Shaped from an early age: behavioural and hormonal phenotypes in juvenile male guinea pigs living in distinct social environments

Melanie Gleske [a, b, *], Carolin Mundinger [a], S. Helene Richter [a, b, c] Sylvia Kaiser [a, b, c]

[a] Department of Behavioural Biology, University of Münster, Badestr. 13, 48149 Münster, Germany

[b] Münster Graduate School of Evolution, University of Münster, Hüfferstr. 1a, 48149 Münster, Germany

[c] Joint Institute for Individualisation in a Changing Environment (JICE), University of Münster, Münster, Germany and Bielefeld University, Bielefeld, Germany

* Corresponding author (melanie.gleske@uni-muenster.de)

Abstract

The individualised social niche results from interactions of an individual with its social environment. The social environment can change during lifetime. Thus, individuals need to be able to conform to different individualised social niches over lifetime. Our goal was therefore to elucidate when and how social niche conformance in guinea pigs occurs. We focused on juvenility, an important developmental phase characterized by prominent changes of the social environment, since the focus on social interactions shifts from parents to peers. For this approach, juvenile male guinea pigs (*Cavia aperea* f. *porcellus)* lived in two distinct social environments: while males of both groups lived in heterosexual pairs, males of one group were socially stimulated (e.g., an unfamiliar individual is introduced into the focus males home enclosure for 10 minutes) regularly whereas males of the other group were not. This procedure increased the number of social interactions, which is a crucial factor for constituting individualised social niches. Socially stimulated males showed different adjustments to their social environment in comparison to non-socially stimulated males. They displayed an initially increased stress response, enabling them to adequately react to the unpredictable social encounters. Over time, males then adjusted to this challenging environment and displayed a decrease in stress response again. Moreover, only socially stimulated males showed a significant increase of courtship and sexual behaviour with age. Taken together, these findings demonstrate that already in juvenile male guinea pigs the social environment induced hormonal adjustments and behavioural changes, thereby laying the grounds for social niche conformance.

Keywords

Behavior, behavioral development, cortisol responsiveness, juvenility, niche conformance, social interactions, social niche, testosterone

1. Introduction

The individualised social niche results from the interactions of an individual with its social environment (Kaiser et al., 2024; Saltz et al., 2016). These interactions are conceptualized as the NC^3 processes niche choice, niche construction, and niche conformance (Trappes et al., 2022). All these processes change the phenotype-environment match and an individual's inclusive fitness (Kaiser et al., 2024). We here focus on social niche conformance, defined as individuals adjusting to an existing social environment, for example by adjustments of the behavioural phenotype. The effects of the social environment on behavioural phenotypes were demonstrated in several species. In birds, fish and mammals for example, individuals from social environments with many opportunities for social interaction reacted less aggressively towards potential competitors than individuals from social environments with only a few interaction partners (Lilie et al., 2022; Nyman et al., 2017; Zimmermann et al., 2017). These findings emphasize how conspecifics, especially potential mating partners and competitors, shape an individual's social environment and are thus an important driver for realizing individualized social niches (Bergmüller and Taborsky, 2010). Such behavioural adjustments can happen through underlying endocrine mechanisms (Müller et al., 2020), for example through shaping of the principal neuroendocrine stress response system, namely the hypothalamic-pituitary-adrenocortical (HPA) axis, which is regulating glucocorticoid secretion and thus stress response (Jacobson & Sapolsky, 1991; Koolhaas et al. 2001; Sachser et al. 2011). The relationship between HPA axis and behaviour was intensively studied in guinea pigs (*Cavia aperea* f. *porcellus*), revealing causal links between social environment and stress responsiveness, for example (Sachser et al., 2013). In addition to that, guinea pigs have a high flexibility regarding their social organization (Sachser, 1998) and are a thus well-suited model organisms for studying social niche conformance.

Evidence for social niche conformance in different species were already found for the prenatal phase (Kaiser & Sachser, 2001; Kaiser & Sachser, 2005; Sachser & Kaiser, 1996), adolescence (Lürzel et al., 2010; Lürzel et al., 2011a; Ruploh et al., 2013; Rystrom et al., 2024a; Rystrom et al., 2024b; Zimmermann et al., 2017) and adulthood (Mutwill et al., 2020). More specifically, regarding the prenatal phase, hormones secreted by pregnant females can cross the placenta (Brust et al., 2015; Kaiser et al., 2003) and directly affect intra-uterine development, because the HPA is susceptible to prenatal programming (Brunton & Russell, 2010). In rodents, for example, prenatal social stress was associated with increased HPA activity in the offspring (Brunton & Russell, 2010; Creutzberg et al., 2021). Also in pigs, daughters from mothers who experienced social stress during pregnancy displayed an overreactive stress phenotype in later life (Jarvis et al., 2006). Such enhanced responses to stress could reflect greater vigilance to environmental threats (Brunton, 2013). In guinea pigs, an unstable social environment during pregnancy led to an adaptive and sex-specific shaping of the behavioural phenotypes of offspring

(Kaiser & Sachser, 2011; Kaiser & Sachser, 2005; Sachser & Kaiser, 2001; Siegeler et al., 2017).These adjustments are in consequence able to increase the fitness of the individual, which is a crucial factor for social niche conformance (Kaiser et al., 2024).

During adolescence, phenotypes are adaptively shaped in response to new information about the (social) environment (Fawcett & Frankenhuis, 2015; Sachser et al., 2018). Furthermore, individuals transition from infancy to adulthood and prominent alterations in the endocrine system and neural circuitry occur (Yurgelun-Todd, 2007), like activity and organization of the HPA axis (McCormick & Mathews, 2010). Male guinea pigs raised in large mixed-sex groups during adolescence frequently engaged in diverse social interactions, which triggered increased testosterone levels. Elevated testosterone in turn inhibited HPA activity, ergo reducing cortisol responsiveness (Seale et al., 2004; Sachser et al., 2013), which is the main glucocorticoid in guinea pigs (Fujieda et al., 1982). This resulted in a low-aggression phenotype, facilitating integration into unfamiliar groups with several adult males and females. In contrast, males housed with only a same-age female during adolescence experienced fewer social interactions, leading to lower testosterone levels, higher cortisol responsiveness and a high-aggression phenotype incompatible with unfamiliar males (Mutwill et al., 2020; Sachser et al., 2013). When formerly colony- and pair-housed males were placed as pairs into a competitive reproductive situation with two unknown females, pair-housed males displaying a more aggressive phenotype also had higher reproductive success, demonstrating the fitness consequences of social niche conformance (Zimmermann et al., 2017). Similar results were found in zebra finches: males raised in a group during adolescence showed the lowest courtship behaviour and lowest aggressiveness, whereas males raised with a single female during adolescence showed the most intense courtship behaviour and highest aggressiveness and were most attractive to females (Ruploh et al., 2013). Thus, the social environment during adolescence directed the development of the males' social behaviour to be adaptive in their likely future environment (Sachser et al., 2020).

Only a few studies investigated social niche conformance during adulthood. In guinea pigs, males were either housed in mixed-sex colonies or in heterosexual pairs (Mutwill et al., 2020). In adulthood, the males were then individually transferred to pair-housing with a female. This way, a social niche transition was induced in the formerly colony-housed males. Before transfer, adult colony-housed males showed significantly higher testosterone levels and lower cortisol responsiveness than pair-housed males. One month after the transfer, the hormonal phenotype of these males changed towards the one of pair-housed males.

Besides the previously mentioned phases, there is also the juvenile phase i.e., the time between weaning and adolescence. Studies investigating juvenility are however lacking. During this time, the social environment changes a lot since the focus on social interactions shifts from the parents to peers.

There is also evidence that during juvenility the HPA axis displays a heightened sensitivity to stress from the environment and thus social niche conformance processes could occur. In rats for example, stress experienced during juvenility affected behaviour in later life (Toledo-Rodriguez & Sandi, 2007) and similar effects could be mimicked by applicating corticosterone during juvenility (Jacobson-Pick & Richter-Levin, 2010).

In order to approach social niche conformance during ontogeny holistically, it is therefore important to also regard the juvenile phase. Our goal in this study was therefore to close this gap and investigate how juvenile male guinea pigs adjust to two different social environments. To analyse when exactly social niche conformance occurs, we repeatedly measured hormone concentrations and observed home-enclosure behaviour. We hypothesized that male guinea pigs realising different social niches differ in their behavioural and hormonal phenotypes. Since juvenile guinea pig males are sexually immature, direct fitness consequences in the form of reproductive success could not be measured. Instead, body weight as fitness proxy was assessed. To analyse the stability of hormone concentrations and body weight over time, a repeatability analysis was conducted.

2. Material and methods

2.1 Animals and housing conditions

All animals used for this study were bred from a breeding program of multi-coloured shorthaired guinea pigs (*Cavia aperea* f. *porcellus*) at the Department of Behavioural Biology at the University of Münster. They were born and reared in a total of six to eight harem groups within one breeding room, each consisting of one male, one to three females and their pre-weaned offspring. The offspring was routinely taken out of the harems after weaning at post-natal day (PND) 21 (±1) and adults were removed and replaced at around 18-24 months of age. Each harem was kept in wooden enclosures with a base area of approximately 1.5 m^2 and a wall height of 0.5 m. The enclosures were filled with wood shavings (Tierwohl Super, J. Rettenmaier & Söhne GmbH + Co KG, Rosenberg, Germany) as bedding and enriched with red plastic shelters and wooden bridges.

The experimental animals were transferred to enclosures in a different housing room after weaning at PND 21 (±3). These enclosures had a base area of 0.5 m^2, a wall height of 0.5 m, were also filled with wood shavings and enriched with a big and a small red plastic shelter. Food (hasfit Cavia C pellets, EQUOVIS GmbH, Münster, Germany) and water mixed with vitamin C were available *ad libitum*. Additionally, hay was replenished daily and fresh fodder (carrots, cucumbers, apples) was fed regularly. All guinea pig housing rooms were kept under controlled conditions with a 12 h: 12 h light/ dark cycle (lights on at 07:00), temperature of approximately 22 °C and relative humidity of approximately 48 %.

2.2 Experimental design

For this study, twenty guinea pig males were used. The experimental phase started after weaning at PND 21 (±3) and lasted six weeks, meaning the animals were 60 (±3) days of age when the experiments ended. In guinea pig males, sexual maturity is usually reached around PND 70 (Trillmich et al., 2006). Each male was paired with a female which was the same age. The male and his respective female partner stem from different harem groups, meaning they were neither half nor full siblings. To investigate the influence of distinct social environments on behavioural and hormonal phenotypes, they were randomly assigned to one of two treatment groups. Males of both groups lived in heterosexual pairs, but males of one group were socially stimulated (see 2.3) regularly (pair-housed male with social stimulation; PM+S group), while males of the other group were not (pair-housed male without social stimulation; PM-S group).

In total, four cortisol response tests (CRTs) to measure basal and reaction cortisol values (*see assessment of endocrine phenotypes*) were conducted within the six week long experimental phase (**Fig. 1**). The first CRT was conducted before the social stimulation treatment started and is thus referred to as CRT0. CRT0 was conducted in the first experimental week, CRT1 and CRT2 followed 14 (±2) days after the preceding one, while CRT3 was carried out 7 (±2) days after CRT2 (**Fig. 1**). Social stimulation and recording of home enclosure behaviour were each conducted three times per week during the whole experimental phase.

Please note: as part of another project, a battery of behavioural tests to further evaluate social and risk-taking behaviour plus fur swabbing with PMDS tubes to analyse chemical fingerprints was conducted in the last week.

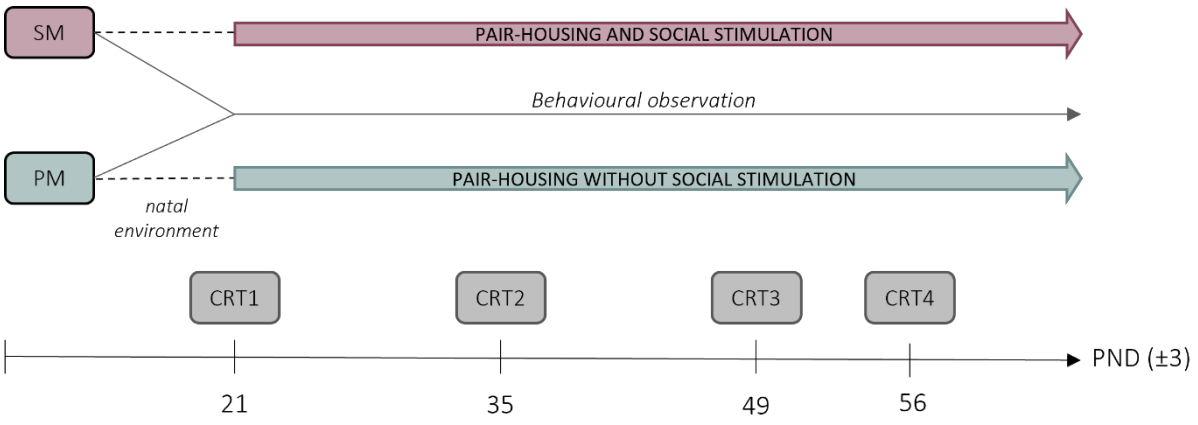


Figure 1: Procedure of behavioural observations in the home enclosure and cortisol response tests (CRT). Focal males were housed with a female partner. One group (PM+S) was regularly stimulated by introducing other individuals into the home enclosure while the other group (PM-S) was not. This social stimulation started after CRT0 and lasted until the experimental phase was finished at post-natal day (PND) 60±3.

2.3 Social stimulation

The social stimulation treatment for the stimulated males (PM+S) started after the first CRT. Upon then, social stimulation was applied three times per week for the whole experimental phase. More in details, an unknown individual was introduced into the home enclosure of the focus male and his female partner for a maximum of ten minutes. In each week, two of these stimulations were done with another male and one with a female. In total, the focus males had a total of twelve social stimulation sessions with another male and six social stimulation sessions with a female. The female stimulation animals always came from the harems to ensure they were pregnant and thus in the same reproductive stadium. The pool of stimulation males included around twelve individuals and the pool of stimulation females around six individuals. They were replaced at irregular intervals. If the focus male was stimulated more than once with the same stimulus animal, there was always a minimum interval of seven days between these stimulation sessions.

There was a time interval of at least 24 h between two stimulation sessions. The day and time of the sessions were randomized. Before the stimulation itself begun, the red plastic shelters were temporarily removed from the home enclosure of the focus male and the video camera was turned on, since all stimulation sessions were recorded. After the stimulation animals were introduced into the home enclosure, a timer was started as the sessions had maximum length of ten minutes. When males displayed escalated aggressive behaviour, the sessions were aborted beforehand to minimise the risk of injury.

2.4 Assessment of behavioural parameters

To analyse how distinct social environments influence (social) behaviour, the home enclosure behaviour of the focus males in both treatment groups was observed by filming them 2-3 times per week for one hour each. For this purpose, a video camera (Panasonic HC-V785 or SONY HDR-CX405) was installed approximately 1.5 m above each experimental home enclosure. The day and time (usually between 09:00 and 15:00) at which the videos were recorded was randomized. In total, 12 h to 18 h of home enclosure behaviour was collected for each individual.

The subsequent analysis was done with the program Interact (Interact, Lab Suite Version 2022, Program version 20.8.3.0, Mangold International GmbH, Arnstorf, Germany). The videos were blinded and randomized, ensuring ID and treatment of the respective individual as well as the time of recording were unknown to the observer.

The observed behaviours were summarized into the following categories: courtship and sexual behaviour, sociopositive behaviour, agonistic behaviour, play and other. The full ethogram can be found in the supplementary material (Tab. S1).

2.5 Assessment of hormone concentrations

Hormones were measured using blood samples obtained in cortisol response tests (CRTs). Therefore, the male guinea pigs were exposed to the stressor of exposure to a novel environment (Hennessy et al. 2006) and stress responses at different time points were assessed by sampling blood. The test started between 12:30 and 13:30. Prior to that, the animals were undisturbed for one hour.

At the start of the CRT the male was taken out of his home enclosure and placed on the experimenter's lap outside of the housing room. To facilitate blood flow, a muscle salve (Finalgon® Wärmesalbe DUO, Zentiva Pharma GmbH, Frankfurt am Main, Germany) for expanding the blood vessels was applied to the guinea pig's ear and wiped off again. After that, the marginal ear vessel was punctured with a lancet (Solofix® Blutlanzetten, B. Braun Melsungen AG, Melsungen, Germany) and blood was collected in heparinized capillary tubes (Capillary tubes for microhaematocrits, 100 µl, Paul Marienfeld GmbH & Co KG, Lauda Königshofen, Germany) to later on determine basal cortisol (c0) and basal testosterone (t) levels. This procedure had to be completed within 3 minutes (cortisol) or 6 minutes (testosterone) respectively after starting the test to avoid the sampling process from influencing the hormone values in the obtained sample itself (Sachser 1994). Then, the guinea pig was singly placed into an unfamiliar enclosure in a different housing room where it stayed for a total of two hours. This enclosure had a size of 1 m^2, wall height of 0.5 m and was equipped with wood shavings, food and water. Exactly one and two hours after the first one, blood sampling was repeated to determine first (c1) and second (c2) cortisol response values. The guinea pigs were weighed after each blood sampling and returned to their home enclosure after the last one.

To separate the blood plasma, the sample was centrifugated (13,000 × g for 5 min), transferred into a 1.5 mL Eppendorf tube and deep frozen at -20°C until assayed. Hormone concentrations were determined in duplicate using enzyme-linked immunosorbent assays (ELISA) (cortisol: RE52061, IBL International, Hamburg, Germany; antibody cross-reactivity: cortisol (100%), prednisolone (30%), 11-deoxycortisol (20%), cortisone (10.7%), prednisone (6.5%), 17 α-hydroxyprogesterone (5.4%), 6β-hydroxycortisol (4.4%), corticosterone 3.8%, desoxycorticosterone (1.8%); testosterone: RE52151, IBL International, Hamburg, Germany; antibody cross-reactivity: testosterone 100%, 11β-OH-testosterone 8.7%, 11α-OHtestosterone, 3.2%, dihydrotestosterone 1.9%). Intra- and inter-assay CVs were determined 2.09% and 3.98% for cortisol and 4.7% and 5.7% for testosterone.

In some cases, it was not possible to collect a sufficient amount of blood for the ELISA, resulting in a decreased sample size. For each CRT, the sample size per group ranged between n = 4 and n = 10.

2.6 Statistical analysis

Data analysis was carried out with RStudio version 2022.07.0 (R Core Team, 2022). A priori sample-size calculation was conducted using the software G*Power version 3.1.9.7 (Faul et al., 2007). The calculations were based on baseline and response cortisol values. Previous studies showed that effects of the social environment on cortisol concentrations are large, with estimated effect size of f = 0.69 (Hennessy et al., 2006; Kaiser et al., 2023). To detect effects with f = 0.69 with an α error probability of 0.05 and a power of 80% a total sample size of at least 19 animals would be needed. Thus, we decided to use a total sample size of n = 20 animals with n = 10 animals per treatment group.

Linear mixed-effect models were used to analyse the influence of the social stimulation treatment on hormone concentrations using the *lme4* (Bates et al., 2015) and *lmerTest* package (Kuznetsova et al., 2017). In total, four models were fit with 1) baseline cortisol, 2) baseline testosterone, 3) cortisol responsiveness after 1 hour and 4) cortisol responsiveness after 2 hours as a respective response variable. To improve model fit, all response variables were square root transformed. Treatment (social stimulation versus no social stimulation) was added as a fixed effect. To investigate changes in hormone concentrations over time, we also included the variable CRT, representing the first, second and third CRT conducted after treatment, as a fixed effect. We excluded data from the CRT conducted before the treatment (CRT0) from the analyses. However, hormone concentrations at CRT0 were still compared between the treatment groups using Wilcoxon rank-sum test to confirm there were indeed no differences between the groups prior to treatment. Furthermore, the continuous variable body weight was first mean-centered and then included as a fixed effect. We also added an interaction between treatment and CRT to determine whether effects of treatment varied across the three CRTs. Last, we fitted ID as a random effect. We used the *performance* (Lüdecke et al., 2021) and *DHARMa* package (Hartig, 2022) to check model assumptions. Marginal and conditional R^2 values were calculated using the *performance* package (Lüdecke et al., 2021), while partial R^2 values for individual predictors were calculated using the *sensemakr* package (Cinelli et al., 2020). Pair-wise comparisons for treatment, CRT and treatment*CRT interaction were done by applying Tukey's adjustment for multiple comparison using the *emmeans* package (Lenth, 2021).

Another linear-mixed effect model was fitted to analyse whether treatment affected body weight. Body weight measured after the first blood sampling in CRT1, CRT2 and CRT3 was modelled as a continuous response variable. The interaction between treatment and CRT was used as fixed effect to investigate the influence of treatment over time. ID was included as random effect. Pairwise comparison and R^2 estimations were conducted as described for the hormone concentrations. Also, body weight at CRT0 was compared between the treatment groups using Wilcoxon rank-sum tests to confirm there were indeed no differences prior to treatment. Additionally, the relationship between body weight and

cortisol responsiveness after 1 hour was examined by calculating Pearson's correlation coefficients for each treatment group separately. Body weight was mean-centered for each time point (CRT1, CRT2, CRT3) and the correlation coefficients were then calculated across all time points and for each time point separately. To determine whether the correlations for each time point differed significantly between treatment groups, Fisher's z-test was conducted using the *cocor* package (Diedenhofen & Musch, 2015).

Adjusted repeatability estimates of hormone concentrations and body weight were calculated for each of the treatment groups using the *rptR* package (Stoffel et al., 2017). 95% confidence intervals were determined by parametric bootstrapping (N = 1000), and likelihood ratio tests were used for significance testing. The models used to estimate adjusted repeatability were the same as mentioned before, with the only exception that treatment was removed as fixed effect.

For the analysis of the home enclosure behaviour, count data of behaviours from the coded videos was transformed into frequencies (occurrence per hour). Several behaviours were observed in only a few individuals, resulting in a zero-inflation of data which was detected using the *performance* package (Lüdecke et al., 2021). Therefore, we pooled behaviour into three categories: courtship and sexual behaviour, social behaviour and play. Agonistic behaviour was excluded from the analysis since it only occurred in a single individual. Generalized linear mixed-effect models with negative binomial distribution accounting for the zero-inflated data were fit for each behavioural category using the *lme4* package (Bates et al., 2015). Again, interaction between treatment and time was used as fixed effect in the models to investigate the influence of treatment over time. Time was categorized into "Phase 1" (1^{st} and 2^{nd} experimental week), "Phase 2" (3^{rd} and 4^{th} experimental week) and "Phase 3" (5^{th} and 6^{th} experimental week). ID was again fitted as a random effect. Model assumptions as well as the estimation of the different R^2 values were conducted in the same manner as for the analysis of hormone concentrations. Pair-wise comparisons for treatment, phase and treatment*phase interaction were done by applying Tukey's adjustment for multiple comparison using the *emmeans* package (Lenth, 2021).

Model summaries and detailed test statistics can be found in the supplementary material.

3. Results

Descriptive statistics for all hormone measurements, body weight and behaviour for each respective time point and over the entire time period can be found in the supplementary material (**Tab. S2-4**).

3.1 Effects of social environment on hormone concentrations and body weight

The comparison of hormone concentrations (c0, c1, c2, t) at CRT0 using Wilcoxon rank-sum tests revealed no significant differences between the treatment groups prior to treatment.

Regarding baseline testosterone and cortisol levels (**Fig. 2**), neither a significant effect of treatment or time (CRT), nor a significant treatment-by-time interaction effect was found.

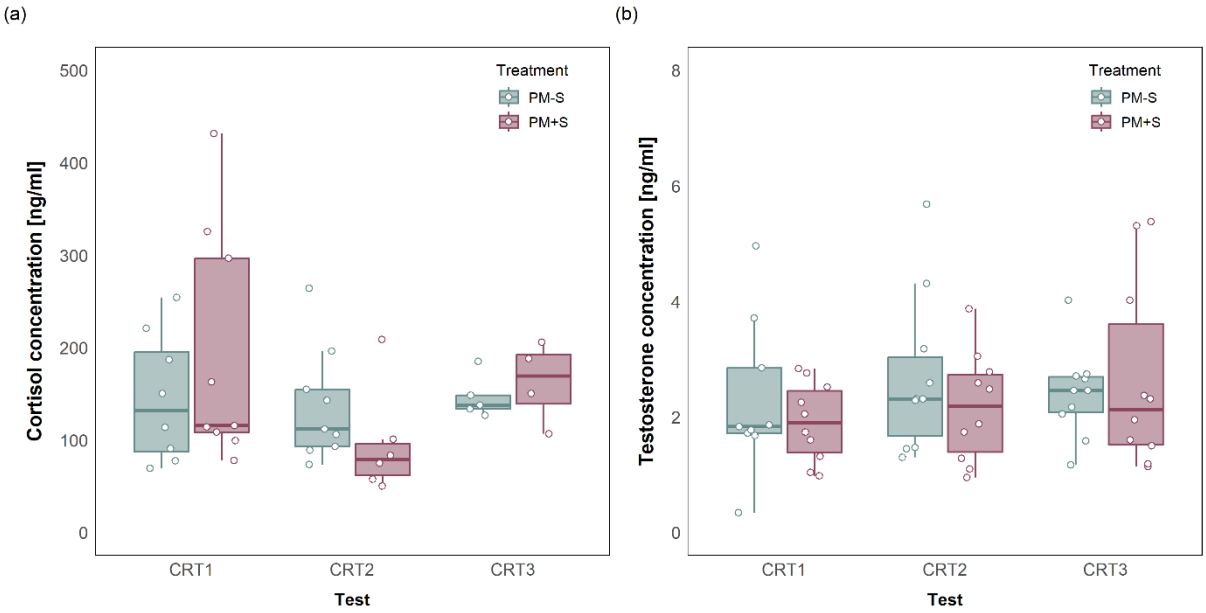


Figure 2: Baseline cortisol **(a)** and testosterone **(b)** concentrations (ng ml^{-1}) two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are medians (horizontal marks), first to third quartiles (boxes), whiskers and all data points. Statistics: (a) Multiple comparisons of LMM; PM-S: $n_{CRT1} = 8$, $n_{CRT2} = 9$, $n_{CRT3} = 5$, PM+S: $_{nCRT0} = 8$, $n_{CRT1} = 9$, $n_{CRT2} = 6$, $n_{CRT3} = 4$. (b) Multiple comparisons of LMM; PM-S: $n_{CRT1} = 9$, $n_{CRT2} = 10$, $n_{CRT3} = 10$, PM+S: $n_{CRT1} = 10$, $n_{CRT2} = 10$, $n_{CRT3} = 10$.

Regarding cortisol responsiveness at 1 hour (c1) of exposure to a novel environment, a significant treatment effect was found at CRT1 (ß = -2.44 ± 1.11, t = -2-2, p = 0.03), where PM+S had significantly higher c1 values than PM-S (**Fig. 3a**). We also found a significant treatment-by-time interaction effect between CRT1 and CRT2 (ß = 3.92 ± 1.34, t = 2.92, p = 0.006) as well as between CRT1 and CRT3 (ß = 2.94 ± 1.34, t = 2.19, p = 0.035), where c1 values decreased for the PM+S group. Additionally, a significant effect of mass was found (ß = -0.03 ± -0.01 t = -4.32, $p < 0.001$). For cortisol responsiveness at 2 hours (c2) of exposure to a novel environment (**Fig. 3b**), a significant treatment-by-time interaction

effect between CRT1 and CRT2 (ß = 4.2 ± 1.7, t = 2.46, p = 0.019) was found, with c2 values strongly decreasing for the PM+S group.

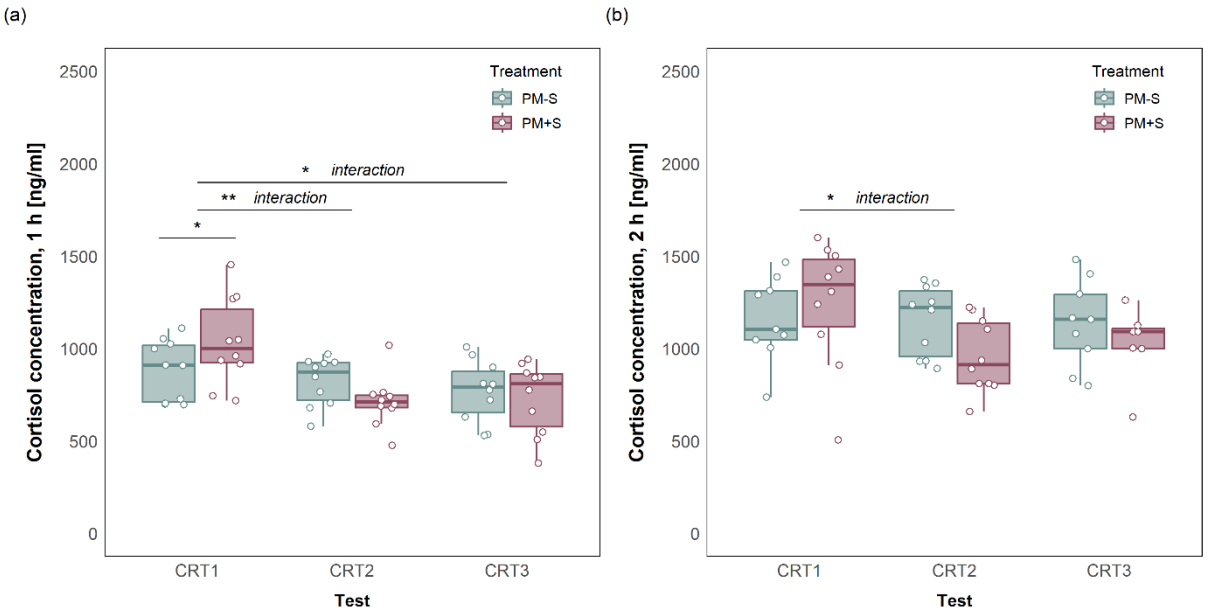


Figure 3: Cortisol concentrations (ng ml^{-1}) at one hour **(a)** and two hours **(b)** of exposure to a novel environment two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are medians (horizontal marks), first to third quartiles (boxes), whiskers and all data points. Statistics: (a) Multiple comparisons of LMM; n_{CRT1} = 10, n_{CRT2} = 10, n_{CRT3} = 10, PM+S: n_{CRT0} = 10, n_{CRT1} = 10, n_{CRT2} = 10, n_{CRT3} = 10; *p < 0.05 **p < 0.01 (b) Multiple comparisons of LMM; PM-S: n_{CRT1} = 9, n_{CRT2} = 10, n_{CRT3} = 9, PM+S: n_{CRT1} = 10, n_{CRT2} = 10, n_{CRT3} = 7; * p < 0.05.

Regarding body weight, the comparison at CRT0 using a Wilcoxon rank-sum test revealed no significant differences between the treatment groups prior to treatment. A significant effect of time was found for the PM-S group from CRT1 to CRT2 (ß = -101.2 ± 6.62, t = -15.28, p < 0.001), CRT1 to CRT3 (ß = -143.1 ± 6.62, t = -21.61, p < 0.001) and CRT2 to CRT3 (ß = -41.9 ± 6.62, t = -6.33, p < 0.001), as well as for the PM+S group from CRT1 to CRT (ß = -116.1 ± 6.62, t = -17.53, p < 0.001), CRT1 to CRT3 (ß = -151.7 ± 6.62, t = -22.91, p < 0.001) and CRT2 to CRT3 (ß = -35.6 ± 6.62, t = -5.38, p < 0.001) (**Fig. 4**). In each case, body weight was increasing.

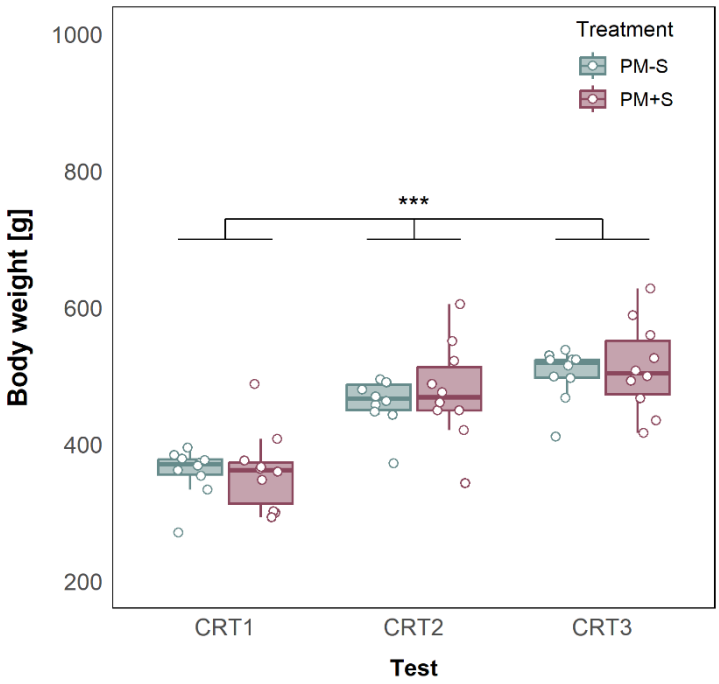


Figure 4: Body weight measured two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are medians (horizontal marks), first to third quartiles (boxes), whiskers and all data points. Statistics: Multiple comparisons of LMM; PM-S: $n_{CRT1} = 10$, $n_{CRT2} = 10$, $n_{CRT3} = 10$, PM+S: $n_{CRT1} = 10$, $n_{CRT2} = 10$, $n_{CRT3} = 10$; *** $p < 0.001$.

The statistical analysis of hormone concentrations showed that c1 concentrations are significantly affected by body weight. At CRT1, body weight and c1 had a significant, strong negative correlation in the PM+S group (r = -0.81, t = -3.88, p = 0.005) and a weak negative correlation in the PM-S group (r = -0.26, t = -0.76, p = 0.472). At CRT2, body weight and c1 had a significant, moderate negative correlation in the PM+S group (r = -0.69, t = -2.67, p = 0.028) and a weak, negative correlation in the PM-S group (r = -0.11, t = -0.31, p = 0.762). At CRT3, body weight and c1 had a significant, strong correlation in the PM+S group (r = -0.74, t = -3.13, p = 0.014) and a significant, strong correlation in the PM-S group (r = -0.71, t = -2.89, p = 0.02). Comparisons between the correlations of the treatment groups were however not significant for any time point. These correlation between body weight and c1 concentrations over all timepoints (CRT1, CRT2, CRT3) are displayed in **Figure 5**.

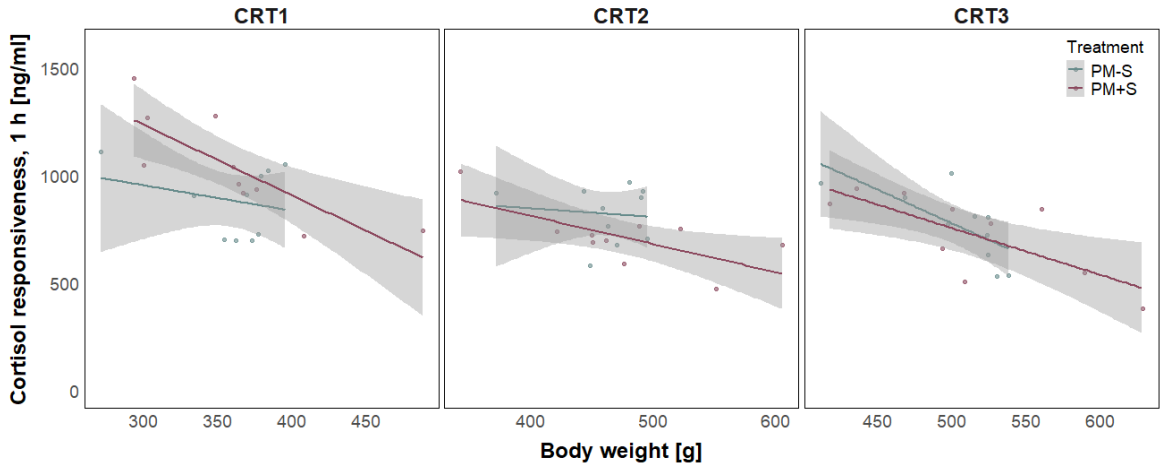
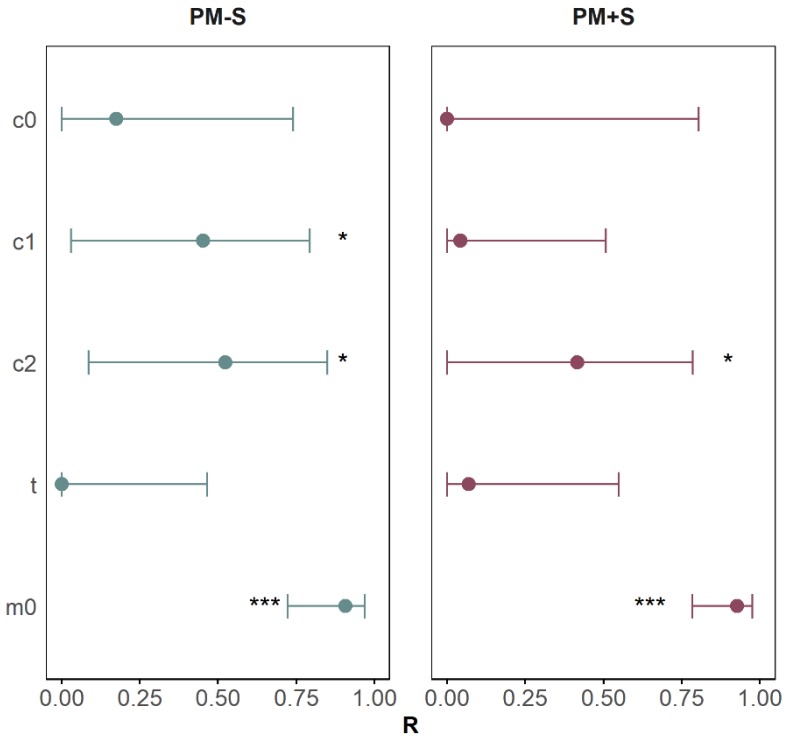


Figure 5: Correlation between cortisol concentrations (ng ml^{-1}) at one hour of exposure to a novel environment and body weight two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are regression lines, confidence intervals and all data points. PM-S: $n_{CRT1} = 10$, $n_{CRT2} = 10$, $n_{CRT3} = 10$, PM+S: $n_{CRT1} = 10$, $n_{CRT2} = 10$, $n_{CRT3} = 10$.

Adjusted repeatability was analysed for hormone concentrations (baseline cortisol, baseline testosterone, cortisol responsiveness after 1 and 2 hours) and body weight in both treatment groups (**Fig. 6**). Baseline cortisol (c0) was not repeatable in the PM+S group (R = 0, CI = [0, 0.81], p = 1). In the PM-S group, baseline cortisol had a low repeatability (R = 0.18, CI = [0, 0.74], p = 0.331). Baseline testosterone (t) had a low repeatability in the PM+S group (R = 0.07, CI = [0, 0.55], p = 0.44) and was not repeatable in the PM-S group (R = 0, CI = [0, 0.47], p = 1). Cortisol responsiveness after 1 hour (c1) had a low repeatability in the PM+S group (R = 0.04, CI = [0, 0.51], p = 0.495) and a moderate repeatability in the PM-S group (R = 0.45, CI = [0.03, 0.79], p = 0.014). Cortisol responsiveness after 2 hours (c2) was moderately repeatable in the PM+S group (R = 0.42, CI = [0, 0.55], p = 0.04) and in the PM-S group (R = 0.52, CI = [0.09, 0.85], p = 0.015). Body weight (m0) was highly repeatable in the PM+S group (R = 0.93, CI = [0.78, 0.98], p < 0.001) and in the PM-S group (R = 0.91, CI = [0.72, 0.97], p < 0.001).



391

392 **Figure 6**: Repeatability (R) of baseline cortisol (c0), cortisol responsiveness after 1 (c1) and 2 hours (c2) of exposure to a novel
393 environment, baseline testosterone (t) and body weight (m0). Males in heterosexual pairs were either socially stimulated
394 (PM+S) or not (PM-S). Plotted are adjusted repeatability (data points) and confidence intervals (whisker). Statistics:
395 repeatability analysis using permutation testing; PM-S: $n_{c0} = 22$, $n_{c1} = 30$, $n_{c2} = 28$, $n_t = 29$, $n_{m0} = 30$, PM+S: $n_{c0} = 19$, $n_{c1} = 30$,
396 $n_{c2} = 27$, $n_t = 30$, $n_{m0} = 30$; *p < 0.05, *** p < 0.001.

397

398 ## 3.2 Effects of social environment on social behaviour

399 For sociopositive behaviour a significant effect of time (phase) was found for the PM-S group between
400 phase 1 and phase 3 (ß = -0.92 ± 0.34, z = -2.74, p = 0.017) and for the PM+S group between phase 1
401 and phase 3 (ß = -1.29 ± 0.33, z = -3.9, p < 0.001). In both groups, the frequency of sociopositive
402 behaviour increased over time (**Fig. 7a**). Furthermore, a significant increase of courtship and sexual
403 behaviour was only found in the PM+S group (ß = -2.38 ± 0.76, z = -3.13, p = 0.005) (**Fig. 7b**).

404

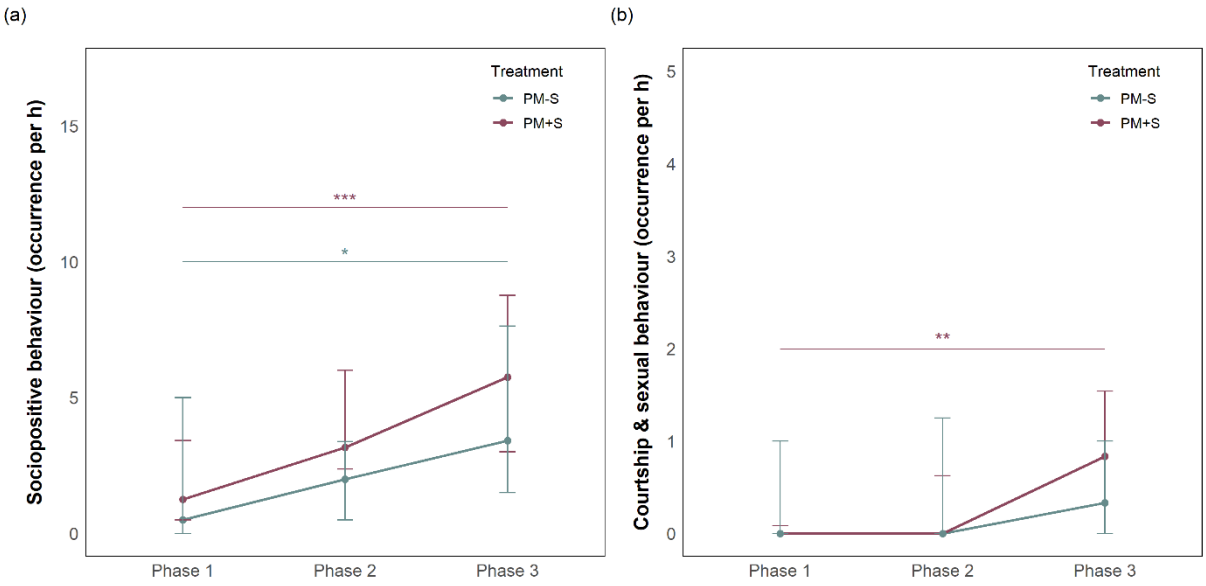


Figure 7: Frequency (occurrence per h) of **(a)** sociopositive behaviour and **(b)** courtship and sexual behaviour in the first (phase 1), second (phase 2) and third (phase 3) two weeks of treatment. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are medians (data points) and first to third quartiles (whiskers). Statistics: Multiple comparisons of GLMM. PM-S: $n_{Phase\ 1} = 20$, $n_{Phase\ 2} = 20$, $n_{Phase\ 3} = 20$, PM+S: $n_{Phase\ 1} = 20$, $n_{Phase\ 2} = 20$, $n_{Phase\ 3} = 20$; *$p < 0.05$, ** < 0.01, *** $p < 0.001$.

4. Discussion

In this study, we investigated how juvenile male guinea pigs adjust to distinct social niches through possible shaping of behavioural and hormonal phenotypes. By repeatedly analysing behavioural and hormonal parameters during juvenility, we aimed to explore when and how social niche conformance occurs in this early phase. For this purpose, male guinea pigs kept under pair-housing conditions with one female only (PM-S) were compared with males who lived with one female and received additional social stimulation by interactions with unfamiliar males and females (PM+S). Stimulated males showed an initially increased cortisol responsiveness which decreased again over time, as well as an increase in courtship and sexual behaviour over time. Moreover, cortisol responsiveness was significantly affected by body weight, this finding was however independent of treatment group.

4.1 Social niche conformance through adjustments of cortisol responsiveness

Interestingly, cortisol responsiveness was different between treatment groups. More specifically, in the cortisol response test (CRT) conducted two weeks after start of social stimulation, cortisol responsiveness after one hour was higher in stimulated males than in non-stimulated males. Since baseline cortisol values did not differ between males of both treatment groups, social stimulation per se did not lead to prolonged higher stress levels. However, animals confronted with unpredictable interactions with unfamiliar conspecifics live in a much more challenging environment. Under such

conditions, a higher endocrine responsiveness to stressors in such a situation could be adaptive. This reactivity provides the organism with energy and shifts it into a state of heightened reactivity which is a prerequisite for responding to environmental challenges in an appropriate way. This has already been demonstrated in birds, where individuals with higher corticosterone responses are more successful in unpredictable conditions and thus better able to cope with environmental change (Cockrem, 2007; Cockrem, 2013). Consequently, the heightened stress response to this unpredictable environment presumably constitutes a niche conformance process in stimulated males. Interestingly, in the subsequent CRTs, cortisol responsiveness significantly decreased in stimulated males. At the end of the experimental phase cortisol responsiveness of stimulated and non-stimulated males almost converged. This indicated juvenile males could adjust to the situation and a conformance process occurred. A stress-induced HPA activation is metabolically costly. Thus, it is adaptive for an organism to reduce HPA activity to stressors without harm (Grissom & Bhatnagar, 2009).

Baseline cortisol levels did not differ between the treatment groups. Other studies have also reported no differences in baseline cortisol levels in guinea pigs living in different social environments (Lürzel et al., 2011a; Mutwill et al., 2020) or of different social status (Sachser & Lick, 1991). These findings suggest no influence of the social environment on baseline cortisol in guinea pigs, unlike in other species, such as mice, where plasma glucocorticoids can be affected by social interactions (Williamson et al., 2017). These differences may be due to differences in the social organization of these species. While male guinea pigs are able to integrate into unfamiliar groups with several adult males and females (Sachser et al., 2013), male mice aggressively defend their territory and monopolize several females (Crowcroft & Rowe, 1963; König et al., 2015; Lidicker, 1976). Another possible explanation is the sample size of baseline cortisol, which might have been too small to detect differences, since collecting a sufficient amount of blood was sometimes not possible.

Finally, the results from the repeatability analysis are in line with a meta-analysis, showing that repeatability estimates tend to be higher for peak hormone levels than for baseline levels (Fanson & Biro, 2019; Taff et al., 2018). The reason for this might elevated hormone responses (e.g., through stress) capturing a more defined aspect of endocrine function, while baseline hormone levels can represent multiple different biological functions (Fanson & Biro, 2019). However, the results obtained here should be interpreted with caution, as the confidence intervals were wide and either close to or included zero (Nakagawa & Cuthill, 2007; Nakagawa & Schielzeth, 2010).

4.1 Social environment affected courtship and sexual behaviour, but not testosterone concentrations

Sociopositive behaviour significantly increased from the beginning to the end of the experimental phase in both treatment groups, suggesting a social relationship has been established between the males and their respective female partners (Sachser, 1998). Sexual and courtship behaviour, however, only significantly increased over time in socially stimulated males. This finding leads to the consideration of socially stimulated males reaching sexual maturity earlier than non-stimulated males. Usually, sexual maturity is accompanied by a peak in testosterone concentration in male rodents (Bell, 2018; Guanga et al., 2020) and studies in Syrian hamsters have also shown most pronounced effects of testosterone on the organization of neural behavioural circuits and thus sexual behaviour are most pronounced during adolescence (Schulz & Sisk, 2006; Schulz et al., 2009). Yet, we neither found differences in testosterone levels between stimulated- or non-stimulated males, nor did testosterone levels significantly increase over time in stimulated males in this study. Thus, an early onset of sexual maturity is unlikely to explain the significant increase in courtship and sexual behaviour. Instead, we favour a different explanation: stimulated males were able to observe such behaviour from adult stimulus males courting the focus male's female partner during the stimulation sessions. Immature guppies, for example, also learn courtship behaviour by observing experienced male conspecifics (Guevara-Fiore, 2012).

The lack of significant differences in testosterone levels between the treatment groups is also surprising for another reason: studies in adolescent male guinea pigs have demonstrated a causal relationship between the frequency of social interactions and increased testosterone concentrations (Lürzel et al., 2011a; Sachser et al., 2018). Yet, it is also known that for adolescent males specifically courting and agonistic encounters are responsible for increased testosterone levels (Hirschenhauser & Oliveira, 2006; Sachser et al., 2013). For juvenile male guinea pigs, however, it is unclear whether male-male interactions are really agonistic, and if male-female interactions are really sexual, when the male has not reached sexual maturity yet. In a study where baseline testosterone levels between colony-housed and individually-housed males were measured repeatedly from juvenility until adulthood, significant differences were also only found from an age of 90 days (i.e., adolescence), but not an age of 30 or 60 days (i.e., juvenility) (Sachser & Pröve, 1988).

4.3 Body weight as fitness proxy and its negative effect on cortisol responsiveness

While reproductive success is a direct measurement of fitness, body weight as an index of body condition can be used as fitness proxy (Wilder et al., 2016). Body weight is related to many life history parameters, such as reproduction, survival and longevity. Animals with higher body weight have more

494 body fat and in consequence more stored excess energy, which is beneficial for several reasons. They
495 are better able to withstand harsher environmental conditions, and the development and expression of
496 secondary sexual traits are often dependent on body condition (Barnett et al., 2015). A larger body
497 weight can also indirectly influence reproduction via a link to higher dominance status in social systems,
498 as it was already demonstrated in guinea pigs and cavies (Asher et al., 2008; Mutwill et al., 2021).
499 However, no differences regarding body weight were found between the treatment groups in this study.
500 Furthermore, repeatability was very high in both stimulated and non-stimulated males, indicating that
501 body weight is a stable individual trait independent of social environment.

502 More interestingly, body weight had a significant negative effect on cortisol responsiveness after 1 hour.
503 The relationship between stress and body weight in animals has been studied a lot. The effects of acute
504 stress on metabolic phenotypes can range from stress-induced anorexia (Calvez et al., 2011) to
505 increased food intake and thus obesity (McMillan, 2013) and are influenced by factors like animal model
506 and type of stress (Patterson & Abizaid, 2013). Regarding stress response, studies indicated high stress
507 responsiveness is linked to obesity (Levin et al., 2000; Hewagalamulage et al., 2016). However, the
508 animals in this study were not only non-obese, but also a negative relationship was found between body
509 weight and cortisol response, most presumably hinting at a different physiological process is involved
510 here. Even though no statistical differences between treatment groups could be found, this effect
511 seemed to be more pronounced in socially stimulated males, since they had more negative and
512 significant correlations for all time points. At the last time point, however, the correlation between body
513 weight and cortisol responsiveness in non-stimulated males was almost as high as in stimulated males
514 and also significant. This indicates an earlier onset of the effect that causes higher body weight to
515 negatively influence cortisol responsiveness in socially stimulated males, possibly due to prior shaping
516 of the HPA axis. This might also constitute a mechanism of the niche conformance process.

517 Furthermore, it is particularly interesting that only cortisol responsiveness after 1 hour, but not after 2
518 hours, was affected by body weight. In guinea pigs, maximum cortisol responsiveness is usually reached
519 after 2 hours, so cortisol responsiveness after 2 hours can be characterised as magnitude of stress
520 response and cortisol responsiveness after 1 hour as speed of stress response (Rystrom et al., 2024b;
521 Taff et al., 2022). Speed and magnitude of stress response are correlated and especially speed of stress
522 response is an important factor and possible target of selection (Taff et al., 2022), as it determines how
523 quickly individuals can adjust to changes (Taff & Vitousek, 2016). The observation of only cortisol
524 responsiveness after 1 hour, but neither baseline cortisol levels nor cortisol responsiveness after 2 hours
525 being negatively affected by body weight, indicates guinea pig males with higher body weights have a
526 slower cortisol response. This would mean the maximum stress response might not be different
527 between bigger and smaller individuals, but the time it takes to reach this maximum. Reasons for this

could involve body weight dependent differences in the adrenal gland and availability or secretion of cortisol or cortisol binding globulins. Still, these hypotheses cannot yet be verified or explained, since studies investigating the exact physiological mechanisms involved in stress response in guinea pigs are lacking.

5. Conclusions

Socially stimulated males showed different adjustments to their social environment: at the beginning of the experimental phase, they displayed an increased stress response to be able to adequately react to the unpredictable social encounters. However, since such increases in stress are metabolically costly and social stimulation were not actually dangerous, the males then adjusted to this challenging environment and displayed a decrease in stress response again. Furthermore, body weight was found to have a significant, negative impact on speed of cortisol reactivity. These findings indicate the speed of cortisol reactivity is a flexible trait and able to adjust to external (social environment) and internal (body weight) parameters and thus forming the basis for individualised niches. Moreover, social stimulation did not only affect endocrine parameters, but also behaviour: while males of both treatment groups displayed a significant increase of sociopositive behaviour over time, only males with additional social stimulation also displayed a significant increase of courtship and sexual behaviour over time. Taken together, these findings demonstrate that already in juvenile guinea pigs the social environment induced hormonal adjustments and behavioural changes and hereby laying the grounds for social niche conformance. For future studies repeating these experiments with adolescent males to investigate social niche conformance throughout ontogeny, we would expect the effects found here are further pronounced and persistent since social interactions become even more meaningful once the individuals reach sexual maturity.

Ethics

All procedures complied with the regulations covering animal experimentation within Germany (Animal Welfare Act) and the EU (European Communities Council Directive 2010/ 63/ EU), and were approved by the local and federal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen “LANUV NRW”, reference number: 81-02.04.2022.A080).

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CRediT authorship contribution statement

Melanie Gleske: Methodology; writing – original draft; investigation; formal analysis; visualization; data curation. S. Helene Richter: Conceptualization; Writing – review and editing. Sylvia Kaiser: Conceptualization; methodology; supervision, writing – review and editing; funding acquisition. Carolin Mundinger: Formal analysis. All authors critically revised the manuscript and gave final approval for publication.

Declaration of competing interests

The authors declare no conflict of interests.

Data availability

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Supplementary material

Materials and methods: Ethogram

Table S1: Ethogram used for the observation of home enclosure behaviour. The abbreviation "FA" stands for "focus animal", e.g., the experimental male.

Category	Behaviour	Description
Courtship and sexual behaviour	Ano-genital licking	The FA stretches its snout towards or touches another animals' ano-genital region and lick or nuzzles the other animals' genital region. The distance between the two animals is less than one snout-width.
Courtship and sexual behaviour	Chin-rest	The FA lays the bottom of its head on another animals' torso.
Courtship and sexual behaviour	Mounting	The FA moves the forepart of its body onto the back of another animal from behind.
Courtship and sexual behaviour	Pelvic thrust	The FA mounts the other animal and moves the lower part of its body fast and rhythmically.
Courtship and sexual behaviour	Mating attempt	The FA puts at least one of its forepaws on another animal and tries to mate with the other animal, but the other animal prevents this.
Courtship and sexual behaviour	Rumba	The FA approaches the other animal slowly and visibly shifts its weight from one hind leg to the other and back, it can also move forward while doing so. This is often accompanied by a low purring noise. Behaviour ends when the FA stops for more than 3s.
Courtship and sexual behaviour	Flank	The FA walks parallel to another animal, touches its side with its own and slightly raises the hind leg on the side that is touching the other animal while moving forward.
Courtship and sexual behaviour	Chin-rump following	The FA walks or runs behind another animal with its nose towards the other animal's rear, trying to make contact with the chased animal. There is a maximum of 1 body length of distance between the two animals. Behaviour ends when the FA stops chasing for at least 3s.
Sociopositive behaviour	Naso-nasal sniffing	The FA stretches its nose towards another animal's nose or snout. The distance between the two animals is less than one snout-width.
Sociopositive behaviour	Naso-anal sniffing	The FA stretches its nose towards or touches another animals' anal region with its nose. The distance between the two animals is less than one snout-width.
Sociopositive behaviour	Social resting	The FA rests next to another animal at least 3s with a distance of less than a half a body length. Behaviour ends when not shown for at least 3s.
Play	Play	The FA makes one or a series of upward leaps and turns the head or foreparts sharply while in the air, or the FA starts with a short and fast run and then stops suddenly and changes the direction.
Agonistic behaviour	Displace	The FA approaches another animal or shows agonistic behaviour towards it, causing the other animal to move at least one body length away from the FA.

Agonistic behaviour	Evade	The FA moves at least one body length away from another animal that approached or interacted otherwise with it..
Agonistic behaviour	Head-thrust	The FA abruptly moves its head towards another animal, hitting or narrowly missing it, or biting it. The distance between the two animals is maximum half a body length.
Agonistic behaviour	Fight	A prolonged agonistic interaction of at least 3s between at least two animals. Head-thrusts, kicks and attack lunges can occur. The behaviour ends when one or both animals back away.
Agonistic behaviour	Kick	The FA abruptly moves one of its hind legs towards another animal.
Agonistic behaviour	Paw	The FA repeatedly moves one or both of its front paws across the bedding without moving in any direction.
Agonistic behaviour	Urine spray	The FA slightly arches its back and, with a small jolt, squirts urine behind it, usually towards another animal, which often reacts by stopping and cleaning itself. The urine squirt itself is not always directly or indirectly (wet spots on the enclosure wall) visible.
Agonistic behaviour	Curved body posture	The FA is standing within a distance of one body length in front of or sideways to another animal. Its body is usually curved with head and rump directed to the other animal, which is also displaying the same behaviour. This behaviour is often accompanied by growling and teeth chattering.
Agonistic behaviour	Head up	The FA is standing still but lifts its head up in such a way that the chin is facing upwards and towards another animal. The distance between both animals is maximum one body length.
Agonistic behaviour	Attack lunge	The FA jumps on or towards another animal, with the landing happening within one body length of the other animal.
Agonistic behaviour	Chase	The FA follows another animal over a distance of at least one body length. This happens with high velocity. During this interaction, the distance between both animals never exceeds two body lengths. Chasing is terminated, if the distance between the animals exceeds to body lengths for more than 3 s.
Other	Being under the house	The FA has moved under the small hideout with at least half of its body. Behaviour ends when the FA has moved at least half of its body out from under the hideout.
Other	Time-out	The FA is not visible.

Results: Descriptive statistics

Table S2: Descriptive statistics for baseline cortisol (c0), cortisol responsiveness after 1 hour (c1) and 2 hours (c2) of exposure to a novel environment and baseline testosterone (t).

Treatment	Hormone	Time point	n	mean	SD	min	max
PM+S	c0	CRT0	8	520.70	298.84	199.23	979.73
		CRT1	9	192.44	126.20	78.06	431.48
		CRT2	6	95.93	58.19	50.22	208.83
		CRT3	4	162.71	44.02	106.60	205.88
		Overall	27	263.85	244.88	50.22	979.73
	c1	CRT0	10	1583.90	365.44	968.58	2110.78
		CRT1	10	1036.95	236.58	718.33	1454.35
		CRT2	10	712.83	137.68	475.50	1017.43
		CRT3	10	729.12	193.55	380.30	942.00
		Overall	40	1015.70	429.22	380.30	2110.78
	c2	CRT0	9	1736.53	525.90	732.33	2296.40
		CRT1	10	1249.38	336.44	505.60	1599.15
		CRT2	10	959.57	197.91	660.11	1222.77
		CRT3	7	1029.25	196.66	630.45	1262.53
		Overall	36	1247.86	451.37	505.60	2296.40
	t	CRT0	7	1.00	0.44	0.66	1.87
		CRT1	10	1.91	0.68	0.98	2.84
		CRT2	10	2.17	0.94	0.95	3.87
		CRT3	10	2.68	1.63	1.14	5.38
		Overall	37	2.02	1.17	0.66	5.38
PM-S	c0	CRT0	9	414.35	184.21	141.28	712.00
		CRT1	8	145.56	69.33	69.57	254.20
		CRT2	9	136.86	61.10	73.58	264.23
		CRT3	5	146.43	23.05	126.85	185.23
		Overall	31	221.21	164.38	69.57	712.00
	c1	CRT0	9	1438.96	221.49	1032.23	1635.13
		CRT1	10	883.41	163.18	696.33	1110.35
		CRT2	10	822.30	131.59	580.20	969.28
		CRT3	10	768.61	166.59	530.18	1009.53
		Overall	39	966.51	312.68	530.18	1635.13
	c2	CRT0	9	1645.85	175.58	1370.85	1835.45
		CRT1	9	1158.78	227.61	736.08	1467.13
		CRT2	10	1155.06	189.08	892.17	1373.48
		CRT3	9	1136.01	234.90	799.63	1481.10
		Overall	37	1270.71	293.48	736.08	1835.45
	t	CRT0	9	1.18	0.87	0.25	3.22
		CRT1	9	2.30	1.35	0.34	4.96
		CRT2	10	2.69	1.38	1.30	5.68
		CRT3	10	2.40	0.76	1.17	4.02
		Overall	38	2.16	1.22	0.25	5.68

Table S3: Descriptive statistics for body weight.

Treatment	Time point	n	mean	SD	min	max
PM+S	CRT0	10	254.20	44.17	211	359
	CRT1	10	360.60	58.31	293	488
	CRT2	10	476.70	72.12	343	605
	CRT3	10	512.30	66.15	417	628
	Overall	40	400.95	118.47	211	628
PM-S	CRT0	10	263.50	32.76	193	313
	CRT1	10	359.80	35.49	271	395
	CRT2	10	461.00	36.16	372	495
	CRT3	10	502.90	38.18	411	538
	Overall	40	396.80	100.13	193	538

Table S4: Descriptive statistics for behaviour.

Treatment	Behaviour	Time point	n	mean	SD	min	max
PM+S	Sociopositive	Phase 1	20	2.12	2.02	0	6.50
		Phase 2	20	4.65	3.78	0.50	15.00
		Phase 3	20	7.78	7.27	0	26.00
		Overall	60	4.85	5.33	0	26.00
	Courthsip and sexual	Phase 1	20	0.15	0.29	0	1.00
		Phase 2	20	0.48	0.72	0	2.33
		Phase 3	20	1.70	3.16	0	13.50
		Overall	60	0.78	1.96	0	13.50
	Play	Phase 1	20	0.54	1.80	0	7.50
		Phase 2	20	0.48	1.27	0	5
		Phase 3	20	0.66	1.30	0	4.50
		Overall	60	0.56	1.45	0	7.50
PM-S	Sociopositive	Phase 1	20	2.52	3.75	0	10.67
		Phase 2	20	3.43	5.08	0	20.50
		Phase 3	20	5.98	8.06	0	36.33
		Overall	60	3.97	5.99	0	36.33
	Courthsip and sexual	Phase 1	20	0.60	1.05	0	3.33
		Phase 2	20	1.23	2.86	0	12.50
		Phase 3	20	1.72	4.41	0	19.67
		Overall	60	1.18	3.07	0	19.67
	Play	Phase 1	20	0.25	0.79	0	3
		Phase 2	20	0.08	0.18	0	0.50
		Phase 3	20	0.48	1.34	0	5
		Overall	60	0.27	0.90	0	5

Results: Wilcoxon test for treatment comparisons of hormone concentrations and body weight at CRT0

Table S5: Wilcoxon rank sum test of hormone concentrations and body weight calculated for the first cortisol response test (CRT) conducted before treatment.

Wilcoxon rank sum test (CRT0)	W	r	p-value
Baseline cortisol	30	0.118	0.596
Cortisol responsiveness, 1h	31	0.246	0.270
Cortisol responsiveness, 2h	31	0.178	0.427
Baseline testosterone	35.5	0.083	0.711
Body weight	62.5	0.203	0.364

Results: Model summaries of linear mixed effect models for hormone concentrations

Table S6: Model summary from mixed effect model (individual ID as random effect) to determine overall effect of time (CRT), treatment, time*treatment interaction and body weight on baseline cortisol. CRT1 (time) and SM-S (treatment) were set as reference level by default.

Fixed effects		Estimate	Std. error	[95% CI]	t-value	p-value	R^2
Baseline cortisol (Transformation: sqrt(x)) N = 41						*Full model: Marginal R^2*	0.165
						Full model: Conditional R^2	
	Intercept	11.298	1.106	[9.051, 13.546]	10.217	< 0.001	
Treatment		1.541	1.418	[-1.341, 4.423]	1.087	0.285	0.034
CRT1 - CRT2		0.982	1.793	[-2.662, 4.627]	0.548	0.587	0.009
CRT1 - CRT3		2.084	2.255	[-2.500, 6.667]	0.924	0.362	0.025
Body weight		-0.011	0.010	[-0.031, 0.008]	-1.164	0.253	0.038
Treatment*CRT1-CRT2		-3.117	2.115	[-7.416, 1.181]	-1.474	0.150	0.060
Treatment*CRT1-CRT3		-1.033	2.419	[-5.948, 3.883]	-0.427	0.672	0.005

Table S7: Model summary from mixed effect model (individual ID as random effect) to determine overall effect of time (CRT), treatment, time*treatment interaction and body weight on cortisol responsiveness after 1 hour of exposure to a novel environment. CRT1 (time) and SM-S (treatment) were set as reference level by default. Significant ($p < 0.05$) results are indicated in bold.

Fixed effects		Estimate	Std. error	[95% CI]	t-value	p-value	R^2
Cortisol responsiveness 1h (Transformation: sqrt(x)) N = 60						*Full model: Marginal R^2*	0.511
						Full model: Conditional R^2	0.642
	Intercept	28.320	0.837	[26.631, 30.010]	33.836	< 0.001	
Treatment		2.435	1.106	[0.207, 4.662]	2.201	**0.033**	0.085
CRT1 - CRT2		2.312	1.221	[-0.137, 4.761]	1.894	0.064	0.071
CRT1 - CRT3		2.674	1.444	[-0.234, 5.583]	1.852	0.071	0.079
Body weight		-0.033	0.008	[-0.049, -0.017]	-4.315	**< 0.001**	0.374
Treatment*CRT1-CRT2		-3.917	1.342	[-6.641, -1.194]	-2.918	**0.006**	0.105
Treatment*CRT1-CRT3		-2.936	1.339	[-5.654, -0.218]	-2.192	**0.035**	0.062

Table S8: Model summary from mixed effect model (individual ID as random effect) to determine overall effect of time (CRT), treatment, time*treatment interaction and body weight on cortisol responsiveness after 2 hours of exposure to a novel environment. CRT1 (time) and SM-S (treatment) were set as reference level by default. Significant ($p < 0.05$) results are indicated in bold.

Fixed effects		Estimate	Std. error	[95% CI]	t-value	p-value	R^2
Cortisol responsiveness 2h (Transformation: sqrt(x)) N = 55						*Full model: Marginal R^2*	0.175
						Full model: Conditional R^2	0.543
	Intercept	32.914	1.245	[30.383, 35.444]	26.430	< 0.001	
Treatment		1.414	1.609	[-1.856, 4.684]	0.879	0.386	0.011
CRT1 - CRT2		2.031	1.707	[-1.402, 5.465]	1.190	0.240	0.045
CRT1 - CRT3		2.547	2.112	[-1.720, 6.813]	1.206	0.235	0.056
Body weight		-0.017	0.012	[-0.042, 0.008]	-1.431	0.167	0.149
Treatment*CRT1-CRT2		-4.198	1.702	[-7.675, -0.722]	-2.467	**0.020**	0.056
Treatment*CRT1-CRT3		-3.101	1.832	[-6.839, 0.636]	-1.693	0.101	0.032

Table S9: Model summary from mixed effect model (individual ID as random effect) to determine overall effect of time (CRT), treatment, time*treatment interaction and body weight on baseline testosterone. CRT1 (time) and SM-S (treatment) were set as reference level by default.

Fixed effects		Estimate	Std. error	[95% CI]	t-value	p-value	R^2
Baseline testosterone (Transformation: sqrt(x)) N = 59						*Full model: Marginal R^2*	0.053
						Full model: Conditional R^2	
	Intercept	1.475	0.130	[1.215, 1.736]	11.364	< 0.001	
Treatment		-0.094	0.171	[-0.437, 0.249]	-0.549	0.585	0.006
CRT1 - CRT2		0.087	0.197	[-0.307, 0.482]	0.445	0.658	0.004
CRT1 - CRT3		0.001	0.219	[-0.439, 0.441]	0.003	0.997	0.000
Mass		0.001	0.001	[-0.001, 0.002]	0.567	0.573	0.006
Treatment*CRT1-CRT2		-0.070	0.239	[-0.549, 0.409]	-0.293	0.771	0.002
Treatment*CRT1-CRT3		0.130	0.239	[-0.349, 0.609]	0.545	0.588	0.006

Results: Multiple comparisons of linear mixed effect models of hormone concentrations

Table S10: Multiple comparisons (Tukey's) of linear mixed effect model to determine effects of time (CRT), treatment and time*treatment interaction on baseline cortisol.

Baseline cortisol	Estimate	Std. error	df	[95% CI]	t-value	p-value
Pair-wise comparison (between treatment groups)						
CRT1	-1.541	1.438	33.951	[-4.463, 1.381]	-1.072	0.291
CRT2	1.577	1.605	33.909	[-1.686, 4.839]	0.982	0.333
CRT3	-0.508	2.044	33.965	[-4.663, 3.646]	-0.249	0.805
Pair-wise comparison (within treatment groups)						
CRT 1 - CRT 2 (PM-S)	-0.982	1.844	33.998	[-5.502, 3.537]	-0.533	0.856
CRT 1 - CRT 3 (PM-S)	-2.084	2.357	30.936	[-7.886, 3.719]	-0.884	0.654
CRT 2 - CRT 3 (PM-S)	-1.101	1.728	26.512	[-5.391, 3.188]	-0.637	0.801
CRT 1 - CRT 2 (PM+S)	2.135	2.161	33.034	[-3.168, 7.439]	0.988	0.590
CRT 1 - CRT 3 (PM+S)	-1.051	2.335	29.630	[-6.811, 4.709]	-0.450	0.895
CRT 2 - CRT 3 (PM+S)	-3.186	1.944	26.604	[-8.010, 1.637]	-1.639	0.247
Interaction contrasts (treatment*CRT)						
CRT1 - CRT2	3.117	2.152	24.259	[-1.321, 7.556]	1.449	0.160
CRT1 - CRT3	1.033	2.513	29.198	[-4.105, 6.170]	0.411	0.684
CRT2 - CRT3	-2.085	2.604	25.472	[-7.443, 3.273]	-0.801	0.431

Table S11: Multiple comparisons (Tukey's) of linear mixed effect model to determine effects of time (CRT), treatment and time*treatment interaction on cortisol responsiveness after 1 hour of exposure to a novel environment. Significant ($p < 0.05$) results are indicated in bold.

Cortisol responsiveness, 1h	Estimate	Std. error	df	[95% CI]	t-value	p-value
Pair-wise comparison (between treatment groups)						
CRT1	-2.435	1.106	45.448	[-4.662, -0.207]	-2.201	**0.033**
CRT2	1.483	1.113	45.143	[-0.758, 3.724]	1.333	0.189
CRT3	0.502	1.109	45.339	[-1.731, 2.734]	0.452	0.653
Pair-wise comparison (between CRTs)						
CRT 1 - CRT 2 (PM-S)	-2.312	1.229	52.686	[-5.275, 0.651]	-1.882	0.154
CRT 1 - CRT 3 (PM-S)	-2.674	1.457	45.455	[-6.205, 0.856]	-1.835	0.170
CRT 2 - CRT 3 (PM-S)	-0.362	1.000	42.549	[-2.791, 2.066]	-0.362	0.930
CRT 1 - CRT 2 (PM+S)	1.605	1.305	50.823	[-1.546, 4.757]	1.230	0.441
CRT 1 - CRT 3 (PM+S)	0.262	1.509	43.677	[-3.398, 3.922]	0.174	0.984
CRT 2 - CRT 3 (PM+S)	-1.344	0.985	40.817	[-3.740, 1.053]	-1.364	0.369
Interaction contrasts (treatment*CRT)						
CRT1 - CRT2	3.917	1.343	36.269	[1.195, 6.640]	2.918	**0.006**
CRT1 - CRT3	2.936	1.339	35.960	[0.220, 5.653]	2.192	**0.035**
CRT2 - CRT3	-0.981	1.339	35.888	[-3.696, 1.734]	-0.733	0.468

Table S12: Multiple comparisons (Tukey's) of linear mixed effect model to determine effects of time (CRT), treatment and time*treatment interaction on cortisol responsiveness after 2 hours of exposure to a novel environment. Significant ($p < 0.05$) results are indicated in bold.

Cortisol responsiveness, 2h	Estimate	Std. error	df	[95% CI]	t-value	p-value
Pair-wise comparison (between treatment groups)						
CRT1	-1.414	1.611	35.989	[-4.681, 1.853]	-0.878	0.386
CRT2	2.784	1.589	34.510	[-0.444, 6.013]	1.752	0.089
CRT3	1.687	1.731	40.019	[-1.812, 5.186]	0.975	0.336
Pair-wise comparison (between CRTs)						
CRT 1 - CRT 2 (PM-S)	-2.031	1.727	47.495	[-6.208, 2.145]	-1.177	0.473
CRT 1 - CRT 3 (PM-S)	-2.547	2.144	41.493	[-7.757, 2.664]	-1.188	0.467
CRT 2 - CRT 3 (PM-S)	-0.515	1.320	39.457	[-3.730, 2.699]	-0.390	0.920
CRT 1 - CRT 2 (PM+S)	2.167	1.844	44.515	[-2.303, 6.637]	1.175	0.474
CRT 1 - CRT 3 (PM+S)	0.555	2.225	37.425	[-4.875, 5.985]	0.249	0.966
CRT 2 - CRT 3 (PM+S)	-1.612	1.382	38.321	[-4.981, 1.756]	-1.167	0.480
Interaction contrasts (treatment*CRT)						
CRT1 - CRT2	4.198	1.704	31.886	[0.728, 7.669]	2.464	**0.019**
CRT1 - CRT3	3.101	1.838	32.550	[-0.640, 6.843]	1.687	0.101
CRT2 - CRT3	-1.097	1.813	31.909	[-4.790, 2.596]	-0.605	0.549

Table S13: Multiple comparisons (Tukey's) of linear mixed effect model to determine effects of time (CRT), treatment and time*treatment interaction on baseline testosterone.

Baseline testosterone	Estimate	Std. error	df	[95% CI]	t-value	p-value
Pair-wise comparison (between treatment groups)						
CRT1	0.094	0.171	51.967	[-0.250, 0.437]	0.548	0.586
CRT2	0.164	0.167	51.932	[-0.171, 0.499]	0.980	0.332
CRT3	-0.036	0.167	51.955	[-0.370, 0.298]	-0.218	0.828
Pair-wise comparison (between CRTs)						
CRT 1 - CRT 2 (PM-S)	-0.087	0.198	50.284	[-0.565, 0.390]	-0.443	0.898
CRT 1 - CRT 3 (PM-S)	-0.001	0.220	51.403	[-0.533, 0.531]	-0.003	1.000
CRT 2 - CRT 3 (PM-S)	0.087	0.171	38.726	[-0.330, 0.504]	0.507	0.868
CRT 1 - CRT 2 (PM+S)	-0.018	0.200	51.749	[-0.501, 0.465]	-0.088	0.996
CRT 1 - CRT 3 (PM+S)	-0.131	0.221	50.309	[-0.664, 0.403]	-0.592	0.825
CRT 2 - CRT 3 (PM+S)	-0.113	0.170	37.724	[-0.527, 0.301]	-0.667	0.784
Interaction contrasts (treatment*CRT)						
CRT1 - CRT2	0.070	0.239	35.905	[-0.415, 0.555]	0.292	0.772
CRT1 - CRT3	-0.130	0.239	35.737	[-0.614, 0.354]	-0.545	0.589
CRT2 - CRT3	-0.200	0.235	35.050	[-0.677, 0.278]	-0.850	0.401

Results: Model summary of linear mixed effect model for body weight

Table S14: Model summary from mixed effect model (individual ID as random effect) to determine overall effect of time (CRT), treatment and time*treatment interaction on body weight. CRT1 (time) and SM-S (treatment) were set as reference level by default. Significant ($p < 0.05$) results are indicated in bold.

Fixed effects		Estimate	Std. error	[95% CI]	t-value	p-value	R^2
Body weight N = 60						*Full model: Marginal* R^2	0.585
						Full model: Conditional R^2	0.968
	Intercept	359.8	16.834	[324.683, 394.917]	21.374	< 0.001	
Treatment		0.8	23.807	[-48.862, 50.462]	0.034	0.974	< 0.001
CRT1 - CRT2		101.2	6.623	[87.768, 114.632]	15.280	**< 0.001**	0.251
CRT1 - CRT3		143.1	6.623	[129.668, 156.532]	21.606	**< 0.001**	0.401
Treatment*CRT1-CRT2		14.9	9.366	[-4.096, 33.896]	1.591	0.120	0.004
Treatment*CRT1-CRT3		8.6	9.366	[-10.396, 27.596]	0.918	0.365	0.001

Results: Multiple comparisons of linear mixed effect model of body weight

Table S15: Multiple comparisons (Tukey's) of linear mixed effect model to determine effects of time (CRT), treatment and time*treatment interaction on body weight. Significant ($p < 0.05$) results are indicated in bold.

Body weight	Estimate	Std. error	df	[95% CI]	t-value	p-value
Pair-wise comparison (between treatment groups)						
CRT1	-0.800	23.807	19.982	[-50.462, 48.862]	-0.034	0.974
CRT2	-15.700	23.807	19.982	[-65.362, 33.962]	-0.659	0.517
CRT3	-9.400	23.807	19.982	[-59.062, 40.262]	-0.395	0.697
Pair-wise comparison (within treatment groups)						
CRT 1 - CRT 2 (PM-S)	-101.200	6.623	36	[-117.389, -85.011]	-15.280	**< 0.001**
CRT 1 - CRT 3 (PM-S)	-143.100	6.623	36	[-159.289, -126.911]	-21.606	**< 0.001**
CRT 2 - CRT 3 (PM-S)	-41.900	6.623	36	[-58.089, -25.711]	-6.326	**< 0.001**
CRT 1 - CRT 2 (PM+S)	-116.100	6.623	36	[-132.289, -99.911]	-17.530	**< 0.001**
CRT 1 - CRT 3 (PM+S)	-151.700	6.623	36	[-167.889, -135.511]	-22.905	**< 0.001**
CRT 2 - CRT 3 (PM+S)	-35.600	6.623	36	[-51.789, -19.411]	-5.375	**< 0.001**
Interaction contrasts (treatment*CRT)						
CRT1 - CRT2	-14.900	9.366	36	[-33.896, 4.096]	-1.591	0.120
CRT1 - CRT3	-8.600	9.366	36	[-27.596, 10.396]	-0.918	0.365
CRT2 - CRT3	6.300	9.366	36	[-12.696, 25.296]	0.673	0.505

Results: Correlation between body weight and cortisol responsiveness after 1 hour

Table S16: Calculation of correlation coefficient (Pearson) and significance testing for correlations (z-test) between body weight and cortisol responsiveness after 1 hour of exposure to a novel environment. Significant ($p < 0.05$) results are indicated in bold.

Correlation between c1 and body weight	r	t-value	p-value
Within treatment groups			
CRT1 (PM+S)	-0.808	-3.883	**0.005**
CRT2 (PM+S)	-0.687	-2.671	**0.028**
CRT3 (PM+S)	-0.742	-3.131	**0.014**
Overall (PM+S)	-0.586	-3.829	**< 0.001**
CRT1 (PM-S)	-0.258	-0.755	0.472
CRT2 (PM-S)	-0.110	-0.314	0.762
CRT3 (PM-S)	-0.714	-2.885	**0.020**
Overall (PM-S)	-0.350	-1.979	0.058
Comparison between treatment groups		**z-value**	**p-value**
CRT1		-1.606	0.108
CRT2		-1.367	0.172
CRT3		-0.111	0.911
Overall		-1.125	0.261

Results: Adjusted repeatability analysis of hormone concentrations and body weight

Table S17: Adjusted repeatability analysis of linear mixed effects models of baseline cortisol (c0), cortisol responsiveness after 1 hour of exposure to a novel environment (c1), cortisol responsiveness after 2 hours of exposure to a novel environment (c2), baseline testosterone (t) and body weight (m0). Significant ($p < 0.05$) results are indicated in bold.

Repeatability	PM+S				PM-S			
	Std. error	[95% CI]	R	p-value	Std. error	[95% CI]	R	p-value
c0	0.254	[0, 0.805]	0	1	0.232	[0, 0.74]	0.175	0.331
c1	0.155	[0, 0.509]	0.042	0.495	0.196	[0.03, 0.793]	0.453	**0.014**
c2	0.210	[0, 0.786]	0.416	**0.040**	0.194	[0.085, 0.849]	0.523	**0.015**
t	0.164	[0, 0.549]	0.069	0.440	0.143	[0, 0.466]	0	1
m0	0.057	[0.784, 0.977]	0.927	**< 0.001**	0.069	[0.723, 0.97]	0.908	**< 0.001**

Results: Model summaries of generalized linear mixed effect models for behaviour

Table S18: Model summary from generalized linear mixed effect model (individual ID as random effect) to determine overall effect of time (Phase), treatment and time*treatment interaction on sociopositive behaviour. Phase 1 (time) and SM-S (treatment) were set as reference level by default. Significant ($p < 0.05$) results are indicated in bold.

Fixed effects		Estimate	Std. error	[95% CI]	z-value	p-value	R^2
Sociopositive behaviour N = 60						*Full model: Marginal R^2*	0.213
						Full model: Conditional R^2	0.376
	Intercept	0.726	0.294	[0.150, 1.303]	2.469	0.014	
Treatment		-0.004	0.407	[-0.801, 0.794]	-0.009	0.993	< 0.001
Phase 1 - Phase 2		0.311	0.350	[-0.374, 0.996]	0.890	0.374	0.002
Phase 1 - Phase 3		0.917	0.335	[0.261, 1.573]	2.741	**0.006**	0.035
Treatment*Phase 1 - Phase 2		0.430	0.484	[-0.518, 1.378]	0.890	0.374	0.004
Treatment*Phase 1 - Phase 3		0.376	0.471	[-0.548, 1.299]	0.797	0.426	0.007

Table S19: Model summary from generalized linear mixed effect model (individual ID as random effect) to determine overall effect of time (Phase), treatment and time*treatment interaction on sexual and courtship behaviour. Phase 1 (time) and SM-S (treatment) were set as reference level by default.

Fixed effects		Estimate	Std. error	[95% CI]	z-value	p-value	R^2
Courtship and sexual behaviour N = 60						*Full model: Marginal R^2*	0.304
						Full model: Conditional R^2	0.373
	Intercept	-0.607	0.472	[-1.532, 0.318]	-1.286	0.198	
Treatment		-1.344	0.811	[-2.933, 0.245]	-1.658	0.097	0.003
Phase 1 - Phase 2		0.622	0.598	[-0.551, 1.795]	1.040	0.298	0.005
Phase 1 - Phase 3		0.988	0.579	[-0.146, 2.122]	1.707	0.088	0.016
Treatment*Phase 1 - Phase 2		0.555	1.001	[-1.406, 2.516]	0.555	0.579	0.001
Treatment*Phase 1 - Phase 3		1.391	0.950	[-0.471, 3.254]	1.464	0.143	0.001

Table S20: Model summary from generalized linear mixed effect model (individual ID as random effect) to determine overall effect of time (Phase), treatment and time*treatment interaction on play behaviour. Phase 1 (time) and SM-S (treatment) were set as reference level by default.

Fixed effects		Estimate	Std. error	[95% CI]	z-value	p-value	R^2
Play behaviour N = 60						*Full model: Marginal R^2*	0.143
						Full model: Conditional R^2	0.277
	Intercept	-1.567	0.784	[-3.103, -0.031]	-2.000	0.045	
Treatment		0.444	1.048	[-1.611, 2.499]	0.424	0.672	0.005
Phase 1 - Phase 2		-1.176	1.209	[-3.545, 1.194]	-0.972	0.331	0.002
Phase 1 - Phase 3		0.590	0.980	[-1.331, 2.511]	0.602	0.547	0.003
Treatment*Phase 1 - Phase 2		1.203	1.509	[-1.754, 4.160]	0.797	0.425	< 0.001
Treatment*Phase 1 - Phase 3		-0.222	1.331	[-2.830, 2.387]	-0.167	0.868	< 0.001

Results: Multiple comparisons of generalized linear mixed effect models of behaviour

Table S21: Multiple comparisons (Tukey's) of generalized linear mixed effect model to determine effects of time (Phase), treatment and time*treatment interaction on sociopositive behaviour. Significant ($p < 0.05$) results are indicated in bold.

Sociopositive behaviour	Estimate	Std. error	[95% CI]	z-ratio	p-value
Pair-wise comparison (between treatment groups)					
Phase 1	0.004	0.407	[-0.794, 0.801]	0.009	0.993
Phase 2	-0.427	0.381	[-1.174, 0.321]	-1.119	0.263
Phase 3	-0.372	0.362	[-1.081, 0.337]	-1.028	0.304
Pair-wise comparison (within treatment groups)					
Phase 1 - Phase 2 (PM-S)	-0.311	0.350	[-1.131, 0.508]	-0.890	0.647
Phase 1 - Phase 3 (PM-S)	-0.917	0.335	[-1.702, -0.133]	-2.741	**0.017**
Phase 2 - Phase 3 (PM-S)	-0.606	0.329	[-1.378, 0.165]	-1.842	0.156
Phase 1 - Phase 2 (PM+S)	-0.742	0.334	[-1.524, 0.041]	-2.220	0.068
Phase 1 - Phase 3 (PM+S)	-1.293	0.331	[-2.070, -0.516]	-3.900	**< 0.001**
Phase 2 - Phase 3 (PM+S)	-0.551	0.307	[-1.271, 0.169]	-1.795	0.171
Interaction contrasts (treatment*CRT)					
Phase 1 - Phase 2	-0.430	0.484	[-1.378, 0.518]	-0.890	0.374
Phase 1 - Phase 3	-0.376	0.471	[-1.299, 0.548]	-0.797	0.426
Phase 2 - Phase 3	0.055	0.449	[-0.826, 0.936]	0.122	0.903

Table S22: Multiple comparisons (Tukey's) of generalized linear mixed effect model to determine effects of time (Phase), treatment and time*treatment interaction on courtship and sexual behaviour. Significant ($p < 0.05$) results are indicated in bold.

Courtship and sexual behaviour	Estimate	Std. error	[95% CI]	z-ratio	p-value
Pair-wise comparison (between treatment groups)					
Phase 1	1.344	0.811	[-0.245, 2.933]	1.658	0.097
Phase 2	0.789	0.653	[-0.491, 2.069]	1.208	0.227
Phase 3	-0.047	0.560	[-1.145, 1.050]	-0.084	0.933
Pair-wise comparison (within treatment groups)					
Phase 1 - Phase 2 (PM-S)	-0.622	0.598	[-2.025, 0.780]	-1.040	0.552
Phase 1 - Phase 3 (PM-S)	-0.988	0.579	[-2.344, 0.368]	-1.707	0.202
Phase 2 - Phase 3 (PM-S)	-0.366	0.557	[-1.672, 0.941]	-0.656	0.789
Phase 1 - Phase 2 (PM+S)	-1.177	0.800	[-3.053, 0.698]	-1.472	0.305
Phase 1 - Phase 3 (PM+S)	-2.379	0.758	[-4.156, -0.603]	-3.139	**0.005**
Phase 2 - Phase 3 (PM+S)	-1.202	0.592	[-2.588, 0.185]	-2.032	0.105
Interaction contrasts (treatment*CRT)					
Phase 1 - Phase 2	-0.555	1.001	[-2.516, 1.406]	-0.555	0.579
Phase 1 - Phase 3	-1.391	0.950	[-3.254, 0.471]	-1.464	0.143
Phase 2 - Phase 3	-0.836	0.818	[-2.440, 0.767]	-1.022	0.307

Table S23: Multiple comparisons (Tukey's) of generalized linear mixed effect model to determine effects of time (Phase), treatment and time*treatment interaction on play behaviour.

Play behaviour	Estimate	Std. error	[95% CI]	z-ratio	p-value
Pair-wise comparison (between treatment groups)					
Phase 1	-0.444	1.048	[-2.499, 1.611]	-0.424	0.672
Phase 2	-1.647	1.209	[-4.016, 0.722]	-1.363	0.173
Phase 3	-0.223	0.946	[-2.076, 1.631]	-0.235	0.814
Pair-wise comparison (within treatment groups)					
Phase 1 - Phase 2 (PM-S)	1.176	1.209	[-1.658, 4.009]	0.972	0.594
Phase 1 - Phase 3 (PM-S)	-0.590	0.980	[-2.887, 1.707]	-0.602	0.819
Phase 2 - Phase 3 (PM-S)	-1.766	1.164	[-4.494, 0.963]	-1.517	0.283
Phase 1 - Phase 2 (PM+S)	-0.027	0.910	[-2.160, 2.106]	-0.030	1.000
Phase 1 - Phase 3 (PM+S)	-0.368	0.891	[-2.456, 1.719]	-0.413	0.910
Phase 2 - Phase 3 (PM+S)	-0.341	0.886	[-2.419, 1.737]	-0.385	0.922
Interaction contrasts (treatment*CRT)					
Phase 1 - Phase 2	-1.203	1.509	[-4.160, 1.754]	-0.797	0.425
Phase 1 - Phase 3	0.222	1.331	[-2.387, 2.830]	0.167	0.868
Phase 2 - Phase 3	1.425	1.461	[-1.439, 4.289]	0.975	0.330