American Journal of Primatology, in press, 2019.

Individual, social and environmental factors affecting salivary and fecal cortisol levels in captive pied tamarins (*Saguinus bicolor*)

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Dedication: In memory of Tine Griede

Running head: Fecal and salivary cortisol in pied tamarins

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Abstract

Pied tamarins (*Saguinus bicolor*) are endangered New World primates, and in captivity appear to be very susceptible to stress. We measured cortisol in 214 saliva samples from 36 tamarins and in 227 fecal samples from 27 tamarins, and investigated the effects of age, sex, pregnancy, rearing history, social status, weight, group composition and enclosure type using generalized linear mixed models. There was no effect of age on either fecal or salivary cortisol levels. Female pied tamarins in late pregnancy had higher fecal cortisol levels than those in early pregnancy, or non-pregnant females, but there was no effect of pregnancy on salivary cortisol. Females had higher salivary cortisol levels than males, but there was no effect of rearing history. However, for fecal cortisol, there was an interaction between sex and rearing history. Hand-reared tamarins overall had higher fecal cortisol levels, but while male parent-reared tamarins had higher levels than females who were parent-reared, the reverse was true for hand-reared individuals. There was a trend towards lower fecal cortisol levels in subordinate individuals, but no effect of status on salivary cortisol. Fecal but not salivary cortisol levels declined with increasing weight. We found little effect of group composition on cortisol levels in either saliva or feces, suggesting that as long as tamarins are housed socially, the nature of the group is of less importance. However, animals in off-show enclosures had higher salivary and fecal cortisol levels than individuals housed on-show. We suggest that large on-show enclosures with permanent access to off-exhibit areas may compensate for the effects of visitor disturbance, and a larger number of tamarins of the same species housed close

together may explain the higher cortisol levels found in tamarins living in off-show accommodation, but further research is needed.

Key words: callitrichid, cortisol, *Saguinus bicolor*, stress, welfare

Research highlights

- Hand-reared pied tamarins have higher fecal cortisol levels than parent-reared tamarins.
- Pied tamarins living off-show with more conspecific groups per building have higher salivary and fecal cortisol levels than tamarins housed on-show in large enclosures with access to off-exhibit areas.

Introduction

Animal welfare science in zoos is a long-established field of study (Powell & Watters, 2017). The highest standards of welfare in zoo-housed animals are essential, particularly given the growing role of captive breeding programs in conservation strategies for threatened species, and as zoos increasingly seek to educate as well as to entertain their visitors (EAZA, 2013; Kagan, Carter, & Allard 2015). Zoos are also challenged regularly about the ethics of keeping animals in captivity (Gross, 2015; Keulartz, 2015), and thus appropriate measures of welfare are needed. Behavioral studies are often used for this purpose (Dawkins, 2004), but measurements of glucocorticoid hormones such as cortisol are increasingly common as a means of assessing wellbeing (Möstl & Palme, 2002; Keay, Singh, Gaunt, & Kaur, 2006; Clark et al., 2012; Hart, 2012).

As well as being influenced by factors such as social and reproductive status (Abbott et al., 2003; Bales, French, Hostetler, & Dietz, 2005; Bales, French, McWilliams, Lake, & Dietz, 2006), cortisol is produced by the hypothalamus–pituitary–adrenal axis (HPA) when an individual is exposed to a stressful situation (Möstl & Palme, 2002; Beehner & Bergman, 2017). Animals in natural environments can usually respond adaptively (either behaviorally and or physiologically) to stressors and ameliorate the stress (Morgan & Tromborg, 2007). Acute stress may therefore not have a negative effect on fitness (Beehner & Bergman, 2017). However, if it is not possible to respond in such a way as to reduce the effect of a stressor, as is often the case in animals living in captivity (Mason, 2010), stress can become chronic and the individual may develop problems such as stereotypic behaviors (e.g. Mason, Clubb, Latham, & Vickery, 2007), poor health (e.g. Munson et al., 2005), excessive weight loss (e.g. Tamashiro, Nguyen, & Sakai, 2005), reduced reproductive success (e.g. Clubb, Rowcliffe, Lee, Mar, Moss, & Mason, 2008), suppressed immunity (Martin, 2009), and impaired cognitive function (Teixeira et al., 2015). A growing number of studies in a variety of mammal species living in zoos has pointed to many factors that can affect cortisol levels, including rearing history, social situation, enclosure size and type, noise or other disturbance (e.g. from zoo visitors), access to outdoor areas, and season (Carlstead, Brown, & Seidensticker, 1993; McCallister, 2005; Carlstead & Brown, 2005; Powell, Carlstead, Tarou, Brown, & Monfort, 2006; Clark et al., 2012; Pirovino et al., 2011; Rajagopal, Archunan, & Sekar, 2011; Cerda-Molina et al., 2012; Kaplan et al., 2012; Shepherdson, Lewis, Carlstead, Bauman, & Perrin, 2013; Schumann, Guenther, Jewgenow, & Trillmich, 2014; Sherwen et al., 2015; Pauling, Lankford, & Jackson, 2017).

Our study evaluates factors that might affect cortisol levels in the pied tamarin (*Saguinus bicolor*), a callitrichid primate that is endemic to the Brazilian Amazonian rain forest near the city of Manaus. Pied tamarins are classed by the IUCN as endangered (Mittermeier, Boubli, Subirá, & Rylands, 2008), and their population continues to decline because of severe habitat loss and fragmentation (Gordo, Calleia, Vasconcelos, Leite, & Ferrari, 2013). Along with in-situ conservation measures, one of the main goals of the conservation action plan for the species is to create a stable, healthy captive population (ICMBio, 2011). However, this has been challenging as pied tamarins appear to be particularly sensitive to the conditions of captivity (Wormell, Brayshaw, Price, & Herron, 1996; Holm, Priston, Price, & Wormell, 2012; Armstrong &

Santymire, 2013). “Wasting syndrome”, characterized by severe weight loss, diarrhea, and alopecia, has been a particular problem in captive populations (Ialeggio & Baker, 1995; Wormell, 2000; Smithyman, 2012; Cabana, Maguire, Hsu, & Plowman, 2018). Pied tamarins are also behaviorally different from other callitrichids in some respects, e.g. in frequently giving birth during the day (Price, Payne & Wormell, 2016). Thus, to adapt management and improve welfare, as much species-specific information as possible is needed about the extent to which pied tamarins experience stress from various sources, as assumptions based on information from other species may not be valid.

The main aims of this research were therefore to determine which factors influence cortisol levels in zoo-housed pied tamarins and to gain an insight into potential sources of stress. We analyzed cortisol in feces and saliva, as samples can be collected non-invasively (Queyras & Carosi, 2004; Heistermann, 2010), and may also give different pictures of the hormonal status of an individual (Cook, 2012). Cortisol reaches saliva in a matter of minutes after it is secreted and as such reflects an acute response to an event. Fecal sampling of cortisol, however, represents a timescale of hours or even days, and thus repeated fecal sampling may provide insight into underlying, longer-term sources of variation in cortisol levels.

We had access to relatively large samples in a single institution, and so were able to look at the effects of eight factors that might affect cortisol levels in this species, including variables at the individual level (sex, age, weight, rearing history, pregnancy), the social level (social status, group composition) and the environmental level (enclosure type). We tested the following predictions:

1. *Cortisol levels in pied tamarins will increase with age*: Previous research has found variable effects of age on cortisol levels in primates, and in several studies there is little or no relationship (e.g. Bergman, Beehner, Cheney, Seyfarth, & Whitten, 2005; Bales et al., 2006; Pirovino et al., 2011; though see Laudenslager, Jorgensen, & Fairbanks, 2012; Fourie, Jolly, Phillips-Conroy, Brown, & Bernstein, 2015a). However, as older tamarins appear to be more susceptible to wasting syndrome (Smithyman, 2012), we predicted that older pied tamarins would have higher cortisol levels.
2. *Individuals with a higher body mass will have lower cortisol levels:* Weight is sometimes used as an indicator of stress (Schumann et al., 2014). We therefore predicted a negative relationship between weight and cortisol levels.
3. *Cortisol levels will increase in female pied tamarins in late pregnancy:* Pregnancy has been found to affect cortisol levels in other callitrichid species, with lower levels of cortisol in breeding females during early pregnancy, and higher levels in late pregnancy (Bales et al., 2005; Ziegler, 2013).
4. *Female pied tamarins will have higher cortisol levels than males:* Previous work (Wark et al., 2016, Armstrong & Santymire, 2013) has found higher cortisol levels in female callitrichids than in males.
5. *Hand-reared tamarins will have higher cortisol levels than parent-reared individuals:* Hand-rearing can have negative consequences for the behavior and reproduction of primates in adulthood (Beck & Power, 1988; King & Mellen, 1994; Ryan, Thompson, Roth, & Gold, 2002), and hand-reared pied tamarins often show higher aggression towards keepers than parent-reared animals

(Coe, 2014). They may therefore be more stressed by contact with people. As hand-reared female pied tamarins never successfully rear their own infants (Price et al., 2016), whereas a hand-reared male did become a competent parent (pers. obs.), we also tested for an interaction between rearing and sex in cortisol levels.

6. *Subordinate pied tamarins will not have higher cortisol levels than dominant individuals:* Tamarin groups vary considerably in composition and mating system, but especially in captivity, are most often composed of a breeding pair plus their offspring and sometimes other individuals (Anzenberger & Falk, 2012). The latter may remain in the group for considerable periods, but rarely breed successfully (Price & McGrew, 1991; Savage, Giraldo, Soto & Snowdon, 1996; Saltzman, Liedl, Salper, Pick & Abbott, 2008; Henry, Hankerson, Siani, French & Dietz, 2013). While in some primate species, subordinate status is associated with higher cortisol levels (Abbott et al., 2003), several studies have found that in the callitrichids, in which intragroup relationships are predominantly affiliative rather than agonistic (Schaffner & Caine, 2000), either dominant individuals have higher cortisol levels than subordinates, or there is no effect of status (Ziegler, Scheffler, & Snowdon, 1995; Saltzman, Prudom, Schultz-Darken, Wittwer, & Abbott, 2004; Bales et al., 2005, 2006).
7. *Tamarins living in mixed-species groups will have higher cortisol levels than those living with conspecifics:* Callitrichids live in close social groups with a sophisticated cooperative rearing system (Tardif et al., 1986; Goldizen, 1987; Price, 1992; Garber, 1997). Therefore, to avoid the potentially negative effects

of single housing, pied tamarins at Jersey Zoo that could not be housed with conspecifics were usually mixed with individuals of other callitrichid species. However, unlike some *Saguinus* species (Heymann & Buchanan-Smith, 2000), pied tamarins are not usually sympatric with other callitrichids, and although pied tamarins can be housed successfully with other species (pers. obs.), this is not always the case (e.g. Gentry & Margulis, 2008).

8. *Tamarins housed in enclosures on show to the public will have higher cortisol levels than tamarins living in off-show enclosures:* Several studies have shown that the presence of visitors increases the stress levels of both wild and zoo animals (Hosey, 2000; Shepherdson, Carlstead, & Wielebnowski, 2004; Behie, Pavelka & Chapman, 2010; Quadros, Goulart, Passos, Vecci, & Young, 2014; Fourie et al., 2015b), and callitrichids in free-ranging environments that could retreat further from the public had lower urinary cortisol levels than caged conspecifics (McCallister, 2005).

Methods

Subjects and management

A total of 42 pied tamarins, all housed at Jersey Zoo in the Channel Islands, took part in the study (see Table 1); some individuals were included in studies of both salivary and fecal cortisol, while others contributed to only one data set. All tamarins involved in the present study were deemed healthy at the time the samples were collected, and were in stable social situations.

Tamarins all had permanent access to large indoor and outdoor areas, predominantly in buildings housing 3–5 callitrichid groups (Wormell & Brayshaw, 2000). Indoor cages were of broadly similar size; minimum dimensions were approximately 2.25 m high x 1.53 m wide x 2.45 m deep. All indoor areas received natural light via skylights or windows. In addition, artificial lighting was provided via strip lights and heat lamps from 0800 to 1800. In the winter months, supplementary UV lighting was also put in place (López, Wormell, & Rodríguez, 2001). Outside enclosures were 16–63 m^2 in area and approximately 4 m high, and were planted with extensive natural vegetation as well as being furnished with ropes, branches and platforms. The design of the buildings meant that tamarins had no visual contact while in their indoor areas, and no or very limited visual contact outside. Levels of auditory and olfactory contact were similar in all buildings.

Three buildings were on show to the public, but only the outdoor areas were accessible to visitors, and there were standoff barriers averaging 1 m from the front of each enclosure, reducing the opportunity for visitors to touch the animals or the mesh cage fronts. Three other buildings were off-show.

Pied tamarins were fed three times daily, at approximately 0800–0830 (primate pellet mix), 1200–1300 (fruit, vegetables and a protein item such as egg), and 1530–1700 (insect feed). Tamarin enclosures were cleaned in the morning, and excess food removed in the late afternoon. Tamarins were trained to sit on scales within their enclosures and were weighed at least weekly.

Sample collection

Salivary cortisol sampling and analysis

Saliva samples were collected between January and June 2007 by keeping staff using the technique described by Cross, Pines, & Rogers (2004). Samples were collected on a weekly basis. All samples included in the analysis were collected in the morning, as cortisol levels decrease during the day (Cross & Rogers, 2004). Individuals were encouraged to chew on 1–2 cotton buds for up to a minute at a time to obtain the required volume of saliva (50µl); a single food incentive, honey, was used on the cotton bud. The cotton buds were then centrifuged at 3200 rpm to extract the saliva and frozen at –20° C until analysis by the Central Science Laboratory in York, UK, using commercially available ELISA test kits and previously described methods (Cross et al., 2004; Cross & Rogers, 2004; Gladwell & Pick, 2007).

Fecal cortisol sampling and analysis

Fecal samples were collected between May and November 2008 at the first check in the morning (between 0800 and 0900), to control for circadian variation. Beetroot juice was used as a fecal marker and was given to tamarins via syringe in the evening prior to fecal deposition and collection the next morning. The animals were not disturbed by sample collection. Samples were frozen at –20°C within 60 min of collection.

Each sample was dried in a fan-assisted oven at 55°C for 7.5 h and was refrozen until needed for further extraction at –20°C. 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 3ml aliquot of 90% (v/v) methanol was added to a 0.1 g portion of the resulting powder and mixed vigorously for 3 h (Heidolphtitramax 100, 1350 rpm, 1.5 mm orbit). This was then centrifuged at 2000 rpm for 15 min 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 (N_2) administered using a Pierce Reacti-Therm Heating Module at 40°C. The residue was resuspended in 0.5 ml phosphate buffer saline buffer containing 0.1% (w/v) Bovine Serum Albumin (BSA) and microcentrifuged for 2 min at 6500 rpm to remove any remaining solid particles. The resulting supernatant was stored at –20° C until needed for measurement. Hormone values were expressed as pg/50µl of fecal extract.

Cortisol enzyme immunoassay

Immunological validation: A modified version of an enzyme immunoassay described by Armstrong & Santymire (2013) was used to quantify levels of fGC. The assay was immunologically validated for quantification of fGC levels in our population of captive pied tamarins using a representative sample pool consisting of 50µL of extract taken from all samples (Diamandis & Christopoulos, 1996). The antibody (R4866, raised against a steroid bovine albumin in rabbit (Munro & Stabenfeldt, 1985)) was diluted to 1:12000 in coating buffer, and the cortisol horseradish peroxide was diluted to 1:22000 in phosphate buffer solution containing 0.1% (w/v) BSA. Samples were run in duplicate at 1: 100 dilution.

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 & Stabenfeldt, 1985). Assay specificity was demonstrated twice by parallel displacement curves of serial dilutions of cortisol standard and the pied tamarin pool over the 10–90% binding range (ANCOVA; $F_{3, 48} = 1.074$, n.s.; $F_{3, 41} = 1.053$, n.s.). Recovery of the standards (halving dilutions in the range from 500 to 7.8 pg) added to a 1:100 dilution of a mixed fecal pool was 96.16 ± 14.91% inferring good accuracy ($r = 0.996$, $F_{1,5} = 595.63$, $P < 0.0001$). Intra-assay coefficients of variation for low, medium and high concentration quality controls were 1.95, 4.79 and 4.93%, respectively. Interassay coefficients of variation for low and high concentration quality controls were 10.03% (n=5 plates) and 18.55% (n=7 plates). The sensitivity of the assay was approximately 1.95 pg/ml.

Biological validation: To determine whether our assay detected biologically meaningful changes, we assessed fGC levels in the morning and afternoon under control conditions to test for circadian variation this hormone. Samples were collected in the morning before 1100 (n = 83) and in the afternoon after 1400 (n = 97) from a mix of adult males and females over a 5-month period. Log transformed cortisol levels were compared between samples deposited in the morning versus the afternoon using an independent t test. Levels of fecal cortisol were significantly raised in the afternoon compared to the morning in accordance with the typical circadian variation in levels of excreted cortisol ($t = 5.128$, d.f. = 179, $P < 0.001$; mean ± S.E.M in the morning: 564.76 ± 116.08 ng/ml and the afternoon: 1027.37 ± 117.03 ng/ml; Sousa & Ziegler, 1998).

Data analysis

General information about the samples, including dates, times, individuals, and any events such as illness, social tension or catch-ups that might affect stress levels, was recorded in daily diaries. For each sample, the most recent weight from that individual prior to sample collection was included in the analysis. We excluded data from individuals who had recently undergone any potentially stressful procedures (e.g. medical treatment, moves to new enclosures, etc.). The final data sets included 214 saliva samples from 36 individuals (mean number of samples per individual = 5.94 ± 3.76 SD), and 227 fecal samples from 27 individuals (mean number of samples per individual = 8.41 ± 3.33 SD).

Age (in years) and weight (in g) were included as continuous variables in the analysis. For saliva samples, the mean age of tamarins at the time of sampling was 5.45 years ± 4.00 SD, range 0.21 – 16.43 years; for fecal samples, the mean age was 5.66 years ± 4.75 SD, range 0.72 – 21.88 years. Although wild pied tamarins typically weigh around 430 g (Ford, 1994), weights obtained during this study averaged 501 g + 81 SD, which is comparable to weights obtained from the global captive population of pied tamarins (Species360, 2018).

In addition to sex, we also included the following categorical variables:

- Rearing history: either hand-reared or parent-reared.
- Enclosure type: either on-show (with permanent access to off-exhibit areas) or off-show. All enclosures had both indoor and outdoor areas to which tamarins had permanent access.
- Social status: dominant or subordinate. Tamarins who were breeding or potentially breeding (i.e. living with a conspecific of the opposite sex) were classed as dominant. Offspring living with their parents were classed as subordinate. Other individuals (i.e. those in single-sex or mixed-species groups) were classified as either dominant or subordinate based on keepers' knowledge of behavior and relationships in each group at the time of sampling (e.g. priority of access to food).
- Group composition, categorized as: breeding pair without offspring, family (pair plus offspring), single-sex conspecific group, or mixed-species group (one or two pied tamarins with one or two individuals of another species. Species involved were *Leontopithecus chrysopygus*, *L. chrysomelas*, *L. rosalia* and *Callithrix geoffroyi.*)
- For females in breeding situations, we counted back from the birth of infants to the date each sample was collected. Assuming a gestation period of 160 days (Heistermann, Pröve, Wolters, & Mika, 1987), we classed early pregnancy as 1–80 days gestation and late pregnancy as 81–160 days gestation.

The dependent variable, cortisol level (in ng/ml for saliva samples, and pg/50µl for fecal samples), was not normally distributed in either case and was therefore natural log-transformed before analysis. We examined the transformed data and standardized residuals graphically to test the assumptions of normality and homoscedasticity, and removed outliers. Final sample sizes for each categorical variable are given in Table 1. We used separate generalized linear mixed models (GLMMs) for each sample type, including individual as a random factor to control for the fact that several samples were obtained from many of the tamarins. Data were analyzed using the statistical software R (version 3.5.1; R Core Development Team, 2018) and the packages nlme (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2018), lme4 (Bates, Maechler, Bolker & Walker, 2015) and MuMIn (Barton, 2015).

We first tested for an effect of pregnancy in breeding and potentially breeding females. We examined log-transformed cortisol levels using a GLMM for each sample type, with individual as a random factor, and pregnancy status (early pregnancy, late pregnancy, or not pregnant) as a fixed factor, and adjusted the data sets for subsequent analysis if necessary (see Results).

We then ran separate GLMMs for each sample type, including main effects plus an interaction between sex and rearing history, with individual as a random factor. We followed the method outlined by Grueber, Nakagawa, Laws, & Jamieson (2011) for model averaging of GLMMs, and considered models with $\Delta AIC_c < 2$ as having strong support, and those with a ΔAIC_c of >2 to have less support (Burnham and Anderson, 2002). We constructed a standardized global model containing all factors of interest, and then used the dredge function in MuMIn to obtain a list of all models with a ΔAIC_c <2 from the best model. We then used model averaging to obtain estimates and 95%

confidence intervals for each factor. Finally, means were back-transformed to display graphically. The figures were produced in Microsoft Excel.

Ethical statement

We confirm that the methods used in this study conformed to the UK's Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039), the American Society of Primatology's Principles for the Ethical Treatment of Non-human Primates, and to the Animal Welfare (Jersey) Law 2004, and met the requirements of Durrell Wildlife Conservation Trust's Ethics Committee.

Conflict of interest statement

The authors confirm that they have no conflicts of interest to declare.

Results

Salivary cortisol

We found no effect of pregnancy on cortisol levels in saliva, and so we used all female samples in subsequent analyses. The final GLMM for log cortisol levels in saliva included only two fixed factors, enclosure type and sex; no other model had a ΔAIC_c <2. The influence of enclosure type was in the opposite direction to the predicted one: individuals housed in off-show enclosures had higher salivary cortisol levels than tamarins in on-show exhibits (β = -0.4399 ± 0.1118 SE, 95% CI = -0.6887, -0.2196;

Figure 1A). Female tamarins had higher salivary cortisol levels than males (β = -0.2905 ± 0.1122 SE, 95% CI = -0.5177, -0.0566; Figure 1B).

Fecal cortisol

Although we had few samples from females in late pregnancy, we found some effect of stage of pregnancy on log cortisol levels in fecal samples ($F_{2,55}$ = 2.684, P = 0.077; Figure 2). Examination of paired contrasts showed that females in late pregnancy had significantly higher cortisol levels than those in early pregnancy (P = 0.033), but the other two pairs did not differ significantly. For further analysis, we removed samples from females in late pregnancy (n = 3 samples from two females) from the data set.

The MuMIn dredge function produced a set of 12 models with a ΔAIC_c <2 from the best model (Table 2). The averaged model (Table 3) showed that fecal cortisol levels tended to be lower in subordinate individuals (Figure 3A). There was an interaction between sex and rearing history: hand-reared tamarins of both sexes had higher fecal cortisol levels than parent-reared individuals, but while male parent-reared tamarins had higher cortisol levels than females who were parent-reared, the reverse was true for hand-reared individuals (Figure 3B). Tamarins in off-show enclosures had higher levels than those living in on-show exhibits (Figure 3C), and enclosure type was the only predictor to occur in all 12 models (see Table 2). There was little effect of group composition on cortisol levels (Figure 3D), and this predictor occurred in only one of the averaged models (Table 2). Finally, as individual weight increased, cortisol levels decreased (Figure 4).

Discussion

As pressure grows on wild populations, captive management will play an increasing role in saving species from extinction, both in terms of maintaining captive populations as insurance and a source for reintroduction, and also in circumstances which require rescue, temporary captivity and translocation to protected environments (Griffiths & Pavajeau, 2008; Baker, Lacy, Leus, & Traylor-Holzer, 2011; Traylor-Holzer, Leus, & Byers, 2018). Understanding the implications of housing, management, hand-rearing and other factors for the wellbeing of these animals is therefore crucial to conservation success (Dickens, Delehanty, & Romero, 2010). In order to obtain robust data, large samples are needed, but this is often difficult in zoo settings as, typically, collections hold only one or two groups of any given species, and cross-institutional studies must incorporate many additional variables to account for differences in the way in which the animals are housed and managed (e.g. Shepherdson et al., 2013). We were able to study a large number of tamarins in a single collection, and therefore draw more reliable conclusions.

We found several factors that affected cortisol levels in zoo-housed pied tamarins. However, the results were somewhat different depending on whether cortisol was measured in saliva or feces. Only sex and enclosure type had an effect on salivary cortisol in pied tamarins, while sex, pregnancy, rearing history, social status, weight and enclosure type all influenced fecal cortisol levels. Fecal cortisol may give a better picture of baseline cortisol levels as it is less affected by daily acute events and as such represents the effects of underlying individual, social and environmental factors affecting cortisol production (Millspaugh & Washburn, 2004; Heistermann, 2010). It is also the easiest type of sample to collect for analysis. As salivary cortisol represents an individual's immediate reaction to an event (Kuhar, Bettinger, & Laudenslager, 2005; Laudenslager, Bettinger, & Sackett, 2006), it may therefore be of most help in investigating the impact of acute stressors on the wellbeing of pied tamarins. Unfortunately we were not able to make direct comparisons between fecal and salivary cortisol levels in individuals, as the two sets of samples were collected at different times.

It is also possible that, since samples from the two media were collected at different times of the year (saliva: January–June, feces: May–November), cortisol levels were differentially affected by weather conditions – both temperature and precipitation have been shown to influence cortisol (de Bruijn and Romero, 2018). Finally, previous research suggests that salivary and fecal cortisol may manifest different response patterns to stress. For example, most empirical research with callitrichids has found increased fecal cortisol following a stressor (e.g. Galvão-Coelho, Silva, & De Sousa, 2012; Pizzuto et al., 2015), whereas recent studies in the common marmoset *Callithrix jacchus* have reported that salivary cortisol following exposure to a stressor may either decrease (Ash et al., 2018; Cross and Rogers, 2006) or increase (Kaplan et al., 2012).

Individual factors affecting cortisol levels

We found no effect of age on cortisol in either feces or saliva. Prediction 1, that older pied tamarins would exhibit higher cortisol levels as they may be more susceptible to wasting syndrome (Smithyman, 2012), was therefore not supported. Similar results have been reported by Bales et al. (2006) for fecal cortisol in male golden lion tamarins, *Leontopithecus rosalia*. Studies in other primate taxa have produced inconsistent results: for example, Erwin, Tigno, Gerzanich, & Hansen (2004) found a positive correlation between age and plasma cortisol in *M. mulatta*, while in the same species, Dettmer, Novak, Suomi, & Meyer (2012) reported a decrease in hair cortisol with age, and Fourie et al. (2015a) found that cortisol levels in two baboon species (*Papio anubis*, *P. hamadryas*) were lowest in adulthood, and higher in both young and older age groups. It is likely that other factors, such as the species' social system (Abbott et al., 2003), housing conditions, and individual reproductive status, have a greater influence on cortisol levels than age.

Weight loss is one of the most consistent and pronounced changes during exposure to stress (Tamashiro et al., 2005). We found no effect of weight on salivary cortisol, but fecal cortisol decreased significantly as weight increased, as we expected (Prediction 2). Links have been found in a number of taxa between poorer body condition or lower weight, and higher baseline cortisol levels (e.g. Macbeth, Cattet, Obbard, Middel, & Janz, 2012; Cattet et al., 2014; Trevisan et al., 2017) or higher glucocorticoid reactivity (Breuner & Hahn, 2003; Pereyra & Wingfield, 2003). Changes in weight may therefore give an early indication of both increased stress levels and the possibility of wasting syndrome, and thus regular non-disruptive weighing, as used in our colony, is an important tool in monitoring health and wellbeing in tamarins in captivity. We excluded data from tamarins with symptoms of illness, but it is possible that individual health parameters could have confounded cortisol titers in our subjects.

Pregnancy had no effect on cortisol levels in saliva, but as expected, cortisol in feces was highest in females in late pregnancy (Prediction 3). Although our sample size for females in late pregnancy was small, this is consistent with the typical pattern in primates, including humans (Ziegler et al., 1995; Leung et al., 2001; Bales et al., 2005). It is well known that the reproductive status of the female in addition to pregnancy influences cortisol titers, e.g. lactation (Starling, Charpentier, Fitzpatrick, Scordato, & Drea, 2010). In the closely related common marmoset, *Callithrix jacchus*, cortisol levels vary reliably across the ovarian cycle and are significantly raised around the peri-ovulatory phase (Saltzman, Schultz-Darken, Wegner, Wittwer, & Abbott, 1998). In our study we were only able to control for early and late pregnancy, and did not take into account other reproductive phases such as ovulation that could have influenced cortisol titers. Furthermore, in several primate species, baseline cortisol levels of males are also affected by the reproductive status of the females in the group, in particular ovulation – something that we could not take into account when analyzing male cortisol values but which may have affected our data (Surbeck, Deschner, Weltring, & Hohmann, 2012; Schoof, Jack & Ziegler, 2014).

We found that female pied tamarins had higher salivary cortisol levels than males (Prediction 4). This agrees with a previous study of four zoo-housed pied tamarins that found significantly higher fecal cortisol levels in the two females (Armstrong &

Santymire, 2012), and with studies of urinary and fecal cortisol in other callitrichid species (Smith & French, 1997a; Wark et al. 2016).

The effect of sex on cortisol in feces in our study, however, depended on the rearing history of the individual. Overall, hand-reared tamarins had higher fecal cortisol levels than parent-reared individuals, supporting Prediction 5, but while hand-reared females did have higher cortisol levels than hand-reared males, male parent-reared tamarins had higher cortisol levels than females who were parent-reared (Predictions 4 and 5). This contrasts with the results reported for *Saguinus geoffroyi* by Kuhar, Bettinger, Sironen, Shaw, & Lasley (2003), who found no differences in cortisol between hand-reared and parent-reared individuals.

It is important to note that the number of hand-reared individuals included in our study was small (n = 5). However, the generally higher fecal cortisol levels that we found in hand-reared pied tamarins are consistent with the greater incidence of negative behavior towards humans that we have observed in hand-reared tamarins (Coe, 2014). Similar results have been reported for pileated gibbons, *Hylobates pileatus*, by Pirovino et al. (2011): hand-reared gibbons had higher levels of fecal glucocorticoid metabolites and exhibited more abnormal behavior than parent-reared individuals. Hand- or nursery-rearing of primates is known to affect allostatic load (a composite measure of stress; Edes, Wolfe, & Crews, 2016), brain development (Bogart, Bennett, Schapiro, Reamer, & Hopkins, 2014), behavior, including parenting (Mallapur & Choudhury, 2003; Niebruegge & Porton, 2006; Vermeer & Devreese, 2015) and response to stressors in later life (Dettmer et al., 2012), and in chimpanzees, the higher the level of conspecific as opposed to human interaction throughout life, the lower the level of cortisol in hair samples (Jacobson, Freeman, Santymire & Ross, 2018).

Hand-reared tamarins are often poor parents, probably in part because they are less likely to have had experience caring for infants as helpers in family groups (Tardif, Richter & Carson, 1984; but see Baker & Woods, 1992, for an exception). Interestingly, while hand-reared female pied tamarins in our colony have invariably been incompetent parents (Price et al., 2016) we have documented adequate parental care in one hand-reared male. Although our sample is too small to draw any firm conclusions, the relationships between rearing history, cortisol levels and parenting, and how they may differ between the sexes, will be important to investigate further. The possibility that these relationships may differ from one species to another should also be borne in mind.

Our results also raise questions about the role and ethics of hand-rearing pied tamarins. Attitudes towards hand-rearing primates in zoos have changed since the 1950s, when it was frequently practiced in order to increase survival rates (Porton & Niebruegge, 2006); realization of the importance of parental rearing, in particular for social development, has led to a decrease in the number of primates removed by zoos for hand-rearing and an improved understanding of the need for early socialization with conspecifics. However, for threatened species such as pied tamarins, hand rearing has remained an important tool to increase captive population sizes. Although in modern zoos, hand-reared infants are socialized from a very early age, work remains to be done in developing rearing and socialization methods that result in adults that are behaviorally and physiologically indistinguishable from parent-reared individuals.

Social factors affecting cortisol levels

We found no effect of social status on salivary cortisol, but there was a trend for subordinate tamarins to have lower fecal cortisol levels than tamarins classed as dominant. These results supports Prediction 6 and are in agreement with previous studies of a number of callitrichid species using various methods of measuring cortisol levels, all of which have found either no effect of status, or reduced cortisol levels in subordinate individuals (Baker, Abbott & Saltzman, 1999; Saltzman et al. 2004: plasma cortisol in captive *Callithrix jacchus*; Smith & French, 1997b: urinary cortisol in *C. kuhli*; Huck, Löttker, Heymann, & Heistermann, 2005: fecal cortisol in wild *Saguinus mystax*; Bales et al., 2005, 2006: fecal cortisol in wild *Leontopithecus rosalia*). In a synthesis of published data on cortisol and status in primates in relation to social systems, Abbott et al. (2003) pointed out that the frequency and rate at which subordinates receive aggression, the stability of access to resources and social relationships, and coping strategies, may all be as important as low rank itself in mediating the stress response. In callitrichids, social life is characterized by cooperation, usually among close relatives, and a high level of affiliative interactions (Schaffner & Caine, 2000), and thus subordinates are not subject to frequent stressors (Abbott et al., 2003). Our results from pied tamarins support this view. However, in some species, rank-related patterns of glucocorticoid excretion are only evident under certain conditions, such as during times of food shortages (*L. catta;* Cavigelli, 1999), the mating season (e.g. *Brachyteles arachnoides hypoxanthus*; Strier, Lynch & Ziegler, 2003), pregnancy (semi-free-ranging provisioned *L. catta*; Starling et al., 2010) or lactation (*Cercopithecus mitis*; Foerster, Cords & Monfort, 2011). Our study might have yielded different relationships between cortisol and social rank under different social or environmental conditions such as following a birth or aggression.

in contrast to other primate studies in which variations in group composition such as group size (Pride, 2005) or number of males in a group (Smith, McCusker, Stevens & Elwood, 2015) have been shown to have a significant impact on cortisol levels, we found little influence of group composition on cortisol levels in either saliva or feces. Our expectation that pied tamarins would exhibit higher cortisol levels if they were housed with members of other species was therefore not supported (Prediction 7). This suggests that as long as an individual is housed socially, it does not make a great deal of difference whether it is living in a mixed-sex family or pair, or a single-sex group, or even with individuals from another species, and is encouraging in terms of management strategies for captive tamarins. However, it is important to remember that each situation is different, and any new grouping, whether of conspecifics or not, should be closely monitored (Buchanan-Smith, 2012).

Environmental factors affecting cortisol levels

We found that in both saliva and feces, cortisol levels were significantly higher in tamarins living in enclosures that were not on show to the public (Prediction 8). This was unexpected – the presence of visitors has often been highlighted as a source of stress and a cause of abnormal behavior in zoo primates (Hosey, 2000), and an earlier study (Armstrong & Santymire, 2012) found that a pair of pied tamarins on exhibit had higher levels of fecal glucocorticoid metabolites than a pair living off-show. However, our results are in agreement with previous behavioral studies of pied tamarins at Jersey Zoo, which found that visitors had no effect on levels of stress-related behaviors (Holm et al., 2012), and that tamarins housed on-show were less vigilant, and vocalized and scent-marked less often than tamarins in off-show enclosures (Steinbrecher, 2016). We suggest that this may be because tamarins on show to the public at the zoo have high and relatively naturalistic enclosures with stand-off barriers preventing direct contact with the public (Figure 5), and they are also able to retreat indoors whenever they wish. Off-show enclosures are similar in design, but typically house more groups of pied tamarins per building (though fewer groups overall) than the on-show enclosures. Crowding has been linked to elevated cortisol in several primate species and may have contributed to the raised levels in the tamarins off-show (Dettmer, Novak, Meyer, & Suomi, 2014; Gabriel, Gould, & Cook, 2018; Pearson, Reeder & Judge, 2015). Interestingly, Pirovino et al. (2011) also found that cortisol values varied across pileated gibbons not only living in different institutions, but across animals residing in different enclosures within the same institution, illustrating the mixture of factors that may affect cortisol at a local level.

Cabana et al. (2018) reported that the likelihood of wasting syndrome developing in zoo-housed callitrichids was lower if there were visual barriers between visitors and monkeys, and if the animals had safe areas to which they could retreat. Another study at Jersey Zoo demonstrated that the level of noise in a building (mostly due to other animals housed there) was positively related to cortisol levels in *S. bicolor* (Simpkins, Routh, Wormell, & Price, 2013). Similarly, Kuhar et al. (2003) found that colony housed *S. geoffroyi* showed higher levels of aggression and lower activity levels than non-colony-housed tamarins, and higher levels of physical activity have been shown to mitigate stress responses in women (Puterman et al. 2011). It would therefore be interesting to investigate activity levels in tamarins housed on- and offshow, and to monitor changes in both behavior and cortisol levels in groups that are moved from one enclosure type to another. Further research into how enclosure design, management, housing density and proximity to conspecifics versus other species influence behavior and stress levels in pied tamarins and other callitrichids is therefore needed.

Although our sample size was comparatively large, we did not have sufficient data to be able to take into account other non-stress related and stress-related factors that might affect HPA function and thus cortisol levels, such as personality (Martin and Reale, 2008; Shepherdson et al., 2013), which influences cortisol in another callitrichid species, the common marmoset (Inoue-Murayama et al., 2018). Variations in metabolic rate (Goymann 2012), activity levels (Smith, McGreer-Whitworth, & French, 1998) and amount of keeper interaction (Carlstead, Paris, & Brown, 2018) were not controlled in this study, but may also affect cortisol levels. Finally, seasonal variation in temperature or poor weather (which could reduce visitor numbers, but potentially increase intragroup tension as tamarins stayed indoors for longer periods) may also have had an effect on cortisol in this study. For example, it would be interesting to investigate levels of salivary cortisol when tamarins are indoors versus outdoors. Focused studies on these possibilities, and further research into the factors we investigated, would contribute a great deal to the successful management of this and other threatened callitrichid species both in captivity, and in cases where intensive management of free-living populations becomes necessary.

Conclusions

1. Fewer factors affect cortisol in saliva than in feces in captive pied tamarins; salivary cortisol is therefore likely to be of more value in assessing the immediate impact of stressful events than in understanding underlying sources of chronic stress.
2. Female pied tamarins have higher fecal cortisol levels in late pregnancy.
3. Female tamarins have higher salivary cortisol levels than males.
4. Female hand-reared tamarins have higher fecal cortisol levels than males, but the reverse is true for parent-reared tamarins.
5. Hand-reared pied tamarins have higher levels of fecal cortisol overall and this is consistent with the higher levels of abnormal and aggressive behavior seen in hand-reared tamarins.
6. Fecal cortisol levels increase in pied tamarins as weight decreases, which can be an indicator of wasting syndrome in this species. Regular non-disruptive weighing may help to identify tamarins at risk.
7. Pied tamarins housed on show had lower cortisol levels in both saliva and feces than tamarins living in off-show enclosures with a higher number of conspecifics in a given area, and therefore may not be affected by

disturbance from visitors as long as their contact with people is minimized by the use of barriers and access to off-exhibit areas.

8. Age does not affect levels of cortisol in this species.

Acknowledgements

We thank Fiona Gladwell at the Central Science Laboratory for analysis of saliva samples; Philippa Blanco, Will Masefield and the many other members of the Mammal Department at Jersey Zoo who helped to collect samples; Valerie Harding for assistance in compiling data from daily diaries; and Lianne Concannon, Tine Griede, and Henry Kuijpers for advice on the statistical analyses. We are also grateful to Karen Bales and two anonymous reviewers for constructive comments on previous drafts.

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Table 1. Sample sizes for each factor included in the GLMMs.

	Saliva samples		Fecal samples	
	No. of samples	No. of individuals	No. of samples	No. of individuals
Sex				
Female	78	16	102	12
Male	136	20	125	15
Rearing history				
Hand reared	36	5	48	5
Parent reared	178	31	179	22
Group composition*				
Family	68	17	140	18
Pair	37	10	42	6
Single sex	21	2	19	2
Mixed species	87	15	26	4
Social status*				
Dominant	147	27	151	17
Subordinate	67	11	76	10
Enclosure type*				
Off-show	159	28	134	21
On-show	55	9	93	12
Pregnancy state (breeding females only)				
Early pregnancy	1	1	14	3
Late pregnancy	3	2	3	2
Not pregnant	26	6	47	7
Total	214	36	227	27

*Number of individuals may sum to more than overall total as some tamarins contributed samples under more than one condition.

Table 2. Models with ΔAIC_c <2 for log cortisol in fecal samples.

Model	df	AIC_c	ΔAIC_c	Weight
Enclosure + rearing + sex + social status + weight + rearing:sex	9	710.39	0.00	0.14
Enclosure + social status + weight	6	710.74	0.36	0.11
Enclosure + rearing + sex + weight + rearing:sex	8	710.77	0.38	0.11
Enclosure + rearing + weight	6	710.91	0.53	0.10
Enclosure + weight	5	711.12	0.73	0.09
Enclosure + rearing + social status + weight	7	711.27	0.88	0.09
Enclosure + rearing + sex + social status + age + weight + rearing:sex	10	711.61	1.22	0.07
Enclosure + rearing + sex + weight	7	711.82	1.43	0.07
Enclosure + sex	5	712.05	1.66	0.06
Enclosure + sex + social status + weight	7	712.27	1.89	0.05
Enclosure + rearing + sex + social status + weight	8	712.31	1.93	0.05
Enclosure + social status + group composition + weight	9	712.33	1.94	0.05

Table 3. Estimates, standard errors and 95% confidence intervals for predictor variables in averaged model for log fecal cortisol. The first level listed for each variable is the reference level.

			Confidence intervals	
Variable	Estimate (β)	SE	2.5 %	97.5 %
Enclosure (offshow–onshow)	-0.5197	0.2275	-0.9680	-0.0715
Weight	-0.5482	0.2693	-1.1132	-0.0520
Rearing (hand–parent)	-0.4181	0.3497	-1.1071	0.2709
Social status (dominant–subordinate)	-0.4585	0.2925	-1.0348	0.1179
Age	-0.3104	0.3130	-0.9274	0.3067
Sex (female–male)	0.2794	0.2587	-0.2305	0.7893
Rearing:sex	1.3365	0.6609	0.0339	2.6392
Group composition (family–mixed)	0.7580	0.3695	0.0297	1.4864
Group composition (family–pair)	0.3760	0.3221	-0.2591	1.0120
Group composition (family–single sex)	-0.1267	0.4640	-1.0414	0.7880
Group composition (mixed–pair)	-0.3821	0.4431	-1.2555	0.4913
Group composition (mixed–single sex)	-0.8848	0.5433	-1.9557	0.1862
Group composition (pair–single sex)	-0.5027	0.5116	-1.5111	0.5058

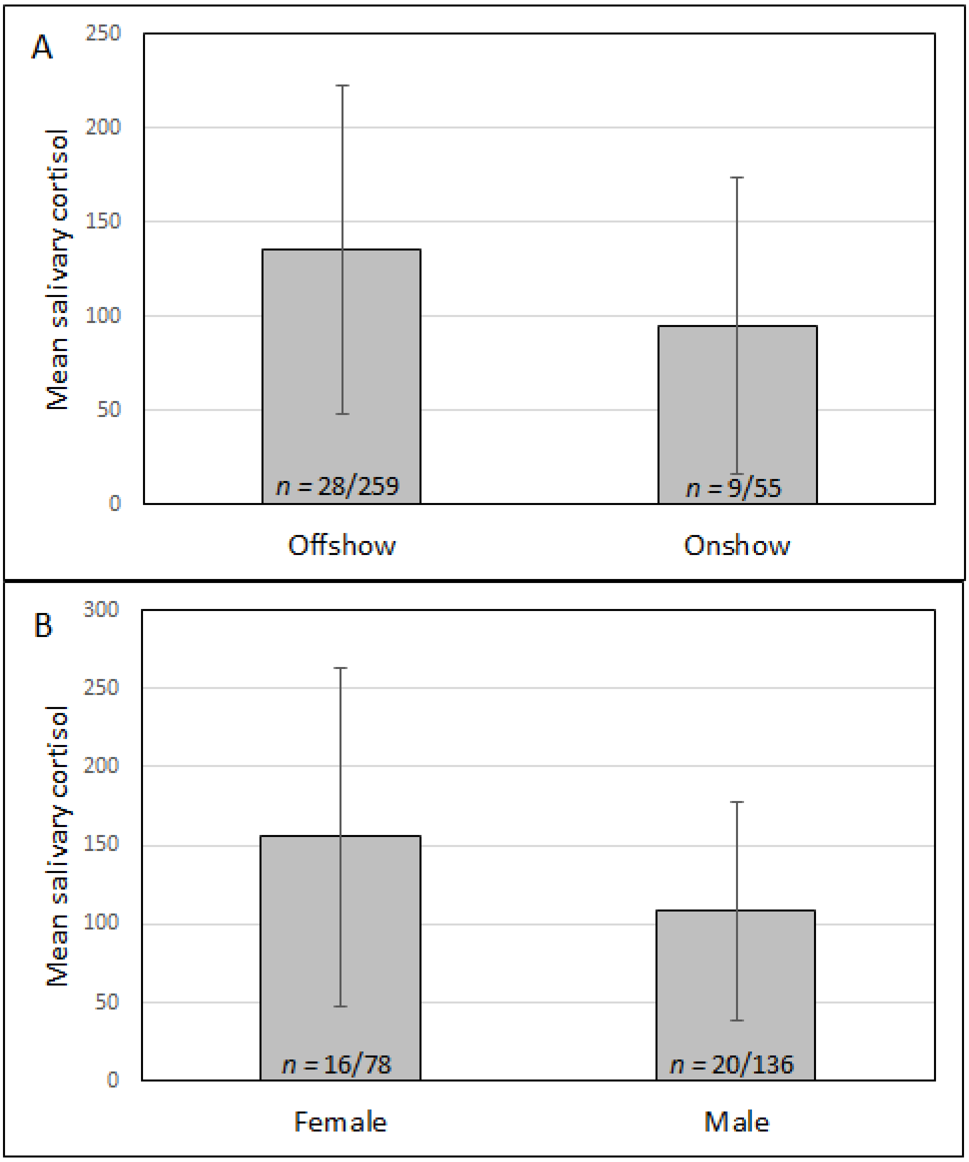

Figure 1. A: Mean salivary cortisol in pied tamarins housed in different enclosure types. B: Mean salivary cortisol in male and female pied tamarins. Other factors did not appear in the final GLMM and are not illustrated. Means were back-transformed from mean log cortisol values. Vertical bars represent coefficient of variation. Sample sizes (number of individuals/number of samples) are given at the base of each bar.

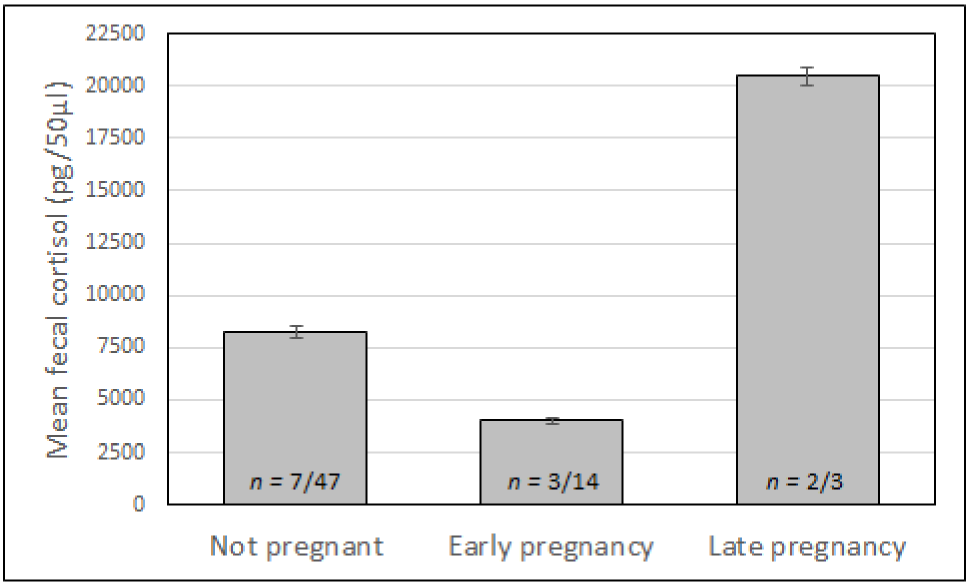

Figure 2. Effect of pregnancy stage on mean fecal cortisol in breeding females. Means were back-transformed from mean log cortisol values. Vertical bars represent coefficient of variation. Sample sizes (number of individuals/number of samples) are given at the base of each bar.

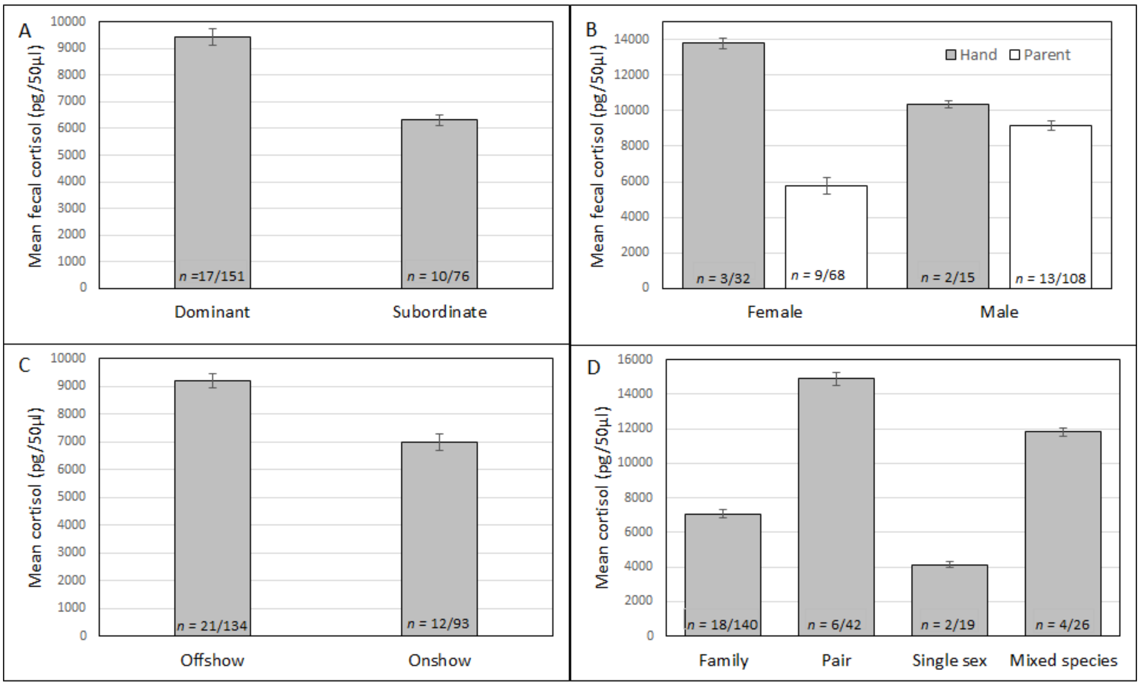

Figure 3. A: Mean fecal cortisol in dominant and subordinate pied tamarins. B: Effect of sex and rearing history on mean fecal cortisol in pied tamarins. C: Mean fecal cortisol in pied tamarins housed in different enclosure types. D: Mean fecal cortisol in pied tamarins housed in different group types. Means were back-transformed from mean log cortisol values; vertical bars represent coefficient of variation. Sample sizes (number of individuals/number of samples) are given at the base of each bar.

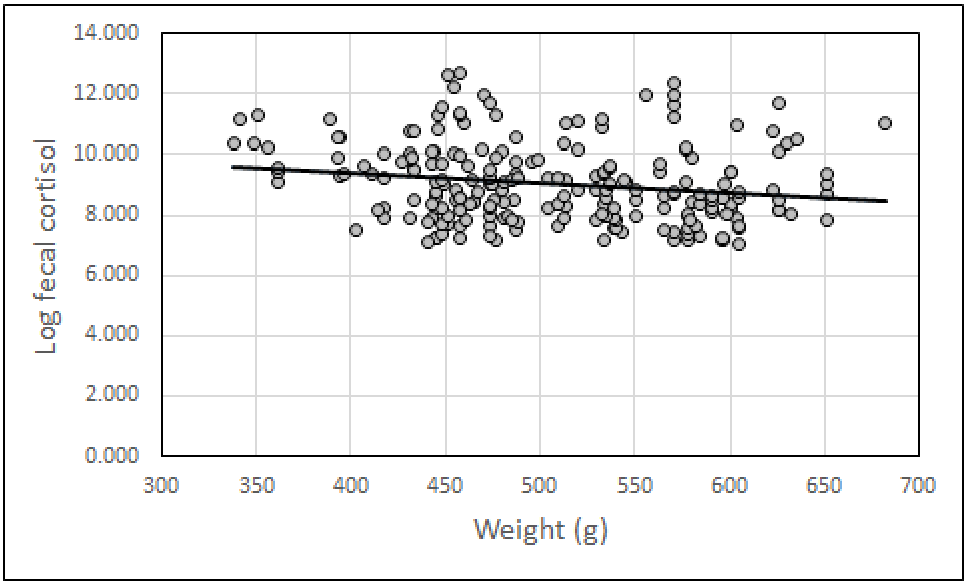

Figure 4. Relationship between log-transformed fecal cortisol levels and weight in pied tamarins.

Figure 5. Large on-show enclosure for pied tamarins at Jersey Zoo, showing stand-off barrier and planting to reduce contact with visitors. A pied tamarin is visible in the center of the image.