Odonatologica 37(2): 111-118 *June 1, 2008*

DIFFERENCES IN IMMUNE ABILITY DO NOT CORRELATE WITH PARASITIC BURDEN IN TWO ZYGOPTERA SPECIES (CALOPTERYGIDAE, COENAGRIONIDAE)

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Received June 27, 2007 / Revised and Accepted October 4, 2007

Differences in phenoloxidase (PO) and hydrolytic enzymes (HE) activity, two key components in insect immune ability, were investigated in *Hetaerina americana* and *Argia tezpi*, to see if they are correlated with patterns of gregarine and mite infection. The prediction was that the sp. with the more robust immune responses would show a less intense parasitic burden. Fully mature adults of both sexes were used. No clear pattern was found: *H. americana* had higher PO activity while *A. tezpi* had higher HE activity but the latter sp. had a higher parasitic load for both parasites. Several possible explanations are discussed. However, it seems most likely that either the immune responses measured may be traded-off with other non-immune functions in which both spp. differ in investment or that both immune components may be traded-off with each other.

INTRODUCTION

The reason why individuals vary in parasite defense has recently been illuminated by the emerging discipline of ecological immunity (ROLFF & SIVA-JOTHY, 2003; SCHMID-HEMPEL, 2005; LAWNIZACK et al., 2007). This is based on the fact that immunity is costly to produce and, therefore, not all individuals can afford an effective immune response. The cost can be either environmental

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or genetic-restricting components and, similar to to any other life-history trait, would restrict an organisms response to the parasite's co-evolutionary responses and pressure (SCHMID-HEMPEL, 2005).

It is expected that how much a particular species invests in immunity will reflect the parasite pressure that that species is facing (e.g. FELLOWES & GODFRAY, 2000). Given that resources to produce an immune response are limited, if parasitism risk is low then a reduced investment to immune defense would be expected. For example, a recent study in birds suggested that those species that are more susceptible to be parasitized showed higher antibody values (LINDSTRÖM et al., 2004). An excellent insect group on which to test this hypothesis is the Zygoptera for the following reasons: (a) they can be easily collected and followed in the field; (b) the most common parasites in the whole Odonata order - intestinal gregarines and ectoparasitic mites - can be easily quantified in/on adult hosts (CORBET, 1999); (c) these parasites have been shown to have detrimental effects on damselfly fitness (e.g. SIVA-JOTHY & PLAISTOW, 1999) and vary in intensity among damselfly species (e.g. ÅBRO, 1974), which suggests differential investment in host immunity; (d) recent developments in damselfly immunity have already documented the type of immune responses that can be costly and linked to parasite defense (e.g. SIVA-JOTHY, 2000; CÓRDOBA-AGUILAR et al., 2006; CONTRERAS-GARDUÑO et al., 2007).

In this paper we have tested the idea that interspecific differences in two key immune arms, phenoloxidase (PO) hydrolytic enzymes (HE), will reflect different pressure by gregarine and ectoparasitic parasites on the two zygoptera species, *Hetaerina americana* and *Argia tezpi*. Immediately after pathogen recognition, PO converts phenols into quinones which lead to the formation of melanin, which either circumvents the parasite's body (cellular encapsulation) or is added to the pathogen's surface (humoral melanization) (CERENIUS & SÖDERHÄLL, 2004; CHRISTENSEN et al., 2005) leading to the formation of toxic substances on the parasite (LANZ-MENDOZA et al., 2002; NAPPI & CHRISTENSEN, 2005). On the other hand, HE functions during immune response activation (i.e. proteases; CHENG, 1992; TZOU et al., 2002; HETRU et al., 2003) by modifying the bacterial membrane and, therefore, allowing pathogen recognition by phagocytic cells or by being released from the cells to degrade the pathogen's membrane (CHENG, 1992; CAJARAVILLE et al., 1995). *H. americana* and *A. tezpi* are ideal for our aims as our previous results suggested that they have extreme parasitic burdens (CÓRDOBA-AGUILAR et al., 2006). Furthermore, the biology of these two species is relatively well-known. In the case of *H. americana*, males defend riverine territories where females arrive to mate. They have a red spot on the basis of each wing (CONTRERAS-GARDUÑO et al., 2006), the size of which correlates with male energetic condition (in the form of lipid muscle reserves) so that those males with larger spots are more likely to win a territorial contest and defend a territory (CONTRERAS-GARDUÑO et al., 2006; SERRANO-MEN-

ESES et al., 2007). As for *A. tezpi*, although less well documented, the males are non-territorial and both sexes aggregate in riverine places to mate (CÓRDOBA--AGUILAR et al., 2006). In this study, our specific prediction is that the species with the higher parasitic burdens will show higher values of PO and HE.

MATERIAL AND METHODS

Hetaerina americana males (N = 20) and females (N = 20) were obtained from the Tehuixtla river (Morelos, Mexico) in May, 2004. *Argia tezpi* males (N = 20) and females (N = 20) were collected at La Buena Mujer Dam (Baja California Sur, Mexico) between May-June, 2004. Given that recently emerged damselfly adults bear low parasitic burdens (e.g. SIVA-JOTHY & PLAISTOW, 1999), all animals were fully mature at the time of capture assessed by their overtly clear reproductive activities at the time of collection (i.e. mating and/or oviposition).

PREPARATION OF ZYGOPTERA HOMOGENATE SUPERNATANT – Each sample (abdomen and thorax only) was homogenised using a polyethylene plastic micro pestle (for 5 minutes) in pre-cooled Eppendorf tubes containing 1 ml of Tris-buffered saline (TBS; 50 mM Tris, hydroxymethyl aminomethane, 1000 mM NaCl). The homogenate was centrifuged at 18,000 g for 10 min at 4°C using a Beckman centrifuge. The clear supernatant was removed and 1 ml of TBS was added to the pellet to be homogenised and centrifuged again twice under the same conditions. Supernatants (3 ml) were pooled from each sample and used immediately in the assays or frozen (-80°C) until use.

ASSAYS FOR PO ACTIVITY – PO activity (in quadruplicate, obtaining an average of the four measurements) was measured spectrophotometrically by recording the formation of dopachrome from $_{L}$-dihydroxyphenylalanine($_{L}$-DOPA, Sigma). 50 µl of sample were mixed on a micro-well plate with 50 µl of $_{L}$-DOPA (3mg/ml of TBS) as substrate. Thereafter, 50 µl of buffer were added and optical density at 490 nm was recorded every 5 min for 1 h, using a micro-plate reader (Model 350, Bio-Rad). As sample blanks, 100 µl of buffer were mixed with either 50 µl of $_{L}$-DOPA. Enzyme activity was expressed as units, where one unit represents the change in absorbance min^{-1} (SÖDERHALL & HÄLL, 1984). Mean PO activity was obtained as a function of time. For logistic reasons, only 10 males and 10 females of *H. americana* were used for PO analysis.

API ZYM SYSTEM – We used the API ZYM commercial kit (BioMerieux, Inc.) which detects the following 19 hydrolytic enzymes: leucyl arylamidase, valyl arylamidase, cystyl arylamidase, trypsin, α-chymotrypsin (all proteases); lipase esterase (C8) and lipase (C14) (all lipases); α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase (all glycosidases); esterase (C1); and phosphatases: alkaline phosphatase, acid phosphatase and naphthol phosphohydrolase (all esterases).

65 microliters of each supernatant sample were added to the reaction strips. These were incubated at 37°C for 4 h. Ten minutes after the addition of the reagents of the API ZYM kit at room temperature, the resulting colours could be estimated and recorded from 0 to 5, according to a colour scale given by the manufacturer. Results were transformed to nM quantity of hydrolysed substrate. Enzyme activities were expressed as units, where one unit represents the hydrolysed substrate in nM/mg of protein.

PROTEIN DETERMINATION – The Bradford (1976) method was used to determine protein concentration in samples; this controls for body size differences between species. Bovine serum albumin (Sigma®) was used as standard. For this, 20 µl of sample and 200 µl of buffer were added into individual wells of a 96-well plate. 40 µl of Bradford reagent was added and after 10 minutes, absorbance was read at 595 nm. As a control, 220 µl of buffer plus 40 µl of Bradford was used.

PARASITIC BURDEN – Fully mature adults of both sexes and species were collected during the second week of each of the following months: *H. americana* from September 2004 to June 2005 and from October 2006 to April 2007 (in total, 20 females and 79 males), and *A. tezpi* from Novem-

ber 2004 to February 2005 (in total, 20 females and 20 males). Once animals were collected they were preserved in 70 % ethanol. By gently removing the entire gut with fine forceps and scissors under a dissecting microscope, gregarines were looked for and counted. These parasites can be distinguished by being attached to the gut lumen. We also counted those mites attached to the cuticle or wing veins, or the scars the stylostoman mite left before unattachment (FORBES, 1991).

ESTIMATORS OF INFECTION – The next estimators of the infection defined by BUSH et al. (1997) were used: Prevalence (percentage of hosts parasitized by one parasite species), abundance (mean of the individuals per parasite species/host species examined) and mean intensity (mean number of individuals of one parasite species in a parasitized host).

STATISTICS – Enzyme values are given as U/mg of protein. For the use of parametric tests, parasite, enzyme, protease and protein concentration values were transformed using the formula $\sqrt{(x + 0.5)}$. Results are reported as mean ± STD unless stated otherwise.

RESULTS

IMMUNE COMPONENTS

There were differences in PO activity between species (t test = 9.3, $P < 0.0001$; Fig. 1a) with *H. americana* having more PO (722.76 ± 53.85 U/μg of protein, N = 20) than *A. tezpi* (206.15 ± 13.25 U/μg of protein, N = 40). There were also differences in HE activity between species (student t test = 6.53, $P < 0.0001$; Fig. 1b). However, in this case *A. tezpi* showed more HE activity (73.71 ± 10.8 U/μg of protein, N = 38) than *H. americana* (3.07 ± 0.41 U/μg of protein, $N = 40$).

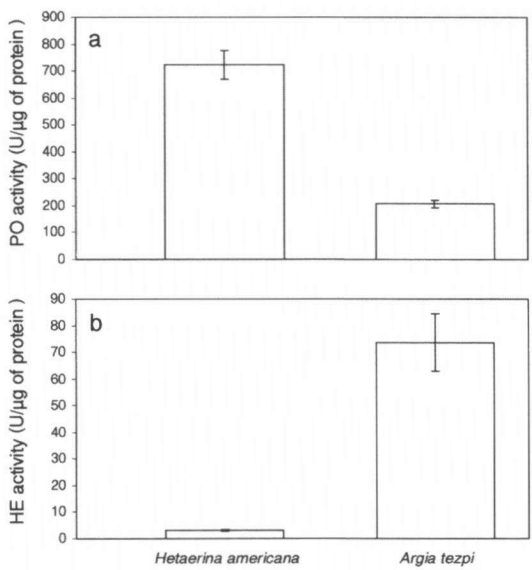

Fig. 1. Differences (mean ± SE) in (a) PO and (b) HE in *Hetaerina americana* and *Argia tezpi*.

Only 3 out of 19 enzymes were not found in both species: lipase, cystyl arylamidase and acid phosphatase. Protein concentration did not differ between species (*H. americana*: 0.75 ± 0.12; *A. tezpi* males: 0.79 ± 0.11; student t test. = 0.18, P = 0.85)

PARASITE OCCURRENCE AND INTENSITY

A significant difference between species prevalence occurred. *A. tezpi* had more gregarines than *H. americana* ($X^2 = 19.37$, $P = 0.0000$) being the same result with mites although no *H. ameri-*

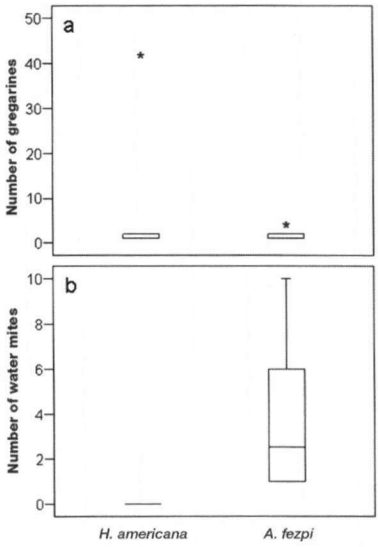

Fig. 2. Abundance (medians and quartiles) of: (a) gregarines and (b) water mites between species. Asterisks are outliers.

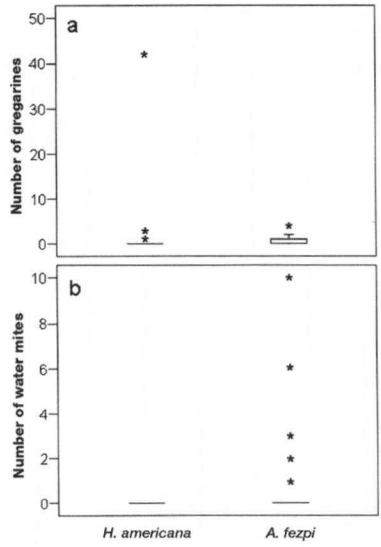

Fig. 3. Intensity (medians and quartiles) of: (a) gregarines and (b) water mites between species. Asterisks are outliers.

cana individual was infested with these parasites. Similarly a greater abundance of gregarines and mites was found in *A. tezpi* (for gregarines, *A. tezpi* rank sum = 6355; *H. americana* rank sum: 3375; Mann-Whitney *U* test: 1405, $P = 0.0007$; Fig. 2a; for mites, *A. tezpi* rank sum = 6633; *H. americana* rank sum: 0; Fig. 2b). Mean intensity did not differ between species in gregarine number (*A. tezpi* rank sum = 149; *H. americana* rank sum: 61; Mann-Whitney *U* test: 40, $P = 0.86$; Fig. 3). As water mites were not found in *H. americana* the test for mean intensity was not performed.

DISCUSSION

Contrary to our prediction, the species that had more gregarine and mite parasites, *A. tezpi*, did not show consistently higher immune responses than the species that had considerably fewer parasites, *H. americana* with *A. tezpi* only showing higher values than *H. americana* in HE. Possible explanations are: (a) HE is more linked to gregarine and mite defence than PO which explains why *A. tezpi* had higher values of HE. This is unlikely given that PO takes a key role at the onset of an immune response (SÖDERHÄLL & CERENIUS, 1998) which does not really depend on which of the two parasites was attacking. Indeed, a contrary result should be expected as HE is more linked to bacterial than metazoan infections. – (b) By being larger in size, *H. americana* may have more PO than HE

compared to *A. tezpi*, given that PO participates also in non-immune functions such as, for example, exoskeleton and egg production (SÖDERHÄLL & CERENIUS, 1998). This hypothesis is also unlikely as we controlled for body size differences in the form of protein concentration in our samples and our results did not reveal significant differences between species in protein concentration. – (c) There is a trade-off between the immune components we measured and other non-immune functions which are different in each species. For example, it may be that, in *H. americana*, there are high energetic costs for traits linked to mate competition (such as male territorial behaviour and the male red wing spot) which can affect HE production and which does not take place in *A. tezpi*. This hypothesis has some support as territorial defence has a detrimental effect on immune-related melanization ability in *H. americana* (CONTRERAS-GARDUNO et al., 2006). It is not clear, however, why *A. tezpi* are not released from sexual selection pressure (as, although the males are not territorial, they still have to look for females so that immune components may be compromised) and then show at least relatively large PO values. This last hypothesis can be tested by elucidating the components that PO and HE are made of and then track whether these components participate on (and, therefore, trade off) the functions where both species spend more energy on. One area worth exploring in this respect is that *A. tezpi* females oviposit more frequently than *H. americana* females (J. Contreras-Carduño, pers. obs.). Given that PO participates in egg production (LI & LI, 2006), the reduced PO found in *A. tezpi* may be related to this higher oviposition frequency. However, given that our PO measurement does not distinguish between immune-related PO and PO that participates in other functions, we should have found large PO values in *A. tezpi*, which was not the case. – (d) Other pathogens, different from those we looked for, may be present and occur in different intensities in the two species, thereby explaining differential patterns of immune investment. Although this hypothesis may apply for HE, it would not be the case for PO, which is a general immune component that participates in both humoral and cellular responses and so would cover the entire pathogen community (SÖDERHÄLL & CERENIUS, 1998). – (e) A sort of trade-off between immune components may occur in such a way that, somehow, each species is investing more on a certain component (e.g. *H. americana* more on PO and *A. tezpi* more on HE). Trade-offs among immune components are not unusual and are actually expected (e.g. RANTALA & ROFF, 2006). One way this can be tested is by exposing hosts to a particular pathogen to elicit a certain immune component (e.g. HE) and then to another to elicit a different component (e.g. nitric oxide), and then have another group with the inverse manipulation. If a trade-off between immune components is taking place, the production of HE in the second group will be higher compared to the first group where HE production was firstly elicited. – (f) More immune measures may be needed as the two we recorded do not provide a complete picture of the immune condition. Although poten-

tially true, the more measures that are made, the more difficult the interpretation of the results becomes and this is why it is recommended that a few, direct (in terms of being linked to the specific pathogen defence) measures should be taken (ADAMO, 2004) which is what we did here. – (g) The robust immune production of PO may reduce parasite infection. Although this seems logical, it is not clear why *A. tezpi* still produces more HE than *H. americana*.

In summary, although our results do not follow the predictions we made, it seems that trade offs between immune and non-immune functions and within immune parameters can explain our results. Future research should test these hypotheses.

ACKNOWLEDGEMENTS

JC-G is in debt with the following people: M.A. PÉREZ-FLORES for his hospitality in Tehuixtla and help in many ways; R. BOLAÑOS-GARCÍA for her hospitality and help in La Paz; J. MANJARREZ for his logistic help; J. CANALES-LAZCANO, L.B. RIVERA-RODRÍGUES, C.S. VENEGAS-BARRERA and K.A. DOMÍNGUES-LINARES for their help in the collection trips; N. ESTRADA, M. RODRÍGUEZ OJEDA and M. ROMERO GERALDO for their laboratory support; and to M. VALDEZ-FUENTES for her attentions and support. Financial support was obtained from PAPIIT (UNAM-IN230603).

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