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1 Sex-specific density-dependent secretion of glucocorticoids in lizards: insights
2 from laboratory and field experiments

3

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24

25

26 **Abstract**

27 Negative density feedbacks have been extensively described in animal species and involve
28 both consumptive (i.e., trophic interactions) and non-consumptive (i.e., social interactions)
29 mechanisms. Glucocorticoids are a major component of the physiological stress response and
30 homeostasis, and therefore make a good candidate for proximate determinants of negative
31 density feedbacks. Here, we combined laboratory and field experiments with enclosed
32 populations to investigate the relationship between density, social stress and plasma
33 corticosterone levels in the common lizard *Zootoca vivipara*. This species exhibits strong
34 negative density feedbacks that affect females more than males, and its life history is sensitive
35 to experimentally-induced chronic elevation of corticosterone plasma levels. We found that
36 prolonged crowding in the laboratory can trigger a chronic secretion of corticosterone
37 independent from food restriction. In the field experiments, corticosterone levels of females
38 were not affected by population density. Corticosterone levels of males increased with
39 population density but only during the late activity season in a first field experiment where we
40 manipulated density. They also increased with density during the mating season but only in
41 populations with a female-biased sex ratio in a second field experiment where we crossed
42 manipulated density and adult sex ratio. Altogether, our results provide limited evidence for a
43 role of basal corticosterone secretion in density feedbacks in this species. Context and
44 density-dependent effects in males may arise from changes in behavior caused by competition
45 for resources, male-male competition, and mating.

46 **Keywords:** chronic social stress, competition, density, sex ratio, *Zootoca vivipara*

47 **Running title:** Density-dependent stress response in a lizard

48

49 **Introduction**

50 Population dynamics are influenced by a complex interplay between stochastic and
51 deterministic components including negative density feedbacks, which result from the
52 negative effects of population density on demographic rates (Herrando-Pérez et al. 2012). A
53 dominant ecological theory is that negative density feedbacks are primarily caused by trophic
54 interactions. In addition, non-consumptive mechanisms, for example social stress due to
55 competition for territories, may also be involved in negative density feedbacks (Christian
56 1970, Boonstra et al. 1998, Edeline et al. 2010, Herrando-Pérez et al. 2012). Heightened
57 frequency of social interactions and limited food supplies at high densities may both cause a
58 chronic social stress response and downstream physiological and behavioral effects that shape
59 the population dynamics (reviewed in Creel et al. 2013).

60 In vertebrates, responses to environmental stressors are mediated by the activation of
61 the hypothalamo–pituitary–adrenal (HPA) axis, which triggers a short-term release of
62 glucocorticoids (Harvey et al. 1984, Sapolsky et al. 2000, Wingfield and Kitaysky 2002).
63 From an energetic point of view, an acute increase in glucocorticoids diverts bioenergetic
64 resources away from “non-essential” physiological functions and shifts the animals into an
65 emergency life-history stage (Wingfield and Kitaysky 2002). In the short term, increased
66 levels of glucocorticoids may allow individuals to restore a positive energy balance for
67 example by suppressing reproduction (Silverin 1998, Moore and Jessop 2003), social
68 activities (DeNardo and Licht 1993), or by increasing activity and foraging (e.g., Tataranni et
69 al. 1996, Cote et al. 2006). Therefore, the short-term individual benefits of elevated
70 glucocorticoids secretion may ultimately reduce population size as a result of diminished
71 reproduction and/or elevated mortality. Given the multiple and profound whole-organism
72 effects of glucocorticoids under chronic stressful social conditions, these hormones could play
73 an important role in negative density feedbacks.

74 Yet, despite wide agreement from laboratory studies over the existence and pathological
75 consequences of chronic glucocorticoids secretion (e.g., Christian 1956, Bhatnagar and
76 Vining 2003, Vegas et al. 2006), studies in wild populations have produced evidence of a
77 variable link between the HPA axis function and density feedbacks (e.g., Boonstra 2013,
78 Creel et al. 2013). This is particularly true in wild populations of rodents, birds and reptiles in
79 which corticosterone is the main adrenal glucocorticoid mediating stress responses (e.g.,
80 Meylan et al. 2003 and references therein, Cote et al. 2006, Creel et al. 2013). For instance, in
81 rodents, circulating corticosterone levels increase with population density in several territorial
82 species, while other factors such as breeding stage or predation risks are more important
83 determinants of corticosterone levels in others (reviewed in Creel et al. 2013). There is also
84 very limited evidence of density-dependent chronic secretion of corticosterone in birds (but
85 see Raouf et al. 2006, Viblanc et al. 2014) and in squamate reptiles (but see Comendant et al.
86 2003). Besides potential methodological differences in the characterization of HPA axis
87 regulation (Breuner et al. 2013), such inconsistencies may come from the limited range of
88 density variation in observational studies. At the same time, experimental studies may not
89 always reflect the natural densities to which animals are exposed and may not allow for
90 behavioral compensations, including spatial avoidance or changes in microhabitat use.
91 Moreover, individual factors such as age, sex and social rank and external factors such as
92 seasonal conditions or predation risks can affect glucocorticoids levels and change the
93 intensity of social stress (Creel et al. 2013). Finally, a chronic social stress could suppress
94 subsequent physiological responses to acute and social stressors and reduce baseline
95 corticosterone levels (Rich and Romero 2005, Cyr and Romero 2007), thus contributing to the
96 lack of ‘a consensus endocrine profile for chronically stressed wild animals’ (Dickens and
97 Romero 2013). There is therefore a strong need for field studies that examine the HPA axis
98 regulation through time under chronic social stress across a relevant range of population

99 densities while accounting for potentially confounding factors at the individual and
100 population levels (reviewed in Creel et al. 2013). Here, we present the results of such a study
101 in the common lizard (*Zootoca vivipara*) where we manipulated the density of experimental
102 populations of lizards maintained in outdoor enclosures over a wide range of densities (from
103 below to above the carrying capacity) and measured plasma levels of corticosterone before,
104 during and after the experiment.

105 Previous field experiments in the common lizard have demonstrated negative density
106 dependence for body growth (with stronger effects in females than in males), for female age
107 at maturation and female reproductive effort and for immunity in both sexes (Mugabo et al.
108 2013, Mugabo et al. 2015) and therefore strong compensatory density regulation (Mugabo et
109 al. 2013). However, the underlying mechanisms of these density feedbacks still remain
110 unclear (González-Suárez et al. 2011, Mugabo et al. 2013). Accumulating evidence suggests
111 that chronic corticosterone releases affect several aspects of the common lizard's behavior
112 and life history such as food consumption, activity, basking behavior, immunity, and
113 reproduction (e.g., Meylan and Clobert 2005, Cote et al. 2006, Meylan et al. 2010). Thus,
114 activation of the HPA axis by chronic social stress could be involved in density feedbacks in
115 this species. Furthermore, the intensity of social stress at high densities should vary with the
116 adult sex ratio due to male aggressions toward females during the mating season and male-
117 male competition for breeding (Fitze et al. 2005, Le Galliard et al. 2005, Le Galliard et al.
118 2008).

119 Based on this knowledge, we predicted that corticosterone secretion increases in
120 response to chronic social stressors in this species (Prediction 1) and therefore that plasma
121 corticosterone levels should increase with population density due to increased levels of social
122 stress (Prediction 2). Stress response to density could be higher in females than in males due
123 to a stronger sensitivity to density than males (as seen in body growth, Mugabo et al. 2013), a

124 lower social dominance and repeated harmful social interactions with males during the
125 breeding season (Le Galliard et al. 2005, Le Galliard et al. 2008). We also expected the
126 increase of corticosterone levels with density to be stronger in male-biased than in female-
127 biased populations due to competition for mates in males and male aggressions on females
128 during mating (Prediction 3). To test these three predictions, we conducted two laboratory
129 experiments and two successive field experiments in semi-natural conditions. First, we tested
130 prediction 1 by comparing the patterns of temporal variations of plasma corticosterone levels
131 following an acute disturbance stress and a chronic social stress (prolonged social
132 confinement) under controlled laboratory conditions. Second, we tested prediction 2 by
133 manipulating population density in female-biased populations maintained in semi-natural
134 conditions. We then tested prediction 2 and 3 by cross manipulating density and adult sex
135 ratio in a second field experiment. We monitored populations for a year in both field
136 experiments and measured corticosterone levels in males and females before, during and after
137 each experiment. Altogether, these experiments enabled us to investigate whether levels of
138 circulating corticosterone are affected by chronic social stress and whether population density
139 triggers chronic secretion of corticosterone in a species exhibiting strong negative density
140 feedbacks.

141 **Methods**

142 *Model species*

143 *Zootoca vivipara* is a small (adult snout-vent length < 75 mm) ovoviviparous lizard inhabiting
144 humid habitats across northern Eurasia. Natural populations can be structured in three age
145 classes: juveniles (newborn individuals), yearlings (1-2 years old) and adults. In natural
146 populations from where experimental individuals originated and in our study site (CEREEP
147 research center, Saint-Pierre-lès-Nemours, France) basal plasma corticosterone levels vary

148 from 1 to 181 ng.ml⁻¹ in adults and are similar between sexes (Meylan et al. 2003, Cote et al.
149 2006).

150 ***Experimental protocols***

151 *Stress response in laboratory conditions*

152 During June and July 2010, we conducted two laboratory experiments to test our prediction
153 that corticosterone secretion increases in response to chronic social stress in this species
154 (Prediction 1). First, we carried out an acute disturbance stress experiment to produce a
155 baseline stress response to compare to chronic social stressors, knowing that acute and
156 chronic stressors can trigger very distinctive patterns of glucocorticoid responses (Carere et
157 al. 2003, Rich and Romero 2005). We then carried out a chronic social stress experiment
158 where the chronic social stressor was a prolonged social confinement during which pairs of
159 males shared a single basking and shelter site in a terrarium under *ad libitum* food conditions.
160 More specifically, this experiment enabled us to test for the effect of social interactions and
161 competition for a shelter and microhabitat for optimal thermoregulation on corticosterone
162 secretion independently of trophic interactions.

163 *Experiment 1: response to an acute disturbance stress*

164 In the “acute disturbance stress” experiment, 15 adult males were placed individually in an
165 empty terrarium and a soft paint brush was waved in front of them during 10 minutes (stress
166 group). The remaining 15 adults males were left undisturbed (control group). All individuals
167 in the stress group exhibited an escape behavior in response to the paint brush *stimuli*. Three
168 successive blood samples were collected as follow from each lizard in the control and in the
169 stress group: 5 days before the acute stress experiment to measure basal plasma corticosterone
170 levels, immediately after and one day after the acute stress.

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171 *Experiment 2: response to a chronic social stress*

172 In the “chronic social stress” experiment, 16 males were maintained by pairs for 10 days in a
173 terrarium containing a single shelter and basking site (stress group) while 15 males were left
174 alone in their individual terrarium (control group, one male of the control group died before
175 the beginning of the experiment). Pairs of individuals of similar body size were created to
176 avoid the establishment of a size-based hierarchy. Five repeated measurements of
177 corticosterone levels were carried out. First, basal corticosterone levels were measured from
178 blood samples collected 6 days before individuals from the stress group were paired. Then,
179 three sets of blood samples were collected 1 day, 3 days and 9 days after the beginning of the
180 experiment. After 10 days, lizards from the stress group were transferred back into their
181 individual terrarium and a last blood sample was collected 4 days later. During both
182 experiments, lizards were fed daily *ad libitum* and maintained in 25 × 15 × 15 cm³ terraria
183 under optimal laboratory conditions for light, water availability and temperature (see Le
184 Galliard et al. 2003 for more details on lizards’ husbandry). All individuals were weighted to
185 the nearest 0.01 g immediately after each blood sampling. Change in mass throughout the
186 experiments was monitored to control for potential effects of stressors on food consumption.

187 *Stress response to density in semi-natural conditions*

188 We conducted two field experiments in order to test our predictions that plasma
189 corticosterone levels increase with population density due to increased levels of social stress,
190 potentially more so in females than in males (Prediction 2), and that this increase should be
191 stronger in male-biased than in female-biased populations due to competition for mates in
192 males and male aggressions during mating in females (Prediction 3).

193 *Experiment 3: response to population density*

194 During June and July 2008, we manipulated the initial density of 24 populations maintained
195 in 10 × 10 m outdoor enclosures located in a natural meadow at the CEREEP research center

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196 in Saint-Pierre-lès-Nemours, France (48°17'N, 2°41' E). Enclosures provided lizards with
197 wild preys and their abundance was negatively affected by the density of lizards (González-
198 Suárez et al. 2011). Populations were established post-breeding following a gradient of five
199 density levels ranging from 7 to 35 adults and yearlings (equivalent to 700 to 3,500 lizards per
200 ha) and 10 to 50 juveniles. Density level 1 had 3 adults, 4 yearlings and 10 juveniles. Density
201 levels 2 to 5 differed from density level 1 by a multiplicative factor of 2 to 5 respectively. All
202 populations were female-biased with a sex ratio of 0.43 (calculated as the proportion of
203 yearling and adult males with 1:2 adult and 1:1 yearling males and females) and had a similar
204 age-structure (Mugabo et al. 2013). Lizards ($n = 162$ adults, 216 yearlings and 549 juveniles)
205 were randomly assigned to experimental populations and were released in outdoor enclosures
206 in June-July 2008. All yearling and adult males and non-reproductive females were released
207 between June 11 and 13 and all reproductive females and their juveniles were released within
208 two days post parturition from June 11 to July 27. Blood samples were collected from adult
209 males and yearling males and females prior to release in the enclosures in June-July 2008
210 (most adult females were still pregnant at this time and were therefore not sampled;
211 potentially reproductive yearling females were kept until their non-reproductive status was
212 confirmed before being sampled for blood up to July 25). Blood samples were then collected
213 in all enclosures during 3 successive recapture sessions in late June 2008, September 2008
214 and April-May 2009. Finally, all surviving individuals were recaptured in May-June 2009 and
215 blood samples were collected in laboratory conditions on all individuals except pregnant
216 females (Table S2). Individuals were measured for body mass after the collection of blood
217 samples in the laboratory.

218 *Experiment 4: response to population density and sex ratio*

219 During June and July 2009, we cross manipulated the initial population density and sex ratio
220 in 24 populations. Populations were established post-breeding according to 3 density levels

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221 and 2 sex ratio levels, i.e., female-biased *versus* male-biased populations. Density level 1 had
222 4 adults, 6 yearlings and 12 juveniles and density levels 2 and 3 differed from density level 1
223 by a multiplicative factor of 2 and 3 respectively. All populations had similar age-structures
224 (see Table S1). Female-biased populations had a sex ratio of 0.4 with 1:3 adult and 3:3
225 yearling males and females and male-biased populations had a sex ratio of 0.7 with 3:1 adult
226 and 2:4 yearling males and females. Juvenile sex ratio was balanced in all treatments. Lizards
227 ($n = 164$ adults, 246 yearlings and 492 juveniles) were randomly assigned to experimental
228 populations and released in outdoor enclosures in June-July 2009 (see Table S1 for more
229 details). Three sets of blood samples were collected: prior to release in laboratory conditions
230 in June-July 2009, in all enclosures in April 2010 and in laboratory conditions after capture in
231 June 2010 (Table S2). All individuals were measured for body mass after blood sampling.

232 Blood sampling and measurements of plasma corticosterone levels

233 Except in the acute stress group, blood samples were taken within 1 minute after capture to
234 avoid an increase of plasma corticosterone levels due to handling. During the capture sessions
235 in the enclosures, observers only spent few minutes in each enclosure to capture wild lizards
236 in order to avoid stressing the individuals by repeatedly trying to catch them. However,
237 corticosterone levels in the field were not affected by the time spent in the enclosures prior the
238 captures in a recent study that was carried out in the same experimental system (Mell et al.
239 2016). About 40-60 μL of whole blood was collected from the post-orbital sinus using 20 μL
240 microhematocrit tubes. Immediately after sampling, blood was centrifuged and the plasma
241 was stored at -30°C . Plasma corticosterone levels were later determined using a competitive
242 enzyme-immunoassay procedure (IDS corticosterone EIA kit, ref AC-14F1, IDS EURL Paris,
243 France). This method provides a quantitative determination of total corticosterone
244 concentration in a set volume of plasma using a polyclonal corticosterone antibody and is
245 based on a colorimetric assay (absorbance read at 450 nm). The sensitivity of the

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246 corticosterone EIA kit is 0.55 ng.ml^{-1} . For all samples, $10 \mu\text{L}$ of plasma were diluted 10 times
247 in $90 \mu\text{L}$ of the sample diluent provided in the EIA kit except for 10 samples which were
248 diluted 20 times due to low volumes of plasma and for which $5 \mu\text{L}$ of plasma were used
249 instead (see Table S2 for sample size). Intra-plate repeatability was estimated by comparing
250 the concentrations of blood samples run twice on the same plate and inter-plate repeatability
251 was estimated by comparing the concentrations of blood samples run twice on two different
252 plates. Measurements were highly repeatable (intra-plate repeatability: $n = 49$, $F_{1,47} = 495.78$,
253 $P < 0.0001$, intra-class correlation coefficient $r = 0.91 \pm 0.03$; inter-plate repeatability: $n = 46$,
254 $F_{1,44} = 112.15$, $P < 0.0001$, intra-class correlation coefficient $r = 0.79 \pm 0.06$).

255 Statistical analyses

256 *Experiment 1*

257 Levels of plasma corticosterone and change in body mass in response to acute disturbance
258 stress were analyzed with mixed-effects linear models in R 2.15.2 (<http://cran.r-project.org/>)
259 following Pinheiro & Bates (2000). The change in mass of an individual was defined as the
260 difference in mass from its average mass throughout the experiment. For both corticosterone
261 levels and change in mass, the fixed part of the models included a group effect (stress versus
262 control), a sampling session effect and their two-way interaction. Models of corticosterone
263 levels also included the fixed effects of the average mass and the change in mass. Individual
264 identity was included as a random effect in all models to account for repeated measurements
265 on the same individual and quantify inter-individual variation in corticosterone levels and
266 change in mass.

267 *Experiment 2*

268 Changes in corticosterone levels in response to a chronic social stress in laboratory conditions
269 were analyzed with generalized additive mixed effects models (GAMMs). GAMMs were
270 used to model the non-linear relationship between corticosterone levels and time due to the

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271 occurrence and disappearance of a chronic social stressor. The fixed part of the models
272 included a smooth function on the number of days since the beginning of the experiment, a
273 group effect (stress versus control) and their two-way interaction as well as an effect of mass
274 and change in mass. Change in mass in response to chronic social stress was analyzed as in
275 Experiment 1. Individual identity was included as a random effect in models of corticosterone
276 levels and change in mass to account for repeated measurements on the same individual.

277 *Experiments 3 and 4*

278 Effects of population treatments on corticosterone levels were analyzed independently for
279 each blood sampling session (i.e., 5 sessions in the density experiment, 3 sessions in the
280 density and sex ratio experiment, see Table S3) since the same individuals were not always
281 captured in each session. In the data analysis of the density experiment, fixed effects were
282 density (density level as a continuous variable), sex, age class and two-way interactions with
283 density to test for sex and age-specific effects of density. In the data analysis of the density
284 and sex ratio experiment, females were not included since only 21 were sampled in April
285 2010 and none were measured in June 2010. Thus, fixed effects of the models were density
286 (categorical variable), sex ratio and their two-way interaction in order to test for an interaction
287 between density and sex ratio on corticosterone levels in males. Age effects were not included
288 because the numbers per age class in each treatment were too low. However, no significant
289 age effects were found in the density experiment (see Table 1).

290 Since a significant relationship between corticosterone levels and density was only
291 found in males (see results), we used the number of males per enclosure as a covariate
292 (quadratic regression) to further investigate if corticosterone levels were influenced by the
293 intensity of male-male interactions. We also used the number of females per enclosure as a
294 covariate (quadratic regression) to investigate if corticosterone levels in males were
295 influenced by the number of potential sexual partners in the population. These tests were run

296 separately to avoid multicollinearity between the covariates number of males and number of
297 females. Finally, we investigated if corticosterone levels in the 21 females recaptured in April
298 2010 were influenced by the number of males in the population (linear regression).

299 Additional fixed effects included in all analyses of field data were body mass (when
300 measured), sampling date within each sampling session (continuous variable in the density
301 experiment and categorical variable in the density and sex ratio experiment), time since
302 release in enclosures (in days) and time of the day (in hours, quadratic regression). These
303 variables were included in the models to account for known confounding factors of
304 glucocorticoid responses to chronic social stressors (reviewed in Creel et al. 2013). Enclosure
305 identity was included as a random effect.

306 *General methods for all statistical analyses*

307 All linear mixed effects models and generalized additive mixed effects models were fitted
308 using the maximum likelihood approach in the *lme* (package *nlme*) and *gamm* (package *mgcv*)
309 procedures respectively and fixed effects were tested with marginal F tests (Pinheiro and
310 Bates 2000). A minimum adequate model was obtained by backward elimination of non-
311 significant terms. Assumptions of normality were fulfilled (based on diagnostic plots of the
312 normality of the residuals of the full models and of the relationship between fitted values and
313 the residuals) but some Bartlett tests revealed significant variance heterogeneity between
314 groups that we accounted for with a *varIdent* function in the procedures *lme* and *gamm*
315 (Pinheiro and Bates 2000, results not shown). All estimates are provided with standard errors
316 unless otherwise stated.

317 **Results**

318 *Stress response in laboratory conditions*

319 In both experiments, inter-individual variation was highly significant for corticosterone levels
320 (random effect: *lme*: LRT = 42.71, df = 1, $P < 0.0001$, $n = 30$, and *gamm*: LRT = 43.79, df =
321 1, $P < 0.0001$, $n = 31$ for the acute stress and social stress experiments, respectively).

322 *Experiment 1: response to an acute disturbance stress*

323 Corticosterone levels increased immediately after the acute stress relative to the control group
324 and returned to a basal level after one day (Figure 1A, *lme*: interaction group \times sampling
325 session: $F_{2,56} = 6.77$, $P = 0.002$). Individuals gained mass over the course of the experiment
326 (*lme*: sampling session: $F_{2,58} = 18.32$, $P < 0.0001$, estimates of change in mass in session 1 = -
327 0.08 ± 0.02 , session 2 = -0.03 ± 0.03 and in session 3 = 0.11 ± 0.03) and change in mass was
328 not affected by acute stress (*lme*: F tests, all $P > 0.74$). Body mass did not influence
329 corticosterone levels ($F_{1,55} = 1.75$, $P = 0.20$)

330 *Experiment 2: response to a chronic social stress*

331 In the social stress experiment, the dynamics of corticosterone levels through time differed
332 between the stress and control groups (Figure 1B). In the stress group, corticosterone levels
333 increased after lizards were paired and levels remained high during up to 3 days. After 9 days,
334 corticosterone levels had returned to a basal level and were not affected by the return of
335 lizards into their individual terrarium (*gamm*: approximate significance of smooth parameter,
336 $F_{2,52,121} = 6.14$, $P = 0.02$; Figure 1B). In the control group, corticosterone levels were stable
337 over the course of the experiment (*gamm*: approximate significance of smooth parameter,
338 $F_{1,121} = 1.77$, $P = 0.19$, Figure 1B). Corticosterone levels also decreased linearly with change
339 in mass (*lme*: β coefficient = -33.17 ± 5.54 , $F_{1,121} = 36.33$, $P < 0.001$) and change in mass
340 through time was affected by an interaction between time and treatment (*lme*: $F_{1,122} = 6.20$, P

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341 = 0.01). In the control group, individuals lost weight (*lme*: β coefficient = -0.004 ± 0.002)
342 while mass remained stable in the stress group (*lme*: β coefficient = 0.003 ± 0.003).

343 ***Stress response to density in semi-natural conditions***

344 *Experiment 3: response to population density*

345 Corticosterone levels were not affected by population density 11 days after release in the
346 outdoor enclosures (Table 1, June 19-26 2008 session, $n = 97$). However, about 3 months
347 after release, corticosterone levels were affected by an interaction between density and sex
348 (Table 1, September 2008 session, $n = 97$). Corticosterone levels increased with density in
349 males (β coefficient = 1.57 ± 1.09) but not in females (β coefficient = -0.74 ± 0.85) so that
350 corticosterone levels were lower in males than in females at low but not at high densities
351 (Figure 2). Male corticosterone levels in September 2008 responded similarly to the number
352 of males and of females per enclosure (number of males: intercept = 12.34 ± 3.19 , β
353 coefficient = 0.66 ± 0.30 ; number of females: intercept = 12.76 ± 3.02 , β coefficient = $0.46 \pm$
354 0.21 ; see Figure S1, supporting information). Corticosterone levels measured during the next
355 spring (i.e., in April ($n = 64$) and June ($n = 65$) 2009) were not affected by density (Table 1).

356 *Experiment 4: response to population density and adult sex ratio*

357 In the density and sex ratio factorial manipulation, male corticosterone levels were affected by
358 an interaction between population density and sex ratio 10 to 12 months after release in the
359 enclosures (April ($n = 106$) and June ($n = 166$) 2010 sessions, Table 2). At low density, male
360 corticosterone levels were higher in male-biased than in female-biased populations while the
361 opposite was observed at high density. Intermediate corticosterone levels were observed for
362 both sex ratios at medium density (Figure 3A and 3B, see Table S3 for model parameter
363 estimates). Male corticosterone levels in April 2010 increased with the number of adult males
364 per enclosure to up to 10 males and then reached a plateau to up to 19 males (Figure 3C, *lme*:
365 linear β coefficient = 1.97 ± 0.70 , $F_{1,21} = 7.79$, $P = 0.01$; quadratic β coefficient = $-0.09 \pm$

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366 0.03, $F_{1,21} = 7.00$, $P = 0.01$). On the contrary, male corticosterone levels in June 2010
367 decreased linearly with the number of males (Figure 3D, *lme*: β coefficient = -0.50 ± 0.23 ,
368 $F_{1,22} = 4.85$, $P = 0.04$). Male corticosterone levels in April 2010 also decreased with the
369 number of females in male-biased populations (*lme*: number of females \times sex ratio: $F_{1,20} =$
370 19.48 , $P = 0.0003$, β coefficient = -1.47 ± 0.74 , *post hoc* test: $F_{1,12} = 7.51$, $P = 0.02$) and
371 increased with the number of females in female-biased populations (*lme*: β coefficient = 1.81
372 ± 0.34 , *post hoc* test: $F_{1,8} = 20.99$, $P = 0.002$; Figure S2A). Male corticosterone levels in June
373 2010 were not affected by the number of females per enclosure (*lme*: F tests, all $P > 0.25$;
374 Figure S2B). In the 21 females recaptured in April 2010, corticosterone levels tended to
375 increase with the number of males per enclosure (Appendix S1, Figure S3, *lme*: linear β
376 coefficient = 1.41 ± 0.73 , $F_{1,12} = 3.70$, $P = 0.08$). In both experiments, corticosterone levels
377 varied importantly between sampling sessions (see Appendix S1, Figure S4).

378 Discussion

379 Using a combination of laboratory and field experiments in the common lizard *Zootoca*
380 *vivipara*, we investigated the relationship between laboratory forced social interactions or
381 population density and plasma levels of corticosterone. Our experiments revealed a strong
382 inter-individual variation in basal corticosterone levels as well as effects of internal factors
383 such as body mass and external factors such as time of the year and time of the day. These
384 results confirm that the activity of the HPA axis is highly plastic (Evans et al. 2006). More
385 importantly, our study revealed complex patterns of corticosterone response to chronic social
386 stress. The laboratory experiment provided strong evidence of a socially-mediated chronic
387 stress due to forced social interactions in the absence of a food restriction. However, in the
388 field experiments, plasma corticosterone levels increased with density only in males from
389 populations characterized by a female-biased adult sex ratio. Complementary analyses further
390 suggested that stress in males was mildly affected by the number of male competitors for

391 mates and increased with the number of reproductive females during the mating season in
392 female-biased populations. Altogether, our results thus provide limited evidence for a role of
393 basal corticosterone secretion in density feedbacks in the common lizard.

394 *Plasma corticosterone levels as a measure of the intensity and nature of stressors*

395 In the laboratory with *ad libitum* food supply, corticosterone levels increased within a day of
396 the onset of confinement of pairs of males and remained high for up to 3 days before
397 returning to baseline levels although group confinement was maintained. This adjustment to a
398 chronic stress could be due to a diminution of aggressive interactions when males become
399 familiar or to habituation. Similar effects of crowding on the HPA stress axis have been well
400 documented in other laboratory studies (e.g., Glennemeier and Denver 2002, Nephew and
401 Romero 2003), and suggest that heightened frequency of social interactions, including
402 aggressiveness and dominance, is sufficient to induce a chronic elevation of plasma
403 glucocorticoids in the absence of a food restriction.

404 Furthermore, the response of corticosterone levels to social stress differed from the
405 response to the acute disturbance stress. First, the response to the social confinement was
406 slightly lower than the one following the acute stress (see Figure 1). Also, the range of
407 increase of corticosterone levels in response to social stress in the field was of the same order
408 of magnitude than in the laboratory social stress experiment. Therefore, our laboratory
409 experiments demonstrate that a chronic social stressor, here due to a prolonged social
410 confinement with direct competition for a shelter and basking site, can induce a moderate
411 chronic corticosterone response in the common lizard compared to the strong short-term
412 response that an acute stress triggers. This result is in accordance with findings by other
413 studies that compared acute and chronic stresses (Carere et al. 2003, Rich and Romero 2005).
414 Second, the relationship between corticosterone levels and body mass differed between the
415 two laboratory experiments. In the acute stress experiment, the gain in mass did not differ

416 between the control and stressed groups and body mass did not influence corticosterone
417 levels. In the social stress experiment, corticosterone levels decreased linearly with a positive
418 change in mass throughout the experiment and mass decreased during the experiment in the
419 control group while it remained stable in the stressed group. This result further suggests that
420 chronic corticosterone secretion could be associated with changes in the energy balance in
421 accordance with our previous demonstration that experimentally-enhanced chronic
422 corticosterone levels increase foraging behavior and food consumption (Cote et al. 2006).

423 *Sex specific effects of density on stress response*

424 We predicted that physiological stress responses to density due to social stress should be
425 stronger in females than in males based on previous field studies in the common lizard
426 showing negative density feedbacks in female reproductive performances and stronger
427 density-dependent effects on body growth in females than in males (Mugabo et al. 2013) and
428 on the species' social and mating system (Le Galliard et al. 2005, Le Galliard et al. 2008).
429 Our findings contradict these predictions as corticosterone levels in the field only increased
430 with density in males, while corticosterone levels did not change significantly with population
431 density in females. In males, the density-dependent increase in corticosterone levels was seen
432 during the late summer of the density experiment but not immediately after release and not
433 during the next spring. It was also significant during spring in the density and sex ratio
434 experiment but solely in female-biased populations. These results indicate stronger effects of
435 density on basal corticosterone secretion in males than in females, even though the growth
436 and survival of adult males were not density-dependent (Mugabo et al. 2013). Previous
437 studies of the reactivity of the HPA axis in non-social species of mammals, birds and
438 amphibians have generally uncovered a positive effect of population density on plasma levels
439 of glucocorticoids, but this pattern has been found to vary across species and its link with the
440 population dynamics still remains unclear (reviewed in Creel et al. 2013).

441 Sex differences in physiological responses to stressors are regularly interpreted as
442 adaptive differences in the HPA activity and reactivity associated with the different life-
443 history tactics of males and females (e.g., Wingfield et al. 1994, Edwards et al. 2013). For
444 instance, female birds in a restricted habitat can suppress their stress response to avoid the
445 loss of a clutch (Wingfield et al. 1994). In this study, the absence of density-dependent HPA
446 response in females could not be explained by a strategy to ensure high quality reproduction
447 (Mugabo et al. 2013), but it could be a survival mechanism in females. Sex differences could
448 also be caused by differences in social interactions and space use behavior between males and
449 females. In the common lizard, adult males are socially dominant and more aggressive than
450 lizards from other age and sex classes, and thus may engage more in social interactions at
451 high densities, especially with other males during the mating season. Yet, during the density
452 experiment, the increase in corticosterone levels was seen in the late summer just before the
453 beginning of hibernation and outside the mating period. Late summer corresponds to the
454 period when male lizards complete the storage of the energetic reserves necessary for their
455 survival in early spring after the wintering period, when other age and sex classes are still
456 hibernating (Bauwens 1981). This period might therefore involve intense intra-specific
457 competition for food, basking sites for thermoregulation and shelters in crowded
458 environments. Indeed, we found that corticosterone levels in males responded similarly to the
459 number of males and of females at that time of the year, strongly suggesting that population
460 density *per se* triggered chronic stress responses in males before hibernation. The results of
461 our chronic social stress experiment in the laboratory, where pairs of males competed for
462 access to a single shelter and basking site also suggest that competition for limited resources
463 at high population densities can increase social stress in males.

464 In the density and sex ratio experiment, male corticosterone levels during the spring
465 season increased more with density in female-biased than in male-biased populations. In

466 addition, male corticosterone levels during the mating period slightly increased with the
467 number of males to up to 10 males and then reached a plateau, whereas corticosterone levels
468 right after the mating season decreased linearly with the number of males. This indicates that
469 male-male competition can increase social stress experienced by males like we predicted, but
470 only during the mating period. In addition, male corticosterone levels increased with the
471 number of adult females in female-biased populations during the mating period but not during
472 the post-mating period. This result suggests that social interactions with females, such as
473 more exploratory behaviors and mating attempts at the highest female densities, also
474 influence the activity of the HPA axis in males during the mating period. Unfortunately, the
475 small range of variation in the number of females per enclosure from male-biased treatments
476 and the lack of overlap with female-biased populations prevented us from drawing solid
477 conclusions about this relationship. In addition, it remains difficult to understand clearly this
478 pattern with our data since the number of males per enclosure was negatively correlated with
479 the number of females. To better understand the role of male-male competition and male
480 mating behaviors on the activity of the HPA, independent, factorial manipulations of the
481 density of adult males and adult females during the mating season should be conducted.

482 Regarding females, corticosterone levels were not significantly related to population
483 density. Thus, given the decline in the abundance of preys with lizard density (González-
484 Suárez et al. 2011), the negative density-dependent feedbacks in reproductive performances
485 and body growth seen in earlier studies (e.g., Mugabo et al. 2013) were more likely caused by
486 direct, energetic effects of food restriction rather than by other physiological effects mediated
487 by basal corticosterone secretion. This decoupling between environmental food restriction and
488 basal corticosterone secretion is supported by a previous laboratory study in the same species
489 (Cote et al. 2010). The relationship between food availability and corticosterone secretion has
490 been investigated only recently in free-living animals, especially seabirds (Jenni-Eiermann et

491 al. 2008, Kitaysky et al. 2010, Barrett et al. 2015), and current results are contrasted (Creel et
492 al. 2013).

493 The lack of a relationship between food restriction and corticosterone secretion in
494 females may be explained by the allostasis model in which the amount of available energy is a
495 crucial mediator of the stress response (Wingfield 2005, McEwen and Wingfield 2010). In
496 this model, plasma glucocorticoid levels increase with energetic demands and reach very high
497 levels only when the required energy by an individual to cope with environmental changes is
498 greater than the energy available in the environment. When the environmental change induces
499 an energetic demand below the amount of energy available in the environment, glucocorticoid
500 secretion should also increase but would reach lower levels. In our case, the severity of
501 nutritional stress in high density populations might not have been high enough to induce
502 strong, detectable differences in corticosterone secretions in females. For example, density did
503 not influence the survival of adult females and the quality of their offspring (Mugabo et al.
504 2013), suggesting little starvation among surviving females.

505 Preliminary data collected in the few adult females recaptured during the mating
506 period in the density and sex ratio experiment further suggested that their corticosterone
507 levels increased with the number of adult males during the mating season. This is in
508 accordance with our initial prediction of an effect of the number of males on social stress
509 experienced by females due to harmful interactions during mating (Le Galliard et al. 2008).
510 This elevation may be caused by repeated mating attempts of males and repeated copulations,
511 since both events are associated with aggressive male behaviors including physical fights,
512 biting and wounding (see Fitze et al. 2005, Le Galliard et al. 2005). We note however that this
513 trend was seen in a small sample of females and only in one of our two field experiments.
514 Unfortunately, less than 30 adult and yearling males in total survived up to the mating season
515 in the density experiment (see Table S1) preventing us from confirming this trend. This result

516 should therefore be confirmed with additional data and experiment focusing explicitly on
517 social stress during mating in females.

518 ***Conclusions***

519 Altogether, our data provide little support to the hypothesis that a chronic corticosterone
520 secretion is primarily involved in the negative density feedbacks in the common lizard due to
521 social stress. This could be because of the occurrence of behavioral compensations, including
522 spatial avoidance or changes in microhabitat use at high densities which would reduce the
523 intensity of the social stress experienced by individuals. These behavioral changes are more
524 likely to occur in sub-dominant (females and yearlings in the common lizard) and subordinate
525 individuals (juveniles) and could have contributed to the sex-specific patterns we observed.
526 However, our findings suggest that density and adult sex ratio interact to influence the
527 intensity of social stress, with sex-specific responses due to the different roles of males and
528 females in the social and mating system of the common lizard. The increase in plasma
529 corticosterone in males seen at higher population densities may have long-term effects on
530 their longevity that remain to be investigated. In addition, we speculate that male harassment
531 during the mating season, rather than population density *per se*, may cause social stress in
532 females with substantial effects on their life history and population dynamics (see Le Galliard
533 et al. 2005, Le Galliard et al. 2008).

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539 Île-de-France (files p3/2008/008, p3/2009/07 and Ce5/2010/039).

540 **Data accessibility**

541 All data will be made accessible on Dryad repository upon publication.

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646 **Tables**

647 **Table 1.** Effect of population density, sex, age class, body mass, date, time since release and time of the day (quadratic regression) on corticosterone
 648 levels at each sampling session before, during and after the density manipulation. Results are from backward elimination of non-significant effects.
 649 Significant effects are in bold. Marginally significant effects are in italic. Sampling date was included as a continuous variable in all models.

Fixed effects	June 7-8 2008		June 19-26 2008		September 9-15 2008		April 27 – May 1 2009		May 20-July 4 2009	
	F_{ndf,ddf}	<i>P</i> value	F_{ndf,ddf}	<i>P</i> value	F_{ndf,ddf}	<i>P</i> value	F_{ndf,ddf}	<i>P</i> value	F_{ndf,ddf}	<i>P</i> value
Density	F _{1,22} = 1.55	0.23	F _{1,22} = 1.78	0.20	F _{1,21} = 0.75	0.40	F _{1,20} = 0.18	0.68	F _{1,19} = 0.02	0.88
Sex	F _{1,192} = 0.02	0.89	F _{1,70} = 1.73	0.19	F_{1,70} = 9.74	0.003	F _{1,37} = 0.19	0.66	F _{1,38} = 0.0009	0.98
Age class	-	-	-	-	F _{1,68} = 0.02	0.89	F _{1,39} = 0.55	0.46	F _{1,40} = 0.53	0.47
Sex × age class	-	-	-	-	F _{1,67} = 2.28	0.14	F _{1,35} = 0.02	0.88	F _{1,36} = 0.12	0.73
Density × sex	F _{1,191} = 0.22	0.64	F _{1,68} = 0.10	0.75	F_{1,70} = 4.48	0.04	F _{1,33} = 0.001	0.97	F _{1,37} = 0.51	0.48
Density × age class	-	-	-	-	F _{1,66} = 0.15	0.70	F _{1,38} = 0.52	0.48	F _{1,39} = 0.56	0.46
Body mass (g)	F_{1,195} = 15.87	0.0001	-	-	-	-	-	-	F _{1,41} = 2.71	0.11
Sampling date (d)	F_{1,195} = 5.87	0.02	F_{1,71} = 5.20	0.03	F _{1,69} = 1.79	0.18	F _{1,40} = 3.24	0.08	F _{1,34} = 0.06	0.8
Time since release (d)	-	-	F _{1,67} = 0.08	0.78	F_{1,70} = 6.36	0.01	F _{1,34} = 0.06	0.81	F _{1,35} = 0.03	0.86
Time of the day (h)	F _{1,194} = 1.46	0.23	F_{1,71} = 14.89	0.0002	F_{1,70} = 16.01	0.0002	F_{1,41} = 6.90	0.01	F _{1,43} = 0.04	0.84
Time of the day ² (h)	F _{1,193} = 2.57	0.11	F _{1,69} = 0.55	0.46	F _{1,65} = 0.05	0.82	F _{1,36} = 0.06	0.81	F _{1,42} = 1.93	0.17

650 g = grams, d = days, h = hour.

651

Density-dependent stress response in a lizard

652 **Table 2.** Effect of population density and sex ratio, body mass, date, time since release and time
 653 of the day (quadratic regression) on corticosterone levels at each sampling session in the density
 654 and sex ratio experiment. Results are from backward elimination of non-significant effects.
 655 Significant effects are in bold. Sampling date was included as a categorical variable (3 days of
 656 sampling in June-July 2009 and 2 in April and June 2010).

657

Fixed effects	June 21 –July 6 2009		April 27-28 2010		June 1-2 2010	
	F_{ndf,ddf}	<i>P</i> value	F_{ndf,ddf}	<i>P</i> value	F_{ndf,ddf}	<i>P</i> value
Density	F _{2,21} = 0.29	0.75	F_{2,18} = 11.75	0.0005	F _{2,18} = 2.09	0.15
Sex ratio	F _{1,20} = 0.25	0.62	F_{1,18} = 11.38	0.0003	F _{1,18} = 2.77	0.11
Density × sex ratio	F _{2,18} = 0.28	0.76	F_{2,18} = 10.74	0.0009	F_{2,18} = 4.03	0.04
Body mass (g)	F _{2,62} = 3.39	0.07	F_{1,58} = 4.40	0.04	F _{1,137} = 1.66	0.20
Sampling date (d)	F _{2,60} = 0.04	0.96	F_{1,58} = 4.13	0.05	F_{1,138} = 6.66	0.01
Time since release (d)	-	-	F _{1,57} = 1.81	0.18	F_{1,138} = 7.18	0.01
Time of the day (h)	F _{1,63} = 0.01	0.92	F_{1,58} = 17.63	0.0001	F _{1,138} = 0.15	0.70
Time of the day ² (h)	F_{1,63} = 10.47	0.002	F _{1,56} = 0.06	0.80	F_{1,138} = 14.62	0.0002

658 g = grams, d = days, h = hour.

659

660 **Figure legends**

661 **Figure 1.** Corticosterone levels of male common lizards in response to a acute stress (A) and to
 662 a chronic social stress in laboratory conditions (B). Data are mean \pm se. In (B) component
 663 smooths (solid lines) and standard errors (dashed lines) are from a generalized additive mixed
 664 effects model (GAMM) in the stress (thick lines) and control groups (thin lines). GAMMs
 665 included a non-parametric smoother on day from start (the smoothness was constrained to a
 666 spline of 3 degrees of freedom, using the argument $k = 4$). In (B), arrows indicate the day when
 667 individuals from the stress group were put by pairs per terrarium and the day when they were
 668 put back into their initial individual terrarium. A: $n = 15$ per group. B: $n = 15$ in the control
 669 group and 16 in the social stress group. Adjusted $R^2 = 0.23$ in (A) and 0.14 in (B).

670

671 **Figure 2.** Corticosterone levels of common lizards according to population density and sex in
 672 late summer 2008 (i.e., 3 months after the start of the density manipulation). Data are mean
 673 corticosterone levels \pm se of wild animals with regressions lines obtained from the minimum
 674 adequate model selected (see main text). $n = 44$ males and 53 females. M: males, F: females.
 675 Adjusted $R^2 = 0.31$.

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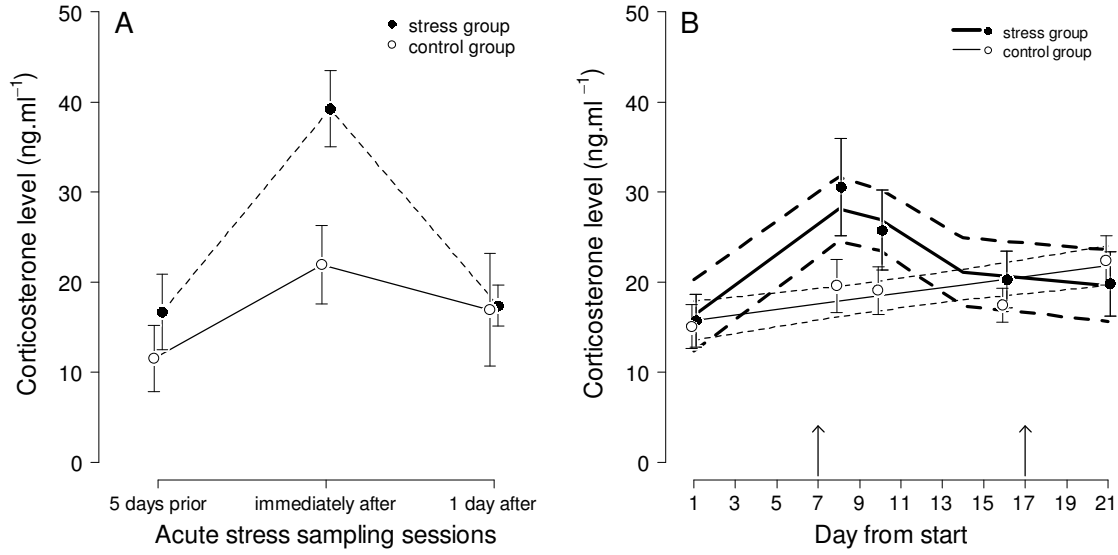
677 **Figure 3.** Corticosterone levels of male common lizards recaptured in April (A, C) and in June
 678 2010 (B, D), i.e., 10 and 12 months after the start of the manipulation of population density and
 679 sex ratio. Data are plotted according to treatment groups (A, B) and according to the number of
 680 adult males in each population (C, D). (A, B) Data are mean corticosterone levels \pm se. (C-D)
 681 Raw data are plotted with the regression line (solid) and associated error lines (dotted lines)
 682 from the minimum adequate model (see main text). A, C: $n = 85$. B, D: $n = 166$. MB: male-
 683 biased, FB: female-biased. Adjusted $R^2 = 0.27$ in (A), 0.19 in (B), in 0.29 (C) and 0.23 in (D).

Density-dependent stress response in a lizard

684 **Figures**

685 **Figure 1**

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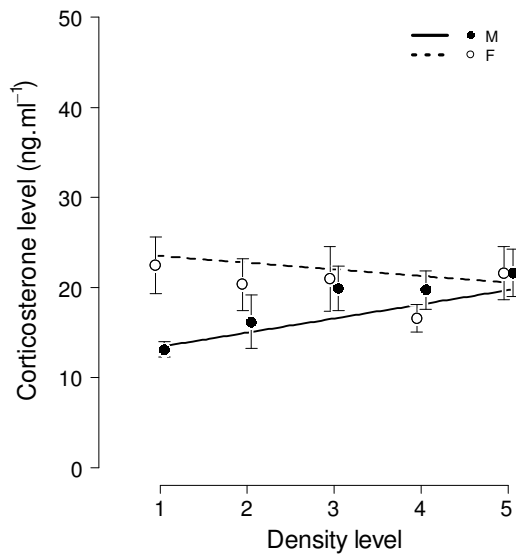
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Density-dependent stress response in a lizard

689 **Figure 2**

690



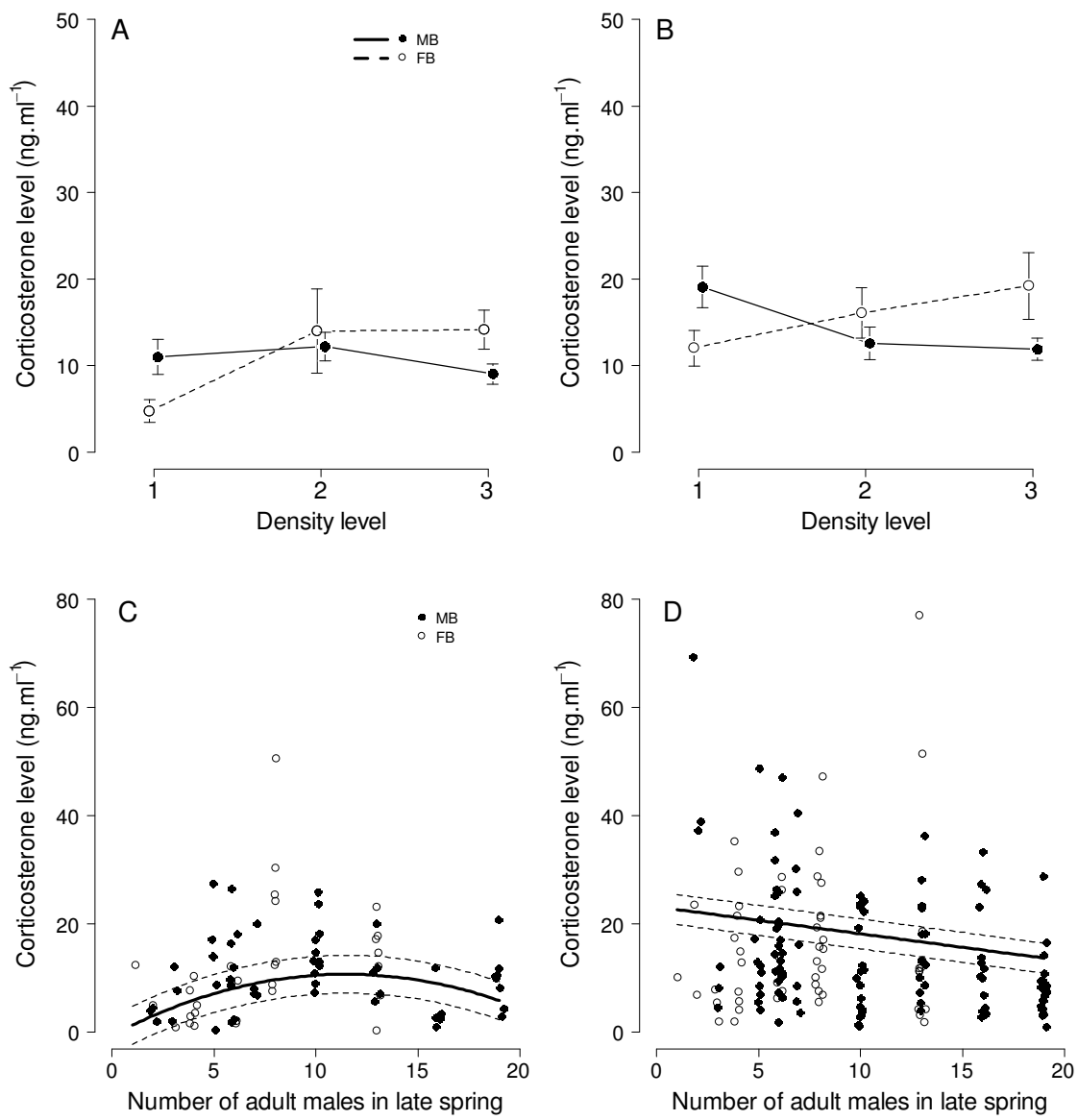
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Density-dependent stress response in a lizard

693 **Figure 3**

694



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