

Seasonal changes in parasite load and a cellular immune response in a colour polymorphic lizard

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Abstract Permanent colour polymorphisms may be maintained by complex interactions between physiological traits (e.g. immunity) and environmental pressures. In this study we investigate morph specific variation in parasite load and cellular immune response (induced by a Phytohaemagglutinin, PHA injection) in a colour polymorphic population of the Dalmatian wall lizard (*Podarcis melisellensis*), where adult males have bright white, yellow or orange throats and ventral sides. Orange males have larger heads and can bite harder than the others. To examine seasonal effects, analyses were performed at an early and late stage in the reproductive season (May and September). Infection with mites and ticks did not differ among morphs, but was more severe at the end of the reproductive season. Fewer orange individuals were infected with

haemogregarines at the end of the season, but white males were always more infected (higher number of haemogregarines in their blood) than other morphs. White and yellow males showed an increased PHA response towards the end of the season, but PHA response decreased in the orange morph. Finally, across all morphs, a relationship was found between ectoparasite load and PHA response. Our study provides indications of alternative life-history strategies among colour morphs and evidence for an up-regulation of the immune function at the end of the reproductive season.

Keywords Parasites · Colour polymorphism · Immune defence · Seasonal differences · Lacertidae

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Introduction

The vertebrate immune system constitutes an effective defence against parasites and pathogens, but it is also expensive to develop and maintain. Therefore, investment in immune defence must be traded off with other fitness components (Schmid-Hempel and Ebert 2003): animals that invest heavily in traits such as parental care (Moreno et al. 2001), mating behaviour (McKean and Nunney 2001) and sexual ornamentation (Verhulst et al. 1999) may opt to tolerate an infection rather than to fight it (Sheldon and Verhulst 1996). Compounding to the complexity, the vertebrate immune system itself consists of several components that may act in synergy but may also compete for available resources (Braude et al. 1999; Zuk and Stoehr 2002; Buchanan et al. 2003; Gasparini et al. 2009). Such trade-offs may help to explain the ecologically and evolutionary important intraspecific variation in immune function that is being observed in a growing number of taxa

(e.g. fish: Clotfelter et al. 2007; frogs: McCallum and Trauth 2007; lizards: Svensson et al. 2001; Calsbeek et al. 2008; birds: Pryke et al. 2007; mammals: Cutrera et al. 2010).

Polymorphic species provide excellent opportunities to study the interplay between immune defence and other life history traits. Morphs are thought to represent different fitness optima, produced by correlational selection on alternative suits of life-history traits. In many cases of male polymorphism, one morph pursues an aggressive, dominant reproductive strategy, often by physically defending a territory. The other morph then takes a more tranquil approach, behaves less aggressively towards conspecifics, and employs alternative mating tactics such as sneaking to obtain copulations. The dominant morph must invest in morphological, physiological and behavioural traits that help to maintain its superior rank but are also likely to trade-off with its immune function. The immunocompetence handicap hypothesis proposes that the association between a dominant mating strategy and reduced immunocompetence arises from the dual effect of testosterone, which promotes secondary sexual traits such as ornaments, bright colours and aggressive behaviour, but is simultaneously immunosuppressive (Folstad and Karter 1992, but see Roberts et al. 2004). Alternatively, the trade-off may simply reflect differential energy allocation: if pursuing a dominant reproductive strategy is energetically demanding, dominant morphs may not have sufficient reserves to mount the immune response, especially towards the end of the reproductive season (Duckworth et al. 2001; Alonso-Alvarez et al. 2007; Roberts and Peters 2009).

Female polymorphism is much less documented (Svensson et al. 2009), but in the few cases studied, female morphs tend to differ in reproductive traits such as clutch size and egg mass, and the success of either morph depends strongly on the social environment (Svensson et al. 2001; Vercken et al. 2007). Here too, differences in immune function between morphs may arise from direct energetic trade-offs, or via the adverse effects of hormones (e.g. elevated levels of corticosterone may minimize the effects of stressors but at the same time reduce immunocompetence: Comendant et al. 2003).

Several recent studies have found empirical support for the prediction that morphs should exhibit differential investment in immune function. For instance, in the Gouldian finch (*Erythrura gouldiae*) aggressive red-headed males exhibit immunosuppression in socially competitive environments, while the non-aggressive black heads do not (Pryke and Griffith 2006). In the side-blotched lizard (*Uta stansburiana*), orange-throated females experience a higher immunosuppressive effect from social crowding than yellow-throated conspecifics (Svensson et al. 2001; see also

Vercken et al. 2007; Calsbeek et al. 2008). In the tawny owl (*Strix aluco*) the interaction between the innate and humoral branches of the immune system is synergistic in dark nestlings, but antagonistic in pale nestlings (Gasparini et al. 2009). These theoretical and empirical considerations corroborate the idea that immune function may play an important role in the maintenance of colour polymorphisms.

The Dalmatian wall lizard, *Podarcis melisellensis*, also shows colour polymorphism. Male *P. melisellensis* differ in throat and ventral colouration, which can be bright white, yellow or orange. The colour morphs also differ in several other traits that seem relevant for their respective reproductive strategy. Orange males are on average larger, they have relatively larger heads, they can bite harder and behave more aggressively than the other morphs (Huyghe et al. 2009a, b). As a result, they enjoy a higher dominance status (Huyghe et al. unpublished data). We here test the hypothesis that this advantage is balanced by a decreased immunocompetence. We expect that high testosterone levels and/or high investment in territorial defence will exhaust orange males as the breeding season advances, resulting in lowered immune responses and increased susceptibility to infectious disease.

Our main objectives are (1) to investigate the relationships between immune defence function (cellular immune response), parasite loads and colour, (2) to detect morph-specific variation in immune function, which could provide insights into the physiological mechanisms maintaining colour polymorphism within this species, and (3) to test whether there is a seasonal effect on immune function for the different morphs. Therefore, we measured immune function both early (May) and late (September) in the reproductive season in a polymorphic population of *P. melisellensis*.

Materials and methods

Study species

Podarcis melisellensis is a small lacertid lizard, endemic to the Adriatic coastline and islands in the Adriatic Sea. In 2008, sexually mature males (snout-vent length, SVL > 55 mm) were captured by noose on the island Lastovo (Croatia) early during the reproductive season (May, $N = 74$) and at the end (September, $N = 38$). Lizards were kept individually in cloth bags and transported to a field based laboratory, where the following measurements were taken. Within 48 h following capture, lizards were returned to the field in seemingly good health. Individuals were categorized in morphs by visual assessment of their colour (white, yellow or orange).

Morphometrics

Each individual's SVL was measured using digital callipers (Mitutoyo, accuracy: 0.01 mm) and each was weighed with a Pro Scout balance (to the nearest 0.1 g). As a condition index residual values of a linear regression analysis of mass (dependent) over SVL (independent) were used (both \log_{10} transformed). Tail type was scored as intact, broken or regenerated.

Parasites

The degree of ectoparasite infestation was assessed by brushing each lizard for 5 min with a soft paint brush over a white paper, so that ticks (fam. Ixodidae) and mites (fam. Trombiculidae) could be collected in a tube filled with 70% aqueous methanol. This resulted in 100% removal of visible ectoparasites. Later, ectoparasites were counted using a binocular loupe at 100 \times magnification.

To assess infestation by haemoparasites, a small amount of blood was collected with a heparinized capillary tube by puncturing the postorbital sinus. Thin blood smears were made on glass microscope slides. Smears were air dried and fixed for 15 min in absolute methanol and later these were Hemacolor-stained (Merck, Darmstadt, Germany) and sealed to facilitate long-term storage. Blood smears showed similar concentrations of red blood cells and were surveyed for 15 min, counting the number of haemogregarines (*Karyolysis* sp.) at 500 \times magnification using a light microscope.

Cellular immune response

We quantified the delayed cutaneous hypersensitivity response (Belliere et al. 2004; Oppliger et al. 2004) as an indicator of one aspect of immunity, at the cellular level. This response was assessed by injecting one foot of every individual with a 20 μ L solution containing 50 mg of phytohaemagglutinin (PHA; Sigma–Aldrich, L-8754) in 10 mL phosphate buffered saline (PBS). PHA influences a variety of cell types and, therefore the response to PHA injection is complex, but can serve as an index for heightened immune cell activity (Kennedy and Nager 2006; Martin et al. 2006). Thickness of the foot was measured before injection and 24 h later, using digital callipers (Mitutoyo, accuracy: 0.01 mm). The other foot was treated in the same way, but injected with 20 μ L of PBS serving as a control. The immune response was calculated as the change in thickness of the PHA injected foot minus the change in the control foot. Larger localized swelling indicated an increased immune activity at the cellular level. Measurements were made in triplicate and the median was used in the analyses.

Statistical analyses

We investigated parasite prevalence (proportion of individuals infected) using hierarchical loglinear analyses, testing for possible differences between times in the breeding season (early vs. late), among colour morphs, and the interaction effect of season \times morph. Generalized linear models were used to test for effects of season and colour, and their interaction on the degree of parasite infection (the number of parasites per individual) and on tail type (intact, broken or regenerated). The variation in PHA response and body condition among morphs, season and their interaction was tested using analyses of variance. Chi square (χ^2) and significance (P) values are only reported for the significant effects. To test the predictability of infection with parasites, logistic regression analyses were used (infected vs. uninfected). Relationships among traits were investigated using correlation analyses.

Results

Differences among morphs across the reproductive season

A greater proportion of the population was infected with ectoparasites at the end of the season than at the beginning: 63% in May versus 100% in September ($\chi^2 = 26.61$, $df = 1$, $P < 0.001$), but prevalence was equal for all morphs ($\chi^2 = 0.26$, $P = 0.88$, Fig. 1a). Accordingly, ectoparasite load (mean number of parasites per lizard) was more severe late in the reproductive season ($\chi^2 = 41.08$, $df = 1$, $P < 0.001$), but it did not differ among morphs ($\chi^2 = 0.34$, $df = 2$, $P = 0.85$; Fig. 2a).

The proportion of individuals infected with haemogregarines differed significantly among morphs across the season ($\chi^2 = 7.98$, $df = 2$, $P = 0.02$): infestation increased for the white and yellow morphs during the season, but decreased for the orange morph (Fig. 1b). The degree of infection (mean number of haemogregarines per lizard) changed across the season ($\chi^2 = 31.41$, $df = 1$, $P < 0.001$) and differed among morphs ($\chi^2 = 8.74$, $df = 2$, $P = 0.02$): infection was more severe at the beginning of the reproductive season and white males had a higher number of haemogregarines in their blood (Fig. 2b).

The response to injection with PHA differed significantly among morphs across the season (interaction effect season \times colour: $F_{2,84} = 4.79$, $P = 0.01$). White and yellow males showed an increased response later in the season, while the immune response of orange males remained the same (Fig. 3).

Body condition did not differ between colour morphs ($F_{2,107} = 0.20$, $P = 0.82$) or season ($F_{1,107} = 0.029$,

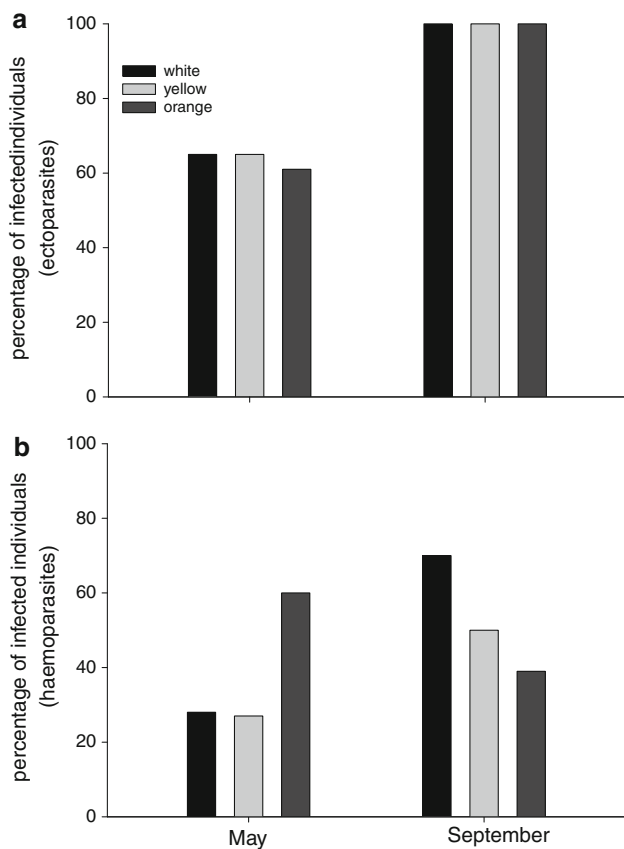


Fig. 1 Percentage of individuals infected with parasites (infestation) for the different morphs early (May, $N = 74$) and late (September, $N = 38$) in the reproductive season. **a** Ectoparasites: more individuals were infected in September, no differences among morphs, **b** Haemogregarines: more white and yellow individuals infected in September, more orange in May

$P = 0.87$). Tail break frequencies were not different among morphs ($\chi^2 = 0.070$, $df = 2$, $P = 0.97$) and were the same early and late in the season ($\chi^2 = 0.019$, $df = 1$, $P = 0.89$).

Relationships among health factors

We documented a significant correlation between presence and absence of ectoparasites with PHA response (logistic regression analysis, $\chi^2 = 16.72$, $df = 3$, $P = 0.001$). PHA response was the sole significant predictor variable in the model ($B = 21.16$, $\chi^2 = 8.73$, $df = 1$, $P = 0.003$); SVL and mass did not explain a significant proportion of the variance of infection (both $\chi^2 < 2.79$, both $P > 0.10$). Moreover, when taking only the infected individuals into account, a correlation was found between the numbers of ectoparasites and PHA response (Spearman's $\sigma = 0.34$, $P = 0.001$, Fig. 4a), but not with SVL or mass (both $\sigma < 0.15$, both $P > 0.17$). Lizards carrying more ectoparasites seemed to have a higher PHA response, but when the

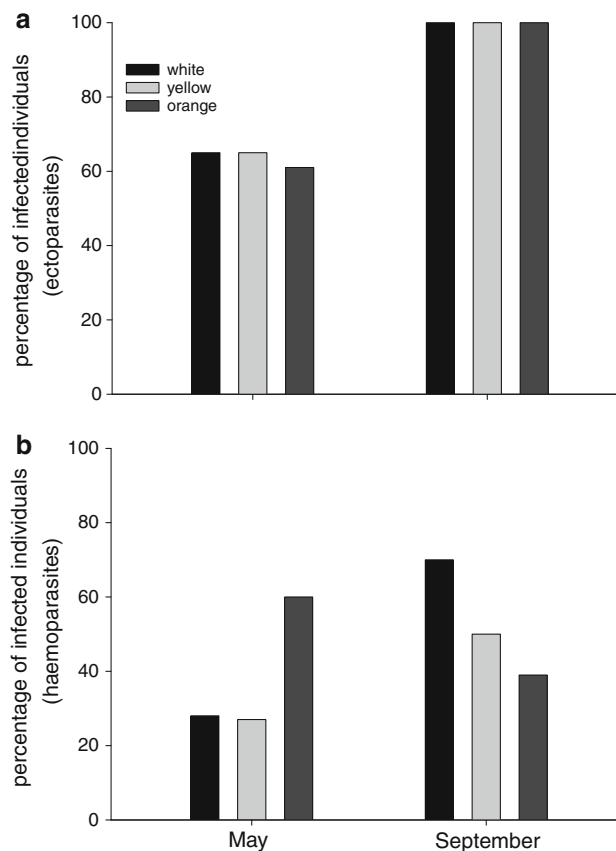


Fig. 2 Mean and standard error of number of parasites for the different morphs early (May) and late (September) in the reproductive season. **a** Ectoparasites: more severe infection in September, no differences among morphs **b** Haemogregarines: more severe infection in May, a higher number of haemogregarines was found in white males

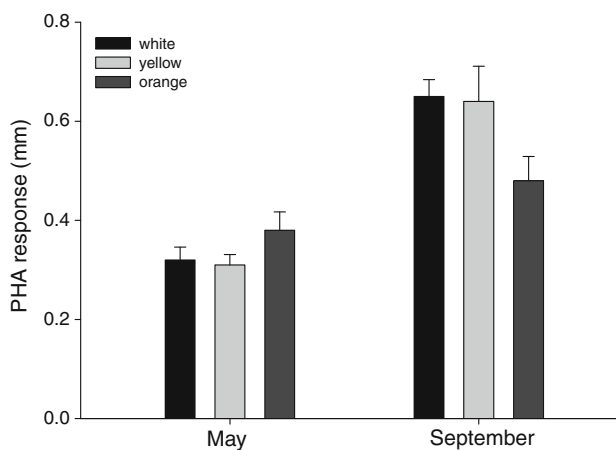
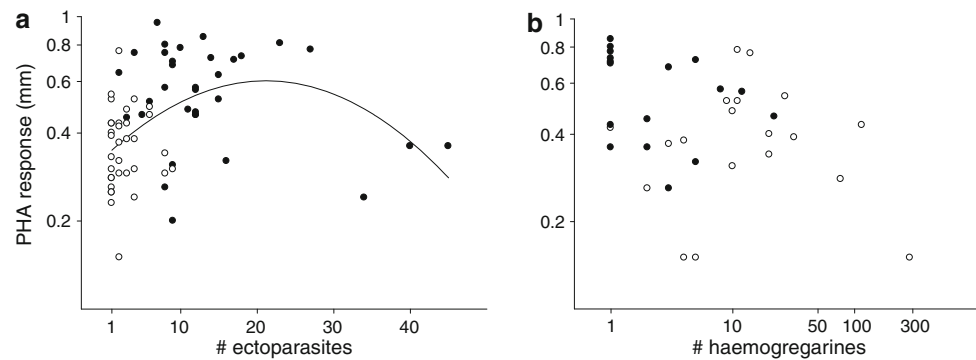


Fig. 3 Mean and standard errors of the response to PHA injection, corrected for injection with PBS (in mm) for the different morphs early (May) and late (September) in the reproductive season. White and yellow males showed an increased response later in the season, while the immune response of orange males remained the same

Fig. 4 Relationship between the response to a PHA injection (in mm), corrected for injection with PBS, and the number of parasites. *Open circles* early season (May) and *closed circles* late season (September). **a** Ectoparasites. **b** Haemogregarines in blood



number of ectoparasites increases, the capacity to respond to a PHA injection appears to drop again (Fig. 4a: quadratic fit line, $r^2 = 0.24$ vs. linear fit line, $r^2 = 0.087$). A quadratic regression produced a better fit than the linear regression (extra sum-of-squares F test, $F_{1,83} = 24.02$, $P < 0.0001$). This correlation did not differ among morphs across the season (three-way interaction colour \times season \times PHA, dependent = # ectoparasites not significant: $\chi^2 = 0.11$, $P = 0.95$).

There was a marginally significant relationship between infestation by ectoparasites and haemogregarines (Hierarchical loglinear analysis: $\chi^2 = 3.96$, $P = 0.06$).

The presence or absence of haemogregarines in the blood could be predicted by mass ($B = -16.11$, $\chi^2 = 4.52$, $P = 0.03$), but not SVL or PHA (both $\chi^2 < 3.66$, both $P > 0.06$); a significant model was generated in a logistic regression analysis (model $\chi^2 = 8.36$, $P = 0.04$), showing that individuals with a low mass have a higher probability of carrying haemogregarines in their blood. The number of haemogregarines was not correlated with SVL ($\sigma = -0.15$, $P = 0.33$), mass ($\sigma = -0.14$, $P = 0.34$) or PHA response ($\sigma = -0.25$, $P = 0.14$, Fig. 4b).

There was no correlation between body condition and PHA response ($r = 0.037$, $P = 0.74$).

Discussion

At the onset of this study, we hypothesized that male *P. melisellensis* morphs might differ in their ability to withstand challenges of their immune system, and that this difference may become apparent near the end of the reproductive season. We found that seasonal changes in different aspects of the immune function were not similar for all morphs.

All morphs showed an increased ectoparasite prevalence (proportion of individuals infected) and infection rate (the number of ectoparasites) as the season progressed (Figs. 1a and 2a). The finding that ectoparasite load was equal for the different morphs is in line with earlier findings that

morphs do not show different activity patterns (Huyghe et al. 2007). Susceptibility to ectoparasite infection is partly related to activity level in several species (e.g. Bouma et al. 2007). Morphs may show similar so-called avoidance behaviour, a non-specific immune defence technique where infection with pathogens is ‘avoided’. Parasite load increased for all morphs during the breeding season, which can be related to an increased presence of parasites and/or increased activity towards the end of the season, and thus a higher probability of becoming infected. Alternatively, the increase in parasite load could be an indirect effect of e.g. decreased survival of uninfected individuals.

The number of individuals with haemogregarines in their blood was lower at the end of the season than at the beginning for orange, but higher for white and yellow males (Fig. 1b). Within infected lizards, the number of haemogregarines found in the blood was higher at the beginning of the season, and white males were always infected to a greater extent (Fig. 2b). The finding that fewer orange individuals suffered from haemoparasite infection in September could indicate several things. In the lizard *Lacerta vivipara* for example, haemoparasite load was positively correlated with reproductive effort (Sorci et al. 1996), and two different pathways were suggested that could lead to this relation. When haemoparasites have a severe impact on the host’s survival, a selective benefit should exist when maximizing investment in current reproduction. Alternatively, a reduced anti-parasite defence could be the consequence of higher reproductive investment. Reproductive investment is lower near the end of the breeding season, explaining the lower levels of haemoparasite load (number of haemogregarines per lizard) within infected individuals. *P. melisellensis* morphs could also differ in timing of their reproductive effort and consequently in parasite infestation (number of individuals infected) and parasite load (number of parasites). On the other hand, lizards become infected with blood parasites by taking up the vectors for haemogregarines as food items (i.e. ticks and mites) (Smallridge and Bull 1999), and as the numbers of these vectors increase in the environment as the

season progresses, we would expect a concordant increase in haemoparasite infection (number of parasites per infected individual). Possibly, the infected individuals with high haemoparasite loads did not survive and were, therefore, not found in September.

A correlation between tail regeneration and blood parasitism was found in *Lacerta vivipara* (Oppliger and Clobert 1997): tailless infected lizards had a slower rate of tail regeneration than parasite-free lizards. The period of tail regeneration is crucial for lizards, as predation may increase, and the longer the time required for regeneration, the greater the risk of predation and other costs associated with tail loss. In the present study, we found no differences in tail break frequency among morphs and between times in the reproductive season. The variation in blood parasite infestation and load were thus not accompanied with variation in tail break frequencies. Although we have no data on tail regeneration itself in *P. melisellensis*, our data suggest do not support the correlation between parasites and tail regeneration.

The elevated PHA-response of orange males at the beginning of the season (Fig. 3) may partly be a consequence of a higher incidence of injuries (acquired immunity). Orange males are more aggressive than the others and, therefore, may be more involved in competitive interactions, possibly resulting in injuries (Huyghe et al. 2007), especially during the breeding season. During these interactions, males tend to bite each other fiercely on the head and neck region. The ability to mount a cell-mediated immune response to a mitogenic stimulus such as PHA may have important fitness consequences, because it constitutes a generalized short-term response to allergens and wounds (Zuk and Johnsen 1998). Therefore, individuals with a higher response have an advantage and a higher survival probability (Gonzalez et al. 1999). Differences in PHA response have been reported for other polymorphic species, such as *Podarcis muralis*, where yellow morphs have a lower response (Sacchi et al. 2007), and the female morphs of *Anolis sagrei* (Calsbeek et al. 2008). White and yellow male *P. melisellensis* showed an increased PHA response near the end of the breeding season, which could mean that they, as the season progresses, become increasingly involved in aggressive interactions. In this case, morphs would ‘divide’ the breeding season, with orange males being more aggressive and more successful in the beginning, and the other morphs at the end. Currently no information is available on the possible fluctuations in aggressive behaviour, male-male competition, and success during the breeding season. We only know that, early in the season, orange males are more aggressive and have a higher probability of winning inter-male competitive events (Huyghe et al. unpublished data).

In *Podarcis muralis* lizards, the immune response to PHA-injection was negatively correlated with the intensity of haemogregarine infection (Amo et al. 2005), but this relation was not found in *P. melisellensis*. In birds, PHA swelling is traded off with other physiological functions, indicated by the fact that PHA swellings are weaker when concurrent with other costly activities (Martin et al. 2006). The amount of swelling in response to a PHA injection showed in *P. melisellensis* however a quadratic relation with ectoparasite load (Fig. 4a): individuals carrying more ectoparasites seemed to have a higher PHA response, but when ectoparasite infection becomes more severe, the capacity to respond to a PHA injection seems to drop again. Individuals which were not carrying any ectoparasite had a lower PHA response than the ones which were. Seemingly, infection by ectoparasites is related to the ability to mount a cellular immune response to a mitogen injection, but in an unexpected way. PHA swelling in our study could perhaps be an indication for high quality individuals which are able to survive high levels of ectoparasite infection and which are capable of responding more effectively to PHA, compared to individuals with lower ectoparasite loads and lower swelling responses. Alternatively, there may be a priming effect, in which those with high loads of ectoparasites may already be primed for immunological responses (PHA swelling) because their immune system is constantly challenged, and those with decreased ectoparasite loads may exhibit a lowered swelling response because their immune systems have not been primed in the same way. When ectoparasite load was very high, PHA responses dropped again, indicating a threshold beyond which animals cannot bear the costs of severe parasite infection and mounting a swelling response.

Our study provides two strong indications of alternative life-history strategies among different colour morphs of the lizard species *Podarcis melisellensis*. Firstly, we found a differential haemogregarine parasitemia infection, with white males having more severe infections than males of other colours. Secondly, the response to PHA injection differed among morphs and seasons, with yellow and white males showing an increase in response as the season progressed. It remains unclear whether these differences are caused by immunological factors, tolerance or behavioural differences. Furthermore, the results of our study indicate an up-regulation of the immune function at the end of the breeding season, when lower numbers of haemogregarines were found and the PHA response increased in two morphs. This may be an important strategy to survive stressful conditions during winter. As this phenomenon is poorly studied in reptile species, more research is necessary to gain insights in this mechanism and its consequences on survival.

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