

Immune expression in a damselfly is related to time of season, not to fluctuating asymmetry or host size

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Abstract. 1. Variation in immune responsiveness within and among species is the subject of the emerging field of ecological immunology. The work reported here showed that individuals of *Lestes forcipatus* Rambur differ in their likelihood of mounting immune responses, and in the magnitude of those responses, against a generalist ectoparasite, the water mite *Arrenurus planus* Marshall.

2. Immune responses took the form of melanotic encapsulation of mite feeding tubes, occurred in the few days after host emergence, and resulted in mites dying without engorging. Such immune responses were more probable and stronger for hosts sampled later rather than earlier in the season. Such responses may act as selection affecting seasonal patterns of egg hatching and larval abundance of mites.

3. Contrary to expectation, metrics of host size (wing length) and wing cell fluctuating asymmetry were not related to the likelihood of immune responses.

4. The importance of season on immune expression of insects has not been explored in detail. These results suggest possible trade-offs in allocation of melanin (or its precursors) to maturation versus immunity, and indicate the need for studies on the synergistic effects of weather and parasitism on host species that use melanotic encapsulation to combat parasites and pathogens.

Key words. *Arrenurus*, damselflies, generalist, *Lestes*, melanotic encapsulation, mites.

Introduction

Emerging theory on ecological immunology treats immune expression as being subject to trade-offs (Sheldon & Verhulst, 1996; Schalk & Forbes, 1997). One question of interest is why individuals, populations, or species should show variation in immune responses. Researchers have shown genetic variability for resistance within and among animal populations (Gregory *et al.*, 1990; Fellowes *et al.*, 1998a) but genetics probably do not capture the full variation in immune expression seen in nature. The work reported here examined the extent to which individuals of *Lestes forcipatus* (Rambur) damselflies (Lestidae: Zygoptera) show differential immune responses to a generalist parasite, the ectoparasitic water mite *Arrenurus planus* Marshall (Arrenuridae: Hydrachnida). The study examined whether time of season, wing cell asymmetry, or a metric of host size was related to adult males or females

responding immunologically to naturally varying numbers of mites.

Trade-offs are expected between immune system function and life-history traits linked to growth and reproduction (Folstad *et al.*, 1989; Sheldon & Verhulst, 1996). Empirical work has demonstrated costs of resistance (Fellowes *et al.*, 1998b, 1999a; Fellowes & Godfray, 2000). The costs and benefits of responding to parasites (or maintaining systems that can respond) probably depend on other factors. These factors include the probability of being parasitised, intensity of parasitism, damage that parasites might cause, and costs of resistance in the absence of parasitism (e.g. Forbes, 1993; Sheldon & Verhulst, 1996). Such factors may differ considerably within and among species because individuals can differ in exposure to parasites or in the costs of mounting immune responses. One main goal of within-species studies is to understand which hosts (or when hosts) should commit resources, time, and energy to defence.

The principal form of immune response in damselflies is melanotic encapsulation of mite feeding tubes (Åbro, 1982; but see Forbes *et al.*, 1999). Melanin has other uses, including

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hardening of the cuticle during the pre-reproductive period and thermoregulation (Corbet, 1999), both of which could depend on weather or time of season, which can influence metrics of damselfly fitness (Thompson, 1990). For temperate zone odonates, individuals that emerge earlier in the season tend to be subjected to more bouts of cool, rainy weather than individuals that emerge later (Corbet, 1999). Whether emergence time influences commitment of melanin resources to immunity rather than other functions has never been investigated.

It might also be predicted that individuals in better condition are better able to resist infection or infestation. This reasoning requires that immunity is costly. There are demonstrations of a cost of immunity for insects. These include studies observing insects varying in a task, then recording responses to simulated parasitism such as König and Schmid-Hempel's (1995) work on bumble-bees, and one study on reproduction of damselflies limiting immune responses (Siva-Jothy *et al.*, 1998). Other studies on *Drosophila* (Fellowes *et al.*, 1998b, 1999a; Fellowes & Godfray, 2000) have demonstrated direct costs of immunity. These include reduced female size and fecundity, potential decreased tolerance to desiccation, and increased attack from other parasitoids.

This study examined whether a metric of body size (wing length) was related to the probability of mounting an immune response. Wing cell fluctuating asymmetry was also scored. Fluctuating asymmetry of both continuous and meristic characters has been linked to size, condition, parasitism, and fitness in many animals including damselflies (Bonn *et al.*, 1996; Leung & Forbes, 1996). Furthermore, wing cell asymmetry has been related to timing of emergence in at least one damselfly (Hardersen *et al.*, 1999), however strengths of fluctuating asymmetry relationships across species are highly heterogeneous and weak overall (Leung & Forbes, 1996).

Importantly, melanotic encapsulation of feeding tubes of *Arrenurus* spp. mites by damselflies occurs early in adult life before host reproduction (Åbro, 1979, 1982; see below). Thus, any variable responses should not be confounded by differences in timing of parasitism or variation in reproductive effort. Some observational and experimental studies support a relationship between reproductive effort and either increased parasitism or reduced immunity (reviewed by Møller, 1997; Siva-Jothy *et al.*, 1998).

Relevant natural history of the host–parasite association

Larvae of *L. forcipatus* damselflies are found in the same habitats (ephemeral ponds) as *A. planus* (Walker, 1953; Wiggins *et al.*, 1980). Colonisation of odonate hosts by *Arrenurus* spp. mites occurs in a series of steps during which the hosts may mount defences. Larval mites first colonise odonate larvae; host larvae groom, presumably to reduce attachment rates (Smith, 1988; Forbes & Baker, 1990; Baker & Smith, 1997; Leung *et al.*, 1999). Larval mites are phoretic on odonate larvae; once larval hosts emerge and start to eclose, the larval mites abandon the cast exoskeleton and crawl onto the newly formed imago. The mites cling to the host with their

palpal claws and soon pierce the host's cuticle using their chelicerae (Smith, 1988).

Anchored to the host by the chelicerae, the mite secretes an acellular mucopolysaccharide that forms a stylostome (a blind-ended feeding tube; Smith, 1988). As mentioned, some odonate hosts attempt to neutralise one or more stylostomes through melanotic encapsulation (Åbro, 1979, 1982). In other insect species, such as the dragonfly *Sympetrum internum*, the host appears to aggregate haemocytes at the site of stylostome formation (Forbes *et al.*, 1999), similar to other aquatic insects parasitised by water mites (cf. Davids, 1973). This response appears to result in collapse of the feeding tube without melanotic encapsulation (Forbes *et al.*, 1999). Therefore, this study first assessed whether *L. forcipatus* showed melanotic encapsulation typical of other odonates and the degree to which the death of mites resulted from immune responses.

The *L. forcipatus*–*A. planus* association is similar to other odonate–*Arrenurus* mite associations. If the mite engorges successfully, it drops off the host when the host returns to the water for reproduction. It continues through its nymphal and adult stages, with its active stadia being free-living aquatic predators on microcrustaceans (Smith, 1988). Host detachment is simultaneous, thus parasitised hosts usually have mites or have lost mites. Evidence of past parasitism or *scarring* is clearly visible on some species (Forbes, 1991).

Materials and methods

Damselflies were surveyed to elucidate patterns of infestation by mites and to score engorgement success. Collections were made at Yezerinac's Pond, Ontario, Canada (44°32'13"N, 79°22'55"W). Fifty-one trips were made to the pond between 28 May and 5 August 1998. On each trip, damselflies were netted, identified as *L. forcipatus* (following Walker, 1953), and their mites were counted, using a 20× loupe.

Gender and approximate age were recorded for each damselfly (cf. Walker, 1953). Teneral had soft, shiny wings, a soft abdomen, and little dark pigmentation in the exoskeleton. Young to old adults had rigid, dull wings. Young adults had melanin deposits in their appendages only whereas older adults showed body pigmentation, and the oldest adults (for males) were pruinose. It was difficult to score accurately whether mites on tenerals were alive or dead so tenerals were excluded from comparisons. Hosts that were not parasitised at the time of capture were also excluded from comparisons. This exclusion was necessary. Although it was possible to ascertain whether older adults had been parasitised (presence of mite scars), it was difficult to enumerate scars and obtain an accurate count of mites previously parasitising those hosts.

Similar to other work on *A. planus* on other host species, dead mites on mature adults had a flattened body that was silver in colour (Forbes *et al.*, 1999). Mites starting to engorge had a red to orange body with their legs still visible. Mites were fully engorged if their legs were obscured entirely by their swollen body, and scored as live and partially engorged if the legs were only obscured partially by their bodies, which were not shrivelled (cf. Forbes *et al.*, 1999). For the purposes

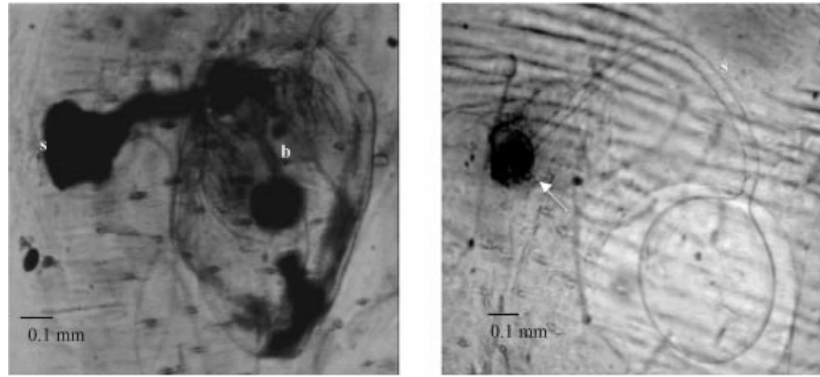


Fig. 1. Light micrograph (scale bars indicated) showing the variation in melanotic encapsulation of *Arrenurus planus* stylostomes by *Lestes forcipatus*. In the left panel, the body of a dead mite (b) and its jointed legs (above b) are visible. s refers to the encapsulated stylostome, which runs from the left margin of the panel to the gnathosoma of the mite (not visible). Melanisation is apparent in the body of the mite (two patches below b). In the right panel, only the stylostome is shown: the live engorged mite had detached from the host cuticle during preparation, leaving behind a scar (arrow). The stylostome (a bubble and long tube, labelled 's') is fully formed and the bulb is surrounded by a clear space, indicating that the stylostome was functional.

of this study, mites were recorded as dead and failing to engorge or as engorging or engorged. None of 400 mites was recorded as having engorged and later dying, indicating that mite death occurred early in the host's adult life.

Seventy-two *L. forcipatus* adults (41 males and 31 females) fulfilled the criteria of current parasitism where engorged or engorging mites versus dead mites could be scored accurately. Of these, 47 were marked using non-toxic permanent Sanford Sharpie® pens (Sanford Corporation, Bellwood, Illinois) on their wings, to preclude re-sampling after release (following Forbes, 1991). All 72 damselflies were processed by enumerating live and dead mites, and by counting the number of wing cells between the nodus and pterostigma for both the left and right forewings (to obtain a measure of wing cell asymmetry). Length of the right forewing was also measured from nodus to wing tip using digital calipers (accurate to 0.01 mm).

Twenty-five damselflies (14 females and 11 males) with one or more dead mites were brought back to the laboratory and prepared for viewing mite feeding tubes. These damselflies were first stored in glassine envelopes and refrigerated at $\approx 5^\circ\text{C}$ for up to 48 h. Preparation involved removing the head, legs, wings, and abdomen, leaving only the thorax. Thoraxes were placed in a 4.5×1.5 -cm glass vial and covered with Andre's solution (1:1:1 chloral hydrate, acetic acid, and water by weight) for a minimum of 48 h at $\approx 20^\circ\text{C}$ before further dissection. To further dissect, the top half of the thorax (including the wing muscles) was removed using 00 insect pins and a 2.5-cm, 22-gauge syringe needle. The ventral half of the thorax was placed on a microscope slide (exocuticular surface down). After applying glycerol and a cover slip, each slide was examined at $100\times$ (phase contrast) and scanned for mites and feeding tubes.

Statistical analyses were carried out using Systat 2.0® (Wilkinson, 1991) and JMP 3.0® (SAS Institute Inc., 1995).

Results

Patterns of mite infestation

Mites were invariably found on the venter of the thorax on a tubercle, or near or on the coxal plates of the legs. Numbers (and log-transformed numbers) of mites on hosts were not distributed normally (using Kolmogorov–Smirnov tests), precluding parametric tests. A Kruskal–Wallis test showed that mite intensities did not differ between females and males ($\chi^2 = 0.50$, $P = \text{NS}$). Females had a median intensity of four mites (interquartile range was two to seven mites; full range was one to 27 mites). Males had a median intensity of five mites (interquartile range was two to nine mites, full range was one to 17 mites).

Causes of mite death

To determine whether mites died due to immune responses, the feeding tubes of 86 dead mites from 25 hosts were examined. All of these mites had completely or almost completely melanised stylostomes (Fig. 1, left panel), i.e. none had fully formed stylostomes and appeared to have died from other causes. Thus, both male and female damselflies mounted immune responses to mites but many carried both live and dead mites. Mites, alive before being placed in the Andre's solution, had perfectly formed stylostomes with no evidence of melanisation on the stylostome itself (Fig. 1, right panel).

Immune responsiveness in relation to gender

Parasitised damselflies were scored as either responders (if they had one or more dead mites) or as non-responders. The

Table 1. Comparison of Julian dates of capture (1 = 28 May 1998) and wing lengths (mean \pm SE) between responders and non-responders (males and females considered separately). Sample sizes for male responders and non-responders were 30 and 11 respectively. Samples sizes for female responders and non-responders were 25 and six respectively.

	Mean \pm SE	<i>F</i>	d.f.	<i>P</i>
<i>Julian dates</i>				
Males				
Responders	52.3 \pm 1.9	9.40	1,39	<0.01
Non-responders	41.0 \pm 2.9			
Females				
Responders	50.9 \pm 1.9	3.10	1,29	0.08
Non-responders	42.2 \pm 6.3			
<i>Wing lengths (mm)</i>				
Males				
Responders	14.25 \pm 0.10	2.27	1,39	0.14
Non-responders	13.95 \pm 0.17			
Females				
Responders	15.34 \pm 0.11	0.009	1,29	0.92
Non-responders	15.37 \pm 0.23			

Table 2. Comparisons of absolute wing cell asymmetry for male and female responders and non-responders. Individuals within a category (e.g. male responders) with absolute asymmetry scores >0 were combined for contingency analyses.

	Absolute wing cell asymmetry			χ^2	d.f.	<i>P</i>
	0	1	2			
Males						
Responders	4	6	1	0.40	1	0.80
Non-responders	13	13	4			
Females						
Responders	3	3	0	0.2	1	0.65
Non-responders	10	12	3			

proportion of responders did not differ between males (30 of 41, 73.2%) and females (25 of 31, 80.6%; $\chi^2=0.55$, d.f.=1, $P=NS$).

Immune responsiveness of females and males in relation to other factors

A significant effect of date of sampling was found on likelihood that males responded immunologically, using a logistic regression ($\chi^2=9.0$, d.f.=1, $P<0.01$). The 11 non-responders were sampled significantly earlier in the season, compared with the 30 responders (Table 1). For females, the six non-responders were sampled earlier than the 25 responders but this result was not significant (Table 1). Responders did not differ from non-responders with respect to wing length

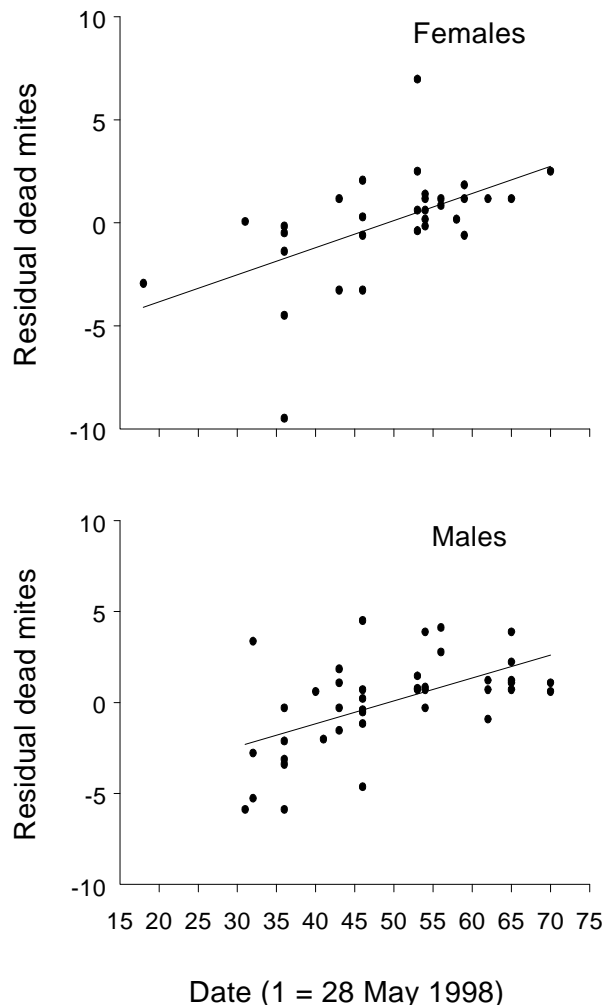


Fig. 2. Relationships between residual numbers of dead mites (from regressions of dead mites versus total mites, see text) and date of sampling. Two types of analysis were performed. (1) Both non-responders and responders were included in analyses to determine residual dead mites and relationships between residual dead mites and date of sampling, for each sex separately. (2) Non-responders were excluded from initial and subsequent analyses. Results are shown only for responders. Males: $-6.2+0.13 \times$ date of sample ($F=17.6$, $P<0.001$, $n=41$); females: $-6.4+0.13 \times$ date of sample ($F=11.9$, $P<0.025$, $n=31$). When non-responders were excluded, males: $-3.3+0.06 \times$ date of sample ($F=4.7$, $P<0.05$, $n=30$); females: $-7.2+0.14 \times$ date of sample ($F=7.1$, $P<0.025$, $n=25$). For both males and females, individuals with higher than expected numbers of dead mites were sampled later in the season.

or wing cell fluctuating asymmetry, for either males or females (Tables 1 and 2).

The effect of season on residual numbers of dead mites, controlling for total number of mites on hosts, was also examined. The number of dead mites was related linearly to the total mites on both males and females. For males, dead mites = $0.33+0.62 \times$ total mites ($F=43.7$, $P<0.001$, $n=41$), whereas for females, dead mites = $-0.95+0.78 \times$ total mites

($F=64.4$, $P<0.001$, $n=31$). If non-responders were excluded, there were still significant relationships between numbers of dead mites and total mites. For males, dead mites = $0.21 + 0.70 \times$ total mites ($F=95.5$, $P<0.001$, $n=30$), whereas for females, dead mites = $-0.54 + 0.77 \times$ total mites ($F=56.6$, $P<0.001$, $n=25$).

These relationships were due to the necessary positive relationship between numbers of dead mites and numbers of dead plus live mites. More importantly, residuals from these relationships were related positively to date of sampling for both males and females, irrespective of whether non-responders were included in, or excluded from, initial and subsequent analyses (Fig. 2). Thus, individuals with higher residual numbers of dead mites were more likely to be sampled later in the season (Fig. 2).

Discussion

Several studies have indicated that mites are costly to their insect hosts. For example, Polak (1996) showed that parasitism by *Macrocheles subbadius* Berlese mites was associated with reduced fecundity of female *Drosophila nigrospiracula* Patterson and Wheeler. For insects, including damselflies, water mites and other parasites have been associated with reductions in flight ability, fat accumulation, fecundity, mating success, and/or survivorship, in both observational and experimental studies (e.g. Lanciani, 1983; Smith, 1988; Forbes, 1991; Forbes & Baker, 1991; Reinhardt, 1996; Léonard *et al.*, 1999; Siva-Jothy & Plaistow, 1999). In other studies, there was no link between parasitism and lowered damselfly fitness (Andrés & Cordero, 1998; Rolff *et al.*, 2000) or the link was ameliorated by maternal effects (Rolff, 1999).

Importantly, *A. planus* mites engorge to 140 times their original volume compared with only five to 35 times for other species in this study region (J. M. Moran and B. P. Smith, pers. obs.). As such, fitness costs of parasitism and immune responses towards *A. planus* might be expected, however fitness costs are only one of several factors expected to be important in maintaining or mounting immunity. In the present study, date of sampling was the only predictor of immunity and degree of that response. Why should this be the case?

The pre-reproductive period of damselflies is prolonged by cool weather (Corbet, 1999; cf. Thompson, 1990). Damselflies emerging later in the season may divert more melanin or precursors of melanin (see Siva-Jothy *et al.*, 1998) away from maturation and towards immunity. It is interesting that the earliest-emerging *Lestes* species at the study site (*Lestes dryas* Kirby) was wholly susceptible to *A. planus*. In fact, all 93 mites found on 36 males and females engorged successfully (C. P. Yourth, pers. obs.). At present, there are no data on whether early emerging species are less likely to surrender melanin resources to immune responses, or are simply incapable of elevated immune responses.

In the present study, time of season was more important than metrics of fluctuating asymmetry and host size in determining probability and degree of immune responses. This is interesting, given that lepidopteran damselflies emerging later in the season

are time-constrained and may actually sacrifice body size for earlier emergence time (Johansson & Rowe, 1999). In fact, time-constrained or later-emerging individuals are smaller in several other species of temperate damselfly (e.g. Forbes & Baker, 1991; Corbet, 1999; Plaistow & Siva-Jothy, 1999). If later-emerging *L. forcipatus* were actually in poorer absolute condition, these results would seem counter-intuitive as these individuals invested more in immunity.

This apparent paradox could be resolved if melanin resources were not in as much demand for maturation (or temperature-dependent activities) later in the season. In a previous study controlling for time of season, Léonard *et al.* (1999) showed that maturation time for females of the damselfly *Enallagma ebrium* (Hagen) was slowed with experimental additions of parasitic mites, however it is not known whether this reflected melanin allocation away from thermoregulation and/or hardening of the cuticle or whether this response would change seasonally. Alternatively, higher mean temperatures later in the season could result in higher encapsulation rates. This argument is supported by experimental work on *Drosophila* subjected to two of three parasitoid species (Fellowes *et al.*, 1999b); however, encapsulation responses to the third parasitoid showed the opposite trend (Fellowes *et al.*, 1999b).

These results cannot be explained by age differences between responders and non-responders. In this study, all damselflies were old adults that had matured successfully. Additionally, the responses are known to occur in the first few days of life. The authors are unaware of any investigations of whether melanotic responses of insect hosts are affected by natural variation in weather in relation to time of season. Such results will have a bearing on the synergistic effects of weather and parasitism on host fitness, a topic that has not received much attention. Such seasonal effects may also place selection on female mites to lay clutches relatively early. In *A. planus*, small increases in larval abundance at the start of the season (as mites start hatching) are followed by large seasonal declines (Forbes *et al.*, 1999).

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