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

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Abstract

Individuals can adjust to different social environments via plastic shaping of behavioural and endocrine phenotypes. As the social environment can change at any time, individuals need to be able to adjust throughout their lives. Our goal was therefore to elucidate when and how behavioural and hormonal adjustments in guinea pigs occur. 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. Socially stimulated males showed different adjustments to their social environment in comparison to non-socially stimulated males. They displayed an initially increased stress responsiveness, 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 responsiveness 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 juvenility the social environment induced hormonal adjustments and behavioural changes in male guinea pigs, thereby highlighting how early-life social experiences can shape individuals' phenotypes.

Keywords

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

1. Introduction

Individuals can adjust to different social environments via plastic shaping of behavioural and endocrine phenotypes [1, 2], likely to result in an optimized phenotype-environment match [1]. Individuals failing to adjust to their (social) environment can experience a variety of different negative consequences [3, 4]. Such maladaptive responses to the social environment have been reported in non-human primates, mice and rats, where incompatible groups resulted in severe aggression, impaired immune function and elevated stress levels [5]. In guinea pig males, for example, different social environments (high vs. low population density) during adolescence shape different behavioural reproductive strategies. While colony-reared male guinea pigs could integrate into an unknown mixed-sex colony through a low-aggressive queuing strategy, males reared in heterosexual pairs initially failed to integrate as they were persistently courting females and fighting males (high-aggressive resource defense strategy) [6]. This high-aggressive tactic led to risk of injury, elevated stress levels and significantly declined body weight [6]. On the other hand, when formerly colony- and pair-housed males were placed as pairs into a competitive reproductive situation with unknown females, pair-housed males displaying such a high-aggressive tactic also had higher reproductive success [7], demonstrating the fitness consequences of phenotype-environment match. These behavioural tactics as well as adjustments to the social environment in general can be mediated through underlying endocrine mechanisms [8]. In this context, the hypothalamic-pituitary-adrenocortical (HPA) axis has a central function. The HPA axis is one of the most important neuroendocrine stress systems and regulating the secretion of glucocorticoids [9–11]. Glucocorticoids are metabolic hormones and have important functions regarding stress response since they are involved in the regulation of metabolic processes, immune functions, behaviour and cognitive functions [12]. Hence, the stress response affects the activation and development of multiple traits, is environmentally sensitive [13] and thus plays a major role for plastic responses to the environment. There are several studies exemplifying how the social environment influences the HPA axis and behaviour. In male guinea pigs for example, frequent social interactions in colony-housed males are associated with reduced HPA activity and a low-aggression phenotype to facilitate group integration, whereas pair-housed males with limited options for social interactions show an increased HPA activity which promotes aggressiveness [7, 14]. In male mice, isolated individuals displayed an enhanced HPA reactivity and thus increased anxiety-like behaviour in comparison to socially housed individuals [15–17]. While exposure to chronic stress is typically associated with various negative consequences, such enhanced endocrine and behavioural responses to stress can reflect greater vigilance to environmental threats, which can promote survival [18].

These examples demonstrate how plastic adjustments of behavioural and endocrine phenotypes allow individuals to effectively cope with their social environment. However, the social environment is rarely

91 static throughout life time but can change at any time. In consequence, individuals should be able to
92 adjust to their social environment throughout life time, too, meaning plasticity of behavioural and
93 endocrine phenotypes should be found in all ontogenetic phases. Still, most research on the shaping of
94 endocrine and behavioural phenotypes through the social environment focused on the early life phase
95 (prenatal and lactation phase) and adolescence. These phases are referred to as sensitive windows of
96 enhanced plasticity [7, 19, 20] and it was often assumed that this plasticity declines with age and can no
97 longer be found beyond adolescence. This assumption was historically based on the belief that the
98 development of the adult brain is completed and that neuronal plasticity thus no longer exists [21].
99 However, it is known for several decades now that adult neurogenesis, i.e., the addition of new neurons
100 to the brain, occurs [21]. In consequence, plasticity of behavioural and endocrine phenotypes in
101 response to the (social) environment is also possible in adult individuals and has been reported across
102 multiple taxa [22–26].

103 On the other hand, it is known that the levels of neurogenesis are significantly higher during the juvenile
104 phase than in adulthood [27]. Juvenility, i.e., time between weaning and adolescence, is an important
105 developmental phase where the focus on social interactions starts to shifts from parents to peers. Social
106 exploration and play behaviour increase during juvenility, suggesting this phase represents an important
107 window for socialization [27]. While a lot of studies are investigating the effects of the early social
108 environment on behaviour and hormones in later life [19, 28–31], research on the effects on juvenile
109 individuals themselves are still scarce. One of the few studies shows that in great tits, for example,
110 nestlings from small broods showed a stronger stress response than nestlings from normal-sized broods
111 [32]. In African cichlid fish, pair-reared juveniles were less active and more subordinate than group-
112 reared juveniles and glucocorticoid receptors were elevated in the latter [33]. These findings further
113 indicate that the juvenile phase represents another sensitive window for plastic shaping of behavioural
114 and endocrine phenotypes. However, comparable evidence from mammals is still lacking. Guinea pigs
115 are a well-suited model organism to address this since they have a high flexibility regarding their social
116 organization [34] and plastic shaping of behavioural and endocrine phenotypes in other life phases has
117 already been investigated in this species [7, 19, 26, 30, 35–39]. The aim of the present study was
118 therefore to investigate how juvenile male guinea pigs adjust to two different social environments
119 through shaping of their behavioural and endocrine phenotype.

120 For this purpose, juvenile male guinea pigs were housed in the following two groups: one group was
121 housed in heterosexual pairs; thus, they had only one female interaction partner. The other group was
122 also housed in heterosexual pairs, but in addition, was socially stimulated by regular encounters with
123 unfamiliar animals of both sexes to increase the amount of opportunities to interact with conspecifics.
124 We repeatedly measured hormone concentrations (cortisol responsiveness, baseline cortisol, baseline

testosterone) as well as body weight and observed home-enclosure behaviour (sexual/ courtship, sociopositive, play and agonistic behaviour) throughout the experiment.

Regarding cortisol responsiveness, earlier studies applying a similar approach in adolescent male guinea pigs found that social stimulation significantly affected stress (cortisol) responsiveness [35, 36]. Thus, also in this study social stimulation was hypothesized to shape cortisol responsiveness differently than regular pair-housing in non-stimulated males. We also examined testosterone concentrations and compared them between treatment groups. Previous research in adolescents indicated that frequent social interactions can increase testosterone levels, which might have inhibiting effects on the HPA axis and thus decrease cortisol responsiveness [11, 35, 36, 40, 41]. Moreover, we assessed baseline cortisol concentrations and body weight, since animals that are not adjusted to their (social) environment can show increased cortisol levels and decreased body weight [4]. In consequence, differences in baseline cortisol levels and/ or body weight between treatment groups could indicate that the respective individuals did not adjust to their social environment. Additionally, body weight served as a fitness proxy. The early-life (social) environment can influence juvenile body weight [42, 43], which in turn predicts various fitness-related traits, such as juvenile survival [42, 44], age at maturity [45], age at first reproduction [46] or longevity [47].

Besides group comparisons, we also conducted repeatability analyses of hormone concentrations and body weight by determining the proportion of variance attributable to individual differences. The measure of repeatability is typically calculated to assess the stability of a trait within individuals by quantifying the consistency across repeated measurements. While only a few studies have addressed how environmental factors shape repeatability of such physiological traits, there is evidence that social complexity can influence individual stability of endocrine phenotypes in guinea pigs [48]. In consequence, traits that are both individually stable and environmentally sensitive are particularly relevant for studying how individuals adjust to different social environments. Therefore, we investigated whether repeatability of such traits differed between the two housing conditions, too.

For the analysis of behavioural phenotypes, we assessed sexual and courtship behaviour as well as sociopositive, play and agonistic behaviour. As it is known that these behaviours can be influenced by the early social environment [7, 29, 33, 49], we hypothesized that these behaviours also differ in stimulated and non-stimulated males.

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.

158 They were born and reared in a total of six to eight harem groups within one breeding room, each
159 consisting of one male, one to three females and their pre-weaned offspring. The offspring was routinely
160 taken out of the harems after weaning at post-natal day (PND) 21 (±1) and adults were removed and
161 replaced at around 18-24 months of age. Each harem was kept in wooden enclosures with a base area
162 of approximately 1.5 m^2 and a wall height of 0.5 m. The enclosures were filled with wood shavings
163 (Tierwohl Super, J. Rettenmaier & Söhne GmbH + Co KG, Rosenberg, Germany) as bedding and enriched
164 with red plastic shelters and wooden bridges.

165 The experimental animals were transferred to enclosures in a different housing room after weaning at
166 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
167 wood shavings and enriched with a big and a small red plastic shelter. Food (hasfit Cavia C pellets,
168 EQUOVIS GmbH, Münster, Germany) and water were available *ad libitum*. Since guinea pigs are
169 incapable of synthesizing ascorbic acid [50, 51] and therefore prone to vitamin C deficiency [52], a
170 vitamin C supplement (100% L-ascorbic acid, altapharma, Dirk Rossmann GmbH, Burgwedel, Germany)
171 was added to the water three times per week (approximately 120 mg vitamin C in 900 ml water shared
172 between the focus male and his female partner). Additionally, hay was replenished daily and fresh
173 fodder (carrots, cucumbers, apples) was fed regularly. All guinea pig housing rooms were kept under
174 controlled conditions with a 12 h: 12 h light/ dark cycle (lights on at 07:00), temperature of
175 approximately 22 °C and relative humidity of approximately 48 %.

176 ## 2.2 Experimental design

177 For this study, twenty male guinea pigs were used. The experimental phase started after weaning at
178 PND 21 (±3) and lasted six weeks, meaning the animals were 60 (±3) days of age when the experiments
179 ended. In guinea pig males, sexual maturity is usually reached around PND 70 [45]. Each male was paired
180 with a female which was the same age. The male and his respective female partner stem from different
181 harem groups, meaning they were neither half nor full siblings. To investigate the influence of distinct
182 social environments on behavioural and hormonal phenotypes, they were randomly assigned to one of
183 two treatment groups. Males of both groups lived in heterosexual pairs, but males of one group were
184 socially stimulated (see 2.3) regularly (pair-housed male with social stimulation; PM+S group), while
185 males of the other group were not (pair-housed male without social stimulation; PM-S group). The
186 twenty males were organized into ten experimental pairs, each consisting of one PM+S male and one
187 PM-S male. To control for variability in housing conditions the home enclosures of both males within an
188 experimental pair- each housed together with their respective female partner- were placed side by side
189 in the same housing room. All experimental procedures (except for social stimulations) were conducted
190 in parallel within each experimental pair to minimise variability in timing of the experiments.

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**). The shorter interval between CRT2 and CRT3 was chosen because guinea pigs reach the end of the juvenile phase and the onset of early adolescence around PND 55 [35]. Extending the interval to 14 days would have meant that some males might already have reached sexual maturity. Social stimulation and recording of home enclosure behaviour were each conducted three times per week during the whole experimental phase. Both procedures were randomly distributed across the week to avoid possible habituation effects and to observe behaviour on different day times. As a results, it was possible that CRTs, social stimulations and video recordings happened on the same day.

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. These procedures took place in the same week as CRT3. The procedural order of CRT3, behavioural tests and fur swabbing varied across individuals, but was identical within each experimental pair. The focus males never experienced more than one of these procedures per day. More detailed descriptions of the behavioural tests can be found in the supplementary material (**Tab. S1**).

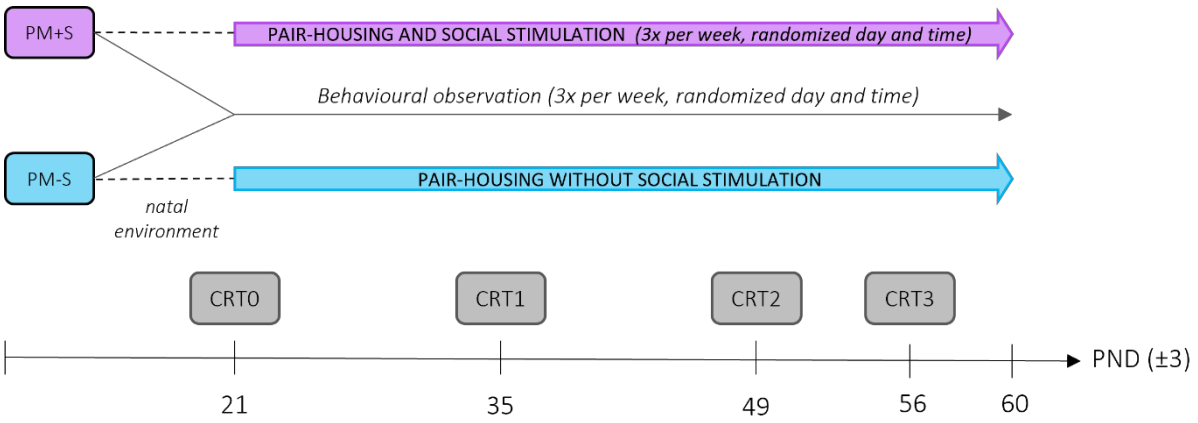

Figure 1: Procedure of behavioural observations in the home enclosure and cortisol response tests (CRT). Focal males were housed with a female partner. One group (PM+S) was regularly stimulated by introducing other individuals into the home enclosure while the other group (PM-S) was not. This social 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 procedure applied in the present study was adapted from Lürzel and colleagues [35, 36], where social stimulation successfully influenced hormonal profiles in adolescent guinea pig males. The social stimulation treatment for the stimulated males (PM+S) started after the first CRT. From then on, social stimulation was applied three times per week for the whole experimental phase.

Social stimulation was conducted by introducing an unknown individual 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, preventing a confounding influence of oestrus. While stimulus females were always adult, the age of stimulus males ranged from 44 to 994 days in total. However, the vast majority of social stimulations with stimulus males was conducted with a male that was at least 100 days old and thus adult. In only six out of 120 cases in total, three focus males were stimulated with a male younger than 100 days. Still, they were always older than the focus male. The pool of stimulus males for each PM+S male included eight to twelve individuals and the pool of stimulus females four to six individuals. The overall pool of stimulus animals changed over time as stimulus animals left the experiment and new ones were added at irregular intervals. In total, 29 stimulus animals were used for the whole experiment. Among those, kinship relations existed. Specifically, the overall pool of stimulus animals included four full sibling pairs, 25 half-sibling pairs and five parent-offspring pairs. However, not all stimulus animal were related, and no focus male was stimulated exclusively with stimulus animals that were all related to each other. 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.

PM+S males never experienced more than one social stimulation session per day and the day and time of day at which social stimulation occurred was varied in order to avoid possible habituation effects by introducing unpredictability. 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. Out of a total of 120 stimulation sessions using males as stimulus animals, eight had to be terminated because aggression escalated.

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 at least two 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, a minimum of

12 h of home enclosure behaviour was collected for each individual, with some animals contributing up to 18 h. The total observation time was the same for one PM+S male and one PM-S male within an experimental pair. Since behaviour was analysed as frequency per hour, variation in total observation time was accounted for. 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 (Tab. 1).

Table 1: 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.

2.5 Assessment of hormone concentrations

Hormones were measured using blood samples obtained in cortisol response tests (CRTs), a standardized test used to measure the endocrine stress response to a challenge [53]. The male guinea pigs were exposed to the stressor of exposure to a novel environment [54] and stress responses at different time points were assessed by sampling blood. The test started between 12:30 and 13:30, as plasma cortisol concentrations fluctuate throughout the day and a peak is observed at 13:00 [55]. 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 [55]. 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 responsiveness. 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 [56]. A priori sample-size calculation was conducted using the software G*Power version 3.1.9.7 [57]. 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 [54, 58]. 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* [59] and *lmerTest* package [60]. 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 for the following reasons: first, the focus of this study was to analyse the effects of social stimulation on hormone concentrations over time, and CRT0 was conducted before social stimulation started. Second, it is known that young animals generally show higher corticosteroid concentrations due to the ongoing maturation of the HPA axis [30]. In their study, Kaiser and Sachser [30] found high baseline cortisol levels in guinea pigs at 20 days of age, which declined by day 34 and remained constant thereafter, marking the completion of HPA axis maturation. Since CRT0 was performed when the experimental animals were around 21 days of age, including this data in the analyses would likely have introduced outliers caused

by these maturation processes, potentially confounding the interpretation of treatment-related effects. 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* [61] and *DHARMa* package [62] to check model assumptions. Marginal and conditional R^2 values were calculated using the *performance* package [61], while partial R^2 values for individual predictors were calculated using the *sensemakr* package [63]. Pair-wise comparisons for treatment, CRT and treatment*CRT interaction were done by applying Tukey's adjustment for multiple comparison using the *emmeans* package [64].

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 [65].

Adjusted repeatability estimates of hormone concentrations and body weight were calculated for each of the treatment groups using the *rprR* package [66]. 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 [61]. Therefore, we pooled behaviour into three categories: courtship and sexual behaviour, social behaviour and play, with individual behaviours being summed within each category. 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 [59]. 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" (1st and 2nd experimental week), "Phase 2" (3rd and 4th experimental week) and "Phase 3" (5th and 6th 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 [64].

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 (see supplementary material, Tab. S5).

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.

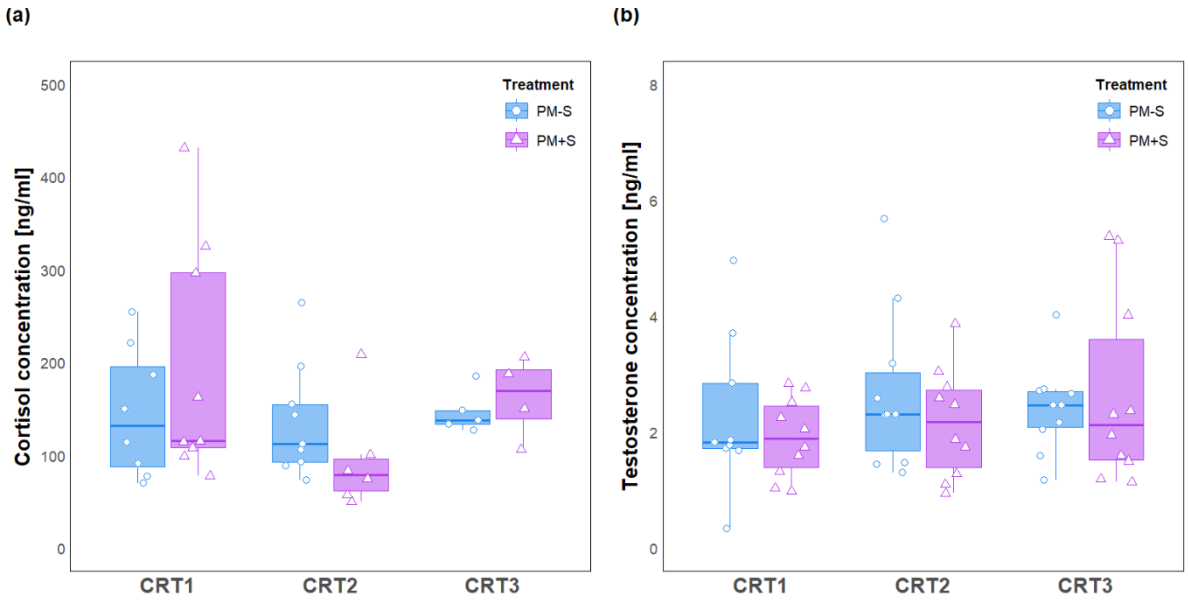

Figure 2: Baseline cortisol **(a)** and testosterone **(b)** concentrations (ng ml^{-1}) two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are medians (horizontal marks), first to 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. 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.

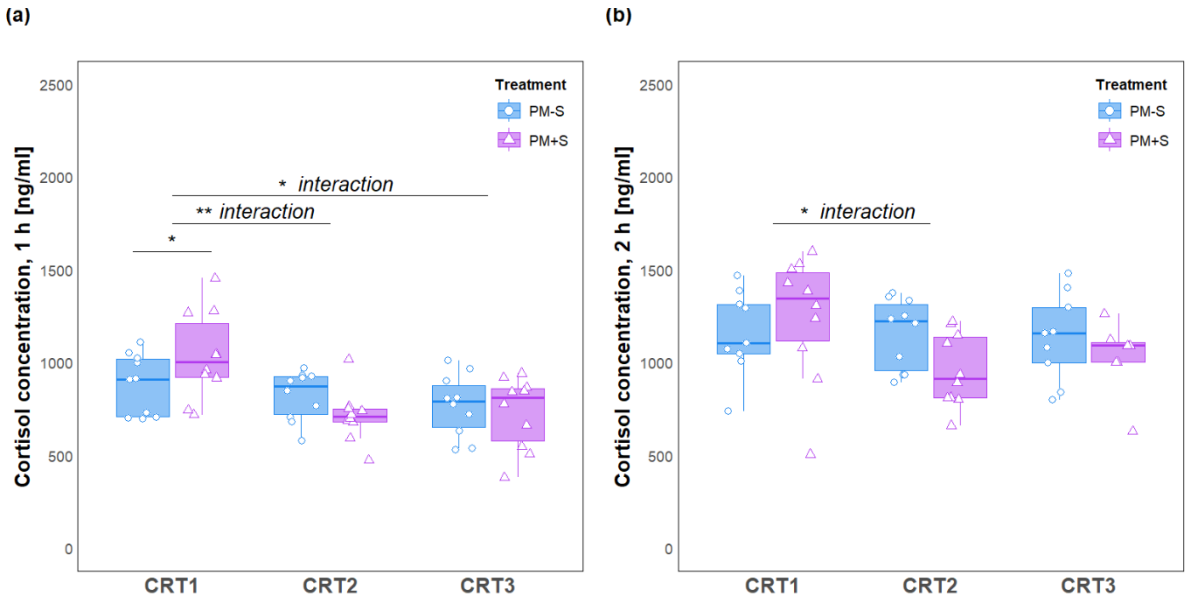

Figure 3: Cortisol concentrations (ng ml^{-1}) at one hour **(a)** and two hours **(b)** of exposure to a novel environment two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are medians (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 (see supplementary material, **Tab. S5**).In both treatment groups, body weight significantly increased over time: In the PM-S group, significant effects occurred 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). In the PM+S group, significant effects also occurred from CRT1 to CRT2 (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**).

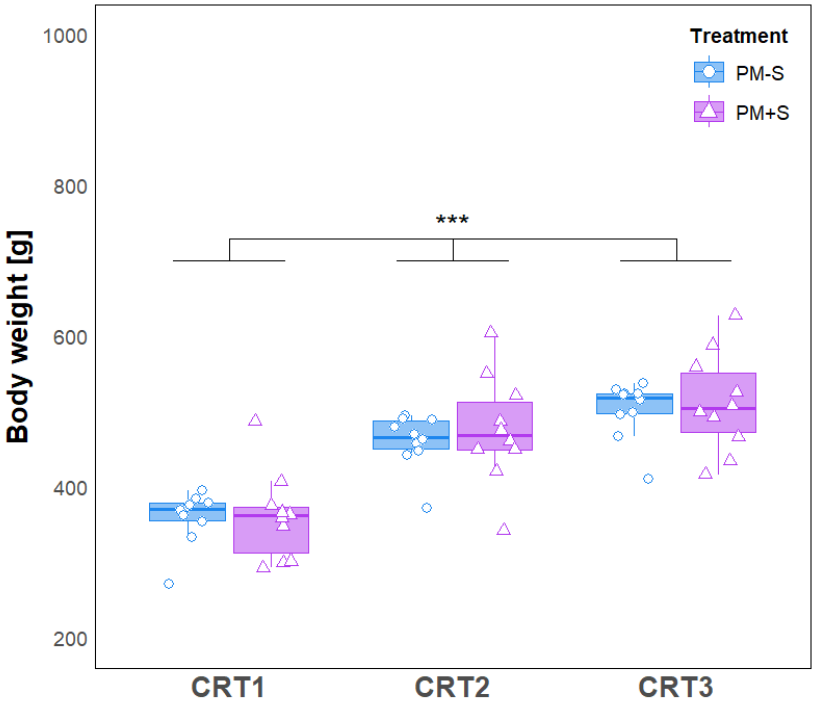

Figure 4: Body weight measured two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are medians (horizontal marks), first to third quartiles (boxes), whiskers and all data points. Statistics: 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 (ß = -0.03 ± -0.01 t = -4.32, $p < 0.001$). 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 negative correlation in the PM+S group ($r = -0.74$, $t = -3.13$, $p = 0.014$) and a significant, strong negative 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**.

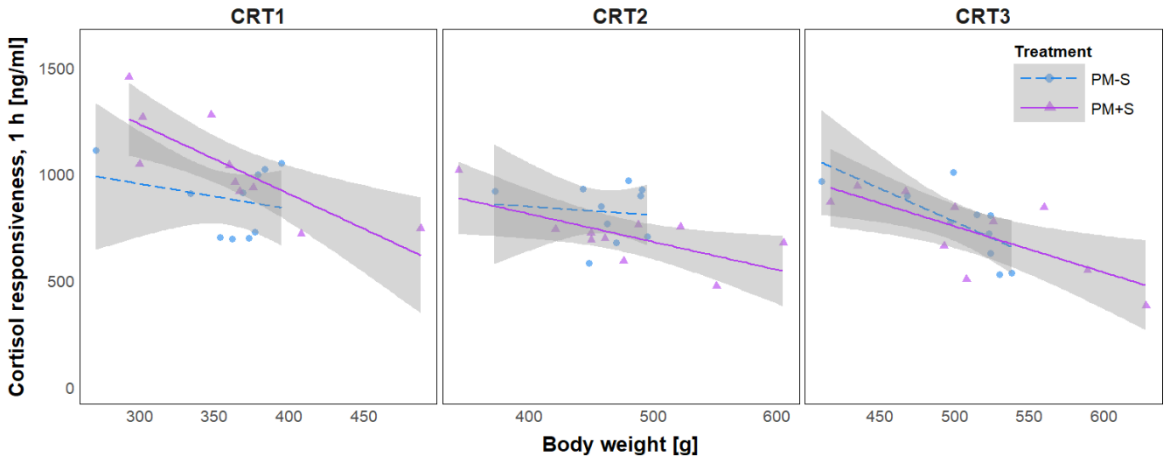

Figure 5: Correlation between cortisol concentrations (ng ml^{-1}) at one hour of exposure to a novel environment and body weight two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted 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) and had a low repeatability (R = 0.18, CI = [0, 0.74], p = 0.331) in the PM-S group. 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 both 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 both 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).

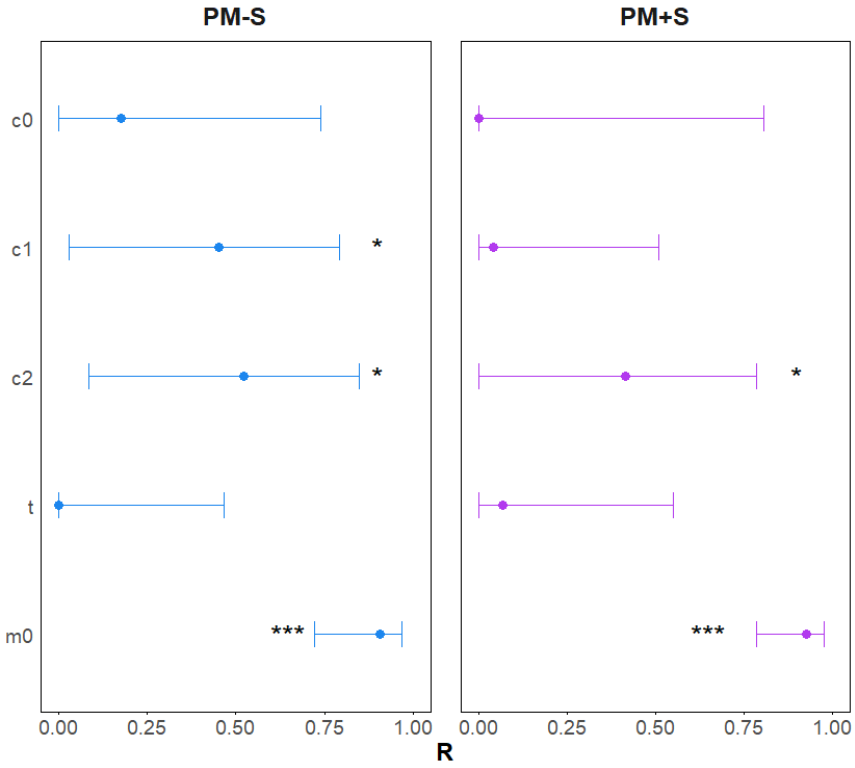

Figure 6: Repeatability (R) of baseline cortisol (c0), cortisol responsiveness after 1 (c1) and 2 hours (c2) of exposure to a novel environment, baseline testosterone (t) and body weight (m0). Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are adjusted repeatability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

3.2 Effects of social environment on social behaviour

For sociopositive behaviour, there was a significant effect of time (phase) between phase 1 and phase 3 for both the PM-S group (ß = -0.92 ± 0.34, z = -2.74, p = 0.017) and the PM+S group (ß = -1.29 ± 0.33, z = -3.9, p < 0.001)." In both groups, the frequency of sociopositive behaviour increased over time (**Fig. 7a**). Furthermore, a significant increase of courtship and sexual behaviour was only found in the PM+S group (ß = -2.38 ± 0.76, z = -3.13, p = 0.005) (**Fig. 7b**). Play behaviour was observed in both treatment groups (see supplementary material, **Tab. S4**), however, neither a significant effect of treatment or time (phase), nor a significant treatment-by-time interaction effect was found.

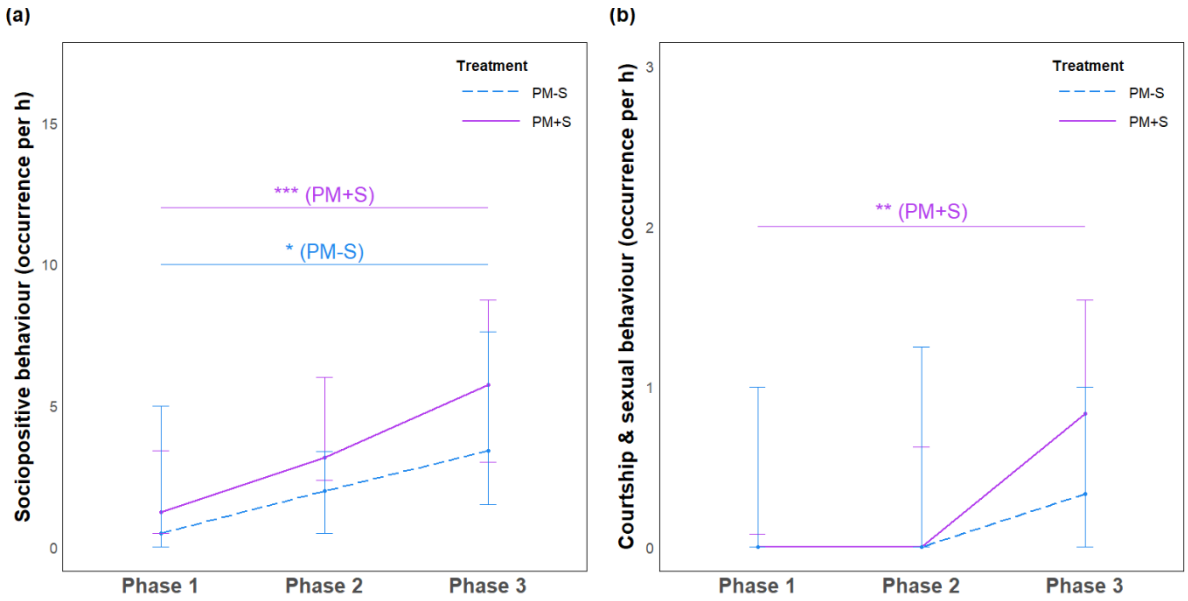

Figure 7: Frequency (occurrence per h) of **(a)** sociopositive behaviour and **(b)** courtship and sexual behaviour in the first (phase 1), second (phase 2) and third (phase 3) two weeks of treatment. Males in heterosexual pairs were either socially stimulated (PM+S) or not (PM-S). Plotted are medians (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 environment through possible shaping of behavioural and hormonal phenotypes. By repeatedly analysing behavioural and hormonal parameters during juvenility, we aimed to explore when and how adjustments through plastic shaping occur 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, too, but 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 Modulation of cortisol responsiveness by the social environment

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. In earlier studies, social stimulation led to a significantly decreased cortisol responsiveness in adolescent male guinea pigs compared to non-stimulated males [35, 36]. Those studies however compared cortisol responsiveness before and after the end of the social stimulation treatment, whereas the heightened stress response

in stimulated males in this study occurred during the treatment phase. One might argue that two weeks of social stimulation constituted a stressful environment, as shown in other rodent studies where short-term (one to two weeks) exposure to chronic mild or unpredictable stress increased basal serum corticosterone levels [67, 68]. But in this study, baseline cortisol values did not differ between males of both treatment groups, suggesting 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 [69, 70]. Consequently, the heightened stress response to this unpredictable environment presumably constitutes an adjustment process in stimulated males. This adjustment could also be interpreted as a process of *social niche conformance*. The concept of individualized social niches has recently gained prominence in behavioural biology and can be understood as "unit consisting of a focal individual and only those social interactions with other conspecific individuals that influence the focal individual's inclusive fitness" [1]. Within this framework, social niche conformance describes the process where individuals adjust to an existing social environment, for example by adjustments of the behavioural or hormonal phenotype [1, 8, 71, 72]. In line with this interpretation, the significant decrease in cortisol responsiveness found in stimulated males in the subsequent CRTs could also reflect such a conformance process. At the end of the experimental phase cortisol responsiveness of stimulated and non-stimulated males almost converged, indicating juvenile males could adjust to the challenging situation. A stress-induced HPA activation is metabolically costly. Thus, it is adaptive for an organism to reduce HPA activity to stressors without harm [73]. Taken together, these findings support our assumption that cortisol responsiveness is plastic and shaped by the social environment in juveniles as well.

Finally, we investigated the repeatability of hormone concentrations because previous studies suggested that the (social) environment can influence the repeatability of physiological traits [48, 74–76]. In this study, repeatability estimates did not differ systematically between treatment groups. However, these results should be interpreted with caution, as the confidence intervals were wide and either close to or included zero [77, 78]. Nevertheless, our findings align with a general pattern reported in a meta-analysis, showing that repeatability estimates tend to be higher for peak hormone levels than for baseline levels [79]. The reason for this might be 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 [74, 80].

4.2 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 [34]. Moreover, the occurrence of play behaviour is an indicator of positive affective state [81] and of environmental conditions that are perceived as safe and non-stressful [82]. The results for baseline cortisol already suggest that social stimulation per se did not lead to prolonged higher stress levels. The occurrence of play behaviour in both treatment groups and the almost complete absence of agonistic behaviour further support this conclusion [82–84].

Regarding sexual and courtship behaviour, we hypothesized that stimulated and non-stimulated males show differences in these behaviours, based on previous research demonstrating that early social experience influences sexual behaviour. For example, male guppies reared with adult males showed earlier and more frequent courtship displays than males reared with adult females only [29]. In our study, this hypothesis was only partly supported: although no significant differences between treatment groups were found, sexual and courtship behaviour significantly increased over time in stimulated males only. An earlier onset of sexual maturity in those males seems unlikely, as this is typically accompanied by a peak in testosterone in male rodents [85, 86]. In this study, however, 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 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) [87].

Instead, two non-exclusive explanations could account for the results obtained here. First, it is possible that 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 [29]. Second, dramatic changes in testosterone levels are not necessarily required for the development of sexual behaviour. In rats, males pair-housed during adolescence displayed more sexual behaviour than single-housed males, regardless of pubertal testosterone levels [88]. This suggests that social experiences during critical developmental phases can organize neural structures that mediate sexual behaviour [88, 89]. Similarly, social stimulation during the juvenile phase may have promoted the development of neural circuits underlying sexual and courtship behaviour through increased opportunities to observe and practice courtship behaviour. Thus, the observed increase in sexual and courtship behaviour in stimulated males likely

reflects a social niche conformance process, helping individuals to adjust to a more complex social environment with multiple potential mating opportunities.

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 [90]. A meta-analysis in mammals and birds has shown that this also applies to juvenile animals as juvenile body weight was found to be a reliable indicator of juvenile survival [44]. A larger body weight can also indirectly influence reproduction via a link to higher dominance status in social systems, as it was already demonstrated in guinea pigs and cavies [91, 92]. Juvenile body weight can be shaped by the (social) environment [42, 43]. In Florida Scrub-Jays for example, brood size and natal group size determined body mass, which in turn was a significant positive predictor of juvenile survival [42]. Thus, we hypothesized that juvenile body weight would also differ between stimulated and non-stimulated males. However, no differences regarding body weight were found between the treatment groups in this study. Furthermore, repeatability was very high in both stimulated and non-stimulated males, indicating that body weight in guinea pigs is a stable individual trait independent of social environment.

Interestingly, body weight was significantly negatively correlated with cortisol responsiveness after 1 hour. In guinea pigs, cortisol responsiveness after 1 hour reflects the speed of the stress response, whereas cortisol responsiveness after 2 hours indicates its magnitude [38, 93]. The observation of only cortisol responsiveness after 1 hour, but neither baseline cortisol levels nor cortisol responsiveness after 2 hours being negatively affected by body weight, indicates guinea pig males with higher body weights have a slower cortisol response. This would mean the maximum stress response might not be different between bigger and smaller individuals, but the time it takes to reach this maximum. In rats, diet-induced obese animals were hyporesponsive to chronic stress [94] and had a blunted stress response following psychosocial stress exposure [95]. Reasons for this could involve body weight dependent differences in the adrenal gland [96] and availability or secretion of cortisol or cortisol binding globulins [97, 98]. However, as the animals in this study were neither obese nor under chronic stress, it remains unclear whether similar physiological processes were involved here.

Even though no statistical differences between treatment groups could be found (which might become detectable with a larger sample size), the negative correlation between body weight and cortisol responsiveness seemed to be more pronounced in socially stimulated males. At the end of the experimental phase, however, the correlation between body weight and cortisol responsiveness in non-stimulated males was almost as high as in stimulated males and also significant. This might suggest an earlier onset of the effect that causes higher body weight to negatively influence cortisol responsiveness in socially stimulated males. Previous studies in mice and rats have shown that social factors can for

example influence adrenal gland weight and thus HPA reactivity [99–101], but the mechanisms underlying such patterns in guinea pigs remain largely unexplored and need further investigation. For such an approach, future studies should not only consider body weight but also incorporate measures such a leptin or body composition analysis [102, 103] to distinguish whether fat or muscle mass accounts for observed differences.

4.4 Conclusion

The concept of social niche conformance describes how individuals change their phenotype in response to the social environment and thus modify their phenotype-environment match [1]. In this study, social niche conformance processes were evident in juvenile male guinea pigs: social stimulation shaped both cortisol responsiveness and sexual/ courtship behaviour to promote adjustments to a more complex social environment. These findings emphasize juvenility as another important phase during which early-life experiences can shape hormonal and behavioural phenotypes.

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

Open data/code are not available yet but will be accessible with peer-reviewed publication.

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Appendix A. Supplementary data

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Supplementary material

Material and Methods:

Table S1: Descript*ion of the behavioural tests conducted in the last experimental week.*

Behavioural test	Category	Description
Step-down test	Risk-taking behaviour	The focus male was placed on a sheltered, square platform (900 cm^2) positioned 23 cm above the ground. The platform was inside a test enclosure (1 m^2) filled with wood shavings and located in a different housing room than the focus male's home enclosure. After the focus male was put on the platform, the experimenter gently held him for a few seconds until the initial flight response subsided. The experimenter then stepped back and stopped the time until the focus male jumped of the platform within a maximum time of 15 minutes.
Social initiative test	Social behaviour	The test was conducted in an enclosure (1 m^2) located in a different housing room than the focus male's home enclosure. The enclosure was filled with wood shavings and contained two grated metal baskets (25 x 19 x 14 cm) in its middle. The metal baskets were turned upside down and spaced approximately 30 cm from each other. One basket was empty and the other contained a pre-weaned male guinea pig which was placed there prior to the test. The focus male was put in the enclosure and the experimenter left the room. After 15 minutes of visual and olfactory contact between the animals, the metal baskets were removed, allowing free interaction. The experimenter left the room during the following additional 15 minutes, after which the test was concluded. The entire session was videotaped.
Male-female interaction test	Social behavior	The test was conducted in an enclosure (1 m^2) located in a different housing room than the focus male's home enclosure. The enclosure was initially divided into two equal halves through a mesh partition. Prior to the test, a pregnant adult female guinea pig was placed in one half of the enclosure. The focus male was then placed in the other half and the experimenter stepped back. After one minute of visual and olfactory contact between the animals, the mesh was removed, allowing free interaction. The experimenter left the room during the following 30 minutes, after which the test was concluded. The entire session was videotaped.

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 PM-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 PM-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 PM-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 PM-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 PM-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 PM-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 PM-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 PM-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