

## Carotenoid-based coloration, oxidative stress and corticosterone in common lizards

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### SUMMARY

**Environmental factors including stressors, health status and social context significantly affect carotenoid-based coloration. For instance, stressors may induce the diversion of carotenoids from pigmentation pathways, potentially explaining why stressed animals often exhibit reduced coloration. However, we recently showed that high blood corticosterone concentrations, which are part of the physiological stress response, are associated with increased redness of the belly in the common lizard (*Lacerta vivipara*). This result clearly contrasts with the findings of many studies of carotenoid-based coloration because corticosterone is believed to increase oxidative stress. Here, we examined whether these positive effects are influenced by differences in food availability. We tested the effect of high corticosterone levels on carotenoid-based coloration, antioxidant enzyme activity and oxidative damage in common lizards subject to low and high food availability. Food restriction abolished the carotenoid-based color enhancement when corticosterone concentrations in animals were high. We discuss how carotenoid-based color can honestly signal individual quality in this species and how the increased redness induced by corticosterone could be a terminal investment in an environment where long-term survival prospects are poor but not when immediate survival is endangered.**

Key words: corticosterone, oxidative damage, food deprivation, ornaments, *Lacerta vivipara*, antioxidant enzymes.

### INTRODUCTION

The evolution of condition-dependent coloration typically involves honest signaling because it is controlled by trade-offs between color pigments, antioxidant capacities and immune defenses (Faivre et al., 2003a; Olson and Owens, 1998). For instance, carotenoids have multiple functions in addition to their role in pigmentation, including antioxidant (Alonso-Alvarez et al., 2004; Blount et al., 2001) and immune enhancer (Bendich, 1989) activities. Carotenoids cannot be synthesized *de novo* by animals (Goodwin, 1984); their multiple functions mean that there must be trade-offs in carotenoid-limited animals between ornamental carotenoid pigmentation and health functions, such as fulfilling nutritional requirements (Faivre et al., 2003b; Fitze et al., 2003; Hill and Montgomerie, 1994; Tschirren et al., 2003) and protection against parasites (Brawner et al., 2000; McGraw and Hill, 2000; Milinski and Bakker, 1990; Saks et al., 2003). Indeed, during an immune challenge, carotenoids may preferentially be used for immune functions rather than for coloration (Blount et al., 2003; Cote et al., 2010a; Faivre et al., 2003a; McGraw and Ardia, 2003). Therefore, carotenoid-based coloration commonly indicates individual health (von Schantz et al., 1999) and makes a contribution to mate choice. Similarly, environmentally stressed animals often exhibit reduced coloration (Belthoff et al., 1994; Brawner et al., 2000; Loiseau et al., 2008a; Meylan et al., 2007; Milinski and Bakker, 1990) that may be a consequence of stressor-induced physiological changes diverting carotenoids from coloration (Landys et al., 2006; Loiseau et al., 2008a).

In many cases, stressful events involve the production of glucocorticoids that can mediate changes in physiological and behavioral pathways [i.e. emergency life-history stages (Romero, 2004; Wingfield, 2003)]. For example, increased glucocorticoid

concentrations can suppress reproductive behavior (Moore and Jessop, 2003; Silverin, 1998) and social activity (DeNardo and Licht, 1993), partially regulate the immune system (Berger et al., 2005; Morici et al., 1997), and increase activity and foraging (Breuner et al., 1998; Cote et al., 2006; Gleeson et al., 1993; Tataranni et al., 1996). For example, fasting increases the production of glucocorticoids (Loiseau et al., 2008b; Lynn et al., 2003), which decreases the hepatic glucose output, increases fat and protein degradation to produce glucose (mobilization of energy that could enhance anaerobic metabolism) and decreases blood triglyceride concentration. By mobilizing energy, glucocorticoids could therefore positively affect foraging and locomotor activity and food intake (Breuner et al., 1998; Cote et al., 2006; Gleeson et al., 1993; Tataranni et al., 1996). These hormone-mediated stress responses constitute a set of adaptive changes that promote immediate survival (Breuner et al., 2008; Cote et al., 2006; Romero, 2004; Wingfield, 2003). However, high blood corticosterone concentrations, especially if they remain elevated over long periods, have a wide range of negative consequences, including reproductive suppression (Sapolsky, 1992), reduced immunocompetence (Bartolomucci et al., 2005; McEwen et al., 1997), decreased insulin production, neural degeneration (Bremner, 1999) and increased oxidative stress (Lin et al., 2004). The production of corticosterone may therefore mediate the equilibrium between processes favoring short-term survival and those favoring long-term survival.

Consequently, color changes may be directly induced by environmental stressors or by the activation of the physiological stress response (Fitze et al., 2009; Loiseau et al., 2008a). In the common lizard (*Lacerta vivipara*), the ventral red coloration of the male lizard plays an important role in male–male interactions for

the accession to females and is involved in sexual attractiveness and female mate choice (Fitze et al., 2009). Indeed, Fitze et al. [(Fitze et al., 2009) see Discussion] demonstrated, using staged mating experiments excluding intra-sexual interaction (Fitze et al., 2008), that redder males copulated with more females. These results strongly suggest a female mate choice based on the ventral coloration of males (Fitze et al., 2009). In this species, carotenoids are responsible for the yellow–orange coloration of the male belly, and the carotenoid content of the skin can be predicted from measurements of its coloration (Fitze et al., 2009). In this species, as in other reptiles (Olsson et al., 2008), carotenoid supplementation does not induce a color change even if it increases blood carotenoid content. These results suggest that carotenoid availability does not explain the observed condition-dependency of coloration in this species. Indeed, both correlative and experimental data show that when excess food is available, high blood corticosterone concentrations are associated with increased redness, suggesting that carotenoids are allocated to coloration when the animal is stressed (Fitze et al., 2009). This observation clearly contrasts with the findings of numerous studies investigating carotenoid-based coloration. Indeed, corticosterone is commonly believed to increase oxidative stress, at least during the acute stress response (Lin et al., 2004; Lin et al., 2006). The mechanism by which corticosterone positively affects carotenoid-based coloration of common lizards is unknown. It would be informative to determine whether these positive effects also apply under food restriction because in this food environment the potentially adverse effects of corticosterone cannot be compensated by increased food intake. For instance, some studies showed that corticosterone-induced modifications strongly depend on body condition (Angelier et al., 2007; Loiseau et al., 2008a), suggesting the effect of energetic conditions on stress responses. As suggested by Breuner et al. (Breuner et al., 2008), we believe that chronic elevation of corticosterone concentrations may lead to these positive effects [e.g. increased coloration (Fitze et al., 2009) and increased short-term survival (Cabezas et al., 2007; Cote et al., 2006)] only if energetic resources (i.e. food) are not limited. According to this hypothesis, we recently showed that, under food restriction, an increase of corticosterone does not induce the corticosterone-induced behavioral and physiological changes observed when food is largely available (Cote et al., 2010b).

We report an investigation of the effects of high corticosterone levels on carotenoid-based coloration, antioxidant enzyme activity and oxidative damage in common lizards (*L. vivipara*) in both low and high food availability conditions. Food deprivation generates oxidative stress mainly due to a depletion of organ antioxidant stores and an increase in free radical production especially in the liver (Pascuala et al., 2003; Robinson et al., 1997). Using an experimental approach, we address two questions: does high corticosterone concentration increase carotenoid-based color expression irrespective of food availability? How does high corticosterone concentration affect oxidative stress?

## MATERIALS AND METHODS

### Species, study site and breeding conditions

The common lizard (*Lacerta vivipara* Jacquin 1787) is a small lacertidae (adult snout–vent length: males 40–60 mm, females 45–75 mm) inhabiting humid habitats in Eurasia. This species feeds on small insects, spiders and earthworms (Avery, 1962) and has a cryptic behavior that is difficult to observe under natural conditions (Clobert et al., 1994). The ventral coloration of males ranges from yellow to red with dark spots. After birth, juveniles are melanistic and start developing their yellow–orange coloration during their first

year of life. The fully developed coloration usually appears after the first hibernation or at latest after the second hibernation (Vercken et al., 2007). The adult coloration is determined partly genetically and partly environmentally (Cote et al., 2008; Vercken et al., 2007).

Before this experiment, in June 2006, we collected 84 adult (>2 years old) males from three different but neighboring populations (less than 1 km apart) over four days in southern France (all sites were on the Mont Lozère, France, 44°27'N, 3°44'E); we collected males from three separate populations rather than one because there were insufficient numbers of males at any single population. We thereby shortened the time needed (to 4 days) to capture the number of males required for the study. After capture we brought the lizards to the laboratory. There, we weighed them and we measured their body length (snout–vent length). We also estimated the quantity of ectoparasites. *Lacerta vivipara* is the host of haematophagous mites (Sorci et al., 1997). These are temporary parasites of several species of reptiles, living on the ground and climbing on the host to take a blood meal. We classified individuals in four categories (0=no parasitized, 3=most parasitized). We provided each lizard with a similar standardized (as concerns food, water, heat, social interactions) environment: the lizards were individually housed in plastic terrariums [25 cm × 15.5 cm × 15 cm (Le Galliard et al., 2003) containing 3 cm-deep litter]. In one corner of the terrarium a bulb provided heat for thermoregulation from 09:00 h to 12:00 h and from 14:00 h to 17:00 h, providing a gradient from room temperature (19–24°C night–day) to 35–37°C (below the bulb), which covers the thermal breadth of this species (Van Damme et al., 1986). An egg carton was added, allowing lizards to hide. Lizards were able to behave normally, and behavior associated with escaping (e.g. scratching on the walls) was rarely observed. The authors attest the adherence to *The National Institutes of Health Guide for Care and Use of Laboratory Animals*. After capture, all lizards were kept for four days in these standardized conditions before experimentation (Fig. 1). The experimental model used involved a manipulation of food consumption, a transdermal administration of corticosterone for 21 days to the common lizard (*L. vivipara*) and an analysis of color change in treated and control groups. Finally, we measured a set of physiological variables reflecting antioxidant enzyme activity.

### Experimental corticosterone application

Forty-two of the captured males were randomly allocated to corticosterone treatment and the other 42 served as controls. Size,

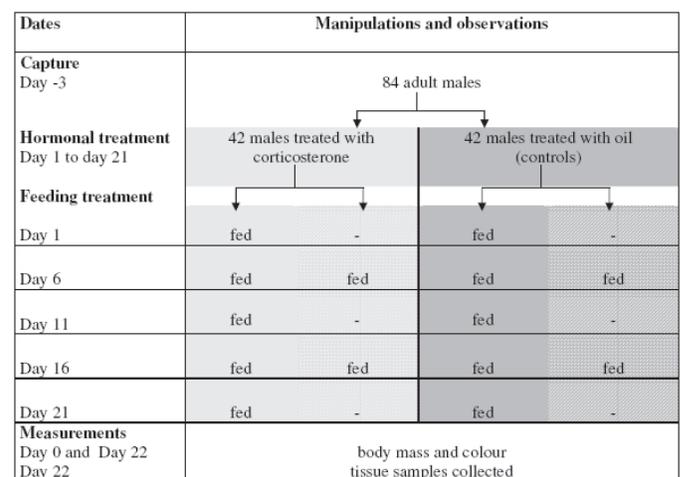


Fig. 1. Experimental design.

condition and date of capture were not significantly different between the two groups (snout–vent length:  $F_{1,80}=1.05$ ,  $P=0.31$ ; body condition:  $F_{1,79}=0.20$ ,  $P=0.66$ ; and capture date:  $F_{1,80}=0.01$ ,  $P=0.94$ ). The corticosterone treatment consisted of a daily application of 4.5  $\mu\text{l}$  of sesame oil mixed with corticosterone (3  $\mu\text{g}$  of corticosterone per 1  $\mu\text{l}$  of oil). Control lizards were treated with 4.5  $\mu\text{l}$  of sesame oil alone (for details, see Cote et al., 2006; Meylan et al., 2003). The treatment was applied on the dorsal surface of the lizard every evening for 21 days (starting on day 1, Fig. 1).

This method of corticosterone administration is similar to that described by Knapp and More (Knapp and More, 1997). It leads to a fivefold to tenfold increase of the basal blood corticosterone concentration (equivalent to an absolute increase of about 100  $\text{ng ml}^{-1}$ ) above that found in natural populations [2 days to 20 days treatment (Meylan et al., 2003; Cote et al., 2006); 10 days treatment (Cote et al., 2010b)]. The mean basal corticosterone concentration in blood from individuals housed for one day in the laboratory day was 21.64  $\text{ng ml}^{-1}$  for females [max. 101.97  $\text{ng ml}^{-1}$  (Meylan et al., 2003)] and 77.03  $\text{ng ml}^{-1}$  for males [max. 181  $\text{ng ml}^{-1}$  (Cote et al., 2006)]. The treatment therefore results in a corticosterone concentration similar to that naturally occurring in response to stressors, which can increase the blood corticosterone concentration of reptiles by more than tenfold from basal levels (Tyrrell and Cree, 1998).

#### Feeding treatments

Half of each hormone-treatment group (corticosterone and control) was randomly allocated to well-fed and food-restricted conditions. Size, condition and date of capture were not significantly different between food-availability groups (snout–vent length:  $F_{1,80}=0.06$ ,  $P=0.81$ ; body condition:  $F_{1,79}=0.52$ ,  $P=0.48$ ; and capture date:  $F_{1,80}=0.01$ ,  $P=0.94$ ). Food availability was based on three previous studies with this species (Cote et al., 2006; Le Galliard et al., 2004; Massot and Clobert, 1995). At the start of the experiment (on day 1) and every 10 days (on day 11 and on day 21), each lizard of the well-fed group was supplied one *Ptychocheilus farinalis* larva; lizards of the food-restricted group received no larvae (Fig. 1). On day 6 and on day 16, all lizards were supplied one larva (Fig. 1). All larvae fed to the lizards were of similar body mass [254  $\pm$  12.64 mg ( $\pm$ s.e.)]. This feeding protocol, with single large items of a single type, allowed standardization of the amount of food provided. Living larvae were presented to the lizards between 11:30 h and 12:30 h and were usually immediately attacked and eaten; in all cases the larvae were eaten by the evening. Terraria were covered with fine mesh to avoid animals obtaining additional prey accidentally. Water was available *ad libitum*.

#### Body mass and ventral coloration

The body mass and belly coloration of each lizard was measured on the first day (the day before the first feeding) and on the 22nd day of the experiment. The lizard's belly coloration was measured using a miniature spectroradiometer (USB2000, Ocean Optics Inc., Dunedin, FL, USA) and a Xenon light source (PX-2 and R400-7-UV/VIS, Ocean Optics Inc.), over the visual spectrum (300–700 nm). Reflectance was measured relative to a diffuse white standard (WS-1, Ocean Optics Inc.) uniformly reflecting 98–100% over the spectral range studied. For each lizard, we took a color measurement on the breast and in the middle of the belly, avoiding black spots. The color measurements corresponded to the average coloration over a surface area of approximately 1 mm  $\times$  1 mm.

We restricted the analyses to the human visible spectrum (400–700 nm). Using Endler's (Endler, 1990) segment

classification method, we derived objective estimates of hue (0–360 deg: 0 deg=red; 60 deg=yellow) in the area from 400 nm to 700 nm. Even if UV reflectance is important in animal vision and should be integrated in color measurement (Cuthill et al., 1999), we restricted our analyses to the hue from this spectrum for two reasons. First, the hue in the area from 400 nm to 700 nm is a good indication of the composition and concentration of carotenoid pigments incorporated into the integument and should be positively related to pigment concentration in both saturated and unsaturated colors (Andersson and Prager, 2006). It is worth noting that, in the common lizard, the skin's carotenoid concentration was negatively correlated with the lizard's hue and the skin's carotenoid concentration explained 47.2% of the variance in hue. However, there was no significant correlation between the lizard's chroma or the lizard's brightness and the lizard's skin carotenoid concentration (Fitze et al., 2009). Second our main goal was to explore new explanations for the previous and surprising results found in this species (Fitze et al., 2009). Therefore, we decided to use the same methodology to be able to compare the results from the two studies. We used the mean coloration of the two measured body parts for the analyses. Color measurement by this protocol gives repeatable results, as shown previously by repeating the same measurements three times on 218 lizards [hue:  $F_{216,434}=6.99$ ,  $P<0.0001$ ,  $R=0.66$  (Lessells and Boag, 1987)].

#### Antioxidant enzyme activities and oxidative damages

We measured a set of physiological variables reflecting antioxidant enzyme activity [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)] and oxidative damage (lipid peroxidation). Antioxidant defenses include enzymatic (SOD, CAT, GPX, glutathione reductase and many others), as well as non-enzymatic free radical scavengers (vitamins C and E, uric acid, melatonin, cysteine, glutathione), and minerals (selenium, zinc) (Fang et al., 2002; Prior and Cao, 1999). Even if recently discovered peroxiredoxin enzymes also play an important reactive oxygen species (ROS) scavenging role in the mitochondria (Balaban et al., 2005), three main antioxidant enzymes located in the cell counteract the effects of the superoxide anion and its damaging derivatives. SOD acts on the superoxide anion to produce hydrogen peroxide and singlet oxygen. Both of these products are themselves potentially damaging ROS. Two further enzymes (CAT, located in peroxisomal subcompartment, and the more widely distributed GPX) convert hydrogen peroxide to water. The mitochondria represent the major ROS source for the cell but ROS formation in extramitochondrial locations, in peroxisomes, by cytochrome P450, and NADPH oxidase reaction, also exists. The activity assessments of the three main antioxidant enzymes (SOD, CAT and GPX) allow more precise ideas about (i) the origin of ROS, and (ii) the response of the complete chain allowing the conversion of superoxide anion to hydrogen peroxide and finally to water.

At the end of the experiment (22nd day of the experiment), we sampled tissues (1 cm of tail) for assaying antioxidant enzyme activities. We restricted this study to forty males for ethical reasons: it required removing a part of the tail (1 cm). Tissue samples were frozen immediately after collection. The day of analyses, frozen tissue samples were rapidly weighed and homogenized with a Elvehjem potter (Bioblock, Illkirch, France) at 4°C, in buffer containing  $\text{KH}_2\text{PO}_4$  (100  $\text{mmol l}^{-1}$ ), dithiothreitol (DTT) (1  $\text{mmol l}^{-1}$ ) and EDTA (2  $\text{mmol l}^{-1}$ ), pH 7.4. The samples were centrifuged (3000 g, for 5 min) and the supernatant was collected and used for enzymatic assays.

In this work, each enzymatic assay [SOD, GPX, CAT and malonaldehyde (MDA)] was purchased with a within-day run avoiding the calculation of between-day coefficient of variation (c.v.). The repeatability and the intermediate precision (within-lab reproducibility) were thus determined by the replicate analysis ( $N=3$ ) of each sample. These within-day determinations gave c.v.'s in the range 5–10%.

### SOD

SOD activity was assayed spectrophotometrically (at 550 nm) by monitoring the rate of acetylated cytochrome *c* reduction by superoxide radicals generated by the xanthine–xanthine oxidase system at 25°C in reaction buffer (0.5 mmol l<sup>-1</sup> xanthine, 0.2 mmol l<sup>-1</sup> cytochrome *c*, 50 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.1 mmol l<sup>-1</sup> EDTA, pH 7.8) (Flohe and Otting, 1984). One activity unit of SOD was defined as the amount of enzyme that inhibits the rate of acetylated cytochrome *c* reduction by 50%. To distinguish manganese-SOD (MnSOD), exclusively located in mitochondrial matrix, from cupro-zinc-SOD (CuZnSOD), which is primarily located in the cytosol, SOD activity was determined after incubation with cyanide (1 mmol l<sup>-1</sup> NaCN). At this concentration, cyanide inhibits the CuZn isoform of the enzyme but does not affect the MnSOD isoform (Flohe and Otting, 1984). SOD activity is expressed as U mg<sup>-1</sup> of total protein.

### GPX

Total GPX activity was assayed by measuring spectrophotometrically at 340 nm the reduction of NADPH ( $\epsilon=6.22 \cdot 10^3$  l mol<sup>-1</sup> cm<sup>-1</sup>) with cumene hydroperoxide (1 mg ml<sup>-1</sup>) as the substrate (Tappel, 1978). Measurements were made at 37°C in the following reaction buffer: 0.25 mmol l<sup>-1</sup> glutathione (GSH), 0.12 mmol l<sup>-1</sup> NADPH, 1 U ml<sup>-1</sup> glutathione reductase (GR) and 10 mmol l<sup>-1</sup> NaCN. GPX activity is expressed as mU mg<sup>-1</sup> of total protein.

### CAT

CAT activity was determined by the method of Aebi (Aebi, 1984). Each supernatant (200 µl) was incubated for 30 min at 0°C with ethanol (95%, 2 µl). Triton X-100 (1%, 2 µl) was added and each sample was centrifuged at 5000 g for 5 min. The supernatant was used for measurement of CAT activity by using the first-order rate constant of the decomposition of hydrogen peroxide by tissue CAT at 20°C in buffer (pH 7.4) containing 40 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 60 mmol l<sup>-1</sup> HNa<sub>2</sub>PO<sub>4</sub>. CAT activity was calculated using the formula:  $k=(2.3/dt)(\log A_1/A_2)$ , where  $k$  is CAT activity,  $dt$  is change in time,  $A_1$  is initial absorbance, and  $A_2$  is final absorbance. CAT activity is expressed in mK mg<sup>-1</sup> of total protein.

### Lipid peroxidation

The concentration of MDA was estimated by the thiobarbituric acid reactive substances (TBARS) method described by Ohkawa et al. (Ohkawa et al., 1979).

### Statistics

The initial ventral coloration and the initial body mass were analyzed using general linear models (Proc GLM, SAS v8.02, SAS Institute, Cary, NC, USA), and the quantity of ectoparasites was analyzed using a generalized linear model (Proc GENMOD, SAS v8.02) with a multinomial distribution and a cumulative logit. The full model included the population of origin for all these analyses, the initial body mass and the initial body length for the analyses of initial ventral coloration and quantity of parasite, plus the initial coloration for the analysis of quantity of parasites. The changes in

body mass and in ventral coloration during the experiment (value after the end of the treatment minus value before the beginning of the treatment), the antioxidant enzyme activities and the value of lipid peroxidation after the end of the treatments were analyzed using general linear model (Proc GLM, SAS v8.02). The assumptions in all models presented were verified on the residuals and were fulfilled. The full model included the treatments (feeding and hormonal treatments), the initial body mass, the population of origin and all interactions between factors. All models were simplified by backward elimination of the non-significant interactions and factors (when not part of significant interactions). The significance level was set at  $P=0.05$ . Differences in sample sizes for changes in ventral coloration reflect missing values, and differences in SOD activity reflect one reduced tissue sample.

## RESULTS

### Pre-experimental body mass, ventral coloration and parasites load

The initial body mass did not depend on the population of origin ( $F_{1,81}=2.23$ ,  $P=0.12$ ) while at the beginning of the experiment, ventral coloration differed significantly between the three populations ( $F_{1,79}=14.05$ ,  $P<0.0001$ ). Initial ventral coloration also was negatively correlated with body length ( $F_{1,79}=14.05$ ,  $P=0.04$ ) but was independent of body mass ( $F_{1,79}=0.007$ ,  $P=0.93$ ). The quantity of ectoparasites was not related to body length ( $\chi^2_1=0.13$ ,  $P=0.72$ ), body mass ( $\chi^2_1=1.26$ ,  $P=0.26$ ) or population of origin ( $\chi^2_2=4.69$ ,  $P=0.10$ ) but was negatively correlated to initial hue values ( $\chi^2_1=7.78$ ,  $P=0.005$ ). It means that redder individuals had fewer parasites (Fig. 2).

### Changes in body mass and ventral coloration

The mean body mass of the lizards at the start of the treatment was  $2.5 \pm 0.06$  g ( $\pm$ s.e.). In captivity, body mass usually decreases in the first two to three weeks (personal observation). The change in body mass during the experiment differed significantly between the three populations ( $F_{1,77}=6.37$ ,  $P=0.003$ ). During the experiment, the body mass of well-fed lizards decreased significantly less than that of lizards in the food-restricted group ( $F_{1,77}=30.89$ ,  $P<0.0001$ ); also, the body mass change was negatively correlated with the initial body mass ( $F_{1,77}=51.03$ ,  $P<0.0001$ ), which is probably due to the regression toward the mean. Consistent with previous reports (Cote

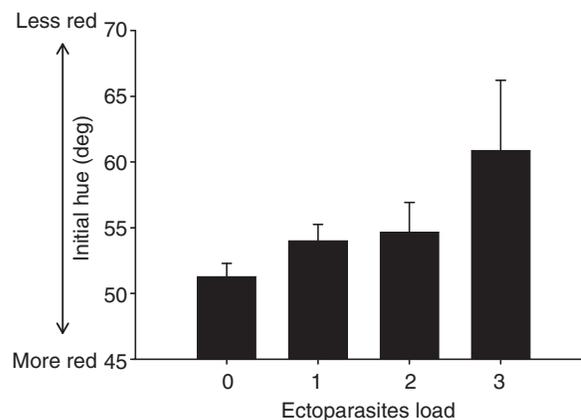


Fig. 2. Mean ( $\pm$ s.e.) ventral hue before the experiment according to ectoparasites load. Ectoparasites are haematophagous mites, temporary parasites of several species of reptiles, living on the ground and climbing on the host to take a blood meal. We classified individuals in four categories (0=not parasitized, 3=most parasitized).

et al., 2006), corticosterone treatment negatively affected body mass ( $F_{1,77}=3.73$ ,  $P=0.05$ ) but the interaction between corticosterone and feeding treatments was at the limit of the significance threshold ( $F_{1,77}=3.46$ ,  $P=0.06$ ). Corticosterone treatment decreased body mass in the well-fed group (contrasts:  $F_{1,77}=7.20$ ,  $P=0.009$ ; corticosterone-treated males:  $-0.20\pm 0.03$  g; control males:  $-0.09\pm 0.03$  g) but had no significant effect on body mass in the food-restricted group (contrasts:  $F_{1,77}=0.003$ ,  $P=0.96$ ; corticosterone-treated males:  $-0.30\pm 0.03$  g; control males:  $-0.30\pm 0.03$  g).

During the experiment, the hue of the lizards' ventral coloration increased on average from  $53.1\pm 0.9$  deg ( $\pm$ s.e.) to  $58.4\pm 0.9$  deg ( $\pm$ s.e.) (less red coloration;  $P\leq 0.0001$ ). The interaction between corticosterone and feeding treatments was significant (Table 1). Corticosterone treatment had no effect on ventral coloration changes in the food-restricted group (contrast:  $F_{1,76}=0.79$ ,  $P=0.38$ , Fig. 3) but well-fed corticosterone-treated lizards maintained a redder ventral coloration than well-fed control lizards (contrast:  $F_{1,76}=21.87$ ,  $P<0.0001$ , Fig. 3). There were no significant interactions between population of origin and the other factors (all  $P>0.1$ ). As we sampled only 40 males for the analyses of oxidative stress, we verified that the color changes in this sub-group were similar to those in the study groups as a whole. Indeed, the effects of corticosterone treatment and feeding treatment were similar in the sub-group (corticosterone treatment:  $F_{1,36}=7.36$ ,  $P=0.01$ ; feeding treatment:  $F_{1,36}=3.85$ ,  $P=0.06$ ; interaction:  $F_{1,36}=3.60$ ,  $P=0.06$ ).

#### Antioxidant enzyme activities and oxidative damages

Both corticosterone and feeding treatments affected the SOD activity (Table 2, Fig. 4A,B). Cytosolic SOD activity depended on corticosterone treatment in well-fed lizards but not in food-restricted lizards (Table 2; contrasts for corticosterone treatments; well-fed:  $F_{1,33}=9.82$ ,  $P=0.004$ ; food-restricted:  $F_{1,33}=0.05$ ,  $P=0.82$ ; Fig. 4A). In the well-fed group, corticosterone-treated lizards had lower cytosolic SOD activity than control lizards. Furthermore, cytosolic SOD activity was lower in food-restricted lizards than in well-fed lizards only in the control group. For mitochondrial SOD activity, the interaction between corticosterone and feeding treatments was at the limit of significance (Table 2). The pattern of effects on mitochondrial SOD activity was similar to that on cytosolic SOD activity (contrasts for corticosterone treatment; well-fed:  $F_{1,33}=4.05$ ,  $P=0.05$ ; food-restricted:  $F_{1,33}=0.23$ ,  $P=0.63$ ; Fig. 4B). GPX activity at the end of the experiment was higher in corticosterone-treated lizards than in control lizards (Table 2, Fig. 3C). Feeding treatments had no effect on GPX activity (Table 2, Fig. 4C). CAT activity at the end of the experiment did not depend on corticosterone treatment but depended on the feeding treatment: food-restricted lizards had lower CAT activity than well-fed lizards (Fig. 4D). Antioxidant enzyme activities did not depend on body mass (Table 2).

Table 1. Effect of corticosterone and feeding treatments on ventral coloration changes (hue values)

	Hue changes	
	Estimates $\pm$ s.e.	Statistical test result
Intercept	14.19 $\pm$ 4.29	
Corticosterone (Cort)	-1.40 $\pm$ 0.50	$F_{1,76}=7.71$ , $P=0.007$
Feeding treatment (HF)	-1.39 $\pm$ 0.51	$F_{1,76}=7.58$ , $P=0.008$
Corticosterone $\times$ feeding treatment	-1.27 $\pm$ 0.51	$F_{1,76}=6.26$ , $P=0.015$
Initial hue	-0.18 $\pm$ 0.08	$F_{1,76}=4.91$ , $P=0.03$

Estimates are given for males of the high food availability group (HF) treated with corticosterone.

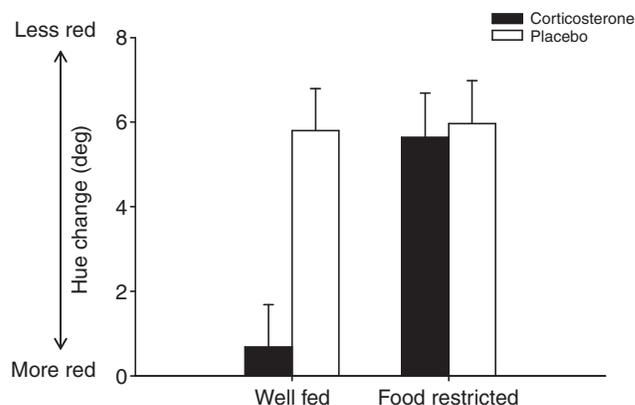


Fig. 3. Mean ( $\pm$ s.e.) ventral hue change (ventral hue at the end of the experiment – ventral hue at the start of the experiment) in male lizards according to corticosterone and feeding treatments.

At the end of the experiment, oxidative damage (i.e. lipid peroxidation) was greater in corticosterone-treated lizards than in control lizards (Table 2, Fig. 5). Food restriction decreased oxidative damage (Table 2, Fig. 5). Body mass was negatively correlated with oxidative damage at the end of the experiment (estimate:  $-13.26\pm 4.87$ ; Table 2).

#### Ventral coloration in relation to oxidative stress

Antioxidant enzyme activities may be linked to oxidative stress in various ways; we used MDA as a marker of oxidative stress. We included this parameter as a factor in the analysis of ventral coloration change. Oxidative damage alone was not related to coloration changes but the interaction between oxidative damage and corticosterone treatments was significant ( $F_{1,36}=8.93$ ,  $P=0.005$ ; Fig. 6). The relationship between hue changes and oxidative damage was positive in control lizards and negative in corticosterone-treated lizards (contrasts for corticosterone treatments; corticosterone-treated:  $F_{1,36}=5.83$ ,  $P=0.02$ ; control:  $F_{1,36}=4.04$ ,  $P=0.05$ ; Fig. 6A,B). Therefore, control males becoming redder throughout the experiment displayed less oxidative damages at the end of the experiment whereas the opposite was observed for corticosterone-treated males.

## DISCUSSION

### Carotenoid-based coloration in common lizards: honest signaling?

Carotenoid-based coloration is thought to provide important signals concerning individual quality (Fitze et al., 2003; Hill and Montgomerie, 1994; Kodric-Brown, 1996; Loiseau et al., 2008a; Olsson, 1994). The ventral red coloration of the male common lizard is made up of carotenoids (Fitze et al., 2009) but it does not necessarily mean that carotenoid-based coloration honestly signal individual quality. In adult common lizards, redder individuals (males and females) are the bigger or heavier individuals (present study) (Fitze et al., 2009). These studies only used adults (>3 years old) excluding the confusion with color development. In this species, body size/condition commonly predict both competitive abilities and energetic resources in this species (Bauwens and Verheyen, 1987; Le Galliard et al., 2004). Moreover, body size is strongly related to the age of individuals. It means that, through its relationship with body size/mass, redder colorations could signal better competitive abilities, higher energetic reserves and/or older age. Moreover, we found that redder males had fewer ectoparasites.

Table 2. Effects of corticosterone treatment, feeding treatment and body mass on antioxidant enzyme activities and oxidative damage

	Antioxidant enzyme activities				Oxidative damage
	Cytosolic SOD	Mitochondrial SOD	GPX	CAT	MDA
Corticosterone	$F_{1,35}=4.62, P=0.038$	$F_{1,35}=1.11, P=0.30$	$F_{1,36}=8.46, P=0.01$	$F_{1,37}=0.24, P=0.62$	$F_{1,36}=4.57, P=0.039$
Food treatment	$F_{1,35}=5.96, P=0.020$	$F_{1,35}=0.02, P=0.89$	$F_{1,36}=0.11, P=0.75$	$F_{1,38}=4.21, P=0.047$	$F_{1,36}=3.57, P=0.067$
Corticosterone $\times$ food treatment	$F_{1,35}=4.42, P=0.042$	$F_{1,35}=3.35, P=0.075$	$F_{1,36}=0.80, P=0.38$	$F_{1,36}=0.15, P=0.70$	$F_{1,35}=0.31, P=0.58$
Initial body mass	$F_{1,34}=0.59, P=0.45$	$F_{1,34}=2.47, P=0.12$	$F_{1,35}=0.64, P=0.43$	$F_{1,35}=0.01, P=0.93$	$F_{1,36}=7.51, P=0.01$

SOD=superoxide dismutase, GPX=glutathione peroxidase, CAT=catalase, MDA=malondialdehyde.

We previously found this result using individuals (males and females) from semi-natural populations (J. Cote, unpublished data). We released lizards in semi-natural populations after removing all ectoparasites (ticks are the major parasite in these semi-natural populations) and taking color measurements. After one year, we captured 73 survivors and we found that initial hue values positively predicted the number of ticks ( $R^2=0.10, F_{1,71}=7.69, P=0.007$ ). Initially redder individuals got fewer ticks over the year. Moreover, at the end of the present study, we ran some preliminary tests to look at phytohaemagglutinin responses (PHA) on several individuals (data not included here). On the 14 well-fed control lizards tested, PHA response was negatively related to hue values at the end of the experiment ( $R^2=0.40, F_{1,12}=8.08, P=0.015$ ). Redder individuals had a higher PHA response. We are aware of the difficulties to interpret PHA responses as a measure of immunocompetence (Kennedy and Nager, 2006). However, this result along with lower parasites loads for redder lizards makes us believe that ventral coloration might reflect, partly, immunocompetence. Furthermore, in the present study, redder control individuals received less

oxidative damage over the experiment (but see the opposite relationship for corticosterone-treated males). Previous studies also showed that the hue or chroma of ventral coloration is influenced by population density (Meylan et al., 2007) and population structure (Cote et al., 2008; Vercken et al., 2007). For example, females living in male-biased populations are more sexually harassed and become subsequently less red (Cote et al., 2008). In this species, ventral coloration is partly a context-dependent signal (see Vercken et al., 2007) that might reflect, in some ways, individual quality. Finally, this context-dependent signal plays a role in male–male interactions for the accession to females and is thus likely to be involved in sexual attractiveness (Fitze et al., 2009). Even if we are missing some direct measurements of quality, ventral coloration is likely to be a sexually- or socially-selected signal associated with individual quality or strategy.

#### Coloration, corticosterone and food

Several studies have demonstrated a link between coloration variation and environmental stress (Belthoff et al., 1994; Brawner

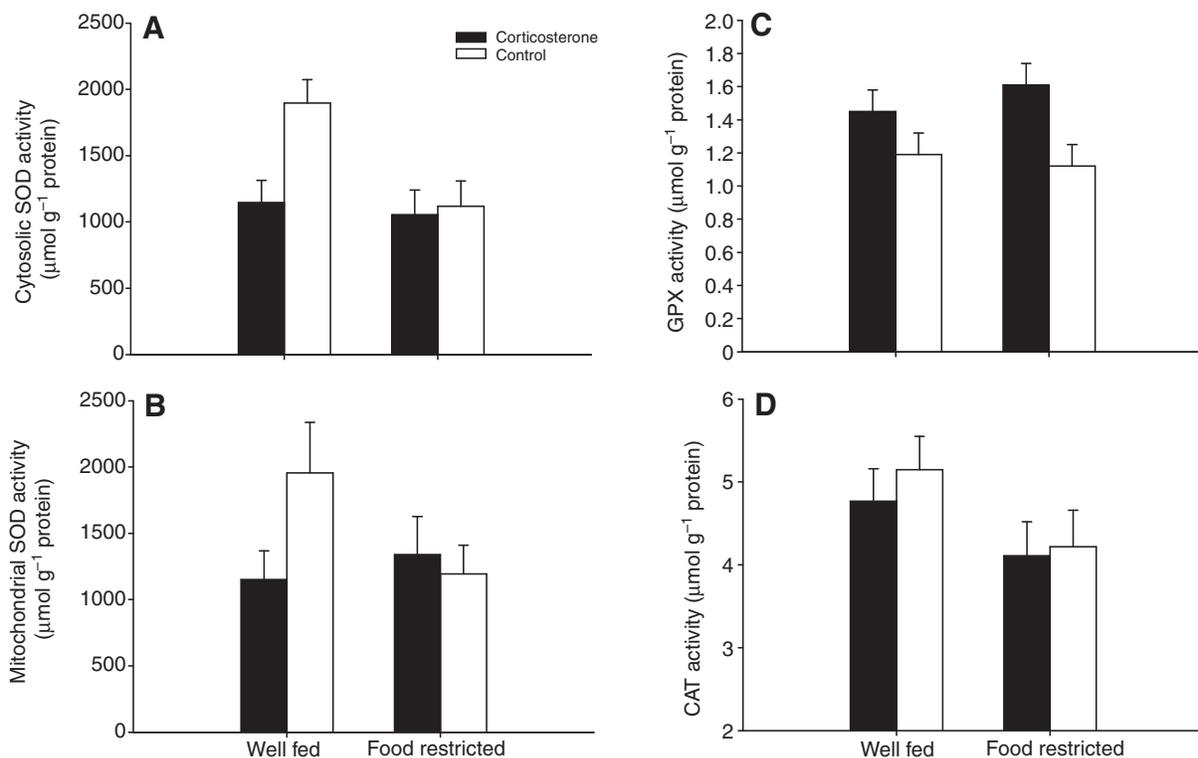


Fig. 4. Antioxidant enzyme activities in male lizards according to corticosterone and feeding treatments. (A) Mean cytosolic superoxide dismutase (SOD) activity ( $\mu\text{mol g}^{-1} \text{protein} \pm \text{s.e.}$ ) is shown for the various treatment groups. (B) Mean mitochondrial SOD activity ( $\mu\text{mol g}^{-1} \text{protein} \pm \text{s.e.}$ ) for the various treatment groups. (C) Mean glutathione peroxidase (GPX) activity ( $\mu\text{mol g}^{-1} \text{protein} \pm \text{s.e.}$ ) for the various treatment groups. (D) Mean catalase (CAT) activity ( $\mu\text{mol g}^{-1} \text{protein} \pm \text{s.e.}$ ) for the various treatment groups.

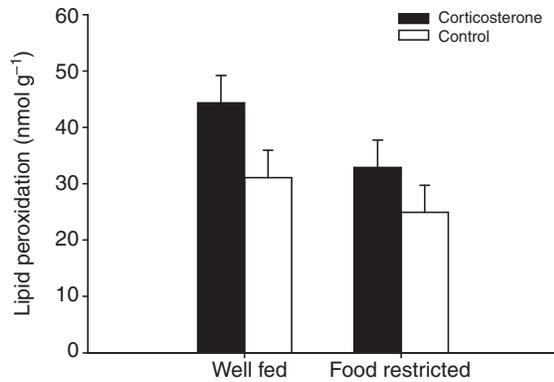


Fig. 5. Lipid peroxidation in male lizards according to the corticosterone and feeding treatments. Mean TBAR (thiobarbituric acid reactive substance) level ( $\mu\text{mol g}^{-1} \pm \text{s.e.}$ ) for the various treatment groups is shown.

et al., 2000; Loiseau et al., 2008a; Meylan et al., 2007). For example, in the lizard *Sceloporus*, acute stress induces a rapid decrease in coloration (Smith and John-Adler, 1999). Such rapid changes are often related to crypsis, a predator-avoidance tactic. Chronic stress, however, may reflect more durable change in the external or internal environment of the individual (Dufty et al., 2002). If the environmental stressor indeed results in decreasing individual quality, the hormone-mediated stress response might help the individual to adopt a relevant strategy with respect to the stressor. We previously showed that increased circulating corticosterone in males enhances food intake, thermoregulation and survival (Cote et al., 2006). How then should coloration be affected by corticosterone? If corticosterone simply reflects individual quality, it would have a negative effect on redness. If corticosterone is the mediator of some allostatic change, i.e. of a change in the strategy used by the individual, then a more complex relationship than a simple diminution in redness would be expected. In particular, it has been predicted that such changes would not occur when the food supply is not enough to provide the energy required to adopt the changes (Breuner et al., 2008; Cote et al., 2010b). Our results seem to corroborate a more complex model. Indeed, redness was significantly more intense at the end of the experiment for corticosterone-treated than control males provided with an abundant food supply whereas when the food supply was limited, redness did not differ between control and corticosterone-treated groups. As expected for an adaptive but energetically costly physiological stress response, the response was inhibited in a low food environment. If redness is a signal of male quality (Fitze et al., 2009), the stress response would increase signaling in a high food environment but suppress it in a low food environment. One might wonder whether the feeding treatments are confounded with differences in carotenoid consumption. From Fig. 2, it is clear that feeding regimes did not change ventral coloration in control males. This result corroborates previous results showing that food consumption has no effect on hue values (Fitze et al., 2009). This lack of effect can be explained by low carotenoid content in the larvae provided (note that nothing is known about the carotenoid content of these commercial larvae) or by the fact that carotenoid ingestion does not induce a color change in this species. Only few studies have examined the link between carotenoids, oxidative stress and coloration in reptiles (Fitze et al., 2009; Olsson et al., 2008). Interestingly, none found an effect of carotenoid supplementation on coloration while the link between coloration and oxidative stress exists (Olsson et al., 2008) (present

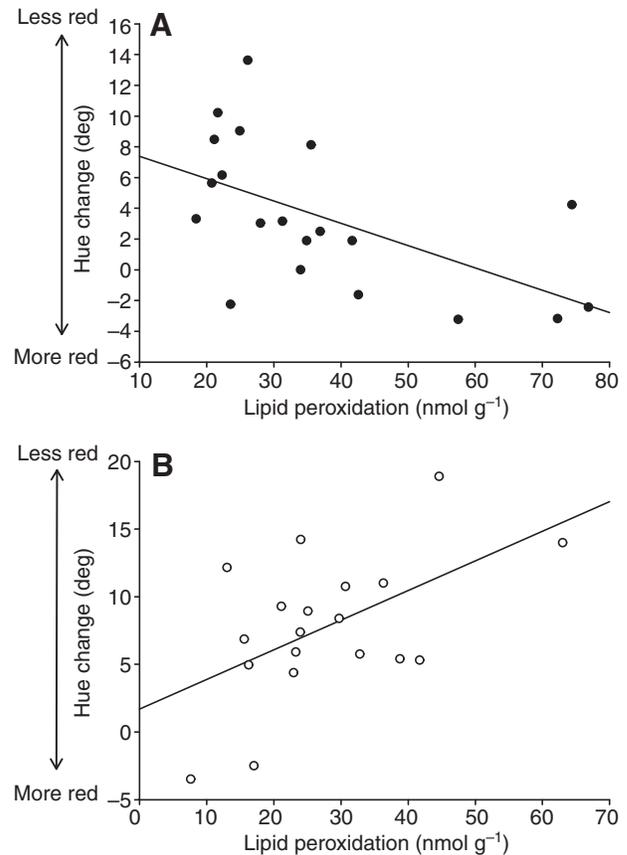


Fig. 6. Relationship between lipid peroxidation and ventral hue change according to corticosterone treatment. Individual values and regression lines are shown. (A) Corticosterone-treated lizards. (B) Control lizards.

study). It suggests that other factors are involved in the links between coloration and oxidative stress. We thus believe that food consumption mediated corticosterone-induced color changes independently of carotenoid ingestion. However, the physiological mechanisms underlying these effects remain unknown. We therefore examined the effects of corticosterone on oxidative stress and the link between oxidative stress and coloration.

#### Coloration, corticosterone and oxidative stress

Under chronic environmental stress, males increase their redness, which can confer advantages both in male–male interactions and for being chosen by females, and thereby increase reproductive success (Fitze et al., 2009). These stress responses, however, are only detectable in a food-rich environment most probably because the allostatic state can only be changed if sufficient food is available. Increasing short-term survival and reproductive success is certainly not without cost. Classical theory concerning the cost of reproduction suggests that increasing the immediate reproductive success is at the expense of future success (Nilsson and Svensson, 1996; Roff, 1992). Unfortunately, no data on long-term survival and reproductive success of males of this species are currently available to allow this prediction to be tested. However, the findings we report provide information about oxidative stress that is often associated with aging and senescence processes (Finkel and Holbrook, 2000; Harman, 1981). We found that high corticosterone levels were associated with higher levels of lipid peroxidation at the end of the experiment, suggesting an increase of oxidative damage. The imbalance between antioxidant

defenses and increased free radical production leads to oxidative stress and then to oxidative damages (e.g. lipid peroxidation). The increase of oxidative damages induced by high corticosterone levels may be the result of increased free radical production or decreased antioxidant defenses. Indeed, high corticosterone levels are believed to enhance free radical production as both food intake and activity are increased. However, the oxygen consumption rates of squamates are known to be reduced by food restriction (McCue, 2007) and exogenous corticosterone (Andrews and Pough, 1985; Miles et al., 2007). We could have expected a reduction of the SOD activity together with a consequent decrease in GPX and CAT activities. Our study shows multiple effects of corticosterone on antioxidant enzyme activities. Irrespective of the changes in the activity of antioxidant enzymes, the oxidant-antioxidant balance is biased toward oxidative stress after an increase in corticosterone level. Work with many other species shows that this shift in oxidative damage is linked to changes in carotenoid-based coloration. In control lizards in our study, redness was negatively correlated to lipid peroxidation (Fig. 5B), indicating that redder individuals are those suffering less oxidative damage (i.e. high quality individuals). By contrast, in corticosterone-treated lizards, redness was positively correlated to lipid peroxidation (Fig. 5A), indicating that the reddest individuals are also those displaying the highest oxidative damages. Even if we do not have measurements of reproductive success, these results might suggest a terminal investment. In an environment that does not ensure good long-term survival prospects (e.g. stressing environment), individuals might increase their immediate reproductive success through increased redness at the expense of their future reproductive success through decreased longevity induced by the increased oxidative damage. However, when immediate survival prospects (e.g. stressing environment with low food availability), such investments might too costly without even any prospects of reproduction. The change in allostatic state in this model concerns re-organisation of the individual's strategy to maximize its reproductive success over time. Of course, this model is one of the explanations and has to be confirmed by more direct measurements of the consequences of chronic stress on individual lifetime reproductive success and longevity. The positive correlation between redness and oxidative damage seems difficult to explain in the light of carotenoid/oxidative stress theory. As Lin et al. showed high corticosterone levels induce complex modifications of the oxidant-antioxidant balance (Lin et al., 2004). In broiler chickens, corticosterone administration initially induces the formation of ROS and thus an increase in lipid peroxidation (Lin et al., 2004). However, chronic corticosterone treatment enhances non-enzymatic antioxidant capacity and thereby prevents the development of more severe oxidative injury (Lin et al., 2004). In our study, we measured lipid peroxidation in the tissues only at the end of the experiment. It was thus impossible to observe the kinetics of corticosterone-induced oxidative damages. Indeed, increased lipid peroxidation may have occurred only during the first days of the treatment. The role of carotenoids in scavenging free radicals has been recently reconsidered (Costantini and Moller, 2008; Hartley and Kennedy, 2004; Isaksson et al., 2007). Carotenoid-based sexual traits may indicate the antioxidant status independent of the plasma carotenoid concentration. Effective antioxidant machinery (enzymatic and non-enzymatic) can prevent carotenoids being altered and destroyed through oxidation. Stronger antioxidant defenses thus allow the organism to allocate unbleached carotenoids to coloration (Hartley and Kennedy, 2004). Consistent with this view, the increased availability of a non-pigmented antioxidant enhanced the expression of carotenoid-based sexual traits but had no effect on the amount of circulating carotenoids (Bertrand et al., 2006). Our corticosterone

treatment may have thus increased both lipid peroxidation (initially) and the availability of carotenoids for coloration (later on), leading to the positive correlation between oxidative damage and redness.

We aimed to determine whether high corticosterone concentrations increase carotenoid-based color expression irrespective of food availability and the effects of high corticosterone concentration on oxidative stress. We clearly show that food availability substantially affects carotenoid-based color expression when animals are subjected to high corticosterone concentrations, and that high corticosterone levels increase oxidative stress partially due to a decrease of SOD activity. More generally, we demonstrate a complex and unusual interaction between the environment and carotenoid-based coloration in a reptile.

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