

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

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7

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11 **Abstract**

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

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

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

## 34 **Introduction**

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

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

55 In animals, dietary protein function as building blocks and as an energy source for  
56 several physiological needs (Simpson & Raubenheimer, 2012), and is inevitably partitioned  
57 amongst different life-history traits. Additionally, since protein availability, acquisition and  
58 requirement considerably varies across an animals developmental stages, proteins also need to  
59 be partitioned temporally between immediate and speculative needs (Llandres et al., 2015).  
60 However, under conditions of protein scarcity animals may face dilemma in doing so, and  
61 may resolve this dilemma to some extent by preferentially allocating proteins towards traits  
62 that confer immediate fitness benefits such as growth and reproduction rather than store them  
63 for anticipatory needs (somatic maintenance, immunity and repair of pathological insults).  
64 Such plasticity in protein allocation may be even more enhanced in holometabolus insects  
65 such as *Drosophila*, where adult traits like body size, reproduction, somatic maintenance and  
66 immunity are largely determined by the dietary proteins acquired as larvae (Llandres et al.,  
67 2015). In populations facing chronic protein malnutrition over several generations, natural  
68 selection should likely favour allocation of the acquired proteins between growth and  
69 anticipatory somatic maintenance to extents that maximises Darwinian fitness (King & Roff,  
70 2010). It might hence seem logical that such populations would allocate proteins to both  
71 growth and somatic maintenance, as an optimal solution. Nevertheless, such populations  
72 might prefer to invest more into growth rather than storage for the following reasons. Firstly,  
73 fitness benefits of investment into growth is immediately realised, while those from storing  
74 resources is speculative. Secondly, on ephemeral resources, the stress is on faster

75 development and maturation (Kolss et al., 2009) and hence individuals may invest all  
76 acquired resources into growth neglecting future needs. Thirdly, allocation to growth and  
77 storage could be hierarchical (Worley et al., 2003), that is allocation threshold for growth  
78 could determine when and how much proteins are redirected towards future needs. Lastly,  
79 biosynthesis and storage of protein metabolites could be physiologically costly (Bourg et al.,  
80 2017).

81         The replicate populations of *Drosophila melanogaster* I used for this investigation  
82 were derived from a single base population. They were reared as larvae on two dietary  
83 regimes: standard diet (six controls populations) and poor-quality diet (six selected  
84 populations) for 180 generations in an experimental evolution setup (Kolss et al., 2009). The  
85 adults in both regimes were maintained on standard diet. Over these generations the selected  
86 populations evolved increased tolerance to chronic malnutrition that were mediated through  
87 several physiological and behavioural adaptations (Kolss et al., 2009, Vijendravarma et al.,  
88 2011, Vijendravarma et al., 2012b, Vijendravarma et al., 2012c, Vijendravarma et al., 2013).  
89 However, concomitantly the selected populations suffered increased susceptibility to  
90 intestinal infection by *Pseudomonas entomophila* (Vijendravarma et al., 2015). Such trade-  
91 offs have been traditionally attributed to reallocation of nutrients required for immune  
92 functions to other life-history traits. However, when immunological responses to *P.*  
93 *entomophila* infection were assayed, populations from both regimes were immunologically  
94 competent to a similar extent (Vijendravarma et al., 2015). Further investigation revealed that  
95 the increased vulnerability of the selected populations to *P. entomophila* was due to their  
96 inability to maintain intestinal epithelium integrity upon pathogen-induced damage  
97 (Vijendravarma et al., 2015), possibly leading to sepsis (Rera et al., 2012). It is however  
98 unclear if reallocation of proteins required for maintaining intestinal integrity to other traits  
99 underlies this trade-off.

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

## 119 **Materials and methods**

120 The experimentally evolved *D. melanogaster* populations (control and selected) and  
121 the selection regimes used to generate them are described in detail elsewhere (Kolss et al.,  
122 2009). Briefly, six control and six selected populations originated from a single base  
123 population were reared on standard larval food (15 g agar, 30 g sucrose, 60 g glucose, 12.5 g  
124 dry yeast, 50 g cornmeal, 0.5 g MgSO<sub>4</sub>, 0.5 g CaCl<sub>2</sub>, 30 mL ethanol, 6 mL propionic acid and

125 1 g nipagin per litre of water (Kolss et al., 2009) and poor larval food respectively for over  
126 180 generations. The poor larval food contained 1/4<sup>th</sup> the amounts of sugars, cornmeal and  
127 yeast as in standard food. Adults from both regimes were maintained on standard food. The  
128 populations were maintained at 25 °C, 70 % humidity and at a density of 200 eggs/30 mL  
129 food. Prior to the assays reported below, all populations were reared on standard larval food  
130 for two generations to remove effects of maternal environment.

131 Adults from both regimes were allowed to oviposit on juice/ agar medium. The eggs  
132 were collected and reared at a density of 200 eggs/30 ml food, on two larval diets that differed  
133 in their protein to carbohydrate (P:C) ratio: the standard diet and the high P:C ratio diet  
134 (standard diet with 1/4<sup>th</sup> the amounts of sugars and cornmeal). Eight rearing bottles per  
135 population per diet were set-up and the eclosing females were used for three separate  
136 standardised assays: susceptibility to intestinal infection (Vodovar et al., 2005); intestinal  
137 integrity upon infection (Vijendravarma et al., 2015); and adult body weight (Vijendravarma  
138 et al., 2011). First, to assay the effect of high protein diet on susceptibility to intestinal  
139 infection in the selected and control populations, groups of 30 females per bottle from three  
140 bottles were starved for 2 hours in empty vials. The flies were then transferred to agar vials  
141 layered with a filter-paper moistened with a mixture of 70 µL of bacterial suspension  
142 (overnight culture of *P. entomophila* in Luria-Bertani broth at 1/4<sup>th</sup> dilution of OD<sub>600</sub> nm ≈  
143 200) and 70 µL of 5 % sucrose solution, and incubated for 18 hours. The flies were then  
144 transferred to fresh vials with standard food; mortality was recorded at regular intervals until  
145 54 hours from the onset of infection treatment. The proportion of flies alive in each treatment  
146 at the final time point was arcsine-square root-transformed and analysed with a nested  
147 ANOVA, with larval diet ('standard' vs. 'high P:C') and regime as fixed factors, and replicate  
148 population as a random factor nested within selection regime. Next, to assay intestinal  
149 integrity upon infection flies eclosing from two bottles were collected and maintained on

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

163

## 164 **Results**

165 Irrespective of being raised as larvae on standard or high P:C ratio diet females from the  
166 selected populations suffered mortality after infection to a similar extent (Fig. 1a, b;  $F_{1,10} =$   
167  $0.267, p = 0.62$ ). The females from the control populations on the other hand suffered slightly  
168 lesser mortality after infection when they were raised as larvae on high P:C diet than on  
169 standard food (Fig. 1a;  $F_{1,10} = 4.18, p = 0.068$ ). This difference was marginally significant  
170 owing to two of the six replicate control populations having similar mortality on the two diets  
171 (Fig. 1b). However, the selected populations suffered significantly higher mortality than in  
172 control populations on both diets (Fig. 1; regime:  $F_{1,10} = 35.07, p = 0.0001$ ; regime x diet:  
173  $F_{1,10} = 3.19, p = 0.105$ ). These differences in susceptibility to intestinal infection between  
174 control and selected populations paralleled with the extent to which their intestine’s had been

175 damaged. After infection similar proportions of ‘smurf’ females were present in selected  
176 populations that were raised on the two diets (Fig. 2a;  $F_{1,10} = 0.25$ ,  $p = 0.63$ ). However, the  
177 control populations when reared on high P:C diets had fewer ‘smurf’ females than on standard  
178 diet (Fig. 2a;  $F_{1,10} = 8.05$ ,  $p = 0.018$ ), and as reported earlier (Vijendravarma et al., 2015) the  
179 selected populations had more ‘smurf’ individuals than in the control populations (Fig. 2a;  
180 regime:  $F_{1,10} = 11.09$ ,  $p = 0.008$ ; regime x diet:  $F_{1,10} = 10.51$ ,  $p = 0.0088$ ). Upon questioning  
181 whether the regimes differ in their allocation of dietary proteins to growth, I found that the  
182 selected population females reared on high P:C diets were heavier than those reared on  
183 standard food (Fig. 2b;  $F_{1,10} = 19.57$ ,  $p < 0.0001$ ), while the control populations showed no  
184 effect of larval diet on body weight (Fig. 2b;  $F_{1,10} = 0.38$ ,  $p = 0.54$ ). The selected populations  
185 were however lighter than the controls on both diets (Fig. 2b; regime:  $F_{1,10} = 48.78$ ,  $p <$   
186  $0.0001$ ; regime x diet:  $F_{1,10} = 5.68$ ,  $p = 0.038$ ).

## 187 **Discussion**

188 Although existence of fitness trade-offs mediated through resource allocation and their  
189 role in shaping evolutionary trajectories is beyond doubt, how such trade-offs evolve is  
190 relatively understudied (Ng’oma et al., 2017, Roff & Fairbairn, 2012). Nevertheless,  
191 considering resource allocation amongst different functions as an evolvable trait by itself  
192 raises several interesting questions. Are optimal resource allocation preferences determined  
193 genetically or is it merely a physiological (plastic) response to the quality and quantity of  
194 resources available? What factors determine or limit resource investment amongst different  
195 traits? Can preferential resource allocation evolve in response to chronic changes in resource  
196 availability? Answers to such questions would have major implications of our understanding  
197 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).

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

252 It is a new consensus in evolutionary medicine that understanding how natural  
253 selection leaves organisms vulnerable to a disease is as equally important as determining its  
254 proximate causes, to find better cures (Nesse, 2011). The vicious cycle between malnutrition  
255 and disease vulnerability is evident across several species including humans (Katona &  
256 Katona-Apte, 2009) and has been well investigated at a physiological level, yet evolutionary  
257 explanations for the same are limited. The recent ‘mismatch’ hypothesis, that relates  
258 vulnerability to disease to differences between ancestral and current environment  
259 (Raubenheimer et al., 2012, Godfrey et al., 2007), provides an evolutionary explanation for  
260 how maladaptive traits that leave bodies vulnerable to disease have evolved. my findings here  
261 empirically demonstrates and highlights how evolved changes in resource allocation can  
262 underlie such a mismatch and consequently lead to disease (Rauw, 2012, Raubenheimer et al.,  
263 2012). This study provides insights on evolutionary basis of human intestinal disorders like  
264 Chron’s disease, and possibly explains how inclusion of modern diets in certain countries  
265 might be leading to the rapid change observed in the epidemiology of Chron’s disease  
266 worldwide (Alhagamhmad et al., 2015).

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274

275

276 **Table 1:**

| Factor                 | Denominator MS    | <i>d.f.</i> | Survival   | Smurf     | Body weight |
|------------------------|-------------------|-------------|------------|-----------|-------------|
| <i>Both regimes</i>    |                   |             |            |           |             |
| 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 *     |
| <i>Control regime</i>  |                   |             |            |           |             |
| Diet                   | error             | 1,34        | 4.178 †    | 8.051 *   | 0.38        |
| <i>Selected regime</i> |                   |             |            |           |             |
| 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

283 **Figures**

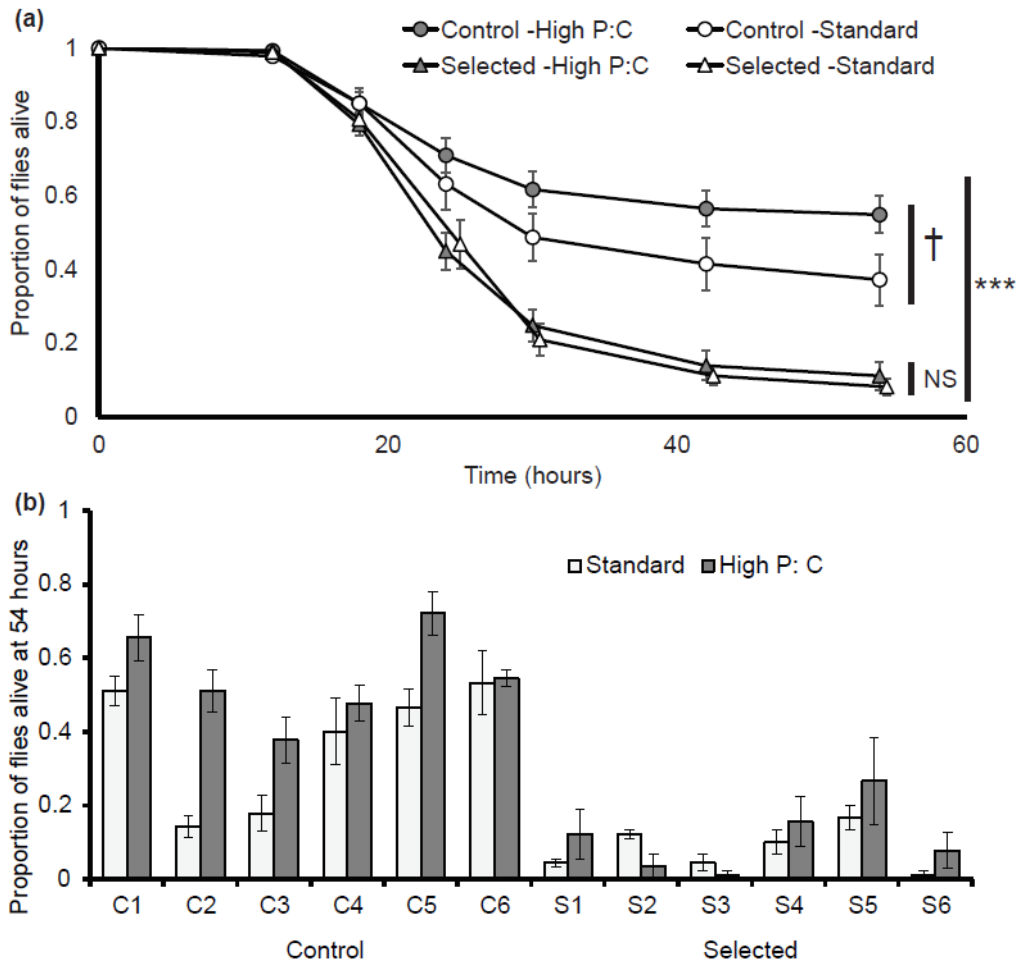


Figure 1.

284

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

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

295

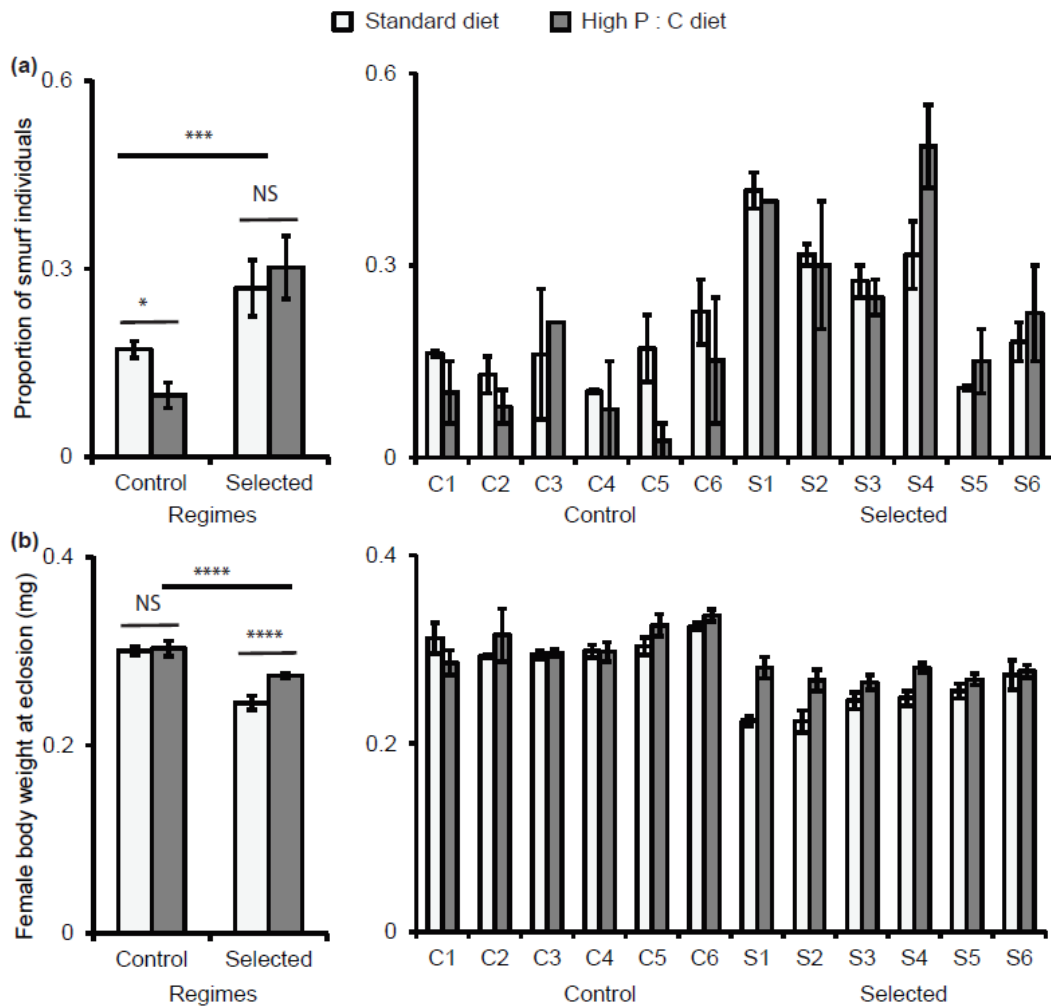


Figure 2.

296

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

306



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