Immunocompetence and resource holding potential in the damselfly, *Calopteryx virgo* L.

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It is generally believed that resource holding potential reliably reflects male quality, but empirical evidence showing this is scarce. Here we show that the outcome of male-male competition may predict male immunocompetence in the territorial damselfly, *Calopteryx virgo* (Odonata: Calopterygidae). We staged contests between 27 pairs of males and found that winners of the contests showed higher immunocompetence, measured as encapsulation response, compared with that of losers. Furthermore, the winners had larger fat reserves. We also collected 29 males that had not been used in staged contests, and found that in these males encapsulation response correlated positively with an individual's fat reserves. Both immunocompetence and resource holding potential seem to depend on energy reserves, suggesting a trade-off between parasite resistance and energetically costly territorial behavior. The results suggest that the outcome of male-male contest can be used to predict male quality in terms of immune defense. *Key words:* damselfly, encapsulation rate, fat, hemocyte, immunocompetence, male-male competition, resource holding potential. *[Behav Ecol 15:169–173 (2004)]*

Both male-male competition and female choice have been subject to considerable research, and understanding of both processes is well developed (see Andersson, 1994). By contrast, their interaction has received little attention and is poorly understood (Qvarnström and Forsgren, 1998; but see Candolin, 1999). It is generally believed that male dominance will reliably reflect certain aspects of mate quality, because only the males of relatively high quality are able to bear the costs of dominance (see Grafen, 1990; Qvarnström and Forsgren, 1998; Zahavi, 1975). However, empirical evidence showing that mate quality is reflected by dominance is scarce (Qvarnström and Forsgren, 1998).

One aspect of mate quality is immunocompetence (see Johnsen et al., 2000). It has been suggested that because success in contests probably depends on male condition and overall health (Borgia, 1979), females could avoid infection and gain genetic benefits in terms of more viable offspring by mating with dominant males (Berglund et al., 1996; Cox and LeBoeuf, 1977). In this study, and in animal behavior generally, dominance is defined as success in contests (see Qvarnström and Forsgren, 1998). Hence, dominance is synonymous for resource holding potential, a term more commonly used in previous studies on odonata (see Marden and Waage, 1990; Plaistow and Siva-Jothy, 1996).

The term immunocompetence is often used to refer to the ability or probability of an individual's immune system to resist and control pathogens and parasites. In insects, one of the most informative ways to assay immunocompetence is to measure the magnitude of the encapsulation response to a novel and standardized antigen such as a nylon monofilament (see König and Schmid-Hempel, 1995; Rantala et al., 2000; Ryder and Siva-Jothy, 2000; Siva-Jothy, 2000). Encapsulation is a cellular immune response through which insects defend themselves against macroscopic foreign bodies (Salt, 1970) and that may also play a role in defense against viruses (Washburn et al., 1996). Together with phagocytosis and humoral processes, this cellular encapsulation constitutes the invertebrate immune system (Gupta, 1986).

Calopteryx (Odonata: Calopterygidae) damselflies are multiple mating sexually dimorphic insects. Males defend territories containing one or more oviposition sites (emergent aquatic vegetation) where pair-formation and guarded oviposition occur (see Pajunen, 1966; Waage, 1973). Territory ownership is settled by aerial contests that can become highly escalated and prolonged between closely matched contestants (Marden and Rollins, 1994; see also Fitzstephens and Getty, 2000). The most important factor affecting the outcomes of the contests are energy reserves, with winners having significantly more fat reserves after the contests than do losers (Marden and Waage, 1990; Plaistow and Siva-Jothy, 1996). Because winners of the territorial contests have larger energy reserves they should be able to allocate more resources to other tasks such as immune defense, which is an important component of fitness and is generally regarded as costly (Barnes and Siva-Jothy, 2000; Sheldon and Verhulst, 1996; Westneat and Birkhead, 1998).

In the present study, we asked whether the outcome of male-male competition could predict male immunocompetence in *Calopteryx virgo* L., a damselfly with male territories containing oviposition sites (emergent aquatic vegetation) for females (for details, see Pajunen, 1966). We hypothesized that winners of the damselfly territorial contests, owing to their expected larger energy reserves (Marden and Waage, 1990; Plaistow and Siva-Jothy, 1996), should have better postcontest immunocompetence compared with that of losers. To test this, we staged contests between *C. virgo* males, collected winners and losers, and measured their fat content, immunocompetence, and eugregarine parasite burden (see Siva-Jothy and Plaistow, 1999). Furthermore, to examine the relationship between age, immunocompetence, and energy reserves, we collected a sample of males that had not been in the staged contests and measured their age, fat content, and immunocompetence. We assayed immunocompetence by measuring the magnitude of the cellular encapsulation response to a nylon monofilament and hemocyte density because previous studies have suggested that high density of

hemocytes may enhance encapsulating ability and may be costly to maintain (see Kraaijeveld et al., 2001).

METHODS

Fighting experiments

We staged the contests between 2 and 15 July 1999 at Mustajoki Creek and Vispiläjoki Creek close to the city of Jyväskylä (62° 16′ N; 25° 30′ E) in central Finland. We marked males individually on the hind wings, with an enamel pen (see Plaistow and Siva-Jothy, 1996; Rantala et al., 2001). After this, we staged contests between neighboring males by gradually merging artificial territories consisting of clumps of floating vegetation (see Marden and Waage, 1990; Waage, 1988). Merging of territories causes both males to act as residents of the same territory, thereby removing the normal resident-intruder asymmetry (Waage, 1988) and permitting analysis of the relationships between fighting success and physiological features of the males (Marden and Waage, 1990). The contest duration varied widely from 0.5–83 min. On average, the contest duration was 33 min (SD = 35). Our experimental design ensured that both contestants behaved as residents. Consequently, every contest in this study determined a chance in residency. Winners and losers were easy to define, because after the contest the winner perched in the territory while loser left the territory. Hence, the contests were not resident-intruder situations and not merely boundary disputes (see also Waage, 1988). We staged 10 contests at Mustajoki Creek and 17 contests at Vispiläjoki Creek between 1000 h and 1600 h Altogether, we collected winners and losers of 27 contests.

Because we did not commence our observations at Vispiläjoki Creek at the beginning of the flying season, it was not possible to determine the absolute age of the contestant males by the date of marking. Males were therefore aged by assigning them to one of four age categories defined by the stiffness of the leading edge of the wings, which increases with age (see Plaistow and Siva-Jothy, 1996). All males used in contests belonged to the age class 2 (see Plaistow and Siva-Jothy, 1996).

Observational data

To examine the relationship between age, energy reserves, and immunocompetence, we collected 29 males that had not been used in staged contests, from the Mustajoki Creek on 15 July. The minimum estimate of age (based on marking the males when they first appeared at the stream) of these males varied from 0–29 days. Unfortunately, when collecting these males, we could not obtain reliable information about male territorial status because of cloudy weather as *C. virgo* is active only in sunny weather.

Measurements

In the field, we kept the collected damselflies in individual black plastic containers in a coldbox and took them into the laboratory (where all measurements were done) in the afternoon. The winner and loser of each pair of males used in the staged contests spent the same duration in the coldbox. To obtain a measure of individual size, we measured fresh mass to the nearest 0.1 mg with electronic balance (Hambascgb-1, A&D Instruments).

It has become a commonly used method to estimate encapsulating ability of insect immune system by measuring the response produced toward a synthetic substrate (see Rantala et al., 2000; Ryder and Siva-Jothy, 2000). Because

encapsulating cell mass contains dark pigment melanin (Carton and Nappi, 1997), we estimated the amount of encapsulating material by measuring the darkness of the encapsulated implant, a method similar to that used by König and Schmid-Hempel (1995) and Schmid-Hempel and Schmid-Hempel (1998). Melanogenesis and hemocyte encapsulation are highly linked immune responses made against endoparasites, and melanin synthesis is essential for successful encapsulation response (Carton and Nappi, 1997). In damselflies the rate of melanin synthesis seems to be limited by the availability of substrate (tyrosine, which is derived from the essential amino acid phenylalanine; Siva-Jothy, 2000). Melanin is also used in male damselflies' wing pigmentation, and therefore, it has been suggested that its precursor phenylalanine may be the limiting resource on which a trade-off between the signal trait and the immune system operates (Hooper et al., 1999; Siva-Jothy, 2000).

To challenge the damselfly immune system, we inserted a 1.7–1.8-mm-long piece of nylon monofilament (diameter = 0.1 mm) through the fourth abdominal pleura on the dorsal side of the sternal tergal margin. The male immune system was allowed to react to the implant for 14 h (based on our preliminary study, which showed that 14 h produces the largest differences between individuals), while the insects were kept in individual black film roll cans at constant room temperature (22 ± 1°C). The implant was then removed and air-dried. Not all implants could be recovered, resulting in encapsulation measurements from 22 pairs of males.

The removed implants were photographed under a microscope from three different directions. Photographs of the implants were taken with "control implants" (an unused piece of nylon to correct for differences in film development) and analyzed with a densitometer. In the case of males used in contests, there were three implants in each picture: control implant and implants from winner and loser. We divided implants into four sections, and to determine the degree of encapsulation rate of each implant, the optical densities of red (R; filter = 700 nm), green (G; filter = 546.1 nm), and blue (B; filter = 435.8 nm) were measured at those four sections. Total color density (R + G + B) was calculated for each section, and the appropriate measures for implants were calculated from mean value of the all three different photographs. To measure repeatability, we took another set of photographs from 18 randomly chosen implants and independently analyzed them with a densitometer. The repeatability (R; see Krebs, 1989) of this method was high ($R = 0.98$, $F_{17,18} = 105.11$, $p < .001$).

To measure hemocyte density, we counted the relative numbers of circulating hemocytes in the hemolymph. Just before removing the implant, damselflies were punctured at the fifth abdominal pleura on the dorsal side of the sternal-tergal margin with a sterile needle to obtain hemolymph, and the hemolymph was collected from the puncture by using a glass micropipette rinsed with heparin. After that, we measured the volume of hemolymph at the micropipette. From the micropipette, hemolymph was smeared on a slide, air-dried, and fixed using methanol. The slides were stained using Diff Quick® (Dade Diagnostika GmbH), and hemocytes were then counted by using a microscope. All hemocyte types encountered were pooled to total hemocyte counts. We counted density of hemocytes by dividing number of hemocytes by volume of hemolymph in the micropipette. We used $\log_{10}$ transformation for the hemocyte density, after which the data satisfied the assumptions of the statistical tests. Hemolymph was obtained from 21 pairs of contestants and 27 males that had not been in the staged contests. The methods of measuring encapsulation and hemocyte density were similar to those used by Rantala et al. (2000).

Table 1

Average fresh mass, body fat, hemocyte density, and encapsulation rate of winner and loser *C. virgo* males after contest

	Winner		Loser		Paired *t* test			
	Mean	SD	Mean	SD	*n*	*t*	df	*P*
Fresh mass (mg)	112.4	12.1	110.4	9.0	27	0.88	26	.39
Hemocyte density (cells/μl)	2464.0	1356.0	3888.0	3327.0	21	−1.99	20	.071
Body fat (mg)	3.0	1.5	2.1	1.2	25	2.16	24	.041
Encapsulation rate	1.33	0.44	0.92	0.38	22	4.98	21	.001

To measure parasite burden of winners and losers, we assessed the number of eugregarine trophozoites in a male's mid-gut by removing the entire gut carefully and making light microscope thin-section preparations (see Siva-Jothy and Plaistow, 1999). We did not assess parasites of the males that had not been in staged contests.

Fat was extracted from the thorax and abdomen for 4 h with refluxing chloroform in a Soxhlet apparatus (for details, see Marden, 1989). Fat content (in milligrams) was measured by subtracting dry fatless body mass (thorax plus abdomen) from dry body mass (thorax plus abdomen). This value is not presented as the residual from a regression of total fat content over dry fatless body mass (see Marden and Rollins, 1994) because the relationship was not significant in the observational data set ($F_{1,26} = 0.45$, $p = .51$). Fat was extracted from 25 pairs of contestants and 26 males that had not been in the staged contests.

RESULTS

Contestants

Winners had stronger encapsulation responses than did losers (paired samples *t* test, $t = 4.98$, df $= 21$, $p < .001$) (Table 1), with 20 of the 22 contests won by a male with a stronger postcontest encapsulation response (Figure 1). On average losers' encapsulation response was 70% of winners' encapsulation response (Table 1). Winners were fatter than losers (paired samples *t* test, $t = 2.16$, df $= 24$, $p = .041$) (Table 1); in 19 of the 25 contests, winners were fatter than losers (Figure 2). On average, winners had 3 mg body fat, and losers had 2.1 mg (Table 1). However, there was no difference in the fresh body mass or hemocyte density between winning and losing males (Table 1). The partial correlation (after controlling for differences in the duration that the males spent in the coldbox after capture) between fat content and encapsulation rate was not statistically significant ($r = .30$, df $= 25$, $p = .13$). We did not find any ectoparasites or eugregarines from *C. virgo* males.

Observational males

In the males that had not been used in staged contests, encapsulation rate correlated positively with (log$_{10}$-transformed) fat content ($r = .44$, df $= 26$, $p = .025$) (Figure 3). Fat content correlated positively with age ($r = .44$, df $= 26$, $p = .018$). We did not find correlation between age and encapsulation response ($r = .015$, df $= 26$, $p = .94$) or between hemocyte density (log$_{10}$-transformed) and encapsulation response ($r = .11$, df $= 26$, $p = .58$).

DISCUSSION

In this study, we staged territorial contests between males of the damselfly, *C. virgo*. The main result was that winners showed stronger postcontest encapsulation response than did losers, indicating that winners have better immunocompetence. Recent studies have shown that male wing pigmentation may provide a reliable signal of immune function in calopterygid damselflies (Rantala et al., 2000; Siva-Jothy, 2000), and these wing ornaments affect female reproductive decisions (Córdoba-Aguilar, 2002, Siva-Jothy, 1999). Our results indicate that it may be possible to predict male immune function simply by the outcome of male-male competition. If the outcome of male-male competition can be used to predict male quality, this may enable females to avoid the costs of mate choice (see Pomiankowski, 1987) and indirectly gain superior sires simply by mating only with males defending high-quality territories (i.e., "passive mate choice," Thornhill and Alcock, 1983).

Our results suggest that both dominance and immunocompetence may depend on individual condition. Winners of our staged contests had bigger postcontest fat reserves, as previously found in other *Calopteryx* species (Marden and Waage, 1990; Plaistow and Siva-Jothy, 1996). Moreover, in males that had not been in staged contests, there was a positive
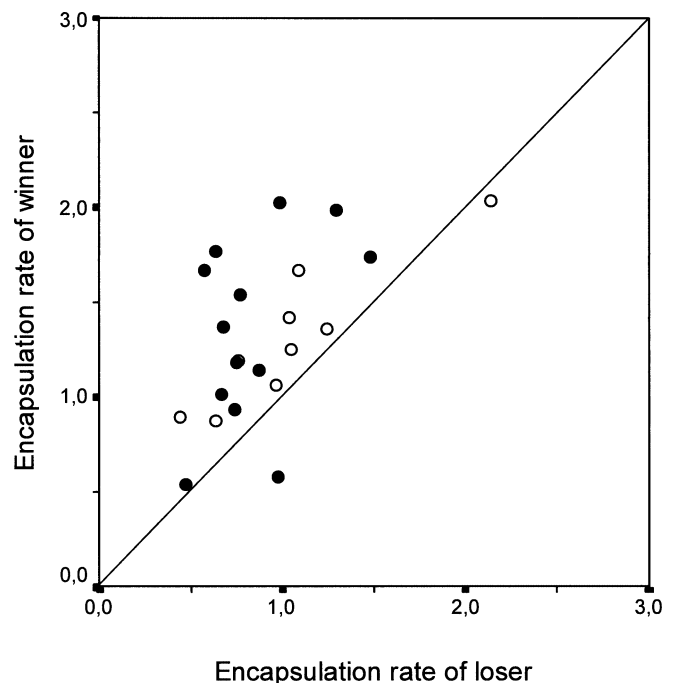

Figure 1
Encapsulation rate of winners and losers of territorial contests in *C. virgo* males. The symbols above the line represent contests in which winner had stronger encapsulation rate. The symbols below the line represent contests in which loser had stronger encapsulation rate. Open dots refer to Mustajoki Creek; filled dots, Vispiläjoki Creek.
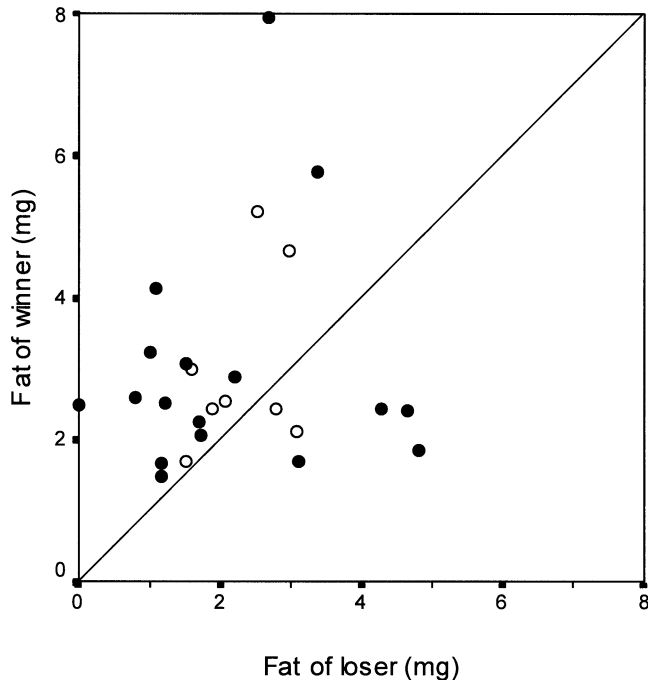
Figure 2
Fat content of winners and losers of territorial contests in *C. virgo* males. Symbols above the line represent contests in which winner had more fat. Symbols below the line represent contests in which loser had more fat. Open dots refer to Mustajoki Creek; filled dots, Vispiläjoki Creek.

correlation between fat content and encapsulation response. It is also possible that males that have originally poor immunocompetence get infected, which results in smaller energy reserves and lower fighting ability. Infection with the eugregarine trophozoites during the teneral life-history stage

is known to affect the ability of damselflies to accumulate fat and, consequently, reduce their ability to be territorial when they become reproductively active (Siva-Jothy and Plaistow, 1999). Surprisingly, we did not find any macroparasites from *C. virgo* in this study—not even eugregarines, which are cosmopolitan mid-gut parasites of damselflies (Åbro, 1996).

The positive relationship between male energy reserves and immunocompetence is consistent with the idea that immune function is costly, as recently demonstrated by Moret and Schmid-Hempel (2000) in a study performed on bumblebees (*Bombus terrestris*). Likewise, Suwanchaichinda and Paskewitz (1998) showed, using the mosquito *Anopheles gambie* Giles, that nutritional deprivation during larval stages decreased adult's melanization response. If both immunocompetence and resource holding potential depend on energy reserves, there may be a trade-off between disease resistance and energetically costly territorial behavior. However, in a previous study, Siva-Jothy et al. (1998) found no change in encapsulation response in fighting males of the calopterygid damselfly, *Matrona basilaris japonica* Fester. They concluded that the result might be a statistical consequence of the small sample size. However, males and females showed a significant reduction in encapsulation response after reproductive activity (copulation and oviposition), which suggests the costliness of these activities (Siva-Jothy et al., 1998).

In contrast to a previous study (Rantala et al., 2000) performed on *C. splendens*, in the present study we did not find any relationship between encapsulation rate and the density of hemocytes. It is possible that in *C. virgo* encapsulation rate is limited by some other factor than hemocyte density. Another surprising result of the current study was the positive correlation between age and fat reserves in reproductively mature *C. virgo* males. Previous studies performed on *C. maculata* (Marden and Waage, 1990) and *C. splendens xanthostoma* (Plaistow and Siva-Jothy, 1996) have found a negative correlation between age and fat reserves. Possibly, our results were contrary to previous studies because of seasonal effects. For example, in the present study the
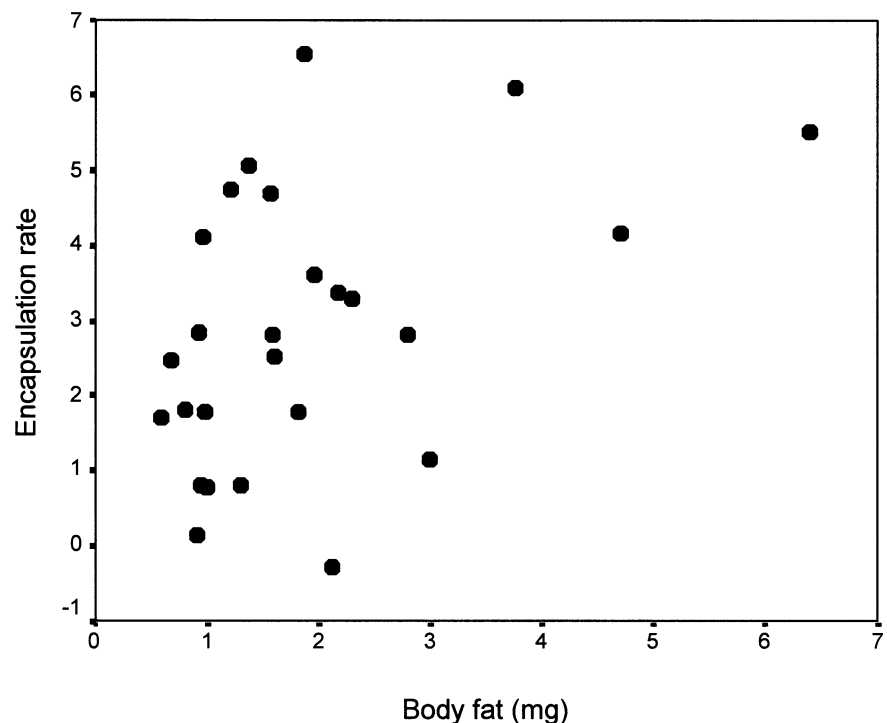

Figure 3
Relationship between fat content and encapsulation rate in *C. virgo* males that were not used in staged contests.

younger males may have been exposed to poor environmental conditions (such as bad weather) during the prereproductive teneral life-history stage when damselflies accumulate fat reserves.

In conclusion, the present study has shown that dominant males are in better condition and have better immunocompetence in the damselfly *C. virgo*, suggesting that male immunocompetence can be predicted by the outcome of male-male competition. We also suggest that future studies should further investigate the possible trade-off between immune function and energetically expensive territorial behavior in general.

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