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Research article

Identification of genetic modifiers of lifespan on a high sugar diet in the *Drosophila* Genetic Reference Panel

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ABSTRACT

Genome-wide association studies (GWAS) have become beneficial in identifying genetic variants underlying susceptibility to various complex diseases and conditions, including obesity. Utilizing the *Drosophila* Genetic Reference Panel (DGRP), we performed a GWAS of lifespan of 193 genetically distinct lines on a high sugar diet (HSD). The DGRP analysis pipeline determined the most significant lifespan associated polymorphisms were within loci of genes involved in: neural processes, behavior, development, and apoptosis, among other functions. Next, based on the relevance to obesity pathology, and the availability of transgenic RNAi lines targeting the genes we identified, whole-body *in vivo* knockdown of several candidate genes was performed. We utilized the GAL4-UAS binary expression system to independently validate the impacts of these loci on *Drosophila* lifespan during HSD. These loci were largely confirmed to affect lifespan in that HSD setting, as well as a normal diet setting. However, we also detected unexpected dietary effects of the HSD, including inconsistent diet effects on lifespan relative to a normal diet and a strong downregulation of feeding quantity.

1. Introduction

Obesity is caused by excess energy intake and insufficient energy expenditure, resulting in an energy imbalance. This energy imbalance results in accumulation of adipose tissue, which becomes excessive for organismal body size if the imbalance is chronic. Obesity can result in pathogenesis, presenting as a low-grade inflammatory, metabolic, and endocrine dysfunctional state that elevates the risk of type 2 diabetes mellitus, cardiovascular disease, neurological decline, certain forms of cancer, respiratory complications, osteoarthritis and mortality. The adipose tissue in the obese mammalian body can induce inflammatory responses. Pro-inflammatory adipokines: tumor necrosis factor α (TNFα), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and resistin are elevated in obesity. Meanwhile, the levels of anti-inflammatory marker adiponectin are decreased in obese individuals (Ellulu et al., 2017). The imbalance between the inflammatory and anti-inflammatory markers can lead to insulin resistance (hyposensitivity), a prerequisite for type II diabetes and a major contributor to other pathologies including hypertension, hyperlipidemia, and atherosclerosis (Kahn and Flier, 2000). However, not all obese individuals are metabolically unhealthy. There is a transition from "obese-healthy" to "obese-unhealthy" that exists, and limited information exists to date on the underlying mechanisms of the transition.

The factors that drive a state of chronically positive energy balance in humans are mainly behavioral and genetic (Adult Obesity Causes & Consequences | Overweight & Obesity | CDC, 2019). Polygenic obesity is the most common form of obesity, which develops due to an interaction between genetic polymorphism and obesogenic lifestyle. This type of obesity is not caused by one allele, but rather the effect of multiple alleles at specific gene loci that each modestly increase the susceptibility to obesity (Hetherington and Cecil, 2010). There are more than 100 such human loci harboring genetic variants associated with Body Mass Index that have been identified so far (Hinney and Hebebrand, 2008). Through genome wide association studies (GWAS), single nucleotide polymorphisms (SNPs) have been identified as obesity-associated in the Melanocortin-4 receptor gene (*MC4R*) and the fat mass and obesity associated gene (*FTO*) (Hinney and Hebebrand, 2008). GWAS utilizes SNPs and other small-scale genetic variants to examine their contribution, and the contribution of genes presumably impacted by these variants or a nearby variant, to conditions such as bipolar disorder, coronary heart disease, Crohn's disease, hypertension, arthritis, obesity, and diabetes (The Wellcome Trust Case Control Consortium, 2007).

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The central metabolic pathways and several aspects of energy homeostasis are largely conserved across animal life. Additionally, it is estimated that 75% of known disease-causing genes in humans are evolutionarily conserved in *Drosophila* (Bier, 2005). The major organs in the fruit fly involved in regulating metabolism and energy homeostasis that are analogous in mammals include the fat body (functions as white adipose tissue and liver), malphigian tubules (functions as kidneys), and oenocytes (equivalent to hepatocyte-like cells). Also included is the gastrointestinal tract, which entails the crop, midgut and hindgut (similar to the mammalian stomach, small and large intestine respectively). Insulin-like peptide producing cells (IPCs) in the median neurosecretory cells (mNSC) are equivalent to the pancreatic β cells. Corpora cardiaca (CC) cells are reminiscent of pancreatic α cells and are located in the ring gland, where they secrete adipokinetic hormone (AKH, analog of human glucagon) (Apidianakis and Rahme, 2011; Bharucha, 2009). Like mammals, excess chemical energy is stored as glycogen and lipids in the *Drosophila* fat body, among other organs (Yamada et al., 2018). The adult fat body in flies and mammals also serves as an endocrine and immune organ. As an immune organ, the adult fat body is responsible for the production of antibacterial and antifungal peptides following septic injury (Vass and Nappi, 2001). As stated previously, a pathogenic interplay between immunity and energy homeostasis in mammals is partly mediated by cytokines (such as IL-6 and TNFα). This has shown parallels in recent *Drosophila* studies in which JNK (Jun N-terminal kinase)-mediated secretion of the JAK/STAT activating cytokines Unpaired 1–3 (Upd 1–3) from damaged tissues and active immune cells is a critical step in the innate immune response with implications in metabolism (Karpac et al., 2011). Upd3 production in macrophages is enhanced in response to a chronic excess lipid diet resulting in insulin resistance, decreased glucose metabolism and reduced lifespan (Woodcock et al., 2015).

In addition, there are more than 100,000 neurons in flies that form neuronal circuits to regulate various behaviors, including feeding, circadian rhythms, sleep, learning and memory (Pandey and Nichols, 2011). The human brain regulates feeding behavior and satiety in discrete neural centers, which can each be related to similar neuronal feeding circuits in the *Drosophila* brain that regulate energy homeostasis through chemosensory systems, including olfactory and gustatory circuits (Bader et al., 2007).

High sugar diet (HSD)-induced obesity in fruit flies has been achieved by increasing glucose, fructose or sucrose content in the standard solid fly medium relative to yeast content. It has been reported that HSD obesity in *Drosophila* approximates human obesity and some of the associated diseases such as diabetes and cardiovascular disease. Previous studies have shown that larvae and adult flies exposed to a HSD develop increased triglyceride content, dyslipidemia, insulin resistance, decreased fertility and cardiovascular disease (Musselman et al., 2018). There have been varying effects of HSD on lifespan due to the lack of a "standardized" formula in preparing the medium. In some studies, HSD has been reported to reduce lifespan, for instance adult fruit flies on a diet of 2%–20% sucrose had their mean life spans shortened by 13%–27% in comparison with those that consumed food containing fructose or glucose in the same concentration range (Lushchak et al., 2014; Na and Cagan, 2013). However, reverse effects of HSD on lifespan have also been reported. For instance, there has also been an extension of 31% in adult lifespan reared on a HSD prepared by adding *D*-glucose to the standard fly medium (Galenza et al., 2016). The extension of lifespan in some of these HSD flies implies that adiposity itself is not the only contributing factor to reduced lifespan in obesity (Gáliková and Klepsatel, 2018). Besides the impact on metabolism and lifespan, excess dietary sugar increases ER (endoplasmic reticulum) stress, disrupts gut homeostasis and alters the population of commensal bacteria in flies (Musselman et al., 2018; Zhang et al., 2014). Furthermore, transcriptomic profiling of head tissue of flies exposed to HSD (sucrose w/v 20%) displayed enrichment of genes coding for CHK (checkpoint kinases), a group of kinases involved in cell cycle control in response to apoptosis or DNA damage (Hemphill et al., 2018).

Mapping genotypic variation to phenotypic variation in model organisms can identify quantitative trait loci for complex traits that can help understand underlying mechanisms of the same traits in humans (Flint and Mackay, 2009). Human GWAS are limited by how many SNPs and subjects can be tested, an inability to control the heterogenous environment of test subjects, and long lifespans of test subjects when examining mortality as an indicator of pathogenesis. Large human linkage disequilibrium (LD) blocks also mean that a single trait-associated variant may indicate multiple susceptible trait gene loci (only one of which is truly involved in the trait) (Moore et al., 2010; Trinh and Boulianne, 2013; Wangler et al., 2017).

The Drosophila Genetic Reference Panel (DGRP) is a population of 205 distinct and naturally polymorphic genetic lines (Ivanov et al., 2015). The DGRP is suited for GWAS because: (i) all DGRP lines have been sequenced and a total of 4,565,215 naturally occurring variants have been identified, (ii) average LD in the DGRP drops rapidly with physical distance between SNPs relative to mammals, (iii) they are rendered to homozygosity through inbreedingand (iv) the lines can be examined in controlled environments (Mackay and Huang, 2018; Mackay et al., 2012). Phenotypic traits such as lifespan, triglyceride storage, climbing behavior, starvation resistance, irradiation resistance, behavioral aggression, alcohol tolerance and response to infections have been subjected to GWAS in the DGRP (Bou Sleiman et al., 2015; Garlapow et al., 2015; Morozova et al., 2015; Shorter et al., 2015; Unckless et al., 2015; Vaisnav et al., 2014). Additionally, available genetic tools in *Drosophila*, including inducible RNA interference (RNAi) via the GAL4-UAS binary expression system, coupled with the short life span of this invertebrate, offer high-throughput functional screening of candidate genes underlying obesity-associated mortality and pathogenesis (Baranski et al., 2018).

In the present study we utilize the experimental genetic strengths and analogous physiological systems in *Drosophila melanogaster* to investigate genetic components influencing HSD-associated mortality. First, we performed a GWAS on lifespan of mated females exposed to HSD utilizing 193 DGRP lines. The HSD state was induced by providing sucrose in a disproportionate amount to yeast in their solid diet, providing sucrose as a nutrient source compared to protein in a ratio of 5:1 w/v. This concentration of sucrose has been previously shown to induce indicators of an obesity-like state in flies (Hemphill et al., 2018; Skorupa et al., 2008). Next, based on the physiological relevance to obesity pathology, and the availability of transgenic RNAi lines of the genes identified from the GWAS, whole-body *in vivo* knockdown of several candidate genes was performed utilizing the GAL4-UAS binary expression system. This was done in order to independently validate the functional impacts of these loci on *Drosophila* lifespan during HSD, while also assaying a number of energy homeostasis traits, including feeding quantity and triglyceride storage.

The DGRP analysis identified several lifespan loci in a HSD setting, and these loci were largely confirmed to affect lifespan in that HSD setting upon independent validation. However, it is important to note that we also detected unexpected dietary effects of the HSD, including inconsistent diet effects on lifespan relative to a normal diet and a strong downregulation of feeding quantity. The loci we tested via RNAi were not limited in impact on lifespan to the HSD, with the majority also impacting lifespan on a normal diet, except for one candidate-*axo*. Also interestingly, while the loci we identify do appear to modulate lifespan on a HSD, there was also a coincident modulation of feeding quantity for the majority of the tested loci.

2. Results

2.1. There is variation in lifespan among the DGRP lines exposed to HSD

Of the 200 DGRP lines, only 193 lines were assayed due to a combination of stock availability through the BDSC and reproductive viability. There was variation of lifespan among the 193 lines on HSD, with DGRP 913 surviving the longest for an average of 74.3 days, and

DGRP 832 the shortest for an average of 10.2 days, after adjustment for block effect (Figure 1). The overall block adjusted mean lifespan of the 193 lines on HSD was 31.1 days. Block-adjusted mean lifespans (Table S1) were entered into the DGRP analysis pipeline (Mackay et al., 2012).

2.2. The known DGRP covariates have no significant effect on HSD lifespan in mated females

Prior to GWAS analysis, the traits of analytical interest are adjusted for cryptic genetic relatedness, chromosomal inversion effects and *Wolbachia* infection status, as unadjusted data shows the potential for systematic inflation or deflation of test statistics (Huang et al., 2014). Four inversions: *In(2L)t*, *In(2R)NS*, *In(3R)P* and *In(3L)P*, termed "common cosmopolitan," have been identified in almost all *Drosophila* populations worldwide. *In(3R)Mo* and *In(3R)K* have been identified as "rare cosmopolitan" inversions because they occur throughout the *Drosophila* species range, but usually at frequencies below 5%, and may be absent in many populations (Corbett-Detig et al., 2012; Krimbas and Powell, 1992). In addition, approximately 53% of the DGRP lines carry the endosymbiotic bacterium, *Wolbachia pipientis*, that can have impact on reproduction and fitness in the host (Mackay et al., 2012). In our case, the major chromosomal inversions *In(2L)t, In(2R)NS, In(3R)P, In(3R)K, and In(3R)Mo* and *Wolbachia* infection status had no significant effects on block-adjusted HSD lifespan in mated females ($P > 0.05$; Table 1).

2.3. GWAS analysis of HSD lifespan in mated females

Upon entering the block-adjusted data into the DGRP analysis pipeline, GWAS of HSD mean lifespan was performed utilizing >2,000,000 polymorphisms with MAF >5%. A quantile-quantile (QQ plot) was generated by the pipeline to display the quantile distribution of observed marker-lifespan association P-values versus the distribution of expected P-values by chance (Figure 2). The QQ plot revealed that beginning with P-values of significance level 10^{-2} (Figure 2) polymorphisms become more associated with HSD lifespan than expected under a null hypothesis, which would be represented by a uniform distribution of P-values. Furthermore, the lifespan-associated polymorphisms adhered to the null distribution pattern at higher P-values.

2.4. Polymorphisms most highly associated with lifespan on a HSD were located near physiologically relevant genes

We used the DGRP-suggested significance threshold of P-value of 10^{-5} to increase the likelihood of identifying true positive associations and reduce the likelihood of multiple testing false positives (Mackay et al., 2012). We identified 50 polymorphisms with P-values ranging between 10^{-8} to 10^{-5} and MAF >5% as associated with HSD lifespan in mated females (Table S2). The HSD lifespan-associated polymorphisms included single nucleotide polymorphisms (SNPs), insertions, deletions and multiple nucleotide polymorphisms (MNPs) that were near or within genes physiologically relevant to obesity (Table 2). The associated polymorphisms tagged genes involved in cell signaling, neural processes, behavior, sensation, development, and regulation of apoptosis (Table 3). The magnitude of the minor allele effect on the direction of HSD lifespan (decreasing or increasing, respectively) is also shown in Table 2. The magnitude of the minor allele effect on the direction of HSD lifespan was generally a decreasing effect of 3–9 days (Table 2). There was only one tagged SNP, 3R_2389634_ SNP, that favored an increase of HSD lifespan for an average of 4.295 days (Table 2).

Upon an initial literature search and retrieval of the gene records from Flybase, the highlighted genes in Table 3 had functions that we determined could most feasibly be involved in the pathology of obesity, and also had readily available UAS-RNAi lines at the BDSC, and therefore were selected to verify their functional impact on lifespan and potentially energy homeostasis.

2.5. Functional validation of GWAS candidate genes

One major advantage of *Drosophila* as a model system is that transgenic fly lines carrying UAS-RNAi constructs have been generated and are readily available to allow targeted knockdown of specific genes in a specific tissue. For our case, we performed *in vivo* whole-body knockdown of *rdgA, olf413, axo, caps* and *mub* (as highlighted in Table 3) utilizing a GAL4 driver that ubiquitously expresses GAL4 under the control of the alphaTub84B promoter (Liu and Lehmann, 2008). To serve as experimental controls, UAS-Controls that are genetically identical to the UAS-RNAi lines except lacking the RNAi construct within the attP sites (site-specific recombination sites) were utilized.

2.6. Validation of rdgA

rdgA was a highly interesting candidate gene from the HSD lifespan GWAS screen (Table 2) due to its previously published ability to extend lifespan upon RNAi knockdown in the developing eye (Lin et al., 2014). *rdgA* had the highest number of HSD lifespan-associated polymorphisms from the GWAS (Table 2) near its gene sequence.

in vivo whole-body RNAi knockdown of *rdgA* reduced lifespan, particularly on a HSD (ANOVA: $P = 0.0005$, Figure 3A). Unexpectedly, HSD increased lifespan relative to NM for all genotypes (ANOVA: $P = .0207$, Figure 3A). It is important to note here that this is most likely due to the lack of standardized HSD across studies, but it is consistent with

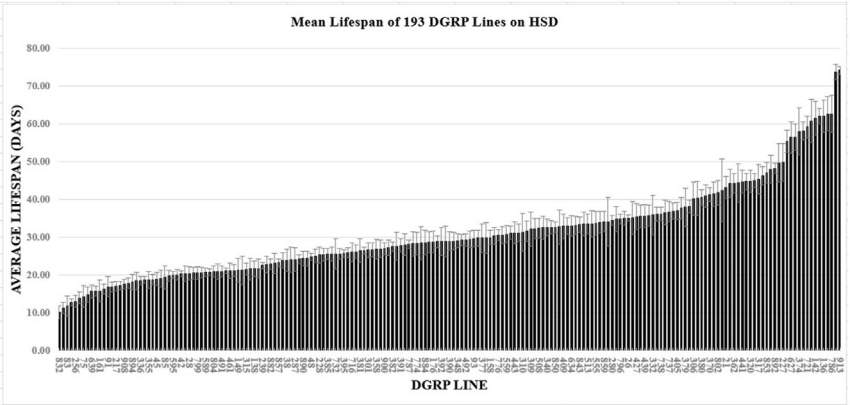

Figure 1. The distribution of mated female mean lifespan for 193 DGRP lines on HSD. The 193 assayed DGRP lines are indicated on the x-axis and correspond to Bloomington Drosophila Stock Center designations for these lines, also corresponding to "Raleigh" (RAL) designations. The error bars represent standard error of the mean (SEM). Each DGRP genotype data point consists of 20 mated females exposed to HSD.

Table 1. Type III ANOVA table showing the effects of five major chromosomal inversions and *Wolbachia (wolba)* infection status on HSD lifespan.

Covariate	Df	Sum of Sq	RSS	AIC	F value	Pr (>F)
factor (*wolba*)	1	479.68	27370	978.22	3.23	0.074
factor (In_2L_t)	2	210.05	27100	974.31	0.707	0.495
factor (In_2R_NS)	2	359.17	27250	975.37	1.21	0.301
factor (In_3R_P)	2	73.20	26964	973.33	0.246	0.782
factor (In_3R_K)	2	365.24	27256	975.41	1.23	0.295
factor (In_3R_Mo)	2	55.52	26946	973.21	0.187	0.830

Legend: Df: degrees of freedom; RSS: residual sum of squares; AIC: Akaike information criterion; F: F statistic and Pr (>F): p-value associated with the F statistic.

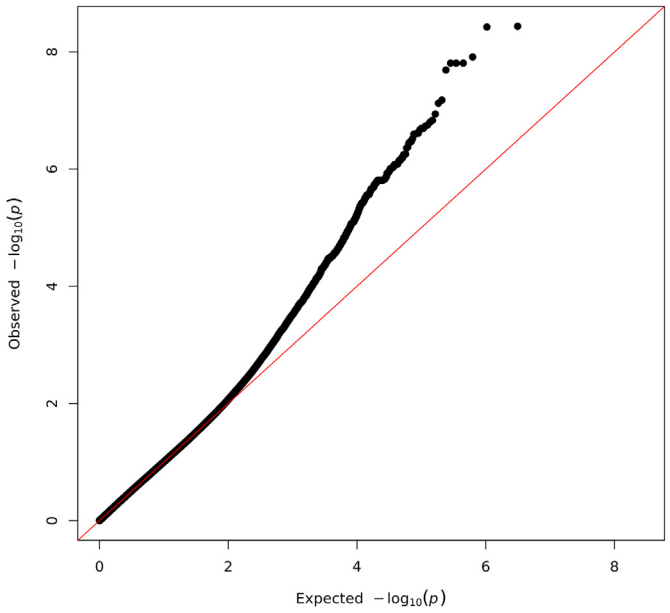

Figure 2. QQ plot of the distribution of the observed variant associations with HSD lifespan against an expected distribution under a null hypothesis. Association is represented by -$\log_{10}$ P-value outcomes of linear mixed models. Beginning with P-values of significance level 10^{-2}, variant association with HSD lifespan (represented by the black dots) becomes more associated with HSD lifespan than expected under a null hypothesis. The straight diagonal line is the distribution of P-values expected if the observed values equal the expected values under a uniform distribution.

some previous findings, and we find here that a low protein to carbohydrate ratio extends lifespan in mated females (Bruce et al., 2013; Lee et al., 2008). This, however, is in direct contrast to results describing a HSD model of obesity as consistently reducing adult fly lifespan (Lushchak et al., 2014; Na and Cagan, 2013). Overall, reducing the expression of *rdgA* reduces lifespan in a HSD setting, in agreement with the effect of tagging variants in the GWAS.

We measured triglyceride content to assess the impact of whole-body knockdown on fat storage in the flies after 7 days on NM and HSD. Both *rdgA* whole-body RNAi knockdown flies and control flies exhibited an increase in their triglyceride content on a HSD compared to the flies fed on NM (ANOVA: P = 0.0040, Figure 3B). *rdgA* whole-body knockdown flies on a HSD for 7 days had the highest amount of triglyceride content, and it is the increase in fat storage for the knockdown flies with HSD that drives the effect of diet we detected. There were no significant differences in triglyceride storage between genotypes within either diet (ANOVA: P = 0.7886; Figure 3B). The genotype*diet interaction was significant (ANOVA: P = 0.0275) and was driven by the significant increase in triglyceride content in *rdgA* whole-body knockdown flies on a HSD in relative to the *rdgA* whole-body knockdown flies on NM (Figure 3B). Overall, although there was a trend toward increased fat content in the HSD, there was no significant impact on triglyceride content in mated female flies that experienced whole-body knockdown of *rdgA*.

As metabolism and behavioral responses are physiologically coordinated, we quantified food intake by exposing mated female flies to 1% Brilliant blue fly media after 7 days of NM and HSD (described in the Methods section). HSD reduced feeding quantity in both the whole-body *rdgA* knockdown and the controls compared to those flies fed on NM (ANOVA: P = 0.0002, Figure 3C). In general, HSD reduced feeding quantity in both genotypes, but the *rdgA* knockdown flies did not display a significant reduction compared to the control flies within either diet (ANOVA: P = 0.1094, Figure 3C).

To assess the mobilization of energy stores during a starved state, we transferred flies to 1% agar after 7 days of exposure to NM and HSD. HSD increased starvation resistance in both the *rdgA* knockdown flies and the controls (ANOVA: P < 0.0001, Figure 3C). *rdgA* whole-body knockdown flies survived a noticeably shorter time than the control flies when subjected to starvation on 1% agar after being fed on a NM, but not HSD (ANOVA: P < 0.0001, Figure 3D).

2.7. Validation of olf413

olf413 was tagged by one HSD lifespan-associated SNP in DGRP mated females (3L_22167626_ SNP).

olf413 whole-body knockdown flies showed a reduction of lifespan compared to the controls on both diets (ANOVA:P = 0.0005, Figure 4A). *olf413* knockdown flies survived for an average of 28.07 days relative to the control flies that survived for an average of 39.45 days on HSD. Tukey's post hoc analysis (P = 0.0138) also showed that the above genotypic difference within the HSD was significant (Figure 4A). No significant difference was observed in lifespan when comparing NM to HSD (ANOVA: P = 0.9503, Figure 4A). In general, reducing the expression of *olf413* reduced lifespan, with an especially significant effect shown on a HSD.

olf413 knockdown flies had a reduced level of triglyceride content compared to the controls after 7 days of exposure on both the NM and the HSD (ANOVA: P < 0.0001, Figure 4B). Flies fed the HSD had an increased level of triglyceride storage relative to the flies fed the NM (ANOVA: P = 0.0019, Figure 4B). The decrease in the amount of triglyceride content in the *olf413* knockdown flies on both diets compared to the respective controls was also revealed to be significant when followed up with Tukey's post hoc analysis (Figure 4B).

Measuring feeding quantity in the *olf413* knockdown and control flies after 7 days on HSD and NM displayed a reduction of feeding quantity in the flies fed the HSD (ANOVA: P = 0.0001, Figure 4C). *olf413* knockdown flies had a decreased feeding quantity on both diets compared to the controls (ANOVA: P = 0.0018, Figure 4C). Thus, reducing the expression of *olf413* decreased feeding quantity in mated female flies, including on a HSD.

After 7 days of exposure to NM and HSD, both genotypes were subjected to starvation by transferring the flies to vials consisting of 1% agar.

Table 2. Commonly occurring polymorphisms (minor allele frequency >0.05) most associated with lifespan of the DGRP lines on a HSD and their location/mode of impact relative to physiologically relevant genes.

Position and Type of Variant	Minor Allele/ Major Allele	MAF in DGRP	Gene Symbol of Nearest Gene	Location Relative to Gene	P-Value of Association Test	Effect size (days)	Lifespan Direction
3R_2759723_ SNP	A/T	0.279	*Antp*	Intron	6.38E-06	-4.505	Decrease
3R_2724626_ SNP	T/A	0.276	*Antp*	Intron	1.07E-05	-4.299	Decrease
3L_4685943_ DEL	ACATTGAG/AACATTGAG	0.090	*axo*	Intron	2.27E-06	-6.898	Decrease
3L_4685614_ SNP	A/G	0.098	*axo*	Intron	2.32E-06	-7.129	Decrease
3L_4685447_ SNP	G/T	0.086	*axo*	Intron	6.48E-06	-6.779	Decrease
3L_4685432_ SNP	A/C	0.086	*axo*	Intron	7.89E-06	-6.721	Decrease
3L_13245644_ SNP	T/C	0.227	*caps*	Intron	4.41E-07	-5.063	Decrease
3L_17691275_ SNP	T/G	0.366	*Ccn*	Intron	1.93E-06	-4.425	Decrease
3R_2389634_ SNP	G/A	0.379	*dpr11*	Intron	2.33E-06	4.295	Increase
2R_17762938_ SNP	T/C	0.427	*Fili*	Intron	1.04E-05	-3.923	Decrease
3L_21897475_ SNP	C/T	0.082	*mub*	Intron	1.64E-08	-8.622	Decrease
3L_21868544_ SNP	A/C	0.103	*mub*	Intron	9.04E-06	-6.537	Decrease
3L_22167626_ SNP	A/T	0.341	*olf413*	Intron	9.29E-06	-4.127	Decrease
X_8874811_ SNP	G/A	0.068	*rdgA*	Intron	8.91E-07	-8.407	Decrease
X_8874815_ SNP	A/T	0.073	*rdgA*	Intron	1.32E-06	-7.98	Decrease
X_8874819_ SNP	C/A	0.074	*rdgA*	Intron	1.32E-06	-7.983	Decrease
X_8874813_ SNP	G/A	0.073	*rdgA*	Intron	1.34E-06	-7.978	Decrease
X_8874756_ SNP	A/C	0.058	*rdgA*	Intron	2.22E-06	-8.842	Decrease
X_8874693_ SNP	G/A	0.078	*rdgA*	Intron	3.44E-06	-7.419	Decrease
X_8874367_ SNP	A/T	0.103	*rdgA*	Intron	3.70E-06	-7.014	Decrease
X_8874799_ SNP	G/A	0.069	*rdgA*	Intron	4.02E-06	-7.921	Decrease
3R_2674803_ INS	TTCTC/T	0.331	*Scr*	Intron	1.34E-06	-4.525	Decrease
3R_2674814_ MNP	AAA/TTT	0.356	*Scr*	Intron	8.39E-06	-4.069	Decrease
3R_2664893_ SNP	G/T	0.353	*Scr*	Intron	7.03E-06	-4.417	Decrease
3R_2676435_ SNP	T/A	0.397	*Scr*	Upstream	1.32E-05	-3.889	Decrease
3R_2676301_ SNP	A/G	0.456	*Scr*	Upstream	1.37E-05	-3.869	Decrease
3R_2676473_ SNP	T/C	0.380	*Scr*	Upstream	5.56E-06	-3.922	Decrease

Note: Variant description format: chromosome arm_ physical coordinate in the genome_polymorphism type. The magnitude of the minor allele effect on the direction of HSD lifespan are shown in the last two columns.
Legend: SNP: single nucleotide polymorphism; DEL: deletion; INS: insertion; MNP: multi-nucleotide polymorphism and MAF: minor allele frequency.

Table 3. Summarized function of the genes identified from the GWAS analysis of HSD lifespan in *Drosophila melanogaster*.

Gene name	Symbol	Function
Antennapedia	*Antp*	DNA binding, heart development; anterior/posterior axis specification; specification of segmental identity, antennal segment; muscle cell fate specification; lymph gland development; neuroblast development; thorax; ventral cord development; midgut development.
Axotactin	***axo***	**Signaling-Transmission of nerve impulse, determines the membrane electrical properties of axons.**
Capricious	***caps***	**Cellular organization/biogenesis, development, stimulus response and signaling. Synapse formation and axon guidance.**
Ccn	*Ccn*	Cell adhesion, cell signaling, negative regulation of cell death and signal transduction.
Defective proboscis extension response 11	*dpr11*	Photoreceptor cell axon guidance; regulation of neuromuscular junction development; detection of temperature stimulus involved in sensory perception of pain; synapse organization; establishment of synaptic specificity at neuromuscular junction; sensory perception of chemical stimulus
Fish-lips	*Fili*	Regulation of apoptotic process; axonogenesis; adult chitin-containing cuticle pigmentation
Mushroom body	***mub***	**Regulation of alternative mRNA splicing, via spliceosome; sleep; thermosensory behavior.**
olf413	***olf413***	**Copper ion binding; dopamine beta-monooxygenase activity. It is involved in the biological process described with: oxidation-reduction process; norepinephrine biosynthetic process; octopamine biosynthetic process; dopamine catabolic process.**
Retinal degeneration A	***rdgA***	**Contributes to phospholipase C based signaling reactions. Stimulus response and signaling. (Also known as Diacylglycerol Kinase)**
Sex combs reduced	*Scr*	DNA binding, development and reproduction

Note: All functional annotations of the genes are based on Flybase 2.0. The bolded genes were ultimately knocked down utilizing the GAL4-UAS system in this study to verify effects on HSD lifespan and various energy homeostasis-related phenotypes.

Flies that were fed a HSD were more resistant to starvation and survived longer than the flies that were on a normal diet (ANOVA: $P < 0.0001$, Figure 4D). *olf413* knockdown flies were less resistant to starvation compared to the controls on both diets (ANOVA: $P < 0.0001$, Figure 4D). Tukey's post hoc analysis affirmed that the differences in starvation resistance that existed within diets between the two genotypes was significant ($P < 0.0001$, Figure 4D).

2.8. Validation of axo

axo was tagged by 4 polymorphisms (Table 2) in the HSD lifespan GWAS in mated females of the DGRP.

axo whole-body knockdown flies survived for an average of 18.23 days compared to the controls that lived for ~40.91 days on the HSD. Tukey's post hoc analysis revealed that this difference was significant

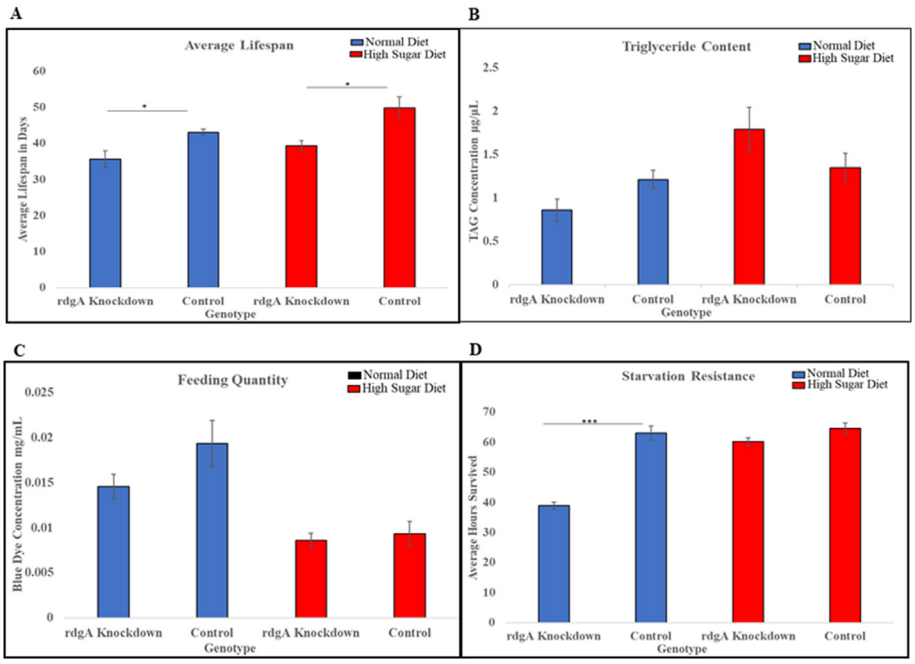

Figure 3. Impacts of *in vivo* whole-body knockdown of *rdgA* on lifespan and energy homeostasis on NM and HSD in mated female flies. (A) Effect of whole-body RNAi knockdown of *rdgA* on lifespan of mated female flies on NM and HSD. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,401.41} = 18.95$, $P = 0.0005$; Diet: $F_{1,139.39} = 6.58$, $P = 0.0207$ and Genotype*Diet: $F_{1,11.70} = 0.55$, $P = 0.4680$. Further analysis with student's post hoc t-test revealed that the difference in lifespan between the *rdgA* knockdown flies and controls on NM was significant, $P = 0.0213$. Error bars represent standard error of mean (SEM). N = 5, 20 flies per replicate. (B) Effect of whole-body knockdown of *rdgA* on triglyceride content of mated female flies on NM and HSD. HSD significantly increased triglyceride storage levels relative to the NM. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,0.02} = 0.02$, $P = 0.7886$; Diet: $F_{1,2.82} = 9.44$, $P = 0.0040$ and Genotype*Diet: $F_{1,1.58} = 5.28$, $P = 0.0275$. Error bars represent standard error of mean (SEM). N = 15 flies per condition. (C) Impact of whole-body knockdown of *rdgA* on feeding quantity of mated female flies on NM and HSD. HSD fed flies exhibited a significant reduction in feeding quantity compared to the NM fed flies. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,0.000038} = 2.87$, $P = 0.1094$; Diet: $F_{1,0.00031} = 23.83$, $P = 0.0002$ and Genotype*Diet: $F_{1,0.000021} = 1.55$, $P = 0.2308$. Error bars represent standard error of mean (SEM). N = 5, 5 flies per replicate, 25 flies per condition. (D) Impact of whole-body knockdown of *rdgA* on starvation resistance of mated female flies on NM and HSD. HSD increased starvation resistance significantly relative to the normal diet. *rdgA* knockdown flies exposed to NM displayed a decrease in starvation resistance compared to the control flies on the same diet. There was no significant genotypic difference in starvation resistance that existed between the flies subjected to our HSD. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,663.55} = 47.62$, $P < 0.0001$; Diet: $F_{1,1019.59} = 73.17$, $P < 0.0001$ and Genotype*Diet: $F_{1,484.13} = 34.74$, $P < 0.0001$. Error bars represent standard error of mean (SEM). N = 5, 100 flies per condition. Comparison bars drawn only to illustrate within diet differences. *P < 0.05, **P < 0.01 and ***P < 0.001. *rdgA* Knockdown mated female flies: alphaTub84B-GAL4/UAS-*rdgA*; Control mated female flies: alphaTub84B-GAL4/UAS-control.

(ANOVA: $P < 0.0001$, Figure 5A), while there was no such difference between genotypes on a NM (Figure 5A). There was no consistent difference in lifespan between flies fed on NM and HSD ($P = 0.8471$, Figure 5A). Overall, the reduction of the expression of this neuronal gene led to decrease in lifespan on a HSD.

axo whole-body knockdown flies showed no change in triglyceride storage levels compared to the control flies on either diet (ANOVA:$P = 0.9522$, Figure 5B). HSD significantly increased triglyceride content of both genotypes compared to NM (ANOVA:$P < 0.0001$, Figure 5B).

Feeding quantity in the *axo* knockdown flies was reduced compared to the control flies after 7 days on either media (ANOVA: $P < 0.0001$, Figure 5C). HSD fed flies had a significant reduction in feeding quantity relative to the NM fed flies (ANOVA: $P = 0.0002$, Figure 5C). *axo* knockdown flies fed a HSD showed a significant reduction (0.003 mg/mL blue dye concentration) in feeding quantity compared to controls exposed to HSD (0.007 mg/mL blue dye concentration) upon follow up with Tukey's post hoc analysis ($P = 0.0018$, Figure 5C). Therefore, whole-body knockdown of *axo* reduces feeding quantity, most apparently on our HSD.

Measuring starvation resistance in the *axo* knockdown and control flies after 7 days on their respective diets showed no significant difference existed between the two genotypes (ANOVA: $P = 0.4227$, Figure 5D). However, flies exposed to the HSD survived significantly longer than the flies fed a NM upon starvation (ANOVA: $P = 0.0006$, Figure 5D).

2.9. Validation of *caps*

caps was tagged by one SNP (3L_13245644_ SNP).

Whole-body knockdown of *caps* reduced lifespan on both the normal and high sugar diet, (ANOVA: $P < 0.0001$). *caps* knockdown flies exposed to HSD survived for an average of 19.36 days compared to the controls

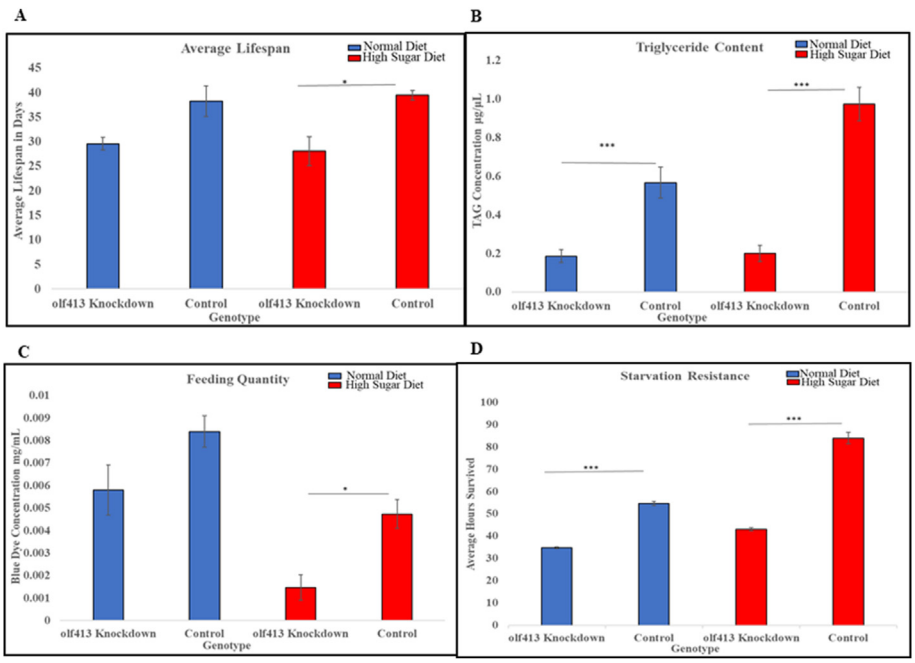

Figure 4. Impacts of *in vivo* whole-body knockdown of *olf413* on lifespan and energy homeostasis on NM and HSD in mated female flies. (A) Effect of whole-body knockdown of *olf413* on lifespan of mated female flies on NM and HSD. *olf413* whole-body knockdown flies displayed a reduction of lifespan on both diets. Tukey's post hoc analysis displayed that the reduction of lifespan in the *olf413* knockdown flies on a HSD was significant compared to the controls on HSD. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,499.50} = 19.02$, $P = 0.0005$; Diet: $F_{1,0.11} = 0.0040$, $P = 0.9503$ and Genotype*Diet: $F_{1,9.59} = 0.37$, $P = 0.5541$. Further analysis with student's post hoc t-test revealed that the difference in lifespan between the *olf413* knockdown flies and controls on NM was also significant, $P = 0.0172$. Error bars represent standard error of mean (SEM). N = 5, 20 flies per replicate. Comparison bars drawn only to illustrate within diet differences. (B) Effect of whole-body knockdown of *olf413* on triglyceride content of mated female flies on NM and HSD. *olf413* whole-body knockdown flies had a lower triglyceride storage relative to the control flies. HSD significantly increased triglyceride content compared to the NM. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,4.99} = 79.46$, $P < 0.0001$; Diet: $F_{1,0.66} = 10.58$, $P = 0.0019$ and Genotype*Diet: $F_{1,0.57} = 9.14$, $P = 0.0038$. Error bars represent standard error of mean (SEM). N = 15 flies per condition. (C) Impact of whole-body knockdown of *olf413* on feeding quantity of mated female flies on NM and HSD. *olf413* knockdown flies displayed a reduction in feeding quantity compared to the control flies with the difference only reaching significance on HSD. HSD decreased feeding quantity relative to normal diet. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,0.000043} = 14.02$. $P = 0.0018$; Diet: $F_{1,0.00008} = 26.06$, $P = 0.0001$ and Genotype*Diet: $F_{1,0.00} = 0.18$, $P = 0.6762$. Error bars represent standard error of mean (SEM). N = 5, 5 flies per replicate, 25 flies per condition. (D) Impact of whole-body knockdown of *olf413* on starvation resistance of mated female flies on NM and HSD. *olf413* knockdown flies survived a shorter duration than control flies when starved. Flies on a HSD displayed a significant increase in starvation resistance compared to the flies on a NM. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,4599.54} = 438.87$, $P < 0.0001$; Diet: $F_{1,1757.81} = 167.72$, $P < 0.0001$ and Genotype*Diet: $F_{1,548.10} = 52.30$, $P < 0.0001$. Error bars represent standard error of mean (SEM). N = 5, 100 flies per condition. Comparison bars drawn only to illustrate within diet differences. *P < 0.05, **P < 0.01 and ***P < 0.001. *olf413* Knockdown mated female flies: alphaTub84B-GAL4/UAS-*olf413*; Control mated female flies: alphaTub84B-GAL4/UAS-control.

that survived for 39.45 days (Figure 6). Tukey's post hoc analysis further confirmed that this difference was significant ($P < 0.0001$).

Further evaluation of the impacts of *in vivo* knockdown of *caps* on energy homeostasis was not performed due to developmental delay caused by the whole-body knockdown of the gene, yielding too few flies for analysis at this time.

2.10. Validation of *mub*

mub is involved in the regulation of alternative mRNA splicing via spliceosome. The gene is involved in thermosensory behavior, learning, and memory (Thurmond et al., 2019). Whole-body knockdown of *mub* utilizing the alphaTub84B-GAL4 driver was lethal. Thus, we were not

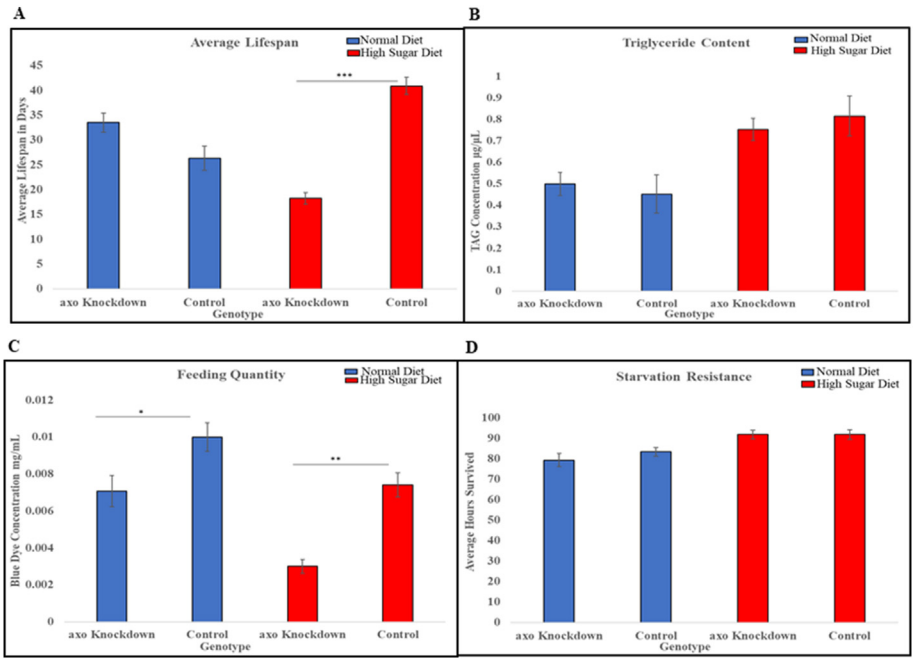

Figure 5. Impacts of *in vivo* whole-body knockdown of *axo* on lifespan and energy homeostasis on NM and HSD in mated female flies. (A) Effect of whole-body knockdown of *axo* on lifespan of mated female flies on NM and HSD. *axo* whole-body knockdown flies experienced a reduction in lifespan compared to the controls on HSD (Tukey's post hoc analysis, $P < 0.0001$). There was no consistent difference in lifespan when comparing NM to HSD. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,300.70,} = 17.33$, $P = 0.0007$; Diet: $F_{1,0.67} = 0.04$, $P = 0.8471$ and Genotype*Diet: $F_{1,1113.78} = 64.17$, $P < 0.0001$. Error bars represent standard error of mean (SEM). N = 5, 20 flies per replicate. (B) Effect of whole-body knockdown of *axo* on triglyceride content of mated female flies on NM and HSD. Flies fed a HSD experienced a significant increase in the level of triglyceride content compared to the flies exposed to the NM. Overall, genotype showed no significant impact on triglyceride content. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,0.0003} = 0.0036$, $P = 0.9522$; Diet: $F_{1,1.51} = 17.94$, $P < 0.0001$ and Genotype*Diet: $F_{1,0.11} = 1.29$, $P = 0.2613$. Error bars represent standard error of mean (SEM). N = 15 flies per condition. (C) Impact of whole-body knockdown of *axo* on feeding quantity of mated female flies on NM and HSD. *axo* knockdown flies exhibited a decrease in feeding quantity compared to the controls on both NM and HSD. Feeding quantity was reduced on a high sugar diet relative to the normal diet. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,0.000067} = 28.33$, $P < 0.0001$; Diet: $F_{1,0.000056} = 23.42$, $P = 0.0002$ and Genotype*Diet: $F_{1,0.000003} = 1.13$, $P = 0.3028$. Error bars represent standard error of mean (SEM). N = 5, 5 flies per replicate, 25 flies per condition. (D) Impact of whole-body knockdown of *axo* on starvation resistance of mated female flies on NM and HSD. *axo* knockdown flies showed no difference in starvation resistance compared to the control flies. HSD fed flies exhibited an increased starvation resistance relative to flies fed the normal diet. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,20.81} = 0.68$, $P = 0.4227$; Diet: $F_{1,551.25} = 17.94$, $P = 0.0006$ and Genotype*Diet: $F_{1,20.81} = 0.68$, $P = 0.4227$. Error bars represent standard error of mean (SEM). N = 5, 100 flies per condition. Comparison bars drawn only to illustrate within diet differences. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. *axo* Knockdown mated female flies: alphaTub84B-GAL4/UAS-*axo*; Control mated female flies: alphaTub84B-GAL4/UAS-control.

able to evaluate the impact of knockdown of the gene on lifespan or energy homeostasis profiling assays presently.

2.11. Synopsis of the analysis of the GWAS-candidate genes

The empirical effects on lifespan and energy homeostasis traits of the *in vivo* whole-body knockdowns in the context of HSD are summarized in Table 4. HSD either had no impact or increased lifespan (not uniformly decreasing it) relative to the NM. Mated female flies exposed to HSD generally exhibited an increase in triglyceride content and decreased feeding quantity (Table 4).

There was a general reduction of lifespan in the knockdown mated female flies for most tested DGRP-identified genes. Feeding quantity was shown to decrease in a majority of the knockdown flies on HSD, except for *rdgA* knockdown (Table 4). Triglyceride content and starvation resistance were less consistently affected by knockdown on HSD.

3. Discussion

3.1. Genetic architecture of HSD lifespan

Previous GWAS of virgin female lifespan utilizing 197 DGRP lines on a standard cornmeal agar-molasses medium displayed a considerable variance in lifespan in agreement with this study (Ivanov et al., 2015). Variation in lifespan is a requisite for identifying polymorphisms associated with HSD lifespan in a DGRP GWAS approach (Figure 1). The

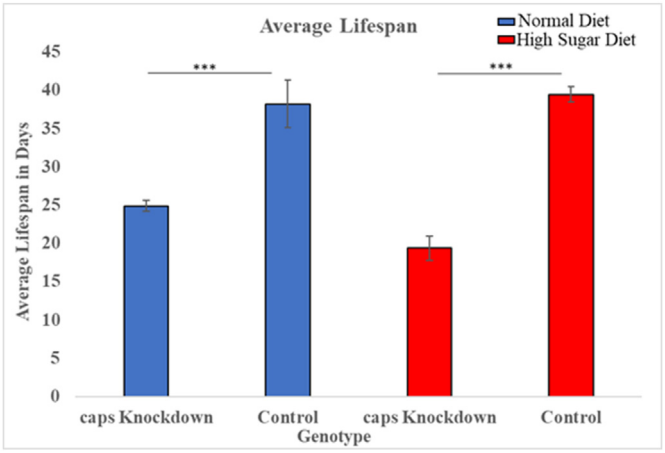

Figure 6. Effect of whole-body knockdown of *caps* on lifespan of mated female flies on NM and HSD. *caps* knockdown flies had a significant decrease in lifespan compared to the control flies on both diets. There was no impact of HSD on lifespan relative to NM. Tukey's post hoc analysis affirmed that the lifespan differences within diets were significant. ANOVA results of the polynomial effect of Genotype, Diet and Genotype*Diet using the Fit Model were as follows, Genotype: $F_{1,1396.12} = 82.24$, $P < 0.0001$; Diet: $F_{1,22.90} = 1.35$, $P = 0.2625$ and Genotype*Diet: $F_{1,57.12} = 3.37$, $P = 0.0853$. Error bars represent standard error of mean (SEM). N = 5, 20 flies per replicate. Comparison bars drawn only to illustrate within diet differences. *P < 0.05, **P < 0.01 and ***P < 0.001. *caps* Knockdown mated female flies: alphaTub84B-GAL4/UAS-*caps*; Control mated female flies: alphaTub84B-GAL4/UAS-control.

departure from the uniform distribution in our QQ plot (Figure 2) implies that the HSD lifespan associated polymorphisms were more associated than expected under the null hypothesis. This is suggestive of a reduced possibility of type I error. QQ plots are also used to depict whether population stratification can inflate the test statistic for non-associated variants if the population structure correlates with the phenotype (He et al., 2014). If population stratification were evident, little or no adherence to the uniform distribution at high P-values would be observed. Our results do show adherence to null expectations at higher P-values.

The polymorphisms associated with HSD lifespan that were near physiologically relevant genes to obesity were mostly intronic except for three SNPs that were near sex combs reduced (*scr*) and were upstream (Table 2). The known segregating variants in the DGRP are mostly intronic (38.2%) or intergenic (25.2%) (Mackay and Huang, 2018). It remains unclear whether intronic variants are of direct functional significance in mammals, but they have been speculated to play a role in regulating alternative splicing (Cooper, 2010). It has also been reported that SNPs are capable of inducing *in vivo* variant structural folds in mRNA (Buratti and Baralle, 2004). Additionally, rSNPs (non-coding regulatory SNPs) can modulate gene expression through multiple mechanisms including transcription factor binding, DNA methylation, and miRNA recruitment (Gu et al., 2017). Some intronic SNPs associated with type 2 diabetes and obesity in mammals play a role in long-range gene regulation, rather than the expression of those genes which actually harbor them (Cooper, 2010). Average LD in the DGRP drops very rapidly with distance between SNPs to an average squared correlation $r^2 < 0.2$ at just 10 base pairs on the autosomes. This rapid deterioration in LD is favorable for identifying causal genes and possibly variants in flies, as *Drosophila* are less likely to exhibit many forms of long-range gene regulation (Huang et al., 2014; Mackay et al., 2012).

A recent study utilizing the DGRP to map the genetic architecture of lifespan in 1–3 day old adult female flies housed with males on a molasses, yeast, and cornmeal diet identified the same genes we identify as associated with HSD lifespan but the variants tagging those genes were different (shown in Table S2). Specifically, this overlap between studies included *axo, CG14082, fz2, olf413*, and *rdgA*. However, the same genes did not show significant overlap when compared with two other published GWA analyses of lifespan in the DGRP, one of which used mated females housed with a male on a standard cornmeal, yeast, and sucrose diet, and another of which used virgin females on a standard cornmeal, agar, and molasses diet (Durham et al., 2014; Huang et al., 2020; Ivanov et al., 2015). The most recent DGRP study concluded that the genetic architecture of lifespan is highly context dependent with substantial genotype-by-sex interaction (GSI) and genotype-by-environment (GEI) for lifespan (Huang et al., 2020). Our results relative to the other DGRP studies of lifespan support this conclusion. Further complicating cross-study comparison is the range of lifespan monitoring conditions utilized in each study, with the study that showed significant overlap with ours utilizing several different temperatures, the presence of males in monitoring vials along with the females, and a smaller number of flies per replicate. Although unclear as to its significance without further investigation, it is also interesting that the study with significant gene overlap used approximately a 3:1 ratio of molasses to yeast (vs. 5:1 ratio of sucrose to yeast presently), with a higher concentration of cornmeal relative to our dietary media for the GWAS (Huang et al., 2020). Due to the availability of the raw lifespan data for the Huang et al. (2020) and Ivanov et al. (2015) studies, the lines showing lifespans in the highest quartile were compared for overlap (includes ~46–50 lines in each quartile). The Huang et al., 2020 GWAS highest quartile of lines showed the most overlap with the lines in the highest quartile in the present study with ~46% of the lines being shared. Meanwhile, the Ivanov et al. (2015) GWAS highest quartile of lines showed a lesser overlap with the lines in the highest quartile of the present study at ~28% of the lines being shared. When we consider the present study, the Huang et al. (2020) study, and the Ivanov et al. (2015) study for overlap for lines in the highest quartile of lifespans, only about 22% of the lines were shared between all 3 studies.

Table 4. A Summary of the effects of *in vivo* whole-body RNAi Knockdowns (relative to controls) on lifespan and energy homeostasis traits on a HSD.

	Lifespan	Triglyceride Content	Feeding Quantity	Starvation Resistance
rdgA Knockdown	Decrease	No Effect	No Effect	No effect
HSD Effect	Increase	Increase	Decrease	Increase
olf413 Knockdown	Decrease	Decrease	Decrease	Decrease
HSD Effect	No effect	Increase	Decrease	Increase
axo Knockdown	Decrease	No effect	Decrease	No effect
HSD Effect	No effect	Increase	Decrease	Increase
caps Knockdown	Decrease	N/A	N/A	N/A
HSD Effect	No effect	N/A	N/A	N/A
mub Knockdown	N/A	N/A	N/A	N/A

Note: The HSD effect (relative to NM) on lifespan and energy homeostasis has also been shown to draw comparisons of genotype and diet effect. The direction of the summarized effects are based on the ANOVA results of the polynomial effect of Genotype and Diet.

Based on the number of polymorphisms associated with the genes, potential relevance in the pathology of obesity, and availability of RNAi lines, we selected *rdgA, olf413, axo, caps* and *mub* for testing our hypothesis that the genes had independently verifiable implications for lifespan, and possibly for energy homeostasis, on the HSD. Ultimately, the direction and magnitude of the effects estimated for polymorphisms in the GWA analyses were approximately the same as for the effects of whole-body RNAi knockdown of the selected GWAS-candidate genes (Table 4).

3.2. Functional validation of rdgA

*rdgA (*Retinal degeneration A) was tagged by 8 SNPs and encodes for an eye-expressed diacylglycerol kinase (DGK) in *Drosophila* that inactivates diacylglycerol (DAG) by converting it to phosphatidic acid (PA) (Masai et al., 1993). DAG accumulation in the cell membrane affects numerous intracellular signaling pathways, including those regulating cell growth, differentiation, and membrane trafficking (Mérida et al., 2008). Considering the numerous metabolic pathways in which DAG is implicated, cells must control the production and clearance of DAG. Additionally, dysregulation of DAG metabolism has been linked to the pathophysiology of several human diseases, such as diabetes and malignant transformation (Carrasco and Mérida, 2007). *rdgA* is involved in phospholipase C (PLC) signaling pathways, controlling the actions of transient receptor potential (TRP) channels and protein transport. Members of the TRP superfamily cation channels are involved in sensory physiological functions such as phototransduction, thermosensation, olfaction, hearing and touch (Montell, 2005). The *Drosophila* genome encodes for three TRP channels (TRPCs): TRP, TRPL and TRPγ. TRP and TRPL are involved in phototransduction, while TRPγ contributes to visual response (Chorna-Ornan et al., 2001). In the context of gene function, reducing the expression of *rdgA* in *Drosophila* has previously been shown to extend lifespan and enhance oxidative stress response through altered metabolism of DAG. The attenuation of DAG reduces PA levels, decreasing TOR (Target of rapamycin) signaling, an important signaling cascade involved in nutrient sensing that controls metabolism and growth (Lin et al., 2014). In our case, whole-body knockdown *rdgA* mated female flies exhibited a reduction of lifespan on both the NM and HSD (Figure 3A) compared to the controls. There were no significant effects of whole-body knockdown of *rdgA* on triglyceride content, feeding quantity and starvation resistance (Table 4).

3.3. Functional validation of olf413

olf413 is predicted to be involved in oxidation-reduction processes, octopamine biosynthetic processes, dopamine catabolic processes and norepinephrine biosynthetic processes. These predicted functions are based on the oxidoreductase activity of dopamine beta-monooxygenase that catalyzes a redox reaction in dopamine (DA) is converted into norepinephrine (NE) in vertebrates (Keller and Robertson, 2009). In invertebrates, the octopamine (OA) biosynthetic process results in the formation of octopamine, an epinephrine analog. OA is an important neuroactive molecule in *Drosophila* that plays a role as a neurotransmitter, neuromodulator, or neurohormone (Monastirioti et al., 1996).

In vivo whole-body knockdown of *olf413* in *Drosophila* reduced lifespan on both the HSD and NM. No significant differences in lifespan were observed between NM and HSD (Figure 4A). This supports the previous finding that virgin female mutant flies lacking the expression of octopamine experienced a reduction of lifespan on a standard fly medium (Li et al., 2016). Measuring the feeding quantity showed that *olf413* knockdown mated female flies exhibited a reduction in feeding quantity compared to the controls on both diets (Figure 4C). HSD fed flies also showed a significant decrease in the feeding quantity relative to the flies fed a NM (Figure 4C and Table 4). The reduction of feeding quantity in the *olf413* knockdown flies exposed to HSD agrees with a previous study that showed quantifying food intake over 24-hour periods using the CAFE (Capillary Feeder assay) in OA deficient fruit flies showed a reduction of daily food intake by approximately 30% (Li et al., 2016). This might be partly related to octopamine's role in sugar learning. OA deficient flies have shown to be severely impaired for sugar memory, but not general learning (Schwaerzel et al., 2003). Furthermore, dopamine-containing neurons have been implicated as downstream mediators of sweet sensory input for other behavioral outputs, including proboscis extension (Linford et al., 2015). Triglyceride storage levels in the *olf413* knockdown mated female flies were significantly lower relative to the control flies on both diets (Figure 4B). HSD fed flies exhibited an increase in the triglyceride content, but there is an almost indistinguishable difference specifically between the *olf413* knockdown flies on HSD and NM (Figure 4B). OA has a direct effect on the fat body, causing the release of fatty acids into the hemolymph, synergistic to the effect of adipokinetic hormone (Roeder, 1999). Mutant flies with altered octopamine signaling have previously been shown to have a decreased triglyceride content, and it is elevated in flies with increased activity of octopaminergic neurons (Erion et al., 2012). Starvation resistance followed a similar pattern as the triglyceride content: *olf413* knockdown flies survived shorter than the controls when starved on 1% agar (Figure 4D). HSD increased starvation resistance in both genotypes compared to the flies fed a NM diet. This is consistent with the previous findings where flies with an increased OA activity exhibited a higher triglyceride level and survived longer on starvation medium (2% agar) compared to the flies with decreased octopamine signaling (Erion et al., 2012).

Overall, *olf413* appeared in our GWAS screen of HSD lifespan, was confirmed to impact lifespan in an independent knockdown, and other metabolic parameters are in strong agreement with prior studies. Because of OA's role in sugar learning/memory, there is also strong evidence for this gene's role in the behavioral response to nutrient excess.

3.4. Functional validation of axo

Axotactin (*axo)* encodes for the neurexin protein, AXO, that is secreted by the glia and which is involved in determining the membrane electrical properties of target axons (Yuan and Ganetzky, 1999). Unfortunately, this gene is yet to be explored in the context of metabolism and there are no existing studies that can corroborate our findings. Of the four RNAi knockdowns of the GWAS candidate genes that we performed, *axo* knockdown mated female flies displayed a reduction of lifespan on HSD only, in contrast to the other genes that impacted lifespan on both HSD and NM (Figure 5A). It has not escaped notice that most loci we tested having an impact on both HSD and NM is indicative that the genes we identified are not “HSD exclusive” in their relevance for lifespan. However, metabolic and physiological factors that influence lifespan on a normal diet can exist in multiple dietary settings. In the case of some of our GWAS-identified genes, if we had not seen an impact on lifespan in a normal dietary setting it would be in contradiction to previous studies that we have described that show their physiological involvement in such a setting (*rdgA* and *olf413*). The identification of loci that impact lifespan on both a HSD and NM, being identified presently in a HSD setting via the DGRP GWAS, expands the number of environments in which that gene is significant.

It has previously been shown that *axo* mutants have a normal development of the embryonic nervous system, implying that the lack of *axo* affects functional properties rather than structure (Yuan and Ganetzky, 1999). Furthermore, in *Drosophila* the glial cells of the blood-brain barrier specifically take up sugars and they utilize glycolysis to secrete alanine and lactate to fuel neuronal mitochondria. Lack of glial glycolysis causes neurodegeneration in the adult brain (Volkenhoff et al., 2015). Thus, to speculate, reducing the expression of *axo* might be

reducing glial-neuronal signaling, also potentially impacting the uptake of sugar in the glial cells and reducing glial glycolysis. This could be particularly pathogenic in the HSD setting with additional plausible impacts on behavior. Feeding quantity was significantly reduced in the HSD fed flies with *axo* knockdown flies exhibiting a lower feeding quantity than the control flies (Figure 5C).

3.5. Functional validation of caps

Capricious (*caps*) encodes for a transmembrane protein receptor with leucine-rich repeats that is expressed in subsets of muscles and motor neurons. *caps* gene product is involved in synapse formation, axon guidance and morphogenesis of multiple tissues, such as wings, legs and the tracheal system (Shishido et al., 1998; Thurmond et al., 2019). Being a developmental gene, not many functional studies have been performed in the context of metabolism. From our previous study that involved detecting behavioral and transcriptional expression changes in the fly heads of w^{1118} mated females exposed to a high fat diet, *caps* was one of the genes that was upregulated due to high fat diet (Rivera et al., 2019). In our case, the *in vivo* whole-body knockdown of the gene led to a developmental delay, yielding only few flies. This is consistent with a previous study where loss of function mutant alleles lacking the first *caps* exon caused mutants to die late in embryogenesis or soon after eclosing, with only a few surviving to adulthood (Shishido et al., 1998). However, we were able to determine the impact of *caps* whole-body knockdown on lifespan with the few flies that survived to adulthood. *caps* knockdown mated female flies displayed a significant reduction of lifespan compared to controls on both diets (Figure 6). However, diet showed no significant impact on lifespan.

3.6. Functional validation of mub

The whole-body knockdown of *mub* (mushroom body) led to a lethal phenotype, generating only flies with the stubble (*sb*) marker, indicating that any progeny lacked the inheritance of the RNAi construct. *mub* is involved in the regulation of alternative mRNA splicing via spliceosome. The gene is involved in thermosensory behavior learning and memory based on its expression in the mushroom bodies (Thurmond et al., 2019). The gene encodes for the MUB protein that is homologous to the human and mouse poly (rC)-binding proteins: PCBP-1 and PCPB2 (Grams and Korge, 1998). These proteins are expressed in a wide range of tissues, including all major internal organs. The PCBPs are involved in mRNA stabilization, translational enhancement, determinants of transcriptional controls and apoptotic pathways (Grams and Korge, 1998; Makeyev and Liebhaber, 2002). However, it has also been shown that *mub* is not necessary for the development of the mushroom bodies, but rather has a more general role in development and embryogenesis. The mushroom body in the fruit fly is an associative brain center that translates odor representations into learned behavioral responses. Mutants of mushroom bodies: *mushroom bodies deranaged* (*mbd)* and *mushroom bodies miniature* (*mbm*) have deformed mushroom bodies and fail in olfactory learning tests, displaying the function of mushroom bodies as neural centers for learning and memory with respect to olfactory signals (Heisenberg et al., 1985). The expression of *mub* has also been detected in other cells of the embryonic brain hemispheres, some of which might be precursor cells of antennal lobes (Grams and Korge, 1998).

3.7. Varying effects of HSD-induced obesity

Flies on the HSD survived longer than the flies on the NM, which contradicts some previous findings of reduced lifespan on HSD (Lushchak et al., 2014; Na and Cagan, 2013). High dietary carbohydrate-to-protein ratio (16:1) has been shown to increase longevity in mated *Canton-S* female flies (Lee et al., 2008). In addition, studies that have utilized glucose instead of sucrose as the source of carbohydrate have shown that increased amount of glucose in the fly medium increased median lifespan of adult w^{1118} female flies by 31% (Galenza et al., 2016). Contrarily, other studies have reported that high dietary sugar (1 mol per deciliter) shortened lifespan in adult flies by increasing cardiac arrythmia and heart deterioration, but this is a concentration that is higher than that which we used (Na and Cagan, 2013). Thus, lack of a standardized HSD recipe across labs has led to varying implications for lifespan and the drawing of different conclusions.

A previous study utilizing w^{1118} and *plin2* (*perilipin2*) mutant flies, which are genetically lean flies lacking a gene essential for fat mobilization, exhibited an increase in meal size and duration when exposed to a 20% sucrose diet. The study suggests that obesity is not the only factor that can alter feeding behavior, but dietary sugar and changes in sweet sensation can also have a direct impact on feeding behavior in flies (May et al., 2019). Additionally, a study investigating the impact of specific dietary components, showed that wildtype (w^{1118} or *Canton-S*) female flies had a significant increase in food intake when subjected to a high carbohydrate (sugar) diet, and flies fed a high protein (yeast) diet exhibited a significant decrease in food intake. The study also reported that the increased lifespan due to reduced total caloric intake had a marginal statistical significance relative to the flies that displayed a reduction in lifespan when exposed to a sugar- (carbohydrate) or yeast (protein)-enriched diet (Skorupa et al., 2008). Another study showed that media with different sugar concentrations (low or high), but the same yeast concentration had a non-significant impact on median lifespan (low sugar: 38 days, high sugar: 34 days) of mated *Canton-S* females. Furthermore, the study displayed that flies survived about 13 days longer on a low yeast/high sugar diet than on a high yeast/low sugar diet despite both diets being energetically equivalent (Min et al., 2007). This is indicative that nutritional composition has an effect on lifespan rather than simply calorie intake. There was a significant amount of overlap of the lifespan loci identified presently and the lifespan loci identified in a recent DGRP GWAS, and in both cases a higher amount of carbohydrate content relative to yeast was apparently used (Huang et al., 2020). There was also a substantial sharing of DGRP lines in the highest quartile of lifespans when the present study and the Huang et al. (2020) study are compared. More investigation is needed on the significance of the ratio of carbohydrate to yeast content in HSD studies and its impact on the relevance of particular lifespan loci in the DGRP.

However, overall, the present HSD increased triglyceride content, decreased feeding quantity and increased starvation resistance in both the knockdown and control flies (Table 4). It is intriguing to speculate that one reason why an impact on lifespan by HSD is not observed presently is because the flies are simply eating less of a potentially detrimental diet, but even if so, they do not escape the anabolic consequences of HSD in terms of energy homeostasis (having increased triglycerides, starvation resistance in Table 4). This combination of outcomes implies that the HSD we used, while it does not decrease lifespan in mated females, does apply a nutritional stress that results in a consistent physiological response.

Although speculative, this implies that the loci identified from our GWAS could be involved in that dynamic behavioral and physiological response to excess nutrient access. There is precedent for loci in the fly for which when gene function is experimentally manipulated there is an impact on the ability to properly respond to nutrient availability, such as *takeout.* Flies that lack proper *to* function are inappropriately hyperphagic when food is abundant and consume a reduced amount relative to control flies when food is provided to them after a starvation period. The TO protein is produced in response to circadian rhythm, is lipophilic, and has an apparent ability to impact juvenile hormone function, sleep patterns, activity level, and gustatory neuron sensitivity to nutrient environment change (Meunier et al., 2007). Putative whole fly knockdown of *olf413* presently resulted in reduced lifespan, feeding quantity, triglyceride content, and starvation resistance most apparently on a HSD (Figure 4). Given the likely role of *olf413* in octopamine production and the body of functional evidence for this gene's impact in energy homeostasis that was described previously, it is possible that knockdown of *olf413* results in an

overadjustment to this HSD environment in downregulation of feeding and triglyceride storage, which in turn impacts lifespan and starvation survival. Putative whole fly knockdown of *axo* presently resulted in reduced lifespan and feeding quantity on HSD, but equivalent content of triglyceride and starvation resistance to that of controls (Figure 5). Given that the triglyceride content and starvation resistance were elevated on HSD for the *axo* knockdowns, the same argument regarding over-adjustment to HSD during feeding downregulation as an explanation for the impact on lifespan seems less likely. It is certainly possible that *axo* knockdown results in a neurological impact, given its discussed function, and this presents several mechanisms through which lifespan and feeding quantity may be impacted, but not triglyceride storage or starvation resistance. These mechanisms are not addressed with assays presently, but might include metabolic rate, behavioral changes, developmental changes, or locomotive changes.

4. Conclusions

The DGRP identified several lifespan loci in a HSD setting, and these loci were largely confirmed to affect lifespan in that HSD setting upon independent validation of candidate genes with an RNAi approach, although the majority of the lifespan loci identified were also relevant in an NM setting. The reduction in lifespan on HSD with RNAi knockdown was usually accompanied by reduction in feeding quantity. We speculate that the quantitative trait loci identified from this DGRP GWAS of HSD lifespan in mated females may be involved in the response to heightened nutritional stress, based on the consistent pattern of feeding downregulation and lifespan reduction with ubiquitous knockdown, coupled with increased fat storage and generally decreased feeding quantity during a HSD.

5. Materials and methods

5.1. Media

All flies were reared or housed on a standard solid diet consisting of 5.2% (w/v) cornmeal, 5.0% (w/v) yeast extract, 1.0% (w/v) agar, 3.0% (w/v) sucrose, 1.5% (v/v) tegosept (20% w/v in 70% ethanol), 0.3% (v/v) propionic acid and 0.3% (v/v) tetracycline (10 mg/mL). The experimental high sugar diet (HSD) consisted of 2.6% (w/v) cornmeal, 4.0% (w/v) yeast extract, 0.8% (w/v) agar, 20% sucrose (w/v), 1.5% (v/v) tegosept (20% w/v in 70% ethanol), and 0.3% (v/v) propionic acid. The normal diet (NM) utilized as a control diet during validation of GWAS-suggested candidate genes was identical to the HSD except that the sucrose content was reduced from 20% to 3%. The rearing media, NM, and HSD formulas utilized in this study were derived from studies that have investigated the impact of varying concentrations of yeast and sucrose on a range of adult-specific *Drosophila* phenotypes including lifespan, fecundity and energy storage (triglyceride and protein content) (Bass et al., 2007; Hemphill et al., 2018; Skorupa et al., 2008).

5.2. Drosophila stocks and husbandry

Fly stocks utilized in the various experiments were originally received from the Bloomington *Drosophila* Stock Center (BDSC) at Indiana University. We utilized 193 out of the 200 DGRP lines available at the BDSC. The flies were allowed to mate and incubated under uniform humidity at 25 °C until larvae development was seen. The parents were then transferred onto new media, and the experimental flies were allowed to emerge. Virgin female flies were harvested from each line and allowed to age for 2–3 days. The virgin females of each genotype were mated with Bloomington Stock 1 (*Canton-S*) males for 48 h in a 3:1 female to male ratio. Males were removed and 20 mated females of each genotype were then exposed to HSD under uniform humidity at 23 °C on a 12-hour light/dark cycle.

For the GWAS-suggested candidate gene studies, we utilized available *in vivo* whole-body RNAi knockdown constructs of *rdgA* (BDSC# 29435), *olf413* (BDSC# 29547), *mub* (BDSC# 55913), *axo* (BDSC# 65911) and *caps* (BDSC# 28020) to verify their impacts on lifespan and energy homeostasis. To serve as experimental controls, genetically homogenous lines (BDSC# 36303 and 36304) lacking the RNAi constructs were utilized. Both the transgenic RNAi and the isogenic control lines were crossed with the GAL4 driver (BDSC# 5138) that ubiquitously expresses GAL4 under the control of the alphaTub84B promoter (Liu and Lehmann, 2008). All the experimentally utilized virgin female knockdown and control flies were housed at a population density of no more than 30 flies per vial and aged for 3–5 days before being mated with the Bloomington stock 1 (*Canton-S*) males for 48 h in a 3:1 ratio. Males were then removed, and the mated females were transferred to NM or HSD under uniform humidity at 23 °C on a 12-hour light/dark cycle. Knockdown and control flies were exposed to NM and/or HSD typically for 7 days before being assayed for all experiments except lifespan, which required continual monitoring.

5.3. Lifespan

The lifespan of the 193 DGRP lines was determined by placing 20 mated females (as described in the *Drosophila* stocks and husbandry section) of each DGRP line on the HSD under uniform humidity at 23 °C on a 12-hour light/dark cycle. Mortality was recorded daily for each genotype until all 20 flies faced mortality. Media was changed every day for the first three days, as the females laid many eggs and changed the solidity of the media. Afterward, media was changed every 3 days.

The lifespan for the whole-body knockdown flies, and respective controls was monitored by subjecting mated females no older than 7 adult days to HSD and NM. 5 replicates of 20 mated females per replicate, a total of 100 flies, were set up for each condition under uniform humidity at 23 °C on a 12-hour light/dark cycle and mortality was recorded daily for each vial until all 100 flies died out. Flies were transferred onto fresh respective media daily for the first three days and then after every three days.

5.4. DGRP pipeline Analysis/GWA analysis of HSD-induced mortality

The average lifespan and standard error of the mean (SEM) of the 193 DGRP lines was computed in Microsoft Excel, as the DGRP pipeline utilizes trait means for GWAS analysis. The average lifespan was first adjusted for any significant block effect as the DGRP lines were received and assayed in four intervals. This was done by utilizing the formula generated by (Arya et al., 2015): Adjusted Mean Lifespan= (Raw Mean of each line-Block Mean) + Grand Mean of all 193 lines. The block-adjusted mean of lifespan was then entered into the DGRP analysis pipeline created by Mackay and colleagues (Mackay et al., 2012) at http://dgrp2.gnets.ncsu.edu. The minimum lifespan was computed as 1 day if it was less than 0. Utilizing linear mixed model ANOVA (analysis of variance), the pipeline adjusts for known DGRP covariates: chromosomal inversion status (*In(2L)t, In(2R)NS, In(3R)P, In(3R)K, and In(3R)Mo*), *Wolbachia* infection status, and cryptic relatedness, in the process notifying the user of any association of each covariate with the trait mean (Mackay et al., 2012). Next, the pipeline utilizes >2,000,000 SNPs (single nucleotide polymorphisms) with minor allele frequency (MAF) > 5% to determine association with lifespan through running the linear mixed model: $Y = \mu + M + L(M) + \varepsilon$, where Y= Lifespan, μ = Grand mean of all 193 lines, M = The effect of the polymorphic marker, L = The DGRP Line (Random) and ε = The environmental error variance.

Functional annotation (gene ontology) of loci near each of the top ($P < 10^{-5}$) variants associated with the mean HSD lifespan was also provided as an output, with all annotations based on Flybase 2.0 (Thurmond et al., 2019).

5.5. Triglyceride content

After 7 days exposure to NM and HSD, 15 flies per experimental condition were first anesthetized with triethylamine (FlyNap®, Carolina

Biological). Individual whole flies were then homogenized in 200 μL PBT (1X PBS containing .1% Tween-20) using an electric pestle. The homogenate was heat inactivated at 70 °C for 5 min and then centrifuged at 13,200 RPM (Labnet Prism™ R Microcentrifuge) for 3 min at 4 °C. The supernatant was extracted and 20 μL of each sample was then pipetted into three-96 well plates in a randomized order. 100 μL of room temperature triglyceride reagent (Infinity™ Triglycerides) was then added to each well and incubated at 37 °C for 20 min. This was repeated for all three 96-well plates and the total absorbance change was measured at 540nm in a plate reader (Synergy HT Biotek), which is proportional to the concentration of triglycerides in each sample and interpolated with triolein standards diluted in PBT (Tennessen et al., 2014).

5.6. Feeding behavior

After 7 days exposure to NM and HSD, five replicates of 5 adult female mated flies (N = 5, 5 per replicate, 25 flies per experimental condition) were transferred on to their respective diets containing 1% Brilliant Blue dye solution (FD&C blue No.1, Spectrum). The dye was incorporated into both diets by diluting the 20% w/v brilliant blue dye solution to a final concentration of 1% in the media. Flies were left undisturbed to feed on the brilliant blue media for 4 h at 23 °C. Flies were snap frozen at -80 °C for 5 min prior to decapitation to avoid eye pigment interference. The flies were then homogenized in 400 μL PBT using an electric pestle and the homogenate was centrifuged for 20 min at 13,000 RPM. The supernatant was extracted and a triplicate of 100 μL of each sample was pipetted into a 96 well plate in a randomized order. The amount of food ingested was quantified by measuring the absorbance of the supernatant extracted at 625 nm in the Biotek plate reader and interpolating the concentration of the blue dye with brilliant blue standards in PBT (Xu et al., 2008).

5.7. Starvation resistance

After 7 days on NM and HSD, 100 flies per experimental condition were anesthetized using CO_2 and transferred into vials containing 1% agar. The 1% agar does not have any nutritional content and serves as a source of hydration for the flies. Flies were housed in a group of 20 flies per vial and mortality was recorded every 6 h until all flies died out (N = 5, 20 per replicate, 100 flies per condition).

5.8. Statistical analysis

All data was analyzed in JMP statistical software by performing ANOVA (Analysis of Variance) using the Fit Model function. The polynomial effect of the following variables on each mean phenotype was determined: Genotype, Diet, and Genotype*Diet. Tukey's HSD (Honestly Significant Difference) post hoc analysis was performed to determine direction and magnitude of significant differences between groups if the model indicated overall significance. Student's post hoc t-test was further utilized in lifespan data analysis to identify differences within diet due to the *a priori* hypothesis of within diet effects if Tukey's HSD did not reveal any existing differences. In all cases, a P-value < 0.05 was considered indicative of a significant difference.

Declarations

Author contribution Statement

Sumit P Patel: Performed the experiments, Analyzed and interpreted the data, Wrote the paper.

Matthew Talbert: Conceived and designed the experiments, Performed the experiments, Analyzed and interpreted the data, Contributed reagents, materials, analysis tools or data, Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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