NO FIRM EVIDENCE OF IMMUNOLOGICAL COSTS OF INSECT OVIPOSITION AND COPULATION: A TEST WITH DRAGONFLIES (ZYGOPTERA)

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The immune response is a costly trait as investment in immunity is frequently traded off against life history components. In insects, for example, experimental tests have provided evidence that oviposition and copulatory activities impair immune ability in the form of encapsulation ability. Here such tests are replicated by using four zygopteran spp., viz. *Argia joergenseni*, *Calopteryx splendens, C. virgo* and *Hetaerina americana* having encapsulation, phenoloxidase and nitric oxide activity − three key components in the insect immune response − as dependent variables. The results provide no consistent results. Only in *A. joergenseni* there was any evidence of oviposition activity (or, in the case of *H. americana*, submergence) affecting encapsulation, but neither in *C. splendens* nor in *H. americana* did copulation have any such effect. In *H. americana,* nitric oxide activity was lower in ♀♀ that had been submerged but there was no effect on phenoloxidase activity. Thus, former observations indicating that oviposition and copulation negatively affect the immune response, cannot be generalized.

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INTRODUCTION

Current evolutionary ecology theory has included the cost of producing and maintaining an immune response as a potential source of trade offs between this function and life history traits. A number of empirical works have supported this, with immunity expression covarying negatively with growth, reproduction and survival (reviewed by SCHMID-HEMPEL, 2005; SADD & SCHMID-HEMPEL, 2009; SCHULENBERG et al., 2009). The underlying logic for this relationship is that, similar to other resource-based trade offs, resources are finite for an organism, thus leading to resource allocation conflicts between costly functions. Two classical cases of immunity costs derived from costly behavioural activities are oviposition and copulation. Such costs have been documented in Zygoptera (SIVA-JOTHY et al., 1998), crickets (ADAMO et al., 2001; BASCUÑÁN-GARCÍA et al., 2010) and beetles (ROLFF & SIVA-JOTHY, 2002). In these cases, oviposition and copulatory activities have negatively affected the immune response and vice versa. The underlying physiological link is the juvenile hormone whose action mediates gamete and accessory gland production, restricting the allocation of resources to immunity (ROLFF & SIVA-JOTHY, 2002). Periods of juvenile hormone action may lead to immune-suppression, rendering animals more vulnerable to infections (ROLFF & SIVA-JOTHY, 2002). In these studies, two immunological parameters were used: phenoloxidase activity (PO - an enzyme that gets activated at the onset of immune response; CERENIUS & SODERHÄLL, 2004) and encapsulation (a defense mechanism against parasitic protozoans and metazoans, fungi and parasitoids such as wasp eggs or larvae (GILLESPIE et al., 1997; LAVINE & STRAND, 2002; BRENNAN & ANDERSON, 2004). These parameters have usually been acknowledged as good indicators of immune capacity (i.e. CONTRERAS-GARDUÑO et al., 2007; RANTALA & ROFF, 2007). Here we have re-addressed the question of whether oviposition and copulation bear an immunological cost in insects, using four species of damselflies as study subjects. Odonates have been widely used in ecological and evolutionary studies (CÓRDOBA-AGUILAR, 2008). Regarding ecological immunity, a wealth of studies in this group have provided key results, indicating that sexual activities and functions are coupled with immune condition in a trade off fashion (e.g. SIVA-JOTHY et al., 1998; CONTRERAS-GARDUÑO et al., 2006, 2007, 2008; CÓRDOBA-AGUILAR & MÉNDEZ, 2006). Here we have included encapsulation ability, PO and nitric oxide (NO) activity as dependent variables. This last variable has not been included in previous studies of oviposition and copulatory costs. NO is a reactive free radical gas that suppresses protein catalytic activity with protein and has harming effects on pathogens' DNA (RIVERO, 2006). This characteristic makes NO another key indicator of immunological condition (RIVERO, 2006) and studies on Zygoptera have corroborated this (i.e. CÓRDOBA-AGUILAR et al., 2009). The use of more than one immunological parameter

has been recommended as a single measure cannot be used to cover the entire host's immune ability (BOA-AMPONSEN et al., 1999; ADAMO, 2004). Our aim was to see the extent of the supposed physiological costs imposed by insect oviposition and copulatory activities. The four species we used in our study show considerable differences in their mating systems: a non-territorial species (*Argia joergenseni*) and three territorial species (*Calopteryx splendens*, *C. virgo* and *H. americana*). This diversity is a significant sample of the sexual biology spectrum found in the Zygoptera (SERRANO-MENESES et al., 2008) and thus makes our selection valid for the aim of our study.

We followed the damselfly experimental protocol of SIVA-JOTHY et al., (1998) in which females that ended oviposition were immune challenged and males were also immune challenged but after copulation. Oviposition behaviour in Zygoptera may include costs other than that of laying eggs *per se*, such as the ovipositing posture, male harassment and/or water immersion (CORBET, 1999). Thus it is important to determine what in particular is potentially affecting the immune response. Therefore, in our experimental protocol, we disentangled the costs of laying eggs from the behaviour that accompanies this activity.

MATERIAL AND METHODS

Argia joergenseni was collected in Cabalango (31°1'S, 64°34'W), Sierras Chicas, Córdoba Province, Argentina, from November 2008-January 2009. *Calopteryx splendens* and *C. virgo* were studied in the Creeks Mynäjoki (60°38'N, 21°55'E) close to Turku, Finland from June-July 2009. *Hetaerina americana* was studied in the Amacuzac river, south Mexico city (18°32'56"N, 99°16'23"W) in June 2003 and September 2009.

EFFECTS OF OVIPOSITION BEHAVIOUR ON IMMUNITY − Thirteen in-tandem *A. jorgenseni* females were allowed to lay eggs in a plastic container filled with water and containing the aquatic plant (*Elodea* sp.) obtained from the damselfly collection site. Oviposition was interrupted to immune challenge the females. This challenge consisted of inserting a previously-disinfected (in 100% ethanol) piece of nylon (1.5-2.0 mm long and 0.5 mm wide), which was left within the animal for 12 h. Control females were 13 in-tandem animals that were not allowed to oviposit and that were immune-challenged for the same 12 h. In both groups, implants were extracted and preserved in 70% ethanol for encapsulation measurement (see below).

Females *C. splendens* (N = 4) and *C. virgo* (N = 5) were allowed to oviposit until they finished. After oviposition, each female was caught. Additionally, non-ovipositing females were also caught (N = 4 and 5 for *C. splendens* and *C. virgo*, respectively). Age of both female sets was similar as judged from the body colour and wing aspect (for a rationale of age differences see CÓRDOBA-AGUILAR, 2009). Both female sets were placed individually in cylindrical plastic containers (75 mm height and 45 mm diameter), which were then placed in a cool box until a nylon challenge. A sterile 2 mm length of nylon monofilament (diameter 0.18 mm) was inserted into the fourth abdominal pleura on the dorsal side of the sternal-tergal margin of all females. Each insect was then returned to its plastic container, and was left for 24 h at constant room temperature (22°C).

In 2003, 22 fully mature *H. americana* females (as judged from their hardened exoskeleton and developed body and wing coloration; CÓRDOBA-AGUILAR, 2009) were collected and gently tethered (with a thread on their thorax, to avoid obstructing wing movements) to a wooden stick, allowing for ca. 5 cm of thread. Females were able to grab the stick and when they did so, were submerged to

the river water for 15 minutes. This situation resembles that of natural oviposition behaviour in this species (CÓRDOBA-AGUILAR, 2009). A previously disinfected nylon implant (1 mm length, 0.2 mm diameter) was inserted through the fourth abdominal pleura on the ventral mid-line using fine forceps. As control animals, 22 fully mature females were collected and a nylon implant with similar characteristics was inserted in the same region as in the experimental females. Ages were similar in both groups as judged from body colour and wing aspect. Implants were left for 8 h. in both groups. We did not use ovipositing females as, unlike the other species in our study and those used in previous studies (i.e. SIVA-JOTHY et al., 1998), we wanted to disentangle the effect of laying eggs from the accompanying behaviour. After insertion, each female was put in a plastic, transparent container (4.5 × 1.4 cm) with a piece of wood for perching and a piece of humid cotton. Containers were maintained within a dark, cool box in the shadow, to reduce the animals' activity (so that they did not incur an unnecessarily high energy expenditure).

In 2009, 11 fully mature *H. americana* females were treated similar to those we submerged in 2003 and 28 fully mature females were used as control individuals. Again, females of both sets showed a similar age as indicated by their colour and aspect. Each of these animals was treated for PO and NO activity (see below).

For all females of the four species, the length of the right forewing was measured (in mm) as an indicator of body size.

EFFECTS OF COPULATION ON IMMUNITY − Sixteen *C. splendens* territorial males were captured soon after they finished copulation. These males were treated similar to those ovipositing *C. splendens* females that were nylon-challenged (described above). As a control group, 16 territorial males that were not copulating were also nylon challenged. For encapsulation measurement, these males were treated as the control *C. splendens* females used for the oviposition experiment and described above. Both sets of males had similar ages according to the criteria of PLAISTOW & SIVA-JOTHY (1996).

In July 2000, 12 *H. americana* males that had finished copulation were captured, separated from the females and challenged in a similar fashion to those *H. americana* ovipositing females described above. As a control group, another set of 12 fully sexually mature males (as judged from their territorial behaviour and brilliant body and wing colours) was captured and were immune challenged similar to the copulating males. As with the experimental *H. americana* females that were nylon-challenged in 2003, implants were left for 8 hrs while the animals were placed individually in a plastic container with a perching piece and humid cotton, in the dark. Encapsulation was measured.

As an indicator of size, the length of the right forewing was measured (in mm) for both species.

MEASURES OF ENCAPSULATION, PO AND NO ACTIVITY − For *A. jorgenseni* and *H. americana*, and while the animals were still alive, each nylon implant was retrieved by carefully removing the abdominal cuticle around the implant under a dissecting microscope. The implant was preserved in ethanol (70%) for 7 days and re-hydrated during 24 h. Implants were photographed using a digital camera attached to a microscope. The encapsulation area around the nylon was measured using the software Image Tool ®. The relative encapsulated value was obtained in relation to the whole implant area. For *C. splendens* and *C. virgo*, each implant was gently removed from the insect and frozen for later analysis. Each implant was photographed from two different angles under a light microscope with a digital camera. Images were then analysed using the computer program Image Pro ®. As a measure of encapsulation rate, we used the mean of the grey values of reflecting light from the two digital pictures of each implant. The data were transformed by subtracting the observed grey values from a control value. The control value was obtained by photographing a haphazardly selected new (i.e. non-used) implant. The darkest grey values corresponded to the highest encapsulation rates. Despite the methodology differences of encapsulation measurements between American and European species, the fact that the comparison is made within species (but not between species) does not invalidate our study aims.

For PO activity, seven mL of phosphate buffer saline containing protease inhibitors (PBS-IP) were injected in the mid region of each damselfly thorax. Heads were then removed and the thorax was

gently pushed to obtain 2 μL of haemolymph/PBS-PI per animal. To these 2 μL per animal 100 μl of PBS-PI were added and PO activity measured spectrophotometrically twice by recording dopa-chrome formation from $_L$-dihydroxyphenylalanine ($_L$-DOPA, Sigma). From this last mixture (102 μL of haemolymph/PBS-PI plus PBS-PI), twenty five μL of sample with a concentration of 10 μg/lL of protein (see this method in Contreras-Garduño et al. 2007) were added to 150 μL of PBS-PI and mixed on a micro-well plate with 25 μL of $_L$-DOPA (3mg/ml of PBS-PI) as substrate (in total 200 μL of sample, PBS and substrate were added per animal). Optical density was registered at 490 nm using a micro-plate reader (Model 350, Bio-Rad). As blanks, 175 μL of PBS were mixed with 25 μL of $_L$-DOPA and the optical density was recorded also at 490 nm. Enzyme activity is expressed as units, where one unit represents the change in absorbance min^{-1} (SÖDERHÄLL & HÄLL, 1984). Three readings of PO were taken every 15 minutes so that each datum represents the mean PO activity for the three recordings. For NO activity, the Griess reaction was used. For this, 50 μl of each haemo-lymph sample (from the 102 μL of haemolymph/PBS-PI plus PBS-PI indicated above) was mixed with 50 μl of sulfanilamide and 50 μl of 0.1 % naphthylethylenediamine (Sigma, St. Louis, MO, USA). This mixture was incubated for 10 min at room temperature. Using a plate reader and at 540 nm, absorbance was recorded after 15 and 30 minutes and an average for each individual was obtained for both recordings. NO was quantified using a NaNO$_2$ (1-100 μM) standard reference curve for each assay. Results are provided as nitrite and nitrate concentrations.

STATISTICAL ANALYSES − When encapsulation values were measured as proportions, data were transformed with the following equation: encapsulation = arcsin (square root(value)). If data were amenable for transformation or were normally distributed, general linear models were used in which treatment (experimental and control animals) was entered as a factor, immune responses (en-capsulation, PO and NO) were entered as response variables and size was entered as a covariate. When general linear models were used, interactions were tested and reported, but they were not included in the final model when they were non-significant. Analyses were carried out in SPSS 15.0. Results are reported as mean ± STD unless stated otherwise. Mann Whitney U tests were used when normality or homogeneity of variances assumptions were not satisfied after transformation.

RESULTS

EFFECTS OF OVIPOSITION ON IMMUNITY

In *A. joergenseni*, encapsulation response was lower in females that were allowed to oviposit (16.30 ± 7.58 %) than in females that did not oviposit (37.87 ± 20.48 %; t test = 3.613, P = 0.002, N = 26; Fig. 1). There was no correlation between wing length and encapsulation response either in ovipositing ($r_{pearson}$ = 0.396, P = 0.180, N = 13) or non-ovipositing females ($r_{pearson}$ = -0.228, P = 0.454, N = 13). In *C. splendens*, there was no difference in encapsulation response in females that oviposited (39.17 ± 25.17) vs. females that did not oviposit (47.58 ± 19.55; $F_{1,6}$ = 0.085 P = 0.782, N = 8). Wing length was not related to encapsulation activity ($F_{1,6}$ = 1.140, P = 0.335, N = 8) and there was no effect of the interaction group (oviposition/no oviposition) − wing length ($F_{1,5}$ = 1.382, P = 0.305, N = 8).

In *C. virgo*, there was no difference in encapsulation response in females that oviposited (44.07 ± 10.85) vs. females that did not oviposit (51.99 ± 17.12; $F_{1,7}$ = 1.371, P = 0.280, N = 10) and wing length did not have any effect ($F_{1,7}$ = 0.643, P = 0.449, N = 10). The interaction group (oviposition/ no oviposition) − wing length
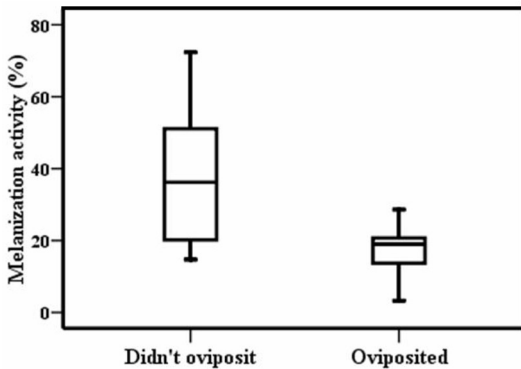
Fig. 1. Difference in encapsulation response in ovipositing and non-ovipositing *Argia joergenseni* females.

was also non-significant in the model ($F_{1,6}$ = 0.005, P = 0.947, N = 10).

In *H. americana*, encapsulation response did not differ between experimental (N = 16; 10.18 ± 2.42 %) and control females (N = 14; 11.19 ± 2.81 %; $F_{1,27}$ = 0.173, P = 0.681). The response was positively correlated with female size ($F_{1,27}$ = 6.101, P = 0.020) but this positive trend was independent of the treatment (treatment − size, $F_{1,27}$ = 0.034, P = 0.855). PO activity did not differ between experimental (N = 11; 0.131 ± 0.147 U / mg protein) and control females (N = 28; 0.055 ± 0.034 U / mg protein; Mann-Whitney U = 104.00; P = 0.124; N = 39), and the non-significant trend remained after removing two outliers from each group (Mann-Whitney U = 87.001, P = 0.271, N = 35). PO activity was not related to female size in submerged ($r_{spearman}$ = -0.530, P = 0.115) or control ($r_{spearman}$ = -0.233, P = 0.233) females. NO activity was lower in submerged females (0.015 ± 0.016 nitrite / nitrate) than in control females (1.05 ± 1.14 nitrite / nitrate; Mann-Whitney U = 10.001, P < 0.001, N = 38), even excluding three outliers from the control group (Mann-Whitney U = 10.000, P < 0.001, N = 35; Fig. 2). There was no relationship between female size and NO activity in control ($r_{spearman}$ = -0.237, P = 0.233, N = 27) or submerged females ($r_{spearman}$ = 0.232, P = 0.518, N = 10).
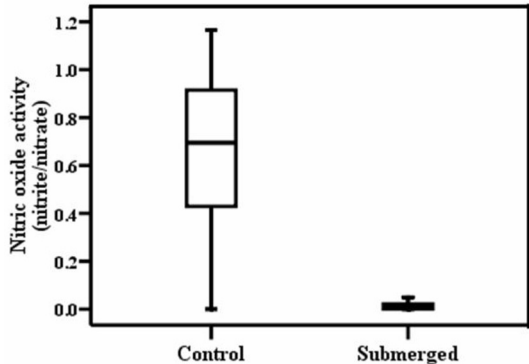

Fig. 2. Difference in NO activity between experimental and control *Hetaerina americana* females.

EFFECTS OF COPULATION ON IMMUNITY

In. *C. splendens*, there was no difference in encapsulation activity of males that copulated (48.02 ± 14.65) and males that did not copulate (43.87 ± 14.89; $F_{1,29}$

= 1.753, P = 0.196, N = 32). Wing length was not related with encapsulation activity ($F_{1,29}$ = 3.603, P = 0.068) and there was no effect of the interaction group (copulated/did not copulate) − wing length ($F_{1,28}$ = 0.633, P = 0.433).

In *H. americana*, there was no difference in encapsulation activity of males that copulated (11.23 ± 1.17 %) and males that did not copulate (10.75 ± 1.30 %; $F_{1,20}$ = 4.070, P = 0.057, N = 24). Wing length was not related with encapsulation activity ($F_{1,20}$ = 0.003, P = 0.954) and the interaction group − wing length was marginally significant ($F_{1,20}$ = 4.240, P = 0.053). When excluding the interaction group − wing length from the model, group and wing length remained non-significant (group: $F_{1,21}$ = 0.905, P = 0.352; wing length: $F_{1,21}$ = 0.086, P = 0.772).

DISCUSSION

In only one species, *A. joergenseni*, did we find that females laying eggs paid the costs of such activity via a reduction in melanization ability. This result was not replicated in the other three calopterygids, in which both laying eggs and the behaviour that accompanies this were investigated. Furthermore, the assumed costs of copulation were not found. Thus, despite previous results in insects, which include one odonate species, regarding immunological costs of copulation and oviposition activities, we did not find support for this. Our experimental framework is robust considering: (1) that more than a single immunological component was used; (2) the immune components used have been frequently identified as indicators of immune condition; (3) our experimental approach replicated experimental procedures used in previous studies (which separate the possible differential costs of females laying eggs and females carrying out ovipositing behaviour but not laying eggs); and 4) we used four good representative species of the sexual diversity found in the Zygoptera. Given these considerations, one can safely generalize that the previously identified immunological costs are unlikely to apply to other odonates. It remains to be explored whether our results also apply to insects in general.

One alternative explanation for our results is that the costs of both copulation and oviposition activities are not high enough to be detected. Previous evidence in odonates regarding the costs of some behavioural activities has been widely documented. For example, it has been found that fighting for territories in males of *C. virgo* and *H. americana* imposes a reduction in encapsulation ability (KO-SKIMÄKI et al., 2004; CONTRERAS-GARDUÑO et al., 2006), while sexual harassment reduces PO activity (CÓRDOBA-AGUILAR, 2009) and fat reserves (CÓRDOBA-AGUILAR & GONZÁLEZ-TOKMAN, 2011) in females of *H. americana*. This implies that these activities entail higher energetic costs than oviposition and copulation. Another explanation is that such costs will emerge based on the animal's condition. For example, in *H. americana*, an animal that is sexually active the entire year, there is seasonal variation in male energetic and

immunological condition (CÓRDOBA-AGUILAR et al., 2009). This implies that some activities may be more costly in some months compared to others. In fact, after a bacterial challenge, males die sooner in seasons when animals are in worse condition (CÓRDOBA-AGUILAR et al., 2009). However, although this may apply to *H. americana*, this is not the case for the other three species given that their sexual activities are seasonal.

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