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Larval food quality affects adult (but not larval) immune gene expression independent of effects on general condition

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Abstract

The potential effect of larval condition on adult immunity in holometabolous insects is rarely considered. We show here that larval food composition can impact adult immunity independent from effects on general condition of the animal. Rather, our data indicate a plastic allocation of resources to immunity in high-protein environments. Specifically, we found that increasing the nutritional yeast (protein) available to larval *Drosophila melanogaster* increased the adult's constitutive transcription of two genes encoding defensive antimicrobial peptides. Adult dry weight was not significantly affected by larval food composition, while adult fat content decreased when larval yeast increased. Larval immune activity was unaffected by alterations of larval diet, indicating a lack of covariation in this trait across life-stages. We conclude that the nutritional environment of insect larvae can affect adult immunity by influencing plastic allocation of resources. These influences are less predictable than constraints linked to general condition would be.

Keywords: *Drosophila melanogaster*, insect immunity, larval environment, life stage

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Introduction

The environment experienced by an animal or a plant during its development can greatly influence its phenotype, a phenomenon coined phenotypic plasticity (Pigliucci 2001). This can occur through a variety of mechanisms, including differential energetic expenditure on specific physiological processes and differential development of various tissue types. In organisms that have several life stages, such as holometabolous insects, effects of the larval environment are sometimes manifested in adult traits (Boggs & Freeman 2005; Amitin & Pitnick 2007; McGraw *et al*. 2007). The strength of immune defence is thought to be a trait important to fitness in most organisms, and one that has particular relevance to humans with respect to insects that can vector disease. Immunological variations in metamorphosing insects have traditionally been studied as being pure traits of either the adult or the larva, although there is accumulating evidence that larval environment can impact adult immunity. For example, larvae of *Anopheles gambiae* mosquitoes reared in water with unautoclaved clay are more susceptible to malaria parasites as adults than are adults that developed in water with clay that has been sterilized (Okech *et al*. 2007). In another example, bumblebee larvae reared by workers with artificially activated immune systems develop into adults with elevated phenoloxidase activity (a component of insect immunity) (Moret & Schmid-Hempel 2001). Some reports showing effects of larval condition on adult immunity involve variation in larval food. Halving the amount of food provided to larvae of the damselfly, *Lestes viridis*, strongly reduces both phenoloxidase activity and the number of adult hemocytes (circulating immune-competent cells) (Rolff *et al*. 2004). Again in *A. gambiae* mosquitoes, adults have increased capacity to melanize foreign

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particles with increasing larval food (Suwanchaichinda & Paskewitz 1998).

The reasons why larval food affects adult immunity remain largely unknown. Two distinct mechanisms could be involved. On the one hand, larval food could influence the proportion of resources allocated to immunity independently of its effect on general 'condition', or overall health of the insect. Mechanistically, this could arise from higher availability of a metabolic resource that is limiting specifically for immunity, or from enhanced allocation to growth and development of tissues with immunological function. On the other hand, more or better food could simply improve overall health in a non-specific way, leading to generally higher performance in all fitness traits, including immunity. This second possibility is supported by the two studies on damselflies and mosquitoes referenced above. Indeed, in both experiments reducing larval food not only reduced adult immune function but also produced smaller adults, which in the case of damselflies also had reduced reserves of fat (Suwanchaichinda & Paskewitz 1998; Rolff *et al.* 2004). However, both of these studies make use of partial starvation, which might be more probably expected to affect overall condition. We hypothesize that nutritional effects across life stages can be more nuanced, and in particular that certain nutrients can have specific immunological effects independent of general health. Such idiosyncratic effects of larval food would be more complex to predict than the consequences of poor general condition. To test this hypothesis, we reared *Drosophila melanogaster* across a gradient of larval protein availability and tested whether adult immune function was correlated with other indicators of general health, or whether adult immunity could be influenced by larval nutrition independent of general condition.

We manipulated larval nutritional environment by feeding the larvae media containing increasing concentrations of yeast. This ingredient is the sole source of protein available to *D. melanogaster* under standard laboratory conditions (Begg & Robertson 1950), and its abundance has been shown to affect reproduction (Skorupa *et al.* 2008), ageing (Piper & Partridge 2007) and immunity (McKean *et al.* 2008). We assayed constitutive immune system activity (i.e. in the absence of experimental immune challenge) in flies by measuring transcription levels of genes encoding two antimicrobial peptides (AMPs), *Diptericin A* and *Metchnikowin*. The products of these genes defend *D. melanogaster* against bacterial and fungal infections (Lemaitre & Hoffmann 2007). Their expression during infection is regulated by the two major humoral immune signalling pathways in *Drosophila*, so their joint expression levels should give solid inference into antimicrobial immune levels. Transcription levels of defence genes are a frequently used measure of invertebrate immune quality (Peng *et al.* 2005; Freitak *et al.* 2007; Wigby *et al.* 2008). Assaying constitutive immunity by measuring AMP expression in our study has the additional benefit that it can be performed in an identical fashion in adults and larvae. Therefore, we were able to test for phenotypic covariation in immune traits across life stages, something that has not been done in previous studies. We measured dry weight (a proxy for size) and fat content in adult flies as indicators of general health (Simmons & Bradley 1997). Fat content also serves as a crude estimate of the size of the fat body, the primary tissue of induce immunological activity in *Drosophila* (Lemaitre & Hoffmann 2007). These traits are analogous to the ones reported in the two previous studies that linked general condition and adult immunity (Suwanchaichinda & Paskewitz 1998; Rolff *et al.* 2004).

Material and methods

Larvae were reared in vials with 8 mL of standard food media (*ad libitum*) containing 0.47 g of glucose, 0.075 g of agar, 7.5 mL of water and either 0.235 g, 0.47 g, 0.94 g or 1.88 g of dead yeast, thus setting the yeast to sugar ratio to 0.5, 1, 2 or 4. Adults were kept on a medium with a yeast to sugar ratio of 1.33. All medium contained 0.3% propionic acid and 0.03% phosphoric acid to inhibit microbial growth.

In each vial, one virgin male and one virgin female were allowed to produce eggs for 2 to 3 days. Larval density was low and uncrowded, ranging from 15 to 50 larvae per vial. Emerging adults were transferred daily to identical vials and grouped by vial of origin. Mortality during maintenance was negligible. Flies were reared in a controlled temperature chamber at 24 °C under a 12:12 h light:dark cycle.

To assay immunity, we collected from each vial (i) 6 actively foraging third instar larvae and (ii) 3 to 10 adult females. Adult flies were collected 5 days after the last adult in the vial emerged from its pupal case. Samples were frozen at −80 °C prior to analysis. To measure dry weight and fat content, we collected up to 3 adult males and 3 adult females per vial and stored them at −20 °C.

We conducted our experiment on several genotypes of flies in order to make sure that our results reflected general properties of *D. melanogaster* as opposed to quirks of a specific genotype. We employed 3 lines founded from females collected in 2004 in Ithaca, New York, USA, (hereafter referred to as Ith 37, 148 and 172 lines), 1 line that had been used for previous studies of fly immunity (referred to as Bpl 6) (Lazzaro *et al.* 2004) and several crosses between males and females from

different representatives of theses lines (referred to as 'Heterozygotes'). For each combination of fly genotype and larval food, we prepared up to 3 vials, but, for practical reasons, all genotypes were not tested on every diet. Overall, we measured AMP expression in 57 groups of flies (larvae and adults) from 29 vials (10, 8, 8 and 3 vials for the 0.5, 1, 2 and 4 yeast to sugar ratio treatments, respectively). We also assayed dry weight and triglyceride content of 122 individual flies (32, 37, 35 and 18 flies for the 0.5, 1, 2 and 4 yeast to glucose ratio treatments, respectively).

Molecular methods

We used quantitative PCR (qPCR) to quantify the constitutive expression of *Metchnikowin* and *Diptericin A*, standardized to the expression of a housekeeping gene, *rp49*. We used standard protocols for RNA extraction, reverse transcription, and qPCR amplification (Fiumera *et al.* 2005; McGraw *et al.* 2007). Briefly, total RNA of each group of larvae or adults collected from a given vial was extracted using Trizol (Invitrogen Corp.), and oligo-dT primed cDNA was synthesized by reverse transcription (reagents from Promega). This cDNA served as the template for the qPCR reaction. AMP transcript abundance was measured on an ABI Prism 7000 according to the manufacturer's protocols, with amplification products visualized using fluorescent probes. Gene expression was quantified as a function of the number of PCR cycles necessary to reach a fluorescence threshold (CT value; see Statistical Methods below). The primer sequences used in qPCR amplification are as follows. *Metchnikowin*: Forward 5′ CTGCCTG TCCGGAAGATACAA 3′, Reverse 5′ TCCCTCCTCCTT GCACACA 3′ and Probe 5′ GCTGGGTGTGATGG 3′; *Diptericin*: Forward 5′ GCGGCGATGGTTTTGG 3′, Reverse 5′ CGCTGGTCCACACCTTCTG 3′ and Probe 5′ TTTGCAGTCCAGGGTC 3′; *rp49*: Forward 5′ AGGC CCAAGATCGTGAAGAA 3′, Reverse 5′ GACGCACTC TGTTGTCGATACC 3′ and Probe 5′ AGCTGTCGCACA AAT 3′.

Biochemical methods

Triglyceride content was measured with a colorimetric method derived from (Clark & Gellman 1985) that is frequently used for insect studies (Ohtsu *et al.* 1992; Montooth *et al.* 2003). Briefly, we dried the flies (12 h at 60 °C), homogenized them individually in 0.2 mL of Tris-EDTA buffer and incubated 0.05 mL of the homogenate with 0.01 mL of lipoprotein lipase (Sigma Corp.) for 30–60 min to transform triglycerides into glycerol. The solution was briefly centrifuged to remove carcass debris, 0.05 mL of the supernatant was incubated with 0.05 mL of glycerol reagent (Sigma Corp.) for 5 min at 37 °C, and the optical density at 540 nm was taken as a measure of glycerol (and therefore triglyceride) content in the sample.

Statistical methods

We analyzed gene expression of *Diptericin A* and *Metchnikowin* with linear mixed effect models and the REML (Restricted Estimate Maximum Likelihood) method in JMP 6.0.3. This method allows the robust analysis of experimental designs with unequal sample sizes and missing treatments within interactions between factors (Pinheiro & Bates 2000). CT values were used as response variables. We modelled the yeast:sugar ratio as ordinal since the relationships between this factor and the CT values were not linear (Fig. 1). This greatly increased the fit of the models compared to modelling yeast:sugar ratio as a continuous factor (e.g. model R^2 for *Metchnikowin* analysis is 0.51 when yeast is modelled as continuous but 0.76 when it is modelled as ordinal). The initial model contained the sugar:yeast ratio in the larval diet as an ordinal factor, and as nominal factors whether the measure had been performed on larvae or adults, the genotype of the flies, and the identity of the rearing vial. Within-vial density of flies and the CT values of the house-keeping gene were included as continuous covariates. Housekeeping gene expression was measured to account for variation in the number of flies included in RNA extraction pools, variation in individual body size, and the efficiency of RNA extraction and cDNA synthesis. Genotype, rearing vial (nested within diet and genotype) and their interactions with other terms were treated as random factors in our statistical models. We started from models containing all the factors above and their 2- and 3-way interactions, then backward eliminated the non-significant terms ($P > 0.1$). The significance of each random factor was estimated by comparing the difference of the -2 log-likelihood of the models with and without the factor to a chi-square distribution with one degree of freedom (Pinheiro & Bates 2000; Crawley 2002). The significances of fixed factors were tested with *F* tests. Homoscedasticity and normality of the residuals were checked to comply with the model's assumptions.

Dry weight was measured using models similar to those used to measure AMP gene expression, only the response variable was raw dry weight and the CT value of the housekeeping gene was not included. As dry weight was measured in individual adults, the sex of the measured flies was added as a nominal, fixed term. For the analysis of triglyceride content, we used the logarithm of triglyceride mass divided by dry weight as the response variable, which, despite being a proportion,

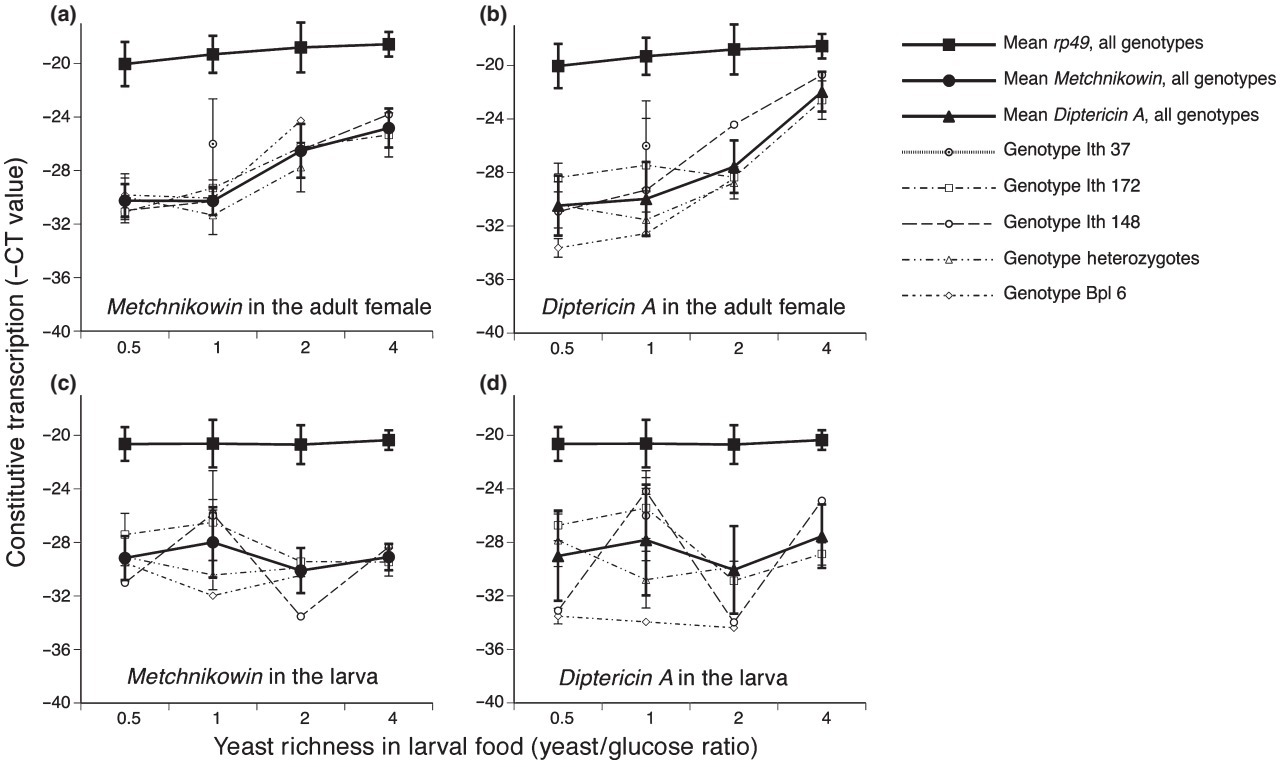

Fig. 1 Effect of larval food richness in yeast on the constitutive transcription of genes encoding two antimicrobial peptides, (a and c) *Metchnikowin* and (b and d) *Diptericin A*, and of a house-keeping gene, *rp49*, in (a and b) adult females (c and d) larvae. The *y*-axis is the qPCR cycle at which fluorescence reaches the critical threshold (the CT value). Every unit decrease in CT value indicates a two-fold increase in gene transcription. *Rp49* expression is expected to be constant over the experiment. It is measured to reflect variation in total cDNA yield in each extraction. The observed variations of larval transcription are mainly due to extraction yield differences (analysis not shown). Vertical bars are standard deviations.

had a distribution suitable for analysis with a linear model. As with dry weight, sex, but not the CT value of the house-keeping gene, was included in the analysis of triglyceride content. In order to optimize statistical power whenever it was possible, we modelled yeast: sugar ratio as continuous since the relationship between this factor and ln(triglycerides content) was linear. Indeed, modelling this factor as ordinal did not improve the fit of the model: R^2 of models with yeast as continuous or ordinal were extremely similar (0.63 and 0.64 respectively). Accordingly, we present the effect of yeast:sugar ratio on fat as a linear regression (Fig. 2c, d).

Results

Immunity

Increasing larval food richness in yeast increased the constitutive transcription of *Diptericin A* and *Metchnikowin* in the adults, but not in the larvae (Table 1, Fig. 1). When adults and larvae were analyzed separately, the monotonic increase in expression over increasing yeast was significant in adults (*Diptericin A*: $F_{3,\ 18} = 10.0$, $P < 0.001$; *Metchnikowin*: $F_{3,\ 21} = 14.8$, $P < 0.001$) but not in larvae (*Diptericin A*: $F_{3,\ 19} = 10.0$, $P = 0.35$; *Metchnikowin*: $F_{3,\ 23} = 2.12$, $P = 0.13$), resulting in the highly significant interaction effect between diet and life stage on expression (Table 1; *Diptericin A*: $F_{3,\ 24} = 5.63$, $P = 0.005$; *Metchnikowin*: $F_{3,\ 24} = 14.1$, $P < 0.001$) but weakly non-significant main effects of diet (Table 1; *Diptericin A*: $F_{3,\ 20} = 2.99$, $P = 0.056$; *Metchnikowin*: $F_{3,\ 23} = 2.44$, $P = 0.091$). Analyses excluding the highest yeast concentration gave similar results. The genotype of the fly also affected the expression of *Diptericin A*, as Bpl6 and heterozygote flies had a lower expression than Ith lines. Genotype did not significantly affect *Metchnikowin* expression. Genotype did not interact significantly with any other factor (all interaction *P* values >0.1).

General condition

Increasing larval food richness in yeast did not significantly increase the overall dry weight of adults (Table 2, Fig. 2a, b). There was a significant interaction between larval diet and sex that was mainly due to the low weight of the adult females that were fed medium

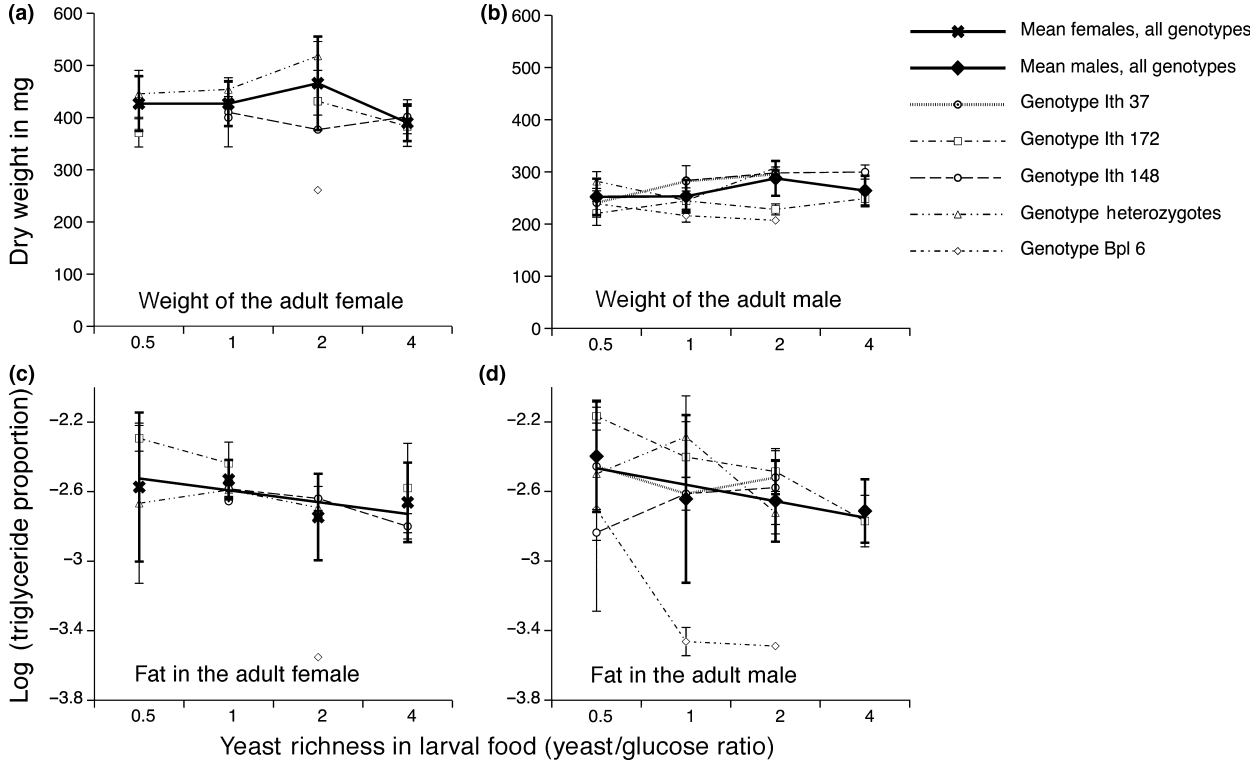

Fig. 2 Effect of larval food richness in yeast on dry weight of (a) adult females and (b) adult males and on the proportion of triglyceride (fat) in (c) adult females and (d) adult males. Proportion of triglyceride is measured as the absolute amount of triglyceride divided by the dry mass of the fly and log-transformed. The absolute amount of triglyceride shows the same decreasing pattern. We plot the linear regression between Log(yeast:sugar) and Log(Triglyceride proportion) as larval food is modelled as a continuous factor in our analysis of fat content. Vertical bars are standard deviations.

the richest in yeast (Fig. 2a). The genotypes significantly differed in their weight, and there was a significant interaction between genotype and sex (Table 2) because the size difference between males and females varied among genotypes. The identity of the vial in which the flies were reared significantly affected size (Table 2), further illustrating the sensitivity of this trait to environmental conditions.

Increasing larval food richness in yeast significantly decreased the triglyceride content of the adult flies (Table 2, Fig. 2c, d). Males and females did not differ significantly in fat content, although the genotypes did. Neither the interaction between larval diet and genotype nor the one between larval diet and sex were significant. As for dry weight, rearing vial identity significantly affected triglyceride content (Table 2).

Discussion

Adult expression of immunity genes and indicators of general health responded differently to variations of larval food composition. Increasing yeast (protein) levels in larval food increased the constitutive transcription of the antibacterial peptide genes *Diptericin A* and *Metchnikowin* in the adults, although larval expression of these genes was unaffected (Fig. 1). In contrast, adult dry weight was not substantially affected by larval food (Fig. 2a, b), and larvae that were raised with abundant yeast produced adults with slightly less fat than when food was poor in yeast (Fig. 2c, d). We therefore found no apparent covariation between immune capacity and adult general health. Although we observed some genetic variation for all traits measured, all tested genotypes were affected similarly by larval food composition. In particular, there was no interaction between genotype and larval diet for any trait studied. This suggests that the observed effect of larval diet on adult investment into immunity is a general property of *D. melanogaster*, and not an artefact specific to any particular genotype. In total, our data suggest that the effect of larval food quality on adult immune potential is not due to an indirect effect on the flies' general condition, but instead is due to specific reallocation of resources on protein-rich diets.

We do not currently know the mechanism by which increased protein availability leads to higher AMP expression, but we can envision two main hypotheses. First, if protein limitations constrain the synthesis of the antimicrobial peptides, and therefore expression of the

Table 1 Final statistical models describing antimicrobial peptide gene expression, from mixed models with the REML method. Non-significant terms ($P > 0.1$) are not shown, except for *genotype* and *rearing vial identity* that control for the non-independence of some observations

Trait	D.F. (Num, Denom)	Test-statistic	*P* value
Diptericin A transcription			
Fixed factors		*F*	
Larval diet	3, 20	2.99	0.056
Life stage	1, 23	4.03	0.057
Larval diet × Life stage	3, 24	5.63	0.005
House-keeping gene transcription	1, 43	6.73	0.013
Random factors		χ^2	
Rearing vial	1	0.12	0.73
Genotype	1	7.18	0.007
Metchnikowin transcription			
Fixed factors		*F*	
Larval diet	3, 23	2.44	0.091
Life stage	1, 24	5.16	0.032
Larval diet × Life stage	3, 24	14.1	<0.001
House-keeping gene transcription	1, 44	9.12	0.004
Random factors		χ^2	
Rearing vial	1	1.15	0.28
Genotype	1	1.07	0.30

Table 2 Final statistical models describing general condition of adult flies, from mixed models with the REML method. Non-significant terms ($P > 0.1$) are not shown, except for *genotype* and *rearing vial identity* that control for the non-independence of some observations

Trait	D.F. (Num, Denom)	Test-statistic	*P* value
Dry weight			
Fixed factors		*F*	
Larval diet	3, 17	2.14	0.133
Sex	1, 5	12.1	0.018
Larval diet × Sex	3, 99	5.18	0.002
Random factors		χ^2	
Rearing vial	1	22.0	<0.001
Genotype	1	6.63	0.010
Genotype × Sex	1	30.1	<0.001
Triglyceride content			
Fixed factors		*F*	
Larval diet [i.e. log (yeast to sugar ratio)]	1, 16	6.59	0.021
Random factors		χ^2	
Rearing vial	1	15.4	<0.001
Genotype	1	10.12	0.001

genes encoding them, then increased protein availability in the diet would allow a greater investment in AMP production. This hypothesis is supported by the recent observation by Povey *et al.* (2009) that increasing protein proportion in the food of caterpillars increases the protein levels as well as their antibacterial and phenoloxidase activities of their hemolymph. Were this the case in our system, however, we would have expected to see enhancement of the overall quality of the fly, and specifically increased mass as a function of increasing dietary protein. The fact that larval yeast content had no effect on adult mass and only small effect on fat storage (Fig. 2c, d) suggests that nutrients were not limiting in our study. A second hypothesis is that dietary manipulation during larval development affects the relative allometry of tissues in the adult, and that increased protein allowed enhancement of tissues that constitutively express AMPs. One might imagine the responsible tissue to be the fat body, which is the primary site of AMP expression in response to infection (Lemaitre & Hoffmann 2007), but the slightly negative relationship between AMP expression and fat reserves argues against they hypothesis of increased fat body as explanatory of our data. However, several other tissues, including gut epithelia, reproductive tissues and subcuticular epithelia express AMP genes (Lemaitre & Hoffmann 2007), and our data do not allow conclusions to be drawn about the relative sizes or activities of these tissues. Distinguishing between the proposed mechanistic hypotheses and identifying the potentially adaptive origin of his pattern will require further focused study.

Surprisingly, there was no effect of larval food on AMP expression in the larva (Fig. 1c, d). This is in contrast to a recent study showing that increasing protein proportion in food increases antibacterial activity in a caterpillar (Povey *et al.* 2009). Our experiment carries the caveat that adult transcription was measured in females only, whereas larval transcription was measured in unsexed pools. Since immunity is known to be different in males and female adult *D. melanogaster* (McKean & Nunney 2005; Taylor & Kimbrell 2007), it is conceivable that male and female larvae responded differently to food composition. If this were true, analyzing the sexes together would have hidden any larval sex variation and weakened the power of our experiment. However, given the very low sexual dimorphism in larvae, we believe sex-specific effects in larvae are likely to be minor, and they would not affect our main conclusions regarding the impact of larval nutrition on adult immune investment.

Our results are in contrast with previous reports on mosquitoes and damselflies, where better immunity resulted from better general condition: in both studies the immunologically strongest adults were also larger and, for damselflies, contained greater reserves of fat

(Suwanchaichinda & Paskewitz 1998; Rolff *et al*. 2004). Importantly, the finding in our study that adult immunity can be independent from general condition means that the effects of larval food on immune potential need not be trivial, and may be difficult to generally predict in the absence of empirical data specific to the study system in question. While the influence of larval food on adult immunity may sometimes be due to nutritional effects on general health, we conclude that varying larval food can also drive a plastic allocation of (sufficient) resources that affects the determination of adult traits.

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