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

Eluned Price^{1*}, Rob Coleman², Judith Ahsmann^{1,3}, Gale Glendewar¹, Jenna Hunt¹, Tessa Smith³, and Dominic Wormell¹

¹Durrell Wildlife Conservation Trust

²University of Chester

³University of Applied Sciences Van Hall Larenstein

Dedication: In memory of Tine Griede

Running head: Fecal and salivary cortisol in pied tamarins

Address for correspondence:

Eluned C. Price

Durrell Wildlife Conservation Trust

Les Augrès Manor, Trinity

Jersey, JE3 5BP

+44 1534 860044

Eluned.Price@durrell.org

1 Abstract

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

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

25

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

27 Research highlights

28

29 • Hand-reared pied tamarins have higher fecal cortisol levels than parent-reared
30 tamarins.

31 • Pied tamarins living off-show with more conspecific groups per building have
32 higher salivary and fecal cortisol levels than tamarins housed on-show in large
33 enclosures with access to off-exhibit areas.

34

35 Introduction

36

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

48 As well as being influenced by factors such as social and reproductive status
49 (Abbott et al., 2003; Bales, French, Hostetler, & Dietz, 2005; Bales, French, McWilliams,
50 Lake, & Dietz, 2006), cortisol is produced by the hypothalamus–pituitary–adrenal axis
51 (HPA) when an individual is exposed to a stressful situation (Möstl & Palme, 2002;
52 Beehner & Bergman, 2017). Animals in natural environments can usually respond
53 adaptively (either behaviorally and or physiologically) to stressors and ameliorate the
54 stress (Morgan & Tromborg, 2007). Acute stress may therefore not have a negative
55 effect on fitness (Beehner & Bergman, 2017). However, if it is not possible to respond
56 in such a way as to reduce the effect of a stressor, as is often the case in animals living
57 in captivity (Mason, 2010), stress can become chronic and the individual may develop

58 problems such as stereotypic behaviors (e.g. Mason, Clubb, Latham, & Vickery, 2007),
59 poor health (e.g. Munson et al., 2005), excessive weight loss (e.g. Tamashiro, Nguyen,
60 & Sakai, 2005), reduced reproductive success (e.g. Clubb, Rowcliffe, Lee, Mar, Moss, &
61 Mason, 2008), suppressed immunity (Martin, 2009), and impaired cognitive function
62 (Teixeira et al., 2015). A growing number of studies in a variety of mammal species
63 living in zoos has pointed to many factors that can affect cortisol levels, including
64 rearing history, social situation, enclosure size and type, noise or other disturbance
65 (e.g. from zoo visitors), access to outdoor areas, and season (Carlstead, Brown, &
66 Seidensticker, 1993; McCallister, 2005; Carlstead & Brown, 2005; Powell, Carlstead,
67 Tarou, Brown, & Monfort, 2006; Clark et al., 2012; Pirovino et al., 2011; Rajagopal,
68 Archunan, & Sekar, 2011; Cerda-Molina et al., 2012; Kaplan et al., 2012; Shepherdson,
69 Lewis, Carlstead, Bauman, & Perrin, 2013; Schumann, Guenther, Jewgenow, &
70 Trillmich, 2014; Sherwen et al., 2015; Pauling, Lankford, & Jackson, 2017).

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

81 Santymire, 2013). “Wasting syndrome”, characterized by severe weight loss, diarrhea,
82 and alopecia, has been a particular problem in captive populations (Ialeggio & Baker,
83 1995; Wormell, 2000; Smithyman, 2012; Cabana, Maguire, Hsu, & Plowman, 2018).
84 Pied tamarins are also behaviorally different from other callitrichids in some respects,
85 e.g. in frequently giving birth during the day (Price, Payne & Wormell, 2016). Thus, to
86 adapt management and improve welfare, as much species-specific information as
87 possible is needed about the extent to which pied tamarins experience stress from
88 various sources, as assumptions based on information from other species may not be
89 valid.

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

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

- 104 1. *Cortisol levels in pied tamarins will increase with age:* Previous research has
105 found variable effects of age on cortisol levels in primates, and in several
106 studies there is little or no relationship (e.g. Bergman, Beehner, Cheney,
107 Seyfarth, & Whitten, 2005; Bales et al., 2006; Pirovino et al., 2011; though see
108 Laudenslager, Jorgensen, & Fairbanks, 2012; Fourie, Jolly, Phillips-Conroy,
109 Brown, & Bernstein, 2015a). However, as older tamarins appear to be more
110 susceptible to wasting syndrome (Smithyman, 2012), we predicted that older
111 pied tamarins would have higher cortisol levels.
- 112 2. *Individuals with a higher body mass will have lower cortisol levels:* Weight is
113 sometimes used as an indicator of stress (Schumann et al., 2014). We
114 therefore predicted a negative relationship between weight and cortisol levels.
- 115 3. *Cortisol levels will increase in female pied tamarins in late pregnancy:*
116 Pregnancy has been found to affect cortisol levels in other callitrichid species,
117 with lower levels of cortisol in breeding females during early pregnancy, and
118 higher levels in late pregnancy (Bales et al., 2005; Ziegler, 2013).
- 119 4. *Female pied tamarins will have higher cortisol levels than males:* Previous work
120 (Wark et al., 2016, Armstrong & Santymire, 2013) has found higher cortisol
121 levels in female callitrichids than in males.
- 122 5. *Hand-reared tamarins will have higher cortisol levels than parent-reared*
123 *individuals:* Hand-rearing can have negative consequences for the behavior and
124 reproduction of primates in adulthood (Beck & Power, 1988; King & Mellen,
125 1994; Ryan, Thompson, Roth, & Gold, 2002), and hand-reared pied tamarins
126 often show higher aggression towards keepers than parent-reared animals

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

132 6. *Subordinate pied tamarins will not have higher cortisol levels than dominant*
133 *individuals*: Tamarin groups vary considerably in composition and mating
134 system, but especially in captivity, are most often composed of a breeding pair
135 plus their offspring and sometimes other individuals (Anzenberger & Falk,
136 2012). The latter may remain in the group for considerable periods, but rarely
137 breed successfully (Price & McGrew, 1991; Savage, Giraldo, Soto & Snowdon,
138 1996; Saltzman, Liedl, Salper, Pick & Abbott, 2008; Henry, Hankerson, Siani,
139 French & Dietz, 2013). While in some primate species, subordinate status is
140 associated with higher cortisol levels (Abbott et al., 2003), several studies have
141 found that in the callitrichids, in which intragroup relationships are
142 predominantly affiliative rather than agonistic (Schaffner & Caine, 2000), either
143 dominant individuals have higher cortisol levels than subordinates, or there is
144 no effect of status (Ziegler, Scheffler, & Snowdon, 1995; Saltzman, Prudom,
145 Schultz-Darken, Wittwer, & Abbott, 2004; Bales et al., 2005, 2006).

146 7. *Tamarins living in mixed-species groups will have higher cortisol levels than*
147 *those living with conspecifics*: Callitrichids live in close social groups with a
148 sophisticated cooperative rearing system (Tardif et al., 1986; Goldizen, 1987;
149 Price, 1992; Garber, 1997). Therefore, to avoid the potentially negative effects

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

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

164 Methods

165 Subjects and management

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

171 Tamarins all had permanent access to large indoor and outdoor areas,
172 predominantly in buildings housing 3–5 callitrichid groups (Wormell & Brayshaw,
173 2000). Indoor cages were of broadly similar size; minimum dimensions were
174 approximately 2.25 m high x 1.53 m wide x 2.45 m deep. All indoor areas received
175 natural light via skylights or windows. In addition, artificial lighting was provided via
176 strip lights and heat lamps from 0800 to 1800. In the winter months, supplementary
177 UV lighting was also put in place (López, Wormell, & Rodríguez, 2001). Outside
178 enclosures were 16–63 m² in area and approximately 4 m high, and were planted with
179 extensive natural vegetation as well as being furnished with ropes, branches and
180 platforms. The design of the buildings meant that tamarins had no visual contact while
181 in their indoor areas, and no or very limited visual contact outside. Levels of auditory
182 and olfactory contact were similar in all buildings.

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

187 Pied tamarins were fed three times daily, at approximately 0800–0830 (primate
188 pellet mix), 1200–1300 (fruit, vegetables and a protein item such as egg), and 1530–
189 1700 (insect feed). Tamarin enclosures were cleaned in the morning, and excess food
190 removed in the late afternoon. Tamarins were trained to sit on scales within their
191 enclosures and were weighed at least weekly.

192

193 Sample collection

194 Salivary cortisol sampling and analysis

195 Saliva samples were collected between January and June 2007 by keeping staff
196 using the technique described by Cross, Pines, & Rogers (2004). Samples were
197 collected on a weekly basis. All samples included in the analysis were collected in the
198 morning, as cortisol levels decrease during the day (Cross & Rogers, 2004). Individuals
199 were encouraged to chew on 1–2 cotton buds for up to a minute at a time to obtain
200 the required volume of saliva (50µl); a single food incentive, honey, was used on the
201 cotton bud. The cotton buds were then centrifuged at 3200 rpm to extract the saliva
202 and frozen at –20° C until analysis by the Central Science Laboratory in York, UK, using
203 commercially available ELISA test kits and previously described methods (Cross et al.,
204 2004; Cross & Rogers, 2004; Gladwell & Pick, 2007).

205

206 Fecal cortisol sampling and analysis

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

213 Each sample was dried in a fan-assisted oven at 55°C for 7.5 h and was refrozen
214 until needed for further extraction at –20°C. Following thawing, each dry sample was
215 ground with a pestle and sifted through a fine wire mesh to remove seeds and fibrous
216 material (Wasser et al., 1993). A 3ml aliquot of 90% (v/v) methanol was added to a 0.1
217 g portion of the resulting powder and mixed vigorously for 3 h (Heidolph Titramax 100,
218 1350 rpm, 1.5 mm orbit). This was then centrifuged at 2000 rpm for 15 min following
219 an adapted version of the methods used by Wasser et al. (1993). The supernatant was
220 poured into a glass test tube and the ethanol evaporated using compressed oxygen
221 free nitrogen gas (N₂) administered using a Pierce Reacti-Therm Heating Module at
222 40°C. The residue was resuspended in 0.5 ml phosphate buffer saline buffer containing
223 0.1% (w/v) Bovine Serum Albumin (BSA) and microcentrifuged for 2 min at 6500 rpm
224 to remove any remaining solid particles. The resulting supernatant was stored at –20°
225 C until needed for measurement. Hormone values were expressed as pg/50µl of fecal
226 extract.

227 Cortisol enzyme immunoassay

228 *Immunological validation:* A modified version of an enzyme immunoassay
229 described by Armstrong & Santymire (2013) was used to quantify levels of fGC. The
230 assay was immunologically validated for quantification of fGC levels in our population
231 of captive pied tamarins using a representative sample pool consisting of 50µL of
232 extract taken from all samples (Diamandis & Christopoulos, 1996). The antibody
233 (R4866, raised against a steroid bovine albumin in rabbit (Munro & Stabenfeldt, 1985))
234 was diluted to 1:12000 in coating buffer, and the cortisol horseradish peroxidase was

235 diluted to 1:22000 in phosphate buffer solution containing 0.1% (w/v) BSA. Samples
236 were run in duplicate at 1: 100 dilution.

237 Cross-reactivity of the cortisol antibody was 100% with cortisol. Cross-reactivity
238 with similar steroids was 9.9% with prednisolone, 6.3% with prednisone, 5.0% with
239 cortisone, 0.7% with corticosterone and <0.3% with various other steroids (Munro &
240 Stabenfeldt, 1985). Assay specificity was demonstrated twice by parallel displacement
241 curves of serial dilutions of cortisol standard and the pied tamarin pool over the 10–
242 90% binding range (ANCOVA; $F_{3,48} = 1.074$, n.s.; $F_{3,41} = 1.053$, n.s.). Recovery of the
243 standards (halving dilutions in the range from 500 to 7.8 pg) added to a 1:100 dilution
244 of a mixed fecal pool was $96.16 \pm 14.91\%$ inferring good accuracy ($r = 0.996$, $F_{1,5} =$
245 595.63 , $P < 0.0001$). Intra-assay coefficients of variation for low, medium and high
246 concentration quality controls were 1.95, 4.79 and 4.93%, respectively. Interassay
247 coefficients of variation for low and high concentration quality controls were 10.03%
248 (n=5 plates) and 18.55% (n=7 plates). The sensitivity of the assay was approximately
249 1.95 pg/ml.

250 *Biological validation:* To determine whether our assay detected biologically
251 meaningful changes, we assessed fGC levels in the morning and afternoon under
252 control conditions to test for circadian variation this hormone. Samples were collected
253 in the morning before 1100 (n = 83) and in the afternoon after 1400 (n = 97) from a
254 mix of adult males and females over a 5-month period. Log transformed cortisol levels
255 were compared between samples deposited in the morning versus the afternoon using
256 an independent t test. Levels of fecal cortisol were significantly raised in the afternoon

257 compared to the morning in accordance with the typical circadian variation in levels of
258 excreted cortisol ($t = 5.128$, $d.f. = 179$, $P < 0.001$; mean \pm S.E.M in the morning: 564.76
259 ± 116.08 ng/ml and the afternoon: 1027.37 ± 117.03 ng/ml; Sousa & Ziegler, 1998).

260

261 Data analysis

262 General information about the samples, including dates, times, individuals, and
263 any events such as illness, social tension or catch-ups that might affect stress levels,
264 was recorded in daily diaries. For each sample, the most recent weight from that
265 individual prior to sample collection was included in the analysis. We excluded data
266 from individuals who had recently undergone any potentially stressful procedures (e.g.
267 medical treatment, moves to new enclosures, etc.). The final data sets included 214
268 saliva samples from 36 individuals (mean number of samples per individual = $5.94 \pm$
269 3.76 SD), and 227 fecal samples from 27 individuals (mean number of samples per
270 individual = 8.41 ± 3.33 SD).

271 Age (in years) and weight (in g) were included as continuous variables in the
272 analysis. For saliva samples, the mean age of tamarins at the time of sampling was
273 5.45 years ± 4.00 SD, range 0.21 – 16.43 years; for fecal samples, the mean age was
274 5.66 years ± 4.75 SD, range 0.72 – 21.88 years. Although wild pied tamarins typically
275 weigh around 430 g (Ford, 1994), weights obtained during this study averaged 501 g +
276 81 SD, which is comparable to weights obtained from the global captive population of
277 pied tamarins (Species360, 2018).

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

- 279
- Rearing history: either hand-reared or parent-reared.
- 280
- Enclosure type: either on-show (with permanent access to off-exhibit
- 281
- 282
- 283
- Social status: dominant or subordinate. Tamarins who were breeding or
- 284
- 285
- 286
- 287
- 288
- 289
- 290
- Group composition, categorized as: breeding pair without offspring,
- 291
- 292
- 293
- 294
- 295
- For females in breeding situations, we counted back from the birth of
- 296
- 297
- 298
- 299

300 The dependent variable, cortisol level (in ng/ml for saliva samples, and pg/50µl
301 for fecal samples), was not normally distributed in either case and was therefore

302 natural log-transformed before analysis. We examined the transformed data and
303 standardized residuals graphically to test the assumptions of normality and
304 homoscedasticity, and removed outliers. Final sample sizes for each categorical
305 variable are given in Table 1. We used separate generalized linear mixed models
306 (GLMMs) for each sample type, including individual as a random factor to control for
307 the fact that several samples were obtained from many of the tamarins. Data were
308 analyzed using the statistical software R (version 3.5.1; R Core Development Team,
309 2018) and the packages nlme (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2018),
310 lme4 (Bates, Maechler, Bolker & Walker, 2015) and MuMIn (Barton, 2015).

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

316 We then ran separate GLMMs for each sample type, including main effects plus an
317 interaction between sex and rearing history, with individual as a random factor. We
318 followed the method outlined by Grueber, Nakagawa, Laws, & Jamieson (2011) for
319 model averaging of GLMMs, and considered models with $\Delta AIC_c < 2$ as having strong
320 support, and those with a ΔAIC_c of >2 to have less support (Burnham and Anderson,
321 2002). We constructed a standardized global model containing all factors of interest,
322 and then used the dredge function in MuMIn to obtain a list of all models with a ΔAIC_c
323 <2 from the best model. We then used model averaging to obtain estimates and 95%

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

326

327 Ethical statement

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

333 Conflict of interest statement

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

335 Results

336 Salivary cortisol

337 We found no effect of pregnancy on cortisol levels in saliva, and so we used all
338 female samples in subsequent analyses. The final GLMM for log cortisol levels in saliva
339 included only two fixed factors, enclosure type and sex; no other model had a ΔAIC_c
340 < 2 . The influence of enclosure type was in the opposite direction to the predicted one:
341 individuals housed in off-show enclosures had higher salivary cortisol levels than
342 tamarins in on-show exhibits ($\beta = -0.4399 \pm 0.1118$ SE, 95% CI = -0.6887, -0.2196;

343 Figure 1A). Female tamarins had higher salivary cortisol levels than males ($\beta = -0.2905$
344 ± 0.1122 SE, 95% CI = -0.5177, -0.0566; Figure 1B).

345 Fecal cortisol

346 Although we had few samples from females in late pregnancy, we found some
347 effect of stage of pregnancy on log cortisol levels in fecal samples ($F_{2,55} = 2.684$, $P =$
348 0.077 ; Figure 2). Examination of paired contrasts showed that females in late
349 pregnancy had significantly higher cortisol levels than those in early pregnancy ($P =$
350 0.033), but the other two pairs did not differ significantly. For further analysis, we
351 removed samples from females in late pregnancy ($n = 3$ samples from two females)
352 from the data set.

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

365 Discussion

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

380 We found several factors that affected cortisol levels in zoo-housed pied tamarins.
381 However, the results were somewhat different depending on whether cortisol was
382 measured in saliva or feces. Only sex and enclosure type had an effect on salivary
383 cortisol in pied tamarins, while sex, pregnancy, rearing history, social status, weight
384 and enclosure type all influenced fecal cortisol levels. Fecal cortisol may give a better
385 picture of baseline cortisol levels as it is less affected by daily acute events and as such
386 represents the effects of underlying individual, social and environmental factors

387 affecting cortisol production (Millspaugh & Washburn, 2004; Heistermann, 2010). It is
388 also the easiest type of sample to collect for analysis. As salivary cortisol represents an
389 individual's immediate reaction to an event (Kuhar, Bettinger, & Laudenslager, 2005;
390 Laudenslager, Bettinger, & Sackett, 2006), it may therefore be of most help in
391 investigating the impact of acute stressors on the wellbeing of pied tamarins.
392 Unfortunately we were not able to make direct comparisons between fecal and
393 salivary cortisol levels in individuals, as the two sets of samples were collected at
394 different times.

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

406

407 Individual factors affecting cortisol levels

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

421 Weight loss is one of the most consistent and pronounced changes during
422 exposure to stress (Tamashiro et al., 2005). We found no effect of weight on salivary
423 cortisol, but fecal cortisol decreased significantly as weight increased, as we expected
424 (Prediction 2). Links have been found in a number of taxa between poorer body
425 condition or lower weight, and higher baseline cortisol levels (e.g. Macbeth, Cattet,
426 Obbard, Middel, & Janz, 2012; Cattet et al., 2014; Trevisan et al., 2017) or higher
427 glucocorticoid reactivity (Breuner & Hahn, 2003; Pereyra & Wingfield, 2003). Changes
428 in weight may therefore give an early indication of both increased stress levels and the

429 possibility of wasting syndrome, and thus regular non-disruptive weighing, as used in
430 our colony, is an important tool in monitoring health and wellbeing in tamarins in
431 captivity. We excluded data from tamarins with symptoms of illness, but it is possible
432 that individual health parameters could have confounded cortisol titers in our subjects.

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

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

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

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

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

474 the level of conspecific as opposed to human interaction throughout life, the lower the
475 level of cortisol in hair samples (Jacobson, Freeman, Santymire & Ross, 2018).

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

486 Our results also raise questions about the role and ethics of hand-rearing pied
487 tamarins. Attitudes towards hand-rearing primates in zoos have changed since the
488 1950s, when it was frequently practiced in order to increase survival rates (Porton &
489 Niebruegge, 2006); realization of the importance of parental rearing, in particular for
490 social development, has led to a decrease in the number of primates removed by zoos
491 for hand-rearing and an improved understanding of the need for early socialization
492 with conspecifics. However, for threatened species such as pied tamarins, hand rearing
493 has remained an important tool to increase captive population sizes. Although in
494 modern zoos, hand-reared infants are socialized from a very early age, work remains
495 to be done in developing rearing and socialization methods that result in adults that
496 are behaviorally and physiologically indistinguishable from parent-reared individuals.

497

498 Social factors affecting cortisol levels

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

519 provisioned *L. catta*; Starling et al., 2010) or lactation (*Cercopithecus mitis*; Foerster,
520 Cords & Monfort, 2011). Our study might have yielded different relationships between
521 cortisol and social rank under different social or environmental conditions such as
522 following a birth or aggression.

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

535

536 Environmental factors affecting cortisol levels

537 We found that in both saliva and feces, cortisol levels were significantly higher in
538 tamarins living in enclosures that were not on show to the public (Prediction 8). This
539 was unexpected – the presence of visitors has often been highlighted as a source of
540 stress and a cause of abnormal behavior in zoo primates (Hosey, 2000), and an earlier

541 study (Armstrong & Santymire, 2012) found that a pair of pied tamarins on exhibit had
542 higher levels of fecal glucocorticoid metabolites than a pair living off-show. However,
543 our results are in agreement with previous behavioral studies of pied tamarins at
544 Jersey Zoo, which found that visitors had no effect on levels of stress-related behaviors
545 (Holm et al., 2012), and that tamarins housed on-show were less vigilant, and vocalized
546 and scent-marked less often than tamarins in off-show enclosures (Steinbrecher,
547 2016). We suggest that this may be because tamarins on show to the public at the zoo
548 have high and relatively naturalistic enclosures with stand-off barriers preventing
549 direct contact with the public (Figure 5), and they are also able to retreat indoors
550 whenever they wish. Off-show enclosures are similar in design, but typically house
551 more groups of pied tamarins per building (though fewer groups overall) than the on-
552 show enclosures. Crowding has been linked to elevated cortisol in several primate
553 species and may have contributed to the raised levels in the tamarins off-show
554 (Dettmer, Novak, Meyer, & Suomi, 2014; Gabriel, Gould, & Cook, 2018; Pearson,
555 Reeder & Judge, 2015). Interestingly, Pirovino et al. (2011) also found that cortisol
556 values varied across pileated gibbons not only living in different institutions, but across
557 animals residing in different enclosures within the same institution, illustrating the
558 mixture of factors that may affect cortisol at a local level.

559 Cabana et al. (2018) reported that the likelihood of wasting syndrome developing
560 in zoo-housed callitrichids was lower if there were visual barriers between visitors and
561 monkeys, and if the animals had safe areas to which they could retreat. Another study
562 at Jersey Zoo demonstrated that the level of noise in a building (mostly due to other
563 animals housed there) was positively related to cortisol levels in *S. bicolor* (Simpkins,

564 Routh, Wormell, & Price, 2013). Similarly, Kuhar et al. (2003) found that colony
565 housed *S. geoffroyi* showed higher levels of aggression and lower activity levels than
566 non-colony-housed tamarins, and higher levels of physical activity have been shown to
567 mitigate stress responses in women (Puterman et al. 2011). It would therefore be
568 interesting to investigate activity levels in tamarins housed on- and offshow, and to
569 monitor changes in both behavior and cortisol levels in groups that are moved from
570 one enclosure type to another. Further research into how enclosure design,
571 management, housing density and proximity to conspecifics versus other species
572 influence behavior and stress levels in pied tamarins and other callitrichids is therefore
573 needed.

574 Although our sample size was comparatively large, we did not have sufficient data
575 to be able to take into account other non-stress related and stress-related factors that
576 might affect HPA function and thus cortisol levels, such as personality (Martin and
577 Reale, 2008; Shepherdson et al., 2013), which influences cortisol in another callitrichid
578 species, the common marmoset (Inoue-Murayama et al., 2018). Variations in
579 metabolic rate (Goymann 2012), activity levels (Smith, McGreer-Whitworth, & French,
580 1998) and amount of keeper interaction (Carlstead, Paris, & Brown, 2018) were not
581 controlled in this study, but may also affect cortisol levels. Finally, seasonal variation in
582 temperature or poor weather (which could reduce visitor numbers, but potentially
583 increase intragroup tension as tamarins stayed indoors for longer periods) may also
584 have had an effect on cortisol in this study. For example, it would be interesting to
585 investigate levels of salivary cortisol when tamarins are indoors versus outdoors.
586 Focused studies on these possibilities, and further research into the factors we

587 investigated, would contribute a great deal to the successful management of this and
588 other threatened callitrichid species both in captivity, and in cases where intensive
589 management of free-living populations becomes necessary.

590

591 Conclusions

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

609 disturbance from visitors as long as their contact with people is minimized
610 by the use of barriers and access to off-exhibit areas.
611 8. Age does not affect levels of cortisol in this species.

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619

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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 $\Delta AIC_c < 2$ 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.

Variable	Estimate (β)	SE	Confidence intervals	
			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

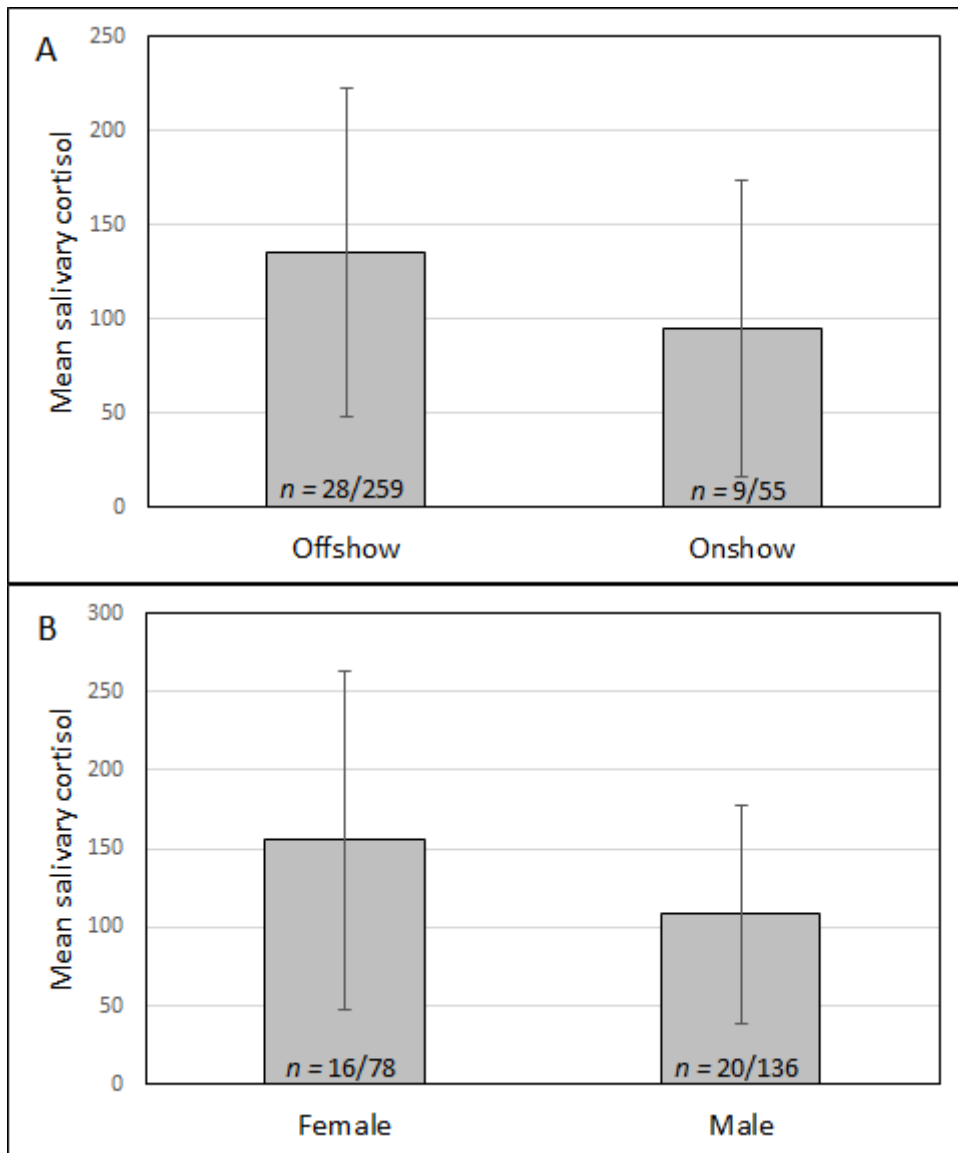


Figure 1. A: Mean salivary cortisol in pied tamarins housed in different enclosure types. B: Mean salivary cortisol in male and female pied tamarins. Other factors did not appear in the final GLMM and are not illustrated. Means were back-transformed from mean log cortisol values. Vertical bars represent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

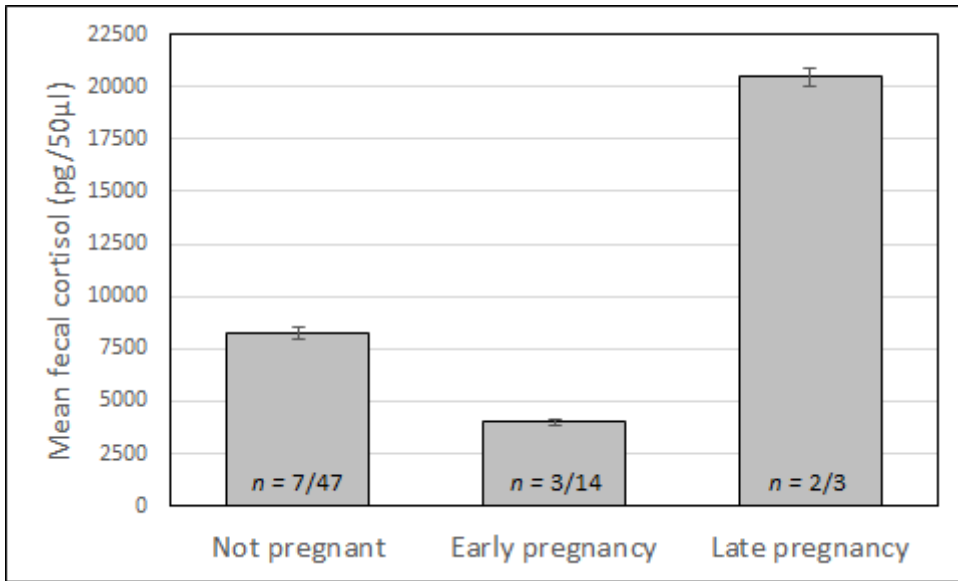


Figure 2. Effect of pregnancy stage on mean fecal cortisol in breeding females. Means were back-transformed from mean log cortisol values. Vertical bars represent coefficient of variation. Sample sizes (number of individuals/number of samples) are given at the base of each bar.

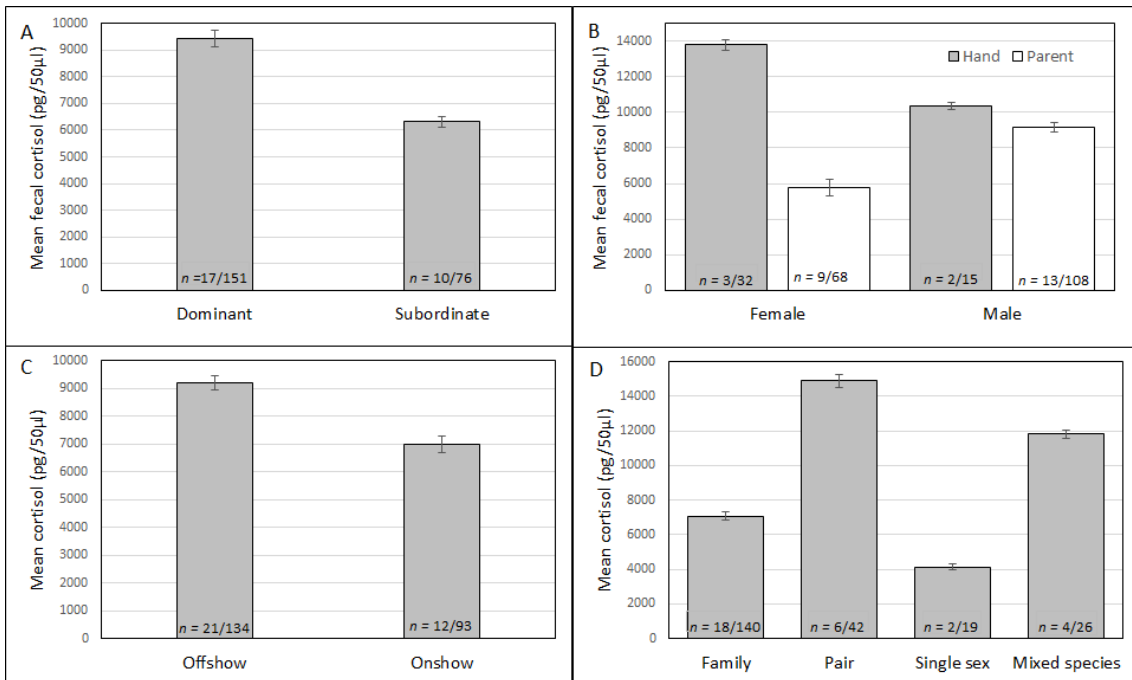


Figure 3. A: Mean fecal cortisol in dominant and subordinate pied tamarins. B: Effect of sex and rearing history on mean fecal cortisol in pied tamarins. C: Mean fecal cortisol in pied tamarins housed in different enclosure types. D: Mean fecal cortisol in pied tamarins housed in different group types. 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.

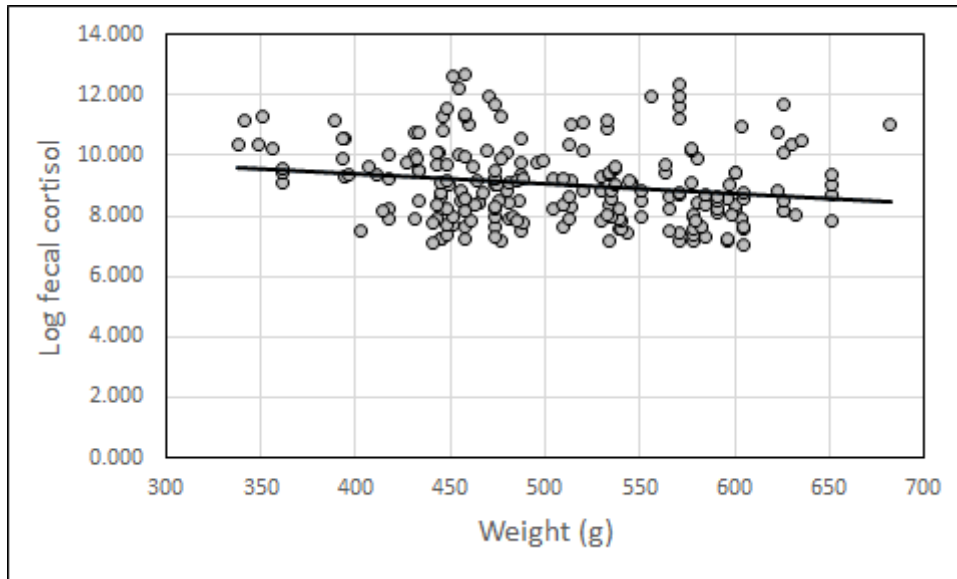


Figure 4. Relationship between log-transformed fecal cortisol levels and weight in pied tamarins.



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.