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Original Article

Experimental adaptation to larval malnutrition maintains male postcopulatory sexual success despite reduced investment in accessory glands

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Abstract

We report the consequences of >330 generations of experimental evolution under larval undernutrition for male postcopulatory success and traits thought to mediate it in *Drosophila melanogaster*, comparing them to phenotypically plastic responses. Males from populations evolved on standard diet showed a >30% plastic reduction in the size of accessory glands (AG) when raised on a nutrient-poor larval diet. Experimental evolution on the poor diet led to a further reduction that was more pronounced in smaller individuals, changing the allometric slope between AG and wing size. Rather than the expected reduction in seminal fluid protein expression, we observed a plastic increase on poor diet in investment in *Acp36DE* and *Acp62F* at the expense of *Acp26Aa* and *SP*, and an evolutionary shift in the time course toward lower virgin and higher postmating seminal fluid protein expression. Surprisingly, neither the plastic nor evolved reduction in AG was associated with impaired performance in reproductive output from matings with excess females, the ability to induce female oviposition or performance in sperm competition ("sperm defense"), except when poor diet-adapted males were raised on standard diet. Thus, larval nutrient shortage favors reduced investment in AG development, but this is compensated for by other mechanisms, minimizing consequences for postcopulatory success.

Keywords: dietary restriction, experimental evolution, sexual conflict, seminal fluid proteins, sexual selection, sperm competition

Introduction

Postcopulatory sexual selection favors male traits that improve success in sperm competition, inhibit female remating, or stimulate short-term egg production (Lüpold et al., 2020; Parker, 1970; Ramm, 2020; Wigby et al., 2020). These traits often result in reduction of female lifetime fitness, thus mediating sexual conflict (Chapman et al., 2003; Rice & Holland, 1997). At the same time, investment in sperm competition [e.g., in quantity and quality of sperm, seminal fluid proteins (SFPs), nutrient-bearing spermatophores or mating plugs] is costly in terms of energy and amino acid supply (Dewsbury, 1982; Perry et al., 2013; Wedell et al., 2002). We can therefore expect such investments to be sensitive to environmental stressors such as nutrient limitation (Chapman & Partridge, 1996; Janicke et al., 2015; Rostant et al., 2020). This hypothesis is largely supported in vertebrates and arthropods, but there are many exceptions (Macartney et al., 2019). Interestingly, the negative effects of juvenile undernutrition on sperm and ejaculate traits tend to be larger than those of adult nutrient shortage (Macartney et al., 2019). However, they are usually less severe than the consequences of juvenile undernutrition for adult body size (Macartney et al., 2019). This pattern implies that investment during development in traits mediating postcopulatory sexual selection is relatively buffered against nutritional stress. This likely occurs at the expense of other fitness-related functions, such as somatic maintenance, growth, or survival, but also possibly of traits under precopulatory sexual selection (Simmons et al., 2017).

The effects of larval nutrition on postcopulatory sexual traits appear quite heterogeneous in *Drosophila*. In some studies, nutritional stress during the larval stage (due to reduced nutrient concentration and/or increased larval density) reduced testis size and sperm counts (Kapila et al., 2022; McGraw et al., 2007), accessory gland (AG) size or SFP production (Kapila et al., 2022; von Hellfeld et al., 2025; Wigby et al., 2016), or success in sperm competition (Kapila et al., 2025; McGraw et al., 2007). Even paternal larval diet has been reported to affect sons' success in sperm competition (Zajitschek et al., 2017). However, other studies detected no effect of diet on the expression of SFP and sperm competitiveness (Patlar et al., 2023) or ejaculate proteome (Zeender et al., 2023), even though the latter study detected substantial genotype × diet interaction. Finally, some studies found that males raised under larval nutritional stress invested more in traits mediating sperm competition (Amitin & Pitnick, 2007; De Nardo et al., 2021). These discrepancies between experiments may result from differences in details of diets and protocols and/or from genotype × diet interactions. It is difficult to ascertain which of these phenotypically plastic responses are adaptive (in the sense of making the best of a bad situation) rather than being maladaptive consequences of physiological stress.

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Experimental evolution offers a direct approach to testing which phenotypic and genetic changes are favored—as direct or correlated response to selection—by particular conditions (Garland & Rose, 2009; Kawecki et al., 2012). This approach has been highly successful in demonstrating that stronger sexual selection favors greater investment in testes or AG size and/or improves males' success in sperm competition (Crudgington et al., 2009; Dore et al., 2021; Hollis et al., 2019; Hosken et al., 2001; Linklater et al., 2007; Simmons & García-González, 2008), even if there are some exceptions (Chechi et al., 2017; Reuter et al., 2008). In contrast, only two evolutionary experiments, both on *Drosophila melanogaster*, appear to have employed experimental evolution to study the consequences of nutritional stress on male investment in traits mediating sexual success. In one, males evolved under lower yeast content in adult diet were less effective in inhibiting female remating, but no differences in a number of other traits were found (Bath et al., 2023; Dore et al., 2021; Sepil et al., 2022). In the other, males evolved under larval crowding showed reduced "sperm defense" ability (P1, i.e., paternity retained by the first male after the female mates with another male); however, the traits mediating this trade-off are unclear as no effects of evolutionary history of larval crowding on testis and AG size was detected (Kapila et al., 2022).

In this study, we used experimental evolution to explore how long-term evolutionary adaptation to developmental malnutrition influences male reproductive traits in *D. melanogaster*, with a specific focus on postcopulatory investment. By integrating physiological and functional aspects of reproductive investment, we aimed to reveal the evolutionary consequences of nutrient limitation for male reproductive success and sexual selection. Our study focused on six *Drosophila* populations ("Selected" populations) that had adapted to a very nutrient-poor larval diet for over >330 generations of experimental evolution, comparing them to six "Control" populations originating from the same gene pool and maintained in parallel on a standard larval diet (Kawecki et al., 2021; Kolss et al., 2009). On this poor diet, nonadapted larvae take twice as long to reach metamorphosis than on the standard diet, and still the surviving adults emerge at half of normal body size (Erkosar et al., 2025; Kolss et al., 2009). Owing to their adaptation to nutrient limitation, the Selected larvae grow and develop on the poor diet faster than the Controls, mediated by improved nutrient acquisition and apparently greater metabolic efficiency (Cavigliasso et al., 2020, 2023). The Selected larvae also initiate their metamorphosis at a smaller larval size than Controls (Vijendravarma et al., 2012), which allows them to complete the development with less accumulated biomass; as a consequence, they are smaller as adults than Control flies, irrespective of larval diet (Cavigliasso et al., 2023; Kolss et al., 2009). Even though the malnutrition regime is limited to the larval stage, adaptation to it has consequences for adult physiology that go beyond the impact of smaller body size (Erkosar et al., 2023). These differences are associated with a shift along a "power-endurance" trait syndrome linking traits from gene expression, energy metabolism, behavior, and life history (Erkosar et al., 2025). Perhaps most interestingly, females of the Selected population raised on poor diet—to which they are more tolerant as larvae—have lower fecundity than Control females that managed to complete development on the poor diet encountering it for the first time in their evolutionary history (Erkosar et al., 2023). Thus, adaptation to larval undernutrition in these populations has apparently traded-off with adult reproductive performance of females.

Here, we study the consequences of this experimental adaptation to larval malnutrition for proxies of male investment in SFPs, key determinants of postcopulatory success, and sexual conflict (Patlar & Civetta, 2022; Wigby et al., 2009). We assessed this investment in terms of AG size and the expression of four SFP genes. The final AG size is largely determined before adult emergence (Box et al., 2024) and thus reflects an important aspect of investment of resources acquired during the larval stage in the future postcopulatory competitiveness of the adult. The SFP expression, which we quantified in whole bodies, is a proxy of allocation of transcriptional resources in SFPs relative to their allocation in housekeeping at the level of the whole organism and may be limited by available metabolic resources but also by the size of the AG. We measured the SFP expression during sexual maturation in the first 2 days of adult life, as well as in mature males that were replenishing their SFPs after mating. We combined these physiological measurements with mating experiments to assess male fertility, the ability to induce female oviposition, and male sperm defense ability (P1). We assayed males from all Selected and Control populations raised on either nutrient-poor or standard diet, allowing us to study both the evolutionary, genetically based response to long-term exposure to poor larval diet and the phenotypic plasticity induced by the larval diet experienced by the assayed individuals.

Despite the inconsistency of published results on the plastic response of AGs and SFPs to larval nutrient shortage (summarized above), we expected that males raised on the poor diet would show reduced AG size and SFP expression because the nutrient (in particular yeast) content of our poor diet was several-fold below that used in other studies (Bath et al., 2023; De Nardo et al., 2021; McGraw et al., 2007; Zeender et al., 2023). We considered alternative hypotheses as to how these traits would evolve over generations under larval undernutrition. On the one hand, owing to the evolutionary adaptation of their metabolism and improved nutrient acquisition (see above), larvae from the Selected populations should be less nutrient-limited on the poor diet than Control larvae, having an overall greater pool of resources, potentially allowing them to invest more in development of sexually selected traits. On the other hand, the strong selection to survive, grow, and develop fast on the poor larval diet may have favored diversion of resources away from the developing AG, leading to their smaller final size and reduced ability to produce SFPs. Irrespective of their direction, we also expected that phenotypically plastic and evolutionary changes AG size would be associated with changes in performance in male fertility, inducement of oviposition, and sperm competition. Finally, our factorial design also allowed us to explore evolutionary change in phenotypically plastic response to diet, assessing to what degree adaptation to the poor larval diet relies on modifying responses to undernutrition rather than on diet-independent phenotypic shifts. In the same optics, we also tested for changes in the allometric relationship between AG size and wing size, which we measured on the same individual males.

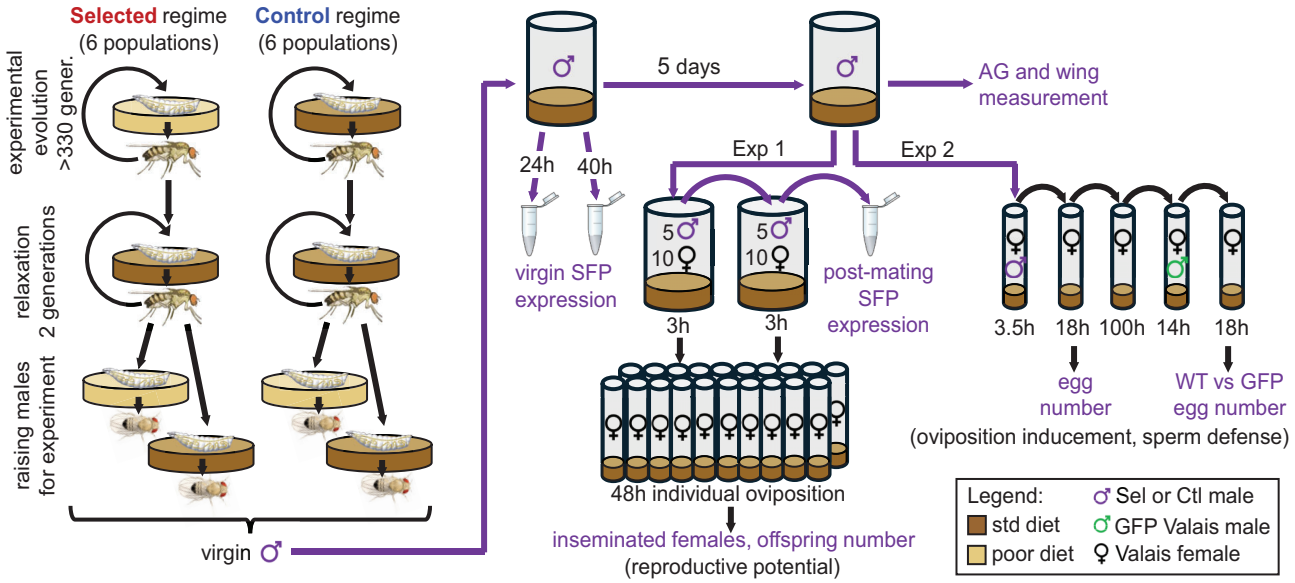

Figure 1. Evolutionary history of the populations and experimental design. Purple arrows indicate transfer or sampling of focal Selected and Control males, purple font refers to collected data.

Materials and methods

Experimental populations and diets

We compared *D. melanogaster* males from six "Selected" populations experimentally evolved under larval undernutrition and six "Control" populations. All originated from a population collected in 1999 in Basel (Switzerland) and maintained on standard diet for several years before the evolution experiment started in 2005 (Kolss et al., 2009). Since then, Control populations were raised at the larval stage on a standard diet (12.5 g dry brewer's yeast, 30 g sucrose, 60 g glucose, 50 g cornmeal, 0.5 g CaCl2, 0.5 g $MgSO_4$, 10 ml 10% Nipagin, 6 ml propionic acid, 20 ml ethanol, and 15 g of agar per liter of water). The larvae of Selected populations were raised on a poor diet, containing one fourth of the concentrations of yeast, sugars, and cornmeal of the standard diet. Larval density was controlled at 200–250 eggs per bottle with 40 ml of food. After emergence, adult flies from both regimes were transferred to bottles containing standard diet until the start of the next generation (generation time 3 weeks). The experiments reported here were performed after more than 330 generations of experimental evolution. Prior to all experiments, all populations were raised on standard diet for two generations to minimize the effects of (grand)parental environment (Vijendravarma et al., 2010). Males used in the experiments were raised on either standard or poor diet as described above, inoculated with 0.5 ml of feces ($OD_{600} = 0.5$) pooled from both Selected and Control populations to standardize the microbiome (Cavigliasso et al., 2020). Egg laying was staggered to match the age of adult flies, compensating for differences in the development time between Control and Selected populations.

To avoid confounding the consequences of male diet adaptation with those of female diet adaptation or male–female coadaptation, we used females from a different population, collected in Valais, Switzerland, in 2007 and maintained on standard diet in a population cage with overlapping generations. We also used competitor with a genetic construct expressing *GFP* under the control of two promoters (pBac{Ubnls-EGFP, ProtB-EGFP}; courtesy of Stefan Lüpold, University of Zürich; Manier et al., 2010) backcrossed into Valais background for five generations. Virgin flies were collected 6–8 h postemergence and maintained in single-sex groups until used in the experiment. All populations were maintained and experiments performed at 25°C, 55% humidity and 12:12 h day–night cycle.

Experimental design

The data were collected in two different experiments performed several weeks apart. Both experiments were factorial, assaying males from the six Selected and six Control populations raised on each of the two larval diets (poor versus standard) (Figure 1). In the first experiment, we measured (1) accessory AG size and wing length of mature virgin males (the latter as a proxy of body size), (2) expression of several SFP genes in young virgin males (at 24 and 40 h postemergence) and in 5-day-old males that depleted their SFPs in multiple matings, and (3) mating potential and fertility when presented with surplus of virgin females. In the second experiment, we assayed (4) the males' ability to induce female egg laying after a single mating and (5) their success in sperm competition in terms of the first male's paternity share (P1). Because of a marked difference in developmental time on poor versus standard diet, data from males raised on poor and standard diet were obtained several days apart. This implies that the effects of diet treatment may be potentially confounded with those of temporal variation.

AG and wing measurements

We measured the AG size (area) and wing length in 5-day-old virgin males from Selected and Control populations raised on either diet. Males at this age are fully mature and the dimension and protein content of their AGs have reached a plateau after increasing over the first 3 days postemer-

gence (Koppik et al., 2018). For method details see the Supplementary methods.

SFP gene expression

We quantified the expression of four SFP genes: *Acp26Aa* (*ovulin*), which stimulates egg laying, sex peptide (*SP*), which reduces female receptivity after mating and affects oviposition, *Acp62F*, a trypsin inhibitor that promotes sperm storage in the spermatheca, and *Acp36DE*, which assist the movement of sperm into female spermatheca and the seminal receptacle after mating (Avila et al., 2011; Neubaum & Wolfner, 1999; Wigby et al., 2009). We used three housekeeping reference genes: *αTub84B*, *eEF1α2*, and *RpL32*. We extracted RNA from entire bodies (rather than from dissected AGs) because we aimed to estimate SFP transcription at the level of the whole organism, relative to its housekeeping transcriptional activity across all tissues and structures. Each sample consisted of five pooled males, with three samples from each population and diet at three timepoints/conditions: virgin males at 24 and 40 h postemergence, to quantify the initial investment in SFPs (Hollis et al., 2019; Koppik et al., 2018), and 5-day-old mature males collected 30 min after they had an opportunity to mate with surplus females (see below). This last measurement reflects investment in SFP replenishment following depletion. The 30 min interval after the end of mating opportunity should be sufficient to induce the expression of SFP genes (Herndon et al., 1997) but not yet to refill the AG with replenished SFPs (Sirot et al., 2009); it should thus reflect the transcriptional response to SFP depletion. Log_2(expression) of each target gene was calculated relative to the geometric mean of the reference genes (Vandesompele et al., 2002). Four of the 216 biological replicates were removed as outliers. For details of the protocol and data preprocessing, see the Supplementary methods, for primers Table S1.

Reproductive potential

In this experiment (Exp 1 in Figure 1), we assayed male reproductive potential in the presence of surplus of females (i.e., more females that the males could mate with during the available time). we also obtained freshly mated males for the last timepoint of SFP expression (see above). Groups of five Selected or Control males (5-day-old), raised on either larval diet, were transferred with 10 virgin Valais females (4–5-day-old) to vials with standard diet. After 3 h, we paired the same group of 5 males with a new group of 10 virgin females for another 3 h. 30 min after the end of the second mating period the males were frozen for SFP expression. The 20 females were individually transferred to new vials with standard diet and allowed to lay eggs for 48 h; we counted the resulting adult offspring 14 days later. For each replicate group of five males ($N = 3$ per population and diet) we analyzed the total number of offspring, as well as the number of inseminated females (those that produced at least one offspring) and the mean number of offspring per inseminated female, separately by the mating period. Additionally, to account for possible biases due to potential differences in egg-do-adult survival, we analyzed the numbers of offspring from this experiment corrected by estimates of egg-to-adult survival of offspring of Selected and Control males obtained in a parallel experiment (see Supplementary methods).

Oviposition inducement and sperm competition

Here, we aimed to quantify the ability of males to induce female oviposition and to retain paternity after the female has had an opportunity to remate (Exp 2 in Figure 1). A single 5-day-old male was paired in a vial with standard diet with a single virgin female aged 4–5-day-old for up to 3.5 h (40 pairs per population and diet); mating was monitored to ensure that the females mated only once. After the mating, males and nonmated pairs were discarded (around 10 pairs per population and diet), and mated females were allowed to lay eggs in the same vial for 18–20 h (the onset of this period varied depending on how soon mating occurred). After this time, we transferred females to fresh vials and froze the old vials to count the eggs later. 120 h after the first mating, each female was placed together with three 7–8-day-old GFP-expressing virgin Valais males (around 30 pairs per population and diet). We initially monitored remating for 8 h; however, it was occurring at a very slow rate, confirming our earlier observations that Valais females are recalcitrant to remating. Therefore, the males and females were left to mate freely overnight for a total of 14 h. To mimic the conditions under which mating occurs in the experimental regimes, until this point in the experiment females were only fed standard diet with no live yeast supplement. This explains the low number of eggs laid by the females; it also potentially makes the role of male inducement of egg laying more important, compared to a situation where females are well-fed on live yeast and bursting with eggs. After the mating opportunity with GFP males the females were transferred with light CO_2 to small petri dishes (~4 cm of diameter) containing orange juice and agar with a drop of yeast liquid paste in the center, and they were allowed to lay eggs for 20 h. After this time, we froze the eggs and later assessed paternity at the embryo stage through fluorescence distinguishing between GFP and wildtype eggs.

Data analysis

Statistical analyses were performed using R (version 4.1.0) and RStudio (1.4.1717). Depending on the expected distribution of the data, we fitted linear mixed models (LMM) or generalized linear mixed models (GLMM) with functions *lmer* and *glmer*, respectively, or in one case a zero-inflated GLMM using *glmmTMB* package (Brooks et al., 2017). The evolutionary regime (Selected versus Control) and the developmental diet (poor versus standard) on which the experimental flies were raised, plus their interaction, were included as the focal fixed effects, with timepoint being another fixed factor for gene expression. We included replicate populations (which are the main level of replication) as a random effect in the model and, where applicable, random interaction of the population with diet and/or timepoint. We tested for the significance of model effects with likelihood ratio tests as implemented in function *mixed* of the *afex* package (Singmann et al., 2025). Marginal means and slopes were estimated with functions *emmeans* and *emtrends* and compared with the *pairs* function of the *emmeans* package (Lenth et al., 2025). The *contrast* function of *emmans* was used to test specific hypotheses. Model suitability was assessed in all cases using diagnostic plots generated with the *DHARMa* package (Hartig et al., 2024). For details of statistical analysis see the Supplementary methods.

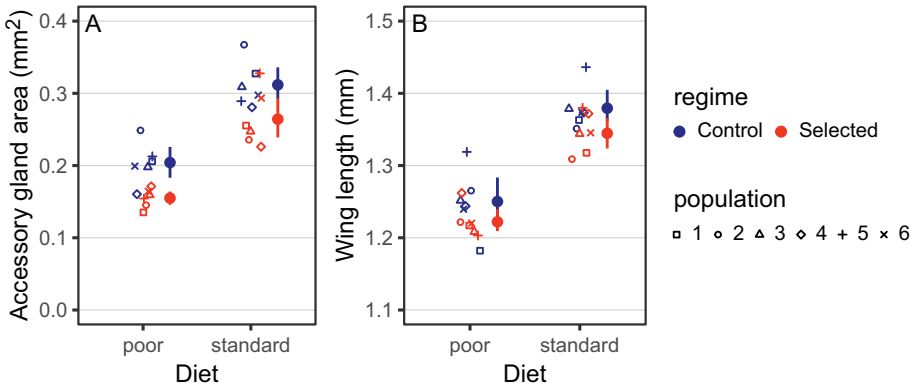

Figure 2. AG size (A) and wing length (B) of males from the Selected and Control populations raised on the poor and standard diet. The large symbols are regime means ± 95% confidence intervals, the small symbols are means of individual populations ($N = 10$ males per population, regime, and diet). (Selected and Control populations are not paired, even if they share the same symbol shape.).

Results

AG size

The AGs of 5-day-old males raised on poor diet were about one-third smaller (in terms of image area) than of those raised on standard diet ($\chi^2_1 = 41.4$, $p < 0.0001$), and Selected males had smaller AGs than Controls ($\chi^2_1 = 9.1$, $p = 0.0026$, Table S2, Figure 2A). There was no interaction between the phenotypically plastic and the evolutionary response to diet (regime × diet $\chi^2_1 = 0.01$, $p = 0.94$). However, in proportional terms, the estimated difference between Selected and Control males was 15% when raised on standard diet and 24% when raised on poor diet (pairwise contrasts both $p < 0.01$, Table S2).

The mean wing length was reduced in males raised on poor compared to standard diet by about 9% ($\chi^2_1 = 45.4$, $p < 0.0001$) and was about 3% smaller in Selected than Control populations ($\chi^2_1 = 3.9$, $p = 0.048$), with the two factors again being mostly additive (regime × diet, $\chi^2_1 = 0.2$, $p = 0.66$, Table S2, Figure 2B). We explored how AG size scales with wing length (which can be interpreted as a proxy for structural size) and whether this relationship differs between regimes and diets. A joint LMM on males raised on both diets revealed that even accounting for allometry with wing size, for males with average wing length those raised on poor diet had about 22% smaller AGs than males of the same wing length raised on standard diet ($\log_{10}$ difference of -0.11, $\chi^2_1 = 25.1$, $p < 0.0001$, Table S3A). This model also revealed a difference between diet conditions in the allometric (log-log) slopes linking the two traits (diet × log(wing length), $\chi^2_1 = 11.7$, $p < 0.001$, Table S3A); we thus proceeded to analyze the two dietary conditions separately.

For flies raised on the standard diet, we found no evidence for difference in the slope between Selected and Control populations (regime × log(wing length), $\chi^2_1 = 0.5$, $p = 0.48$, Table S3B). AG size scaled with wing length with a joint log-log slope of $b = 1.47 \pm 0.45$, with a marginally significant difference in elevation ($\chi^2_1 = 3.5$, $p = 0.069$), corresponding to glands of estimated means of Selected males being 13% smaller than those of Control males with the same wing length (Figure 3B).

By contrast, for flies raised on the poor diet AG sizes scaled more steeply with wing length in Selected than Control males (3.94 ± 0.43 versus 2.24 ± 0.40; regime × log(wing length), $\chi^2_1 = 8.5$, $p = 0.003$; Figure 3A). While the AG size of Selected and Control populations converged for males with the longest wings, for males with average wing length, the AGs of Selected males were 19% smaller ($\chi^2_1 = 7.6$, $p = 0.006$, Table S3B), with the difference even more pronounced for the smallest males (Figure 3A). In fact, the 3.94 allometric slope for Selected males raised on poor diet was significantly greater than 2 ($t_{120} = 4.6$, $p < 0.0001$), which is the slope expected under isometric (proportional) scaling (because the gland area has been measured as an area while the wing measurement is linear). Assuming that individual variation wing and AG size reflects variation in larval nutritional and physiological condition, this result implies that adaptation to the poor diet led to AG size of Selected males being more sensitive to individual condition than wing size.

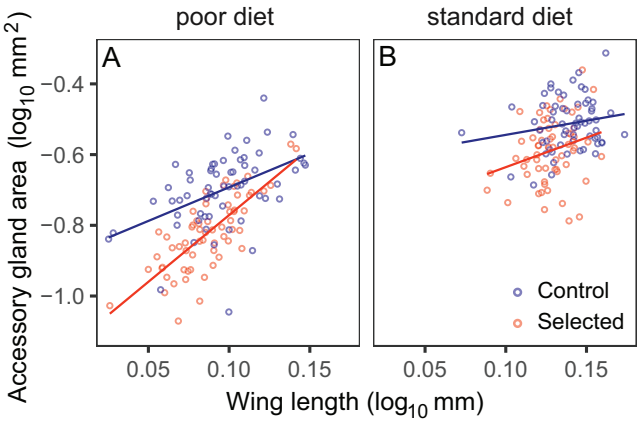

Figure 3. The allometric relationship between wing length AG size (area) in mature virgin males raised on (A) poor and (B) standard diet. Each symbol corresponds to a single male (mean size of the two gland and two wings), lines are predictions from LMMs, population identity is not indicated (but included in the models as a random factor).

Expression of SFPs

We quantified the relative expression of four SFPs in whole bodies of males raised on either diet, at 24 and 40 h posteclosion in unmated males, and in 5-day-old males just after they had been allowed to mate for 6 h with a surplus of virgin females. The expression of all four genes showed consistent regime × timepoint interaction (all $\chi^2_1 \geq 19.3$, p

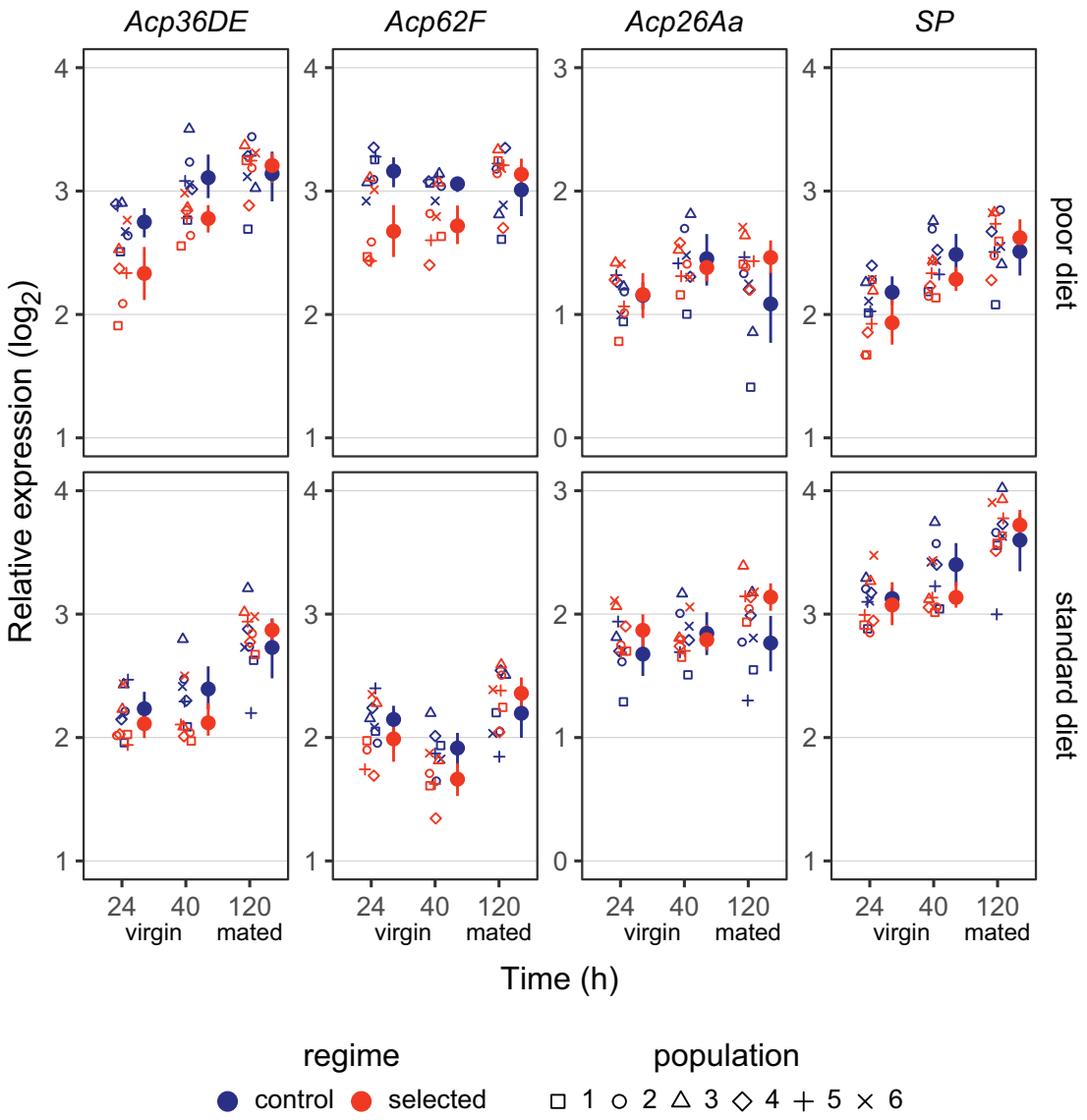

Figure 4. The expression of four SFP genes in males of Selected and Control populations raised on either larval diet, at three timepoints (at 24 and 40 h postemergence in virgin males, and in mature 5-day-old males following mating with surplus females). The expression is in whole body and relative to the geometric mean of three housekeeping genes (i.e., relative $\log_2$ expression of 1 means mRNA abundance 2-fold higher than the mean of the housekeeping genes). The large circles represent, for each regime, the estimated marginal means ± 95% CI; the small symbols are means of the replicate populations. $N = 3$ samples of 5 males per combination of regime, population, diet, and timepoint.

< 0.001) and diet × timepoint interaction (all $\chi^2_1 \geq 17.3$, $p \leq .001$, LMM, Table S4A, Figure 4). We used a priori contrast analysis within the LMM to make sense of these complex patterns (see the section "Methods"). The contrasts indicated that the regime × timepoint interaction was driven by a difference of the regime effect between the post-mating timepoint and the two premating timepoints: for all genes, the Selected—Control difference was greater (by $\log_2$ fold-change of about 0.3–0.4) postmating than pre-mating (contrast regime × timepoint pre- vs postmating: all $t_{29} \geq 4.8$, $p < 0.0001$, Table S4B). In other words, Selected males increased their SFP expression more (or reduced it less) between early adult life and the post-mating status than Control males. We detected no differences in the regime effect between the two pre-mating time points (contrast regime × timepoint 24 vs 40h: all $t_{29} \leq 1.5$, $p > 0.13$, Table S4B). Premating, the expression of the two SFPs involved in sperm competition (*Acp36DE* and *Acp62F*) was lower in Selected than Control populations by 17% and 19%, respectively ($t_{17} = 2.8$, $p = 0.013$ and $t_{17} = 2.9$, $p = 0.010$, contrast regime@pre-mating, Table S4). Postmating, Selected males tended to show a higher expression than Control males for all four SFPs, but the difference was only significant for *Acp26Aa/ovulin* (a 33% increase, regime@post-mating, $t_{23} = 3.7$, $p = 0.001$, Table S4).

Even though the diet × timepoint interaction implies that the magnitude of the effect of larval diet varied across timepoints, the sign of the diet effect for each gene was completely consistent across timepoints and between Selected and Control populations (this is best seen in Figure S1). However, strikingly, these effects were of opposite signs for two pairs of genes: while males raised on poor diet showed a lower expression of *Acp26Aa* and *SP* (the latter more than 2-fold), they had a substantially higher expression of *Acp36D*

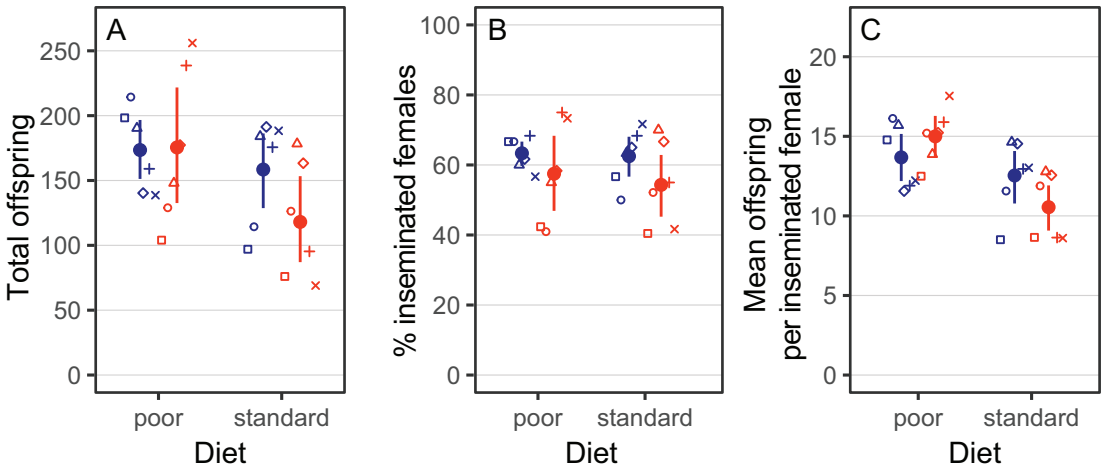

Figure 5. Reproductive potential of males. (A) The total number of offspring sired by groups of 5 focal males allowed to mate with 10 virgin females over 3 h and with a new set of 10 virgin females for another 3 h mating period. (B) The fraction of the 20 females that produced offspring (i.e., were successfully inseminated). (C) The mean number of offspring per inseminated female. $N = 3$ groups of 5 males per population, regime, and diet. The large symbols are estimated marginal means ± 95% confidence intervals for each regime, the small symbols are means of replicate populations. For the same results split between the two 3 h mating periods see Figure S2.

and *Acp62F* than males raised on standard diet (Figure S1). Thus, rather than a general change in investment in SFPs, the phenotypically plastic response to diet appears to have involved a shift of relative allocation to different SFPs. Figure S1 also makes apparent the pattern responsible for the diet × timepoint interaction pattern: for all genes the (signed) poor—standard diet difference was greater at the 40 h timepoint than either 24 or 120 h.

Reproductive potential

To quantify male fertility in terms of the potential to father offspring with access to surplus females, we housed groups of five males with 10 virgin females for 3 h and then replaced the females with another set of 10 virgin females and let them interact for another 3 h; later, we counted the number of offspring produced by each female over the following 48 h. We first analyzed the total number of offspring produced by all 20 females of a particular replicate. While there was a trend for Selected males to have fewer offspring than Control males when both were raised on standard diet (estimated marginal means 118 ± 19 versus 158 ± 19, EMMs ± SE, $t_{28.8} = 1.5$, $p = 0.14$), there was no hint of a difference between the two regimes when the males were raised on poor diet (Control 174 ± 19 versus Selected 176 ± 19, $t_{28.8} = 0.1$, $p = 0.94$, Table S5, Figure 5A).

The above measure of reproductive potential combines the proportion of the 20 available females that became inseminated and the number of offspring produced by those mated females. We thus analyzed these two variables separately, taking producing at least one offspring as the criterion of the females having been successfully inseminated. We also included the mating period (the first 3 h versus the last 3 h) as a factor in the analysis. The proportion of inseminated females declined more steeply between the first and second mating period for males raised on the poor diet than for those raised on standard diet (diet × mating period interaction, $\chi^2_1 = 31.2$, $p < 0.0001$; Table S6). However, pooled across the two mating periods, the mean fraction of inseminated females was nearly identical between the diets (Figure 5B; $\chi^2_1 = 0.8$, $p = 0.96$, Table S6). We detected no effect of regime, even if Selected males tended to be slightly less successful than Controls ($\chi^2_1 = 1.2$, $p = 0.27$, Table S6).

The mean number of offspring per inseminated female showed a somewhat complex pattern of interactions (Table S7) which was driven by the low number of offspring produced by females inseminated by Selected males raised on standard diet during the first mating period (Figure 5C, contrast diet@Selected $t_{124} = 4.8$, $p < 0.0001$, Table S7). Diet did not detectably affect this trait for Control males (diet@Control $t_{124} = 0.6$, $p = 0.53$). We detected no effect of regime on this trait for males raised on poor diet (Regime@poor diet $t_{52.3} = 1.0$, $p = 0.30$); if anything, Selected males actually tended to sire more offspring per inseminated female than Control males. Thus, overall, we find no evidence that either plastic response or evolutionary adaptation to poor larval diet reduces male fertility; we find some evidence that Selected males perform less well when transplanted back to standard larval diet.

In a parallel experiment we measured egg-to-adult survival of offspring fathered with Selected and Control males with Valais females and raised on standard diet (Table S8). We did not detect a difference in survival probability between offspring of Selected and Control fathers raised on the poor diet (0.932 ± 0.010 versus 0.947 ± 0.009, back-transformed EMMs ± SE; $z = 1.1$, $p = 0.28$, Table S8). However, for fathers raised on standard diet, offspring of Selected males had lower survival than those of Controls (0.879 ± 0.015 versus 0.927 ± 0.012, $z = 2{,}5$, $p = 0.013$). Correcting the total reproductive output or the number of offspring per inseminated female by these estimated survival probabilities (see the Supplementary methods) had little effect on the outcome of statistical tests (columns labelled "corrected" in Tables S5 and S7) and did not change any of the above conclusions.

Oviposition inducement and sperm competition

We conducted a "sperm defense" assay that also allowed us to assess male ability to induce female oviposition. Of the

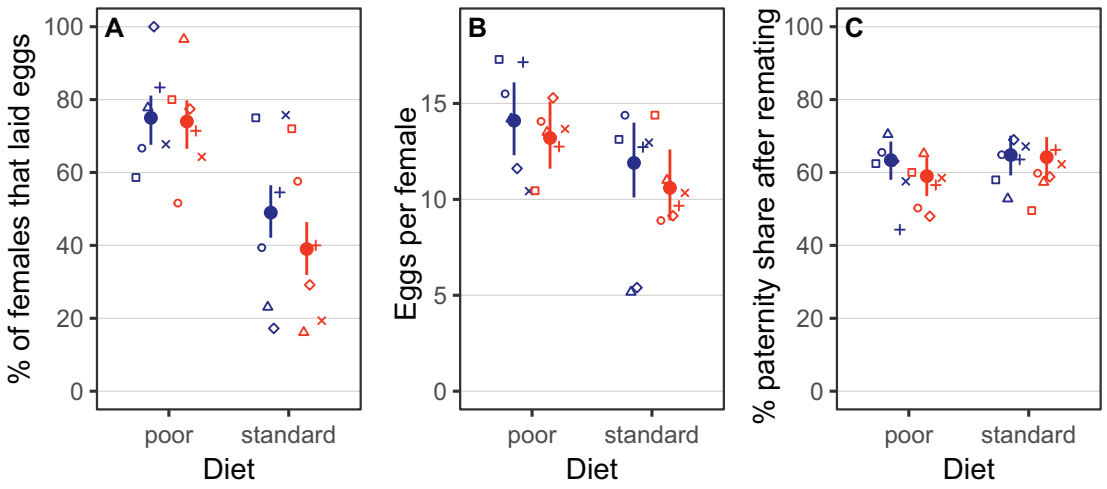

Figure 6. Inducement of egg laying and sperm defense. (A) The proportion of initially virgin females that laid at least one egg within 20 h of a single mating with a focal male. (B) The mean number of eggs per female within 20 h of the single mating (excluding females that lad no eggs). (C) Paternity share of the initial focal male following opportunity of the female to remate with three GFP-carrying males (as% of wildtype vs. GFP embryos); $N = 21$–33 per population, regime, and diet. The large symbols are estimated marginal means ± 95% confidence intervals, the small symbols are means of individual populations.

694 females assayed, 289 did not lay any eggs within 20 h of a single mating with a focal male despite a copulation having been observed. Strikingly, the failure to lay any eggs was much more frequent for females mated to males raised on standard diet—less than half laid any eggs, compared to about 75% of females mated to males raised on poor diet (Figure 6A; diet $\chi^2_1 = 66.2$ $p < 0.0001$, zero-inflated part, Table S9). Among females that did lay eggs, those mated with males reared on the standard diet laid fewer eggs (Figure 6B; $\chi^2_1 = 6.1$, $p = 0.014$, count part, Table S9) than those reared on the poor diet. Among males raised on standard diet, Selected males were less effective than Controls in inducing females to lay any eggs (contrast regime@standard diet, $z = 1.97$, $p = 0.049$ for the zero-inflated part, Table S9); otherwise, we detected no differences between regimes, despite some trends for Selected males to perform slightly less well (Table S9).

We then assessed the paternity of the first male (P1) after the females were housed with three GFP-marked males 5 days later for 22 h and scoring wildtype versus GFP-expressing embryos; 603 females produced offspring after the second mating. Although Selected males tended to show a lower proportional paternity share than Control males (0.634 ± 0.027 versus 0.590 ± 0.027, EMMs ± SE), we detected no significant effects of either evolutionary regime ($\chi^2_1 = 0.8$, $p = 0.38$) or dietary treatment ($\chi^2_1 = 1.3$, $p = 0.25$; Figure 6C, Table S10). This analysis includes all females that laid eggs after the second mating, including those that only produced wildtype offspring; thus, the P1 measure combines the ability to inhibit female remating and success in sperm competition following remating.

Discussion

We set up this study expecting that the phenotypically plastic response to larval undernutrition would involve a reduction of investment in AGs and SFPs, resulting in lower reproductive potential and reduced sperm competition performance. We also formulated alternative hypotheses concerning consequences of experimental evolutionary adaptation to larval undernutrition. Either, improved nutrient acquisition and metabolic adaptations would allow poor diet-raised Selected males to increase their investment in these traits compared to poor diet-raised Control males, with concomitant improvement in postcopulatory performance. Or, conversely, selection under nutrient shortage would favor diversion of resources from developing AG to other vital functions, resulting in poorer postcopulatory performance. Our results showed that both phenotypic plasticity and experimental adaptation in response to chronic larval undernutrition affect AG size and SFP expression, but the pattern was more complex than what we had expected.

Ancestral phenotypic plasticity

We interpret the difference between mean phenotypes expressed by Control populations raised on standard versus poor diet as representative of the phenotypic plasticity of the ancestral population, subject the caveat that these phenotypes were not measured at the same time (see the section "Methods"). As we had predicted, the AGs of Control males were 34% smaller when raised on poor rather than standard diet. Because the AGs were measured in terms of area on two-dimensional images, the actual difference in volume was likely proportionally larger; a rough rescaling assuming volume ~ area$^{3/2}$ would suggest a 46% reduction in AG volume. In contrast, the wing length was only reduced by 9%; assuming wing area ~ wing length^2, this agrees well with previously reported 17% reduction in wing area (Vijendravarma et al., 2011). Thus, AG size was more strongly affected by larval undernutrition than wing size. However, AG size is not more affected than overall body size; although we did not measure it in this study, body mass and total body protein content of poor-diet raised flies are less than half of those of flies raised on standard diet (Erkosar et al., 2025; Kolss et al., 2009). A reduction in AG size under our poor larval diet is consistent with previously reported effects of larval crowding conditions (Kapila et al., 2022; Wigby et al., 2016). The latter are most likely mediated by nutrient limitation because higher

larval density without nutrient limitation does not lead to smaller AG size (Bretman et al., 2016; Patlar et al., 2025). The plastic reduction in AG size in our study is also consistent with the relationship between juvenile diet and investment in AG and traits such as seminal fluid quantity or testes size observed across other arthropods (Macartney et al., 2019).

In line with the reduction of AG size in males raised on poor diet, we expected a globally reduced investment in SFP that would be reflected in a lower expression of SFP genes. Instead, we observed a complex response: males raised on the poor diet showed a marked increase in relative expression of the two genes involved in sperm transfer and storage (*Acp36DE* and *Acp62F*) and concomitant similarly large decrease in expression of genes known for their action on the female nervous system and affecting their physiology and behavior (*Acp26Aa/ovulin* and *SP*). While translational and posttranslational regulation can decouple differences in mRNA abundance from protein production, these mechanism mainly appear to serve to accelerate responses to changes in conditions (Liu et al., 2016). At a steady-state, which we expect for the synthesis of secreted proteins by specialized glands, typically 50%–80% of variation in protein abundance is explained by variation in mRNA abundance (Liu et al., 2016; Riba et al., 2019). Thus, it is likely that these shifts in SFP expression do reflect at least roughly changes in SFP production, implying a shift in the relative composition of the SFP secretions.

Contrasting effects of larval nutritional environment on the expression of different SFPs appear not to have been reported. (Wigby et al., 2016) found that males raised under high larval density exhibited reduced abundance of *Acp26Aa* and *SP* proteins in AG (quantified by ELISA). This is consistent with the reduced expression of corresponding genes we observed, but the SFPs that increased in expression in our study were not measured in that study. In a contradiction to our results, Patlar et al. (2023) found no effect of larval density on the expression of three SFPs, including *Acp36DE* and *SP*, although the degree of nutritional limitation in that study was rather mild. Similarly, using a proteomic approach, (von Hellfeld et al., 2025) found that high larval density did not significantly affect the composition of the SFP proteome inside the AG. However, they found that larval diet differentially affected the composition of SFPs transferred to the females, as did (Zeender et al., 2023), while Hopkins et al. (2019) reported such changes in response to male-male competition. Such adjustments of ejaculate SFP composition are expected to be accompanied by shifts in the relative rates at which different SFPs are synthesized, consistent with contrasting changes in SFP gene expression such as those we found.

Despite their highly reduced body and AG size, poor-diet raised Control males had as high reproductive potential and similar sperm defense ability as those raised on standard diet and even produced a greater number of offspring from a single mating to a virgin female. We do not have any evidence linking this to changes in SFP expression; if anything, an association of reduced expression of *Acp26Aa* and *SP* with increased induction of female fecundity is counterintuitive, given their role in stimulating egg production and laying. However, while we did not measure SFP transfer, several studies report that males developed under nutritional stress transfer a greater fraction of their SFP stores per mating (although not necessarily a greater absolute volume) (von Hellfeld et al., 2025; Wigby et al., 2016; Zeender et al., 2023). This should lead to faster depletion of SFPs in poor-diet males, consistent with the steeper decline of number of offspring per inseminated female between the first and second mating period in our assay of reproductive potential. Our findings together with those cited above thus support the notion that nutritional stress during the larval stage induces shifts in relative investment in different SFPs and their allocation per ejaculate in ways that alleviate the developmental consequences of undernutrition (Hopkins et al., 2019).

The apparent absence of negative effects of developmental nutritional stress on postcopulatory performance under developmental nutritional stress in our study parallels the findings by De Nardo et al. (2021) and Patlar et al. (2023). Some other studies (e.g., Amitin & Pitnick, 2007; McGraw et al., 2007) did find such negative effects; discrepancies between studies are likely due to the details of experimental protocols or the genotypes of the flies. The apparent effort to preserve postcopulatory performance despite suffering larval undernutrition in our Control populations is likely to trade-off with other fitness traits, in particular competitive mating success, as has been reported by De Nardo et al. (2021). Still, the fact that the poor-diet raised males could maintain performance in key aspects of postcopulatory sexual selection despite great reduction in body and AG size is remarkable.

Evolutionary adaptation to poor larval diet

In spite of being genetically adapted to the poor larval diet and able to develop and grow on it considerably faster than Controls (Cavigliasso et al., 2023), the Selected males raised on poor diet had even smaller AGs than poor diet-raised Controls (24% smaller in AG area, or 34% in volume assuming volume $\sim$ area$^{3/2}$). This is a greater reduction than would be explained by body size: much of the difference remains when wing length is used as covariate. Furthermore, on the poor diet Selected flies are only 13% smaller in terms of whole-body protein content (Erkosar et al., 2025). This implies a reduction not only in terms of absolute but also in relative investment in the development of AG. Combining the phenotypically plastic and evolutionary response to the poor diet, Selected males raised on the poor diet had AGs only of about 35% of the volume of AG of Control males raised on standard diet.

Even though the volume of AG increases over the first few days postemergence (Koppik et al., 2018), this increase mainly reflects accumulation of SFPs—cell division in AG ceases before emergence (Box et al., 2024). Thus, the smaller AG size of males from the Selected populations is largely determined during larval and pupal stage, reflecting reduced allocation of metabolic resources during development. This result parallels the reduced number of ovarioles in the ovaries of Selected females compared to Controls (Erkosar et al., 2023), which is also determined during larval stage (Bergland et al., 2008). We cannot exclude that these changes are nonadaptive pleiotropic consequences of selection on other traits or genes. However, they are consistent with the hypothesis that selection on the Selected larvae to develop under extreme nutrient limitation favored diversion of resources from the developing reproductive structures (AG in

males, ovaries in females), likely to allocate them to other structures and functions. The fact that the evolutionary response of AG size was in the same direction as its phenotypic plasticity suggests that the phenotypically plastic reduction of AG size in response to undernutrition is also a part—possibly a cost—of an adaptive response (Ghalambor et al., 2007).

We also observed an evolutionary shift in SFP expression timing. Evolution under nutrient limitation led to lower expression of at least two SFPs by young virgin males, especially those raised on the poor diet, but it favored similar or higher expression after SFP stores were depleted by mating. While mature flies use exclusively nutrients obtained during the adult stage for reproduction, the reproductive effort of very young males is likely to depend on nutrients acquired during larval stage (this has been shown for *Drosophila* females; Aguila et al., 2013). Yet, following development on the poor diet, freshly eclosed Selected flies have depleted metabolic reserves; in particular their fat to protein ratio is 0.9 compared to 1.4 for Control (Erkosar et al., 2025). Availability of free amino acids is also likely lower because Selected populations depend to a greater degree than Controls on using amino acids as energy source to fuel metamorphosis (Cavigliasso et al., 2023). Even after 3 days of feeding on standard diet Selected flies (females) are depleted for most essential amino acids relative to Controls (Erkosar et al., 2023). Furthermore, gene expression data also suggest that Selected flies invest more heavily than Controls in cuticle and trachea development during the first days of adult life (Erkosar et al., 2023), presumably because these structures are less mature upon emergence. This would leave freshly eclosed Selected males little metabolic resources to invest in SFPs before the male has sufficiently replenished them by feeding as adult, offering a hypothetical explanation for the reduced expression of some SFPs by young Selected males.

Yet, despite much smaller AGs and the reduced initial expression of at least some SFPs, poor-diet raised Selected males were not significantly inferior to poor diet-raised (or standard diet-raised) Control males for any of aspect of postcopulatory performance that we investigated, with trend in either direction depending on the trait. Whether this is in part mediated by the differences in SFP expression is unclear; many aspects of adult physiology and metabolism differ between Selected and Control adults raised on the poor diet (at least in females; we do not have data for males) (Erkosar et al., 2023, 2025). Notably, the rate of protein synthesis, including SFP, not only depends on gene expression (mRNA abundance); it may be limited by the availability of ribosomes, amino acids or ATP. Still, in contrast to what we found in males here, the reproductive output in poor-diet raised Selected females is reduced by one third compared to poor-diet raised Controls, more than what could be explained by the 8% reduction in ovariole number (Erkosar et al., 2023). Thus, with the caveat that we only examined a few aspects of male reproductive performance, these results suggest that Selected males were mostly spared the tradeoff between tolerance to larval undernutrition and adult reproductive performance that we observe in females. It should be noted, however, that our results appear to contradict those from an experimental evolution study of adaptation to larval crowding in *D. melanogaster*. There, no differences in AG size were found between populations evolved under high versus low density (Kapila et al., 2022), but the former showed a reduced success in sperm defense (Kapila et al., 2025). Thus, the consequences of adaptation to larval nutritional stress may depend on the specific nature of the stress (nutrient-poor diet versus overcrowding), the specific composition of the diet, the initial gene pool, and/or on the details of the protocol. What is consistent between these two experimental evolution studies is that changes (or their absence) in AG size did not predict changes (or their absence) in postcopulatory performance; a similar discrepancy has been found in populations evolved under different mating systems (Hollis et al., 2019).

Evolution of phenotypic plasticity

Our factorial design allowed us to compare the plastic response of Control and Selected populations to larval diet. We found that the differences in AG size and SFP expression between Selected and Control males raised on standard diet were similar to those observed on the poor diet. This implies that adaptation to the poor diet did not specifically act on the response to nutrient shortage but has largely been mediated by mechanisms that are independent of nutritional conditions (i.e., mainly affected the elevation rather than slope of the reaction norms). This result parallels the largely additive effects of the evolutionary regime and current developmental diet on genome-wide gene expression and metabolite abundance observed in these populations (Erkosar et al., 2023). For performance traits, we have some evidence that the Selected males raised on standard diet are inferior to Control males, both for reproductive output of mating with surplus females and inducement of oviposition following a single mating. This likely reflects evolutionary trade-offs of adaptation to the poor diet, of which we have detected several instances for other fitness-related traits (Erkosar et al., 2023; Kolss et al., 2009).

Nonetheless, the proportional magnitude of the differences in AG size and in part in SFP expression was larger for males raised on poor than on standard diet. Similarly, at the individual level, AG size decreased with decreasing wing size more steeply in Selected than Control males raised on the poor diet. Thus, rather than being better buffered against nutrient shortage, the investment of Selected male larvae in developing AG evolved to be more sensitive to individual condition/resource availability than that of Controls (at least relative to the sensitivity of the wing size). This finding to some degree resembles a result reported from another evolution experiment, where females evolved on a poor adult diet showed a greater sensitivity in their ovariole number to poor larval diet than females not adapted to nutrient restriction (Bath et al., 2023). It is unclear whether this reflects an adaptive change in allocation strategy or is a byproduct of other changes in body architecture resulting e.g., from differences in the rate of growth and development.

Conclusions

Overall, our results suggest that both phenotypically plastic and evolutionary responses to larval undernutrition in holometabolous insects can go a long way to protect male postcopulatory performance against the consequences of nutrient shortage, even if the size of a key organ—the AGs—is strongly affected. As a corollary, they also suggest that when it comes to male sexual success, the size of AGs or other rel-

evant organs is not necessarily a reliable predictor of post-copulatory success.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

Data are available at https://doi.org/10.5281/zenodo.19055654.

Author contributions

T.J.K. and B.C.D. conceptualized the research; B.C.D. performed all fly experiments and dissections; C.L.M. performed RNA extraction and RT-qPCR; B.C.D., Y.H., and T.J.K. curated the data, performed the statistical analysis, and wrote the paper.

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Conflict of interest

The authors declare no conflict of interest.

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