Title: Experimental evolution demonstrates evolvability of preferential nutrient allocation to competing traits in response to chronic malnutrition.

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Abstract

Investigating the evolutionary origins of disease vulnerability is an important aspect of evolutionary medicine that strongly complements our current understanding on proximate causes of disease. Life history trade-offs mediated through evolutionary changes in resource allocation strategies could be one possible explanation to why suboptimal traits that leave bodies vulnerable to disease exist. For example, *Drosophila melanogaster* populations experimentally evolved to tolerate chronic larval malnutrition succumb to intestinal infection despite eliciting a competent immune response, owing to the loss of their intestinal integrity. Here, I test if evolved changes in resource allocation underlies this trade-off, by assaying preferential allocation of dietary protein towards growth and tissue repair in the same populations. Using two phenotypic traits: regeneration of intestinal epithelium post-pathogenic infection and body weight, I show that in accordance to the dynamic energy budget theory (DEB) dietary protein acquired during the larval phase is allocated to both growth and adult tissue repair. Furthermore, by altering the ratio of protein and carbohydrates in the larval diets I demonstrate that in comparison to the control populations, the evolved

(selected) populations differ in their protein allocation strategy towards these two traits. While the control populations stored away excess protein for tissue repair, the selected populations invested it towards immediate increase in body weight rather than towards an unanticipated tissue damage. Thus, I show how macronutrient availability can alter resistance, and provide empirical evidence that supports the 'mismatch hypothesis', wherein vulnerability to disease is proposed to stem from the differences between ancestral and current environment.

Keywords: P:C ratios, geometric framework of nutrition, smurf, *D. melanogaster*, mismatch hypothesis.

Introduction

Optimal allocation of resources, especially nutrients across important life history traits is a fundamental assumption of life-history theory, and since nutrients utilized by one trait can no longer be used for other traits, trade-offs are inevitable (Leroi, 2001; McDade, 2005; Van Straalen and Roelofs, 2006; Roff, 2007). Theoretically, when resource acquisition is constant, selection for higher fitness through efficient resource allocation should result in intermediate optimal values for various fitness traits (Stearns, 1992, Parker & Smith, 1990). However, in populations under directional selection for specific traits, resources may be preferentially reallocated in ways that increase these traits beyond their optimal value, and should lead to a concomitant decrease in resource availability for other traits (Roff & Fairbairn, 2012). Several studies, especially those involving experimental evolution, artificial selection and animal breeding have attributed fitness trade-offs amongst growth, reproduction and maintenance (somatic and immunological) to preferential reallocation of resources to a given trait (Zera & Harshman, 2001). Furthermore, researchers in evolutionary medicine now propose that such changes in resource allocation that occur through natural selection could result in traits that leave organisms vulnerable to disease. (Nesse, 2011). However, we have very little

understanding of: a) how such preferential resource allocation evolve; and b) how such evolved allocation strategies constrain optimal utilization of novel resources. In this paper, I use experimental evolution (Kawecki et al., 2012) to demonstrate how resource allocation of dietary protein evolves under nutritional stress, consequently leading to an evolutionary trade-off between tolerating chronic malnutrition and tolerating intestinal pathogens.

In animals, dietary protein function as building blocks and as an energy source for several physiological needs (Simpson & Raubenheimer, 2012), and is inevitably partitioned amongst different life-history traits. Additionally, since protein availability, acquisition and requirement considerably varies across an animals developmental stages, proteins also need to be partitioned temporally between immediate and speculative needs (Llandres et al., 2015). However, under conditions of protein scarcity animals may face dilemma in doing so, and may resolve this dilemma to some extent by preferentially allocating proteins towards traits that confer immediate fitness benefits such as growth and reproduction rather than store them for anticipatory needs (somatic maintenance, immunity and repair of pathological insults). Such plasticity in protein allocation may be even more enhanced in holometabolus insects such as *Drosophila*, where adult traits like body size, reproduction, somatic maintenance and immunity are largely determined by the dietary proteins acquired as larvae (Llandres et al., 2015). In populations facing chronic protein malnutrition over several generations, natural selection should likely favour allocation of the acquired proteins between growth and anticipatory somatic maintenance to extents that maximises Darwinian fitness (King & Roff, 2010). It might hence seem logical that such populations would allocate proteins to both growth and somatic maintenance, as an optimal solution. Nevertheless, such populations might prefer to invest more into growth rather than storage for the following reasons. Firstly, fitness benefits of investment into growth is immediately realised, while those from storing resources is speculative. Secondly, on ephemeral resources, the stress is on faster

development and maturation (Kolss et al., 2009) and hence individuals may invest all acquired resources into growth neglecting future needs. Thirdly, allocation to growth and storage could be hierarchical (Worley et al., 2003), that is allocation threshold for growth could determine when and how much proteins are redirected towards future needs. Lastly, biosynthesis and storage of protein metabolites could be physiologically costly (Bourg et al., 2017).

The replicate populations of *Drosophila melanogaster* I used for this investigation were derived from a single base population. They were reared as larvae on two dietary regimes: standard diet (six controls populations) and poor-quality diet (six selected populations) for 180 generations in an experimental evolution setup (Kolss et al., 2009). The adults in both regimes were maintained on standard diet. Over these generations the selected populations evolved increased tolerance to chronic malnutrition that were mediated through several physiological and behavioural adaptations (Kolss et al., 2009, Vijendravarma et al., 2011, Vijendravarma et al., 2012b, Vijendravarma et al., 2012c, Vijendravarma et al., 2013). However, concomitantly the selected populations suffered increased susceptibility to intestinal infection by *Pseudomonas entomophila* (Vijendravarma et al., 2015). Such trade-offs have been traditionally attributed to reallocation of nutrients required for immune functions to other life-history traits. However, when immunological responses to *P. entomophila* infection were assayed, populations from both regimes were immunologically competent to a similar extent (Vijendravarma et al., 2015). Further investigation revealed that the increased vulnerability of the selected populations to *P. entomophila* was due to their inability to maintain intestinal epithelium integrity upon pathogen-induced damage (Vijendravarma et al., 2015), possibly leading to sepsis (Rera et al., 2012). It is however unclear if reallocation of proteins required for maintaining intestinal integrity to other traits underlies this trade-off.

I aimed to understand this trade-off from a physiological perspective and determine the extent to which resource allocation contributes towards it. For this, I manipulated the amount of dietary protein available to larvae from the control and selected populations, assessed their susceptibility to *P. entomophila*, and assessed if this could be attributed to changes in their intestinal integrity (Vijendravarma et al., 2015). Since dietary protein intake is known to affect both an organism's ability to resist pathogens and its ability to repair damaged tissues (Lee et al., 2008), increasing dietary proteins should improve tolerance to *P. entomophila* and support better intestinal integrity upon infection in the control populations. If the trade-off is not mediated through protein reallocation, then one would expect the susceptibility to *P. entomophila* in our selected populations to be mitigated by the increased dietary proteins to some extent. Alternatively, the susceptibility of selected populations might remain unaltered, if protein reserves necessary for containing pathogen-induced intestinal damage is reallocated to other traits as an adaptation to chronic malnutrition. However, to rule out the possibility that selected populations underutilize the ingested proteins and to demonstrate that these proteins are indeed being reallocated elsewhere, it would be essential to screen for correlated changes in other life-history traits that might compete for the ingested proteins. Positive correlation between adult body weight and larval dietary protein has been reported in several studies (Kristensen et al., 2011), I hence tested if body weight at eclosion (a proxy for growth) competes for the excess dietary protein acquired as larvae.

Materials and methods

The experimentally evolved *D. melanogaster* populations (control and selected) and the selection regimes used to generate them are described in detail elsewhere (Kolss et al., 2009). Briefly, six control and six selected populations originated from a single base population were reared on standard larval food (15 g agar, 30 g sucrose, 60 g glucose, 12.5 g dry yeast, 50 g cornmeal, 0.5 g $MgSO_4$, 0.5 g $CaCl_2$, 30 mL ethanol, 6 mL propionic acid and

1 g nipagin per litre of water (Kolss et al., 2009) and poor larval food respectively for over 180 generations. The poor larval food contained 1/4th the amounts of sugars, cornmeal and yeast as in standard food. Adults from both regimes were maintained on standard food. The populations were maintained at 25 °C, 70 % humidity and at a density of 200 eggs/30 mL food. Prior to the assays reported below, all populations were reared on standard larval food for two generations to remove effects of maternal environment.

Adults from both regimes were allowed to oviposit on juice/ agar medium. The eggs were collected and reared at a density of 200 eggs/30 ml food, on two larval diets that differed in their protein to carbohydrate (P:C) ratio: the standard diet and the high P:C ratio diet (standard diet with 1/4th the amounts of sugars and cornmeal). Eight rearing bottles per population per diet were set-up and the eclosing females were used for three separate standardised assays: susceptibility to intestinal infection (Vodovar et al., 2005); intestinal integrity upon infection (Vijendravarma et al., 2015); and adult body weight (Vijendravarma et al., 2011). First, to assay the effect of high protein diet on susceptibility to intestinal infection in the selected and control populations, groups of 30 females per bottle from three bottles were starved for 2 hours in empty vials. The flies were then transferred to agar vials layered with a filter-paper moistened with a mixture of 70 µL of bacterial suspension (overnight culture of *P. entomophila* in Luria-Bertani broth at 1/4th dilution of OD_{600} nm ≈ 200) and 70 µL of 5 % sucrose solution, and incubated for 18 hours. The flies were then transferred to fresh vials with standard food; mortality was recorded at regular intervals until 54 hours from the onset of infection treatment. The proportion of flies alive in each treatment at the final time point was arcsine-square root-transformed and analysed with a nested ANOVA, with larval diet ('standard' vs. 'high P:C') and regime as fixed factors, and replicate population as a random factor nested within selection regime. Next, to assay intestinal integrity upon infection flies eclosing from two bottles were collected and maintained on

standard food for three days. Groups of 20 female flies per bottle were infected with *P. entomophila* as described above for 10 hours and were then transferred onto standard food containing the blue dye (2.5% w/v) for 8 hours. The proportion of individuals showing the 'smurf' phenotype (Vijendravarma et al., 2015) was subsequently recorded 10 hours later. The arcsine-square root transformed proportion of individuals showing 'smurf' was compared between the regimes and diets using nested ANOVA. Finally, to compare adult body weight on the two diets; I randomly collected groups of 12 eclosing females from three bottles per population per diet. Upon eclosion the flies were collected in Eppendorf tubes and snap frozen in liquid nitrogen. The flies were then dried at 70 °C in an oven for 3 days and then weighed as a group to the nearest microgram. The average body weight per female was calculated, log transformed and analysed using a nested ANOVA. The data were analysed using JMP (version 10) software. The factors included in the nested analysis of variance, the *F*-statistic and significance for the three traits have been tabulated in Table 1.

Results

Irrespective of being raised as larvae on standard or high P:C ratio diet females from the selected populations suffered mortality after infection to a similar extent (Fig. 1a, b; $F_{1,10} = 0.267$, $p = 0.62$). The females from the control populations on the other hand suffered slightly lesser mortality after infection when they were raised as larvae on high P:C diet than on standard food (Fig.1a; $F_{1,10} = 4.18$, $p = 0.068$). This difference was marginally significant owing to two of the six replicate control populations having similar mortality on the two diets (Fig. 1b). However, the selected populations suffered significantly higher mortality than in control populations on both diets (Fig.1; regime: $F_{1,10} = 35.07$, $p = 0.0001$; regime x diet: $F_{1,10} = 3.19$, $p = 0.105$). These differences in susceptibility to intestinal infection between control and selected populations paralleled with the extent to which their intestine's had been

damaged. After infection similar proportions of 'smurf' females were present in selected populations that were raised on the two diets (Fig. 2a; $F_{1,10} = 0.25$, $p = 0.63$). However, the control populations when reared on high P:C diets had fewer 'smurf' females than on standard diet (Fig. 2a; $F_{1,10} = 8.05$, $p = 0.018$), and as reported earlier (Vijendravarma et al., 2015) the selected populations had more 'smurf' individuals than in the control populations (Fig. 2a; regime: $F_{1,10} = 11.09$, $p = 0.008$; regime x diet: $F_{1,10} = 10.51$, $p = 0.0088$). Upon questioning whether the regimes differ in their allocation of dietary proteins to growth, I found that the selected population females reared on high P:C diets were heavier than those reared on standard food (Fig. 2b; $F_{1,10} = 19.57$, $p < 0.0001$), while the control populations showed no effect of larval diet on body weight (Fig. 2b; $F_{1,10} = 0.38$, $p = 0.54$). The selected populations were however lighter than the controls on both diets (Fig. 2b; regime: $F_{1,10} = 48.78$, $p < 0.0001$; regime x diet: $F_{1,10} = 5.68$, $p = 0.038$).

Discussion

Although existence of fitness trade-offs mediated through resource allocation and their role in shaping evolutionary trajectories is beyond doubt, how such trade-offs evolve is relatively understudied (Ng'oma et al., 2017, Roff & Fairbairn, 2012). Nevertheless, considering resource allocation amongst different functions as an evolvable trait by itself raises several interesting questions. Are optimal resource allocation preferences determined genetically or is it merely a physiological (plastic) response to the quality and quantity of resources available? What factors determine or limit resource investment amongst different traits? Can preferential resource allocation evolve in response to chronic changes in resource availability? Answers to such questions would have major implications of our understanding on how resource allocation is regulated and the evolution of ensuing life history trade-offs.

198 This study addresses the above questions to some extent by assaying the extent to
199 which dietary proteins are allocated to two competing traits: body weight at eclosion (a
200 function of growth) and maintenance of intestinal integrity upon infection (a function of
201 somatic maintenance), in response to chronic larval malnutrition over several generations
202 (Vijendravarma et al., 2015). Given that the control and selected populations do not differ in
203 their larval feeding rate (acquisition of dietary protein) (Vijendravarma et al., 2012a) the
204 classical resource acquisition-allocation model (the Y model) (van Noordwijk & de Jong,
205 1986) would attribute any changes in the two competing traits to differential protein
206 allocation. Convincingly, in contrast to the control flies the selected flies allocated the surplus
207 dietary protein to growth (Fig. 2b) rather than somatic maintenance (Fig. 2a), and
208 consequently showing no change in their susceptibility to intestinal infection (Fig. 1). This
209 study thus empirically demonstrates a nutrition-dependent context in which preferential
210 resource allocation can evolve and suggests that resource allocation as a trait must have a
211 genetic basis.

212 Investigating resource allocation in *Drosophila* a holometabolus insect provides an
213 excellent system, wherein a) resource acquisition strikingly differs between their life stages
214 (Simpson & Raubenheimer, 2012), and b) such acquired resources can be both allocated
215 within and between life stages. Two recent theoretical concepts have provided major insights
216 into the evolution of nutrient mediated trade-offs in such system: first, the dynamic energy
217 budget theory (DEB) (Llandres et al., 2015); and second the geometric framework of nutrition
218 (Simpson & Raubenheimer, 2012). This study empirically included certain aspects from the
219 above two theories: a) Proteins acquired as larvae were allocated to adult traits and b) the
220 trade-off was assayed on two diets that differed in their protein carbohydrate ratios, providing
221 a deeper understanding on how nutrients are acquired during developmental stages are
222 allocated in holometabolus insects. Furthermore, investigating experimentally evolved

223 *Drosophila* populations in this context, provided a unique opportunity to study how resource
224 allocation evolves in response to nutritional stress.

225 Animals can respond to protein scarcity both within and across generations through
226 either plastic or adaptive changes in their behavioural and physiological traits that facilitate
227 increased protein acquisition (Simpson & Raubenheimer, 2012). Likewise, the selected
228 populations investigated here have evolved increased propensity to cannibalize conspecific
229 larvae to supplement their protein requirement (Vijendravarma et al., 2013). Interestingly,
230 despite such adaptive changes, the selected populations have simultaneously evolved
231 mechanisms that allocate proteins preferentially to growth rather than storage (somatic
232 maintenance). The extent to which these populations allocate proteins to growth in preference
233 to other competing traits like reproduction in preference remains unknown, but since the
234 results here supports hierarchical allocation (Worley et al., 2003), we could speculate that
235 they do so. Furthermore, storage of nutrients might be costly (Bourg et al., 2017), explaining
236 why resources may be preferentially allocated to other traits like growth (this study) or to
237 reproduction (Simmons & Bradley, 1997). Recent findings have additionally shown that
238 organisms not only vary their allocation strategies between traits to maximise fitness in
239 response to resource availability (Clark et al., 2015), but can also evolve plasticity in doing so
240 when temporal variation in resource availability is predictable (King & Roff, 2010). Thus, the
241 evolution of preferential allocation I report here is likely to have been shaped by the selection
242 regime these flies were reared in for over 180 generations: only larvae and not adults of our
243 selected populations were reared on poor diet in the experimental evolution set-up for several
244 generations (Kolss et al., 2009).

245 The data on intestinal integrity (smruf assay) upon infection clearly demonstrates that
246 high P:C larval diet facilitated better regeneration of the gut in control but not selected
247 populations (Fig 2a), leading to increased survival only in the control populations (Fig. 1a,b).

However, while we know that other immune traits (antimicrobial peptides, ROS activity, etc.) of control and selected populations do not differ on standard diet (Vijendravarma et al., 2015), the extent to which high P:C larval diet would alter these adult immune traits specifically in our populations (Fellous & Lazzaro, 2010) needs to be assayed.

It is a new consensus in evolutionary medicine that understanding how natural selection leaves organisms vulnerable to a disease is as equally important as determining its proximate causes, to find better cures (Nesse, 2011). The vicious cycle between malnutrition and disease vulnerability is evident across several species including humans (Katona & Katona-Apte, 2009) and has been well investigated at a physiological level, yet evolutionary explanations for the same are limited. The recent 'mismatch' hypothesis, that relates vulnerability to disease to differences between ancestral and current environment (Raubenheimer et al., 2012, Godfrey et al., 2007), provides an evolutionary explanation for how maladaptive traits that leave bodies vulnerable to disease have evolved. my findings here empirically demonstrates and highlights how evolved changes in resource allocation can underlie such a mismatch and consequently lead to disease (Rauw, 2012, Raubenheimer et al., 2012). This study provides insights on evolutionary basis of human intestinal disorders like Chron's disease, and possibly explains how inclusion of modern diets in certain countries might be leading to the rapid change observed in the epidemiology of Chron's disease worldwide (Alhagamhmad et al., 2015).

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276 **Table 1:**

Factor	Denominator MS	*d.f.*	Survival	Smurf	Body weight
Both regimes					
Regime	Population	1,10	35.067 ***	11.087 **	48.769 ***
Diet	Population X Diet	1,10	7.731 *	2.678	9.835 *
Regime x diet	Population X Diet	1,10	3.186	10.511 **	5.684 *
Control regime					
Diet	error	1,34	4.178 †	8.051 *	0.38
Selected regime					
Diet	error	1,34	0.267	0.2482	19.57 ***

277 †p < 0.1, *p < 0.05, **p < 0.01, ***p < 0.001; all the remaining P > 0.1.

278 **Table 1.** Summary of analysis of variance (*F*-Statistic and its significance) on the three traits,
279 analysed jointly for both regimes and separately for each regime. For *F*-tests on both regimes
280 *d.f.* = 1, 10; population is a random factor nested within the selection regime; tests for
281 population and its interactions are not reported.

282

Figures

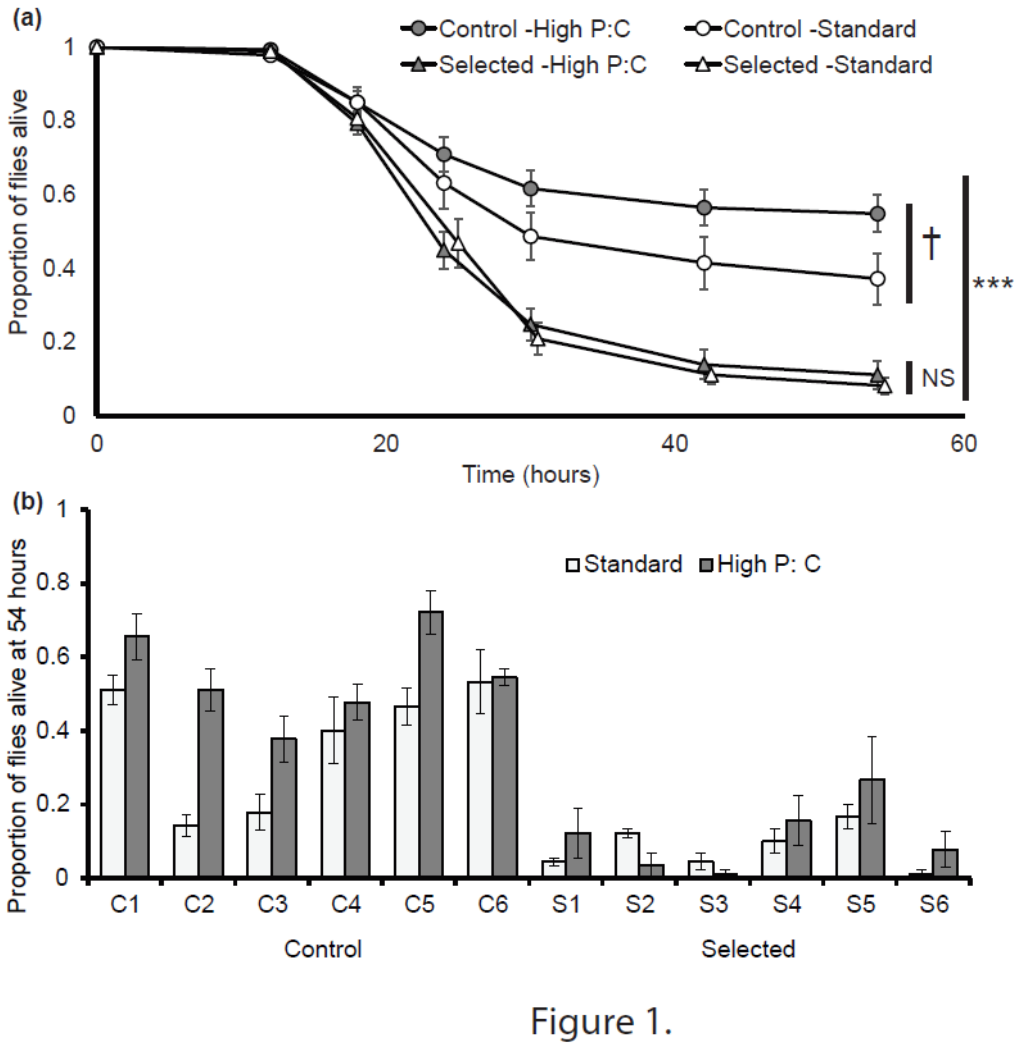

Figure 1.

Figure 1: Effect of high P:C larval diet on adult susceptibility to *P. entomophila* intestinal infection in *Drosophila* populations adapted to chronic malnutrition. **(a)** Survival curves of control (circle symbols) and selected (triangle symbols) populations upon infection with *P. entomophila*, when reared on standard (open symbols) and high P:C (closed symbols) larval diets; each data point indicates the mean ± SE of six independent replicate populations evolved under each regime. Flies were fed *P. entomophila* until 18 hours and subsequently maintained on standard diet for rest of the assay. **(b)** Number of females surviving 54 hours after onset of infection (last time-point in the survival curve above); mean ± SE of six

independent replicate populations evolved under each regime on standard (light bar) and high P:C (dark bar) larval diets. ***P < 0.001; †P < 0.1; ns: P > 0.1.

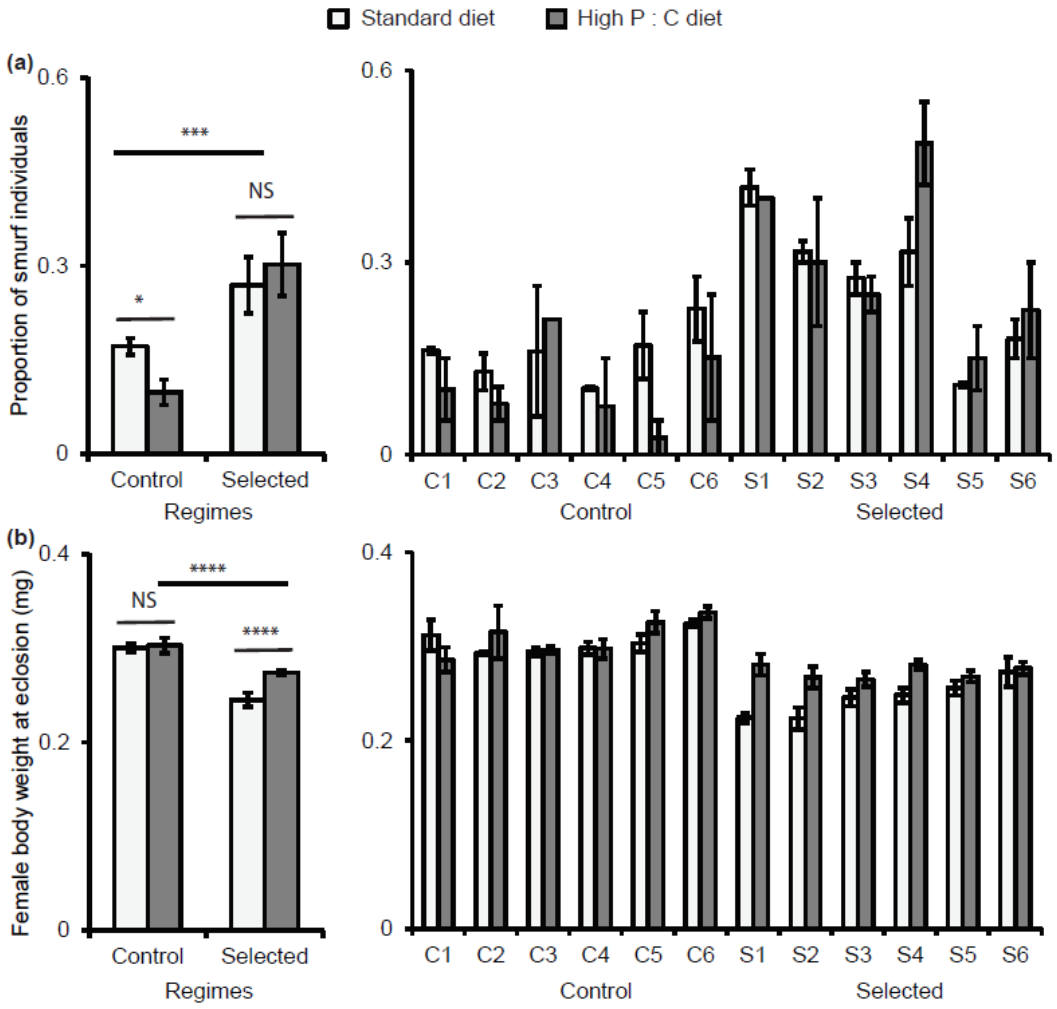

Figure 2.

Figure 2: Effect of high P:C larval diet on adult body weight and infection mediated intestinal dysfunction in *Drosophila* populations adapted to chronic malnutrition. **(a)** The proportion of smurf flies (individuals with loss of gut wall integrity upon infection) in control and selected populations when reared as larvae on standard (light bar) and high P:C (dark bar) diets. **(b)** Female body weight at eclosion in control and selected populations when reared as larvae on

302 standard (light bar) and high P:C (dark bar) diets. Each data point in ‘a’ and ‘b’ indicates the
303 mean of the six replicate populations ± SE based on variation among populations within the
304 regime and is presented in the respective adjacent panels. ***P < 0.001, **P < 0.01, *P <
305 0.05, NS: P > 0.1.

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