Different roles of *eye absent* in the basal ovarian follicle and germarium of developing cockroach ovaries

Ramos, S.[1], Chelemen, F.[1], Pagone, V. [1], Elshaer, N.[1,2], Irles, P.[1,3], Piulachs, M.D.[1,*]

[1]Institut de Biologia Evolutiva (UPF-CSIC), Passeig Maritim de la Barceloneta, 37, 08003 Barcelona, Spain. [2] Department of Plant Protection, Faculty of Agriculture, Zagazig University, Egypt. [3] Instituto de Ciencias Agronómicas y Veterinarias, Universidad de O´Higgins, Chile.

*Corresponding author:

MDP mdolors.piulachs@ibe.upf-csic.es

Phone: +34 932 309 648

Fax: + 34 932211011

Abstract

Eye absent (Eya) is a protein which has been structurally conserved from hydrozoans to humans which has two functions: it is both a transcription cofactor and a protein tyrosine phosphatase. Eya was first described in the fly *Drosophila melanogaster* for its role in eye development, and the same functions were also later reported in less derived insects. Studies on the involvement of Eya in insect oogenesis are limited to *D. melanogaster*, which has meroistic ovaries. In this fly, Eya plays a fundamental role in the first stages of ovarian development because Eya mutations abolish gonad formation.

In this present work we studied the function of Eya in the panoistic ovary of the cockroach *Blattella germanica*. We demonstrated that Eya is essential for correct ovary development also in this ovary type. In *B. germanica* ovaries, Eya affects both somatic and germinal cells in the germarium and the vitellarium, acting differently in different ovarian regions. Development of the basal ovarian follicles is arrested BgEya-depleted females, while in the germaria, BgEya helps to the maintain the correct number of somatic and germinal stem cells by regulating the expression of ecdysteroidogenic genes in the ovary.

KEYWORDS: panoistic ovary, ecdysone, Halloween genes, cell proliferation, insect oogenesis, Notch

1. Introduction

Maintaining the stability of stem cells is crucial in every organism, and this is especially important in the case of germ stem cells. Oogenesis describes the process of ovary development from the time of germ stem cell differentiation until oocyte maturation. During oogenesis, oocytes must synthesize and accumulate all maternal factors needed by embryos to complete their development, thus ensuring reproductive success.

In insect ovaries, germ stem cells are located in niches in the germarium of each ovariole. The control of their proliferation and differentiation has been thoroughly studied, mainly in species with meroistic polytrophic ovaries such as the fruit fly *Drosophila melanogaster* (see Ameku et al., 2017; Belles and Piulachs, 2015; Dai et al., 2017). In contrast, the number of genes involved in regulating oogenesis so far identified in less modified panoistic ovaries such as those of the cockroaches including *Blattella germanica* is very limited. This prevents study of the mechanisms that control oocyte growth and maturation in these basal insects. *B. germanica* is emerging as a choice model in which to study panoistic ovaries. In this cockroach, each ovary has around 20 ovarioles and only the most basal ovarian follicle of each ovariole matures during a given gonadotrophic cycle, a process that starts early in the last nymphal instar. The basal ovarian follicles are almost ready to mature in freshly ecdysed females, whereas the development of the remaining ovarian follicles of each ovariole is arrested until these basal ones are oviposited.

In previous contributions dealing with the regulation of oogenesis in panoistic ovaries, we have studied the function of Notch in the ovary of *B. germanica* and its interactions with the EGFR signalling pathway (Elshaer and Piulachs, 2015; Irles et al., 2016; Irles and Piulachs, 2014). Given that the Notch pathway participates in the control of germ cell proliferation, here we postulated that the main effectors of this function would be downstream genes in the same pathway, and thus *eye absent* (*eya*) might play a key role in this process.

The *Eya* gene has been structurally conserved from hydrozoans to humans (Duncan et al., 1997; Graziussi et al., 2012; Jemc and Rebay, 2007). It was first described in *D. melanogaster* for its role in eye development by determining cell fates (differentiation or death) in early stages of postembryonic development (Bonini et al., 1993).

Subsequently, *eya* orthologues have been found in vertebrates and in other phyla (Duncan et al., 1997; Graziussi et al., 2012; Zimmerman et al., 1997), and a wide range of functions have been reported for its corresponding protein.

The Eya protein has two functions: It was first described as transcriptional cofactor which is recruited to transcriptional complexes via the eya domain (ED), a conserved C-terminal motif that interacts with the Six family DNA binding proteins (Jemc and Rebay, 2007). Different research groups subsequently reported that the ED has intrinsic protein tyrosine phosphatase activity, leading eya to be described as an example of a new class of eukaryotic protein phosphatases (see Rebay, 2015 and references therein).

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

In contrast, studies on the functions of *eya* in insect oogenesis are limited to *D. melanogaster*, and its possible role in other insect ovary types remains untested. Therefore, in this present work we aimed to provide further information about the role of this peculiar protein in panoistic ovaries. We used the cockroach *B. germanica* as a model, and focussed on the regulation of stem cell proliferation and differentiation in this species.

2. Material and Methods

2.1. Cockroach colony and sampling

Adult females of the cockroach *B. germanica* (L.) were obtained from a colony fed *ad libitum* on Panlab dog chow and water, and reared in the dark at 29 ± 1ºC and 60–70% relative humidity. Freshly ecdysed adult females were selected and used at appropriate ages. Mated females were used in all experiments (the presence of spermatozoa in the spermatheca was assessed to confirm that mating had occurred). All dissections and tissue samplings were performed on carbon dioxide-anaesthetized specimens.

2.2. RNA extraction and expression studies

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

The expression pattern of the different *B. germanica* genes was determined by quantitative real time PCR (qRT-PCR) in ovaries from sixth instar nymph and adults. One ovary pair, for adults, or pools of two ovary pairs for nymphs, for every chosen age were used. The expression levels in treated individuals were quantified individually. PCR primers used in qRT-PCR expression studies were designed using the Primer3 v.0.4.0 (Rozen and Skaletsky, 2000). The actin-5c gene of *B. germanica* (Accession number AJ862721) was used as a reference for expression studies. qRT-PCR reactions were made using the iTaq Universal SYBR Green Supermix (BioRad) containing 200 nM of each specific primer (performed in triplicate). Amplification reactions were carried out at 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 30 s, using MyIQ Single Color RTPCR Detection System (BioRad). After the amplification phase, levels of mRNA were calculated relative to BgActin-5c. Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA. The primer sequences used to quantify gene expression are indicated in table S1.

2.3. RNAi experiments

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

2.4. 20-Hydroxyecdysone treatments

Newly emerged last instar nymphs or adult females, were injected with 1µL of a 10mM 20-Hydroxyecdysone (20E) (10% Ethanol) just after the moult to the corresponding stage. Nymphs were dissected when they were 6-day-old, just when ecdysone in the hemolymph reaches the highest levels, or when they were 8-day-old just before the moult to adult. Adult females were dissected when they were 5-day-old, before choriogenesis begins.

2.5. Immunochistochemistry

After dissection, ovaries were immediately fixed in paraformaldehyde (4% in PBS) for 2h. Washing samples and antibody incubations were performed as previously described (Irles and Piulachs, 2014). The primary antibody employed were rabbit antibody anti-PH3 (Cell Signaling Technology, Denver, MA; dilution 1:250), and mouse antibody anti-Eya, (deposited to the DSHB by Benzer, S. / Bonini, N.M.; product eya10H6; dilution 1:50) as nuclear marker of germ cells. However, we cannot asses a BgEya labelling, since there is not decrease of BgEya labelling in dsRNA treated animals. The secondary antibodies used were Alexa-Fluor 647 conjugated donkey anti-rabbit IgG, or Alexa-Fluor 647 conjugated goat anti-mouse IgG (Molecular Probes, Carlsbad, CA). Ovaries were incubated at room temperature for 20 min in 300 ng/ml phalloidin-TRITC (Sigma) and then for 5 min in 1 µg/ml DAPI (Sigma) PBT. After three washes with PBT, ovaries were mounted in Mowiol (Calbiochem, Madison, WI, USA) and observed using a Zeiss AxioImager Z1 microscope (Apotome) (Carl Zeiss MicroImaging).

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

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

2.5. Statistics

Quantitative data are expressed as mean ± standard error of the mean (S.E.M.). Statistical differences between morphometric data were evaluated using the ANOVA or the T-student test using IBM SPSS statistics software. Comparisons of gene expression between treatment and control groups were made using the Pair-Wise Fixed Reallocation Randomization Test (which makes no assumptions about distributions) (Pfaffl et al., 2002), employing REST 2008 v. 2.0.7 software (Corbett Research).

3. Results

3.1. Eya in *Blattella germanica* ovaries and efficiency of RNAi treatments

In the *B. germanica* ovaries, BgEya is expressed through the gonadotrophic cycle (Figure 1A); its expression is remarkably variable in the ovaries of last instar nymphs, but peaks just after they moult into adults. BgEya expression in the ovary then begins to decline and bottoms out when choriogenesis starts. This expression pattern suggests that BgEya plays important functions in the early steps of oogenesis. To examine this possibility we started by using RNAi to investigate the possible functions of BgEya in this process. Newly emerged last instar nymph females, were treated with dsBgEya (n = 36) or dsMock (n = 40). All the treated nymphs correctly reached the adult stage and so we inferred that this treatment did not affect moulting. However, all of the dsBgEya-treated adult females failed to oviposit (Table S2). Thus, we treated new batches of 0-day-old sixth instar nymphs with dsBgEya and transcript depletion was assessed in their ovaries at different ages. The BgEya mRNA levels were lowest (74 % decrease; P(H1) = 0.097), at the end of the nymphal stage (8-day-old sixth instar nymphs), but also remained low in 3-day-old and 5-day-old adult dsBgEya-treated females (P(H1) = 0.031 and P(H1) = 0.0001, respectively, Figure 1B).

196

197 **3.2. *Eya* is involved in basal ovarian follicles growth and maturation.**

198 The growth of the basal ovarian follicles in BgEya-depleted females was slowed, and
199 moreover, their general shape became spherical (Figure 1C, D and E). Furthermore,
200 there were significantly fewer follicular cells in the basal ovarian follicles of BgEya-
201 depleted females compared to dsMock-treated females (Figure 2A), because fewer
202 mitotic divisions had occurred in the follicular epithelia (Figure 2B and C).

203 The nuclei size, cell shape, and distribution of these follicular cells within the epithelia
204 was also affected in BgEya-depleted females (Figure 2D and F). Additionally, F-actins
205 appeared to be concentrated at the junctions between follicular cell membranes, which
206 could explain the variations we have observed in cell morphology (Figure 2E and G).

207 These morphological changes became more conspicuous over time and so in more
208 mature females, basal ovarian follicles with different degrees of affectation were
209 observed in the same ovary in more mature females (Figure 3A-C). In 5-day-old adult
210 dsMock-treated females, all the follicular cells were binucleated and polyploid, and no
211 further cell divisions had occurred (Figure 3D – D'), while in dsBgEya-depleted
212 females F-actins were distributed on the cell membranes and appeared concentrated in
213 the expansions connecting adjacent cells (Figure 3D'').

214 This occurs in a peculiar moment of the follicular epithelium development, when the
215 follicular cells contract to leave large intercellular spaces (a phenomenon called
216 patency, see Davey and Huebner, 1974), thus allowing the vitellogenic proteins to reach
217 the oocyte membrane to be uptaken by the oocyte through a specific receptor. In
218 addition, in these 5-day-old adult BgEya-depleted females, only a few of the follicular
219 cells were binucleated, therefore indicating that they were unsynchronised (Figure 3E,
220 E'). In addition, these cells showed a high variability in size and shape, but never
221 showed signs of patency (Figure 3E'').

222 Furthermore, vitellogenin receptor (VgR) mRNA was upregulated in ovaries from
223 BgEya-depleted females at all of the three ages studied: the last day of last nymphal
224 instar (8-day-old) and in 3-day- and 5-day-old adults ($P(H1) = 0.012$, $P(H1) = 0.022$,
225 and $P(H1) = 0.001$; Figure S1). This is, when the levels of VgR mRNA levels are

already currently high in the ovaries of newly emerged last instar nymphs, although they subsequently decreases to very low levels in adult females (Ciudad et al., 2006).

The modification of basal ovarian follicle shape, together with the phenotypes observed in follicular cells and the unexpected increase in VgR expression in 5-day-old adult ovaries, indicates that although these ovarian follicles seemed ready to mature, they would not have grown and appeared to starting along an apoptotic pathway. To test this conjecture, we measured the expression of the effector *Caspase-1* in ovaries from BgEya-depleted females. In both, 6-day-old and 8-day-old sixth instar nymphs, the levels of ovarian *Caspase*-1 mRNA were similar in dsMock-treated and BgEya-depleted females (Figure S2). However, *Caspase-1* expression significantly increased in the ovaries of 5-day-old adult BgEya-depleted females (Figure S2), thus confirming that basal ovarian follicles are compromised at this developmental stage.

3.3. BgEya depletion affects somatic and germinal cells, increasing the rate of ovarian follicles differentiation.

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

In this line, BgEya depletion resulted in changes at the germarium level and consequently, also in the vitellarium. Compared to dsMock-treated females, at least two extra ovarian follicles differentiated and were released into the vitellarium in ovaries from 8- day-old BgEya-depleted sixth instar nymphs (Figure 4A and D-E; Table S3). These extra ovarian follicles were maintained in adult females and even in 5-day-old BgEya-depleted adult females (Figure 4A and F). The vitellarium of some ovarioles contained as many as ten ovarian follicles in BgEya-depleted females. This concurs with the phenotype observed in adult females with depleted Notch (BgN) expression (Figure 4A and G), which is not surprising because BgN depletion also reduce BgEya expression (Irles et al., 2016; Irles and Piulachs, 2014).

In contrast, depletion of BgEya did not affect BgN expression. However, Delta (BgDl) and Serrate (BgSer), the main ligands of Notch were upregulated. In addition, the expression of Hippo (BgHpo) and Yorkie (BgYki), two of the main components of the Hippo pathway, significantly increased in the ovaries of BgEya-depleted females (Figure 4H-I), which suggests that the cell fate is modified and also the degree of cell proliferation. Moreover, the germ cell markers *nanos* (Bg*nos*), *vasa* (Bg*vas*), and *fs(1)Yb* (Bg*Yb*, also called *Tudor 12*; Figure 4), which all appear to be crucial in modulating germinal and somatic stem cell proliferation in *D. melanogaster* (King et al., 2001; Wang and Lin, 2004), were overexpressed in BgEya-depleted females of *B. germanica*.

Taken together, all these results clearly indicate that BgEya affects both somatic and germinal cells, and acts at different levels in the ovary.

3.4. Ecdysone signalling and the differentiation of ovarian follicles

The formation and differentiation of ovarian follicles in *D. melanogaster* is induced by 20E signalling. Therefore, we assumed that this regulatory mechanism was ancestral and might also operate in *B. germanica*. In adult females the ovary is the only source of ecdysteroids (Pascual et al., 1992; Romaña et al., 1995). Conversely, the prothoracic glands are the only described source of ecdysteroids in nymphs. To unveil the possible action of 20E the *B. germanica* ovary, we measured the expression of E75A and HR3 (two early genes in the ecdysone signalling pathway), in the ovaries of last instar nymphs and adult females. These results showed that the E75A and HR3 expression pattern in these ovaries correlated with the ecdysone/20E titter in the hemolymph (Figure S3). The expression of *E75A* and *HR3* at least demonstrates that the *B. germanica* ovary may be able to respond to ecdysone signalling during the last nymphal instar.

Thus, to determine the capacity of the *B. germanica* ovary to synthesise ecdysone during oogenesis, we measured the expression of the ecdysteroidogenic genes *Neverland* (Bg*Nev*), *Spookiest* (Bg*Spot*), *Phantom* (Bg*Phm*), *Shadow* (Bg*Sad*) and *Shade* (Bg*Shd*), in *B. germanica* ovaries throughout the gonadotrophic cycle (Figure S3). These genes are expressed in the ovaries of last instar nymphs and adults, although

287 their expression is higher in the former. The expression of Bg*Shd* correlated with the
288 hemolymph ecdysone/20E titre (Figure S3) and so, given that *Shad*e converts ecdysone
289 into 20E (the active form), these ovaries were at least capable of responding to
290 ecdysone.

291 Based on this, we also measured the expression of Bg*Nvd*, Bg*Spot* and Bg*Shd*, in
292 ovaries from BgEya-depleted females (Figure 5A). While the expression of Bg*Nvd* did
293 not seem to be affected by BgEya depletion, Bg*Spot* and Bg*Shd* expression levels were
294 higher in 8-day-old BgEya-depleted sixth instar nymphs, and significantly increased in
295 5-day-old adult females (Figure 5A).

296 These results suggest that BgEya represses ecdysone biosynthesis in ovaries, thus
297 affecting the differentiation of stem cells in the germaria.

298 To assess the possible action of ecdysone upon ovarian follicle differentiation, 20E was
299 applied to newly emerged last instar nymphs (Figure 5B). A significant increase ($p <$
300 0.002) in the number of differentiated ovarian follicles was observed in 6-day-old last
301 instar nymphs, but two days later, the number of ovarian follicles localised in the
302 vitellaria in 8-day-old nymphs was very variable compared to the controls (Figures 5B,
303 E and F). This number ranged from 3 to 11 ovarian follicles (Figure 5B, G, H and H'),
304 suggesting that some ovarian follicles enter in cell death.

305 Moreover, the effect of 20E on ovarian follicle differentiation was not instar specific.
306 The newly emerged adult females treated with 20E also produced more differentiated
307 ovarian follicles 5 days after treatment than their age-matched controls ($p< 0.002$;
308 Figure 5B). Expression of BgEya in the ovaries of these 8-day-old 20E-treated last
309 instar nymphs was not affected by treatment with ecdysone (Figure 5C) and the
310 expression of ecdysteroidogenic genes did not significantly changes after 20E treatment.

311

312 **4. Discussion**

313 In hemimetabolan species, the primary function of *eya* was originally described by its
314 involvement in eye development which was related to cell proliferation (Dong and
315 Friedrich, 2010; Takagi et al., 2012). In the present work we also demonstrated the
316 involvement of *eya* in ovary development in a hemimetabolan species: depletion of this

317 protein in *B. germanica* precludes the completion of the gonadotrophic cycle, thus
318 making females of this species sterile.

319 The phenotypes observed after depletion of BgEya in last instar nymphs indicates that
320 this gene functions early in the gonadotrophic cycle and acts differently in the different
321 regions of the ovary, affecting both somatic and germ cells. Indeed, the development of
322 basal ovarian follicles, which usually start to grow and mature during the last nymphal
323 instar, arrested in BgEya-depleted females and they also lost their elliptical morphology
324 to become spherical.

325 Similar to observations in BgN-depleted females, this phenotype becomes more
326 conspicuous as the females aged. Thus, this aspect of the BgN phenotype can also be
327 attributed to a concomitant decrease in BgEya (Irles and Piulachs, 2014). Conversely,
328 the disappearance of the stalks between all ovarian follicles in the ovariole, which was
329 described in females treated with dsBgN, cannot be attributed to *Eya*. In ovaries from
330 BgEya-depleted females the stalk was always present between the basal and subbasal
331 ovarian follicles, although the stalk between the youngest ovarian follicles was
332 frequently absent or undifferentiated.

333 Of note, the arrest in oocyte growth in BgEya-depleted females, was not attributable to
334 significant changes in vitellogenesis, because the fat body expressed vitellogenin at
335 similar levels to the controls (results not shown). Despite this, vitellogenin was not
336 incorporated into the growing oocytes. Furthermore, we also observed that VgR
337 transcripts accumulated in ovaries of BgEya-depleted females, when usually its
338 expression decrease as the oocyte growth, coinciding with the increase of the
339 vitellogenin receptor in the membranes of basal oocytes (Ciudad et al., 2006). This
340 suggests that vitellogenin was not incorporated into the growing oocytes of BgEya-
341 depleted females because its receptor is absent.

342 However, the most remarkable phenotype observed in ovarioles from BgEya-depleted
343 females was the uncontrolled stem cell proliferation and differentiation in the germaria,
344 and the resulting increase in the number of differentiated ovarian follicles produced.
345 Interestingly, along with a notable swelling of the germaria, this phenotype is
346 reminiscent of those described in *D. melanogaster eya*-null mutants (Bai and Montell,
347 2002; Leiserson et al., 1998). The aforementioned swollen appearance occurred in these
348 eya-null mutant flies because the maturing egg chambers development arrested but the

germaria continued to proliferate (Bonini et al., 1998). This resemblance suggests that the role of eya in the control of stem cell differentiation and proliferation is conserved between cockroaches and flies.

In *D. melanogaster*, the formation and differentiation of ovarian follicles are controlled by 20E (see Belles and Piulachs, 2015; Hsu et al., 2019; König et al., 2011; Uryu et al., 2015). This type of regulation may be ancestral and could also operate in insects where juvenile hormone plays the role of gonadotropic hormone (Bellés et al., 2000; Comas et al., 2001; Treiblmayr et al., 2006).The only source of ecdysone in adult *B. germanica* females is the ovary. In adults the main function of this hormone is to promote chorion synthesis in mature basal ovarian follicles (Pascual et al., 1992; Romaña et al., 1995). However, ecdysteroidogenic genes are expressed in the ovary of last instar nymphs of *B. germanica* and so we cannot rule out the possibility that immature ovaries of this species can synthesise ecdysone

In *D. melanogaster* germarium ecdysone signalling controls de quantity, but not the differentiation status of germinal stem cells. In fact, this latter may be mediated by Notch pathway. Ecdysone signalling induces *Dl* expression at the terminal filament in cell membranes, which activates N and determines the fate of these cells, which can become cap or escort cells (Ameku et al., 2017; Green et al., 2011; Hsu et al., 2019). Our results in *B. germanica* suggest that similar signalling networks occur in panoistic ovaries. When BgEya is depleted BgDl expression significantly increase and N expression is activated, again suggesting an increase in ecdysone levels. This signalling follows a pathway which is equivalent to the one already described *D. melanogaster*, and also results in swollen germaria in the ovarioles of Eya-depleted cockroaches.

The overexpression of ecdysteroidogenic genes in the ovaries of BgEya-depleted nymphs, indicates that BgEya regulates the proliferation of ovarian follicles in the *B. germanica* ovary by repressing the ecdysteroidogenic pathway. This idea correlates with the expression of BgEya in adult ovaries, in which the decrease of BgEya levels at the end of the gonadotrophic cycle coincides with the increase of ecdysone in the ovaries at this age (Romaña et al., 1995). This increase induces the chorion synthesis in mature basal ovarian follicles (Pascual et al., 1992).

Ectopic treatment with 20E gave results similar to those obtained after BgEya depletion: ovarian follicle proliferation, and swelling of the germaria. However, BgEya expression

was not modified by ecdysone treatment, indicating that *eya* regulates the activity of the ecdysteroidogenic pathway but is not controlled by 20E.

In summary, *ey*a has two different functions in the panoistic ovary of *B. germanica*. On the one hand, is a downstream component of the Notch pathway in basal ovarian follicles and modulates proliferation the correct proliferation and fate of follicular cells. On the other hand, *eya* acts on somatic and germinal stem cells to regulate their differentiation and proliferation, by controlling ecdysone signalling in the germaria and in the terminal filament. Of note, both these functions fit with the functional duality of *eya* already reported for *D. melanogaster*.

Acknowledgments

We thank the financial support from the Ministry of Science and Innovation, Spain for grants BFU2011-22404 and CGL2016-76011-R that also received financial assistance from the European Fund Economic and Regional Development (FEDER) And from the Catalan Regional Government (grant 2017 SGR 1030). PI is received the financial support from *Apoyo al Retorno de Investigadores desde el Extranjero* program (*Convocatoria* 2013, 821320046, PAI, CONICYT). The funders had no role in the design of the study, data collection or analysis, the decision to publish, or in the preparation of the manuscript.

References

Ameku, T., Yoshinari, Y., Fukuda, R., Niwa, R., 2017. Ovarian ecdysteroid biosynthesis and female germline stem cells. Fly (Austin). 11, 185–193. doi:10.1080/19336934.2017.1291472

Bai, J., Montell, D., 2002. EYA represses polar cell fate in the Drosophila ovary. Development 129, 5377–5388. doi:10.1242/dev.00115

Belles, X., Piulachs, M.D., 2015. Ecdysone signalling and ovarian development in insects: from stem cells to ovarian follicle formation. Biochim. Biophys. Acta 1849, 181–6. doi:10.1016/j.bbagrm.2014.05.025

Bellés, X., Piulachs, M.D., Pascual, N., Maestro, J.L., Martín, D., 2000. On the role of Juvenile Hormone in vitellogenesis in cockroaches: A reply to Holbrook et al.

412 Physiol. Entomol. 25, 207–210. doi:10.1046/j.1365-3032.2000.00199.x
413 Bonini, N.M., Leiserson, W.M., Benzer, S., 1998. Multiple roles of the eyes absent gene
414 in Drosophila. Dev. Biol. 196, 42–57. doi:10.1006/dbio.1997.8845
415 Bonini, N.M., Leiserson, W.M., Benzer, S., 1993. The eyes absent gene: Genetic
416 control of cell survival and differentiation in the developing Drosophila eye. Cell
417 72, 379–395. doi:10.1016/0092-8674(93)90115-7
418 Ciudad, L., Piulachs, M.-D., Bellés, X., 2006. Systemic RNAi of the cockroach
419 vitellogenin receptor results in a phenotype similar to that of the Drosophila
420 yolkless mutant. FEBS J. 273. doi:10.1111/j.1742-4658.2005.05066.x
421 Comas, D., Piulachs, M.-D., Bellés, X., 2001. Induction of vitellogenin gene
422 transcription in vitro by juvenile hormone in Blattella germanica. Mol. Cell.
423 Endocrinol. 183, 93–100. doi:10.1016/S0303-7207(01)00589-5
424 Dai, W., Peterson, A., Kenney, T., Burrous, H., Montell, D.J., 2017. Quantitative
425 microscopy of the Drosophila ovary shows multiple niche signals specify
426 progenitor cell fate. Nat. Commun. 8, 1–14. doi:10.1038/s41467-017-01322-9
427 Davey, K., Huebner, E., 1974. The response of the follicle cells of Rhodnius prolixus to
428 juvenile hormone and antigonadotropin in vitro. Can J Zool. 52, 1407–1412.
429 Dong, Y., Friedrich, M., 2010. Enforcing biphasic eye development in a directly
430 developing insect by transient knockdown of single eye selector genes. J. Exp.
431 Zool. Part B Mol. Dev. Evol. 314 B, 104–114. doi:10.1002/jez.b.21313
432 Duncan, M.K., Kos, L., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., Tomarev, S.I.,
433 1997. Eyes absent: a gene family found in several metazoan phyla, Mammalian
434 Genome. Spfinger-VerlagNew York Inc.
435 Elshaer, N., Piulachs, M.D., 2015. Crosstalk of EGFR signalling with Notch and Hippo
436 pathways to regulate cell specification, migration and proliferation in cockroach
437 panoistic ovaries. Biol. Cell 107, 273–85. doi:10.1111/boc.201500003
438 Graziussi, D.F., Suga, H., Schmid, V., Gehring, W.J., 2012. The “Eyes absent” (eya)
439 Gene in the Eye-Bearing Hydrozoan Jellyfish Cladonema radiatum: Conservation
440 of the Retinal Determination Network. J. Exp. Zool. Part B Mol. Dev. Evol. 318,
441 257–267. doi:10.1002/jez.b.22442
442 Green, D.A., Sarikaya, D.P., Extavour, C.G., 2011. Counting in oogenesis. Cell Tissue
443 Res. 344, 207–212. doi:10.1007/s00441-011-1150-5
444 Hsu, H.-J., Bahader, M., Lai, C.-M., 2019. Molecular control of the female germline
445 stem cell niche size in Drosophila. Cell. Mol. Life Sci. 1, 3. doi:10.1007/s00018-

019-03223-0

Irles, P., Elshaer, N., Piulachs, M.-D., 2016. The Notch pathway regulates both the proliferation and differentiation of follicular cells in the panoistic ovary of Blattella germanica. Open Biol. 6. doi:10.1098/rsob.150197

Irles, P., Piulachs, M.D., 2014. Unlike in Drosophila Meroistic Ovaries, hippo represses notch in Blattella germanica Panoistic ovaries, triggering the mitosis-endocycle switch in the follicular cells. PLoS One 9, e113850. doi:10.1371/journal.pone.0113850

Jemc, J., Rebay, I., 2007. The Eyes Absent Family of Phosphotyrosine Phosphatases: Properties and Roles in Developmental Regulation of Transcription. Annu. Rev. Biochem. 76, 513–538. doi:10.1146/annurev.biochem.76.052705.164916

King, F.J., Szakmary, A., Cox, D.N., Lin, H., 2001. Yb Modulates the Divisions of Both Germline and Somatic Stem Cells through piwi- and hh-Mediated Mechanisms in the Drosophila Ovary. Mol. Cell 7, 497–508. doi:10.1016/S1097-2765(01)00197-6

König, A., Yatsenko, A.S., Weiss, M., Shcherbata, H.R., 2011. Ecdysteroids affect Drosophila ovarian stem cell niche formation and early germline differentiation. EMBO J. 30, 1549–1562. doi:10.1038/emboj.2011.73

Leiserson, W.M., Benzer, S., Bonini, N.M., 1998. Dual functions of the Drosophila eyes absent gene in the eye and embryo. Mech. Dev. 73, 193–202. doi:10.1016/S0925-4773(98)00052-5

Pascual, N., Cerdá, X., Benito, B., Tomás, J., Piulachs, M.D., Bellés, X., 1992. Ovarian ecdysteroid levels and basal oöcyte development during maturation in the cockroach Blattella germanica (L.). J. Insect Physiol. 38, 339–348. doi:10.1016/0022-1910(92)90058-L

Pfaffl, M., Horgan, G., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res.

Rebay, I., 2015. Multiple Functions of the Eya Phosphotyrosine Phosphatase. Mol. Cell. Biol. 36, 668–77. doi:10.1128/MCB.00976-15

Romaña, I., Pascual, N., Belles, X., 1995. The ovary is a source of circulating ecdysteroids in Blattella germanica. Eur. J. Entomol. 92, 93–103.

Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365–386.

Takagi, A., Kurita, K., Terasawa, T., Nakamura, T., Bando, T., Moriyama, Y., Mito, T.,

Noji, S., Ohuchi, H., 2012. Functional analysis of the role of eyes absent and sine oculis in the developing eye of the cricket Gryllus bimaculatus. Dev. Growth Differ. 54, 227–240. doi:10.1111/j.1440-169X.2011.01325.x

Treiblmayr, K., Pascual, N., Piulachs, M.D., Keller, T., Belles, X., 2006. Juvenile hormone titer versus juvenile hormone synthesis in female nymphs and adults of the German cockroach, Blattella germanica. J. Insect Sci. 6.

Uryu, O., Ameku, T., Niwa, R., 2015. Recent progress in understanding the role of ecdysteroids in adult insects: Germline development and circadian clock in the fruit fly Drosophila melanogaster. Zool. Lett. 1, 1–9. doi:10.1186/s40851-015-0031-2

Wang, Z., Lin, H., 2004. Nanos Maintains Germline Stem Cell Self-Renewal by Preventing Differentiation. Science (80-.). 303, 2016–2019. doi:10.1126/science.1093983

Yang, X., ZarinKamar, N., Bao, R., Friedrich, M., 2009. Probing the Drosophila retinal determination gene network in Tribolium (I): The early retinal genes dachshund, eyes absent and sine oculis. Dev. Biol. 333, 202–214. doi:10.1016/J.YDBIO.2009.02.040

Zimmerman, J.E., Bui, Q.T., Steingrimsson, E., Nagle, D., Fu, W., Genin, A., Spinner, N., Copeland, N., Jenkins, N., Bucan, M., Bonini, N.M., 1997. Two, Cloning and Characterization of Eyes, Vertebrate Homologs of the Drosophila Gene, Absent. Genome Res. 7, 128–141.

Figure Legends

Figure 1. Expression of BgEya. **A**. Pattern of BgEya expression, in ovaries in sixth nymphal instar and adult females during the first gonadotrophic cycle. The black dashed line indicates the moult to adult. Profiles of ecdysone (green dashed) and juvenile hormone (red line) on hemolymph, and ecdysone content in ovary (blue line) are represented. **B**. Expression of BgEya in ovaries from females treated with dsBgEya on N6D0. Data represent copies of mRNA per 1000 copies of Bg*Actin-5c* (relative expression) and are expressed as the mean ± S.E.M. (n = 3). Expression on N6D8, AdD3 and AdD5 were downregulated (P(H1) = 0.097, 0.031 and 0.0001, respectively). **C**. Width (BOF-W) and the length (BOF-L) of basal ovarian follicle in BgEya-depleted females. (statistical differences to respective control are indicated *: $p < 0.0001$). **D**. Ovariole from an 8-day-old dsMock-treated nymph. **E**. Ovariole from an 8-day-old BgEya-depleted nymph. Scale 200 µm. N6D8: 8-day-old sixth instar female; AdD3: 3-day-old adult female; AdD5: 5-day-old adult female.

Figure 2. BgEya in 8-day-old sixth instar nymphs. **A**. number of follicular cells in basal ovarian follicles from dsMock- and dsBgEya-treated females. N6D8: 8-day-old sixth instar nymph; AdD3: 3-day-old adult; AdD5: 5-day-old adult (statistical differences to respective control are indicated *: $p<0.002$; **: $p < 0.0001$). **B**. Ovariole from 8-day-old dsMock-treated nymph in which the follicular cells are actively dividing, labelled with anti-phospho-histone 3 (PH3) antibody (in B', the isolated channel showing the PH3 labelling is displayed). **C**. Ovarioles from BgEya-depleted female showing a scarce number of cells dividing (in C' the isolated channel showing the PH3 labelling is displayed). **D-E**. Follicular epithelia from dsMock-treated nymphs, in D the follicular cells nuclei are show and some mitotic figures are visible (arrowheads). In E the F-actins microfilaments appear uniformly distributed in the cell membranes are showed. **F-G**. Follicular epithelia from BgEya-depleted nymphs, in F the follicular cells nuclei are show evidencing differences in size and form, with an absence of mitosis. In G the F-actins microfilaments in basal ovarian follicles nymphs display a non-uniform distribution. In all images the anterior pole of the basal ovarian follicle is forward the top-right. BOF: basal ovarian follicle. Nuclei stained with DAPI

and F-actins microfilaments with TRICT-Phalloidin. In B and C, the PH3 appear in magenta. Scale bar in B-C: 100 µm, in D-G: 10 µm.

Figure 3. BgEya in 5-day-old adults. A. Ovariole from a dsMock-treated female. **B-C**. Ovariole from BgEya-depleted females showing different degree of affectation. **D**. Follicular epithelia from dsMock-treated females showing the binucleated cells and the patency between cells. In D' are displayed the nuclei stained with DAPI are show and in D'' the cytoskeleton of F-actins. **E**. Follicular epithelia from BgEya-depleted females showing cells of different size and morphology, mostly mononucleated and few binucleated (arrowheads). In **E**' are show the nuclei of different sizes stained with DAPI and in **E**'' the cytoskeleton of F-actins with a uniform distribution on cell membranes and without signs of patency. Scale in A-C: 200 µm; D and E: 20 µm.

Figure 4. BgEya on ovarian follicle differentiation. **A**. Number of ovarian follicles in ovarioles from dsMock- and dsBgEya-treated females. Ovarian follicles in the vitellaria were quantified including the subbasal and all released from the germarium. Data from 5-day-old dsBg*N*-depleted ovarioles were included in the graph (data was obtained from Irles et al., 2016; Irles and Piulachs, 2014). Data is expressed as the mean ± S.E.M (n = 13-50), see also Table S3. Different letters indicate significant differences ($p < 0.0001$). N6D0, N6D6 and N6D8: 0-day-old, 6-day-old and: 8-day-old sixth instar female respectively; AdD3 and AdD5: 3-day-old and 5-day-old adult female. **B**. Ovariole from dsMock-treated N6D8. **C.** Vitellarium and germarium from dsMock-treated AdD5 ovariole. **D**. Ovarioles from N6D8 BgEya-depleted. **E.** Vitellarium and germarium from a N6d8 BgEya-depleted. **F**. Ovariole from an AdD5-dsBgEya-depleted.**G**. Ovariole from AdD5 Bg*N*-depleted in N6D8 (see (Irles et al., 2016). Nuclei from follicular cells were stained with DAPI, F-actins microfilaments with TRICT-Phalloidin and the nucleus from germinal cells with eya10H6 antibody. **H.** Expression of the main components of Notch pathway in ovaries from 5-day-old BgEya-depleted adults. **I.** Expression of Bg*Hpo* and Bg*Yki* in ovaries from 5-day-old BgEya-depleted adults. **J.** Expression of Bg*nos*, Bg*vas* and Bg*Yb* in ovaries from 8-day-old BgEya-depleted nymphs and 5-day-old treated adults. In H-J, data represent copies of mRNA per 1000 copies of Bg*Actin-5c* (relative expression) and are expressed as the mean ± S.E.M. (n =

572 3). Scale bars in B, D, F and G: 100 µm; in C: 200 µm; in E: 50 µm. Asterisk indicates
573 statistical differences ($p < 0.02$).

574

575 **Figure 5. Ecdysone in ovary of *B. germanica*. A.** Expression levels of Bg*Nvd*, Bg*Spot*
576 and Bg*Shd* in ovaries from BgEya-depleted females. Statistical differences to respective
577 control are indicated by an * ($p < 0.002$). Data represent copies of mRNA per 1000
578 copies of Bg*Actin-5c* (relative expression) and are expressed as the mean ± S.E.M. (n =
579 6-10). **B**. Box plot representing the number of ovarian follicles localized in the
580 vitellarium in, Control (C: 10% EtOH) and 20E-treated females (20E: 10µM 20-
581 Hydroxyecdysone). N6D6: 6-day-old sixth instar nymph; N6D8: 8-day-old sixth instar
582 nymph; AdD5: 5-day-old adult female. Last instar nymphs and adult females were
583 treated at the day of emergence. Statistical differences to respective control are
584 indicated *: $p < 0.002$; n.s. no significant (n= 20-50)**. C**. Expression of BgEya in
585 nymphal ovaries treated with 10 µM of 20E. **D**. Expression levels of Bg*Nvd*, Bg*Spot*,
586 Bg*Pha* and Bg*Sad* in ovaries from N6D6 treated with 10 µM 20E. In C and D, data
587 represent copies of mRNA per 1000 copies of Bg*Actin-5c* and are expressed as the
588 mean ± S.E.M. (n = 3-4). **E**. Ovariole from a Control N6D8. **F**. Vitellarium and
589 germarium from a Control N6D8. **G**. Ovariole from a 20E treated N6D8 showing a
590 reduced number of ovarian follicles released from the germarium. In the insert the
591 germarium is show at higher magnification. **H.** Ovariole from a 20E treated N6D8
592 showing a high number of ovarian follicles released from the germarium, in **H'** is
593 showed a detail of the germarium at higher magnification, where is possible to see the
594 differentiated ovarian follicles. Scale bars in F-H: 100 µm, in H': 50 µm. Ovarioles
595 were stained with TRITC-Phalloidin.

596

597

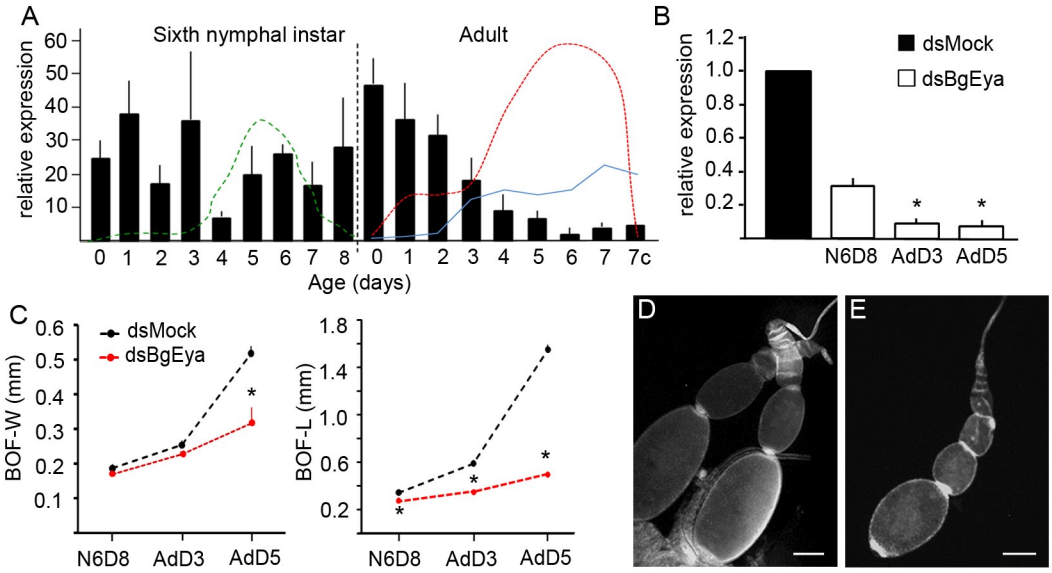

Figure 1

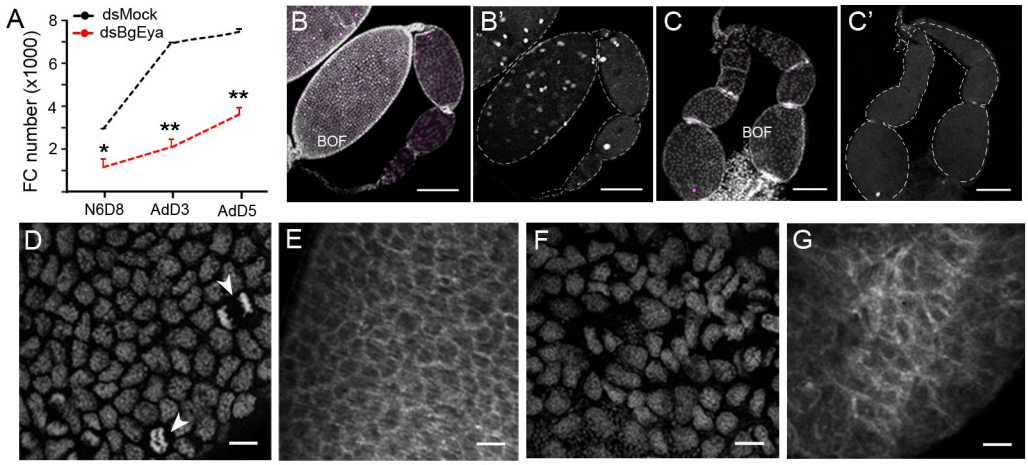

Figure 2

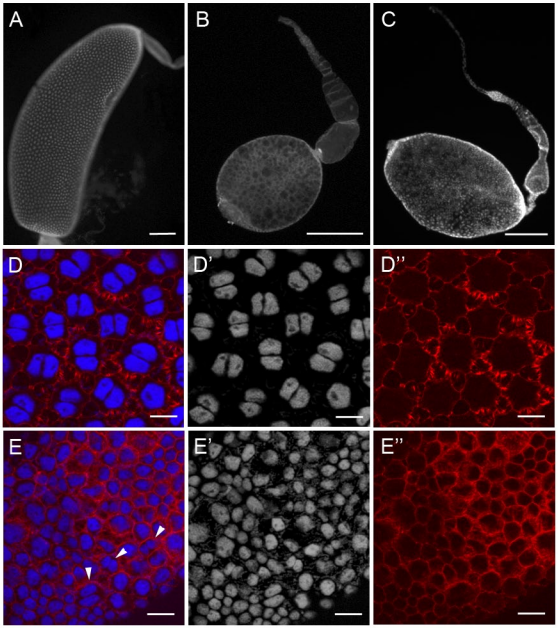

Figure 3

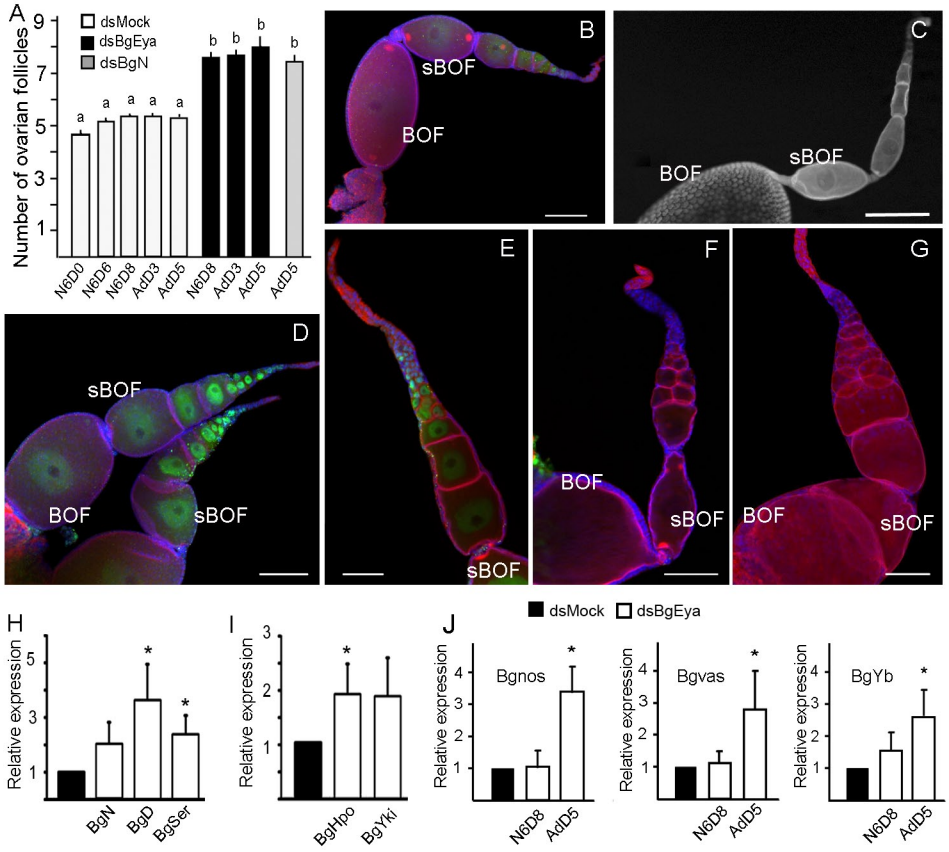

Figure 4

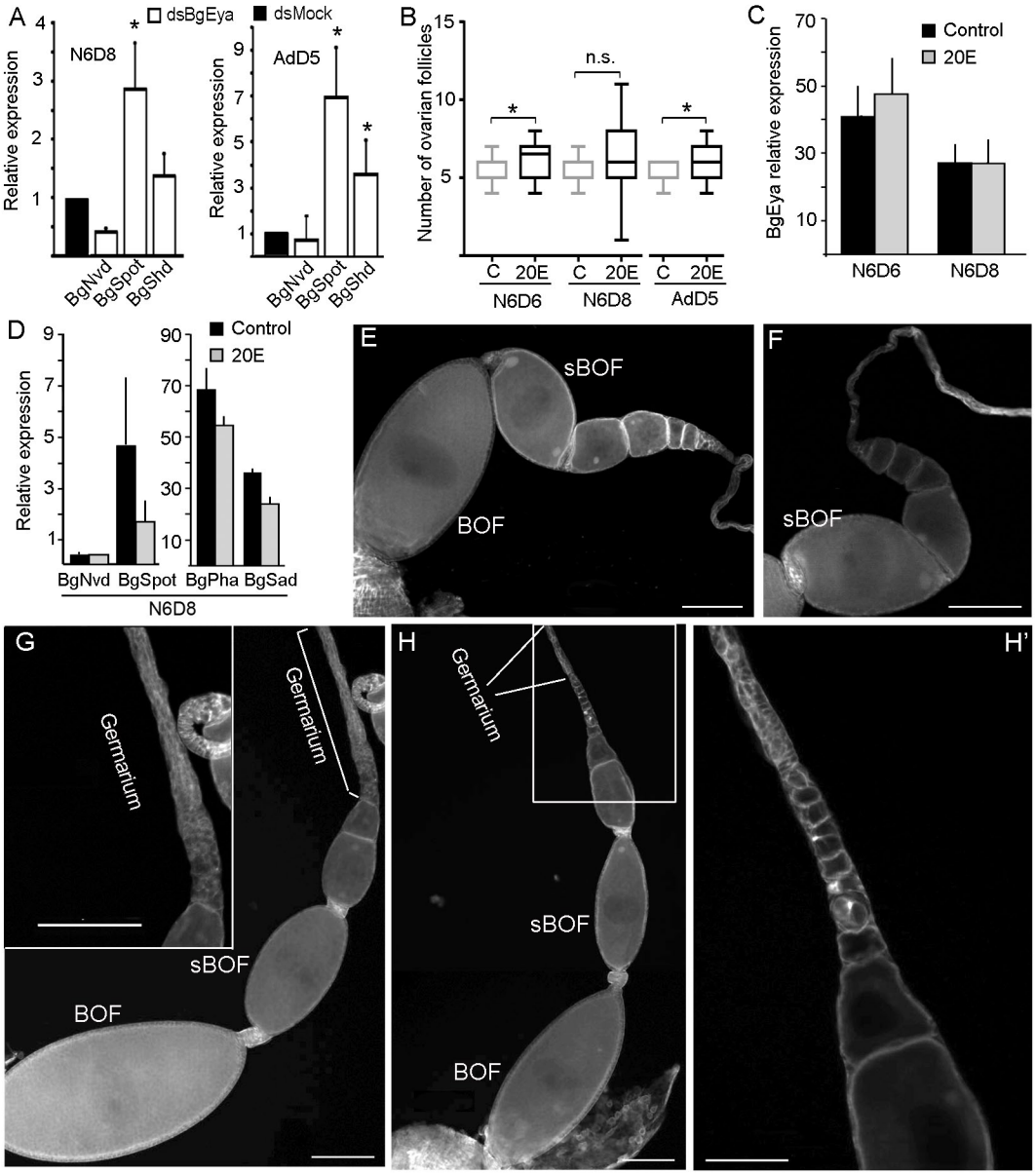

Figure 5

Table S1: Accession Number of Studied Sequences and Primer Sequence Used for qRT-PCR and RNAi experiments.

F: Primer forward - R: Primer reverse

Primer name			**Primer Sequence (5'-3')**	**Accession number**
BgEya	dsRNA1	F R	TTTTGGATCTGACGGCTTTC GCAAGGGCTGGAACTAACTG	PSN54252.1
BgEya	dsRNA2	F R	GGCTCTTAGGCACAAAACGA GCAGCTTCTTCATCCTGTCC	PSN54252.1
BgEya	qRTPCR	F R	GAGGCATTTTTCCGATTGAA GCAGCTTCTTCATCCTGTCC	PSN54252.1
Bg Caspase1	qRTPCR	F R	AAGCGGAAGGATTCATACCA GATGACTGCCTTGCCTCTTC	CEP28036.1
BgVgR	qRTPCR	F R	ACCAACTCCACAAGGACCAC AACGGATCTGCACCTGTAGC	CAJ19121.1
BgVasa	qRTPCR	F R	GAAACGAACCGCTGACTTTAT CACTCCCATTCGTCCATTCT	PSN55909.1
Bgnos	qRTPCR	F R	ATTGTCCAGAGTTTCAACTTAAT CCTGTTTCTTTGAACGCTTCTT	PSN32832.1
BgYb	qRTPCR	F R	CGAAACAACTCCACCGTTTT CTCCGCATGCCATTTTAACT	PSN55645.1
BgN	qRTPCR	F R	GCTAAGAGGCTGTTGGATGC TGCCAGTGTTGTCCTGAGAG	HF969255
BgDl	qRTPCR	F R	CCACTACAAGTGTTCGCCAA TACCTCTCGCATTCGTCACA	HF969256
BgSer	qRTPCR	F R	TCCTCTTGGCAGTGCATTTG CTTGATCACAGAGGATGCCG	HG515375.1
BgHpo	qRTPCR	F R	GACATTTGGAGCCTTGGCAT AGGTTTCCCTTCAGCCATTTC	HF969251
BgYki	qRTPCR	F R	TCCCTACCACACACACCAGA GACCATCCAATGTTGCCATA	HF969253
BgSad	qRTPCR	F R	ATGAGGAGGTTCAGGGTGTG CTGGCCAGAAGTCATTTGGT	PSN51657.1
BgPhm	qRTPCR	F R	CTAGGCACCAGAGCACCTTC GCAAGCACTGTGTCTTCCAA	PSN36025.1
Bg Spot	qRTPCR	F R	GAAGTTCAAATGCGAGCACA GCAATGGAACTGTCCTGGTT	PSN53270.1
BgShd	qRTPCR	F R	CACAGAGGCGCACAAGTTTA GTTCCCCTTCAAAGTCCACA	PSN43891.1
BgNvd	qRTPCR	F R	CTGGGGCCAGTCACAATACT GCAGGGGCTTGTCAATGTAT	PSN31862
BgE75A	qRTPCR	F R	GTGCTATTGAGTGTGCGACATGAT TCATGATCCCTGGAGTGGTAGAT	CAJ87513.1
BgHR3	qRTPCR	F R	GATGAGCTGCTCTTAAAGGCGAT AGGTGACCGAACTCCACATCTC	CAJ90621
BgActin-5c	qRTPCR	F R	AGCTTCCTGATGGTCAGGTGA TGTCGGCAATTCCAGGGTACATGGT	AJ862721

Table S2. Effect of Bg*Eya* depletion in oviposition. Newly emerged sixth instar nymphs (N6D0) females were treated with dsBg*Eya* or dsMock and left to oviposit. The day of oviposition (counted 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	% Fertil oothecae
dsMock (N6D0)	40	7.68 ± 0.13	100	17.92 ± 0.11	100
dsBg*Eya* (N6D0)	36	0	0	0	0

Table S3. The ovarian follicles (OF) in the vitellaria from dsMock-, dsBg*Eya*- and 20E treated females, were quantified at different ages. The counted ovarian follicles were those from the subbasal to the youngest that has left the germarium, both included. The ovarian follicles were measured from different ovarioles (n) belonging to 7-10 females.

Treatment	Age	n	OF (mean ± SEM)
dsMock (N6D0)	N6D0	22	4.68 ± 0.166
	N6D6	50	5.48 ± 0.131
	N6D8	30	5.37 ± 0.122
	AdD3	31	5.32 ± 0.169
	AdD5	28	5.32 ± 0.126
dsBg*Eya* (N6D0)	N6D8	50	7.60 ± 0.191
	AdD3	13	7.69 ± 0.208
	AdD5	13	8.00 ± 0.408
20E (N6D0)	N6D6	18	6.28 ± 0.289
	N6D8	39	6.10 ± 0.319
20E (AdD0)	AdD5	28	6.20 ± 0.205

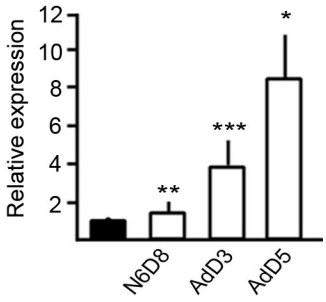

Figure S1. Expression of Vitellogenin Receptor in ovaries of newly emerged sixth instar nymphs females, treated with dsBg*Eya*. N6D8: 8-day-old sixth instar female; AdD3: 3-day-old adult female; AdD5: 5-day-old adult female. Data represent copies of mRNA per 1000 copies of Bg*Actin-5c* (relative expression) and are expressed as the mean ± S.E.M. (n = 3). Asterisk indicate the statistical differences to respective control: *: p(H1)< 0.02; ** p(H1)< 0.01; *** p(H1)<0.001.

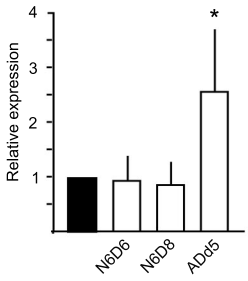

Figure S2. Expression of *Caspase 1* in ovaries of newly emerged sixth instar nymphs treated with dsBg*Eya*. N6D6: 6-day-old sixth instar female; N6D8: 8-day-old sixth instar female; AdD5: 5-day-old adult female. Data represent copies of mRNA per 1000 copies of Bg*Actin-5c* (relative expression) and are expressed as the mean ± S.E.M. (n = 3). Asterisk indicate the statistical differences to respective control: * p(H1)<0.001.

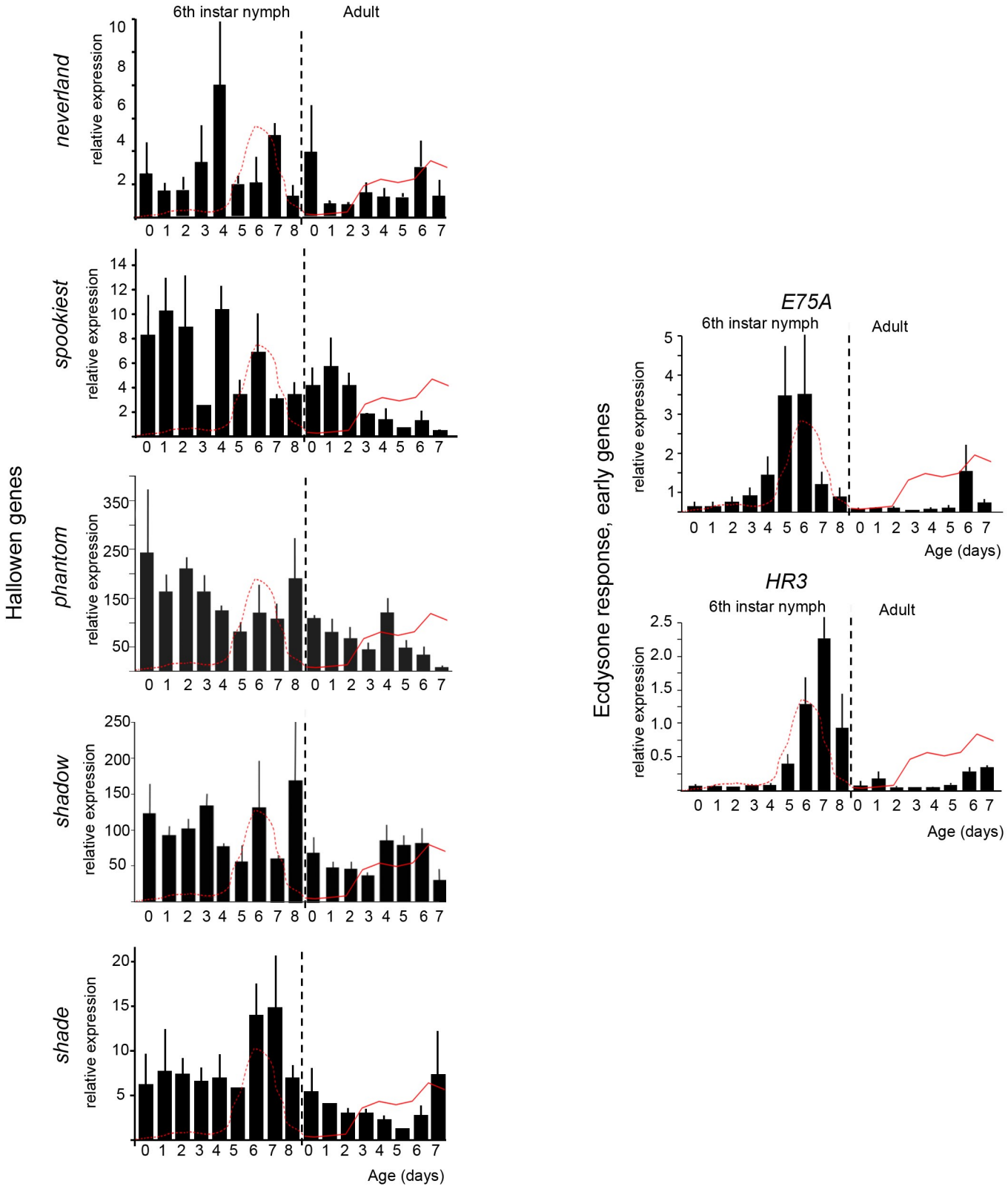

Figura S3. Expression pattern of the Ecdysone-related genes in ovaries of *Blattella germanica*: mRNA expression patterns in ovaries of *Neverland*, *spookiest*, *phantom*, *shadow* and *shade* (five Hallowen genes involved in the ecdysone biosynthesis pathway) and early genes *E75* and *HR3* were measured in ovaries of sixth nymphal instar and adult females. The black dashed line indicates the moult to adult. The dashed red line displays the profile of ecdysone in hemolymph and the solid read line the profile of ecdysone in ovaries. Data represent copies of mRNA per 1000 copies of Bg*Actin-5c* (relative expression) and are expressed as the mean ± S.E.M. (n= 3-6).