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Effects of Altering Insulin Signaling Pathway Genes on Sex Specific Growth in *Drosophila melanogaster*

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Abstract

Introduction: The mechanisms of growth and development in humans are becoming better understood by studying *Drosophila melanogaster*. Although the presence of two major nutrient-sensing pathways and their intracellular signaling molecules have been identified, sex-specific effects of altering these pathways are poorly understood. This study aims to use *Drosophila* strains to confirm the expression patterns of 5 GAL4 strains (r4, PPL, Lsp, da, nubbin) with green fluorescent protein (GFP), and observe the sex-specific phenotypic responses in a tissue-specific and systemic manner.

Methods: Each GAL4 strain was crossed with a UAS-GFP NLS (upstream activation sequence-nuclear localization sequence) reporter to view GFP expression patterns and to UAS-insulin receptor dominant negative (InR DN) and constitutively active (InR CA) reporters to assess phenotypic response. Sex-specific phenotypic responses were observed by measuring wing size and thorax length of both male and female flies.

Results: It was observed that all 5 GAL4 strains exhibited expression patterns consistent with their tissue specific promoters. In addition, when all 5 GAL4 strains were crossed with the UAS-InR DN and UAS-InR CA, sex-specific phenotypic responses were observed in terms of tissue-specific and systemic growth.

Discussion: Confirming the expression patterns of all 5 GAL4 strains is necessary when looking at tissue-specific and systemic phenotypic responses, as it ensures that phenotypic responses are due to altering of InR and not of non-functioning GAL4 strains themselves. Interestingly, the mean thorax lengths for the InR CA were consistently smaller than the InR DN for all GAL4 strains.

Conclusion: Although this study found promising results, more research is required to truly understand sexual size dimorphism in growth patterns. A next step is using the UAS-GAL4 system to alter genes of other signaling molecules within the IIS or TOR pathway. By looking at different key players within the pathway, we can understand how all these molecules work together and which ones have a greater sex-specific effect.

Keywords: insulin signaling pathway; sexual dimorphism; growth; UAS-GAL4 system; *Drosophila melanogastor*

Introduction

In *Drosophila*, growth is modulated in response to nutrient availability via nutrient-sensing pathways [1]. The regulation of tissue and body growth maintains optimal fitness, thus, this system is tightly controlled and coordinated at both a cellular and systemic level [1]. Cellular nutrient-sensing is mediated through two main pathways: the insulin and insulin-like growth factor signaling (IIS) pathway [2] and target of rapamycin (TOR) signaling pathway [3]. Systemic nutrient-sensing is mediated through extracellular hormones and growth factors that circulate the body and provide information regarding nutritional status to individual cells [1]. This allows for coordination between growth rates of adjacent tissues and consumption of energy stores to facilitate systemic growth [1].

Different types of tissues work together to regulate growth in response to nutrient availability in *Drosophila*. The brain has insulin producing cells (IPCs) that produce insulin-like peptides (ILPs). ILPs circulate throughout the body and bind to the insulin receptors (InRs) within the plasma membrane. ILPs function analogously to mammalian insulin, which stimulates overall growth by upregulating cellular processes like transcription, translation, and protein synthesis [4]. The fat body, a major storage site for macromolecules, plays a central role in the exchange of metabolites under different developmental and environmental conditions. It integrates multiple nutritional and hormonal signals into regulatory molecules in the form of fat body signals (FBSs). FBSs regulate ILP synthesis and their release from IPCs in the brain in order to control the IIS pathway [5]. The prothoracic gland (PG) is a specialized neuroendocrine organ located in the head region of *Drosophila*. It produces and secretes ecdysone, a steroid hormone and key regulator of developmental timing. A peak in ecdysone secretion signals

an end of the larval stage and induces pupation. *In vitro* experiments on insects show that low concentrations of ecdysone stimulate growth by regulating the cell cycle and increasing the rate of mitosis, whereas at high levels, it causes differentiation of cells and tissues [6]. The fat body monitors levels of circulating ecdysone. The prothoracicotropic hormone, a neurosecretory hormone that is produced by the medial neurosecretory cells of the brain promotes the synthesis of ecdysone in the PG and its release into the hemolymph [7]. *Drosophila* imaginal discs are larval epithelial cells that form the adult appendages of the fly. Each imaginal disc starts as a cluster of cells which exponentially proliferate during the larval phase, after which tightly integrated patterning processes ensure appropriate fates are assigned to cells during tissue growth. Pattern regulators inform disc cells about their identity and position within the wing disc [8]. Several growth effectors also play a role in wing growth, and they can be directly or indirectly regulated.

Current literature shows that disrupting the IIS pathway reduces larval growth, cell size, and cell number [9]. The IIS pathway also regulates growth, development, and metabolic homeostasis [10]. Many of the functions of both IIS and TOR nutrient sensing pathways are deeply researched. However, sex-specific effects of these patterns are poorly understood. Therefore, this study will observe these sex-specific effects using the UAS-GAL4 system, which alters gene expression and function. This bipartite model includes two strains of flies: (1) a strain with the GAL4 transcription factor and its tissue-specific promoter and (2) a strain with the upstream activation sequence (UAS) enhancer specific for the GAL4 transcription factor and coupled with a downstream gene of interest [11]. The GAL4 transcription factor is a positive regulator of galactose induced genes [11]. When both strains are crossed with each other, the GAL4 transcription factor binds to the UAS enhancer and activates its coupled gene of interest, occuring within the tissue that the GAL4 promoter is specific for [11]. This project will use 5 GAL4 strains: r4-GAL4, PPL-GAL4, Lsp-GAL4, da-GAL4 and nubbin-GAL4. The nubbin-GAL4 strain was chosen to identify a tissue-specific phenotypic response as it was only expressed in the wing imaginal disk. Alternatively, the other strains were chosen to identify a systemic phenotype response as the r4-GAL4, PPL-GAL4, and Lsp-GAL4 were expressed in the fat body and da-GAL4 was expressed ubiquitously.

This study has two aims: (1) whether all tissue-specific GAL4 strains show correct expression patterns when crossed with a UAS-GFP.NLS reporter, and (2) whether there a phenotypic response when the GAL4 strains are crossed with UAS-InR DN and UAS-InR DN CA to alter insulin receptor expression. Two reporters will be used to alter InR function in this experiment: InR dominant negative (DN) and InR constitutively active (CA). The InR DN inhibits insulin receptor function and InR CA maintains insulin receptor function with or without the presence of its ligands. It is hypothesized that considering the GAL4 strains are expressed at the correct location, each strain crossed with the InR CA will exhibit greater tissue-specific and systemic growth compared to InR DN, and that there will be a display of sexual size dimorphism within each strain.

Methods

This project used 5 GAL4 strains: r4-GAL4, PPL-GAL4, Lsp-GAL4, da-GAL4 and nubbin-GAL4, and 4 UAS strains: UAS-GFP Lac7 NLS, UAS-GFP NLS.14, UAS-InR DN, and UAS-InR CA. All strains were ordered from the Bloomington Drosophila Stock Centre.

First Set of Crosses: GAL4 Check with GFP

BSC 33832: *y^{1}w*;P{w^{+};r4-GAL4}3*	X	***BSC 6451***: y'w';*UAS-GFP Lac7 NLS*
BSC 33832: *y^{1}w*;P{w^{+};r4-GAL4}3*	X	***BSC 4775***: w^{1118};.*UAS-GFP NLS.14.*
BSC 58768: *w*;P{w^{+};PPL-GAL4}2*	X	***BSC 6451***: y'w';*UAS-GFP Lac7 NLS*
BSC 58768: *w*;P{w^{+};PPL-GAL4}2*	X	***BSC 4775***: w^{1118};.*UAS-GFP NLS.14.*
BSC 6357: *y^{1}w^{1118};P{w^{+};Lsp-GAL4}3*	X	***BSC 6451***: y'w';*UAS-GFP Lac7 NLS*
BSC 6357: *y^{1}w^{1118};P{w^{+};Lsp-GAL4}3*	X	***BSC 4775***: w^{1118};.*UAS-GFP NLS.14.*
BSC 55851: *w*;P{w^{+};da-GAL4}3*	X	***BSC 6451***: y'w';*UAS-GFP Lac7 NLS*
BSC 55851: *w*;P{w^{+};da-GAL4}3*	X	***BSC 4775***: w^{1118};.*UAS-GFP NLS.14.*
BSC 25754: *SAM w;SAM;P{w^{+};nubbin-GAL4}2*	X	***BSC 6451***: y'w';*UAS-GFP Lac7 NLS*
BSC 25754: *SAM w;SAM;P{w^{+};nubbin-GAL4}2*	X	***BSC 4775***: w^{1118};.*UAS-GFP NLS.14.*

Figure 1. First Set of 10 Crosses to Check GAL4 Expression Patterns with GFP (Created with Microsoft Word).

Flies were grown at 24°C. Figure 1 shows crosses of all GAL4 strains with UAS-GFP NLS (nuclear localization of green fluorescent protein). The 3rd instar larvae (5 for each cross) of the F1 progeny were dissected and mounted on a microscope slide using phosphate-buffered saline. Larval tissue was imaged under the Olympus BX43 Microscope at 4x or 10x magnification with the X-Cite 120 LED light source.

Second Set of Crosses: Tissue-specific and Systemic Growth

BSC 33832: *$y^1w^*;P\{w^+;r4\text{-}GAL4\}3$*	X	***BSC 8253***: SAM w;SAM;SAM *UAS-InR DN*
BSC 33832: *$y^1w^*;P\{w^+;r4\text{-}GAL4\}3$*	X	***BSC 8248***: SAM w;SAM *UAS-InR CA*
BSC 58768: *$w^*;P\{w^+;PPL\text{-}GAL4\}2$*	X	***BSC 8253***: SAM w;SAM;SAM *UAS-InR DN*
BSC 58768: *$w^*;P\{w^+;PPL\text{-}GAL4\}2$*	X	***BSC 8248***: SAM w;SAM *UAS-InR CA*
BSC 6357: *$y^1w^{1118};P\{w^+;Lsp\text{-}GAL4\}3$*	X	***BSC 8253***: SAM w;SAM;SAM *UAS-InR DN*
BSC 6357: *$y^1w^{1118};P\{w^+;Lsp\text{-}GAL4\}3$*	X	***BSC 8248***: SAM w;SAM *UAS-InR CA*
BSC 55851: *$w^*;P\{w^+;da\text{-}GAL4\}3$*	X	***BSC 8253***: SAM w;SAM;SAM *UAS-InR DN*
BSC 55851: *$w^*;P\{w^+;da\text{-}GAL4\}3$*	X	***BSC 8248***: SAM w;SAM *UAS-InR CA*
BSC 25754: *SAM w;SAM;$P\{w^+;nubbin\text{-}GAL4\}2$*	X	***BSC 8253***:SAM w;SAM;SAM *UAS-InR DN*
BSC 25754: *SAM w;SAM;$P\{w^+;nubbin\text{-}GAL4\}2$*	X	***BSC 8248***: SAM w;SAM *UAS-InR CA*

Figure 2. Second Set of 10 Crosses to Visualize Tissue-Specific and Systemic Growth (Created with Microsoft Word).

Flies were grown at 24°C. Figure 2 shows crosses of all GAL4 strains with UAS-InR DN and UAS-InR CA. Adult flies of the F1 generation (10 male and 10 female) were stored in 70% ethanol and imaged under the Leica M125 Microscope in greyscale at 5.0 (50x total) magnification with a DFC Camera. All thorax measurements were taken with ImageJ and the data was transferred to RStudio as a .csv file for statistical analysis.

Table 1. Fly Food Recipe

Batch Size / *Ingredients*	**Single**	**Double**	**Triple**	**Quadruple**
ddH_20 (l)	4.25	8.5	12.75	17
Molasses (g)	390	780	1170	1560
Carageenam (g)	27	54	81	108
Cornmeal (g)	245	490	735	980
Yeast (g)	50	100	150	200
Propionic acid (ml)	12	24	36	48
10% Methyl paraben (ml)	25	50	75	100

Note: courtesy of the Dworkin Lab.

Results

GAL4 Check with GFP

All 5 GAL4 strains were crossed with GFP to confirm whether their actual expression pattern is consistent with their tissue-specific promoter. When viewing the larvae under a fluorescent microscope, all 5 GAL4 strains showed correct expression patterns (Figure 3). The r4-GAL4 was expressed in the fat body and salivary glands, the

PPL-GAL4 was expressed in the fat body, salivary glands, and the gut, the Lsp-GAL4 was expressed in the fat body and salivary glands, the da-GAL4 was expressed ubiquitously, except for the wing imaginal discs, and the nubbin-GAL4 was only expressed in the wing imaginal discs.

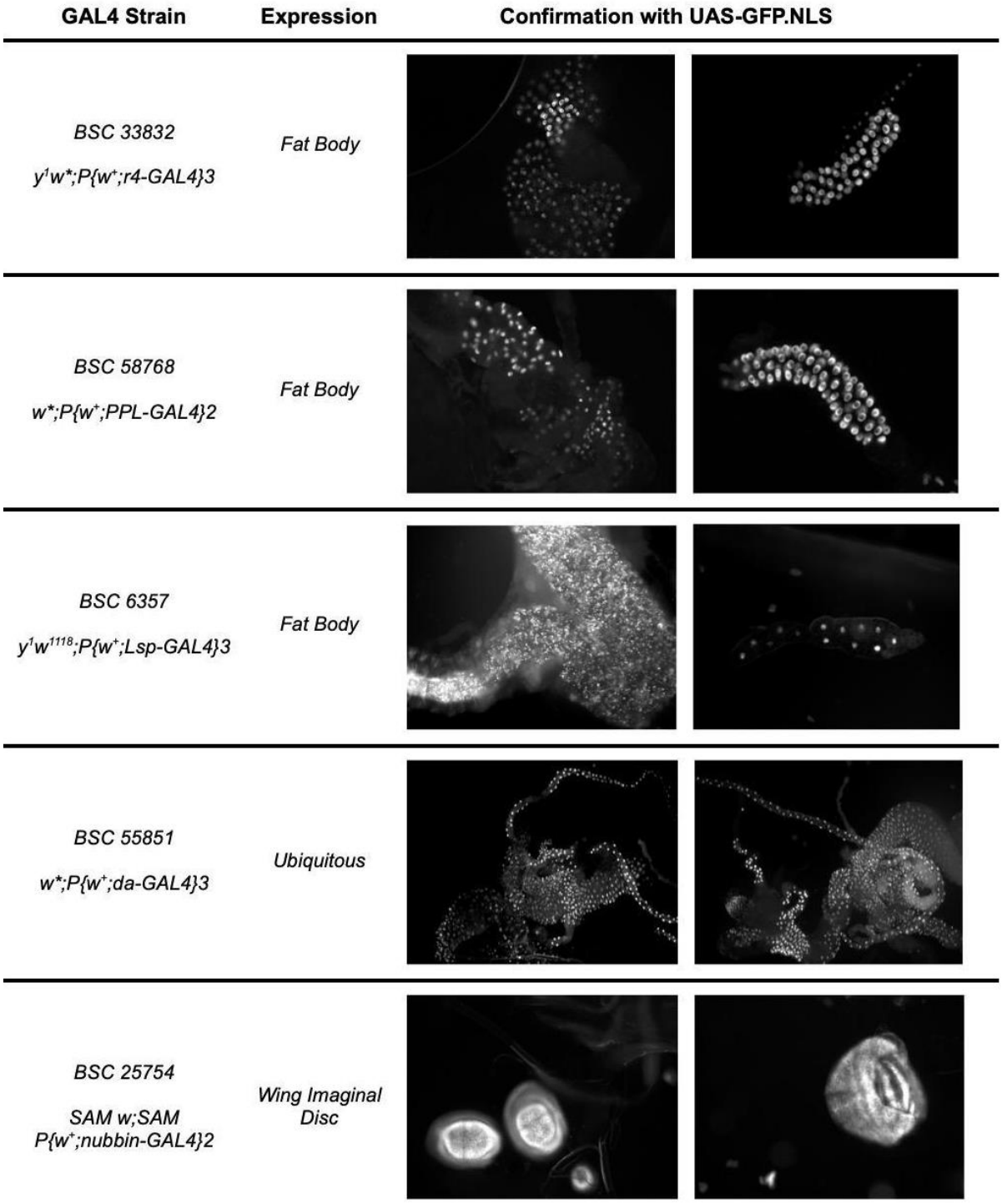

Figure 3. List of GAL4 strains, expression of their tissue-specific promoters, and fluorescent images to confirm their expression pattern. All GAL4 strains correctly expressed GFP (images taken with the Leica M125 Microscope in greyscale at 5.0 (50x total) magnification with a DFC Camera; created with Microsoft Word).

Tissue-specific Phenotypic Response: Wing Size

Tissue-specific growth was determined by looking at wing images of the nubbin-GAL4 strain and comparing the control (nubbin-GAL4 only), nubbin-GAL4 with InR DN, and nubbin-GAL4 with InR CA. Compared to the size of the control, wings of the InR DN were smaller for both male and female flies at 18°C and female flies at 24°C. Compared to the size of the control, wings of the InR CA were larger for both male and female flies at 18°C and male flies at 24°C.

Systemic Phenotypic Response: Thorax Length

Systemic growth was determined by looking at the thorax lengths of male and female flies for the second set of crosses (see *Methods*). The mean thorax lengths were plotted to compare the sex-specific differences (Figure 4). For all 10 crosses, the mean female thorax length was larger than the male. The mean thorax lengths for the InR CA were consistently smaller than the InR DN for all GAL4 strains.

To compare the sexual size dimorphism for each cross, a graph plotted the difference between female and male mean thorax length (Figure 5). The da-GAL4 with the InR DN shows the greatest size difference and the PPL-GAL4 with the InR CA shows the least.

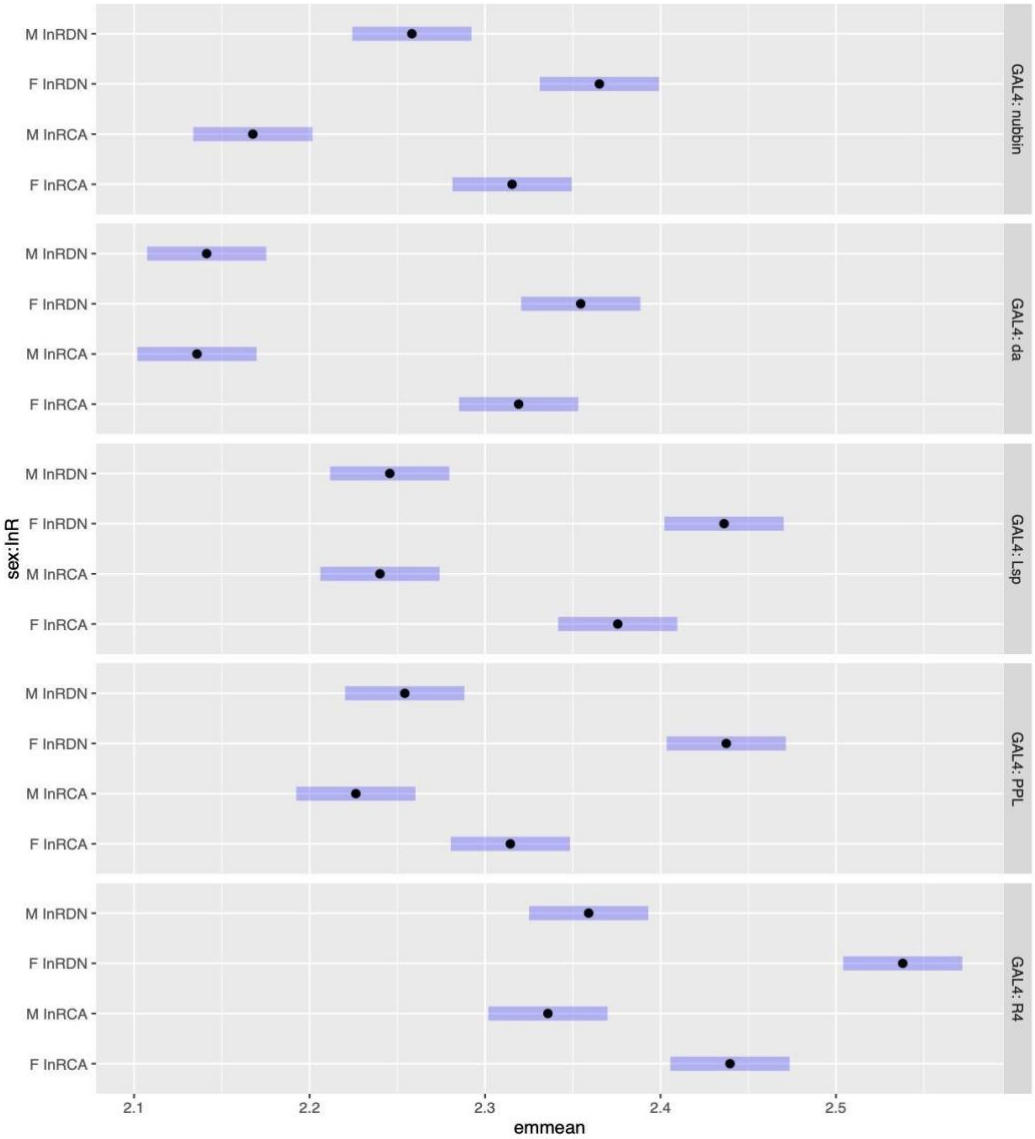

Figure 4. Mean Thorax Length for Male (N=10) and Female (N=10) Flies for Each GAL4 Strain with InR DN, and with InR CA (created with R software).

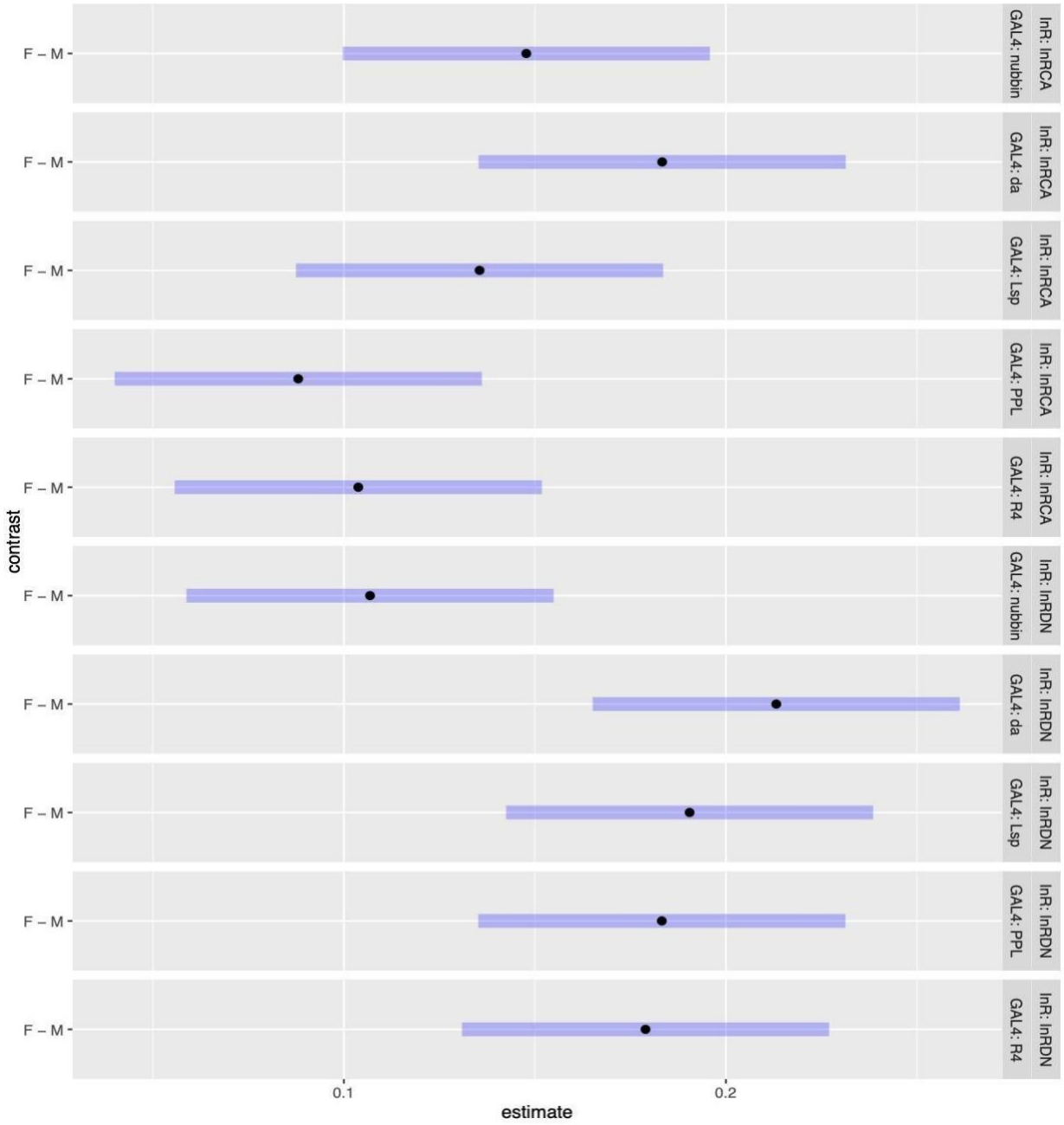

Figure 5. Difference in Female and Male Mean Thorax Length for Each GAL4 Strain with InR DN, and with InR CA (Created with R software).

Discussion

To observe the sex-specific patterns of growth in *Drosophila*, genes of the IIS pathway were altered using the UAS-GAL4 system. The expression patterns of 5 GAL4 stains (r4, PPL, Lsp, da, nubbin) were checked with GFP NLS to confirm whether they were consistent with the location of their respective tissue-specific promoter. Although all GAL4s showed correct expression of GFP, for the r4, PPL, and Lsp strains, expression was seen in unexpected tissues as well (i.e., salivary glands). Being aware of all expressed tissues was important when looking at tissue-specific and systemic phenotypic responses. This ensured that the phenotypic responses were due to altering of InR and not of non-functioning GAL4 strains.

Sexual size dimorphism was observed when looking at wing size of the nubbin-GAL4 and thorax lengths of all 5 GAL4s. However, the InR CA, despite maintaining InR function, had a consistently smaller mean thorax length than the InR DN. To ensure no mistake was made when performing the crosses, the nubbin-GAL4 wing sizes for both InR DN and CA were checked. As expected, the wing size

for the InR CA was significantly larger than the InR DN, confirming correct crossing. Additionally, performing a cross with the UAS-InR without the presence of the GAL4 would confirm whether the anomaly was due to the UAS-InR strain itself or the interaction between the UAS-GAL4 and its effect on the InR. Another possible explanation could be that due to the complexity of the IIS pathway and its tightly regulated structure, perhaps altering one aspect of the nutrient-sensing pathway (i.e., InR) may not be enough to portray a systemic response. This may be why a clear difference between wing sizes of the nubbin-GAL4 was observed, but for thorax lengths, the InR DN and InR CA did not behave as expected.

Overall, the experiment has two main findings: (1) the 5 tested GAL4 strains all show correct expression patterns and can be used for further experimentation, and (2) there is a sex-specific effect on growth in both a tissue-specific and systemic manner.

Conclusion

In summary, all tested GAL4 strains exhibit a correct expression pattern, specific to their tissue of interest. Nubbin-GAL4 crossed with InR DN and with InR CA displayed a tissue-specific phenotypic response in the wing. All 5 GAL4 strains crossed with InR DN and InR CA displayed a systemic phenotypic response, determined by measuring thorax length. Both phenotypic responses depict sexual size dimorphism, with some more significant than others for mean thorax length. The mean thorax lengths of the InR CA were smaller than the InR DN, otherwise, all other results were consistent with the proposed hypothesis. However, the observations of this study should be interpreted considering a major limitation. As the sample size was very small, statistical analysis was not conducted to determine significance. Thus, it is important for future studies to use a much larger sample size to derive more definitive conclusions. Although this project found some promising results, more research is required to truly understand sexual size dimorphism in growth patterns. A next step is using the UAS-GAL4 system to alter genes of other signaling molecules within the IIS or TOR pathway, like the mTORC1, mTORC2, FoxO, PI3K, or AKT. By looking at different key players within the pathway, we can understand how all these molecules work together and which ones have a greater sex-specific effect.

List of Abbreviations

AKT: protein kinase B
CA: constitutively active
DN: dominant negative
FBS: fat body signal
FoxO: Forkhead Box class O
GFP: green fluorescent protein
IIS: insulin and insulin-like growth factor signaling
InR: insulin receptor
IPC: insulin producing cell
ILP: insulin-like peptide
NLS: nuclear localization sequence
PI3K: phosphatidylinositide 3-kinase
PDK: phosphoinositide-dependent protein kinase 1
PG: prothoracic gland
TOR: target of rapamycin
UAS: upstream activation sequence

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval/Participant Consent

No ethics approval required since no human participants were involved in the study.

Authors' Contributions

SP: made contributions to the design of the study, collected and analyzed data, drafted the manuscript and gave final approval of the version to be published.
ID: made contributions to the design of the study, analyzed data, edited the manuscript, and gave final approval of the version to be published.

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