x familiarity condition	1,30.051	0.049	0.826	1,14	0.019	0.892
Number of head turns	Sex	1,25.614	0.292	0.594	1,14.855	1.201	0.291
	Familiarity condition	1,27.233	2.779	0.107	1,15.559	0.488	0.495
	Sex x familiarity condition	1,27.353	2.689	0.112	1,14.354	0.323	0.579
Time perching alone	Sex	-	-	-	1,14	1.445	0.249
	Familiarity condition	-	-	-	1,14	0.088	0.771
	Sex x familiarity condition	-	-	-	1,14	0.623	0.443

Table A.5. Output from models analysing the effects of adolescent housing condition in Chapter 3 on behavioural measures taken prior to exposure to an unfamiliar environment split by social context of testing. All comparisons were not significant (p > 0.05).

Dependent variable	Independent variable	Individual context			Group context		
		df	F	p	df	F	p
Number of hops	Sex	1,50.870	0.045	0.832	1,20.586	1.845	0.189
	Housing condition	2,49.878	0.364	0.696	2,20.511	0.535	0.593
	Sex x housing condition	2,48.818	0.463	0.632	2,19.841	0.577	0.571
Number of head turns	Sex	1,50.301	0.792	0.378	1,52	0.844	0.363
	Housing condition	2,48.733	0.051	0.950	2,52	0.295	0.734
	Sex x housing condition	2,47.454	1.157	0.323	2,52	0.351	0.621
Time perching alone	Sex	-	-	-	1,23	0.039	0.845
	Housing condition	-	-	-	2,23	0.361	0.701
	Sex x housing condition	-	-	-	2,23	0.053	0.948

Chapter 3: Behaviour prior to exposure to an unfamiliar object

Number of hops in an individual context were each subject to a square root transformation prior to analysis in the familiarity conditions model. Number of hops in an individual context, number of hops in a group context, and number of head turns in an individual context were also square root transformed prior to analysis in the housing conditions model. Raw data was used when analysing all other variables. Raw data for each variable split by adolescent housing condition are summarised in Table A.6. and the output from the models are summarised in Table A.7. (familiarity conditions model) and Table A.8. (housing conditions model).

Table A.6. Behavioural measures taken prior to exposure to an unfamiliar object in Chapter 3 split by adolescent condition and social context of testing. Data presented are mean (standard error of the mean).

Measure	Adolescent condition			
	LN	LN/C	HN/LD	HN/HD
Individual context				
Number of hops	54.06 (6.42)	62.56 (7.59)	58.37 (6.97)	59.50 (6.93)
Number of head turns	39.89 (3.57)	40.28 (4.51)	42.58 (5.10)	46.20 (7.38)
Group context				
Number of hops	58.61 (4.82)	57.17 (5.57)	51.35 (5.52)	55.80 (4.98)
Number of head turns	34.94 (4.70)	30.78 (3.64)	29.90 (4.11)	29.90 (3.92)
Time perching alone	238.33 (16.65)	265.00 (15.71)	254.70 (12.63)	258.65 (11.37)

Table A.7. Output from models analysing the effects of adolescent familiarity condition in Chapter 3 on behavioural measures taken prior to exposure to an unfamiliar object split by social context of testing. All comparisons were not significant ($p > 0.05$).

Dependent variable	Independent variable	Individual context			Group context		
		df	F	p	df	F	p
Number of hops	Sex	1,31.611	0.910	0.347	1,60.773	0.141	0.709
	Familiarity condition	2,25.030	0.803	0.379	1,60.773	0.047	0.830
	Sex x familiarity condition	1,31.879	0.307	0.583	1,60.773	0.123	0.727
Number of head turns	Sex	1,31.396	1.851	0.183	1,32	0.181	0.674
	Familiarity condition	1,28.118	0.269	0.608	1,32	0.447	0.508
	Sex x familiarity condition	1,30.428	4.154	0.060	1,32	0.006	0.937
Time perching alone	Sex	-	-	-	1,14.803	0.037	0.85
	Familiarity condition	-	-	-	1,15.425	0.639	0.436
	Sex x familiarity condition	-	-	-	1,14.425	0.23	0.639

Table A.8. Output from models analysing the effects of adolescent housing condition in Chapter 3 on behavioural measures taken prior to exposure to an unfamiliar object split by social context of testing. All comparisons were not significant ($p > 0.05$).

Dependent variable	Independent variable	Individual context			Group context		
		df	F	p	df	F	p
Number of hops	Sex	1,48.898	0.167	0.684	1,52	0.487	0.488
	Housing condition	2,47.925	0.127	0.881	2,52	0.601	0.552
	Sex x housing condition	2,45.799	0.941	0.398	2,52	0.547	0.582
Number of head turns	Sex	1,51	0.035	0.853	1,52	0.250	0.619
	Housing condition	2,51	0.082	0.921	2,52	0.458	0.635
	Sex x housing condition	2,51	0.118	0.889	2,52	0.387	0.681
Time perching alone	Sex	-	-	-	1,23	0.017	0.897
	Housing condition	-	-	-	2,23	0.365	0.698
	Sex x housing condition	-	-	-	2,23	0.093	0.912

Appendix B: supplementary data tables

Chapter 2 behaviour-hormone correlations

Table B.1. Correlations between latency to enter the dosing box across the five adolescent trails with measures of corticosterone concentration taken in response to a capture and restraint stressor in adolescence. All correlations were not significant (p > 0.0025).

Dosing box entry latency for each trial	Basal CORT (ng/ml)		10 min CORT (ng/ml)		30 min CORT (ng/ml)		Peak CORT (ng/ml)	
	r	p	r	p	r	p	r	p
1	-0.220	0.076	0.097	0.440	0.079	0.529	0.113	0.367
2	-0.097	0.438	0.034	0.785	0.101	0.419	0.084	0.500
3	0.009	0.943	0.050	0.688	0.083	0.509	0.091	0.466
4	-0.096	0.442	0.074	0.554	-0.038	0.764	0.005	0.970
5	-0.143	0.251	0.128	0.307	-0.076	0.542	-0.056	0.568

Table B.2. Correlations between male corticosterone and testosterone concentrations with behavioural and neural variables in which a significant effect of group was found. All correlations were not significant correlations (Bonferroni corrected α = 0.0013).

Variable	Basal CORT (ng/ml)		10 min CORT (ng/ml)		30 min CORT (ng/ml)		Peak CORT (ng/ml)		Testosterone (ng/ml)	
	r	p	r	p	r	P	r	p	r	p
Individual context unfamiliar environment										
Latency to enter unfamiliar environment (min)	0.068	0.692	-0.113	0.511	-0.246	0.148	-0.212	0.214	-0.332	0.052
Time perching in unfamiliar environment (sec)	-0.082	0.633	-0.223	0.191	-0.184	0.283	-0.330	0.049	-0.004	0.984
Number of head turns	-0.085	0.622	-0.413	0.012	-0.177	0.301	-0.264	0.120	0.154	0.378
Unfamiliar conspecifics										
Time perching alone (sec)	0.011	0.949	0.063	0.714	0.076	0.661	0.087	0.612	0.221	0.203
Number of times test bird displaces unfamiliar bird	-0.159	0.353	0.155	0.504	-0.073	0.670	-0.018	0.915	0.139	0.427
Courtship factor score	0.057	0.740	0.309	0.067	0.174	0.309	0.273	0.107	0.042	0.811
Glucocorticoid receptor										
Hypothalamus	0.003	0.987	0.364	0.052	0.141	0.465	0.318	0.092	0.176	0.361
Hippocampus	0.040	0.824	0.183	0.308	0.045	0.804	0.168	0.350	-0.062	0.732

Table B.3. Correlations between female corticosterone and estradiol concentrations with behavioural variables in which a significant effect of group was found. All correlations were not significant correlations (Bonferroni corrected α = 0.0013).

Variable	Basal CORT (ng/ml)		10 min CORT (ng/ml)		30 min CORT (ng/ml)		Peak CORT (ng/ml)		Estradiol (pg/ml)	
	r	p	r	p	r	p	r	p	r	p
Individual context unfamiliar environment										
Latency to enter unfamiliar environment (min)	0.035	0.854	0.226	0.229	-0.297	0.111	-0.248	0.187	0.008	0.968
Time perching in unfamiliar environment (sec)	0.276	0.140	-0.327	0.078	-0.294	0.115	-0.331	0.074	-0.195	0.301
Number of head turns	0.145	0.446	0.169	0.371	0.008	0.966	0.115	0.544	0.253	0.178
Unfamiliar conspecifics										
Time perching alone (sec)	-0.227	0.228	0.014	0.943	-0.286	0.126	-0.239	0.203	0.256	0.172
Number of times test bird displaces unfamiliar bird	0.072	0.706	-0.162	0.392	0.016	0.935	0.071	0.708	0.083	0.661
Courtship factor score	0.264	0.159	0.065	0.732	0.096	0.613	0.130	0.493	0.296	0.112
Glucocorticoid receptor										
Hypothalamus	-0.127	0.573	-0.110	0.626	0.335	0.128	0.308	0.164	0.333	0.130
Hippocampus	-0.084	0.667	-0.115	0.552	-0.091	0.640	-0.089	0.646	0.232	0.227

Chapter 3 behaviour-hormone correlations

Table B.4. Correlations between corticosterone concentrations during adolescent capture and restraint and individual behaviour recorded during adolescent unfamiliar social interactions. All correlations were not significant (p > 0.003).

Variable	Basal CORT (ng/ml)		15min CORT (ng/ml)		45min CORT (ng/ml)		CORT 15-45min change (ng/ml)	
	r	p	r	p	r	p	r	p
Female								
Duration perching alone (sec)	0.053	0.759	0.188	0.273	-0.193	0.259	-0.092	0.595
Number of head turns	-0.074	0.784	-0.081	0.776	0.025	0.927	0.084	0.757
Male								
Duration perching alone (sec)	-0.041	0.802	-0.267	0.096	-0.038	0.817	-0.204	0.207
Number of head turns	0.085	0.722	-0.114	0.634	0.025	0.917	0.053	0.823

Table B.5. Correlations between male hormone variables (corticosterone concentrations during adult capture and restraint, basal estradiol concentration in adulthood) with female behaviour that were affected by adolescent condition in the group context unfamiliar environment and unfamiliar object tasks, as well as the unfamiliar conspecifics task. All correlations were not significant ($p > 0.0014$).

Variable	Basal CORT (ng/ml)		15min CORT (ng/ml)		45min CORT (ng/ml)		CORT 15-45min change (ng/ml)		Estradiol (pg/ml)	
	r	p	r	p	r	p	r	p	r	p
Group context unfamiliar environment										
Time perching in unfamiliar environment (min)	0.351	0.067	0.113	0.565	0.083	0.675	-0.084	0.669	0.248	0.204
Duration perching alone (sec)	0.134	0.496	0.223	0.255	0.374	0.050	0.209	0.287	-0.046	0.815
Group context unfamiliar environment										
Time perching on unfamiliar object (sec)	0.089	0.651	0.116	0.558	0.181	0.357	0.082	0.678	-0.017	0.932
Duration perching alone (sec)	0.085	0.668	0.008	0.967	0.396	0.037	0.311	0.107	-0.223	0.254
Unfamiliar conspecifics										
Duration on ground alone (sec)	0.283	0.145	0.047	0.814	-0.106	0.592	-0.134	0.496	0.133	0.498
Number of times test bird displaces unfamiliar bird	0.033	0.869	0.167	0.396	-0.155	0.431	-0.198	0.314	0.021	0.917

Table B.6. Correlations between female hormone variables (corticosterone concentrations during adult capture and restraint, basal estradiol concentration in adulthood) with female behaviour that were affected by adolescent condition in the group context unfamiliar environment and unfamiliar object tasks, as well as the unfamiliar conspecifics task. All correlations were not significant ($p > 0.0014$).

Variable	Basal CORT (ng/ml)		15min CORT (ng/ml)		45min CORT (ng/ml)		CORT 15-45min change (ng/ml)		Testosterone (ng/ml)	
	r	p	r	p	r	p	r	p	r	p
Group context unfamiliar environment										
Time perching in unfamiliar environment (min)	-0.166	0.381	-0.109	0.567	-0.013	0.944	0.022	0.907	0.236	0.079
Duration perching alone (sec)	-0.053	0.780	0.286	0.126	-0.254	0.176	-0.308	0.097	-0.012	0.952
Group context unfamiliar environment										
Time perching on unfamiliar object (sec)	-0.080	0.673	0.365	0.048	0.155	0.414	0.002	0.993	0.398	0.029
Duration perching alone (sec)	0.116	0.540	-0.212	0.260	-0.113	0.552	-0.028	0.883	0.211	0.263
Unfamiliar conspecifics										
Duration on ground alone (sec)	0.126	0.506	-0.024	0.901	0.179	0.343	0.076	0.688	0.158	0.405
Number of times test bird displaces unfamiliar bird	0.055	0.772	0.348	0.059	0.394	0.031	0.164	0.385	-0.234	0.214
Courtship factor score	0.296	0.112	0.229	0.224	-0.160	0.400	-0.333	0.072	0.111	0.558