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Plasticity of immune function and condition under the risk of predation and parasitism

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ABSTRACT

Ecological immunology attempts to elucidate the causes of the large variation in immunity and resistance observed in natural populations. Here we report on a novel experiment that investigated how the risks of parasitism and predation altered investment in immunity and condition in insects during larval development. The study organism is the damselfly *Coenagrion puella*, the parasite is a water mite and the predators are encaged *Aeshna cyanea* dragonflies. Our experiments show that females increase their investment in a cellular as well as a humoral component of the immune system in the presence of natural enemies. By contrast, males do not show such alteration. However, males show altered condition under the risks of parasitism and predation. Our results highlight the importance of species interactions for the plasticity of immune function.

Keywords: dragonflies, ecological immunology, differences between the sexes, natural enemies, plasticity, water mites.

INTRODUCTION

In the wild, organisms face multiple natural enemies, which are often present simultaneously (Crawley, 1992; Sih *et al.*, 1998). Facing multiple enemies can result in trade-offs, such that avoiding one enemy results in a higher encounter rate with another enemy (Decastaeker *et al.*, 2002). However, only a few studies have examined the impact of multiple enemies simultaneously. These have focused on behavioural as well as life-history changes (e.g. Baker and Smith, 1997; Eklöv and Werner, 2000; Decastaeker *et al.*, 2002; Lass and Bittner, 2002). Our aim was to examine the effects of multiple natural enemies on the plasticity of immune function and condition (see Rolff and Joop, 2002, for the use of condition) in larval insects.

We concentrate on these traits because they are closely linked to fitness and to one another (Plaistow and Siva-Jothy, 1996; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003). Furthermore, we included sex in our analysis. Differences between the sexes are often

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neglected in such ecological studies, even though they have been shown to be important (Rolff, 2002; Zuk and Stoehr, 2002, and references therein), as males and females take different routes to fitness (Bateman, 1948). To the best of our knowledge, only one experiment has studied the impact of multiple enemies with regard to immunity (Rigby and Jokela, 2000). The present study builds on that of Rigby and Jokela (2000) in four important aspects:

1. Animals are exposed to a perceived risk of parasitism and predation, respectively.
2. Allocation during larval development is looked at.
3. We include condition.
4. We examine differences between the sexes as opposed to hermaphrodites.

Insects with aquatic larvae and terrestrial adults undergo an extreme habitat shift (Johansson and Rowe, 1999; Plaistow and Siva-Jothy, 1999). During this habitat shift, damselflies are probably at the most vulnerable stage of their life history (Corbet, 1999). Differences in immunity and condition caused earlier in their larval stage should become more pronounced and determine the success of the habitat shift.

We used the damselfly *Coenagrion puella* as the study organism, the predators were larvae of the dragonfly *Aeshna cyanea* and the parasites were larvae of the water mite *Arrenurus cuspidator*. Insect predators have been shown to influence the choice of microhabitat (Suhling and Lepkojus, 2001), reduced survival rates and changes in activity in larval damselflies and dragonflies (Stoks *et al.*, 1999). The presence of predators imposes stress that can cause malnutrition (Johansson and Leonardsson, 1998) or reduce digestive efficiency (Stoks and McPeek, 2003). The risk of predation can easily be exerted with caged predators (Koperski, 1997). Parasites also affect the life history and behaviour of their host (Moore, 2002). Water mites have been shown to reduce, among other traits, the survivorship of adult damselflies (Braune and Rolff, 2001). Water mite larvae settle on damselfly larvae in the final instar but do not parasitize until the larvae emerge (Rolff, 2001). This enabled us to manipulate the risk of parasitism of adult dragonflies. Larval water mites induce behavioural changes in larval damselflies (Baker and Smith, 1997; Leung *et al.*, 1999). Such changes have been interpreted as parasite avoidance behaviour (Baker and Smith, 1997). Hence larval dragonflies are capable of perceiving the risk of parasitism of their adult stage. We concentrated on the late larval instars of the damselflies, as this is the time when they share their microhabitats with the predator (Inden-Lohmar, 1997) and parasite (Mitchell, 1967) used in this study.

Innate immunity has been defined as 'a set of disease-resistance mechanisms that are not specific to a particular pathogen' (e.g. due to anatomy, physiological, phagocytotic and inflammatory mechanisms (Goldsby *et al.*, 2000)). We studied two different components of the insect innate immune system: cellular defence and an important part of humoral defence, the phenoloxidase cascade (Gillespie *et al.*, 1997). Haemocytes, insect blood cells, play an important role in insect immune defence. They recognize pathogens and are involved in encapsulation and phagocytosis (Kurtz *et al.*, 2000). There is also good evidence that haemocyte count is positively related to successful immune defence (Fellowes and Godfray, 2000; Kurtz *et al.*, 2000). Phenoloxidase is an enzyme present in most arthropods' haemolymph and cuticle (Sugumaran, 2002). Phenoloxidase catalyses the production of melanin, which is involved in wound repair and encapsulation. Using *Drosophila* mutants, Braun *et al.* (1998) demonstrated that phenoloxidase activity is positively correlated with

resistance against a variety of pathogens. We measured condition by assessing different physiological traits (Rolff and Joop, 2002) – fat content, muscle mass and skeletal size – all of which are related to fitness in damselflies (Marden, 1989; Plaistow and Siva-Jothy, 1996; Sokolovska *et al.*, 2000).

Here, we examine the notion of whether insects alter their allocation to immune function and condition in the presence of natural enemies. Because of the parasites' life cycle and the caging of the predators, we were able to manipulate the perceived risk of parasitism and predation.

MATERIALS AND METHODS

Study organisms

The target species *C. puella* (Odonata, Insecta) is a common and well-studied (e.g. Corbet, 1999; Rolff, 2001) damselfly of northern and central Europe, usually occurring in small ponds. Larvae hibernate in later instars. Shortly before emergence, the damselfly larvae move to shallow water regions. The damselfly larvae were caught in the first 2 weeks in April 2001 in the area of 'Klei' near Braunschweig (Lower Saxony, Germany, 52°21′N, 10°35′E), when they were in the last three instars.

Arrenurus cuspidator (Hydrachnellae, Acarina) is a common ectoparasite on damselflies in central Europe (Rolff, 2001). After the damselfly's emergence, the mites climb from the exuvia to the newly emerged adult. The mite larvae then pierce the cuticle and feed on haemolymph and tissues. During oviposition, mites from male and female damselflies detach and complete their life cycle (Rolff, 2001).

Aeshna cyanea (Aeshnidae, Odonata) is a widespread and abundant dragonfly species in Europe, and its habitat overlaps strongly with that of *C. puella* (Inden-Lohmar, 1997). The larval stage lasts 2 years (Inden-Lohmar, 1997); last instars of *A. cyanea* can predominantly be found close to the water surface, an area which is preferred by late instars of *C. puella* as well.

Experimental design

All damselfly larvae were held under the same temperature, feeding conditions and light. We used a full factorial design with the following non-lethal risk treatments, each replicated four times with 25 *C. puella* larvae per tank: (a) control, (b) with parasite (approximately 400 *A. cuspidator* larvae), (c) with predator (one caged *A. cyanea* larva), (d) with parasite and predator (later referred to as two risks). Unfortunately, one predation replicate became contaminated with *A. cuspidator*. This tank was therefore excluded from the analyses.

Each of the 16 tanks was filled with gravel to a height of 2.5 cm and with tap water to a height of 16 cm (total water volume of 6.7 litres). Each tank was initially stocked with 25 damselfly larvae, resulting in a density of about 250 larvae per square metre, which is comparable to natural conditions (Banks and Thompson, 1987). As the larvae were in different instars, their head width was measured. Instars were distributed equally among tanks, but at random within instars. The tanks were placed randomly. The experiment began on 24 April 2001.

Aeshnid larvae in the ante-penultimate stadium F-2 were caged ($17.6 \times 7.5 \times 7.5$ cm) to avoid predation (Koperski, 1997). The cages were covered with mesh and gauze (mesh

width = 0.5 mm). This allowed water to flow through the cage and the damselfly larvae to detect the predator visually and chemically (Koperski, 1997). To ensure that the presence of a cage had no influence on damselfly behaviour, cages were placed in each tank. Other furniture included a plastic plant and a climbing structure.

Johansson (1996) showed that feeding six *Daphnia* sp. to a coenagrionid damselfly larva every other day guarantees feeding to satiation. If the damselflies are fed to satiation, they may not be resource limited; therefore, they were fed at a lower level. Because of the rising temperatures from April to May, the damselflies were fed at changing intervals, as can be seen in Table 1. *Aeshna cyanea* were fed biweekly with *Chironomus* sp. *Arrenurus cuspidator* larvae do not feed during the free-living stage.

From 17 May 2001, an emergence structure was presented to the damselfly larvae, for the first 19 days for 2 h per day (10.00–12.00 or 12.00–14.00 h) and thereafter for 4 h per day (10.00–14.00 h). When a larva climbed out of the water, it was caught with tweezers and placed separately into ice-cold water to prevent emergence (see Rolff, 1999). This slows down all physiological reactions. We did not collect the damselflies after emergence because after eclosion the mites become parasitic and interfere with the immune system of the host. The animals were stored until 14.00 h in the cold water on ice and the following measurements were conducted.

Morphometric and physiological measurements

The larvae were removed from the water and gently dried with paper tissue before being placed in a micro-centrifuge tube and kept on ice for chilling. Their fresh weight was measured to the nearest 100 μg (Mettler PM 480 DeltaRange). After chilling them on ice again, blood samples were taken (see below), the damselflies were sexed and the head width measured. A *camera lucida* and a digital vernier calliper (Mitutoyo, Digimatic) were used for measuring head width. Head width is a common indicator of size in damselflies (Banks and Thompson, 1987).

Following the protocol of Barnes and Siva-Jothy (2000), haemolymph extracts were obtained by perfusing with 0.3 ml cacodylate buffer (0.01 M Na-Coc, 0.005 M $CaCl_2$). The last abdominal segment was cut off and the buffer injected into the ventral side of the first thoracic segment (syringe: Beckton Dickinson, Micro-fine, 0.3 ml, 0.33×12.7 mm). The sample was aliquoted for haemocyte counting and the assaying of phenoloxidase activity (see below). The bled larvae were frozen at −90°C and kept for analyses of fat content and muscle mass.

Twenty microlitres of each blood sample were pipetted into one well of a multi-well slide coated with poly-D-lysin (0.01 mg per 1 ml) (M.T. Siva-Jothy, personal communication).

Table 1. Feeding levels during the experiment

Duration	Average max. room temperature (°C)	Frequency	Number of *Daphnia* sp. per damselfly
24/04 to 30/04 2001	13.14	Every third day	4
01/05 to 12/05 2001	14.46	Every second day	4
13/05 to 05/07 2001	18.35	First day, second day, alternating	4, 2

The slides were incubated in a humid chamber at room temperature for 1 h. Then, 1 μl DAPI (4,6-diamidino-2-phenylindol dihydrochloride) solution was added as a fluorescent stain and the slides were left to dry in a dark box over night. Haemocytes were counted using a Leitz-Diaplan microscope with an image analyser (optimas 6.1, Optimas Corporation). As it was not possible to count a whole well, three pictures were taken at random and the average cell counts determined (magnification × 20) (Ryder and Siva-Jothy, 2001).

To analyse phenoloxidase activity, the samples were thawed in ice water and the cell walls removed via centrifugation (4°C, 6500 rpm, 15 min, Eppendorf centrifuge 5417R). Then, 0.2 ml of the supernatant was added to 0.6 ml L-DOPA (10 mM in Na-Coc buffer). Readings of the absorbance were recorded every minute for 30 min at 30°C and the slope was calculated from these, using SWIFT II analysis software (see Barnes and Siva-Jothy, 2000, for details).

The frozen larvae were thawed and freeze-dried for 24 h (LSL SECFROID, LYOLAB B), and their dry mass was measured to the nearest 10 μg (Mettler AE 160). Fat extraction followed the protocol of Plaistow and Siva-Jothy (1996, and see references within), using a Soxhlett extractor, for 8 h of continuous reflux (Plaistow and Siva-Jothy, 1996; Rolff and Joop, 2002). After fat extraction, the damselflies were freeze-dried again and re-weighed. The calculated difference between dry-weight and fatless-weight was taken as fat content.

The fatless and dried bodies were covered with 0.35 M sodium hydroxide (NaOH) for 24 h at room temperature in 1.5-ml micro-centrifuge tubes. After soaking, the hydroxide was removed, replaced with distilled water for an hour, and the bodies freeze-dried for 24 h (see Marden, 1989). Muscle mass was calculated from the difference between the fatless mass and the muscle-less mass. Cuticle weight is the remaining weight after fat and muscle extraction has been performed.

Statistical analysis

We analysed the data using full-factorial analyses of covariance. The factors were predation and parasitism, and tank means of the traits under study were used as the response variable. Size and day of emergence were entered as covariates. Day of emergence measures the time at which individuals within one tank were on average exposed to treatments. The data were analysed separately for sex (see below for examining differences between the sexes), because physiological differences were expected *a priori* (e.g. Kurtz *et al.*, 2000; Braune and Rolff, 2001). The other physiological traits – wet weight, dry weight, cuticle weight, fat content and muscle mass – were highly correlated. All these traits are related to condition in *C. puella* (Rolff and Joop, 2002). We defined condition as the 'status of physiological reserves that are essential and available for the traits under study' (Rolff and Joop, 2002). They were collapsed into two variables using a principal component analysis based on the correlation matrix (which accounts for different scaling of the data). We ran separate principal component analyses for males and females, because we knew *a priori* of differences between the sexes. Subsequent tests were performed using the principal components as response variables. Parasite × predator interactions were removed if non-significant.

The sexes differ in immune traits in *C. puella* (G. Joop, unpublished data). However, our prime interest was not differences between the sexes *per se*, but to determine whether the plasticity of immune function is different in different environments (i.e. treatments). We first

calculated differences in immune trait expression between male and female tank means. We then ran a model with these calculated differences as response (dependent) variables and the risks of parasitism and predation as factors. This was not possible for the other physiological traits, because different traits loaded in a different manner on the principal components extracted.

RESULTS

Haemocyte density in females was affected by the risks of parasitism and predation (see Table 2, Fig. 1). By contrast, male haemocyte density was not affected by the risks of

Table 2. Results of analyses of covariance for haemocytes, phenoloxidase activity and condition by sex

	Males			Females		
Source	d.f.	*F*	*P*	d.f.	*F*	*P*
Haemocytes						
Predator	1	0.034	0.857	1	0.348	0.570
Parasite	1	1.190	0.301	1	1.586	0.240
Day of emergence	1	1.251	0.270	1	7.426	0.023
Head width	1	0.002	0.969	1	4.269	0.069
Predator × parasite				1	9.922	0.033
Residuals	10			9		
Phenoloxidase						
Predator	1	0.691	0.427	1	3.478	0.095
Parasite	1	0.127	0.730	1	0.145	0.712
Day of emergence	1	2.667	0.137	1	2.162	0.176
Head width	1	1.103	0.321	1	0.555	0.475
Predator × parasite	1			1	7.319	0.024
Residuals	10			9		
PC1						
Predator	1	4.844	0.052	1	0.061	0.810
Parasite	1	5.837	0.036	1	0.721	0.416
Day of emergence	1	3.640	0.085	1	1.583	0.237
Head width	1	0.529	0.484	1	1.562	0.240
Residuals	10			10		
PC2						
Predator	1	0.008	0.931	1	1.583	0.240
Parasite	1	5.879	0.038	1	0.379	0.553
Day of emergence	1	0.514	0.491	1	0.688	0.428
Head width	1	3.754	0.085	1	0.537	0.482
Predator × parasite	1	3.523	0.093	1	2.931	0.121
Residuals	9			9		

Note: PC1 and PC2 for males are not the same as for females (see Table 3).

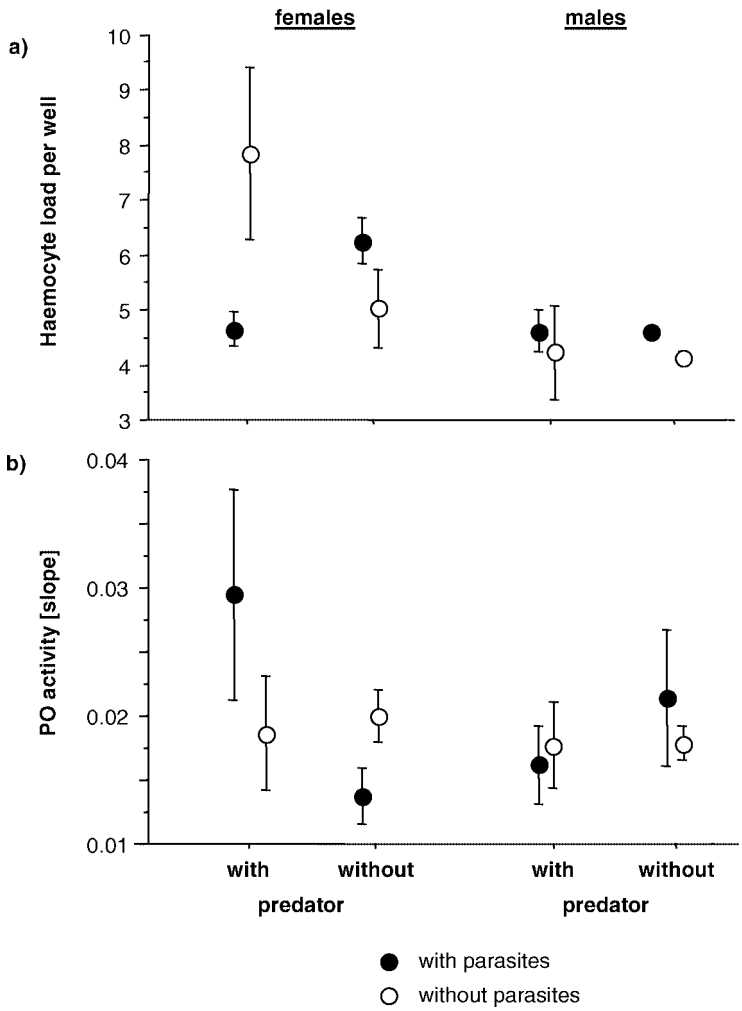

Fig. 1. The effects of treatment on (a) female and male haemocyte load (square root-transformed) and (b) female and male phenoloxidase (PO) activity (log-transformed). Error bars represent standard errors of the mean.

parasitism and predation (Table 2, Fig. 1). The plasticity of male and female haemocyte density differed significantly across treatments (predator × parasite: $F_{1,9} = 13.4$, $P = 0.005$, response variable (female tank mean – male tank mean); see 'Materials and methods' for the structure of the model).

Females showed an altered phenoloxidase activity under the risks of parasitism and predation (see Table 2, Fig. 2). The phenoloxidase activity in males was not altered by treatment (Table 2, Fig. 2). Male and female plasticity in phenoloxidase activity differed significantly (predator × parasite: $F_{1,9} = 6.9$, $P = 0.028$; see 'Materials and methods' for the structure of the model).

We extracted two principal components (PCs) for males and females, respectively (Table 3). For males, PC1 correlates highly positively with muscle mass, dry weight and

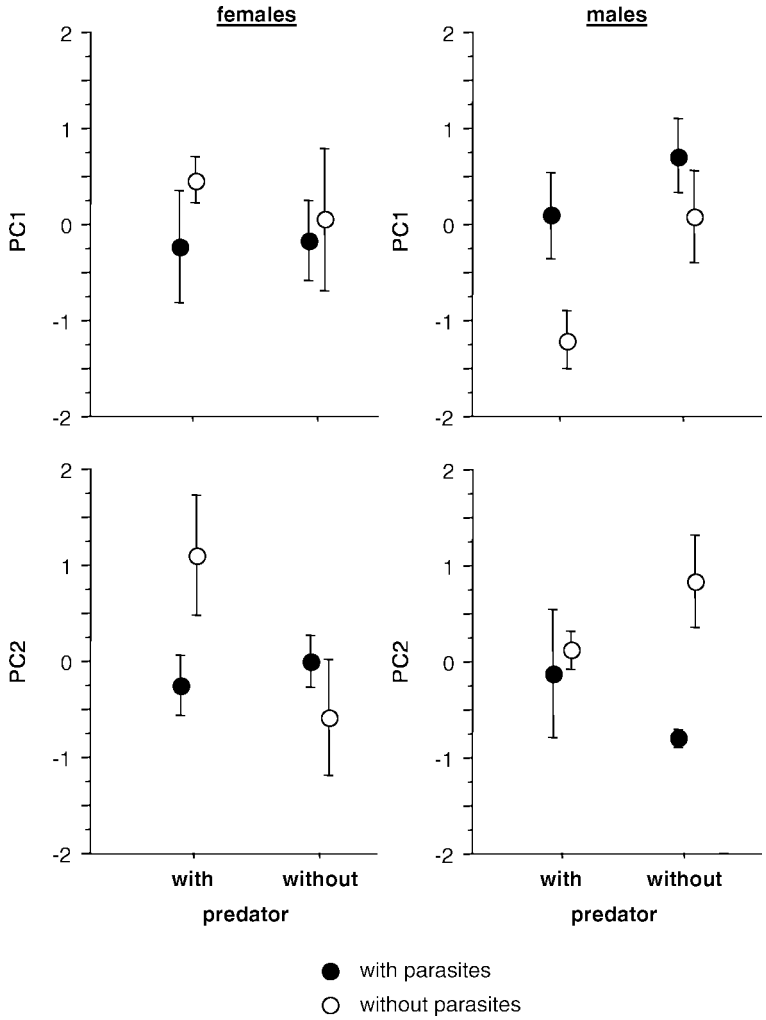

Fig. 2. Treatment effects on condition for males and females as measured by PC1 (upper panels) and PC2 (lower panels). Note that both PC1 and PC2 are estimated separately for females and males (see Table 3). Error bars represent standard errors of the mean.

cuticle weight and explains 57.95% of the variance. PC2 correlates highly positively with fat content and fresh weight and explains 26.64% of the variance. Together, PC1 and PC2 explain 84.59% of the variance. Predators and parasites have an effect on PC1 such that condition is altered under the risk of parasitism and we also found a nearly significant effect of predation risk (Table 2). PC2 (mainly related to fat content) is decreased in the presence of parasites.

For females, PC1 correlates highly positively with muscle mass, dry mass, fat content and wet mass and explains 64.14% of the variance. PC2 correlates highly positively with cuticle mass, but less so with fat content and dry mass, and explains 18.20% of the variance. Together, PC1 and PC2 explain 82.34% of the variance. In females, condition is neither altered by the risk of parasitism nor by the risk of predation (Table 2).

Table 3. Factor loadings of the physiological traits on the principal components

	Males		Females	
Trait	PC1	PC2	PC1	PC2
Fresh weight	0.166	0.847	0.847	–0.010
Dry weight	0.859	0.489	0.853	0.492
Cuticular weight	0.876	0.028	0.088	0.951
Fat content	0.073	0.916	0.651	0.475
Muscle mass	0.918	0.086	0.922	0.144

Note: separate principal components were calculated for males and females.

DISCUSSION

The results show that the perceived risks of predation and parasitism significantly alter the components of immune function and condition of larval *C. puella*. The experimental set-up allowed for independent manipulation of parasitism and predation risks, as well as these two factors combined. Females altered the expression of the two immunological traits studied, haemocyte density and phenoloxidase activity. Both were increased in the presence of natural enemies. However, haemocyte load was increased only in the presence of a single natural enemy. Phenoloxidase activity only increased in the simultaneous presence of both natural enemies. By contrast, male immune function was neither influenced by the risk of predation nor the risk of parasitism. Furthermore, females have a higher haemocyte density than males. This confirms findings in other insect species (see Kurtz *et al.*, 2000; Rolff, 2002, for references).

Even though both sexes are exposed to the same risks of parasitism and predation, their reaction differs. The increase in components of the immune system, such as haemocyte number, in the presence of predators can be useful because these mechanisms are involved in wound repair (Sugumaran, 2002). Wounding occurs regularly in damselfly larvae (Baker and Dixon, 1986). Predators often remove lamellae from their prey without actually killing it (Stoks, 1999). Such wounds are potential sites for pathogen invasions. Why, however, do females invest either in phenoloxidase or in haemocytes? And why do males not react to the presence of natural enemies in this way? One could postulate that females might possess more plasticity in immune function, in the case of haemocytes simply because they have more than males. Females may also differ in their microhabitat use as well as in the efficiency of food digestion. Furthermore, there are differences in life-history trajectories, as males maximize fitness by increasing the mating rate (Bateman, 1948), whereas for females the main predictor for fitness is longevity (Banks and Thompson, 1987). Males are more plastic in late larval instars (Baker *et al.*, 1992). Females put more weight on between emergence and sexual maturity than males (Anholt *et al.*, 1991). Because of the physiological analyses, we were unable to determine whether females trade-off immunity against, for example, fecundity later in life. We can, however, state that parasitized females produce smaller clutches (Rolff, 1999) and show higher mortality (Braune and Rolff, 2001) than parasitized males. Therefore, there may be a greater selection pressure on females to invest in immune components (Rolff, 2002). *Coenagrion puella* does not exhibit successful

resistance against the water mite *A. cuspidator* (Rolff, 2001). However, because of the haemolymph loss and opportunistic infections, investment in immune function might pay off in the long term. This argument is based on the assumption that females need to invest more in immunity than males (Rolff, 2002; Zuk and Stoehr, 2002). All these reasons may help to explain why females invest more in immunity in the presence of natural enemies. However, why they increase their haemocyte number in the presence of one but not two natural enemies, but increase phenoloxidase activity in the simultaneous presence of predator and parasite (two-risk treatment), is unclear.

We expected condition to be lower in the presence of predators. It has been shown that in the presence of fish or invertebrate predators, damselflies such as *Enallagma cyathigerum* (Koperski, 1997) and *Lestes sponsa* (Stoks *et al.*, 1999) decrease activity and digestive efficiency (Stoks and McPeek, 2003). In contrast to the immune traits, only males reacted to the presence of natural enemies. Males showed a decrease in condition, in the principal component (PC1) that mainly comprises muscle mass and dry weight, in the presence of predators. However, in the presence of parasites only, they showed an ambiguous reaction. PC2, the component that is mainly related to fat content and fresh weight, decreased. In contrast, PC1 increased. As survivorship after emergence depends on condition (Braune and Rolff, 2001), this shift in condition could be interpreted as adaptive. Males showed more plasticity in condition in the presence of predators or parasites than females. This might be explained by the fact that male coenagrionid dragonflies are more active in the last instars (Baker *et al.*, 1992). As activity is usually reduced in the presence of predators, the reduction in male condition could be stronger, even though there are currently no published data available on this issue.

The study of how plasticity of immune function is shaped in an ecological context, especially during the interaction with natural enemies, is only in its infancy (Rolff and Siva-Jothy, 2003). The few studies conducted to date have shown that social environment (Traniello *et al.*, 2002), habitat (Kurtz *et al.*, 2002), density (Wilson *et al.*, 2001) and diet (Feder *et al.*, 1997) influence the expression of the immune response. However, we are still a long way from a thorough understanding of how the environment shapes the investment in immune function (Rolff and Siva-Jothy, 2003) and alters allocation rules (Worley *et al.*, 2003). In this study, we experimentally combined two major sources of variability in immune function – differences between the sexes and interspecific interactions (Schmid-Hempel, 2003). We have shown that males and females differ in their investment in components of immunity and condition if they perceive risks posed by natural enemies.

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