Evolution of foraging behaviour in response to chronic malnutrition in *Drosophila melanogaster*

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ABSTRACT

Chronic exposure to food of low quality may exert conflicting selection pressures on foraging behaviour. On the one hand, more active search behaviour may allow the animal to find patches with slightly better, or more, food; on the other hand, such active foraging is energetically costly and thus may be opposed by selection for energetic efficiency. Here we test these alternative hypotheses in *Drosophila* larvae. We show that populations which experimentally evolved improved tolerance to larval chronic malnutrition have shorter foraging path length than unselected control populations. A behavioural polymorphism in foraging path length (the rover-sitter polymorphism) exists in nature and is attributed to the *foraging* locus (*for*). We show that a sitter strain (for^{s2}) survives better on the poor food than the rover strain for^{R}, confirming that the sitter foraging strategy is advantageous under malnutrition. Larvae of the selected and control populations did not differ in global *for* expression. However, a quantitative complementation test suggests that the *for* locus may have contributed to the adaptation to poor food in one of the selected populations, either through a change in *for* allele frequencies, or by interacting epistatically with alleles at other loci. Irrespective of its genetic basis, our results provide two independent lines of evidence that sitter-like foraging behaviour is favoured under chronic larval malnutrition.

Keywords: competition, experimental evolution, feeding, nutritional stress, PKG, rover-sitter, starvation.

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1. INTRODUCTION

Food that animals consume is ultimately metabolized to release energy for survival and reproduction; however, foraging for food is itself energetically expensive and risky, and the optimal balance between the benefits and costs is likely to be environment-dependent. Foraging success and efficiency may be affected by metabolic and physiological traits [1, 2], but in particular depend on behaviour. One of the main aspects of foraging behaviour is whether and when to leave the currently exploited food patch and look for potentially greener pastures [3]. Here we focus on the mobility aspect of foraging in *Drosophila* larvae in the context of chronic nutritional stress.

Drosophila melanogaster larvae may face different forms of nutritional stress during their development on ephemeral habitats in nature, which can be to some degree imitated under laboratory conditions. When reared at high densities, *Drosophila* larvae experience a temporally variable food source: as a result of intraspecific competition and accumulation of waste products, the initially rich and plentiful food deteriorates in both quality and quantity as the larvae develop. Larvae must crawl longer distances around other larvae, drowned pupae and adults to reach a nearby food patch. Additionally, high competition favours the ability to find the patches that still remain relatively underexploited or unpolluted. It is thus not surprising that *Drosophila* populations maintained under high larval density over multiple generations evolve increased mobility while foraging, compared to populations reared at low-densities [4]. Other feeding-associated behaviours favoured under larval crowding include an increase in larval competitive ability and a higher feeding rate [5, 6].

A difference in mobility during foraging has been reported between natural allelic variants at the gene *foraging* (*for*) encoding the PKG (cGMP-dependent protein kinase) [7, 8]. Larvae with the rover (for^R) allele travel significantly longer distances while feeding on yeast paste and move more between food patches than larvae that are homozygous for the sitter (for^S) allele. However, even though this difference in path lengths of these natural variants is consistently observed on high- and low quality food, they do not differ in their rate of locomotion in the absence of food [7-11]. The *for* locus also contributes to the difference in foraging path lengths among

populations experimentally evolved under high and low densities [4]. In natural populations the polymorphism is presumably maintained by density- and frequency-dependent selection operating at the larval stage [12]. Taken together, those results consistently indicate that crowded conditions on nutritionally rich food favour greater mobility while foraging in *Drosophila* larvae.

In this study, we investigated if a change in the mobility aspect of larval foraging behaviour in *D.melanogaster* is favoured by another form of nutritional stress: chronic malnutrition under a low larval density. In our malnutrition regime, larvae are reared on a food medium that is extremely poor in nutrition, such that larvae not adapted to those conditions survive poorly, take twice as long to develop from egg to pupa, and the resulting adults are less than half the weight of flies reared on standard food [13, 14]. However, this poor food is available at a large quantity, such that the total energetic content per larva is at least ten times the energetic content of a large pre-pupation larva [14]. This chronic malnutrition scenario is thus in several ways different from larval crowding implemented in the studies cited above: food is poor from the start and does not deteriorate further in quality as rapidly as under the larval crowding regime. Because larval density is low, the concentration of metabolic waste likely remains low compared to the crowded regimes.

We hypothesized that these poor food conditions would result in conflicting selection pressures on larval foraging mobility. On the one hand, one could expect that greater mobility would allow the larvae to better exploit heterogeneities in food quality, resulting from both the initial imperfection of food homogenization and from the foraging activity of other larvae. This, as with larval crowding, would favour rover-like phenotypes. On the other hand, locomotion in *Drosophila* larvae is energetically expensive [15], so selection for metabolic efficiency would favour sitter-like phenotypes, which move less while feeding, and thus conserve energy.

To test these alternative hypotheses, we first studied the foraging-related mobility (i.e., the foraging path length) in six *D.melanogaster* populations which, in the course of over 80 generations of experimental evolution, have become adapted to the chronic malnutrition conditions. As the control we used six unselected populations originally derived from the same base population [13]. Because the malnutrition-tolerant populations turned out to have a shorter foraging path, we independently verified the adaptive significance of this trait on the poor food by comparing viability and developmental rate of a rover (for^{R}) and a sitter (for^{s2}) strain on both

poor and standard food. In parallel to the selected (i.e., malnutrition-tolerant) populations [13], we expected the sitter strain to show higher viability than the rover strain on the poor food, and faster development on both food types. Furthermore, increased expression of at least some *for* transcripts induces longer foraging path [7] and other rover-like behaviours [16, 17]. Reduced *for* expression is thus one possible mechanism mediating the evolution of shorter foraging path in our malnutrition-tolerant selected populations. To address this possibility, we compared larval *for* expression between the selected and control populations using quantitative RT-PCR. Finally, we used a quantitative complementation test [18] on one selected and one control population in an attempt to verify if the *for* locus contributes to the difference in malnutrition tolerance between them. A failure to complement would either indicate that the phenotypic difference between populations is at least in part owing to different allele frequency at the *for* locus, or that the *for* locus interacts epistatically with loci responsible for the difference [18].

2. MATERIALS AND METHODS

(a) Fly stocks and maintenance

Six replicate *D. melanogaster* populations originating from a single base population were maintained on poor larval food for over 80 generations; six control populations derived from the same base population were concurrently maintained on standard 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) [13]. The poor larval food contained 25% of the amounts of sugars, yeast and cornmeal of the standard food. Adult flies from both regimes upon emergence were always maintained on standard food with supplemented yeast. Prior to the assays described below, all populations were reared for two to three generations on standard food to eliminate maternal effects.

To minimize differences in genetic background between rover and sitter strains, we used the natural rover strain (for^R) isolated in Toronto and sitter mutant (for^{s2}) strain generated on rover genetic background [7, 19]; both strains were maintained on standard food. All populations were maintained and assays were performed at 25ºC, 70% humidity.

(b) Larval foraging assay

The length of the path traversed by larvae while feeding on yeast paste was measured for six control and six selected populations after 84 generations of selection using the method described in [9]. Briefly, eggs from the control and selected populations were collected and reared on standard food for 92 ± 2 hours at the density of approximately 200-250 larvae per bottle containing 30 ml of standard food. The foraging larvae were collected, rinsed with water and individually placed on a thin layer of yeast paste (2:1 ratio by weight of water : live yeast) filled in a shallow circular well (0.5 mm depth and 85 mm diameter) and covered with Petri dish lids. Each larva was allowed to feed the yeast paste for 5 minutes and the path traversed by the larvae was traced on to a transparency sheet. The traced foraging path of larvae was scanned and digitized using a custom made software to calculate the distance traversed (path-length in pixels) for each larva. The experiment was split into four blocks and per block path-lengths for 10 larvae per population (12 lines in total) were measured; 480 larvae were assayed in total.

To exclude any differences in larval locomotion that might confound the measurements of foraging behaviour described above we measured the path traversed by larvae from control and selected populations in the absence of food after 86 generations of selection. Larvae were raised in the same way and of the same age as in the foraging assay. Ten foraging larvae per population were used for this assay. Each larva was placed in an individual 5 cm Petri plate lined with 2% agar and its movement was traced for 2 minutes. The path traversed was scanned and its length digitally measured. The foraging path-length and locomotion path-length data was analysed using mixed-model ANOVA in JMP 8. The selection regime was included as a main effect while the replicate populations were nested within the selection regime and was treated as a random effect in the model; block was treated as a random effect for the foraging data.

(c) *for* polymorphism and larval malnutrition

To compare the fitness of rover and sitter strains, which differ in their foraging strategies, on our standard and poor food we used a rover strain homozygous for a natural allele for^R derived from a natural population, and a sitter strain homozygous for allele for^{s2}, a mutant derived from the for^R genetic background [7, 19]. Eight bottles were set-up for each strain; four with 30ml of standard food and four with equal volume of poor food, each bottle was seeded with 200 eggs. The 16 bottles were incubated and the number of flies emerging in each bottle was recorded every 24 hours. Egg-to-adult viability and the developmental rate for each strain/ food vial were

calculated. We compared the viability and developmental rate of for^R and for^{s2} on both diets (standard and poor) using a two-way ANOVA.

(d) *for* gene expression

We used quantitative RT-PCR to compare the pooled relative expression of *for* transcripts in control and selected populations (after 123 generations of selection). Larvae were raised in the same way and were of the same age as in the foraging assay. Two groups of 25 larvae per population from two replicate bottles were collected and snap frozen within 1.5 ml eppendorf tubes containing ~15-20 glass beads. These larvae were homogenized at 4°C in a tissue homogenizer and its total RNA was extracted using RNA kit (RNeasy mini kit, Qiagen). The quality and quantity of the RNA extracted was estimated on an Agilent Bioanalyzer 2100. Reverse transcription (cDNA synthesis) was performed in a 10 µl reaction using 1µl total RNA (0.6ng) using Supercript II (Invitrogen). The cDNA templates were then diluted to 1/10 in water (milliQ).

The quantitative PCR was performed using Sybr GREEN I (Invitrogen) on Applied Biosystems 7900HT SDS. The primers used (forward: 5'-GTA GAG CTG GTC CAA ACT AAT GG-3', reverse: 5'- AAC TCT CCA TCA GCA TGT ACA GG-3') amplify a 202 bp fragment in the catalytic domain (2L:3623601..3623803) common to all known transcripts of the *for* gene (Aaron Allen and Marla B. Sokolowski, personal communication; www.flybase.org). We used three reference genes (a) *rp49* (forward: 5'-GAC GCT TCA AGG GAC AGT ATC TG-3' , reverse 5'-AAA CGC GGT TCT GCA TGA G -3') [20], (b) *ef1α* (forward 5'-GTG AAG CAG CTG ATC GTT GGT-3' ,reverse 5'-CAT AAC GGG CCT CGC TGT AT -3') (U. Friedrich, unpublished) and (c) *actin5c* (forward 5'-CAA GTG CGA GTG GTG GAA GTT-3', reverse 5'-GCA GGT GGT TCC GCT CTT T-3') [21].Three technical replicates were set-up on the 384-well PCR plate for each of the two biological samples and each gene.

The cycle threshold values (Ct) were analysed with qBasePLUS (Biogazelle). The primer efficiency of all primer pairs were >1.98 and were hence set to a default value of 2. The Normalized Relative Quantities (NRQ's) for each biological replicate were calculated by the software using average Ct values across the technical replicates, relative to the three reference genes. One biological replicate for control population C6 was excluded from the analysis as its

expression levels were 11-fold higher than all other samples. The log-transformed NRQ's of the control and selected populations were compared using a mixed-model ANOVA as described for the foraging and locomotion assays above.

(e) Quantitative complementation assay

The design of the quantitative complementation followed [18]. We crossed virgin females from one control population (C1) and one selected population (S1) with males from either the rover strain for^R or the sitter strain for^{s2}, and assayed the viability of the resulting offspring on the poor food. for^{s2} is a recessive allele, characterized by a highly reduced PKG activity [7, 16]. Therefore, a smaller phenotypic difference between C1 × for^R and S1 × for^R crosses than between C1 × for^{s2} and S1 × for^{s2} crosses would imply either that some alleles at the *for* locus contribute to the divergence between C1 and S1 populations, or that the *for* locus interacts epistatically with other loci responsible for that divergence [18].

The quantitative complementation assay was carried out after 96 generations of selection. In addition to the four crosses discussed above, we assayed in parallel the four parental lines (i.e., C1, S1, for^R and for^{s2}). This was performed in two blocks two generations apart; for each block four bottles with 30ml poor food were set up per parental strain or cross. Each bottle was seeded with 200 eggs. The number of flies emerging in each bottle was recorded every day. Egg-to-adult viability for each parental strain or cross was calculated as the proportion of eggs that survived to produce an adult. The proportions were arcsine-square root transformed. The complementation hypothesis was tested with an ANOVA as the interaction between the maternal strain (selected or control) and the paternal strain (sitter or rover). Additional planned comparisons were carried out as contrasts; block effect was included as a random factor.

3. RESULTS

(a) Larval foraging assay

Larvae from the selected populations traced on average a shorter path while feeding on yeast paste than larvae from the control populations (Fig. 1a; $F_{1,10} = 8.7$, $p = 0.015$), although there was significant variation among replicate populations ($F_{10,465} = 3.8$, $p < 0.0001$). In particular, four of the selected populations consistently showed very short foraging path, while the means of the

remaining selected populations (S2 and S3) overlapped with the range of variation of the control lines (Fig. 1a). In contrast, the path-length traversed by larvae in absence of food, (i.e. on agar surface) did not differ between the selection regimes (Fig. 1b) ($F_{1,10} = 1.1$, $p = 0.33$), although there was variation among populations ($F_{10,108} = 2.7$, $p = 0.005$). A significant block effect was found in the foraging assay ($F_{3,465} = 8.1$, $p < 0.0001$).

(b) *for* polymorphism and larval malnutrition

On poor food the sitter strain for^{s2} had a higher egg to adult viability than the rover strain for^{R} (Fig. 2a; $F_{1,6} = 9.4$, $p = 0.02$), while the reverse was true on the standard food ($F_{1,6} = 47.0$, $p = 0.0005$), indicating a strong genotype × environment interaction ($F_{1,12} = 25.4$, $p = 0.0003$). The sitter strain developed more slowly than the rover strain on both food types (Fig. 2b; poor food: $F_{1,6} = 17.9$, $p = 0.006$; standard food: $F_{1,6} = 71.4$, $p<0.0001$; interaction $F_{1,12} = 0.032$, $p = 0.86$).

(c) *for* gene expression

Reduction in *for* expression in the selected populations was one hypothetical mechanisms underlying their sitter-like foraging. We found no support for it; if anything, the expression of pooled *for* transcripts tended to be higher in selected than in control populations ($F_{1,10} = 3.1$, $p = 0.11$, Fig. 1c). Even though there was significant variation among populations within both regimes ($F_{10,23} = 4.6$, $p = 0.009$), it was not correlated across populations with the foraging path ($r = -0.4$, $p = 0.10$).

(d) Quantitative complementation assay

The pattern of egg-to adult viability on poor food in the four crosses involved in the quantitative complementation test (Fig. 3a) qualitatively conformed to the expectation under failure to complement. There was an interaction between the maternal strain (selected versus control) and the *for* allele ($F_{1,27} = 9.2$, $p = 0.005$). F1 offspring of the selected population crossed with sitter strain for^{s2} had a significantly higher viability than progeny of control population crossed with sitter strain for^{s2} ($F_{1,27} = 32.7$, $p<0.0001$). However, no difference in viability was found between the progeny of the selected and the control population crossed with the rover strain for^{R} ($F_{1,27} = 2.1$, $p = 0.16$). This pattern was observed in both blocks; the block effect was not significant ($F_{1,27} = 0.2$, $p = 0.63$).

Nonetheless, the differences in viability among the crosses were much smaller than the difference (assayed in parallel) between the selected and the control line, or between the sitter and the rover strain (Fig. 3b). All crosses survived as well, or almost as well, as the selected line, and clearly better than the control line or the rover strain (Fig 3a, b). Again, this was observed in both blocks of the experiment.

The egg-to-adult viability of the four parental strains (Fig. 3b) confirmed previous findings. The sitter strain for^{s2} had higher viability than the rover strain for^{R} on poor food ($F_{1,27} = 113$, $p < 0.0001$) and as expected the selected population had higher viability than the control population ($F_{1,27} = 158$, $p < 0.0001$; Fig 3b). The block effect was significant ($F_{1,27} = 21.4$, $p < 0.0001$).

4. DISCUSSION

We demonstrate here the evolution of a shorter foraging path in four of our six selected *D.melanogaster* populations which had adapted to chronic larval malnutrition, paralleling the well-described sitter phenotype [16, 19]. Consistent with this result, a sitter strain homozygous for the for^{s2} allele showed higher viability on the poor food than the rover strain for^{R}, while the reverse was true on the standard food. Thus, two independent lines of evidence indicate that sitter-like behaviour is favoured under our poor food regime, possibly because it is more energy efficient: locomotion is energetically costly, and less food is ingested while the larva roves. The poor food medium is initially fairly homogeneous, and even though some heterogeneity presumably arises as a result of larval feeding and excretion, it is apparently not large enough to compensate for the costs of search [15].

Our results seem to contradict an earlier study where rovers survived better under poor food conditions [16]. It is difficult to identify the reasons for this apparent discrepancy, as the composition of the media used in that study differs from that used here. In particular, our poor food contained less than half of the lowest yeast concentration used in [16] (3.1 versus 7.5 g per litre). Physiological studies [16, 22, 23] throw some light on the complex relationship between nutritional level and the rover-sitter foraging polymorphism. On good food rovers have higher glucose absorption rate despite having lower feeding rate than the sitters. On poorer foods both rovers and sitters increase their feeding rate to a common maximum where rovers in addition maintain their higher absorption rates [16]. However, when subjected to short bouts of starvation,

rovers show a greater reduction in blood (haemolymph) sugar than sitters, and when allowed to feed again rovers had reduced feeding rate and slower recovery than the sitters [24]. Possibly, the importance of these pleiotropic effects of the rover-sitter polymorphism depends in a subtle way on the details of the food composition and other experimental conditions, tipping the balance in favour of one or another strain for reasons independent of the difference in foraging behaviour.

Enhanced *for* expression leads to longer foraging path [7], and natural rover genotypes show a higher *for* PKG activity in larval bodies [16]. Hence, a reduced expression of *for* might have been one candidate mechanism mediating the shorter foraging path of the selected populations. This hypothesis was not supported by the quantitative RT-PCR data; if anything, the global expression of pooled *for* transcripts tended to be higher in the selected populations. This does not preclude a potential role of subtle differences in the expression of specific transcripts in specific cell types, or differences at a post-transcriptional regulation. *for* is a complex gene with at least 11 transcripts, neither function nor expression pattern of which has been elucidated (www.FlyBase.ch; Marla B. Sokolowski personal communication).

Lack of changes in genes expression does not preclude a contribution of a locus to an evolved phenotypic change [18]. Interestingly, the quantitative complementation test offers some tentative support for the involvement of the *for* locus; either allelic variants at the *for* locus directly would have contributed to the divergence, or some other polymorphisms underlying the divergence would interact epistatically with the *for* locus [18]. However, the conclusion of the quantitative complementation test is weakened by the unexpected heterosis shown by the crosses. The simplest explanation for this heterosis - high degree of inbreeding of the control lines and hence restoration of heterozygosity at many loci - seems not very likely. The viability of the control lines on the poor food was as low as in this study, already early in the experiment, before any substantial inbreeding would have occurred [13]. Furthermore, we have not observed heterosis in crosses between replicate populations (R. K. Vijendravarma, unpublished data). Rather, we suspect that this may reflect heterozygote advantage at a specific locus or complementation between some specific alleles in the genetic backgrounds of our populations and both *for* strains. Thus, while suggestive, the quantitative complementation do not provide compelling evidence for the involvement of the *for* locus in the divergence between the selected and control lines. Possibly, the evolution of a shorter foraging path in the selected lines has other genetic basis.

The parallelism between the selected populations and the sitter strain in our study did not extend to developmental time. Rather, sitters developed more slowly than rovers on both food types, whereas the selected populations evolved faster development, possibly in response to the cut-off on time to emergence during selection [13, 25]. Rovers have been reported to develop faster than sitters, particularly on poor food [16], and increased foraging path has been reported as a correlated response to selection for accelerated development [26]. However, developmental rate is a highly polygenic trait and a net outcome of the effects of many underlying traits. In particular, a major mechanism of the faster development of our selected population is their smaller critical size at which metamorphosis is initiated [25]. Thus, our finding that the selected populations develop faster and show sitter-like foraging does not necessarily contradict the existence of mechanisms that tie long foraging path with faster development, such as feeding rate [26] or the rate of food absorption in the gut [16].

Our results support the argument that low quality food conditions at low population density favour different adaptations than crowded conditions on limited high-quality food. In contrast to results reported here, in two separate evolutionary experiments high larval densities favoured rover-like foraging phenotype, i.e., greater foraging path length [4, 5]. Both studies also reported an increased feeding rate; in contrast, our selected populations do not differ in their feeding rates from the control populations [27]. Other differences between experimental adaptation to chronic malnutrition and adaptation to crowded conditions are discussed in [25, 27]. Thus, although both larval crowding and larval malnutrition cause nutritional stress during development and the plastic responses for both are similar (reduced size at emergence, slower development and low viability), the evolutionary forces acting on populations under these scenarios are different or even opposite.

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27. Vijendravarma R.K., Narasimha S., Kawecki T.J. 2012, in press Adaptation to abundant low quality food improves the ability to compete for limited rich food in *Drosophila melanogaster*. *PLoS one*.

FIGURE LEGENDS

Figure 1: Larval traits of the selected (dark grey bars) and control (light grey bars) populations; for each trait left panel indicate selection regime means ± SE and the right panel indicates the means ± SE of individual replicate populations within each selection regime. (a) Path-length traversed by larvae during foraging on yeast in five minutes. (b) Path-length traversed by larvae on agar in two minutes. (c) Relative expression level of *for* transcripts.

Figure 2: Developmental traits (mean ± SE) of foraging strains for^{R} (light grey bars) and for^{s2} (dark grey bars) on poor and standard larval food. (a) Egg-to-adult viability. (b) Egg-to adult developmental rate. Both strains were maintained for three generations on standard food before the assay.

Figure 3: Quantitative complementation assays measuring Egg-to-adult viability on poor larval food (a) of four crosses between selection populations (one control population (C1), one selected population (S1)) and foraging strains (for^{R} and for^{s2}); (b) of the four parental strains used for the crosses.

Figure 1:

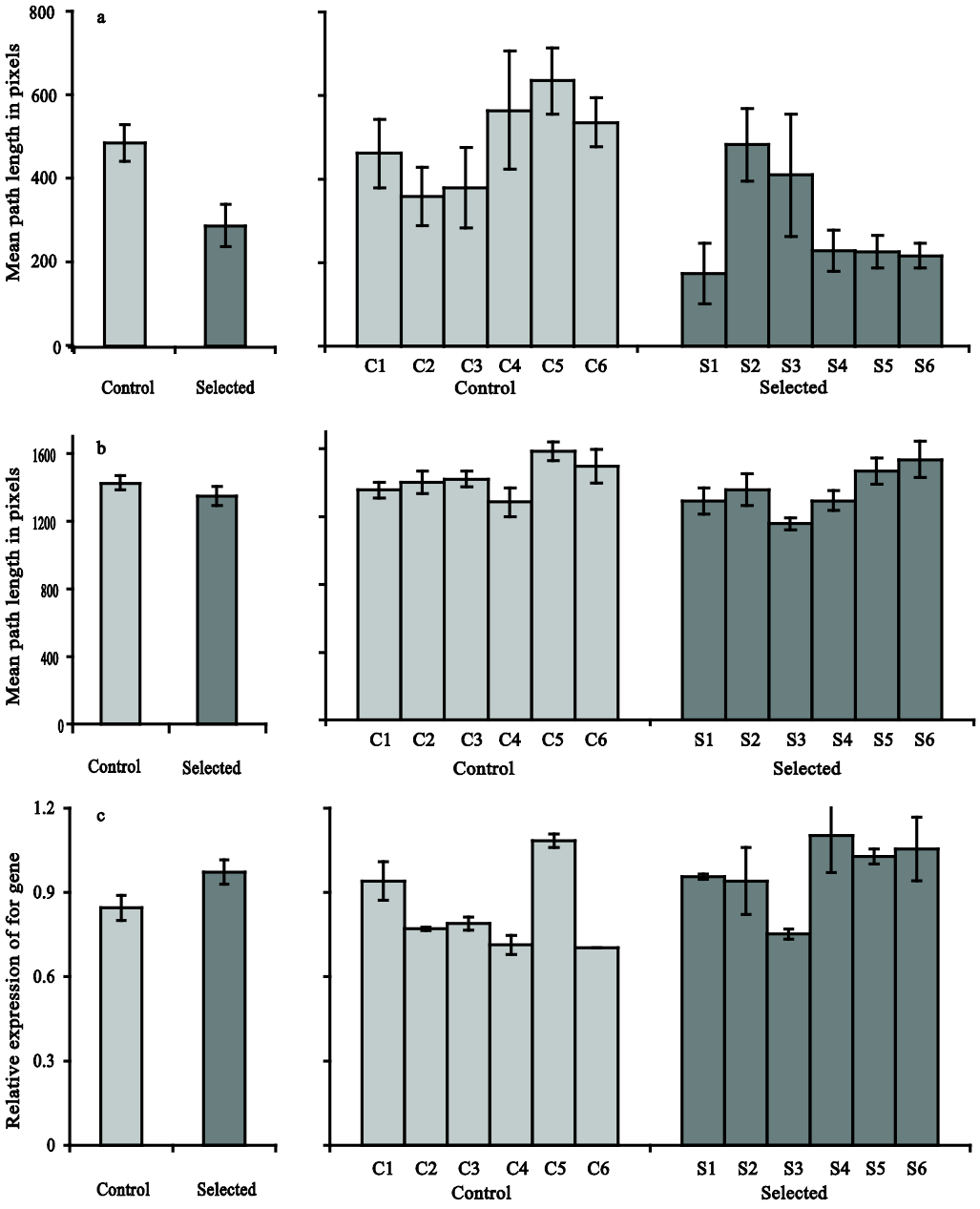

a
Mean path length in pixels
800
600
400
200
0
Control
Selected
C1 C2 C3 C4 C5 C6
Control
S1 S2 S3 S4 S5 S6
Selected
b
Mean path length in pixels
1600
1200
800
400
0
Control
Selected
C1 C2 C3 C4 C5 C6
Control
S1 S2 S3 S4 S5 S6
Selected
c
Relative expression of for gene
1.2
0.9
0.6
0.3
0
Control
Selected
C1 C2 C3 C4 C5 C6
Control
S1 S2 S3 S4 S5 S6
Selected

Figure 2:

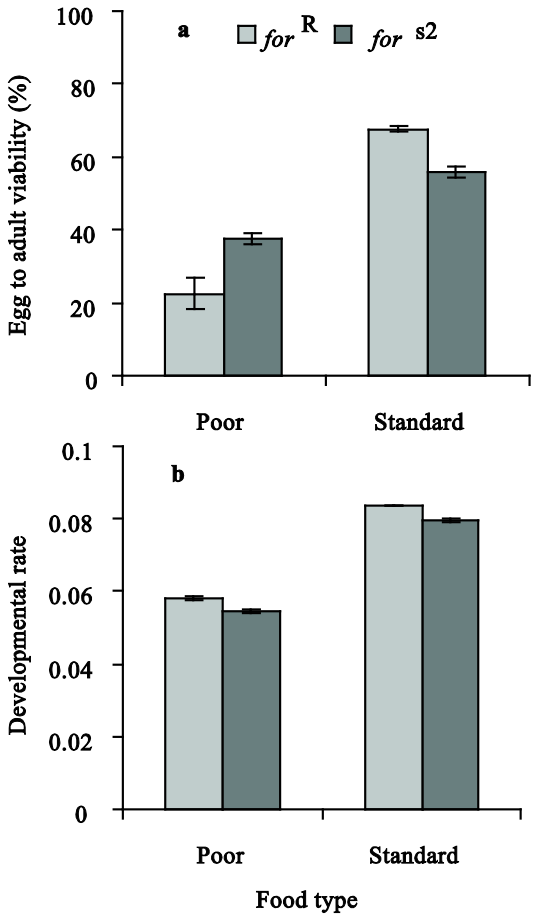
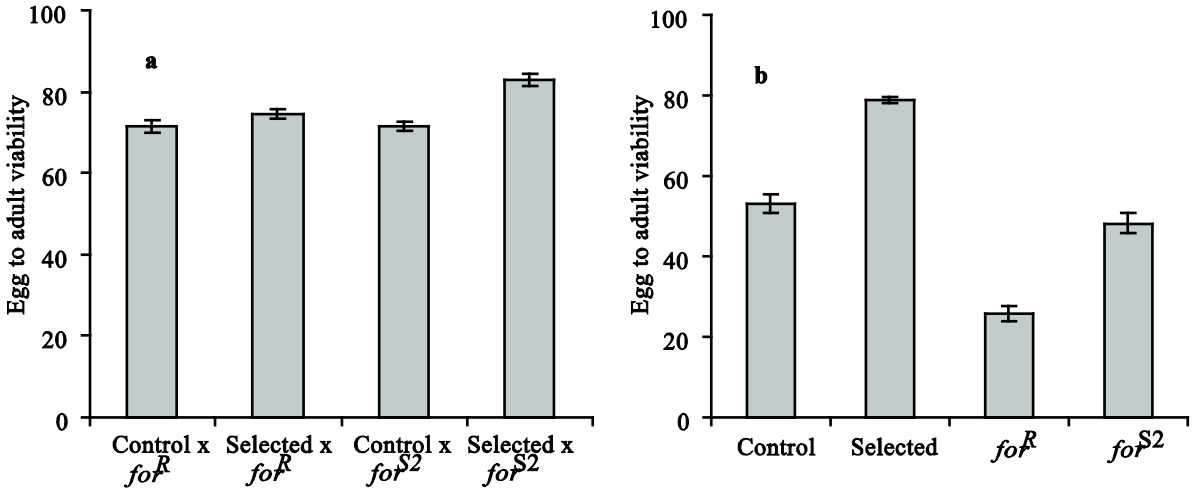

a
for R
for s2
Egg to adult viability (%)
100
80
60
40
20
0
Poor
Standard
b
Developmental rate
0.1
0.08
0.06
0.04
0.02
0
Poor
Standard
Food type

Figure 3:


a
Egg to adult viability
100
80
60
40
20
0
Control x for^R
Selected x for^R
Control x for^S2
Selected x for^S2
b
Egg to adult viability
100
80
60
40
20
0
Control
Selected
for^R
for^S2