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

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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. Our work brings 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: 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 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 seasonality in the phenotype (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 largely 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 genes that were tested (*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). Lgr3 GAL4 lines have also been shown to be expressed in neurons innervating the oviduct and in posterior follicle cells, which could suggest autocrine and paracrine signalling by Dilp8 could promote fecundity and ovulation in mated

flies (Liao and Nässel 2020).

Here, we use genetic and tissue-specific RNA interference studies 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 supersede the requirement for the Dilp8-Lgr3 pathway for ovulation. Follicle-cell vesicle secretion inhibition experiments suggest that ovulation promotion is achieved by Dilp8 secretion from follicle cells into the oviduct. Our work shows that frequent spontaneous ovulation (short oocyte retention times) in *Drosophila* virgins is an actively promoted process. We provide evidence that Dilp8 promotes oocyte quality by at least one mechanism: by eliminating aged oocytes by spontaneous ovulation. By demonstrating that Dilp8 antagonizes systemic insulin signaling, we propose that it might contribute to promoting oocyte quality via an additional mechanism, which involves restricting new oocyte production to optimal nutritional conditions when enough oocytes are already stocked in the ovary. Our results provide a framework to understand virgin oocyte retention regulation and evolution and demonstrate that relaxin-like signaling has a very ancient function in reproductive physiology in bilaterian animals, most notably in promoting ovulation.

RESULTS

Dilp8 is expressed and secreted in 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 imaginal disc stainings with this antibody (Colombani, Andersen, and Léopold 2012) and follicle cell stainings with an independent antibody (Liao and Nässel 2020).

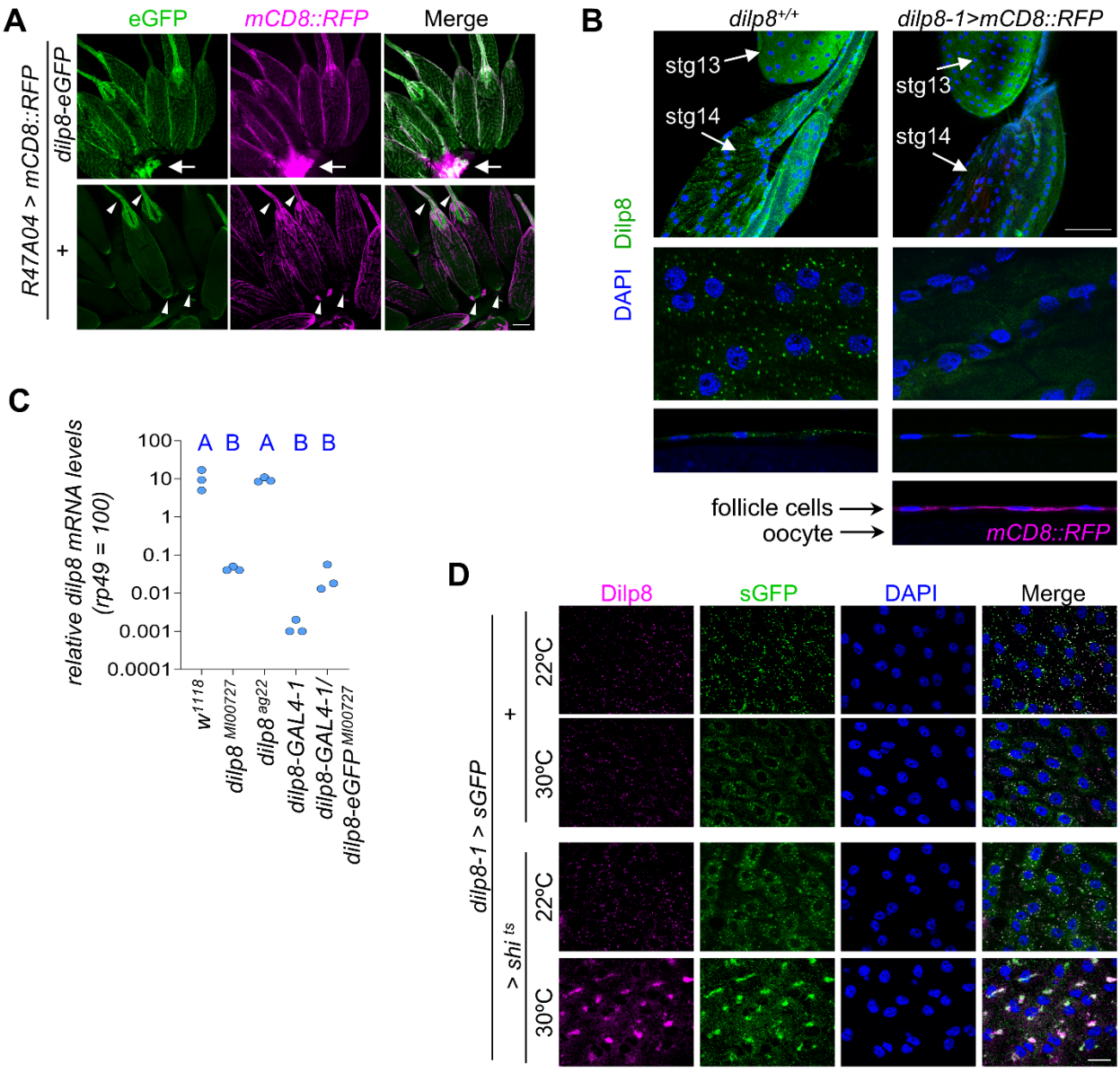

Fig 1. Dilp8 is expressed in and secreted from mature, stage 14 follicle cells. **(A)** The *R47A01-GAL4* line is expressed in stage 14 ovarian follicles, which also show eGFP fluorescence from the *dilp8-eGFP[MI00727]* transcriptional reporter. The arrow points to the residual body (lower panel). The dorsal appendages and the posterior end of stage 14 follicles are autofluorescent under blue light (arrowheads). 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]* ($dilp8^{MI00727}$) 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.

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

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

162

163 *Dilp8 mutant virgins retain oocytes in the ovary*

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

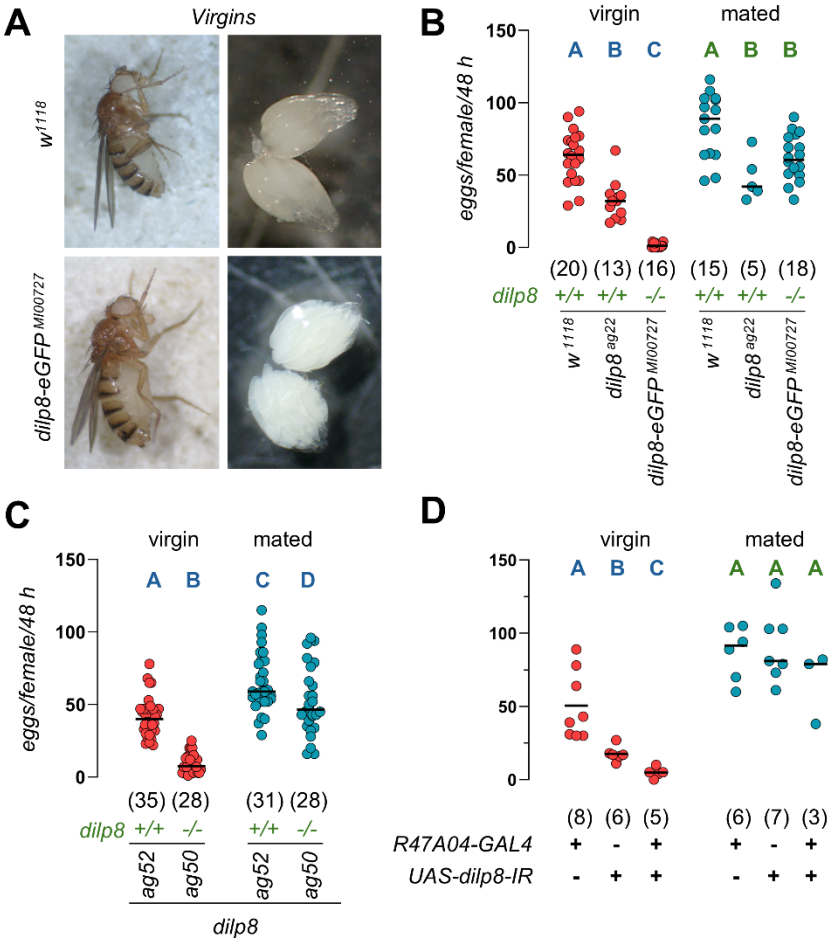

Fig 2. Dilp8 is required in virgin females for ovulation. **(A)** The abdomen of *dilp8* mutant flies was bloated due to the accumulation of eggs in the ovaries. **(B)** Virgin females lacking *dilp8* (*dilp8-eGFP*^{MI00727}) laid fewer eggs than w^{1118} and *dilp8*^{ag22} control flies. The effect 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 of 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.

173 *dilp8-eGFP*^{MI00727} with the *dilp8*^{ag22} precise excision and partially rescued relative to *w*^{1118} controls (**Figure**
174 **2B**). This is in contrast to previous reports suggesting that mated (and non-backcrossed) *dilp8-eGFP*^{MI00727}
175 mutant animals had reduced fecundity (Liao and Nässel 2020; Colombani, Andersen, and Léopold 2012;
176 Haomiao Li et al. 2023), suggesting that this discrepancy could be an effect of heterogeneous backgrounds.
177 Using the amount of unfertilized eggs laid as virgins as a proxy, we found similar results as above with an
178 independent *dilp8* null allele, *dilp8*^{ag50} (Heredia et al. 2021), when compared to its wild-type background
179 allele, *dilp8*^{ag52} (**Figure 2C**), or with RNA interference (RNAi)-mediated knockdown of *dilp8* [*UAS-dilp8-*
180 *IR* (Dietzl et al. 2007; Perkins et al. 2015)] specifically in follicle cells (*R47A04>dilp8-IR*) (**Figure 2D**).
181 Again, even though a small significant effect on fecundity was detectable in mated *dilp8*^{ag50} null alleles,
182 the effect size (21% reduction) was visibly smaller than that of virgins (81% reduction), and it was not
183 detectable in mated *R47A04>dilp8-IR* animals. We concluded that follicle cell-derived Dilp8 positively
184 influences the amount of unfertilized eggs laid in virgin females, and that mating renders this influence
185 largely redundant.

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

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

204 To quantitatively test this assumption, we estimated ovulation time, oviduct oocyte transit time, and uterus
205 residency time in *dilp8* mutants and controls in virgin and mated conditions using a previously established
206 method (Beard, Oramas, and Sun 2023). Our results showed that control virgins ovulated on average once
207 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.

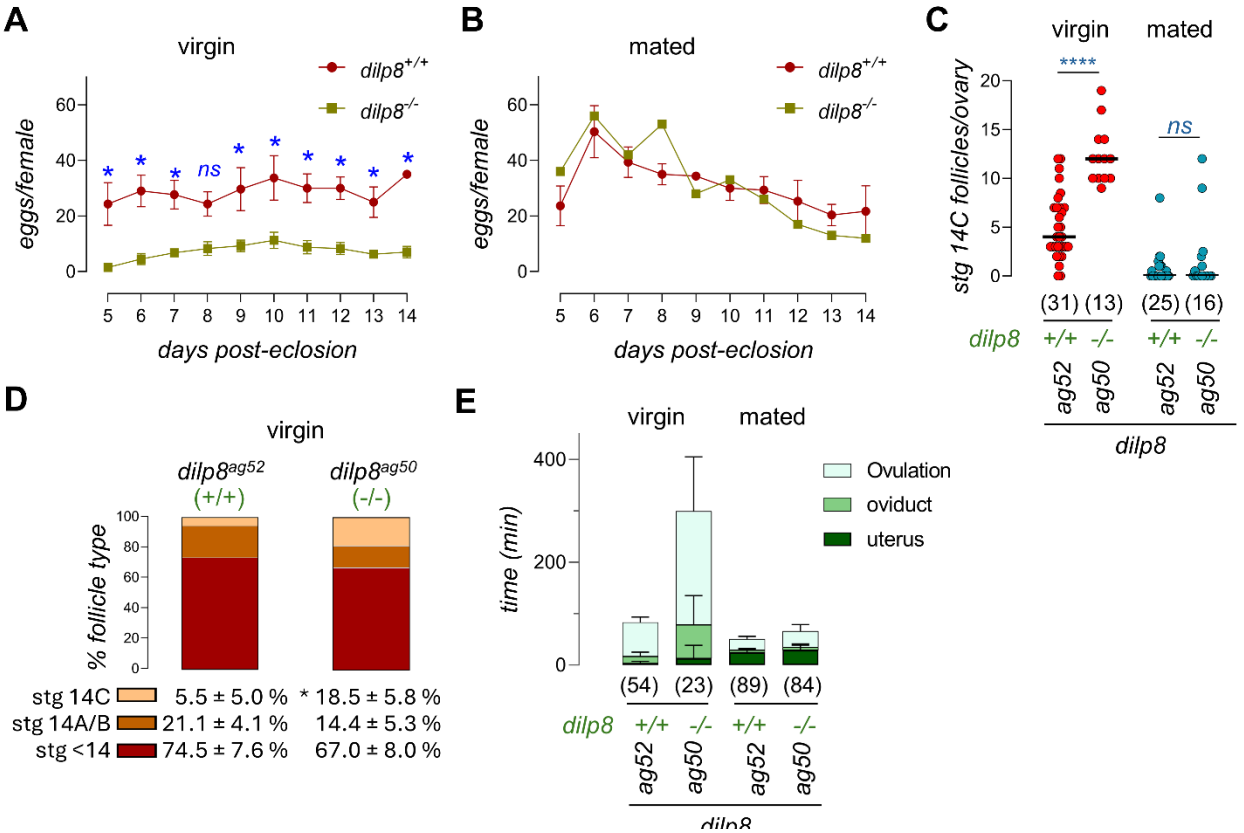

Fig 3. Dilp8 regulates ovulation rate. **(A)** Unfertilized egg laying evaluated for ten days is constantly lower in virgin *dilp8* mutant flies (*dilp8*$^{-/-}$ = *dilp8*^{ag50}) when compared with background control (*dilp8*$^{-/-}$ = *dilp8*^{ag52}). The difference was statistically significant for 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 minutes ($P < 0.0005$, Z-score test) and uterus transit time (dark green) from 3.1 ± 4.2 to 13.0 ± 25.5 minutes ($P < 0.005$, Z-score test) in mutant flies compared to background controls of the depicted genotypes, while oviduct transit time (medium green) it does not show a statistically significant difference. There was no statistically-significant difference between mated mutants and controls regarding the three times evaluated ($P > 0.05$, 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 in virgins. This explains why *dilp8* mutant virgins lay less unfertilized
229 eggs than control animals and accumulate late stage oocytes in their ovaries.

230

231 *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

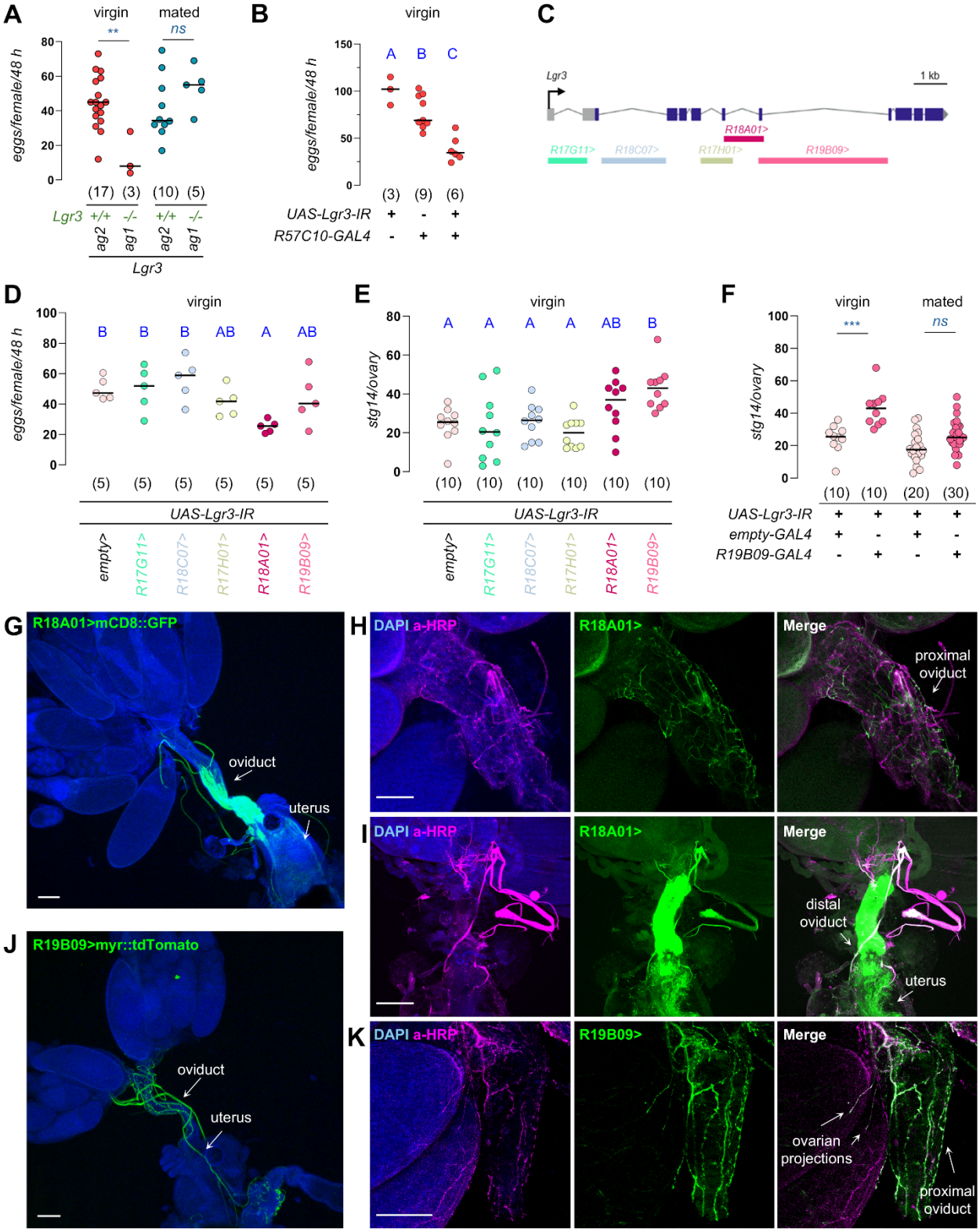

Fig 4. Lgr3 is required for virgin ovulation and egg laying in at least two different neuronal populations. **(A)** Virgin females lacking Lgr3 (*Lgr3*^{ag1}), the Dilp8 receptor, laid fewer eggs than their background controls (*Lgr3*^{ag2}). The effect was lost upon mating. Number of replicates, each 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>*) affect 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

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

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

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

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

Just like 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 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 is 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 of eggs than that of *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) relative to 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.

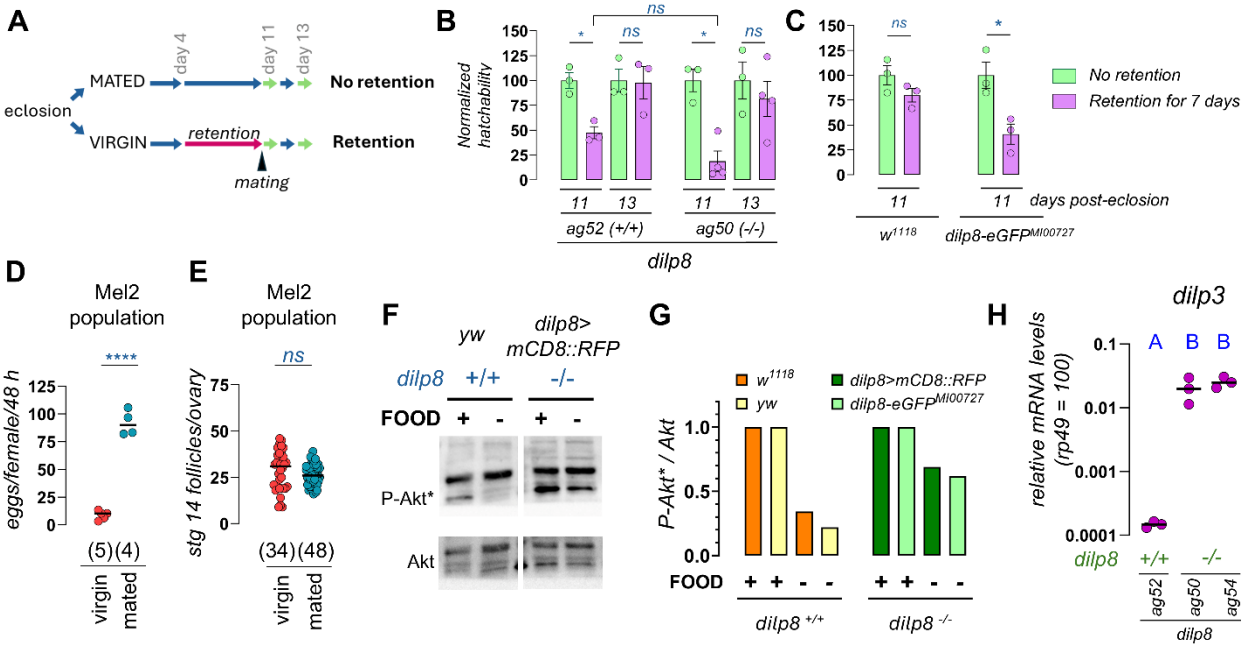

Fig 5. Egg retention leads to reduced hatchability. **(A)** Schematic representation of the protocol used to induce egg retention. Newly eclosed females of the "No retention" condition were mated and kept on food supplemented with yeast. Hatchability was evaluated on the eggs laid at days 11 and 13 after 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 *Drosophila 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 are 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

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

Fourth, virgin and mated females of the Mel2 population accumulated stage 14 oocytes in their ovaries to a similar extent [28.5 ± 10.7 (virgins, $N = 34$ ovaries) vs. 26.7 ± 5.7 (mated, $N = 48$ ovaries), $P > 0.05$, Student's t-test] (**Figure 5E)**. This indicates that despite laying even fewer unfertilized eggs per day than virgin *dilp8* mutants (which lay ~5-10 unfertilized eggs per day), virgins of the Mel2 population do not accumulate more than 1-2 stage 14 follicles per ovariole (considering ~20 ovarioles per ovary [as a reference, the DGRP lines have 19.4 ± 2.84 average ± standard deviation ovarioles per ovary, $N = 200$ lines (Lobell et al. 2017)]. This is coherent with what has been reported for control backgrounds of other genotypes and laboratories (Boulétreau-Merle 1990; Boulétreau-merle and Fouillet 2002; King and Sang 1959); Bloch Qazi, Heifetz, and Wolfner 2003; Richards 2007; Greenblatt and Spradling 2018; Greenblatt et al. 2019). In fact, long retention virgin females, such as Mel2 population females, do not accumulate stage 14 follicles beyond 2 per ovariole because they abruptly reduce new follicle production and vitellogenesis via increased resorptions (Boulétreau-Merle 1990; Boulétreau-merle and Fouillet 2002). This suggests the existence of an oogenesis progression checkpoint that is sensitive to the amount of stage 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 clearly 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 *would* arrest oogenesis *if* they did
421 not ovulate copious amounts of oocytes. In the latter case, short-retention virgins would require Dilp8 to
422 ensure the checkpoint, since they fail to arrest oogenesis when *dilp8* is absent. This interpretation aligns
423 with the second alternative presented at the end of the previous section: that Dilp8 could have an
424 independent role promoting oocyte quality, which involves some other role on ovarian physiology.

425

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

427 The negative feedback by which long-retention animals halt new mature stage 14 follicle production kicks
428 in after they accumulate ~2 stage 14 follicles per ovary (Boulétreau-Merle 1990; Boulétreau-merle and
429 Fouillet 2002), strongly suggesting that it responds to the amount of stage 14 follicles in the ovary. As
430 Dilp8 is a secreted factor expressed specifically in this follicle stage in the ovary, we hypothesized it
431 participates in signaling the negative feedback. This negative feedback pathway is reminiscent of the
432 nutrition-dependent checkpoint, which prevents development of vitellogenic oocytes beyond stage 8/9 in
433 the absence of protein-rich food sources (Greenblatt and Spradling 2018; Greenblatt et al. 2019; Richard et
434 al. 2005; Drummond-Barbosa and Spradling 2001) and the gating mechanism that limits egg production
435 while conditions are suboptimal in stressful conditions (Meiselman et al. 2017; Meiselman et al. 2018).
436 These likely correspond to the same checkpoint which is controlled at multiple levels, including by mating
437 (Soller et al. 1999). Indeed, oogenesis progression is subject to extensive endocrine control by the
438 insulin/insulin-like growth factor (hereafter, insulin), ecdysone, ecdysis triggering hormone, and juvenile
439 hormone signaling pathways (Soller et al. 1999; Drummond-Barbosa and Spradling 2001; Richard et al.
440 2005; Liu et al. 2008; Grutenko et al. 2010; Meiselman et al. 2017; Meiselman et al. 2018; Greenblatt and
441 Spradling 2018; Greenblatt et al. 2019). The balance between ecdysone and juvenile hormone, mediated
442 by ecdysis triggering hormone, dictates whether oogenesis progresses or not, with juvenile hormone likely
443 sitting at the bottom of the cascade (Liu et al. 2008; Grutenko et al. 2010; Meiselman et al. 2017; Meiselman
444 et al. 2018). The nutrient-sensitive insulin signaling pathway can modulate ecdysone and juvenile hormone
445 levels and also play an independent role in controlling the vitellogenic stage checkpoint, placing upstream
446 and in parallel to the ecdysone-ecdysis triggering hormone-juvenile hormone endocrine axis (g; Greenblatt
447 and Spradling 2018; Greenblatt et al. 2019; Richard et al. 2005; Drummond-Barbosa and Spradling 2001).
448 Here, we hypothesized that the Dilp8-Lgr3 pathway beyond promoting ovulation and egg transit through

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

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

480

481 **DISCUSSION**

482 *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 in this case peri-oocytic Dilp8 would only reach Lgr3-positive neurons following follicle rupture at

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

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

548 A similar role has been proposed for ecdysis triggering hormone, which is secreted from trachea-associated
549 endocrine cells (INKA cells) in optimal growth conditions, acting on octopaminergic neurons to promote
550 ovulation (Meiselman et al 2017; Meiselman et al 2018). In stressful conditions (starvation, heat stress) its
551 secretion is inhibited by ecdysone signaling, reducing octopamine-dependent ovulation levels. It will be
552 interesting to study the interaction between the Dilp8-Lgr3 signaling pathway and the ecdysis triggering

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

557

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

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

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

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

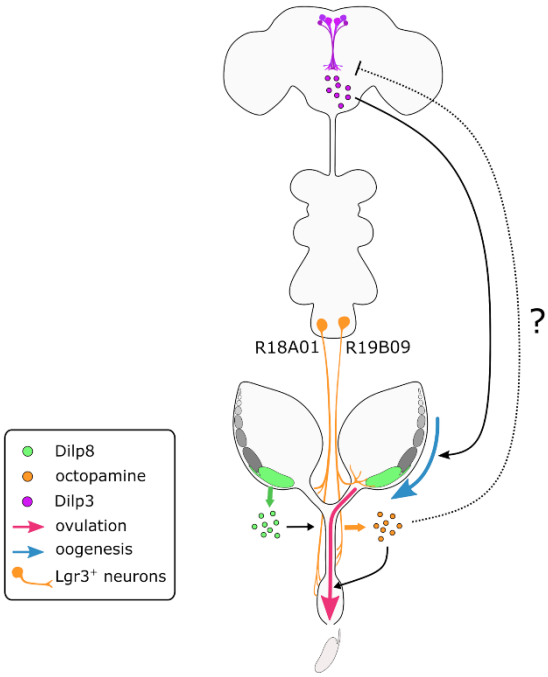

Figure 6. Model for the role of the Dilp8-Lgr3 signaling pathway in reproductive physiology control. Dilp8 peptide (green), produced and continuously secreted from follicle cells surrounding fully mature follicles, acts on two different populations of Lgr3-expressing neurons (orange neurons) that innervate 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 oocyte 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 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 (R. A. D. 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 (R. A. D. Bathgate et al. 2013; Gontijo and Garelli 2018; Imambocus et al. 2022; Heredia et al. 2021), making relaxins potential therapeutic targets for multiple diseases (Ross A. D. 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)

672

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

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

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

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

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

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

695

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

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

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

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

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

712

713 **Follicle classification**

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

722

723 **Egg-laying time assay**

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

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

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

Induction of egg retention

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

Blockage of vesicle release in follicle cells

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

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

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

768 **Immunofluorescence analyses**

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

780

781 **qRT-PCR.**

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

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

799 *rp49*_F 5´TTGAGAACGCAGGCGACCGT 3´

800 *rp49*_R 5´CGTCTCCTCCAAGAAGCGCAAG 3´

801 *dilp8*_F 5´CGACAGAAGGTCCATCGAGT 3´

802 *dilp8*_R 5´GATGCTTGTTGTGCGTTTTG 3´

803 *dilp3*_F 5´CCGAAACTCTCTCCAAGCTC 3´

804 *dilp3*_R 5´ GCCATCGATCTGATTGAAGTT 3´

805 *CG15279*_F 5' TCGTCTATCCAAATTGGTCTTACTC 3'

806 *CG15279*_R 5' AATGGCCACGATCATCCA 3'

807 *CG1698*_F 5' GTCTACGACTTTTCACCCATCA 3'

808 *CG1698*_R 5' AACGTGGCGTAGTAGGTGGT 3'

809 *CG3759*_F 5' AAACTGTAGGCGAATCAGCAG 3'

810 *CG3759*_R 5' CTCCTTTCGATAAGTGGTGTTGT 3'

811 *CG6508*_F 5' AGGTCCAGGCGGATAACAAT 3'

812 *CG6508*_R 5' TTACCCTGCAAGTATGTGAAGC 3'

813 *Oamb*_F 5' GGAGAAAACTGGCCAGAACTAA 3'

814 *Oamb*_R 5' TTACCCTGCAAGTATGTGAAGC 3'

815

816 **Western Blot**

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

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

826

827 **Statistical analysis**

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

836 **AUTHORS CONTRIBUTIONS**

837 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,
838 phenotypic, molecular, and behavioral experiments. Y.V., F.H., A. Garelli and A.M. Gontijo designed
839 research. F.H., A. Garelli and A.M. Gontijo supervised the work. A. Garelli and A.M. Gontijo contributed
840 to image and data analyses and wrote the manuscript with the help of all authors.

841

842 **ACKNOWLEDGMENTS**

843 We thank Élio Sucena for the Mel2 population, Pierre Léopold for the a-Dilp8 antibody, Rita Teodoro and
844 Carolos Ribeiro for stocks, Maria Dominguez for discussions and support in the initial stages of this work,
845 and Ana Margarida Moreira and Maria João Cruz for help in initial experiments of this work. We thank lab
846 members for discussions and the Jianjun Sun and team for discussions and for sharing unpublished results
847 prior to publication. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537),
848 the Kyoto Drosophila Stock Center, and the Vienna Drosophila Resource Center (VDRC) at Vienna
849 BioCenter Core Facilities (VBCF), member of the Vienna BioCenter (VBC), Austria, were used in this
850 study. Work in the Integrative Biomedicine Laboratory was supported by the Fundação para Ciência e
851 Tecnologia (FCT; PTDC/BIA-BID/31071/2017; EXPL/BIA-BID/1524/2021; EXPL/BIA-
852 COM/1296/2021; 10.54499/2022.03859.PTDC; 2023.15344.PEX), by the Research Units iNOVA4Health
853 (10.54499/UIDB/04462/2020) and cE3c - Centre for Ecology, Evolution and Environmental Changes
854 (10.54499/UIDB/00329/2020), by the The Associate Laboratory Life Sciences for a Healthy and

Sustainable Future (LS4FUTURE) and CHANGE financed by the FCT/MCTES (Ministério da Ciência, Tecnologia e Ensino Superior), by Congento LISBOA-01-0145-FEDER-022170, co-financed by FCT/Lisboa2020; UID/Multi/04462/2019; and by the Microscopy Facility of the Faculdade de Ciências da Universidade de Lisboa (PPBI-POCI-01-0145-FEDER-022122). Work in the Garelli lab was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT; PICT 2017-0254 and PICT 2020-01568), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; PIP11220150100182CO) and Universidad Nacional del Sur PGI 24/B288. AMG and FH were individually supported by grants 10.54499/CEECINST/00102/2018/CP1567/CT0031 and 10.54499/DL57/2016/CP1457/CT0016, respectively. AG and YV are CONICET researchers, MSP holds a CONICET PhD fellowship. All data are available in the manuscript or the supporting information. All reagents and fly strains generated in this study are available from the corresponding authors without restriction

DECLARATION OF INTERESTS

The authors declare no competing interests.

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