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Phenology determines seasonal variation in ectoparasite loads in a natural insect population

Running title: Phenology drives parasitism of odonates

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Abstract

1. The extent to which individuals are parasitised is a function of exposure to parasites and the immune response, which in ectotherms may be associated with temperature.

2. We test the hypothesis that seasonal variation in ectoparasite burden is driven by temperature using an extensive mark-release-recapture study of adult *Coenagrion puella* (L.) (Zygyoptera) as a model system. Mite counts were taken both at capture and on a subset of subsequent recaptures over two entire, consecutive breeding seasons.

3. Emergence date was the most significant factor in determining individual differences in mite burden, and mean counts for individuals emerging on the same days showed strong unimodal relationships with time of season. Subsequent recounting of mites on a subset of individuals showed that patterns of loss of mites were similar between seasons.

4. While, temperature did not significantly affect mite burdens within seasons and ectoparasite prevalence was very similar across the two seasons, intensity of infection and rate of mite gain in unparasitised individuals were significantly higher in the cooler season.

5. We demonstrate that, while temperature may modulate the invertebrate immune response, this modulation does not manifest in variations in mite burdens in natural populations.

Key words. Immunology, invertebrates, Odonata, damselfly, *Coenagrion puella*, mites, *Arrenurus*, temperature, phenology

Introduction

A species' response to parasitism is crucial for its survival (i.e. its ultimate fitness), and therefore acts as a key evolutionary driver. As such, considerable effort has been directed towards understanding the mechanisms behind host-parasite coevolution and, in particular, host defence. The insect immune system, understudied compared to the immune system of vertebrates, is capable of mounting a range of responses to different types of pathogens and parasites (Siva-Jothy et al., 2005). While genetic factors certainly influence the strength of the immune response (Hughes & Sokolowski, 1996), it is now recognised that environmental factors, such as diet, temperature and reproductive behaviour, also contribute (Schmid-Hempl, 2004). Investment in the immune response constitutes a potentially substantial expenditure of resources on the part of the individual (Sheldon & Verhulst, 1996). Since environmental conditions are a key determinant of the available energy budget in ectotherms, the implication is that immune investment will vary with the environment.

Odonates (dragonflies and damselflies) are important component of both aquatic and terrestrial ecosystems (Knight et al., 2005). As a taxon with tropical origins (Pritchard & Leggott, 1987) their ecology, including distributions (Hickling et al., 2006), phenology (Hassall et al., 2007), trophic interactions (Thompson, 1978), and body size (Johansson, 2003), is largely temperature driven. Odonata are parasitized by a range of endo- (principally Protozoa, Cestoda and Trematoda) and ectoparasites (for a review see Corbet, 1999 sections 5.2.3 and 8.5.3). Ectoparasitism of damselflies (Odonata: Zygoptera) occurs most frequently by water mites (Hydrachnida) and is common wherever both taxa coexist (Smith, 1988; Smith & Oliver, 1986). This relationship is an important component of odonate life history, having been implicated in a range of fitness components including flight ability, survivorship and mating success (for a review see Forbes & Robb, 2008).

The most common and best-studied group of Hydrachnida parasitizing odonates is the family Arrenuridae, particularly the genus *Arrenurus* (Corbet, 1999). Most work suggests that arrenurid mites cling to the final larval instar of the host prior to eclosion to adults, insert a feeding tube through the soft cuticle of the imago shortly after emergence, and then proceed to consume haemolymph. The host immune system delivers one of two responses: either an aggregation of haemocytes around the site of formation of the feeding tube (Forbes et al., 1999) or, more commonly, melanotic encapsulation of feeding tubes over a few days immediately following emergence, leading to death of the mite (Yourth et al., 2001, 2002). If permitted to feed, the mite life cycle is completed, after approximately 10-20 days (although engorgement can be accelerated at higher temperatures (Harris & Harrison, 1974)), by detaching when the host comes into proximity to water, particularly during oviposition (Rolff, 1997). Therefore, it is commonly-assumed that mite-load is greatest at emergence and thereafter decreases during the reproductive season. However, some species of *Arrenurus* can attach to mature individuals (Åbro, 1990; Imamura & Mitchell, 1967), permitting an increase in mite load during an individual's lifetime. Thus it is perhaps not surprising that conflicting patterns of the intensity of ectoparasitism on damselflies has been reported during the flight season, with studies finding both increases (Andrés & Cordero Rivera, 1998) and decreases (Yourth et al., 2002) in mite burdens. More recently, this putative temporal effect was shown to be associated with temperature, with higher temperatures facilitating a greater immune response (Robb & Forbes, 2005). Thus, while seasonal dynamics of parasitism and laboratory responses to temperature treatments have been studied, the link between environmental temperature in the field and observations of ectoparasitism has yet to be demonstrated conclusively. Also, while

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studies such as these have determined patterns of population-level changes in mite abundance over a season, we are not aware of any studies reporting temporal variation in mite number with age on individually-marked damselflies. Previous studies have also taken place within a single year, rather than comparing different seasons.

In this study we present evidence of the absence of a link between temperature and intensity of ectoparasitism in a natural population of the damselfly *Coenagrion puella* (Odonata: Zygoptera) over two entire breeding seasons. The intensity of parasitism is instead related to the date on which each individual emerged. *Arrenurus* spp. infecting *C. puella* elicit reduced longevity (Braune & Rolff, 2001) and lower numbers of offspring, while increasing offspring size (Rolff, 1999). Moreover, arrenurid mite parasitism is negatively correlated with fat content (Rolff et al., 2000) and positively correlated with fluctuating asymmetry (Bonn et al., 1996), but may (Forbes 1991) or may not affect mating success (McKee et al., 2003; Rolff et al., 2000). Male *C. puella* infected by unidentified mites showed greater dispersal than uninfected individuals, although this effect was not seen in females (Conrad et al., 2002). This and other work suggests that the particular impacts of parasitism may depend on environmental conditions (Braune & Rolff, 2001). The present study suggests that observed effects of temperature on the immune response in the laboratory are not manifested in the field as variations in mite burdens. Additionally, we demonstrate contrasting patterns of mite temporal dynamics following pre- and post-maturation parasitism.

Material and methods
Field work
Two extensive mark-release-recapture studies were carried out on *C. puella* during the summers of 2005 and 2006 at a small, isolated pond in southern England (Queen Elizabeth Country Park, 50°57'39"N, 0°58'41" W, elevation 104 m). The pond was visited daily and both the first and last mature individuals on the wing were recorded. No attempt was made to capture and mark teneral individuals due to their fragility. On capture, individuals were marked using a paint pen on the dorsal surface of the thorax and a unique alphanumeric code was written on the wing using a waterproof, non-toxic marker. The right hindwing (an indicator of body size) of all individuals was measured on capture using digital callipers.

Numbers of mites on all captured individuals were counted. Additional mite counts were performed occasionally on subsequent recaptures such that 21%, 12% and 13% of individuals in the 2005 season and 27%, 20% and 22% of individuals in the 2006 season had 2, 3 or >4 mite counts, respectively. Specimens with mites exhibiting noticeably different morphologies were sent away for identification by experts. All mites of all observed morphotypes were confirmed as being *Arrenurus* spp. (*A. bruzelli* (Koenike), *A. cuspidator* (Müller) and *A. papillator* (Müller)), but specific identification was not possible in the field.

Data processing
Climate data (maximum and minimum temperature, sunshine hours and precipitation) were obtained from a UK Meteorological Office weather station situated 37km from the pond (Leckford, station identification code 852). Mean daily temperature was estimated as the average of the maximum and minimum temperature. Sex-specific durations of the pre-reproductive period for *C. puella* are given by Banks and Thompson (1985): 13.2 ±0.22 (SE) (rounded to 13) days for males and

16.5 ±0.34 (rounded to 17) days for females. These numbers were subtracted from the first day of sighting at the pond to give estimated emergence dates for each individual. The mean of each of the three weather variables was calculated for the date of emergence and the two days preceding and following that date (five days in total).

Statistical analysis

For each season, generalised linear models (GLMs) were constructed to explain variation in initial mite counts across individuals. Predictor variables comprised mean temperature, mean precipitation and mean daily hours of sun during the emergence period (see above), emergence date and its quadratic term, sex and wing length as a proxy for body size. Individual mite counts were also pooled for individuals emerging on the same day and the mean calculated to reduce the noise due to highly skewed mite prevalence. These data were analysed using reduced GLMs involving the same predictors but without wing length and sex. Differences in temperature were examined between seasons both by comparing the mean temperatures across the flight season (i.e. periods in each season during which *C. puella* adults were present) and comparing the temperatures in the two seasons between 01-May and 31-Aug (paired t-test). The first test compares the temperatures to which adult damselflies were exposed while the second tests for a difference in temperature between the same period in each year.

The rate of loss of mites was analysed by calculating the proportional decline in mite numbers in relation to the initial mite count for each individual over its lifespan. Individuals with fewer than 20 mites were excluded, leaving 212 individuals in 2005 and 160 individuals in 2006, as proportional variations in small numbers of mites had disproportionate effects on the results. Data were analysed for mite counts recorded up to 12 days after initial capture of each individual. Differences between season in mite loss were tested for using ANOVA with season, days since marking and season*days as terms. A proportion of individuals in both seasons which were initially recorded as not having any mites were later recorded with mites. The incidence of this post-maturation parasitism was investigated using χ^2 to look for associations with sex and mating behaviour. Mating behaviour involves both individuals approaching the water during tandem oviposition (more common in *C. puella* than solitary oviposition (Martens, 1994)) and the female more so due to her need to be in contact with vegetation at the water surface which forms the oviposition substrate. Patterns of increasing mite numbers are analysed by comparing the mean mite counts of individuals initially recorded as being unparasitized and the proportion of those individuals recorded with parasites at each day after capture. All statistical tests were carried out in R (R Development Core Team, 2006).

Results

In GLMs explaining individual variation in mite burdens at first capture, the only consistent parameters that were significant were the date of emergence, the quadratic of that term and the mean hours of sun (Table 1). The unimodal relationships between emergence and mite burden in 2006 are clear from Fig. 1. In 2005 there is a weakly significant effect of body size (wing length) on the number of mites with larger individuals having fewer ectoparasites. This runs contrary to experimental studies in the laboratory (Robb & Forbes, 2005). When individuals were pooled for each emergence day, GLMs explained 34.5% of the variance in mean daily mite count in 2005 and 59.4% of the variance in 2006 (Table 2). Date of emergence was positively and significantly

correlated with temperature in 2005 (r=0.640, P<0.001) and 2006 (r=0.606, P<0.001) so if mite burdens were negatively affected by increases in temperature then an effect of emergence temperature on mite load should be significant and negative – this is not the case (Tables 1 and 2). The average number of hours of sunshine is significantly associated with mite burden, however, this relationship is positive, suggesting that damselflies emerging on sunnier days were more heavily infected with mites, and the number of hours of sunshine do not correlate with mean temperature in either season (2005: r=0.184, P=0.107; 2006: r=0.076, P=0.577). The cause of the relationship between the number of hours of sunshine and the number of mites an individual was parasitized by is unclear.

There was a clear difference between seasons, with mean daily temperature significantly higher in 2006 than in 2005 (two-sample t-test; t=4.259, P<0.001; Fig. 2a). The temperature between the two seasons was also significantly higher in 2006 (paired t-test; t=-2.198, P=0.029). Individuals emerged significantly earlier in 2005 (two sample t-test; t=-10.283, P<0.001) where the mean ordinal emergence date was 152.6 (± 0.604 (S.E.); 152 = 1-Jun) compared to 160.7 (± 0.505; 160 = 9-Jun) in 2006. While the proportion of the population carrying ectoparasitic mites was almost exactly equal across seasons (2005: 0.744; 2006: 0.752; Fig. 2b), the median number of mites parasitizing individuals in 2006 was significantly lower (Mann-Whitney U-test; U=101269, P<0.001; Fig. 2c).

The proportion of mites still present on individuals showed similar patterns of decay in 2005 and 2006 (ANOVA: season, $F_{1,254}$=1.580, P=0.210; day since first sighting, $F_{10,254}$=5.316, P<0.001; season*day, $F_{10,254}$=0.824, P=0.606; Fig. 3). In both seasons the same pattern exists of approximately linear decline in median mite numbers over the three days immediately post-capture followed by a plateau with little or no further loss of mites. This plateau appears to be at between 0.05-0.10 of the initial mite count and is consistently higher in 2005 relative to 2006.

138 and 242 individuals were recorded as being unparasitized on first capture in 2005 and 2006, respectively. Of these, 30 (22%) and 91 (38%) were recorded as being parasitized by mites on subsequent captures. Neither sex nor mating behaviour was important in 2005 but both had a significant effect in 2006 (χ^2 analysis, sex vs. post-maturation parasitism: χ^2_1=4.877, P=0.027; mating vs. post-maturation parasitism: χ^2_1=9.725, P=0.002); females were more likely to exhibit post-maturation parasitism than males and mated individuals were more likely to exhibit post-maturation parasitism than unmated individuals.

Further analysis of trends in the abundance of mites on previously unparasitized individuals showed significant, positive relationships between age and mite number (Fig. 4a). The relationship between mite counts and age in 2005, the cooler year, was much stronger and steeper. Levels of prevalence increased asymptotically with age (Fig. 4b). In 2005 all individuals which had been recorded as unparasitized at first capture were parasitized by 12 days after first capture, while in 2006 this threshold was reached at 16 days after first capture.

Discussion
Temperature has been implicated as an important factor in determining parasite burdens in ectotherms. This study failed to find any evidence of this effect in a natural insect population. Within flight seasons, the intensity of ectoparasitic infection of *C. puella* by *Arrenurus* spp. is

determined by emergence phenology and not by temperature, as previously suggested. However, a greater overall abundance of mites in the cooler year, a greater rate of mite accumulation after maturation in the cooler year and an earlier asymptote of prevalence in post-maturation parasitism in the cooler year suggest that inter-season variations in mite burdens might be influenced by temperature. We also demonstrate evidence of an effect of sex and behaviour on post-maturation parasitism.

Temperature is one of a range of ecological factors that affect both the immune response of invertebrate hosts (Fellowes et al., 1999; Rolff & Siva-Jothy, 2003) and the fitness of parasites that exploit them (Vale et al., 2008), although correlations between temperature and other biologically important environmental factors (particularly oxygen concentrations and salinity) potentially confound results in aquatic systems. However, even in terrestrial environments the relationship between temperature and encapsulation is inconsistent. For example, encapsulation has also been shown to be higher in cooler seasons (Flanders & Bartlett, 1964; Hu, 1939) and a review of the effects of temperature on encapsulation in Coccoidea (Homoptera) highlighted that higher temperatures can cause increases, decreases or no change in encapsulation, as well as presenting evidence of unimodal responses (Blumberg, 1996). This inconsistency is also present in field observations of ectoparasite infection in odonates (cf. Andrés & Cordero Rivera, 1998; Yourth et al., 2002), although the only experimental study to investigate the phenomenon in odonates showed increased encapsulation with increased temperature (Robb & Forbes, 2005). The response to ectoparasitic mites involves the phenyloxidase-melanocyte (PO-M) component of the invertebrate immune response during encapsulation. Interestingly, enhanced immune function in female *C. puella* (higher haemocyte counts and phenyloxidase activity) was not manifest as reduced mite abundance (Joop et al., 2006), though temperature effects were not considered. This may explain the lack of variation in mite counts with temperature observed in our study.

We have presented data which do not support the hypothesis that temperature influences the outcome of damselfly-mite interactions once those interactions have begun. However, temperature also plays a role in determining odonate phenology (Dingemanse & Kalkman, 2008; Hassall et al., 2007). It has been proposed that mites have adapted to exploit the developmental timing of their hosts (Rolff, 2001), although generalist mite species do not appear to exhibit synchrony with seasonal hosts (Smith, 1988 and references therein). Whether this will continue to be the case when the host community as a whole is advancing phenology will require further investigation. There is some evidence that seasonal variations in mite abundance in an odonate community are affected by phenology (Zawal, 2006). In putative generalist mite taxa, such as *Arrenurus* spp., a change in odonate emergence phenology may result in an opportunistic shifting of host preference to whichever species happen to coincide with the parasitic stage of the mite life cycle. For example, Conrad et al. (2004) detected greater incidence of mite parasitism in *C. puella* than *I elegans*, with the latter emerging earlier in the season, which is contrary to our finding that mite intensity was higher in a season with earlier emergence (2005). Some data indicate that, rather than being broad generalists, arrenurid mites survive better on certain species even though they exhibit no host preference (Lajeunesse et al., 2004). Changes in phenology may alter host abundance, to the extent that mites will experience a greater prevalence of a host that they are less well adapted to, or even cannot survive on. Given the changing climate, mite survival will depend on an ability to track host

phenology and/or (better) exploit alternate hosts, either through true generalism or via local adaptation.

Despite extensive research into ectoparasitism, quantifying the level of variation in the degree of parasitism with age of host in natural populations is rarely considered for odonates, and indeed more broadly with respect to insect taxa. Certainly an immune response to ectoparasitic mites is mounted within 24 hours of emergence in *Lestes* species, when the feeding tube is first inserted through the cuticle (Robb & Forbes, 2005). It is possible that arrenurid mites remaining until the beginning of the reproductive period (i.e. first sighting in the study) had successfully engorged and that subsequent loss of mites through the season from this point is related to active detachment at the water body. Since approximately 90-95 % of mites become detached from their hosts, this implies extremely effective transmission between parasitic and free-living life history stages in mites that survive the host immune system. Of course, we present evidence that some mites attach during maturation. Only two published studies have reported post-maturation parasitism by arrenurid mites, affecting *Cercion hieroglyphicum* (Brauer) (Imamura & Mitchell, 1967), and *Lestes sponsa* (Hansemann) and *Pyrrhosoma nymphula* (Sulzer) (Åbro, 1990).

Rolff (2000), working on *C. puella*, and McKee et al. (2003), working on *Xanthocnemis zealandica* (McLachlan), found no difference in parasitism between sexes at emergence (i.e. teneral / immature individuals). Due to our intensive marking methodology and the isolated location of the study site, we are confident that the first sightings of individuals occurred at the very beginning of the mature period and therefore that the mite burdens at this point reflect the ectoparasite load at emergence as the animals had not been in proximity to water. Thus our study supports these earlier results. Sheridan et al. (2000) suggest that a general absence of sex-biased parasite loads in arthropods (cf. vertebrates where males tend to be more heavily parasitized) reflect the absence of endocrine-immune interactions; although a few studies have uncovered a different response to parasitism between sexes in some arthropods, including odonates (Braune & Rolff 2001; Joop et al. 2006), Yourth et al. (2001) reported no apparent gender bias to the immune response of *Lestes* spp. exposed to mites. Nonetheless, in odonates, greater numbers of mites often occur on mature males (e.g. Lajeunesse et al., 2004; McKee et al., 2003), and this may be driven by behavioural factors. McKee et al. (2003) hypothesised that because adult females make direct contact with water to oviposit, for example, they should have fewer mites than adult males. However, this mechanism of mite detachment provides a potential route for additional parasitism as well and, through this, an explanation for the bias we observed towards additional parasitism in females and mated individuals. An extensive study by Zawal (2006) also identified greater numbers of *Arrenurus* spp. on female damselflies, supported by results from a study of *Lestes disjunctus* (Selys) (Robb & Forbes, 2006). Robb and Forbes (2006) propose two mechanisms that may contribute to female-biased mite loads: (i) females spend more time as final instar larvae and are therefore more likely to encounter mites, or (ii) increased feeding leading to higher growth rates in females involves less grooming. While odonates are generally protandrous (Corbet, 1999), male *C. puella* have been found to emerge both earlier (Lowe et al., 2009) and later (Banks & Thompson, 1985) in the season than females. The absence of protandry, and the resulting exposure to cooler, early season temperatures in males, may explain why there is no difference between the sexes in the degree of parasitism in this species. However, despite the putative survival (i.e. fitness) consequences of parasitism (e.g. Braune & Rolff, 2001), parasite load does not always negatively affect direct correlates of fitness, such as mating

activity (Andrés & Cordero Rivera, 1998; McKee et al., 2003; Rolff et al., 2000) or ejaculate volume (Rolff et al., 2001). Of course, that we detected this sex/behavioural preferences in one year only highlights the deficiencies of studies carried out over one or few seasons as the potential influences of the environmental cannot be partitioned out.

The use of natural enemies to control insect pests is commonplace and this relies on a firm understanding of the interaction between the host immune system and the natural enemy being employed. Thermal biology is rarely incorporated into this understanding, despite having significant consequences for the degree of control that the enemy is capable of exerting on the target pest (Thomas & Blanford, 2003). Where enemy release through introduction of a species to a new region has caused that species to become a pest, a novel thermal regime in the new region may render useless existing knowledge about the interaction between the pest and candidate biocontrol agents. This may result either from temperature-mediated variation in the immune response of the host, as shown by some other studies, or variations in phenology between species which affects temporal overlap. Such variations in phenology have been shown to decouple trophic interactions in both terrestrial (Visser et al., 2006) and aquatic (Winder & Schindler, 2004) systems. A similar effect has been proposed in parasitoids (Godfray et al., 1994). Selective pressures exist for parasites to match their phenology to hosts and for hosts to alter their phenology to minimise exposure to parasites. It has been speculated that *Gynaephora groenlandica* (Wocke) (Lepidoptera: Lymantriidae) undergoes a period of dormancy when parasitoid abundances are high as a result of such a pressure (Hance et al., 2007).

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Tables

Table 1. Results of generalised linear models for individual differences in mite counts at first capture in *Coenagrion puella* in 2005 (n=425) and 2006 (n=617).

	2005 season		2006 season	
Predictor	t	*P*	t	*P*
Emergence date	**-4.630**	**<0.001**	**4.355**	**<0.001**
Emergence date^2	**4.392**	**<0.001**	**-4.620**	**<0.001**
Emergence sun	**2.775**	**0.006**	**3.629**	**<0.001**
Winglength	**-2.107**	**0.036**	-1.459	0.145
Emergence temperature	1.513	0.131	0.333	0.739
Emergence precipitation	0.119	0.906	-0.049	0.961
Sex	-1.251	0.212	-0.922	0.357
r^2	6.0%		15.3%	

Table 2. Results of generalised linear models for variation in mite counts at first capture in *Coenagrion puella* emerging on different days.

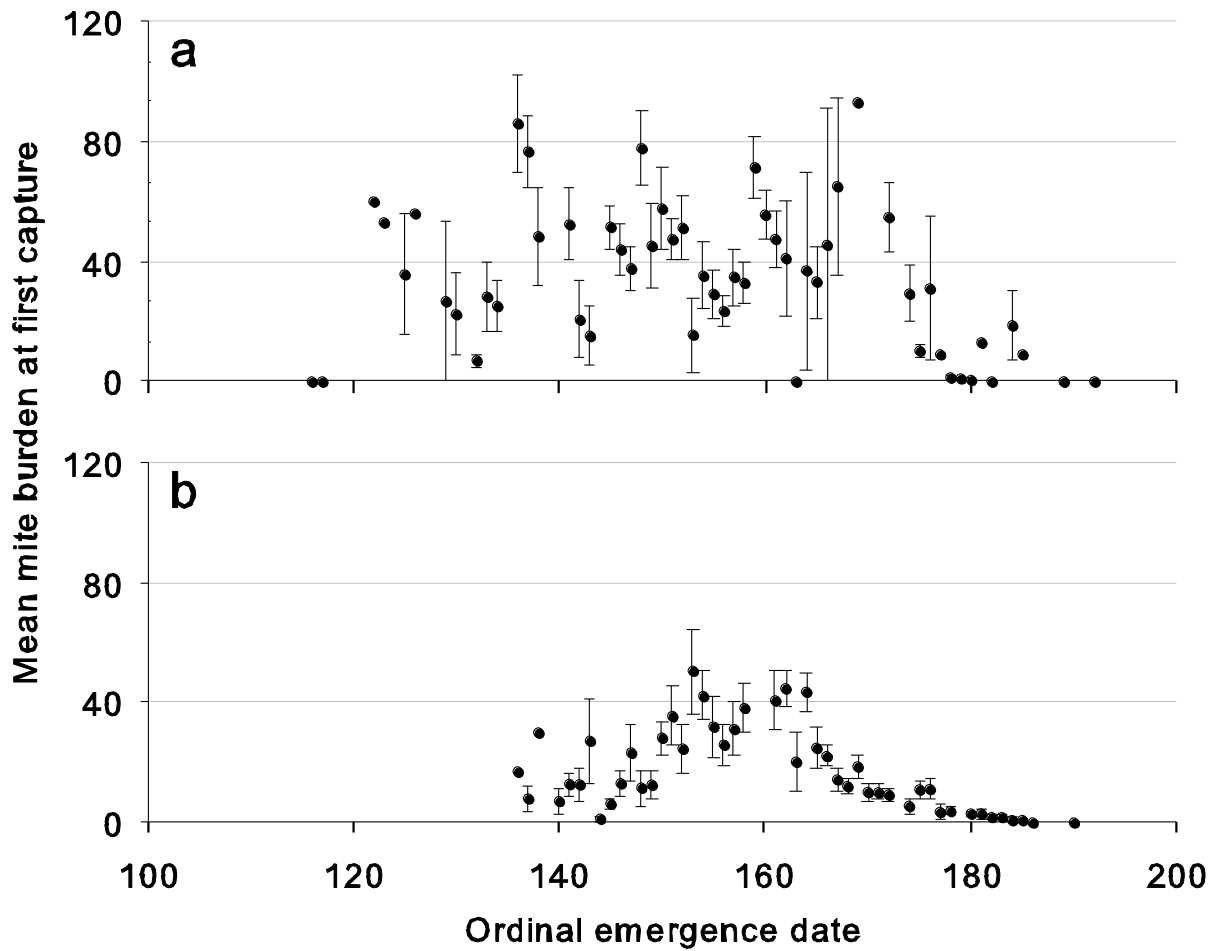| Predictor	2005 season t	2005 season *P*	2006 season t	2006 season *P*
Emergence date	**3.715**	**0.001**	**2.481**	**0.017**
Emergence date^2	**-3.798**	**<0.001**	**-2.774**	**0.008**
Emergence temperature	0.543	0.590	0.203	0.840
Emergence sun	-0.948	0.348	**2.212**	**0.033**
Emergence precipitation	**-2.453**	**0.018**	-1.036	0.306
r^2		34.5%		59.4%

Fig. 1. Variation in initial mite counts for individuals of *Coenagrion puella* with estimated emergence date in (top) 2005 and (bottom) 2006.
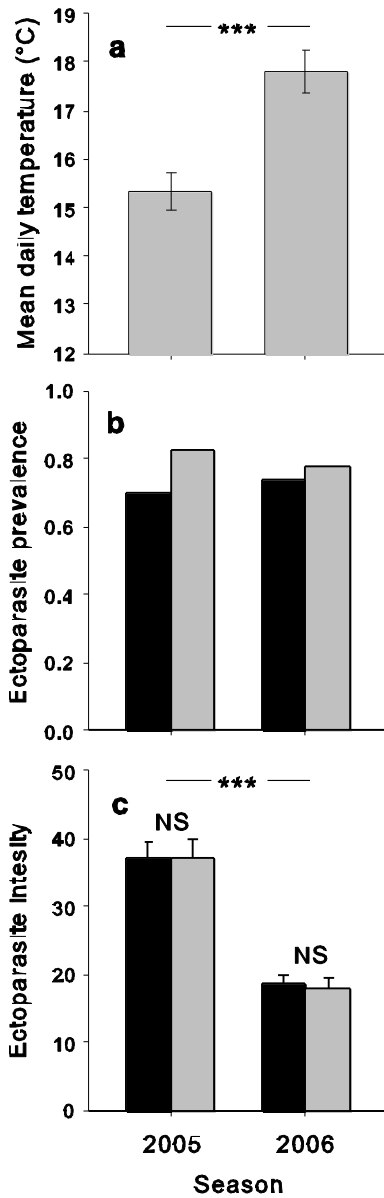
Fig. 2. Variation in (a) mean daily temperature over the entire flight period, and (b) prevalence and (c) intensity (mean mite count ± SE) of ectoparasitism by mites on male (black bars) and female (grey bars) *Coenagrion puella* in 2005 and 2006. *** = *P*<0.001, NS = non-significant.
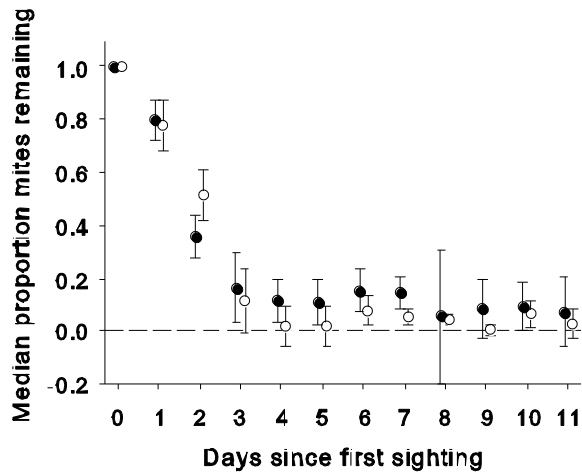
Fig. 3. Rate of loss of mites with days since first sighting in *Coenagrion puella* during 2005 (filled circles) and 2006 (open circles). Data are the proportions of the initial mite number (recorded at first capture) remaining on each day ± SE.
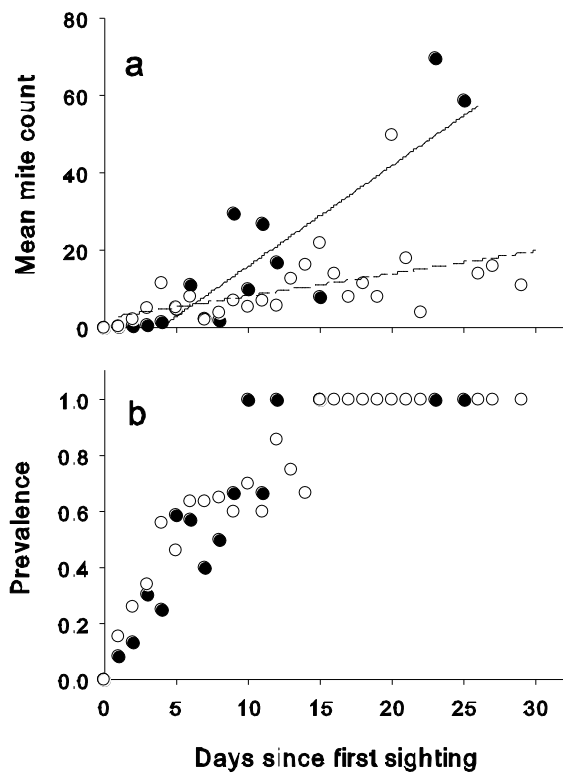
Fig. 4. Patterns of (a) mite infection intensity and (b) mite prevalence (proportion of individuals recorded as being parasitized) in individuals with initial mite counts of zero. Linear regressions in (a): 2005 mite count, $F_{2,14}$=49.7, P<0.001, r^2=0.765; 2006 mite count, $F_{2,24}$=7.61, P=0.011, r^2=0.209.