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A Biological Validation Procedure for the Measurements of Fecal Outputs and Fecal Cortisol Metabolites in Male Syrian Hamsters

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Abstract

Monitoring fecal outputs and fecal cortisol metabolites (FCM), a noninvasive technique, has been used to investigate physiological responses to stress and relationships between hormones and behavior in an increasing number of species. The aim of this study was to investigate whether measurements of fecal outputs and FCM can be used as indexes to repeatedly and precisely monitor stress levels in male Syrian hamsters using a social defeat as a biological validation method. The feces voided by each animal were collected every 3 h for at least 1 day before and after experiencing a single fighting interaction, and the extracted FCM during the pre- and post-fight phases was quantified by enzyme immunoassays. During the pre-fight baseline phase, both the number of fecal pellets and the FCM levels fluctuated throughout the whole day. Although the number of fecal pellets did not differ between the dark and light cycles, the levels of FCM were significantly higher during the dark cycle than during the light cycle. During the post-fight phase, the experience of fighting did not result in a significant difference in the number of fecal pellets per hour between the winner and loser groups, but did considerably increase the total amount of fecal outputs in both groups. The level of FCM was significantly higher in the loser group than in the winner group during the 1st and 7th 3-h collection periods after the fight, which indicated that the experience of defect affected the behavioral and physiological responses of the losers. Our findings suggest that measurement of FCM is sensitive enough to distinguish the stress levels between winners and losers after experiencing a fight. The measurements of fecal outputs and FCM levels provide new opportunities to longitudinally and frequently monitor behavioral and hormonal responses to stress in hamsters and other small laboratory animals.

Key Words: male hamsters, fecal pellets, fecal cortisol metabolites, social defeat, non-invasive method, biological validation

Introduction

Stressors are environmental stimuli that lead to an imbalance of homeostasis in an organism, which results in the induction of stress responses (14). Stress responses include behavioral and physiological changes that involve the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis (4, 14). The SNS, which is also known as the adrenergic system, is responsible for what is classically called the fight-or-flight response. The HPA axis, which is a feedback loop that consists of the hypothalamus, pituitary gland and adrenal cortex, is a major component of the neuroendocrine system that controls reactions to stress, and it acts more slowly than the SNS pathway (5, 20). In the HPA axis, the

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adrenal cortex produces and secretes a variety of steroid hormones, including glucocorticoids. Hormones of this type, such as cortisol, are involved in the coordination of circadian cycles, glucose metabolic processes and stress responses in particular. In the majority of animals including humans and hamsters, cortisol is the main glucocorticoid (11, 23). The release of cortisol increases blood pressure and levels of blood glucose, and simultaneously reduces immune responses (19). After the metabolism of cortisol and other steroids, which follows species-specific pathways and occurs mainly in the liver, the degradation products are eliminated through the urine or bile as conjugates. In the intestine, these metabolites can be reabsorbed by the enterohepatic circulation, then decomposed by bacteria, and excreted in the feces (20, 22).

Many studies have successfully used plasma/serum levels of cortisol or corticosterone to quantify the stress response in primates and laboratory rodents (11, 12, 25, 30). Frequently-used methods for blood sampling include the implantation of indwelling catheters, tail vein nicking, tail clipping, orbital sinus blood sampling, blood collection from the saphenous vein and intracardiac puncture. Despite many advantages of measuring plasma/serum levels of cortisol, the optimal sampling method to measure stress hormones with minimal stress to the animal is frequently debated. The collection of blood can be stressful to an animal, which potentially increases SNS/HPA activities and enhances the stress responses (30, 33). Blood sampling usually requires the confinement or handling of animals, which again might be stressful and might confound the accuracy of the results (8). In consideration of small blood volumes and welfare of small animals or endangered species, it is relatively difficult to perform repeated or frequent assessment of plasma hormones in these animals by this method. Thus, it is of great value for researchers to develop alternative and noninvasive methods to longitudinally and repeatedly monitor hormone fluctuations in animals.

Indeed, some non-invasive sampling procedures, such as the measurement of glucocorticoid (or its metabolites) levels in urine, saliva or milk have been developed to monitor and assess physiological responses to stress in an increasing number of species (20, 22, 28). Among these non-invasive methods, fecal samples offer additional advantages in that (i) feces can be collected easily and over a long period of time, (ii) individuals can be used as their own baseline controls, and (iii) these procedures are nearly physiological feedback-free or stress-free (20, 22). In humans and experimental animals, physical or psychological stress has been reported to increase outputs of fecal pellets or diarrhea *via* stimulation of autonomic neurons and colonic motility (18, 24), and the number of fecal pellets reflects the degree of stress (1, 21). The quantification of fecal glucocorticoid metabolites, *e.g.*, fecal cortisol metabolites (FCM), has become an increasing popular technique as a potential indicator of animal welfare or wildlife wellness in many free-ranging or intractable animals and in some domestic livestock and pet animals (6, 20, 26, 28, 31). In contrast, studies that apply this method in small laboratory rodents are relatively limited (27, 28). The use of fecal samples should be promoted further and used to monitor long-term stress responses in various types of laboratory animals.

Among small laboratory rodents, hamsters are widely used for behavioral and stress-related studies in the laboratory (10) and in the field (7). Measuring the activity of the HPA axis and levels of stress hormones, in particular cortisol, in hamsters can be a useful method for assessing how an animal copes with stressful events. Indeed, it has been reported that acute and chronic social defeat increases plasma cortisol levels and decreases plasma testosterone and humoral immunity in male Syrian hamsters (12, 13). Quantification of daily fecal outputs and daily production of FCM were also used to assess stress response after ovariectomy in female hamsters (2). Taking these findings into consideration, it is of great interest to apply such noninvasive methods to monitor stress-induced alterations of FCM levels in male Syrian hamsters and to provide biological validation of this method in hamsters. Serial samples before and after a stressful event (*e.g.*, restraint, injections, blood sampling, or social defeat) can be used to evaluate the biological relevance of the measurement (*i.e.*, a biological validation) as suggested in the guidelines for measuring fecal steroids (22). In the present study, a single fighting interaction was applied as a stressful event in male hamsters and their behaviors were closely monitored during the fight. Thus, using a social defeat as a biological validation, the objectives of this study were to [1] repeatedly monitor fecal pellet output before and after experiencing a single fighting interaction and [2] monitor changes in FCM levels before and after the social conflict as indexes of stress responses in male hamsters.

Materials and Methods

Animals

Twelve male adult Syrian hamsters (*Mesocricetus auratus*), 4-6 months of age, body weights 130-150 g and without prior sexual or aggressive experience were used as subjects in this experiment. Additional males, 7 months of age, 160-170 g, that

had previously won a number of fights were used as fighters to increase the sample size of defeated individuals. All hamsters were descended from a stock of the National Laboratory Animal Center in Taipei, Taiwan, and were housed within the animal rooms of the Psychology Department, National Taiwan University from 2 months of age. Each hamster was individually housed in Polysulfone individually ventilated cages (34 × 22 × 16 cm) that contained corn cob bedding with food and water available *ad libitum*. The animal colony was maintained on a reverse 12:12 light:dark cycle with lights off at 8 AM and a temperature of 22 ± 2°C. Animals were handled and weighed daily seven days before the behavioral experiment. All animal procedures were performed according to protocols approved by the appropriate Animal Care and Use Committees established by National Taiwan University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Procedures for a Single Fighting Interaction

The protocol for fighting was performed at 2 PM during the dark cycle under dim illumination (30 lux) in behavioral testing rooms that were separated from the animal colony. A Plexiglas cage (46 × 26 × 15 cm) with a wire-mesh barrier and a glass lid was used as the interaction chamber. On the day of the procedure, each animal was paired with another male. Each member of the pair was placed on one side of the interaction chamber and separated by the wire-mesh barrier in the middle of the chamber. Each pair was allowed to smell, touch and explore the chamber or the other male across the wire-mesh barrier for 10 sec. The wire-mesh barrier was then removed quickly and the experimenter waited for one hamster to step into the opposite side of the chamber. Once both hamsters were on the same side, the wire-mesh barrier was replaced to facilitate interaction and a glass lid was placed on top to avoid escape. The two males were allowed to interact with each other directly and fight for territorial dominance until they were stopped by the experimenter. The fight was stopped when dominance was established (*i.e.*, the loser showed an obviously submissive postures such as lying on its back or fleeing) and maintained for one additional minute. The sequence of fighting behaviors was relatively consistent among all pairs, and no obvious wounding or bleeding was found. Their fighting patterns were very similar to those reported previously (15). The behavioral performances of each subject during the fighting interaction were videotaped with a JVC Hard Disk camcorder (Victor Company, Japan). The latency from attack to flight (defined as the time from the initial attack or bite until the loser fled) in losers and latency to attack (defined as the time from opening of the metal barrier until the initial bite) were recorded with a stopwatch. All subjects were returned to their home cages immediately after the fighting experience.

Feces Collection Procedure

The feces voided by each subject were collected from the home cages of the subjects for 5 consecutive days. The 5-day feces collection procedure can be divided into three experimental phases: [1] a 2-day habituation phase; [2] a 24-h pre-fight baseline phase, and [3] a 30-h post-fight phase. During the habituation phase, to acclimate all subjects to the feces collection process, each subject remained undisturbed in its home cage from 2 PM to 8 AM (lights off at 8 AM) on the first 2 days; the bedding was changed at 2 PM and the accumulated feces were collected at 8 AM. The pre-fight baseline phase was started on Day 3 at 2 PM and accumulated feces from each subject were collected every 3 h for a total of 24 h (*i.e.*, eight 3-h fecal outputs). Immediately after the end of the pre-fight baseline phase, all animals were subjected to the single fighting interaction at 2 PM on Day 4 as described above. On the basis of the outcomes of the fights, the subjects were divided into two groups: a winner group and a loser group. At the end of the fighting, all subjects were returned to their previous home cages with new bedding. During the post-fight phase, feces were collected every 3 hours for a total of 30 h (*i.e.*, ten 3-h fecal outputs). Thus, over the 5 days of fecal collection, one 24-h (*i.e.*, eight 3-h fecal outputs) pre-fight baseline procedure and one 30-h (ten 3-h fecal outputs) post-fight collection procedure were conducted.

At the beginning of each fecal collection period, in order to reduce the collection time and unnecessary interference to the subject, each subject was moved temporarily from its original home cage into a clean cage. After the old, dirty bedding had been replaced with new, clean corn cob bedding, each subject was placed gently back in its original home cage. The whole process took less than 30 sec. Each subject remained in its home cage with food and water available *ad libitum* between collections. The same procedure was then performed again and the dirty bedding was removed quickly into a clean cage for collection. All feces voided by each subject during each collection period were collected manually into a clean tiny zipper bag with the exception of feces contaminated by urine, which might contain high concentrations of cortisol metabolites (22). The entire number of fecal pellets was counted to give the number of fecal pellets and all samples were stored quickly in a freezer at -80°C to avoid the possible

influence of environmental temperature and moisture on the steroid concentrations.

Fecal Extraction

All fecal samples obtained from each subject during each collection period were weighed and homogenized (they were not lyophilized because hamster feces are very dry). The fecal extraction procedure was established by the Laboratory of Animal Physiology of Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan. An aliquot of 0.04 g feces from each homogenized sample was mixed and extracted with 9 volumes (*i.e.*, 0.04 g feces in 0.36 ml) of 88.9% methanol for 30 min at 1800 rpm in a mixer. The feces-methanol mixture was then mixed with 0.6 volume (*i.e.*, 0.36 ml mixture added 0.216 ml) of petroleum ether for 15 sec in a mixer. After centrifugation (15 min at 825 g), the supernatant was decanted and the remaining extraction phase was transferred into a new tube. All extracts were kept frozen at -20°C until assayed.

Determination of Concentrations of FCM

Serial dilutions of a sample were assayed to identify the best dilution for analyzing the samples. A competitive enzyme immunoassay (EIA) for detecting fecal cortisol metabolites (FCM), using an rabbit-polyclonal antibody, was developed by the Laboratory of Animal Physiology of Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan and the Department of Medical Technology and Institute of Biotechnology, Yuanpei University of Science and Technology, Hsinchu, Taiwan (3). The EIA was used to determine the concentration of FCM at each fecal collection period. Briefly, 50 µl of diluted sample and 150 µl of cortisol conjugated to horseradish peroxidase were added to each well of a 96-wells EIA plate (Costar 9018) coated with polyclonal rabbit anti-FCM antibody. After the samples had been incubated at room temperature for 15 min and washed twice with phosphate buffer saline (PBS), the color was developed by incubating with 200 µl of 2.2 mM o-phenylenediamine with 0.003% H_2O_2 at room temperature for 30 min. The reaction was stopped by adding 50 µl of 8 N sulphuric acid. Absorbance at 490 nm was compared with a cortisol standard curve and the concentration of FCM in the samples was calculated. All samples were analyzed in duplicates and the results are expressed as ng/g of fecal weight. The intra-assay and inter-assay variation coefficients were within the range 1.5-3.7% and 5.4-6.3%, respectively. The sensitivity of this assay was 1.3 ng/ml.

Data Analysis and Statistical Analysis

We calculated the mean number of fecal pellets per hour (means ± SEM) and mean concentration (means ± SEM ng/g of feces) of FCM in each 3-h fecal collection period. We chose number of fecal pellets over fecal weights as another index of stress because moisture content of each fecal pellet varies over time which might affect the total weights. All data were normally distributed (Kolmogorov-Smirnov Test, data not shown). Using StatView 5.0.1. (SAS Institute Incorporated, Cary, NC, USA), data were analyzed by repeated measures analysis of variance (ANOVA), paired Student's *t*-test or Pearson correlation analyses where appropriate. *F* values reaching significant difference ($P < 0.05$) were evaluated further by *post-hoc* analysis using the Fisher's protected least significant difference (PLSD) test. Pearson correlation analyses were performed to evaluate the relationships between fecal cortisol concentration and the number of fecal pellets. It should be noted that, in order to perform one sample paired *t*-tests between different collection time periods, missing data points that were due to lack of defecation during certain time periods in only a few animals, were replaced with the amount of mean change of the group between the previous point and the missing point.

Results

Behaviors during the Single Fighting Interaction

During the fighting interaction, the sequence of behaviors was relatively consistent among all pairs. Males started by sniffing each other, they then circled one another and engaged in upright and sideways postures, and finally fought. Fighting behaviors involved being locked together at right angles to one another and trying to bite the flank or ventral surface of the other hamster. Eventually, one male fled and ran around at speed, *i.e.*, fleeing behavior. Dominance was established within 60 seconds and the loser was frequently chased by the winner and exhibited submissive or defensive behaviors until the fight was stopped by the experimenter. The latency from attack to flight for the losers was 24.9 ± 16.2 seconds and the latency to attack was 18.4 ± 12.4 seconds. No observable differences were found between the losers that were defeated by experienced winners and the losers that were defeated by untried males.

Number of Fecal Pellets and FCM Levels during the Pre-fight Baseline Phase

Before the fighting, no significant difference was found for any measurement between the winner

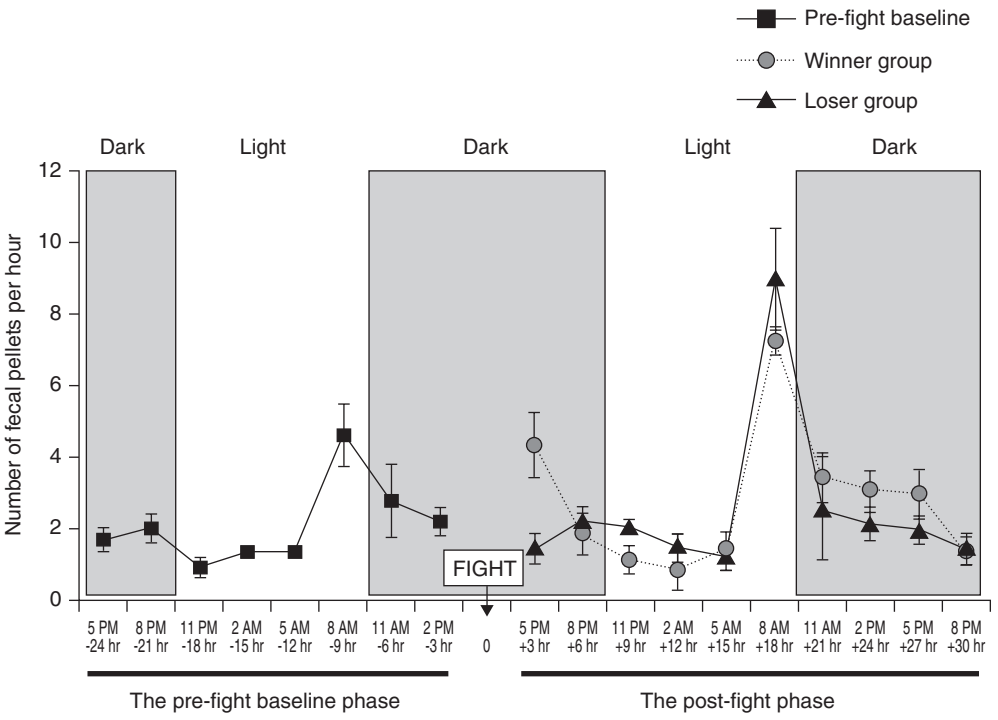

Fig. 1. The means ± SEM number of fecal pellets per hour during each 3-h collection period during the dark cycles (gray areas) and light cycles of the pre-fight baseline and post-fight phases. The black squares represent the pooled data from the winner and loser groups (n = 12) during the pre-fight baseline phase. The gray circles represent the data from the winner group (n = 5) during the post-fight phase and the black triangles represent the data from the loser group (n = 7) during the post-fight phase.

and loser groups. There was no significant difference between the two groups in the number of fecal pellets during either the total 24-h period or any of the 3-h collection periods during the pre-fight baseline phase. Therefore, the data for the winner and loser groups were pooled (n = 12). Within this 24-h period, the mean number of fecal pellets per hour was 2.1 ± 0.1 for each subject (the averages for the winner group and the loser group were 2.0 ± 0.3 and 2.2 ± 0.1, respectively). The mean number of fecal pellets per hour appeared to fluctuate across the eight 3-h collection periods. The highest number was 4.6 ± 0.9 per hour during the 5 AM-8 AM period (the last 3 h of the light cycle) and the lowest number was 0.9 ± 0.2 per hour during the 8 PM-11 PM period (the first 3 h of the light cycle) (Fig. 1). The total mean number of fecal pellets per hour during the 12-h dark cycle and the 12-h light cycle was 2.2 ± 0.3 and 2.0 ± 0.3 respectively, and there was no significant difference in the total number of fecal pellets between the dark and light cycles [$t(11) = 0.272$, $P > 0.05$].

In addition, the levels of FCM appeared to fluctuate across the eight 3-h collection periods too (Fig. 2). The highest level of FCM (623.2 ± 73.4 ng/g) was detected during the 5 PM-8 PM period of the dark cycle, *i.e.*, the last period of the dark cycle. In contrast, the lowest level of FCM (314.0 ± 26.5 ng/g) was detected during the 11 PM-2 AM period of the light cycle, which corresponded to the second period of the light cycle. Furthermore, the total mean concentrations of FCM during the 12-h dark cycle and the 12-h light cycle were 545.2 ± 30.8 ng/g and 390.5 ± 26.7 ng/g, respectively. The mean level of FCM was significantly higher during the dark cycle than during the light cycle [$t(11) = 5.518$, $P < 0.001$].

Number of Fecal Pellets and FCM Levels during the Post-Fight Phase

Immediately after the single fighting interaction, animals were assigned to the winner group or the loser group according to the outcome of the fight. After fighting, the mean number of fecal pellets per hour over the entire post-fight 30 h was similar in both the winner group and the loser group (2.9 ± 0.1 *vs*. 2.8 ± 0.1). Repeated measures ANOVA revealed that there was a significant difference in the ten fecal collection periods ($F(9, 90) = 15.33$, $P < 0.01$) but no significant difference was found between the two groups across the ten fecal collection periods [$F(1, 90) = 2.69$, $P > 0.05$] (Fig. 1). However, the mean number of fecal pellets per hour in both groups after the fight was significantly increased compared to their mean numbers during the pre-fight baseline

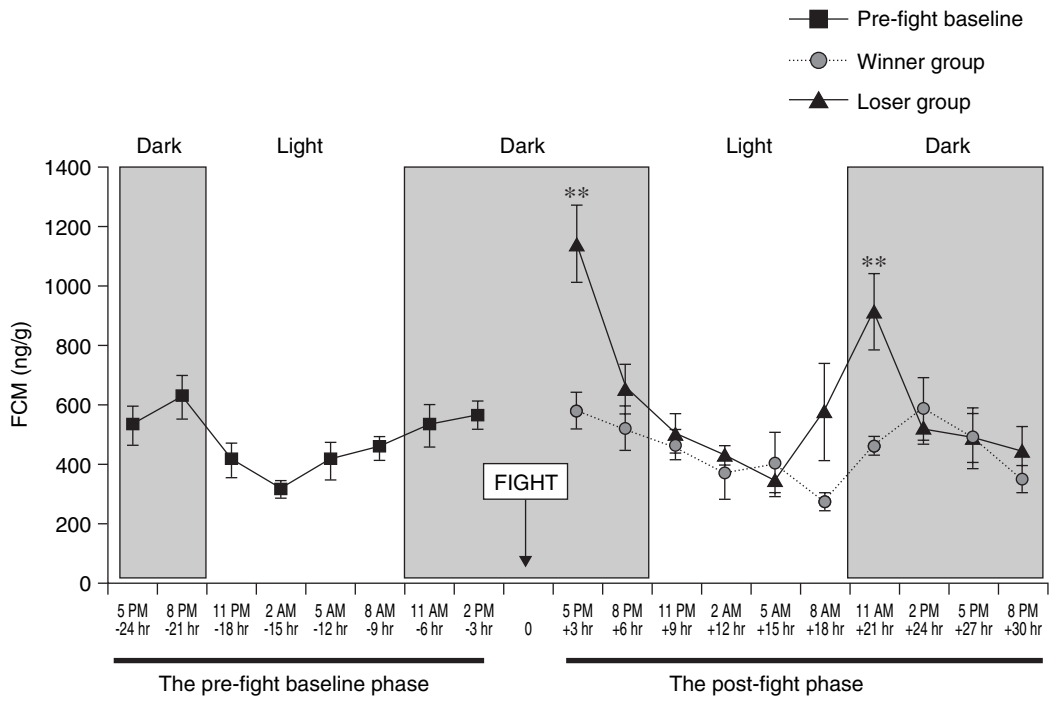

Fig. 2. The means ± SEM level of FCM (ng/g) during each 3-h collection period during the dark cycles (gray areas) and light cycles of the pre-fight baseline and post-fight phases. The black squares represent the pooled data from the winner and loser groups (n = 12) during the pre-fight baseline phase. The gray circles represent the data from the winner group (n = 5) during the post-fight phase and the black triangles represent the data from the loser group (n = 7) during the post-fight phase. ** - $P <$ 0.01 between the winner and loser groups.

phase [2.9 ± 0.1 *vs*. 2.0 ± 0.3, $t(4) = 3.630$, $P < 0.05$ for the winner group; 2.8 ± 0.1 *vs*. 2.2 ± 0.1, $t(6) = 3.348$, $P < 0.05$ for the loser group], which indicated that the experience of fighting affected the behavioral responses and frequency of defecation in both groups.

In addition, the experience of fighting appeared to affect the levels of FCM, especially in the loser group (Fig. 2). Repeated measures ANOVA revealed that there were significant differences in the ten fecal collection periods [$F(9, 90) = 7.04$, $P < 0.01$] and between the two groups across the ten collection periods ($F(1, 90) = 8.261$, $P < 0.05$). There was also a significant interaction effect between groups and collection periods [$F(1, 90) = 3.533$, $P < 0.05$]. Fisher's PLSD *post hoc* analysis revealed that males in the loser group had a significantly higher level of FCM than males in the winner group during the 2 PM-5 PM period of the dark cycle, which corresponded to the first 3-h period after the fight (1,132.0 ± 131.5 *vs*. 573.3 ± 64.1 ng/g, $P < 0.01$), and during the 8 AM-11 AM period, which corresponded to the seventh 3-h period after the fight or the first 3-h period in the dark cycle (907.8 ± 128.3 *vs*. 456.2 ± 32.6 ng/g, $P < 0.01$). In addition, the levels of FCM in the loser group were elevated significantly during these two periods compared to the levels detected in the equivalent periods during the pre-fight baseline phase [for the 2 PM-5 PM period: 1,132.0 ± 131.5 *vs*. 579.2 ± 109.1 ng/g, $t(6) = 2.769$, $P < 0.05$; for the 8 AM-11 AM period: 907.8 ± 128.3 *vs*. 482.6 ± 70.4 ng/g, $t(6) = 2.469$, $P <$ 0.05]. In contrast, for the winner group, no significant difference was found between any collection period before and after the fight.

Correlation between Fecal Cortisol Concentration and Number of Fecal Pellets

During the pre-fight baseline phase, the correlation coefficient between the number of fecal pellets and the concentration of FCM was 0.020 ($P = 0.849$), which indicated that there was no correlation. Further analysis revealed that there was also no correlation between the number of fecal pellets and the concentration of FCM during the dark cycle ($r = -0.113$, $P =$ 0.458) or the light cycle ($r = 0.161$, $P = 0.295$). These results indicated that the concentration of FCM was not affected by the number of fecal pellets.

Discussion

Among small experimental rodents, Syrian hamsters are a prime model for investigating the behavioral mechanisms and stress-related responses in the laboratory and in the field (7, 9, 15, 32). The

experience of social defeat, a stressful experience with higher ecological and ethological validity compared to other stressors used in the laboratory, has been applied to hamsters to investigate behavioral, pharmacological, hormonal and neurobiological mechanisms of social conflict (10, 13) as well as social learning and memory (16). In this study, the measurement of the number of fecal pellets together with the quantification of FCM levels offers several advantages over more invasive methods to measure levels of cortisol metabolites as indexes for stress responses, and such non-invasive techniques appear to be useful in detecting stress levels in male hamsters. Our findings indicate that the measurement of FCM levels is more sensitive than the measurement of fecal pellets to reveal stressful responses in defeated hamsters and there is no correlation between these two measurements. In order to achieve correct results, a careful consideration of various confounding factors and a controlled application of this FCM method are necessary (22). On the one hand, it is better to collect a fecal sample shortly after defecation to avoid contamination with urine or degradation of FCM, because fecal steroids are not stable and undergo further metabolism by bacterial enzymes after defecation (20, 22). On the other hand, a relatively prolonged interval between collections (*e.g.*, > 3-h fecal collection periods) can be beneficial, because it reduces unwanted disturbance to the living conditions and behavioral responses of the animal, which is highly favorable for studying stress responses and physical reactions in learning and memory. Therefore, the advantages and drawbacks of different collection intervals should be taken into consideration before any fecal collection process is performed. A relatively longer habituation period before the actual experiment is advisable to avoid confounding effects of sampling stress.

In hamsters, FCM levels have been used as a quantitative index of activity of the HPA axis in field and laboratory studies (2, 6). In the present study, we collected fecal pellets every 3 h and conducted an EIA to monitor the alterations of FCM in male Syrian hamsters. Both the number of fecal pellets and FCM levels varied over the whole day. Such findings indicate biological validation as measuring whether the naturally occurring diurnal variation of glucocorticoid metabolites in a given species can indicate biological relevance as suggested previously (28). Although we found no significant differences between the winner group and the loser group in the number of fecal pellets after the fight, the mean number of fecal pellets during the post-fight phase was significantly increased in both groups compared to the number during their pre-fight phase. Such elevation indicates the experience of fighting significantly affected the behavioral responses and frequency of defecation in both winners and losers, which is in line with previous studies that the number of fecal pellets reflects the degree of stress (1, 21, 24). Nevertheless, our results suggest that the measurement of fecal pellets might not be as sensitive as the measurement of FCM level to distinguish the stress levels between losers and winners after a fighting interaction.

During the post-fight phase of the present study, losers displayed significantly elevated FCM levels as compared with those males in the winner group and with their baseline FCM levels during the pre-fight phase. It has been reported that acute and chronic social defeat immdiately increases plasma cortisol levels in male Syrian hamsters (12, 13). In female hamsters, it was also found that the mean weights of 24-h fecal outputs was significantly decreased one day after ovariectomy but the mean 24-h production of FCM was significantly increased two and four days after the surgery (2). Regardless of differences in sex, stressor and experimental procedures, our findings in male hamsters are similar with these studies and further provide temporal alterations of FCM levels and number of fecal pellets after experiencing a defeated stress. Our findings not only indicate that measurement of FCM is a useful method to assess stress level after a social defeat but also provide a biological validation of this method in hamsters.

Interestingly, by using this temporal fecal collection method, our results revealed that significantly elevated FCM levels in the loser group, as compared with the winner group, were detected especially during the first and seventh 3-h fecal collection periods after the fight. Using blood sampling immediately after experiencing an acute defeat or chronic social defeats, it was reported that submissive, but not dominant, male hamsters exhibited elevated plasma levels of adrenocorticotropin (ACTH) and cortisol (12). The results of previous studies also revealed that maximum levels of radioactivity in the feces of mice due to ^{3}H-labeled corticosterone occurred during the dark and light cycles at approximately 4 and 10 h, respectively, after intraperitoneal injection (29) and that, in male hamsters, plasma cortisol levels during the light cycle were increased significantly 2.5 h after the first intramuscular injection of 1 IU ACTH (17). Given the fact that the respiratory and heart rates of male hamsters are relatively slower than those of male mice (approximately 74 breaths/min and 286-400 beats/min in hamsters *vs*. 100 breaths/min and 300-600 beats/min in mice; data from the National Laboratory Animal Center of Taiwan), the second peak, *i.e.*, 21 h after the fight, is more likely to represent a direct and event-specific consequence of the fight that was caused by the activation of HPA axis in male hamsters. This finding seems to be in line with a

previous study that female hamsters recovered from ovariectomy and anesthetization displayed elevated daily production of FCM level two days after the surgery (2). Additional physiological or pharmacological validation, *e.g*. ACTH challenge, measuring circulating cortisol or measuring the time delay between FCM excretion and circulating cortisol, could be helpful to serve as relevant proofs. But these additional measurements do not directly correspond to FCM changes induced by a defeated stress. Future studies are needed to further elucidate these speculations.

Taken together, the current study demonstrates the biological relevance of FCM levels with respect to stress by repeatedly and frequently monitoring the alterations of FCM levels before and after experiencing a single fighting interaction. The measurement of the number of fecal pellets indicated time-specific increases in both groups after the fight and the measurements of FCM levels further revealed time-specific elevations of FCM concentrations in the loser group after a stressful defeat. Such a non-invasive technique offers several advantages with minimal disturbance or stress to the animals. It provides new opportunities to monitor long-term physical and hormonal responses to stress in hamsters and other small animals in a more ethical and humanitarian manner.

Acknowledgments

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