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Fathers and sons: Physiological stress in male Zaisan mole voles, *Ellobius tancrei*

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ABSTRACT

The social environment can be stressful for at least some group members, resulting in elevated levels of glucocorticoid stress hormones (GC). Patterns of the relationships between social rank and GC levels vary between species. In carnivores, primates and birds that live in permanent cooperative groups, helpers do not usually display physiological indicators of stress. Very little is known about status-related GC differences within cooperative groups of rodents. In this laboratory study, we compared GC concentrations in dominant (fathers) and subordinate (natal sons) males of a cooperative subterranean vole, *Ellobius tancrei.* The assessment of adrenocortical activity by measuring urine glucocorticoid metabolites (UGM) was previously validated for this species through an ACTH challenge test. We observed clear peaks of UGM in the second or third urine samples taken after the administration of ACTH (lag time equal to 2.5–3 h). Thus, UGM is suitable to estimate physiological stress in *Ellobius*. Postpubertal sons living in natal groups had significantly higher UGM concentrations than their fathers. The average UGM levels of sons were positively associated with their ages and paternal body masses, and negatively associated with paternal ages. Hence, son-father interactions rather than just younger ages of sons appear to contribute to GC differences. The revealed pattern was not consistent with that reported for most cooperative species from other taxa, highlighting the importance of comparative studies.

1. Introduction

The social environment can be stressful for at least some group members, resulting in elevated levels of glucocorticoid stress hormones (GC). Chronically elevated GC concentrations were generally thought to characterise subordinance, but today, it is known that patterns of the relationship between social rank and stress hormone levels vary between species. This diversity seems to reflect, among other things, the variety of social systems. In carnivores, primates and birds that live in permanent cooperative groups, subordinates do not usually display physiological indicators of stress (see Abbott et al., 2003; Goymann and Wingfield, 2004; Creel, 2005; Sapolsky, 2005; Creel et al., 2013 for review). Surprisingly, very little is known about the factors that determine GC differences among same-sex individuals within cooperative groups in rodents (Armitage, 1991; Arnold and Dittami, 1997; Clarke and Faulkes, 1998). From the comparative perspective, monogamous cooperative breeders phylogenetically related to the well-studied polygamous species, such as brown rat (*Rattus norvegicus*) and house mouse (*Mus musculus*), are of special interest. Whereas other aspects of the stress physiology of monogamous muroid rodents have received great attention (see Beery and Kaufer, 2015 for review), the link between social status and GC has not been investigated. Mole voles (*Ellobius*) are specialised subterranean muroid rodents that form stable monogamous or polyandrous groups including offspring of two or three generations (Meklenburtsev, 1937; Slastenina, 1963; Davydov, 1988; Evdokimov, 2001). Delay of natal dispersal, singular breeding, intense intra-sexual competition among females and female-biased sexual dimorphism (Slastenina, 1963; Davydov, 1988; Smorkatcheva and Kuprina, 2018) allow mole voles to be designated as cooperative breeders. In this article, we compare GC concentrations in dominant (fathers) and subordinate (natal sons) male Zaisan mole voles, *E. tancrei.* The non-invasive method used for the assessment of adrenocortical activity was validated for this species in a separate experiment.

2. Materials and methods

This study was conducted at Saint Petersburg State University. Focal animals were the descendants of eight mole voles captured in Tajikistan (third through sixth generations). Animals were kept in family groups of a breeding pair and their offspring (see Smorkatcheva et al., 2016 for

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details of housing conditions).

2.1. Validation of urine steroid measures

To validate the assessment of adrenocortical activity in *E. tancrei* by measuring urine glucocorticoid metabolites (UGM), we performed an ACTH challenge test. The subjects were four adult males and three adult females from four family groups. We used each animal as its own control on the Pre-treatment (PT) Day and injected the animal with ACTH on the Treatment (T) Day. The PT and T days were separated by 48 h. Urine samples were collected from 09 h00 to 19 h00 on both days. Blood samples were taken from the *retro*-orbital sinus with heparinised Pasteur pipette tubes at 10 h00–10 h20, 12 h00–12 h20 and 17 h00–17 h20 on both PT and T days. All blood collection procedures were completed within 3 min. Each animal was injected intraperitoneally with a synthetic ACTH dissolved in sterile isotonic saline solution (Synacthen, Ciba-Geigy AG, Basel, Switzerland, dosage 2 μg/100 g body weight) between 11 h00 and 11 h15 of the T day. We did not inject animals with saline solution on the PT Day as we wanted to ensure contrast in the level of stress between PT and T conditions. During the whole validation experiment, the focal animals stayed in their home cages. To collect urine samples, we took advantage of the fact that captive mole voles always urinated in the opposite corner from their nest. The researcher intercepted an animal on its way to the toilet corner and transferred it into a clean container. The animal was returned to the nest immediately after urination, and urine was collected from the floor of the container with a pipette. If an individual urinated during the blood collection or ACTH injection procedure, the urine was also collected. The exact time of urination was noted.

2.2. Collection of urine samples from fathers and natal sons

Subjects of this experiment were housed under the same conditions as the other animals in our laboratory colony. Each focal group (n = 17) consisted of a pair of breeders and their one to four weaned offspring, including a focal postpubertal son, up to two of his female littermates and up to three prepubertal siblings. Group composition remained unchanged during the experiment. The collection of urine samples started when the focal sons were between 3.5 and 5.5 months of age. To obtain urinary samples, we simultaneously placed each of the two focal males of the same group into an individual clean plastic container. If the animal urinated, the urine was collected immediately in a syringe pipette, and stored at −20 °C until hormone measurement. If a male did not urinate in 15 min, he was returned to the home cage, and the next attempt at urine collection was performed two days later. At least 7 days separated successive collection dates. All samples were collected between 12 h00 and 15 h00. We ended urine collection six weeks after obtaining the first sample.

2.3. Plasma corticosterone and urine corticosterone metabolite assays

Plastic scintillation vials with urine were stored at −20 °C. The level of corticosterone, which is the main stress hormone in mole voles (Moshkin et al., 2002), was measured both in plasma (PC) and urine using an enzyme immunoassay, according to the instructions for the corticosterone EIA kit (Enzo Life Sciences, USA). The cross-reactivity was 28.6% with deoxycorticosterone and 1.7% with progesterone. Testosterone, aldosterone, cortisol, and estradiol had cross-reactivities of less than 0.2%. The sensitivity of the kits was 26.9 pg/ml. To determine the parallelism, a five-point two-fold dilution series of pooled urine and plasma samples was prepared and compared with the standard curve for corticosterone. There were no significant differences between the slope of the standard curve and the slopes of lines generated from the plasma and urine samples of the assayed voles ($p > 0.5$). The inter- and intra-assay variations were 9.6% and 1.8%, respectively. UGM concentrations were indexed with creatinine to account for variations in water excretion and expressed as mass per 1 μg of creatinine. The creatinine concentrations in samples were measured with the Clinical Chemistry reagent kits Creatinine FL (Vital Diagnostics, Russia).

2.4. Data analysis

In all analyses, hormone data were normalised using a decimal logarithm transformation.

2.4.1. Validation of urine steroid measures

To ensure that the ACTH injection affected PC concentrations, we first performed an ANOVA with DAY and HOUR of blood collection as within-subject variables and SEX as a between-subject variable. Then, we compared the PC levels in samples taken at 12 h00–12 h20 of the PT and T days with a Repeated ANOVA. The effect of the ACTH injection on UGM levels was first evaluated by a visual inspection of individual UGM dynamics on PT and T days, and then proven by a statistical comparison with a mixed ANOVA. In this analysis, we compared the influence of DAY on UGM levels for the samples taken from 12 h00 to 16 h00 (the potentially ACTH-affected period, mid-day samples) with that for the samples taken from 09 h00 to 12 h00 (morning samples) and from 16 h00 to 19 h00 (evening samples). Individual identity was introduced as a random effect.

2.4.2. Comparison of UGM concentrations in fathers and sons

Only the samples obtained from two kin males on the same day or, in several cases, separated by 1–2 days were analysed. The final set included 51 pairs of urinary samples for 34 males from 17 families. The mean number of samples per male was [X ± SD] 3.0 ± 1.3; ages of sons and fathers at first sampling ranged from 101 to 163 days and from 347 to 1686 days, respectively.

First, we used a mixed ANOVA to test for the influence of the male status on UGM (log-transformed); family affiliation was treated as a random factor effect. Next, we tested which of the following factors, if any, contributed to the inter-group variation in UGM levels of each male category: son's and father's ages (sAGE and fAGE, respectively), son's and father's body masses (sBM and fBM, respectively), and group size (number of weaned animals). Body masses (in g) were measured at first sampling and ages (in days) were determined on sampling dates. UGM concentrations as well as ages were averaged for each individual after log-transformation. To start with, we ran two all subset regression analyses (the package *"leaps"*, R 3.5.1, with adjusted R^2 as a criterion statistic) with all candidate predictors, plus a number of samples used for individual mean calculations as independent variables. Either son's or father's average UGM concentrations (sUGM and fUGM, respectively) were used as response variables. The fits of the yielded models were estimated using Akaike information criterion corrected for a small sample size (AICc, package *sme* R 3.5.1). The assumptions of the models were proven using the function *gvlma* (package *gvlma,* R 3.5.1*)*. We reported standardised coefficients of the models to highlight the relative importance of the different independent variables. All tests were two tailed and the α level of significance was 0.05.

3. Results and discussion

3.1. Validation of urine steroid measures

We found a significant influence of HOUR ($F_{2,10} = 32.7$; $p < 0.001$) as well as an HOUR X DAY interaction ($F_{2,10} = 5.10$; $p = 0.030$) on PC concentrations. Neither the main effect of DAY nor SEX, nor any other interaction effect was significant ($p = 0.341$–0.760). The interaction effect was due to increased PC levels after ACTH injection observed in all animals except one female (Fig. 1). PC levels were significantly higher in samples taken at 12 h00–12 h20 of the T Day than at the same time on the PT Day ($F_{1,6} = 12.4$; $p = 0.012$). Each

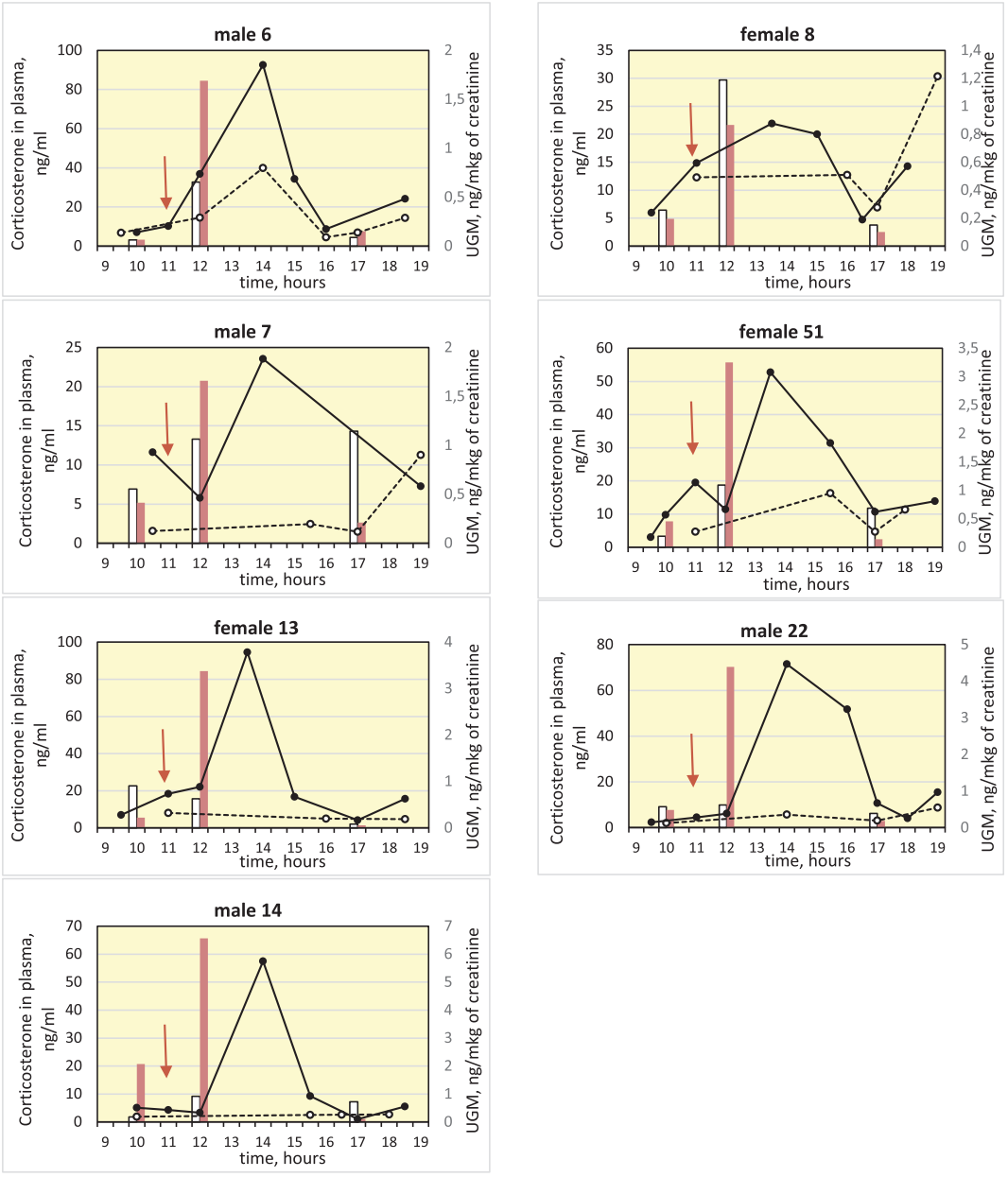

Fig. 1. Corticosterone concentrations in plasma (bars) and concentrations of glucocorticoid metabolites in urine samples (lines and circles) taken from individual Zaisan mole voles on Pre-treatment and Treatment days. Pre-treatment: light bars, dotted line and open circles; Treatment: red/grey bars, solid line, and closed circles. Arrays indicate the time of ACTH injection on the Treatment Day.

of the six animals that displayed elevations in PC on the T Day demonstrated a clear UGM peak in the second or third urine sample taken after the administration of ACTH (lag time equal to 2.5–3 h, Fig. 1). The effects of DAY ($F_{1,35} = 9.7$; $p = 0.020$), sampling period ($F_{2,35} = 9.9$; $p = 0.002$) and their interaction ($F_{1,35} = 9.4$; $p = 0.002$) on UGM concentration were significant whereas all other effects were not significant ($p = 0.077$–0.981).

The results of this experiment suggest that elevations in UGM are indicative of a physiological stress response in Zaisan mole voles. The observed lag time is within the range inferred from the published data for several muroid species (Lepschy et al., 2007; Kalliokoski et al., 2010; Thorpe et al., 2014; Sipari et al., 2017). The successful validation enables the application of this non-invasive method in future studies of mole vole behaviour and endocrinology.

3.2. Comparison of UGM concentrations in fathers and their sons

UGM levels were significantly affected by male status ($F_{1,66} = 8.4$; $p = 0.009$; Fig. 2) and family identity ($F_{16,66} = 2.7$; $p = 0.026$); the interaction effect was marginally significant ($F_{16,66} = 1.8$; $p = 0.053$). In most groups, sons were more physiologically stressed than their fathers. Age asymmetry, asymmetry in the frequency of agonistic interactions, and asymmetry in the availability of a mate partner may be responsible for the revealed difference. We used our data to test predictions about the relationships between inter-group variation in sons' UGM and variation in several variables made by each hypothesis.

In birds and mammals, more pronounced/protracted stress responses are usually characteristic of juvenile or periadolescent animals (Naidenko and Erofeeva, 2005; Mateo, 2006; Hiadlovská et al., 2015; Romeo et al., 2016). Although all our sons were older than the reported age of male puberty for *E. tancrei* (~3 months; Smorkatcheva et al.,

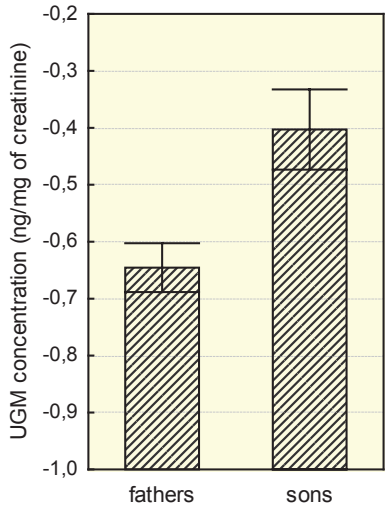

Fig. 2. UGM concentrations (log-transformed, mean ± SEM) in Zaisan mole vole fathers and natal sons.

Table 1
Model selection to predict average urine glucocorticoid metabolite concentrations in natal sons of *Ellobius tancrei,* and fitted parameters.

Predictors included	Adj.R^2,	p	AICc	ΔAICc
fBM + sAGE + fAGE	**0.40**	**0.021**	**19.98**	**0**
fBM + sAGE	**0.20**	**0.082**	**22.05**	**2.07**
son AGE	**0.08**	**0.144**	**22.14**	**2.16**
fBM + sAGE + fAGE + N	0.39	0.038	23.84	3.86
fBM + sAGE + fAGE + sBM + N	0.39	0.056	28.35	8.37
fBM + sAGE + fAGE + sBM + GS + N	0.34	0.110	35.76	15.78

fBM, sBM = Father's and son's body masses (in g).
fAGE, sAGE – Father's and son's ages (in days, log-transformed).
N – Number of urine samples.
Adj.R^2 – Adjusted coefficient of determination.
p – Significance of a model.
AICc = Akaike's Information Criterion adjusted for small sample sizes.
ΔAICs = difference between AICc and most parsimonious model's AICc.
Best models are in bold. Models within six AICc values of the best model with equal or lesser number of parameters are considered equally good. For further details, see Section 2.

2016), we cannot rule out that some of them, most likely the younger individuals, had immature HPA system, resulting in higher sUGM levels. Thus, the age asymmetry hypothesis predicts that if there is a relationship between sUGM levels and sAGE, it is negative. By contrast, if sons are stressed by their inability to either establish pair bonds or disperse, one can expect a positive, if any, association between sUGM and sAGE. Finally, the positive association of sUGM with group size and any association with fathers' individual attributes would provide support for the agonistic interactions hypothesis.

The all subsets regression analysis with sUGM as a response variable yielded six models (Table 1). The AICc-selected model supported the effects of sAGE (β = 0.25 ± 0.09; t = 2.88; p = 0.013), fBM (β = 0.22 ± 0.08; t = 2.62; p = 0.021), and fAGE (β = -0.21 ± 0.09; t = -2.39; p = 0.033) on the average UGM of sons. This model explained 40% of the variation in sUGM. Two additional models were close to the best model based on ΔAICc (2.1 and 2.2). Model 2 included fBM (β = 0.17 ± 0.09; t = 1.81; p = 0.092) and sAGE (β = 0.17 ± 0.09; t = 1.87; p = 0.083); it was marginally significant (p = 0.082) and explained 20% of the variation in the response variable. Model 3 contained only sAGE (β = 0.15 ± 0.10; t = 1.54; p = 0.144) and poorly explained sUGM (8% of the variation). The remaining three models were rejected as they had weak evidence ratios and were more complex compared to the best model (Richards, 2008; Burnham et al., 2011). The all subsets regression analysis revealed that none of the examined independent variables influenced fUGM: even the best model with sBM as a predictor accounted for only 3.4% of the variation in the response variable.

Contrary to the expectations of the age asymmetry hypothesis, average UGM concentrations were higher in older sons compared to younger sons. One possible explanation for this pattern is a higher propensity of older sons to challenge fathers' dominance. However, this seems unlikely given the independence of fUGM from sAGE and sBM. Alternatively, the inability to implement an endogenous ontogenetic programme may be stressful for mature offspring, with stress intensity increasing with age. Finally, older sons may be subject to more frequent aggression imposed by dominants than younger sons. According to our observations, direct aggression is very rare in intact families, but offspring are sometimes tugged and engaged into incisor fencing by breeders. Despite being infrequent, these contacts may be stressful, at least for the recipient. The associations between sUGM and two fathers' characteristics appear to be consistent with the intra-sexual antagonism hypothesis. Heavier (being in better physical condition) as well as younger (possibly having higher testosterone levels; Hardy and Schlegel, 2004) dominants may be more aggressive toward their mature sons. We recognise that specially designed experiments are required for the discrimination between possible proximate causes of the status-related difference in stress levels in male mole voles.

To our knowledge, this is the first study to examine intra-group endocrine variation in males of any monogamous muroid rodent. The revealed pattern was not consistent with that reported for most cooperative species from other taxa, showing once again that the stress-dominance relationship cannot be predicted from the social system alone, and highlighting the importance of comparative studies. In conclusion, we would like to draw attention to the genus *Ellobius* as a promising rodent model for investigating cooperative breeding.

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