# Relaxin signaling is critical for virgin female reproductive physiology in *Drosophila*

**Yanel Volonté[a,c], Fabiana Heredia[b,c], Rebeca Zanini[b,c], Juliane Menezes[b,c], María Sol Perez[a], Mafalda Gualdino[b,c], Lara Lage[b,c], Leonor da Silva Luz[b,c], Andreia P. Casimiro[c], Andrés Garelli[a,b,c,1], Alisson M. Gontijo[b,c,1]**

a Instituto de Investigaciones Bioquímicas de Bahía Blanca, Consejo Nacional de Investigaciones Científicas y Técnicas, and Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Bahía Blanca B8000FWB, Argentina.

b cE3c - Centre for Ecology, Evolution and Environmental Changes & CHANGE, Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, 1749-016, Lisbon, Portugal.

c iNOVA4Health, Nova Medical School, NMS, Universidade Nova de Lisboa, Lisbon 1150-082, Portugal

1 Corresponding authors: agarelli@inibibb-conicet.gob.ar and amgontijo@ciencias.ulisboa.pt

**Significance statement:**

Ovulation enables mature oocytes to exit the ovary for potential fertilization. In *Drosophila*, ovulation is induced by mating but also occurs spontaneously in virgins, with rates varying widely in natural populations: short oocyte retention is ancestral, while longer retention is favored in colder climates. The molecular regulation of virgin ovulation remains unclear. Here, we show that disrupting the *Drosophila* relaxin/insulin-like peptide Dilp8 or its receptor Lgr3—an orthologue of vertebrate RXFP1/2—in follicle cells or specific neurons delays ovulation, slows egg transit, and reduces oocyte quality. Mating largely rescues these defects, suggesting the pathway is dispensable post-mating. Our findings reveal a molecular mechanism for oocyte retention in *Drosophila* virgins and support an ancient, conserved role for relaxin-like signaling in ovulation.

## ABSTRACT

Ovulation allows mature oocytes to exit the ovary for potential fertilization. In *Drosophila*, ovulation can be induced by mating or occur spontaneously in lower amounts in virgin females. Virgin ovulation rates show high populational variation, with short oocyte retention times being ancestral, and long retention being selected for in colder climates. The molecular mechanisms controlling virgin ovulation rates are poorly understood. Here, we show that reduced activity of the Drosophila insulin-like peptide 8 (Dilp8), a relaxin-like peptide secreted from terminal follicle cells of the ovary, slows virgin ovulation and ovulated egg genital tract transit by a factor of ~5-6. These phenotypes are largely rescued by mating, suggesting other mechanisms override the Dilp8-Lgr3 pathway post-mating. Knockdown of the Dilp8 receptor, Lgr3—an orthologue of vertebrate relaxin RXFP1/2 receptors—in at least two subsets of reproductive-tract-innervating neurons, one of which are octopaminergic, produces similar phenotypes. Dilp8-Lgr3 signaling in virgin females ensures high oocyte quality by promoting the elimination of aging oocytes and by systemically antagonizing insulin signaling, which is critical for oogenesis progression beyond previtellogenic stages. We hypothesize this latter Dilp8 activity factors in oocyte stock size in the nutrient-availability-based decision to invest in producing more oocytes or not, ensuring oocyte overstocking in virgins only occurs in highly favorable nutritional conditions. Our findings provide a molecular basis for oocyte retention time regulation in *Drosophila* virgins, and bring further support for an ancient, conserved role for relaxin-like signaling in regulating ovulation and overall female reproductive physiology.


## INTRODUCTION

Ovulation is a fundamental biological process in which a mature oocyte sheds its surrounding follicle cells and leaves the ovary, enabling the possibility of fertilization (Bloch Qazi, Heifetz, and Wolfner 2003; Richards 2007). Ovulation can be profoundly influenced by environmental and internal states, such as seasons, lunar cycles, stress, pheromones, mating status, and pregnancy in ways that are many times species-specific (Rekwot et al. 2001; Bloch Qazi, Heifetz, and Wolfner 2003; Yamamoto, Kosinski, and Greenstein 2006; Richards 2007; Mercier and Hamel 2009; Brown 2018). In *Drosophila*, like in other species with induced ovulation [*e.g.,* cats and camels (Bakker and Baum 2000; Ratto et al. 2012)], ovulation is largely triggered by mating. This occurs via at least two processes in *Drosophila*: 1) a seminal protein transferred from the male, ovulin (Heifetz et al. 2000; Bloch Qazi, Heifetz, and Wolfner 2003), increases ovulation rates in a process that is dependent on the monoamine hormone octopamine (Rubinstein and Wolfner 2013), and 2) female spermathecal secretory cell factors secreted via a non-canonical secretory pathway also contribute to increasing postmating ovulation rate (Schnakenberg, Matias, and Siegal 2011; Sun and Spradling 2013).

During sexual maturation, virgin *Drosophila* females accumulate mature oocytes and ovulate significantly less than mated females throughout their lives, although the difference becomes smaller in older females (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002). The timing of the onset of virgin ovulation (also called oocyte retention or virgin egg retention phenotype) is a life-history trait that varies clinally and seasonally in natural populations and has a genetic basis (Boulétreau-Merle, Fouillet, and Terrier 1992; Akhund-Zade et al. 2017; Horváth and Kalinka 2018). Short retention phenotypes (where laying of unfertilized eggs starts as soon as 2 days after adult emergence) are considered the ancestral state of *Drosophila melanogaster*, while long retention phenotypes (where the first unfertilized eggs are laid up to 15 days after adult emergence) are considered adaptations to cold environments and overwintering (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002). In the latter case, at the population level, both phenotypes are maintained via balancing selection, which is thought to contribute to the seasonality of the phenotypes (Boulétreau-Merle, Fouillet, and Terrier 1992; Boulétreau-Merle and Fouillet 2002). Whereas many species, such as murines and primates, also ovulate spontaneously (Bakker and Baum 2000), energy investment in oogenesis is considered relatively higher for *Drosophila* females, so that early and numerous virgin ovulations are thought to be a residual and unregulated process, and are considered a pointless loss of reproductive potential (Akhund-Zade et al. 2017; Horváth and Kalinka 2018; Boulétreau-Merle and Fouillet 2002). Genomewide association studies using Drosophila Genetic Reference Panel (DGRP) inbred lines (Mackay et al. 2012) have pointed out several genes with variation associated with virgin egg retention, and mutations in four of the genes that could be experimentally verified (*GfrI/mun*, *T48*, *mes-4,* and *klp67A*), caused longer virgin retention times when compared to controls (Akhund-Zade et al. 2017), suggesting that cold-adaptation could involve loss of traits that are otherwise selected for in warmer environments and seasons. The genes that promote ovulation in warmer conditions remain to be defined and studied in detail.

Drosophila insulin-like peptide 8 (Dilp8) is a relaxin-like peptide that promotes developmental stability and pupariation behavior progression during early and late larval stages, respectively (Garelli et al. 2012; Heredia et al. 2021; Colombani, Andersen, and Léopold 2012; Gontijo and Garelli 2018). In both cases, Dilp8 acts via a conserved neuronal relaxin-receptor-like receptor, the Leucine rich repeat containing G protein coupled receptor 3, Lgr3, the orthologue of vertebrate RXFP1/2 receptors (Garelli et al. 2015; Vallejo et al. 2015; Colombani et al. 2015; Jaszczak et al. 2016). Dilp8 is highly expressed in the adult ovary (Chintapalli, Wang, and Dow 2007; Garelli et al. 2012), specifically in terminal-stage follicle cells coating mature oocytes (Liao and Nässel 2020; Hongjie Li et al. 2022; Haomiao Li et al. 2023). Follicle-cell derived Dilp8 has been proposed to generally regulate fecundity, ovulation, and metabolism in adults (Liao and Nässel 2020; Haomiao Li et al. 2023). Endogenous *Lgr3* has also been proposed to regulate fecundity (Haomiao Li et al. 2023). Moreover, a subset of sexually-dimorphic *Lgr3*-positive neurons has been shown to regulate female receptivity and fecundity (Meissner et al. 2016). Finally, expression of Lgr3 GAL4 lines was found in posterior follicle cells and in neurons innervating the oviduct, which could suggest

that Dilp8 promotes fecundity and ovulation in mated flies via both autocrine and paracrine signaling mechanisms (Liao and Nässel 2020).

Here, we use genetic and tissue-specific RNA interference coupled with reproductive behavior monitoring and ovulation assays to show that Dilp8 and Lgr3 are indeed critical for ovulation control, but mostly in virgin females. In the absence of *dilp8* in follicle cells or *Lgr3* in at least two subpopulations of neurons that collectively innervate the ovary, oviduct, and uterus, ovulation is significantly slower in virgin females, so that these animals retain oocytes in the ovary for ~5-6 times longer than controls. Furthermore, the virgin oocyte retention phenotype is largely rescued by mating, suggesting that other mechanisms can supersede the requirement for the Dilp8-Lgr3 pathway for ovulation. Inhibition of vesicle secretion in follicle cells indicates that ovulation is promoted by the release of Dilp8 from these cells into the oviduct. Collectively, this suggests that frequent spontaneous ovulation (short oocyte retention times) in *Drosophila* virgins is an actively-induced and modulated process. In addition, we find that Dilp8 promotes optimal oocyte quality by at least one mechanism: by eliminating aged oocytes by spontaneous ovulation. Finally, we show that Dilp8 can antagonize systemic insulin signaling, which is critical for the progression of oogenesis beyond pre-vitellogenic stages. This could suggest that Dilp8 also promotes oocyte quality by a second, negative-feedback mechanism, where the accumulation of mature oocytes in the ovary increasingly inhibits the production of new mature oocytes, most effectively so under suboptimal nutrient conditions. Our results provide a framework to understand virgin oocyte retention regulation and evolution, and demonstrate that relaxin-like signaling has an ancient function in reproductive physiology in bilaterians, most notably in promoting ovulation.

## RESULTS

### *Dilp8 is expressed in and secreted from mature, stage 14, follicle cells*

Previous work has shown that *dilp8* mRNA is highly enriched in the ovary of *D. melanogaster* and other flies (Garelli et al. 2012; Liao and Nässel 2020; Chintapalli, Wang, and Dow 2007; Haomiao Li et al. 2023). In the ovaries, this expression is detectable in the follicle cells of stage 14 (choriogenic) follicles in genomewide expression studies (Tootle et al. 2011; Hongjie Li et al. 2022), using the transcriptional reporter and loss of function allele *dilp8-enhanced Green Fluorescent Protein (eGFP)*$^{MI00727}$ (Garelli et al. 2012; Venken et al. 2011; Liao and Nässel 2020; Haomiao Li et al. 2023), two enhancer traps *dilp8-GAL4* lines (*dilp8-1-GAL4 and dilp8-2-GAL4*) (Hayashi et al. 2002) (**Figure 1A**), or with an anti-Dilp8 antibody (**Figure 1B**) (Colombani, Andersen, and Léopold 2012) . The anti-Dilp8 staining is specific as it is lost in animals carrying two copies of *dilp8–1-GAL4* (**Figure 1B**), which strongly reduces *dilp8* mRNA levels proportionally to levels found in *dilp8-eGFP*$^{MI00727}$ homozygotes (**Figure 1C**). Follicle cell Dilp8 is cytoplasmic and vesicular, as expected from stainings of imaginal discs with this antibody (Colombani, Andersen, and Léopold 2012) and of follicle cells with an independent antibody (Liao and Nässel 2020).

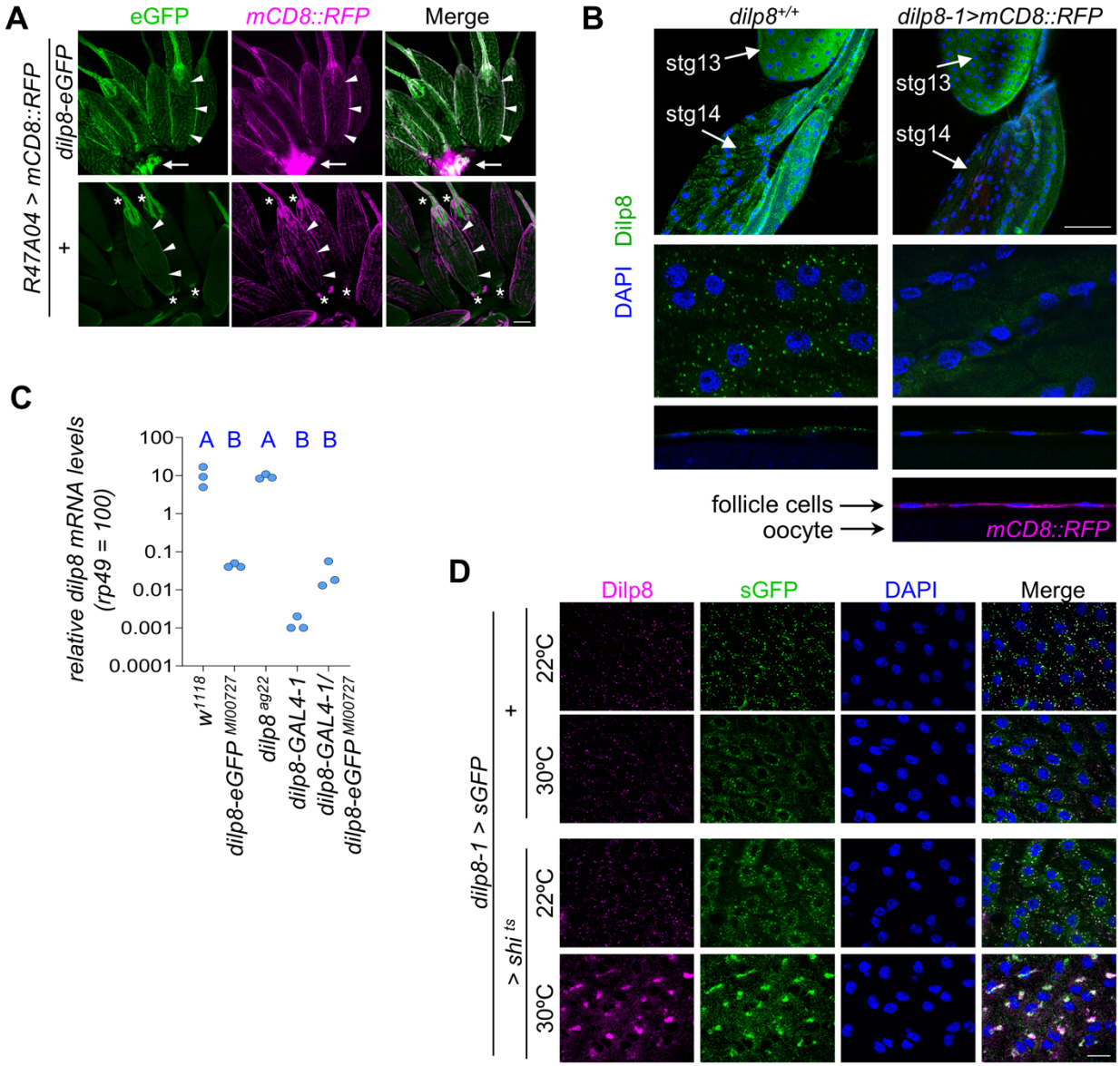


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**Fig 1. Dilp8 is expressed in and secreted from mature, stage 14 follicle cells**. **(A)** The *R47A04-GAL4* line is expressed in follicle cells of stage 14 ovarian follicles (*UAS-mCD8::RFP* expression; magenta, arrowheads) which also show eGFP fluorescence from a *dilp8-eGFP[MI00727]* transcriptional reporter (green, arrowheads, top panel). Follicles from control animals not carrying the *dilp8-eGFP[MI00727]* reporter have no detectable eGFP expression (green, bottom panel). The arrow points to the residual body (top panel). The dorsal appendages and the posterior end of stage 14 follicles are autofluorescent under blue light (asterisks). Scale bar = 100 µm. **(B)** Dilp8 immunostaining shows a punctate pattern in stage 14 follicle cells that is absent in mature follicles from a female carrying two copies of *dilp8-GAL4-1* (*dilp8-1>mCD8::RFP*). Earlier follicles show unspecific fluorescence, that is also present in animals that lack *dilp8*. Scale bar = 50 µm. **(C)** Ovaries from homozygous *dilp8-eGFP[MI00727]* (*dilp8-eGFP*$^{MI00727}$) animals have reduced *dilp8* expression that is restored to wild type levels upon precise excision of the *Mi{MIC}* element (resulting in the new wild-type allele, *dilp8*$^{ag22}$). Homozygous *dilp8-GAL4-1* animals also show reduced *dilp8* mRNA levels. Each dot is an independent ovary sample. Same blue letter, $P > 0.05$, ANOVA and Tukey's HSD test. **(D)** Seven-day-old flies that carry a temperature sensitive allele of *shibire* (*>shi*$^{ts}$) expressed in mature follicle cells with *dilp8-GAL4-1* (*dilp8-1>*) lose the Dilp8 vesicular pattern upon shifting them from 22 ºC to 30 ºC for one day. A genetically encoded secreted GFP (*sGFP*) protein, also driven by *dilp8-1>*, was enriched in the same compartments as Dilp8, both at 22 ºC and 30 ºC, confirming the effective blockage of vesicle recycling and accumulation of Dilp8 and sGFP in the perinuclear region. Dilp8 and sGFP distribution is not affected by temperature in follicle cells of control flies that lack *UAS-shi*$^{ts}$. Scale bar = 10 µm.

132 Following ovulation, follicle cells form a transient body, called residual body, which is an anatomically
133 and functionally analogous structure to the vertebrate corpus luteum (Pfeiffer et al. 2008; Deady et al. 2015;
134 Buning 2012). To test if Dilp8 is secreted from follicle cells or whether it is only secreted at the residual
135 body stage following ovulation, we inhibited secretory vesicle recycling in stage 14 follicle cells of well-
136 fed 7 day-old virgins by expressing the temperature-sensitive mutant of the *Drosophila* Dynamin-encoding
137 gene, *shibire*$^{ts}$ (*shi*$^{ts}$) (Kitamoto 2001) under the control of *dilp8-1-GAL4* (*dilp8>shi*$^{ts}$) and by exposing the
138 animals to different temperatures for one day. Shi$^{ts}$ expression at >29°C is expected to cause secretory
139 vesicle depletion in follicle cells with preexisting vesicle pools only if vesicles fuse to the plasma membrane
140 to release their cargo during the temperature-shift period, due to the inability of the mutant Dynamin to
141 participate in the proper recycling of plasma-membrane-associated secretory vesicle membranes. New
142 vesicles are also not expected to be generated since Dynamin is likely also required for vesicle budding
143 from the trans-Golgi network (Jones et al. 1998). Results revealed no observable Dilp8-positive vesicles in
144 follicle cells at >29°C, in contrast to 22°C (**Figure 1D**). Instead, Dilp8 was enriched in a perinuclear
145 compartment, showing that its trafficking to the membrane was abrogated, as expected.

146 To confirm that our manipulations interfered with secretion, we expressed sGFP–a genetically encoded
147 secreted fusion protein composed of GFP and Decapentaplegic (Dpp) sequences including Dpp cleavage
148 and secretory transport domains (Entchev, Schwabedissen, and González-Gaitán 2000)–in follicle cells
149 using *dilp8-1-GAL4*. At permissive temperatures, follicle-expressed sGFP labels most, but not all, Dilp8-
150 positive vesicles at different intensity levels, supposedly due to its overexpression using the GAL4-UAS
151 system. At >29°C, sGFP was found enriched in the same perinuclear compartment as Dilp8 (**Figure 1D**),
152 confirming the efficient blockage of the secretory pathway. These findings are consistent with secretion of
153 Dilp8 from the follicle cells in the ovary, while they are still surrounding mature oocytes. In this experiment,
154 *dilp8>shi*$^{ts}$ animals did not lay unfertilized eggs during the temperature shift, so we know that the stage 14
155 follicles surveyed had been previously generated during the 7 day oocyte retention period. In fact,
156 *dilp8>shi*$^{ts}$ flies or flies expressing *shi*$^{ts}$ in follicle cells using the *R47A04-GAL4* line (Pfeiffer et al. 2008;
157 Deady et al. 2015) quantitatively lay drastically fewer unfertilized eggs than controls (**Supplementary**
158 **figure 1**), consistent with a model where constitutive Dilp8 secretion from stage 14 follicles is required for
159 proper virgin ovulation. It is unclear whether Dilp8 is secreted basally towards the oviduct and/or
160 ensheathing ovariole muscles or apically towards the underlying mature oocyte, or in both directions. While
161 we did not assay for secretion in residual bodies/corpora lutea, it is conceivable that Dilp8 continues to be
162 produced and secreted at that stage.

163

164 *Dilp8 mutant virgins retain oocytes in the ovary*

165 Virgins that are homozygous for the hypomorphic allele *dilp8-eGFP*$^{MI00727}$ and backcrossed to the $w^{1118}$
166 background (hereafter, *dilp8-eGFP*$^{MI00727}$) have bloated abdomens relative to control $w^{1118}$ virgins (**Figure**
167 **2A**). Bloating in *dilp8-eGFP*$^{MI00727}$ homozygous virgins is due to a large amount of late stage oocytes in
168 the ovary and a lower number of unfertilized eggs laid (**Figure 2A-B**). To demonstrate that the phenotype
169 was indeed caused by the *dilp8* insertion and not by another linked mutation, we removed the *eGFP*$^{MI00727}$
170 cassette from the *dilp8* locus by transposon remobilization, generating the new WT allele *dilp8*$^{ag22}$ (see
171 Methods). This restored *dilp8* mRNA levels to similar levels as $w^{1118}$ controls (**Figure 1C**), and resulted in
172 an increase in the number of unfertilized eggs laid as virgins (**Figure 2B**), suggesting that the virgin egg
173 retention phenotype is linked to *dilp8* levels. Strikingly, the reduced egg-laying phenotype was absent in

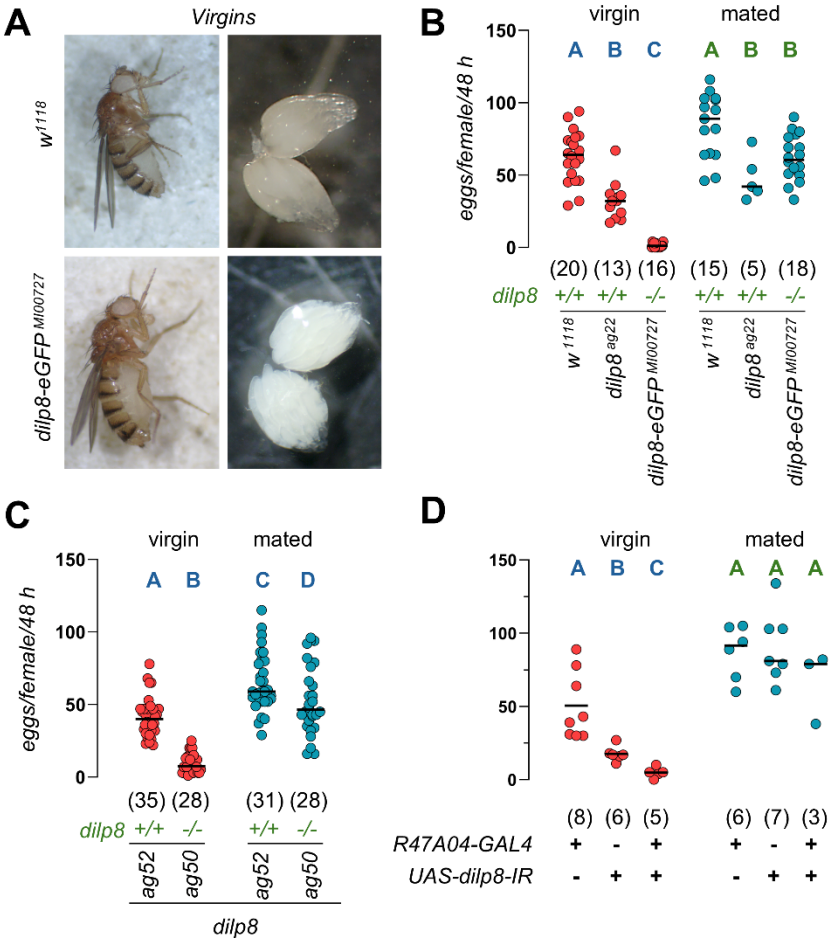


**Fig 2. Dilp8 is required in virgin females for ovulation**. **(A)** The abdomen of *dilp8* mutant flies was bloated due to the accumulation of eggs in the ovaries. **(B)** Virgin females lacking *dilp8* (*dilp8-eGFP*$^{MI00727}$) laid fewer eggs than $w^{1118}$ and *dilp8*$^{ag22}$ control flies. The effect was lost upon mating. Number of replicates, each with five individuals is indicated between parentheses. Same letter $P > 0.05$, ANOVA and Tukey's HSD test. **(C)** Virgin females carrying a null allele of *dilp8* (*dilp8*$^{ag50}$) laid fewer eggs than their background controls (*dilp8*$^{ag52}$). The effect size is reduced upon mating. Number of replicates, each with five individuals is indicated between parentheses. Same letter $P > 0.05$, ANOVA and Tukey's HSD test. **(D)** Dilp8 is required in follicle cells surrounding the mature oocyte to facilitate ovulation in virgin females. Knockdown of *dilp8* in follicle cells with the stage-14-specific driver reduces the number of eggs laid by virgin females but not mated females. Same letter, $P > 0.05$, Brown-Forsythe and Welch ANOVA and Dunnett's T3 test. Number of replicates, each with five individuals is indicated between parentheses.

174 mated females when comparing *dilp8-eGFP*$^{MI00727}$ with the *dilp8*$^{ag22}$ precise excision and partially rescued
175 relative to *w*$^{1118}$ controls (**Figure 2B**). This is in contrast to previous reports suggesting that mated (and
176 non-backcrossed) *dilp8-eGFP*$^{MI00727}$ mutant animals had reduced fecundity (Liao and Nässel 2020;
177 Haomiao Li et al. 2023), suggesting that this discrepancy could be an effect of heterogeneous backgrounds.
178 Using the amount of unfertilized eggs laid as virgins as a proxy, we found similar results as above with an
179 independent *dilp8* null allele, *dilp8*$^{ag50}$ (Heredia et al. 2021), when compared to its wild-type background
180 allele, *dilp8*$^{ag52}$ (**Figure 2C**), or with RNA interference (RNAi)-mediated knockdown of *dilp8* [*UAS-dilp8-*
181 *IR* (Dietzl et al. 2007; Perkins et al. 2015)] specifically in follicle cells (*R47A04>dilp8-IR*) (**Figure 2D**).
182 Again, even though a small significant effect on fecundity was detectable in mated *dilp8*$^{ag50}$ null alleles,
183 the effect size (21% reduction) was visibly smaller than that of virgins (81% reduction), and it was not
184 detectable in mated *R47A04>dilp8-IR* animals. We concluded that follicle cell-derived Dilp8 positively
185 influences the amount of unfertilized eggs laid in virgin females, and that mating renders this influence
186 largely redundant.

187 Dilp8 could increase unfertilized egg laying in many ways, such as by increasing the rate of oogenesis,
188 oocyte maturation, or ovulation. The data above likely rule out any critical role for Dilp8 exclusively
189 modulating the rates of oogenesis or oocyte maturation, as egg laying is largely unaffected in mated
190 animals, which implies oocytes in *dilp8* mutants are being generated and maturing at rates similar to those
191 in controls. To confirm this, we first followed unfertilized egg laying in well-fed virgin *dilp8*$^{ag50}$ mutants
192 and control (*dilp8*$^{ag52}$) animals for 10 days starting 5 days after eclosion in well-fed conditions. Results
193 showed that unfertilized egg laying was constantly lower (~3-5-fold) in *dilp8* mutant animals than in control
194 *dilp8*$^{ag52}$ animals throughout the experiment (**Figure 3A**). In contrast, when females were mated, no
195 difference in egg laying (fecundity) was observed between *dilp8* mutant and control females during the
196 same time-span (**Figure 3B**).

197 Dissecting the ovaries of virgin females at day 6-7 showed that the number of fully mature stage (14C)
198 follicles (Deady, Li, and Sun 2017; Beard, Oramas, and Sun 2023) in the ovaries of virgin *dilp8*$^{ag50}$ mutants
199 was ~3-fold higher than controls **(Figure 3C).** This came at the cost of earlier stage 14 follicles (14A/B),
200 but less so of follicles of other stages, which represented a similar fraction of the total follicles in *dilp8*
201 mutants and controls (**Figure 3D**). These results suggested that control animals were laying unfertilized
202 eggs at a faster rate than *dilp8* mutants, so that the stage 14 follicles did not spend enough time in the ovaries
203 of control animals to accumulate a sizable population of stage 14C follicles. In other words, ovulation is
204 slower in *dilp8* mutants.

205 To quantitatively test this assumption, we estimated ovulation time, oviduct oocyte transit time, and uterus
206 residency time in *dilp8* mutants and controls in virgin and mated conditions using a previously established
207 method (Beard, Oramas, and Sun 2023). Our results showed that control virgins ovulated on average once
208 each ~80 min. Oviduct and uterus residency times lasted together ~15 min on average (~20% of total

ovulation time), so that the ovarian ovulation process took ~65 min (~80% of total ovulation time) (**Figure 3E and Supplementary table I**). In mated animals, there was one ovulation each 50 min (a ~20% reduction in time compared to virgin animals), but oocytes stayed most of their time (~30 min) in transit in the oviducts or in the uterus, and only ~20 min in the ovary (**Figure 3E and Supplementary table I**). These results showed that the life-history of an ovulated oocyte within the reproductive system is profoundly affected by mating.

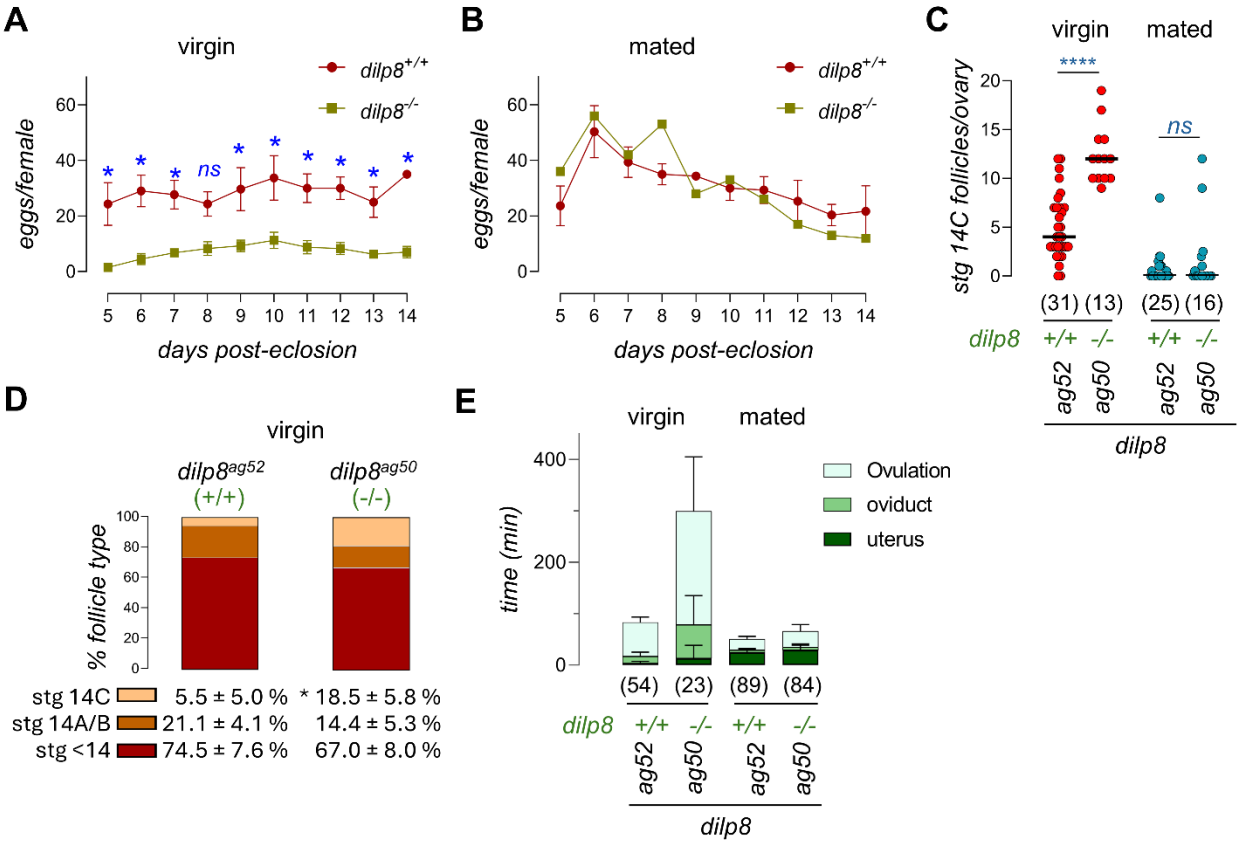


**Fig 3. Dilp8 regulates ovulation rate**. **(A)** Unfertilized egg laying evaluated for ten days is constantly lower in virgin *dilp8* mutant flies (*dilp8*$^{-/-}$ = *dilp8*$^{ag50}$) when compared with background control (*dilp8*$^{-/-}$ = *dilp8*$^{ag52}$). The difference was statistically significant for the time course of the evaluation, $P < 0.05$ (*), ANOVA and Bonferroni's multiple comparison test. Shown is the average ± SEM (error bars) of $N = 3$ repeats with five females each. **(B)** The effect of *dilp8* mutation on egg laying in virgin females is lost after mating, $P > 0.05$, ANOVA and Bonferroni's multiple comparison test. Shown is the average ± SEM (error bars) of $N = 3$ repeats with five females each. **(C)** The amount of fully mature follicles (stage 14C follicles) is increased in virgin females carrying a null allele of *dilp8* (*dilp8*$^{ag50}$). Upon mating, the number of mature follicles stored in the ovary is reduced in mutant and control flies. $P < 0.0001$ (****), ANOVA and Tukey's HSD test. *ns*, $P > 0.05$. Each dot represents an ovary. Number of ovaries is indicated between parentheses. **(D)** Proportion of fully mature (stage (stg) 14C), early-14 (stage 14A/B) and earlier stages (1 to 13) in the ovaries of control (*dilp8*$^{ag52}$) and *dilp8* mutant (*dilp8*$^{ag50}$) flies. The proportion of fully mature follicles is higher in mutant virgin flies. $P = 0.017$, Student's t–test with Bonferroni correction. $N = 8$ and 6 ovaries, for *dilp8*$^{ag52}$ and *dilp8*$^{ag50}$ flies, respectively. **(E)** Total ovulation time is increased in *dilp8* mutant virgin flies, but not in mated ones. The analysis of individual stages shows that ovulation time (lightest green) increases from 66.1 ± 10.3 to 221.2 ± 105.7 min ($P < 0.0005$, Z-score test) and uterus transit time (dark green) from 3.1 ± 4.2 to 13.0 ± 25.5 min ($P < 0.005$, Z-score test) in mutant flies compared to background controls of the depicted genotypes, while oviduct transit time (medium green) does not show a statistically significant difference. There was no statistically-significant difference in the duration of the three evaluated stages between mated mutants and controls ($P > 0.05$, Z-score tests; see also Supplementary Table I). Number of flies evaluated is indicated between parentheses.

215 In contrast to control virgins, *dilp8*$^{ag50}$ mutant virgins ovulated once every ~300 min, but their oocytes spent
216 a similar proportion of time in each compartment of the reproductive apparatus as controls (80% of the
217 time in the ovary and 20% of the time in the oviduct and uterus) (**Figure 3E and Supplementary Table**
218 **I**). Clearly, the whole ovulation process seems to be slower in *dilp8* virgins than in controls, including
219 ovarian ovulation, oviduct transit, and uterus residency, suggesting that Dilp8 modulates either a broad
220 process or multiple processes influencing ovulation and genital tract transit rates in virgins. In mated *dilp8*
221 mutants, however, although ovulation rate was also slightly slower, residency times were not statistically
222 significantly different from those in control mated females (**Figure 3E and Supplementary Table I**). This
223 is consistent with the fact that mating largely overrides the requirement for Dilp8 for fast ovulation rates.
224 Furthermore this is also consistent with the overall lack of correlation between genes associated with
225 oocyte/egg retention in virgin and mated females in the DGRP GWAS studies (Akhund-Zade et al. 2017;
226 Horváth and Kalinka 2018), suggesting that the two traits are under different genetic control. These results
227 strongly suggest that virgin females use Dilp8 signaling for efficient ovulation, transit, and extrusion of
228 unfertilized eggs from the uterus. This explains why *dilp8* mutant virgins lay less unfertilized eggs than
229 control animals and accumulate late stage oocytes in their ovaries.

230

231 *The Dilp8 receptor, Lgr3, is required non-autocrinally for ovulation and egg laying in virgins*

232 During larval and prepupal stages, Dilp8 acts via its neuronal receptor Lgr3, an ortholog of vertebrate
233 relaxin RXFP1/2 receptors (Garelli et al. 2015; Vallejo et al. 2015; Colombani et al. 2015; Jaszczak et al.
234 2016; Gontijo and Garelli 2018; Heredia et al. 2021). To test if Lgr3 is required for ovulation control in
235 adult female virgins, we profiled unfertilized egg laying in *Lgr3* null mutants (*Lgr3*$^{ag1}$) and their respective
236 background controls (*Lgr3*$^{ag2}$) (Garelli et al. 2015). Consistent with the results obtained for *dilp8* mutants,
237 *Lgr3* mutants also laid fewer unfertilized eggs as virgins than controls, and the effect was rescued by mating
238 (**Figure 4A**). Using *Lgr3-GAL4* lines to infer *Lgr3* expression, a previous report had suggested that *Lgr3*
239 is expressed at the distal posterior tip of the stage 14 follicles (Liao and Nässel 2020), a region which
240 overlays the aeropyle, a specialized structure of the eggshell involved in gas exchange (Margaritis, Kafatos,
241 and Petri 1980; Dobens and Raftery 2000). Here, using a translational *Lgr3* reporter (*superfolder GFP*
242 (*sfGFP*)::*Lgr3*, *sfGFP::Lgr3*$^{ag5}$*]*) (Garelli et al. 2015), we found no expression in this region. Instead, we
243 find similar patterns of endogenous autofluorescence of chorionic layers of the aeropyle in controls lacking
244 any transgene (*e.g., Lgr3*$^{ag11}$, a genetic background control) (Garelli et al. 2015) (**Supplementary Figure**
245 **2; see also Figure 1A, arrowheads**). We conclude that Lgr3 is not expressed at detectable levels in the
246 posterior tip follicle cells of mature follicles. Hence, it is likely that Dilp8 acts non-cell-autonomously to
247 regulate ovulation and ovulated egg transit in the genital tract.

To further test for an autocrine role of Dilp8 in stage 14 oocytes in the ovary, we verified if it regulated other genes associated with oocyte maturation. We compiled a list of genes with enriched expression in stage 14 follicles (**Supplementary Table II**) (Tootle et al. 2011), and performed quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assays with ovaries from virgins of *dilp8* mutants and controls of a subset of these genes (namely, *Oamb, CG15279, CG1698*, *CG3759,* and *CG6508*). Our results

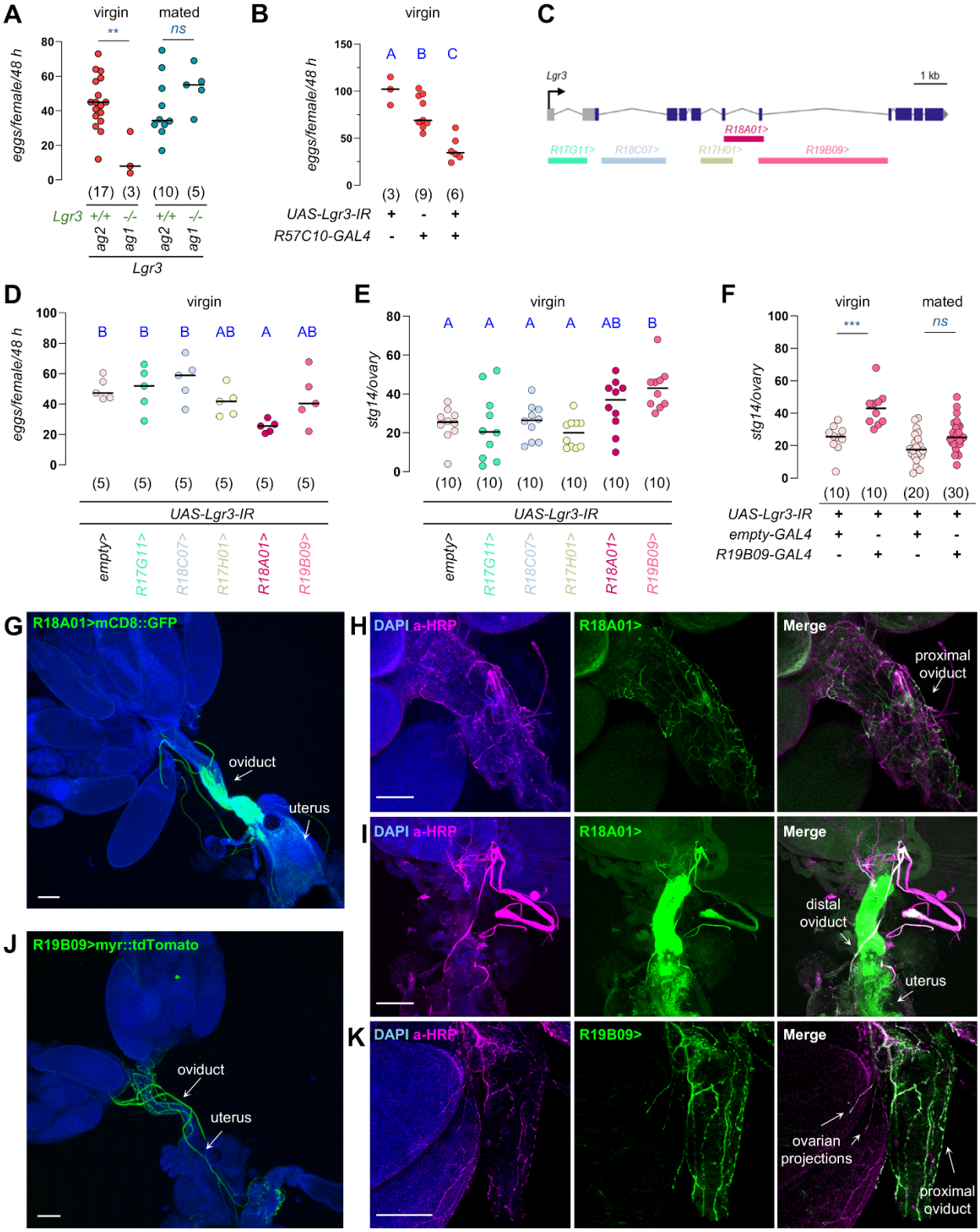

**Fig 4. Lgr3 is required for virgin ovulation and egg laying in at least two different neuronal populations.** **(A)** Virgin females lacking Lgr3 (*Lgr3*$^{ag1}$), the Dilp8 receptor, laid fewer eggs than their background controls (*Lgr3*$^{ag2}$). The effect was lost upon mating. Number of replicates, each with five individuals, is indicated between parentheses. (*) $P < 0.05$; *ns,* $P > 0.05$, ANOVA and Tukey's HSD test. **(B)** Panneuronal downregulation of *Lgr3* by RNAi reduces the number of eggs laid by virgin females. Number of replicates, each with five individuals, is indicated between parentheses. Same blue letter, $P > 0.0001$, ANOVA and Tukey's HSD test. **(C)** Schematic representation of the *Lgr3* locus depicting the regions spanned by the different *cis* regulatory motifs (CRMs) that drive GAL4 expression in *CRM-Lgr3-GAL4* lines. **(D)** Effect of the knockdown of *Lgr3* expression with individual *CRM-Lgr3-GAL4* lines on the number of eggs laid by virgin females. Silencing *Lgr3* in *R18A01-GAL4* expressing cells reduces the number of eggs laid. Same blue letter, $P > 0.05$, ANOVA and Tukey's HSD test. The number of replicates, each with five individuals, is indicated between parentheses. **(E)** Effect of the knockdown of *Lgr3* expression with individual *CRM-Lgr3-GAL4* lines on the number of mature oocytes stored in the ovaries of virgin females. Silencing *Lgr3* in *R19B09-GAL4* cells increases the number of mature follicles stored per ovary. Each dot represents one ovary. Total number of ovaries between parentheses. Same blue letter, $P > 0.05$, ANOVA and Tukey's HSD test. **(F)** Lgr3 *RNAi* with *R19B09-GAL4* increases the number of mature follicles in the ovary of virgin females, but not in mated females. The virgin data is the same as in panel E; reproduced for comparison. Same blue letter, $P > 0.05$, ANOVA and Tukey's HSD test. Each dot represents one ovary. Total number of ovaries between parentheses. (***) $P > 0.0005$, ANOVA and Tukey's HSD test. **(G-I)** Confocal Z-stack projections of the reproductive organs of *R18A01-GAL4 UAS-mCD8::GFP* and **(J-K)** *R19B09-GAL4 UAS-myr::tdTomato* female flies. Fluorescent reporter expression is false colored in green. Nuclei are stained with DAPI (blue) and neuronal projections with anti-HRP antibody (magenta). **(H)** *R18A01>mCD8::GFP* marks neurons innervating the proximal basis of the oviduct. **(I)** Expression of *R18A01>mCD8::GFP* in the epithelial cells of the distal portion of the oviduct, and in neurons that project to the lower reproductive tract. **(K)** Expression of *R19B09>myR::tdTomato* in neurons that innervate oviduct and ovarian base. Scale bar = 100 µm.

show that expression of none of these mature follicle marker genes was consistently affected by loss of *dilp8* (**Supplementary Figure 3**). These results suggest that *dilp8* does not consistently affect stage 14 follicle maturation and are consistent with a non-autocrine role of *dilp8* on ovulation processes via other *Lgr3*-positive cells.

*Lgr3 is required in different neuronal populations for ovulation and egg laying in virgins*

As mentioned above, Lgr3 is required in neurons in two instances where a clear function for Dilp8 has been demonstrated in growing larva and pupariating animals (Garelli et al. 2015; Vallejo et al. 2015; Colombani et al. 2015; Jaszczak et al. 2016; Heredia et al. 2021). To test if *Lgr3* is also required in neurons for ovulation control, we knocked-down *Lgr3* in neurons using RNAi and the panneuronal driver *R57C10-GAL4* (Pfeiffer et al. 2008). Results showed that *Lgr3* is required in neurons for proper laying of unfertilized eggs in virgins (**Figure 4B**). Although no function for endogenous *Lgr3* has been genetically or physiologically defined in adults, a subset of sexually-dimorphic *Lgr3*-positive neurons labelled by the *Lgr3 cis-regulatory motif R17H01-GAL4* (*R17H01>*) affects female receptivity and fecundity (Meissner et al. 2016), while another subset of brain *Lgr3* neurons labeled by the *Lgr3 cis-regulatory motif R19B09-GAL4* (*R19B09>*) affects sugar appetite in mated females (Laturney, Sterne, and Scott 2023). Furthermore, some neurons labeled by an *Lgr3-GAL4* line (Meissner et al. 2016) can innervate the proximal oviduct (Liao and Nässel 2020). Based on these findings it has been proposed that *Lgr3* has functions controlling female reproductive physiology

271 and metabolism. It is unclear, however, if the neuronal activities are mediated by or require *Lgr3* at all, and
272 if the neurons that control fecundity are the same as those innervating the proximal oviduct.

273 To gain insight into the subpopulation of neurons potentially receiving the follicular Dilp8 signal and thus
274 supposedly controlling ovulation and egg transit in the reproductive tract, we knocked-down *Lgr3* in
275 different *Lgr3*-positive populations using a well-defined panel of five *cis-regulatory motifs* from the *Lgr3*
276 locus (Garelli et al. 2015; Vallejo et al. 2015; Colombani et al. 2015; Jaszczak et al. 2016; Meissner et al.
277 2016; Heredia et al. 2021; Jenett et al. 2012; Zanini et al. 2024) (**Figure 4C**) and quantified unfertilized
278 egg laying and the amount of stage 14 follicles in the ovary in virgins. Knockdown of *Lgr3* in *R18A01-*
279 *GAL4* (*R18A01>*) cells (*R18A01>Lgr3-IR*) significantly affected the number of unfertilized eggs laid by
280 virgins (**Figure 4D**). Knockdown in *R17H01>* and *R19B09>* cells had a marginal or variable effect. *Lgr3*
281 knockdown in *R19B09>* cells led to a significant increase in the number of mature oocytes in the ovaries
282 of virgins, while knockdown in *R18A01>* cells had a marginal effect (**Figure 4E**). The *R19B09>Lgr3* effect
283 on oocyte retention was reduced by mating (**Figure 4F**). Importantly, proper ovulation does not require
284 *Lgr3* in *R17H01>* neurons, which had been shown to regulate fecundity when activated (Meissner et al.
285 2016), indicating that these neurons either regulate other Lgr3-dependent reproductive processes or do not
286 require *Lgr3* for that regulation. Collectively, our results show that Dilp8 acts on Lgr3 in at least two
287 subpopulations of neurons marked by the *R18A01>* the *R19B09>* lines to control ovulation and/or egg
288 transit in virgins. Why *Lgr3* in *R19B09>* neurons strongly affects oocyte retention, but only marginally
289 affects unfertilized egg laying in virgins remains to be defined, but it could suggest additional activities of
290 *Lgr3 R19B09>* neurons that promote oocyte production, apart from promoting ovulation. Regardless, these
291 experiments suggest that the effects Dilp8 has on ovulation and genital tract egg transit likely arise from its
292 collective activity on multiple neuronal populations. The expression patterns of these lines have not been
293 previously profiled in the female genital tract, although the *R19B09>* line has been previously shown to be
294 sexually-dimorphic in the central nervous system (Meissner et al. 2016).

295

296 *Neurons requiring Lgr3 to control ovulation and egg transit innervate the reproductive tract*

297 To verify the neuroanatomy of the Lgr3 neurons in the reproductive system, we drove membrane-tagged
298 reporters (*UAS-mCD8::GFP* or *UAS-myR::tdTomato*) in virgin and mated females and observed their
299 reproductive tracts with confocal microscopy. We found reporter expression in neurons with different
300 branching patterns for each driver in the female reproductive tract. *R18A01>mCD8::GFP* is weakly
301 expressed in neurons innervating the proximal basis of the oviduct (**Figure 4G, H**) and the lower
302 reproductive tract (a region that contains the uterus) (**Figure 4G, I**). *R18A01>* is also strongly expressed
303 in epithelial cells of oviduct, most prominently at its distal portion (**Figure 4G, I**). On the other hand,
304 *R19B09>myR::tdTomato* is strongly expressed in neurons innervating the whole oviduct, starting from the

base of the ovary down to the distal oviduct (**Figure 4J, K**). These results lead to the conclusion that *Lgr3* controls virgin ovulation and unfertilized egg laying from at least two different cell populations.

Although we cannot exclude that *Lgr3* expression in other cells or tissues contribute to ovulation and/or genital tract egg transit in virgins, *Lgr3* expression is undetectable in oviducts in different single-cell RNA sequencing data efforts (Hongjie Li et al. 2022; Thayer et al. 2024), whereas it is readily detectable in adult neurons by sequencing (Hongjie Li et al. 2022) or by *in situ* hybridization (Meissner et al. 2016). Considering that panneuronal *Lgr3* knockdown phenocopies *Lgr3* mutation (**Figure 4B)** and that both *R18A01>* and *R19B09>* are expressed in neurons innervating the genital tract, we conclude that Lgr3 is required in neurons to control ovulation and genital tract egg transit in virgins in response to follicle-cell derived Dilp8. Do both neuronal populations control both aspects of virgin egg laying? From the expression pattern and different activities of each group of cells, we favor the hypothesis that Lgr3 in *R19B09>* neurons controls ovulation and oviduct transit, whereas Lgr3 in *R18A01>* neurons controls ovulation, oviduct transit, and uterus extrusion, leading to a stronger unfertilized egg laying phenotype than *R19B09>*.

*Dilp8 indirectly promotes oocyte quality by facilitating ovulation of older oocytes*

What would be the function of promoting ovulation and unfertilized egg laying in virgins? *Drosophila* oogenesis is a relatively energy-intensive process and unfertilized egg laying is considered a pointless loss of reproductive potential (Akhund-Zade et al. 2017; Horváth and Kalinka 2018; Boulétreau-Merle and Fouillet 2002). However, this must be contrasted with the observations that prolific unfertilized egg laying (*i.e.,* short retention) phenotypes are selected for in warmer climates in wild populations and the potential for expressing such phenotypes remains present in cold-adapted populations via balancing selection (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002; Boulétreau-Merle, Fouillet, and Terrier 1992). Hence, it is likely that short ovulation times are subject to selective pressures, just like longer retention ones are subject to pressures for overwintering.

Similarly to human oocytes (te Velde and Pearson 2002) and those of the nematode *Caenorhabditis elegans* (Luo et al. 2010), older mature oocytes in *Drosophila* display reduced viability (hatchability), which degrades proportionally to the time the oocyte spends in retention in the ovary (Greenblatt and Spradling 2018; Greenblatt et al. 2019). This alone could provide a selective force for the Dilp8-Lgr3 pathway to promote ovulation in virgins: to frequently purge the ovary of older, less viable oocytes. However, previous data suggest that *D. melanogaster* oocytes do not lose viability in the first 5 days of retention, even though it is unclear if virgin egg laying was factored in these assays (Greenblatt and Spradling 2018; Greenblatt et al. 2019). Regardless, this would suggest that laying unfertilized oocytes before day 4-5–which is something short retention flies do copiously in the wild (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002; Boulétreau-Merle, Fouillet, and Terrier 1992)–makes very little sense, as it would not

promote any further egg viability upon mating, and instead just waste precious resource investment. Hence, we asked if Dilp8 has any additional role in promoting oocyte quality by assaying for further reduced egg viability (hatchability) in *dilp8* mutants when compared to controls in a paradigm of egg retention.

For this, we grew well-fed *dilp8*$^{ag50}$ mutants and their controls for 4 days in yeast-enriched food and then switched them to hardened agar apple-juice plates for 7 days. This regimen drastically reduces virgin egg-laying of controls to approximately one egg per week on average from day 4 on, leading to mature oocyte aging during the 7-day retention period. At day 11, these flies were mated and we scored the % of eggs that hatched into larvae (hatchability) as a readout of oocyte quality. We found that, as expected, *dilp8* mutants that retained oocytes for 7 days as virgins had 18.2 % of the hatchability of *dilp8* mutants that had been mating and feeding on yeast throughout the 7 days (**Figure 5A**). However, two days after mating, the difference was no longer observed, showing that the loss of oocyte quality is due to the prolonged retention of the older oocytes in the ovary. Forcing oocyte retention for 7 days in wild-type animals also led to reduced hatchability rates in our assay: to 47.5% of that of mated controls (**Figure 5B**), in line with previous reports (Greenblatt and Spradling 2018; Greenblatt et al. 2019). Although this was more than twice the hatchability rate than that of eggs from *dilp8* mutants which retained oocytes, the difference was not statistically significant, probably because of the low statistical power of the experimental design, with a smaller *N* of repetitions (3-4) than the number of conditions. Parallel assays comparing *dilp8-eGFP*$^{MI00727}$ homozygous mutants with their $w^{1118}$ controls gave similar results: a ~50% reduction of hatchability in *dilp8* mutants relative to controls, which did not reach statistical significance (**Figure 5C**). We concluded that oocyte quality is lost with time in both *dilp8* and WT mutant animals, and that there is a tendency for further loss in hatchability rates when *dilp8* is absent. These results could be explained in two ways: 1) if we posit that mature oocytes lose quality with time at an equal rate in *dilp8* mutant and control contexts, it is possible that, under our experimental setup, mature oocytes are slightly older in *dilp8* mutants when we measure their quality at day 11 because *dilp8* mutants start accumulating mature oocytes from day 0 due to their ovulation defect. On the other hand, controls spontaneously ovulate more oocytes up to day 4, when oocyte retention starts. Hence, control oocytes could be 3-4 days younger and thus have a higher quality than those of *dilp8* mutants. This would suggest that Dilp8 has no additional direct role in promoting oocyte quality than that of facilitating their elimination via virgin ovulation in normal conditions. Alternatively, 2) Dilp8 could have an independent role promoting oocyte quality, which involves some other role on ovarian physiology. Regardless, these results showed that Dilp8 promotes oocyte quality indirectly by facilitating the elimination of older oocytes. Further work is required to determine unequivocally if Dilp8 has an additional role in promoting oocyte quality.

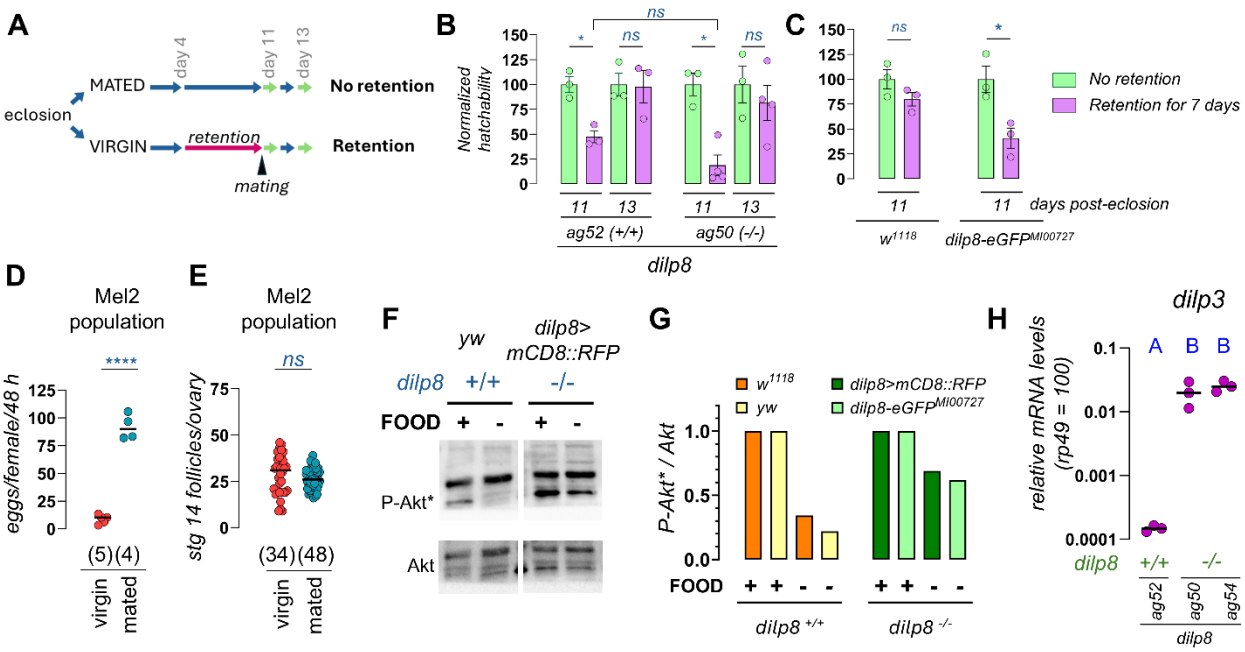


**Fig 5. Egg retention leads to reduced hatchability**. **(A)** Schematic representation of the protocol used to induce egg retention. Newly eclosed females of the "No retention" condition were mated and kept on food supplemented with yeast. Hatchability was evaluated on the eggs laid at days 11 and 13 after eclosion. Virgin females of the "Retention" condition were fed yeast for four days, then transferred to hard agar plates without yeast to induce egg retention. Seven days later, they were mated and fed yeast. Hatchability was evaluated on the eggs laid at days 11 and 13 after eclosion. Eggs laid at day 13 after eclosion are expected to be produced after mating and considered non-retained. **(B)** Egg viability is reduced after seven days of oocyte retention in the ovary of *dilp8* mutant flies (*dilp8*$^{ag50}$) and its background control (*dilp8*$^{ag52}$). Hatchability of newly generated oocytes is restored to control values two days later. Each dot represents an independent biological replicate with five flies. Asterisk: $P < 0.01$, Student's t-test with Bonferroni correction for multiple comparisons. ns, $P > 0.05$. **(C)** Egg viability after seven days of oocyte retention in the ovary of *dilp8* mutant flies (*dilp8-eGFP*$^{MI00727}$). The size effect in control $w^{1118}$ flies is smaller and the difference is not statistically significant from "No Retention" controls. Asterisk: $P < 0.025$, Student's t-test with Bonferroni correction. ns, $P > 0.05$. **(D)** Females from an outbred *D. melanogaster* population display two modes of ovulation; spontaneous laying of a small number of unfertilized eggs as virgins (virgin, red dots), and mating-induced egg laying (mated, blue dots). Number of replicates, each with five individuals, is indicated between parentheses. Asterisks $P < 0.0001$, unpaired Student's t-test. **(E)** Virgin females from an outbred population do not accumulate unfertilized oocytes in their ovaries. Depicted is the number of stage 14 follicles per ovary in virgin and mated females. This gives on average less than 2 stage 14 follicles per ovariole, considering ~20 ovarioles per ovary. The number of ovaries assayed is depicted in parentheses. ns, $P > 0.05$, unpaired Student's t-test. **(F)** Phosphorylation levels of Akt at serine 305 (P-Akt), specifically of a fast-migrating band (P-Akt*), are reduced upon 24 h starvation in the body (head and thorax) of wild type animals. The effect of starvation on P-Akt* is less prominent in *dilp8* mutant flies. Complete blots are shown in Supplementary figure 4. **(G)** Quantification of P-Akt* relative to total Akt in two different control and *dilp8* mutant genotypes. **(H)** Female virgins homozygous for different *dilp8* mutant alleles, *dilp8*$^{ag50}$ or *dilp8*$^{ag54}$, show increased levels of *dilp3* mRNA, as evaluated by qRT-PCR, compared to the background control *dilp8*$^{ag52}$. Each dot represents a biological replicate (5-day old female virgins, whole body). Same blue letter: $P > 0.05$, ANOVA and Tukey's HSD test.

373 *dilp8 mutants are in a short-retention background: implications for oogenesis and oocyte quality control*

374 Whereas using laboratory stocks, such as $w^{1118}$ "controls", allows us to better control for genetic
375 backgrounds, it is not clear how much of their ovulation profiles reflects the reality of wild fly populations
376 in our experimental settings and conditions. To get a better perspective of this, we profiled virgin and mated
377 egg-laying behavior in a previously-characterized outbred *D. melanogaster* population (Mel2) from

378 Portugal (temperate climate) (Martins et al. 2013; Faria and Sucena 2017) (see Methods). Our results
379 showed that well-fed virgins of the Mel2 population spontaneously laid 4.3 ± 2.0 (mean ± standard
380 deviation, $N = 5$ groups of 5 animals) unfertilized eggs per day (about 1 ovulation every 5-6 h), which rose
381 to 45.9 ± 5.7 eggs per day (1 ovulation every 0.5 h) upon mating (**Figure 5D)**. This result has many
382 implications. First, it confirms that in our settings, *D. melanogaster* has two modes of ovulation:
383 spontaneous and induced; and that in this representative outbred population, spontaneous ovulation is about
384 an order of magnitude less frequent than mating-induced ovulation, yet it is still highly robust and
385 quantifiable. Second, it brings further support to the idea that spontaneous ovulation is neither a negligible
386 process nor an artifact of laboratory stocks such as the $w^{1118}$ "controls", which have been kept under
387 laboratory conditions for decades. Instead, our results are in line with previous reports carried out with flies
388 recently collected from the wild or with their established populations (Boulétreau-Merle 1990; Boulétreau-
389 Merle and Fouillet 2002; Boulétreau-Merle, Fouillet, and Terrier 1992) and surveys of DGRP lines ($N$ =
390 90), all of which displayed virgin egg laying (Akhund-Zade et al. 2017; Horváth and Kalinka 2018;
391 Boulétreau-Merle and Fouillet 2002). Third, it shows that while Mel2 population virgins align more with
392 the long-retention phenotype, which is selected for in temperate climates (Boulétreau-Merle 1990;
393 Boulétreau-Merle and Fouillet 2002), the virgins of the various laboratory controls we profiled herein lay
394 20-30 unfertilized eggs per day (**Figures 2B-D, 3A, 4A-B, D**), which is a rate ~5-6 times higher than that
395 of Mel2 population virgins. This suggests that our lab stocks are more representative of short retention
396 types, which start laying unfertilized eggs more quickly and more plentifully than flies with long retention
397 phenotypes (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002). This genetic background
398 obviously (and serendipitously) facilitated the detection of a role for the Dilp8-Lgr3 pathway in promoting
399 ovulation and egg transit through the genital tract.

400 Fourth, virgin and mated females of the Mel2 population accumulated stage 14 oocytes in their ovaries to
401 a similar extent [28.5 ± 10.7 (virgins, $N = 34$ ovaries) vs. 26.7 ± 5.7 (mated, $N = 48$ ovaries), $P > 0.05$,
402 Student's t-test] (**Figure 5E)**. This indicates that despite laying even fewer unfertilized eggs per day than
403 virgin *dilp8* mutants (which lay ~5-10 unfertilized eggs per day), virgins of the Mel2 population do not
404 accumulate more than 1-2 stage 14 follicles per ovariole (considering ~20 ovarioles per ovary [as a
405 reference, the DGRP lines have 19.4 ± 2.84 average ± standard deviation ovarioles per ovary, $N = 200$ lines
406 (Lobell et al. 2017)]). This is coherent with what has been reported for control backgrounds of other
407 genotypes and laboratories (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002; King and Sang
408 1959; Bloch Qazi, Heifetz, and Wolfner 2003; Richards 2007; Greenblatt and Spradling 2018; Greenblatt
409 et al. 2019). In fact, long retention virgin females, such as Mel2 population females, do not accumulate
410 stage 14 follicles beyond 2 per ovariole because they abruptly reduce new follicle production and
411 vitellogenesis via increased resorptions (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002).
412 This suggests the existence of an oogenesis progression checkpoint that is sensitive to the amount of stage
413 14 follicles per ovariole. On the opposite hand, virgins with short retention phenotypes lay copious amounts

414 of unfertilized eggs and do not reach the "2 mature oocyte/ovariole" threshold in normal conditions,
415 showing continuously higher follicle generation and vitellogenesis without oocyte accumulation. In fact,
416 as the threshold is not easily reached in short-retention virgins, it is not clear whether or not they have a
417 competent checkpoint or not to begin with. As the *dilp8* mutant alleles we are studying herein are in an
418 apparent short-retention background ($w^{1118}$), this could suggest either that we only detect oocyte
419 accumulation in *dilp8* mutants in this background because the checkpoint for halting stage 14 follicle
420 accumulation is defective, or that virgins with short-retention phenotypes have an effective checkpoint and
421 *would* arrest oogenesis *if* they did not ovulate copious amounts of oocytes. In the latter case, short-retention
422 virgins would require Dilp8 to ensure the checkpoint, since they fail to arrest oogenesis when *dilp8* is
423 absent. This interpretation aligns with the second alternative presented at the end of the previous section:
424 that Dilp8 could have an independent role promoting oocyte quality, which involves some other role on
425 ovarian physiology.

426

427 *Evidence that the Dilp8-Lgr3 pathway antagonizes insulin/IGF-like signaling to block vitellogenesis*

428 The negative feedback by which long-retention animals halt new mature stage 14 follicle production kicks
429 in after they accumulate ~2 stage 14 follicles per ovary (Boulétreau-Merle 1990; Boulétreau-Merle and
430 Fouillet 2002), strongly suggesting that it responds to the amount of stage 14 follicles in the ovary. As
431 Dilp8 is a secreted factor expressed specifically in this follicle stage in the ovary, we hypothesized it
432 participates in signaling the negative feedback. This negative feedback pathway is reminiscent of the
433 nutrition-dependent checkpoint, which prevents development of vitellogenic oocytes beyond stage 8/9 in
434 the absence of protein-rich food sources (Greenblatt and Spradling 2018; Greenblatt et al. 2019; Richard et
435 al. 2005; Drummond-Barbosa and Spradling 2001) and the gating mechanism that limits egg production
436 while conditions are suboptimal in stressful conditions (Meiselman et al. 2017; Meiselman et al. 2018).
437 These likely correspond to the same checkpoint which is controlled at multiple levels, including by mating
438 (Soller et al. 1999). Indeed, oogenesis progression is subject to extensive endocrine control by the
439 insulin/insulin-like growth factor (hereafter, insulin), ecdysone, ecdysis triggering hormone, and juvenile
440 hormone signaling pathways (Soller et al. 1999; Drummond-Barbosa and Spradling 2001; Richard et al.
441 2005; Liu et al. 2008; Grutenko et al. 2010; Meiselman et al. 2017; Meiselman et al. 2018; Greenblatt and
442 Spradling 2018; Greenblatt et al. 2019). The balance between ecdysone and juvenile hormone, mediated
443 by ecdysis triggering hormone, dictates whether oogenesis progresses or not, with juvenile hormone likely
444 sitting at the bottom of the cascade (Liu et al. 2008; Grutenko et al. 2010; Meiselman et al. 2017; Meiselman
445 et al. 2018). The nutrient-sensitive insulin signaling pathway can modulate ecdysone and juvenile hormone
446 levels and also play an independent role in controlling the vitellogenic stage checkpoint, placing it upstream
447 and in parallel to the ecdysone-ecdysis triggering hormone-juvenile hormone endocrine axis (Greenblatt
448 and Spradling 2018; Greenblatt et al. 2019; Richard et al. 2005; Drummond-Barbosa and Spradling 2001).

449 Here, we hypothesized that the Dilp8-Lgr3 pathway, beyond promoting ovulation and egg transit through
450 the reproductive tract, also negatively affects insulin signaling, participating in ensuring the checkpoint
451 when late stage 14 oocytes accumulate in the ovary. This would mean that insulin signaling would be
452 somehow increased in *dilp8* mutants. To test this, we first assayed for a systemic increase in insulin
453 signaling in the body of *dilp8* mutants relative to control animals using an antibody against phosphorylated
454 Akt (pAkt), which increases with insulin signaling (Burgering and Coffer 1995; Franke et al. 1995; Scanga
455 et al. 2000). Control animals starved in 2% agar-water plates had a visible reduction in pAkt levels relative
456 to well-fed animals (kept on yeast-food), as expected, showing that our assays were reliably detecting
457 insulin signaling (**Figure 5F**). While yeast-fed *dilp8* mutants had similar levels of pAkt as controls, two
458 different *dilp8* mutants showed a clear persistent enrichment of pAkt during starvation relative to control
459 levels, suggesting that systemic insulin signaling is likely active in *dilp8* mutants (**Figure 5F,G**).

460 A major source of insulin-like peptides that convey systemic insulin signaling in adults are the *pars*
461 *intercerebralis* neurosecretory cells (insulin producing cells), which express different *dilp*s (*e.g., dilp2,*
462 *dilp3,* and *dilp5*) (Broughton et al. 2005; Cao and Brown 2001; Belgacem and Martin 2002). A previous
463 report had shown that ectopic Dilp8 expression in Dimmed-positive secretory neurons led to a reduction in
464 the level of Drosophila insulin-like peptide 3 (Dilp3) levels in insulin producing cells of the brain of adults
465 of unspecified sex and mating status (Liao and Nässel 2020). Reduced levels of Dilps and other peptides in
466 secretory cells could reflect either reduced production or increased secretion of the peptides [*e.g.*,
467 (Géminard, Rulifson, and Léopold 2009)]. A hint that Dilp3 production might have been reduced at the
468 transcript level comes from RNA-sequencing experiments done with 3-day old virgins, which revealed a
469 small (2.6-fold), yet statistically significant, upregulation of *dilp3* mRNA levels in non-backcrossed *dilp8-*
470 *eGFP*$^{MI00727}$ mutants relative to $w^{1118}$ animals (Haomiao Li et al. 2023). To confirm that endogenous Dilp8
471 signaling negatively regulated *dilp3* expression in virgin animals in a more controlled and homogeneous
472 background, we performed qRT-PCR assays of whole virgin females from our previously published *dilp8*
473 allele series (Garelli et al. 2015; Vallejo et al. 2015; Colombani et al. 2015; Jaszczak et al. 2016; Heredia
474 et al. 2021; Fernandez-Acosta et al. 2025) and found that *dilp3* mRNA is upregulated by a factor of 100-
475 200 fold in two independent *dilp8* mutant alleles (*dilp8*$^{ag50}$ and *dilp8*$^{ag54}$ relative to their wild-type *dilp8*$^{ag52}$
476 controls (**Figure 5H)**. These results suggest that the Dilp8-Lgr3 pathway could negatively affect systemic
477 insulin signaling in virgins via *dilp3*. We hypothesize that this ectopic insulin signaling promotes
478 vitellogenesis checkpoint bypass. This would lead to a model where oocyte quality is also negatively
479 affected in *dilp8* mutants because late stage 14 follicles could be generated in suboptimal nutritional
480 conditions due to deregulated (nutrient-independent) insulin signaling.

481

482 **DISCUSSION**

*The Dilp8-Lgr3 pathway promotes ovulation and unfertilized egg transit through the genital tract in virgins*

A role for Dilp8 in controlling ovulation, fecundity, and metabolism in mated females had been previously proposed based on RNAi studies and a hypomorphic mutation (Liao and Nässel 2020; Haomiao Li et al. 2023). Here, we show that the Dilp8-Lgr3 relaxin-like pathway indeed modulates ovulation and genital tract egg transit, but also oocyte quality, and it does so more clearly, or in some contexts exclusively, in virgin females. We show that these roles are less visible and consistent in mated animals, suggesting that mating provides redundant inputs that largely override the requirement of the Dilp8-Lgr3 pathway. It is likely that sperm derived molecules, such as ovulin, and/or female spermathecal secretory cell factors participate in this bypass of Dilp8-Lgr3 (Heifetz et al. 2000; Bloch Qazi, Heifetz, and Wolfner 2003; Schnakenberg, Matias, and Siegal 2011; Sun and Spradling 2013; Rubinstein and Wolfner 2013).

Dilp8 is strongly upregulated in the very last stage, stage 14, of follicle maturation, which immediately precedes ovulation. Dilp8 was proposed to act autocrinally on cells at the posterior tip of the stage 14 follicle, which supposedly expressed the Lgr3 receptor, based on the expression of fluorescent reporters driven by Lgr3 regulatory regions (Liao and Nässel 2020). In contrast, we show that the posterior tip of stage 14 follicles is strongly autofluorescent due to the underlying chorionic structure, called aeropyle, and find no additional detectable expression of Lgr3 using an extensively used endogenously-tagged Lgr3 protein reporter construct (Garelli et al. 2015; Heredia et al. 2021; Fernandez-Acosta et al. 2025). Additional evidence that Dilp8 has no apparent autocrine role on stage 14 follicle maturation *per se*, was suggested by our expression profiling of *dilp8* mutant ovaries, which showed a lack of any effect on the expression of oocyte maturation genes, and also by our observation that *dilp8* mutants had no major egg laying defects when mated, suggesting that ovary dynamics proceeds similarly to wild-type animals in the absence of *dilp8* in mated animals. These results suggested that Dilp8 must act in a paracrine manner in other nearby tissues, rather than in an autocrine manner on the follicle cells themselves. Consistently, we showed that Lgr3 is required in neurons, more specifically in neurons marked by *R18A01* and *R19B09 cis-regulatory motifs*, which do not seem to coincide in their expression patterns, indicating that Lgr3 is required in at least two different neuronal populations for proper reproductive physiology. Whereas *R19B09* is expressed in oviduct-innervating neurons, *R18A01* is additionally expressed in neurons innervating the lower reproductive tract, including the uterus. This could suggest that Dilp8 acts as an endocrine signal on such relatively more distant neurons.

A conceptually important finding is that Dilp8 is secreted from stage 14 follicle cells when they still reside in the ovary. An alternative scenario would have been that it is only secreted upon ovulation. This observation by itself has major implications on our model of how the Dilp8-Lgr3 pathway influences female reproductive physiology. We still do not know where Dilp8 is secreted to, but we can make informed assumptions. Dilp8 could be secreted basally into the ovariole/oviduct space and/or apically towards the oocyte (peri-oocytic space). We can likely exclude the possibility that the latter occurs exclusively, because

518 in this case peri-oocytic Dilp8 would only reach Lgr3-positive neurons following follicle rupture at
519 ovulation, making a role for Dilp8 and Lgr3 in promoting ovulation after it occurs more difficult to explain.
520 Instead, we favor a model where Dilp8 is also or exclusively secreted basally/basolaterally into the
521 ovariole/oviduct space, after which it could easily reach the various Lgr3-positive neurons that innervate
522 the reproductive tract, such as those around the base of ovary and oviduct, or even reach the hemolymph
523 and act systemically.

524 The neuroanatomy of the female reproductive tract innervation has been previously described (Deshpande
525 et al. 2022; Demerec 1994; Maria Monastirioti 2003; Rodríguez-Valentín et al. 2006; Pauls et al. 2018;
526 Middleton et al. 2006; Cole et al. 2005; M. Monastirioti, Linn, and White 1996). These neurons originate
527 from the abdominal ganglia of the VNC and reach the reproductive tract via the median abdominal nerve,
528 which emits two bilateral branches towards the oviducts and uterus. The first branch innervates the ovary
529 and oviduct, while the latter innervates the uterus. A cluster of eight to ten octopaminergic neurons of the
530 abdominal ganglion innervate the base of the ovary (the only fibers known to innervate the ovary), oviduct,
531 and the uterus, forming modulatory Type II synapses (Middleton et al. 2006; Pauls et al. 2018), whereas
532 other non-octopaminergic neurons innervate the oviduct and uterus, forming Type I neuromuscular
533 boutons, which are likely glutamatergic. The neuroanatomical properties of the *Lgr3* neurons we describe
534 herein are consistent with *R19B09>* neurons being of the octopaminergic-type, forming octopaminergic
535 Type II boutons, whereas *R18A01>* neurons are likely of the glutamatergic type, forming Type I
536 connections. This is further supported by the fact that both *R19B09>* and *R18A01>* drivers are expressed
537 in the abdominal ganglia, most notably and strongly *R19B09>* (Pfeiffer et al. 2008; Jenett et al. 2012)
538 (**Supplementary Figure 5**). The ovary and oviduct octopaminergic neurons (*R19B09>* equivalent) have
539 been proposed to contract the ovary and relax the oviduct (Deshpande et al. 2022; Demerec 1994; Maria
540 Monastirioti 2003; Rodríguez-Valentín et al. 2006; Pauls et al. 2018; Middleton et al. 2006), thus promoting
541 ovulation and egg transit, whereas the non-octopaminergic (likely glutamatergic) neurons (*R18A01>*
542 equivalent), have been proposed to promote contractions of the oviduct (following relaxation), also
543 promoting egg transit (Deshpande et al. 2022). Interestingly, these oviduct-innervating non-
544 octopaminergic, glutamatergic neurons are likely responsive to octopamine, as they express an octopamine
545 receptor (Deshpande et al. 2022). These known activities align well with the functions we defined for Dilp8
546 and for Lgr3 in these neurons, which is to facilitate ovulation and egg laying in virgins. Hence Lgr3 is
547 likely to act synergistically with octopamine, either modulating its release, stability, or modulating the
548 responsiveness of underlying octopamine receptor expressing cells.

549 A similar role has been proposed for ecdysis triggering hormone, which is secreted from trachea-associated
550 endocrine cells (INKA cells) in optimal growth conditions, acting on octopaminergic neurons to promote
551 ovulation (Meiselman et al 2017; Meiselman et al 2018). In stressful conditions (starvation, heat stress) its
552 secretion is inhibited by ecdysone signaling, reducing octopamine-dependent ovulation levels. It will be

553 interesting to study the interaction between the Dilp8-Lgr3 signaling pathway and the ecdysis triggering
554 hormone pathway and how these signals, which originate from distinct tissues (ovary and INKA cells,
555 respectively), might integrate different endocrine readouts (environmentally-favorable conditions: high
556 insulin and low ecdysone levels) with differentiation signals (mature oocyte-derived Dilp8) to promote
557 ovulation upstream of octopamine.

558

559 *The Dilp8-Lgr3 pathway ensures optimal oocyte quality by two possible mechanisms*

560 The promotion of spontaneous ovulation by the Dilp8-Lgr3 pathway in virgin females increases average
561 oocyte quality by discharging older oocytes which have lower hatchability rates. This could be enough to
562 justify selection of short-ovulation phenotypes in warmer climates and seasons (Boulétreau-Merle 1990;
563 Boulétreau-Merle and Fouillet 2002). However, the accumulation of extra mature oocytes, beyond the
564 typical maximum 2 per ovariole of wild-type animals, in animals with reduced *dilp8* or *Lgr3* function
565 suggested that the Dilp8-Lgr3 pathway could regulate an additional aspect of reproductive biology
566 upstream of ovulation. Specifically, that Dilp8 would participate in the oogenesis checkpoint that
567 antagonizes further investment in mature oocyte development once 2 mature oocytes per ovariole are
568 developed. The expression of Dilp8 in the last follicle-development stage and the fact that it is secreted
569 from these follicles places it as the perfect quantitative readout of the amount of mature oocytes per
570 ovariole. This suggests that there is a threshold system quantitatively responding to Dilp8 levels, and that
571 Dilp8 could help maintain the balance between oogenesis and ovulation rates.

572 We present evidence that Dilp8 antagonizes Akt phosphorylation in the female body and *dilp3* mRNA
573 production, both activities which place it upstream of a systemic signaling system, the insulin signaling
574 pathway, that is known to participate in a nutritionally- and stress-sensitive checkpoint that promotes
575 vitellogenesis progression only when nutritional conditions are optimal (Drummond-Barbosa and
576 Spradling 2001; Richard et al. 2005; Grutenko et al. 2010; Meiselman et al. 2017; Meiselman et al. 2018;
577 Greenblatt and Spradling 2018; Greenblatt et al. 2019). Critically, this provides a second mechanism by
578 which the Dilp8-Lgr3 pathway could ensure optimal oocyte quality: by limiting mature oocyte production
579 to situations with optimal nutrient availability. In the absence of Dilp8-Lgr3 signaling, vitellogenesis could
580 be more easily bypassed, leading to the production of lower quality oocytes.

581 It remains to be defined if this negative effect on insulin signaling is mediated by follicle cell-derived Dilp8
582 or not, and through which population of Lgr3 neurons (or other non-neuornal cells) it is conveyed.
583 Nevertheless, clues of how this pathway could work come from similarities of the ovarian phenotype of
584 *dilp8* mutants (**Figure 1A**) with those of mutants partially deficient in the octopamine pathway: a drastic
585 accumulation of >2 stage 14 oocytes per ovariole that is primarily attributed to ovulation defects
586 (Deshpande et al. 2022; Demerec 1994; Maria Monastirioti 2003; Rodríguez-Valentín et al. 2006; Pauls et

al. 2018; Middleton et al. 2006; Cole et al. 2005; M. Monastirioti, Linn, and White 1996). Since Dilp8 acts on Lgr3 neurons, a subset of which are certainly octopaminergic neurons [based on the fact that the single fibers that innervate the ovary are octopaminergic (Deshpande et al. 2022; Demerec 1994; Maria Monastirioti 2003; Rodríguez-Valentín et al. 2006; Pauls et al. 2018; Middleton et al. 2006; Cole et al. 2005; M. Monastirioti, Linn, and White 1996)], the simplest model that explains our findings is that the Dilp8-Lgr3 pathway achieves its adult activities by modulating octopamine signaling (synergistically) (**Figure 6**). This would imply that octopamine also inhibits the insulin pathway, which is in fact largely supported by the literature in *Drosophila* and *C. elegans* (Crocker et al. 2010; Damrau et al. 2017; Y. Li et al. 2016; Tao et al. 2016), and leads to the prediction that if ovulation could be rescued in octopamine mutants, their oocytes would also be of reduced quality.

Curiously, the dual control of ovulation and oogenesis by the Dilp8-Lgr3 pathway draws further parallel between this and the ecdysis triggering hormone pathway, which also affects both processes (Meiselman et al. 2018). However, whereas ecdysis triggering hormone controls ovulation via octopamine, as we propose Dilp8-Lgr3 also does, ecdysis triggering hormone promotes oogenesis progression via the juvenile hormone, by acting directly on the corpora allata, the juvenile hormone-producing organ. Females with ablated INKA cells, hence, with no detectable *ecdysis triggering hormone* mRNA production, accumulate

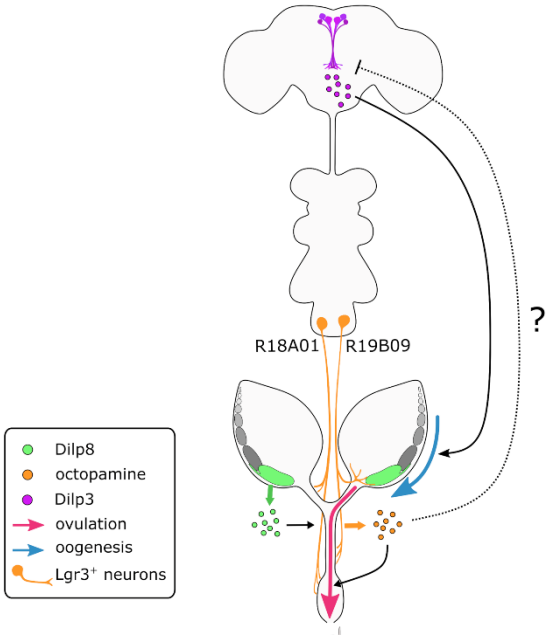


**Figure 6. Model for the role of the Dilp8-Lgr3 signaling pathway in reproductive physiology control.** Dilp8 peptide (green), produced and continuously secreted from follicle cells surrounding fully mature follicles, acts on two different populations of Lgr3-expressing neurons (orange neurons) that innervate the lateral oviduct and uterus (R18A01), and the base of the ovary and the lateral and common oviducts (R19B09), facilitating ovulation and egg transit through the reproductive tract (large red arrow), and oogenesis progression in virgin females (blue arrow). Dilp8 inhibits *dilp3* transcription in a still uncharacterized way (dashed line), which might involve release of octopamine (orange arrow, orange dots) from octopaminergic Lgr3+ neurons (R19B09, right orange neurons). Dilp3 peptide, likely secreted from insulin producing cells in the brain (magenta neurons), is hypothesized to facilitate oogenesis progression. The requirement of Dilp8 for fast ovulation is rendered largely redundant upon mating.

late stage oocytes due to their ovulation defect, but not more than 2 mature oocytes per ovariole, even when ectopic juvenile hormone is provided (Meiselman et al. 2018). This suggests that part or all of the mature-oocyte-number-sensitive checkpoint is still active in the absence of ecdysis triggering hormone and that juvenile hormone alone is not sufficient to bypass the checkpoint. This would be consistent with the fact that these animals would still have the Dilp8-Lgr3 signaling pathway active and that these pathways might have distinct and complementary roles.

Virgin egg laying is completely inhibited by increasing the stiffness of the egg laying substrate. Clearly, this manipulation overrides the cues for spontaneous ovulation in control animals, showing that spontaneous ovulation in virgins is also under environmental (sensorial) control, likely upstream of the Dilp8-Lgr3 pathway. It would be interesting to determine at which level this regulation occurs. Determining the precise nature of the Lgr3 neurons controlling ovulation and genital tract egg transit will surely contribute to the understanding of these processes. On the other hand, our work suggests that genes and regulatory regions required for Dilp8-Lgr3 pathway activity in the reproductive system would be good candidates for loss-of-function variation that promotes long oocyte retention phenotypes, associated with cold adaptation and overwintering.

In conclusion we have revealed new roles for the Dilp8-Lgr3 relaxin signaling in female reproductive physiology. Our profiling of a wild population and elegant previous work on virgin egg retention in wild populations (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002) suggests our discoveries were likely only possible because–or were largely facilitated by the fact that–laboratory stocks show mostly short-retention behavior as virgins. This way we could reveal the pro-ovulatory activity of the Dilp8-Lgr3 pathway. The fact that the Dilp8-Lgr3 pathway is modulatory and not absolute, like the octopamine pathway, also allowed the determination of downstream activities on facilitating unfertilized egg transit through the ovulatory tract, which would not have been trivial to determine if oocytes remained trapped in the ovary.

The relaxin pathway (Bathgate et al. 2013) has been historically associated with female reproduction since it was first demonstrated to promote relaxation of the interpubic ligament in pregnant guinea pigs and rabbits (Hisaw 1926) and to be produced by the corpus luteum (Fevold, Hisaw, and Meyer 1930). It has become clear that the relaxin pathway was present in the last bilaterian ancestor (Mita et al. 2009; Gontijo and Garelli 2018; Feng et al. 2025), and that it has evolved multiple functions that go beyond female reproduction, including functions on tissue remodelling, multi-tissue physiology regulation, behavior, stress regulation, and development (Bathgate et al. 2013; Gontijo and Garelli 2018; Imambocus et al. 2022; Heredia et al. 2021), making relaxins potential therapeutic targets for multiple diseases (Bathgate et al. 2018). Although relaxins have mostly been associated with late pregnancy events in vertebrates [reviewed in (Klein 2016)], relaxin is also expressed in follicles and other ovarian cells in many vertebrates, including during the nonpregnant oestrus cycles, and a direct role in promoting ovulation is clear in horses and rats,

where relaxin induces the secretion of enzymes that stimulate follicle cell rupture [reviewed in (Klein 2016)]. A role for relaxin signaling in ovulation/spawning control was also recently demonstrated in starfish (Feng et al. 2025). Our study in *Drosophila*, a protostome, therefore allows for the conclusion that the modulation of ovulation is an ancestral role of the relaxin pathway in bilaterians.

## MATERIALS AND METHODS

### *Drosophila* husbandry and stocks

The following stocks were used in this study:

*w*; P{UAS-shi$^{ts}$1.K}3* was a gift from Rita Teodoro. The *Lgr3$^{ag1}$* mutant, the *sfGFP::Lgr3$^{ag5}$* reporter, and *Lgr3$^{ag2}$* and *Lgr3$^{ag11}$* background controls were previously described (Heredia et al. 2015). The *dilp8$^{ag50}$* and *dilp8$^{ag54}$* mutant alleles and their background control *dilp8$^{ag52}$* were previously described (Heredia et al. 2021). *UAS-dilp8* is *UASt-dilp8::3xFLAG*, and was previously described (Garelli et al. 2012). *P{UAS-dpp.sGFP} (UAS-sGFP)* was a gift from Pierre Leopold (Entchev, Schwabedissen, and González-Gaitán 2000). *empty-GAL4* is *pBDP[GAL4] attp2 (III)* and was a gift from Carlos Ribeiro. Our *w$^{1118}$* stock can be tracked back to the Dominguez lab, Alicante, Spain. *UAS-Lgr3-IR* is *y$^{1}$ sc$^{*}$ v$^{1}$ sev$^{21}$; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.GL01056}attP2* (BL36887) and was obtained from the Bloomington Drosophila Stock Center, Indiana University Bloomington (BDSC; BL, stock number). *dilp8-1-GAL4* is *y* w*; P{GawB}NP0007* and *dilp8-2-GAL4* is *w*; P{w$^{+mW}$.hs=GawB}NP0778* and were obtained from the Kyoto Drosophila Stock Center. *dilp8-1-GAL4* was recombined with *w$^{*}$; P{y$^{+t7.7}$ w$^{+mC}$=10XUAS-IVS-mCD8::RFP}attP40* (BL32219) or *UAS-dilp8* and then backcrossed to *w$^{1118}$* to get *w$^{1118}$; dilp8-1>mCD8:RFP* and *w$^{1118}$; dilp8-1-GAL4 UAS-dilp8*, respectively, as described below. *y$^{1}$ w$^{*}$; Mi{MIC}Ilp8$^{MI00727}$* (BL33079) was backcrossed to *w$^{1118}$* to get *w$^{1118}$; dilp8-1-GAL4 UAS-dilp8*, as described below. *UAS-dilp8-IR* is a recombinant of *y$^{1}$ v$^{1}$; P{y$^{+t7.7}$ v$^{+t1.8}$=TRiP.HMS06016}attP40* (BL36726) with *P{KK112161}VIE-260B (v102604)*, obtained from the Vienna Drosophila Resource Center, respectively.

The following stocks were also obtained from the BDSC:

- *w$^{1118}$; P{y$^{+t7.7}$ w$^{+mC}$ =GMR47A04-GAL4}attP2* (BL50286)
- *w$^{1118}$; P{y$^{+t7.7}$ w$^{+mC}$=GMR57C10-GAL4}attP2* (BL39171)
- *w$^{1118}$; P{y$^{+t7.7}$ w$^{+mC}$=GMR19B09-GAL4}attP2* (BL48840)
- *w$^{1118}$; P{y$^{+t7.7}$ w$^{+mC}$ =GMR17G11-GAL4}attP2* (BL49275)
- *w$^{1118}$; P{y$^{+t7.7}$ w$^{+mC}$=GMR18C07-GAL4}attP2* (BL48806)
- *w$^{1118}$; P{y$^{+t7.7}$ w$^{+mC}$=GMR18A01-GAL4}attP2* (BL48791)
- *w$^{1118}$; P{y$^{+t7.7}$ w$^{+mC}$=GMR17H01-GAL4}attP2* (BL48786)
- *y$^{1}$ w$^{*}$; P{w$^{+mC}$=UAS-mCD8.mRFP.LG}10b* (BL27399)
- *w$^{*}$; P{y$^{+t7.7}$ w$^{+mC}$=10XUAS-IVS-myr::tdTomato}attP40* (BL32222)
- *w$^{1}$; P{y$^{+t7.7}$ w$^{+mC}$=10XUAS-IVS-myr::GFP}attP40* (BL32198)
- *y$^{1}$ w$^{67c23}$* (BL6599)

673

674 Stocks are maintained at low densities at 18 °C in a 12-h light/dark cycle. All experiments were done at 25
675 ºC in a 12-h light/dark cycle, or at a different temperature when specified.

676 Backcrossed stocks had their 1st and 2nd chromosomes substituted for those of the *w[1118]* stock using
677 directional crossing and balancer chromosomes (CyO), while the 3rd chromosome was backcrossed 5x in
678 total.

679 An outbred *D. melanogaster* population (Mel2) was obtained from the Sucena lab (Martins et al. 2013;
680 Faria and Sucena 2017). The population is maintained in laboratory cages at a census of 1500-2000
681 individuals, under constant temperature (25°C) and humidity (60-70%) with a 12-h light/dark cycle. All
682 experimental flies were generated from egg lays with controlled density.

683 Food: Animals are fed with cornmeal-agar medium, consisting of 4.5% molasses, 7.5% sugar, 7%
684 cornflour, 2% granulated yeast extract, 1% agar, and 0.25% nipagin, mixed in distilled water.

685 **Precise excision of the *Mi{MIC}* element from *dilp8-eGFP$^{MI00727}$***

686 The precise excision of the *Mi{MIC}* element from *dilp8-eGFP$^{MI00727}$* (Venken et al. 2011), which we
687 named *dilp8$^{ag22}$*, was obtained by crossing virgin *dilp8-eGFP$^{MI00727}$* females with males carrying the heat
688 shock-inducible MiET1 transposase on a *Cy* balancer chromosome (*w$^{1118}$; If/Cy-Minos; MKRS/TM6B*) and
689 transferred to new vials every day. After 48 h of development and until pupariation, the F1 progeny was
690 given a 1-h 37 °C heat shock daily to induce the expression of the MiET1 transposase. *Cy-Minos/+; dilp8-*
691 *eGFP$^{MI00727}$/MKRS* or *TM6B* male adults were selected and individually crossed to the balancer strain *w$^{1118}$;*
692 *If/CyO; MKRS/TM6B*. Deletion of the *Mi{MIC}* element was confirmed by PCR with primers
693 dilp8MMIC_F CAATAGATCAGTAGGCACGTCGAT and dilp8MMIC_R
694 CAGCAAGTCCAGCAGCATGT. The restoration of wild type *dilp8* expression level was
695 confirmed by qRT-PCR (**Figure 1C**).

696

697 **Egg-laying assay and hatchability assessment**

698 Flies used to evaluate oogenesis and oviposition were reared under controlled conditions. Females laid eggs
699 overnight on apple juice egg-laying plates (25% apple juice, 2.5% sucrose, 2% agar, 5% nipagin in a 60-
700 mm diameter petri dish). The next day, L1 larvae were transferred to vials with fly food at low density (~30
701 larvae per vial) and kept at 25 °C. Virgin females reared under these conditions were placed in groups of
702 ~10-15 per vial with paste yeast supplemented food until they were used for egg-laying assays four-five
703 days later. At that time, they were transferred to egg-laying plates supplemented with yeast paste, at a rate

704 of five females per cage. Egg-laying plates were replaced every 24 h for a total of 48 h, except in long-term
705 oviposition experiments, where flies were evaluated for 10 d.

706 The number of eggs laid on the plates was counted at the time of plate replacement and used to determine
707 the average egg-laying time. When oviposition was evaluated in mated females, virgin females were
708 collected following the indicated procedure, mated at day 1 and transferred to egg laying plates on day 4-
709 5.

710 For hatchability assessment, plates with eggs were kept at 25 °C, and L1 larvae were counted daily until no
711 newly hatched larvae were observed. Hatchability was expressed as the percentage of eggs that successfully
712 completed embryonic development and hatched as larvae.

713

714 **Follicle classification**

715 Ovaries were dissected in cold PBS, fixed for 30 min in 4% paraformaldehyde, rinsed with PBS containing
716 Triton (0.3%) (PBST), stained with DAPI for 30 min, rinsed with PBS, and mounted in 50% PBS glycerol
717 or Fluoromount-G (Southern Biotech). Follicles were staged following published criteria (Deady, Li, and
718 Sun 2017; Beard, Oramas, and Sun 2023; Jia et al. 2016). Briefly, mature follicles were identified by their
719 long dorsal appendage and by DAPI staining, as they lack nurse cell nuclei in the anterior region of the
720 follicle. We classified stage 14 follicles as 'early' if they lacked nurse cells and their nuclei were randomly
721 distributed, and as 'fully mature' if the nuclei were aligned in a row, as shown in **Supplementary figure 6**.
722 *dilp8-eGFP*$^{MI00727}$ is only active in fully mature stage 14 follicles (**Figure 1**).

723

724 **Egg-laying time assay**

725 The egg-laying process can be divided into three stages: ovulation, transit through the lateral and common
726 oviduct, and transit through the uterus and oviposition. The total time required to lay a single egg can be
727 estimated from the number of eggs laid per female per day. The duration of each stage can be inferred based
728 on the assumption that the probability of finding an egg in a specific region of the reproductive tract at any
729 given time is proportional to the duration of that stage. The time required for each stage is then calculated
730 by multiplying the total egg-laying time by the frequency of eggs found within each reproductive tract
731 region (Beard, Oramas, and Sun 2023). To quantify the distribution of eggs within the reproductive tract,
732 5-d old virgin females were treated as in the Egg-Laying Assay, mated, and frozen at -80 °C for five minutes
733 six hours after mating. They were then stored frozen until further analysis. Females were dissected, and the
734 position of the egg within the reproductive tract was recorded as follows:

735 ● None (N) – No egg found in the reproductive tract
736 ● Bilateral (B) – More than 75% of an egg in the lateral oviduct
737 ● Common (C) – Egg entirely within the common oviduct
738 ● Uterus (U) – Egg located in the uterus
739 ● Ejected (E) – Egg expelled from the uterus

740 To calculate ovulation time, oviduct time, and uterus time, the total egg-laying time was multiplied by the
741 percentage of eggs in ovulation (%N), oviduct (%B + %C), and uterus (%U + %E), respectively. The egg-
742 laying time is calculated using the number of eggs/female/day (Beard, Oramas, and Sun 2023).

743

744 **Induction of egg retention**

745 Egg retention was induced by placing females on hard-agar (5% agar) egg-laying plates without yeast paste.
746 Briefly, virgin females were collected as described above and placed in vials with yeast-paste-supplemented
747 food to induce oocyte production for four days. On day four, females were transferred to hard-agar egg-
748 laying plates for seven days. Under these conditions, females laid very few eggs, regardless of genotype.
749 On day 11 after eclosion, virgin females were mated and placed on standard egg-laying plates supplemented
750 with yeast paste. Hatchability of laid eggs was evaluated on the first and third day after retention. Eggs laid
751 during the first 24-h period corresponded to those retained in the ovary during the experimental retention
752 period. Eggs laid on the third day were expected to be mostly produced after mating and, therefore,
753 considered non-retained. In parallel, virgin females were collected in the same way but mated on day 1
754 after eclosion and continuously housed with males until the end of the experiment served as the "no-
755 retention" control. They were kept on yeast-supplemented standard food for four days and transferred to
756 regular egg-laying plates supplemented with yeast on day five. On day 11, they followed the same regime
757 as retained females.

758

759 **Blockage of vesicle release in follicle cells**

760 Virgin *dilp8-1-GAL4 UAS-shi$^{ts}$ UAS-sGFP* females, were developed at room temperature (RT) and
761 maintained at the same temperature after eclosion. On day 7, they were shifted to 30 ºC for 24 h, or kept at
762 RT as controls. Control flies without *UAS-shi$^{ts}$* received the same temperature regime. Ovaries were
763 dissected as indicated below and stained with a-Dilp8 and a-GFP antibodies.

764 To assess the effect of the blockage of vesicle release on egg laying, flies were developed at 18 ºC. Virgin
765 females were maintained at 30 ºC on vials with regular food. On day five after eclosion, flies were
766 transferred to egg-laying plates supplemented with a smear of yeast paste and allowed to lay eggs for 48 h.

767 Egg-laying plates were replaced every 24 h at 30 °C, depending on the corresponding condition. Flies were
768 assayed in groups of five individuals.

769 **Immunofluorescence analyses**

770 Ovaries were dissected in Schneider Medium (Biowest – cat. #L0207 or Gibco - cat. #21720-024), fixed
771 for 30 min in 4% paraformaldehyde, rinsed with PBS with Triton (0.3%) (PBST), incubated with primary
772 antibody for 24 h and with fluorescently labeled secondary antibody for 24 h in PBST with 1% bovine
773 serum albumin. Samples were washed 3× for 30 min each in PBST after each antibody incubation. Nuclei
774 were counterstained with DAPI (Sigma) and tissues were mounted in Fluoromount-G (Southern Biotech)
775 or Dabco mounting medium (Sigma-Aldrich). Primary antibodies used were: Rat anti-Dilp8, 1:500
776 (Colombani, Andersen, and Léopold 2012), Rabbit Anti-GFP, 1:200 (Life Technologies, A11122), Mouse
777 Living Colors DsRed, 1:500 (Clontech, 632496). Secondary antibodies: Donkey Anti-Rat Alexa Fluor 647,
778 1:250 (Jackson 712-605-153), Goat Anti-Rabbit Alexa Fluor 488, 1:250 (Invitrogen A11008), Goat Anti-
779 Mouse Alexa Fluor 568, 1:250 (Life Technologies, A11036), Goat Anti-Horseradish Peroxidase (HRP)
780 Alexa Fluor 647, 1:250 (Jackson Immuno Research, 123-605-021).

781

782 **qRT-PCR**

783 The material used for the qRT–PCR experiments were obtained from 5 staged animals, depending on the
784 experiment, or 20 ovaries per biological replica, and was macerated using pellet pestles and homogenized
785 in 800 µl of TRI Reagent or NZYol and centrifuged at 12,000 × g for 1 min, to lower tissue debris. After
786 the centrifugation, half volume of absolute ethanol was added to the supernatant and mixed well. Then, the
787 sample was loaded in a binding column of the RNA extraction kit. An extra DNAse treatment (Turbo DNA-
788 free kit, Ambion, Life Technologies) was performed to reduce gDNA contamination. cDNA synthesis was
789 performed using the Maxima First Strand cDNA Synthesis Kit for RT–quantitative PCR (Thermo
790 Scientific) or NZY First-Strand cDNA Synthesis Kit, following manufacturer's instructions.

791 qRT–PCR experiments were performed as described previously (Heredia et al. 2021). Briefly, the
792 experiments were performed in a LightCycler® 96 Instrument (Roche) or in a CFX96$^{TM}$ (Bio-Rad) using
793 the FastStart Essential DNA Green Master dye and polymerase (Roche). The final volume for each reaction
794 was 10 µl, consisting of 5 µl of dye and polymerase (master mix), 2 µl of cDNA sample (diluted to an
795 estimated 1-10 ng/µl equivalent of RNA) and 3 µl of the specific primer pairs. The efficiency of the primers
796 for the qPCR was verified by analyzing a standard curve with 3 serial dilutions of gDNA from w1118
797 animals and the production of primer dimer was checked by analyzing the melting curve. Only primers
798 with more than 90% of efficiency and with no primer dimer formation were used. qRT-PCR results were
799 expressed as mRNA levels relative to *rp49* (*rp49* = 100). Primer pairs used for *D. melanogaster* genes:

800 *rp49*_F 5´TTGAGAACGCAGGCGACCGT 3´

801 *rp49*_R 5´CGTCTCCTCCAAGAAGCGCAAG 3´

802 *dilp8*_F 5´CGACAGAAGGTCCATCGAGT 3´

803 *dilp8*_R 5´GATGCTTGTTGTGCGTTTTG 3´

804 *dilp3*_F 5´CCGAAACTCTCTCCAAGCTC 3´

805 *dilp3*_R 5´ GCCATCGATCTGATTGAAGTT 3´

806 *CG15279*_F 5' TCGTCTATCCAAATTGGTCTTACTC 3'

807 *CG15279*_R 5' AATGGCCACGATCATCCA 3'

808 *CG1698*_F 5' GTCTACGACTTTTCACCCATCA 3'

809 *CG1698*_R 5' AACGTGGCGTAGTAGGTGGT 3'

810 *CG3759*_F 5' AAACTGTAGGCGAATCAGCAG 3'

811 *CG3759*_R 5' CTCCTTTCGATAAGTGGTGTTGT 3'

812 *CG6508*_F 5' AGGTCCAGGCGGATAACAAT 3'

813 *CG6508*_R 5' TTACCCTGCAAGTATGTGAAGC 3'

814 *Oamb*_F 5' GGAGAAAACTGGCCAGAACTAA 3'

815 *Oamb*_R 5' TTACCCTGCAAGTATGTGAAGC 3'

816

817 **Western Blot**

818 Female pupae reared at controlled conditions were transferred to a new vial and fed on food supplemented
819 with yeast paste for four days after eclosion. On day four, flies were separated into two groups. Control
820 flies were kept in vials with yeasted food while starved flies were transferred to 2% agar in water. One day
821 later, the thorax and head were dissected in ice-cold PBS and stored in 250 µl of ice-cold modified RIPA
822 buffer with NaF, NaVO4 and SpI proteases and phosphatases inhibitors. Each sample contained material
823 from five female flies. Protein concentration was measured with Bradford assay and 30 µg of protein
824 separated on a 12% SDS-PAGE gel, transferred and analyzed with anti Pospho-Drosophila Akt (Ser505)

825 and Akt antibodies from Cell Signaling. P-Akt/Akt was calculated as the ratio between the nutrition-
826 sensitive fast migrating p-Akt band (p-Akt*), over total Akt.

827

828 **Statistical analysis**

829 For all tests, alpha was set at 0.05 a priori. For quantitative data, comparison between multiple conditions
830 was done using ANOVA when samples had normal distribution (Shapiro-Wilk test and equal variance). If
831 the result of these tests was statistically significant, then Tukey's post-hoc tests were applied for the
832 assessment of statistical significance of pairwise comparisons. When samples had unequal standard
833 deviations, the Brown-Forsythe and Welch ANOVA and Dunnett's T3 test was used instead. Comparisons
834 between two conditions were done using one-tailed unpaired (unless noted otherwise) Student's t-test, when
835 samples had normal distribution and equal variance. Comparison between proportions was performed using
836 Student's t –test with Bonferroni correction. Z-score test was used for egg-laying time analysis.

837 **AUTHORS CONTRIBUTIONS**

838 Y.V., F.H., R.Z., J.M., M.S.P., M.G., L.L., L.S.C., A.P.C., A. Garelli, and A.M. Gontijo performed genetic,
839 phenotypic, molecular, and behavioral experiments. Y.V., F.H., A. Garelli and A.M. Gontijo designed
840 research. F.H., A. Garelli and A.M. Gontijo supervised the work. A. Garelli and A.M. Gontijo contributed
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842

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## DECLARATION OF INTERESTS

The authors declare no competing interests.

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