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Repeatability of behaviour and cortisol concentrations during the oestrus cycle in guinea pigs

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– This work is dedicated to my daughter –

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ABSTRACT

Characterised by a spontaneous ovulation and a subsequent active luteal phase with significantly increased progesterone secretion, the oestrus cycle in female guinea pigs has many similarities to reproduction in humans. Despite this fact, there are not many studies investigating the influence of oestrus on physiology and behaviour in guinea pigs. Therefore, the aim of this study was to examine to what extent the cycle phase influences cortisol concentrations and behaviour. In addition, the parameters were compared in two consecutive cycles to determine whether both physiological parameters and behaviour are consistent. Daily cycle monitoring was performed in twelve female guinea pigs by visual inspection of the vaginal membrane and body mass changes. Saliva samples to analyse cortisol concentrations were taken before and after the 15-minute dark-light test. A dark-light test, a step-down test and a sociality test were performed to analyse exploration, anxiety, risk-taking and social interest. Oestrus was characterised by significantly higher cortisol concentrations and significantly lower body mass compared to dioestrus. In the dark-light test, the cycle phase had no significant influence on the behaviour. The step-down test could only be analysed descriptively, as different females stepped down the platform in only eight out of 48 test runs. Locomotion in the sociality test seems to be partly influenced by the cycle phase, but inconsistently, and it could be a consequence of the interaction with the conspecific. The interaction with the conspecific was significantly increased during oestrus compared to dioestrus, at least in the first cycle. Regarding the repeatability, all behaviours shown in the dark-light test and the sociality test were repeatable over time, but independently of the cycle phase. The results of this study show that oestrus can influence physiology and, to some extent, behaviour in female guinea pigs. However, the individual differences in behaviour indicate that personality has a stronger influence on behaviour than the cycle itself. This study contributes to a research area that offers great potential.

Keywords: guinea pigs, oestrus cycle, personality, cortisol, social interest, emotionality

1. INTRODUCTION

The oestrus cycle of female guinea pigs is increasingly coming to the forefront of current research (e.g., Glenk et al., 2018; Nemeth et al., 2023, 2018), which is highly important because of many similarities in reproduction compared to humans (e.g., Enders and Blankenship, 1999). The oestrus cycle in guinea pigs lasts around 16 days (Selle, 1922), which is relatively long compared to other rodents such as rats, showing a cycle length of 4-5 days (Freeman, 1988). Like in other species, the oestrus cycle of guinea pigs can be divided into four stages: oestrus, metoestrus, dioestrus, and prooestrus (Selle, 1922). Accompanied by an oestrogen peak around oestrus (Joshi et al., 1973; Nemeth et al., 2018), ovulation occurs spontaneously in guinea pigs (Enders and Blankenship, 1999) and is followed by an active luteal phase (Blatchley et al., 1976) with significantly increased progesterone concentrations 5-13 days later (Nemeth et al., 2018).

In addition to these hormonal changes, there are also changes in other physiological parameters throughout the oestrus cycle in guinea pigs, for example body mass is significantly decreased during oestrus compared to dioestrus (Nemeth et al., 2023, 2018), probably due to reduced food intake at this time (Czaja and Goy, 1975). Furthermore, metabolic rates measured through mean oxygen consumption and resting metabolic rates were significantly increased during oestrus compared to dioestrus (Nemeth et al., 2023). The oestrus cycle might also influences the cortisol concentrations, whereby the baseline cortisol concentrations at the time of oestrus are significantly increased in comparison to dioestrus (Nemeth et al., 2023, 2018). These changes during oestrus indicate altered homeostasis and energy balance. Beside the well-studied hormonal and physiological changes, only a few studies have investigated the behaviour of female guinea pigs during their cycle or only very selective behavioural parameters were examined (Birke, 1981; Glenk et al., 2018).

Sinchak and Wagner (2012) reviewed that cyclic changes in oestrogen concentrations during the oestrus cycle strongly influence behaviour, leading to differences in behaviour during oestrus compared to dioestrus. Golden hamsters had shown an increase in activity (Richards, 1966) and rats had displayed less anxiety-like behaviour (Mora et al., 1996) and more exploratory behaviour (Martin and Bättig, 1980) during oestrus. During female-female interactions, guinea pigs spent more time interacting

with the conspecific and displayed more locomotion during oestrus (Birke, 1981). However, the results in guinea pigs are not entirely homogeneous, as a more recent study had shown that females showed less social interest during oestrus as indicated by more flight behaviour and less sit side by side behaviour (Glenk et al., 2018). Anyway, the same study had reported an increase in cortisol concentrations during the conducted female-female confrontation in dioestrus but not in oestrus (Glenk et al., 2018), indicating an effect of the cycle stage on (social) stress responses.

Under stressful situations, the activity of the hypothalamic-pituitary-adrenocortical (HPA) axis plays an important role by ensuring the production of corticotropin-releasing hormone (CRH) in the hypothalamus and thus the release of adrenocorticotropic hormone (ACTH) into the pituitary gland. This involves the initial hormonal regulation of the stress response (Kleine and Rossmanith, 2007). ACTH is transported via the blood circulation into the adrenal cortex, where it ensures the synthesis and release of glucocorticoids, the steroid hormones corticosterone and cortisol (Ehlert and von Känel, 2010). Glucocorticoid stress response can not only differ between reproductive phases (Nemeth et al., 2023), but can also vary according to sex, season, and body condition. In addition, there can be strong individual differences in the stress response (Cockrem, 2013).

Individuality is characterised by animals differing from their conspecifics in their physiological and behavioural traits (Lathe, 2004). The term ‘personality’ assumes that individual behavioural differences are consistent over time and/or across contexts (Dall et al., 2004). If a behaviour has a relatively low within-individual variance, it is more repeatable than a behaviour with a high between-individual variance. This means that a behaviour is repeatable if an individual behaves consistently over time and its behaviour differs from other conspecifics (Bell et al., 2009). Thus, repeatability describes the extent of individual variation in a behavioural (or physiological) trait compared to the overall variation within a population (Nakagawa and Schielzeth, 2010). A previous study in guinea pigs had shown that the socio-positive and aggressive behaviour towards an unknown conspecific of the same sex as well as boldness in the novel object test in male and female guinea pigs was significantly repeatable over a period of four weeks and independent of sex (Brust and Guenther, 2017). Furthermore, the dominance rank index of guinea pigs in their housing group is also stable over six to eight weeks and therefore repeatable (Rystrom et al., 2022;

Zipser et al., 2013). In addition, sexual and courtship behaviour is repeatable in male guinea pigs (Zipser et al., 2013). In contrast to the baseline cortisol concentration, the cortisol responsiveness after both one and two hours in an open-field test is repeatable in both sexes of guinea pigs (Rystrom et al., 2022; Zipser et al., 2013).

Our study aim was to investigate the influence of the cycle phase on cortisol concentrations, exploratory behaviour, anxiety-like behaviour, risk-taking behaviour, and social interest. In addition, the extent to which the corresponding parameters were consistent at an individual level was also examined. We are not aware of any study that has investigated this so far as the oestrus cycle was also not considered in previous studies on repeatability in behavioural and physiological traits in guinea pigs. Furthermore, this study would be the first to compare the behaviour of two consecutive cycles in order to investigate to what extent the influence of the cycle phase on behavioural and physiological traits in both cycles are consistent. A comparison of two cycles should provide more robust results and thus correspondingly more reliable statements can be made about the oestrus cycle. The present study is therefore intended to increase the knowledge about possible behavioural changes during the oestrus cycle in guinea pigs in order to be able to conduct more specific studies in the future or to draw conclusions relevant to human medicine.

2. ANIMALS, MATERIALS, AND METHODS

2.1 Animals

The female guinea pigs used for this study were descendants of a domesticated, heterogeneous, and multicoloured guinea pig population (*Cavia aperea f. porcellus*) that was bred at the Department of Behavioral and Cognitive Biology at the University of Vienna. The animals were all adult, between six and 18 months old, sexually mature, and accustomed to the daily presence and handling by humans. The animals could be distinguished by their individual fur colour and scheme.

2.2 Animal Husbandry

The twelve female guinea pigs used in this study were kept in two groups of nine females each; three further females in both groups were not part of the study but were kept together with the focal animals.

The enclosures of the two groups had the same structure and equipment. They had a size of 3.2 m² (1.6 m x 2 m). The walls of the enclosures, which were 0,5 m high, were made of laminated chipboards. The floor of the enclosures was covered with bedding material. Both enclosures were equipped with two laminated chipboard shelters. Guinea pig pellet food (ssniff V2233, ssniff Spezialdiäten GmbH, Soest, Germany) was provided *ad libitum* in two feeding bowls per enclosure. Hay was distributed daily in one hay rack per group to ensure abrasion of the teeth and to support intestinal activity. Water was accessible in three water bottles per enclosure, refilled daily, and available *ad libitum*.

The enclosures were cleaned twice a week. The average room temperature was 22 ± 1 °C and the average humidity in this room was 50 ± 5 %. There was a twelve-hour day-night cycle, so that the light turned on at 7:00 am and turned off at 7:00 pm. The animals were kept together in a room with other guinea pig enclosures, so that the females were exposed to olfactory and acoustic stimuli from other females and males.

2.3 Study Design

Data collection for this study took place over a five-week period (30.08.2023 – 02.10.2023). Already two weeks prior to the testing period, daily cycle monitoring was carried out on each of the twelve females. For this purpose, the vagina of each female was visually inspected. This is a suitable method to observe changes in the vaginal membrane, as it opens and closes depending on the stage of the oestrus cycle. It is closed during the latent period of the oestrus cycle and open at the time of ovulation (Wilson et al., 2021). The cycle monitoring was performed according to the procedure of Wilson et al. (2021). The first day on which the vagina was visibly open was defined as oestrus. Dioestrus was defined as eight to ten days later. Following the monitoring, each female was weighed by using a standard household kitchen scale (accuracy ± 1 g) and then returned to the home enclosure.

After cycle monitoring, the following procedure was done on each day of the study, if applicable. Females in oestrus and/or dioestrus were sampled for saliva and then tested at 30-minute intervals in (1.) a dark-light test, (2.) a step-down test, and (3.) a sociality test. After the dark-light test, another saliva sample was taken. Saliva samples were collected to determine differences in cortisol concentrations associated with the cycle stage and potential stress responses to the dark-light test. The behaviour during the dark-light test, step-down test, and sociality test were analysed to see if there were differences in the behaviour depending on whether the females were in oestrus or dioestrus. Each female was tested in two consecutive cycles thereby covering two oestrus and two dioestrus stages of each female. Based on the cycle monitoring before the onset of the actual testing phase, six females were tested first in oestrus and six females first in dioestrus.

2.4 Behavioural Tests

The behavioural tests took place in a different room than the guinea pig housing room, but in which the same conditions prevailed as in the housing room (cf. 2.2 Animal Husbandry). The dark-light test and the sociality test were video recorded using GoPro HERO3 cameras. The video cameras were already switched on before the animals were placed in the enclosure. The step-down test was observed live.

2.4.1 Dark-Light Test

The dark-light test is a common test, primarily in mice and other rodents, to test exploratory behaviour in a new environment and anxiety-like behaviour (Hascoët et al., 2001). Mora et al. (1996) had shown that female Sprague-Dawley female rats show less anxiety-like behaviour during oestrus compared to dioestrus. Whether there were differences in the exploratory and anxiety-like behaviour during oestrus and dioestrus should be found out by means of locomotion and the spatial stays in the dark-light test.

2.4.1.1 Procedure of the Dark-Light Test

The test enclosure had a size 60 x 120 cm and the floor of the enclosure was covered with bedding material. The walls of the enclosure, which were 40 cm high, were made of white laminated fibreboard. The enclosure was separated by a wall into two equally sized areas. One area was darkened by a movable cover made of fibreboard. A sliding door in the separating wall allowed to open the dark area so that the females could explore the light area.

One video camera was mounted on a metal rail 2 m over the centre of the enclosure. Another video camera was installed in the movable cover of the dark area without allowing light to pass through, so that the behaviour in the dark area after opening the sliding door could also be analysed.

Before the test, a saliva sample (*cortisol pre*) was taken from the focal female. The animal was then placed in the dark area, with the sliding door remaining closed so that the animal could acclimatise for one minute while the experimenter left the room. After one minute, the sliding door was opened and the test began. The test always lasted 15 minutes. Subsequently, another saliva sample (*cortisol post*) was taken and the female was returned to its home enclosure until the female participated approximately 30 minutes later in the step-down test.

2.4.2 Step-Down Test

The step-down test is a suitable method to test risk-taking behaviour in guinea pigs (Zipser et al., 2013). Whether there were differences in the behaviour during oestrus and dioestrus should be found out by means of latency to step down the platform as a measure of risk-taking.

2.4.2.1 Procedure of the Step-Down Test

The experimental set-up approximately followed the protocol of Zipser et al. (2013). The test enclosure had a size of 1 m^2 (100 x 100 cm) and the floor of the enclosure was covered with bedding material. The walls of the enclosure, which were 50 cm high, were made of fibreboard. The platform from which the animals should step down had a base area of 30 x 30 cm and a height of 20 cm and was placed in the middle of a wall of the test enclosure.

At the beginning of the test, the female was placed at the edge of the platform. The experimenter withdrew immediately afterwards and set a stopwatch. The test lasted a maximum of five minutes, or until the animal stepped down during this period. Subsequently, the female was returned to its home enclosure until the female participated approximately 30 minutes later in the sociality test.

2.4.3 Sociality Test

During social confrontations, the animals are confronted with a new environment on the one hand and an unknown conspecific on the other. This can be a stressful situation for the animals (Nemeth et al., 2014), but social partners can also attenuate the stress caused by the unfamiliar environment (Michael B. Hennessy et al., 2006). This type of confrontation can lead to both socio-positive and aggressive contact between the animals (Brust and Guenther, 2017). During oestrus, female guinea pigs showed more locomotion and more social interest in female conspecifics in social confrontations (Birke, 1981), but also more flight behaviour (Glenk et al., 2018). Whether there were

differences in the social interest in same-sex conspecifics during oestrus and dioestrus should be found out by the amount of interaction time with a conspecific in the sociality test.

2.4.3.1 Procedure of the Sociality Test

The test took place in an enclosure with a size of 60 x 120 cm. The floor of the enclosure was covered with bedding material. The walls of the enclosure, which were 40 cm high, were made of white fibreboards. The right area of the enclosure remained empty and a grid was placed in the centre of the left area. The grid had a diameter of 25 cm and was just large enough for a conspecific to fit in and turn around its own axis so that the focal animal could walk around the grid and approach the conspecific (see fig. 1). The conspecifics were from a different female housing group that were not unknown to the focal animals, but they had not been in direct contact for a month.
At the beginning of the test, the conspecific was placed in the grid of the left area. The focal female was then placed in the right-hand corner of the right area of the enclosure with her snout pointing towards the enclosure wall. Once the animals were in the enclosure, the test lasted 15 minutes. Afterwards, the animals were returned to their home enclosures.

Figure 1.: Test enclosure of the sociality test.

2.4.4 Video Analysis

By using BORIS (Behavioral Observation Research Interactive Software, program version v.7.13.9; Friard and Gamba, 2016), the videos of the dark-light test and the

sociality test were analysed. BORIS is a software that can be used to evaluate uploaded videos. With this software, single keys on the laptop keyboard can be coded as subjects and behaviours. Modifiers can be added for single behaviours so that the behaviour can be categorised. This enabled a count to be made for each female of all the behaviours it displayed. In addition, behaviours can be marked as point or state events, so that the frequency on the one hand and the duration of a behaviour on the other hand can be counted.

The start time of the video evaluation can be set manually. The analysis of the videos from the dark-light test began one minute after one female was placed in the test enclosure as soon as the sliding door was opened. The analysis of the videos from the sociality test began when one female was placed in the test enclosure. During the evaluation, the video speed could be manually increased or decreased, or if the females had not shown any behaviour, it was possible to fast-forward. It was also possible to rewind the video if a behaviour was overlooked or the behaviour was not clearly visible.

2.4.4.1 Behavioural Analysis of the Dark-Light Test

The following behaviours were analysed: duration of locomotion in the dark area, duration of locomotion in the light area, latency to leave the dark area, frequency of entering the light area, and time spent in the centre of each area.

Locomotion was measured as soon as the female moved more than one body length. The *frequency the animal entered the light area* was counted as soon as the female was in the light area with more than half the body length. If a female entered the light area in this way for the first time, the *latency to leave the dark area* was recorded. The *duration spent in the centre* was registered as soon as the female was one body length away from the walls of the enclosure.

2.4.4.2 Behavioural Analysis of the Step-Down Test

It was analysed whether the females stepped down or not (yes/no). The latency to *step down* was defined as the time point at which the animal left the platform and touched the ground of the enclosure with all four paws. If the females stepped down, the *latency to step down* was also noted.

2.4.4.3 Behavioural Analysis of the Sociality Test

The following behaviours were analysed: time spent in the left area (containing the conspecific in the grid), time spent in the right area (empty), duration of locomotion in the left area, duration of locomotion in the right area, and duration of the interaction time with the conspecific.

The *time spent (…)* was measured as soon as the female was more than one body length in the corresponding area. *Locomotion* was analysed as soon as the female moved more than one body length. *Interaction time* was defined as visible interaction with the conspecific, for example by sniffing, gnawing or climbing on the grid.

2.5 Saliva Collection and Hormone Analysis

Besides blood sampling (Sachser et al., 1998) , saliva sampling is also a suitable method to measure cortisol concentrations in guinea pigs (Nemeth et al., 2016). Therefore, the less invasive method of saliva sampling was chosen for this experiment. Previous studies have already shown that cortisol concentrations in female guinea pigs fluctuate during the oestrus cycle, with cortisol concentrations being higher during oestrus compared to dioestrus. (Nemeth et al., 2023, 2018). To find out to what extent cortisol concentrations differ at the time of oestrus or dioestrus, saliva samples were taken before the dark-light test for the baseline values (*cortisol pre*). Saliva samples were also taken after the dark-light test (*cortisol post*) to determine the possible influence of the test on the cortisol concentrations and thereby a stress response.

Saliva samples were taken with a standard cotton swab. To do this, the cotton swab was carefully placed in the mouth of each animal and gently twisted around in the mouth. The whole procedure took on average no longer than one minute per individual. Afterwards, the females were placed in the test enclosure of the dark-light test or returned to their home enclosures. The saliva samples were prepared for the hormonal analysis directly after the saliva sampling. For this purpose, the cotton swabs with the saliva samples were placed in a halibated pipette tip that had previously been placed in a 1.5 mL Eppendorf tube. The stalk of the cotton swab was then cut off and the Eppendorf tubes were closed so that these could be centrifuged at 10,000 rpm for ten minutes. The samples were then frozen at -20 °C and only defrosted again for analysis.

2.5.1 Analysis of the Saliva Samples

By the common method of the so-called enzyme-linked immunosorbent assay the concentration of cortisol in the saliva could be determined (Cortisol ELISA, RE52061, IBL International GmbH, Hamburg, Germany). Samples were diluted 1:7 and analysed following the manufacturer's instructions. The hormone analysis was run in duplicates and the exclusion criterion was if the duplicates had a deviation of more than 15 %. Intra and inter coefficients of variance of control samples were 3.34 % and 4.12 % respectively.

2.6 Statistics

All data were analysed with the software R version 4.2.2 (R Core Team, 2022). The statistics were carried out with the additional packages nlme (Pinheiro et al., 2023) to perform linear mixed models, lme4 (Bates et al., 2015) for generalised linear mixed models, and emmeans (Lenth, 2023) for post-hoc analyses. Analyses of body mass, cortisol concentrations, and behaviour in the dark-light test were performed by using linear mixed models and behaviour in the sociality was analysed by using generalised linear mixed models (with binomial errors) always including the 'cycle phase' (oestrus and dioestrus) and 'cycle number' (cycle 1 and cycle 2) as fixed effects and individual ID as random effect to correct for repeated measurements. When analysing the cortisol

concentration, the time of measurement before or after the dark-light test (cortisol pre and cortisol post) was also included as a fixed effect. Shapiro-Wilk normality test and Levene's test for variance homogeneity were used to analyse normal distribution and homogeneity of variance of all model residuals. Some of the data was log-transformed. Post-hoc analyses were Bonferroni-corrected. The significance level was set at 0.05. The package rptR (Stoffel et al., 2017) was used to estimate adjusted repeatability for body mass, cycle duration, duration of the open vaginal membrane, cortisol concentrations, and behaviour in the behavioural tests (dark-light test, step-down test, and sociality test). The significance level was set at 0.05.

2.7 Ethical Statement

This study was performed in accordance with European Union (EU) Directive 2010/63/EU for animal experiments and national laws. The study has been checked and approved by the institutional ethics committee of the Faculty of Life Sciences of the University of Vienna (Nr. 2023-011) and the Austrian Federal Ministry of Science and Research (2023-0.514.989).

3. RESULTS

3.1 Physiology

3.1.1 Cycle length and opening of vaginal membrane

The average cycle length of the females was 16.75 ± 2.71 days in the first cycle and 17.92 ± 2.93 days in the second cycle. During the first cycle the vaginal membrane of the females was open for an average of 4.58 ± 2.06 days and during the second cycle for an average of 4 ± 1.83 days. Both durations did not differ between the cycles and also no repeatability was found (cycle duration: $F_{1,11} = 0.940$, $p = 0.353$; $R = 0$, $CI = 0 – 0.52$, $p = 1$; duration vaginal opening: $F_{1,11} = 0.494$, $p = 0.497$; $R = 0$, $CI = 0 – 0.53$, $p = 1$).

3.1.2 Body mass

Body mass was in general significantly lower during oestrus than during dioestrus, and overall lower in the first compared to the second cycle (cycle phase: $F_{1,33} = 11.898$, $p = 0.002$; cycle number: $F_{1,33} = 4.776$, $p = 0.036$; cycle phase x cycle number: $F_{1,33} = 0.388$, $p = 0.538$) (fig. 2).

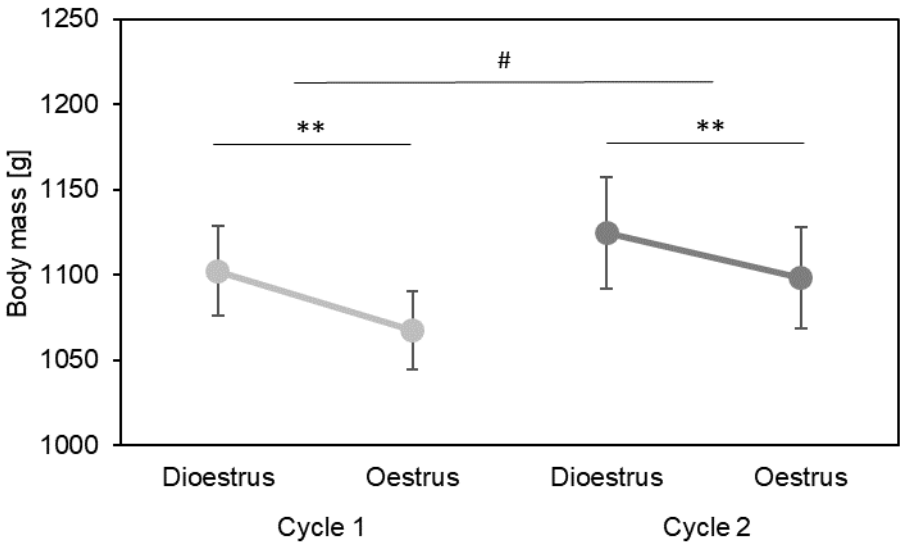

Figure 2.: Body mass [g] in dioestrus and oestrus during both cycles. Data are the mean ± s.e.m., ** $p < 0.01$ comparing dioestrus and oestrus; # $p < 0.05$ comparing cycle 1 and cycle 2

3.1.3 Saliva cortisol concentrations

Corrected for a significant effect of cycle number (log-transformed; $F_{1,78} = 8.198$, $p = 0.005$), with generally higher cortisol concentration in the second cycle, cycle phase significantly affected cortisol (log-transformed; $F_{1,78} = 20.813$, $p \leq 0.001$). Cortisol concentrations were significantly higher during oestrus compared to dioestrus. Timing of measurement (pre vs. post) was not significant and was therefore removed from the model (log-transformed; $F_{1,78} = 0.001$, $p = 0.975$) (fig. 3).

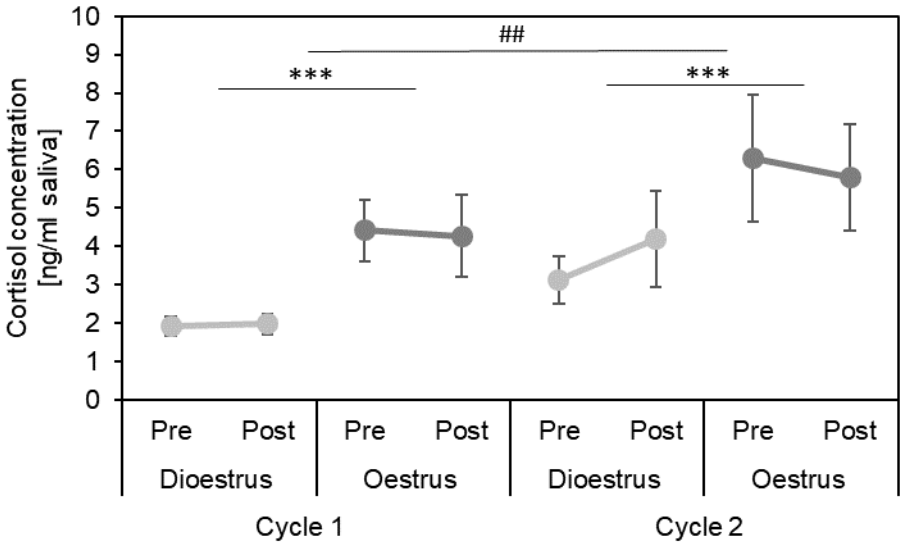

Figure 3.: Cortisol concentrations [ng/ml saliva] in dioestrus and oestrus during both cycles. Data are the mean ± s.e.m., *** $p < 0.001$ comparing dioestrus and oestrus; ## $p < 0.01$ comparing cycle 1 and cycle 2

3.2 Behavioural Tests

3.2.1 Dark-Light Test

Overall, there was no significant influence of the cycle phase on any of the measured behavioural parameters in the dark light test. In general, the females had displayed more locomotion in the dark area in the first compared to the second cycle (log-transformed; cycle phase: $F_{1,33} = 1.955$, $p = 0.171$; cycle number: $F_{1,33} = 4.599$, $p = 0.04$; cycle phase x cycle number: $F_{1,33} = 1.156$, $p = 0.290$) (fig. 4).

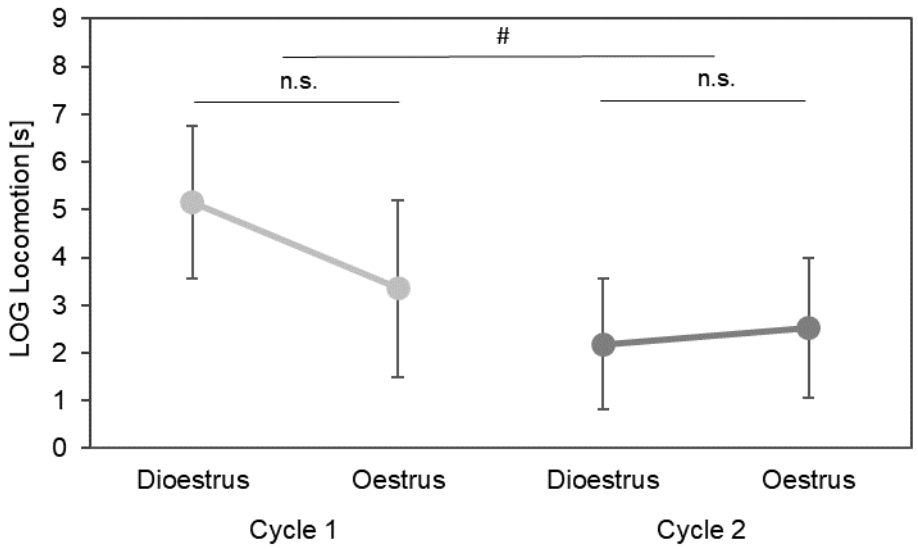

Figure 4.: Locomotion [s] in the dark area of the dark-light test in dioestrus and oestrus during both cycles. Data are the mean (log-transformed) ± s.e.m., n.s. not significant comparing dioestrus and oestrus; # $p < 0.05$ comparing cycle 1 and cycle 2

Regarding the time in the centre of the dark area, no significant effect was found for either the cycle phase or the cycle number (log-transformed; cycle phase: $F_{1,33} = 2.424$, $p = 0.129$; cycle number: $F_{1,33} = 2.177$, $p = 0.150$; cycle phase x cycle number: $F_{1,33} = 3.237$, $p = 0.081$) (fig. 5).

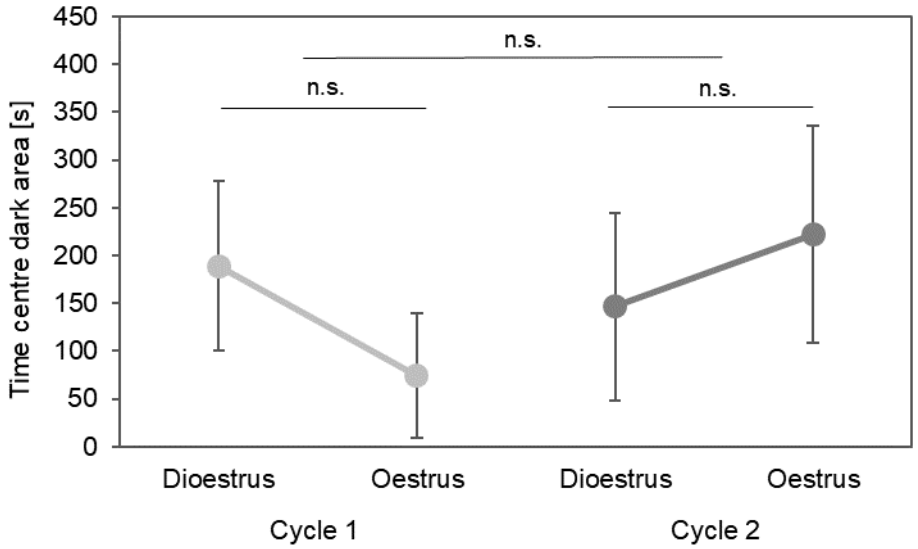

Figure 5.: Time in the centre of the dark area [s] of the dark-light test in dioestrus and oestrus during both cycles. Data are the mean ± s.e.m., n.s. not significant comparing dioestrus and oestrus and comparing cycle 1 and cycle 2

As only three females left the dark area during the entire experiment, the duration of locomotion in the light area, the latency to leave the dark area, and the frequency the animal entered the light area were not statistically analysed further. The average time until leaving the dark area was 210.41 ± 93.17 seconds in these animals.

3.2.2 Step-Down Test

Out of a total of 48 test runs, only in eight runs, different females stepped down the platform. The results of the step-down test were therefore only analysed descriptively and the latency to step down was not taken into further account. In the first cycle, two out of twelve females stepped down the platform during dioestrus and three out of twelve during oestrus. In the second cycle, two out of twelve females stepped down in both cycle phases. Only one female had stepped down twice, once in oestrus and once in dioestrus.

3.2.3 Sociality Test

The proportion of time spent in the left area (containing the conspecific) was significantly affected by the interaction of cycle phase and cycle number (cycle phase: $Chi^2 = 9.486$, $p = 0.002$; cycle number: $Chi^2 = 15.943$, $p < 0.001$; cycle phase x cycle number: $Chi^2 = 1054.072$, $p < 0.001$). The females spent significantly more time in the left area during oestrus compared to dioestrus in the first cycle ($z = - 25.210$, $p < 0.001$), but less time there during oestrus in the second cycle ($z = 21.048$, $p < 0.001$). A comparison of the cycles revealed that the females spent significantly more time in the left area in oestrus during the first cycle than in oestrus during the second cycle ($z = 20.249$, $p < 0.001$). In dioestrus it was the opposite, with less time in the left area in the first cycle and more time in the second cycle ($z = - 25.976$, $p < 0.001$) (fig. 6).

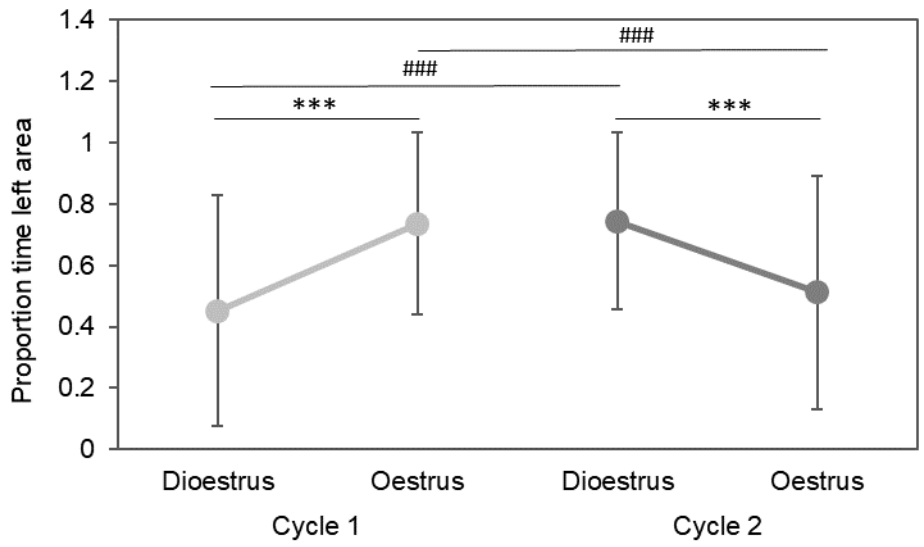

Figure 6.: Proportion of time spent in the left area (containing the conspecific) in relation to the total time in the sociality test in dioestrus and oestrus during both cycles. Data are the mean ± s.e.m., *** $p < 0.001$ comparing dioestrus and oestrus; ### $p < 0.001$ comparing cycle 1 and cycle 2

Locomotion in the left area (as proportion on the total time in the left area) was significantly affected by the cycle phase and cycle number (cycle phase: $Chi^2 = 32.145$, $p < 0.001$; cycle number: $Chi^2 = 38.126$, $p < 0.001$; cycle phase x cycle number: $Chi^2 = 2.554$, $p = 0.11$), whereby the females in general displayed significantly more locomotion in the first cycle as well as during oestrus compared to dioestrus in both cycles (cycle 1: $z = - 5.401$, $p < 0.001$; cycle 2: $z = - 2.268$, $p = 0.023$) (fig. 7, A). The proportion of locomotion in the right area (empty) was significantly affected by the interaction of cycle phase and cycle number (cycle phase: $Chi^2 = 0.002$, $p = 0.966$; cycle number: $Chi^2 = 0.738$, $p = 0.390$; cycle phase x cycle number: $Chi^2 = 12.314$, $p < 0.001$). In the first cycle, the females showed significantly more locomotion in the right area in oestrus than in dioestrus ($z = - 2.266$, $p = 0.023$). In the second cycle it was the other way round, with the females displaying significantly less locomotion in oestrus compared to dioestrus ($z = 2.695$, $p = 0.007$). There was no significant difference between the cycle numbers with regard to oestrus ($z = 1.531$, $p = 0.126$). Regarding dioestrus, the females showed more locomotion in the right area in the second cycle than in the first cycle ($z = - 3.234$, $p = 0.001$) (fig. 7, B).

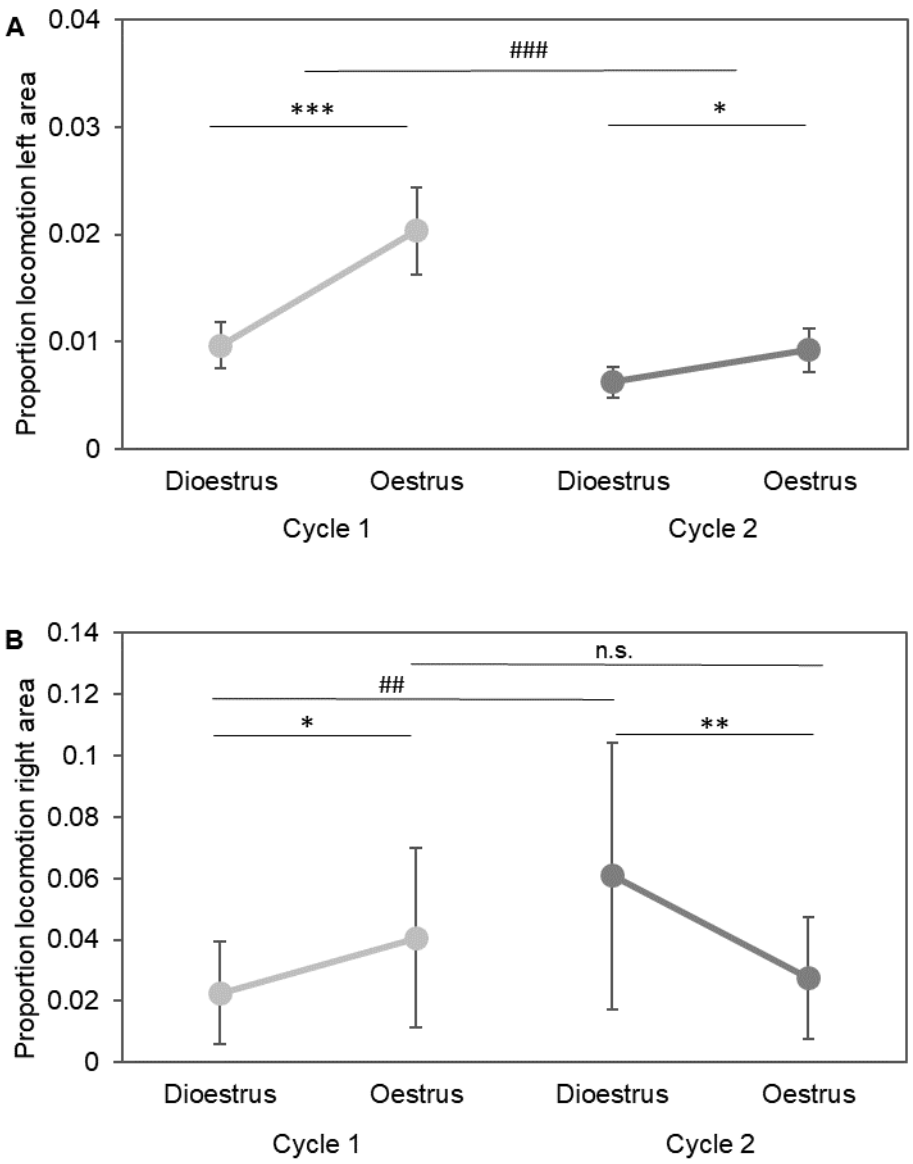

Figure 7.: Proportion of the locomotion displayed in (A) the left area (containing the conspecific) and (B) the right area (empty) in relation to the total time in the corresponding area of the sociality test in dioestrus and oestrus during both cycles. Data are the mean ± s.e.m., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing dioestrus and oestrus; n.s. not significant, ## $p < 0.01$, ### $p < 0.001$ comparing cycle 1 and cycle 2

The interaction time with the conspecific (as proportion on the total time in the left area) was significantly affected by the cycle phase interacting with cycle number (cycle phase: $Chi^2 = 70.201$, $p < 0.001$; cycle number: $Chi^2 = 4.802$, $p = 0.028$; cycle phase x cycle number: $Chi^2 = 58.280$, $p < 0.001$). The females interacted significantly more with the conspecific during oestrus compared to dioestrus in the first cycle ($z = - 11.309$, $p < 0.001$), but there was no significant difference between the cycle phases in the second cycle ($z = - 0.532$, $p = 0.595$). A comparison of the cycles

revealed that the females interacted significantly more in oestrus during the first cycle compared to oestrus during the second cycle ($z = 6.620$, $p < 0.001$). In dioestrus it was the opposite, with less interaction time in the first cycle and more interaction time during dioestrus in the second cycle ($z = -4.417$, $p < 0.001$) (fig. 8).

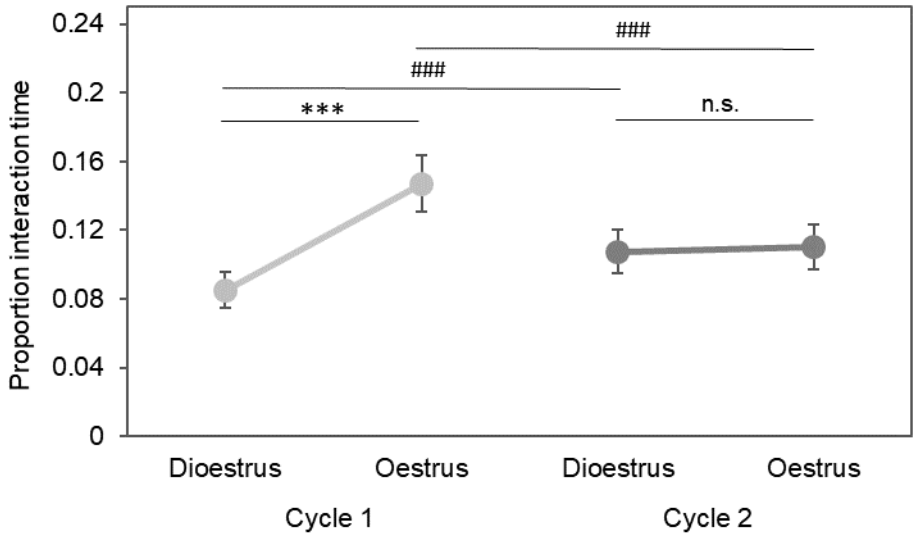

Figure 8.: Proportion of interaction time in relation to the total time spent in the left area of the sociality test in dioestrus and oestrus during both cycles. Data are the mean ± s.e.m., n.s. not significant, *** $p < 0.001$ comparing dioestrus and oestrus; ### $p < 0.001$ comparing cycle 1 and cycle 2

3.3 Repeatability

In terms of physiology, only body mass was repeatable over the two cycles ($R = 0.935$, $CI = 0.83 – 0.97$, $p < 0.001$), correspondingly, neither cortisol concentrations before the dark-light test ($R = 0.092$, $CI = 0 – 0.41$, $p = 0.245$) nor after the dark-light test ($R = 0.036$, $CI = 0 – 0.35$, $p = 0.386$) were repeatable over time. Regarding the dark-light test, both locomotion displayed in the dark area ($R = 0.392$, $CI = 0.06 – 0.68$, $p = 0.002$) and time spent in the centre of the dark area ($R = 0.579$, $CI = 0.27 – 0.81$, $p < 0.001$) were repeatable over the two cycles. All behaviours displayed in the sociality test were likewise repeatable over time, including the time in the left area ($R = 0.656$, $CI = 0.34 – 0.84$, $p < 0.001$), locomotion in the left area (log-transformed; $R = 0.501$, $CI = 0.15 – 0.75$, $p < 0.001$), locomotion in the right area (log-transformed; $R = 0.449$,

CI = 0.12 – 0.72, p < 0.001), and interaction time with the conspecific (R = 0.289, CI = 0 – 0.58, p = 0.014) (fig. 9).

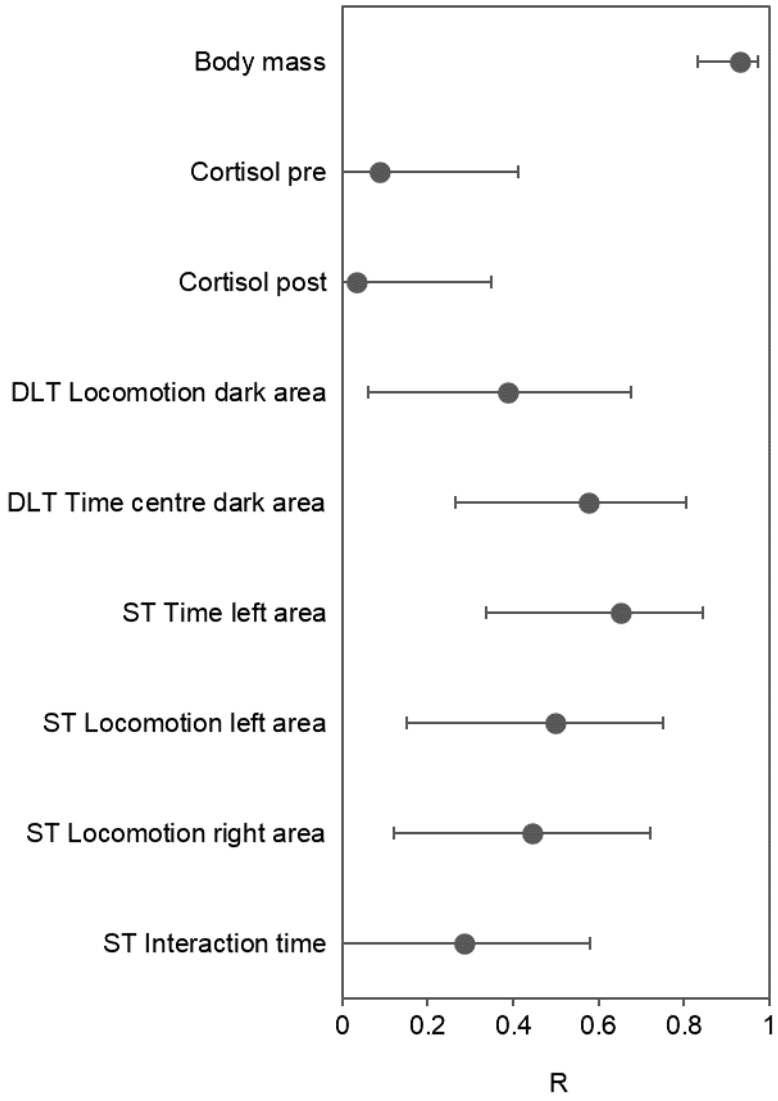

Figure 9.: Forest plot summarising repeatability estimates and 95 % confidence intervals. ST: sociality test; DLT: dark-light test

4. DISCUSSION

This study compared two consecutive oestrus cycles and investigated the extent to which behavioural and physiological parameters differ between the stages of oestrus and dioestrus in female guinea pigs. In the present study, we showed that body mass, cortisol concentrations and, to some extent, social interest in conspecifics are influenced by the oestrus cycle. In contrast, emotionality such as anxiety-like behaviour or risk-taking did not appear to be influenced by the cycle. Based on the definition of repeatability (see Bell et al., 2009) and the fact that all behaviours investigated and analysed here were repeatable over time, our findings indicate that personality has a much stronger influence on behaviour than the oestrus cycle in female guinea pigs.

In general, guinea pigs are suitable for investigating influences of the oestrus cycle, as cycle monitoring can be carried out by visual inspection of the vaginal membrane (Wilson et al., 2021), whereas in rats (or mice) vaginal smears including staining and evaluation must be used (Ajayi and Akhigbe, 2020). The average cycle length of the females in the present study is consistent with previous studies (Czaja and Goy, 1975; Selle, 1922). At around four to six days, the opening of the vaginal membrane was slightly higher than the 48-72 hours recorded in the study by Wilson et al. (2021). Both durations did not differ between the cycles and also no repeatability was found, indicating that cycle length and vaginal opening have a high within-individual variance in guinea pigs.

Additionally to the vaginal opening, body mass was controlled during the whole cycle as it is an important indicator of oestrus (Nemeth et al., 2023). At the time of vaginal membrane opening, which is associated with oestrus, feed intake in guinea pigs is lowest (Czaja and Goy, 1975) and oestrogen concentrations are highest (Nemeth et al., 2018) compared to other stages of the ovarian cycle. Since neither was investigated in the present study, it can only be assumed that oestrogen has a suppressive effect on the animals' feeding behaviour and thus on body mass, which is probably why the body mass of the females in this study was significantly lower at the stage of oestrus compared to dioestrus in both cycles. In addition, body mass was significantly lower in the first cycle than in the second cycle. This was probably because the animals' food was switched to *ad libitum* two weeks prior the study so that the

animals had unlimited access to food. Body mass itself was highly repeatable over the two cycles, indicating that it varied less within than among individuals.

In addition to body mass, cortisol concentrations were as well influenced by the cycle stage, with significantly higher cortisol concentrations during oestrus compared to dioestrus. Effects on cortisol concentrations align with the results by Nemeth et al. (2023, 2018). Oestrus seems to be energetically demanding and stressful since cortisol concentrations are high and body mass is low. Regarding the stress-induced cortisol concentrations, no significant differences between oestrus and dioestrus were found in previous studies (Glenk et al., 2018; Nemeth et al., 2023). This is largely consistent with the present findings, which revealed no effect of the 15-minute dark-light test on cortisol concentrations in oestrus or dioestrus. The stress-induced cortisol concentrations in the blood are reached approximately after 10-30 minutes (Kirschbaum et al., 2000). Within 2-3 minutes, the released cortisol is transferred into the saliva (Kirschbaum and Hellhammer, 1994). Furthermore, it has already been proven that saliva samples are just as suitable for analysing cortisol concentrations as blood samples although concentrations in saliva are much lower (Nemeth et al., 2016). It is therefore assumed that the methods used here should have shown a stress-induced increase in cortisol concentrations. Nevertheless, it cannot be ruled out that the animals were not stressed at all, as previous studies in guinea pigs measured cortisol concentrations in a stressful situation only after 30-60 minutes (e.g., Mutwill et al., 2023; Nemeth et al., 2016; Rystrom et al., 2024, 2022) but none provided earlier measurements.

The so-called open-field test is commonly used to expose guinea pigs to an unknown environment and thereby induce an increase in circulating cortisol. The novel environment functions as a psychological stressor evoking a stress response (Hennessy et al., 2006; Kaiser et al., 2007; Mutwill et al., 2023; Rystrom et al., 2024, 2022). Therefore, the dark-light test used in the present study, as a test that investigates exploratory and anxiety-like behaviour in a novel environment (Hascoët et al., 2001), is likely to be a suitable stressor to induce a stress response in the animals. However, in contrast to the open-field test, the guinea pigs had the opportunity to remain in the dark area during the dark-light test. Although the test enclosure was still an unfamiliar environment for the animals, this test may have appeared to be less stressful than an open-field test perhaps due to the dark area, resulting in no difference

in the cortisol concentration before and after the test. It might have been better to take saliva samples before and after the sociality test and thus obtain more robust results, since social confrontations are stressful situations for guinea pigs (Sachser and Lick, 1989). In the study by Glenk et al. (2018) cortisol concentrations were significantly higher after a social confrontation that lasted ten minutes compared to baseline during dioestrus, meaning that an increase can be detected after a short time. Since cortisol concentrations did not change during the dark-light-test in both cycle phases in the present study, but still appeared to be elevated during oestrus compared to dioestrus, it can be concluded that increased cortisol concentrations were caused by oestrus itself and not, for example, by increased activity or social interactions in the group.

With regard to the repeatability, the baseline cortisol concentrations were not repeatable over the two oestrus cycles in the present study. We know from previous studies that baseline cortisol concentrations are not stable over time in both female and male guinea pigs (Rystrom et al., 2022; Zipser et al., 2013). In male guinea pigs, there are rather contradictory results, because a more recent study showed that there was a repeatability in the baseline cortisol concentration in group-housed males, although with a high within-individual variation (Mutwill et al., 2023). Glucocorticoids, like cortisol, are involved in metabolic processes and are responsible for controlling energy homeostasis (Ehlert and von Känel, 2010). In general, the activity of the HPA-axis depends on different factors, for example activity patterns of the animal (Koolhaas et al., 2011). It cannot be excluded that the females followed their natural behaviour, for example, sleeping or interacting with their housing partners before the dark-light test, thereby influencing the baseline cortisol concentration. Perhaps because of higher within-individual variation due to inconsistent behaviour of the individuals prior to saliva sampling, the baseline cortisol concentration was not repeatable over two oestrus cycles. As with the baseline cortisol concentration, no repeatability of the stress-induced cortisol concentration was found across the two cycles. This was perhaps because of higher within-individual variation and no pronounced individual responses, as the 15-minute dark-light test did not cause a significant increase in cortisol concentration at any stage of the oestrus cycle in the females. However, previous studies have shown that the stress response is stable over a certain period of time in both females and males (Mutwill et al., 2023; Rystrom et al., 2022; Zipser et al., 2013).

In contrast to cortisol concentrations, there were no differences between oestrus and dioestrus in the behaviour of the females during the dark-light test. We are not aware of any comparable studies in guinea pigs that investigated effects of the oestrus cycle on explorative and anxiety-like behaviour during the oestrus cycle before. However, female rats, for example, had displayed less anxiety-like behaviour in the elevated plus-maze (Mora et al., 1996) and more exploratory behaviour in the Dashiell type maze (Martin and Bättig, 1980) during oestrus. In contrast to rats, explorative and anxiety-like behaviour as an indicator of emotionality does not appear to be influenced by the cycle phase in guinea pigs, as the females showed no significant difference in their locomotion and the duration they spent in the centre in the dark area between oestrus and dioestrus in the dark-light test. Another study has found increased locomotion during oestrus, but in a social context (Birke, 1981). Social confrontations can be an enormous challenging situation for guinea pigs, leading to body mass loss and increased cortisol concentrations (Sachser and Lick, 1989). Therefore, it is quite possible that the animal's behaviour varies in different contexts. However, the females showed significantly less locomotion in the second cycle compared to the first. In general, they showed less locomotion the more often they were tested in the dark-light test. This could indicate a habituation effect (see e.g., Blumstein, 2014) independent of the oestrus cycle. During this study, however, only a total of three different females left the dark area of the dark-light test. This could mean that the test reflected the anxiety-like behaviour of the females as they very rarely left the dark area. Furthermore, the behaviour in the dark-light test was repeatable over the two cycles but independently of the cycle phase. The individuals either have shown a lot locomotion and stayed in the centre of the dark area for a longer time or little to no locomotion and tended to stay near the enclosure walls in the test. This is contrary to what has been found for emotional behaviour in male guinea pigs, as they showed no repeatability over time neither in the open-field test nor in the dark-light test (Zipser et al., 2013). However, the results of the present study should be viewed with caution, because the repeatability estimation was low with large confidence intervals. Moreover, it has to be noted that repeatability was found in parameters that are not necessarily decisive in the test and with regard to anxiety.

Additionally, to exploratory and anxiety-like behaviour, risk-taking behaviour as an indicator of emotionality also appears to be not influenced by the oestrus cycle in

guinea pigs. During the entire experiment, only a total of eight different females stepped down in the step-down test. In the animals that stepped down, no regularity or preference for a particular cycle stage could be determined, as they stepped down equally in oestrus and dioestrus. Only one female had stepped down twice, once in oestrus during the first cycle and once in dioestrus during the second cycle. It is also possible that the females would have needed an incentive such as food to step down the platform and take the risk. In the study by Brust and Guenther (2017), the guinea pigs were trained to knock over an object for a novel object test in order to reach the treat underneath. Here, boldness in the novel object test was repeatable in both males and females over four weeks (Brust and Guenther, 2017). Boldness covaries with risk-taking in voles (Eccard et al., 2022), meaning that boldness and risk-taking behaviour could be comparable traits. Since the study by Zipser et al. (2013) also did not use a reward in the step-down test, it would be interesting to find out to what extent food as an incentive would influence the risk-taking behaviour of both male and female guinea pigs and whether the behaviour in females is then influenced by the cycle. As with the dark-light test, domesticated guinea pigs seem to be generally less risk-taking and overall highly anxious. In comparison, male cavies (wild guinea pigs) displayed significantly more explorative behaviour and less anxiety-like behaviour in both the dark-light test and open-field test (Zipser et al., 2014), and in addition, they were more bold in the novel object task compared to domesticated guinea pigs (Brust and Guenther, 2015). Therefore, the process of domestication may have reduced exploration and risk-taking to such an extent that, under certain circumstances, these behaviours no longer occur in female guinea pigs.

During the sociality test the females displayed more locomotion during oestrus compared to dioestrus in both cycles, at least in the left area which contained the conspecific. This is consistent with the study by Birke (1981) showing increased locomotion of oestrus females in a social context. In the right area without the conspecific, results were not that obvious as females more locomotion in the first cycle and less locomotion in the second cycle during oestrus compared to dioestrus. Locomotion seems to be partly influenced by the cycle phase in guinea pigs but inconsistently and it might be at least partly a consequence of interaction with the conspecific. Furthermore, at least in the first cycle the females interacted more with the conspecific during oestrus than dioestrus. This is again consistent with the study by

Birke (1981), but in contrast to the results of the study by Glenk et al. (2018), where the females showed less sit side by side behaviour and more flight behaviour, which could indicate less social interest. However, this should be viewed with caution, as there were no differences between the cycle phases in any other social parameters. In addition, in the present study there was no recognisable pattern in the duration the females spent in the area with the conspecific. In the first cycle they spent significantly more time in the left area during oestrus compared to dioestrus and in the second cycle it was the other way around. Regarding the repeatability of the behaviour in the sociality test, all behaviours were repeatable over the two oestrus cycles but independently of the cycle phase. Our findings agree with another study, where guinea pigs were consistent in their socio-positive and aggressive behaviour (Brust and Guenther, 2017). Temporal stability in social behaviours is not surprising, as guinea pigs are highly social animals (Kaiser et al., 2010) and form stable dominance hierarchies in different social organised groups, for example, harem groups (Brust and Guenther, 2017), only female groups (Rystrom et al., 2022), and only male groups (Zipser et al., 2013). Therefore, social traits seem to be personality traits. However, as with the dark-light test, the results should be viewed with caution, because of low repeatability estimates with large confidence intervals.

In conclusion, our results indicate that the oestrus cycle in guinea pigs appears to have an influence on body mass, cortisol concentration, and to some point on social behaviour but not on emotionality. The current state of research in this field is very limited, as there are few comparable studies and the studies that do exist show inconsistent results. This means that more studies investigating cycle influences on guinea pig behaviour and physiology are needed. Furthermore, we found stable individual differences in body mass and the behaviour in the dark-light test and sociality test. Body mass and behavioural traits were repeatable across all cycle phases and were therefore not influenced by the cycle, which means personality has a much stronger influence on behaviour of female guinea pigs than the oestrus cycle. In summary, the current state of research does not adequately report on the oestrus cycle of guinea pigs and associated differences in behaviour and cortisol concentrations. However, there is a lot of potential in this research topic, to which this study could certainly contribute.

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ZUSAMMENFASSUNG

Gekennzeichnet durch eine spontane Ovulation und einer darauffolgenden aktiven lutealen Phase mit signifikant erhöhter Progesteronausschüttung, weist der Östruszyklus bei weiblichen Meerschweinchen viele Gemeinsamkeiten zur Reproduktion bei Menschen auf. Trotz dieser Tatsache gibt es nicht viele Studien, die den Einfluss des Östrus auf die Physiologie und das Verhalten bei Meerschweinchen untersuchen. Deshalb war es das Ziel dieser Studie herauszufinden, inwieweit die Zyklusphase Einfluss auf die Cortisolkonzentrationen und das Verhalten nimmt. Darüber hinaus wurde die Parameter in zwei aufeinanderfolgenden Zyklen miteinander verglichen, um zu ermitteln, ob sowohl physiologische Parameter als auch das Verhalten konsistent sind. Um den Östruszyklus zu bestimmen, wurde bei zwölf weibliche Meerschweinchen täglich Zyklusmonitoring, durch visuelle Inspektion der vaginalen Membran, durchgeführt und das Körpergewicht überprüft. Speichelproben zur Analyse der Cortisolkonzentrationen wurden vor und nach dem 15-minütigen Dark-Light Test durchgeführt. Der Dark-Light Test, der Step-Down Test und der Sociality Test wurden durchgeführt, um Exploration, Ängstlichkeit, Risikobereitschaft und soziales Interesse zu analysieren. Der Östrus war charakterisiert durch signifikant höhere Cortisolkonzentrationen und signifikant niedrigeres Körpergewicht im Vergleich zum Diöstrus. Im Dark-Light Test nahm die Zyklusphase keinen signifikanten Einfluss auf das Verhalten. Der Step-Down Test konnte nur deskriptiv analysiert werden, da von den insgesamt 48 Testläufen nur in acht Läufen verschiedene Weibchen von der Plattform gesprungen sind. Lokomotion im Sociality Test schien teilweise von der Zyklusphase beeinflusst, allerdings inkonsistent und könnte mitunter eine Folge der Interaktion mit dem Artgenossen sein. Die Interaktion mit dem Artgenossen war während dem Östrus im Vergleich zum Diöstrus signifikant erhöht, zumindest im ersten Zyklus. Was die Konsistenz des Verhaltens (repeatability) anbelangt, so waren alle im Dark-Light Test und im Sociality Test gezeigten Verhaltensweisen über die Zeit hinweg konsistent, jedoch unabhängig von der Zyklusphase. Die Ergebnisse dieser Studie zeigen, dass der Östrus die Physiologie und zum Teil auch das Verhalten bei weiblichen Meerschweinchen beeinflussen kann. Die individuellen Unterschiede im Verhalten deuten jedoch darauf hin, dass die Persönlichkeit einen stärkeren Einfluss

auf das Verhalten nimmt, als die jeweilige Zyklusphase. Diese Studie leistet einen Beitrag zu einem Forschungsbereich, welcher großes Potenzial bietet.

Schlagwörter: Meerschweinchen, Östruszyklus, Persönlichkeit, Cortisol, soziales Interesse, Emotionalität