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

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

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

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

Yet, despite wide agreement from laboratory studies over the existence and pathological consequences of chronic glucocorticoids secretion (Christian 1956, Bhatnagar and Vining 2003, Vegas et al. 2006), studies

in wild populations have produced evidence of a variable link between the HPA axis function and density feedbacks (Boonstra 2013, Creel et al. 2013). This is particularly true in wild populations of rodents, birds and reptiles in which corticosterone is the main adrenal glucocorticoid mediating stress responses (Meylan et al. 2003, Cote et al. 2006, Creel et al. 2013). For instance, in rodents, circulating corticosterone levels increase with population density in several territorial species, while other factors such as breeding stage or predation risks are more important determinants of corticosterone levels in others (reviewed by Creel et al. 2013). There is also very limited evidence of density-dependent chronic secretion of corticosterone in birds (but see Raouf et al. 2006, Viblanc et al. 2014) and in squamate reptiles (but see Comendant et al. 2003). Besides potential methodological differences in the characterization of HPA axis regulation (Breuner et al. 2013), such inconsistencies may come from the limited range of density variation in observational studies. At the same time, experimental studies may not always reflect the natural densities to which animals are exposed and may not allow for behavioral compensations, including spatial avoidance or changes in microhabitat use. Moreover, individual factors such as age, sex and social rank and external factors such as seasonal conditions or predation risks can affect glucocorticoids levels and change the intensity of social stress (Creel et al. 2013). Finally, a chronic social stress could suppress subsequent physiological responses to acute and social stressors and reduce baseline corticosterone levels (Rich and Romero 2005, Cyr and Romero 2007), thus contributing to the lack of 'a consensus endocrine profile for chronically stressed wild animals' (Dickens and Romero 2013). There is therefore a strong need for field studies that examine the HPA axis regulation through time under chronic social stress across a relevant range of population densities while accounting for potentially confounding factors at the individual and population levels (reviewed by Creel et al. 2013). Here, we present the results of such a study in the common lizard *Zootoca vivipara* where we manipulated the density of experimental populations of lizards maintained in outdoor enclosures over a wide range of densities (from below to above the carrying capacity) and measured plasma levels of corticosterone before, during and after the experiment.

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

Based on this knowledge, we predicted that corticosterone secretion increases in response to chronic social stressors in this species (prediction 1) and therefore that plasma corticosterone levels should increase with population density due to increased levels of social stress (prediction 2). Stress response to density could be higher in females than in males due to a stronger sensitivity to density than males (as seen in body growth, Mugabo et al. 2013), a lower social dominance and repeated harmful social interactions with males during the breeding season (Le Galliard et al. 2005, 2008). We also expected the increase of corticosterone levels with density to be stronger in male-biased than in female-biased populations due to competition for mates in males and male aggressions on females during mating (prediction 3). To test these three predictions, we conducted two laboratory experiments and two successive field experiments in semi-natural conditions. First, we tested prediction 1 by comparing the patterns of temporal variations of plasma corticosterone levels following an acute disturbance stress and a chronic social stress (prolonged social confinement) under controlled laboratory conditions. Second, we tested prediction 2 by manipulating population density in female-biased populations maintained in semi-natural conditions. We then tested prediction 2 and 3 by cross manipulating density and adult sex ratio in a second field experiment. We monitored populations for a year in both field experiments and measured corticosterone levels in males and females before, during and after each experiment. Altogether, these experiments enabled us to investigate whether levels of circulating corticosterone are affected by chronic social stress and whether population density triggers chronic secretion of corticosterone in a species exhibiting strong negative density feedbacks.

Methods

Model species

Zootoca vivipara is a small (adult snout-vent length <75 mm) ovoviviparous lizard inhabiting humid habitats across northern Eurasia. Natural populations can be structured in three age classes: juveniles (newborn individuals), yearlings (1–2 years old) and adults. In natural populations from where experimental individuals originated and in our study site (CEREEP research center, Saint-Pierre-lès-Nemours, France) basal plasma corticosterone levels vary from 1 to 181 ng ml^{-1} in adults and are similar between sexes (Meylan et al. 2003, Cote et al. 2006).

Experimental protocols

Stress response in laboratory conditions

During June and July 2010, we conducted two laboratory experiments to test our prediction that corticosterone secretion increases in response to chronic social stress in this species (prediction 1). First, we carried out an acute disturbance stress experiment to produce a baseline stress response to compare to chronic social stressors, knowing that acute

and chronic stressors can trigger very distinctive patterns of glucocorticoid responses (Carere et al. 2003, Rich and Romero 2005). We then carried out a chronic social stress experiment where the chronic social stressor was a prolonged social confinement during which pairs of males shared a single basking and shelter site in a terrarium under ad libitum food conditions. More specifically, this experiment enabled us to test for the effect of social interactions and competition for a shelter and microhabitat for optimal thermoregulation on corticosterone secretion independently of trophic interactions.

Experiment 1: response to an acute disturbance stress

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

Experiment 2: response to a chronic social stress

In the 'chronic social stress' experiment, 16 males were maintained by pairs for 10 days in a terrarium containing a single shelter and basking site (stress group) while 15 males were left alone in their individual terrarium (control group, one male of the control group died before the beginning of the experiment). Pairs of individuals of similar body size were created to avoid the establishment of a size-based hierarchy. Five repeated measurements of corticosterone levels were carried out. First, basal corticosterone levels were measured from blood samples collected six days before individuals from the stress group were paired. Then, three sets of blood samples were collected one day, three days and nine days after the beginning of the experiment. After 10 days, lizards from the stress group were transferred back into their individual terrarium and a last blood sample was collected four days later. During both experiments, lizards were fed daily ad libitum and maintained in $25 \times 15 \times 15$ cm^3 terraria under optimal laboratory conditions for light, water availability and temperature (see Le Galliard et al. 2003 for more details on lizards' husbandry). All individuals were weighted to the nearest 0.01 g immediately after each blood sampling. Change in mass throughout the experiments was monitored to control for potential effects of stressors on food consumption.

Stress response to density in semi-natural conditions

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

Experiment 3: response to population density

During June and July 2008, we manipulated the initial density of 24 populations maintained in 10×10 m outdoor enclosures located in a natural meadow at the CEREEP research center in Saint-Pierre-lès-Nemours, France (48°17′N, 2°41′E). Enclosures provided lizards with wild preys and their abundance was negatively affected by the density of lizards (González-Suárez et al. 2011). Populations were established post-breeding following a gradient of five density levels ranging from 7 to 35 adults and yearlings (equivalent to 700 to 3500 lizards per ha) and 10 to 50 juveniles. Density level 1 had three adults, four yearlings and 10 juveniles. Density levels 2 to 5 differed from density level 1 by a multiplicative factor of 2 to 5 respectively. All populations were female-biased with a sex ratio of 0.43 (calculated as the proportion of yearling and adult males with 1:2 adult and 1:1 yearling males and females) and had a similar age-structure (Mugabo et al. 2013). Lizards ($n = 162$ adults, 216 yearlings and 549 juveniles) were randomly assigned to experimental populations and were released in outdoor enclosures in June–July 2008. All yearling and adult males and non-reproductive females were released between 11 and 13 June and all reproductive females and their juveniles were released within two days post parturition from 11 June to 27 July. Blood samples were collected from adult males and yearling males and females prior to release in the enclosures in June–July 2008 (most adult females were still pregnant at this time and were therefore not sampled; potentially reproductive yearling females were kept until their non-reproductive status was confirmed before being sampled for blood up to July 25). Blood samples were then collected in all enclosures during three successive recapture sessions in late June 2008, September 2008 and April–May 2009. Finally, all surviving individuals were recaptured in May–June 2009 and blood samples were collected in laboratory conditions on all individuals except pregnant females (Supplementary material Appendix 1 Table A2). Individuals were measured for body mass after the collection of blood samples in the laboratory.

Experiment 4: response to population density and sex ratio

During June and July 2009, we cross manipulated the initial population density and sex ratio in 24 populations. Populations were established post-breeding according to 3 density levels and 2 sex ratio levels, i.e. female-biased versus male-biased populations. Density level 1 had four adults, six yearlings and 12 juveniles and density levels 2 and 3 differed from density level 1 by a multiplicative factor of 2 and 3 respectively. All populations had similar age-structures (Supplementary material Appendix 1 Table A1). Female-biased populations had a sex ratio of 0.4 with 1:3 adult and 3:3 yearling males and females and male-biased populations had a sex ratio of 0.7 with 3:1 adult and 2:4 yearling males and females. Juvenile sex ratio was balanced in all treatments. Lizards ($n = 164$ adults, 246 yearlings and 492 juveniles) were randomly assigned to experimental populations and released in outdoor enclosures in June–July 2009 (see Supplementary

material Appendix 1 Table A1 for more details). Three sets of blood samples were collected: prior to release in laboratory conditions in June–July 2009, in all enclosures in April 2010 and in laboratory conditions after capture in June 2010 (Supplementary material Appendix 1 Table A2). All individuals were measured for body mass after blood sampling.

Blood sampling and measurements of plasma corticosterone levels

Except in the acute stress group, blood samples were taken within one minute after capture to avoid an increase of plasma corticosterone levels due to handling. During the capture sessions in the enclosures, observers only spent few minutes in each enclosure to capture wild lizards in order to avoid stressing the individuals by repeatedly trying to catch them. However, corticosterone levels in the field were not affected by the time spent in the enclosures prior the captures in a recent study that was carried out in the same experimental system (Mell et al. 2016). About 40–60 µl of whole blood was collected from the post-orbital sinus using 20 µl microhematocrit tubes. Immediately after sampling, blood was centrifuged and the plasma was stored at –30°C. Plasma corticosterone levels were later determined using a competitive enzyme-immunoassay procedure (IDS corticosterone EIA kit, ref AC-14F1, IDS EURL Paris, France). This method provides a quantitative determination of total corticosterone concentration in a set volume of plasma using a polyclonal corticosterone antibody and is based on a colorimetric assay (absorbance read at 450 nm). The sensitivity of the corticosterone EIA kit is 0.55 ng ml^{-1}. For all samples, 10 µl of plasma were diluted 10 times in 90 µl of the sample diluent provided in the EIA kit except for 10 samples which were diluted 20 times due to low volumes of plasma and for which 5 µl of plasma were used instead (see Supplementary material Appendix 1 Table A2 for sample size). Intra-plate repeatability was estimated by comparing the concentrations of blood samples run twice on the same plate and inter-plate repeatability was estimated by comparing the concentrations of blood samples run twice on two different plates. Measurements were highly repeatable (intra-plate repeatability: $n = 49$, $F_{1,47} = 495.78$, $p < 0.0001$, intra-class correlation coefficient $r = 0.91 \pm 0.03$; inter-plate repeatability: $n = 46$, $F_{1,44} = 112.15$, $p < 0.0001$, intra-class correlation coefficient $r = 0.79 \pm 0.06$).

Statistical analyses

Experiment 1

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

Experiment 2

Changes in corticosterone levels in response to a chronic social stress in laboratory conditions were analyzed with generalized additive mixed effects models (GAMMs). GAMMs were used to model the non-linear relationship between corticosterone levels and time due to the occurrence and disappearance of a chronic social stressor. The fixed part of the models included a smooth function on the number of days since the beginning of the experiment, a group effect (stress versus control) and their two-way interaction as well as an effect of mass and change in mass. Change in mass in response to chronic social stress was analyzed as in experiment 1. Individual identity was included as a random effect in models of corticosterone levels and change in mass to account for repeated measurements on the same individual.

Experiment 3 and 4

Effects of population treatments on corticosterone levels were analyzed independently for each blood sampling session (i.e. five sessions in the density experiment, three sessions in the density and sex ratio experiment, Supplementary material Appendix 1 Table A3) since the same individuals were not always captured in each session. In the data analysis of the density experiment, fixed effects were density (density level as a continuous variable), sex, age class and two-way interactions with density to test for sex and age-specific effects of density. In the data analysis of the density and sex ratio experiment, females were not included since only 21 were sampled in April 2010 and none were measured in June 2010. Thus, fixed effects of the models were density (categorical variable), sex ratio and their two-way interaction in order to test for an interaction between density and sex ratio on corticosterone levels in males. Age effects were not included because the numbers per age class in each treatment were too low. However, no significant age effects were found in the density experiment (Table 1).

Since a significant relationship between corticosterone levels and density was only found in males, we used the number of males per enclosure as a covariate (quadratic regression) to further investigate if corticosterone levels were influenced by the intensity of male-male interactions. We also used the number of females per enclosure as a covariate (quadratic regression) to investigate if corticosterone levels in males were influenced by the number of potential sexual partners in the population. These tests were run separately to avoid multicollinearity between the covariates number of males and number of females. Finally, we investigated if corticosterone levels in the 21 females recaptured in April 2010 were influenced by the number of males in the population (linear regression).

Additional fixed effects included in all analyses of field data were body mass (when measured), sampling date within each sampling session (continuous variable in the density experiment and categorical variable in the density and sex ratio experiment), time since release in enclosures (in days)

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

	7–8 June 2008		19–26 June 2008		9–15 September 2008		27 April–1 May 2009		20 May–4 July 2009	
Fixed effects	$F_{ndf,ddf}$	p-value	$F_{ndf,ddf}$	p-value	$F_{ndf,ddf}$	p-value	$F_{ndf,ddf}$	p-value	$F_{ndf,ddf}$	p-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 day² (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

and time of the day (in hours, quadratic regression). These variables were included in the models to account for known confounding factors of glucocorticoid responses to chronic social stressors (reviewed by Creel et al. 2013). Enclosure identity was included as a random effect.

General methods for all statistical analyses

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

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.pm86h> (Mugabo et al. 2016).

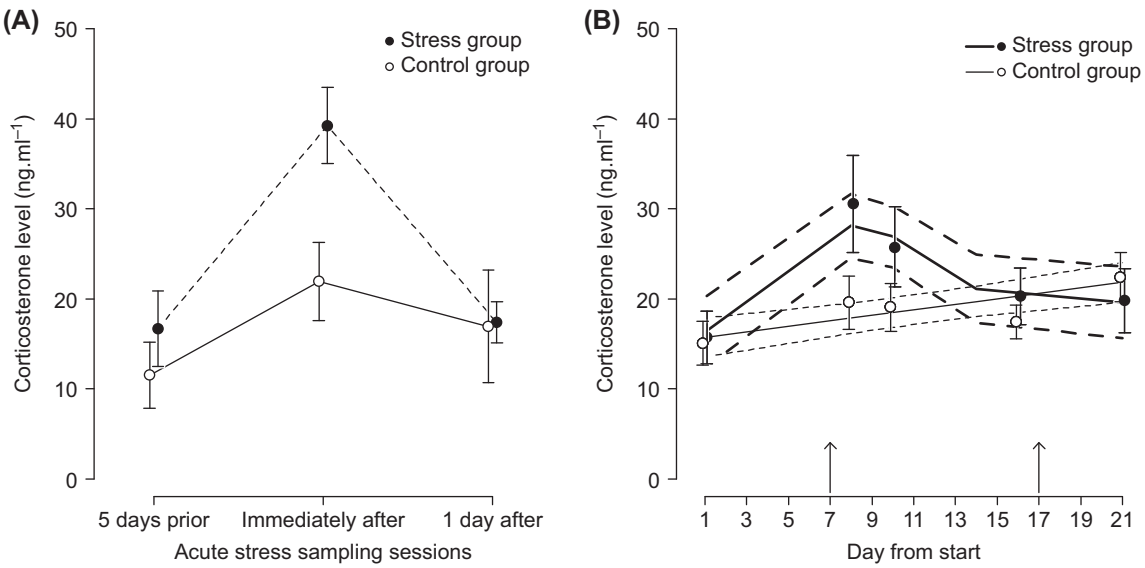

Figure 1. Corticosterone levels of male common lizards in response to a acute stress (A) and to a chronic social stress in laboratory conditions (B). Data are mean ± SE. In (B) component smooths (solid lines) and standard errors (dashed lines) are from a generalized additive mixed effects model (GAMM)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

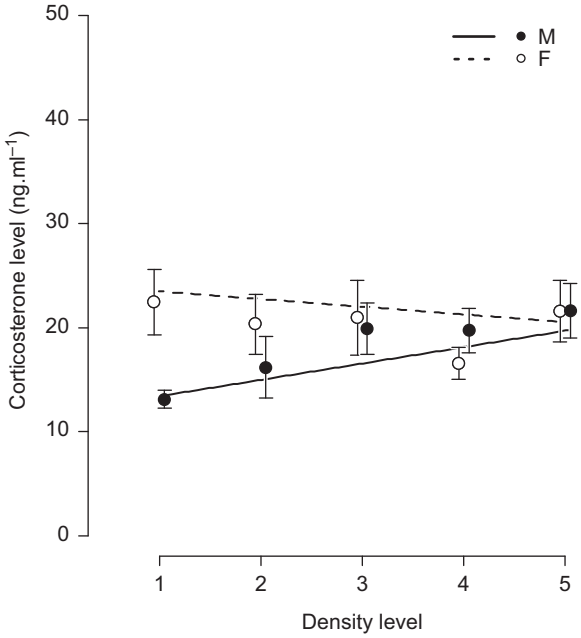

Figure 2. Corticosterone levels of common lizards according to population density and sex in late summer 2008 (i.e. three months after the start of the density manipulation). Data are mean corticosterone levels ± SE of wild animals with regressions lines obtained from the minimum adequate model selected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

Results

Stress response in laboratory conditions

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

Experiment 1: response to an acute disturbance stress

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

Experiment 2: response to a chronic social stress

In the social stress experiment, the dynamics of corticosterone levels through time differed between the stress and control groups (Fig. 1B). In the stress group, corticosterone levels increased after lizards were paired and levels remained high during up to three days. After nine days, corticosterone levels had returned to a basal level and were not affected by the return of lizards into their individual terrarium (*gamm*: approximate significance of smooth parameter, $F_{2.52,121} = 6.14$, $p = 0.02$; Fig. 1B). In the control group, corticosterone levels were stable over the course of the experiment (*gamm*: approximate significance of smooth parameter, $F_{1,121} = 1.77$, $p = 0.19$, Fig. 1B). Corticosterone levels also decreased linearly with change in mass (*lme*: β-coefficient = –33.17 ± 5.54, $F_{1,121} = 36.33$, $p < 0.001$) and change in mass through time was affected by an interaction between time and treatment (*lme*: $F_{1,122} = 6.20$, $p = 0.01$). In the control group, individuals lost weight (*lme*: β-coefficient = –0.004 ± 0.002) while mass remained stable in the stress group (*lme*: β-coefficient = 0.003 ± 0.003).

Stress response to density in semi-natural conditions

Experiment 3: response to population density

Corticosterone levels were not affected by population density 11 days after release in the outdoor enclosures (Table 1, 19–26 June 2008 session, n = 97). However, about three months after release, corticosterone levels were affected by an interaction between density and sex (Table 1, September 2008 session, n = 97). Corticosterone levels increased with density in males (β-coefficient = 1.57 ± 1.09) but not in females (β-coefficient = –0.74 ± 0.85) so that corticosterone levels were lower in males than in females at low but not at high densities (Fig. 2). Male corticosterone levels in September 2008 responded similarly to the number of males and of females per enclosure (number of males: intercept = 12.34 ± 3.19, β-coefficient = 0.66 ± 0.30;

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

	June 21–July 6 2009		April 27–28 2010		June 1–2 2010	
Fixed effects	$F_{ndf,ddf}$	p-value	$F_{ndf,ddf}$	p-value	$F_{ndf,ddf}$	p-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 day² (h)	**$F_{1,63} = 10.47$**	**0.002**	$F_{1,56} = 0.06$	0.80	**$F_{1,138} = 14.62$**	**0.0002**

number of females: intercept = 12.76 ± 3.02, β-coefficient = 0.46 ± 0.21; Supplementary material Appendix 1 Fig. A1). Corticosterone levels measured during the next spring (i.e. in April (n = 64) and June (n = 65) 2009) were not affected by density (Table 1).

Experiment 4: response to population density and adult sex ratio

In the density and sex ratio factorial manipulation, male corticosterone levels were affected by an interaction between population density and sex ratio 10 to 12 months after release in the enclosures (April (n = 106) and June (n = 166) 2010 sessions, Table 2). At low density, male corticosterone levels were higher in male-biased than in female-biased populations while the opposite was observed at high density. Intermediate corticosterone levels were observed for both sex ratios at medium density (Fig. 3A–B, see Supplementary material Appendix 1 Table A3 for model parameter estimates). Male corticosterone levels in April 2010 increased with the number of adult males per enclosure to up to 10 males and then reached a plateau to up to 19 males (Fig. 3C, *lme*: linear β-coefficient = 1.97 ± 0.70, $F_{1,21} = 7.79$, p = 0.01; quadratic β-coefficient = –0.09 ± 0.03, $F_{1,21} = 7.00$, p = 0.01). On the contrary, male corticosterone levels in June 2010 decreased linearly with the number of males (Fig. 3D, *lme*: β-coefficient = –0.50 ± 0.23, $F_{1,22} = 4.85$, p = 0.04). Male corticosterone levels in April 2010 also decreased with the number of females in male-biased populations (*lme*:

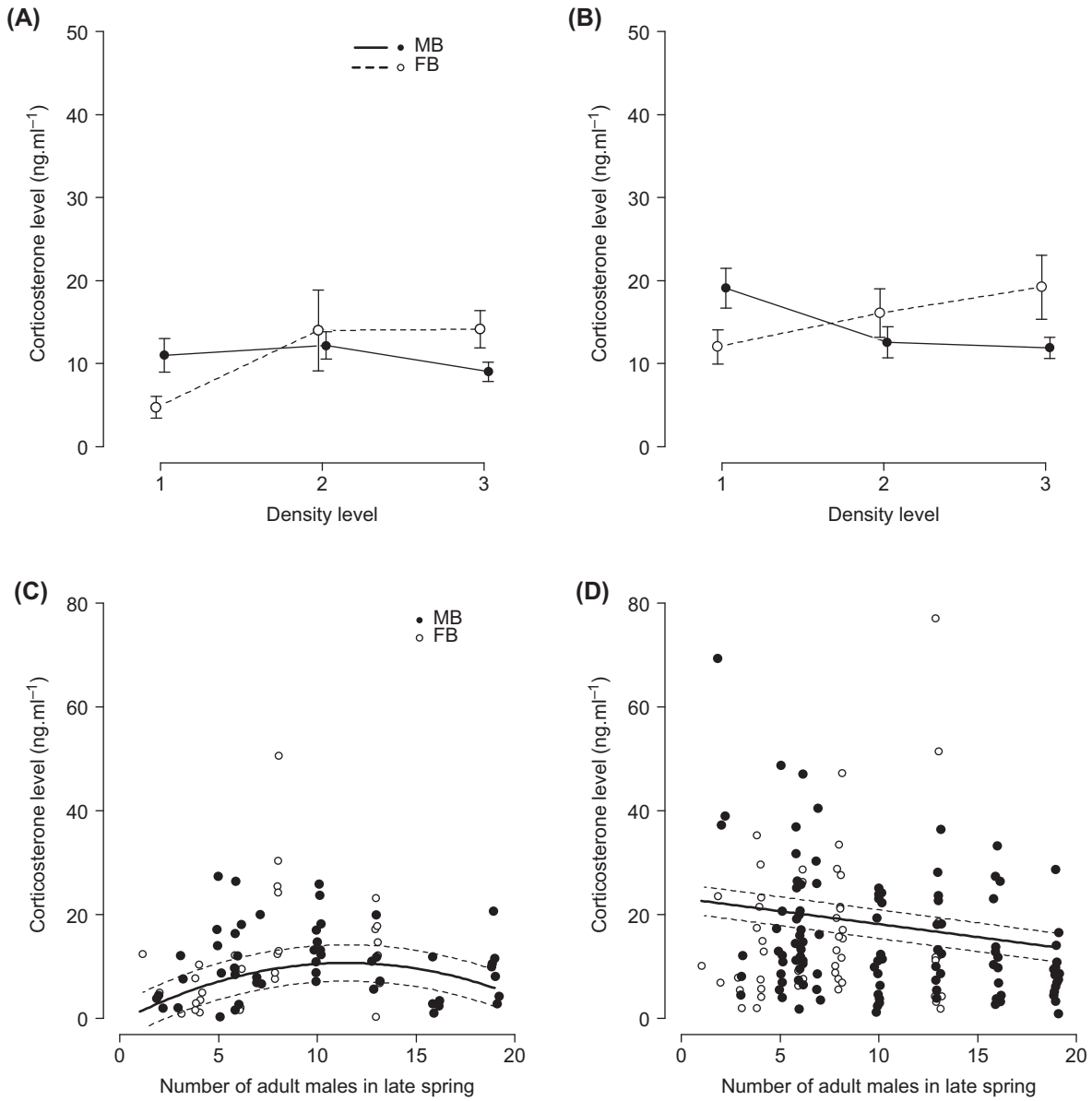

Figure 3. Corticosterone levels of male common lizards recaptured in April (A, C) and in June 2010 (B, D), i.e. 10 and 12 months after the start of the manipulation of population density and sex ratio. Data are plotted according to treatment groups (A, B) and according to the number of adult males in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

number of females × sex ratio: $F_{1,20} = 19.48$, $p = 0.0003$, β-coefficient = -1.47 ± 0.74, post hoc test: $F_{1,12} = 7.51$, $p = 0.02$) and increased with the number of females in female-biased populations (*lme*: β-coefficient = 1.81 ± 0.34, post hoc test: $F_{1,8} = 20.99$, $p = 0.002$; Supplementary material Appendix 1 Fig. A2A). Male corticosterone levels in June 2010 were not affected by the number of females per enclosure (*lme*: F-tests, all $p > 0.25$; Supplementary material Appendix 1 Fig. A2B). In the 21 females recaptured in April 2010, corticosterone levels tended to increase with the number of males per enclosure (Supplementary material Appendix 1 Fig. A3, *lme*: linear β-coefficient = 1.41 ± 0.73, $F_{1,12} = 3.70$, $p = 0.08$). In both experiments, corticosterone levels varied importantly between sampling sessions (Supplementary material Appendix 1 Fig. A4).

Discussion

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

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

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

Furthermore, the response of corticosterone levels to social stress differed from the response to the acute disturbance stress. First, the response to the social confinement was slightly lower than the one following the acute stress (Fig. 1). Also, the range of increase of corticosterone levels in response to social stress in the field was of the same order of magnitude than in the laboratory social stress experiment. Therefore, our laboratory experiments demonstrate that a chronic social stressor, here due to a prolonged social confinement with direct competition for a shelter and basking site, can induce a moderate chronic corticosterone response in the common lizard compared to the strong short-term response that an acute stress triggers. This result is in accordance with findings by other studies that compared acute and chronic stresses (Carere et al. 2003, Rich and Romero 2005). Second, the relationship between corticosterone levels and body mass differed between the two laboratory experiments. In the acute stress experiment, the gain in mass did not differ between the control and stressed groups and body mass did not influence corticosterone levels. In the social stress experiment, corticosterone levels decreased linearly with a positive change in mass throughout the experiment and mass decreased during the experiment in the control group while it remained stable in the stressed group. This result further suggests that chronic corticosterone secretion could be associated with changes in the energy balance in accordance with our previous demonstration that experimentally-enhanced chronic corticosterone levels increase foraging behavior and food consumption (Cote et al. 2006).

Sex specific effects of density on stress response

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

Sex differences in physiological responses to stressors are regularly interpreted as adaptive differences in the HPA activity and reactivity associated with the different life-history

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

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

Regarding females, corticosterone levels were not significantly related to population density. Thus, given the decline in the abundance of preys with lizard density (González-Suárez et al. 2011), the negative density-dependent feedbacks in reproductive performances and body growth seen in earlier studies (Mugabo et al. 2013) were more likely caused by direct, energetic effects of food restriction rather than by other physiological effects mediated by basal corticosterone secretion. This decoupling between environmental food restriction and basal corticosterone secretion is supported by a previous laboratory study in the same species (Cote et al. 2010). The relationship between food availability and corticosterone secretion has been investigated only recently in free-living animals, especially seabirds (Jenni-Eiermann et al. 2008, Kitaysky et al. 2010, Barrett et al. 2015), and current results are contrasted (Creel et al. 2013).

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

Preliminary data collected in the few adult females recaptured during the mating period in the density and sex ratio experiment further suggested that their corticosterone levels increased with the number of adult males during the mating season. This is in accordance with our initial prediction of an effect of the number of males on social stress experienced by females due to harmful interactions during mating (Le Galliard et al. 2008). This elevation may be caused by repeated mating attempts of males and repeated copulations, since both events are associated with aggressive male behaviors including physical fights, biting and wounding (Fitze et al. 2005, Le Galliard et al. 2005). We note however that this trend was seen in a small sample of females and only in one of our two field experiments. Unfortunately, less than 30 adult and yearling males in total survived up to the mating season in the density experiment (Supplementary material Appendix 1 Table A1) preventing us from confirming this trend. Thisresult should therefore be confirmed with additional data and experiment focusing explicitly on social stress during mating in females.

Conclusions

Altogether, our data provide little support to the hypothesis that a chronic corticosterone secretion is primarily involved in the negative density feedbacks in the common lizard due

to social stress. This could be because of the occurrence of behavioral compensations, including spatial avoidance or changes in microhabitat use at high densities which would reduce the intensity of the social stress experienced by individuals. These behavioral changes are more likely to occur in sub-dominant (females and yearlings in the common lizard) and subordinate individuals (juveniles) and could have contributed to the sex-specific patterns we observed. However, our findings suggest that density and adult sex ratio interact to influence the intensity of social stress, with sex-specific responses due to the different roles of males and females in the social and mating system of the common lizard. The increase in plasma corticosterone in males seen at higher population densities may have long-term effects on their longevity that remain to be investigated. In addition, we speculate that male harassment during the mating season, rather than population density per se, may cause social stress in females with substantial effects on their life history and population dynamics (Le Galliard et al. 2005, 2008).

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Supplementary material (available online as Appendix oik-03701 at <www.oikosjournal.org/appendix/oik-03701>).
Appendix 1.