Evolution under juvenile malnutrition impacts adult metabolism and fitness

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Keywords: experimental evolution, dietary adaptation, Drosophila, metabolome, thrifty phenotype, developmental programming, nutrient restriction, trade-off

Abstract

Juveniles are often first to suffer from nutrient shortage, and juvenile undernutrition is likely an important force of natural selection shaping animal physiology, with consequences potentially extending into adulthood. We combined RNAseq, targeted metabolomics and genomics to study the consequences of experimental evolution under juvenile undernutrition for metabolism of reproductively active adult females of *Drosophila melanogaster*. Compared to six Control populations maintained on standard diet, six Selected populations evolved for over 230 generations on a nutrient-poor larval diet showed major changes in adult gene expression and metabolite abundance. In particular, Selected flies were relatively deficient in essential amino acids and purine nucleotides, but showed overabundance of several non-essential amino-acids involved in purine synthesis and overexpression of multiple enzymes catalyzing this pathway. Selected flies also accumulated medium-chain acylcarnitines suggestive of congestion in beta-oxidation, possibly linked to deficiency of electron transporters. Some aspects of the metabolic profile of Selected flies resembled that of flies subject to starvation. Furthermore, differences between Selected and Control populations in adult gene expression were in general positively correlated with differences in larval expression, consistent with pleiotropy in gene regulation between the life stages. Finally, Selected flies were less fit in terms of fecundity than Controls even when both were raised under the conditions under which the Selected populations evolved. These results suggest that evolutionary adaptation to juvenile undernutrition has large pleiotropic consequences for adult metabolism, and that they are costly rather than adaptive for adult fitness.

Introduction

Nutrient shortage is an important and widespread ecological stressor for animals, and a driver of natural selection on animal physiology. Juveniles are often the first to suffer from undernutrition, as they are usually competitively inferior to adults. Prolonged nutrient shortage at a juvenile stage usually results in reduced adult size, but it also often has profound effects on adult physiology that do not seem mediated by smaller body size. These effects include changes in epigenetic marks (Lillycrop and Burdge 2015), gene expression (May and Zwaan 2017; Szostaczuk, et al. 2020) metabolome (Agnoux, et al. 2014), triglyceride accumulation (Lukaszewski, et al. 2013; Klepsatel, et al. 2020), blood pressure (de Brito Alves, et al. 2014), behavior (Akitake, et al. 2015) and longevity (Davis, et al. 2016). In humans, prenatal undernutrition is correlated with negative consequences for diverse aspects of metabolic function and health at old age (Roseboom, et al. 2006; de Rooij, et al. 2010).

In contrast to such phenotypically plastic within-generation responses, we know much less about how adult physiology changes over evolutionary time in response to natural selection driven by repeated exposure to juvenile undernutrition. In a few experimental evolution studies on *Drosophila* evolutionary adaptation to poor larval nutrition (nutrient-poor diet or larval crowding) was associated with changes in adult traits such as fecundity (May, et al. 2019), lifespan (Shenoi, et al. 2016; May, et al. 2019), starvation resistance (Kawecki, et al. 2021), pathogen resistance (Vijendravarma, et al. 2015) or heat tolerance (Kapila, et al. 2021). However, changes in gene expression and metabolism underlying these correlated responses of adult phenotypes to selection for larval undernutrition tolerance have not been explored. Insights into if and how selection driven by juvenile undernutrition shapes adult metabolism would shed light on the importance of development for understanding adult physiology, with potential implications for human health (Neel 1962; Prentice, et al. 2005).

We used experimental evolution (Kawecki, et al. 2012) to study the consequences of evolutionary adaptation to juvenile (larval) undernutrition for gene expression, metabolite abundance and reproductive performance of adult *Drosophila melanogaster* females. We compare six replicate populations (the "Selected" populations) evolved for over 230 generations on a very nutrient-poor larval diet, to six populations of the same origin maintained on a standard larval diet (the "Control" populations). Importantly, in the course of experimental evolution flies of both sets of populations were always transferred to fresh standard diet soon after emergence, maintained on this diet for 5-6 days, and additionally fed live yeast before reproduction. Thus, every generation of Selected populations experienced a switch from the poor larval diet to a standard adult diet. Compared to the Controls, larvae of the Selected populations evolved higher viability, faster development and faster growth on the poor diet (Kolss, et al. 2009; Vijendravarma and Kawecki 2013; Cavigliasso, et al. 2020). The Selected larvae invest more in protein digestion (Erkosar, et al. 2017), are more efficient in extracting amino acids from the poor diet (Cavigliasso, et al. 2020), and are able to initiate metamorphosis at a smaller size (Vijendravarma, et al. 2012); the traits that likely contribute to their improved performance on the poor diet. The Selected populations also evolved reduced dependence on microbiota for development on poor diet (Erkosar, et al. 2017) and a greater susceptibility to an intestinal pathogen both as larvae and adults (Vijendravarma, et al. 2015); as adults they are less resistant than Controls to starvation (Kawecki, et al. 2021). Evolutionary divergence between Selected and Control populations is highly polygenic, involving over 100 genomic regions enriched in hormonal and metabolic genes (Kawecki, et al. 2021).

In this study we address the following questions about the consequences of the evolutionary divergence between the Selected and Control populations for the adult stage:

First, do the Selected and Control populations show major differences in adult gene expression and metabolite abundance, implying that the physiological consequences of evolution under larval undernutrition extend to the adult stage? If yes, which pathways are affected?

Second, do these genetically-based evolutionary responses resemble aspects of the phenotypically plastic responses of adult metabolism to poor larval diet? Have the plastic responses themselves evolved? Plastic responses that program metabolism adaptively for poor nutritional environment in adulthood would be misdirected in the Selected populations because the adults were switched to standard diet; evolution should thus counteract them. Conversely, plastic responses that alleviate the consequences of having developed on a poor diet irrespective of adult diet should have become amplified as a result of evolution on the poor diet.

Third, are differences in gene expression between Selected and Control adults similar in terms of genes affected and direction of change to those previously reported for larvae (Erkosar, et al. 2017)? Such similarity would suggest that genetic variants underlying adaptation to larval diet have pleiotropic effects of on gene expression at the two life stages, and thus that their physiology is not free to evolve independently.

Fourth, do the metabolic profiles of Selected and Control flies differ along a similar axis as the profiles of fed versus starving flies? Selected flies are less resistant to starvation than Controls and the genomic architecture of divergence between them shares many genes with starvation resistance (Kawecki, et al. 2021). This question addresses the link between these two findings, and the answer would aid in functional interpretation of metabolic changes evolved by the Selected populations.

Finally, do adult flies of the Selected perform better in terms of fitness than Controls when both are raised on the poor larval diet? This would be expected if most changes in their adult physiology evolved because they improve performance of adults raised on poor diet. In contrast, if the metabolic changes observed at the adult stage primarily reflect pleiotropic effects of genetic variants that improve larval growth and survival on the poor diet, Selected adults should not have an advantage or even be inferior to Controls even when raised under the conditions under which the former evolved.

Results

Gene expression patterns point to evolutionary changes in metabolism

To explore long-term (evolutionary) consequences of nutrient-poor larval diet on adult metabolism and compare them to within-generation (plastic) responses, we studied the gene expression patterns of 4 day old adult females from the six Selected and six Control populations, each raised on either poor or standard larval diet. These females were transferred to standard diet upon emergence from the pupa and maintained in mixed-sex group; thus, they were mated and reproductively active. We focused on the abdominal fat body, the key metabolic organ combining the functions of mammalian liver and adipose tissue. Because a clean dissection of adult fat body is difficult, we performed RNAseq on "carcass", consisting of the fat body together with the attached abdominal body wall and any embedded neurons, but excluding the digestive, reproductive and most of the excretory organs.

Based on the estimation of true nulls (Storey and Tibshirani 2003), the evolutionary regime (i.e., Selected versus Control) affected the expression of about 26 % of all 8701 genes included in the analysis while the current larval diet affected about 45%. Allowing for 10% false discovery rate (FDR), we identified 219 genes as differentially expressed between Selected and Control populations and 827 between flies raised

on standard or poor diet (Supplemental Table S1). The magnitude of the changes in gene expression was moderate, with the significant genes showing on average about 1.76-fold difference (in either direction) between Selected and Control populations and about 1.30-fold difference between flies raised on poor versus standard diet. While first three principal components did not differentiate the evolutionary regimes or larval diets (not shown), PC4 and PC5 did (Figure 1A). The divergence of gene expression patterns between Selected and Control populations as well as between flies raised on poor and standard diet was supported by MANOVA on the first five principal components, jointly accounting for 67% of variance (Wilks' $\lambda = 0.052$, $F_{6,5} = 15.1$, $P = 0.0046$ for regime and Wilks' $\lambda = 0.057$, $F_{6,5} = 13.9$, $P = 0.0055$ for diet). We detected no interaction between the evolutionary regime and current diet either in the PCA (Wilks' $\lambda = 0.59$, $F_{6,5} = 0.6$, $P = 0.41$) or in the gene-by-gene analysis (100 % of genes estimated as true nulls for the interaction, lowest $q = 99.9\%$). Thus, both the evolutionary regime and the larval diet experienced within the same generation had substantial effects on adult gene expression patterns, but these effects were largely independent of each other (i.e., they were additive).

The strongest statistical signal from gene ontology (GO) enrichment analysis on genes differentially expressed between Selected and Control flies points to genes involved in cuticle development and maturation, including several structural cuticle proteins (Fig. 1B, Supplemental Table S2A). Most of these genes are expressed in epidermis (including in the tracheal system), and nearly all show higher expression in the Selected compared to Control populations. This may reflect differences in the relative amount of the epidermal tissues or in the rate of cuticle maturation. Several other enriched GO terms (phagocytosis, pigment metabolic process, iron ion homeostasis, response to fungus) include multiple genes expressed mainly in hemocytes; these genes are downregulated in Selected flies, suggesting lower abundance of hemocytes.

The other top enriched GO terms point to metabolic processes, notably oxidation/reduction, biosynthesis of purine compounds and amino acid catabolism. Being defined by the type of catalyzed reaction rather than biological function, the oxidative/reductive GO term includes a diverse set of differentially expressed genes, including some involved in amino acid and purine metabolism. The products of several of genes in this GO term, both up- and down-regulated, are involved in mitochondrial function. In addition this GO term includes several cytochromes involved in synthesis (*shd*) and degradation (*Cyp6a9*, *Cyp9h1*, *Cyp6d5*, *Cyp6a20*) of insect hormones, all downregulated in the Selected populations.

Differentially expressed genes involved in amino acid catabolism included enzymes involved in catabolism of arginine (*Oat*), serine (*Shmt*), branched-chain amino acids (*CG17896, CG6638*, *Dbct*), tryptophan (*Trh*), asparagine (*CG7860*) and tyrosine (*CG1461*). Further genes coding for enzymes involved in cysteine (*CG5493*), phenylalanine (*Dhpr*), proline (*slgA*) and methionine (*CG10621*) metabolism were differentially expressed at nominal $P < 0.01$ but did not pass the 10 % FDR. Thus, rather than being centered on a specific pathway, the signal of enrichment in amino acid metabolism was based on multiple differentially expressed genes distributed over multiple branches of amino acid metabolism. A few more genes involved in amino acid catabolism overexpressed in the Selected populations link the aspartate-glutamate metabolism with the synthesis of purine compounds, which also showed a strong signal of enrichment in differentially expressed genes. We examined evolutionary changes in aspartate-glutamate and purine metabolism in more detail below.

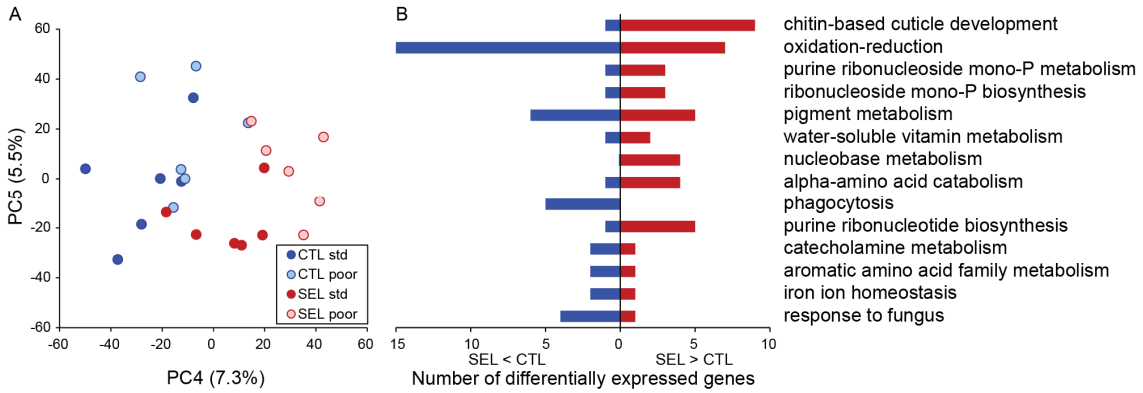

Figure 1. Adaptation to larval undernutrition leads to divergence in gene expression patterns in carcasses of adult females. (A) Sample score plot of the 4th and 5th principal components on the expression levels of 8701 genes of flies from Selected (SEL) and Control (CTL) populations raised on poor and standard (std) larval diet. Each point represents a replicate population × diet combination. (B) Biological process GO-terms enriched (at $P < 0.01$) in genes differentially expressed between Selected and Control populations, with the number of genes upregulated (red) and downregulated (blue) in Selected populations. For details of the GO-term enrichment results see Supplemental Table S2.

Plastic response of gene expression to larval diet does not predict evolutionary response

As for genes differentially expressed between Selected and Control populations, the top GO term enriched for genes differentially affected by the larval diet treatment was "chitin-based cuticle development" (Table S2b). This is consistent with differences in body size: flies raised on the poor diet are much smaller than those raised on the standard diet (Kolss, et al. 2009), and thus likely characterized by a greater surface-to-volume ratio. This is, however, the only similarity between the effects of evolutionary regime and of the diet treatment for most enriched GO-terms. No GO terms related to metabolism of amino acids, purines or any other basic metabolic processes were significantly enriched for the effect of diet treatment. Instead, genes differentially affected by the diet treatment were enriched for many terms related to development, reproduction, nervous system and behavior (Supplemental Table S2B).

This absence of parallelism between the evolutionary (genetically-based) and within-generation (phenotypically plastic) responses to the poor larval diet is also visible at the level of individual genes. Although the number of genes that are differentially expressed at 10 % FDR for both of those factors (42) is greater than the 20.8 expected under independence (Fisher's exact test $P < 0.0001$), at least 18 of them are involved in integumentary (i.e., cuticle, epidermis or tracheal) development. Most of the remaining ones play a role in the nervous system or transmembrane transport. Of the genes involved in amino acid and purine metabolism that are differentially expressed at 10 % FDR between Selected and Control files, *Pfas* (an enzyme in purine synthesis) is the only one that is also differentially expressed between diet treatments (poor > standard). Of 235 genes that pass the less stringent criterion of being differentially expressed at nominal (not adjusted) $P < 0.05$ for both factors, 123 show the same sign of expression difference between poor and standard diet as between Selected and Control populations, and 112 show the opposite sign, not different from what would be expected at random ($P = 0.13$, Fisher's Exact Test). Thus, except for genes involved in the cuticle maturation, the plastic (within-generation) response of adult gene expression pattern to larval undernutrition did not predict the evolutionary, genetically-based response.

Much of evolutionary change in gene expression is conserved between life stages

The Selected and Control populations evolved under different larval diets but the adult diet they experienced was the same. Thus while the divergence between them in larval gene expression might be interpreted as driven by immediate nutrient availability, selection at the adult stage likely resulted from the need to compensate for carry-over effects of larval diet. We thus used previously published data on whole-body gene expression divergence in the larvae (Erkosar, et al. 2017) to explore the degree to which the divergence in gene expression between Selected and Control populations is correlated between the two life stages. Of 424 genes that were differentially expressed at both stages at nominal $P < 0.05$, 377 (89%) showed the same direction of change, more than expected at random (Fisher's Exact Test, $P < 0.0001$). Eighty-four genes passed more stringent criterion of 10 % FDR at both stages; of those, 78 (93%) showed the same direction of change in the two stages (Fisher's Exact Test, $P < 0.0001$). The estimates of log-fold change were even correlated across all 8437 genes shared between the data sets ($r = 0.42$, $P < 0.0001$). Thus, many of the differences between Selected and Control populations in the adult gene expression patterns paralleled those observed in the larvae. This is despite the fact that the larval RNAseq was performed on the whole body and without sexing the larvae.

There were, however, also notable differences. In particular, the enrichments in cuticle development, purine biosynthesis and amino acid catabolism are specific to genes that are differentially expressed in the adults but not in the larvae (Table S2D). Genes that are differentially expressed both in the adults and in the larvae are enriched in several terms related to transmembrane transport and iron homeostasis (Table S2C). Conversely, fatty acid beta oxidation and insulin signaling pathway show a strong signature of differentiation between Selected and Control larvae (Erkosar, et al. 2017; Cavigliasso, et al. 2020), but we found no differentiation for any of those genes in the adults.

Thus, while for many genes evolutionary adaptation to larval diet has led to correlated changes in expression in larvae and adult flies, changes linked to some physiological processes were apparently specific to either the larval or the adult stage.

Metabolome analysis points to evolutionary changes in amino acid and purine metabolism

Differences in gene expression between Selected and Control flies pointed to the consequences of diet adaptation for adult metabolism. We therefore used a broad-scale targeted metabolomics approach to measure the abundance of key polar metabolites involved in multiple core pathways in central metabolism. As for gene expression, the analysis was carried out on the fat body with some adjacent cuticle and neural tissue (the "carcass") of 4 day old females from Selected and Control population, each raised on either poor or standard diet. Additionally, we also performed metabolome analysis on carcasses of flies that were subject to 24 h of starvation, resulting in a 3-way design (2 evolutionary regimes each with 6 populations × 2 current larval diets × fed vs starved flies, with 2 biological replicates, a total of 96 samples). After filtering for data quality, we retained 113 metabolites that were measured (normalized to protein content) in all samples. We first describe the effects of evolutionary regime and current larval diet on the metabolome of fed flies; the effect of starvation is described later.

The first two principal components extracted from the metabolome data clearly differentiated the Selected versus Control populations, as well as flies raised on poor versus standard diet (Fig 2; MANOVA on PC1

and PC2 scores, Wilks' $\lambda = 0.185$, $F_{2,9} = 19.8$, $P = 0.0005$ and Wilks' $\lambda = 0.055$, $F_{2,9} = 77.3$, $P < 0.0001$, respectively). Of the 113 metabolites, 57 were found to be differentially abundant between Selected and Control flies at 10% FDR, and also 57 differentially abundant between flies raised on poor versus standard diet. We found no interaction between these two factors either in the univariate analysis (no metabolite passing 10% FDR, lowest $q = 0.39$) or in the PCA (MANOVA, Wilks' $\lambda = 0.918$, $F_{2,9} = 0.4$, $P = 0.68$). Thus, both the evolutionary history of exposure to poor versus standard diet and the within-generation diet treatment had major effects on the metabolome, but, as for gene expression, these effects were largely additive. Therefore, we present the results as a heat map corresponding to effects of these two factors on abundance of individual metabolites (the first two columns in Fig 2; the third column corresponds to the effect of starvation discussed in a later section).

We focused first on the evolutionary response (i.e., the difference between Selected and Control population). Evolutionary adaptation to larval malnutrition resulted in a striking shift in proteinogenic amino acid concentrations in adult flies: eight essential amino acids were less abundant in the Selected than Control flies, while the reverse was the case for four non-essential ones (glutamine, asparagine, serine and aspartate) (Fig 2A). No link is apparent between the differences in abundance of different amino acids and their role in energy metabolism as glucogenic versus ketogenic. However, three of these four overabundant amino acids are substrates in de novo purine synthesis (Scaraffia, et al. 2006; Salway 2018). Yet, multiple purine compounds were less abundant in the Selected than Control flies, including both purine nucleobases and nucleosides and the key electron carriers NAD, NADP and FAD; an exception is ADP, which was more abundant in the Selected flies (Fig 2B; ATP has not been reliably measured). No such general trend for reduced abundance was observed for pyrimidines (Fig 2C), implying that the pattern observed for purines is not mediated by changes in synthesis or degradation of RNA.

While fatty acids were not included in the targeted metabolome analysis, we did quantify the abundance of acyl-carnitines, i.e., fatty acid residues attached to carnitine, the molecule that transports them in and out of mitochondria. Compared to the Controls, the Selected flies show accumulation of medium chain (C6 – C12) acyl-carnitines (Fig 2D). In contrast, acyl-carnitines with long-chain (C16 – C18) residues typical for insect triglycerides (Stanley-Samuelson, et al. 1988) are less abundant in the Selected than Control flies (Fig 2D). Accumulation of medium-chain acyl-carnitines suggests a bottleneck in beta-oxidation; in humans it is a hallmark of medium chain acyl-CoA dehydrogenase (MCAD) deficiency, a disorder causing dangerous hypoglycemia during fasting (Reis de Assis, et al. 2004). However, none of the enzymes with predicted MCAD activity showed any differences in expression between Selected and Control populations, although one of the genes involved in the next step in beta oxidation (3-hydroxyacyl-CoA dehydrogenase *Had2*) was less expressed in Selected flies. Also overabundant in Selected flies was isovalerylcarnitine, which carries the residue of deamination of branched-chain amino acids (rather than an intermediate of beta-oxidation of fatty acids).

Selected flies had lower levels of trehalose (the principal sugar circulating in hemolymph) and of lactate than Controls (Fig 2E), but a higher concentration of UDP-glucose, an intermediate in the synthesis of trehalose and glycogen. They also showed an increased concentration of compounds linking glycerol with glycolysis (glycerol-3-phosphate, glyceraldehyde-3-phosphate and 2-/3-phosphoglycerate), plus a non-significant trend towards higher concentration of phosphoenolpyruvate, with no indication of differences in concentration of upstream and downstream glycolysis intermediates (Fig 2E). Together with an increase of glycerate this pattern suggests that the Selected flies may be catabolizing glycerol to a greater extent than Controls. The lower concentration of succinate in Selected flies is difficult to interpret, given the lack of differences in other TCA cycle intermediates (Fig 2E). It might be linked to the fact that of 18 genes coding for enzymes of the TCA cycle in the RNAseq data, the only gene that is significantly less

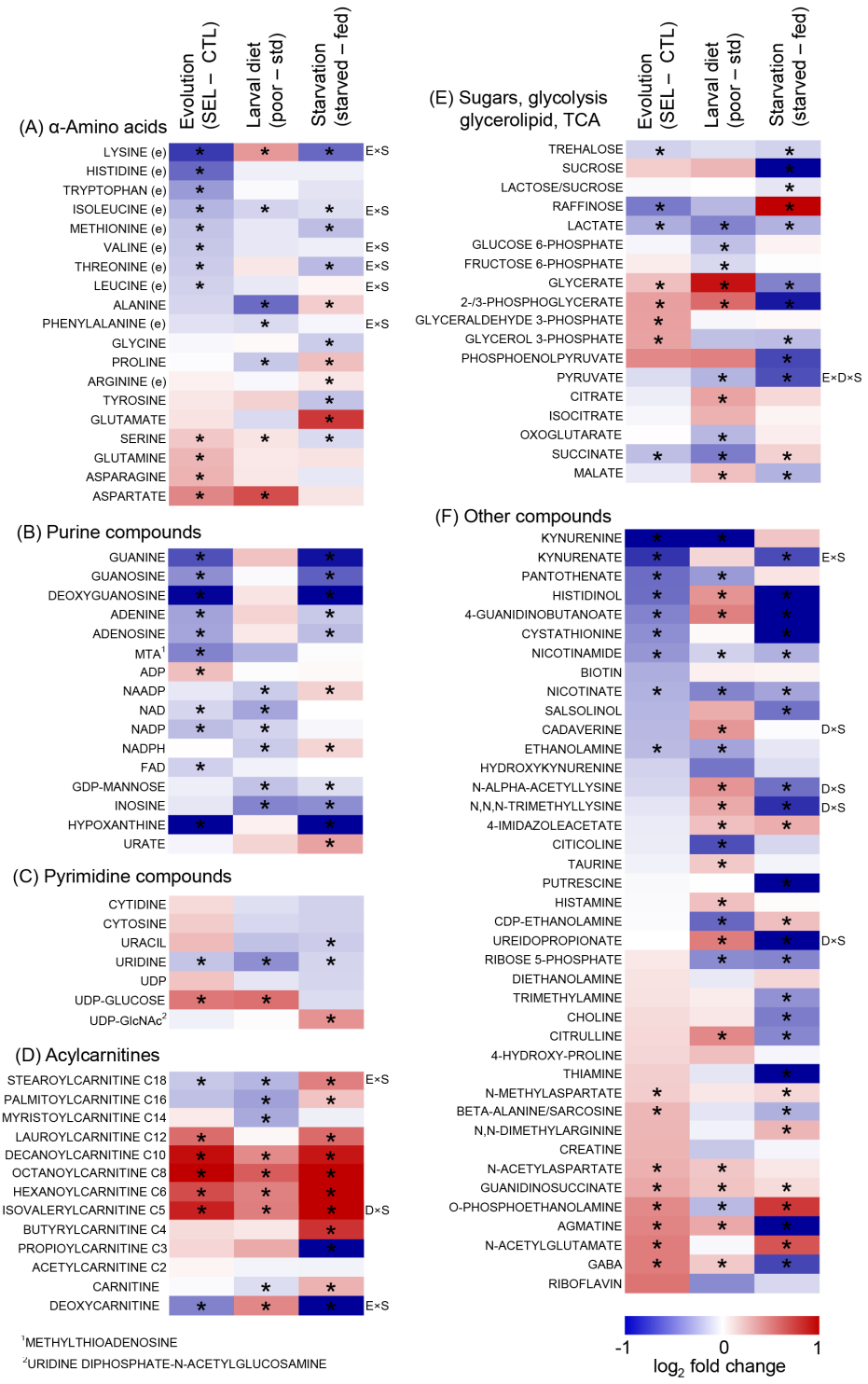

Figure 2. The effect of experimental factors on metabolite abundance. The first two columns of the heat maps indicate, respectively, the effect of evolutionary regime (least square mean difference Selected – Control) and the within-generation (phenotypically plastic) effect of larval diet (least square mean difference poor – standard diet) on the relative concentration of metabolites in fed flies. The third column indicates the main effect of the starvation treatment (least square mean difference between starved and fed flies) on metabolite concentrations in flies from both Selection regimes raised on both diets. Thus red (blue) indicates that a compound is more (less) abundant in Selected, poor diet-raised and starved flies than in Control, standard diet-raised and fed flies, respectively. Asterisks indicate effects significant at $q < 0.1$. Annotations to the right of the heat maps indicate interactions significant at $q < 0.1$: E×S = evolutionary regime × starvation treatment, D×S = larval diet × starvation treatment, E×D×S = three-way interaction; (e) indicates an essential amino acid, C2-C18 for acylcarnitines refers to the length of the acyl chain.

expressed in the Selected flies is *ScsbetaG*, a subunit of succinyl-coA synthetase that catalyzes a step in synthesis of succinate from alpha-ketoglutarate.

A number of other metabolites showed differential abundance between Selected and Control flies (Fig 2F). More than half of them are derivatives of amino acids. Compounds less abundant in Selected flies include kynurenine and kynurenate, tryptophan derivatives involved in the synthesis of NAD+; cystathionine (an intermediate in synthesis of cysteine from methionine), histidinol and ethanolamine (products of degradation of, respectively, histidine and serine), and vitamins involved in synthesis of coenzymes: nicotinate and nicotinamide (NAD+), and pantothenate (coenzyme A). Compounds more abundant in Selected than Control flies include several derivatives of glycine, arginine, aspartate and glutamate. Some of them (GABA, N-methylaspartate/NMDA and N-acetylaspartate/NAA, agmatine) are involved in neurotransmission; the others do not appear to be functionally linked. Differences in abundance of those amino acid derivatives between Selected and Control flies reinforce the notion that adaptation to the poor larval diet has been associated in wide-ranging changes in amino acid metabolism.

Some aspects of plastic response of metabolome are recapitulated by evolution

To what degree was the genetically-based evolutionary response of metabolome anticipated by the phenotypically plastic response to the diet experienced by the assayed individuals? Thirty metabolites were differentially abundant at 10% FDR for both evolutionary regime and diet treatment, close to what would be expected from a random overlap. However, 25 of the 30 had the same sign of the difference between Selected and Control flies as the difference between flies raised on the poor vs standard diet, significantly more than expected (Fisher's Exact Test, $P = 0.0005$). Thus, for metabolites that were affected by both the evolutionary and the plastic response to poor larval diet, the evolutionary response tended to reinforce rather than counteract the phenotypically plastic change. In particular, compared to flies raised on standard diet, flies raised on the poor diet showed a deficiency of long-chain acyl-carnitines but an excess of medium-chain ones (Fig 2D, middle column), and a deficiency of NAD+ and NADP (Fig 2B) and their precursors nicotinate and kynurenine (Fig 2F), paralleling the patterns in Selected relative to Control flies. Some of the plastic effects of the poor diet treatment on sugar metabolism were also similar to those of evolutionary response, notably a deficiency of lactate and succinate, and an excess of UDP-glucose and of 2-/3-phosphoglycerate and glycerate, the latter accentuated by the deficiency of upstream and downstream glycolysis intermediates (Fig 2E).

Other aspects of the plastic response to larval diet were not recapitulated in the evolutionary response. The plastic response affected the balance of different TCA cycle intermediates, two being more and two less abundant in flies raised on poor diet (Fig 2E). More than a dozen modified amino acids and products of amino acid degradation were also affected, but these changes were not correlated with corresponding differences between the Selected and Control flies. Although several proteinogenic amino acids showed differential abundance depending on the diet treatment, these differences were also not correlated to those due to the evolutionary regime, and did not seem to show a consistent relationship with the properties of the amino acids. Finally, with the exception of inosine, the plastic response to diet did not detectably affect the abundance of purine nucleobases and nucleotides that do not contain a nicotinamide group (Fig 2B). These results are consistent with the absence of signal of the effect of diet on amino acid and purine metabolism in gene expression patterns presented above.

The apparent absence of interaction between regime and diet treatment implies that the metabolome of the Selected and Control flies responds in the same way to the larval diet treatment, i.e., that the sign and magnitude of the plastic response to diet did not change as a result of the evolutionary adaptation.

Metabolic profile of Selected flies tends towards starved-like state

In addition to assessing the metabolome of flies directly sampled from the (standard) adult diet, we also assessed the metabolome of Selected and Control flies after they have been subject to 24 h of total food deprivation (moisture was provided). Our motivation was twofold. First, a comparison of fed and starved flies would reveal how the metabolome changes under an ongoing nutritional stress, and thus potentially help interpret metabolome changes driven by genetically-based adaptation to recurrent nutrient shortage at the larval stage (i.e., the effect of evolutionary regime). Second, adult flies from Selected populations are less resistant to starvation than Controls (Kawecki, et al. 2021). Hence, we expected that the metabolome of the Selected and Control populations might throw some light on the mechanisms underlying these differences in starvation resistance.

As expected, 24 h of starvation had a strong effect on fly metabolome. Principal component analysis cleanly separated starved from fed samples (MANOVA on the first two PC scores $F_{2,9} = 834.8$, $P < 0.0001$; Figure 3A, Supplementary Table S4). Seventy-four out of the 113 metabolites were detected to be differentially abundant (at 10% FDR) between fed and starved flies (in terms of the main effect of starvation treatment in the full factorial model, Supplementary Table S3). Of 39 metabolites affected by both starvation treatment and the evolutionary regime, 29 showed the same direction of change between fed and starved flies as between Control and Selected flies, significantly more than expected by chance ($P = 0.006$, Fisher's Exact Test). In particular, in parallel to Selected flies relative to Controls, starved flies showed a decrease in multiple purine metabolites and two forms of vitamin B3 (nicotinate and nicotinamide), and an increase in medium-chain acyl-carnitines (Figure 2B,D). For some other metabolites the effect of starvation differed markedly from that of the evolutionary regime. In particular, several intermediates of the glycolysis pathway were markedly less abundant in starved than fed flies (Fig. 2E) whereas the surplus of acyl-carnitines also applied to those with long acyl chains. This likely reflects a shift to catabolizing lipid stores for ATP generation following the depletion of carbohydrates.

The pattern for proteogenic amino acids also appeared rather different (Fig. 2A). However, the focus on the main effects of starvation treatment on amino acid abundance is somewhat misleading because for six of the ten essential amino acids the response to starvation differed between the Selected and Control flies. For all six the sign of the interaction was the same: while the levels of all these amino acids decreased in Control flies when they starved ($q < 0.1$), their abundance declined less (lysine), remained similar (threonine, isoleucine) or even increased (phenylalanine, leucine, valine; $q < 0.1$) in starving Selected flies (Fig 3B). The other three metabolites with significant regime × starvation interaction (kynurenine, stearoylcarnitine, deoxycarnitine) showed different patterns (Figure 3B). There were also six metabolites with a significant larval diet × starvation or an interaction among all three experimental factors; they did not appear to be functionally connected or to share a common pattern (Supplemental Figure S4).

These results imply that evolutionary adaptation of the Selected populations to larval undernutrition had consequences for the way their essential amino acid metabolism responds to nutrient shortage at the adult stage. Furthermore, even though in a normal fed state the Selected flies appeared deficient in most essential amino acids, they could maintain or increase their levels during 24 h of food deprivation. This suggests that their low concentrations in the fed state are not due to their absolute shortage but to differential allocation and use. In total, abundance of seven essential amino acids declined significantly upon starvation in Control flies, making the parallel with the difference between Selected and Control flies in fed state more evident. Indeed, the evolved differences in abundance of all proteogenic amino acids between Selected and Control flies were significantly positively correlated with the presumably

ancestral response of standard diet-raised Control to starvation; the same was the case for purine compounds and acyl carnitines (Figure 3C).

Differential response of the Selected and Control populations to starvation is supported by MANOVA on the first two PC scores (regime × starvation interaction $F_{2,9} = 8.4$, $P = 0.0089$). Also, PC1 scores of fed flies from Selected populations were lower (i.e., closer to starved flies) than those of fed flies from the Controls (Figure 4A; ANOVA on PC1 scores from fed flies, $F_{1,10} = 8.9$, $P = 0.014$). This further supports the notion that some aspects of the adult metabolic profile of Selected populations evolved in the direction resembling effects of starvation.

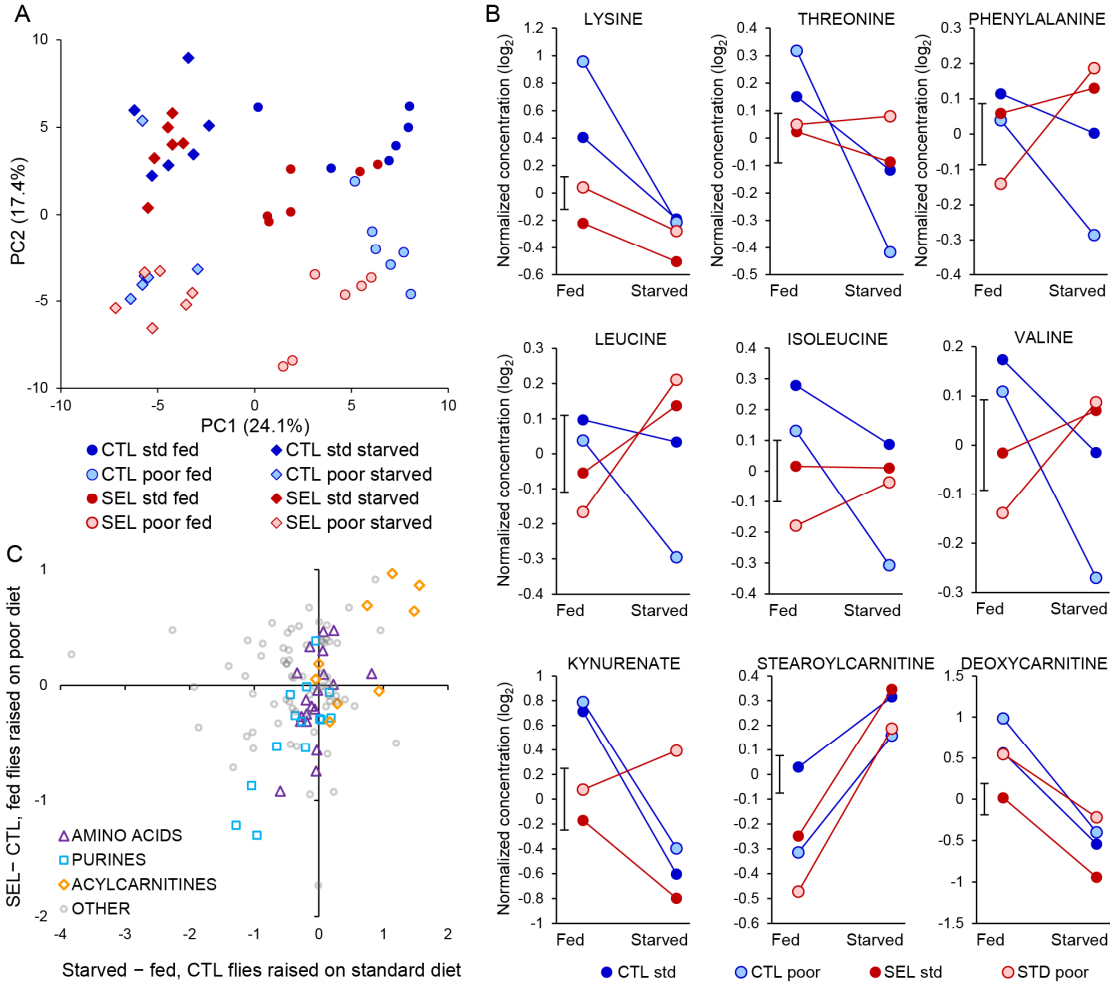

Figure 3. (A) Principal component score plot on metabolite abundance for all conditions (Selected vs Control, poor vs standard larval diet, fed vs starved flies). The two replicate samples from the same population × diet × starvation treatment combination were averaged. (B) Metabolites that show significant ($q < 0.1$) interaction between the evolutionary regime and starvation treatment. Plotted are least-square means obtained from the full factorial general mixed model. The bar next to the Y-axis indicates the standard error of the means (identical for all treatments as they were estimated from the model). (C) The relationship between the effect of 24 h starvation (starved minus fed flies) on metabolite abundance in Control flies raised on the standard diet and the evolutionary change (Selected minus Control) quantified in fed flies raised on poor diet. Three categories of metabolites of interest are highlighted; "acylcarnitines" only include those with even-numbered acyl chain (resulting from catabolism of triglycerides) but not those with 3C and 5C chains (which are products of amino acid catabolism). Pearson's correlations: amino acids $r = 0.50$, $N = 19$, $P = 0.030$; purine compounds $r = 0.76$, $N = 16$, $P = 0.0006$; acylcarnitines $r = 0.74$, $N = 9$, $P = 0.022$; other metabolites $r = 0.02$.

Genomic, transcriptomic and metabolomic imprint on purine synthesis

In an attempt to integrate metabolite, gene expression and genome sequence changes associated with adaptation to the poor diet we performed joint pathway analysis in Metaboanalyst v. 5.0 (Xia, et al. 2009). In addition to the metabolites differentially abundant and genes differentially expressed between the Selected and Control populations, we included in this analysis 771 genes previously annotated to single nucleotide polymorphisms (SNPs) differentiated in frequency between these two sets of populations (Kawecki, et al. 2021). This analysis confirmed that changes in amino acid and purine metabolism contributed to adaptation to poor larval diet (supplemental table S5). While most of the nine pathways identified in this analysis were only or mainly enriched in differentially abundant metabolites, two – purine metabolism and alanine, aspartate and glutamate metabolism – involved a mix of metabolites and expression and SNP candidate genes (supplemental table S5).

We therefore examined the evolutionary changes in these two pathways (imported from the KEGG database; (Kanehisa and Goto 2000)). The alanine-aspartate-glutamate pathway contains multiple links among three of the four amino acids that are overabundant in the Selected flies. However, few of the many enzymes involved in those links showed a signal of differentiation between Selected and Control lines (Figure 4A, supplemental table S3). In contrast, purine metabolism showed a striking pattern of upregulation of multiple key enzymes involved in de novo purine synthesis in the Selected population, combined with overabundance of the three amino acids (glutamine, serine and aspartate) that act as donors of four nitrogen atoms that form the basic purine structure (Kastanos, et al. 1997; Salway 2018) and provide (aspartate) a further amino group in the synthesis of AMP from IMP (Figure 4B). Despite this, compared to the Control flies, the Selected flies appeared depleted of nucleosides and nucleobases derived from IMP (the purine-nucleoside phosphorylase CG16758 that catalyzed conversion between them was overexpressed). We also found that multiple genes involved in AMP-ATP-cAMP conversion were associated with candidate SNPs differentiated in frequency between the Selected and Control populations. While difficult to interpret functionally, this adds to the evidence that selection driven by the dietary regime targeted purine metabolism.

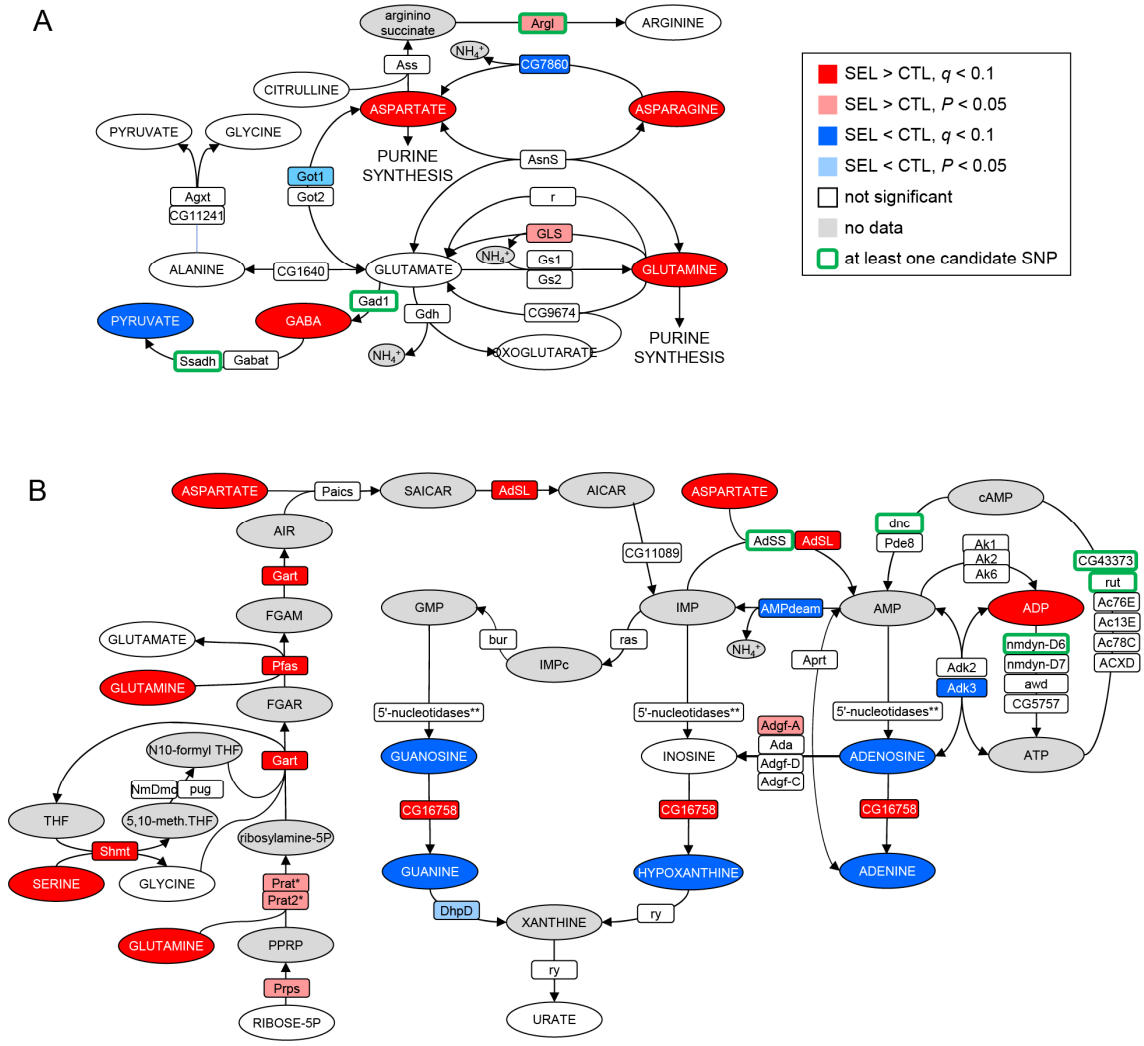

Figure 4. Imprint of evolutionary adaptation to poor larval diet on (A) alanine, glutamate and aspartate metabolism and (B) purine metabolism pathways of adult female flies. Highlighted in color are metabolites differentially abundant and enzymes differentially expressed between the Selected and Control populations, as well as enzymes to which at least one SNP differentiated in frequency between the sets of populations has been annotated. Enzymes differentially expressed at the nominal $P < 0.05$ but not passing the 10% FDR are also indicated. Metabolites in grey were either not targeted, not detected, or the data did not pass quality filtering. The pathways have been downloaded from KEGG (https://www.genome.jp/kegg/pathway.html). For the sake of clarity some substrates and products of depicted reactions that were not differentially abundant or absent from the data, as well as cofactors, have been omitted. Only genes expressed in female fat body have been included. *Prat and Prat2 were significantly overexpressed when analyzed jointly but not individually (see Supplemental Figure S3). **5'-nucleosidases: *cN-IIB*, *NT5E-2*, *Nt5B*, *veil*, *CG11883*; none was differentially expressed.

Adult cost of larval adaptation

The above results demonstrate that evolutionary adaptation to nutrient-poor larval diet has led to significant changes in adult metabolism. It is not clear, however, to what degree these changes may have evolved to improve fitness of adults that experienced poor diet as larvae, rather than being carry-over effects or trade-offs of changes driven by selection acting on the larval stage. If the former, adults from the Selected populations should perform better than those from the Control populations when both are

raised under the conditions of the poor larval diet regime. If the latter, the fitness of Selected adults raised on poor diet should be similar to or even lower than the fitness of Control adults developed on the same poor diet.

To test these alternative predictions, we compared the fecundity of females from Selected and Control populations raised on the poor larval diet, transferred to standard diet after eclosion, supplemented with ad-libitum live yeast for 24 h before oviposition, and allowed to oviposit overnight. These conditions mimicked those under which Selected populations have been maintained and propagated in the course of their experimental evolution. The rate at which females can convert nutrients into eggs within this short time window is thus arguably a key measure of their fitness, and is inherently dependent on the metabolism of nutrients, in particular proteins, lipids and nucleic acids. Despite having evolved under these conditions, Selected females laid only about 2/3 of the number of eggs produced by the Control females, whose ancestors did not face the poor larval diet ($F_{1,8} = 44.5$, $P < 0.001$; Fig. 5A). Fly ovaries consist of a variable number of branches called ovarioles; in each ovariole eggs are produced sequentially one by one. The rate of egg production may thus be limited by the number of ovarioles (Schmidt, et al. 2005), which is determined during larval development (Bergland, et al. 2008). Indeed, we found that poor diet-raised females of the Selected populations tended to have slightly fewer ovarioles than their Control counterparts ($F_{1,10} = 4.7$, $P = 0.056$; Fig. 5B). However, even adjusted for the difference in ovariole number, the Selected flies produced substantially fewer eggs than Controls ($F_{1,10} = 17.1$, $P = 0.002$; Fig. 5C).

Drosophila are "income breeders" in that they directly convert acquired nutrients into eggs rather than relying on previously accumulated reserves (O'Brien, et al. 2008). The fecundity difference thus implies that the Selected populations are less effective than Controls at converting dietary nutrients into the nutritional and/or generative components of the egg even when raised under the conditions under which the former but not the latter evolved during more than 200 generations.

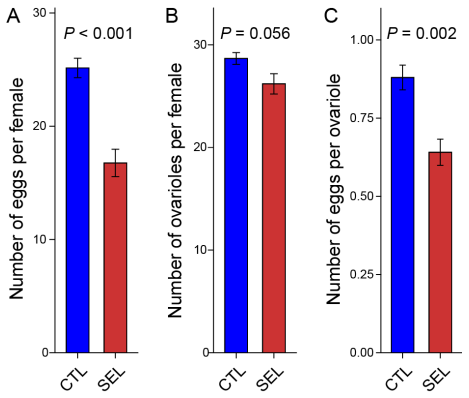

Figure 5. Selected populations pay a fecundity cost for larval adaptation to poor diet. (A) The number of eggs laid overnight by females from the Selected and Control. (B) The number of ovarioles (left + right ovary) per female. (C) The mean number of eggs per ovariole. The females experienced the dietary conditions, under which the Selected populations evolved: the larvae were raised on the poor diet; the adults were transferred to the standard diet and additionally provided live yeast 24 h before oviposition. Values are means ± SEM.

Discussion

Experimental evolution under chronic larval undernutrition resulted in major shifts in gene expression patterns and metabolite abundance at the adult stage, replicable across six independent experimental (Selected) populations of *Drosophila melanogaster*. This occurred even though adults of all populations

experienced standard diet in the course of the experimental evolution. This demonstrates that adult and juvenile metabolism are not evolutionarily independent from each other, even in a species in which these life stages are separated by a complete metamorphosis.

The evolutionary changes in gene expression did not in general recapitulate the phenotypically plastic responses of adult expression to larval diet. The abundance of a small but distinct subset of metabolites (nicotinate/nicotinamide and acyl-carnitines) did evolve in the direction that mimicked the plastic response, but this was not the case for most of the other metabolites. Contrary to our expectations, we also found no evidence of changes in the magnitude – not to mention the direction – of the plastic responses to larval diet. Rather, the effects of evolutionary past and current larval diet on both gene expression and metabolite abundance were essentially additive, with no evolutionary regime × larval diet interaction. For those genes and compounds that showed both a plastic and an evolutionary response, evolution essentially shifted the reaction norm of the plastic response without significantly changing its slope (Supplementary Figure S4). This suggest either that these plastic responses are of little consequence for fitness of adults raised on poor larval diet and subsequently transferred to standard diet, or that the slopes of these reaction norms are not very evolutionarily malleable, at least in the short term (Scheiner 1993; Ghalambor, et al. 2007).

Differences in adult gene expression between Selected and Control populations were broadly positively correlated with differences between them in larval gene expression, even though the gene expression was quantified in whole body in larvae and in dissected carcasses in adults. This correlation might have in principle been a result of larvae and adults being subject to similar selection pressures. This seems unlikely – while Selected and Control populations evolved on different larval diets, both sets of populations were exposed to the same adult diet. A more plausible explanation is that most genetic variants available for evolution affected gene expression of larvae and adults similarly, resulting in positive genetic correlation across the life stages. Under this scenario, many of the adult gene expression changes would represent correlated responses to selection on the larval stage rather than the response to selection on adults. While at the macroevolutionary time scale the ability to decouple juvenile and adult phenotypes is thought to be a major factor in the success of holometabolous insects (Moran 1994; Rolff, et al. 2019), surprisingly little is known to what degree larval and adult physiological traits are constrained from evolving independently in the short term (Collet and Fellous 2019).

Such constrains, mediated by cross-stage pleiotropic effects of underlying genetic variants, would lead to fitness trade-offs if larval and adult stage were exposed to contrasting environments (Collet and Fellous 2019). Consistent with this prediction, when raised on the poor diet and transferred to the standard diet as adults the Selected flies were less effective than Controls in converting dietary nutrients into eggs, even though these were the conditions under which the former had evolved (an analogous result on fecundity has been reported in another evolution experiment (May, et al. 2019)). These results suggest that strong selection imposed by the poor larval diet led to changes in gene expression patterns that helped larvae cope with nutrient shortage, but the underlying genomic changes also affected adult gene expression, resulting in changes in metabolism that were largely maladaptive.

Fecundity in *Drosophila* is limited by essential amino acids (Grandison, et al. 2009), in particular those in short supply relative to the needs of fly protein synthesis (Piper, et al. 2017). Thus, lower abundance of most essential amino acids in the Selected flies is consistent with their lower fecundity. In addition to being limiting substrates for protein synthesis, several essential amino acids (methionine, valine, isoleucine and in particular leucine) activate TOR complex 1, which promotes protein synthesis (Wolfson, et al. 2016; Antikainen, et al. 2017). Much of the protein synthesis activity in adult female fat bodies is directed towards synthesizing egg proteins (Piper, et al. 2017). Less is known about the potential limiting role of purines for fecundity. However, at least the first 10 embryonic divisions rely entirely on

ribosomes, and tRNA of maternal origin (Qin, et al. 2007) and a mutant with impaired rRNA synthesis has a lower egg production (Mohan and Ritossa 1970). It is therefore possible that deficiency of purine nucleotides might also contribute to reduced fecundity.

But why should essential amino acids be less abundant in Selected flies compared to Controls? The Selected flies do not show a general deficiency in amino acids – several non-essential amino acids are overabundant. The pattern differences in amino acid abundance also cannot be explained by a mismatch between dietary amino acids and the needs of protein synthesis. The proportions of different amino acids in yeast mostly match the composition of *Drosophila* proteins in general and of the yolk proteins particular (Piper, et al. 2017). Even though there is an excess of glutamine in yeast in relation to *Drosophila* needs, this is not the case for the other three amino acids that were more abundant in Selected than Control populations – serine is in fact the most deficient amino acid in yeast relative to *Drosophila* protein composition (Piper, et al. 2017). Similarly, while tryptophan and lysine are overabundant in the yeast diet relative to *Drosophila* needs, leucine is deficient (Piper, et al. 2017); yet, all three are deficient in Selected flies compared to Controls. Thus, rather than dietary amino acid acquisition or use in protein synthesis, the differences in amino acid abundance between Selected and Control populations is consistent with differential use of amino acids in metabolism – their interconversion, catabolism and use in the synthesis of other metabolites.

Another striking consequence of evolutionary adaptation to poor larval diet is accumulation of medium-chain acyl-carnitines in Selected flies relative to Controls. This metabolic phenotype is also evident in flies raised on poor relative to standard larval diet, and in flies subject to starvation relative to fed flies (Fig. 3). Although it is a classic symptom of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, we found no signal of reduced expression of genes involved in this and subsequent steps of beta oxidation. Beta oxidation is prone to congestion because of competition between longer and shorter acyl-CoA molecules for the same dehydrogenase enzymes, also leading to moderate accumulation of acylcarnitines. Such congestion can result from increased supply of long-chain fatty acids to mitochondria, insufficient capacity of the TCA cycle, or deficiency of oxidized forms of the key electron carriers FAD and NAD^+ (van Eunen, et al. 2016; Gnainsky, et al. 2021). The latter may in turn result from a backlog of the reduced forms $FADH_2$ and NADH at the electron transport chain or from general deficiency of those cofactors. We have no indication of reduced expression of TCA cycle enzymes in the Selected flies, but they are deficient in both NAD^+ and FAD (Fig. 2B). In contrast, starving flies do not show reduced abundance of the electron carriers, but they are expected to have mobilized their lipid reserves, thus increasing the supply of fatty acids to mitochondria (Lee and Jang 2014). The similarity of the medium-chain acylcarnitine phenotype between Selected and starved flies may thus have different underlying causes.

Nonetheless, there are other aspects of metabolome where the evolved differences between Selected and Control flies resemble the effect of 24 h of starvation, notably the deficiency of several essential amino acids, purine metabolites and multiple amino acid derivatives (Figure 2, Figure 3C). We have previously shown the genomic architecture of differentiation between the Selected and Control populations includes many candidate genes for starvation resistance; yet, the Selected flies are less resistant to starvation than Controls (Kawecki, et al. 2021). It is tempting to speculate that, as a byproduct of genetic adaptation to poor larval diet, adult flies have become programmed to express a nutritionally-deprived metabolic phenotype, which might explain both their low fecundity and low starvation resistance. This, together with other results discussed above, supports the notion that the evolution of adult metabolism may be to a large degree shaped by pleiotropic effects of genetic variants favored by selection driven by nutritional conditions experienced by juveniles.

Methods

Diets, experimental evolution and fly husbandry

Two diets were used for this study. The "standard" diet consisted of 12.5 g dry brewer's yeast, 30 g sucrose, 60 g glucose, 50 g cornmeal, 0.5 g $CaCl_2$, 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 "poor" diet contained 1/4 of the concentrations of yeast, sugars and cornmeal, but the same concentrations of the other ingredients. All experiments were carried out at 25°C and 12:12 h LD cycle.

Six Selected and six Control populations were all derived from the same base population originally collected in 1999 in Basel (Switzerland) and maintained on the standard food for several years before the evolution experiment started in 2005 (Kolss, et al. 2009). By the time of the experiments reported in this paper the Selected populations had been maintained on the poor larval diet for over 230 generations; the Control populations were maintained in parallel on the standard larval diet. Larval density was controlled at 200-250 eggs per bottle with 40 ml of food medium. Adults of both regimes were transferred to standard diet within 14 days of eggs laying (sometimes a day or two later when not enough adults have emerged from the poor diet) and additionally fed live yeast 24 h before egg collection to stimulate egg production. The target adult population size was 180-200 individuals.

Flies used in the experiments reported here were raised using similar procedures. Prior to all experiments reported in this study all populations were raised on the standard diet for at least 2 generations to minimize the effects of maternal environment (Vijendravarma, et al. 2010). For RNAseq and metabolome analyses flies of both Selected and Control populations were raised on both larval diets. Eggs to establish the next generation were collected by allowing flies to oviposit overnight on orange juice agar sprinkled with live yeast. During the egg collection for the experiments eggs were washed with water to remove any traces of diet at the surface – a procedure that also removes much of microbiota. To control the colonization by microbiota, eggs were re-inoculated using parental feces from a mix of all 12 populations as described in (Erkosar, et al. 2017). All experiments are performed on females, aged 4-6 days old from eclosion. Selected larvae develop on the poor diet faster than Controls (Kolss, et al. 2009; Erkosar, et al. 2017). To ensure that the females from Selected and Control populations can be collected synchronously, we initiated the larval cultures in a staggered manner over several days, so that we could collect females from around peak of emergence and at the same time for Selected and Control populations despite the difference in developmental time.

RNAseq on adult carcasses

Flies of all six Selected and six Control populations were raised on both poor and standard larval diet; adults of both sexes were transferred to fresh standard diet four days before collection and allowed to mate freely. Female flies were collected in the morning and dissected in PBS. The abdomen was separated, and the gonads, the gut and the bulk of Malpighian tubes were removed, leaving the "carcass", consisting of the abdominal fat body attached to the body wall, as well as any hemolymph, oenocytes, neurons and fragments of Malpighian tubes that remained embedded in the fat body. Precise dissection of the adult fat body without disrupting it is very difficult, which is why carcass is typically used instead (e.g. modEncode project, (Brown, et al. 2014)). From each of the 12 populations raised on either diet we collected a sample of 10 carcasses, i.e., 24 samples in total. The samples were snap-frozen in liquid nitrogen and stored at –80°C.

RNA was extracted from the carcass samples using "Total RNA Purification Plus" by Norgen Biotek (#48300, 48400). cDNA libraries (TrueSeq Standard RNA) were generated and sequenced on two lanes

of Illumina HiSeq4000 (single read, 150 bp) by the Genomic Technologies Facility of the University of Lausanne following manufacturer's protocols. Reads were mapped (pseudoaligned) to *D. melanogaster* reference genome BDGP6.79 using *kallisto* (Bray, et al. 2016), with 22 to 31 million mapped reads per sample. Read counts per transcript feature output from *kallisto* were converted to counts per gene using *tximport* (Soneson, et al. 2016). We filtered out genes with very low expression in that we only retained genes with read count per million greater than 2 in at least 6 samples. We further detected 45 pairs of genes with identical counts across all samples; from each pair we retained only the gene with a lower FBgn number, leaving 8701 unique genes.

Differential expression analysis was performed with *limma-voom* (Law, et al. 2014; Ritchie, et al. 2015), with evolutionary regime (Selected versus Control), with *EdgeR* normalization (Robinson, et al. 2010) implicit in the voom algorithm. Larval diet (poor versus standard) and their interaction were the fixed factors in the *limma* model; replicate population was a random factors modelled with *duplicateCorrelation* function of *limma*. Adjustment of *P*-values for multiple comparison was performed using Storey's FDR *q*-values (Storey and Tibshirani 2003) as implemented in procedure MULTTEST option PFDR of SAS/STAT software v. 9. 4 (Copyright © 2002-2012 by SAS Institute Inc., Cary, NC, USA). Genes with expression different at $q < 0.1$ were considered as significant for enrichment analyses. GO-term enrichment analysis was carried out with bioconductor 3.12 package *topGO* v. 2.42.0.

Because the FDR threshold focuses on minimizing false positives, it leaves out many genes that have truly differ in expression. Thus, the number of genes that pass the FDR threshold greatly underestimates the number of genes that truly differ in expression, and the relationship between the two depends greatly statistical power. Therefore, we also estimated the number of genes that differed in expression due to each factor in the analysis as the total number of genes minus the estimated number of "true nulls" (Storey and Tibshirani 2003) (https://bioconductor.org/packages/topGO/), using the *weight* method and Fisher's exact test.

To study to which degree the gene expression profiles of flies from the two regimes raised on the two larval diets were separable in a multivariate space we performed principal component analysis (PCA) on the correlation matrix of the log-normalized expression data for all genes. To test for the separation of the samples in this multivariate space we performed a multivariate analysis of variance (MANOVA) on the PCA scores, with regime, diet and their interaction as the factors (using procedure GLM of SAS/STAT software).

Larval gene expression analysis

The comparison of adult to larval gene expression differences was based on previously published data from a RNAseq study of whole larvae from the Selected and Control populations raised either in germ-free or microbiota colonized state; all larvae were raised on the poor diet in that experiment (Erkosar, et al. 2017). To maximize the compatibility of the adult and larval data sets we remapped the larval reads using the same *kallisto* algorithm as for adult carcasses, and analyzed the expression of the 11475 genes with the same *limma-voom* approach, with evolutionary regime, microbiota treatment and their interaction as the factors. We used the main effect of evolutionary regime from this study for the comparison with the adult results.

Broad-scale targeted metabolomics

Metabolite abundance was measured in Selected and Control flies using multiple pathway targeted analysis. Mated 4-day old females from all six Selected and all six Control populations were raised on

both poor and standard diet and their carcasses were dissected and stored in the same way as for the RNAseq experiment described above. From each population raised on each diet we obtained two samples of 10 carcasses (fed flies). Two additional samples of 10 carcasses per population and larval diet were obtained from females subject to 24 h of starvation on nutrient-deprived agarose (starved flies). The two samples for each population, larval diet and adult starvation treatment (i.e. fed or starved) were dissected by two experimenters, resulting in two experimental batches of 48 samples each. The two batches were also processed for metabolite extraction and analysis separately, on different days.

Extracted samples were analyzed by Hydrophilic Interaction Liquid Chromatography coupled to tandem mass spectrometry (HILIC - MS/MS) in both positive and negative ionization modes using a 6495 triple quadrupole system (QqQ) interfaced with 1290 UHPLC system (Agilent Technologies). Data were acquired using two complementary chromatographic separations in dynamic multiple reaction monitoring mode (dMRM) as previously described (van der Velpen, et al. 2019; Medina, et al. 2020). Data were processed using MassHunter Quantitative Analysis (for QqQ, version B.07.01/Build 7.1.524.0, Agilent Technologies). Signal intensity drift correction was performed on the pooled QC samples and metabolites with CV > 30% were discarded (Dunn, et al. 2011; Tsugawa, et al. 2014; Broadhurst, et al. 2018). In addition, a series of diluted quality controls (dQC) were used to evaluate the linearity of metabolite response; peaks with correlation to dilution factor $R^2 < 0.75$ were discarded.

Metabolome analysis

The peak area data for each compound were log-transformed, zero-centered and Pareto-scaled separately for the two experimental batches. To test for differential abundance of single compounds we fitted general mixed models (GMM) to these Pareto-scaled relative metabolite values using procedure MIXED of SAS/STAT software v. 9.4 (Copyright © 2002-2012 by SAS Institute Inc., Cary, NC, USA). For each compound we first fitted a full model, with evolutionary regime (Selected versus Control), larval diet (poor versus standard), starvation treatment (starved versus fed) and all their interactions as fixed factors, and population nested within regime, diet × population and starvation × population as random factors. Inspecting the residuals from this model we detected 13 data points (out of 10848) with externally Studentized residuals greater in magnitude than ±4.0; these data points were removed as outliers and the model was refitted.

The residuals for three of the 113 compounds (creatine, hydroxykynurenine and trehalose) deviated from normality at 10% FDR (Wilk-Shapiro test); we still report the results for these three compounds but they should be treated with caution. Because the main focus of the paper is on the metabolism of flies in their normal fed state, we also analyzed the data from the 48 samples obtained from fed flies separately, fitting GMM with regime, diet and their interaction as well as experimental batch as fixed effects, and population nested in regime and diet × population interaction as random effects. The fixed factors in the GMM were tested with type 3 *F*-tests, using Satterthwaite method to estimate the denominator degrees of freedom. *P*-values for each factor were adjusted for multiple comparison as for differentially expressed genes.

To visualize and test for multivariate differentiation of the metabolome we performed PCA on log-abundances of the 113 metabolites. The values from the two batches were averaged before this analysis so there was one point per population × diet combination × starvation treatment. As for gene expression patterns, sample scores from the PCA were tested for the effects of experimental factors and their interaction in a MANOVA implemented in procedure GLM of SAS/STAT software.

We also explored the relationship between the evolutionary change in metabolite abundance and the response to starvation for several categories of compounds. We specifically examined if the difference in

metabolite abundance between Selected and Control flies raised on poor food, in their normal fed state, was correlated (Pearson's *r*) with the difference between starved and fed Control flies raised on the standard diet. These two variables are functions of non-overlapping sets of measurements, thus avoiding spurious correlations due to non-independence of errors.

Fecundity and ovariole number

Fecundity was only assayed in females raised on poor larval diet, as described above. Male genotype and in particular the seminal fluid proteins males transfer to females during mating affect the short-term fecundity of the female. To ensure that any differences in egg number among populations are driven by female physiology and not by differences between males to which those females were mated, females from all populations were mated to males from a single laboratory population, originally collected from another site in Switzerland (Valais) in 2007. From each population, we collected 25-35 virgin females at the peak day of emergence and transferred them to standard food. Three days later we split the females in replicates of 10-12 females (2-3 replicates depending on the total number of females remaining alive) and placed them together with 10-12 young males on orange juice-agar medium supplemented with live yeast; they were allowed to feed and mate for 24 h. Subsequently males were discarded (to prevent potential fecundity reduction due to male harassment) and the groups of females allowed to oviposit overnight in new bottles with fresh orange juice-agar supplemented with live yeast. Eggs were washed from the medium surface with tap water, collected on a fine nylon mesh and transferred to a well of a 12-well cell culture plate containing 3 mL of 1% sodium dodecyl sulfate (SDS, Sigma) to facilitate egg dispersion. A photograph of each well was taken under a Leica stereomicroscope with a Canon 60D camera with manual exposure programming, automatic white balance, 3.2 sec of exposure time and ISO 400.

The number of eggs was estimated automatically with Codicount ImageJ plugin (https://www6.paca.inrae.fr/institut-sophia-agrobiotech_eng/CODICOUNT), following (Perez 2017). This approach is based on automatically quantifying the area corresponding to the eggs on the image, based on color contrast between the eggs and the background. This total egg area was then converted to an estimate of the number of eggs by dividing it by the area of a single egg, estimated from a separate sample (the same standard egg area was used for all images). Thus estimated number of eggs was divided by the number of females in a particular replicate. In one replicate one of ten females was found dead at the end of oviposition; for the analysis we set the number of females in this bottle at 9.5.

Flies were raised on the poor larval diet. While the number of ovarioles is fixed by the time of emergence, they are better visible and easier to count if filled with developing eggs (Bergland, et al. 2008). We therefore allowed 25 freshly emerged females to mate with 25 males on standard diet for three days, and subsequently to feed on ad libitum live yeast for two days, before collecting and storing them at –80°C until dissections. Six females from each population were haphazardly chosen for ovary dissection. We dissected the ovaries by separating the abdomen and cutting its posterior end, opening the abdomen laterally and removing the ovaries. We then dipped the ovaries briefly in a solution of crystal violet to improve visual contrast, opened them and counted the number of ovarioles in each ovary. Dissections and counting were done blindly with respect to the identity of the sample. To normalize the fecundity of each population relative to ovariole number we divided the mean number of eggs per female in this population by the mean number of ovarioles.

Egg number and ovariole number per female were analyzed with a GMM, with regime as a fixed factor and population nested in regime as a random factor. For the number of eggs per ovariole we only had one data point per population, these were compared between regimes with a one-way linear model.

Acknowledgements

This project has been supported by the Swiss National Science Foundation (grant 31003A_162732 to T.J.K.) and by research funding from the University of Lausanne. We thank Tony Teav, a research technician at Metabolomics Platform at UNIL for his contribution to sample preparation and data processing.

Author contributions

B.E. and T.J.K designed the study; C.D. F.C., H.G.A. and J.I. B.E. contributed to design of experiments. B.E., C.D., F.C. L.S. and L.K. carried out the experiments. H.G.A. and J.I. performed metabolite analysis. B.E. and T.J.K. analyzed the data and wrote the paper, with contributions from other authors.

Data availability

The raw and processed data from the RNAseq on adult carcasses are available from NCBI GEO (accession number GSE193105). Raw data for the previously published larval RNAseq are available from NCBI SRA (accession numbers SAMN07723150-SAMN07723173). The metabolite abundance data and the fecundity and ovariole number data are provided in supplementary material (Supplemental Table S6 and S7).

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