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Relationship between exploratory activity and adrenocortical activity in the black rat (*Rattus rattus*)

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Abstract

The relationship between physiological and behavioral stress markers is documented in several rodent species. However, there is no information regarding the role of adrenocortical activity in behavior of the black rat (*Rattus rattus*). Therefore, we hypothesize that the adrenocortical activity of black rats varies between individuals and is related to some of the behaviors in a novel environment. To test this hypothesis, we (i) validated a method for quantifying glucocorticoid metabolites from feces (fGCMs) with an enzyme immunoassay (EIA); (ii) examined variation and diurnal rhythms of feces and GCM production; and (iii) examined the relationship between GCM levels and exploratory behavioral traits. We fulfilled the first aim (i) by successfully performing an ACTH challenge test to validate the use of a 5α-pregnane-3β,11β,21-triol-20-one EIA for measuring fGCMs. Second (ii) we detected considerable consistent interindividual variability in production of both feces and glucocorticoids. The peak production of feces occurred in the first hour of the dark cycle, the peak of fGCMs occurred approximately 3 h later. Lastly, (iii) there was no clear relationship between behavior in the hole board test and GCMs. Grooming, a typical behavioral stress marker, was negatively associated with stress reactivity, while head-dipping in the hole-board test (traditionally considered an exploratory behavior independent of stress) was not correlated with the GCMs. This study offers a first look at GCMs in the black rat, successfully validates a method for their measurement and opens possibilities for future research of the relationship between glucocorticoids and exploratory behavior in this species.

KEYWORDS

ACTH challenge test, coping styles, feces, hole-board test, ship rat

1 | INTRODUCTION

Previous research has repeatedly shown that levels of glucocorticoids are correlated with observable behavioral traits (Martins et al., 2007; Sangenstedt et al., 2017; Spruijt & Gispen, 1986). Glucocorticoids mediate energy mobilization and regulate carbohydrate metabolism (MacDougall-Shackleton et al., 2019) and besides other things, they can be used as a parameter of relative stress levels of animals (Möstl & Palme, 2002). Animal personality research often uses novel environment tests to ascertain which behavioral traits, if any, mirror the stress levels

Rupert Palme and Daniel Frynta: contribution of these authors is equal.

of the animal (Archer, 1973). Some of the behavioral traits, for example, freezing or avoiding the open arms in an elevated plus maze, are so widely accepted as behavioral stress markers that they are an integral part of breeding programs for high- and low anxiety lines of laboratory rats, for example, the Roman high and low avoidance rats (Claudio Carere et al., 2014). Exploratory and anxiety-related behavior can be used to predict responses to chronic or sub-chronic stress (Castro et al., 2012). Such correlations allow assessing which animals are more stressed than others without the need for physiological testing.

In this study, we expand on some previous work (Žampachová et al., 2017), which focused on the exploratory behavior of the black rat (*Rattus rattus*). It described repeatable behavior in novel environment tests and interpreted some behavioral traits (e.g., grooming) as behavioral markers of stress. However, these results raised a question whether there is a physiological correlate of this behavior, which would strengthen this interpretation. The best candidate for such a correlate seemed to be glucocorticoids. Researchers have previously demonstrated that glucocorticoids mediate coping mechanisms in novel environment tests (Denenberg, 1969; Guindre-Parker, 2018; Lendvai et al., 2011; Levine et al., 1967).

When measuring glucocorticoid concentration, one option is to analyze blood samples taken shortly after a stimulus. This approach has its disadvantages, mainly connected to the acquisition of the blood sample, which is stressful by itself, has to be performed quickly to avoid confusion of the results by the procedure and is not suitable for longer monitoring (Palme, 2019; Sheriff et al., 2011). An increasingly popular alternative is measuring the levels of glucocorticoid metabolites (GCMs) in feces, which is noninvasive and allows repeated sampling without the need to manipulate the animal and therefore possibly influence the results (Möstl & Palme, 2002; Palme, 2019). This method is especially suitable for studies focusing on long-term rhythms of glucocorticoid levels in the organism (Fraňková et al., 2012; Nováková et al., 2008).

However, before the first use of such a method in a given species, a physiological (or biological) validation is required (Palme, 2019; Touma & Palme, 2005). This validation usually takes the form of an adrenocorticotropic hormone (ACTH) challenge test, where the hypothalamic–pituitary–adrenal (HPA) axis is artificially stimulated with a dose of ACTH and a subsequent peak of fecal GCMs is observed. Such a test also allows us to discover the species-specific time-lag between the peak in glucocorticoid secretion and the excretion of their metabolites via the feces (Palme, 2019). Especially in rodents the excretion of GCMs in feces is subject to a large diurnal variation, which reflects the diurnal variation of glucocorticoids in the blood stream (Lepschy et al., 2007). This diurnal rhythm can confound the results and must be considered.

The methods for measuring fecal GCMs have been validated for laboratory rats (Lepschy et al., 2007) and mice (Touma et al., 2004), but not for our focal species, the black rat (*R. rattus*). The black rat is a rodent species with a large impact on humans as a pest (Capizzi et al., 2014) and a vector of zoonoses (Lapuz et al., 2008; Matthias et al., 2008; McCormick, 2003; Nitatpattana et al., 2002), as well as an invasive species with major impact on island habitats (Towns et al., 2006). Even though the black rat is closely related to the Norway rat (*R. norvegicus*), it is a distinct species with observable behavioral differences (Foster et al., 2011). Therefore, any method validated for the Norway rat cannot be blindly used on the black rat without a proper validation.

After a method for measuring GCMs is validated, it can be used to detect some characteristics of adrenocortical activity of the animals. GCM levels can be, among other things, heavily influenced by social environment (Bartolomucci, 2007). As we had no way of determining the social status of the experimental animals, we used the size of testes and seminal vesicles as an approximation. For example, male African striped mice (*Rhabdomys pumilio*) have smaller testes when housed in family groups, and also have higher corticosterone levels (Schradin et al., 2009).

Both the baseline adrenocortical activity (Bonier et al., 2009; Madliger & Love, 2016) and intensity of a reaction to a stressful stimulus (Lendvai et al., 2015; Taff & Vitousek, 2016) are subject to expectable interindividual variation. These two characteristics are not necessarily correlated, should be considered as separate traits (Guindre-Parker, 2018), and might cause different behaviors to manifest in novel environment. Stress in rodents is in general associated with grooming, freezing, or thigmotaxis. These behaviors are often labeled anxiety or boldness. As ACTH administration was shown to induce grooming (Dunn et al., 1981; Spruijt & Gispen, 1986), we expect grooming to be associated with an animal's reactivity to stress. Another behavior considered to be a stress marker is thigmotaxis (moving only along the walls of the arena) and a tendency to spend time in the corners of the arena (Lynn & Brown, 2009; Ossenkopp et al., 1994; Prut & Belzung, 2003).

Exploratory behavior is considered as a separate set of traits, defined as a reaction to novelty and not related to stress reactivity (Hughes, 1997; Réale et al., 2007). In a hole-board test, rearing or head-dipping (inspecting the holes in the ground) are often used as markers of exploratory behavior (Abel, 1995; Casarrubea et al., 2009; Hooks & Kalivas, 1995; Lever et al., 2006). However, anxiety inhibits head-dipping behavior in mice (Takeda et al., 1998), therefore another subject of investigation was a relationship between stress and traditional "exploratory" behavioral traits.

Therefore, to determine the patterns of GCM production in the black rat and to assess the potential relationship between glucocorticoid metabolite levels and behavior, we set three goals for this study. We aimed to (1) validate a method of measuring GCMs from fecal samples of the black rat (*Rattus rattus*); (2) characterize the production of feces and variation of GCMs during the day; and (3) test for possible links between GCM levels (baseline or stress induced) and either behavioral traits extracted from the hole board test or morphological characteristics, namely body weight, and size of testes and seminal vesicles.

2 | MATERIAL AND METHODS

2.1 | Animals and housing

The tested subjects were 23 wild adult male black rats (*R. rattus*), originating from Central Bohemia (caught in a piggery in the village

Palecek) with a mean weight of 153 g (range: 106–213 g). They were housed individually in wire cages (20 × 30 × 24 cm) with mesh floor with 12 L:12D regime and temperature set to 21 ± 1°C and given 3 weeks to acclimatize. The cages were put close to each other to allow the animals to socialize. The animals were fed with standard laboratory food pellets (ST1, Velaz Ltd.). Dry bread, food pellets and water were provided ad libitum.

2.2 | Collection and processing of fecal samples

After the 3-week acclimatization period, the cages were elevated approximately 2 cm above the surface by detachable legs, and a clean filtration paper was placed under the cage for fecal boli collection with minimal disturbance of the animal. The article was changed at least once a day or when it was contaminated by urine. Fecal boli were collected every hour with tweezers, stored in Eppendorf tubes and immediately frozen at −20°C. The boli contaminated with urine were counted, but neither stored nor used in GCM analysis. During the dark phase, the red light was on to enable sample collection.

For a timeline of sample collection, see Figure 1. We started with 2-day monitoring (Monitoring Day 1 and 2) of the diurnal rhythm of feces production. The collection of samples started on Monitoring Day 1 at 07:00 and continued for 48 h, ending at 06:00, at the end of Monitoring Day 2. After these two Monitoring Days, there was a 5-day break, after which the experiment began with Control Day. The collection of samples started at 07:00 and lasted until 11:00 of the following day. These samples were used as control measures of GCM levels for the ACTH challenge test; therefore, we called these 28 h a "Control Day."

At 11:00, after the last sampling of Control Day, during a period of low feces production, we intraperitoneally injected ACTH (Synacthen, Ciba-Geigy, Basel, 250 µg/L) in saline solution (0.8 ml per kg of the weight of the animal, which equals 0.02 IU/kg) to 13 rats (the "ACTH group"). The remaining 10 rats (the "Saline group") received only the saline solution (0.8 ml per kg of the weight of the animal). The complete manipulation with animals was under 3 min for each individual to minimize a confounding stress reaction.

After the injection, we continued the hourly sampling for the next 24 h (Experimental Day). These samples reflect the effect of the ACTH challenge test. After the Experimental Day, we continued the sampling procedure for another 24 h, further referred to as Monitoring Day 3, to collect further data on feces production.

2.3 | Measurement of GCMs

The samples were weighed, homogenized with mortar, and pestle and a portion of 0.08 g was weighed into an Eppendorf tube. Afterwards 1.6 ml of 80% methanol was added. If the sample weighed less than 0.08 g (range: 0.013–0.077 g; 147 cases from 688 samples), the corresponding amount of methanol was added. The samples were shaken for 15 min on a multivortex and centrifuged (11,500×*g*) for 2 min. Then the supernatant (1.2 ml) was transferred to a new Eppendorf tube and stored at −20°C until further analysis (Palme et al., 2013). To measure fecal GCMs we used a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA), which was first described by Touma et al. (2003; see reference for assay specificities), and successfully validated for laboratory rats (Lepschy et al., 2007, 2010). Intra- and inter-assay coefficients of variation of low and high concentration pool samples were less than 10% and less than 15%, respectively.

2.4 | Behavioral testing and morphological parameters

Two weeks after the last feces collection, we performed a hole-board test twice for each animal on two consecutive days. The hole-board test was a glass arena (60 × 60 × 50 cm) covered with a Perspex board to prevent the animal from escaping. At the bottom, there was a chipboard panel with 16 holes (diameter 6 cm and depth 4 cm), coated with nontoxic paint for easier washing. The behavior was recorded with a video camera under very low illumination (5–8 lx) and in the experimenter's absence to minimize disturbance. Every test lasted 10 min and afterward the arena was cleaned using 96% ethanol to neutralize the odor. After the second hole board test, the animals were euthanized using CO_2 and we measured and weighed their testicles and measured the seminal vesicles.

We evaluated the hole board test in the ACTIVITIES software (Vrba & Donát, 1993). We observed latency, frequency, and duration of the following behavioral traits: ambulation (along the wall or in the

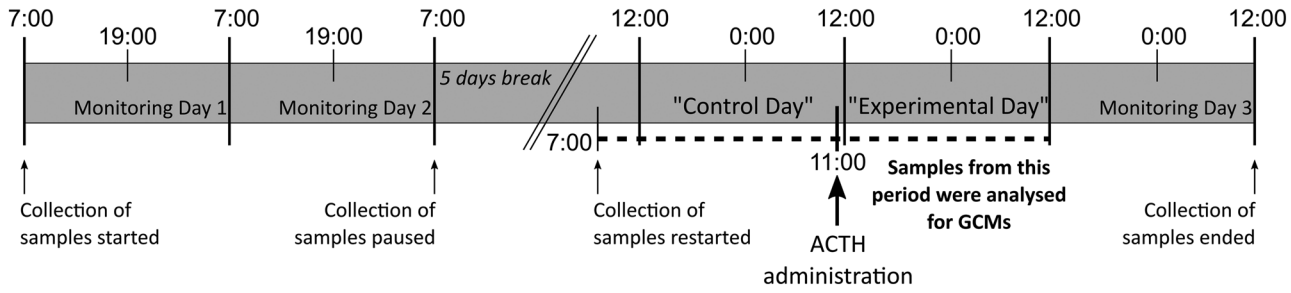

FIGURE 1 Timeline of the fecal sample collection

central part of the arena), rearing (against the wall or in the central part of the arena), sitting and grooming (in the corner, next to the wall or in the central part of the arena), jumping, freezing, and head-dipping (in the outer ring of the holes or in the central ring).

2.5 | Statistical procedures

The production of fecal boli was measured during the three Monitoring Days plus the Control Day. We used Kendall's coefficient of concordance (W) to establish the interindividual variability of production of fecal boli and GCM levels (R, command KendallW, package DescTools v0.99.19). Furthermore, we assessed the repeatability of GCM levels (package rptR), using the guidelines of Stoffel et al. (2017).

For the ACTH challenge test, we evaluated the GCM levels from the period of 6–10 h after the injection. We chose this critical time window based on the delay between ACTH injection and increase of fecal GCMs in laboratory mice, which was 8–10 h (Touma et al., 2004), and in laboratory rats, which was 4–12 h (Lepschy et al., 2007). Therefore, we established the critical time window in the middle of the interval observed in the laboratory rat without the extremes. This critical time window was also supported by our empirical data (see Section 3). We also determined peak values for each individual, represented by the mean of the three highest values of GCM of the critical time window.

We performed paired *t*-test comparisons of the values in this critical time window before and after injection, separately for the ACTH group and Saline group. The samples from the Control Day were taken in the corresponding time window of the Experimental Day (between 18:00 and 22:00). Because the critical time window was chosen a priori based on the delay observed in the laboratory rat, we also performed a separate paired t-test comparison for each hour of collection separately to determine the delay time of fecal peak GCMs excretion after the ACTH injection.

We also compared the GCM values between the groups of animals (ACTH and Saline) during the critical time window after injection. However, when we used the whole time window for the comparison, the results were affected by large individual variation. Therefore, we decided to also compare the peak values (see above) using one-tailed analysis of covariance. We added the mean GCM level of the previous day as a covariate, therefore we eliminated the confounding effect of interindividual variability in GCM secretion.

We used a linear model to assess the relationship between GCM levels and behavior. The behavioral variables were chosen based on the results of the previous work concerning the exploratory behavior of the black rat (Žampachová et al., 2017). The full model included the following variables as fixed factors: grooming (duration), sitting (duration), ambulation (duration), rearing (count), jumping (count), head-dipping (count), and time spent in the central part of the arena (duration). The variables were averaged across the two repetitions of the hole-board test. The dependent variable was the mean of GCM levels of the dark phase of the Control Day (18:00–05:00). We used only the dark phase levels because the data from the light phase were heavily influenced by the large number of missing values. We then reduced the model, using the AIC ("step" function in R). To approximate stress reactivity, we used the same model with the same fixed factors and the peak values of the Experimental Day (mean of the three highest GCM levels of the Experimental Day) as a dependent variable. We also included the peak values of the Control Day as the first fixed factor. This way we tested the effect of behavioral variables only on residual variability after explaining the variability caused by the intrinsic differences between individuals (the Control Day peaks). Therefore, this procedure allowed us to assess the relative reaction to stressful stimuli. This analysis was performed separately for the ACTH and Saline group.

In addition, we used the linear model to explore GCM levels and morphological parameters with baseline GCMs (mean value of all the GCMs during the Control Day) as a dependent variable and the weight of the animal, length of seminal vesicles, and weight of the testes as fixed factors.

3 | RESULTS

3.1 | Production of feces

Considering the large amount of samples, we used only the number of fecal boli and not the total weight (wet weight of samples ranged from 0.013 to 1.155 g). The average daily production of an individual black rat was 71.6 fecal boli (*SE* = 2.6; 95% CI 66.5–76.7, coefficient of variance *v* = 0.35). The highest production (mean = 9.8) was detected between 18:00 and 19:00 (in the first hour of the dark part of the cycle), the lowest production was between 13:00 and 14:00 (mean = 0.3), which is roughly the middle of the light part of the cycle. There was a high interindividual but low intraindividual variability in the diurnal rhythms of fecal boli production during the four monitored days (Kendall's $W = 0.38$, $p < .001$).

3.2 | Diurnal rhythm in GCM levels

The average GCM level was 2.87 μg/g wet weight (WW; *SD* = 1.76, coefficient of variance *v* = 0.61), the highest GCM levels were present at 22:00 (mean = 4.00, *SD* = 2.2, see Figure 2). There was one exceptional average level at 06:00 (mean = 4.02, *SD* = 2.50). However, this seems to be an isolated incident caused by three unusually high values (>7 μg/g WW), not corresponding with the overall trend. The lowest GCM levels occurred at 12:00 (mean = 0.78, *SD* = 0.57).

The individual repeatability of GCM levels, corrected for the hour of collection, was high ($r = 0.51$; $p < 0.001$), suggesting high intra-individual consistency. Kendall's coefficient of concordance tested the agreement in the diurnal levels of GCM's among the individuals. The value of this coefficient was not very high ($W = 0.23$, $p < 0.001$), probably because of the high variability among the individuals.

3.3 | ACTH challenge test

We compared the GCM levels in the critical time window between 18:00 and 22:00 of the Control and Experimental Day (see Figure 3). The peak values in the ACTH group ranged from 2.00 to 9.63 μg/g WW (mean 3.21 μg/g) before the injection (Control Day) and 2.08–10.00 μg/g WW (mean 4.41 μg/g) after the injection (Experimental Day). The paired *t*-test comparison showed that in the ACTH group GCM levels after administration differed significantly from those of the same animals before the injection ($t = -4.43$; $p < 0.001$). Such a difference was not present in the Saline group ($t = -1.82$; $p = 0.075$). Because the critical time window was set a priori, we also tested the difference between Experimental and Control Day for each hour of collection separately to establish the delay of fecal GCMs increase. The delay between the ACTH injection and fecal GCMs increase was 6–8 h—the *t*-test comparisons were significant at 19:00 ($t = -2.32$; $p = 0.045$) and 20:00 ($t = -3.03$; $p = 0.016$).

GCM levels of the ACTH and Saline group in the critical time window after the administration were not significantly different ($t = 1.08$, $p = 0.282$). However, when we compared only peak values (mean of the three highest levels during the critical time window), we found a significant difference ($F = 3.39$; $p = 0.04$).

We also examined the relationship between behavior in the hole-board test and the mean of the three highest GCM levels in the critical time window (the peak values). The linear model representing relationship between stress reactivity included the peak values of the Control Day as the first factor. Therefore, variability explained by other factors was already corrected for the interindividual differences in GCM levels. The reduced model for ACTH group (intercept = −8.14) included the Control Day peak ($F = 27.32$; $p = 0.002$), rearing ($F = 6.31$; $p = 0.046$), and nonsignificant variables grooming, sitting, and jumping. The Saline group model (intercept = 1.48) contained the Control Day peak ($F = 10.76$; $p = 0.017$), grooming ($F = 13.80$; $p = 0.011$) and nonsignificant time spent in the center. For detailed results of the models, see Table 1.

Another linear model was used to assess the link between baseline GCM levels (see above) and the animal's weight, length of seminal vesicles, and weight of testes. The mean weight of testes was 1.31 g ($SE = 0.03$, coefficient of variance $v = 0.11$), the mean length of seminal vesicles was 17.94 mm ($SE = 0.76$, coefficient of variance $v = 0.20$). When we analyzed the effect of testes weight, seminal vesicles length, and body weight on the baseline GCMs, the reduced model (intercept = 6.22) showed only a significant negative effect of the animal's weight ($F = 5.25$; $p = 0.033$, estimate = −0.02).

3.4 | Relationship between GCMs and exploratory activity and morphological parameters

We used a linear model to assess the relationship between mean GCMs and behavior. The reduced model (intercept = −2.44) contained three nonsignificant effects associated with the baseline GCMs: grooming, ambulation, and time spent in the center.

4 | DISCUSSION

We successfully validated a method for measuring fecal GCMs in the black rat, demonstrated the presence of a diurnal rhythm of both feces production and GCM levels, and found some connections between GCM levels and behavior in a novel environment test.

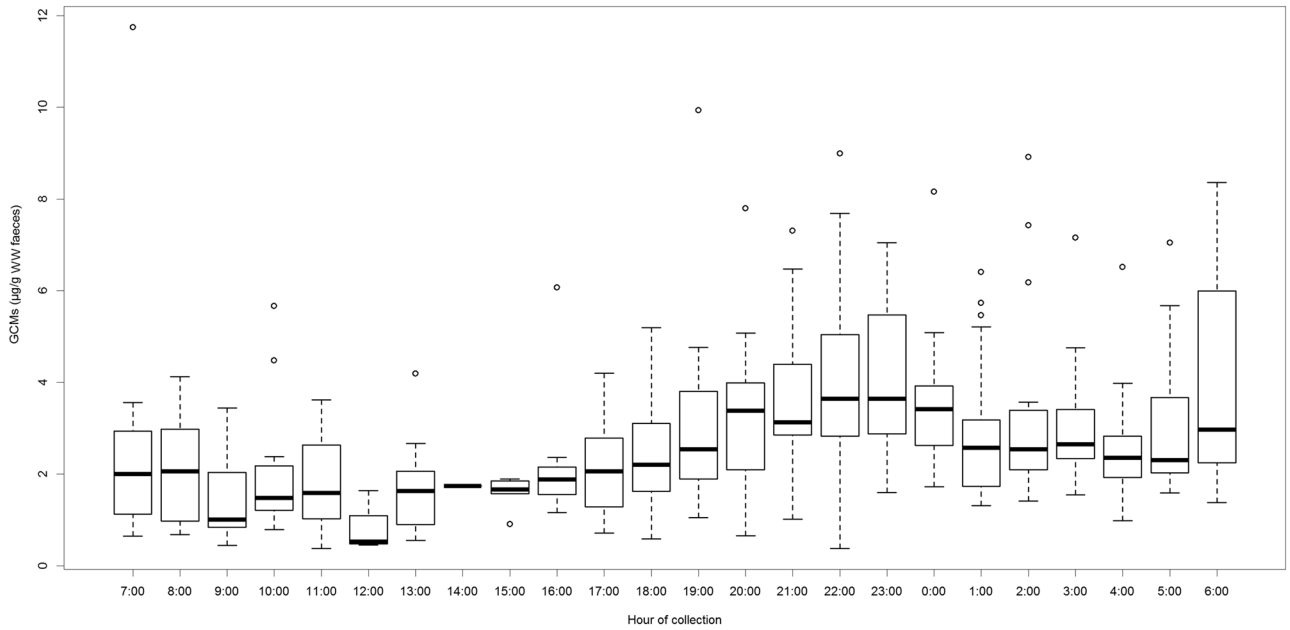

FIGURE 2 Diurnal variation in glucocorticoid metabolite levels during the Control Day. Data are presented as boxplot graphs (medians, quartiles and outliers are indicated)

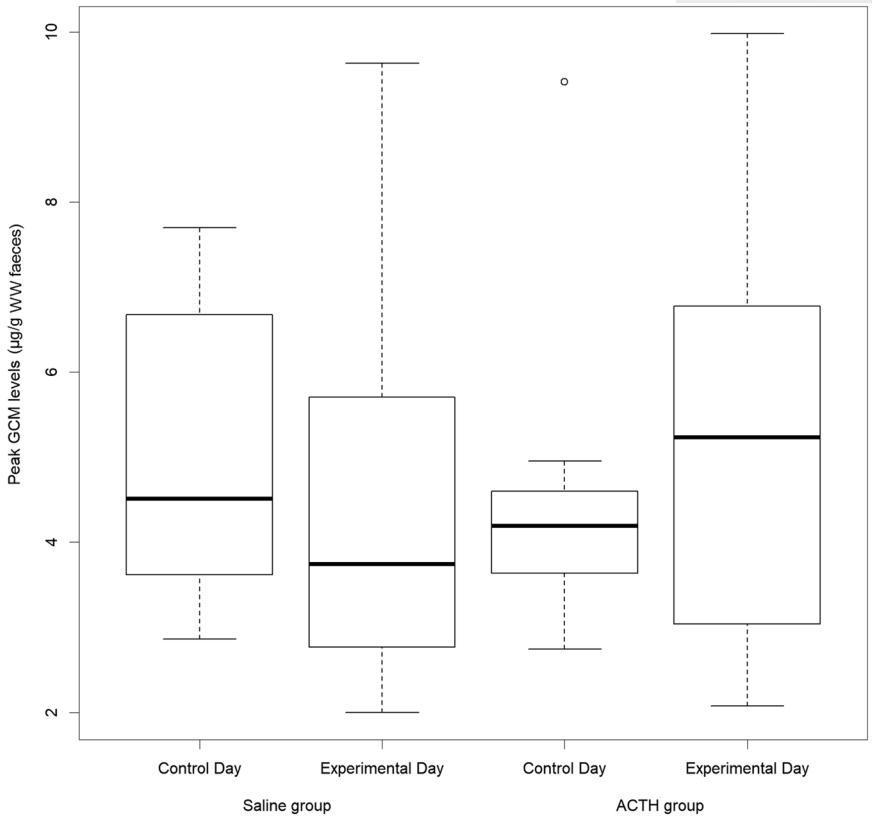

FIGURE 3 Comparison of the three highest GCM levels (mean of each animal) from the critical time window (18:00–22:00) between the Control Day and Experimental Day, separate for Saline group and ACTH group. Data are presented as boxplot graphs (medians, quartiles and outliers are indicated). ACTH, adrenocorticotropic hormone; GCM, glucocorticoid metabolite

4.1 | Diurnal variation in feces production and GCMs

The production of feces in the present study was 71.6 fecal boli per day. It was higher than in a previous study (Fraňková et al., 2019) where the mean value was 52 fecal boli. This might have been caused by the fact that rats in the previous study were provided with a novel food, which had lower nutritional value. Also, the novelty of the food could lead to lowered food intake, leading to a lower-than-normal production of fecal boli.

We detected a large diurnal variation in both production of feces and GCM levels, in agreement with a study performed on rats (Lepschy et al., 2010). The time lag between the peak in blood corticosterone and peak of fecal GCMs in laboratory rats was approximately 4–12 h (Lepschy et al., 2007), in the black rat we established it at 6–8 h. The natural peak of GCM in the black rat occurred about 3 h after the beginning of the dark period. Considering the time lag, this result suggests that blood corticosterone levels peaked during the second half of the light phase.

The production of GCMs in the black rat also proved to be consistently different between individuals, which suggests that it might be connected to animal personality. Such a relation between consistent individual differences in GCM levels and animal personality has already been reported in other species, for example, in the yellow-bellied marmot, *Marmota flaviventris* (Smith et al., 2012) or the great tit, *Parus major* (C. Carere et al., 2003; Stöwe et al., 2010).

4.2 | Validation of the EIA method

GCM levels following the ACTH administration were significantly higher than respective baseline concentrations, but were not significantly different after saline injection. However, there was also a significant difference in GCM levels between the ACTH and Saline group after the administration. In our opinion these results provide

TABLE 1 The output of reduced linear models, determining which behavioral traits are associated with GCM measures

Dependent variable	Explanatory variable	Estimate	*F*	*p*
Baseline GCMs	Grooming	4.59	1.89	0.186
	Ambulation	7.42	1.5	0.237
	Time in the center	−6.34	2.74	0.115
Peak values (ACTH group)	Control Day peak	0.66	27.32	**0.002**
	Grooming	3.48	0.82	0.4
	Sitting	7.6	0.95	0.367
	Rearing	0.54	6.31	**0.046**
	Jumping	0.1	1.86	0.221
Peak values (Saline group)	Control Day peak	0.5	10.76	**0.017**
	Grooming	−1.97	13.8	**0.011**
	Time in the center	1.23	2.32	0.179

Note: Significant *p*-values are marked in bold.

Abbreviations: ACTH, adrenocorticotropic hormone; GCM, glucocorticoid metabolite.

sufficient evidence that the 5α-pregnane-3β,11β,21-triol-20-one EIA can be used to evaluate adrenocortical activity in the black rat by measuring fecal GCMs. We are aware that the difference between the ACTH and Saline group is rather small, though significant. There are three factors that might have confounded the effect of ACTH. First, the stress caused by the administration itself might have nonsignificantly elevated the GCM levels in the Saline group as well. For example, in mice a saline injection and different blood sampling procedures have been found to increase GCM levels (Meyer et al., 2020; Touma et al., 2004). The second factor might be individual differences in sensitivity of the HPA axis, documented in laboratory mice and rats (Gentsch et al., 1982; Veenema et al., 2004, 2005), which might have affected the resulting GCM levels in ACTH group. Finally, the intraperitoneal injection in a wild animal is a difficult procedure and it cannot be ruled out that in some of the experimental animals the ACTH did not enter the peritoneum and therefore did not have the desired effect.

The comparison between two groups of individuals might also be influenced by large interindividual variability (as demonstrated by the high repeatability of fecal GCMs, as well as the relatively low coefficient of concordance). Therefore, we attribute more importance to the comparison of the individual GCM levels before and after the injection. In this case, the animal serves as its own control, as recommended earlier (Palme, 2019).

4.3 | Relationship between exploratory behavior and GCMs

We tested the relationship between behavior in a novel environment and three different measures of adrenocortical activity in the black rat, to cover different aspects of endocrine plasticity (Guindre-Parker, 2018). First, we evaluated baseline GCM levels, which might be considered a personality trait. The second measure is a reaction to a stressful stimulus (saline injection), which might reflect a general reactivity. This measure includes the complex reaction to stress, possibly including stress perception and regulatory mechanisms. The third is the reaction to artificial stress (ACTH injection), which reflects the reaction of the HPA axis to a physiological stimulus. Therefore, we call this measure "ACTH sensitivity."

We found that grooming, one of the most widely used behavioral stress markers in rodents (van Erp et al., 1994), was connected to all three GCM measures, although it was a significant factor only for the model for the Saline group. Moreover, in the Saline group the relationship to stress reactivity was a negative one, which contradicts the usual interpretations. Rearing, usually considered a measure of exploratory behavior (for overview of interpretations, see Žampachová et al., 2017) was positively associated with ACTH sensitivity. Stress is usually associated with immobility (De Boer & Koolhaas, 2003; Kalueff et al., 2008), however, Denenberg (1969) showed that activity during the first day of an open field test correlates positively with emotional reactivity. During subsequent repetitions of the open field test, the correlation between activity and emotional reactivity is negative (Denenberg, 1969). The positive effect of rearing in our results is in concordance with this conclusion. It could also explain the negative relationship between grooming and stress reactivity. If the stress reactive animal also reacted with heightened activity, it would reduce the time spent by grooming.

Other behavioral variables were not significant, however could not be excluded from the models, because the model excluding these variables had higher AIC than the model including them. One of them was thigmotaxis, another behavioral stress marker (Archer, 1973; Lynn & Brown, 2009; Ossenkopp et al., 1994; Prut & Belzung, 2003), which was connected positively to baseline GCMs and negatively to stress reactivity. Ambulation remained in the model for baseline GCMs, while jumping and sitting remained in the model for ACTH sensitivity. The effect of ambulation could be explained by findings of Denenberg (1969; see above). The combination of jumping and sitting could mean that the ACTH sensitive animals do not explore the environment, but either proactively try to escape the arena by jumping or simply stay immobile. A previous study (Žampachová et al., 2017) interpreted jumping as exploratory behavior, based on the importance of vertical activity for the black rat (Foster et al., 2011) and its correlation with ambulation and rearing. However, our current results are in favor of the interpretation of jumping as a stress response, which have been observed in mice (*Mus musculus*, Bridgman et al., 2013) and Brandt's voles (*Lasiopodomys brandtii*, Hegab et al., 2014). The discrepancy in the interpretations concerning both ambulation and jumping further supports the need for more detailed research of exploratory behavior in the black rat, including vertical activity and physiological correlates of behavioral traits.

The specific attribute of the hole-board test is the possibility to investigate the holes in the ground, which is expressed as head-dipping. This is considered a better measure of exploratory behavior than the traditional "open field" measures like ambulation or rearing (Abel, 1995; Casarrubea et al., 2009). Exploratory behavior is considered independent of the stress reaction, which was underlined by our models where head-dipping was not included in any of our final ones. Therefore, our results are in concordance with the interpretation of head-dipping as a suitable marker of exploratory behavior. Rearing, the traditional exploratory behavioral trait extracted from the open field test, was positively associated with ACTH sensitivity, which suggests that it might not be independent of stress and therefore an inferior measure of exploratory behavior than head-dipping. Previous work showed that these two behaviors were independent in the black rat (Žampachová et al., 2017) and therefore might provide us with different information about the animal.

Similarly, in our experimental data, we demonstrated that in the black rat stress reactivity (the peak values after a stressful stimulus) and baseline GC are not interchangeable variables (Guindre-Parker, 2018). In search for behavioral correlates of stress, it is important to determine whether the behavioral trait is correlated with baseline adrenocortical activity or reactivity. These results suggest that the research of exploratory behavior, which is one of the key components of contemporary animal personality research, would

greatly benefit from endocrine flexibility framework suggested by (Taff & Vitousek, 2016).

We found no significant effect of testes weight or seminal vesicle length on baseline fecal GCMs. This may be explained by low variability in testes weight within the examined sample. Although all the examined animals were adults, there was sufficient variability in body weight, presumably reflecting the individual growth trajectories and age. We found a negative relationship between baseline fecal GCMs and body weight. This supports the conclusion that chronic stress contributes to a body weight reduction (Harris et al., 2004). We had no information about the age of the animals. Therefore, we cannot safely comment on a relationship between GCMs and age.

5 | CONCLUSION

In conclusion, we met our first objective and successfully validated the EIA to measure fecal GCMs in the black rat. Similarly, we characterized the diurnal variation of both GCM levels and production of feces. As for our third objective, the results are more complex. We found that the relationships between behavioral traits and baseline GCMs were non-significant. Grooming was only significantly associated with stress reactivity. Moreover, we found that the correlation with rearing (for ACTH sensitivity) was positive, meaning the more "stress sensitive" the individual was, the more it reared. Head-dipping, considered by some as more reliable exploratory behavior unaffected by a stress response, was not connected to any of the GCM variables. However, our design did not include measurements of plasma glucocorticoids immediately after the hole board test. This analysis might have revealed a relationship between behavioral traits and glucocorticoids, which might not necessarily be present between baseline GCMs or general reactivity and behavior in the novel environment. This highlights that even though measuring rapid endocrine response is logistically challenging, it cannot be replaced with baseline GCM measures.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

Data are published on Mendeley Data, under the link https://doi.org/10.17632/37dydzpb3m.1

ETHICAL STATEMENT

All animal procedures were carried out in strict accordance with the law of the Czech Republic. The experiments were approved by the local Institutional Animal Care and Use Committee of the Ministry of Agriculture of the Czech Republic (permit number 41545/2017-MZE-17214).

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