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In vitro temperature dependent activation of T-lymphocytes in Common wall lizards (*Podarcis muralis*) in response to PHA stimulation

Roberto Sacchi[1,*], Enrica Capelli[1], Stefano Scali[2], Daniele Pellitteri-Rosa[1], Michele Ghitti[1], Emanuele Acerbi[1], Elena Pingitore[1]

[1] *Dipartimento di Scienze della Terra e dell'Ambiente, Università degli Studi di Pavia, Via Ferrata 9, I-27100 Pavia, Italy. *Corresponding author.* E-mail: roberto.sacchi@unipv.it
[2] *Museo Civico di Storia Naturale, C.so Venezia 55, I-20121 Milano, Italy*

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Abstract. Ecological immunology attempts to explain the variability of immune response among individuals by invoking costs and trade-offs, which may optimize the immune defence against pathogens. In ectotherms body temperature is correlated to that of the surrounding environment, so that their entire physiology, including immune functions, is influenced by the environmental temperature. We used *in vitro* phytohaemoagglutinin (PHA) stimulation in order to assess the effects of temperature on cell mediated adaptive response in male and female Common wall lizards (*Podarcis muralis*). Cell cultures were prepared from blood samples, inoculated with PHA and incubated at 22°C, 25°C, 32°C, and 38°C for three days. PHA stimulation caused proliferation of T-lymphocytes, but the effect depended on the incubation temperature. Lymphocyte proliferation was significantly impaired at both 22°C and 38°C compared to 32°C, which represented the highest levels of activation. Furthermore, lymphocyte activation was more variable in males while females were less immune suppressed than males at low temperatures. Differences between sexes suggest a possible influence of steroid hormones.

Keywords. Ecological immunology, adaptive immune response, temperature dependent effects, phytohaemoagglutinin PHA, T-lymphocyte proliferation, ectotherms.

INTRODUCTION

Ecological immunology investigates the physiological or molecular basis of immune responses, by placing them in the context of ecology and adaptation (Sheldon and Verhulst, 1996; Norris and Evans, 1999; Sadd and Schmid-Hempel, 2009). There is extensive evidence that immune defence is costly, requiring investment of energy, nutrients, and time, during the development, maintenance, and use of the immune system (Klasing and Leshchinsky, 1999; Lochmiller and Deerenberg, 2000). When resources are limited, allocation of energy to immune defences may be modulated by the need to spend energy on other functions such as growth, reproduction, and maintenance (Nelson and Demas, 1996). Thus, multiple trade-offs occurring between immune functions and other compartments and life-processes cause severe constraints on the evolution of immune response and the other fitness related traits.

Costs of development, maintenance and use of immune defence have been widely investigated on homeotherm vertebrates, and especially birds (Norris and Evans, 1999; Martin et al., 2008). By contrast, ecthotermic vertebrate have been less extensively studied (see Zimmerman et al., 2010), even if a large amount of researches had been done on fish (e.g., Bly and Clem, 1992; Collazos et al., 1996; Köllner and Kotterba, 2002; Ndong et al., 2007; Prophete et al., 2009). Ectotherms are particularly interesting under an eco-immunological perspective, as body temperature is correlated to that of

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the surrounding environment, so that their entire physiology, including immune functions, is influenced by the environmental temperature (Zimmermann et al., 2010). This offers an interesting opportunity to investigate the costs of the immune response, as the trade-offs mediated by the immune function are likely to have different settings at different environmental temperatures. Both endotherms and ectotherms show seasonal variation in immune-response, but ectotherms display higher variability due to their dependence on environmental temperatures as heat source (Zimmermann et al., 2010).

The immune system of ectotherms has been shown to respond across a wide range of temperatures, but strongest responses occur at species-specific temperature ranges, with impaired responses at temperatures above and below the optimal threshold (Le Morvan et al., 1998; Mondal and Rai, 2001; Merchant et al., 2003; Merchant and Britton, 2006; Raffel et al., 2006). For example, experiments carried out on carp (*Cyprinus carpio*) immunized against bovine serum albumin, revealed that the primary antibody response is suppressed at low temperatures (Avtalion, 1969), particularly that mediated by T-helper cells (Le Morvan et al., 1996). Similarly, Mondal and Rai (2001) reported that the highest levels of phagocytosis and cytotoxicity of splenic macrophages from *Hemidactylus flaviviridis* occurred at 25°C, with impaired macrophage function at both higher and lower temperatures.

In reptiles, the studies on temperature effects on immune system chiefly focused on the seasonal variation of immune response in both innate (Mondal and Rai, 2001; Merchan et al., 2003, 2004) and adaptive immunity (Muñoz and De la Fuente, 2001; but see Zimmermann et al., 2010 for a review). Surprisingly, no one has specifically investigated the response of the immune system to short time variation of temperature such that occurring daily or with weather instability. Short time effects of temperature are particularly interesting under an eco-immunological perspective, since pathogen ability to infect ectotherms can be heavily affected by fast temperature-dependent fluctuations of host immune system (Wright and Cooper, 1981), with direct consequences on the behavioural, ecological and physiological strategies that individuals adopt to optimize fitness.

In the present study we measured the T-cell mediated immune response of Common wall lizards *Podarcis muralis* under different temperatures, covering the typical thermal-activity range of the species. To do this, we set up cell cultures from blood samples that were activated with phytohaemagglutinin (PHA) and incubated at four different temperatures. PHA is a lectin found in plants, especially legumes, which causes local swelling and oedema, driven by mitogenesis and infiltration of T-lymphocytes into the injected tissue (Goto et al., 1978). The Common wall lizard is a small lizard (snout-vent length, SVL, 45-75 mm) widespread in southern and central Europe, which mates multiply and produces two clutches per year on average (Sacchi et al., 2012) during its life (max lifetime 5 years, Barbault and Mou, 1988; pers. obs.). Breeding season starts from late February and ends in July (Sacchi et al., 2012), and body temperature during activity is near 33°C, being slightly higher (33-36°C) in warmer regions (e.g. Central Italy) and lower (32°C) in mountain areas (Avery, 1978; Braña, 1991; Tosini and Avery, 1994). The immune-system has been previously investigated in relation to polymorphic ventral colouration (Sacchi et al., 2007; Galeotti et al., 2010) or immunocompetence handicap hypothesis (Oppliger et al., 2004), but earlier study have not examined the influences of the environmental factors on the immune system response.

MATERIALS AND METHODS

Individual collection

From 6 July to 4 August 2012, we collected by noosing 24 (12 males and 12 females) adult Common wall lizards (snout-vent length, SVL > 50 mm, Sacchi et al., 2012) in a farm in the surrounding of Pavia (Northern Italy, 45°11'31"N, 9°9'11"E). We carried out five sampling sessions in which at least one male and one female were collected. After capture, lizards were measured by a digital calliper (accuracy ± 0.1 mm) for SVL and transferred in cloth bags to the laboratory at the University of Pavia.

Blood sampling and cell cultures

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. Cell suspension was then subdivided into two 7 ml sub-cultures, one of which was inoculated with 1% PHA solution (PHA-P Sigma L-8754, 50 mg in 10 ml phosphate buffered saline (Oppliger et al., 2004; Sacchi et al., 2007). The remaining solution (1 ml) was used to assess starting cell concentration (SCC) using a Neubauer chamber, and only live lymphocytes were counted. Each sub-culture was then distributed in four 1.5 ml culture tubes, and incubated at 22°C, 25°C, 32°C, and 38°C for 3 days. Afterwards, cell were collected, re-suspended and newly counted. This second count involved only proliferating lymphocytes. Stimulation of T-cell after incubation was evaluated by determining the colony-forming units per ml (CFU) and the ratio between the total cell recovery to SCC (growth index, GI). Unfortunately, 30 samples incubated at 32°C were lost because the stove broke up during experiment. So our final sample included 48 cultures for 22°C, 25°C and 38°C, but only 18 for 32°C.

Statistical analyses

Preliminarily, we ran a t-test for matched pairs to check for differences in lymphocyte stimulation between the PHA-treated and the control cultures at each of the four incubation temperatures. The CFU in the full sample showed a Poisson-like distribution and thus its relationship with sex, treatment (PHA vs control) and temperature (analysed as a four levels factor) was investigated by a GLMM with Poisson error distribution and log link function. The number of colonies counted after the three day incubation was the dependent variable, while the individual identity entered the model as random factor. All main effects and two-way interactions sex × temperature, sex × treatment and temperature × treatment were included in the models as fixed effects. We also included the SVL as covariate in order to account for possible effects of individual size on immune-response. The growth index GI was slightly skewed, and achieved normality after four root transformation. The relationship between GI and sex, treatment and temperature was thus analysed using a linear mixed model including individual identity as random factor, and the same fixed factors, covariate and their interactions used in the analysis of CFU. Both models were then optimized by removing all non-significant terms (using likelihood-ratio tests) until only significant terms were retained (Zuur et al., 2009). All tests were performed using R 2.13.1 statistical package (R Development Core Team, 2010), and unless otherwise stated, values reported are means ± SE.

RESULTS

Peripheral blood lymphocytes of both sexes were actually stimulated by PHA at all incubation temperatures, as both CFU and GI were significantly far higher in activated cultures than in controls. However, the difference between the GI values of females in PHA-activated and control recorded at 32°C was marginally not significant, probably because of the small sample (see Table 1 for statistics). PHA-activated lymphocytes showed the characteristic activated morphology with enlarged size (2-3 times) and spikes and cell aggregates (clones) containing from few to several dozen cells (Fig. 1).

The GLMM confirmed the capacity of PHA to activate lymphocytes proliferation, but the intensity of the response was highly affected by both incubation temperatures and sex (Fig. 2a). Indeed, the CFU significantly increased in PHA treated cultures with respect to controls, but strictly depending on the incubation temperature (temperature × treatment: LRT-χ^2 = 29.34, *df* = 3, *P* < 0.0001, Fig. 2a). The highest activation was recorded at 32°C (8056 ± 1197 colonies/ml), while the minimum occurred at both 25°C (2760 ± 489 colonies/ml) and 38°C (2617 ± 531 colonies/ml). Moreover, males showed on average a significant higher activation than females, irrespective of the incubation temperatures (males: 3583

Table 1. *In vitro* effects of PHA administration on T-lymphocyte proliferation assessed by colony-forming units (CFU) and growth index (GI) in male and female Common wall lizards at four different temperatures.

	PHA-activated	Controls	N	t	*P*
CFU					
Males					
22°C	2643 ± 694	26 ± 26	12	3.774	0.0031
25°C	2890 ± 692	0	12	4.176	0.0015
32°C	8489 ± 1767	1484 ± 1484	6	3.000	0.030
38°C	2760 ± 787	0	12	3.505	0.0049
Females					
22°C	4414 ± 791	0	12	5.581	0.00016
25°C	2630 ± 721	26 ± 26	12	3.570	0.0044
32°C	7187 ± 1005	0	3	7.155	0.019
38°C	2473 ± 746	195 ± 195	12	2.970	0.013
GI					
Males					
22°C	5.85 ± 1.16	1.09 ± 0.22	12	3.906	0.0024
25°C	6.17 ± 1.24	1.25 ± 0.45	12	6.578	0.00039
32°C	16.7 ± 2.18	1.28 ± 0.67	6	3.416	0.019
38°C	4.95 ± 0.93	0.91 ± 0.29	12	4.958	0.00043
Females					
22°C	11.26 ± 3.58	1.58 ± 0.67	12	5.745	0.00012
25°C	9.20 ± 3.07	1.56 ± 0.57	12	5.437	0.00020
32°C	6.32 ± 1.05	0.82 ± 0.81	3	3.237	0.084
38°C	5.30 ± 1.53	1.47 ± 0.54	12	3.487	0.0051

± 523 colonies/ml, females: 3482 ± 452 colonies/ml; sex × treatment: LRT-χ^2 = 13.74, *df* = 1, *P* = 0.00021), even though PHA-activation was higher in females than in males when cultures were incubated at 22°C (sex × temperature: LRT-χ^2 = 40.97, *df* = 3, *P* < 0.0001, Fig. 2a, Table 1). Finally, the size of individuals did not significantly affect the activation of lymphocytes (p-value at removal > 0.66).

The final model for GI included only the interaction temperature × treatment (LRT-χ^2 = 13.24, *df* = 3, *P* = 0.0041), confirming that cell proliferation was higher at 32°C (GI = 13.27 ± 2.26) than in all other temperatures (GI22°C = 8.56 ± 1.93, GI25°C = 7.69 ±1.65, GI38°C = 5.13 ± 0.88, Fig. 2b). The GI was higher in males than in females at 32°C (Fig. 2b), but the effect of sex was not significant (*P* > 0.81), and was removed from the final model. As for CFU, the size of individuals had no significant effects on immune-response.

Finally, the intensity of the lymphocyte activation was highly variable among individuals, as both models revealed a highly significant effect of the random factor

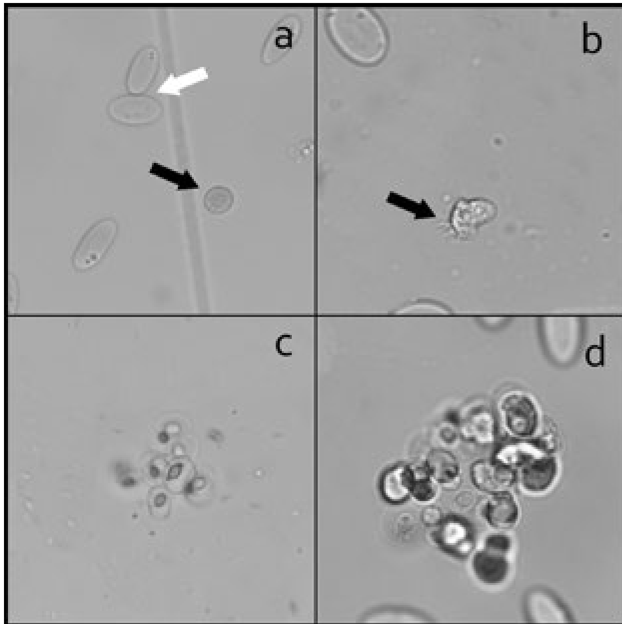

Fig. 1. Morphology of lymphocytes of Common wall lizards in blood cultures: a) a single lymphocyte (black arrow) among erythrocytes (white arrow); b) a lymphocyte with spikes and a small (c) and a large (d) cell aggregates.

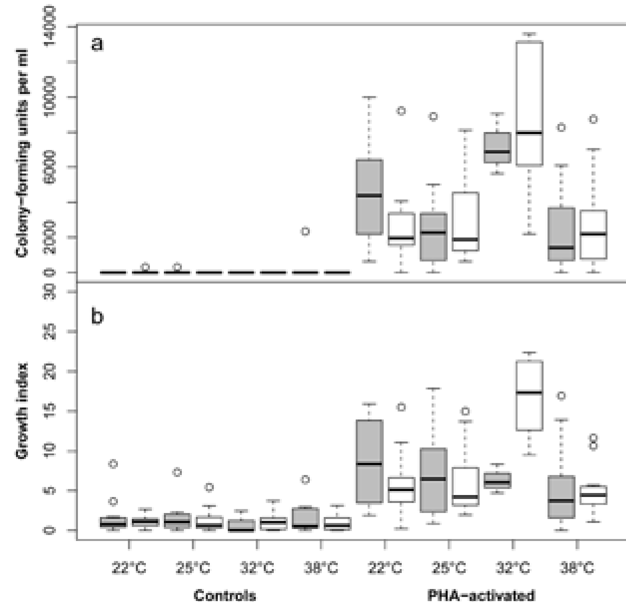

Fig. 2. The *in vitro* effect of temperature on colony-forming unit (CFU, a) and growth index (GI, b) of T-lymphocytes of male (white) and female (gray) Common wall lizards incubated with (PHA-activated) or without (Controls) phytohaemoagglutinin.

(CFU: LRT-χ^2 = 406.38, df = 1, P < 0.0001, GI: LRT-χ^2 = 22.01, df = 1, P < 0.0001).

DISCUSSION

In this paper we analysed the effect of short-time variation of temperature on the lymphocyte activation in male and female Common wall lizards, assessed by PHA injection in blood cultures. Despite the fact that temperature is expected to affect the immune response more extensively in ectotherms than in endotherms, the susceptibility to sudden changes of environmental temperature by immune response of the same individual, to our knowledge, had never been performed in a terrestrial reptile. Previous immunological studies in reptiles used PHA injection to examine the negative effect of steroids on cell-mediated immunity (Belliure et al., 2004; Oppliger et al., 2004; Berger et al., 2005; Huyghe et al., 2009), morph-specific differences in immune response (Sacchi et al., 2007; Huyghe et al., 2009), or female preference for males with better immune response (López and Martín, 2005).

Several components of the defence mechanisms of reptiles including phagocyte bactericidal activity, leukocyte mobilization and humoral mediators of inflammation have been reported to be temperature-dependent (Farag and El Ridi, 1984; Muñoz and De la Fuente, 2001; Merchant et al., 2003, 2004, Keller et al., 2005). However, most studies did not analysed the response of the same individual to different temperatures, rather compared the responses of different groups of individuals to different temperatures (Farag and El Ridi, 1984; Muñoz and De la Fuente, 2001). So, these findings cannot actually be considered a demonstration of a direct effect of temperature on the immune system, since several other factors may affect immune-response (e.g., hormones Mondal and Rai, 2002; Belliure et al., 2004; Huyghe et al., 2009). The in vitro activation of lymphocytes enabled us to set up an experimental design with repeated measures within individual, in which every lizard has been exposed to all the incubation temperatures. This design allowed us to actually evaluate the change in the immune function of a given individual in response to the variation of thermal condition. Thus any significant change in lymphocytes proliferation can be attributed to the direct effect of environmental temperature.

In this study we found that the lymphocytes proliferation following PHA stimulation was significantly impaired in cultures kept at both 22°C and 38°C compared to that kept at 32°C, which represented the highest levels of activation in both sexes. Since the ability of lymphocytes to proliferate following a mitogenic stimulus *in vitro* is an indication of immune response *in vivo*

(Clem et al., 1984), our data suggest that Common wall lizards keep a temperature range in which immune response is optimal, while temperatures above and below this range exert a suppressive effect. Our data agree with past researches on thermal activity, which indicated that Common wall lizards reach the maximum efficiency near to 33°C (Avery, 1978; Braña, 1991; Tosini and Avery, 1994), and suggest that physiological processes in this species are optimized near this temperature. This conclusion agrees also with previous findings in other species of reptiles: the highest levels of phagocytosis and cytotoxicity of splenic macrophages of *Hemidactylus flaviviridis* occurred at 25°C, with impaired macrophage function at both higher (37°C) and lower temperatures (7°C and 15°C, Mondal and Rai, 2001). In *Alligator mississippiensis* antimicrobial and amoebacidal activity occurred between 5°C and 40°C, and was significantly reduced at temperatures below 15°C and above 30°C (Merchant et al., 2003, 2004). Accordingly, most physiological processes are optimized near 30-31°C (Merchant et al., 2003, 2004).

Earlier reports on the effects of temperature on the immune system were aimed to sustain the hypothesis that reptiles might be immunosuppressed during the winter months, when body temperatures are far below the optimum. In our study, however, we found that immune suppression may already occur during the breeding season, in coincidence with cold periods, or even during night time. Indeed, body temperatures of lizards remain remarkably constant during the day, but decrease substantially both in the early morning and in the late evening (Braña, 1991). These findings have relevant consequences in the perspective of ecological-immunology, since short-time fluctuations of temperature might severely affect the trade-offs between immune function and other compartments and life-processes, with remarkable effects on behavioural and physiological fitness-related traits. For example, temperature-dependent suppression of immune function might increase the susceptibility to parasite infections during the breeding season and consequently reduce the reproductive efforts of less resistant individuals. Furthermore, the immunocompetence handicap hypothesis (Folstad and Karter, 1992) states that androgens have a dual effect in stimulating the expression of the secondary sexual characters while suppressing the immune functions. The reduction in the immune response following unpredictable fluctuations of temperature below or above the optimum, might amplify the suppressive action of androgens, with negative effects on the expression of secondary sexual traits of males. However, these additional "thermal" costs might be paid differentially by the different individuals; for example lizards keeping less quality territories including low quality basking sites might suffer the highest levels of temperature-dependent immune suppression. Actually, both individuals settled in marginal territories and subordinate individuals might spend much more time to reach optimal body temperature than dominant individuals occupying the best basking sites, and therefore might be exposed for longer time to the consequences of temperature-dependent immune suppression. The effects of temperature on the immune functions in ectotherms should, ultimately, increase the variance of male quality, and consequently amplify the difference in the expression of the secondary sexual traits among them. In this scenario, endotherms are not expected to pay additional costs in immunocompetence due to fluctuations of environmental temperatures, since they use metabolic heat to maintain body temperature within the optimal physiological range. So, under high variable temperature regimes (e.g. in temperate climates) endotherms might reveal a lower variability in male secondary sexual traits than ectotherms, and this difference should be reduced in more stable climatic conditions (e.g. in tropical regions).

A second relevant result of this study was the difference in temperature-dependent immune suppression between males and females. Earlier studies in mammals have well established sex differences in cell-mediated immune responses, with females generally having enhanced immune-reactivity than males (Ansar Ahmed et al. 1985; Grossman, 1989; Cannon and St. Pierre, 1997; Klein, 2004). Among reptiles, sex differences in immune response have been reported in the Striped sand snake *Psammophis sibilans* and in the Yellow-bellied house gecko *H. flaviviridis,* in which females have higher response than males (Saad, 1989; Mondal and Rai, 1999, 2002). In both cases, sex-associated immune differences have been related to sex steroids, even if experimental studies have shown that immunosuppressive effects of sex hormones act mainly on innate immunity (Mondal and Rai, 1999, 2002). Indeed, gonadectomy of both males and females causes a considerable increase in percentage phagocytosis, while *in vitro* administration of dihydrotestosterone (DHT) and 17β-estradiol (E2) suppresses phagocytosis, cytotoxic activity of splenic macrophages and IL-1 secretion (Mondal and Rai, 1999, 2002). On the other hand, Con A and PHA stimulations did not cause significant differences between sexes in the T-lymphocytes proliferation in both Caspian pond turtle and loggerhead sea turtles (Muñoz and De la Fuente, 2001; Keller et al., 2005), suggesting that adaptive response is similar in males and females. Contrary to these latter, we showed that PHA stimulation in Common wall lizards causes dependent activation of T-lymphocytes, suggesting that adaptive immune response might be actually

affected by sex steroids. However, this difference between sexes varied at different temperatures, and immunosuppression was more severe on males at temperatures below the optimum and more severe on females at temperatures above the optimal. This different response by sexes might be explained by different and opposite effects of male and female hormones on the immune function, but future researches are needed to highlight the specific effect of hormones on lymphocyte activation in this species. In conclusion, *in vitro* activation of immunoresponse is a powerful and effective tool to investigate the effect of biotic and abiotic factors on the immune function in natural populations of reptiles. In particular, the possibility to analyse immune-response *in vitro* might allow researchers to repeatedly test the same individual, improving the opportunities to focus on the currencies that mediate the cost of immune responses in life history trade-offs.

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