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Cis-regulatory polymorphism at *fiz* ecdysone oxidase contributes to polygenic evolutionary response to malnutrition in *Drosophila*

Fanny Cavigliasso[1], Mikhail Savitsky[2], Alexey Koval[3], Berra Erkosar[1¤a], Loriane Savary[1¤b], Hector Gallart-Ayala[4], Julijana Ivanisevic[4], Vladimir L. Katanaev[3], Tadeusz J. Kawecki[1]*

1 Department of Ecology and Evolution, Faculty of Biology and Medicine, University of Lausanne, Lausanne, Switzerland, **2** HumanaFly Facility, Faculty of Medicine, University of Geneva, Geneva, Switzerland, **3** Translational Research Centre in Oncohaematology, Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva, Geneva, Switzerland, **4** Metabolomics Unit, Faculty of Biology and Medicine, University of Lausanne, Lausanne, Switzerland

¤a Current address: E.B. FIND, Data Science Unit, Geneva, Switzerland
¤b Current address: S.L. Agence Régionale pour la Qualité et l'Hygiène Alimentaire, Montagny, Switzerland
* tadeusz.kawecki@unil.ch

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Abstract

We investigate the contribution of a candidate gene, *fiz* (*fezzik*), to complex polygenic adaptation to juvenile malnutrition in *Drosophila melanogaster*. Experimental populations maintained for >250 generations of experimental evolution to a nutritionally poor larval diet (Selected populations) evolved several-fold lower *fiz* expression compared to unselected Control populations. Here we show that this divergence in *fiz* expression is mediated by a *cis*-regulatory polymorphism. This polymorphism, originally sampled from a natural population in Switzerland, is distinct from a second *cis*-regulatory SNP previously identified in non-African *D. melanogaster* populations, implying that two independent *cis*-regulatory variants promoting high *fiz* expression segregate in non-African populations. Enzymatic analyses of Fiz protein expressed in *E. coli* demonstrate that it has ecdysone oxidase activity acting on both ecdysone and 20-hydroxyecdysone. Four of five *fiz* paralogs annotated to ecdysteroid metabolism also show reduced expression in Selected larvae, implying that malnutrition-driven selection favored general downregulation of ecdysone oxidases. Finally, as an independent test of the role of *fiz* in poor diet adaptation, we show that *fiz* knockdown by RNAi results in faster larval growth on the poor diet, but at the cost of greatly reduced survival. These results imply that downregulation of *fiz* in Selected populations was favored by selection on the nutritionally poor diet because of its role in suppressing growth in response to nutrient shortage. However, they suggest that *fiz* downregulation is only adaptive in combination with other changes evolved by Selected populations, which ensure that the organism can sustain the faster growth promoted by *fiz* downregulation.

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Author summary

Juvenile animals are particularly vulnerable to famines, which result in stunted growth and often lead to lifelong consequences for health and Darwinian fitness. Natural selection can alleviate consequences of juvenile undernutrition by improving the acquisition of scarce nutrients through more efficient digestion or minimizing their "waste" for non-essential tasks. However, mechanisms that regulate protein synthesis and cell division must also be fine-tuned so that the organism can grow as fast as is sustainable with the available nutrients without endangering survival. We show that the evolution of digestive and metabolic adaptations in experimental *Drosophila* populations exposed for >250 generations to a nutritionally poor diet was accompanied by a regulatory mutation in a gene (*fiz*) that modulates insect steroid hormones. Reduced expression of the *fiz* gene resulting from this change promotes faster larval growth on the poor diet. However, in the absence of other adaptations, the faster growth promoted by *fiz* downregulation cannot be sustained by larvae confronted with poor diet, resulting in very high mortality. Thus, evolution of improved tolerance to juvenile malnutrition appears to rely on a combination of multiple coordinated changes that may be detrimental in isolation.

Introduction

Full understanding of adaptive evolution requires elucidation of molecular, developmental and physiological links between specific changes in the genome and fitness-determining traits, such as morphology, behavior or life history. The last two decades brought increasingly detailed insights into a growing number of cases of adaptive evolution mediated by single or few genes. Examples include adaptive variation in color in mammals [1], repeated evolution of changes in armor plates in sticklebacks [2,3], sensory basis of host shift in *Drosophila sechellia* [4], and a trade-off between larval growth and adult flight performance in a butterfly mediated by polymorphism in a respiratory enzyme [5]. It is more challenging to identify how specific genetic changes contribute to complex adaptations that involve changes in multiple molecular pathways and physiological mechanisms, and that are mediated by polymorphisms in a large number of genes. Genomics screens and whole genome expression studies have identified numerous candidate polymorphisms and genes involved in traits such as heat tolerance, starvation resistance, fecundity or longevity [6–8]. However, rather few of these candidates have been verified experimentally [e.g., 7,9,10], and for most we only have rudimentary understanding of the mechanisms linking genetic variation to phenotypic traits that directly determine fitness.

In this study we explore the contribution of a *cis*-regulatory polymorphism at a single candidate gene to highly polygenic and multifaceted adaptation to juvenile malnutrition in *Drosophila melanogaster*. Nutrient shortage is a fact of life in many animal species, and juveniles in particular are vulnerable, because they usually cannot interrupt their growth and development to wait out the bad times. Furthermore, in many species (including *Drosophila* and most other insects) juveniles are considerably less mobile than adults and thus constrained from looking for food elsewhere. Thus, natural selection imposed by period of nutrient shortage during juvenile development is likely to have shaped animal physiology.

Our study stems from a laboratory evolution experiment in which six *D. melanogaster* populations (subsequently referred to as "Selected" populations) have been raised on a nutritionally poor larval diet for >250 generations (>15 years). In parallel, six Control populations originating from the same base population have been maintained on a standard diet. In the

course of this experimental evolution the Selected populations have evolved high tolerance to larval undernutrition, manifested in their higher egg-to-adult survival, shorter developmental time and higher larval growth rate on the poor diet compared to Control populations [11–13]. This improvement has been in part mediated by an increased rate of amino acid acquisition from the poor diet, traded off for reduced rate of absorbing dietary sugars [13]. This shift in macronutrient acquisition has been associated with higher activity of digestive proteases and lower activity of amylases, although these differences are more apparent in germ-free larvae [11]. Metabolome analysis indicated divergence in ways in which the acquired nutrients are used by the Selected and Control larvae. Notably, in spite of their faster acquisition from the diet, the concentrations of multiple free amino acids, including seven essential, are lower in Selected than Control larvae raised on poor diet [14]. This is consistent with a more expeditious use of amino acids for protein synthesis underpinning the faster growth of Selected larvae. However, we have also shown that Selected larvae use a greater fraction of acquired amino acids for energy generation [14]. The Selected populations also evolved a smaller critical size at which metamorphosis is initiated, allowing them to complete development with a lower total accumulated biomass [15]; hence, in spite of their faster larval growth they emerge as smaller adults than Controls [14]. Furthermore, the Selected larvae move less while foraging [16] and show increased cannibalistic tendencies [17]. Consistent with this multitude of phenotypic traits associated with evolutionary adaptation to the poor diet, more than 1400 genes were found to differ in expression between Selected and Control larvae (when both raised on poor diet) [11]. And, over 3000 single nucleotide polymorphisms (SNPs) in more than 100 genomic regions, in or near about 700 genes, diverged in frequency between Selected and Control populations, implying a highly polygenic genetic architecture [18].

To identify most promising candidates for genes causally mediating the evolutionary adaptation to nutritional conditions, we examined genes that were both differentially expressed between Selected and Control populations in a previously published RNAseq data [11,19] and had at least one candidate SNP within their sequence boundaries or 5kb upstream and downstream [18]. Of 102 such genes, the one with by far the largest difference in expression was *fiz* (*fezzik*, CG9509, FBgn0030594). Its expression was several-fold lower in the Selected than Control larvae (Fig A in S1 Appendix). Furthermore, a non-synonymous SNP in the gene (Glu133Lys), a SNP in the promoter region, and several further SNPs within 3 kb upstream of the gene were significantly differentiated in frequency between the Selected and Control populations. Further inspection of the sequence data revealed that all six Selected populations were fixed (or nearly fixed) for one allele at each of these candidate SNPs. Conversely, four of the six Control populations were fixed for the other allele, whereas Control populations C1 and C3 showed intermediate allele frequencies (Fig 1A). The same two Control populations were also intermediate for the level of *fiz* expression (Fig 1B). This concordance between allele frequency and gene expression is suggestive of *cis*-regulatory divergence, likely driven by selection due to nutritional conditions.

fiz is mainly expressed in Malpighian tubules [20,21] and, based on homology with *Spodoptera littoralis* ecdysone oxidase and the *Drosophila* gene *Eo*, has been predicted to code for an ecdysone oxidase [22]. Ecdysone, or more precisely its active form 20-hydroxyecdysone (20E), is an insect hormone whose pulses trigger transitions between larval instars and the metamorphosis to the adult stage [23]. However, it also appears to be involved in regulating growth between molts in response to nutrient shortage [24]. Ecdysone oxidases catalyze the conversion of ecdysone and 20E to 3-dehydroecdysone (3DE) and 3-epi-20-hydroxyecdysone (3D20E), respectively [25–27]. Even though the physiological role of this step in ecdysteroid metabolism in *Drosophila* remains unclear (see Discussion), downregulation of *fiz* expression promotes larval growth under normal nutritional conditions, resulting in larger adults with no

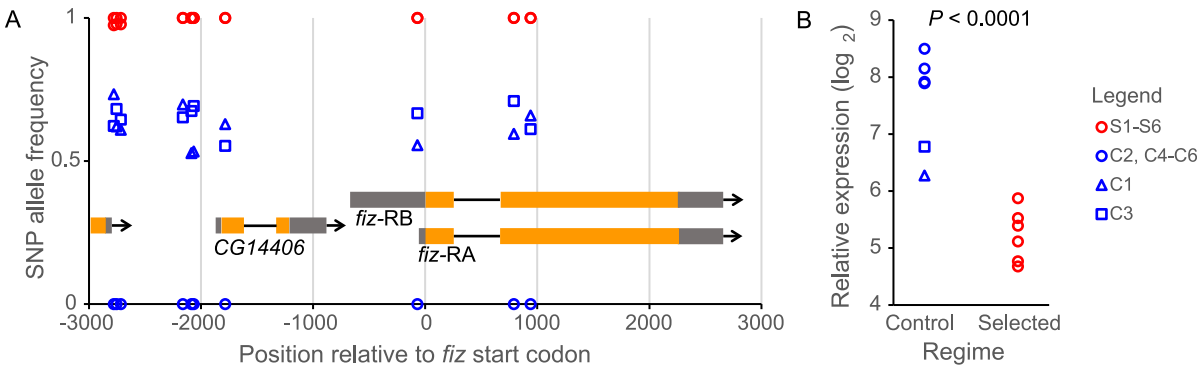

Fig 1. Genomic and expression differentiation of *fiz* gene between Control (C1-C6) and Selected (S1-S6) populations. A. Positions and allele frequencies at candidate SNPs in the genomic vicinity of *fiz* relative to gene transcripts. Allele frequencies are estimated from pooled whole genome sequencing [18]; for each SNP the frequency plotted is that of the allele more frequent in the Selected than in Control populations. All Selected populations are (nearly) fixed for one allele at all SNPs, and four Control populations are fixed for the alternative allele. Only SNPs assessed as significantly differentiated between Selected and Control populations are plotted; for a full list of polymorphisms in this region see S1 Table. B. Expression of *fiz* in third instar larvae raised on the poor diet, based on previously published RNAseq data [11,19]. The values are averages from the two conditions used in that study (germ free and *Acetobacter*-inoculated); the expression in the two conditions was highly correlated across populations ($r = 0.99$). *CG14406* is a gene of unknown function immediately upstream of *fiz*; it is not differentially expressed between Selected and Control populations ($P = 0.75$).

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change in developmental time [28]. Furthermore, downregulation of *fiz* appears to enhance starvation resistance, at least in male flies [29], and the gene has been implicated in microbiota-mediated promotion of larval growth on valine-deficient artificial diet [30], suggesting a role in regulating response to nutritional stress. Finally, many non-African *D. melanogaster* populations are polymorphic for a *cis*-regulatory genetic element with a large effect on *fiz* expression, apparently maintained by some form of balancing selection [28,31], implying that such natural genetically determined differences in *fiz* expression may have adaptive consequences.

Therefore, we set out to investigate how changes in expression of *fiz* might contribute to adaptation to poor diet in our experimental populations. We first verified whether Fiz protein indeed has ecdysone oxidase activity. We then characterized the dynamics of *fiz* expression in Selected and Control populations across development and tested whether the differential expression could be compensated by a divergence in expression of other confirmed or putative ecdysone oxidase genes. We tested whether differences in the expression of *fiz* were *cis*-regulatory and aimed to identify the most likely SNP responsible for this divergence. Finally, to independently verify the role of *fiz* in adaptation to poor diet, we investigated how knockdown of *fiz* affects development, growth and survival of larvae on the poor diet.

Results

Fiz is an ecdysone oxidase

As the first step towards understanding the potential role of *fiz* in adaptation to larval undernutrition, we verified the bioinformatic prediction that Fiz protein shows ecdysone oxidase activity, i.e., that it catalyzes the reaction ecdysone + O_2 ⇌ 3-dehydroecdysone + H_2O_2 [22]. To verify this prediction, we obtained purified Fiz protein by expressing it in *E. coli and* tested it for oxidase activity using both ecdysone (α-ecdysone) and its activated form 20-hydroxyecdysone (20E, β-ecdysone) as substrates. Incubation of Fiz with either ecdysone or 20E resulted in production of H_2O_2 as predicted from the reaction (Fig 2A and 2B and Table A in S1 Appendix). Furthermore, in an independent in vitro experiment the amount of ecdysone and

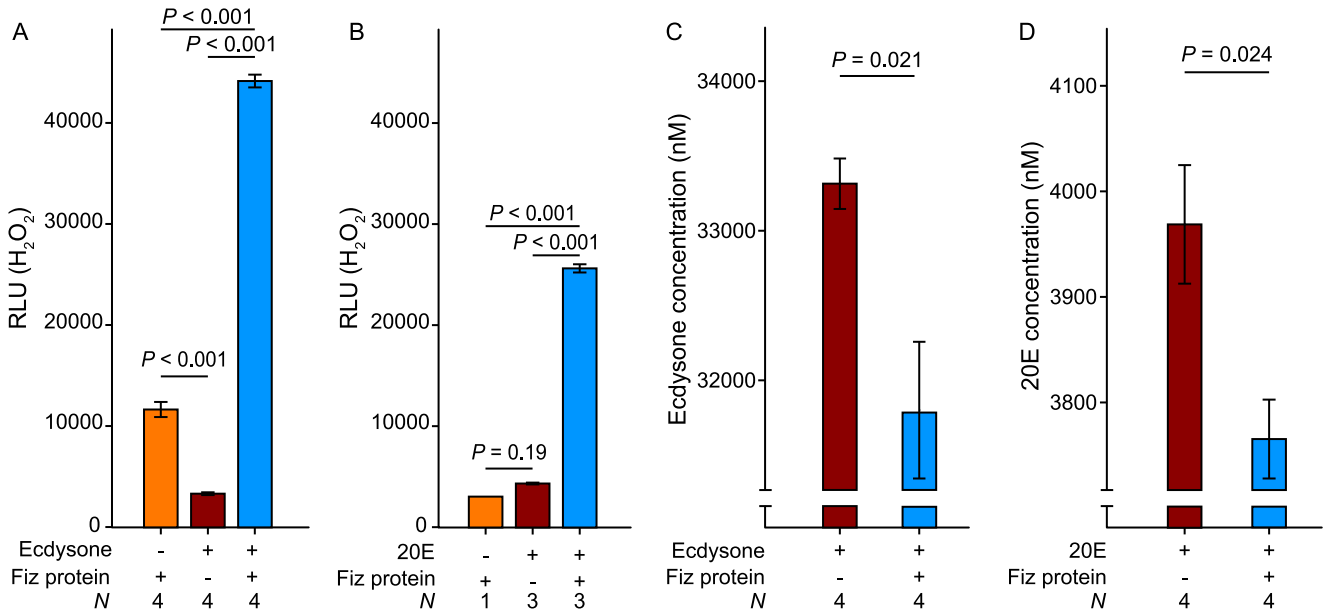

Fig 2. In vitro activity of purified Fiz protein on ecdysone and 20E. A and B. Production of H_2O_2 by Fiz protein incubated with ecdysone (A) and 20E (B) as substrates, compared to Fiz protein alone or substrate alone, in relative units. C and D. Reduction of the relative concentration of ecdysone (C) and 20E (D) following incubation with Fiz protein. Bars correspond to the means ± SE of N = number of replicate reactions; "+" and "-" indicate the presence *versus* absence of a particular compound.

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20E was reduced when incubated with purified Fiz protein compared to the condition without Fiz (Fig 2C and 2D, $F_{1,6} = 9.7$, $P = 0.021$ for ecdysone; $F_{1,6} = 9.1$, $P = 0.024$ for 20E). Taken together, these results demonstrate that Fiz has an ecdysone oxidase activity and is able to use both ecdysone and 20E as substrates.

fiz and its paralogs are less expressed in Selected than Control populations

The RNAseq experiment that detected the difference in *fiz* expression between Selected and Control populations [11] was based on a single time point during the 3rd larval instar (L3). If *fiz* expression occurred in pulses in response to pulses of ecdysone [23,32], that difference might be due to a time shift of the pulse rather than a consistent difference in the level of expression. To address this possibility, we measured the expression of *fiz* at several time points in larvae, prepupae and young adults. To keep the workload manageable, we carried this and the following assays on three Selected and three Control populations fixed for alternative alleles at *fiz* candidate SNPs. This excluded Control populations C1 and C3, which were polymorphic at these SNPs (Fig 1); among the remaining populations we chose at random Control populations C2, C4, C6 and Selected populations S1, S2, S3. The expression of *fiz* was consistently lower in these Selected populations compared to Controls, regardless of the developmental stage or diet (Fig B and Table B in S1 Appendix). In addition, the expression of *fiz* was relatively stable over time in Controls whereas a temporary drop was observed in prepupae of Selected populations. The levels of *fiz* expression are not directly comparable between diets in the larvae, as the developmental stages may not correspond. However, prepupae and adults of both sexes (which were sampled at the same stage) raised on the poor diet consistently showed lower *fiz* expression than their counterparts raised on standard diet (all $P < 0.05$; Table C in S1 Appendix). This suggests that the expression of *fiz* is also modulated by diet.

Of more than a dozen *fiz* paralogs the two with the highest homology to *fiz* (and to *Spodoptera littoralis* ecdysone oxidase) are *Eo*; (FBgn0030597), and *CG9512* (FBgn0030593) [22]. Like *fiz* and unlike other paralogs, these two genes are mainly expressed in Malpighian tubules (FlyAtlas2, [20]); the product of *Eo* has been shown to have ecdysone oxidase activity while it had been predicted for *CG9512* [22]. The downregulation of *fiz* in Selected populations might thus potentially be compensated by upregulation of these two other ecdysone oxidase genes. This was clearly not the case for prepupae and adults–the expression levels of *Eo* of *CG9512* in Selected populations at these stages were similar to or lower than in Controls (Fig B and Table B in S1 Appendix). However, the results for the larvae were inconsistent across the time points. This could reflect the fact that, because of differences in their rate of development [11–13], the Selected and Control larvae of the same age were at different developmental stages.

We thus quantified the expression of (verified or predicted) ecdysone oxidase genes in larvae that were developmentally synchronized (see Methods), focusing on the first three quarters of the third larval stage between the molt to L3 and the onset of the wandering stage. This is the period when most body mass is acquired; it also encompasses the pulse of ecdysone that initiates transcriptional changes that mediate the transition to the wandering stage, ultimately leading to metamorphosis (which still requires three further pulses of ecdysone) [23,33]. We did this only on larvae raised on standard diet because patterns of expression of ecdysone oxidase genes were broadly similar between diets (Fig B in S1 Appendix) and because on the poor diet the Selected and Control larvae develop at different rates, and even individuals in the same bottle become desynchronized in their developmental stage. Even though *fiz*, *Eo*, *CG9512* each showed different temporal dynamics, all were consistently less expressed in Selected larvae compared to Controls (Fig 3, $P < 0.001$ for the four genes, Table D in S1 Appendix). The same was the case for two other *fiz* paralogs with a putative function in steroid metabolism expressed in larvae, *CG9521* and *CG12539* (Fig 3 and Table D in S1 Appendix). A fifth paralog, *CG45065*, a putative ecdysone oxidase mainly expressed in the tracheal system, also tended to be downregulated in Selected mid/late third instar larvae compared to Controls, but this difference was not statistically supported (Fig 3 and Table D in S1 Appendix). Taken together, these results suggest that at least in the first three quarters of the 3rd larval stage, there was no compensation for the downregulation of *fiz* in Selected larvae; rather all known or predicted ecdysone oxidases were (or tended to be) downregulated in the malnutrition-adapted Selected larvae compared to Controls.

In addition to ecdysone oxidases, products of two other genes are known to directly regulate the active form of ecdysone in peripheral tissues in *Drosophila*: *shade* (*shd*), which converts ecdysone to 20E [34] and *Cyp18a1* which deactivates 20E by hydroxylation [35, 36]. We did not detect differences in expression of these two genes between Selected and Control third instar larvae; while the trends observed in the data do not allow us to exclude some differences, they would be much smaller than differences in the expression of ecdysone oxidase genes (Fig 3 and Table D in S1 Appendix).

Based on the enzymatic function of ecdysone oxidases in oxidizing ecdysone and 20E, one might expect that their lower expression in Selected larvae would affect ecdysone signaling. The concentration of the active form of ecdysone 20E in third instar *Drosophila* larvae is below or at detection threshold until shortly before pupariation [32]. The level of 20E at this stage is thus often quantified indirectly by measuring expression of genes activated by 20E, such as *Eip74EF* and *broad* [28,37,38]. Expression of both *Eip74EF* and *broad* increased over the course of the third larval instar, consistent with the expected increase in 20E levels. However, the expression of these two genes did not differ between Selected and Control larvae, although it tended to be lower in Selected larvae at individual time points (Fig 3 and Table D in S1

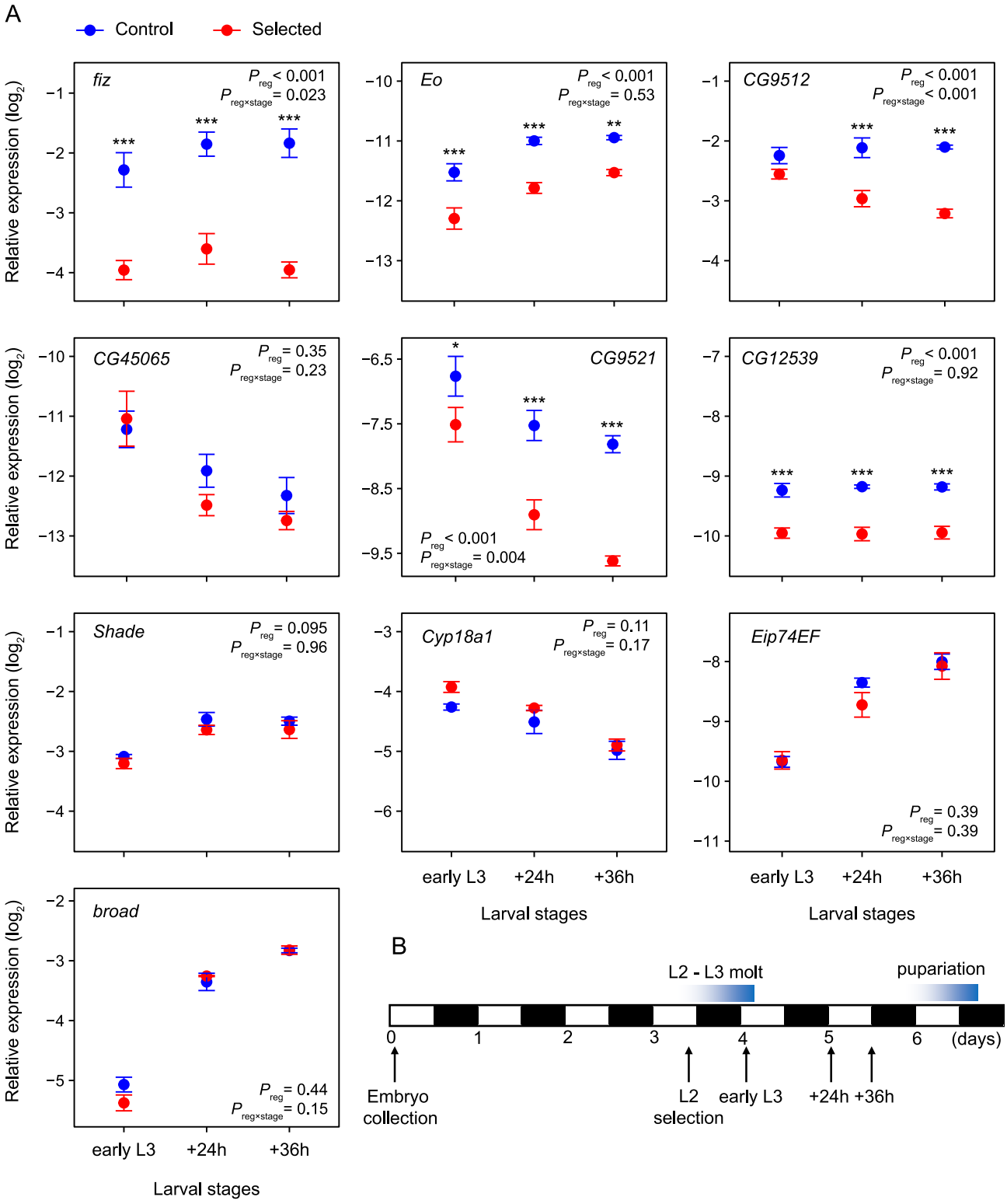

Fig 3. (A) Relative expression of focal genes in third instar larvae raised on standard diet (means ± SE from qPCR relative to three reference genes). (B) Timing of the sampling points relative to larval development (white and black bars indicate the light and darkness periods). 3.5-day-old L2 larvae were collected, those that molted to L3 overnight were collected next morning ("early L3"), with further samples taken 24h and 36h later. For each gene and diet, the P_{reg} and $P_{reg×stage}$ refer to the main effect of the evolutionary regime (i.e., Selected versus Control) and to regime × stage interaction, respectively. Asterisks indicate a significant stage-specific pairwise difference between Control and Selected populations after *P*-value correction (sequential Bonferroni adjustment for stages). $N = 3$ populations × 3 replicates of 10 pooled larvae per evolutionary regime and stage.

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Appendix). Thus, we have no indication of 20E reaching different levels in Selected than in Control larvae, despite lower expression of *fiz* and other ecdysone oxidase genes.

Difference in *fiz* expression is *cis*-regulatory and not sex-specific

The existence of candidate SNPs upstream of *fiz* opens the possibility that the difference in expression between the Selected and Control populations is mediated by one (or more) of these variants in a *cis*-regulatory manner. *Cis*-regulatory elements act on genes on the same chromosome (and not on their counterparts on the homologous chromosome). Therefore, if the difference in *fiz* expression were *cis*-regulatory, heterozygous individuals carrying one chromosome each from a Selected and a Control population would express the *fiz* copy on the "Selected" chromosome much less than the copy on the "Control" chromosome [39]. To quantify the relative contribution of the two alleles to the pool of *fiz* transcripts in heterozygotes, we took advantage of a polymorphic site in the coding region of *fiz* (G/A at position 791 after start codon). Based on pool sequencing data [18] and verified at the time of the present experiment (see Methods), the three focal Control populations are fixed for allele "A" and the three Selected populations are fixed for allele "G", allowing us to quantify the relative contributions of the two alleles to the transcript pool using cDNA amplicon sequencing [39]. We did this in F1 female late 3rd instar larvae from three independent crosses (in both directions) between Control and Selected populations (C2 × S1, C4 × S2 and C6 × S3; we used females because *fiz* is on the X chromosome). Regardless of the direction of the cross, these F1 females showed a highly allele-biased expression, with the transcript of the "Control" allele "A" being over 5-fold more abundant than the transcript of the "Selected" allele "G" (Fig 4A, $t_4 = 31.8$, $P < 0.001$, Table F in S1 Appendix). This demonstrates a large *cis*-regulated component of the expression difference [39]. To ascertain how much of the difference in *fiz* expression between Selected and Control larvae is explained by the *cis*-regulated component, we quantified the proportion of the transcripts of the two *fiz* alleles in samples containing equal amounts of cDNA from Selected and Control female larvae [39]. The proportion of the "Selected" *vs* "Control" allele in the transcript pool of these mixed samples ("Mix" in Fig 4A) was similar to that in F1 females. This implies that all or nearly all of the difference in *fiz* expression between Selected and Control populations is *cis*-regulatory [39].

We used the same approach to test the *cis*-regulatory nature of differences in gene expression in another (predicted) ecdysone oxidase, *CG9512*. Its transcript begins less than 1kb downstream from the end of *fiz*, it shows a similar level of expression in larvae as *fiz* (Fig 3) and it also harbors a SNP (T/C) in the coding region of *fiz* (position 904 after start codon) that is fixed for alternative alleles in the three Selected from the three Control populations. Even though the Selected allele tended to be slightly less abundant than the Control allele in the *CG9512* transcript pool, the difference was much smaller than that between their relative abundances in the 50:50 mix of Selected and Control larvae (Fig 4B and Table F in S1 Appendix). Thus, in contrast to *fiz*, the difference in expression of *CG9512* appears to be mainly *trans*-regulatory.

As *fiz* and *CG9512* are on the X-chromosome, males only inherit the maternal copies of these genes. For *fiz*, consistent with *cis*-regulatory nature of the expression difference, male larvae from crosses between Selected and Control populations showed *fiz* expression similar to that of their maternal population and very different from each other (Fig 4C and Table G in S1 Appendix). These results also confirmed that larvae of both sexes showed a similar difference in *fiz* expression, validating results obtained without sexing of the larvae (which is impractical before mid-3rd larval stage). As with *fiz*, the expression of *CG9512* also depended on the direction of the cross, with the expression in each cross being similar to that in its maternal

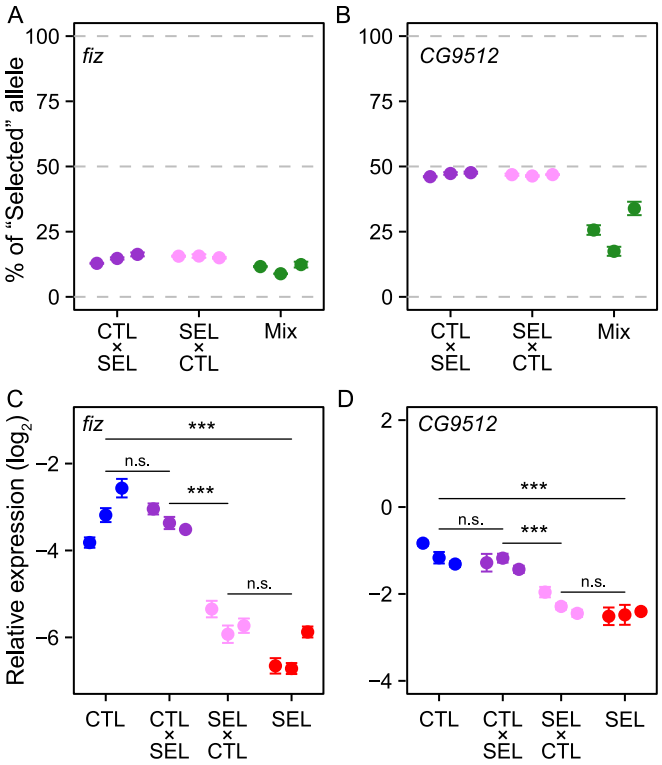

Fig 4. Expression of alleles of *fiz* and *CG9512* in F1 crosses between Control and Selected populations. A,B: Proportional contribution of the "Selected" allele to the transcript pool of *fiz* (A) and *CG9512* (B) in heterozygous F1 female larvae, determined by amplicon sequencing. The proportion of the "Selected" allele in 50:50 mix of cDNA from parental lines provides a reference. C,D: Relative expression (from RT-qPCR) of *fiz* and *CG9512* in F1 male larvae (hemizygous for both X chromosome genes) compared to the parental populations. SEL: Selected; CTL: Control; F1 crosses are represented as maternal × paternal population. The three symbols per category correspond to three independent population pairs (C2 × S1, C4 × S2, C6 × S3). Each symbol indicates mean ± SE of N = 2 to 4 (A-B) or 4 (C-D) replicates per population or cross; each replicate consisted of a pool of seven late third instar larvae. ***$P < 0.001$, n.s. $P > 0.05$.

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population (Fig 4D and Table G in S1 Appendix). While in principle this could mean that, in contrast to females, the difference in *CG9512* expression in males is *cis*-regulatory, a more parsimonious explanation is that the expression difference is mediated by a *trans*-regulatory element located on the X chromosome.

The responsible polymorphism is distinct from previously identified *cis*-regulatory SNP

We examined previously published pool seq data [18] from our populations for candidate polymorphisms potentially responsible for these *cis*-regulatory differences in *fiz* expression (Fig 1A). The genetic variation of these populations had been sampled from a natural population in Basel, Switzerland. We thus also explored population genetic data sets in natural populations to get insights into the evolutionary history of candidate polymorphisms [40,41]. A previous study identified a *cis*-regulatory C/G SNP at genomic position X:14909071 with a large effect on *fiz* expression; Sub-Saharan populations are fixed for the "low-expression" ancestral allele C whereas cosmopolitan populations harbor a derived "high expression" allele G at frequencies between 0.20 to 0.60 in most populations [28,31,40] (S2 Table). This

polymorphism (referred to as "SNP 67" in [28]) is absent in our populations–all are fixed for the "low expression" C allele [18]. The same study identified two other SNPs further upstream (at X: 14910020 and X: 14910124) with minor effects on *fiz* expression, where the cosmopolitan populations appear to be fixed for the higher expression alleles [28], they are also not polymorphic in our populations.

Hence, the large divergence in *fiz* expression in our study must be mediated by an alternative *cis*-regulatory polymorphism. A likely candidate is a G/T SNP just 5 bp away, at genomic position X:14909076, 70 bp upstream of the start codon. At this new SNP all Selected populations are fixed for the G allele, Control population C2, C4, C5, C6 are fixed for the T allele, and populations C1 and C3 –which show intermediate *fiz* expression—harbor intermediate frequencies (Fig 1 and S1 Table). This results in a nearly perfect correlation across populations between allele frequency at this SNP and *fiz* expression ($r = 0.96$, $P < 0.001$). This polymorphism occurs in multiple populations across the cosmopolitan range of *D. melanogaster* [31], with frequencies of allele T in European populations typically below 10% (S2 Table). The T variant appears to be absent from Sub-Saharan Africa–in a collection of 280 sequences from across the region (Drosophila Genome Nexus [40,41]) all carry the G allele. This suggests that the G allele is ancestral, which is further supported by *D. simulans* carrying the same base in the homologous sequence. The two derived alleles in the *fiz* promoter–G at X:14909071 and T at X:14909076 are found in multiple sequences from outside of Africa, including coexisting in the same populations, but never in the same sequence, consistent with their independent origins (S3 Table).

Our candidate SNP at X:14909076 and the previously identified *cis*-regulatory SNP at X:14909071 [28] are both within a predicted *fiz* promoter (Eukaryotic Promoter Database, [42]) and within the same transcription factor binding hotspot (TFBS_HSA_036116) identified by chromatin immunoprecipitation [43]. A search of transcription factor databases JASPAR [44] and OnTheFly [45] revealed that the ancestral sequence at positions X:14909077.. X:14909070 corresponds to the binding motif of the transcription factor *Adf1*. Substitution of the derived variant at either of the two SNP sites is predicted to cause a complete loss of this binding affinity (Fig C in S1 Appendix). Nonetheless, whether and how *Adf1* might control *fiz* expression is unknown.

Ten other SNPs and three short (2–7 bp) indels had been found within 1 kb upstream of the *fiz* gene (and further seven SNPs in the intron of the gene, S1 Table), but none of them has been identified as a candidate for targets of selection [18]. However, *fiz* lies within an apparent 355 kb genomic block containing 218 candidate SNPs, many of which show essentially identical allele frequencies to the SNP in *fiz* promoter in our populations [18]. Several such candidate SNPs are located between 1.7 and 2.3 kb upstream of *fiz* (Fig 1A), and like the SNP in the promoter region, their frequencies are highly correlated ($r > 0.95$) among populations with *fiz* expression levels. Thus, even though the SNP in *fiz* promoter is a highly promising candidate, the *cis*-regulatory element responsible for differences in *fiz* expression in our populations may well be farther away from the gene. Even though we cannot with confidence identify the responsible polymorphism, the differences in *fiz* expression are clearly not mediated by the *cis*-regulatory SNPs previously identified by ref. [28].

fiz knockdown promotes growth but impairs survival on poor diet

The *cis*-regulatory divergence in *fiz* expression between the Selected and Control populations is suggestive of its contribution to dietary adaptation. However, *fiz* lies within a genomic region where many SNPs show highly parallel changes in allele frequency, presumably due to linkage disequilibrium [18]. This region contains about 40 genes. Hence, rather than being

favored by selection, the divergence between Selected and Control populations in the expression of *fiz* may have evolved as a byproduct of selection on linked polymorphisms affecting expression or functioning of other genes. Therefore, as an independent test of the role of reduced *fiz* expression in adaptation to undernutrition, we studied the effect of an RNAi knockdown of *fiz* (in all tissues) on larval performance traits. *fiz* knockdown has been previously shown to promote larval growth on a nutrient-rich diet, resulting in larger adults with no change in developmental time [28]. If *fiz* expression divergence between the Selected and Control populations were adaptive, we would expect *fiz* knockdown to have positive effects on performance on at least some aspects of performance on the poor diet.

The knockdown considerably reduced egg to adult survival on both poor and standard diet (Fig 5A; $\chi^2 = 17.7$, $P < 0.001$ and $\chi^2 = 14.3$, $P < 0.001$, respectively). It also accelerated egg-to-adult development of both sexes on poor diet (Fig 5B, $F_{1,6} = 18.9$, $P = 0.005$ and Table H in S1 Appendix)[28], but not on standard diet (Fig 5B, $F_{1,8} = 0.3$, $P = 0.59$ and Table H in S1 Appendix). Furthermore, regardless of the diet, *fiz* knockdown flies were larger in terms of adult body mass, and this was not mediated by prolonged development. Even though body weight

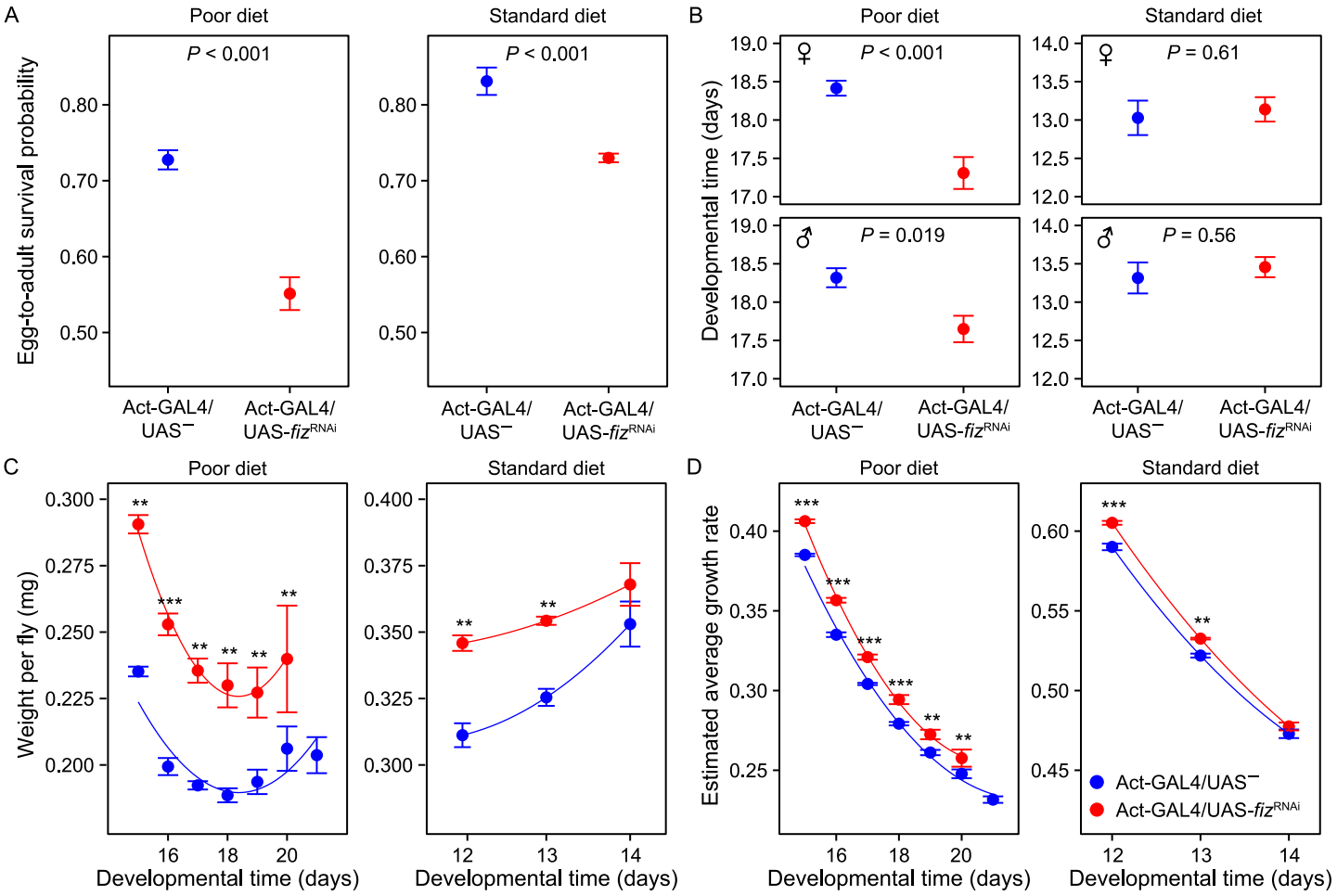

Fig 5. Effect of ubiquitous *fiz* RNAi knockdown on egg-to-adult survival probability (A), developmental time (B), female weight (C) and estimated female growth rate (D) on poor and standard diets. Symbols indicate means ± SE. $N = 4$ replicate bottles for poor and 5 bottles for standard diet (each bottle initially contained 200 eggs). Red and blue lines in C and D represent predictions from statistical models (including the linear and quadratic components).

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varied with developmental time, for any duration of development the knockdown females were heavier than the controls that eclosed at the same time (Fig 5C; $F_{1,11} = 25.1$, $P < 0.001$ on poor diet; $F_{1,21} = 13.0$, $P = 0.002$ on standard diet, Table I in S1 Appendix). This implies that the *fiz* knockdown improved the growth rate: even though (as is typical in *Drosophila*) slower development was associated with slower growth, for any length of development the estimated average larval growth rate of knockdown larvae was higher than that of controls (Fig 5D and Table J in S1 Appendix).

The RNAi knockdown reduced *fiz* expression in early 3rd instar larvae by about 1.9–2.5-fold (Fig D in S1 Appendix), less than the about 3-5-fold difference between Selected and Control populations (Fig 3). Thus, the effects of the knockdown–in particular the high larval mortality–are unlikely to be due to excessive reduction in *fiz* expression. These results thus indicate that reduction of *fiz* expression by RNAi in a non-adapted genetic background was sufficient to increase the average larval growth rate and adult size not only on a nutrient-rich standard diet (as has already been reported [28]), but also on the poor diet. Furthermore, these effects were greater on the poor diet than standard one (Table K in S1 Appendix), and development was accelerated only on poor diet. However, this happened at the expense of reduced survival on both diets, possibly explaining why a low expression of *fiz* had not been favored in Control populations.

Discussion

Enhanced *fiz* expression evolved twice in out-of-Africa populations

We discovered a several-fold evolutionary divergence in the expression of *fiz* between our "Selected" and "Control" populations of *D. melanogaster*. This divergence has been driven by laboratory natural selection imposed by diet quality and mediated by a *cis*-regulatory polymorphism. Previous studies reported a geographical pattern of variation in *fiz* expression in *D. melanogaster*. While populations from the ancestral range in sub-Saharan Africa show low expression, many cosmopolitan populations (from Europe, North Africa, Central and South-East Asia) contain a substantial frequency of genetic variants with several-fold higher *fiz* expression [28,31,46]. Increased *fiz* expression is associated with reduced adult size and reduced wing loading, which might have facilitated *D. melanogaster* expansion out of Africa [28]. However, most cosmopolitan populations have remained polymorphic, with both high- and low-expression variants likely maintained by a combination of fluctuating and sexually antagonistic selection [29]. In the course of evolutionary adaptation to a nutrient-poor larval diet, our Selected populations reverted to the Sub-Saharan low expression phenotype of *fiz*, while the Control populations maintained on standard diet retained the polymorphism or became fixed for the high-expression variant.

A single nucleotide polymorphism in the promoter region has previously been identified as responsible for much of variation in *fiz* expression across *D. melanogaster range*, with two additional SNP variants with smaller effects [28]). However, these polymorphisms are absent from our populations. The divergence in *fiz* expression between Selected and Control populations must therefore be mediated by a different genetic polymorphism, a likely candidate being the SNP within *fiz* promoter at position X:14909076, with further potential candidates upstream. Thus, irrespective of the forces favoring it, increased *fiz* expression appears to have evolved independently twice during out-of-Africa expansion of *D. melanogaster*, a case of convergent evolution at the molecular level. Such cases of independent evolution of the same molecular phenotype based on different mutations of the same gene are rather scarce in animals [e.g., 47,48].

Downregulation of *fiz* reverses undernutrition-induced growth inhibition

We found that *fiz* knockdown resulted in faster larval growth, shorter development and larger adult size when larvae were raised on a nutrient-poor diet. We also confirmed an earlier finding [28] that *fiz* knockdown increased adult size without a change in developmental on a standard diet. The *fiz* knockdown also induced high larval mortality on both diets. This is likely the reason (or at least a major reason) why downregulation of *fiz* was favored in the course of experimental evolution of our Selected populations on the poor diet. Faster growth under nutritional restriction can be mediated by two complementary mechanisms: enhanced acquisition of dietary nutrients resulting in greater availability of raw materials (amino acids, lipids, etc.) and energy, and promotion of growth via signaling pathways that regulate the rate at which these raw materials and energy are used for growth. While adaptation of the Selected populations to undernutrition involves both mechanisms [11,13,14], our results and preexisting knowledge indicate that *fiz* downregulation contributed to their adaptation via the latter mechanism, i.e., changes in growth regulation. *fiz* has been identified as growth inhibitor in *Drosophila* larvae [28]; in particular it mediates growth inhibition by diet with unbalanced amino acid content [30]. We demonstrated that, consistent with bioinformatic prediction, purified Fiz protein has ecdysone oxidase activity in vitro, acting on both ecdysone and 20E. This supports the notion that *fiz* acts via modulation of ecdysone signaling [28]. Ecdysone is a key insect hormone that triggers molts and metamorphosis; however, it also acts as a negative regulator of systemic growth throughout development, in particular in response to nutrient shortage [23,49–51] and under mild hypoxia [52]. It is unlikely that the downregulation of *fiz* contributes to better nutrient acquisition. Ecdysone or ecdysteroids in general are not known to mediate nutrient acquisition, and the high mortality of *fiz* knockdown larvae on the poor diet suggests that their fast growth drains nutrients and energy from life-sustaining processes. In other words, *fiz* knockdown on its own appears to perturb an adaptive adjustment of growth rate to low nutrient availability.

In addition to *fiz*, four out of five *fiz* paralogs expressed in larvae and annotated as oxidoreductases involved in ecdysteroid metabolism were also downregulated in Selected larvae compared to Controls (Fig 3). This suggests that selection imposed by the poor diet generally acted to reduce ecdysone oxidase activity. Understanding how this might modify ecdysone signaling and promote growth is hindered by the dearth of knowledge about the biological function of ecdysone oxidases in *Drosophila*. Based on research in Lepidoptera, it has been proposed that ecdysone oxidases act to deactivate 20E [26,53,54]. However, although the predicted products of ecdysone oxidase acting on ecdysone and 20E (3DE and 3D20E, respectively) are a part of ecdysteroid metabolism in *Drosophila* [25], 3-oxidation does not appear to be the main pathway leading to ecdysone deactivation and elimination in this species [35,36,55]. The growth-repressing function of *fiz* is also inconsistent with its acting to deactivate 20E – 20E is also a growth inhibitor [56], and so its deactivation would promote growth rather than repressing it. Intriguingly, several older studies report that 3D20E has a signaling activity in *Drosophila* larvae [57–59]. In particular, while 20E is thought to act mainly via ecdysone receptor EcR [60], 3D20E has been reported to mediate a second, EcR-independent signaling pathway via the HR38 receptor [59] essential for metamorphosis [61]. In *Anopheles gambiae*, male-derived 3D20E transferred with the ejaculate also acts as a remating-suppressing hormone in females [62]. Furthermore, in silkworm 3D E appears to serve as a reservoir of ecdysteroids that are converted rapidly to 20E during embryonic development [27], and a similar conversion of 3D20E to 20E has been reported in the epidermis of *Manduca* larvae. Such "recycling" of ecdysone and 20E deactivation products has also been proposed as a major source of 20E peak during a late stage of *Drosophila* metamorphosis [63]. Our results suggest the intriguing

possibility that the products of ecdysone/20E oxidation directly play an active role in regulating larval growth in *Drosophila* or can be recycled into active compounds that do so. If the growth-promoting effects of downregulation of *fiz* were mediated by modulating the titer of 3D20E rather than 20E, this might explain why we did not see differences in expression of 20E-induced genes *Eip74EF* and *broad* between Selected and Control populations (although, given the dynamic nature of their expression, it is possible that we missed such differences). However, although we demonstrated ecdysone oxidase activity in vitro, we cannot exclude the possibility that *fiz* catalyzes other reactions or is involved in other pathways in vivo.

Adaptation to malnutrition involves coadapted traits

The adaptation of Selected populations to poor diet is manifested in both faster larval growth and higher egg-to-adult survival compared to Control populations [11,12,14]. In contrast, even though *fiz* knockdown resulted in substantially enhanced larval growth on the poor diet (thus recapitulating one aspect of adaptation in Selected populations), this occurred at a great cost to survival. The survival cost was likewise apparent on standard diet. This suggests that on its own a reduction in *fiz* expression is maladaptive on either diet under the conditions implemented in this evolutionary experiment. If so, how to explain the divergence in expression and the apparent fixation of a low-expression *cis*-regulatory variant in all Selected populations? The prevalence of a high expression *fiz* variant in Control populations could reflect a high initial frequency of this variant when sampled from the field and/or selection in favor of this variant on standard diet under laboratory conditions. However, both Selected and Control populations were derived from a base population that had already been maintained–and subject to natural selection–in the laboratory on standard food for >100 generations [12]. Thus, if these conditions favor high *fiz* expression, the high expression variant would have had ample time to reach an appreciable frequency already in the base population, even if this variant had been rare in the gene pool initially sampled from nature. This makes it unlikely for the low expression variant to have been fixed in all six Selected populations by genetic drift. We cannot exclude the possibility that the low expression variant was fixed in Selected populations by selection on a linked genetic variant, given the apparent strong linkage disequilibrium around *fiz* [18]. However, the fact that several putative ecdysone oxidases likewise show reduced expression in Selected populations suggests selection for low ecdysone oxidase activity, whereas results from natural populations [28,29] imply that *cis*-regulatory polymorphisms of *fiz* have adaptive relevance.

Therefore, a more parsimonious explanation for the low *fiz* expression in Selected populations is that, despite not being adaptive on its own, it was favored by selection under the poor diet regime in combination with evolutionary changes in other traits, mediated by other genes. Given that *fiz* acts as growth inhibitor, one may speculate that downregulation in the Selected larvae allowed them to better realize their growth potential despite nutrient shortage. This potential may have been enhanced by improved assimilation of dietary amino acids [13] or changes in metabolism [14]. In contrast, downregulation of *fiz* in otherwise non-adapted larvae appears to cause them to overextend themselves trying to grow at a rate that their metabolism cannot sustain. Furthermore, the faster growth of *fiz*-knockdown larvae compels them to accumulate more biomass before attempting metamorphosis, which becomes increasingly challenging as the condition of the diet deteriorates over time as the nutrients are extracted and metabolic waste products accumulate. Such attempts to grow beyond what the available means permit might explain their high mortality. In contrast, owing to their reduced critical size for metamorphosis initiation, the Selected larvae convert their faster growth into fast development and actually emerge smaller as adults than Controls [14,15]. Only with these

other facets of adaptation to undernutrition would a reversal of *fiz*-mediated growth inhibition be adaptive. Thus, while the precise mechanisms remain to be elucidated, our results indicate that adaptation to larval undernutrition is not only polygenic, but also involves epistatic interactions and multiple co-adapted traits.

Materials and methods

Synthesis and purification of *Fiz* protein

We amplified the coding region of the *fiz* gene from cDNA clone (FI24119, *Drosophila Genomics Resource Center)* with primers (forward: GATCTCACCATCACCATCACCATTCACTA GACGGTGGCCAGAAT; reverse: AAGCTTGTCGACGGAGCTCGGCCAATCGAAACGA GTTCTGTGT) using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich USA, cat. #M0530S). The vector pET-23b_RGS_6xHis_Go [64] was used for subcloning amplified *fiz* sequence, and this vector was digested with NcoI and EcoRI to remove Go sequence. We purified the vector by gel extraction using GeneJet Gel extraction kit (K0692, ThermoFisher Scientific, USA). NcoI-EcoRI fragment containing backbone plasmid and N-terminal 6xHis tag was assembled with amplified *fiz* sequence. Cloning step was performed using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, cat. #E5520S). The cloning was verified by restriction analysis and sequencing.

We transformed *Escherichia coli* strain DE3-Rosetta Gami with our plasmid (pET-23b_RGS_6xHis_fiz) and grew overnight at 37˚C on a Petri dish. Pre-culture initiated with two independent colonies were then grown overnight at 37˚C in LB solution. These precultures were mixed 1:1, and we subsequently inoculated a small volume of this pre-culture with a mix of 1 × LB, 100 μg/mL ampicillin, 10 μg/L polypropylene glycol (pre-culture:medium ratio of 1:100) and grew at 37˚C and 180 rpm to reach an OD_{600} = 0.6–0.8. Cultures were then cooled down for 30 min at 17˚C before a further 8h overnight incubation at 17˚C with 1 mM isopropyl-1-thio-d-galactopyranoside (IPTG). After 10 min centrifugation at 6000 × g, we resuspended cell pellets in 1 × TBS (20mM Tris-HCl pH 7.4, 150mM NaCl) and 1 mM PMSF, before lysing them with a Cell disruptor (Constant systems). We removed debris by centrifugation at 15,000 × g/15 min/4˚C. We collected and mixed the supernatant with 30 mM imidazol and 50 μl of drained beads (HisPur Ni-NTa Resin) per liter of initial culture before incubating at RT for 1 h on a rotating shaker and centrifuging at 1,000 × g/1 min/4˚C. The pellet was washed five times. Each washing consisted of adding 50 mL of washing buffer (1 × TBS and 10 mM imidazole) before incubation on ice for 10–15 min to settle the beads and remove the supernatant. For the third washing, the 10 min incubation on ice has been replaced by an incubation at 4˚C for 1 h on a rotating shaker. The elution was done with 1 × TBS and 300 mM imidazole. The elution volume corresponded to two volumes of drained beads. After a short centrifugation, the supernatant was collected, and the purity of the protein was checked on a gel (Fig E in S1 Appendix). We then performed a Bradford assay (Roti-Quant, K015.3, Roth) to estimate the protein concentration. Because Fiz is predicted to be a flavoprotein (UniprotKB, automatic annotation, [21]), we confirmed that our purified Fiz contained FAD (Table L in S1 Appendix), which is a proxy for proper folding of flavoproteins.

Enzymatic activity of *Fiz* protein in vitro

Ecdysone oxidases (KEGG EC 1.1.3.16) use O_2 to convert ecdysone substrate into 3-dehydroecdysone and H_2O_2; this reaction is thought to deactivate the hormone [22]. No chemical standard of 3-dehydroecdysone was available, precluding an assay based on its detection/quantification. Thus, to test whether Fiz has ecdysone oxidase activity, we used two other methods, one based on quantifying H_2O_2 production with a fluorescent enzymatic assay and the other

using liquid chromatography–mass spectrometry (LC-MS) to measure the decrease of the ecdysone substrate. In addition to ecdysone (a.k.a. α-ecdysone, KEGG C00477), in both methods we also used the active form of ecdysone, 20-hydroxyecdysone (20E, a.k.a. β-ecdysone or β-ecdysterone; KEGG C02633) as a potential substrate.

For the first method, the H_2O_2 production was quantified with ROS-Glo H_2O_2 assay kit (#G8820, Promega, Switzerland). We incubated 28 μg of purified Fiz with 20 μL of 125 μM ecdysone or 20E and Tris-HCl (pH = 8) to reach a final volume of 80 μL, and with 20 μl of H_2O_2 substrate from the kit for 1 h. We then incubated the reaction with ROS-Glo detection solution for 20 min. The amount of H_2O_2 was estimated by measuring relative luminescent values with a plate reading luminometer (Hidex Sense Plate Reader, Labgene, Switzerland). Two negative controls were done for each assay: one with the substrate (Ecdysone or 20E) and without the Fiz protein (buffer only), and one with the Fiz protein and without the substrate. Four and three replicate reactions were quantified for each condition involving Ecdysone or 20E, respectively. Five negative controls with only Fiz protein (no ecdysone/20E) were performed, four simultaneously with the Ecdysone assay and together with the 20E assay.

For the second method, we incubated 28 μg of purified Fiz with 20 μL of ecdysone 125 μM or 20E 125 μM and Tris-HCl pH 8 (final volume of 80 μL) for 80 min. As negative control, we replaced Fiz with its TBS buffer. The reaction was then quenched with 320 μL of 80% methanol and stored at −80˚C. Alpha-ecdysone and 20-hydroxyecdysone (beta-ecdysone) were quantified by LC-MS/MS analysis in positive ionization mode using a TSQ Altis triple quadrupole system (QqQ) interfaced with Vanquish Horizon UHPLC system (Thermo Scientific). The chromatographic separation was carried out in a Accucore aQ column (2.6 μm, 100 mm × 2.1 mm I.D) (Thermo Scientific). Mobile phase was composed of A = 0.1% acetic acid in water and B = 0.1% acetic acid in ACN at a flow rate of 250 μL/min. Column temperature was 40˚C and sample injection volume 2μL. The linear gradient elution starting from 2% to 100% of B (in 5 min) was applied and held until 6 min. The column was then equilibrated to initial conditions. ESI source conditions were set as follows: voltage 3500 V in positive mode, Sheath Gas (Arb) = 50, Aux Gas (Arb) = 10, Sweep Gas (Arb) = 1 and Ion Transfer Tube Temperature 275˚C. Nitrogen was used as the nebulizer and Argon as collision gas (1.5 mTor). Vaporizer Temperature was set to 350˚C. Selected Reaction Monitoring (SRM) was used as acquisition mode with a total cycle time of 400 ms. Optimized collision energies for each metabolite were applied. Raw LC-MS/MS data was processed using the Thermo Scientific Xcalibur software. External calibration curves (spanning the wide concentration range from 1nM to 20μM) were used to report the estimated concentrations of ecdysone and 20E.

The activity of purified Fiz was analyzed by fitting a linear model (LM) with either the amount of H_2O_2 or substrate (Ecdysone or 20E) as response variable and the condition (purified protein + substrate (E or 20E) or purified protein only or substrate only (E or 20E)) as a fixed factor. We used a type 2 F-test and when applicable, pairwise comparisons were performed with *emmeans* and *pairs* functions in R.

Experimental evolution

This study is based on a long-term laboratory evolution experiment, in which six Selected populations of *Drosophila melanogaster* have been evolving on a nutritionally poor larval diet for over 15 years, with six Control populations maintained in parallel on standard diet. These populations were originally derived from a lab-adapted base population collected in Basel, Switzerland in 1999 and subsequently maintained in the lab on the standard diet (15 g agar, 30 g sucrose, 60 g glucose, 12.5 g dry brewer's yeast, 50 g cornmeal, 0.5 g CaCl2, 0.5 g MgSO4, 10 mL Nipagin 10%, 6 mL propionic acid, 20 mL ethanol per liter of water) until the evolution

experiment commenced in 2005. The Control populations have continued to be maintained on the standard diet. The six Selected populations have been maintained on a diluted larval diet, containing one-fourth of the amounts of sugars, yeast and cornmeal of the standard diet; we refer to this diet as "the poor diet". All populations were maintained at a controlled density of approximately 200–250 eggs for 40 mL of food, with a generation cycle of 3 weeks and census size of about 180–200 breeding adults. After their emergence, adult flies of both selection regimes were transferred to standard diet and additionally fed ad libitum live yeast to allow mating and egg laying. Fly stock maintenance and all experiments were performed at 25˚C with 50–70% humidity and 12:12 light cycle. Details of the fly maintenance are described elsewhere [12].

For this study, we chose a subsample of three Control (C2, C4, C6) and three Selected (S1, S2, S3) populations. This allowed us to limit the workload so that despite multiple timepoints or crosses we could still process all populations at the same time. Furthermore, these three Selected and three Control populations were fixed for alternative alleles at candidate SNPs associated with *fiz* and showed clear differences in *fiz* expression, which was not the case for some of the populations left out of this study, notably C1 and C3 (see Results).

Before each experiment, we reared all populations on standard diet for at least two generations (relaxed selection) to limit environmental maternal effects. To obtain larvae for each experiment, we let approximately 200 adult flies from a given population lay eggs overnight on an orange juice-agar plates supplemented with yeast. The desired number of eggs was then transferred onto the experimental media (see below) and inoculated with feces suspension (OD_{600} = 0.5) from a pool of flies from all populations to ensure homogeneity of larval microbiota [14]. The assays reported here were performed after 258 to 290 generations of experimental evolution.

Expression of ecdysone oxidase and ecdysone-dependent genes across development

We used RT-qPCR to compare the expression of ecdysone oxidase genes and several other genes of interest between the Control (C2, C4, C6) and Selected (S1, S2, S3) populations in two independent experiments. For both experiments, the food was homogenized with a blender before adding eggs to facilitate subsequent larvae collection at different time points.

The first experiment aimed to test for consistent differences in expression throughout much of the development and into the adult stage. From each population we transferred approximately 100 eggs into each of four bottles (three bottles for larvae–one bottle per collection day; one bottle for prepupae and adults) with about 15 mL of either poor or standard diet. From the poor diet, we collected larvae at day 4 and 5 post egg laying as well as at day 7 for Controls only (at day 7, Selected larvae have already started to pupariate). From the standard diet larvae were sampled at day 3, 4 and 5 post egg-laying. From both diets we also sampled white prepupae with everted spiracles and virgin male and female adult flies (two samples of seven individuals per population, diet and stage). For each timepoint we examined another sample of larvae to determine if they were in the second or third instar (based on the shape of anterior spiracles). See Fig B in S1 Appendix for the correspondence between larval stage and age.

In the second experiment we aimed to test for differences in gene expression during the first three quarters of the third larval instar. This was only done on the standard diet, which allowed for good synchronization of the developmental stage. Approximately 200 eggs were transferred to a bottle with 40 mL of standard diet. After 78–80 hours, from each bottle we sampled around 100 second instar larvae and transferred them to a new bottle containing 15

mL of fresh standard diet. 17 h later, from each bottle we collected one sample of 10 third instar larvae. At this time, we also transferred two sets of 20 3rd instar larvae in two new bottles with standard diet and collected samples of 10 larvae 24 and 36 hours later. Three replicate samples per population and stage were collected from different sets of bottles. In this experiment, we also measured the expression of two other paralogs of *fiz* (*CG9521* and *CG12539*), two genes involved in activation/deactivation of 20E (*shade* and *Cyp18a1*) as well as two ecdysone-dependent genes (*broad* and *Eip74EF*).

The samples were flash-frozen in liquid nitrogen and stored at –80˚C. We extracted RNA with the RNeasy Plus mini Kit (#74134, Qiagen, Switzerland) following manufacturer's protocol and quantified with a spectrophotometer (DS-11 FX, DeNovix, Bucher Biotec AG, Switzerland); 300 ng was used as template for the reverse transcription using the PrimeScript RT Master mix (#RR036A, Takara, France). The qPCR was performed with a QuantStudio 6 Flex system equipped with a 384-well block and the SspAdvanced Universal SYBR Green Supermix (#1725272, BioRad, Switzerland) under the following conditions: 95˚C for 30 sec, 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec. The melt-curve analysis was between 65 and 95˚C with a 0.5˚C increment at 5 sec per step. Primer efficiency was estimated on the same device. All PCR primers used in this paper (listed in Table E in S1 Appendix) were designed to avoid any SNPs (candidate or not) previously identified in these populations [18]. In addition to the target genes, we amplified three reference genes (*αTub84B*, *eEF1α2*, *RpL32*), chosen for their stability with diets [65]. For the analysis of gene expression, we took the median of the technical replicates. Two to four technical replicates were done depending on the experiment and genes.

We quantified the expression of each gene (GOI) of interest relative to the (unweighted) geometric mean of the three reference genes [66] and expressed it on $\log_2$ scale:

$$\log_2(\text{relative expression of a GOI}) = \text{Mean}[\log_2(\frac{(E_{GOI}+1)^{-Ct\ GOI}}{GeoMean[(E_{REF}+1)^{-CtREF}]})]$$

where E is the primer efficiency, *Ct* is the number of cycles until the threshold, and Mean stands for arithmetic mean.

To compare the relative expression of genes of interest ($\log_2$ transformed), we fitted for each gene a linear mixed model (LMM) with Type 3 F-tests and using R function *lmer* [67]. The selection regime (Control or Selected), the developmental stage and their interaction were included as fixed factors. The replicate populations nested within the selection regime in interaction with stage were included as random factors. Pairwise comparisons were performed with *emmeans* and *pairs* functions in R. P-values of pairwise comparisons were adjusted with sequential Bonferroni [68].

Allele-specific expression of *fiz* and *CG9512* in F1 female larvae

To test for *cis*- or *trans*-regulatory nature of difference in *fiz* and *CG9512* expression, we used amplicon sequencing to assess the relative contribution of the alleles originating from the Selected and Control populations to mRNA pool in heterozygous larvae [39]. With *fiz* and *CG9512* being located on the X chromosome, these larvae had to be female. Because phenotypic sexing of live larvae, based on presence or absence of developing testes, is only practical in late third larval instar, we focused on this stage. We generated three types of SEL × CTL F1 crosses by crossing virgin females of populations S1, S2 and S3 with males from Control populations C2, C4 and C6, respectively. Similarly, CTL × SEL F1 crosses were obtained by making the same crosses in the reverse direction. The F1 larvae were raised on standard diet as described above; larvae of the parental Control and Selected populations were raised in parallel.

We collected samples of seven late (but not yet wandering) third instar larvae (5 days-old) judged to be female based on the apparent absence of developing testes (four samples per population or cross; four samples of male larvae were also collected for the assay described in the next subsection). The samples were flash-frozen and stored at −80˚C. We extracted both RNA and DNA from these presumed-female larval samples. The samples were homogenized in 1 mL of TRIzol Reagent (#15596018, ThermoFisher Scientific, Switzerland) solution and both DNA and RNA were extracted from each sample using the manufacturer's recommendations. The quality of nucleic acids was assessed with a Fragment Analyzer (Agilent, Switzerland) at the Lausanne Genomic Technologies Facility of the University of Lausanne.

Sexing of larvae as female is prone to errors because it based on not seeing developing testes, which can be poorly visible (e.g., embedded in the fat body). Thus, to ensure the samples only contained female larvae, we tested for the presence of the Y chromosome by performing PCR on DNA extracted from those samples with primers specific to two Y chromosome genes, *FDY* and *Pp1-Y2* [69,70]. A further PCR on the autosomal gene *Act42A* served as a positive control for the quality of DNA. Of the 48 samples, one sample where extraction failed and eight samples in which an amplicon for at least one of these two Y-chromosome genes was detected were discarded (Table M and Fig F in S1 Appendix). This resulted in two to four replicates per cross retained for amplicon sequencing.

RNA from these retained samples was reverse-transcribed into cDNA using 250 ng as a template and the PrimeScript RT Master mix (#RR036A, Takara, France). To provide a comparison point for the allele-specific expression in F1 larvae, we also generated 50:50 mixed parental samples by mixing equal amounts of cDNA from a Selected and a Control sample, using the same population pairings as those used to generate the crosses. The relative amounts of transcripts originating from the two populations in the 50:50 mix should thus correspond to the relative differences in expression between the populations.

To generate the amplicon, PCR was performed on the cDNA with the enzyme 2X KAPA HiFi Hotstart ReadyMix (#KK2602, Roche Diagnostic, Switzerland) and the following conditions: 95˚C for 3 min, 35 cycles of 98˚C for 20 sec, 60˚C for 15 sec and 72˚C for 15 sec and a final extension step at 72˚C for 1 min. cDNA libraries were prepared according to the 16S Metagenomic Sequencing Library Preparation protocol (15044223 Rev. B, Illumina). Libraries were indexed using Nextera XT Index Kit (FC-131-1002/Illumina). They were subjected to Illumina MiSeq paired-end sequencing (MiSeq v2 PE250, Illumina, Switzerland) in one lane at the Genomic Technologies Facility of the University of Lausanne. The primers used here differed from those used for qPCR (Table E in S1 Appendix). They contained the overhang and were located within one exon whereas for qPCR, when possible, one primer was exon spanning. *fiz* and *CG9512* primers bonded the second exon and amplified a region containing one and three SNPs, respectively. For simplicity, for *CG9512*, we did the analysis focusing on only one of the three SNPs.

After de-multiplexing, the reads were first sorted to each gene based on the sequence using a custom script. Subsequently, the reads identified as corresponding to the allele originating from the Selected and Control population were counted. This was done based on the base identity at the coding SNPs, at which the Selected and Control populations were fixed for alternative alleles: G/A at position 791 after start codon (X:14908215) for *fiz* and C/T at position 904 (X:14904032) for *CG9512* [18]. With at least 23,000 reads covering each SNP in any sample we obtained precise estimates of relative abundance of the allelic transcripts.

We verified that the parental populations were indeed fixed for alternative alleles at the focal coding SNPs by sequencing genomic DNA amplicons from the female larval samples from populations C2, C4, C6, S1, S2, S3. Although some alleles in each of these genomic DNA amplicons were attributed to the allele supposedly absent from the population, the highest

frequency was 1.5%, well below the lowest non-zero frequency of 1/14 = 7.1% possible in a sample of 7 individuals. Thus, these "wrong" alleles likely represent sequencing errors.

For each SEL × CTL, CTL × SEL and mixed parental line sample, we calculated the ratio of each allele as log(number of reads allele 1/number of reads allele 2), with allele 1 and 2 being arbitrary chosen for each gene. This ratio was then analyzed with a LMM with the cross type (SEL × CTL or CTL × SEL or mixed parental line) as a fixed factor and the pairing population nested within the cross type as a random factor. Custom contrasts using emmeans R package were then applied to test if the ratio in both F1 together deviated from 50:50, which would indicate a *cis*-regulatory component to their difference in expression. Furthermore, we compared the ratio of the F1 larvae with those in the mixed parental lines; if the proportion in F1 larvae deviated less from 50:50 than that of the mixed samples it would indicate a contribution of a trans-regulatory component to the expression difference.

Expression of ecdysone oxidase genes in Selected, Control and F1 male larvae

To test whether the differential expression of *fiz*, *CG9512*, *Eo* and *CG45065* between selection regime was also observed in males, we quantified (with RT-qPCR) the expression of male Selected and Control late third instar larvae. Furthermore, we quantified the expression of these genes in larvae from F1 crosses between Selected and Control populations. Because the male larvae only carried maternal X chromosome, a difference in expression between reciprocal crosses that resembled the difference between their maternal populations would indicate that the difference in expression is mediated by (*cis*- or *trans*-acting) genetic variants located on the X chromosome.

We used samples of seven male larvae collected from the same larval cultures as the female larval samples used in amplicon sequencing (see above). RNA was extracted and cDNA generated as described above. RT-qPCR was performed with SsoAdvanced Unversal SYBR Green Supermix (#1725272, BioRad, Switzerland) and the CFX96 Touch System (BioRad, Switzerland) under the following conditions: 95˚C for 30 sec, 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec. The melt-curve analysis was between 65 and 95˚C with a 0.5˚C increment at 5 sec per step. We analysed the melting curves with Bio-Rad CFX Maestro software and ensured that a single product was amplified. We measured the expression of *fiz* and *CG9512* relative to three reference genes (*αTub84B*, *eEF1α2*, *RpL32*). The relative expression of the genes of interest ($\log_2$ transformed) were calculated as in 4.4. section and similar LMM were done for each gene with the population type (the selection regime (Control or Selected) and the type of F1 (CTL×SEL or SEL×CTL) as fixed factor. The replicate populations nested within the population type were included as a random factor. Pairwise comparisons were performed with *emmeans* and *pairs* functions in R.

Effect of *fiz* knockdown on larval performance

To knockdown *fiz* expression, we expressed a UAS-fiz^{RNAi} construct under the control of ubiquitously expressed Act-GAL4 driver. We crossed >100 virgin Act-GAL4/CyO, *twi*-UAS-GFP females with 20–40 males homozygous for UAS-fiz^{RNAi} (ID: 107089; Vienna Drosophila Resource Center, Austria). As the control, we used offspring from the cross between Act-GAL4/CyO, *twi*-UAS-GFP females and males from a line containing an empty vector at the same genomic location (UAS^{-}; ID: 60100; Vienna Drosophila Resource Center, Austria). The female driver line being heterozygous, only half of the offspring would carry the driving Act-GAL4 haplotype; the other half would carry CyO, *twi*-UAS-GFP and thus express GFP. We thus sorted embryos based on the GFP phenotype (6 h after the end of oviposition to give

them enough time for the GFP to become visible) and transferred groups of 200 non-GFP eggs to bottles with either standard or poor diet (N = 4–5 replicate bottles per cross and diet; for logistic reasons, the two diets were tested in separate experiments).

For each bottle of each cross and diet, we estimated the egg-to-adult survival probability (i.e., the number of eclosed adults divided by the number of eggs) and analyzed it by fitting a generalized linear mixed model (GLMM) for each diet with a binomial error distribution with the number of eclosed *versus* non-eclosed flies as the response variable, the genotype (control or *fiz*-knockdown) as fixed factor and the bottles as random factor. GLMM were fitted using the mixed function in *afex* R package [71] and significance of fixed factor was tested with Likelihood Ratio test (LRT) method. Eclosing adults of either sex were scored daily to estimate egg-to-adult developmental time (we used the inverse of the development time for the analysis). Females eclosed on each day were collected, dried at 70˚C for 24h and weighed as a group with a precision balance (Mettler Toledo, MT5) to the nearest 1 μg. Growth rate was calculated as in [14]: growth rate = ln(female weight/egg weight)/larval developmental time, where egg weight was assumed to be 5 μg [12] and the larval developmental time was estimated as the time from egg laying to the day on which the females were collected minus 5 days to account for the time needed for embryonal development and metamorphosis. To analyze the effect of fiz-knockdown on these performance traits, we fitted a LMM for each diet with inverse of developmental time or female weight or female growth rate as response variable, the genotype (control *versus fiz*-knockdown) and when applicable, the sex or the developmental time, quadratic development time and their interaction with genotype as fixed factors. For all these models, the replicate bottles were used as random factors. We tested for studentized residuals and removed one outlier data point (studentized residual > 3) for weight and growth rate.

In each experiment an additional bottle with non-GFP embryos on standard diet was prepared to test the effectiveness of the knockdown. Five days later, late third instar larvae were collected in three replicates and *fiz* expression was measured with RT-qPCR as described above, using *αTub84B*, *eEF1α2* and *RpL32* as reference genes. As previously, to test the effectiveness of *fiz*-knockdown in each block, we fitted a LM and a type 2 F-test with the genotype (control *versus fiz*-knockdown) as fixed factor.

Identifying transcription factor binding motifs

To find out whether the candidate SNP in the *fiz* promoter region may be part of a transcription factor binding motif (TFBM), we used the *Tomtom* tool of the *MEME Suite* v. 5.5.5 [72], with a 21 bp sequence centered on the candidate SNP as the query. We used an FDR value of $q = 0.05$ as a cutoff and limited the search to the (–) strand, on which *fiz* is located.

Statistical analysis

Statistical analyses were performed in R statistical software, as described above. The R scripts are available in S1 Scripts.

Supporting information

S1 Appendix. Figs A-F and Tables A-M.
(PDF)

S1 Table. Polymorphisms (SNPs and indels) within 3kb upstream and downstream of *fiz* start codon identified in whole genome pooled sequencing and their allele frequencies in the Control and Selected populations.
(XLSX)

S2 Table. The frequencies of derived alleles at SNP positions X:14909076 and X:14909071 in European and North American populations of Drosophila melanogaster.
(XLSX)

S3 Table. Genotypes at the SNP positions X:14909076 and X:14909071 in the Drosophila Genome Nexus collection.
(XLSX)

S1 Data. Original data.
(ZIP)

S1 Scripts. R scripts used in data analysis.
(ZIP)

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Author Contributions

Conceptualization: Fanny Cavigliasso, Mikhail Savitsky, Berra Erkosar, Hector Gallart-Ayala, Julijana Ivanisevic, Vladimir L. Katanaev, Tadeusz J. Kawecki.

Data curation: Fanny Cavigliasso, Hector Gallart-Ayala.

Formal analysis: Fanny Cavigliasso, Hector Gallart-Ayala, Tadeusz J. Kawecki.

Funding acquisition: Vladimir L. Katanaev, Tadeusz J. Kawecki.

Investigation: Fanny Cavigliasso, Mikhail Savitsky, Alexey Koval, Berra Erkosar, Loriane Savary, Hector Gallart-Ayala.

Methodology: Mikhail Savitsky, Loriane Savary, Hector Gallart-Ayala, Tadeusz J. Kawecki.

Project administration: Tadeusz J. Kawecki.

Visualization: Fanny Cavigliasso, Tadeusz J. Kawecki.

Writing – original draft: Fanny Cavigliasso, Tadeusz J. Kawecki.

Writing – review & editing: Fanny Cavigliasso, Mikhail Savitsky, Hector Gallart-Ayala, Julijana Ivanisevic, Vladimir L. Katanaev, Tadeusz J. Kawecki.

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