Experimental Evolution and Heart Function in *Drosophila*

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ABSTRACT

Drosophila melanogaster is a good model species for the study of heart function. However, most previous work on *D. melanogaster* heart function has focused on the effects of large-effect genetic variants. We compare heart function among 18 *D. melanogaster* populations that have been selected for altered development time, aging, or stress resistance. We find that populations with faster development and faster aging have increased heart dysfunction, measured as percentage heart failure after electrical pacing. Experimental evolution of different triglyceride levels, by contrast, has little effect on heart function. Evolved differences in heart function correlate with allele frequency changes at many loci of small effect. Genomic analysis of these populations produces a list of candidate loci that might affect cardiac function at the intersection of development, aging, and metabolic control mechanisms.

Keywords: Drosophila, aging, heart function, experimental evolution.

Introduction

Maintaining normal heart performance is crucial to both survival and quality of life in humans (Roger et al. 2012). Recent genome-wide association studies have identified several loci that may contribute to human coronary artery disease (IBC 50K CAD Consortium 2011; Prins et al. 2012) and cardiac arrhythmia (Ellinor et al. 2012). However, these loci explain only a small percentage of cardiovascular disease risk. In large mammalian populations, most cases of heart disease are unlikely to result only from deleterious alleles that are of major effect, even in congenital heart disease (for recent studies in mice, see Winston et al. 2012; Schulkey et al. 2015). Moreover, most cases of heart disease arise later in adult life, when cardiovascular disease as a whole is quite common (Olivetti et al. 1991; Lakatta and Levy 2003; Strait and Lakatta 2012; Dickinson et al. 2014). Such widespread aging-associated heart disease is unlikely to result only from deleterious alleles of major effect. Rather, at least some of it must be the result of alleles of small to intermediate effect (Cookson et al. 2009; Rau et al. 2015). This in turn may explain why the genetic analysis of human heart disease is complicated by differences in genetic backgrounds, especially between ethnicities (Charles et al. 2014). Heart disease risk is further modified by environmental factors, such as diet and toxins (Parnell et al. 2010; Amin et al. 2013). These complexities suggest that basic research on heart disease should benefit from studies of simpler model organisms in a controlled laboratory setting.

Drosophila is a biomedically useful model for the study of the genetic mechanisms of both cardiac development and cardiac function. The *Drosophila melanogaster* heart is a tubular structure that pumps hemolymph in an open circulatory system, is made up of two major cell types (cardiac cells and pericardial cells), and forms at the dorsal midline of the embryo. Despite the morphological differences between the *D. melanogaster* heart and the vertebrate heart, the development of the heart initiates through molecular mechanisms that are evolutionarily conserved (reviewed in Bodmer and Venkatesh 1998). There is genetic conservation of loci affecting heart function between *Drosophila* and vertebrates (Bodmer 1995; Cripps and Olson 2002; Bodmer and Frasch 2010; Ocorr et al. 2014). Moreover, there is extensive information concerning *D. melanogaster* heart anatomy (Miller 1950; Rizki 1978; Curtis et al. 1999; Dulcis and Levine 2005; Lehmacher et al. 2012) and development (Molina and Cripps 2001; Zeitouni et al. 2007; Bodmer and Frasch 2010). Several studies have shown that single mutations can induce fly heart disease phenotypes similar to those of humans (Ocorr et al. 2007*a*; Cammarato et al. 2008; Taghli-Lamallem et al. 2008; Qian and Bodmer 2009; Bloemink et al. 2011; Viswanathan et al. 2014; Martinez-Morentin et al. 2015). On the basis of studies of heart disease in wild-caught *Drosophila*, heart disease in flies can also have a polygenic basis involving multiple chromosomes (Ocorr et al. 2007*b*; Zhang et al. 2013). The fly heart disease resembling restrictive cardiomyopathy in humans, described by Zhang et al. (2013), is an example of a rare heart phenotype with

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a complex genetic basis, which suggests that the genetic mechanisms underlying other heart phenotypes may also be complex.

Laboratory selection on *D. melanogaster* can result in extreme functional differentiation from naturally segregating variation (e.g., Rose et al. 2004). This differentiation provides tremendous opportunities to investigate phenotypes from a physiological as well as a genetic perspective (Burke and Rose 2009). The genomic material of selected populations can provide insight into the genetic basis of phenotypes that respond to laboratory selection, especially as genomic technologies have become accessible. Pool-seq (whole-genome sequencing of pools of individuals) is a particularly effective method for associating phenotypic divergence resulting from laboratory selection with allele frequency changes genome-wide (Schlötterer et al. 2014). This method involves extracting nucleic acids from many individuals collected from a common selection treatment. Next-generation sequence data from these pools provide frequency estimates of polymorphisms across the genome for entire populations, which is particularly useful when these populations have evolved under different selection regimes. Pool-seq has been used to identify candidate alleles involved in several *D. melanogaster* phenotypes, including development time (Burke et al. 2010), domestication to the laboratory (OrozcoterWengel et al. 2012), body size (Turner et al. 2011), courtship song (Turner and Miller 2012; Turner et al. 2013), and resistance to viral infection (Martins et al. 2014).

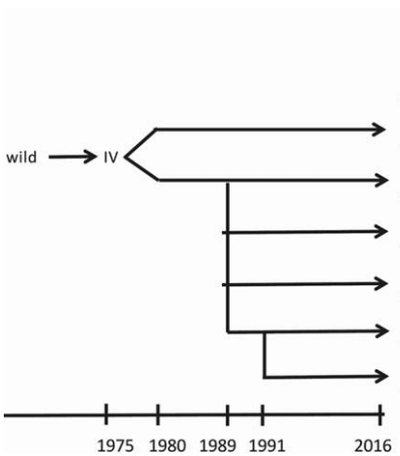
We characterized the differentiation of heart function among populations of *D. melanogaster* that were laboratory selected for differences in development time, aging rates, or stress resistance. Our prediction was that fly populations that have shorter life spans will have worse heart function than longer-lived populations. Our results supported this prediction in that selection for faster development led to the evolution of less robust hearts and selection for delayed aging led to the evolution of more robust hearts. On the other hand, selection on stress resistance had no significant effect on heart robustness. We probed a pool-seq data set collected from some of these populations to evaluate extant candidate genes for heart function and found that many had indeed evolved to different allele frequencies in populations with altered hearts.

Methods

Populations Used

The *Drosophila melanogaster* populations used in this study (fig. 1) share as a common ancestor the IV population (Ives 1975) and have all been maintained as large populations with a minimum of 1,000 individuals per generation. The IV population is maintained in 8-dr glass vials at densities of 60–80 flies per vial on discrete 14-d generation cycles at ~25°C with constant illumination. Five replicate populations called B_{1-5} were separated from the IV stock but maintained under the IV conditions (Rose 1984). Simultaneously, another five replicate populations called O_{1-5} were separated from the IV stock and selected for postponed reproduction (Rose 1984). The O populations are maintained on discrete 70-d generation cycles and have evolved increased longevity, slower aging rates, and increased resistance to various environmental stressors compared with their control B populations (Rose et al. 2004).

The O_{1-5} populations are ancestral to five replicate populations called CO_{1-5}, where subscripts indicate common ancestry in this case (Rose et al. 1992). The CO populations are maintained on discrete 28-d generation cycles and otherwise are kept under identical conditions as the O populations. CO populations are not as long lived as the O populations but are longer lived than the B populations, reflecting their intermediate generation time.



selection treatment name	selection regime	generation time (days)	triglycerides (TG/mg fly) female	triglycerides (TG/mg fly) male	replicate populations tested for heart function	replicate populations tested for heart activity
B	control for O	14	NA	NA	4	NA
O	postponed reproduction	70	NA	NA	4	NA
C	control for D	21	168 ± 11	60 ± 5	3	2
D	desiccation resistance	21	108 ± 2	35 ± 1	3	2
CO	control for ACO	28	114 ± 7	35 ± 4	3	3
ACO	accelerated development	9-10	69 ± 5	17 ± 2	3	3

Figure 1. *Drosophila melanogaster* populations used in this study. All populations were derived from an ancestral IV population, which was collected from the wild. The populations differ in generation time and triglyceride levels as well as other phenotypes not summarized here. Each selection treatment is fivefold replicated. Triglyceride levels shown are the mean and the SE of the mean for the replicates. The last two columns show the number of replicate populations from each selection treatment that were used in the heart function assays. NA = not available.

The CO_{1-5} populations are ancestral to five replicate populations called ACO_{1-5}, where subscripts again indicate common ancestry (Chippindale et al. 1997). The ACO populations are maintained on discrete 9–10-d generation cycles; these populations develop faster, have higher aging rates, and have decreased longevity than the B, CO, and O populations. In addition, the ACO flies have stunted growth and a reduction in viability and stress resistance (Chippindale et al. 1997, 2004).

The O populations are also ancestral to another five replicate populations that were selected for desiccation resistance, called the D_{1-5} populations, and to five corresponding control populations for these desiccation-resistant populations, called the C_{1-5} populations (Rose et al. 1992). The D flies are more resistant to desiccation stress and store more water and glycogen than the C flies (Rose et al. 2004). Both the D and the C flies are more resistant to starvation stress than their ancestral O populations (Rose et al. 2004).

Each of the above-described selection treatments is replicated fivefold, and thus phenotypic and genotypic differences that are consistent within selection treatment but differ from those of other selection treatments are likely to have resulted from adaptation instead of genetic drift or inbreeding. Before every experimental assay, the populations used were put through two identical 14-d generation cycles to eliminate maternal effects and the effects of environmental acclimation. The experiments were staggered in time, but pairs of treatment and control populations with the same subscript were always tested together.

Triglyceride Measurement

Triglyceride measurements were performed as described in Birse et al. (2010). In brief, adult flies were placed into empty vials for 30 min and then placed into Eppendorf tubes in batches of 12 and weighed for immediate quantification or frozen at −80°C for later processing. Flies were then homogenized in 100 μL of a 1:1 mixture of phosphate-buffered saline for 1 min at 10,000 rpm using a high-throughput ball-bearing homogenizer (Talboys). The homogenates were then spun at 4,000 rpm on a centrifuge (Eppendorf 5810R) for 5 min, and 5 μL of supernatant was transferred into 170 μL of triglyceride reagent (Thermo Electron catalogue no. TR22421/2780-250). The reaction mixture was incubated in an Environ shaker at 300 rpm at 37°C for 10 min; the optical density at 550 nm was measured using a SpectraMax M2e microplate reader (Molecular Devices) and compared with a standardized curve. Triglycerides were measured for the ACO_{1-3}, CO_{1-3}, $D_{1\text{ and }3}$, and $C_{1\text{ and }3}$ populations.

Heart Function

Pacing Assay. Flies from the B_{1-4}, O_{1-4}, C_{1-3}, D_{1-3}, CO_{1-3}, and ACO_{1-3} populations were tested for stress-induced heart dysfunction using an electrical pacing assay of intact flies (Wessells and Bodmer 2004; Wessells et al. 2004). Specifically, at each age 80 flies per sex were sampled from population cages (which were used to age flies at densities of 400 males and 400 females). Flies were anesthetized with triethylamine as a 50% solution (FlyNap, Carolina Biological Supply). Each fly was placed on its dorsal side on a microscope slide with the tip of its head and the lower abdomen touching conductive gel and its wings moved up next to the head. The gel on either end of the fly was in contact with strips of aluminum foil, which were connected to two electrodes. Stimulation then occurred at 40 V and 6 Hz for 30 s. The fly heart was viewed under an inverted microscope before, during, and after pacing. Heart failure was operationally defined as a fly heart remaining in fibrillation or cardiac arrest 2 min after the end of pacing.

Semiautomated Optical Heartbeat Analysis (SOHA) Heart Function Assay. Flies from $C_{1,2}$, $D_{1,2}$, CO_{1-3}, and ACO_{1-3} populations were monitored for heart function in an in situ denervated heart preparation, described elsewhere (Ocorr et al. 2007*a*; Fink et al. 2009). Sample flies (15–20 flies per sex per age per population) were taken from the population cages (which were used to age flies at densities of 400 females and 400 males per cage). In brief, the abdominal heart tube was surgically exposed in anesthetized flies under oxygenated artificial hemolymph (Ocorr et al. 2007*a*) for high-resolution video microscopy (Vogler and Ocorr 2009). High-speed 30-s movies of the beating hearts in the A3 section (one of the abdominal sections of the fruit fly body) were taken at a rate of 100–150 frames per second using a Hamamatsu EM-CCD digital camera on a Leica DM LFSA microscope with a 10 × immersion lens. All measurements were made after 15–20 min of equilibration at room temperature. The videos were acquired using SimplePCI imaging software and analyzed using custom MatLab code (Ocorr et al. 2007*b*; Fink et al. 2009).

Heart Function Parameters. Systolic interval is defined here as the duration of the contraction (until the end of relaxation). Diastolic interval is defined as the pause in the movement of the heart wall after relaxation from contraction and before the next contraction. Heart period is the time from the beginning of a contraction until the beginning of the next contraction or the time from the end of one diastolic interval until the end of the next. Heart period may become increasingly more variable with age or disease, resulting from prolonged diastolic intervals (bradycardia) or systolic intervals (indicative of sustained contraction), which often results in an increase in the SD of the heart period. We used the SD of the heart period as a measure of arrhythmia and normalized it to the median heart period to obtain what has been called the arrhythmia index (for details, see Ocorr et al. 2007*a*; Fink et al. 2009). Diastolic and systolic diameters were obtained by marking the edges of the heart tube during diastole and systole in still images from the 30-s movies. These values were then used to estimate the contractility of the heart tube as percent fractional shortening, where $\%FS = (\text{diastolic diameter} - \text{systolic diameter})/\text{diastolic diameter} \times 100$. Due to overall body size differences between the ACO and CO populations, a standardized set of diastolic and systolic diameter measurements were calculated by correcting for heart length. This was done by measuring the length of the A3 section of the heart (the same heart section that was filmed in the SOHA assay)

of 10 ACO and 10 CO flies from each sex for every age in every replicate population.

Three-Day Survival and Reproduction after Electrical Pacing

In our pacing assay described above and the pacing assays of previous studies, flies were discarded 2 min after the end of electrical pacing. Therefore, there was no information available about whether flies that are electrically paced can survive and reproduce after pacing. To test for longer-term survival and reproduction after pacing, we aged ~10,000 flies in cages at densities of ~400 males and 400 females per cage for each of the B_{1-3} and O_{1-3} populations. Once a week for 4 wk, we sampled 800 female flies per population from the cages by taking approximately equal numbers of females from each cage. Half of the flies (400 females per population) were anesthetized with FlyNap and electrically paced as described above. The other half were handled in an identical manner, but the stimulator was never turned on. The electrically paced and control group flies were then removed from the conductive gel using soft forceps and placed inside separate cages. Within hours, the flies inside the population cages resumed what looked like normal activity as they groomed the gel off of them and the FlyNap anesthesia wore off. Mortality and fecundity were then compared among the B and O populations for 3 d. Dead flies were removed from the cages daily and counted. Fecundity was measured by counting the eggs laid by females on food plates in their cage every day for 3 d. The plates were then maintained for 4 d after removal from the cage and observed for the presence of larvae; all treatment and control groups laid viable eggs, but we did not quantify the viability.

Statistical Methods

Triglycerides per milligram fly data were analyzed using ANOVA in R (ver. 3.1.2; www.r-project.org). Adult heart function was tested at t weeks of age ($t = 1$, 3, 5, and 7), and cardiac arrest or fibrillation within 2 min of electrical pacing was recorded. The probability of cardiac arrest/fibrillation increased with age in a linear fashion. Tests were done on matched pairs of selection regimes, for example, ACO versus CO and C versus D. We used a linear logistic regression function to model these probabilities, $p_i(t)$, over adult age and for each selection regime i ($i = 1$ [ACO or CO] or 2 [C or D]) as

$$\log\left(\frac{p_i(t)}{1-p_i(t)}\right) = \beta_0 + \delta_i\alpha_0 + (\beta_1 + \delta_i\alpha_1)t, \qquad (1)$$

where $\delta_i = 0$ if $i = 1$ and 1 otherwise. Thus, the β_i parameters are the ACO (or C) regression parameters, and the α_i parameters represent the incremental changes due to the (CO or D) selection. Parameters of equation (1) were estimated by the glm function in R (R Development Core Team 2015), which provides maximum likelihood estimates of the parameters in equation (1) and accounts for the binomial distribution of the experimental data.

Variation in heart phenotypes, heart period, arrhythmia index, fractional shortening, diastolic interval, systolic interval, diastolic diameter, and systolic diameter have three sources of variation: selection regime, population, and individual. Among-population variation arises from genetic differences that may be caused by random genetic drift. Individuals vary due to segregating genetic variation within populations, measurement errors, and microenvironmental sources of variation that cannot be experimentally controlled. Selection regime is a fixed effect and is the primary source of variation we are using these statistical tests to examine. Given the limited number of ages, a linear description of phenotype change with age is most congruent with these data. Accordingly, the appropriate statistical model for the heart phenotypes are linear mixed-effects models (Pinheiro and Bates 2000, chaps. 1–2).

For each of the seven phenotypes—heart period, arrhythmia index, fractional shortening, diastolic interval, systolic interval, diastolic diameter, and systolic diameter—we let y_{ijkt} be the measured phenotype for individual k ($k = 1, 2, \ldots, n_{ijt}$) from selection regime i (e.g., $i = 1$ if ACO [or C] and 2 if CO [or D]), population j ($j = 1, 2, \ldots, 6$), and age t weeks. The linear model of heart phenotypes is then

$$y_{ijkt} = \alpha + \delta_i\beta + \pi t + \delta_i\mu t + b_j + \varepsilon_{ijkt}, \qquad (2)$$

where $\delta = 0$ if $i = 1$ and 1 otherwise and b_j and ε_{ijkt} represent random variation from populations and individuals, respectively, and are assumed to be normally distributed with a mean of 0 and variances σ_j^2 and σ_k^2, respectively. To test for differences between the paired selection regimes (ACO vs. CO and C vs. D), we used equation (2) to predict the phenotypes at all ages and then compute the age-specific differences between selection regimes. By placing simultaneous 95% confidence intervals on these predictions, the type I error of any predicted difference being significantly greater than 0 is kept at ≤5% (Miller 1966, chap. 3).

As an example, the predicted phenotypic value of a 5-wk ACO fly is $\alpha + 5\pi$, and for a CO fly it is $\alpha + \beta + 5(\pi + \mu)$ (eq. [2]). The difference, CO − ACO, is then $\beta + 5\mu$. The variance of the difference is $\mathrm{Var}(\beta) + 25\,\mathrm{Var}(\mu) + 10\,\mathrm{Cov}(\mu, \beta)$. Since we were conducting four or five tests (for each week), we used a Bonferroni correction to control the type I error rate and computed 99% confidence intervals on the difference to test for significance. The lme function in R was used to estimate the fixed and random effects from equation (2).

Ad Hoc Genomic Sequence Investigation

We surveyed genomic differences between the ACO and CO treatments to look for evidence of enrichment for genes previously reported to be important for heart function and obesity. Burke et al. (2010) provided a list of alleles that are highly differentiated between the ACO and CO treatments. For clarity, we refer to the differentiation statistic used by Burke et al. (2010) as a FET score, and any FET score above 3 indicates an allele frequency that is statistically significantly differentiated

between the CO and ACO populations (for a complete discussion of this test statistic, refer to the source paper, wherein the statistic is referred to as $L_{10}FET_{5\%}$). Burke et al. (2010) found that a subsample of alleles with high FET scores share differentiation among replicate populations and suggested that differentiation between the ACO and CO treatments is highly repeatable, both at the phenotypic level and at individual loci. Neely et al. (2010) published a list of putative cardiac genes identified by a genome-wide RNA interference (RNAi)–silencing screen (cf. table S2 in Neely et al. 2010). We cross-referenced these two lists, taking a particular interest in the single-nucleotide polymorphisms (SNPs) identified by Burke et al. (2010) that occur within transcript regions of candidate cardiac genes identified by Neely et al. (2010). First, we provide a list of all of these SNPs and their associated significance scores (table S1; tables S1, S2 are available online). Table S1 presents only SNPs that occur in the Neely et al. list of candidate cardiac genes and are also significant in our ACO/CO pool-seq comparison. Second, we look for enrichment of significant FET scores in candidate cardiac transcript regions compared with randomly sampled transcript regions. This was done by evaluating the FET scores associated with 100-kb regions centered on the midpoint of each putative cardiac transcript. This approach takes into account reported levels of linkage disequilibrium in these populations (up to 100 kb; cf. Teotonio et al. 2009) as well as potentially informative noncoding genomic regions. These scores are compared with those from an equal number of randomly chosen transcripts, sampled from all annotated transcripts in the *Drosophila* genome (dmel-all-transcript-r6.06.fasta; www.flybase.org). To visualize whether FET scores appear to be enriched in cardiac candidates compared with random genes, we compared the distribution of these scores associated with each set (fig. S1*A*; fig. S1 is available online). We took this same approach to identify significantly differentiated alleles between the ACO and CO populations that fall within candidate obesity genes (cf. Pospisilik et al. 2010; table S2; fig. S1*B*).

Results

Comparison of Populations Selected for Accelerated Development (ACO) and Their Control Populations (CO)

We compared cardiac physiology among *Drosophila* populations that experienced different evolutionary histories (fig. 1). First, we tested heart function in three replicate ACO populations and their three paired controls via electrical pacing methods (e.g., Wessells and Bodmer 2004). ACO populations take less time to develop and have smaller body size relative to all other populations studied here. Smaller body size has sometimes been correlated with reduced heart robustness (Zarndt et al. 2015). The $ACO_{1–3}$ populations experienced significantly more heart failure after pacing than the control $CO_{1–3}$ populations (fig. 2*A*). All populations experienced an age-dependent increase in heart failure that persisted throughout the life spans of both males and females.

We next compared the $ACO_{1–3}$ and $CO_{1–3}$ populations for heart rate, heart period, arrhythmia, systolic and diastolic intervals and diameters, and contractility using methods described in Ocorr et al. (2007*a*, 2007*b*) and Fink et al. (2009). The $ACO_{1–3}$ populations had significantly longer heart periods (figs. 2*B*, 4*A*) due to much longer diastolic intervals (figs. 2*D*, 4*D*) than the $CO_{1–3}$ populations. Heart diameters in systole and diastole were similar between the $ACO_{1–3}$ and $CO_{1–3}$ populations (figs. 2*E*, 4*E*, 4*F*). However, the ACO flies are smaller than the CO flies and the ACO hearts are shorter than the CO hearts by a ratio of 0.93 to 1 (where 1 means identical size), so we conducted a second analysis by standardizing systolic and diastolic diameters by heart size. After standardizing for heart length, there were still no differences between the heart diameters of these populations. The diastolic diameters of ACO hearts tended to be narrower, even after normalization of heart length of the smaller ACO flies compared with CO flies, but this difference was not significant (fig. 2*E*). Narrow heart phenotype, akin to diastolic dysfunction, has previously been observed under stress conditions that also cause smaller body size, that is, under hypoxia selection (Zarndt et al. 2015). The $ACO_{1–3}$ and $CO_{1–3}$ populations did not differ in fractional shortening, a measure of contractility (figs. 2*F*, 4*G*). At young ages, ACO flies tend to have a somewhat larger arrhythmia index than CO flies (fig. 2*C*), but the differences are not significant (fig. 4*B*) and lie within the normal range of laboratory wild-type strains (arrhythmia index: 0.1–0.2 at young ages; see Fink et al. 2009). Thus, the main differences between the ACO and CO treatments were that the ACO flies experienced more heart dysfunction in terms of both a higher propensity to fail when stressed by pacing and prolonged heart periods at every age.

In an ad hoc genomic sequence investigation, we found 489 significantly differentiated SNPs in the ACO-CO data set that occur within the 506 candidate cardiac transcripts ("heart genes") from Neely et al. (2010). The 489 SNPs localize to 103 unique genes, and 44 of these SNPs in 12 genes are predicted to result in a nonsynonymous amino acid change (table S1). Notably, however, transcripts associated with heart genes are not more likely to harbor variants with elevated levels of differentiation than a randomly chosen group of transcripts. When evaluating average FET scores of individual genes (determined by averaging FET scores from all SNPs within 50 kb of the midpoint of individual transcripts), the distribution of these scores for candidate cardiac transcripts is not qualitatively different from the distribution of scores for an equal number of randomly chosen transcripts (fig. S1*A*). However, we do observe a small hump in this distribution of cardiac genes to random genes; eight genes are associated with mean FET scores >5. These eight genes are highlighted in table S1, although there are no obvious shared properties among them.

One of the evolved differences between the ACO and CO treatments is that the ACO flies have lower triglyceride levels (fig. 1). Therefore, we carried out the ad hoc analysis described in the previous paragraph on an independently generated set of candidate adult "obesity" genes, which were identified by triplicate screens for whole-fly triglyceride content following RNAi induction in >11,000 different UAS-RNAi transgenic lines (table S2 of Pospisilik et al. 2010). Pospisilik et al. (2010)

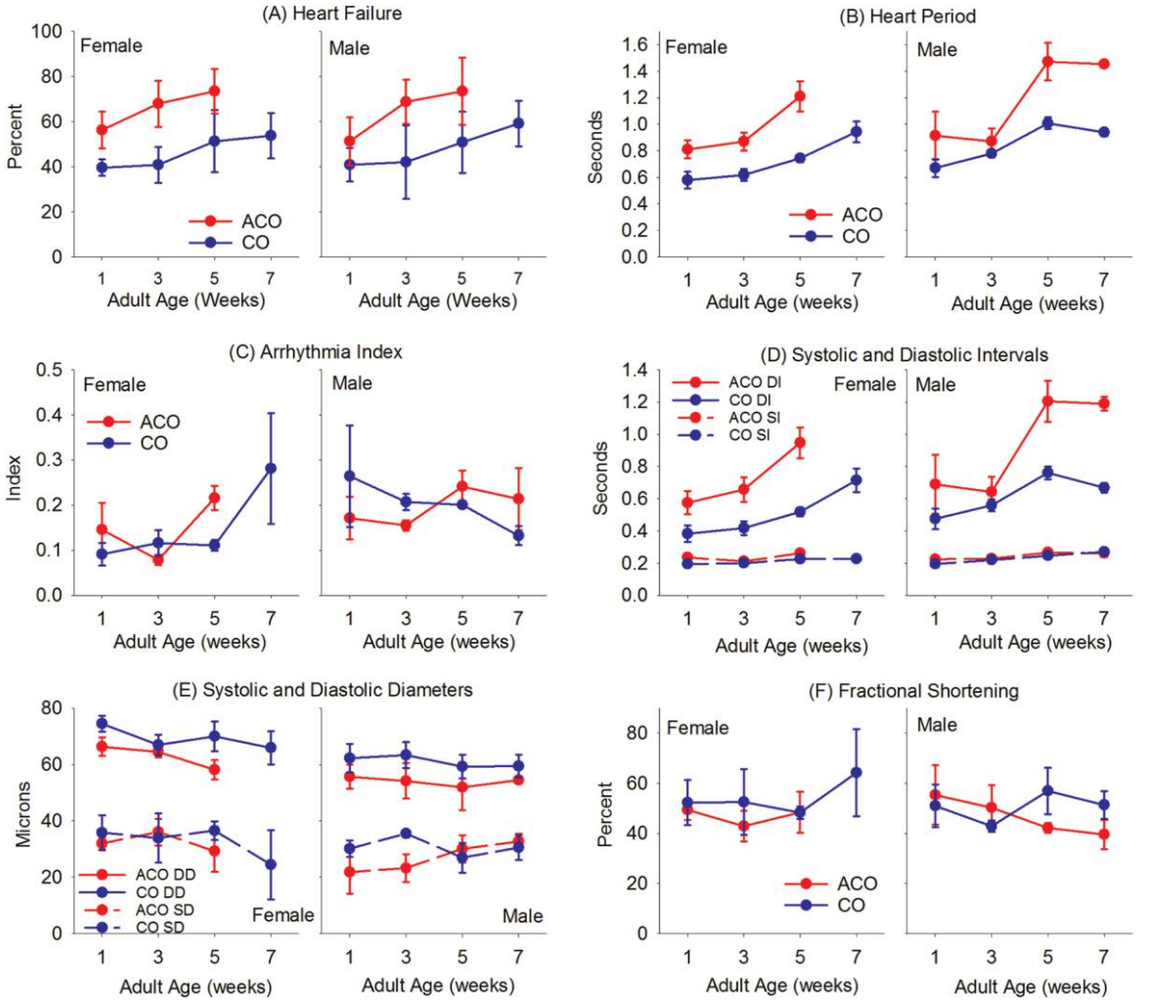


Figure 2. Comparison of heart phenotypes for the accelerated development treatment (ACO, red) and its control (CO, blue). Points represent means of the phenotypic measure across replicate populations, and bars show the SE of the mean across replicate populations. *A*, ACO_{1-3} populations experienced significantly more heart failure after pacing than CO_{1-3} populations (general linear model in R). For each replicate, 80 flies per sex per age were tested; 2*B*–2*E* were obtained from 30-s high-speed movies of the heart tube of 15–20 dissected flies for each sex, age, and replicate population. Heart phenotype data were analyzed using a linear mixed-effects model in R (see fig. 4). *B*, ACO_{1-3} populations had longer heart periods than CO_{1-3} populations. Heart period was longer in males than in females. *C*, The arrhythmia index did not differ between ACO_{1-3} and CO_{1-3} populations. The arrhythmia index is the SD of the heart period normalized to the median heart period. *D*, Diastolic intervals (solid lines) and systolic intervals (dashed lines). ACO_{1-3} populations had longer diastolic intervals than CO_{1-3} populations, but the treatments did not differ in systolic intervals. *E*, Diastolic diameters (solid lines) and systolic diameters (dashed lines). Systolic and diastolic diameters did not differ between ACO_{1-3} and CO_{1-3} populations, but females had larger systolic and diastolic diameters than males. *F*, Percent fractional shortening, or contractility, did not differ between treatments. Percent fractional shortening, which provides an estimate of the contractility of the heart tube, is calculated as %FS = (diastolic diameter − systolic diameter)/diastolic diameter × 100.

identified 508 unique candidate obesity genes; 507 SNPs significantly differentiated in the ACO-CO data set occur within these candidate obesity transcripts and localize to 128 unique genes (table S2). Thirty-three of these SNPs in 22 genes are predicted to result in nonsynonymous amino acid substitution. Significantly differentiated SNPs are, again, not enriched in regions of the genome associated with obesity transcripts compared with a random sample of transcripts (fig. S1*B*).

Comparison of Populations Selected for Increased Desiccation Resistance (D) and Their Control Populations (C)

Despite the D and C populations having the same generation time and similar longevity, they are highly differentiated in triglyceride levels (fig. 1; see also Djawdan et al. 1998). It should be noted that the C populations have been weakly selected for starvation resistance, while the D populations have been strongly

selected for desiccation resistance (Rose et al. 1992, 2004). Thus, the C populations have long had significantly higher levels of triglycerides than the D populations, while the D populations have had much higher water content (Rose et al. 2004). Despite these differences in body composition, we found no differences in stress-induced heart dysfunction among the D and C populations, contrary to the commonplace association between circulating lipid levels and cardiovascular disease in human populations. The D_{1-3} and C_{1-3} populations all showed an increase in heart dysfunction with age in the electrical pacing assays (fig. 3*A*). The slope of this increase was higher in the D populations than in the C populations ($P = 0.0019$), but the slope was not affected by sex ($P = 0.72$), nor was there a significant sex by selection treatment interaction ($P = 0.28$). The D_{1-3} and C_{1-3} populations did not show any statistically significant differences for any of the seven other heart phenotypes (figs. 3*B*–3*F*,

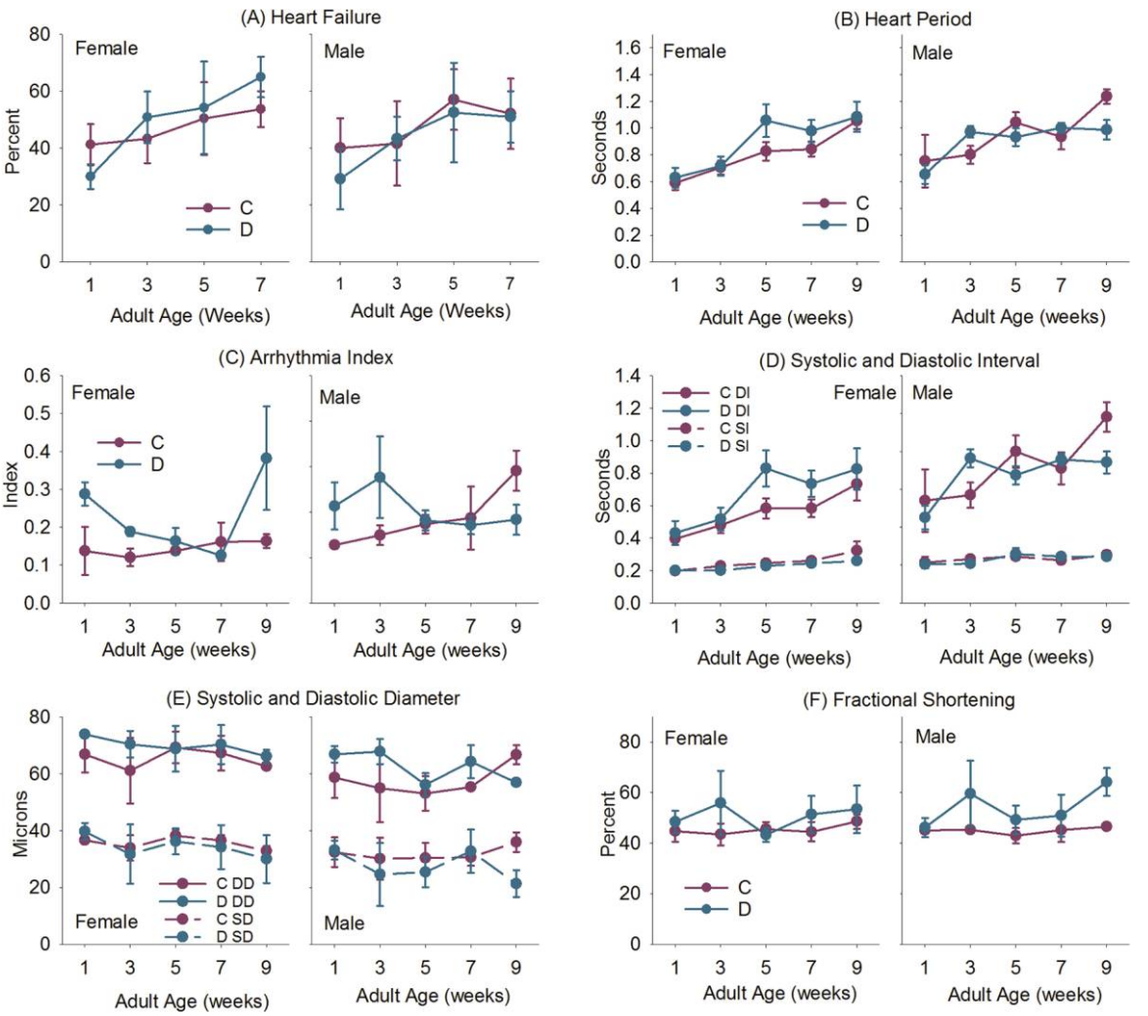


Figure 3. Comparison of heart phenotypes in the desiccation-resistant treatment (D, cyan) and its control (C, purple). Points represent means of the phenotypic measure across all replicate populations, and bars show the SE of the mean across replicate populations. *A*, D_{1-3} and C_{1-3} populations show a significant difference in the slope of heart failure with age ($P = 0.0019$). The heart failure rate rises more steeply with age in the D populations than in the C populations. For each replicate, 80 flies per sex per age were tested; 3*B*–3*E* were obtained from 30-s high-speed movies of the heart tube of 15–20 dissected flies for each sex, age, and replicate population. Heart phenotype data were analyzed using a linear mixed-effects model in R (see fig. 4). *B*, $D_{1,2}$ populations and $C_{1,2}$ populations did not differ in heart period. *C*, The arrhythmia index did not differ between $D_{1,2}$ and $CO_{1,2}$ populations. The arrhythmia index is the SD of the heart period normalized to the median heart period. *D*, Diastolic intervals (solid lines) and systolic intervals (dashed lines). $D_{1,2}$ and $C_{1,2}$ populations did not differ in diastolic or systolic intervals. *E*, Diastolic diameters (solid lines) and systolic diameters (dashed lines). Diastolic diameters did not differ between $D_{1,2}$ and $C_{1,2}$ populations, but there was an age by treatment effect for systolic diameters. Females had larger diastolic and systolic diameters than males. *F*, Percent fractional shortening increased with age in the $D_{1,2}$ populations but not in the $C_{1,2}$ populations. Percent fractional shortening, which provides an estimate of the contractility of the heart tube, is calculated as %FS = (diastolic diameter − systolic diameter)/diastolic diameter × 100.

4*A*–4*G*). Thus, despite the difference in desiccation resistance and triglyceride content between the C and D populations, heart function seems to be largely unaltered.

Comparison of Populations Selected for Postponed Reproduction (O) and Their Control Populations (B)

We found that females and males of the long-lived O_{1-4} populations had better heart function than the B_{1-4} populations in that they were less prone to heart failure under electrical pacing stress (fig. 5*A*). Thus, these findings parallel the results of our comparison between the ACO and CO populations (fig. 2*A*); in both cases, shorter-lived populations had more heart dysfunction after electrical pacing at every chronological age, and all exhibited a similar age-dependent increase. Unlike the case of the ACO-CO contrast, however, the B-O contrast is not confounded by a large body weight difference.

Examining Three-Day Survival and Reproduction after Electrical Pacing

The electrical pacing method applied in the experiments above has been widely used in the field (e.g., Wessells et al. 2004), but the long-term effects on fitness of the pacing assay remained unknown because flies had been discarded following electrical pacing. To test the longer-term effects of this assay, we maintained flies after electrical pacing and recorded their subsequent survival and fecundity. Specifically, we followed 3-d survival and

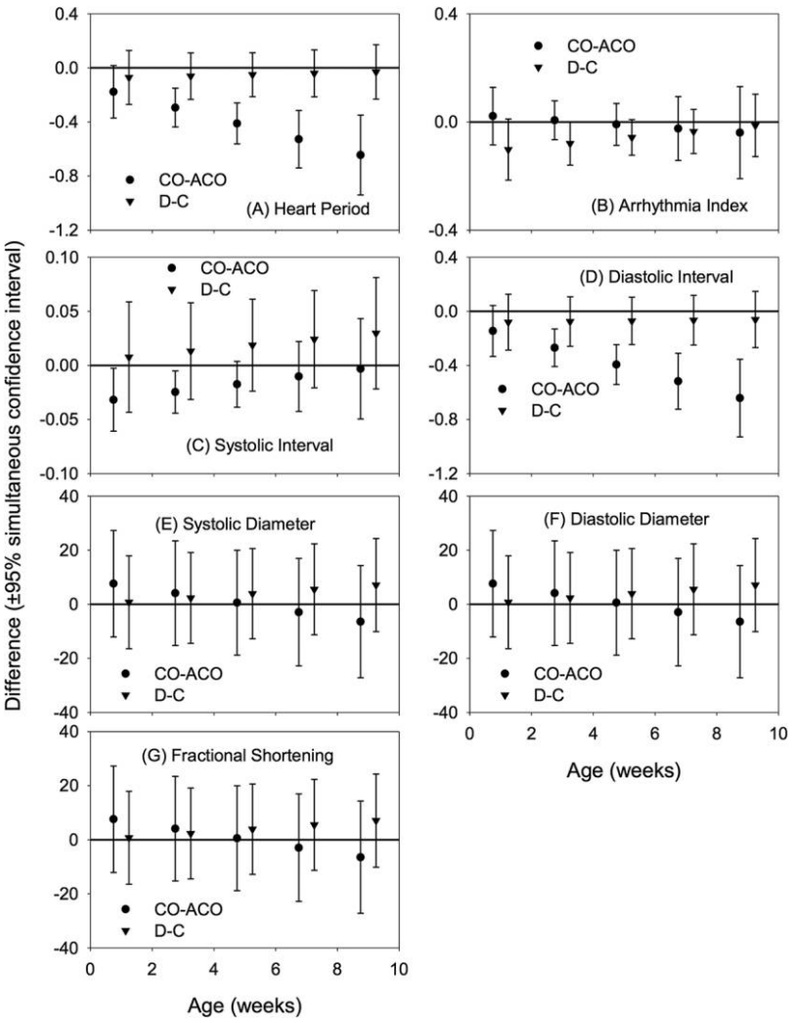


Figure 4. Statistical analysis of heart phenotypes. Shown are age-specific phenotypic differences between the CO and ACO populations (circles) and the D and C populations (triangles). The error bars are simultaneous 95% confidence intervals. Any interval that does not include 0 indicates a statistically significant difference for that phenotype and pair of populations. The measured heart phenotypes are indicated in each graph. See "Statistical Methods" for details.

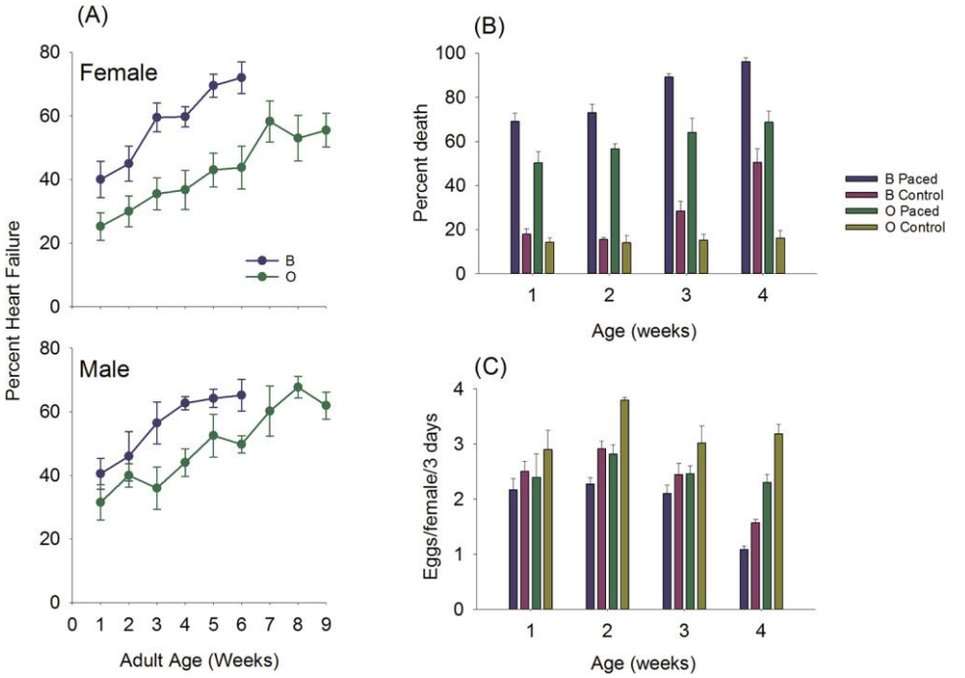


Figure 5. Heart function comparison of populations selected for delayed reproduction (O, green) and their controls (B, blue). *A*, B_{1-4} populations experienced significantly more heart failure initially after pacing than O_{1-4} populations ($P < 10^{-6}$). In addition, the heart failure rate rose more slowly in the O populations than in the B populations ($P = 0.003$). Points show the average rate of heart failure of four replicate populations, and bars show the SE of the mean across the replicate populations. For each replicate population, at every age 80 flies per sex were tested. For *B* and *C*, each bar shows the average phenotype of three replicate populations for the B and O treatments, and the error bars show the SE of the mean across the replicates. *B*, Percentage of female flies that died, of 400 female flies in total, in the 3 d after electrical pacing (B electrically paced = blue; O electrically paced = green) or equal handling without electrical pacing (B control = purple; O control = dark yellow). In week 1, B_{1-3} females had higher mortality resulting from electrical pacing than O_{1-3} females, while control mortality was the same in B_{1-3} and O_{1-3} females. B_{1-3} mortality increased with age faster than O_{1-3} mortality for electrically paced and control females. *C*, Number of eggs laid on day 3 after electrical pacing (B electrically paced = blue; O electrically paced = green) or equal handling (B control = purple; O control = dark yellow). Control females laid more eggs than electrically paced females in all populations. Egg-laying decreased with age more quickly in B_{1-3} populations compared with the age-specific decrease in O_{1-3} populations.

fecundity of 400 electrically paced and 400 control females of each of the O_{1-3} and B_{1-3} populations. Immediately after handling, we placed all electrically paced and control flies into population cages (regardless of the observed effect on the heart in the short term after pacing). Most flies regained what looked like normal locomotive activity within an hour of replacing them in the prehandling environment. We did not observe individual flies under the microscope during pacing to determine which flies experienced heart failure. But presumably many of the flies that died within the first 24 h after handling may have been those that experienced heart failure initially. For three consecutive days we removed and counted dead flies and recorded fecundity. Flies from the O_{1-3} population survived the electrical pacing better than B_{1-3} flies, showing lower total mortality over 3 d (fig. 5*B*). The O_{1-3} females laid more eggs than the B_{1-3} females on day 3 after pacing (fig. 5*C*), measured by counting eggs laid on a food plate that was placed inside the population cage on day 2 and removed 24 h later. The number of eggs laid per female was near zero for all groups on the first day after handling, but counts increased over the first 3 d after handling. In all cases, the flies produced viable eggs, but we did not quantify the viability.

Discussion

In this study, we used outbred *Drosophila melanogaster* populations that were divergently selected for various traits under laboratory conditions (fig. 1; Rose et al. 2004). We first found that populations selected for accelerated development had evolved differences in heart function. Specifically, they showed increased heart failure under pacing and longer heart periods compared with their ancestral control populations throughout adulthood. Therefore, these two phenotypes, heart failure and heart period, were our focus in our tests for associations between heart function and regions of identified genomic segregation of alleles. We found that while such associations exist, they are not more common than associations between randomly chosen transcripts and heart function. This suggests that conventional candidate heart genes identified in previous research are not necessarily causal factors in the differentiation of heart

function in populations selected for rapid development. The faster-developing populations have smaller body size and proportionally smaller hearts compared with their control populations.

The increased heart failure of populations that had evolved accelerated development raised the question of whether such heart function differences might also arise in other pairs of populations that differ in longevity but not in developmental period. We found that populations that have evolved to be long-lived had improved heart function under pacing stress compared with their control populations at all ages even though these populations take comparable amounts of time to develop from eggs into mature reproductive adults. Thus, it appears that selection for prolonged life span leads to enhanced heart function with age, as previously found for single life-prolonging mutations in insulin-TOR or integrin signaling (Wessells et al. 2004, 2009; Nishimura et al. 2014), while selection for accelerated development, which shortens life span, leads to increased heart dysfunction even at young ages. These results underscore previous inferences concerning the relationship between aging and heart function drawn from longitudinal age-dependent correlations and studies of large-effect alleles or mutants (Wessells et al. 2004; Ocorr et al. 2007*b*, 2007*c*; Cammarato et al. 2008; Nishimura et al. 2014).

It is worth noting that the diastolic diameters in the faster-developing populations appeared constricted beyond the proportionality, albeit not statistically significantly. Previous work has found that hypoxia, which leads to smaller size in flies, produces constricted hearts, which is akin to diastolic dysfunction (Zarndt et al. 2015). Levels of triglycerides also differ between the faster-developing populations and their controls, with the faster-developing populations having lower triglyceride levels. Despite lower triglyceride levels, the faster-developing flies exhibited impaired response to heart pacing. Moreover, we find that flies that have evolved moderately higher lipid levels do not show increased heart dysfunction compared with their controls. These findings challenge earlier results concerning the impact of lipids on *Drosophila* heart dysfunction (cf. Birse et al. 2010; Birse and Bodmer 2011; Lim et al. 2011; Diop and Bodmer 2012; Diop et al. 2015; Hardy et al. 2015), where fly obesity models involve either strong selection for starvation resistance to increase lipid content or exposure to high-calorie diets. Flies with much higher lipid content generally have decreased heart robustness, reduced cardiac contractility, blocked heart electrical conduction, and severe structural pathologies (Birse et al. 2010; Hardy et al. 2015). Birse et al. (2010) and Birse and Bodmer (2011) have argued that the buildup of fat in the heart itself is the key mechanism of heart dysfunction in obesity-model flies. But Hardy et al. (2015) do not find such cardiac steatosis, concluding instead that increased storage of triglycerides in the *Drosophila* fat body has a pathophysiological impact on heart function. Previous research has implicated a number of genetic pathways and molecular mechanisms in cardiac dysfunction that could involve metabolic reserves like fat, such as the SREBP pathway, the insulin-TOR pathway, the hexosamine biosynthetic pathway, and PGC-1/*stargel* expression (Birse et al. 2010; Lim et al. 2011; Na et al. 2013; Diop et al. 2015). For example, Birse et al. (2010) showed that reducing the activity of the insulin-TOR pathway prevents lipid accumulation in *Drosophila* fed a high-fat diet as well as protects against heart dysfunction. Evidently, much remains to be resolved with respect to the role played by lipids in the etiology of heart disease, but apparently the level of overall lipid accumulation in the heart or elsewhere is not the only critical variable.

Our genome-wide analysis provides many polymorphisms that could potentially be linked to cardiac phenotypes. But these alleles are not overrepresented in published lists of candidate genes underlying heart function and vice versa. Neely et al. (2010) argue that their candidate heart genes have functional consequences on the basis of cardiac RNAi knockdown experiments with transformant lines, but we find no significant overlap of the genes with allele frequency changes in our experimentally evolved *Drosophila* populations. Our findings are more like those of Reed et al. (2014), who find a disparity between results obtained from functional studies of inbred lines and results obtained from evolve-and-resequence experiments involving diet. Like Reed et al. (2014), we caution against using RNAi lines or inbred lines for inferring the genetics of complex traits in natural populations. Our failure to identify a signature of allele frequency change at SNPs in cardiac genes could well be a consequence of the particular selection regimes implemented here. The ACO populations were selected for rapid development, and while this selection happens to produce an interesting cardiac phenotype, genetic variants with large effects for either rapid development or heart failure may not be the same. Put another way, future work involving direct selection for improved cardiac function may recruit more of the cardiac genes currently reported in the literature. That being said, our observation that cardiac physiology evolves as a side effect of selection for rapid development implies a phenotypic correlation between these two complex traits, and thus we find the absence of a signature of a genetic correlation here interesting. Moreover, in our study shorter-lived populations (B) have higher rates of heart failure than longer-lived populations (O) even when the short-lived populations have not experienced selection for rapid development, suggesting that the heart failure differences do not result exclusively from phenotypic correlations between heart function and development. Both selection treatment pairs (ACO-CO and B-O) have resulted in differences in aging rates, with shorter-lived populations aging faster (Rose et al. 2002). Thus, it is possible that the higher rate of heart failure in the shorter-lived populations in both of these comparisons is related to aging patterns.

It is notable that experimental evolution and resequencing studies do not always implicate canonical genes that can affect a trait in the laboratory. For example, the response to longevity selection described in Burke et al. (2010) did not include all genes known to extend life span when mutated, such as *methuselah* and *Indy*. Body size selection in Turner et al. (2011) did not capture some of the genes that affect body size when mutated in the laboratory, such as *chico* and *insulin receptor*. We advocate combining experimental evolution with genome-wide sequencing as a key approach to identifying the

specific loci that underpin the evolution of complex physiologies that do not necessarily focus on, or even involve, large-effect regulatory genes of developmental and homeostatic processes. The present study marks a beginning for this approach to determine the evolutionary features of the genomics of heart physiology.

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