

Boldness in anti-predator behaviour and immune defence in field crickets

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ABSTRACT

Questions: Are prey animals' behavioural responses against predation associated with their ability to resist parasites and pathogens, and if so, how is this adjusted in different populations?

Methods: We studied the association between anti-predator behaviour and immune defence (encapsulation response and lytic activities of the haemolymph) using laboratory-reared male field crickets originating from two wild populations that differ in predation and parasitism risk. The anti-predator behaviours that we measured were as follows: (1) the animal's latency to become active inside a safe refuge, (2) its latency to emerge from a safe refuge after disturbance, and (3) the animal's freezing time following a separate alarm cue.

Results: Crickets originating from a high-parasitoid, high-predation risk population (Arizona) had higher encapsulation responses than crickets from a low-parasitoid, low-predation risk population (California). Lytic activity correlated negatively with freezing time. Encapsulation response was positively correlated with the latency to become active and latency to emerge from a safe refuge in the high-predation risk population, but not in the low-predation risk population.

Conclusion: Predation and parasitoids may increase the cricket's investment in parasite resistance, despite the potential costs of anti-predator behaviour.

Keywords: anti-predator response, cost, field cricket, *Gryllus*, immunocompetence.

INTRODUCTION

Responses of prey to predation risk include defences such as specialized morphological traits and behaviours that have been adjusted by natural selection over time (Endler, 1980; Lima and Dill, 1990; Chivers and Smith, 1998). Presumably, natural selection acts to favour stronger anti-predator behaviour in populations with higher predation risk (Riechert and Hedrick, 1990; Chivers *et al.*, 2001), contributing to co-evolutionary cycles between predator and prey [cf. the 'Red Queen' hypothesis of Van Valen (1973)]. Numerous experimental studies have

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demonstrated that predation can have an effect on various fitness-related traits (e.g. Huntingford, 1982; Hedrick and Dill, 1993; Chivers and Smith, 1998; Cooper, 1999; Grostal and Dicke, 1999; Hedrick, 2000; Rigby and Jokela, 2000; Lewkiewicz and Zuk, 2004).

According to life-history theory, organisms are expected to show trade-offs between different life-history traits, including behavioural traits (Stearns, 1992). An effective anti-predator behaviour can be costly in many ways. For example, increased vigilance (i.e. time spent hiding or immobile when avoiding predation) could mean less time for foraging or competing for resources. These types of costs of anti-predator behaviour are well documented (e.g. Lima and Dill, 1990; Cooper, 2000; Cooper and Perez-Mellado, 2004). Some forms of anti-predator behaviour, such as extended flight from predators, are energetically demanding, and therefore incur trade-offs with other traits (Ydenberg and Dill, 1986).

Recently, trade-offs between immunological defence and life-history traits have been identified as particularly important (for reviews, see Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; see also Viney *et al.*, 2005). The host immune system fights against parasites and diseases to reduce the fitness costs of parasitism (Goater and Holmes, 1997), but this may reduce investment in other energy-demanding functions, such as self-maintenance. Thus, if anti-predator behaviour limits an individual's access to and competition for nutritional resources (e.g. through time constraints), this should detract from its capability to respond immunologically against parasites and pathogens. Previous data on insects demonstrate that a poor nutritional environment can have a negative effect on immune response (Vass and Nappi, 1998; Siva-Jothy and Thompson, 2002; Rantala *et al.*, 2003). Only a few studies have examined the potential immunological costs of anti-predator behaviour. Rigby and Jokela (2000) demonstrated that increased predator-avoidance behaviour in a freshwater snail (*Lymnaea stagnalis*) reduced the snail's ability to defend against potential pathogens. In general, if this type of predator-mediated negative response in an individual's immunocompetence exists, even a short-term increase in anti-predator behaviour could result in an increased risk of becoming infected by parasites and pathogens, and also a lower tolerance of already existing infections (Rigby and Jokela, 2000).

In the present study, we examined the association between cricket anti-predator behaviour and immune defences in two populations of field crickets, to establish potential immunological costs of anti-predator behaviour. Although variation between individuals in the amount of resources available to invest in immunocompetence and other traits often makes direct relationships difficult to observe at the population level (Van Noordwijk and de Jong, 1986; Reznick *et al.*, 2000), our aim was to describe these patterns to form a base for further experimental work.

Insects are useful models for studying trade-offs between immune function and different phenotypic traits (e.g. morphological, physiological, behavioural, and life-history traits) (Rolff and Siva-Jothy, 2003). The immune defence system of insects is notably less complex than the immune system of vertebrates, but includes many homologous components (Vilmos and Kurucz, 1998; Rolff and Siva-Jothy, 2003). Most insects do not show a well-developed acquired immunity; for example, insects do not possess lymphocytes or immunoglobulins (Gillespie *et al.*, 1997). The main characterization of insect immunity includes both the inducible expression of a large array of anti-microbial peptides, and the constitutive melanization-encapsulation response. Encapsulation is a non-specific, constitutive response through which insects fight against several multicellular pathogens such as fungi, nematodes, and parasitoids (Gillespie *et al.*, 1997). In addition, the encapsulation response plays a role in defence against viruses (Washburn *et al.*, 1996). In detail, during the encapsulation process, haemocytes recognize an object as foreign

and cause other haemocytes to aggregate and form a capsule. A cascade of biochemical reactions then leads to the deposition of melanin and hardening of the capsule (Gillespie *et al.*, 1997). As a result, the enclosed intruder dies from suffocation or from the release of necrotizing compounds (Nappi *et al.*, 1995). In invertebrates, one of the most informative ways to assay this reaction is to measure the magnitude of the encapsulation response to a novel and standardized antigen such as a nylon monofilament (e.g. König and Schmid-Hempel, 1995; Rantala *et al.*, 2003; Ahtiainen *et al.*, 2004). The ability to encapsulate abiotic material is strongly related to the ability to encapsulate a parasite (Paskewitz and Riehle, 1994; Gorman *et al.*, 1996). Furthermore, the ability to encapsulate a nylon monofilament is associated with the ability to resist entomopathogenic fungi (M.J. Rantala, unpublished). Invertebrates also produce several anti-microbial peptides (Hetru *et al.*, 1998). An enzyme thought to be important in the non-specific immune response of insects against bacterial infection is lysozyme, which hydrolyses β -1,4 linkages in the peptidoglycan of bacterial cell walls (Götz and Trenczek, 1991). Thus, lysozyme activity of insect haemolymph can be easily assayed by the clearance rate of bacterial suspension by an individual's haemolymph (Rantala and Kortet, 2003, 2004).

Field crickets (*Gryllus* spp.) frequently occur in high densities in California and Arizona in the USA. Natural field cricket populations in these locations are notably different from one another in the risk of parasitism and predation (Luong *et al.*, 2005; Hedrick and Kortet, 2006). We previously demonstrated that more predators occur in the Arizona population's habitat than in the California population's habitat, using tethering experiments that were conducted at both habitats. We also found more parasitoid flies, which are attracted to male song and lay their maggots on crickets, in Arizona than in California (Hedrick and Kortet, 2006).

In the present study, we looked for potential immunological costs of anti-predator behaviour by examining associations between cricket anti-predator behaviour and immune defences in the two populations. We hypothesized that crickets that invest more in anti-predator behaviour than their conspecifics cannot invest as much in immune defence as crickets that invest less in anti-predator behaviour (i.e. they may have different life-history strategies). To test this hypothesis, we first measured various behavioural traits that are potentially important as anti-predator responses in male field crickets originating from two populations (in California and in Arizona) that differ in predation and parasitism risk (Hedrick and Kortet, 2006). We then measured a cricket's immunological response against a nylon implant, and determined the lytic activities of his haemolymph.

METHODS

Crickets

We used two cricket species, *G. integer* and another, larger, undescribed species that occurs sympatrically with the Arizona population of *G. integer*. According to preliminary mtDNA studies (D. Gray, unpublished data), this undescribed species is closely related to the field cricket *G. lineaticeps* and is tentatively denoted as '*Gryllus 15*' (D. Weissman, unpublished data). *Gryllus 15* shares endoparasitic nematode species with *G. integer* (Luong *et al.*, 2005), shows similar behaviour to *G. integer*, and has similar times of peak abundance (A.V. Hedrick and R. Kortet, unpublished data). We used the two related *Gryllus* species from Arizona to provide additional information about the association between anti-predator behaviour and investment in immune defences, because this could reveal possible differences between species within one habitat.

In Aguila, Arizona, predation risk is high and parasitoid flies, which are attracted to male song and lay their maggots on crickets, are common (Hedrick and Kortet, 2006). In contrast, in Davis, California, predation risk is much lower and the crickets have a very low risk of parasitism by parasitoid flies (Hedrick and Kortet, 2006). These differences between populations offer an opportunity to undertake population-level comparisons of the association between parasite resistance and anti-predator behaviour.

In both populations, males call from cracks in the ground to attract sexually receptive females, and females travel above-ground to find males (Hedrick and Dill, 1993). Typically, males fight aggressively for and defend cracks and females (Kortet and Hedrick, 2005). Males sing at the entrance of the crack with their heads outside the crack entrance, but abruptly stop calling if they sense a predator approaching and run further into the crack (Hedrick, 2000). Although they do leave cracks to forage, they appear reluctant to do so, presumably because this increases predation risk (Hedrick, 2000). Similar hiding behaviour occurs in many taxa and is generally recognized as an anti-predator tactic (Dill and Fraser, 1997; Hugie, 2003, 2004; Jennions *et al.*, 2003). Females are also sensitive to predation risk: they adjust their mate-choice decisions in response to perceived predation risk (Hedrick and Dill, 1993).

Known predators of adult crickets include spiders, toads, lizards, bats, voles, mice, rats, and birds (Hedrick and Kortet, 2006). In addition to hiding behaviour, anti-predator responses of field crickets include decreasing or changing behavioural activity to avoid visually and acoustically hunting predators and parasitoids (e.g. Hedrick, 2000; Kortet and Hedrick, 2004; Lewkiewicz and Zuk, 2004). Previous work on the high-predation, high-parasitism risk population (Arizona) and low-predation, low-parasitism risk population (California) revealed differences in anti-predator behaviour. Male crickets from Arizona had longer latencies to emerge from a safe refuge in a novel environment than males from California (Hedrick and Kortet, 2006).

The crickets used in this experiment were the first laboratory generation derived from wild-caught mothers (August 2003) that had been inseminated in the field before capture. *Gryllus integer* females were collected from both Davis, California and Aguila, Arizona, whereas *G. 15* females were collected only from Aguila, Arizona. We used mature, virgin male crickets that were approximately 2 weeks past the final moult on the first day of the experiment to control for potential age- and gender-dependence in behavioural traits (Hedrick and Kortet, 2006) and immune defence (e.g. Vainio *et al.*, 2004). We studied 53 *G. integer* from California, 33 *G. integer* from Arizona, and 35 *G. 15* from Arizona.

Laboratory crickets were maintained at $25 \pm 1^\circ\text{C}$ with *ad libitum* food (Purina chick starter) and water, under a 12:12 h light/dark photoperiod. Experimental males were removed from bulk family boxes as nymphs (approximately 1/4 adult size) and reared individually (also with *ad libitum* food and water) in waxed cardboard cups. Individuals were physically, but not acoustically, isolated from other cricket individuals to ensure virginity and control for experience.

Anti-predator trials

Anti-predator trials were designed to measure an animal's latency to become active and emerge from a safe refuge when placed in a novel environment. These trials (methods modified from Hedrick, 2000) were conducted in a sound-proof, temperature-controlled dark room chamber ($26 \pm 1^\circ\text{C}$), containing a computer, desk, and red filter light (25-W red incandescent bulb, 60 cm distance from the arena). The red light was used to mimic nocturnal conditions.

At the beginning of a trial, an experimental male was placed in a clean experimental vial (clear translucent plastic vial covered with a layer of Scotch tape to make it semi-opaque, 4 cm in diameter and 6½ cm long), set upright in an experimental arena (Rubbermaid food storage container; 17 × 17 × 10 cm high). The cricket inside the vial was given 2 min to acclimate to the environment. After acclimation, we began the trial by carefully placing the vial down lengthwise in the arena and setting a plexi-glass cover over the top of the arena. We then recorded three time variables: 'activity', 'head', and 'body'. 'Activity' was defined as the number of seconds from trial initiation until the cricket's first movement within the vial. 'Head' was defined as the time when the cricket's head first emerged from the vial (broke the plane of the vial opening). 'Body' was defined as the time when the cricket's entire body exited the vial. For all crickets, the time at which the cricket's head emerged was highly correlated with the time at which all of the body emerged (Spearman $r = 0.982$, $P < 0.001$), so hereafter we report only the 'head' times as a measure of emergence (hiding time). We excluded values of 2 s or less because these probably did not reflect hiding behaviour, but rather escape behaviour.

The trial lasted for 10 min; if a cricket had not moved within 10 min, the trial was ended. Times were entered into the computer during the trials using the AV Bio-Statistics Professional 4.5 program (available at <http://www.cc.jyu.fi/~ansvain/avbs/>) (© Anssi Vainikka). Crickets were weighed after the trials to ensure that handling did not have any effect on their behaviour (fresh body mass to the nearest 0.0001 g). No cricket was used in more than one trial.

After a cricket had exited the vial, we performed a second test of anti-predator behaviour by presenting the cricket with a simulated predator cue. To simulate a predator, we dropped a small petri dish (diameter 37 mm) from a height of 10 cm, 30 cm outside of the arena onto a large, overturned plastic box (bottom thickness 4 mm) on which the arena rested so that crickets could sense the vibrations transmitted through the bottom of the arena. The crickets initially responded to this cue by ceasing motion (i.e. freezing). We recorded the time it took for the cricket to resume activity after freezing. This freezing test was conducted only for crickets that emerged from a vial within 10 min.

Immune assays

After the behavioural experiments, the crickets were anaesthetized by chilling them on ice in a freezer for 3 min. To measure encapsulation rate, a 2.0 ± 0.1 mm long piece of nylon monofilament (diameter 0.18 mm, rubbed with sandpaper, knotted at one end) was inserted through a puncture in the pleural membrane between the second and third sternite. After insertion of the monofilament, crickets were placed in individual vials and kept at a constant temperature ($28 \pm 1^\circ\text{C}$) for 24 h to allow for an immune response. Our preliminary experiments demonstrated that this was a suitable period for detecting variation in the encapsulation response among individuals. At the end of this period the implant was removed, and the monofilament was later photographed from three different angles under a light microscope with a digital video recorder. These pictures were analysed using the Image J program (version 1.34s). The degree of encapsulation response was quantified as grey values of reflecting light from implants. The Image J program gives the grey values from the selected area of the picture. As a measure of encapsulation rate, we used the average grey values of three video pictures. The data were transformed so that the darkest grey values would correspond to the highest encapsulation rate. This transformation was done by

subtracting the observed grey values from the control grey value (clear implant) (see Rantala and Kortet, 2003; Rantala *et al.*, 2003). The repeatability of this method is high in crickets (see Rantala and Roff, 2006). Note that this method for measuring encapsulation response is targeted to capture an aspect of the encapsulation, melanization, that is closely associated with its energetic cost (e.g. Simmons *et al.*, 2005).

During the dissection to remove the nylon monofilaments, we collected 5 μl of haemolymph from each cricket from a puncture in its abdomen. The haemolymph was then mixed with 50 μl phosphate-buffered saline solution (pH 6.4). Frozen samples were stored at -80°C . Lysozyme activity against *Micrococcus lysodeikticus* was assayed turbidometrically using methods similar to those of Rantala and Kortet (2003, 2004). A solution of 80 μl of 0.35 $\text{mg} \cdot \text{ml}^{-1}$ freeze-dried *M. lysodeikticus* buffer (pH 6.4) was mixed with 50 μl of the defrosted buffered haemolymph and placed in a plastic multicuvette (Labsystems cliniplate). The optical density of the mixture was measured at 490 nm at 25°C at 20-s intervals for 5 min with a plate reader (Softmax pro). All samples were analysed in random order. Lytic activity was expressed as total change in optical density, measuring the clearance rate of bacterial solution by haemolymph. The repeatability of this method is high in crickets (see Rantala and Roff, 2006). The results were converted to the standard unit values using dilution series of lysozyme from hen egg white (Merck, 50,000 units $\cdot \text{mg}^{-1}$). The haemolymph samples were converted to lysozyme-like activities (i.e. $\text{ng} \cdot \text{m}^{-1}$ equivalents of hen egg white lysozyme).

Statistical analyses

First, we studied normality of the parameters using the Kolmogorov-Smirnov test, then performed multivariate analysis of covariance (MANCOVA) with cricket weight as a covariate and male group as an independent variable for those variables that were normally distributed and had equal variances. Hiding time ('head') was not a normally distributed variable and did not fit the assumptions for MANCOVA, so it was analysed using Kaplan-Meier survival analysis (Crowley and Breslow, 1984) for population differences and non-parametric Spearman correlations for correlation analyses. We did not include freezing time in the MANCOVA because we measured this only for crickets that emerged from a vial within the 10-min experimental time. Correlation analyses between immune variables and behavioural variables in anti-predator trials (excluding hiding time) were performed using Pearson correlations. Two of the *G. 15* males died during the experiment on encapsulation response. Moreover, some of the study animals lost their implants during the experiment and some did not yield enough haemolymph for lytic assays. Thus, the sample sizes for statistical tests are smaller than the original numbers of animals studied.

RESULTS

The results of the MANCOVA indicated significant differences between groups of males (California *G. integer*, Arizona *G. integer*, and Arizona *G. 15*) in dependent variables (encapsulation response, lytic activity and behavioural cautiousness, measured as the first activity; Wilks' $\lambda = 0.218$, $F = 20.942$, $P < 0.001$). However, we observed no significant relationships between the covariate cricket weight and the dependent variables (Wilks' $\lambda = 0.973$, $F = 0.837$, $P = 0.477$). Univariate tests (Table 1) revealed that groups of males differed in encapsulation response and in behavioural cautiousness (the first activity), but not in lytic activity (see mean values in Table 2).

Table 1. Results of MANCOVA for the factor 'group' (California *G. integer*, Arizona *G. integer*, Arizona *G. 15*) for male crickets

Parameter	Details of test	<i>P</i>
Immune defence		
Encapsulation response	$F_{2,91} = 5.024$	0.009
Lytic activity	$F_{2,91} = 0.058$	0.944
Cautiousness		
Activity	$F_{2,91} = 4.118$	0.019

Note: The effect of the covariate weight was not significant in any of the tests.

Table 2. Mean values ($\pm$ one standard error) for the studied immune parameters in three groups of male field crickets from two habitats (California and Arizona)

	California, <i>G. integer</i>	Arizona, <i>G. integer</i>	Arizona, <i>G. 15</i>
Encapsulation response	68.55 ± 4.27 $n = 41$	86.23 ± 6.18 $n = 28$	91.75 ± 6.16 $n = 26$
Lytic activity (lysozyme-like activity values)	181.7 ± 21.7 $n = 41$	178.8 ± 15.1 $n = 28$	168.3 ± 27.1 $n = 26$

Pairwise Bonferroni-adjusted comparisons revealed that *G. integer* males in California had weaker encapsulation responses than *G. integer* males and *G. 15* males in Arizona, but the two cricket groups in Arizona (*G. integer* and *G. 15*) did not differ from one another (see mean values in Table 2). *Gryllus integer* males in California were less cautious (mean first activity time $\pm$ standard error: 146.2 ± 25.4 s, $n = 41$) than *G. integer* males in Arizona (293.8 ± 40.8 s, $n = 28$), while *G. 15* males in Arizona (221.9 ± 41.8 s, $n = 26$) did not differ from *G. integer* males in Arizona or in California.

When we analysed hiding times for all three groups of males using survival analysis, we found that overall differences were statistically significant – that is, crickets from the different groups recorded significantly different hiding times before emerging from the vial (Kaplan-Meier survival analysis, Gehan-Breslow test statistic = 7.599, d.f. = 2, $P = 0.022$) (Fig. 1). Planned comparisons between the three groups of males showed a significant difference in hiding times between *G. integer* in California (mean hiding time $\pm$ standard error = 357.2 ± 29.4 s, $n = 53$) and *G. integer* in Arizona (458.5 ± 38.9 s, $n = 33$) (Kaplan-Meier survival analysis, $P = 0.042$), and between *G. integer* in Arizona and *G. 15* in Arizona (292.1 ± 43.2 s, $n = 35$) (Kaplan-Meier survival analysis, $P = 0.008$), but *G. integer* in California did not differ from *G. 15* in Arizona (Kaplan-Meier survival analysis, $P = 0.234$).

In *G. 15* from Arizona, lytic activity was significantly negatively correlated with encapsulation responses ($n = 28$, Pearson two-tailed $r = -0.454$, $P = 0.015$), but not in *G. integer* from Arizona ($n = 28$, Pearson two-tailed $r = -0.333$, $P = 0.083$). Among California crickets

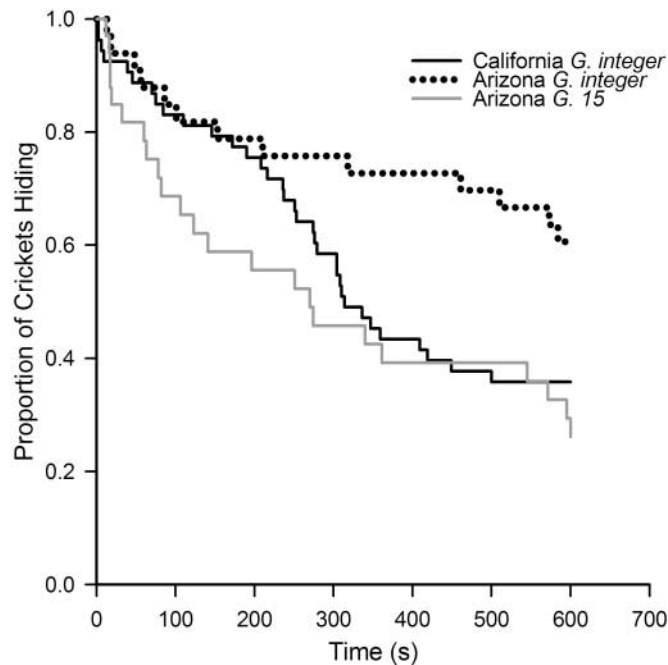


Fig. 1. Kaplan-Meier survival analysis of the emergence time in anti-predatory trials (proportion of crickets still hiding versus time) for California (*G. integer*) and Arizona (*G. integer* and *G. 15*) field crickets. Survival curves for all data from 0 to 600 s. The groups of males differed significantly (Kaplan-Meier survival analysis, Gehan-Breslow test statistic = 7.599, d.f. = 2, $P = 0.022$).

there was no significant correlation between lytic activity and encapsulation response ($n = 41$, Pearson two-tailed $r = -0.066$, $P = 0.340$). Over all male groups, lytic activity was significantly negatively correlated with freezing time ($n = 59$, Pearson two-tailed $r = -0.288$, $P = 0.026$), but not with the first activity ($n = 104$, Pearson two-tailed $r = -0.068$, $P = 0.480$) or hiding time ($n = 111$, Spearman two-tailed $r = 0.005$, $P = 0.962$).

Correlations between immune variables and behavioural cautiousness in anti-predator trials revealed different patterns in crickets from the two habitats (Table 3). In California *G. integer*, immune parameters were not related to male cautiousness in anti-predator trials (Table 3). However, in Arizona *G. integer*, encapsulation response was positively associated with a cricket's hiding time ($n = 30$, Spearman two-tailed $r = 0.423$, $P = 0.020$), and a similar trend occurred for the association between encapsulation and the first activity ($n = 30$, Pearson two-tailed $r = 0.359$, $P = 0.052$). However, these patterns were not detectable among *G. 15* in Arizona (Table 3).

DISCUSSION

The present results indicate notable population differences in encapsulation response, which is a major mechanism used by insects to defend themselves against multicellular pathogens and parasites such as parasitoid flies (Gillespie *et al.*, 1997). *Gryllus integer* males in the Davis, California population had weaker encapsulation responses than *G. integer* males in the Aguila, Arizona population. *Gryllus 15* in Arizona also had stronger encapsulation

Table 3. Two-tailed correlations (P = Pearson, S = Spearman) between immune defence parameters and cautiousness in anti-predator trials in male field crickets from two habitats (California and Arizona)

	Anti-predator behaviour		
	Activity	Emergence	Freezing time
California, <i>G. integer</i>			
Encapsulation response	−0.033 (P) <i>n</i> = 44, <i>P</i> = 0.415	−0.015 (S) <i>n</i> = 44, <i>P</i> = 0.462	0.051 (P) <i>n</i> = 27, <i>P</i> = 0.399
Lytic activity	−0.079 (P) <i>n</i> = 50, <i>P</i> = 0.293	0.117 (S) <i>n</i> = 50, <i>P</i> = 0.209	−0.302 (P) <i>n</i> = 30, <i>P</i> = 0.052
Arizona, <i>G. integer</i>			
Encapsulation response	0.359 (P) <i>n</i> = 30, <i>P</i> = 0.052	0.423 (S) * <i>n</i> = 30, <i>P</i> = 0.020	−0.009 (P) <i>n</i> = 10, <i>P</i> = 0.486
Lytic activity	−0.204 (P) <i>n</i> = 31, <i>P</i> = 0.271	−0.69 (S) <i>n</i> = 31, <i>P</i> = 0.169	−0.661 (P) * <i>n</i> = 11, <i>P</i> = 0.027
Arizona, <i>G. 15</i>			
Encapsulation response	0.234 (P) <i>n</i> = 29, <i>P</i> = 0.222	0.056 (P) <i>n</i> = 29, <i>P</i> = 0.773	0.199 (P) <i>n</i> = 18, <i>P</i> = 0.430
Lytic activity	−0.092 (P) <i>n</i> = 30, <i>P</i> = 0.631	−0.036 (P) <i>n</i> = 30, <i>P</i> = 0.849	−0.330 (P) <i>n</i> = 18, <i>P</i> = 0.181

* Significant correlations (*P* < 0.05).

responses than *G. integer* in California. We have previously shown that both the risk of parasitism by parasitoid flies and the risk of predation is much higher in Arizona than in California (Hedrick and Kortet, 2006). In the present study, we found that our *G. integer* study populations also differed in the association between male encapsulation response and behavioural cautiousness: males with higher encapsulation responses behaved less actively and hid longer in the Arizona population, but not in the California population. Taken together, these results suggest that the presence of predators and parasites selects for an increase in the crickets' investment in the encapsulation response, even in populations where the insects are confronted with higher risks of predation. This is opposite from the result expected if there are trade-offs between encapsulation response and anti-predator behaviour within individuals, as predicted by life-history theory, and these trade-offs appear at the population level. In general, however, variation between individuals in the amount of resources available to invest in immunocompetence and other traits can make direct relationships difficult to observe at the population level (Van Noordwijk and de Jong, 1986; Reznick *et al.*, 2000). Also, the data presented here are purely correlational, and the trade-off might be revealed in a carefully planned experiment using animals of similar phenotypic condition and size, in which some crickets are directly presented with predators and/or parasitoids, and others serve as controls.

We found indicative support for the immunological costs of anti-predator behaviour, since lytic activity was negatively correlated with freezing time, even though lytic activity

was not correlated with the other behavioural variables we studied. Cautious crickets that are less active and hide for longer periods than bolder crickets should have less time for foraging and competing for resources than bolder crickets. Thus, if everything else is equal, cautious crickets should be less able to invest in their immune defence, as our results on lytic activity and freezing time suggest. Anti-microbial peptides such as lysozyme are likely costly to produce, since body fat is needed when they are synthesized (e.g. Hetru *et al.*, 1998). Lysozyme hydrolyses β -1,4 linkages in the peptidoglycan of bacterial cell walls and it has been suggested to play a major role in the humoral immune response of insects against bacterial infection (Götz and Trenczek, 1991). However, work by Adamo (2004) suggests that baseline lytic activity may not always be correlated with the ability of crickets to fend off experimental challenges. In our study, a haemolymph sample was taken after the removal of the implant from a male. Thus, the lytic activities reported here are not pure baseline values, since a cricket's body had 24 h to react and potentially raise its lysozyme level after the implant was inserted. Moreover, we believe that our results represent normal lytic activity in the crickets, since we did not observe any diseases or bacterial epidemics in the laboratory during the experiments and all the study animals were behaving normally and looked healthy. Previous work on snails by Rigby and Jokela (2000) demonstrated notable immunological costs (a depression of the percentage of phagocytic haemocytes) of anti-predator behaviour. However, in contrast to field crickets, the anti-predator response of the snails involves a direct physiological cost, since it consists of retreating deeply within the shell after expelling blood.

The positive correlation we found between encapsulation response and behavioural cautiousness in Arizona field crickets also suggests that the encapsulation response could be linked to additional behavioural traits in these crickets. Previously, we demonstrated that *G. integer* males that won more intra-sexual fights, indicating their potential for dominance, hid for shorter times and behaved less cautiously than their subordinate contestants (Kortet and Hedrick, in press). This suggests that there may be a 'behavioural syndrome' (*sensu* Sih *et al.*, 2004) for aggressiveness and activity in *G. integer*, and that aggressive males could have weaker encapsulation responses (but see Rantala and Kortet, 2004; Väänänen *et al.*, 2006). It is also possible that the males of the Arizona population that behaved more cautiously in anti-predator trials were in better condition than the males that behaved more boldly. A male in good condition does not have to take risks to achieve more nutritional resources compared with a male in poor condition. In Arizona, predation and parasitism may strongly select for crickets that are both good at collecting resources and that have high immune function, resulting in a positive correlation between long hiding times and strong encapsulation responses.

Generally, immune functions seem to be condition-dependent (e.g. Siva-Jothy and Thompson, 2002; Rantala *et al.*, 2003; Koskimäki *et al.*, 2004), which may have yielded the observed positive correlation between encapsulation response and behavioural cautiousness. Unfortunately, we did not measure condition or body fat of the crickets used in this study. It would be interesting to repeat this work, adding a starvation treatment to manipulate body condition (see, for example, Moret and Schmid-Hempel, 2000; Rantala *et al.*, 2003). Often negative correlations only appear under poor conditions or when background variation is removed.

We found a species difference in emergence times between Arizona *G. integer* and Arizona *G. 15*: *G. integer* behaved more cautiously. It is possible that bolder *G. 15* might compensate for the risk of being parasitized by a fly by stronger encapsulation responses (as indicated by our results). These results indicate that species within a single habitat can have different

behavioural strategies against predation measured as hiding time. Alternatively, it is possible that our measure of time spent hiding measures exploratory behaviour, rather than boldness towards predators. However, we think that both these types of behaviours are related, since an actively exploring animal is more likely to be detected and captured by predators than his less active conspecifics.

In conclusion, increased pressure by predators and parasitoids may shape the cricket's investment in immune response, perhaps through the potential costs of anti-predator behaviour. However, more detailed experimental studies are needed to investigate this interesting topic in evolutionary ecology.

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