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Sex differences in immune defenses and response to parasitism in monarch butterflies

Elizabeth Lindsey · Sonia Altizer

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Abstract Host susceptibility and patterns of infection are predicted to differ between males and females due to sex-based tradeoffs between the demands of reproduction and costly immune defenses. In this study, we examined immune defenses and the response to experimental infection by a protozoan parasite, *Ophryocystis elektroscirrha*, in male and female monarch butterflies, *Danaus plexippus.* We quantified two measures of immunity in late instar larvae: the concentration of circulating hemocytes and mid-gut phenoloxidase activity, and also quantified final parasite loads, body size, longevity, and wing melanism of adult butterflies. Results showed that females had greater average hemocyte counts than males in the absence of infection; males, but not females, showed an increased concentration of hemocytes in the presence of infection. However, higher hemocyte concentrations in larvae were not significantly correlated with lower adult parasite loads, and mid-gut phenoloxidase activity was not significantly associated with hemocyte counts or parasite treatments. Among unparasitized females, greater hemocyte concentrations were costly in terms of reduced body size, but for parasite-treated females, hemocyte concentrations and body size were positively associated. Across all monarchs, unparasitized butterflies showed greater wing melanism (darker forewings) than parasitized monarchs. Overall, this study provides support for differential costs of immune defenses in male and female monarch butterflies, and a negative association between parasite infection and monarch wing melanism.

Keywords Hemocytes · Insect immunity · Phenoloxidase · *Danaus plexippus* · *Ophryocystis elektroscirrha* · Tradeoffs

E. Lindsey (✉) · S. Altizer
Graduate Division of Biological and Biomedical Sciences, Population Biology, Ecology, and Evolution, Emory University, Atlanta, GA 30322, USA
e-mail: efriedl@emory.edu

S. Altizer
Odum School of Ecology, University of Georgia, Athens, GA 30602, USA

Introduction

A persistent theme in studies of ecological immunity is that host defenses can be costly in terms of reductions in other fitness components (Norris and Evans 2000; Rolff and Siva-Jothy 2003; Viney et al. 2005) and that tradeoffs arising from these fitness costs can maintain variation in host susceptibility in natural populations despite the obvious benefits of resistance (Boots and Begon 1993; Schmid-Hempel and Ebert 2003). The costs and benefits of host resistance could differ greatly between males and females due to their differential reproductive strategies (Zuk 1990; Zuk and McKean 1996; Yourth et al. 2002; Zuk et al. 2004). Specifically, females may benefit more from greater immunity because their fitness tends to be limited by longevity and the number of offspring they can rear, whereas males are more often limited by the number of mates they can inseminate (Andersson 1994; Moore and Wilson 2002). Thus, conventional wisdom predicts that males should show lower measures of immune defenses, in part due to a tradeoff between investing in longer-term survival versus competition for increased mating opportunities (McKean and Nunney 2001; Zuk and Stoehr 2002). On the other hand, when longevity is equally important for males and females, or if the impact of parasites on host condition is greater for males, sexual selection could favor greater male investment in immunity (Stoehr and Kokko 2006; Stoehr 2007).

While the majority of support for sex differences in immunity is found in mammals, insects are well-suited for studies of the costs and benefits of immune function and their correlation with other host traits (Rolff and Siva-Jothy 2003; Schmid-Hempel 2005). Many ecological and evolutionary studies of insect resistance have focused on two generalized immune effector traits: hemocyte concentration and melanic encapsulation. The concentration of circulating hemocytes reflects the number of cells available to recognize and phagocytize foreign particles. Hemocytes also participate in cellular encapsulation, in which hemocytes adhere to parasites in layers, and melanic encapsulation, which involves the production and deposition of melanin onto foreign particles (Gillespie et al. 1997; Silva et al. 2002). Melanic encapsulation (hereafter called melanization) occurs through the activation of the phenoloxidase (PO) enzymatic cascade, and can be measured through the enzyme kinetics of PO using hemolymph or tissue (Siva-Jothy 2000; Schmid-Hempel 2005). A number of studies have quantified hemocyte concentration, melanization and cellular encapsulation to infer levels of parasite defense by insect hosts (Paskewitz, Brown et al. 1989; Dunphy 1991; Eslin and Prevost 1996; Nigam et al. 1997; Cotter and Wilson 2002; Rolff and Siva-Jothy 2004) and to examine costs of insect immune defenses via tradeoffs with other fitness components (Moret and Schmid-Hempel 2000; Adamo et al. 2001; McKean and Nunney 2001). However, although a few correlational analyses indicate that greater measures of immune effector traits (Paskewitz et al. 1989; Adamo 2004) and external melanism (Wilson et al. 2001) are associated with resistance to some pathogens, the effectiveness of these mechanisms against a range of naturally-occurring parasites in insect hosts remains relatively unknown.

Sex differences in host defenses, where females show greater immunity or resistance to infection, have been demonstrated for several arthropods including scorpionflies, crickets, shrimp, dragonflies, and damselflies (Gray 1998; Radhika et al. 1998; Kurtz et al. 2000; Kurtz and Sauer 2001; Rolff 2001; Yourth et al. 2002; Adamo 2004; Fedorka et al. 2004). In many cases, females show greater levels of hemocytes (da Silva et al. 2000; Kurtz et al. 2000) or PO activity (Gray 1998; Kurtz et al. 2000; Adamo et al. 2001; Rolff 2001). However, other studies showed greater measures of immune effector traits in males (Stoehr 2007), including when food resources were limited (Zuk et al. 2004; McKean and Nunney 2005), suggesting that immune effector traits are phenotypically plastic and vary under resource limitations.

In some insect species, differences in immunity also relate to sexual dimorphism in body pigmentation and sexually selected traits. Specifically, cuticular melanism (darker body pigmentation) has been associated with greater measures of PO activity and melanin production in beetles and Lepidoptera (Barnes and Siva-Jothy 2000; Wilson et al. 2001; Cotter et al. 2004a). In damselflies, larger wingspots and more symmetrical wings of males correlated with increased immunocompetence (Rantala et al. 2000); other work on damselflies showed that darker wing pigmentation correlated with parasite resistance and hence could signal higher male quality to potential mates (Siva-Jothy 2000; Yourth et al. 2002). Variations in melanism are particularly interesting because of the direct role melanin plays in insect immunity. Sexual dimorphism in wing pigmentation has been observed in monarch butterflies (Davis et al. 2005), such that females have darker wings than males. Importantly, this is the first study to explore the relationship between immune effector traits, parasite infection and wing pigmentation in monarchs.

In this study, we quantified two immune effector traits (hemocyte concentration and phenoloxidase activity) in male and female monarch butterflies (*Danaus plexippus*) following experimental infection with the naturally occurring protozoan parasite *Ophryocystis elektroscirrha*. We predicted that monarchs exposed to parasites would show greater measures of immunity, provided that the immune defenses we examined are inducible. We also predicted that innate host resistance could be evolutionarily beneficial for both male and female monarch butterflies because this parasite causes wing deformities, decreased body size, reduced longevity, poorer flight ability and decreased mating success (Leong et al. 1992; Leong et al. 1997; Altizer and Oberhauser 1999). This is particularly true for monarchs in eastern N. America that undergo extreme long-distance migrations and endure a long wintering period prior to the onset of breeding activity (Brower and Malcolm 1991; Brower et al. 1995). Hence, even small reductions in survival or flight ability caused by infection could preclude reproductive success the following spring. We further predicted that the costs of parasite infection, and hence the benefits of immunity, might be greater for female monarchs than for males. This is because overwintering males that are in poor condition can mate and transfer spermatophores to females rather than re-migrate north in the spring (Van Hook 1993; Van Hook 1996; Oberhauser and Frey 1999); females in poor condition do not have this option, and must remigrate >500 km to oviposit on host plants. Previous work has also shown that female body size at eclosion is positively related to lifetime fecundity and egg size (Oberhauser 1997; Oberhauser 2004); thus, parasite-induced reductions in host body size could have additional negative effects on female reproduction, beyond reduced longevity and mating success. Finally, we examined the relationship between host defenses and adult fitness components in the presence and absence of infection to investigate their potential costs and benefits to both males and females, and we quantified dark pigmentation on the wings of adult butterflies to test the prediction that external melanism correlates positively with measures of parasite resistance.

Methods and materials

General methods

The study system

Monarch butterflies (*Danaus plexippus)* are geographically widespread (Ackery and Vane-Wright 1984); all populations examined to date are infected by the neogregarine

protozoan *Ophryocystis elektroscirrha*, and prevalence varies dramatically among populations with different migratory strategies (Altizer et al. 2000). The life cycle of *O. elektroscirrha* is closely correlated with host development (McLaughlin and Myers 1970). Transmission occurs when larvae ingest spores scattered by infected adults onto eggs and foliage. Parasite spores lyse in the larval gut, and emerging sporozoites penetrate and pass through the gut wall, migrate to the hypoderm, and undergo vegetative schizogony during the host's late larval and early pupal stages. Several days prior to adult butterfly eclosion, parasites undergo sexual reproduction and haploid spore formation. Infected butterflies emerge covered with dormant parasite spores on the outside of their bodies (McLaughlin and Myers 1970; Leong et al. 1992).

Monarch sources and mating design

Monarchs used in this experiment were the offspring of 11 wild-caught females mated with 4 wild-caught males collected from Giles County (Virginia, USA) during June 2005. All monarchs were examined for the presence of *O. elektroscirrha* (Altizer et al. 2000) and only uninfected individuals were used to obtain progeny. After mating, female monarchs were placed in 2 separate 0.6 m^3 cages supplied with potted greenhouse-reared *Asclepias incarnata*. Plants were transferred to a laboratory and maintained at 24°C after 60 or more eggs were laid on a single plant.

Inoculation and host rearing

Larvae were randomly divided into two groups: parasite treated ($N = 107$) and control ($N = 110$). Parasite spores were obtained from an eastern N. America monarch infected with an eastern strain of *O. elektroscirrha* (collected in Clarkston, GA, USA). We inoculated second instar larvae individually by feeding them 1 cm^2 *A. incarnata* pieces to which 10–15 parasite spores had been manually transferred. Control larvae were fed pieces of *A. incarnata* free of parasite spores. Larvae were maintained singly in 10 cm diameter Petri dishes until they consumed all of the plant material.

After inoculation, we transferred larvae to individual 0.47 l plastic containers with mesh screen lids. We fed fresh cuttings of greenhouse-raised *A. incarnata* to larvae daily, removed frass, changed the lining of the container, and kept the lining moist. *A. incarnata* was sterilized by soaking it in a 20% bleach solution for 20 min and rinsing it in tap water prior to use. All rearing containers were kept on laboratory benches (24°C) and their positions were rotated daily. After an individual pupated, the container was moved to a separate laboratory (26°C). We recorded the dates of pupation and eclosion and placed each adult butterfly into a glassine envelope 6–12 h post-eclosion. Following eclosion, we recorded sex and time to death in days based on holding monarchs inactive in the laboratory at 26°C in envelopes without access to food. The measure of longevity reflects both the duration of adult life as well as the amount of stored energy reserves.

Immunity, infection, and adult morphology

Immune parameters

We measured two immune effector traits in late instar larvae: concentration of circulating hemocytes and phenoloxidase activity of the mid-gut tissue. Samples were collected from 5th (final) instar larvae. Hemolymph was extracted from the second to last proleg of a

random subset of parasite-treated and control group larvae ($N = 149$); 10 µl of hemolymph was placed into 50 µl of an anti-coagulant and anti-melanization PBS 1× [NaCl 1.37 mM, KCl 0.268 mM, Na_2HPO_4 1.08 mM, KH_2PO_4 0.147 mM, EDTA 10 mM, Citric Acid 10 mM, PTC 0.0197 mM, pH 7.4 stored at 4°C] solution on ice. Hemocytes were counted on the day of collection using a 6.6 µl chamber hemocytometer under 400×. We performed counts from each of two separate hemocytometer chambers and calculated the average number of cells per µl.

For a subset of larvae sampled for hemocyte concentration (65 out of 149), we extracted the mid-gut immediately after hemolymph collection. The mid-gut section of the larva was removed, rinsed in 4°C anti-coagulant PBS 1× [NaCl 1.37 mM, KCl 0.268 mM, Na_2HPO_4 1.08 mM, KH_2PO_4 0.147 mM, EDTA 10 mM, Citric Acid 10 mM, pH 7.4], and placed in a 1.5 ml microcentrifuge tube with a 1 ml solution of anti-coagulant PBS and 20 µl of 3% paraformaldehyde. Samples were homogenized in the tube and placed at −20°C. The mid-gut sample was thawed to 4°C, vortexed, and 20 µl of 3% paraformaldehyde was added. We homogenized and vortexed samples a second time and centrifuged at 9000 rpm for 1 min. 20 µl of sample was added to 140 µl of distilled water, 20 µl of PBS (1×) pH 7.4, and 20 µl L-Dopa (4 mg/ml). We measured the absorbance at 490 nm every 27 s at 30°C for 300 measures (02:14:33) using a Biotek microplate reader and KC Junior software. We calculated both the slope of the kinetic curve (absorbance per hour) and the final absorbance during linear phase (verified visually for each sample) to estimate the rate of production of the precursor to melanin, Dopachrome (Barnes and Siva-Jothy 2000; Siva-Jothy 2000; Cerenius and Soderhall 2004). Each sample was assayed twice and the average of the two samples was used for analysis.

Parasite assessment

For all monarchs that survived to adulthood (and not sacrificed for measures of mid-gut phenoloxidase activity $N = 65$), we quantified parasite reproduction based on the number of *O. elektroscirrha* spores on adult butterflies ($N = 83$). Upon death, the abdomen of each monarch was removed and placed into a vial containing 5 ml of deionized water. We vortexed each sample for 5 min at maximum speed (Vortex Genie II) and counted the number of dislodged *O. elektroscirrha* spores in two replicate hemocytometer chambers at a magnification of 400×. Average counts per monarch were multiplied by 5×10^5 to estimate the total number of spores per individual.

Body size and melanism

Adult body length (from tip of head to end of abdomen, in mm) was measured using a digital caliper ($N = 83$). We used digital image analysis to measure adult wing size and the external melanism ($N = 79$). Upon death we removed and scanned monarch forewings using a flatbed HP scanner set to 300 dpi using the same exposure for each scan. Measurements were made using Adobe Photoshop software with the Image Processing Tool Kit plugin (Reindeer Graphics, Inc.). Total forewing area (in mm^2) and two measures of wing melanism were obtained for both forewings of each adult butterfly following Davis, Farrey et al. (2005). We quantified the proportion of area on each forewing encompassed by black pigmentation. We also estimated the density of black pigmentation as an indicator of the overall intensity or opacity of black, with the units of density set so that lower values corresponded to an increased intensity of black. Average measures per individual were based on results for L and R forewings.

Statistical analysis

Count variables were log transformed (number of spores, development time, and adult longevity) or square root transformed (hemocyte concentration) prior to analysis to normalize the error variance. We tested for equal variances between parasite treatment groups using Fisher's *F*-test, for normally distributed data, and Levene's Test for non-normally distributed but continuous data, at significance levels of $\alpha = 0.05$. Unequal variances based on Levene's Test at $\alpha = 0.05$ were observed for adult longevity. We used two-tailed Pearson's correlations to explore the relationships between immune effector traits and morphological measures within and between parasitized and control monarchs. Directed *t*-tests were used to test the specific prediction that immune parameters were greater in parasite-treated versus control monarchs (Rice and Gaines 1994). Directed tests allocate a greater probability to the tail of the distribution in the predicted direction (γ), while retaining a smaller probability in the opposite tail to detect unexpected deviations opposite to predictions ($\delta < \gamma$; where $\delta + \gamma = \alpha$). We followed the guidelines in Rice and Gaines (1994) by setting γ/α to 0.8, giving values of $\gamma = 0.04$ and $\delta = 0.01$.

We ran ANCOVA using GLM in SPSS 13.0 to examine the effects of hemocyte concentration, sex and parasite treatment on monarch longevity, parasite load, body length, wing area, and wing melanism (Full model: Dependent variable = parasite + sex + hemocyte concentration + all two- and three-way interactions). We followed Crawley (2002) in performing model simplification and used Akaike's information criterion adjusted for small sample sizes (AICC) for model comparison. Predictor variables with *P*-values greater than 0.10 were sequentially deleted from the full model starting with the variable with the highest *P*-value, and then model fit was evaluated at each step. In cases where missing data affected sample sizes amongst statistical models, we used adjusted-R^2 over AICC for model comparison. If a factor with $P > 0.10$ affected the significance ($\alpha = 0.05$) of another variable it was retained in the final model. We tested for normal distributions of the residuals and all of the minimum adequate models showed normally distributed residuals.

Results

General results

A total of 36 parasitized (48.6%) and 47 control (62.7%) monarchs survived to adulthood (excluding those sacrificed to measure PO activity). Differences in pre-adult mortality of parasitized and control monarchs were not significant (Mann–Whitney $Z = -0.534$, $P = 0.593$). Of the monarchs surviving to adulthood, 21 parasitized and 23 control monarchs were sampled for circulating hemocyte concentration as larvae. Although there was greater mortality among individuals sampled for hemocyte concentration the difference was not significant (Mann-Whitney $Z = -0.532$, $P = 0.595$). All monarchs in the parasite-treated group were infected with *O. elektroscirrha*, but no monarchs in the control treatment were infected. Average parasite loads removed from the abdomens of inoculated monarchs were 1.29×10^6 spores (range: 0.32×10^6–5.13×10^6). Average parasite loads did not differ between male and female butterflies ($t_{80} = 0.291$, $P_{2\text{-tailed}} = 0.772$).

Immune parameters

Across all monarchs sampled for hemolymph, hemocyte concentrations were higher for parasite-treated (29 ± 12 SD; $N = 74$) versus control monarchs (23 ± 11 SD; $N = 75$) and

this difference was significant ($t_{147} = -2.991$, $P_{\text{Directed}} = 0.002$), supporting the notion that hemocyte levels increased upon recognition of the parasite. Similar but non-significant differences were observed among the subset of monarchs that survived to adulthood ($t_{42} = -1.40$, $P_{\text{Directed}} = 0.106$). No significant difference was found between parasitized monarchs sampled for hemocytes that survived to adulthood and those that did not survive to adulthood ($t_{40} = 0.512$, $P = 0.612$). Females had similar hemocyte concentrations across control and parasite treatments, whereas control males had lower hemocyte concentrations than parasitized males (Fig. 1a). A two-way ANOVA did not provide support for effects of treatment or sex on hemocyte concentrations (treatment: $F_{1,78} = 1.378$, $P = 0.248$; sex: $F_{1,78} = 0.054$, $P = 0.818$; treatment*sex: $F_{1,78} = 1.192$, $P = 0.282$). However, directed *t*-tests demonstrated that hemocyte counts were significantly lower in control versus parasitized males ($t_{23} = -1.79$, $P_{\text{Directed}} = 0.044$), whereas concentrations in parasitized and control females were similar ($t_{16} = -0.052$, $P_{\text{Directed}} = 0.480$).

Within infected females, parasite loads decreased with increased hemocyte concentrations, and the opposite pattern was observed for males. However, statistical analysis indicated that parasite load was not significantly associated with sex or hemocyte concentration, and no interactions involving parasite treatment, sex and hemocyte concentration were retained in the final model (Table 1).

Two measures of larval mid-gut PO activity—final absorbance and the slope of increasing product over time—were highly correlated with one another (Pearson correlation:

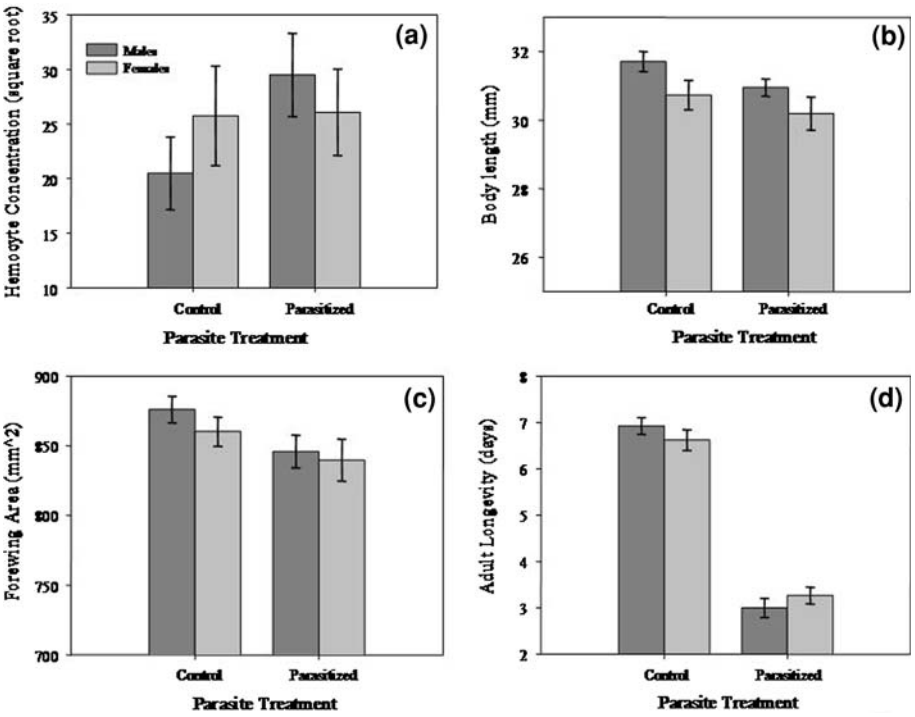

Fig. 1 Effects of parasite treatment and sex on (**a**) hemocyte concentration of larvae (cells per μl of hemolymph, square-root transformed), (**b**) adult body length (in mm), (**c**) adult forewing area (in mm^2), and (**d**) adult longevity (in days). In each figure, data for males are shown in black and females are shown in grey. Error bars represent standard error

$R = 0.684$; $N = 65$; $P = 0.000$). However, neither measure of mid-gut PO activity was correlated with hemocyte concentration (e.g., for reaction slope, Pearson correlation: $R = -0.152$; $N = 65$; $P = 0.227$). Mid-gut PO activity measures were similar for parasitized and control larvae (Parasitized: mean slope $= 0.0639 \pm 0.0058$ SD; Control: mean slope $= 0.0648 \pm 0.0079$ SD) and there was no significant effect of parasite treatment on PO activity ($t_{63} = 0.534, P = 0.595$). Mid-gut PO activity could not be compared between males and females because sex was not recorded for dissected larvae.

Adult body size and longevity

Adult longevity, forewing area and body length were lower among parasite-treated versus control monarchs and males incurred a greater proportional decrease in each of these measures relative to females (Fig. 1b–d). However, for adult longevity neither sex nor the three way interaction between sex, parasite infection and hemocyte concentration were statistically significant (Table 1) Among control females, hemocyte concentrations were negatively associated with both body length and wing area, whereas parasitized females showed the opposite trend (Fig. 2a, only body length shown). Among males, both control and parasite-treated monarchs showed a positive association between hemocyte concentration and measures of body size (Fig. 2b). These patterns were supported by statistical analyses showing a significant 3-way interaction between sex, parasite infection and hemocyte concentration for analyses involving both body length and forewing area (Table 1).

Wing melanism

Females had a greater proportion of black pigmentation on their forewings (65.86 ± 2.60 SD; $N = 35$) than males (56.20 ± 1.96 SD; $N = 44$) and also showed a higher intensity (i.e., density) of black pigmentation (Females: 133.80 ± 7.80 SD; $N = 36$; Males: 147.02 ± 8.29 SD; $N = 46$; lower density scores are associated with more intense black). Control females had the greatest proportion of black pigmentation on their forewings (66.71 ± 1.98 SD; $N = 21$), followed by parasitized females (64.59 ± 2.95 SD; $N = 14$), control males (56.80 ± 2.13 SD; $N = 26$), and parasitized males (55.33 ± 1.29 SD; $N = 18$). The main effects of parasite treatment and sex on the proportion of black pigmentation were significant, but not the two-way interaction (Table 1), consistent with the observation that greater wing melanism among uninfected monarchs was consistent for both males and females. Within the subset of parasitized females, final spore loads were negatively associated with the proportion of black on adult forewings, although this relationship was not significant (Pearson's correlation: $R = -0.260$, $N = 14$, $P = 0.370$). No relationship was observed between wing melanism and parasite loads for males, and we also observed no relationship between hemocyte concentration and measures of wing melanism (Table 1).

Discussion

Our results showed that average hemocyte concentrations, but not measures of mid-gut phenoloxidase (PO) activity, were greater among larvae experimentally challenged with the neogregarine protozoan *O. elektroscirrha*. Among monarchs surviving to adulthood, only males, showed evidence of greater hemocyte production following parasite infection.

Table 1 Analysis of variance of dependent variables (column headings) and independent variables included in the final minimum adequate models

Source	Parasite load	Adult longevity	Body length	Forewing area	Proportion black	Density black
Parasite treatment	$F_{1,80} = 4786.107$, **$P = 0.000$** ↑	$F_{1,80} = 292.319$, **$P = 0.000$** ↓	$F_{1,35} = 6.204$, **$P = 0.018$** ↓	$F_{1,35} = 2.180$, $P = 0.149$	$F_{1,76} = 13.367$, **$P = 0.000$** ↓	$F_{1,79} = 1.961$, $P = 0.165$
Sex			$F_{1,35} = 0.741$, $P = 0.395$	$F_{1,35} = 0.510$, $P = 0.480$	$F_{1,76} = 410.714$, **$P = 0.000$** ↑♀	$F_{1,79} = 54.361$, **$P = 0.000$** ↑♀
Hemocytes			$F_{1,35} = 3.956$, **$P = 0.055$** ↑	$F_{1,35} = 3.897$, **$P = 0.056$** ↑		
Sex*parasite treatment			$F_{1,35} = 2.398$, $P = 0.130$	$F_{1,35} = 3.483$, $P = 0.070$		
Sex*hemocytes			$F_{1,35} = 0.108$, $P = 0.744$	$F_{1,35} = 0.010$, $P = 0.922$		
Parasite treatment*hemocytes			$F_{1,35} = 2.612$, $P = 0.115$	$F_{1,35} = 0.531$, $P = 0.471$		
Sex*parasite treatment*hemocytes			$F_{1,35} = 4.155$, **$P = 0.049$**	$F_{1,35} = 4.845$, **$P = 0.034$**		
Adjusted R^2	.983	.782	.233	.085	.844	.406

Analyses began with the full model: Response variable = parasite treatment + sex + hemocyte concentration + treatment*sex + hemocyte*treatment + hemocyte*sex + treatment*sex*hemocyte + error term. Model simplification was performed as described in Methods text. *F*- and *P*-values are shown only for explanatory variables retained in the final model; adjusted R^2 is shown for the final reduced models. The direction of the effect of independent variables on dependent variables is indicated as follows: ↑ increase/positive relationship and ↓decrease/positive relationship, and ↑♀ greater in females. For the independent variable "Parasite Treatment" the direction indicated is for the parasitized monarchs

Bold indicates significant and nearly significant results; *P* values less than 0.06

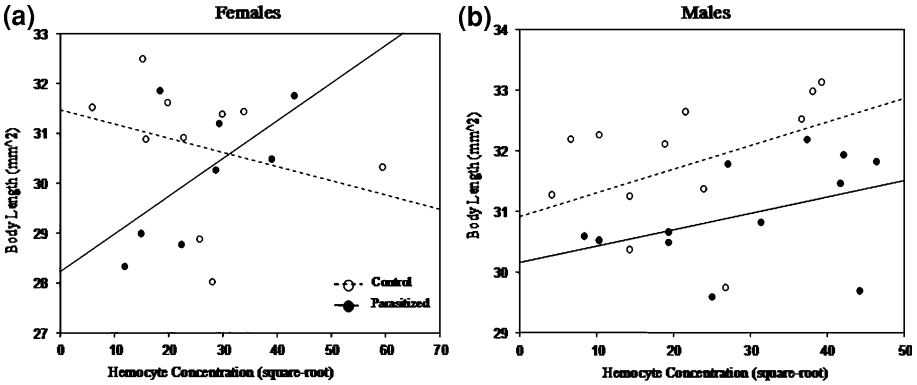

Fig. 2 Relationship between hemocyte concentration of larvae (square-root transformed) and adult body length for (**a**) females and (**b**) males. Separate trend lines are shown for unparasitized (dashed line, open circles) and parasitized (solid line, closed circles) treatment groups. Linear regression slopes and adjusted R^2 values based on simple regression models are as follows: for control females, slope $= -0.303$, $t_9 = -0.899$, $P = 0.395$; $R^2 = 0.092$; for parasitized females, slope $= 0.615$, $t_7 = 1.913$, $P = 0.104$; $R^2 = 0.379$; for control males, slope $= 0.441$, $t_{12} = 1.630$, $P = 0.131$; $R^2 = 0.195$; for parasitized males, slope $= 0.411$, $t_{11} = 1.428$, $P = 0.184$; $R^2 = 0.169$

Together, these findings suggest that (1) production of hemocytes increased in the presence of a debilitating parasite, and (2) males and females may differ in baseline hemocyte production and response to infection. Previous work has demonstrated that insects can show increased expression of immune defenses, including hemocyte concentrations, following acute challenges by foreign materials (Freitak et al. 2003; Robb and Forbes 2006) although patterns vary with insect species and the specific immune effector traits assayed (Siva-Jothy et al. 2001; Yourth et al. 2002; Adamo 2004; Chernysh et al. 2004; Armitage and Siva-Jothy 2005). The increased hemocyte production observed here indicates that this immune effector trait might be an inducible (rather than constitutive) defense under some circumstances.

Although we predicted that mid-gut defenses could be important during the initial phase of parasite infection when *O. elektroscirrha* sporozoites penetrate the gut wall (McLaughlin and Myers 1970), there was no association between mid-gut PO activity and parasite treatment. However, it is possible that mid-gut PO activity responds to infection at an earlier life stage than we examined here. Because larvae were sampled destructively for the mid-gut assay, we were unable to examine how mid-gut PO activity affects the final outcome of infection, or how this measure differed between males and females. Moreover, our results provided no evidence for a correlation between hemocyte concentrations and mid-gut PO activity, which appears to be consistent with a general phenomenon that associations between different immune effort traits vary among insect species, with some studies showing positive associations between multiple measures of immune defenses e.g., in desert locusts, crickets, scorpionflies and Egyptian cotton leafworms (Gillespie et al. 2000; Kurtz et al. 2000; Adamo 2004; Cotter et al. 2004b), and others showing no association between measures of immune defenses e.g., in crickets and dameslflies (Adamo et al. 2001; Rolff 2001).

The finding that parasitized males had greater average hemocyte counts than control males, but that females showed similar concentration of hemocytes following infection, was supported by directed t-tests of hemocyte concentrations in relation to parasite

treatment. One explanation for this finding is that females could benefit more from maintaining higher baseline immune defenses than males (Zuk 1990; Zuk and McKean 1996; Yourth et al. 2002; Zuk et al. 2004). Greater investment or benefits from immunity by females have been demonstrated for other insect species including milkweed beetles (Abbot and Dill 2001), wolf spiders (Ahtiainen et al. 2005), scorpionflies (Kurtz et al. 2000; Kurtz and Sauer 2001), field crickets (Adamo et al. 2001), house crickets (Gray 1998), and damselflies (Rolff 2001; Yourth et al. 2002). Alternatively, counterpoints to this pattern have been demonstrated in *Drosophila* (McKean and Nunney 2005), crickets (Zuk et al. 2004), earwigs (Rantala et al. 2007), butterflies (Stoehr 2007), and in a meta-analysis of studies from arthropod hosts (Sheridan et al. 2000). For example, Stoehr (2007), demonstrated that sex differences in the encapsulation response of adult cabbage butterflies changed with age, such that younger males, but older females had higher rates of encapsulation.

If host defenses are costly, then greater investment in immune defenses should correlate negatively with host longevity and body size in the absence of infection. Among control females, greater hemocyte concentrations were indeed associated with reduced body length and forewing area. The opposite trend was observed for parasite-treated females; monarchs with greater hemocyte concentrations suffered less from the negative effects of *O. elektroscirrha*. These trends are consistent with the idea that female monarchs benefit from maintaining a costly defense if the risk of infection is sufficiently high. This finding is important in light of previous work that demonstrated negative effects of *O. elektroscirrha* on adult monarch body size (Altizer and Oberhauser 1999); indeed, de Roode, Gold et al. (2007) showed that although male and female butterflies showed similar longevity reductions in response to parasite infection, the negative effect of parasitism on adult body size was much greater for females. Because female body size in monarchs is known to correlate positively with lifetime fecundity and egg size (Oberhauser 1997; Oberhauser 2004), the consequences of infection could be more substantial for females than for males. Moreover, in monarch populations that migrate long distances, such as in eastern N. America, selection pressures on female longevity and greater body size could be even more intense. This is because males in poor condition can mate before the wintering season ends (Van Hook 1996; Oberhauser and Frey 1999), but females must fly hundreds of kilometers to oviposit on milkweed host plants in the southeastern United States. Heavily parasitized females are likely to have an extremely low probability of reproducing under such demanding conditions, leading to greater benefits for potentially costly immune defenses in females as compared to males.

In monarchs, females have darker forewings than males, in terms of the proportion of wing area covered by scales with black pigmentation (Davis et al. 2005). Our study confirmed this difference in wing melanism between males and females, and further showed that wing melanism correlates with monarch butterfly infection status. Specifically, control monarchs had darker forewings than parasitized monarchs, and this difference was observed among both males and females. It is therefore possible, as demonstrated for other insect systems (Barnes and Siva-Jothy 2000; Wilson et al. 2001; Cotter et al. 2004a), that resistance to parasitism is correlated with external melanism on monarch forewings, and that wing pigmentation could be an indicator of the infection status of potential mates. Two further explanations for darker wings among unparasitized butterflies are that (1) resources for melanin production are limited and may be allocated to only one function (i.e. wing melanism or parasite defense; (Hooper et al. 1999; Talloen et al. 2004; Freitak et al. 2005), or that (2) coloration/melanism is influenced by the general overall "health" of an individual (Stoehr 2006).

In summary, this study provides support for differential costs and consequences of immune defenses in male and female monarch butterflies following infection by *O. elektroscirrha*. Further work incorporating a range of parasite doses and larger samples sizes is needed to evaluate the degree to which males and females differ in their baseline and induced defenses, and the degree to which different immune effector traits influence parasite replication. To our knowledge, this study is the first to quantify immune defenses of a butterfly species in response to a naturally-occurring protozoan parasite, and further emphasizes the need to understand how insect species balance investment in potentially costly immune defenses with other life history demands in the face of uncertain infection risk.

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