

1 Social experience during adolescence in female rats increases 50 kHz ultrasonic vocalisations in
2 adulthood, without affecting anxiety-like behaviour

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4 **Running Head:** Adolescent social experience enhances female adult rat 50kHz call rate

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1 **Abstract**

2 Adolescents are highly motivated to engage in social interactions, and researchers have
3 hypothesised that positive social relationships during adolescence can have long-term, beneficial
4 effects on stress reactivity and mental wellbeing. Studies of laboratory rodents provide the
5 opportunity to investigate the relationship between early social experiences and later behavioural
6 and physiological responses to stressors. In this study, female Lister-hooded rats (N=12 per group)
7 were either i) provided with short, daily encounters (10 minutes per day) with a novel partner
8 during mid-adolescence (postnatal day 34-45; 'social experience', SE, subjects) or ii) underwent
9 the same protocol with a familiar cagemate during mid-adolescence ('control experience', CE,
10 subjects), or iii) were left undisturbed in the homecage (non-handled 'control', C, subjects). When
11 tested in adulthood, the groups did not differ in behavioural responses to novel environments
12 (elevated plus-maze, open field, light-dark box) or in behavioural and physiological (urinary
13 corticosterone) responses to novel social partners. However, SE females emitted significantly
14 more 50kHz ultrasonic vocalisations than control subjects both before and after social separation
15 from a familiar social partner, which is consistent with previous findings in male rats. Thus,
16 enhanced adolescent social experience appears to have long-term effects on vocal communication
17 and could potentially modulate adult social relationships.

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19 **Keywords:** adolescence, social novelty, neophobia, ultrasonic vocalisations, glucocorticoids

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1 **1. Introduction**

2 Adolescents exhibit a strong motivation to engage in social interactions with peers (Nelson *et al.*,
3 2016; Trezza *et al.*, 2011). Although some social interactions, such as peer rejection, can be a
4 major source of stress during this stage of life (Conley & Rudolf, 2009), positive social experiences
5 can act as a buffer against the deleterious effects of these stressors (Gunnar & Hostinar, 2015).
6 These positive social interactions could have long-term, as well as immediate, impacts on stress
7 reactivity and emotionality. For instance, strong adolescent friendship networks amongst human
8 beings have been linked with lower anxiety and depression symptoms in early adulthood (Narr *et*
9 *al.*, 2017). During adolescence, a number of brain areas undergo significant developmental change,
10 including regions involved in emotional and social processing, such as the amygdala and prefrontal
11 cortex (Tottenham & Galván, 2016). The hypothalamic-pituitary-adrenal (HPA) axis, a
12 neuroendocrine system that mediates responses to possible threats, also exhibits plasticity during
13 adolescence (Romeo *et al.*, 2016). Adolescent social contact may improve emotional wellbeing
14 via enhanced prefrontal cortex functioning (Bell *et al.*, 2010) and diminished HPA axis reactivity
15 (Terranova *et al.*, 1999), whilst adolescent social stress may impair wellbeing by modulating
16 amygdala development (Tsai *et al.*, 2014) and potentiating HPA axis activity (Isgor *et al.*, 2004).
17 Adolescent social interactions, via a range of neural and physiological processes, are therefore able
18 to have long-term effects on affective and social responses (Sachser *et al.*, 2013).

19 Research on laboratory rodents has investigated how social experiences during adolescence
20 influence later behavioural, neural and endocrine responses to stressors (McCormick *et al.*, 2015).
21 In many species, adolescents exhibit high levels of social play (e.g. male rats: Klein *et al.*, 2010;
22 mice: Terranova *et al.*, 1993). Social play is rewarding for adolescent rodents (Trezza *et al.*, 2011)
23 and enhances the development of adult social skills and emotional competencies (Himmler *et al.*,
24 2016; Pellis & Pellis, 2017). Depriving adolescent rodents of the opportunity to interact socially,
25 via isolation housing, has been shown to have long-term dampening effects on adult social

1 interactions and to increase anxiety-like behaviour and disrupt fear extinction (e.g., male rats:
2 Seffer *et al.*, 2015; Skelly *et al.*, 2015; van den Berg *et al.*, 1999; see Burke *et al.*, 2017a).
3 Adolescent social isolation has also been reported to have effects on later HPA axis functioning,
4 such as elevated circulating glucocorticoid concentration (e.g., rats: Lukkes *et al.*, 2009; Pisu *et*
5 *al.*, 2106; McCormick *et al.*, 2015). Diminished social contact during adolescence may result in
6 greater HPA axis activity; for instance, fewer opportunities for play has been linked to diminished
7 functioning of the medial prefrontal cortex (Bell *et al.*, 2010), an area that typically exerts an
8 inhibitory effect on the HPA axis (Herman *et al.*, 2003).

9 Additional evidence for the long-term effects of adolescent social experience is provided
10 by the literature on environmental enrichment, where group-housed animals are commonly
11 described as experiencing ‘social enrichment’ (Girbovan & Plamondon, 2013; Simpson & Kelly,
12 2011). This research has shown that rodents housed with three or more peers during adolescence
13 exhibit reduced anxiety-like behaviour and enhanced social behaviour in adulthood compared to
14 controls (e.g., rats: Brenes *et al.*, 2016; Harati *et al.*, 2013; Peña *et al.*, 2009). Social and physical
15 enrichment potentially acts as a mild stressor that produces an adult phenotype that is better able
16 to cope with subsequent novel situations (Crofton *et al.*, 2015), and this hypothesis is supported
17 by evidence that adolescent social enrichment reduces HPA activation when exploring a novel
18 environment in adulthood (e.g., male guinea pigs: Lürzel *et al.*, 2010; 2011; rats: Peña *et al.*, 2009).
19 Adolescent isolation housing and social enrichment therefore do not just shape social behavior,
20 but also appear to have substantial effects on anxiety-like responses and stress physiology in later
21 life.

22 Adolescent social instability has also been used to examine how adverse experiences
23 during early life can have long-term detrimental effects (McCormick *et al.*, 2015). A standard
24 social instability paradigm has been developed for adolescent rodents, which involves a
25 combination of repeated daily isolation (e.g., 1 hour) followed by pairing with a new social partner

1 each day (e.g., postnatal day, PND, 30-45; McCormick, 2010). Adolescent social instability has
2 been shown to have adverse outcomes, such as increased anxiety-like responses in novel
3 environments and reduced social interactions with unfamiliar peers in adulthood (e.g., mice:
4 Caruso *et al.*, 2017; Schmidt *et al.*, 2007; male rats: Green *et al.*, 2013; Hodges *et al.*, 2017; see
5 McCormick & Green, 2013; McCormick *et al.*, 2015). However, evidence for long-term effects of
6 adolescent social instability on HPA functioning has been inconsistent, with adult rodents exposed
7 to social instability during adolescence exhibiting blunted, similar or raised corticosterone levels
8 relative to control subjects (e.g., mice: Caruso *et al.*, 2017; Schmidt *et al.*, 2007; rats: Mathews *et*
9 *al.*, 2008; McCormick *et al.*, 2008; see Brown & Spencer, 2013).

10 The long-term effects of adolescent social experience are likely influenced by a range of
11 factors (Hodges *et al.*, 2018), including whether the subjects are male or female. Most studies of
12 adolescent social experience have used male rodents (Burke *et al.*, 2017a), even though, in human
13 beings, a female-biased prevalence in affective disorders emerges during this stage of life (Kessler
14 *et al.*, 2005). Adolescent social interactions can modulate development in female rats (Bell *et al.*,
15 2010; Schneider *et al.*, 2016), but the long-term effects of manipulating social experience during
16 adolescence on anxiety-like behaviour often differ between female and male rodents, depending
17 on the experimental paradigm; for instance, unlike males, female rats singly housed during
18 adolescence do not differ from controls in their anxiety-like behaviour in adulthood (e.g., Butler
19 *et al.*, 2014; Jahng *et al.*, 2012; Weintraub *et al.*, 2010) and adolescent social instability has a
20 stronger negative impact on females than males (e.g., Bourke & Neigh, 2011; Caruso *et al.*, 2018;
21 Weathington *et al.*, 2012). Sex differences in response to adolescent single housing and social
22 instability emphasise that adolescent social experiences are not always adverse, but may have
23 beneficial or negligible effects depending on whether male or female animals are tested.

24 Male rodents that have undergone social isolation or social instability during adolescence
25 exhibit reduced testosterone levels and impaired sexual behaviour in adulthood compared to

1 control subjects (e.g., McCormick *et al.*, 2013; Ward & Reed, 1985). In addition, male rats that
2 have experienced adolescent social instability are less preferred as a sexual partner (McCormick
3 *et al.*, 2017), spend less time interacting with novel same-sex conspecifics (Green *et al.*, 2013) and
4 exhibit more aggressive behaviour towards cage-mates during food competition (e.g., Cumming
5 *et al.*, 2014), compared to control subjects. These findings are consistent with the hypothesis that
6 lack of appropriate social experience during adolescence impairs later social competence. Vocal
7 communication is an important component of social interactions (Wöhr *et al.*, 2015). Male rodents,
8 for example, can emit 50 kHz ultrasonic vocalisations (USVs) in order to solicit social contact and
9 socio-sexual interactions (Brudzynski, 2005) and prevent playful interactions from escalating into
10 aggression (Burke *et al.*, 2017b). Adolescent rats also emit 50kHz vocalisations during play and
11 such calls can, in turn, facilitate further playful interactions (Burke *et al.*, 2018). Consistent with
12 the notion that more adolescent social experiences can enhance social competency, male rats that
13 are group housed during adolescence emit more 50kHz ultrasonic vocalisations (USVs) in
14 response to female partners and exhibit greater approach behaviour to playbacks of 50kHz USVs
15 when in adulthood compared to adult rats that were single housed in adolescence (Inagaki *et al.*,
16 2013; Seffer *et al.*, 2015; see Wöhr *et al.*, 2015). In comparison, relatively little is known about
17 the effects of adolescent social experience on later sexual and social competence, including vocal
18 communication, in female rodents (Burke *et al.*, 2017a).

19 The aim of this study was to examine the long-term effects of enhanced social experience
20 during adolescence on later anxiety-like behaviour and social behaviour in female rats. Our design
21 used short-term social encounters that involved pairing female rats with a novel same-sex partner
22 for 10 minutes once per day over a 12-day period (PND 34-45, i.e., mid-adolescence; Lynn &
23 Brown, 2009). Such short-term encounters have been widely used in adult rats to quantify social
24 exploration (File & Seth, 2003), and the adolescent age range in which rats in the current study
25 were manipulated are comparable to previous research investigating adolescent social instability

1 (e.g. McCormick *et al.*, 2010). In comparison to social instability tests in which rats are briefly
2 single housed then pair-housed with a new partner on each subsequent day (e.g. McCormick *et al.*,
3 2010), adolescent rats in the current study were not single housed and had a familiar home-cage
4 partner to return to each day. Short-term social encounters during adolescence may therefore be
5 expected to act as a form of social enrichment that potentially enhances social competencies and
6 diminishes anxiety-like behaviour when in adulthood, as occurs in response to other forms of
7 social enrichment (Girbovan & Plamondon, 2013; Simpson & Kelly, 2011). During adolescence
8 the amount of time spent interacting with a novel or familiar social partner was recorded, and, in
9 adulthood, we recorded behavioural responses to novel environments (elevated plus-maze and
10 light-dark box), social preference for a novel male conspecific, and USV production in response
11 to separation from a same-sex cage-mate. In addition, urinary corticosterone levels were measured
12 following social separation to examine the effects of adolescent social experience on HPA activity,
13 given that environmental enrichment during adolescence has previously been shown to dampen
14 the HPA axis (Morley-Fletcher *et al.*, 2003). Female rats experiencing enhanced social experience
15 were predicted to exhibit lower levels of anxiety-like behaviour, lower corticosterone production,
16 and higher levels of social engagement than controls when in adulthood.

17 **2. Methods**

18 *2.1. Ethical statement*

19 Ethical guidelines as set out in the Principles of Laboratory Animal Care (NIH, Publication No.
20 85–23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986 were
21 adhered to under Project Licence 60/4354 and Personal Licence IDFA58352.

22 *2.2. Animals and housing*

23 The subjects were 36 female Lister-hooded rats, bred in-house from nine litters (stock animals
24 from Envigo, UK). An additional 14 females and 6 males from these litters were used as social

1 interaction partners ('playmates'). All pups were reared by their mothers in plastic and wire-mesh
2 cages (52 x 40 x 26 cm, length x depth x height), then weaned on PND 26 into same-sex sibling
3 pairs, which were housed in plastic and wire-mesh cages (45cm x 28cm x 21cm cages, length x
4 depth x height). All animals had *ad libitum* access to pellet food (DBM Food Hygiene Supplies
5 Ltd, Scotland) and water, and the cages were located in a single holding room on a 12:12 light-
6 dark cycle (lights on at 07:00) with regulated temperature ($20\pm 1^\circ$) and humidity ($55\pm 5\%$). All
7 subjects were weighed once per week during development, including at the start and end of the
8 adolescent social manipulation, to assess whether all rats were growing at comparable rates.

9 2.3. Experimental design

10 i) Adolescent social experience

11 The three sets of female subjects (N=12 per condition, housed as 6 pairs) differed in the amount
12 of social experience and experimenter handling during adolescence, as follows: i) 'social
13 experience' (SE) subjects were provided with the opportunity to interact socially with a similar-
14 aged (± 1 day) female conspecific ('playmate') in an arena for 10 minutes per day for a 12-day
15 period during adolescence (PND 34-45) (i.e., each SE subject interacted with 12 novel partners in
16 total); ii) 'control experience' (CE) subjects were placed into an arena with their familiar cage-
17 mate for 10 minutes per day for the same 12-day period; and iii) non-handled 'control' (C) subjects
18 were left undisturbed in their home cages during this period. Rats from each litter were distributed
19 across the conditions, with no more than two individuals from a litter used in the same condition,
20 and 'playmates' were not from the same litter as the subject undergoing testing.

21 Two identical test arenas were used for the social interaction sessions. Each arena consisted
22 of an open-topped box constructed from perspex (48cm x 47cm x 44 cm, length x height x depth)
23 with black cardboard on the outside of the walls and sawdust on the floor. The arenas were located
24 adjacent to one another in a testing room and were surrounded by a black curtain. For the SE

1 condition, a pair of subjects and a pair of playmates were transported separately to the testing
2 room, and one of the subjects was placed into each of the arenas, followed by a playmate. For CE
3 subjects, a pair of subjects was transported to the testing room, and both animals were placed into
4 one arena. At the end of each session (i.e., after 10 minutes had elapsed), all animals were returned
5 to the home-cages, and the apparatus was cleaned with 70% alcohol solution.

6 During social interaction sessions, behaviour was observed via a ceiling-mounted camera
7 projecting to a computer monitor, and behavioural data were entered by an observer using an in-
8 house software programme. The arena was visually divided into four equal quadrants, and the
9 following behavioural measures were calculated: i) *duration of time both rats were in the same*
10 *quadrant* (seconds), ii) *number of play behaviour bouts* (nape attacks, boxing, pinning or evasion),
11 and iii) *number of investigative sniffs* (facial, flank and anogenital sniff) (definitions follow Cirulli
12 *et al.*, 1996 and Klein *et al.*, 2010). As the ceiling-mounted camera was located above only one
13 arena, both sets of animals could not be observed in the SE sessions; therefore, behavioural data
14 were collected for only one pair of animals (i.e. data were collected of six out of twelve sessions
15 for each SE subject), and the same number of sessions were therefore observed for CE pairs. C
16 pairs were left in their home cages during adolescence and their social behavior were therefore not
17 recorded.

18 *ii) Adulthood behavioural testing*

19 In adulthood, all subjects underwent three periods of testing. During the first period (PND 96-
20 111), the subjects were tested in: a) an elevated plus-maze, b) a light-dark box, and c) a social
21 novelty task (see '*Response to novelty*'). The order of testing was counter-balanced across
22 conditions, and each subject was tested on one task per week (i.e., on PND 96/97, PND 103/104
23 and PND 110-111). The second period (PND 122-132) involved assessment of urinary
24 corticosterone concentrations following a 3-hour period of housing in a novel home cage with an

1 unfamiliar female partner or familiar partner (i.e., cage-mate), with all subjects experiencing both
2 conditions on consecutive days and with the order counterbalanced across conditions (see '*CORT*
3 *response to social interactions*'). The third period of testing (PND 156-169) involved a social
4 separation task, in which ultrasonic vocalisations were recorded before, during and after separation
5 from a familiar partner (i.e., cagemate) (see '*Ultrasonic vocalisations before, during, and after*
6 *social separation*').

7 2.4. Apparatus and measures

8 i) Response to novelty

9 All subjects were tested individually. In all tests, the apparatus was surrounded by a black curtain
10 and cleaned with 70% alcohol solution after each animal, and behavioural data were recorded via
11 a ceiling-mounted camera.

12 a) The *elevated-plus maze (EPM)* consisted of a wooden plus-shaped maze that was raised 56cm
13 off the floor using a metal frame. The four wooden arms (51cm x 11cm, length x width) extended
14 out from a central area (11cm x 11 cm). Two of the arms had walls ('closed' arms; 40cm height),
15 and two did not have walls ('open' arms). At the start of the test, the subject was placed into the
16 central area, facing an open arm, and each test lasted 5 mins. The animal was recorded as entering
17 a new area when all four paws crossed onto a new arm or into the central area, and the following
18 scores were calculated: i) *total number of entries into closed and open arms*, and ii) *percentage of*
19 *time spent on the open and closed arms*.

20 b) The *light-dark box (LDB)* consisted of a perspex arena (119cm x 44cm x 47 cm; length x width
21 x height), which was divided into two compartments using an opaque plastic divider with an
22 opening at floor level (11cm diameter archway). The smaller, 'dark' compartment (49cm x 44cm
23 x 47cm cm; length x width x height) had a wooden lid and black card on the exterior walls, while
24 the larger, 'light' compartment (70cm x 44cm x 47cm; length x width x height) was lit from above

1 by an artificial white-light source (150lux) and had white cards on the exterior walls. At the start
2 of the test, the subject was placed into the dark compartment, and the test lasted for 5 mins. The
3 animal was recorded as entering a new area when all four paws crossed into another compartment,
4 and the following scores were calculated: (i) *latency to enter the light compartment (seconds)*, ii)
5 *number of entries into the light compartment*, and iii) *percentage of time spent in the light*
6 *compartment*.

7 c) The *social novelty (SN) task* was conducted in a perspex arena (119cm x 44cm x 47cm (length
8 x width x height) with black card on the exterior walls. Two perforated transparent perspex boxes
9 (24cm x 21cm x 46cm; length x width x height) were placed into opposite corners of the arena.
10 During testing, one box ('social box') contained an unfamiliar male rat (i.e., non-siblings), while
11 the other box ('object box') contained a novel object (five objects were used, e.g., a glass jar filled
12 with grey stones and a blue plastic bottle, which were counterbalanced across conditions). At the
13 start of the test, which lasted 5 mins, the subject was placed into the central area. Animals were
14 considered to be in contact with the stimulus box when touching the box with head, body, or paws,
15 and the following scores were calculated: i) *number of contacts with the social box and object box*,
16 and ii) *percentage of time in contact with the social box and object box (seconds)*.

17 ii) *CORT response to social interaction*

18 A pair of subjects was transported to a small testing room, and a urine sample was collected from
19 each subject by holding the animal over a clean, plastic cagebase (max. 10 mins). A minimum of
20 50 µl of urine was collected from the floor using a pipette (i.e. pre-interaction sample), then stored
21 at -20°C until assayed. The subjects were then moved to another testing room and pair-housed
22 with either a familiar (i.e. cagemate) or unfamiliar same-aged female rat (cages measured 39cm x
23 57cm x 26cm, length x width x height, and contained *ad libitum* pellets and water). After three
24 hours, the rats were moved back to the original testing room, and a second urine sample was

1 collected (i.e. post-interaction sample), and all animals were then returned to home cages. Three
2 hours is sufficient for determining changes in urinary CORT concentration (Bamberg *et al.*, 2001),
3 and is sufficiently long to induce a physiological stress response (Terranova *et al.*, 1999). All rats
4 underwent urine sampling under both social conditions (i.e. housed with familiar or unfamiliar
5 rats).

6 Creatinine concentrations in the urine samples were quantified using an enzyme
7 immunoassay kit (Creatinine (urinary) Colorimetric Assay, Cayman Chemical, USA). 5µl urine
8 samples were diluted to a concentration of 1:20, and samples were run in duplicate across two
9 plates, which were read on a Biochrom Anthos 2010 Microplate Reader (Biochrom Ltd., UK).
10 Intra-plate coefficients of variation were 9.74% and 11.52%, and inter-plate coefficient of
11 variation was 10.63%. CORT concentrations were quantified using radioimmunoassay, following
12 a previously described protocol (Spencer *et al.*, 2009). All samples were run in duplicate across
13 two assays. 50% binding (ng/ml) for each assay was 0.71 and 0.75. Intra-assay coefficients of
14 variation were 14.35% and 11.33%, and inter-assay coefficient of variation was 12.84%. In order
15 to scale CORT concentrations for urine concentration, urinary CORT concentration was expressed
16 as a ratio between CORT and creatinine concentrations, i.e. 10^{-5} CORT (ng/ml)/creatinine (mg/dl).

17 *iii) Ultrasonic vocalisations before, during, and after social separation*

18 The apparatus consisted of a perspex arena, which was divided into two compartments (each
19 measuring 32cm x 44cm x 47cm, length x width x height) using a perforated transparent barrier
20 that allowed visual, olfactory and vocal communication. Two ultrasound microphones
21 (CM16/CMPA, Avisoft Bioacoustics, Germany) were positioned above the arena, one on either
22 side of the barrier. The analogue microphone output was digitized using an Edirol FA101 sound
23 card (Roland Corp., Japan; 192kHz sampling rate in 24-bit format), which was operated using
24 open source software (Audacity, version 2.0.5), and stored as .wav files. Ultrasonic vocalisations

1 (USVs) were visualised as spectrograms in Audacity and categorised as 50kHz (frequency range
2 30-80kHz) or 22kHz USVs (frequency range 20-25kHz) (based on definitions in Wright *et al.*,
3 2010). Only 50 kHz calls are presented, as fewer than 10% of rats emitted 22kHz UVSs.

4 The social separation task consisted of three phases: pre-separation, separation, and
5 reunion. During pre-separation, a pair of rats that were from the same home cage were placed into
6 the arena, with one rat in each compartment, for five minutes, and USVs were recorded. One of
7 the subjects was then placed into a cage (39cm x 57cm x 26cm, length x width x height, with *ad*
8 *libitum* pellets and water) in the testing room, and the other subject was placed into a similar cage
9 in another testing room. The microphones were moved so that one was suspended above each
10 cage, and USVs were recorded for the first five minutes of a 60 minute separation period. The
11 subjects were then placed back into the original arena, with one animal per compartment with a
12 microphone, and USVs were recorded for a 5 minute period, after which the subjects were returned
13 to the home cage. To control for any potential effects of being moved to a different room, each
14 subject was tested twice (once remaining in the original testing room and once being moved to the
15 other room), with seven days between the tests, and test order was counter-balanced across
16 conditions.

17 2.6. Data analysis

18 All analyses were conducted using R (version 1.1.463). Adolescent social behaviour, body weight,
19 EPM and SN data were analysed using linear mixed models (LMMs), with litter size entered as a
20 random factor in all models and repeated measures used where appropriate (e.g., session number
21 in the adolescent social interaction data and arm type in the EPM data), using the ‘nlme’ package
22 (Pinheiro *et al.*, 2018). LDB data were analysed using a multivariate analysis of variance. Change
23 in urinary CORT/creatinine concentration (i.e. pre-interaction minus post-interaction
24 CORT/creatinine concentration) was calculated to determine the change in CORT in response to

1 familiar or unfamiliar rats, and analysed using a LMM with partner adolescent condition and litter
2 size entered as random factors. Pre-interaction 10^{-5} CORT (ng/ml)/creatinine (mg/dl) did not differ
3 with adolescent condition (general linear model: $F_{2,69} = 0.236$, $p = 0.791$) and was entered as a co-
4 variate in the model. Ultrasonic calls were analysed using a generalized linear model with data
5 fitted to a gamma error distribution using the ‘glm2’ package (Marschner, 2011). Prior to analyses,
6 residuals were checked for normality (Shapiro-Wilk) and variables that were positive skewed were
7 transformed (square-root or log10) to achieve normality or analysed using generalized linear
8 models. An alpha value of $p < 0.05$ was used throughout, with significant main effects and
9 interactions further explored using Bonferroni *post-hoc* tests. Cohen’s d was calculated as a
10 measure of effect size for all significant *post-hoc* pairwise comparisons. All data are presented as
11 means \pm one standard error. Data available on request from the authors.

12 **3. Results**

13 *3.1. Body weight*

14 The subjects gained weight between the start and end of the adolescent social manipulation (age:
15 $F_{1,33} = 9630.55$, $p < 0.001$, $d = 11.391$), and body weight did not differ on average between the
16 conditions (condition: $F_{2,33} = 3.165$, $p = 0.055$; age x condition: $F_{2,33} = 0.168$, $p = 0.846$; PND 32:
17 SE = 56.4 ± 1.81 g; CE = 60.8 ± 1.76 g; C = 55.1 ± 1.66 g; PND 45: SE: 150.2 ± 2.41 g; CE: 153.0 ± 3.02 g;
18 C = 147.0 ± 2.83 g).

19 *3.2. Adolescent social interactions*

20 The results confirmed that SE females spent a greater proportion of time in the same quadrant as
21 their social partner than did CE females ($F_{1,17} = 23.554$, $p < 0.001$, $d = 1.26$; SE = $54.80 \pm 0.91\%$;
22 CE = $44.70 \pm 1.40\%$), an effect that did not differ with test session (session: $F_{5,79} = 0.138$, $p = 0.714$;
23 session x condition: $F_{5,79} = 0.977$, $p = 0.326$). SE pairs also engaged in more play behaviour than
24 CE pairs ($F_{1,17} = 14.653$, $p < 0.001$; SE = 5.56 ± 0.53 play interactions per session; CE = 2.31 ± 0.37

1 play interactions per session), and this difference depended upon the test session ($F_{5,79} = 9.281$, p
2 < 0.001 ; **Figure 1**): the difference between conditions was only apparent in sessions 4, 5 and 6
3 (session 4: $p = 0.02$, $d = 1.154$; session 5: $p < 0.001$, $d = 2.144$; session 6: $p < 0.001$, $d = 1.888$),
4 and the main effect of session was also significant ($F_{5,79} = 6.685$, $p < 0.001$). SE pairs also engaged
5 in more sniffing behaviour than CE pairs ($F_{1,17} = 83.929$, $p < 0.001$, $d = 1.881$; $SE = 20.67 \pm 0.72$
6 sniffs per session; $CE = 10.11 \pm 0.73$ sniffs per session), which did not depend upon test session
7 (session: $F_{5,79} = 1.481$, $p = 0.205$; session x condition: $F_{5,79} = 1.503$, $p = 0.198$).

8 *3.3. Adult behavioural responses to unfamiliar environments*

9 *Elevated-plus maze.* Rats entered the open arms more than the closed arms ($F_{2,33} = 102.607$, $p <$
10 0.001 ; **Figure 2a**) and spent a greater proportion of time on the open arms than the closed arms
11 ($F_{1,33} = 67.925$, $p < 0.001$, $d = 2.542$; **Figure 2b**). However, number of arm entries and proportion
12 of time spent on each arm did not differ with adolescent condition (entries: $F_{2,33} = 2.064$, $p = 0.143$;
13 time: $F_{2,33} = 0.350$, $p = 0.707$), and the interaction between condition and arm type was also not
14 significant for either variable (entries: $F_{2,33} = 1.150$, $p = 0.329$; time: $F_{2,33} = 0.368$, $p = 0.695$).

15 *Light-dark box.* Regardless of adolescent condition, rats entered the light compartment after a
16 similar duration of time ($F_{2,29} = 0.102$, $p = 0.903$), entered the light compartment a similar number
17 of times ($F_{2,29} = 0.463$, $p = 0.634$), and spent comparable durations of time in the light compartment
18 ($F_{2,29} = 0.052$, $p = 0.949$) (**Table 1**).

19 *Social novelty.* Subjects were in contact with the social box more than the object box ($F_{1,32} =$
20 90.174 , $p < 0.001$; **Figure 3a**) and spent a greater proportion of time in contact with the social box
21 than the object box ($F_{1,32} = 151.581$, $p < 0.001$, $d = 3.041$; **Figure 3b**). Number of times, and
22 duration of time, in contact with a stimulus box did not differ with adolescent condition (contacts:
23 $F_{2,32} = 1.708$, $p = 0.197$; duration: $F_{2,32} = 3.139$, $p = 0.057$), and the interaction between condition

1 and stimulus box type was not significant for either variable (contacts: $F_{2,32} = 0.834$, $p = 0.444$;
2 duration: $F_{2,32} = 0.686$, $p = 0.511$).

3 *3.4. Corticosterone response to familiar and unfamiliar conspecifics*

4 Change in urinary CORT/creatinine ratio did not differ with adolescent condition or with partner
5 familiarity (condition: $F_{2,34} = 0.534$, $p = 0.470$; partner familiarity: $F_{1,34} = 0.426$, $p = 0.512$;
6 condition x partner familiarity: $F_{2,34} = 1.606$, $p = 0.214$; **Table 2**).

7 *3.5. USVs before, during, and after social separation*

8 The total number of 50kHz USVs emitted by subjects depended on task phase ($F_{2,91} = 49.795$, $p <$
9 0.001 ; pre-separation v separation: $p < 0.001$; pre-separation v reunion: $p < 0.001$; separation v
10 reunion: $p = 0.002$) and adolescent condition ($F_{2,91} = 4.417$, $p = 0.015$), and the interaction between
11 task phase and condition was also significant ($F_{4,91} = 4.181$, $p = 0.004$; **Figure 4**). Post-hoc
12 analyses revealed that, during pre-separation, SE subjects emitted more calls, on average, than
13 both groups of controls (C vs. SE: $p = 0.007$, $d = 0.986$; CE vs. SE: $p = 0.008$, $d = 1.02$; C vs. CE:
14 $p = 0.825$). The number of 50kHz USVs produced during separation did not differ between
15 conditions (C vs. CE, $p = 0.495$; C vs. SE, $p = 0.4296$; CE vs. SE, $p = 0.300$). During reunion, SE
16 subjects emitted more USVs, on average, than C subjects ($p = 0.013$, $d = 0.832$) but not CE subjects
17 ($p = 0.354$), and C and CE females emitted similar number of USVs ($p = 0.162$).

18 **4. Discussion**

19 In this study, female rats that experienced enhanced levels of social interaction during adolescence
20 did not differ from control females in their responses to novel environments or novel, opposite-
21 sex partners during adulthood. In addition, no differences were found between groups in urinary
22 corticosterone levels following social separation from a familiar, same-sex partner. These findings
23 suggest that, in contrast to adolescent social instability procedures that involve repeated rehousing
24 with novel conspecifics (McCormick *et al.*, 2015), short-term social encounters during

1 adolescence do not appear to have long-term effects on behavioural responses to novel
2 environments and opposite-sex partners, or urinary corticosterone levels, in female rats. However,
3 females that had enhanced social experience during adolescence produced more 50kHz USVs than
4 controls, both before social separation and following reunion with a familiar partner. 50kHz USVs
5 in rats have an important role in co-ordinating and enhancing affiliative and socio-sexual
6 interactions (e.g. Burke *et al.*, 2017c; Inagaki *et al.*, 2013). Our finding that adolescent social
7 stimulation modulates patterns of vocal communication in adulthood in female rats is consistent
8 with a previous study of male rats (Inagaki *et al.*, 2013) and potentially reflects long-term effects
9 on social competence.

10 In our design, SE subjects were placed into an arena with an unfamiliar female for 10 mins
11 per day for a 12-day period of mid-adolescence (PND 34-45), while CE subjects were placed into
12 the arena with their cagemate. Previous studies have reported that adolescent rats exhibit more
13 social play behaviour with novel than familiar partners (e.g., Cirulli *et al.*, 1996; Hodges &
14 McCormick, 2015), and, in line with these studies, SE subjects interacted with their social partners
15 at a higher frequency than did CE subjects during these sessions, both in terms of social play and
16 time spent in physical proximity. The difference in frequency of social play between SE and CE
17 subjects was only observed during the later sessions, corresponding to the second half of the
18 exposure days, which potentially reflects a habituation effect to the arena, although other studies
19 have reported that adolescent rats interact more with novel than familiar partners during one-off
20 sessions (Cirulli *et al.*, 1996; Hodges & McCormick, 2015; Reinhart *et al.*, 2006). Regardless of
21 potential habituation effects, these data confirm that the experimental design successfully resulted
22 in adolescent SE subjects interacting with novel partners.

23 In adulthood, SE subjects did not differ from control subjects in terms of response to novel
24 environments (i.e., EPM and LDB). These findings contrast with previous studies that report an
25 increase in adult anxiety-like behaviour in response to adolescent social instability that involves

1 single housing then pairing adolescent subjects with a new social partner each day (e.g., less time
2 spent on the open arms of the EPM: Caruso *et al.*, 2018; Schmidt *et al.*, 2007, but see McCormick
3 *et al.*, 2008). Our results suggest that enhanced social contact does not have significant long-term
4 effects on the willingness of female subjects to explore potentially aversive areas of novel
5 environments. Whether our experimental design would produce long-term effects in male rats
6 remains to be tested. One reason for the absence of effects could be that the social experiences
7 across all conditions provide sufficient social stimulation for the typical behavioural responses to
8 novelty to develop. Adolescent rats experience close contact with siblings (e.g. Thiels *et al.*, 1990)
9 and occasional interactions with unfamiliar rats when living in colonies (e.g. Calhoun, 1962).
10 Perhaps only social interactions sufficiently above or below these typical experiences, such as
11 social instability or single housing, modify adult behavioural responses to unfamiliar stimuli. A
12 sufficiently high number and duration of novel social interactions during adolescence are needed
13 to modulate adult novelty responses in guinea pigs (Lürzel *et al.*, 2010; 2011), and such effects
14 could also be present in rats.

15 Adult SE subjects also did not differ from adult control females in the amount of time spent
16 in contact with a transparent barrier that separated the subject from a male conspecific. Although
17 SE subjects spent the highest average amount of time in contact with this barrier, the difference
18 between groups of females was not significant. For all groups, the absolute amount of time spent
19 next to the barrier with the male was very similar to a previous study using the same apparatus in
20 which the position of the two sexes was reversed (Brown *et al.*, 2015), suggesting that female rats
21 are as motivated as males to investigate an opposite-sex conspecific in this task. Previous studies
22 have shown that adult female and male rats that have been group-housed during adolescence
23 engage in more social exploration with same- and opposite-sex rats than do rats that have been
24 pair-housed or single-housed during this stage of life (e.g., Brenes *et al.*, 2016; Molenda-Figueira
25 *et al.*, 2017; Peña *et al.*, 2009), while male rats exposed to adolescent social instability exhibit

1 impaired social interactions with same-sex and opposite-sex peers in adulthood (Green *et al.*, 2013;
2 McCormick *et al.*, 2013; Ward & Reed, 1985). In the current study, females were not provided
3 with the opportunity to interact directly with males and oestrous cycle stage was not recorded, so,
4 in the future, direct social interactions at specific stages of fertility could be examined to determine
5 whether our adolescent social manipulation enhances social exploration in later-life and under
6 which reproductive states.

7 The HPA axis is known to undergo considerable development during adolescence and is
8 sensitive to a range of both stressful and non-stressful events during this stage of life (Romeo *et al.*
9 *et al.*, 2016). Adolescent rodents that have experienced social isolation exhibit greater glucocorticoid
10 secretion than group-housed animals in response to stressors in adulthood (e.g., Lukkes *et al.*,
11 2009; Weintraub *et al.*, 2010), while adolescent social enrichment decreases later glucocorticoid
12 secretion (e.g., Peña *et al.*, 2009). In the current study, urine samples were collected from subjects
13 before and after being paired with an unfamiliar or familiar female for three hours. No differences
14 were found between SE, CE and C females in terms of either absolute corticosterone levels or the
15 change in corticosterone from baseline to post-housing. In addition, no overall effect of partner
16 familiarity was found, in contrast to studies of male rats reporting higher circulating corticosterone
17 levels in males that have been housed with an unfamiliar conspecific compared to those paired
18 with a familiar partner (Caruso *et al.*, 2014; Hodges *et al.*, 2014); these studies measured serum
19 and faecal corticosterone levels, which potentially reflected HPA functioning more accurately than
20 the urine sampling used in the current study.

21 In the final test, 50kHz USVs were recorded from subjects immediately prior to one hour
22 of separation from the cage mate, as well as during the first five minutes of the separation period
23 and after being reunited with the partner. SE females produced higher average numbers of 50kHz
24 USVs than CE and C subjects during the pre-separation period, as well as higher average numbers
25 of 50kHz USVs than C subjects during reunion. CE subjects exhibited intermediate levels of USVs

1 during reunion relative to the other two groups, which suggests that the handling experienced by
2 these control subjects potentially had subtle long-term effects, but this hypothesis would require
3 further investigation. For all subjects combined, the levels of 50kHz USV production were highest
4 during separation, in line with previous evidence that social separation elicits USV production in
5 adult rats (e.g., Wöhr *et al.*, 2008). Differences between the groups in pre-separation 50kHz call
6 rate could reflect different affective responses to the novel testing environment (Knutson *et al.*,
7 2002); however, no behavioural differences between groups were found in the novel environment
8 tasks. Instead, 50kHz USVs are likely reflect differences in sociality, as these calls have an
9 important communicative role in rats (Wöhr *et al.*, 2015). 50 kHz USVs are considered to reflect
10 social motivation (e.g., Börner *et al.*, 2016) and male rats that have been singly housed during
11 adolescence have been reported to display avoidance behaviour in response to playbacks of 50kHz
12 USVs (Seffer *et al.*, 2015). 50kHz USVs also have an important role in socio-sexual competency
13 (Brudzynski, 2005; but see Ågno & Snoeren, 2015), with male rats singly housed in adolescence
14 showing deficits in adult sexual interactions (Molenda-Figuera *et al.*, 2016) and also exhibiting
15 reduced rates of 50kHz USV production when exposed to female conspecifics in adulthood
16 (Inagaki *et al.*, 2013). The findings in the current study that SE rats emit more 50 kHz USVs than
17 controls therefore most plausibly indicate that, for female rats, a greater quantity of social
18 interactions during adolescence may enhance social motivation and/or sexual competency in later-
19 life via, or alongside, changes in vocal communication. However, further work is needed to
20 document the behavioural correlates of 50kHz USV production to test the hypothesis that
21 adolescent social stimulation in the current study enhances social and/or sexual competencies.

22 In summary, providing female rats with the opportunity to engage in social interactions,
23 including social play behaviour, with novel partners during adolescence did not have long-term
24 effects on later behavioural responses to novelty or glucocorticoid activity following social
25 separation. However, females that had gained this additional social experience during adolescence

1 emitted higher numbers of 50kHz USVs both prior to, and following, a period of social separation
2 compared to control groups. These findings suggest that enhanced adolescent social experience
3 affects later social interactions in female rats and is consistent with evidence that adolescent social
4 experience alters later vocal and social behaviour in male rats (e.g., Cumming *et al.*, 2014; Green
5 *et al.*, 2013; Inagaki *et al.*, 2013; Seffer *et al.*, 2015). Social interactions during adolescence have
6 been hypothesised to have long-term effects on social competence (Pellis & Pellis, 2007), defined
7 as the ability of an individual to respond appropriately to social cues (Taborsky & Oliveira, 2012),
8 and, in human beings, positive social interactions during adolescence potentially underpin healthy
9 emotional and social development (Gray, 2011).

10

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15 **Data Availability Statement**

16 The data that support the findings from this study are available from the corresponding author
17 upon reasonable request.

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1 **Legends**

2 **Figure 1.** Number of play behavior interactions occurring within SE and CE pairs during
3 adolescent social interaction sessions (* = $p < 0.05$).

4 **Figure 2.** Number of entries into the arms (a) and proportion of time spent on the arms (b) of the
5 EPM by C, CE and SE subjects (* = $p < 0.05$).

6 **Figure 3.** Number of contacts with the stimulus boxes (a) and proportion of time spent in contact
7 with the stimulus boxes (b) in the OSN (* = $p < 0.05$).

8 **Figure 4.** Total number of 50kHz USVs emitted in response to social separation and reunion
9 averaged (mean) across test sessions (Pre = pre-separation, Sep = separation, Reu = reunion; * =
10 $p < 0.05$).

11 **Table 1.** Behavioural measures in the LDB split by adolescent condition.

12 **Table 2.** Change in 10^{-5} CORT concentration (ng/ml) scaled for creatinine concentration (mg/dl)
13 in response to social interaction with a familiar or unfamiliar conspecific (post-interaction minus
14 pre-interaction).

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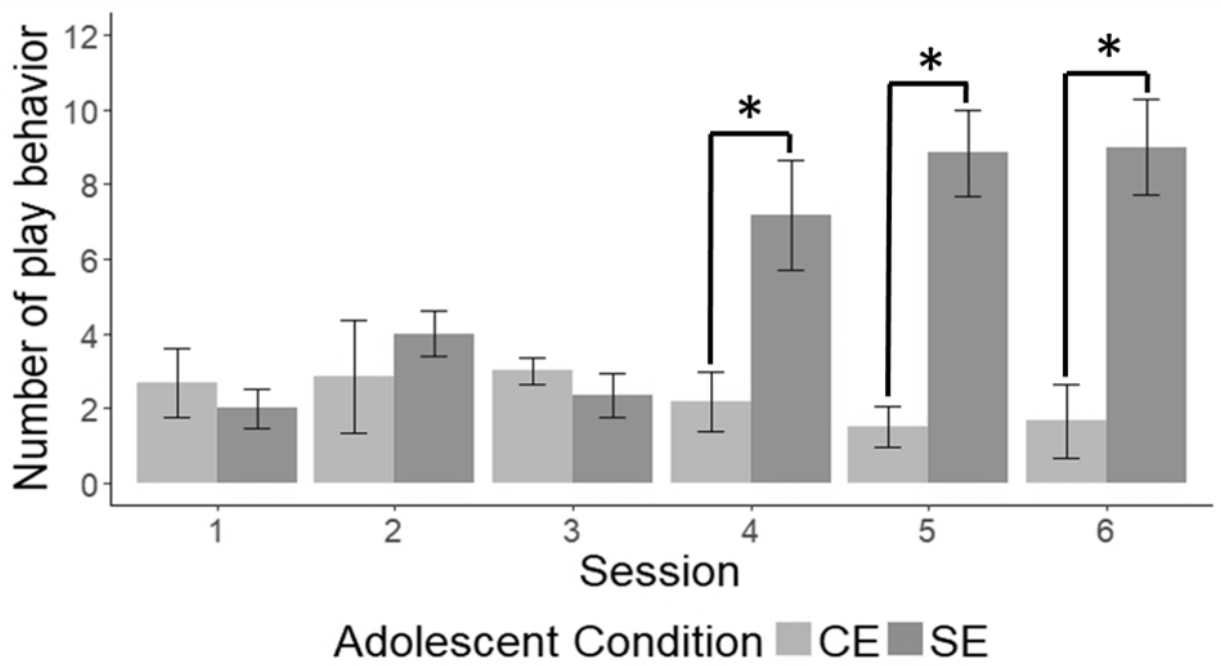
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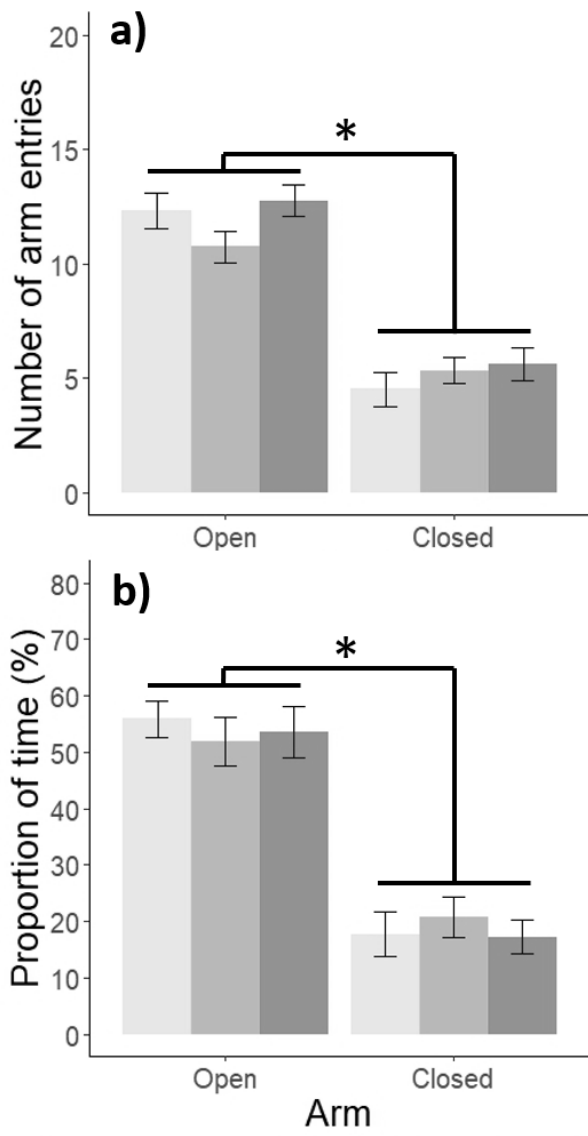
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1 **Figure 1**



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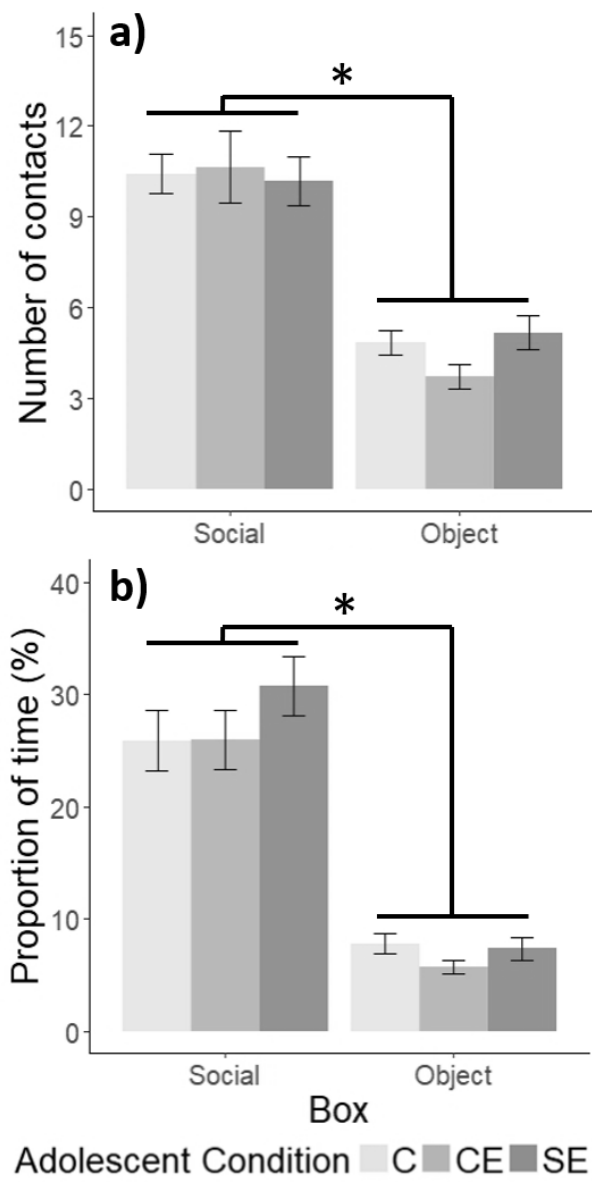
1 **Figure 2**



Adolescent Condition C CE SE

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1 **Figure 3**



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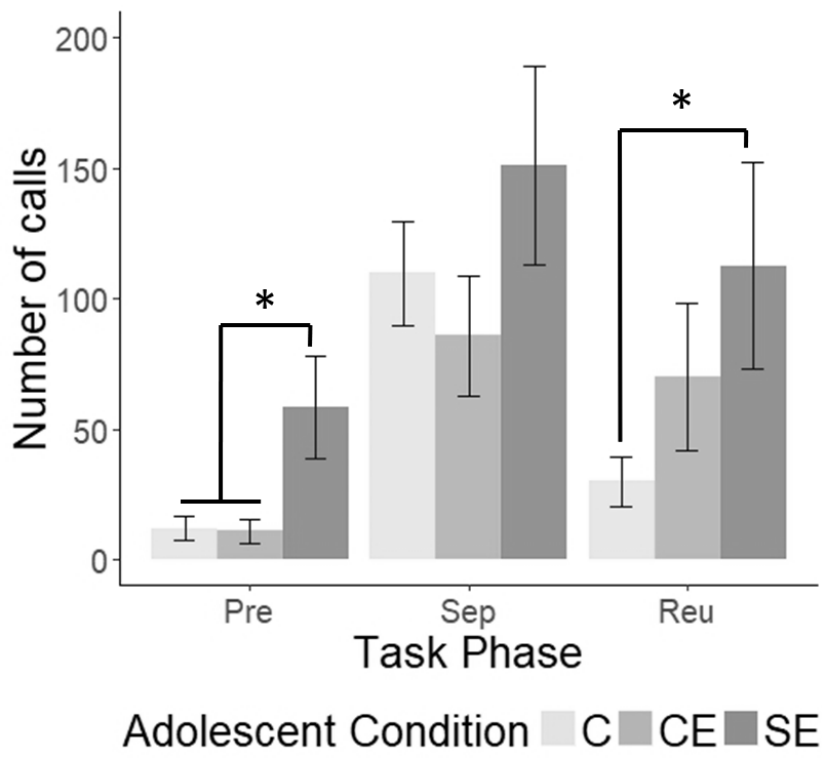
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1 **Figure 4**



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1 **Table 1**

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| Measure | Adolescent Condition | | |
|---|----------------------|---------------|---------------|
| | C (M±SEM) | CE (M±SEM) | SE (M±SEM) |
| Latency to enter light compartment (sec) | 52.48 (24.42) | 46.8 (20.58) | 49.68 (28.58) |
| Number of light compartment entries | 3.91 (0.65) | 4.00 (0.45) | 4.70 (0.76) |
| Proportion of time in light compartment (%) | 26.24 (5.19) | 24.64 (4.20) | 23.20 (4.69) |

1 **Table 2**

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| Urinary Corticosterone | Adolescent Condition | | | | | |
|---------------------------|----------------------|-----------------------|---------------------|-----------------------|---------------------|-----------------------|
| | C (M±SEM) | | CE (M±SEM) | | SE (M±SEM) | |
| | Familiar Partner | Unfamiliar Partner | Familiar Partner | Unfamiliar Partner | Familiar Partner | Unfamiliar Partner |
| Pre-interaction | 8.52 (1.37) | 10.33 (2.52) | 9.93 (2.21) | 14.89 (2.71) | 12.85 (3.30) | 12.06 (3.20) |
| Post-interaction | 14.29 (3.46) | 11.39 (2.90) | 14.46 (3.46) | 14.83 (3.70) | 11.84 (3.38) | 14.94 (3.38) |
| Change | 5.77 (3.65) | 1.06 (1.81) | 4.53 (2.61) | -0.06 (4.2) | -1.01 (4.10) | 2.88 (3.57) |

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