

**Patterns of behaviour, group structure and reproductive status predict levels of glucocorticoid metabolites in zoo-housed ring-tailed lemurs, *Lemur catta***

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## ABSTRACT

In ring-tailed lemurs, *Lemur catta*, the factors modulating hypothalamic-pituitary adrenal (HPA) activity differ between wild and semi-free-ranging populations. Here we assess factors modulating HPA activity in ring-tailed lemurs housed in a third environment: the zoo. First we validate an enzyme-immunoassay to quantify levels of glucocorticoid (GC) metabolites in the faeces of *L. catta*. We determine the nature of the female-female dominance hierarchies within each group by computing David's scores and examining these in relation to faecal GC (fGC). Relationships between female age and fGC are assessed to evaluate potential age related confounds. The associations between fGC, numbers of males in a group and reproductive status are explored. Finally, we investigate the value of seven behaviours in predicting levels of fGC. The study revealed stable linear dominance hierarchies in females within each group. The number of males in a social group together with reproductive status, but not age, influenced fGC. The seven behavioural variables accounted for 68% of the variance in fGC. The amount of time an animal spent locomoting and in the inside enclosure, were both negative predictors of fGC. The study highlights the flexibility and adaptability of the HPA system in ring-tailed lemurs.

Keywords: Dominance, social rank, glucocorticoids, *Lemur catta*, zoo, non-invasive

## INTRODUCTION

The social environment in which an individual resides has a major impact on the function of the hypothalamic-pituitary-adrenal (HPA) axis across numerous taxa including primates (Cavigelli and Caruso, 2015). The HPA axis is activated in response to negative or positive challenges in the environment (Sapolsky, 2002). Stimulation of the HPA axis causes a cascade of physiological events culminating in the release of glucocorticoids (GC) into the blood which in turn trigger adjustments to enable the animal to cope with the event. For individuals residing in a group, both baseline and reactive HPA function will be dependent on their position in the social hierarchy, since this will determine an array of factors such as access to resources (Foerster et al., 2011), availability of social support (Abbott et al., 2003) and amount of aggression or threats received (Setchell et al., 2010).

Several studies have explored the interaction between the activity of the HPA axis and social rank in wild (e.g., Emery Thompson et al., 2010), semi-free-ranging (e.g., Starling et al., 2010) and laboratory-housed non-human primates (e.g., Tung et al., 2012). As to be expected in light of species-differences in life history parameters, the associations differ across species and environment (see reviews by Abbott et al., 2003; Cavigelli and Caruso, 2015; Creel, 2001; Goyman and Wingfield, 2004). In some species, subordinate individuals experience the highest levels of activity in the HPA axis (e.g., female *Cercopithecus mitis albogularis*, Foerster and Monfort, 2010), whereas in other species dominant individuals have heightened activity in the HPA axis (e.g., male *Propithecus verreaux*, Fichtel et al., 2007). Other species show no relationship between rank and activity in the HPA axis (e.g., female *Cebus capucinus*, Carnegie et al., 2011). A mixture of the above patterns was observed in a wild population of male *Papio cynocephalus*: an inverse relationship was observed between social rank and cortisol in

males, with the exception of the highest ranking males who had significantly elevated cortisol levels (Gesquiere et al., 2011). These studies generate a complex picture of factors influencing activity in the HPA axis in social animals and suggest that although dominance status can play a role in the function of the HPA axis, the extent of the relationship is dependent on other factors such as experimental setting (Calisi and Bentley, 2009), sex (Cavigelli, 1999; Gould et al., 2005), season (Ostner et al., 2008), reproductive status (Starling et al., 2010), food availability (Cavigelli, 1999), age (Emery Thompson et al., 2010), group stability (Setchell et al., 2010) and even study design (Edwards et al., 2013).

Wild *L. catta* reside in multi-male multi-female groups of 10-20 individuals, in which females are dominant over males (Kappeler, 1993; Sauther et al., 1999). Females exhibit philopatry and males emigrate. Female (and male) social rank is not inherited via the matriline and instead aggressively acquired (Kappeler, 1990; Sauther et al., 1999). The link between GC levels and social status in female *L. catta* has been studied in the wild and under semi-free-ranging provisioned conditions (Cavigelli, 1999; Cavigelli et al., 2003; Pride, 2005; Starling et al., 2010). As in other species, this association is modified by a host of social and environmental factors. In wild *L. catta*, factors linked to GC output varied across study groups and included access to food and amount of food consumed, amount of aggression initiated (for dominant females) or received (for subordinate females), reproductive state, proximity to a group member, predation risk, presence versus absence of an infant and the extent of inter-group encounters (Cavigelli, 1999; Cavigelli et al., 2003; Pride, 2005). Similarly, in semi-free-ranging groups, inter-group differences in the factors modulating GC levels in females were found, and included reproductive status and group structure (Starling et al., 2010).

The goal of the current study is to assess the factors that influence the HPA axis in female *L. catta* residing in social groups in a third environment, the zoo - with particular focus on the effects of social rank. Zoos are characterized by the regular presence of large numbers of unfamiliar humans, restricted space and a highly managed environment which excludes important experiences shown to modulate HPA function in animals in the wild such as food availability and predator threat (Hosey, 2005). In this way, zoos differ from the other two environments in which the link between social rank and HPA function has been studied in *L. catta*. Comparing our data to that of studies conducted with *L. catta* in the wild and semi-natural habitats can contribute to our understanding of factors influencing the HPA axis in this species (Calisi and Bentley, 2009). Furthermore, the study has important welfare implications for captive ring-tailed lemurs in light of the established link between HPA functioning and health in primates (Sapolsky, 2005).

For this study, we first immunologically and biologically validate an enzyme-immunoassay (EIA) to quantify levels of fGC metabolites excreted by zoo-housed *L. catta*. Immunological validation assesses specificity, accuracy, precision and sensitivity of the assay (Diamandus and Christopoulos, 1996). Biological validation examines the extent to which our biochemical techniques were able to detect biologically meaningful changes in cortisol excretion. All mammals studied to date exhibit a circadian rhythm in cortisol excretion, with elevated levels of cortisol observed upon waking reflecting heightened activity in the HPA axis at this time (Coe and Levine, 1995). If our methods do accurately reflect levels of activity in the HPA axis and concomitant release of GCs our assay would detect higher levels of fGC excreted by our subjects in the morning compared to the afternoon period.

Our study tested six hypotheses as detailed below:

**Hypothesis one (H1):** Social rank will impact fGC levels in female *L. catta*.

Female dominance hierarchies were examined by recording the distribution of aggressive and submissive behavioural interactions in our established, zoo-based groups. Since our groups are organized into stable linear dominance hierarchies of a despotic nature, similar to those observed in wild and semi-free-ranging populations (Cavigelli, 1999; Starling et al., 2010), we predict (P1) high fGC levels in dominant animals compared to subordinate animals reflecting the physiological demands on dominant females of maintaining high rank via physical aggression (i.e. the stress of domination hypothesis; Sapolsky, 2005).

**Hypothesis two (H2):** There will be a relationship between age and levels of fGC in our subjects.

Cortisol titres change across age in some primate species potentially confounding experimental results evaluating HPA function (Ebner et al., 2015). We predict (P2) a positive correlation between cortisol levels and age as shown in other primate species (e.g. *Homo sapiens*, Nater et al., 2013).

**Hypothesis three (H3):** The number of males in a social group will affect fGC levels.

In both captive and wild groups of ring-tailed lemurs (Pride 2005; Starling et al., 2010) high numbers of males in a group are positively associated with increased GC levels in both males and females. We therefore predict (P5) higher cortisol levels in females in Zoo B as this group contains five adult males compared to the other zoos which contains zero, two or three adult males (zoo D, both zoos A and C, and zoo E respectively).

**Hypothesis four (H4):** Reproductive status will modify fGC levels.

In light of the positive association between reproductive steroids and GC demonstrated in many primate species reproductively active females in our study are

predicted (P4) to have higher fGC levels than non-reproductively active females (Bales et al., 2005; Carnegie et al., 2011; Smith and French 1997; Starling et al., 2010; Weingrill et al., 2004).

**Hypothesis five (H5):** Behaviour will have a significant impact on HPA function in *L. catta* females residing in a zoo.

The behavioural correlates of dominance, such as the amount of aggression given, can be more accurate and biologically relevant predictors of position in the hierarchy than rank *per se* in primates (Edwards et al., 2013; Ray and Sapolsky, 1992; Virgin and Sapolsky, 1997). Since dominance status is aggressively acquired in lemurs (Kappeler, 1990), we predict (P5a) that the amount of aggression given and or received (P5b) will relate positively to fGC titres as shown for other primate species (e.g., Cavigelli et al., 2003; Arlet et al., 2009). Relationships between social rank and GC levels can be modulated by the amount of social support available (Abbott et al., 2003). As *L. catta* are highly social animals, we predict (P5c) the amount of grooming given by our captive subjects will be negatively linked to fGC levels and time spent alone (P5d) to be positively linked to fGC levels as shown previously for wild *L. catta*, as well as other primate species (Foerster et al. 2011; Kappeler 1990; Sonnweber et al., 2015). Indices pertinent to life in captivity (e.g., the amount of time animals spend in different parts of the enclosure such as indoors versus outdoors) influence GC levels in primates and are thus also included as a variable in our analyses (e.g., Amrein et al., 2014). We predict (P5e) cortisol levels to be lower the more time subjects spent indoors due to increased opportunities for close contact behaviours shown previously to reduce cortisol levels such as grooming (Sonnweber et al., 2015). The amount of time subjects spend eating is included as a predictor variable in the analysis since this behaviour is linked to cortisol output in an array of species, including ring tailed lemurs (Cavigelli, 1999; Emery

Thompson et al., 2010; Pride 2005; Weingrill et al., 2004). The ability of *L. catta* to survive in diverse ecological habitats has been attributed to their ‘intelligent’ feeding behaviour emphasising the significance of this behaviour for the species (Gould et al., 2015). A positive correlation between time spent feeding and fGC levels is predicted (P5f) as a reflection of increased feeding competition. Finally, we predict (P5g) a positive relationship between levels of activity and fGC titres reflecting the physiological demands of locomotion (Smith et al. 1998).

## **METHODS**

### **Subjects**

Subjects were 25 captive adult female *L. catta* (aged between 2 and 23 years at the start of the study; see Ichino et al., 2015) housed in five well-established groups, kept in five different zoological institutions in the United Kingdom, referred to as zoos A, B, C, D and E to maintain anonymity (Table 1). The group structure of all groups was similar to that found in the wild (Jolly et al., 2002). All zoos were licensed under the amended Zoo Licensing Act, 1981 [(Amendment England and Wales) Regulations 2002 and Zoo Licensing Regulations (Northern Ireland) 2003] regulated in England by Department for Environment, Food and Rural Affairs and in Northern Ireland by the Department of the Environment. The research complies with the legal and ethical requirements as per the host institutions (i.e. University of Chester and Queens University of Belfast). Only group members older than 22 months were used as focal animals since this is the age at which reproduction is possible in a provisioned group (Parga and Lessnau, 2005). All data were collected by one researcher. The zoos were studied in succession in alphabetical order between April 2003 and October 2004.



## **Husbandry**

All subjects had access to a heated indoor enclosure (20 – 22<sup>0</sup>C) with shelves for resting (plus ropes in zoos B, C and D) and an outdoor enclosure containing climbing and resting apparatus e.g., trees, ropes, wooden planks, shelves and structural beams. All subjects had natural dirt and grass flooring. In particular we note (a) subjects at zoo A were free-ranging, with the public, in the zoological gardens (9.7 hectares) and (b) subjects at zoo B resided on an island they shared with one female black and white ruffed lemur (*Varecia variegata*). Interactions between the two species occurred occasionally, but they generally occupied different areas of the island.

All subjects received a varied diet (fruit, vegetables, Milupa baby food, primate pellets and egg, cheese and meal worms periodically) twice daily (once in the morning and once in the afternoon), water *ad libitum* and received periodic feeding enrichment. Enclosures were cleaned twice daily after the faecal samples had been removed. Indoor areas were washed, disinfected and bedding was replaced weekly, where appropriate.

## **Reproductive status**

The extent to which the zoo-housed subjects exhibited regular breeding periods (as is evident in the wild and the semi-free-ranging subjects studied to date) was unknown. Females were classified as either reproductive or non-reproductive at the time of study. Reproductive females were pregnant, gave birth, or lactated during the study period (N = 13), whereas non-reproductive females did not show any of these signs (N = 12).

## **Collection of behaviour data**

Focal animal observations (767 hours) were made on the 25 female subjects. Behavioural data were collected from all institutions but zoo B during five days a week over a minimum period of five weeks. Observations were equally distributed among three time slots, defined as morning (08:00 – 11:00 hours), midday (11:01 – 14:00 hours) and afternoon (14:01 – 17:00 hours). At Zoo B, data were collected two to three times a week over a six-week period. Data were not collected within 30 minutes of feeding from any institution.

Behaviour data were collected using five-minute focal animal samples with a sample period of 20 seconds. For each focal observation, instantaneous and one-zero behaviour scores were corrected for time spent 'out of sight'. The latter were used to compute a mean behaviour score for each behaviour for each subject encompassing their whole study period.

## **Behaviour definitions**

Behaviour definitions were taken from Cavigelli (1999), Cavigelli et al. (2003), Jolly (1966), and Pereira and Kappeler (1997). 'Initiating aggression' was recorded when an animal elicited a submissive action from another group member by directing one or several of the following behaviour(s): stare, approach, touch, charge, chase, lunge, bite, nip or cuff (Pereira and Kappeler, 1997). A subject was scored as having 'received aggression' when it performed the following submissive actions in response to a group member: look away, turn away, spat vocalization, crouch, depart, flee or jump away (Pereira and Kappeler, 1997). We recorded 'grooming with others' when the subject licked or tooth combed another animal or received licking or tooth-combing. 'Inside' was recorded when an animal was in the indoor area of the enclosure and 'eat'

constituted licking, chewing or swallowing. An animal was defined as 'alone' when it was over 5m from the nearest group member. 'Locomotion' defined an animal changing location using two or four limbs and included walking, running and jumping and when an animal could not be seen by the observer it was defined as 'out of sight'. All behaviours were expressed as rate per five minutes and correct for time spent 'out of sight'.

### **Determination of the dominance hierarchy**

In each institution, the nature of female dominance status was explored for the social group. Female-female aggressive and submissive interactions were recorded. When an aggressive or submissive interaction was observed, focal animal sampling was suspended for the duration of and for two minutes following the interaction. The aim was to record the frequency of aggressive and submissive dyadic interactions and the participants (per five minute observation). An interaction was recorded when a group member (defined as the winner) elicited a clear submissive response from another group member without themselves displaying any submissive signals (Kappeler, 1993). The loser was the individual that responded to the winner with a clear submissive signal. The winner was assigned a score of one for each successful dyadic win.

### **Rank order analysis**

The dominance matrix was analysed with MatMan (de Vries et al., 1993) and reordered to find an order most consistent with a linear hierarchy by the I&SI method which minimizes the number of inconsistencies (I) and the strength of inconsistencies (SI) (de Vries, 1998). The index of linearity  $h$  (Appleby, 1983) was calculated and tested against the null hypothesis that linear ranking occurred by chance by means of a randomisation

test in MatMan (de Vries, 1995). Since there were tied or unknown relationships, the improved index of linearity (h') rather than Landau's index was calculated and tested by means of a randomization test with the aid of MatMan (Appleby, 1983; de Vries, 1995). The directional consistency index (DC) gives the frequency with which the behaviour occurred in its more frequent direction relative to the total number of times the behaviour occurred (Van Hooff and Wensing, 1987). The total number of times the behaviour occurred in the direction of the higher frequency (H) minus the number of times in the less frequent direction (L) is divided by the total frequency:  $DC = (H - L) / (H + L)$ . As another descriptive measure, the number of one-way relationships was counted, i.e. the number of dyads in which the behaviour was shown in one direction only, irrespective of the frequency of interaction within the dyads. In two-way relationships, dyadic dominance interactions occurred at least once in both directions (from A to B and from B to A).

We calculated David's scores for each individual female (David, 1987; Gammell et al., 2003), based on the dyadic dominance index (DDI) (de Vries, 1998) as an additional measure of dominance that takes the relative strength of the opponents into account. By normalising these David's scores, they range from 0 to  $n-1$ , in which  $n$  is the number of animals. These Normalised David's scores, based on the dyadic dominance index (called NDS-DDI values) can then be linearly regressed on the rank numbers of the individuals to calculate the steepness of a hierarchy (de Vries et al., 2006). The slope of this linear regression varies from zero to one, in which zero is considered indicative of an egalitarian dominance hierarchy, with small rank distances, while a slope of one indicates a strictly despotic dominance hierarchy with large rank differences (van Schaik, 1989).

### **Faecal sample collection and extraction**

Fresh faecal samples were collected from all subjects opportunistically between 08:00 – 11:00 h to control for circadian variation. The animals were not disturbed by the sample collection. Samples collected from subjects at zoos A, B and E were frozen at  $-20^{\circ}\text{C}$  within 15 minutes of deposition. Samples from subjects at zoos C and D were collected and frozen up to 7.5 hours following deposition. We have shown previously that fGC levels do not differ between portions of a sample collected and frozen immediately versus portions of the same sample left at ambient temperature for up to eight hours before freezing (McCusker, 2006). A mean  $\pm$  S.E.M. of  $6.1 \pm 0.25$  faecal samples per subject contributed to the analyses (Table 1).

A 2 g portion of each sample was dried in a fan assisted oven at  $55^{\circ}\text{C}$  for 7.5 hours and refrozen at  $-20^{\circ}\text{C}$  until needed for further extraction. Following thawing, each dry sample was ground with a pestle and sifted through a fine wire mesh to remove seeds and fibrous material (Wasser et al., 1993). A 0.2 g portion of the resulting powder was mixed vigorously with 3 ml of 99.8% ethanol for three hours [Heidolph Titramax 100, 1350 rpm, 1.5 mm orbit] and then centrifuged at 2000 rpm for 15 minutes following an adapted version of the methods used by Wasser et al. (1993). The supernatant was poured into a glass test tube and the ethanol evaporated using compressed oxygen free nitrogen gas ( $\text{N}_2$ ) administered using a Pierce Reacti-Therm Heating Module at  $40^{\circ}\text{C}$ . The residue was resuspended in 1ml Phosphate Buffer Saline buffer and the resulting solution micro-centrifuged for two minutes at 6500 rpm to remove any remaining solid particles. The supernatant was removed and stored at  $-20^{\circ}\text{C}$  until needed for measurement.

### **Cortisol Enzyme-immunoassay**

### **Immunological validation**

A modified version of an enzyme-immunoassay EIA described by Smith and French (1997) was used to quantify levels of fGC. The assay was immunologically validated for quantification of fGC levels in captive *L. catta* using a representative sample pool collected from adult males (n=8) and females (n=9) residing in three institutions (Diamandus and Christopoulos, 1996). The antibody [R4866, raised against a steroid bovine albumin in rabbit (Munro and Stabenfeldt, 1985)] was diluted to 1:12,000 in coating buffer and the cortisol horseradish peroxidase was diluted to 1:22,000 in phosphate buffer solution.

Cross reactivity of the cortisol antibody was 100 % with cortisol. Cross reactivity with similar steroids was 9.9 % with prednisolone, 6.3 % with prednisone, 5.0 % with cortisone, 0.7 % with corticosterone and < 0.3 % with various other steroids (Munro and Stabenfeldt, 1985). Linear regressions of the displacement curves of serial dilutions of cortisol standard and the pool mixed of the mentioned faecal samples above over the 10 – 90 % binding range did not differ significantly inferring parallelism and assay specificity [ANCOVA;  $F(3,29) = 0.3$ , NS]. Recovery of the standards (halving dilutions in the range 500 – 3 pg) added to a 1:4 dilution of a mixed faecal pool was  $95.16 \pm 8.74$  % inferring good accuracy ( $r=0.990$ ,  $P<0.0001$ ,  $y=10.442x+1.124$ ,  $N=13$ ). Intra-assay coefficients of variation for low and high concentration quality controls were 4.6 % and 1.0 %, respectively ( $N=29$  plates). Inter-assay coefficients of variation for low and high concentration quality controls were 24.6 % and 23.4 %, respectively ( $N = 29$  plates). Sensitivity of the assay was approximately 7.8 pg/ml.

### **Extraction efficiency for fGC**

To assess the efficiency of the extraction procedure, ten dry faecal samples (a mix of male and female samples from zoo A; 0.2 g each) and four control tubes were spiked in duplicate with 600  $\mu$ l of tritiated cortisol (approximately 3000 cpm; Amersham, Buckinghamshire, UK) and extracted as described above. Scintillation cocktail (4 ml, Optiphase, 'hi-safe 2', PerkinElmer, Wellesley, Massachusetts, USA) was added to the samples, and the controls and counts per minute assessed to compute the percentage of procedural loss during extraction. Extraction efficiency was 74.4 %.

### **Biological validation**

To determine whether or not our assay detected biologically meaningful circadian variation in fGC levels we used a repeated measures design to assess levels of fGC levels as excreted across the day in six females from three institutions (Table 1). Samples were collected during three time slots across the day over a two week period, and levels of fGC were quantified (08:00-11:00 hours, 11:01-14:00 hours and 14:01-17:00 hours). A mean GC level was obtained for each female from each time slot and entered in a one factor repeated measures ANOVA. A mean number  $\pm$  S.E.M. of  $2.33 \pm 0.21$ ,  $2.67 \pm 0.91$  and  $2.17 \pm 0.48$  samples from each female were collected in each time slot.

### **Statistics**

All fGC levels were log-transformed to normalize the data. Proportional behavioural data were square root arcsine transformed (Sokal and Rohlf, 1995). Planned post hoc comparisons were conducted using the Tukey honestly significant difference test. Alpha was set at 0.05. To avoid pseudoreplication, we computed a mean fGC level for each female for use in the analyses using each female's repeated fGC measures.

Potential circadian variation in fGC levels (computed in this study to assess biological validity) was assessed using a one factor repeated measures ANOVA. Potential relationships between social rank (as reflected in David's scores) and transformed fGC level were examined using Spearman rank correlation (H1). Pearson product moment correlation was used to explore any link between female age and transformed fGC levels (H2). A two-factor ANOVA was used to assess the impact of number of adult males in a group (H3) and reproductive status (reproductively active or non-reproductive, H4) on fGC levels. As with previous similar studies in the field, we conducted a standard multiple regression (Tabachnick and Fidell, 2001) to explore behavioural regulation of GC excretion in female captive ring-tailed lemurs (H5). A total of seven behaviours were chosen as independent predictor variables, and individual log transformed mean fGC level was the dependent variable. Standard regression was used due to the low case-to-IV ratio. Predictor variables included: aggressive attacks initiated, aggressive attacks received, grooming with other group members, amount of time spent alone, time spent in the indoor enclosure and eating together with rate of locomotion. Data were analysed using IBM SPSS Statistics 21.

## **RESULTS**

### **Assay Validation: Circadian variation in fGC level**

fGC levels were highest between 08:00-11:00 hours and then decreased significantly across the two remaining time blocks in the day [ANOVA:  $F(2, 10) = 4.3$ ,  $P < 0.05$ ] (Figure 1). The observed circadian variation in levels of fGC measured by our assay confirms the biological validity of our techniques.

### **Dominance analyses**



In three of the five groups, the dominance hierarchy was highly linear. The directional consistency index was high to very high in all groups, suggesting stable dominance relationships. In most groups there were but a few unknown relationships, and dominance interactions mainly occurred in one-way (Table 2). Steepness was high in groups from zoos A, D, E, reflecting despotic groups. Steepness was lower in groups B and C, suggesting that these groups harboured relatively egalitarian relationships (although with indices of linearity of 0.70 and 0.77 respectively groups B and C probably tended more towards despotic than egalitarian). Groups B and C were the only two groups with unknown relationships and in the case of group B, a very low number of observations upon which to construct a David's Score. Additional data on groups B and C is probably needed therefore in order to accurately classify the patterning of dominance within the groups. Contrary to our prediction (P1), there was no significant relationship between social rank and cortisol levels in any groups although there was a non-significant trend towards increasing cortisol with higher social rank (H1, Figure 2 and Table 3).

### **The relationship between age, housing institution and reproductive status and individual fGC levels**

There was no significant correlation between fGC levels and female age (Pearson product moment correlation:  $r = 0.329$ ,  $N = 25$ , NS, H2). The number of males in a group had a significant impact on fGC levels [ $F(3, 24) = 32.926$ ,  $P < 0.000$ ; Figure 3, H3]. Females residing in groups with five males had significantly elevated levels of fGC compared to females residing in zoos with less than five males ( $P < 0.01$ ). fGC levels were significantly higher in reproductively active compared to non-reproductively active females [ANOVA:  $F(1, 24) = 5.328$ ,  $P < 0.03$ ; Figure 4, H4]. There was no

interaction between number of males in a group and reproductive status [ $F(1, 24) = 0.501$ , NS].

A total of 68% of variance in fGC levels was accounted for by the seven predictor variables: [standard multiple regression:  $F(7, 24) = 5.205$ ,  $r^2=0.682$ ,  $P < 0.003$ , Table 4, H5]. The amount of time an animal was in the inside enclosure and spent locomoting were both negative contributors to the model (Table 4).

## **DISCUSSION**

The current study revealed that activity in the HPA axis of zoo-housed female *L. catta* was regulated by the number of males in a group and reproductive status, but probably not social rank. Behavioural parameters also predicted a significant amount of variance in fGC levels. The amount of time subjects spent indoors was a significant negative predictor of fGC levels, as was the amount of time spent locomoting.

Our study reveals that HPA activity in our subjects was modulated by factors both similar and disparate to those shown to regulate HPA activity in conspecifics in previous research residing in different environments. These differences may in part be due to differences in the relative costs of social status and availability of coping strategies in our captive subjects compared to wild and or semi-free-ranging subjects. Differences may also be due to experimental design and sampling methods (Edwards et al., 2013). For example, our study used average fGC levels in the analyses which manifest different associations with social and environmental factors than temporally matched samples (Edwards et al., 2013).

### **Determinants of fGC levels in captive ring-tailed lemurs**

Females residing in the group with five adult males (i.e. zoo B) had significantly higher cortisol levels than females residing in groups with zero, two or three adult males as predicted. The number of males in a group has been linked previously to cortisol output in adults of both sexes in captive and wild groups of ring-tailed lemurs (Pride 2005; Starling et al., 2010). Since female *L. catta* are dominant over males, one might not expect the numbers of males in the group to have any impact on female physiology in this species. It could be argued however that the excessive mating attempts from a large number of males together with the indirect corollaries of male intra-sexual competition may have elevated HPA function in females in zoo B as suggested for other primate species. High levels of male-male competition activated the female HPA axis in wild *Cebus capucinus* (Carnegie et al., 2011). Furthermore, the strongest predictor of female cortisol levels in a wild group of *Pan troglodytes* was the amount of aggression they received from males (Emery Thompson et al., 2010). One cannot rule out however the impact of additional factors unique to the group in zoo B, on fGC levels, such as enclosure design and husbandry procedures (which we were not able to examine in the current study). These factors have been shown previously to influence cortisol release in captive animals (Amrein et al., 2014; Fontani et al., 2014; Pirovino et al., 2011; Smith and French, 1997; Zaragoza et al., 2011).

High cortisol levels in zoo B may also be explained by the observation that all females in the group were reproductively active at the time of study – a physiological state associated with high cortisol in wild and semi-free-ranging groups of *L. catta* (Cavigelli, 1999; Starling et al., 2010), as also shown in our captive females and several other primate species (e.g., *Leontopithecus rosalia*, Bales et al., 2005; *Cebus capucinus*, Carnegie et al., 2011; *Callithrix kuhli*, Smith and French 1997; *Papio ursinus*, Weingrill et al., 2004). The high fGC levels observed in reproductively active females in our

study were not surprising in light of the established interaction between the HPA axis and hypothalamic-pituitary gonadal system (Rivier and Rivest, 1991) driven by the varying physiological demands across the ovarian and pregnancy cycles. Due to logistical reasons the current study was unable to make fine distinctions across the female reproductive cycle (see also Cavigelli, 1999) and as such was unable to explore relationships between HPA function and significant reproductive phases such as oestrus. The presence of oestrus females in a group can have a significant modulatory effect on the HPA axis of both the females themselves (e.g. *Homo sapiens*, Wolfram et al., 2011) and resident males (e.g. *Pan paniscus*, Surbeck et al., 2012) and therefore would have been an interesting component to the current study had it been possible. The demographics of our study groups did not allow us to adequately separate the effect of reproductive status from number of males in the group (and potentially other factors unique to zoo B) – an important research goal for future studies.

### **Comparable fGC levels in females of different social rank**

There was no significant relationship between fGC levels and David's score, i.e. social rank, in any group as shown previously for females of other primate species (e.g., *Macaca fuscata*, Takeshita et al., 2014) and some wild and free-ranging groups of *L. catta* (Pride, 2005; Starling et al., 2010). This was surprising in light of the highly linear, stable hierarchies, identified in our captive groups. The result would suggest that maintaining high social rank in our subjects was not in itself stressful as we had predicted based on the 'stress of domination' hypothesis (Creel, 2001). Equally, being the target of aggression in a confined space with limited opportunities for escape was also not stressful in the groups. Females in despotic groups of *Macaca mulatta* similarly showed comparable cortisol values (Qin et al., 2013). In contrast to our results, Cavigelli (1999) and Cavigelli et al.

(2003) report high GC output in dominant and middle rank *L. catta* females compared to low rank females (wild and semi-free-ranging provisioned groups). These divergent results illustrate the sensitivity of the lemur HPA axis to social and environmental factors. The comparable fGC levels across females of all social ranks in our captive subjects suggests that the social and or environmental stressors causing high cortisol concentration in some dominant females of this species in the wild and semi-free-ranging groups (such as feeding competition or predation risk) are either absent or do not have a significant impact on our captive subjects. The HPA axis of several captive non-primate species has been shown to behave differently to that of conspecifics in the wild (see Calisi and Bentley, 2009). The current research is the first, to our knowledge, that has compared the behaviour of the primate HPA axis across different environments within a single species. Differences between the outcome of similar studies conducted in disparate environments such as the current study can elucidate the flexibility of a biological system and how it functions in response to different physical and social constraints

Non-significant trends of increasing fGC with higher social rank were observed in three of the current groups, which is similar to the results of Cavigelli (1999) and Cavigelli et al. (2003). Future research incorporating more zoo-based groups and a longer sampling period during which behaviour and cortisol data are more finely linked may help to clarify the factors modulating HPA function in captive *L. catta*. It has been speculated recently (Cavigelli and Caruso, 2015) that the temporal patterning of maximum and minimum GC values across individuals may be important for understanding how the HPA axis functions and thus should be considered for future studies of this kind.

In some species, rank related patterns of GC excretion in females are only observed under a particular set of conditions such as during times of limited food

resources (*L. catta*, Cavigelli, 1999), the mating season (e.g., *Brachyteles arachnoides hypoxanthus*, Strier et al., 2003), pregnancy (semi-free-ranging provisioned *L. catta*, Starling et al., 2010) or lactation (*Cercopithecus mitis*, Foerster et al., 2011). Studies of wild and semi-free-ranging ring-tailed lemurs suggest that rank related patterns of GC output (i.e. high cortisol in dominant animals) may indeed be linked to food availability. One study on wild ring-tailed lemurs identified daily food intake as a major predictor of cortisol levels in all groups studied (Pride, 2005) whereas another field study identified feeding effort as a predictor of cortisol for a group of ring-tailed lemurs residing in a dry, harsh forest area but not for a second group living in a lush environment presumably with abundant food (Cavigelli, 1999). Since lemurs have a high reliance on obtaining food from the environment as opposed to utilising stored fat resources (Gould et al., 2011), one would predict high levels of feeding competition in this species during periods of low food availability. Food provisioning in our captive subjects may ameliorate feeding competition and diminish associated rank related differences in HPA axis activation (although see Altmann and Muruthi, 1988).

Social instability has a potent impact on rank related differences in cortisol excretion in males of some primate species e.g., *Papio hamadryas ursinus* (Bergman et al., 2005), *Papio h. anubis* (Sapolsky, 1983) and *Mandrillus sphinx* (Setchell et al., 2010). Instability in female social dynamics may similarly impact female rank related patterns in GC output. For example, one would predict female intra-sexual competition and social instability to have different physiological consequences for females of different social rank due to their differing priorities, dissimilar access to social support and varying amounts of aggressive challenges experienced. The apparently stable dominance hierarchies evident in our social groups may partially explain the comparable cortisol titres across differently ranked females.

### **Behavioural predictors of fGC levels**

The strongest behavioural predictor of cortisol levels was the amount of time subjects spent inside. As predicted, we found a negative relationship between cortisol levels and time spent indoors. It could be speculated that by spending time indoors, the zoo-housed ring-tailed lemurs were able to reduce the physiological effort required to maintain homeostasis in cold conditions. Free-ranging and zoo-housed primates have high cortisol levels in the cold season compared to the warmer seasons (Beehner and McCann, 2008; Takeshita et al., 2014), which probably reflects the augmented physiological stress required to sustain homeostasis (Follenius et al., 1982). However since the study used data collected across both winter and summer months a more parsimonious explanation could be that when indoors, subjects would probably have had increased opportunities to engage in close contact behaviour such as grooming, which has been shown previously to be associated with low cortisol levels in primates (Foerster et al., 2011; Shutt et al., 2007; Sonnweber et al., 2015). Our results suggest the indoor areas at the zoos were large enough to minimise the negative social interactions (and increased GC levels) typically observed in primates under crowded conditions (Pearson et al., 2015).

Contrary to our prediction, there was a significant, negative relationship between GC levels and the amount of time spent locomoting. From an energy perspective this result is surprising as one might have expected the physiological demands of locomotion to raise cortisol titres as shown for other primate species (e.g., *Callithrix kuhli*, Smith et al., 1998). A link between cortisol and locomotion is not seen in all species e.g., *C. jacchus* and such an association may only manifest itself under certain environmental or social conditions that were not apparent in our study (Galva"O-Coelho et al., 2012). It is

also feasible that high GC levels in some subjects were associated with behaviours not measured in our study, such as auto-grooming that are incompatible with high levels of locomotion. Alternatively, high GC output may have been concomitant with a passive coping style to a stressor as seen in some primate species (Feng et al., 2011; Mahar et al., 2014).

In conclusion, we have shown that the HPA axis of zoo-housed ring-tailed lemurs is modulated by factors both similar to those that modulate HPA axis function in wild and semi-free-ranging conspecifics (e.g., reproductive function and number of males in a group) and distinct from those regulating HPA function of conspecifics (e.g., amount of time spent in an enclosed area and the amount of time spent locomoting). Our results highlight the flexibility and adaptability of this physiological system in ring-tailed lemurs.



## **ACKNOWLEDGEMENTS**

The authors thank the management and staff of the participating zoos i.e. Belfast, Edinburgh, Blackpool, Dublin zoos and one anonymous zoo for their support of the research. We express particular thanks to the animal care staff for their cooperation, help and flexibility. Reagents for the enzyme-immunoassay were provided by Coralie Munro of the University of California. We thank Rob Coleman for his assistance with the biochemical analyses. Valuable comments on the manuscript were provided by Dr Verena Behringer, Dr Karl Hard and two anonymous reviewers. Financial support was provided by the Department for Employment and Learning, Northern Ireland and the University of Chester.

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## **TABLES**

### Table 1

Information on the 25 female subjects. Abbreviations; F = female, M = male, RA = reproductively active, Non-RA = non-reproductively active. \* Contributed faecal samples to the analysis of circadian variation in faecal cortisol levels.

### Table 2

Overview of the dominance hierarchies in each of the study groups, for more information regarding the dominance measures, see text.

### Table 3

Sperman rank correlation between David's score and fGC levels for subjects in each group in the five zoos.

### Table 4

Statistical results from the multivariate linear regression for the seven behaviour variables.

## **FIGURES**

Figure 1. Mean  $\pm$  S.E.M. levels of fGC (ng/g dry weight) excreted by n = 6 female *Lemur catta* housed in three institutions in three time slots across the day. Bars with different letters are significantly different to each other ( $P < 0.05$ ).

Figure 2. Scatter plots detailing the associations between levels of fGC and David's Score for groups in zoos A to E. Increasing David's Score represents increasing social rank.

Figure 3. Mean  $\pm$  S.E.M. levels of fGC (ng/g dry weight) for females residing in groups with zero, two, three or five males.

Figure 4. Mean  $\pm$  S.E.M. levels of fGC (ng/g dry weight) for reproductively active (Reprod.) and non-reproductively active (Non-reprod.) females.