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Immune responses vary with parasite burden in an insular lizard

Mario Garrido and Valentín Pérez-Mellado

Department of Animal Biology, University of Salamanca, Campus Unamuno, Salamanca, Spain

ABSTRACT

Background: Immunological studies are often context-independent. However, an alternative is emerging in the form of eco-immunology, which focuses on the natural variation in immune functions of free-living organisms in relation to their ecological constraints and evolutionary context. Immunological research also tends to study only parasite resistance – that is, mechanisms by which hosts prevent infection or reduce parasite growth. But eco-immunology addresses tolerance as well as resistance. Through tolerance, hosts defend themselves by minimizing the damage caused by parasites instead of reducing parasite infection.

Goal: To determine the immune responses of free-living lizards that have varying parasite loads and body conditions.

Organism: A wild, insular population of *Podarcis lilfordi*.

Method: Immune response was measured as the bactericidal strength of plasma, and the immune response was examined with respect to parasite load and to body condition.

Results: Immune responses were affected by an interaction between parasite load and body condition. In lightly infected lizards, the immune response was positively correlated with body condition. But in heavily infected lizards, this correlation was not evident. Individuals in the population studied seem to exhibit different strategies of parasite defence. Some appear to resist parasite infection, whereas others rely on tolerance.

Keywords: bacterial killing assay, haemogregarine, immune response, immunological strategies, insularity, parasitic infestation, resistance, tolerance.

INTRODUCTION

Predators and pathogens can exert a major impact on the fitness of organisms (Caro, 2005; Schmid-Hempel, 2011), even reducing it to zero, so hosts have developed a set of anti-parasite strategies. Among them, the immune system is the most sophisticated, and the biological fitness of individuals is critically dependent on it (Wakelin and Apanius, 1997; Schulenburg *et al.*, 2009). As a result of this, there is strong selection favouring the evolution of the immune system and other anti-parasitic strategies. However, the maintenance of effective immune defences as well as mounting an immune response are costly and compete with other energetically

Correspondence: M. Garrido, Department of Animal Biology, University of Salamanca, Campus Unamuno, 37007 Salamanca, Spain. e-mail: gaiarrido@usal.es

demanding functions such as growth and reproduction (reviewed in Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000). The activity of the immune system is also costly in specific resources such as proteins and amino acids (Schmid-Hempel, 2011).

In recent decades, the field of ecological immunology has addressed the study of immune function in an evolutionary and ecological context (Sheldon and Verhulst, 1996). The main eco-immunological premise is that host defences should be employed or evolved only when costs are acceptable and are outweighed by benefits (Baucom and De Roode, 2011). If ecological demands are great, or if fitness can be maximized via growth or reproduction, immune defences may be compromised, lowered or altered (Martin *et al.*, 2011). In this sense, hosts are able to fight parasites using two strategies: (1) *tolerance* decreases or alleviates fitness reduction owing to parasite infection, but without reducing parasite infection or growth (Schulenburg *et al.*, 2009; Baucom and De Roode, 2011); (2) *resistance* prevents infection or reduces parasite growth by confining parasite spread or investing in immune components that sequester parasites or protect and repair host tissues (Rynkiewicz *et al.*, 2013). The two strategies are not necessarily mutually exclusive within an individual, and, at certain burden thresholds, individuals may switch from a resistance strategy to one of tolerance (Schmid-Hempel, 2011). Resistant hosts may be as fit as tolerant ones but have low parasite loads (Råberg *et al.*, 2007; Baucom and de Roode, 2011).

Here, parasitic infection and immune response were studied in the insular lizard *Podarcis lilfordi* on Aire Island (Balearic Islands, Spain). On Aire, scarce resources are the norm, like other insular ecosystems of the Mediterranean basin (Pérez-Mellado and Corti, 1993), and rates of parasitism by haemogregarines and mites are among the highest to be reported in Mediterranean lizards (Garrido and Pérez-Mellado, 2013a, 2013b). As immune responses are costly, we expect hosts to optimize their fitness in a way that minimizes the combined cost of parasite damage itself and of mounting a parasite defence. In this sense, we expect an internal trade-off where investment in immune defence is to compromise other physiological demands.

MATERIALS AND METHODS

Study area and species

Podarcis lilfordi (Günther 1874) is a medium-sized lacertid lizard, with a snout-to-vent length (SVL) of up to 81 mm in males and 75 mm in females (Pérez-Mellado, 1998). The present study was conducted from 16 to 23 June 2011 on Aire Island, a small islet off Menorca. Aire is a typical Mediterranean coastal islet, its vegetation highly influenced by its environmental characteristics, strong winds, high salinity, and long periods of drought. As on other Mediterranean islands, food resources are scarce (Pérez-Mellado and Corti, 1993). Lizard densities can be very high, more than 4000 individuals per hectare (Pérez-Mellado *et al.*, 2008).

Adult male lizards ($n = 25$) were captured by noose on sunny days, between 08.00 and 12.00 GMT, when lizards were most active. For each individual, we recorded SVL and body weight.

Body condition has been broadly used as an estimate of foraging success, and ultimately biological fitness (Jakob *et al.*, 1996). In this study, body condition was estimated from the residuals of the regression of natural log-transformed body weight on natural log-transformed SVL. Using a monocle 5× magnification, we counted the number of ecto-parasites on the body surface of lizards immediately after capture. To obtain blood samples,

again immediately after capture, we made a slight longitudinal cut in the dorsal and proximal side of the tail with a sterile scalpel. Blood samples for immune analysis (see below) were collected in a heparinized microcapillary tube. Then, we conducted a blood smear to estimate blood parasite load. Blood smears were placed on microscope slides and air dried in the field. Specimens were always released at the site of capture. Once in the laboratory, slides were fixed with 100% methanol for 10 min and then stained with modified Giemsa for 20 min. Samples were analysed using an optical microscope 400× magnification. Similar to previous studies, the only blood parasites identified were haemogregarines (Garrido and Pérez-Mellado, 2013a, 2013b). Haemogregarines were assigned to the genus *Hepatozoon* (A. Marzal, unpublished data and personal communication). All ectoparasites observed were assigned to the family Trombiculidae [larvae of chigger mites (M. Moraza, personal communication)]. The intensity of parasitism was estimated based on a total of 2000 counted cells per sample. Prevalence was estimated as the percentage of infected individuals.

Blood samples for immune response assays were stored on ice until centrifugation at 3600 rpm for 12 min, and plasma was separated within 8 hours of collection. We obtained approximately 30 μL of plasma from each lizard, which was kept frozen at −20°C until immune assays were conducted within the next 24 hours.

Immune assay

Based on the review of Demas *et al.* (2011), the immune response of individuals was assessed by bacterial killing assay (BKA). This assay, which characterizes a functionally relevant immune response that involves the action of phagocytes, opsonizing proteins, and natural antibodies (French *et al.*, 2010), contributes to an innate immune response to intracellular pathogens (Plüddemann *et al.*, 2011), previously used to study ectoparasite infestation (Rynkiewicz *et al.*, 2013). Bacterial killing assay evaluates the ability to remove an actual pathogen and provides a functionally relevant evaluation of host innate immune function by injecting relatively artificial antigens or mitogens (Demas *et al.*, 2011). This assay provides a more functional assessment of immune function than assays of isolated immune components or monitoring techniques, which can vary quantitatively (Norris and Evans, 2000; Demas *et al.*, 2011). For these reasons, we consider BKA a suitable method to investigate how parasitic infections are related using immunological strategies.

Bacterial killing assay is used to measure a functional response by an animal's innate immune system against a relevant pathogen, *Escherichia coli* (French *et al.*, 2010; Ruiz *et al.*, 2011). Immune response was determined following a similar protocol to that developed by Ruiz *et al.* (2010, 2011). We prepared a bacterial stock solution by diluting 2×10^{-6} times a saturated concentration of *E. coli* in a CO_2-independent media with 2.34 mg of L-glutamine (Sigma-Aldrich, St. Louis, MO). Based on pilot trials (unpublished results), this concentration was considered the best for obtaining quantifiable positive controls (avoiding bacterial background lawn) but with enough colonies to estimate killing capabilities of plasma. Then, we diluted plasma samples in 200 μL of the working bacterial solution. These plasma/bacteria cocktails were incubated at 37°C for 30 min. Subsequently, we plated each sample on agar plates, including two positive controls (with only bacteria) and a negative control (without bacteria). Plates were incubated overnight at 37°C to allow colony growth. We then counted the number of colonies on each plate and compared them with the positive control plates in order to calculate the percentage of bacteria killed by each lizard plasma sample [percentage of killed bacteria = 100 – (number of colonies on sample plate/average number of colonies

on positive control plates) × 100]. No colonies were formed on negative control plates. We discarded plates on which we detected contamination by other bacterial colonies. In this way, we were able to calculate the *ex vivo* bacterial killing capability of collected plasma exposed to *E. coli*.

Statistical analyses

The statistical analyses were performed using R v.2.12.1 (R Development Core Team, 2010). We carried out a multiple linear regression analysis (Quinn and Keough, 2002) to determine which variables were related to immune response. Immune response was square-root transformed and both haemogregarine and mite load values were natural-log transformed to meet the assumption of normality. In accordance with previous studies, the ecological relationships between parasitism and body condition were found to be complex on Aire Island, and we obtained no evidence to support a correlation between variables in the population under study (Garrido and Pérez-Mellado, 2013a, 2013b, 2014). In the present study, no collinearity was observed among the explanatory variables. The variance inflation factor (VIF) was lower than 3 (Zuur *et al.*, 2010) for all variables: haemogregarine load = 1.15; mite load = 1.15; body condition = 1.16. Thus, none of the variables was discarded.

We fitted an additive model to consider the effects of all independent variables and then, using forward selection, we added the variables with the largest *F*-value (Quinn and Keough, 2002). Next, we fitted a multiplicative model including interaction terms of the variables retained in the additive model. To select the best model, we considered at each step the highest adjusted r^2 along with the lowest AIC (Akaike's Information Criterion) and BIC (Bayesian Information Criterion) values (Quinn and Keough, 2002). For significant continuous predictors included in the final model, effect sizes were reported as partial correlation coefficients (r) (Nakagawa and Cuthill, 2007).

Frequently in multiple regression models with several continuous variables, the effects of the predictors are considered additive. Although additivity is appropriate in many cases, there are times when it does not apply (Dallal, 2001). Sometimes, the purpose of a study is to formally test whether additivity holds. Perhaps the way a response variable varies with one predictor depends on another predictor. One way to investigate this is by including an interaction term in the model (Dallal, 2001). In the present study, interaction terms between continuous variables were interpreted following the method of Aiken and West (1991) and Cohen *et al.* (2003). This is a specific statistical technique to interpret results in order to obtain an understanding of the real effects of the variables under study. The purpose is to determine the values of one continuous predictor for which another continuous predictor has an effect on the response variable (Aiken and West, 1991; Cohen *et al.*, 2003).

RESULTS

Following the forward selection procedure, we began with the null model and introduced, at each step, the predictor with the highest partial *F*-value (Quinn and Keough, 2002). Hence, we first added ectoparasite load ($F = 13.51$), followed by haemogregarine load ($F = 0.27$), and then body condition ($F = 0.02$). Up to this point, having included each predictor, the AIC and BIC values were reduced and the value of adjusted r^2 was increased (Table 1). The multiplicative model including all possible interactions between previously introduced variables (mite and haemogregarine loads and body condition) indicated poorer values for

Table 1. Adjusted r^2, AIC (Akaike's Information Criterion), and BIC (Bayesian Information Criterion) values at different stages of the forward selection procedure (Quinn and Keough, 2002) to select the minimal adequate model of immune response (BKA) in an insular population of the Balearic lizard, *Podarcis lilfordi*

	Adjusted r^2	AIC	BIC
Null model		132.34	134.78
Mite load	0.34	122.79	126.44
Mite load + Haemogregarine load	0.35	112.76	117.31
Mite load + Haemogregarine load + Body condition	0.39	104.08	109.30
Maximal model	0.42	105.41	114.81
Mite load + Haemogregarine load + Body condition + Haemogregarine load*Body condition	0.51	100.23	106.49

Note: Immune response was square-root transformed and mite and haemogregarine load values were log-transformed to meet the assumption of normality.

Table 2. Results of the multiple regression (minimal adequate model) for innate immune response, estimated by bacterial killing capability of blood plasma, in adult *Podarcis lilfordi* males on Aire Island

	d.f.	SS	MS	F	Pr(>F)
Mites	1	99.05	99.05	19.30	<0.0001*
Haemogregarines	1	0.86	0.86	0.17	0.687
Body condition	1	2.08	2.08	0.40	0.533
Haemogregarines*Body condition	1	26.40	26.40	5.14	0.038*
Residuals	16	82.10	82.10		

Note: Immune response was squared-root transformed and mite and haemogregarine load values were log-transformed to meet the assumption of normality.

the three estimators (adjusted $r^2 = 0.42$, AIC = 105.41, BIC = 114.81) and, consequently, this model was discarded (Quinn and Keough, 2002). Only by including the interaction between haemogregarine load and body condition was the additive model improved (Table 1). The remaining interactions showed a P-value above 0.65 (all $F < 0.35$).

The minimal adequate model ($F_{4,16} = 6.26$, $P < 0.005$; Table 2) revealed that immune response increased with diminished mite load (slope = −1.49; Fig. 1) but was not related to blood parasite load or body condition. However, both were retained in the model (Table 2). Finally, the interaction of log-transformed values of haemogregarine load and body condition was significantly related to the immune response of hosts (Table 2). This interaction showed a medium-sized effect ($r = 0.49$), with mite load showing a large effect ($r = 0.73$).

Since it was involved in an interaction term of the GLM model, and given constant values of the remaining variables, the relation of body condition and immune response was different for different values of haemogregarine load (Dallal, 2001; Sweet and Martin, 2011). We analysed the interaction between continuous variables (condition and blood parasite load) following Aiken and West (1991) and Cohen *et al.* (2003). To generate simple regression

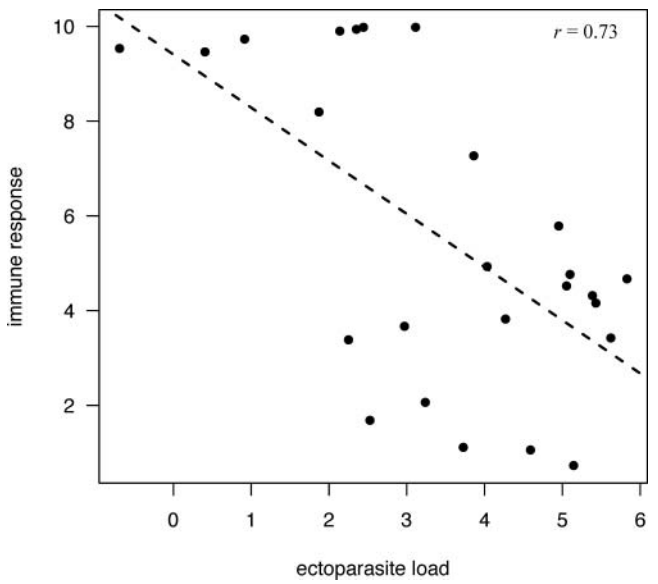

Fig. 1. Relationship between bacterial killing capability of plasma and ectoparasite load in the Balearic lizard, *Podarcis lilfordi*, on Aire Island (Menorca, Spain). Immune response is square-root transformed and mite load values are natural log-transformed to meet the assumption of normality. Effect sizes are reported as partial correlation coefficients (r) (Nakagawa and Cuthill, 2007).

Table 3. Results of t-tests for the slopes of the simple regression equations (see Aiken and West, 1991) calculated for the relation between body condition and immune response at different values of haemogregarine load

	Simple slope	Standard error	t-test	d.f.	P-value
HL_H	$b_H = -3.95$	$s_H = 5.19$	$t = -3.95/5.19 = -0.76$	16	$P = 0.23$
HL_M	$b_M = 1.01$	$s_M = 4.35$	$t = 1.01/4.35 = 0.23$	16	$P = 0.41$
HL_L	$b_L = 15.56$	$s_L = 7.08$	$t = 15.56/7.08 = 2.20$	16	$P = 0.02$*

Note: medium (HL_M), high (HL_H), and low (HL_L) haemogregarine load.

equations recommended by these authors, we chose three different values of haemogregarine load (HL): the median (i.e. the value that divides the sample into two equal parts), one standard error above the median, and one standard error below the median. We refer to these as the medium (HL_M), high (HL_H), and low (HL_L) haemogregarine load respectively; as the median was 12 and the standard error 10, these parasite load values were $HL_M = 12$, $HL_H = 22$, and $HL_L = 2$. Since we divided sample size based on the median value, we can consider lizards with more than 12 haemogregarines per 2000 cells to be 'highly infected', and lizards with fewer than 12 haemogregarines per 2000 cells to be lightly infected.

After the generation of simple regression equations for HL_M, HL_H, and HL_L, we computed t-tests for the slopes (Table 3). The t-tests revealed that only for the HL_L simple regression was the slope statistically different from zero. Hence, we can conclude that body condition was positively correlated with immune response in 'lightly infected' individuals

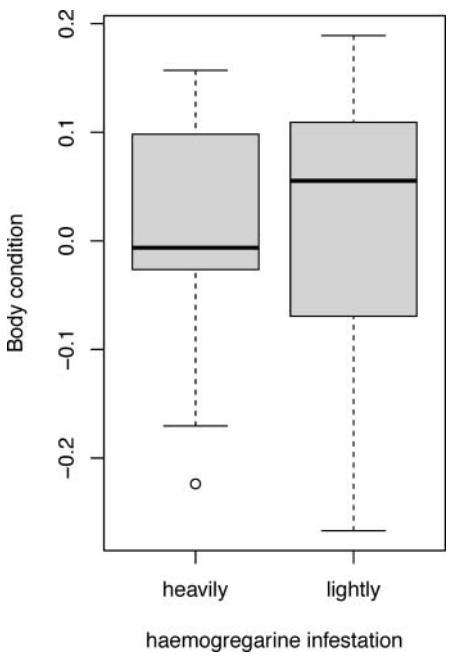

Fig. 2. Median body condition (± interquartile range) of *Podarcis lilfordi* did not differ significantly ($P = 0.74$) between lightly and heavily haemogregarine-infected individuals on Aire Island (Menorca, Spain).

only. No relationship was apparent for highly infected lizards. Furthermore, body condition was similar between lightly and highly infected individuals (Fligner test for comparison of variances: $\chi^2 = 0.18$, d.f. = 1, $P = 0.67$; one-way ANOVA, $F_{1,19} = 0.11$, $P = 0.74$; Fig. 2).

DISCUSSION

The results of the bacterial killing assay and parasitic infections partially support our predictions for the existence of both immunological strategies – tolerance and resistance – in Aire Island lizards. As in previous studies, infestation by mites showed a negative correlation with immune response (Cooper *et al.*, 1985; Sorci, 1995; Smallridge and Bull, 2000; Madsen *et al.*, 2005). In contrast, the effects of haemogregarine infestation on immune response differed dramatically from those of mites. The significant interaction term revealed that immune response varies with body condition depending on haemogregarine load. Ruiz *et al.* (2010), in an experimental manipulation study, observed a context-dependent response of testosterone on the relation between food intake (related to condition) and bacteria killing capability. Food supplementation increased immune response and plasma testosterone levels. Furthermore, testosterone treatment enhanced immune response in food-supplemented animals (Ruiz *et al.*, 2010). Similarly, our results revealed that individuals in better condition had a better immune response, but only among lightly parasitized lizards.

Because resource availability and pathogens fluctuate over time and space (Nelson *et al.*, 2002) and because immune defence is costly (see Martin *et al.*, 2008 and references therein), animals must adaptively allocate resources among physiological systems to maximize fitness (Stearns, 1992;

Ricklefs and Wikelski, 2002). As a result, individuals within a population may exhibit different immunological strategies: some individuals will prioritize the immune system ahead of other physiological requirements, while other individuals will first satisfy other demands. In the former, there is a greater immunological effort to remove parasites (resistance strategy). Those lizards prioritize immune defence and should mount a better anti-parasite response. In the present study, using the methodology of Aiken and West (1991) and Cohen *et al.* (2003), we revealed that among lightly infected lizards (individuals showing a parasite load below the median value), body condition was positively correlated with immune response, suggesting that such individuals opt for 'resistance' (remove parasites). Lifjeld *et al.* (2002) established that immune response is condition-dependent in that animals in better condition or having a greater endogenous energy/resource reserve, typically maintain better immune responses than those in poor body condition. In addition, loss of body condition would mean a reduction in resources for defence against parasitic infection, thus reducing the ability of lizards to develop an immune response to infection (e.g. Cooper *et al.*, 1985; Smallridge and Bull, 2000). Hence, among lightly infected individuals in the present study body condition was positively related to immune response; those with better condition showed a stronger immune response and a lower parasite load. Amo *et al.* (2007) reported that in a population of *P. muralis* from Spain, lizards with poorer body condition had lower cell-mediated immune responses and a higher blood parasite load.

In contrast, among heavily infested lizards we found no relationship between immune response and body condition. Since a good immune defence is costly and host resources are limited, there is a trade-off between the immune response and other physiological demands, such as growth or reproduction (Sheldon and Verhulst, 1996; Møller *et al.*, 1999). That is, some individuals opt not to prioritize the immune system. As an alternative strategy, they give priority to other physiological requirements and may tolerate a higher parasite burden (tolerance strategy). In these lizards, body condition was not related with immune response. Tolerance can only be adopted in populations where the negative consequences are not too costly – that is, where such a strategy is effective in terms of fitness. The prevailing environmental conditions in some Mediterranean populations, as on Aire Island, may favour the adoption of tolerance as an immune strategy. In a general context, predators mainly capture the sickest and most parasitized prey individuals (Navarro *et al.*, 2004; Genovart *et al.*, 2010). Thus, one of the major risks of not prioritizing so highly the immune response is the increased risk of predation. These individuals are therefore removed from the population and, consequently, are not detectable by researchers. In a population with low predation pressure, as on Aire Island (Cooper and Pérez-Mellado, 2012), this would not happen and the two strategies could co-exist. In addition, the comparison of body condition between individuals opting for tolerance and those opting for resistance also supports our hypothesis. We observed no differences in biological fitness, estimated as body condition (Jakob *et al.*, 1996), between individuals adopting one or other of these two strategies. Recently, Rynkiewicz *et al.* (2013) argued that resistant hosts have lower parasite loads than tolerant ones due to highly effective removal of parasites, but that resistant individuals may be as fit as tolerant individuals (see also Råberg *et al.*, 2007; Baucom and de Roode, 2011).

The co-existence of different ecological or physiological strategies is not new. It is in fact common in many other components of life-history traits of individuals within a population. For example, there are consistent differences in many behavioural traits, including boldness, exploration, sociability, and aggressiveness (Rodríguez-Prieto *et al.*, 2011), foraging tactics (Trillmich and Trillmich, 1986), and mating and reproductive strategies (Gross, 1996) among

individuals. The ecological and evolutionary consequences of such immunological variation must be considered. Plants would be either resistant or tolerant, but not at the same time (see Weinig, 2003 and references therein). Because of the costs associated with both strategies, plants may opt for one or other strategy depending on different environmental factors, creating temporally or spatially fluctuating selection regimes for resistance and tolerance, modifying the relative costs and benefits of resistance and tolerance (see Weinig, 2003 and references therein). In the same way, animals could change their strategy according to changes in biotic and abiotic conditions, or physiological requirements. It is important, however, that our results are interpreted with caution, since the combined influence of parasite load and body condition on immune response has yet to be studied thoroughly.

On the other hand, parasitic infestation by mites was negatively correlated with bactericidal capability of plasma. By applying the same approximation that we used for haemogregarines, we should expect that all individuals exhibit a resistance strategy against infestation by ectoparasites. Hence, immune response would depend directly on body condition (Lifjeld *et al.*, 2002) and the most parasitized individuals should exhibit a lower body condition. However, the lack of a correlation between mite load and body condition (see Materials and Methods) does not support this result. It could be that lizards with a strong immune response are directly suppressing ectoparasites. Alternatively, the immunosuppressive effect of ectoparasite saliva could be responsible for the direct correlation between immune response and ectoparasite load (Ribeiro *et al.*, 1985, 1990; Titus and Ribeiro, 1990; Moore, 2002), and not a host's decision regarding investment in the immune system, as might be expected from an eco-immunological perspective.

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