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1 **Social stress in female Columbian ground squirrels: density-independent**
2 **effects of kin contribute to variation in fecal glucocorticoid metabolites**

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16 data. CuB, RB conducted laboratory analyses. SS analyzed the data. RP provided antibodies and
17 expertise on FCM measurement. SS and VAV wrote the manuscript. All authors commented on the
18 paper.

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31

32

33 **ABSTRACT**

34 Social interactions among conspecifics can have marked effects on individual physiology, especially
35 through its modulation of the stress axis by affecting the production of adrenal glucocorticoids (GCs).
36 Previous research has focused on how individual GC levels may be influenced by social status, but
37 few studies have considered how the balance between positive (e.g. cooperation) and negative (e.g.
38 competition) social interactions shape individual GC levels. A lack of association between individual
39 GC levels and social factors may be confounded by opposite effects of social competition on the one
40 hand, and social cooperation on the other. We tested for these effects in the Columbian ground squirrel
41 (*Urocitellus columbianus*), a colonial rodent. During the breeding season, females are exposed to
42 territorial unrelated neighbors and to territorial, but more tolerant, close kin. On the one hand,
43 territoriality and competition for resources led us to predict a positive association between local
44 colony density and female GC levels. On the other hand, higher tolerance of philopatric kin females
45 and known fitness benefits led us to predict a negative association between kin numbers and female
46 GC levels. We compared levels of fecal cortisol metabolites (FCMs) in females at two different spatial
47 scales during lactation: local (a female's core territory during lactation, 30m-radius about her nest
48 burrow) and colony-wide. At the local scale, female FCM levels were neither related to colony density
49 nor to the number of co-breeding female kin, but FCM levels increased with age. At the colony scale,
50 female FCM levels varied in a quadratic fashion with female kin numbers. FCM levels decreased from
51 0 to 1 co-breeding kin present and increased with >1 kin present. Among females that had only one
52 co-breeding kin present, daughters (and littermate sisters and mothers, but not significantly) led to a 14%
53 reduction in FCM levels compared with females that had no kin. Our results reject the idea that local
54 colony density is associated with increased GC levels this species, but indicate subtle (positive and
55 negative) effects of kin on individual GC secretion. They further call into question the importance of
56 the nature of social relationships in modulating the stress experienced by individuals.

57 **Keywords:** Age, glucocorticoids, kinship, population density, social environment, stress

58 **SIGNIFICANCE STATEMENT**

59 Few studies have tested how the balance between positive (e.g. cooperation) and negative (e.g.
60 competition) social interactions shape individual stress and glucocorticoid (GC) levels in group-living
61 animals. In colonial Columbian ground squirrels, breeding females are exposed to territorial neighbors,
62 and to more tolerant close kin. We show that kin numbers have subtle (positive and negative) effects
63 on female GC levels. Compared to breeding females with no kin, female GC levels decrease by 15%
64 with the presence of a single co-breeding close relative, but increase with the presence of more than
65 one co-breeding related female. Among females that have only one co-breeding kin, the presence of
66 daughters (and littermate sisters and mothers, but not significantly) leads to a 14% reduction in female
67 GC levels. Our results highlight how GC levels may be influenced by the specific nature of social
68 relationships in group-living animals.

69

70

71 INTRODUCTION

72 In social organisms, the interactions resulting from regular contact with related and unrelated
73 animals may have profound effects on individual physiology, health, and fitness (Sapolsky 1992;
74 Bartolomucci 2007; Razzoli et al. 2018). Studies have highlighted both positive and negative effects of
75 social interactions (or lack thereof) on individual metabolic rate (Stefanski and Engler 1998; Sloman
76 et al. 2000; Willis and Brigham 2007; Cao and Dornhaus 2008), immunity (Stefanski and Engler 1998;
77 de Groot et al. 2001), and oxidative stress (Nation et al. 2008; Zhao et al. 2013; Beaulieu et al. 2014;
78 Lardy et al. 2016), as well as gene regulation and cellular maintenance (Kotrschal et al. 2007; Tung et
79 al. 2012; Aydinonat et al. 2014). In particular, the so-called stress axis (the hypothalamic-pituitary-
80 adrenal axis, HPA) may provide insight into these positive and negative effects (Harris 2020). The
81 HPA is one of the key physiological systems mediating the relationship between the organism and its
82 environment, permitting short-term adaptations to acute stressors, such as social conflict, and long-
83 term evolutionary responses to particular ecological and environmental challenges. The HPA axis is a
84 vital regulator of adaptation, with the glucocorticoid (GC) hormones from the adrenal glands
85 influencing the expression of approximately 10% of the genome and its targets including genes that
86 control metabolism, growth, repair, reproduction, and the management of resource allocation (Le et al.
87 2005). Because of its central role in maintaining homeostasis via the action of GC hormones
88 (Sapolsky et al. 2000; Wingfield and Romero 2001), a large number of studies have considered the
89 effects of the social environment (i.e. social interactions between conspecifics, territoriality,
90 population density, social status, etc.) on HPA axis activation (Boonstra and Boag 1992; Creel 2001;
91 DeVries 2002; Carere et al. 2003; Goymann and Wingfield 2004; Dantzer et al. 2013; reviewed in
92 Boonstra et al. 2007; Creel et al. 2013)

93 On one hand, social competition and conflict may increase individual stress and the activity of
94 the HPA axis, which can often be assessed through increases in individual GCs (Goymann and
95 Wingfield 2004; Ostner et al. 2008; Rubenstein and Shen 2009). On the other hand, social cooperation
96 may help to alleviate individual stress and decrease the activity of the HPA axis, through affiliative
97 social networks (e.g. Wittig et al. 2008, 2016), or social and emotional support (Turner-Cobb et al.

98 2000; Scheiber et al. 2009; Young et al. 2014), reducing or stabilizing individual GC levels. Positive
99 effects of the social environment on decreasing the activity of the HPA axis are expected where group-
100 living or social cooperation among individuals is known to have positive effects on fitness, for
101 instance by providing anti-predator benefits (Hare et al. 2015), or by decreasing rates of inter-
102 individual aggression and/or affecting the outcome of aggression (Frigerio et al. 2005). This is likely
103 to happen in species where stable cooperative alliances can form among social members (e.g. Young
104 et al. 2014), where cooperative family groups are the essential units of the social system, or where
105 tolerant kin co-occur. The direction in which the social environment affects the activity of the HPA
106 axis is thus complex and may be subtle. A lack of association between social factors and individual
107 stress load may result from the antagonistic effects of social competition on the one hand (increasing
108 individual GCs) and social cooperation on the other (decreasing individual GCs). In this regard,
109 concurrently evaluating the relationship between individual GC levels and aspects of the social
110 environment pertaining both to competition (e.g. number of territorial or dominant conspecifics) and
111 cooperation (e.g. number of social allies or nepotistic kin individuals) is likely to provide valuable
112 information on the physiological costs and benefits of sociality.

113 We tested for opposing (positive and negative) effects of the social environment on the
114 activity of the HPA axis in female Columbian ground squirrels (*Urocitellus columbianus*). Columbian
115 ground squirrels are colonial rodents living in colonies of up to over 100 individuals (Festa-Bianchet
116 and Boag 1982; Murie and Harris 1988). They are a hibernating species, with a short (3-4 mo.) active
117 season, during which, mature females (typically >1 year old) breed, raise a single litter, and actively
118 forage and fatten before subsequent hibernation (Murie and Harris 1982; Dobson et al. 1992). We
119 specifically focused on females during lactation for three reasons. First, females are the philopatric sex
120 in Columbian ground squirrels (King 1989a; Arnaud et al. 2012), allowing for social familiarity
121 among colony members (Hare 1992, 1994; Raynaud et al. 2008) and the establishment of social
122 relationships. Second, females during lactation are specifically territorial, defending a core-territory
123 limited to a ca. 30-m radius around individual nest burrows that are used for raising young (Festa-
124 Bianchet and Boag 1982; Murie and Harris 1988). Territorial aggression is expected to have

125 physiological effects on individuals, among which is the activation of the HPA axis, and the secretion
126 of GC hormones (Boonstra and Boag 1992; see Creel et al. 2013 for a review). Although females
127 defend a core territory during lactation, they regularly range throughout the entire colony in their daily
128 foraging activities during this period, and are subject both to local and colony-wide (up to *ca.* 2-3 ha
129 on our study sites) social environments (FSD et al., personal observations). Third, in Columbian
130 ground squirrels, female kin overlap both spatially and temporally (King and Murie 1985; Murie and
131 Harris 1988). The philopatry of kin provides both direct (Viblanco et al. 2010) and indirect (Dobson et
132 al. 2012) fitness benefits for breeding females. Direct kin-related fitness benefits appear to occur
133 mostly via increased breeding success through the production of larger litters and greater survival of
134 young to yearling age, both at first breeding (Neuhaus et al. 2004), and over a lifetime (Viblanco et al.
135 2010; Dobson et al. 2012). In turn, those fitness benefits likely arise because female kin are less
136 aggressive to one another (King 1989b; Viblanco et al. 2016a). Lowered aggression may facilitate the
137 acquisition/maintenance of breeding territories (Harris and Murie 1984; Neuhaus et al. 2004; Arnaud
138 et al. 2012), thus providing a safer environment for raising offspring (i.e. diminished risks of
139 infanticide by unrelated females; Stevens 1998), and allowing females to invest more energy into
140 reproduction (Viblanco et al. 2016b).

141 Given the above, we hypothesized that both local colony density and the presence of female
142 co-breeding kin should affect the activity of the HPA axis in breeding females in opposite directions.
143 The first hypothesis is supported by the peak in female territoriality during gestation and lactation
144 (Festa-Bianchet and Boag 1982), the potential for female-related infanticide during lactation (Dobson
145 1990; Stevens 1998), the importance of food resources in regulating population size (Dobson and
146 Kjelgaard 1985; Dobson 1995; Dobson and Oli 2001), and reported dispersal occurrences of females
147 from high to low local density areas (Arnaud et al. 2012). Thus, we predicted that local conspecific
148 density would be positively associated with female GC levels. The second, kin-related hypothesis is
149 supported by the higher tolerance of females towards individual kin (King 1989b; Viblanco et al.
150 2016a), and positive kin effects on female investment in reproduction (Viblanco et al. 2010, 2016b).

151 Here, we predicted that increasing numbers of co-breeding kin should decrease female-female
152 competition and be negatively associated with female GC levels.

153 We assessed female GC levels during the territorial period of lactation by analyzing for fecal
154 cortisol metabolites (FCMs). Metabolized GCs excreted in the feces provide a useful non-invasive
155 method for assessing individual stress in Columbian ground squirrels (Bosson et al. 2009), and they
156 reflect free, biologically active levels of plasma GCs (Sheriff et al. 2010; Fauteux et al. 2017). FCMs
157 provide a more integrated measure of individual GC levels than can be obtained through acute plasma
158 measures, and are less prone to researcher-induced biases (Sheriff et al. 2011; Palme 2019). In red
159 squirrels (*Tamiasciurus hudsonicus*), individual perception of social density is reflected in their FCM
160 levels (Dantzer et al. 2013). Thus, FCMs should provide a rigorous method for testing relationships
161 between the social environment and GC levels in female Columbian ground squirrels.

162

163 **METHODS**

164 **Study sites and demographic monitoring**

165 Data were collected over two consecutive years (2013 and 2014) in the Sheep River Provincial Park
166 (Alberta, Canada), in three different colonies of Columbian ground squirrels monitored as part of
167 long-term studies on the behavior and ecology of these animals: meadow A (50°38'19.80"N;
168 114°39'46.47"W; 1520m; 3.4ha), meadow B (50°38'10.73"N; 114°39'56.52"W; 1524m; 2.3ha), and
169 Dot (50°38'59.74"N; 114°39'41.79"W; 1545m; 3.0ha). It was not possible to record data blind because
170 our study involved focal animals in the field. In each year, entire ground squirrel populations (mean \pm
171 SD = 118 \pm 68 individuals, range = 60 – 226) were trapped using 13 x 13 x 40 cm³ live-traps
172 (Tomahawk Live Trap, Hazelhurst, WI, USA) baited with a knob of peanut butter (taken from the tip
173 of a knife) as individuals emerged from hibernation (Skippy®, Hormel Foods, LLC). Each ground
174 squirrel was given a pair of uniquely numbered ear tags (Monel #1 National Band & Tag Co.,
175 Newport, KY, USA) for permanent identification. In addition, each individual was given a unique
176 dorsal mark using black human hair dye (Clairol® Hydrience N°52 Black Pearl, Clairol Inc., New

177 York, USA) for identification during field observations. Each female was monitored from emergence
178 of hibernation through the first emergence of the pups from nest burrows at about the time that they
179 were weaned. Details on the long-term monitoring of the colonies are given elsewhere (Hare and
180 Murie 1992; Raveh et al. 2010, 2011; Rubach et al. 2016). Briefly, identification of the mating date for
181 all breeding females allowed estimation of the timing of parturition (+24 days after mating; Murie et al.
182 1998) and weaning (+27 days after birth; Murie and Harris 1982). In the field, nest burrows were
183 identified from repeated visual observations of females entering burrows with mouthfuls of dry grass
184 nesting material, and complete litters were caught and marked at these burrows near the time of
185 weaning (Raveh et al. 2010). For virtually all adult females, we recorded complete information on
186 individual age and life history since the time of birth.

187

188 **Feces sampling and FCM assays**

189 *Sample collection:* Fecal samples were collected during lactation by baiting live-traps with a
190 small amount of peanut butter and deploying them close to focal individuals (see above). Traps were
191 systematically cleaned before being deployed, to ensure fecal samples corresponded to targeted
192 individuals. Fecal samples were always collected within minutes of capture, animals on the study sites
193 being target-trapped. In Columbian ground squirrels, an acute stressor causes FCM levels to increase 7
194 \pm (SE) 0.82 hours later (the gut passage time) (Bosson et al. 2009). Thus, we are confident that GC
195 levels measured in these animals were not affected by trapping, since time of capture invariably put
196 FCM-critical timing to the previous night or morning (samples collected at the first capture of the day).
197 Fecal samples were most often collected either directly into 2-mL sterile vial as the female defecated,
198 or from the floor of the trap. In this latter case, the female was always observed defecating in the trap
199 and the feces collected immediately. We insured no fecal sample was contaminated by urine upon
200 collection. Samples that were contaminated were systematically discarded. Hence, there was no
201 possible confusion of fecal samples nor cross-contamination in the field. We systematically recorded
202 hour of sample collection. Because FCM levels are likely to vary according to the time of sampling in
203 the day, we insured that sampling hour had no significant effect on FCM levels prior to analyses ($t = -$

204 0.09; $P = 0.93$). Fecal samples were immediately stored on ice packs when in the field, and transferred
205 to a -20°C freezer within no more than a couple of hours after sampling. At the end of the field season,
206 samples were shipped on dry ice to the University of Toronto and stored at -80°C until analyses.
207 Overall, we were able to acquire 126 fecal samples for 92 females.

208 *FCM assays:* Fecal cortisol metabolites were determined as previously validated and described
209 in Columbian ground squirrels (Bosson et al. 2009). Briefly, lyophilized fecal samples were frozen in
210 liquid nitrogen and pulverized with a small grinding pestle. We weighed 0.030 ± 0.001 g of the sample,
211 and extracted FCMs by vortexing it (30 min at 1 450 rpm; Barnstead Thermolyne Maxi-Mix III, IA) in
212 1 mL of 80% methanol (v/v). FCMs (ng/g dried feces) were determined using a 5α -pregnane-
213 $3\beta,11\beta,21$ -triol-20-one enzyme immunoassay (EIA), specifically designed to measure metabolites
214 with a 5α - $3\beta,11\beta$ -diol structure (Touma et al. 2003). Cross-reactivities of the antibody used in this EIA
215 are given elsewhere (Touma et al. 2003). All samples were run in duplicate on fifteen 96 well plates.
216 Low value ($\sim 70\%$ binding) and high value ($\sim 30\%$ binding) pooled samples were run on each plate as
217 controls. Intra-assay coefficients of variation were $5.9 \pm 1.1\%$ (low pool) and $4.6 \pm 1.3\%$ (high pool),
218 and the mean inter-assay coefficient of variation based on the pools was $5.5 \pm 1.2\%$.

219

220 **Kin numbers**

221 For each breeding female, we used long-term matrilineal genealogies to calculate her number
222 of co-reproductive close kin or non-kin. We counted as close kin her mother, daughter(s), and
223 littermate sister(s). Among sisters, we only considered littermates as close kin (i.e. females born in the
224 same litter) based on previous findings that non-littermate sisters do not appear to be recognized as
225 close kin in this species (Hare and Murie 1996), and only littermates, mothers, and daughters, appear
226 to provide genial neighbor benefits in terms of direct and indirect fitness (Viblanco et al. 2009; Dobson
227 et al. 2012). During lactation, female Columbian ground squirrels actively defend a core territory of ca.
228 30-m surrounding their nest-burrows to protect their young. During this period however, they
229 regularly range throughout the entire colony in their daily foraging activities (FSD et al., personal

230 observations). Females are thus exposed both to local and colony-wide social environments, and for
231 each female, we calculated the number of co-reproductive close kin and the overall number of
232 conspecifics (including close kin) occurring at these two different spatial scales: local and colony-
233 wide. The local scale comprised a radius of 30-m around a given female's nest burrow. For this, we
234 used the location of female nest burrows and, for a given female, counted all the kin and non-kin nest
235 burrows located within a 30-m radius of her own. The second spatial scale was global, and we counted
236 all co-reproductive kin present in the colony (colonies ranged from 2.3 to 3.4 ha in our study). We
237 subsequently evaluated the relationships between social environments and female FCM levels at those
238 two different scales.

239

240 **Data analyses**

241 *Variation in female FCM levels related to local conspecific and co-breeding kin numbers*

242 We used a linear mixed model (LMM) to test for the relationships between female FCM levels and
243 local conspecific and local co-reproductive kin numbers within a 30-m radius. Breeding female FCM
244 levels (ln-transformed) was specified as the dependent variable, and conspecific and co-breeding kin
245 numbers were specified as independent variables in the model. We further included female age to test
246 for potential age-related effects on female GC levels. Female ID within colony and year were included
247 as random factors in the model to account for repeated measures on individuals in different years, and
248 repeated measures within the same colony. Thus, the model was specified as:

$$249 \quad \ln(\text{FCM}) \sim n_{\text{conspecifics}} + n_{\text{kin}} + \text{age} + (1|\text{colony:ID}) + (1|\text{year})$$

250

251 *Variation in female FCM levels related to overall co-breeding kin numbers*

252 A similar LMM was used to test for the relationships between female FCM levels and co-breeding kin
253 numbers at a colony scale. In this model, it made little sense to test for a population density effect on
254 female FCM levels, since the number of conspecifics at the population level is identical for all females

255 in a given year and meadow. Although we originally predicted a negative linear effect of kin numbers
256 on female FCM levels, visual inspection of the data suggested a non-linear effect of kin numbers on
257 female FCM levels. A non-linear effect might occur if there is some optimal kin number such that
258 increasing kin numbers up to that optimal point allows decreasing territorial aggression (King 1989;
259 Viblanc et al. 2016) and reducing the activity of the HPA axis, but results in kin competition (e.g. for
260 food resources; Dobson and Kjelgaard 1985; Dobson 1990) and increased activation of the HPA axis
261 beyond. Thus, we included a quadratic term for kin numbers as an independent variable in the model.
262 Female ID within colony and year were included as random factors in the model to account for
263 repeated measures on individuals in different years, and repeated measures within the same colony.
264 Here, the model was specified as:

$$265 \quad \ln(\text{FCM}) \sim n_{\text{kin}} + n_{\text{kin}}^2 + \text{age} + (1|\text{colony:ID}) + (1|\text{year})$$

266

267 *Nature of kin environment and relationship with female FCM levels*

268 In light of the previous analyses, potentially highlighting a special effect of having one kin present in
269 the population on female FCM levels (see Results), we tested if FCM levels varied depending on the
270 nature of the 1 kin relationship to breeding females. For all females that had only one close-kin co-
271 breeder, we identified whether that individual was a mother, a daughter or a littermate sister. We then
272 ran a LMM including female FCM levels as the dependent variable of interest, the nature of the kin
273 relationship (mother, daughter, littermate sister, no kin) as the independent variable. Here also, female
274 ID within colony and year were included as random factors in the model to account for repeated
275 measures on individuals in different years, and repeated measures within the same colony. The model
276 was thus specified as:

$$277 \quad \ln(\text{FCM}) \sim \text{kin category}_{[\text{No kin/mother/daughter/littermate sister}]} + \text{age} + (1|\text{colony:ID}) + (1|\text{year})$$

278

279 All analyses were performed in R 3.6.2. (R Core Team 2019). The approach with linear mixed
280 models was conducted using the ‘lme4’ v. 1.1.20 package (Bates et al. 2015) with the alpha level set
281 to 0.05. FCM levels were ln-transformed prior to analyses to meet normality assumptions. However,
282 average values in the text are reported based on the raw data. Conditional and marginal R^2 values for
283 mixed-effect models were computed with the ‘MuMIn’ package v. 1.42.1 (Barton 2019). The marginal
284 R^2 represents the variance explained by fixed factors in the model whereas the conditional R^2
285 represents the variance explained by both fixed and random factors in the model. For all models, we
286 insured that residual distribution did not substantially deviate from normal distributions using qq-plots
287 (‘fitdistrplus’ package in R; Delignette-Muller and Dutang 2015). Independent variables were checked
288 for collinearity using Variance Inflation Factors (VIFs) (suggested cut-off $VIF > 3$; Zuur et al. 2010).
289 Results are provided as means \pm 1 SE.

290

291 **RESULTS**

292 **Variation in female FCM levels in relation to conspecifics and kin numbers at a local scale**

293 Contrary to our predictions, within a 30-m radius of a female’s nest burrow, a female’s FCM level was
294 not positively related to local conspecific density, or negatively to local kin density (Table 1, see
295 Online Supplementary Material 1). Female age however, was positively related to female FCM levels
296 (Table 1): the older a female, the higher her FCM levels. At a local spatial scale, a female’s age was
297 not significantly associated with kin density (Pearson’s correlation; $r = 0.12$, $t = 1.38$, $p = 0.17$), or
298 conspecific density ($r = 0.03$, $t = 0.39$, $p = 0.69$). Although co-breeding close kin numbers and
299 conspecific density were obviously correlated at the local scale ($r = 0.40$, $t = 4.91$, $p < 0.001$), there
300 was no indication of substantial collinearity in the model (all VIFs < 1.23). Indeed, testing for the
301 relationship between kin/conspecific density and female FCMs levels (accounting for age) in separate
302 models led to the same results (see Online Supplementary Material 2).

303

304 **Variation in female FCM levels in relation to overall kin numbers at a colony scale**

305 At a colony scale, once controlling for female ID and colony as random factors, 12.68% of residual
306 variance in female FCM levels was explained by the number of co-breeding close kin and female age
307 (marginal $R^2 = 12.68\%$; conditional $R^2 = 33.91\%$). Breeding female FCM levels varied in a quadratic
308 fashion (estimate \pm SE: -0.15 ± 0.07 kin + 0.06 ± 0.03 kin²) with the number of close co-breeding kin
309 present in the population (Table 2A; Fig. 1). FCM levels decreased by 15.11% on average between 0
310 (613.9 ± 24.8 ng FCM/g) and 1 (521.2 ± 23.4 ng FCM/g) close kin, but increased rapidly thereafter, by
311 12.91% on average, between 1 and 2 (588.5 ± 34.6 ng FCM/g) close kin present. Here also, female
312 FCM levels were positively related to female age (Table 2A; Fig. 2). It is noteworthy that few females
313 had 3 to 4 co-breeding close kin present, so that sample sizes for those categories were small (Fig. 1).
314 However, an analysis on females that had only 0, 1 or 2 kin present led to a similarly significant
315 quadratic effect of kin numbers on female FCM levels (Table 2B; see Online Supplementary Material
316 3).

317

318 **Nature of the kin environment and relationship with female FCM levels**

319 For females that had only one co-breeding kin present in the colony and for which we had FCM levels,
320 19 had a co-breeding mother, 17 a co-breeding sister and 9 a co-breeding daughter. Females with
321 different types of close kin exhibited different values of FCMs during the lactation period (Table 3).
322 Whereas the presence of a mother or sister did not seem to significantly affect female FCM levels,
323 females with a co-breeding daughter present had 14% lower FCM levels than females that had no kin
324 present in the population (Table 3; Fig. 3).

325

326 **DISCUSSION**

327 Within animal groups social conflict and cooperation might impose different tolls on
328 individuals, with varying consequences on the functioning of the HPA axis and the secretion of GC,
329 so-called “stress”, hormones (reviewed in Creel et al. 2013). Whereas numerous studies have
330 considered the positive (e.g. Scheiber et al. 2009; Frigerio et al. 2005; Young et al. 2014; Ludwig et al.

331 2017) or negative (e.g. Goymann and Wingfield 2004; Ostner et al. 2008) relationships between social
332 environments and individual GC levels in group-living species, few have concurrently investigated the
333 joint effect of socially aggressive and socially tolerant environments on the stress axis of free-living
334 vertebrates (Dantzer et al. 2017). Here, we tested the hypothesis that local colony density and the
335 presence of co-breeding kin should affect female HPA axis activity in opposite directions in the
336 Columbian ground squirrel. We expected that female GC levels would increase with high local
337 conspecific density (a reflection of increased competition), and decrease with more co-breeding kin (a
338 reflection of increased cooperation). Such effects could be expected because of: (1) local competition
339 on one hand (high female territoriality, risks of infanticide carried out by lactating females, importance
340 of food resources in regulating demographics, and reported dispersal of females from high density
341 areas (Festa-Bianchet and Boag 1982; Dobson and Kjelgaard 1985; Dobson 1990; Arnaud et al.
342 2012)); and (2) kin-related direct and indirect fitness benefits on the other (Neuhaus et al. 2004;
343 Viblanc et al. 2010; Dobson et al. 2012), likely through reduced rates of aggression (King 1989;
344 Viblanc et al. 2016a), kin acting as ‘genial neighbors’ to one another.

345 Contrary to our predictions, our analysis conducted at the local spatial scale of a 30m-radius
346 around a female’s nest burrow (viz. the area actively defended during lactation; Festa-Bianchet and
347 Boag 1982) did not suggest that an increase in local colony density resulted in an increase in female
348 FCM levels. Similarly, at a local scale, increasing co-breeding close kin numbers did not seem to be
349 associated with a decrease in FCM levels. Interestingly however, though the kin effect was lacking at
350 a local scale, it existed at a population scale, though unexpectedly, this effect was not linear. Whereas
351 we expected a negative relationship between kin numbers and female FCM levels, the data showed a
352 negative quadratic effect of the social kin environment on female FCM levels. Female FCM levels
353 were high when no kin were present, decreased by 15% when 1 co-breeding close kin was present, and
354 increased when more than one kin were present. Although a quadratic function appeared to provide
355 the best fit to the data, it should be noted that the sample size of individuals with 3 ($n = 4$) and 4 ($n =$
356 1) close kin was low. Nonetheless, when considering only female with 0, 1 and 2 kin present (for
357 which there was adequate sample sizes), the quadratic effect remained, but was weaker.

358 The fact that the kin effect was not clear at a local level, but appeared at a colony level,
359 suggests that kin advantages extend beyond the reduction of territorial aggression on female core
360 territories per se. Indeed, although the core of female aggression is located within a 30-m radius
361 (Festa-Bianchet and Boag 1982), females may encounter kin individuals well beyond 30-m of their
362 nest burrow (Viblanco et al. 2010; Arnaud et al. 2012). At a colony scale, kin environments may not
363 only reduce aggression rates during daily commutes to and from foraging sites (King 1989; Viblanco et
364 al. 2016a), but also facilitate emigration movements and territorial establishment (Arnaud et al. 2012).
365 This advantage is likely to occur even over the course of a single breeding season, as females are
366 known to change their nest burrow locations, sometimes more than once during lactation (FSD et al.,
367 pers. obs.). On the other hand, the positive relationship between female FCMs and kin numbers
368 beyond one kin is likely to reflect social competition for resources (Dobson and Kjelgaard 1985;
369 Dobson 1995), and an up-regulation of the HPA axis (but see caveat expressed above).

370 In our study, the negative effect of kin numbers on female FCMs was limited to the presence
371 of one kin. This is consistent with previous findings that the greatest effect of kin in mediating
372 changes in energy allocation from somatic towards reproductive allocation occurred from a shift of
373 having no kin to having one kin present (Viblanco et al. 2016b). Considering the nature of the kin
374 relationship for females having only one co-breeding kin, we found that females with a co-breeding
375 daughter experienced a decrease in their FCM levels compared to females with no kin. Thus, co-
376 breeding daughters, but not littermate sisters or mothers, appeared to provide a substantial advantage
377 in terms of decreasing GC levels. It should be noted however, that females with co-breeding sisters
378 had 23% (though not significantly) lower FCM levels than females with no kin around. One
379 explanation for this result is that part of the variation in FCM levels was accounted by year effects,
380 since most females with co-breeding sisters occurred in 2014 ($n = 12$) vs. 2013 ($n = 5$) whereas other
381 categories were fairly balanced between years (see Table 3), and overall FCM levels were slightly
382 lower in 2014 ($\ln\text{-FCM}_{2013} = 6.37 \pm 0.03$ ng/g dried faeces vs. $\ln\text{-FCM}_{2014} = 6.24 \pm 0.04$ ng/g dried faeces).
383 Previous studies in Columbian ground squirrels have suggested that kin advantages mediated through
384 mother-daughter relationships might include territory bequeathal, mothers dispersing to avoid

385 competition with philopatric daughters (Harris and Murie 1984). However, evidence from female
386 dispersal movements suggests that mothers are more likely to accommodate and tolerate daughters
387 (Wiggett and Boag 1992; Arnaud et al. 2012) than provide advantages in terms of territory bequeathal.
388 Thus, differences between kin in terms of FCM levels may perhaps be explained by age-related social
389 dominance patterns, with dominant mothers being equivalent to not having any kin around, and
390 subordinate daughters or equally ranked sisters posing a lowered source of stress for breeding females.
391 This idea is supported by the fact that received aggression decreases but elicited aggression generally
392 increases with age, suggesting a pattern of age-related dominance in female Columbian ground
393 squirrels (Viblanco et al. 2016a).

394 Interestingly, investigations into the relationships between individual GC levels and the
395 presence of kin in the social environment have led to mixed results in other social mammals. In
396 closely related Richardson ground squirrels (*Urocitellus richardsonii*) for instance, the disappearance
397 of mothers from the population had no impact on FCM levels of their offspring shortly after weaning,
398 and removing related neighbors from adjacent territories did not appear to substantially affect the
399 FCM levels of breeding females (Freeman et al. 2019). In contrast, for cooperatively breeding
400 meerkats (*Suricata suricatta*), when parents are the dominant breeding pair, subordinate individuals
401 seem to benefit from living in social groups in the form of lower GC levels; in comparison with
402 subordinate individuals that live in social groups with an unrelated dominant pair (Dantzer et al.
403 2017). In our study species and overall, the effects of kin on female FCM levels were rather limited,
404 and detectable only over a narrow range and context of close kin availability at the colony, but not
405 local, scale. These findings reinforce previous suggestions that Columbian ground squirrel societies
406 are typified by somewhat egalitarian and inclusive social constructs that transcend boundaries dictated
407 by kinship alone (Hare 1992, 1994; Hare and Murie 1996, 2007; Fairbanks and Dobson 2010). Taken
408 together, those results raise the intriguing question of the extent to which variation in social lifestyles
409 (i.e. from more egalitarian to despotic constructs) may shape the stress load experienced by
410 individuals. In this regard, a comparative inter-specific study of GCs in relation to the social
411 environment may be useful to evaluate the physiological toll imposed by various social constructs on

412 individuals, all while controlling for other sources of GC variation such as climate, food availability or
413 predation (Rubenstein and Shen 2009; Dantzer et al. 2017). For instance, positive effects of kin on
414 individual GC levels may be more pronounced in matrilineal species where social systems are
415 characterized by more despotic relationships, or stronger dominance hierarchies than in the Columbian
416 ground squirrel. Amongst others, the diversity of social systems in rodents, and particular sciurids,
417 make them good models for future investigations into such questions (Wolff and Sherman 2008).

418 FCM levels increased with age in female Columbian ground squirrels. In vertebrates,
419 increasing GC levels with age have been suggested to reflect two concurrent mechanisms: (1)
420 increasing metabolic and reproductive demands with age (Crespi et al. 2013); and (2) progressive
421 deterioration of the HPA axis in senescing individuals (Gupta and Morley 2011). For instance,
422 perturbed regulation of the HPA axis leading to high GC production have been documented with
423 increasing age in humans (Sherman et al. 1985; Van Cauter et al. 1996; Chahal and Drake 2007), dogs
424 (Reul et al. 1991), and rats (Scaccianoce et al. 1990). Our results in Columbian ground squirrels are
425 likely to reflect those two concurrent mechanisms: reproductive effort in breeding females is known to
426 increase until about 5 years of age, with females of 6 years old and above starting to exhibit
427 reproductive senescence (Broussard et al. 2003). This is consistent with the pattern of increase in FCM
428 levels observed in this study that could be linked to increased metabolic demands up to a point, and
429 reflect senescence of the HPA axis beyond that point.

430 Increased circulating GC levels are often viewed as indicative of chronic stress with
431 potentially detrimental effects (but see Boonstra (2013)), such as on the immune system (Sapolsky et
432 al. 2000) or oxidative stress (Costantini et al. 2011). However, the primary function of glucocorticoids
433 under acute conditions is energy mobilization (Sapolsky et al. 2000) and increased levels of maternal
434 glucocorticoids may have adaptive transgenerational consequences (Avishai-Eliner et al. 2001;
435 Cottrell and Seckl 2009; Jensen 2013; Sheriff and Love 2013). In red squirrels, females subject to both
436 experimental and natural increases in conspecific density are known to exhibit increased FCM levels
437 compared to controls, with positive consequences on their offspring growth rates and survival in a
438 competitive social environment (Larsen and Boutin 1994; Dantzer et al. 2013). Thus, increased

439 maternal FCM levels may appear as advantageous for the young to adapt to future social environments,
440 despite potentially negative impacts on maternal immunity, oxidative stress, and fitness due to
441 pleiotropic effects of GCs. This may also be the case in the Columbian ground squirrel, and remains to
442 be tested.

443 To conclude, our results suggest complex relationships between the social kin environment
444 and individual stress levels in a wild colonial mammal, revealing the existence of a social trade-off
445 between advantages and costs to social conspecifics in terms of GC levels. Whether those complex
446 relationships translate into significant fitness costs or set a threshold for optimal group size remain to
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448

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473

474 **DATA AVAILABILITY**

475 The data related to this paper are accessible as figshare doi: 10.6084/m9.figshare.11949078

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689

690 **TABLES**

691 **Table 1** Linear mixed model estimates for the relationships between female FCM levels and female
 692 age, number of conspecifics and number of close kin within a 30-m radius of the nest burrow. Female
 693 identity within colony, and year, were included as random effects in the model. σ^2 = within-group
 694 variance; τ_{00} = between-group variance, ICC = intraclass correlation coefficient. Sample size along
 695 with marginal and conditional R^2 are presented

| ln FCMs (ng per g dried feces) | | | | |
|------------------------------------|-----------------------|---------------|------------------|------------------|
| <i>Fixed effects</i> | | | | |
| | <i>Estimates ± SE</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> |
| (Intercept) | 6.10 ± 0.09 | 5.92 – 6.28 | 66.11 | <0.001 |
| Local kin numbers | 0.01 ± 0.06 | -0.12 – 0.13 | 0.15 | 0.883 |
| Local colony density | 0.01 ± 0.01 | -0.01 – 0.03 | 0.71 | 0.482 |
| Age | 0.05 ± 0.01 | 0.02 – 0.07 | 3.63 | <0.001 |
| <i>Random Effects</i> | | | | |
| σ^2 | | 0.06 | | |
| $\tau_{00\text{colony:ID}}$ | | 0.01 | | |
| $\tau_{00\text{year}}$ | | 0.01 | | |
| ICC _{colony:ID} | | 0.15 | | |
| ICC _{year} | | 0.10 | | |
| Fecal samples / Individuals | | 126 / 92 | | |
| Marginal R^2 / Conditional R^2 | | 0.097 / 0.323 | | |

696

697 **Table 2** Linear mixed model estimates for the relationships between female FCM levels and female age, number of close kin and number of close kin² in the
698 colony. Female identity within colony, and year, were included as random effects in the model. σ^2 = within-group variance; τ_{00} = between-group variance, ICC
699 = intraclass correlation coefficient. A) Model including all females. B) Model restricted to females that had 0, 1 or 2 kin individuals present, removing
700 potential outlier effects of low sample sizes for females with 3 (N= 4) or 4 (N=1) kin present. Sample size along with marginal and conditional R² are
701 presented

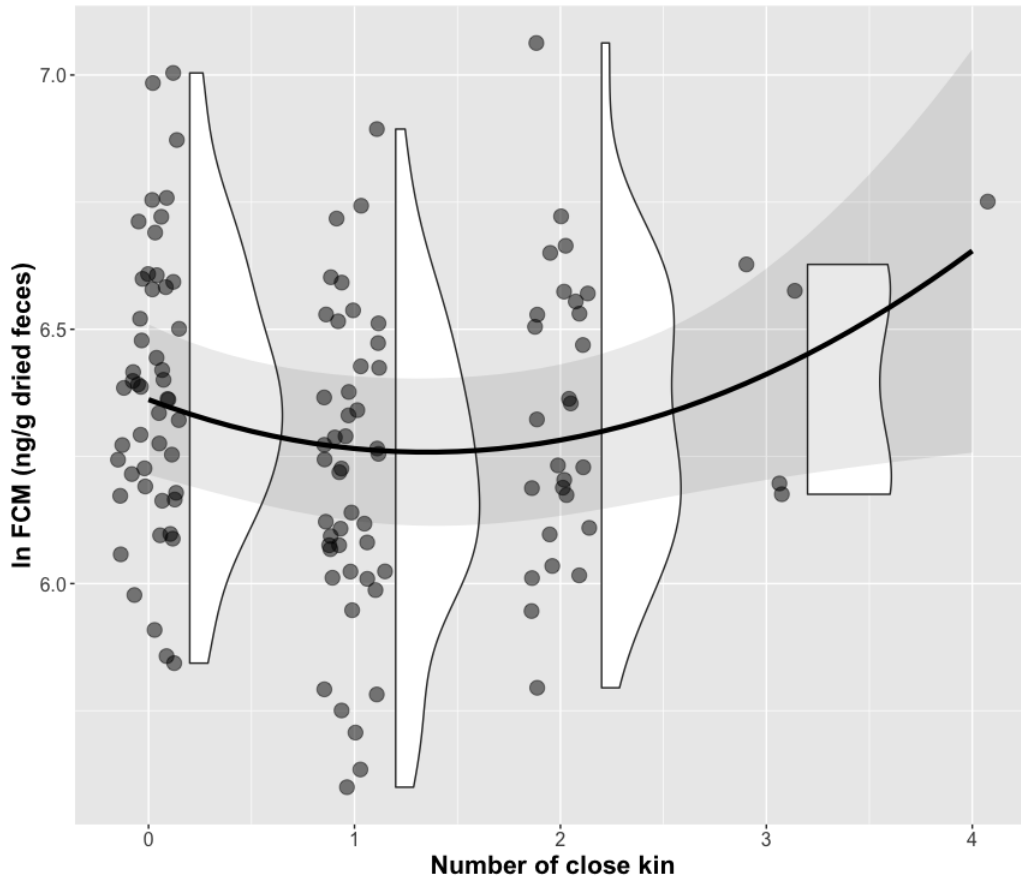
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| | A) Model with all values of co-breeding close kin | | | | B) Model with a maximum value of co-breeding close kin of 2 | | | |
|--|---|---------------|------------------|------------------|---|---------------|------------------|------------------|
| | <i>Fixed effects</i> | | | | | | | |
| | <i>Estimates ± SE</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> | <i>Estimates ± SE</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> |
| (Intercept) | 6.19 ± 0.09 | 6.01 – 6.38 | 66.59 | <0.001 | 6.21 ± 0.09 | 6.04 – 6.38 | 71.09 | <0.001 |
| Kin numbers | -0.15 ± 0.07 | -0.29 – -0.02 | -2.25 | 0.026 | -0.24 ± 0.11 | -0.45 – -0.03 | -2.25 | 0.026 |
| Kin numbers ² | 0.06 ± 0.03 | 0.01 – 0.11 | 2.24 | 0.027 | 0.11 ± 0.05 | 0.00 – 0.21 | 1.98 | 0.050 |
| Age | 0.04 ± 0.01 | 0.02 – 0.07 | 3.20 | 0.002 | 0.04 ± 0.01 | 0.01 – 0.07 | 2.97 | 0.004 |
| | <i>Random Effects</i> | | | | | | | |
| σ^2 | | 0.06 | | | | 0.06 | | |
| τ_{00} colony:id | | 0.01 | | | | 0.02 | | |
| τ_{00} year | | 0.01 | | | | 0.01 | | |
| ICC _{colony:id} | | 0.14 | | | | 0.19 | | |
| ICC _{year} | | 0.11 | | | | 0.07 | | |
| Fecal samples / Individuals | | 126 /92 | | | | 121 / 91 | | |
| Marginal R ² /Conditional R ² | | 0.127/0.339 | | | | 0.117/0.347 | | |

704 **Table 3** Mean values (\pm SE) for fecal cortisol metabolites (FCM) for females that had only one co-
 705 breeding kin in the colony. The nature of the one kin relationship was either the mother, a daughter or
 706 a littermate sister. Values not significantly different at $p < 0.05$ share the same letter (Tukey Honest
 707 Significant differences test). Individual fecal sample sizes are given (N)

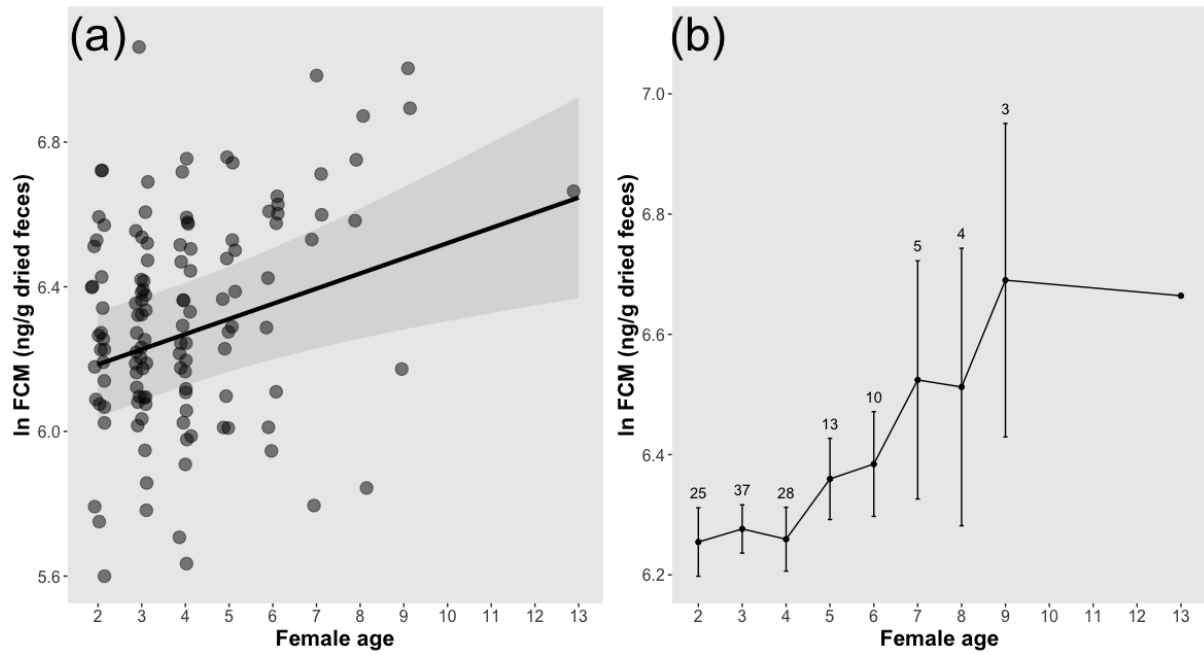
| Kin category | FCMs (ng per g dried feces) | ln (FCMs) | Tukey HSD | Individual fecal samples | |
|--------------------------|-----------------------------|-----------------|-----------|--------------------------|------|
| | | | | per year (N) | |
| | | | | 2013 | 2014 |
| No kin | 613.92 \pm 24.83 | 6.38 \pm 0.04 | a | 29 | 20 |
| Mother | 563.68 \pm 32.96 | 6.30 \pm 0.06 | a,b | 9 | 10 |
| Daughter | 527.03 \pm 66.04 | 6.21 \pm 0.11 | b | 4 | 5 |
| Littermate sister | 470.59 \pm 34.17 | 6.12 \pm 0.07 | a,b | 5 | 12 |

708



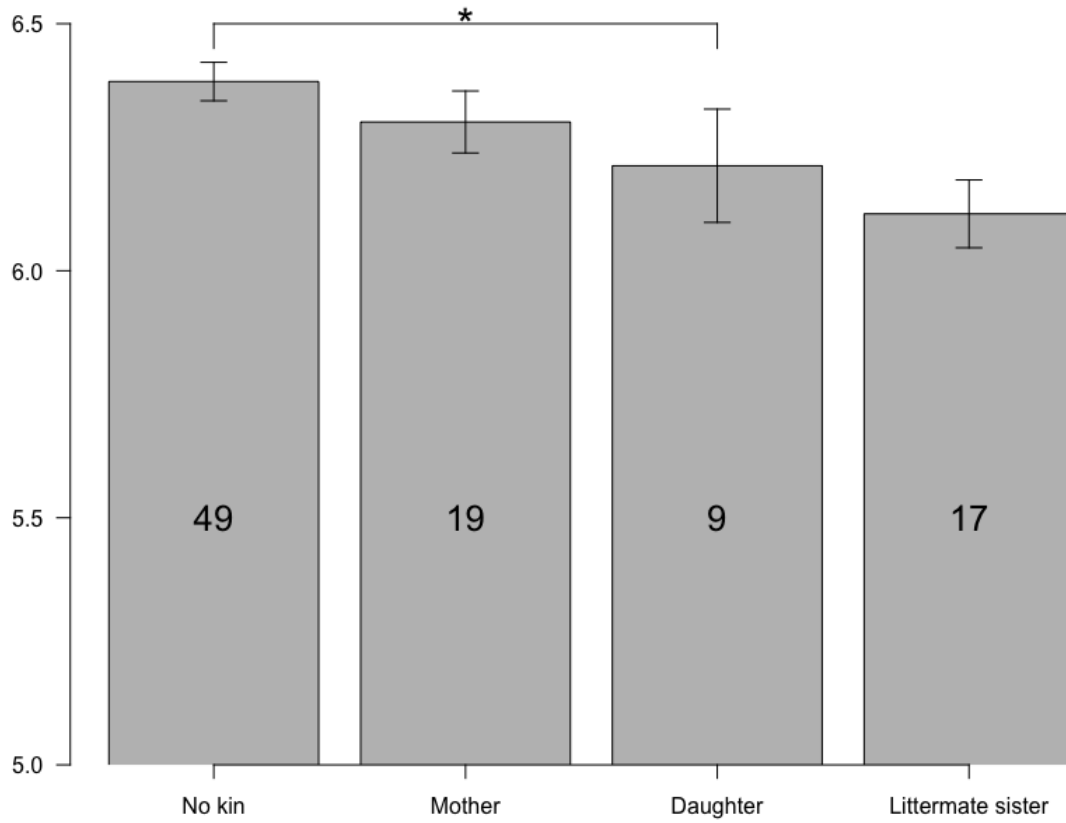
710

711 Fig. 1 Quadratic relationship between close kin numbers and lactating female fecal cortisol metabolite
712 (FCM) levels (ln-transformed) in Columbian ground squirrels (*Uroditellus columbianus*). The
713 estimated effect and 95% CI from the linear mixed model is plotted. Violin plots show the distribution
714 of data (n = 126 fecal samples; N = 92 females)



715

716 Fig. 2 Relationships between female age and fecal cortisol metabolite (FCM) levels (ln-transformed)
 717 in Columbian ground squirrels (*Urocitellus columbianus*). a) estimated effect and 95% CI from the
 718 linear mixed model (n = 126 fecal samples; N = 92 females); b) mean levels \pm SE (fecal sample sizes
 719 are indicated above the bars). Note different scales on the y-axis



721

722 Fig. 3 Female fecal cortisol metabolite (FCM) levels (ln-transformed) for females with zero or one co-
723 breeding kin in the study (either no kin, a mother present, a daughter present, or a littermate sister
724 present). Values are given as means \pm SE. Significant differences to no-kin levels are given by the
725 asterisk. All other values were not significantly different from each other (Tukey HSD; see Table 4).
726 Fecal sample sizes are indicated in the bars (n = 94 fecal samples; N = 74 females)

727



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