

1 *eyes absent* in the cockroach panoistic ovaries regulates proliferation and differentiation  
2 through ecdysone signalling.

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5 Ramos, S.<sup>1</sup>, Chelemen, F.<sup>1</sup>, Pagone, V.<sup>1</sup>, Elshaer, N.<sup>1,2</sup>, Irlles, P.<sup>1,3</sup>, Piulachs, M.D.<sup>1,\*</sup>

6 <sup>1</sup>Institut de Biologia Evolutiva (CSIC- Universitat Pompeu Fabra), Passeig Maritim de  
7 la Barceloneta, 37, 08003 Barcelona, Spain. <sup>2</sup> Department of Plant Protection, Faculty  
8 of Agriculture, Zagazig University, Egypt. <sup>3</sup> Instituto de Ciencias Agronómicas y  
9 Veterinarias, Universidad de O'Higgins, Chile.

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11 \*Corresponding author:

12 MDP [mdolors.piulachs@ibe.upf-csic.es](mailto:mdolors.piulachs@ibe.upf-csic.es)

13 Phone: +34 932 309 648

14 Fax: + 34 932211011

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20 **Abstract**

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22 Eyes absent (Eya), is a protein structurally conserved from hydrozoans to humans, for  
23 which two basic roles have been reported: it can act as a transcription cofactor and as a  
24 protein tyrosine phosphatase. Eya was discovered in the fly *Drosophila melanogaster* in  
25 relation to its function in eye development, and the same function was later reported in  
26 other insects. Eya is also involved in insect oogenesis, although studies in this sense are  
27 limited to *D. melanogaster*, which has meroistic ovaries, and where *eya* mutations  
28 abolish gonad formation.

29 In the present work we studied the function of *eya* in the panoistic ovary of the  
30 cockroach *Blattella germanica*. We show that *eya* is essential for correct development  
31 of panoistic ovaries. In *B. germanica*, *eya* acts at different level and in a distinct way in  
32 the germarium and the vitellarium. In the germarium, *eya* contributes to maintain the  
33 correct number of somatic and germinal cells by regulating the expression of  
34 steroidogenic genes in the ovary. In the vitellarium, *eya* facilitates follicle cells  
35 proliferation and contributes to regulate the cell program, in the context of basal ovarian  
36 follicle maturation. Thus, *eya*-depleted females of *B. germanica* arrest the growth and  
37 maturation of basal ovarian follicles and become sterile.

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44 **KEYWORDS:** panoistic ovary, ecdysone, 20E, Halloween genes, cell proliferation,  
45 insect oogenesis, Notch

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## 48 **1. Introduction**

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50 Maintaining the stability of stem cells is crucial in every organism, and this is especially  
51 important in the case of germinal stem cells. Oogenesis entails the process of ovary  
52 development from the time of germinal stem cell differentiation until oocyte maturation.  
53 During the oogenesis of many species, oocytes support a high level of transcription that  
54 is crucial not only for the growth of the oocyte, but also for the zygote activation, thus  
55 ensuring successful reproduction (Song and Wessel, 2005). In insects with meroistic  
56 ovaries, the transcriptional activity is mainly executed by the specialized nurse cells.  
57 Conversely, in panoistic ovaries the germinal vesicle is the responsible to maintain the  
58 oocyte itself, and provide the necessary materials to ensure embryogenesis  
59 (Bogolyubov, 2007; Büning, 1994).

60 In insect ovaries, germinal stem cells are located in niches in the germarium of each  
61 ovariole. The control of their proliferation and differentiation has been thoroughly  
62 studied in species with meroistic polytrophic ovaries, such as the fruit fly *Drosophila*  
63 *melanogaster* (see Ameku et al., 2017; Belles and Piulachs, 2015; Dai et al., 2017). In  
64 contrast, the knowledge of genes involved in regulating oogenesis in panoistic ovaries is  
65 very limited. Oocyte growth and maturation in panoistic ovaries has been systematically  
66 studied in the cockroach *Blattella germanica* (Elshaer and Piulachs, 2015; Herraiz et al.,  
67 2014; Irls et al., 2016; Irls and Piulachs, 2011; Tanaka and Piulachs, 2012), which is  
68 emerging as a choice model to study this ovary type. In *B. germanica*, each ovary has  
69 around 20 ovarioles, but only the most basal ovarian follicle of each ovariole matures  
70 during a given gonadotrophic cycle. The basal ovarian follicles are almost ready to  
71 mature in freshly ecdysed females, which means that the first gonadotrophic cycle starts  
72 early in the last nymphal instar. Importantly, the development of the remaining ovarian  
73 follicles of each ovariole is arrested until these basal ones are oviposited (Irls and  
74 Piulachs, 2014).

75 In previous contributions dealing with the regulation of oogenesis in panoistic ovaries,  
76 we have studied the function of *Notch* (*N*) in the ovary of *B. germanica* and its  
77 interactions with the EGFR signalling pathway (Elshaer and Piulachs, 2015; Irls et al.,  
78 2016; Irls and Piulachs, 2014). Given that the Notch pathway participates in the control

79 of germinal cell proliferation, here we postulated that the main effectors of this function  
80 would be downstream genes in the same pathway. Thus, we focused on *eyes absent*  
81 (*eya*) as a candidate to play a key role in this process.

82 The *eya* gene is structurally conserved from hydrozoans to humans (Duncan et al.,  
83 1997; Graziussi et al., 2012; Jemc and Rebay, 2007). It was discovered in *D.*  
84 *melanogaster* for its role in determining cell fates (differentiation or death) in  
85 postembryonic development in relation to eyes formation (Bonini et al., 1993).  
86 Subsequently, *eya* orthologues have been found in vertebrates and in other phyla  
87 (Duncan et al., 1997; Graziussi et al., 2012; Zimmerman et al., 1997), and a wide range  
88 of functions in development have been reported for the corresponding protein.

89 Two basic roles have been reported for Eya. It was first described as transcriptional  
90 cofactor that is recruited to transcriptional complexes via the Eya domain (ED), a  
91 conserved C-terminal motif that interacts with the Six family DNA binding proteins  
92 (Jemc and Rebay, 2007). Subsequently, different research groups reported that the ED  
93 has intrinsic protein tyrosine phosphatase activity, establishing Eya as an example of a  
94 new class of eukaryotic protein phosphatases (see Rebay, 2015 and references therein).

95 In insects, the function of *eya* in eye development has been reported both in  
96 holometabolan species like *D. melanogaster* and the red flour beetle *Tribolium*  
97 *castaneum* (Yang et al., 2009), as well as in hemimetabolan species. In the later, Dong  
98 and Friedrich (2010) studied *eya* in the post-embryonic development of the locust  
99 *Schistocerca americana* and Takagi and co-workers (2012) investigated its role in eye  
100 development in embryos and nymphs of the cricket *Gryllus bimaculatus*.

101 Research on *eya* functions in insect oogenesis has been limited to *D. melanogaster*,  
102 where it was studied during gonad formation in embryogenesis (Boyle et al., 1997), and  
103 in the adult during egg chambers differentiation (Bonini et al., 1998). From the early  
104 stages of ovarian development (stage 2a) *eya*, is expressed in follicle cells until stage 10  
105 of egg chamber, when follicle cells start the migration over the oocyte. An inappropriate  
106 follicle cell development results in sterility due to the arrest of egg chamber  
107 development (Bonini et al., 1998). More recently it has also been demonstrated that *eya*  
108 expression in the *D. melanogaster* ovary is essential to regulate polar and stalk cell  
109 fates. Thus, loss of *eya* transforms epithelial follicle cells in polar cells, while repression  
110 of *eya* is required for stalk cell formation (Bai and Montell, 2002).

111 In contrast to the research carried out in *D. melanogaster*, the possible role of *eya* in  
112 other insect ovary types remains unstudied. Therefore, in this work we aimed at  
113 addressing the role of this important protein in panoistic ovaries. We used the cockroach  
114 *B. germanica* as a model, and focussed on the regulation of cell proliferation and  
115 differentiation in the germarium.

116

## 117 **2. Material and Methods**

### 118 **2.1. Cockroach colony and sampling**

119 Adult females of the cockroach *B. germanica* (L.) were obtained from a colony fed *ad*  
120 *libitum* on Panlab dog chow and water, and reared in the dark at  $29 \pm 1^\circ\text{C}$  and 60–70%  
121 relative humidity. Freshly ecdysed adult females were selected and used at appropriate  
122 ages. Mated females were used in all experiments (the presence of spermatozoa in the  
123 spermatheca was assessed at the end of the experiment to confirm mating. All  
124 dissections and tissue samplings were performed on carbon dioxide-anaesthetized  
125 specimens.

### 126 **2.2. RNA extraction and expression studies**

127 Total RNA was isolated using the GenElute Mammalian Total RNA Kit (Sigma,  
128 Madrid, Spain). A total of 300 ng from each RNA extraction was treated with DNase  
129 (Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse  
130 transcriptase (Invitrogen, Carlsbad CA, USA) and random hexamers (Promega). RNA  
131 quantity and quality were estimated by spectrophotometric absorption at 260/280 nm in  
132 a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies, Wilmington, DE,  
133 USA).

134 The expression pattern of the examined *B. germanica* genes was determined by  
135 quantitative real time PCR (qRT-PCR) in ovaries from sixth instar nymph and adults.  
136 One ovary pair, for adults, or pools of two ovary pairs for nymphs, for every chosen age  
137 were used. The expression levels in treated individuals were quantified individually.  
138 PCR primers used in qRT-PCR expression studies were designed using the Primer3  
139 v.0.4.0 (Rozen and Skaletsky, 2000). The *actin-5c* gene of *B. germanica* (Accession  
140 number AJ862721) was used as a reference for expression studies. qRT-PCR reactions

141 were made using the iTaq Universal SYBR Green Supermix (BioRad) containing 200  
142 nM of each specific primer (performed in triplicate). Amplification reactions were  
143 carried out at 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 30 s, using  
144 MyIQ Single Color RTPCR Detection System (BioRad). After the amplification phase,  
145 levels of mRNA were calculated relative to *actin-5c*. Results are given as copies of  
146 mRNA per 1000 copies of *actin-5c* mRNA. The primer sequences used to quantify gene  
147 expression are indicated in Table S1.

148

### 149 **2.3. RNAi experiments**

150 To deplete the expression of *eya*, two dsRNA (*dsey*) were designed targeting the C-  
151 terminal domain of *eya* (318 and 325 bp each). As the same ovary phenotype was found  
152 using both dsRNA, we will refer to the RNAi treatments as *dsey*. A dsRNA (*dsMock*)  
153 corresponding to 307-bp of the *Autographa californica* nucleopolyhedrovirus sequence  
154 was used as control. The dsRNAs were synthesized in vitro as we previously described  
155 (Ciudad et al., 2006). The dose used was 1 µg for either *dsey* or *dsMock*, and they  
156 were injected into the abdomen of 0-day-old sixth nymphal instar or in 0-day-old adult  
157 females.

### 158 **2.4. 20-Hydroxyecdysone treatments**

159 Newly emerged last instar nymphs or adult females, were injected with 1µL of a 10mM  
160 20-hydroxyecdysone (20E) (10% ethanol). Nymphs were dissected when they were 6-  
161 day-old, just when ecdysteroids in the hemolymph reaches the highest levels (Cruz et  
162 al., 2003), or when they were 8-day-old, thus just before the molt to adult stage. Adult  
163 females were dissected when they were 5-day-old, before choriogenesis begins.

### 164 **2.5. Immunohistochemistry**

165 After dissection, ovaries were immediately fixed in paraformaldehyde (4 % in PBS) for  
166 2 h. Washing samples and antibody incubations were performed as previously described  
167 (Irles and Piulachs, 2014). The primary antibody employed were rabbit antibody anti-  
168 PH3 (Cell Signaling Technology, Denver, MA; dilution 1:250), rabbit antibody anti-  
169 cleaved Caspase-3 (*Asp-175*, Cell Signaling Tech; dilution 1:50), and mouse antibody  
170 anti-*Eya*, (deposited to the DSHB by Benzer, S. / Bonini, N.M.; product *eya10H6*;

171 dilution 1:50) as nuclear marker of germinal cells. However, were unable to assess Eya  
172 labelling, since there is not decrease of protein labelling in dsRNA treated insects. The  
173 secondary antibodies used were Alexa-Fluor 647 conjugated donkey anti-rabbit IgG, or  
174 Alexa-Fluor 647 conjugated goat anti-mouse IgG (Molecular Probes, Carlsbad, CA).  
175 Ovaries were also incubated at room temperature for 20 min in 300 ng/ml phalloidin-  
176 TRITC (Sigma) and then for 5 min in 1 µg/ml DAPI (Sigma) PBT, to show the F-actin  
177 and nuclei, respectively. After three washes with PBT, ovaries were mounted in Mowiol  
178 (Calbiochem, Madison, WI, USA) and observed using a Zeiss AxioImager Z1  
179 microscope (Apotome) (Carl Zeiss MicroImaging).

180 The number of cells in the follicular epithelia was estimated applying the function  
181 described in (Pascual et al., 1992).

182 We considered that an ovarian follicle has been released from the germarium when it is  
183 possible to identify the cell membrane surrounding the oocyte. The most basal follicle  
184 was excluded when quantifying ovarian follicles in the vitellarium.

## 185 **2.5. Statistics**

186 Quantitative data are expressed as mean ± standard error of the mean (S.E.M.).  
187 Statistical differences between morphometric data were evaluated using the ANOVA or  
188 the Student's t-test using IBM SPSS statistics software. Comparisons of gene  
189 expression between treatment and control groups were made using the Pair-Wise Fixed  
190 Reallocation Randomization Test (which makes no assumptions about distributions)  
191 (Pfaffl et al., 2002), employing REST 2008 v. 2.0.7 software (Corbett Research).

192

## 193 **3. Results**

### 194 **3.1. *eya* in *Blattella germanica* ovaries and efficiency of RNAi treatments**

195 In the *B. germanica* ovaries, *eya* is expressed through the gonadotropic cycle (Figure  
196 1A). The expression is remarkably variable in the last nymphal instar, although a clear  
197 peak can be observed just after the imaginal molt. Then, the expression begins to  
198 decline and reaches the lowest values on day 6, when choriogenesis starts. This pattern  
199 suggests that *eya* plays important functions in the early steps of oogenesis. To test this  
200 hypothesis, we used RNAi approaches. Thus, newly emerged sixth instar female

201 nymphs, were treated with *dseya* (n = 36) or dsMock (n = 40). All the *dseya* treated  
202 nymphs molted to the adult stage, which indicates that the treatment does not affect the  
203 image molt, but all resulting adult females failed to oviposit (Table S2). To assess the  
204 efficiency of the RNAi, we did new treatments of freshly emerged sixth instar female  
205 nymphs with *dseya*, and transcript decrease was examined in the ovaries at different  
206 ages. At the end of the nymphal stage (8-day-old sixth instar nymphs), *eya* mRNA  
207 levels were depleted ( $p = 0.097$ ), then, the expression kept decreasing in 3- and 5-day-  
208 old adult females ( $p = 0.031$  and  $p = 0.0001$ , respectively, Figure 1B).

209

### 210 **3.2. *eya* is involved in growth and maturation of basal ovarian follicles.**

211 The growth of the basal ovarian follicles in *eya*-depleted females was slowed (Figure  
212 1C), and their general shape became spherical (Figure 1D and E). There were  
213 significantly fewer follicular cells in the basal ovarian follicles of *eya*-depleted females  
214 compared to dsMock-treated females (Figure 2A). In 8-day-old sixth instar nymphs,  
215 labelling with PH3 antibody revealed fewer mitotic divisions in the follicular epithelia  
216 from *eya*-depleted females (Figure 2B and C), and the follicular cells within the  
217 epithelia in basal ovarian follicles showed a remarkable variation of nuclei size (Figure  
218 2D and F). Additionally, F-actin appeared to concentrate at the junctions between  
219 follicular cell membranes, which could explain the changes in cell shape, and  
220 distribution through the epithelia (Figure 2E and G).

221 These morphological changes in the ovaries became more conspicuous over time during  
222 the adult period, and basal ovarian follicles with different degrees of malformation were  
223 observed in the same ovary (Figure 3A-C). In 5-day-old adult control females, all  
224 follicular cells are binucleated and polyploid, and no further cell divisions occurs  
225 (Figure 3D and D'). F-actin are distributed on the cell membranes and appear  
226 concentrated in the expansions connecting adjacent cells (Figure 3D''). This occurs  
227 when the follicular cells contract and leave large intercellular spaces (a phenomenon  
228 called patency, see Davey and Huebner, 1974), thus allowing the vitellogenic proteins  
229 to reach the oocyte membrane, and to be internalized into the oocyte through a specific  
230 receptor. Conversely, in 5-day-old *eya*-depleted adult females, only a few follicular cells  
231 were binucleated (Figure 3E, arrowheads), thus indicating that they became  
232 desynchronised. Besides, these cells showed high variability in size and shape, F-actins

233 appeared concentrated in cell membranes (Figure 3E''), and the follicular epithelia  
234 never showed signs of patency.

235 Furthermore, the expression of the *vitellogenin receptor* (*VgR*) was upregulated in  
236 ovaries from *eya*-depleted females at the three ages examined: the last day of last  
237 nymphal instar (8-day-old) and in 3- and 5-day-old adults ( $p = 0.012$ ,  $p = 0.022$ , and  $p =$   
238  $0.001$  respectively; Figure S1). Usually, *VgR* mRNA levels are high in the ovaries of  
239 newly emerged last-instar nymphs. The expression subsequently decreases until  
240 reaching very low levels in adult females at the end of vitellogenesis (Ciudad et al.,  
241 2006). The modification of basal ovarian follicle shape, together with the phenotypes  
242 observed in follicular cells and the unexpected increase in *VgR* expression in 5-day-old  
243 adult ovaries, indicate that although these ovarian follicles seemed ready to mature, they  
244 did not grow. Instead, we presumed that these ovaries might be degenerating through a  
245 programmed cell death process. To test this conjecture, we measured the expression of  
246 the effector *caspase-1* (*casp1*) in the ovaries of *eya*-depleted females. In 6- and 8-day-  
247 old sixth instar nymphs, the mRNA levels of ovarian *casp1* in *eya*-depleted insects were  
248 similar to those measured in controls (Figure S2A). However, *casp1* expression was  
249 significantly upregulated in the ovaries of 5-day-old adult *eya*-depleted insects with  
250 respect to controls (Figure S2A). In addition, labelling for the executioner *casp3* in  
251 basal ovarian follicles of 5-day-old *eya*-depleted insects appeared concentrated in the  
252 nucleus of follicular cells (Figure S2B and C), while in controls *casp3* labelling is very  
253 faint and mainly distributed throughout the cytoplasm of follicular cells. Taken together,  
254 the data suggest that basal ovarian follicles of *eya*-depleted insects are compromised at  
255 this developmental stage.

256

### 257 **3.3. *eya* depletion affects somatic and germinal cells, increasing the rate of ovarian** 258 **follicle differentiation.**

259 In *B. germanica* females, the number of ovarian follicles in the vitellarium is established  
260 early in the last nymphal instar, and this number is maintained during the remainder of  
261 the first gonadotrophic cycle (Table S3 and Figure 4A-C). After oviposition, a new  
262 ovarian follicle is released from the germarium to the vitellarium. This suggests that  
263 specific mechanisms maintain the number of differentiated ovarian follicles in *B.*  
264 *germanica*.

265 *eya* depletion resulted in changes in the germarium and, as a consequence, also in the  
266 vitellarium. Compared to control females, at least two extra ovarian follicles were  
267 released into the vitellarium in ovaries from 8- day-old *eya*-depleted sixth instar nymphs  
268 (Figure 4A and D-E; Table S3). These extra ovarian follicles were maintained in adult  
269 females and they were observed even in 5-day-old *eya*-depleted adult females (Figure  
270 4A and F). In a few *eya*-depleted adult females, the vitellarium of some ovarioles  
271 contained as many as ten ovarian follicles. This concurs with the phenotype observed in  
272 *Notch* (*N*)-depleted adult females (Figure 4A and G), which is not surprising, as *N*  
273 depletion reduces *eya* expression (Irles et al., 2016; Irles and Piulachs, 2014).

274 In contrast, *N* expression is not affected in the ovaries of 5-day-old *eya* depleted insects,  
275 although *Delta* (*Dl*) and *Serrate* (*Ser*), the main ligands of *N*, appear upregulated  
276 (Figure 4H). In addition, the expression of *hippo* (*hpo*) and *yorkie* (*yki*), two important  
277 components of the Hippo pathway, significantly increases in the ovaries of *eya*-depleted  
278 females (Figure 4I). Moreover, expression of *nanos* (*nos*), *vasa* (*vas*), and *fs(1)Yb* (*Yb*),  
279 which are crucial in the modulation of germinal and somatic stem cell proliferation in  
280 *D. melanogaster* (King et al., 2001; Wang and Lin, 2004), results significantly  
281 upregulated in the ovaries of 5-day-old *B. germanica* *eya*-depleted insects (Figure 4I).  
282 Taken together, the results indicate that *eya*-depletion affects differently the distinct  
283 regions of the ovary. In basal ovarian follicles, their development becomes arrested, and  
284 they tend to die (Figure S2), whereas in the germarium, *eya*-depletion triggers an  
285 increase in differentiated cells, thus raising the number of ovarian follicles in the  
286 vitellarium (Figure 4).

### 287 **3.4. Ecdysone signalling and the differentiation of ovarian follicles**

288 In *D. melanogaster*, the formation and differentiation of ovarian follicles are triggered  
289 by 20E signalling (see Ameku et al., 2017; Belles and Piulachs, 2015; Hsu et al., 2019;  
290 König et al., 2011; Uryu et al., 2015). Therefore, we presumed that this mechanism  
291 might also operate in *B. germanica*. The prothoracic gland is the main source of  
292 ecdysteroids in *B. germanica* nymphs whereas the ovary is its main source in the adult  
293 female, as the prothoracic gland degenerates after metamorphosis (Belles, 2020; Pascual  
294 et al., 1992; Romaña et al., 1995). However, it has not been described whether *B.*  
295 *germanica* nymphal ovaries can produce ecdysone/20E, and/or respond to it, and  
296 regulate developmental processes.

297 To examine the response of nymphal ovaries to an ecdysone/20E signal, the expression  
298 of one ecdysone-dependent early gene, *E75A* (Mané-Padrós et al., 2008), was measured  
299 in ovaries of *B. germanica* during the sixth nymphal instar and the adult. The expression  
300 pattern of *E75A* correlates with the profile of ecdysteroids titre in the haemolymph in  
301 last nymphal instar, and with the levels of ecdysteroids in the adult ovary, where the  
302 peak of ecdysone during choriogenesis coincides with the maximal expression of *E75A*  
303 (Figure 5A).

304 Furthermore, the expression of *E75A* was measured in ovaries from 8-day-old *eya*-  
305 depleted last instar nymphs. Results showed that *E75A* expression was upregulated on  
306 average, although the differences between controls and *dseya*-treated were not  
307 statistically significant (Figure 5B). This suggests that *eya*-depletion triggered an  
308 increase of ecdysone signalling in the nymphal ovaries.

309 Although the *B. germanica* adult ovary is able to produce ecdysteroids (Cruz et al.,  
310 2003; Pascual et al., 1992), the expression of steroidogenic genes in the adult ovary has  
311 not been explored. Hence, we measured the expression of *neverland* (*nvd*), *spookiest*  
312 (*spot*), *phantom* (*phm*), *shadow* (*sad*) and *shade* (*shd*), during the sixth nymphal instar  
313 and the adult. The results (Figure 5C) showed that the expression patterns of the  
314 different genes do not correlate with each other, or with the profiles of ecdysteroids.  
315 However, the expression levels are higher in the last nymphal instar than in the adult, in  
316 general. Moreover, the highest expression levels of *nvd* and *shd* appear to coincide with  
317 the maximum peak of ecdysteroids, in the last nymphal instar, and with the highest  
318 content of ecdysteroids in the adult ovary (Figure 5C).

319

320 Furthermore, in the ovaries of 8-day-old *eya*-depleted last instar nymphs, we measured  
321 the expression of *nvd*, *spot* and *shd*, three genes that represent three characteristic steps  
322 of the biosynthesis of 20E, an early step (*nvd*), a step in the so called black box (*spot*)  
323 and that of the transformation of ecdysone into 20E (*shd*) (Niwa and Niwa, 2016; Ou et  
324 al., 2016). Results indicated that the expression of *nvd* and *shd* was not significantly  
325 affected by *eya* depletion, whereas that of *spot* was upregulated (Figure 6A). These  
326 results suggest that *eya* represses the expression of at least *spot*, which might  
327 compromise the ecdysone biosynthesis in the ovaries, thus possibly affecting oogenesis  
328 processes.

329 To test the effects of ecdysteroids on *E75A* expression in the ovaries, we applied 20E to  
330 newly emerged last instar nymphs. Then, we measured the expression of *E75A* in the  
331 ovaries of 8-day-old last instar nymphs. Results showed that *E75A* expression was  
332 significantly upregulated in the ovaries of 20E-treated insects, with a fold change close  
333 to 30 (Figure 6B). This indicates that *E75A* readily respond to 20E in nymphal ovaries,  
334 which suggest that its expression can be used as readout of ecdysteroid changes, as used  
335 in other studies (Colombani et al., 2012; Li et al., 2016).

336 Additionally, results showed that *eya* expression in the ovaries of the 8-day-old last  
337 instar nymphs was not affected by the 20E treatment (Figure 6C). Similarly, the  
338 expression of the steroidogenic genes did not significantly change after 20E treatment  
339 (Figure 6D). Regarding the ovarian follicles in the vitellarium, its number increased  
340 after the 20E treatment (Figure 6E; Table S3). In 6-day-old last instar nymphs, a  
341 significant increase ( $p < 0.002$ ) in the number of differentiated ovarian follicles was  
342 observed. However, two days later, the number of ovarian follicles found in the  
343 vitellarium was very variable (ranging between 3 and 11; Figure 6F and G-H')  
344 compared to the controls (Figures 6E and F), which suggests that some ovarian follicles  
345 underwent cell death. Finally, the effect of 20E on ovarian follicle differentiation was  
346 not limited to the last nymphal instar. Newly emerged adult females that had been  
347 treated with 20E also showed a higher number of differentiated ovarian follicles in  
348 comparison with the respective controls ( $p < 0.002$ ; Figure 6E).

349

#### 350 **4. Discussion**

351 In hemimetabolan species, *eya* was originally described by its involvement in eye  
352 development, which was related to cell proliferation (Dong and Friedrich, 2010; Takagi  
353 et al., 2012). In the present work we have shown the involvement of *eya* in ovary  
354 development in a hemimetabolan species. Depletion of *eya* in the cockroach *B.*  
355 *germanica* prevents the completion of the gonadotrophic cycle, which derives in  
356 females sterility.

357 The phenotypes observed after *eya* depletion in last instar nymphs of *B. germanica*  
358 indicate that this gene acts early in oogenesis, playing distinct roles in different regions  
359 of the panoistic ovary. Indeed, the development of basal ovarian follicles, which usually

360 start to grow and mature during the last nymphal instar, is arrested in *eya*-depleted  
361 females. Moreover, the basal ovarian follicles lost their typical elliptical morphology  
362 and become spherical. A similar phenotype was observed in *N*-depleted females of *B.*  
363 *germanica* (Irles and Piulachs, 2014), and, intriguingly, this phenotype becomes more  
364 conspicuous as the females aged. The fact that *eya* depletion partially phenocopies *N*-  
365 depletion is no surprising, as *N* depletion results in a decrease of *eya* expression (Irles  
366 and Piulachs, 2014). In contrast, the disappearance of the stalks between the ovarian  
367 follicles in the ovariole, which is an additional consequence of *N* depletion (Irles and  
368 Piulachs, 2014), was not observed in *eya*-depleted insects, whose ovaries show a well  
369 formed stalk between the basal and sub-basal ovarian follicles, although the stalk  
370 between the youngest ovarian follicles was frequently absent or undifferentiated.

371 The fat body of *eya*-depleted females is expressing vitellogenin at similar levels than in  
372 controls (results not shown). However, the observations in *eya*-depleted females suggest  
373 that vitellogenin is not incorporated into the growing oocytes, which might be due to the  
374 absence of the corresponding receptor, *VgR*. Intriguingly, abundant *VgR* transcripts  
375 accumulate in ovaries of *eya*-depleted females, whereas the expression of *VgR* mRNA  
376 levels decreases in control females as the oocyte growth, a decrease that coincides with  
377 the increase of *VgR* protein levels in the membrane of basal oocytes (Ciudad et al.,  
378 2006). Taken together, the data suggest that *VgR* translation is prevented in *eya*-depleted  
379 females, explaining why the basal oocytes do not incorporate vitellogenin.

380 The most remarkable phenotype observed in ovarioles from *eya*-depleted females was  
381 the uncontrolled cell proliferation and differentiation in the germaria, resulting in an  
382 increase in the number of differentiated ovarian follicles produced. Interestingly, they  
383 show a notable swelling of the germaria, a phenotype that is reminiscent of that  
384 described in *D. melanogaster eya*-null mutants (Bai and Montell, 2002; Bonini et al.,  
385 1998). The aforementioned swollen shape occurred in these *eya*-null mutant flies  
386 because the development of the maturing egg chamber arrested, but the germaria  
387 continued proliferating (Bonini et al., 1998). This concurrence suggests that the role of  
388 *eya* in the control of stem cell differentiation and proliferation has been evolutionarily  
389 conserved between cockroaches and flies.

390 In *D. melanogaster*, the formation and differentiation of ovarian follicles is triggered by  
391 20E (see Ameku et al., 2017; Belles and Piulachs, 2015; Hsu et al., 2019; König et al.,

392 2011; Uryu et al., 2015). The triggering effect of 20E on somatic and germinal cells in  
393 the germarium, appear ancestral and also operating in less modified insects, where  
394 juvenile hormone plays a gonadotropic role (Belles et al., 2000; Comas et al., 2001;  
395 Treiblmayr et al., 2006). The source of ecdysone in adult *B. germanica* females is the  
396 ovary, and at this age, their main function is to promote chorion synthesis in mature  
397 basal ovarian follicles (Pascual et al., 1992; Romaña et al., 1995). However,  
398 steroidogenic genes are expressed in ovaries of last instar nymphs of *B. germanica*,  
399 which suggest that immature ovaries of this species also synthesise ecdysone.

400 In the germarium of *D. melanogaster*, ecdysone signalling controls de quantity, but not  
401 the differentiation status of germinal stem cells. In fact, the latter may be mediated by  
402 the Notch pathway. Ecdysone signalling induces *Dl* expression at the terminal filament  
403 in cell membranes, which activates *N* and determines the fate of these cells, which can  
404 become cap or escort cells (Ameku et al., 2017; Green et al., 2011; Hsu et al., 2019).  
405 Our results in *B. germanica* suggest that similar signalling networks can occur in  
406 panoistic ovaries. When *eya* is depleted *Dl* expression significantly increase and *N*  
407 expression becomes activated, again suggesting that ecdysone levels had increased.  
408 Thus, the signalling pathway found in *B. germanica* appears equivalent to that described  
409 in *D. melanogaster*, and, significantly, *eya*-depletion in cockroaches results in swollen  
410 germaria in the ovarioles, as occurs in the fruit fly.

411 The overexpression of steroidogenic genes in the ovaries of *eya*-depleted nymphs  
412 suggests that *eya* regulates the proliferation of ovarian follicles in the *B. germanica*  
413 ovary by controlling the steroidogenic pathway. This idea is in line with the expression  
414 of *eya* in adult ovaries, in which the decrease of *eya* levels at the end of the  
415 gonadotrophic cycle coincides with the increase of ovarian ecdysone at this age  
416 (Romaña et al., 1995). This ecdysone increase triggers the production of chorion  
417 proteins in mature basal ovarian follicles (Pascual et al., 1992).

418 Ectopic treatment with 20E gave similar results to those obtained after *eya* depletion:  
419 ovarian follicle proliferation, and swelling of the germaria. However, *eya* expression  
420 was not modified by 20E treatment, which indicates that *eya* regulates the activity of the  
421 steroidogenic pathway but is not controlled by 20E.

422 In summary in *B. germanica*, *eya* as a downstream component of the Notch pathway,  
423 regulates the correct cell proliferation and cell fate in the follicular epithelia of basal

424 ovarian follicles. While in the germarium and the terminal filament, *eya* acts on somatic  
425 and germinal cells regulating their differentiation and proliferation, by controlling  
426 ecdysone signalling. Important from an evolutionary point of view, these functions are  
427 equivalent, possibly homologous, to the functional duality of *eya* reported in the most  
428 derived fly *D. melanogaster*.

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438

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582  
583

## 584 **Figure Legends**

585 **Figure 1. Expression of *eya* in the ovaries of *Blattella germanica* and effects of *eya***  
586 **depletion. A.** Expression pattern of *eya* in ovaries in sixth instar nymphs and adult  
587 females during the first gonadotropic cycle. Data represent copies of *eya* mRNA per  
588 1000 copies of *actin-5c* and are expressed as the mean  $\pm$  S.E.M. (n= 3-6). Profiles of  
589 ecdysteroids titer in the haemolymph (grey dashed line), and ecdysteroids content in the  
590 ovaries (grey solid line) are also shown (ecdysteroids data from Cruz et al., 2003;

591 Pascual et al., 1992; Romaña et al., 1995). **B.** Expression of *eya* in ovaries of 8-day-old  
592 (N6D8) sixth instar nymph; 3-day-old adult (Add3) and 5-day-old adult (Add5) that  
593 were treated with *dseya* in freshly emerged sixth nymphal instar. Data represent  
594 normalized values against dsMock-treated insects (reference value 1), and are expressed  
595 as the mean  $\pm$  S.E.M. (n = 3). The asterisks indicate statistically significant differences  
596 with respect to controls: \*, p = 0.031 and \*\*, p= 0.0001). **C.** Width (BOF-W) and the  
597 length (BOF-L) of basal ovarian follicle in *eya*-depleted N6D8, Add3 and Add5  
598 females. The asterisk indicates statistically significant differences with respect to  
599 controls: \*, p < 0.0001). **D.** Ovarioles from N6D8 dsMock-treated female. **E.** Ovariole  
600 from an N6D8 *dseya*-treated female.

601

602 **Figure 2. Effects of *eya* depletion in 8-day-old sixth instar nymphs of *Blattella***  
603 ***germanica*.** **A.** Number of follicular cells in basal ovarian follicles from dsMock- and  
604 *dseya*-treated females; N6D8: 8-day-old sixth instar nymph; Add3: 3-day-old adult;  
605 Add5: 5-day-old adult; the asterisks indicate statistically significant differences with  
606 respect to controls: \*, p = 0.002 and \*\*, p= 0.0001). **B.** Ovariole from an 8-day-old  
607 dsMock-treated nymph; the actively dividing follicular cells are labelled with anti-  
608 phospho-histone 3 (PH3) antibody (in B', the isolated channel showing the PH3  
609 labelling is shown; the outline of the ovarioles has been highlighted with a dashed line  
610 for clarity). **C.** Ovarioles from an 8-day-old *dseya*-treated nymph showing a few number  
611 of cells dividing (in C' the isolated channel showing the PH3 labelling is shown; the  
612 outline of the ovarioles has been highlighted with a dashed line for clarity); BOF: basal  
613 ovarian follicle. **D-E.** Follicular epithelia from 8-day-old dsMock-treated nymphs; In D,  
614 the follicular cell nuclei, stained with DAPI, are shown; some mitotic figures are visible  
615 (arrowheads); In E, the F-actin microfilaments, stained with TRITC, appear uniformly  
616 distributed in the cell membranes. **F-G.** Follicular epithelia from 8-day-old *dseya*-  
617 treated nymphs. In F, the follicular cell nuclei, stained with DAPI, are shown  
618 evidencing differences in size and form with absence of mitosis; in G, the F-actin  
619 microfilaments stained with TRITC, in basal ovarian follicles display a non-uniform  
620 distribution.

621 **Figure 3. Effects of *eya* depletion in 5-day-old adult ovary of *Blattella germanica*.**  
622 **A.** Ovariole from a dsMock-treated female. **B-C.** Ovarioles from *dseya*-treated females

623 showing different degrees of malformation; BOF: Basal ovarian follicle. **D.** Follicular  
624 epithelia of dsMock-treated females showing the binucleated cells and a high degree of  
625 patency; D' shows the nuclei and D'' the cytoskeleton of F-actin. **E.** Follicular epithelia  
626 of dseyatreated females showing cells of different size and morphology, mostly  
627 mononucleated; E' shows the nuclei stained with DAPI, and E'' the cytoskeleton of F-  
628 actin, showing a uniform distribution on cell membranes and no signs of patency;  
629 arrowheads indicate a few binucleated cells.

630 **Figure 4. Effects of *eya* depletion on ovarian follicle differentiation in *Blattella***  
631 ***germanica*.** **A.** Number of ovarian follicles in the vitellaria from dsMock- and dseyatreated  
632 females; ovarian follicles were quantified including the subbasal and all released  
633 follicles from the germarium; data from 5-day-old *N*-depleted ovarioles is also shown  
634 (obtained from Irls et al., 2016 and Irls and Piulachs, 2014); data is expressed as the  
635 mean  $\pm$  S.E.M (n = 13-50) (see also Table S3); different letters indicate statistically  
636 significant differences with respect to controls (p < 0.0001). N6D0, N6D6 and N6D8: 0-  
637 day-old, 6-day-old and: 8-day-old sixth instar nymphs respectively; AdD3 and AdD5:  
638 3-day-old and 5-day-old adult female, respectively. **B.** Ovariole from a N6D8 dsMock-  
639 treated female; BOF: basal ovarian follicle; sBOF: subbasal ovarian follicle. **C.**  
640 Vitellarium and germarium of a dsMock-treated AdD5 ovariole. **D.** Ovarioles from  
641 N6D8 dseyatreated female. **E.** Vitellarium and germarium from an N6d8 dseyatreated  
642 female. **F.** Ovariole from an AdD5 dseyatreated female. **G.** Ovariole from AdD5; ds*N*  
643 was applied on N6D8 (see Irls et al., 2016); from B to G, the nuclei from follicular  
644 cells were stained with DAPI, F-actin microfilaments with TRICT-Phalloidin and the  
645 nucleus from germinal cells were labelled with *eya* 10H6 antibody (Anti-*Eya*). **H.**  
646 Expression of the main components of Notch pathway in ovaries from 5-day-old dseyatreated  
647 adults. **I.** Expression of *hpo* and *yki* in ovaries from 5-day-old dseyatreated  
648 adults. **J.** Expression of *nos*, *vas* and *Yb* in ovaries from 8-day-old dseyatreated nymphs  
649 and 5-day-old treated adults; from H to J data represent normalized values against  
650 dsMock (reference value 1), and are expressed as the mean  $\pm$  S.E.M. (n = 3); the  
651 asterisk indicates statistical significant differences with respect to controls (p < 0.02).

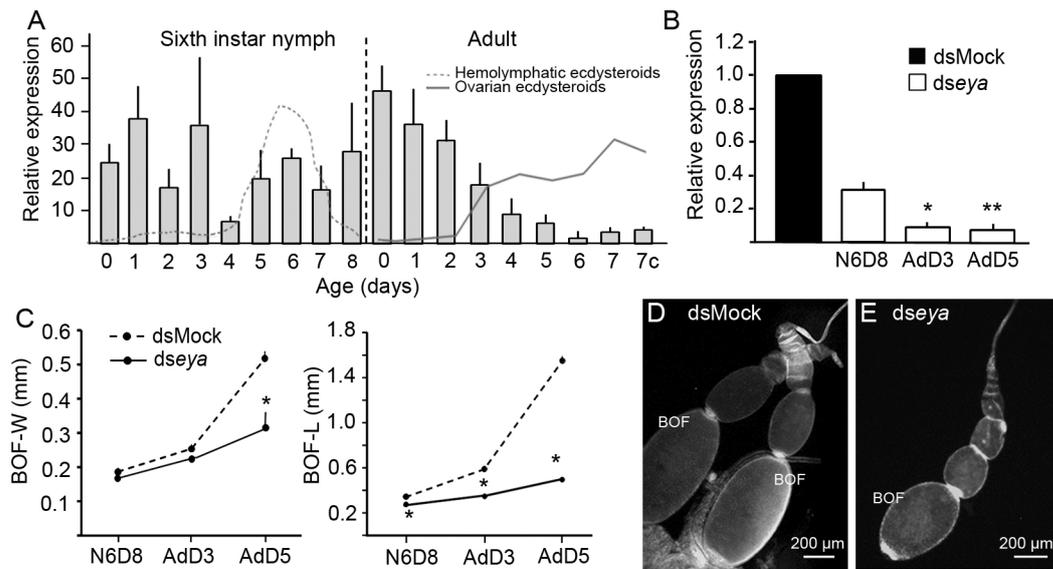
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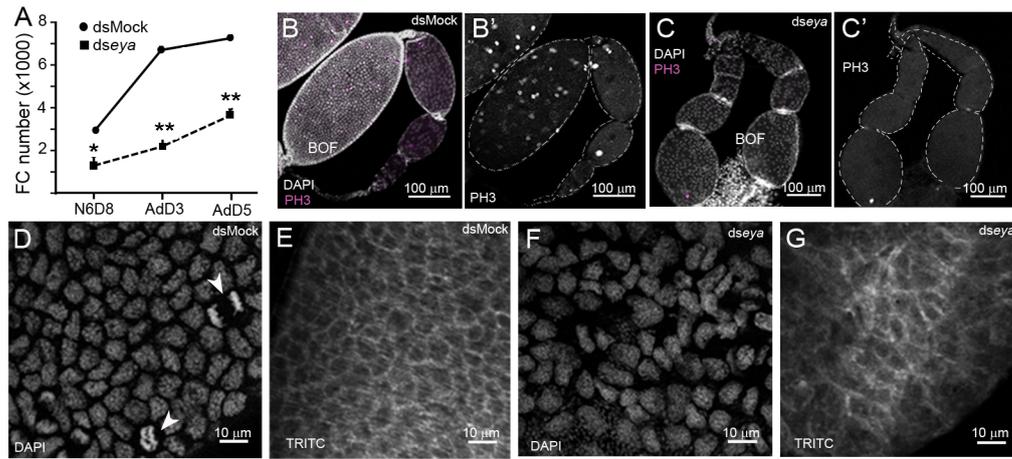
653 **Figure 5. Expression of *E75A* and steroidogenic genes in the ovaries of *Blattella***  
654 ***germanica*.** **A.** The expression pattern of *E75A* in ovaries of sixth instar nymphs and  
655 adult females. **B.** Expression of *E75A* in ovaries from 8-day-old dseyatreated nymphs

656 (N6D8); data represent normalized values against dsMock (reference value 1), and are  
657 expressed as the mean  $\pm$  S.E.M. (n = 3). **C.** The mRNA expression patterns of  
658 *neverland*, *spookiest*, *phantom*, *shadow* and *shade*, in ovaries of sixth instar nymphs and  
659 adult females; in A and C, the profiles of ecdysteroid titer in the haemolymph (grey  
660 dashed line), and ecdysteroid content in the ovaries (grey solid line) are also shown  
661 (data from Cruz et al., 2003; Pascual et al., 1992; Romaña et al., 1995); data represent  
662 copies of mRNA per 1000 copies of *actin-5c* (relative expression) and are expressed as  
663 the mean  $\pm$  S.E.M. (n= 3-6).

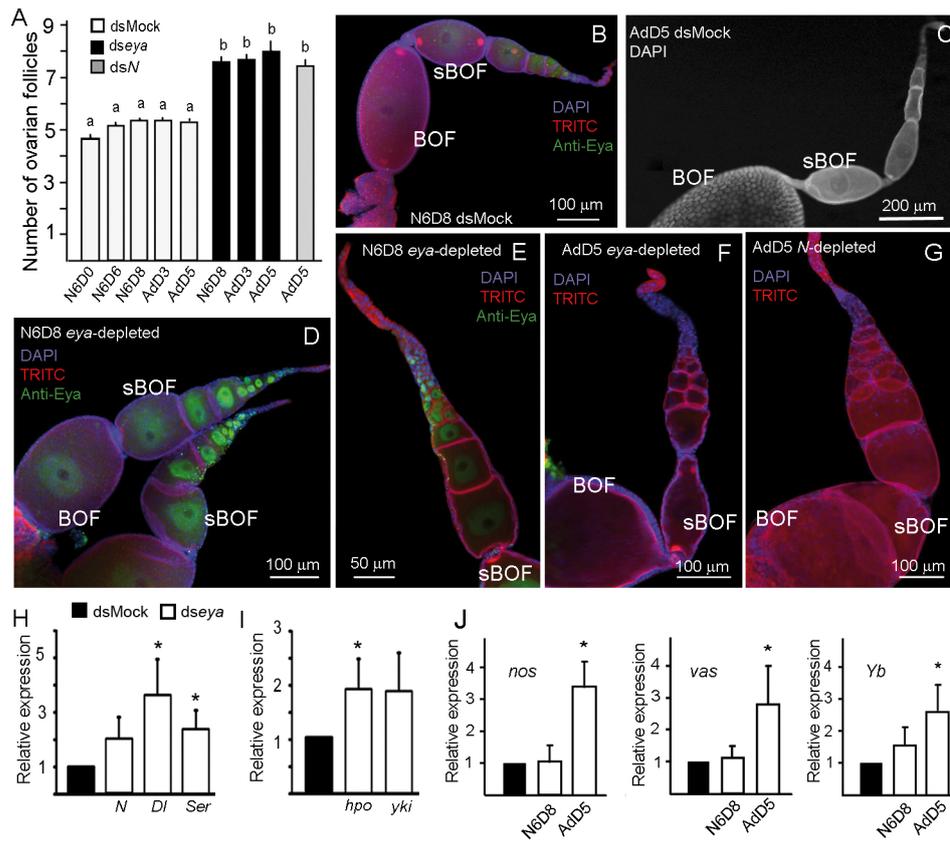
664 **Figure 6. Effects of 20E treatment on ovarian development in *Blattella germanica*.**

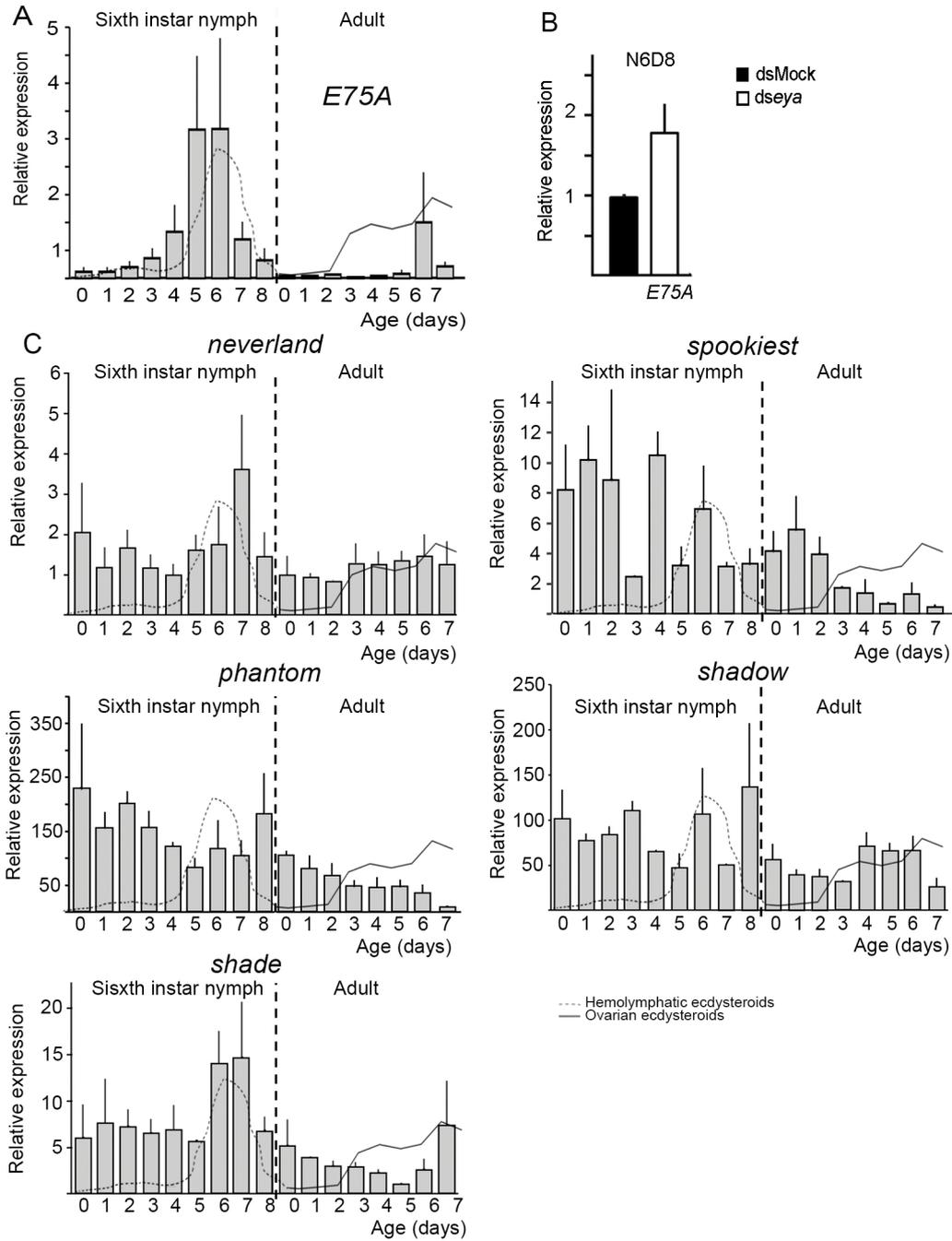
665 **A.** Expression levels of *nvd*, *spot* and *shd*, in ovaries from N6D8 dseya-treated females;  
666 data represent normalized values against the control (reference value 1) and are  
667 expressed as the mean  $\pm$  S.E.M. (n = 4 -10); the asterisk indicates statistical significant  
668 differences with respect to controls (p <0.002). **B.** Expression levels of *E75A* in ovaries  
669 from N6D8 treated with 20E; data represent normalized values against the control  
670 (reference value 1) and are expressed as the mean  $\pm$  S.E.M. (n = 4 - 10); the asterisk  
671 indicates statistical significant differences with respect to controls (p <0.001). **C.**  
672 Expression of *eya* in nymphal ovaries from females treated with 20E. **D.** Expression  
673 levels of *nvd*, *spot*, *phm* and *sad* in ovaries from N6D8 treated with 20E; data represent  
674 copies of mRNA per 1000 copies of *actin-5c* and are expressed as the mean  $\pm$  S.E.M. (n  
675 = 3-4). **E.** Box plot representing the number of ovarian follicles localized in the  
676 vitellarium and germarium in 20E-treated females, the asterisk indicates statistical  
677 significant differences with respect to controls (p < 0.002; n.s. no significant;n= 20-50).  
678 **F.** Ovarirole from a control N6D8; BOF: Basal ovarian follicle, sBOF: subbasal ovarian  
679 follicle. **G.** Ovarirole from a N6D8 20E-treated female, showing a reduced number of  
680 ovarian follicles released from the germarium; in the inset, the germarium is show at  
681 higher magnification. **H.** Ovarirole from a N6D8 20E-treated female, showing a high  
682 number of ovarian follicles released from the germarium. **I.** Detail of the germarium  
683 from panel H, at higher magnification showing the differentiated ovarian follicle;  
684 samples were stained with phalloidin-TRITC to show the F-actin microfilaments. From  
685 B to I, last instar nymphs and adult females were treated with 10  $\mu$ M 20E at the day of  
686 emergence to the respective instar, whereas controls were treated with a solution of 10%  
687 EtOH; N6D6: 6-day-old sixth instar nymph; N6D8: 8-day-old sixth instar nymph;  
688 AdD5: 5-day-old adult female.

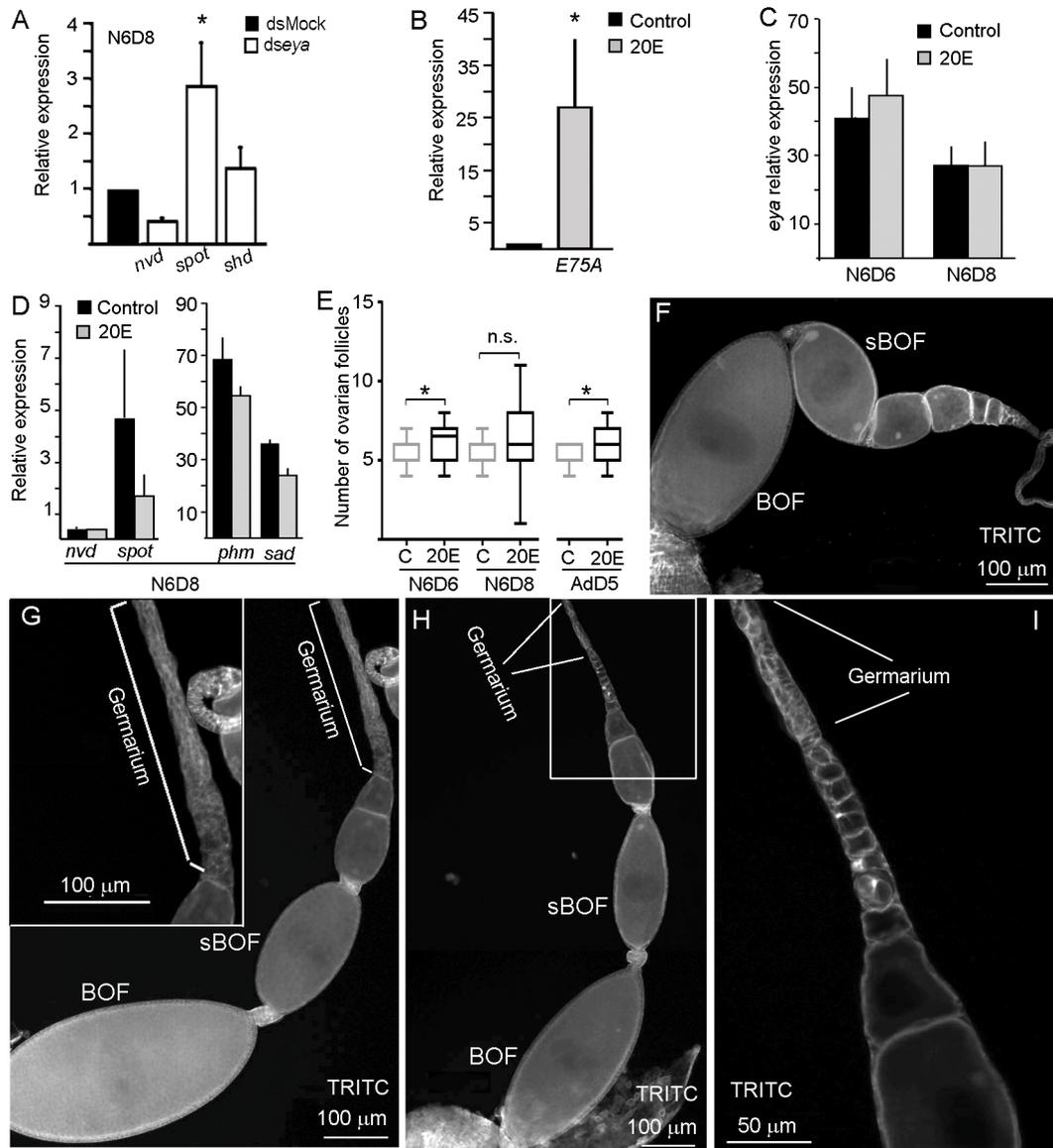


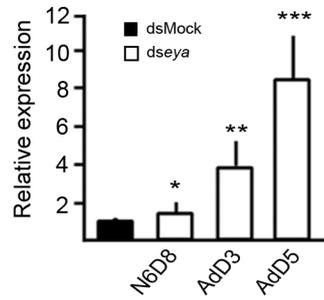




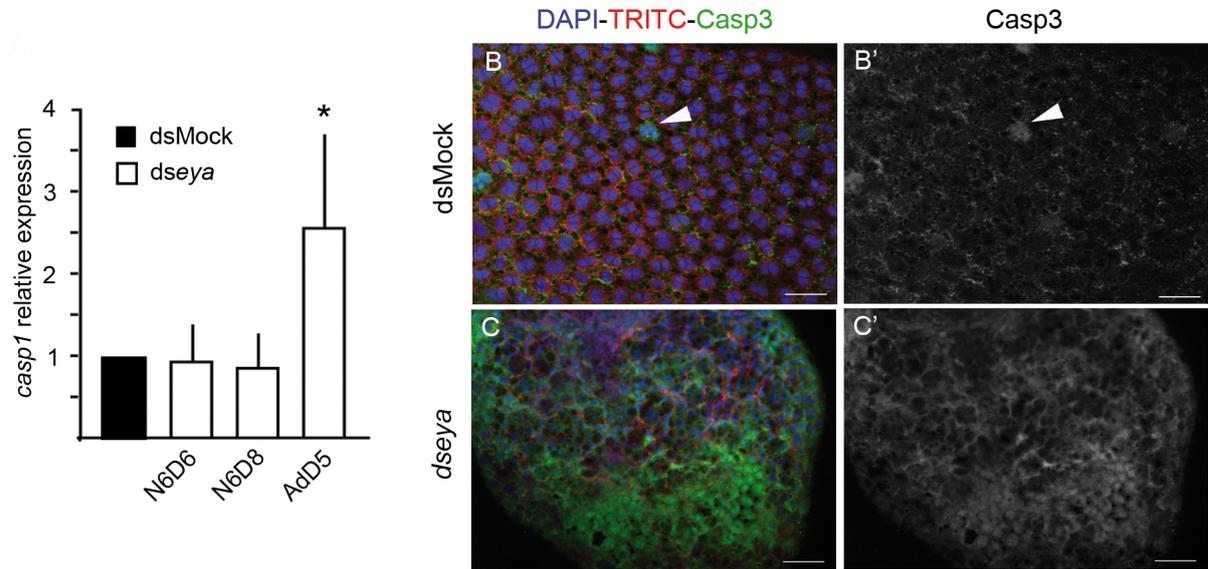








**Figure S1.** Expression of *Vitellogenin receptor* in ovaries from *eya*-depleted females. Newly emerged last instar nymphs were treated with *dseya* and ovaries dissected at different ages. N6D8: 8-day-old sixth instar nymph; AdD3: 3-day-old adult; AdD5: 5-day-old adult. Data represent normalized values against the control (reference value 1) and are expressed as the mean  $\pm$  S.E.M. (n = 3). The asterisks indicate statistically significant differences (t-test) with respect to controls: \*, p = 0.012; \*\*, p = 0.022; \*\*\*, p = 0.001.



**Figure S2. Caspase activity in *eya*-depleted ovaries.** A. Expression of *caspase 1* (*casp1*) in ovaries from *eya*-depleted females. Newly emerged last instar nymphs were treated with *dseya* RNA, and ovaries dissected at different ages. N6D6: 6-day-old sixth instar female; N6D8: 8-day-old sixth instar female; AdD5: 5-day-old adult female. Data represent normalized values against the control (reference value 1) and are expressed as the mean  $\pm$  S.E.M. ( $n = 3$ ). The asterisks indicate statistically significant differences (t-test) with respect to controls:  $p < 0.001$ . B. Follicular epithelium in basal ovarian follicle from a 5-day-old dsMock-treated female. The uniform distribution of binucleated follicular cells is shown. Only some sporadic nuclei (arrowhead) appeared labelled by the anti-Casp3 antibody (green). In B' the labelling for Casp3 is shown. C. Follicular epithelium in basal ovarian follicle from a 5-day-old *eya*-depleted female, most of the nuclei appeared labelled for Casp3. In C' nuclei labelled for Casp3 are shown. In all images, the posterior pole of the basal follicle is towards the bottom. Scale bars: 50  $\mu$ m.

**Table S1:** Primer sequences used for quantitative real-time PCR (qRT-PCR) and RNAi experiments. Actin-5c is the housekeeping gene use in expression studies. F: Primer forward; R: Primer reverse

Primer name			Primer Sequence (5'-3')	Accession number
<i>eya</i>	dsRNA1	F R	TTTTGGATCTGACGGCTTTC GCAAGGGCTGGAACACTACTG	PSN54252.1
<i>eya</i>	dsRNA2	F R	GGCTCTTAGGCACAAAACGA GCAGCTTCTTCATCCTGTCC	PSN54252.1
<i>eya</i>	qRT-PCR	F R	GAGGCATTTTTCCGATTGAA GCAGCTTCTTCATCCTGTCC	PSN54252.1
<i>VgR</i>	qRT-PCR	F R	ACCAACTCCACAAGGACCAC AACGGATCTGCACCTGTAGC	CAJ19121.1
<i>casp1</i>	qRT-PCR	F R	AAGCGGAAGGATTCATACCA GATGACTGCCTTGCCCTCTTC	CEP28036.1
<i>vas</i>	qRT-PCR	F R	GAAACGAACCGCTGACTTTAT CACTCCCATTCGTCCATTCT	PSN55909.1
<i>nos</i>	qRT-PCR	F R	ATTGTCCAGAGTTTCAACTTAAT CCTGTTTCTTTGAACGCTTCTT	PSN32832.1
<i>Yb</i>	qRT-PCR	F R	CGAAACAACCTCCACCGTTTT CTCCGCATGCCATTTTAACT	PSN55645.1
<i>N</i>	qRT-PCR	F R	GCTAAGAGGCTGTTGGATGC TGCCAGTGTTGTCCTGAGAG	HF969255
<i>Dl</i>	qRT-PCR	F R	CCACTACAAGTGTTCGCCAA TACCTCTCGCATTTCGTCACA	HF969256
<i>Ser</i>	qRT-PCR	F R	TCCTCTTGGCAGTGCATTTG CTTGATCACAGAGGATGCCG	HG515375.1
<i>hpo</i>	qRT-PCR	F R	GACATTTGGAGCCTTGGCAT AGGTTTCCCTTCAGCCATTTT	HF969251
<i>yki</i>	qRT-PCR	F R	TCCCTACCACACACACCAGA GACCATCCAATGTTGCCATA	HF969253
<i>sad</i>	qRT-PCR	F R	ATGAGGAGGTTTCAGGGTGTG CTGGCCAGAAGTCATTTGGT	PSN51657.1
<i>phm</i>	qRT-PCR	F R	CTAGGCACCAGACACCTTC GCAAGCACTGTGTCTTCCAA	PSN36025.1
<i>spot</i>	qRT-PCR	F R	GAAGTTCAAATGCGAGCACA GCAATGGAAGTGTCTGGTT	PSN53270.1
<i>shd</i>	qRT-PCR	F R	CACAGAGGCGCACAAGTTTA GTTCCCCTTCAAAGTCCACA	PSN43891.1
<i>nvd</i>	qRT-PCR	F R	CTGGGGCCAGTCACAATACT GCAGGGGCTTGTCAATGTAT	PSN31862
<i>E75A</i>	qRT-PCR	F R	GTGCTATTGAGTGTGCGACATGAT TCATGATCCCTGGAGTGGTAGAT	CAJ87513.1
<i>actin-5c</i>	qRT-PCR	F R	AGCTTCCTGATGGTCAGGTGA TGTCGGCAATTCCAGGGTACATGGT	AJ862721

**Table S2. *eya* depletion in oviposition.** Newly emerged sixth instar nymphs (N6D0) females were treated with *dseya* or *dsMock* and left to oviposit. The day of oviposition (from the day of adult emergence), the number of females that oviposit, the days that the oothecae were transported and the number of fertile oothecae were recorded.

Treatment (day of treatment)	n	Days to oviposit	% Females that oviposit	Days of oothecae transport	% Fertile oothecae
<i>dsMock</i> (N6D0)	40	7.68 ± 0.13	100	17.92 ± 0.11	100
<i>dseya</i> (N6D0)	36	-	0	-	-

**Table S3.** Number of ovarian follicles (OF) in the vitellaria from dsMock-, *dseya*- and 20E treated females, were quantified at different ages. Data was recorded from different ovarioles (n) belonging to 7-10 females. Considering those OF from the subbasal position to the youngest that has left the germarium, both included.

Treatment	Age	n	OF (mean $\pm$ SEM)
<b>dsMock (N6D0)</b>	N6D0	22	4.68 $\pm$ 0.166
	N6D6	50	5.48 $\pm$ 0.131
	N6D8	30	5.37 $\pm$ 0.122
	AdD3	31	5.32 $\pm$ 0.169
	AdD5	28	5.32 $\pm$ 0.126
<b>dseya (N6D0)</b>	N6D8	50	7.60 $\pm$ 0.191
	AdD3	13	7.69 $\pm$ 0.208
	AdD5	13	8.00 $\pm$ 0.408
<b>20E (N6D0)</b>	N6D6	18	6.28 $\pm$ 0.289
	N6D8	39	6.10 $\pm$ 0.319
<b>20E (AdD0)</b>	AdD5	28	6.20 $\pm$ 0.205