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Colour variation in the polymorphic common wall lizard (*Podarcis muralis*): An analysis using the RGB colour system

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ABSTRACT

Intra-specific colour variation may either reflect a discrete polymorphism, potentially related to life-history strategies; a continuous signal, which may be related to individual quality; or a combination of both. Understanding the true nature of this colour variation may thus help to identify the possible selective mechanism producing it. The common wall lizard (*Podarcis muralis*) exhibits polymorphic colouration, both sexes showing three pure morphs differing in throat and belly colouration (white, yellow and red) and three intermediate morphs (white-yellow, white-red, and yellow-red). In this study we used digital photography and image analysis (RGB system) to investigate colour variation among morphs, sexes and populations. We found that colouration of the six phenotypes results from the combination of three discrete colour tinges (white, red and yellow): pure morphs express a single colour, and no continuous variation exists among them. Similarly, ventral parts of the intermediate white-red and yellow-red morphs present a mixture of two discrete colours resulting from the concurrent expression in different scales of the same colours showed by the corresponding pure morphs. Finally, the white-yellow intermediates show the same colour tinge than pure yellow morph, but with a lower intensity. The finding that white-yellow lizards are smaller than all other ones suggests that they might be subadults not yet expressing the full yellow colouration. Morph colouration significantly varied among sexes, suggesting a possible role for hormone plasma levels in controlling colour expression. It is noteworthy that RGB values varied among sites, indicating that colour expression in this species holds some level of environmental plasticity. Therefore, our results confirmed the presence of discrete colour morphs, which may be genetically based in both sexes of common wall lizards. However, our findings also showed intra-individual variation in colour expression within a morph that is associated with habitat occupancy, which suggests phenotypic plasticity in morphs.

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1. Introduction

Intra-specific colour variation is a widespread phenomenon in animals, stemming from the combined action of genetic and environmental factors (e.g. Hill and Montgomerie, 1994; Hoffman and Blouin, 2000; Roulin, 2004). In some species, colour variation represents a discrete polymorphism, implying the coexistence of at least two sharply distinct and genetically determined colour morphs in the same population, the rarest of which is too frequent to be solely due to recurrent mutation (Ford, 1945; Huxley, 1955). These morphs are often associated with alternative behavioural strategies (e.g. reproductive, territorial, habitat use) maintained by different evolutionary mechanisms (e.g. negative frequency

dependent selection, disruptive selection, apostatic selection and assortative mating, Galeotti et al., 2003; Roulin, 2004; Gray and McKinnon, 2007). Therefore, morphs may represent alternative, locally adapted optima within a single species (reviewed in Sinervo and Svensson, 2002), which may be at the root of sympatric speciation processes through morph loss and phenotype fixation of a single allele or set of alleles in a newly colonized environment ("morph speciation", see West-Eberhard, 1989; Corl et al., 2010 and references therein for reviews). On the other hand, colouration is often a continuous trait whose expression changes with body condition, health status and environmental conditions (e.g. Hill and Montgomerie, 1994; Fitze et al., 2009). In this case, colouration represents a condition-dependent trait honestly signalling individual quality, as a result of trade-offs between colour pigment, antioxidant capacities and immune-competence (Olson and Owens, 1998; Cote et al., 2008). Under this scenario, colouration may be a plastic phenotypic trait subject to sexual selection.

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Relationships between colour morphs, alternative behavioural strategies, and rapid phenotypic evolution have been better investigated in lizards (e.g. Sinervo and Lively, 1996; Sinervo, 2001; Sinervo and Zamudio, 2001; Vercken et al., 2007; Corl et al., 2010). In the side blotched lizard (*Uta stansburiana*), a North American trimorphic species, six colour phenotypes are associated with three male and two female alternative breeding strategies, which underlie a single-locus/three alleles genetic control (Sinervo and Lively, 1996; Sinervo et al., 2000a, 2001). In this species, alternative, morph-related strategies coexist in the same population, no strategy being evolutionary stable per se, but maintained by negative frequency-dependent selection and density dependent selection (Sinervo and Lively, 1996; Sinervo et al., 2000a). Similarly, female colour morphs of the European common lizard (*Zootoca vivipara*) exhibit density-dependent breeding strategies with morph frequencies regularly cycling over time (Vercken et al., 2007, 2008). However, the existence of discrete colour morphs in this species has been questioned (Cote et al., 2008), by showing that colour underwent a continuous variation between the colour morphs claimed by Vercken et al. (2007). Understanding the true nature of colour variation is therefore not a trivial question, because if colouration varies gradually, the “alternative” phenotypes should be considered as the more frequent colours within a continuous variation linking each morphotype, and different colour phenotypes might mainly represent the expression of phenotypic plasticity rather than the result of micro-evolutionary processes.

The common wall lizard (*Podarcis muralis*) is a widespread European lacertid that exhibits a marked colour polymorphism (i.e. CP; Cheylan, 1988; Sacchi et al., 2007a,b; Calsbeek et al., 2010), adults of both sexes showing six morphs that differ in throat and belly colouration: three pure phenotypes (i.e. white, yellow and red) and three intermediate phenotypes (white-red, yellow-red and white-yellow, Sacchi et al., 2007b; Calsbeek et al., 2010, Fig. 1). In recent years, the CP of this species has been the subject of several studies, which found some phenotypic differences associated with each morph. For example, divergence among morphs emerged in body size (Sacchi et al., 2007b; Calsbeek et al., 2010); immune-competence and stamina (Sacchi et al., 2007a; Martin et al., 2008; Calsbeek et al., 2010); homing behaviour (Scali et al., 2012) and reaction to stress (Galeotti et al., 2010). By contrast, no difference in aggression and fighting ability apparently exists (Sacchi et al., 2009), but only dominance hierarchies based on body size (Edsman, 1990; Sacchi et al., 2009). Despite these behavioural and life-history correlations, the mechanisms driving this polymorphism remain unclear.

The three visually pure colour phenotypes are markedly different from each other, since all ventral scales are of a unique, sharply distinct colour. Among intermediate colour morphs, white-red and yellow-red individuals are clearly dichromatic, showing a mosaic of distinct white (or yellow) and red scales irregularly distributed on throat and belly; sometimes, both colours are expressed in different portions of the same scale (Fig. 1). However, it should be emphasized that these intermediates primary colours never mix to produce pink or orange. By contrast, white-yellow intermediates do not show the basic two colours on alternative scales, but exhibit a lighter shade of yellow in all ventral scales, as pure morphs do. Thus, a major topic for the study of CP in this species is the analysis of different colour patterns shown by individuals, in order to understand if there are six discrete colour morph classes, or a continuum of colours. In particular, the understanding of colour expression in intermediate individuals could lend insights into the selective mechanism producing and maintaining CP in the populations of this species, as well as into the relative contribution of genetic make-up and phenotypic plasticity underlying colour expression in different morphs.

Here, by using digital photography and colour analysis in the RGB system, we provide a quantitative assessment of colour variation of male and female common wall lizards in five different populations. Digital photography is an effective tool in animal colour analysis alternative to spectrophotometry that is becoming increasingly used because of the flexibility and apparent precision that digital imaging provides (reviewed in Stevens et al., 2007). Despite this, the images recorded by a camera are dependent not only on the characteristics of the object photographed, the ambient light, and its geometry, but also upon the characteristics of the camera device (Barnard and Funt, 2002; Westland and Ripamonti, 2004; Stevens et al., 2007). A possible solution to address these problems is to reconstruct the reflectance spectrum of the object by including in each image a pantone with a set of reference colours (Villafuerte and Negro, 1998; Bergmann and Beehner, 2008).

Using this colour system we tested the existence of a discrete colour polymorphism by estimating the distributions of the RGB colour values. If the observed morphs exhibit discrete colour classes, RGB values should cluster consistently with these visually identified groups. We also analyzed the colour variability among and within morphs in response to sex, size, body region (throat vs belly) and microgeographic pattern of populations.

2. Materials and methods

2.1. Individual collection and colour measure

Adult common wall lizards (snout-vent length, SVL > 54 mm, Barbault and Mou, 1986, 1988) were collected by noosing during spring and summer 2009 in five sites in the surroundings of Pavia (Northern Italy, 45°11'31" N, 9°9'11" E, Fig. 2). Overall, we captured 202 individuals (114 males and 88 females), of which 71 were white, 53 yellow, 19 red, 12 white-red, 17 yellow-red, and 30 white-yellow. At each site, we captured on average 40 lizards (34–56, see Table S1 for details) including at least one individual for each phenotype (Fig. 2), and multiple captures of the same individual were avoided by photographic identification (Sacchi et al., 2010). Each individual was measured to record SVL to the nearest 0.1 mm and weighed to the nearest 0.1 g. The ventral parts (throat and belly) of all lizards were photographed adjacent to a GretagMacBeth Mini ColorChecker chart (24 colour references, 5.7 cm × 8.25 cm) using a Nikon Coolpix 4300 (resolution 2272 × 1704 pixels). Lizards were then released at the site of capture.

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcz.2013.03.001>.

To determine whether our visual colour morph classifications were compatible with discrete categories, we used the RGB values of both throat and belly obtained following the method proposed by Bergmann and Beehner (2008). The RGB system is an additive colour method and is as effective as spectrophotometry in analysing pure morphs (Villafuerte and Negro, 1998; Bergmann and Beehner, 2008). In addition, contrary to spectrophotometry, which is unable to capture the discrete and heterogeneous spatial distribution of bicolour ventral scales (Vercken et al., 2008; Calsbeek et al., 2010), the RGB system is very efficient in resolving this kind of “mosaic” intermediate phenotype. We used the Camera plug-in for Adobe Photoshop CS3 to create a new colour profile that adjusted the colour in the photographs (in the jpeg format) to the known colour levels in each square of the ColorChecker chart. For each lizard, we measured the colour of throat and belly by selecting the areas of all scales showing the colouration (i.e. black spots were excluded) using the ‘magic wand’ tool (on average near 2200 and 9200 pixels for throat and belly respectively), and recording the RGB levels using the histogram palette. Finally, the RGB colour values were rearranged in the Hue, Saturation and Brightness (HSB) system,

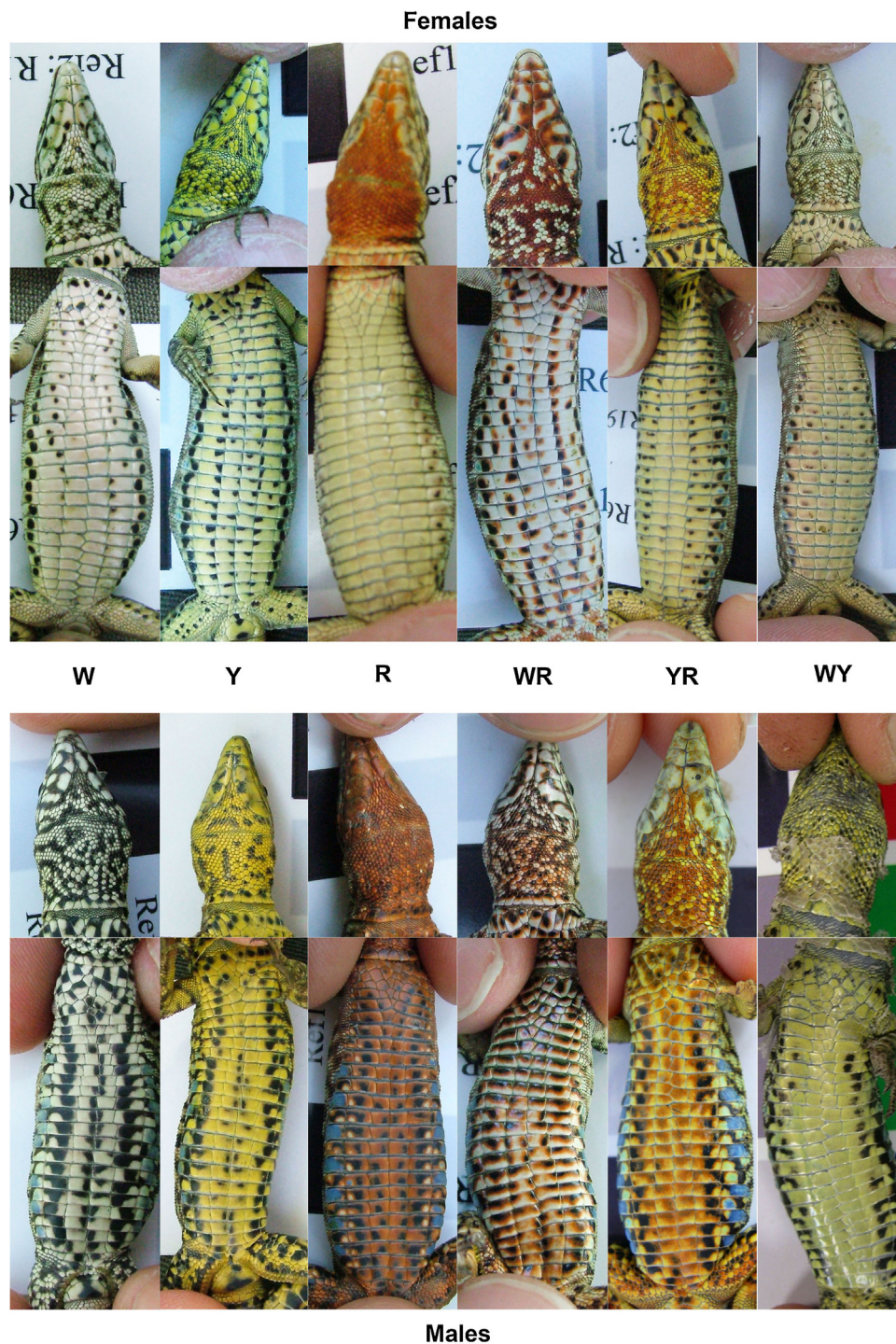


Fig. 1. Colour morphs in females (a) and males (b) of common wall lizards; W, Y, and R indicate the three pure phenotypes (i.e. white, yellow and red), whereas WR, YR, and WY indicate the three intermediate phenotypes (white-red, yellow-red and white-yellow).

which is the most common, intuitive and perceptually relevant representation of points in an RGB colour model. In the HSB system the cubic geometry of the RGB colour space is rearranged in a cylindrical-coordinate system in which the hue is the angle around the vertical axis and corresponds to the colour lights, the saturation is the distance from the axis, and the brightness corresponds to the distance along the axis.

For the three pure morphs and white-yellow intermediates ($n = 173$) two measures, one for each body region, were recorded, while for the mosaic intermediates (i.e. white-red and yellow-red

morphs, $n = 29$) we made four measures, one for each colour in both body regions. However, we were not able to collect colour data for all individuals, since some females expressed colours only on the throat, whereas in some red-yellow mosaic intermediates one of the two colours was expressed in only small areas and was not measured. Thus the final sample included 231 colour measures (173 from monochromatic morphs, and 58 from mosaic intermediates) for the throat, and 219 colour measures for the belly (174 from monochromatic morphs and 45 from mosaic intermediates).

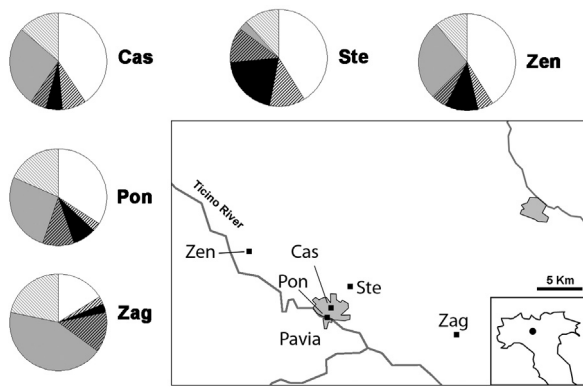


Fig. 2. Map of the five populations of common wall lizard considered in this study. Pie graphs show the relative abundance of the six colour morphs of each population: pure red: black, white-red intermediate: black and white, pure white: white, white-yellow intermediate: white and grey, pure yellow: grey, yellow-red intermediate: black and grey. Site acronyms: CAS: Pavia castle, PON: Bridge on the Ticino River, STE: Stefanini farm, ZAG: Zagonara village, ZEN: San Zeno farm in Bereguardo village.

2.2. Statistical analyses

Unlike other morphs, red-white and red-yellow bicolor intermediates had two series of HSB values, one for each colour, which cannot be averaged to obtain a single “mean” colour. Therefore, we considered these mosaic intermediates as two differently monochromatic individuals distinct from both the red, white and yellow pure morphs and from the white-yellow intermediate one. The red-white morph was associated to two new “virtual” morphs, the former accounting the red component of the mosaic colouration (hereafter red_{white} morph), the latter accounting for the white component of colouration (hereafter $white_{red}$ morph). In a similar way, the red-yellow morph was associated to the red_{yellow} and $yellow_{red}$ new morphs accounting respectively for the red and yellow components of mosaic colouration.

In order to check for differences in colouration between morphs, sexes and body regions and test the hypothesis of population variation in colour patterns, we used three linear mixed models – one for hue, saturation and brightness respectively – including the morph \times sex \times body region interaction, all two-way interactions and main effects as fixed terms, and morph within site as random effect, which allowed for different intercepts for morphs within each site. Normality of residuals was achieved, but the residual plots showed some slight deviation from homogeneity of variances. These deviations were accounted for by assuming different variances for sexes within each morph (Zuur et al., 2007). The initial model was then subjected to a stepdown simplification procedure, where non-significant terms ($P > 0.05$) were sequentially removed, starting from the interaction terms, until a minimal adequate model, including only significant variables, was obtained (Zuur et al., 2007).

To test if colour morphs differed in size, we used a linear mixed-model including SVL as the dependent factor, and morph, sex and their interaction as fixed factors. The site entered the model as a random factor in order to incorporate the auto-correlation of lizards belonging to the same population. The distribution of residuals was normal, but some slight deviation from homogeneity of variances occurred. These deviations were accounted for by assuming different variances for morphs (Zuur et al., 2007). The initial model was then optimized using the same procedure as in the previous analyses. We subsequently used a discriminant function analysis (DFA) to assess if the visually classified colour morphs could be reliably recognized from each other using the RGB values extracted from the images. Leave-one-out cross validation was then used to evaluate the accuracy of DFA in colour morph classification.

Finally, we examined whether the colouration of morphs were affected by body size. To do this, we analyzed the effect of SVL on hue, saturation and brightness in red (i.e. pure-red, red_{white} and red_{yellow}), and yellow morphs (i.e. pure-yellow, white-yellow, and $yellow_{red}$) separately. For white morphs (i.e. pure-white and $white_{red}$) only the brightness was considered. For each them, we carried out three linear mixed model ANOVA – one for hue, saturation and brightness respectively – including the interaction SVL \times sex \times body region as fixed effect (which accounted for different slopes for sex and body region), and site as random effect. Normality of residuals was achieved in all models, but different variance function structures (i.e. for site in models of red morphs and for sex within site in models of yellow morphs) were used in order to account for slight deviations from homogeneity of variances (Zuur et al., 2007). As in previous analyses, minimal adequate models including only significant variables were obtained by removing non significant terms.

All tests were performed using the R 2.12.1 statistical package (R-Development Core Team, 2010), and unless otherwise stated, values reported are means \pm SE.

3. Results

3.1. Variability of hue among morphs

The final model obtained for hue included the interactions morph \times sex (likelihood-ratio test, $L = 36.61$, $df = 7$, $P < 0.0001$) and morph \times body region ($L = 18.42$, $df = 7$, $P = 0.012$), suggesting that colouration differ among morphs depending on sex and body region. Indeed, pure red females had higher hues (i.e. were more orange) than corresponding males ($t_{394} = 4.44$, $P < 0.0001$, Fig. 3), while the opposite occurred in white-yellow ($t_{394} = 3.93$, $P = 0.0001$) and pure yellow morphs ($t_{394} = 17.49$, $P < 0.0001$), in which females expressed a lighter yellow than males (Fig. 3). By contrast, no significant sex based differences emerged in hues of all other morphs (Fig. 3). On the other hand, pure-red lizards had significantly higher hue in throat than belly ($t_{28} = 6.79$, $P = 0.0001$), whereas no significant difference was found in all other morphs (statistics not shown, Fig. 3).

In both sexes, the hue of throats did not significantly differ among red morphs (statistics not shown, Fig. 3), with the only exception of red_{yellow} males, which slightly differed from pure red ones ($t_{28} = 2.64$, $P = 0.013$, Fig. 3). Significant differences were also lacking among the belly hues of red morphs in both sexes (statistics not shown, Fig. 3). On the other hand, the hues of red male morphs significantly differed from that of all other morphs for both throat and belly (statistics not shown, Fig. 3) with the only exception of the $white_{red}$ males, whose belly hue was not different from that of red_{yellow} ones ($t_{28} = 1.93$, $P = 0.06$, Fig. 3). The throat hues of the three red morphs were also significantly different from those of all other morphs in females (Fig. 3). However, less difference occurred in the comparison of belly hues between red females and those of all other morphs. Indeed, significant differences in this case occurred only with respect to pure-yellow ones ($t_{28} = 2.07$, $P = 0.047$, Fig. 3), whereas red_{yellow} and red_{white} females did not express a different colour with respect to $white_{red}$ ones ($t_{28} = 1.84$, $P = 0.076$ and $t_{28} = 1.79$, $P = 0.084$, respectively, Fig. 3). The hue did not significantly differ between pure-white and $white_{red}$ morphs in neither males (throat: $t_{28} = 0.14$, $P = 0.88$; belly: $t_{28} = 0.15$, $P = 0.87$), nor females (throat: $t_{28} = 0.10$, $P = 0.92$; belly: $t_{28} = 0.54$, $P = 0.59$, Fig. 3). In addition, the hues of white morphs did not differ from those of yellow morphs in males (Fig. 3) with the only exception of the throat of pure white lizards, which differed from the pure yellow ($t_{28} = 2.44$, $P = 0.021$) and white-yellow ones ($t_{28} = 2.81$, $P = 0.009$). No significant differences emerged among white and yellow for

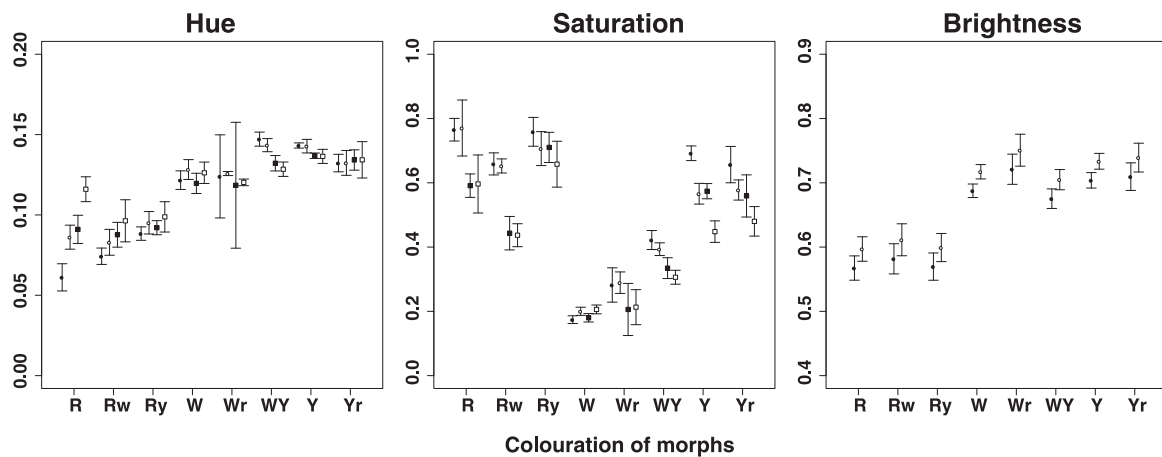


Fig. 3. Effects (means and 95% confidence intervals) of morph, sex, and body region on the hue, saturation and brightness of scale colouration in Common wall lizards. Symbols represent (●) male throat, (○) male belly, (■) female throat, and (□) female belly.

female morphs for both throat and belly (Fig. 3). No significant differences occurred among the throat and belly hues of pure-yellow, white-yellow, and yellow-red morphs of both sexes (statistics not shown, Fig. 3). Finally, significant differences between throat and belly hues occurred only in the pure-red morph ($t_{394} = 3.85$, $P = 0.00013$), particularly in females, which usually do not express colouration on the belly (Fig. 3).

The random effects at both site and morph within site levels were highly significant (respectively, $L = 33.37$, $P < 0.0001$, and $L = 79.71$, $P < 0.0001$), suggesting that the features of sites had a noticeable effect on the colours expressed by lizards. Standard deviations were, respectively, $\sigma = 0.008$ and $\sigma = 0.012$. Thus the variability of morphs within site is 50% larger than that of sites, suggesting that there is a relevant morph specific variability in addition to the site-to-site variability. Plots of the prediction intervals of the random effects (Fig. 4) confirm that the random effects for sites has less variability than morphs, and the variability among sites of white and yellow morphs is much higher than that in red ones.

3.2. Variability of colour saturation among morphs

The minimal model obtained for the saturation included the same interactions selected for the hue, i.e. morph \times sex ($L = 24.35$, $df = 1$, $P < 0.0001$) and morph \times body region ($L = 14.82$, $df = 1$, $P < 0.0001$). Therefore, the intensity of the colouration also varied among morphs depending on both sex and body region. Indeed, throats and bellies showed a more saturated colour in males than females in all morphs but red_{-yellow}, pure-white and white_{-red} ones (statistics not shown, Fig. 3). Males and females expressed a more saturated colour in the throat than belly in pure-yellow ($t_{28} = 19.65$, $P < 0.0001$) and yellow-red (even if in this last the test was near to the significant threshold, $t_{28} = 1.90$, $P = 0.057$). Surprisingly, pure-white lizards showed a higher colour saturation in belly than in throat ($t_{28} = 2.54$, $P = 0.011$), whereas no significant differences occurred in all other morphs (Fig. 3).

Among red morphs, the throat of red_{-white} lizards of both sexes expressed a colour slightly, but significantly, less saturated than that shown by pure-red (males: $t_{28} = 2.46$, $P = 0.020$; females: $t_{28} = 2.25$, $P = 0.032$, Fig. 3) and red_{-yellow} ones (males: $t_{28} = 2.21$, $P = 0.036$; females: $t_{28} = 2.59$, $P = 0.015$, Fig. 3). The same differences occurred among colour saturations of bellies (statistic not shown, Fig. 3), but the divergence between red_{-white} and red_{-yellow} males was not significant ($t_{28} = 1.18$, $P = 0.25$, Fig. 3). By contrast, no difference in colour saturations occurred between pure-red and red_{-yellow} lizards (statistics not shown, Fig. 3).

In white morphs, no significant differences in colour saturation occurred between females (throat: $t_{28} = 0.54$, $P = 0.59$; belly: $t_{28} = 0.13$, $P = 0.89$), whereas white_{-red} males showed a slightly more saturated colouration with respect to pure-white males in both throat ($t_{28} = 2.54$, $P = 0.017$) and belly ($t_{28} = 2.10$, $P = 0.045$, Fig. 3).

The colour saturations of throat and belly did not significantly differ between pure-yellow and yellow_{-red} lizards of both sexes (statistics not shown, Fig. 3). By contrast, white-yellow lizards of both sexes expressed a more saturated throat colouration than did both pure-white (males: $t_{28} = 8.14$, $P < 0.0001$; females: $t_{28} = 5.34$, $P = 0.0001$) and white_{-red} (males: $t_{28} = 3.04$, $P = 0.005$; females: $t_{28} = 2.60$, $P = 0.014$) morphs, and a less saturated colouration than did both pure-yellow (males: $t_{28} = 7.83$, $P < 0.0001$; females: $t_{28} = 6.74$, $P < 0.0001$) and yellow_{-red} (males: $t_{28} = 5.00$, $P < 0.0001$; females: $t_{28} = 5.85$, $P < 0.0001$, Fig. 3). The same differences occurred among colour saturations of bellies (statistics not shown, Fig. 3), even the divergence between white-yellow and white_{-red} females was not significant ($t_{28} = 1.76$, $P = 0.089$, Fig. 3).

The random effects at the site and morph within site levels were highly significant ($L = 10.05$, $P = 0.0015$, $L = 15.51$, $P < 0.0001$), but the estimated standard deviation for the morph ($\sigma = 0.034$) was much higher (58%) than for the site ($\sigma = 0.022$), suggesting that also for colour saturation, the site-to-site variability explained less variation than the morph specific variability.

Plots of the prediction intervals of the random effects (Fig. 4) confirm this impression in that all the prediction intervals for the random effects for sites contain zero; in addition, morph variability was more pronounced in white and yellow than in red morphs.

3.3. Variability of colour brightness among morphs

The final model obtained for brightness included only the main effects of sex ($L = 7.94$, $df = 1$, $P = 0.0048$) and morph ($L = 33.32$, $df = 7$, $P < 0.0001$), while the body region had no significant effect (P -value at removal > 0.11). Males had less bright colouration than females ($t_{409} = 2.93$, $P = 0.0035$, Fig. 3), whereas red morphs had less bright colouration than all other morphs (statistics not shown, Fig. 3). No significant differences in brightness occurred among pure-red, red_{-yellow}, and red_{-white} morphs, nor among pure-white, white_{-red}, pure-yellow, white-yellow, and yellow_{-red} ones (statistics not shown, Fig. 3).

Both random effects were highly significant (site: $L = 50.60$, $df = 1$, $P < 0.0001$; morph within site: $L = 14.56$, $df = 1$, $P = 0.0001$), and had similar standard errors (site: $\sigma = 0.052$; morph within site: $\sigma = 0.045$). Plots of the prediction intervals of the random effects (Fig. 4) confirm that site-to-site variability in brightness is similar

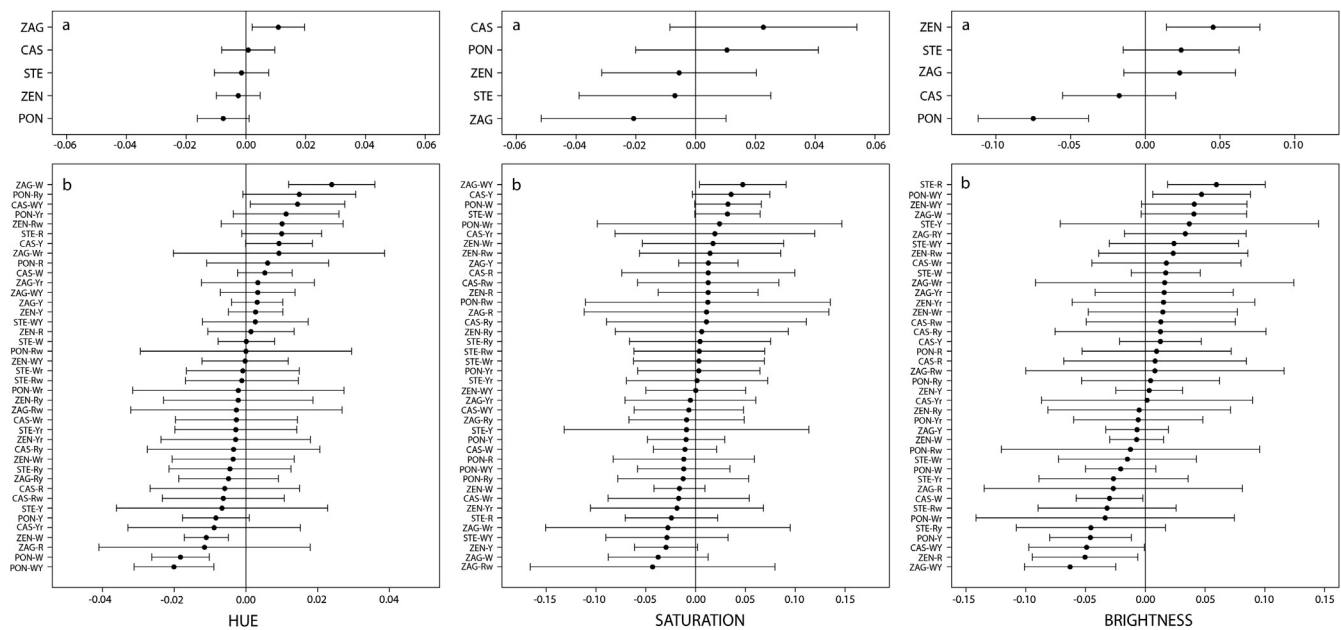


Fig. 4. Prediction intervals on the random effects for the hue, saturation and brightness models at the (a) farm level and (b) morph within farm level.

to that among morphs within site. Finally, red and yellow morphs appeared more variable than white ones (Fig. 4).

3.4. Visual classification of colour morphs

The discriminant function analysis performed on the colour values extracted from the images confirmed that the six visually classified morphs were highly statistically different from each other ($F=57.37$, $df=21.16$, $P<0.0001$). The first discriminant function accounted for 59% of colour variability, and clearly separated the white colouration expressed by both pure white and white_{-red} morphs from all others. The second discriminant function, which accounted for 40% of colour variability, better separated red colouration (expressed by pure red, red_{-white} and red_{-yellow} lizards) from yellow colouration (expressed by yellow and yellow_{-red} morphs). White-yellow lizard occurred at intermediate values of the two discriminant functions. Leave-one-out cross-classification matrix (Table 1) clearly provide support for different groups and 86.2% of colouration were correctly classified, although classification accuracy varied among morphs. Noteworthy, white-yellow lizards partially overlapped white and pure yellow ones.

3.5. Body size variation among colour morphs

The final model included only the main effects of sex ($L=11.15$, $df=1$, $P<0.0001$) and morph ($L=17.01$, $df=5$, $P=0.0045$), whereas

Table 1
Leave-one-out cross validation of morph colouration according with the discriminant analysis performed on RGB values.

Morphs	Predicted colouration			
	Red	White	White-yellow	Yellow
Red	41 (82%)	5 (10%)	3 (6%)	1 (2%)
Red _{-white}	21 (84%)	3 (12%)	0	1 (4%)
Red _{-yellow}	32 (89%)	0	1 (3%)	3 (9%)
White	0	163 (99%)	2 (1%)	0
White _{-red}	2 (8%)	21 (84%)	1 (4%)	1 (4%)
White _{-yellow}	0	32 (48%)	17 (26%)	17 (26%)
Yellow	1 (1%)	8 (6%)	14 (11%)	103 (82%)
Yellow _{-red}	3 (9%)	1 (3%)	2 (6%)	29 (82%)

the interaction was removed ($P>0.40$) suggesting that differences in size among morphs were independent of sex. The main effect of sex reflected the well known sexual dimorphism of the species (males being larger than females, $t_{191}=3.39$, $P=0.0009$, Fig. 5). More interestingly, the significant effect of morph depended exclusively on the white-yellow and pure yellow individuals (Fig. 6). The former were significantly smaller than both pure-yellow ($t_{191}=3.79$, $P=0.0002$), and red-white lizards ($t_{191}=2.49$, $P=0.014$), the latter were also significantly larger than pure-white ones ($t_{191}=2.20$, $P=0.029$). By contrast, no differences in size were found between pure white and white-red lizards, nor among pure red, white-red and yellow-red (Fig. 5).

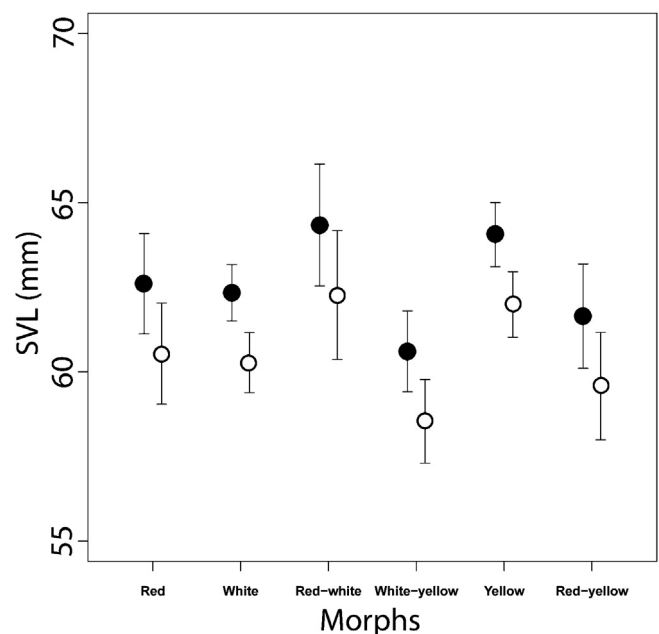


Fig. 5. Variation of SVL among male (●) and female (○) morphs of common wall lizards.

3.6. Effect of body size on colouration in red and yellow morphs

Red colouration was significantly affected by the body size of lizards, as the minimal adequate models for both hue and saturation included the main effect of SVL (hue: $L=10.71$, $df=1$, $P=0.0011$; saturation: $L=4.71$, $df=1$, $P=0.030$). By contrast, lizard size had no effect on colour brightness (P -value at removal >0.45). Irrespective of sex and body region, larger red lizards had lower hues ($\beta = -0.0009 \pm 0.0002$) and higher saturations ($\beta = 0.0044 \pm 0.0020$), suggesting that red colour became more intense with increasing body size. In addition, the random effect was not significant in any of the three models (hue: $P > 0.99$; saturation: $P > 0.98$; brightness: $P > 0.34$), suggesting that among-site variability within red morphs is low.

Yellow was also affected by the size of lizards, but only in terms of colour saturation, as the minimal models of both hue and brightness did not include SVL (P -values at removal being >0.31 and >0.15 respectively). Irrespective of sex and body region, the yellow saturation significantly increased with increasing SVL ($\beta = 0.011 \pm 0.002$; $L = 19.97$, $df = 1$, $P > 0.0001$), suggesting that larger lizards express a more intense colouration than smaller ones.

However, the random effect was significant in all the three models obtained for yellow morphs (hue: $L = 84.71$, $df = 1$, $P < 0.0001$; saturation: $L = 3.89$, $df = 1$, $P = 0.048$; and brightness: $L = 9.00$, $df = 1$, $P = 0.0027$), confirming that the expression of the yellow colouration is highly dependent on the site features where lizards live. Finally, no effect of SVL on colour brightness in white morphs was found (P -value at removal >0.40).

4. Discussion

In this study we analyzed the throat and belly colouration in males and females of common wall lizards in order to ascertain how colour changes depending on morph, sex, site and body region. Despite the high variability, our analyses showed that colour morphs of common wall lizards basically express only three main and discrete colour types, i.e. red, white, yellow. These three colours correspond to as many alternative scale colouration, which combined generate the six phenotypes. Indeed, we clearly showed that pure and intermediate red morphs express the same kind of red colour, as the pure white and white-red intermediate individuals share the same kind of white on their scales. More interestingly, we found that the pure yellow and white-yellow colouration did not differ for hue but only for saturation, suggesting that these two morphs expressed the same kind of yellow, but with different intensity. In other words, the yellow and light-yellow of these two morphs does not represent two different tinges, but rather two different intensities of the same colour, which can be reliably distinguished solely on visual parameters (human vision or digital image). In this scenario, the finding that pure yellow individuals of both sexes are larger than white-yellow ones is of particular interest, since the size in general is a reliable measure of age in reptiles, although stress levels and food availability can impact growth rates. Therefore, white-yellow individuals might not be a different morph than pure yellow ones, but rather represent a cohort of younger yellow individuals (e.g. individuals in the first reproductive year) that have not yet completely expressed their scale colouration.

4.1. Ontogenetic variation of morph colouration

The increase of colour intensity with age is a common phenomenon in reptiles (Rand and Andrews, 1975; Werner, 1978; Cooper and Greenberg, 1992; Carpenter, 1995). For example, the throat colouration of juveniles of the polymorphic tree lizard

(*Urosaurus ornatus*) is not fixed, and changes in males as they mature (Carpenter, 1995). Orange is the colour most frequently seen on both young male and female throats, but the sequences of colour changes leading to adult morphs vary among individuals (Hover, 1985; Carpenter, 1995). So, developmental sequences may be different among individuals having the same adult morph (Carpenter, 1995). It is possible that ontogenetic colour change in tree lizards occurs during developmental stages in which colour badges may not be completely functional (Carpenter, 1995). With less complicated patterns, throat and belly colouration might show ontogenetic change also in common wall lizards. If the intensity of the colouration promotes aggressive interactions among yellow individuals, the initial light-yellow throat of younger lizards might inhibit adult aggression, and protect them from the attacks by dominant larger males. Although aggressive behaviour does not differ among colour morphs (Sacchi et al., 2009), we recently showed that homing behaviour significantly differs among yellow morphs, pure yellow males being more prone to return to their territories than light-yellow ones, after moving up to 200 m (Scali et al., 2012). Consequently, light-yellow males might be less territorial and also less aggressive than pure yellow males. Alternatively, white and red morph may adopt reproductive strategies not involving age advantage, as occurring in yellow one.

4.2. Factors controlling the expression of morph colouration

To date, a genetic basis for colour expression in polymorphic lizards is well-supported by several recent studies (Sinervo et al., 2000a, 2001; Olsson et al., 2007; Calsbeek et al., 2009; Runemark et al., 2010; Huyghe et al., 2010). The genetic system governing the expression of polymorphisms in these species are quite simple, involving one or few loci: for example, colour morphs of *U. stansburiana* segregate as a single Mendelian locus with three alleles (Sinervo et al., 2000a, 2001, 2002), and the same occurs for the Australian painted dragon (*Ctenophorus pictus*, Olsson et al., 2007). By contrast, colour morphs of *Anolis sagrei* are inherited on the basis of two loci with epistatic interactions (Calsbeek et al., 2009). Even if we have no data on the inheritance of throat and belly colours in common wall lizards, preliminary analyses of intra-population genetic structure performed with microsatellite markers show a significant genetic divergence for the yellow morph from both the white and the red individuals as a result of disruptive selection enhanced by concomitant assortative mating among yellow morphs (Bellati, 2012). In this scenario, the result that red and white colour of intermediates did not differ from the respective pure morphs leads to the hypothesis that alternative alleles control the expression of different colour morphs. However, in both red intermediates the two colours appear on distinct scales or within different portion of the same scales, while in the white-yellow morph all scales show the same light-yellow colour. These findings suggest that the expressions of red and yellow colourations might be under two different and independent genetic controls. The white should represent the basal colour (young individuals being uniformly white on throat and belly at hatching), while the two genetic systems could drive the transitions of white towards red and yellow respectively.

A dual control of colour expression in these lizards (one for the red and one for the yellow) agrees with the organization in layers of pigments within lizard skin. In lizards yellow colouration usually appears to be due to carotenoids, while red colouration usually arises from pteridines (Bagnara et al., 1968; Bagnara and Hadley, 1973; Morrison et al., 1995). However, several exceptions to this dichotomy exist, and red carotenoid-based colouration have been described in lizards (e.g. Fitze et al., 2009). Carotenoids and pteridines are stored in specific chromatophores in the outermost layer of dermal unit (i.e. xanthophores containing both

carotenoids and pteridines and erythrophores containing only pteridines, Grether et al., 2004). Thus different physiological origins for yellow and red colour of the six phenotypes in common wall lizards might imply an interaction between different loci controlling for the distribution and storing of carotenoids and pteridines in the skin.

On the other hand, scale colours in lizards are under endocrine regulation (Sinervo et al., 2000b; Knapp et al., 2003; Sinervo and Calsbeek, 2003; Mills et al., 2008). Hormones can generate pleiotropic and epistatic effects on colour expression (Sinervo and Svensson, 1998), so a single endocrine locus can epistatically couple colour traits, given that colour is controlled by hormones. Therefore, the phenotypes of common wall lizards might be controlled by a single multiallelic genetic factor modulated by the endocrine system, rather than by the epistatic interactions among multiple loci. A relationship between colour morphs and hormones has been actually found in *Podarcis melisellensis*, which shows a polymorphic ventral colouration quite similar to that of *P. muralis*.

A relevant result of our study is the finding of the sex-based variation of colouration, which might imply a possible effect of sexual hormones on colour expression. In fact, we found that males were generally more intensely coloured than females, whereas females' red and yellow colouration only rarely extend over the belly. These differences might arise from the effects of steroid hormones (Sinervo et al., 2000b; Mills et al., 2008). A possible link between hormones and colouration has been located by Sinervo and Calsbeek (2003) in the action of hormones on plasma carotenoid-binding protein controlling for the deposition of carotenoids within dermal scales. Thus sexual hormones might have pleiotropic effects on colour expression (particularly on the yellow) significantly affecting both the amount of pigments stored within skin and the body regions where pigments have to be deposited.

Irrespective of a specific mechanism linking colour phenotype to endocrine system, our data indicate that sexual hormones are probably related with sexual dimorphism in colour expression, and may therefore be involved also in the different behaviour and physiology of the morphs (Sinervo and Zamudio, 2001).

4.3. Geographic variation of morph colouration

A further result of this study was the colour variation among sites within morph, which was particularly relevant for yellow morphs. This geographic variation of the RGB values in both sexes suggests that colour expression in common wall lizards holds some level of environmental plasticity. Such plasticity might reflect the among-site variation in the availability of the essential resources for colour expression. For example, carotenoids cannot be synthesized by animals and have to be ingested by diet (Grether et al., 1999). Thus, the within sex variability of colouration among sites we observed in common wall lizards may reflect the availability and composition of carotenoids of sampled localities. Like other vertebrates, lizards obtain carotenoids through the herbivorous insects they consume, and in some species a correlation between male colour and carotenoid pigments has been shown (Kwiatkowski and Sullivan, 2002). However, the results about dietary limitation on carotenoid-based colouration in lizards are controversial, and some other studies have shown that dietary limitation of carotenoids does not reduce male carotenoid-base advertisement colouration (Olsson et al., 2008; Fitze et al., 2009). An alternative and non-exclusive explanation to the dietary limitation hypothesis is the existence of variation in the ability of lizards to acquire carotenoids by diet, and allocate it to colouration. Indeed, differences among sites in some ecological features, such as basking sites, food resources, predation risk, or population density, which can be physiologically stressful and modify the hormonal profiles of

individuals, may alter the intensity of colouration. Consistent to this last hypothesis, Martin et al. (2008) found that less parasitized lizards had a brighter, more yellow ventral colourations.

Furthermore, environmental conditions (light levels, humidity, etc.) may render some type of signals more effective than others in different microhabitats (Endler, 1992). Indeed, natural selection is expected to favour signals and signalling behaviours that maximize the probability to receive and detect a signal relative to background noise. Therefore, colour variation among sites in common wall lizards could reflect an adaptation to local conditions of light and colour substrate in order to optimize intra- and inter-morph communication.

Finally, although the occurrence of a marked sexual dimorphism, across-site variation appeared to be quite consistent between sexes, suggesting a potential genetic underlying effect. Thus, geographic variability of colour expression by morphs may also be the results of a geographic structure of genetic variation among populations.

In conclusion, our results support the hypothesis that both sexes of common wall lizard exhibit a true colour polymorphism, with discrete colour phenotypes, likely under genetic control. However, environmental condition and sexual dimorphism represent a relevant component in colour expression by morphs, while among site variation represents the phenotypic variation across resource availability and sexual dimorphism represents the phenotypic response across sexes due to hormones and genes. Even though colouration has an environmental component, our results suggest that genetic control is probably prevailing, particularly for red colouration. These findings have important consequences for the understanding of evolutionary processes that control and maintain colour variation in *P. muralis* morphs. Indeed, discrete colour morphs observed in this species suggest the occurrence of a genetic control and might correspond to different alternative, locally adapted optima in both sexes (Calsbeek et al., 2010; Galeotti et al., 2010).

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