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„Biological validation of steroid hormone analysis in guinea pig saliva samples“

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Abstract

A careful validation of new methods is necessary to avoid biases in the further usage. It was attempted to validate commercially available antibodies for steroid hormone measurements in human saliva for the usage in guinea pigs. For this, known biological stimuli for cortisol, testosterone, 17β-oestradiol and progesterone were used, namely social confrontations with and without the presence of a female and the physiological processes throughout the female oestrus cycle.

In experiment 1 social confrontations of two male individuals were performed to induce a rise in cortisol concentrations. No such significant increase took place, instead, a high individual variance occurred, with older animals showing higher cortisol concentrations. However, correcting cortisol concentrations for aggressive behaviour resulted in significantly increased concentrations during social confrontations. In experiment 2, saliva samples of females were collected in oestrus and dioestrus. Additionally, their interest in males was analysed by confronting them with a male. Neither 17β-oestradiol, nor progesterone showed significant differences between oestrus and dioestrus. A correlation between 17β-oestradiol and progesterone and a slight influence of 17β-oestradiol on female behaviour during oestrus was found. In experiment 3, testosterone and cortisol concentrations were measured during social confrontations of two males and an additional female to stimulate competition and sexual behaviour. Neither testosterone nor cortisol showed an increase during the confrontations. However, correcting for aggressive behaviour again resulted in increased hormone concentrations. Testosterone correlated positively with aggression and sexual behaviour.

In summary there were some correlations with the used stimuli found, but a clear validation was not possible.

1) Introduction

Many biological and physiological processes are strongly related to the hormone system. Reproduction for example relies heavily on the production and secretion of specific hormones to facilitate sexual behaviour as well as the maturation of gametes (Nelson & Kriegsfeld 2017). Steroid hormones are a group of hormones which derivate from cholesterol. They have various effects on physiological processes and behaviour and can further be subdivided into different groups like corticosteroids, androgens or oestrogens.

Measuring steroid hormone concentrations is an important tool for studies in various fields. Glucocorticoid analyses, for example, can provide information about an animal´s reaction to a stressor, but specific sampling methods have to be evaluated before routinely used in scientific or medical studies. A careful validation of a method for each species is necessary to avoid systematic biases due to species-specific physiological responses (Touma & Palme 2005). Biological validation is a possibility to evaluate non-invasive hormone measurements based on the animals´ natural physiological processes and reactions.

1.1) Glucocorticoids and Stress Response

Corticosteroids are involved in the physiological response to a stressor. One subgroup are glucocorticosteroids, which have a major influence on the glucose metabolism. They stimulate glucagon and gluconeogenesis to cover the higher energy demand during the stress response. After the occurrence of a stressor, the HPA-axis (hypothalamic-pituitary-adrenal-axis) gets activated and glucocorticoids are secreted from the adrenal cortex (Nelson & Kriegsfeld 2017; Wasserman 2009b). Physiological stress responses lead to a temporarily readjustment of the body in order to promote processes which are necessary to cope with the stressor. This includes an increase of cardiovascular tone and muscle-supply, an enhancement of cognitive activities and apart from the activation of stored energy also the inhibition of further storage (Sapolsky et al. 2000). Experimentally induced absence of glucocorticoids in rats lead to an impairment of gluconeogenesis which could be counterbalanced by administration of dexamethasone (a synthetic glucocorticoid) (Friedmann et al. 1967). During a stressful

situation glucocorticoids suppress different reactions in the body, for example in the immune system (Munck & Náray-Fejes-Tóth 1994). Glucocorticoids were also linked to cognitive functions and memory, which can also be seen as an adaption to cope with the specific stressor in the future. In rats it was shown that stressed males are more likely to succumb in an aggressive encounter and that stress seems to have reinforcing effects on memory and recognition, leading to more stable social hierarchies over time (Cordero & Sandi 2007; Timmer & Sandi 2010). In guinea pigs, social confrontations, especially among unfamiliar males, lead to an immediate increase in glucocorticoid concentrations (Sachser 1987). When the social environment is stable, guinea pigs form hierarchies with dominant and subdominant males. Although there are differences between dominant and subdominant animals concerning behaviour, there are not necessarily differences concerning stress load or cortisol levels in such a stable situation. When a guinea pig is isolated, cortisol levels increase. This effect can be reduced by social support in the presence of the preferred partner (Sachser et al. 1998).

1.2) Testosterone, Dominance and Sexual Behaviour

Testosterone is an androgen produced in the male testes. It plays an important role during adolescence, in the formation of male characteristics and the development of sexual behaviour, as well as the regulation of sexual behaviour in adult males (Damassa et al. 1977; McGinnis & Dreifuss 1989; Nelson & Kriegsfeld 2017; Wasserman 2009b).

The presence of a female can lead to elevated testosterone concentrations in rodents (Batty 1978; Purvis & Haynes 1973; Shulman & Spritzer 2014). Castration causes a sharp decline in sexual behaviour and subsequent replacement of testosterone can re-establish this behaviour (Valenstein & Young 1955). Testosterone concentrations are also linked to dominant and aggressive behaviour in different species like rabbits (Briganti et al. 2003; Girolami et al. 1997), rats (Albert et al. 1986; Breuer et al. 2001; Schuurman 1980) and mice (Machida et al. 1981; Oyegbile & Marler 2005). The most dominant male is not necessarily the one having the highest testosterone level. In guinea pigs the individual experience during adolescence (Sachser et al. 2018) and the size and stability of the social group play an important role. In big mixed-sex groups dominant males monopolized small areas and several females and showed the highest plasma testosterone titers, whereas in smaller groups all present males

established a linear hierarchy and the second highest ranked males showed highest testosterone concentrations with exception of one situation of social instability. In this single case of instability, the position of a dominant male was challenged, which led to elevated testosterone concentrations in this individual (Sachser & Pröve 1986). Confrontations of male guinea pigs with unfamiliar conspecifics in general and foreign males in particular lead to an immediate rise in testosterone concentrations and aggressive behaviour (Lürzel et al. 2010; Nemeth et al. 2021; Sachser 1987; Sachser & Lick 1989; Sachser & Pröve 1984). This effect is influenced by territory ownership: when a dominant male is confronted with an unfamiliar male in his home cage, the increase in testosterone is higher and he is more likely to win the encounter than the intruder (Sachser & Pröve 1984). Differences in testosterone concentration as a result of the amount of physical conflicts that a male encounters have been documented by Machatschke and collegues (Machatschke et al. 2008), but were not found in other studies with different rodents (Dessi-Fulgheri et al. 1976; Huhman et al. 1991; Sachser 1987). With advancing age, testosterone levels decline gradually in male guinea pigs (Rigaudiere et al. 1976).

1.3) Oestrogen, Progesterone and the Oestrus Cycle

Female guinea pigs show a relatively long oestrus cycle of 16 days on average (Ishii 1920; Stockard & Papanicolaou 1917; Young et al. 1935). Because of the similarities between the reproductive cycles, female guinea pigs have been considered as good model organisms for research related to human reproduction (Hammarström et al. 1992). A speciality of the female guinea pig is the existence of a vaginal membrane, which is closing the crescent orifice of the vagina during the greater part of the animal´s lifetime. It ruptures at the beginning of the oestrus and stays open for three to four days on average. Afterwards this membrane grows again between the vulvar lips, a process that can be compared to the healing of an injury. When the vaginal membrane is intact, a copulation is not possible, which probably serves as an additional mechanism for the timing of copulation and ovulation (Kelly & Papanicolaou 1927). This is possibly necessary in guinea pigs, because the rather long oestrus phase can again be divided into four stages, during which a sequence of swelling of the epithelium, mucus secretion, destruction of the epithelium and emergence of leucocytes takes place in the uterus and to a lesser degree also in the vagina (Stockard & Papanicolaou 1917, 1919). To

confirm and narrow down the time of ovulation, vaginal smears can be made. Cornificated cells are dominating the smears during oestrus and at the point of ovulation leucocytes appear, but in a rather small account (Durrani et al. 1985; Harned & Casida 1972; Stockard & Papanicolaou 1917). Due to the presence of the vaginal membrane, obtaining vaginal smears from inside the vagina is not possible at other phases of the guinea pigs oestrus cycle, unless the membrane is ruptured artificially, which causes pain and bleeding (Kelly & Papanicolaou 1927). Nevertheless, Young and colleagues questioned the usefulness of vaginal smears for the determination of ovulation and emphasized the altered behaviour at oestrus (higher activity before oestrus and the willingness to accept an approaching male as a sexual partner) (Young et al. 1935).

Female guinea pigs show a typical pattern of hormone fluctuations throughout the cycle. Oestrogen concentrations show a peak before oestrus and remain low during the rest of the cycle. Progesterone shows the opposite pattern and is typically elevated during dioestrus, approximately from day 4 to day 15 of the cycle (Blatchley et al. 1976; Hammarström et al. 1992; Joshi et al. 1973; Nemeth et al. 2018). High concentrations of oestradiol lead to an increase in LH (luteinizing hormone), which stimulates the maturation of the follicles and results in ovulation. When no pregnancy occurs, the follicle becomes a corpus luteum, a structure which secretes progesterone (Wasserman 2009a). Experimental administration of oestradiol leads to an earlier and longer oestrus (Goy & Young 1956), whereas progesterone can be used to block ovulation in guinea pigs and to help in artificial synchronization of the oestrus cycles in a group of females (Grégoire et al. 2012; Labhsetwar & Diamond 1970).

1.4) Aims and Experimental Approaches

The aim of the present study was to analyse cortisol, testosterone, oestradiol (17β-oestradiol) and progesterone in guinea pig saliva samples with commercial enzyme-linked immunosorbent assay (ELISA) kits developed for human saliva samples. This was done using mostly non-invasive biological methods of validation (relations to natural biological stimuli known to correlate with steroid hormone concentrations in guinea pigs).

In most of the previous studies, steroid hormones were measured in blood plasma. Measurement of steroid hormones in saliva samples is an important non-invasive alternative to gain insight into the physiological reactions and processes. Cortisol, oestrogens and

progesterone have been successfully measured in guinea pig faecal samples (Bauer et al. 2008; Nemeth et al. 2018). Cortisol was also analysed reliably in saliva samples (Fenske 1996, 1997; Nemeth et al. 2016). Attempts to measure testosterone in guinea pig faeces or saliva samples failed (Fenske 1996, Bauer et al. 2008). Measurements of oestradiol and progesterone in saliva samples were found to be reliable in humans (Lu et al. 1999), but have not been validated in female guinea pigs so far.

Social confrontations of unfamiliar male guinea pigs can serve as a stressor in experiments. In prior studies this setting lead to an increase in cortisol levels in the males, which could be measured after 30 minutes (Nemeth et al. 2016, 2021), 20 minutes (Haemisch 1990) or even when the confrontations lasted only 10 minutes (Sachser 1987). As mentioned above, social confrontations, the presence of a female and respective sexual interactions also lead to an increase of testosterone levels in guinea pigs (Sachser 1987).

Therefore, in this study, social confrontations were used to elicit increases in cortisol and testosterone in male guinea pigs and the concentrations between the basal condition of group housing and the experimental condition of social confrontation were compared. In females, 17β-oestradiol and progesterone were measured in oestrus and dioestrus, expecting the typical pattern of those hormones during the oestrus cycle.

To develop non-invasive methods for measuring these four important steroid hormones further can contribute to the improvement of behavioural studies. Taking plasma samples has many advantages, but the sampling procedure per se can cause pain and stress to the animal and therefore the results may be biased (Cook 2012; Vahl et al. 2005). Saliva samples are an opportunity to improve future studies both concerning the reliability of the results and the situation of the tested animals.

2) Materials & Methods

2.1) Animals

The study was conducted with 33 domestic guinea pigs (*Cavia aperea f. porcellus*), 20 males (held in two groups) were used for the analysis of cortisol and testosterone and 13 females for the measurement of oestrogen (17β-oestradiol) and progesterone.

All animals used were bred at the Department of Behavioural and Cognitive Biology of the University of Vienna. The stock was established at 2013 and new animals were added regularly.

An individual recognition was possible due to the natural colour and pattern of the fur. Males were kept in two groups of ten individuals each and body mass ranged between 577g and 1174g at the onset of the experiment (Group 1: 577g to 1174g; Group 2: 688 to 1109g), females were all kept in one group, body mass ranging between 758g and 1270g. There was an age difference between the two groups of males. Group 2 included younger individuals (four to nine months) than Group 1 (four months to three years). Males were randomly assigned to the two housing groups, all older individuals being in the same group. Females were between four months and three years old.

Experiments started on January 18th and ended on February 26th. All experimental treatment started at 9 a.m. before the animals were fed and lasted approximately until 12 a.m.

2.2) Housing Conditions

The animals were kept in single-sex groups, which were established months before the onset of the experiment, therefore the animals were habituated to this situation.

The guinea pigs were kept in enclosures with walls built of laminated chipboard and wood shavings as bedding material. Houses and tunnels were provided as hiding opportunities.

Males were kept in two groups of 10 animals each in enclosures of 2m x 2.4m with two houses and a tunnel in each group.

Females were kept in one large group. The size of their enclosure was 2m x 3.6m and two houses and two tunnels were provided.

All animals were kept in the same room at a light circle of 12 hours light and 12 hours darkness (light from 7 a.m. to 7 p.m.). The room temperature was 22° C (+/- 1°). Air humidity in the room was 50% (+/- 5%).

All animals were fed with standard food pellets for Guinea pigs (Ssniff V2233-0, ssniff Spezialdiäten GmbH, Soest Germany). Females received 25g per individual and males 30g per individual. Additionally, they received hay, approximately 200g per day and group, provided in hayracks. Water was provided *ad libitum* in drinking bottles for rodents.

2.3) Experimental Set-Up

2.3.1) Experiment 1 – Cortisol

The cortisol concentrations of 20 male guinea pigs during basal group housing conditions (see housing conditions) and social confrontations of two unfamiliar individuals were compared. In both conditions, saliva samples were collected for cortisol analysis.

During group housing, the animals were video recorded for half an hour in the morning (starting at 9:00 a.m.) on three consecutive days for later analysis. For this a GoPro Hero 7 was used. The camera was attached above the enclosure in a way that the whole area of the enclosure was covered. For recording houses, tunnels and food were removed, but the guinea pigs had still access to water.

For the basal cortisol concentration saliva samples were taken during group housing on January 21st, one day before the social confrontations were started. Standard cotton buds were used for this. Additionally, oral glucose tolerance tests (OGTT) were performed during group housing and social confrontation. For this, the blood glucose level in a sober condition was measured and afterwards 2 g glucose (dissolved in water) per kg bodyweight was administered orally using 1 ml syringes. One hour and two hours after this treatment the blood glucose level was measured again. For this measurement, the ear was pricked with a lancet to obtain a small drop of blood. Glucose levels were then measured using a common glucose meter with single-use test strips. By holding the test strip onto the animal´s ear, the blood could be absorbed by it and the measurement was made automatically by the glucose meter (blood glucose measured in mg/dl).

On the next day the animals were transferred to an adjacent testing room for the social confrontations and stayed there for 48 hours (January 22nd to 24th). Each male was confronted with a male of the other group in a squared arena of 1 m². Opponents were matched according to their bodyweight to establish preconditions as equal as possible. All ten confrontations were carried out at the same time.

The arenas contained wood shavings as a bedding material. The walls of the arenas were made of fibreboards (40 cm high). No houses or tunnels were provided during the social confrontation. Animals were fed with the same pellets like in group housing, 30g per day and individual. Water in water bowls was available *ad libitum*.

During the social confrontations the animals were also video recorded. The first recording took place for half an hour, immediately after they were transferred into the arenas. They were recorded again for half an hour after 24 and 48 hours.

Saliva samples were taken after each recording (30 minutes, 24 hours and 48 hours after beginning of the social confrontation) and the animals were weighed. After 48 hours of social confrontation a second oral glucose tolerance test (OGTT) was carried out, before the animals were returned into their social groups.

2.3.2) Experiment 2 – 17β-Oestradiol & Progesterone

Starting on January 18th, all females were weighed daily and checked for vaginal opening. After observing one full oestrus cycle (observing two consecutive vaginal openings) in each female, it was predicted when the following oestrus phases will occur.

Saliva samples were taken in the oestrus and dioestrus phase. Two saliva samples were taken (one for 17β-oestradiol, one for progesterone) in each phase.

Half of the animals were sampled during oestrus first, the other half was tested during dioestrus first. For oestrus phase, sampling started one day before the next expected opening of the vagina. This was repeated daily until the actual vaginal opening, where again two saliva samples were taken for hormone-concentrations at oestrus time. Body mass was monitored for confirmation of the time of oestrus (It was shown the body weight drops shortly before oestrus (Nemeth et al. 2018)). For the dioestrus phase, animals were sampled 10 days after vaginal opening.

Saliva samples were taken with pieces of Salimetrics Children´s Swabs (Salimetrics® Salimetrics, LLC. ©2021).

Additionally, vaginal smears were taken in oestrus and dioestrus phases. The smears were taken outside the vagina to prevent the possibility of interference with the cycle (during dioestrus the vaginal membrane would otherwise have to be pierced through). Standard cotton buds moistened with 2-3 drops of saline solution were used and the cell material/fluids immediately smeared onto a microscopic slide. The smear was affixed with a fixation-spray and dyed at a later date (dying protocol after Papanicolaou 1942) and analysed using a light microscope.

A behavioural test was performed to analyse possible differences in the females´ behaviour throughout the oestrus cycle. For this, a squared arena of $1m^2$ was used and divided into three compartments using metal grids. Each female was placed in the middle section for ten

minutes, with a randomly selected male on one side, while the other side was left blank. Then it was analysed how much time the female spent nearby the male conspecific (defined as time the female spent in the left half of the middle section) and how much behaviour was shown towards the male. Behaviours included sniffing at the grid, or towards the male while standing at the grid, and biting at the grid (interpreted as effort to reach the male). This test was done in oestrus and dioestrus phases.

2.3.3) Experiment 3 – Testosterone

Testosterone measurements were done with the same 20 male guinea pigs as used for the cortisol experiment. Basal testosterone concentrations during group housing and social confrontations were compared.

The animals were again recorded three times on consecutive days (February 21st to 23rd) for 30 minutes each, as described in experiment 1. On February 23rd the animals were weighed and two saliva samples were taken (one for testosterone, one again for cortisol). Both samples were taken with pieces of Salimetrics Children´s Swabs (Salimetrics®, LLC. ©2021).

The animals were transferred into squared arenas (1m^2) for social confrontation (as described in experiment 1) and stayed there for 24 hours. Five different confrontations were done simultaneously. The confrontations were made from 24th to 26th February. Opponents were the same as in the previous confrontations for cortisol measurements. Additionally, a female was added to each group to stimulate sexual behaviour. All those females were in dioestrus to ensure equal situations for all males and prevent pregnancies.

The animals were recorded for half an hour after they were put into the arenas. Afterwards saliva samples were taken from the males. This procedure was repeated 24 hours later and then the animals were returned to their former groups.

2.4) Hormone Samples and Analysis

All saliva samples were collected by inserting the cotton bud/ Children´s Swab into the mouth of the guinea pig and moving it around for a while to stimulate salivation. Children´s Swabs were cut into smaller pieces that fitted into the animal´s mouths and held with tweezers during saliva collection. Immediately afterwards the samples were centrifuged (10 000

rotations per minute for 5 minutes) and the pieces of Children´s Swab/ cotton bud removed. After this, the samples were frozen at -20° C and stored for later analysis.

To test whether antibodies developed for hormone analysis in human saliva samples are also suitable for guinea pig saliva samples, ELISA test kits from IBL (IBL International GmbH, D-22335 Hamburg, Germany) for cortisol, testosterone, progesterone and 17β-oestradiol were used. Samples were defrosted and serial dilutions made to determine when 50% binding of hormones occurs. For cortisol and progesterone samples dilutions of 1:10 were determined. testosterone and 17β-oestradiol samples were diluted 1:4.

After this, the instructions of the different ELISA test kits were followed. 50µl of each sample were used for the analysis and all samples were double analysed. All hormone concentrations were denoted in pg/ml.

According to the manufacturer´s guidelines, each analysis was performed with internal controls. Moreover, intra- and inter-CVs of the standards were as following: cortisol: intra 4.6%, inter 4.6%; testosterone: intra 6.3%, 17β-oestradiol: intra 3.3%; progesterone: intra 4.4%.

2.5) Behavioural Analysis

The video recordings of group housing and social confrontations were analysed using the software BORIS (Version 8.0.9.; Friard & Gamba 2016).

Sociopositive and agonistic behaviour was analysed, as well as locomotion (forward movement only). Nose-nose contact, naso-anal sniffing, social grooming and resting side by side (resting with direct body contact) were defined as sociopositive behaviour. Agonistic behaviour was divided into dominant and aggressive behaviour. Displacement of another guinea pig, as well as actually sexual behaviour like mounting or rumba-rumble among males were considered as dominance behaviour. Aggressive behaviour included: fighting, biting, stand threat, teeth chattering, head butts, chasing and generally attacks towards another guinea pig.

Additionally, sexual behaviour (of the males) was analysed in experiment 3. This category included sniffing of the females anogenital region, mounting and chin-rump-following, rumba rumble and behaviour like marking of the female. The female´s behaviour was also analysed,

using principally the same categories as for the male animals (sociopositive and -negative) (for more detailed description of guinea pig behaviour see: Rood 1972).

2.6) Statistical Analysis

Statistical analysis was made using the software R (version 4.1.0). Data was tested for normal distribution (Shapiro-Wilk normality test) and homogeneity of variance (Levene´s test).
In experiment 1, the change in body mass was analysed by performing an ANOVA (repeated measurements). For the analysis of the oral glucose tolerance test (OGTT, Experiment 1) a linear mixed model was performed (hours 0, 1 and 2 after glucose administration included as fixed effect). Cortisol concentrations in experiment 1 were compared between group housing (basal condition) and social confrontation by a repeated measurements ANOVA (data log-transformed). Cortisol changes in Experiment 1 were further analysed by a linear regression of the cortisol response (defined as the difference of cortisol concentration in group housing and after 0.5 hours of social confrontation) and the cortisol concentration in group housing. Then cortisol concentrations between age groups were compared using a linear mixed model with day (Group housing and 0.5 hour, 24 hours and 48 hours of social confrontation) and age as fixed effects. The number of aggressive interactions throughout the experiment was also analysed with a Friedman-Test. Cortisol concentrations were then corrected for aggressive interactions. For this the number of aggressive interactions was included as a covariate in a linear mixed model (analysing the change of cortisol concentrations throughout the experiment) and new means (of cortisol concentrations) were estimated. For this the data was log-transformed. The estimated means calculated by the model were then transformed back for representation.
For experiment 2, changes in body mass throughout the oestrus cycle were also analysed by performing an ANOVA (repeated measurements). 17β-oestradiol and progesterone at day -1, 0 and 10 of the respective oestrus cycle were compared via Friedman-test. 17β-oestradiol and progesterone were correlated on day 0 and day 10 using Pearson´s product-moment correlation. To analyse the influence of 17β-oestradiol on female behaviour throughout the oestrus cycle, linear regressions were made (number of behaviours of females towards males on day 0 and day 10).
For experiment 3, cortisol and testosterone concentrations were log-transformed and then compared between group housing and social confrontation by t-tests. The body mass between

group housing and social confrontation was also analysed by a t-test. The change in the number of aggressive interactions throughout the experiment was analysed with a Wilcoxon-Test. Both, testosterone and cortisol, were corrected for aggressive interactions in the same way as in experiment 1. Again, the predicted means were transformed back for representation. A linear regression was carried out to test for the influence of testosterone on aggressive behaviour in group housing and a second linear regression to test its influence on sexual behaviour during social confrontation (with a female present).

2.7) Ethical Statement

Animal keeping and experimentation were performed in accordance with EU Directive 2010/63/EU for animal experiments and were approved and permitted by the institutional board on animal ethics and experimentation (Faculty of Life Sciences, University of Vienna; no. 2021 – 001).

3) Results

3.1) Experiment 1 – Cortisol

Mean body mass in males changed significantly during the experiment ($F_{3,57}$ = 34.755, p< 0.0001) with a significant drop after 24 hours of social confrontation (figure 1A).

Mean basal blood glucose levels were approximately the same in group housing and social confrontation. During the oral glucose tolerance tests the mean blood glucose levels differed between the two conditions ($F_{2,95}$ = 3.162, p = 0.047), while basal glucose concentrations did not (t= 0.563, p= 0.575). One hour after glucose administration, concentrations increased during both conditions, but were slightly higher during social confrontations (t=1.761, p=0.081) than during group housing. Two hours after glucose administration concentrations decreased again, but remained higher during social confrontations (t= 2.930, p= 0.004; figure 1B).

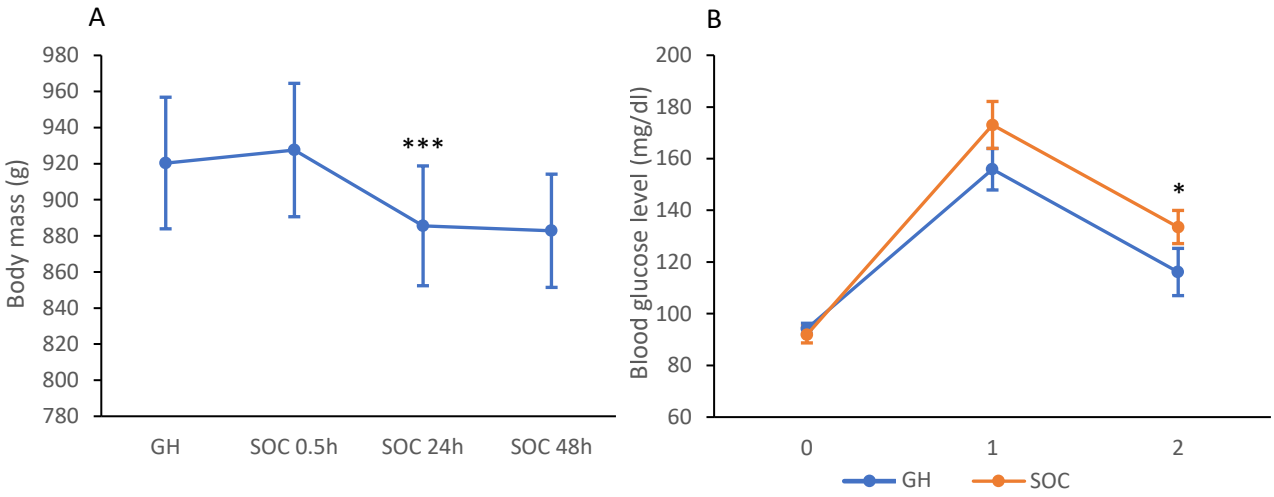

Figure 1: (A) Mean body mass (g) in group housing (GH) and after 0.5, 24 and 48 hours in social confrontation (SOC); (B) Mean blood glucose concentrations during oral glucose tolerance tests in group housing (GH) and social confrontation (SOC), before glucose administration (0), one (1) and two (2) hours afterwards (+/- standard error of the mean); n= 20; Mean +/- SEM; * $p \leq 0.05$, *** $p \leq 0.001$

Mean cortisol concentrations showed no significant change during the experiment ($F_{3,57}$ = 1.590, p = 0.202; figure 2). There was, however, a high individual variance in cortisol concentrations both during group housing and social confrontations (figure 2,3).

The cortisol response from group housing to the first day of social confrontation was significantly dependent on cortisol concentration in group housing ($F_{1,18}$= 35.528, p< 0.001). Animals with high cortisol concentrations in group housing showed a decrease on the first day of social confrontation, whereas animals with low cortisol levels had higher levels in the social confrontation (figure 3). Cortisol response correlated with aggressive behaviour on the first day of the social confrontation (r_S: 694.41, p= 0.033, figure: 4).

However, the cortisol concentrations were affected by age. In group housing older individuals (34 to 36 months old) had significantly higher cortisol concentrations (mean= 16187.5 +/- 4414.299 pg/ml) than younger individuals (four to nine months, mean= 2633.75 +/- 384.076 pg/ml) ($F_{1,14}$= 13.329, p= 0.003). During the first 30 minutes of social confrontation the cortisol levels of the older animals decreased significantly and younger individuals had then significantly higher cortisol levels (always $p < 0.05$, mean cortisol concentrations after 30 minutes: older individuals: 6125 +/- 1308.176 pg/ml, younger individuals: 5250 +/- 1542.895 pg/ml). After 24 hours and 48 hours there was no significant difference between older and younger individuals.

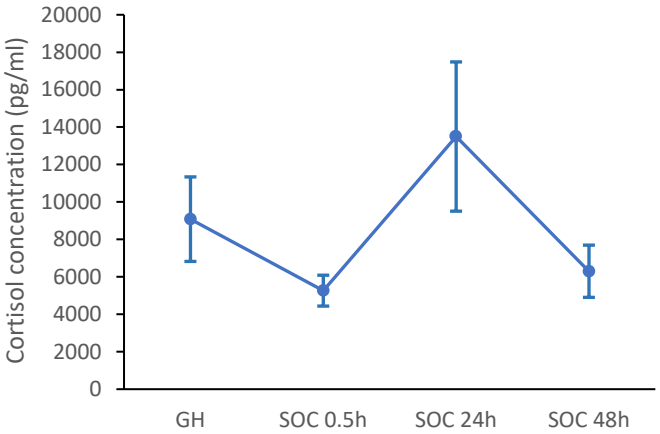

Figure 2: Mean cortisol concentrations in group housing (GH) and 0.5, 24 and 48 hours after beginning of social confrontation (SOC); n= 20; Mean +/- SEM

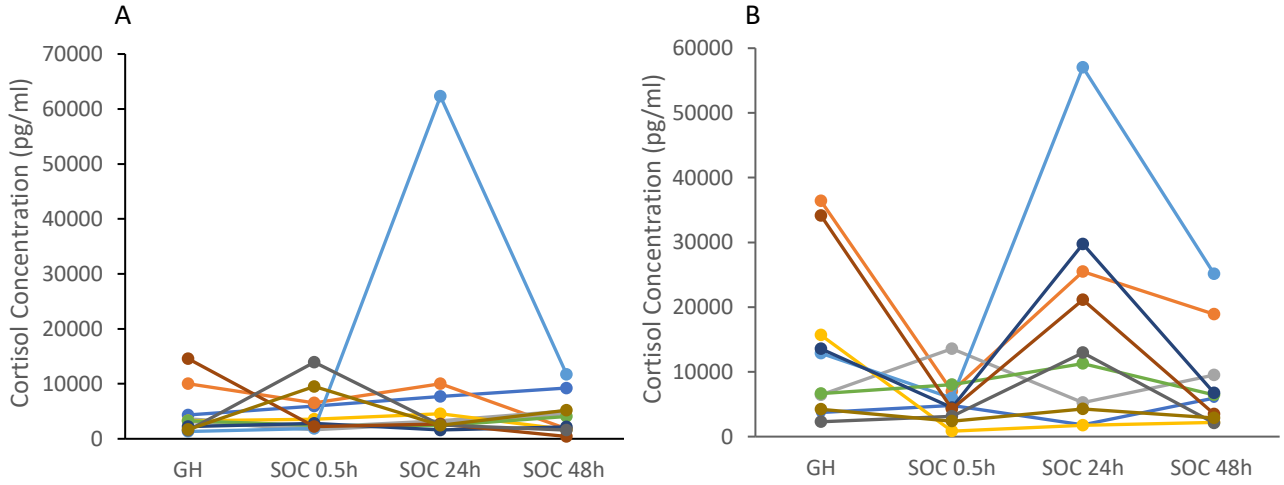

Figure 3: Individual cortisol concentrations in group housing (GH) and 0.5, 24 and 48 hours after beginning of social confrontation (SOC); (A) individual cortisol levels of males in group 1; (B) individual cortisol levels of males in group 2; each group: n= 10

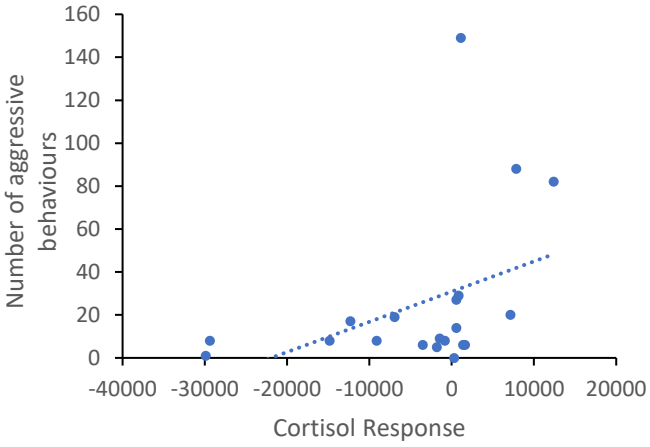

Figure 4: Cortisol Responses (change in cortisol between group housing and social confrontation; cortisol given in pg/ml) correlated with numbers of aggressive behaviours on the first day of social confrontation, n= 20

The number of aggressive interactions changed significantly during the experiment. In the first 30 minutes of the social confrontations there were on average as many interactions as during group housing. After 24 hours the number decreased significantly (Friedman Chi^2= 48.688, df= 3, $p < 0.001$) and remained low until the end of the experiment (figure 5A).

When corrected for aggressive interactions, the cortisol levels after 24 hours of social confrontation were significantly elevated ($F_{3,53}$= 3.713, p= 0.017; figure 5B).

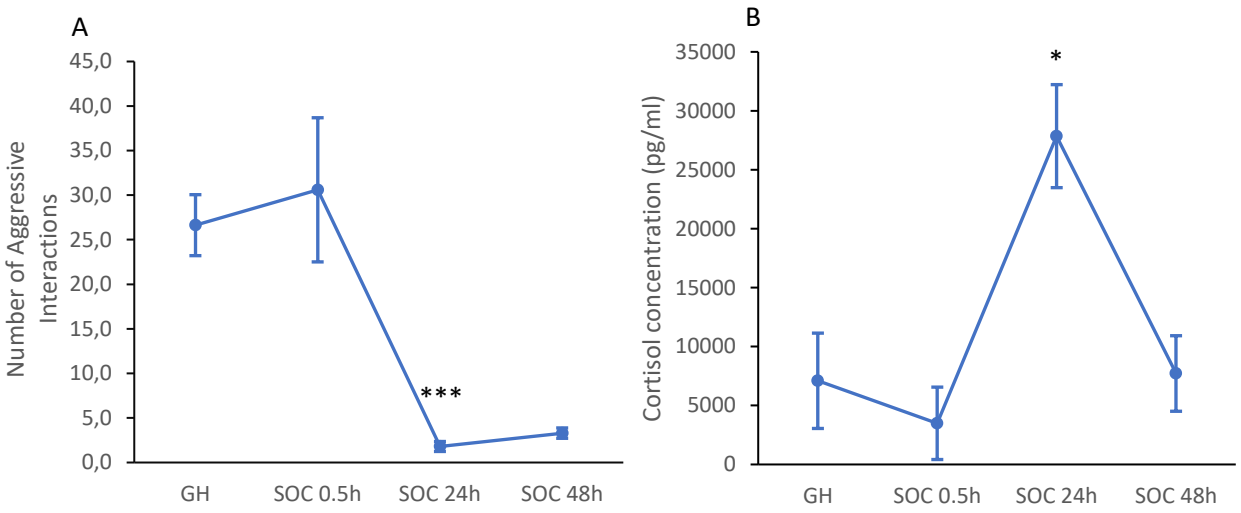

Figure 5: (A) Frequency of aggressive interactions and (B) cortisol concentrations corrected for aggressive interactions in group housing (GH) and 0.5, 24 and 48 hours after beginning of social confrontation (SOC); n=20; Mean +/- SEM; * $p \leq 0.05$, *** $p \leq 0.001$

3.2) Experiment 2 – 17β-Oestradiol & Progesterone

Female guinea pigs showed a significant drop in body mass at day 0, the day of vaginal opening ($F_{2,23}$= 3.989, p= 0.033; figure 6).

The vaginal smears differed in cellular composition between day 0 and day 10. On day 10 only keratinized epithelial cells were present (100%), no leucocytes or nucleated epithelial cells. On day 0, all of these cells were present in the smears. Between 23.20% and 100% of cells were keratinized epithelial cells, between 0.40% and 55.24% were nucleated epithelial cells. Leucocytes were present in two smears in a very different quantity (8.64% and 76.30%).

Mean 17β-oestradiol concentrations showed no significant differences throughout the oestrus cycle (Friedman Chi^2= 0.666, df= 2, p= 0.716); figure 7A). Progesterone concentrations also did not change during the experiment (Friedman Chi^2= 1.167, df= 2, p= 0.558; figure 7B).

There was a high individual variation in 17β-oestradiol and progesterone concentrations throughout the experiment (figure 7C and D).

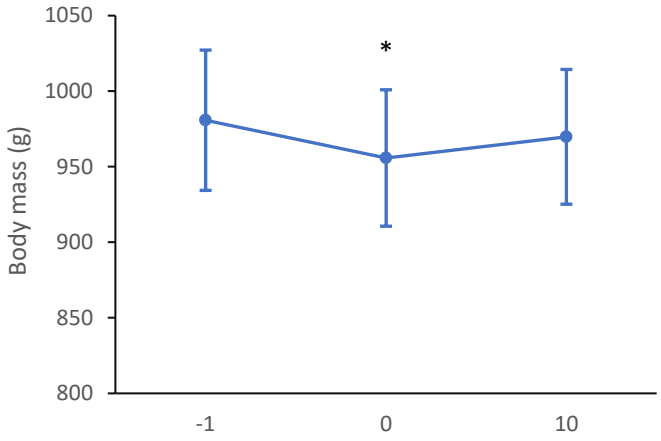

Figure 6: Body mass of female guinea pigs (n= 13) during oestrus cycle; measured on the day before vaginal opening (-1), at the first day of vaginal opening (0) and 10 days afterwards (10); n= 13; Mean +/- SEM; * p ≤ 0.05

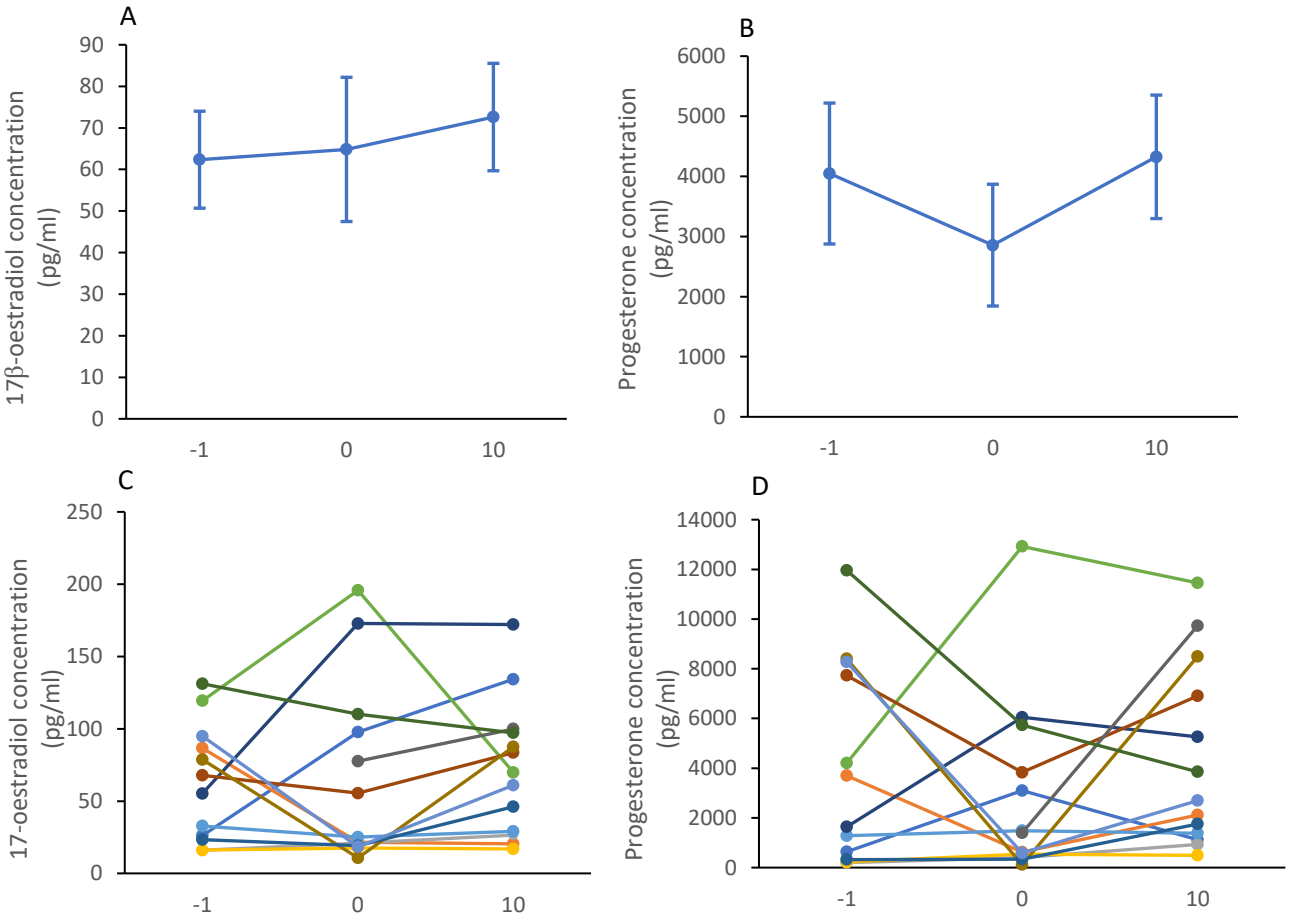

Figure 7: 17β-oestradiol and progesterone concentrations during the oestrus cycle, measured on the day before vaginal opening (-1), the first day of vaginal opening (0) and 10 days afterwards (10); (A) mean 17β-oestradiol concentrations, (B) mean progesterone concentrations, (C) individual 17β-oestradiol concentrations, (D) individual progesterone concentrations; n= 13; Mean +/- SEM (A/B)

17β-oestradiol and progesterone concentrations were positively correlated on day -1 (r= 0.838, p= 0.001, figure 8A) and day 0 (r= 0.915, p= 0.00002, figure 8B). However, they did not correlate on day 10 (r= 0.388, p= 0.190; figure 8C).

In the behavioural test there was no significant difference between day 0 and day 10 concerning the behaviour of the females towards the males (t= 0.376, df= 12, p= 0.713). There was also no difference in the time spent in the left section of the cage which was next to the present male when comparing day 0 and day 10 (V=46, p= 1). The total number of behaviours shown by the female towards the male on day 0 was slightly influenced by the 17β-oestradiol levels ($F_{1,11}$= 4.024, p= 0.07). On day 10 there was no such influence ($F_{1,11}$= 0.110, p= 0.746; figure 9).

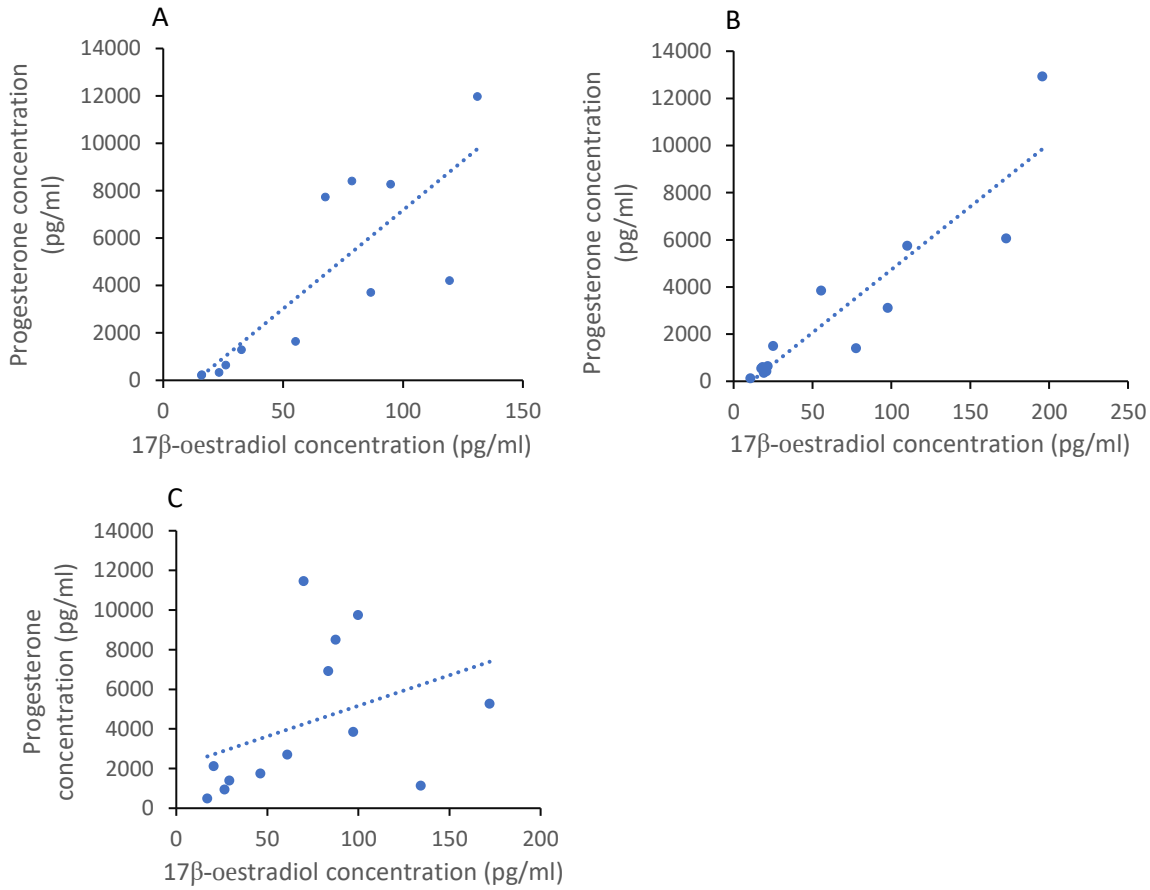

Figure 8: Correlations of 17β-oestradiol and progesterone during the oestrus cycle; (A) day -1 (one day before vaginal opening), (B) day 0 (day of vaginal opening) and (C) day 10 (10 days after vaginal opening); n= 13

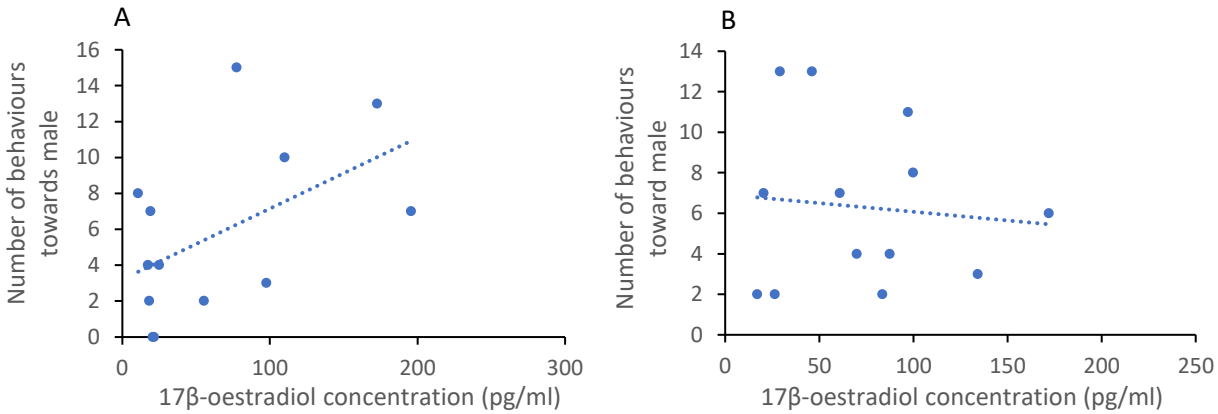

Figure 9: Correlation of 17β-oestradiol concentration and number of behaviours of female guinea pigs towards males on (A) day 0 and (B) day 10; n= 13

3.3) Experiment 3 – Testosterone

There was no difference in mean testosterone concentrations throughout the experiment (t= 1.044, n= 20, p= 0.309; figure 10A). In addition, cortisol concentrations showed no significant difference between group housing and social confrontations (t= 1.341, n= 20, p= 0.196) and there was a high individual variation during the social confrontation (figure 10B).

Mean body mass decreased significantly during the social confrontations (t= 8.508, n=20, $p < 0.001$; figure 11A).

The number of aggressive interactions was significantly lower during social confrontations compared to group housing (V= 169, p= 0.003; figure 11B).

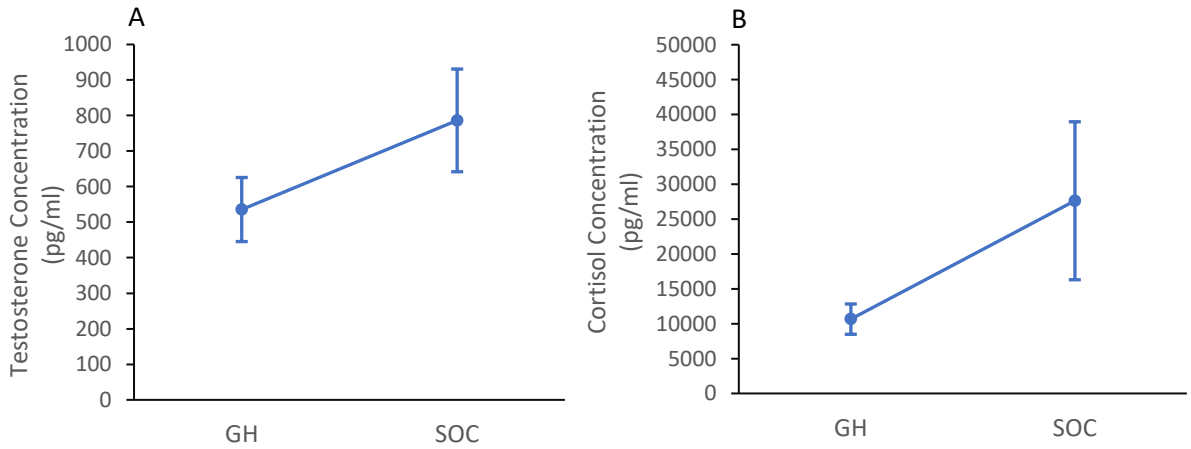

Figure 10: (A) mean testosterone concentrations and (B) mean cortisol concentrations of males in group housing (GH) and during social confrontation (SOC); n= 20; Mean +/- SEM

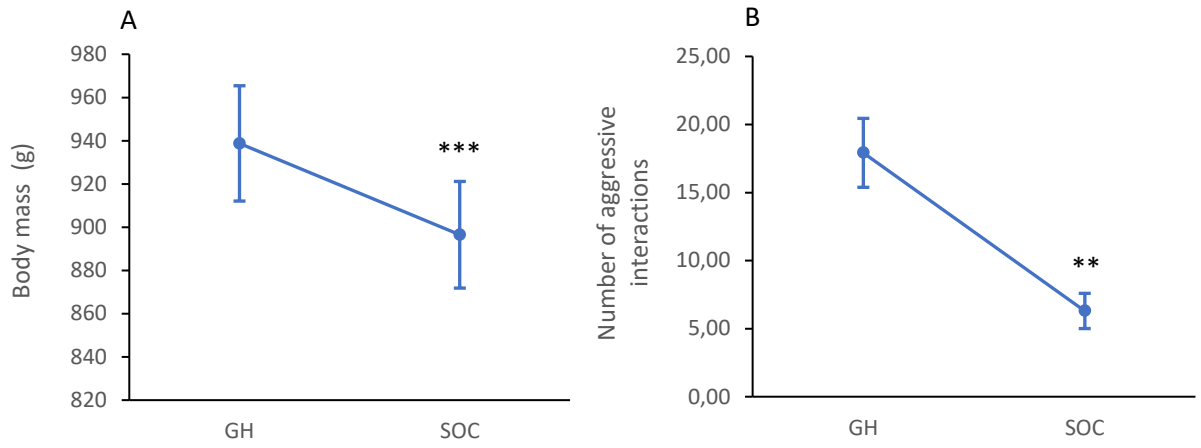

Figure 11: (A) mean body mass and (B) mean number of aggressive interactions (active and passive) in group housing (GH) and social confrontation (SOC); n= 20; Mean +/- SEM; ** $p \leq 0.01$, *** $p \leq 0.001$

Including the number of aggressive interactions as covariate in the analysis of testosterone and cortisol concentrations led to a significant difference between group housing and social confrontation in both cases. Both hormones showed a significant increase in social confrontations under the consideration of aggressive interactions (testosterone: $F_{1,17}$= 5.161, p= 0.036; cortisol: $F_{1.17}$= 4.136, p= 0.058; figure 12 A and B).

In group housing, testosterone concentrations were positively correlated with aggressive interactions ($F_{1,18}$= 4.822, p= 0.041, figure 13A). During social confrontations the number of sexual behaviours positively affected testosterone concentrations ($F_{1,17}$= 4.937, p= 0.039; figure 13B).

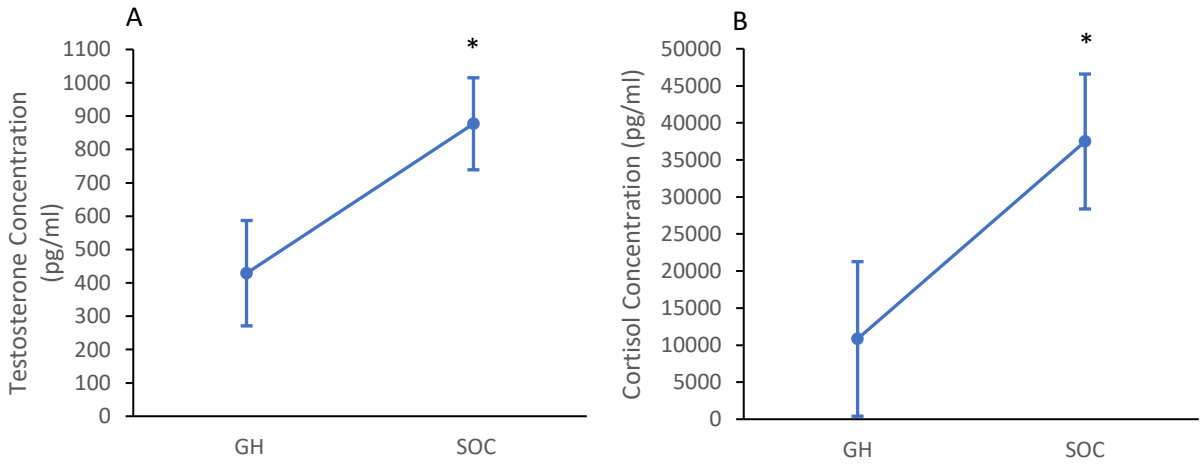

Figure 12: (A) Mean testosterone and (B) cortisol concentrations corrected for aggressive interactions in group housing (GH) and social confrontation (SOC); n= 20; Mean +/- SEM; * $p \leq 0.05$

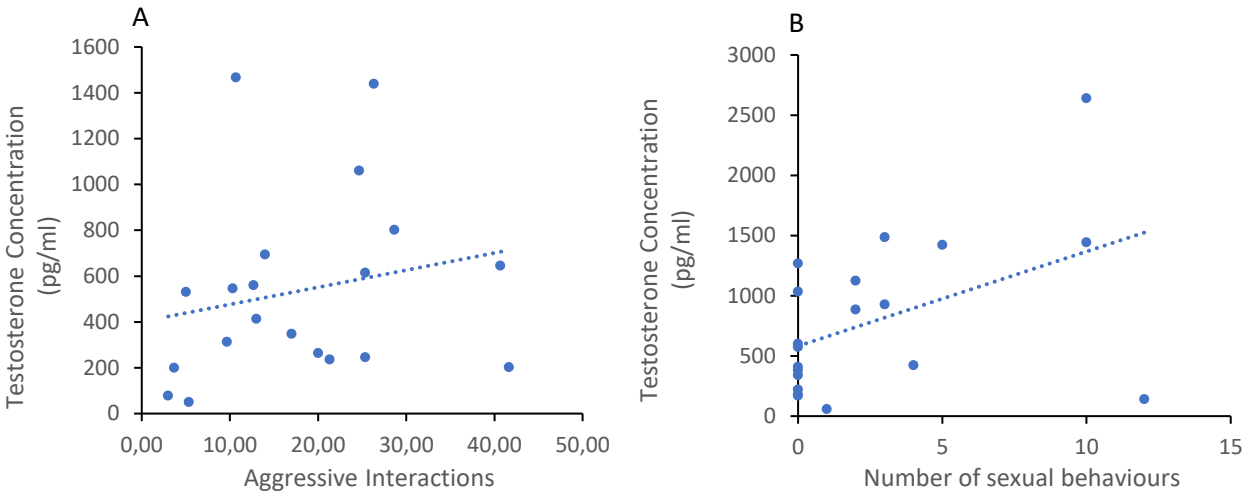

Figure 13: (A) Testosterone concentrations correlated with aggressive interactions in group housing (data were ln transformed for statistical analysis) and (B) testosterone concentrations correlated with number of sexual behaviours in social confrontation; n= 20

4) Discussion

The aim of this study was to validate antibodies for steroid hormone analysis in human saliva samples for the usage in guinea pigs. In three experiments, increases of cortisol, testosterone, 17β-oestradiol and progesterone concentrations should have been triggered by biological stimuli or correlate with physiological conditions. The results have not been as expected, for example no clear increase in cortisol during the social confrontation was measured, although changes in body weight and an altered reaction during the oral glucose tolerance test indicate that the confrontation did serve as a stressor. In summary, none of the measured hormone concentrations showed clearly the expected patterns. Nevertheless, some of the results fit well to the respective physiological context. This indicates that the used antibodies possibly are still adequate for the usage in guinea pigs.

4.1) Experiment 1 – Cortisol

Social challenges in guinea pigs are characterized by various physiological and behavioural changes related to increased stress loads. The mean body mass dropped after 24 hours of social confrontation. This supports the idea that the confrontations served as a stressor and that the physiological stress response of the body led to a higher energy demand (Sapolsky et al. 2000). Another support for this can be found in the results concerning the blood glucose levels. Although the blood glucose levels in sober conditions did not differ when comparing group housing and social confrontations, the increase after administration of glucose during the oral glucose tolerance test (OGTT) was significantly higher during the social confrontation. It is a known effect of glucocorticoids to reduce the storage of glucose, which stays in the circulatory system, thereby providing more energy for further physiological processes (Sapolsky et al. 2000).

If the social confrontation served as a stressor, the cortisol concentrations should have increased during the experiment, but this was not the case. There was a high individual variance and a difference between older and younger animals concerning their basal cortisol levels and cortisol responses. Older individuals had higher cortisol concentrations during group housing. Basically, this is not coherent with other studies in which younger guinea pigs had higher cortisol concentrations than older ones, (see e.g. Fazekas et al. 1974; Hennessy et al. 2006). However, these findings are limited to rather young animals, since all used guinea pigs in the mentioned study were less than one year old, while the animals´ age in our study ranged from four months to three years. It is possible that some stressful situation took place during the group housing phase of this study, for example during night time, when the guinea pigs were unobserved. Another possibility is that the housing condition of a rather large group of only male animals is more challenging for older males.

The high basal cortisol concentrations in older animals probably resulted in reduced responsiveness during the social confrontation. High cortisol concentrations over a longer period may impair the possibility of an individual to react to an unforeseen new stressor, as stress responses can only be maintained for a certain time, depending on the specific stress load (Romero et al. 2009). According to this hypothesis, the younger males may have been able to cope better with the situation because their basal stress level was lower. This could also account for the correlation of the cortisol response and aggressive behaviour on the first

day of social confrontation. Animals with a positive cortisol response (increase of cortisol concentration) had lower cortisol levels beforehand (during group housing). This is coherent with previous findings (e.g. Nemeth et al. 2021), supporting the reliability of our measurements. Their better initial physiological state allowed them to react more efficiently during the confrontation, both hormonally and behaviourally, which enhanced their chances to gain the higher hierarchical status, as also suggested by previous findings in rats (Cordero & Sandi 2007).

After 24 hours of social confrontation the difference between young and old individuals vanished. At this time there were hardly any aggressive interactions observed, indicating that hierarchical relationships were settled. It can be assumed that the situation at this time was not as stressful as at the beginning of the social confrontation, which makes it remarkable that there was no significant decrease of cortisol concentrations towards the end of social confrontations.

Moreover, when the cortisol concentrations were corrected for the influence of aggressive behaviour, the results after 24 hours of social confrontations were significantly higher compared to the other sampling points in time. A social confrontation is primarily a behavioural stimulus with two males trying to cope with the unknown conspecifics by immediate fighting (or at least threatening) and by that forming a dominance relationship. This process usually includes high aggressiveness and an immediate rise in cortisol concentrations (see e.g. Nemeth et al. 2016; Sachser 1987). Behaviour and hormonal status are closely linked and can influence each other. Nevertheless, there was no significant change in cortisol concentrations during the social confrontation. Including the aggressive interactions as a behavioural influence on the hormonal situation is appropriate in the case of a social stressor like a social confrontation.

4.2) Experiment 2 – 17β-Oestradiol & Progesterone

The guinea pig oestrus cycle can easily be followed by simple measurements, like tracking body mass and checking for vaginal opening.

There was a significant decrease in body mass on the day of vaginal opening. It was shown that such a drop takes place before ovulation and is triggered by oestrogens (Czaja & Goy 1975), therefore this serves as a proof of an intact oestrus cycle in the females. Another

indirect proof for this was provided through the vaginal smears. During dioestrus there were only keratinized epithelial cells in the smears. This is due to the fact that the vaginal membranes were left intact during this study. In other studies with female guinea pigs, vaginal smears were either only obtained when the membrane was naturally open (Burgos & Wislocki 1956; Harned & Casida 1972) or it was opened artificially (Lilley et al. 1997; Selle 1922; Stanberry et al. 1986). When the membrane is closed, it covers the vaginal orifice tightly. The absence of other cells than keratinized ones indicates that there is no cell material from inside the vagina going through the barrier of the membrane. Tearing the vaginal membrane apart seems to be a necessity, if reliable vaginal smears are to be obtained during the dioestrus phase. In this study this was not done because of the possible influences of an artificial rupture on the hormonal state and sequence of the oestrus cycle.

The vaginal smears obtained during the vaginal opening were very divers. Keratinized and nucleated epithelial cells could be found in very different quantities. In two smears also leucocytes were present. This outcome is probably due to the fact that the oestrus phase in guinea pigs is rather long (three to four days) and can again be subdivided into different stages. According to Stockard and Papanicolaou (1917, 1919) a special sequence occurs, that causes specific histological changes. It was argued that this sequence serves as additional timing mechanism for insemination. Only one aspect of this process is to be discussed here, namely the inflow of leucocytes into the vagina during the late oestrus. This alters the chemical environment in a way that destroys remaining sperm. Harned & Casida (1972) also described that shortly after the vaginal opening mainly keratinized epithelial cells are present, while leucocytes start to occur when ovulation takes place. The different results of the vaginal smears are possibly due to this very long and diverse oestrus phase in guinea pigs. Females were checked daily in the morning for vaginal opening. The openings could have occurred recently beforehand or hours before (potentially almost a day before), which seems to have a major influence on the physiological state of the animal and therefore also on physiological measurements in relation to the oestrus cycle. This could also be a factor for the hormone measurements. Neither 17β-oestradiol nor progesterone concentrations showed the expected pattern throughout the oestrus cycle. The methods used for monitoring the oestrus cycle are maybe not exact enough to guarantee reliable measurements representing the changes throughout the oestrus cycle in saliva samples.

Another factor may have been a pathological one, connected with the different ages of the used animals. Female guinea pigs have a strong tendency to develop cysts in the genital tract and the probability increases with age. It was shown that females who are 3 years old have genital cysts with a probability of 40% (Bertram et al. 2018). Other studies showed that such cysts can cause reproductive failure and irregularities in the oestrus cycle (Keller et al. 1987; Pilny 2014). This could also account to some degree for the high individuality of 17β-oestradiol and progesterone concentrations in this study. A prolonged oestrus cycle can possibly also be a sign of a pathological state. Some of the female guinea pigs in this study had rather long or irregular cycles, so it is possible that not all animals were completely healthy in this regard.

There are also findings suggesting that guinea pigs can have oestrus cycles with two phases of follicular maturation, which is connected with a more complex pattern of hormone secretion, for example a second peak of oestrogens during the time in which dioestrus is supposed to take place (Hutz et al. 1990). Progesterone peaks before ovulation were also measured in guinea pigs (Feder et al. 1968; Nemeth et al. 2018).

Taken together the hormonal pattern of female guinea pigs may be more complicated than thought and a high variance can be found when hormones are measured. It is therefore possible that the findings of this experiment are reliable, although this cannot be said with certainty. Since no pattern was found at all, it cannot be said that the used antibodies were validated successfully.

One of the only hints for valid measurements was a correlation of 17β-oestradiol and progesterone on the day of vaginal opening and one day beforehand, at the onset of oestrus phase. This is probably due to processes of biosynthesis (Nelson & Kriegsfeld 2017), progesterone being a predecessor of oestrogens. During dioestrus, this relationship was absent.

Additionally, behavioural tests were made to detect whether the females show different behaviour towards males in oestrus and dioestrus phase. It was expected, that the females would show more interest in their male conspecifics when being in oestrus, but no such relationship was found.

During oestrus phase there was a slight influence of 17β-oestradiol on the behaviour of the females found. The different hormonal changes during oestrus are known to lead to male-seeking behaviour. The attention and reaction towards stimuli are altered and sexual behaviour is promoted (Nofrey et al. 2008; Young et al. 1935; Nelson & Kriegsfeld 2017). A

connection between oestrogens and behaviour during oestrus makes sense – while ovulation is initiated, the coherent behaviour is triggered, which helps finding a mate and initiating copulation. Consequentially, such a relationship between 17β-oestradiol and behaviour towards males was absent during dioestrus. These last findings indicated that the hormonal measurements are possibly reliable. Nevertheless, neither a definite difference in behaviour, nor in 17β-oestradiol or progesterone concentrations between oestrus and dioestrus was found, therefore the reliability of the measurements remains questionable. This is also supported by the fact that there was no difference between the hormonal courses of older and younger females (data not shown).

4.3) Experiment 3 – Testosterone

Social confrontations of male guinea pigs should not only lead to physiological stress responses, but also to aggressive behaviour and a rise in testosterone concentrations (e.g. Lürzel et al. 2010).

Neither testosterone nor cortisol concentrations showed a significant increase during social confrontations in experiment 3, similar to cortisol in experiment 1. Again, an explanation could be that the situation of the social confrontation was not as stressful as intended, despite the additionally present female. The males were confronted with the same opponent as in experiment 1, so it is possible that the situation was too familiar this time. However, the mean body mass decreased, which is indicative of a physiological stress reaction. Being taken out of the normal housing group and getting transferred into arenas for social confrontations should in itself be a stressor, even when this was already experienced before (Nemeth et al. 2021). It has been shown that the testosterone concentration can rise, when the social position of a male guinea pig is challenged (Sachser 1987). It can be argued that because the opponent was not unfamiliar this time, social instability was not perceived as strongly as during the first social confrontation (during experiment 1). The hierarchy between the opponents, which was established during experiment 1 was probably still relevant during the new encounter, which has also been shown in previous studies (Nemeth et al. 2021). This is supported by the fact that there was less aggressiveness during the second social confrontation (data not shown). Nevertheless, the additional female must have altered the situation, serving per se as a

stimulus for sexual behaviour and testosterone, but also as a reason for the opponents to combat, which again should trigger aggressiveness, testosterone and cortisol secretion.
Also this time, the number of aggressive interactions was much lower after 24 hours of social confrontation than in group housing. Testosterone is linked to aggressiveness, therefore it seems consistent that if there is no substantial rise in testosterone, no increase in the amount of aggressiveness takes place. Nevertheless, when testosterone concentrations were corrected for aggressive interactions, a significant increase was identified. The same was true for cortisol in this experiment. Again, it can be argued that the confrontation represents a behavioural stimulus, and if this behavioural aspect is considered, the results fit our original expectations.
The more important factor in this experiment was the presence of a female during the social confrontation. All females were in the phase of dioestrus, among other things to prevent pregnancies. Anyway, they should have still represented a sexual stimulus, because the males were not accustomed to the presence of females. And indeed, while during group housing, testosterone concentrations correlated with aggressive behaviour, testosterone correlated with sexual behaviour during the social confrontations. The present female was the relevant new stimulus during the social confrontation, not the other male. These results make sense and may indicate that the hormone measurements in this experiment are plausible to some degree, but again do not fully confirm that the used antibodies lead to reliable results.

4.4) Conclusion

The stimuli and natural physiological processes used in the experiments did generally not lead to any direct changes in the hormone concentrations. This indicates that the validation of the used antibodies was not successful. Nevertheless, some tendencies and correlations were found, that show some validity of the used antibodies, for example the relationship between cortisol concentrations in group housing and cortisol response in experiment one, the connection between 17β-oestradiol and female behaviour during oestrus or the correlation of testosterone with aggression and sexual behaviour. An additional collection of blood samples would have potentially provided more clarity as to whether the used antibodies are appropriate for guinea pigs, but the aim of this study was to use only biological methods of validation and to avoid invasive procedures.

For all four hormone measurements it has to be mentioned that although some of the results were making sense, in summary no definite validation was possible. Further experiments are necessary for clarification.

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Deutsche Zusammenfassung

Eine sorgfältige Validierung von neuen Methoden ist notwendig, um Fehler in der künftigen Nutzung zu vermeiden. Ziel der vorliegenden Arbeit war eine biologische Validierung von Antikörpern zur Steroidhormonanalyse, welche für humane Speichelproben entwickelt wurden, für die Verwendung bei Speichelproben von Meerschweinchen. Dafür wurden bekannte biologische Stimuli für Cortisol, Testosteron, 17β-Estradiol und Progesteron verwendet – soziale Konfrontationen mit und ohne Weibchen und die physiologischen Prozesse während des Östruszyklus. In Experiment 1 wurden soziale Konfrontationen zwischen jeweils zwei Männchen durchgeführt, um die Cortisol Konzentrationen zu erhöhen. Es fand keine deutliche Erhöhung statt, stattdessen trat eine hohe individuelle Varianz auf, wobei ältere Tiere eine höhere Cortisol Konzentration zeigten. Wurden die Werte auf den Einfluss von Aggressivität korrigiert, ergaben sich signifikant erhöhte Cortisol Konzentrationen während der sozialen Konfrontation. In Experiment 2 wurden Speichelproben von Weibchen im Östrus und Diöstrus gesammelt. Zusätzlich wurde deren Interesse an Männchen analysiert, indem sie mit einem Männchen konfrontiert wurden. Weder 17β-Estradiol, noch Progesteron zeigten einen signifikanten Unterschied zwischen Östrus und Diöstrus. Während des Östrus wurden eine Korrelation von 17β-Estradiol und Progesteron und ein schwacher Einfluss von 17β-Estradiol auf das Verhalten festgestellt.

In Experiment 3 wurden Testosteron und Cortisol während sozialer Konfrontationen gemessen, bei denen zusätzlich ein Weibchen als Stimulus für Konkurrenz und sexuelles Verhalten anwesend war.

Weder Testosteron, noch Cortisol zeigten einen Anstieg während der Konfrontationen. Die Werte wurden jedoch wiederum auf Aggression korrigiert, was erhöhte Hormonkonzentrationen ergab. Testosteron korrelierte positiv mit Aggression, sowie sexuellem Verhalten. In Summe war, trotz einiger Korrelationen, keine klare Validierung möglich.