# Ovarian ecdysteroid biosynthesis and female germline stem cells

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## Abstract

The germline stem cells (GSCs) are critical for gametogenesis throughout the adult life. Stem cell identity is maintained by local signals from a specialized microenvironment called the niche. However, it is unclear how systemic signals regulate stem cell activity in response to environmental cues. In our previous article, we reported that mating stimulates GSC proliferation in female *Drosophila*. The mating-induced GSC proliferation is mediated by ovarian ecdysteroids, whose biosynthesis is positively controlled by Sex peptide signaling. Here, we characterized the post-eclosion and post-mating expression pattern of the genes encoding the ecdysteroidogenic enzymes in the ovary. We further investigated the biosynthetic functions of the ovarian ecdysteroid in GSC maintenance in the mated females. We also briefly discuss the regulation of the ecdysteroidogenic enzyme-encoding genes and the subsequent ecdysteroid biosynthesis in the ovary of the adult *Drosophila*.

## Introduction

In many animals, sperm and egg production requires a robust stem cell system that balances self-renewal with differentiation.[1] Germline stem cells (GSCs) produce progeny germ cells that differentiate into gametes and replicate themselves to maintain the generative cell population. The balance between self-renewal and differentiation of GSCs is important because perturbation of this balance causes germ cell depletion, infertility or tumorigenesis.[1,2] GSCs are maintained by a specialized microenvironment called the niche.[3] The niche provides local signals to maintain stem cell identity.[4] Furthermore, GSC number is also controlled by systemic signals, including the insect steroid hormone ecdysteroids,[5–11] which are also known as "molting hormones". In the larval stage, ecdysteroids are biosynthesized from dietary cholesterol through several catalyzed steps in the specialized endocrine organ called the prothoracic gland. Recently, molecular studies have identified several ecdysteroidogenic enzymes such as Noppera-bo,[12–14] Neverland,[15–17] Non-molting glossy/Shroud,[18] CYP307A1/Spook,[19,20] CYP307A2/Spookier,[19] CYP306A1/Phantom,[21,22] CYP302A1/Disembodied,[23–25] CYP315A1/Shadow,[24] and CYP314A1/Shade [26] (Fig. 1A). Molecular genetics has revealed that ecdysteroid signaling is indeed active in adult insects, and is involved in controlling multiple steps during adult oogenesis, including egg chamber development and vitellogenesis,[27,28] follicle growth and survival,[9,29] and stem cell niche formation.[9] In addition, certain genes encoding the ecdysteroidogenic enzymes are required for egg development after stage 8,[19,26] egg production,[30] and border cell migration.[31]

It is well-documented that for the regulation of molting and metamorphosis, the biosynthesis and signaling of ecdysteroids are coordinately modulated in response to various environmental cues such as nutrition, photoperiod, and temperature.[32,33] Therefore, it is possible that the environmental cues are also reflected in egg production processes such as in the control of GSC number. However, the mechanism by which ecdysteroids regulate GSCs in

response to environmental cues is unclear.

One of the major environmental cues that affect egg production is the mating stimulus. In the *Drosophila* female, mating induces dramatic changes in reproductive behavior such as increased egg laying and decreased mating behavior.[34,35] The post-mating response is triggered by the male's Sex peptide (SP), which is present in the seminal fluid and transferred to the female during copulation.[36–38] Because mating functions as a switch for reproductive activation, the demand for gametes increases during mating to generate more offspring. Therefore, it is possible that mating modulates GSC activity to activate gametogenesis and increase the supply of eggs. This is indeed the case, as our previous study has demonstrated that GSC number increases in response to mating.[30] Moreover, we also found that the mating-induced GSC increase is mediated by ovarian ecdysteroids.[30] In contrast, the underlying mechanisms that control ovarian ecdysteroid biosynthesis in virgin and mated females are still unknown.

In this Extra View, we extend our previous findings by characterizing the expression pattern of the ecdysteroidogenic enzyme-encoding genes in the ovary in the post-eclosed and post-mated periods. In addition, we show that ovarian ecdysteroid biosynthesis has a long-term effect on GSC maintenance in the mated female flies.

## Results and Discussion

### Expression of ecdysteroidogenic enzyme-encoding genes in the ovary of virgin females

In our original paper, we have demonstrated that ovarian ecdysteroid biosynthesis is activated by mating stimuli, and the level of the ovarian ecdysteroid in the mated females is significantly higher than that in the virgin females.[30] In addition, our data suggest that this activation, at least in part, results from transcriptional up-regulation of ecdysteroidogenic enzyme-encoding

genes.[30] Moreover, several previous studies have reported that the ovarian ecdysteroid is detected in virgin females.[39,40] Especially, Tu et al. describe the changes in ecdysteroid level in the wild type ovary 0–48 hours after eclosion without mating. Interestingly, a peak in the levels of the ovarian ecdysteroid is observed in virgin female flies approximately 18 hours after eclosion, which may be required for initiating oogenesis.[40] This observation suggests that the expression of the genes encoding the ecdysteroidogenic enzymes fluctuate temporally even in virgin females. However, this scenario has not yet been tested.

We therefore investigated the transcription pattern of the ecdysteroidogenic enzyme-encoding genes, including *noppera-bo (nobo)*, *neverland (nvd)*, *shroud (sro)*, *spook (spo)*, *phantom (phm)*, *disembodied (dib)*, *shadow (sad)*, and *shade (shd)* (Fig. 1A) in virgin females. The ovaries of the virgin females were dissected at 3-hour intervals within the first 6 to 27 hours post-eclosion. We observed that the expression levels of certain genes, namely, *nobo*, *nvd*, *sro*, and *phm*, gradually decreased after eclosion, while there was no significant change in the temporal expression of *dib* and *sad* (Fig. 1B). However, we observed a significant increase in the *spo* and *shd* mRNA abundance at 15–21 hours post-eclosion (Fig. 1B). Taken together, most of the genes were highly expressed until 18 hours after eclosion. We speculate that the expression of the ecdysteroidogenic enzyme-encoding genes might be a preparation to achieve the highest level of ovarian ecdysteroids at 18 hours post-eclosion.[40] In contrast, most of the genes involved in biosynthesis showed lower expression levels 18–21 hours after eclosion and later, when the virgin females had lower ecdysteroid levels in the ovary.[40] These results suggest that the ecdysteroidogenic enzyme-encoding gene expression is regulated not only in the mated females but also in the virgin females, implying that unknown tropic stimuli, other than the mating stimuli, may be involved in controlling the ovarian ecdysteroid biosynthesis in the post-eclosion period. However, it should be noted that the physiological relevance of the temporal change in individual ecdysteroidogenic enzyme-coding genes is unclear so far.

## Sex peptide and its receptor up-regulate the expression of the ecdysteroidogenic enzyme-encoding genes differently.

We have previously found that the mating-induced ecdysteroid biosynthesis is mediated by the male-derived SP and its receptor SPR, the components of a canonical neuronal pathway that induces a post-mating behavioral switch in females.[30,37,41,42] Moreover, we have described that flies with a loss of SPR function exhibit significant reduction in *nvd* and *phm* expression.[30] To further investigate how SP signaling affects the expression of the ecdysteroidogenic enzyme-encoding genes in the ovary, we examined their expression levels in wild-type female flies that were mated with the *SP* null mutant males (the ligand mutant).[41] We found that the female flies that mated with the control males showed increased expression of certain ecdysteroidogenic enzyme-encoding genes, including *sro*, *spo*, *phm*, *sad*, and *shd*, compared to those in the virgin female flies (Fig. 1C). However, mating with the *SP* null mutant males did not induce any increase in transcript levels of most of the genes except for *spo* (Fig. 1C). These results suggest that mating up-regulates the transcription of the ecdysteroidogenic enzyme-encoding genes in the ovary via SP from the male's seminal fluid. In addition, it is noteworthy that SP appears to influence the expression of more ecdysteroidgenic enzyme-encoding genes than SPR.[30] These results imply that SP might affect the expression of the ecdysteroidogenic enzyme-encoding genes in the ovary via both the SPR-dependent pathway and an unknown SPR-independent pathway.

## Ovarian ecdysteroid biosynthesis and GSC maintenance

While our previous study has revealed an indispensable role of ecdysteroids in GSC proliferation within 24 hours after mating, other studies have demonstrated that the ovarian ecdysteroid signaling and its downstream cascade are essential for many aspects of

oogenesis,[9,27–29,43] particularly GSC maintenance,[5–10] over a week and more after mating. Therefore, we examined the effect of ecdysteroid biosynthesis on oogenesis, including stem cell regulation, after mating and over a longer period post-mating. We have previously reported that mating increases GSC number and this increase is maintained after 6 days from the first mating.[30] To confirm the role of the ovarian ecdysteroid in GSC maintenance for over a week, we dissected ovaries from 2-week-old females at 1 week after mating (Fig. 2A).

To generate the ovary in which ecdysteroid biosynthesis is impaired, we knocked down *nvd*, which encodes the ecdysteroidogenic enzyme responsible for catalyzing the first step of the ecdysteroid biosynthesis pathway,[16,17] by transgenic RNA interference (RNAi) with the *c587-GAL4* driver. While the *c587-GAL4* driver is known to be active in adult ovarian somatic cells, including escort cells and follicle cells, but not in nurse cells, we have previously found that the *c587-GAL4*-driven *nvd* RNAi (*c587>nvd* RNAi) efficiently leads to a significant reduction of NVD protein levels in both follicle and nurse cells for unknown reasons.[30] In addition, we have reported that *c587>nvd* RNAi leads to reduction in ovarian ecdysteroid levels compared to those in control animals.[30]

In the experimental flies that underwent the mating protocol shown in Fig. 2A, we found that the *c587>nvd* RNAi female flies had significantly less GSCs (1.90 GSCs on average) compared to that in the control female flies (2.13 GSCs on average) (Fig. 2D, left). To eliminate the possibility of developmental defects in oogenesis caused by this genotype (*c587-GAL4* is already active in somatic cells at the larval stage),[44] we confirmed that the number of GSCs in the female flies were not affected by the *c587>nvd* RNAi condition 1 day after eclosion compared to those in the control flies (Fig. 2D, right). Second, there were no differences in GSC number between the *c587>nvd* RNAi and control pre-mating flies, which were at 1 week after eclosion (Fig. 2D, right). These results suggested that *c587>nvd* RNAi does not affect GSC establishment during either pre-adult oogenesis or pre-mating ovarian

maturation. In other words, our data strongly support our hypothesis that ovarian ecdysteroid biosynthesis in the adult stage is required for GSC maintenance in the mated females. We also tested whether ecdysteroid biosynthesis affects the process of germ cell differentiation in the germarium. However, we did not observe any changes in the number of germ cells in the cystoblast, 2-cell cyst, 4-cell cyst, 8-cell cyst, and 16-cell cyst (Fig. 2B) in the *c587>nvd* RNAi female flies (Fig. 2E). In addition, the number of egg chambers in each stage (Fig. 2C) was not affected by the down-regulation of *nvd* in the ovary (Fig. 2F). Taken together, ovarian ecdysteroid biosynthesis controls the number of GSC, but not the number of differentiating germ cells and stage of the egg chamber.

We next examined whether the GSC maintenance phenotype in the *c587>nvd* RNAi is caused by a reduction in ovarian ecdysteroid levels. We measured the ovarian ecdysteroid levels in the *c587>nvd* RNAi female flies. We found that knocking down of *nvd* resulted in reduced ovarian ecdysteroid levels compared to that in the control female flies (Fig. 2G). To confirm whether this reduction is caused by a decrease of NVD enzymatic activity, we performed a transgenic rescue experiment in the *c587>nvd* RNAi background. As expected, the levels of the ovarian ecdysteroid were restored upon overexpression of the wild-type *nvd* ortholog of the silkworm *Bombyx mori* (*nvd-Bm[wt]*), but not its enzymatically dead form (*nvd-Bm[H109A]*) (Fig. 2G), suggesting that the reduction in ecdysteroid level is not caused by any off-target effects of the transgenic RNAi. Consistent with this data, the GSC phenotype in the *c587>nvd* RNAi flies was also rescued by co-expression of the wild-type *nvd-Bm*, but not the enzyme-dead form (Fig. 2H). To further investigate the role of ecdysteroid on the regulation of GSC maintenance, we performed a feeding rescue experiment using 7-dehydrocholesterol (7dC), the downstream metabolite generated by NVD. We found that the *c587>nvd* RNAi females fed with 7dC did not show a significant decrease in GSC number than the control female flies. (Fig. 2I). In addition, the GSC number in *c587>nvd* RNAi flies was rescued by the

oral administration of 20-hydroxyecdysone (20E), the biologically active ecdysteroid. These data suggest that ovarian ecdysteroid biosynthesis plays an important role in controlling GSC proliferation and long-term GSC maintenance in the mated female flies (Fig. 3).

## Outlook

In conjunction with our previous study,[30] our data suggest that ecdysteroid biosynthesis in the ovary is differentially regulated in the different life-stages of the female adult fly, including the post-eclosion and pre-mating stage, the post-mating early stage, and the post-mating late stage (Fig. 3). In every stage, ecdysteroid biosynthesis plays essential roles in controlling oogenesis, especially GSC proliferation and/or maintenance. We have confirmed that ecdysteroid-dependent GSC proliferation in the post-mating stage is controlled by the SP-SPR signaling pathway, which stimulates the ovarian ecdysteroid biosynthesis via regulation of the expression of the ecdysteroidogenic enzyme-encoding genes. In contrast, the identity of the genes and signaling pathways that influence the expression of the enzyme-encoding genes and the subsequent ecdysteroid biosynthesis in the ovary are unclear. Moreover, the cause of the fluctuation in ovarian ecdysteroid biosynthesis during the female adult lifespan is not yet clear. This is in contrast to the fluctuation of ecdysteroid titer that is observed during the embryonic, larval, and pupal development.[32,33,45] It should be remembered that studies on the role of steroid hormone biosynthesis in sexual maturation and gametogenesis in the postnatal stage of mammals has received more attention.[46] In this sense, further studies on ovarian ecdysteroid biosynthesis in *Drosophila* and other insects would be intriguing to comprehensively understand the roles of steroid hormone biosynthesis across the animal phyla in the future.

## Materials and Methods

The flies were raised on cornmeal-agar-yeast media at 25°C. *yw* was used as the control strain. $SP^0$ and $SP^\Delta$ (ref. [41]) were gifts from Nobuaki Tanaka (Hokkaido University, Japan). *c587-GAL4* [47,48] was a gift from Hiroko Sano (Kurume University, Japan). Other strains used were *UAS-nvd-IR*, *UAS-nvd-Bm [wt]*, *UAS-nvd-Bm [H190A]* (ref. [16]). Staining of GSCs with the 1B1 antibody,[49] quantitative reverse transcription-polymerase chain reaction, and ecdysteroid measurements were performed as previously described.[30]

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Figure legends

**Figure 1.** Transcriptional regulation of ecdysteroidogenic enzyme genes in the ovary. (A) The ecdysteroid biosynthesis pathway. Cholesterol is converted into 20-hydroxyecdysone (active form of ecdysone) by several ecdysteroidogenic enzymes (Shown in bold). (B) Temporal changes in ecdysteroidogenic enzyme genes in virgin female flies in post-eclosion period (n=4). Most of the genes showed higher expression levels at 6 hours or 15–21 hours post-eclosion (*nobo*, *nvd*, *sro*, *spo*, *phm*, and *shd*). (C) Relative changes in ecdysteroidogenic enzyme gene expression in ovary. Ovaries were dissected from age-matched virgin and mated females at 16 hours post-mating. Some genes showed significant increase in mated female flies compared to virgin female flies (*sro*, *spo*, *phm*, *sad*, and *shd*). These increased expressions after mating were suppressed when female flies mated with *SP* null male flies (except for *spo*). Values are presented as the mean with standard error of the mean in B. For statistical analysis, t-test with Holm's correction was used for B, Student's t-test was used for C. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, NS, non-significant ($P > 0.05$).

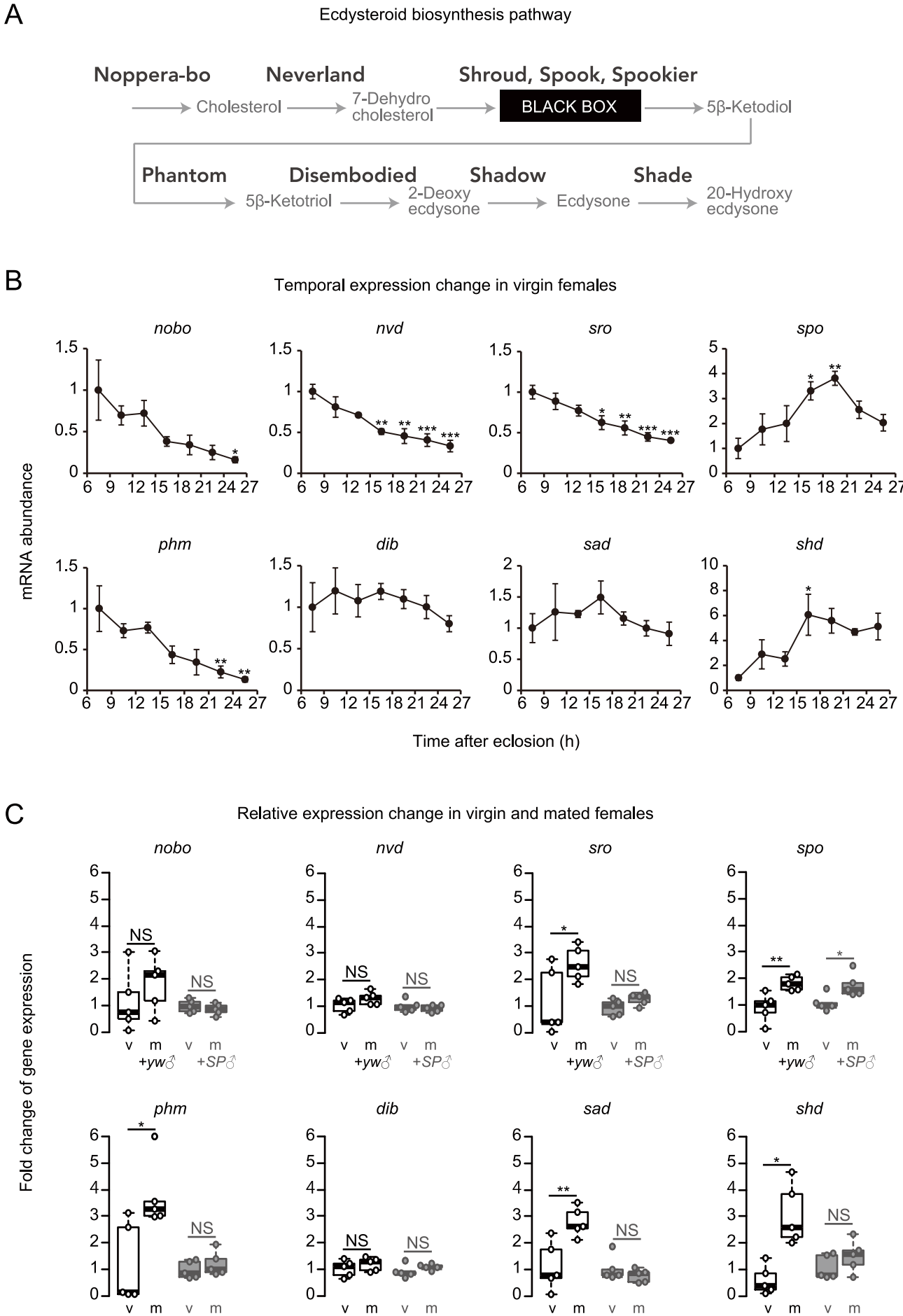
**Figure 2.** The role of ecdysteroid biosynthesis on the regulation of GSC maintenance. (A) Protocol for all experiments in this figure. One-week-old females were mated with males and used for the assay, 1 week after mating. (B) *Drosophila* germarium. Germline stem cell (GSC) resides in a niche, comprising somatic cells called cap cells, terminal filament, and escort stem cells. GSCs are identifiable by their typical spectrosome morphology and their location (adjacent to the niche cells). GSC produces one self-renewing daughter and one cystoblast (CB) that differentiates into a germline cyst. The cystoblast divides four times with incomplete cytokinesis (2 cc: 2-cell cyst, 4 cc: 4-cell cyst, 8cc: 8-cell cyst and 16 cc: 16-cell cyst). (C) *Drosophila* ovary is composed of 15–20 ovarioles. The continuous developing egg chamber is divided into 14 stages. (D) Left: Frequencies of germaria containing zero, one, two

and three GSCs (left y-axis), and average number of GSCs per germarium (right y-axis) in mated females. Ovarian *neverland* (*nvd*) knockdown in ovarian somatic cells (escort cells and follicle cells, using *c587-GAL4*) reduced average GSC number as compared to the control ($P$ = 0.006145). Right: Temporal change in GSC number in virgin females (1-day-old and 1-week-old) and mated females (2-week-old), ($n \geq 94$). (E and F) The average number of germline cyst (E) and egg chamber in each stage (F) was not changed in ovarian *nvd* RNAi female flies (*c587>nvd RNAi*). (G) *UAS-nvd-Bm [wt]* and *UAS-nvd-Bm [H190A]* were used for overexpressing the wild-type form and enzymatic inactive form of *Bombyx mori nvd* transgenes, respectively. Ovarian ecdysteroid decreased in *c587>nvd RNAi* female flies as compared to the control flies ($P$ = 0.0233). This reduction was restored by overexpressing *UAS-nvd-Bm [wt]* but not *UAS-nvd-Bm [H190A]*. (H) GSC phenotype in *c587>nvd RNAi* animals was restored by overexpressing *UAS-nvd-Bm [wt]* but not *UAS-nvd-Bm [H190A]*. (I) GSC phenotype in *c587>nvd RNAi* flies was rescued by oral administration of 20E or 7dC. Values are presented as the mean with standard error of the mean in G. The numbers of samples examined are indicated in parentheses in D, E, F, H and I. For statistical analysis, Wilcoxon rank sum test was used for D, E and F. t-test with Holm's correction was used for G. Steel-Dwass test was used for H and I. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, NS, non-significant ($P > 0.05$).

**Figure 3.** Model for this study. Ecdysteroid biosynthesis in the ovary is differentially regulated in different adult life stages, including the post-eclosion and pre-mated stage (upper column), the post-mating early stage, and the post-mating late stage (lower column). In post-eclosion stage, ecdysteroidogenic enzyme gene expression is regulated by unknown tropic stimuli and may be involved in controlling the ovarian ecdysteroid biosynthesis to initiate oogenesis. In post-mated early stage, SP stimulates ecdysteroid biosynthesis via up-regulation of

453 biosynthesis enzyme gene expression, which control GSC proliferation. Ovarian ecdysteroid
454 biosynthesis is also required for GSC maintenance in post-mated late stages.

# Figure 1.


A
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Neverland
Shroud, Spook, Spookier
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7-Dehydro cholesterol
BLACK BOX
5β-Ketodiol
Phantom
Disembodied
Shadow
Shade
5β-Ketotriol
2-Deoxy ecdysone
Ecdysone
20-Hydroxy ecdysone
B
Temporal expression change in virgin females
nobo
nvd
sro
spo
phm
dib
sad
shd
mRNA abundance
Time after eclosion (h)
C
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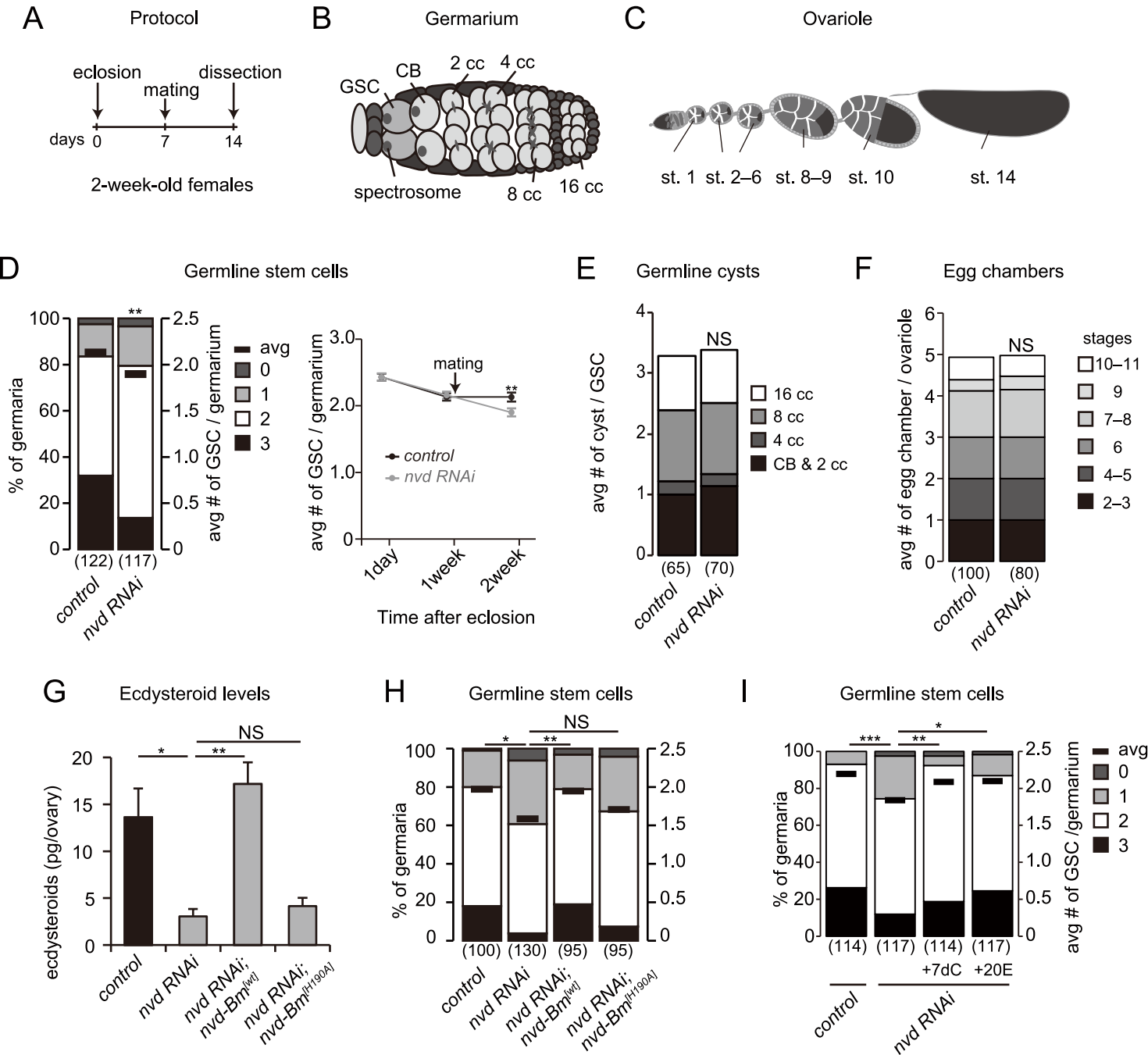
Fold change of gene expression
NS
v m v m
+yw♂ +SP♂

# Figure 2.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mating
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B
Germarium
GSC
CB
2 cc
4 cc
spectrosome
8 cc
16 cc
C
Ovariole
st. 1
st. 2–6
st. 8–9
st. 10
st. 14
D
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% of germaria
avg # of GSC / germarium
avg
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1
2
3
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(117)
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nvd RNAi
mating
control
nvd RNAi
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1week
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E
Germline cysts
avg # of cyst / GSC
NS
16 cc
8 cc
4 cc
CB & 2 cc
(65)
(70)
control
nvd RNAi
F
Egg chambers
avg # of egg chamber / ovariole
NS
stages
10–11
9
7–8
6
4–5
2–3
(100)
(80)
control
nvd RNAi
G
Ecdysteroid levels
ecdysteroids (pg/ovary)
*
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control
nvd RNAi
nvd RNAi; nvd-Bm[wt]
nvd RNAi; nvd-Bm[H190A]
H
Germline stem cells
% of germaria
*
**
NS
(100)
(130)
(95)
(95)
control
nvd RNAi
nvd RNAi; nvd-Bm[wt]
nvd RNAi; nvd-Bm[H190A]
I
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% of germaria
avg # of GSC /germarium
avg
0
1
2
3
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*
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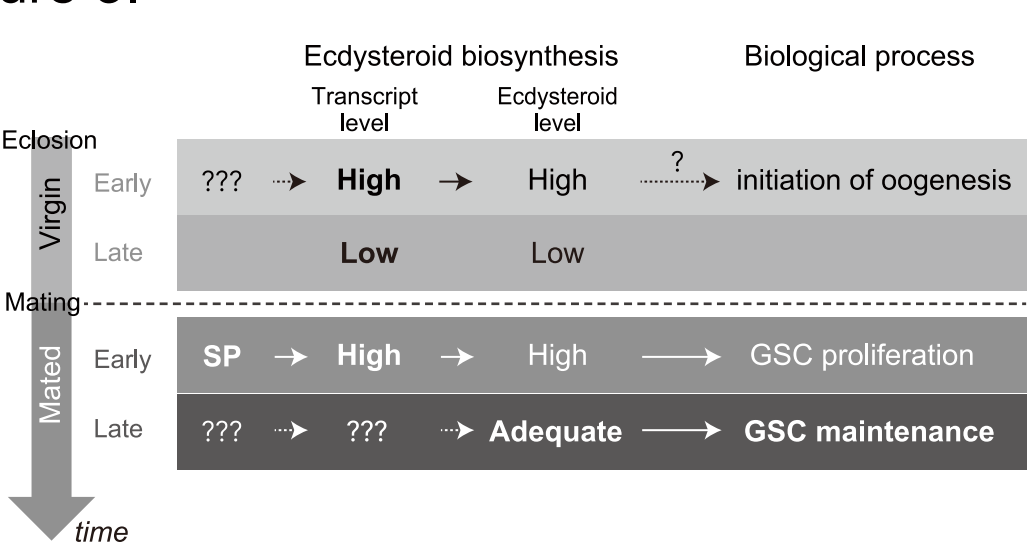
(117)
(114)
(117)
+7dC
+20E
control
nvd RNAi

# Figure 3.


Ecdysteroid biosynthesis
Biological process
Transcript level
Ecdysteroid level
Eclosion
Virgin
Early
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High
High
?
initiation of oogenesis
Late
Low
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Mating
Mated
Early
SP
High
High
GSC proliferation
Late
???
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Adequate
GSC maintenance
time