A single nucleotide variant in the PPARγ-homolog *Eip75B* affects fecundity in *Drosophila*

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ABSTRACT

Still little is understood about the nucleotide changes that underlie variation in complex phenotypes. Variation in the PPARγ-homolog *Eip75B* has previously been suggested to be associated with longevity and life-history differences in the fruit fly *Drosophila melanogaster*. Using RNAi knockdown, we first demonstrate that reduced expression of *Eip75B* in adults affects lifespan, egg laying rate and egg volume. To then test the effect of a naturally occurring SNP variant within a cis-regulatory domain of *Eip75B*, we screened wildtype lines with alternative alleles and conducted precise genome editing using CRISPR/Cas9. These experiments revealed that this natural polymorphism has a significant effect on fecundity and egg-to-adult viability, but not on longevity or other life-history traits. These results provide a rare functional validation for the role of a natural allelic variant in adaptation of life-history traits directly linked to fitness at the single nucleotide level.

KEYWORDS: pleiotropy, *Drosophila*, ageing, reproduction, CRISPR/Cas9, RNAi

32 **INTRODUCTION**

33 Single nucleotide polymorphisms (SNPs) are the major constituent of genetic variation and
34 fuel for evolutionary adaptation (Suh & Vijg, 2005). High-throughput sequencing
35 technologies propelled the association of natural variants with complex, quantitative traits
36 (Schlötterer, Tobler, Kofler, & Nolte, 2014), but functional tests demonstrating a causal
37 effect at base-pair resolution are rare, especially for multicellular organisms (Mokashi et al.,
38 2021; Ramaekers et al., 2019). Instead, functional analyses are often done at the gene level
39 by generating (null) mutants or using RNAi in model organisms (Fabian et al., 2018; Huang et
40 al., 2020; Martins et al., 2014; Parker et al., 2020; Zhou et al., 2011). Functional effects of
41 natural alleles may, however, be more subtle or different altogether from mutant alleles
42 (Mokashi et al., 2021; Stern, 2011). The aim of this study was to conduct functional analysis
43 of a natural polymorphism of *Ecdysone-induced protein 75B* (*Eip75B*), a promising candidate
44 gene for ageing and life-history evolution in fruit flies.

45 *Eip75B* encodes a nuclear hormone receptor homologous to peroxisome
46 proliferator-activated receptor gamma (PPARγ), a key regulator of adipogenesis and
47 pregnancy-induced modifications in lipid metabolism in mice and humans (Hong & Park,
48 2010; Waite et al., 2000; Zipper, Jassmann, Burgmer, Gorlich, & Reiff, 2020). Nuclear
49 hormone receptors, such as *Eip75B*, are evolutionarily conserved regulatory hub genes that
50 integrate environmental factors, hormonal signaling, and metabolism and relay these inputs
51 to downstream targets (Hoffmann & Partridge, 2015; Jagannathan & Robinson-Rechavi,
52 2011; Palanker et al., 2006). In the fruit fly, *Eip75B* also acts as an early-response gene in the
53 ecdysone signaling pathway, which plays a critical role in the regulation of development,
54 physiology and life-history of arthropods, similar to thyroid hormones in vertebrates (Belles
55 & Piulachs, 2015; Bialecki, Shilton, Fichtenberg, Segraves, & Thummel, 2002; Caceres et al.,

56 2011; Flatt, Moroz, Tatar, & Heyland, 2006; Galikova, Klepsatel, Senti, & Flatt, 2011;
57 Jaumouillé, Machado Almeida, Stähli, Koch, & Nagoshi, 2015; Magwire et al., 2010; Simon,
58 Shih, Mack, & Benzer, 2003; Terashima & Bownes, 2006; Toivonen & Partridge, 2009;
59 Yamanaka, Rewitz, & O'Connor, 2013).

60 *Eip75B* has been identified as an "ageing" candidate gene by two independent
61 genome-wide association studies (Huang et al., 2020; Pallares et al., 2020) and three
62 experimental evolution experiments (Carnes et al., 2015; Hoedjes et al., 2019; Parker et al.,
63 2020), but each study detected different candidate SNPs. Experimental evolution of
64 longevity has been intricately linked to changes in fecundity, suggesting a shared genetic
65 basis of these two traits likely due to antagonistic pleiotropy (Carnes et al., 2015; Fabian et
66 al., 2018; Flatt, 2020; May et al., 2019; Remolina, Chang, Leips, Nuzhdin, & Hughes, 2012).
67 Moreover, evolutionary changes in longevity can be associated with changes in viability,
68 development time, immune function, stress resistance, and even learning ability (e.g.,
69 (Baldal, Brakefield, & Zwaan, 2006; Chippindale, Hoang, Service, & Rose, 1994; Fabian et al.,
70 2018; Flatt, 2020; Mery & Kawecki, 2005). Given its central role in hormonal signaling and its
71 pleiotropic effects on numerous developmental and physiological processes, *Eip75B* may
72 thus be involved in the concerted evolution of longevity and correlated life-history traits.

73 In this study, we first used RNAi silencing of *Eip75B* in adults to determine the effects
74 of the gene on lifespan, egg laying and egg volume. We then selected a naturally occurring
75 SNP in a cis-regulatory domain of *Eip75B*, previously identified as a candidate in an
76 experimental evolution study (Hoedjes et al., 2019), for functional analyses by applying a
77 combination of SNP-specific association studies and precise genomic modification using
78 CRISPR/Cas9. Our results demonstrate that a single nucleotide variant can have a major
79 effect on a complex fitness-related trait, namely fecundity.

80

81 **RESULTS**

82 ***RNAi knock-down of Eip75B in adults reduces longevity, egg laying rate and egg volume***

83 Recent studies have found that weak, constitutive knockdown of *Eip75B* throughout
84 development and the adult stage increases lifespan in both males and females (Huang et al.,
85 2020), whereas tissue-specific knockdown in the amnioserosa, ovaries and accessory glands
86 causes decreased lifespan but increased fecundity in females (Parker et al., 2020). Given
87 that *Eip75B* might have potentially confounding pleiotropic developmental effects on adult
88 life-history, we investigated the effect of gene knockdown specifically in adults only. The
89 effects of *Eip75B* knockdown on longevity, fecundity (measured as daily egg laying rate per
90 female) and egg volume were assessed with four independent RNAi constructs expressed in
91 the adult stage using the inducible, constitutive *daughterless*-GS-GAL4 driver (Osterwalder,
92 Yoon, White, & Keshishian, 2001). RT-qPCR confirmed a significant, partial knockdown of
93 *Eip75B* for three of the four constructs, E01, E03, and E44, but not for the fourth construct
94 (E10) (Table 1). The fourth construct was nonetheless included in the study as it had been
95 observed to affect lifespan and fecundity in previous studies (Huang et al., 2020; Parker et
96 al., 2020).

97 Knockdown of *Eip75B* expression resulted in a significant reduction in lifespan only
98 for constructs E01 (females) and E44 (both sexes), although we also observed a similar trend
99 in lifespan reduction for construct E03 (Figure 1A). The effects of *Eip75B* knockdown on egg
100 laying rate and egg volume were more consistent, with a significant reduction in both egg
101 laying rate and egg volume for constructs E01, E03, and E44, and a weak trend for construct
102 E10 (Figure 1B and 1C).

These results confirm the role of *Eip75B* in affecting lifespan and fecundity, in line with previous studies, but also suggest that the direction and the magnitude of the observed phenotypic effects depend on the timing, the tissue, and the strength of the genetic modification (Huang et al., 2020; Parker et al., 2020).

A natural polymorphism at Eip75B correlates with variation in fecundity

The *Eip75B* SNPs which have been identified as potentially affecting ageing and life-history are located throughout the gene, mostly in non-coding regions (Carnes et al., 2015; Hoedjes et al., 2019; Huang et al., 2020; Jha et al., 2015; Pallares et al., 2020; Parker et al., 2020; Turner, Stewart, Fields, Rice, & Tarone, 2011). *Eip75B* is a large (>100 kb) gene comprising seven transcript variants that are expressed from alternative promotors. Isoforms have different temporal and spatial expression patterns and fulfill different, even opposing, functions in insects (Bernardo, Dubrovskaya, Jannat, Maughan, & Dubrovsky, 2009; Cruz, Mane-Padros, Zou, & Raikhel, 2012; Keshan, Hiruma, & Riddiford, 2006; Li et al., 2016; Terashima & Bownes, 2006; Zipper et al., 2020). Given this complex genomic architecture and the pleiotropic effects of *Eip75B,* independent SNPs may have different regulatory functions. We focused on a set of 16 significantly diverged, intronic SNPs that were detected by experimental evolution of lifespan extension by selecting on the age at reproduction (Hoedjes et al., 2019; May et al., 2019) (at FDR<0.0005). One of these SNPs (3L:18026199, genome release 6) is located within a predicted cis-regulatory motif. This SNP is bi-allelic, with the "T" variant being more common in the late-reproducing, long-lived populations (average frequency: 0.84 "T", 0.16 "G") as compared to the early-reproducing populations (average frequency: 0.52 "T", 0.48 "G") (Hoedjes et al., 2019). These frequencies fall within

126 the range of frequencies observed in natural populations of *D. melanogaster* in Europe and
127 North America (Appendix 1).
128 To test if the "T" allele is associated with a longer lifespan and/or changes in
129 fecundity as compared to the "G" allele, we used a targeted SNP association study in which
130 we assessed a set of F1 crosses of distinct lines of the *Drosophila Genetic Reference Panel*
131 (DGRP) (Mackay et al., 2012) differing in allelic state ("T" vs. "G"). This heterozygous F1
132 approach was chosen as the DGRP strains are inbred and may thus suffer from reduced
133 fitness due to recessive deleterious alleles. Previous studies of wildtype lines or
134 recombinant outbred populations with alternative allelic states have already indicated
135 significant effects for several natural SNP alleles in life-history evolution (Betancourt et al.,
136 2021; Durmaz et al., 2019; Glaser-Schmitt & Parsch, 2018; Glaser-Schmitt, Wittmann,
137 Ramnarine, & Parsch, 2021).
138 F1 crosses homozygous for the "G" allele (and with a diverse genomic background)
139 showed a significantly and consistently higher egg laying rate than crosses homozygous for
140 the "T" allele (Figure 2). However, there was no significant association between the
141 candidate SNP and longevity. These findings suggest that the "G" allele may have played a
142 role in facilitating a higher (early) fecundity in the populations selected for early
143 reproduction, but that this SNP is not responsible for differences in lifespan and late
144 reproduction previously reported in the experimental evolution experiment (May et al.,
145 2019).
146
147 ***CRISPR/Cas9 editing reveals pleiotropic effects of the Eip75B SNP on fecundity and***
148 ***viability but not longevity***

149 To minimize potential confounding effects of genetic background on lifespan and fecundity,
150 we used CRISPR/Cas9-induced genomic editing to modify the candidate SNP in an isogenic
151 background without introducing any markers or off-target modifications that might
152 interfere with gene function in the genomic region surrounding the focal SNP. We
153 constructed two edited lines with the "G" allele and two lines with the "T" allele. The lines
154 carrying the "G" allele exhibited a significantly higher egg laying rate and higher egg-to-adult
155 viability as compared to lines with the "T" allele (Figure 3). Lines with the "G" allele also
156 showed a small but significant reduction in starvation resistance in females. No significant
157 differences between the two allelic states were observed for lifespan, egg-to-adult
158 development time, adult dry weight, and egg volume.
159

160 **DISCUSSION**

161 ***The PPARγ-homolog Eip75B plays a significant role in the evolution of fecundity***

162 Our results demonstrate a clear pleiotropic effect of a naturally occurring nucleotide
163 polymorphism at the *Eip75B* locus on egg-to-adult viability and fecundity. The effect of this
164 SNP on reproduction may be linked to the known role of *Eip75B* in oogenesis (Ables, Hwang,
165 Finger, Hinnant, & Drummond-Barbosa, 2016; Belles & Piulachs, 2015), but could also
166 indicate that allelic variants at this locus modify nutrient availability or metabolic processes
167 required for egg production. Ecdysone titers in the ovaries themselves control overall food
168 intake, but *Eip75B* also plays a role in the mating-induced remodeling of the gut, which
169 prepares the female body for the increasing energy demands associated with egg laying
170 (Carvalho-Santos & Ribeiro, 2018; Reiff et al., 2015; Zipper et al., 2020). Further functional
171 analyses of the two allelic variants studied here are necessary to disentangle the mode of
172 action of this polymorphism.

173

174 ***Functional assays of natural alleles are required for understanding longevity and life-***

175 ***history evolution***

176 Even though RNAi-induced knockdown of *Eip75B* affects longevity, as shown here and by

177 others (Huang et al., 2020; Parker et al., 2020), no effect on lifespan was observed for a

178 natural allelic variant previously identified as an "ageing" candidate in an experimental

179 evolution study (Hoedjes et al., 2019). Instead, we found that this polymorphism affects

180 viability and fecundity. No effects on various other life-history traits, known to be regulated

181 by the *Eip75B*, were detected for this SNP, perhaps suggesting that natural alleles have

182 fewer pleiotropic effects than null alleles of the same gene (Stern, 2011). Hence, other

183 allelic variants of *Eip75B* may play a role in longevity. In addition, the effects of an individual

184 SNP – like the one we have tested here – might depend on the haplotype and genetic

185 background, given the major role linkage and epistasis can play in affecting quantitative

186 traits (Mackay, 2014; Magwire et al., 2010).

187 In conclusion, a vast number of studies have identified naturally occurring candidate

188 polymorphisms associated with complex traits, but experimental demonstrations of direct,

189 causal effects of such variants still remain scarce (Mokashi et al., 2021; Ramaekers et al.,

190 2019). Our study provides a rare example of functional characterization of a natural

191 nucleotide polymorphism with pleiotropic effects on two complex fitness-related traits,

192 viability and fecundity. Our results further suggest that this molecular polymorphism has, as

193 one might expect for regulatory variants (Stern 2011), fewer pleiotropic effects than the

194 knockdown of the entire gene or null alleles at this locus.

195

196 **METHODS**

197 **RNAi**

198 Transgenic RNAi was performed using the ubiquitously expressing, mifepristone (RU486)-
199 inducible *daughterless* (*da*)-GeneSwitch(GS)-GAL4 (Tricoire et al., 2009) (courtesy of
200 Véronique Monnier, Paris) to drive the expression of *Eip75B* UAS-RNAi constructs during the
201 adult stage only, to avoid adverse effects on development. Two independent UAS-RNAi
202 constructs obtained from the Vienna *Drosophila* RNAi Center (VDRC) (#108399 [E10] and
203 #44851 [E44]), and two additional independent constructs obtained from Bloomington
204 Drosophila Stock Center (BDSC) (#TRiP.GLC01418 [E01] and #TRiP.JF02257 [E03]) were used.
205 These constructs target a domain of *Eip75B* that is shared by all isoforms. The isogenic host
206 strain for the VDRC RNAi library, w^{1118} (#60000), was used to test for the potential effects of
207 mifepristone treatment. All lines were kept and assays were performed at 25°C, 65%
208 humidity and 12h:12h light:dark cycle, on a cornmeal-yeast-sucrose-agar medium (per 1
209 liter of food: 7 g agar, 50 g sucrose, 50 g cornmeal, 50 g yeast, 6 ml propionic acid, 10 ml of a
210 20% nipagin stock solution). After emergence, flies were kept on medium containing either
211 100 or 200 µg/ml (233 or 466 µM, respectively) mifepristone dissolved in ethanol, or on
212 control medium (i.e., ethanol without mifepristone).

213 Quantitative real-time PCR (qRT-PCR) was used to confirm knockdown efficiency of the
214 different constructs. For this, cohorts of F1 offspring between *da*-GS-GAL4 females and
215 males carrying one of the UAS-RNAi constructs were collected upon emergence, and placed
216 in vials with either control medium or medium with mifepristone (100 or 200 µg/ml) for four
217 days in groups of 10 females and 10 males. The flies were then flash frozen in liquid
218 nitrogen and three females were pooled together per sample and homogenized for whole-
219 body RNA extraction. RNA extractions were done using a MagMax robot, using the

220 MagMax-96 total RNA isolation kit (ThermoFisher Scientific), following the instructions of
221 the manufacturer. cDNA was prepared following the GoScript Reverse Transcriptase system
222 (Promega); qRT-PCR was done on a QuantStudio 6 real-time PCR machine (ThermoFisher
223 Scientific), using Power SYBR Green PCR Master mix (ThermoFisher Scientific). We analyzed
224 ten biological samples per construct and mifepristone treatment, with three technical qRT-
225 PCR replicates each. The expression levels of *Eip75B* and four reference genes (*TBP*, *Cyp1*,
226 *Ef1α48D* and *Rap2l*; see Table 2 for primer sequences) were measured. The reference genes
227 had a stability of M<0.25 as determined a priori from a set of female whole-body samples of
228 various strains and mifepristone concentrations using the GeNorm algorithm incorporated
229 in qbasePLUS v.1.5 (Biogazelle). The three technical replicates were analyzed for
230 reproducibility, and measurements were accepted if none or only one of the technical
231 replicates differed >0.5 Cq from the other two replicates. In the latter case the divergent Cq
232 value was removed from the dataset. All measurements fulfilled these criteria. The technical
233 replicates were averaged and a single gene expression value per gene per sample was
234 calculated using qbasePLUS v.1.5. The gene expression levels of the four reference genes
235 were then averaged geometrically to obtain an accurate normalization factor for each
236 sample (Vandesompele et al., 2002). The relative gene expression levels of *Eip75B* for the
237 four RNAi constructs were then calculated compared to the expression of the control
238 treatment (i.e. no mifepristone) for each construct separately, respectively. The gene
239 expression levels were analyzed in R (v.3.3.1) using ANOVA with mifepristone concentration
240 as a fixed effect (continuous variable).
241 We detected a significant reduction in whole-body *Eip75B* expression three out of
242 four constructs when being fed mifepristone (E01: $F = 6.11$, $P = 0.020$; E03: $F = 19.66$, $P =$
243 0.00013; E44: $F = 4.51$, $P = 0.043$) (Figure 1 and Table 1). The average reduction in *Eip75B*

244 expression was 18%, 28%, and 18%, respectively, at a mifepristone concentration of 200
245 µg/ml. No significant reduction in the expression of *Eip75B* was detected for construct E10
246 (F = 1.95, P = 0.04), even though phenotypic effects on lifespan had been observed when
247 using this construct in previous studies (Huang et al., 2020)

248 We examined the effects of *Eip75B* knockdown on lifespan, fecundity and egg
249 volume. To measure the effects of lifespan, cohorts of F1 offspring between crosses of *da*-
250 GS-GAL4 virgin females and males carrying one of the four UAS-RNAi constructs or the
251 isogenic control strain were collected within a 24-hour window; the flies were sexed under
252 mild CO_2 exposure and transferred at random to 1-liter demography cages with food vials
253 (with 0, 100, or 200 µg/ml mifepristone) attached to the cages. For each genotype and
254 mifepristone concentration, we set up three replicate cages, each containing 75 flies per
255 sex. Dead flies were scored and fresh food was provided every two days. Differences in
256 lifespan between mifepristone-induced RNAi and uninduced controls were analyzed in R
257 (v.3.3.1) for the two sexes separately using mixed-effects Cox (proportional hazards)
258 regression with mifepristone concentration as fixed effect and with 'replicate cage' as a
259 random effect using the R package *coxme* (v2.2-5) for each construct separately. In addition,
260 to test the statistical significance of similar trends in lifespan changes across the four
261 constructs, we used a mixed-effects Cox regression on all data, with mifepristone
262 concentration, construct, and their interaction as fixed effects and with ‘replicate cage’ as
263 random effect. Bonferroni correction was applied to account for multiple testing (0.05/8 =
264 0.00625).

265 The effect of *Eip75B* knockdown on fecundity was assessed by measuring daily egg
266 laying rates. For this, virgin females were collected from the F1 offspring of *da*-GS-GAL4
267 females and males from a UAS-RNAi strain or the isogenic control strain. After 24 hours, two

268 virgin females and two w^{1118} males were placed together in vials with 0 (i.e. control) or 100
269 µg/ml mifepristone. The analyses were limited to mifepristone concentrations of 100 µg/ml,
270 as a significant effect of 200 µg/ml of mifepristone on the control was observed
271 (Mifepristone: $\chi^2 = 28.9$, $P = 5.3e^{-7}$; Mifepristone*Day: $\chi^2 = 22.6$, $P = 2.0e^{-6}$) (Figure 1 – figure
272 supplement 1). Ten replicate vials were prepared per genotype and mifepristone
273 concentration. The flies were left for 48 hours to ensure mating and consumption of
274 mifepristone before the start of the experiment. Then the flies were transferred to a fresh
275 vial with food (with 0 or 100 mifepristone, respectively) to lay eggs for 24 hours. The
276 number of eggs laid per pair of flies was counted under a microscope. The daily egg laying
277 rate was measured at day 3, 6, 9, 12 and 15.
278 After day 15, all flies were transferred to vials with medium without mifepristone for
279 24 hours. The eggs laid were collected and 30 randomly chosen eggs per strain and
280 mifepristone concentration were photographed with a Canon EOS 60D camera under a
281 Leica dissection microscope. The length and the width of each egg were measured using
282 ImageJ (v.1.51h) software and the volume of each egg was approximated following the
283 formula described by (Jha et al., 2015): volume = $1/6\pi(\text{width})^2*\text{length}$. Variation in egg
284 laying rates was analyzed in R (v.3.3.1) using generalized linear mixed models with a Poisson
285 distribution and with mifepristone concentration, day and their interaction as fixed effects
286 and with 'replicate vial' as a random effect using the R package *lme4* (v.1.1-13). Egg volume
287 was analyzed in R using ANOVA with mifepristone concentration as fixed effect. A
288 Bonferroni correction was applied to account for multiple testing (0.05/4 = 0.0125).
289 Evaluation of a *da*-GS-GAL4/w^{1118} indicated that that the mifepristone
290 concentrations used did not significantly affect lifespan, egg laying rate and egg volume
291 (Figure 1 – figure supplement 1)

292

293 **SNP association studies**

294 Strains from the *Drosophila* Genetic Reference Panel (DGRP), obtained from the
295 Bloomington *Drosophila* Stock Center (BDSC) were used for a SNP association experiment.
296 The SNP genotypes of our candidate SNP in *Eip75B* of each line were obtained from the
297 genome database available for this panel. The genotype was confirmed by sequencing a
298 small genomic region surrounding the SNP using Sanger sequencing. We generated ten
299 independent F1 crosses from twenty DGRP strains per allelic variant, and then assessed
300 lifespan and fecundity of these crosses. The ten F1 crosses per allelic variant were generated
301 by crossing virgin females of one strain with males from another strain (Table 3). The F1
302 crosses were reared and assays were performed at 25°C, 65% humidity and 12h:12h
303 light:dark cycle, on a cornmeal-yeast-sucrose-agar medium as described above.

304 Lifespan was assessed in demography cages as described earlier with the regular
305 medium provided in food vials. Flies that emerged within a 24-hour window were collected
306 and 75 males and 75 females were placed in a single demography cage. For the experiment
307 using F1 crosses, a single demography cage per cross was setup. Differences in lifespan
308 between the two allelic states of each SNP were analyzed in R (v.3.3.1) using mixed-effects
309 Cox (proportional hazards) regression with allele, sex and their interaction as fixed effects
310 and with 'F1 cross' as a random effect using the R package *coxme* (v2.2-5).

311 Egg laying rate over a period of 30 days after emergence was measured to provide
312 insight in both early (peak) and late (post-peak) fecundity. Again, flies that emerged within a
313 24-hour window were collected, and two females and two males were placed together in a
314 vial containing regular medium. The experiment with F1 crosses, three replicate vials per
315 cross were setup. Every third day (i.e. day 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30), the number

316 of eggs laid per pair of flies during a period of 24 hours was counted under a microscope.
317 Variation in egg laying rates was analyzed in R (v.3.3.1) using generalized linear mixed
318 models with a Poisson distribution and with allelic state, day and their interaction as fixed
319 effects and with 'replicate vial' as a random effect using the R package *lme4* (v.1.1-13).
320

321 **CRISPR/Cas9 induced SNP modification**

322 For editing the SNP in *Eip75B* at position 3L:18026199, plasmids containing the gRNA
323 sequence and dsDNA donor templates were prepared and injected into *yw*;*nos*-Cas9 (II-
324 attP40)/*CyO* embryos. This strain has a "T" allele at position 3L:18026199, hence the goals
325 of the experiment was to modify it to a "G" allele. In addition to editing the candidate
326 *Eip75B* SNP, a dsRED marker was inserted to chromosome 2L, into the C-terminus of *I(2)gl*,
327 to facilitate screening. For this, gRNA sequences were cloned into bicistronic pDCC6
328 plasmids (*Eip75B*: 5' CACTGCTTGCAATAAGTGATGGG 3'; *I(2)gl*: 5'
329 TGTTAAAATTGGCTTTCTTCAGG 3'). Homology directed repair was facilitated by constructing
330 donor templates matching the flanking sequences of the injection line for 500bp on either
331 side of the dsDNA cleavage point (i.e. 1 kb in total). These donor templates were cloned into
332 pcDNA3.1. The injection mix consisted of 35 µg of the *Eip75B* donor plasmid, 35 µg of the
333 *I(2)gl* dsRED donor plasmid, 15 µg I2gl gRNA plasmid, and 15 µg *Eip75B* plasmid per 100µl.
334 The CRISPR-Cas9 editing was done by InDroso (Rennes, France). A total of 300 embryos
335 were injected, resulting in 60 surviving larvae and 30 fertile adults. The flies were crossed to
336 a balancer line (*w*;+;*Dr*/*TM6*,*Sb*,*Tb*) and their offspring was screened visually for the dsRED
337 marker and molecularly, by sequencing, for the *Eip75B* candidate SNP. A total of seven
338 fertile lines with the desired modification in *Eip75B* ("G") were obtained in the F1. Lines that
339 were positive for the dsRED marker, but negative for the modification in *Eip75B* (hence,

340 they carried the "T" allele) were kept to serve as controls. The *Eip75B*-modified and control
341 lines were backcrossed to the balancer line for three generations to remove the dsRED
342 marker and to obtain homozygous lines for the candidate SNP: *w*;;*Eip75B*$^{T18'026'199G}$ (edited)
343 and *w*;;*Eip75B*^{T} (control). We generated two homozygous edited lines with the "G" allele
344 ("E5" and "E6") and two lines with the "T" allele ("C4" and "C6"). The sequence surrounding
345 the *Eip75B* SNP, including the full length of the homology arms, were sequenced to confirm
346 the identity of the SNP and to monitor for any potential undesired genomic modifications.
347 No genomic modifications, apart for the candidate SNP, were detected in any of the lines.
348 An additional set of control lines was generated by backcrossing uninjected *yw*;*nos*-Cas9 (II-
349 attP40)/*CyO* flies into a w^{1118} background, using the balancer line and the same crossing
350 scheme as applied for the injected flies.
351 The following phenotypes were tested in the CRISPR-edited and control lines:
352 lifespan, egg laying rate, egg volume, egg-to-adult development time, egg-to-adult viability,
353 starvation resistance, and adult dry weight. Before the onset of phenotyping experiments
354 flies that were used to generate the cohorts for the experiments were also screened for the
355 identity of the *Eip75B* SNP using PCR-based screening. For this, gDNA was isolated from
356 individual flies using Chelex extraction (Biorad). Flies were placed individually in 100 ul
357 Chelex solution (5g of Chelex mixed with 95ml TE buffer) with 5 ul proteinase K (Promega),
358 and incubated overnight at 56ºC. The resulting gDNA was then analyzed with two
359 alternative reverse primers to distinguish the two allelic states: forward primer 5'
360 CCGGGCACAGTCTCAGTGTT 3', in combination with reverse primers 5'
361 GCGCCAAAGCTTTCCCGTC**A** 3' or 5' GCGCCAAAGCTTTCCCGTC**C** 3'. The PCR was done with
362 GoTaq2 polymerase following the instructions of the manufacturer (Promega) and the
363 following PCR protocol*:* 94°C for 3 min; 32 cycles with 94°C for 15 sec, 62°C for 30 sec, and

364 68°C for 1 min; 68°C for 6 min. This procedure facilitated rapid and high-throughput
365 screening by gel electrophoresis. A subset of the samples was sequenced using Sanger
366 sequencing for confirmation. All phenotyping experiments were done in parallel using the
367 same cohort of flies.

368 Lifespan was measured as described above, by placing 75 females and 75 males that
369 emerged in a 24h window in demography cages with standard medium provided. Five
370 replicate cages were set-up per strain. Differences in lifespan between the two allelic states
371 in *Eip75B* were analyzed in R (v.3.3.1) using mixed-effects Cox (proportional hazards)
372 regression with allele, sex and their interaction as fixed effects and with 'line' and 'replicate
373 cage' as random effects using the R package *coxme* (v2.2-16).

374 Egg laying rate was measured as described above. Two females and two male flies,
375 emerged within a 24-hour window, were placed together in a vial containing standard
376 medium. On days 5, 10, 15, 20, 25, and 30 the number of eggs laid per pair of flies during a
377 period of 24 hours was counted under a microscope. Egg laying rates was analyzed in R
378 (v.3.3.1) using generalized linear mixed models with a Poisson distribution and with allelic
379 state, day and their interaction as fixed effects and with 'line' and 'replicate vial' as random
380 effects using the *R* package *lme4* (v.1.1-26). The effect of allelic state for each day separately
381 were analyzed in models with only allelic state as fixed effect and 'replicate vial' as random
382 effect.

383 Egg volume was assessed by collecting eggs from females, age 7 days after
384 emergence, laid overnight. We randomly picked 30 eggs, which were photographed and
385 measures as described above. Egg volume was analyzed in *R* using ANOVA with allele as
386 fixed effect.

387 To assay egg-to-adult development time ~400 flies were placed in bottles with
388 regular medium and yeast added to lay eggs during two consecutive periods of 3 hours
389 ("batch 1" and "batch 2"). Eggs were collected and distributed to vials at density of 40 eggs
390 per vial, with 16 replicate vials per line. Emergence of adult flies was scored three times per
391 day, at 08:00 ("lights on"), 14:00, and 20:00 ("lights off"). For each vial, the mean time of
392 emergence per sex per vial was calculated and analyzed using linear mixed models with
393 allele, sex and their interaction as fixed effects and batch and line as random effects using
394 the *R* package *lme4*. The egg-to-adult viability was calculated as the proportion of flies that
395 emerged from 40 eggs per vial. It was analyzed using linear mixed model with allele as fixed
396 effect and batch and line as random effects using *lme4*.
397 Starvation resistance was measured by collecting flies from a 24h window of
398 emergence and placing them in vials with standard medium at a density of 10 females and
399 10 males for 5 days. Then the two sexes were placed in separate vials. At day 7 after
400 emergence, the flies were transferred to vials with 0.5% agar. Survival was scored three
401 times daily (08:00 ("lights on"), 14:00, and 20:00 ("lights off")) until all flies has died. The
402 mean time to death was calculated per vial and analyzed for both sexes separately using
403 linear mixed models with allele as fixed effect and line as random effect.
404 Adult dry weight was assayed by collecting flies from a 24h window of emergence
405 and placing them in vials with standard medium at a density of 10 females and 10 males. On
406 day 7 after emergence, the flies were frozen at -20°C. Flies were dried individually by placing
407 them in 96-wells plates in an incubator at 65°C for three days. This was done in two batches.
408 Adult dry weight was then determined in pools of six flies, with 16 pools of flies per line and
409 sex, on a Mettler Toledo MT5 microbalance. The mean time to death was calculated per vial

410 and analyzed for both sexes separately using linear mixed models with allele as fixed effect
411 and line as random effect.
412

413 **ACKNOWLEDGEMENTS**

414 We thank Tadeusz Kawecki for generously allowing us to use his *Drosophila* facilities; the
415 Flatt and Keller labs for discussion and support; Véronique Monnier for the *da*-GS-GAL4
416 strain; and the Bloomington *Drosophila* Stock Center (BDSC) and the Vienna *Drosophila* RNAi
417 Center (VDRC) for providing stocks. This work was supported by H2020-MSCA-IF-2015 (grant
418 701949 to KMH), the European Research Council (grant 741491 to LK), and the Swiss
419 National Science Foundation (grants PP00P3_165836, PP00P3_133641/1, and 310030E-
420 164207 to TF, grant 310030B_176406 to LK).
421

422 **COMPETING INTERESTS**

423 The authors declare that there are no competing interests
424

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609 **TABLES**

610

611 **Table 1: Confirmation of knockdown of *Eip75B* by RNAi.** The relative expression of *Eip75B*
612 after mifepristone application (100 µg/ml or 200 µg/ml**)**, was compared to the expression
613 level in the control (no mifepristone) using quantitative real-time PCR.

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Acronym	**Construct**	***Eip75B* relative expression**		***F***	***P***
		100 µg/ml	**200 µg/ml**		
E01	TRiP.GLC01418	0.86	0.82	6.11	0.020
E03	TRiP.JF02257	0.83	0.72	19.66	0.00013
E10	VDRC #108399	1.08	0.87	1.95	0.17
E44	VDCR #44851	0.85	0.82	4.51	0.043

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620 **Table 2: Primers used to quantify *Eip75B* knockdown using quantitative real-time PCR.**

Gene	Forward primer	Reverse primer	Amplicon (bp)	Primer efficiency*
Eip75B	GGAGGATGTCCCCTGCGCAACC	GAGTATTCCGATCTGCTGCTGCTG	168	1.88
TBP	CGGTTTCCCTGCAAAGTTCCTCGA	CACGATTCGAGGTCGCACCATACG	172	1.92
Cyp1	GTCGGCAGCGGCATTTCAGAT	CTGCACGCTGACGAAGCTAGG	78	1.91
Ef1a48D	TGCCACACCGCTCACATTGCT	CACGCACAGGGGCTTAGAGG	150	1.96
Rap2l	TATTGGACACCGCGGGCACA	TGGGTGCTGGCTGACTTCCT	168	1.96

**qPCR protocol: 95°C for 10 min; 40 cycles with 95°C for 15 sec and 60°C for 1 min; melting analysis from 60°C until 95°C (+0.05 °C/s).*

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623 **Table 3: F1 crosses setup for assessing the correlation of the focal SNP identity with**

624 **lifespan and fecundity.** Ten F1 crosses were setup per allelic variant. The area surrounding

625 this SNP was sequenced to confirm SNP identity. The table indicates from which DGRP lines

626 females and males were taken to generate the F1 crosses.

Allele	Cross	Females	Males
GG	1	142	787
GG	2	304	852
GG	3	319	379
GG	4	356	821
GG	5	386	324
GG	6	555	105
GG	7	73	377
GG	8	796	832
GG	9	83	790
GG	10	908	158
TT	1	239	362
TT	2	306	208
TT	3	340	375
TT	4	385	508
TT	5	391	853
TT	6	405	129
TT	7	426	566
TT	8	443	843
TT	9	716	355
TT	10	819	761

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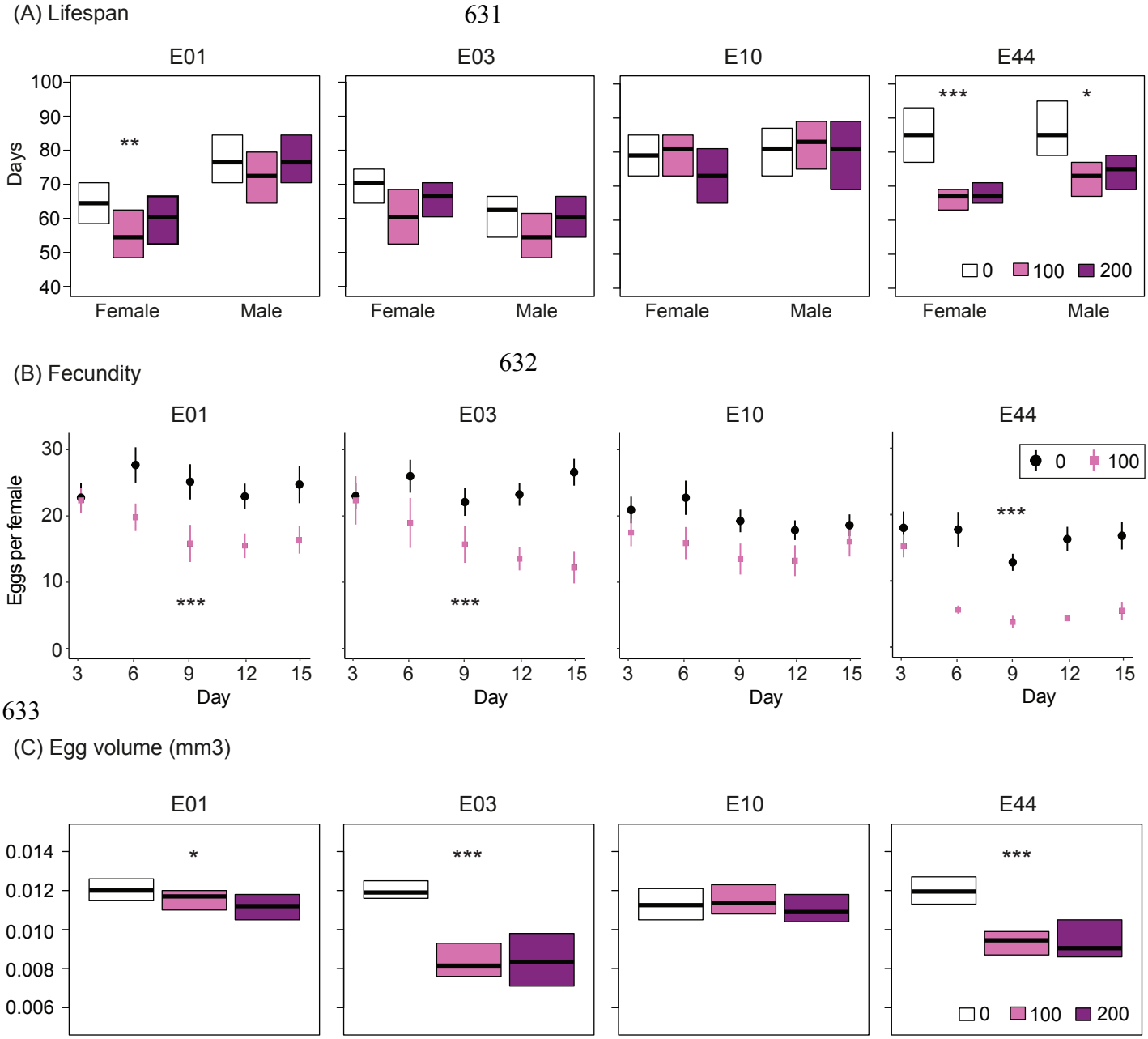
630 **FIGURES**



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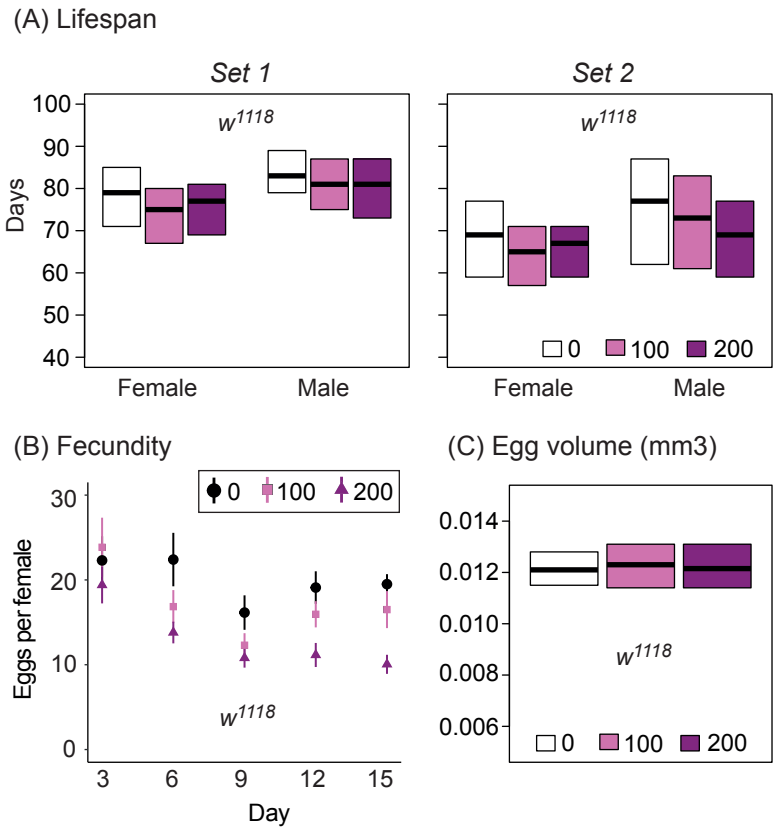
635 **Figure 1: Effects of RNAi knockdown of *Eip75B* on lifespan, fecundity, and egg volume** Effects of
636 RNAi on *Eip75B* in the adult stage were assessed using four different UAS-RNAi constructs (E01, E03,
637 E10, and E44) under control of the mifepristone-inducible *da*-GS-GAL driver. The effects of
638 mifepristone concentration (0, 100, or 200 µg/ml), as measure for RNAi knockdown, on lifespan
639 were considered significant when P<0.0042 (Bonferroni: 0.05/12), whereas effects on egg laying rate
640 and egg volume were considered significant when P<0.01 (Bonferroni: 0.05/5); asterisks indicate

641 significant results. (A) *Eip75B* knockdown resulted in a significant reduction in lifespan for the
642 constructs E44 (female: $P < 0.0001$, male: $P = 0.0021$) and E01 (female: $P = 0.0002$, male: $P = 0.22$),
643 but not E03 (female: $P = 0.44$, male: $P = 0.1$) and E10 (female: $P = 0.071$, male: $P = 0.28$). (B) *Eip75B*
644 knockdown had a significant effect on fecundity for constructs E01 ($P < 0.0001$), E03 ($P < 0.0001$) and
645 E44 ($P < 0.0001$), but not E10 ($P = 0.18$). (C) Also, *Eip75B* knockdown had a significant effect on egg
646 volume for constructs E01 ($P = 0.0028$), E03 ($P < 0.0001$) and E44 ($P < 0.0001$), but not E10 ($P =$
647 0.066).
648



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650 **Figure 1 – figure supplement 1: No significant effects of mifepristone application on *da*-GS-**

651 **GAL4/*w*^{1118}.** Mifespristone application did not have a significant effect on lifespan (Batch 1 - female:

652 $P = 0.039$, male: $P = 0.37$; Batch 2 - female: $P = 0.3$, male: $P = 0.0052$), nor on egg volume ($P = 0.44$).

653 A mifepristone concentration of 200 µg/ml did, however, have a significant effect on egg laying rates

654 ($P < 0.0001$). The concentration of mifepristone was therefore limited to 100 µg/ml for the fecundity

655 experiments ($P = 0.045$). The effects of mifepristone concentration (0, 100, or 200 µg/ml) were

656 considered significant when P<0.0042 (Bonferroni: 0.05/12), whereas effects on egg laying rate and

657 egg volume were considered significant when P<0.01 (Bonferroni: 0.05/5).

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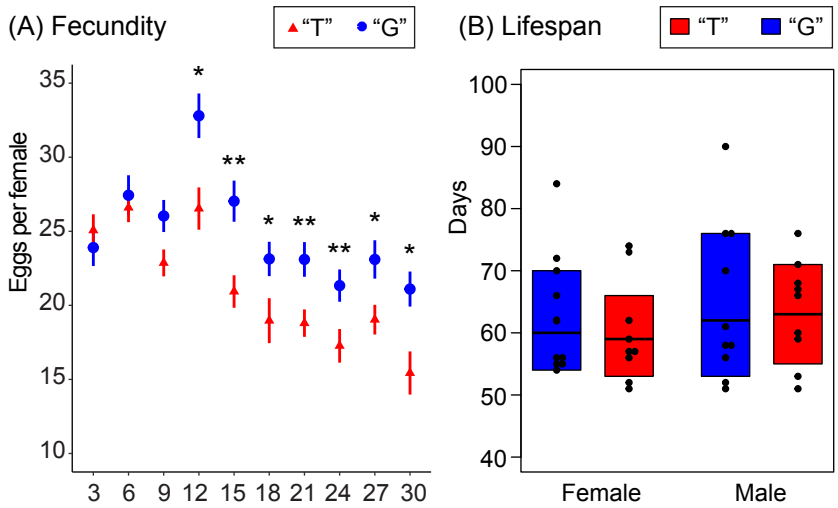

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662 **Figure 2: Association of a SNP in a cis-regulatory motif of *Eip75B* with (A) fecundity and (B) lifespan**

663 **in wildtype lines.** Correlation of the "T" versus "G" identity of the biallelic *Eip75B* SNP at position

664 3L:18026199 with fecundity and lifespan was tested by measuring F1 crosses of the *Drosophila*

665 *Genetic Reference Panel*. (A) There was a significant effect of SNP identity on fecundity (allele: P

666 <0.0001, allele*day: P <0.0001), but (B) not on lifespan (female: P = 0.47; male: P = 0.66). Asterisks

667 indicate significant results.

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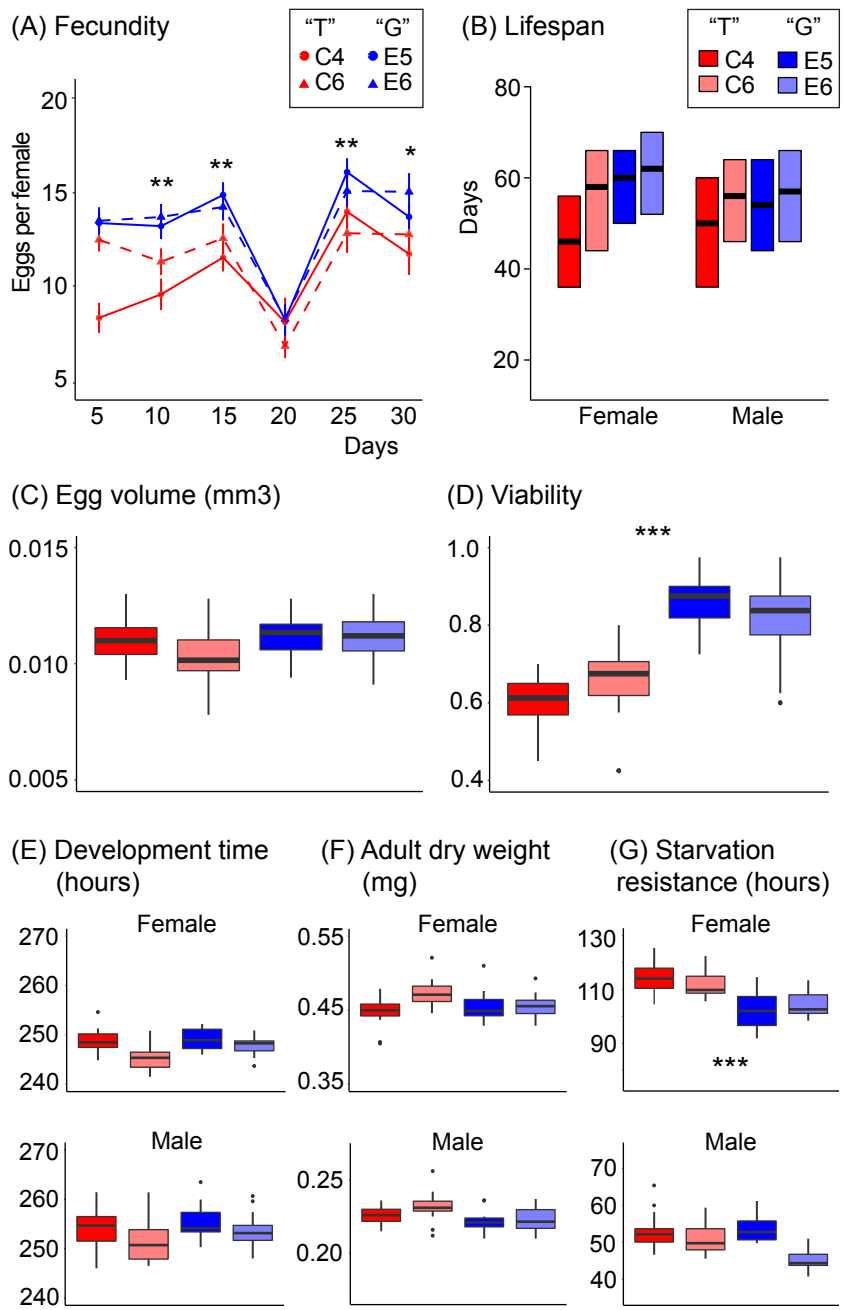

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671 **Figure 3**: **CRISPR-Cas9 modification of the *Eip75B* SNP in an isogenic background reveals its effects**

672 **on life history.** Two lines with the "G" allele (E5 and E6) and two lines with the "T" allele (C4 and C6)

673 were constructed using CRISPR-Cas9 and tested for differences in a range of life history traits known

674 to be regulated by *Eip75B* from mutant analyses and RNAi studies. (A) There was a significant effect

675 of SNP identity on fecundity, as measured by daily egg laying rates per female up to day 30 after

676 emergence (allele: $P = 0.0013$, allele*day: $P = 0.052$), Also, there was a significant effect on (D) egg-

677 to-adult viability (*P* = 0.0004), and (G) starvation resistance in females (females: *P* = 0.0006; males: *P*

678 = 0.41). This SNP allele did not have an effect on (B) lifespan (females: *P* = 0.0006; males: *P* = 0.41),

679 (C) egg volume (*P* = 0.12), (E) egg-to-adult development time (females: *P* = 0.16; males: *P* = 0.24),

680 and (F) adult dry weight (females: *P* = 0.58; males: *P* = 0.052). Asterisks indicate significant results.

681

A single nucleotide variant in the PPARγ-homolog *Eip75B* affects fecundity in *Drosophila*

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APPENDIX 1

Population genetic analysis of the candidate SNP in Eip75B

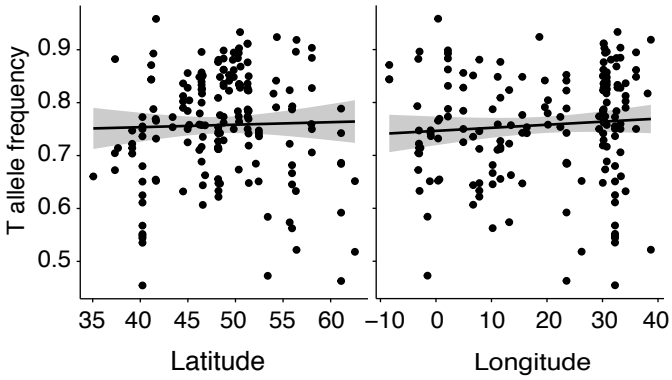
Methodology

The frequency of the "T" allele of the candidate SNP in *Eip75B* (3L:18026199, release 6) ranged from an average of 0.52 to 0.84 in early reproducing *versus* late reproducing, long-lived EE populations, respectively (Hoedjes et al., 2019). These allele frequencies were compared to the allelic variation that exists in populations from Europe, North-America, and Africa similar to (Hoedjes et al, in prep). For this, we analyzed whole genome sequencing data from multiple geographic populations available from the DEST dataset (Kapun et al., 2021). We included both single individual sequencing data and Pool-Seq data from various sources. For the single individual sequencing data, the “T” allele frequency of the *Eip75B* allele was calculated when at least ten individuals were sequenced for a given population. Data from pooled populations was included when the coverage for each *Eip75B* SNP was at least 10. We tested the data from the 173 samples collected in Europe for clinal distribution patterns of the allele frequencies. For this, we used generalized linear models (GLMs) based on the allele counts of the *Eip75B* SNP, with either longitude or latitude as explanatory factor. False discovery rate was estimated by testing clinal distribution patterns among a set

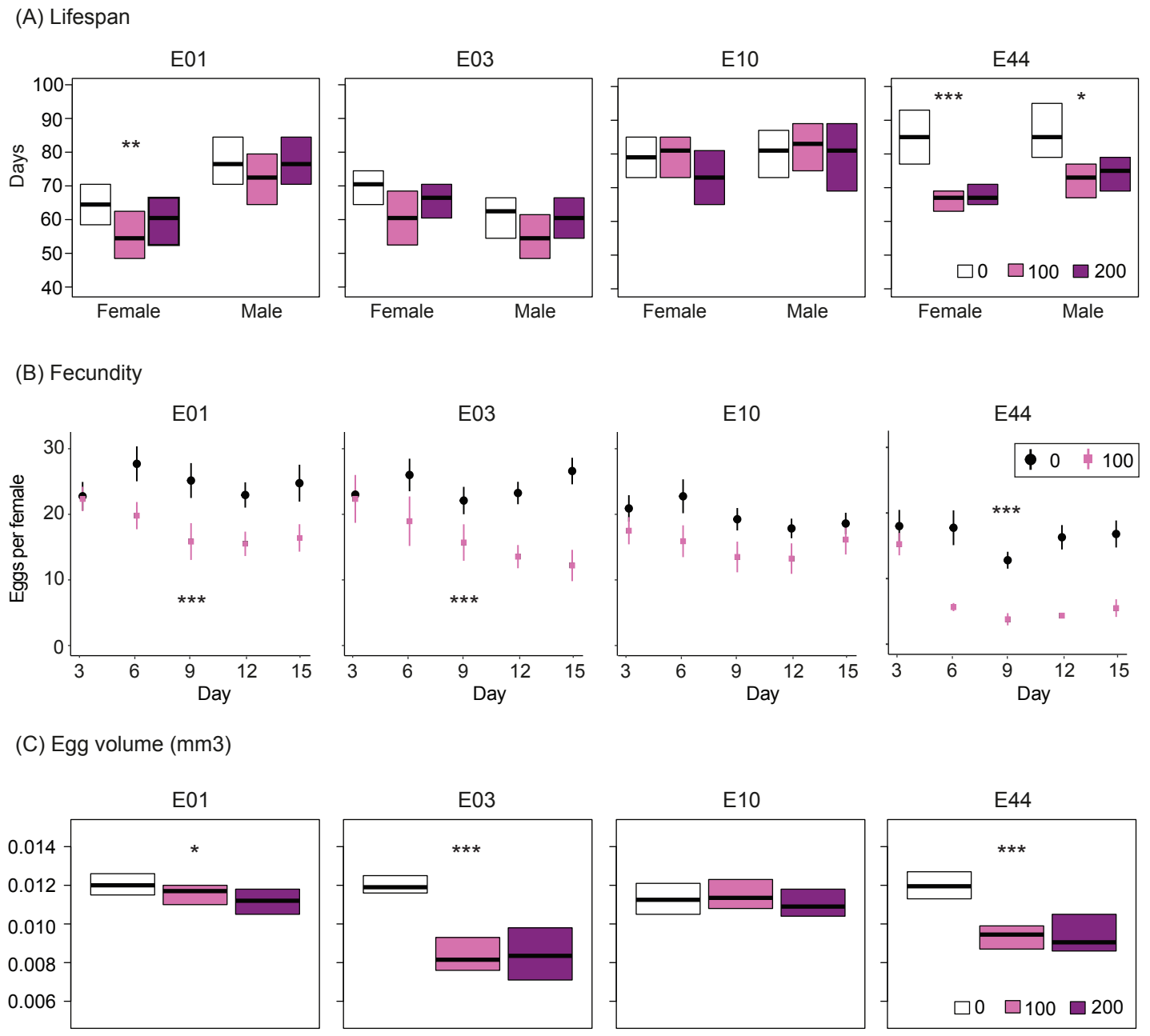
of 21'008 neutral SNPs (Clemente & Vogl, 2012; Parsch, Novozhilov, Saminadin-Peter, Wong, & Andolfatto, 2010). Empirical cumulative density functions (ECDF) were generated based on the *P* values of these neutral SNPs. The area of the upper tail confined by the $\chi 2$ values of the *Eip75B* SNP indicates the percentile of neutral SNPs with $\chi 2$ values equal or higher than the *Eip75B* SNP. This parameter provides a corrected estimate of significance of clinality of each SNP compared to genome-wide neutral estimates (Ramaekers et al., 2019).

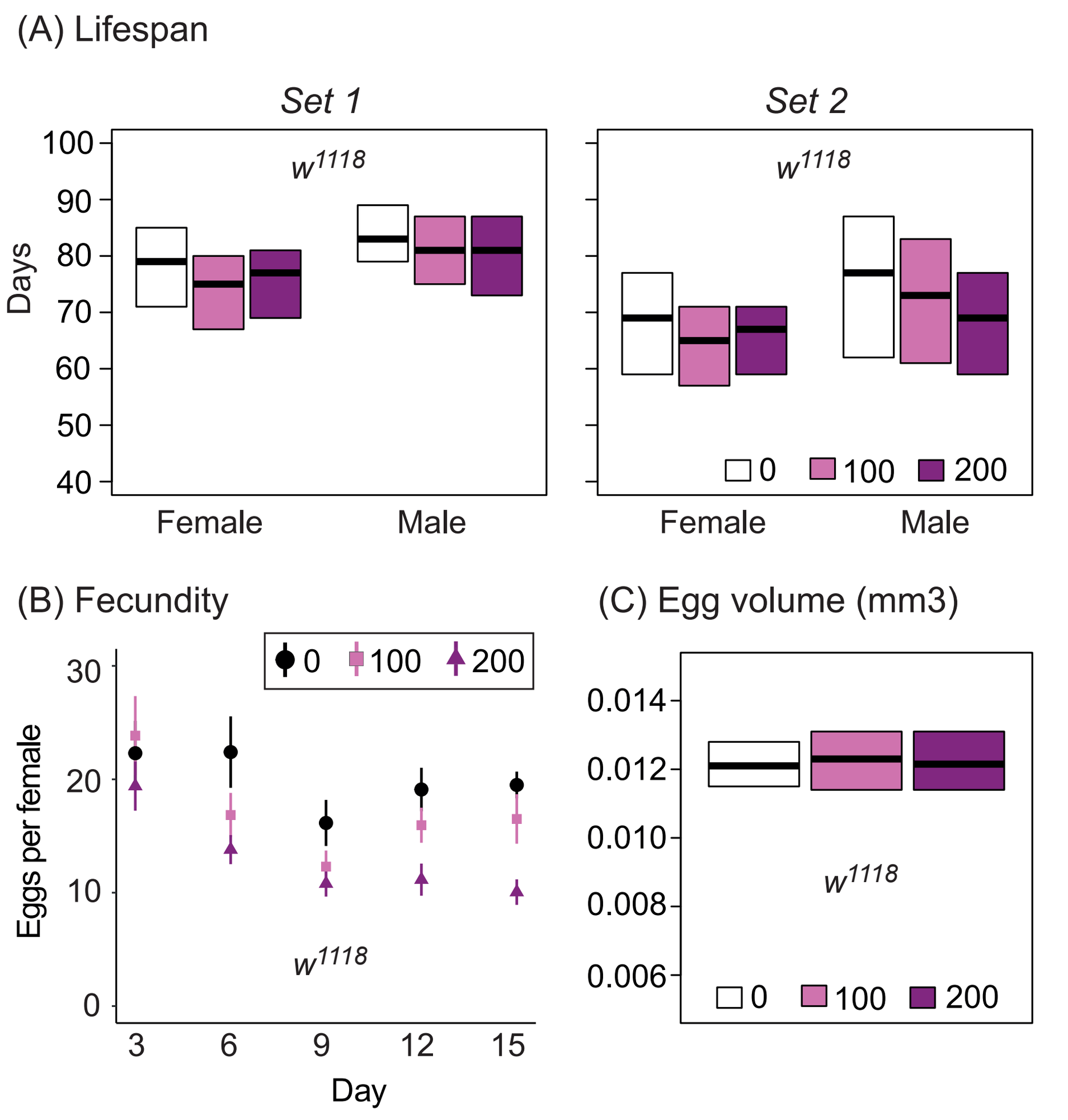
Distribution of the Eip75B allele

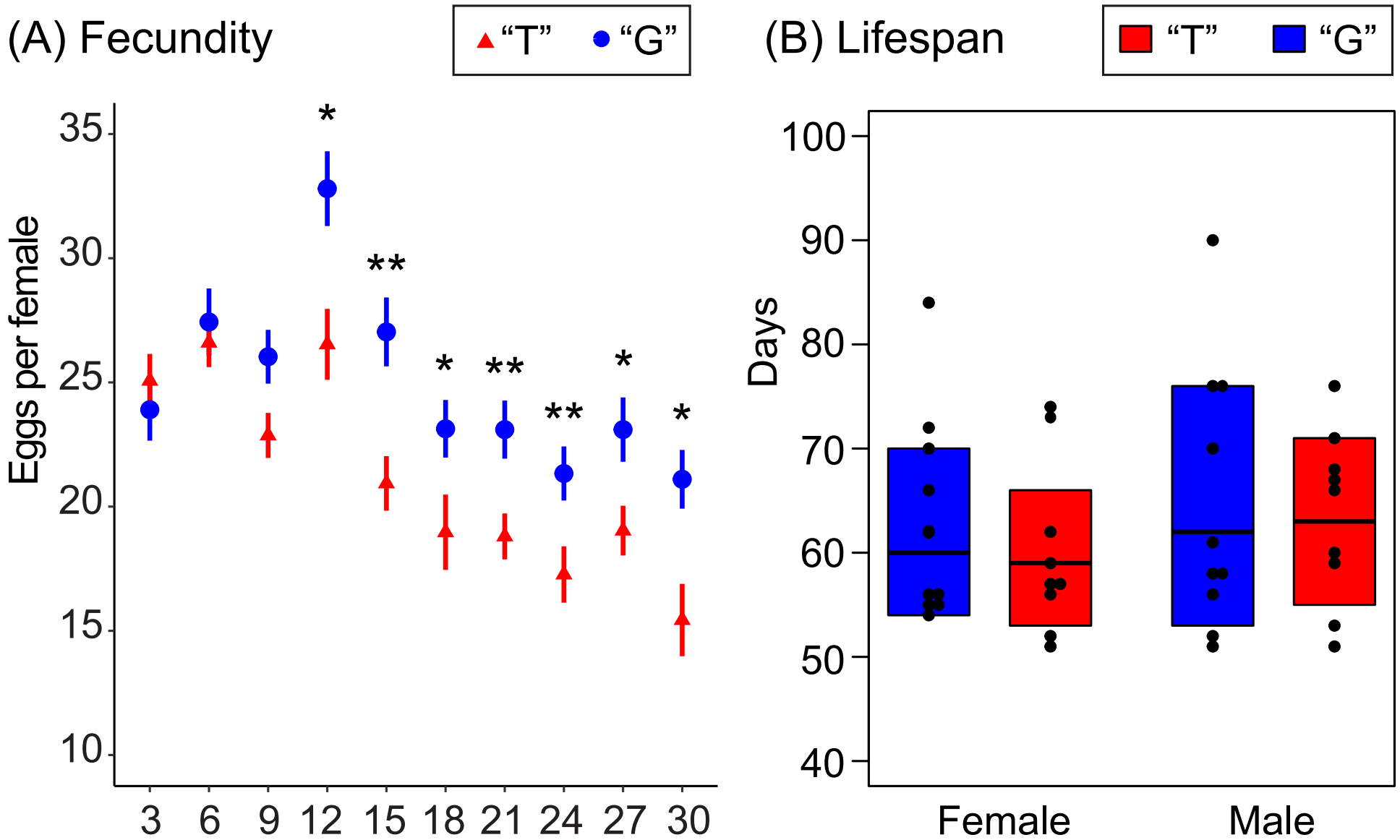
Analysis of the allele frequencies of the *Eip75B* SNP in multiple geographic populations from Europe (173 samples, "T" allele frequency: 0.53-0.92) and North America (55 samples: 0.53-0.82) indicates a variable allele frequency for this SNP in natural populations, and demonstrates that the allele frequencies observed by (Hoedjes et al., 2019) fall within the range of naturally occurring variation in Europe and North America (Supplementary Table 1). Limited data on the allele frequencies of the candidate EiP75B SNP in African populations suggests that both alleles were present in ancestral populations already, although the frequency of the "G" allele may have been limited ("T" allele frequency: 0.8 in Gabon, 1.0 in Cameroon, 0.99 in Zambia). Analysis of the allele frequency data from the European populations provided no indication for a clinal distribution of the *Eip75B* SNP (latitude: χ^2 = 1.13, $P = 0.29$, $P_{corrected} = 0.61$; longitude: $X^2 = 9.45$, $P = 0.002$, $P_{corrected} = 0.43$).

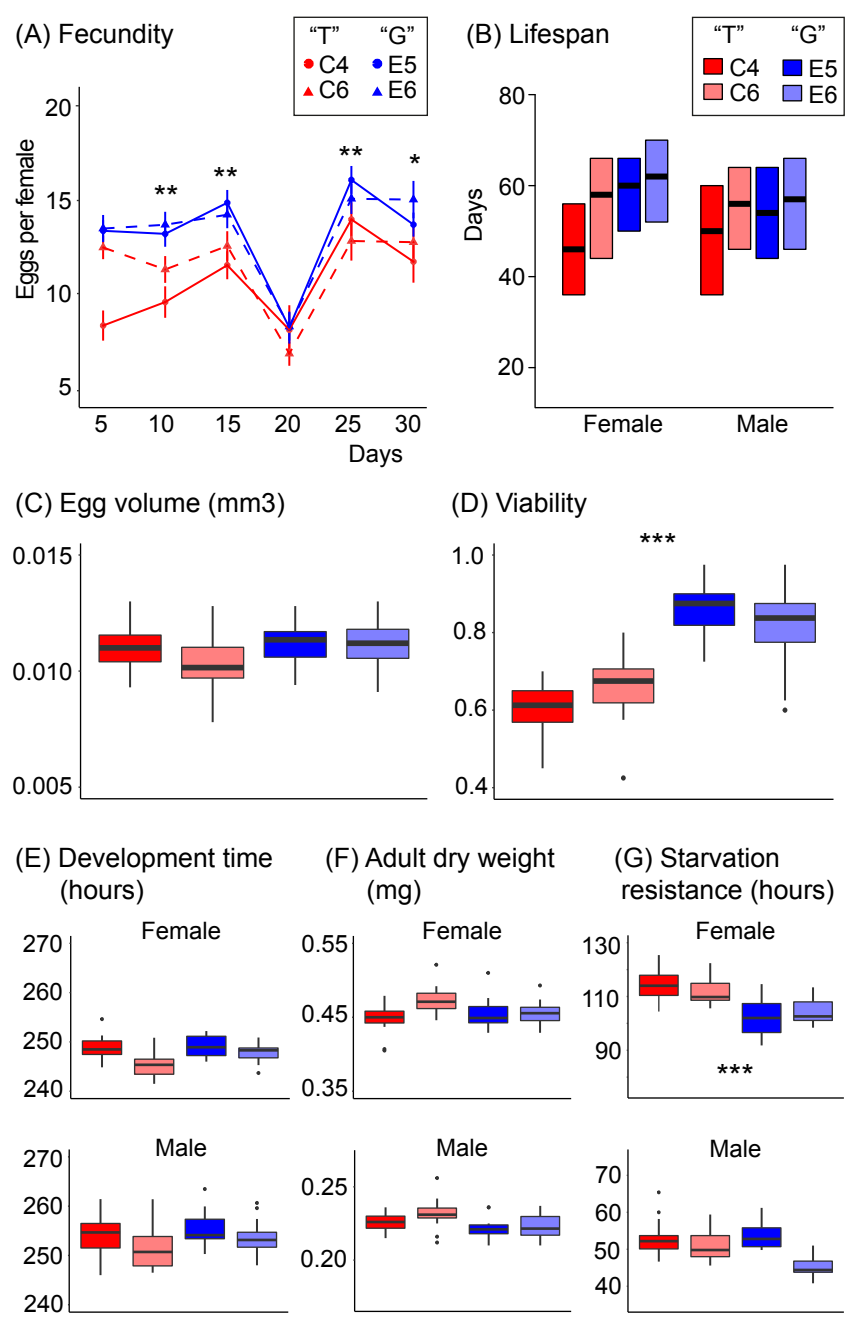


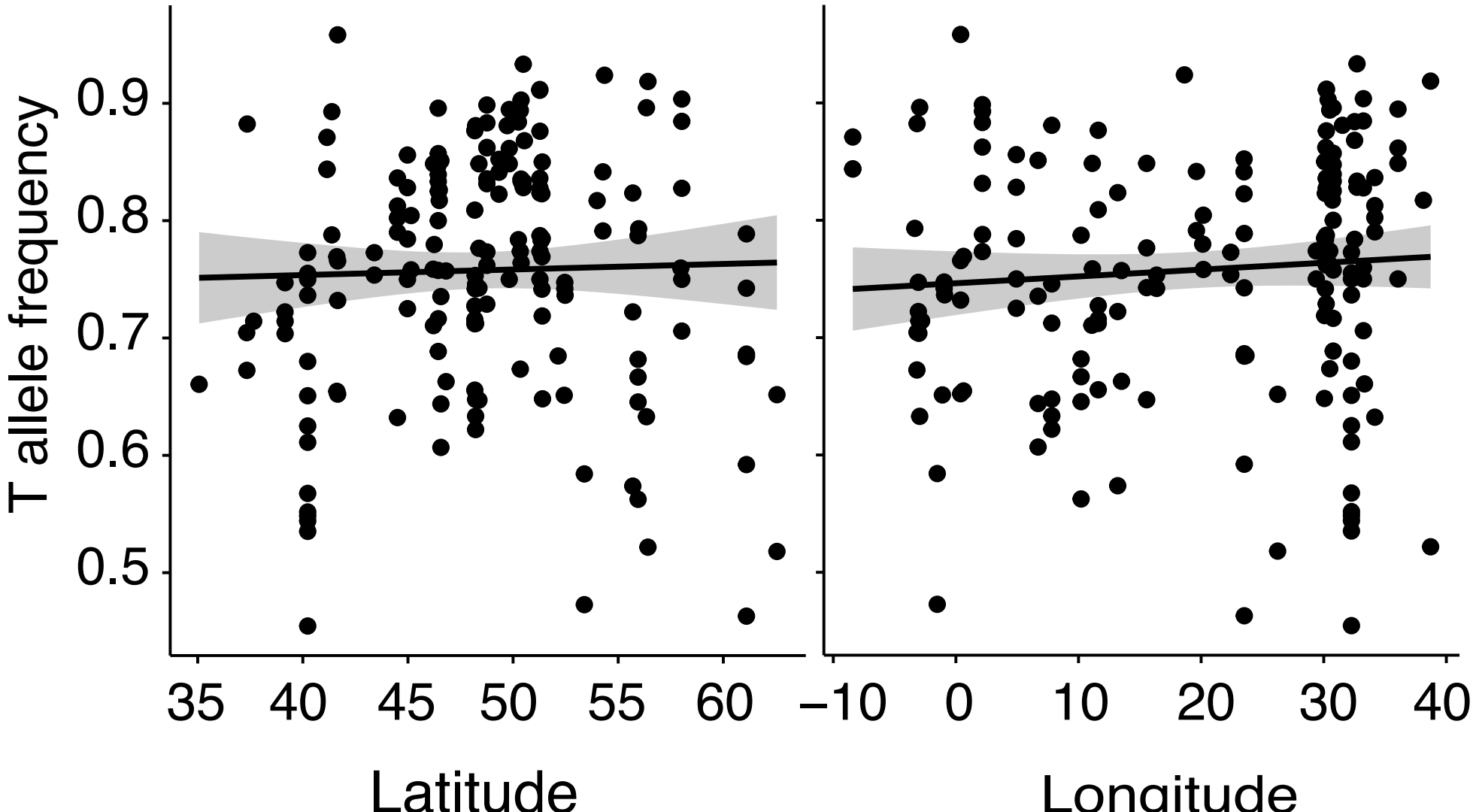
Appendix 1 – figure 1: Allele frequency distribution of the "T" allele at position 3L: 18026199 across Europe. The samples are ordered by latitude (left) and longitude (right).


(A) Lifespan
E01
E03
E10
E44
Days
100
90
80
70
60
50
40
**
***
*
Female
Male
0
100
200
(B) Fecundity
Eggs per female
30
20
10
0
***
3
6
9
12
15
Day
(C) Egg volume (mm3)
0.014
0.012
0.010
0.008
0.006
*


(A) Lifespan
Set 1
Set 2
Days
100
90
80
70
60
50
40
w1118
w1118
0
100
200
Female
Male
Female
Male
(B) Fecundity
0
100
200
Eggs per female
30
20
10
0
w1118
3
6
9
12
15
Day
(C) Egg volume (mm3)
0.014
0.012
0.010
0.008
0.006
w1118
0
100
200


(A) Fecundity
"T"
"G"
Eggs per female
35
30
25
20
15
10
*
**
*
**
**
*
*
3
6
9
12
15
18
21
24
27
30
(B) Lifespan
"T"
"G"
Days
100
90
80
70
60
50
40
Female
Male


(A) Fecundity
"T"
"G"
C4
E5
C6
E6
Eggs per female
Days
**
**
**
*
(B) Lifespan
Days
Female
Male
(C) Egg volume (mm3)
(D) Viability
***
(E) Development time (hours)
(F) Adult dry weight (mg)
(G) Starvation resistance (hours)
Female
Male
***


T allele frequency
0.9
0.8
0.7
0.6
0.5
35
40
45
50
55
60
Latitude
−10
0
10
20
30
40
Longitude