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Obesity-associated cardiac dysfunction in starvation-selected *Drosophila melanogaster*

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Hardy CM, Birse RT, Wolf MJ, Yu L, Bodmer R, Gibbs AG. Obesity-associated cardiac dysfunction in starvation-selected *Drosophila melanogaster*. *Am J Physiol Regul Integr Comp Physiol* 309: R658–R667, 2015. First published July 1, 2015; doi:10.1152/ajpregu.00160.2015.—There is a clear link between obesity and cardiovascular disease, but the complexity of this interaction in mammals makes it difficult to study. Among the animal models used to investigate obesity-associated diseases, *Drosophila melanogaster* has emerged as an important platform of discovery. In the laboratory, *Drosophila* can be made obese through lipogenic diets, genetic manipulations, and adaptation to evolutionary stress. While dietary and genetic changes that cause obesity in flies have been demonstrated to induce heart dysfunction, there have been no reports investigating how obesity affects the heart in laboratory-evolved populations. Here, we studied replicated populations of *Drosophila* that had been selected for starvation resistance for over 65 generations. These populations evolved characteristics that closely resemble hallmarks of metabolic syndrome in mammals. We demonstrate that starvation-selected *Drosophila* have dilated hearts with impaired contractility. This phenotype appears to be correlated with large fat deposits along the dorsal cuticle, which alter the anatomical position of the heart. We demonstrate a strong relationship between fat storage and heart dysfunction, as dilation and reduced contractility can be rescued through prolonged fasting. Unlike other *Drosophila* obesity models, the starvation-selected lines do not exhibit excessive intracellular lipid deposition within the myocardium and rather store excess triglycerides in large lipid droplets within the fat body. Our findings provide a new model to investigate obesity-associated heart dysfunction.

obesity; heart disease; *Drosophila melanogaster*; starvation selection; laboratory natural selection

OBESITY IS A LEADING RISK factor for many diseases, including type-II diabetes, cardiovascular disease, and certain types of cancer (9, 38). With worldwide obesity rates at an all-time high, attention has focused on understanding the etiology of obesity-associated diseases. Recently, *Drosophila melanogaster* has emerged as an excellent model to investigate the genetic causes and physiological consequences of obesity. In the laboratory, *Drosophila* can be made obese through dietary changes, genetic manipulation, and adaptation to evolutionary stress (10, 11, 16–18, 22, 25, 26, 29). These models recapitulate many aspects of obesity, and some have provided the tools to investigate how increased lipids affect the heart. For instance, *Drosophila* fed lipogenic diets high in fat or sugar, store higher levels of triglycerides (TG) and exhibit a range of associated cardiomyopathies (11, 29). Genetic mutations that alter lipid homeostasis can also induce heart dysfunction. For example, hyperactivation of TOR signaling through reduction of sestrin function or mutation of downstream components of TOR, such as PGC-1 and ATGL lipase, lead to increased total TG and heart dysfunction, including arrhythmia, reduced cardiac contractility, and defects in structural integrity (17, 25). Additionally, genetic dysregulation of phospholipid metabolism results in increased expression of *Drosophila* sterol regulatory element-binding protein (*dSREBP*), leading to higher levels of stored fat and alterations to the structure and function of the heart (26). Another study found that the loss of one copy of the gene coding for the *Drosophila* fatty acid transport protein (*dFatp*) results in obese flies, which exhibit low cardiac stress resistance (46).

Common among many of these models is the increase in intracellular accumulation of lipids within the myocardium, hypothesized to cause structural and functional damage to the heart (11, 17, 26, 29, 46). This lipotoxic effect is well documented in mammals and occurs when TG levels surpass the storage capacity for lipids within adipose tissue (9). In *Drosophila*, the functional equivalent of adipose tissue is the fat body, a heterogeneous tissue responsible for nutrient storage and utilization amidst many other functions (6). Within the fat body, highly conserved intracellular lipid droplets act as the primary site of TG synthesis, storage, and breakdown (24). In lipotoxic conditions, lipid droplets within the adipose tissue become oversaturated, which leads to ectopic fat deposition in nonadipose tissues. In mammals, excess accumulation of lipids in nonadipose tissues is strongly correlated with organ degeneration, leading to disrupted structure and function (48). In *Drosophila* this may result in reduced lifespan (29), altered carbohydrate homeostasis (11, 29), and heart dysfunction (11, 17, 26, 29, 46).

While obesity has many pathophysiological effects, the ability to store excess lipids may have an ecological advantage in the context of starvation resistance. In nature, lipids stored within the fat body may be used as a vital resource to survive periods of low nutrient availability (2, 37). Indeed, natural variation for starvation-resistance in *Drosophila simulans* positively correlates with stored fats (8). In the laboratory, selection for starvation-resistance in *Drosophila melanogaster* leads to correlated increases in stored lipids (16, 22). However, adaptation to starvation-resistance incurs evolutionary costs, including reductions in metabolic rate, locomotion, and longevity (42). While these phenotypes are similar to the in-

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creased lipids, impaired activity levels (via geotaxic response) and decreased longevity reported in other obesity models (11, 29), to our knowledge there have been no investigations into the physiological consequences of starvation selection on the heart.

We selected replicated populations of *Drosophila melanogaster* for starvation resistance for over 65 generations. In response to selection, the Starvation-selected (hereafter "S") populations evolved an obese condition, storing nearly twice the level of total lipids compared with their ostensibly unselected Fed controls (hereafter "F" populations) (41). The stored lipids are metabolized upon exposure to starvation, and utilized by the S populations to survive nearly 14 days without food. The excess lipids in the S lines accumulate during an extended larval feeding period (41). However, S flies remain obese in adulthood despite consuming fewer calories than their unselected controls when fed ad libitum (27). There are many evolutionary tradeoffs associated with increased adiposity in the S lines including decreases in developmental rate, fecundity, metabolic rate, activity levels, and flight performance and disrupted sleeping patterns (12, 27, 41). Because many of these phenotypes are suggestive of metabolic disorder, we questioned whether starvation-selected *Drosophila* also exhibited heart dysfunction.

Here, we demonstrate using multiple approaches that starvation-selected *Drosophila melanogaster* have dilated, less-contractile hearts than their unselected controls. Our data and observations suggest that this dysfunction is in part related to expanded fat body tissue which physically interferes with the anatomical position of the heart. We also demonstrate that heart dysfunction in the S populations is associated with increased fat storage, as cardiac enlargement and contractile impairment can be rescued through prolonged fasting. Unlike most obesity models in *Drosophila*, our data suggest S flies do not ectopically store lipids within the myocardium. Instead S flies have adapted by increasing the storage capacity of lipids within the fat body by increasing lipid droplet volume. These findings shed light into the evolutionary origins of obesity and provide a unique model to study the genetics and physiology of obesity-associated cardiac disease.

METHODS

Laboratory natural selection for starvation resistance. The starvation-selected ("S") populations were derived from wild caught *D. melanogaster* collected in Terhune, New Jersey, in 1998 as previously described (27). In 2007, subpopulations were derived from these lines and subjected to selection for starvation resistance. Each generation, 4-day-old posteclosion adults were given noncaloric agar and maintained until 80–90% had died. The survivors were fed and used as founders for the next generation. Fed control ("F") populations were given unrestricted access to food but otherwise handled in parallel with the starvation-selected lines. Each cohort (starvation-selected and fed control) is composed of 3 independent replicates. Experiments conducted here were from generations 65–80. For all experiments the S populations were removed from selection for one generation, maintained and treated in parallel to the controls to remove potential parental effects. All experiments were performed on females.

Semiautomated optical heartbeat analysis (SOHA). To analyze cardiac contractions we used previously described video microscopy techniques (19, 33–35). For this method it is necessary to surgically expose the fly's heart to make it accessible for high-resolution filming (49). In brief, dissections to expose the fly's heart within the abdomen were kept in oxygenated saline and high-speed digital movies were taken and analyzed using custom software (sohasoftware.com) for end-diastolic (EDD), end-systolic (ESD) diameter, heart period intervals and arrhythmia (19, 33–35). Fractional shortening (FS) was calculated as (EDD − ESD)/EDD × 100.

Optical coherence tomography (OCT). Cardiac function was also measured using a custom-built OCT microscopy system (Bioptigen, Durham, NC) as previously described (52, 54). Flies were briefly anesthetized using CO_2, immobilized, and allowed to awaken prior to performing OCT. M-mode images, resulting from a method that measures heart wall movement during the cardiac cycle, were acquired, processed, and analyzed to calculate EDD and ESD, determined from 3 consecutive heart beats. Heart rates were calculated from m-mode images and expressed as the period in seconds per heart beat.

Rescue experiments. We performed two sets of experiments to reduce TG content in S flies in an attempt to rescue heart function. For adult rescue experiments, both F and S flies were aged 4–5 days, then placed on media containing either noncaloric 1% agar or standard cornmeal. S lines were evenly split between these two conditions while F flies were transferred only to fresh standard cornmeal medium, with care taken to maintain similar densities between S and F cohorts. Flies on all treatments were aged for 7 days, then subjected to TG measurements, SOHA, or OCT.

The larval rescue protocol has been previously described (27). In short, F and S larvae were reared in parallel. When F larvae began to wander, 3rd instar S larvae were picked from their media and placed in either fresh standard cornmeal medium or 1% agar. Eclosing flies were placed on standard cornmeal medium and aged 4–5 days, then subjected to TG measurements and SOHA.

Triglyceride and protein measurements. Individual flies were homogenized using a motorized pestle in 120 μl of Western lysis buffer (previously described; 3) on ice. Homogenates were incubated at 70°C for 5 min to heat-kill lipases. Samples were centrifuged and the supernatant was used to measure either total TG or total protein levels. For TG, a 1:10 dilution of supernatant to Infinity Triglycerides Reagent (Thermo Scientific, Middletown, CA.) was mixed in a 96-well plate. The plate was covered with parafilm to prevent evaporation and incubated for 30 min at 37°C, then was measured using a μQuant Microplate Spectrophotometer (BioTek Instruments, Winooski, VT) at 540 nm. For total protein, a 1:5 solution of supernatant to dye (dye: 1 part 4% $CuSO_4$ to 49 parts bicinchoninic acid) was mixed in a 96-well plate, covered and incubated at 25°C for 12 h, and assayed for absorbance at 562 nm. Final concentrations for all samples were calculated compared with a standard curve of either Triglyceride Standard (Pointe Scientific, Ann Arbor, MI) or bovine serum albumin for protein standards. The number and age of flies assayed varied depending on experiment and are labeled in the figure legends.

For heart-specific assays, 15–30 individual hearts were exposed in 1× PBS. Excess fat and associated cells were removed from the sides of the heart using a finely pulled glass micropipette attached to a vacuum. Samples were washed 3 times with 1× PBS, then dissected into 15–30 μl of Western lysis buffer. Samples were homogenized with a motorized pestle, briefly sonicated, then incubated for 5 min at 70°C. Solutions were treated as stated above to determine the final concentrations of heart-specific TG or protein. Nine such samples were analyzed per selection treatment.

Histology. Histological sections were obtained as previously described (54). Briefly, adult female flies (4–5 days posteclosion) were collected and placed in Telly's fixation buffer (60% ethanol, 3.33% formalin, 4% glacial acetic acid) for at least 1 wk at 4°C prior to paraffin embedding. Serial 8-μm sections were stained with hemotoxylin and eosin (H&E), then analyzed using a Leica DM2500 microscope equipped with a Leica DFC310FX digital camera. For each selection treatment, 3–5 serial sections from 13 animals were scored for the presence and degree of fat body accumulation between the heart and the dorsal cuticle. Data were analyzed using the

Freeman-Halton extension of the Fisher's Exact Test with the R software package (39). Images were cropped and annotated to demonstrate anatomical displacement.

Heart structure. Hearts were dissected, stained, and imaged as previously described (4). Briefly, flies were dissected in oxygenated artificial hemolymph, and then the exposed hearts were chemically relaxed, fixed, and stained with a 1:1,000 dilution of GFP-conjugated phalloidin (Alexa Fluor 488 phalloidin, Grand Island, NY). Images were taken with a Zeiss Apotome in conjunction with the Axiovision software package. Image stacks were processed using the Fiji software package (43) to visualize the circumferential fibers of the cardiomyocytes. Images were randomly labeled and blindly scored for structural defects; 25–32 hearts were scored per selection treatment, and data were analyzed with Fisher's Exact Test using the R software package (39).

Lipid droplet size. Adult flies, ~4–5 days old, were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA), frozen, then cut longitudinally into 30-μm sections using a Vibratome UltraPro 5000 Cryostat (The Vibratome, St. Louis, MO). When the cryostat approached the midline of the animal, sections were transferred to a glass slide and mounted in a medium containing 10 μl Nile Red Stock Solution (0.1% wt/vol, Nile Red/acetone), 10 μl Triton X-100, 5 ml glycerol, and 5 ml 1× PBS. Samples were immediately imaged (<2 h) using a Nikon A1R Confocal (Nikon Instruments, Melville, NY) at 40× with a 1.5× digital zoom. Z-stacks were taken and used to generate maximum-intensity projections. Lipid droplet size was quantified based off of previously described techniques (50). Briefly, the 5 largest droplets per section were measured for 2D area using the Fiji software package (43), for a total of 19 animals per selection treatment.

Statistical analysis. All analyses were performed using either Statistica 7.1 software package (StatSoft, Tulsa, OK) or the R software package (39). Fisher's Exact Test was used to quantify qualitative analyses, and variations of a nested ANOVA were used where appropriate. Our standard model to compare the S and F populations is a nested ANOVA with selection regime and replicate population as independent variables with replicate population nested within selection regime. Because the individual replicate populations are independent, they are treated as a random effect. In the case of the rescue experiments, the replicate populations were not independent as one replicate was split into either a control or rescued group. In this situation replicate was treated as a fixed effect. Statistics for the rescue experiments were generated by pairwise variations of the nested ANOVA's between the 3 groups (F, S, SR) with replicate population treated as a fixed or random effect where appropriate (see Figs. 4, *B–H* and 5, *B–E*). Fisher's Exact Tests were performed in R. The standard test was used for the 2×2 contingency table generated when looking at heart structure (see Fig. 6*D*), however, the 2×3 contingency tables generated by the histological analysis required the Freeman-Halton Extension of Fisher's Exact Test (see Fig. 3*B*).

RESULTS

Selection for starvation resistance leads to a dilated heart phenotype. To test whether selection for starvation resistance leads to cardiac dysfunction in *Drosophila melanogaster*, we employed two methods. First, we surgically exposed the hearts of adult flies in oxygenated artificial hemolymph in a denervated, purely myogenic state and recorded cardiac contractions using high-speed video microscopy (SOHA) (15, 19, 33–35). End-systolic and -diastolic diameters of the heart wall were measured from these recordings at a defined point in the 2nd heart chamber in the A3 abdominal segment and used to calculate fractional shortening. Custom software (19) was used to analyze high-speed movies for the quantification of heart period (duration of each cardiac cycle), contraction intervals, arrhythmia, and fractional shortening. We found no significant differences between the F and S populations for heart beat length (heart period, Fig. 1*A*), arrhythmia, or contraction intervals (Fig. 2, *A–C*). We observed an overall reduction in fractional shortening ($P < 0.03$), a measure of relative heart contractility, that was the result of a significant increase in the systolic diameter, suggesting systolic dysfunction (Fig. 1*A*). The diastolic diameters also tended to be larger than the controls, although this increase did not reach statistical significance. Representative M-modes, which display heart wall movements over time, from the starvation-selected lines also demonstrated a dilated, less contractile heart with preserved systolic as well as diastolic intervals (Fig. 1*C*, bottom panel; Fig. 2, *B* and *C*).

The *Drosophila* heart runs along the cuticle of the dorsal abdomen, tethered to the cuticle by sets of alary muscles. Many times upon dissection, we observed that the hearts in the S lines were loosely bound to the dorsal cuticle and appeared distended due to large fat body deposits. These hearts would often detach from the cuticle during preparation and could not be used in the video analysis. Because this phenomenon was not observed in the F lines, we hypothesized that the abnormal heart parameters in the S lines represented the lower limits of heart dysfunction, since cardiac function in a subset of S flies could not be measured.

To validate our results with a complementary method, we performed optical coherence tomography (OCT), which measures heart function in intact flies, which includes innervation. OCT is a noninvasive, nondestructive imaging modality that produces image information similar to the M-modes obtained with SOHA (Fig. 1, *C* and *D*), or to echocardiography in mammals, including humans (52, 53). Although resolution is lower in OCT than in SOHA, OCT captured a more inclusive range of variation within the S populations, as it was not affected by the surgical limitations found in the S flies. Both end-systolic and -diastolic diameters, measured in this case at a defined point in the first heart chamber (conical chamber), were increased ($P < 3.3 \times 10^{-3}$ and $P < 5.1 \times 10^{-3}$, respectively) (Fig. 1*B*), again resulting in an overall reduction in fractional shortening ($P < 6.8 \times 10^{-4}$). Similar to the findings of surgically prepared specimens, we found no significant changes in heart period (Fig. 1*B*). Transversely oriented M-modes provided a visual representation of the differences in end-diastolic and -systolic diameters (Fig. 1*D*).

Increased fat body mass physically interferes with the heart. Alary muscles are responsible for anchoring the heart to the cuticle and assisting in relaxation during diastole (23). In the starvation-selected lines the alary muscles appeared to be compromised, presenting as a loose attachment of the heart to the dorsal cuticle upon dissection. This appeared to be caused by large deposits of fat body morphologically altering the position of the heart towards the ventral abdomen. To investigate this possibility, we performed histology and evaluated serial longitudinal sections of adult flies. Many S flies had gross anatomical displacement of the heart towards the ventral abdomen with large deposits of adipose tissue between the heart and the dorsal cuticle (Fig. 3*A*, *right* panel). Fat body deposits between the dorsal cuticle and heart were observed in ~85% of S flies compared with ~31% of F flies ($P < 0.02$) (Fig. 3*B*). Of the S flies with fat between the dorsal cuticle and the heart, ~27% had a severe phenotype where the heart was

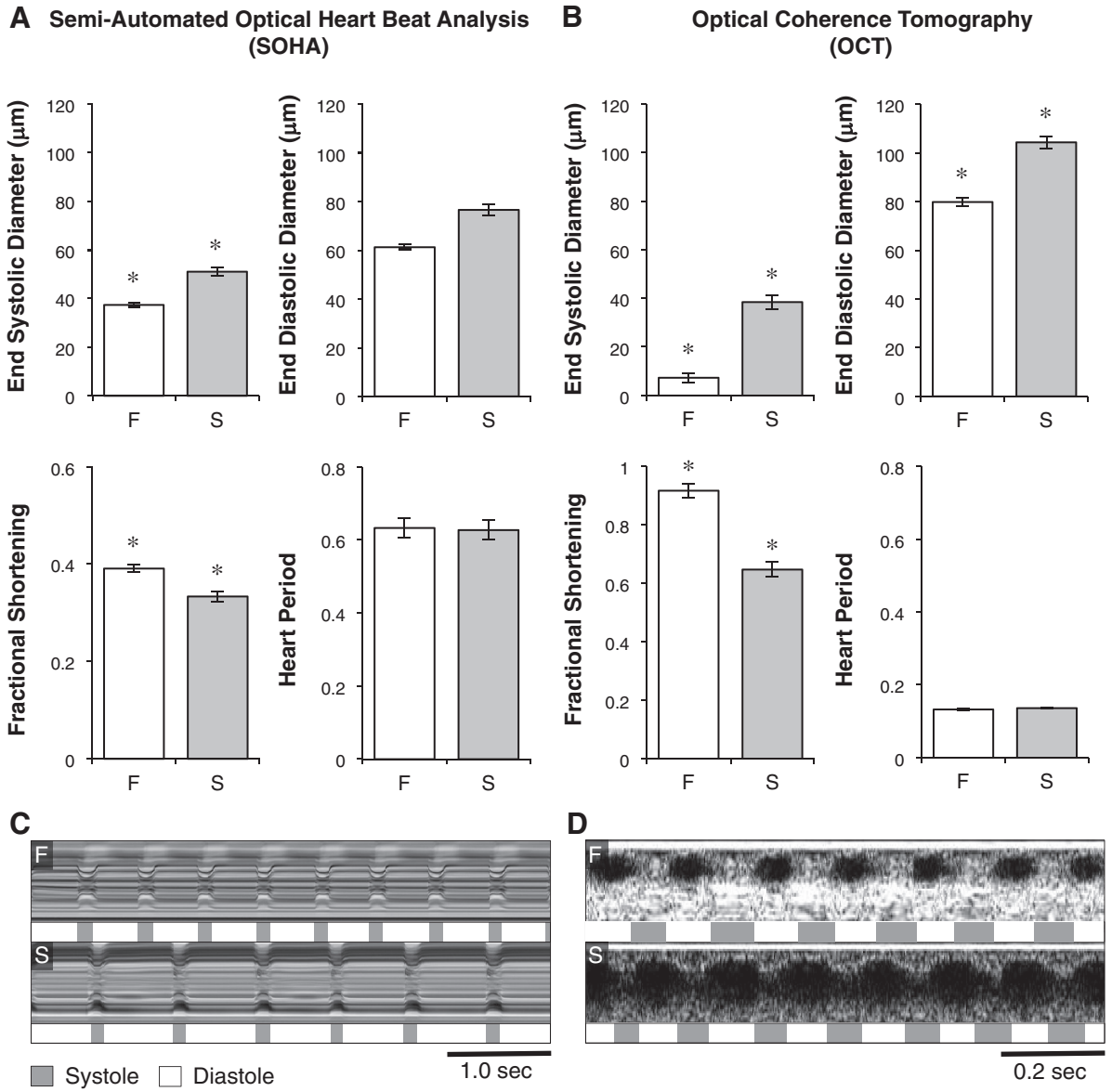

Fig. 1. Starvation-selected hearts are dilated and less contractile than their unselected controls. *A*: heart parameters measured by semiautomated optical heartbeat analysis (SOHA) showed that the S populations have higher systolic diameter ($*P < 0.05$), trending but not statistically significantly higher diastolic diameter ($P > 0.06$), lower fractional shortening ($*P < 0.03$), and no difference in heart period ($P > 0.86$) compared with the F populations. Nested ANOVA, $N = 57–74$. *B*: heart parameters measured from optical coherence tomography (OCT) largely agreed with SOHA as S populations showed higher systolic diameter ($*P < 3.3 \times 10^{-3}$), higher diastolic diameter ($*P < 5.1 \times 10^{-3}$), lower fractional shortening ($*P < 6.8 \times 10^{-4}$), and no difference in heart period ($P > 0.14$). Nested ANOVA, $N = 42–68$. Representative M-modes where the *Y*-axis is a 1-pixel-width slice of the original video viewed over time (*x*-axis) in either SOHA (*C*) or OCT (*D*) demonstrate a S heart that is dilated and less contractile than the F controls.

drastically distended towards the ventral abdomen (Fig. 3*A*, *right* panel). The severe phenotype was not observed in the F populations (Fig. 3*B*).

Heart dysfunction in the starvation-selected lines is dependent on increased fat storage. Many phenotypic differences between the S and F lines are likely genetic, caused by alterations in allelic frequencies in response to selection for starvation resistance over many generations. These genetic differences may directly lead to heart dysfunction or may affect the heart as a secondary consequence of increased fat storage. To address this question, we performed experiments to decouple fat accumulation and heart dysfunction. In the first experiment, we transferred 4-day-old adults to noncaloric 1% agar for 7 days (Fig. 4*A*). After 1 wk of starvation the "rescued" S lines (hereafter "SR") had reduced TG levels compared with their age-matched S controls ($P < 1.2 \times 10^{-10}$) (Fig. 4*B*). TG levels in the SR lines were not significantly different from the age-matched F controls suggesting that starvation for 7 days was sufficient to rescue the S lines from obesity. To investigate the relationship between fat storage and cardiac dysfunction, we measured heart parameters on the SR lines using SOHA and OCT. SOHA demonstrated that lean SR flies had lower diastolic and systolic diameters than their age-matched S controls ($P < 0.04$ and $P < 1.6 \times 10^{-4}$, respectively) (Fig. 4, *C* and *D*) as well as increased contractility as measured through fractional shortening ($P < 1.4 \times 10^{-4}$)

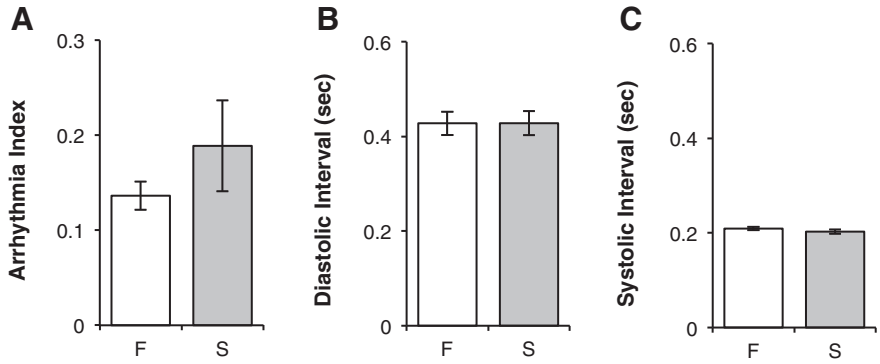

Fig. 2. Starvation-selected *Drosophila* show no difference in contraction intervals or arrhythmia index. The S populations showed no difference in arrhythmia index ($P > 0.21$) (*A*), diastolic interval ($P > 0.92$) (B), or systolic interval ($P > 0.49$) (*C*) compared with their unselected controls. Nested ANOVA, $N = 57–74$.

(Fig. 4*E*). While the end-diastolic and -systolic diameters failed to fully recover to F levels, fractional shortening levels in the SR lines were rescued, as they did not differ from the F controls ($P > 0.39$) (Fig. 4*E*). The OCT data largely supported the results obtained by SOHA. After a 7-day diet, SR flies had lower end-diastolic and -systolic diameters ($P < 1.5 \times 10^{-11}$ and $P < 3.3 \times 10^{-4}$, respectively) (Fig. 4, *F* and *G*) as well as greater fractional shortening ($P < 2.1 \times 10^{-3}$) (Fig. 4*H*). Average diastolic diameter was higher in the S lines compared with the age-matched F controls, but the data failed to reach significance ($P > 0.12$) (Fig. 4*F*). Differences in the absolute values between Figs. 1 and 4 are likely a function of age and generations of selection.

Larval development time in the S populations is ~24 h longer than in the F populations, during which they accumulate lipids available for adult starvation resistance (41). In additional rescue experiments, we transferred S larvae to noncaloric 1% agar when the F larvae began to wander, to standardize larval feeding time (Fig. 5*A*). Adults were aged 4–5 days with food and assayed for heart dysfunction by SOHA. Rescued SR adults had lower TG content compared with the S controls ($P < 2.0 \times 10^{-7}$) and were not significantly different from the F flies (Fig. 5*B*). We found this improved the contractility of the heart to F levels as SR fractional shortening was significantly increased compared with the S controls ($P < 1.1 \times 10^{-4}$) (Fig. 5*E*). This improvement in fractional shortening was a function of lower systolic diameter in the SR lines ($P < 0.04$) (Fig. 5*D*) as diastolic diameter was not different from the S controls ($P > 0.87$) (Fig. 5*C*).

Starvation-selected lines do not ectopically store lipids in the heart but instead increase storage capacity of lipids within the fat body. Drosophila challenged with lipogenic diets store TG ectopically in nonadipose tissues such as the heart (11, 29). Intracellular lipid accumulation in the cardiomyocytes appears to disrupt myofibrillar architecture, and can lead to severe cardiac defects including partial conduction blocks, dysfunctional ostia, and decreases in fractional shortening (11). To test the hypothesis that heart dysfunction in the S lines is caused by intracellular fat accumulation, we dissected hearts and measured their TG contents in F and S flies. Despite higher whole body levels of TG in the S flies ($P < 0.03$) (Fig. 6*A*), we found no significant changes in heart-specific TG levels (standardized to total heart protein, $P > 0.84$), indicating no difference in cardiac lipids between the S and F lines (Fig. 6*B*). Intramyocardial lipid deposition is further hypothesized to lead to structural changes in the myofibrillar architecture of the cardiomyocytes (11, 26). To address this possibility we examined hearts for gross structural changes. Adult hearts were surgi-

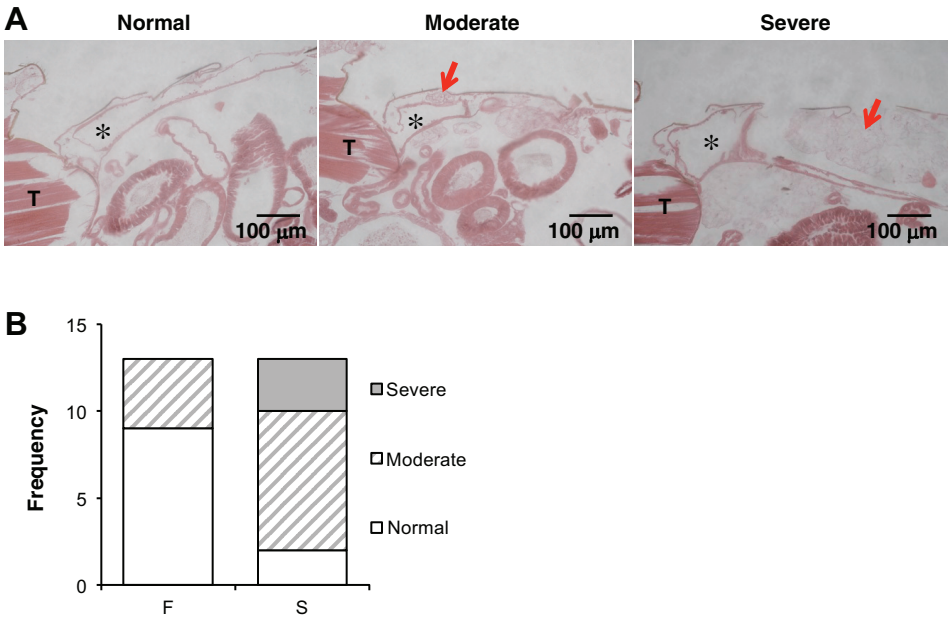

Fig. 3. Fat body physically interferes with the starvation-selected heart. *A*: longitudinal histological sections (hematoxylin and eosin stain). The asterisk indicates the lumen of the conical chamber of the heart, "T" indicates location of the thorax, and the red arrows point to fat body deposits. Normal sections (*left*) show the heart closely associated with the dorsal cuticle. Moderate sections (*center*) have small deposits of fat body between the dorsal cuticle and heart. Severe sections (*right*) have large fat body deposits between the heart and dorsal cuticle. *B*: starvation-selected lines have higher instance of moderate and severe phenotypes ($P < 0.02$). Fisher's Exact Test (Freeman-Halton Extension), $N = 13$.

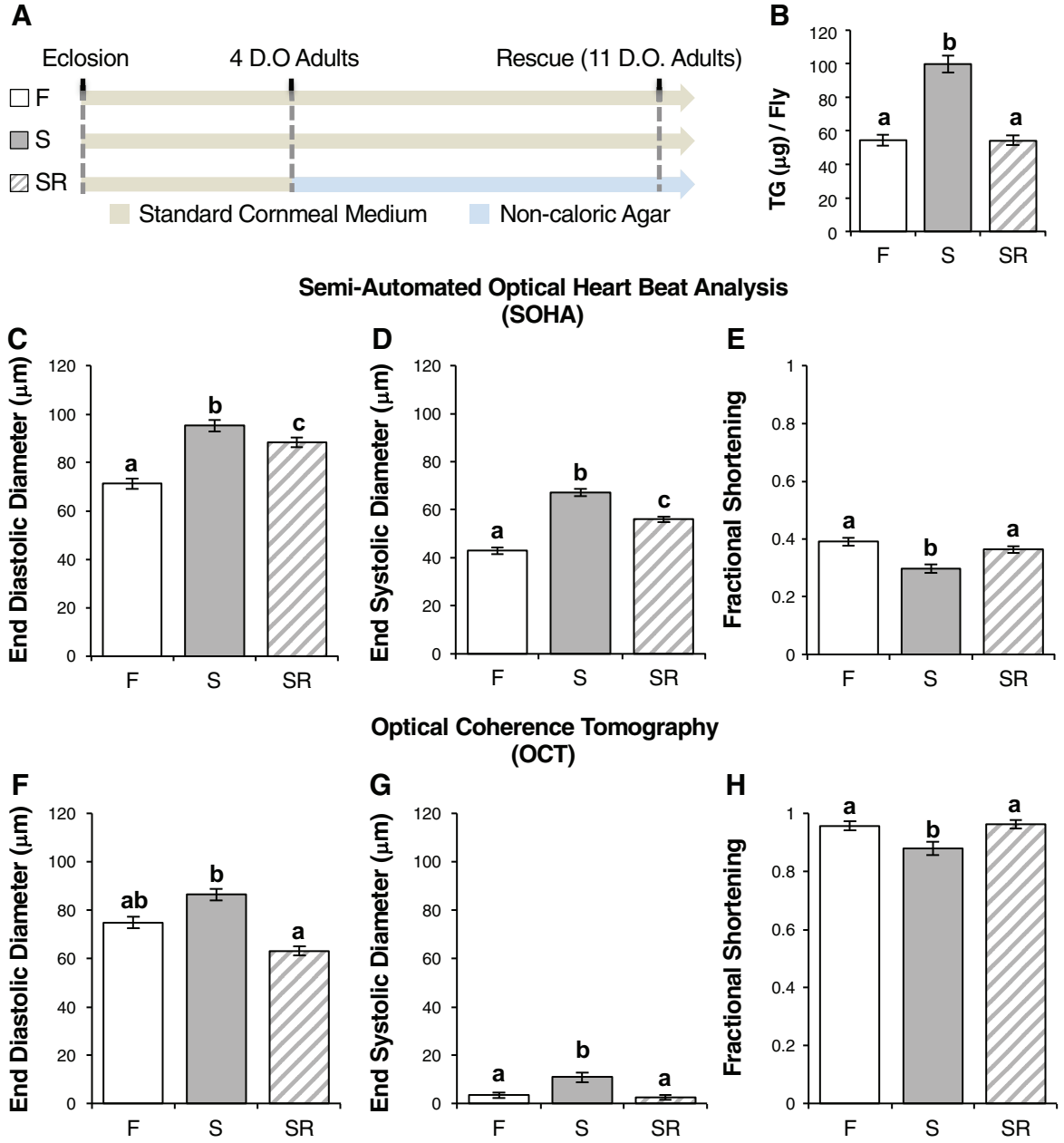

Fig. 4. The heart phenotype can be partially rescued through a 1-wk-long diet. *A*: experimental design. A cohort of the starvation-selected lines were made lean ("SR" Group) by a 1-wk-long diet of 1% noncaloric agar. *B*: the diet significantly reduced total triglycerides (TGs) compared with the age-matched S control lines ($P < 1.2 \times 10^{-10}$, $N = 24$) and were nonsignificantly different from the F lines. Results from SOHA showed SR populations had correlated improvements in diastolic (*C*) and systolic diameters (*D*) as well as improved fractional shortening (*E*) compared with the S populations ($P < 0.04$, $P < 1.6 \times 10^{-4}$, and $P < 1.4 \times 10^{-4}$, respectively, $N = 45–46$). OCT measurements showed SR populations had correlated improvements in diastolic (*F*) and systolic diameters (*G*) as well as improved fractional shortening (*H*) compared with the S populations ($P < 1.5 \times 10^{-11}$, $P < 3.3 \times 10^{-4}$, and $P < 2.1 \times 10^{-3}$, respectively, $N = 44–45$). Differing letters a, b, and c denote significant differences between groups ($P < 0.05$). Pairwise nested ANOVAs (F vs. S, F vs. SR, S vs. SR) were used for these assays (see METHODS for details).

cally exposed and stained with GFP-conjugated phalloidin to visualize the actin fibers of the cardiomyocytes. We found low levels of myofibrillar disruption in the cardiomyocytes of both the F and S flies, but quantitatively the frequency of disruption was not significantly different (Fig. 6*D*) ($P > 0.21$).

The S populations harbored higher whole body TG ($P < 0.02$) (Fig. 7*A*), but total protein levels were not significantly different from the F controls ($P > 0.54$) (Fig. 7*B*). Despite comparable protein levels, S flies have visibly large, distended abdomens (27) and during dissection we observed extensive fat body tissue in the S abdomen. Adipose tissue has a limited storage capacity for fats and the fat-mediated lipotoxic effects on the heart have been attributed to conditions when lipids are in excess of storage capacity. We hypothesized that the S lines had adapted to starvation selection by increasing TG storage capacity within the fat body. We examined lipid droplets within the fat body using Nile Red staining. In the S lines, longitudinal sections of the fat body near the dorsal cuticle had

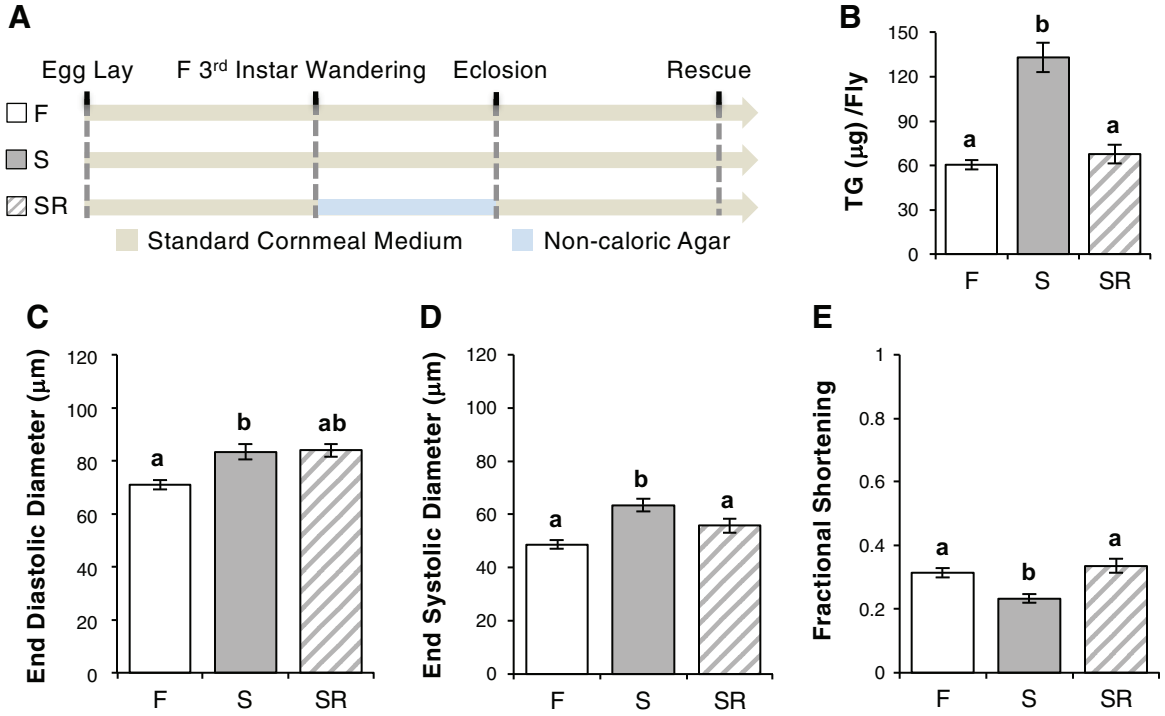

Fig. 5. The heart phenotype can be partially rescued by removing nutrient sources during extended larval development in the S lines. *A*: experimental design. S flies are deprived of nutrients during their extended larval feeding period. This standardizes their nutrient availability to that of the F lines leading to reduced total TG/fly in the rescued S (SR) populations ($P < 2.0 \times 10^{-7}$) (*B*). Nested ANOVA, $N = 18$. *C*: the treatment did not affect diastolic diameter ($P > 0.87$); however, systolic diameter (*D*) was significantly reduced in the S populations ($P < 0.04$), leading to an overall improvement in contractility as measured through fractional shortening ($P < 1.1 \times 10^{-4}$) (*E*). Pairwise nested ANOVA, $N = 53$–58. Differing letters a, b, and c denote significant differences between groups ($P < 0.05$).

large lipid droplets (Fig. 6*E*, *bottom* panel). The area of the largest droplets was ~65% larger than the area of the largest lipid droplets from the F populations ($P < 0.04$) (Fig. 6*F*). This corresponded to an approximate 117% increase in lipid droplet volume.

DISCUSSION

Obesity-associated heart dysfunction in starvation-selected Drosophila. Many recent studies have demonstrated the merits of using *Drosophila* as a model to study the genetics and physiology of obesity-induced heart dysfunction (11, 17, 25, 26, 29, 46). Here we present the first study to investigate the effects of obesity on the heart in laboratory-evolved populations. We found that long-term laboratory selection for starvation resistance leads to obese flies with a correlated tradeoff in heart function. Using independent methods we demonstrated that starvation-selected hearts are dilated and less contractile than their unselected controls.

While the data from SOHA and OCT largely agree, there are some important technical differences that affect their individual interpretation. The surgical preparation required for SOHA severs the ventral thorax and head from the abdomen, eliminating the effects of neurogenic innervation on the heart from the central nervous system, leaving a purely myogenic heart that will beat for hours in oxygenated artificial hemolymph. Therefore these data are useful in understanding the myogenic properties of the heart. On the other hand, OCT is an in vivo assay that measures the heart parameters of adult flies which have their entire nervous system intact. These differences are observed in the data as heart rate in OCT is nearly 4 times larger than with SOHA (Fig. 1), which may be expected in a neurologically innervated vs. completely myogenic heart (13) and has been documented in *Drosophila* (34). Because of the unique physiology of the S populations, we lost many S hearts during dissection for SOHA which may have limited our ability to statistically resolve the increased diastolic diameter observed in the OCT data. Furthermore, the surgical preparation for SOHA requires the partial removal of fat body to better visualize the heart. By removing this tissue, this procedure may provide a slight, acute improvement in contractility, leading to reduced differentiation between the F and S populations. Despite their inherent differences, both methods consistently agreed on the relative differences in diameter and contractility between the F and S populations.

Genetic basis of heart dysfunction in starvation-selected Drosophila is correlated with disrupted lipid homeostasis. The genetic basis of heart dysfunction in the S lines could be due to pleiotropic effects of starvation-selected alleles, which directly affect the development or structure of the heart. Many genetic mechanisms could explain maladaptive heart phenotypes in the context of selection for starvation-resistance. For example, linkage disequilibrium (LD) between positively selected starvation-resistance genes and heart-specific loci could have caused increased frequency of particular alleles with direct negative effects on the heart. Alternatively, rearing flies in small cages with plentiful food and lack of predation may have lifted selective restraints on the heart, allowing genetic drift towards dysfunction. In addition, selected alleles may have

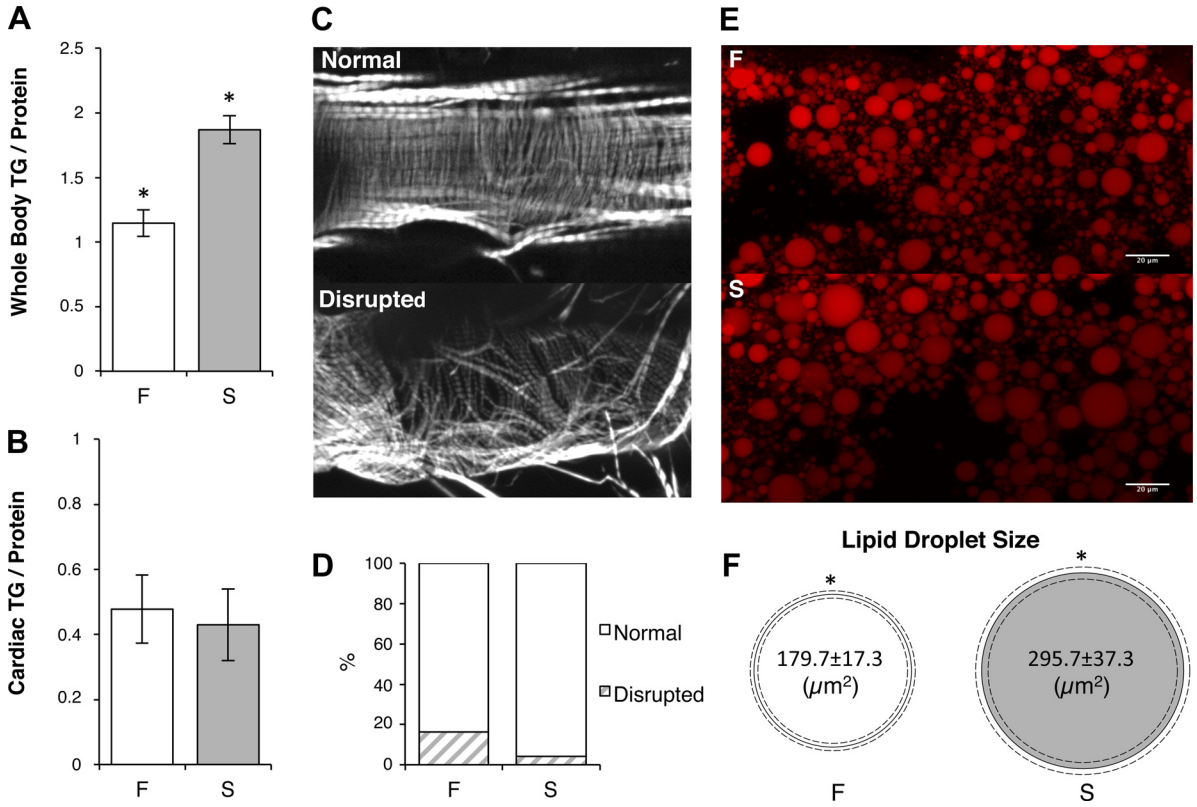

Fig. 6. Starvation selected lines resist ectopic lipid storage in the heart. *A*: the S populations store higher whole body levels of TGs than the F populations ($P < 0.03$, standardized to total protein). Nested ANOVA, $N = 24$. *B*: the excess lipids in the S populations are not ectopically stored within the heart ($P > 0.84$, standardized to total heart protein). Nested ANOVA, $N = 9$. *C*: we then screened for disrupted myofibrils in our populations. Normal circumferential fibers (*top*) are oriented vertically, perpendicular to the heart tube. Disrupted fibers (*bottom*) have gaps between these fibers. *D*: we found low levels of disruption in both F and S populations, but no difference between selection treatments ($P > 0.21$). Fisher's Exact Test, $N = 25$–32. *E*: we stained the lipid droplets of the adult fat body in the F and S populations with Nile Red. *F*: the 2-dimensional area per lipid droplet was on average much larger in the S populations ($^*P < 0.04$). Nested ANOVA, $N = 19$.

tissue-specific effects with alleles favoring starvation-resistance in one tissue causing off-target effects in other cell types like the heart, which may not be crucial to starvation survival. Conversely, starvation-selected alleles may affect the heart as a secondary consequence of disrupted lipid homeostasis. Our data support the latter, as we demonstrated that heart function could be improved by reducing total TG through a 7-day starvation period. We interpret these findings to suggest that heart dysfunction is not caused by alleles which directly disrupt the development or function of the heart, but by a fat-dependent mechanism.

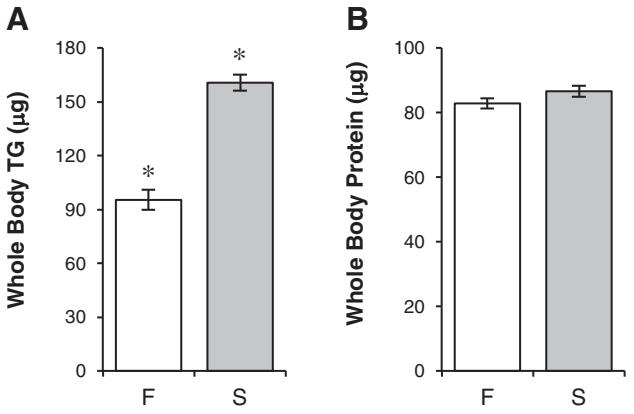

Fig. 7. Whole body TG and total protein levels. *A*: the S populations have higher levels of whole body TG than F populations ($^*P < 0.02$). *B*: there were no differences in whole body protein levels between the S and F populations ($P > 0.54$). Nested ANOVA, $N = 24$.

One of the most important factors leading to obesity-associated heart dysfunction in *Drosophila* is cardiac steatosis (11, 17, 26, 29, 46). Our data suggest this is not the case in starvation-selected *Drosophila*, as these flies do not ectopically store TG in the heart. Instead we believe starvation-selected *Drosophila* have adapted to the extreme context of their ecology by increasing the storage capacity of lipids within their fat body. Histological analysis confirmed the presence of expansive fat body tissue in the S lines, and further examination revealed this tissue contained large lipid droplets. The expanded tissue morphologically altered the proper anatomical placement of the heart along the dorsal cuticle. Our data suggest that this physical interference contributes to heart dysfunction in the S lines.

An alternative mechanism for obesity-induced heart disease is the chronic inflammation of adipose tissue leading to changes in cytokine production and cellular signaling in responding tissues (47, 51). Cross talk between the fat body and other organs has been well documented in *Drosophila* (1, 5, 7, 40). Our data do not exclude the possibility that the fat body is inflamed in the S populations, leading to changes in fat body-

heart signaling. This intriguing possibility requires further investigation that is beyond the scope of the current study.

Perspectives and Significance

Many theories have been proposed to explain the evolutionary origins of obesity in humans (20). Of many interesting ideas, our data are consistent with the thrifty genotype hypothesis, which argues that genetic variants which promote the efficient utilization and storage of nutrients are selected for during periods of famine and starvation (30). While this theory has faced criticism (21, 44, 45) it is important to note its plausibility in the context of extreme selection pressures across repeated generations in our starvation selected populations, which are unmatched in the anthropological record. Other theories argue that epigenetic factors may play a role in driving obesity, as gene/environment interactions in the womb may lead to changes in chromatin structure (45). This has recently been supported in *Drosophila* as both maternal and paternal high-sugar diets have been shown to cause transgenerational effects on metabolism and macronutrient homeostasis (14, 36). However, our preliminary tests have revealed no such parental effects on lipid storage, fecundity or starvation-resistance in our populations (Gibbs AG, unpublished data). In addition our flies are removed from selection for one generation and reared with identical environmental conditions as the F populations prior to each experiment. This suggests that genetics plays the primary role in the evolved characteristics of the S populations.

In mammals it is hypothesized that a larger capacity to store fats in subcutaneous adipose tissue (SAT) limits the harmful effects of ectopic fat deposition in other tissues (9). This helps explain why low SAT levels in individuals with lipodystrophic disorders leads to high levels of ectopic fat accumulation and metabolic disorders (31, 32). Selection on genetic variants that increase the storage capacity of adipose tissue may then be an adaptive response to provide the organism with a larger reservoir to store neutral fats. Evidence for the protective role of adipose tissue has been demonstrated in *Drosophila*, where the fat body has been shown to protect against hyperglycemia in response to a high-sugar diet by switching to a lipogenic expression profile (28). In nature the ability to store excess lipids in adipose tissue may provide a protective buffer against periods of famine while also avoiding metabolic disorders caused when fats are stored ectopically. Counterselective forces (e.g., predation, decreased mobility) that result in decreased dispersal ability or increased predation would likely balance allelic frequencies to an adaptive state. In our experiments some of these counterforces are removed, reducing selective restraint against increased adipose storage capacity. This resulted in S flies that store so much fat that the tissue appears to physically interfere with the normal function of the heart. This unique mechanism of adaptation may provide S flies with increased energy reserves vital to survive starvation while dampening the lipotoxic heart phenotype traditionally associated with obesity. It will be interesting to further examine this hypothesis and test whether the S populations are resistant to other lipotoxic phenotypes traditionally associated with obesity, including insulin resistance and reduced longevity, in future studies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.M.H., R.T.B., and A.G.G. conception and design of research; C.M.H., R.T.B., M.J.W., and L.Y. performed experiments; C.M.H. and A.G.G. analyzed data; C.M.H., R.T.B., M.J.W., R.B., and A.G.G. interpreted results of experiments; C.M.H. prepared figures; C.M.H. and A.G.G. drafted manuscript; C.M.H., R.T.B., M.J.W., L.Y., R.B., and A.G.G. edited and revised manuscript; C.M.H., R.T.B., M.J.W., L.Y., R.B., and A.G.G. approved final version of manuscript.

REFERENCES

1. **Agaisse H, Perrimon N.** The roles of JAK/STAT signaling in *Drosophila* immune responses. *Immunol Rev* 198: 72–82, 2004.
2. **Aggarwal DD.** Physiological basis of starvation resistance in *Drosophila leontia*: analysis of sexual dimorphism. *J Exp Biol* 217: 1849–1859, 2014.
3. **Aguila JR, Hoshizaki DK, Gibbs AG.** Contribution of larval nutrition to adult reproduction in *Drosophila melanogaster*. *J Exp Biol* 216: 399–406, 2013.
4. **Alayari NN, Vogler G, Taghli-Lamallem O, Ocorr K, Bodmer R, Cammarato A.** Fluorescent labeling of *Drosophila* heart structures. *J Vis Exp* 2009 Oct 13;(32). pii: 1423. doi:10.3791/1423.
5. **Andersen DS, Colombani J, Léopold P.** Coordination of organ growth: principles and outstanding questions from the world of insects. *Trends Cell Biol* 23: 336–344, 2013.
6. **Arrese EL, Soulages JL.** Insect fat body: energy, metabolism, and regulation. *Annu Rev Entomol* 55: 207–225, 2010.
7. **Bai H, Kang P, Tatar M.** Drosophila insulin-like peptide-6 (*dilp6*) expression from fat body extends lifespan and represses secretion of Drosophila insulin-like peptide-2 from the brain: *dilp6* expression increases *Drosophila* lifespan. *Aging Cell* 11: 978–985, 2012.
8. **Ballard JWO, Melvin RG, Simpson SJ.** Starvation resistance is positively correlated with body lipid proportion in five wild caught *Drosophila simulans* populations. *J Insect Physiol* 54: 1371–1376, 2008.
9. **Bastien M, Poirier P, Lemieux I, Després JP.** Overview of epidemiology and contribution of obesity to cardiovascular disease. *Prog Cardiovasc Dis* 56: 369–381, 2014.
10. **Birse RT, Bodmer R.** Lipotoxicity and cardiac dysfunction in mammals and *Drosophila*. *Crit Rev Biochem Mol Biol* 46: 376–385, 2011.
11. **Birse RT, Choi J, Reardon K, Rodriguez J, Graham S, Diop S, Ocorr K, Bodmer R, Oldham S.** High-fat-diet-induced obesity and heart dysfunction are regulated by the TOR pathway in *Drosophila*. *Cell Metab* 12: 533–544, 2010.
12. **Brewer ML.** *Kinematic Analysis of Axial Rotations and the Effects of Stress Selection on Takeoff Flight Performance* [Online] (PhD Thesis). Las Vegas, NV: UNLV, 2013. http://digitalscholarship.unlv.edu/thesesdissertations/1806.
13. **Brooks CM, Lange G.** Interaction of myogenic and neurogenic mechanisms that control heart rate. *Proc Natl Acad Sci USA* 74: 1761–1762, 1977.
14. **Buescher JL, Musselman LP, Wilson CA, Lang T, Keleher M, Baranski TJ, Duncan JG.** Evidence for transgenerational metabolic programming in *Drosophila*. *Dis Model Mech* 6: 1123–1132, 2013.
15. **Cammarato A, Ocorr S, Ocorr K.** Enhanced assessment of contractile dynamics in *Drosophila* hearts. *Biotechniques* 58: 77–80, 2015.
16. **Chippindale AK, Chu TJF, Rose MR.** Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster*. *Evolution* 50: 753, 1996.
17. **Diop SB, Bisharat-Kernizan J, Birse RT, Oldham S, Ocorr K, Bodmer R.** PGC-1/Spargel counteracts high-fat-diet-induced obesity and cardiac lipotoxicity downstream of TOR and brummer ATGL lipase. *Cell Rep* 10: 1572–1584, 2015.

18. **Diop SB, Bodmer R.** *Drosophila* as a model to study the genetic mechanisms of obesity-associated heart dysfunction. *J Cell Mol Med* 16: 966–971, 2012.
19. **Fink M, Callol-Massot C, Chu A, Ruiz-Lozano P, Belmonte J, Giles W, Bodmer R, Ocorr K.** A new method for detection and quantification of heartbeat parameters in *Drosophila*, zebrafish, and embryonic mouse hearts. *Biotechniques* 46: 101–113, 2009.
20. **Genné-Bacon EA.** Thinking evolutionarily about obesity. *Yale J Biol Med* 87: 99–112, 2014.
21. **Hales C, Barker D.** Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Int J Epidemiol* 42: 1215–1222, 2013.
22. **Harshman LG, Hoffmann AA, Clark AG.** Selection for starvation resistance in *Drosophila melanogaster*: physiological correlates, enzyme activities and multiple stress responses. *J Evol Biol* 12: 370–379, 1999.
23. **Klowden MJ.** *Physiological Systems in Insects* (2nd ed). Amsterdam: Elsevier/Academic, 2007.
24. **Kuhnlein RP.** Lipid droplet-based storage fat metabolism in *Drosophila*. Lipid droplet synthesis and metabolism: from yeast to man. *J Lipid Res* 53: 1430–1436, 2012.
25. **Lee JH, Budanov AV, Park EJ, Birse R, Kim TE, Perkins GA, Ocorr K, Ellisman MH, Bodmer R, Bier E, Karin M.** Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science* 327: 1223–1228, 2010.
26. **Lim HY, Wang W, Wessells RJ, Ocorr K, Bodmer R.** Phospholipid homeostasis regulates lipid metabolism and cardiac function through SREBP signaling in *Drosophila*. *Genes Dev* 25: 189–200, 2011.
27. **Masek P, Reynolds LA, Bollinger WL, Moody C, Mehta A, Murakami K, Yoshizawa M, Gibbs AG, Keene AC.** Altered regulation of sleep and feeding contributes to starvation resistance in *Drosophila melanogaster*. *J Exp Biol* 217: 3122–3132, 2014.
28. **Musselman LP, Fink JL, Ramachandran PV, Patterson BW, Okunade AL, Maier E, Brent MR, Turk J, Baranski TJ.** Role of fat body lipogenesis in protection against the effects of caloric overload in *Drosophila*. *J Biol Chem* 288: 8028–8042, 2013.
29. **Na J, Musselman LP, Pendse J, Baranski TJ, Bodmer R, Ocorr K, Cagan R.** A *Drosophila* model of high sugar diet-induced cardiomyopathy. *PLoS Genet* 9: e1003175, 2013.
30. **Neel JV.** Diabetes mellitus: A "thrifty" genotype rendered detrimental by "progress"? *Am J Hum Genet* 14: 353–362, 1962.
31. **Nelson MD, Victor RG, Szczepaniak EW, Simha V, Garg A, Szczepaniak LS.** Cardiac steatosis and left ventricular hypertrophy in patients with generalized lipodystrophy as determined by magnetic resonance spectroscopy and imaging. *Am J Cardiol* 112: 1019–1024, 2013.
32. **Nolis T.** Exploring the pathophysiology behind the more common genetic and acquired lipodystrophies. *J Hum Genet* 59: 16–23, 2014.
33. **Ocorr K, Akasaka T, Bodmer R.** Age-related cardiac disease model of Drosophila. *Mech Ageing Dev* 128: 112–116, 2007.
34. **Ocorr K, Reeves NL, Wessells RJ, Fink M, Chen HSV, Akasaka T, Yasuda S, Metzger JM, Giles W, Posakony JW, Bodmer R.** KCNQ potassium channel mutations cause cardiac arrhythmias in Drosophila that mimic the effects of aging. *Proc Natl Acad Sci USA* 104: 3943–3948, 2007.
35. **Ocorr K, Reeves NL, Wessells RJ, Fink M, Chen HSV, Akasaka T, Yasuda S, Metzger JM, Giles W, Posakony JW, Bodmer R.** KCNQ potassium channel mutations cause cardiac arrhythmias in Drosophila that mimic the effects of aging. *Proc Natl Acad Sci USA* 104: 3943–3948, 2007.
36. **Öst A, Lempradl A, Casas E, Weigert M, Tiko T, Deniz M, Pantano L, Boenisch U, Itskov PM, Stoeckius M, Ruf M, Rajewsky N, Reuter G, Iovino N, Ribeiro C, Alenius M, Heyne S, Vavouri T, Pospisilik JA.** Paternal diet defines offspring chromatin state and intergenerational obesity. *Cell* 159: 1352–1364, 2014.
37. **Parkash R, Aggarwal D, Kalra B.** Coadapted changes in energy metabolites and body color phenotypes for resistance to starvation and desiccation in latitudinal populations of *D. melanogaster. Evol Ecol* 26: 149–169, 2012.
38. **Pergola G de, Silvestris F.** Obesity as a major risk factor for cancer. *J Obes* 2013: 1–11, 2013.
39. **R Core Team.** *R: A Language and Environment for Statistical Computing*[Online]. R Foundation for Statistical Computing. http://www.R-project.org/.
40. **Rajan A, Perrimon N.** *Drosophila* cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion. *Cell* 151: 123–137, 2012.
41. **Reynolds LA.** *The Effects of Starvation Selection on Drosophila Melanogaster Life History and Development* (PhD Thesis). Las Vegas, Nevada: UNLV, 2013.
42. **Rion S, Kawecki TJ.** Evolutionary biology of starvation resistance: what we have learned from *Drosophila*: starvation resistance in *Drosophila*. *J Evol Biol* 20: 1655–1664, 2007.
43. **Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A.** Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9: 676–682, 2012.
44. **Speakman JR.** Thrifty genes for obesity, an attractive but flawed idea, and an alternative perspective: the "drifty gene" hypothesis. *Int J Obes* 32: 1611–1617, 2008.
45. **Stöger R.** The thrifty epigenotype: An acquired and heritable predisposition for obesity and diabetes? *Bioessays* 30: 156–166, 2008.
46. **Sujkowski A, Saunders S, Tinkerhess M, Piazza N, Jennens J, Healy L, Zheng L, Wessells R.** dFatp regulates nutrient distribution and long-term physiology in *Drosophila*. *Aging Cell* 11: 921–932, 2012.
47. **Sun S, Ji Y, Kersten S, Qi L.** Mechanisms of inflammatory responses in obese adipose tissue. *Annu Rev Nutr* 32: 261–286, 2012.
48. **Unger RH, Clark GO, Scherer PE, Orci L.** Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochim Biophys Acta* 1801: 209–214, 2010.
49. **Vogler G, Ocorr K.** Visualizing the beating heart in *Drosophila. J Vis Exp* 2009 Sep 28;(31). pii: 1425. doi:10.3791/1425.
50. **Wang C, Liu Z, Huang X.** Rab32 is important for autophagy and lipid storage in *Drosophila*. *PLoS ONE* 7: e32086, 2012.
51. **Wang Z, Nakayama T.** Inflammation, a link between obesity and cardiovascular disease. *Mediators Inflamm* 2010: 1–17, 2010.
52. **Wolf MJ, Amrein H, Izatt JA, Choma MA, Reedy MC, Rockman HA.** From the cover: *Drosophila* as a model for the identification of genes causing adult human heart disease. *Proc Natl Acad Sci USA* 103: 1394–1399, 2006.
53. **Wolf MJ, Rockman HA.** *Drosophila*, genetic screens, and cardiac function. *Circ Res* 109: 794–806, 2011.
54. **Yu L, Daniels JP, Wolf MJ.** Vascular and cardiac studies in *Drosophila* [Online]. In: *Manual of Research Techniques in Cardiovascular Medicine*, edited by Ardehali H, Bolli R, Losordo DW. Wiley, p. 432–439. http://doi.wiley.com/10.1002/9781118495148.ch50 [14 Apr 2015].