Natural variation in phospholipase IPLA2-VIA contributes to starvation sensitivity in *Drosophila melanogaster*

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Abstract

Resistance to starvation is a classic complex trait where genetic and environmental variables can greatly modify an animal's ability to survive without nutrients. Genetic analyses in *Drosophila* have shown that starvation resistance is highly polygenic with different genetic architectures in different mapping populations. In this study we sought to dissect the genetic basis of starvation resistance among a set of mitonuclear genotypes carrying different mtDNAs paired with specific nuclear genomes from the Drosophila Genetic Reference Panel (DGRP). We focused on differences between one of the most sensitive strains (DGRP-765) and a strain with more moderate resistance (DGRP-315) whose starvation phenotypes appeared to be modified by alternative mtDNAs. Using complementary pooled-sequencing and forward genetic mapping approaches, we identified regions of chromosomes 2L, 3L and 3R contributing to starvation sensitivity and localize a major effect locus modifying starvation resistance to the coding region of phospholipase iPLA2-VIA. These analyses further confirm that the alternative mtDNAs had little influence on variation in starvation resistance between the genotypes studied. The sensitive line shows a starvation-dependent depletion of glucose and glycogen that is modified by hemi- and heterozygosity in the iPLA2-VIA region. These findings contribute to our understanding of the complex genetic relationship between resistance to starvation stress and nutrient metabolism.

42 # Introduction

43 Coping with periods of low nutrient availability is a fundamental fitness component of all
44 organisms. In animals, multiple metabolic processes are required to convert dietary
45 nutrients into usable resources to successfully cope with periods of starvation. Energy
46 that is not needed immediately has to be converted into a form amenable for efficient
47 storage and that stored energy has to be accessible for utilization during periods of
48 starvation. For these reasons, the genetics of starvation resistance provides a compelling
49 model for understanding the integrative aspects of animal physiology (Brown *et al.* 2019).
50 Furthermore, a better understanding of the genetic basis of nutrient catabolism and
51 storage will also shed a light on genes relevant to metabolic diseases and fitness traits in
52 nature (Hardy *et al.* 2018).
53
54 Much of the information on the genetics of starvation resistance comes from studies in
55 Drosophila employing either direct selection on experimental outbred populations or
56 genome wide association studies (GWAS) across samples of inbred lines. Variation in
57 starvation resistance is an appealing trait for studies of quantitative and evolutionary
58 genetics due to its highly polygenic nature and its correlation with other fitness and
59 metabolic traits (Service and Rose 1985; Chippindale *et al.* 1996; Harshman *et al.* 1999;
60 Hardy *et al.* 2018; Everman *et al.* 2019). GWAS approaches using either the Drosophila
61 Genetic Reference Panel (DGRP;(Mackay *et al.* 2012)) or the Drosophila Synthetic
62 Population Resource (DSPR;(Everman *et al.* 2019)) have identified a number of candidate
63 loci influencing variation in starvation resistance confirming the complexity of its polygenic
64 nature (Harbison *et al.* 2004; Sorensen *et al.* 2007; Huang *et al.* 2012; Mackay *et al.* 2012;
65 Everman and Morgan 2018; Everman *et al.* 2019). Importantly, there are clear differences

among studies in the location of QTLs and the putative candidate loci identified (HUANG *et al.* 2012; MACKAY *et al.* 2012; EVERMAN AND MORGAN 2018; EVERMAN *et al.* 2019). These differences have been attributed to the distinct genetic composition of the mapping populations and presumably unique epistatic interactions among loci influencing starvation resistance (*ops. cit.*). Notably, none of the QTL identified in the two subsets of the mapping lines used by (EVERMAN *et al.* 2019) overlapped, likely due to the different founder lines used to build each subset (DSPR pA and pB). Despite these differences, there are shared loci that emerge from a comparison of multiple studies (table S9 in (EVERMAN *et al.* 2019)).

A growing literature has identified a role for mitochondrial functions in stress responses at multiple levels (UMA NARESH *et al.* 2022; ERKOSAR *et al.* 2025). Given the central roles that mitochondria play in both anabolic and catabolic functions, the complex interactions between mitochondrial and nuclear genomes may play important roles in adaptive traits, and particularly in metabolically demanding conditions such as starvation, oxidative stress and animal performance (RAND *et al.* 2004; HILL 2015). In previous work, we demonstrated the impact of mitonuclear epistasis in development time among a panel of 72 mitonuclear genotypes constructed from 6 mtDNAs introgressed into 12 nuclear genomes from the Drosophila Genetic Reference Panel (DGRP) (MOSSMAN *et al.* 2016). We selected a subset of these lines to examine starvation resistance based on significant differences in development time between mtDNAs in each of several DGRP backgrounds. These analyses revealed a putative mitonuclear epistatic interaction influencing starvation resistance and its relation to diet composition. In the current study, we sought to use a

89 combination of quantitative genetic and classical genetic mapping approaches to examine
90 the genetic basis of starvation resistance between two DGRP lines carrying different
91 mtDNAs (DGRP-765 and -315). Genomic sequencing of sensitive and resistant pools of
92 adults from advanced intercross populations derived from these lines identified regions
93 of allelic differentiation. Chromosome segregation and recombination mapping identified
94 major factors on chromosome 3L, 3R and 2L, but not chromosome X, 2R or the
95 mitochondrial genome. Deficiency mapping indicated that the starvation sensitivity in
96 DGRP-315 localizes in part to the phospholipase iPLA2-VIA gene with associated effects
97 of carbohydrate metabolism. The correspondence of these approaches contributes to our
98 understanding of the polygenic nature of starvation resistance and points to
99 polymorphisms in the iPLA2-VIA region that motivate further study of the associations
100 between starvation sensitivity, lipid metabolism and carbohydrate metabolism.
101

102 # Materials and Methods

103 ***Fly stocks***

104 The current study focuses on the differences in starvation resistance between specific
105 mitonuclear genotypes carrying alternative mtDNAs placed onto nuclear genomic
106 backgrounds of the Drosophila Genetic Reference Panel (DGRP;(MACKAY *et al.* 2012)).
107 In a previous study we constructed a panel of 72 mitonuclear genotypes using balancer
108 chromosome substitution to place six mtDNAs onto each of 12 DGRP nuclear
109 backgrounds to study mitonuclear epistasis for development time and its dietary
110 modification (MOSSMAN *et al.* 2016). From these 72 lines we quantified starvation
111 resistance of 12 mitonuclear genotypes consisting of four different mtDNAs placed on

112 three DGRP nuclear backgrounds (lines DGRP-765, -315 and -820). The four mtDNAs
113 included two from *D. simulans* (*siI and siII*) and two from *D. melanogaster* (*OregonR* =
114 *OreR* and *Zimbabwe53* = *Zim53*). These mtDNAs were chosen to test for the impact of
115 mtDNA polymorphism and divergence on starvation resistance. The DGRP
116 backgrounds were chosen based on published starvation data showing that DGRP-765,
117 -315 and -820 had low, medium and high values of starvation resistance, respectively
118 (AYROLES *et al.* 2009; MACKAY *et al.* 2012). The genotype notation used is
119 *mtDNA;nuclearDNA*, e.g., *Zim53;315* carries the *D. melanogaster Zim53* mtDNA on a
120 homozygous *DGRP-3*15 chromosomal background.
121
122 The results of this starvation assay are shown in Figure 1. The resistance levels were
123 consistent with previously published rankings of the three nuclear genotypes (820 > 315
124 > 765). The results further showed that one unique combination, *siI;765,* (a *D. simulans*
125 *siI* mtDNA on the *DGRP-765* nuclear background) had a significant increase in
126 starvation resistance compared to the other mitonuclear genotypes in the *DGRP-765*
127 background. The goal of the current study was to determine whether the apparent
128 'rescue' of starvation resistance was due to the introgression of the *siI* mitochondrial
129 haplotype or due to a nuclear factor or factors. The focal mitonuclear genotypes are
130 shown in Table 1. Additional genotypes for segregation, recombination and deficiency
131 mapping are listed in Table S1.
132
133 ***Starvation Stress***

134 Survival during starvation stress was measured by placing approximately 125 female flies
135 per genotype in each of three replicate demography cages. Flies were collected and
136 sorted over CO_2 and allowed to recover in quart-sized plastic demography cages affixed
137 with a 25x95 mm vial containing one of three different isocaloric food media: high sugar
138 food (15% sucrose, 5% Yeast, 2% agar), balanced food (10% sucrose, 10% yeast, 2%
139 agar) or low sugar food (5% sucrose, 15% yeast, 2% agar; referred to as food type V, II
140 and IV, respectively, in (ZHU *et al.* 2014)). The three replicate cages for each genotype
141 were conditioned on these diets for 7 days unless otherwise indicated, with new food vials
142 added every other day. After 7 days, any dead flies were removed, and the vial for each
143 demography cage was replaced with 2% Difco Bacto-agar prepared with deionized H_20
144 to initiation starvation. The number of dead flies was recorded every 12 hours and
145 removed from each demography cage until all flies were dead. As starvation resistance
146 values for the different genotypes were most distinct on the high sugar diet "V", most of
147 the genetic mapping was carried out on that diet. The survival data from each cage were
148 generally consistent so counts from each replicate cage were pooled and the combined
149 data modeled using a Kaplan-Meir survival model implemented in R (THERNEAU AND
150 GRAMBSCH 2000). Significant differences in survivals were determined using a log rank
151 test.
152
153 ***Forward genetic mapping of starvation sensitivity***
154 *Chromosomal localization of resistance*. To determine if different mtDNAs were
155 associated with the difference in starvation resistance reciprocal crosses were done
156 between sil;765 and Zim53;765 as follows: virgin female sil;765 x male Zim53;765 and

157 virgin female Zim53;765 x male siI;765). Maternal inheritance of mtDNA provides a test
158 for the role of the *siI* mtDNA in the rescue of starvation sensitivity. To localize nuclear
159 factors associated with starvation sensitivity we generated genotypes that were
160 selectively heterozygous for different combinations of *Zim53;765* autosomes. Twenty
161 *Zim53;765* virgin females were first crossed to 20 males from the double-balancer stock
162 ($wg^{Sp-1}/CyO; Dr^{1}/TM3, Sb^{1}$). Twenty F1 male progeny with both the 2nd chromosome
163 *CyO* and 3rd chromosome *Dr* markers present were sorted over CO_2 and crossed to 20
164 *Zim53;765* virgin females in eight replicate 20-female x 20-male crosses. Progeny from
165 this second cross were segregated into four groups based on the presence or absence
166 of the *CyO* and *D*r markers. The four groups were: reconstituted wild-type *Zim53;765*,
167 chromosome 2 heterozygotes (*CyO/765*), chromosome 3 heterozygotes (*Dr/765*) and
168 heterozygotes for both chromosome 2 and 3 (*CyO/765;Dr/765*). Flies from each group
169 were moved to 3 replicate demography cages with approximately 125 flies each. The
170 survival of each group under starvation stress was assayed as described earlier.
171
172 ***Whole genome sequencing of sensitive and resistant pools from Advanced***
173 ***Intercross Populations***
174 *Population Cages.* Five replicate advanced intercross populations (AIPs) were
175 established between the sensitive (*Zim53;765*) and resistant (*Zim53;315*) lines. Each
176 replicate population was initiated using 25 *Zim53;315* virgin females crossed to 25
177 *Zim53;765* males, in addition to the reciprocal cross, to yield a total number of 50 male
178 and 50 female parents of each line as the founders of each of the five replicate cages.
179 These parents were introduced into Plexiglas cages (20 x 20 x 20cm) and allowed to lay

180 eggs in four 125mL culture bottles with 30mL of Drosophila medium in each bottle. The
181 four bottles in each cage were left with their tops open for 3 days, and then each bottle
182 was plugged to allow flies to develop. Prior to eclosion, the plugs were removed from
183 each bottle, and all flies were allowed to emerge into the cage and mate at random. Four
184 new culture bottles replaced the old bottles in each replicate cage, and the adults were
185 allowed to lay eggs for 48 hours. After egg laying, adults were cleared from each bottle,
186 plugs were inserted, and cultures were allowed to develop for 7 days following the
187 termination of egg laying, after which the plugs were removed from all bottles to allow
188 eclosion of the next generation into each cage. Population sizes exceeded 1000
189 individuals after the first generation. All five cages were maintained at large population
190 sizes (>2000 adults) for 10 generations to allow recombination to take place between the
191 sensitive (*Zim53;765*) and resistant (*Zim53;315*) DGRP nuclear genotypes. These two
192 DGRP strains are homozygous for the standard (ST) chromosome arrangement
193 (http://dgrp2.gnets.ncsu.edu/) so this lack of inversions should have allowed free
194 recombination across the genome in these populations.
195
196 *Starvation Stress.* The female flies from each generation-10 population cage were
197 collected over CO_2 and moved to demography cages with a density of approximately 150
198 flies per cage. The flies were allowed to recover for 7 days with access to high sugar food
199 (Type V: 15% sucrose, 5% Yeast, 2% agar) and then subjected to starvation conditions
200 by replacing the food vials with a 2% agar and water mix. Every six hours from the start
201 of starvation, all the dead flies were collected, flash frozen and stored at -80C. The high
202 sugar treatment enhanced the difference among genotypes in starvation resistance. The

203 genotypic differences in starvation were also evident with moderate or low sugar
204 treatments but mapping experiments were more effective with the high sugar treatment
205 so that was used throughout these analyses.
206
207 *Pool-sequencing.* Pools of approximately 50 dead flies were made by combining flies from
208 the two earliest time points when deaths were first observed (24 hour and 30 hour) and
209 the last two time points when the last live flies were present (96 hour and 102 hour). DNA
210 from each replicate pool was isolated using the DNeasy DNA purification kit from Qiagen
211 (Cat No 69506). Each sample was RNase treated prior to DNA elution using a 10-minute
212 RNase A (Cat No 19101) treatment. Libraries were prepared and sequenced using the
213 DNBseq platform by the Beijing Genomics Institute (BGI).
214
215 *Estimation of genetic differentiation using Popoolation2.* Raw reads were mapped to the
216 dm6 build of the *Drosophila* genome using *bwa* version 0.7.15 (LI AND DURBIN 2009).
217 Variants were called using *samtools* version 1.9 (LI *et al.* 2009). Allele frequency
218 differences and estimates of population subdivision (F_{ST}) were determined using
219 *Popoolation2 (*KOFLER *et al.* 2011*)*, and the significance of differences in allele frequencies
220 between the early-dying and late-dying sample across the five replicate populations was
221 determined using the Cochran-Mantel-Haenszel test. P-values were corrected using the
222 Bonferroni method and differences with FDR-corrected p-values < 0.05 were considered
223 to be significant. Linkage disequilibrium was estimated from paired end reads, following
224 the procedure described in (FEDER *et al.* 2012). The expectation of this AIP pool-seq
225 experiment is that recombination would segregate major factors responsible for the

226 difference in starvation resistance between these strains so that extreme-phenotype
227 mapping by pooled sequencing might identify chromosomal regions associated with this
228 difference. We acknowledge that 10 generations, moderate populations sizes (~2000)
229 and founding samples of 50 extreme-phenotype adults may not have the power to
230 generate high resolution recombinants for localizing individual loci, but our intention was
231 to use this as a means to complement other classical mapping approaches (chromosome
232 segregation, marker-recombination assays and deficiency mapping). The use of five
233 replicate populations provided some level of replication that was appropriate for the
234 budget available at the time.
235
236 ***Chromosome 3 recombination***
237 The chromosome segregation experiments indicated that the majority of the difference in
238 starvation resistance between the lines mapped to chromosome 3 and the pooled
239 sequence analyses of the AIP cages identified two broad candidate regions on
240 chromosome 3, one each on 3L and 3R. We performed recombination mapping using
241 three successive crosses to marked mapping stocks to further localize candidate regions
242 (cross 1, 2, 3; see Fig. S1). For cross 1 we attempted to generate *Zim53;765* chromosome
243 3 recombinants by crossing 20 *Zim53;765* virgin females to 20 males from a multiply
244 marked recessive stock obtained from the Bloomington Stock Center (BSC_576 = ru^1
245 hry^1 $Diap^{th\text{-}1}$ st^1 cu^1 sr^1 e^s ca^1 hereafter referred to as BSC_576 for convenience). The
246 progeny from this cross however failed to eclose. As an alternative cross 1, we crossed
247 20 *sil;765* virgin females to 20 *BSC_576* males in an effort to allow recombination across
248 the *DGRP-765* 3rd chromosomes that are present in the *sil;765* stock. The *sil;765*

249 genotype was suspected to be heterozygous for the *DGRP-765* 3rd chromosome and a
250 3rd chromosome from the *TM3* balancer stock that may have recombined with the *DGRP-*
251 *765* 3rd chromosome during the construction of the *sil;765* original stock since *sil;765*
252 lacked the *TM3* marker Sb^1 (Sb is at 89B4, band 58). This suspicion of balanced
253 heterozygosity was based on the observation that several generations of backcrossing of
254 male DGRP-765 to virgin female *sil;765* did not reduce starvation resistance implying that
255 the *sil;765* carried some heterozygosity and was in some way resistant to the impact of
256 recurrent backcrossing.
257 For cross 2, 20 F_1 virgin *sil;765* / BSC_576 females were collected and crossed to 20
258 males from a multiply marked recessive stock with the same recessive markers as
259 BSC_576 in addition to a dominant marker present on one copy of chromosome 3
260 balanced over the TM6 balancer (BSC_1711: ru^1 hry^1 $Diap^{th-1}$ st^1 cu^1 sr^1 e^s Pri^1 ca^1 /
261 *TM6B,* Bri^1, Tb^1). This cross was done in three replicate 20-female x 20-male vials. F_1
262 males from this second cross were collected and segregated into distinct recombinant
263 classes based on the recessive and dominant markers present.
264 For cross 3, males from each of these recombinant categories were each mated to virgin
265 starvation resistant *sil;765* females and to virgin starvation sensitive *Zim53;765* females
266 to generate offspring with different segments of the 3rd chromosome that was
267 homozygous for the starvation-sensitive *DGRP-765* chromosome. Comparison of the
268 offspring from these two different mitonuclear lines that showed significant differences in
269 starvation (*sil;765* and *Zim53;765*) could further resolve regions of chromosome 3
270 associated with starvation sensitivity. Due to the presumed heterozygosity of the *sil;765*
271 3rd chromosome we expected that recombination between the multiple-marked 3rd

272 chromosome with the *sil;765* 3rd chromosomes would be confounded if: 1) the
273 chromosome suspected to be the homologue of the 3rd chromosome in *sil;765* was a
274 broken balancer chromosome, or 2) if that homologue was some other wild type
275 chromosome that had been recombining with the *DGRP-765* 3rd chromosome. This would
276 reduce the efficiency of the recombination mapping as only an unknown fraction of the
277 offspring would have been derived from the actual pairing of the *sil;765* 3rd chromosome
278 derived from DGRP-765 and the recombinant-marked chromosomes derived from the F1
279 female parent. We acknowledge that this was not ideal but collected the mapping data to
280 see if it provided useful information consistent with the independent chromosome
281 segregation mapping and AIP pool-seq data.
282
283 Five classes of recombinant offspring phenotypes were designated based on the visible
284 markers present reflecting different breakpoints along the chromosome: class i*: ru, h*;
285 class ii: *ru, h, Diap, st*; class iii: *ru, h, Diap, st, cu*; class iv: *ru, h, Diap, st, cu, sr*; and class
286 v: *ru, h, Diap, st, cu, sr, e*. Twenty males from each group were crossed to 20 *Zim53;765*
287 virgin females and allowed to mate for 48 hours in three replicate 20-female x 20-male
288 crosses (the *Zim53;765* genotype was confirmed to be homozygous for the DGRP-765
289 chromosome). The males from each of these crosses were removed and then re-mated
290 to *sil;765* virgin females. Female offspring from each of these two sets of crosses were
291 collected over CO_2. Sorted by recombinant class and conditioned for starvation assays
292 on high sugar food. Sufficient flies for a starvation stress survival assay were only
293 available from the class i, class ii and class v crosses, so only these were considered.
294 The survival of these females under starvation stress was measured as described above.

295

296 ***Chromosome 3L Deficiency Screen***

297 The recombination mapping and the AIP pool-seq data implicated chromosome 3L as
298 harboring a major starvation factor, so deficiency mapping was pursued. Twenty males
299 from each deficiency line (Supplemental Table 1) were crossed to 20 *Zim53;765* virgin
300 females in eight replicate 20-female x 20-male crosses. F_1 females were collected and
301 sorted over CO_2. Females were separated based on the presence of the respective
302 balancer marker (flies heterozygous for the deficiency and the *765* chromosome do not
303 have the balancer present) and moved to 3 replicate demography cages with
304 approximately 125 flies each, for each genotype. The flies were allowed to recover for 7
305 days on high sugar food and survival under starvation stress measured as described
306 above.

307

308 ***iPLA2-VIA Complementation***

309 The chromosome 3L deficiency mapping, in conjunction with the available DGRP
310 sequences, identified *iPLA2-VIA* as a candidate gene based on nucleotide
311 polymorphisms private to *DGRP-765* and starvation sensitivity phenotypes of *iPLA2-VIA*
312 alleles (Lin *et al.* 2018). Deficiencies within that gene were obtained from the Bloomington
313 Stock Center for further complementation mapping: *BSC_80133* = y^1 w^*; *iPLA2-VIA*^{Delta174}
314 and *BSC_80134* = y^1 w^1; *iPLA2-VIA*^{Delta192} hereafter *80133* and *80134* respectively.
315 These deficiencies were generated by imprecise excision of a P-element from *iPLA-VIA*
316 that removed segments of 5' noncoding sequence and exons 1-5 and -7 (Lin *et al.* 2018).
317 Twenty *Zim53;765* flies were crossed in reciprocal to 20 flies from each of these two *iPLA-*

318 *VIA* coding region deficiencies. Each cross was done in replicates of 4. As a control,
319 males from each *iPLA2-VIA* deficiency stock were also crossed to *Zim53;315* females
320 with the expectation that the *DGRP-315* nuclear genome would complement the iPLA2-
321 VIA deficiencies and restore starvation to normal levels . F_1 females were collected and
322 sorted over CO_2 and moved to 3 replicate demography cages with approximately 125
323 flies each. The flies were allowed to recover for 7 days on high sugar food and survival
324 under starvation stress measured as described above.
325
326 ***Glucose Assay***
327 The free glucose levels, as described in (TENNESSEN *et al.* 2014), were measured for five
328 genotypes: *Zim53;765*, *Zim53;315*, *iPLA2-VIA*^{Delta174}, *Zim53;315* crossed to *iPLA2-*
329 *VIA*^{Delta174} and *Zim53;765* crossed to *iPLA2-VIA*^{Delta174}. 120 female flies from each
330 genotype were collected and sorted over CO_2 and allowed to recover in demography
331 cages affixed with 25x95 mm vials of high sugar food (15% sucrose, 5% Yeast, 2% agar)
332 for five days. Three replicates were done for each genotype. After five days, any dead
333 flies were removed and half of the remaining live flies were removed from the demography
334 cage, immediately flash frozen in liquid N_2 and stored at -80°C. The food supplied to each
335 demography cage was replaced with 2% Difco Bacto-agar prepared with deionized H_20.
336 After 12 hours, dead flies were discarded and the remaining live flies were removed from
337 the demography cage, immediately flash frozen in liquid N_2 and stored at -80°C. Six
338 frozen flies from each replicate were transferred to a 1.5uL micro-centrifuge tube and
339 homogenized in 120uL of Phosphate Buffered Saline (PBS) using a steel bead and
340 Qiagen TissueLyser II set to 30Hz for 2 minutes. A 10uL aliquot was removed to measure

341 protein content using the Pierce™ BCA Protein Assay Kit (23225). The remaining
342 homogenate was heat treated for 10 minutes at 70°C. The homogenate was diluted 1:4
343 with PBS. The free glucose was measured with the Glucose Oxidase (GO) kit (MAK097-
344 1KT) by following the protocol for free glucose measurement laid out in (TENNESSEN *et al.*
345 2014).
346

347 **Data Availability**

348 Drosophila strains, code and raw data are available at the Rand Lab GitHub site for this
349 publication (https://github.com/DavidRandLab/Williams-et-al.-Starvation-Genetics-2021).
350 The authors affirm that all analyzed data necessary for confirming the conclusions of the
351 article are present within the article, figures, tables and GitHub site. Raw sequences for
352 the pool-seq mapping are available at BioProject accession number: PRJNA1130456.
353

354 # Results

355 **Starvation resistance among mitonuclear genotypes**

356 Twelve mitonuclear genotypes from a panel of 72 previously-reported genotypes
357 (MOSSMAN *et al.* 2016) were chosen for starvation assays as described in the Methods
358 section. Each of these 12 mitonuclear introgression lines was subjected to starvation
359 assays on three different isocaloric diets: high sugar food (15% sucrose, 5% Yeast, 2%
360 agar), balanced food (10% sucrose, 10% yeast, 2% agar) or low sugar food (5%
361 sucrose, 15% yeast, 2% agar; referred to as food type V, II and IV, respectively (ZHU *et*
362 *al.* 2014)). The mitonuclear genotypes on the *DGRP-765* nuclear background were of
363 particular interest because the *DGRP-765* line represents one of the least resistant lines
364 in terms of starvation resistance in the DGRP, and it appeared that introgression of the

365 *sil* mitochondrial DNA haplotype significantly improved resistance to starvation only in
366 the *DGRP-765* nuclear background (Figure 1). To test whether the rescue of starvation
367 resistance mapped to a cytoplasmic or nuclear factor, we performed reciprocal crosses
368 to examine if maternal inheritance of mtDNA was the source of the rescue of starvation
369 sensitivity. When we crossed *Zim53;765* to *sil;765*, the direction of the cross (*Zim53;765*
370 *female* crossed *sil;765* males vs. *sil;765* females to *Zim53;765 males*) made no
371 difference to starvation resistance of its progeny. This implied that the *sil* mtDNA
372 haplotype was not the source of the rescue, as would be expected if the trait was being
373 inherited maternally (Fig. S2).
374
375 We analyzed the reads from an RNA-sequencing data set from each fly line and
376 compared the genotype calls for each SNP present in *Zim53;765* reads (restricted to the
377 coding regions) and the genotype calls at the same locations in *sil;765*. For the vast
378 majority of these sites, the *sil;765* was heterozygous for the *DGRP-765* allele and an
379 alternative allele (in most cases the reference allele) (Fig. S2A,B). We did a similar
380 comparison of *sil;765* to an alternate mitonuclear genotype (*Zim53;315*) and did not see
381 a similar overlap between the *sil;765* allele and the *Zim53;315* allele (Fig. S2A,B).
382 Based on these results we concluded that the *sil;765* genotypes was heterozygous
383 across the *DGRP-765* chromosomes. This suggested to us that starvation sensitivity in
384 *Zim53;765,* and the two other *mito;765* combinations (*Ore;765* and *siII;765*), were
385 homozygous recessive and the improvement in resistance to starvation seen in *sil;765*
386 was due to its nuclear heterozygosity. Indeed, we observed that the offspring from a

387 *Zim53;765* to *Zim53;315* cross were significantly more resistant to starvation than
388 *Zim53;765* (see Figure 6).

389 **Chromosome 3 heterozygosity partially suppresses *Zim53;765* starvation**
390 **sensitivity**
391
392 Based on the suppression of starvation sensitivity seen in *Zim53;765* heterozygotes,
393 (Zim53;765 x Zim53:315 F1 hybrids) we reasoned that starvation sensitivity phenotypes
394 in *Zim53;765* and other genotypes were homozygous recessive traits and that we could
395 localize the effect locus/loci to chromosomes using chromosome segregation tests and
396 observing which chromosome segregants masked the trait. Virgin females from three
397 different genotypes (the original *DGRP-765, Zim53;765* and *Zim53;315*) were mated to
398 males from the marked double-balancer stock ($wg^{Sp-1}/CyO; Dr^{1}/TM3, Sb^{1}$). F1 males
399 heterozygous for the maternal autosomes paired with the paternal *CyO* and Dr^{1} markers
400 were mated back to virgin females from the three maternal genotypes, generating the
401 four 2-autosome segregant populations of F1 flies as follows: heterozygous for
402 chromosome 2, heterozygous for chromosome 3, heterozygous for both chromosome 2
403 and 3, or neither (the latter being reconstituted homozygous wild type 2nd and 3rd
404 chromosomes of the original maternal genotypes: *DGRP-765, Zim53;765* and
405 *Zim53;315*). *DGRP-765* and *Zim53;765* flies heterozygous for chromosome 3 had a
406 ~200% increase in mean survival relative to the original stock (Figure 2A; Table
407 S2:SummaryTable_figure2a) and flies heterozygous for both chromosomes 2 and 3
408 realized additional increases in mean survival. Heterozygosity for chromosome 2 alone
409 contributed ~75% increase (range 50-116%; see Table S2:SummaryTable_figure2a).
410 For Zim53:315, chromosome 3 heterozygosity contributed nothing alone but a small
411 effect in combination with chromosome 2.

412 A repeat segregation experiment with *Zim53;765* and the heterozygous *sil;765*
413 genotype was conducted and revealed comparable results for *Zim53;765* (80-100%
414 increase from chromosome 3 heterozygosity with additional effects of chromosome 2)
415 and very different chromosomal influences for *sil;765*, as expected from its
416 heterozygous condition (Figure 2B; see Table S2:SummaryTable_figure2b; raw data
417 are in other tabs of Table S2). Taken together, these results suggest that the main
418 effect locus/loci controlling *Zim53;765* and DGRP-765 starvation sensitivity are present
419 on chromosome 3 with a secondary locus/loci present on chromosome 2.

420 **Sites on chromosome 2R and 3 have significant differences in allele frequency**
421 **between early and late dying flies.**
422
423 To understand the genetic basis of 765 starvation sensitivity, we sought to identify genetic
424 differences characteristic of starvation sensitive flies. We created replicate advanced
425 intercross populations (AIPs) in which *Zim53;765* was allowed to recombine with
426 *Zim53;315*. We set up five independent populations where *Zim53;765* flies were crossed
427 to *Zim;315* in reciprocal and the populations maintained for 10 generations at population
428 sizes >1000 adults. Following this, females from each population were subjected to
429 starvation stress and dead flies were collected every six hours until all flies were dead.
430 Flies that died during the first three and last two six-hour intervals were separately
431 combined into early- and late-dying pools of adult females (18-24, 24-30 and 30-36 hours
432 vs. 90-96 and 96-102 hours). Total genomic DNA was sequenced from each of the five
433 replicates from these two pooled samples, hereafter 'early' and 'late' dying pools.
434 Consistent differences in allele frequencies across the early and late dying populations
435 were determined using the Cochran-Mantel-Haenszel test implemented in Popoolation2
436 (KOFLER *et al.* 2011). There was an enrichment in significantly different variants across

437 chromosome 3 and a localized region of chromosome 2R (Figure 3). These results
438 suggest that *Zim53;765* and as a consequence *DGRP-765*, starvation sensitivity is based
439 on variants present on chromosome 3 and chromosome 2. Importantly, these advanced
440 intercross pool-seq analyses are consistent with the chromosome segregation analyses
441 where factors on chromosomes 3 and 2 were responsible for starvation sensitivity of the
442 *Zim;765* genotype.
443

444 **Heterozygosity of chromosome 3L region partially suppresses Zim53;765**
445 **starvation sensitivity**
446

447 To further localize the main effect loci on chromosome 3 we used recombination and
448 deficiency mapping across chromosome 3. As there are over 200 deficiencies spanning
449 chromosome 3, we first generated a series of recombinants with break points at different
450 locations along chromosome 3. Cross 1 used *sil;765* virgin females mated to a multiply
451 marked recessive stock (ru^1 hry^1 $Diap^{th-1}$ st^1 cu^1 sr^1 e^s ca^1 hereafter referred to as
452 BSC_576 for convenience). Cross 2 used heterozygous F_1 virgin females from cross 1
453 mated to males from a similarly marked recessive stock with one dominant marker added
454 to distinguish progeny (BSC_1711: ru^1 hry^1 $Diap^{th-1}$ st^1 cu^1 sr^1 e^s Pri^1 ca^1 / *TM6B,* Bri^1,
455 Tb^1). The F1 offspring from cross 2 revealed recombinant males that were pooled into
456 three classes of recombinants (classes i, ii, v) that were selected based on the presence
457 of visible recessive markers and sufficient flies for starvation analyses (Figure 4A).
458 Recombinant class i is likely heterozygous for the region of 3L from the telomere up to at
459 least the regions marked by *h,* with recombination breakpoints existing anywhere
460 between h and the *cu* gene. Recombinant class ii is heterozygous for most of 3L and up

461 to *cu* gene on 3R, with breakpoints existing anywhere between the *cu* gene and the *ca*
462 gene. Recombinant class v is likely heterozygous for most of chromosome 3 with
463 breakpoints existing from the *ca* gene up to the telomere of 3R. Other classes of
464 recombinants did not emerge in sufficient numbers to conduct the final mapping crosses.
465 It is important to note that because these recombinants were generated from *sil;765*, the
466 pool of recombinants will carry both the *DGRP-765* alleles from that chromosome which
467 was substituted onto the *D. simulans sil* mtDNA background during original stock
468 construction and alternate, unknown alleles from a chromosome that was likely
469 introduced during the balancer substitution process during original line construction. A
470 *TM3 Sb*^{1} 3rd chromosome balancer was used in these crosses and may have recombined
471 with segments of the *DGRP-765* 3rd chromosome during the replacement crosses (see
472 Methods).
473 To reveal the localization, cross 3 used males from the three recombinant classes
474 emerging from cross 2, each of which were mated to virgin *Zim53;765* females, and then
475 the same males were mated to virgin *sil;765* as a control. The starvation sensitivity of the
476 female offspring of the *Zim53;765* and recombinant males was compared to the female
477 offspring of the *sil;765* and recombinant male controls. While we cannot discern genotype
478 at the individual fly level, *DGRP-765* chromosome 3 alleles will be enriched in the progeny
479 from the *Zim53;765* vs recombinant cross, while the alternative will be true for the *sil;765*
480 vs recombinant cross. We expected that flies homozygous for the causal *Zim53;765* allele
481 would die earlier during starvation stress, thus revealing the region of *Zim53;765*
482 chromosome 3 responsible for its starvation sensitivity. Although the heterogeneity of
483 respective offspring populations will mask this effect, the enrichment of the *DGRP-765*

484 allele in the *Zim53;765* vs recombinant cross population and the alternate allele in the
485 *sil;765* vs recombinant cross population should result in appreciable differences in deaths
486 early on during starvation stress. Indeed, we observed a significant difference between
487 the starvation sensitivity of recombinant class i offspring, but not in the case of the class
488 ii offspring; class v offspring showed a small but significant difference (see Figure 4B;
489 Table S3). These results indicate that heterozygosity of a region between the *h* marker of
490 recombinant class i and the *cu* marker of recombinant class ii carries a factor required to
491 suppress *Zim53;765* starvation sensitivity.

492 **Hemizygosity of 3L: 9812381 – 9899255 prevents partial suppression of *Zim53;765***
493 **starvation sensitivity**
494
495 We employed a deficiency screen across the region between recombinant class i and
496 recombinant class ii markers. The objective of this screen was to identify the region(s) of
497 chromosome 3L that failed to complement *Zim53;765* starvation sensitivity. *Zim53;765*
498 virgin females were crossed to a series of flies deficient for various parts of chromosome
499 3L (Table S1). The deficient region of each line is maintained over a balancer
500 chromosome so the resulting offspring can be positive for the balancer, indicating
501 complete heterozygosity of chromosome 3, or offspring lacking the balancer are
502 hemizygous across the deficiency region and heterozygous for the rest of chromosome
503 3. Significant differences in the starvation resistance of the two classes of offspring
504 indicates that heterozygosity of the deficiency region is required for the suppression of
505 starvation sensitivity. We initially screened 15 deficiency lines and identified nine where
506 the starvation sensitivity was not suppressed (failed to complement) in the partial
507 chromosome 3L hemizygote (Table S4A and Fig.S3). Several of the deficiency stock
508 crosses with the most significant difference between Df and wt chromosome pairings

509 shared a common region of chromosome 3 between 3L:9812381 – 3L:9899255 (Fig. S3
510 and Figure 5), indicating that heterozygosity in this region is required for the suppression
511 of *Zim53;765* starvation sensitivity. Two deficiencies mapped to similar locations but
512 showed conflicting evidence for suppression of starvation sensitivity (Df282 and Df 283;
513 Fig. S3 and Table S1). The latter contained additional genes (lncRNA:CR45878,
514 CG46387) in the proximal region of the deficiency; the functional consequences of these
515 mapping differences were unclear. Three deficiencies on either side of the putative
516 overlap region also showed significant suppression of sensitivity (BSC113, 4470 and
517 4488). Despite the variation among the deficiency data, they were consistent with the
518 three broader-scale approaches of chromosome segregation, pool-seq and
519 recombination mapping in identifying candidate genes in this region of chromosome 3L.
520

521 **Variation in iPLA2-VIA as candidates for *Zim53;765* starvation sensitivity**

522 Next, we sought to identify *Zim53;765* variants in the chromosome 3L region that might
523 underlie its sensitivity to starvation. We utilized the available genomic sequencing data of
524 each DGRP line to identify nucleotide variants unique to the *DGRP-765* sequence
525 (MACKAY *et al.* 2012). As the causal variant is deleterious, we focused our initial analysis
526 on low frequency variants (present in less than 10 % of the 200 DGRP2 sequences). We
527 identified 83 variants which fit this frequency cutoff and only thee SNPs were predicted to
528 lead to non-synonymous changes (Fig. S4 and Table 2). A C-to-A mutation, predicted to
529 result in a G-to-V amino acid residue change in the conserved GXGXXG DNA binding
530 motif of the phospholipase *iPLA2-VIA*, appeared as a strong candidate. Mutations in
531 *iPLA2-VIA* have previously been shown to cause starvation sensitivity and reduced

532 lifespan in *Drosophila* (KINGHORN *et al.* 2015; KINGHORN AND CASTILLO-QUAN 2016; LIN *et al.*
533 2018). In addition, PolyPhen-2 predicted the mutation as probably damaging, with a score
534 of 0.999 out of 1 (ADZHUBEI *et al.* 2010). Based on this analysis, we performed finer
535 resolution deficiency mapping in the *iPLA2-VIA* gene.

536 **$iPLA2\text{-}VIA^{Del174}$ fails to complement *Zim53;765*/ DGRP-765 starvation sensitivity**
537 **and glucose homeostasis**
538 To determine if the starvation phenotype of *Zim53;765* and as a consequence *DGRP-*
539 *765*, results from a defective copy of *iPLA2-VIA*, we sought to determine if the *Zim53;765*
540 copy of *iPLA2-VIA* gene could complement *iPLA2-VIA* null mutations. We crossed
541 *Zim53;765* virgin females to two previously characterized *iPLA2-VIA* null lines ($iPLA2\text{-}VIA^{Del174}$,
542 $iPLA2\text{-}VIA^{Del192}$), generated by imprecise excision of a mobile element; Table
543 S1 (LIN *et al.* 2018). To rule out the potential effects of mtDNA haplotype on starvation
544 resistance, we also utilized the original *DGRP-765* strain obtained from the Bloomington
545 Stock center. We found that progeny from *DGRP-765* crossed to $iPLA2\text{-}VIA^{Del174}$ or
546 $iPLA2\text{-}VIA^{Del192}$ were also starvation sensitive (Figure 6). In contrast, crossing $iPLA2\text{-}$
547 VIA^{Del174} or $iPLA2\text{-}VIA^{Del192}$ to the starvation resistant strain *Zim53;315* resulted in the
548 suppression of starvation sensitivity (i.e., restored starvation resistance; Figure 6).
549

550 **Depletion of glucose and glycogen are associated with starvation sensitivity in**
551 ***Zim53;765* and *DGRP-765***. Ablation or pharmaceutical inhibition of calcium independent
552 phospholipases have been shown to reduce insulin secretion in response to a glucose
553 challenge (SONG *et al.* 2005; ALI *et al.* 2013). This raised the question of whether *Zim53;765*
554 and *iPLA2-VIA* share defects in glucose metabolism. We observed that following 12 hours
555 of starvation, *Zim53;765* and the original *DGRP-765* line significantly deplete their levels

556 of free glucose and glycogen in contrast to *Zim53;315,* suggesting improper regulation of
557 glucose during acute starvation (Figure 7). Interestingly, this phenotype was not observed
558 in *iPLA2-VIA*^{Del174} homozygotes (Figure 7). However, we found that progeny from
559 *Zim53;765* crossed to *iPLA2-VIA*^{Del174} also significantly deplete their available free
560 glucose following 12 hours of starvation (Figure 7; Table S6a,b,c). Taken together, these
561 results indicate shared genetic bases of *iPLA2-VIA* and *Zim53;765* in starvation sensitivity
562 but distinct roles in glucose metabolic effects on starvation. Notably these effects follow
563 a week of conditioning on high sucrose foods that may influence the time course of sugar
564 depletion during starvation and its impact on survival during starvation.
565

566 ## Discussion

567 In the current study we sought to identify the genetic basis of a striking difference in
568 starvation resistance between one particular genotype among a set of related mitonuclear
569 genotypes (see Figure 1). Given the central role that mitochondria play in both anabolic
570 and catabolic processes related to energy stores, the possibility that combinations of
571 mitochondrial and nuclear genes might contributed to quantitative variation for starvation
572 resistance warranted additional genetic analysis. This is further motivated by the highly
573 polygenic nature of starvation resistance and its correlation with other fitness and
574 metabolic traits (SERVICE AND ROSE 1985; CHIPPINDALE *et al.* 1996; HARSHMAN *et al.* 1999;
575 HARDY *et al.* 2018; EVERMAN *et al.* 2019). Our early crosses ruled out an effect of the mtDNA
576 on the strong differences in starvation between *sil;765* and *Zim53;765* which refocused
577 the analyses to the genetic architecture of starvation resistance between distinct nuclear
578 genomes. Because the nuclear genomes used to build the mitonuclear lines were part of

579 the well-characterized DGRP we sought to use the classical tools of the Drosophila model
580 and the genomic information available for the DGRP to carry out a quantitative trait locus
581 search between two distinct homozygous lines from the DGRP. The motivation was
582 twofold: the difference between the sensitive mitonuclear lines (in *DGRP-765*
583 backgrounds) and resistance lines (in *DGRP-315* or *-820* backgrounds) was very distinct,
584 and the sensitive *mitonuclear-765* lines also appeared less sensitive to the high
585 sugar:protein ratio in the food used prior to the starvation assay (Figure 1). Applying
586 forward genetic approaches to this classic quantitative genetic system offered the
587 opportunity to test the hypothesis that starvation sensitivity was associated with sugar
588 metabolism.
589

590 **Polygenic basis of *Zim53;765* starvation sensitivity**

591 The chromosomal segregation analyses and the Advanced Intercross Population (AIP)
592 pool-seq analyses show parallel results at a chromosomal scale and further confirm the
593 polygenic basis of the differences between the *DGRP-315* and *-765* genomes. Figure 2
594 shows a clear main effect of chromosome 3 and some additive or epistatic effects from
595 chromosome 2. Chromosome 3 heterozygosity was necessary to suppress *Zim53;765*
596 starvation sensitivity but heterozygosity of chromosome 2 and 3 together further improved
597 starvation resistance. Notably, chromosome 2 heterozygosity in isolation had limited
598 effect on suppressing *Zim53;765* starvation sensitivity (Figure 2A, B). This indicates that
599 the chromosome 2 locus/loci acts as a modifier that genetically interacts with the
600 chromosome 3 locus/loci. Importantly, *Zim53;315* has a distinct genetic basis of starvation
601 resistance, with chromosome3 having little effect (Figure 2A)

602

603 The pool-seq results from the AIP design (Figure 3) show corresponding patterns for
604 chromosomes 2 and 3: strong evidence on chromosome 3 for allele frequency differences
605 between early-and late dying pools of flies, but limited evidence for this on chromosome
606 2. Figure 2 shows a weak signal of starvation suppression by chromosome 2 for the very
607 last-dying flies but it is not significant. This could reflect heterozygosity of the few loci that
608 just pass significance on chromosome 2L in the pool-seq data (Figure 3), but this remains
609 speculative.

610

611 We note that the AIP analyses involved recombination between the focal line at the
612 extreme end of the starvation resistance spectrum (*Zim53;765*, most sensitive) and a line
613 more central in the distribution of starvation scores (Zim;315, with the DGRP-315 nuclear
614 chromosomes). Though a compelling case could be made for recombining Zim;315 with
615 a line at the opposite end of the distribution (most resistant; e.g., Zim;820 with the *DGRP-*
616 *820* nuclear chromosomes), our starvation data did not show a large difference between
617 the *-315* and *-820* mitonuclear genotypes, but the *-315* mitonuclear genotypes had
618 somewhat tighter variance of starvation scores on the different sugar:protein diets, so we
619 chose that background (Figure 1). The AIP pool-seq data show strong allelic
620 differentiation across one broad peak on chromosome 3L and additional peaks on 3R,
621 with fewer significant peaks on chromosome 2L (4 SNPs above threshold). The resolution
622 of these analyses is admittedly low and resolving specific candidate loci was not an
623 expected goal of the AIP pool-seq. It is likely that many of these significant variants are
624 in linkage disequilibrium since the populations recombined over a period of only 10

625 generations. Nevertheless, the clustering of significant variants on chromosomes 2 and
626 3 are consistent with a polygenic basis for *Zim53;765* starvation sensitivity. The AIP
627 analyses served the important goal of confirming the results of the chromosome
628 segregation analyses and identifying where to focus subsequent higher resolution
629 recombination and deficiency mapping.
630

631 **Dissecting the 3L candidate region**

632 We sought to narrow the target region, by generating recombinants across chromomere
633 3 between *Zim53;765* and the multiply marked recessive stocks, as this would provide a
634 more localized region for deficiency analyses. We however failed to do this directly as
635 crossing *Zim53;765* females to males from each of the multiple marked recessive stocks
636 failed to yield an appreciable number of offspring. We use the *sil;765* to generate
637 recombinants because *sil;765* was constructed using balancer substitutions and
638 backcrosses to *DGRP-765* and thus likely carried *DGRP-765* chromosomes, and it
639 produced sufficient offspring to proceed with the remaining two recombination crosses.
640 Despite learning that *sil;765* was a hybridized version of *765* nuclear chromosomes, a
641 fact confirmed by comparing the alleles of *Zim53;765* variants to *sil;765*'s, it remained
642 possible that the recombinant offspring would help localize genetic effects between
643 chromosome arms 3L or 3R, which proved true. The 15 different deficiencies used for
644 mapping were chosen to span the regions of chromosome 3L that were most likely to
645 carry a candidate gene or genes contributing to the starvation differences between the
646 DGRP chromosomes present in *Zim;765* and *Zim;315*. The preponderance of the
647 evidence from these data supported the hypothesis that heterozygosity of the region of

648 3L 9812381 – 9899255 was required for suppression of *Zim53;765* starvation sensitivity,
649 with deficiencies BSC392, -394 and -673 being clear examples (Figures 5 and S3). Again,
650 however, the deficiency data included some inconsistent results. When *Zim53;765* was
651 crossed with Df(3L)BSC282, there was only a modest difference in the starvation
652 resistance of the heterozygous and homozygous progeny, and when crossed with
653 Df(3L)BSC283, there was no statistical difference in the starvation resistance (Fig. S3).
654 The two additional genes missing in Df(3L)BSC283 relative to Df(3L)BSC282 may explain
655 these conflicting data, but neither gene has any known function reported in Flybase
656 (lncRNA:CR45878, CG46387).
657
658 **Complementation points to Zim53;765 iPLA2-VIA**
659 There are over 30 genes in the candidate regions defined by 3L 9812381 – 9899255 (see
660 Table S1). Of all the variants in this region, the nonsynonymous mutation present in the
661 calcium-independent phospholipase iPLA2-VIA immediately stood out, due to the location
662 of the mutation in the functional domain of the enzyme, and the phenotypes previously
663 associated with iPLA2-VIA loss of function. Mutations in the human homolog PLA2G6
664 gene are characteristic of multiple autosomal recessive disease (Guo *et al.* 2018). The
665 amino acid mutation in the Drosophila iPLA2-VIA protein that is private to *DGRP-765* lies
666 in the GXGXXG consensus nucleotide binding motif similar to those found in other protein
667 kinases. This motif contributes to the binding of ATP to iPLA2-VIA which is essential for
668 its catalytic function (Ramanadham *et al.* 2015). Several studies have independently
669 characterized starvation sensitivity in *iPLA2-VIA* mutants (Kinghorn *et al.* 2015; Iliadi *et*
670 *al.* 2018; Lin *et al.* 2018; Mori *et al.* 2019). In each case, targeted mutations were introduced

671 in *iPLA2-VIA* to generate null models, with the goal of better understanding its role in
672 neurodegeneration. These studies identified aberrant lipid metabolism, lipid peroxidation
673 and mitochondrial dysfunction as potential causes of stress sensitivity and
674 neurodegeneration. We should not necessarily expect point mutations in *iPLA2-VIA* to
675 universally have the same effect as these deletion loss of function models, as mutations
676 in PLA2G6 have varying effects on catalytic activity (ENGEL *et al.* 2010). However, we can
677 expect that if mutation(s) in *iPLA2-VIA* do in fact underlie *Zim53;765* starvation resistance,
678 an *iPLA2-VIA* null should fail to complement *Zim53;765* starvation sensitivity. We showed
679 that two separate nulls generated by (LIN *et al.* 2018) do in fact fail to complement
680 *Zim53;765* starvation sensitivity, indicating that natural variants in iPLA2-VIA contribute
681 some of the starvation sensitivity of *Zim53;765* and *DGRP-765*.
682
683 The correlation between starvation resistance and lipid content has been demonstrated
684 across species and as associated responses to selection for starvation resistance
685 (CHIPPINDALE *et al.* 1996; HARSHMAN *et al.* 1999; HARDY *et al.* 2018; EVERMAN *et al.* 2019).
686 While our current study does not have details on lipid content, there may be a link to the
687 glucose and glycogen utilization we report (Figure 7), and to other possible candidate
688 genes in the 3L 9812381 – 9899255 region: there are several insulin-like peptides (*Ilps*)
689 ~40-100kb distal to the 3L 9812381 – 9899255 that show polymorphism among the
690 DGRP lines. Some phospholipases have been associated with modulation of insulin
691 secretion in response to glucose by releasing arachidonic acid (AA), and exogenous AA
692 supplementation is sufficient to promote insulin release from rat islet cells (METZ 1988a).
693 This insulin secretion is believed to promote phospholipid hydrolysis and accumulation of

694 phospholipid-derived intermediates (METZ 1988b). This turnover of phospholipids could
695 promote insulin release, creating an interesting dynamic between glucose and lipid
696 metabolism, with phospholipases at the center. The premature depletion of glucose and
697 glycogen in *Zim53;765* in the early stages of starvation could be one consequence of the
698 SNP(s) in iPLA2-VIA or an interaction with *Ilps*. Consistent with this, we found that *iPLA2-*
699 *VIA*^{Del174} fails to complement the premature glucose and glycogen depletion phenotypes
700 associated with *Zim53;765* (Figure 7). What is especially interesting is that the iPLA2-VIA
701 null itself does not display this phenotype. This raises the possibility that affected
702 pathways which ultimately result in starvation sensitivity differ between the deletion
703 mutation of *iPLA2-VIA*^{Del174} and the point mutation unique to *Zim53;765*. It will be an
704 interesting target of a future study to characterize the additional molecular bases
705 underlying the starvation sensitivity of *Zim53;765,* as the amino acid mutation in the
706 conserved GXGXXG motif of *iPLA2-VIA* could potentially highlight novel functions of
707 *iPLA2-VIA*.
708

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713 resistance, which provided the impetus for this work. Dr. Jim Mossman assisted with the
714 analyses of DGRP polymorphisms. Yevgeniy Raynes and Leah Darwin provided helpful
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716

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Conflicts of Interest

None declared.

726

Stock Name (mtDNA;nuclearDNA)	Mitochondrial haplotype	Nuclear haplotype	Phenotype
Zim53;765	*D. melanogaster Zim53*	DGRP-765	Starvation sensitive
sil;765	*D. simulans sil*	DGRP-765 recombinant	Starvation resistant
DGRP-765	From original DGRP-765 line	DGRP-765	Starvation sensitive
Zim53;315	*D. melanogaster Zim53*	DGRP-315	Starvation resistant

727

728 **Table 1. Mitonuclear stocks used for genetic analyses in this study.** A table of

729 additional stocks used for chromosome substitution, recombination mapping and

730 deficiency mapping are listed in supplemental file 1.

731

Chr.	start	end	REF	ALT	CONSEQUENCE	REFDNA	VARDNA	REFAA	VARAA	SYMBOL
chr3L	9824166	9824166	C	T	nonsynonymous	CCA	TCA	P	S	CG43897
chr3L	9833935	9833935	G	A	synonymous	AGC	AGT	S	S	RasGAP1
chr3L	9854881	9854881	C	A	nonsynonymous	GGC	GTC	G	V	iPLA2-VIA
chr3L	9880027	9880027	A	G	synonymous	TTT	TTC	F	F	Taf2
chr3L	9880564	9880564	G	T	synonymous	ATC	ATA	I	I	Taf2
chr3L	9881643	9881643	C	A	nonsynonymous	GAG	GAT	E	D	Taf2
chr3L	9884329	9884329	A	T	synonymous	ATA	ATT	I	I	CalpB
chr3L	9884605	9884605	A	G	synonymous	GGA	GGG	G	G	CalpB

732

733 **Table 2. Sequence details of candidate SNPs in the genomic region identified by**
734 **classical mapping approaches**. See text and Fig. S4 for details.
735
736

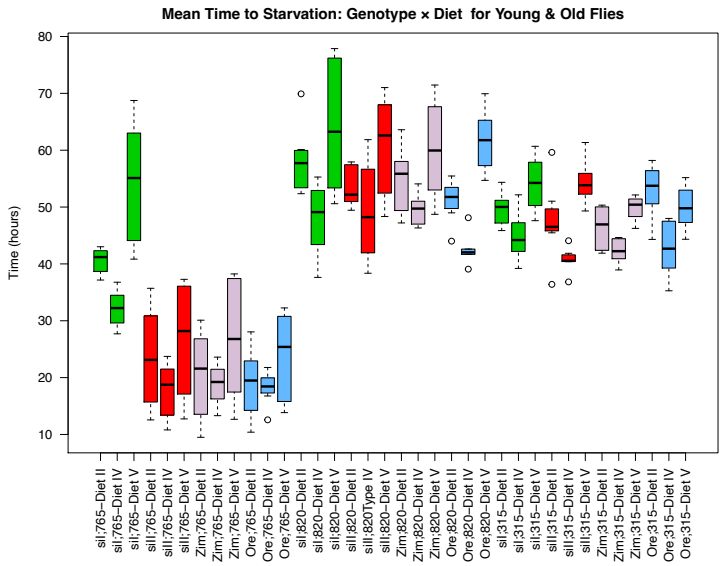


Figure 1. Starvation time among mitonuclear genotypes on different diets. The x-axis lists the genotypes with the notation: mtDNA;nucDNA-Diet, with Diets I, IV and V being balanced, low and high sugar:yeast ratios, respectively (10g:10g, 5g:15g, 15g:5g; see Methods).

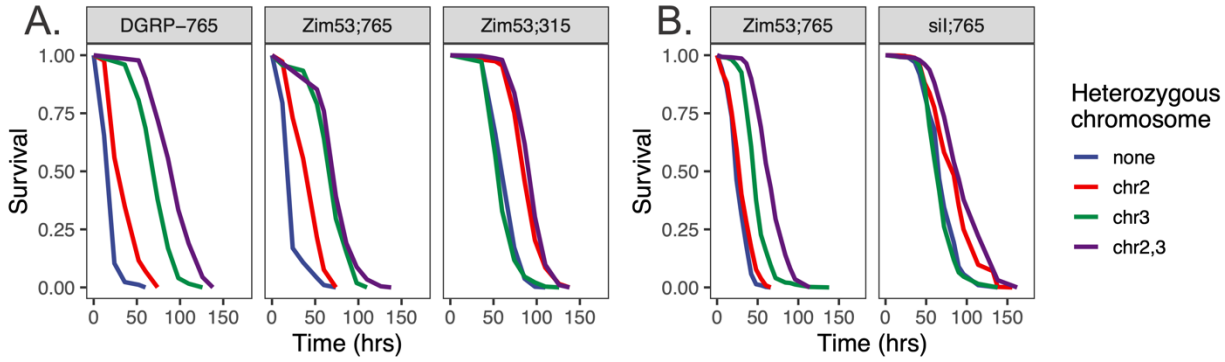


Figure 2. Chromosome 3 suppresses *Zim53;765* starvation sensitivity
(A) *DGRP-765* and *Zim53;765* flies are starvation sensitive while *Zim;315* flies are more resistant. In both *DGRP-765* and *Zim53;765*, heterozygosity for chromosome 3 (chr3) rescues the majority of starvation sensitivity with chromosome 2 heterozygotes (chr2) contributing additional resistance. In *Zim53;315* heterozygosity on chromosome 2 is the primary source of increases in starvation resistance, with little additional contribution from chromosome 3. (B) A replicate experiment with *Zim53;765* shows a similar major effect of chromosome 3 with additional effect of chromosome 2, while the *siI;765* stock with nuclear heterozygosity has increased starvation resistance and distinct chromosomal contributions. Differences in survival between pairs of genotypes were determined using a log-rank test (implemented in the R survival package) and included in Table_S2_Chromosome Segregation Mapping.

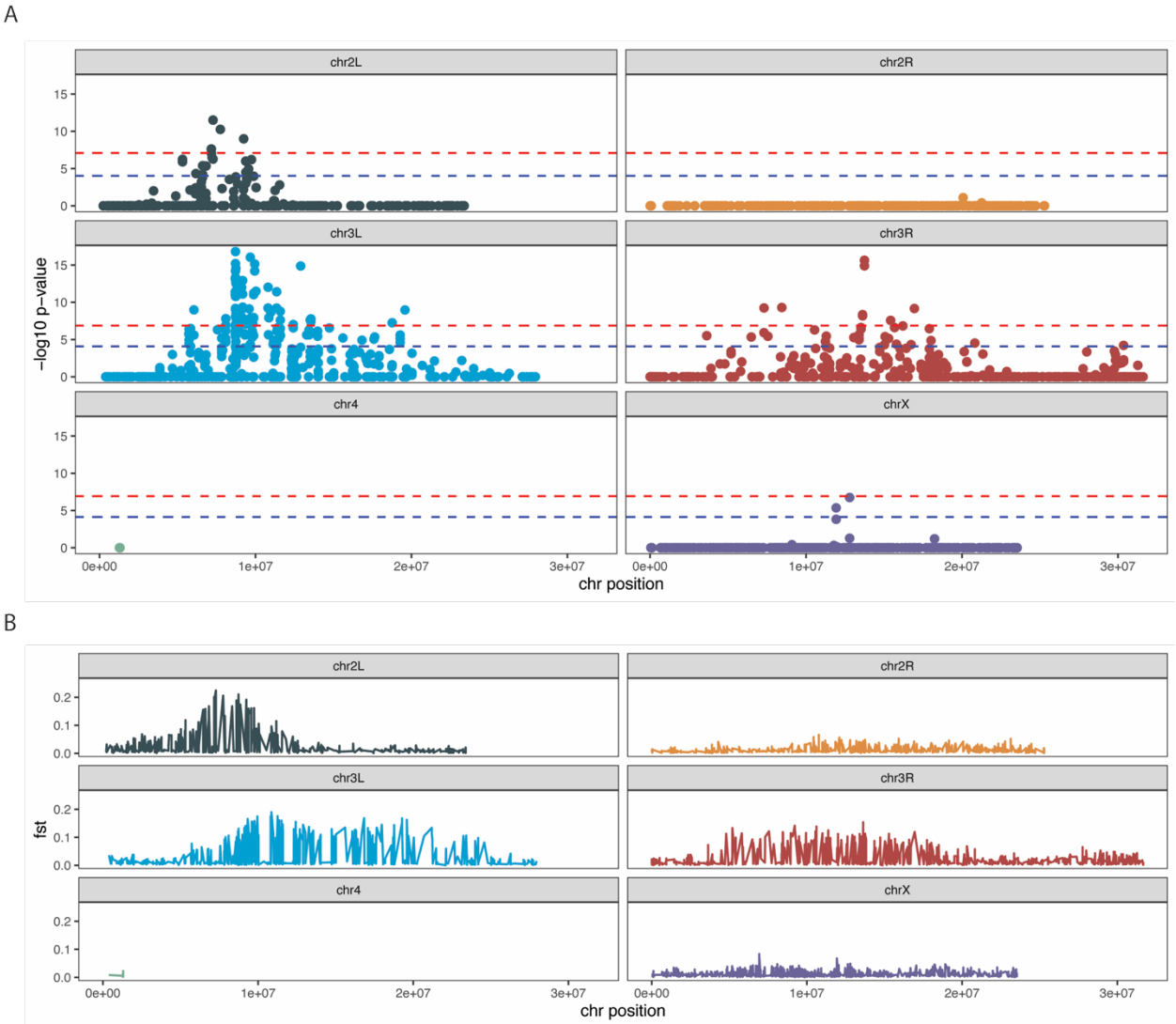


Figure 3. Significant allelic frequency differences between the tails of replicate advanced intercross populations subjected to starvation stress.
An advanced intercross population was created by crossing *Zim53;765* and *sil;315* in reciprocal. The hybrids were allowed to recombine freely for 10 generations before being subjected to starvation stress. Dead flies were collected every 6 hours and dead flies from the first 3 timepoints were combined and subjected to sequencing analysis. The same was done with the last two timepoints. In total 5 replicates AIP populations were generated and sequenced. (A) Consistent differences in allele frequencies across replicates were determined using the Cochran-Mantel-Haenszel (CMH) test and Popoolation2. The y-axis is the negative log-transformed, Bonferroni corrected p-value of each SNP (represented by each circle). The x-axis is the position of each SNP along the indicated chromosome. Blue and red lines highlight corrected p-values above $1x10^{-5}$ and $1x10^{-6}$ respectively. (B) F_{ST} between pools for each site averaged across replicate populations. The location of elevated FST values is generally concordant with significant CMH test statistics.

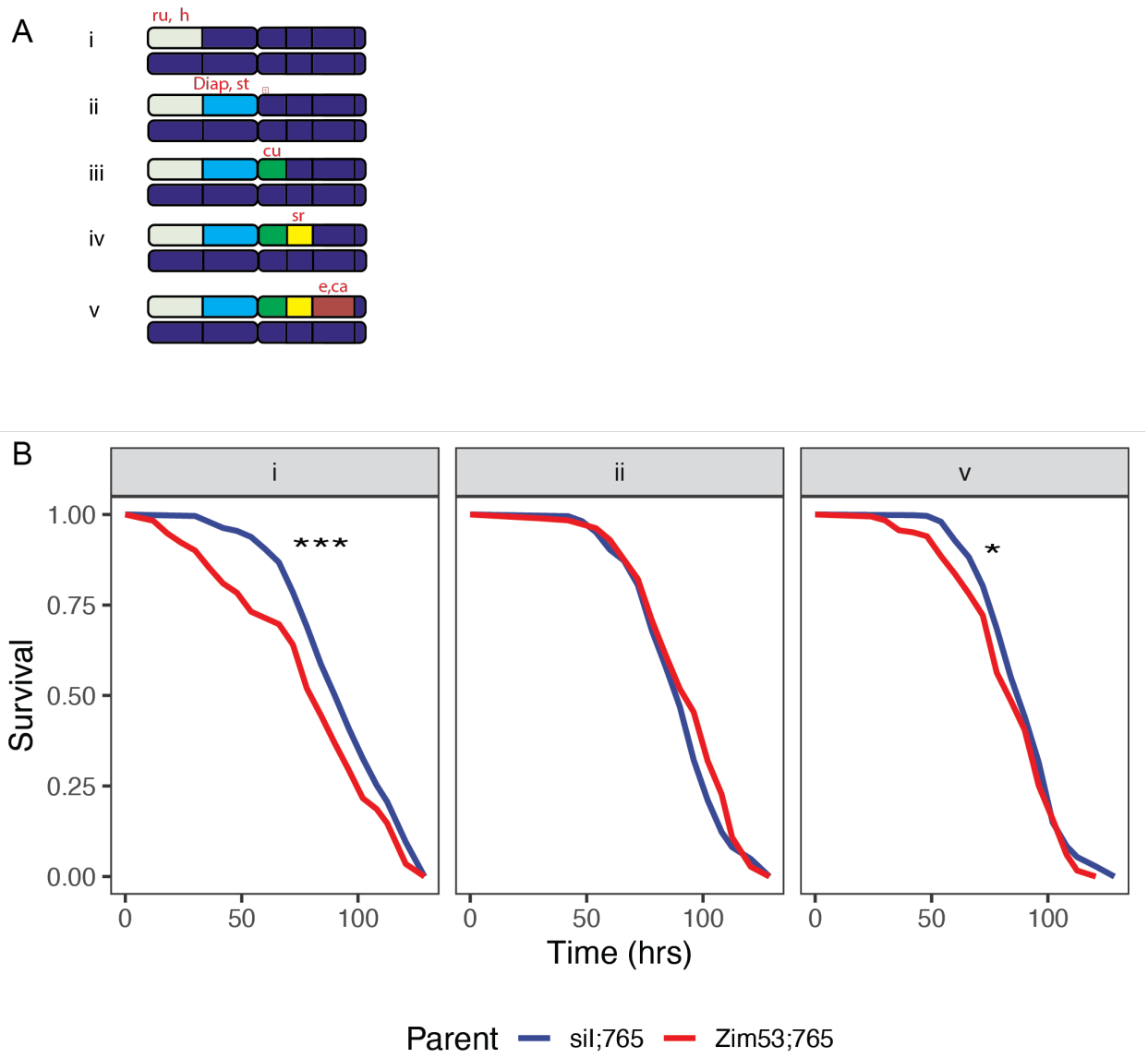


Figure 4. Recombination mapping localizes the majority of *Zim53;765* starvation sensitivity to chromosome 3L

(A) Cartoon representation of recombination breakpoints for each recombinant class generated through meiotic recombination of the chromosome 3 mapping line. (B) Survival of female offspring produced by crossing each recombinant class to *Zim53;765* homozygotes or heterozygotes. Differences in survival were determined using a log-rank test (implemented in the R survival package; *** = p , 0.001, * = p<0.05) and included in Table_S3_Meiotic Mapping Survival.

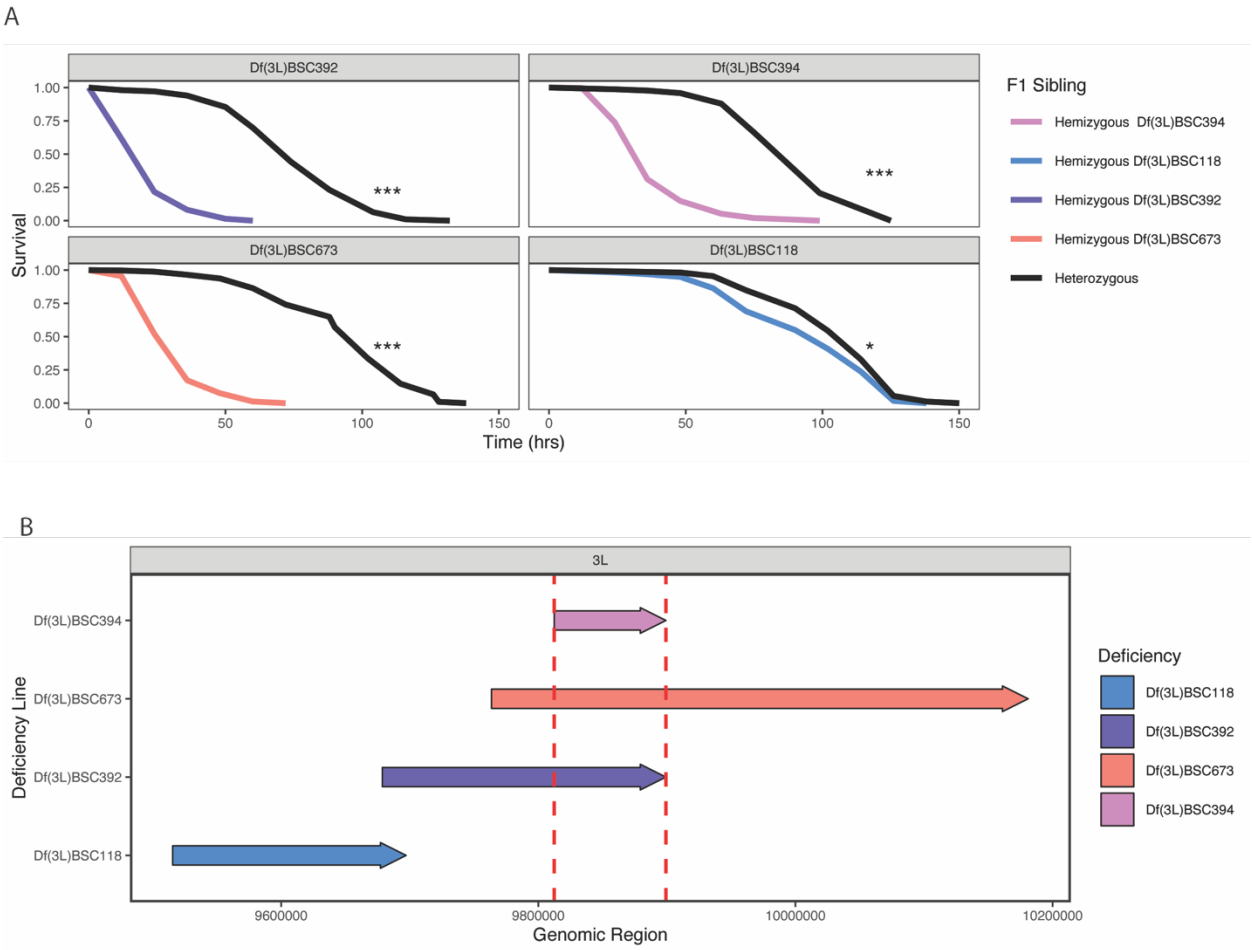


Figure 5. Deficiency mapping identifies the 3L: 9812381 – 9899255 region as a component of *Zim53;765* starvation sensitivity

(A) Survival of offspring from the cross between female *Zim53;765* and male deficiency stocks following starvation stress. Heterozygous refers to progeny that inherited the marked balancer chromosome, hemizygous refers to progeny that inherited the deficient chromosome. Line numbers correspond to Bloomington Stock Center names (see table S1). Differences in survival were determined using a log-rank test (implemented in the R survival package) and included in Table S4_Deficiency mapping: summary and statistics tabs. (B) Linear representation of hemizygous regions of respective deficiency lines. Highlighted region is common to the three deficiency regions which failed to suppress *Zim53;765* starvation sensitivity. See Fig. S3 for additional crosses

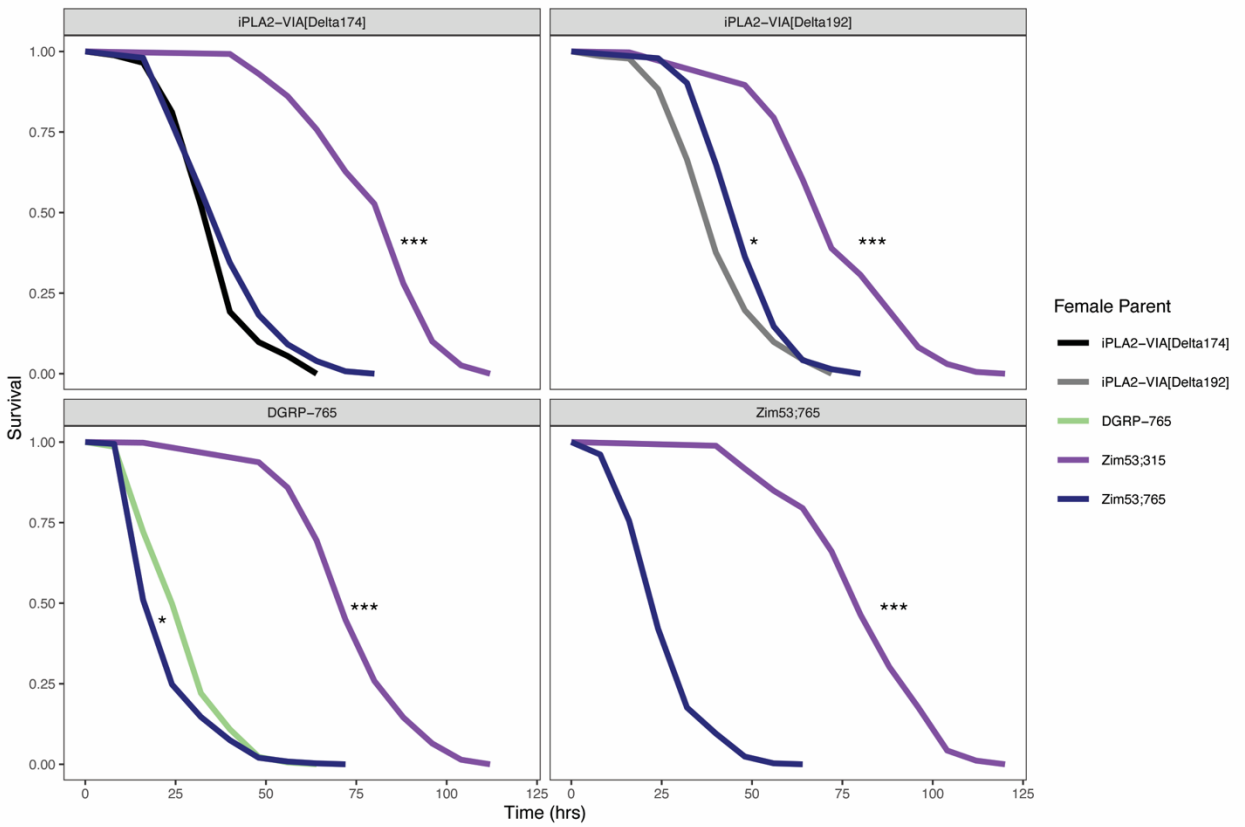


Figure 6. *Zim53;765* fails to complement iPLA2-VIA null lines.
Survival of female offspring from indicated crosses following starvation stress. Each panel represents the male parent, and colored lines identify the female parent. *Zim;765* is starvation sensitive as a homozygote and as a heterozygote over two different *iPLA2-VIA* deficiency null alleles. Differences in survival were determined using a log-rank test (implemented in the R survival package) and included in Table S5_iPLA-VIA complementation).

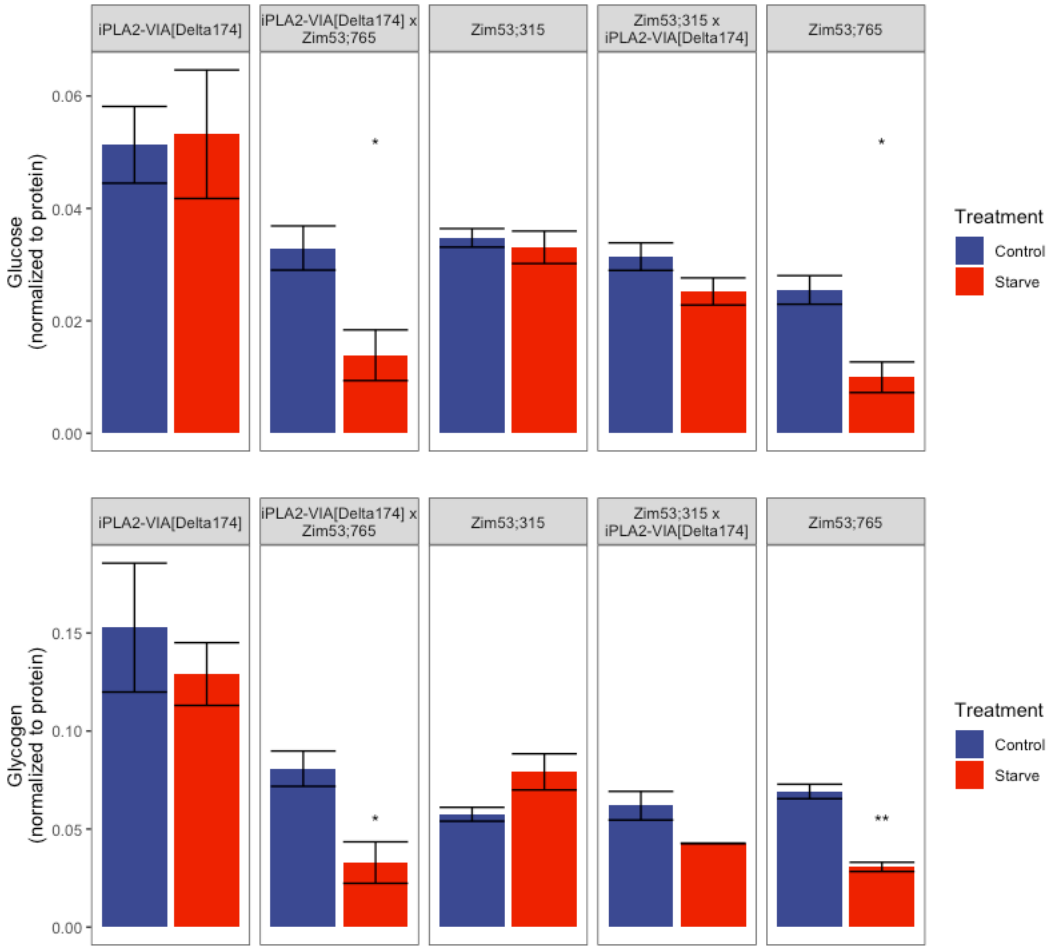
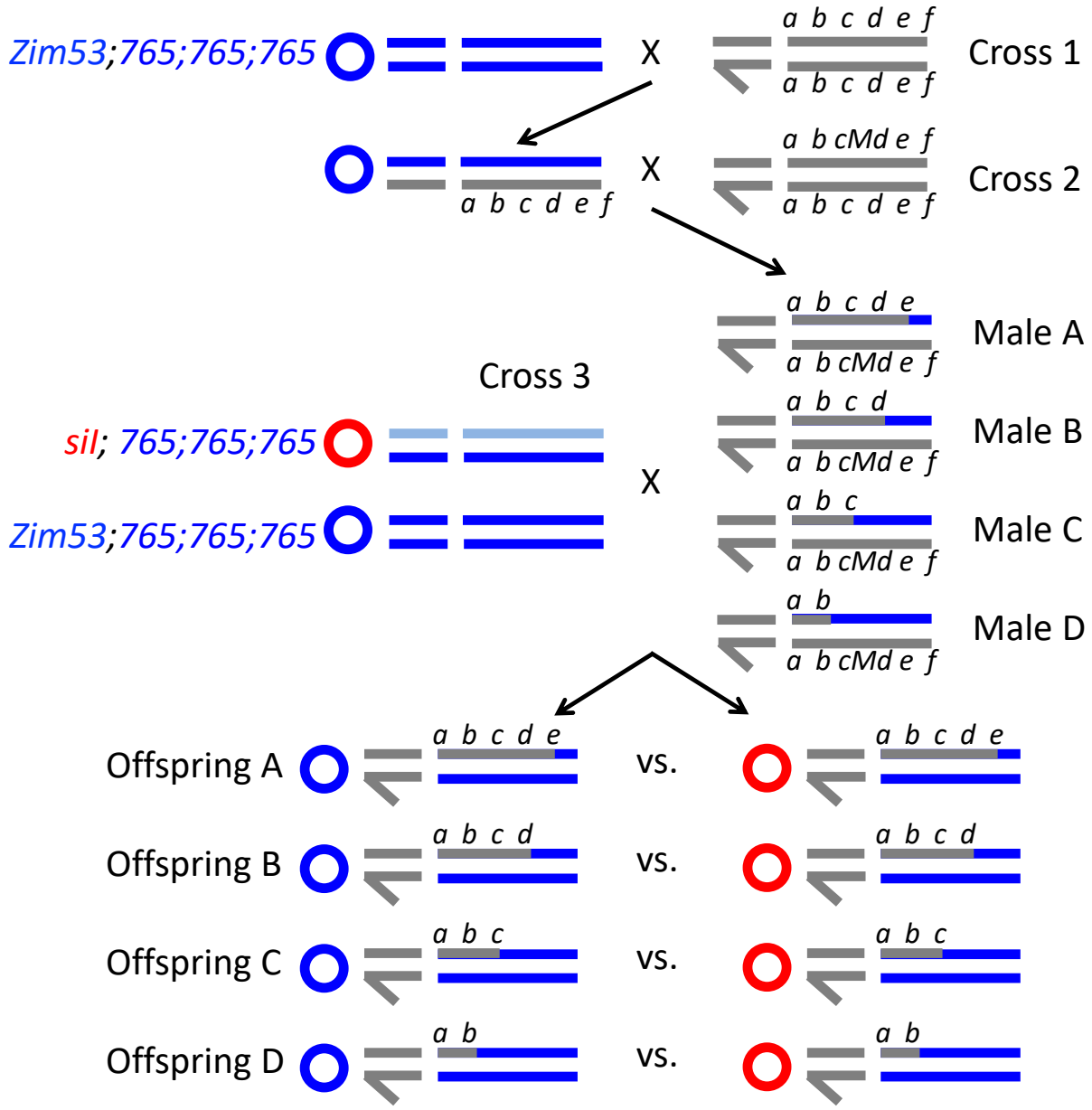
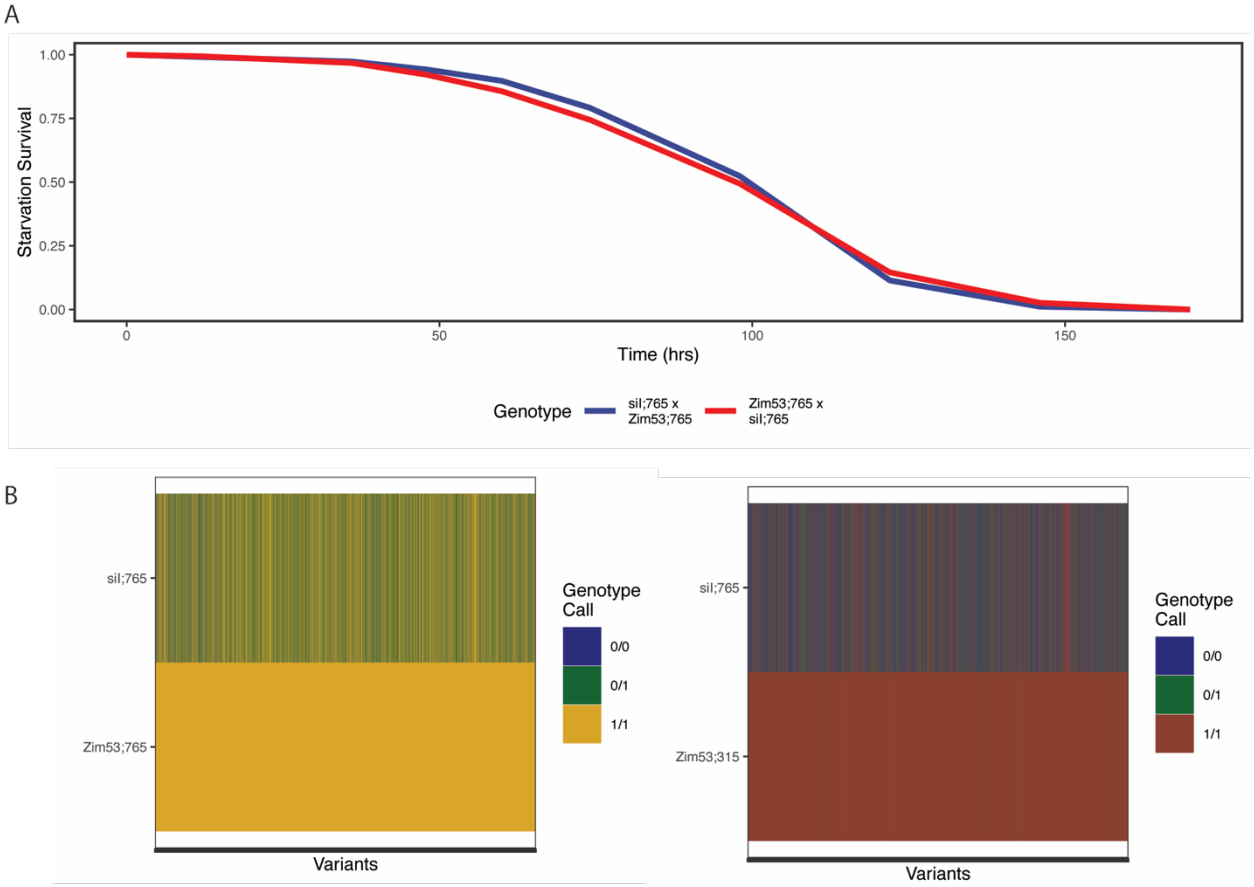


Figure 7. iPLA2-VIA[Delta174] fails to complement glucose and glycogen depletion phenotype of *Zim53;765*

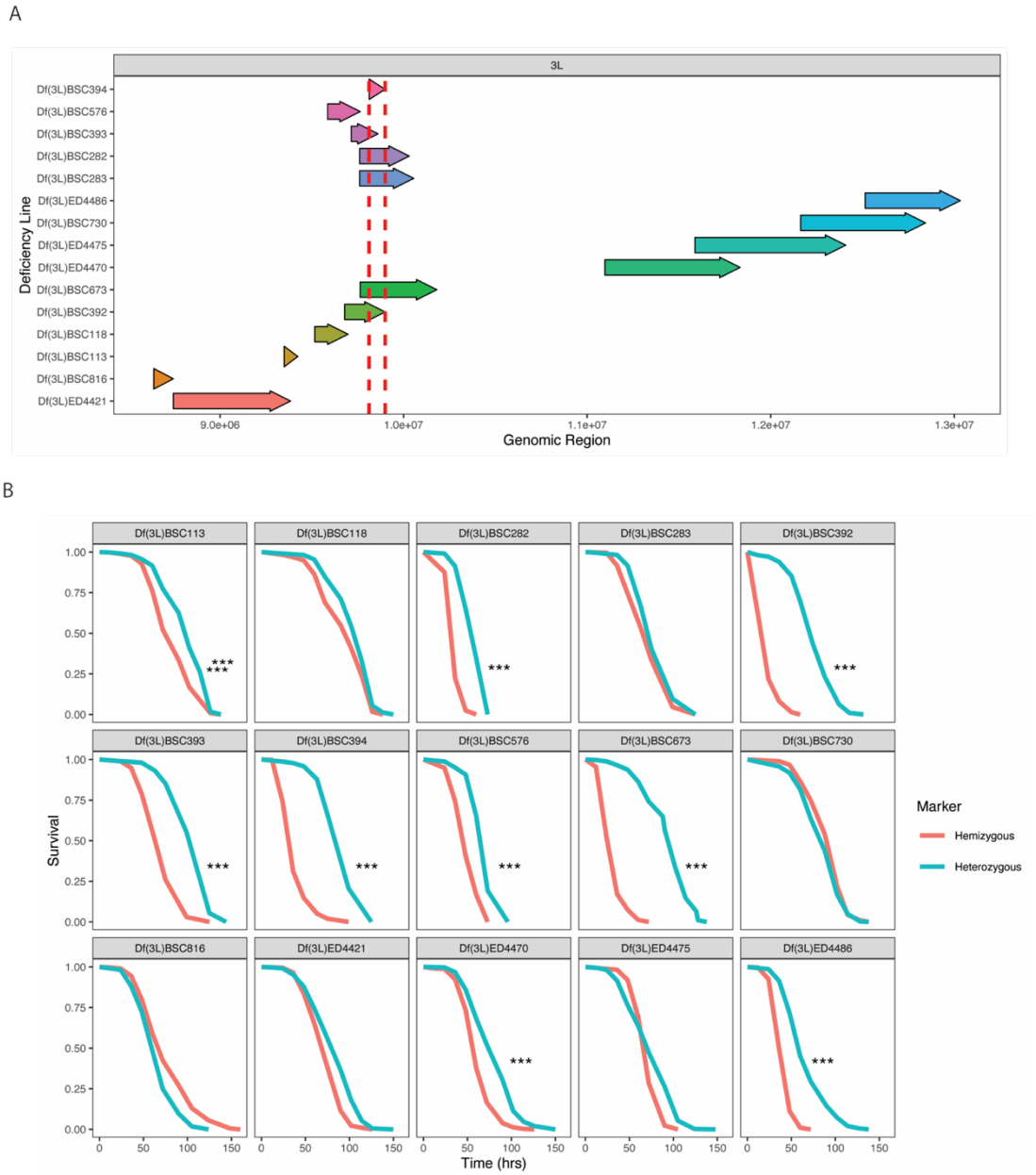
The free glucose and total glycogen levels were measured for *Zim53;765*, *Zim53;315*, iPLA2-VIA[Delta174], *Zim53;315* crossed to iPLA2-VIA[Delta174] and *Zim53;765* crossed to iPLA2-VIA[Delta174]. Values in the un-starved (control) was compared to the values in the starved samples (12 hours) for 3 replicates of 6 flies using a student's t-test. Significant differences are indicated by * <0.05, ** <0.01.



Supplemental Figure S1. Recombination scheme for meiotic mapping. The scheme includes three sequential crosses using the focal lines described in the text and two multiply marked stocks to resolve recombination breakpoints. Note that *Zim53;765* is homozygous for DGRP-765 chromosomes and is starvation sensitive while *sil;765* is starvation resistant but has nuclear heterozygosity is starvation resistant (see Figure 1).

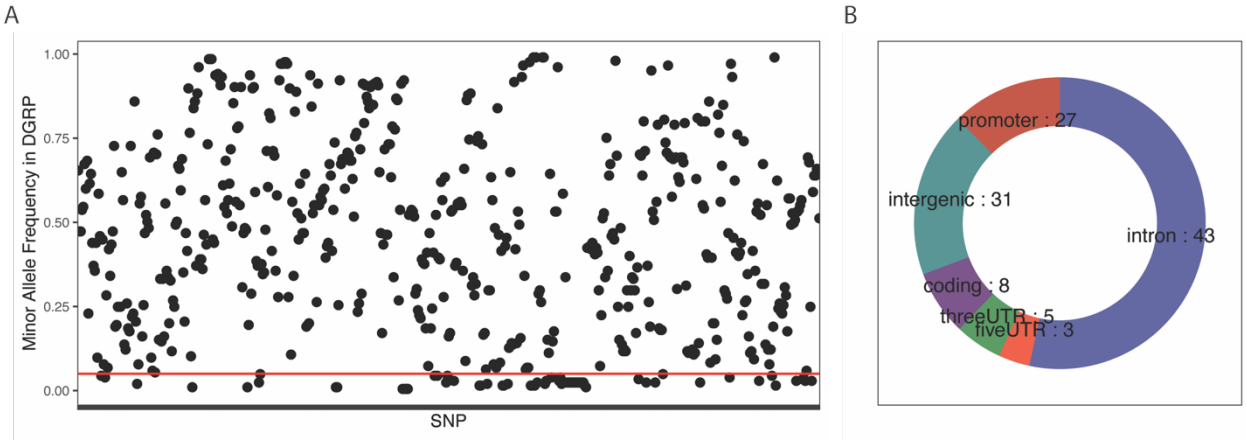


Supplemental Figure S2. *sil;765* is a hybrid of DGRP-765
(A) Survival of female offspring of *Zim53;765,* and *sil;765* crossed in reciprocal. There is no statistical difference in survival based on a log-rank test for significance. (B and C) Genotype of *sil;765* at homozygous variant sites in RNA sequencing reads from (C) *Zim53;765* and (D) *Zim53;315* Green indicates that *sil;765* was heterozygous for both the variant and the reference allele, blue indicates that *sil;765* was homozygous for the reference allele.



Supplemental Figure S3

(A) Linear representation of deficient region of respective deficient lines in region identified in meiotic recombination mapping. (B) Survival of offspring from the cross between *Zim53;765* and the respective deficient male following starvation stress. Differences in survival were determined using a log-rank test (implemented in the R survival package) and included in TableS4_Deficiency mapping. Heterozygous refers to progeny that inherited the balancer chromosome, hemizygous refers to progeny that inherited the deficient chromosome. Line numbers correspond to Bloomington Stock Center names.



Supplemental Figure S4. Frequency of DGRP-765 variants among the Drosophila Genetic Reference Panel

(A) Allele frequency of *DGRP-765* variants in the 3L candidate region among the lines of the Drosophila Genetic Reference Panel. Variants found below the red line are present in less than ten percent of the lines. (B) Proportions of low frequency (<10%) variants in different functional categories for in the 3L candidate region present in DGRP-765.

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