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Infection risk dictates immunological divergence among populations in a Mediterranean lizard

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Abstract

The ability of vertebrates to evolve different defense strategies in response to varying parasitism regimes remains poorly understood. Hosts may adopt two different strategies to defend themselves against parasites: tolerance (hosts alleviate the negative fitness consequences of parasite infection) and resistance (hosts strengthen their immune response as parasite burden increases). Both strategies are effective but fitness has been reported to decline faster in less tolerant individuals. Here, we assessed the number of splenocytes and the cell-mediated response (proxies for resistance) and body condition (a proxy for tolerance) in four populations of a Greek endemic lizard (*Podarcis gaigeae*), each exposed to different infection risk (defined as the cumulative effect of parasite burden and duration of exposure). We anticipated that populations with heavy parasite burden would enhance the efficacy of their immune response (resistance) compared to lizards deriving from parasite-poor habitats. We also predicted that populations with longer exposure to parasites would be adopted and be more tolerant. Each factor (duration of exposure and parasite burden) had a distinct effect on the immune response and thus, our results where rather complicated. Lizards with heavy parasite burden and aperiodic exposure demonstrated resistance, whereas lizards with heavy parasite burden and chronic exposure were more tolerant. Populations with low parasite burden and minimal exposure were more resistant. Our results suggest that the development of some immunological strategies may be differentiated under different infection risks, even within the same species.

Keywords: Cell-mediated response, Islands, Mixed Lymphocyte Reaction, Reptiles, Immune Resistance, Immune Tolerance

Introduction

In natural populations, parasites are considered as powerful means guiding selection, as they influence hosts' overall fitness and alter the composition of species in ecological communities (Altizer et al., 2003, Beldomenico et al., 2008). To compensate the negative effects of parasites, vertebrates have evolved an efficient immune system that includes a plethora of non-specific and specific mechanisms to fend parasites off (Altizer et al., 2003, Elgert, 1996).

Parasites deprive energy and nutrients from their host, induce detrimental effects on host fitness and modulate host density (Wikelski et al., 2004, Bize et al., 2010). To confront parasites' infestation, hosts can employ the two main, complementary components of immune defense response: resistance (i.e., hosts strengthen their immunological response as parasite burden increases; the lower infection intensity the higher the resistance) and tolerance (i.e., hosts alleviate the reduction in fitness due to parasite infection, without reducing parasite infection) (Råberg et al., 2009, Baucom & de Roode, 2011). Resistance, though effective and direct, is energetically costly and may affect numerous life-history traits of the host (Lochmiller & Deerenberg, 2000, Martin et al., 2006). On the contrary, tolerance may not protect the host against parasites as effectively, but induces no collateral damages (Schneider & Ayres, 2008, Råberg et al., 2009). Hence, hosts that are capable to fight parasites are not always the healthiest ones and, conversely, hosts with high parasite burden might retain their good condition (Bize et al., 2008). The defense strategy and the magnitude of the immune response that hosts will adopt are affected by infection risk and parasite prevalence (Lindström et al., 2004, Schmid-Hempel, 2003, Bordes et al., 2012). Resistance and

tolerance are equally used by animals (Råberg et al., 2009, Sorci, 2013). However the majority of ecological studies dealing with wild populations tend to directly examine the extend of immune response (resistance) and neglect the impact of parasite burden on body condition and other fitness traits (assessed by tolerance) (e.g. Amo et al., 2006, Kalbe & Kurtz, 2006, Lindström et al., 2004, Lee et al., 2006, Schneider & Ayres, 2008, Svensson & Råberg, 2010).

In Mediterranean ecosystems periodic disturbances by traditional land-use practices, such as livestock grazing, limit landscape diversity and create nutrient-poor sites (Fleischner, 2002). On top of that, livestock transfer mites and ticks that, besides their direct effects, such as blood removal and decrease of reproductive performance (Chilton & Bull, 1993, Vaclav et al., 2007), are potential vectors of blood parasites (Fleischner, 1994, Telford, 2008). Such problems further deteriorate in insular taxa, which are more susceptible to parasites that decrease hosts' body condition and health status (Beadell et al., 2007, Huyghe et al., 2010, Garrido & Pérez-Mellado, 2013).

The immune response of vertebrates may be affected by the parasitism regime in a certain habitat (Bordes et al., 2012, Lindström et al., 2004, Rynkiewicz et al., 2013). Here, we investigated the role of infection risk (defined as the cumulative effect of parasite burden and duration of exposure to parasites) in driving immunological variation using as model organism the Skyros wall lizard (Skyros Archipelago, Greece). We tested four populations that are subjected to different grazing regimes (none, aperiodic or chronic; Fig. 1) and experience different levels of parasitism (Pafilis et al., 2013). We assessed the T cell-mediated response and the number of splenocytes (proxies for resistance) and body condition (a proxy for tolerance) and assessed them in accordance to parasite burden. We hypothesized that heavily parasitized lizards from the grazed biotopes would invest more in active immune defense (resistance) than lizards with low parasite burden. We also anticipated that lizards that are exposed for longer periods to parasites would be more tolerant due to long-term host-parasite interactions and the high energetic cost of resistance (Lindström et al., 2004, Rynkiewicz et al., 2013, Schmid-Hempel & Ebert, 2003).

Materials and Methods

Study system

The Skyros wall lizard (*Podarcis gaigeae*) is an insectivorous, small-bodied (snout-to-vent length - SVL: 40-73 mm), lacertid lizard that lives on Skyros Island and on 21 surrounding islets [Central Aegean Sea, Greece; (Valakos *et al.*, 2008)]. In this study we used exclusively adult male lizards to eliminate any age- or sex-related effects on immunity (Saad & Deeb, 1990, Smith & John-Alder, 1999). Sampling was conducted in accordance with the Greek National Legislation (Presidential Decree 67/81). Lizards (36 in total) were collected in spring 2011 from the islets Diavates (N 38°47'20, E 24°30'46; 9 individuals; no grazing) and Lakonissi (N 38°50'56, E 24°28'30; 9 individuals; aperiodic grazing), as well as from two different locations of Skyros Island, Palamari (N 38°57'40, E 24°30'32; 9 individuals; no grazing) and Nyfi (N 38°49'22, E 24°34'02; 9 individuals; chronic grazing) (Fig. 1). All individuals were transferred to the animal facilities of the Faculty of Biology at the University of Athens. Lizards were housed individually in vitreous terraria (20 × 25 × 15 cm) under a controlled photoperiod (12 h light: 12 h dark), had access to water *ad libitum* and were fed once every other day with mealworms (*Tenebrio molitor*) coated with mineral powder (TerraVit Powder, JBL GmbH & Co. KG). For each lizard we recorded body length (SVL) and body mass using a digital caliper (Silverline 380244, accurate to 0.01 mm) and a digital scale (Ohaus, Scout-TM, accurate to 0.01g), respectively. Additionally, we estimated body condition as the residuals of the linear regression of $\log_{10}$-transformed weight against $\log_{10}$-transformed SVL (proxy for energy reserves; Băncilă et al., 2010, Labocha et al., 2014).

Parasite prevalence and infestation levels

A blood sample from each lizard was obtained by clipping off the tail tip. Blood smears were prepared on microscope slides to examine the presence of intra-erythrocytic parasites. Smears were air dried, fixed in absolute methanol for 10 min and stained in Giemsa (Sigma-Aldrich) diluted 1:9

(v/v) with phosphate buffer (pH 7.2) for 20 min. Samples were analyzed using an optical microscope at 100×. The haemoparasites identified were haemogregarines. The infestation levels for haemogregarines were estimated for each lizard as the percentage of infected cells on a total of 2000 red blood cells counted (hereafter 'haemogregarine infestation levels') (Amo *et al.*, 2007). Haemogregarine prevalence was estimated as the percentage of infected lizards (i.e., the relative frequency of infected individuals within the population). We also recorded the presence/absence of ticks (*Ixodes ricinus*) for each lizard captured in the field and estimated tick infestation levels (mean number of ticks per lizard) and tick prevalence (Pafilis *et al.*, 2013).

Preparation and storage of spleen cells

Due to the small body size of *Podarcis* species, withdrawal of sufficient quantity of peripheral blood cells was not possible and thus, we used spleen cells (Valakos *et al.*, 2007). Spleen cells were counted in a Neubauer chamber using Trypan blue and adjusted to 2×10^6 splenocytes/mL complete medium (CM) [consisting of RPMI-1640 (Gibco, NY, USA) supplemented with 1% fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Sigma-Aldrich), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), 5×10^{-5} M 2-mercaptoethanol and 1% penicillin–streptomycin (all from Gibco)]. Splenocytes were used either fresh or frozen. For cryopreservation, spleen cell suspensions were centrifuged at 1500 rpm for 5 min at 4°C; the cell pellet was diluted in 90% (v/v) FBS, 10% (v/v) dimethyl-sulfoxide (DMSO) and stored at -80°C. After thawing, splenocytes were examined for viability and proliferative response (see Supplementary Material 1).

Determination of T cell-mediated responses

Cell-mediated responses were assessed using one-way xenogeneic mixed lymphocyte reaction (MLR) that determines T-cell proliferation (Lightbody et al., 1971, Turka & Lechler, 2009). In one-way MLR, stimulating/donor cells are inactivated after treatment with mitomycin-C (mit-C; Bach and Voynow

1966). Mit-C arrests proliferation of the cells, but allows them to maintain their antigenicity (Tomasz et al., 1987). As a result, the proliferation measured is due to responder T-cell activation.

As responder cells, we used freshly thawn spleen cells from Diavates, Nyfi and Lakonissi lizards (for details see Supplementary Material 1) and freshly isolated splenocytes of Palamari lizards. Responder splenocytes were co-cultured with inactivated splenocytes (stimulators) of a donor *P. erhardii* lizard. For inactivation *P. erhardii* splenocytes were treated with 25 μg/mL mit-C (Kyowa, Japan; for experimental details see Supplementary Material 2). Mit-C was deactivated with the addition of FCS and stimulators were washed thrice in CM by centrifugation at 1500 rpm for 5 min at 25°C. Two × 10^5 responder splenocytes from each animal in 100 μL aliquots were mixed with an equal number of stimulators at a final volume of 200 μL/well in U-bottomed 96-well tissue culture plates (Costar, Cambridge, MA, USA). Unmixed (2×10^5 cells/well) responders (to quantify basal cell-proliferative responses of each animal) and stimulators were used as controls.

All cultures were set up in triplicates and incubated for five days at 37°C (Valakos et al., 2007). During the last 18 h of incubation, 50 μl of RPMI-1640 containing 1 μCi [^{3}H]-thymidine were added per well (Valakos *et al.*, 2007). Cultures were harvested in a semi-automated cell harvester (Skatron Inc., Tranby, Norway). The radioactivity incorporated into cellular DNA was determined by liquid scintillation counting. Data were expressed as counts per minute (cpm) and stimulation index (S.I.) was calculated according to the equation:

$$\text{S. I.} = \frac{cpm \text{ of co} - \text{cultured spleen cells}}{sum\ of\ cpm \text{ of unmixed responder and stimulator spleen cells}}$$

Statistical approach

Cell-mediated responses were expressed as S.I. values. We first examined the heteroscedasticity and normality of the data. Whenever parametric assumptions were not met, data were transformed. If transformations were unsuccessful, non-parametric tests were performed, otherwise we used parametric tests. To assess the differences in body length, body mass, body condition, S.I. values and number of cells per spleen, we used one-way ANOVA, coupled with post-hoc Tukey *HSD* test. As fixed effect, we used the locality of individuals. To reduce the within-group error in cell-mediated response caused by the effect of body size (Amo et al., 2006, Sacchi et al., 2007, Meylan et al., 2013), we repeated the latter analysis using the residuals of S.I. values against SVL as the dependent variable. We conducted a chi-square test to assess the differences in parasite (ticks and haemogregarines) prevalence between populations. Kruskal-Wallis ANOVA coupled with Median test was used to examine infestation levels.

To explain the variation of resistance for the raw data as estimated by S.I. values and the number of cells per spleen, we employed generalized linear mixed model (GLMM) using the type of grazing (aperiodic, long-term, none), tick and haemogregarine infestation levels and body condition as fixed effects, and including locality and habitat (islet or island site) as nested random effects. The statistical significance of each predictor was determined by likelihood ratio tests (LRT). This analysis was carried out separately for *P. gaigeae* populations subjected to different grazing regimes. Similarly, to elucidate the variation in body condition between individuals of the same population, we used mixed models fitted by maximum likelihood using tick and haemogregarines infestation levels, the number of splenocytes and S.I. values as fixed effects, including locality and habitat nested random effects when needed. All statistical analyses were conducted in R (v. 2.15.3) software (R Development Core Team, 2013). GLMM were conducted using the package *lme4* (Bates *et al.*, 2015).

Results

Effects of locality on body size, body weight and body condition

Lizards from Diavates were heavier (ANOVA; $F_{3,32} = 204.62$, $P < 0.001$) and longer (ANOVA; $F_{3,32} = 28.63$, $P < 0.001$) compared to those from Lakonissi, Palamari and Nyfi (Table 1). Lakonissi lizards were located in the middle of this gradient (Tukey *HSD* test; *P*s < 0.05). Diavates lizard populations had higher mean body condition index compared to Lakonissi conspecifics (ANOVA; $F_{3,36} = 3.39$, $P = 0.029$). Besides the latter finding, no further differences were registered (Table 1).

Effects of locality and grazing on parasite burden

Tick and haemogregarine infestation levels were significantly higher in Lakonissi and Nyfi lizards (grazed biotopes) compared to Diavates and Palamari (goat-free sites) conspecifics (Kruskal-Wallis; $H_{3,36} = 11.78$, $P = 0.008$ and $H_{3,36} = 11.38$, $P = 0.010$, respectively; Table 1 and Fig. 2). Similarly, tick and haemogregarine prevalence were higher in Lakonissi and Nyfi populations (χ^2 test, Fisher exact test, $P < 0.05$; Table 1). The GLM showed that parasite burden was positively related with the grazing regime, but not with locality and habitat ($F_{3,32} = 9.82$, $P < 0.001$).

Effects of locality and grazing on immunological response

The comparison of S.I. values revealed statistically significant differences among populations (ANOVA, $F_{3,32} = 54.144$, $P < 0.001$), with Diavates lizards demonstrating substantially higher splenocyte proliferation compared to Lakonissi, Nyfi and Palamari lizards (Fig. 3A). Nevertheless, given the major effects of body size to cell-mediated response ($r^2 = 0.81$, $F_{1,34} = 142.90$, $P < 0.001$), we reanalysed the data using the residuals of cell-mediated response to SVL. After correcting for SVL, we found that the Nyfi population showed a lower cell-mediated response compared to Diavates,

Palamari and Lakonissi (ANOVA, $F_{3,32} = 6.70$, $P = 0.001$; Fig. 3B). Lakonissi lizards had also significantly more splenocytes compared to the other three *P. gaigeae* populations (ANOVA; $F_{3,32} = 14.36$, $P < 0.001$; Table 1). However, these differences were not related to SVL ($r^2 = 0.001$, $F_{1,34} = 0.023$, $P = 0.881$).

The variation of T-cell mediated responses for the raw data as expressed by S.I. values could be explained by host body condition (log likelihood = -71.58, *df* = 4, $P < 0.001$; Table 2). The number of splenocytes was related to body condition and grazing regime (log likelihood = 303.36, *df* = 4, $P = 0.001$; Table 2). Tick and haemogregarine infestation levels did not significant influence neither S.I. values nor the number of splenocytes (Table 2).

When the analysis was carried out separately among populations subjected to different infection risk using the two components of cell-mediated response (indices for resistance) as the dependent varibles, we found that S.I. values were negatively related to body condition (log likelihood = -25.85, *df* = 3, $P = 0.040$) under no grazing (in Palamari and Diavates) (Table 3). To the contrary, the number of splenocytes was not related with any variable (log likelihood = 158.36, *df* = 3, $P = 0.441$; Table 3). In Lakonissi (aperiodic grazing) the variation in resistance as estimated by S.I. values was negatively correlated with body condition (log likelihood = -37.27, *df* = 3, $P < 0.001$), whereas haemogregarine infestation levels were positively correlated with the number of splenocytes (log likelihood = 58.23, *df* = 3, $P <0.001$; Table 3). Finally, in lizards under chronic grazing (Nyfi), S.I. values (log likelihood = -16.704 *df* = 3, $P = 0.972$) and the number of splenocytes (log likelihood = 72.66, *df* = 3, $P = 0.594$) were not correlated by any variable (Table 3).

Similarly, when body condition used as the dependent variable we found that this was negatively related with S.I. values (log likelihood = -42.34, *df* = 4, *P* = 0.046; Table 4) for Palamari/Diavates lizards (no grazing). Moreover, Lakonissi lizards' body condition (aperiodic grazing) was negatively related to S.I. values and parasite load (log likelihood = -44.29, *df* = 4, *P* < 0.001; Table 4). Finally, body condition of Nyfi lizards (chronic grazing) was positively related to haemogregarine infestation levels (log likelihood = -25.88, *df* = 4, *P* = 0.042; Table 4).

Discussion

In this study, we aimed to investigate the role of parasite burden and period of exposure to parasites as drivers of immune defense mechanisms in a Mediterranean lizard. However, our results did not fully support our initial assumptions and revealed a rather complex relation between infection risk and immune response induced. Heavily parasitized lizards that were aperiodically exposed to parasites (Lakonissi) invested more in cell-mediated response and demonstrated resistance. Lizards with high parasite burden and chronic exposure to parasites (Nyfi) were more tolerant. Finally, lizards with low parasite burden and, at the same time, minimal exposure (Diavates and Palamari) were more resistant. Our findings shed light on the relationship between grazing, body condition and infection risk: livestock presence directly increases parasite burden that negatively affects body condition. The duration of grazing though has debatable effects on body condition.

Nyfi lizards demonstrated high tolerance in response to their long exposure to parasites and the high infection risk due to grazing, and their body condition was positively correlated with haemogregarine infestation level but not with ticks (Table 4). Previous researchers suggested that chronic exposure to parasites dictates hosts to evolve higher tolerance, in order to reduce the severity of parasite consequences and minimize the impact of infection on their fitness (Bordes et al.,

2012, Rynkiewicz et al., 2013), and, thus, reduce their cell-mediated response. These hosts usually evolve strong innate and humoral responses, while the magnitude of cellular response decreases (Ardia et al., 2011, Lindström et al., 2004, Mallon et al., 2003, Rynkiewicz et al., 2013). In other words, hosts living in parasite-rich environments choose to avoid a cost-efficient type of defense (resistance) without experiencing a fitness reduction caused by parasites (Lindström et al., 2004, Schneider & Ayres, 2008). Though our findings support this idea and Nyfi lizards withstand the effects of parasitism on body condition (Fig. 3), a more generalized conclusion cannot be drawn at present, as we only used one assay (MLR) and estimated only the cell-mediated response as well as the parasite range tested was narrow. The high tolerance status of Nyfi lizards was only observed for haemogregarine and not for tick infestation. Therefore, more data including the evaluation of humoral immunity and a broader range of parasites will shed more light in the interactions between immune defense and infection risk.

Contrary to Nyfi population, Lakonissi lizards, that had also high parasite burden (Fig. 2) but aperiodic exposure, were more resistant (Table 3). This reflects a greater investment in cell-mediated response and enhanced activation of the immune system due to parasitism (Bordes et al., 2012, Rynkiewicz et al., 2013). Lakonissi islet is subjected to irregular grazing and thus, host-parasite interactions are discontinuous and only for a short period (Pafilis et al., 2013). Therefore, Lakonissi lizards would be expected to benefit by investing in cell-mediated response whenever infection risk and hence, parasite burden increases, in order to defend themselves against parasites (Gasparini et al., 2001, Schmid-Hempel & Ebert, 2003). The high parasite burden, however, negatively affected body condition (Table 4), indicating lower tolerance of Lakonissi lizards. This observation is in agreement with the concept that resistance and tolerance are two different components of immune defense usually engaged in antagonistic relation (Råberg et al., 2007, Schneider & Ayres, 2008).

Interestingly, though tick and haemogregarine prevalence and infestation levels were considerably low in Diavates and Palamari lizards (Fig. 2), where no grazing occur and infection risk is low, spleen cell proliferation (S.I. values) was high and statistically similar to the values recorded for Lakonissi population (Fig. 3). This contradicts the traditional concept of investment on active immunity, according to which hosts would strengthen their immunological response only when parasites numerically increase (Altizer *et al.*, 2003). We have to denote however, that in our study we focused on a small fraction of the wide parasite fauna (which additionally includes gastrointestinal and lung parasite communities, mites, helminths, etc) that infest lizards (Roca et al., 2009, Yildirimhan et al., 2011). Therefore, the observed reduced immune response against parasites (i.e. no correlation) in Diavates and Palamari (Table 3) must be approached with caution, due to the limited taxa of parasites monitored herein. Hence, the observed high cell-mediated response of Diavates and Palamari lizards might be linked to other parasites that were not investigated.

Resistance to parasites and the development of an effective immune response are energetically costly and an organism has to divert energy from other biological traits and physiological processes (eg. reproduction, locomotor capacity, tail regeneration, growth). However, energy reallocation could lead to trade-offs (Bordes et al., 2012, Lochmiller & Deerenberg, 2000, van der Most et al., 2011, Amo et al., 2006, Huyghe et al., 2010). We believe that a scenario like this could explain the decline in body condition that was observed in relation to the increasing immunological response and parasite burden (Table 4) in Lakonissi, Diavates and Palamari lizards (but not in Nyfi), because of the "price paid" to develop a highly effective response to control parasites. Moreover, the decrease in lizards' fitness in Lakonissi population could also explain the significant decline in the density of this population that has previously been reported during the years of grazing (Pafilis *et al.*, 2013).

Taken as a whole, our findings suggest that the type of defense a host will adopt is more complicated than what it was previously thought. In agreement with previous studies, we found that resistance is beneficial when parasites reduces hosts' fitness (Bordes et al., 2012, Rynkiewicz et al., 2013, Apanius et al., 2000), while tolerance may be activated when hosts experiencing high parasite burden and the infection risk is high (Bordes et al., 2012, Rynkiewicz et al., 2013, Lindström et al., 2004). Nevertheless, we showed that under different infection risks, populations within a single species could evolve different defense strategies that may have divergent effects on population's dynamics and host-parasite interactions (Rausher, 2001, Svensson & Råberg, 2010, Schneider & Ayres, 2008). Nyfi lizards experience high and constant infection risk and have evolved tolerance. On the other hand, irregular host-parasite interactions (Lakonissi) and low parasite burden (Diavates and Palamari) restrict lizards to invest more in immunity (resistance) when necessary. Nevertheless, resistance, as translated to increased cell-mediated responses, seems to trade-off for tolerance (Simms & Triplett, 1994, Schneider & Ayres, 2008) and lizards from these three sites experience a significant reduction in their body condition. Today the introduction of exotic parasites on wildlife populations through human activities is increasing (Wikelski et al., 2004, Daszak et al., 2000). Thus, the understanding of the variation in immune responsiveness in different populations turns out to be quite important.

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Supporting Information

Supporting Information 1. Storage of reptilian spleen cells and evaluation of their proliferative response and viability.

Supporting Information 2. Determination of the optimal concentration of mit-C to arrest stimulator cell proliferation.

Figure legends

Figure 1. Grazing pressure and parasite abundance for the four *Podarcis gaigeae* populations studied during a period of six years (2006-2011). The graphical representation is based on the study of Pafilis et al. (2013).

Figure 2. Boxplot of (A) tick infestation levels (mean number of ticks per lizard) and (B) haemogregarine infestation levels (percentage of infected cells on a total of 2000 red blood cells counted for each lizard) between the four populations under study.

Figure 3. Comparison of S.I. values between populations subjected to different grazing regimes as acquired from (A) analysis of variance and (B) after correcting for SVL (using the residuals of S.I. values against SVL).

Tables

Table 1. Descriptive statistics of body size (SVL), body mass, infestation levels, mean number of blood parasites and mean number of cells per spleen ($\times 10^5$) for the four populations included in the study. Body condition was estimated as the residuals of the linear regression of $\log_{10}$-transformed weight against $\log_{10}$-transformed SVL. Mean values ± 1 SE are given for each parameter. The sample size (*N*) for each variable and populations was *N=9*.

Table 2. Results of generalized linear mixed models as regard the variation of resistance expressed by S.I. values and/or the number of cells in the spleen of individuals. As fixed effects we used the type of grazing, tick and haemogregarine infestation levels, and body condition, while locality and environment were treated as nested random effects. Models were fitted by maximum likelihood. *P* values less than 0.05 are in bold.

Table 3. Results of generalized linear mixed models as regard the variation of resistance expressed by S.I. values and/or the number of cells in the spleen according to host locality. As fixed effects we used the tick and haemogregarine infestation levels and body condition. Models were fitted by maximum likelihood. *P* values less than 0.05 are in bold.

Table 4. Results of generalized linear mixed models regarding tolerance as assessed by body condition according to environmental grazing pressure. As fixed effects we used tick and haemogregarine infestation levels, S.I. values and the number of splenocytes. Models were fitted by maximum likelihood. *P* values less than 0.05 are in bold.

Table 1.

Trait	Nyfi (N)	Palamari (P)	Lakonissi (L)	Diavates (D)
SVL (mm)	59.39 ± 1.88	59.12 ± 1.41	65.83 ± 3.33	83.97 ± 1.57
Weight (g)	6.03 ± 0.30	5.94 ± 0.31	7.64 ± 0.47	18.07 ± 0.51
Body condition	-0.02 ± 0.02	-0.02 ± 0.02	-0.03 ± 0.04	0.07 ± 0.02
Tick infestation	2.11 ± 0.65	0.11 ± 0.11	1.56 ± 0.47	0.22 ± 0.15
Tick prevalence	67%	11%	67%	22%
Haemogregarine infestation	9.33 ± 2.35	1.33 ± 0.80	3.44 ± 1.23	0.78 ± 0.43
Haemogregarine prevalence	78%	33%	67%	33%
Cells/spleen	119.00 ± 3.31	105.44 ± 5.09	144.22 ± 5.85	104.00 ± 5.07

Table 2.

Dependent variable	Independent variable	Estimate	Standard error	*t* value	*P* value
S.I. values	Tick infestation levels	0.005	0.009	0.512	0.609
	Haemogregarine infestation levels	0.004	0.003	1.569	0.117
	Body condition	-0.790	0.134	-5.887	**<0.001**
	Grazing	0.158	0.091	1.733	0.083
Splenocytes	Tick infestation levels	1.137	2.074	0.548	0.583
	Haemogregarine infestation levels	-0.129	0.521	-0.248	0.804
	Body condition	-61.201	28.629	-2.138	**0.003**
	Grazing	-16.914	2.999	-5.640	**<0.001**

Table 3.

Grazing/ Population	Dependent variable	Independent variable	Estimate	Standard error	*t* value	*P* value
No grazing/	S.I. values	Tick infestation levels	0.023	0.063	0.378	0.705
Diavates & Palamari		Haemogregarine infestation levels	-0.006	0.012	-0.543	0.587
		Body condition	-0.865	0.314	-2.754	**0.006**
	Splenocytes	Tick infestation levels	-7.872	12.084	-0.651	0.514
		Haemogregarine infestation levels	-1.189	2.497	-0.476	0.634
		Body condition	-57.830	48.437	-1.194	0.232
Aperiodic	S.I. values	Tick infestation levels	0.015	0.010	1.509	0.191
grazing/ Lakonissi		Haemogregarine infestation levels	-0.002	0.004	-0.498	0.639
		Body condition	-0.941	0.120	-7.852	**<0.001**
	Splenocytes	Tick infestation levels	1.795	2.011	0.892	0.413
		Haemogregarine infestation levels	3.418	0.913	3.743	**0.013**
		Body condition	-25.909	24.152	-1.073	0.332
Chronic	S.I. values	Tick infestation levels	0.001	0.014	0.060	0.954
grazing/ Nyfi		Haemogregarine infestation levels	0.001	0.006	0.192	0.855
		Body condition	-0.261	0.615	-0.424	0.689
	Splenocytes	Tick infestation levels	0.189	1.952	0.097	0.927
		Haemogregarine infestation levels	-1.022	0.833	-1.227	0.274
		Body condition	124.611	88.067	1.415	0.216

Table 4.

Grazing/ Population	Dependent variable	Independent variable	Estimate	Standard error	*t* value	*P* value
No grazing/ Diavates & Palamari	Body condition	Tick infestation levels	-0.052	0.038	-1.355	0.175
		Haemogregarine infestation levels	0.001	0.007	0.104	0.917
		S.I. values	-0.268	0.123	-2.177	**0.029**
		Splenocytes	-0.001	0.001	-1.241	0.214
Aperiodic grazing/ Lakonissi	Body condition	Tick infestation levels	0.009	0.007	1.193	0.298
		Haemogregarine infestation levels	-0.019	0.006	-2.998	**0.040**
		S.I. values	-1.164	0.107	-10.924	**<0.001**
		Splenocytes	0.005	0.002	2.717	0.063
Chronic grazing/ Nyfi	Body condition	Tick infestation levels	0.001	0.008	0.072	0.946
		Haemogregarine infestation levels	0.007	0.002	3.202	**0.032**
		S.I. values	-0.315	0.283	-1.111	0.329
		Splenocytes	0.003	0.002	1.754	0.154