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SEXUAL DIMORPHISM IN IMMUNITY: A TEST USING INSECTS (COLEOPTERA, DIPTERA, LEPIDOPTERA, ODONATA)

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Evolutionary theory indicates that ♂♂ should allocate more resources to increase mating efficiency trading off longevity while ♀♀ would actually do the reverse to gain in egg production and laying. One recent hypothesis dictates that these differences would lead ♀♀ to invest more in immunity (to increase longevity) than ♂♂ (which will invest more in courtship traits). This difference should be more accentuated in spp. whose ♀♀ mate multiply, in which ♂♂ will invest less to immunity than in spp. where ♀♀ mate once. Here, this was tested by using 8 insect spp. with varying sexual selection pressure, that belong to 4 orders. For each order, one sp. was used in which ♀♀ accept one (or close to) mating during their life and another in which ♀♀ mate multiply. Encapsulation ability, phenoloxidase activity and hydrolytic enzymes were examined. Animals were virgin, sexually mature and well-fed. Comparative analyses provided restricted support as for ♀♀ having higher immune values and that this pattern should be more evident in relation to sexual selection intensity when both pairs of spp. per order and all spp. were analyzed. This study calls for a reformulation of current assumptions of immune costs in relation to gender life history differences.

INTRODUCTION

Males gain in reproductive success by mating with as many females as possible while in females the number of matings does not correlate with reproductive

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success (BATEMAN, 1948). This fundamental difference has evolutionary affected the administration of resources devoted to reproduction in each sex to increase fitness. To what life history theory goes, one prediction derived from these sexual differences is that males will invest more in traits that increase mating efficiency while females will invest in traits that favor longevity to produce more eggs (BATEMAN, 1948; TRIVERS, 1972; CLUTTON-BROCK, 1983). Since resources are frequently scarce, this difference means that other functions may be energetically impaired. In this case, it has been suggested that the ability to defend against pathogens and/or parasites is one very likely candidate for two reasons: it is costly to maintain and it is directly related to both sexes' reproductive needs (reviewed by SHELDON & VERHULST, 1996; ROLFF & SIVA-JOTHY, 2002; SCHMID-HEMPEL, 2005; LAWNIZAK et al., 2007). Furthermore, a recent hypothesis that envisages a conflict in resources devoted to immunity states that investment to immune defense will be higher in females due to selection acting on increased longevity (ROLFF, 2002) in contrast to males where resources needed for courtship traits will impair those devoted to immunity (ZUK, 1990; FOLSTAD & KARTER, 1992; WEDEKIND & FOLSTAD, 1994; SHELDON & VERHULST, 1996; ZUK & MCKEAN, 1996). According to these ideas, as species depart from monogamy, resource allocation pressures (between sexual traits and immunity) will be less conflicting, while more polygamic species will show more unequal sexually dimorphic immune ability with males investing much less in immunity (ZUK, 1990).

A number of species-specific studies have indeed shown a trend in which females seem to have a more robust immune system based on quantitatively higher immune responses either in the presence (e.g. GRAY, 1998; KURTZ et al., 2000; ADAMO et al., 2001; JOOP & ROLFF, 2004; RANTALA & ROLFF, 2006) or absence (e.g. RADHIKA et al., 1998; YOURTH et al., 2002; ARMITAGE & SIVA-JOTHY, 2005; SCHWARZENBACH et al., 2005; POMFRET & KNELL, 2006) of infective agents (for contrary patterns or no biases at all, however, see for example FEDORKA et al., 2004; ZUK et al., 2004; MCKEAN & NUNNEY, 2005; CÓRDOBA-AGUILAR et al., 2006). This evidence, however, has not been supported by sexual differences in parasitic burden rates in a multi-species study (SHERIDAN et al., 2000) although such an analysis is debatable on the basis that non-immune based differences can also explain parasitic sex biases (for a number of explanations for this see ROLFF, 2002; SCHWARZENBACH et al., 2005).

Despite this seemingly more female-biased immune ability, one can still argue that sex-specific differences in immunity can be explained not by the reasons outlined above but by other three hypotheses. One such hypothesis indicates that one may expect females to show the same or lower immunocompetence than males due to the fact that egg production is extremely costly especially in situations when eggs have to be produced in a single episode and lifespan is short (ZUK et al., 2004). A second hypothesis states that sexual differences are more plastic,

condition- and social-dependent (MCKEAN & NUNNEY, 2005). This means that, for example, either sex may become immunocompromised if it engages in energetically costly reproductive behavior. To illustrate this with an example, a study with *Drosophila melanogaster* showed that when food was abundantly provided, sexes did not differ in immune ability against a bacterial infection in the absence of inter-sexual interactions (MCKEAN & NUNNEY, 2005). However, compared to males, immune ability of females was more affected by food than by inter-sexual interactions (MCKEAN & NUNNEY, 2005). These examples show that immune ability shows condition-dependence which may widen immune response limits with no clear sexual differences. A third hypothesis further states that longevity may be positively selected in males for whose reasons they may invest substantially in immunity (STOEHR & KOKKO, 2006). Furthermore, there may be selection to invest in immunity at least until courtship trait production is finished (STOEHR & KOKKO, 2006). According to this, sexes possibly will not differ in immunity.

According to the hypothesis that predicts male reduced immune ability (MRIA), one can expect that sexual differences in immunity will be more evident if sexual selection pressure is more intense. In the same way that, for example, sexual color dimorphism is more evident in species in which sexual selection pressure is more intense (ANDERSSON, 1994), one would predict that males will invest less in immunity if sexual selection is more intense compared to males of species in which this selection is less intense (for a similar rationale but for parasite burden between the sexes see HAMILTON & ZUK, 1982). One similar approach was followed by KLEIN & NELSON (1998, 1999), who compared sexual differences in immunity in polygynous versus monogamous vole species. The results of their study were that sexual differences indeed emerged when individually-housed animals were tested but not in the predicted direction as males had higher immune responses than females in the polygynous species being the contrary for the monogamous species (KLEIN & NELSON, 1998). However, sexual differences reverted in the polygynous voles but not in monogamous voles when both sexes were housed together (KLEIN & NELSON, 1998). In this paper, we have extended this test this by carrying out a multi-species investigation looking first at whether both sexes indeed show a difference in immune ability and whether this is more evident in species where sexual selection intensity is higher. We have tested some predictions that specifically follow to the MRIA hypothesis that females will be more immunocompetent than males and that this difference will be more evident when sexual selection intensity (=female mating multiply) is higher. We have used eight species belonging to four orders (Coleoptera, Diptera, Lepidoptera and Odonata) with a pair of species per order, whose conditions are that females of each species pair will vary in the number of times they mate in their lifetime to have monandric (or close to) and polyandric species. Two reasons make us think that these conditions are convenient for our study. First, this difference in female

mating rate may have imposed distinct sexual opportunities on males with males investing less in immunity than in courtship traits in species whose females are polyandric, while this differential investment will be less extreme in species whose females are monandric. Second, it would be expected that the more times a female mates, the more resources she will have to allocate to immunity for reasons such as sexually-transmitted diseases (e.g. REINHARDT et al., 2003) and injury due to male mating attempts (e.g. CRUDGINGTON & SIVA-JOTHY, 2000; BLACKENHORN et al., 2002). According to these reasons, if a male has more opportunities to mate, he will invest less in immunity as he is investing already and substantially in courtship traits and, along with the fact that females are investing more in immunity, the sexual dimorphism in immunity in a species with these characteristics will be higher. To assess immune ability, we used three parameters which give a general and good idea of both cellular and humoral immune capacity: (1) encapsulation, the act of cell covering and killing of large invaders (TZOU et al., 2002; CERENIUS & SÖDERHÄLL, 2004; CHRISTENSEN et al., 2005), which can presumably conflict the allocation of resources in the insect host (ARMITAGE et al., 2003) and that has been shown to be a good indicator of pathogen resistance ability (RANTALA & ROFF, 2007); (2) phenoloxidase, an enzyme that participates in a number of insect cellular and humoral defense traits such as cuticle melanization, wound repair, cytotoxin production near pathogens and the encapsulation of relatively large pathogens (SÖDERHÄLL & CERENIUS, 1998; SUGUMARAN, 2002), which has been used profusely in insect literature (reviewed in SCHMID-HEMPEL, 2005) and whose production has been shown to be costly (e.g. SIVA-JOTHY, 2000); and (3) hydrolytic enzymes which participate in the immune response activation (CHENG, 1992; TZOU et al., 2002; HETRU et al., 2003) and/or modify the membrane of Gram+ bacteria allowing phagocytic cells to recognize a pathogen (CHENG, 1992; CAJARAVILLE et al., 1995). Although this last parameter has been rarely used in the literature that concerns the evolutionary ecology of immune defense (however, see CÓRDOBA-AGUILAR et al., 2006 and CONTRERAS-GARDUÑO et al., 2007 for examples in the odonate species used here), it is pertinent here as it shows one aspect of cellular response as an indicator of phagocytic activity.

MATERIAL AND METHODS

SPECIES USED. – ***Verres corticola*** **(Coleoptera: Passalidae).** These beetles are common in different tropical areas of Southern Veracruz, Mexico. Individuals have been reared for research purposes in the Instituto de Ecología, A. C. (Xalapa, Veracruz, Mexico). Similar to other passalids, both sexes' cooperation to rear the broods has restricted mating opportunities to males and females. This has presumably led both sexes to practice monogamy at least for parental care periods which may last several months (CASTILLO & REYES-CASTILLO, 1997; SCHUSTER & SCHUSTER, 1997). Animals were obtained in May, 2005.

Tenebrio molitor **(Coleoptera: Tenebrionidae).** With its worldwide distribution, the reproductive biology of this species has been well studied. Both sexes' promiscuity has been actually useful for differ-

ent studies, particularly sperm competition (e.g. WORDEN & PARKER, 2005; ROLFF et al., 2005). Animals were used from a collection in the Instituto de Ecología, UNAM and utilized in February 2005.

***Rhagoletis zoqui* (Diptera: Tephritidae).** A parasite of walnuts, from Central Mexico (BUSH, 1966). In the only published study of mating behavior, this fruit fly has a strong tendency to monandry: under artificially crowded conditions, females paired on average 1.6 times over a 9-day period (ALUJA et al., 2001). This fact supposes that female mating rates in the field should be actually lower (ALUJA et al., 2001). Mating rate in males is also extremely low, approaching one mating during lifetime (J. Rull, unpubl. data). Field collected individuals were reared through diapause under insectary conditions at the Instituto de Ecología, A. C. (Xalapa, Veracruz, Mexico) in July, 2005.

***Rhagoletis pomonella* (Diptera: Tephritidae).** A parasite of hawthorns (*Crataegus* sp.) from Mexico and North America (RULL et al., 2006). Both sexes mate multiply. Under semi-natural conditions, in a field cage, OPP & PROKOPY (2000) recorded an average of a mating per day for both sexes over a 14 day observational period, with some females mating up to eight times per day and some males up to six times a day. Field collected animals were reared through diapause under insectary conditions at the Instituto de Ecología, A. C. (Xalapa, Veracruz, Mexico).

***Callophrys xami* (Lepidoptera: Lycaenidae).** This butterfly has a distribution that includes Mexico, southern Arizona and Texas. Females have a strong tendency to monandry: in insectary conditions, only 5% of 120 females accepted a second mating, a situation that parallels to what occurs in natural conditions (ABUNDIS, 2006). On the other hand, males rarely mate more than once in their lifetime (in a two year study which included two reproductive seasons, and having observed 159 males, only 27 copulated of which only three mated more than once (CORDERO et al., 2000), which means they are close to being monogamous). Animals were collected in the natural preserve "El Pedregal de San Angel" of the Universidad Nacional Autónoma de México (UNAM), Mexico, D. F. The first generation of non-related animals was used for analysis (for rearing conditions see JIMENEZ & SOBERON, 1989) in May, 2005.

***Heliconius ismenius* (Lepidoptera: Heliconiidae).** It is present from southern and Central America to southern USA; unlike other *Heliconius* species, *H. ismenius* males and females have been documented to mate multiply (MENDOZA, 2005). Individuals were collected in the Francisco Javier Clavijero Botanical Garden (Xalapa, Veracruz, Mexico), from September to November, 2005.

***Hetaerina americana* (Odonata: Calopterygidae).** This damselfly has a wide distribution which includes Southern USA and central, tropical areas of Mexico (ABBOTT, 2005). Males are territorial but rarely gain more than one mating in their life and a similar pattern seems to account for females which allows to suggest that both tend to be monogamous (CÓRDOBA-AGUILAR, 2009).

***Argia tezpi* (Odonata: Coenagrionidae).** A damselfly usually located from Mexican to Central American regions (ABBOTT, 2005). Males are nonterritorial and both sexes mate at least more than five times in their life (unpub. obs. of over 56 males and 17 females followed during a 26-day period; A. Córdoba-Aguilar, unpub. data). Collection was carried out in the same site and dates as *H. americana*.

GENERAL PROTOCOL FOR IMMUNE QUANTIFICATION. – Sexually mature, virgin adults of both sexes were used for each species. Food *ad libitum* was provided previous to immune testing as described below to correct for possible differences in condition caused by previous environmental influences such as food shortage or social stressing conditions which may affect survival during immune tests especially after filament insertion (see below). This assumption is founded as it comes from our previous research with the damselfly species (J. Contreras-Garduño & A. Córdoba-Aguilar, unpub. data). Although it may be thought that this may mask potential sexual differences in immunity, if both sexes have rooted differences in this parameter, these differences should arise and persist even when resources are fully satisfied. Food was provided as follows: for *V. corticola* and *T. molitor*, recently emerged individuals were fed with wood debris and flour for five and six days respectively; *R. zoqui* and *R. pomonella* immature stages were fed approximately for two months with walnut and

hawthorn fruits respectively until emergence which is when immune tests were carried out; for butterflies, recently emerged adults were of both sexes were fed with water and sugar for three days; for Odonata, teneral individuals of both sexes were marked (using a permanent ink pen and writing an individual number on their wings) and collected when they had their first visit to the river. In these animals, males had finished the development of their sexual body and wing coloration typical of a sexually mature individual while females had already produced eggs but had no sperm in the sperm storage organs. Damselflies were fed with food *ad libitum* (providing as many *Drosophila melanogaster* flies as animals could eat) for three days. Flies were provided manually using forceps.

ENCAPSULATION. – This was quantified by totally inserting a 2 mm length nylon filament in the medium, dorsal region of the abdomen (in the case of Coleoptera the insertion was done in the 5th abdominal segment; in the 4th abdominal segment for Diptera and Odonata; and in the 7th abdominal segment for Lepidoptera) of both sexes in each species. The filament was previously disinfected by immersing this in 70% ethanol for one hour and the insertion was carried out using fine dissecting forceps. After insertion, *V. corticola, T. molitor* and *C. xami* animals were individually separated in petri dishes (1 cm height × 10 cm diameter); *R. pomonella* and *R. zoqui* in plastic containers (15 cm height × 9.5 cm diameter); *H. ismenius* individuals in envelopes; and the odonates in plastic tubes (4.5 height × 1.4 width) with a wooden piece as a perching place and a humid cotton piece. Animals were then left for 10 h and 12:12 h dark-light conditions at constant temperature (25°C). Although there is no established consensus as for how long a nylon implant should remain inside the animal, previous evidence in *H. americana* (CONTRERAS-GARDUNO et al., 2006; CONTRERAS-GARDUNO et al., 2007) and *A. tezpi* used here (A. Córdoba-Aguilar unpubl. data) suggest that by ten hours immune response after nylon insertion has been completed. During this time, food was not delivered as for some species (e.g. butterflies) due to logistic reasons as animal movements had been already restrained and, if released, they would have not eaten. With this situation, we had to limit food to all animals and species to provide the same conditions. Animals were preserved in 70% ethanol and the filament was obtained by gently removing the insect cuticle with dissecting forceps under a stereoscopic microscope. Three photographs of each filament (previously changing the position of it) were taken to avoid biases in cell patches irregularly located on the implant. Photographs were taken keeping the same distance from the microscope lens to the filament. Encapsulation was quantified by obtaining the average of the three photographs per species after manually selecting the covered area that appeared on the implant using the Image Tool (version 3.0) software. This measurement methodology has become a standard technique in the ecological immunology literature (e.g. RYDER & SIVA-JOTHY, 2000; SIVA-JOTHY, 2000). To see our consistency in applying this protocol, the inter- and intra-observer reliability of this measurement methodology was calculated. For the inter-observer reliability, two different people were asked to measure 30 filaments coming from a sample of 30 *H. americana* males. For the intra-observer, one of these people was asked to measure twice a different sample of 24 filaments without knowing the order of the filaments. There was a close correlation for both the inter- ($r = 0.68$, $P < 0.0001$) and intra-observer ($r = 0.98$, $P < 0.0001$) measurements which suggests this methodology is consistent.

PHENOLOXIDASE AND HYDROLYTIC ENZYMES. – Individual males and females were allocated in Eppendorf tubes containing 1ml of phosphate buffered saline (140 Mm NaCl, 2.6 mM KCl, 1.5 Mm KH_2PO_4 pH 7.4) to prevent sample decomposition and immune related enzyme activation. Each individual was macerated in the buffer to obtain a homogeneous sample which was centrifuged (ALC PK121R) at 18,000 *g* at 4°C during 10 minutes. The supernatant was removed and another 1 ml of buffer was added for further homogenization and centrifuging for three times. Phenoloxidase was quantified using a microplate reader (Bio-Rad Laboratories, Richmond, Calif.). For this, 50 µl of the sample were mixed with 50 µl of L-DOPA (3 mg/ml of buffer). Readings were done at 490 nm to see phenoloxidase activity (RISHAN et al., 2005; LUNA-GONZÁLEZ et al., 2004). Only one reading was taken after 15 min, time at which the enzyme activity reaches its highest point for all species (A. Córdoba-Aguilar, unpub. data). The maceration followed by repeated ho-

mogenization was carried out to get more trustable readings as otherwise the sample may precipitate to the eppendorf bottom and, when pippeted, the actual reading can produce much higher values (J. Contreras-Garduño, unpubl data). Although this method of using the entire animal to measure PO bears the criticism of not only measuring hemolymph PO (which is where immune components are) but PO from other body regions, our previous measurements in both damselfly species suggest that, despite increased PO levels when using the whole animal, still total body and hemolymph PO show a positive correlation (CÓRDOBA-AGUILAR et al., 2006; CONTRERAS-GARDUÑO et al., 2007). As a control, 50 µl of buffer and 50 µl of L-DOPA buffer (3 mg/ml of buffer) were used. Hydrolytic enzymes were quantified by using the APYZYM® (bioMériux) kit. This allows the detection and quantification of 19 hydrolytic enzymes (proteases: leucyl arylamidase, valyl arylamidase, cystyl arylamidase, trypsin, α-chymotrypsin; lipases: lipase esterase [C8] and lipase [C14]; glycosidases: α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase; esterases: esterase [C1]; and phosphatases: alkaline phosphatase, acid phosphatase and naphthol phosphohydrolase). Sixty five microliters of sample were added to the reaction strips and incubated for 4 hours at 37° C. After 10 min, change in colour was quantified by using the manual provided by the manufacturer. Results were transformed to nM of substrate hydrolysed. Enzyme activities were expressed as units, where one unit is the hydrolyzed substrate in nM/mg of protein. All measurements were performed in triplicate (3 strips) and the average of the three results was used.

PROTEIN DETERMINATION. – We used the BRADFORD (1976), method for determining protein concentration in samples. Bovine serum albumin (Sigma®) was used as standard to which 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. 220 µl of buffer plus 40 µl of Bradford were used as control.

STATISTICAL ANALYSIS. – Since most data followed non-normal distributions, they were log_{10} transformed. To compare sexual immune dimorphism in encapsulation, we used General Linear Models when data were normally distributed and Generalized Linear Models when data were not normally distributed. In these analyses, encapsulation was the dependent variable, sex was a factor, and body size was a covariate (this procedure controls for allometric effects on encapsulation). In each model we tested the interaction term sex – body size, nevertheless, no interaction term was significant ($P > 0.05$ in all cases). Therefore these interaction terms were removed from the models. To compare sexual immune dimorphism in phenoloxydase and hydrolytic enzymes, we used T-tests or Mann-Whitney U tests, depending on the distribution of the data.

To compare sexual dimorphism in the immune response among species with high and low levels of sexual selection intensity, male and female immune response (encapsulation, phenoloxydase, hydrolytic enzymes) were log_{10} transformed prior to analyses. Therefore, sexual dimorphism in the immune response was calculated as log_{10} (male immune response) – log_{10} (female immune response) since *log* differences tend to be symmetric around zero, and less likely to violate the assumptions of parametric tests than other indices (i.e. SMITH, 1999). T-tests were then used to compare sexual dimorphism in the immune response at species level.

PHYLOGENETIC ANALYSIS. – Phylogeny – We use a composite phylogeny that includes all 8 species (Fig. 1), since no single comprehensive phylogenetic hypothesis is yet available that would include the species we used in this study. Our composite phylogeny was built using the nucleotide sequence data from the 18S small subunit rRNA gene of insects (KJER, 2004) and each species was augmented to this phylogeny according to the position of each insect order. Since branch lengths were not known for the taxa used due to the composite nature of our phylogeny, we set branch length to unit.

Changes in the immune response between males and females – To test evolutionary changes in the immune response between males and females, we used phylogenetic independent contrasts (FELSENSTEIN, 1985) as implemented by CAIC (PURVIS & RAMBAUT,

1995) to control for the phylogenetic non-independence of species (HARVEY & PAGEL, 1991). Our data were consistent with the assumption of this contrast method that the standardized contrasts should be independent from their estimated nodal values (FELSENSTEIN, 1985).

We tested the evolutionary changes in the immune response between males and females by fitting major axis regressions (MA, model II regression, SOKAL & ROHLF, 1981) between female (dependent variable) and male immune response (independent variable) using phylogenetic independent contrasts. In these analyses, a slope not significantly different from 1 suggests that male and female response have evolved in a correlated fashion. MA was forced through zero when using phylogenetic independent contrasts (HARVEY & PAGEL, 1991; GARLAND et al., 1992). We provide the slopes of these regressions and their 95% confidence intervals (lower CI – upper CI). Slopes and confidence intervals of major axis regressions were calculated by bootstrapping the contrasts using R (R Development Core Team, http://www.R-project.org).

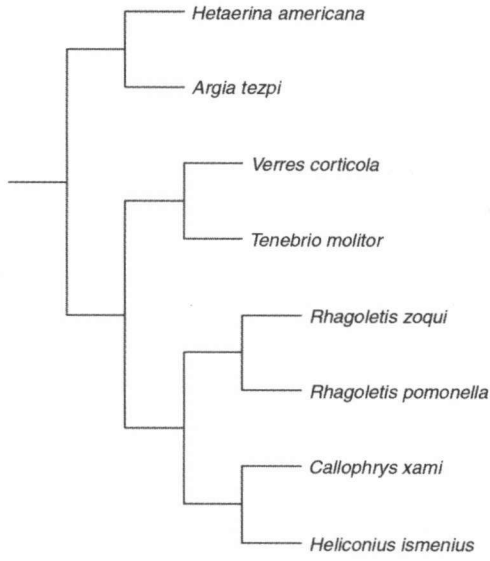

Fig. 1. Composite phylogeny of Insecta using KJER's (2004) molecular phylogeny.

Changes in the immune response among species – To test sexual differences in the immune response among species with high and low levels of sexual selection, we used Generalised Least Squares (GLS; PAGEL, 1997, 1999; GARLAND & IVES, 2000; FRECKLETON et al., 2002). GLS is a phylogenetic comparative method that incorporates the phylogenetic autocorrelation of the data in the structure of errors (variance-covariance matrix; MARTINS & HANSEN, 1997; FRECKLETON et al., 2002). The structure of variance-covariance matrix was determined from the composite phylogeny of insects. GLS was used to test the maximum likelihood of the evolutionary regression coefficient between two traits (PAGEL, 1997, 1999). In order to improve the fit of our data to the model, we estimated the maximum likelihood value of the weighting parameter λ (see FRECKLETON et al., 2002), and used this λ to correct for phylogenetic effect in linear models (PAGEL, 1997, 1999).

Phenoloxidase and hydrolytic enzyme values are given as U/mg of protein. Analyses were carried out using R (ver. 2.4.1; R Development Core Team, http://www.R-project.org). GLS modelling were carried out in R (R Development Core Team, http://www.R-project.org) using codes written by Robert P. Freckleton.

RESULTS

SEXUAL DIMORPHISM IN IMMUNE ABILITY WITHIN SPECIES

At species level certain species showed significant differences in the immune response: in 10 out of 24 comparisons (Tab. I). Females were somehow more

immunocompetent than males in 8 cases. Nevertheless, phylogenetic analyses revealed that evolutionary increases in male encapsulation were correlated with evolutionary increases in female encapsulation, since the slope between male and female encapsulation contrasts did not differ significantly from 1 (b = 11.085, 95% confidence intervals (CIs), lower-upper, -11.791 – 56.126, N = 7 contrasts). This trend was similar for male and female phenoloxidase response (b = 1.119, 95% CIs 0.692 – 1.800, N = 7 contrasts) and male and female hydrolytic enzymes response (b = 1.030, CIs -5.535 – 2.538, N = 7 contrasts).

COMPARISON OF SEXUAL DIMORPHISM IN IMMUNE RESPONSE IN SPECIES THAT VARY IN SEXUAL SELECTION INTENTSITY

Sexual dimorphism in encapsulation did not differ among species with high or low levels of sexual selection ($T\text{-test}_6$ = 0.486, P = 0.644). This trend among species with high and low levels of sexual selection was similar for sexual dimorphism in phenoloxidase ($T\text{-test}_6$ = 1.593, P = 0.162) and hydrolytic enzymes ($T\text{-test}_6$ = 0.396, P = 0.706).

These results were consistent using GLS since there were no evolutionary changes in sexual differences in the immune response between species with high and low levels of sexual selection (Tab. II).

DISCUSSION

We found dubious support for sexual dimorphism within species with the *a priori* expectations that males should invest less to immunity, and with sexual selection intensity making this dimorphism more evident between species. This is contrary to the MRIA hypothesis which suggests that females (especially in polygamic species) will have more robust immune defenses than males due to increased longevity (ROLFF, 2002) or to the action of sexual selection on males (ZUK, 1990; FOLSTAD & KARTER, 1992; WEDEKIND & FOLSTAD, 1994; SHELDON & VERHULST, 1996; ZUK & MCKEAN, 1996; MOORE & WILSON, 2002). It is also contrary to single species-based studies in which indeed females have been shown to be better immunocompetent or having a reduced parasite/pathogen burden compared to males (see references in the introduction). These studies have measured a number of components which have usually included one (e.g. ZUK et al., 2004; SCHWARZENBACH et al., 2005), two (e.g. POMFRET & KNELL, 2006) but rarely three immune parameters (however see FEDORKA et al., 2004) as it is the case of our study. Although there is nothing stated in terms of how many parameters should be measured (MATSON et al., 2006), the fact is that the more measures (which desirably should include both cellular and humoral components as in our study), the more complete the picture in terms of immune robustness as the measure of pathogen defense and resistance (BOA-AMPOSSEN et

Table I

Comparisons of sexual dimorphism within species in three immune parameters. PO = phenoloxidase. Difference denotes whether there were statistical significant differences in immune response, outlining the sex that had a larger immune response in those cases that this was the case. * Determined using Generalized Linear Models (see Material and Methods)

Insect order	Species	Encapsulation	Difference	PO	Difference	Hydrolytic enzymes	Difference
Coleoptera	*V. corticola*	Sex $F_{1,11} = 1.345$, P = 0.271 Size $F_{1,11} = 0.057$, P = 0.815	No	T test$_{14} = 1.344$, P = 0.200	No	T test$_{14} = 0.259$, P = 0.800	No
	T. molitor	$Sex_{1,15}$ P = 0.001 $Size_{1,15}$ P = 0.246	Males*	T test$_{18} = 2.041$, P = 0.056	No	T test$_{18} = 2.368$, P = 0.029	Females
Diptera	*R. zoqui*	Sex $F_{1,16} = 28.704$, P = 0.001 Size $F_{1,16} = 0.085$, P = 0.774	Males	U test$_{(males=10, females=10)} = 33.5$, P = 0.218	No	T test$_{16} = 0.553$, P = 0.588	No
	R. pomonella	Sex $F_{1,10} = 3.024$, P = 0.113 Size $F_{1,10} = 1.119$, P = 0.315	No	T test$_{18} = 2.671$, P = 0.016	Females	T test$_{18} = 0.164$, P = 0.872	No
Lepidoptera	*C. xami*	Sex $F_{1,14} = 25.553$, P = 0.001 Size $F_{1,14} = 6.501$, P = 0.023	Females	T test$_{13} = 4.287$, P = 0.001	Females	T test$_{13} = 1.459$, P = 0.168	No
	H. ismenius	Sex $F_{1,4} = 2.801$, P = 0.170 Size $F_{1,4} = 8.453$, P = 0.044	No	T test$_{9} = 0.423$, P = 0.682	No	T test$_{9} = 0.192$, P = 0.852	No
Odonata	*H. americana*	$Sex_{1,17}$ P = 0.387 $Size_{1,17}$ P = 0.363	No*	U test$_{(males=20, females=20)} = 0$, P = 0.001	Females	U test$_{(males=17, females=17)} = 42$, P = 0.001	Females
	A. tezpi	Sex $F_{1,17} = 1.718$, P = 0.207 Size $F_{1,4} = 2.186$, P = 0.158	No	U test$_{(males=19, females=19)} = 0$, P = 0.001	Females	U test$_{(males=16, females=15)} = 38$, P = 0.001	Females

al., 1999; ADAMO, 2004a). It has to be mentioned, however, that immune measures are frequently difficult to interpret, particularly the relationships among them (ADAMO, 2004a) and thus direct tests of resistance should be carried out to support the use of different immune measures (ADAMO, 2004a). In fact, a test like this in which encapsulation via a nylon filament challenge was just carried out and showed that this is a good indicator of pathogen resistance (RANTALA & ROFF, 2007). Paradoxically, MUCKLOW et al. (2004), and ADAMO (2004b), showed recently, in *Daphnia* and crickets respectively, that not always PO is a good indicator of pathogen resistance.

Multiple species comparisons using sexual selection intensity are not common in the literature. The strongest evidence as for sexual selection intensity being related to one aspect of immune ability - parasitic infections - was put forward by MOORE & WILSON (2002). These authors found that males were in general more parasitized than females in mammals but that male-biased mortality was more intense in polygynous species compared to monogamous species. One explana-

tion, as these authors assert, is that high investment to courting traits may render males to be less robust in immune ability. However, there are three different hypotheses, unlike the MRIA hypothesis, that do not base their assumptions on males being consistently less immune robust and which were outlined in the introduction. A recent paper by MCKEAN & NUNNEY (2005) suggests that, although sexual selection is related to resources invested to immunity, immune ability is possibly more affected by resource availability such as food. These latter authors recently documented this with a series of experiments showing that sexual dimorphism in immune ability can be sometimes reversed in *D. melanogaster*. According to their study, immunocompetence can be predicted in terms of condition dependence. At distinct resource- (diet for females) and social-demanding (sexual activity for males) situations, both sexes varied highly in their immune response to extremes in which sometimes females resulted less able to deal with bacterial infections compared to males. These authors explained these results on the basis that each sex does not have fixed limits in immune and defense expression with respect to the other sex but that there is high plasticity in how much each sex invests in immunity. This plasticity can go to such extremes that situations may be found in which both sexes may show no differences or where even males may show higher immune ability values than females. These results are supported by the fact that increased longevity is not correlated with egg production if dietary resources are provided (FOWLER & PARTRIDGE, 1989; CHAPMAN & PARTRIDGE, 1996). Related to this, ZUK et al. (2004), have argued that female insects may reduce their investment to immunity given the high cost that egg production entails which may render females to be less or similar immune robust than males. Furthermore, a more recent view has even challenged the assumption that longevity is not selected in males, and that, due to this, males may therefore invest heavily

Table II

Relationships between immune response, dependent variable, *log*(male immune response) - *log*(female immune response), and measures of sexual selection (low, high) using Generalised Least Squares in insects. λ is the weighting parameter in GLS (see FRECKLETON et al., 2002)

Models	Regression coefficient ± SE	t	P
Model 1 - Encapsulation			
Sexual selection intensity	0.526 ± 0.952	0.553	0.600
Model 2 - Phenoloxidase			
Sexual selection intensity	0.214 ± 0.145	1.468	0.192
Model 3 - Hydrolytic enzymes			
Sexual selection intensity	-0.063 ± 0.075	0.836	0.434

Model 1: $\lambda = 0.00006$. $r^2 = 0.048$, $F_{2,6} = 0.305$, $P = 0.600$
Model 2: $\lambda = 0.00007$. $r^2 = 0.264$, $F_{2,6} = 2.156$, $P = 0.192$
Model 3: $\lambda = 0.99993$. $r^2 = 0.104$, $F_{2,6} = 0.699$, $P = 0.434$.

in immunity to procure more matings (STOEHR & KOKKO, 2006). According to this hypothesis, sexes may invest differentially to immunity depending on how much males invest in courtship trait expression (for example, how intense condition dependent these traits are). Unlike the MRIA hypothesis, these alternative explanations "share" the only prediction that sexual differences in immune investment are difficult to predict at the multi-species level which is the case of our results. We measured the immune ability with which each sex will start its reproductive life (although animals were captured when they were young, they were sexually mature when immune tests were carried out so that the immune assessment was performed during the reproductive window). At this time, males, however, should have allocated resources to sexual traits which would have affected resource administration to immunity according to the MRIA hypothesis. This was not the general case for the species we used here although in some cases (*R. pomonella*, *H. ismenius*), one criticism is that we had a reduced sample size. Still, the MRIA hypothesis does not apply in general which includes several species whose males develop striking courtship traits or behaviors such as, for example, wing pigmentation and territorial defense in *H. americana* (GRETHER 1996a, b; CONTRERAS-GARDUNO et al., 2006), fighting behavior in *R. pomonella* (PROKOPY & PAPJ, 2000), and female-attracting pheromones in *T. molitor* (RANTALA et al., 2003). Furthermore, MRIA hypothesis applied to a restricted set even in those cases in which the immune parameter has been previously documented to be resource-demanding and, therefore, energetically costly (e.g. PO, SIVA-JOTHY, 2000; encapsulation, ARMITAGE et al., 2003). In the absence of energetically demanding situations (as animals were virgin and not exposed to socially-demanding situations; e.g. fighting and oviposition, SIVA-JOTHY et al., 1998) and when resource allocation should not put animals in a conflict (as animals were well fed prior to immune measurements; e.g. MORET & SCHMID-HEMPEL, 2001; SIVA-JOTHY & THOMPSON, 2002), our results can hardly be confounded with factors that have been documented to negatively affect immune response. This means that our results are close to what animals, in terms of energy devoted to immune ability, start with when they face sexual opportunities which is when the presumable conflict in resource allocation has emerged. This does not mean that these conflicts do not occur but that the conflict does not take the extremes predicted by the MRIA hypothesis. Although our work was not designed to test the alternative hypotheses, the factors that these hypotheses consider relevant (e.g. food resources or social situations being extremely important for both sexes' immunity; costly egg production reducing investment to immunity; longevity and immunity being positively selected in males) may also explain our results. The particular and very different biology of each species, however, prevents us from exploring and discussing the bases of each alternative hypothesis.

Considering the results obtained, is there any prediction actually related to the biology of each species? Immune differences may be interpreted as the different

requirements with which each sex, compared to the other sex, would start its reproductive life in the face of pathogen and/or parasite attacks. For example, the null immune ability differences in all species suggest that both sexes experience similar immune challenge pressures. This may mean that potential infective agents are similarly attacking both sexes. Unfortunately, there is no available information as for parasite/pathogen agents in most species used in our study as is the case in other animals, for example, mammals (MOORE & WILSON, 2002). For the few species in which detailed data are available of parasites and their fitness effects on hosts are the damselflies. In these animals, it has been shown that gregarines (Protozoa) negatively affect survival and mating opportunities (e.g. SIVA-JOTHY, 1999; CÓRDOBA-AGUILAR, 2002; CÓRDOBA-AGUILAR et al., 2003). One previous study in the two damselfly species has shown that both sexes show similar gregarine and mite patterns despite females being more immunocompetent in phenoloxidase and hydrolytic enzymes (CÓRDOBA-AGUILAR et al., 2006). Interestingly, this consistency in immune parameters persisted even when in that previous study, older animals were used (CÓRDOBA-AGUILAR et al., 2006). This means that age has little effect on immune values which gives partial support to the MRIA hypothesis. However, given that the alternative hypotheses cannot provide clear predictions with respect to which sex should show higher immune values, both studies in damselflies cannot be used to reject these hypotheses. Still, it would be desirable that parasitic and immune comparisons like this can be carried out with other species to unravel if parasite/pathogen pressure is likely to predict sexual immune differences (FORBES, 2007). This should be the case for those species in which we found differences in immune values.

If patterns of pathogen and/or parasite pressures are correlated with the tendencies we have shown here, this may mean that sexual immune values are possibly related to immune preparation at the reproductive life onset (especially if at this time there is a niche transition where a different set of parasites and pathogens will be present as, for example, from the aquatic to the aerial stage in damselflies). How relevant both infective agents and sexual selection intensity are must be individually tested in controlled experiments with varying degrees of both evolutionary aspects. In any case, our result calls for a reformulation of the presumable intrinsic immune sexual differences assumed by the MRIA hypothesis.

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