JOURNAL OF ANIMAL BEHAVIOUR AND BIOMETEOROLOGY

RESEARCH ARTICLE
Published Online: April 11, 2022
https://doi.org/10.31893/jabb.22019

Non-invasive assessment of physiological stress in captive common palm civets (*Paradoxurus hermaphroditus*) in Vietnam

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Abstract Common Palm Civet (*Paradoxurus hermaphroditus*) is a rare mammal appearing in South and Southeast Asia, first described in 1777. An expensive coffee is made of beans eaten and rejected by civets. In Vietnam, the animals are increasingly captured and kept captive to sell these beans, which has adversely affected their population. Because of the detrimental effects of stress, studying stress responses is important for this wildlife conservation. The stress response of the Common Palm Civet was investigated through the concentration of glucocorticoid hormones in feces under different conditions such as translocation and forced-pairing during the breeding season, among others. This is a non-invasive method for measuring glucocorticoid hormones in captive Common Palm Civet in Vietnam. Our results validated that fecal glucocorticoid metabolites (FGM) accurately reflect circulating glucocorticoid stress hormones in Common Palm Civet. FGM increased under adrenocorticotropic hormone stimulation and during the breeding season (April and November-December). FGM during estrus and parturition was high as well in female civets. Stress response was also observed in case of forced-pairing or residence change. These data could be useful for research and the conservation of this species.

Keywords: animal behavior, fecal glucocorticoid metabolite, glucocorticoids, corticotropin, corticosterone

1. Introduction

Paradoxurus hermaphroditus, also known as Common Palm Civet, was described in 1777 and belongs to the *Viverridae* family and the *Carnivora* order (Krishnakumar et al 2002). This civet species is mainly distributed in the tropical and subtropical regions of South Asia and Southeast Asia, in countries such as Vietnam, Laos, China, etc (Nakabayashi et al 2016; Seamons and Grzimek's 2003; Rabinowitz 1991). Common Palm Civet plays an unexpected role in producing a special coffee. The biological fermentation of coffee beans in its digestive system is considered a special process creating a unique, high-quality, and expensive beverage that many people like (Parikesit et al 2019). The economic value of this animal ensuing has increased its poaching and breeding to obtain these fermented beans. Combined with the loss of habitat, it has greatly affected its long-term survival (Winaya et al 2020). Vietnam has included this species in group IIB of the list of rare and precious animals that need to be protected. Therefore, research on civets is essential to help protect them. Animal behavior and responses studies are of particular interest in animal conservation because animal stress can lead to many diseases (Hien 2020). The responses to stress are often expressed through animal behavior and can also be seen by measuring biochemical markers (Hing et al 2016; Martin et al 2011). In vertebrates, the stress response occurs from an important mechanism through the secretion of glucocorticoids derived from the adrenal gland (Martin et al 2011). It has been known for many years that glucocorticoid hormones are important stress hormones, and they are therefore used as an indicator in the investigation of stress in animals (Davis et al 2018; Whirledge and Cidlowski 2010).

The hypothalamic-pituitary-adrenal axis regulates glucocorticoids released from the adrenal glands. The adrenal glands synthesize and release glucocorticoids in response to physiological and stress signals (Whirledge and Cidlowski 2010). After being released from the adrenal gland into the blood, glucocorticoids will contact target tissues to regulate physiological processes such as immune function, skeletal growth, cardiovascular function, reproduction, cognition, among others (Sapolsky et al 2000). Glucocorticoids are released under stressful conditions to help the organism defend itself against the stressor (Whirledge and Cidlowski 2010; Sapolsky et al 2000). Short-term release of these hormones will increase fitness through energy mobilization. However, prolonged periods of high glucocorticoid concentrations are known to be detrimental because of their negative influence on reproductive function, immune system, growth, muscle development, and metabolism (Whirledge and Cidlowski 2010; Sapolsky et al

2000; Busch and Hayward 2009; Dekkers et al 2011; Inoue et al 2021).

In this study, we examine the role of glucocorticoids in effective adaptation to stress by assessing glucocorticoid concentrations in fecal samples of civets bred under different conditions and at different stages of their development. Using feces as a study material allows linking an animal's endocrine status with its behavior or other life-history features with minimal impact on the animal's natural behavior, especially without invasive sampling. For animals caught in the wild and kept in captivity for conservation, invasive sampling should be limited as much as possible to research while minimizing the stress level of animals (Kumar et al 2014; Kumar et al 2019).

2. Materials and Methods

2.1. Animal experiments

The Common Palm Civets were housed under comparable conditions in separate pens in Dong Nai Biotechnology Center farm, Cam My district, Dong Nai province, Vietnam. Civets were randomly arranged in the experiment and periodic health checks were performed. The tags were used for individual monitoring during the study.

Standard diet for individuals weighting around 3 kg for a day: 400 - 700 grams of food providing 450 Kcal, 18 grams of crude protein, 3 grams of lipid, and 105 grams of dried matter. Animals proactively drink water in prepared bowls. The pens were cleaned daily and disinfected once a month. All procedures were performed according to the laboratory animal care protocol of the Institute of Vaccines and Medical Biologicals (IVAC), Vietnam (Approved number SD-CN2/009 20/05).

2.2. Adrenocorticotropic hormone challenge test

The adrenocorticotropic hormone (ACTH) was injected intramuscularly at the dose of 62.5 µg tetracosactide per total body weight to determine the biological relevance of glucocorticoid metabolites excreted in feces. Fecal samples were collected every day for a week, starting two days before ACTH administration. To acquire data from a control group, we simply injected saline solution instead of ACTH into the animals.

2.3. Sample preparation and extraction of fecal glucocorticoid metabolites

About 5 grams of fecal samples were collected in a 200x140x0.04 mm plastic bag (Seisan Nippon, Japan) and stored at -20°C until analysis. The hormone was isolated using the procedure described previously (Brown et al 1994). Between 0.2 and 0.3 g of dried, mixed, and pulverized feces were boiled in 5 ml of 90% aqueous ethanol for 20 min. After centrifugation at 500 g for 10 min, the supernatant was recovered, and the pellet resuspended in 5 ml of 90% aqueous ethanol, vortexed for 1 min, and recentrifuged to recover the supernatant. Both supernatants were combined, dried in an oven at 40 °C, resuspended in 1 ml of absolute methanol by vortexing for 1 min, sonicated for 30 s, and then stored at -20 °C until further processing.

2.4. Measurement of fecal glucocorticoid metabolites

The fecal glucocorticoid metabolites (FGM) were determined using a cortisol ELISA kit (DRG International, Gemany). The procedure was carried out automatically by using ELISA Dynex DS2 device (Dynex, USA).

2.5. Data analysis

The tests were performed at least three times. Data were statistically analyzed using GraphPad Prism version 9.0.0. Data were presented as mean ± SD. Two-way ANOVA with Bonferroni post-tests was also performed using this package. The level of significance was $P < 0.05$.

3. Results and Discussion

This study presents the results of the first analysis of FGM concentration measurements in Common Palm Civet in Vietnam. We found an influence of breeding season on FGM concentrations, during which females had the highest concentrations of FGM during the estrus and parturition periods. In addition, the influence on the FGM concentrations of various contextual, social and environmental conditions that the captive animals experienced was investigated and shown to be significant.

3.1. Adrenocorticotropic hormone challenge

Glucocorticoids, including cortisol and corticosterone, play a role in metabolism (Whirledge and Cidlowski 2010). The interaction of glucocorticoids and its receptor in the brain mediates the response to stress (Sapolsky et al 2000). The action of glucocorticoids in stress response is also strongly related to inflammatory or neurodegenerative processes (Sapolsky et al 2000). Under normal conditions, glucocorticoids are mainly involved in homeostasis, and their concentration rapidly increases in response to stress (Whirledge and Cidlowski 2010; Sapolsky et al 2000; Busch and Hayward 2009). The release of ACTH plays a crucial role in glucocorticoid synthesis, a reversible process due to the inhibition of ACTH excretion when glucocorticoids are at a high concentration (Dekkers et al 2011; Inoue et al 2021). In this study, an ACTH challenge assay was performed to evaluate the response of the relevant hormones, including glucocorticoids, to get data on the physiology of the animals (Kumar et al 2014; Kumar et al 2019). Three adult male civets between 19 and 33 months and three female civets between 25 and 31 months received ACTH injections and we monitored their glucocorticoids levels before and after injection using their feces.

Our results show that the FGM increased sharply at the time of ACTH injection, peaked one day later, and decreased in the following days. There was no statistical difference between the gender groups at any point in our measures. On the fifth day after ACTH injection, the FGM returned to normal value compared to the control group. The

control groups tended to exhibit stable levels of FGM before and after saline solution injections (Figure 1). However, FGM also increased in the control group on the day of saline injection. We believe this is simply because catching the animals for injection is a stressful experience regardless of what is injected. The FGM value in the control group returned to normal in the following days, whereas it reached its maximum value in the experimental group. ACTH challenge studies, therefore, revealed that our FGM measures reflect adrenal biological activity and that monitoring alterations in FGM concentrations can be used as a reliable measure of adrenal activity. This was also observed in other species (Kumar et al 2014; Kumar et al 2019; Brown et al 1994; Keay et al 2006).

3.2. FGM concentration in fecal samples

The concentration of glucocorticoids in Common Palm Civet was investigated for 12 months by measuring the FGM of 20-month-old female and 18-month-old male civets with three samplings a month. As shown in Figure 2, the FGM concentration (ng/g) increased twice a year, in April and from November to December, from 12.4 to 14.8 ng/g; Meanwhile, for the rest of the year, the FGM concentration remained in the range of 5.2 to 8.2, regardless of gender.

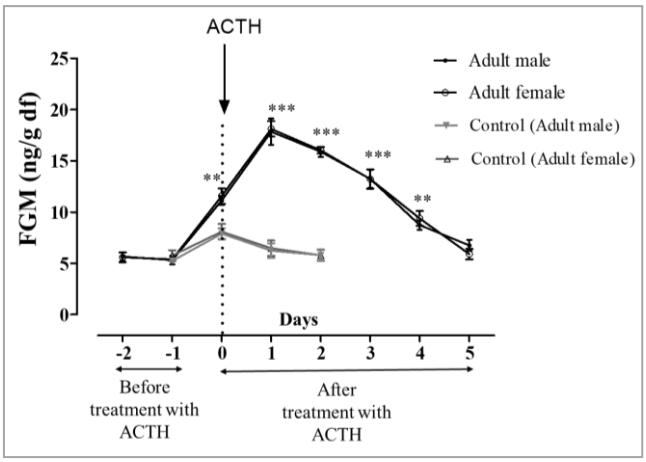

Figure 1 FGM concentrations (ng/g dried fecal (df)) under ACTH stimulation. Three adult male civets and three adult female civets received ACTH injections. The fecal samples were collected each day for a week, starting two days before the injection. The control groups received saline solution injections. Asterisks indicate significant differences between the group that received the treatment and the control (***$P < 0.001$; ** $P < 0.01$).

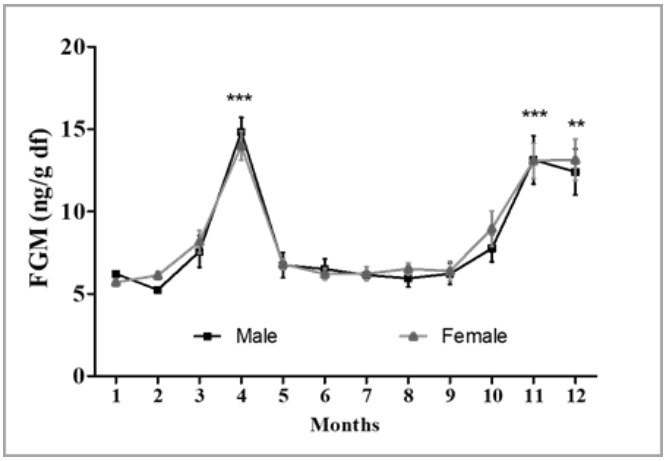

Figure 2 FGM concentrations (ng/g dried fecal (df)) monitoring over the year**.** The experiment was performed for 12 months. The fecal samples were collected 3 times/month; values (ng/g) are mean ± SD. Asterisks indicate significant differences between months (***$P < 0.001$; **$P < 0.01$).

Our other study recently showed that the highest number of newborn civets was in April (more than 40%) and in the October - December period (about 20%) (Nguyen et al 2017). Besides, testosterone levels of male civets were also investigated and reached their highest level during these periods (Nguyen et al 2020). This suggests that glucocorticoid hormones levels may be closely related to the estrous cycle in civets. We assessed FGM levels in reproductive and non-reproductive female civets of different ages to clarify this.

We investigated the FGM concentrations in females during: (a) estrus (mean 3-6 days), (b) parturition (pregnancy + birth + suckling ≈ 120 days), and (c) non-breeding season. Our results showed a significant difference between groups of female civets tested during breeding and non-breeding season: in animals over 12 months (*i.e.,* sexually mature animals), FGM concentrations during the breeding season are much higher than during the non-breeding season (Figure 3A). Specifically, our results showed that the FGM concentration of female civets during estrus (16.3 ± 0.8 ng/g) and parturition (14.8 ± 1.3 ng/g) was significantly higher than during pregnancy (6.8 ± 0.6 ng/g) and non-breeding season (6.5 ± 0.6 ng/g) (Figure 3B). The breeding season has a higher energetic cost due to the activation of catabolic processes associated with hypothalamic-pituitary-adrenal axis activation, ovulation, and pregnancy (Cavigelli et al 2003; Saltzman et al 1998), all of which lead to higher glucocorticoid metabolites in various mammal species (Edwards and Boonstra 2018). Few hare females with a very high gestagen metabolites level (≥ 3000 ng/g) were observed during the breeding period in the study of Rehnus and Palme (2021). Gestagen metabolites have a positive effect on glucocorticoid metabolites, the strength of which depends on the pregnancy states of the females (Edwards and Boonstra 2018).

In August, the non-breeding pairing was also carried out with two male and two female civets sexually mature to test the stress response created. The FGM was measured two days before pairing, during two days of pairing, and three days after detachment (Figure 4). Intense stress response was noticed during the pairing period with negative behaviors such as growling or biting, leading us to separate them after two days of pairing. Because they only interact with their congeners during mating season, the Common Palm Civets face stress under forced-pairing conditions (Brown et al 1994).

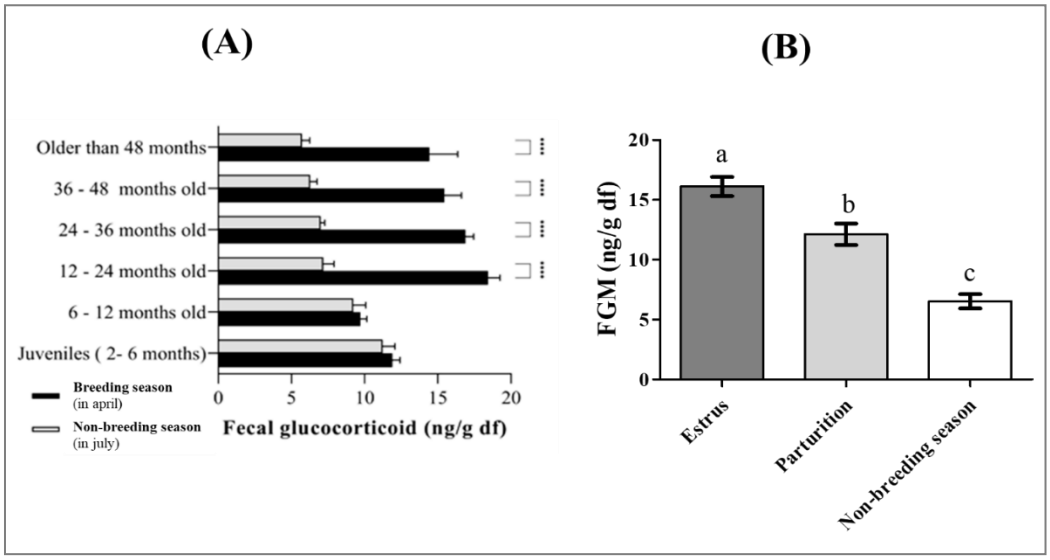

Figure 3 FGM concentrations (ng/g dried fecal (df)) in civets according to the stage of the breeding season and the age of the animals. **(A)** For each age range, a group consisting of two male and two female was tested during the breeding season (April) and non-breeding season (July). Fecal samples were collected from each individual 3 times/month. Asterisks indicate significant differences between breeding and non-breeding seasons (***$P < 0.001$). **(B)** A group consisting of 3 female civets was tested during the non-breeding, estrus, and parturition stages. The experiment was carried out from 6/2018 to 3/2020. Fecal samples were collected from each individual 3 times/month; values (ng/g) are mean ± SD. Different letters indicate significant differences between non-breeding and other stages at $P < 0.05$.

The concentration of FGM during the pairing time was recorded from 18.3 to 21.3 ng/g, which was the highest concentration observed among the experiments in this study. The stress status began to decrease gradually after the civets were separated and nearly returned to normal after three days of detachment (according to pre-pairing data). The documented results of animal stress under forced-pairing have also been reported or mentioned previously in other animals such as primates or birds (Keay et al 2006; Rehnus and Palme 2021; Palme et al 2005).

Translocation of animals in conservation work is inevitable and certainly leads to stress for the animals that may cause a state of chronic stress (Bhattacharjee et al 2015). In nature, short-term acute stress responses are essential for the survival and development of animals, whereas chronic stress often ends up causing diseases in the long term (Whirledge and Cidlowski 2010; Möstl 2014). The civets were translocated from Ho Chi Minh City to Binh Duong province: 26 kilometers traveled in 2 hours by motorbike. The animals were placed in a sealed cage to protect them from direct sunlight. The fecal samples were collected each day starting two days before the move and in the new accommodation for a week. The FGM was measured in two groups, including juvenile and adult civets of both genders (Figure 5).

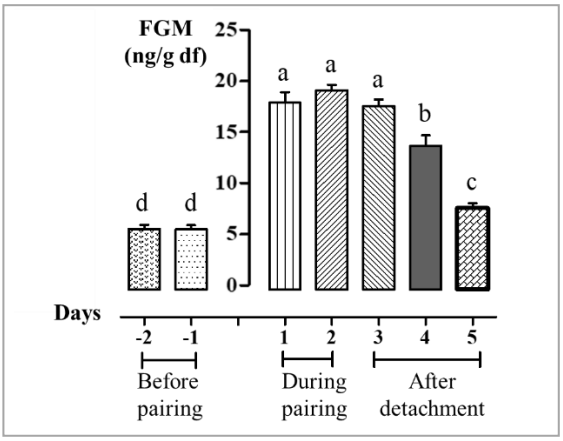

Figure 4 FGM concentrations (ng/g dried fecal (df)) under forced-pairing. The experiment was performed with two male and two female civets, all sexually mature. The fecal samples were collected each day starting two days before pairing, during the two days of pairing, and three days after detachment. The experiment was performed three times; values (ng/g) are mean ± SD. Different letters indicate significant differences between days at $P < 0.05$.

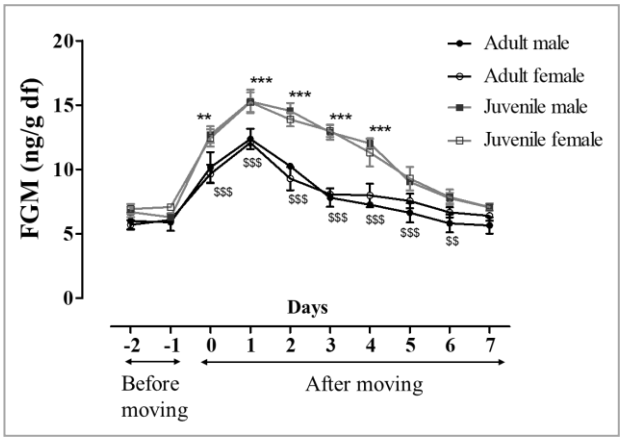

Figure 5 FGM concentrations (ng/g dried fecal (df)) under translocation. The fecal samples were collected two days before moving in the new accommodation and each day for a week after moving. The experiment was performed three times; values (ng/g) are mean ± SD. Asterisks indicate significant differences between adult male civets and juvenile male civets; Dollar symbols indicate significant differences between adult female civets and juvenile female civets (***$P < 0.001$; **$P < 0.01$).

The stress response differed between the two groups and was independent of gender. The stress process started at the beginning of the translocation and gradually returned to normal after a week in the new house. The FGM concentration curve also shows that the stress response was stronger in juveniles than in adults. This does not come as a surprise as there is evidence that juvenile animals are more sensitive than adults (Möstl 2014). Berger et al (2007) showed that younger, unhabituated animals have significantly higher baseline glucocorticoid levels in areas with novel or occasional stressors. Increased stress may impact animal health, fitness, conservation of populations, and emerging infectious diseases (Angelier and Wingfield 2013; Johnstone et al 2014). Therefore, the managers and legislation should attempt to minimize all forms of activity around important breeding colonies, limited movement, and frequent changes of residence.

4. Conclusions

The glucocorticoid-mediated stress response of Common Palm Civet was evaluated by measuring this metabolic hormone in feces. The results showed that the FGM concentration increased during specific periods, including the reproductive stage, especially when giving birth, non-breeding pairing, and when translocated. We hope that this information will prove valuable for the conservation of this rare animal.

Acknowledgments

We would like to thank the farm of Dong Nai Biotechnology Center, Cam My district, Dong Nai province, Vietnam, for their support during the experiments.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

This research received no external funding.

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