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A non-coding indel polymorphism in the *fruitless* gene of *Drosophila melanogaster* exhibits antagonistically pleiotropic fitness effects

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The amount of genetic variation for fitness within populations tends to exceed that expected under mutation–selection–drift balance. Several mechanisms have been proposed to actively maintain polymorphism and account for this discrepancy, including antagonistic pleiotropy (AP), where allelic variants have opposing effects on different components of fitness. Here, we identify a non-coding indel polymorphism in the *fruitless* gene of *Drosophila melanogaster* and measure survival and reproductive components of fitness in males and females of replicate lines carrying each respective allele. Expressing the *fruitless* region in a hemizygous state reveals a pattern of AP, with one allele generating greater reproductive fitness and the other conferring greater survival to adulthood. Different fitness effects were observed in an alternative genetic background, which may reflect dominance reversal and/or epistasis. Our findings link sequence-level variation at a single locus with complex effects on a range of fitness components, thus helping to explain the maintenance of genetic variation for fitness. Transcription factors, such as *fruitless*, may be prime candidates for targets of balancing selection since they interact with multiple target loci and their associated phenotypic effects.

1. Introduction

Genetic variation for fitness provides the raw material for selection and genetic drift to cause the genetic evolution of populations [1]. The action of both forces, however, tends to reduce genetic variation. This is particularly relevant in the case of traits that are closely linked to fitness and therefore, by definition, under strong directional selection. The classic explanation for the presence of heritable variation for fitness in populations is mutation–selection–drift balance, where standing variation is maintained at an equilibrium between the generation of new variation by recurrent mutation and its reduction through selection and drift [2,3]. Yet most populations typically harbour considerable amounts of genetic variation for traits and fitness—and more than can be accounted for by mutation–selection–drift balance alone [4]. This discrepancy between theoretical expectations and empirical data constitutes a central and perennial puzzle in evolutionary biology [4,5].

One possible resolution of this paradox is that fitness variation is actively maintained by balancing selection. Initially popularized by Dobzhansky [6], balancing selection is a force actively maintaining two or more allelic variants at a locus. The active maintenance of polymorphism requires that the selective value of an allele depends on the context in which it finds itself [7,8]. Allelic fitness effects can depend on the genetic context within an individual, as in the case of overdominance [9] or reciprocal sign epistasis [10], or the genetic context in the

population, as with negative frequency-dependent selection [11] or variable environmental conditions (fluctuating selection, [12]). In the case of antagonistic selection, polymorphism is maintained because the fitness effect of an allele depends on the sex of the carrier (sexual antagonism, [13,14]), or on an individual's life-history stage (antagonistic pleiotropy, [15]).

Antagonistic pleiotropy (AP) occurs when mutations have a beneficial effect on one fitness component but a deleterious effect on another. Initially conceived in the 1950s [15,16], AP has become a major hypothesis for the evolution of ageing, where mutations that increase fitness early in life are proposed to cause deterioration and increased mortality later in life [15,17]. AP could maintain genetic variation if, for example, one allele confers increased early life fitness and a shorter lifespan, while the other causes a more even reproductive output over a longer life, with both strategies providing similar long-term fitness pay-offs and greater fitness than an intermediate strategy [18,19]. Despite some empirical evidence of pleiotropic trade-offs [20], modelling has shown that the conditions under which AP generates balancing selection and maintains polymorphism are quite restrictive [18,21–23]. This, combined with relatively few empirical examples of AP in nature, has led researchers to question whether AP is a major contributor to the maintenance of genetic variation for fitness [22,24].

However, recent theoretical and empirical studies have reignited interest in AP as a mechanism generating balancing selection. Models of metapopulation structure in fungi [25] and viability and fertility selection in flowering plants [26] have demonstrated a crucial role of AP in maintaining genetic variation for fitness in wild populations. Similarly, Mérot *et al*. [24] found that AP in fitness effects and the resulting variation in life-history trade-offs is most likely responsible for the maintenance of an inversion polymorphism in the seaweed fly *Coelopa frigida*. More recent theoretical models have further shown that the conditions required for AP to generate balancing selection are less stringent than initially believed. For example, taking into account sex-specific fitness effects or even small variations in dominance between traits or over time may be enough for AP to generate balancing selection under a wider range of conditions [27]. Furthermore, AP may generate excess fitness variance (relative to unconditionally deleterious mutation–selection balance) by slowing the removal of deleterious variation, rather than maintaining it *per se* [8,27]. Together these developments suggest that the proportion of AP genetic variation (and possibly balanced variation) has been historically underestimated [4], underscoring the need for further experiments that link sequence-level polymorphism with measurements of fitness components at different life stages, ideally in both sexes.

In this study, we describe AP fitness effects associated with a polymorphism in a non-coding region of the *fruitless* gene (*fru*) of *Drosophila melanogaster*. The *fru* gene is a key component of the sex-determination cascade and is responsible for sex-specific nervous system development and courtship behaviour [28–30]. In line with its crucial functions, *fru*'s protein-coding sequence is conserved across insect taxa [31]. Contrasting with the evolutionary constraint that is evident at the phylogenetic level, *fru* also exhibits evidence of positive selection [32]. In line with this evidence for ongoing selection, we identify here a polymorphism within the 5′ non-coding region of the *fru* gene. The polymorphism consists of an indel and linked SNPs that segregate at intermediate frequencies across worldwide populations of *D. melanogaster*. To investigate why this locus is unusually polymorphic, we assess the consequences of each respective allele for multiple fitness components in both sexes. We find that one allele confers higher reproductive fitness in both sexes, while the alternative allele results in greater larval survival and, in some cases, greater adult longevity. These effects further depend on the genetic background in which the alleles are expressed, suggesting that dominance reversal and/or epistasis may also contribute to the maintenance of this polymorphism. Our study adds to the growing body of evidence for a reassessment of the role played by AP, and possibly balancing selection, in maintaining individual allele polymorphisms and genetic variation for fitness.

2. Methods

(a) Identification of an indel in a polymorphic region of *fru*

A polymorphic region of *fru* was identified by investigating signatures of balancing selection in population genomic data from two collections of wild flies from Raleigh, US ($N = 205$; [33]) and Zambia ($N = 197$; [34]), using metrics of genetic diversity (nucleotide diversity, Tajima's D) and linkage disequilibrium (LD, quantified as Kelly's ZnS) (electronic supplementary material, Methods S1). Based on these analyses, a 1000 bp region of elevated polymorphism and LD was identified. To characterize this region further, we performed Sanger sequencing on a 400 bp stretch within this region from chromosomes sampled from LH_M, a laboratory-adapted North American population of fruit flies [35], revealing a polymorphic indel in *fru*, with a long (L) and short (S) allele (electronic supplementary material, Methods S1).

(b) Fly culture and husbandry

Unless otherwise stated, flies were maintained on corn-agar-molasses medium with a powdering of live yeast in either vials (8 ml of media) or bottles (50 ml) in 25°C constant temperature rooms at 50% humidity on a 12 : 12 h light–dark cycle. When required, flies were collected as virgins, every 0–6 h post-eclosion until sufficient numbers were obtained. Flies were anaesthetised using a CO_2 pad for short periods of time and manipulated using a fly aspirator.

(c) Creation of allelic lines

We created allelic lines, which carried S or L alleles in an isogenic genomic background. Allelic lines were created through initial identification of LH_M individuals carrying the S or L allele (electronic supplementary material, Methods S1), and then backcrossing these individuals into a $Df(3R)fru^{4-40}/TM6B$ stock. Flies of this stock carry chromosomes of an isogenic Canton-S genetic background, except for the third chromosome, where they are heterozygotes for a Canton-S chromosome carrying a deletion covering the *fru* locus ($Df(3R)fru^{4-40}$) [36], and the TM6B balancer chromosome. TM6B contains multiple and nested inversions and carries several homozygous lethal mutations, as well as dominant marker mutations which produce phenotypes for identification, including *Tubby* (*Tb*) that causes a distinct shape of the pupa [37]. Backcrossing was performed over seven generations using the pupal phenotype *Tb* as a marker (for full details of the crossing scheme, see electronic supplementary material, Methods S2 and figure S1). We used this approach to generate three independent lines each for the S (S1–3) and L (L1–3) alleles.

(d) Generating focal flies

We performed fitness assays on 'focal' flies generated by crossing individuals from the allelic lines to flies from the *Df(3R)fru*$^{4-40}$/*TM6B* stock. The resulting individuals carried the *fru* allele (L or S) of a line complemented either by the *Df(3R)fru*$^{4-40}$ deficiency (*D*) or by the TM6B balancer chromosome (B). Since the deleted region of the *Df(3R)fru*$^{4-40}$ chromosome extends over the *fru* locus, flies which inherit this chromosome (D) are hemizygous for whichever *fru* allele they inherit. The *fru* alleles can, therefore, be studied in isolation in D flies. The B chromosome (TM6B) was genotyped (see electronic supplementary material, Methods S1) and found to carry the S allele. The contrast of allelic fitness effects between flies complemented with the D deficiency or the B chromosome thus allows us to gain information on dominance effects of the *fru* alleles and epistatic interactions with the genetic background. The cross to generate focal flies also ensures that line-specific recessive deleterious alleles are masked by complementing with both B and D chromosomes, so as to minimally affect fitness measurements associated with the *fru* alleles. Before crossing, flies were maintained for multiple (greater than 10) generations in bottles containing molasses media, at a population size of 200–300 flies per bottle and three bottles per line.

For each line (S1–3 and L1–3), crosses were performed by setting up replicate vials containing 10 virgin allelic line females and 10 *Df(3R)fru*$^{4-40}$/*TM6B* males. These vials were left overnight for the flies to mate. To limit larval densities, we twice transferred flies to fresh vials for 4 h egg lays (approx. 10.00–14.00 and approx. 14.00–18.00). To establish focal flies carrying the *fru* allele paired with either the D complement (wild-type pupal phenotype) or the B complement (*Tb* pupal phenotype), emerging pupae were sorted into separate vials based on their phenotype. Twelve total line sets were thus established, i.e. lines S1–3 and L1–3 in D or B background, referred as S/D, S/B, etc. when referring collectively to all three lines carrying a particular allele.

(e) Fitness assays

(i) Reproductive success

Focal females were mated to males from their own vial before being placed as triplets at 3 days old into vials containing 1% agar and fed by a capillary tube through the stopper containing a 4 : 1 yeast to sugar solution (6.5 g yeast extract and 1.625 g sugar per 100 ml) at 25°C and 80% humidity, with new food capillaries supplied daily. Triplets were maintained until the focal females were 4–5 days old, since females are initially reluctant to lay in this novel environment and need time to grow accustomed to it. Triplets were then transferred to new agar vials (this time 0.8% agar was used since a lower agar % enabled clearer photos) at approximately 16.00 and allowed to lay eggs for 18 h. Vials were photographed using webcamSeriesCapture (github.com/groakat/webcamSeriesCapture) software and a Logitech HD Pro webcam C920. We used the machine learning program *QuantiFly* (github.com/dwaithe/quantifly) [38] to count the eggs in each picture. Vials where a female died or where bubbles, debris or other contaminants caused counting problems were removed from further analysis. Fitness was assayed in three experimental blocks. In total, 863 successful female fecundity trials were performed.

Focal males were reared on standard food in vials of 30 mixed-sex flies until 4–5 days old. To assay male mating success, focal males were paired with a competitor male from the *Df(3R)fru*$^{4-40}$/*TM6B* stock. Pairs of males were held in vials overnight. The next morning a virgin *Df(3R)fru*$^{4-40}$/*TM6B* female was added to the vial without CO_2 anaesthesia and the two males competed for mating. The males were allowed to compete for 90 min, thereby maximizing the likelihood of a single mating while keeping the rate of double matings negligible. The males were then removed and the female left to lay eggs over a period of several days. Once the larvae pupated, paternity was scored using the pupal phenotype. If all pupae displayed the *Tb* phenotype then paternity was assigned to the competitor (*Df(3R)fru*$^{4-40}$/*TM6B*) male. If pupae were a mixture of wild-type and *Tb*, paternity was assigned to the focal male. Only vials with greater than 10 pupae were included in further analysis, to ensure that the probability of not observing any wild-type pupae among the offspring of a wild-type male would be minimal ($0.5^{10} = 0.001$) and paternity could be reliably scored. We obtained data on mating success for 1149 males across three experimental blocks.

(ii) Larval survival, sex ratio and development time

Fifty virgin females from the *fru* allelic lines and fifty males from the *Df(3R)fru*$^{4-40}$/*TM6B* line were placed together into egg-laying chambers (approx. 2.5 cm diameter, 5 cm height) to mate and lay eggs. The floor of these chambers was composed of a grape juice/agar mixture (172 ml concentrated grape juice per litre) with a small quantity of yeast as a protein source. After 48 h, once they had acclimatized to the conditions, the flies were transferred to an identical chamber with the same food source and left for a further 24–30 h to lay the eggs which would become the 'focal' larvae assessed in this assay. Newly hatched, 1st instar larvae were picked and placed in groups of 50 into vials containing standard medium and left to develop. Newly formed pupae were removed from the vial and placed into new vials depending on their phenotype (*Tb* or wild-type). For each vial and line, we recorded the number of eclosing flies of each sex, the proportion of surviving larvae and the sex ratio (once all flies eclosed). Development time was recorded as the number of days from when larvae were placed in the vial until eclosion as an adult. Complete data on larval survival, sex ratio and development time was collected for 2052 flies (1049 females and 1003 males) from 180 vials.

(iii) Lifespan

Due to the larger number of flies required for this assay compared to previous assays, focal flies were generated using a slightly different method. Groups of 100 *fru* allelic line females and 100 *Df(3R)fru*$^{4-40}$/*TM6B* line males were placed together in an enclosure containing a petri dish filled with corn-agar-molasses medium and left to lay eggs overnight. The next day, small sections of the media, each containing a similar number of eggs, were cut out and placed into individual vials. The eggs were then left to hatch and the larvae to develop. As pupae emerged the flies were separated into vials depending on the pupal phenotype (*Tb* or wild-type). The vials were checked daily until sufficient flies for the experiment eclosed on the same day, which occurred 10 days after eggs were laid. All flies used in the assay were virgins and varied in age by no more than 24 h. Newly eclosed flies were anaesthetised with CO_2, separated by sex, and placed in vials in groups of 10. Every other day (Monday, Wednesday, Friday), flies were transferred to a new vial without anaesthesia. The number of dead flies at each transfer was recorded and dead flies removed. If a fly escaped this was recorded and included in the analysis by censoring. This process was continued until all flies had died. Complete lifespan data were collected for 1659 flies, with partial data obtained for another 257 flies.

(f) Statistical analyses

All statistical analyses were performed in *RStudio* [39]. Mixed-effects models were fitted using the package *lme4* [40]. All mixed-effects models included the flies' line ID (S1–3 or L1–3) as a random variable. If the assay was carried out in multiple blocks, this was also included as a random effect. *p*-values for each model term were calculated using parametric bootstrapping (package *pbkrtest* [41]) based on 1000 simulations.

Egg count output from the *QuantiFly* program was square-root transformed (to achieve better model fitting) and analysed using a linear mixed-effects model (LMM) with Gaussian error.

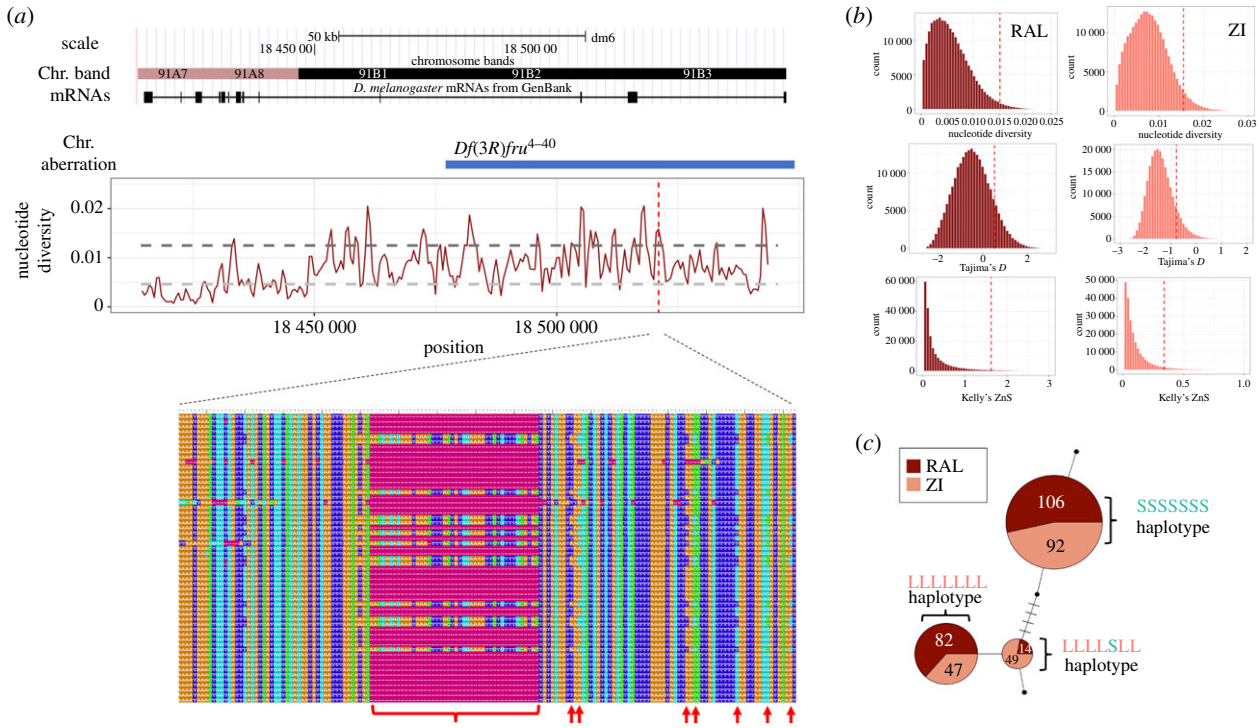

Figure 1. Population genetic signatures of elevated polymorphism in the *fru* gene. (*a*) Map of the *fru* gene, including breakpoints of chromosome bands, gene model, approximate span of the *Df*(*3R*)*fru*$^{4–40}$ deletion, nucleotide diversity (in RAL) in 1000 bp windows (grey horizontal lines = median genome-wide nucleotide diversity; dark grey horizontal lines = 95% quantile of genome-wide nucleotide diversity) and position of the *fru* indel (vertical red dashed line). Alignments of a subset of the approximately 400 bp region spanning the *fru* indel (brackets) obtained through Sanger sequencing of LH_M-derived chromosomes are also shown, with closely linked SNPs (used to construct the haplotype network shown in *c*) shown as red arrows. (*b*) Histograms of nucleotide diversity, Tajima's D and Kelly's ZnS for all 1000 bp windows across the genome in RAL and ZI populations, with the vertical red dashed line representing the 1000 bp window encompassing the *fru* indel. (*c*) Haplotype network constructed from SNPs closely linked to the *fru* indel (red arrows in *a*) in RAL and ZI populations. (Online version in colour.)

The model included the *fru* allele (L or S), chromosomal complement (B or D) and their interaction as fixed effect parameters.

Male mating success was recorded by scoring paternity (focal versus competitor male) as a binary response variable. A GLMM (generalized linear mixed-effects model) with logit link function and binomial error structure was then fitted for this variable, containing the male's *fru* allele, its chromosomal complement, and the interaction between the two, as fixed effects. We also included a random block effect in the model.

Larval survival was measured as the number of adult flies emerging from each vial. An LMM with Gaussian error was applied to the log-transformed number of surviving offspring as a response variable. This produced a better fit according to log-likelihood and AIC than using a GLMM with a Poisson error distribution. The offspring's *fru* allele and chromosomal complement were included in the model as fixed effects. An additional random variable was added to account for the identity of the vial housing each fly before separation at the pupal stage. Sex ratio was calculated as the number of males divided by the total number of flies which emerged from each vial and square-root transformed. A Gaussian LMM was applied to the sex ratio values which included *fru* allele and chromosomal complement as fixed effects and an additional random variable to account for differences between individual vials.

Development time was analysed using a Gaussian LMM including *fru* allele, chromosomal complement, sex and their interactions as fixed effects and larval vial and fly line as random effects. Development time was log-transformed to improve the model fit.

Lifespan data were analysed using Cox proportional hazard (CPH) models from the R package *survival* [42]. A model was constructed including *fru* allele, sex and chromosomal complement as explanatory variables. The significance of model terms was assessed with sequential likelihood ratio tests. Additional models were run with single explanatory variables on either the entire or stratified datasets to estimate hazard ratios for significant model terms. Kaplan–Meier survival curves were fitted using functions from the *survminer* package [43].

3. Results

(a) *Fru* polymorphism

Our population genetic analysis revealed variation in polymorphism levels and LD across *fru* (figure 1*a*, top). One region exhibited elevated polymorphism and LD, both in a Zambian population sample from the ancestral distribution range of *D. melanogaster* and in the DGRP, a population sample from the recently colonized North American range of the species (Raleigh, USA) (figure 1*b*; electronic supplementary material, Results S1). Sanger sequencing of this region (using flies from the North American LH_M population) further revealed an indel polymorphism, with some chromosomes carrying a 43 bp insertion that is in perfect LD with seven SNPs in the flanking sequence (figure 1*a*, bottom). Given that the flanking SNPs occur at intermediate frequencies in the two distantly related worldwide populations (Raleigh: f(L) = 0.475, f(S) = 0.525—Zambia: f(L) = 0.511, f(S) = 0.489; figure 1*c*; electronic supplementary material, Results S1) and given the very close proximity (approx. 10–80 bp) and perfect linkage between flanking variants and the indel in LH_M, we can infer that L (insertion-carrying) and S (deletion-carrying) alleles of the *fru* indel segregate at intermediate frequencies in these two worldwide populations as well.

(b) Reproductive success

There was no effect of the *fru* allele alone on the number of eggs laid ($\chi^2_1 = 2.62$, $p = 0.189$; figure 2*a*). However, there was

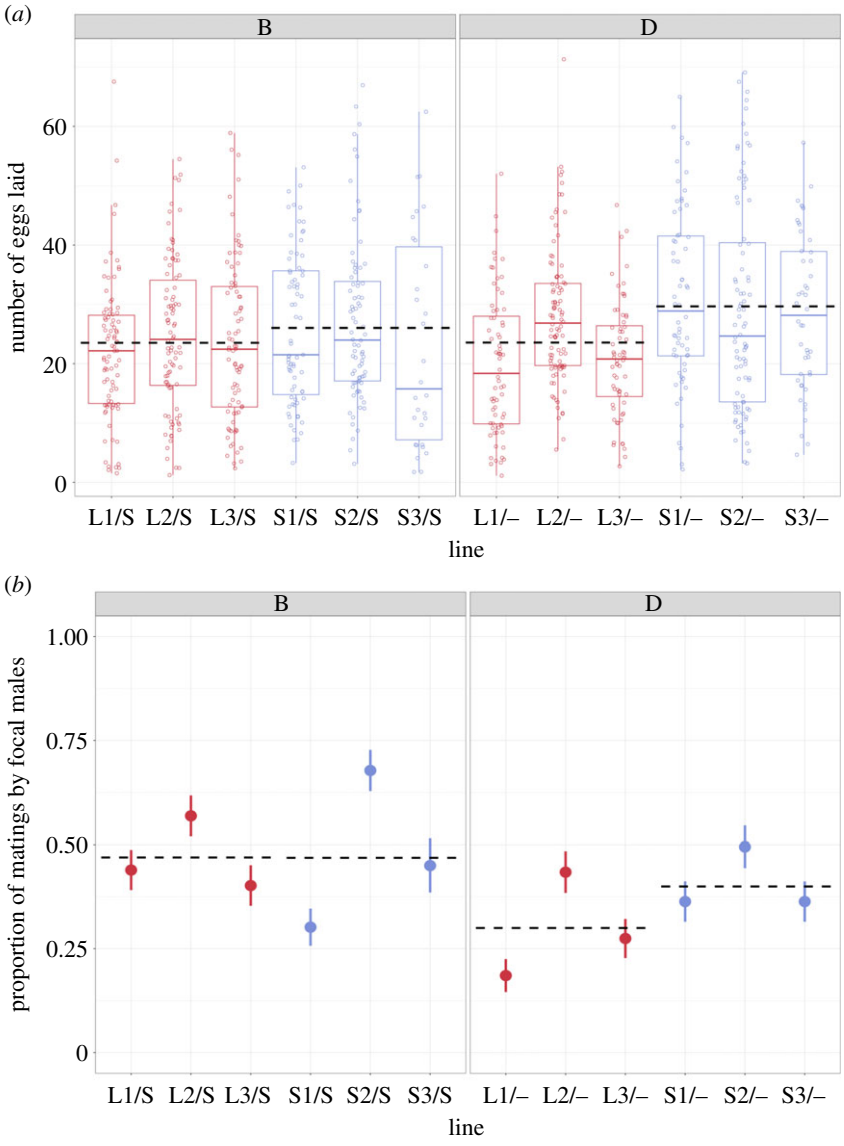

Figure 2. (*a*) Number of eggs laid by triplets of focal females from each line (L1–3 and S1–3) and chromosomal complement (B and D). over an 18 h period. Allelic means represented by dashed lines (L/B: 23.57 ± 0.79; S/B: 26.03 ± 1.06; L/D: 23.57 ± 0.78; S/D: 29.67 ± 1.13). (*b*) Proportion of matings (±standard error) obtained by focal males for each line (L1–3 and S1–3) and chromosomal complement (B and D). Allelic means represented by dashed lines (L/B: 0.47 ± 0.028; S/B: 0.468 ± 0.031; L/D: 0.299 ± 0.027; S/D: 0.407 ± 0.029). Individual data points are not shown in (*b*), as the response is binary (taking only values of 0 and 1). (Online version in colour.)

an effect on fecundity due to the chromosomal complement, with D females laying 7.3% more eggs than B females ($\chi^2_1 = 4.31$, $p = 0.041$; figure 2*a*). Furthermore, there was a significant allele-by-complement interaction, whereby S/D flies laid more eggs (21.6% excess) than all other genotypes ($\chi^2_1 = 4.29$, $p = 0.031$; figure 2*a*).

There was no effect of the *fru* allele on male mating success ($\chi^2_1 = 0.49$, $p = 0.562$; figure 2*b*). The success rate of B males was 32.5% higher than that of D males ($\chi^2_1 = 17.38$, $p = 0.001$; figure 2*b*). There was a clear difference between the alleles when in a hemizygous state (D complement) with S/D males achieving 35.8% more matings than L/D males, though the allele-by-complement interaction was not statistically significant ($\chi^2_1 = 3.52$, $p = 0.058$).

(c) Larval survival and sex ratio

A greater number of L allele larvae survived to adulthood compared to S allele larvae (a 51.2% survival benefit of the L allele; $\chi^2_1 = 7.64$, $p = 0.016$; figure 3) and more larvae inheriting the D chromosome survived to adulthood than those inheriting the B chromosome (22.56% more D than B larvae survived; $\chi^2_1 = 17.95$, $p < 0.001$; figure 3). There was no evidence for an interaction between *fru* allele and chromosomal complement ($\chi^2_1 = 1.25$, $p = 0.275$; figure 3). There were also no significant effects on the sex ratio of emerging adult flies due to either *fru* allele ($\chi^2_1 = 0.054$, $p = 0.809$), chromosomal complement ($\chi^2_1 = 2.14$, $p = 0.158$) or their interaction ($\chi^2_1 = 2.89$, $p = 0.097$; electronic supplementary material, figure S2).

(d) Development time

Females developed 2.1% faster than males across all genotypes ($\chi^2_1 = 98.69$, $p = 0.001$; electronic supplementary material, figure S3) and the B chromosome lead to faster development than the D chromosome by 2.5% ($\chi^2_1 = 9.21$, $p = 0.003$). Yet, the *fru* allele had no significant effect on development time ($\chi^2_1 = 0.36$, $p = 0.655$), nor was there support for two-way interactions between any of the variables (allele-by-sex: $\chi^2_1 = 0.91$, $p = 0.357$; allele-by-chromosome: $\chi^2_1 = 0.038$, $p = 0.848$; chromosome-by-sex: $\chi^2_1 = 2.52$, $p = 0.106$) nor

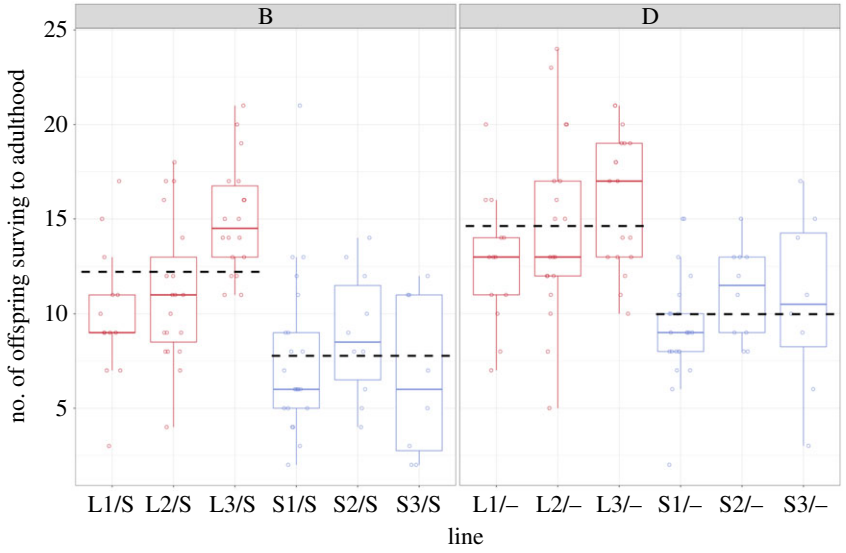

Figure 3. Number of offspring surviving from egg to adulthood for each line (L1–3 and S1–3) and chromosomal complement (B and D). Allelic means represented by dashed lines (L/B: 12.22 ± 0.57; S/B: 7.78 ± 0.64; L/D: 14.62 ± 0.6; S/D: 9.98 ± 0.51). (Online version in colour.)

between all three variables ($\chi^2_1 = 0.012$, $p = 0.921$) (electronic supplementary material, figure S3).

(e) Lifespan

A global analysis across the entire dataset did not reveal a significant effect of allele ($p = 0.71$; figure 4). We did find, however, a significant effect of complement ($p < 0.001$), with greater lifespan (smaller hazard) in flies with the D than the B complement ($HR_{D/B} = 0.72$), and sex ($p < 0.001$), with greater lifespan in males ($HR_{M/F} = 0.82$). The latter effect is probably largely driven by a significant complement-by-sex interaction ($p < 0.001$), where the direction of the sex-difference in survival is reversed between the D complement ($HR_{M/F} = 1.27$) and the B complement, with a large drop in survival of B females ($HR_{M/F} = 0.50$, figure 4). In addition, we found significant pairwise interactions between allele and complement ($p = 0.001$; D complement: $HR_{S/L} = 0.84$; B complement: $HR_{S/L} = 1.14$) and between allele and sex ($p = 0.028$; females: $HR_{S/L} = 1.04$; males: $HR_{S/L} = 0.93$). The three-way interaction was not significant ($p = 0.25$).

4. Discussion

In this study, we identified an indel polymorphism in the *fruitless* gene and measured the performance of allelic lines for a number of relevant fitness components, in both sexes. The data provide evidence for complex allelic fitness effects (table 1 for a summary), with variation in the impact of the *fru* alleles between fitness components, sexes and chromosomal complements.

For cases where the *fru* allele was present in a hemizygous state (paired with the D chromosome) the effects are compatible with AP, in which alleles affect fitness in different and opposing ways (table 1). Thus, flies inheriting the S allele outperformed L flies in assays of male and female adult reproductive fitness, with S females laying more eggs than L females and S males tending to have greater competitive mating success than L males. Conversely, flies inheriting the L allele had greater larval survival than those with the S allele in both sexes. These contrasting effects on reproductive fitness and survival suggest that allelic variants at the *fru* locus act antagonistically, contributing to a major life-history trade-off.

In addition to AP effects, we also find evidence for interactions between the focal *fru* alleles and their chromosomal complement, which is either a wild-type chromosome carrying the deficiency *Df(3R)fru*$^{4-40}$ (D) or a balancer chromosome *TM6B* (B). Because the latter carries an S allele, such that L/B flies are L/S heterozygotes while S/B flies are S/S homozygotes, the comparison between the genotypes in the two complements allows us to make some inferences about dominance. Estimates of phenotypic means from our data suggest dominance for two traits, male mating success and larval survival. For male mating success, S/B (S/S) and L/B (L/S) males perform equally well while S/– males have greater mating success than L/– males (figure 2*b*, significant allele-by-complement interaction), suggesting the dominance of the S allele. For larval survival, in contrast, the difference in eclosion rate between S/S and S/L individuals is similar to the difference between S/– and L/– individuals (figure 3; significant allelic effect but no allele-by-complement interaction), suggesting that the L allele is dominant for this phenotype. These findings of trait-specific dominance raise the intriguing possibility of dominance reversal, where the beneficial allele is dominant for both traits.

Yet there is also evidence for more complex genetic interactions. Thus, there was no difference between the effect of the two alleles on adult mortality when paired with the D chromosome, but in females L flies had lower adult mortality than S flies when paired with the B chromosome. This pattern is indicative of epistatic interactions between the focal polymorphism and the genetic background (as well as the sex-determining pathway). It is not surprising that such interactions should be apparent in our data, given the large number of sequence differences that will be present between the B and D chromosomes. What is less clear is to what degree these effects are biologically meaningful, given the presumably unnaturally high deleterious mutation load on the balancer chromosome. Nevertheless, the fact that epistatic allelic differences for particular fitness components arise in the presence of both complements makes it plausible that similar, albeit potentially weaker, effects would occur in interactions of *fru* alleles with naturally occurring polymorphisms elsewhere in the genome.

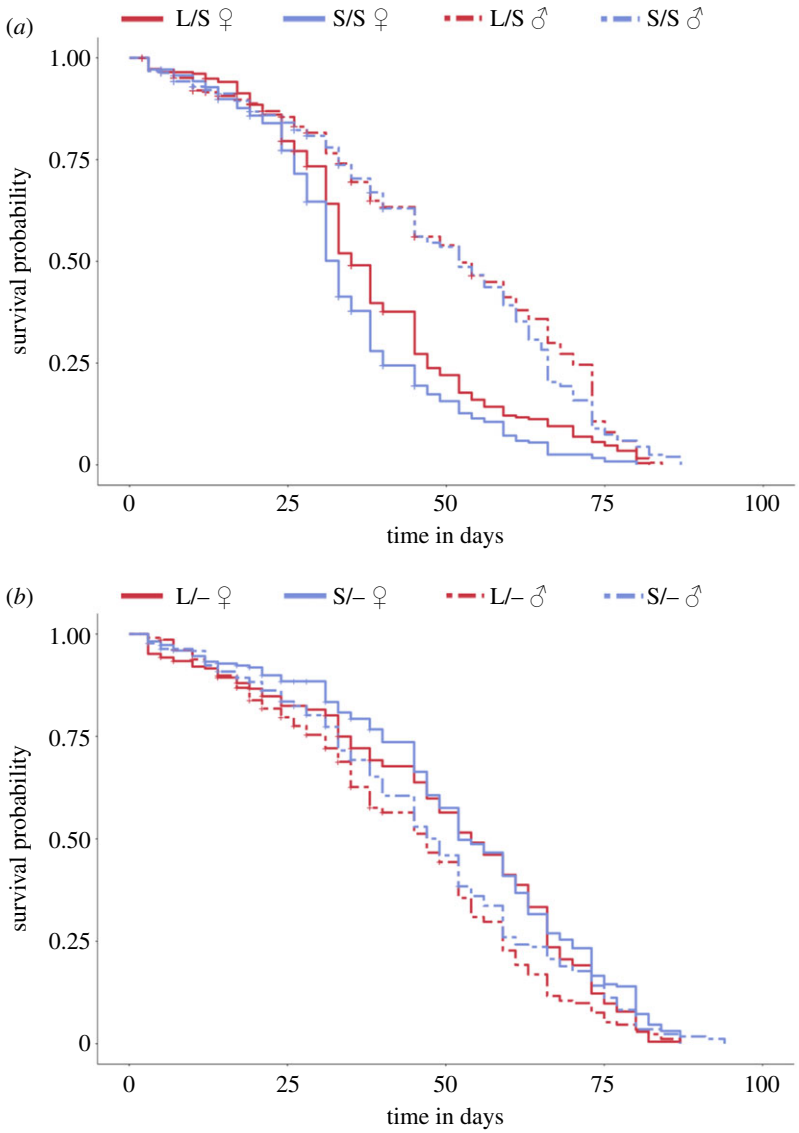

Figure 4. Kaplan–Meier survival curves of flies carrying the B complement (*a*) and D complement (*b*). Line colour designates *fru* genotype (red = L allele and blue = S) and line type indicates sex (solid line = females and dashed line = males). For example, the blue dashed line represents S allele males. (Online version in colour.)

Table 1. Summary of the effects of *fru* alleles S and L on fitness components, in each sex and chromosome complement. The table indicates instances where, based on data, the S allele or the L allele resulted in greater or smaller values (S > L and S < L, respectively) or similar values (S = L) for measures of a fitness component. NA denotes cases where a trait could not be measured.

	B♂	B♀	D♂	D♀
female fecundity	NA	S > L	NA	S > L
male mating success	S = L	NA	S > L	NA
larval survival	S < L	S < L	S < L	S < L
development time	S = L	S = L	S = L	S = L
lifetime	S = L	S < L	S = L	S = L

Life-history traits, such as adult fecundity and survival probability [18,21] that we measured here, are often thought to be associated with genetic trade-offs [19]. In such cases, an increase in performance in one fitness component leads to concurrent decreases in performance in another, for example, due to resource allocation. Within this framework, AP is likely to occur when mutations affect the allocation that underlies the trade-off. AP effects can sometimes maintain genetic polymorphism in general models [18,21], models replicating the properties of specific natural systems [25,26] and in empirical observations [24]. Similarly, the antagonistic fitness relationship we have discovered between the two *fru* alleles may maintain genetic variation at the *fru* locus.

Supporting this interpretation, our findings contradict some of the arguments that had been put forward against a plausible role of AP in maintaining polymorphism through balancing selection [22,23]. For example, classic theory predicts that in order for AP to maintain polymorphism, fitness effects need to be large and similar across fitness components, leading to doubts about the ability for AP as a source of balancing selection based on the assumption that fitness effects are small (less than or equal to 1%) in most cases [5,22]. Interestingly, however, the fitness differences we observe are considerable. In D flies, where AP is evident, S females lay 25.1% more eggs than L females (29.67 versus 23.57) and S males achieve a third more matings than L males (40% versus 30%), while L flies of both sexes survive to adulthood

with a probability that is 46.5% greater than that of S flies (14.62% versus 9.98%). The efficacy of AP-selection would also be weakened if fitness effects were limited to one sex [22,23]. But this again is not the case here: we observe similar effects in both sexes for both reproductive fitness and egg-to-adult survival, although we find no reversal of fitness effects between the sexes (sexual antagonism), which could have further facilitated maintenance of polymorphism in conjunction with AP [27]. Another property that aids the maintenance of polymorphism via AP is dominance reversal, where the beneficial effect of each allele on a given fitness component is dominant [23]. Interestingly, our data provide some evidence for such a pattern, with the S allele exhibiting a dominant beneficial effect on male mating success (see figure 2*b*), while the L allele exhibits a dominant beneficial effect on larval survival (see figure 3 and discussion above). The aggregate heterozygote advantage produced by these two effects will generate balancing selection that helps stabilize the polymorphism at *fru*. In addition, genetic variation could be further stabilized by epistatic interactions [8] such as those observed in fly survival (figure 4) and discussed above. Theoretical models do not often consider epistatic effects in regards to AP, but models have shown that epistasis can help maintain polymorphism at sexually antagonistic loci [44] and similar processes could, in principle, affect AP loci.

Beyond evolutionary dynamics, our results raise the question of how genetic variation at the *fru* locus generates phenotypic effects across the different fitness components we measure. The FRU protein is a BTB–zinc-finger transcription factor and is produced in multiple isoforms, some of which are sex-limited [29,30,36]. The sequence differences between the L and S alleles are upstream of the coding regions, close to the sex-specific promotor P1. Accordingly, the differences observed here between the alleles must arise due to differences in expression levels rather than coding changes, and potentially due to the relative concentrations of different sex-limited and shared isoforms. Both the absolute and relative concentrations of different isoforms could potentially have important consequences on organismal function and phenotypes, given *fru*'s role as a top-level transcription factor. The number of its targets (between 217 and 291 depending on the particular isoform, [45]) would be expected to generate considerable trickle-down effects through the regulatory cascade. Even slight initial differences in *fru* expression between L and S alleles could potentially result in major, and pleiotropic, effects on a range of phenotypes. For example, mutations in *fru* can result in drastic changes in male mating behaviour and brain development [28,29,46]. The large number of target sites also provides a potential mechanism for the epistatic interactions we observe, depending on the interplay between the abundance of the different FRU isoforms, the specific sites they bind to and the regulation that results from that binding. It is difficult to make inferences about these regulatory effects. But an investigation of the sites which interact with *fruitless* is ongoing [45] and together with a more detailed knowledge of how the target loci are involved in behavioural and morphological traits, this will shed light on the mechanism(s) that link *fru* to downstream traits.

In addition to the effects of allelic variants, complements and their interaction, we observed a significant amount of fitness variation between individual lines carrying the same genotype. The method of introgression used to create the allelic lines involved naturally occurring, stochastically placed break points. As a consequence, introgressing a specific allelic variant into the region of interest will also introduce some flanking sequence of unknown size. Variation in the extent of that flanking sequence can generate differences in phenotype between lines carrying a given genotype in the target region. In principle, variation in flanking sequence could also produce systematic differences between S and L lines. In this case, however, the causative variation would require high LD with the S and L alleles.

Notwithstanding these caveats, our study provides a rare manipulative experimental test of the hypothesis that AP maintains polymorphic variation at an individual candidate gene. Our results provide evidence for allelic variants at the *fru* locus generating AP between fitness components where one allele (L) enhances survival and the other allele (S) enhances reproduction. Since the *fru* polymorphism influences multiple fitness components, and each allele is beneficial in some instances and deleterious in others, our data support the idea that the *fru* polymorphism is maintained through large antagonistic effects on fitness components, in conjunction with dominance reversal. Our results complement recent findings in other systems [24], indicating that AP is a plausible mechanism for maintaining genetic variation for fitness.

Data accessibility. Data and R scripts used in the analyses are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.9zw3r22dm [47].

Authors' contributions. M.D.J.: data curation, formal analysis, funding acquisition, investigation, methodology, software, validation, visualization, writing-original draft, writing-review and editing; F.R.: conceptualization, formal analysis, funding acquisition, methodology, resources, software, validation, visualization, writing-review and editing; C.D.: data curation, investigation, methodology; K.F.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing-review and editing; M.R.: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, writing-review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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