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

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Abstract

Individuals can adjust to different social environments via 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 examine potential effects of different social environments on the endocrine and behavioural phenotype in male guinea pigs during juvenility, an important developmental phase characterized by prominent changes of the social environment. For this approach, twenty domestic juvenile male guinea pigs (*Cavia aperea* f. *porcellus)* were housed in two distinct social environments: while males of both groups lived in heterosexual pairs, males of one group were additionally 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. We hypothesized males from the two social conditions to differ in their hormonal and behavioural phenotype. Indeed, only males with additional social stimulation displayed an initially increased stress responsiveness, enabling them to adequately react to the unpredictable social encounters. Over time, males then conformed to this challenging environment and displayed a decrease in stress responsiveness again. Moreover, only males with additional social stimulation showed a significant increase of courtship and sexual behaviour with age. Taken together, these findings suggest that already in juvenility the social environment induced hormonal adjustments and behavioural changes in male guinea pigs, thereby highlighting how juvenile social experiences can shape individuals' phenotypes.

Keywords

Basal cortisol; basal testosterone; cortisol responsiveness; courtship behavior; early social environment; juvenility

1. Introduction

Individuals can adjust to different social environments via shaping of endocrine and behavioural 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 negative consequences [3,4]. Maladaptive responses to the social environment have been reported in non-human primates, mice and rats, where group incompatibility resulted in severe aggression, impaired immune function and elevated stress levels [5]. In guinea pig males (*Cavia aperea* f. *porcellus*), for example, different social environments (high vs. low population density) during adolescence shape different behavioural reproductive strategies. Males reared in large groups, i.e., in a high population density, could integrate into an unknown large mixed-sex colony through a low-aggressive queuing strategy [6]. Males reared in heterosexual pairs, i.e., in a low population density, initially failed to integrate as they 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]. Yet, when formerly colony- and pair-housed male guinea pigs were placed as pairs into a competitive reproductive situation with unknown females, pair-housed males displaying such a high-aggressive tactic had the higher reproductive success [7], demonstrating the fitness consequences of phenotype-environment match. These behavioural tactics and 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 systems regulating the secretion of glucocorticoids [9,10]. On a continuous low baseline level glucocorticoids are released for maintenance of metabolism and homeostasis [11]. Higher amounts are secreted into the blood stream in response to unpredictable challenges. This stressor-induced HPA function, i.e., HPA reactivity, activates distinct pathways including gluconeogenesis and immune redistribution before levels return to baseline by negative feedback [11–14]. In social encounters, glucocorticoids mainly function to mobilise energy and modulate different behaviours [15–18]. On the one hand, glucocorticoid concentrations can acutely change in response to the current situation. For instance, formerly pair-housed guinea pig males showed elevated baseline glucocorticoid levels when they were introduced into an unknown colony [6]. HPA reactivity on the other hand, can also be shaped by environmental factors in the long term [19,20]. In male guinea pigs for example, frequent social interactions in colony-housed males are associated with a reduced HPA reactivity and a low-aggression phenotype to facilitate group integration, whereas pair-housed males with limited options for social interactions show an increased HPA reactivity which promotes aggressiveness [7,21]. In laboratory male mice (*Mus musculus* f. *domestica*), isolated individuals displayed an enhanced HPA reactivity and increased anxiety-like behaviour in comparison to socially housed individuals [22–24].

These enhanced endocrine and behavioural responses can reflect greater vigilance to environmental threats, which can promote survival [25].

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 static throughout life time but can change at any time. In consequence, individuals should be able to adjust to their social environment throughout life time, too. This would mean plasticity of behavioural and endocrine phenotypes should be found in all ontogenetic phases. Still, most research on the shaping of endocrine and behavioural phenotypes by the social environment focused on early life phases (prenatal and lactation phase) and adolescence. These phases are referred to as sensitive windows of enhanced plasticity [7,26,27] which was often assumed to decline with age and no longer to be found beyond adolescence [28]. This assumption was historically based on the belief that the development of the adult brain is completed and that at this point neuronal plasticity no longer exists [29]. However, it is known for several decades now that adult neurogenesis, i.e., the addition of new neurons to the brain, occurs [29]. In consequence, plasticity of behavioural and endocrine phenotypes in response to the (social) environment is also possible in adult individuals and has been reported across multiple taxa [19,30–32].

Nonetheless, it is known that the levels of neurogenesis are significantly higher during the juvenile phase than in adulthood [33]. Juvenility, i.e., time between weaning and adolescence, is an important developmental phase where the focus of social interactions starts to shift from parents to peers. Social exploration and play behaviour increase during juvenility, suggesting this phase represents an important window for socialization [33]. While a lot of studies are investigating the effects of the early social environment on behaviour and hormones in later life [26,34–36], research on the effects on juvenile individuals themselves are still scarce. One of the few studies shows that in African cichlid fish (*Astatotilapia burtoni*) pair-reared juveniles were less active and more subordinate than group-reared juveniles and glucocorticoid receptors were elevated in the latter [37]. In laboratory mice, juveniles displayed a higher vulnerability for social defeat stress regarding glucocorticoid activation and social avoidance behaviour compared to adults [38]. These findings indicate that the juvenile phase represents another sensitive window for shaping of behavioural and endocrine phenotypes. Guinea pigs are a well-suited model organism to address this topic further since they are highly flexible in their social organization [39] and plastic shaping of behavioural and endocrine phenotypes in other life phases has already been investigated in this species [6,7,19,26,40–44].

The aim of the present study was therefore to examine potential effects of different social environments on the endocrine and behavioural phenotype in juvenile male guinea pigs. In this regard, we were also

interested in potential interactions between time and social environment. This contributes to a better understanding of possible adjustment processes during this developmental phase.

As established in former work [41,42], the distinct social environments used in the present study were males in heterosexual pairs versus males in heterosexual pairs that received additional social stimulation via regular encounters with unfamiliar animals of both sexes (for details see methods).

Regarding endocrine phenotypes, baseline testosterone and cortisol (stress) responsiveness are known to be shaped by the social environment, as shown previously in adolescent and adult male guinea pigs [19,41,42]. Baseline cortisol and body weight in turn, often reflect more immediate responses to socially challenging environments [4,6]. For these reasons, we tested the hypothesis that baseline cortisol, baseline testosterone, cortisol responsiveness and body weight differ between males from the two social conditions.

Concerning behavioural phenotype, we considered courtship and sexual behaviour as well as sociopositive and agonistic behaviour, since these behaviours have been formerly shown to be influenced by the early social environment [35,37,38,45]. In addition, we examined play behaviour, which occurs predominantly during juvenility [46] and is typically observed in social environments that are perceived as safe and non-stressful [47]. We tested the hypothesis that the frequencies of these behavioural measures differ between males from the two social conditions. We had no a priori expectation in which direction an interaction of time and social conditions would shape endocrine and behavioural phenotype.

Above and beyond the relation between social environment and endocrine and behavioural phenotype detectable by group comparisons, recent work indicates that the social environment can further influence the stability of trait expression [48]. The relative stability in trait expression is commonly quantified by the *repeatability R* that is the proportion of total phenotypic variance attributable to between-individual differences [49,50]. Studies in guinea pigs provided first evidence that social complexity can affect the repeatability of hormonal traits [48]. Examining repeatability therefore offers insight into how consistently individuals express their endocrine phenotype under different social conditions, and whether additional social stimulation affects this stability. Against this background, we determined the repeatability of hormonal phenotype within the two social conditions and tested the hypothesis that repeatability of baseline cortisol, baseline testosterone, and cortisol responsiveness would differ between them.

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 were available *ad libitum*. Since guinea pigs are incapable of synthesizing ascorbic acid [51,52] and therefore prone to vitamin C deficiency [53], a vitamin C supplement (100% L-ascorbic acid, altapharma, Dirk Rossmann GmbH, Burgwedel, Germany) was added to the water three times per week (approximately 120 mg vitamin C in 900 ml water shared between the focus male and his female partner). 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 male guinea pigs 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 [54]. 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 social conditions. Males of both groups lived in heterosexual pairs, but males of one group were additionally socially stimulated (see 2.3 Social stimulation) regularly (pair-housed male with additionally social stimulation; PM+S condition), while males of the other group were not (pair-housed male without additional social stimulation; PM-S condition). The twenty males were organized into ten experimental

pairs, each consisting of one PM+S male and one PM-S male. To control for variability in housing conditions the home enclosures of both males within an experimental pair- each housed together with their respective female partner- were placed side by side in the same housing room. All experimental procedures (cortisol response tests, body weight measurements, video recordings) were conducted 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 2.4 Assessment of hormone concentrations) 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 [41]. 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 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**).

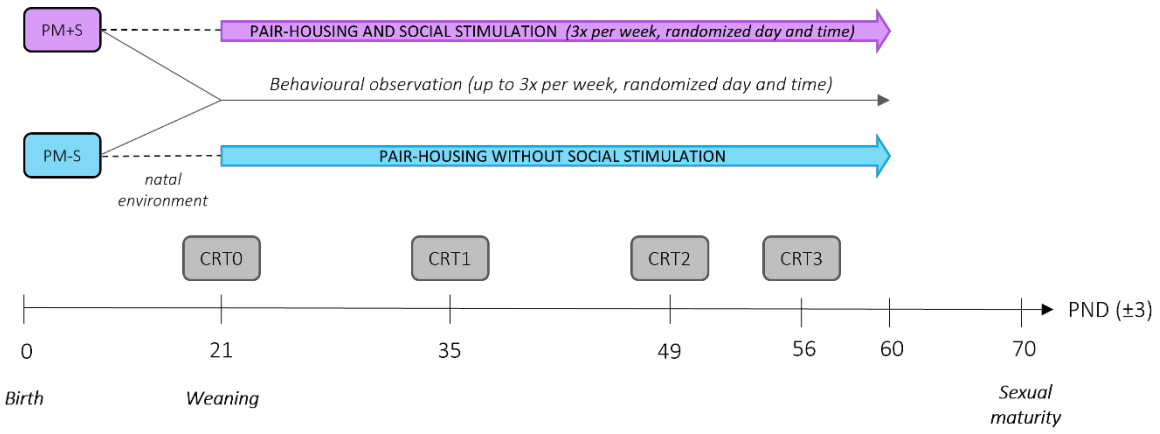

Figure 1: Procedure of behavioural observations in the home enclosure and cortisol response tests (CRT). Focal males were housed with a female partner. One group (PM+S) was regularly additionally stimulated by introducing other individuals into the home enclosure while the other group (PM-S) was not. This social stimulation started after CRT0 and lasted until the experimental phase was finished at post-natal day (PND) 60±3. Developmental milestones are indicated along the timeline: birth (PND 0), weaning (PND 21), and sexual maturity (around PND 70).

2.3 Social stimulation

The social stimulation procedure applied in the present study was adapted from Lürzel and colleagues [41,42], where additional social stimulation successfully influenced hormonal profiles in adolescent guinea pig males. The social stimulation treatment for the respective males (PM+S) started after CRT0. 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 hormone concentrations

Hormones were measured using blood samples obtained in cortisol response tests (CRTs), a standardized test in which the endocrine stress response was assessed at different time points following exposure to a novel environment as stressor [55,56]. 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 [57]. 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. For cortisol, this procedure had to be completed within 3 minutes to prevent the sampling process itself from influencing the hormone values in the obtained sample itself [57]. Such stress-induced changes in hormone values appear later in testosterone than in cortisol [57] and collecting a sufficient amount of blood for testosterone assays requires slightly more time. Therefore, blood samples for testosterone were collected within 6 minutes, which represents a compromise between sample quality and animal welfare and follows the procedure applied in earlier studies (e.g., [6,7,19,58]). 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.

2.5 Assessment of behavioural parameters

To analyse how distinct social environments influence (social) behaviour, the home enclosure behaviour (i.e., outside social stimulation sessions for PM+S males) of the focus males in both conditions 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 and play. The full ethogram can be found in the supplementary material (Tab. S2).

2.6 Statistical analysis

Data analysis was carried out with RStudio version 2022.07.0 [59]. A priori sample-size calculation was conducted using the software G*Power version 3.1.9.7 [60]. 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 [55,61]. 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 condition on hormone concentrations using the *lme4* [62] and *lmerTest* package [63]. 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. To investigate changes in hormone concentrations over time, we added the interaction between social condition (additional social stimulation versus no additional stimulation) and the variable CRT, representing the first, second and third CRT conducted after

311 treatment, as a fixed effect. We excluded data from the CRT conducted before the treatment (CRT0)
312 from the analyses for the following reasons: first, the focus of this study was to analyse the effects of
313 social stimulation on hormone concentrations over time, and CRT0 was conducted before social
314 stimulation started. Second, it is known that young animals generally show higher corticosteroid
315 concentrations due to the ongoing maturation of the HPA axis [40]. In their study, Kaiser and Sachser
316 [40] found high baseline cortisol levels in guinea pigs at 20 days of age, which declined by day 34 and
317 remained constant thereafter, marking the completion of HPA axis maturation [40]. Since CRT0 was
318 performed when the experimental animals were around 21 days of age, including this data in the
319 analyses would likely have introduced outliers caused by these maturation processes, potentially
320 confounding the interpretation of treatment-related effects. However, hormone concentrations at
321 CRT0 were still compared between the treatment groups using Wilcoxon rank-sum test to confirm there
322 were indeed no differences between the groups prior to treatment. Furthermore, the continuous
323 variable body weight was first mean-centered and then included as a fixed effect because earlier studies
324 in guinea pigs have shown that body weight can influence hormone concentrations [43,48]. Last, we
325 fitted ID as a random effect. We used the *performance* [64] and *DHARMa* package [65] to check model
326 assumptions. Marginal and conditional R^2 values were calculated using the *performance* package [64],
327 while partial R^2 values for individual predictors were calculated using the *sensemakr* package [66]. Pair-
328 wise comparisons for treatment, CRT and treatment*CRT interaction were done by applying Tukey's
329 adjustment for multiple comparison using the *emmeans* package [67].

330 Another linear-mixed effect model was fitted to analyse whether treatment affected body weight. Body
331 weight measured after the first blood sampling in CRT1, CRT2 and CRT3 was modelled as a continuous
332 response variable. The interaction between treatment and CRT was used as fixed effect to investigate
333 the influence of treatment over time. ID was included as random effect. Pairwise comparison and R^2
334 estimations were conducted as described for the hormone concentrations. Also, body weight at CRT0
335 was compared between the treatment groups using Wilcoxon rank-sum tests to confirm there were
336 indeed no differences prior to treatment. As previously mentioned, body weight was included as a fixed
337 effect in the linear mixed-effect models for hormone concentrations. Any significant effect of body
338 weight was further examined by calculating Pearson's correlation coefficients for each treatment group
339 separately. Body weight was mean-centered for each time point (CRT1, CRT2, CRT3) and the correlation
340 coefficients were then calculated across all time points and for each time point separately. To determine
341 whether the correlations for each time point differed significantly between treatment groups, Fisher's
342 z-test was conducted using the *cocor* package [68].

343 Adjusted repeatability estimates of hormone concentrations and body weight were calculated for each
344 of the treatment groups using the *rprR* package [69]. 95% confidence intervals were determined by
345 parametric bootstrapping (N = 1000), and likelihood ratio tests were used for significance testing. The

346 models used to estimate adjusted repeatability were the same as mentioned before, with the only
347 exception that treatment was removed as fixed effect.

348 For the analysis of the home enclosure behaviour, count data of behaviours from the coded videos was
349 transformed into frequencies (occurrence per hour). Several behaviours were observed in only a few
350 individuals, resulting in a zero-inflation of data which was detected using the *performance* package [64].
351 Therefore, we pooled behaviour into three categories: sociopositive behaviour, courtship and sexual
352 behaviour and play behaviour, with individual behaviours being summed within each category. Agonistic
353 behaviour was excluded from the analysis since it only occurred in a single individual. Generalized linear
354 mixed-effect models with negative binomial distribution accounting for the zero-inflated data were fit
355 for each behavioural category using the *lme4* package [62]. Again, interaction between treatment and
356 time was used as fixed effect in the models to investigate the influence of treatment over time. Time
357 was categorized into "Phase 1" (1st and 2nd experimental week), "Phase 2" (3rd and 4th experimental
358 week) and "Phase 3" (5th and 6th experimental week). ID was again fitted as a random effect. Model
359 assumptions as well as the estimation of the different R^2 values were conducted in the same manner as
360 for the analysis of hormone concentrations. Pair-wise comparisons for treatment, phase and
361 treatment*phase interaction were done by applying Tukey's adjustment for multiple comparison using
362 the *emmeans* package [67].

363 Model summaries and detailed test statistics can be found in the supplementary material.
364

365 ## 3. Results

366 Descriptive statistics for all hormone measurements, body weight and behaviour for each respective
367 time point can be found in the supplementary material (Tab. S3-S5).

368 ### 3.1 Effects of social environment on hormone concentrations and body weight

369 The comparison of hormone concentrations (c0, c1, c2, t) at CRT0 using Wilcoxon rank-sum tests
370 revealed no significant differences between the social conditions prior to treatment (see supplementary
371 material, Tab. S6).

372 Regarding baseline cortisol and testosterone levels (Fig. 2), neither a significant effect of treatment
373 condition or time (CRT), nor a significant treatment-by-time interaction effect was found (see
374 supplementary material, Tab. S11 + S14).

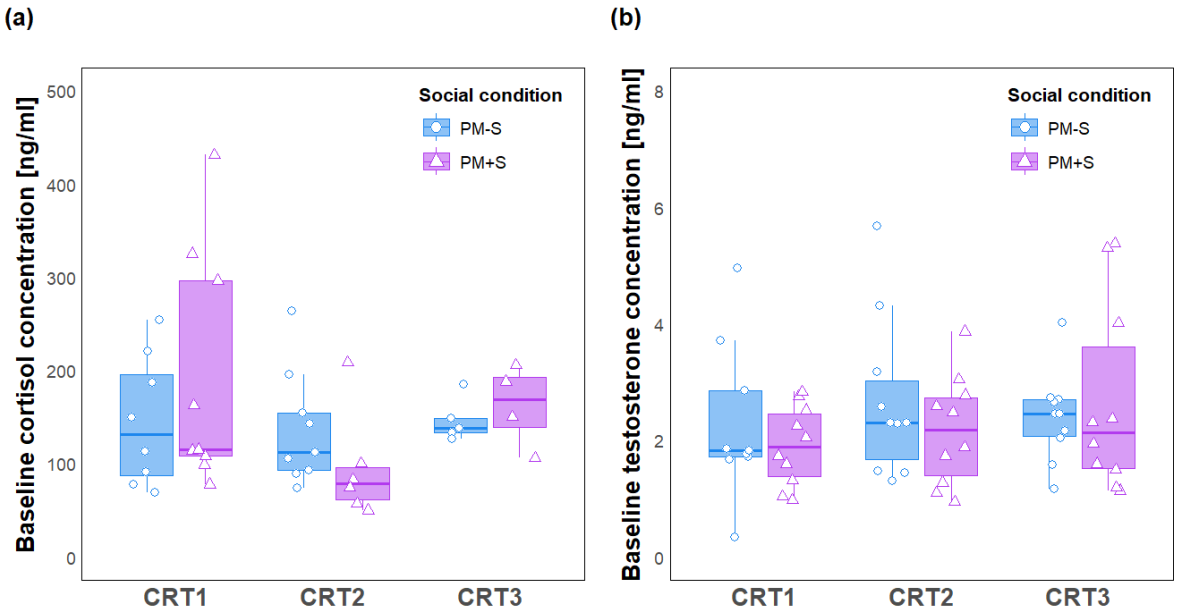

Figure 2: Baseline cortisol **(a)** and testosterone **(b)** concentrations (ng ml^{-1}) two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males were either additionally socially stimulated (PM+S) or not (PM-S). Plotted are medians (horizontal lines), first to third quartiles (boxes), whiskers and all data points.

375 Regarding cortisol responsiveness at 1 hour (c1) of exposure to a novel environment a significant
376 treatment-by-time interaction effect was found between CRT1 and CRT2 (ß = 3.92 ± 1.34, t = 2.92, p =
377 0.006) as well as between CRT1 and CRT3 (ß = 2.94 ± 1.34, t = 2.19, p = 0.035), where c1 values
378 decreased for the PM+S group (**Fig. 3a**). For cortisol responsiveness at 2 hours (c2) of exposure to a
379 novel environment (**Fig. 3b**), a significant treatment-by-time interaction effect between CRT1 and CRT2
380 (ß = 4.2 ± 1.7, t = 2.46, p = 0.019) was found, with c2 values decreasing for the PM+S group.

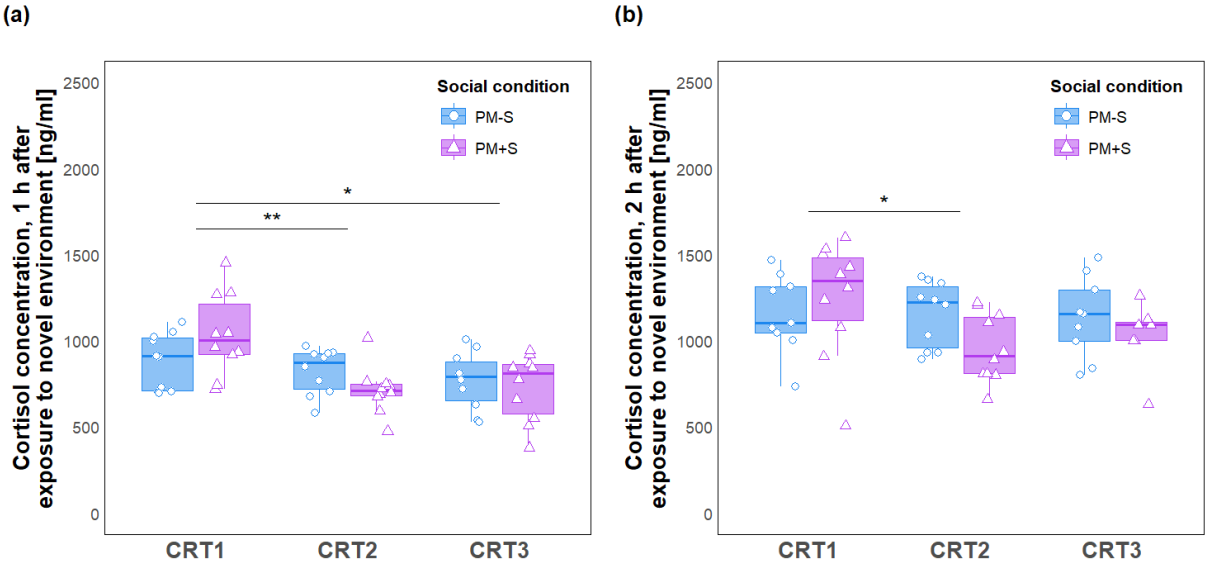

Figure 3: Cortisol concentrations (ng ml^{-1}) at one hour **(a)** and two hours **(b)** of exposure to a novel environment two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males were either additionally socially stimulated (PM+S) or not (PM-S). Plotted are medians (horizontal lines), first to third quartiles (boxes), whiskers and all data points. *p < 0.05 **p < 0.01.

Regarding body weight, the comparison at CRT0 using a Wilcoxon rank-sum test revealed no significant differences between the social conditions prior to treatment (see supplementary material, **Tab. S6**). In both social conditions, 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, $p < 0.001$). The statistical analysis of hormone concentrations showed that c1 concentrations are significantly related to body weight (ß = -0.03 ± -0.01 t = -4.32, $p < 0.001$), indicating that males with higher body weight displayed lower c1 concentrations. This relationship was further examined in more detail. 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 social conditions were however not significant for any time point (see supplementary material, **Tab. S17**). These correlations between body weight and c1 concentrations over all timepoints (CRT1, CRT2, CRT3) are displayed in **Figure 4**.

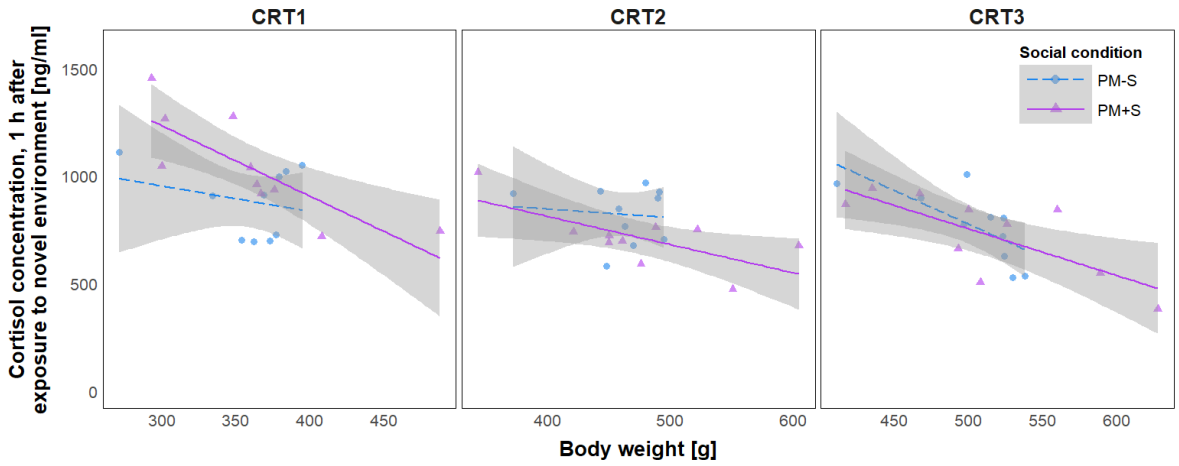

Figure 4: Correlation between cortisol concentrations (ng ml^{-1}) at one hour of exposure to a novel environment and body weight two weeks (CRT1), four weeks (CRT2) and five weeks (CRT3) after treatment start. Males were either additionally socially stimulated (PM+S) or not (PM-S). Plotted are regression lines, confidence intervals and all data points.

Adjusted repeatability was analysed for hormone concentrations (baseline cortisol, baseline testosterone, cortisol responsiveness after 1 and 2 hours) in both social conditions (**Fig. 5**).

Baseline cortisol (c0) was not significantly repeatable under any social condition (PM+S group: R = 0, CI = [0, 0.81], p = 1; PM-S group: R = 0.18, CI = [0, 0.74], p = 0.331). Similarly, also baseline testosterone (t) was not repeatable in either of the two social conditions (PM+S group: R = 0.07, CI = [0, 0.55], p = 0.44; PM-S group (R = 0, CI = [0, 0.47], p = 1) In contrast to this, repeatability of cortisol responsiveness after 1 hour (c1) differs between the two social conditions: while it showed very low and non-significant repeatability in the PM+S group (R = 0.04, CI = [0, 0.51], p = 0.495), it was significantly repeatable in the PM-S group with moderate repeatability estimates (R = 0.45, CI = [0.03, 0.79], p = 0.014). For cortisol responsiveness after 2 hours (c2) we found moderate and significant repeatability under both social conditions (PM+S group: R = 0.42, CI = [0, 0.55], p = 0.04; PM-S group: R = 0.52, CI = [0.09, 0.85], p = 0.015). Please note, that the significant p-value for c2 in the PM+S group has to be interpreted with caution as the confidence intervals included zero [49,70].

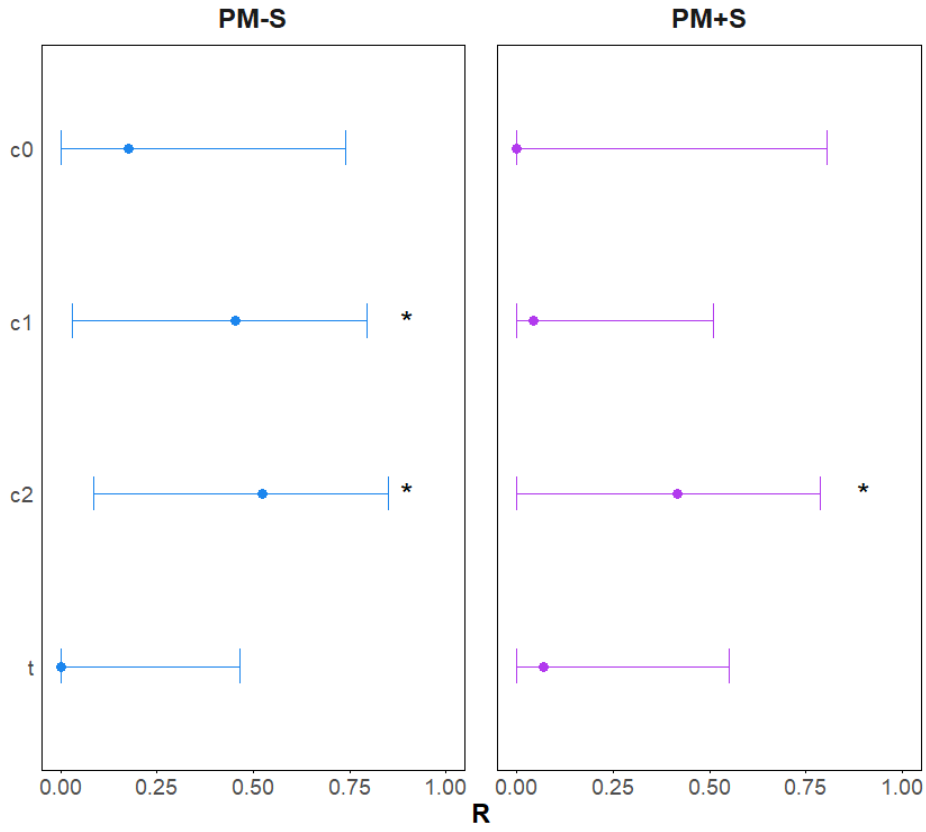

Figure 5: Repeatability (R) of baseline cortisol (c0), cortisol responsiveness after 1 (c1) and 2 hours (c2) of exposure to a novel environment andbaseline testosterone (t). Males were either additionally socially stimulated (PM+S) or not (PM-S). Plotted are adjusted repeatability (data points) and confidence intervals (whisker). *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. 6a**). 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. 6b**). Play behaviour was observed in both social conditions (see supplementary material, **Tab. S5**), however, neither a significant effect of treatment or time (phase), nor a significant treatment-by-time interaction effect was found (see supplementary material, **Tab. S24**).

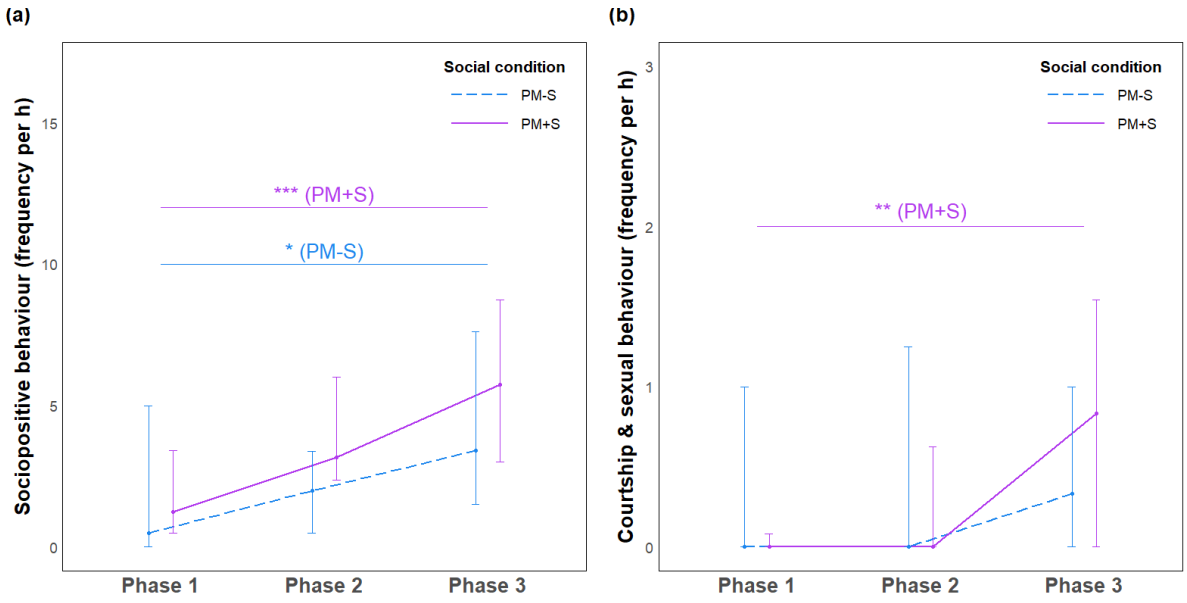

Figure 6: Frequency (per h) of **(a)** sociopositive behaviour and **(b)** courtship and sexual behaviour in the first (phase 1), second (phase 2) and third (phase 3) two weeks of treatment. Males were either additionally socially stimulated (PM+S) or not (PM-S). Plotted are medians (data points) and first to third quartiles (whiskers). *p < 0.05, ** < 0.01, *** p < 0.001.

4. Discussion

In this study, we examined potential effects of different social environments on the endocrine and behavioural phenotype in juvenile male guinea pigs. By repeatedly analysing hormonal and behavioural parameters during juvenility, we aimed to explore how time and social environment might interact in the way they affect these phenotypes during 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). PM+S males showed an initially increased cortisol responsiveness which decreased again over time. Cortisol responsiveness was also significantly affected by body weight, this finding was however independent of social condition. Baseline cortisol and baseline testosterone concentrations, by contrast, were not affected by social condition. Repeatability analyses revealed that baseline cortisol and testosterone were not repeatable in either social condition, whereas cortisol responsiveness was repeatable in the PM-S condition and partly repeatable in the PM+S condition. Regarding behaviour, the frequency of sociopositive behaviour significantly increased over time in both social conditions, while an increase in the frequency of courtship and sexual behaviour was shown in the PM+S condition only. Play behaviour was not affected by social condition.

4.1 Modulation of cortisol responsiveness by the social environment

Cortisol responsiveness was different between social conditions. Males without additional social stimulation (PM-S) showed no changes in cortisol responsiveness over time, indicating a stable stress response across the experimental phase. This pattern is in line with earlier studies in adolescent [41,42] and adult guinea pigs [19], where pair-housed males without additional social stimulation likewise showed no difference in cortisol responsiveness before and after the treatment phase.

Males with additional social stimulation (PM+S), however, displayed an initially elevated cortisol responsiveness. More specifically, in the cortisol response test (CRT) conducted two weeks after start of social stimulation, cortisol responsiveness was higher in PM+S males than in PM-S males. In earlier studies, additional social stimulation led to a significantly decreased cortisol responsiveness in adolescent male guinea pigs compared to males without additional social stimulation [41,42]. Those studies however compared cortisol responsiveness before and after the end of the social stimulation treatment, whereas the heightened stress response in PM+S males in this study occurred during the experimental 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 [71–74]. But in this study, baseline cortisol values did not differ between males of both social conditions, 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 more challenging environment than pair-housed males without additional social stimulation [48]. Under such conditions, a higher endocrine responsiveness to social stressors in such a situation could be beneficial. 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 [75]. 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 [76,77]. Consequently, the initially heightened stress responsiveness in PM+S males could hint towards a possible endocrine adjustment process to the unpredictable and socially challenging environment. In the subsequent CRTs cortisol responsiveness in PM+S males significantly decreased again. Hence, at the end of the experimental phase cortisol responsiveness of PM+S and PM-S males almost converged. This finding could also be explained in terms of endocrine adjustment. As previously mentioned, additional social stimulation did not elevate baseline stress levels and only eight out of 180 social stimulation sessions had to be terminated due to escalated aggression. Thus, the PM+S males might have learned that social stimulations were not inherently harmful. A stress-induced HPA activation (i.e., heightened cortisol responsiveness) is metabolically costly [18] and it is therefore beneficial for an organism to

reduce HPA activity to stressors without harm [78]. Endocrine adjustments to the social environment have already been reported in multiple studies in adolescent and adult guinea pigs (e.g., [6,7,19,41,42,79]). More recently, such findings have been conceptually integrated into the framework of *social niche conformance*, which has gained prominence in behavioural ecology [1,8,80]. The individualized social niche describes the unit that is shaped by social interactions of a focal individual with conspecifics [1,48], whereas social niche conformance refers to the process by which individuals adjust to an existing social environment through shaping of their hormonal and/ or behavioural phenotype [1,8,80,81]. This concept has also been applied to guinea pigs, where endocrine mechanisms are known to mediate adjustments to the social environment [8]. The results support our hypothesis that cortisol responsiveness differs between the two social conditions during juvenility. Additionally, they could be viewed as consistent with the social niche conformance concept. Males with additional social stimulation displayed an initially increased stress responsiveness, enabling them to adequately react to the unpredictable social encounters. Over times, they then conformed to this challenging environment and displayed a decrease in cortisol responsiveness again.

Finally, we investigated the repeatability of hormone concentrations to assess the stability of endocrine trait expression within individuals and to examine whether social environment affects this stability, as suggested by previous studies [48,82–84]. In this study, repeatability estimates did not differ systematically between treatment groups, indicating that the stability of endocrine trait expression was not affected by social condition. However, these results should be interpreted with caution, as the confidence intervals were wide and either close to or included zero [49,70]. 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 [85]. 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 [82,86].

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 [39]. Moreover, the occurrence of play behaviour is an indicator of positive affective state [87] and of environmental conditions that are perceived as safe and non-stressful [47]. The results for baseline cortisol, which did not differ between the two social conditions, already suggest that social stimulation per se did not lead to prolonged higher stress levels. Therefore, the occurrence of play behaviour in both treatment groups and the almost complete absence of agonistic behaviour further support this conclusion [47,88,89].

Regarding courtship and sexual behaviour, we hypothesized that males with and without additional social stimulation show differences in these behaviours, based on previous research demonstrating that early social experience influences sexual behaviour. For example, male guppies (*Poecilia reticulata*) reared with adult males showed earlier and more frequent courtship displays than males reared with adult females only [35]. In our study, this hypothesis was only partly supported: although no significant differences between treatment groups were found, courtship and sexual behaviour significantly increased over time in PM+S 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 [90,91]. In this study, however, we neither found differences in testosterone levels between PM+S and PM-S males, nor did testosterone levels significantly increase over time in PM+S 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) [92]. Instead, two non-exclusive explanations could account for the results obtained here. First, it is possible that PM+S 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 [35]. Second, dramatic changes in testosterone levels are not necessarily required for the development of sexual behaviour [93]. In laboratory rats (*Rattus norvegicus* f. *domestica*), males pair-housed during adolescence displayed more sexual behaviour than single-housed males, regardless of pubertal testosterone levels [93]. This suggests that social experiences during critical developmental phases can organize neural structures that mediate sexual behaviour [93,94]. In another rat study for example, single-housed males showed smaller volume and neuronal soma size in a sexually relevant brain nucleus and, in parallel, displayed less frequent and delayed sexual behaviours compared to group-housed males [94]. Similarly, social stimulation during the juvenile phase may have promoted the development or reorganization of neural circuits underlying courtship and sexual behaviour [93,94] through increased opportunities to observe and practice courtship behaviour [35]. The influence of social environment on behavioural phenotypes has also been discussed within the framework of social niche conformance. For example, in zebra finches (*Taeniopygia guttata*), males adjusted their courtship and competitive behaviour to the social environment without corresponding changes in testosterone or corticosterone levels, suggesting that such adjustments of behavioural phenotype can occur independently of endocrine modulation [81]. In guinea pigs, living in different social environments during adolescence leads to adaptive shaping of the behavioural phenotype [1,8,19,79]. Males conform to the respective social environment (low vs. high population density) using either a high-aggressive resource defense or low-aggressive queuing strategies, thereby realizing different individualized social niches [1,8,79]. In this context, the observed increase in courtship and

541 sexual behaviour in PM+S males in the present study could also reflect a social niche conformance
542 process, helping individuals to adjust to a more complex social environment with multiple potential
543 mating opportunities.

544 ### 4.3 Body weight and its negative effect on cortisol responsiveness

545 Body weight can reflect immediate responses to socially challenging environments [4,6]. In adolescent
546 guinea pigs, for example, males reared in heterosexual pairs showed significant weight loss during the
547 initial days in an unfamiliar mixed-sex colony, whereas body weight of males reared in such colonies
548 remained unaffected [6]. Thus, we hypothesized that juvenile body weight would also differ between
549 males with and without additional social stimulation. However, no differences regarding body weight
550 were found between males of the two social conditions in this study. This finding further supports the
551 assumption that social stimulation per se did not lead to prolonged higher stress levels, which are often
552 associated with decreased body weight resulting from reduced food intake and enhanced catabolic
553 processes through elevated glucocorticoid secretion [4,95].

554 Interestingly, body weight was significantly negatively correlated with cortisol responsiveness after 1
555 hour. In guinea pigs, cortisol responsiveness after 1 hour reflects the speed of the stress response,
556 whereas cortisol responsiveness after 2 hours indicates its magnitude [43,96]. The observation of only
557 cortisol responsiveness after 1 hour, but neither baseline cortisol levels nor cortisol responsiveness after
558 2 hours being negatively affected by body weight, suggests guinea pig males with higher body weights
559 have a slower cortisol response. This would mean the maximum stress response might not be different
560 between bigger and smaller individuals, but the time it takes to reach this maximum. In rats, diet-
561 induced obese animals were hyporesponsive to chronic stress [97] and had a blunted stress response
562 following psychosocial stress exposure [98]. Reasons for this could involve body weight dependent
563 differences in the adrenal gland [99] and availability or secretion of cortisol or cortisol binding globulins
564 [100,101]. However, it remains unclear whether similar physiological processes were involved here, as
565 the animals in this study were neither obese nor under chronic stress, and findings from such studies
566 are therefore difficult to directly compare with our results.

567 Even though no statistical differences between treatment groups could be found (which might become
568 detectable with a larger sample size), the negative correlation between body weight and cortisol
569 responsiveness seemed to be more pronounced in PM+S males. At the end of the experimental phase,
570 however, the correlation between body weight and cortisol responsiveness in PM-S males was almost
571 as high as in PM+S males and also significant. This might suggest an earlier onset of the effect that causes
572 higher body weight to negatively influence cortisol responsiveness in PM+S males. Previous studies in
573 mice and rats have shown that social factors can for example influence adrenal gland weight and thus
574 HPA reactivity [102–104], but the mechanisms underlying such patterns in guinea pigs remain rather

unexplored. Earlier studies in guinea pigs have also reported associations between body weight and cortisol responsiveness [43,48]. However, these effects vary in their direction and the underlying causes remain unclear, thus highlighting the need for further investigations.

4.4 Conclusion

The present study demonstrates that distinct social environments during juvenility influence both hormonal and behavioural phenotypes in male guinea pigs. Juvenile males exposed to additional social stimulation displayed a decrease in cortisol responsiveness and an increase in courtship and sexual behaviour over time, which could possibly promote niche conformance processes to a more complex social environment. These findings emphasize juvenility as another important developmental 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: Description 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.

Table S2: 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.

Results: Descriptive statistics

Table S3: 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). All hormones were measured as concentration in blood plasma (ng/ml).

Social condition	Hormone	Time point	n	mean	median	SD	min	max
PM+S	c0	CRT0	8	520.70	425.13	298.84	199.23	979.73
		CRT1	9	192.44	115.50	126.20	78.06	431.48
		CRT2	6	95.93	79.17	58.19	50.22	208.83
		CRT3	4	162.71	169.18	44.02	106.60	205.88
	c1	CRT0	10	1583.90	1606.60	365.44	968.58	2110.78
		CRT1	10	1036.95	1001.34	236.58	718.33	1454.35
		CRT2	10	712.83	710.19	137.68	475.50	1017.43
		CRT3	10	729.12	809.46	193.55	380.30	942.00
	c2	CRT0	9	1736.53	1824.05	525.90	732.33	2296.40
		CRT1	10	1249.38	1347.59	336.44	505.60	1599.15
		CRT2	10	959.57	913.23	197.91	660.11	1222.77
		CRT3	7	1029.25	1092.23	196.66	630.45	1262.53
	t	CRT0	7	1.00	0.85	0.44	0.66	1.87
		CRT1	10	1.91	1.90	0.68	0.98	2.84
		CRT2	10	2.17	2.18	0.94	0.95	3.87
		CRT3	10	2.68	2.13	1.63	1.14	5.38
PM-S	c0	CRT0	9	414.35	478.83	184.21	141.28	712.00
		CRT1	8	145.56	132.01	69.33	69.57	254.20
		CRT2	9	136.86	112.05	61.10	73.58	264.23
		CRT3	5	146.43	137.93	23.05	126.85	185.23
	c1	CRT0	9	1438.96	1484.93	221.49	1032.23	1635.13
		CRT1	10	883.41	909.91	163.18	696.33	1110.35
		CRT2	10	822.30	874.00	131.59	580.20	969.28
		CRT3	10	768.61	790.94	166.59	530.18	1009.53
	c2	CRT0	9	1645.85	1700.43	175.58	1370.85	1835.45
		CRT1	9	1158.78	1105.53	227.61	736.08	1467.13
		CRT2	10	1155.06	1223.49	189.08	892.17	1373.48
		CRT3	9	1136.01	1158.83	234.90	799.63	1481.10
	t	CRT0	9	1.18	1.01	0.87	0.25	3.22
		CRT1	9	2.30	1.83	1.35	0.34	4.96
		CRT2	10	2.69	2.31	1.38	1.30	5.68
		CRT3	10	2.40	2.46	0.76	1.17	4.02

Table S4: Descriptive statistics for body weight (g).

Social condition	Time point	n	mean	median	SD	min	max
PM+S	CRT0	10	254.20	247.50	44.17	211	359
	CRT1	10	360.60	362.00	58.31	293	488
	CRT2	10	476.70	468.50	72.12	343	605
	CRT3	10	512.30	504.00	66.15	417	628
PM-S	CRT0	10	263.50	259.50	32.76	193	313
	CRT1	10	359.80	371.00	35.49	271	395
	CRT2	10	461.00	466.50	36.16	372	495
	CRT3	10	502.90	519.00	38.18	411	538

Table S5: Descriptive statistics for sociopositive, sexual and courtship and play behaviour. All behaviours were measured as frequencies per hour.

Social condition	Behaviour	Time point	n	mean	median	SD	min	max
PM+S	Sociopositive	Phase 1	20	2.12	1.25	2.02	0	6.50
		Phase 2	20	4.65	3.17	3.78	0.50	15.00
		Phase 3	20	7.78	5.75	7.27	0	26.00
	Sexual and courtship	Phase 1	20	0.15	0	0.29	0	1.00
		Phase 2	20	0.48	0	0.72	0	2.33
		Phase 3	20	1.70	0.83	3.16	0	13.50
	Play	Phase 1	20	0.54	0	1.80	0	7.50
		Phase 2	20	0.48	0	1.27	0	5
		Phase 3	20	0.66	0	1.30	0	4.50
PM-S	Sociopositive	Phase 1	20	2.52	0.5	3.75	0	10.67
		Phase 2	20	3.43	2	5.08	0	20.50
		Phase 3	20	5.98	3.42	8.06	0	36.33
	Sexual and courtship	Phase 1	20	0.60	0	1.05	0	3.33
		Phase 2	20	1.23	0	2.86	0	12.50
		Phase 3	20	1.72	0.33	4.41	0	19.67
	Play	Phase 1	20	0.25	0	0.79	0	3
		Phase 2	20	0.08	0	0.18	0	0.50
		Phase 3	20	0.48	0	1.34	0	5

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

Table S6: Wilcoxon rank sum test of hormone concentrations and body weight calculated for the first cortisol response test (CRT) conducted before treatment (additional social stimulation).

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 S7: Model summary from mixed effect model used to analyse baseline cortisol. Data was square root transformed (N = 41). The model included the interaction between treatment (social condition) and time (CRT) and body weight as fixed effects, with individual ID as random effect. CRT1 (time) and PM-S (treatment) were set as reference level by default.

Baseline cortisol	**Estimate**	**Std. error**	**[95% CI]**	**t-value**	**p-value**	**R^2**
					Full model: Marginal R^2	0.165
					Full model: Conditional R^2	*NA*
Intercept	11.298	1.106	[9.051, 13.546]	10.217	< 0.001	
Fixed effects						
Treatment (social condition)	1.541	1.418	[-1.341, 4.423]	1.087	0.285	0.034
CRT1 - CRT2 (time)	0.982	1.793	[-2.662, 4.627]	0.548	0.587	0.009
CRT1 - CRT3 (time)	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 S8: Model summary from mixed effect model used to analyse cortisol responsiveness after 1 hour of exposure to a novel environment. Data was square root transformed (N = 60). The model included the interaction between treatment (social condition) and time (CRT) and body weight as fixed effects, with individual ID as random effect. CRT1 (time) and PM-S (treatment) were set as reference level by default.

Cortisol responsiveness 1h	**Estimate**	**Std. error**	**[95% CI]**	**t-value**	**p-value**	**R^2**
					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	
Fixed effects						
Treatment (social condition)	2.435	1.106	[0.207, 4.662]	2.201	0.033	0.085
CRT1 - CRT2 (time)	2.312	1.221	[-0.137, 4.761]	1.894	0.064	0.071
CRT1 - CRT3 (time)	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 S9: Model summary from mixed effect model used to analyse cortisol responsiveness after 2 hours of exposure to a novel environment. Data was square root transformed (N = 55). The model included the interaction between treatment (social condition) and time (CRT) and body weight as fixed effects, with individual ID as random effect. CRT1 (time) and PM-S (treatment) were set as reference level by default.

Cortisol responsiveness 2h	**Estimate**	**Std. error**	**[95% CI]**	**t-value**	**p-value**	**R^2**
					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	
Fixed effects						
Treatment (social condition)	1.414	1.609	[-1.856, 4.684]	0.879	0.386	0.011
CRT1 - CRT2 (time)	2.031	1.707	[-1.402, 5.465]	1.190	0.240	0.045
CRT1 - CRT3 (time)	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 S10: Model summary from mixed effect model used to analyse baseline testosterone. Data was square root transformed (N = 59). The model included the interaction between treatment (social condition) and time (CRT) and body weight as fixed effects, with individual ID as random effect. CRT1 (time) and PM-S (treatment) were set as reference level by default.

Baseline testosterone	Estimate	Std. error	[95% CI]	t-value	p-value	R^2
					Full model: Marginal R^2	0.053
					Full model: Conditional R^2	*NA*
Intercept	1.475	0.130	[1.215, 1.736]	11.364	< 0.001	
Fixed effects						
Treatment (social condition)	-0.094	0.171	[-0.437, 0.249]	-0.549	0.585	0.006
CRT1 - CRT2 (time)	0.087	0.197	[-0.307, 0.482]	0.445	0.658	0.004
CRT1 - CRT3 (time)	0.001	0.219	[-0.439, 0.441]	0.003	0.997	0.000
Body weight	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 S11: Multiple comparisons (Tukey's) of linear mixed effect model to determine effects of treatment (social condition), time (CRT) and treatment*time interaction on baseline cortisol.

Baseline cortisol	Estimate	Std. error	df	[95% CI]	t-value	p-value
Pair-wise comparison (between social conditions)						
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 (between time points)						
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 (social condition*time point)						
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 S12: Multiple comparisons (Tukey's) of linear mixed effect model to determine effects of treatment (social condition), time (CRT) and treatment*time 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 social conditions)						
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 time points)						
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 (social condition*time point)						
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 S13: Multiple comparisons (Tukey's) of linear mixed effect model to determine effects of treatment (social condition), time (CRT) and treatment*time 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 social conditions)						
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 time points)						
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 (social condition*time point)						
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 S14: Multiple comparisons (Tukey's) of linear mixed effect model to determine effects of treatment (social condition), time (CRT) and treatment*time interaction on baseline testosterone.

Baseline testosterone	Estimate	Std. error	df	[95% CI]	t-value	p-value
Pair-wise comparison (between social conditions)						
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 time points)						
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 (social condition*time point)						
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 S15: Model summary from mixed effect model used to analyse body weight. Data was square root transformed (N = 41). The model included the interaction treatment (social condition) and time (CRT) and body weight as fixed effects, with individual ID as random effect. CRT1 (time) and PM-S (treatment) were set as reference level by default.

Body weight	Estimate	Std. error	[95% CI]	t-value	p-value	R^2
					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	
Fixed effects						
Treatment (social condition)	0.8	23.807	[-48.862, 50.462]	0.034	0.974	< 0.001
CRT1 - CRT2 (time)	101.2	6.623	[87.768, 114.632]	15.280	< 0.001	0.251
CRT1 - CRT3 (time)	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 S16: Multiple comparisons (Tukey's) of linear mixed effect model to determine treatment (social condition), time (CRT) and treatment*time 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 social conditions)						
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 (between time points)						
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 (social condition*time point)						
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 of exposure to a novel environment

Table S17: 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 at different time points			
Within social conditons	**r**	**t-value**	**p-value**
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 social conditions		**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 S18: 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) and baseline testosterone (t). 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	0.232	[0, 0.74]	0.175	0.331	0.254	[0, 0.805]	0	1
c1	0.196	[0.03, 0.793]	0.453	**0.014**	0.155	[0, 0.509]	0.042	0.495
c2	0.194	[0.085, 0.849]	0.523	**0.015**	0.210	[0, 0.786]	0.416	**0.040**
testosterone	0.143	[0, 0.466]	0	1	0.164	[0, 0.549]	0.069	0.440

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

Table S19: Model summary from generalized linear mixed effect model used to analyse sociopositive behaviour (N = 60). The model included the interaction between treatment (social condition) and time (phase) with individual ID as random effect. Phase 1 (time) and PM-S (treatment) were set as reference level by default.

Sociopositive behaviour	**Estimate**	**Std. error**	**[95% CI]**	**z-value**	**p-value**	**R^2**
					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	
Fixed effects						
Treatment (social condition)	-0.004	0.407	[-0.801, 0.794]	-0.009	0.993	< 0.001
Phase 1 - Phase 2 (time)	0.311	0.350	[-0.374, 0.996]	0.890	0.374	0.002
Phase 1 - Phase 3 (time)	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 S20: Model summary from generalized linear mixed effect model used to analyse courtship and sexual behaviour (N = 60). The model included the interaction between treatment (social condition) and time (phase) with individual ID as random effect. Phase 1 (time) and PM-S (treatment) were set as reference level by default.

Sexual and courtship behaviour	**Estimate**	**Std. error**	**[95% CI]**	**z-value**	**p-value**	**R^2**
					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	
Fixed effects						
Treatment (social condition)	-1.344	0.811	[-2.933, 0.245]	-1.658	0.097	0.003
Phase 1 - Phase 2 (time)	0.622	0.598	[-0.551, 1.795]	1.040	0.298	0.005
Phase 1 - Phase 3 (time)	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 S21: Model summary from generalized linear mixed effect model used to analyse play behaviour (N = 60). The model included the interaction between treatment (social condition) and time (phase) with individual ID as random effect. Phase 1 (time) and PM-S (treatment) were set as reference level by default.

Play behaviour	**Estimate**	**Std. error**	**[95% CI]**	**z-value**	**p-value**	**R^2**
					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	
Fixed effects						
Treatment (social condition)	0.444	1.048	[-1.611, 2.499]	0.424	0.672	0.005
Phase 1 - Phase 2 (time)	-1.176	1.209	[-3.545, 1.194]	-0.972	0.331	0.002
Phase 1 - Phase 3 (time)	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 S22: Multiple comparisons (Tukey's) of generalized linear mixed effect model to determine effects of treatment (social condition), time (phase) and treatment*time 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 social conditions)					
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 (between time points)					
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 (social condition*time point)					
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 S23: 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.

Sexual and courtship behaviour	Estimate	Std. error	[95% CI]	z-ratio	p-value
Pair-wise comparison (between social conditions)					
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 (between time points)					
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 (social condition*time point)					
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 S24: 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 social conditions)					
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 (between time points)					
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 (social condition*time point)					
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