1 MATERNAL CORTICOSTERONE DEPOSITION IN AVIAN YOLK:
2 INFLUENCE OF LAYING ORDER AND GROUP SIZE IN A
3 JOINT-NESTING, COOPERATIVELY BREEDING SPECIES¹

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21 **Abstract**

22 Glucocorticoid hormones play a key role in day-to-day adjustments to fluctuating metabolic needs.
23 These hormones also mediate physiological and behavioral responses to stressful events, allowing
24 individuals to cope with stressors. Various environmental insults, such as a food shortages, predation
25 attempts, and agonistic encounters often elevate plasma glucocorticoid levels in vertebrates. Because
26 exposure to maternally-derived (via circulation or egg) glucocorticoids may be detrimental to the
27 developing embryo, maternal stress can have negative carryover effects on offspring fitness. We
28 examined corticosterone, the primary avian glucocorticoid, concentrations in egg yolk in a plural-
29 breeding, joint-nesting species, the smooth-billed ani (*Crotophaga ani*), in which females compete
30 among themselves to lay eggs in the final incubated clutch. We investigated whether yolk
31 corticosterone levels varied with laying order and group size. Because egg-laying competition leads to
32 physiological and social stress that is intensified with group size and laying order, we predicted that
33 yolk corticosterone levels should increase from the early to the late egg-laying period and from single
34 female to multi-female groups. In this two-year field study, we found that yolk corticosterone levels of
35 late-laid eggs within the communal clutch were higher in multi-female groups than in single female
36 groups. Results from this study suggest that laying females experience higher levels of stress in multi-
37 female groups and that this maternal stress influences yolk corticosterone concentrations. This study
38 identifies a novel cost of group-living in plural-breeding cooperatively breeding birds, namely an
39 increase in yolk corticosterone levels with group size that may result in detrimental effects on offspring
40 development.

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43 Keywords: Corticosterone; Yolk steroid hormone deposition; Smooth-billed ani; Communal breeding;
44 Social Stress; Radioimmunoassay.

45 **1. Introduction**

46

47 Avian eggs contain maternally-derived hormones that can have significant effects on
48 embryonic development, post-hatching growth, and offspring survival (for reviews see Groothuis et al.,
49 2005; Groothuis and Schwabl, 2008; Williams and Groothuis, 2015). The allocation of hormones to
50 the egg may vary in response to a number of factors including laying order, paternal quality, and food
51 availability (Gil, 2003; Groothuis et al., 2005; von Engelhardt and Groothuis, 2011). For instance, yolk
52 testosterone levels vary with laying order and can affect nestling begging behavior, growth, and social
53 status or interactions among nest-mates (Gil, 2003; Groothuis et al., 2005; Schwabl, 1993, 1996).
54 Some researchers interpret these studies to hypothesize that females use yolk hormone deposition to
55 adjust adaptively their reproductive investment in response to the current conditions (Schwabl et al.,
56 1997; Stoleson and Beissinger, 1995; see von Engelhardt and Groothuis, 2011; and Williams and
57 Groothuis, 2015 for reviews). While findings from research in this area are exciting, caution and
58 further studies are needed before one can assume causal links between yolk hormone levels and a
59 variable of interest, given contradictory findings across studies and the relatively few published works
60 from which to draw meaningful conclusions.

61 In his seminal paper, Schwabl (1993) reported that egg yolks contain measurable amounts of
62 sex steroid hormones, such as androstenedione, testosterone, 5 α -dihydrotestosterone, and 17 β -
63 estradiol, as well as low levels of the adrenal steroid hormone, corticosterone (CORT). Because
64 relatively low levels of CORT are present in eggs, this steroid hormone has received much less
65 attention than androgens (Groothuis et al., 2005), with a small number of published studies that have

66 focused on yolk CORT levels (Larsen et al., 2015; Love et al., 2005, 2008; Saino et al., 2005;
67 Vallarino et al., 2012). Furthermore, our understanding of yolk CORT effects may be further limited
68 by the methodology that has been commonly used to measure CORT. For instance, Rettenbacher et al.
69 (2009) suggest that yolk CORT levels may have been overestimated in various studies, as much of the
70 CORT measured in eggs may actually be progestens, as they exhibit considerable cross-reactivity with
71 the antibodies that have commonly been used. Given that progesterone is a common early product in
72 the production pathway of all steroids, it and related progestens can be expected to be abundant in yolk
73 (Lipar et al., 1999; Paitz and Bowden, 2010; Rettenbacher et al., 2009).

74 Knowledge of how CORT is incorporated into the egg yolk and whether or how it subsequently
75 affects development is limited, despite the fact that corticosterone is an important hormone that can
76 influence both maternal and offspring phenotype (Groothuis and Schwabl, 2008; Poisbleau et al., 2009;
77 Rettenbacher et al., 2005; Schoech et al., 2011; Williams and Groothuis, 2015). Pre- or peri-natal
78 exposure to elevated levels of maternal or endogenous glucocorticoids can have negative effects, both
79 short-term effects upon embryonic development (Eriksen et al., 2003; Heiblum et al., 2001; Love et al.,
80 2008; Mashaly, 1991), and long-term effects upon other traits, such as offspring body mass (Love et
81 al., 2005, 2008; Saino et al., 2005), growth (Hayward and Wingfield, 2004; Heiblum et al., 2001; Love
82 et al., 2005), sensitivity to stress (Hayward and Wingfield, 2004; Schoech et al., 2011; Welberg and
83 Seckl, 2001), and behavior (Lesage et al., 2004; Rubolini et al., 2005; Schoech et al., 2009). Although
84 the zona reticularis of the adrenal cortex is a known source of androgen production, no evidence of
85 ovarian CORT production has been reported (Groothuis and Schwabl, 2008). Thus, any CORT within
86 the yolk almost certainly is of adrenal origin that has moved to the ovary, and hence the yolk, via the
87 maternal circulatory system. Therefore, yolk CORT levels should, to some degree, reflect maternal
88 plasma levels of CORT given that it is a lipid-soluble hormone that likely enters the yolk by passive

89 diffusion (Almasi et al., 2012; Groothuis et al., 2005; Hayward and Wingfield, 2004; but see
90 Rettenbacher et al., 2005). However, the lipid-rich composition of yolk combined with the highly
91 vascularized nature of ovarian follicles and the lipophilic nature of steroid molecules establishes a
92 scenario where egg yolk could in effect serve as a CORT ‘sink’. Because the affinity of CORT for yolk
93 is much greater than it is for aqueous plasma, CORT might have a propensity to move into the yolk,
94 thus concentrating therein (Groothuis and Schwabl, 2008). Yolk CORT levels almost certainly
95 represent accumulation over the several day period of yolk deposition following recruitment of a given
96 follicle. As a result, yolk CORT may represent a time-integrated measure of maternal CORT (Cook,
97 2012). Therefore, yolk CORT levels needn’t necessarily reflect a laying female’s current state, rather,
98 levels within the egg may reflect the environmental conditions that the female experienced during yolk
99 formation (Love et al., 2005; Saino et al., 2005). For example, female barn swallows (*Hirundo rustica*)
100 that were exposed to a predator during egg-laying produced eggs with higher concentrations of CORT
101 than controls (Saino et al., 2005). Further, embryos that were exposed to higher CORT levels were less
102 likely to successfully hatch, and of those that hatched and subsequently fledged, the young were
103 smaller than controls (Saino et al., 2005). Therefore, stressful conditions experienced by mothers may
104 influence egg quality and offspring phenotype, which in turn, may negatively affect maternal fitness
105 (Rubolini et al., 2005; Saino et al., 2005; Sinervo, 1998).

106 In this study, we examined yolk corticosterone concentrations in the smooth-billed ani
107 (*Crotophaga ani*), a plural-breeding, joint-nesting communally breeding species. Anis are
108 characterized by an unusual breeding system wherein groups of between one and seven socially
109 monogamous pairs share a single nest (Quinn and Startek-Foote, 2000). Intense egg-laying competition
110 for access to the final incubated clutch of eggs occurs in multi-female groups with eggs laid early
111 having high probabilities of loss because of egg tossing and burial beneath nest materials and

112 subsequently laid eggs (Quinn and Startek-Foote, 2000; Schmaltz et al., 2008a). This egg-laying
113 competition intensifies as group size increases and both the number of eggs laid and lost per female
114 increase with group size (Schmaltz et al., 2008a). We investigated whether yolk CORT levels varied
115 with group size and laying order (i.e., laying order for all eggs within a specific communal clutch). We
116 predicted an increase in yolk CORT levels from the early to the late laid eggs because costs associated
117 with egg production and the ensuing physiological stress likely increase during egg production
118 (Salvante and Williams, 2003; Williams, 2005). Such an increase in yolk CORT levels with laying
119 order has been reported recently in lesser black-backed gulls (*Larus fuscus*; Larsen et al., 2015).
120 Females in single-female ani groups do not experience the potentially stressful agonistic within-group
121 interactions than their multi-female counterparts experience. Further, females in single female groups
122 lay fewer eggs than their multi-female counterparts. Therefore, we also predicted that when compared
123 to multi-female groups, yolk CORT levels in single-female groups should (1) be generally lower and
124 (2) not exhibit marked laying order increases.

125

126 **2. Material and Methods**

127

128 **2.1 Study sites**

129

130 We studied smooth-billed anis at the Cabo Rojo (17°59'N, 67°10'W, elevation from 2 to 42 m)
131 and Laguna Cartagena (18°01'N, 67°06'W, elevation from 55 to 71 m) National Wildlife refuges in
132 south-western Puerto Rico during the rainy season when most ani breeding occurs (September-
133 January, 2003-2004 and 2004-2005, hereafter denoted as the 2003 and 2004 seasons, respectively).
134 The south-western portion of Puerto Rico has a dry tropical climate and both Cabo Rojo (587 acres)

135 and Laguna Cartagena (794 acres) refuges contain second-growth dry scrubland, open, and disturbed
136 habitats that attract breeding anis (Loflin, 1983; Quinn and Startek-Foote, 2000).

137

138 **2.2 Field procedures**

139

140 Details about group size determination and nest check regime are provided elsewhere
141 (Schmaltz et al., 2008a). In brief, we checked every active nest daily during the egg-laying period and
142 marked every egg with non-toxic markers to indicate egg number in the laying sequence of a given
143 nest. We sampled eggs that were among the first or last 20 % of a group's clutch (i.e., early- *versus*
144 late-laid eggs) and did the same in nests with single female breeders. Therefore, from this point
145 onwards, we use the terminology 'group egg-laying order' instead of 'egg-laying order'. We collected
146 yolk samples from freshly laid, yet to be incubated eggs by inserting a 25 gauge needle through the
147 eggshell at the middle of the long axis of the egg, following the method pioneered by Schwabl (1993).
148 We inserted the needle halfway within the egg to reach the center of the yolk (see Lipar et al., 1999)
149 and extracted a small sample (approximately 40 mg) of yolk with a 1 ml disposable syringe. We sealed
150 the insertion hole with New-Skin[®] liquid bandage (Medtech, Jackson, WY, USA) and returned the egg
151 to its nest. We transferred yolk samples to 1.5-ml Eppendorf[®] tubes that were kept cool on ice until
152 return to the lab where they were frozen and stored until shipment overnight on ice to the University of
153 Memphis for assay (see below). To verify the uniformity of the sampling method, we also collected
154 samples by yolk biopsies from 14 freshly laid intact eggs that had been recently 'tossed' from nests not
155 under study (0-2 day-old eggs). We froze these 14 eggs, and then, separated the yolk from the
156 albumen. We subsequently thawed and homogenized the whole yolk, and collected another small
157 sample (approximately 40 mg) for assay from the same eggs. Because both Lipar et al. (1999) and

158 Rettenbacher et al. (2005) found differential levels of hormones across yolk layers, we compared
159 hormone values for each egg from homogenized whole egg yolks with those obtained from the yolk
160 center of the same egg using a biopsy (see below).

161

162 **2.3 Radioimmunoassay**

163

164 All yolk samples were analyzed for the presence of CORT with a competitive-binding
165 radioimmunoassay (Ball and Wingfield, 1986; Schoech et al., 1996; Wingfield and Farner, 1975; also
166 see Schwabl, 1993 for specific methods that pertain to extraction of steroids from yolk). We weighed a
167 sub-sample, approximately 40 mg, to the nearest 0.01 mg, added 500 µl distilled water and three glass
168 beads to each sample and vortexed them repeatedly to homogenize the yolk-water mixture. We added
169 approximately 2,000 cpm of [³H]corticosterone (PerkinElmer Life and Analytical Sciences, Inc.,
170 Waltham, MA) to each yolk sample to allow calculation of the recovery percentages after extraction
171 and chromatography. After further mixing, we let the homogenate equilibrate for at least 12 hr at 4°C
172 prior to extraction. The steroids were extracted from the aqueous phase by adding 3 ml of a
173 petroleum:diethyl ether (30%:70%) solution, that was vortexed, then snap-frozen, and the ether
174 containing the steroids was then decanted. This was done twice for each sample and the resultant 6 ml
175 of ether solution was then evaporated in a water bath (37°C) under a stream of N₂ gas. We then added
176 1 ml of 90% ethanol to the tubes, vortexed, and then stored overnight at -20°C (Schwabl, 1993). We
177 separated the precipitated proteins and lipids from the ethanol fraction by centrifugation at 2000 rpm
178 for 15 min. The ethanol phase was decanted and subsequently dried under N₂. Samples were
179 resuspended in 0.5 ml of a solution of 10% ethyl acetate in isooctane, vortex, and then added to hand-
180 packed chromatography columns that consisted of a celite:water (3 g:1 ml) lower phase and a

181 celite:ethylene glycol:propylene glycol (6 g:1.5 ml:1.5 ml) upper phase. Increasingly polar volumes of
182 solvent mixtures (ethyl acetate:isooctane) that were forced through the columns by pressurized N₂
183 allowed collection of the different steroid-containing fractions based upon their polarity. After the
184 Progesterone (P), DHT, T, and E₂ fractions were removed, we collected the CORT fraction from each
185 column with 4 ml of solution containing equal parts of ethyl acetate and isooctane. This
186 chromatography fractionation renders the potential problems of antibody cross-reactivity to progestens
187 noted by Rettenbacher et al. (2009) a non-issue. The recovered T fractions were analyzed, along with
188 plasma T levels in adults, in a separate study (Schmaltz et al., 2008b). Each elute containing the CORT
189 was dried under N₂, the sample was then reconstituted in buffer, and stored overnight at 4°C. We
190 compared duplicate values of each sample to a standard curve that ranged in concentration from 2000
191 to 7.81 pg. Yolk samples were spread across three different assays. The intra-assay coefficients of
192 variation based on six standards within each of the three assays were 5.7%, 9.6%, and 10.3%, and the
193 inter-assay coefficient of variation was 8.7%. Corticosterone antibody and standard for assay
194 procedures were obtained from Biogenesis and Sigma, respectively.

195

196 **2.4 Data and statistical analysis**

197

198 For each nest, we averaged CORT values from different eggs laid during the early (first 20%)
199 period and late (last 20%) period, producing two average CORT values per nest (early and late). In
200 multi-female nests, each data point (early or late) represents an average from different eggs that were
201 laid within 1.4 d (± 0.29) of one another on average. Eggs belonging to the same category (early or
202 late) within a given nest were, therefore, almost certainly laid by different females as ani females lay
203 an egg every other day during the laying period (Quinn and Startek-Foote, 2000). Because females in

204 single-female groups lay 5.3 ± 0.3 eggs on average, only the first and last laid eggs were included in
205 the early and late egg-laying categories, respectively. Therefore, no averaging was necessary for 9
206 nests. For the other 17 groups, an average of 1.8 eggs with a range of 1 to 4 eggs (early laying
207 category) and an average of 1.5 eggs with a range of 1 to 3 eggs (late laying category) were collected.
208 For any given smooth-billed ani nesting group, membership and group sizes differed between years
209 (Schmaltz et al., 2008a); therefore, groups were treated as independent units. We studied a total of 17
210 groups in 2003 and 9 groups in 2004. Of these 26 groups, 15 were located at Cabo Rojo, and 11 at
211 Laguna Cartagena. The mean lay-date for the early and late egg-laying periods were respectively 60.4
212 (± 6.5) and 70.0 (± 6.8) days from the beginning of the reproductive season (set as September first).
213 Because of nest failures due to depredation or destruction by high winds, we were unable to sample
214 late-laid eggs from four groups. Therefore, we only include early egg data for those four nests (26
215 groups in the early category vs 22 groups in late category). Note that exclusion of these four early-laid
216 data points does not qualitatively change our results. We monitored and analyzed a total of 86 fresh
217 eggs from 26 different ani groups during the two years of the study. Of these, a total of 53 eggs had
218 been either tossed or buried. This percentage of egg loss is comparable to other studies in which egg
219 puncturing was not performed (Schmaltz et al., 2008a). For the factor group-size, groups of 4 and 5
220 females were merged to one category (> 4 females) because of low samples size of these groups. For
221 multi-female groups, the variable day of year was calculated by averaging the laying dates of the
222 different eggs belonging to a laying category (early or late). Yolk CORT values were rank-transformed
223 to meet the requirements of parametric tests. To check for the assumptions of linearity of model and
224 normally distributed and homogeneous residuals, we visually inspected the residuals plotted against
225 fitted values and QQ plots. We used a mixed effect model with normal distribution to analyze variation
226 in yolk CORT by including group egg-laying order (early *versus* late), year, site location, and group

size as fixed factors, group ID as random factor, and day of year as a covariate. We tested for all main effects and two-way interactions in the model. We tested group size effects in the early and late egg-laying categories with a post hoc test (least significant difference: LSD). We used a t-test to look at possible yolk CORT differences between retained and lost (buried or tossed) eggs. For the t-test, we did not assume equality of variances as Levene's test was significant. This explains the below change in degrees of freedom from 84 to 57. We examined the relationship between CORT levels from both sampling methods (puncturing and whole yolk) with a Spearman rank correlation. All tests were performed in SPSS 23.0 (SPSS Inc.) as two-tailed tests with an α of 0.05. Means are provided with standard errors (SE). The standardized effect sizes (Cohen's d) are reported followed by their 95% confidence intervals (CI). Cohen's d and CI values were calculated using the 'effect size calculator' (David B. Wilson; www.campbellcollaboration.org/resources/effect_size_input.php).

3. Results

CORT levels from egg puncture samples were significantly correlated with levels from the homogenized whole yolk samples ($r_s = 0.86$, $N = 14$, $P < 0.001$). There was no significant overall effect of group egg-laying order (early *versus* late) on yolk levels of CORT ($F_{1,38} = 1.47$, $P = 0.232$; Fig. 1), with a small effect size Cohen's d value of 0.09 (-0.48 to 0.65). However, levels increased with group size ($F_{3,25} = 4.94$, $P = 0.008$), with a large effect size Cohen's d value of 0.79 (0.15 to 1.43) for single vs multi-female groups. Yolk CORT levels did not differ between years ($F_{1,27} = 1.36$, $P = 0.254$; Cohen's d = 0.14 (-0.45 to 0.73); Fig. 1), or days within years ($F_{1,28} = 3.33$, $P = 0.079$), but did differ between our two field location ($F_{1,27} = 7.26$, $P = 0.012$) with a small effect size Cohen's d value of 0.23 (-0.34 to 0.80). There were no significant two-way interaction terms in the model. Within the early

egg-laying order category, yolk CORT levels did not differ between the four different group sizes ($F_{3,19} = 0.33$, $P = 0.803$; Fig. 2). However, yolk CORT levels in late-laid eggs were higher in multi-female groups when compared to single-female groups ($F_{3,15} = 3.99$, $P = 0.028$; Fig. 2). This yolk CORT increase in late-laid eggs was most pronounced from single-female groups up to groups of three females as levels dropped in the largest group of four females (Fig. 2). There was no significant difference between yolk CORT levels from eggs that were retained and eggs that were lost to either egg tossing or burial (t-test: $t_{57} = -1.78$, $P = 0.08$).

4. Discussion

We found different patterns of yolk CORT in single- and multi-female groups, suggesting that increased group size acted as a source of stress that was expressed in the late laid eggs. The effect size of group size was much higher than the effect size of laying order. With this finding, we postulate that the increased competition experienced by female anis in multi-female groups induced a social and physiological stress that, in turn, increased adrenal CORT secretion which subsequently resulted in increased yolk CORT levels.

The increased CORT in late-laid eggs in multi-female ani groups could in theory result from both behavioral and physiological factors, both of which could cause increases in a female's circulating CORT levels. These factors are likely to be important in a joint nesting species like the smooth-billed ani given the intense scramble competition that females experience (Schmaltz et al., 2008a). First, at the behavioral level, competitive interactions, for access to the nest, representation in the final clutch of eggs, and in general (e.g., for food, social interactions, etc.), are expected to be higher in multi-female ani groups than for females that nest singly. Such increased competitive

273 interactions could act as a social stressor that would result in increased glucocorticoid levels, as has
274 been found in other social species (Abbott et al., 2003; Goymann and Wingfield, 2004; Sapolsky et al.,
275 2000; Wingfield et al., 1997). Our findings that the effect size due to group size was large and that the
276 effect of laying order was apparent in multi-female groups, but not in single female groups support the
277 idea that the effect of sociality was important. Second, elevated CORT titers could result from the
278 physiological events that accompany egg-laying. For example, egg production, ovulation, and
279 oviposition may be accompanied with increased CORT levels (Hegner and Wingfield, 1986; Kitaysky
280 et al., 1999; Silverin and Wingfield, 1982; Wingfield and Farner, 1978), and experimental evidence
281 links egg production with elevated plasma CORT levels (Etches et al., 1984; Salvante and Williams,
282 2003; Williams et al., 1985). While all females, irrespective of group size, would be expected to
283 experience any CORT increases that result from these physiological mechanisms, on average females
284 in multiple-female groups lay more eggs over a longer period than females in single-female groups
285 (Schmaltz et al., 2008a). That we detected no evidence of an increase in late- versus early-laid eggs in
286 single-female groups may be due to insufficient sample size or reflect that group size results in greater
287 stress than do the above noted physiological mechanisms. In addition, the significant difference
288 observed between our two field locations also point toward a link between egg production and CORT
289 levels.

290 Our Laguna Cartegana field site experiences higher rainfall than our Cabo Rojo site (Weaver
291 and Schwagerl, 2009). This likely translates into a higher food abundance (insects, spiders, lizards) for
292 anis at Laguna Cartagena since rainfall often correlates with insect abundance in dry tropical scrubland
293 (Strong and Sherry, 2000; Dugger et al. 2004). Yolk CORT levels were significantly higher at Cabo
294 Rojo ($3.6 \text{ pg/mg} \pm 0.7$) than at Laguna Cartegana ($2.3 \text{ pg/mg} \pm 0.5$). Dry years and low food
295 abundance have been associated with higher baseline levels of CORT in other species, such as the

296 cooperatively breeding Florida scrub-jay (*Aphelocoma coerulescens*, Schoech et al., 2009). The lower
297 food abundance at Cabo Rojo may translate into higher egg-laying costs for female anis and explain
298 the elevated yolk CORT levels at that site.

299 Based on the large effect size found for group size and the fact that laying order effects were
300 small, especially for females nesting alone, it is evident that sociality is an important factor in the
301 breeding biology of anis. We found an unexpected drop in yolk CORT levels in late laid eggs in
302 groups of 4-5 females. This finding could potentially be explained by the small sample size for these
303 groups (N = 4). Although speculative, it is not unreasonable to hypothesize that the combination of
304 behavioral and physiological differences might explain the differences in the patterns of CORT in the
305 yolks of later laid-eggs in single- and multiple-female ani groups.

306 Ideally, we would have been able to measure plasma CORT levels in female anis to examine
307 whether the above postulated patterns linking circulating and yolk CORT levels hold. Unfortunately,
308 the difficulty of catching anis necessitated the use of a trapping method that precluded measurement of
309 baseline plasma levels of CORT (see Schmaltz et al., 2008b). As a result, we are unable to compare
310 plasma CORT levels of females among groups, or to compare yolk and plasma hormone levels as we
311 were able to do for testosterone in this species (see Schmaltz et al., 2008b). There was a trend for
312 higher CORT levels in retained eggs; possibly because eggs lost to either tossing or burial were more
313 likely to belong to the early egg-laying category.

314

315 **5. Conclusions**

316

317 Yolk CORT levels increased with group size in late laid eggs from females nesting alone to
318 females nesting with one or two other conspecifics. Although the influence of group size and social

319 stress on circulating CORT levels of individuals have been established in previous work (Abbott et al.,
320 2003; Goymann and Wingfield, 2004; Sapolsky et al., 2000; Wingfield et al., 1997), few studies have
321 investigated whether social and environmental variables affect CORT deposition in eggs (Love et al.,
322 2008; Saino et al., 2005). We know of no other study that has examined the influence of both group
323 size and laying order on yolk CORT levels. Results from this study suggest a potential cost of group-
324 living in cooperatively breeding birds, namely that the increase in yolk corticosterone levels might
325 shape the phenotype of the offspring. Alternatively, it is possible that such a higher CORT
326 environment during development is beneficial and serves to match offspring phenotype with the
327 postnatal environment that they will experience after hatching (Crino and Breuner, 2015). The
328 potential effects of glucocorticoids on chick behavior are the subject of on-going work in smooth-
329 billed anis.

330

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332

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344

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Fig 1.PDF

Fig 2.PDF

Fig. 1. Mean ($\pm$ SE) concentration of corticosterone in egg yolks as a function of year and group egg-laying order. The early laying order category corresponds to eggs laid during the first 20% of the clutch while the late category corresponds to eggs laid during the last 20% of the clutch. Group egg-laying order categories that differ significantly are denoted by different letters above the bars. The numbers above the x axis in the figure represent the number of nests in each category.

Fig. 2. Mean ($\pm$ SE) concentration of corticosterone in egg yolks as a function of group egg-laying order and group size (i.e., GS; the number of laying females in a group) combined across both years of the study. The early laying order category corresponds to eggs laid during the first 20% of the group clutch while the late category corresponds to eggs laid during the last 20% of the group clutch. Group egg-laying order categories that differ significantly are denoted by different letters above the bars. The line above bars represents groups that are not significantly different from one another (i.e., no differences of the early clutches with group size).