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Sex differences in insect immune function: a consequence of diet choice?

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Abstract Males often have reduced immune function compared to females but the proximate mechanisms underlying this taxonomically widespread pattern are unclear. Because immune function is resource-dependent and sexes may have different nutritional requirements, we hypothesized that sexual dimorphism in immune function may arise from differential nutrient intake (acquisition hypothesis). To test this hypothesis, we examined patterns of phenoloxidase (PO) activity in relation to nutrient consumption in Queensland fruit flies (Q-flies). In the first experiment, flies were allowed to choose their preferred nutrient intake. Compared with males, female Q-flies had higher PO activity, consumed more calories, and preferred a higher protein:carbohydrate (P:C) diet, suggesting that differential acquisition could explain sex differences. In the second experiment, we restricted flies to one of 12 diets varying in protein and carbohydrate concentrations and mapped PO activity for each sex onto a nutritional landscape. Counter to our hypothesis, females had higher PO activity than males at any given level of nutrient intake. Both carbohydrate and protein intake affected PO activity in females but only protein affected PO activity in males. Our results indicate that sex differences in Q-fly immune function are not solely explained by sex differences in nutrient intake, although nutrition does contribute to the magnitude of these sex differences.

Keywords *Bactrocera tryoni* · Geometric framework · Immunity · Nutritional ecology

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Introduction

Sexual dimorphism in immune function has been reported across diverse taxa, with females generally having greater immune function (Folstad and Karter 1992; Nunn et al. 2009). At an evolutionary level, sex differences in immune function are thought to reflect differences in life history strategy (reviewed in Zuk and Stoehr 2010). Males and females often differ in how they allocate resources to maximize fitness (Trivers 1972). Males are usually limited by the availability of receptive females whereas females are limited by the number of offspring that they can produce and rear (Trivers 1972; McKean and Nunney 2005). Accordingly, the optimal allocation strategy for males is to trade off lifespan-enhancing traits (e.g., immune function) for increased reproductive effort, and the optimal strategy for females is to balance reproductive effort and lifespan (McKean and Nunney 2005; Stoehr and Kokko 2006; Zuk and Stoehr 2010). Therefore, sex differences in this immunity-reproduction trade-off may arise because females allocate a greater proportion of their resources toward lifespan-extending immune function (Rolff 2002; Stoehr and Kokko 2006; Zuk and Stoehr 2010).

The strategic allocation paradigm has precipitated research focusing on identifying physiological mechanisms, with special attention to hormones (Ahmed et al. 2010; Klein and Huber 2010). In particular, there is some evidence that sex-specific hormones (e.g., testosterone (Folstad and Karter 1992) may be immunosuppressive in male vertebrates (Muehlenbein and Bribiescas 2005; Ahmed et al. 2010). However, insects lack such sex-specific hormones, but nonetheless exhibit a parallel pattern of comparatively lower immune function in males (Rolff 2002; Nunn et al. 2009).

To date, greater emphasis has been placed on allocation strategies, but trade-offs can also be mediated by variation in resource acquisition. In the traditional resource-allocation model (e.g. van Noordwijk and de Jong 1986), trait expression is affected by both the quantity of resources acquired and how these resources are allocated. Therefore, even if both sexes use identical allocation strategies, they may differ in immune function if they differ in resource acquisition (e.g., both sexes allocate 20 % of resources to immune function, but females acquire more resources). Stoehr and Kokko (2006) modelled sex differences in immune function by assuming males and females have the same energetic intake but different patterns of resource allocation. In contrast, Houston et al. (2007) developed a state-based model that allowed animals to adjust both resource acquisition and allocation. Though optimal allocation strategies for immune function were the main focus of their analysis, their model suggested that animals might also adjust resource acquisition to improve immune function.

Besides dietary energy intake, trait expression may also be driven by diet composition. Recent advances in nutritional ecology have revised our conventional view of phenotypic trait expression by establishing a nutrient-explicit framework. This framework correlates trait expression with the intake of specific nutrients, such as protein and carbohydrates (Raubenheimer and Simpson 1993, 1999; Simpson et al. 2004; Raubenheimer et al. 2009). Traits have been found to vary quite widely in their response to nutrient composition of the ingested diet, suggesting that different traits have different nutritional requirements (Simpson et al. 2004; Lee et al. 2006, 2008b; Cotter et al. 2010; Fanson and Taylor 2012). If a pair of traits has non-overlapping nutritional requirements, trade-offs between these traits can only occur at the acquisition level, not at the allocation level. In accordance, animals can alter their nutrient intake (e.g. by choosing a high protein diet) to increase the expression of one trait at the expense of another. For instance, females of some flies prefer a high protein diet, which increases reproduction but also decreases lifespan (Lee et al. 2008a; Fanson et al. 2009, 2012). This framework demonstrates that trade-offs can be

modified not only by altering internal allocation mechanisms, but also by altering external nutrient acquisition strategies. The prevalence of acquisition as a mechanism in mediating trade-offs among traits is relatively unknown, though recent studies with insects have suggested that nutrient composition of the diet can be highly influential (Lee et al. 2008a; Cotter et al. 2010; Fanson et al. 2012).

This study examines how nutrients mediate sexual dimorphism in immune function. We tested the resource acquisition hypothesis using the Queensland fruit fly (*Bactrocera tryoni*; 'Q-fly'). This hypothesis proposes that optimal nutrient targets for traits differ, and if an animal chooses to maximize one trait through resource acquisition, the other trait may be compromised. In this context, we are specifically interested in the potential role of resource acquisition in shaping the immunity-reproduction trade-off. Male and female Q-flies differ in nutritional requirements for reproduction; females maximize lifetime egg production on protein-rich diets (Fanson and Taylor 2012) whereas males require little protein for reproduction (Perez-Staples et al. 2007, 2008; Prabhu et al. 2008), but require carbohydrate to fuel energetically expensive wing calling (Mankin et al. 2008). Since protein is an important building block for many components of the immune system (Good 1981; Hansen et al. 1982; Li et al. 2007), female diet may also support higher immune function.

To examine patterns of immune function, we quantified levels of phenoloxidase (PO) activity. PO is a key component of the constitutive immune system of insects and is involved in several defence mechanisms, including encapsulation response, cellular defence, blood clotting and wound repair (Cerenius et al. 2008). PO activates the process of melanogenesis as well as a host of intermediate products (e.g. quinones, superoxide, hydrogen peroxide) effective against fungal, viral and bacterial pathogens (reviewed in González-Santoyo and Córdoba-Aguilar 2012). PO is costly to produce, which suggests that there is a commensurate benefit for investing in PO and that PO expression will be involved in trade-offs with traits requiring common resources (González-Santoyo and Córdoba-Aguilar 2012). Furthermore, a recent meta-analysis found PO activity to be the only immune component that is consistently higher in female insects across a wide range of species (Nunn et al. 2009), which is particularly relevant to this study. In studies that have considered sex effects, the increased PO activity in females has been associated with decreased susceptibility to pathogens (e.g. Adamo et al. 2001; Joop et al. 2006).

To elucidate the role of nutrition in mediating sex differences in PO levels, we developed nutritional landscapes (e.g. Simpson et al. 2004) for each sex to visualize how PO levels change with nutrient consumption (Fig. 1). If resource acquisition mediates sex differences, PO response surfaces across the nutritional landscape should be similar for both sexes. That is, if nutrient acquisition mediates PO activity, then when males and females are forced to consume similar diets, their PO activity should be similar (Fig. 1). In contrast, if resource allocation mediates PO activity, then females are predicted to have higher PO activity even when similar diets are consumed, resulting in different response surfaces for males and females.

Methods

General methods

Study species

Q-flies were obtained as pupae from the Elizabeth Macarthur Agricultural Institute (New South Wales, Australia). Flies were transferred to individual 70 ml specimen containers

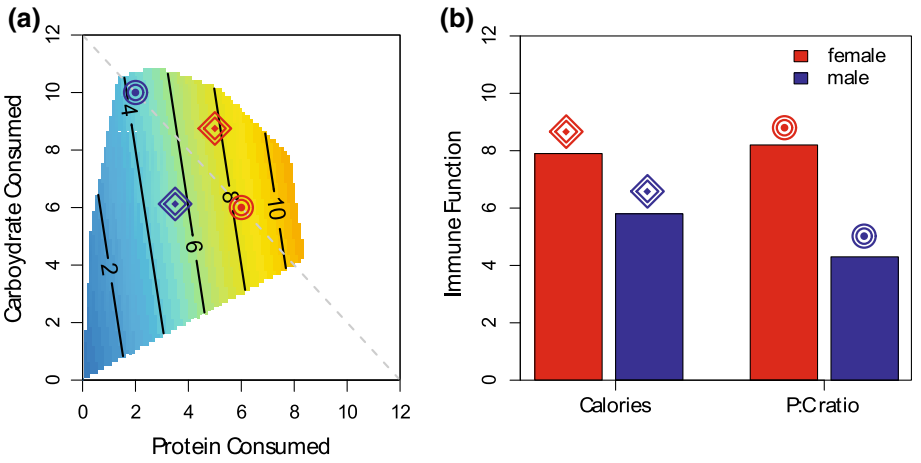

Fig. 1 Schematic of the relationship between resource acquisition and immune function. Assuming similar patterns of resource allocation across the nutritional landscape (**a**), there are two different patterns of nutrient consumption that can result in sex differences in immune function (**b**). One possibility is that males (*blue diamond*) and females (*red diamond*) prefer the same P:C ratio but females consume more calories. An alternative scenario is that males (*blue circle*) prefer a lower P:C ratio than females (*red circle*), but both sexes consume the same amount of calories (*dotted line* in **a** represents *isocaloric line*). (Color figure online)

within 24–28 h post-emergence. For all experiments, temperature was 25.9 ± 0.9 °C, relative humidity was 81.2 ± 1.4 %, and the light schedule was 14L:10D.

Experimental diets

Adult flies were fed a liquid, chemically defined diet comprising amino acids, sucrose and micronutrients (Supplemental Table 1). Diets were dispensed using micropipette tips (2–200 µl) that were filled with 50, 35, or 25 µl of diet for diet concentrations of 30, 100, or 330 g/l, respectively. The quantity of diet was adjusted to account for different rates of consumption across concentrations. Over 95 % of the pipettes were completely depleted in 3 days. Micropipettes were checked twice daily and refilled when depleted or when 5 days passed since refilling. Any remaining diet was measured and then corrected for evaporation following Fanson et al. (2009).

Phenoloxidase (PO) extraction and assay

Frozen flies were weighed on a micro-balance (Sartorius ME5) to the nearest 0.01 mg. The head and wings were removed and the body was placed in a 1.5 ml microcentrifuge vial with 600 µl of ice-cold Tris-buffered saline (TBS; Sigma T5030; 0.05 M Tris, 0.15 M NaCl, pH 7.6). The body was crushed using a micro-pestle and centrifuged for 5 min (10,000 g, 4 °C). Flies were then further homogenized by sonication (Cole Parmer Ultra-Sonic Processor CP 750) in an ice-water bath at 80 % power for two 15-s pulses. Following a second centrifugation, the supernatant was decanted into a clean microcentrifuge vial and assayed immediately.

PO activity was quantified using previously described methods (Gliksman and Yuval 2010). Briefly, 50 µl of supernatant was added to a chilled microplate with 50 µl TBS and 50 µl of saturated L-DOPA solution (L-dihydroxyphenylalanine; Sigma D9628; 4 mg/ml).

The plate was shaken for 5 min and optical density at 490 nm was measured every 1 min for 10 min using a micro-plate reader (BioTek ELx808, Winooski, VT, USA). The plate-reader maintained a constant temperature of 36 °C throughout the reading. Blanks, consisting of 50 µl L-DOPA and 100 µl TBS, were run on each plate to control for possible spontaneous oxidation of the substrate. To control for variability in the colour of the fly extract, we ran a control for each sample that consisted of 50 µl supernatant and 100 µl TBS. Both blanks and controls were subtracted from the sample values to obtain final optical density measurements. For the analysis, we used the slope of the change in optical density over time (Vmax: linear phase between 5 and 15 min) as an estimate of PO activity.

During initial pilot tests, we repeated the above protocol with the addition of α-chymotrypsin, which converts all prophenoloxidase (proPO) remaining in the supernatant to PO (Bailey and Zuk 2008). Results were near identical, suggesting that nearly all proPO is converted to PO during freezing and the preparation of the sample.

Experiment 1: choice diet

To test for sex differences in PO activity and preferred nutrient intake, flies were allowed to self-regulate consumption. Each fly was provided with three micropipette tips (2–200 µl), containing water, protein-rich diet, and carbohydrate-rich diet. To assess how flies regulated intake (Lee et al. 2008a), they were given one of two diet concentrations (30 or 100 g/l) for each nutrient, resulting in four diet combinations. For each sex by diet combination, there were initially 15 replicates. At day 20, remaining flies were frozen at −80 °C.

Experiment 2: no-choice diet

To create response surfaces of the relationship between immune function and nutrient consumption for each sex, flies were supplied with two micropipette tips; one containing 100 µl of water and another containing a liquid diet varying in P:C ratio (0:1, 1:8, 1:4, 1:1) and concentration (30, 100, 330 g/l). There were 27 replicates per diet treatment for each sex. At day 15 post-emergence, nine flies from each treatment group were frozen, and at day 20 any remaining flies were frozen for later assays of PO levels. Two separate time points were sampled to test for consistency of results during the main reproductive period.

Data analysis

All statistical analyses were conducted in SAS (v9.1). Parameter estimates are listed as $\beta \pm$ SE.

Experiment 1: choice diet

First, we conducted a general linear model testing for sex differences in PO activity. For this model, we included sex, concentration of protein-rich diet, concentration of carbohydrate-rich diet, and all interactions to predict PO activity at day 20. Fly weight was included as a covariate. Non-significant interactions ($P > 0.10$) were removed using backward selection process. PO activity was log-transformed to meet statistical assumptions.

Next, to test for sex differences in nutritional intake, we compared total protein and carbohydrate consumption between sexes by performing a MANOVA. Sex, diet concen-

tration, and all interactions were treated as predictors. Fly weight was included as a covariate. For this analysis, total protein and carbohydrate consumption were square root transformed. Only flies that lived to day 20 were included in these analyses.

Additionally, we described the degree of nutrient trajectories (i.e., P:C ratio) for each sex on each different diet treatment using a general linear model (Littell et al. 2006). The ratio of carbohydrate to protein was converted to radians using an arctangent transformation and then to degrees from radians. Using degrees as the response variable, we conducted linear model in which we included concentration of carbohydrate and protein diets, fly sex, and all possible interactions.

Experiment 2: no-choice diet

To describe patterns in PO activity in relation to nutrient intake, we performed a second-order surface analysis with sex, protein and carbohydrate consumption as predictor variables. We centred protein and carbohydrate consumption and included all first- and second-order effects for this model (Myers et al. 2009). Initially, we also included age (day 15 or 20), but no significant differences ($P > 0.1$) were found so we removed this variable. PO activity was log-transformed to meet statistical assumptions.

Results

Experiment 1: sexual dimorphism in PO and nutritional intake

Female Q-flies had higher PO activity than males (Fig. 2a, $F_{1,65} = 7.21$, $P = 0.009$), though the magnitude of this difference depended on the protein concentration of the diet (Fig. 2a, $F_{1,65} = 6.00$, $P = 0.017$). Sex differences were larger when females were provided with choice diets including concentrated protein diets (Fig. 2a). PO activity was not significantly affected by carbohydrate concentration (Fig. 2a, $F_{1,65} = 2.73$, $P = 0.10$). Finally, there was a positive relationship between body mass and PO activity ($\Delta b = 0.16 \pm 0.06$ ln(PO activity)/mg; $F_{1,65} = 5.96$, $P = 0.017$).

As for nutritional intake, female flies ingested more calories than males by consuming more protein (difference in least-square means $\Delta b = 0.43 \pm 0.07$ mg$^{0.5}$, $t_{64} = 6.29$, $P < 0.001$) and more carbohydrates ($\Delta b = 0.22 \pm 0.07$ mg$^{0.5}$, $t_{64} = 3.22$, $P = 0.002$) over 20 days (Fig. 2b). For flies fed a higher concentration of protein, both carbohydrate ($\Delta b = 0.56 \pm 0.07$ mg$^{0.5}$, $t_{64} = 8.24$, $P < 0.001$) and protein ($\Delta b = 0.80 \pm 0.07$ mg$^{0.5}$, $t_{64} = 11.83$, $P < 0.001$) consumption increased. On the other hand, flies fed a higher concentration of carbohydrate increased carbohydrate ($\Delta b = 0.66 \pm 0.07$ mg$^{0.5}$, $t_{64} = 9.74$, $P < 0.001$) but decreased protein ($\Delta b = -0.51 \pm 0.07$ mg$^{0.5}$, $t_{64} = -7.54$, $P < 0.001$) consumption.

In addition to consuming more calories, females consumed a slightly higher P:C ratio than males ($\Delta b = 3.8° \pm 0.7°$, $P < 0.001$) for all diet combinations. The P:C ratio targeted by flies was affected by concentration of both diets. Increasing the concentration of the carbohydrate-rich diet (from 30 to 100 g/l) caused a decrease in the ratio of P:C consumed in both sexes ($\Delta b = 13.2° \pm 0.70°$, $P < 0.001$). Similarly, increasing the concentration of the protein-rich diet increased P:C ratio for both sexes ($\Delta b = 5.9° \pm 0.7°$, $P = 0.007$).

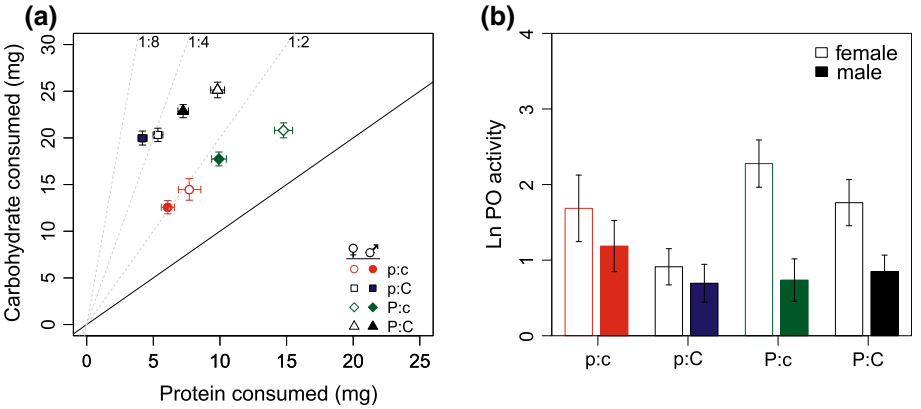

Fig. 2 PO activity (**a**) and total nutrient consumption (**b**) for Q-flies fed one of four choice diets for 20 days. *Filled bars/symbols* represent males and *open bars/symbols* represent females. *Different colours/shapes* indicate different choice diet combinations: *uppercase* 'P' and 'C' indicate high concentration (100 g/l) of protein-rich and carbohydrate-rich diets, respectively, and *lowercase letters* indicate low concentration (30 g/l). *Solid line* indicates the right boundary (1:1 P:C) of the nutrient surface. *Dashed lines* indicate different P:C trajectories for reference. Estimates are least-square means and *error bars* represent one SE

Experiment 2: nutritional landscapes for PO activity

The nutritional landscapes of PO activity clearly showed that males and females differed not only in overall PO activity, but also in how nutrition affects PO activity (Table 1; Fig. 3a, b). PO activity was higher in females, even after controlling for the effects of body mass, protein consumption, and carbohydrate consumption. Increasing protein consumption increased PO activity in both sexes (Table 1). In contrast, increased carbohydrate consumption increased PO activity only in females (Table 1).

Discussion

We tested the hypothesis that sex differences in PO level are explained proximately by sex differences in acquisition. As predicted by the acquisition hypothesis, female Q-flies had higher PO activity and consumed more nutrients, especially protein, than males. However, comparisons of PO activity across a nutritional landscape revealed that nutrition affects PO activity differently in males and females. Females consistently had higher PO activity than males, even when both sexes were forced to consume similar amounts of protein and carbohydrate. Therefore, although the differential intake hypothesis does not solely explain the sex difference in PO activity of Q-flies it does account for part of these differences.

Our results demonstrated sex-specific plasticity in the effect of resource acquisition on immune function. For males, PO levels did not change much across the nutritional landscape, indicating that they allocate a relatively fixed amount of resources to PO production and suggesting that additional resources are allocated to other traits (e.g. reproduction). In contrast, females adjusted PO levels in relation to diet, suggesting that when more resources are available, females invest more into PO production. This additional investment in PO by females suggests that PO levels are important to female fitness. These

Table 1 Parameter estimates for surface regression in which average daily carbohydrate and protein consumption predicts PO activity

	Estimate ± SE	DF	*F*	*P*
Body size	**0.067 ± 0.015**	**1, 423**	**20.21**	**<0.001**
Sex (female)	**0.966 ± 0.112**	**1, 423**	**74.86**	**<0.001**
Protein (P)	**2.448 ± 0.736**	**1, 423**	**26.78**	**<0.001**
Carbohydrate (C)	−0.821 ± 0.356	1, 423	0.16	0.69
P × sex	−0.103 ± 0.933	1, 423	0.01	0.91
C × sex	**1.459 ± 0.442**	**1, 423**	**10.90**	**0.001**
C × C	0.267 ± 1.476	1, 423	0.01	0.93
P × P	**−9.156 ± 4.021**	**1, 423**	**8.93**	**0.003**
P × C	3.474 ± 4.609	1, 423	0.27	0.60
C × C × sex	−0.691 ± 1.821	1, 423	0.14	0.70
P × P × sex	4.777 ± 4.526	1, 423	1.11	0.29
P × C × sex	−4.157 ± 5.327	1, 423	0.61	0.44

Bolding indicates significant variables ($P < 0.05$)

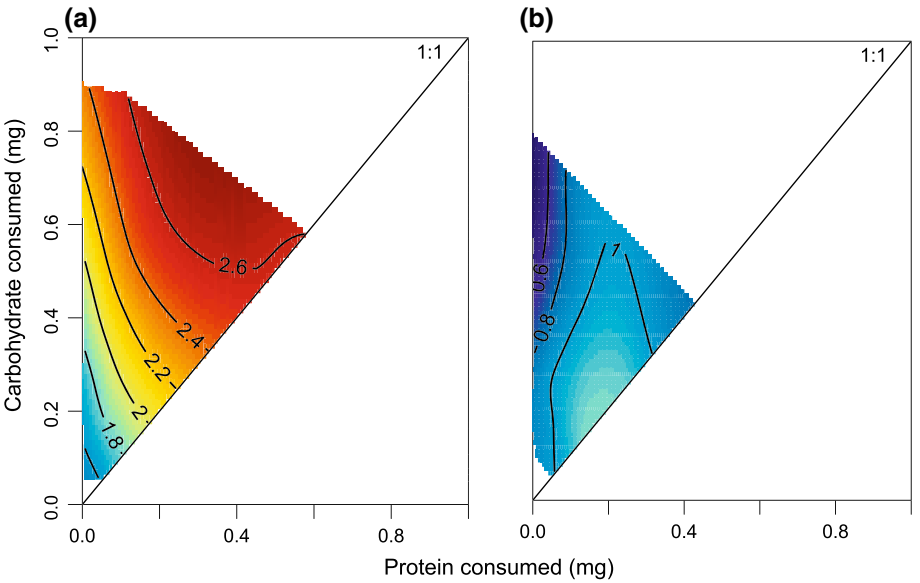

Fig. 3 Phenoloxidase activity in relation to daily protein and carbohydrate consumption for female (**a**) and male (**b**) flies fed a fixed diet (no choice experiment). *Blue colours* indicate low PO activity and *red* indicates high PO activity. Surface *plots* were fitted to predicted values from second-order regressions. (Color figure online)

results are consistent with "Bateman's principle of immunity" (Rolff 2002; Nunn et al. 2009) that predicts higher immune function in females due to the greater effect of survival on lifetime reproductive success.

Female Q-flies are likely under strong selection for increased survival due to their natural history. Female flies take longer than males to achieve reproductive maturity (Perez-Staples et al. 2007). Furthermore, since females have limited egg production

capacity per day, a female flies' fitness is tightly linked to post-mating survival (Meats and Leighton 2004; Fanson et al. 2009). In contrast, male Q-flies can transfer enough sperm to fertilize thousands of eggs in a single copulation event that only takes a few hours (Perez-Staples et al. 2007). Therefore, male fitness is less linked to survival and may partly explain why female Q-flies have longer lifespans than males (Fanson et al. 2012).

Our PO results coincide well with the few other studies exploring links between specific nutrients and PO activity. Similar to our results for males, diet quantity and quality had only a small effect on PO activity for non-immune challenged caterpillars (Lee et al. 2006, 2008b; Cotter et al. 2010). However, in immune-challenged caterpillars (sex was not known—Cotter et al. 2010), higher protein and carbohydrate consumption resulted in increased PO activity, matching well with our results for female Q-flies. Perhaps female Q-flies maintain PO activity in preparation for an immune-challenge, such as mating (Gliksman and Yuval 2010) which exposes females to pathogens (Lockhart et al. 1996; Fedorka and Zuk 2005).

There may be costs to females for maintaining elevated PO levels. During an infection, activated PO produces toxic quinones and reactive oxygen species (ROS) that destroy the invading pathogen; however, these non-specific molecules may also cause tissue damage to the host (Sadd and Siva-Jothy 2006). Thus, an overly aggressive immune response may result in increased rates of damage accumulation (Dowling and Simmons 2009). Recently, it was shown that female Q-flies (Fanson and Taylor 2012), field crickets (Maklakov et al. 2008) and *Drosophila melanogaster* (Lee et al. 2008a) reared in an innocuous lab environment have reduced lifespan when fed protein-rich diets. These results have been attributed to toxicity associated with protein consumption, possibly due to nitrogenous waste or enhanced production of ROS (Simpson and Raubenheimer 2009). Since nutrient-dependent increase in PO activity maps quite well onto nutrient-dependent decrease in lifespan of female Q-flies (Fanson et al. 2009), it may be that toxic effects of PO underlie the decrease in lifespan of female Q-flies fed protein-rich diets. However, a recent study showed that male Q-flies had similar decreases in lifespan when fed protein-rich diets (Fanson et al. 2012), but male Q-flies have relatively constant PO levels.

Although nutrient intake is linked to PO activity, differential intake is not sufficient to fully explain sex differences in Q-fly PO activity but does indicate that differential acquisition can play a role in the magnitude of the sex differences. Furthermore, our findings did suggest that males have a relatively fixed strategy of investment in PO activity, whereas females adaptively adjust PO activity to their nutritional environment. These results imply that immune function plays a more important role in female life history strategies (Arizmendi et al. 2008) and nutritional acquisition and allocation are important mechanisms for mediating immune function.

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