A low sugar diet enhances *Drosophila* body size in males and females via sex-specific mechanisms

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SUMMARY STATEMENT

A low sugar diet in *Drosophila* promotes growth in both males and females, but the underlying genetic, transcriptional, and metabolic mechanisms are not fully shared.

ABSTRACT

In *Drosophila*, changes to dietary protein elicit different body size responses between the sexes. Whether these differential body size effects extend to other macronutrients remains unclear. Here, we show that lowering dietary sugar (0S diet) enhanced body size in male and female larvae. Despite an equivalent phenotypic effect between the sexes, we detected sex-specific changes to signaling pathways, transcription, and whole-body glycogen and protein. In males, the low sugar diet augmented insulin/insulin-like growth factor signaling pathway (IIS) activity by increasing insulin sensitivity, where increased IIS was required for male metabolic and body size responses in 0S. In females reared on low sugar, IIS activity and insulin sensitivity were unaffected, and IIS function did not fully account for metabolic and body size responses. Instead, we identified a female-biased requirement for the target of rapamycin pathway in regulating metabolic and body size responses. Together, our data suggest the mechanisms underlying the low sugar-induced increase in body size are not fully shared between the sexes, highlighting the importance of including males and females in larval studies even when similar phenotypic outcomes are observed.

INTRODUCTION

In *Drosophila*, dietary nutrients impact larval growth to influence final body size. Nutrient quantity promotes growth during larval development, as nutrient-rich conditions favour larger body sizes (Edgar, 2006; Hietakangas and Cohen, 2009; Nijhout et al., 2014). Nutrient quality also regulates larval growth, as individual macronutrients differ in their body size effects. For example, while dietary protein promotes a larger body size across a wide concentration range (Britton and Edgar, 1998; Britton et al., 2002; Edgar, 2006; Shingleton et al., 2017), moderate or high levels of dietary sugar inhibit growth and reduce body size (Musselman et al., 2011; Pasco and Léopold, 2012; Reis, 2016). This suggests a complex relationship between individual macronutrients and body size.

One factor that influences the magnitude of nutrient-dependent changes to *Drosophila* body size is biological sex (McDonald et al., 2021; Millington et al., 2021a; Shingleton et al., 2017; Stillwell et al., 2010; Teder and Tammaru, 2005). For example, manipulating nutrient quantity by altering dietary protein and carbohydrates causes sex-biased trait size effects (Shingleton et al., 2017). Male and female phenotypic responses to nutrient quality also differ, as the magnitude of protein-dependent changes to body size are larger in females (Millington et al., 2021a). Due to the widespread use of mixed-sex groups in larval growth studies, however, it remains unclear whether sex-specific body size responses to dietary protein extend to other macronutrients, such as sugar.

Our examination of larval development revealed that lowering dietary sugar augmented the rate of growth and increased body size in both males and females. Indeed, the largest body size in each sex was observed in a diet with no added sugar (0S). Despite an equivalent low sugar-induced increase in body size, signaling pathway activation, transcriptional responses, and metabolic changes were not fully shared between the sexes. In males, our data show that the low sugar-induced changes to metabolism and body size were triggered by higher insulin/insulin-like growth factor signaling pathway (IIS) activity, where increased IIS was due to improved insulin sensitivity. In females, there was no change in IIS activity or insulin sensitivity in 0S. Instead, females showed transcriptional responses consistent with increased anabolic metabolism, and genetic studies indicated a role for the target of rapamycin (TOR) pathway in regulating the metabolic and body size effects of the low sugar diet. Together, our data suggest male and female larvae achieve a larger body size in 0S via distinct mechanisms. This highlights the importance of including both sexes in larval growth studies, as different mechanisms may underlie similar phenotypic responses.

RESULTS AND DISCUSSION

A low sugar diet promotes an increased rate of growth and larger body size

To determine the body size effects of dietary sugar in each sex, we measured pupal volume in *white*^{1118} (*w*; FBgn0003996) male and female larvae reared in diets with different quantities of sugar. Because dietary sugar represses growth in a mixed-sex larval group (Musselman et al., 2011; Pasco and Léopold, 2012), we started with a

widely-used diet (1S) (Lewis, 1960) and removed sugar in a stepwise manner until none remained (0S). In w^{1118} females, body size was significantly larger in larvae cultured on a diet with half (0.5S), or one-quarter (0.25S), the amount of sugar found in 1S (Fig. 1A). Interestingly, the largest body size was found in larvae reared in 0S (Fig. 1A). In w^{1118} males, body size was similarly larger in larvae reared on 0.5S, 0.25S, and 0S compared with larvae raised on 1S (Fig. 1B). Given that the body size effect of a low sugar diet was equivalent between the sexes (Fig. 1C; Supplemental file 1), the phenotypic responses to dietary sugar were not different between males and females. Although we reproduced this finding using adult weight (Fig. 1D), more studies are needed to understand why the effect of sugar on adult weight was smaller than on pupal volume. More work is also needed to determine whether these growth effects were mediated by dietary sugar alone, as removing cornmeal from 1S to match the caloric content of 0S did not affect body size (Millington et al., 2021a), or by changing the protein to carbohydrate ratio, as both factors affect larval development (Kim et al., 2020; Matzkin et al., 2011; Musselman et al., 2011; Pasco and Léopold, 2012). Nevertheless, we extend prior knowledge by demonstrating an equivalent body size response between the sexes to a low sugar diet, and by showing a non-sex-specific increase in the larval growth rate (Fig. 1E-H).

A low sugar diet augments IIS activity only in males due to improved insulin sensitivity

IIS has emerged as a key regulator of *Drosophila* nutrient-dependent growth (Gokhale and Shingleton, 2015; Grewal, 2009; Koyama and Mirth, 2018; Lecuit and Le Goff, 2007; Teleman, 2010). Indeed, high levels of IIS activity promote a larger body size (Böhni et al., 1999; Britton et al., 2002; Brogiolo et al., 2001; Chen et al., 1996; Fernandez et al., 1995; Ikeya et al., 2002; Patel et al., 2003; Poltilove et al., 2000). Given the larger body size of males and females cultured on 0S, we measured diet-induced changes to IIS activity. To quantify IIS activity, we measured mRNA levels of genes coregulated by transcription factor Forkhead box, sub-group O (Foxo; FBgn0038197) (*e.g. Insulin receptor* [*InR*; FBgn0283499], *brummer* [*bmm;* FBgn0036449], *eukaryotic initiation factor 4E-binding protein* [*4E-BP*; FBgn0261560]). Foxo's transcriptional activity is normally repressed by IIS; thus, high IIS activity inhibits Foxo and reduces mRNA levels of Foxo target genes (Alic et al., 2011; Gershman et al., 2007; Jünger et al., 2003; Puig and Tjian, 2005; Zinke et al., 2002).

In w^{1118} females, mRNA levels of Foxo target genes were not different between larvae reared in 1S and 0S (Fig. 2A). In contrast, mRNA levels of Foxo targets were significantly lower in w^{1118} male larvae in 0S (Fig. 2B). Given that we confirmed the accuracy of our larval sorting by detecting the correct male- and female-specific isoforms of sex determination genes (Fig. S1A), these findings suggest that a low sugar diet enhanced IIS activity in males but not females. To confirm this, we monitored the subcellular localization of green fluorescent protein (GFP) fused to a pleckstrin

homology (PH) domain (GFP-PH), which shows increased membrane localization when IIS activity is high (Britton et al., 2002). No diet-dependent change in membrane GFP-PH localization occurred in w^{1118} females (Fig. 2C); however, male larvae cultured in 0S had higher membrane GFP-PH than males raised on 1S (Fig. 2D). Because feeding behaviour was not different between the sexes (Fig. 2E), our data support a model in which the low sugar diet augments IIS activity in male but not female larvae.

One potential reason for higher IIS activity in males reared on 0S is improved insulin sensitivity, as moderate levels of dietary sugar cause insulin resistance in a mixed-sex larval group (Musselman et al., 2011; Lourido et al., 2021; Pasco and Léopold, 2012). To test this, we monitored insulin sensitivity by quantifying GFP-PH membrane localization in larval fat bodies with and without human insulin stimulation (Pasco and Léopold, 2012). While insulin stimulation significantly enhanced GFP-PH membrane localization in females reared on both diets (Fig. 2F), insulin stimulation only augmented GFP-PH membrane localization in males reared on 0S (Fig. 2G). This suggests males reared on 0S had higher insulin sensitivity than males reared on 1S, whereas females were insulin sensitive in both contexts. Supporting this, we observed a significant downregulation of *puckered* (*puc*; FBgn0243512), a gene that is lower in insulin sensitive larvae (Lourido et al., 2021; Pasco and Léopold, 2012), in males but not females reared on 0S (Fig. 2H). Thus, higher IIS activity in male larvae reared in 0S was caused by improved insulin sensitivity, an effect we did not observe in females. This extends our understanding of sex differences in the nutrient-dependent regulation of IIS (Millington et al., 2021a), and shows that in flies, as in mammals (Guerre-Millo et

al., 1985; Mittendorfer, 2005; Macotela et al., 2009; Yki-Järvinen, 1984), females show higher insulin sensitivity than males in some contexts.

Male-biased requirement for IIS in promoting the low sugar-induced increase in body size

Given IIS's known role in regulating body size, we asked whether the male-specific increase in IIS activity between 1S and 0S was required for the low sugar-induced increase in body size. To test this, we measured body size in male and female larvae carrying mutations in IIS that support normal growth, but which blunt high levels of IIS activation (*InR*^{E19}/+) (Chen et al., 1996; Millington et al., 2021a; Rideout et al., 2015). While body size was larger in *w*^{1118} control males reared on 0S (Fig. 3A), 100% of the low sugar-induced increase in body size was blocked in *InR*^{E19}/+ male larvae (Fig. 3A; genotype:diet interaction $p<0.0001$). This suggests the low-sugar induced increase in IIS activity was required in males to achieve a larger body size. In females, only 48% of the low sugar-induced increase in body size was blocked in *InR*^{E19}/+ larvae (Fig. 3B). Given that the magnitude of genotype effects on the body size response to diet was larger in males than in females (sex:diet:genotype interaction $p=0.0114$), our data indicates a male-biased phenotypic response to reduced IIS function, an effect we replicated across independent experiments despite modest interexperiment variation in the magnitude of the low sugar-induced increase in body size (Fig. S2A-D).

We further reproduced this male-biased body size effect in larvae lacking the coding sequences for *Drosophila insulin-like peptide 3* (*dilp3*; Fbgn0044050) (Fig. 3C, D; sex:diet:genotype interaction: p=0.0003), *Drosophila insulin-like peptide 2* (*dilp2*; Fbgn0036046) (Fig. 3C, D; sex:diet:genotype interaction: p=0.0627), and in flies lacking the coding sequences for *dilp2,3, and Drosophila insulin-like peptide 5* (*dilp5*; Fbgn0044038) (genotype *dilp2-3,5*) (Fig. S3A, B; sex:diet:genotype interaction p<0.0001); however, loss of *dilp5* had no effect on the low sugar-induced increase in body size in either sex (Fig. 3C, D). Together with the male-specific increase in IIS activity between 1S and 0S, the male-biased body size effect of reduced IIS in a low sugar context confirms that the signaling and genetic mechanisms that promote larval growth in 0S are not fully shared between the sexes.

A low sugar diet causes differential transcriptional and metabolic responses in males and females

IIS influences body size by triggering profound changes in gene expression (Alic et al., 2011; Bülow et al., 2010; Grewal, 2009; Guertin et al., 2006; Li, Edgar, and Grewal 2010; Musselman and Kühnlein, 2018; Teleman et al., 2008; Tiebe et al., 2015; Webb et al., 2016; Zinke et al., 2002). Because we observed sex-specific regulation of IIS in a low sugar diet, we performed an unbiased analysis of transcriptional changes in males and females reared on 1S and 0S. We found significant differences in the transcriptional response to a low sugar diet between the sexes (Fig. 4A), and show that diet affects sexual dimorphism in gene expression (Fig. 4B), consistent with prior reports (Camus,

Piper, and Reuter, 2019; Jaime et al., 2017). Interestingly, the proportion of genes differentially regulated by a low sugar diet was higher in males (Supplemental file 2): 298 (8.2%) of differentially expressed genes were unique to females and 1832 (50.3%) unique to males (Fig. 4C). A low sugar diet therefore causes a distinct transcriptional response in each sex (Fig. S4A, B). Indeed, the majority (58%) of enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were different between males and females ($p_{adj} < 0.05$; Fig. 4D). For example, genes in the "FoxO signaling pathway" category were only downregulated in males in 0S (Fig. 4D; bolded text). Given that Foxo is repressed when IIS activity is high, this further supports a male-specific IIS increase in 0S.

One overrepresented biological process in both sexes was metabolic regulation: 80.7% of genes differentially expressed in a low sugar context were linked with metabolism (Fig. 4D). We therefore examined several metabolic parameters in male and female larvae in 1S and 0S to determine the physiological significance of these sex-specific gene expression responses. While we found non-sex-specific changes to whole-body triglyceride and glucose levels on a low sugar diet (Fig. S5A, B), we observed sex-specific effects on whole-body protein, glycogen, and trehalose levels. Whole-body protein levels were significantly higher only in w^{1118} females reared in 0S (Fig. 4E), whereas whole-body glycogen and trehalose were significantly higher only in males reared on 0S (Fig. 4F, G). Importantly, these metabolic changes cannot be attributed to sex differences in the relationship between organ and body size (Fig. S6A).

Instead, the male-specific increase in glycogen was likely due to increased IIS activity in 0S, as the low sugar-induced increase in glycogen was blunted in *InR*^{E19}/+ males (Fig. 4H), aligning with IIS's known role in regulating carbohydrate metabolism (Mattila and Hietakangas, 2017). Thus in males, the low sugar diet augments IIS activity due to improved insulin sensitivity, leading to higher whole-body glycogen levels and a larger body size. In females, these mechanisms are not fully shared: the low sugar diet caused no change in insulin sensitivity, and reduced IIS function did not block the female-specific increase in body size or whole-body protein levels (Fig. S7A).

To gain deeper insight into the phenotypic effects of a low sugar diet in females, we focused on female-specific metabolic and transcriptional changes. The main metabolic phenotype was increased whole-body protein (Fig. 4E), and female-specific transcriptional changes included higher mRNA levels of genes related to glycolysis and gluconeogenesis, folate biosynthesis, biosynthesis of amino acids, and the pentose phosphate pathway, and lower mRNA levels of genes linked with lysosomes, arachidonic acid metabolism, and fatty acid degradation (down). Together, these changes indicate a general upregulation of anabolic processes in 0S. This anabolic regulation is unlikely to depend on *Mondo* (FBgn0032940) and *bigmax* (FBgn0039509), two key regulators of sugar sensing and tolerance (Mattila et al., 2015; Havula and Hietakangas, 2012; Havula et al., 2013; Havula et al., 2018), as we observed non-sex-specific changes to known *Mondo-bigmax* target genes (Fig. S8A-D; Supplemental file 2). Another key regulator of anabolic metabolism is the TOR pathway (Wullschleger et al., 2006). In multiple organisms, TOR promotes mRNA translation and protein synthesis, the pentose phosphate pathway, and glycolytic flux (Dunlop and Tee, 2009;

LaPlante and Sabatini, 2013; Wullschleger et al., 2006), and represses catabolic processes such as autophagy and lysosomal biogenesis (Puertollano, 2014).

Given that the female-specific metabolic and transcriptional changes in 0S are consistent with a potential role for TOR, we asked whether TOR function was required in females for the metabolic and body size responses to a low sugar diet. In female larvae with reduced TOR function (*Target of rapamycin, Tor*; FBgn0021796; genotype $Tor^{\Delta P}$/+), the low sugar-induced increase in whole-body protein was blocked (Fig. 4I). This provides genetic evidence that TOR plays a role in regulating the diet-dependent increase in protein levels, aligning with TOR's well-known effect on protein synthesis (Wullschleger et al., 2006). Because TOR controls growth in part due to regulation of protein synthesis (Killip and Grewal., 2012; Marshall et al., 2012; Rideout et al., 2012; Ghosh et al., 2014; Terada et al., 1995; Barbet et al., 1996; Zhang et al., 2000), we monitored the low sugar-induced increase in body size in larvae with reduced TOR function. In w^{1118} females, larvae reared on 0S were significantly larger than genotype-matched larvae raised on 1S (Fig. 4J); however, 100% of the low sugar-induced increase in body size was blocked in $Tor^{\Delta P}$/+ female larvae (Fig. 4J; genotype:diet interaction $p<0.0001$). This suggests TOR function in females contributes to their larger body size in 0S. In males, while the low sugar-induced increase in body size was 50% blocked in $Tor^{\Delta P}$/+ larvae (Fig. 4K; genotype:diet $p<0.0001$), the magnitude of genotype effects on the diet-induced increase in body size was greater in females than in males (sex:diet:genotype interaction $p=0.0303$). This female-biased effect was robust, as we reproduced the sex:diet:genotype interaction in $Tor^{\Delta P}$/+ larvae across multiple biological

replicates (Fig. S9A-D). This data provides genetic evidence that TOR plays a female-biased role in regulating the metabolic and body size responses to a low sugar diet.

Based on these metabolic and body size effects, we next examined TOR regulation in males and females in 1S and 0S. Our RNAseq data showed that mRNA levels of two TOR-responsive genes, *unkempt* (*unk*; FBgn0004395) and *cabut* (*cbt*; FBgn0043364) (Guertin et al., 2006; Ingaramo et al., 2020; Tiebe et al., 2015), were differentially regulated in a low sugar diet in females but not in males (Fig. 4L). The low sugar diet-induced decrease in both *unk* and *cbt* mRNA is consistent with increased TOR activity (Guertin et al., 2006; Ingaramo et al., 2020; Tiebe et al., 2015), suggesting that a low sugar diet augments TOR in females but not males. When we examined levels of phosphorylated Ribosomal protein S6 kinase (S6k; FBgn0283472) (p-S6k), one of TOR's downstream targets related to ribosomal biogenesis (Miron et al., 2003; Radimerski et al., 2002; Rintelen et al., 2001; Wullschleger et al., 2006; Zhang et al., 2000), we found a trend toward increased p-S6k levels in both sexes ($p=0.0625$) potentially related to an increase in total S6k levels, as the trend was abolished when we monitored the ratio of p-S6k/t-S6k (Fig. S10A-C). While this S6k regulation did not align with the sex-biased regulation of TOR transcriptional targets or our genetic data supporting a female-biased role for TOR in a low sugar context, TOR has many downstream targets that mediate its effects on diverse cellular processes. Thus, future studies will need to investigate TOR targets beyond S6k for sex-biased regulation, and test whether additional targets mediate the female-biased changes to metabolism and body size in a low sugar context. This highlights the importance of studying IIS and TOR

biology in both sexes to gain a deeper understanding of how these key nutrient-sensing pathways couple metabolic regulation with diet.

Overall, our findings support a clear role for the upregulation of IIS as a key mechanism underlying the metabolic and body size responses in male larvae reared on a low sugar diet. In females, TOR had female-biased effects on the low sugar-induced changes to metabolism and body size. Together with the differential transcriptional responses in 0S between males and females, these data suggest that the mechanisms underlying the low sugar-induced increase in body size are not fully shared between the sexes. This highlights the importance of including both males and females in larval growth studies, as not all mechanisms will be shared between the sexes even in contexts where the phenotypic response is equivalent.

MATERIALS AND METHODS

Data availability. Original images are available upon request. Raw values for all data collected and displayed in this manuscript are available in Supplemental File 3. All data necessary for confirming the conclusions of the article are present within the article, figures, tables, and Supplemental files.

Fly husbandry. Our 1S diet consists of 20.5 g/L sucrose, 70.9 g/L D-glucose, 48.5 g/L cornmeal, 45.3 g/L yeast, 4.55 g/L agar, 0.5g $CaCl_2•2H_2O$, 0.5 g $MgSO_4•7H_2O$, 11.77 mL acid mix (propionic acid/phosphoric acid). Our 0S diet consists of 48.5 g/L cornmeal, 45.3 g/L yeast, 4.55 g/L agar, 0.5g $CaCl_2•2H_2O$, 0.5 g $MgSO_4•7H_2O$, 11.77 mL acid mix. Details of 0.75S, 0.5S, and 0.25S diets can be found in Supplemental file 4. Larvae

were raised at a density of 50 animals per 10 mL food at 25°C, and sexed by gonad size as mid-third instar larvae at the time of collection for gene expression or metabolic experiments (108 hr after egg-laying). For pupal volume experiments, animals were separated by sex between 0-12 hr after puparium formation. Adult flies were maintained at a density of twenty flies per vial in single-sex groups. For all metabolic, gene expression, imaging, insulin stimulation, and Western blotting experiments, larvae were collected at 108 hr after egg-laying.

Fly strains. The following fly strains from the Bloomington *Drosophila* Stock Center were used: w^{1118} (#3605), InR^{E19} (#9646), $Tor^{\Delta P}$ (#7014). Additional fly strains include: *dilp2, dilp3, dilp5,* and *dilp2-3,5* (Grönke et al., 2010). All fly strains were backcrossed for at least 6 generations, in addition to extensive prior backcrossing (Grönke et al., 2010; Millington et al., 2021a; Millington et al., 2021b).

Body size. Pupal volume and adult weight were measured as previously described (Delanoue et al., 2010; Millington et al., 2021a; Millington et al., 2021b; Rideout et al., 2015). For pupal volume one biological replicate consists of one pupa, for adult weight one biological replicate consists of one tube of 10 flies.

Feeding behaviour. Feeding behavior was quantified as number of mouth-hook contractions per 30 s. One biological replicate represents one larva.

Developmental timing. Time to pupariation was measured as previously described (Millington et al., 2021a). Time to 50% pupariation was calculated per replicate and used for quantification and statistical analysis. One biological replicate consists of 50 animals in one vial, with pupae sexed at 12 hr intervals to determine pupariation in both sexes.

Metabolism assays. One biological replicate consists of ten female or male larvae. Larvae were frozen on dry ice, and homogenized in appropriate buffers to measure whole-body lipid, protein, glucose, glycogen, and trehalose levels. All assays were performed as described in Tennessen et al. (2014) and Wat et al. (2020) and according to manufacturer's instructions.

RNA extraction and cDNA synthesis. RNA extraction and cDNA synthesis were performed as previously described (Marshall et al., 2012; Rideout et al., 2012; Rideout et al., 2015; Wat et al., 2020). Briefly, each biological replicate consists of ten w^{1118} larvae frozen on dry ice and stored at -80°C. Each experiment contained 3-4 biological replicates per sex, and each experiment was performed at least twice. RNA was extracted using 500 μl Trizol (Thermo Fisher Scientific: #15596018) and precipitated using isopropanol and 75% ethanol. Pelleted RNA was resuspended in 200 μl molecular biology grade water (Corning, 46-000-CV) and stored at -80°C until use. For cDNA synthesis, an equal volume of RNA per reaction was DNase-treated and reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, 205314).

Quantitative real-time PCR (qPCR). qPCR was performed as previously described (Marshall et al., 2012; Rideout et al., 2012; Rideout et al., 2015; Wat et al., 2020). Each biological replicate consists of 10 larvae. Values displayed in each graph represent the fold change for a gene's mRNA, normalized to *Act5c* and β-*tub*, housekeeping genes that were not differentially regulated between the sexes or between 1S and 0S. A complete primer list is included as Supplemental file 5.

Preparation of protein samples, SDS-PAGE, and Western blotting. Samples were generated as previously described (Millington et al., 2021a). 20 μg of protein was loaded per lane, separated on a 12% SDS-PAGE gel in SDS running buffer, and transferred onto a nitrocellulose membrane (Bio-Rad) for 2 hr at 40 V on ice. Membranes were incubated for 24 hr in blocking buffer at 4°C (5% milk or 5% BSA in TBST 0.1%) and subsequently incubated with primary antibodies overnight at 4°C. Anti-pS6k (#9209, Cell Signaling), anti-tS6k (gift from A. Teleman), and anti-Actin (#8432, Santa Cruz) were used at 1:1000. After 3 x 2 min washes in 0.1% TBST, HRP-conjugated secondary antibodies were used at 1:5000 for pS6k (#65–6120; Invitrogen) and 1:3000 for actin (#7076; Cell Signaling). Membranes were washed (3 x 2 min, 2 x 15min) in 0.1% TBST, washed 1 x 5 min in TBS, and finally Pierce ECL was applied according to the manufacturer's instructions (#32134, Thermo Scientific). To measure the concentration of all proteins loaded in a sample, we used the stain-free labeling system from BioRad (Cat# 1610185) according to manufacturer's instructions.

GFP-PH localization and insulin sensitivity. To measure IIS activity in the fat body, late third instar larvae were collected at 108 hours AEL and washed in PBS. Twelve to fifteen larvae were collected for each batch and inverted in PBS; inverted carcasses were fixed in 4% paraformaldehyde in PBS on a rocking platform at room temperature for 40 min, then washed twice in PBS. Fixed fat bodies were mounted in SlowFade Diamond mounting media (S36972, Thermo Fisher Scientific) prior to imaging. To measure insulin sensitivity, late third instar larvae were collected at 108 hours AEL and washed in PBS once. The larvae were then moved into a dish with ice-cold Schneider's *Drosophila* Media (21720024, Thermo Scientific) and inverted. Inverted carcasses (with fat body) were immediately moved to a separate dish with fresh Schneider's. Once all dissections were complete, Schneider's media was replaced with 2 mL of fresh Schneider's *Drosophila* Media containing a final concentration of 0.5 µM recombinant human insulin (I2643, Sigma-Aldrich) and incubated at room temperature for 20 min. Carcasses were then washed once with PBS and fixed with 4% paraformaldehyde for 40 min, washed twice with PBS, and mounted in SlowFade Diamond mounting media (S36972, Thermo Fisher Scientific). Images were acquired on a Leica SP8 confocal microscope with 20X objective, and quantified using Fiji (Schindelin et al., 2012). For each cell, the average GFP intensity of a short stretch of cell membrane and a small stretch of adjacent were quantified, and the ratio of membrane to cytosolic GFP was calculated.

RNA sequencing and analysis. RNA from w^{1118} males and females reared on either the 1S or 0S diet was sequenced to determine gene expression. Quality control of samples was performed using the Agilent 2100 Bioanalyzer to confirm sample quality. Sequencing was performed using on a NextSeq 500 sequencer. Sequence quality was assessed using FastQC on Illumina BaseSpace**.** Reads were aligned to *Drosophila melanogaster* genome (UCSC dm3) using STAR aligner with default settings. All raw sequencing data has been deposited and is publicly available through the NCBI Gene Expression Omnibus. For differential expression analysis, fragments per kilobase of transcript per million mapped reads were calculated using the DESeq2 normalization method. Log fold changes and significance for differential expression were determined and a significance threshold of 0.05 was selected for adjusted *p* values determined with Bonferroni correction. Differentially expressed genes are presented in Supplemental file 2.

Statistical analysis. GraphPad Prism (GraphPad Prism version 8.4.3 for Mac OS X) was used for all statistical tests, and for figure preparation. For normally distributed data with comparisons between two experimental groups, a Student's *t*-test was used to determine significance. For normally distributed data with comparisons between more than two experimental groups, one-way ANOVAs were used to determine significance. In order to determine significant interactions between sex, diet, and genotype, multivariate ANOVAs (2-way, and 3-way ANOVAs) were used on normally distributed data. For non-normally distributed data, we used Wilcoxon signed rank tests for

comparisons between two groups. A complete list of *p*-values and full details of statistical tests is provided in Supplemental file 1.

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Figures

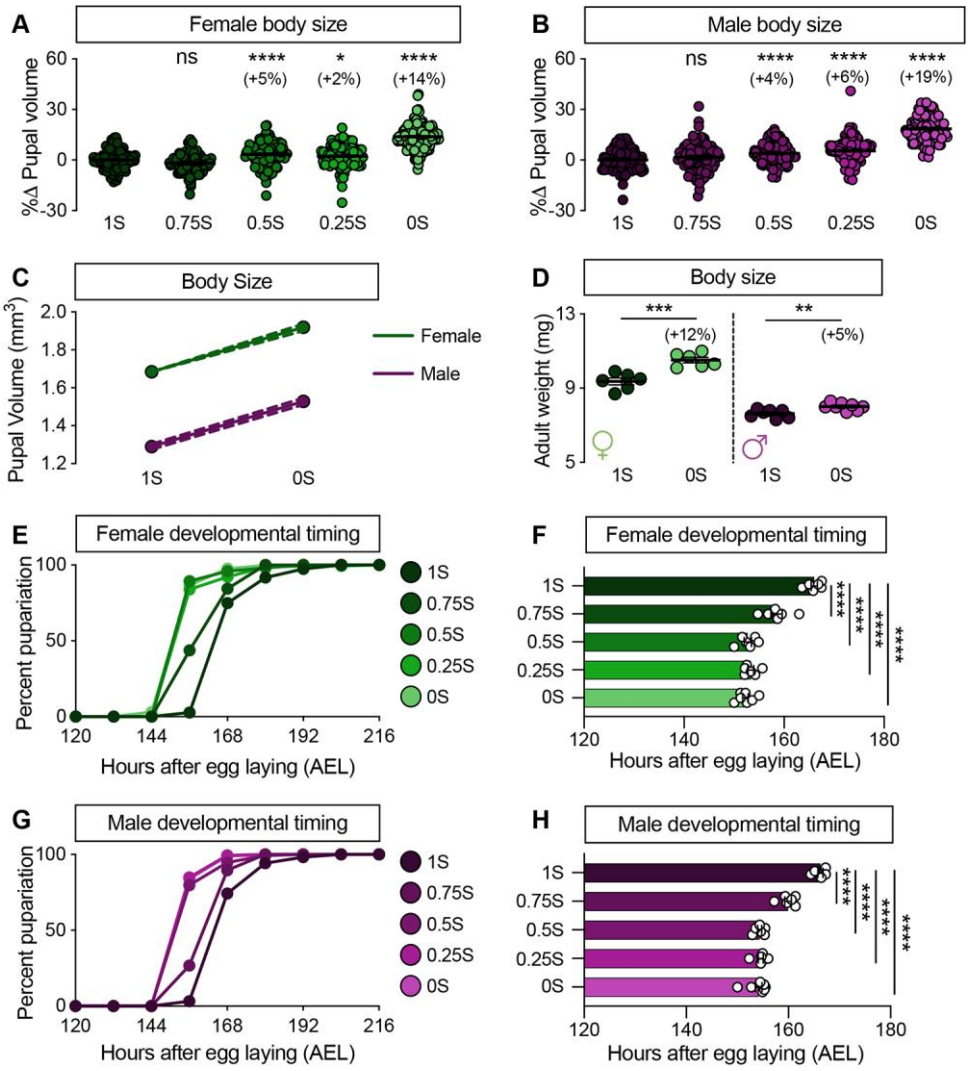

Figure 1. A low sugar diet promotes an increased rate of growth and larger body size. (A) Pupal volume in *w*^{1118} females cultured on 1S, 0.75S, 0.5S, 0.25S, and 0S (one-way ANOVA, Dunnett's multiple comparison test). n=100-160 pupae. (B) Pupal volume in *w*^{1118} males cultured on 1S, 0.75S, 0.5S, 0.25S, and 0S (one-way ANOVA, Dunnett's multiple comparison test). n=100-121. (C) Reaction norms for pupal volume in

both sexes plotted using 1S and 0S data from A and B (two-way ANOVA). (D) Adult weight in w^{1118} female and male flies reared on 1S and 0S (Student's *t* test). n=6-8 replicates of 10 adult flies each. (E, F) Time to pupariation in w^{1118} females cultured on 1S, 0.75S, 0.5S, 0.25S, and 0S (one-way ANOVA, Tukey HSD test). n=6-8 biological replicates of 50 larvae. (G, H) Time to pupariation in w^{1118} males cultured on 1S, 0.75S, 0.5S, 0.25S, and 0S (one-way ANOVA, Tukey HSD test). n=6-8 biological replicates of 50 larvae. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; ns indicates not significant; error bars indicate SEM; dashed lines indicate 95% confidence interval. To make percent change in pupal volume whole numbers, decimals <0.5 were rounded down, and decimals >0.5 were rounded up. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

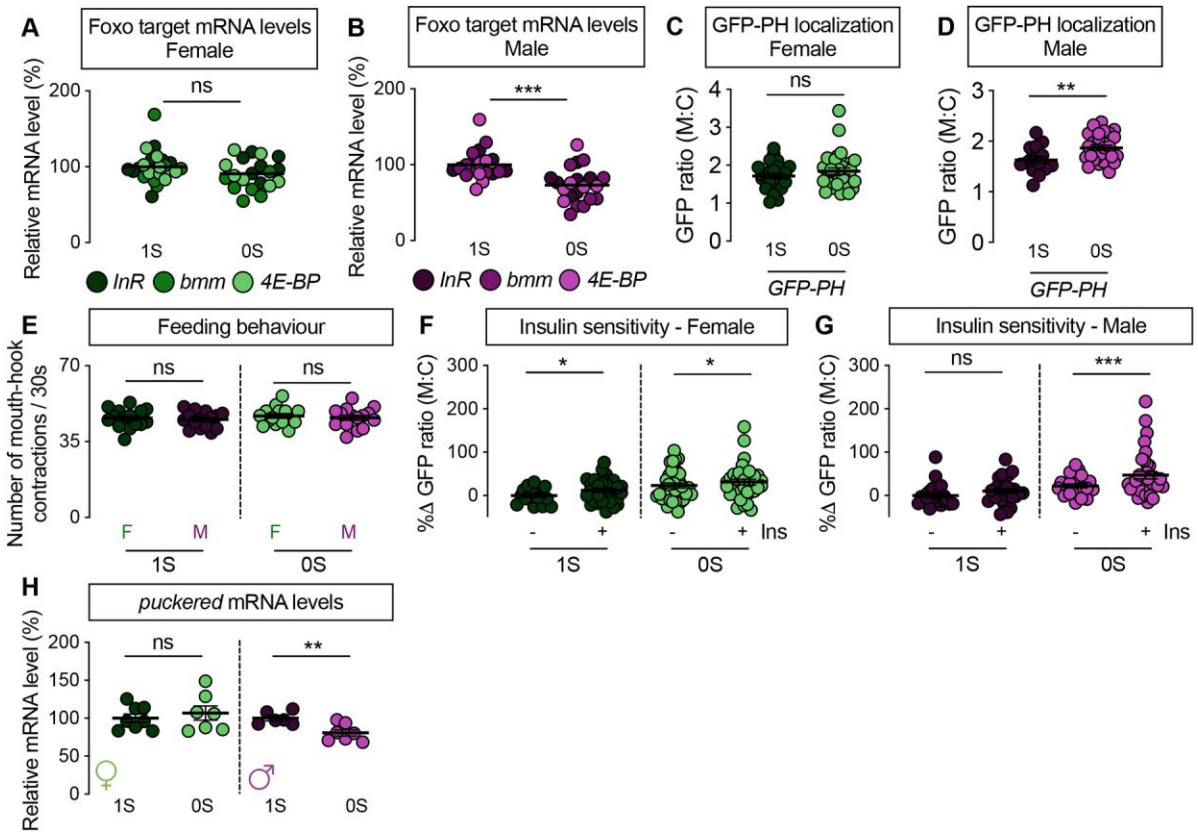

Figure 2. A low sugar diet has sex-specific effects on IIS pathway activity via regulation of insulin sensitivity. (A) mRNA levels of Foxo target genes (*InR*, *bmm*, and *4E-BP*) in female larvae reared on 1S or 0S (Student's *t* test). n=7-8 biological replicates. (B) mRNA levels of Foxo target genes in male larvae reared on 1S or 0S (Student's *t* test). n=7 biological replicates. (C) Ratio of cell surface membrane-associated GFP-PH and cytoplasmic GFP-PH (GFP ratio [M:C]) in dissected fat bodies of female larvae. The ratio was not significantly different between 1S and 0S (Student's *t* test). n=23-30 biological replicates. (D) In males, GFP-PH M:C ratio was significantly higher in males cultured on 0S than 1S (Student's *t* test). n=15-26. (E) Mouth-hook contractions in w^{1118} female and male larvae raised on 1S or 0S (Student's *t* test). n=15 larvae. (F) In females, GFP-PH M:C ratio was significantly higher in females cultured on

0S and 1S when treated with insulin (two-way ANOVA, Bonferroni's multiple comparisons test). n=16-37. (G) In males, GFP-PH M:C ratio was significantly higher in males cultured on 0S but not 1S when treated with insulin (two-way ANOVA, Bonferroni's multiple comparisons test). n=22-37. (H) mRNA levels of *puc* in female and male larvae raised in 1S or 0S (Student's *t* test). n=6-8 biological replicates. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; ns indicates not significant; error bars indicate SEM. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

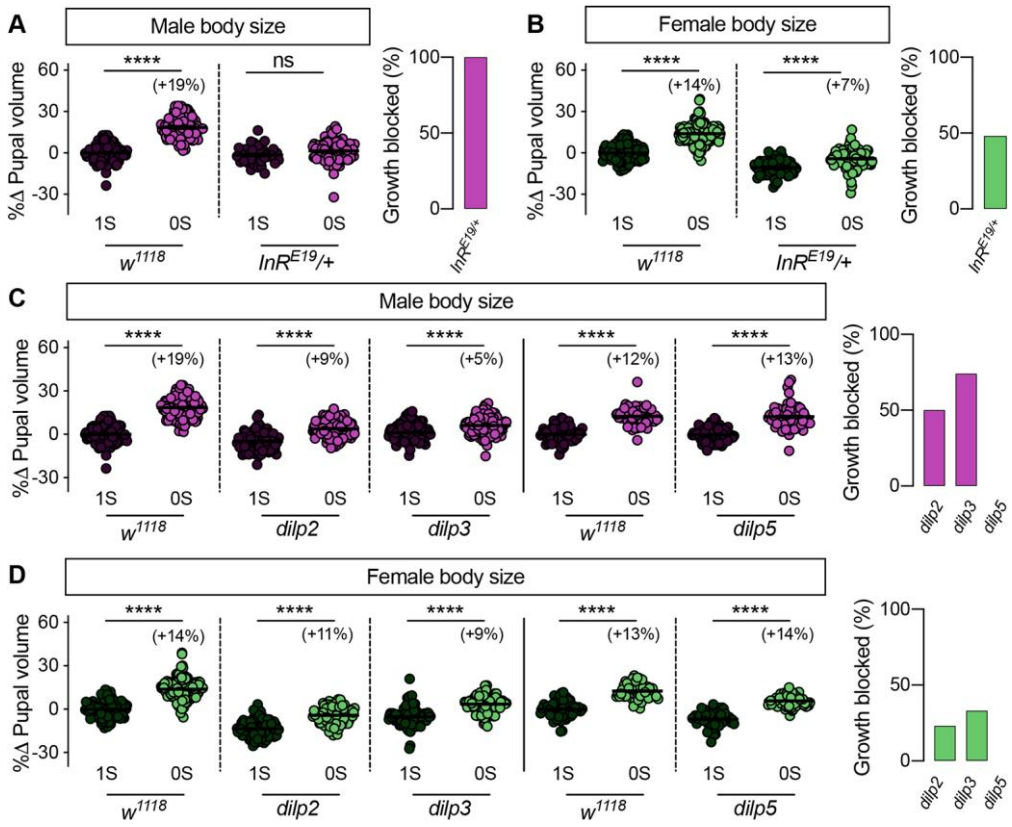

Figure 3. Male-biased requirement for IIS and *Drosophila* insulin-like peptides in promoting the low sugar-induced increase in body size. (A) Pupal volume in w^{1118} and InR^{E19}/+ males cultured on 1S or 0S (two-way ANOVA, Tukey HSD test). n=36-120. (B) Pupal volume in w^{1118} and InR^{E19}/+ females cultured on 1S or 0S (two-way ANOVA, Tukey HSD test). n=73-160. (C) Pupal volume in w^{1118}, *dilp2* mutant, *dilp3* mutant, and *dilp5* mutant males reared on 1S or 0S (two-way ANOVA, Tukey HSD test). n=69-120. (D) Pupal volume in w^{1118}, *dilp2* mutant, *dilp3* mutant, and *dilp5* mutant females reared on 1S or 0S (two-way ANOVA, Tukey HSD test). n=55-160. To calculate sex:diet:genotype interactions three-way ANOVAs were used. * p<0.05; *** p<0.001; **** p<0.0001; ns indicates not significant; error bars indicate SEM. To make percent change in pupal volume whole numbers, decimals <0.5 were rounded down, and

decimals >0.5 were rounded up. *p*-values, samples sizes, and statistical tests are in Supplemental file 1. Note: parallel data collection means that w^{1118} control data in 0S and 1S are the same in Fig. 1A, B, 3A-D.

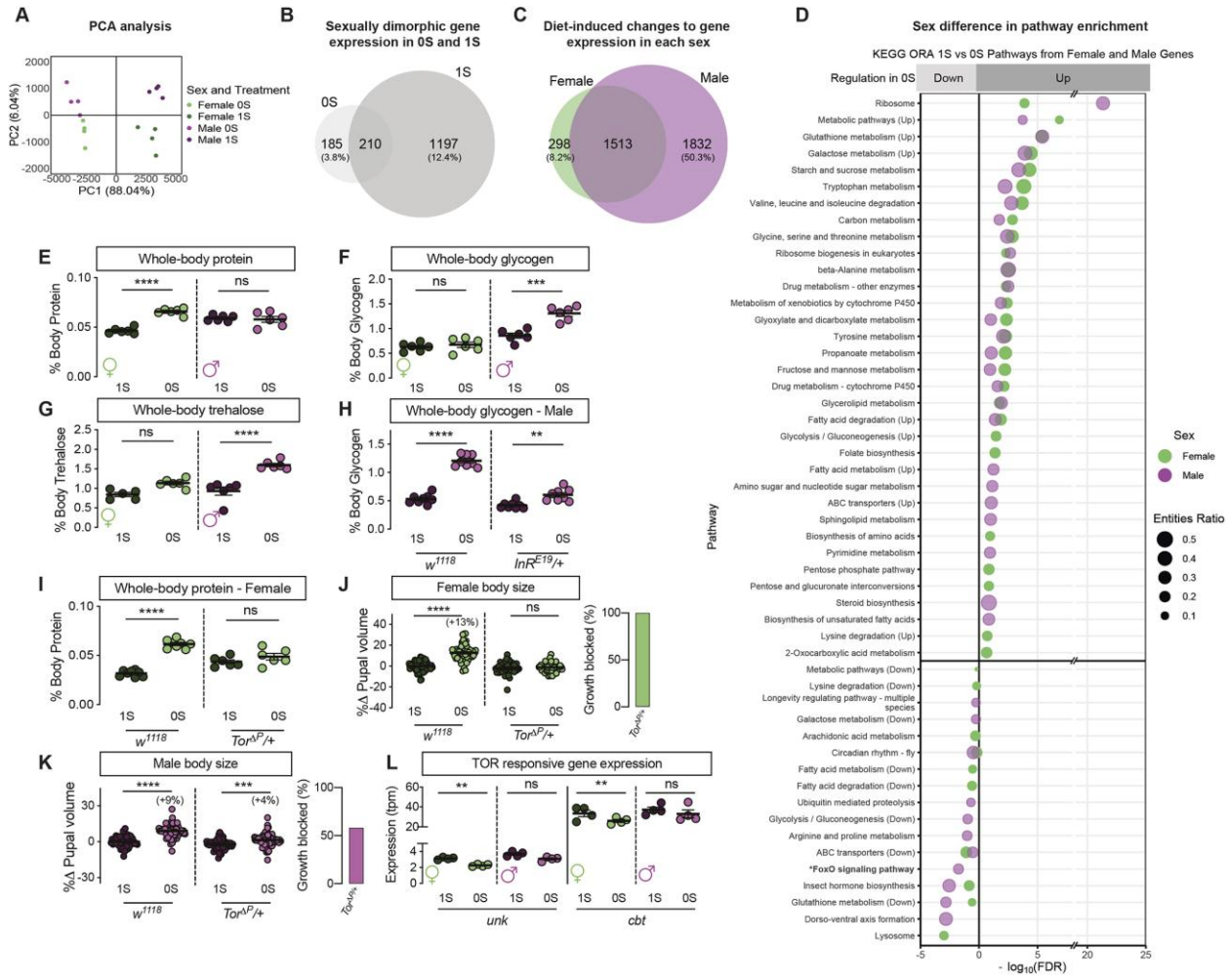

Figure 4. A low sugar diet has sex-biased effects on metabolic gene expression and metabolism. (A) PCA plot for male and female larvae reared on 1S or 0S diets separates by sex and diet. (B) Venn diagram of sexually dimorphic gene expression in larvae reared on 0S or 1S. (C) Venn diagram of diet-induced changes to gene expression in each sex. (D) KEGG enrichment analysis shows pathways differentially regulated between 0S and 1S in males and females. (E) Whole-body protein levels in w^{1118} female and male larvae raised on 1S or 0S (Student's *t* test). n=6 biological replicates. (F) Whole-body glycogen levels in w^{1118} female and male larvae reared on 1S or 0S (Student's *t* test). n=6 biological replicates. (G) Whole-body trehalose levels in

w^{1118} female and male larvae cultured on 1S or 0S (Student's *t* test). n=5-6 biological replicates. (H) Whole-body glycogen levels in w^{1118} and InR^{E19}/+ male larvae reared on 1S or 0S (two-way ANOVA, Tukey HSD test). n=8 biological replicates. (I) Whole-body protein levels in w^{1118} and $Tor^{\Delta P}$/+ female larvae reared on 1S or 0S (two-way ANOVA, Tukey HSD test). n=6-8 biological replicates. (J) Pupal volume in w^{1118} and $Tor^{\Delta P}$/+ females cultured on 1S or 0S (two-way ANOVA, Tukey HSD test). n=58-98. (K) Pupal volume in w^{1118} and $Tor^{\Delta P}$/+ males cultured on 1S or 0S (two-way ANOVA, Tukey HSD test). n=58-69. (L) Expression of *unkempt* and *cabut* in females and males raised in 1S and 0S from RNA-seq data (adjusted *p* values calculated in DESeq2). * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001; ns indicates not significant; error bars indicate SEM. To make percentage change pupal volume whole numbers, decimals <0.5 were rounded down, and decimals >0.5 were rounded up. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

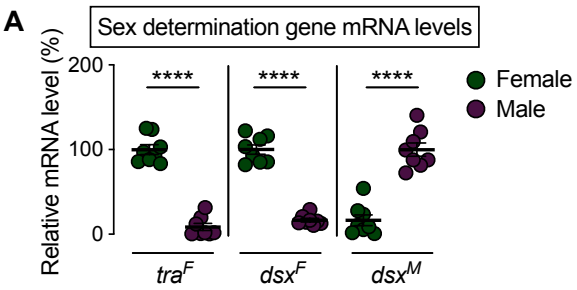

Fig. S1. Sex determination gene expression in sexed larvae. (A) mRNA levels of sex-specific isoforms of sex determination genes in male and female larvae (Student's *t* test). Females are expected to show expression of the female-specific isoforms of *transformer* (tra^F) and *doublesex* (dsx^F), whereas males are expected to show expression of the male-specific isoform of *doublesex* (dsx^M) . **** p<0.0001; error bars indicate SEM. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

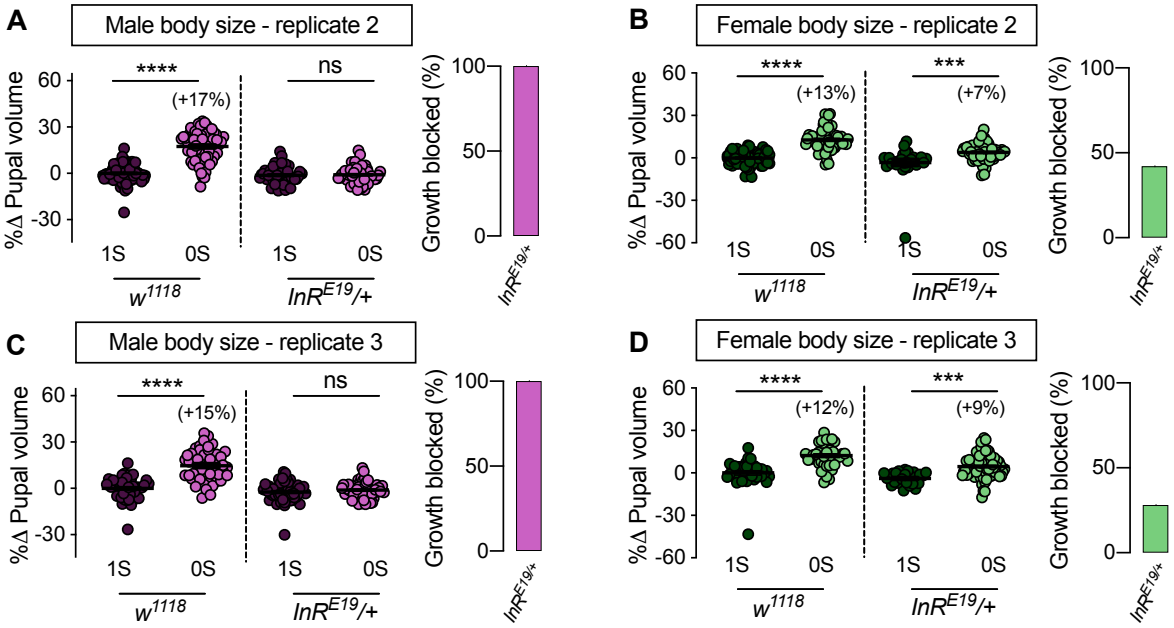

Fig. S2. Male-biased requirement for IIS in promoting the low sugar-induced increase in body size. (A) Pupal volume in w^{1118} and InR^{E19}/+ females cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 27-61. (B) Pupal volume in w^{1118} and InR^{E19}/+ males cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 48-74. (C) Pupal volume in w^{1118} and InR^{E19}/+ females cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 26-73. (D) Pupal volume in w^{1118} and InR^{E19}/+ males cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 45-51. To calculate sex:diet:genotype interactions three-way ANOVAs were used. *** p<0.001; **** p<0.0001; ns indicates not significant; error bars indicate SEM. To make percentage change pupal volume whole numbers, decimals <0.5 were rounded down, and decimals >0.5 were rounded up. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

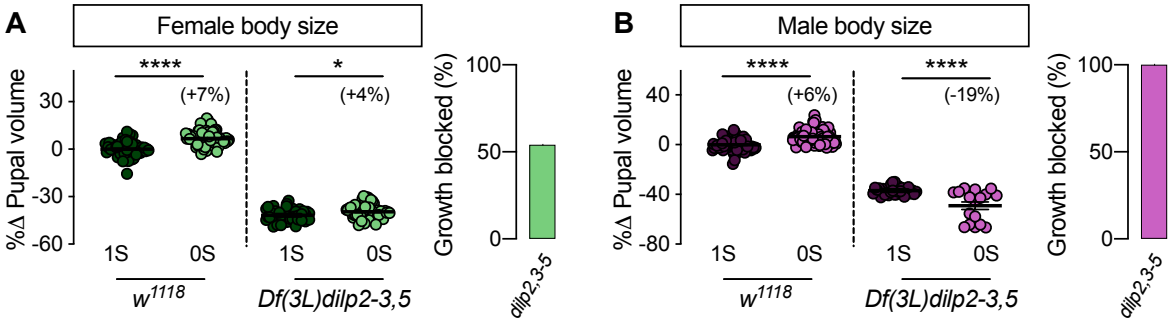

Fig. S3. Sex-biased requirement for *Drosophila* insulin-like peptides in promoting the low sugar-induced increase in body size. (A) Pupal volume in w^{1118} and *dilp2-3,5* females cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 50-69. (B) Pupal volume in w^{1118} and *dilp2-3,5* males cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 17-97. To calculate sex:diet:genotype interactions three-way ANOVAs were used. * p<0.05; **** p<0.0001; ns indicates not significant; error bars indicate SEM. To make percentage change pupal volume whole numbers, decimals <0.5 were rounded down, and decimals >0.5 were rounded up. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

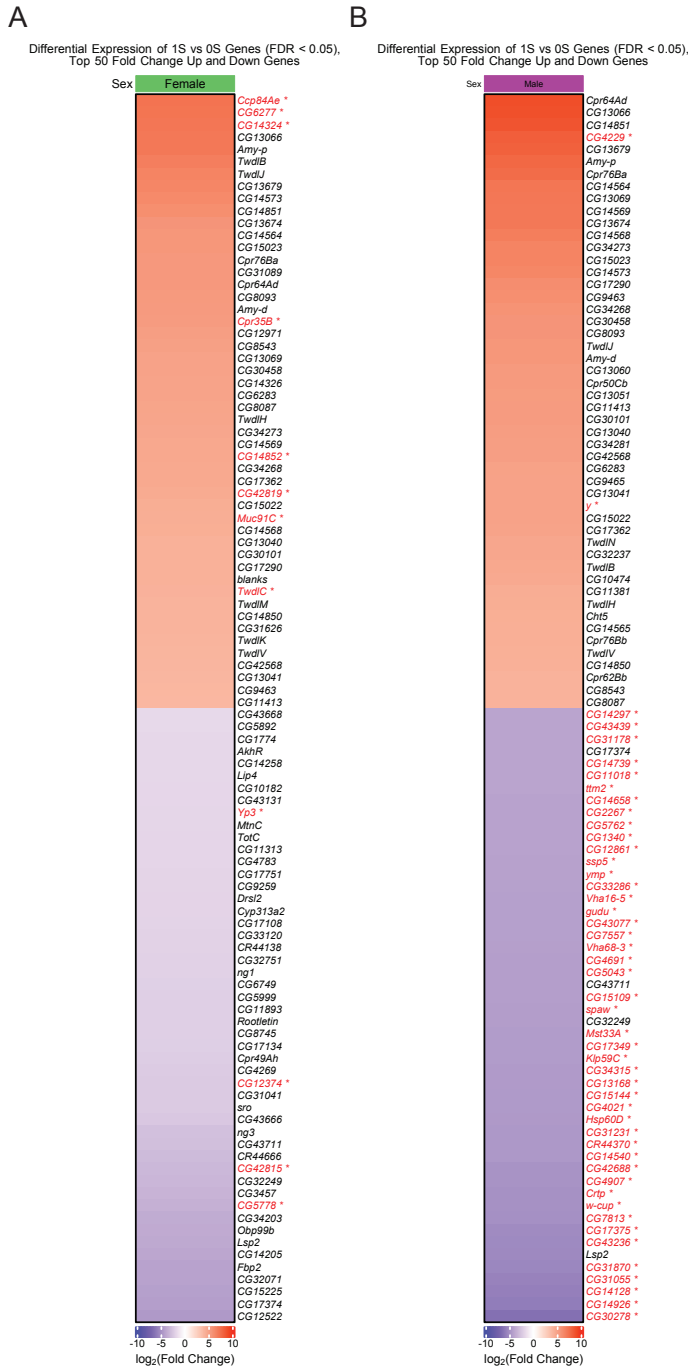

Fig. S4. Sex-specific changes in gene expression in response to a low sugar diet. (A) Top 50 differentially expressed upregulated and downregulated genes in females reared on a 0S diet. Differentially expressed genes unique to females are labelled in red. (B) Top 50 differentially expressed upregulated and downregulated genes in males reared on a 0S diet. Differentially expressed genes unique to males are labelled in red. A list of all differentially expressed genes is provided in Supplemental file 2.

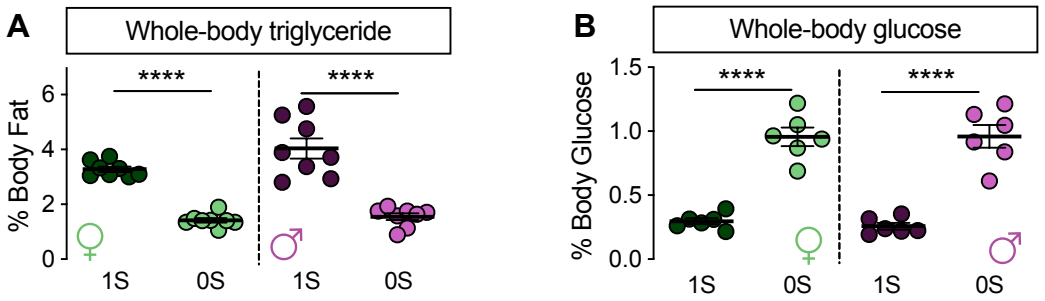

Fig. S5. Non-sex-specific changes to whole body triglyceride and glucose levels in larvae raised on a low-sugar diet. (A) Whole-body triglyceride levels in w^{1118} female and male larvae reared on 1S or 0S (Student's *t* test). n = 8 biological replicates. (B) Whole-body glucose levels in w^{1118} female and male larvae cultured on 1S or 0S (Student's *t* test). n = 6 biological replicates. **** p<0.0001; error bars indicate SEM. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

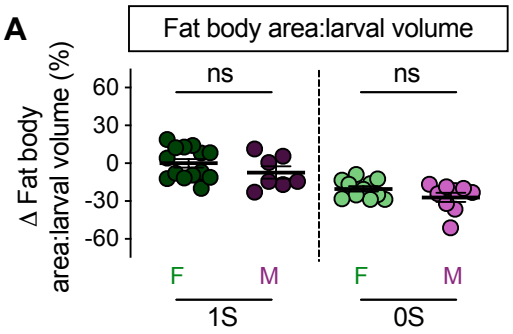

Fig. S6. Scaling between fat body area and body size does not differ between males and females reared on 0S or 1S. (A) The ratio of fat body area to larval volume is not different between females and males reared on either 1S or 0S (Student's *t* test). n = 7-14. ns indicates not significant; error bars indicate SEM. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

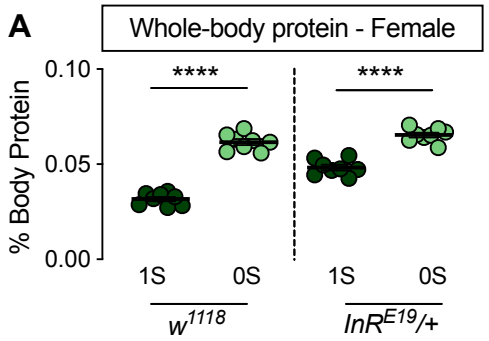

Fig. S7. Reduced IIS function does not block the female-specific increase in whole-body protein in 0S. (A) Whole-body protein levels in w^{1118} and InR^{E19}/+ female larvae reared on 1S or 0S. n = 8 biological replicates (two-way ANOVA followed by Tukey HSD test). **** p<0.0001; ns indicates not significant; error bars indicate SEM. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

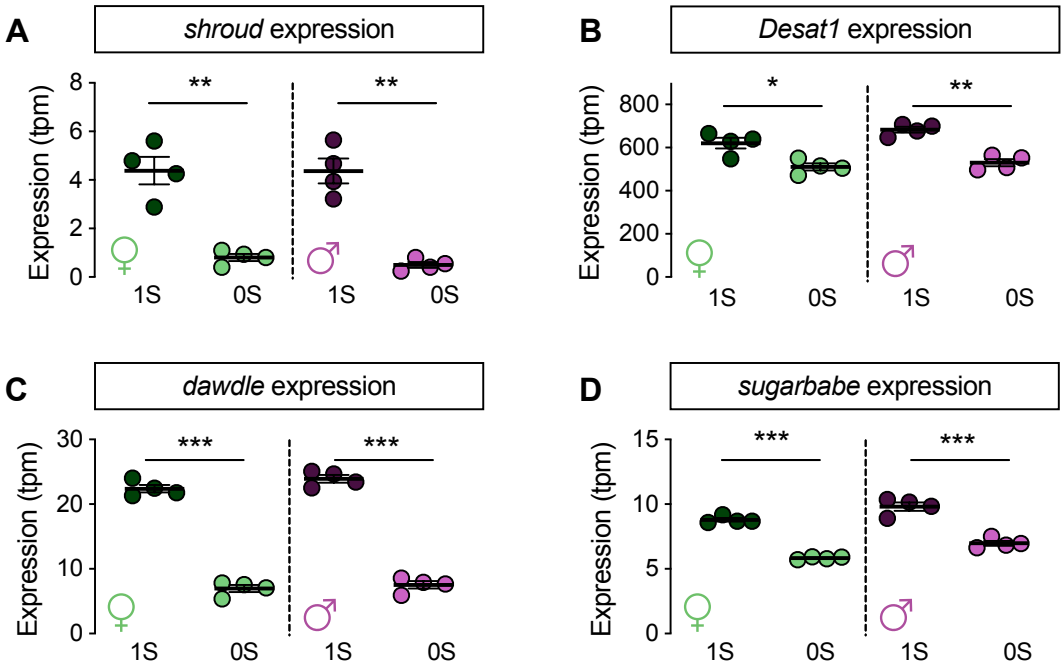

Fig. S8. Non-sex-specific changes to *Mondo*/*bigmax* target expression in larvae raised on a low-sugar diet. (A) Expression of *shroud* in females and males raised in 1S and 0S from RNA-seq data (Analyzed using adjusted *p* values calculated in DESeq2). (B) Expression of *Desat1* in females and males raised in 1S and 0S from RNA-seq data (Analyzed using adjusted *p* values calculated in DESeq2). (C) Expression of *dawdle* in females and males raised in 1S and 0S from RNA-seq data (Analyzed using adjusted *p* values calculated in DESeq2). (D) Expression of *sugarbabe* in females and males raised in 1S and 0S from RNA-seq data (Analyzed using adjusted *p* values calculated in DESeq2). ** p<0.01; *** p<0.001; ns indicates not significant; error bars indicate SEM. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

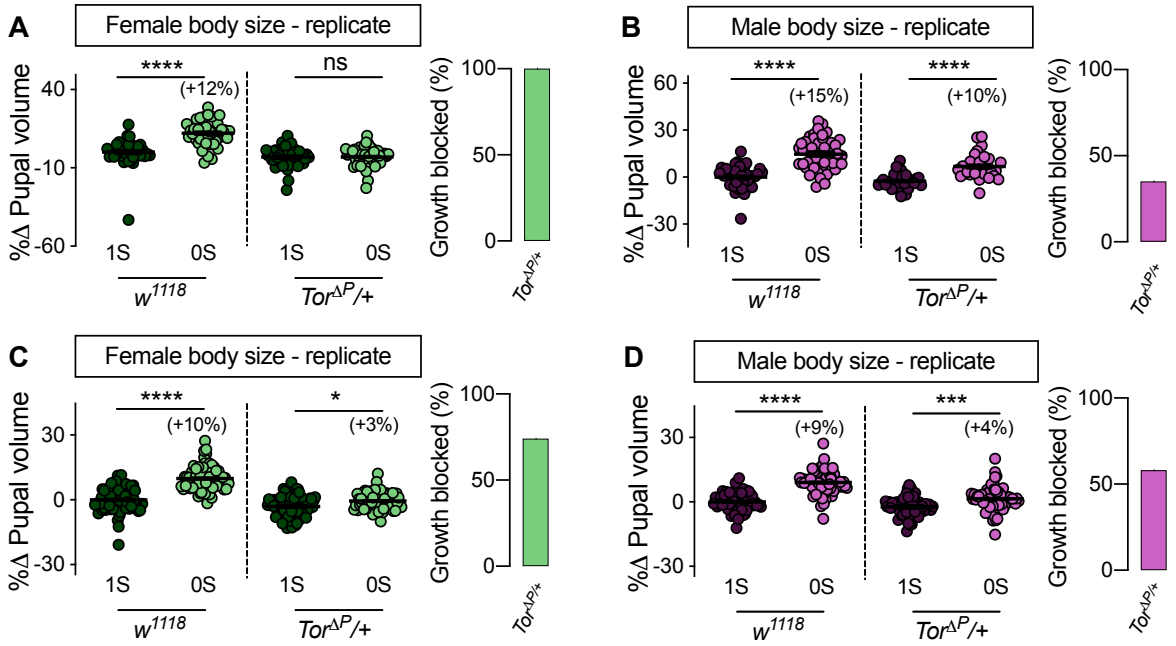

Fig. S9. Female-biased requirement for Tor in regulating the low sugar-induced increase in body size. (A) Pupal volume in w^{1118} and $Tor^{\Delta P}$/+ females cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 39-53. (B) Pupal volume in w^{1118} and $Tor^{\Delta P}$/+ males cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 29-49. (C) Pupal volume in w^{1118} and $Tor^{\Delta P}$/+ females cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 49-61. (D) Pupal volume in w^{1118} and $Tor^{\Delta P}$/+ males cultured on 1S or 0S (two-way ANOVA followed by Tukey HSD test). n = 56-74. To calculate sex:diet:genotype interactions three-way ANOVAs were used. *** p<0.001; **** p<0.0001; ns indicates not significant; error bars indicate SEM. To make percent change in pupal volume whole numbers, decimals <0.5 were rounded down, and decimals >0.5 were rounded up. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

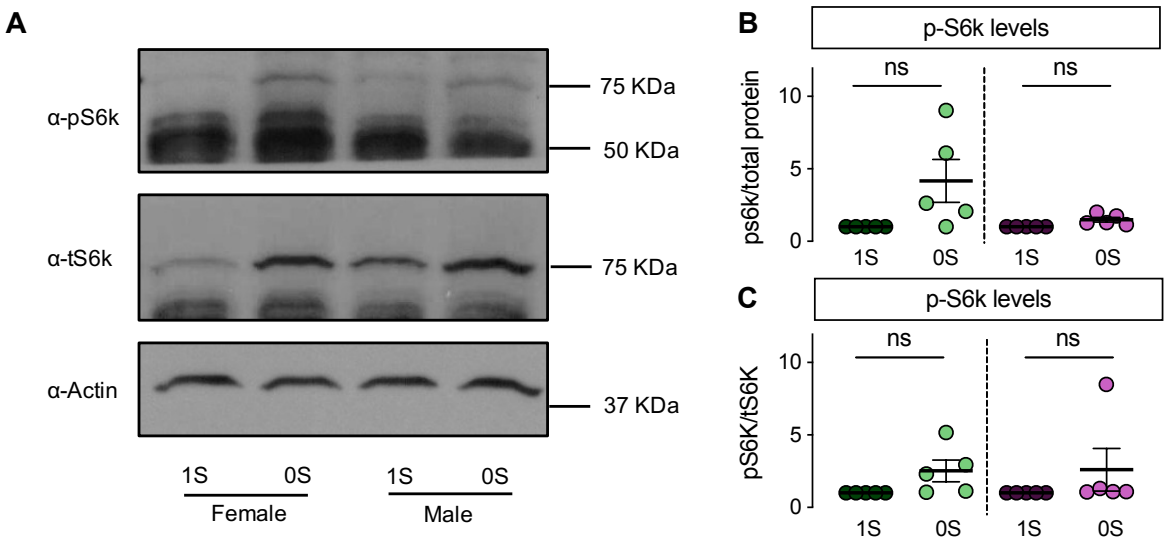

Fig. S10. Non-sex-specific trend toward increased phosphorylated S6k in a low sugar diet. (A) Representative blots of phospho-S6k, total S6K, and actin in male and female larvae raised in 1S or 0S diets. (B) Quantification of p-S6k/total protein levels in male and female larvae raised in 1S or 0S (Wilcoxon signed rank test). (C) Quantification of p-S6K/t-S6k levels in 1S or 0S (Wilcoxon signed rank test). ** $p<0.01$; ns indicates not significant; error bars indicate SEM. *p*-values, samples sizes, and statistical tests are in Supplemental file 1.

Table S1.

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Table S2.

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