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Selection on insect immunity in the wild

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The strength of selection on immune function in wild populations has only been examined in a few vertebrate species. We report the results from a study measuring selection on a key insect immune enzyme, phenoloxidase (PO), in a wild population of the damselfly *Calopteryx xanthostoma*. We followed individually marked males from the pre-reproductive adult phase and recorded their lifetime mating success. We found positive selection on PO activity in response to an immune insult, but no selection on wing-spot quality, a trait actively displayed to females during courtship. We suggest that positive selection on PO activity in the year of study may be explained by annual fluctuations in parasite loads.

Keywords: ecological immunology; *Calopteryx xanthostoma*; positive selection; phenoloxidase

1. INTRODUCTION

Understanding the role of selection in shaping evolutionary changes in the wild is a prerequisite to understanding the adaptations of extant populations (Endler 1986). Phenotypic selection has been widely studied since the advent of appropriate techniques for quantifying selection on quantitative characters (Lande & Arnold 1983; Arnold & Wade 1984; Rausher 1992). However, the use of these powerful techniques has been mainly restricted to estimating the strength and nature of selection on morphological or life-history traits (Kingsolver *et al.* 2001; Hoekstra *et al.* 2001). Very few data exist for the strength and nature of selection on physiological traits, presumably because such traits are more difficult to measure in the wild. Such traits are rarely incorporated into studies of life-history evolution despite their relevance to such studies (Finch & Rose 1995; Zera & Harshman 2001; Rolff & Siva-Jothy 2003). Of particular recent relevance to studies of evolutionary ecology is immune function, a trait that links parasite resistance, reproductive investment and fitness (e.g. Zuk & Stoehr 2002; Rolff & Siva-Jothy 2003; Schmid-Hempel 2003).

The strength of selection on components of the immune system has been quantified only in two bird species (Saino *et al.* 1997; Råberg & Stjernman 2003) and in lizards (Svensson *et al.* 2001). Råberg & Stjernman (2003) reported positive directional and stabilizing selection on immune responsiveness against tetanus and diphtheria, respectively. Svensson *et al.* (2001) found positive directional selection on antibody responsiveness in one colour morph of the side-blotched lizard (*Uta stansburiana*), but negative directional selection in the other morph. Given that immune function is costly (Behnke *et al.* 1992; Schmid-Hempel 2003; Rolff & Siva-Jothy 2003), we predict that it should be subject to stabilizing selection. However, fluctuations in parasite pressure or condition dependence could result in either form of directional selection (Råberg & Stjernman 2003).

We examine the strength of phenotypic selection on an important component of immune function in a wild population of the damselfly *Calopteryx xanthostoma*. Male and female *C. xanthostoma* show a main emergence peak in late June/early July. Between 5 and 6 days after emergence, males have developed and fixed their characteristic melanic wing pigmentation pattern and a few days later begin reproductive activity at the river (see Siva-Jothy & Plaistow 1999). Males compete for, and defend, patches of the water plant *Ranunculus penicillatus*, the preferred oviposition substrate of females. Territoriality is costly (Plaistow & Siva-Jothy 1996) and the outcome of intrasexual competition has been shown to be dependent on the degree of wing pigmentation in another calopterygid (Grether 1996). Territorial males court arriving females (Siva-Jothy 1999) and, if successful, copulate and subsequently guard their ovipositing mate. High levels of last-male sperm precedence (Siva-Jothy & Hooper 1996), immediate access to oviposition sites and post-copulatory guarding behaviour ensure that a territorial male fertilizes all eggs his recent mate lays during the guarded oviposition bout. This results in a 1000-fold fitness advantage over males that have no territory (Plaistow & Siva-Jothy 1996).

The insect immune system comprises defence mechanisms modulated by haemocytes (insect blood cells) and humoral defence such as antibacterial peptides and the phenoloxidase (PO) enzyme cascade (Gillespie *et al.* 1997). Both of these arms of the innate insect immune system provide powerful defence against parasites. We concentrate on PO, a key enzyme in the melanization of the cuticle, wound repair, the production of cytotoxins near pathogens and encapsulating larger pathogens (Sugumaran 2002). Moreover, its function in the context of parasite-mediated sexual selection in the damselfly *C. xanthostoma* has already been documented (Siva-Jothy 1999, 2000). Briefly, the enzyme is responsible for producing the sexually dimorphic, fixed, melanic wing spot of males that is prominently displayed during the stereotyped and relatively complex courtship of this insect. Quantitative and qualitative aspects of the wing spot affect female reproductive decisions in *C. xanthostoma* (Siva-Jothy 1999). Moreover, regulation of the enzyme's activity in the insect's haemolymph during an immune challenge has been shown to be correlated with (i) the ability of males to resist fitness-reducing parasites, and (ii) the nature of the fixed sexually dimorphic melanic wing spot (Siva-Jothy 2000).

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Since *C. xanthostoma* is a well-studied field system, where it is possible to measure the activity of PO without sacrificing individuals (Siva-Jothy 2000), we were able to estimate immune function at the start of the study and to follow the lifetime mating success of individuals with known PO activity.

2. MATERIAL AND METHODS

(a) *Study site and species*

Observations and manipulations of a natural population of *C. xanthostoma* were carried out in July 2002 (coincident with peak population densities of reproductively active individuals) on a non-manipulated stretch of the Vidourle river in southern France (43°52′ N, 04°03′ E).

(b) *Marking and observation*

Pre-reproductive, adult males were caught and aged using the method of Siva-Jothy & Plaistow (1999): all study animals belonged to the same age category. Animals were individually marked with unique combinations of coloured acrylic paint dots on their thorax and abdomen. Male behaviour on all territories in the observation area (a *ca.* 100 m stretch of the river) was observed continuously from 10.30 to 17.00 (the period in which at least 95% of all reproductive activity occurs) each day for 21 days. A spell of bad weather stopped all reproductive activity 22 days after the start of the study from which point no marked males were resighted. Observers noted all reproductive activity and timed the duration of oviposition following observed copulations. A census of all marked individuals on territories, on the bank side vegetation and in the surrounding woodland was undertaken every hour.

(c) *Measuring wing-pigment characteristics*

All marked males had their wing spots photographed using fixed trans-illumination with a Nikon D1x digital camera set up to take the same exposures of every wing. Images were analysed using Optimas (v. 6.1) image analysis software. The grey-scale values (0–255, with 0 = black and 255 = white) were set so that the pigmented area in the image of the wing spot was 'selected' whereas the non-pigmented areas were not. The lower value represents the darkest pixel in the image of the wing pigment. These threshold values, as well as the area of pigment thus defined, were recorded. After measuring the sample population, the upper threshold values were analysed and the lowest of these upper thresholds (i.e. the darkest value for the threshold that defined the lightest pixels of pigmentation) was used to re-analyse all wings. The result of this procedure is that the wings with the darkest, most homogeneously distributed pigment showed a single, software-defined, area of pigmentation. Wings that were lighter and/or with more heterogeneous pigment distribution now showed several smaller software selected areas of pigmentation defined by the new threshold. Our measure of wing-pigment heterogeneity (WSH) was the mean number of areas in the pigmented region of the wings of an individual after re-analysis with the new threshold values (dark threshold = 35, light threshold = 85) multiplied by the inverse of the proportion of wing area defined after application of the new threshold values compared with the area bounded by pigment. The result of this analysis is that a value of '1' indicates a dark homogeneous wing (relative to the others) whereas larger integers indicate wings with increasingly light and heterogeneous wing pigmentation distribution. This technique has significant repeatability (Siva-Jothy 2000). Because we minimized handling of individuals we used wing-spot area (as measured from the image used to calculate WSH) as a surrogate for body size. This trait is tightly correlated with body length and body mass (M. T. Siva-Jothy, unpublished data).

(d) *Phenoloxidase assay*

Previous work (Siva-Jothy 2000) has shown that PO levels 24 h after a standardized immune insult are significantly correlated with parasite resistance ability. Consequently, we challenged every male at the time of marking with a 1.5 mm piece of sterile nylon monofilament (diameter 0.18 mm) injected into the abdominal haemolymph. Males were released and collected 24 h later when a hind limb was severed for PO analysis (this procedure had no observable effect on male behaviour and was applied to all focal individuals in the population). There is a strong positive correlation between haemolymph PO levels in an adult hind limb and the haemolymph extracted from the body cavity at the same time (Siva-Jothy 2000). Leg PO samples were collected by placing a chilled hind leg, severed at the coxa, in 0.5 ml of ice-cold sodium cacodylate buffer (0.01M sodium cacodylate, 0.005M $CaCl_2$). Samples were homogenized for 20 s with a motorized micro-pestle followed by centrifugation (4 °C, 2800*g*, 5 min). Supernatants were removed and 0.5 ml of 3 mM L-DOPA in sodium cacodylate solution was added, and the reaction allowed to proceed at 30 °C in a spectrophotometer (Pharmacia Biotech Ultraspec 2000) for 20 min. Readings were taken every minute at 490 nm and analysed using Swift II software (Pharmacia Biotech). Enzyme concentration was measured as the slope of the reaction curve during the linear phase of reaction (between 5 and 15 min after the reaction mix was made; personal observation).

(e) *Analysis*

To estimate the intensity of selection on immune function and wing-spot quality we used regression techniques (Lande & Arnold 1983; Endler 1986; Brodie *et al.* 1995). Prior to analysis, the fitness measurement, here the number of copulations, is transformed into relative fitness (individual number of copulations/mean number of copulations) (Lande & Arnold 1983; Endler 1986; Brodie *et al.* 1995). The traits of interest are standardized (Lande & Arnold 1983; Endler 1986; Brodie *et al.* 1995). We calculated univariate models to estimate linear (β_{uni}) and nonlinear (quadratic) selection (γ_{uni}) intensities. Linear (β_{uni}) selection intensities were estimated from a model that included only linear terms. As noted by Endler (1986) the univariate coefficients measure direct and indirect selection. Ideally, the multivariate coefficients measure direct selection on each trait. This is achieved because correlation with other traits is statistically removed. However, this hinges on the assumption that all the important traits have been studied, which is very difficult to verify. To estimate multivariate linear (β_{multi}) and multivariate nonlinear (quadratic) selection (γ_{multi}) gradients we used multivariate models that included the number of days we observed a male as a covariate to control for the correlation between male presence and mating success. Therefore, the multivariate model included WSH, PO activity and days. Two separate models were run to estimate multivariate linear (β_{multi}) and multivariate nonlinear (quadratic) selection (γ_{multi}) (Endler 1986; Råberg & Stjernman 2003).

3. RESULTS

On any one day the proportion of animals with nylon implant recaptured for assaying PO activity exceeded 85%. Within these recaptured individuals PO activity and WSH did not differ between resighted individuals and those that disappeared (PO: $F_{1,97} = 0.042$, $p = 0.838$; WSH: $F_{1,97} = 0.011$, $p = 0.918$). The number of days of

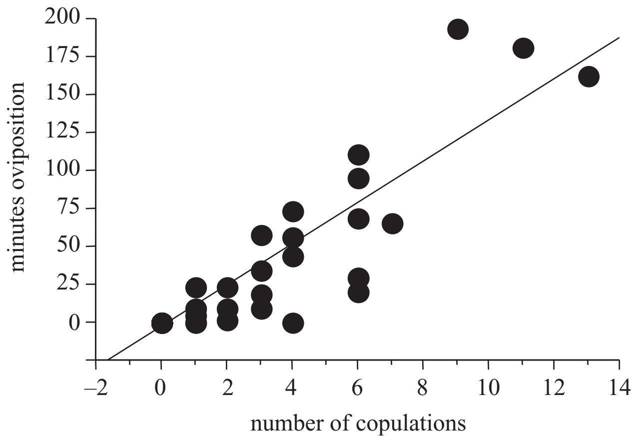

Figure 1. The relationship between number of copulations of male *Calopteryx xanthostoma* and total oviposition time of the females with which they mated.

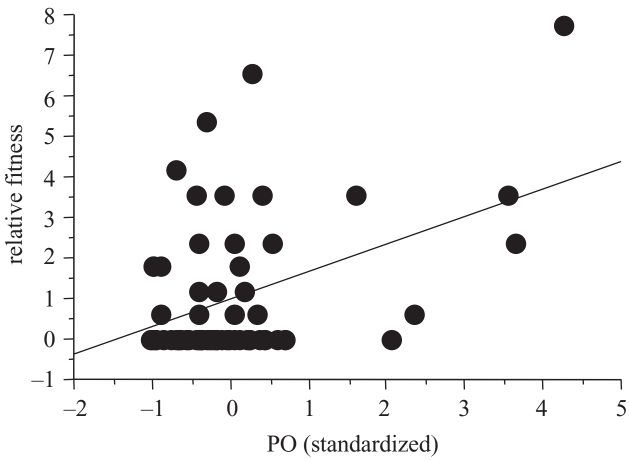

Figure 2. Relative fitness of *Calopteryx xanthostoma* males as a function of PO activity.

observation was not correlated with PO ($r = 0.16$, $p = 0.216$). There was no correlation between body size and WSH ($r = -0.134$, $p = 0.186$) or PO activity ($r = -0.009$, $p = 0.926$). Therefore, body size and days of observation were not included in the subsequent analyses.

In total we obtained lifetime mating success data for 64 males. The number of copulations was tightly correlated with the time a female spent ovipositing after being inseminated by a known male ($r = 0.907$, $p < 0.0001$; figure 1). Since we were not able to mark all females in the study population, we used the number of copulations as a fitness surrogate. Ovipositing females were sometimes interrupted by predators or other males, after which they left the site. Because mating success is correlated with the number of days an individual was observed, the latter is included as a covariate (using daily mating rate, number of matings/days of individual observation yielded the same outcome; data not shown) in the multivariate models.

We found positive directional selection in both univariate models and the linear multivariate model on PO activity but not on WSH (figure 2; table 1). (Despite the fact that we found a significant quadratic selection coefficient in the nonlinear univariate model, i.e. indicative of disruptive selection, the data in figure 2 show there were few low (negative) values of standardized PO activity: such data would be expected if disruptive selection was operating). Furthermore, fitting a univariate quadratic model did not increase the fit of the model. We also fitted the interaction between wing-spot quality and PO activity to investigate possible correlational or curvilinear selection. However, we found no significant interaction term ($F_{3,58} = 0.558$, $p = 0.458$).

4. DISCUSSION

We found positive directional selection on immune function (PO activity) in a wild population of the damselfly *C. xanthostoma*. The only other insect study to examine this relationship (Baer & Schmid-Hempel 2003) also reported a positive correlation between immune function and fitness in 'semi-natural' bumble-bee colonies, although it does not report selection intensities or gradients. Vertebrate studies of selection on immune function in the wild also report positive directional selection (Saino *et al.* 1997; Svensson *et al.* 2001; Råberg & Stjernman 2003).

Why did we find positive selection on PO activity? Given that immune function is costly (Rolff & Siva-Jothy 2003; Schmid-Hempel 2003), one might expect stabilizing selection: parasites exert positive directional selection and the costs of immune function exert negative directional selection. Two hypotheses can explain positive directional selection on immune function: an increase in the prevalence of parasites that are tackled partly by the PO cascade, and/or that immune function is a condition-dependent trait (Råberg & Stjernman 2003).

PO activity in *C. xanthostoma* is related to refractoriness against eugregarine gut parasites (Siva-Jothy 2000). These protozoan parasites decrease the ability of adults to assimilate nutrients (Siva-Jothy & Plaistow 1999) and are the commonest parasite in this study population: their prevalence fluctuates over the years (Siva-Jothy & Plaistow 1999; M. T. Siva-Jothy, unpublished data). In 2002, the prevalence of parasites was 46% with an average of 4.4 parasites per infected host (M. T. Siva-Jothy, unpublished data). This was among the highest parasite intensity observed over the past 7 years in this study population. This high level of gregarine infection may therefore explain positive selection in our study. Unfortunately, since gregarine loads could not be scored for the individuals in this study, because hosts need to be dissected in order to score them, we cannot make a stronger inference.

The second explanation for positive selection on immune function is condition dependence (e.g. Råberg & Stjernman 2003). Mating success in male *C. xanthostoma* is condition dependent: males with more fat reserves have higher mating success because they can sustain energetically costly escalated fights (Plaistow & Siva-Jothy 1996). Furthermore, the expression of the PO cascade in insects is also known to be condition dependent (Siva-Jothy & Thompson 2002). Following this reasoning, males in better condition should have a higher PO activity, and hence better resistance against gregarines, while at the same time they should be more successful in establishing territories. We are not currently in a position to refute either of these non-exclusive explanations for the positive selection on PO activity found in our study. However,

Table 1. Univariate linear (β_{uni}) and nonlinear (γ_{uni}) selection intensities and multivariate linear (β_{multi}) and nonlinear (γ_{multi}) selection gradients for PO activity and WSH in *Calopteryx xanthostoma* males.
(The multivariate estimates are controlled for the number of days a male was observed. Standard errors are in parentheses. Significant selection intensities and gradients are in bold. If necessary, the significance test was conducted with an appropriate model not based on least-squares estimates (analysis not shown).)

	β_{uni}	γ_{uni}	β_{multi}	γ_{multi}
PO	**0.68** (0.18)	**0.47** (0.22)	**0.53** (0.18)	0.44 (0.24)
WSH	−0.09 (0.20)	0.11 (0.21)	−0.13 (0.16)	0.04 (0.20)

given the high parasite prevalence and intensity in this study year we feel the first explanation is more likely.

Surprisingly, we did not detect selection on WSH. Experimental manipulation of wing-spot quality revealed sexual selection on daily mating success in *C. xanthostoma* (Siva-Jothy 1999). There are three mutually non-exclusive explanations for our failure to detect selection on wing-spot quality. (i) The experimental manipulation of Siva-Jothy (1999) produced a bimodal distribution of WSH compared with the continuous distribution in our non-manipulated dataset. (ii) In relation to this, our dataset is relatively small compared with other selection datasets from the wild (e.g. Kingsolver *et al.* 2001). Sample size might therefore have been insufficient to estimate selection on wing-spot quality, even though it proved sufficient to estimate the strength of selection on PO activity. (iii) Because of randomization in the experiment of Siva-Jothy (1999), factors such as differences in condition are eliminated. Such factors might, however, have been important in our study (see above).

In summary, we were able to measure selection on PO activity, an important component of resistance against the most prevalent parasite, in the wild. However, we did not find selection on the fixed melanic wing spots, the expression of which is dependent on PO activity earlier in ontogeny.

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