SEX-SPECIFIC PLASTICITY AND THE NUTRITIONAL GEOMETRY OF INSULIN-SIGNALING GENE EXPRESSION IN *DROSOPHILA MELANOGASTER*

Jeanne M.C. McDonald[1,2] *, Pegah Nabili[2] *, Lily Thorsen[2] *, Alexander Shingleton[2,3]

[1] Department of Ecology and Evolutionary Biology, Cornell University, Corson Hall Ithaca, NY 14853

[2] Department of Biology, Lake Forest College, 555 North Sheridan Road, Lake Forest, IL 60045

[3] Department of Biological Sciences, University of Illinois at Chicago, 840 W Taylor St, Chicago, IL 60607

* These authors contributed equally to this study

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14 # 1. Abstract

15 **Background:** Sexual-size dimorphism (SSD) is replete among animals, but while the selective pressures
16 that drive the evolution of SSD have been well studied, the developmental mechanisms upon which these
17 pressures act are poorly understood. Ours and others' research has shown that SSD in *Drosophila* reflects
18 elevated levels of nutritional plasticity in females versus males, such that SSD increases with dietary
19 intake and body size, a phenomenon called sex-specific plasticity (SSP). Additional data indicate that
20 while body size in both sexes responds to variation in protein level, only female body size is sensitive to
21 variation in carbohydrate level. Here we explore whether these difference in sensitivity at the
22 morphological level are reflected by differences in how the insulin/IGF-signaling (IIS) and TOR-signaling
23 pathways respond to changes in carbohydrates and proteins in females versus males, using a nutritional
24 geometry approach.

25 **Results:** The IIS-regulated transcripts of *4E-BP* and *InR* most strongly correlated with body size in
26 females and males respectively, but neither responded to carbohydrate level and so could not explain the
27 sex-specific response to body size to dietary carbohydrate. Transcripts regulated by TOR-signaling did,
28 however, respond to dietary carbohydrate in a sex-specific manner. In females, expression of *dILP5*
29 positively correlated with body size, while expression of *dILP2,3* and *8,* was elevated on diets with a low
30 concentration of both carbohydrate and protein. In contrast, we detected lower levels of dILP2 and 5
31 protein in the brains of females fed on low concentration diets. We could not detect any effect of diet on
32 *dILP* expression in males.

33 **Conclusion:** Although females and males show sex-specific transcriptional responses to changes in
34 protein and carbohydrate, the patterns of expression do not support a simple model of the regulation of
35 body-size SSP by either insulin- or TOR-signaling. The data also indicate a complex relationship between
36 carbohydrate and protein level, *dILP* expression and dILP peptide levels in the brain. In general, diet

quality and sex both affect the transcriptional response to changes in diet quantity, and so should be considered in future studies that explore the effect of nutrition on body size.

2. Background

Sexual Size Dimorphism (SSD), the difference in body size between males and females, is perhaps the most familiar and widespread form of sexual dimorphism. This condition is extremely variable among species. For example, a female blanket octopi can weigh 10,000- 20,000 times more than male [1], while a male southern elephant seals can weigh seven times more than a female [2]. Further, the degree of SSD is highly evolutionarily labile, and can vary between closely related species or among populations within species, sometimes dramatically. For example, among populations of the Australian carpet python, females range from being less than 1.5x to more than 10x the size of males [3]. While intraspecific variation in SSD is likely due, in part, to genetic differences between populations, it may also be a consequence of sex-specific differences in the phenotypic plasticity of body size. Environmental variation can account for the vast majority of variation in body size within a population [4, 5], and if males and females differ in the extent to which the environment affects body size, this will generate changes in SSD across environments [6]. While there is considerable evidence that males and females differ in the extent of their body size response to a variety of environmental variables – a phenomenon called *Sex-Specific Plasticity* (SSP)– the role that SSP plays in the developmental generation and evolution of sexual size dimorphism is largely unknown. This is compounded by a general lack of knowledge regarding the proximate developmental mechanisms that generate differences in body size between males and females.

Perhaps the most important, and certainly the best understood, environmental factor that regulates body size is developmental nutrition. In all animals where it has been studied, the nutritional regulation of growth is mediated via the insulin/IGF-signaling (IIS) pathway, a highly conserved receptor tyrosine

kinase pathway, the components of which pre-date the Metazoa [7]. Insulin-like peptides are released in response to nutrition and bind to receptors of dividing cells to initiate a signal-transduction cascade that regulates the expression of positive and negative growth regulator. The IIS pathway positively regulates the activity of TOR-signaling, which also responds directly to cellular levels of amino acid. Activation of IIS/TOR-signaling results in an increase in both growth rate and (typically) adult body size at high levels of nutrition, while deactivation of IIS/TOR-signaling does the opposite at low levels of nutrition.

Although both males and female typically display nutritional plasticity, in many species one sex is more nutritionally plastic than the other. In birds and mammals, male growth tends to be affected more by nutritional stress than female growth [8], while in arthropods, the reverse appears to be true [9]. This is accompanied by the general trend of male-biased SSD in birds and mammals versus female-biased SSD in arthropods [10, 11]. Generally, therefore, the larger sex tends to be more nutritionally plastic than the smaller sex, and SSD increases with body size within a population [11]. This suggests that the mechanisms that generate SSD at least partially overlap with those that generate sex-specific nutritional plasticity.

Several adaptive hypotheses that explain why selection acts differently on males versus females may explain a relationship between SSD and SSP [9]. Under the adaptive canalization hypothesis [12], traits that are under strong stabilizing selection should be more canalized and less plastic than traits that are not. Thus, if there were strong stabilizing selection on the body size of one sex but not the other, then this could lead to differences in size plasticity. In contrast, the condition dependence hypothesis predicts that traits under strong directional sexual selection will exhibit greater sensitivity to environmental condition [13, 14] Consistent with this latter hypothesis is the observation that exaggerated traits used by males to

83 compete for and attract mates are often typically highly plastic relative to the same trait in females (e.g.
84 [14]). The condition-dependence hypothesis was formulated to explain the condition dependence of
85 individual traits within the body and their response to directional sexual selection. Whether it can explain
86 the correlation between SSP and SSD for the body as a whole, and where body size is not obviously
87 subject to sexual selection (for example for female-biased SSD) is unclear.

88

89 While these adaptive hypotheses provide an ultimate evolutionary mechanism for a correlation between
90 SSD and SSP, several studies have hinted at the proximate developmental mechanism linking the two
91 phenomena. A study by Emlen et al [15] found that exaggerated male traits – specifically the horns of the
92 beetle *Trypoxylus dichotomus* – are more insulin sensitive than other traits in the body. This accounted for
93 both the trait's increase in size in males relative to females, and their elevated nutritional plasticity.
94 Consequently, the trait becomes more sexually dimorphic as trait size increases. In *Drosophila*
95 *melanogaster*, where females are larger and more nutritionally plastic than males, SSD also appears to be
96 regulated by the IIS pathway. This is based on three pieces of evidence: (1) SSD is eliminated in flies with
97 suppressed IIS through hypomorphic mutation of the insulin receptor (*InR*) [16]; (2) well-fed females
98 have higher IIS activity than males [17], and; (3) SSD requires sex-specific difference in the neurosecretory
99 cells that produce insulin-like peptides (dILPs), the hormone that activates the IIS pathway [18]. Because
100 SSD is eliminated in flies with a loss of InR activity, it follows that female body size is more sensitive to
101 changes in insulin-signaling than male body size. Consequently, as for the beetle horn, SSP of body size
102 in *Drosophila* may result from sex-specific differences in insulin-sensitivity. What is unknown, however, is
103 whether the differences in nutritional plasticity between males and females is evident at the level of
104 IIS/TOR-signaling activity itself.

105

106 To address this question, we looked at the expression of genes that regulate and are transcriptionally
107 regulated by the IIS and TOR-signaling pathways across a nutritional landscape, where nutritional
108 quality and quantity varies. In a previous study [19], we looked at the effect of changes in protein-to-
109 carbohydrate ratio (diet quality) and total food concentration (diet quantity) on the size of the wing,
110 maxillary palp, femur of the first leg and thorax. For all traits, female trait size was more sensitive to
111 changes in either diet quality or quantity than male trait size. Intriguingly, however, these sex-specific
112 differences could be attributed to the each trait's size response to changes in carbohydrate versus protein
113 concentration: males and female trait sizes were equally sensitive to changes in protein concentration, but
114 only female trait size was detectibly sensitive to changes in carbohydrate concentration. Here we test the
115 hypothesis that these observed sex-specific differences in body-size plasticity are reflected by
116 corresponding differences in response to diet at the level of the IIS/TOR-signaling pathways.
117

118 ## 3. Results

119 ### *3.1. Female and male body size responds differently to changes in carbohydrates but not*
120 *proteins.*

121 We found that, consistent with prior results [19], males and females differed in their response to dietary
122 carbohydrate versus protein (Figure 1, Supplementary Figure 1, Table 1). Specifically, both male and
123 female body size responded to changes in dietary protein as a negative quadratic, with body size
124 increasing as protein concentration increased, but at a decreasing rate (Table 1). In contrast, only female
125 body size responded to dietary carbohydrate concentration, this time as a positive quadratic, such that
126 body size declined with increasing carbohydrate, but at a decreasing rate (Table 1). Correspondingly,
127 including an interaction between sex and carbohydrate when modeling the relationship between diet and
128 body size significantly improved model fit, while including an interaction between sex and protein did

not further improve fit (Table 2). Thus male and female body size appears to respond differently to carbohydrate but not to protein. As a result, sexual size dimorphism (SSD) varied across the nutritional geometry landscape (Figure 1, Supplementary Figure 1).

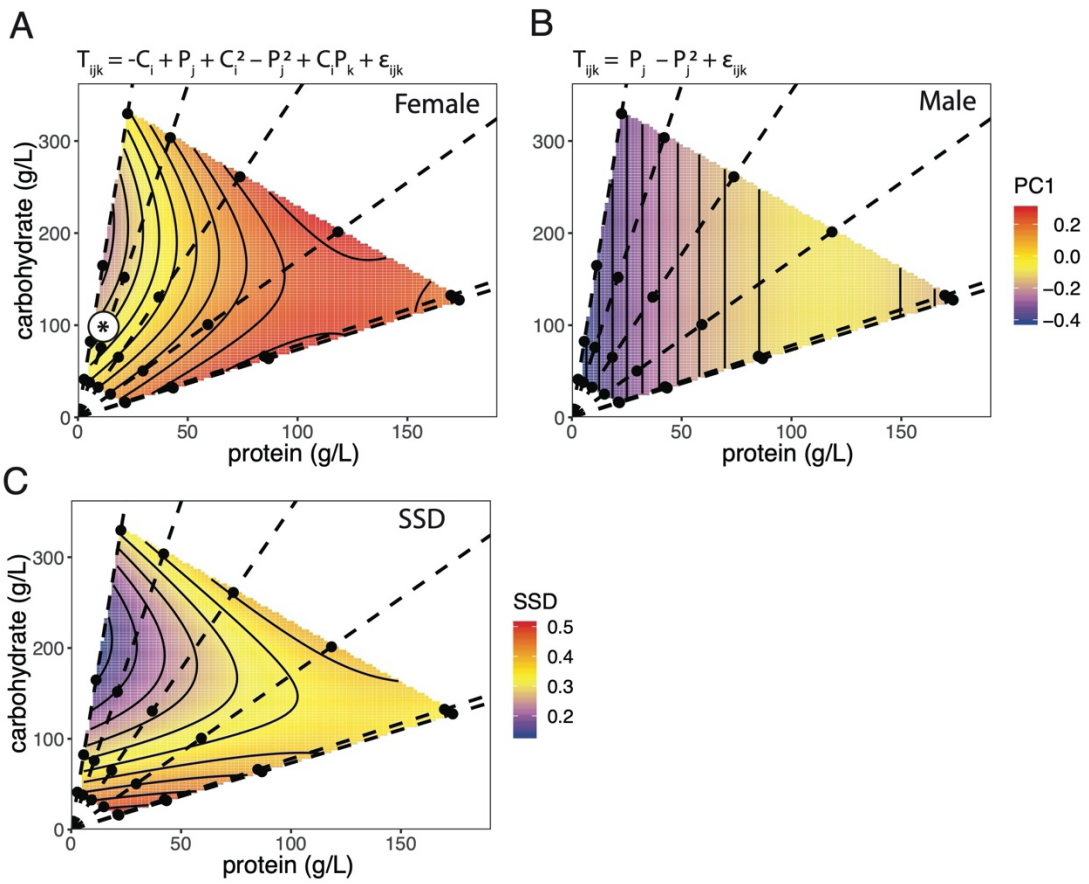


Figure 1: The effect of protein and carbohydrate concentration on female and male body size and sexual size dimorphism (SSD). (A, B) Surfaces show the fitted relationship between body size, carbohydrate level and protein level in female and male flies, based on the statistical model specified by the equation above each chart. T = body size, P = protein, C = carbohydrate, ε = error, subscripts refer to levels within each factor. (C) Surface shows the difference in female and male body size (SSD) across the same nutritional landscape, using fitted values from A and B. Points indicate diets tested and dotted lines connect diets with equal protein-to-carbohydrate ratios (1:14.6, 1:7.2, 1:3.5, 1:1.7, 1.3:1, 1.4 :1). * indicates approximate composition of standard cornmeal-molasses medium. Corresponding thin-plate spline plots are shown in Supplementary Figure 1.

142 ### *3.2. Female and male IIS/TOR gene expression responds differently to changes in diet*

143 We collected expression data for eight genes that either regulate or are regulated by the IIS/TOR signaling
144 pathway: insulin receptor (*InR*); eukaryotic translation initiation factor 4E binding protein (*4E-BP/Thor*);
145 Drosophila insulin like peptides 2, 3, 5 and 8 (*dILP 2, 3, 5,8),* Absent, small, or homeotic discs-like protein
146 (*Ash2L*), and *CG3071* (the Drosophila homolog of UTP15/SAW). Expression of both *InR* and *4E-BP* is
147 negatively regulated by the IIS pathway [26], *dILPs 2,3,5* encode peptides that ostensibly activate the IIS
148 pathway, while *dILP8* retards development by inhibiting the synthesis and release of ecdysone [27].
149 Finally expression of both *Ash2L* and *CG3071* has been shown to be negatively and positive regulated by
150 *TOR* signaling, respectively [28]. Expression of only a few of these genes correlated directly with body
151 size: *4EBP* and *dILP5* negatively and positively correlated with body size in females, respectively, while
152 expression of *InR* negatively correlated with body size in males (Table 3). Nevertheless, a multivariate
153 analysis of gene expression across all diets supported a significant interaction between the effects of sex
154 and carbohydrate, as a linear factor, and sex and protein, as a quadratic factor, on gene expression (Table
155 4). We therefore explored how sex and diet affected the expression of individual genes. Specifically, we
156 looked to see whether sex-specific responses in the expression of genes that either regulate or are
157 regulated by the IIS/TOR-signalling pathways reflected the sex-specific response of body size to changes
158 in diet.

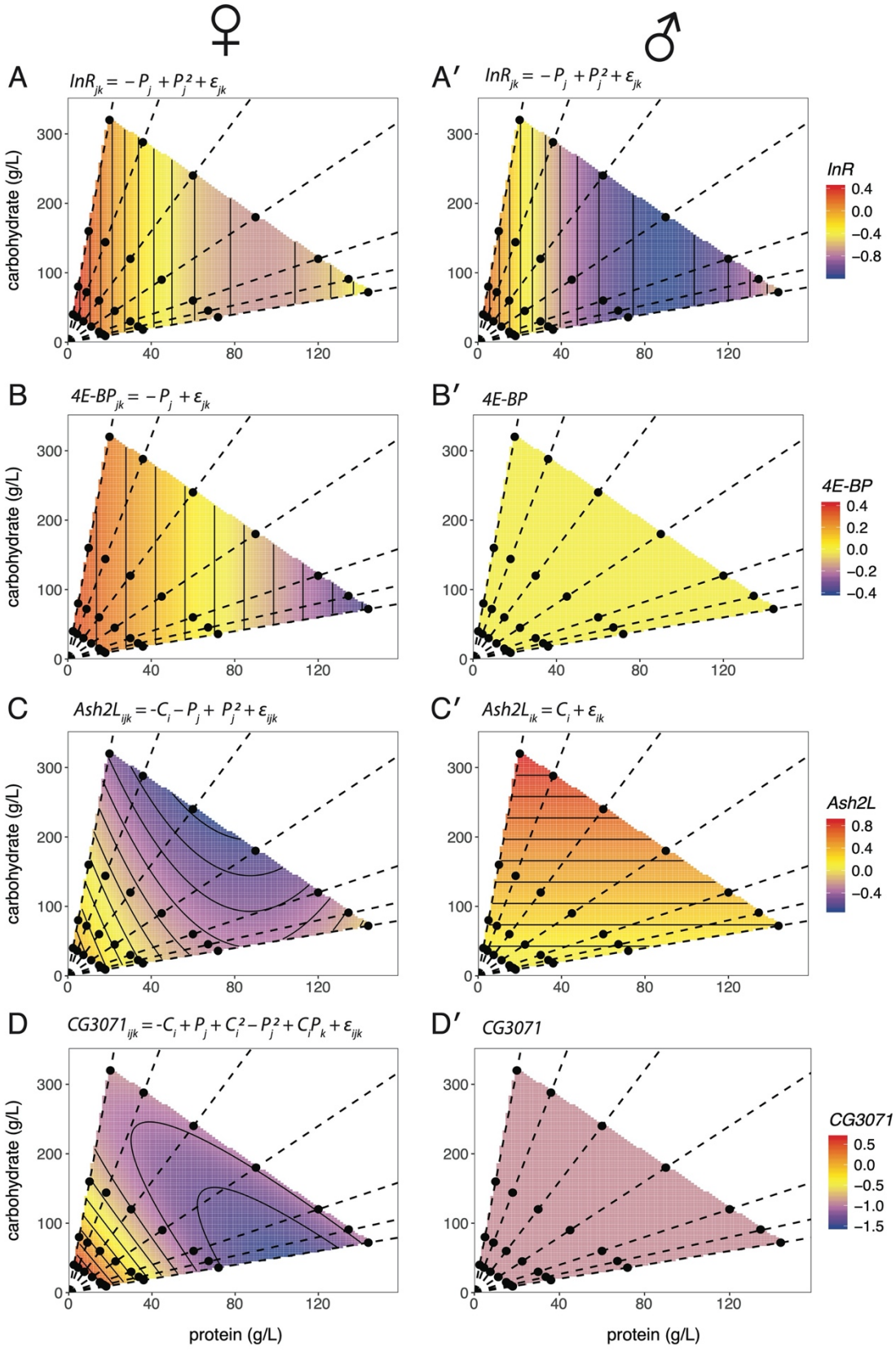
159

160 ***Genes Regulated by IIS***. Both *InR* and *4E-BP* expression responded to changes only in dietary protein but
161 not carbohydrate (Figure 2). *InR* expression in males and females had a positive quadratic response to
162 protein level, decreasing as protein level increased, but at a declining rate (Table 5). The expression of *InR*
163 was lower across all diets in males than in females, but there was no significant interaction between the
164 effects of sex and protein level on gene expression (Table 5). In contrast, *4E-BP* expression was sensitive

165 to protein level only in females, decreasing linearly as protein increased, but was unaffected by diet in
166 males (Table 4). Consequently, there was a significant interaction between the effect of sex and protein
167 level on *4E-BP* expression (Table 5).

168

169 ***Genes Regulated by TOR Signaling.*** Expression of both *Ash2L* and *CG3071* had a complex and sex-
170 specific response to diet. Carbohydrate significantly affected *Ash2L* expression as a linear relationship,
171 but in opposite directions in either sex: Increased carbohydrate increased *Ash2L* expression in males but
172 decreased *Ash2L* expression in females (Figure 2, Table 4). *Ash2L* expression was not affected by protein
173 in males, but had a positive quadratic response to protein in females, decreasing as protein increased but
174 at a declining rate. There was therefore a significant interaction between sex and diet on the expression of
175 *Ash2L,* although only between sex and carbohydrate and not sex and protein (Table 5). *CG3071* also
176 showed a sex-specific expression response to diet. In females, *CG3071* expression had a negative
177 quadratic response to protein level (increasing at a declining rate as protein increased), a positive
178 quadratic response to carbohydrate level (decreasing at a declining rate as carbohydrate increased), with
179 a significant carbohydrate: protein interaction (Table 4). In contrast, *CG3071* expression was not affected
180 by diet in males, which lead to a significant diet-by-sex interaction effect on *CG3071* expression (Table 5).

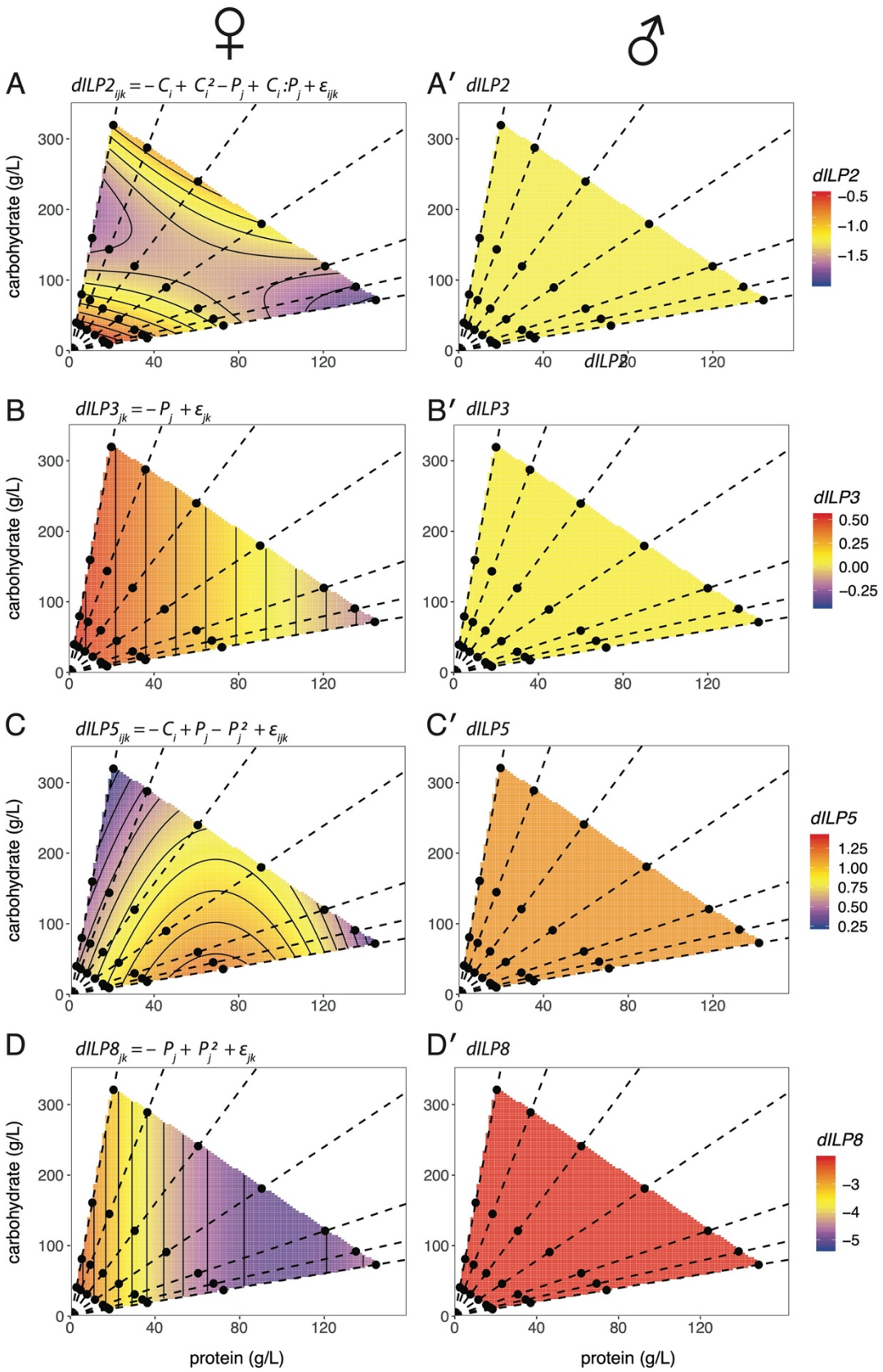

♀
♂
A
$lnR_{jk} = -P_j + P_j^2 + \varepsilon_{jk}$
A′
$lnR_{jk} = -P_j + P_j^2 + \varepsilon_{jk}$
lnR
0.4
0.0
−0.4
−0.8
B
$4E\text{-}BP_{jk} = -P_j + \varepsilon_{jk}$
B′
4E-BP
4E-BP
0.4
0.2
0.0
−0.2
−0.4
C
$Ash2L_{ijk} = -C_i - P_j + P_j^2 + \varepsilon_{ijk}$
C′
$Ash2L_{ik} = C_i + \varepsilon_{ik}$
Ash2L
0.8
0.4
0.0
−0.4
D
$CG3071_{ijk} = -C_i + P_j + C_i^2 - P_j^2 + C_iP_k + \varepsilon_{ijk}$
D′
CG3071
CG3071
0.5
0.0
−0.5
−1.0
−1.5
carbohydrate (g/L)
protein (g/L)
0
100
200
300
40
80
120

181

182 **Figure 2: The effect of protein and carbohydrate concentration on the expression of IIS and TOR**
183 **transcriptionally-regulated genes in females and males.** Surfaces show the fitted relationship between
184 gene expression, carbohydrate level and protein level in female and male flies, based on the statistical
185 model specified by the equation above each chart (Table 4). *P* = protein, *C* = carbohydrate, ε = error,
186 subscripts refer to levels within each factor. Expression of (A, A′) *InR*, and (B, B′) *4E-BP*, both negatively
187 regulated by the activity of the IIS via the Forkhead transcription factor FOXO. (C, C′) Expression of
188 *Ash2L*, ostensibly negatively regulated by the activity of TOR signaling. (D, D′) Expression of *CG3071*,
189 ostensibly positively regulated by the activity of TOR signaling. Points indicate diets tested and dotted
190 lines connect diets with equal protein-to-carbohydrate ratios (1:16, 1:8, 1:4, 1:2, 1:1, 2:1). Corresponding
191 thin-plate spline plots are shown in Supplementary Figure 2.

192

193 ***Genes That Regulate IIS/TOR Signaling.*** The expression of *dILP2* showed a significant response to diet in
194 females, declining linearly with increasing protein, and at a decreasing rate with increasing carbohydrate,
195 as a positive quadratic. There was also a significant carbohydrate-by-protein interaction effect on *dILP2*
196 expression in females. There was no significant effect of diet on *dILP2* expression in males, although this
197 appears to be a consequence of low statistical power, due to large amount of within-diet variation in
198 *dILP2* expression among male samples compared to females sample. Fitting a one-way ANOVA of *dILP2*
199 expression against diet, where diet is each protein-to-carbohydrate ratio at each concentration, the
200 residual variance (a measure of average variance within diets) was significantly higher in males than in
201 females, by a ratio of 8.68 (Supplementary Table 1). Correspondingly, we had very low power (0.22) to
202 detect a significant sex-by-diet interaction for *dILP2* expression, based on the observed effect size
203 (Cohen's f^2 = 0.023, see Supplementary Material for details). Further, there was no evidence that
204 expression of *dILP2* was different between males and females, independent of diet (Table 5).


♀
♂
A
$dILP2_{ijk} = -C_i + C_i^2 - P_j + C_i{:}P_j + \varepsilon_{ijk}$
A′ dILP2
B
$dILP3_{jk} = -P_j + \varepsilon_{jk}$
B′ dILP3
C
$dILP5_{ijk} = -C_i + P_j - P_j^2 + \varepsilon_{ijk}$
C′ dILP5
D
$dILP8_{jk} = -P_j + P_j^2 + \varepsilon_{jk}$
D′ dILP8
carbohydrate (g/L)
protein (g/L)
dILP2
−0.5
−1.0
−1.5
dILP3
0.50
0.25
0.00
−0.25
dILP5
1.25
1.00
0.75
0.50
0.25
dILP8
−3
−4
−5

205

206 **Figure 3: The effect of protein and carbohydrate concentration on the expression of dILPs in females**
207 **and males.** Surfaces show the fitted relationship between gene expression, carbohydrate level and protein
208 level in female and male flies, based on the statistical model specified by the equation above each chart
209 (Table 4). *P* = protein, *C* = carbohydrate, ε = error, subscripts refer to levels within each factor. (A) *dILP2*.
210 (B) *dILP3.* (C) *dILP 5.* (D) *dILP8*. Points indicate diets tested and dotted lines connect diets with equal
211 protein-to-carbohydrate ratios (1:16, 1:8, 1:4, 1:2, 1:1, 2:1). Corresponding thin-plate spline plots are
212 shown in Supplementary Figure 3.

213

214 The expression of *dILP3* decreased linearly with protein in females, but there was no detectable effect of
215 diet on *dILP3* expression in males (Figure 2, Table 4). There was also no detectable difference in
216 expression between the sexes (Table 5). As for *dILP2*, variation in expression of *dILP3* among samples
217 within diets was significantly higher in males than in females (Supplementary Table 1), and a power
218 analysis gave a power of only 0.05 to detect a sex-by-protein interaction on *dILP3* expression, based on the
219 observed effect size (Cohen's f^2 <0.001).

220

221 The expression of *dILP5* was only marginally affected by diet in females, with a significant negative
222 quadratic effect of protein. We could detect no significant effect of either carbohydrate or protein on
223 *dILP5* expression in males. As for *dILP2* and *3*, *dILP5* expression was more variable among male samples
224 within diets than among female samples (Supplementary Table 1), and a power analysis gave a power of
225 only 0.23 to detect a sex-by-protein interaction on *dILP5* expression, based on the observed effect size
226 (Cohen's f^2 = 0.022). Nevertheless, there was sex-specific expression independent of diet, such that *dILP5*
227 expression was higher in males than in females (Table 5).

228

229 The expression of *dILP8* responded as a positive quadratic in response to protein in females, declining as
230 protein increased at a decreasing rate, but did not show any response to any aspect of diet in males
231 (Figure 2, Table 4). The lack of a corresponding response to protein in males was not due to a lack of

232 statistical power: a power analysis suggested that the power to detect the female effect size of protein on
233 *dILP8* expression (Cohen's f^2 = 0.46) in males was 0.99. Further, *dILP8* expression was not more variable
234 among male samples within diets than among female samples (Supplementary Table 1). Consequently,
235 we detected a significant sex-by-protein interaction on *dILP8* expression (Table 5).

236

237 ### *3.3. dILP protein levels in the brain increase with increasing food concentration*

238 Our finding that *dILP* expression in females was generally higher in larvae fed on lower food
239 concentrations was surprising, given that dILPs (apart from dILP8) are canonically positive regulators of
240 growth in response to nutrition. We therefore explored the relationship between diet, *dILP* expression
241 and the levels of dILP peptide in the insulin-producing cells (IPCs) of the brain. Staining for dILP2 and
242 dILP5 peptide was higher in larvae fed a higher food concentration (360g/l) versus a lower food
243 concentration (45g/l), with a 1:2 protein: carbohydrate ratio (Figure 4).

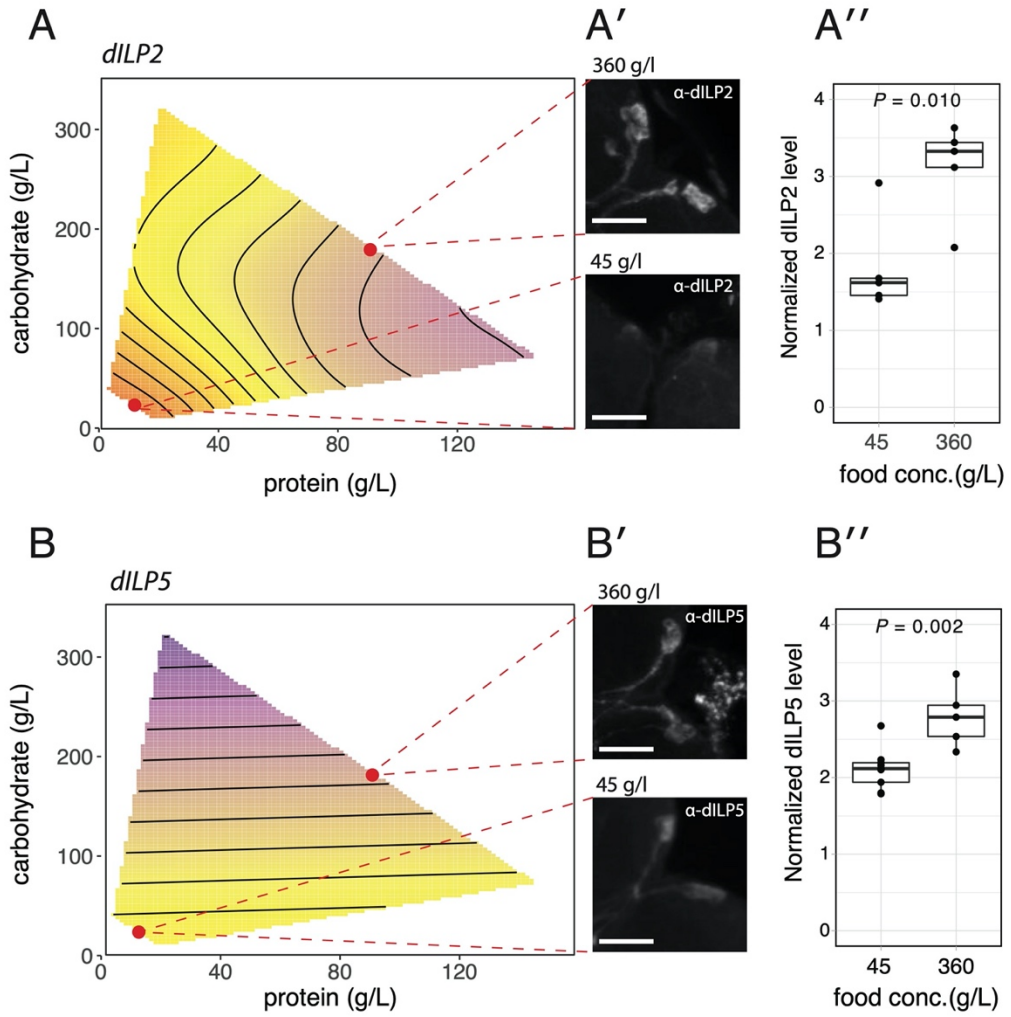


Figure 4: The effect of diet on dILP2/5 expression and peptide levels in the brain in females and males. (A,B) The relationship between diet and *dILP2* and *dILP5* expression. Points indicate 45 and 360 g/l food concentration at 1:2 protein: carbohydrate ratio. (A′, B′) Representative images of dILP2 and dILP5 levels in the insulin-producing cells (IPCs) of female larvae reared on different diets. (A″, B″) Mean normalized level of dILP2 and dILP5 in the IPCs of larvae reared on different diets. *P*-values are for a pooled t-test.

4. Discussion

Previous studies indicate that in *Drosophila,* body size is more nutritionally plastic in females than in males, a phenomenon referred to sex-specific plasticity (SSP) [19]. Nutritional plasticity is canonically regulated by the insulin/IGF-signaling and TOR signaling pathways in *Drosophila* and most other

255 animals. We hypothesized, therefore, that differences in nutritional plasticity between the sexes reflects
256 sex-specific differences in the activation and activity of these pathways. In this study, we applied a
257 nutritional geometry framework to test this hypothesis, and explored the sex-specific effects of protein
258 and carbohydrates on body size and the expression of genes that activate and are transcriptionally
259 regulated by IIS/TOR. Our results indicate that, broadly, gene expression is more nutritionally sensitive in
260 females than in males, although the effect of nutrition on the expression of IIS and TOR-signaling genes is
261 both quantitively and qualitatively different between males and females.

262

263 The simplest hypothesis to account for sex-specific differences in the nutritional plasticity of body size in
264 *Drosophila* is that females have the same response to nutrition as males, but at a higher sensitivity. Under
265 this hypothesis, the response surfaces of body size and gene expression across a nutritional landscape of
266 varying protein and carbohydrate concentrations should be the same shape in both sexes, but with lower
267 gradients in males. This would be evident as lower parameter values in males versus females. This is not
268 supported by the data, however. In particular, body size in males shows more or less the same sensitivity
269 to changes in protein concentration as females, but has no detectable sensitivity to changes in
270 carbohydrate concentration. This in turn suggests that an important aspect of sex-specific nutritional
271 plasticity in *Drosophila* is differences in the response to variation in dietary carbohydrates.

272

273 The best understood regulator of body size with respect to nutrition are the insulin/IGF-signaling and
274 TOR-signaling pathways [29, 30]. The IIS pathway is activated by circulating dILPs, some of which are
275 released in a nutrition-dependent manner [27]. The TOR-signaling pathway is in part regulated by IIS,
276 but also responds directly to circulating amino acids [31–33]. Because the SSP of body size in flies appears
277 to primarily driven by a differential response to carbohydrates but not to protein, we might expect that

278 the effect of diet on body size is mediated primarily by IIS rather than TOR-signaling. Our data do not
279 support this hypothesis. We used the expression of *InR* and *4E-BP* as a measure of IIS activity: Both are
280 transcriptionally regulated by the forkhead transcription factor FOXO, which is activated when nutrition
281 and IIS activity is low [26, 34]. If sex-specific plasticity is mediated by IIS then we would expect to see sex-
282 specific differences in *Inr* and *4EBP* expression in response to changes in carbohydrate but not to protein.
283 In both males and females, however, *InR* expression responded only to protein level – and to the same
284 extant – but did not respond to carbohydrate level. Expression of *4E-BP* only responded to protein level
285 in females, but did not respond to carbohydrate level in either sex. Collectively, variation in the
286 expression of *4E-BP* and *InR* were the strongest correlates with body size in females and males
287 respectively (Table 3), consistent with the hypothesis that the IIS is the major regulator of body size with
288 respect to nutrition in *Drosophila*. However, the expression of these genes do not reflect sex-specific
289 differences in the effect of carbohydrate on body size, and so do not explain sex-specific plasticity.

290

291 In contrast to *InR* and *4E-BP*, the expression of *Ash2L* and *CG3071* both responded to carbohydrates in a
292 sex-specific manner. However, the response is not consistent with a previous report on the regulation of
293 the these genes' expression by TOR-signaling [28]. This study reported that *Ash2L* is negatively regulated
294 by TOR-signaling while *CG3071* is positively regulated. If TOR-signaling increases with protein, then we
295 would expect *Ash2L* expression to decrease correspondingly, which is true in females. However, *Ash2L*
296 expression also decreases with increasing carbohydrate, which is difficult to reconcile with the negative
297 effects that carbohydrate has on female growth. Even more challenging to interpret is the observation that
298 *Ash2L* expression increases with carbohydrate but not protein in males, even though male do not appear
299 to have a growth response to carbohydrate. Thus the relationship between *Ash2L* expression and body
300 size is not a simple one. The expression pattern of *CG3071* is similarly unclear. While *CG3071* also has a
301 carbohydrate and protein response in females, this response is qualitatively similar to the response of

302 *Ash2L,* which is not expected if TOR-signaling positively regulates *CG3071* expression but negatively
303 regulates *Ash2L* expression. Thus the role that TOR signaling plays in regulating SSD and SSP is
304 equivocal based on our data. Problematically, the mechanism by which TOR-signaling regulates *Ash2L*
305 and *CG3071* expression has not been fully explored, which makes interpreting changes in *Ash2L* and
306 *CG3071* expression with diet even more challenging.

307

308 Our data suggests a complex relationship between sex, diet, *dILP* expression and dILP retention levels in
309 the IPCs. In females, there were stark differences in the pattern of expression among the dILPS. Of all the
310 dILPs, *dILP5* expression was most strongly correlated with body size, increasing with increasing protein
311 level but decreasing with increasing carbohydrate level. We also found that dILP5 levels in the IPCs were
312 lower in larvae fed on lower food concentrations. Thus, chronic reductions in nutrition reduce *dILP5*
313 expression and dILP5 IPCs level. This is consistent with dILP5 being a positive regulator of growth in
314 response to nutrition [35]. The negative effect of low nutrition on *dILP5* expression has been observed in
315 previous studies [30, 35, 36]. However, at least one of these studies reported an increase in dILP5
316 retention in the IPCs of larvae that were either starved for 24 hours or reared on a low protein diet since
317 birth [36], which is inconsistent with our data. The low protein diet in this study was generated by
318 reducing the amount of protein from ~16g/l to ~0.8g/l, while keeping the concentration of carbohydrate at
319 ~60g/l, thereby decreasing the protein-to-carbohydrate ratio. In our study we reduced overall food
320 concentration from 360g/l to 45g/l while retaining a 1:2 protein-to-carbohydrate ratio. It is possible,
321 therefore, that the retention of dILP5 in the IPCs in the earlier study is due to a low protein-to-
322 carbohydrate ratio. Indeed, a second earlier study showed that a chronic high sugar diet with low
323 protein-to-carbohydrate ratio also increased dILP5 levels in the IPCs [37], although this time
324 accompanied by an increase in *dILP5* expression. Thus the relationship between *dILP5* expression and

325 dILP5 levels in the IPCs may depend not only on the overall food concentration, but also on food
326 composition, in particular the protein-to-carbohydrate level.
327
328 Previous studies suggest that *dILP2* expression is unaffected by reduced nutrition [30, 36] (although see
329 supplementary data in [38]), but that dILP2 peptides are retained in the IPCs in larvae that have been
330 starved 24h or are reared on a low protein diet [36, 39, 40]. A high sugar diet is also associated with
331 increased retention of dILP2 peptide in the IPCs, but with an increase in *dILP2* expression [37]. In our
332 study, however, we found that *dILP2* expression declined with an increase in both protein and
333 carbohydrate, and that dILP2 peptide was lower in the IPCs of larvae fed on lower food concentrations.
334 Our observed expression of *dILP3* was also inconsistent with previous studies that found that acute
335 starvation reduced expression [30], while rearing on a high protein diet increased expression [41]. In
336 contrast, we found that, as for *dILP2*, *dILP3* expression declined with increasing protein, although was
337 unaffected by carbohydrate level, which is surprising given that circulating sugars promote the release of
338 dILP3 from the IPCs [40]. Our finding that *dILP2* and *dILP3* expression is lowest on low protein diets that
339 restrict growth, and that dILP2 peptides levels are higher when *dILP2* expression is lower, suggests that
340 dILP2 and dILP3 may negatively regulate their own expression, which is a common characteristic of
341 hormone regulation.
342
343 Finally, the expression of *dILP8* showed a similar pattern to *dILP3,* increasing with a decrease in protein
344 concentration. dILP8 is involved in regulating growth and developmental timing through its inhibitory
345 effects on ecdysone synthesis [42, 43]. It is released from imaginal discs in response to damage or growth
346 perturbation, as well as showing periodic changes in expression throughout development . Ecdysone
347 synthesis is also inhibited by low nutrition early in the third larval instar, leading to a delay in

348 metamorphosis [44]. The observation that females on low protein diets also show elevated levels of *dILP8*
349 expression, suggests that dILP8 may play a role in regulating growth and/or development in response to
350 nutrition, at least in females.

351

352 We did not detect an effect of diet on the expression of any of the *dILP*s in male larvae. This did not,
353 however, translate into a significant interaction between diet and sex on *dILP* expression, and therefore
354 does not help explain the sex-specific differences in nutritional plasticity of body size. This was because
355 variation in *dILP 2,3,5* and *8* expression among samples within diets was significantly higher for male
356 samples than for females samples (Supplementary Table 1), reducing the statistical power to detect diet-
357 by-sex interactions. The only exception was for *dILP8,* such that *dILP8* expression was significantly more
358 plastic in females than in males. Nevertheless, *dILP8* expression in females did not correlate with
359 carbohydrate level, and so is unlikely to explain the differential effect of carbohydrate on body size in
360 females versus males. The elevated variation in *dILP* expression among male samples was not seen for
361 expression of *4eBP*, *InR*, *Ash2L*, or *CG3072*, and is therefore not likely due to sample degradation. It is also
362 not due to lower levels of expression in males relative to females. It is possible, therefore, that *dILP*
363 expression levels are more developmentally dynamic in males then females, and that we are capturing
364 aspects of that instability by measuring gene expression at only a single point in development.

365

366 Collectively, there appears to be no simple explanatory relationship between the sex-specific nutritional
367 geometry of body size with the sex-specific nutritional geometry of IIS/TOR-signaling gene expression.
368 Further, many of our findings do not replicate what has been reported in previous studies. An earlier
369 study looking at *dILP* expression in adults across a nutritional landscape saw similarly complex and non-
370 intuitive relationships between diet and gene expression [23]. What is very clear from both studies is that

371 the protein-to-carbohydrate ratio affects the transcriptional response of IIS/TOR-signaling genes to
372 changes in total diet, and our study indicates that it does so in a sex-specific way. This has important
373 implications for interpreting those studies that have looked at changes in the expression of IIS/TOR-
374 signaling genes in response to changes in nutrition. First, many studies simply dilute diet to reduce
375 overall nutrition. Problematically, while most *Drosophila* labs use 'standard' food recipes, the use of
376 different types of yeast, cornmeal, mollasses *etc* means that the composition of these diets may be unique
377 to most research groups [45]. Our data indicate that the effect of diet dilution on both body size and
378 IIS/TOR-signaling gene expression – and potentially the activity of the IIS/TOR-signaling pathways – will
379 depend on the P:C ratio of that specific diet. Second, these responses are sex-specific, and studies that do
380 not consider sex may come to different conclusions than studies that do. Third, the relationship between
381 *dILP* expression, dILP retention and diet is similarly complex, and may also depend on the composition
382 of the diet being manipulated.

383

384 One important caveat with our, and almost all other studies of IIS/TOR-signaling gene expression during
385 development, is that we measured expression at a single developmental time point, at the very beginning
386 of larval wandering. Previous studies have shown that the activity of the IIS and TOR-signaling pathways
387 change dynamically during development, and that different dILPs are expressed at different life stages.
388 This may also account for differences between our and other studies of the effects of nutrition on
389 IIS/TOR-signaling gene expression. Ideally, one would like to conduct a multidimensional study of gene
390 expression across both time and a nutritional landscape, and to tie this with a corresponding study of
391 dILP levels both in the IPCs and circulating in the hemolymph. Such a study would not only help
392 elucidate the relationship between IIS/TOR-signaling and the sex-specific effects of nutrition on body
393 size, but also help us understand better how nutrition regulates the transcription, translation, storage and
394 release of dILPs.

395 ## 5. Conclusions

396 Our study provides a foundation for future work that looks at the sex-specific effects of diet quantity and
397 quality on body size and on the developmental mechanisms that regulate body size. Our data show
398 significant differences in the effect of diet on gene expression in males and females, although they do not
399 suggest a simple explanatory relationship between gene expression and body size. Future studies on sex-
400 specific plasticity in *Drosophila*, and other animals, should therefore consider not only the nutritional
401 geometry of gene expression, but also of protein levels, pathway activity and other developmental
402 parameters such as growth rate and growth duration, ideally at multiple time points throughout
403 development.

404 ## 6. Materials and Methods

405 The goal of the study was to test the hypothesis that sex-specific differences in body-size plasticity across
406 a nutritional landscape in *Drosophila* correspond to sex-specific differences in the response to diet at the
407 level of the IIS/TOR-signaling pathways, using the expression of genes that regulate or are regulated by
408 IIS/TOR-signaling to assay pathway activity.

409 ### *6.1. Fly Stocks and Maintenance*

410 Flies used for the nutritional geometry of body size were as described in [19]. Flies used for the
411 nutritional geometry of gene expression were an isogenic line of white-eyed but otherwise wild-type flies
412 (VDRC 60000). Flies were maintained as a stock at 17˚C on standard on standard media of 45 g of
413 molasses, 75 g of sucrose, 70 g of cornmeal, 20 g of yeast extract, 10 g of agar, 1100 ml of water, and 25 ml
414 of a 10% Nipagin solution per liter of fly food, under constant light.

415 ### 6.2. *Diet Manipulation for Nutritional Geometry of Body Size*

416 Flies were reared on 24 diets: six protein-to-carbohydrate ratios (1:14.6, 1:7.2, 1:3.5, 1:1.7, 1.3:1, 1.4 :1), at
417 four food concentrations: (45, 90, 180 and 360 g l^{-1}), with six replicate vials per diet, as described in [19].
418 We measured four non-genital traits in each fly: the length of the first femur, the area of the wing, the
419 area of the maxillary palp and the length of the thorax (see [19] for details). Data were collected from, on
420 average, 17 flies from each sex at each diet, with no more than 30 flies coming from any one vial. To assay
421 the effect of diet on overall body size, we conducted a principle component analysis on all log-
422 transformed non-genital morphological data from all flies, and used each fly's value for the first principle
423 component (PC1) as a proxy for overall body size [20, 21].

424 ### 6.3. *Diet Manipulation for Nutritional Geometry of Gene Expression*

425 Flies were reared on 28 different diets: seven protein-to-carbohydrate ratios (1:16, 1:8, 1:4, 1:2, 1:1, 1.5:1
426 and 2:1) at four food concentrations (45, 90, 180, and 360 g/l). To generate the diets we made an instant-
427 yeast solution (Lesaffre SAF-Instant Red: 44% protein, 33% carbohydrate) and a sucrose solution
428 (Mallinokrodt, Paris, Kentucky) at each food concentration. We also added 0.5% agar, 10% nipagen (10%
429 p-hydroxy benzoic acid methyl ester in 95% ethanol) and 1% propionic acid (Sigma Life Science, St.
430 Louis, Missouri) to each solution, to control consistency and prevent fungal growth. The solutions were
431 autoclaved and mixed at different ratios to achieve the desired protein-to-carbohydrate ratios for each
432 diet (supplemental Table 1). 10 ml of each of the 28 different diets were aliquoted into separate 25 x 95
433 mm vials.

434 Populations of 50 flies were placed in mating cages with 60mm petri dishes of standard lab food. The flies
435 were allowed to lay eggs for 24 hrs. The adult flies were removed and the eggs were left on the petri dish
436 for another 24hrs until the larvae had hatched. The first instar larvae were then transferred in groups of
437 50 into each food vial. There were at least five replicate vials for each diet.

438 All cultures were maintained at 25˚C at constant light under 60-70% humidity. Larvae were collected
439 from the food vials when they had just initiated wandering, to ensure that all larvae were collected at the
440 same developmental stage regardless of diet. All larvae were sexed and flash-frozen to -80˚C within five
441 minutes of being removed from the vial. The larvae were stored at -80˚C until they were used for RNA
442 extractions.

443 ### *6.4. Gene Expression Quantification*

444 We used qPCR to assay gene expression in male and female larvae collected at the beginning of larval
445 wandering. Larvae were pooled into cohorts of 15 individuals, and we collected 3 cohorts of each sex for
446 each of the 28 diets, with the exception of the 1:16 360g/L diet, which only generated enough larvae for 2
447 cohorts of each sex. For each sample, RNA was extracted using Trizol (Invitrogen, Grand Island, NY,
448 USA) and treated with DNase 1 (Invitrogen) before being reverse-transcribed with High Capacity cDNA
449 Reverse Transcription Kit synthesis according to manufactures instructions (Thermo Fischer Scientific,
450 Carlsbad, CA, USA). We used SYBR Green PCR master mix (Thermo Fischer Scientific, Carlsbad, CA,
451 USA) to conduct qPCR using a CFX Connect Real-time System (Bio-Rad Laboratories, Inc., Hercules, CA).
452 mRNA abundance was calculated using the standard curve method [22]. Relative gene expression was
453 calculated by comparing the measured mRNA abundance of the gene of interest to that of ribosomal
454 protein 49 (RP49). Prior studies have indicated that RP49 is insensitive to changes in nutritional
455 conditions [23]. In two samples (1:16 180g and 1:4 45g male), gene expression levels were an order of
456 magnitude above all other samples across all amplicons. Data from these samples was therefore excluded
457 from subsequent analysis. The complete protocol is provided in the supplementary material, along with
458 primer sequences.

459 ### *6.5. dILP2 and dILP5 Levels in the Brain*

460 The following antibodies were used: dILP2 rat (1:800); dILP5 rabbit (1:800) (both gifts of P. Leopold);
461 AlexaFluor 594 Donkey anti-Rat IgG (1:500); AlexaFluor 594 Goat anti-Rabbit IgG (1:500) (both from Life
462 Technologies).

463 Larvae were reared on either 45g/l or 360g/l at a protein:carbohydrate ratio of 1:2, as described above.
464 Brains were dissected in 1X PBS on ice from wandering female third instar larvae. The brains were fixed
465 for 30 minutes in 4% paraformaldehyde and washed in 0.3% Triton-X in PBS (PBT) overnight at 4°C. They
466 were then washed for 1 hr in 2% NGS in 0.2% I-Block in PBT (BBT/NGS) and incubated with primary
467 antibody diluted in BBT/NGS overnight at 4°C. Subsequently, brains were washed four time in 0.2%
468 BSA/I-Block in PBT (BBT), 15 minutes per wash. Brain were then incubated in secondary antibody at
469 room temperature for 2 hours. Finally, brains were washed in four times in PBT for 15 minutes per wash
470 and mounted with vector shield + DAPI.

471 We acquired images using Olympus Fluoview FV101 confocal microscope. For both 45g/L and 360g/L
472 dILP2 images, 35% DAPI and 35% Alexa 594 intensity settings were used. For both 45g/L and 360g/L
473 dILP5 images, 30% DAPI and 30% Alexa 594 intensity settings were used. We used a 1.5 micron step size
474 to capture a Z-stack of images for each IPC (one per brain), which was flattened to a single image using a
475 maximum-intensity projection in FIJI software. The intensity of the IPC in both the Alexa 594 and DAPI
476 channel was then used to generate a normalized measure of the level of dILP2 or dILP5 in each brain. We
477 repeated this for five brains for dILP2 and dILP5 at each diet level (nine for dILP5 at 45g/l). dILP levels
478 were then compared between diets using a standard pooled *t*-test.

6.6. *Statistical Analysis*

Nonlinear response surfaces are routinely modelled using the second-order polynomial regression [19, 23–25]. To determine the effect of protein and carbohydrate level on body size or gene expression we therefore fit the model:

$$T_{ijk} = C_i + P_j + C_i^2 + P_j^2 + C_iP_j + \varepsilon_{ijk}$$

where *T* is trait (body size or expression level), *C* is carbohydrate concentration, *P* is protein concentration and ε is error (subscripts are levels within variables). Models for body size included replicate vial as a random factor and were fit using *lmer* in the *lme4* package in *R*. Models for expression level were fit using *lm* in the *base* package of *R*. For each trait, the significance of each parameter was tested with a type III ANOVA using the *Anova* function in the *car* package for standard models and the *summary* function in the *lmerTest* package for mixed models. Any non-significant parameters were subsequently removed from the model before the data were reanalyzed using the simplified model. Models that did not contain interactions were analyzed with a type II ANOVA. We subsequently plotted the fitted values of the simplest model against carbohydrate and protein levels. We also plotted thin-plate splines of the raw measurements against carbohydrate and protein levels. Finally, we plotted sexual size dimorphism (SSD) as the difference between the female and male fitted values against carbohydrate and protein level, as well a thin-plate splines of the difference between mean female and male values at each diet.

To test whether the response to carbohydrates or protein differed between the sexes we fit two models:

$$\text{Model 1: } T_{ijk} = S_k + (C_i + P_j + C_i^2 + P_j^2 + C_iP_j) + \varepsilon_{ijk}$$

$$\text{Model 2: } T_{ijk} = S_k*(C_i + P_j + C_i^2 + P_j^2 + C_iP_j) + \varepsilon_{ijk}$$

where *S* is sex. Models were compared using ANOVA (for standard models of expression level) or parametric boostrapping (for mixed models of body size). If inclusion of sex as an interactive factor rather

501 than an additive factor significantly improved the fit of the model, we concluded that the relationship
502 between trait and diet varied between males and females. If the relationship between trait and diet could
503 be explained by a simpler model, we tested whether inclusion of sex as an interactive rather than an
504 additive factor in the simplified model improved the fit of the model. We also conducted a similar
505 multivariate analysis on the effect of diet and sex on the expression of multiple genes, using MANOVA to
506 compare models. Finally, we used the fitted values from the relationship between diet and body size to
507 test the relationship between body size and the expression of each gene, using the linear model:

508 $$T_{ij...p} = A_i + B_j + \ldots + H_p + \varepsilon_{ij...p}$$

509 where *T* is body size (fitted values from the linear mixed model of the relationship between body size and
510 diet) and *A* through *H* is the expression of the genes measured in our analysis.

511 All analyses were conducted in *R* and the data and scripts for the analyses are provided on Dryad. Gene
512 expression levels were log-transformed prior to analysis. For all analyses, we plotted residual against
513 fitted values to confirm homogeneity of variance and generated a QQ plot to confirm that the residuals
514 were approximately normally distributed.

515

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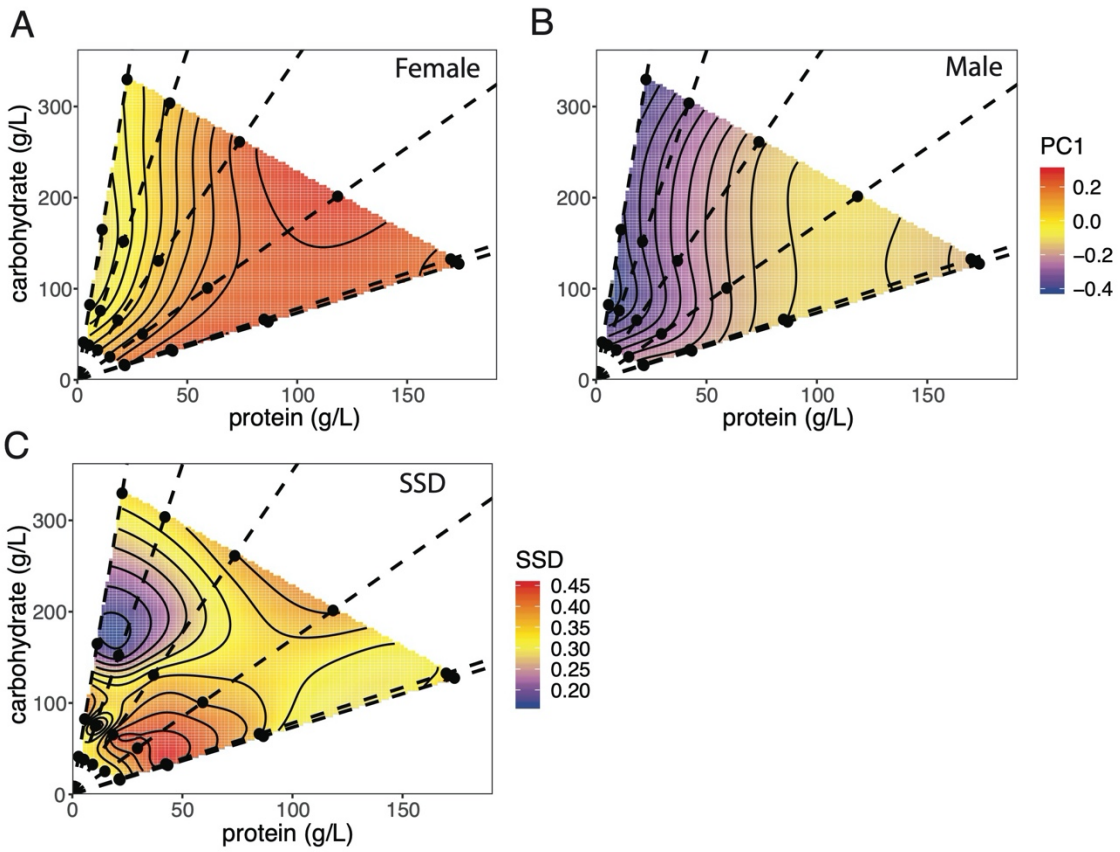
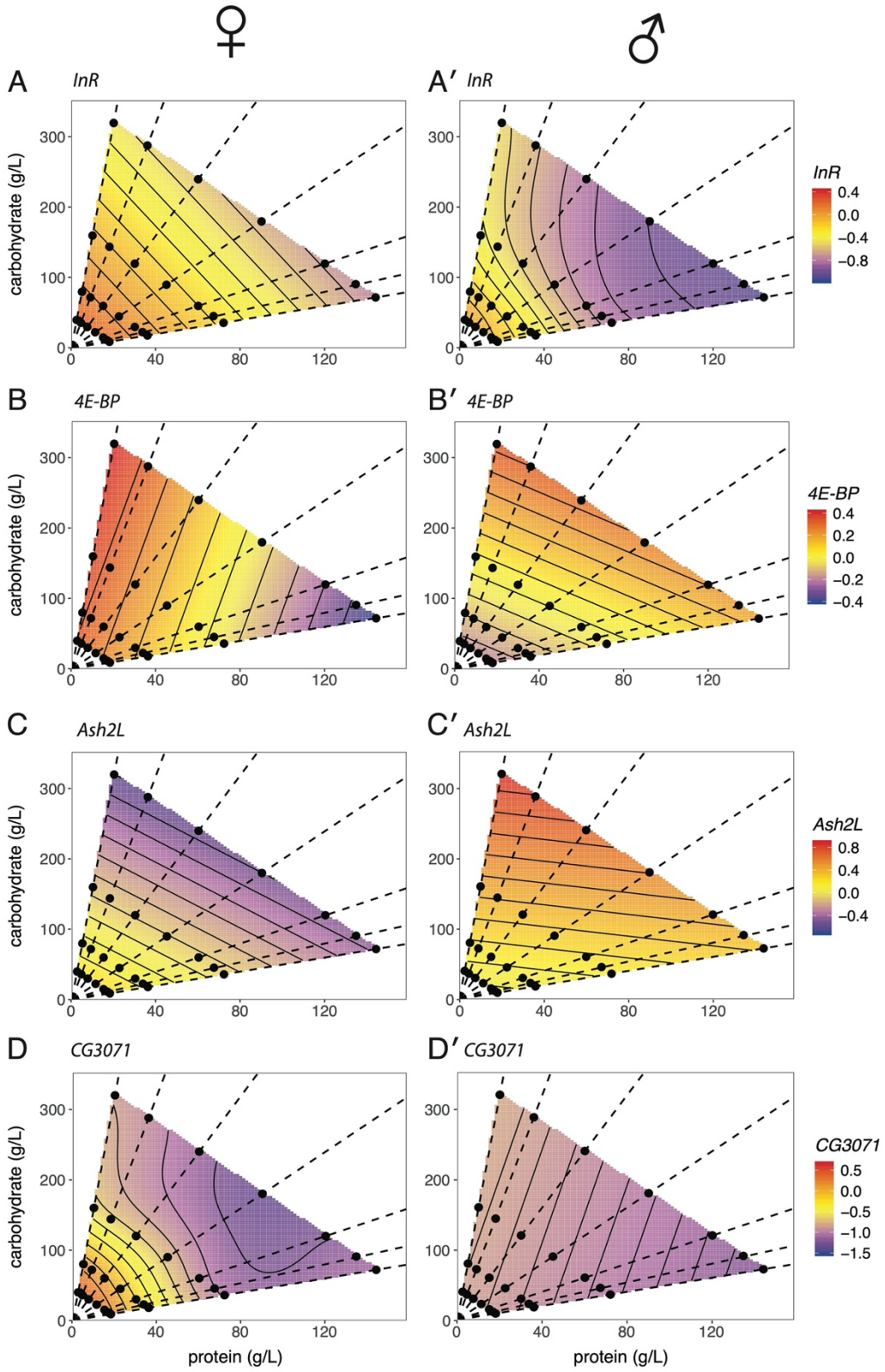
615

8. Supplementary Figures

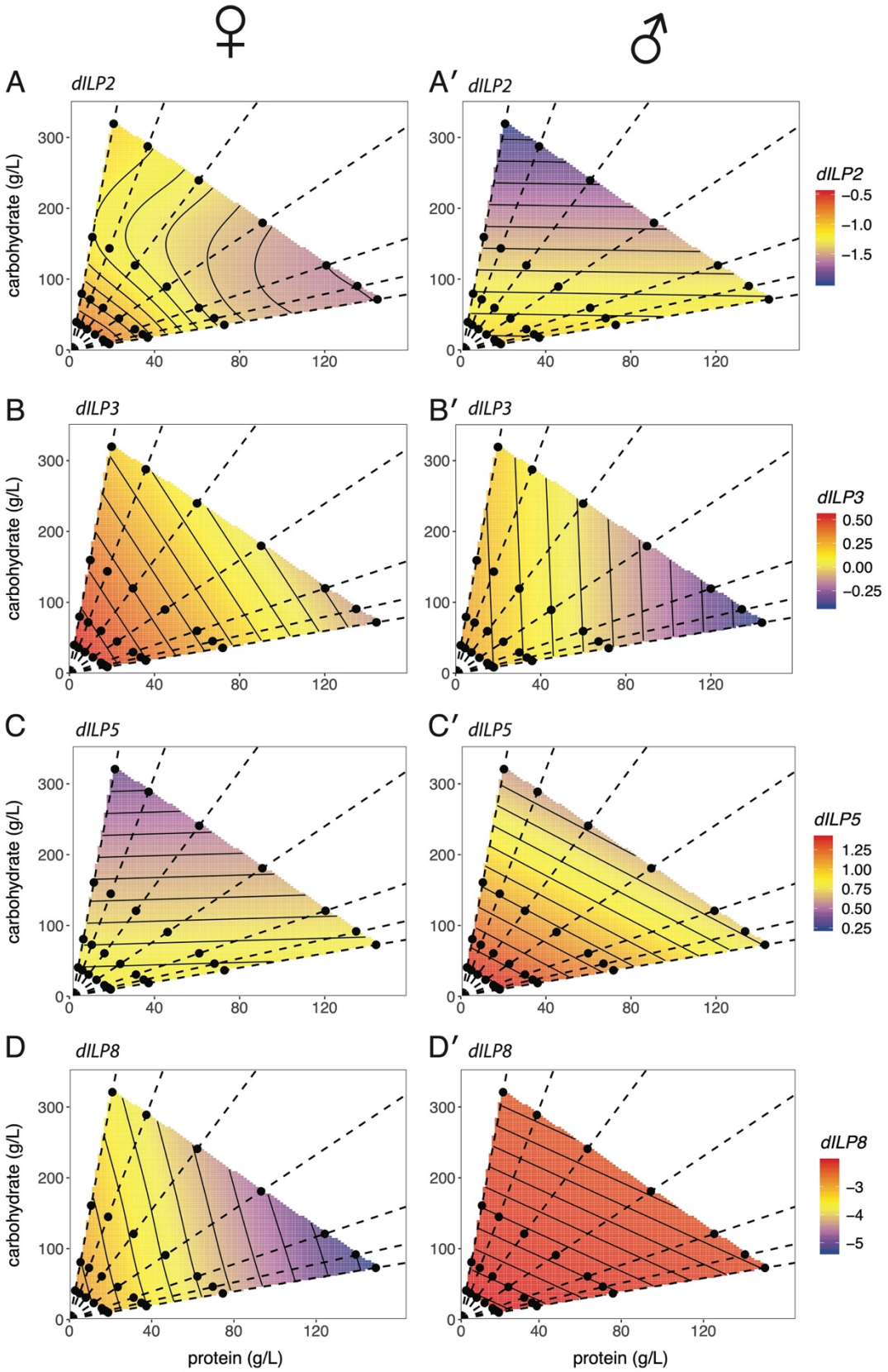


Supplementary Figure 1: Thin plate spline of the effect of protein and carbohydrate concentration on female and male body size and sexual size dimorphism (SSD). (A, B) Surfaces shows the relationship between body size, carbohydrate level and protein level in female and male flies (C) Surface shows a thin plate spline of the difference in female and male body size (SSD) across the same nutritional landscape, using fitted values from A and B. Points indicate diets tested and dotted lines connect diets with equal protein-to-carbohydrate ratios (1:14.6, 1:7.2, 1:3.5, 1:1.7, 1.3:1, 1.4 :1).


♀
♂
A
InR
A′
InR
B
4E-BP
B′
4E-BP
C
Ash2L
C′
Ash2L
D
CG3071
D′
CG3071
carbohydrate (g/L)
protein (g/L)
0
40
80
120
100
200
300
InR
0.4
0.0
−0.4
−0.8
4E-BP
0.4
0.2
0.0
−0.2
−0.4
Ash2L
0.8
0.4
0.0
−0.4
CG3071
0.5
0.0
−0.5
−1.0
−1.5

624

625 **Supplementary Figure 2: Thin plate spline of the effect of protein and carbohydrate concentration on**
626 **the expression of IIS and TOR transcriptionally-regulated genes in females and males.** Surfaces shows
627 the relationship between gene expression, carbohydrate level and protein level in female and male flies.
628 Expression of (A, A′) *InR*, and (B, B′) *4E-BP*, both negatively regulated by the activity of the IIS via the
629 Forkhead transcription factor FOXO. (C, C′) Expression of *Ash2L*, ostensibly negatively regulated by the
630 activity of TOR signaling. (D, D′) Expression of *CG3071*, ostensibly positively regulated by the activity of
631 TOR signaling. Points indicate diets tested and dotted lines connect diets with equal protein-to-
632 carbohydrate ratios (1:16, 1:8, 1:4, 1:2, 1:1, 2:1).


♀
♂
A
dILP2
A′
dILP2
B
dILP3
B′
dILP3
C
dILP5
C′
dILP5
D
dILP8
D′
dILP8
carbohydrate (g/L)
protein (g/L)
0
40
80
120
100
200
300
dILP2
−0.5
−1.0
−1.5
dILP3
0.50
0.25
0.00
−0.25
dILP5
1.25
1.00
0.75
0.50
0.25
dILP8
−3
−4
−5

633

634 **Supplementary Figure 3: Thin plate spline of the effect of protein and carbohydrate concentration on**
635 **the expression of dILPs in females and males.** Surfaces shows the relationship between gene expression,
636 carbohydrate level and protein level in female and male flies. (A) *dILP2*. (B) *dILP3.* (C) *dILP 5.* (D) *dILP8*.
637 Points indicate diets tested and dotted lines connect diets with equal protein-to-carbohydrate ratios (1:16,
638 1:8, 1:4, 1:2, 1:1, 2:1). Corresponding thin-plate spline plots are shown in Supplementary Figure 1.
639

9. Tables

Table 1: Effects of protein (P), carbohydrate (C), and their squares and product on body size in females and males.

SEX[A]		C	P	C^2	P^2	C*P
FEMALE	β^B	-0.0044	0.0068	0.00001	-0.00003	0.00001
	X^2	17.04***	0.02^{NS}	21.45***	36.25***	4.80*
MALE	β^B		0.0081		-0.00004	
	X^2		11.408***		11.513**	

[A] For each sex, we fitted a linear mixed effect orthogonal polynomial regression with replicate vial as a random factor and removed non-significant parameters.

[B] Parameter values for the response surface are for the non-orthogonal polynomial regression to facilitate interpretation

[C] Type III Wald chi-square test with associated P-values: NS $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

651 **Table 2: Effect of including sex as an interactive versus additive term when modeling the influence of**
652 **diet on body size**

653

MODEL[A]	DF[B]	AIC[C]	BIC[C]	LL	MODEL COMPARSONS	LRT [D]	*P* [E]
A: $T_{ijkl} = S_k + C_i + C_i^2 + P_j + P_j^2 + C_iP_j + R_l$	9	-1059.0	-1017.3	538.48			
B: $T_{ijkl} = C_i + C_i^2 + S_k (P_j + P_j^2) + C_iP_j + R_l$	11	-1057.7	-1006.8	539.85	A. v. B	2.73	0.256
C: $T_{ijkl} = S_k (C_i + C_i^2) + P_j + P_j^2 + C_iP_j + R_l$	11	-1066.7	-1015.8	544.34	A. v. C.	11.71	**0.006**
D: $T_{ijkl} = S_k (C_i + C_i^2 + P_j + P_j^2 + C_iP_j) + R_l$	14	-1067.7	-1002.9	547.85	C. v. D.	7.03	0.071

654
655 [A] *T* is the body size, *S* is sex, *C* is carbohydrate, *P* is protein, *R* is replicate vial (random factor). The models differ by
656 having sex as an interactive versus an additive factor.
657 [B] Estimated degrees of freedom for each model.
658 [C] AIC, BIC, log likelihood (LL) calculated using ML fit.
659 [D] Likelihood ratio test (LRT) statistic is for model comparisons.
660 [E] *P*-value for LRT is calculated by parametric bootstrapping using ML fit. Significant *P*-values are shown in bold.
661

662 **Table 3: Coefficients and partial-eta-squared for multiple regression of body size against gene**
663 **expression in males and females.**

664

GENE	FEMALE			MALE		
	Coefficient [A]	η_p^2	***P*** [B]	**Coefficient**	η_p^2	***P***
InR	-0.014	0.004	0.638	-0.038	0.168	**0.047**
4E-BP	-0.123	0.147	**0.003**	0.018	0.013	0.593
Ash2L	0.014	0.003	0.660	0.019	0.034	0.391
CG3071	-0.053	0.038	0.137	-0.020	0.017	0.545
dILP2	0.039	0.018	0.308	-0.019	0.072	0.206
dILP3	-0.010	0.001	0.866	-0.018	0.080	0.181
dILP5	0.054	0.071	**0.042**	0.047	0.146	0.066
dILP8	-0.025	0.076	0.035	<0.001	<0.001	0.970

665 [A] Coefficients for the multiple regression $T_{ij...p} = A_i + B_j + \ldots + H_p$ where T is body size (fitted values from the linear
666 mixed model of the relationship between body size and diet) and A through H is the expression of the genes
667 measured in our analysis.
668 [B] Significant P-values are shown in bold.

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674 **Table 4: Effect of including sex as an interactive versus additive term when modeling the influence of**
675 **diet on the expression of multiple genes**

676

MODEL[A]	DF [B]	VAR [C]	*PILLAI*[D]	*F* [E]	*P* [F]
$\boldsymbol{T}_{ijk} = S_k + (C_i + P_j + P_j^2)$	92	0.53979			
$\boldsymbol{T}_{ijk} = S_k\ (C_i + P_j + P_j^2)$	89	0.51856	0.5284	2.1254	**0.002**
$\boldsymbol{T}_{ijk} = S_k\ (C_i + C_i^2 + P_j + P_j^2 + C_iP_j)$	85	0.51583	0.3791	1.0599	0.384

677

678 [A] ***T*** is a matrix of expression levels across eight genes, *S* is sex, *C* is carbohydrate, *P* is protein, *R* is replicate vial
679 (random factor). The models differ by having sex as an interactive versus an additive factor.
680 [B] Estimated degrees of freedom for each model.
681 [C] Generalized variance
682 [E] Pillai's Trace is against model specified in row above.
683 [D] Aproximate *F*-statistic is against model specified in row above.
684 [E] *P*-value for LRT is calculated by parametric bootstrapping using ML fit. Significant *P*-values are shown in bold.

685

686

687 **Table 5: Effects of protein (*P*), carbohydrate (C), and their squares and product on gene expression in**
688 **females and males.**

GENE	SEX		C	C^2	P	P^2	C*P
InR	female	β^A			-0.025	0.0001	
		t-value[B]			**-3.883*** **	**2.904** **	
	male	β			-0.033	0.0002	
		t-value			**-2.466***	**2.347***	
4E-BP	female	β			-0.005		
		t-value			-3.614***		
	male	β					
		t-value					
Ash2L	female	β	-1.64258		-0.018	0.0001	
		t-value	**-2.180***		**-2.160***	**2.076***	
	male	β	1.7724				
		t-value	**2.651** **				
CG3071	female	β	-0.014	0.00002	-0.049	0.0002	0.00013
		t-value	**-4.252*** **	**2.083***	**-5.137 *** **	**4.313*** **	**3.175** **
	male	β					
		t-value					
dILP2	female	β	-0.016	0.00004	-5.255		0.00012
		t-value	**-2.692** **	**3.013** **	**-3.095** **		**2.524***
	male	β					
		t-value					
dILP3	female	β			-0.005		
		t-value			**-2.950** **		
	male	β					
		t-value					
dILP5	female	β	-1.5897		0.019	-0.0001	
		t-value	-1.573		0.156	**-2.087***	
	male	β					
		t-value					
dILP8	female	β			-0.054	0.0003	
		t-value			**-5.101*** **	**2.917** **	
	male	β					
		t-value					

689

690 [A] Parameter values for the response surface are for the non-orthogonal polynomial regression to facilitate
691 interpretation.
692 [B] t-tests are for orthogonal polynomials. [NS] $P > 0.05$, * $P < 0.05$, * $P < 0.01$, *** $P < 0.001$. Significant *t*-values are shown
693 in bold.

694

695

696 **Table 6: Effect of including sex as an interactive versus additive term when modeling the influence of**
697 **diet on the expression of individual genes.**

GENE	MODEL[A]	DF[B]	RSS[C]	*F*[D]	*P*[E]
InR	$T_{jk} = P_j + P_j^2$	148	100.209		
	$T_{jk} = S_k + P_j + P_j^2$	147	96.398	5.7500	**0.0178**
	$T_{jk} = S_k (P_j + P_j^2)$	145	96.120	0.2098	0.811
4E-BP	$T_{jk} = S_k + P_j$	153	52.219		
	$T_{jk} = S_k P_j$	152	48.604	11.305	**0.001**
Ash2l	$T_{ik} = S_k + C_i$	152	80.009		
	$T_{ik} = S_k * C_i$	151	72.610	15.888	**0.0001**
	$T_{ik} = S_k (C_i + P_j + P_j^2)$	147	68.459	2.229	0.069
CG3071	$T_{ijk} = S_k + C_i + C_i^2 + P_j + P_j^2$	123	52.497		
	$T_{ijk} = S_k (C_i + C_i^2 + P_j + P_j^2)$	118	43.322	4.998	**0.0003**
dILP2	$T_{ijk} = C_i + C_i^2 + P_j$	123	157.57		
	$T_{ijk} = S_k + C_i + C_i^2 + P_j$	122	156.47	0.8623	0.3550
	$T_{ijk} = S_k (C_i + C_i^2 + P_j + C_iP_j)$	119	152.96	0.9097	0.4386
dILP3	$T_{jk} = P_j$	152	136.38		
	$T_{jk} = S_k + P_j$	151	134.35	2.2632	0.1346
	$T_{jk} = S_k P_j$	150	134.35	<0.0001	0.9803
dILP5	$T_{ijk} = C_i + P_j + P_j^2$	148	147.40		
	$T_{ijk} = S_k + C_i + P_j + P_j^2$	147	142.85	4.6873	**0.0320**
	$T_{ijk} = S_k (C_i + P_j + P_j^2)$	144	139.80	1.0458	0.37431
dILP8	$T_{jk} = S_k + P_j + P_j^2$	121	205.98		
	$T_{jk} = S_k (P_j + P_j^2)$	119	190.40	4.8693	**0.0093**

698 [A] *T* is a expression level of gene, *S* is sex, *C* is carbohydrate, *P* is protein. The models differ by having sex as an
699 interactive versus an additive factor. The models chosen are the simplest where one or either sex have significant
700 parameter values.
701 [B] Degrees of freedom for each model.
702 [C] Residual Sum Square
703 [D] *F*-statistic is against model specified in row above.
704 [E] Significant P-values are shown in bold.

705 **Supplementary Table 1: Variation in gene expression among samples within diets for male and female**
706 **samples.**

707

	4E-BP	*INR*	*ASH2L*	*CG3071*	*DILP2*	*DILP3*	*DILP5*	*DILP8*
FEMALE RMSE[A]	0.4184	0.5834	0.5233	0.5070	0.0505	0.4012	0.7208	1.3167
MALE RMSE[A]	0.5041	0.6971	0.6720	0.4876	1.4900	1.2494	0.9775	1.0351
***P*-VALUE[B]**	0.2752	0.3739	0.1016	0.1628	**<0.0001**	**<0.0001**	**0.0389**	**0.0018**

708 [A] Root mean square error from the model $T = D$ where T is expression level of gene and D is diet (protein and
709 carbohydrate combination).
710 [B] P-value for F test comparing residual variance for males and female level of gene expression, after fitting the model
711 $T = D$. Significant P-values are shown in bold

712

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714