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Sex-, morph- and size-specific susceptibility to stress measured by haematological variables in captive common wall lizard *Podarcis muralis*

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ABSTRACT

In polymorphic species of animals, colour morphs may show alternative physiological properties, and hence evolve or be maintained as an indirect response to selection exerted on these physiological attributes. In this study, we investigated if different colour morphs (white, red and yellow) of the polymorphic common wall lizard differed in their physiological responses to a long-term stress by determining variation between capture and release in leukocytes profiles, haemoparasite loads and body condition of male and females maintained in captivity throughout the breeding season. We found that most blood parameters of lizards varied significantly following captivity, and this variation was sex-, morph- and size-dependent. In particular, the heterophil:lymphocyte ratio (H:L), a sensitive measure of immunodepression and long-term stress, varied significantly among yellow females, larger individuals significantly increasing and smaller individuals decreasing their H:L ratio after captivity. This trend was reversed in red females, where smaller individuals presented raised H:L index at release. Our study indicated that response to long-term stressful conditions, such as those induced by captivity, differed among common wall lizard colour morphs, implying a sex-, size-(i.e. age) and morph-specific sensitivity to stress, and hence a different physiological profile of colour morphs, which may contribute to the maintenance of colour polymorphism in this species.

1. Introduction

Identifying the processes maintaining phenotypic/genetic variability in wild populations is a major concern in today's conservation and evolutionary biology, since phenotypic variation is at the heart of natural selection. A recently burgeoning literature (reviewed in Gray and McKinnon, 2007) has been devoted to understand the mechanisms that generate and maintain colour phenotypic variation in animals, i.e. colour polymorphism (hereafter CP), due to their implications in the evolution of reproductive isolation and sympatric speciation (Fisher, 1930; Van Valen, 1965; Van Valen and Grant, 1970). CP is defined as "the coexistence in one interbreeding population of two or more distinct and genetically determined colour forms, the least abundant of which is present in numbers too great to be due solely to recurrent mutation", following the original definition by Huxley (1955).

Several mechanisms can contribute to CP development and maintenance (genetic drift and gene flow, disruptive selection, heterosis, apostatic selection, sexual selection, sensory bias, Galeotti et al., 2003; Roulin, 2004), but we know very little about which mechanism is the most important or how they might interact. To achieve these objectives it is crucial 1) to recognize what ecological factors promote CP, 2) to highlight the physiological and developmental mechanisms underlying morph differentiation, and 3) to elucidate how genotype interacts with environment to affect morph fitness. This is a complex goal to reach, since CP accomplishes a wide range of functions, such as conveying information on social and reproductive status or competitive ability of its bearer (Dawkins and Krebs, 1978; Cooper and Burns, 1987; Thompson and Moore, 1991a,b; Olsson, 1994; Weiss, 2002; Andrés et al., 2002) or supporting ecological functions such as antipredatory, thermoregulatory or habitat-use strategies (Cooper and Greenberg, 1992; Olsson and Madsen, 1998; Martin and Forsman, 1999; Galeotti et al., 2003; Galeotti and Rubolini, 2004; Lopez et al., 2004; Leal and Fleishman, 2004; Stuart-Fox et al., 2006). However, the close link between genotype and phenotype in polymorphic species may allow detailed studies of natural and sexual selection effects on evolutionary processes.

Reptiles offer a good model to investigate the evolution and maintenance of CP as they often show a high intraspecific variability of colour patterns (Cooper and Burns, 1987; Rand, 1992; Thompson et al., 1993; Forsman and Shine, 1995; Sinervo and Lively, 1996; Weiss, 2002; Vercken et al., 2007). In some species this polychromatism may be transient, since colourations are expressed only during the breeding season, or may change during ontogenesis (Rand, 1989, 1990; Martin

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and Forsman, 1999; Baird, 2004). In other species, particularly in lizards, individuals occur in discrete, stable, genetically based colour morphs, which coexist at different equilibrium levels within the same population (Thompson and Moore, 1991a, 1991b; Carpenter, 1995; Sinervo and Lively, 1996; Sinervo and Zamudio, 2001; Sacchi et al., 2007b). Since CP is often expressed only ventrally in many species, rather than accomplishing antipredatory or thermoregulatory functions, it may be associated with alternative behavioural (territorial/reproductive) strategies in males as well as in females, which clearly underlie different physiological profiles due to different genotypes. For example, in the side-blotched lizard (*Uta stansburiana*), the three male morphs (orange, yellow and blue) are associated with territorial, sneaky and mate-guarding strategies respectively, depending on population density and morph-frequency (e.g., Sinervo and Lively, 1996; Sinervo and Zamudio, 2001; Svensson et al., 2001; Sirot et al., 2003; Zamudio and Sinervo, 2003; Svensson et al., 2005). Orange males had higher testosterone plasma levels and behaved more aggressively compared to the other morphs in this species (Sinervo et al., 2000a), as occurs also in tree lizards (*Urosaurus ornatus*, Hover, 1985; Knapp and Moore, 1996). Thus, colour morphs may signal genotype and the consequent physiological and behavioural adaptations to specific environmental conditions, which may be relevant for potential mates and rivals.

The common wall lizard, *Podarcis muralis*, exhibits a polymorphic colouration (Cheylan, 1988; Sacchi et al., 2007b), with both sexes showing three main discrete morphs differing in throat and belly colouration (i.e. red-, yellow- and white-throated, see Fig. 1). Throat and belly colourations develop starting from the second year of life in this species (Cheylan, 1988), and individuals maintain the same colour through their life (Cheylan, 1988; S. Scali and R. Sacchi, unpubl. data). Colour appears to be unimportant in determining contest outcome in intermale fighting (Sacchi et al., 2009), but a recent paper showed a morph-specific immune responsiveness in males of this species, since yellow morphs had a lower immune response compared to both red and white males, whose immune response was similarly high (Sacchi et al., 2007a). This result suggests that immune response could be involved in the processes maintaining colour polymorphism in this species. Thus, colour morphs may differ in their physiological traits as, for example, their haematological profile.

In this study, we aimed at identifying covariations between colour morph and physiological traits in the common wall lizard. To this aim, we determined the haematological profiles of colour morphs in order to 1) test for differences among morphs in body condition, blood parameters and blood parasitism upon capture, and 2) analyse how body condition, blood parameters and parasitism changed in relation to sex, morph and age after a captivity period involving reproduction and egg laying by females.

2. Methods

2.1. Individual collection and marking

We captured sexually mature lizards (>50 mm snout-vent length, SVL) by noosing (i.e., using a slip knot of sewing thread attached to the end of a 2-m pole, Blomberg and Shine, 1996) in 8 different sites located in Pavia town and in the surroundings (Lombardy, North Italy) between February and March 2008. In each site, we carried out two capture sessions: in the first (February), we captured only females, in order to collect virgin individuals (i.e. females that had not mated yet in the current season as determined by the lack of male's bite signs on their belly), whereas in the second session (March) we noosed only males; thus males and females were captured ca. one month apart. Lizards were marked on the back by a unique combination of colours. Each individual was classified as white, yellow or red according to throat and belly colouration (Fig. 1), measured by a digital calliper (accuracy ± 0.1 mm) for SVL, weighed (accuracy ± 0.1 g), and transferred to the laboratory within 2 h from capture.

Overall, we noosed 128 adult lizards (64 males and 64 females) equally distributed among colour morphs (43 white, 43 yellow and 42 red, Table 1).

2.2. Housing condition and measures of female breeding investments

Lizards were initially held separately indoor (females from 13 February to 21 July, males from 3 March to 21 July) under a natural light–dark cycle in individual transparent plastic jars (20 × 30 × 20 cm) provided with a newspaper sheet as substratum, a water tank and a

Fig. 1. The three colour morphs of female (left) and male (right) common wall lizards.

Table 1
Mean values of body mass and haematological parameters of male and female common wall lizards recorded at capture and release (n males = 60, n females = 53, Wilcoxon test for matched samples; see Methods).

	Capture mean ± s.e.	Release mean ± s.e.	Statistic	P
Males				
Body mass	6.70 ± 0.17	7.95 ± 0.16	−11.15[a]	<0.001
Haemoproteus	4.36 ± 1.38	3.10 ± 0.70	−0.25	0.80
Heterophils	5.57 ± 0.54	11.17 ± 0.85	−5.01	<0.001
Eosinophils	5.59 ± 0.41	7.47 ± 0.52	−2.99	0.003
Basophils	3.64 ± 0.37	3.95 ± 0.29	−1.25	0.21
Neutrophils	0.11 ± 0.05	0.13 ± 0.05	−0.39	0.69
Lymphocytes	85.01 ± 0.82	77.25 ± 1.22	−4.90	<0.001
Monocytes	0.09 ± 0.04	0.04 ± 0.02	−1.48	0.14
H:L	0.07 ± 0.01	0.16 ± 0.01	−4.94	<0.001
Females				
Body mass	5.01 ± 0.14	5.50 ± 0.15	−3.51[a]	0.001
Haemoproteus	2.50 ± 0.55	2.72 ± 0.81	−0.09	0.93
Heterophils	5.93 ± 0.61	11.37 ± 1.03	−4.06	<0.001
Eosinophils	4.08 ± 0.42	9.51 ± 1.09	−4.04	<0.001
Basophils	3.24 ± 0.30	3.54 ± 0.41	−0.18	0.86
Neutrophils	0.26 ± 0.10	0.16 ± 0.06	−0.50	0.61
Lymphocytes	86.17 ± 0.87	75.33 ± 1.78	−4.78	<0.001
Monocytes	0.31 ± 0.08	0.09 ± 0.04	−2.48	0.013
H:L	0.07 ± 0.01	0.17 ± 0.02	−4.37	<0.001

[a]Values for paired sample t test.

shelter, and fed with 3 *Tenebrio molitor* larvae each day. Plastic jars were placed in four cabinets (16 jars per cabinet), each subdivided in four levels. Each level was illuminated by an UV-B lamp (18 W) in order to provide the daily UV requirements for calcium and vitamin D fixation, and by four incandescent lamps (25 W) for heating. UV lamps were switched on for 3 h a day (from 10.00 to 13.00 h), while incandescent lamps were set alight for 6 h a day (from 11.00 to 17.00 h). Males and females were then paired according to all possible colour combinations (females of any given morph were mated to males of all three morphs) to carry out breeding experiments, which lied outside the purposes of this study. However, in this paper we used two measures of breeding investments, namely the total clutch size and the total clutch mass produced by each female following mating experiments carried out in 2008, in order to investigate the effect of breeding efforts on female haematological parameters.

At the end of the experiment all lizards were returned to their capture sites. All animals increased significantly their body mass between capture and release (males: +1.25 g on average, females: +0.496 g on average, see Table 1 for statistics).

2.3. Blood sampling and leukocyte counts

Blood samples were collected in the laboratory from the postorbital sinus (MacLean et al., 1973) as smears both at capture and at release for all lizards. No lizards suffered long-term consequences from this procedure. Blood slides were prepared by placing a drop of blood directly onto a glass slide and smearing it with a second slide to produce a blood layer 1 cell thick. Blood smears were air-dried, fixed in methanol and stained by the May–Grunwald/Giemsa staining method. Leukocytes and red blood cells were counted by randomly scanning blood smears at 630× magnification under oil immersion following standard routines (Canfield, 1998; Latimer and Bienzle, 2000). In each microscopic field (50 fields), red blood cells were counted and leukocytes classified as heterophils, eosinophils, neutrophils, basophils, lymphocytes and monocytes. In each smear, 150–200 leukocytes and the corresponding red blood cells were counted; this allowed the number of leukocytes of the different types per 10,000 red blood cells to be calculated. Even when fewer leukocytes were counted, this method has been shown to give significantly repeatable within-blood smear measures of leukocyte concentrations (Saino et al., 1995; Saino et al., 1997). Concentration of different leukocytes is a simple measure of cell-mediated immunity in reptiles; in particular, eosinophil concentrations increase with increasing parasite loads (haemoprotozoan and metazoan) and with plasma corticosterone levels (Frye, 1991; Campbell, 1996; Marcato, 1997; Mader, 2000; Davis et al., 2008). Basophils, neutrophils and monocytes constituted <5% of counted leukocytes and were excluded from subsequent analyses. The heterophil:lymphocyte ratio (H:L) was also calculated as a sensitive measure of immunosuppression and long-term stress; an increase in this ratio may reflect a reduction in the responsiveness of the immune system due to an increase of corticosterone plasma level following a stressful experience (Davis et al., 2008). In addition, contrary to plasma corticosterone levels, which rise within 2–5 min from the stress event (e.g. Langkilde and Shine, 2006), H:L ratio takes many hours or even days before rising in ectothermic animals, and thus values at capture of this index may be considered as the baseline values of individuals (Bennet et al., 1972; Bennet and Reap, 1978; Hoi-Leitner et al., 2001; Case et al., 2005; Davis et al., 2008).

To record the presence of hemoparasites, the whole slide was scanned following a zigzag path and parasites were identified at genus level by L. Sacchi (Parasitology Laboratory, University of Pavia). Only *Haemoproteus* sp. was found in the smears and its load (defined as the number of parasites found per 10,000 red blood cells) was determined by examining 100 fields per slide under oil immersion (630×).

2.4. Statistical analyses

Data were $\log_{10}$-transformed (parasite load and H:L) or arcsine square-root-transformed (concentrations) before applying parametric statistics.

We first ran an exploratory ANOVA model coupled with a post-hoc test (LSD procedure) using collection site as grouping variable on blood parameters recorded at capture for each lizard and considered in this study (concentrations of heterophils, eosinophils and lymphocytes as well as H:L ratio). Since no differences in blood parameters were found among collection sites (all P-values>0.36), we excluded this factor from further analyses.

In further analyses, we used SVL, which is much less variable than body mass, as a measure of body size, whereas as an index of body condition we used body mass while including SVL in the model as a covariate.

To investigate the effect of captivity, sex, colour morph, body size (SVL) on individual blood profile, body condition and parasite load of lizards, mixed model analyses were run by including sampling time (capture and release), sex and colour morph as fixed factors, individual identity as random factor (to take into account the data dependence) and SVL as a covariate; two-, three- and four-way interactions between sampling, sex, morph, and SVL were also included in the initial models. SVL was centered on the mean value, to make easier the interpretation of the fixed effect terms in the models (Kreft et al., 1995).

In addition, to check for the effects of reproductive effort on blood parameters, GLM analyses were run for females only by including the differences in blood parameter values between release and capture as response variables, colour morph as a fixed factor, SVL and two measures of reproductive investments (total number of eggs laid and clutch mass) as covariates; models were run by including the two non-independent measure of reproductive effort separately; two-way interactions between morph and each covariate were included in the initial models.

Finally, to check for relationships between changes in body mass or parasite load and changes in blood parameters across individuals, we ran GLM analyses using as response variables the differences in body mass or parasite load (log-transformed) between release and capture, morph and sex as fixed factors and the differences in (appropriately transformed, see above) blood parameters (including parasite load for body mass) between release and capture as covariates. Models were run

by including each covariate separately. Two-way interactions between morph or sex and each covariate were included in the initial models.

All models were simplified by removing non-significant interaction terms first, starting from higher level interactions. All non-significant interaction terms at a given level were dropped from the model at once. Interactions at lower levels were kept in the model even if non-significant when they were included in statistically significant higher order interactions. Main effects were kept in the models even if non-significant. Analyses were performed using SPSS 16.0. Unless otherwise stated, parameter estimates and mean values are reported together with associated s.e.

3. Results

3.1. Variation in body mass, parasite and blood parameters in relation to sex-, morph- and body-size

Body mass and most blood parameters varied between capture and release (Table 1). In particular, body mass, heterophils, eosinophils and H:L ratio significantly increased, while lymphocytes decreased in both sexes after captivity; monocytes decreased in females only.

The mixed model analysis showed that body mass varied depending on time of sampling × sex × morph × SVL (Table 2): specifically, larger white and yellow females showed poorer conditions at release compared to capture, while the opposite was true for smaller females of the same morphs (Fig. 2). Red females and males of all morphs showed a positive covariation between body mass and SVL that did not differ according to sampling time (Fig. 2).

Parasite load (*Haemoproteus* sp.) varied significantly depending on sampling time × morph × SVL (Table 2); larger red individuals showed increased parasite load at release compared to capture (Fig. 3), while parasite load of the other two morphs did not covary with SVL both at capture and release (Fig. 3).

Heterophil concentration varied significantly depending on the combined effect of sampling time × morph × SVL (Table 2). In particular, larger yellow morphs showed higher heterophil levels than smaller individuals at release, but not at capture (Fig. 4). Conversely, larger red and white morphs showed higher heterophil

Table 2
Mixed model analyses of body mass and haematological parameters of common wall lizards in relation to sampling time, sex, morph, SVL and their interactions (asterisks denote removed terms).

Model	*F*	d.f.	*P*	Model	*F*	d.f.	*P*
Body mass				*Haemoproteus load*			
Sampling time	66.6	1, 101	<0.001	Sampling time	0.03	1, 107	0.86
Sex	108.1	1, 101	<0.001	Sex	0.81	1, 106	0.37
Morph	0.19	2, 101	0.83	Morph	1.72	2, 106	0.18
SVL	161.6	1, 101	<0.001	SVL	0.30	1, 106	0.58
Time × sex	25.1	1, 101	<0.001	Time × sex	0.18	1, 106	0.67
Time × morph	0.70	2, 101	0.50	Time × morph	1.57	2, 107	0.21
Time × SVL	5.57	1, 101	0.020	Time × SVL	0.56	1, 107	0.46
Sex × morph	0.52	2, 101	0.60	Sex × morph	0.15	2, 103	0.86*
Sex × SVL	8.85	1, 101	0.004	Sex × SVL	0.22	1, 103	0.64*
Morph × SVL	3.34	2, 101	0.040	Morph × SVL	1.23	2, 106	0.30
Time × sex × morph	0.57	2, 101	0.57	Time × sex × morph	0.63	2, 103	0.53*
Time × sex × SVL	4.24	1, 101	0.042	Time × sex × SVL	0.57	1, 103	0.45*
Time × morph × SVL	0.54	2, 101	0.59	Time × morph × SVL	3.52	2, 107	0.033
Sex × morph × SVL	0.48	2, 101	0.62	Sex × morph × SVL	1.01	2, 101	0.34*
Time × sex × morph × SVL	3.43	2, 101	0.036	Time × sex × morph × SVL	0.10	2, 101	0.90*
Heterophils				*Eosinophils*			
Sampling time	59.9	1, 107	<0.001	Sampling time	31.1	1, 101	<0.001
Sex	0.19	1, 105	0.66	Sex	0.84	1, 101	0.36
Morph	0.58	2, 105	0.56	Morph	0.96	2, 101	0.39
SVL	0.43	1, 105	0.51	SVL	0.11	1, 101	0.75
Time × sex	0.22	1, 106	0.64*	Time × sex	3.99	1, 101	0.049
Time × morph	0.39	2, 107	0.68	Time × morph	0.41	2, 101	0.67
Time × SVL	0.29	1, 107	0.59	Time × SVL	0.11	1, 101	0.75
Sex × morph	2.28	2, 103	0.11*	Sex × morph	0.45	2, 101	0.64
Sex × SVL	5.79	1, 105	0.018	Sex × SVL	3.14	1, 101	0.08
Morph × SVL	0.19	2, 105	0.83	Morph × SVL	4.06	2, 101	0.020
Time × sex × morph	1.10	2, 103	0.34*	Time × sex × morph	0.21	2, 101	0.81
Time × sex × SVL	1.20	1, 103	0.28*	Time × sex × SVL	2.16	1, 101	0.15
Time × morph × SVL	8.15	2, 107	0.001	Time × morph × SVL	4.26	2, 101	0.017
Sex × morph × SVL	0.55	2, 101	0.58*	Sex × morph × SVL	2.50	2, 101	0.09
Time × sex × morph × SVL	2.62	2, 101	0.08*	Time × sex × morph × SVL	5.86	2, 101	0.004
Lymphocytes				*H:L*			
Sampling time	61.7	1, 112	<0.001	Sampling time	54.8	1, 101	<0.001
Sex	0.01	1, 107	0.97	Sex	0.49	1, 101	0.49
Morph	2.46	2, 107	0.09	Morph	0.80	2, 101	0.45
SVL	0.02	1, 107	0.90	SVL	2.86	1, 101	0.09
Time × sex	0.99	1, 108	0.32*	Time × sex	2.56	1, 101	0.11
Time × morph	0.34	2, 108	0.71*	Time × morph	1.23	2, 101	0.30
Time × SVL	0.01	1, 108	0.96*	Time × SVL	3.21	1, 101	0.08
Sex × morph	1.42	2, 103	0.24*	Sex × morph	1.35	2, 101	0.26
Sex × SVL	5.81	1, 107	0.018	Sex × SVL	11.2	1, 101	0.001
Morph × SVL	2.42	2, 103	0.09*	Morph × SVL	0.13	2, 101	0.88
Time × sex × morph	1.36	2, 103	0.26*	Time × sex × morph	0.34	2, 101	0.71
Time × sex × SVL	1.10	1, 103	0.29*	Time × sex × SVL	3.17	1, 101	0.08
Time × morph × SVL	2.37	2, 103	0.10*	Time × morph × SVL	14.0	2, 101	<0.001
Sex × morph × SVL	1.99	2, 101	0.14*	Sex × morph × SVL	1.52	2, 101	0.22
Time × sex × morph × SVL	2.24	2, 101	0.11*	Time × sex × morph × SVL	7.48	2, 101	0.001

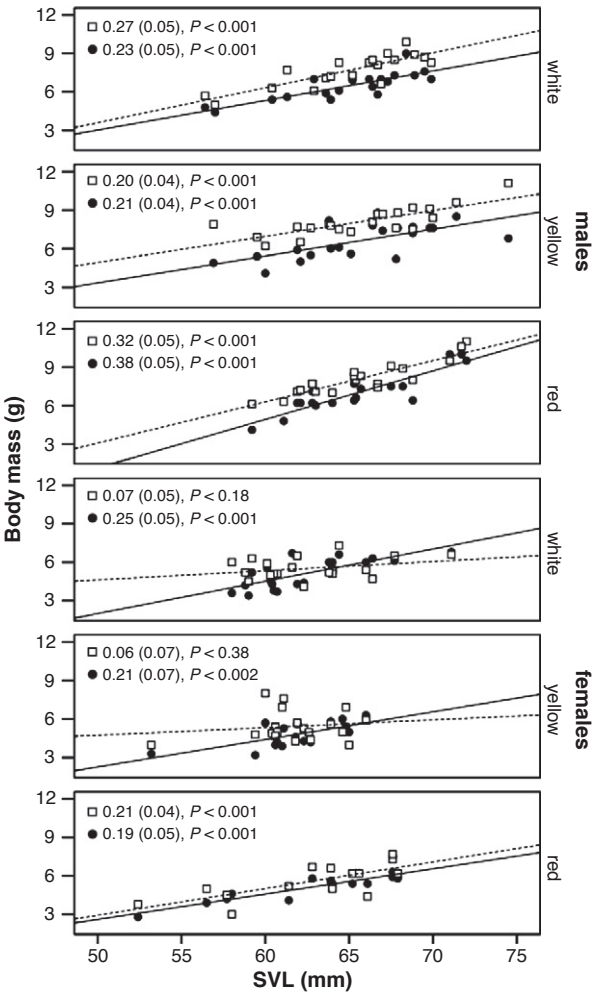

Fig. 2. Variation in body mass according to sampling time (capture, release), sex, colour morph and SVL of lizards (capture: black dots and solid line, release: open squares and broken line). Slopes of regression line (s.e.) are derived from the models shown in Table 2.

concentrations than smaller individuals at capture, while at release all individuals showed similar heterophil levels irrespective of size (Fig. 4). Secondly, there was a significant combined effect of sex × SVL (Table 2): heterophils tended to decrease with SVL in males, whereas they did not vary among females, though both slopes did not differ significantly from 0 (Fig. 5). The GLM analysis of change in heterophil concentration between capture and release carried out on females only while including separately the two measures of reproductive investment as covariates indicated that neither clutch size and clutch mass nor their interaction with colour morph affected heterophil concentrations (all *P*-values > 0.33, details not shown).

Concerning the eosinophils, the mixed model analysis revealed a strong combined effect of sampling time × sex × morph × SVL (Table 2). The effect was apparent in white females, where all individuals showed very similar eosinophil concentration at capture, (Fig. 6) while at release larger females had increased their eosinophil levels (Fig. 6). Conversely, red females, whose eosinophil concentrations increased with SVL at capture (Fig. 6), showed similar eosinophil levels irrespective of size at release (Fig. 6). Finally, yellow females did not show any size-related variation in eosinophils both at capture and release, and a similar pattern was found in males of all morphs (see Fig. 6). The GLM analysis of change in eosinophils concentration between capture and release carried out using females only while including separately the two measures of reproductive investment as covariates showed that clutch size and its interaction with morph did not affect eosinophil concentra-

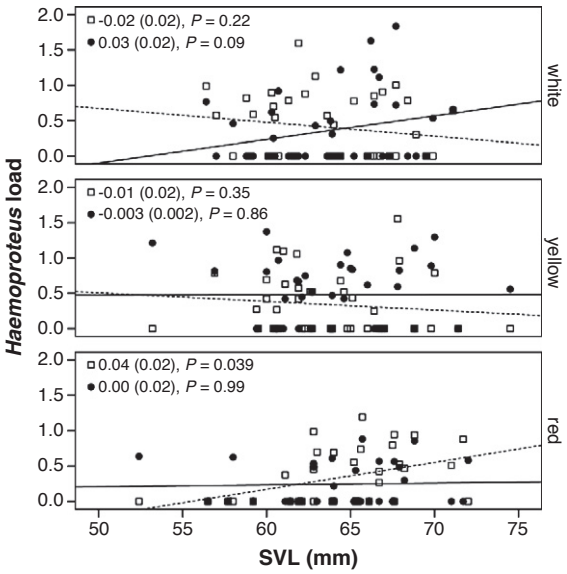

Fig. 3. Variation in *Haemoproteus* load (number of parasites per 10,000 red blood cells, $\log_{10}$-transformed) according to sampling time (capture, release), colour morph and SVL of lizards (capture: black dots and solid line, release: open squares and broken line). Slopes of regression line (s.e.) are derived from the models shown in Table 2.

tions (both *P*-values > 0.09). However, there was a combined effect of clutch mass × morph on change in eosinophils ($F_{2,41} = 4.22$, $P = 0.022$), despite none of the morph-specific regression slopes differed significantly from 0 ($\beta_{white} = 0.060 \pm 0.04$, $t = 1.54$, $P = 0.131$; $\beta_{yellow} = -0.053 \pm 0.03$, $t = -1.69$, $P = 0.099$; $\beta_{red} = 0.070 \pm 0.035$, $t = 1.98$, $P = 0.054$).

Lymphocyte concentrations varied according to sampling time for all individuals and to sex × SVL (Table 2, Fig. 7), larger males tending to show larger lymphocytes concentration than smaller males, whereas the opposite was true for females (Fig. 7). The GLM analysis of change in

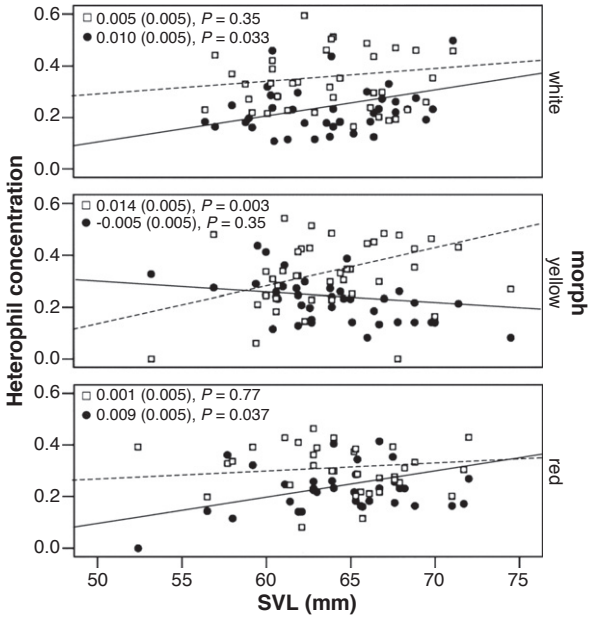

Fig. 4. Variation in heterophil concentration according to sampling time (capture, release), colour morph and SVL of lizards (capture: black dots and solid line, release: open squares and broken line). Slopes of regression line (s.e.) are derived from the models shown in Table 2.

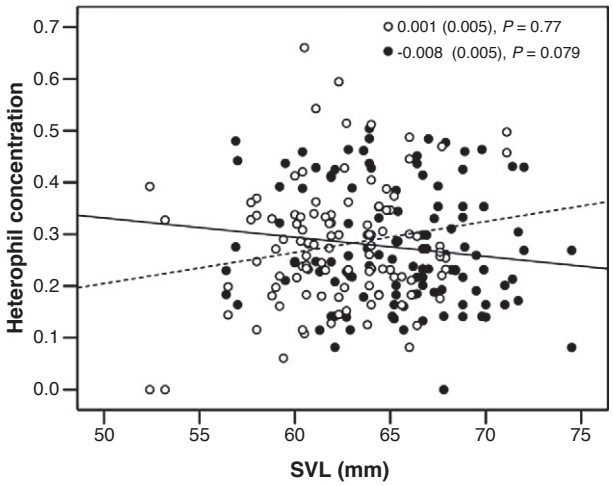

Fig. 5. Relationship between heterophil concentration, sex and SVL (males: black dots and solid line, females: open dots and broken line). Slopes of regression line (s.e.) are derived from the models shown in Table 2.

lymphocyte concentration between capture and release carried out using females only while including separately the two measures of reproductive investment as covariates indicated that neither clutch size and clutch mass nor their interaction with colour morph affected lymphocytes concentrations of females (*P*-values>0.15, details not shown).

The H:L ratio varied significantly according to sampling time × sex × morph × SVL (Table 2, Fig. 8). This variation involved both yellow and red females as well as yellow males: in particular, among yellow females, larger individuals significantly increased (i.e. they get worse) and smaller individuals decreased their H:L index (i.e. they get better) after captivity (Fig. 8). This trend was reversed in red females, where smaller individuals, which showed lower H:L values than larger individuals at capture, presented raised H:L index (i.e. they get worse) at release, while larger individuals maintained their high H:L values (Fig. 8). Finally, larger yellow males showed higher H:L values at release with respect to capture (i.e. they get worse after captivity). H:L values of white males and females and of red males did not show any size-related variation either at capture or release (Fig. 8). Measures of breeding investments by females, i.e. clutch size and clutch mass, and their interaction with morph did not affect change in H:L ratios between capture and release (*P*-values>0.38, details not shown).

Finally, mixed models confirmed that body mass and all blood parameters considered, except parasite load, varied significantly depending on time of sampling (Table 2). Specifically, body mass, heterophils, eosinophils and H:L ratio increased, while lymphocytes decreased between capture and release (Table 2). There was also an effect of sampling time × sex on body mass and eosinophil concentration (Table 2), with males increasing their body mass during captivity more than females, and females increasing their eosinophil concentration more than males (see Table 1). However, neither body mass nor blood parameters, including parasite load, varied depending on colour morph *per se* or in interaction with sampling time and/or sex. (Table 2).

3.2. Relationships between change in body mass, parasite load and blood parameters

Variation in body mass between release and capture was not predicted by variation in blood parameters or their interaction with sex and morph (Table 3), but it was affected by the change in parasite load × sex interaction ($F_{1,107} = 7.19$, $P = 0.009$); in particular, lizard males, but not females, showing an increase in parasite load at release were those that increased less their body mass during captivity ($\beta_{males} = -0.52 \pm 0.22$, $t = -2.39$, $P = 0.019$; $\beta_{females} = 0.28 \pm 0.209$, $t = 1.37$, $P = 0.18$).

On the other hand, variation in parasite load between release and capture was predicted by the change in eosinophils × sex interaction ($F_{1,107} = 4.69$, $P = 0.033$): males that showed a larger increase in

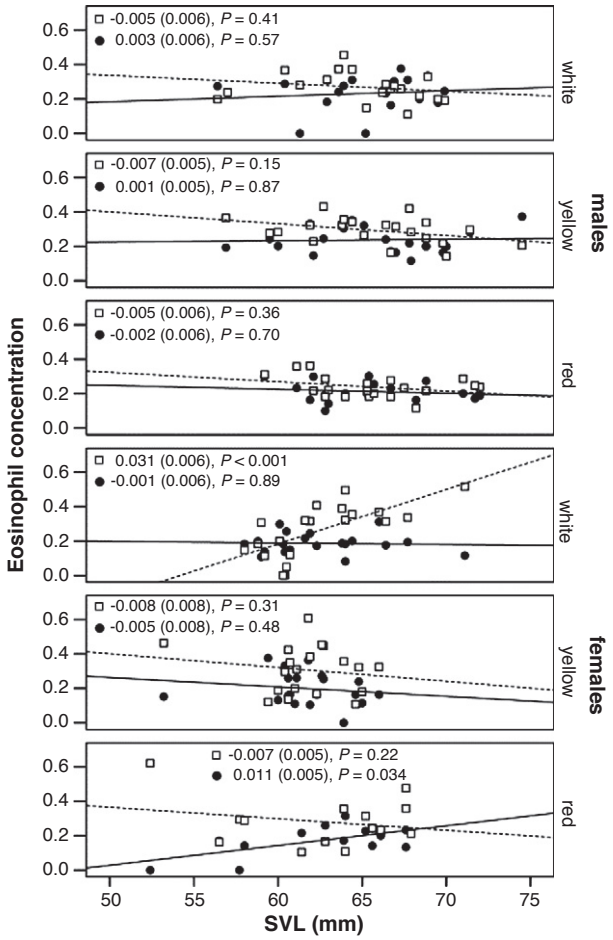

Fig. 6. Variation in eosinophil concentration according to time of sampling, sex, colour morph and SVL of lizards (capture: black dots and solid line, release: open squares and broken line). Slopes of regression line (s.e.) are derived from the models shown in Table 2.

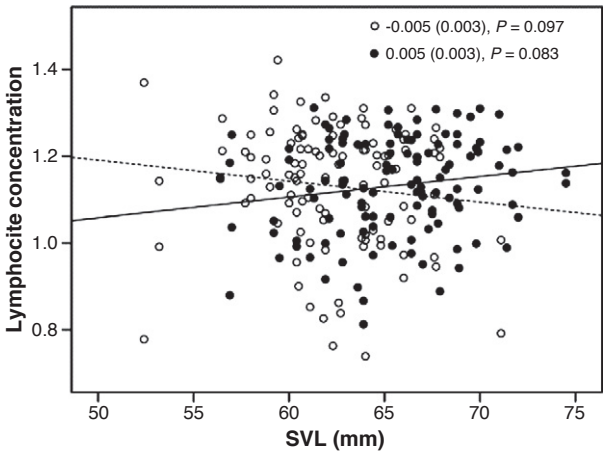

Fig. 7. Variation in lymphocyte concentration according to sex and SVL of lizards (males: black dots and solid line, females: open dots and broken line). Slopes of regression line (s.e.) are derived from the models shown in Table 2.

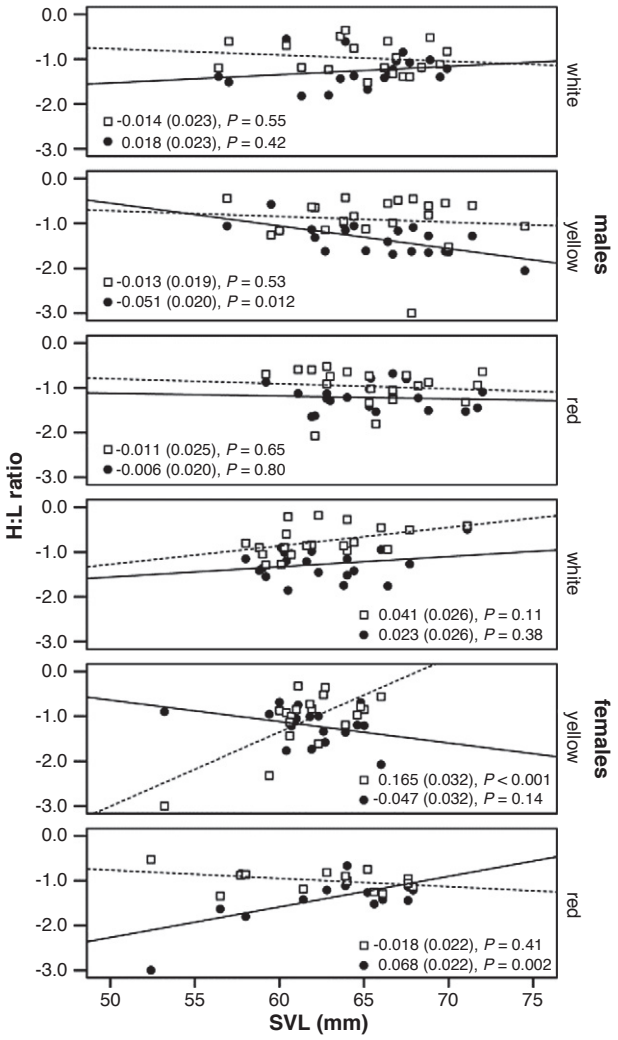

Fig. 8. Variation in H:L ratio according to time of sampling, sex, colour morph and SVL of lizards (capture: black dots and solid line, release: open squares and broken line). Slopes of regression line (s.e.) are derived from the models shown in Table 2.

eosinophil concentration tended to increase parasite load ($\beta_{males} = 1.49 \pm 0.78$, $t = 1.91$, $P = 0.058$), whereas this was not the case among females ($\beta_{females} = -4.72 \pm 0.46$, $t = 1.01$, $P = 0.31$). Changes in other blood parameters did not affect change in body mass or parasite load (Table 3).

In both model sets, the interaction sex × morph was never statistically significant, as well as the main effect of morph, and sex in models of change in parasite load. On the other hand, the main effect of sex was highly significant in models of change in body mass (all $F_{1,108} > 16.0$, $P < 0.001$).

4. Discussion

This study showed that morphs of common wall lizards differed at capture in their haematological parameters depending on sex and size (which reflects age in reptiles, Stamps and Krishnan, 1998), and in their haematological responsiveness to altered life-conditions related to captivity (Table 4). This suggests that different morphs show alternative fitness/physiological optima. Indeed, different haematological profiles and different responsiveness to altered life-conditions may provide a mean to endure various kinds of stressors eventually resulting in differential fitness advantages for different morphs and age-classes. For example, younger (i.e. smaller) yellow females reacted better to altered life-conditions related to captivity than older (i.e. larger) yellow morphs

Table 3
P-values derived from mixed model analyses of differences in body mass and parasite loads between release and capture (Δ Body mass and Δ Parasites, respectively) of common wall lizards in relation to differences in haematological parameters (e.g., Δ Heterophils) and their interactions with morph and sex. *P*-values of non-significant interaction terms are those at removal from the model; *P*-values of the main effect for each model were obtained from models including also sex and morph as main effects (see Methods).

Predictor	Response variables	
	Δ Body mass	Δ Parasites
	P	*P*
Δ Heterophils	0.37	0.78
Δ Heterophils × morph	0.36	0.47
Δ Heterophils × sex	0.64	0.74
Δ Eosinophils	0.19	0.26
Δ Eosinophils × morph	0.21	0.56
Δ Eosinophils × sex	0.44	0.033
Δ Lymphocytes	0.34	0.74
Δ Lymphocytes × morph	0.24	0.22
Δ Lymphocytes × sex	0.29	0.31
Δ H:L	0.85	0.52
Δ H:L × morph	0.69	0.28
Δ H:L × sex	0.88	0.06
Δ Parasites	0.44	
Δ Parasites × morph	0.89	
Δ Parasites × sex	0.009	

of both sexes and red or white females of all sizes, and this could confer them an advantage when facing sudden environmental perturbations or increased population densities. Actually, the yellow female fitness was not affected by density of yellow and orange morphs in the polymorphic side-blotched lizard (Sinervo et al., 2000b), and this might be due to their superior ability to endure the stress deriving from competition with neighbours.

Specifically, our results showed that variation in body condition following captivity was associated with sex, morph and age, because only white and yellow females, which lose or gained weight from capture to release depending on their size (larger got worse, while smaller got better), were involved. Similarly, blood parasite loads changed during captivity according to colour morph and size, with larger red individuals showing an increase in their parasite burdens after captivity. Finally, all blood parameters considered varied significantly between capture and release, and this variation was sex-, morph- and size-specific as well, with larger white and yellow and smaller red females showing the greatest changes in blood parameters following captivity. These changes in females' blood parameters were not due to their breeding effort during captivity, since neither clutch size nor clutch mass affected female blood profiles, except for change in eosinophils, which varied according to morph × clutch mass. However, this morph-specific effect was difficult to interpret because regression slopes of change in eosinophils on clutch mass were not statistically significant in any morph.

Table 4
Summary of variation in haematological parameters of common wall lizard morphs at capture and release derived from statistically significant relationships highlighted by mixed models (see Results and Figs. 2–8).

Morph	Capture	Release
White	More heterophils in larger individuals	More eosinophils in larger females
Yellow	Lower H:L values in larger males	More heterophils in larger individuals Higher H:L values in larger females
Red	More heterophils in larger individuals More eosinophils in larger females Higher H:L values in larger females	Higher parasite loads in larger individuals

Interestingly, the H:L ratio, a sensitive measure of stress and immunosuppression (Ots et al., 1998; Hoi-Leitner et al., 2001; Davis et al., 2008), changed in yellow individuals of both sexes and in red females during captivity, although in a opposite way according to body size; smaller yellow females had lower (they got better), whereas smaller red females had higher H:L values (they got worse) at release compared to capture. Likewise, larger yellow males and females showed increased H:L values at release, thus worsening their condition after captivity.

High H:L ratios in blood samples reliably indicate high glucocorticoid levels (Davis et al., 2008), since this ratio is positively related to the magnitude of the stressors and to the circulating glucocorticoids. Chronically elevated glucocorticoids may therefore cause long-term elevations in H:L ratios (Davis et al., 2008). The increase of H:L ratios between capture and release we documented in our study may thus reflect stressful conditions experienced during captivity, since it is well documented that captivity conditions affect the H:L ratio in other reptiles (e.g. *Terrapene c. carolina*, Case et al., 2005). Alternatively, the observed variation in haematological profiles may reflect a natural seasonal trend, i.e. lizards experienced more stress at the end of breeding season and therefore showed raised H:L ratios. However, the main long-term stressors that can be at work in the wild are competition with neighbours for shelters and basking sites (fighting is rare and mainly concentrates at the start of breeding season; P. Galeotti, pers. obs.), which was excluded in our experimental setting, and reproductive effort, which did not affect physiological changes in our captive setting condition. In addition, a seasonal trend in haematological variables of common wall lizards occurs from summer to winter (Duguy, 1967) rather than within the breeding season; finally, our lizards came from different sites and their breeding cycle was not synchronous; e.g. start of breeding was in early February at one collection site, while in mid-March at a nearby (500 m away) collection site. Thus, if a seasonal pattern of variation in blood parameters according to breeding cycle actually exists in the wild, the use of lizards from different populations should flatten this trend in captivity. Therefore, we may tentatively suggest that variation in haematological parameters of our lizards was likely mainly due to the stress induced by captivity, meaning with this term not only space restraint, but all altered life-conditions linked to captivity, i.e., changes in diet, photoperiod, activity and social interactions.

Our study also allowed us to investigate whether changes in haematological parameters during captivity were associated with changes in body condition and parasite load. We found that the increase in body mass during the captivity period was smaller in males that had an increased parasite burden, whereas change in body mass was not affected by change in parasite load among females. This difference between the sexes may be reconciled with a sex difference in circulating hormone levels, particularly testosterone, that is higher in males than females and is known to be immunosuppressive (Folstad and Karter, 1992). Males showing a greater increase in parasite loads may also have higher circulating testosterone levels (e.g. Klukowski and Nelson, 2001; Oppliger et al., 2004; John-Alder et al., 2009), and may pay higher costs in terms of body condition compared to those with low testosterone levels, perhaps because of increased metabolic rates (Oppliger et al., 2004). On the other hand, among females, a lack of covariation between body mass change and change in parasite load may depend on a lack of covariation between testosterone levels and parasite loads.

Not surprisingly, variation in eosinophil concentrations of males was related, albeit not significantly, to variation in parasite load during captivity. Actually, eosinophil increase is a typical cellular-mediated response to parasite infections (haemoprotozoans and metazoans) in many animals (Frye, 1991; Campbell, 1996; Marcato, 1997; Mader, 2000; Davis et al., 2008). The fact that only males showed this variation may be due to their potential higher susceptibility to parasites, possibly because of their higher testosterone levels, despite no statistically significant sex-differences in parasitism were found in this study (see Tables 1 and 2).

Body colouration of reptiles is determined by the combination of three basic types of pigment cells in the dermal skin: xanthophores, iridophores, and melanophores (Cooper and Greenberg, 1992; Bagnara et al., 1968), which are deposited in different layers: xanthophores (carotenoids) in the uppermost layer, iridophores in the intermediate layer, and melanophores (melanins) in the basal layer (Kuriyama et al., 2006). It is very likely that throat and belly colourations of the common wall lizard could be determined in the same way, and, most probably, the yellow and red colouration may derive from yellow carotenoids, as occurs in side-blotched and common lizards (Fox, 1976; Fitze et al., 2009), and from reddish-brown pheomelanins (Ducrest et al., 2008). Since both carotenoid metabolism and production of melanins are under strong genetic control (Ketterson and Nolan, 1992; Majerus, 1998), carotenoid and melanin-based colouration are often associated with variation in physiological and behavioural traits (e.g. Mosher and Henny, 1976; Roulin et al., 2000, 2001; Armbruster, 2002), stemming from the pleiotropic and epistatic effects of genes governing hormonal regulation and the synthesis of melanins (Sinervo and Basolo, 1996; Zera et al., 1998; Svensson et al., 2002; Ducrest et al., 2008). Colour morphs may indeed show alternative physiological properties, and hence evolve or be maintained as an indirect response to selection exerted on these physiological attributes. This may take place if pigments involved in the production of colour morphs alter physiological processes. For example, melanin pigments are known to display a number of biological properties in pathogen resistance mechanisms (Majerus, 1998; Wilson et al., 2001), potentially explaining why different morphs may vary in the magnitude with which they resisted pathogens and parasites. In addition, the production of colour pigments requires a complex molecular cascade, and the biochemicals involved may have pleiotropic effects on a number of morphological, physiological, and behavioural traits in a way that different morphs display different attributes.

Suggestive evidence for a genetic correlation between CP and other phenotypic traits is increasing in recent years (Roulin, 2004). For example, genetic colour polymorphism is associated with immunocompetence in the barn owl, *Tyto alba* (Roulin et al., 2000, 2001), the side-blotched lizard (Svensson et al., 2001), and the common wall lizard (Sacchi et al., 2007a), with developmental rate in some species of frogs (review in Hoffman and Blouin, 2000), with opioid regulation in the land snail (*Cepaea nemoralis*, Kavaliers, 1992), with resistance to salinity in white sea snails (*Littorina saxatilis*, Sokolova and Berger, 2000) and with regulation of corticosteroids in the lizard *Podarcis melisellensis* and the barn owl (Huyghe et al., 2009; Almasi et al., 2010).

Our study indicated that response to long-term stressful conditions, such as those represented by captivity, differed among common wall lizard colour morphs, implying a sex-, size- (age-) and morph-specific sensitivity to stress in this species, and hence a different physiological profile of the colour morphs. Differential colour-based susceptibility of individuals to environmental stressors, such as temperature, predators, diseases or parasite infections may clearly depend on the genetic background in polymorphic species. This fits well with the hypothesis that different colour morphs signal different genotypes, with different life-history and behavioural strategies as occurs in other lizard species (Sinervo and Lively, 1996; Sinervo and Zamudio, 2001). In fact, morph differences in cellular mediated response to stress (ultimately induced by plasma glucocorticoid levels) may translate in behavioural alterations as well (e.g., increased locomotory activity and foraging, reduced thermoregulatory activity and missing reproductive and aggressive behaviour, Moore and Miller, 1984; DeNardo and Licht, 1993; Belthoff and Dufty, 1998; Belliure and Clobert, 2004), with obvious fitness consequences for different morphs.

What is the relevance of this study for current ideas about evolution and maintenance of colour polymorphism? If female and male common wall lizards respond differently to chronic stress according to colour morph and age, as our results suggested, then it is likely that response to an acute stress may vary in a mostly similar way, and this may have profound implications for the maintenance of CP in this species, since indirect selection may act differentially on physiological attributes (e.g., metabolic rate, immune response, resistance to stressors) that vary among colour morphs.

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