Sex-biased regulation of body size by nutrient-responsive signaling pathways in *Drosophila*

by

JASON WILLIAM MILLINGTON

B.Sc.(hons), University of Nottingham, 2015

A THESIS SUBMITTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Cell and Developmental Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

August 2021

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

Sex-biased regulation of body size by nutrient-responsive signaling pathways in *Drosophila*

submitted by   Jason William Millington   in partial fulfilment of the requirements for

the degree of   Doctor of Philosophy

in   Cell and Developmental Biology

Examining Committee:

Dr. Elizabeth J. Rideout, Cellular and Physiological Sciences, UBC

Supervisor

Dr.Vanessa Auld, Zoology, UBC

Supervisory Committee Member

Dr. Carolyn Brown, Department of Medical Genetics, UBC

University Examiner

Dr. Daniel S. Luciani, Department of Surgery, UBC

University Examiner

Dr. Bruce Edgar, Department of Oncological Sciences, University of Utah

External Examiner

ABSTRACT

Sexual size dimorphism (SSD) is common throughout the animal kingdom. In the fruit fly, *Drosophila melanogaster*, females are ~30% larger than males. Over the past two decades, studies in *Drosophila* have expanded our knowledge of the genetic and dietary requirements for growth. However, it remains incompletely understood how males and females differ in the regulation of growth. The insulin/insulin-like growth factor signaling pathway (IIS) was found to be a key regulator of nutrient-dependent growth and body size. The appropriate coupling of growth with dietary nutrients is known as body size plasticity. Recent studies have implicated both dietary nutrients and IIS in establishing SSD, but the mechanism remains poorly understood. To better understand how males and females differ in growth, I used *Drosophila* to perform a series of studies examining the contribution of nutrients and IIS on growth in both sexes.

In Chapter 2, I found that IIS activity is required for increased female body size. Further, genetically augmenting IIS in males is sufficient for increased body size. In Chapter 3, I build upon this characterization and identify that in a high protein dietary context, females increase IIS activity and body size more than males. This results in increased female body size plasticity. Specifically, when dietary protein is abundant, females produce high levels of the insulinotropic factor Stunted which promotes increased IIS and larger body size. This mechanism was dependent on the sex determination gene *transformer*. These findings elucidate a molecular mechanism underlying the sex difference in body size plasticity. In Chapter 4, I present evidence that in a low-sugar dietary context both sexes increase growth via distinct mechanisms to achieve the same phenotype. Specifically, males increase IIS activity whereas females increase target of rapamycin (TOR) signaling to reach a larger body size. Together, my thesis provides novel mechanistic insight into how males and females differ in their phenotypic response to genetic manipulation and dietary manipulation. This work provides the basis for future studies to identify conserved sex differences in the regulation of nutrient-responsive pathways, and ultimately will inform our knowledge of the sex-biased risk of human metabolic disease.

LAY SUMMARY

In most animals, males and females differ in body size. Dietary nutrients are required for animals to grow, and when nutrients are low males and females no longer differ in body size. However, we don't fully understand the genes involved in how males and females become different in size in response to nutrients. The goal of this thesis was to use the fruit fly to identify these genes involved in growth in both sexes in response to nutrients. We show that the insulin signaling pathway, is crucial for this sex difference in body size. We find that how males and females couple insulin signaling with nutrients is different and this affects the sex difference in body size. These results are important because by understanding how nutrients affect insulin signaling differently in males and females, we can better understand sex differences in the risk of developing metabolic diseases like diabetes.

PREFACE

Chapter 2: **"Genetic manipulation of insulin/insulin-like growth factor signaling pathway activity has sex-biased effects on *Drosophila* body size"**

Work in this chapter resulted in the manuscript published as, "Millington, J.W., Brownrigg, G.P., Basner-Collins, P.J., Sun, Z., Rideout, E.J. 2021. "Genetic manipulation of insulin/insulin-like growth factor signaling pathway activity has sex-biased effects on *Drosophila* body size" *G3: Genes, Genomes, Genetics*, jkaa067." The content of this publication is reprinted and modified for the purposes of this thesis with permission from the publisher. For this publication, I performed all experiments and analysis presented. Elizabeth Rideout and I designed the experiments and the methods of analysis, and I wrote the first draft of the manuscript. George P. Brownrigg aided with the experiments presented in Figure 4 of the final manuscript, Paige J. Basner-Collins aided with the experiments presented in Figure 1 of the final manuscript, and Ziwei Sun aided with the experiments presented in Figure 2. The automated quantification of pupal volume images utilised in this study was developed together with Charlotte Chao.

Chapter 3: **"Female-biased upregulation of insulin pathway activity mediates the sex difference in *Drosophila* body size plasticity"**

Work in this chapter resulted in the manuscript published as, "Millington, J.W., Brownrigg, G.P., Chao, C., Sun, Z., Basner-Collins, P.J., Wat, L.W., Hudry, B., Miguel-Aliaga, I., Rideout, E.J. 2021. "Female-biased upregulation of insulin pathway activity mediates the sex difference in *Drosophila* body size plasticity" *eLife*. 10:e58341." The content of this publication is reprinted and modified for the purposes of this thesis with permission from the publisher. For this publication, I was aided by the following contributions:

- George P. Brownrigg performed western blots on samples I generated which was presented as Figure 3 – figure supplement 1A, and Figure 5 – figure supplement 1A-D

- Charlotte Chao dissected, and imaged larval fat bodies to quantify a fluorescent readout of insulin signaling activity which was presented as Figure 1F and 1H in the final manuscript.
- Ziwei Sun and Paige J. Basner-Collins aided with the separation of male and female flies for body size experiments.
- Lianna W. Wat dissected the wings of flies grown on different diets for size quantification which was presented as Figure 1 – figure supplement 3A in the final manuscript.
- Our collaborators, Bruno Hudry and Irene Miguel-Aliaga, provided us with a strain of flies ($tra^{F\ K\text{-}IN}$) for use in this study.

In all other cases, I performed all experiments and analysis presented. Elizabeth Rideout and I designed the experiments and the methods of analysis, and I wrote the first draft of the manuscript.

Chapter 4: **"A low sugar diet enhances *Drosophila* body size in males and females via sex-specific mechanisms"**

Work in this chapter resulted in the manuscript published as a preprint, "Millington, J.W., Wat, L.W., Sun, Z., Basner-Collins, P.J., Brownrigg, G.P., Rideout, E.J. 2021. "A low sugar diet enhances *Drosophila* body size in males and females via sex-specific mechanisms" *bioRxiv"*. At time of writing, this work is in review.
. For this publication, I was aided by the following contributions:
- Lianna W. Wat performed western blots on samples I generated which was presented as Figure 2F and Figure S1A.
- Ziwei Sun, Paige J. Basner-Collins, and George P. Brownrigg aided with the separation of male and female flies for body size experiments.

In all other cases, I performed all experiments and analysis presented. Elizabeth Rideout and I designed the experiments and the methods of analysis, and I wrote the first draft of the manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

20E	20-hydroxyecdysone
4E-BP	Eukaryotic initiation factor 4E-binding protein
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
AEL	After egg laying
ATP	Adenosine triphosphate
Bmm	Brummer
CCHa2	CCHamide-2
CCHa2-R	CCHamide-2 receptor
dALS	Acid-labile subunit
Dilp1	*Drosophila* insulin-like peptide 1
Dilp2	*Drosophila* insulin-like peptide 2
Dilp3	*Drosophila* insulin-like peptide 3
Dilp4	*Drosophila* insulin-like peptide 4
Dilp5	*Drosophila* insulin-like peptide 5
Dilp6	*Drosophila* insulin-like peptide 6
Dilp7	*Drosophila* insulin-like peptide 7
Dilp8	*Drosophila* insulin-like peptide 8
Dilps	*Drosophila* insulin-like peptides
Doa	Darkener of apricot
Dome	Domeless
Dsx	Doublesex
EGFR	Epidermal Growth Factor Receptor
Egr	Eiger
EMS	Ethyl methanesulfonate
FCG	Four core genotypes
Foxo	Forkhead box, sub-group O
Fru	Fruitless
Gbp1	Growth-blocking peptide 1
Gbp2	Growth-blocking peptide 2
GH	Growth hormone

GIP	Gastric inhibitory polypeptide
Grnd	Grindelwald
ICNs	Insulin-connecting neurons
Idgf1	Imaginal disc growth factor 1
IGF-1	Insulin-like growth factor 1
IGFR	Insulin-like growth factor receptor
IIS	Insulin/insulin-like growth factor signaling
Imp-L2	Ecdysone-induced gene 2
InR	Insulin receptor
IPCs	Insulin-producing cells
ISCs	Intestinal stem cells
JH	Juvenile hormone
L1	1st larval instar
L2	2nd larval instar
L3	3rd larval instar
Mth	Methuselah
NOS	Nitric oxide synthase
Pdk1	Phosphoinositide-dependent kinase 1
PG	Prothoracic gland
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pi3K	Phosphatidylinositol-3-kinase
PIC	Protease-inhibitor cocktail
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
Pten	Phosphatase and tensin homolog
PTTH	Prothoracicotropic hormone
Rdo	reduced ocelli
Rheb	Ras-homolog enriched in brain
S6k	Ribosomal protein S6 kinase
Sdr	Secreted decoy of insulin receptor
Shd	Shade

Slif	Slimfast
Spn88Eb	Serpin 88Eb
Srl	Spargel
SSD	Sexual size dimorphism
Sun	Stunted
Sxl	Sex-lethal
TLR-4	Toll-like receptor 4
Tor	Target of rapamycin kinase
TOR	Target of rapamycin signaling
TORC1	Target of rapamycin complex 1
Tra	Transformer
Tra-2	Transformer-2
Upd2	Unpaired-2

LIST OF SYMBOLS

♀ Female

♂ Male

ACKNOWLEDGEMENTS

DEDICATION

For my parents, John and Yvonne Millington, to whom I owe everything.

1. INTRODUCTION

Sexual dimorphism exists throughout the animal kingdom. While male-female differences are most obvious in primary sexual characteristics, such as the gonads, there are also sex differences in diverse traits that are not involved in reproduction, called secondary sexual characteristics. One secondary sexual characteristic present throughout the animal kingdom is sexual size dimorphism (SSD). Among invertebrates, the female is often larger. This increased female body size may support increased reproductive capacity by facilitating greater egg production. For example, across many species of spider there is a positive relationship between increased female size and larger clutch sizes (Honek, 1993; Prenter et al., 1999). In other animal groups including mammals, males are the larger sex. One reason for this is that increased male size supports male fitness by allowing larger males to outcompete smaller males for mates and other resources such as food (Flatt, 2020). Indeed, higher reproductive success is observed in male elephant seals of a larger size (Modig, 1996). Thus, SSD may exist in part due to reproductive advantages that accompany a specific body size within a sex (Fairbairn et al., 2007; Fairbairn, 2013).

Precisely how SSD is established during development varies between species, but often the larger sex relies on an increased rate of growth during development to achieve a larger body size (Stillwell et al., 2010). One well-studied example of how SSD is established emerges from studies in mammals. Individuals with an XX sex chromosome complement develop ovaries, the female reproductive organs, and produce the sex hormone oestrogen. In XY individuals, the presence of the Y chromosome directs testis development and the production of testosterone. The presence of oestrogen and testosterone contribute to SSD through the somatotropic axis, where the production and secretion of growth hormone (GH) from the somatotropes of the anterior pituitary gland promotes growth. Once in circulation, GH stimulates the production and secretion of insulin-like growth factor 1 (IGF-1) from the liver (Kaplan and Cohen, 2007). Once IGF-1 is in the circulation, it binds to insulin-like growth factor receptor (IGFR) to promote skeletal growth (Kaplan and Cohen, 2007). In females, oestrogen acts in the liver to blunt the induction of IGF-1

1

leading to reduced growth in females (Weissberger, Ho, and Lazarus, 1991; Bellantoni et al., 1996). In males, testosterone plays an important role in promoting increased male body size. First, testosterone promotes the secretion of GH from the pituitary gland (Giustina et al., 1997; Keenan et al., 1993). Second, testosterone augments the GH-dependent induction of IGF-1 in the liver (Gibney et al., 2005). Together this results in increased male body size. This modulation of IGF-1 activity by GH is crucial for SSD as loss of either GH or IGF-1 eliminates SSD (Efstratiadis, 1998; Lupu et al., 2001; Bikle et al., 2001; Liu et al., 2016). Thus, the opposing actions of oestrogen and testosterone on GH and IGF-1 production and action contribute to SSD.

Other than sex hormones, X chromosome number may also contribute to SSD. For example, humans with Klinefelter's syndrome (XXY) are larger than XY individuals (Varella, 1984), suggesting the additional X promotes increased body size. Conversely, individuals with Turner's syndrome (XO) have a smaller body size compared with XX individuals (Clement-Jones et al., 2000). This correlation has been mechanistically explored using the four-core-genotypes (FCG) mouse model, where mice can be bred to have primary male or female sex characteristics but with either an XX or XY sex chromosome complement (Arnold, 2009; Arnold and Chen, 2009; De Vries et al., 2002). In XX or XY mice with either male or female primary sex characteristics, body size is larger in mice with two X chromosomes, independent of gonadal sex and sex hormones (Chen et al., 2012; Link et al., 2020). One explanation for these effects of sex chromosomes on SSD is that while typically X-chromosome inactivation corrects the dose imbalance of X-linked genes between males and females, several X-linked genes escape X-inactivation and may contribute to increased body size in XX individuals, as has recently been shown in mice (Link et al., 2020).

Despite the important role of sex chromosomes and sex hormones in regulating SSD, it remains incompletely understood what the genes and pathways are that lie downstream of these factors to mediate SSD. One approach to resolve this gap in knowledge is to use a model system to advance our understanding of genes and pathways that influence SSD. One model system that has been used

extensively to study the mechanisms that determine body size is the fruit fly, *Drosophila melanogaster.* Studying the regulation of body size in *Drosophila* offers several advantages. The first advantage is that *Drosophila* rapidly progress through a four-stage life cycle: 1) embryonic development; 2) larval/juvenile growth phase; 3) pupa, the transition to sexual maturity; 4) adulthood, the reproductive stage. From embryo to adult, the generation time of *Drosophila* is around ten days at 25°C. This fast rate of development in *Drosophila* is advantageous compared to the relatively slower rate of development in mammalian models (6-8 weeks to sexual maturation). Following embryonic development and hatching, the larval stage consists of high levels of food consumption and growth through three larval instars, or moults (L1-L3). By progressing through these instars, the larva sheds its cuticle allowing for increased growth. Once in the third instar, the larva will reach a 'critical weight' beyond which the larva is committed to undergoing pupariation. Due to the formation of a hard chitinous exoskeleton during metamorphosis, body size will not change through adulthood. Together these features of *Drosophila* development make for a highly tractable system to measure growth and body size in a short period of time.

A second advantage of using *Drosophila* as a model system is that there are many powerful genetic tools. The UAS/GAL4 system is a binary expression system that allows researchers to perform gain- and loss-of-function studies in a spatially and temporally restricted manner (Brand and Perrimon, 1993; Duffy, 2002). There are libraries of UAS-overexpression and UAS-RNAi lines that allow >85% of the genes in the genome to be manipulated. Other commonly used binary expression systems such as LexA/LexAop (Lai and Lee, 2006), and QF/QUAS (Potter et al., 2010) systems are functionally equivalent to the UAS/GAL4 system. Loss-of-function experiments by generating mutants of a candidate gene are now relatively quick and easy in flies with the advent of gene editing techniques such as clustered regularly interspersed short palindromic repeats (CRISPR) optimized for use in flies (Port et al., 2014; Port et al., 2020). Combining these genetic tools further allows for gene interaction studies by determining epistatic relationships, and allows for intersectional analysis of overlapping populations of cells. This ability to manipulate genes and gene expression in a spatially- and temporally-restricted manner has

allowed for the dissection of many complex molecular mechanisms controlling growth and final body size. Indeed, several genetic tools have been exploited to perform both forward and reverse genetic screens to identify gene that impact body size in *Drosophila (*Harvey et al., 2003; Oldham et al., 2000; Zhang et al., 2000; Raisin et al., 2003; Tseng and Hariharan, 2002*)*.

A third advantage of using flies as a model to study the regulation of body size is that the genes and signaling pathways identified in *Drosophila* are likely to be conserved in humans. Approximately 75% of human disease-related genes are conserved, including genes involved in growth (Adams et al., 2000; Banfi et al., 1996; Reiter et al., 2001). Indeed, genes and pathways shown to influence growth and body size in flies have been shown to perform similar roles in other animals, such as mammals. For example, the Hippo/Warts signaling pathway is highly conserved between flies and mammals as a key regulator tissue growth (Fu et al., 2014; Harvey and Tapon, 2007; Moeller et al., 2017; Pan, 2010). Together, these factors establish *Drosophila* as an excellent model to study the regulation of growth and body size, and provide solid foundations to explore the genetic and molecular mechanisms that regulate SSD.

This thesis aims to advance knowledge of the mechanisms underlying body size control in each sex. In Chapter 2 of this thesis, I use loss- and gain-of-function approaches to describe the requirement for the conserved insulin/insulin-like growth factor signaling pathway (IIS) in regulating body size during development in male and female *Drosophila*. In Chapter 3, I reveal the molecular mechanisms underlying the increased ability of female larvae to adjust their body size in a nutrient-rich context. Specifically, we identify a non-cell-autonomous mechanism coupling dietary protein levels with IIS activity through a fat-derived insulinotropic factor in females. Finally, in Chapter 4, I provide data suggesting that male and female *Drosophila* display distinct changes in nutrient-dependent signaling pathways to achieve an equivalent body size phenotype in a low sugar context.

For the remainder of this introduction, I will review two important bodies of literature upon which my thesis will build to examine the molecular mechanisms

underlying *Drosophila* SSD: 1) genetic and molecular mechanisms of nutrient-dependent growth, 2) effects of sex determination genes on body size.

1.1. REGULATION OF GROWTH DURING DEVELOPMENT

1.1.1. *Environmental factors that affect Drosophila body size*

Many extrinsic environmental factors affect growth and final body size in flies. Here, I will describe key biotic and abiotic factors that contribute to growth and final body size.

Nutrients. One key requirement for normal growth during development is nutrient availability. In *Drosophila*, when dietary nutrients are low in abundance, developmental time is longer and final body size is smaller (Beadle et al., 1938; Edgar, 2006; Mirth and Riddiford, 2007; Mirth and Shingleton, 2012; Nijhout, 2003; Robertson, 1963). In nutrient-rich conditions developmental time is shorter and final body size is larger (Beadle et al., 1938; Edgar, 2006; Mirth and Shingleton, 2012; Nijhout, 2003; Robertson, 1963). This is true throughout the animal kingdom from other insect species (Davidowitz et al., 2004) through to humans (de Onis and Branca, 2016). Thus, nutrient quantity plays an important role in regulating body size. Nutrient quality also matters, as specific dietary macronutrients have distinct effects on final body size in *Drosophila* (Raubenheimer, et al., 2009; Raubenheimer et al., 2014; Shingleton et al., 2017). For example, dietary protein is a key source of amino acids that are required for growth and a normal final body size (Britton and Edgar, 1998; Geminard et al., 2009; Robertson, 1963). Indeed, *Drosophila* larvae fail to grow in a diet lacking protein (Britton and Edgar, 1998; Edgar, 2006), and will actively reject a diet lacking essential amino acids, reflecting the necessity of dietary protein for growth (Bjordal et al., 2014). However, it is important to note that more protein is not always beneficial to growth, as extremely high dietary protein results in smaller final body size (Musselman et al., 2011). While dietary carbohydrates are dispensible for *Drosophila* growth, in contrast to protein, carbohydrate concentration does influence body size. For example, a diet rich in sugars, such as glucose and

sucrose, strongly inhibits growth (Musselman et al., 2011; Pasco and Leopold, 2012; Reis, 2016). In addition to the individual effects of dietary macronutrients, recent studies using isocaloric nutritional geometry diets reveal that a complex interaction of both dietary macronutrients contributes to final body size (Raubenheimer, et al., 2009; Raubenheimer et al., 2014; Shingleton et al., 2017).

Temperature. In addition to nutrient availability, environmental temperature is a key determinant of growth and body size. The relationship between temperature and body size in animals has been investigated for over a century, with a larger body size typically associated with cold temperatures in both ectotherms and endotherms (Bergmann, 1847; Neel, 1940; Schreider, 1950). This inverse relationship between temperature and body size is known as the temperature-size rule (Atkinson, 1994) and is seen throughout species from birds (Weeks et al., 2020) to mammals (Freckleton et al., 2003; Ashton et al., 2000). However, there are notable exceptions to the temperature size rule where body size has a positive relationship with temperature, such as in marine ectotherms (Atkinson, 1995; Siikavuopio et al., 2012). The ability to couple final body size with environmental temperature is advantageous as body size scaling alters the surface area to volume ratio, ultimately allowing for more optimal thermoregulation (Atkinson, 1994; Schoener and Janzen, 1968). This is the case in *Drosophila,* where larvae reared in colder temperatures have a longer developmental growth period and reach a larger body size due to a temperature-dependent change in critical weight for pupariation (Ghosh et al., 2013; Karan et al., 1998; Neel, 1940; Nunney and Cheung, 1997; Partridge et al., 1994). Indeed, *Drosophila* are capable of sensing temperature (Gallio et al., 2011; Hamada et al., 2008), and actively increase their growth through a cold-sensing mechanism to promote large final body size (Li and Gong, 2015).

Oxygen. Oxygen is required for growth and oxygen-sensing mechanisms couple oxygen availability and final body size. For example, in humans living at high altitudes, the lower levels of oxygen available (hypoxia) results in short stature and delayed development (Frisancho and Baker, 1970; Frisancho, 2013). This is also the case in *Drosophila*, and other insects, where low oxygen causes a longer developmental time and a smaller final body size (Frazier et al., 2001; Callier and

Nijhout, 2011). Interestingly, the effects of hypoxia on developmental timing and final body size are observed at oxygen concentrations permissive of aerobic respiration. This suggests that the effects of hypoxia are actively controlled before basic metabolic processes become dysfunctional (Harrison et al., 2015; Van Voorhies, 2009). This suggests an adaptive oxygen-sensing response encoded genetically, as opposed to a passive effect of dysfunctional metabolism on final body size under hypoxic conditions. Indeed, recent studies have identified an oxygen sensing mechanism that couples oxygen and body size (Lee et al., 2019; Texada et al., 2019). Specifically, the oxygen dependent remodeling of tracheal airway branching in the developing larva is central to the coupling of oxygen availability and final body size (Texada et al., 2019).

Microbiome. Another important determinant of growth and final body size is the microbiome composition. Both commensal and pathogenic bacteria colonization has effects on growth. For example, in humans, chronic enteropathogenic infection limits growth during development (Rogawski et al., 2018), potentially due to lower levels of IGF-1 (DeBoer et al., 2017). Similarly, in *Drosophila*, pathogenic infection promotes the activation of immune signaling pathways known to affect growth. Specifically, activation of immune signaling pathways during development results in smaller final body size (DiAngelo et al., 2009; Roth et al., 2018; Suzawa et al., 2019). This is mediated by one branch of the innate immune system, the Toll pathway, which inhibits growth by blocking the *Drosophila* ortholog of IGF-1 (DiAngelo et al., 2009; Roth et al., 2018; Suzawa et al., 2019). Interestingly, the activation of Toll signaling to inhibit growth promoting pathway activity is conserved, as Toll-like receptor-4 (TLR-4) expressing macrophages secrete factors to inhibit insulin signaling pathway activity in mammals (Lackey and Olefsky, 2016). Additionally, in *Drosophila*, the absence of a microbiome also has major effects on developmental timing and final body size as IIS and TOR activity is reduced in germ-free flies (Erkosar et al., 2015; Shin et al., 2011; Storelli et al., 2011; Wong et al., 2014).

1.1.2. Hormonal factors that regulate body size

In response to extrinsic environmental factors, a range of hormones are produced in *Drosophila* affect growth and final body size. Here I will describe the key hormonal factors which regulate growth and body size.

Ecdysone. The steroid hormone 20-hydroxyecdysone (20E) is a key determinant of the length of the growth period and body size in many insect species such as *Drosophila* and the tobacco hornworm, *Manduca sexta* (Garen et al., 1977; Nijhout, 1976). The precursor to 20E, ecdysone, is produced in the larval prothoracic gland (PG) and converted to 20E by the CYP450 enzyme Shade (*shd*; FBgn0003388) primarily in the larval fat body (Petryk et al., 2003). This bioactivation of 20E is controlled by nutrient status where high levels of dietary nutrients are required for Shd upregulation and 20E production (Buhler et al., 2018). This active 20E is secreted in a series of pulses to trigger progression through larval instars, culminating in a spike of high 20E titer at the end of L3 to trigger pupariation (Riddiford and Truman, 1993). Bypassing this nutrient-dependent activation of 20E by feeding a 20E-supplemented diet during larval growth is sufficient to inhibit growth and results in a smaller body size (Colombani et al., 2005; Delanoue et al., 2010). Conversely, blunting ecdysone signaling results in increased body size (Colombani et al., 2005; Delanoue et al., 2010). Therefore, when ecdysone signaling is high, body size is smaller, and lower levels of ecdysone signaling results in larger body size.

PTTH. The timing of the pulse of 20E to trigger pupariation is itself controlled by hormonal factors. For example, the neuropeptide prothoracicotropic hormone (PTTH) is derived from a pair of neurosecretory cells in the brain and is required for the production of 20E pulses (McBrayer et al., 2007). PTTH also acts to integrate environmental factors with hormone production. For example, PTTH may mediate the effects of oxygen on developmental timing and body size. Upon hypoxia, nitric oxide is produced and PTTH promotes the expression of genes required for nitric oxide synthase (NOS) activity in the PG (Caceres et al., 2011). In *Drosophila* and other insects such as *Manduca*, this hypoxia-dependent activation of NOS results in

altered 20E production, and earlier pupariation (Callier et al., 2013; Wingrove and O'Farrell, 1999). However, it remains unclear whether hyperoxia has any effect on 20E production, developmental timing, and final body size in *Drosophila*.

     Juvenile Hormone. A third hormone controlling developmental time and body size is juvenile hormone (JH). In both *Drosophila* and *Manduca*, JH production is high during times of low nutrient availability to prevent pupariation. Specifically, during starvation, JH is produced in the corpora allata of developing larvae, and high levels of JH suppress ecdysone production which prolongs the developmental growth period (Cymborowski et al., 1982; Fain and Riddiford, 1975; Nijhout and Williams, 1974; Mirth and Shingleton, 2012; Mirth et al., 2014; Riddiford and Ashburner, 1991; Riddiford et al., 2010). Consequently, in flies lacking JH body size is smaller (Mirth et al., 2014; Riddiford et al., 2010), and feeding of JH delays pupariation (Riddiford and Ashburner, 1991). Therefore, together with ecdysone, JH affects both developmental time and final body size.

1.1.3. Genetic factors that regulate body size.

     Following the identification of environmental and hormonal factors which regulate final body size, studies next turned to identifying genes and pathways required for nutrient-dependent growth. Using genetic screens in a nutrient-rich context to identify modifiers of eye and wing size, several genes controlling body, and tissue, growth were identified (Harvey et al., 2003; Oldham et al., 2000; Zhang et al., 2000; Raisin et al., 2003; Tseng and Hariharan, 2002). For example, "pinhead" mutant flies, generated using EMS-induced mutagenesis, identified a requirement for many *Drosophila* homologs of genes in the target-of-rapamycin (TOR) pathway, where Tor is a conserved regulator of cell growth and proliferation (Oldham et al., 2000; Zhang et al., 2000). Another pathway that emerged as a key regulator of growth from these screens was the IIS pathway. Foundational studies over the past 20 years have expanded and deepened our understanding of how these two conserved signaling pathways, IIS and TOR, regulate growth during development. Activation of either the IIS and TOR pathways promotes growth and increased body size, whereas inhibition of either pathway reduces growth and body size (Boulan et

al., 2015; Edgar, 2006; Hietakangas and Cohen, 2009; Nijhout, 2003; Nijhout et al., 2014).

IIS. Activation of IIS begins with *Drosophila* insulin-like peptide (Dilp) production and secretion from the insulin-producing cells (IPCs) of the larval brain (Brogiolo et al., 2001; Geminard et al., 2009; Ikeya et al., 2002; Rulifson et al., 2002). The IPCs in *Drosophila* are functionally analogous to the pancreatic beta cells which produce and secrete insulin in mammals (Rulifson et al., 2002). Interestingly, the neural production of insulin is observed throughout many invertebrate (Kondo et al., 1996; Pierce et al., 2001; Smit et al., 1998) and vertebrate species (Clarke et al., 1996; Mehran et al., 2012). Given that the specification of IPCs in *Drosophila* shares many similarities to the specification of mammalian beta cells (Wang et al., 2007), this suggests a common neural origin of insulin-producing cells across species.

The IPCs express Dilps1,2,3, and 5 in larvae, which mediate growth in response to dietary nutrient context (Brogiolo et al., 2001; Rulifson et al., 2002; Gronke et al., 2010). The IPCs are the main source of growth-promoting Dilps as IPC ablation results in dramatically reduced body size which can be rescued by transgenic expression of Dilps (Rulifson et al., 2002). However, it is important to note that to date eight Dilps have been identified (Dilps1-8), seven of which can promote growth (Dilps1-7) (Brogiolo et al., 2001; Ikeya et al., 2002). Once secreted into the haemolymph IPC-derived Dilps bind to the insulin receptor (InR; Fbgn0283499) on target tissues, where Dilp binding promotes InR autophosphorylation and recruitment of adaptor proteins to InR at the plasma membrane (Almudi et al., 2013; Böhni et al., 1999; Chen et al., 1996; Poltilove et al., 2000; Werz et al., 2009). As a consequence of adapter protein recruitment, the regulatory and catalytic subunits of phosphatidylinositol-3-kinase (Pi3K21B; FBgn0020622 and Pi3K92E; FBgn0015279, respectively) are further recruited and facilitate the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) (Leevers et al., 1996). This increase in plasma membrane PIP_3 results in the recruitment of signaling kinases Akt (Akt; FBgn0010379) and phosphoinositide-dependent kinase 1 (Pdk1; FBgn0020386) which together control many cellular processes which drive cell, tissue, and organismal growth (**Fig. 1.1**) (Cho et al.,

2001; Rintelen et al., 2001; Verdu et al., 1999). Evidence in support of IIS's key role in promoting growth comes from studies showing loss of many IIS components reduces body size (Böhni et al., 1999; Britton et al., 2002; Chen et al., 1996; Fernandez et al., 1995; Grewal, 2009; Gronke et al., 2010; Teleman, 2009). This role for IIS in promoting growth is highly conserved in other species. For example, in mice (Efstratiadis, 1998; Lupu et al., 2001), dogs (Hoopes et al., 2012; Sutter et al., 2007), and humans with Laron syndrome (Laron et al., 1966; Clemons et al., 1976) reductions in IGF-1 lead to reduced growth.
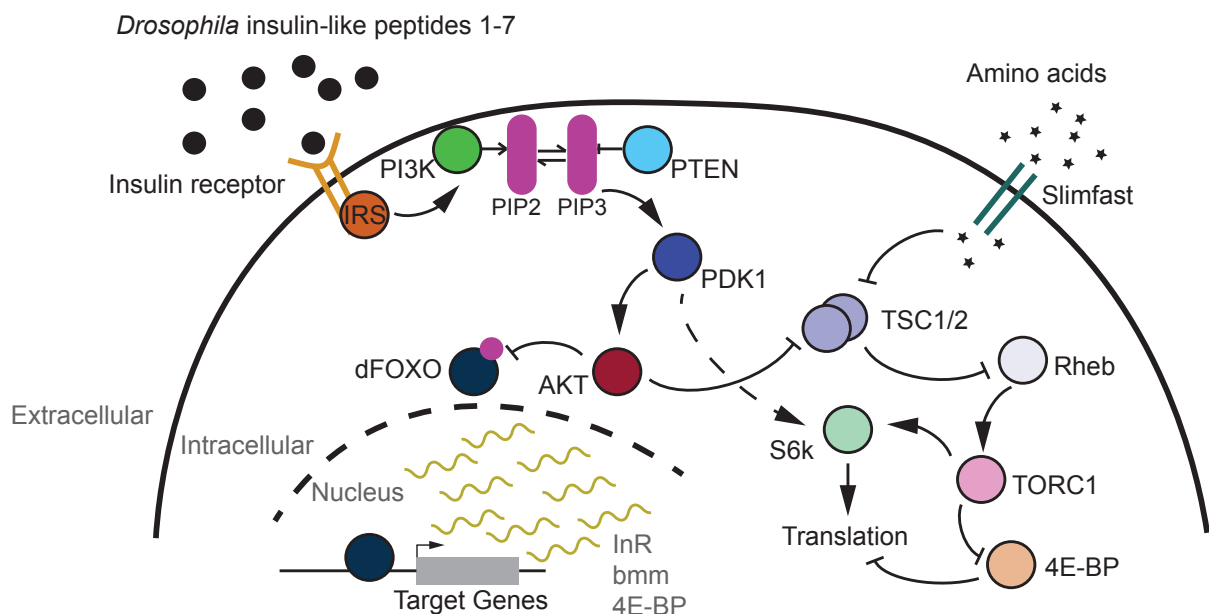
Figure 1.1. Simplified IIS/TOR pathway in *Drosophila*. Intracellular IIS signaling begins with Dilps binding to InR stimulating the production of PIP_3 at the cell membrane. Pdk1 and Akt mediate a range of intracellular processes to control growth, such as Foxo phosphorylation. Increase in intracellular amino acid concentration through amino acid transporter Slimfast results in increased activity of TORC1 complex through Rheb. High levels of TORC1 activity mediates a range of processes that affect growth, such as through mRNA translation. There is crosstalk between IIS and TOR pathway components at multiple levels of the pathway.

TOR. TOR pathway activation is mediated by the cell-autonomous uptake of nutrients to promote growth (Edgar, 2006; Grewal, 2009). Upon feeding, dietary protein is digested into amino acids which enter the haemolymph and are taken into

cells through amino acid transporters (**Fig. 1.1**) (Colombani et al., 2003; Miguel-Aliaga et al., 2018). Amino acid uptake promotes recruitment of the TORC1 complex to the lysosomal membrane, a required step for TORC1 activation (Kim et al., 2008; Sancak et al., 2008; Sancak et al., 2010). TORC1 is the primary complex mediating TOR's effects on cell growth and proliferation (Avruch et al., 2009; Wullschlager et al., 2006; Zhang et al., 2000). Then, the recruited TORC1 complex interacts with the small GTPase Ras homolog enriched in brain (Rheb; FBgn0041191) which activates TORC1 to promote increased cell size and proliferation dependent on Tor kinase (Patel et al., 2003; Saucedo et al., 2003; Stocker et al., 2003).

The activation of Tor kinase in TORC1 by Rheb leads to increased growth by augmenting protein biosynthesis through multiple effectors (Hall et al., 2007). TOR effectors identified to play an important role in body size and cell growth include ribosomal protein S6 kinase (S6k; FBgn0283472), and eukaryotic initiation factor 4E-binding protein (4E-BP; FBgn0261560), respectively. Both S6k and 4E-BP regulate protein biosynthesis, but act in opposing ways. TORC1 phosphorylation of S6k augments protein biosynthesis by facilitating translation initiation and elongation resulting in increased body size (Wang et al., 2001; Holz et al., 2005). However, TORC1-mediated phosphorylation of 4E-BP promotes protein biosynthesis by preventing 4E-BP from inhibiting cap-dependent mRNA translation (Gingras et al., 2001; Hay and Sonenburg, 2004; Miron et al., 2001; Teleman et al., 2005). Both S6k and 4E-BP are key downstream effectors TORC1, however while S6k controls body size, 4E-BP only acts to inhibit cell growth in stress conditions (Montagne et al., 1999; Miron et al., 2001; Teleman et al., 2005).

TOR activity also regulates transcription, at least partly mediated by the post-translational regulation of the transcription factor Myc (Myc; FBgn0262656) (Grewal et al., 2005; Guertin et al., 2006; Orian et al., 2005; Teleman, 2008). Myc regulates many aspects of protein biosynthesis in response to nutrient status such as ribosome biogenesis and mRNA translation (Grewal et al., 2005). Increasing mRNA translation promotes increased body size and genetic manipulations which reduce mRNA translation leads to reduced body size (Grewal et al., 2007; Rideout et al., 2012; Marshall et al., 2012; Ghosh et al., 2014; Sriskanthadevan-Pirahas et al.,

2018a; Sriskanthadevan-Pirahas et al., 2018b). Therefore, these nutrient- and TORC1-dependent changes in ribosome biogenesis, mRNA translation act together to control growth and final body size. Together, these studies demonstrate an important role for TOR signaling in the cell-autonomous regulation of cell size and proliferation in response to cellular amino acid levels. This role for TOR in nutrient-dependent regulation of growth is highly conserved across species (De Virgilio and Loewith, 2006). For example, S6k mutant mice are also significantly smaller than their wild type littermates (Pende et al., 2004; Selman et al., 2009; Shima et al., 1998).

1.1.4. Coupling nutrients with growth

Given that nutrient availability is required for growth during development, and that many genes are required to achieve a normal body size even in a nutrient-rich environment, the next step in understanding the regulation of body size was to identify factors that couple nutrient input with growth. Many studies over the last 20 years show that coupling of nutrients with body size is mediated in part by the function of specific organs within the body that communicate with other organs and tissues by short- and long-range signals. In *Drosophila*, the most well-characterised example of this non-cell-autonomous mechanism of growth is the fat body, which functions as a key nutrient sensor to couple dietary nutrients with larval growth. The first clue that the fat body was a nutrient sensor came from experiments monitoring cell proliferation in endoreplicating and mitotic tissues. Constant nutrition is required to maintain endoreplicative growth in larvae (Britton and Edgar, 1998), and arrested growth is associated with a cessation in endoreplication (Galloni and Edgar, 1999; Zhang et al., 2000). These studies found that input of nutrients stimulated the cell cycles in both endoreplicating and mitotic tissues (Britton and Edgar, 1998). The IIS signaling pathway is key in maintaining endoreplication and growth in larval tissues in response to nutrients through Pi3K (Britton et al., 2002). Similarly, TOR signaling is required to couple nutrients with growth (Zhang et al., 2000; Patel et al., 2003). However, cell cycle stimulation is due in part to factors derived from the fat body

which act at a distance to control growth. Through elegant organ coculture experiments, researchers found that a mitogenic factor is secreted from the fed fat body to promote neuroblast proliferation in the starved brain, independent of the direct sensing of nutrients (Britton and Edgar, 1998).

Further insight into the coupling of nutrients with growth came from research showing that the IPCs secrete Dilps in response to secreted fat body-derived factors (Colombani et al., 2003; Geminard et al., 2009; Arquier et al., 2008). From these studies a straightforward model of how the fat body senses nutrients and controls Dilp secretion from the IPCs to regulate growth has emerged. In a protein-rich dietary context, the amino acid transporter Slimfast (Slif; FBgn0037203) facilitates the uptake of amino acids (Colombani et al., 2003). This increase in intracellular amino acids stimulates fat body TOR activity (Colombani et al., 2003). Fat body TOR activation non-cell-autonomously promotes *Drosophila* insulin-like peptide 2 (Dilp2; FBgn0036046) secretion from the IPCs (Colombani et al., 2003; Geminard et al., 2009). In nutrient-poor conditions, lower levels of amino acid import into the fat results in less TOR activity and Dilp retention in the IPCs, although refeeding rapidly triggers Dilp secretion (Colombani et al., 2003; Geminard et al., 2009). Due to lower levels of Dilp secretion, the production of PIP_3 in target tissues is lower, resulting in diminished levels of Akt and Pdk1 recruitment and a slower rate of growth (Britton et al., 2002; Nowak et al., 2013).

This reduced growth in nutrient-poor conditions can be rescued by the cell-autonomous activation of IIS which rescues the reduced cell size (Britton et al., 2002; Geminard et al., 2009; Nowak et al., 2013). Conversely, in a nutrient-rich context, downregulation of *slif* expression uncouples nutrients from growth and phenocopies amino acid starvation and causes Dilp retention, growth arrest, and lethality (Colombani et al., 2003). However, this can be bypassed by augmenting TOR signaling in the larval fat body in nutrient-poor conditions which promotes Dilp secretion and increased growth, but at the cost of higher rates of lethality (Geminard et al., 2009). Thus, the fat body senses amino acids directly by coupling intracellular amino acid levels with TOR activity to promote Dilp secretion from the IPCs. This role for the fat as a nutrient sensor is indispensable for growth. This is in contrast to

mammals where the insulin-producing pancreatic beta cells directly sense circulating glucose to regulate insulin secretion (Campbell and Newgard, 2021). However, in *Drosophila*, direct sensing of nutrients by the larval IPCs plays a lesser role in coordinating systemic growth (Maniere et al., 2016).

But how does TOR activity in the fat body influence Dilp secretion from IPCs? Recently, a series of studies have identified several humoral factors (known as fat-to-brain signals, or insulinotropic factors) which are secreted from the fat in a TOR-dependent manner to promote Dilp secretion. These fat-to-brain signals respond to distinct nutrient components of the diet. For example, Stunted (Sun; FBgn0014391), Growth-blocking peptide 1 (Gbp1; FBgn0034199), and Growth-blocking peptide 2 (Gbp2; FBgn0034200) are all upregulated and secreted in response to dietary protein consumption (Koyama and Mirth, 2016; Delanoue et al., 2016). In contrast, CCHamide-2 (CCHa2; FBgn0038147), and Unpaired-2 (upd2; FBgn0030904) are induced by dietary sugar feeding to promote Dilp secretion (Rajan and Perrimon, 2012; Sano et al., 2015). However, these fat-to-brain signals may also be responsive to other nutrient components of the diet. For example, CCHa2 is weakly responsive to protein feeding, and Upd2 is induced by fat consumption (Rajan and Perrimon, 2012; Sano et al., 2015). In the absence of dietary nutrients another fat derived factor, Eiger (Egr; FBgn0033483), inhibits Dilp secretion and growth (Agrawal et al., 2016). Consequently, high levels of growth in a nutrient-rich context, and reduced growth in a nutrient-poor context, are both actively regulated by nutrient conditions to fine tune body size.

The mechanisms by which Dilp secretion is induced by fat-to-brain signals differs widely. These can be broadly classified into two groups: direct, and indirect regulation of Dilp secretion. For example, Sun in circulation directly regulates Dilp2 secretion through its receptor Methuselah (Mth; FBgn0023000) expressed on the IPCs (Delanoue et al., 2016). Similarly, CCHamide-2 receptor (CCHa2-R; FBgn0033058) is expressed on the IPCs and regulates Dilp production (Sano et al., 2015), and Egr directly interacts with its receptor Grindelwald (Grnd; FBgn0032682) expressed on the IPCs to inhibit Dilp secretion (Agrawal et al., 2016). In contrast, circulating Upd2 binds to its receptor Domeless (Dome; FBgn0043903) expressed

on GABAergic neurons which inhibit IPC activity (Rajan and Perrimon, 2012). Upd2-dependent stimulation of JAK/STAT signaling in these GABAergic neurons relieves the inhibition of IPC activity thus indirectly promoting Dilp secretion (Rajan and Perrimon, 2012). A similar indirect mechanism promoting Dilp secretion is mediated in response to Gbp1 and Gbp2 through a pair of intermediate inhibitory neurons termed insulin-connecting neurons (ICNs) (Meschi et al., 2019). Gbp1 and Gbp2 bind to the epidermal growth factor receptor (EGFR; FBgn0003731) expressed on the ICNs to promote EGF signaling which relieves the inhibition of IPC activity (Meschi et al., 2019). Therefore, both direct and indirect actions of fat-to-brain signals regulate Dilp production and secretion in response to dietary nutrients.

Together, these studies form a rich foundation of knowledge on the regulation of Drosophila body size during development. However, many of these studies were performed using a mixed-sex population of larvae. As a result, less is known about the requirement for these genes and pathways in regulating body size in each sex.

1.2. SEX DIFFERENCES IN GROWTH DURING DEVELOPMENT

1.2.1. *Mechanisms of sex determination*

A range of sex determination mechanisms have evolved in a variety of species (Manolakou et al., 2006; Bachtrog et al., 2014). While the specifics of sex determination vary, there exist several parallels in sex determination between species. Many species use a sex determination mechanism dependent on sex chromosome complement. One common form of sex determination through sex chromosomes is heterogamy, where one sex bears two identical sex chromosomes and one is heterozygous for a sex-determining locus. In mammals and *Drosophila*, the heterogametic sex is the male possessing an XY sex chromosome complement. However, while mammals and *Drosophila* share an XX/XY sex determination mechanism, the downstream effectors mediating sex-specific development are very different.

1.2.2. Sex determination in Drosophila

In *Drosophila*, the sex determination pathway downstream of sex chromosome complement has been extensively studied. In early embryonic development, sex is determined by the number of X chromosomes (Bridges, 1921 Salz and Erickson, 2010). In XX female embryos, a functional Sex Lethal (*Sxl*; FBgn0264270) protein is produced. Sxl is a master regulator of female development (**Fig. 1.2a-c**). In XY individuals, a functional Sxl protein is not produced, and male development occurs. The main downstream target of Sxl in directing female development is *transformer* (*tra*; FBgn0003741), where Sxl binding to *tra* pre-mRNA introduces a female-specific splice that causes a functional Tra protein to be produced (Tra^F) (Belote et al., 1989; Boggs et al., 1987; Inoue et al., 1990). Tra is a key determinant of female development: lack of Tra 'transforms' females into phenotypic males, whereas ectopic Tra expression in males is sufficient to specify female development (Sturtevant, 1945). Like Sxl, Tra^F is a splicing factor which controls many aspects of female sexual development, reproduction, and behaviour via regulation of two downstream target genes: *doublesex* (*dsx*; FBgn0000504) and *fruitless* (*fru*; FBgn0004652).

Tra^F-dependent alternative splicing of *dsx* pre-mRNA produces a female-specific Dsx isoform (Dsx^F). Dsx^F regulates many aspects of female somatic development such as pigmentation, genital development, and female sexual behaviour (Kopp et al., 2000; Chatterjee et al., 2011; Clough et al., 2014; Camara, Whitworth, and Van Doren, 2008; Williams et al., 2008; Rideout et al., 2010). Tra^F-dependent splicing of *fru* transcripts derived from the P1 promoter (*fru-P1*), on the other hand, results in no *fru-P1* derived protein expression in females due to alternative 5' splice site choice. Consequently, female *fru-P1* transcripts do not encode a functional protein whereas in males a functional Fru^M protein is produced (Ryner et al., 1996; Ito et al., 1996; Heinrichs, Ryner, and Baker, 1998). As loss of *dsx*, or *fru*, results in defects in sexual differentiation and behaviour, the prevailing view is that Tra^F regulates many aspects of female sexual development and behaviour via Dsx^F. In the absence of Tra^F, *dsx* and *fru-P1* pre-mRNAs undergo default splicing, resulting in the production of male-specific isoforms of Dsx (Dsx^M)

17

and Fru (Fru^M). Dsx^M and Fru^M together regulate many aspects of male somatic development and male sexual behaviours such as courtship (Kopp et al., 2000; Williams et al., 2008; Rideout et al., 2010; Chatterjee et al., 2011; Clough et al., 2014; Massey and Wittkopp, 2016; Billeter et al., 2006; Kimura et al., 2008; Shirangi, Taylor, and McKeown et al., 2006).

While *dsx* and *fru* determine many phenotypic sex differences, recent studies have identified male-female differences mediated by Sxl/Tra independent of their canonical downstream effectors. The first identification of a *dsx*- and *fru*-independent role for Tra was in the regulation of body size. Tra is both required for increased body size in females, and sufficient to drive growth in feminized males (Rideout et al., 2015). Since, several studies have identified Sxl/Tra-dependent phenotypes independent of Dsx and Fru. For example, sex differences in stress-induced intestinal stem cell (ISC) proliferation in the adult midgut are cell-autonomously controlled by Sxl/Tra, independent of *dsx*, through *imaginal disc growth factor 1* (*idgf1*; FBgn0020416), *Serpin 88Eb* (*Spn88Eb*; FBgn0038299), and *reduced ocelli* (*rdo*; FBgn0243486) (Hudry et al., 2016; Ahmed et al., 2020). Additionally, testis-derived factors non-cell-autonomously control the sex difference in carbohydrate metabolism gene expression in the adult midgut, independent of intrinsic sex identity in the midgut (Hudry et al., 2019). Recent evidence also suggests novel relationships between Tra and its canonical factors. For example, Tra is required for the production of a population of female-specific Dilp7-expressing motoneurons (Castellanos et al., 2013; Garner et al., 2018). These studies found Tra acts genetically downstream of *fru* splicing to control the production of these motoneurons (Garner et al., 2018). This emerging evidence demonstrates that much remains to be discovered as to how phenotypic sex differences are controlled by sex determination genes in *Drosophila*.
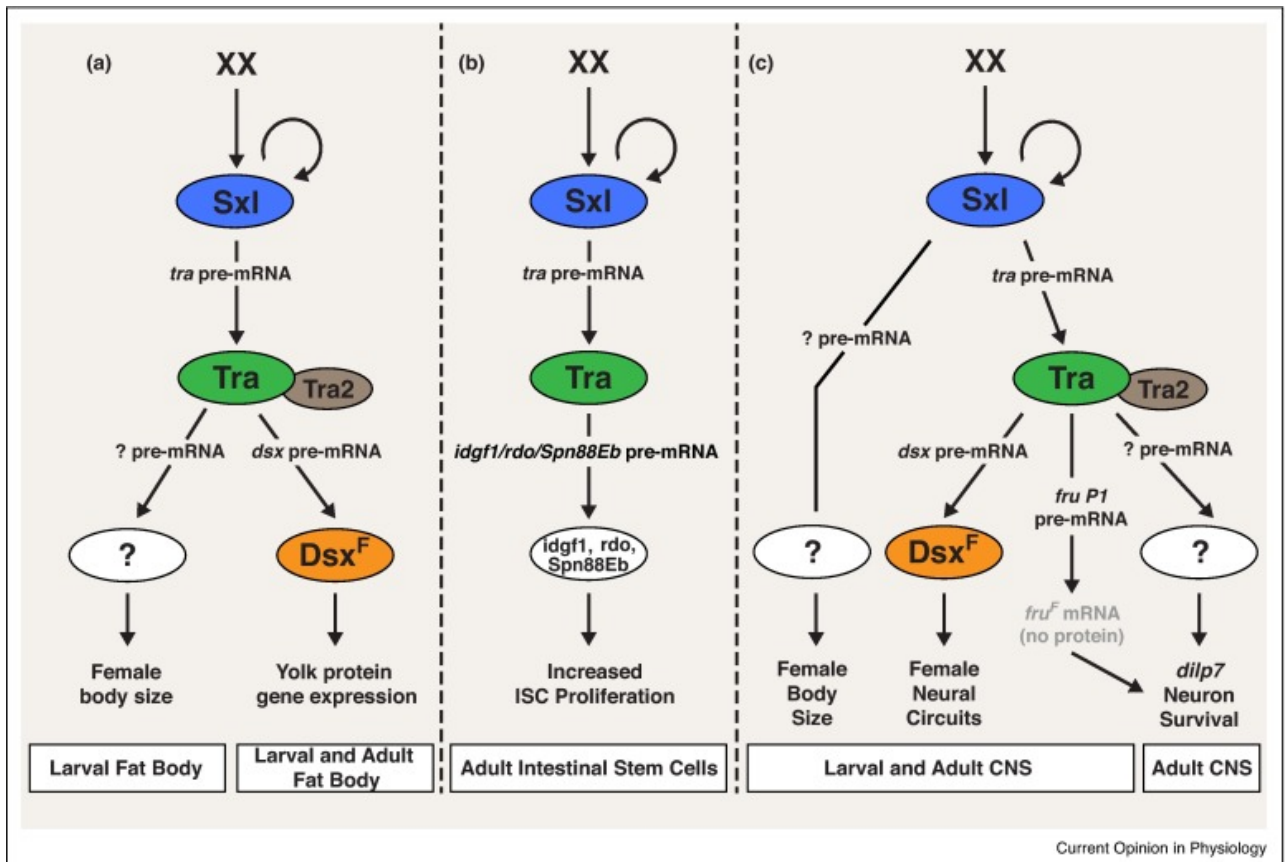
Figure 1.2. New insights into sex determination pathway in *Drosophila*. A working model incorporating findings from recent studies on sex determination pathway activity in multiple larval and adult tissues. Canonically, female sex is determined in *Drosophila* by the presence of two X chromosomes resulting in the production of a functional Sxl protein which splices the pre-mRNA of its main downstream target, *tra*, producing a functional Tra protein in females. Tra, also a splicing factor, acts together with its cofactor Tra2 and splices the pre-mRNA of its downstream targets *dsx* and *fru* producing a female-specific Dsx^F isoform and introducing a stop codon in *fru* transcripts resulting in no functional Fru protein in females. Recent studies have expanded our knowledge of the sex determination pathway by identifying additional branches active in specific tissues and at different times during development. (a) For example, in the larval fat body a Tra-dependent but *dsx*-independent branch regulates body size in female larvae. (b) Further, in the ISCs of the adult midgut, this Tra-dependent but *dsx*-independent branch promotes ISC proliferation in females; however, this function is independent of Tra2. (c) In the larval and the adult central nervous system, both Tra and Tra2 specify female neural circuits via *dsx* and *fru*. However, recent studies

19

have identified both canonical and non-canonical roles for Tra in promoting the survival of female-specific Dilp7-expressing motoneurons. Additionally, a Sxl-dependent but Tra-independent branch also functions in subsets of neurons to promote increased female body size. Adapted from Millington and Rideout, 2018.

1.2.3. Sex differences in Drosophila body size

In *Drosophila*, as in other animals, one important determinant of body size is biological sex (Millington and Rideout, 2018; Stilwell et al., 2010; Teder and Tammaru, 2005). Several factors influence this male-female difference in body size.

Sex determination genes. Until recently, it was incorrectly presumed that only Sxl, not Tra, controls the sex difference in body size (Cline and Meyer, 1996; Cline, 1984; Evans and Cline, 2013). However, evidence that sex determination genes affect body size was first collected in 1961 when Brown and King reported that mutations in *tra* caused a smaller body size in female flies (Brown and King, 1961). Sxl and Tra have recently both been shown to control SSD independent of Dsx and Fru (Rideout et al., 2015; Sawala and Gould, 2017). Loss of either *Sxl* or *tra* results in smaller female body size, but no difference in males (Rideout et al., 2015; Sawala and Gould, 2017). This was suggested to be due to the transformation of gonads, however, subsequent research demonstrated that Tra's effects on body size are independent of gonad transformation (Rideout et al., 2015). Instead, Tra was found to be an important regulator of growth both cell-autonomously and non-cell-autonomously. Loss of Tra in individual cells results in smaller cell size in females, with no effect in males, and overexpression of Tra results in larger cells in males (Rideout et al., 2015). Tra was found to regulate body size non-cell-autonomously from the larval fat body, where loss of Tra in the fat results in smaller females, and overexpression of Tra in the fat in a masculinized *tra* mutant female is sufficient to rescue this reduced body size (Rideout et al., 2015). Following the identification of Tra in regulating SSD, Sxl was identified to be required for increased female cell and body size (Sawala and Gould, 2017). Specifically, Sxl in the neurons is required for increased female body size, acting in the IPCs and the Gad1 producing neurons (Sawala and Gould, 2017). While this study did not identify the mechanism by which

Sxl acts in these neurons to regulate SSD, Sxl in the neurons regulates the entire SSD as females with neuronal loss of Sxl are the same size as males (Sawala and Gould, 2017). Together, these studies demonstrate a crucial role for Sxl and Tra for SSD.

X-linked genes. The X-linked gene *Myc* is expressed at higher levels in females and promotes increased female body size (Mathews et al., 2017). This is due to *Myc* expresseion escaping dosage compensation (Mathews et al., 2017). Typically, Sxl is a master regulator of dosage compensation of X-linked genes, in addition to its role in splicing *tra* pre-mRNA (Bashaw and Baker, 1996; Cline and Meyer, 1996; Salz and Erickson, 2010; Sawala and Gould, 2017). Therefore, this explains why Sxl controls the entire sex difference in body size as loss of Sxl disrupts both *tra* pre-mRNA splicing and dosage compensation (Sawala and Gould, 2017; Mathews et al., 2017). Indeed, simultaneous loss of both Tra and Myc results in equal size males and females (Mathews et al., 2017).

Nutrients. Dietary nutrients are required for SSD as males and females have equal body size in a nutrient-poor context (Rideout et al., 2015). However, the relationship between nutrient-status and SSD remains incompletely understood. Clues as to how nutrients affect growth in both sexes have been reported for almost a century. Early studies of diet and wing size identified a greater magnitude of change to the size of cells in females in a nutrient-poor diet than males (Alpatov, 1930). Recent studies have built on these earlier findings and identified sex-biased growth responses of male and female traits to dietary protein and carbohydrates (Shingleton et al., 2017). Specifically, female trait size is significantly more affected by changes to dietary protein concentration than males (Shingleton et al., 2017). This suggests that the magnitude of nutrient-dependent changes to growth may be higher in females.

Recent progress has been made in identifying how nutrients are coupled with sex differences in growth. For example, recent studies have implicated sex differences in IIS activity as important for SSD (**Fig. 1.3**). Indeed, sex differences exist in the expression of Dilps, IIS activity during the larval growth period, and the secretion of Dilp2 (Rideout et al., 2015; McDonald et al., 2020). Further, strong

reductions in IIS activity through hypomorphic mutation of InR eliminates the sex difference in body size (Testa et al., 2013), and loss of some individual Dilps has sex-specific effects on growth (Gronke et al., 2010). This appears to be specific to IIS as pharmacological inhibition of TOR activity does not have sex-biased effects on body size (Rideout et al., 2015), suggesting that IIS, not TOR, contributes to SSD. This evidence of sex-biased regulation of IIS activity is further bolstered by reports of sex-biased effects of reduced IIS on a range of phenotypes. For example, alterations to diet and IIS activity lead to widespread sex-biased and sex–specific changes in the transcriptome of adult flies (Camus et al., 2019; Graze et al., 2018). Additionally, the sex-biased effects of diet and IIS activity on lifespan have been consistently observed in flies (Bjedov et al., 2010; Clancy, 2001; Giannakou et al., 2004; Grönke et al., 2010; Regan et al., 2016; Tatar et al., 2001; Woodling et al., 2020; Wu et al., 2020), and humans (Van Heemst et al., 2005). However, despite the implication of sex determination genes and nutrients for sex differences in growth, the mechanisms remain unclear how these factors interact to determine SSD.
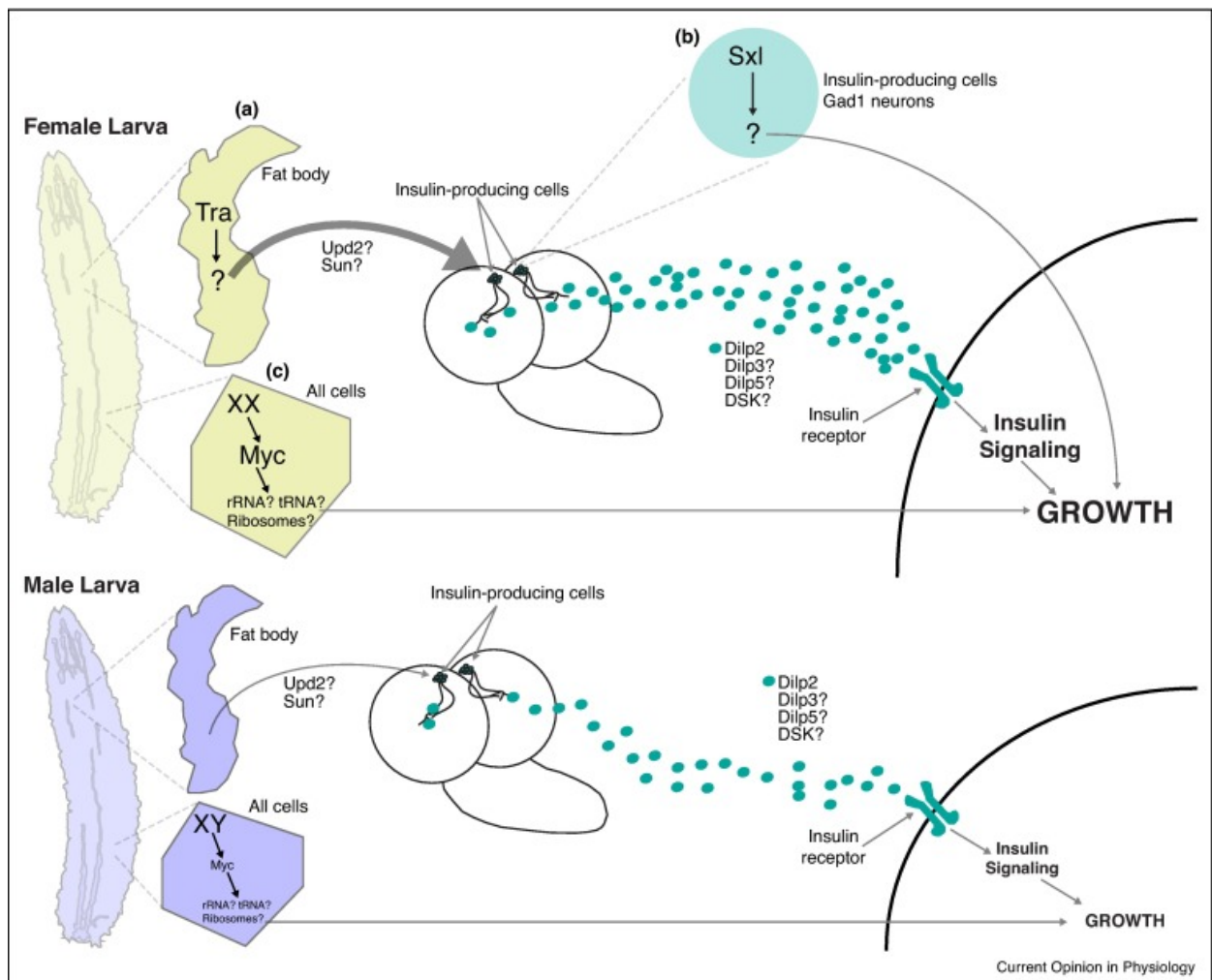
Figure 1.3. Multiple mechanisms contribute to sex differences in growth in *Drosophila*. A working model incorporating findings from recent studies on sex differences in growth. (a) Several recent studies have implicated the IIS pathway in the regulation of sex differences in body size. Previous findings have demonstrated sex differences in Dilp regulation whereby female third instar larvae have higher levels of Dilp2 secretion than males. This may result in increased IIS as females have elevated IIS activity compared to males during this larval stage. Interestingly, the sex determination gene *tra* acts in the larval fat body to control Dilp2 secretion from the IPCs non-cell-autonomously. Which fat-derived factors, such as Sun and Upd2, which mediate this increased Dilp2 secretion is not known. (b) Additionally, Sxl in the IPCs and Gad1 neurons, independent of Tra, promotes increased female body size. (c) Further, in all cells, the growth regulator Myc promotes increased female body size potentially through altered levels of ribosomal RNA, ribosome biogenesis, or tRNAs. Adapted from Millington and Rideout, 2018.

The goal of this thesis is to advance knowledge of the mechanisms underlying the differential control of body size in both sexes in *Drosophila*. In the following three chapters, I present evidence demonstrating that males and females differ in both the genes and pathways that contribute to final body size, and how these genes and pathways mediate nutrient-dependent growth.

2. GENETIC MANIPULATION OF INSULIN/INSULIN-LIKE GROWTH FACTOR SIGNALING PATHWAY ACTIVITY HAS SEX BIASED EFFECTS ON *DROSOPHILA* BODY SIZE

2.1. SYNOPSIS

In *Drosophila* raised in nutrient-rich conditions female body size is approximately 30% larger than male body size due to an increased rate of growth and differential weight loss during the larval period (Testa et al., 2013). While the mechanisms that control this sex difference in body size remain incompletely understood, recent studies suggest that sex-based differences in insulin/insulin-like growth factor signaling pathway (IIS) plays a role in the sex-specific regulation of processes that influence body size during development (Testa et al., 2013; Rideout et al., 2015). In larvae, IIS activity differs between the sexes, and there is evidence of sex-specific regulation of IIS ligands (Rideout et al., 2015). Yet, we lack knowledge of how changes to IIS activity impact body size in each sex, as the majority of studies on IIS and body size use single- or mixed-sex groups of larvae and/or adult flies. The goal of our current study was to clarify the contribution of IIS activity to body size in each sex. To achieve this goal, we used established genetic approaches to enhance, or inhibit, IIS activity, and quantified pupal size in males and females. Overall, genotypes that inhibited IIS activity caused a female-biased decrease in body size, whereas genotypes that augmented IIS activity caused a male-biased increase in body size. These data extend our current understanding of body size regulation by showing that most changes to IIS pathway activity have sex-biased effects, and highlights the importance of analyzing body size data according to sex.

2.2. INTRODUCTION

Over the past two decades, the *Drosophila* larva has emerged as an important model to study the molecular and developmental processes that contribute to final body size. When nutrients are plentiful, one important factor that affects body

size in most *Drosophila* species is whether the animal is male or female: female flies are typically larger than male flies (Alpatov *et al*. 1930; Pitnick *et al*.1995; French *et al*. 1998; Huey *et al*. 2006; Testa *et al*. 2013; Okamoto *et al*. 2013; Rideout *et al*. 2015; Sawala and Gould 2017; reviewed in Millington and Rideout 2018). This increased body size is due to an increased rate of larval growth and sexually dimorphic weight loss in wandering larvae, as the duration of the larval growth period does not differ between the sexes in wild-type flies (Testa *et al*. 2013; Okamoto *et al*. 2013; Sawala and Gould 2017). While the precise molecular mechanisms underlying the male-female difference in body size remain incompletely understood, recent studies have revealed a key role for the insulin/insulin-like growth factor signaling pathway (IIS) in the sex-specific regulation of developmental processes that influence body size (Shingleton *et al*. 2005; Gronke *et al*. 2010; Testa *et al*. 2013; Rideout *et al*. 2015; Liao *et al*. 2020; Millington *et al*. 2020 preprint).

Normally, IIS activity is higher in female larvae than in age-matched males (Rideout *et al.* 2015; Millington *et al*. 2020 preprint). Given that increased IIS activity is known to promote cell, tissue, and organismal size (Grewal 2009; Teleman 2009), this suggests that elevated IIS activity is one reason that females have a larger body size. Indeed, the sex difference in body size was abolished between male and female flies carrying a mutation that strongly reduced IIS activity (Testa *et al*. 2013), and between male and female pupae reared on diets that markedly decrease IIS activity (Rideout *et al*. 2015). In both cases, the sex difference in body size was eliminated by a female-biased decrease in body size (Testa *et al*. 2013; Rideout *et al*. 2015). While these findings suggest that IIS plays a role in sex-specific body size regulation during development, only one genetic combination was used to reduce IIS activity (Testa *et al*. 2013). Therefore, it remains unclear whether the sex-biased effect of reduced IIS activity on body size is a common feature of genotypes that alter IIS activity.

In the present study, we used multiple genetic approaches to either enhance or inhibit IIS activity, and monitored body size in males and females. While previous studies show that the genetic approaches we employed effectively alter IIS activity, the body size effects in each sex remain unclear due to frequent use of mixed-sex or

single-sex experimental groups, and the fact that statistical tests to detect sex-by-genotype interactions were not applied (Fernandez *et al*. 1995; Chen *et al*. 1996; Leevers *et al*. 1996; Böhni *et al*. 1999; Brogiolo *et al*. 2001; Cho *et al*. 2001; Rintelen *et al*. 2001; Ikeya *et al*. 2002; Britton *et al*. 2002; Rulifson *et al*. 2002; Zhang *et al*. 2009; Geminard *et al*. 2009; Gronke *et al*. 2010). Our systematic examination of IIS revealed most genetic manipulations that reduced IIS activity caused a female-biased reduction in body size. In contrast, most genetic manipulations that enhanced IIS activity increased male body size with no effect in females. Together, these findings provide additional genetic support for IIS as one pathway that impacts sex-specific body size regulation in *Drosophila*.

2.3. MATERIALS AND METHODS

2.3.1. Fly husbandry

Drosophila growth medium consisted 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 $CaCl_2 \cdot 2H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 11.77 mL acid mix (propionic acid/phosphoric acid). Diet data was deposited under "Rideout Lab 2Y diet" in the *Drosophila* Dietary Composition Calculator (Lesperance and Broderick 2020). For all experiments, parental flies of appropriate genotypes were crossed and allowed to lay eggs on grape juice agar plates for a period of 12 hours. At 24 hr AEL, larvae were picked off of grape juice agar plates into growth medium and raised at a density of 50 animals per 10 mL food at 25°C. Male and female pupae were sexed by gonad size. Adult flies were maintained at a density of twenty flies per vial in single-sex groups.

2.3.2. Fly strains

The following fly strains from the Bloomington *Drosophila* Stock Center were used: *w[1118]* (#3605), *UAS-rpr* (#5823), *UAS-Imp-L2-RNAi* (#55855), *InR[E19]* (#9646), *InR[PZ]* (#11661), *Df(3R)Pi3K92E[A]* (#25900), *chico[1]* (#10738), *foxo[21]* (#80943), *foxo[25]* (#80944), *r4-GAL4* (fat body), *dilp2-GAL4* (IPCs). Additional fly strains include: *UAS-Kir2.1* (Baines *et al*. 2001), *dilp1, dilp3, dilp4, dilp5, dilp6[41], dilp7, Df(3L)ilp2-3,5,*

Df(3L)ilp1-4,5 (Grönke *et al.* 2010)*, Sdr[1]* (Okamoto *et al.* 2013), *Pi3K92E[2H1]* (Halfar *et al.* 2001), *Pdk1[4]* (Rintelen *et al.* 2001), *Akt1[3]* (Stocker *et al.* 2002)*.* All fly strains except *dilp6[41]* were backcrossed into a *w[1118]* background for 6 generations. All strains without a visible marker were crossed six times to a *w[1118]* strain carrying a balancer chromosome corresponding to the genomic location of the gene. These crosses were in addition to prior extensive backcrossing of *dilp* mutant strains (Grönke *et al.* 2010).

2.3.3. Body size

For pupal volume, larvae of each genotype were synchronized at 24 hr AEL and cultured in *Drosophila* growth medium at a density of 50 larvae per vial to pupariation. Pupal length and width were determined using an automated detection and measurement system. Segmentation of the pupae for automated analysis was carried out using the "Marker-controlled Watershed" function included in the MorphoJ plugin (Klingenberg, 2011) in ImageJ (Schindelin *et al.* 2012; Rueden *et al.* 2017). Briefly, the original image containing the pupae was blurred using the "Gaussian blur" function. A selection of points marking the pupae was then created using the "Find Maxima" function. Next, a new image with the same dimension as the pupae was created, where the individual points were projected onto this original image using the "Draw" function. Then, we labelled each point using the "Connected Components Labeling" function in the MorphoJ plugin (Klingenberg 2011). This image is now the marker image. Upon completion of the marker image, we used the "Morphological Filters" function in the MorphoJ package with the options "operation=Gradient element=Octagon radius =2" to generate a gradient image of the pupae. Using the "Marker-controlled Watershed" function with the gradient image as the input, and the marker image to identify regions of interest outlining the pupae, the width and length of the pupae were obtained by selecting "Fit ellipse" option under the "Set Measurements" menu in ImageJ. Once length and width were determined using this automated measurement system, pupal volume was calculated using this formula: $4/3\pi(L/2)(W/2)^2$ (L, length; W, width) (Delanoue *et al.* 2010; Rideout *et al.* 2012, 2015; Marshall *et al.* 2012; Ghosh *et al.* 2014). To

measure adult weight, 5-day-old virgin male and female flies were collected and weighed in groups of ten on an analytical balance.

2.3.4. Statistical analysis and data presentation

GraphPad Prism (GraphPad Prism version 8.4.2 for Mac OS X) was used to perform all statistical tests and to prepare all graphs in this manuscript. Statistical tests and significance are indicated in figures and figure legends.

2.4. RESULTS

2.4.1. *Reduced IPC function causes a female-biased decrease in body size*

In *Drosophila*, the insulin-producing cells (IPCs) located in the brain are an important source of IIS ligands called *Drosophila* insulin-like peptides (Dilps). In larvae, the IPCs synthesize and release Dilp1 (FBgn0044051), Dilp2 (FBgn0036046), Dilp3 (FBgn0044050), and Dilp5 (FBgn0044048) into the hemolymph (Brogiolo *et al*. 2001; Ikeya *et al*. 2002; Rulifson *et al*. 2002; Lee *et al*. 2008; Geminard *et al*. 2009). When circulating Dilps bind to the Insulin-like Receptor (InR; FBgn0283499) on the surface of target tissues, an intracellular signaling cascade is initiated which ultimately promotes cell, tissue, and organismal size (Chen *et al*. 1996; Böhni *et al*. 1999; Poltilove *et al*. 2000; Britton *et al*. 2002; Werz *et al*. 2009; Almudi *et al*. 2013). The importance of the IPCs in regulating IIS activity and body size is illustrated by the fact that IPC ablation and silencing both reduce IIS activity and decrease overall body size (Rulifson *et al*. 2002; Geminard *et al*. 2009). Yet, the precise requirement for IPCs in body size regulation in each sex remains unclear, as past studies presented data from a mixed-sex population of larvae or reported effects in only a single sex (Rulifson *et al*. 2002; Geminard *et al*. 2009). Because recent studies show that the sex of the IPCs contributes to the sex-specific regulation of body size (Sawala and Gould 2017), we asked how the presence and function of the IPCs affected body size in each sex.

First, we ablated the IPCs by overexpressing proapoptotic gene *reaper* (*rpr*; FBgn0011706) with the IPC-specific GAL4 driver *dilp2-GAL4* (Brogiolo *et al*. 2001; Rulifson *et al*. 2002). This method eliminates the IPCs during development (Rulifson *et al*. 2002). To quantify body size, we measured pupal volume to capture developmental processes such as growth and weight loss that occur during the larval period (Delanoue *et al*. 2010; Testa *et al*. 2013). In females, pupal volume was significantly lower in *dilp2>UAS-rpr* pupae compared with *dilp2>+* and *+>UAS-rpr* control pupae (**Fig. 2.1A**). In males, pupal volume was also significantly lower in *dilp2>UAS-rpr* pupae compared with control *dilp2>+* and *+>UAS-rpr* pupae (**Fig. 2.1A**); however, the magnitude of the decrease in body size was greater in females than in males (sex:genotype interaction $p<0.0001$; two-way ANOVA). Next, to

determine how reduced IPC function affected body size in each sex, we overexpressed the inwardly-rectifying potassium channel *Kir2.1* (Baines *et al*. 2001) using *dilp2-GAL4*. This approach reduces Dilp secretion and lowers IIS activity in a mixed-sex group of larvae (Geminard *et al*. 2009). We found that pupal volume was significantly reduced in *dilp2>UAS-Kir2.1* females compared with *dilp2>+* and *+>UAS-Kir2.1* control females (**Fig. 2.1B**). In males, pupal volume was reduced in *dilp2>UAS-Kir2.1* pupae compared with *dilp2>+* and *+>UAS-Kir2.1* control pupae (**Fig. 2.1B**). Because the magnitude of the decrease in female body size was larger than the reduction in male body size (sex:genotype interaction $p<0.0001$; two-way ANOVA), this result indicates that inhibiting IPC function caused a female-biased reduction in pupal size. Together, these results identify a previously unrecognized sex-biased body size effect caused by manipulating IPC survival and function. Because previous studies show that IPC loss and IPC inhibition affects several developmental processes that impact final body size, these sex-specific body size effects may be due to sex-specific changes in larval growth, growth duration, and larval weight loss (Okamoto *et al*. 2013; Testa *et al*. 2013; Rideout *et al*. 2015; Sawala and Gould 2017).

2.4.2. Loss of IPC-derived Dilps causes a female-biased reduction in body size

Given that the larval IPCs produce Dilp1, Dilp2, Dilp3, and Dilp5 (Brogiolo *et al*. 2001; Ikeya *et al*. 2002; Rulifson *et al*. 2002; Lee *et al*. 2008; Geminard *et al*. 2009), we tested whether the loss of some (*Df(3L)ilp2-3,5)*, or all (*Df(3L)ilp1-4,5)*, of the IPC-derived Dilps affected pupal size in males and females. While a previous study reported how loss of all IPC-derived *dilp* genes affected adult weight, data from both sexes was not available for all genotypes (Gronke *et al*. 2010). In females, pupal volume was significantly smaller in *Df(3L)ilp2-3,5* pupae, which lack the coding sequences for *dilp2*, *dilp3*, and *dilp5* (Gronke *et al*. 2010), compared with w^{1118} control pupae (**Fig. 2.1C**). In males, body size was also significantly reduced in *Df(3L)ilp2-3,5* homozygous pupae compared with w^{1118} controls (**Fig. 2.1C**); however, the decrease in body size was significantly greater in females than in males (sex:genotype interaction $p<0.0001$; two-way ANOVA). When we measured

body size in males and females lacking all IPC-derived Dilps (*Df(3L)ilp1-4,5*), which lack the coding sequences for *dilp1*, *dilp2*, *dilp3*, *dilp4*, and *dilp5* (Gronke *et al*. 2010), we reproduced the female-biased reduction in body size (**Fig. 2.1C**; sex:genotype interaction *p*<0.0001; two-way ANOVA). This reveals a previously unrecognized sex-biased body size effect arising from loss of most, or all, IPC-derived Dilps. Given that several *dilp* genes are known to affect developmental processes that impact body size, these sex-specific body size effects may reflect sex-specific changes in larval growth rate and larval weight loss (Okamoto *et al*. 2013; Testa *et al*. 2013; Rideout *et al*. 2015; Sawala and Gould 2017), and possibly sex-specific effects on the duration of the larval growth period.
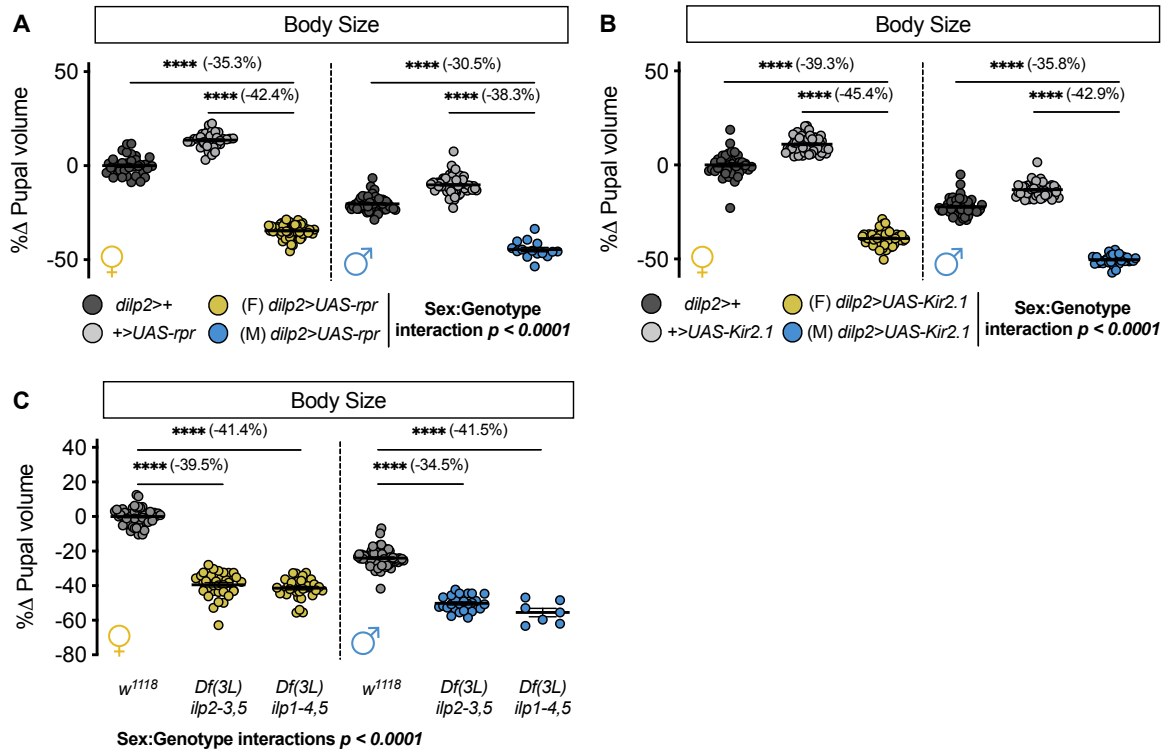
Figure 2.1. IPC ablation, loss of IPC function, and loss of IPC-derived Dilp ligands all cause a female-biased decrease in growth.

(A) Pupal volume in *dilp2>UAS-rpr* females and males compared to *dilp2>+* and *+>UAS-rpr* controls (*p*<0.0001 for all comparisons; two-way ANOVA followed by Tukey HSD test). *n* = 15-71 pupae. (B) Pupal volume in *dilp2>UAS-Kir2.1* females and males compared to both *dilp2>+* and *+>UAS-Kir2.1* controls (*p*<0.0001 for all comparisons; two-way ANOVA followed

by Tukey HSD test). *n* = 31-53 pupae. (C) Pupal volume in *Df(3L)ilp2-3,5* and *Df(3L)ilp1-4,5* homozygous females and males compared with sex-matched *w^1118* controls (*p*<0.0001 for all comparisons; two-way ANOVA followed by Tukey HSD test). *n* = 7-74 pupae. **** indicates *p*<0.0001; error bars indicate SEM. For all panels, females are shown on the left-hand side of the graph and males are shown on the right-hand side. *p*-values for all sex:genotype interactions are indicated on the graphs.

2.4.3. Loss of individual dilp genes causes a female-specific decrease in body size

While Dilp1, Dilp2, Dilp3 and Dilp5 are all produced by the IPCs, previous studies have uncovered significant differences in regulation, secretion, and phenotypic effects of these IPC-derived Dilps (Brogiolo *et al*. 2001; Zhang *et al*. 2009; Okamoto *et al*. 2009; Grönke *et al*. 2010; Cognigni *et al*. 2011; Stafford *et al*. 2012; Bai *et al*. 2012; Linneweber *et al*. 2014; Cong *et al*. 2015; Liu *et al*. 2016; Nässel and Vanden Broeck, 2016; Post *et al*. 2018, 2019; Semaniuk *et al*. 2018; Ugrankar *et al*. 2018; Brown *et al*. 2020). We therefore wanted to determine the individual contributions of IPC-derived Dilps to pupal size in each sex. Further, given that there are non-IPC-derived Dilps that regulate diverse aspects of physiology and behaviour (*dilp4*, FBgn0044049; *dilp6,* FBgn0044047; and *dilp7,* FBgn0044046) (Gronke *et al*. 2010; Castellanos *et al*. 2013; Garner *et al*. 2018), we wanted to determine the requirement for these additional Dilps in regulating pupal size in each sex. While a previous study measured adult weight as a read-out for body size in *dilp* mutants (Gronke *et al*. 2010), we measured pupal volume to ensure changes to adult weight were not due to altered gonad size (Green and Extavour 2014). We found that pupal volume was significantly smaller in female pupae lacking the coding sequences for *dilp1*, *dilp3*, *dilp4*, *dilp5*, and *dilp7,* respectively, compared with *w^1118* control females (**Fig. 2.2A**). This data aligns well with findings from two recent studies showing a female-specific decrease in larval size caused by loss of *dilp2* (Liao *et al*. 2020; Millington *et al*. 2021). In contrast to most *dilp* mutants; however, there was no significant difference in pupal volume between homozygous *y,w,dilp6*[41] female pupae and control *y,w* females (**Fig. 2.2B**). In males, pupal volume was not significantly different between *dilp1*, *dilp3*, *dilp4*, *dilp5*, and *dilp7* mutant pupae and *w^1118* controls (**Fig. 2.2C**); however, pupal volume was significantly reduced in

y,w,dilp6^{41} pupae compared with *y,w* controls (**Fig. 2.2D**). Together, these results extend our current understanding of body size regulation by revealing sex-specific requirements for all individual *dilp* genes in regulating body size. These sex-specific body size effects may be due to a combination of sex-specific effects on larval growth, weight loss in wandering larvae, or growth duration.
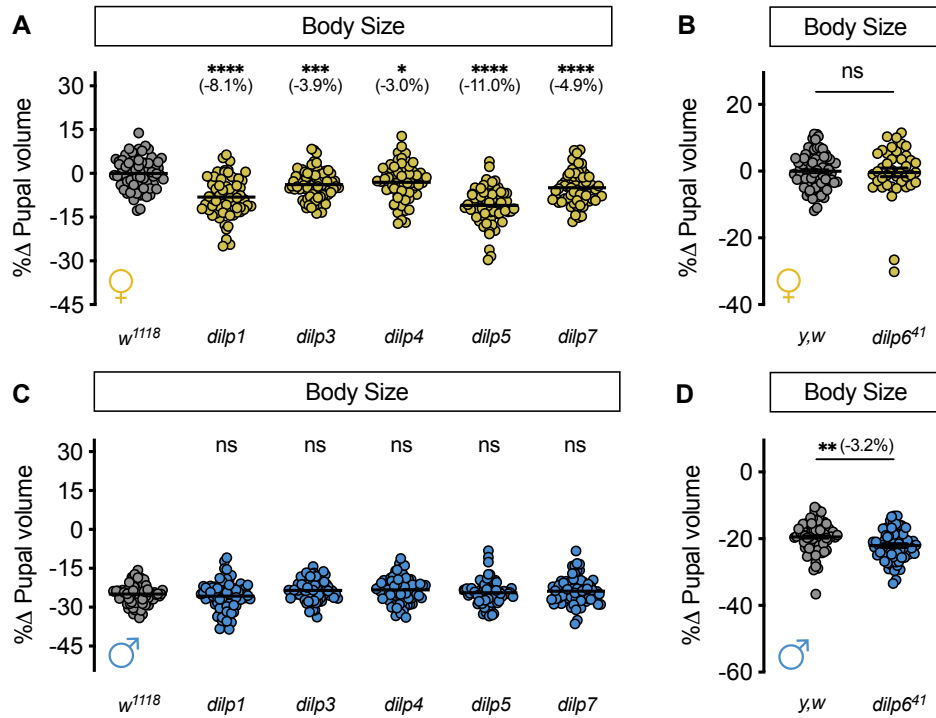
Figure 2.2. Loss of individual *dilp* genes causes sex-biased effects on growth.
(A) In females, pupal volume was significantly reduced compared with *w^{1118}* controls in pupae carrying individual mutations in each of the following genes: *dilp1*, *dilp3*, *dilp4*, *dilp5*, and *dilp7* ($p<0.0001$, $p = 0.0003$, $p = 0.0136$, $p<0.0001$, and $p<0.0001$, respectively; one-way ANOVA followed by Dunnett's multiple comparison test). n = 59-74 pupae. (B) Pupal volume was not significantly different between *y,w* control female pupae and *dilp6^{41}* mutant females ($p = 0.7634$, Student's *t* test). n = 41-74 pupae. (C) In males, pupal volume was not significantly reduced compared with *w^{1118}* controls in pupae carrying individual mutations in each of the following genes: *dilp1*, *dilp3*, *dilp4*, *dilp5*, and *dilp7* ($p = 0.7388$, $p = 0.2779$, $p = 0.1977$, $p = 0.9535$, and $p = 0.4526$, respectively; one-way ANOVA followed by Dunnett's multiple comparison test). n = 66-79 pupae. (D) Pupal volume was significantly reduced in male *dilp6^{41}* pupae compared with *y,w* control males ($p = 0.0017$, Student's *t* test). n = 64-70

pupae. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$; **** indicates $p<0.0001$; ns indicates not significant; error bars indicate SEM. Panels A and B display female data; panels C and D show male data.

2.4.4. Loss of Dilp-binding factor Imp-L2 causes a male-specific increase in body size

Once released into the circulation, the Dilps associate with proteins that modulate their growth-promoting effects. For example, Dilp1, Dilp2, Dilp5 and Dilp6 form a high-affinity complex with fat body-derived *ecdysone-inducible gene 2* (*Imp-L2*, FBgn0001257) and Convoluted/*Drosophila* Acid Labile Subunit (Conv/dALS; FBgn0261269) (Arquier *et al*. 2008; Honegger *et al*. 2008; Alic *et al*. 2011; Okamoto *et al*. 2013), whereas Dilp3 interacts with Secreted decoy receptor of Insulin-like Receptor (Sdr; FBgn0038279) (Okamoto *et al*. 2013). Binding of the Imp-L2/dALS complex to individual Dilps likely reduces Dilp binding to InR, as reduced fat body levels of either Imp-L2 or dALS augment IIS activity and increase body size (Arquier *et al*. 2008; Honegger *et al*. 2008; Alic *et al*. 2011). Similarly, loss of Sdr in flies carrying an amorphic *Sdr* allele (*Sdr^1*), increases IIS activity and increases body size (Okamoto *et al*. 2013). While the Sdr study reported that the magnitude of the increase in adult weight was equivalent in both sexes (Okamoto *et al*. 2013), which we confirm using pupal volume (**Fig. 2.3A**; sex:genotype interaction p = 0.5261; two-way ANOVA), it remains unclear how the Imp-L2/dALS complex affects pupal size in each sex. Given that one source of secreted Imp-L2 is the fat body (other tissues shown to express Imp-L2 include the corpora cardiaca, insulin-producing cells, and a subset of gut enteroendocrine cells) (Honegger *et al*. 2008; Sarraf-Zadeh *et al*. 2013), we overexpressed an RNAi transgene at equivalent levels in each sex (Millington *et al*. 2021) to reduce *Imp-L2* mRNA levels in the fat body. We found that in females, pupal volume was not significantly different between pupae with fat body-specific overexpression of the *Imp-L2-RNAi* transgene (*r4>UAS-Imp-L2-RNAi*) and control *r4>+* and *+>UAS-Imp-L2-RNAi* pupae (**Fig. 2.3B**). In contrast, pupal volume was significantly larger in *r4>UAS-Imp-L2-RNAi* male pupae compared with *r4>+* and *+>UAS-Imp-L2-RNAi* control males (**Fig. 2.3B**). This finding aligns with previous

studies showing that *Imp-L2* loss enhances body size (Honegger *et al*. 2008). Further, this finding extends our knowledge by identifying a male-specific effect of reduced fat body *Imp-L2* on pupal size (sex:genotype interaction *p*<0.0001; two-way ANOVA), a sex-biased effect that may arise due to sex-specific changes in larval growth, larval weight loss, or developmental timing.
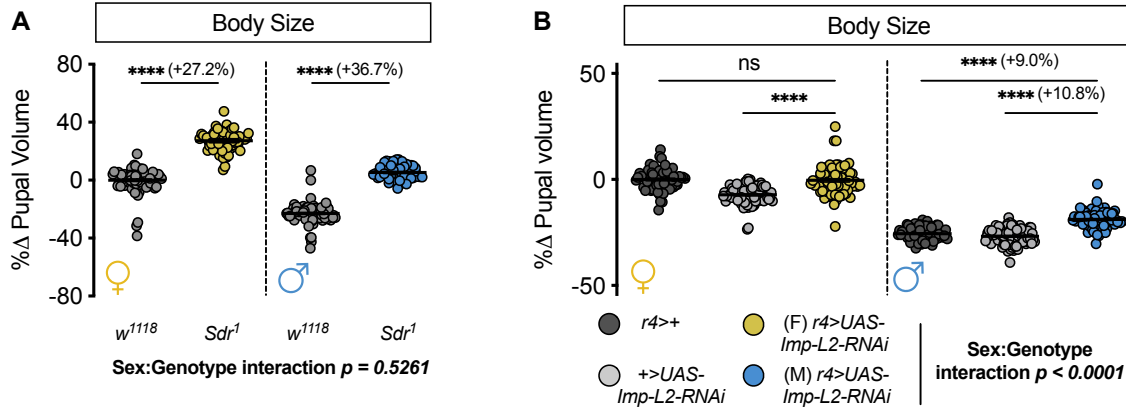
Figure 2.3. Fat body loss of Dilp-binding protein *Imp-L2* has sex-biased effects on growth.

(A) Pupal volume in *Sdr^1* mutant females and males compared with *w^{1118}* control females and males (*p*<0.0001 for both sexes; two-way ANOVA followed by Tukey HSD test). *n* = 52-88 pupae. (B) In females, pupal volume was not significantly different between pupae with fat body-specific knockdown of *Imp-L2* (*r4>UAS-Imp-L2-RNAi*) compared with *r4>+* and *+>UAS-Imp-L2-RNAi* control pupae (*p* = 0.9948 and *p*<0.0001, respectively; two-way ANOVA followed by Tukey HSD test), whereas *r4>UAS-Imp-L2-RNAi* males were significantly larger than *r4>+* and *+>UAS-Imp-L2-RNAi* control males (*p*<0.0001 for both comparisons; two-way ANOVA followed by Tukey HSD test). *n* = 70-92 pupae. **** indicates *p*<0.0001; ns indicates not significant; error bars indicate SEM. For all panels, females are shown on the left-hand side of the graph and males are shown on the right-hand side. *p*-values for all sex:genotype interactions are indicated on the graphs.

2.4.5. *Altered activity of the intracellular IIS pathway causes sex-biased and non-sex-specific effects on body size*

In flies, IIS activity is stimulated by Dilp binding the InR on the surface of target cells (Fernandez *et al*. 1995; Chen *et al*. 1996). This Dilp-InR interaction induces receptor autophosphorylation and recruitment of adapter proteins such as

chico (FBgn0024248), the *Drosophila* homolog of mammalian insulin receptor substrate (Böhni *et al*. 1999; Poltilove *et al*. 2000; Werz *et al*. 2009). The recruitment and subsequent activation of the catalytic subunit of *Drosophila* phosphatidylinositol 3-kinase (*Pi3K92E*; FBgn0015279) increases the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$) at the plasma membrane (Leevers *et al*. 1996; Britton *et al*. 2002), which activates signaling proteins such as Phosphoinositide-dependent kinase 1 (Pdk1; FBgn0020386) and Akt1 (FBgn0010379) (Alessi *et al*. 1997). Both Pdk1 and Akt1 phosphorylate many downstream effectors to promote body size (Verdu *et al*. 1999; Cho *et al*. 2001; Rintelen *et al*. 2001). The importance of these intracellular IIS components in regulating organism size is illustrated by studies showing that the loss, or reduced function, of most IIS components significantly decreases body size (Leevers *et al*. 1996; Chen *et al*. 1996; Böhni *et al*. 1999; Weinkove *et al*. 1999; Brogiolo *et al*. 2001; Rulifson *et al*. 2002; Zhang *et al*. 2009; Geminard *et al*. 2009; Grönke *et al*. 2010; Murillo-Maldonado *et al*. 2011). It is important to note that the effects of intracellular IIS components on body size are due to effects on several developmental processes including larval and pupal growth, larval weight loss, and growth duration (Chen *et al*. 1996; Böhni *et al*. 1999; Shingleton *et al*. 2005; Slaidina *et al*. 2009; Gronke *et al*. 2010; Testa *et al*. 2013). Yet, the majority of studies on the regulation of body size by intracellular IIS components were performed in a single- or mixed-sex population of larvae and/or adult flies, and tests for sex-by-genotype interactions were not applied (Fernandez *et al*. 1995; Chen *et al*. 1996; Leevers *et al*. 1996; Böhni *et al*. 1999; Brogiolo *et al*. 2001; Cho *et al*. 2001; Rintelen *et al*. 2001; Ikeya *et al*. 2002; Rulifson *et al*. 2002; Britton *et al*. 2002; Geminard *et al*. 2009; Zhang *et al*. 2009; Gronke *et al*. 2010). Given that recent studies have demonstrated the sex-specific regulation of IIS components such as Akt1 (Rideout *et al*. 2015), we investigated the requirement for each component in regulating pupal size in males and females. In line with previous results showing a female-biased decrease in adult weight in flies heterozygous for two hypomorphic *InR* alleles (Testa *et al*. 2013), we observed a female-biased pupal volume reduction in pupae carrying an additional combination of hypomorphic *InR*

alleles (**Fig. 2.4A**; sex:genotype interaction $p<0.0001$; two-way ANOVA) (Fernandez *et al*. 1995; Tatar *et al*. 2001).

To expand these findings beyond *InR*, we measured pupal volume in males and females with whole-body loss of individual intracellular IIS components. Given that we did not obtain viable pupae homozygous for an amorphic allele of *chico* (*chico¹*), we measured pupal volume in *chico¹*/+ males and females. In *chico¹*/+ females, pupal volume was significantly reduced compared with control w^{1118} pupae (**Fig. 2.4B**). In *chico¹*/+ males, pupal volume was reduced compared with control w^{1118} pupae (**Fig. 2.4B**). Given that the magnitude of the reduction in pupal volume was similar in males and females (sex:genotype interaction $p = 0.1399$; two-way ANOVA), reduced *chico* did not cause a sex-biased effect on pupal size. In females heterozygous for one predicted null and one loss-of-function allele of *Pi3K92E*, Df(3R)*Pi3K92E^A* and *Pi3K92E^{2H1}*, respectively (Weinkove *et al*. 1999; Halfar *et al*. 2001), pupal volume was significantly reduced compared with control w^{1118} pupae (**Fig. 2.4C**). In Df(3R)*Pi3K92E^A*/*Pi3K92E^{2H1}* males, we observed a significant reduction in pupal volume (**Fig. 2.4C**); however, the magnitude of the decrease in pupal size was larger in females compared with males (sex:genotype interaction $p<0.0001$; two-way ANOVA). This indicates that loss of Pi3K92E caused a female-biased decrease in body size. Similarly, a previous study showed that heterozygous loss of *Phosphatase and tensin homolog* (*Pten*; FBgn0026379), which antagonizes the lipid kinase activity of Pi3K92E to repress growth, also caused a sex-biased increase in pupal volume (Millington *et al*. 2021).

Next, we examined pupal size in males and females homozygous for a loss-of-function allele of *Pdk1* (*Pdk1⁴*). We observed no effect on pupal volume in either sex in *Pdk1⁴* homozygotes (**Fig. 2.4D**). Given that a previous study showed that adult weight was reduced in *Pdk1⁴*/*Pdk1⁵* (Rintelen *et al*. 2001), we additionally measured adult weight in order to make a direct comparison between our findings and past findings. We found an equivalent body size reduction in *Pdk1⁴* males and females compared with sex-matched control w^{1118} flies (**Fig. 2.4E**; sex:genotype interaction $p = 0.5030$; two-way ANOVA). This suggests that reduced *Pdk1* did not cause a sex-biased reduction in pupal size. One important target of *Pdk1* is the

serine/threonine kinase Akt1. In females, homozygous for a hypomorphic allele of *Akt1* (*Akt1³*), pupal volume was significantly reduced compared with control *w¹¹¹⁸* pupae (**Fig. 2.4F**). In *Akt1³* males, we observed a significant reduction in pupal size compared with control *w¹¹¹⁸* pupae (**Fig. 2.4F**). Given that the magnitude of the decrease in pupal size was larger in females than in males (sex:genotype interaction *p*<0.0001; two-way ANOVA), this indicates that loss of Akt1 caused a female-biased effect on pupal size. Together, these findings identify previously unrecognized sex-biased body size effects of reduced *Pi3K92E* and *Akt1*.

One downstream target of IIS that contributes to the regulation of body size is transcription factor *forkhead box, sub-group O* (*foxo*; FBgn0038197). When IIS activity is high, Akt1 phosphorylates Foxo to prevent Foxo from translocating to the nucleus (Puig *et al*. 2003). Given that Foxo positively regulates mRNA levels of many genes that are involved in growth repression and catabolism (Zinke *et al*. 2002; Junger *et al*. 2003; Kramer *et al*. 2003; Slack *et al*. 2011; Alic *et al*. 2011), elevated IIS activity enhances body size in part by inhibiting Foxo (Junger *et al*. 2003; Kramer *et al*. 2003). Because previous studies show increased Foxo nuclear localization and elevated Foxo target gene expression in males (Rideout *et al*. 2015; Millington *et al*. 2021), we examined how Foxo contributes to pupal size in each sex by measuring body size in females and males heterozygous for two different loss-of-function *foxo* alleles (*foxo²¹/foxo²⁵*). In *foxo²¹/foxo²⁵* females and males, pupal volume was not significantly different from sex-matched *w¹¹¹⁸* control pupae (**Fig. 2.4G**). To directly compare our findings with prior reports on body size effects of *foxo* (Kramer *et al*. 2003; Junger *et al*. 2003), we also measured adult weight. In adult females, body weight was not significantly different between *foxo²¹/foxo²⁵* mutants and control *w¹¹¹⁸* flies (**Fig. 2.4H**); however, *foxo²¹/foxo²⁵* adult males were significantly heavier than control *w¹¹¹⁸* males (**Fig. 2.4H**). Because we observed a male-specific increase in body size (sex:genotype interaction *p* = 0.0014; two-way ANOVA), our data suggests that Foxo function normally contributes to the reduced adult weight of males. This reveals a previously unrecognized sex-specific role for Foxo in regulating body size. Taken together, these results identify sex-biased effects on pupal size arising from reduced function of some intracellular IIS

components (*e.g.*, *InR*, *Pi3K92E*, *Akt*, *foxo*). In contrast, other intracellular IIS components have non-sex-specific effects on body size (*e.g.*, *chico*, *Pdk1*). It will be important in future studies to address how different developmental mechanisms (*e.g.*, larval growth, larval weight loss, growth duration) contribute to both sex-biased and non-sex-biased body size effects of individual IIS components.

A Body Size

**** (-61.6%)   **** (-54.8%)

%Δ Pupal volume

♀ w^{1118} InR^{E19}/InR^{PZ} ♂ w^{1118} InR^{E19}/InR^{PZ}

Sex:Genotype interaction *p < 0.0001*

B Body Size

**** (-4.2%)   **** (-8.1%)

%Δ Pupal volume

♀ w^{1118} $chico^1/+$ ♂ w^{1118} $chico^1/+$

Sex:Genotype interaction *p = 0.1399*

C Body Size

**** (-40.6%)   **** (-37.0%)

%Δ Pupal volume

♀ w^{1118} $Pi3K92E^{2H1}/Df(3R)Pi3K92E^A$ ♂ w^{1118} $Pi3K92E^{2H1}/Df(3R)Pi3K92E^A$

Sex:Genotype interaction *p < 0.0001*

D Body Size

ns   ns

%Δ Pupal volume

♀ w^{1118} $Pdk1^4$ ♂ w^{1118} $Pdk1^4$

E Body Size

** (-13.4%)   * (-15.0%)

%Δ Adult weight

♀ w^{1118} $Pdk1^4$ ♂ w^{1118} $Pdk1^4$

Sex:Genotype interaction *p = 0.503*

F Body Size

**** (-51.1%)   **** (-48.8%)

%Δ Pupal volume

♀ w^{1118} Akt^3 ♂ w^{1118} Akt^3

Sex:Genotype interaction *p < 0.0001*

G Body Size

ns   ns

%Δ Pupal volume

♀ w^{1118} $foxo^{21}/foxo^{25}$ ♂ w^{1118} $foxo^{21}/foxo^{25}$

H Body Size

ns   **** (+22.9%)

%Δ Adult weight

♀ w^{1118} $foxo^{21}/foxo^{25}$ ♂ w^{1118} $foxo^{21}/foxo^{25}$

Sex:Genotype interaction *p = 0.0014*

Figure 2.4. Both sex-biased and non-sex-biased effects on growth arise from loss of intracelllular IIS pathway components.

(A) Pupal volume in females and males heterozygous for two hypomorphic *InR* alleles (*InR^{E19}/InR^{PZ}*) compared with sex-matched *w^{1118}* controls (*p*<0.0001 for both sexes; two-way ANOVA followed by Tukey HSD test). *n* = 32-133 pupae. (B) Pupal volume in females and males heterozygous for a null *chico* allele (*chico^1/+*) compared with sex-matched *w^{1118}* controls (*p*<0.0001 for both females and males; two-way ANOVA followed by Tukey HSD test). *n* = 93-133 pupae. (C) Pupal volume in females and males heterozygous for a deficiency and hypomorphic allele of *Pi3K92E (Df(3R)Pi3K92E^A/Pi3K92E^{2H1}*) compared with sex-matched *w^{1118}* controls (*p*<0.0001 for all comparisons in females and males; two-way ANOVA followed by Tukey HSD test). Note: the *Df(3R)Pi3K92E^A/Pi3K92E^{2H1}* pupae were collected and analyzed in parallel with the *InR^{E19}/InR^{PZ}* genotype, so the *w^{1118}* control genotype data is shared between these experiments. *n* = 52-133 pupae. (D) Pupal volume was not significant different in either females or males homozygous for a hypomorphic *Pdk1* allele (*Pdk1^4*) compared with *w^{1118}* controls (*p* = 0.6739 and *p* = 0.7847, respectively; two-way ANOVA followed by Tukey HSD test). *n* = 61-84 pupae. (E) Adult weight in *Pdk1^4* females and males compared with *w^{1118}* controls (*p* = 0.0017 and *p* = 0.0491 for females and males respectively; two-way ANOVA followed by Tukey HSD test). *n* = 5-8 biological replicates of ten adult flies. (F) Pupal volume in females and males homozygous for a hypomorphic *Akt1* allele (*Akt1^3*) compared with sex-matched *w^{1118}* controls (*p*<0.0001 for both sexes; two-way ANOVA followed by Tukey HSD test). *n* = 44-60 pupae. (G) In females and males heterozygous for two hypomorphic alleles of foxo (*foxo^{21}/foxo^{25}*), pupal volume was not significantly different compared with sex-matched *w^{1118}* controls (*p* = 0.8841 and 0.9646, respectively; two-way ANOVA followed by Tukey HSD test). *n* = 110-153 pupae. (H) In *foxo^{21}/foxo^{25}* females, adult weight was not significantly different compared with *w^{1118}* controls (*p* = 0.8786; two-way ANOVA followed by Tukey HSD test). In males, adult weight was significantly higher in *foxo^{21}/foxo^{25}* flies compared with *w^{1118}* control flies (*p*<0.0001; two-way ANOVA followed by Tukey HSD test). *n* = 5-8 biological replicates of 10 adult flies. * indicates *p*<0.05; ** indicates *p*<0.01; **** indicates *p*<0.0001; ns indicates not significant; error bars indicate SEM. For all panels, females are shown on the left-hand side of the graph and males are shown on the right-hand side. *p*-values for all sex:genotype interactions are indicated on the graphs.

2.5. DISCUSSION

An extensive body of work has demonstrated an important role for IIS in promoting cell, tissue, and organismal size in response to nutrient input (Fernandez *et al*. 1995; Chen *et al*. 1996; Böhni *et al*. 1999; Britton *et al*. 2002; Grewal, 2009; Teleman, 2009). More recently, studies suggest that IIS also plays a role in sex-specific body size regulation (Testa *et al*. 2013; Rideout *et al*. 2015; Millington *et al*. 2020 preprint). However, potential links between IIS and the sex-specific regulation of body size were inferred from studies using a limited number of genotypes to modulate IIS activity. The goal of our current study was to determine whether the sex-biased body size effects observed in previous studies represent a common feature of genotypes that affect IIS activity. Overall, we found that the loss of most positive regulators of IIS activity caused a female-biased reduction in body size. On the other hand, loss of genes that normally repress IIS activity caused a male-specific increase in body size. Thus, most changes to IIS activity cause sex-biased, or sex-specific, effects on body size (summarized in Table 2.1), highlighting the importance of collecting and analyzing data from both sexes separately in studies that manipulate IIS activity and/or examine IIS-responsive phenotypes (*e.g.*, lifespan, immunity).

	Genetic Manipulation	*Female-biased*	*Male-biased*	*Non-sex-specific*	*Percent change body size*
Reduced circulating Dilps	IPC ablation	Yes			F – 34.5% M – 30.5%
	IPC silencing	Yes			F – 39.3% M – 35.8%
	dilp2-3,5	Yes			F – 39.5% M – 34.5%
	dilp1-4,5	Yes			F – 41.4% M – 41.5%
	dilp1	Yes			F – 8.1% M – ns
	dilp3	Yes			F – 3.9%

					M – ns
	dilp4	Yes			F – 3.0%
					M – ns
	dilp5	Yes			F – 11.0%
					M – ns
	dilp6		Yes		F – ns
					M – 3.2%
	dilp7	Yes			F – 4.9%
					M – ns
Increased circulating Dilps	*Sdr*			Yes	F – 27.2%
					M – ns
	Fat body Imp-L2		Yes		F – ns
					M + 9.0%
Intracellular IIS pathway	*InR*	Yes			F – 61.6%
					M – 54.8%
	chico^1/+			Yes	F – 4.2%
					M – 8.1%
	Pi3K92E	Yes			F – 40.6%
					M – 37.0%
	Pdk1			Yes	F – 13.4%
					M – 15.0%
	Akt	Yes			F – 51.1%
					M – 48.8%
	foxo		Yes		F – ns
					M + 22.9%

Table 2.1. Summary of sex-biased effects of IIS pathway manipulations on body size.

All data used in this summary table is derived from pupal volume experiments, except for *Pdk1* and *foxo*, where adult weight is shown.

One important outcome from our study was to provide additional genetic support for IIS as an important regulator of the sex difference in body size. Data implicating IIS in the sex-specific regulation of body size first emerged from a

detailed examination of the larval stage of development in wild-type flies of both sexes (Testa *et al*. 2013). In this study, the authors reported a female-biased body size reduction in flies with decreased InR function (Testa *et al*. 2013). A subsequent study extended this finding by uncovering a sex difference in IIS activity: late third-instar female larvae had higher IIS activity than age-matched males (Rideout *et al*. 2015). The reasons for this increased IIS activity remain incompletely understood; however, Dilp2 secretion from the IPCs was higher in female larvae than in males (Rideout *et al*. 2015). Given that Dilp2 overexpression is known to augment IIS activity and enhance body size (Ikeya *et al*. 2002; Geminard *et al*. 2009), these findings suggest a model in which high levels of circulating Dilp2 (and possibly other Dilps) are required in females to achieve and maintain increased IIS activity and a larger body size in nutrient-rich conditions. In males, lower circulating levels of Dilp2 lead to reduced IIS activity and a smaller body size. If this model is accurate, we predict that female body size will be more sensitive to genetic manipulations that reduce Dilp ligands and/or IIS activity. Previous studies provided early support for this model by demonstrating a female-biased reduction in body size due to strong *InR* inhibition and *dilp2* loss (Testa *et al*. 2013; Liao *et al*. 2020; Millington *et al*. 2020 preprint). Now, we provide strong genetic support for this model using multiple genetic manipulations to reduce IIS activity, confirming that *Drosophila* females depend on high levels of IIS activity to promote increased body size. One potential reason for this high level of IIS activity in females is to ensure successful reproduction, as IIS activity in females regulates germline stem cell divisions, ovariole number, and egg production (LaFever and Drummond-Barbosa 2005; Hsu *et al*. 2008; Hsu and Drummond-Barbosa 2009; Gronke *et al*. 2010; Extavour and Green 2014). Unfortunately, this elevated level of IIS activity shortens lifespan, revealing an important IIS-mediated tradeoff between fecundity and lifespan in females (Broughton *et al*. 2005).

A second prediction of this model is that augmenting either circulating Dilp levels or IIS activity will enhance male body size. Indeed, we demonstrate that loss of *Imp-L2*, which increases free circulating Dilp levels (Arquier *et al*. 2008; Honegger *et al*. 2008; Alic *et al*. 2011; Okamoto *et al*. 2013), and loss of *foxo*, which mediates

growth repression associated with low IIS activity (Junger *et al*. 2003; Kramer *et al*. 2003), both cause a male-specific increase in body size. Together, these findings suggest that the smaller body size of male pupae is partly due to low IIS activity. While the reason for lower IIS activity in males remains unclear, studies show that altered IIS activity in either of the two main cell types within the testis compromises male fertility (Ueishi *et al*. 2009; McLeod *et al*. 2010; Amoyel *et al*. 2014; Amoyel *et al*. 2016). Future studies will therefore need to determine how males and females each maintain IIS activity within the range that maximizes fertility. In addition, it will be important to determine whether the female-biased phenotypic effects of lower IIS activity that we observe, and which are prevalent in aging and lifespan studies (Clancy *et al*. 2001; Holzenberger *et al*. 2003; Magwere *et al*. 2004; Van Heemst *et al*. 2005; Selman *et al*. 2008; Regan *et al*. 2016; Kane *et al*. 2018) extend to additional IIS-associated phenotypes (*e.g*., immunity and sleep) (DiAngelo *et al*. 2009; Cong *et al*. 2015; Roth *et al*. 2018; Suzawa *et al*. 2019; Brown *et al*. 2020).

Another important task for future studies will be to gain deeper insight into sex differences in IPC function, as one study identified sex-specific Dilp2 secretion from the IPCs (Rideout *et al*. 2015). Indeed, recent studies have revealed the sex-specific regulation of one factor (*stunted*, FBgn0014391) that influences Dilp secretion from the IPCs (Millington *et al*. 2021), and female-specific phenotypic effects of another factor that influences IPC-derived Dilp expression (Woodling *et al*. 2020). Together, these studies suggest that sex differences in IPC function and circulating Dilp levels exist, and may arise from the combined effects of multiple regulatory mechanisms. Given that our knowledge of IPC function has recently expanded in a series of exciting studies (Meschi *et al*. 2019; Oh *et al*. 2019), more work will be needed to test whether these newly discovered modes of IPC regulation operate in both sexes. Further, it will be important to ascertain how sex differences in the IPCs are specified. One recent study showed that *Sex-lethal* (*Sxl*; FBgn0264270), a key regulator of female sexual development, acts in the IPCs to regulate the male-female difference in body size (Sawala and Gould 2017). By studying how *Sxl* function alters IPC gene expression, activity, and connectivity, it will be possible to gain mechanistic insight into the sex-specific regulation of body size.

Beyond an improved understanding of sex differences in IPC function, it will be essential to study the sex-specific regulation of *dilp* genes and Dilp proteins, as we show female-specific effects on body size in pupae lacking most individual *dilp* genes. While two previous studies report female-biased effects of loss of *dilp2* (Liao *et al.* 2020; Millington *et al.* 2020 preprint), this is the first report of a female-specific role for *dilp1*, *dilp3*, *dilp4, dilp5,* and *dilp7* in promoting growth. While the female-specific effect of *dilp2* loss on pupal size aligns with the fact that female larvae have higher circulating Dilp2 levels (Rideout *et al.* 2015), much remains to be discovered about the sex-specific regulation of most *dilp* genes and Dilp proteins. For example, females have an increased number of *dilp7*-positive cells compared with males (Castellanos *et al.* 2013; Garner *et al.* 2018); however, it is unclear whether these additional *dilp7*-positive cells in females augment circulating Dilp7 levels. A full understanding of the female-specific effects that accompany loss of most individual *dilp* genes will therefore require more knowledge of sex differences in the regulation of *dilp* genes and Dilp proteins. In addition to revealing the female-specific effects of many *dilp* genes on pupal size, we are also the first to report a male-specific body size effect of *dilp6*. Normally, Dilp6 function sustains growth in nonfeeding conditions, and is upregulated in low-nutrient contexts (Slaidina *et al.* 2009). Interestingly, male larvae have lower IIS activity than age-matched females (Rideout *et al.* 2015), where decreased IIS activity phenocopies a low-nutrient environment (Britton *et al.* 2002). Therefore, one potential explanation for the male-specific effect of *dilp6* loss on pupal size is that reduced IIS activity in normal males leads to an increased reliance on Dilp6 to maintain body size. In females, higher levels of potent growth-promoting Dilp2 (Ikeya *et al.* 2002), and possibly other Dilps, promote IIS activity to minimize the requirement for Dilp6 function. This possibility will be important to test in future studies, alongside experiments to address a potential sex-specific role for other regulators of *dilp6*/Dilp6 including steroid hormone ecdysone and the Toll signaling pathway (Slaidina *et al.* 2009; Suzawa *et al.* 2019). Further, as our knowledge of how individual *dilp* genes affect larval development and physiology continues to grow, analyzing data from both sexes will play an important role in

extending knowledge of the mechanisms underlying sex differences in body size and other IIS-associated traits.

In contrast to the female-biased effects of most genetic manipulations that reduced Dilp availability, we observed both sex-biased and non-sex-biased effects on body size in pupae with reduced function of key intracellular IIS components. For example, reduced InR, Pi3K92E, and Akt1 function caused a female-biased reduction in body size, whereas there was an equivalent reduction in male and female body size due to lower *chico* and *Pdk1* function. While more information on larval growth, developmental timing, and larval weight loss are needed to fully understand why different IIS components have sex-biased or non-sex-biased body size effects, one recent study showed that heterozygous loss of *chico* caused insulin hypersecretion (Sanaki *et al*. 2020). Given that hyperinsulinaemia contributes to insulin resistance, and that insulin resistance decreases *Drosophila* body size (Musselman *et al*. 2011, 2018; Pasco and Leopold 2012), more studies will be needed to determine whether the smaller body size of *chico^1/+* male and female pupae, and possibly *Pdk1* mutant flies, can be attributed to insulin resistance. In fact, more knowledge of sex-specific tissue responses to insulin is urgently needed in male and female flies, as studies in mice and humans have identified sex differences in insulin sensitivity (Macotela *et al*. 2009; Geer and Shen 2009). Because *Drosophila* is an emerging model to understand the mechanisms underlying the development of insulin resistance (Musselman *et al* 2011), this knowledge would help determine whether flies are a good model to investigate the sex-biased incidence of diseases associated with insulin resistance, such as the metabolic syndrome and type 2 diabetes (Mauvais-Jarvis 2015).

3. FEMALE-BIASED UPREGULATION OF INSULIN PATHWAY ACTIVITY MEDIATES THE SEX DIFFERENCE IN *DROSOPHILA* BODY SIZE PLASTICITY

3.1. SYNOPSIS

Nutrient-dependent body size plasticity differs between the sexes in most species, including mammals (Badyaev, 2002; Mcdonald et al., 2020; Shingleton et al., 2017; Stilwell and Davidowitz, 2010). Previous work in *Drosophila* showed that body size plasticity was higher in females (Mcdonald et al., 2020; Shingleton et al., 2017), yet the mechanisms underlying increased female body size plasticity remain unclear. Here, we discover that a protein-rich diet augments body size in females and not males because of a female-biased increase in activity of the conserved insulin/insulin-like growth factor signaling pathway (IIS). This sex-biased upregulation of IIS activity was triggered by a diet-induced increase in *stunted* mRNA in females, and required *Drosophila insulin-like peptide 2*, illuminating new sex-specific roles for these genes. Importantly, we show that in females, sex determination gene *transformer* promotes the diet-induced increase in *stunted* mRNA via transcriptional regulator Spargel. Together, these findings provide novel insight into mechanisms underlying the sex difference in nutrient-dependent body size plasticity.

3.2. INTRODUCTION

In insects, the rate of growth during development is influenced by environmental factors such as nutrient availability (Boulan et al., 2015; Edgar, 2006; Hietakangas & Cohen, 2009; Nijhout, 2003; Nijhout et al., 2014). When nutrients are abundant, the growth rate is high and body size is large (Beadle et al., 1938; Edgar, 2006; Mirth & Shingleton, 2012; Nijhout, 2003; Robertson, 1963). When nutrients are scarce, the growth rate is lower and body size is smaller (Beadle et al., 1938; Edgar, 2006; Mirth & Riddiford, 2007; Mirth & Shingleton, 2012; Nijhout, 2003; Robertson, 1963). This ability of an organism or genotype to adjust its body size in line with

nutrient availability is a form of phenotypic plasticity (Agrawal, 2001; Garland & Kelly, 2006). While the capacity of individuals to display nutrient-dependent changes to body size depends on many factors, one important factor that affects phenotypic plasticity is whether an animal is male or female (Stillwell et al., 2010; Teder & Tammaru, 2005). For example, in *Drosophila* the magnitude of changes to wing cell size and cell number in a nutrient-poor diet were larger in females compared with males (Alpatov, 1930). Similarly, the magnitude of protein- and carbohydrate-induced changes to several morphological traits was larger in female flies (Shingleton et al., 2017). While these studies clearly establish a sex difference in nutrient-dependent phenotypic plasticity, the genetic and molecular mechanisms underlying this increased trait size plasticity in females remain unclear.

Clues into potential mechanisms underlying the increased nutrient-dependent phenotypic plasticity in female flies have emerged from over 20 years of studies on nutrient-dependent growth in *Drosophila* (Andersen et al., 2013; Boulan et al., 2015; Edgar, 2006; Koyama & Mirth, 2018; Mirth & Piper, 2017). In particular, these studies have identified the conserved insulin/insulin-like growth factor signaling pathway (IIS) as a key regulator of nutrient-dependent growth in *Drosophila* (Böhni et al., 1999; Britton et al., 2002; Chen et al., 1996; Fernandez et al., 1995; Grewal, 2009; Teleman, 2009). In nutrient-rich conditions, insulin-producing cells (IPCs) in the larval brain release *Drosophila* insulin-like peptides (Dilps) into the circulation (Brogiolo et al., 2001; Géminard et al., 2009; Ikeya et al., 2002; Rulifson et al., 2002). These Dilps bind the Insulin-like Receptor (InR; FBgn0283499) on target cells to induce receptor autophosphorylation and recruitment of adapter proteins (Almudi et al., 2013; Böhni et al., 1999; Chen et al., 1996; Poltilove et al., 2000; Werz et al., 2009). These adapter proteins enable the recruitment of the regulatory and catalytic subunits of the *Drosophila* homolog of phosphatidylinositol 3-kinase (*Pi3K21B*; FBgn0020622 and *Pi3K92E*; FBgn0015279, respectively), which catalyzes the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) from phosphatidylinositol (4,5)-bisphosphate (PIP_2) (Leevers et al., 1996). Increased plasma membrane PIP_3 recruits and activates signaling proteins such as phosphoinositide-dependent kinase 1 (Pdk1; FBgn0020386) and Akt (Akt;

FBgn0010379), which influence diverse cellular processes to enhance cell, tissue, and organismal size (Cho et al., 2001; Grewal, 2009; Rintelen et al., 2001; Verdu et al., 1999).

In contrast, when nutrients are scarce, Dilp release from the IPCs is reduced (Géminard et al., 2009), and plasma membrane Pi3K recruitment, PIP_3 levels, and Pdk1- and Akt-dependent signaling are all reduced (Britton et al., 2002; Nowak et al., 2013). Together, these changes diminish cell, tissue, and organismal size (Arquier et al., 2008; Britton et al., 2002; Géminard et al., 2009; Honegger et al., 2008; Okamoto et al., 2013; Rulifson et al., 2002; Zhang et al., 2009). Indeed, the potent growth-promoting ability of IIS activation is shown by the fact that increased IIS activity augments body size (Arquier et al., 2008; Goberdhan et al., 1999; Honegger et al., 2008; Ikeya et al., 2002; Nowak et al., 2013; Okamoto et al., 2013; Oldham et al., 2002), whereas reduced IIS activity limits cell, organ, and body size (Böhni et al., 1999; Brogiolo et al., 2001; Chen et al., 1996; Colombani et al., 2003; Gao et al., 2000; Grönke et al., 2010; Leevers et al., 1996; Murillo-Maldonado et al., 2011; Rulifson et al., 2002; Weinkove et al., 1999; Zhang et al., 2009). Because increased IIS activity bypasses the reduction in cell size in low-nutrient conditions (Britton et al., 2002; Géminard et al., 2009; Nowak et al., 2013), and mutations that blunt IIS pathway activity reduce size in nutrient-rich contexts (Böhni et al., 1999; Brogiolo et al., 2001; Chen et al., 1996; Leevers et al., 1996), *Drosophila* studies have established IIS as one key pathway that promotes organismal growth downstream of nutrient input. While this highlights the impact of *Drosophila* on our knowledge of how IIS couples nutrient input with growth, it is important to note that most studies used a mixed-sex population of larvae. Given that cell and body size differ significantly between male and female flies (Alpatov, 1930; Brown & King, 1961; Okamoto et al., 2013; Partridge et al., 1994; Rideout et al., 2015; Sawala & Gould, 2017; Testa et al., 2013), more knowledge is needed of nutrient-dependent changes to body size and IIS activity in each sex.

Recent studies have begun to make progress in this area by studying IIS regulation and function in both sexes in a single dietary context (reviewed in Millington & Rideout, 2018). For example, in late third instar larvae there are sex

differences in *dilp* mRNA levels, IIS activity, and Drosophila insulin-like peptide-2 (Dilp2; FBgn0036046) secretion from the IPCs (Rideout et al., 2015; McDonald et al., 2020). Similarly, transcriptomic studies have detected male-female differences in mRNA levels of genes associated with IIS function (Mathews et al., 2017; Rideout et al., 2015), and revealed links between IIS and the sex determination hierarchy gene regulatory network (Castellanos et al., 2013; Chang et al., 2011; Clough et al., 2014; Fear et al., 2015; Garner et al., 2018; Goldman & Arbeitman, 2007). As increasing evidence of sex-specific IIS regulation accumulates, several reports reveal sex-limited and sex-biased phenotypic effects caused by changes to IIS function. Changes to IIS activity in larvae show sex-biased effects on growth and final body size (Grönke et al., 2010; Rideout et al., 2015; Shingleton et al., 2005; Testa et al., 2013; Millington et al., 2020), and there are widespread sex-specific and sex-biased changes to gene expression in adult flies with altered diet and IIS activity (Camus et al., 2019; Graze et al., 2018). Further, sex differences exist in how changes to diet and IIS activity affect life span (Bjedov et al., 2010; Clancy et al., 2001; Giannakou et al., 2004; Grönke et al., 2010; Regan et al., 2016; Tatar et al., 2001; Woodling et al., 2020; Wu et al., 2020). Together, these studies illuminate the utility of *Drosophila* in revealing sex-specific IIS regulation and describing the physiological impact of this regulation. Yet, more studies are needed to discover the molecular mechanisms underlying sex-specific IIS regulation, and to extend these studies beyond a single nutritional context.

Additional insights into male-female differences in the regulation of cell, tissue, and body size arise from studies on sex determination genes. In *Drosophila*, sex is determined by the number of X chromosomes. In XX females, a functional splicing factor called Sex-lethal (Sxl; FBgn0264270) is produced (Bell et al., 1988; Bridges, 1921; Cline, 1978; Salz & Erickson, 2010). Sxl-dependent splicing of *transformer* (*tra;* FBgn0003741) pre-mRNA allows a functional Tra protein to be produced in females (Belote et al., 1989; Boggs et al., 1987; Inoue et al., 1990; Sosnowski et al., 1989). In XY males, the lack of a functional Sxl protein causes the default splicing of *tra* pre-mRNA, and no functional Tra protein is produced in males (Cline & Meyer 1996; Salz & Erickson, 2010; Belote et al., 1989; Boggs et al., 1987;

Inoue et al., 1990; Sosnowski et al., 1989). The presence of functional Sxl and Tra proteins in females accounts for most aspects of female sexual development, behavior, and physiology (Anand et al., 2001; Billeter et al., 2006; Brown & King, 1961; Camara et al., 2008; Christiansen et al., 2002; Clough et al., 2014; Dauwalder, 2011; Demir & Dickson, 2005; Goodwin et al., 2000; Hoshijima et al., 1991; Hudry et al., 2016, 2019; Ito et al., 1996; Millington & Rideout, 2018; Neville et al., 2014; Nojima et al., 2014; Pavlou et al., 2016; Pomatto et al., 2017; Regan et al., 2016; Rezával et al., 2014, 2016; Rideout et al., 2010; Ryner et al., 1996; Sturtevant, 1945; von Philipsborn et al., 2014). Recently, new roles for Sxl and Tra in regulating body size were also described. While female flies are normally larger than males, females lacking neuronal *Sxl* were smaller than control females, and not different in size from males (Sawala & Gould, 2017). Similarly, females lacking a functional Tra protein were smaller than control females; however, these *tra* mutant females were still larger than males (Brown & King, 1961; Mathews et al., 2017; Rideout et al., 2015). Together, these studies indicate that Tra and Sxl are required to promote a larger body size in females; however, much remains to be discovered about the mechanisms by which Sxl and Tra impact body size. Moreover, which sex determination genes contribute to the male-female difference in diet-induced trait size plasticity remains unknown, as studies on sex determination genes used a single diet.

In the present study, we aimed to improve knowledge of the genetic and molecular mechanisms that contribute to male-female differences in nutrient-dependent phenotypic plasticity in *Drosophila.* Our detailed examination of body size revealed increased phenotypic plasticity in females in response to a protein-rich diet, in line with studies on plasticity in other traits (Shingleton et al., 2017). We discovered that a female-biased upregulation of IIS activity was responsible for the larger body size of females raised on a protein-rich diet. Mechanistically, we show that the nutrient-dependent upregulation of *stunted* (*sun*; FBgn0014391) mRNA levels by transcriptional coactivator Spargel (Srl; FBgn0037248) in females triggers the diet-induced increase in IIS activity, as females with reduced *sun* do not augment IIS activity or body size in a protein-rich diet. Importantly, we show that sex

determination gene *tra* is required for the nutrient-dependent increase in *sun* mRNA, IIS activity, and phenotypic plasticity in females, and that Srl represents a key link between Tra and regulation of *sun* mRNA levels. In males, ectopic *tra* expression confers nutrient-dependent body size plasticity via Srl-mediated regulation of *sun* mRNA levels and IIS activity. Together, these results provide new insight into the molecular mechanisms that govern male-female differences in body size plasticity, and identify a previously unrecognized role for sex determination gene *tra* in regulating nutrient-dependent phenotypic plasticity.

3.3. MATERIALS AND METHODS

3.3.1. Fly husbandry

For all experiments, parental flies of appropriate genotypes were crossed and allowed to lay eggs on grape juice agar plates for a period of 12 hours. At 24 hr AEL, larvae were picked off of grape juice agar plates into growth medium and raised at a density of 50 animals per 10 mL food at 25°C. *Drosophila* growth medium consisted of: 0.5x: 5.125 g/L sucrose, 17.725 g/L D-glucose, 12.125 g/L cornmeal, 11.325 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). 1x: 10.25 g/L sucrose, 25.45 g/L D-glucose, 24.25 g/L cornmeal, 22.65 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). 2x: 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). 1Y: 20.5 g/L sucrose, 70.9 g/L D-glucose, 48.5 g/L cornmeal, 22.65 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). 2Y: 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). Details for diets manipulating dietary sugar (1S) and calorie content (2Y calories) are found in Table S3.1. Our diets were also deposited in the *Drosophila* Dietary Composition Calculator (DDCC) (Lesperance and Broderick, 2020). Animals were collected as indicated in figure legends, and sexed by gonad size. When gonad size could not be used to determine sex (*e.g.*, *tra* mutants, *da-*

GAL4>UAS-tra^F), chromosomal females were identified by the presence of an X-linked GFP. Adult flies were maintained at a density of 20 flies per vial in single-sex groups.

3.3.2. Fly strains

The following fly strains from the Bloomington *Drosophila* Stock Center were used: *Canton-S* (#64349), *w^{1118}* (#3605), *tra^1 (#675), Df(3L)st-j7 (#5416), srl^1* (#14965), *InR^{E19}* (#9646), TRiP control (#36303) *UAS-ilp2-RNAi* (#32475), *UAS-upd2-RNAi* (#33949), *UAS-tra^F* (#4590), *y,w* (#1495), *da-GAL4* (ubiquitous), *r4-GAL4* (fat body), *cg-GAL4* (fat body), *dilp2-GAL4* (IPCs), *elav-GAL4* (post-mitotic neurons), *UAS-rheb* (#9688), *UAS-cyt-c-p-RNAi* (#64898), *UAS-Idh-RNAi* (#41708), *mth^1* (#27896). The following fly strains from the Vienna *Drosophila* Resource Center were used in this study: *UAS-sun-RNAi* (GD23685), *UAS-Gbp1-RNAi* (KK108755) *UAS-Gbp2-RNAi* (GD16696), *UAS-CCHa2-RNAi* (KK102257), *UAS-mth-RNAi* (KK106399). Additional fly strains include: *dilp2* (Grönke et al., 2010), *pten^{2L100}*, *UAS-sun, tGPH (GFP-PH), tra^{KO} (Hudry et al., 2016), tra^{F K-IN} (Hudry et al., 2019), y,w;;ilp2HF* (Park et al., 2014).

3.3.3. Body size

Pupal volume was measured in male and female pupae, as previously described, using this formula: $4/3\pi(L/2)(W/2)^2$ (L, length; W, width) (Delanoue et al., 2010; Marshall et al., 2012; Rideout et al., 2012, 2015). For adult weight, 5-day-old virgin male and female flies were weighed in groups of ten in 1.5 ml microcentrifuge tubes on an analytical balance. Wing length was measured as previously described (Garelli et al., 2012).

3.3.4. Developmental timing

Larvae were placed into the experimental diet ±2 hr post-hatching. Percent pupation was calculated by comparing the number of pupae at 12 hr intervals to the total pupae in the vial after all animals pupated.

3.3.5. Feeding behavior

Feeding behavior was quantified in sexed larvae by counting mouth hook contractions for 30 sec.

3.3.6. Protease feeding experiments

We treated larvae with a broad-spectrum protease inhibitor (PIC; Sigma-Aldrich #P2714) or a serine protease-specific inhibitor (AEBSF; Sigma-Aldrich #A8456) by adding the inhibitors to the food at final concentrations of 100 ml of 1x PIC per L, and 4 mM AEBSF as previously described (Erkosar et al., 2015).

3.3.7. RNA extraction and cDNA synthesis

One biological replicate represents ten larvae frozen on dry ice and stored at -80°C. Each experiment contained 3-4 biological replicates per sex, per genotype, and per diet, and each experiment was repeated twice. RNA was extracted using Trizol (Thermo Fisher Scientific; 15596018) according to manufacturer's instructions, as previously described (Marshall et al., 2012; Rideout et al., 2012, 2015; Wat et al., 2020). cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit according to manufacturer's instructions (Qiagen; 205314).

3.3.8. Quantitative real-time PCR (qPCR)

qPCR was performed as previously described (Rideout et al., 2012, 2015; Wat et al., 2020). mRNA levels were normalized to expression of *Actin5C* and *β-tubulin*. To determine changes in Foxo target gene expression, we plotted and analyzed the fold change in mRNA levels for each of three known Foxo target genes (*InR*, *bmm*, and *4E-BP*) together to quantify IIS activity in each sex and dietary context, an established approach to analyze co-regulated genes (Blaschke et al., 2013; Hudry et al., 2019). A complete primer list is available in Table S3.2.

3.3.9. Preparation of protein extract

Dissected fat bodies were prepared for SDS-PAGE by homogenizing sets of ten larval fat bodies 108 hr after egg laying in an appropriate volume of lysis buffer (20

mM Hepes (pH 7.8), 450 mM NaCl, 25% glycerol, 50 mM NaF, 0.2 mM EDTA, 1 mM DTT, 1x Protease Inhibitor Cocktail (Roche, 04693124001), 1x Phosphatase Inhibitor Cocktail (Roche, 4906845001) using the Omni Bead Ruptor (VWR). Cellular fragments were pelleted, and supernatant collected by centrifugation for 5 min at 10000 rpm at 4°C (Thermo Scientific, Heraeus Pico 21 centrifuge). Protein concentration was determined by Bradford assay (Bio-Rad #550-0205) prior to SDS-PAGE.

3.3.10.   SDS-PAGE and Western blotting

A total of 20 μL of sample with 20 μg protein was loaded into each well. Proteins were separated using a 12% gel SDS-PAGE gel in SDS running buffer, and transferred to a nitrocellulose membrane (Bio-Rad) for 2 hr at 40 V on ice. Membranes were incubated for 1 hr in blocking buffer (5% milk or 5% BSA in TBST 0.1%) then incubated with primary antibodies overnight at 4°C. Membranes were washed (3 x 2min) in TBST 0.1% then probed with secondary antibodies in blocking buffer for 1 hr at room temperature. After washes (3 x 2min, 2 x 15min,1 x 5min) in TBST 0.1%, membranes were treated with Pierce ECL (Thermo Scientific #32134) or Immobilon Forte (Millipore #WBLUF0100). Images were quantified using Image Studio (LI-COR). Primary antibodies: Anti-pS6K (#9209; Cell Signalling, and anti–actin (#8432; Santa Cruz), were used at 1:1000. HRP-conjugated secondary antibodies were used at 1:5000 for pS6k (anti-rabbit #65-6120; Invitrogen) and 1:3000 for actin (anti-mouse #7076; Cell Signalling).

3.3.11.   Hemolymph Western blotting

Hemolymph Western blotting was performed as previously described (Delanoue et al., 2016). Briefly, hemolymph from 40 larvae was collected in 40 μL of PBS with protease and phosphatase inhibitors (Roche 04693124001, Roche 4906845001), and hemocytes were removed by centrifugation according to the published protocol (Delanoue et al., 2016). Antibody concentrations used to detect hemolymph proteins were 1:50 for anti-Sun and 1:1000 for anti-Cv-d. Anti-guinea pig HRP-conjugated secondary was used at 1:2000.

3.3.12.   Fecundity and fertility

For female fecundity, single 6-day-old virgin female flies raised as indicated were crossed to three age-matched *CS* virgin males for a 24 hr mating period. Flies were transferred to fresh food vials with blue 2Y food to lay eggs. The number of eggs laid over 24 hr was quantified. For male fertility, single 6-day-old virgin males were paired with three 6-day-old virgin *CS* females to mate, and females were allowed to lay eggs for 24 hr. The number of progeny was quantified by counting viable pupae.

3.3.13.   Microscopy

GFP-PH larvae were picked into 1Y or 2Y food. Larvae were dissected 108 hr after egg laying (AEL) and inverted carcasses were fixed for 30 minutes in 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature. Carcasses were rinsed twice with PBS, once in 0.1% Triton-X in PBS (PBST) for 5 minutes, then incubated with Hoechst (5 µg/mL, Life Technologies H3570), LipidTOX Red (1:100, Thermo Fisher Scientific H34476), and phalloidin fluor 647 (1:1000, Abcam ab176759) in PBST for 40 min. The stained carcasses were washed with PBS and mounted in SlowFade Diamond (Thermo Fisher Scientific S36972). Images were acquired with a Leica SP5 (20X). Mean GFP intensity was quantified at the cell membrane (marked by phalloidin) and in the cytoplasm using Fiji (Schindelin et al., 2012). Three cells per fat body were measured, and at least five fat bodies per sex and per diet were measured.

3.3.14.   Statistics and data presentation

Statistical analyses and data presentation were carried out using Prism GraphPad 6 (GraphPad Prism version 8.4.3 for Mac OS X). Statistical tests and significance are indicated in figures and figure legends.

3.4. RESULTS

3.4.1. *High levels of dietary protein are required for increased nutrient-dependent body size plasticity in females*

Previous studies identified a sex difference in nutrient-dependent plasticity in several morphological traits (Shingleton et al., 2017; Stillwell et al., 2010; Teder & Tammaru, 2005). To determine whether sex differences in nutrient-dependent body size plasticity exist in *Drosophila*, we measured pupal volume, an established readout for *Drosophila* body size (Delanoue et al., 2010), in *white^{1118}* (*w*; FBgn0003996) males and females reared on diets of varying nutrient quantity. We found that pupal volume in *w^{1118}* female larvae raised on the 2-acid diet (1×) (Lewis, 1960) was significantly larger than genotype-matched females raised on a diet with half the nutrient quantity (0.5×) (**Fig. S3.1A**). In *w^{1118}* males, pupal volume was also significantly larger in larvae raised on the 1× diet compared with the 0.5× diet (**Fig. S3.1A**). No significant sex-by-diet interaction was detected using a two-way analysis of variance (ANOVA) (sex:diet interaction p = 0.7048), suggesting that nutrient-dependent body size plasticity was not different between the sexes in this context. We next compared pupal volume in *w^{1118}* males and females raised on the 1× diet with larvae cultured on a diet with twice the nutrient content (2×). Pupal volume in *w^{1118}* females was significantly larger in larvae raised on the 2× diet compared with larvae cultured on the 1× diet (**Fig. S3.1A**). In *w^{1118}* males, the magnitude of the nutrient-dependent increase in pupal volume was smaller compared with female larvae (**Fig. S3.1A**; sex:diet interaction p < 0.0001). This suggests that in nutrient-rich conditions, there is a sex difference in phenotypic plasticity, where nutrient-dependent body size plasticity is higher in females. To represent the normal body size responses of each sex to nutrient quantity, we plotted reaction norms for pupal volume in *w^{1118}* males and females raised on different diets (**Fig. S3.1B**). The body size response to increased nutrient quantity between 0.5× and 1× was not different between the sexes (**Fig. S3.1B**); however, the body size response to increased nutrient quantity between 1× and 2× was larger in females than in males (**Fig. S3.1B**). Importantly, these findings were not specific to pupal volume, as we reproduced our findings using adult weight as an additional readout for body size

(**Fig. 3.1A, B**). Thus, our findings demonstrate that while phenotypic plasticity is similar between the sexes in some nutritional contexts, body size plasticity is higher in females than in males in a nutrient-rich environment.
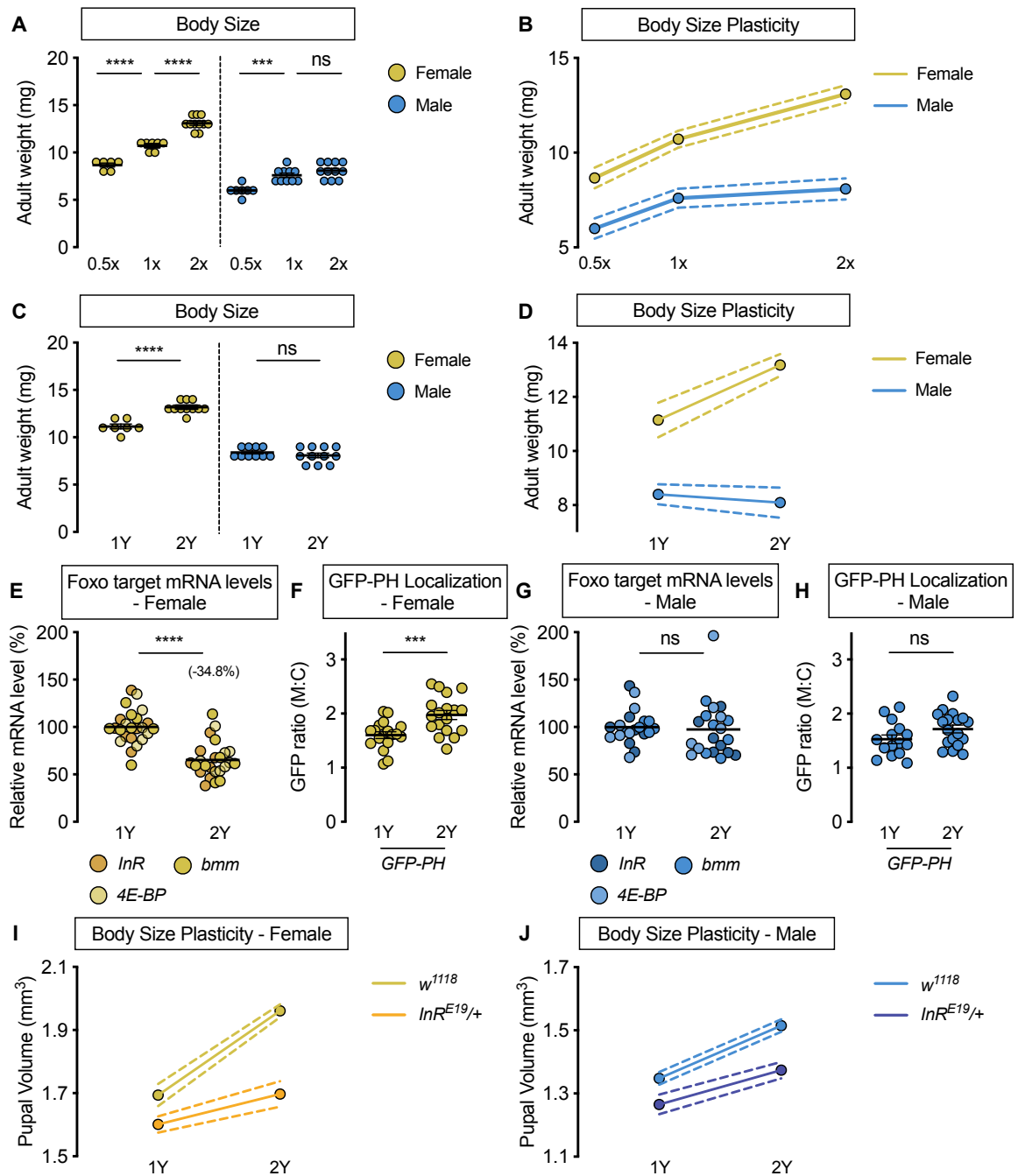
Figure 3.1. Upregulation of IIS activity is required for increased nutrient-dependent body size plasticity in females in a protein-rich diet.

(A) Adult weight was significantly higher in w^{1118} males and females cultured on 1× compared with flies raised on 0.5× ($p<0.0001$ for both sexes; two-way ANOVA followed by Tukey HSD test). The magnitude of this increase in adult weight was the same in both sexes (sex:diet interaction $p = 0.3197$; two-way ANOVA followed by Tukey HSD test). Adult weight was significantly higher in w^{1118} females raised on 2× compared to flies cultured on 1×; however, male adult weight was not significantly increased ($p<0.0001$ and $p = 0.4015$, respectively; two-way ANOVA followed by Tukey HSD test), where the diet-dependent increase in adult weight was higher in females (sex:diet interaction $p = 0.0003$; two-way ANOVA followed by Tukey HSD test). (B) Reaction norms for adult weight in response to changes in nutrient quantity in w^{1118} females and males, plotted using the data presented in panel A. n = 6-11 groups of 10 flies. (C) Adult weight was significantly higher in females cultured on 2Y compared with flies raised on 1Y; however, male adult weight was not significantly higher in flies raised on 2Y compared with males cultured on 1Y ($p<0.0001$ and $p = 0.7199$, respectively; two-way ANOVA followed by Tukey HSD test, sex:diet interaction $p<0.0001$). (D) Reaction norms for adult weight in w^{1118} females and males reared on either 1Y or 2Y, plotted using data from panel C. n = 7-11 groups of 10 flies. (E) In females, mRNA levels of Foxo targets (*insulin receptor* (*InR*)*, brummer* (*bmm*)*,* and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*)), were significantly lower in larvae raised on a protein-rich diet (2Y) compared with larvae raised on a diet containing half the protein content (1Y) ($p<0.0001$; Student's *t* test). n = 8 biological replicates. (F) Quantification of the ratio between cell surface membrane-associated green fluorescent protein (GFP) and cytoplasmic GFP fluorescence (GFP ratio [M:C]) in a dissected fat body of female larvae from the GFP-PH strain. The ratio was significantly higher in female larvae cultured on 2Y compared with larvae raised on 1Y ($p = 0.001$; Student's *t* test). n = 18 biological replicates. (G) In males, there was no significant difference in mRNA levels of Foxo targets between larvae raised on 2Y compared with larvae cultured on 1Y ($p = 0.7323$; Student's *t* test). n = 6-7 biological replicates. (H) In males, the ratio (M:C) for GFP-PH was not significantly different between males cultured on 2Y compared with larvae raised on 1Y ($p = 0.0892$; Student's *t* test). n = 15-18 biological replicates. (I) Pupal volume was significantly higher in both w^{1118} females and InR^{E19}/+ females reared on 2Y compared with genotype-matched females cultured on 1Y ($p<0.0001$ for both genotypes; two-way ANOVA followed by Tukey HSD test); however, the magnitude of the nutrient-dependent increase in pupal volume was

lower in $InR^{E19}/+$ females (genotype:diet interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). n = 58-77 pupae. (J) Pupal volume was significantly higher in both w^{1118} males and $InR^{E19}/+$ males reared on 2Y compared with genotype-matched males cultured on 1Y ($p<0.0001$ for both genotypes; two-way ANOVA followed by Tukey HSD test). While we observed a sex:diet interaction in the w^{1118} control genotype, there was no sex:diet interaction in the $InR^{E19}/+$ genotype ($p<0.0001$ and $p = 0.7104$, respectively; two-way ANOVA followed by Tukey HSD test). n = 47-76 pupae. For body size plasticity graphs, filled circles indicate mean body size, and dashed lines indicate 95% confidence interval. *** indicates p<0.001, **** indicates p<0.0001; ns indicates not significant; error bars indicate SEM.

To narrow down macronutrients that account for the increased body size plasticity in females, we changed individual food ingredients and measured body size in w^{1118} males and females. We first altered dietary yeast, as previous studies show that yeast is a key source of protein and an important determinant of larval growth (Britton et al., 2002; Géminard et al., 2009; Robertson, 1963). In w^{1118} females raised on a diet with yeast content that corresponds to the amount in the 2× diet (2Y diet), pupal volume was significantly larger than in females raised on a diet containing half the yeast content (1Y) (**Fig. S3.1C**). It is important to note that the yeast and calorie content of the 1Y diet was within the range of standard diets used in many larval growth studies (22.65 g/L vs. 21-46 g/L and 586 calories/L vs 459-760 calories/L, respectively) (Ghosh et al., 2014; Koyama & Mirth, 2016; Marshall et al., 2012; Sawala & Gould, 2017), and therefore does not represent a nutrient-restricted diet. In w^{1118} males, the magnitude of the nutrient-dependent increase in pupal volume was smaller than in females (**Fig. S3.1C**; sex:diet interaction $p = 0.0001$) suggesting that nutrient-dependent body size plasticity was higher in females in a yeast-rich context. Indeed, when we plotted reaction norms for pupal volume in both sexes, the magnitude of the yeast-dependent change in pupal volume (**Fig. S3.1D**) and adult weight (**Fig. 3.1C, D**) was larger in females than in males. This sex difference in phenotypic plasticity in a yeast-rich context was reproduced in *Canton-S* (*CS*), a wild-type strain (**Fig. S3.2A, B**), and using wing length as an additional measure of size (**Fig. S3.3A**). Thus, our findings indicate that the male-female

difference in nutrient-dependent body size plasticity persists across multiple genetic backgrounds, and confirms that body size is a robust trait to monitor nutrient-dependent phenotypic plasticity.

Given the sex difference in body size plasticity in response to altered yeast content, we hypothesized that yeast may trigger increased nutrient-dependent body size plasticity in females. To test this, we raised larvae on diets with altered sugar (**Fig. S3.4A**) or calorie content (**Fig. S3.4B**). Because we observed no sex:diet interaction for either manipulation (sex:diet interaction p = 0.6536 and p = 0.3698, respectively), this suggests dietary yeast mediates the sex difference in nutrient-dependent body size plasticity. To test whether protein is the macronutrient in yeast that enables sex-specific phenotypic plasticity, we pharmacologically limited protein breakdown by culturing larvae on the 2Y diet supplemented with either a broad-spectrum protease inhibitor (protease inhibitor cocktail; PIC) or a serine protease-specific inhibitor (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; AEBSF). Previous studies suggest that these inhibitors are specific, as the growth-inhibitory effects of these protease inhibitors was buffered by feeding larvae with bacteria that enhance intestinal protease mRNA levels and gut proteolytic activity (Erkosar et al., 2015). While we found a significant body size reduction in both sexes treated with protease inhibitors (**Fig. S3.5A, B**), in line with previous studies (Erkosar et al., 2015), the magnitude of the inhibitor-induced decrease in pupal volume was larger in female larvae than in males (sex:treatment interaction p = 0.0029 [PIC] and p < 0.0001 [AEBSF]). This indicates that yeast-derived dietary protein is the macronutrient that augments nutrient-dependent body size plasticity in females. While two potential explanations for the male-female difference in body size plasticity are a sex difference in food intake or length of the growth period, we found no differences in either phenotype between w^{1118} male and female larvae cultured on 1Y or 2Y (**Fig. S3.6A-C**). Moreover, the larger body size of female larvae does not explain their increased nutrient-dependent body size plasticity, as a genetic manipulation that augments male body size did not enhance phenotypic plasticity (**Fig. S3.7A, B**). Taken together, our data reveals female larvae have enhanced

body size plasticity in a nutrient-rich context, and identifies abundant dietary protein as a prerequisite for females to maximize body size.

3.4.2. The nutrient-dependent upregulation of IIS activity in females is required to achieve a larger body size in a protein-rich context

In a mixed-sex population of *Drosophila* larvae, IIS activity is positively regulated by nutrient availability to promote growth (Böhni et al., 1999; Britton et al., 2002; Chen et al., 1996; Fernandez et al., 1995; Grewal, 2009; Teleman, 2009). We therefore examined nutrient-dependent changes to IIS activity in larvae raised on 1Y and 2Y (**Fig. 3.1E-H**). Previous studies show that high levels of IIS activity repress mRNA levels of several genes via transcription factor Forkhead box, sub-group O (Foxo; FBgn0038197) (Alic et al., 2011; Jünger et al., 2003; Kang et al., 2017; Puig & Tjian, 2005; Zinke et al., 2002). We therefore assessed mRNA levels of known Foxo target genes *InR*, *brummer* (*bmm*, FBgn0036449), and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*, FBgn0261560) to quantify IIS activity in each sex and dietary context. Given that these genes are coregulated and behave the same in response to dietary manipulation (**Fig S3.8A,B**), we combined these data for our analysis, an established approach to analyze coregulated genes (Blaschke et al., 2013; Hudry et al., 2019). In w^{1118} females, mRNA levels of Foxo target genes were significantly lower in larvae reared on 2Y than in larvae raised on 1Y (**Fig. 3.1E**). This suggests IIS activity is significantly higher in females raised on 2Y than in females cultured on 1Y. To confirm this, we used the localization of a ubiquitously-expressed green fluorescent protein (GFP) fused to a pleckstrin homology (PH) domain (GFP-PH) as an additional readout of IIS activity. Because high levels of IIS activity raise plasma membrane PIP_3, and PH domains bind specifically to PIP_3, larvae with elevated IIS activity show increased membrane localization of GFP-PH (Britton et al., 2002). We observed a significantly higher membrane localization of GFP-PH in females cultured on 2Y than in female larvae raised on 1Y (**Fig. 3.1F**). Together with increased Foxo target gene repression in 2Y, this GFP-PH data indicates that females reared on 2Y have higher IIS activity than females cultured on 1Y. In males, the magnitude of the nutrient-dependent change in Foxo target genes

was smaller than in females (**Fig. 3.1G**). as we detected a significant sex:diet interaction for Foxo target genes (p = 0.0007). Indeed, there was no significant increase in GFP-PH membrane localization between males raised on 2Y and males reared on 1Y (**Fig. 3.1H**). Taken together, these results reveal a previously unrecognized female-biased upregulation of IIS activity in a protein-rich context.

To determine whether increased IIS activity is required in females for the ability to maximize body size on a protein-rich diet, we measured pupal volume in larvae heterozygous for a hypomorphic mutation in the *InR* gene (InR^{E19}/+) that were raised in either 1Y or 2Y. Previous studies have shown that while overall growth is largely normal in InR^{E19}/+ heterozygous animals, growth that requires high levels of IIS activity is blunted (Chen et al., 1996; Rideout et al., 2012, 2015). In w^{1118} control females, larvae cultured on 2Y were significantly larger than larvae raised on 1Y (**Fig. 3.1I**); however, the magnitude of this protein-dependent increase in pupal volume was smaller in InR^{E19}/+ females (**Fig. 3.1I**; genotype:diet interaction p < 0.0001). This suggests that nutrient-dependent body size plasticity was reduced in InR^{E19}/+ females. Indeed, while we observed a sex difference in phenotypic plasticity in the w^{1118} control genotype (sex:diet interaction p < 0.0001), the sex difference in nutrient-dependent body size plasticity was abolished in the InR^{E19}/+ genotype (**Fig. 3.1I, J**; sex:diet interaction p = 0.7104). Together, these results indicate that the nutrient-dependent upregulation of IIS activity in females is required for them to achieve a larger body size in a protein-rich context, and that the sex difference in body size plasticity arises from the female-biased upregulation of IIS activity in a protein-rich context.

3.4.3. dilp2 is required for the nutrient-dependent upregulation of IIS activity and a larger body size in females raised on a protein-rich diet

Previous studies have identified changes to the production and release of Dilps as important mechanisms underlying nutrient-dependent changes to IIS activity and body size (Colombani et al., 2003; Géminard et al., 2009; Zhang et al., 2009). For example, mRNA levels of *Drosophila insulin-peptide-3* (*dilp3*; FBgn0044050)

and *Drosophila insulin-peptide-5* (*dilp5*; FBgn0044048), but not *dilp2*, decrease in response to nutrient withdrawal (Colombani et al., 2003; Géminard et al., 2009; Ikeya et al., 2002), and the release of Dilps 2, 3, and 5 from the IPCs is altered by changes in nutrient availability (Géminard et al., 2009; Kim & Neufeld, 2015). Levels of Dilp2 also fluctuate during larval development (Slaidina et al., 2009). Interestingly, a recent study suggests that late third-instar female larvae have increased Dilp2 secretion compared with age-matched males when the larvae were raised in a diet equivalent to 2Y (Rideout et al., 2015). Given that Dilp2 is an important growth-promoting Dilp (Grönke et al., 2010; Ikeya et al., 2002), we tested whether *dilp2* was required in females for the nutrient-dependent upregulation of IIS activity. In control w^{1118} females, mRNA levels of Foxo target genes were significantly lower in larvae raised on 2Y than in larvae reared on 1Y (**Fig, 3.2A**), suggesting a nutrient-dependent increase in IIS activity. In contrast, mRNA levels of Foxo target genes were not significantly lower in *dilp2* mutant female larvae raised on 2Y compared with genotype-matched females cultured on 1Y (**Fig, 3.2A**), suggesting that loss of *dilp2* in females eliminated the nutrient-dependent increase in IIS activity. The magnitude of the nutrient-dependent decrease in Foxo target gene expression was smaller in w^{1118} males compared with w^{1118} females (**Fig, 3.2B**, sex:diet interaction p = 0.0511), but not in *dilp2* mutant males compared with genotype-matched females (sex:diet interaction p = 0.6754). This indicates that *dilp2* loss blocks the female-biased upregulation of IIS activity in a protein-rich diet.
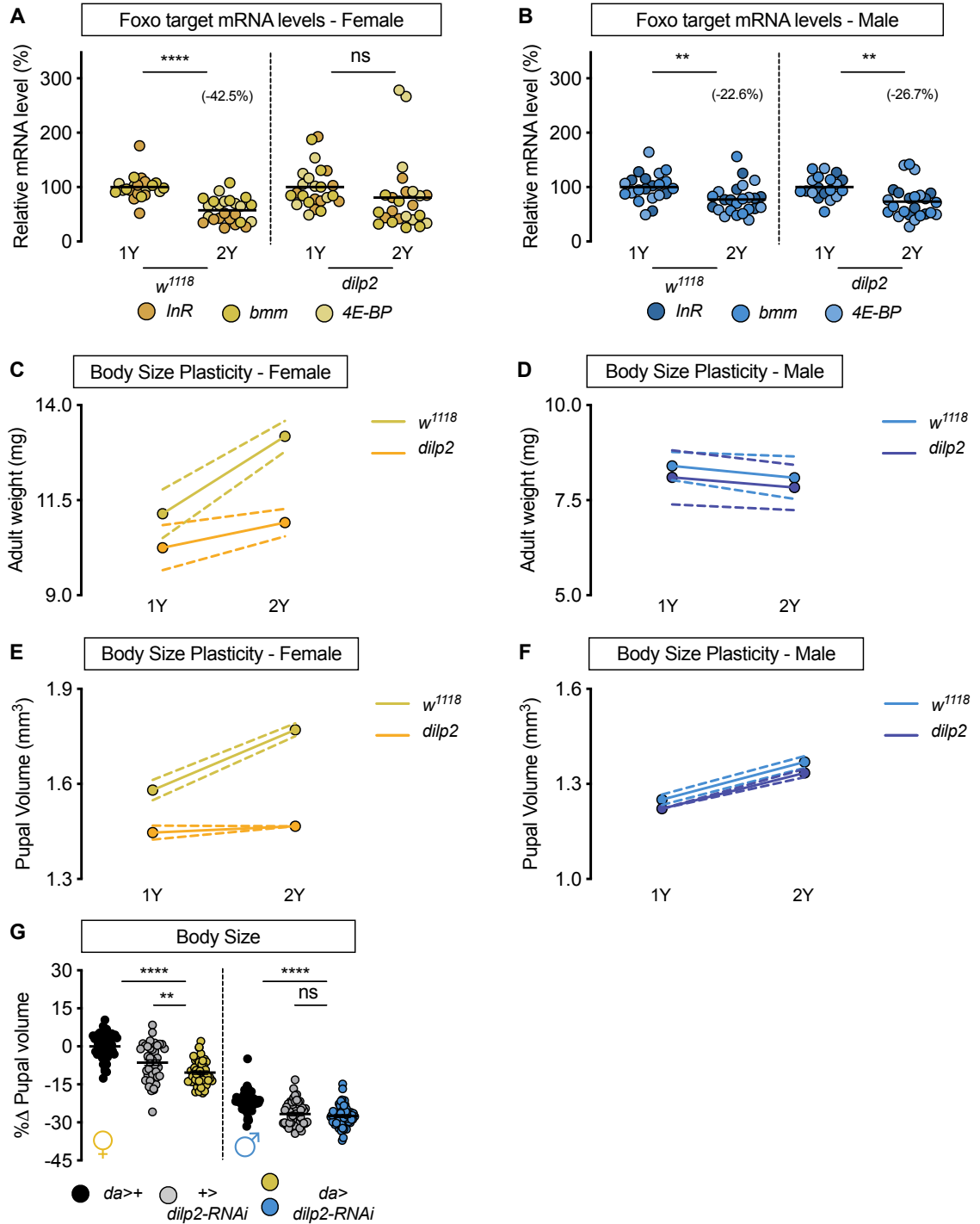
Figure 3.2. *Drosophila* insulin-like peptide 2 is required for the nutrient-dependent upregulation of insulin pathway activity and increased female body size plasticity.
(A) In control *w^1118* females, mRNA levels of Foxo targets (*insulin receptor* (*InR*)*, brummer* (*bmm*)*,* and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*)), were significantly lower in larvae cultured on a protein-rich diet (2Y) compared with larvae raised on a diet

67

containing half the protein content (1Y) ($p<0.0001$; Student's *t* test). In *dilp2* mutant females, there was no significant difference in mRNA levels of Foxo targets in larvae cultured on 2Y compared with larvae raised on 1Y ($p = 0.2231$ Student's *t* test). n = 8 biological replicates. (B) In control *w^1118* and *dilp2* mutant males, mRNA levels of Foxo targets were significantly lower in larvae cultured on 2Y compared with larvae raised on 1Y ($p = 0.0066$ and $p = 0.0023$ respectively; Student's *t* test). n = 7-8 biological replicates; however, the magnitude of the reduction in Foxo target gene expression in *w^1118* males was smaller than in genotype-matched females. (C) Adult weight was significantly higher in *w^1118* females raised on 2Y compared with flies cultured on 1Y ($p<0.0001$; two-way ANOVA followed by Tukey HSD test); however, adult weight was not significantly different between *dilp2* mutant females reared on 2Y versus 1Y ($p = 0.1263$; two-way ANOVA followed by Tukey HSD test). n = 7-11 groups of 10 flies. (D) Adult weight in control *w^1118* and *dilp2* mutant males was not significantly higher in flies reared on 2Y compared with males raised on 1Y ($p = 0.8366$ and $p = 0.8817$, respectively; two-way ANOVA followed by Tukey HSD test). There was a significant sex:diet interaction in the control *w^1118* genotype ($p<0.0001$), but not in the *dilp2* mutant genotype ($p = 0.0827$; two-way ANOVA followed by Tukey HSD test). n = 10-12 groups of 10 flies. (E) Pupal volume was significantly higher in *w^1118* females but not in *dilp2* mutant females reared on 2Y compared with genotype-matched females cultured on 1Y ($p<0.0001$ and $p = 0.6486$ respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was higher in *w^1118* females (genotype:diet interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). n = 74-171 pupae. (F) Pupal volume was significantly higher in *w^1118* males and *dilp2* mutant males reared on 2Y compared with genotype-matched males cultured on 1Y ($p<0.0001$ for both genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was not different between genotypes (genotype:diet interaction $p = 0.6891$; two-way ANOVA followed by Tukey HSD test). n = 110-135 pupae. (G) Pupal volume was significantly reduced in females upon RNAi-mediated knockdown of *dilp2* in 2Y when compared to both control genotypes ($p<0.0001$ [*da>*+], and $p = 0.002$ [+>*UAS-dilp2-RNAi*], respectively; two-way ANOVA followed by Tukey HSD test), but not in males in 2Y ($p<0.0001$ [*da>*+], and 0.9634 [+>*UAS-dilp2-RNAi*], respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the effect of RNAi-mediated knockdown of *dilp2* on pupal volume was higher in females (sex:genotype interaction $p = 0.003$; two-way ANOVA followed by Tukey HSD test). n = 44-59 pupae. For all body size plasticity graphs, filled circles indicate mean body size, and dashed lines indicate 95%

confidence interval. ** indicates $p<0.01$, **** indicates $p<0.0001$; ns indicates not significant; error bars indicate SEM.

To determine whether the inability to augment IIS activity on 2Y affects the nutrient-dependent increase in female body size, we measured body size in w^{1118} and *dilp2* mutant larvae cultured on either 1Y or 2Y. In w^{1118} control females, adult weight was significantly higher in flies cultured on 2Y compared with flies raised on 1Y (**Fig, 3.2C**); however, this nutrient-dependent increase in adult weight was not observed in *dilp2* mutant females (**Fig, 3.2C**; genotype:diet interaction $p = 0.0024$). In w^{1118} control males and *dilp2* mutant males, there was no significant increase in adult weight in flies raised on 2Y compared with genotype-matched flies cultured on 1Y (**Fig, 3.2D**; genotype:diet interaction $p = 0.935$). Indeed, in contrast to the sex difference in nutrient-dependent body size plasticity in the w^{1118} genotype (sex:diet interaction $p < 0.0001$), the sex difference in phenotypic plasticity was abolished in the *dilp2* mutant genotype (sex:diet interaction $p = 0.0827$). Importantly, we replicated all these findings using pupal volume (**Fig, 3.2E, F**), and reproduced the female-specific effects of *dilp2* loss by globally overexpressing a *UAS-dilp2-RNAi* transgene (**Fig, 3.2G**), and show that *dilp2* loss does not alter feeding behavior (**Fig. S3.9A**). While we did not determine a sex difference in circulating Dilp2 levels in larvae with an endogenously tagged *dilp2* allele due to body size plasticity defects in this strain (Park et al., 2014) (**Fig. S3.10A, B**), an experiment that will be important to repeat in future using alternative ways of measuring circulating Dilp2, we show that changes to *dilp* mRNA levels in males and females lacking *dilp2* (**Fig. S3.11A, B**), and nutrient-dependent changes to *dilp* mRNA levels (**Fig. S3.12A, B**), were similar in both sexes. Together, our data reveals a previously unrecognized female-specific requirement for *dilp2* in triggering a nutrient-dependent increase in IIS activity and body size in a protein-rich context.

3.4.4. A nutrient-dependent increase in stunted mRNA levels is required for enhanced IIS activity and a larger body size in females cultured in a protein-rich context

Nutrient-dependent changes in Dilp secretion from the IPCs, and consequently IIS activity, are mediated by humoral factors that are regulated by dietary nutrients (Britton & Edgar, 1998; Delanoue et al., 2016; Koyama & Mirth, 2016; Rajan & Perrimon, 2012; Rodenfels et al., 2014; Sano et al., 2015). For example, in a mixed-sex population of larvae, dietary protein augments mRNA levels of *Growth-blocking peptides 1* and *2* (*Gbp1*, FBgn0034199; *Gbp2*, FBgn0034200), *CCHamide-2* (*CCHa2;* FBgn0038147), *unpaired 2* (*upd2;* FBgn0030904), and *sun* (Delanoue et al., 2016; Koyama & Mirth, 2016; Rajan & Perrimon, 2012; Sano et al., 2015). Increased levels of these humoral factors promote the secretion of IPC-produced Dilps to enhance IIS activity and growth (Delanoue et al., 2016; Koyama & Mirth, 2016; Meschi et al., 2019; Rajan & Perrimon, 2012; Sano et al., 2015). To determine whether any humoral factors contribute to the sex-biased increase in IIS activity in a protein-rich diet, we examined mRNA levels of each factor in larvae of both sexes raised on either 1Y or 2Y. In w^{1118} females, *sun* mRNA levels in larvae reared on 2Y were significantly higher than in larvae cultured on 1Y (**Fig. 3.3A**). In contrast, mRNA levels of *Gbp1*, *Gbp2*, *CCHa2*, and *upd2* were not significantly higher in female larvae reared on 2Y compared with 1Y (**Fig. 3.3B**). Thus, while previous studies have shown that mRNA levels of all humoral factors were severely reduced by a nutrient-restricted diet or nutrient withdrawal (Delanoue et al., 2016; Koyama & Mirth, 2016; Rajan & Perrimon, 2012; Sano et al., 2015), our study suggests that for most factors, augmenting dietary protein beyond a widely-used level does not further enhance mRNA levels. In males, there was no significant increase in *sun* mRNA levels (**Fig. 3.3C**), or any other humoral factors (**Fig. 3.3D**), in larvae reared on 2Y compared with 1Y. Thus, there is a previously unrecognized sex difference in the regulation of *sun* mRNA levels in a protein-rich context, which we confirm leads to a sex difference in circulating Sun levels (**Fig. S3.13A**).

Given that a comprehensive series of genetic, molecular, and organ co-culture experiments have established that Sun promotes IIS activity by enhancing

Dilp2 secretion (Delanoue et al., 2016), we hypothesized that the female-specific increase in *sun* mRNA levels in 2Y triggers the nutrient-dependent upregulation of IIS activity in females. To test this, we overexpressed UAS-*sun-RNAi* in the larval fat body using *r4-GAL4*, and cultured the animals on either 1Y or 2Y. Importantly, overexpression of the *UAS-sun-RNAi* transgene significantly decreased *sun* mRNA levels in both sexes (**Fig. S3.14A, B**), where GAL4 expression was similar between the sexes in 1Y and 2Y (**Fig. S3.14C**). In control *r4>+* and *+>UAS-sun-RNAi* females, we observed a significant decrease in Foxo target gene expression in larvae cultured on 2Y compared with genotype-matched larvae reared on 1Y (**Fig. 3.3E**). In contrast, the nutrient-dependent decrease in Foxo target gene expression was absent in *r4>UAS-sun-RNAi* females (**Fig. 3.3E**; diet:genotype interaction $p < 0.0001$), suggesting *sun* is required in females for the nutrient-dependent increase in IIS activity. In males, the magnitude of the nutrient-dependent decrease in Foxo target gene expression was smaller than in genotype-matched females for the *r4>+* and *+>UAS-sun-RNAi* control strains ($p = 0.0166$ [*r4>+*]; $p = 0.0119$ [*+>UAS-sun-RNAi*]), but not in the *r4>UAS-sun-RNAi* strain (**Fig. 3.3F**) (sex:diet interaction $p = 0.1121$ [*r4>UAS-sun-RNAi*]). Importantly, the lack of a diet:genotype interaction among males indicates that there was no effect of genotype on Foxo target gene expression ($p = 0.1068$). Together, this data suggests that in females a protein-rich diet stimulates a nutrient-dependent increase in *sun* mRNA that promotes IIS activity. In males, the 2Y diet did not augment *sun* mRNA levels, suggesting one reason for the female-biased increase in IIS activity in a protein-rich diet.

We next asked whether the female-specific increase in *sun* mRNA and its impact on IIS activity contribute to the nutrient-dependent increase in female body size in a protein-rich context. In *r4>+* and *+>UAS-sun-RNAi* control females, adult weight was significantly higher in flies cultured on 2Y compared with genotype-matched flies raised on 1Y (**Fig. 3.3G**). In contrast, the nutrient-dependent increase in adult weight was abolished in *r4>UAS-sun-RNAi* females (**Fig. 3.3G**; genotype:diet interaction $p = 0.0014$). This indicates *r4>UAS-sun-RNAi* females have reduced nutrient-dependent body size plasticity, a finding that cannot be explained by changes to feeding behavior (**Fig. S3.15A**). In *r4>+*, *+>UAS-sun-RNAi*,

and *r4>UAS-sun-RNAi* male flies raised on 2Y, adult weight was not significantly higher than in genotype-matched males raised on 1Y (**Fig. 3.3H**; genotype:diet interaction p = 0.9278). Importantly, in contrast to the sex difference in nutrient-dependent body size plasticity we observed in the *r4>+* and *+>UAS-sun-RNAi* control genotypes (sex:diet interaction p = 0.011 and p = 0.0005, respectively), the sex difference in phenotypic plasticity was abolished in the *r4>UAS-sun-RNAi* genotype (sex:diet interaction p = 0.8749), findings we reproduced using pupal volume (**Fig. S3.16A, B**). While we observed no phenotypic plasticity effects in larvae with whole-body, pan-neuronal, or IPC loss of Sun receptor *methuselah* (*mth*; Fbgn0023000; Delanoue et al., 2016) (**Fig. S3.17A-F**), likely due to use of different *dilp2-GAL4* lines, minor variation in rearing conditions, and sex-specific plasticity defects in the *dilp2-GAL4* strain, we reproduced the female-specific effects of *sun* knockdown on body size using an additional fat body GAL4 line (**Fig. S3.18A**). Further, we show that this role for *sun* in mediating the nutrient-dependent increase in female body size in a protein-rich context is unique to *sun*, as no other humoral factors caused sex-specific effects on body size (**Fig. S3.18B, C**).
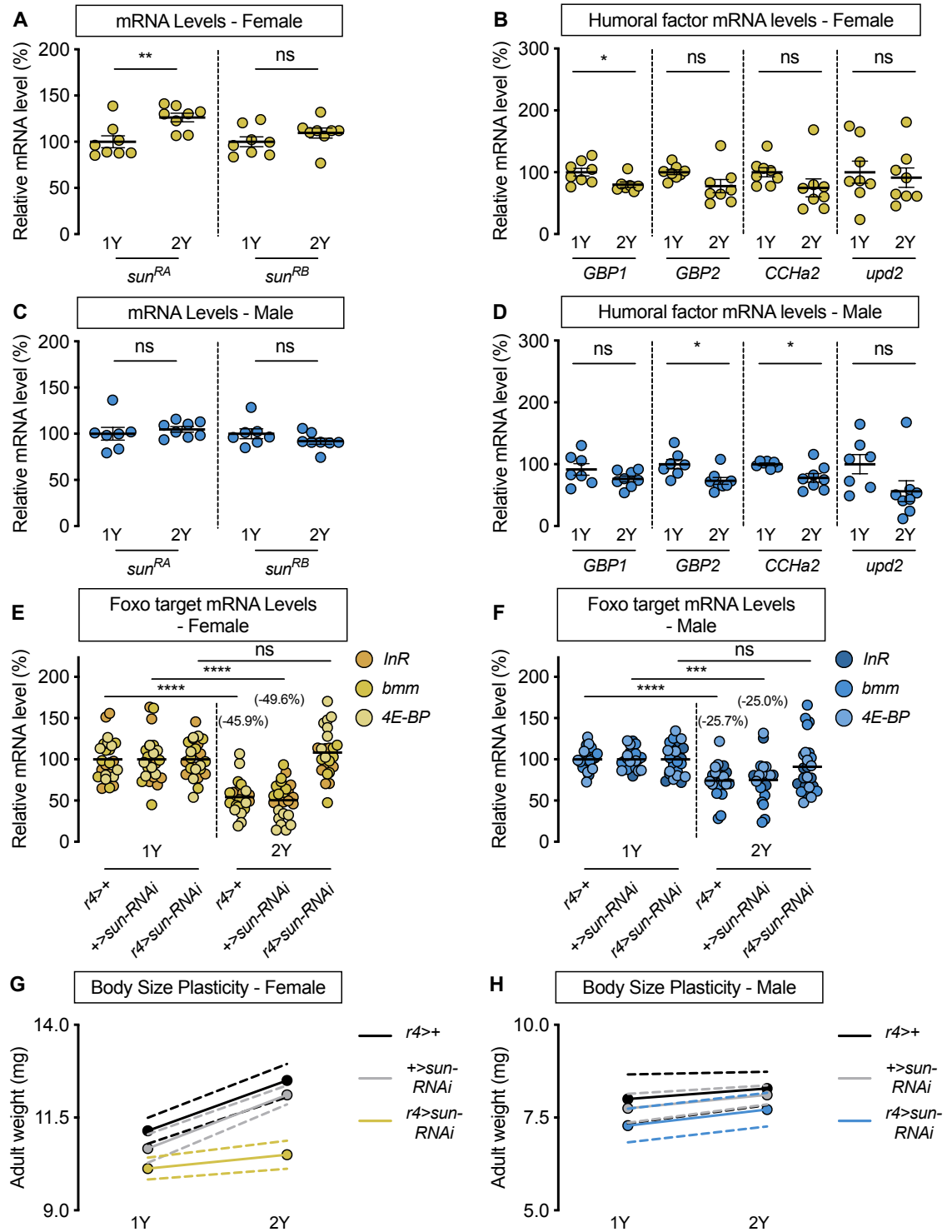
Figure 3.3. *stunted* is required for the nutrient-dependent upregulation of insulin pathway activity and increased female body size plasticity.

(A) In females, mRNA levels of *stunted* (*sun*)^{RA}, but not *sun*^{RB}, were significantly higher in larvae cultured on a protein-rich diet (2Y) compared with larvae raised on a diet containing

half the protein content (1Y) (p = 0.0055 and p = 0.2327, respectively; Student's t test). n = 8 biological replicates. (B) mRNA levels of *Growth-blocking peptide 1* (*Gbp1*) were significantly different in females cultured on a protein-rich diet (2Y) compared with females raised in a diet containing half the protein concentration (1Y) (p = 0.0245; Student's t test); however, mRNA levels of *Growth-blocking peptide 2* (*Gbp2*)*, CCHamide-2* (*CCHa2*)*,* and *unpaired 2* (*upd2*) were not significantly different between female larvae raised on 1Y and 2Y (p = 0.0662, 0.1416, and 0.7171, respectively; Student's t test). n = 7-8 biological replicates. (C) In males, mRNA levels of sun^{RA} and sun^{RB} were not significantly different in larvae raised on 2Y compared with larvae raised on 1Y (p = 0.5832 and p = 0.2017, respectively; Student's t test). n = 7-8 biological replicates. (D) Levels of *Gbp1* and *upd2* were not significantly different between male larvae raised on 2Y compared with larvae reared on 1Y (p = 0.1487, and p = 0.1686, respectively; Student's t test); whereas levels of *Gbp2* and *CCHa2* were significantly different between males raised in 2Y and 1Y (p = 0.0214, and p = 0.0272, respectively; Student's t test). n = 7-8 biological replicates. (E) In control *r4>+,* and *+>sun-RNAi* females, mRNA levels of Foxo targets (*insulin receptor* (*InR*)*, brummer* (*bmm*)*,* and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*)), were significantly lower in larvae cultured on a protein-rich diet (2Y) compared with larvae raised on a diet containing half the protein content (1Y) (p<0.0001, for both comparisons; Student's t test). However, in *r4>sun-RNAi* females, there was no significant difference in Foxo target mRNA levels (p = 0.2792; Student's t test). n = 8 biological replicates. (F) In control *r4>+,* and *+>sun-RNAi* males, mRNA levels of Foxo targets were significantly lower in larvae cultured on 2Y compared with larvae raised on 1Y (p<0.0001 and p = 0.0001, respectively; Student's t test). While *r4>sun-RNAi* males showed no significant difference in Foxo target mRNA levels (p = 0.2469; Student's t test), there was no genotype:diet interaction among males (p = 0.1068), suggesting that genotype had no impact on Foxo target genes. Importantly, there was a significant sex:diet interaction for Foxo target mRNA levels in both the *r4>+* control (p = 0.0166; two-way ANOVA followed by Tukey HSD test) and *+>sun-RNAi* control (p = 0.0119; two-way ANOVA followed by Tukey HSD test), but not in *r4>sun-RNAi* larvae (p = 0.1121; two-way ANOVA followed by Tukey HSD test). n = 7-8 biological replicates. (G) Adult weight was significantly higher in female flies raised in 2Y compared with females raised in 1Y in *r4>+* and *+>UAS-sun-RNAi* controls (p<0.0001 for both genotypes; two-way ANOVA followed by Tukey HSD test); however, adult weight was not significantly different between *r4>UAS-sun-RNAi* females reared on 2Y compared with genotype-matched females raised on 1Y (p = 0.5035; two-way ANOVA followed by Tukey

HSD test). n = 7-10 groups of 10 flies. (H) Adult weight was not significantly higher in male flies reared in 2Y compared with males cultured in 1Y for *r4>+* and *+>UAS-sun-RNAi* controls or *r4>UAS-sun-RNAi* males (p = 0.8883, 0.6317, and 0.554, respectively; two-way ANOVA followed by Tukey HSD test). There was a significant sex:diet interaction in the *r4>+* and *+>UAS-sun-RNAi* control genotypes (p = 0.011 and p = 0.0005, respectively; two-way ANOVA followed by Tukey HSD test), but no sex:diet interaction in the *r4>UAS-sun-RNAi* genotype (p = 0.8749; two-way ANOVA followed by Tukey HSD test). n = 6-9 groups of 10 flies. For all body size plasticity graphs, filled circles indicate mean body size, and dashed lines indicate 95% confidence interval. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ **** indicates $p<0.0001$; ns indicates not significant; error bars indicate SEM.

Our data suggests a model in which the nutrient-dependent increase in *sun* mRNA levels is one important reason that females raised in a protein-rich context have a larger body size. To determine whether increased *sun* mRNA levels could augment body size, we overexpressed *sun* specifically in the fat body in larvae of each sex reared on 1Y and 2Y. We found that fat body *sun* overexpression was sufficient to increase body size in both sexes, in both the 1Y and 2Y diets (**Fig. S3.19A, B**). This demonstrates that increased *sun* mRNA levels are sufficient to enhance body size in these contexts. While this finding contrasts with data from a previous study using a different diet and a mixed-sex experimental group (Delanoue et al., 2016), when we replicated their experimental conditions we found a significant increase in body size that was obscured by pooling data from males and females (**Fig. S3.20A, B**). Together, this data supports a model in which increased fat body *sun* mRNA levels enhance body size in multiple nutritional contexts, an effect that was previously overlooked due to minor variation between lab diets and use of a mixed-sex experimental group. It is important to note, however, that despite the larger body size of *sun*-overexpressing males and females, phenotypic plasticity was not increased in the *sun*-overexpressing larvae (**Fig. S3.19A, B**; diet:genotype interaction p = 0.4959; and p = 0.0895, respectively). This is likely due to the fact that the nutrient-dependent increase in *sun* mRNA levels was still absent in the context of *sun* overexpression in males (**Fig. S3.20C**), as our model suggests it is the ability to upregulate *sun* mRNA in response to dietary protein, rather than

absolute *sun* mRNA levels, that allows females raised on a protein-rich diet to achieve a larger body size.

3.4.5. *Sex determination gene transformer promotes nutrient-dependent body size plasticity in females*

To gain a more complete understanding of the sex difference in phenotypic plasticity, we wanted to identify genetic factors in females that confer the ability to upregulate *sun* mRNA levels in response to dietary protein. One candidate was sex determination gene *tra*, as *tra* was previously found to impact IIS activity and body size in a diet equivalent to 2Y (Rideout et al., 2015; Mathews et al., 2017). Thus, we performed loss- and gain-of-function studies with *tra* and monitored changes to IIS activity, *sun* mRNA, and body size in both the 1Y and 2Y diets. In control w^{1118} females, Foxo target gene expression was significantly lower in larvae raised on 2Y compared with larvae cultured on 1Y (**Fig. 3.4A**); however, this nutrient-dependent decrease in Foxo target gene expression was abolished in *tra* mutant females ($tra^1/Df(3L)st\text{-}j7$) (**Fig. 3.4A**; diet:genotype interaction p = 0.0081). Similarly, while *sun* mRNA levels in w^{1118} control females were significantly higher in larvae raised on 2Y compared with 1Y (**Fig. 3.4B**), this nutrient-dependent increase in *sun* mRNA levels was absent in *tra* mutant females (**Fig. 3.4B**). This indicates that *tra* is required in females for the nutrient-dependent increase in *sun* mRNA levels and IIS activity in a protein-rich context.

To determine whether lack of *tra* also impacts nutrient-dependent body size plasticity, we measured body size in w^{1118} controls and *tra* mutants raised in 1Y and 2Y. In control w^{1118} females, adult weight was significantly higher in flies raised on 2Y compared with flies cultured on 1Y (**Fig. 3.4C**); however, this nutrient-dependent increase in adult weight was blocked in *tra* mutant females (**Fig. 3.4C**; genotype:diet interaction p < 0.0001), a finding we reproduced using pupal volume (**Fig. S3.21A**). Given that we confirmed this result using an additional *tra* mutant allele (tra^{KO}) (Hudry et al., 2016) (**Fig. S3.21B**), and that this finding cannot be explained by changes to food intake (**Fig. S3.21C**), this indicates that *tra* mutant females have reduced nutrient-dependent body size plasticity compared with control females

(genotype:diet interaction $p < 0.0001$ [$tra^1/Df(3L)st\text{-}j7$]; $p < 0.0001$ [tra^{KO}]). In control w^{1118} and tra mutant males, adult weight was not significantly higher in flies raised on 2Y compared with genotype-matched flies reared on 1Y (**Fig. 3.4D**; genotype:diet interaction $p = 0.4507$). Given that we observed a sex difference in nutrient-dependent body size plasticity in the w^{1118} genotype (sex:diet interaction $p < 0.0001$), but not in the tra mutant strains (sex:diet interaction $p = 0.6598$ [$tra^1/Df(3L)st\text{-}j7$]; $p = 0.5068$ [tra^{KO}]), findings we replicated with pupal volume (**Fig. S3.21D, E**), our data reveals a previously unrecognized requirement for tra in regulating the sex difference in nutrient-dependent phenotypic plasticity. To determine whether tra affects phenotypic plasticity via regulation of sun, we overexpressed sun in the fat body of tra mutant females. We found that the reduced body size of tra mutant females in 2Y was rescued by fat body sun overexpression (**Fig. S3.22A**). This supports a model in which the smaller body size of tra mutant females reared in 2Y was due at least in part to lower sun mRNA levels.
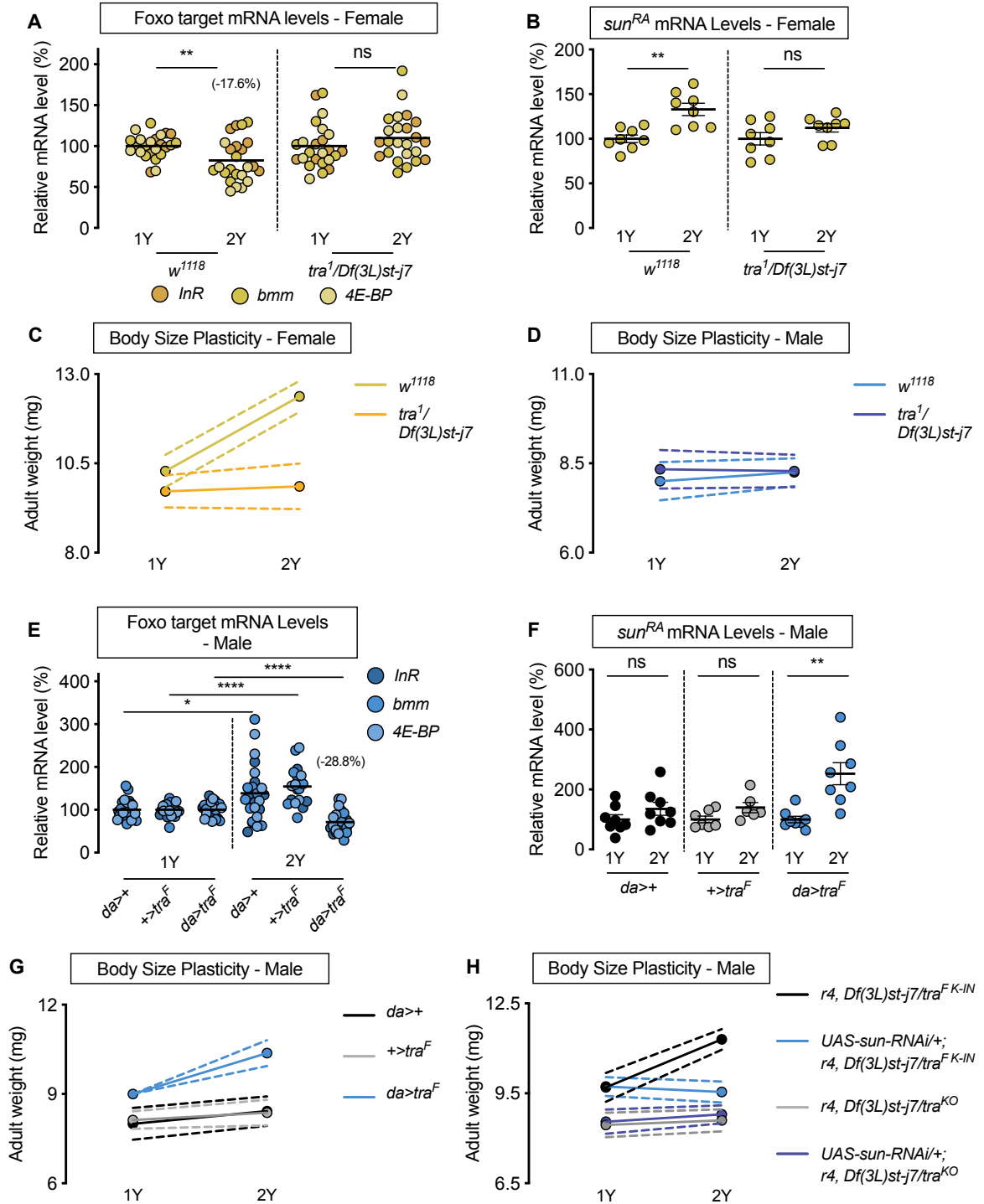
Figure 3.4. Sex determination gene *transformer* (*tra*) regulates increased nutrient-dependent body size plasticity in females.

(A) In control *w^1118^* females, mRNA levels of Foxo targets (*insulin receptor* (*InR*)*, brummer* (*bmm*)*,* and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*)), were significantly lower in larvae cultured on a protein-rich diet (2Y) compared with larvae raised on a diet

containing half the protein content (1Y) (p = 0.0057; Student's t test). In *tra* mutant (*tra^1/Df(3L)st-j7*) females, there was no significant difference in mRNA levels of Foxo targets in larvae cultured on 2Y compared with larvae raised on 1Y (p = 0.2291 Student's t test). n = 8 biological replicates. (B) In control females, mRNA levels of *sun^{RA}* were significantly higher in larvae cultured on 2Y compared with larvae raised on 1Y (p = 0.0011; Student's t test); however, in *tra^1/Df(3L)st-j7* females there was no significant difference in *sun^{RA}* mRNA levels between larvae cultured on 2Y compared with larvae raised on 1Y (p = 0.1644; Student's t test). n = 8 biological replicates. (C) Adult weight was significantly higher in *w^{1118}* females raised on 2Y compared with females reared on 1Y (p<0.0001; two-way ANOVA followed by Tukey HSD test); however, there was no significant difference in adult weight between *tra^1/Df(3L)st-j7* females cultured on 2Y compared with genotype-matched females raised on 1Y (p = 0.9617; two-way ANOVA followed by Tukey HSD test). n = 7-8 groups of 10 flies. (D) Adult weight was not significantly higher in either *w^{1118}* control or *tra^1/Df(3L)st-j7* mutant males in flies raised on 2Y compared with males reared on 1Y (p = 0.7808 and p = 0.9983, respectively; two-way ANOVA followed by Tukey HSD test). There was a significant sex:diet interaction in the *w^{1118}* control genotype (p<0.0001; two-way ANOVA followed by Tukey HSD test); however, there was no sex:diet interaction in the *tra^1/Df(3L)st-j7* genotype (p = 0.6598; two-way ANOVA followed by Tukey HSD test). n = 6-8 groups of 10 flies. (E) In control *da>+,* and *+>tra^F* males, mRNA levels of Foxo targets were significantly higher in larvae cultured on a protein-rich diet (2Y) compared with larvae raised on a diet containing half the protein content (1Y) (p = 0.0108 and p<0.0001, respectively; Student's t test). However, in *da>tra^F* males, there was a significant decrease in Foxo target mRNA levels (p<0.0001; Student's t test). n = 8 biological replicates. Importantly, there was a significant sex:diet interaction for Foxo target mRNA levels in both the *da>+* control (p = 0.0004; two-way ANOVA followed by Tukey HSD test) and *+>tra^F* control (p<0.0001; two-way ANOVA followed by Tukey HSD test), but not in *da>tra^F* larvae (p = 0.3095; two-way ANOVA followed by Tukey HSD test). n = 7-8 biological replicates. (F) In control *da>+* and *+>UAS-tra^F* males, mRNA levels of *sun^{RA}* were not significantly different between larvae cultured on 2Y compared with larvae raised on 1Y (p = 0.2064 and p = 0.0711, respectively; Student's t test). In contrast, *da>UAS-tra^F* males showed a significant increase in mRNA levels of *sun^{RA}* in larvae cultured on 2Y compared with males raised on 1Y (p = 0.0013; Student's t test). n = 6-8 biological replicates. (G) Adult weight was not significantly higher in *da>+* and *+>UAS-tra^F* control males reared on 2Y compared with genotype-matched males flies cultured on 1Y (p = 0.5186 and p = 0.8858, respectively; two-way ANOVA followed by Tukey HSD test);

however, there was a significant increase in adult weight between *da>UAS-tra^F* males cultured on 2Y compared with genotype-matched flies raised on 1Y (*p*<0.0001; two-way ANOVA followed by Tukey HSD test). n = 7-8 groups of 10 flies. (H) Adult weight was significantly higher in *r4-GAL4* control males with *tra^F K-IN*, which express physiological levels of a functional Tra protein, when reared on 2Y compared with 1Y (*p*<0.0001 [*r4,Df(3L)st-j7/tra^F K-IN*]); two-way ANOVA followed by Tukey HSD test). In contrast, the nutrient-dependent increase in adult weight was abolished upon fat body knockdown of *sun* in a *tra^F K-IN* male (*p* = 0.9915 [*UAS-sun-RNAi/+;r4,Df(3L)st-j7/tra^F K-IN*]); two-way ANOVA followed by Tukey HSD test). Adult weight was no different in *tra* mutant *r4-GAL4* males (*r4,Df(3L)st-j7/tra^KO*) reared on 2Y compared with genotype-matched males cultured on 1Y (*p* = 0.9980; two-way ANOVA followed by Tukey HSD test). Adult weight was not further reduced in 1Y with fat body knockdown of *sun* in a *tra* mutant male (*UAS-sun-RNAi/+;r4,Df(3L)st-j7/tra^KO*) (*p* = 0.9998 [*UAS-sun-RNAi/+;r4,Df(3L)st-j7/tra^KO* v *r4,Df(3L)st-j7/tra^KO*]); two-way ANOVA followed by Tukey HSD test). n = 9-11 groups of 10 flies. For all body size plasticity graphs, filled circles indicate mean body size, and dashed lines indicate 95% confidence interval. * indicates *p*<0.05, ** indicates *p*<0.01, **** indicates *p*<0.0001; ns indicates not significant; error bars indicate SEM.

To determine whether lack of a functional Tra protein in males explains their reduced nutrient-dependent body size plasticity, we overexpressed *UAS-tra^F* in all tissues using *daughterless* (*da*)-*GAL4.* We first asked whether Tra overexpression impacted the nutrient-dependent regulation of *sun* mRNA and IIS activity. In control *da>+* and *+>UAS-tra^F* males, there was no significant decrease in Foxo target gene expression in larvae reared in 2Y compared with larvae raised in 1Y (**Fig. 3.4E**). In *da>UAS-tra^F* males, however, there was a significant nutrient-dependent decrease in mRNA levels of Foxo target genes (**Fig. 3.4E**). Because we observed a significant diet:genotype interaction (*p* < 0.0001), the magnitude of the nutrient-dependent increase in IIS activity in the *da>UAS-tra^F* genotype was larger than in control males. Similarly, while *sun* mRNA levels in control *da>+* and *+>UAS-tra^F* males were not significantly different in larvae raised on 2Y compared with larvae reared on 1Y (**Fig. 3.4F**), there was a nutrient-dependent increase in *sun* mRNA levels in *da>UAS-tra^F* males (**Fig. 3.4F**). In *da>+*, *+>UAS-tra^F*, and *da>UAS-tra^F* females, we observed a significant decrease in Foxo target gene expression, and a significant increase in

sun mRNA levels (**Fig. S3.23A, B**). Thus, the presence of a functional Tra protein in males confers the ability to upregulate *sun* mRNA levels and IIS activity, revealing that the lack of Tra in normal males accounts for the lack of a nutrient-dependent increase in *sun* mRNA and IIS activity.

We next tested whether the presence of a functional Tra protein in males would augment nutrient-dependent body size plasticity. We observed a significant increase in adult weight between *da>UAS-tra^F^* males reared on 2Y compared with genotype-matched males raised on 1Y (**Fig. 3.4G**; genotype:diet interaction *p* = 0.0038). This nutrient-dependent increase was not present in either control *da>+* or *+>UAS-tra^F^* males (**Fig. 3.4G**), a finding we reproduced using pupal volume (**Fig. S3.23C**). Because one study suggested high levels of Tra expression may cause lethality (Siera & Cline, 2008), we repeated the experiment using males from a recently published strain of flies in which flies carry a cDNA encoding the female-specific Tra protein knocked into the *tra* locus (*tra^F K-IN^*). These males express Tra at a physiological level (Hudry et al., 2019). As with *da>UAS-tra^F^* males, we found *tra^F K-IN^* males had increased nutrient-dependent body size plasticity compared with control *w^1118^* males and *tra^KO^* males (**Fig. S3.23D**; genotype:diet interaction *p* < 0.0001). Thus, males expressing a functional Tra protein have increased phenotypic plasticity compared with control males, revealing a new role for *tra* in conferring the ability to adjust body size in response to a protein-rich diet. In females, we observed a significant increase in both adult weight and pupal volume in *da>+*, *+>UAS-tra^F^*, and *da>UAS-tra^F^* flies raised on the 2Y diet compared with genotype-matched females cultured on the 1Y diet (**Fig. S3.23E, F**); however, lack of a significant genotype:diet interaction indicates that phenotypic plasticity in *da>UAS-tra^F^* females was not different from controls (*p* = 0.5912), findings we reproduced with the *tra^F K-IN^* allele (**Fig. S3.23G**; genotype:diet interaction *p* < 0.0001). Importantly, the sex difference in nutrient-dependent body size plasticity that we observed in the *w^1118^* control genotype (sex:diet interaction *p* < 0.0001) was abolished between *tra^F K-IN^* males and their genotype-matched females (*p* = 0.3168). To determine whether the nutrient-dependent upregulation of *sun* mRNA was required for Tra to enhance male body size in a protein-rich context, we overexpressed the *UAS-sun-RNAi* transgene

in the fat body of *tra*[F K-IN] males. We found that the nutrient-dependent body size increase in *tra*[F K-IN] males was blocked in males with fat body *sun* loss (**Fig. 3.4H**), a finding we reproduced in *tra*[F K-IN] females (**Fig. S3.23H**). This indicates that the nutrient-dependent upregulation of *sun* mRNA in larvae with a functional Tra protein is required for phenotypic plasticity. Together, these data demonstrate a new role for Tra in regulating the sex difference in nutrient-dependent body size plasticity, and identify fat body *sun* as one downstream factor that mediates Tra's effects on phenotypic plasticity.

3.4.6. Transcriptional coactivator Spargel represents one link between Transformer and regulation of sun mRNA levels

While sex determination gene *tra* impacts sexual differentiation via regulation of confirmed target genes *doublesex* (*dsx*; FBgn0000504) and *fruitless* (*fru*; FBgn0004652), neither *dsx* nor *fru* affect body size (Rideout et al., 2015). Given the key role of *sun* in mediating the nutrient-dependent increase in body size downstream of Tra, we wanted to identify the link between Tra and regulation of *sun* mRNA levels. Previous studies show that transcriptional coactivator *spargel* (*srl*, FBgn0037248), the *Drosophila* homolog of *peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (*PGC-1*) (Tiefenbock et al., 2010), coordinates *sun* mRNA levels with dietary protein (Delanoue et al., 2016). To test whether Srl mediates the sex difference in nutrient-dependent upregulation of *sun* mRNA levels, we examined mRNA levels of *sun* in female larvae heterozygous for a strong hypomorphic allele of *srl* (*srl*[1]/+) (Tiefenbock et al, 2010). In females, we found that the nutrient-dependent upregulation of *sun* mRNA levels in w^{1118} control larvae was blunted in *srl*[1]/+ larvae (**Fig. 3.5A**; diet:genotype interaction $p < 0.0001$). To determine whether a smaller nutrient-dependent increase in *sun* mRNA levels affects the ability of *srl*[1]/+ larvae to achieve a larger body size in a protein-rich context, we raised *srl*[1]/+ larvae on 1Y and 2Y. While we confirmed that *srl*[1]/+ larvae have no generalized developmental defects, as there was no decrease in body size in *srl*[1]/+ female or male larvae reared on 1Y (**Fig. 3.5B, C**), we showed that the nutrient-dependent increase in body size in *srl*[1]/+ females was eliminated (**Fig. 3.5B**). Given that adult weight was significantly higher in control w^{1118} females raised

on 2Y compared with genotype-matched females cultured on 1Y (**Fig. 3.5B**), this indicates that srl^1/+ females have reduced nutrient-dependent body size plasticity (genotype:diet interaction $p < 0.0001$). In control w^{1118} and srl^1/+ males, adult weight was not significantly higher in flies raised on 2Y compared with genotype-matched flies reared on 1Y (**Fig. 3.5C**; genotype:diet interaction $p = 0.8323$). This result suggests that Srl mediates the nutrient-dependent upregulation of *sun* mRNA levels and increased nutrient-dependent body size plasticity in female larvae in a protein-rich context, where future studies will need to determine whether Srl also impacts the sex difference in circulating Sun. Indeed, while Sun is also regulated at the level of secretion by fat body Target-of-Rapamycin (TOR) signaling (Delanoue et al., 2016), we found no sex difference in fat body TOR activity in either 1Y or 2Y (**Fig. S3.24A-D**). Given that TOR activity does not affect *sun* mRNA levels (Delanoue et al., 2016), which we confirm (**Fig. S3.24E**), our data indicates that the sex difference in nutrient-dependent upregulation of *sun* mRNA levels is due to Srl, and not TOR. This aligns with our previous finding that treating larvae with TOR inhibitor
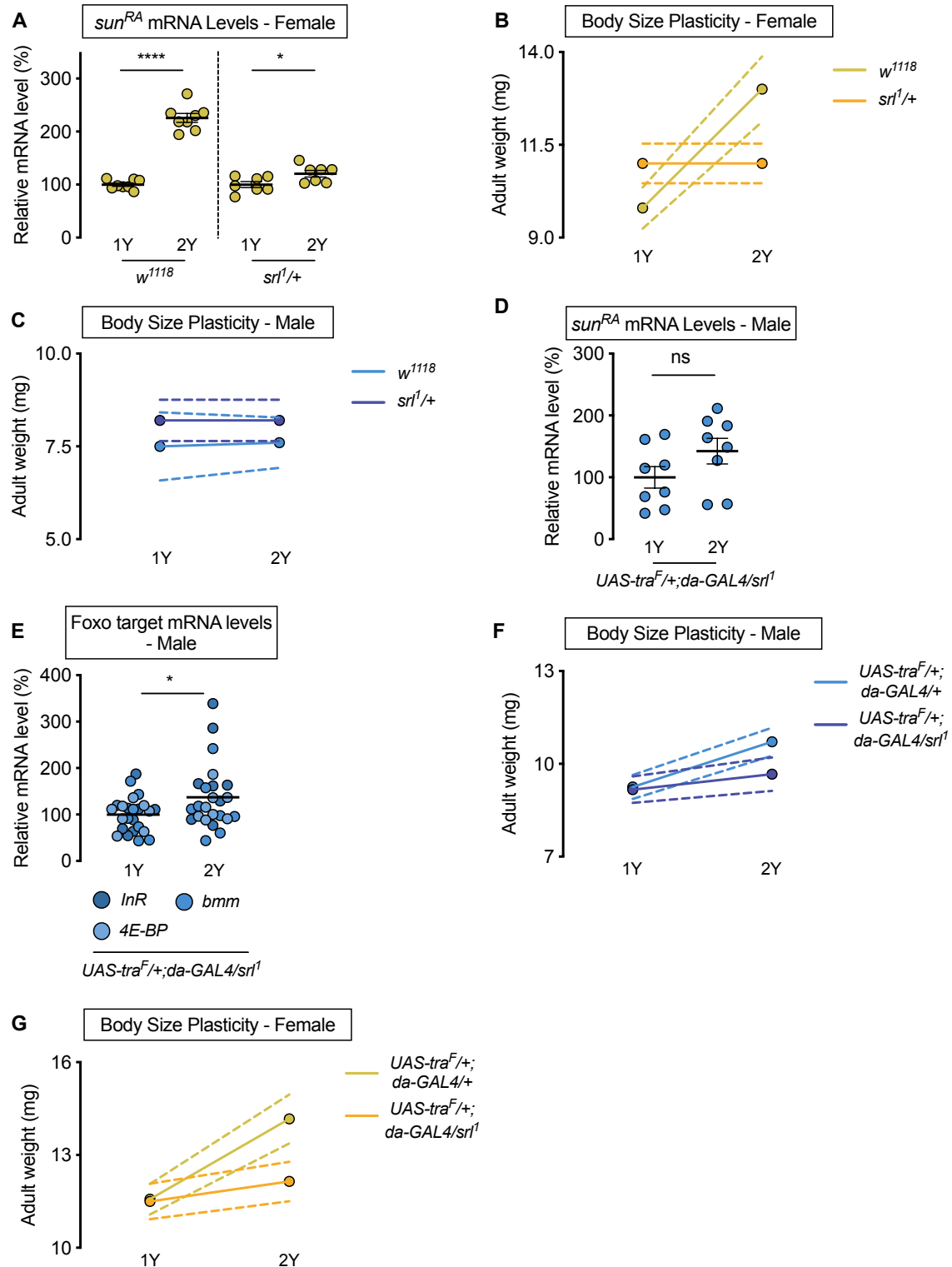
Figure 3.5. Sex determination gene *transformer* (*tra*) requires transcriptional coactivator *spargel* (*srl*) for increased nutrient-dependent body size plasticity in females. (A) In control *w1118* females and females with heterozygous loss of *srl* (*srl1/+*),

mRNA levels of sun^{RA} were significantly higher in larvae cultured on 2Y compared with larvae raised on 1Y ($p<0.0001$ and $p = 0.0301$; Student's t test); however, there was a significant genotype:diet interaction indicating that the protein-dependent upregulation of sun^{RA} was blunted in $srl^1/+$ females ($p<0.0001$; two-way ANOVA followed by Tukey HSD test). n = 7-8 biological replicates. (B) Adult weight was significantly higher in w^{1118} females raised on 2Y compared with females reared on 1Y ($p<0.0001$; two-way ANOVA followed by Tukey HSD test); however, there was no significant difference in adult weight between $srl^1/+$ females cultured on 2Y compared with genotype-matched females raised on 1Y ($p>0.9999$; two-way ANOVA followed by Tukey HSD test). n = 5-7 groups of 10 flies. (C) Adult weight was not significantly higher in either w^{1118} control or $srl^1/+$ mutant males in flies raised on 2Y compared with males reared on 1Y ($p = 0.9906$ and $p>0.9999$, respectively; two-way ANOVA followed by Tukey HSD test). n = 4-5 groups of 10 flies. (D) mRNA levels of sun^{RA} were not significantly different in $da>tra^F$ males with heterozygous loss of srl ($UAS\text{-}tra^F/+;da\text{-}GAL4/srl^1$) cultured on 1Y compared to genotype matched males cultured on 2Y ($p = 0.1405$; Student's t test). n = 8 biological replicates. (E) In control $da>tra^F$ males with heterozygous loss of srl, mRNA levels of Foxo targets (*insulin receptor* (*InR*), *brummer* (*bmm*), and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*)), were significantly higher in larvae cultured on a protein-rich diet (2Y) compared with larvae raised on a diet containing half the protein content (1Y) ($p = 0.0266$; Student's t test). n = 8 biological replicates. (F) Adult weight was higher in $da>UAS\text{-}tra^F$ males raised on a protein-rich diet (2Y) compared with $da>UAS\text{-}tra^F$ males reared on a diet containing half the protein content (1Y) ($p<0.0001$; two-way ANOVA followed by Tukey HSD test). In contrast, the nutrient-dependent increase in adult weight was abolished in $da>UAS\text{-}tra^F$ males heterozygous for srl^1 ($p = 0.2811$; two-way ANOVA followed by Tukey HSD test). n = 6-8 groups of 10 flies. (G) Adult weight was higher in $da>UAS\text{-}tra^F$ females raised on 2Y compared with $da>UAS\text{-}tra^F$ females reared on 1Y ($p<0.0001$; two-way ANOVA followed by Tukey HSD test). In contrast, the nutrient-dependent increase in adult weight was absent in $da>UAS\text{-}tra^F$ females heterozygous for srl^1 ($p = 0.2927$; two-way ANOVA followed by Tukey HSD test). n = 6-7 groups of 10 flies. For all body size plasticity graphs, filled circles indicate mean body size, and dashed lines indicate 95% confidence interval. * indicates $p<0.05$, **** indicates $p<0.0001$; ns indicates not significant; error bars indicate SEM.

Rapamycin did not cause sex-biased effects on larval growth (Rideout et al., 2015).

To determine whether Srl mediates the Tra-dependent regulation of *sun* mRNA levels, we measured *sun* mRNA levels in males with ectopic Tra expression (*da>UAS-tra^F*). While *da>UAS-tra^F* males show a significant nutrient-dependent upregulation of *sun* mRNA levels compared with *da>+* and *+>UAS-tra^F* control males (**Fig. 3.4F**), we found that *sun* mRNA levels were no longer higher in *da>UAS-tra^F* males heterozygous for the *srl^1* allele raised on 2Y compared with genotype-matched males reared on 1Y (**Fig. 3.5D**). Similarly, we observed no decrease in Foxo target genes between *da>UAS-tra^F* males heterozygous for the *srl^1* allele raised on 2Y compared with genotype-matched males reared on 1Y (**Fig. 3.5E**), indicating the nutrient-dependent upregulation of IIS activity in *da>UAS-tra^F* males was abolished in the context of reduced Srl function. Given that we observed no Tra-dependent changes to TOR activity (**Fig. S3.24F, G**), when taken together our data indicates that Srl function is required for the Tra-dependent increase in *sun* mRNA levels in a protein-rich context. Srl therefore represents an additional link between sex determination gene *tra* and the regulation of gene expression. Moreover, we show that the Srl-dependent regulation of *sun* downstream of Tra is significant for phenotypic plasticity, as the nutrient-dependent increase in body size was blocked in *da>UAS-tra^F* females and males heterozygous for the *srl^1* allele (**Fig. 3.5F, G**; genotype:diet interaction p = 0.0146 and p = 0.0008, respectively). While we find that Srl targets other than *sun* were also regulated in a sex-specific manner by nutrients and Tra function (**Fig. S3.25A-D**), other functionally similar Srl targets did not reproduce sex-specific changes to nutrient-dependent body size plasticity that we observed upon loss of fat body *sun* (**Fig. S3.25E-H**). Although we cannot rule out all Srl targets, our data indicates a key role for *sun* among Srl targets in mediating the effects of Tra on nutrient-dependent body size plasticity. This reveals a previously unrecognized role for Srl in mediating sex-specific changes to gene expression, and identifies Srl as a new link between Tra and nutrient-dependent changes to gene expression.

3.4.7. Increased nutrient-dependent body size plasticity in females promotes fecundity in a protein-rich context

Previous studies have shown that plentiful nutrients during development maximize body size to promote fertility in *Drosophila* females (Bergland et al., 2008; Green & Extavour, 2014; Grönke et al., 2010; Hodin & Riddiford, 2000; Klepsatel et al., 2020; Mendes & Mirth, 2016; Robertson, 1957a, 1957b; Sarikaya et al., 2012; Tu & Tatar, 2003), and that high levels of IIS activity are required for normal egg development, ovariole number, and fecundity (Green & Extavour, 2014; Grönke et al., 2010; Mendes & Mirth, 2016; Richard et al., 2005). In line with these findings, w^{1118} female flies reared on 2Y produced significantly more eggs compared with genotype-matched females cultured on 1Y (**Fig. 3.6A**). This aligns with findings from many studies showing that increased nutrients promote fertility (Green & Extavour, 2014; Grönke et al., 2010; Mendes & Mirth, 2016; Richard et al., 2005), and suggests that the ability to augment IIS activity and body size in response to a protein-rich diet allows females to maximize fecundity in conditions where nutrients are plentiful. To test this, we measured the number of eggs produced by InR^{E19}/+ females and w^{1118} controls raised in either 1Y or 2Y. In contrast to w^{1118} females, the nutrient-dependent increase in egg production was absent in InR^{E19}/+ females (**Fig. 3.6A**). Similarly, there was no diet-induced increase in egg production in *dilp2* mutant females (**Fig. 3.6B**). These findings suggest that the nutrient-dependent increase in IIS activity and body size are important to promote fecundity in a protein-rich context. This result aligns with findings from a previous study showing that lifetime fecundity was significantly lower in *dilp2* mutants raised in a yeast-rich diet (Grönke et al., 2010). To extend our findings beyond *dilp* genes, we next examined fecundity in females with an RNAi-mediated reduction in *sun*. We found that the nutrient-dependent increase in egg production in *r4>UAS-sun-RNAi* females was eliminated, in contrast to the robust diet-induced increase in fecundity in *r4>+* and *+>UAS-sun-RNAi* control females (**Fig. 3.6C**). Together, this data suggests that *dilp2* and fat body-derived *sun* play a role in maximizing IIS activity and body size to promote egg production in a protein-rich context. Future studies will need to determine which aspect of ovary development is affected by these genetic manipulations (Green &

Extavour, 2014; Grönke et al., 2010; Mendes & Mirth, 2016; Richard et al., 2005), whether this phenotype is specific to *dilp2,* and whether the effects require *InR* function in the ovary or in other tissues.

In males, which have a reduced ability to augment body size in response to a protein-rich diet, we also investigated the relationship between nutrient content, body size, and fertility. When we compared fertility in *w^1118* males reared on 1Y compared with males raised on 2Y, we found no significant difference in the number of offspring produced (**Fig. 3.6D**). Thus, neither male body size nor fertility were enhanced by rearing flies in a protein-rich environment. Given that previous studies suggest that a larger body size in males promotes reproductive success (Ewing, 1961; Partridge et al., 1987; Partridge & Farquhar, 1983), we next asked whether genetic manipulations that augment male body size also increased fertility. One way
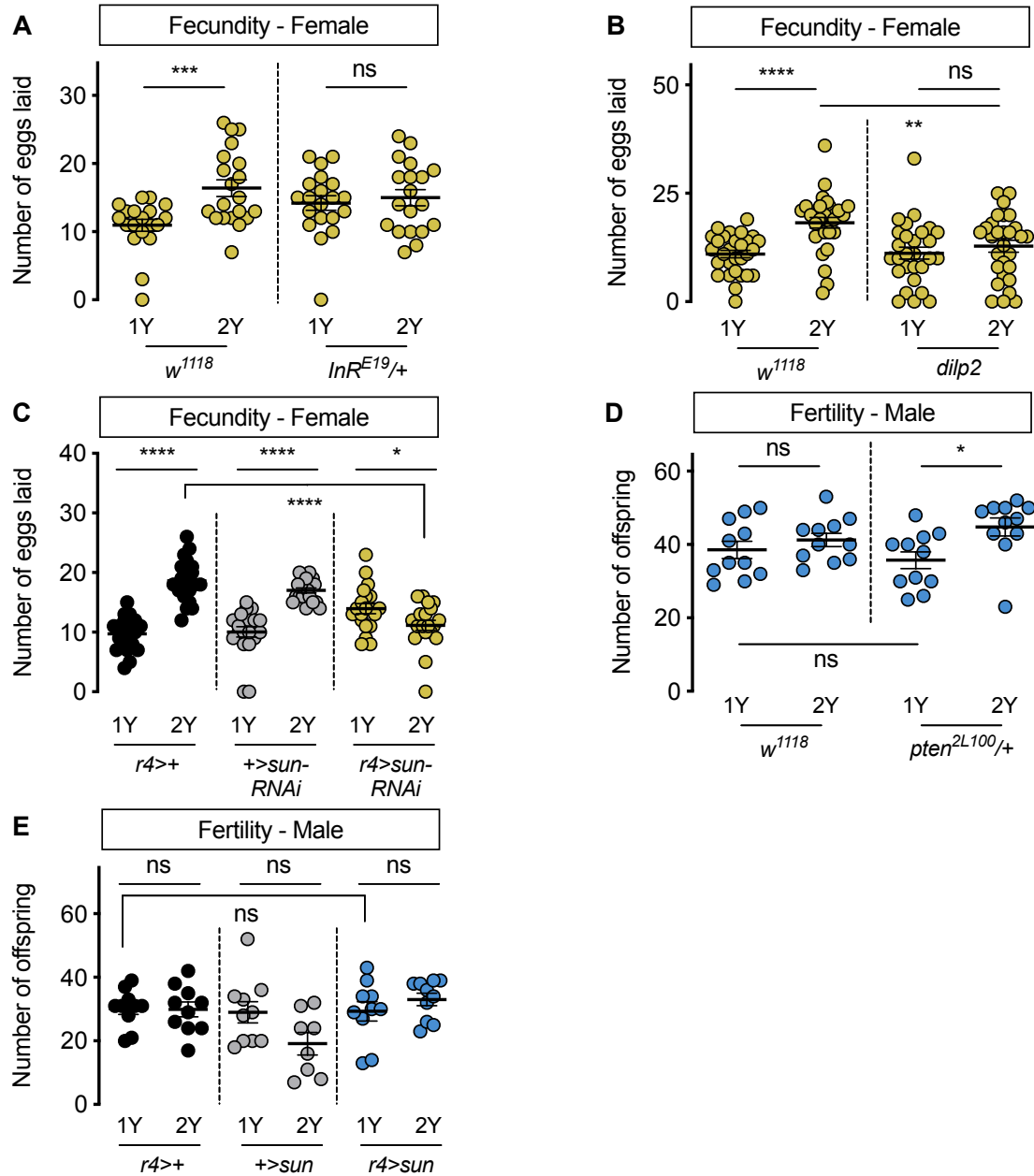
Figure 3.6. Increased nutrient-dependent body size plasticity in females promotes fertility.

(A) In control w^{1118} females there was a significant increase in the number of eggs laid by females raised on 2Y compared with females cultured on 1Y (p = 0.0009; Student's t test); however, there was no significant difference in the number of eggs laid between InR^{E19}/+ females cultured on 2Y compared with genotype-matched females raised on 1Y (p = 0.617; Student's t test). n = 19-20 biological replicates. (B) In control w^{1118} females, there was a

significant increase in the number of eggs laid by females raised on 2Y compared with females cultured on 1Y ($p<0.0001$; Student's *t* test); however, there was no significant difference in the number of eggs laid between *dilp2* mutant females cultured on 2Y compared with females raised on 1Y ($p = 0.4105$; Student's *t* test). n = 28-30 biological replicates. (C) In control *r4>+* and *+>UAS-sun-RNAi* females there was a significant increase in the number of eggs laid by females raised on 2Y compared with control females cultured on 1Y ($p<0.0001$ for both genotypes; Student's *t* test). In *r4>UAS-sun-RNAi* females, the number of eggs laid by females cultured on 2Y was lower than females raised on 1Y ($p = 0.0243$; Student's *t* test). n = 20 biological replicates. (D) In control w^{1118} males there was no significant difference in the number of offspring produced between a 1Y and 2Y diet ($p = 0.3662$; Student's *t* test). There was also no significant difference in the number of offspring produced between control w^{1118} males and males heterozygous for a loss-of-function allele of *phosphatase and tensin homolog* (*pten;* genotype $pten^{2L100}$/+) raised on 1Y ($p = 0.4003$; Student's *t* test). Unlike control males, $pten^{2L100}$/+ males reared on 2Y produced significantly more offspring than genotype-matched males raised on 1Y ($p = 0.0137$; Student's *t* test). n = 11 biological replicates. (E) In control *r4>+* and *+>UAS-sun* and *r4>UAS-sun* males, there was no significant effect on the number of offspring produced between a 1Y and 2Y diet ($p = 0.9222$, 0.0595, and 0.32 respectively; Student's *t* test). There was also no significant difference in the number of offspring produced between control *r4>+, +>UAS-sun* males and *r4>UAS-sun* males raised on 1Y ($p = 0.9723$ and $p = 0.9969$ respectively; one-way ANOVA followed by Tukey HSD test). n = 8-10 groups of 10 flies. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$; ns indicates not significant; error bars indicate SEM.

---

to augment male body size in 1Y is heterozygous loss of *phosphatase and tensin homolog* (*pten*, FBgn0026379; $pten^{2L100}$/+) (**Fig. S3.7B**). Interestingly, fertility was not significantly higher in $pten^{2L100}$/+ males compared with w^{1118} controls raised in 1Y (**Fig. 3.6D**), suggesting that a larger body size does not always augment fertility in males. Similarly, when we measured fertility in *r4>UAS-sun* males, which are larger than control males (**Fig. S3.17B**), fertility was not significantly different from *r4>+* and *+>UAS-sun* control males (**Fig. 3.6E**). Interestingly, when we examined fertility in $pten^{2L100}$/+ and *r4>UAS-sun* males in 2Y, fertility was significantly increased in

pten^{2L100}/+ males compared with genotype-matched controls cultured in 1Y (**Fig. 3.6D**), an observation we did not repeat in *r4>UAS-sun* males (**Fig. 3.6E**). Ultimately, this less robust and more complex relationship between body size and fertility in males suggests a possible explanation for their decreased nutrient-dependent body size plasticity compared with females.

3.5. DISCUSSION

In many animals, body size plasticity in response to environmental factors such as nutrition differs between the sexes (Fairbairn, 1997). While past studies have identified mechanisms underlying nutrient-dependent growth in a mixed-sex population, and revealed factors that promote sex-specific growth in a single nutritional context, the mechanisms underlying the sex difference in nutrient-dependent body size plasticity remain unknown. In this study, we showed that females have higher phenotypic plasticity compared with males when reared on a protein-rich diet, and elucidated the molecular mechanisms underlying the sex difference in nutrient-dependent body size plasticity in this context. Our data suggests a model in which high levels of dietary protein augment female body size by stimulating an increase in IIS activity, where we identified a requirement for *dilp2* and *sun* in promoting this nutrient-dependent increase in IIS activity. Importantly, we discovered *tra* as the factor responsible for stimulating *sun* mRNA levels and IIS activity in a protein-rich context, revealing a novel role for sex determination gene *tra* in regulating phenotypic plasticity. Mechanistically, *tra* enhanced *sun* mRNA levels and body size in protein-rich conditions via transcriptional coactivator Srl, identifying Srl as one link between *tra* and the nutrient-dependent regulation of gene expression. Together, these findings provide new insight into how *Drosophila* females achieve increased nutrient-dependent body size plasticity compared with males.

One key feature of this increased phenotypic plasticity in females was a female-biased increase in IIS activity in a protein-rich context. This reveals a previously unrecognized sex difference in the coupling between IIS activity and dietary protein. In females, there was tight coupling between increased nutrient input

and enhanced IIS activity across a wide protein concentration range in all control genotypes. In males, this close coordination between dietary protein and IIS activity was weaker in a protein-rich context. Our data shows that sex-biased nutrient-dependent change to IIS activity during development is physiologically significant, as it supports an increased rate of growth and consequently larger body size in females but not in males raised on a protein-rich diet. In future studies, it will be important to determine whether the sex difference in coupling between nutrients and IIS activity exists in other contexts. For example, previous studies on the extension of life span by dietary restriction have shown that male and female flies differ in the concentration of nutrients that produces the maximum life span extension, and in the magnitude of life span extension produced by dietary restriction (Magwere et al., 2004; Regan et al., 2016). Similar sex-specific effects of dietary restriction and reduced IIS on life span have also been observed in mice (Holzenberger et al., 2003; Kane et al., 2018; reviewed in Regan & Partridge, 2013; Selman et al., 2008) and humans (van Heemst et al., 2005). Future studies will be needed to determine whether a male-female difference in coupling between nutrients and IIS activity account for these sex-specific life span responses to dietary restriction. Indeed, given that sex differences have been reported in the risk of developing diseases associated with overnutrition and dysregulation of IIS activity such as obesity and type 2 diabetes (Kautzky-Willer et al., 2016; Mauvais-Jarvis, 2018; Tramunt et al., 2020), more detailed knowledge of the male-female difference in coupling between nutrients and IIS activity in other models may provide insights into this sex-biased risk of disease.

In addition to revealing a sex difference in the nutrient-dependent upregulation of IIS activity, our data identified a female-specific requirement for *dilp2* and *sun* in mediating the diet-induced increase in IIS activity in a protein-rich context. While previous studies have shown that both *dilp2* and *sun* positively regulate body size (Ikeya et al., 2002; Grönke et al., 2010; Delanoue et al 2016), we describe new sex-specific roles for *dilp2* and *sun* in nutrient-dependent phenotypic plasticity. Elegant studies have shown that *sun* is a secreted factor that stimulates Dilp2 release from the IPCs (Delanoue et al., 2016). Together with our data, this

suggests a model in which females are able to achieve a larger body size in a protein-rich diet because they have the ability to upregulate *sun* mRNA levels, whereas males do not. Indeed, we show that higher *sun* mRNA levels are sufficient to augment body size. This model aligns well with findings from two previous studies on Dilp2 secretion in male and female larvae. The first study, which raised larvae on a protein-rich diet equivalent to the 2Y diet, found increased Dilp2 secretion in females compared to males (Rideout et al., 2015). The second study, which raised larvae on a diet equivalent to the 1Y diet, found no sex difference in Dilp2 secretion and no effects of *dilp2* loss on body size (Sawala & Gould, 2017). Thus, while these previous studies differed in their initial findings on a sex difference in Dilp2 secretion, our data reconcile these minor differences by identifying context-dependent effects of *dilp2* on body size. It is important to note that absolute confirmation of a sex difference in hemolymph Dilp2 levels will be needed in future studies because the body size plasticity defects in the *dilp2-HF* strain precluded its use as a tool to quantify circulating Dilp2 levels in our study. Future studies will also need to determine whether these sex-specific and context-dependent effects of *dilp2* are observed in other phenotypes regulated by *dilp2* and other *dilp* genes. For example, flies carrying mutations in *dilp* genes show changes to aging, metabolism, sleep, and immunity, among other phenotypes (Bai et al., 2012; Brown et al., 2020; Cong et al., 2015; Grönke et al., 2010; Liu et al., 2016; Nässel & Vanden Broeck, 2016; Okamoto et al., 2009; Okamoto & Nishimura, 2015; Post et al., 2018, 2019; Slaidina et al., 2009; Stafford et al., 2012; Zhang et al., 2009; Brogiolo et al., 2001; Cognigni et al., 2011; Linneweber et al., 2014; Semaniuk et al., 2018; Suzawa et al., 2019; Ugrankar et al., 2018). Further, it will be interesting to determine whether the sex-specific regulation of *sun* is observed in any other contexts, and whether it will influence sex differences in phenotypes associated with altered IIS activity, such as life span.

While our findings on *sun* and *dilp2* provide mechanistic insight into the molecular basis for the larger body size of females reared on a protein-rich diet, a key finding from our study was the identification of sex determination gene *tra* as the factor that confers plasticity to females. Normally, nutrient-dependent body size plasticity is higher in females than in males in a protein-rich context. In females

lacking a functional Tra protein, however, this increased nutrient-dependent body size plasticity was abolished. In males, which normally lack a functional Tra protein, ectopic Tra expression conferred increased nutrient-dependent body size plasticity. While a previous study showed that on the 2Y diet Tra promotes Dilp2 secretion (Rideout et al., 2015), our current study extends this finding in two ways: by identifying *sun* as one link between Tra, Dilp2, and changes to IIS activity, and by showing that Tra regulates *sun* mRNA via conserved transcriptional coactivator Srl. While previous studies discovered Srl as the factor that promotes *sun* mRNA levels in response to dietary protein in a mixed-sex larval population (Delanoue et al., 2016), our findings reveal a previously unrecognized sex-specific role for Srl in regulating transcription. Because loss of Tra reduces Srl transcriptional activity, this new link between Tra and Srl suggests an additional way in which Tra may impact gene expression beyond its canonical downstream targets *dsx* and *fru*. While this builds on recent studies that reveal a number of additional Tra-regulated genes (Clough et al., 2014; Hudry et al., 2016, 2019), it will be important to determine whether these additional Tra-regulated genes including *sun* represent direct targets of Tra/Srl. Future studies will also be needed to elucidate how Tra impacts Srl transcriptional activity in a context-dependent manner, however, uncovering a connection between a sex determination gene and a key regulator of genes involved in mitochondrial function suggests an additional mechanism that may contribute to sex differences in phenotypes affected by mitochondrial function (*e.g.*, lifespan) (Tiefenbock et al., 2010; Cho et al., 2011; Tower, 2015, 2017). In addition, it will be critical to explore how the presence of Tra allows an individual to couple dietary protein with body size. Because the *tra* locus is regulated both by alternative splicing and transcription (Belote et al., 1989; Boggs et al., 1987; Grmai et al., 2018; Inoue et al., 1990; Sosnowski et al., 1989), and Tra protein is regulated by phosphorylation (Du et al., 1998), our study highlights the importance of additional studies on the regulation of the *tra* genomic locus and Tra protein throughout development to gain mechanistic insight into its effects on nutrient-dependent body size plasticity.

       While the main outcome of our work was to reveal the molecular mechanisms that regulate the sex difference in nutrient-dependent body size plasticity, we also

provide some insight into how genes that contribute to nutrient-dependent body size plasticity affect female fecundity and male fertility. Our findings align well with previous studies demonstrating that increased nutrient availability during development and a larger female body size confers increased ovariole number and fertility (Green & Extavour, 2014; Klepsatel et al., 2020; Mendes & Mirth, 2016; Robertson, 1957a, 1957b), as females lacking either *dilp2* or fat body-derived *sun* were unable to augment egg production in a protein-rich context. Given that previous studies demonstrate IIS activity influences germline stem cells in the ovary in adult flies (Hsu et al., 2008; Hsu & Drummond-Barbosa, 2009; Kao et al., 2015; LaFever & Drummond-Barbosa, 2005; Lin & Hsu, 2020; Su et al., 2018), there is a clear reproductive benefit that arises from the tight coupling between nutrient availability, IIS activity, and body size in females. In males, however, the relationship between fertility and body size remains less clear. While larger males are more reproductively successful both in the wild and in laboratory conditions (Ewing, 1961; Partridge & Farquhar, 1983), other studies revealed that medium-sized males were more fertile than both larger and smaller males (Lefranc & Bundgaard, 2000). Given that our study revealed no significant increase in the number of progeny produced by larger males, the fertility benefits that accompany a larger body size in males may be context-dependent. For example, a larger body size increases the ability of males to outcompete smaller males (Flatt, 2020; Partridge et al., 1987; Partridge & Farquhar, 1983). Thus, in crowded situations, a bigger body may provide significant fertility gains. On the other hand, in conditions where nutrients are limiting, an imbalance in the allocation of energy from food to growth rather than to reproduction may decrease fertility (Bass et al., 2007; Camus et al., 2017; Jensen et al., 2015; Wood et al., 2018). Future studies will need to resolve the relationship between body size and fertility in males, as this will suggest the ultimate reason(s) for the sex difference in nutrient-dependent body size plasticity.

4. A LOW SUGAR DIET ENHANCES *DROSOPHILA* BODY SIZE IN MALES AND FEMALES VIA SEX-SPECIFIC MECHANISMS

4.1. SYNOPSIS

In *Drosophila*, changes to dietary protein elicit different body size responses between the sexes (McDonald et al., 2020; Millington et al., 2021; Shingleton et al., 2017). Whether this sex difference in nutrient-dependent body size regulation extends to other nutrients, such as dietary sugar, remains unclear. Here, we show that reducing dietary sugar enhanced body size in *Drosophila* male and female larvae. Indeed, the largest body size was found in larvae reared in a diet without added sugar. Despite the equivalent body size effects of a low sugar diet between males and females, we detected sex-specific changes to the insulin/insulin-like growth factor (IIS) and target of rapamycin (TOR) signaling pathways. Specifically, we found a female-specific increase in TOR pathway activity and a male-specific increase in IIS activity in 0S compared with 1S. Further, we show that the metabolic changes observed in larvae reared on a low sugar diet differ between the sexes. Thus, despite identical phenotypic responses to dietary sugar in males and females, these sex-limited changes to TOR and IIS were associated with distinct changes to whole-body metabolism and were required for the increased body size in each sex. This highlights the importance of including both sexes in all mechanistic studies on larval growth, as males and females may use different molecular and metabolic mechanisms to achieve similar phenotypic outcomes.

4.2. INTRODUCTION

In *Drosophila*, dietary nutrients impact the rate and duration of larval growth to influence final body size. Nutrient quantity promotes growth during larval development, as conditions where nutrients are plentiful favour larger body sizes (Edgar, 2006; Hietakangas and Cohen, 2009; Nijhout et al., 2014). Nutrient quality is also critical in regulating 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., 2020 preprint; 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 altered 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 a reduction in dietary sugar significantly increased the rate of growth and body size in males and females. Indeed, the largest body size was observed in a diet with no added sugar. Despite the equivalent body size increase in males and females, sex-specific mechanisms underlie the larger body size of larvae raised in a low sugar diet. In females, the low sugar diet stimulated increased target of rapamycin (TOR) pathway activity, whereas the activity of the insulin/insulin-like growth factor signaling pathway (IIS) was enhanced in males. Genetic studies confirmed that these female- and male-specific changes to TOR and IIS, respectively, were important for the low sugar-induced increase in body size, and biochemical studies revealed sex-specific changes to metabolic gene expression and metabolism. Together, our findings provide additional mechanistic insight into how dietary sugar affects development by revealing sex-specific changes to cell signaling pathways and metabolism. This highlights the importance of including both sexes in all larval growth studies, as we show that equivalent phenotypic outcomes may be achieved via distinct mechanisms in each sex.

4.3. MATERIALS AND METHODS

4.3.1. Fly husbandry

For all experiments, parental flies of appropriate genotypes were crossed and allowed to lay eggs on grape juice agar plates for a period of 12 hours. At 24 hr AEL, larvae were picked off of grape juice agar plates into growth medium and raised at a density of 50 animals per 10 mL food at 25°C. 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 CaCl2•2H2O, 0.5 g MgSO4•7H2O, 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 CaCl2•2H2O, 0.5 g MgSO4•7H2O, 11.77 mL acid mix. Details of 0.75S, 0.5S, and 0.25S diets can be found in Table S4.1. Larvae were raised at a density of 50 animals per 10 mL food at 25°C, and sexed by gonad size. Adult flies were maintained at a density of twenty flies per vial in single-sex groups.

4.3.2. 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,* and *dilp5* (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,b).

4.3.3. Body size

Pupal volume was measured as previously described using this formula: $4/3\pi(L/2)(W/2)^2$ (L, length; W, width). Adult weight was measured as previously described (Delanoue et al., 2010; Millington et al., 2021a; Millington et al., 2021b; Rideout et al., 2015).

4.3.4. Feeding behaviour

Feeding behavior was quantified as number of mouth-hook contractions per 30 s.

4.3.5. 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.

4.3.6. Metabolism assays

Each biological replicate consists of ten female or male larvae. Larvae were frozen on dry ice, and homogenized for lipid, protein, glucose, glycogen, and trehalose assays. All assays were performed as described in Tennessen et al. (2014) and Wat et al. (2020).

4.3.7. 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).

4.3.8. 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). mRNA levels were normalized to expression of *Actin5C* and *β-tubulin*. Primer list in Table S4.2.

4.3.9. 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), 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 as per manufacturer's instructions (#32134, Thermo Scientific).

4.3.10.   *Statistical analysis*

GraphPad Prism (GraphPad Prism version 8.4.3 for Mac OS X) was used for all statistical tests, and for figure preparation. Statistical tests and significance are indicated in figures and figure legends.

4.4. RESULTS AND DISCUSSION

4.4.1. *A low sugar diet promotes an increased rate of growth and augments body size*

To determine the body size effects of dietary sugar in each sex, we quantified pupal volume in *white*[1118] (*w*; FBgn0003996) male and female larvae reared in diets with different levels of dietary 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 no added sugar 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. 4.1A**). Interestingly, the largest body size was found in female larvae reared in 0S (**Fig. 4.1A**).

In *w*[1118] males, body size was significantly larger in larvae reared on 0.5S and 0.25S compared with larvae raised on 1S (**Fig. 4.1B**). As in females, the largest body size among males was recorded on 0S (**Fig. 4.1B**). Importantly, the body size effects of reduced sugar diets were equivalent between the sexes (**Fig. 4.1C**), a finding we reproduced using adult weight (**Fig. 4.1D**), indicating that phenotypic responses to dietary sugar were not different between males and females. Because a diet with fewer calories has no effect on body size (Millington et al., 2021a), our findings suggest that the larger size of larvae raised in 0S can be attributed to less dietary sugar. This agrees with data from a mixed-sex larval group showing that dietary sugar inhibits growth (Musselman et al., 2011; Pasco and Léopold, 2012), and extends previous findings by showing the body size effects occur in both sexes.
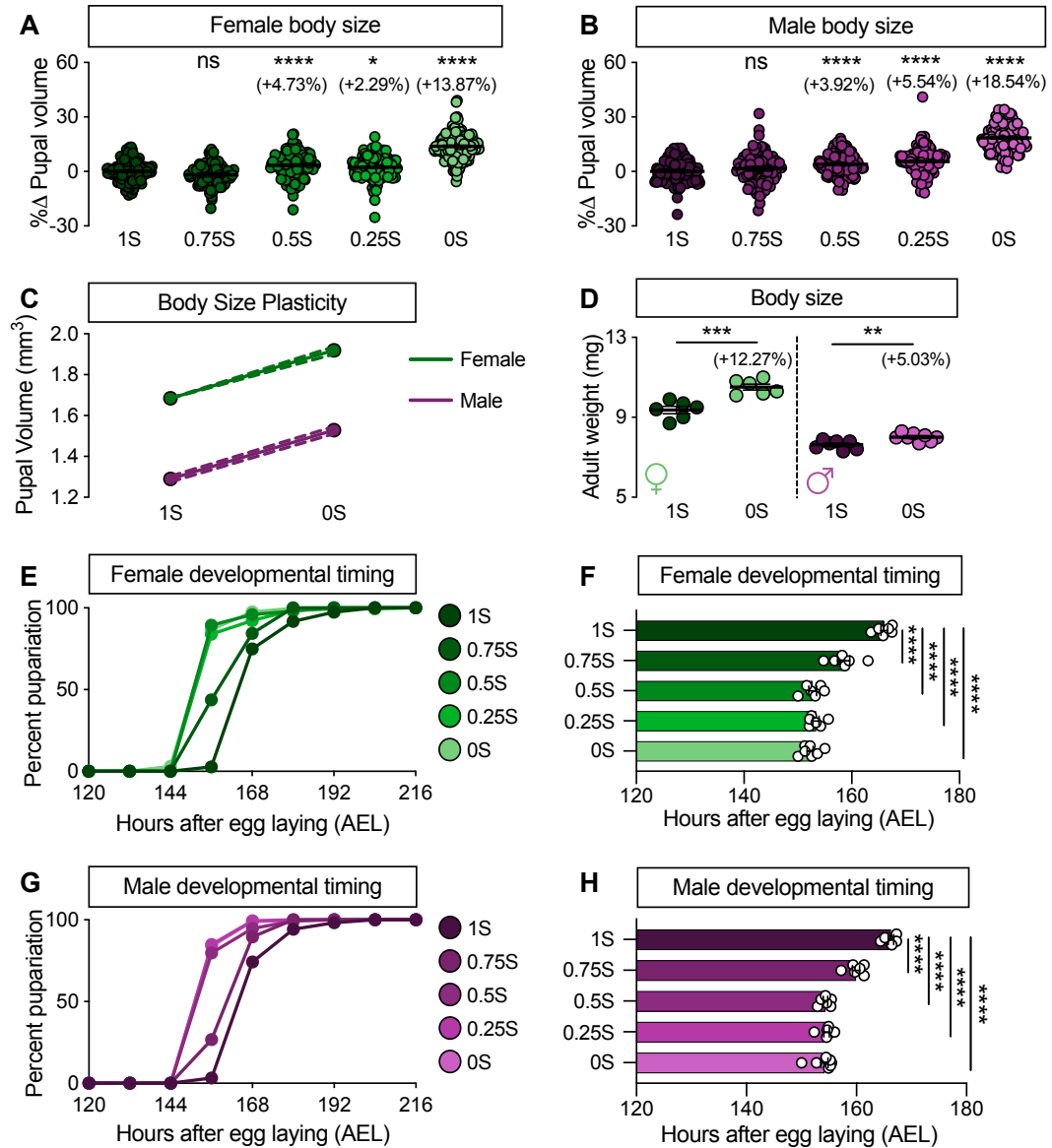
Figure 4.1. A low sugar diet promotes an increased rate of growth and final body size.
(A) Pupal volume was no different in *w^1118^* females cultured on 0.75S diet compared with genotype matched females raised on 1S (*p* = 0.0798; one-way ANOVA followed by Dunnett's multiple comparison test) but was significantly higher in 0.5S, 0.25S, and 0S diets (*p*<0.0001, *p* = 0.0193, *p*<0.0001, respectively; one-way ANOVA followed by Dunnett's multiple comparison test). n = 100-160 pupae. (B) Pupal volume was no different in *w^1118^* males cultured on 0.75S diet compared with genotype matched males raised on 1S (*p* = 0.3099; one-way ANOVA followed by Dunnett's multiple comparison test) but was significantly higher in 0.5S, 0.25S, and 0S diets (*p*<0.0001 for all comparisons; one-way ANOVA followed by Dunnett's multiple comparison test). n = 100-121 pupae. (C) Reaction

norms for pupal volume in both sexes plotted using 1S and 0S data from A and B. (D) Adult weight in w^{1118} female and male flies was significantly increased in flies reared in a sugar-free diet (p = 0.0006, and p = 0.0039, respectively; Student's t test). n = 6-8 groups of 10 adult flies. (E) The time to pupariation was shorter in w^{1118} female larvae cultured on diets with decreasing sugar. n = 126-235 pupae. (F) Time to 50% pupariation was calculated for each replicate using the data in panel E. The time to pupariation was significantly shorter in female larvae cultured on 0.75S, 0.5S, 0.25S, and 0S (p<0.0001 for all comparisons; one-way ANOVA followed by Tukey HSD test). (G) The time to pupariation was shorter in w^{1118} male larvae cultured on diets with decreasing sugar. n = 116-195 (H) Time to 50% pupariation was calculated for each replicate using the data in panel G. The time to pupariation was significantly shorter in male larvae cultured on 0.75S, 0.5S, 0.25S, and 0S (p<0.0001 for all comparisons; one-way ANOVA followed by Tukey HSD test). * 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 determine the growth rate in larvae reared on low sugar diets, we measured the time between egg-laying and pupariation in both sexes. In w^{1118} females, time to 50% pupariation was significantly shorter in larvae raised on each reduced-sugar diet compared with genotype-matched larvae cultured in 1S (**Fig. 4.1E, F**). Time to 50% pupariation was also reduced in w^{1118} males raised on each reduced-sugar diet compared with genotype-matched males reared on 1S (**Fig. 4.1G, H**). Given that diets with less added sugar shorten the larval growth period and increase body size, our data suggests the larval growth rate in each sex was significantly accelerated in a low sugar context.

4.4.2. A low sugar diet has sex-biased effects on insulin/insulin-like growth factor (IIS) and target of rapamycin (TOR) signaling

Many signaling pathways control organismal and tissue growth during development, however, IIS and TOR have emerged as key regulators of 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 and TOR activity promote a larger body size (Böhni et al., 1999; Britton et al., 2002;

Chen et al., 1996; Fernandez et al., 1995; Patel et al., 2003; Poltilove et al., 2000; Zhang et al., 2000). Given the larger body size of males and females cultured on 0S, we examined IIS and TOR activity in larvae reared on 0S and 1S. To measure IIS activity, we quantified mRNA levels of genes that are coregulated by transcription factor Forkhead box, sub-group O (Foxo; FBgn0038197) (*e.g. Insulin receptor* (*InR*;
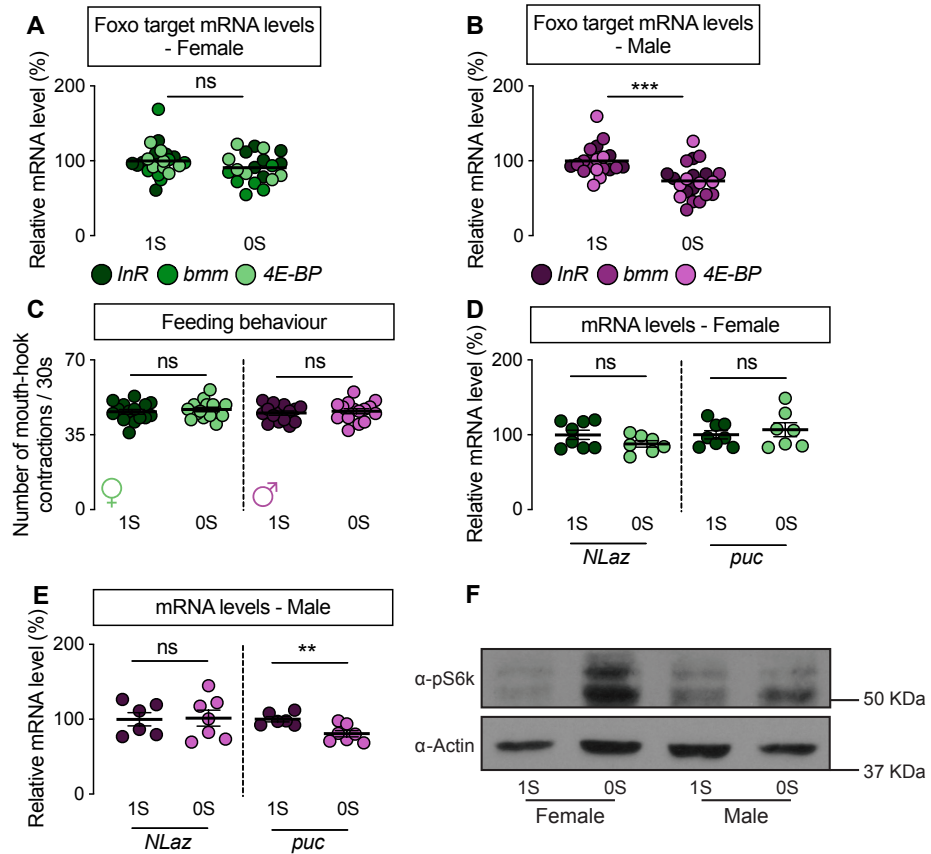
Figure 4.2. A low sugar diet has sex-biased effects on insulin/insulin-like growth factor (IIS) and target of rapamycin (TOR) signaling.

(A) In females, mRNA levels of Foxo targets (*insulin receptor* (*InR*), *brummer* (*bmm*), and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*)) were not significantly different between larvae raised on a diet containing sugar (1S) and larvae cultured on a sugar-free diet (0S) (p = 0.1396; Student's t test). n = 8 biological replicates. (B) In males, mRNA levels of Foxo targets were significantly lower in larvae raised on 0S compared to larvae cultured on 1S (p = 0.0003; Student's t test). n = 6-7 biological replicates. (C) There was no significant difference in mouth hook contractions between w^{1118} control female and male larvae raised on 1S compared to larvae raised on 0S (p = 0.4982, and p = 0.6112, respectively; Student's t test). n = 20 biological replicates. (D) In females, mRNA levels of

Jun-N-terminal kinase pathway targets *Neural Lazarillo* (*NLaz*) and *puckered* (*puc*) were not significantly different between larvae raised in 1S and larvae raised in 0S (p = 0.1385, and p = 0.5321, respectively; Student's *t* test). n = 7-8 biological replicates. (E) In males, mRNA levels of *NLaz* were not significantly different between between larvae raised in 1S and larvae raised in 0S (p = 0.9190; Student's *t* test), but *puc* mRNA levels were significantly lower in larvae raised in 0S compared to larvae cultured in 1S (p = 0.0055; Student's *t* test). n = 6-7 biological replicates. (F) Levels of phosphorylated S6 kinase (pS6k) in females and males raised in 1S or 0S. ** p<0.01; *** p<0.001; ns indicates not significant; error bars indicate SEM.

FBgn0283499), *brummer* (*bmm;* FBgn0036449), and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*; FBgn0261560). For example, when IIS activity is high, Foxo is repressed and mRNA levels of *InR*, *bmm*, and *4E-BP* are low (Alic et al., 2011; 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 0S and 1S (**Fig. 4.2A**), suggesting IIS activity was not altered in females. In contrast, mRNA levels of Foxo target genes were significantly lower in *w^1118^* male larvae in 0S (**Fig. 4.2B**), indicating enhanced IIS activity. Importantly, feeding behaviour was not different between the sexes in either diet (**Fig. 4.2C**). While increased IIS activity in males raised on 0S may be due to improved insulin sensitivity, changes to mRNA levels of two genes upregulated by insulin insensitivity (*Neural Lazarillo* (*NLaz*; FBgn0053126) and *puckered* (*puc*; FBgn0243512) were not consistent with improved insulin sensitivity in either sex (**Fig. 4.2D, E**) (Lourido et al., 2021; Pasco and Léopold, 2012). Indeed, altered *puc* mRNA levels in males likely reflect Foxo activity, as *puc* is a Foxo target (Bai et al., 2013). Together, these findings reveal a previously unrecognized sex difference in IIS regulation in a low sugar context, adding to a growing body of evidence showing sex differences in the nutrient-dependent regulation of IIS in larvae (Millington et al., 2021a).

We next measured TOR activity by monitoring the phosphorylation of TOR's downstream target Ribosomal protein S6 kinase (S6k; FBgn0283472). In *w^1118^*

females, levels of phosphorylated S6k (pS6k) were higher in 0S in multiple biological replicates (**Fig. 4.2F**; **Fig S4.1A**), an effect we did not reproduce in w^{1118} males (**Fig. 4.2F**; **Fig S4.1A**). This suggests that the low sugar diet caused a female-biased increase in TOR activity, revealing a previously unrecognized sex difference in the nutrient-dependent regulation of TOR. Taken together, these findings not only extend knowledge of sex-specific IIS regulation, but also provide the first report of sex-biased TOR regulation.

4.4.3. Sex-biased requirement for IIS, Drosophila insulin-like peptides, and TOR in promoting the low sugar-induced increase in body size

To determine whether sex-biased changes to IIS and TOR play a role in mediating the low sugar-induced increase in body size, we measured body size in male and female larvae carrying mutations in each pathway that blunt high levels of IIS and TOR activation (Chen et al., 1996; Millington et al., 2021a; Rideout et al., 2015; Zhang et al., 2000). To determine the requirement for IIS, we measured pupal volume in w^{1118} larvae, and in larvae heterozygous for a hypomorphic allele of *InR* (*InR^{E19}*/+), in 1S and 0S. While body size was larger in w^{1118} male larvae reared on 0S than 1S (**Fig. 4.3A**), the low sugar-induced increase in body size was blocked in *InR^{E19}*/+ males (**Fig. 4.3A**; genotype:diet interaction $p<0.0001$). Thus, IIS activity was required in males for increased body size in 0S. In contrast, female w^{1118} and *InR^{E19}*/+ larvae reared on 0S were significantly larger than genotype-matched females raised on 1S (**Fig. 4.3B**). While we detected a significant genotype:diet interaction in females ($p<0.0001$), the magnitude of genotype effects on the body size response to low sugar was smaller in females than in males (sex:diet:genotype interaction $p=0.0114$). Thus, reduced IIS function had a male-biased impact on the low sugar-induced increase in body size.

Beyond InR, we reared male and female larvae lacking the coding sequences for *Drosophila insulin-like peptide 2* (*dilp2*; Fbgn0036046), *Drosophila insulin-like peptide 3* (*dilp3*; Fbgn0044050), and *Drosophila insulin-like peptide 5* (*dilp5*; Fbgn0044038) on 0S and 1S (Grönke et al., 2010). These Dilps are produced and secreted by insulin-producing cells in the brain (Brogiolo et al., 2001; Géminard et

al., 2009; Ikeya et al., 2002; Rulifson et al., 2002). Circulating Dilps stimulate IIS activity and growth by binding to InR on target cells (Teleman, 2010). In males, loss of *dilp2* and *dilp3* blunted the low sugar-induced increase in body compared with w^{1118} controls (genotype:diet *p*<0.0001 for both)*; loss of *dilp5* had no effect (genotype:diet *p*=0.9751) (**Fig. 4.3C**). In females, while loss of *dilp2* and *dilp3* (genotype:diet *p*<0.0001 for both), but not *dilp5* (genotype:diet *p*=0.9389), blunted the low sugar-induced increase in body size (**Fig. 4.3D**), the magnitude of genotype effects on the increase in body size were larger in males for *dilp3* (sex:diet:genotype interaction: *p*=0.0003), with a similar trend in *dilp2* (sex:diet:genotype interaction: *p*=0.0627). Thus, we identify a male-biased requirement for several genes that influence IIS activity in regulating the low sugar-induced increase in body size, a finding that aligns with the male-specific increase in IIS activity in 0S.

To determine the requirement for TOR in mediating the low sugar-induced increase in body size, we measured pupal volume in larvae heterozygous for a hypomorphic allele of *Target of rapamycin* (*Tor*; $Tor^{\Delta P}$/+) (Zhang et al., 2000). In w^{1118} females, larvae reared on 0S were significantly larger than genotype-matched larvae raised on 1S (**Fig. 4.3E**); however, this low sugar-induced increase in body size was blunted in $Tor^{\Delta P}$/+ female larvae (Fig. 3E; genotype:diet interaction *p*<0.0001). This suggests the low sugar-induced increase in TOR activity in females was required to achieve a larger body size. In w^{1118} and $Tor^{\Delta P}$/+ males, pupal
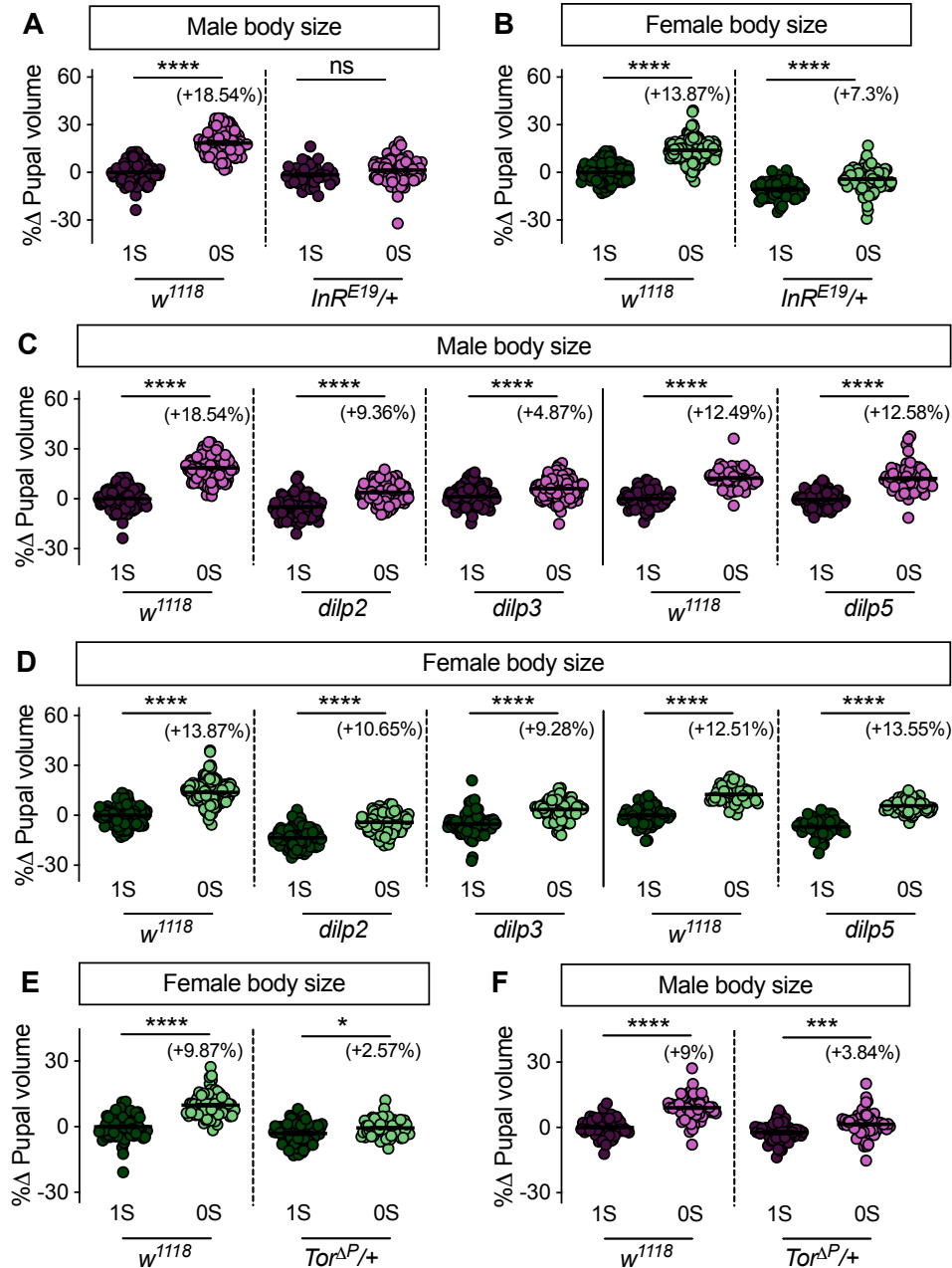
Figure 4.3. Sex-biased requirement for IIS, *Drosophila* insulin-like peptides, and target of rapamycin (TOR) in promoting the low sugar-induced increase in body size.

(A) Pupal volume was significantly higher in w^{1118} male larvae (p<0.0001; two-way ANOVA followed by Tukey HSD test) but not in InR^{E19}/+ heterozygote male larvae reared on a sugar-free diet (0S) compared to males raised on a diet containing sugar (1S) (p = 0.1539; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was higher in w^{1118} males than InR^{E19}/+ heterozygote males (genotype:diet interaction p<0.0001; two-way ANOVA by Tukey HSD test). n = 36-120

pupae. (B) Pupal volume was significantly higher in w^{1118} and InR^{E19}/+ heterozygote female larvae reared on 0S compared to females raised on 1S (p<0.0001 for all comparisons; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was higher in w^{1118} females than InR^{E19}/+ heterozygote females (genotype:diet interaction p<0.0001; two-way ANOVA by Tukey HSD test). n = 73-160 pupae. (C) Pupal volume was significantly higher in w^{1118}, $dilp2$ mutant, $dilp3$ mutant, and $dilp5$ mutant male larvae reared on 0S compared to males raised on 1S (p<0.0001 for all comparisons; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was higher in w^{1118} males than $dilp2$ mutant, and $dilp3$ mutant males (genotype:diet interaction p<0.0001 for all comparisons; two-way ANOVA by Tukey HSD test), but was not different in $dilp5$ mutants (genotype:diet interaction p = 0.9751; two-way ANOVA by Tukey HSD test) n = 69-120 pupae. (D) Pupal volume was significantly higher in w^{1118}, $dilp2$ mutant, $dilp3$ mutant, and $dilp5$ mutant female larvae reared on 0S compared to females raised on 1S (p<0.0001 for all comparisons; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was higher in w^{1118} females than $dilp2$ mutant, and $dilp3$ mutant females (genotype:diet interaction p<0.0001 for all comparisons; two-way ANOVA by Tukey HSD test), but was not different in $dilp5$ mutants (genotype:diet interaction p = 0.9389; two-way ANOVA by Tukey HSD test) n = 55-160 pupae. (E) Pupal volume was significantly higher in w^{1118} and $Tor^{\Delta P}$/+ heterozygote female larvae reared on 0S compared to females raised on 1S (p<0.0001, and p = 0.0135, respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was higher in w^{1118} females than $Tor^{\Delta P}$/+ heterozygote females (genotype:diet interaction p<0.0001; two-way ANOVA by Tukey HSD test). n = 58-98 pupae. (F) Pupal volume was significantly higher in w^{1118} and $Tor^{\Delta P}$/+ heterozygote male larvae reared on 0S compared to males raised on 1S (p<0.0001, and p = 0.0001, respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was higher in w^{1118} males than $Tor^{\Delta P}$/+ heterozygote males (genotype:diet interaction p<0.0001; two-way ANOVA by Tukey HSD test). n = 58-69 pupae. * p<0.05; *** p<0.001; **** p<0.0001; ns indicates not significant; error bars indicate SEM. Note: parallel collection of multiple genotypes and diets means that w^{1118} control data in 0S and 1S are the same in Fig. 1A, B, 3A-D.

---

volume was significantly larger in larvae raised on 0S compared with genotype-matched larvae reared on 1S (**Fig. 4.3F**). While the low sugar-induced increase in

body size was smaller in $Tor^{\Delta P}/+$ males compared with controls (genotype:diet p<0.0001), the magnitude of genotype effects on the body size response were larger in females than in males (sex:diet:genotype interaction (p=0.0303). This reveals a previously unrecognized female-biased requirement for TOR activity in regulating body size in a low sugar context.

4.4.4. A low sugar diet has sex-specific effects on metabolic gene expression and whole-body metabolism

IIS and TOR promote increased body size by regulating diverse aspects of metabolism, (*e.g.* triglyceride storage, protein synthesis, glucose homeostasis) (Grewal, 2009; Musselman and Kühnlein, 2018; Teleman et al., 2008). We therefore measured mRNA levels of a selection of genes implicated in metabolic regulation. In w^{1118} male and female larvae reared on 0S, we found significant changes to mRNA levels compared with larvae reared on 1S, many of which were sex-specific (**Fig. 4.4A**). For genes encoding proteins involved in fat metabolism, 9/14 and 5/14 genes showed low sugar-induced changes to mRNA levels in males and females, respectively (**Fig. 4.4A**). For genes encoding ribosomal proteins, which play integral roles in protein synthesis, 1/12 and 6/12 genes showed low sugar-induced changes to mRNA levels in males and females, respectively (**Fig. 4.4A**). While this examination of mRNA levels includes only a fraction of genes that affect metabolism, this data suggests that a low sugar diet causes sex-biased changes to mRNA levels of metabolic genes.

To determine the physiological significance of these sex-biased changes in mRNA levels (Gershman et al., 2007; Li et al., 2006; Mattila and Hietakangas, 2017; Teleman et al., 2008; Zinke et al., 2002), we measured whole-body levels of several macronutrients in male and female larvae reared in 0S and 1S. In both male and female w^{1118} larvae reared on 0S, triglyceride levels were significantly reduced compared with sex-matched larvae cultured on 1S (**Fig. 4.4B**). Thus, a low sugar diet reduced adiposity in both sexes. In contrast, w^{1118} females reared in 0S had significantly higher protein levels (**Fig. 4.4C**), an effect that was not reproduced in w^{1118} males (**Fig. 4.4C**). This reveals a previously unrecognized sex difference in the

regulation of whole-body protein in a low sugar context. While glucose levels were significantly higher in w^{1118} male and female larvae raised in 0S (**Fig. 4.4D**), the low sugar diet caused a male-specific increase in glycogen and trehalose (**Fig. 4.4E, F**). Thus, we observed both sex-specific and non-sex-specific alterations in whole-body carbohydrate levels in a low sugar context, highlighting the importance of including both sexes when studying diet-induced metabolic changes. Indeed, our findings
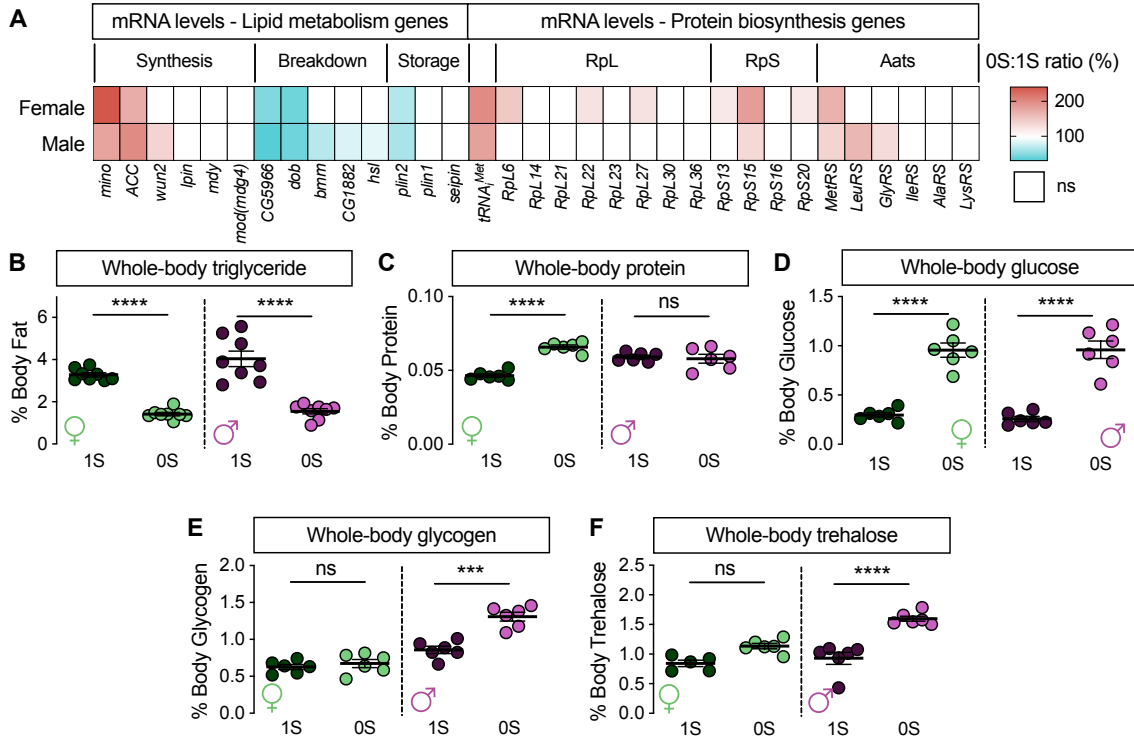
Figure 4.4. A low sugar diet has sex-biased effects on metabolic gene expression and metabolism.

(A) In w^{1118} females raised on a sugar-free (0S) diet, mRNA levels of *mino, ACC, dob, CG5966, plin2, tRNA$_i^{Met}$, RpL6, RpL22, RpL27, RpS15, RpS13, RpS20,* and *MetRS* were significantly different from females reared in 1S (*p* = 0.0005, 0.0019, 0.0041, 0.0006, and 0.0009, 0.0133, 0.0119, 0.003, 0.03, 0.0321, 0.0039, 0.0112, and 0.0428 respectively; Student's *t* test). In w^{1118} males raised on 0S, mRNA levels of *ACC, mino, wun2, CG5966, dob, bmm, CG1882, hsl, plin2, tRNA$_i^{Met}$, RpS15, LeuRS, MetRS,* and *GlyRS* were significantly different from males reared in 1S (*p*<0.0001, <0.0001, 0.0004, 0.0014, <0.0001, 0.0150, 0.0376, 0.0389, <0.0001, 0.0008, 0.018, 0.0009, 0.0122, and 0.013, respectively; Student's *t* test). n = 6-8 biological replicates. (B) Whole-body triglyceride levels in w^{1118} female and male larvae was significantly lower in a sugar-free diet (*p*<0.0001 for both

comparisons; Student's *t* test). n = 8 biological replicates. (C) Whole-body protein levels in w^{1118} female larvae was significantly higher in a sugar-free diet ($p<0.0001$; Student's *t* test), but no different in males ($p = 0.7045$; Student's *t* test). n = 6 biological replicates. (D) Whole-body glucose levels in w^{1118} female and male larvae was significantly higher in a sugar-free diet ($p<0.0001$ for both comparisons; Student's *t* test). n = 6 biological replicates. (E) Whole-body glycogen levels in w^{1118} male larvae was significantly higher in a sugar-free diet ($p = 0.0001$; Student's *t* test), but no different in females ($p = 0.4851$; Student's *t* test). (F) Whole-body trehalose levels in w^{1118} male larvae was significantly higher in a sugar-free diet ($p<0.0001$; Student's *t* test), but no different in females ($p = 0.4924$; Student's *t* test). n = 6 biological replicates. n = 6 biological replicates. *** p<0.001; **** p<0.0001; ns indicates not significant; error bars indicate SEM.

---

suggest sugar-induced changes to carbohydrate metabolism found in previous studies were possibly driven by effects in males (Musselman et al., 2011; Pasco and Léopold, 2012).

In conclusion, our study adds to a growing literature showing sex-specific effects of dietary nutrients on phenotypes such as body size, metabolism, lifespan, and fertility (Green and Extavour, 2014; Hudry et al., 2019; Klepsatel et al., 2020; Millington et al., 2021a; Regan et al., 2016; Wat et al., 2020). Because we show males and females activate distinct signaling pathways and exhibit sex-specific metabolic changes to achieve equivalent phenotypic outcomes, this suggests the absence of a sexually dimorphic phenotype in larval growth studies does not provide sufficient rationale for using single- or mixed-sex groups of animals. Instead, both sexes must be included to draw accurate conclusions regarding the signaling, metabolic, and body size effects of dietary nutrients.

5. DISCUSSION

5.1. OVERVIEW OF FINDINGS

There is a growing appreciation that biological sex is an important variable that must be integrated into biomedical research, from data collection to analysis. In doing so, we will expand our understanding of the molecular mechanisms by which biological sex influences many aspects of development and physiology, and how dysfunction in these physiological processes leads to sex differences in disease. In my thesis, I focused on improving knowledge of the mechanisms underlying both the sex-specific regulation of growth and the sex difference in nutrient-dependent body size plasticity using *Drosophila* as a model. While *Drosophila* has long been used to study the molecular mechanisms underlying growth, the use of mixed-sex populations has precluded a full understanding of how males and females differ in the mechanisms that impact body size. In this thesis, I made three important findings that define key differences in the genetic and molecular mechanisms that impact body size regulation in males and females.

In Chapter 2, I systematically analysed the role of the IIS pathway in regulating growth in each sex. A major finding of this study was that genetically reducing IIS pathway activity has female-biased effects on final body size, whereas genetically increasing IIS activity augmented body size only in males. This represented the first detailed examination of how IIS contributes to growth in both sexes.

In Chapter 3 I identified one mechanism underlying the sex difference in body size plasticity to dietary nutrients in *Drosophila*. I showed that females increase final body size in a nutrient-rich context by upregulating IIS activity, dependent on Dilp2 and Sun. In males, the diet-dependent upregulation of IIS was not as large as in females, resulting in reduced body size plasticity. This provided the first evidence of a role for sex determination factor Tra in regulating body size plasticity, and implicated Srl as a novel link between the sex determination pathway and body size plasticity.

In Chapter 4, I provide the first evidence that differential regulation of the IIS and TOR signaling pathways between the sexes can produce an equivalent body size outcome in a low-sugar diet. While previous research identified dietary sugar as inhibitory for growth (Musselman et al., 2011; Pasco and Leopold, 2012; Reis, 2016), my study shows that the pathways responsible for growth repression are distinct in males and females. This suggests that even when males and females display the same phenotypic outcome due to a dietary manipulation, the underlying molecular mechanisms may not be shared.

Based on these findings, the remainder of the discussion will focus on emergent themes, future directions, and limitations of my studies.

5.2. INSULIN PATHWAY HAS WIDESPREAD SEX-BIASED AND SEX-SPECIFIC EFFECTS

5.2.1. *Loss of IPC function has female-biased effects.*

One key finding of my research was the sex-biased effect of IPC ablation and IPC silencing on body size. While previous studies have demonstrated severe growth restriction due to IPC ablation (Rulifson et al., 2002), it remained unclear whether males and females were equally affected. My finding that females were more affected than males suggests that IPC function is important for females to achieve a larger body size than males. This finding aligns with results from a recent study that shows the sex of the IPCs impacts the male-female difference in body size (Sawala and Gould, 2017). Indeed, a previous study showed that secretion of the most potent growth-promoting Dilp, Dilp2, was higher in female larvae than in males (Rideout et al., 2015). Thus, the sex of the IPCs likely affects their function, perhaps via regulation of Dilp secretion, and causes sex-biased effects on IIS-dependent phenotypes.

Indeed, evidence for sex-biased effects of changes to IIS-associated phenotypes have been documented in many animals. For example, studies in worms, flies, and mice show that reduced IIS extends lifespan in a female-biased manner (hermaphrodite-biased in *C. elegans*) (Clancy et al., 2001; Giannakou et al.,

2004; Honjoh et al., 2017; Hotzi et al., 2018; Hwangbo et al., 2004; Selman et al., 2008; Tatar et al., 2001). Similarly, in flies, IPC ablation is sufficient to extend lifespan, an effect that may be female-biased (Broughton et al., 2005; Haselton et al., 2010). Despite these sex-specific responses to changes in IIS function, many open questions remain.

First, in addition to the IPCs, which cells and tissues mediate these effects? Several studies indicate that IIS activity in several tissues has sex-biased effects on a range of IIS-dependent phenotypes. For example, the fat is a major regulator of many IIS-dependent phenotypes and IIS activity in the fat contributes to lifespan in both flies and mammalian models. Similarly, IIS activity in the neurons contributes to many phenotypes and loss of IIS in mouse neurons has female-specific effects on body weight (Bluher et al., 2002; Bluher et al., 2003; Bruning et al., 2000; Hwangbo et al., 2004; Partridge et al., 2011). In addition, the gut is highly sexually dimorphic and, in *Drosophila,* females have increased levels of intestinal stem cell (ISC) proliferation which contributes to the breakdown of the gut barrier function with age (Ahmed et al., 2020; Hudry et al., 2016; Regan et al., 2016). Interestingly, this age-related gut dysfunction in females was ameliorated by dietary restriction (Regan et al., 2016). Given that one regulator of ISC proliferation is diet and IIS activity (Choi et al., 2011; O'Brien et al., 2011), this suggests that diet-dependent IIS activity may contribute to sex differences in ISC proliferation in the gut and to gut barrier dysfunction during aging. Multiple tissues may therefore contribute to the sex-biased phenotypic effects of changes to IIS activity.

Second, which specific IIS genes contribute to sex differences in IIS-dependent phenotypes? Interestingly, individual Dilps may have sex-biased effects on several phenotypes. For example, loss of *dilp2* may have a greater effect on lifespan extension in females (Gronke et al., 2010). This mirrors the sex-biased effects of Dilp2 loss on female body size; however, while many Dilps cause female-specific effects on body size, previous studies do not observe strongly sex-biased lifespan extension for all these Dilps. Thus, whether altered levels of all Dilps cause sex-biased phenotypic effects depends on the phenotype under examination. Lessons learned in flies will then need to be tested in mammalian models, such as

mice. Further, these lessons will need to be applied to additional IIS-dependent phenotypes, such as metabolism (Hudry et al., 2016; Hudry et al., 2019; Wat et al., 2020). Given the highly-conserved nature of differential insulin signaling activity affecting a range of dimorphic traits between the sexes, mechanisms identified in flies may be relevant for a broad range of mammalian phenotypes.

5.2.2. *Loss of individual dilps has sex-specific effects on body size.*

Another finding from my studies was the sex-specific effects of loss of all growth-promoting Dilps. I identified female-specific effects of loss of each of Dilps 1, 2, 3, 4, 5, and 7 on body size, and a male-specific effect of loss of Dilp6 on body size. Other recent studies have also confirmed the sex-specific effects of loss of Dilps on body size, such as loss of *dilp2* in a nutrient-rich context (Liao et al., 2020). However, while our studies suggest that several Dilps are regulated sex-specifically to control body size in each sex our knowledge of how Dilp regulation differs between males and females remains limited. One recent study found higher levels of Dilp2 secretion from the IPCs in female larvae (Rideout et al., 2015). However, the IPCs produce and secrete other Dilps during development such as Dilps 1,3, and 5 (Brogiolo et al., 2001; Rulifson et al., 2002). Limited clues exist as to how these other IPC-derived Dilps may differ between the sexes. Both Dilps 3, and 5 are secreted in response to different nutrient components of the diet. For example, Dilp3 is secreted in response to circulating sugars in the haemolymph (Kim and Neufeld, 2015), and Dilp5 is responsive to dietary protein levels (Okamoto and Nishimura, 2015). This nutrient-dependent regulation of Dilp3 and 5 may suggest that sex-specific roles for Dilps may be dependent on nutrient context. Interestingly, these IPC-derived Dilps may also be found stored in the same secretory granules and selectively secreted (Kim and Neufeld, 2015). For example, Dilp2 and Dilp3 may be found in the same secretory granules, but Dilp2 secretion is stimulated by dietary protein and Dilp3 by dietary sugar (Kim and Neufeld, 2015). How this selective secretion is controlled, and how, or whether, it differs between males and females remains unknown. By studying these processes in both sexes we will identify how

the sex-specific production, secretion, and activity of Dilps in response to dietary nutrients controls body size in both sexes.

In addition to IPC-derived Dilps, I identified a female-specific requirement for non-IPC-derived Dilps on body size. For example, Dilp4 promotes increased female body size but comparatively little is known about how this Dilps is produced, secreted, and functions to promote growth in larvae. This suggests that the sex-biased regulation of Dilps may be prevalent in many tissues. For example, recent studies have demonstrated that Dilp7 is produced by a larger population of Dilp7-producing motoneurons which are required for female fertility (Castellanos et al., 2013; Garner et al., 2018). The role of these female-specific Dilp7-producing cells may be adult onset and solely related to reproduction. However, whether there may be previously unrecognised sex differences in larval Dilp7 production or Dilp7-producing cells in promoting increased growth remains unknown. More knowledge of how sex differences in production and/or secretion of many Dilps will be needed to understand the female-biased effects of loss of most Dilps. In addition, it will be important to determine whether the milder phenotypic changes we observe in males reflects a lower requirement for Dilp proteins to support larval growth, or whether males have more active mechanisms to compensate for Dilp loss. For example, the transcriptional response to reduced IIS signaling in adults is strongly male-biased (Graze et al., 2018). It will be interesting to determine whether this widespread remodelling of gene expression in males is required to compensate for loss of IIS activity.

5.2.3. *Loss of intracellular IIS pathway components have both sex-biased and non-sex-biased effects on body size.*

Downstream of Dilp production and release, I demonstrated that loss of some intracellular IIS components have female-biased effects on growth and SSD whereas others had a non-sex-biased effect on growth. Thus, it is not a universal feature of mutations that affect IIS activity to have female-biased effects on body size. Why some genes have sex-biased effects and others do not is an open question. One potential explanation for this observation is the different relative ability

of each hypomorphic allele to disrupt the gene product. For example, Akt^3 is a strong hypomorphic allele which causes a female-biased reduction in body size, whereas $Pdk1^4$ is a weakly hypomorphic allele (Rintelen et al., 2001). It will therefore be important to test whether stronger *Pdk1* allelic combinations also have non-sex-biased effects on body size. An alternative possibility is that loss of intracellular IIS signaling causes tissue insulin resistance by blunting the ability of cells and tissues to activate IIS signaling. This has been well-described in mice, where loss of mammalian insulin receptor is used to induce an insulin resistant phenotype in various tissues (Bruning et al., 2000; Michael et al., 2000). Fly tissues can develop insulin resistance (Hirabayashi et al., 2013; Musselman et al., 2011; Pasco and Leopold, 2012), thus, it will be important for future studies to test the relative sensitivity of male and female tissues to insulin in flies and whether loss of IIS pathway components does induce insulin resistance. A final possibility to explain why some and not other IIS components cause sex-biased effects is that there is an adaptor protein *lnk* that acts in parallel with Chico to control IIS activity (Werz et al., 2009). Future studies will therefore be needed to determine which intracellular pathways are activated in each sex when Dilps bind to InR.

5.2.4. Study limitations.

One limitation of our studies is that there are a limited number of assays to directly detect circulating Dilps in the haemolymph of flies. Unlike in mammals, where circulating levels of insulin can be detected at picomolar levels, few equivalents to this sensitive assay exist for Dilp quantification in *Drosophila*. Several approaches have been designed to attempt to measure circulating Dilp levels, primarily focusing on the most potent growth-promoting Dilp, Dilp2. For example, one way to estimate circulating Dilp2 levels is to indirectly infer from the relative intensity of intracellular Dilp2 immunoreactivity in the IPCs whether Dilp2 is being retained (high signal) or secreted (low signal) (Geminard et al., 2009). However, while being the most commonly used method of assessing circulating Dilp2 levels, this technique cannot discriminate between altered Dilp production or secretion.

More recent progress has been made on developing a direct readout of circulating Dilp levels using epitope tagging. One of the first direct measurements of circulating Dilp2 levels was achieved using a FLAG tagged Dilp2 (Dilp2^F) overexpressed using the Dilp2-GAL4 driver (Honegger et al., 2008; Geminard et al., 2009). However, this method still lacks physiological relevance as overexpression of Dilp2^F produces supraphysiological levels of Dilp2, in addition to endogenous Dilp2 production, and is not as bioactive as untagged Dilp2 (Honegger et al., 2008; Geminard et al., 2009; Park et al., 2014). Recent efforts have produced better epitope tagged Dilp transgenes which do not require overexpression. For example, a stable transgenic *dilp2* tagged with both FLAG and HA (*dilp2^{HF}*) expressed under the control of *dilp2* regulatory sequences in a genetic background lacking the endogenous *dilp2* locus recapitulates the bioactivity of endogenous Dilp2 and can be detected in the picomolar range through enzyme-linked immunosorbent assay (ELISA) (Park et al., 2014). A similar approach utilizing CRISPR has also been successful for epitope tagging Dilp6 (Dilp6^{HF}) which has allowed for quantification of circulating Dilp6 in the haemolymph (Suzawa et al., 2019). Despite these tools, I was still unable to measure circulating Dilp2 levels in our hands as Dilp2^{HF} transgenic flies had a defect in body size plasticity to dietary nutrients. This suggests that while epitope tagging of Dilp2 does not interfere with bioactivity, the nutrient dependent regulation of Dilp2^{HF} may be perturbed between a control and high dietary protein context. Therefore, it will be important to develop better, and more direct, ways of measuring circulating Dilp levels for a more precise dissection of Dilp function, both in general and between the sexes.

5.3. SEX DIFFERENCES IN NUTRIENT-DEPENDENT PHENOTYPIC PLASTICITY

My second study was larger in scope, and made several interesting findings that I will discuss in the sections below.

5.3.1. *Sex-specific regulation and function of insulinotropic factor Stunted*

One interesting finding from my second study was that Dilp secretion and IIS are regulated by different secreted factors from the fat body in males and females.

Specifically, I identified a key role for the insulinotropic factor Sun in regulating increased female body size plasticity. My studies identified a role for sex-specific regulation of *sun* mRNA levels, however, Sun protein is also regulated at multiple levels. Sun is a component of the ATP synthase complex and is found in the mitochondria (Cvejic et al., 2004; Kidd et al., 2005), and Sun secretion into the hemolymph is regulated by dietary protein (Delanoue et al., 2016). Yet the precise mechanism by which Sun is secreted into the hemolymph remains unclear. One possible non-canonical secretion mechanism by which Sun may enter circulation is through selective mitophagy. Nutrient status is a key regulator of mitophagy (Webster et al., 2014; Youle and Narendra, 2011), and several differing cell types across species actively increase or decrease intracellular mitochondria number (Hämäläinen et al., 2013; Vafai and Mootha, 2012). Our study identified mRNA levels as one important mechanism the sexes differ in sun regulation, but this does not preclude sex-specific regulation for additional aspects of sun regulation. It will be interesting for future studies to determine all the levels at which Sun is sex-specifically regulated by a protein-rich diet to cause the male-female difference in circulating Sun. Interestingly, sex differences in the secretion of insulinotropic factors have also been observed in mammals. For example, in non-diabetic humans, circulating levels of the incretin gastric inhibitory polypeptide (GIP), a gut derived insulinotropic factor in mammals, are higher in males upon glucose challenge than females (Matsuo et al., 2014).

5.3.2. *The sex determination gene transformer is a plasticity factor*

I presented evidence that the sex determination gene *tra* controls the sex difference in body size plasticity to dietary nutrients. This suggests that Tra is a plasticity factor that allows females to couple dietary protein levels with body size through the IIS pathway. This is an interesting possibility given that several phenotypes regulated by sex determination factors are affected by nutrition. For example, abdominal pigmentation, a secondary sex characteristic controlled by Dsx (Kopp et al., 2000), is reduced in both sexes raised on nutrient-poor conditions (Shakhmantsir et al., 2014). Similarly, re-mating behaviour, under the control of Dsx

expressing neurons, is reduced when food is absent (Harshman et al., 1988). Yet, how Tra acts to couple nutrient status with IIS and growth remains unclear.

One possible mechanism linking nutrient status and Tra would be through the nutrient-dependent regulation of Tra mRNA levels or protein. At present, mRNA levels of sex determination genes have not been linked to nutrient status, but several sex determination factors are post-translationally modified in *Drosophila*. For example, both Tra and its cofactor Tra-2 are phosphorylated by the LAMMER kinase Darkener of Apricot (Doa; FBgn0265998), a kinase known to phosphorylate several SR protein splicing factors (Du et al., 1998). This phosphorylation of Tra and Tra-2 is functionally required for sex determination in *Drosophila* as mutations to Doa result in an intersex phenotype (Du et al., 1998). One potential link between nutrients and Tra would therefore be through nutrient-dependent phosphorylation of Tra by Doa. Indeed, Doa activity has been linked to nutrient status through TORC1 (Tang et al., 2018). Future studies will therefore need to determine whether Tra phosphorylation differs between our 1Y and 2Y diets, and whether this depends on Doa.

Another key finding of my study is the identification of the transcriptional coactivator Srl as required for Tra to promote increased body size plasticity to dietary nutrients. However, the link between Tra and Srl remains unclear. One potential link may be through a physical interaction of Tra and Srl proteins. For example, the phosphorylation of SR proteins, such as Tra, promotes the interaction of SR protein splicing factors to form complexes which mediate many aspects of pre-mRNA processing (Kohtz et al., 1994; Shepard and Hertel, 2009; Wu and Maniatis, 1993). Indeed, the splicing function of Tra and Tra-2 is dependent on its phosphorylation of serine residues in the RS domain (Du et al., 1998). Interestingly, Srl also contains an RS domain (Mukherjee et al., 2014) which may suggest a potential direct interaction between Tra and Srl. However, a lack of available Tra reagents for biochemistry is a limitation for testing this hypothesis. To address this question, it will be important to develop tools to test the interaction of Tra and Srl and whether a direct interaction of Tra and Srl is required for increased female body size plasticity.

My identification of Srl as an important factor for increased female body size plasticity suggests that sex differences in mitochondrial function may underlie the sex difference in body size plasticity. Srl is a key regulator of mitochondrial activity, where Srl promotes the expression of several genes involved in oxidative phosphorylation, and overexpression of Srl is sufficient for increased oxygen consumption and ATP production (Tiefenbock et al., 2010; Rera et al., 2011; Mukherjee and Duttaroy, 2013). Indeed, there are known sex differences in mitochondrial function in *Drosophila*. For example, sex differences exist in mitochondrial Lon protease alternative splicing which contributes to the sex difference in oxidative stress tolerance, dependent on Tra (Pomatto et al., 2017). Given that ATP production and mitochondrial activity have known effects on body size in *Drosophila* (Jacobs et al., 2020; Kidd et al., 2005; Sriskanthadevan-Pirahas et al., 2021), it will be interesting to explore precisely how mitochondria differ in structure and function in females and males in different dietary contexts.

5.3.3. Study limitations

One limitation of my study is that the diets tested differ in calorie content. While my data manipulating calories independent of protein and sugar levels suggest that calories do not account for the sex differences in nutrient-dependent growth, it will be interesting to expand these studies using isocaloric diets altering the proportions of dietary nutrients such as protein and sugar. Indeed, one powerful approach to determine the relative contributions of dietary nutrients to SSD is through a nutritional geometry framework, where using a series of isocaloric diets the relative proportions of dietary nutrients are altered. Nutritional geometry has been used to study sex differences in trait size identifying increased female-trait size plasticity to dietary nutrition, in line with our findings (Shingleton et al., 2017). However, sex differences in body size plasticity in a nutritional geometry framework has only been extrapolated from trait size data (Shingleton et al., 2017; McDonald et al., 2020). Given the high degree of agreement between my studies and those in a nutritional geometry framework this suggests that both approaches are valid, but may be improved by accounting for calories as a variable.

5.4. DISTINCT PATHWAYS MEDIATE SIMILAR PHENOTYPIC OUTCOME IN EACH SEX

In Chapter 4, I identified that a low sugar diet causes equivalent growth phenotypes in both sexes. Surprisingly, my data suggests that sex-specific alterations in IIS and TOR signaling pathway activity mediate these effects. This ability to produce equivalent phenotypic outcomes via distinct mechanisms suggests that mechanistic sex differences may be prevalent, even for non-dimorphic phenotypes. For future studies, this will require the examination of both sexes, even when phenotypic sex differences are not obvious. Previous studies have described how different genotypes can present identical phenotypes, a concept known as robustness or canalisation (Waddington, 1942), but less is known about the molecular mechanisms underlying sex differences in this process. It will be important to identify whether sex-specific mechanisms exist for a range of non-dimorphic phenotypes in males and females.

One important finding from this study that requires more follow-up was that IIS was increased in a low sugar context specifically in males. This male-specific effect was in contrast to the female-biased increase in IIS activity in a protein-rich context. One potential explanation for this finding is that reducing dietary sugar in males may relieve insulin resistance. High levels of dietary sugar cause insulin resistance in developing larvae, triggering reduced growth and a smaller body size (Musselman et al., 2011; Pasco and Leopold, 2012; Reis et al., 2016). However, these diets used very high levels of dietary sugar, far in excess of the levels used in our control (1S) diet. If it is true that the low sugar diet alleviates insulin resistance, however, this will be important for many studies on larval growth that use diets with added sugar, as it suggests that males develop insulin resistance even at relatively low levels of dietary sugar. Indeed, in mammals, males have a higher propensity to develop insulin resistance than females (Macotela et al., 2009).

While the mechanism underlying increased IIS in males in a low sugar context remains unknown, it will be interesting to determine whether this increased IIS mediates the male-specific increase in metabolites such as trehalose and

glycogen. Indeed, IIS signaling is required for glycogen synthesis in the larval fat body (Yamada et al., 2018), consequently it will be important to determine whether IIS activity is required for the male-specific increase in glycogen levels in a low sugar context.

In contrast to the male-specific increase in IIS, we observed a female-specific increase in TOR signaling in a low-sugar context. This was unexpected as a previous study identified that TOR is activated in some contexts by the dietary sugar trehalose (Kim and Neufeld, 2015). While the mechanism underlying the increased TOR activity in a low sugar context remains unclear, the increased TOR signaling we identify may explain the female-specific increase in protein content in a low-sugar context. Given that we observed several female-specific and female-biased increases to ribosomal protein gene expression, this may indicate that females reared in 0S have more ribosomes, protein synthesis, and consequently higher levels of protein. However, as we only examined a selection of ribosomal protein genes, it will be important to perform an unbiased analysis of transcriptomic changes in both sexes reared in our 1S and 0S diets. Because TOR signaling promotes protein biosynthesis by upregulating ribosomal biogenesis and translation, it will be interesting to see if TOR is required for the female-specific increase in protein in females reared in a low sugar diet. Further, it will be interesting to test whether the female-specific increase in TOR enhances mRNA translation as a mechanism to achieve a larger body size (Grewal et al., 2005; Grewal et al., 2007; Rideout et al., 2012; Marshall et al., 2012; Ghosh et al., 2014; Sriskanthadevan-Pirahas et al., 2018a; Sriskanthadevan-Pirahas et al., 2018b).

5.5. CONCLUSIONS

Overall, my thesis identifies critical sex-biased regulation of conserved pathways controlling growth that contributes to SSD and the sex difference in body size plasticity to dietary nutrients. While many unanswered questions arise from my work, my findings provide a solid foundation for future studies to expand our knowledge of the molecular mechanisms by which biological sex contributes to SSD and sex differences in nutrient-dependent body size plasticity. As studies on growth

and body size plasticity had previously determined mechanisms in a single or mixed-sex population, this thesis fills an important gap by providing a comprehensive analysis demonstrating sex differences in IIS pathway activity contributing to SSD, and identifying one mechanism underlying the sex difference in body size plasticity to dietary nutrients. In the future, expanding these studies to mammalian models to identify conserved sex differences in the regulation of these pathways will inform our understanding of the sex-biased risk and incidence of human metabolic disease.

BIBLIOGRAPHY

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., et al. (2000). The genome sequence of Drosophila melanogaster. Science *287*, 2185–2195.

Agrawal, A.A. (2001). Phenotypic plasticity in the interactions and evolution of species. Science *294*, 321–326.

Agrawal, N., Delanoue, R., Mauri, A., Basco, D., Pasco, M., Thorens, B., and Léopold, P. (2016). The Drosophila TNF Eiger Is an Adipokine that Acts on Insulin-Producing Cells to Mediate Nutrient Response. Cell Metabolism *23*, 675–684.

Ahmed, S.M.H., Maldera, J.A., Krunic, D., Paiva-Silva, G.O., Pénalva, C., Teleman, A.A., and Edgar, B.A. (2020). Fitness trade-offs incurred by ovary-to-gut steroid signalling in Drosophila. Nature *584*, 415–419.

Alic, N., Andrews, T.D., Giannakou, M.E., Papatheodorou, I., Slack, C., Hoddinott, M.P., Cochemé, H.M., Schuster, E.F., Thornton, J.M., and Partridge, L. (2011a). Genome-wide dFOXO targets and topology of the transcriptomic response to stress and insulin signalling. Mol Syst Biol *7*, 502.

Alic, N., Hoddinott, M.P., Vinti, G., and Partridge, L. (2011b). Lifespan extension by increased expression of the Drosophila homologue of the IGFBP7 tumour suppressor. Aging Cell *10*, 137–147.

Almudi, I., Poernbacher, I., Hafen, E., and Stocker, H. (2013). The Lnk/SH2B adaptor provides a fail-safe mechanism to establish the Insulin receptor-Chico interaction. Cell Commun Signal *11*, 26.

Alpatov, W.W. (1930). Phenotypical Variation in Body and Cell Size of Drosophila melanogaster. Biological Bulletin *58*, 85–103.

Amoyel, M., Hillion, K.-H., Margolis, S.R., and Bach, E.A. (2016). Somatic stem cell differentiation is regulated by PI3K/Tor signaling in response to local cues. Development *143*, 3914–3925.

Anand, A., Villella, A., Ryner, L.C., Carlo, T., Goodwin, S.F., Song, H.J., Gailey, D.A., Morales, A., Hall, J.C., Baker, B.S., et al. (2001). Molecular genetic dissection

of the sex-specific and vital functions of the Drosophila melanogaster sex determination gene fruitless. Genetics *158*, 1569–1595.

Andersen, D.S., Colombani, J., and Léopold, P. (2013). Coordination of organ growth: principles and outstanding questions from the world of insects. Trends Cell Biol *23*, 336–344.

Arnold, A.P. (2009). Mouse models for evaluating sex chromosome effects that cause sex differences in non-gonadal tissues. J Neuroendocrinol *21*, 377–386.

Arnold, A.P., and Chen, X. (2009). What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues? Front Neuroendocrinol *30*, 1–9.

Arquier, N., Géminard, C., Bourouis, M., Jarretou, G., Honegger, B., Paix, A., and Léopold, P. (2008). Drosophila ALS regulates growth and metabolism through functional interaction with insulin-like peptides. Cell Metab *7*, 333–338.

Ashton, K.G., Tracy, M.C., and Queiroz, A. de (2000). Is Bergmann's Rule Valid for Mammals? The American Naturalist *156*, 390–415.

Atkinson, D. (1994). Temperature and Organism Size—A Biological Law for Ectotherms? In Advances in Ecological Research, M. Begon, and A.H. Fitter, eds. (Academic Press), pp. 1–58.

Atkinson, D. (1995). Effects of temperature on the size of aquatic ectotherms: Exceptions to the general rule. Journal of Thermal Biology *20*, 61–74.

Avruch, J., Long, X., Ortiz-Vega, S., Rapley, J., Papageorgiou, A., and Dai, N. (2009). Amino acid regulation of TOR complex 1. American Journal of Physiology-Endocrinology and Metabolism *296*, E592–E602.

Bachtrog, D., Mank, J.E., Peichel, C.L., Kirkpatrick, M., Otto, S.P., Ashman, T.-L., Hahn, M.W., Kitano, J., Mayrose, I., Ming, R., et al. (2014). Sex Determination: Why So Many Ways of Doing It? PLOS Biology *12*, e1001899.

Badyaev, A.V. (2002). Growing apart: an ontogenetic perspective on the evolution of sexual size dimorphism. Trends in Ecology & Evolution *17*, 369–378.

Bai, H., Kang, P., and Tatar, M. (2012). Drosophila insulin-like peptide-6 (dilp6) expression from fat body extends lifespan and represses secretion of Drosophila insulin-like peptide-2 from the brain. Aging Cell *11*, 978–985.

Bai, H., Kang, P., Hernandez, A.M., and Tatar, M. (2013). Activin Signaling Targeted by Insulin/dFOXO Regulates Aging and Muscle Proteostasis in Drosophila. PLoS Genet *9*.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in Drosophila neurons developing without synaptic transmission. J. Neurosci. *21*, 1523–1531.

Banfi, S., Borsani, G., Rossi, E., Bernard, L., Guffanti, A., Rubboli, F., Marchitiello, A., Giglio, S., Coluccia, E., Zollo, M., et al. (1996). Identification and mapping of human cDNAs homologous to Drosophila mutant genes through EST database searching. Nature Genetics *13*, 167–174.

Bass, T.M., Grandison, R.C., Wong, R., Martinez, P., Partridge, L., and Piper, M.D. (2007). Optimization of dietary restriction protocols in Drosophila. J Gerontol A Biol Sci Med Sci *62*, 1071–1081.

BEADLE, G.W., TATUM, E.L., and CLANCY, C.W. (1938). FOOD LEVEL IN RELATION TO RATE OF DEVELOPMENT AND EYE PIGMENTATION IN DROSOPHILA MELANOGASTER. The Biological Bulletin *75*, 447–462.

Bell, L.R., Maine, E.M., Schedl, P., and Cline, T.W. (1988). Sex-lethal, a Drosophila sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins. Cell *55*, 1037–1046.

Bellantoni, M.F., Vittone, J., Campfield, A.T., Bass, K.M., Harman, S.M., and Blackman, M.R. (1996). Effects of oral versus transdermal estrogen on the growth hormone/insulin-like growth factor I axis in younger and older postmenopausal women: a clinical research center study. The Journal of Clinical Endocrinology & Metabolism *81*, 2848–2853.

Belote, J.M., McKeown, M., Boggs, R.T., Ohkawa, R., and Sosnowski, B.A. (1989). Molecular genetics of transformer, a genetic switch controlling sexual differentiation in Drosophila. Dev Genet *10*, 143–154.

Bergland, A.O., Genissel, A., Nuzhdin, S.V., and Tatar, M. (2008). Quantitative trait loci affecting phenotypic plasticity and the allometric relationship of ovariole number and thorax length in Drosophila melanogaster. Genetics *180*, 567–582.

Bergmann, C. (1848). Über die Verhältnisse der Wärmeökonomie der Thiere zu ihrer Größe.

Bikle, D., Majumdar, S., Laib, A., Powell-Braxton, L., Rosen, C., Beamer, W., Nauman, E., Leary, C., and Halloran, B. (2001). The Skeletal Structure of Insulin-Like Growth Factor I-Deficient Mice. Journal of Bone and Mineral Research *16*, 2320–2329.

Billeter, J.C., Rideout, E.J., Dornan, A.J., and Goodwin, S.F. (2006). Control of male sexual behavior in Drosophila by the sex determination pathway. Curr Biol *16*, R766-76.

Bjedov, I., Toivonen, J.M., Kerr, F., Slack, C., Jacobson, J., Foley, A., and Partridge, L. (2010). Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metab *11*, 35–46.

Bjordal, M., Arquier, N., Kniazeff, J., Pin, J.P., and Léopold, P. (2014). Sensing of Amino Acids in a Dopaminergic Circuitry Promotes Rejection of an Incomplete Diet in Drosophila. Cell *156*, 510–521.

Blaschke, K., Ebata, K.T., Karimi, M.M., Zepeda-Martínez, J.A., Goyal, P., Mahapatra, S., Tam, A., Laird, D.J., Hirst, M., Rao, A., et al. (2013). Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. Nature *500*, 222–226.

Blüher, M., Michael, M.D., Peroni, O.D., Ueki, K., Carter, N., Kahn, B.B., and Kahn, C.R. (2002). Adipose Tissue Selective Insulin Receptor Knockout Protects against Obesity and Obesity-Related Glucose Intolerance. Developmental Cell *3*, 25–38.

Blüher, M., Kahn, B.B., and Kahn, C.R. (2003). Extended Longevity in Mice Lacking the Insulin Receptor in Adipose Tissue. Science *299*, 572–574.

Boggs, R.T., Gregor, P., Idriss, S., Belote, J.M., and McKeown, M. (1987). Regulation of sexual differentiation in D. melanogaster via alternative splicing of RNA from the transformer gene. Cell *50*, 739–747.

Böhni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell *97*, 865–875.

Boulan, L., Milán, M., and Léopold, P. (2015). The Systemic Control of Growth. Cold Spring Harb Perspect Biol *7*.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Bridges, C.B. (1921). TRIPLOID INTERSEXES IN DROSOPHILA MELANOGASTER. Science *54*, 252–254.

Britton, J.S., and Edgar, B.A. (1998). Environmental control of the cell cycle in Drosophila: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. Development *125*, 2149–2158.

Britton, J.S., Lockwood, W.K., Li, L., Cohen, S.M., and Edgar, B.A. (2002). Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. Dev Cell *2*, 239–249.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001). An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Curr Biol *11*, 213–221.

Broughton, S.J., Piper, M.D.W., Ikeya, T., Bass, T.M., Jacobson, J., Driege, Y., Martinez, P., Hafen, E., Withers, D.J., Leevers, S.J., et al. (2005). Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands. PNAS *102*, 3105–3110.

Brown, E.H., and King, R.C. (1961). STUDIES ON THE EXPRESSION OF THE TRANSFORMER GENE OF DROSOPHILA MELANOGASTER. Genetics *46*, 143.

Brown, E.B., Shah, K.D., Faville, R., Kottler, B., and Keene, A.C. (2020). Drosophila insulin-like peptide 2 mediates dietary regulation of sleep intensity. PLoS Genet *16*, e1008270.

Brüning, J.C., Gautam, D., Burks, D.J., Gillette, J., Schubert, M., Orban, P.C., Klein, R., Krone, W., Müller-Wieland, D., and Kahn, C.R. (2000). Role of Brain Insulin Receptor in Control of Body Weight and Reproduction. Science *289*, 2122–2125.

Buchanan, J.L., Meiklejohn, C.D., and Montooth, K.L. (9). Mitochondrial Dysfunction and Infection Generate Immunity-Fecundity Tradeoffs in Drosophila. Integr Comp Biol *58*, 591–603.

Buhler, K., Clements, J., Winant, M., Bolckmans, L., Vulsteke, V., and Callaerts, P. (2018). Growth control through regulation of insulin signalling by nutrition-activated steroid hormone in Drosophila. Development *145*.

Cáceres, L., Necakov, A.S., Schwartz, C., Kimber, S., Roberts, I.J.H., and Krause, H.M. (2011). Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. Genes Dev *25*, 1476–1485.

Callier, V., and Nijhout, H.F. (2011). Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis. PNAS *108*, 14664–14669.

Callier, V., Shingleton, A.W., Brent, C.S., Ghosh, S.M., Kim, J., and Harrison, J.F. (2013). The role of reduced oxygen in the developmental physiology of growth and metamorphosis initiation in Drosophila melanogaster. Journal of Experimental Biology *216*, 4334–4340.

Camara, N., Whitworth, C., and Van Doren, M. (2008). The creation of sexual dimorphism in the Drosophila soma. Curr Top Dev Biol *83*, 65–107.

Campbell, J.E., and Newgard, C.B. (2021). Mechanisms controlling pancreatic islet cell function in insulin secretion. Nature Reviews Molecular Cell Biology *22*, 142–158.

Camus, M.F., Fowler, K., Piper, M.W.D., and Reuter, M. (2017). Sex and genotype effects on nutrient-dependent fitness landscapes in Drosophila melanogaster. Proceedings of the Royal Society B: Biological Sciences *284*, 20172237.

Camus, M.F., Piper, M.D.W., and Reuter, M. (2019). Sex-specific transcriptomic responses to changes in the nutritional environment. ELife *8*, e47262.

Castellanos, M.C., Tang, J.C., and Allan, D.W. (2013). Female-biased dimorphism underlies a female-specific role for post-embryonic Ilp7 neurons in Drosophila fertility. Development *140*, 3915–3926.

Chang, P.L., Dunham, J.P., Nuzhdin, S.V., and Arbeitman, M.N. (2011). Somatic sex-specific transcriptome differences in Drosophila revealed by whole transcriptome sequencing. BMC Genomics *12*, 364.

Chatterjee, S.S., Uppendahl, L.D., Chowdhury, M.A., Ip, P.-L., and Siegal, M.L. (2011). The female-specific Doublesex isoform regulates pleiotropic transcription factors to pattern genital development in Drosophila. Development *138*, 1099–1109.

Chen, C., Jack, J., and Garofalo, R.S. (1996). The Drosophila insulin receptor is required for normal growth. Endocrinology *137*, 846–856.

Chen, X., McClusky, R., Chen, J., Beaven, S.W., Tontonoz, P., Arnold, A.P., and Reue, K. (2012). The Number of X Chromosomes Causes Sex Differences in Adiposity in Mice. PLOS Genetics *8*, e1002709.

Cho, K.S., Lee, J.H., Kim, S., Kim, D., Koh, H., Lee, J., Kim, C., Kim, J., and Chung, J. (2001). Drosophila phosphoinositide-dependent kinase-1 regulates apoptosis and growth via the phosphoinositide 3-kinase-dependent signaling pathway. Proc Natl Acad Sci U S A *98*, 6144–6149.

Choi, N.H., Lucchetta, E., and Ohlstein, B. (2011). Nonautonomous regulation of Drosophila midgut stem cell proliferation by the insulin-signaling pathway. PNAS *108*, 18702–18707.

Christiansen, A.E., Keisman, E.L., Ahmad, S.M., and Baker, B.S. (2002). Sex comes in from the cold: the integration of sex and pattern. Trends Genet *18*, 510–516.

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., Hafen, E., Leevers, S.J., and Partridge, L. (2001). Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. Science *292*, 104–106.

Clarke, D.W., Mudd, L., Boyd, F.T., Fields, M., and Raizada, M.K. (1986). Insulin Is Released from Rat Brain Neuronal Cells in Culture. Journal of Neurochemistry *47*, 831–836.

Clement-Jones, M., Schiller, S., Rao, E., Blaschke, R.J., Zuniga, A., Zeller, R., Robson, S.C., Binder, G., Glass, I., Strachan, T., et al. (2000). The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome. Human Molecular Genetics *9*, 695–702.

Clemons, R.D., Costin, G., and Kogut, M.D. (1976). Laron dwarfism: Growth and immunoreactive insulin following treatment with human growth hormone. The Journal of Pediatrics *88*, 427–433.

Cline, T.W. (1978). Two closely linked mutations in Drosophila melanogaster that are lethal to opposite sexes and interact with daughterless. Genetics *90*, 683–698.

Cline, T.W. (1984). Autoregulatory Functioning of a Drosophila Gene Product That Establishes and Maintains the Sexually Determined State. Genetics *107*, 231–277.

Cline, T.W., and Meyer, B.J. (1996). VIVE LA DIFFÉRENCE: Males vs Females in Flies vs Worms. Annu. Rev. Genet. *30*, 637–702.

Clough, E., Jimenez, E., Kim, Y.A., Whitworth, C., Neville, M.C., Hempel, L.U., Pavlou, H.J., Chen, Z.X., Sturgill, D., Dale, R.K., et al. (2014). Sex- and tissue-specific functions of Drosophila doublesex transcription factor target genes. Dev Cell *31*, 761–773.

Cognigni, P., Bailey, A.P., and Miguel-Aliaga, I. (2011). Enteric neurons and systemic signals couple nutritional and reproductive status with intestinal homeostasis. Cell Metab *13*, 92–104.

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., and Léopold, P. (2003). A nutrient sensor mechanism controls Drosophila growth. Cell *114*, 739–749.

Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carré, C., Noselli, S., and Léopold, P. (2005). Antagonistic Actions of Ecdysone and Insulins Determine Final Size in Drosophila. Science *310*, 667–670.

Cong, X., Wang, H., Liu, Z., He, C., An, C., and Zhao, Z. (2015). Regulation of Sleep by Insulin-like Peptide System in Drosophila melanogaster. Sleep *38*, 1075–1083.

Cvejic, S., Zhu, Z., Felice, S.J., Berman, Y., and Huang, X.-Y. (2004). The endogenous ligand Stunted of the GPCR Methuselah extends lifespan in Drosophila. Nature Cell Biology *6*, 540–546.

Cymborowski, B., Bogus, M., Beckage, N.E., Williams, C.M., and Riddiford, L.M. (1982). Juvenile hormone titres and metabolism during starvation-induced supernumerary larval moulting of the tobacco hornworm, Manduca sexta L. Journal of Insect Physiology *28*, 129–135.

Dauwalder, B. (2011). The roles of fruitless and doublesex in the control of male courtship. Int Rev Neurobiol *99*, 87–105.

Davidowitz, G., and Nijhout, H.F. (2004). The Physiological Basis of Reaction Norms: The Interaction Among Growth Rate, the Duration of Growth and Body Size. Integr Comp Biol *44*, 443–449.

De Virgilio, C., and Loewith, R. (2006). The TOR signalling network from yeast to man. The International Journal of Biochemistry & Cell Biology *38*, 1476–1481.

DeBoer, M.D., Vijayakumar, V., Gong, M., Fowlkes, J.L., Smith, R.M., Ruiz-Perez, F., and Nataro, J.P. (2017). Mice with infectious colitis exhibit linear growth failure and subsequent catch-up growth related to systemic inflammation and IGF-1. Nutrition Research *39*, 34–42.

Delanoue, R., Slaidina, M., and Léopold, P. (2010). The steroid hormone ecdysone controls systemic growth by repressing dMyc function in Drosophila fat cells. Dev Cell *18*, 1012–1021.

Delanoue, R., Meschi, E., Agrawal, N., Mauri, A., Tsatskis, Y., McNeill, H., and Léopold, P. (2016). Drosophila insulin release is triggered by adipose Stunted ligand to brain Methuselah receptor. Science *353*, 1553–1556.

Demir, E., and Dickson, B.J. (2005). fruitless splicing specifies male courtship behavior in Drosophila. Cell *121*, 785–794.

DiAngelo, J.R., Bland, M.L., Bambina, S., Cherry, S., and Birnbaum, M.J. (2009). The immune response attenuates growth and nutrient storage in Drosophila by reducing insulin signaling. PNAS *106*, 20853–20858.

Du, C., McGuffin, M.E., Dauwalder, B., Rabinow, L., and Mattox, W. (1998). Protein phosphorylation plays an essential role in the regulation of alternative splicing and sex determination in Drosophila. Mol Cell *2*, 741–750.

Duffy, J.B. (2002). GAL4 system in drosophila: A fly geneticist's swiss army knife. Genesis *34*, 1–15.

Edgar, B.A. (2006). How flies get their size: genetics meets physiology. Nat Rev Genet *7*, 907–916.

Efstratiadis, A. (2004). Genetics of mouse growth. Int. J. Dev. Biol. *42*, 955–976.

Erkosar, B., Storelli, G., Mitchell, M., Bozonnet, L., Bozonnet, N., and Leulier, F. (2015). Pathogen Virulence Impedes Mutualist-Mediated Enhancement of Host Juvenile Growth via Inhibition of Protein Digestion. Cell Host Microbe *18*, 445–455.

Evans, D.S., and Cline, T.W. (2013). Drosophila switch gene Sex-lethal can bypass its switch-gene target transformer to regulate aspects of female behavior. PNAS *110*, E4474–E4481.

Ewing, A.W. (1961). Body size and courtship behaviour in Drosophila melanogaster. Animal Behaviour *9*, 93–99.

Fain, M.J., and Riddiford, L.M. (1975). Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, manduca sexta (l.). The Biological Bulletin *149*, 506–521.

Fairbairn, D.J. (1997). Allometry for sexual size dimorphism: pattern and process in the coevolution of body size in males and females. Annu Rev Ecol Syst *28*, 659–687.

FAIRBAIRN, D.J. (2007). Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism (Oxford University Press).

FAIRBAIRN, D.J. (2013). Odd Couples: Extraordinary Differences between the Sexes in the Animal Kingdom (Princeton University Press).

Fear, J.M., Arbeitman, M.N., Salomon, M.P., Dalton, J.E., Tower, J., Nuzhdin, S.V., and McIntyre, L.M. (2015). The Wright stuff: reimagining path analysis reveals novel components of the sex determination hierarchy in Drosophila melanogaster. BMC Syst Biol *9*, 53.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., and Schlessinger, J. (1995). The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. EMBO J *14*, 3373–3384.

Flatt, T. (2020). Life-History Evolution and the Genetics of Fitness Components in. Genetics *214*, 3–48.

Frazier, M.R., Woods, H.A., and Harrison, J.F. (2001). Interactive Effects of Rearing Temperature and Oxygen on the Development of Drosophila melanogaster. Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches *74*, 641–650.

Freckleton, R.P., Harvey, P.H., and Pagel, M. (2003). Bergmann's Rule and Body Size in Mammals. The American Naturalist *161*, 821–825.

French, V., Feast, M., and Partridge, L. (1998). Body size and cell size in Drosophila: the developmental response to temperature. Journal of Insect Physiology *44*, 1081–1089.

Frisancho, A.R. (2013). Developmental Functional Adaptation to High Altitude: Review. American Journal of Human Biology *25*, 151–168.

Frisancho, A.R., and Baker, P.T. (1970). Altitude and growth: A study of the patterns of physical growth of a high altitude Peruvian Quechua population. American Journal of Physical Anthropology *32*, 279–292.

Fu, D., Lv, X., Hua, G., He, C., Dong, J., Lele, S.M., Li, D.W.-C., Zhai, Q., Davis, J.S., and Wang, C. (2014). YAP regulates cell proliferation, migration, and steroidogenesis in adult granulosa cell tumors. Endocrine-Related Cancer *21*, 297–310.

Gallio, M., Ofstad, T.A., Macpherson, L.J., Wang, J.W., and Zuker, C.S. (2011). The coding of temperature in the Drosophila brain. Cell *144*, 614–624.

Galloni, M., and Edgar, B.A. (1999). Cell-autonomous and non-autonomous growth-defective mutants of Drosophila melanogaster. Development *126*, 2365–2375.

Gao, X., Neufeld, T.P., and Pan, D. (2000). Drosophila PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways. Dev Biol *221*, 404–418.

Garelli, A., Gontijo, A.M., Miguela, V., Caparros, E., and Dominguez, M. (2012). Imaginal discs secrete insulin-like peptide 8 to mediate plasticity of growth and maturation. Science *336*, 579–582.

Garen, A., Kauvar, L., and Lepesant, J.-A. (1977). Roles of ecdysone in Drosophila development. PNAS *74*, 5099–5103.

Garland, T., and Kelly, S.A. (2006). Phenotypic plasticity and experimental evolution. J Exp Biol *209*, 2344–2361.

Garner, S.R.C., Castellanos, M.C., Baillie, K.E., Lian, T., and Allan, D.W. (2018). female-specific Ilp7 motoneurons are generated by Fruitless-dependent cell death in males and by a double-assurance survival role for Transformer in females. Development *145*.

Geer, E.B., and Shen, W. (2009). Gender differences in insulin resistance, body composition, and energy balance. Gend Med *6 Suppl 1*, 60–75.

Géminard, C., Rulifson, E.J., and Léopold, P. (2009). Remote control of insulin secretion by fat cells in Drosophila. Cell Metab *10*, 199–207.

Gershman, B., Puig, O., Hang, L., Peitzsch, R.M., Tatar, M., and Garofalo, R.S. (2007). High-resolution dynamics of the transcriptional response to nutrition in Drosophila: a key role for dFOXO. Physiological Genomics *29*, 24–34.

Ghosh, A., Rideout, E.J., and Grewal, S.S. (2014). TIF-IA-Dependent Regulation of Ribosome Synthesis in Drosophila Muscle Is Required to Maintain Systemic Insulin Signaling and Larval Growth. PLOS Genetics *10*, e1004750.

Ghosh, S.M., Testa, N.D., and Shingleton, A.W. (2013). Temperature-size rule is mediated by thermal plasticity of critical size in Drosophila melanogaster. Proceedings of the Royal Society B: Biological Sciences *280*, 20130174.

Giannakou, M.E., Goss, M., Jünger, M.A., Hafen, E., Leevers, S.J., and Partridge, L. (2004). Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science *305*, 361.

Gibney, J., Wolthers, T., Johannsson, G., Umpleby, A.M., and Ho, K.K.Y. (2005). Growth hormone and testosterone interact positively to enhance protein and energy metabolism in hypopituitary men. American Journal of Physiology-Endocrinology and Metabolism *289*, E266–E271.

Gingras, A.-C., Raught, B., Gygi, S.P., Niedzwiecka, A., Miron, M., Burley, S.K., Polakiewicz, R.D., Wyslouch-Cieszynska, A., Aebersold, R., and Sonenberg, N. (2001). Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev. *15*, 2852–2864.

Giustina, A., Scalvini, T., Tassi, C., Desenzani, P., Poiesi, C., Wehrenberg, W.B., Rogol, A.D., and Veldhuis, J.D. (1997). Maturation of the Regulation of Growth Hormone Secretion in Young Males with Hypogonadotropic Hypogonadism Pharmacologically Exposed to Progressive Increments in Serum Testosterone*. The Journal of Clinical Endocrinology & Metabolism *82*, 1210–1219.

G.J Bashaw, and Baker, B.S. (1996). Dosage compensation and chromatin structure in Drosophila. Current Opinion in Genetics & Development *6*, 496–501.

Goberdhan, D.C., Paricio, N., Goodman, E.C., Mlodzik, M., and Wilson, C. (1999). Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. Genes Dev *13*, 3244–3258.

Gokhale, R.H., and Shingleton, A.W. (2015). Size control: the developmental physiology of body and organ size regulation. WIREs Developmental Biology *4*, 335–356.

Goldman, T.D., and Arbeitman, M.N. (2007). Genomic and functional studies of Drosophila sex hierarchy regulated gene expression in adult head and nervous system tissues. PLoS Genet *3*, e216.

Goodwin, S.F., Taylor, B.J., Villella, A., Foss, M., Ryner, L.C., Baker, B.S., and Hall, J.C. (2000). Aberrant splicing and altered spatial expression patterns in fruitless mutants of Drosophila melanogaster. Genetics *154*, 725–745.

Graze, R.M., Tzeng, R.Y., Howard, T.S., and Arbeitman, M.N. (2018). Perturbation of IIS/TOR signaling alters the landscape of sex-differential gene expression in Drosophila. BMC Genomics *19*, 893.

Green, D.A., and Extavour, C.G. (2014). Insulin signalling underlies both plasticity and divergence of a reproductive trait in Drosophila. Proc Biol Sci *281*, 20132673.

Grewal, S.S. (2009). Insulin/TOR signaling in growth and homeostasis: a view from the fly world. Int J Biochem Cell Biol *41*, 1006–1010.

Grewal, S.S., Li, L., Orian, A., Eisenman, R.N., and Edgar, B.A. (2005). Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. Nature Cell Biology *7*, 295–302.

Grewal, S.S., Evans, J.R., and Edgar, B.A. (2007). Drosophila TIF-IA is required for ribosome synthesis and cell growth and is regulated by the TOR pathway. J Cell Biol *179*, 1105–1113.

Grmai, L., Hudry, B., Miguel-Aliaga, I., and Bach, E.A. (2018). Chinmo prevents transformer alternative splicing to maintain male sex identity. PLoS Genet *14*, e1007203.

Grönke, S., Clarke, D.F., Broughton, S., Andrews, T.D., and Partridge, L. (2010). Molecular evolution and functional characterization of Drosophila insulin-like peptides. PLoS Genet *6*, e1000857.

Guertin, D.A., Guntur, K.V.P., Bell, G.W., Thoreen, C.C., and Sabatini, D.M. (2006). Functional Genomics Identifies TOR-Regulated Genes that Control Growth and Division. Current Biology *16*, 958–970.

Hall, D.J., Grewal, S.S., de la Cruz, A.F.A., and Edgar, B.A. (2007). Rheb-TOR signaling promotes protein synthesis, but not glucose or amino acid import, in Drosophila. BMC Biology *5*, 10.

Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in Drosophila. Nature *454*, 217–220.

Hämäläinen, R.H., Manninen, T., Koivumäki, H., Kislin, M., Otonkoski, T., and Suomalainen, A. (2013). Tissue- and cell-type–specific manifestations of heteroplasmic mtDNA 3243A>G mutation in human induced pluripotent stem cell-derived disease model. PNAS *110*, E3622–E3630.

Harrison, J.F., Shingleton, A.W., and Callier, V. (2015). Stunted by Developing in Hypoxia: Linking Comparative and Model Organism Studies. Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches *88*, 455–470.

Harshman, L.G., Hoffmann, A.A., and Prout, T. (1988). Environmental Effects on Remating in Drosophila Melanogaster. Evolution *42*, 312–321.

Harvey, K., and Tapon, N. (2007). The Salvador–Warts–Hippo pathway — an emerging tumour-suppressor network. Nature Reviews Cancer *7*, 182–191.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. (2003). The Drosophila Mst Ortholog, hippo, Restricts Growth and Cell Proliferation and Promotes Apoptosis. Cell *114*, 457–467.

Haselton, A., Sharmin, E., Schrader, J., Sah, M., Poon, P., and Fridell, Y.-W.C. (2010). Partial ablation of adult Drosophilainsulin-producing neurons modulates glucose homeostasis and extends life span without insulin resistance. Cell Cycle *9*, 3135–3143.

Havula, E., and Hietakangas, V. (2012). Glucose sensing by ChREBP/MondoA-Mlx transcription factors. Semin Cell Dev Biol *23*, 640–647.

Havula, E., Teesalu, M., Hyötyläinen, T., Seppälä, H., Hasygar, K., Auvinen, P., Orešič, M., Sandmann, T., and Hietakangas, V. (2013). Mondo/ChREBP-Mlx-

regulated transcriptional network is essential for dietary sugar tolerance in Drosophila. PLoS Genet *9*, e1003438.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev. *18*, 1926–1945.

van Heemst, D., Beekman, M., Mooijaart, S.P., Heijmans, B.T., Brandt, B.W., Zwaan, B.J., Slagboom, P.E., and Westendorp, R.G. (2005). Reduced insulin/IGF-1 signalling and human longevity. Aging Cell *4*, 79–85.

Heinrichs, V., Ryner, L.C., and Baker, B.S. (1998). Regulation of Sex-Specific Selection offruitless 5′ Splice Sites by transformerand transformer-2. Molecular and Cellular Biology *18*, 450–458.

Hietakangas, V., and Cohen, S.M. (2009). Regulation of tissue growth through nutrient sensing. Annu Rev Genet *43*, 389–410.

Hirabayashi, S., Baranski, T.J., and Cagan, R.L. (2013). Transformed Drosophila Cells Evade Diet-Mediated Insulin Resistance through Wingless Signaling. Cell *154*, 664–675.

Hodin, J., and Riddiford, L.M. (2000). Different mechanisms underlie phenotypic plasticity and interspecific variation for a reproductive character in drosophilids (Insecta: Diptera). Evolution *54*, 1638–1653.

Holz, M.K., Ballif, B.A., Gygi, S.P., and Blenis, J. (2005). mTOR and S6K1 Mediate Assembly of the Translation Preinitiation Complex through Dynamic Protein Interchange and Ordered Phosphorylation Events. Cell *123*, 569–580.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Géloën, A., Even, P.C., Cervera, P., and Le Bouc, Y. (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature *421*, 182–187.

Honegger, B., Galic, M., Köhler, K., Wittwer, F., Brogiolo, W., Hafen, E., and Stocker, H. (2008). Imp-L2, a putative homolog of vertebrate IGF-binding protein 7, counteracts insulin signaling in Drosophila and is essential for starvation resistance. J Biol *7*, 10.

Honěk, A. (1993). Intraspecific Variation in Body Size and Fecundity in Insects: A General Relationship. Oikos *66*, 483–492.

Honjoh, S., Ihara, A., Kajiwara, Y., Yamamoto, T., and Nishida, E. (2017). The Sexual Dimorphism of Dietary Restriction Responsiveness in Caenorhabditis elegans. Cell Reports *21*, 3646–3652.

Hoopes, B.C., Rimbault, M., Liebers, D., Ostrander, E.A., and Sutter, N.B. (2012). The insulin-like growth factor 1 receptor (IGF1R) contributes to reduced size in dogs. Mamm Genome *23*, 780–790.

Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H., and Shimura, Y. (1991). Control of doublesex alternative splicing by transformer and transformer-2 in Drosophila. Science *252*, 833–836.

Hotzi, B., Kosztelnik, M., Hargitai, B., Takács-Vellai, K., Barna, J., Bördén, K., Málnási-Csizmadia, A., Lippai, M., Ortutay, C., Bacquet, C., et al. (2018). Sex-specific regulation of aging in Caenorhabditis elegans. Aging Cell *17*.

Hsu, H.J., and Drummond-Barbosa, D. (2009). Insulin levels control female germline stem cell maintenance via the niche in Drosophila. Proc Natl Acad Sci U S A *106*, 1117–1121.

Hsu, H.J., LaFever, L., and Drummond-Barbosa, D. (2008). Diet controls normal and tumorous germline stem cells via insulin-dependent and -independent mechanisms in Drosophila. Dev Biol *313*, 700–712.

Hudry, B., Khadayate, S., and Miguel-Aliaga, I. (2016). The sexual identity of adult intestinal stem cells controls organ size and plasticity. Nature *530*, 344–348.

Hudry, B., de Goeij, E., Mineo, A., Gaspar, P., Hadjieconomou, D., Studd, C., Mokochinski, J.B., Kramer, H.B., Plaçais, P.Y., Preat, T., et al. (2019). Sex Differences in Intestinal Carbohydrate Metabolism Promote Food Intake and Sperm Maturation. Cell *178*, 901-918.e16.

Hwangbo, D.S., Gersham, B., Tu, M.-P., Palmer, M., and Tatar, M. (2004). Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature *429*, 562–566.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Curr Biol *12*, 1293–1300.

Inoue, K., Hoshijima, K., Sakamoto, H., and Shimura, Y. (1990). Binding of the Drosophila sex-lethal gene product to the alternative splice site of transformer primary transcript. Nature *344*, 461–463.

Ito, H., Fujitani, K., Usui, K., Shimizu-Nishikawa, K., Tanaka, S., and Yamamoto, D. (1996). Sexual orientation in Drosophila is altered by the satori mutation in the sex-determination gene fruitless that encodes a zinc finger protein with a BTB domain. Proc Natl Acad Sci U S A *93*, 9687–9692.

Jacobs, H.T., George, J., and Kemppainen, E. (2020). Regulation of growth in Drosophila melanogaster: the roles of mitochondrial metabolism. J Biochem *167*, 267–277.

Jensen, K., McClure, C., Priest, N.K., and Hunt, J. (2015). Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in Drosophila melanogaster. Aging Cell *14*, 605–615.

Jünger, M.A., Rintelen, F., Stocker, H., Wasserman, J.D., Végh, M., Radimerski, T., Greenberg, M.E., and Hafen, E. (2003). The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol *2*, 20.

Kane, A.E., Sinclair, D.A., Mitchell, J.R., and Mitchell, S.J. (2018). Sex differences in the response to dietary restriction in rodents. Curr Opin Physiol *6*, 28–34.

Kang, P., Chang, K., Liu, Y., Bouska, M., Birnbaum, A., Karashchuk, G., Thakore, R., Zheng, W., Post, S., Brent, C.S., et al. (2017). Drosophila Kruppel homolog 1 represses lipolysis through interaction with dFOXO. Sci Rep *7*, 16369.

Kao, S.H., Tseng, C.Y., Wan, C.L., Su, Y.H., Hsieh, C.C., Pi, H., and Hsu, H.J. (2015). Aging and insulin signaling differentially control normal and tumorous germline stem cells. Aging Cell *14*, 25–34.

Kaplan, S.A., and Cohen, P. (2007). REVIEW: The Somatomedin Hypothesis 2007: 50 Years Later. The Journal of Clinical Endocrinology & Metabolism *92*, 4529–4535.

Karan, D., Morin, J.P., Moreteau, B., and David, J.R. (1998). Body size and developmental temperature in drosophila melanogaster: analysis of body weight reaction norm. Journal of Thermal Biology *23*, 301–309.

Kautzky-Willer, A., Harreiter, J., and Pacini, G. (2016). Sex and Gender Differences in Risk, Pathophysiology and Complications of Type 2 Diabetes Mellitus. Endocr Rev *37*, 278–316.

Keenan, B.S., Richards, G.E., Ponder, S.W., Dallas, J.S., Nagamani, M., and Smith, E.R. (1993). Androgen-stimulated pubertal growth: the effects of testosterone and dihydrotestosterone on growth hormone and insulin-like growth factor-I in the treatment of short stature and delayed puberty. The Journal of Clinical Endocrinology & Metabolism *76*, 996–1001.

Kidd, T., Abu-Shumays, R., Katzen, A., Sisson, J.C., Jiménez, G., Pinchin, S., Sullivan, W., and Ish-Horowicz, D. (2005). The epsilon-subunit of mitochondrial ATP synthase is required for normal spindle orientation during the Drosophila embryonic divisions. Genetics *170*, 697–708.

Killip, L.E., and Grewal, S.S. (2012). DREF is required for cell and organismal growth in Drosophila and functions downstream of the nutrition/TOR pathway. Developmental Biology *371*, 191–202.

Kim, J., and Neufeld, T.P. (2015). Dietary sugar promotes systemic TOR activation in Drosophila through AKH-dependent selective secretion of Dilp3. Nat Commun *6*, 6846.

Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T.P., and Guan, K.-L. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. Nature Cell Biology *10*, 935–945.

Kimura, K., Hachiya, T., Koganezawa, M., Tazawa, T., and Yamamoto, D. (2008). Fruitless and Doublesex Coordinate to Generate Male-Specific Neurons that Can Initiate Courtship. Neuron *59*, 759–769.

Kohtz, J.D., Jamison, S.F., Will, C.L., Zuo, P., Lührmann, R., Garcia-Blanco, M.A., and Manley, J.L. (1994). Protein–protein interactions and 5'-splice-site recognition in mammalian mRNA precursors. Nature *368*, 119–124.

Kondo, H., Ino, M., Suzuki, A., Ishizaki, H., and Iwami, M. (1996). Multiple Gene Copies for Bombyxin, an Insulin-related Peptide of the SilkmothBombyx mori: Structural Signs for Gene Rearrangement and Duplication Responsible for

Generation of Multiple Molecular Forms of Bombyxin. Journal of Molecular Biology *259*, 926–937.

Kopp, A., Duncan, I., and Carroll, S.B. (2000). Genetic control and evolution of sexually dimorphic characters in Drosophila. Nature *408*, 553–559.

Koyama, T., and Mirth, C.K. (2016). Growth-Blocking Peptides As Nutrition-Sensitive Signals for Insulin Secretion and Body Size Regulation. PLoS Biol *14*, e1002392.

Koyama, T., and Mirth, C.K. (2018). Unravelling the diversity of mechanisms through which nutrition regulates body size in insects. Curr Opin Insect Sci *25*, 1–8.

Kramer, J.M., Davidge, J.T., Lockyer, J.M., and Staveley, B.E. (2003). Expressionof Drosophila FOXO regulates growth and can phenocopy starvation. BMC Developmental Biology *3*, 5.

Lackey, D.E., and Olefsky, J.M. (2016). Regulation of metabolism by the innate immune system. Nature Reviews Endocrinology *12*, 15–28.

LaFever, L., and Drummond-Barbosa, D. (2005). Direct control of germline stem cell division and cyst growth by neural insulin in Drosophila. Science *309*, 1071–1073.

Lai, S.-L., and Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in Drosophila. Nature Neuroscience *9*, 703–709.

Laron, Z., Pertzelan, A., and Mannheimer, S. (1966). Genetic pituitary dwarfism with high serum concentation of growth hormone--a new inborn error of metabolism? Isr J Med Sci *2*, 152–155.

Lee, B., Barretto, E.C., and Grewal, S.S. (2019). TORC1 modulation in adipose tissue is required for organismal adaptation to hypoxia in Drosophila. Nature Communications *10*, 1878.

Lee, K.-S., Kwon, O.-Y., Lee, J.H., Kwon, K., Min, K.-J., Jung, S.-A., Kim, A.-K., You, K.-H., Tatar, M., and Yu, K. (2008). Drosophila short neuropeptide F signalling regulates growth by ERK-mediated insulin signalling. Nature Cell Biology *10*, 468–475.

Leevers, S.J., Weinkove, D., MacDougall, L.K., Hafen, E., and Waterfield, M.D. (1996). The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. EMBO J *15*, 6584–6594.

Lefranc, A., and Bundgaard, J. (2000). The Influence of Male and Female Body Size on Copulation Duration and Fecundity in Drosophila Melanogaster. Hereditas *132*, 243–247.

Lessel, C.E., Parkes, T.L., Dickinson, J., and Merritt, T.J.S. (8). Sex and Genetic Background Influence Superoxide Dismutase (cSOD)-Related Phenotypic Variation in. G3 (Bethesda) *7*, 2651–2664.

Lewis, E.B. (1960). A new standard food medium. Drosophila Information Service *34*, 117–118.

Li, Q., and Gong, Z. (2015). Cold-sensing regulates Drosophila growth through insulin-producing cells. Nature Communications *6*, 10083.

Li, M.V., Chang, B., Imamura, M., Poungvarin, N., and Chan, L. (2006). Glucose-Dependent Transcriptional Regulation by an Evolutionarily Conserved Glucose-Sensing Module. Diabetes *55*, 1179–1189.

Liao, S., Post, S., Lehmann, P., Veenstra, J.A., Tatar, M., and Nässel, D.R. (2020). Regulatory Roles of Drosophila Insulin-Like Peptide 1 (DILP1) in Metabolism Differ in Pupal and Adult Stages. Front Endocrinol (Lausanne) *11*.

Lin, K.Y., and Hsu, H.J. (2020). Regulation of adult female germline stem cells by nutrient-responsive signaling. Curr Opin Insect Sci *37*, 16–22.

Link, J.C., Wiese, C.B., Chen, X., Avetisyan, R., Ronquillo, E., Ma, F., Guo, X., Yao, J., Allison, M., Chen, Y.-D.I., et al. (2020). X chromosome dosage of histone demethylase KDM5C determines sex differences in adiposity. J Clin Invest *130*, 5688–5702.

Linneweber, G.A., Jacobson, J., Busch, K.E., Hudry, B., Christov, C.P., Dormann, D., Yuan, M., Otani, T., Knust, E., de Bono, M., et al. (2014). Neuronal control of metabolism through nutrient-dependent modulation of tracheal branching. Cell *156*, 69–83.

Liu, Y., Liao, S., Veenstra, J.A., and Nässel, D.R. (2016). Drosophila insulin-like peptide 1 (DILP1) is transiently expressed during non-feeding stages and reproductive dormancy. Sci Rep *6*, 26620.

Lourido, F., Quenti, D., Salgado-Canales, D., and Tobar, N. (2021). Domeless receptor loss in fat body tissue reverts insulin resistance induced by a high-sugar diet in Drosophila melanogaster. Sci Rep *11*, 3263.

Lupu, F., Terwilliger, J.D., Lee, K., Segre, G.V., and Efstratiadis, A. (2001). Roles of Growth Hormone and Insulin-like Growth Factor 1 in Mouse Postnatal Growth. Developmental Biology *229*, 141–162.

Macotela, Y., Boucher, J., Tran, T.T., and Kahn, C.R. (2009). Sex and depot differences in adipocyte insulin sensitivity and glucose metabolism. Diabetes *58*, 803–812.

Magwere, T., Chapman, T., and Partridge, L. (2004). Sex differences in the effect of dietary restriction on life span and mortality rates in female and male Drosophila melanogaster. J Gerontol A Biol Sci Med Sci *59*, 3–9.

Manière, G., Ziegler, A.B., Geillon, F., Featherstone, D.E., and Grosjean, Y. (2016). Direct Sensing of Nutrients via a LAT1-like Transporter in Drosophila Insulin-Producing Cells. Cell Rep *17*, 137–148.

Manolakou, P., Lavranos, G., and Angelopoulou, R. (2006). Molecular patterns of sex determination in the animal kingdom: a comparative study of the biology of reproduction. Reproductive Biology and Endocrinology *4*, 59.

Marshall, L., Rideout, E.J., and Grewal, S.S. (2012a). Nutrient/TOR-dependent regulation of RNA polymerase III controls tissue and organismal growth in Drosophila. EMBO J *31*, 1916–1930.

Marshall, L., Rideout, E.J., and Grewal, S.S. (2012b). Nutrient/TOR-dependent regulation of RNA polymerase III controls tissue and organismal growth in Drosophila. EMBO J *31*, 1916–1930.

Massey, J., and Wittkopp, P.J. (2016). The genetic basis of pigmentation differences within and between Drosophila species. Curr Top Dev Biol *119*, 27–61.

Mathews, K.W., Cavegn, M., and Zwicky, M. (2017). Sexual Dimorphism of Body Size Is Controlled by Dosage of the X-Chromosomal Gene Myc and by the Sex-Determining Gene tra in Drosophila. Genetics *205*, 1215–1228.

Matsuo, T., Kusunoki, Y., Katsuno, T., Ikawa, T., Akagami, T., Murai, K., Miuchi, M., Miyagawa, J., and Namba, M. (2014). Response of incretins (GIP and GLP-1) to an

oral glucose load in female and male subjects with normal glucose tolerance. Diabetes Research and Clinical Practice *106*, e25–e29.

Mattila, J., and Hietakangas, V. (2017). Regulation of Carbohydrate Energy Metabolism in Drosophila melanogaster. Genetics *207*, 1231–1253.

Mattila, J., Havula, E., Suominen, E., Teesalu, M., Surakka, I., Hynynen, R., Kilpinen, H., Väänänen, J., Hovatta, I., Käkelä, R., et al. (2015). Mondo-Mlx Mediates Organismal Sugar Sensing through the Gli-Similar Transcription Factor Sugarbabe. Cell Rep *13*, 350–364.

Mauvais-Jarvis, F. (2018). Gender differences in glucose homeostasis and diabetes. Physiology & Behavior *187*, 20–23.

McBrayer, Z., Ono, H., Shimell, M., Parvy, J.-P., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-Villemant, C., Gilbert, L.I., and O'Connor, M.B. (2007). Prothoracicotropic Hormone Regulates Developmental Timing and Body Size in Drosophila. Developmental Cell *13*, 857–871.

McDonald, J.M.C., Nabili, P., Thorsen, L., Jeon, S., and Shingleton, A. (2020). Sex-specific plasticity and the nutritional geometry of insulin-signaling gene expression in Drosophila melanogaster. BioRxiv 2020.11.16.385708.

Mehran, A.E., Templeman, N.M., Brigidi, G.S., Lim, G.E., Chu, K.-Y., Hu, X., Botezelli, J.D., Asadi, A., Hoffman, B.G., Kieffer, T.J., et al. (2012). Hyperinsulinemia Drives Diet-Induced Obesity Independently of Brain Insulin Production. Cell Metabolism *16*, 723–737.

Mendes, C.C., and Mirth, C.K. (2016). Stage-Specific Plasticity in Ovary Size Is Regulated by Insulin/Insulin-Like Growth Factor and Ecdysone Signaling in Drosophila. Genetics *202*, 703–719.

Meschi, E., Léopold, P., and Delanoue, R. (2019). An EGF-Responsive Neural Circuit Couples Insulin Secretion with Nutrition in Drosophila. Dev Cell *48*, 76-86.e5.

Michael, M.D., Kulkarni, R.N., Postic, C., Previs, S.F., Shulman, G.I., Magnuson, M.A., and Kahn, C.R. (2000). Loss of Insulin Signaling in Hepatocytes Leads to Severe Insulin Resistance and Progressive Hepatic Dysfunction. Molecular Cell *6*, 87–97.

Miguel-Aliaga, I., Jasper, H., and Lemaitre, B. (2018). Anatomy and Physiology of the Digestive Tract of Drosophila melanogaster. Genetics *210*, 357–396.

Millington, J.W., and Rideout, E.J. (2018). Sex differences in Drosophila development and physiology. Current Opinion in Physiology *6*, 46–56.

Millington, J.W., Brownrigg, G.P., Basner-Collins, P.J., Sun, Z., and Rideout, E.J. (2021a). Genetic manipulation of insulin/insulin-like growth factor signaling pathway activity has sex-biased effects on Drosophila body size. G3 Genes|Genomes|Genetics.

Millington, J.W., Brownrigg, G.P., Chao, C., Sun, Z., Basner-Collins, P.J., Wat, L.W., Hudry, B., Miguel-Aliaga, I., and Rideout, E.J. (2021b). Female-biased upregulation of insulin pathway activity mediates the sex difference in Drosophila body size plasticity. ELife *10*, e58341.

Miron, M., Verdú, J., Lachance, P.E.D., Birnbaum, M.J., Lasko, P.F., and Sonenberg, N. (2001). The translational inhibitor 4E-BP is an effector of PI(3)K/Akt signalling and cell growth in Drosophila. Nature Cell Biology *3*, 596–601.

Mirth, C.K., and Piper, M.D. (2017). Matching complex dietary landscapes with the signalling pathways that regulate life history traits. Curr Opin Genet Dev *47*, 9–16.

Mirth, C.K., and Riddiford, L.M. (2007). Size assessment and growth control: how adult size is determined in insects. Bioessays *29*, 344–355.

Mirth, C.K., and Shingleton, A.W. (2012). Integrating body and organ size in Drosophila: recent advances and outstanding problems. Front Endocrinol (Lausanne) *3*, 49.

Mirth, C.K., Tang, H.Y., Makohon-Moore, S.C., Salhadar, S., Gokhale, R.H., Warner, R.D., Koyama, T., Riddiford, L.M., and Shingleton, A.W. (2014). Juvenile hormone regulates body size and perturbs insulin signaling in Drosophila. Proc Natl Acad Sci U S A *111*, 7018–7023.

Modig, A.O. (1996). Effects of body size and harem size on male reproductive behaviour in the southern elephant seal. Animal Behaviour *51*, 1295–1306.

Moeller, M.E., Nagy, S., Gerlach, S.U., Soegaard, K.C., Danielsen, E.T., Texada, M.J., and Rewitz, K.F. (2017). Warts Signaling Controls Organ and Body Growth through Regulation of Ecdysone. Current Biology *27*, 1652-1659.e4.

Montagne, J., Stewart, M.J., Stocker, H., Hafen, E., Kozma, S.C., and Thomas, G. (1999). Drosophila S6 Kinase: A Regulator of Cell Size. Science *285*, 2126–2129.

Mukherjee, S., and Duttaroy, A. (2013). Spargel/dPGC-1 Is a New Downstream Effector in the Insulin–TOR Signaling Pathway in Drosophila. Genetics *195*, 433–441.

Mukherjee, S., Basar, M.A., Davis, C., and Duttaroy, A. (2014). Emerging functional similarities and divergences between Drosophila Spargel/dPGC-1 and mammalian PGC-1 protein. Front. Genet. *5*.

Murillo-Maldonado, J.M., Sánchez-Chávez, G., Salgado, L.M., Salceda, R., and Riesgo-Escovar, J.R. (2011). Drosophila insulin pathway mutants affect visual physiology and brain function besides growth, lipid, and carbohydrate metabolism. Diabetes *60*, 1632–1636.

Musselman, L.P., and Kühnlein, R.P. (2018). Drosophila as a model to study obesity and metabolic disease. J Exp Biol *221*.

Musselman, L.P., Fink, J.L., Narzinski, K., Ramachandran, P.V., Hathiramani, S.S., Cagan, R.L., and Baranski, T.J. (2011). A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila. Disease Models & Mechanisms *4*, 842–849.

Musselman, L.P., Fink, J.L., Grant, A.R., Gatto, J.A., Tuthill, B.F., and Baranski, T.J. (2018). A Complex Relationship between Immunity and Metabolism in Drosophila Diet-Induced Insulin Resistance. Molecular and Cellular Biology *38*.

Nässel, D.R., and Vanden Broeck, J. (2016). Insulin/IGF signaling in Drosophila and other insects: factors that regulate production, release and post-release action of the insulin-like peptides. Cell Mol Life Sci *73*, 271–290.

Neel, J.V. (1940). The Interrelations of Temperature, Body Size, and Character Expression in Drosophila Melanogaster. Genetics *25*, 225–250.

Neville, M.C., Nojima, T., Ashley, E., Parker, D.J., Walker, J., Southall, T., Van de Sande, B., Marques, A.C., Fischer, B., Brand, A.H., et al. (2014). Male-specific fruitless isoforms target neurodevelopmental genes to specify a sexually dimorphic nervous system. Curr Biol *24*, 229–241.

Nijhout, H.F. (1976). The rôle of ecdysone in pupation of Manduca sexta. Journal of Insect Physiology *22*, 453–463.

Nijhout, H.F. (2003). The control of body size in insects. Dev Biol *261*, 1–9.

Nijhout, H.F., and Williams, C.M. (1974). Control of moulting and metamorphosis in the tobacco hornworm, Manduca sexta (L.): cessation of juvenile hormone secretion as a trigger for pupation. J Exp Biol *61*, 493–501.

Nijhout, H.F., Riddiford, L.M., Mirth, C., Shingleton, A.W., Suzuki, Y., and Callier, V. (2014). The developmental control of size in insects. WIREs Developmental Biology *3*, 113–134.

Nojima, T., Neville, M.C., and Goodwin, S.F. (2014). Fruitless isoforms and target genes specify the sexually dimorphic nervous system underlying Drosophila reproductive behavior. Fly (Austin) *8*, 95–100.

Nowak, K., Seisenbacher, G., Hafen, E., and Stocker, H. (2013). Nutrient restriction enhances the proliferative potential of cells lacking the tumor suppressor PTEN in mitotic tissues. Elife *2*, e00380.

Nunney, L., and Cheung, W. (1997). The Effect of Temperature on Body Size and Fecundity in Female Drosophila Melanogaster: Evidence for Adaptive Plasticity. Evolution *51*, 1529–1535.

O'Brien, L.E., Soliman, S.S., Li, X., and Bilder, D. (2011). Altered Modes of Stem Cell Division Drive Adaptive Intestinal Growth. Cell *147*, 603–614.

Oh, Y., Lai, J.S.-Y., Mills, H.J., Erdjument-Bromage, H., Giammarinaro, B., Saadipour, K., Wang, J.G., Abu, F., Neubert, T.A., and Suh, G.S.B. (2019). A glucose-sensing neuron pair regulates insulin and glucagon in Drosophila. Nature *574*, 559–564.

Okamoto, N., and Nishimura, T. (2015). Signaling from Glia and Cholinergic Neurons Controls Nutrient-Dependent Production of an Insulin-like Peptide for Drosophila Body Growth. Dev Cell *35*, 295–310.

Okamoto, N., Yamanaka, N., Yagi, Y., Nishida, Y., Kataoka, H., O'Connor, M.B., and Mizoguchi, A. (2009). A fat body-derived IGF-like peptide regulates postfeeding growth in Drosophila. Dev Cell *17*, 885–891.

Okamoto, N., Nakamori, R., Murai, T., Yamauchi, Y., Masuda, A., and Nishimura, T. (2013). A secreted decoy of InR antagonizes insulin/IGF signaling to restrict body growth in Drosophila. Genes Dev *27*, 87–97.

Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M., and Hafen, E. (2002). The Drosophila insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels. Development *129*, 4103–4109.

de Onis, M., and Branca, F. (2016). Childhood stunting: a global perspective. Maternal & Child Nutrition *12*, 12–26.

Orian, A., Steensel, B. van, Delrow, J., Bussemaker, H.J., Li, L., Sawado, T., Williams, E., Loo, L.W.M., Cowley, S.M., Yost, C., et al. (2003). Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. Genes Dev. *17*, 1101–1114.

Pan, D. (2010). The hippo signaling pathway in development and cancer. Dev Cell *19*, 491–505.

Park, S., Alfa, R.W., Topper, S.M., Kim, G.E.S., Kockel, L., and Kim, S.K. (2014). A Genetic Strategy to Measure Circulating Drosophila Insulin Reveals Genes Regulating Insulin Production and Secretion. PLOS Genetics *10*, e1004555.

Partridge, L., and Farquhar, M. (1983). Lifetime mating success of male fruitflies (Drosophila melanogaster) is related to their size. Animal Behaviour *31*, 871–877.

Partridge, L., Ewing, A., and Chandler, A. (1987). Male size and mating success in Drosophila melanogaster: the roles of male and female behaviour. Animal Behaviour *35*, 555–562.

Partridge, L., Barrie, B., Fowler, K., and French, V. (1994). EVOLUTION AND DEVELOPMENT OF BODY SIZE AND CELL SIZE IN DROSOPHILA MELANOGASTER IN RESPONSE TO TEMPERATURE. Evolution *48*, 1269–1276.

Partridge, L., Alic, N., Bjedov, I., and Piper, M.D.W. (2011). Ageing in Drosophila: The role of the insulin/Igf and TOR signalling network. Experimental Gerontology *46*, 376–381.

Pasco, M.Y., and Léopold, P. (2012). High Sugar-Induced Insulin Resistance in Drosophila Relies on the Lipocalin Neural Lazarillo. PLOS ONE *7*, e36583.

Patel, P.H., Thapar, N., Guo, L., Martinez, M., Maris, J., Gau, C.-L., Lengyel, J.A., and Tamanoi, F. (2003). Drosophila Rheb GTPase is required for cell cycle progression and cell growth. Journal of Cell Science *116*, 3601–3610.

Pavlou, H.J., Lin, A.C., Neville, M.C., Nojima, T., Diao, F., Chen, B.E., White, B.H., and Goodwin, S.F. (2016). Neural circuitry coordinating male copulation. Elife *5*.

Pende, M., Um, S.H., Mieulet, V., Sticker, M., Goss, V.L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S.C., and Thomas, G. (2004). S6K1−/−/S6K2−/− Mice Exhibit Perinatal Lethality and Rapamycin-Sensitive 5′-Terminal Oligopyrimidine mRNA Translation and Reveal a Mitogen-Activated Protein Kinase-Dependent S6 Kinase Pathway. Molecular and Cellular Biology *24*, 3112–3124.

Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.-P., Li, Y., Dauphin-Villemant, C., and O'Connor, M.B. (2003). Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. PNAS *100*, 13773–13778.

von Philipsborn, A.C., Jörchel, S., Tirian, L., Demir, E., Morita, T., Stern, D.L., and Dickson, B.J. (2014). Cellular and behavioral functions of fruitless isoforms in Drosophila courtship. Curr Biol *24*, 242–251.

Pierce, S.B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S.A., Buchman, A.R., Ferguson, K.C., Heller, J., Platt, D.M., Pasquinelli, A.A., et al. (2001). Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. Genes Dev. *15*, 672–686.

Poltilove, R.M., Jacobs, A.R., Haft, C.R., Xu, P., and Taylor, S.I. (2000). Characterization of Drosophila insulin receptor substrate. J Biol Chem *275*, 23346–23354.

Pomatto, L.C.D., Carney, C., Shen, B., Wong, S., Halaszynski, K., Salomon, M.P., Davies, K.J.A., and Tower, J. (2017). The Mitochondrial Lon Protease Is Required for Age-Specific and Sex-Specific Adaptation to Oxidative Stress. Curr Biol *27*, 1–15.

Port, F., Chen, H.-M., Lee, T., and Bullock, S.L. (2014). Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. PNAS *111*, E2967–E2976.

Port, F., Strein, C., Stricker, M., Rauscher, B., Heigwer, F., Zhou, J., Beyersdörffer, C., Frei, J., Hess, A., Kern, K., et al. (2020). A large-scale resource for tissue-specific CRISPR mutagenesis in Drosophila. ELife *9*, e53865.

Post, S., Karashchuk, G., Wade, J.D., Sajid, W., De Meyts, P., and Tatar, M. (2018). Insulin-Like Peptides DILP2 and DILP5 Differentially Stimulate Cell Signaling and Glycogen Phosphorylase to Regulate Longevity. Front Endocrinol (Lausanne) *9*, 245.

Post, S., Liao, S., Yamamoto, R., Veenstra, J.A., Nässel, D.R., and Tatar, M. (2019). Drosophila insulin-like peptide dilp1 increases lifespan and glucagon-like Akh expression epistatic to dilp2. Aging Cell *18*, e12863.

Potter, C.J., Tasic, B., Russler, E.V., Liang, L., and Luo, L. (2010). The Q System: A Repressible Binary System for Transgene Expression, Lineage Tracing and Mosaic Analysis. Cell *141*, 536–548.

Prenter, J., Elwood, R.W., and Montgomery, W.I. (1999). SEXUAL SIZE DIMORPHISM AND REPRODUCTIVE INVESTMENT BY FEMALE SPIDERS: A COMPARATIVE ANALYSIS. Evolution *53*, 1987–1994.

Puig, O., and Tjian, R. (2005). Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. Genes Dev *19*, 2435–2446.

Puig, O., Marr, M.T., Ruhf, M.L., and Tjian, R. (2003). Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev. *17*, 2006–2020.

Raisin, S., Pantalacci, S., Breittmayer, J.-P., and Léopold, P. (2003). A new genetic locus controlling growth and proliferation in Drosophila melanogaster. Genetics *164*, 1015–1025.

Rajan, A., and Perrimon, N. (2012). Drosophila cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion. Cell *151*, 123–137.

Raubenheimer, D., Simpson, S.J., and Mayntz, D. (2009). Nutrition, ecology and nutritional ecology: toward an integrated framework. Functional Ecology *23*, 4–16.

Raubenheimer, D., Rothman, J.M., Pontzer, H., and Simpson, S.J. (2014). Macronutrient contributions of insects to the diets of hunter–gatherers: A geometric analysis. Journal of Human Evolution *71*, 70–76.

Regan, J.C., and Partridge, L. (2013). Gender and longevity: why do men die earlier than women? Comparative and experimental evidence. Best Pract Res Clin Endocrinol Metab *27*, 467–479.

Regan, J.C., Khericha, M., Dobson, A.J., Bolukbasi, E., Rattanavirotkul, N., and Partridge, L. (2016). Sex difference in pathology of the ageing gut mediates the greater response of female lifespan to dietary restriction. Elife *5*, e10956.

Reis, T. (2016). Effects of Synthetic Diets Enriched in Specific Nutrients on Drosophila Development, Body Fat, and Lifespan. PLOS ONE *11*, e0146758.

Reiter, L.T., Potocki, L., Chien, S., Gribskov, M., and Bier, E. (2001). A Systematic Analysis of Human Disease-Associated Gene Sequences In Drosophila melanogaster. Genome Res *11*, 1114–1125.

Rera, M., Bahadorani, S., Cho, J., Koehler, C.L., Ulgherait, M., Hur, J.H., Ansari, W.S., Lo, T., Jones, D.L., and Walker, D.W. (2011). Modulation of Longevity and Tissue Homeostasis by the Drosophila PGC-1 Homolog. Cell Metabolism *14*, 623–634.

Rezával, C., Nojima, T., Neville, M.C., Lin, A.C., and Goodwin, S.F. (2014). Sexually dimorphic octopaminergic neurons modulate female postmating behaviors in Drosophila. Curr Biol *24*, 725–730.

Rezával, C., Pattnaik, S., Pavlou, H.J., Nojima, T., Brüggemeier, B., D'Souza, L.A.D., Dweck, H.K.M., and Goodwin, S.F. (2016). Activation of Latent Courtship Circuitry in the Brain of Drosophila Females Induces Male-like Behaviors. Curr Biol *26*, 2508–2515.

Richard, D.S., Rybczynski, R., Wilson, T.G., Wang, Y., Wayne, M.L., Zhou, Y., Partridge, L., and Harshman, L.G. (2005). Insulin signaling is necessary for vitellogenesis in Drosophila melanogaster independent of the roles of juvenile hormone and ecdysteroids: female sterility of the chico1 insulin signaling mutation is autonomous to the ovary. J Insect Physiol *51*, 455–464.

Riddiford, L.M., and Ashburner, M. (1991). Effects of juvenile hormone mimics on larval development and metamorphosis of Drosophila melanogaster. General and Comparative Endocrinology *82*, 172–183.

Riddiford, L.M., and Truman, J.W. (1993). Hormone Receptors and the Regulation of Insect Metamorphosis. Am Zool *33*, 340–347.

Riddiford, L.M., Truman, J.W., Mirth, C.K., and Shen, Y. (2010). A role for juvenile hormone in the prepupal development of Drosophila melanogaster. Development *137*, 1117–1126.

Rideout, E.J., Dornan, A.J., Neville, M.C., Eadie, S., and Goodwin, S.F. (2010). Control of sexual differentiation and behavior by the doublesex gene in Drosophila melanogaster. Nat Neurosci *13*, 458–466.

Rideout, E.J., Marshall, L., and Grewal, S.S. (2012a). Drosophila RNA polymerase III repressor Maf1 controls body size and developmental timing by modulating tRNAiMet synthesis and systemic insulin signaling. Proc Natl Acad Sci U S A *109*, 1139–1144.

Rideout, E.J., Marshall, L., and Grewal, S.S. (2012b). Drosophila RNA polymerase III repressor Maf1 controls body size and developmental timing by modulating tRNAiMet synthesis and systemic insulin signaling. PNAS *109*, 1139–1144.

Rideout, E.J., Narsaiya, M.S., and Grewal, S.S. (2015). The Sex Determination Gene transformer Regulates Male-Female Differences in Drosophila Body Size. PLoS Genet *11*, e1005683.

Rintelen, F., Stocker, H., Thomas, G., and Hafen, E. (2001). PDK1 regulates growth through Akt and S6K in Drosophila. Proc Natl Acad Sci U S A *98*, 15020–15025.

Robertson, F. (1963). The ecological genetics of growth in Drosophila 6. The genetic correlation between the duration of the larval period and body size in relation to larval diet. Genetical Research *4*, 74–92.

Robertson, F.W. (1957a). Studies in quantitative inheritance X. Genetic variation of ovary size inDrosophila. Journal of Genetics *55*, 410–427.

Robertson, F.W. (1957b). Studies in quantitative inheritance XI. Genetic and environmental correlation between body size and egg production inDrosophila Melanogaster. Journal of Genetics *55*, 428.

Rodenfels, J., Lavrynenko, O., Ayciriex, S., Sampaio, J.L., Carvalho, M., Shevchenko, A., and Eaton, S. (2014). Production of systemically circulating Hedgehog by the intestine couples nutrition to growth and development. Genes Dev *28*, 2636–2651.

Rogawski, E.T., Liu, J., Platts-Mills, J.A., Kabir, F., Lertsethtakarn, P., Siguas, M., Khan, S.S., Praharaj, I., Murei, A., Nshama, R., et al. (2018). Use of quantitative molecular diagnostic methods to investigate the effect of enteropathogen infections on linear growth in children in low-resource settings: longitudinal analysis of results from the MAL-ED cohort study. The Lancet Global Health *6*, e1319–e1328.

Roth, S.W., Bitterman, M.D., Birnbaum, M.J., and Bland, M.L. (2018). Innate Immune Signaling in Drosophila Blocks Insulin Signaling by Uncoupling PI(3,4,5)P3 Production and Akt Activation. Cell Rep *22*, 2550–2556.

Rulifson, E.J., Kim, S.K., and Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science *296*, 1118–1120.

Ryner, L.C., Goodwin, S.F., Castrillon, D.H., Anand, A., Villella, A., Baker, B.S., Hall, J.C., Taylor, B.J., and Wasserman, S.A. (1996). Control of male sexual behavior and sexual orientation in Drosophila by the fruitless gene. Cell *87*, 1079–1089.

Salz, H.K., and Erickson, J.W. (2010). Sex determination in Drosophila: The view from the top. Fly (Austin) *4*, 60–70.

Sanaki, Y., Nagata, R., Kizawa, D., Léopold, P., and Igaki, T. (2020). Hyperinsulinemia Drives Epithelial Tumorigenesis by Abrogating Cell Competition. Developmental Cell *53*, 379-389.e5.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to mTORC1. Science *320*, 1496–1501.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., and Sabatini, D.M. (2010). Ragulator-Rag Complex Targets mTORC1 to the Lysosomal Surface and Is Necessary for Its Activation by Amino Acids. Cell *141*, 290–303.

Sano, H., Nakamura, A., Texada, M.J., Truman, J.W., Ishimoto, H., Kamikouchi, A., Nibu, Y., Kume, K., Ida, T., and Kojima, M. (2015). The Nutrient-Responsive

Hormone CCHamide-2 Controls Growth by Regulating Insulin-like Peptides in the Brain of Drosophila melanogaster. PLoS Genet *11*, e1005209.

Sarikaya, D.P., Belay, A.A., Ahuja, A., Dorta, A., Green, D.A., and Extavour, C.G. (2012). The roles of cell size and cell number in determining ovariole number in Drosophila. Dev Biol *363*, 279–289.

Saucedo, L.J., Gao, X., Chiarelli, D.A., Li, L., Pan, D., and Edgar, B.A. (2003). Rheb promotes cell growth as a component of the insulin/TOR signalling network. Nature Cell Biology *5*, 566–571.

Sawala, A., and Gould, A.P. (2017). The sex of specific neurons controls female body growth in Drosophila. PLoS Biol *15*, e2002252.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nature Methods *9*, 676–682.

Schoener, T.W., and Janzen, D.H. (1968). Notes on Environmental Determinants of Tropical Versus Temperate Insect Size Patterns. The American Naturalist *102*, 207–224.

Schreider, E. (1957). Ecological Rules and Body-heat Regulation in Man. Nature *179*, 915–916.

Selman, C., Lingard, S., Choudhury, A.I., Batterham, R.L., Claret, M., Clements, M., Ramadani, F., Okkenhaug, K., Schuster, E., Blanc, E., et al. (2008). Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. FASEB J *22*, 807–818.

Semaniuk, U.V., Gospodaryov, D.V., Feden'ko, K.M., Yurkevych, I.S., Vaiserman, A.M., Storey, K.B., Simpson, S.J., and Lushchak, O. (2018). Insulin-Like Peptides Regulate Feeding Preference and Metabolism in Drosophila. Frontiers in Physiology *9*.

Shakhmantsir, I., Massad, N.L., and Kennell, J.A. (2014). Regulation of cuticle pigmentation in drosophila by the nutrient sensing insulin and TOR signaling pathways. Developmental Dynamics *243*, 393–401.

Shepard, P.J., and Hertel, K.J. (2009). The SR protein family. Genome Biology *10*, 242.

Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kozma, S.C. (1998). Disruption of the p70s6k/p85s6k gene reveals a small mouse phenotype and a new functional S6 kinase. The EMBO Journal *17*, 6649–6659.

Shin, S.C., Kim, S.-H., You, H., Kim, B., Kim, A.C., Lee, K.-A., Yoon, J.-H., Ryu, J.-H., and Lee, W.-J. (2011). Drosophila Microbiome Modulates Host Developmental and Metabolic Homeostasis via Insulin Signaling. Science *334*, 670–674.

Shingleton, A.W., Das, J., Vinicius, L., and Stern, D.L. (2005). The Temporal Requirements for Insulin Signaling During Development in Drosophila. PLOS Biology *3*, e289.

Shingleton, A.W., Masandika, J.R., Thorsen, L.S., Zhu, Y., and Mirth, C.K. (2017). The sex-specific effects of diet quality versus quantity on morphology in Drosophila melanogaster. Royal Society Open Science *4*, 170375.

Shirangi, T.R., Taylor, B.J., and McKeown, M. (2006). A double-switch system regulates male courtship behavior in male and female Drosophila melanogaster. Nature Genetics *38*, 1435–1439.

Siera, S.G., and Cline, T.W. (2008). Sexual back talk with evolutionary implications: stimulation of the Drosophila sex-determination gene sex-lethal by its target transformer. Genetics *180*, 1963–1981.

Slack, C., Giannakou, M.E., Foley, A., Goss, M., and Partridge, L. (2011). dFOXO-independent effects of reduced insulin-like signaling in Drosophila. Aging Cell *10*, 735–748.

Slaidina, M., Delanoue, R., Gronke, S., Partridge, L., and Léopold, P. (2009). A Drosophila insulin-like peptide promotes growth during nonfeeding states. Dev Cell *17*, 874–884.

Smit, A.B., Van kesteren, R.E., Li, K.W., Van minnen, J., Spijker, S., Van heerikhuizen, H., and Geraerts, W.P.M. (1998). Towards Understanding the Role of Insulin in the Brain: Lessons from Insulin-related Signaling Systems in the Invertebrate Brain. Progress in Neurobiology *54*, 35–54.

Sosnowski, B.A., Belote, J.M., and McKeown, M. (1989). Sex-specific alternative splicing of RNA from the transformer gene results from sequence-dependent splice site blockage. Cell *58*, 449–459.

Sriskanthadevan-Pirahas, S., Deshpande, R., Lee, B., and Grewal, S.S. (2018a). Ras/ERK-signalling promotes tRNA synthesis and growth via the RNA polymerase III repressor Maf1 in Drosophila. PLOS Genetics *14*, e1007202.

Sriskanthadevan-Pirahas, S., Lee, J., and Grewal, S.S. (2018b). The EGF/Ras pathway controls growth in Drosophila via ribosomal RNA synthesis. Developmental Biology *439*, 19–29.

Sriskanthadevan-Pirahas, S., Turingan, M.J., Chahal, J.S., Thorson, E., and Grewal, S.S. (2021). Adipose mitochondrial metabolism controls body growth by modulating cytokine and insulin signaling. BioRxiv 2021.04.12.439566.

Stafford, J.W., Lynd, K.M., Jung, A.Y., and Gordon, M.D. (2012). Integration of taste and calorie sensing in Drosophila. J Neurosci *32*, 14767–14774.

Stillwell, R.C., and Davidowitz, G. (2010). Sex differences in phenotypic plasticity of a mechanism that controls body size: implications for sexual size dimorphism. Proceedings of the Royal Society B: Biological Sciences *277*, 3819–3826.

Stillwell, R.C., Blanckenhorn, W.U., Teder, T., Davidowitz, G., and Fox, C.W. (2010). Sex Differences in Phenotypic Plasticity Affect Variation in Sexual Size Dimorphism in Insects: From Physiology to Evolution. Annual Review of Entomology *55*, 227–245.

Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. (2003). Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. Nature Cell Biology *5*, 559–566.

Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., and Leulier, F. (2011). Lactobacillus plantarum Promotes Drosophila Systemic Growth by Modulating Hormonal Signals through TOR-Dependent Nutrient Sensing. Cell Metabolism *14*, 403–414.

Sturtevant, A.H. (1945). A Gene in Drosophila Melanogaster That Transforms Females into Males. Genetics *30*, 297–299.

Su, Y.H., Rastegri, E., Kao, S.H., Lai, C.M., Lin, K.Y., Liao, H.Y., Wang, M.H., and Hsu, H.J. (2018). Diet regulates membrane extension and survival of niche escort cells for germline homeostasis via insulin signaling. Development *145*.

Sutter, N.B., Bustamante, C.D., Chase, K., Gray, M.M., Zhao, K., Zhu, L., Padhukasahasram, B., Karlins, E., Davis, S., Jones, P.G., et al. (2007). A Single IGF1 Allele Is a Major Determinant of Small Size in Dogs. Science *316*, 112–115.

Suzawa, M., Muhammad, N.M., Joseph, B.S., and Bland, M.L. (2019a). The Toll Signaling Pathway Targets the Insulin-like Peptide Dilp6 to Inhibit Growth in Drosophila. Cell Rep *28*, 1439-1446.e5.

Suzawa, M., Muhammad, N.M., Joseph, B.S., and Bland, M.L. (2019b). The Toll Signaling Pathway Targets the Insulin-like Peptide Dilp6 to Inhibit Growth in Drosophila. Cell Reports *28*, 1439-1446.e5.

Tang, H.-W., Hu, Y., Chen, C.-L., Xia, B., Zirin, J., Yuan, M., Asara, J.M., Rabinow, L., and Perrimon, N. (2018). The TORC1-Regulated CPA Complex Rewires an RNA Processing Network to Drive Autophagy and Metabolic Reprogramming. Cell Metabolism *27*, 1040-1054.e8.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Yin, C.M., and Garofalo, R.S. (2001). A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science *292*, 107–110.

Teder, T., and Tammaru, T. (2005). Sexual size dimorphism within species increases with body size in insects. Oikos *108*, 321–334.

Teleman, A.A. (2009). Molecular mechanisms of metabolic regulation by insulin in Drosophila. Biochemical Journal *425*, 13–26.

Teleman, A.A., Chen, Y.-W., and Cohen, S.M. (2005). 4E-BP functions as a metabolic brake used under stress conditions but not during normal growth. Genes Dev *19*, 1844–1848.

Teleman, A.A., Hietakangas, V., Sayadian, A.C., and Cohen, S.M. (2008). Nutritional control of protein biosynthetic capacity by insulin via Myc in Drosophila. Cell Metab *7*, 21–32.

Tennessen, J.M., and Thummel, C.S. (2011). Coordinating Growth and Review Maturation — Insights from Drosophila. Curr Biol *21*, R750–R757.

Tennessen, J.M., Barry, W., Cox, J., and Thummel, C.S. (2014). Methods for studying metabolism in Drosophila. Methods *68*, 105–115.

Testa, N.D., Ghosh, S.M., and Shingleton, A.W. (2013). Sex-specific weight loss mediates sexual size dimorphism in Drosophila melanogaster. PLoS One *8*, e58936.

Texada, M.J., Jørgensen, A.F., Christensen, C.F., Koyama, T., Malita, A., Smith, D.K., Marple, D.F.M., Danielsen, E.T., Petersen, S.K., Hansen, J.L., et al. (2019). A fat-tissue sensor couples growth to oxygen availability by remotely controlling insulin secretion. Nature Communications *10*, 1955.

Thurmond, J., Goodman, J.L., Strelets, V.B., Attrill, H., Gramates, L.S., Marygold, S.J., Matthews, B.B., Millburn, G., Antonazzo, G., Trovisco, V., et al. (2018). FlyBase 2.0: the next generation. Nucleic Acids Research *47*, D759–D765.

Tiefenböck, S.K., Baltzer, C., Egli, N.A., and Frei, C. (2010). The Drosophila PGC-1 homologue Spargel coordinates mitochondrial activity to insulin signalling. The EMBO Journal *29*, 171–183.

Tramunt, B., Smati, S., Grandgeorge, N., Lenfant, F., Arnal, J.F., Montagner, A., and Gourdy, P. (2020). Sex differences in metabolic regulation and diabetes susceptibility. Diabetologia *63*, 453–461.

Tseng, A.-S.K., and Hariharan, I.K. (2002). An overexpression screen in Drosophila for genes that restrict growth or cell-cycle progression in the developing eye. Genetics *162*, 229–243.

Tu, M.P., and Tatar, M. (2003). Juvenile diet restriction and the aging and reproduction of adult Drosophila melanogaster. Aging Cell *2*, 327–333.

Ugrankar, R., Theodoropoulos, P., Akdemir, F., Henne, W.M., and Graff, J.M. (2018). Circulating glucose levels inversely correlate with Drosophila larval feeding through insulin signaling and SLC5A11. Commun Biol *1*, 110.

Vafai, S.B., and Mootha, V.K. (2012). Mitochondrial disorders as windows into an ancient organelle. Nature *491*, 374–383.

Van Voorhies, W.A. (2009). Metabolic function in Drosophila melanogaster in response to hypoxia and pure oxygen. Journal of Experimental Biology *212*, 3132–3141.

Varrela, J. (1984). Effects of X chromosome on size and shape of body: An anthropometric investigation in 47,XXY males. American Journal of Physical Anthropology *64*, 233–242.

Verdu, J., Buratovich, M.A., Wilder, E.L., and Birnbaum, M.J. (1999). Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. Nat Cell Biol *1*, 500–506.

Vries, G.J.D., Rissman, E.F., Simerly, R.B., Yang, L.-Y., Scordalakes, E.M., Auger, C.J., Swain, A., Lovell-Badge, R., Burgoyne, P.S., and Arnold, A.P. (2002). A Model System for Study of Sex Chromosome Effects on Sexually Dimorphic Neural and Behavioral Traits. J. Neurosci. *22*, 9005–9014.

Waddington, C.H. (1942). Canalization of Development and the Inheritance of Acquired Characters. Nature *150*, 563–565.

Wang, S., Tulina, N., Carlin, D.L., and Rulifson, E.J. (2007). The origin of islet-like cells in Drosophila identifies parallels to the vertebrate endocrine axis. PNAS *104*, 19873–19878.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., and Proud, C.G. (2001). Regulation of elongation factor 2 kinase by p90RSK1 and p70 S6 kinase. The EMBO Journal *20*, 4370–4379.

Wat, L.W., Chao, C., Bartlett, R., Buchanan, J.L., Millington, J.W., Chih, H.J., Chowdhury, Z.S., Biswas, P., Huang, V., Shin, L.J., et al. (2020). A role for triglyceride lipase brummer in the regulation of sex differences in Drosophila fat storage and breakdown. PLoS Biol *18*, e3000595.

Webster, B.R., Scott, I., Traba, J., Han, K., and Sack, M.N. (2014). Regulation of Autophagy and Mitophagy by Nutrient Availability and Acetylation. Biochim Biophys Acta *1841*, 525–534.

Weeks, B.C., Willard, D.E., Zimova, M., Ellis, A.A., Witynski, M.L., Hennen, M., and Winger, B.M. (2020). Shared morphological consequences of global warming in North American migratory birds. Ecology Letters *23*, 316–325.

Weinkove, D., Neufeld, T.P., Twardzik, T., Waterfield, M.D., and Leevers, S.J. (1999). Regulation of imaginal disc cell size, cell number and organ size by Drosophila class I(A) phosphoinositide 3-kinase and its adaptor. Curr Biol *9*, 1019–1029.

WEISSBERGER, A.J., HO, K.K.Y., and LAZARUS, L. (1991). Contrasting Effects of Oral and Transdermal Routes of Estrogen Replacement Therapy on 24-Hour Growth

Hormone (GH) Secretion, Insulin-Like Growth Factor I, and GH-Binding Protein in Postmenopausal Women*. The Journal of Clinical Endocrinology & Metabolism *72*, 374–381.

Werz, C., Köhler, K., Hafen, E., and Stocker, H. (2009a). The Drosophila SH2B family adaptor Lnk acts in parallel to chico in the insulin signaling pathway. PLoS Genet *5*, e1000596.

Werz, C., Köhler, K., Hafen, E., and Stocker, H. (2009b). The Drosophila SH2B Family Adaptor Lnk Acts in Parallel to Chico in the Insulin Signaling Pathway. PLOS Genetics *5*, e1000596.

Williams, T.M., Selegue, J.E., Werner, T., Gompel, N., Kopp, A., and Carroll, S.B. (2008). The Regulation and Evolution of a Genetic Switch Controlling Sexually Dimorphic Traits in Drosophila. Cell *134*, 610–623.

Wingrove, J.A., and O'Farrell, P.H. (1999). Nitric Oxide Contributes to Behavioral, Cellular, and Developmental Responses to Low Oxygen in Drosophila. Cell *98*, 105–114.

Wong, A.C.-N., Dobson, A.J., and Douglas, A.E. (2014). Gut microbiota dictates the metabolic response of Drosophila to diet. Journal of Experimental Biology *217*, 1894–1901.

Wood, J.G., Schwer, B., Wickremesinghe, P.C., Hartnett, D.A., Burhenn, L., Garcia, M., Li, M., Verdin, E., and Helfand, S.L. (2018). Sirt4 is a mitochondrial regulator of metabolism and lifespan in Drosophila melanogaster. Proceedings of the National Academy of Sciences *115*, 1564.

Woodling, N.S., Aleyakpo, B., Dyson, M.C., Minkley, L.J., Rajasingam, A., Dobson, A.J., Leung, K.H.C., Pomposova, S., Fuentealba, M., Alic, N., et al. (2020). The neuronal receptor tyrosine kinase Alk is a target for longevity. Aging Cell *n/a*, e13137.

Wu, J.Y., and Maniatis, T. (1993). Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell *75*, 1061–1070.

Wu, Q., Yu, G., Cheng, X., Gao, Y., Fan, X., Yang, D., Xie, M., Wang, T., Piper, M.D.W., and Yang, M. (2020). Sexual dimorphism in the nutritional requirement for adult lifespan in Drosophila melanogaster. Aging Cell *19*, e13120.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR Signaling in Growth and Metabolism. Cell *124*, 471–484.

Youle, R.J., and Narendra, D.P. (2011). Mechanisms of mitophagy. Nature Reviews Molecular Cell Biology *12*, 9–14.

Zhang, H., Stallock, J.P., Ng, J.C., Reinhard, C., and Neufeld, T.P. (2000). Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes Dev *14*, 2712–2724.

Zhang, H., Liu, J., Li, C.R., Momen, B., Kohanski, R.A., and Pick, L. (2009). Deletion of Drosophila insulin-like peptides causes growth defects and metabolic abnormalities. Proc Natl Acad Sci U S A *106*, 19617–19622.

Zinke, I., Schütz, C.S., Katzenberger, J.D., Bauer, M., and Pankratz, M.J. (2002). Nutrient control of gene expression in Drosophila: microarray analysis of starvation and sugar-dependent response. EMBO J *21*, 6162–6173.

APPENDIX

A.1 SUPPLEMENTARY DATA FOR CHAPTER 3
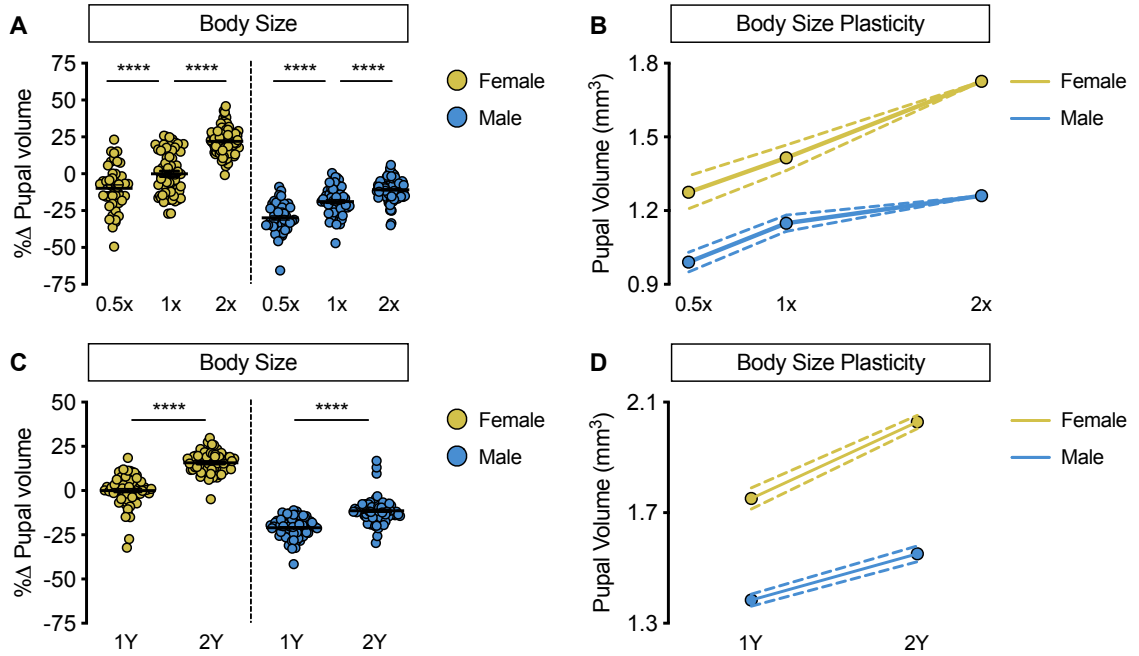
Figure S3.1. Increased female body size plasticity in a protein-rich diet.

(A) Pupal volume was significantly higher in w^{1118} males and females cultured on a widely-used diet (1×) compared with larvae raised on a reduced-nutrient diet (0.5×) ($p<0.0001$ and $p = 0.0006$, respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of this increase in pupal volume was the same in both sexes (sex:diet interaction $p = 0.7048$; two-way ANOVA followed by Tukey HSD test). Pupal volume was significantly higher in w^{1118} males and females raised on a nutrient-rich diet (2×) compared with larvae cultured on 1× ($p<0.0001$ for both; two-way ANOVA followed by Tukey HSD test); however, the magnitude of the increase in body size was significantly larger in females than in males (sex:diet interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). (B) Reaction norms for pupal volume in w^{1118} larvae raised on diets of varying quantity (0.5×, 1×, 2×), plotted using data presented in panel A. n = 43-100 pupae. (C) Pupal volume was significantly higher in both males and females cultured on a yeast-rich medium (2Y) compared with larvae raised on a diet containing half the quantity of yeast (1Y) ($p<0.0001$ for both sexes; two-way ANOVA followed by Tukey HSD test); however, the magnitude of the nutrient-dependent increase in pupal volume was larger in females than in males

(sex:diet interaction p = 0.0001; two-way ANOVA followed by Tukey HSD test). (D) Reaction norms for pupal volume in response to changes in dietary yeast in w^{1118} females and males, plotted using the data presented in panel C. n = 62-80 pupae. For body size plasticity graphs, filled circles indicate mean body size, and dashed lines indicate 95% confidence interval. **** indicates p<0.0001; error bars indicate SEM.
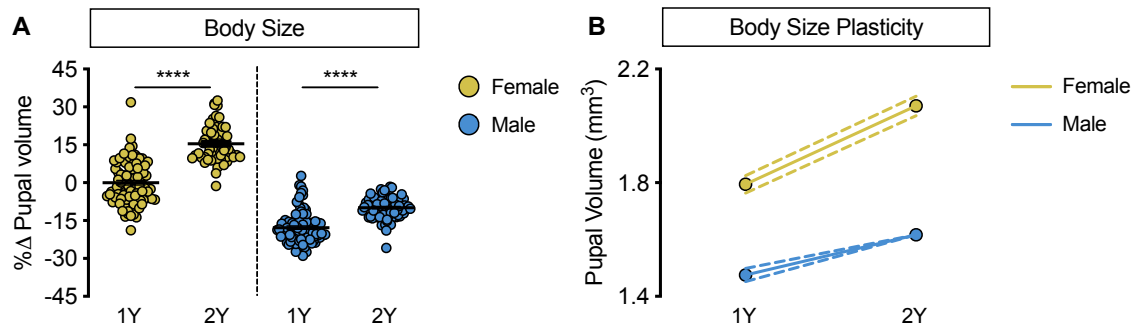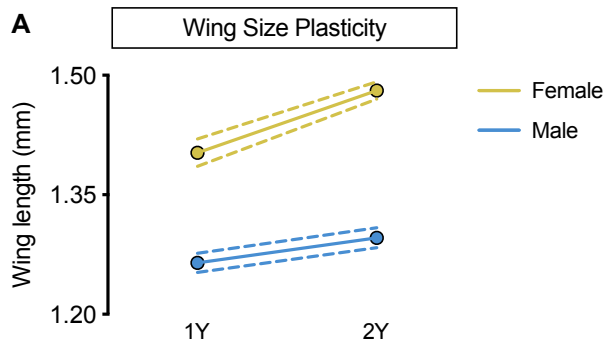
Figure S3.2. Increased nutrient-dependent body size plasticity in *Canton-S* females.
(A) Pupal volume was significantly higher in both *Canton-S* (*CS*) females and males reared on a protein-rich diet (2Y) compared with genotype-matched females and males cultured on a diet containing half the protein concentration (1Y) ($p<0.0001$ for both sexes; two-way ANOVA followed by Tukey HSD test); however, the magnitude of the nutrient-dependent increase in pupal volume was higher in females (sex:diet interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). (B) Reaction norms for pupal volume in response to changes in yeast quantity in *CS* females and males, plotted using the data in panel A. n = 57-95 pupae. For body size plasticity graphs, filled circles indicate mean pupal volume, and dashed lines indicate 95% confidence interval. **** indicates $p<0.0001$; error bars indicate SEM.

Figure S3.3. Increased nutrient-dependent plasticity in female wing size.
(A) Wing length was significantly higher in both *w*[1118] females and males reared on a protein-rich diet (2Y) compared with genotype-matched females and males cultured on a diet containing half the protein content (1Y) ($p<0.0001$ and $p = 0.0018$, respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in wing length was higher in females (sex:diet interaction $p = 0.0004$; two-way ANOVA

followed by Tukey HSD test). n = 16-28 wings. For wing size plasticity graphs, filled circles indicate mean wing length, and dashed lines indicate 95% confidence interval.
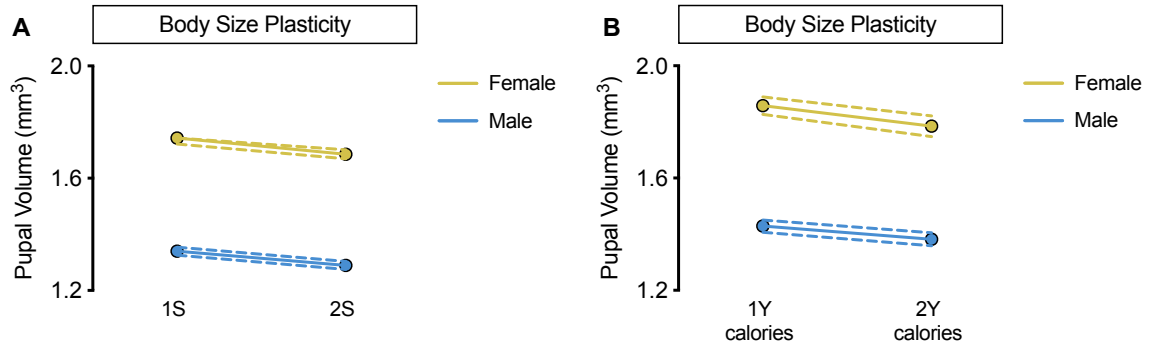
Figure S3.4. No sex-specific effect of altering dietary sugar concentration or calorie content.

(A) Pupal volume was significantly decreased in both w^{1118} females and males reared on a diet with twice the sugar (2S) compared with genotype-matched females and males cultured on a diet with the sugar content of our regular diet (1S) (p<0.0001 and p = 0.0002, respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent decrease in pupal volume was not different between females and males (sex:diet interaction p = 0.6536; two-way ANOVA followed by Tukey HSD test). n = 117-133 pupae. (B) While pupal volume was significantly decreased in w^{1118} females and not males reared on a 2Y calorie-matched diet compared with genotype-matched females and males cultured on a 1Y calorie-matched diet (p = 0.0039 and p = 0.0662 respectively; two-way ANOVA followed by Tukey HSD test), there was no sex:diet interaction indicating that one sex was not more affected than the other (sex:diet interaction p = 0.3698; two-way ANOVA followed by Tukey HSD test). n = 44-74 pupae. For body size plasticity graphs, filled circles indicate mean pupal volume, and dashed lines indicate 95% confidence interval.
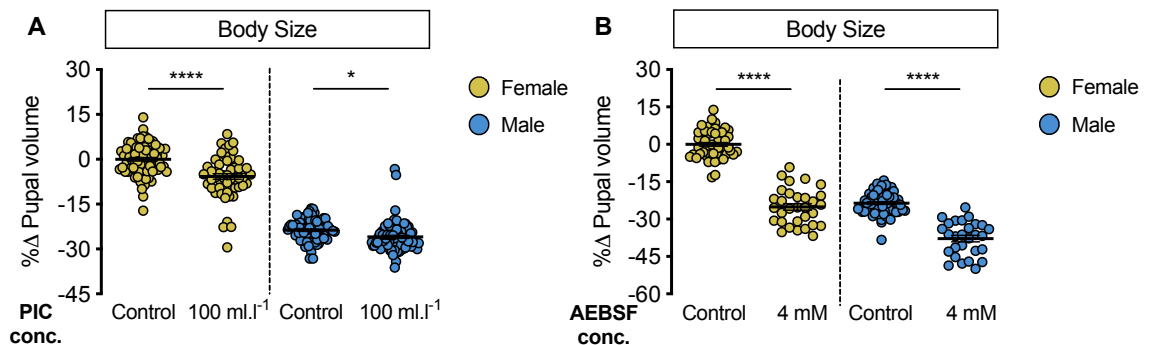
Figure S3.5. Pharmacological inhibition of protein breakdown has female-biased effects on body size.

(A) Pupal volume was significantly higher in both w^{1118} females and males reared on a protein-rich diet (2Y) compared with genotype-matched females and males cultured on 2Y containing a broad-spectrum protease inhibitor cocktail (PIC) ($p<0.0001$ and $p = 0.0185$, respectively; two-way ANOVA followed by Tukey HSD test). Importantly, the magnitude of the effect of inhibiting protein breakdown on pupal volume was higher in females (sex:treatment interaction $p = 0.0029$; two-way ANOVA followed by Tukey HSD test). n = 57-92 pupae. (B) Pupal volume was significantly higher in both w^{1118} females and males reared on 2Y compared with genotype-matched females and males cultured on 2Y containing a serine protease-specific inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) ($p<0.0001$ for both sexes; two-way ANOVA followed by Tukey HSD test); however, the magnitude of the effect of inhibiting protein breakdown on pupal volume was higher in females (sex:treatment interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). n = 28-66 pupae. * indicates $p<0.05$; **** indicates $p<0.0001$; error bars indicate SEM.
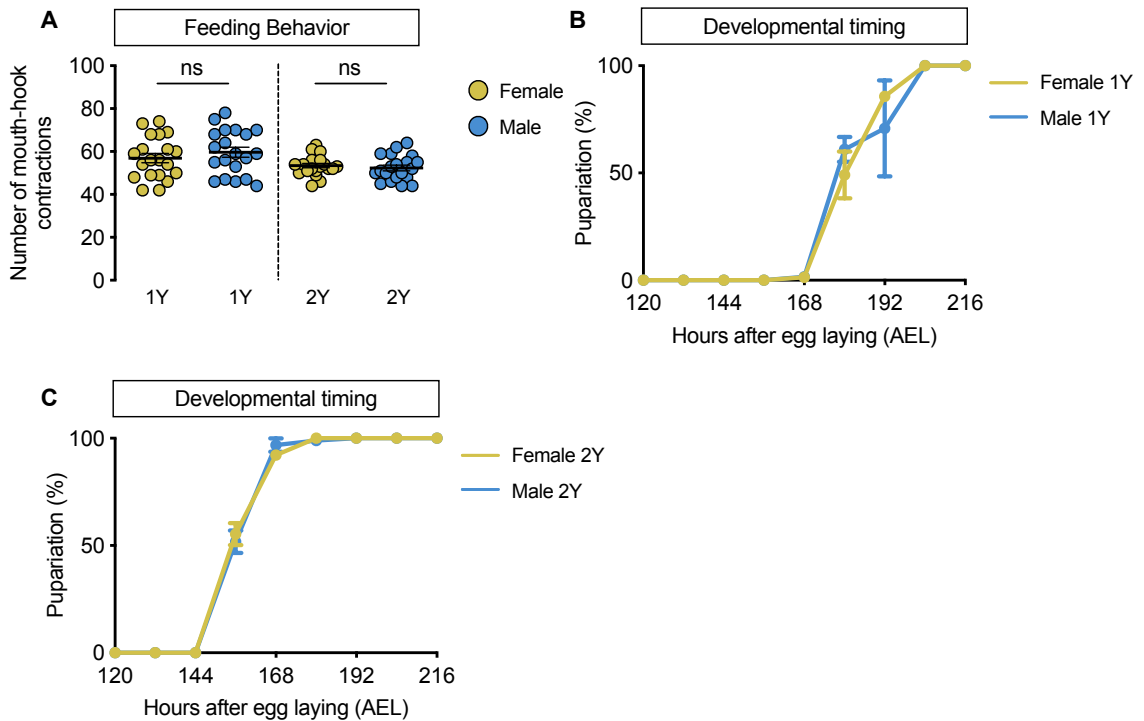
Figure S3.6. No sex difference in food intake or time to pupation.

(A) There was no significant difference in mouth hook contractions between w^{1118} control male and female larvae raised on a diet containing a widely-used protein content (1Y) ($p = 0.3965$; Student's t test), or a protein-rich diet (2Y) ($p = 0.5175$; Student's t test). n = 20 biological replicates. (B) There was no sex difference in the time to pupation between w^{1118}

control male and female larvae when cultured on 1Y. n = 79-93 pupae. (C) There was no sex difference in the time to pupation between w^{1118} control male and female larvae when cultured on 2Y. n = 87-94 pupae. ns indicates not significant; error bars indicate SEM.
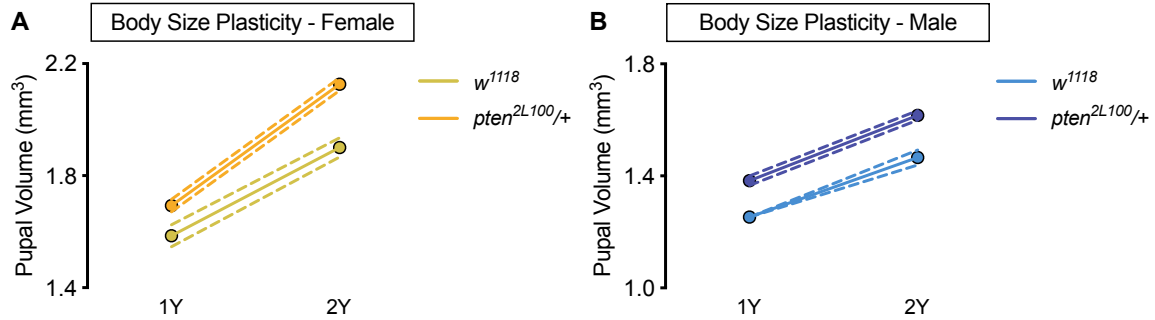

Figure S3.7. Larger body size does not confer increased body size plasticity. (A) Pupal volume was significantly higher in both w^{1118} females and $pten^{2L100}$/+ females reared on a protein-rich diet (2Y) compared with genotype-matched females cultured on a diet containing half the protein content (1Y) ($p<0.0001$ for both genotypes; two-way ANOVA followed by Tukey HSD test). n = 60-89 pupae. (B) Pupal volume was significantly higher in both w^{1118} males and $pten^{2L100}$/+ males reared on 2Y compared with genotype-matched males cultured on 1Y ($p<0.0001$ for both genotypes; two-way ANOVA followed by Tukey HSD test). Importantly, the magnitude of the nutrient-dependent increase in pupal volume was not different between w^{1118} males and $pten^{2L100}$/+ males (genotype:diet interaction p = 0.3557; two-way ANOVA followed by Tukey HSD test). n = 65-88 pupae. For body size plasticity graphs, filled circles indicate mean pupal volume, and dashed lines indicate 95% confidence interval.
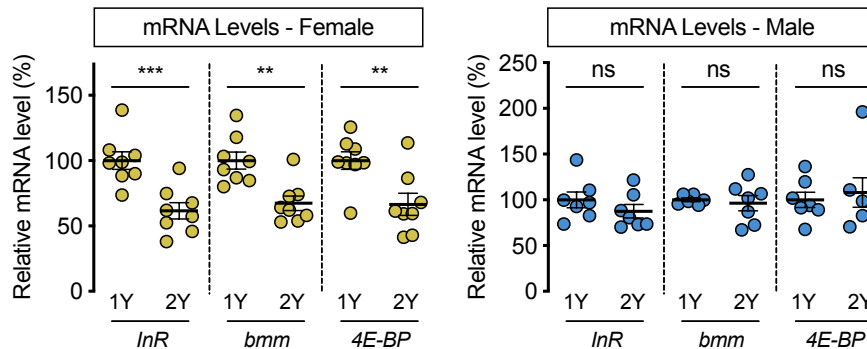

Figure S3.8. IIS activity is upregulated in response to a protein-rich diet in females, not males. (A) In females, mRNA levels of the *InR*, *bmm*, and *4E-BP* were significantly lower in larvae raised on a protein-rich diet (2Y) compared with larvae raised on a diet

171

containing half the protein content (1Y) (p = 0.0009, 0.0019, and 0.0077, respectively; Student's t test). n = 8 biological replicates. (B) In males, there was no significant difference in InR, bmm, or 4E-BP mRNA levels between larvae raised on 2Y compared with larvae cultured on 1Y (p = 0.291, 0.6994, and 0.666, respectively; Student's t test). n = 6-7 biological replicates. * indicates *p*<0.05; *** indicates *p*<0.001; ns indicates not significant; error bars indicate SEM.
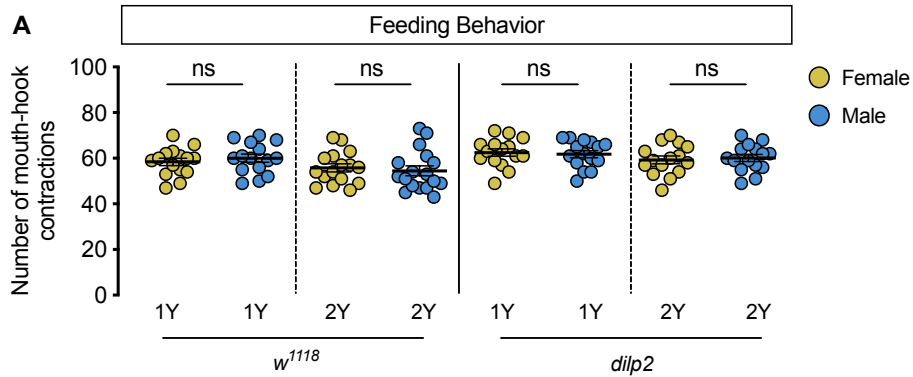

Figure S3.9. No sex difference in food intake in *dilp2* mutant larvae.

(A) There was no significant difference in mouth hook contractions between *w*^{1118} control male and female larvae raised on a diet containing a widely-used protein content (1Y) (*p* = 0.5015; Student's *t* test), or a protein-rich diet (2Y) (*p* = 0.6514; Student's *t* test). There was no significant difference in mouth hook contractions between *dilp2* mutant male and female larvae raised in 1Y (*p* = 0.7667; Student's *t* test), or 2Y (*p* = 0.7101; Student's *t* test). n = 15-17 biological replicates. ns indicates not significant; error bars indicate SEM.
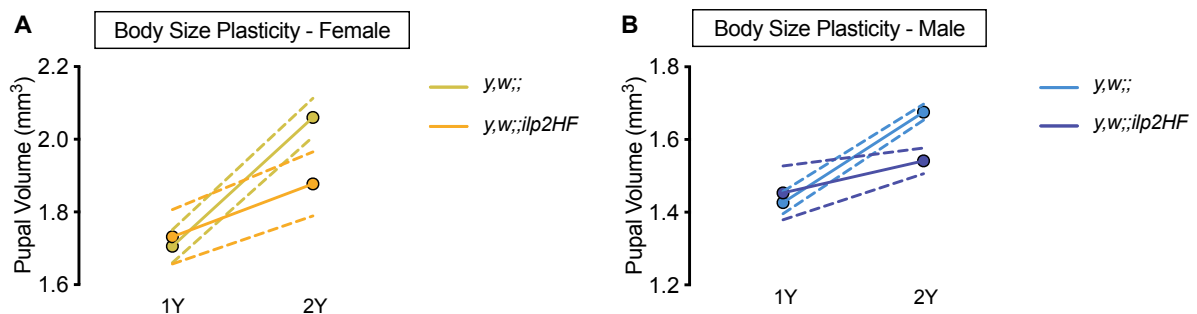

Figure S3.10. HA and FLAG tagged *dilp2* transgenic flies exhibit impaired nutrient dependent body size plasticity.

(A) Pupal volume was significantly higher in *y,w* females and *y,w;;ilp2HF* females reared on 2Y compared with genotype-matched females cultured on 1Y (*p*<0.0001 and *p* = 0.0246

respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was higher in *y,w* females (genotype:diet interaction *p* = 0.001; two-way ANOVA followed by Tukey HSD test). n = 13-36 pupae. (B) Pupal volume was significantly higher in *y,w* males and *y,w;;ilp2HF* males reared on 2Y compared with genotype-matched females cultured on 1Y (*p*<0.0001 and *p* = 0.0354 respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was higher in *y,w* males (genotype:diet interaction *p*<0.0001; two-way ANOVA followed by Tukey HSD test). n = 12-46 pupae. For body size plasticity graphs, filled circles indicate mean pupal volume, and dashed lines indicate 95% confidence interval.
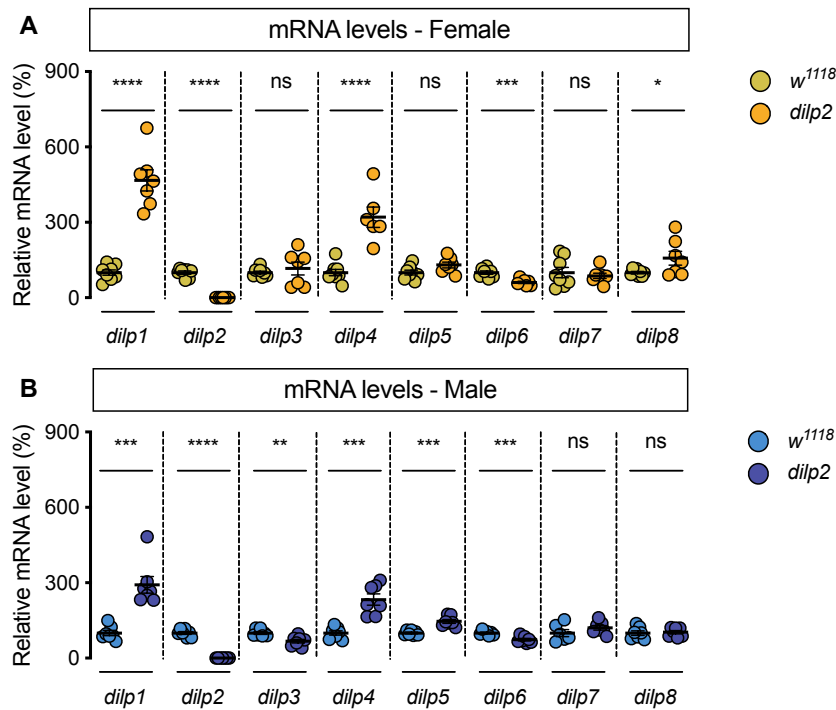

Figure S3.11. Genotype-dependent changes to *dilp* mRNA levels. (A) In *dilp2* mutant females, mRNA levels of *dilp1, dilp2, dilp4, dilp6,* and *dilp8* were significantly different from *w^1118* control females (*p*<0.0001, <0.0001, <0.0001, 0.0003 and 0.0454, respectively; Student's *t* test), but mRNA levels of *dilp3, dilp5,* and *dilp7* were not significantly different (*p* = 0.5142, 0.0574, and 0.605, respectively; Student's *t* test). n = 6-8 biological replicates. (B) In *dilp2* mutant males, mRNA levels of *dilp1, dilp2, dilp3, dilp4, dilp5,* and *dilp6* were significantly different from *w^1118* control males (*p* = 0.0001, <0.0001, 0.0034, 0.0001, 0.0001, and 0.0008, respectively; Student's *t* test), but mRNA levels of *dilp7* and *dilp8* were not significantly different (*p* = 0.2302, and 0.7809, respectively; Student's *t* test). n = 6-7

biological replicates. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$; ns indicates not significant; error bars indicate SEM.
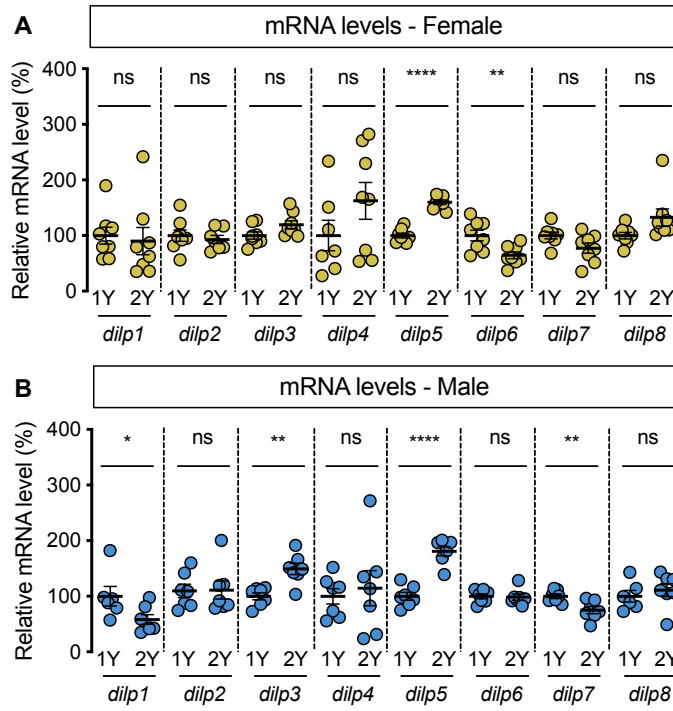

Figure S3.12. Diet-dependent changes to *dilp* mRNA levels.

(A) mRNA levels of *dilp5* and *dilp6* were significantly different between females raised on a protein-rich diet (2Y) compared with female larvae cultured on a diet with half the protein concentration of 2Y (1Y) ($p<0.0001$ and 0.0079, respectively; Student's *t* test), but mRNA levels of *dilp1, dilp2, dilp3, dilp4, dilp7, dilp8* were unchanged (p = 0.7337, 0.5947, 0.0672, 0.1777, 0.0562 and 0.0643, respectively; Student's *t* test). n = 7-8 biological replicates. (B) In males cultured in 1Y, mRNA levels of *dilp1, dilp3, dilp5, dilp7* were significantly different from male larvae raised on 2Y (p = 0.047, 0.0014, <0.0001, and 0.0068, respectively; Student's *t* test); mRNA levels of *dilp2, dilp4, dilp6,* and *dilp8* were unchanged (p = 0.9388, 0.6812, 0.8157 and 0.5054, respectively; Student's *t* test). n = 6-7 biological replicates. * indicates $p<0.05$, ** indicates $p<0.01$, **** indicates $p<0.0001$; ns indicates not significant; error bars indicate SEM.
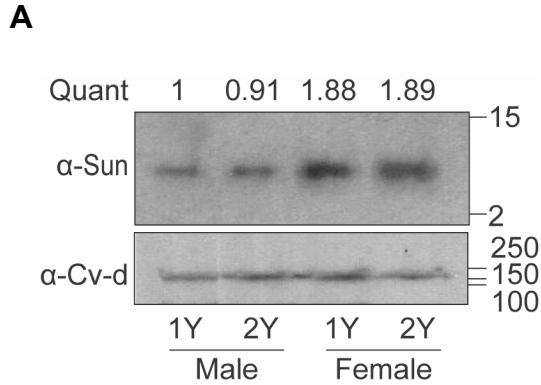
A

Figure S3.13. Increased circulating levels of Stunted (Sun) in females.

(A) Hemolymph levels of Sun in male and female larvae 108 hr after egg laying raised on a protein-rich diet (2Y) compared with male and female larvae cultured on a diet with half the protein concentration of 2Y (1Y). Quantification indicates ratio of Sun protein normalised to loading control Crossveinless-d (Cv-d), relative to male 1Y. n = 1 biological replicates.
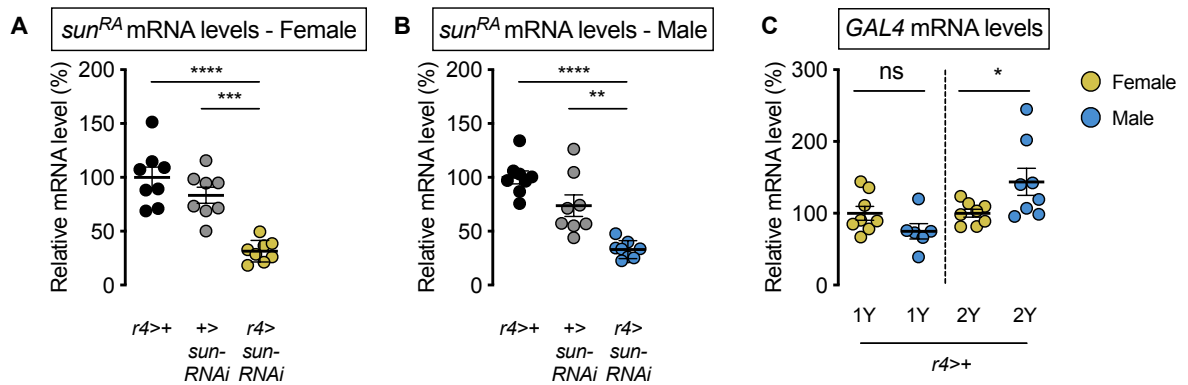

Figure S3.14. Validation of *stunted* (*sun*) knockdown.

(A) mRNA levels of *stunted* (sun^{RA}) were significantly lower in *r4-GAL4>UAS-sun-RNAi* females compared with *r4-GAL4>+* and *+>UAS-sun-RNAi* control females ($p<0.0001$ and p = 0.0001, respectively; one-way ANOVA followed by Tukey HSD test). n = 8 biological replicates. (B) mRNA levels of *stunted* (sun^{RA}) were significantly lower in *r4-GAL4>UAS-sun-RNAi* males compared with *r4-GAL4>+* and *+>UAS-sun-RNAi* control males ($p<0.0001$ and p = 0.0012, respectively; one-way ANOVA followed by Tukey HSD test). n = 8 biological replicates. (C) Levels of *GAL4* mRNA were not significantly different between the sexes in larvae raised in 1Y (p = 0.1105; Student's *t* test), whereas *GAL4* mRNA levels were significantly higher in males in 2Y (p = 0.0428; Student's *t* test). n = 6-8 biological replicates.

175

* indicates *p*<0.05, ** indicates *p*<0.01, *** indicates *p*<0.001, **** indicates *p*<0.0001; ns indicates not significant; error bars indicate SEM.
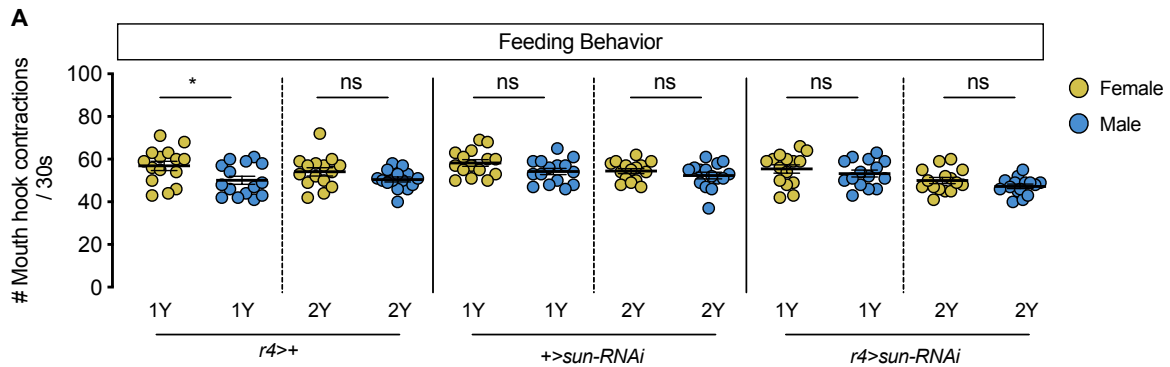

Figure S3.15. No sex difference in food intake in fat body *stunted* (*sun*) knockdown larvae.

(A) There was a significant difference in mouth hook contractions between *r4* >+ control male and female larvae raised on a diet containing a widely-used protein content (1Y) (*p* = 0.025; Student's *t* test), but not on a protein-rich diet (2Y) (*p* = 0.1201; Student's *t* test). There was no significant difference in mouth hook contractions between +>*sun-RNAi* control male and female larvae raised in 1Y (*p* = 0.0725; Student's *t* test), or 2Y (*p* = 0.296; Student's *t* test). There was no significant difference in mouth hook contractions between *r4*>*sun-RNAi* male and female larvae raised in 1Y (*p* = 0.3997; Student's *t* test), or 2Y (*p* = 0.1249; Student's *t* test). n = 15 biological replicates. * indicates *p*<0.05; ns indicates not significant; error bars indicate SEM.
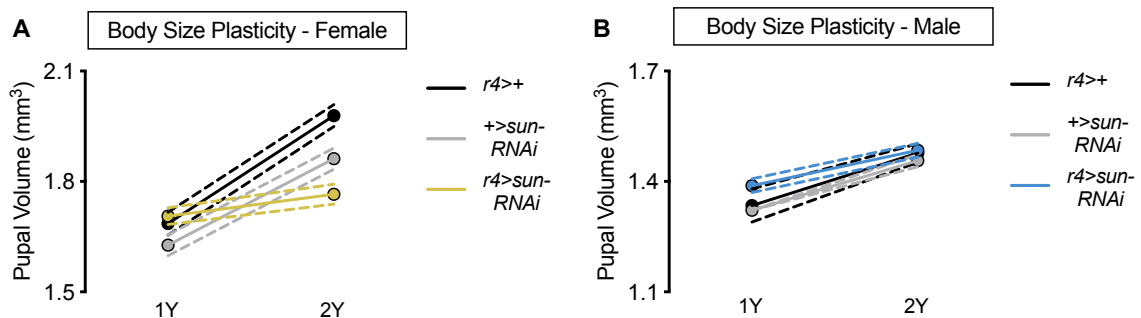

Figure S3.16. Nutrient-dependent increased female body size plasticity requires *stunted* (*sun*).

(A) Pupal volume was significantly higher in *r4*>+, +>*UAS-sun-RNAi*, and *r4*>*UAS-sun-RNAi* females reared on 2Y compared with genotype-matched females cultured on 1Y (*p*<0.0001

176

[*r4>+* and *+>UAS-sun-RNAi*] and *p* = 0.0367 [*r4>UAS-sun-RNAi*]; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was significantly lower in *r4>UAS-sun-RNAi* females (genotype:diet interaction *p*<0.0001; two-way ANOVA followed by Tukey HSD test). n = 69-80 pupae. (B) Pupal volume was significantly higher in *r4>+*, *+>UAS-sun-RNAi*, and *r4>UAS-sun-RNAi* males reared on 2Y compared with genotype-matched males cultured on 1Y (*p*<0.0001 for all genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was not significantly different between *r4>UAS-sun-RNAi* males and control males (genotype:diet interaction *p* = 0.0784; two-way ANOVA followed by Tukey HSD test). n = 44-80 pupae. For body size plasticity graphs, filled circles indicate mean pupal volume, and dashed lines indicate 95% confidence interval.
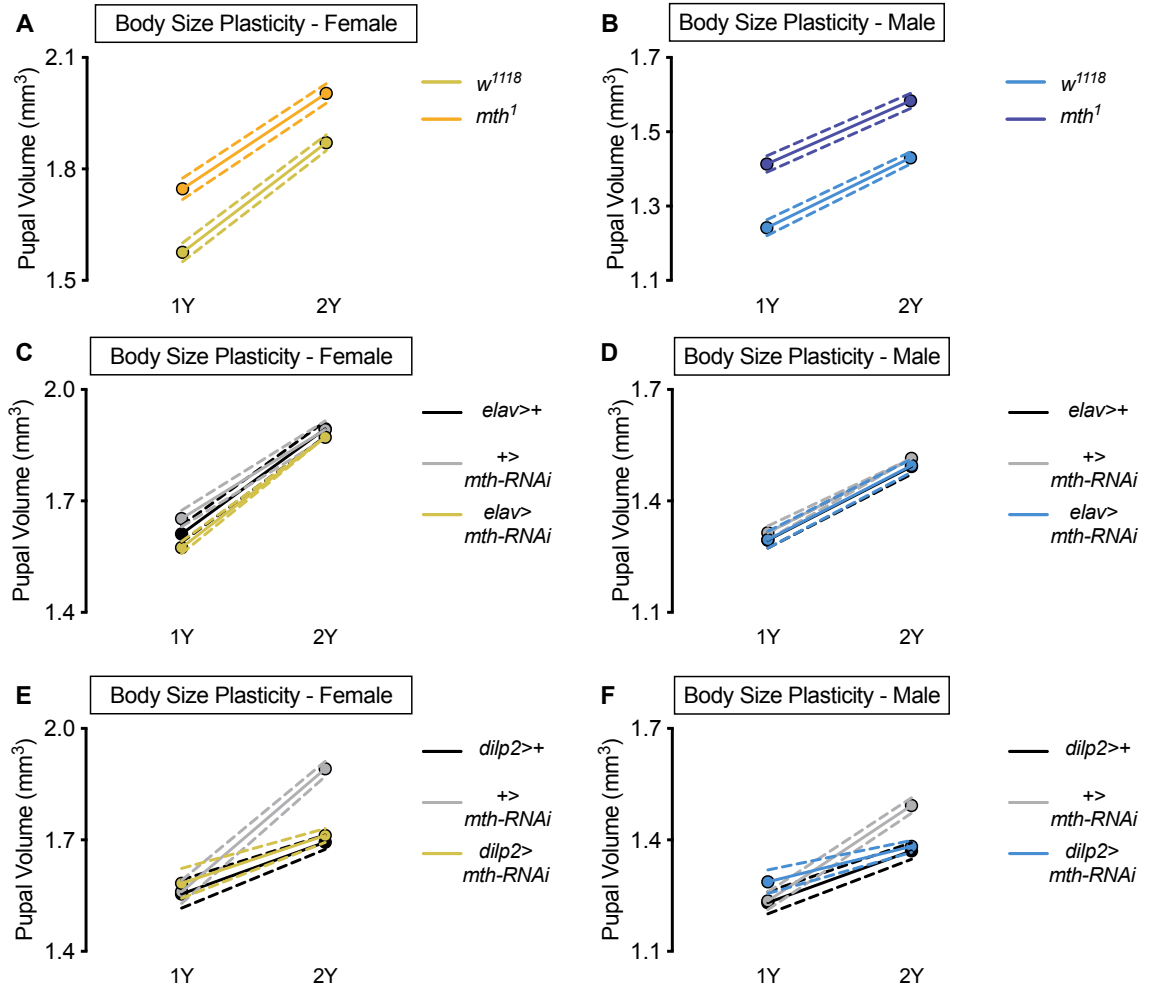

Figure S3.17. *methuselah* (*mth*) is dispensable for nutrient-dependent increased female body size plasticity.

(A) Pupal volume was significantly higher in w^{1118} females and mth^1 mutant females reared on 2Y compared with genotype-matched females cultured on 1Y ($p<0.0001$ for both genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was not significantly different (genotype:diet interaction p = 0.1383; two-way ANOVA followed by Tukey HSD test). n = 59-69 pupae. (B) Pupal volume was significantly higher in w^{1118} males and mth^1 mutant males reared on 2Y compared with genotype-matched males cultured on 1Y ($p<0.0001$ for both genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was not significantly different (genotype:diet interaction p = 0.3697; two-way ANOVA followed by Tukey HSD test). n = 60-75 pupae. (C) Pupal volume was significantly higher in *elav>+*, *+>UAS-mth-RNAi*, and *elav>UAS-mth-RNAi* females reared on 2Y compared with genotype-matched females cultured on 1Y ($p<0.0001$ for all genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was not significantly larger in *elav>UAS-mth-RNAi* females, but was significantly different in *+>UAS-mth-RNAi* females (genotype:diet interaction p = 0.0148; two-way ANOVA followed by Tukey HSD test). n = 53-77 pupae. (D) Pupal volume was significantly higher in *elav>+*, *+>UAS-mth-RNAi*, and *elav>UAS-mth-RNAi* males reared on 2Y compared with genotype-matched males cultured on 1Y ($p<0.0001$ for all genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was not significantly different (genotype:diet interaction p = 0.9947; two-way ANOVA followed by Tukey HSD test). n = 57-86 pupae. (E) Pupal volume was significantly higher in *dilp2>+*, *+>UAS-mth-RNAi*, and *dilp2>UAS-mth-RNAi* females reared on 2Y compared with genotype-matched females cultured on 1Y ($p<0.0001$ for all genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was significantly blunted in *dilp2>+* and *dilp2>UAS-mth-RNAi* females (genotype:diet interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). n = 36-64 pupae. (F) Pupal volume was significantly higher in *dilp2>+*, *+>UAS-mth-RNAi*, and *dilp2>UAS-mth-RNAi* males reared on 2Y compared with genotype-matched males cultured on 1Y ($p<0.0001$ for all genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was significantly blunted in *dilp2>+* and *dilp2>UAS-mth-RNAi* males (genotype:diet interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). n = 34-63 pupae. For body size plasticity graphs, filled circles indicate mean pupal volume, and dashed lines indicate 95% confidence interval.
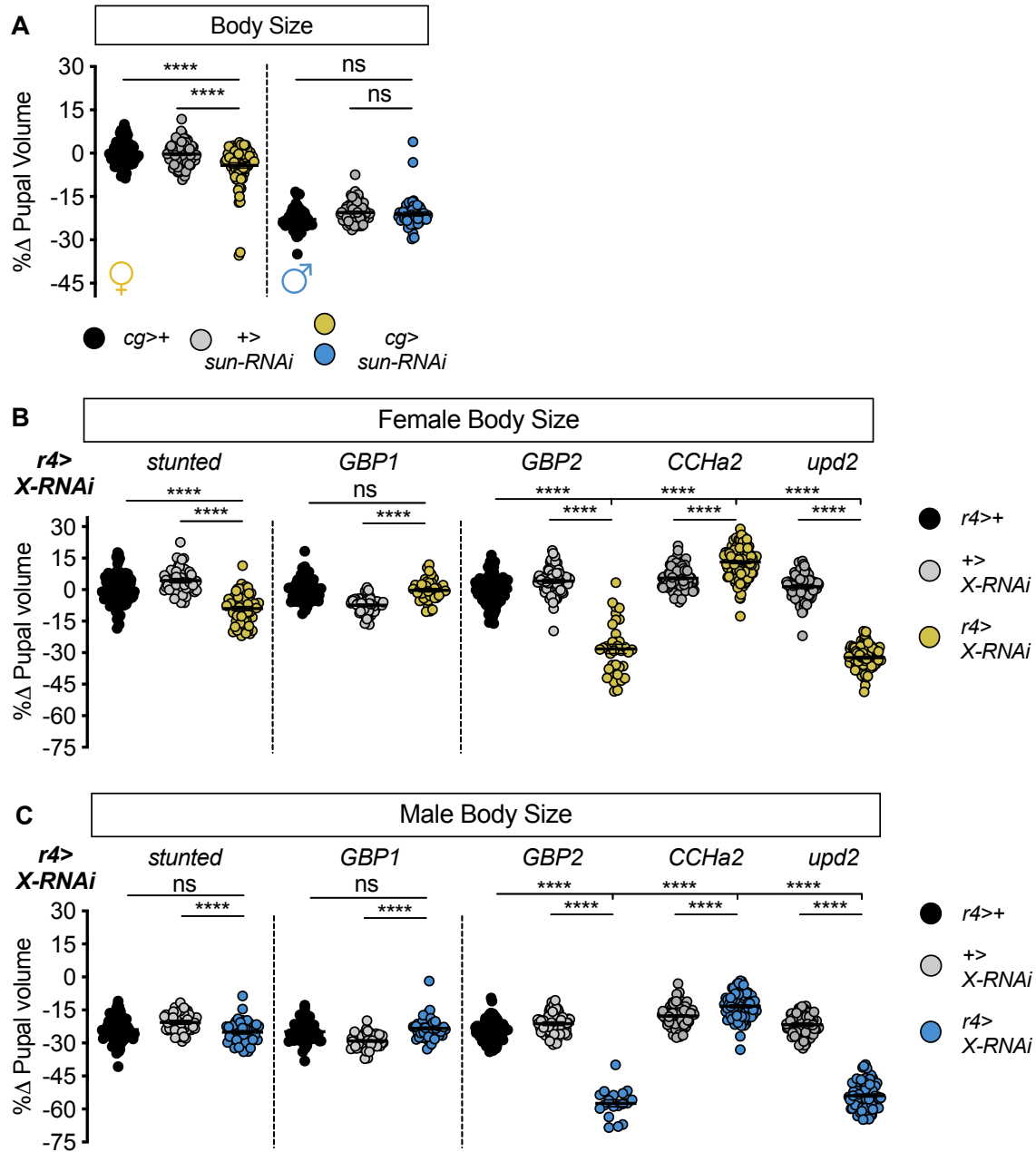
Figure S3.18. Most humoral factors have non-sex-specific effects on body size.

(A) Pupal volume was significantly smaller in females with fat body-specific expression of an RNAi transgene directed against *stunted* (*sun*). Pupal volume was significantly reduced in *cg>UAS-sun-RNAi* females compared with *cg>+* and *+>UAS-sun-RNAi* control females (*p*<0.0001 for both comparisons; two-way ANOVA followed by Tukey HSD test). This decreased pupal volume was not reproduced in *cg>UAS-sun-RNAi* males compared with *cg>+* and *+>UAS-sun-RNAi* control males (*p* = 0.3657 and *p* = 0.9852, respectively; two-way ANOVA followed by Tukey HSD test). RNAi-mediated knockdown of *sun* had larger

effects on pupal volume in females than in males (sex:genotype interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). n = 54-85 pupae. (B) Pupal volume was significantly different in females with fat body-specific expression of RNAi transgenes directed against *sun*, *Growth-blocking peptide 2* (*Gbp2*), *CCHamide-2* (*CCHa2*), *unpaired 2* (*upd2*) compared with *r4>+* and *+>UAS-X-RNAi* control females ($p<0.0001$ for both comparisons [*sun*], $p<0.0001$ for both comparisons [*Gbp2*], $p<0.0001$ for both comparisons [*CCHa2*], $p<0.0001$ for both comparisons [*upd2*]; one-way ANOVA followed by Tukey HSD test); but not upon RNAi-mediated knockdown of *Growth-blocking peptide 1* (*Gbp1*) ($p = 0.9665$ and $p<0.0001$ respectively; one-way ANOVA followed by Tukey HSD test). n = 35-114 pupae. (C) Pupal volume was significantly different in males with fat body-specific expression of RNAi transgenes directed against *Gbp2*, *CCHa2*, and *upd2* compared with *r4>+* and *+>UAS-X-RNAi* control males ($p<0.0001$ for both comparisons [*Gbp2*], $p<0.0001$ for both comparisons [*CCHa2*], $p<0.0001$ for both comparisons [*upd2*]; one-way ANOVA followed by Tukey HSD test); but not reduced in males carrying RNAi transgenes directed against *sun* and *Gbp1* ($p = 0.3513$ and $p<0.0001$, respectively [*sun*]; $p = 0.1274$ and $p<0.0001$, respectively [*Gbp1*]; one-way ANOVA followed by Tukey HSD test). n = 18-100 pupae. For body size graphs, filled circles indicate pupal volume and error bars indicate SEM. **** indicates $p<0.0001$; ns indicates not significant.
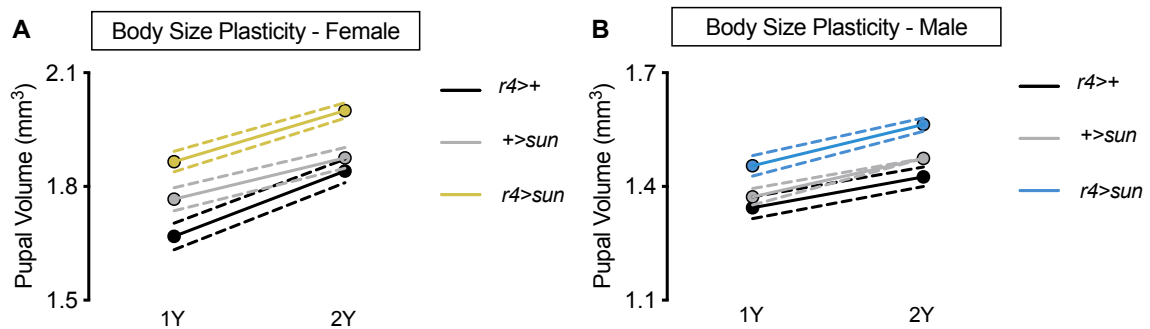

Figure S3.19. *stunted* (*sun*) overexpression augments body size but does not confer increased body size plasticity in males.

(A) Pupal volume was significantly higher in *r4>+*, *+>UAS-sun*, and *r4>UAS-sun* females reared on a protein-rich diet (2Y) compared with genotype-matched females cultured on a diet containing half the protein concentration (1Y) ($p<0.0001$ for all genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was not significantly different between female genotypes (genotype:diet

interaction p = 0.0895; two-way ANOVA followed by Tukey HSD test). n = 43-65 pupae. (B) Pupal volume was significantly higher in *r4>+*, *+>UAS-sun*, and *r4>UAS-sun* males reared on 2Y compared with genotype-matched males cultured on 1Y ($p<0.0001$ for all genotypes; two-way ANOVA followed by Tukey HSD test), but the magnitude of the nutrient-dependent increase in pupal volume was not different between male genotypes (genotype:diet interaction p = 0.4959; two-way ANOVA followed by Tukey HSD test). n = 44-67 pupae. For body size plasticity graphs, filled circles indicate mean pupal volume, and dashed lines indicate 95% confidence interval.
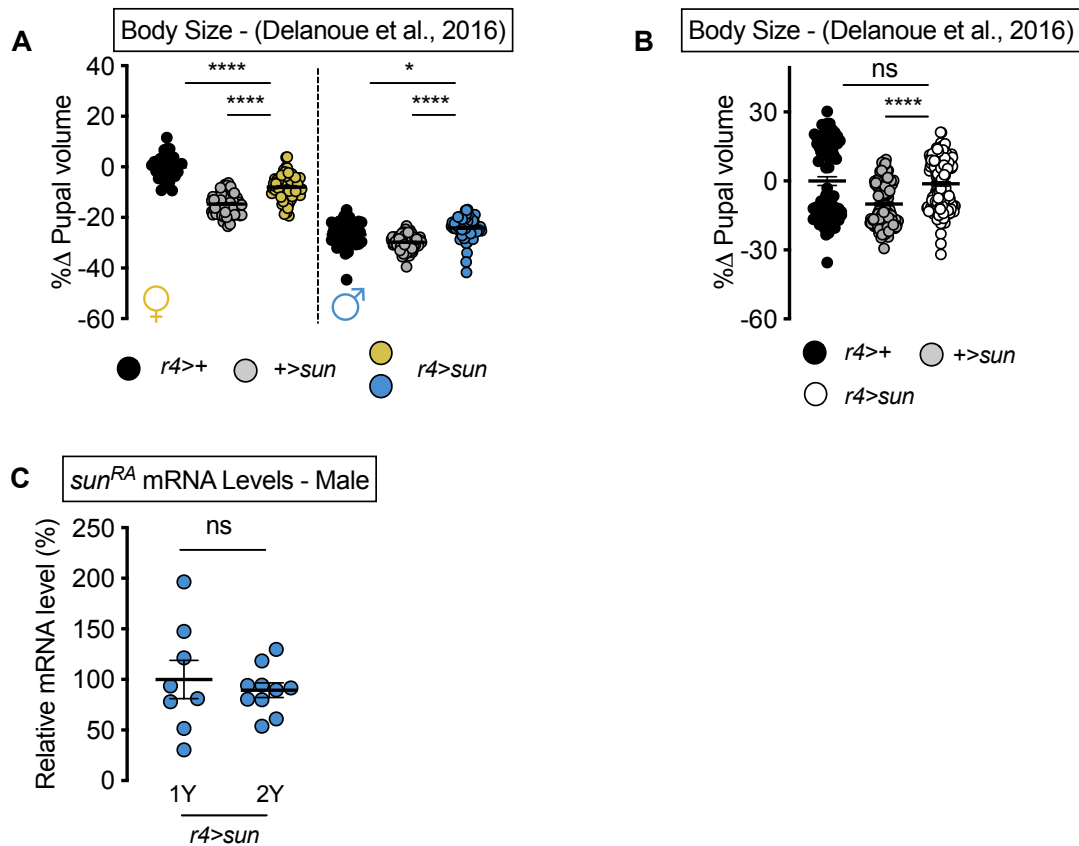
(A)

Figure S3.20. *stunted* (*sun*) overexpression augments body size in the diet used in Delanoue et al. (2016) in males.

(A) Pupal volume was not significantly larger in *r4>UAS-sun* females compared with *r4>+* and *+>UAS-sun* control females cultured on the diet used in Delanoue et al. (2016) ($p<0.0001$ for both comparisons; two-way ANOVA followed by Tukey HSD test). Pupal volume was significantly larger in *r4>UAS-sun* males compared with *r4>+* and *+>UAS-sun* control males cultured on the diet used in Delanoue et al. (2016) (p = 0.0104 and $p<0.0001$,

respectively; two-way ANOVA followed by Tukey HSD test). n = 36-95 pupae. (B) Pupal volume was not significantly different in pooled in *r4>UAS-sun* males and females compared with pooled *r4>+* and *+>UAS-sun* control males and females cultured on the diet used in Delanoue et al. (2016) (*p* = 0.7224 and *p*<0.0001, respectively; two-way ANOVA followed by Tukey HSD test). n = 77-174 pupae. (C) mRNA levels of *sun^{RA}* were not significantly different in *r4>UAS-sun* males cultured on 1Y compared to genotype matched males cultured on 2Y (*p* = 0.5763; Student's *t* test). n = 8-10 biological replicates. For body size graphs, filled circles indicate pupal volume and error bars indicate SEM. * indicates *p*<0.05, **** indicates *p*<0.0001; ns indicates not significant.
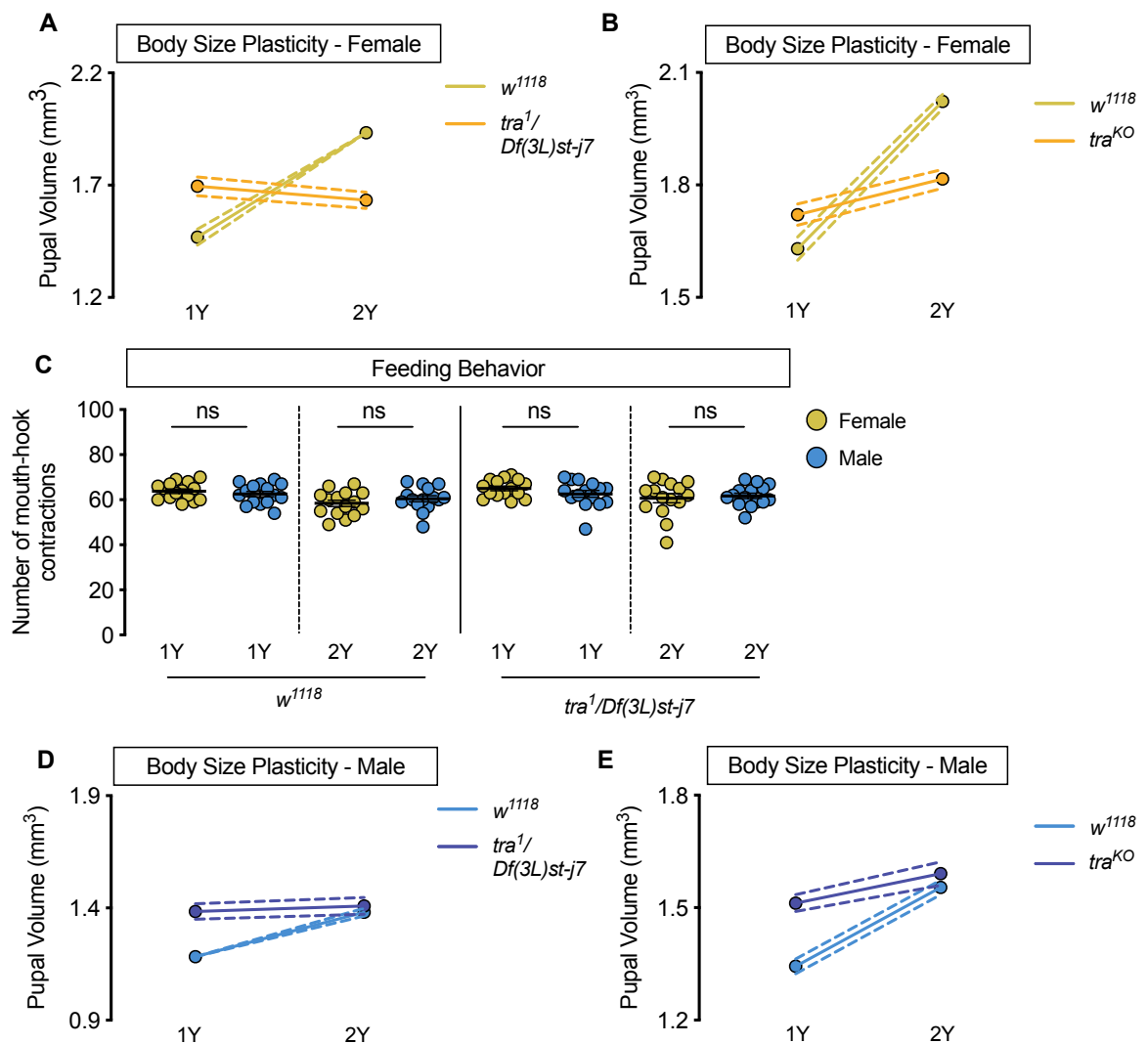

Figure S3.21. Increased nutrient-dependent body size plasticity in females requires *transformer*.

(A) Pupal volume was significantly higher in w^{1118} females reared on a protein-rich diet (2Y) compared with w^{1118} females cultured on a diet containing half the protein concentration (1Y) ($p<0.0001$; two-way ANOVA followed by Tukey HSD test); however, this nutrient-dependent increase in pupal volume was not observed in *transformer* (*tra*) mutant females (*tra^1/Df(3L)st-j7*) (p = 0.1036; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in pupal volume was lower in *tra^1/Df(3L)st-j7* females (genotype:diet interaction $p<0.0001$). n = 39-69 pupae. (B) Pupal volume was significantly higher in w^{1118} females and *tra* mutant females (*tra^{KO}*) reared on a protein-rich diet (2Y) compared with w^{1118} females and *tra^{KO}* females cultured on a diet containing half the protein concentration (1Y) ($p<0.0001$, for both comparisons; two-way ANOVA followed by Tukey HSD test); however, the magnitude of the nutrient-dependent increase in pupal volume was lower in *tra^{KO}* females (genotype:diet interaction $p<0.0001$). n = 71-81 pupae. (C) There was no significant difference in mouth hook contractions between w^{1118} control male and female larvae raised on a diet containing a widely-used protein content (1Y) (p = 0.4103; Student's *t* test), or a protein-rich diet (2Y) (p = 0.2961; Student's *t* test). There was no significant difference in mouth hook contractions between *tra* mutant (*tra^1/Df(3L)st-j7*) male and female larvae raised in 1Y (p = 0.1961; Student's *t* test), or 2Y (p = 0.6732; Student's *t* test). n = 15 biological replicates. (D) Pupal volume was significantly higher in w^{1118} males ($p<0.0001$; two-way ANOVA followed by Tukey HSD test), but not in *tra^1/Df(3L)st-j7* mutant males reared on 2Y compared with genotype-matched males cultured on 1Y (p = 0.6643; two-way ANOVA followed by Tukey HSD test). n = 37-65 pupae. (E) Pupal volume was significantly higher in w^{1118} males and *tra* mutant males (*tra^{KO}*) reared on 2Y compared with genotype-matched males cultured on 1Y ($p<0.0001$, for both comparisons; two-way ANOVA followed by Tukey HSD test). n = 44-80 pupae. For body size plasticity graphs, filled circles indicate mean pupal volume, and dashed lines indicate 95% confidence interval. ns indicates not significant; error bars indicate SEM.
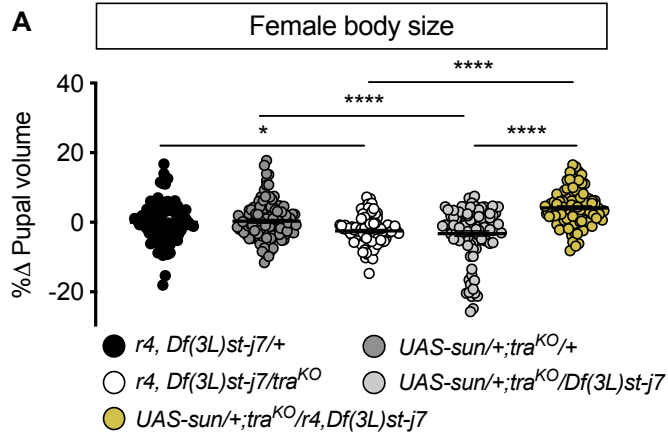
A Female body size

Figure S3.22. Fat body *stunted* (*sun*) overexpression is sufficient to rescue the reduced body size of *transformer* (*tra*) mutant females in a protein-rich (2Y) diet.
(A) In females, pupal volume was significantly smaller in *tra* mutant *r4-GAL4* control females compared to *r4-GAL4* control females with one copy of *tra* (p = 0.0194 [*r4,Df(3L)st-j7/+* v *r4,Df(3L)st-j7/tra^{KO}*]; one-way ANOVA followed by Tukey HSD test). Pupal volume was significantly smaller in *tra* mutant *UAS-sun* control females compared to *UAS-sun* control females with one copy of *tra* ($p<0.0001$ [*UAS-sun/+; tra^{KO}/+* v *UAS-sun/+; tra^{KO}/Df(3L)st-j7*]; one-way ANOVA followed by Tukey HSD test). Pupal volume was significantly larger in *tra* mutant females with fat body overexpression of *sun* compared to *tra* mutant controls ($p<0.0001$, for both comparisons; one-way ANOVA followed by Tukey HSD test). n = 94-117 pupae. For body size graphs, filled circles indicate pupal volume and error bars indicate SEM. * indicates $p<0.05$, **** indicates $p<0.0001$; error bars indicate SEM.
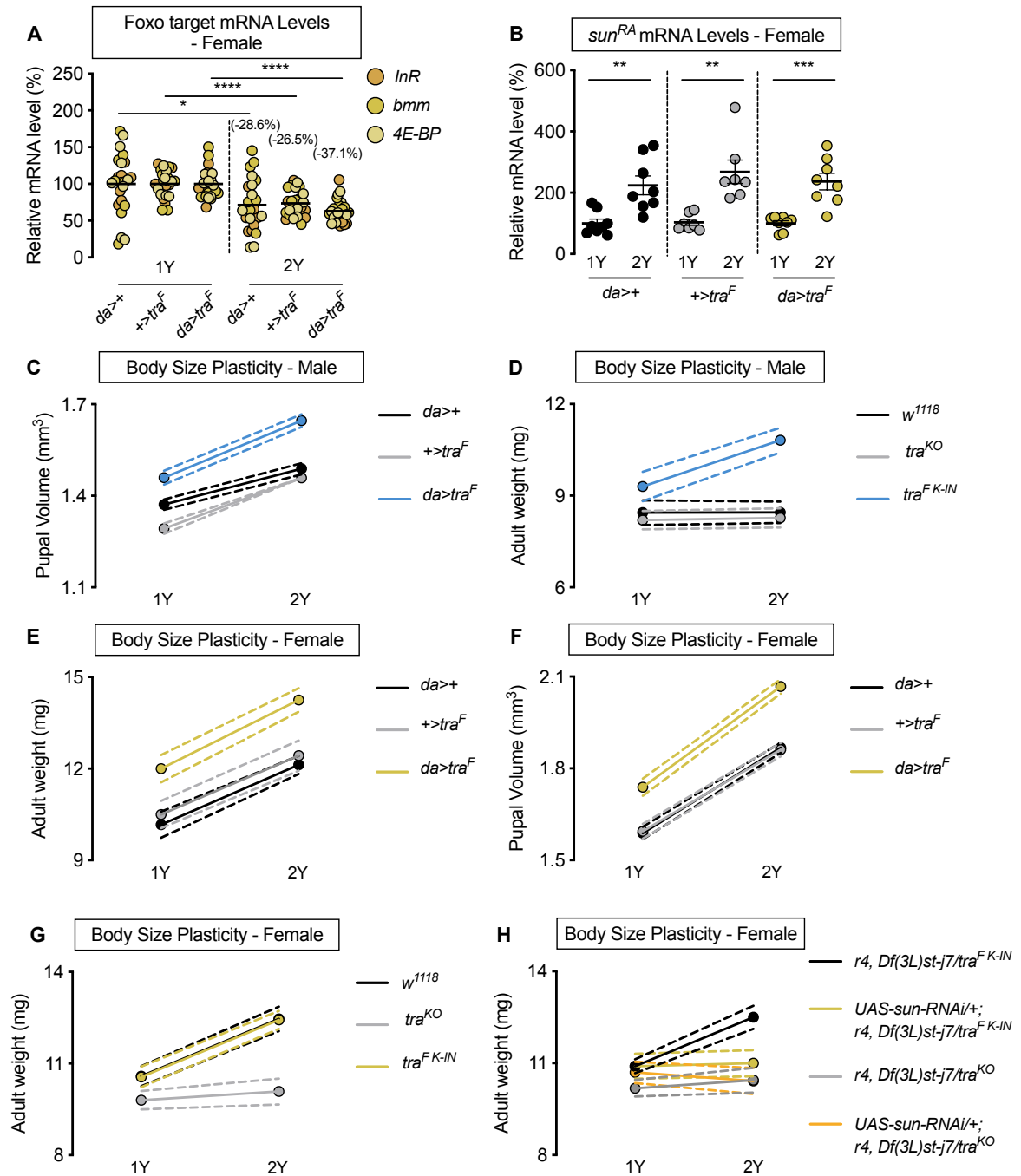
Figure S3.23. Sex determination gene *transformer* (*tra*) regulates increased nutrient-dependent body size plasticity.

(A) In control *da>+, +>UAS-tra^F* and *da>UAS-tra^F* females, mRNA levels of Foxo targets (*insulin receptor* (*InR*)*, brummer* (*bmm*)*,* and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*)), were significantly lower in larvae cultured on a protein-rich diet (2Y) compared with larvae raised on a diet containing half the protein content (1Y) (*p* = 0.0124 [*da>+*],

$p<0.0001$ [+>*UAS-tra^F*], and $p<0.0001$ [*da>UAS-tra^F*], respectively; Student's t test). n = 8 biological replicates. (B) In control *da>+* and +>*UAS-tra^F*, and *da>UAS-tra^F* females, mRNA levels of *sun^{RA}* were significantly higher in larvae cultured on 2Y compared with larvae raised on 1Y ($p = 0.0024$ [*da>+*], $p = 0.0013$ [+>*UAS-tra^F*], and $p = 0.0003$ [*da>UAS-tra^F*], respectively; Student's t test). n = 8 biological replicates. (C) Pupal volume was significantly higher in *da>+*, +>*UAS-tra^F*, and *da>UAS-tra^F* males reared on a protein-rich diet (2Y) compared with genotype-matched males cultured on a diet containing half the protein concentration (1Y) ($p<0.0001$ for all genotypes; two-way ANOVA followed by Tukey HSD test). Importantly, the magnitude of the nutrient-dependent increase in pupal volume was higher in *da>UAS-tra^F* males (genotype:diet interaction $p = 0.0012$; two-way ANOVA followed by Tukey HSD test). n = 70-91 pupae. (D) Adult weight was significantly higher in *tra$^{F\ K-IN}$* males, which express physiological levels of a functional Tra protein, when the males were reared on 2Y compared with genotype-matched males raised on 1Y ($p<0.0001$; two-way ANOVA followed by Tukey HSD test). In contrast, there was no significant increase in adult weight in *w^{1118}* and *tra^{KO}* male flies reared on 2Y compared with genotype-matched males raised on 1Y ($p>0.9999$ and $p = 0.9996$, respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in adult weight was significantly higher in *tra$^{F\ K-IN}$* males compared with *w^{1118}* and *tra^{KO}* male flies (genotype:diet interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). n = 9-11 groups of 10 flies. (E) Adult weight was significantly higher in *da>+*, +>*UAS-tra^F*, and *da>UAS-tra^F* females reared on 2Y compared with genotype-matched females cultured on 1Y ($p<0.0001$ for all genotypes; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in adult weight was not significantly different between *da>UAS-tra^F* females and *da>+* and +>*UAS-tra^F* controls (genotype:diet interaction $p = 0.5912$; two-way ANOVA followed by Tukey HSD test). n = 6-8 groups of 10 flies. (F) Pupal volume was significantly higher in *da>+*, +>*UAS-tra^F*, and *da>UAS-tra^F* females reared on 2Y compared with genotype-matched females cultured on 1Y ($p<0.0001$ for all genotypes; two-way ANOVA followed by Tukey HSD test). n = 68-94 pupae. (G) Adult weight was significantly higher in both *w^{1118}* females, and in females with a knock-in transgene of the female isoform of *tra* (*tra$^{F\ K-IN}$*), when reared on 2Y compared with 1Y ($p<0.0001$ for both genotypes; two-way ANOVA followed by Tukey HSD test). In contrast, the nutrient-dependent increase in adult weight was abolished in *tra* mutant females (*tra^{KO}*) reared on 2Y compared with genotype-matched females cultured on 1Y ($p = 0.864$; two-way ANOVA followed by Tukey HSD test). Importantly, the magnitude of the nutrient-dependent increase in adult weight

was significantly lower in tra^{KO} females, which lack a functional Tra protein, than in w^{1118} and $tra^{F\,K\text{-}IN}$ females (genotype:diet interaction $p<0.0001$; two-way ANOVA followed by Tukey HSD test). n = 10-16 groups of 10 flies. (H) Adult weight was significantly higher in $r4$-GAL4 control females with $tra^{F\,K\text{-}IN}$, when reared on 2Y compared with 1Y ($p<0.0001$ [$r4,Df(3L)st$-$j7/tra^{F\,K\text{-}IN}$]); two-way ANOVA followed by Tukey HSD test). In contrast, the nutrient-dependent increase in adult weight was abolished upon fat body knockdown of sun in a $tra^{F\,K\text{-}IN}$ female ($p = 0.9999$ [UAS-sun-$RNAi/+;r4,Df(3L)st$-$j7/tra^{F\,K\text{-}IN}$]); two-way ANOVA followed by Tukey HSD test). Adult weight was no different in tra mutant $r4$-GAL4 females ($r4,Df(3L)st$-$j7/tra^{KO}$) reared on 2Y compared with genotype-matched females cultured on 1Y ($p = 0.9550$; two-way ANOVA followed by Tukey HSD test). Importantly, adult weight was not further reduced in 1Y with fat body knockdown of sun in a tra mutant female (UAS-sun-$RNAi/+;r4,Df(3L)st$-$j7/tra^{KO}$) ($p = 0.99$ [UAS-sun-$RNAi/+;r4,Df(3L)st$-$j7/tra^{F\,K\text{-}IN}$ v UAS-sun-$RNAi/+;r4,Df(3L)st$-$j7/tra^{KO}$]); two-way ANOVA followed by Tukey HSD test). n = 9-12 groups of 10 flies. For body size plasticity graphs, filled circles indicate mean body size, and dashed lines indicate 95% confidence interval. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$; error bars indicate SEM.

A

α-pS6k

α-Actin

75
50
50
37

M   F
1Y

B *p-S6k* - Protein levels

ns

p-S6k/Actin ratio

M   F
1Y

C

α-pS6k

α-Actin

75
50
50
37

M   F
2Y

D *p-S6k* - Protein levels

ns

p-S6k/Actin ratio

M   F
2Y

E sun^{RA} mRNA Levels - Male

ns
ns

Relative mRNA level (%)

● *r4>+*
○ *+>rheb*
● *r4>rheb*

1Y

F

α-pS6k

α-Actin

75
50
50
37

1Y   2Y   1Y   2Y
w^{1118} tra^{KO}

G Female *p-S6k* - Fat body protein levels

ns   ns

p-S6k/Actin ratio
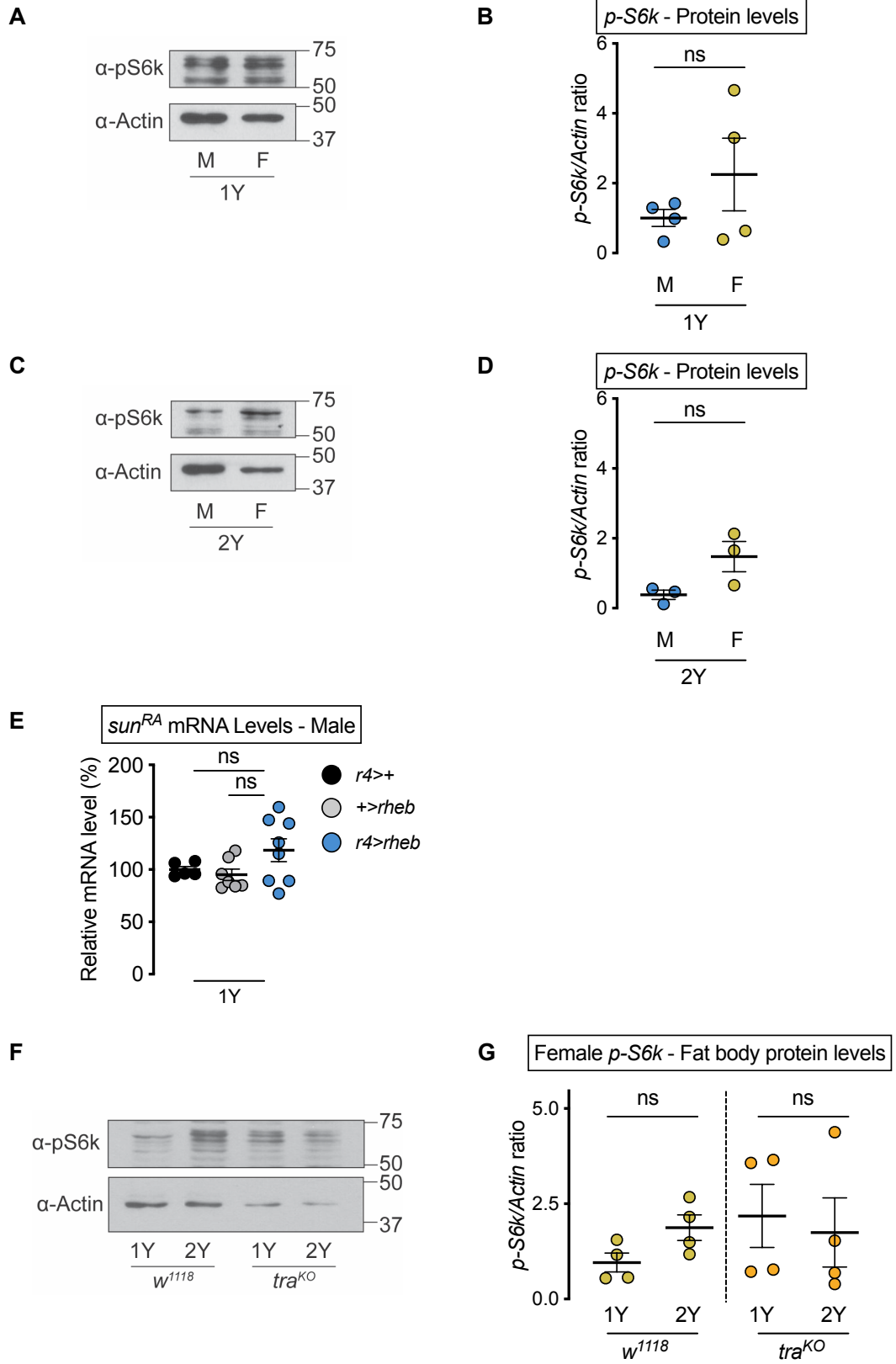
1Y   2Y   1Y   2Y
w^{1118} tra^{KO}

Figure S3.24. No nutrient- or Transformer-dependent sex difference in fat body target-of-rapamycin (TOR) signaling activity.

(A) Levels of phosphorylated S6 kinase (pS6k) were quantified in dissected fat bodies from w^{1118} male and female larvae cultured in 1Y. (B) pS6k levels were not different in males or females in 1Y (p = 0.2896; Student's t test). n = 4 biological replicates. (C) Levels of pS6k were quantified in dissected fat bodies from w^{1118} male and female larvae cultured in 2Y. (D) pS6k levels were not different in males or females in 2Y (p = 0.0732; Student's t test). n = 3 biological replicates. (E) mRNA levels of *stunted* (sun^{RA}) were not significantly different in *r4-GAL4>UAS-rheb* males compared with *r4-GAL4>+* and *+>UAS-rheb* control males (p = 0.3229 and p = 0.1252, respectively; one-way ANOVA followed by Tukey HSD test). n = 5-8 biological replicates. (F) Levels of pS6k were quantified in dissected fat bodies from female w^{1118} and female *transformer* (*tra*) mutant larvae cultured in 1Y and 2Y. (G) pS6k levels were not different in female w^{1118} or female tra^{KO} between 1Y and 2Y (p = 0.0702 and p = 0.737, respectively; Student's t test). n = 4 biological replicates. ns indicates not significant; error bars indicate SEM.
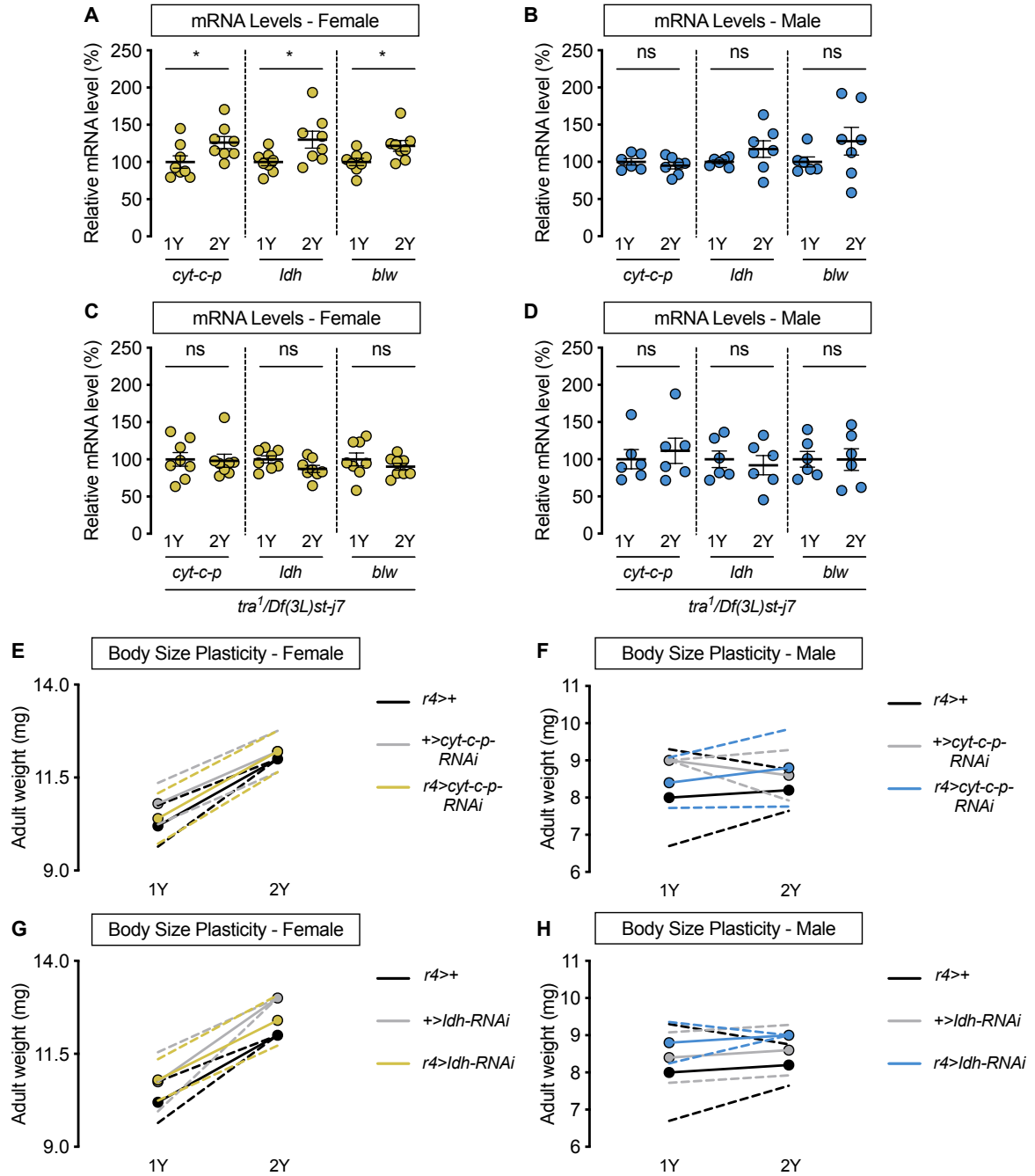
Figure S3.25. *transformer* (*tra*) is required for nutrient dependent upregulation of *spargel* (*srl*) target expression in females, but not all *srl* targets are not required for increased female nutrient-dependent body size plasticity.

(A) In control w^{1118} females, mRNA levels of the *srl* targets *Cytochrome c proximal* (*cyt-c-p*), *Isocitrate dehydrogenase* (*Idh*), and *bellwether* (*blw*) were significantly higher in larvae raised on a protein-rich diet (2Y) compared with larvae raised on a diet containing half the protein content (1Y) (*p* = 0.0409, 0.0307, and 0.0274, respectively; Student's *t* test). n = 8

biological replicates. (B) In control *w*^{*1118*} males, mRNA levels of *cyt-c-p, Idh,* and *blw* were not significantly different in larvae raised on 2Y compared with larvae raised on 1Y (*p* = 0.4316, 0.1906, and 0.2146, respectively; Student's *t* test). n = 6-7 biological replicates. (C) In *tra* mutant females, mRNA levels of *cyt-c-p, Idh,* and *blw* were not significantly different in larvae raised on a protein-rich diet (2Y) compared with larvae raised on a diet containing half the protein content (1Y) (*p* = 0.8865, 0.0731, and 0.334, respectively; Student's *t* test). n = 8 biological replicates. (D) In *tra* mutant males, mRNA levels of *cyt-c-p, Idh,* and *blw* were not significantly different in larvae raised on 2Y compared with larvae raised on 1Y (*p* = 0.6078, 0.6453, and 0.9819, respectively; Student's *t* test). n = 6 biological replicates. (E) Adult weight was significantly higher in *r4>+, +>cyt-c-p-RNAi*, and *r4>cyt-c-p-RNAi* females reared on 2Y compared with genotype-matched females cultured on 1Y (*p*<0.0001 [*r4>+*], *p* = 0.0004 [*+>cyt-c-p-RNAi*], and *p*<0.0001 [*r4>cyt-c-p-RNAi*], respectively; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in adult weight was not significantly different (genotype:diet interaction *p* = 0.4936; two-way ANOVA followed by Tukey HSD test). n = 5 groups of 10 flies. (F) Adult weight was not significantly different in *r4>+, +>cyt-c-p-RNAi*, and *r4>cyt-c-p-RNAi* males reared on 2Y compared with genotype-matched males cultured on 1Y (*p* = 0.9954 [*r4>+*], *p* = 0.8873 [*+>cyt-c-p-RNAi*], and *p* = 0.8873 [*r4>cyt-c-p-RNAi*], respectively; two-way ANOVA followed by Tukey HSD test). n = 4-5 groups of 10 flies. (G) Adult weight was significantly higher in *r4>+, +>Idh-RNAi*, and *r4>Idh-RNAi* females reared on 2Y compared with genotype-matched females cultured on 1Y (*p*<0.0001 for all comparisons; two-way ANOVA followed by Tukey HSD test). The magnitude of the nutrient-dependent increase in adult weight was not significantly different (genotype:diet interaction *p* = 0.2104; two-way ANOVA followed by Tukey HSD test). n = 4-5 groups of 10 flies. (H) Adult weight was not significantly different in *r4>+, +>Idh-RNAi*, and *r4>Idh-RNAi* males reared on 2Y compared with genotype-matched males cultured on 1Y (*p* = 0.9912 [*r4>+*], *p* = 0.9885 [*+>Idh-RNAi*], and *p* = 0.9885 [*r4>Idh-RNAi*], respectively; two-way ANOVA followed by Tukey HSD test). n = 4-5 groups of 10 flies. For body size plasticity graphs, filled circles indicate mean adult weight, and dashed lines indicate 95% confidence interval. * indicates *p*<0.05; ns indicates not significant; error bars indicate SEM.

Table S3.1. Extra fly food recipes used in chapter 3, not detailed in methods.

Ingredient	1S	1Y Calories
H2O (L)	1	1
Sucrose (g)	10.25	20.5
D-glucose (g)	35.45	70.9
Cornmeal (g)	48.5	28.6
Yeast (g)	45.3	45.3
Agar (g)	4.6	4.6
CaCl2 (g)	0.5	0.5
MgSO4 (g)	0.5	0.5

Table S3.2. A complete list of primers used in chapter 3.

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
InR	GCTGCATCTCCTGTCGAAAT	CGTTGGACAGTGGGTGATAC
bmm	GTCCCTTCAGTCCCTCCTTC	TATGAAGCACGCACACAACA
4E-BP	GCTAAGATGTCCGCTTCACC	CCTCCAGGAGTGGTGGAGTA
sunRA	GGGTTTGACGCTGAGCTG	CATTTTGCGCGAGTCCTT
sunRB	CTTTCATGAGCTGGCTTGC	CATTTTGCGCGAGTCCTT
dilp1	CCCCGGAAACCACAAACTCT	TAAAGCCATGGGGACACACC
dilp2	TCCACAGTGAAGTTGGCCC	AGATAATCGCGTCGACCAGG
dilp3	AGAGAACTTTGGACCCCGTGAA	TGAACCGAACTATCACTCAACAGTCT
dilp4	GCGGAGCAGTCGTCTAAGGA	TCATCCGGCTGCTGTAGCTT
dilp5	GAGGCACCTTGGGCCTATTC	CATGTGGTGAGATTCGGAGCTA
dilp6	CGATGTATTTCCCAACAGTTTCG	AAATCGGTTACGTTCTGCAAGTC
dilp7	CAAAAAGAGGACGGGCAATG	GCCATCAGGTTCCGTGGTT
dilp8	GGACGGACGGGTTAACCATT	CATCAGGCAACAGACTCCGA
Gbp1	CATCCTACCGCTGGTCTTCC	ACTGGCAGCACAGTGGTGTT
Gbp2	ACTTCAGCTCGTCCCCAGAA	ACGGTTGACGACCTCCTGAT
CCHa2	GCCTACGGTCATGTGTGCTAC	ATCATGGGCAGTAGGCCATT
upd2	GCGCGGTGGGTTATATCTT	ATCAGAGATCCCGGAGTGG
GAL4	CACCGACGCTAATGATGTTG	TGGAACCTGACTCGAAGACC
cyt-c-p	CTGGTGATGTTGAGAAGGGAAAG	AGATTGGGTCCAACCTTGTGC
ldh	TTGAGCTGCATACCTACGATCT	TCAGCACAGTCAATGGTGACC
blw	CCGTTTCCGTGTGGGAATCAA	AGAGCGGTCTTACCAGTCTGA

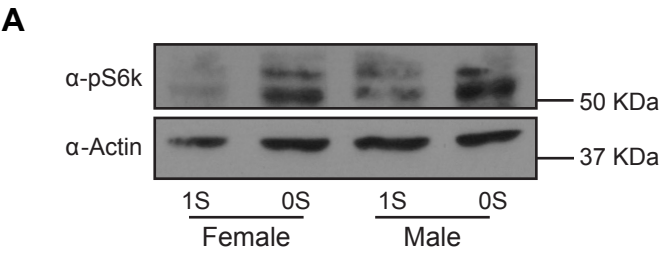## A.2 SUPPLEMENTARY DATA FOR CHAPTER 4

A

Figure S4.1. A low sugar diet has sex-biased effects on target of rapamycin (TOR) signaling.

(A) Levels of pS6k in females and males raised in 1S or 0S.

Table S4.1. Fly food recipes used in chapter 4.

Ingredients (per L)	1S	0.75S	0.5S	0.25S	0S
H2O (L)	1	1	1	1	1
Sucrose (g)	20.5	15.375	10.25	5.125	0
D-glucose (g)	70.9	53.175	35.45	17.725	0
Cornmeal (g)	48.5	48.5	48.5	48.5	48.5
Yeast (g)	45.3	45.3	45.3	45.3	45.3
Agar (g)	4.6	4.6	4.6	4.6	4.6
CaCl2 (g)	0.5	0.5	0.5	0.5	0.5
MgSO4 (g)	0.5	0.5	0.5	0.5	0.5

Table S4.2. A complete list of primers used in chapter 4.

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
Act5c	TTGTCTGGGCAAGAGGATCAG	ACCACTCGCACTTGCACTTTC
B-tub	ATCATCACACACGGACAGGA	GAGCTGGATGATGGGGAGTA
InR	GCTGCATCTCCTGTCGAAAT	CGTTGGACAGTGGGTGATAC
bmm	GTCCCTTCAGTCCCTCCTTC	TATGAAGCACGCACACAACA
4E-BP	GCTAAGATGTCCGCTTCACC	CCTCCAGGAGTGGTGGAGTA
NLaz	TCATACGCCGTCGTCTACAG	GAGGAAGGCCTGGGATACAT
puc	GCAGAATTTGCGCAAGAGCGG	GAGCAGTTACTACCCGCCAG
mino	AGCTGCATCTATGCCGAAAG	TGAGCGACTCACGAGACATC
ACC	CAAAGTACCGAGGATATACATCTCC	GATAGCCCTCACCGAGTTCA
wun2	CCTCTTACTGTGATGCTTGTGG	TGTTCGTGGATAGCTGCTCT
lpin	GATCTGTTTCCCGACAAGGA	ACTGGAATGTTTGGGTCAGC
mdy	CAGTGCCGTCTTCCATGAAT	CCATTATGCACAGAGGCTGA
mod(mdg4)	TTTATTAGCACCGCGGAATC	GTCCACGGTCTCGATCTTGT
CG5966	TCTTTCGAGAGCTTTAAGGACA	AGGGCTTGCTATCTCCAGTC
dob	GTGGTTCAATTTGCCGGAGT	TCCTCGGACCTGTGGAG
CG1882	CGCAGTACATACACCAGTGC	CGCTGCGACTTGATCTTCTC
hsl	AAATTTCACAATGTTATCCAACG	TTGTCGGGGTCTCAGTTCTC
plin2	ATTGGATAGCCGTCCAACTG	AGTCTGGCTGTCAACGGAGT
plin1	CCGCATCATCACAATCTCAC	TGGGTGGCTGAATAATGGTT
seipin	CCCGTTCACATGCAGTTCAA	GCCAACCATCAGGAGTTGC
tRNAiMet	AGAGTGGCGCAGTGGAAG	AGAGCAAGGTTTCGATCCTC
RpL6	CTCTGTACCGCCTGAAGGAC	AGGAGGCCTTGCTCTTCTTC
RpL14	TCTGACCAAGTACCGCATCA	GCAGATGTTCTGTGCCTTGA
RpL21	AGGCATATCATGGCAAAACC	CACTTGGAGTGGTGGATGTG
RpL22	AGCTGATCCCTTCAGTGGAA	GGCTAGCCCGAAGTTTTCTT
RpL23	GCTCAGGAAGAAGGTCATGC	CTTCATTTCGCCCTTGTTGT
RpL27	CCCATTCGCTACTTGTGGTT	CATCACCATAGGCACGTTTG
RpL30	GGTGGCCGTTAAGAAACAAA	GGGTCTTCAAGGTCTGCTTG
RpL36	AAGGATAAGAGGGCCCTGAA	CTCAGCTGGGTGAGGATGTT
RpS13	AGGCAGTGCTCGACTCGTAT	TTCCCGAGGATCTGTACCAC

RpS15	TCGAACAAAATGCCATACGA	CGACTTGCTGCATAGAACGA
RpS16	GAACCCAAGGTCCTGCAATA	TACATGACCACCACCGCTAA
RpS20	ACGGTGCAAAGAACCAGAAC	AGTCTTACGGGTGGTGATGC
MetRS	AAATGCAGCTCCTCACATTG	CGTGCAGAGTCGTACATCCT
LeuRS	GTCCACCTGCAGTCCAAAAT	CGGGATCCCAGTTAACAAGA
GlyRS	TCAAATTGGCAACTCCTTCC	GAGCACGGGATCACAGAAAT
IleRS	GTTGTGGACCCTTGCGTAGT	AACACAGCATGCGAAGTTTG
AlaRS	AAGCAACTTCCCAAGAAGCA	CGAAAAGTGGCACAAACAGA
LysRS	ATCTGCATTTCGGGTTGAAG	GATGATCTTGGCACGGATCT