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RESEARCH ARTICLE

Effects of Colour Morph and Temperature on Immunity in Males and Females of the Common Wall Lizard

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Abstract Colour polymorphism in reptiles is generally associated with the coexistence of alternative reproductive strategies that involve specific trade-offs among different life history traits. Notably, body temperature trades off with immunocompetence: temperature has relevant effects on immune-response, but maintaining the optimal temperature increases both energetic costs and predatory risk. This trade-off gains complexity by sex, since males and females could optimize fitness by different strategies. Given that there is no single solution for trade-offs, different links among alternative evolutionary stable solutions and morphs might evolve independently in each sex. We tested this hypothesis in the common wall lizard (*Podarcis muralis*) by means of in vitro cultures of blood cells in order to examine the response of the immune-system to phytohemo-agglutinin stimulation in male and female morphs at two different temperatures (i.e. 22 and 32 °C), corresponding to the thermal optima of the two sexes. We found (i) morph-specific immunity in both sexes, i.e. yellow lizards suffer immunosuppression with respect to the other morphs, and (ii) sex-specific immunity under hot conditions, i.e. females of all morphs were immunosuppressed with respect to males. Results support the hypothesis that morphs might differently invest in immunocompetence, according to different set-up for the trade-offs between immunity and other life-history traits, resulting in alternative strategies with different fitness optima.

Introduction

The persistence of colour polymorphism (Huxley 1955) within a given population in reptiles is generally associated with the coexistence of alternative reproductive strategies that involve specific trade-offs among behavioural, morphological, physiological, and other life history traits (Sinervo and Lively 1996; Svensson et al. 2001a; McKinnon and Pierotti 2010). Consequently, different morphs may represent alternative locally adapted optima within a single species (reviewed in Sinervo and Svensson 2002). Among reptiles, lizards are particularly interesting as many species keep marked colour polymorphisms at population level (Thompson and Moore 1991a, b, 1992; Carpenter 1995; Sinervo and Lively 1996; Sinervo and Zamudio 2001; Olsson et al. 2007; Sacchi et al. 2007b), thus offering suitable models to investigate how lizard morphs behave in response to trade-offs.

Trade-offs involving immune system are of particular interest, since the ability of dealing with parasites and diseases has severe consequences on the fitness individuals may achieve, but also entails substantial costs for them (Lochmiller and Deerenberg 2000; Demas 2004; Cox et al. 2015). Temperature is one critical environmental factor, as ectotherms control their body temperature behaviorally. Indeed, the immune response of ectotherms is affected by temperature (Wright and Cooper 1981; Zapata et al.

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1992), and temperature-induced variations could have severe implications for the survival of the organism. Typically, the immune response of reptiles shows a species-specific optimal temperature and decreases above and below this optimum (Merchant et al. 2003, 2005; Zimmerman et al. 2010b; Butler et al. 2013; Sacchi et al. 2014). However, the temperature at which the host's immune system is most effective could not match the temperature at which the pathogen is most efficient, with severe consequences on the individual's fitness (Jackson and Tinsley 2002; Pounds et al. 2006; Rohr and Raffel 2010; Kilburn et al. 2011). As a consequence, in some species individuals rise body temperature to have beneficial effects on the immune system as well as on other components, such as the expression of genes involved in pathogen combating (Boltaña et al. 2013), and/or direct negative effects on pathogens (Kluger et al. 1975; Elliot et al. 2005). At the same time, maintaining optimal temperatures, or increasing it, is not free of costs, namely energetic demand and increased predatory risk (Bennett and Ruben 1979; Belliure and Carrascal 1998; Alford and Lutterschmidt 2012; Brewster et al. 2013). For example, metabolic rates in ectotherm escalate with body temperature (Gillooly et al. 2001), and the behavioural regulation of temperature implies elevated and prolonged exposure to predators (Herczeg et al. 2008). Thus, the extent of the immune response might depend on the balance between costs and benefits of this "immunity-temperature" trade-off: when this balance is unfavourable, individuals are expected to lower their response to immune challenge, while they should be able to mount higher immune responses when the temperature-related costs decrease. For example, increased costs of no thermoregulation with elevation modulate the response to lipopolysaccharide injection in *Psammodromus algirus* (Zamora-Camacho et al. 2016), and lowland lizards selected lower body temperature to reduce the energetic costs associated with metabolism and diverting the energy saved to the immune system.

The "immunity-temperature" trade-off gains complexity in the light of another basic and ubiquitous factor, which is known to affect both immune system and thermoregulation, i.e. sex. Indeed, by pursuing different strategies to maximize their own fitness, males and females might find different set up for the balance between costs and benefits intrinsic to the trade-off. Lower body temperature during pregnancy is supposed to be beneficial in females for embryo development (e.g. Shine 1980; Sinervo et al. 1991; Andrews et al. 1997; Schwarzkopf and Andrews 2012), and the consequent reduction of thermoregulation costs might release resources towards the immune system, which may allow for a better long-term reproductive success. Thus, selection might have driven immune function to be better adapted to lower temperature in females with respect to

males. At the opposite, development, body shape, colouration, fertility and behaviours in males are all influenced by androgen steroids (Ketterson and Nolan Jr 1992; Cox et al. 2004; Oliveira 2004; Hau 2007). Experimentally elevated testosterone plasma level fosters a strong motivation to defend the most profitable and largest territories (Moore 1988), as presumable increasing mating success compared to control individuals (Denardo and Sinervo 1994). Nevertheless, testosterone is a double edge sword, as stated by the Immunocompetence Handicap Hypothesis (ICHH, Folstad and Karter 1992), and supported by experimental evidence over different vertebrate taxa (reviewed in Roberts et al. 2004; Foo et al. 2016). High testosterone plasma level can decrease immune functions, favours parasite infections, stimulates risky behaviours, and thus diminishes survival (Olsson et al. 2000; Klukowski and Nelson 2001; Cox and John-Alder 2007), and there is therefore no way for males of maximizing stamina and aggressive behaviours at once.

Given that colour morphs in reptiles associate with alternative reproductive strategies involving specific trade-offs among different life history traits, each morph is expected not to mount an immune response, or even to show immunosuppression whenever costs of immunity outweigh its benefits (e.g., Sacchi et al. 2017). In other words, the suppression of immune responses would be adaptive for a morph-specific strategy when the risk of experiencing costs is higher than the expected costs from the infection itself. Since the setup of the trade-offs revolving around immunity is not unique, there is room for correlational selection to promote links among alternative evolutionary stable solutions and colour morphs, leading to morph specific immunity (Sinervo and Svensson 2002).

Common wall lizard (*Podarcis muralis*) is a medium-sized lacertid species showing ventral and throat polymorphic colouration, with three main morphs, i.e. white, yellow or red (Cheylan 1988; Sacchi et al. 2007b, 2013; Calsbeek et al. 2010). All morphs occur in both sexes and within the same population (Sacchi et al. 2007b). During the last decade, a lot of studies revolved around colour polymorphism of this species, and morph specific correlations have been detected in several life-history traits: for example, body size (Sacchi et al. 2007a; Calsbeek et al. 2010), homing behaviour (Scali et al. 2013), diet (Scali et al. 2016), stress responses (Galeotti et al. 2010), mating strategies (Galeotti et al. 2013), and fighting ability (Abalos et al. 2016). More interestingly, morph specific patterns have emerged in males for both immuno-competence (Sacchi et al. 2007a; Martín et al. 2008; Calsbeek et al. 2010) and seasonal variation of testosterone plasma levels (Sacchi et al. 2017). The immune-response is lower in yellow than in both red and white males (Sacchi et al. 2007a), whereas yellow males maintain significantly higher testosterone plasma levels at the beginning of the breeding season (April) and display a

stronger hormone's decline at the end of the breeding season (July) with respect to the other two morphs (Sacchi et al. 2017). Therefore, a strong initial investment promoted by high testosterone levels may trade-off against immunity according to the ICHH. Consequently, yellow morph on one side, and red and white morphs on the other side, may really represent the two opposite extremes of the trade-off between a "risky-strategy" (low stamina with high testosterone levels) vs a "conservative strategy" (high stamina with low intra-sexual competitive ability).

Temperature might play a relevant role in this scenario, as immune-response (notably adaptive immune response) has been shown to vary depending on it, with males having an higher optima than females (32 vs. 22–25 °C, Sacchi et al. 2014). Consequently, temperature may impose additional costs to individuals for mounting an immune response: the more the temperature departs from the optimal value, the higher this cost is. Furthermore, thermal costs should be particularly heavy for yellow individuals, namely males, which are subjected to the immunosuppressive effects of androgens.

All the above mentioned studies suggest that immunity, hormones and temperature are probably linked through complex interactions with colour morphs in common wall lizard, but a clear picture of those associations is not still possible. Several crucial pieces of information are still lacking, such as immune-response in female morphs as well as the effect of temperature on morph specific immune-response in both sexes. In the current study we tried to answer to these last two questions. By means of in vitro cultures of blood cells we examined the response of the immune-system to phytohemagglutinin (PHA) stimulation in male and female morphs at two different temperatures (i.e. 22 and 32 °C), corresponding to the thermal optima of the two sexes.

Materials and Methods

Individual Collection and Morphological Variables

During spring 2013 and 2014 we collected 51 adult common wall lizards of both sexes (26 males and 25 females; snout-vent length, SVL > 50 mm, Sacchi et al. 2012) by noosing in a farm in the surroundings of Pavia (Northern Italy; 45°11′31″ N, 9°9′11″E). Lizards were easily assigned to the correct colour morph by visual inspection (17 white, 22 yellow, and 12 red), and at least six individuals in each morph for each sex were used. Individuals were measured (to the nearest 0.1 mm using a calliper) for snout-vent length (SVL) and photographed for individual identification as described by Sacchi et al. (2010) in order to avoid pseudoreplication. Body size did

not significantly differ among morphs in males neither in females (two factors ANOVA including SVL as response variable and sex, morph and their interaction as predictors, morph: $F_{2,45} = 0.43$, $P = 0.65$; sex: $F_{1,45} = 8.61$, $P = 0.005$; sex × morph: $F_{2,45} = 0.55$, $P = 0.58$). Finally, we calculated an index of body condition as the residuals from a regression of body mass against SVL.

Blood Sampling, Cell Cultures and Immune-Response Test

In vitro activation of lymphocytes enabled us to repeatedly challenge the immune system of the same individual at different temperatures preventing the adaptive immune response to form an immunological memory (Sacchi et al. 2014). By this way, we evaluated the change in the immune function of a given individual in response to the variation of thermal condition (i.e., any significant change in lymphocytes proliferation can be interpreted as the direct effect of temperature variation). Blood samples (15–20 μl) were collected in heparinized capillary tubes from the postorbital sinus (MacLean et al. 1973) and inoculated in 15 ml of RPMI 1640 medium supplemented with 10% bovine serum (Sacchi et al. 2014). Cell suspension was then subdivided into two 7 ml sub-cultures, one of which was inoculated with 70 μl of a suspension of PHA (PHA-P Sigma L-8754, 50 mg in 10 ml phosphate buffered saline, Oppliger et al. 2004; Sacchi et al. 2007a, 2014). PHA is a lectin found in plants, especially legumes, which causes mitogenesis of T-lymphocytes into the injected tissue (Goto et al. 2015), and has been routinely used for challenging immune system in lizards (Vinkler et al. 2010; Zimmerman et al. 2010). The remaining solution (1 ml) was used to assess starting lymphocyte concentration using a Neubauer chamber (Sacchi et al. 2014). Each sub-culture was then distributed in two 1.5 ml culture tubes, and incubated at 22 and 32 °C for 3 days. Afterwards, cells were collected, re-suspended and newly counted. This second count involved only proliferating lymphocytes. Stimulation of T-cell after incubation was evaluated by the fold change of the PHA sample with respect to the corresponding control.

Statistical Analyses

Differential immune responses of morphs to the experimental treatments were assessed by a linear mixed model including morph × treatment × sex, and morph × treatment × condition as explanatory variables. The individual entered the model as random intercept to account for unexplained variation at individual level (σ^2_{ind}) after we controlled for the explanatory variables. Since previous studies had shown that the yellow morph consistently differs from the others either for immunity, hormone profile,

and behaviour (Sacchi et al. 2007a, 2017; Scali et al. 2013), we modelled the morph's effect (a three level factor) using the Helmert coding, which compares each level of the variable to the mean of the subsequent levels. Hence, the first contrast compares the mean of the dependent variable for the yellow morph (level 1) with the mean of all the other morphs (levels 2 and 3), while the second contrast compares the mean of the dependent variable for the white morph (level 2) with the mean of the red morph (level 3). The initial model was simplified using backward elimination of the nonsignificant terms (likelihood ratio χ^2 test, Zuur et al. 2009), and we checked residuals of the initial model for normality and homoscedasticity (Zuur et al. 2009). Finally, pseudo-R^2 accounting for the variance explained by the model (fixed and random effects combined) and for the fixed effects alone were computed according to Nakagawa and Schielzeth (2013). Analyses were performed using the package lme4 (Bates et al. 2015) and MuMIn (Barton 2016) in R ver. 3.2.4 (R core Team 2016), and otherwise stated, data reported are means ± standard errors.

Results

Peripheral blood lymphocytes of both sexes were actually stimulated by PHA at both 22 and 32 °C treatments, as the fold changes were significantly higher than zero in all cases (males: 2.19 ± 0.30 and 2.54 ± 0.33; females: 2.31 ± 0.28 and 1.66 ± 0.28; one sample t test at 25 and 24 df for males and females respectively: $t > 5.91$, $P < 0.001$).

The final model included the main effects of morph and condition, and the interaction sex × treatment (see Table 1 for statistics). It accounted for 57% of the total variance

Table 1 Variables affecting the immune response of common wall lizards to the PHA-inoculation (fold change with respect to controls) assessed by a linear mixed model after removing not significant terms

| Variables (coefficients)	$\beta \pm SE$	χ^2/t	d.f.	P
Morph		6.37	2	0.041
Yellow vs other morphs	−0.55 ± 0.24	2.33	46	0.024
White vs red morph	−0.28 ± 0.24	1.17	46	0.249
Sex		0.26	1	0.611
Females vs males	−0.23 ± 0.46	0.51	67	0.613
Treatment		1.47	1	0.225
32 vs 22 °C	0.34 ± 0.28	1.21	49	0.231
Condition		4.40	1	0.036
Slope	−0.63 ± 0.30	2.10	46	0.041
Sex × Treatment		6.00	1	0.014
Females vs males at 32 °C	−0.99 ± 0.41	2.45	49	0.018

See methods for details

in the immune response, while the fixed effects alone accounted for 17% of the whole variance. Consistently, the random intercept was also significant (likelihood ratio $\chi^2_1 = 4.22$, $P = 0.04$), and the unexplained variation at individual level (σ^2_{ind}) accounted for 49% of the whole unexplained variation of lizards' immune response after controlling for the explanatory variables. This last result suggested that a relevant amount of the variability in the immune response depends on some individual's features not related with their colour or condition, nor with the two thermal treatments.

Regardless of incubation temperature or body condition, yellow lizards of both sexes showed lower immune-response than males and females of all the other two morphs ($\beta = -0.55 \pm 0.24$, $t_{46} = 2.33$, $P = 0.024$, Fig. 1), while no significant difference occurred between red and white lizards ($\beta = -0.28 \pm 0.24$, $t_{46} = 1.17$, $P = 0.25$, Fig. 1).

Males and females showed a similar response at 22 °C (♂ vs. ♀: $\beta = 0.23 \pm 0.46$, $t_{67} = 0.51$, $P = 0.61$, Fig. 2), but at 32 °C females almost halved their response whereas males slightly increased it, leading to a significant difference between sexes (♂ vs. ♀: $\beta = 1.23 \pm 0.46$, $t_{67} = 2.66$, $P = 0.0098$, Fig. 2).

Finally, lizards of both sexes mounted a significantly lower response with increasing body condition ($\beta = -0.63 \pm 0.30$, $t_{46} = 2.098$, $P = 0.018$, Fig. 3), despite thermal treatments and colour morphs.
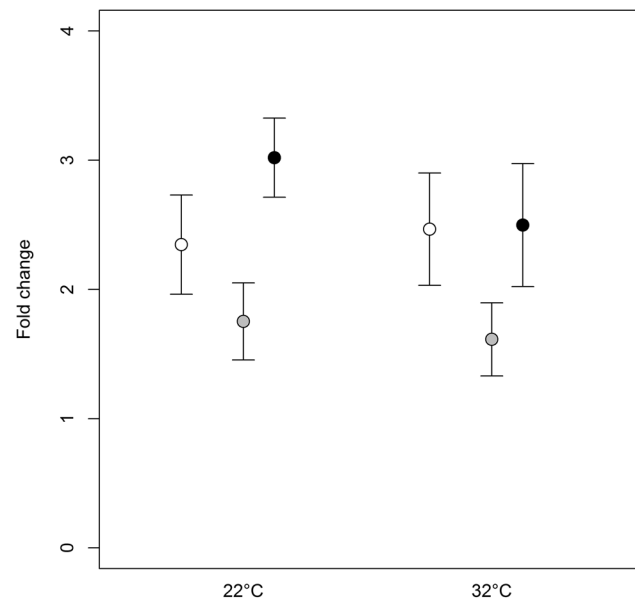
Fig. 1 Immune response of common wall lizard's morphs to the PHA-injection (fold change with respect to controls) after incubation at 22 and 32 °C; *white, grey,* and *black colours* are for *white, grey, red* morphs respectively; *symbols* and *bars* represent means ± 1se. (Color figure online)
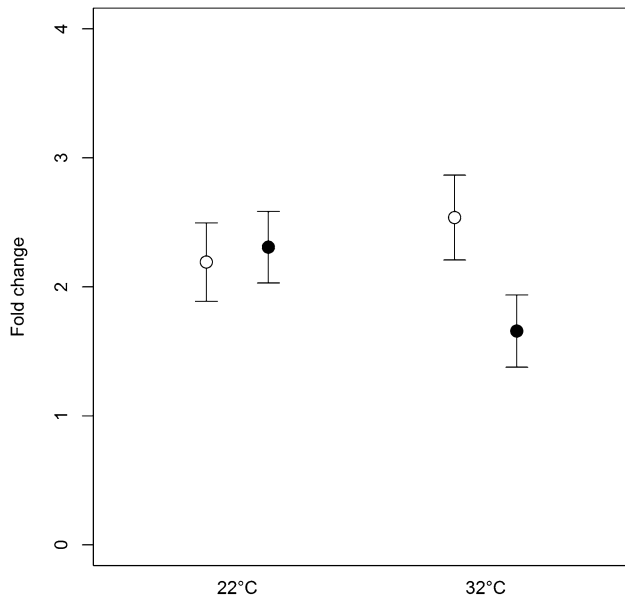
Fig. 2 Immune response of male (*white dots*) and female (*black dots*) common wall lizards to the PHA-injection (fold change with respect to controls) after incubation at 22 and 32 °C; *symbols* and *bars* represent means ± 1se
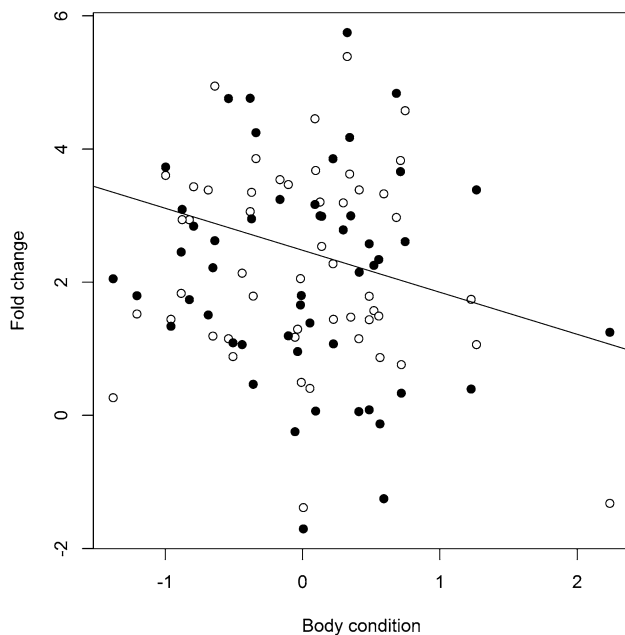
Fig. 3 Relationship between the immune response to the PHA-injection (fold change with respect to controls) and body condition in common wall lizards after incubation at 22 °C (*white dots*) and 32 °C (*black dots*)

Discussion

With the aid of blood cell cultures supplied with PHA, we found a clear morph-specific response in both sexes, which

was independent of the effect of temperature and body condition. In general, yellow individuals were immunosuppressed with respect to red and white ones, which in their turn had a similar response. At the opposite, temperature and sex strictly interacted to control the immune response, with females showing immunosuppression at high temperature. Finally, immune response negatively correlates with body condition (in either hot or cold conditions) in all morphs, suggesting the occurrence of a trade-off between immunity and condition.

Immunosuppression in Yellow Morph

The modulation of an immune challenge is impacted by body size and body condition that are a proxy for fatty reserves (Meylan et al. 2013). Indeed, adipose tissue depots represent a substantial portion of the total energetic budget and play an important role in sustaining physiological processes energetically expensive, including immune function (reviewed in Demas 2004). A reduction in total body fat is in fact correlated with impaired immunity in a wide range of species (Norgan 1997; Spurlock et al. 1997; Klasing 1998). Free fatty acid released by adipose tissue depots represent the main fuel for lymphocytes (Ardawi and Newsholme 1985), and enhance their proliferation (Pond 1996). So, immune-challenged individuals incur in an increase of energetic expenditures and, consequently, cannot simultaneously maintain a high immune response and increase fat reserves because of energetic constraints.

Given that common wall lizards undergo such energetic constraints, a dichotomous strategy associated to colour (yellow/not yellow) has to be invoked, at least for males, by the light of the ICHH. Yellow males have been shown to produce more testosterone at the beginning of the season with respect to both red and white males (Sacchi et al. 2017). This stronger initial investment in hormones may trade-off against immunity according to the ICHH (Folstad and Karter 1992), thus imposing increased costs when dealing with immune-challenge. On the other hand, lower testosterone plasma levels in white and red individuals might allow these males to suffer less for the costs of the immune-response, and do not harshly deteriorate fat reserves. Hence, morphs in males might represent the two opposite extremes of the trade-off depicted by the ICHH. Yellow males might have been selected to follow a 'high reproductive investment strategy', where individuals maximize the expression of the typical traits of intensive and demanding sexual behaviours: increasing movements, boldness, exploratory abilities, and, perhaps, aggressiveness. At the opposite, white and red males might have been selected to adopt a 'healthy and long survival reproductive strategy', where individuals maximize the ability to resist parasites and diseases at the expense of the reproductive

performance. In other words, yellow males might have been selected for high reproductive performances in short time, whereas red and white males for low reproductive performances over long lasting period. Some previous experimental findings support these notions: challenging homing ability, we found that yellow males achieved a higher return success than white and red ones, especially when individuals were released at greater distances (Scali et al. 2013). Yet, high T-levels promote boldness (Raynaud and Schradin 2014), and homing ability is associated with boldness because shy individuals tend to remain sheltered. In addition, high T-levels trigger dispersal in polygynous species and increase home range (Raynaud et al. 2012; Marjamäki et al. 2013; Onyango et al. 2013). Furthermore, T-levels have been shown to rise secretion by femoral pores increasing male appeal to females (Baeckens et al. 2016), and morph-specific secretions have been actually detected in common wall lizards (Pellitteri-Rosa et al. 2014). Thus, yellow males could invest more energy in marking territories to attract females. Seasonal T-level patterns of yellow males (Sacchi et al. 2017) might also indicate that they are especially active early in spring, when the competition for the best territories is at the top, whereas white and red males could benefit of reduced competition later in the season, when yellow males have exhausted their resources.

Morph specific immunosuppression in females may be related to alternative life history strategies as occurring in males, with yellow females investing less in immune function (and/or more in other life-history traits) compared with red and white ones. A general dichotomy between yellow vs both red and white females has already emerged in breeding strategy and home fidelity. Indeed, female common wall lizards adopt alternative r/K breeding strategies (Galeotti et al. 2013), yellow females being selected to lay large clutches of small eggs, while red and white females to lay small clutches of large eggs. Additionally, yellow females get homing faster than other females (Scali et al. 2013), suggesting that they might also adopt a different territorial strategy. Thus, female's morph specific immunity might actually reflect the different pattern of energy allocation proper to the specific strategies adopted by morphs, and natural selection might have differentially acted on combinations of female morph and immunocompetence, favouring high levels of immune function in one morph and low levels of immune function in the other. A similar correlation between morphs and immunocompetence has been previously found in females of *Uta stansburiana* (Svensson et al. 2001b) and *Anolis sagrei* (Calsbeek et al. 2008), in which social environment had different selective effect on morphs, promoting female morphs to be selected for high and low immune function. In both species, social crowding has a higher immunosuppressive effect in one morph, suggesting that the costs of immunity were actually morph-specific.

Alternatively, immunosuppression in yellow females might descend from the morph specific strategies adopted by yellow males, as consequence of an intersexual genetic correlation (Lande 1980). Indeed, selection on a character in one sex may cause an evolutionary change in the selected sex, as well as a correlated response in the other sex because of the effects of pleiotropy of genes affecting the homologous characters of both sexes (Møller 1993). Therefore, immunosuppression caused by higher T-plasma levels in males might also occur in females because of a genetic correlation, even though it may not be advantageous or even be slightly detrimental. In other words, immunosuppression in yellow females should be the cost they pay to allow yellow males to follow a 'high reproductive investment strategy'.

Immunity-Temperature Trade-Off

At 32 °C females showed lower immune response compared to males, whatever the morph is. Notably, ectotherms have the potential for behaviorally controlling their body temperature, which would allow for multiple solutions to the immunity-temperature trade-off; namely "conservative" (maintaining low body temperature, low metabolic rate and reallocating resources to the immune system when needed) or "risk-taking" (preferring high body temperatures and potentially strong immunocompetence at the cost of an erosive metabolic rate, which could undermine an efficient response to infection, and an increased risk of predation). Such trade-offs may help to explain the difference in thermal preference between sexes: the second strategy may be favored in males, as their reproductive success can be maximized by a "risky" strategy of increased sexual activity (i.e. higher temperature and metabolic rate allow for increased activity and exploratory behaviour) but not in females, where the first "conservative" strategy would allow for a better long-term reproductive success. Specifically in females, it is still unclear whether the preference of lower body temperature during pregnancy is beneficial to the current developing embryos, the female future reproductive success, or both (Schwarzkopf and Andrews 2012). Anyway, pregnant females of many species have been demonstrated to regulate at a lower body temperature than males or post parturient females do (Garrick 1974; Beuchat 1986; Andrews and Rose 1994; Amo et al. 2007). A possible reason for that is a trade-off between their own preferred temperature and the lower optimum temperature for embryo development, or, alternatively, an active selection for lower temperatures forced by the physical impairment by the mass of the clutch, which causes reduced sprint

speed and increased predation risk (e.g. Shine 1980; Sinervo et al. 1991; Andrews et al. 1997).

Conclusions

By this study, we confirmed three previous findings on immunity in common wall lizard, i.e., (i) yellow males are immunosuppressed with respect to both red and white males (Sacchi et al. 2007a), (ii) red and white male morphs mount similar immune-responses (Sacchi et al. 2007a), and (iii) females are more efficient in mounting an immune-response at low than high temperature (Sacchi et al. 2014). Results support the hypothesis that morphs (in both sexes) of common wall lizards might invest differently in immunocompetence, possibly reflecting different solutions for the trade-offs between immunity and the other traits, resulting in alternative life history strategies (a risky vs a conservative one) with different fitness optima. Differential investment in life-history traits may lead to correlational selection, which may build genetic correlations over time (Brodie 1989; McGlothlin et al. 2005) within individuals expressing different morphs, and may play a crucial role in the evolution of genetically based polymorphisms. However, the genetic mechanisms of polymorphism in this species have to be still demonstrated, as well as the heritability of the immune function, but there is reliable support that this occurs in other polymorphic lizards (Svensson et al. 2001a).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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