Molecular mechanisms of nutrient-dependent regulation of fecundity in *Drosophila* males

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Contents

1. General introduction

Drosophila melanogaster is known as a model organism, which is broadly used for genetical and developmental biological studies because it has lots of homologous genes in human being.

Structure and functions of transcription factor Dve

The *Drosophila* homeobox gene *defective proventriculus* (*dve*) generates two transcripts, type A (~4.9 kb) and type B (~3.5 kb) from different transcription initiation sites **(Fig 1 A)**. The *dve* gene product isoforms (Dve-A and Dve-B) are a putative transcription factor containing two homeodomains (HD-N and HD-C) and a compass (CMP) domain **(Fig 1 B).** These domains are evolutionarily conserved, and the CMP domain is found in mammalian special AT-rich sequence binding proteins (SATBs) **(Fig 1 C; Bürglin et al., 2002).** SATBs are expressed in several organs and act as a chromatin remodeling factor, which regulates various genes.

Dve is also broadly expressed in various organs; intestine (guts), kidney (malpighian tubules), wing, leg, antenna, neurons (photoreceptor cell and brain), reproductive organs, and so on. The homozygous larvae for the strong loss of function allele dve^{1} are defective in proventriculus morphogenesis, resulting in feeding defect and lethality at first-larval instar **(Nakagoshi et al., 1998).** During midgut development, expression of two Dve isoforms is highly dynamic; Dve-A is a major isoform in the embryonic middle midgut, transiently repressed in copper cells around hatching and re-expressed later. On the other hand, isoform switching from Dve-A to Dve-B occurs in adjacent interstitial cells. Such spatio-temporal isoform switching is crucial for proper intestinal cell development **(Nakagawa et al., 2011).** For morphogenesis of wing and leg, stage-specific repression of Dve is extremely important **(Shirai et al., 2007; Sugimori et al., 2016).** Dve expression is also crucial for regulation of rhodopsin expression in the photoreceptor cells and proper head morphogenesis. Accessory gland, a male reproductive organ also requires the Dve activity for high fecundity **(Figs 2 A-B and 3; Minami et al., 2012).**

Structure and function of accessory gland and seminal fluid proteins

The *Drosophila* male accessory gland has functions similar to those of the mammalian prostate gland and the seminal vesicle, and secretes accessory gland proteins (Acps; ～83 proteins) into the seminal fluid, which are essential for male fecundity **(Fig 2 A-B; Stevison et al., 2004; Chapman et al., 2004; Davies et al., 2004)**. Each of the two lobes of the AG is composed of two types of binucleated epithelial cells: approximately 1,000 main cells and 40~60 secondary cells (SCs) **(Fig 2 A-B; Bertram et al., 1992).** After mating, Acps in the females induce female long-term post-mating responses, such as increased egg laying and reduced sexual receptivity, to increase progeny production for a mated male **(Wolfner 1997; Avila et al., 2011; Mohorianu et al., 2018)**.

Seminal fluid proteins include cysteine-rich secreted proteins (CRISPs), sperm protecting lectin,

proteases which regulate sperm release form sperm storage organs, protease inhibitors for sperm competition, and so on. However, most of their functions are still unknown. There is no human orthologue of identified Acps, but some functional analogues are expressed **(LaFlamme et al., 2012)**.

The possible cause of human prostate dysfunction is disorder of coagulation and liquefaction of seminal fluid, and seminal fluid function for fecundity maintenance is evolutionarily highly conserved. Lots of knowledges of Acps from main cells are already reported. For example, Sex peptide (SP, also known as Acp70A) is secreted from the main cells and induces female long-term post-mating responses, such as increased egg laying and reduced sexual receptivity, to increase progeny production for a mated male **(Chen et al., 1988; Aigaki et al., 1991; Chapman et al., 2003; Liu et al., 2003; Kubli et al., 2003)**. The other example of Acp from main cells is Acp36DE, which is required for efficient sperm storage through induction of morphological changes in female uterus **(Chen et al., 1996; Neubaum et al., 1999; Wolfner 1997; Bloch et al., 2003; Avila, et al., 2009 and 2011; Mohorianu et al., 2018; Laflamme et al., 2013)**.

Though physiological significance of the secondary cells remained unclear, recent research has revealed that both main cells and SCs of the AG are essential for male fecundity. Dve and the transcription factor Abdominal-B (Abd-B) are strongly expressed in adult SCs, and Dve is required for survival of SCs. Both Abd-B and Dve are required for SC maturation. Reduced expression of Dve results in low fecundity with immature or no SCs, indicating that SCs are also essential for male fecundity **(Fig 2 C, Fig 3; Minami et al., 2012; Gligorov et al., 2013).**

Relationship between nutrition and fertility/ fecunedity

In various animals, a significant amount of energy is required in the production of eggs, sperm and seminal fluid for reproduction. Insect study of *Drosophila melanogaster* and *Tribolium castaneum* provides lots of knowledges about interaction between nutrition and fertility/ fecundity **(Xu et al., 2015)**. Under nutrient deprivation (poor diet) conditions, *Drosophila* female oogenesis is blocked by programmed cell death to reduce energy consumption **(Drummond et al., 2001).** In *Drosophila* males, courtship behavior is enhanced in the presence of food odor **(Das et al., 2017)**. Males having larger accessory glands show high fecundity in *Drosophila*, but lots of energy are required for a large amount of Acps **(Fig 4; Wigby et al., 2009).** However, detail of the mechanisms of nutrition and fertility/ fecundity is still unknown.

Steroid hormones-related developmental controls and nutrition sensing

Steroid hormones are synthesized from cholesterol and can bind to specific receptors. Mammalian steroid hormones are grouped into two classes: corticosteroids (typically made in the adrenal cortex, hence cortico-) and sex steroids (typically made in the gonads or placenta). There are five types of steroid hormones according to the receptors to which they bind: glucocorticoids, mineralocorticoids (corticosteroids), androgens, estrogens, and progestogens (sex steroids). Steroid hormones help control

metabolism, inflammation, immune functions, salt and water balance, development of sexual characteristics, and the ability to withstand illness and injury **(Tomkins, 1967; Patel 2018)**.

Ecdysone is a *Drosophila* steroid prohormone, which is secreted from the prothoracic glands and converted to the active form 20-hydroxyecdysone (20E) in the larval fat body (homologous organ to mammal liver). Active ecdysone promotes ecdysis (molting) and metamorphosis **(Fig 5 A).** The ecdysone receptor (EcR) consists of a heterodimer of two nuclear receptors, EcR and Ultraspiracle (USP). The EcR gene in *Drosophila* encodes 3 isoforms (EcR.A, EcR.B1 and EcR.B2) that vary in their N-terminal region and have their common DNA binding and ligand binding domains. The stage- and tissue-specific distribution of the isoforms during metamorphosis suggests distinct functions for the different isoforms **(Fig 5 B and C; Lavorgna et al., 1993; White et al., 1997; Lam et al., 2000; Agawa et al., 2007; Ruaud et al., 2010; Boulanger et al., 2012; Sultan et al., 2014; Akagi et al. ; Yaniv et al., 2016).**

Same as mammalian steroid hormones, ecdysone is also used for fecundity/ fertility control in insects. Proper oogenesis requires ecdysone signaling through EcR isoforms in *Drosophila* female **(Carney et al., 2000).** Under poor nutrition, ecdysone from adult oocyte and fat body is actively changed into 20E, which promotes cell death of mature eggs (abnormal oogenesis) **(Terashima et al., 2005).** In *Drosophila* male, ecdysone is expressed in the accessory gland, and the ecdysone signaling is required for male fecundity **(Hentze et al., 2013; Sharma et al, 2017).**

Prominent increase in the ecdysone titer secreted from the prothoracic glands (ecdysone pulses) are required for all aspects of morphogenesis **(Fig 5 D).** In the recent research, it has been also reported that systemic growth is regulated by the interaction between nutrient signaling and ecdysone signaling **(Francis et al., 2010; Buhler et al., 2018).**

In this thesis, the first section is the results about nutrition-dependent fecundity control in *Drosophila* males, and the second section is the results that such fecundity is regulated by ecdysone signaling.

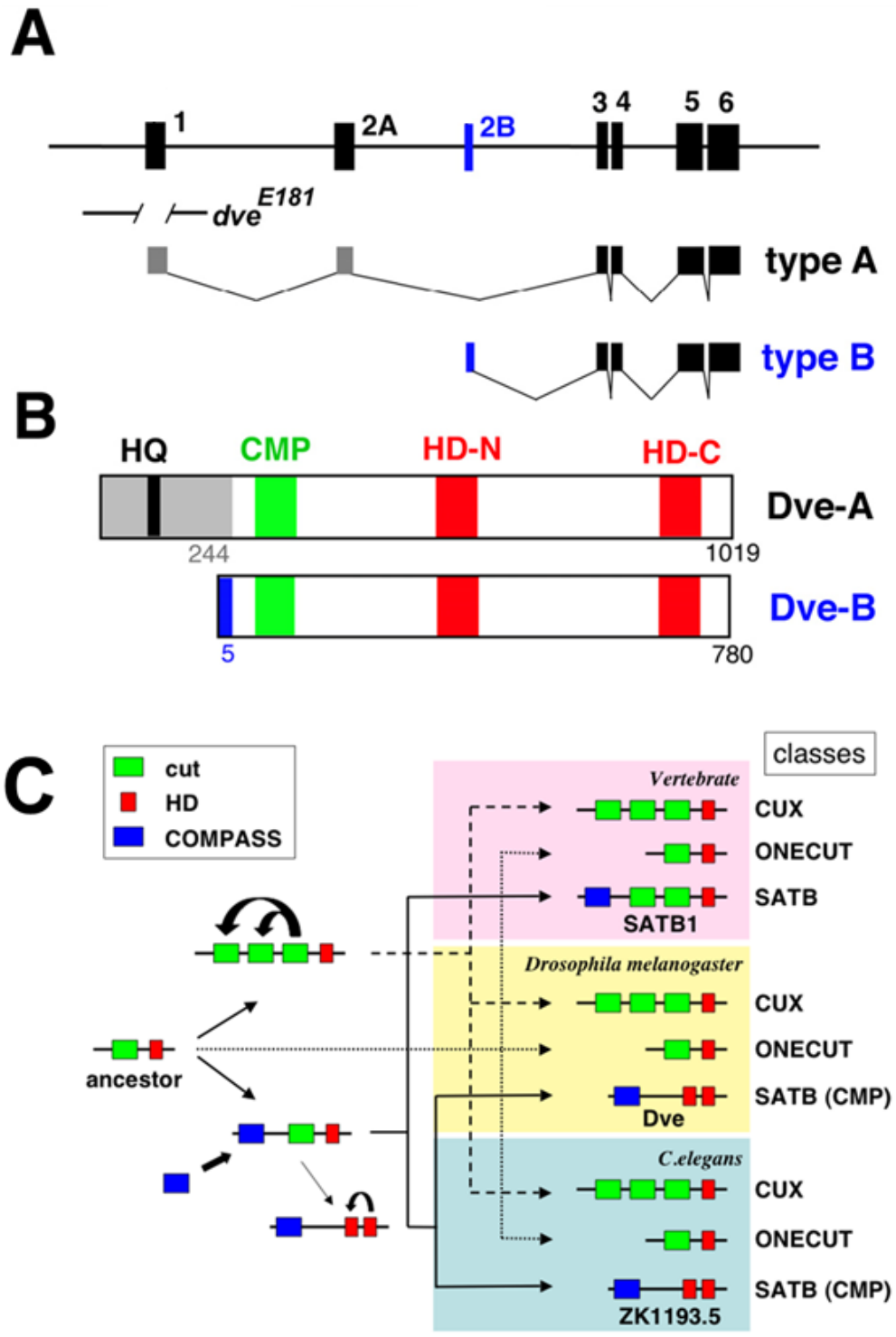
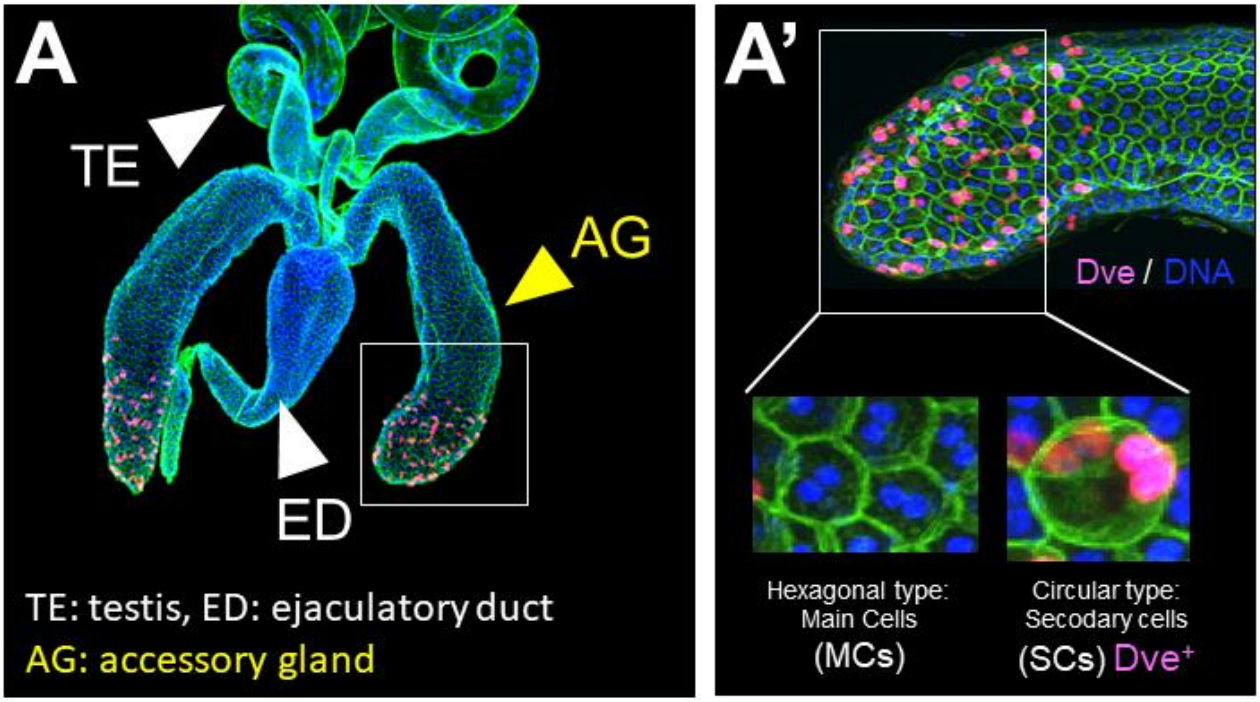


Fig 1. Structure of the *defective proventriculus* (*dve*) gene.

(A) Organization of the *dve* locus. The type-B specific exon 2B is indicated by blue.
(B) Two isoforms of Dve protein. HQ: histidine, glutamine-rich region, CMP: compass domain, HD-N and HD-C: homeodomains located in the N- and C-terminal side, respectively.
(C) Hypothetical model of the evolution of cut superclass homeobox genes from a common ancestor of plants, fungi and animals. The present-day classes are shown on the right. The different domains are color-coded, and more uncertain events are indicated by dashed arrows (adapted from Bürglin et al., 2002).



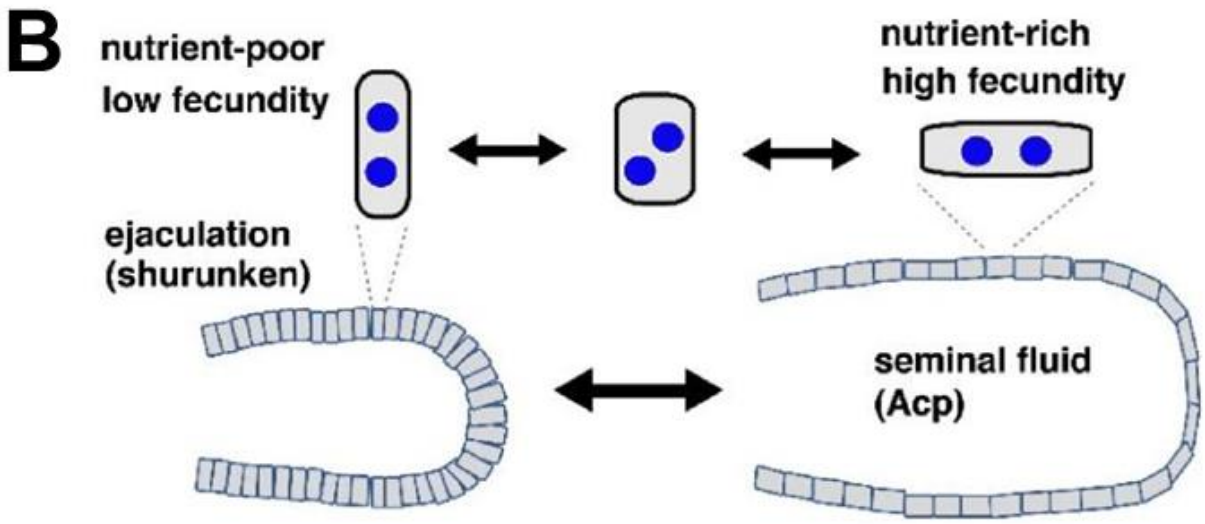


Fig 2. Structure of reproductive organ in *Drosophila* male

(A) Adult male accessory gland (AG) (yellow arrowhead), TE: testis, ED: ejaculatory duct. The boxed region is magnified in A'. (A') MC: main cell, SC: secondary cell. Dve (magenta) is expressed in SCs. Cell boundaries and nuclei (DNA) are marked by Spectrin (green) and TO-PRO3 (blue), respectively.
(B) Plastic changes of the AG size.

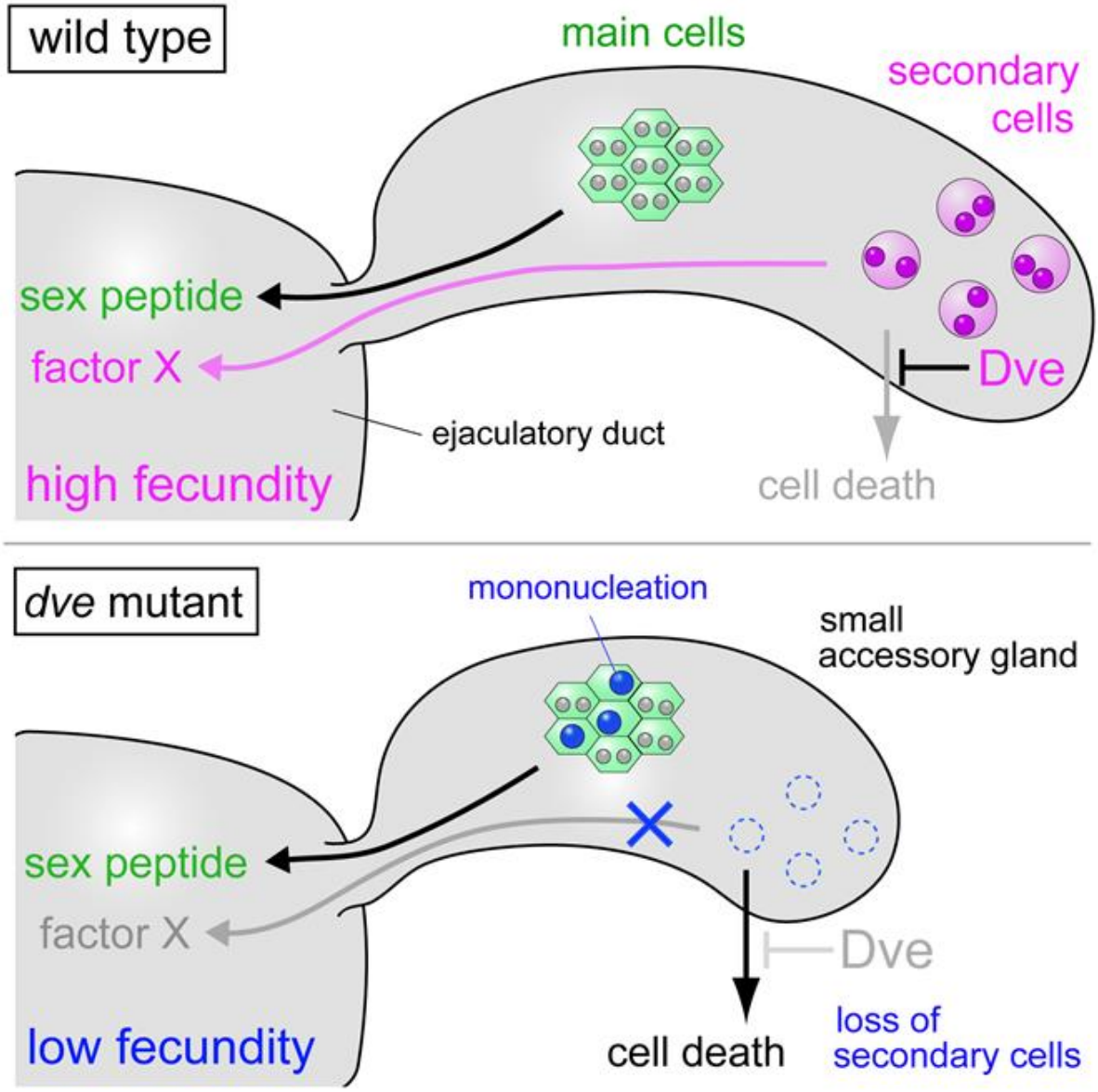


Fig 3. Function of accessory gland cells for male fecundity

Sex peptide (SP) is a major accessory gland protein (Acp) secreted from main cells. Maturation of SCs is regulated by Dve, Abd-B, and the SC maturation is essential for fecundity.

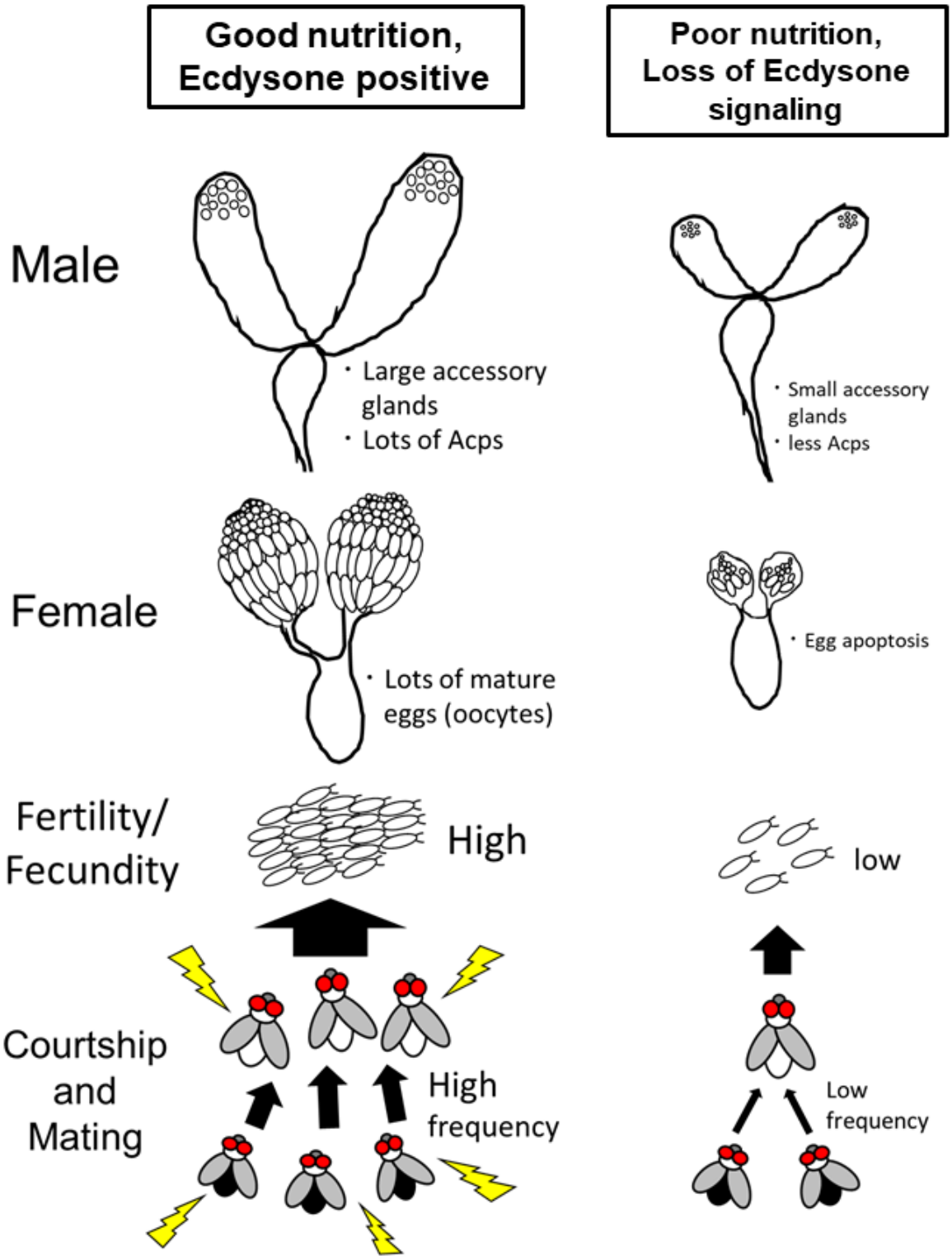


Fig 4. Nutrition and steroid hormone are crucial for fecundity/ fertility acquisition.

Effects of nutritional composition and steroid hormone. Male accessory glands and Female ovary are affected by nutrition and steroid hormone. Under poor nutrition and less steroid hormones, fertility/ fecundity becomes lower. Courtship behavior is also regulated by nutrition.

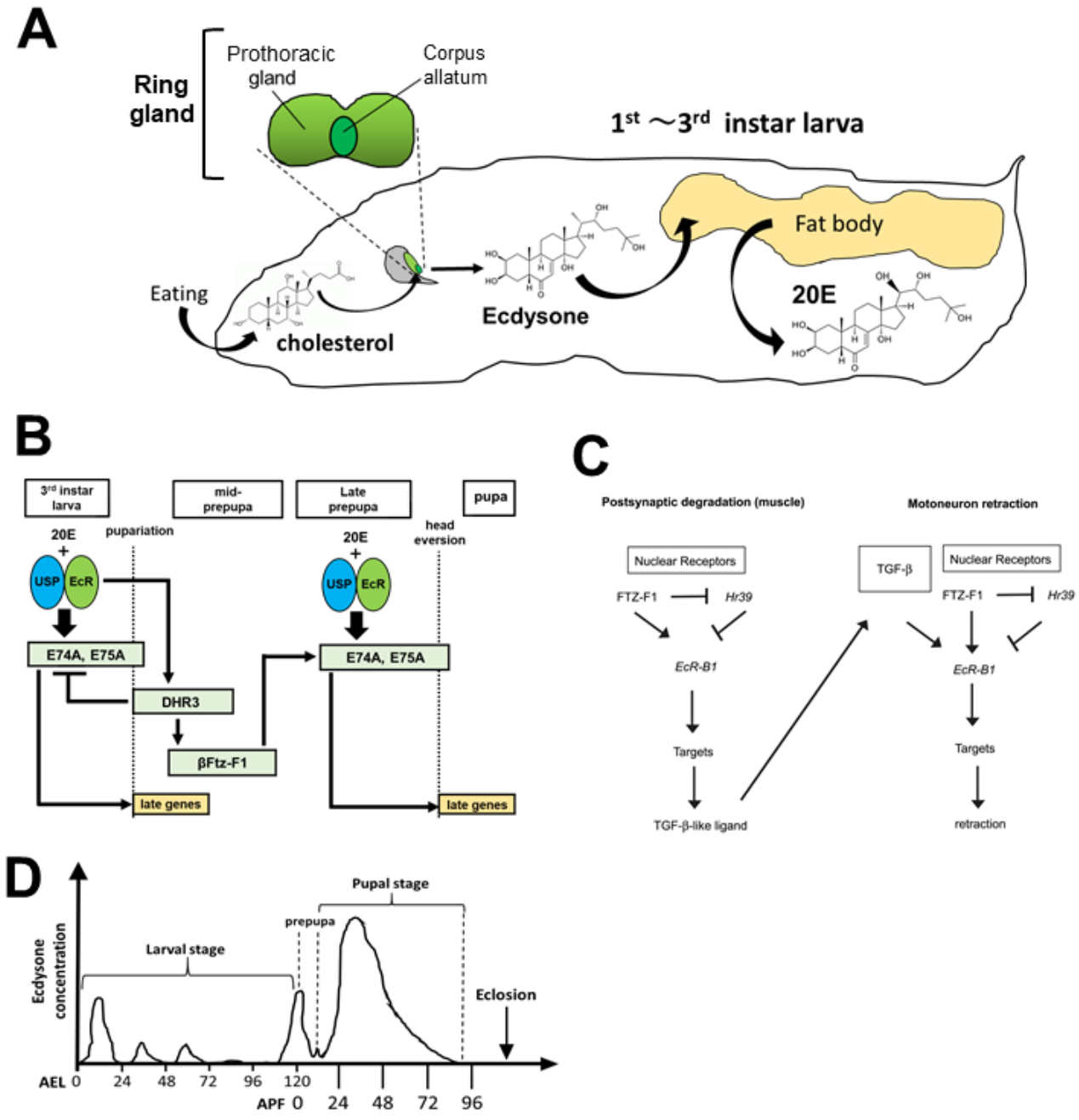


Fig 5. Function of Ecdysone during development.

(A) Production and secretion of Ecdysone in larval stage. Ecdysone is produced from cholesterol in prothoracic gland. Ecdysone is converted into its active form 20-hydroxyecdysone (20E) in the fat body.

(B) 20E can be received by UAS-EcR heterodimer for Ecdysone signal transduction. Ecdysone signaling prevents larval growth and promotes ecdysis (adapted from Ruaud et al., 2010).

(C) FTZ-F1 and HR39 positively and negatively respectively control the EcR-B1 expression at the postsynapse. FTZ-F1-mediated EcR-B1 expression leads to transcriptional regulation of target genes involved in postsynaptic degradation. Then, TGF-β/BMP signaling is supposed to be induced in response to postsynaptic degradation. This signal produced by the dismantling muscle is received by the motor neuron where activation of EcR-B1 expression (by TGF-β signaling and nuclear receptor) triggers the axon retraction. In addition, glial cells and macrophages participate to the neuromuscular junctions (NMJs) dismantling process (adapted from Boulanger et al., 2012).

(D) Ecdysone concentration titration in Drosophila. Ecdysone level is increased around the ecdysis (Ecdysone pulse), and soon after that, active 20E-depenent signaling is transmitted. These are three higher Ecdysone concentration during development; 1st instar larva, prepupa, and early pupal stage.

2. Nutrient conditions sensed by the reproductive organ during development optimize male fecundity in *Drosophila*

Abstract

Nutrient conditions affect the reproductive potential and lifespan of many organisms through the insulin signaling pathway. Although this is well characterized in female oogenesis, nutrient-dependent regulation of fertility/fecundity in males is not known. Seminal fluid components synthesized in the accessory gland are required for high fecundity in *Drosophila* males. The accessory gland is composed of two types of binucleated cells: a main cell and a secondary cell (SC). The transcription factors Defective proventriculus (Dve) and Abdominal-B (Abd-B) are strongly expressed in adult SCs, whose functions are essential for male fecundity. Gene expression of both Dve and Abd-B was down-regulated under nutrient-poor conditions. In addition, nutrient conditions during the pupal stage affected the size and number of SCs. Under nutrient-rich conditions, fecundity is enhanced by increasing the size and the number of SCs. Under nutrient-poor conditions, expression of both Dve and Abd-B in SCs is downregulated along with reduced size of SCs, and fecundity is low. However, these SCs can recover their size under normal diet conditions in the adult stage, and fecundity is also recovered. These morphological changes clearly correlated with fecundity, suggesting that SCs act as nutrient sensors.

Here, this research provides evidence that Dve associates nutrient conditions with optimal reproductive potential in a target of rapamycin signaling-dependent manner, and the results show that physiological changes of SCs regulate male fecundity in a highly plastic manner depending on nutrient conditions.

Introduction

A significant amount of energy is required in the production of eggs, sperm and seminal fluid for reproduction. Environmental cues and diet are integrated with the central nervous system and activate a "reproductive mode" through endocrine signals, whereas dietary restrictions increase survival capacity in poor environmental conditions and leads to a "waiting mode" with an extended lifespan **(Partridge et al., 2005; Tatar et al., 2003).**

The nutrient-sensing pathway mediated by the target of rapamycin (TOR) kinase is activated as a downstream event in the insulin signaling pathway or by the availability of amino acids. The insulin signaling pathway is highly conserved during evolution and regulates various aspects of physiology including oocyte growth and maturation **(Das et al., 2017).** Under nutrient deprivation (poor diet) conditions, *Drosophila* oogenesis is blocked by programmed cell death to reduce energy consumption **(Drummond-Barbosa et al., 2001).** In humans, certain types of ovarian dysfunctions are associated with hypo- or hyperinsulinemia, suggesting a close association between nutrient conditions and female reproduction **(Chang et al., 2005; Colton et al., 2002; Diamanti-Kandarakis et al., 2012).**

In males, the effects of insulin signaling on fertility/fecundity are not known, although the proliferation of germline stem cells is under the control of insulin signaling **(McLeod et al., 2010; Shim et al., 2013; Ueishi et al., 2009).** Several reports support the notion that male fecundity is also regulated by nutrient conditions; (a) nutrient conditions affect testicular size and the rate of sperm production in sheep and goats **(Martin et al., 1995)**; (b) in *Drosophila* males, courtship behavior is enhanced in the presence of food odor **(Grosjean et al., 2011; Vosshall et al., 2007)**; (c) the insulin signaling pathway is required for growth and maturation of the accessory gland in *Drosophila* and *Tribolium castaneum* **(Taniguchi et al., 2012; Xu et al, 2015)**; and (d) males having larger accessory glands show high fecundity in *Drosophila* **(Wigby et al., 2009).**

The *Drosophila* male accessory gland secretes accessory gland proteins (Acps) into the seminal fluid, which are essential for male fertility/fecundity **(Chapman et al., 2004; Wolfner, 2002).** Each of the two lobes of the accessory gland is composed of two types of binucleated cells: approximately 1,000 main cells and 40–60 secondary cells (SCs) **(Bertram et al. 1992).** Adult main cells are flat hexagonal cells, and SCs are large spherical cells interspersed among the main cells at the distal tip of each accessory gland lobe. The monolayer epithelium is surrounded by a thin layer of muscle, which contracts during copulation and ejaculation **(SusicJung et al. 2012, Tayler et al. 2012).** The transcription factors, Defective proventriculus (Dve) and Abdominal-B (Abd-B), are strongly expressed in adult SCs; these transcription factors are essential for male fecundity **(Gligorov et al.,**

2013; Minami et al., 2012). SCs have unusually large vacuoles, which are nonacidic secretory vacuoles (SVs) and acidic mature late endosomal multivesicular bodies or lysosomes (MVBLs) **(Redhai et al., 2016; Rylett et al., 2007).** Exosomes, which are nano-sized vesicles, are generated in the MVBLs, and their secretion is required for a part of female postmating responses **(Corrigan et al., 2014).**

Here, this research provides evidence that nutrient conditions are sensed by the accessory gland cells and that the SCs regulate male fecundity through nutrient-dependent changes in Dve and Abd-B expression and subsequent vacuolar formation.

Results

SCs in the accessory gland are responsive to nutrient conditions

Courtship behavior of *Drosophila* males is enhanced in the presence of food, and fecundity is also enhanced when males have large accessory glands **(Grosjean et al., 2011; Vosshall, 2007; Wigby et al., 2009).** Thus, it was hypothesized that nutrient-rich conditions stimulate accessory gland cells to produce a large amount of seminal fluid components for reproductive success. However, diets high in protein, sugar or fat did not affect the gross morphology of the accessory gland. In contrast, poor diet conditions (0.25 × normal diet) induced a size reduction in some SCs **(Fig 6 a–c, Fig S1).** In these cells, the expression of the transcription factor Dve was greatly reduced, whereas normal-sized cells showed strong Dve expression **(Fig 6 c).**

It has been reported that nutrient conditions are sensed in the fat body, and some adipokines (CCHa2, Stunted and Eiger) are secreted into the hemolymph to act on insulin-producing cells (IPCs) **(Agrawal et al., 2016; Delanoue et al., 2016; Sano et al., 2015).** In addition, Unpaired 2, a fat body-derived cytokine, indirectly activates the IPCs by relieving a tonic inhibition from GABAergic neurons **(Rajan et al., 2012).** *Drosophila* insulin-like peptides (DILPs) secreted from IPCs into the hemolymph regulate target cell physiology through insulin signaling pathways **(Badisco et al., 2013).**

The expression of the *dve-GAL4[30A]* (*dG30A*) driver reflects the endogenous Dve expression in the accessory gland cells **(Minami et al., 2012)** and is also detected in other cell types, including the fat body. To mimic nutrient-poor conditions, the dominant-negative form of target of rapamycin (TOR) or insulin receptor (InR) was induced in these dve-expressing cells (*dG30A>TOR[DN]* or *dG30A>InR[DN]*, respectively). These SCs showed a severe size reduction as observed under the poor diet condition, although the number of SCs was not affected **(Fig 6 d–h).** These results prompted us to determine whether the lack of the fat body-derived factors caused a reduction in the size of SCs.

Targeted inhibition of TOR or insulin signaling in the fat body was induced with *Cg-GAL4* (*Cg>TOR[DN]* and *Cg>InR[DN]*), and the size of SCs was only mildly reduced, although the level of GAL4 expression was greatly increased in *Cg-GAL4* compared to that of *dve-GAL4[30A]* **(Figs S2 and S3)**. Thus, it is assumed that the fat body-derived factors only partially affect the response of SCs. It was hypothesized that the accessory gland cells directly respond to the nutrient conditions such as DILPs and/or amino acids in the hemolymph. Thus, another GAL4 driver line, *paired* (*prd*)-*GAL4* was used; this GAL4 expression was detected in the accessory gland but not in the fat body **(Fig S3).** Induction of TOR[DN] using *prd-GAL4* (*prd>TOR[DN]*) resulted in severe reduction in the size of SCs, suggesting that accessory gland cells directly sense the nutrient conditions through the levels of circulating DILPs and/or amino acids **(Figs S4 B–B" and S4 E)**. In addition, reduced insulin signaling pathway activity (*prd>InR[DN]*) resulted in severe reduction in the size of main cells, leading to the formation of small accessory glands **(Figs S4 C–C")**, which was consistent with the previous reports (Taniguchi et al., 2012). However, these small accessory glands had nearly normal-sized SCs at 25°C

(Fig S4 C"), but the size of SCs was reduced at 30°C **(Fig S4 E)**. Different effects of *dve-GAL4* and *prd-GAL4* drivers appear to reflect their developmental patterns of expression **(See Discussion).**

Apoptosis-mediated regulation of SC Survival

Although the nutrient-poor condition clearly reduced the size of SCs, increased amounts of protein, sugar or fat in the diet did not affect the morphology of the SCs. If the SCs act as nutrient sensors, the direct activation of the insulin signaling pathway should affect their morphology. Thus, the activated form of InR (InR[act]) was induced in the accessory gland cells (*dG30A>InR[act]*). Under this condition, the number of SCs increased to ~100 cells compared to 60 cells in the control **(Fig 7 b and d).** There are two possible explanations for this increased cell number: One is activated proliferation, and the other is inhibition of cell death **(See below)**. In addition, the size of the SCs also increased by 1.8-fold **(Fig 7 b' and e).**

The transcription factor Dve is strongly expressed in the SCs, and Dve activity is required to maintain SCs through the inhibition of cell death **(Minami et al., 2012).** Because the SCs of dve mutants are completely abolished through apoptotic cell death, it was hypothesized that the increased number of SCs with activated insulin signaling was due to the inhibition of apoptosis. To investigate this idea, *Drosophila* inhibitor of apoptosis 1 (DIAP1) was induced in the accessory gland cells (*dG30A>DIAP1*). The number of these SCs was also increased to ~100 cells in the *dG30A>DIAP1* flies, whereas their cell size was unaffected **(Fig 7 c–e).** Thus, these results strongly indicate that the number of SCs was regulated by apoptotic cell death in a nutrient-dependent manner.

Then it was examined whether the increased number of SCs can affect fecundity. Cumulative egg production in test females mated with *dG30A>InR[act]* males or *dG30A > DIAP1* males clearly increased for 14 days in both cases **(Fig 7 f).** In wild-type females, remating does not occur for approximately 7 days due to the action of Acp70A (Sex peptide (SP)). The test females that mated with *dG30A>InR[act]* or *dG30A>DIAP1* males showed a longer period of rejection during the 14 days **(Fig 7 g).** Thus, the targeted activation of insulin signaling in the accessory gland clearly enhances male fecundity through the increase in secondary cell number.

Nutrient-dependent Abd-B expression regulates male fecundity

The reduced size of the SCs was also reported in Abd-B mutant males. In these mutant cells, the maturation of vacuoles was strongly inhibited **(Gligorov et al., 2013).** Thus, it was investigated whether the size reduction in SCs with reduced TOR signaling was also associated with the loss of vacuoles. A visualization of the vacuoles with cytoplasmic GFP expression showed that *dG30A>TOR[DN]* males lacked the vacuoles as observed in Abd-B mutants **(Fig 8 a–b).** This result suggests a regulatory pathway where nutrient conditions affect vacuolar formation through the activity of Abd-B. Thus, it was assessed that Abd-B expression when TOR activity was reduced in the accessory gland. In *dG30A>TOR[DN]* males, Abd-B expression was greatly reduced **(Fig 8 c–d).**

Under a poor diet condition, some SCs showed a size reduction associated with reduced Dve expression **(Fig 6 c).** The SCs are crucial for male fecundity because males mutant for Abd-B or dve have low fecundity due to the reduced size or loss of SCs, respectively **(Gligorov et al., 2013; Minami et al., 2012).** Thus, it is assumed that males with targeted inhibition of TOR signaling in the accessory gland (*dG30A>TOR[DN]*) also have low fecundity through reduced Abd-B expression and the absence of vacuoles. For instance, the cumulative egg production in test females mated with *dG30A>TOR[DN]* males decreased to approximately 77% of that of control females at day 7 **(Fig 8 e).** Moreover, remating activity at 48 hr after the first mating was greatly increased in test females mated with *dG30A>TOR[DN]* males ($p < 0.001$, Fisher's exact test) **(Fig 8 f).** Thus, these results indicate that males with reduced TOR signaling in the accessory gland have low fecundity due to the reduced activity of SCs.

Nutrient conditions during the pupal stage are critical for SC physiology

Changes in nutrient signaling in the accessory gland affect male fecundity. To determine the period for sensing the nutrient conditions, it was carried out temporal inhibition or activation of the nutrient signaling pathway. As expected from the expression profile of *dve-GAL4[30A]*, temporal inhibition of TOR or InR (*dG30A>TOR[DN]* or *dG30A>InR[DN]*, respectively) during the pupal stage greatly reduced the number of SCs **(Fig 9 a–e,h)**, whereas inhibition after eclosion did not affect the morphology of the accessory gland cells **(Figs 4h and S5 A–S5 D).** Thus, the temporally regulated inhibition of TOR or insulin signaling during the pupal stage showed a more severe effect on the number of SCs **(compare Fig 9 h with Fig 6 g).**

These differences seem to be due to enhanced GAL4 expression at 30°C during the pupal stage. In addition, sustained inhibition of InR with *prd-GAL4* (*prd>InR[DN]*) occurs from the pupal to adult stages, and the reduced insulin signaling severely affected main cell growth even at 25°C **(Fig S4 C").** Under this condition, the size of SCs remained normal, indicating that SCs mainly respond to nutrient conditions during the pupal stage. Similar results were obtained in the case of activation. Temporal activation of the insulin signaling pathway during the pupal stage (*dG30A>InR[act]*) remarkably increased the number of SCs **(Fig 9 f–h)**, whereas activation after eclosion had no effect on SC counts **(Figs 9 h and Fig S5 E–F).** Thus, physiological changes in SCs are mainly regulated by nutrient conditions during the pupal stage, but not in the adult stage.

Nutirent-dependent plasticity in the adult stage

Even if nutrient conditions were poor during development, eclosed flies can move to nutrient-rich environment for survival. However, if nutrient conditions during development affected the size of main cells, nutrient-poor conditions should make very tiny AGs such as those of prd mutant males. It is assumed that these AGs need much more energy in the adult stage to recover fecundity. Thus, during development, nutrient dependent changes of SCs rather than main cells can optimize fecundity without

affecting the gross morphology. Therefore, it should be examined whether small SCs under nutrient-poor conditions can recover their size under normal nutrient conditions in the adult stage. Temporal inhibition of TOR or InR during the pupal stage resulted in severe size reduction of SCs, and the eclosed flies were reared under normal nutrient conditions that TOR or InR was functional **(Fig 10 A).** At day 7 of recovery phase, SCs remained small but fully recovered their size at day 14 **(Figs 10 B-F).** Thus, SCs can plastically change their size in response to nutrient conditions even in the adult stage to optimize fecundity **(Fig 11).**

Discussion

The male accessory gland is a nutrient sensor for reproduction

Nutrient availability is sensed in the fat body, and some adipokines are secreted into the hemolymph to mediate DILP secretion from IPCs. The systemic DILPs regulate target cell physiology through insulin signaling pathways **(Nassel et al., 2016).** In *Drosophila* females, nutrient-poor conditions strongly inhibit oogenesis. In contrast, nutrient-rich environments enhance female receptivity and stimulate egg production due to enhanced oogenesis through the insulin signaling pathway **(Drummond-Barbosa et al., 2001; Gorter et al., 2016; Hsu et al., 2009).** Thus, the nutrient-dependent changes in oogenesis appear to be critical to modulate female fertility. It seems likely that these responses are essential for leaving their progeny in a good nutrient environment for reproductive success.

In *Drosophila* males, protein starvation inhibits spermatogenesis through apoptotic cell death of transit-amplifying spermatogonia. The decreased sperm count can reduce fecundity to some extent; however, this starvation-induced elimination is required for maintenance of stem cell function and tissue homeostasis **(Yang et al., 2015).** It is assumed that the more critical factors for male fecundity are sperm storage efficiency and its stability. These physiological features of the sperm are regulated by Acps rather than the amount of ejaculated sperm. Sperm storage is induced by conformational changes of the uterus in response to Acp36DE **(Neubaum et al., 1999)**, and the sperm stability is regulated through the binding of Acp70A (SP) to the sperm **(Peng et al., 2005).** These two Acps are secreted by the main cells and contribute largely to male fecundity. Thus, it is likely that the nutrient-dependent changes in main cell size, leading to changes of the total volume of the accessory gland, are important in determining optimal reproductive potential **(Taniguchi et al., 2012; Wigby et al., 2009).**

Another cell type, SCs, comprises only 5%–6% of the accessory gland epithelial cells; however, these cells are also essential for fecundity. The results clearly indicate that nutrient conditions affect male fecundity through dynamic changes in the size and number of SCs. It should be noted that changes in these minority cells only have a minimal effect on the gross morphology of the accessory gland. Thus, the two types of accessory gland cells respond to nutrient conditions in different ways; the response of main cells is associated with the total volume change, and SCs respond with minimal volume change. It is assumed that these differences allow for highly plastic responses of fecundity to the nutrient conditions after eclosion **(see below).**

Nutrient sensors during development

Inhibition of the nutrient signaling pathway with two GAL4 drivers, *dve-GAL4[30A]* and *prd-GAL4*, affected different cell types in the accessory gland. *prd-GAL4* was continuously expressed from the pupal to adult stages in both cell types, whereas *dve-GAL4* expression was very low in adult main cells but was highly maintained in SCs from the pupal to adult stages. Inhibition of TOR with *dve-GAL4* in the pupal stage resulted in severe size reduction in SCs due to a maturation defect. Similar results were

also observed with *prd-GAL4*, but the effect was mild. In contrast, inhibition of InR with *prd-GAL4*, but not with *dve-GAL4*, resulted in very small accessory glands, presumably due to a growth defect in the main cells after eclosion. In these small accessory glands, the size of SCs was nearly normal at 25°C **(Fig S3 C'').** Thus, these results suggest that nutrient conditions in the pupal stage determine the physiological features of SCs and that nutrient availability after eclosion determines the rate of DILP-dependent cell growth of the main cells. The TOR kinase is activated as a downstream event in the insulin signaling pathway or by the availability of amino acids. Effects of TOR inhibition on SCs were stronger than those of InR inhibition, suggesting that the TOR activity was more important for the physiological changes of SCs during the pupal stage. It is an intriguing possibility that SCs regulate their maturation and survival in response to the levels of amino acids through the Slimfast-TOR pathway **(Colombani et al., 2003).**

It was also investigated the physiological significance of two types of accessory gland cells in response to nutrient conditions at different developmental time points. Nutrient conditions during the larval–pupal stages reflect the nutrient availability just after eclosion. Under nutrient-poor conditions, it was hypothesized that it is an effective way to change the physiology of minority SCs without affecting main cell development during the pupal stage **(Fig S5 G).** If eclosed flies can move to nutrient-rich conditions, their main cells can quickly respond to growth and maturation, leading to a "reproductive mode." In contrast, if nutrient conditions are still poor, eclosed flies have to maintain a "waiting mode" to save energy with small and immature accessory glands. Under nutrient-rich conditions, the increased size and number of SCs during the pupal stage further enhance fecundity mediated by DILP-dependent main cell growth after eclosion. Thus, it is assumed that changing the physiological features of SCs during the pupal stage confers highly plastic responses to nutrient conditions in the adult stage.

Dve- and Abd-B-dependent nutrient sensing

Nutrient conditions strongly affect both the number and size of the SCs. Targeted inhibition of TOR signaling in the accessory gland (*dG30A>TOR[DN]*), as well as Abd-B mutant cells, resulted in size reduction in SCs associated with defective vacuolar formation. In addition, Abd-B expression was greatly reduced when TOR signaling was inhibited, suggesting that nutrient conditions (TOR activity) affect vacuolar formation through the activity of Abd-B **(Fig 10).** It seems likely that Abd-B is a nutrient-responsive transcription factor that regulates the maturation of the SCs, which is essential for male fecundity **(Gligorov et al., 2013; Sitnik et al., 2016).**

With activated insulin signaling (*dG30A>InR[act]*), the number of SCs increases by approximately 1.8-fold compared to that under normal nutrient conditions. Thus, approximately 100 cells can develop into SCs, whereas excess cells are eliminated by apoptotic cell death to save energy. Under normal nutrient conditions, approximately 60 cells can survive through Dve-mediated inhibition of cell death **(Minami et al., 2012).** Moreover, Dve expression seems to be up-regulated in activated insulin

signaling **(Figs 7 b and 9g)**, suggesting that the high activity of Dve enhances the survival of SCs in response to nutrient-rich conditions. Under nutrient-poor conditions, reduced Dve expression associated with defective vacuolar formation was observed in some SCs **(Fig 6c).** These results support the notion that Dve is also a nutrient-responsive transcription factor that affects the physiology of SCs. When TOR activity was reduced in the accessory gland (*dG30A>TOR[DN]*), however, Dve expression was unaffected even in the small SCs with no vacuoles. This result implies that Dve acts upstream of the TOR-Abd-B pathway, and this notion is consistent with Dve-dependent Abd-B expression in the pupal stage **(Minami et al., 2012).** Alternatively, Dve and TOR might respond independently to nutrient conditions and regulate Abd-B expression in a cooperative manner. Thus, the data suggest that nutrient conditions regulate Dve expression and TOR activities in the SCs and that these changes regulate Abd-B expression and subsequent vacuolar formation **(Fig 10).** The results clearly show that a new player, Dve, associates nutrient conditions with optimal reproductive potential. Further characterization of the impacts of regulatory pathways on Dve activity should provide insights into the mechanism underlying nutrient-dependent modulation of fertility/fecundity **(Fig 11).**

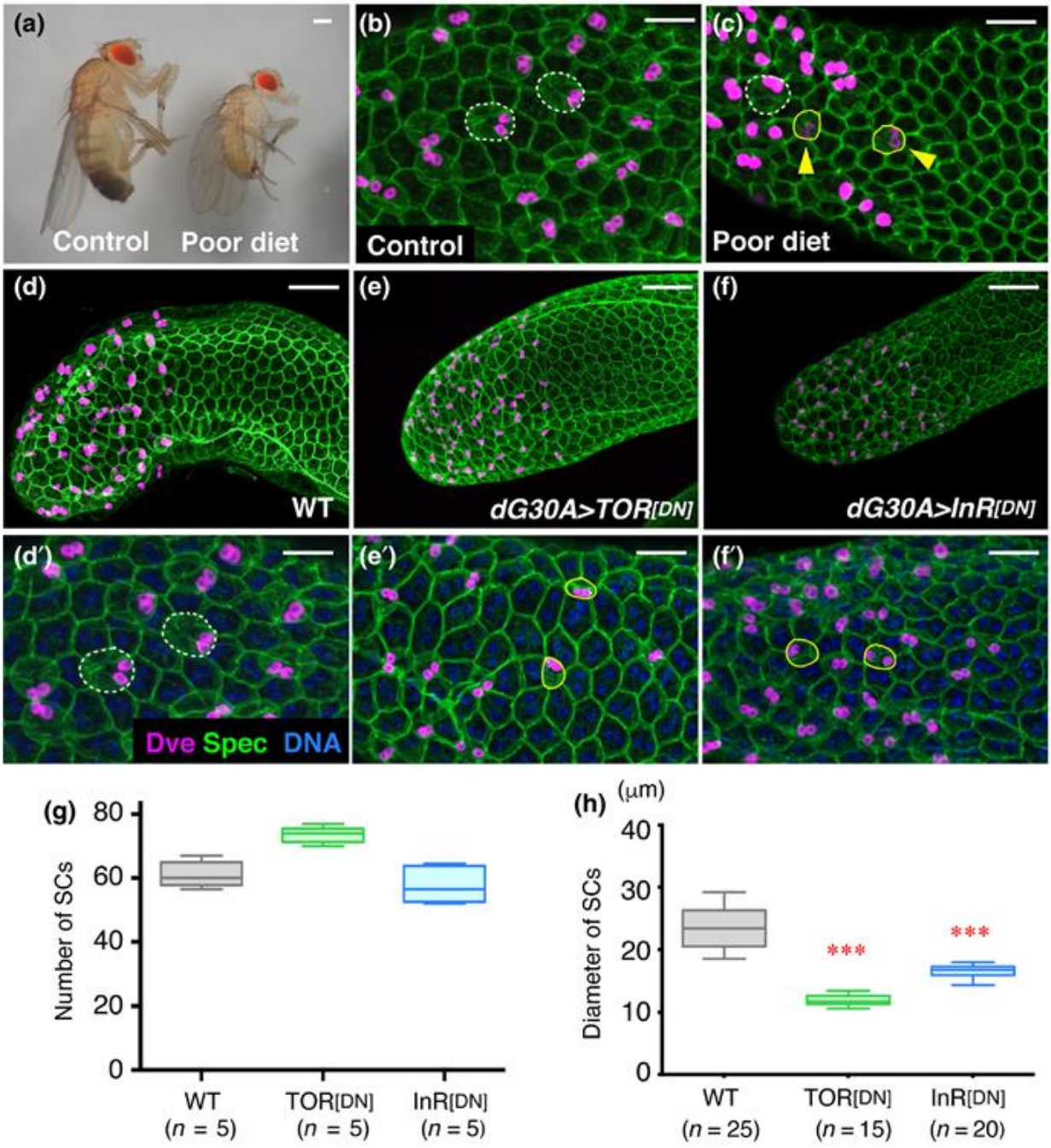


Fig 6. Secondary cells (SCs) in the accessory gland are responsive to nutrient conditions.

(a–c) Effects of nutritional composition. Accessory glands of a wild‐type control (b) and of a fly grown with a poor diet (c). Cell boundaries are labeled with anti‐Spectrin (green in b–f). Nuclei of SCs are labeled with anti‐Dve (magenta in b–f), and some cells are outlined. The SCs with reduced size and reduced Dve expression are indicated by yellow arrowheads in (c). (d–f) Accessory glands of a wild‐type control (d, WT) and with inhibition of TOR (E, *dG30A > TOR[DN]*) or insulin signaling (f, *dG30A > InR[DN]*) are shown. The nuclei are labeled with TOPRO‐3 (blue) in the magnified images (d'–f'). Scale bar is 200 μm (a), 50 μm (d–f) and 20 μm (b,c and d'–f'). (g,h) Morphological changes of SCs. Targeted inhibition of TOR (*dG30A > TOR[DN]*, green) or insulin signaling (*dG30A > InR[DN]*, blue) do not reduce the number of SCs (g), whereas the average size of SCs (h) was greatly reduced under these conditions at 25° C. The number (n) of scored accessory glands (g) or SCs (h) is indicated.

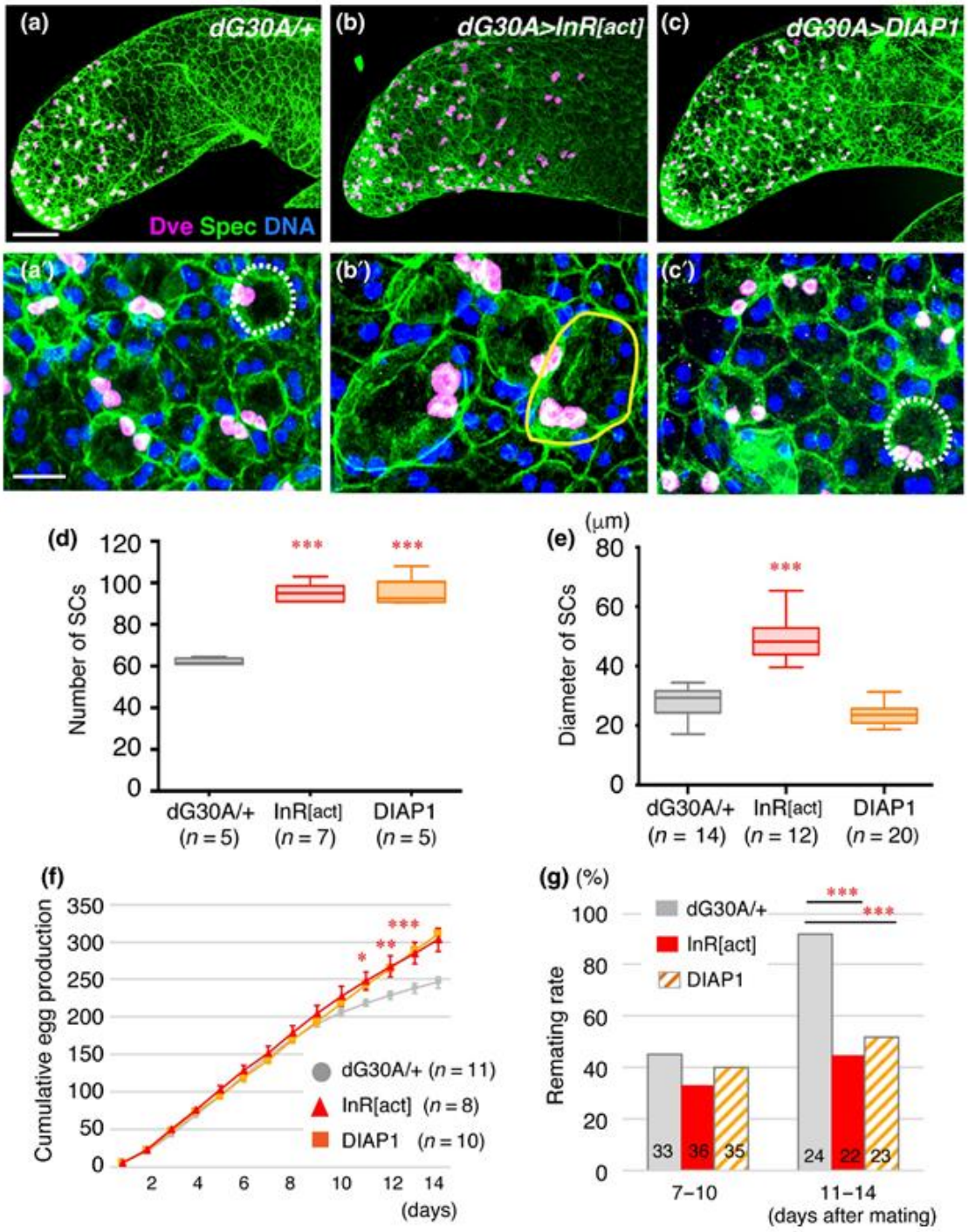


Fig 7. Apoptosis – mediated regulation of secondary cell (SC) survival.

(a–c) Accessory glands of a control male (A, *dG30A/+*), with activated insulin signaling (b, *dG30A>InR[act]*) and with cell death blockade (C, *dG30A > DIAP1*). Magnified images are shown in a'–c', and nuclei of the SCs are labeled with anti – Dve (magenta); some cells are outlined. Cell boundaries are labeled with anti – Spectrin (green). Nuclei are labeled with TOPRO – 3 (blue) in magnified images (a'–c'). Scale bar is 50 μm (a–c) and 20 μm (a'–c'). (d,e) Morphological changes of SCs. (d) The number of SCs increased by approximately 1.8 – fold with activated insulin signaling (red) or cell death blockade (orange). (e) The size of SCs increased only in activated insulin signaling. (f,g) Postmating behavior of females mated with a control male (gray), a male with activated insulin signaling (red) or a male with cell death blockade (orange). The increase in the number of SCs enhanced egg production/female after 11 days (f) and reduced remating activity for longer period (G). The numbers (*n*) of scored accessory glands (D), SCs (e) and mated females (f,g) are indicated.

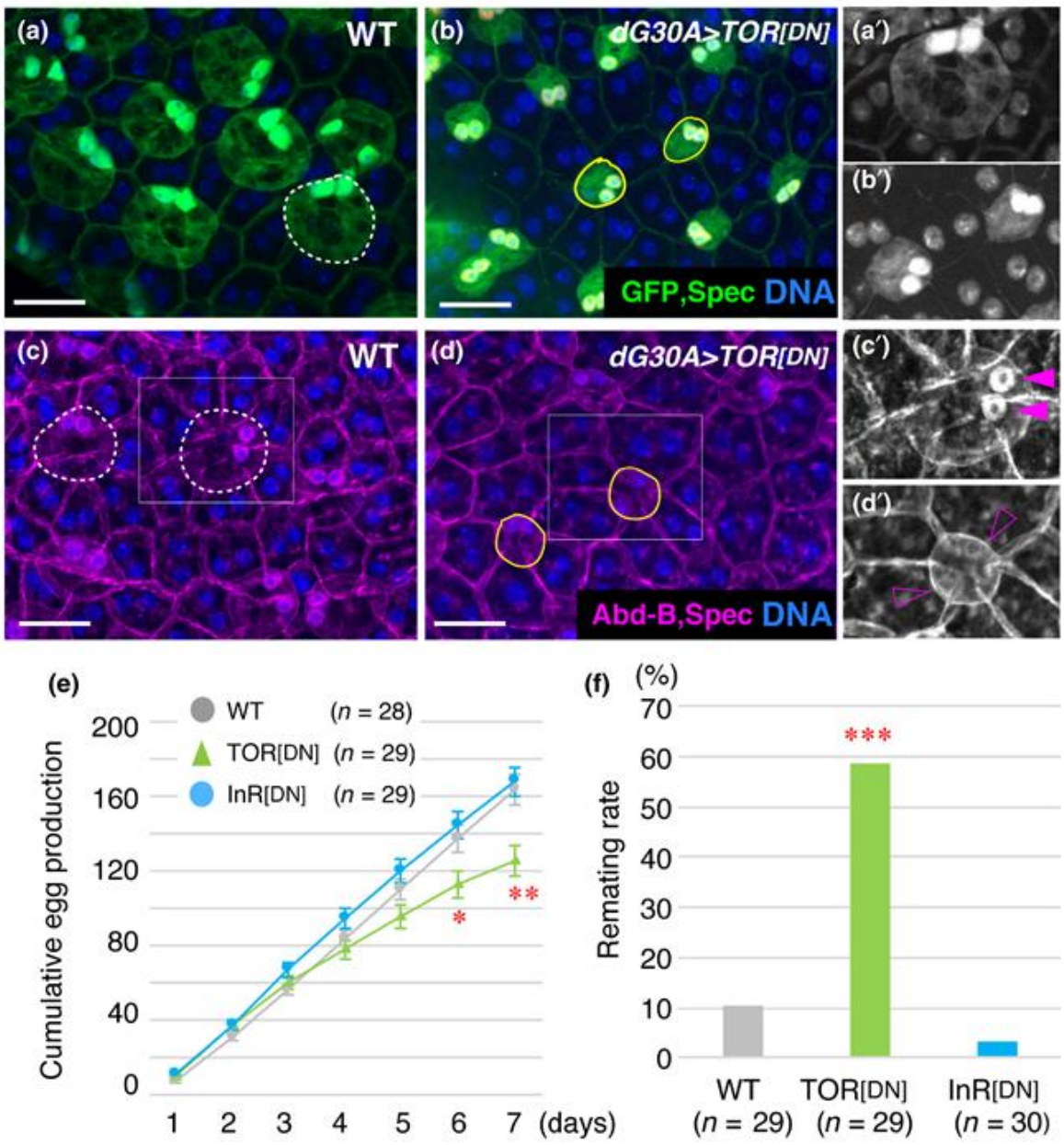


Fig 8. Nutrient‐dependent Abd‐B expression regulates male fecundity.

(a–d) Accessory glands of the wild‐type control (a,c) and with reduced TOR signaling *dG30A > TOR[DN]* (b,d) are shown. Nuclei are labeled with TOPRO‐3 (blue). Vacuoles in the secondary cells (SCs) are visualized by the absence of cytoplasmic GFP expression (green), and cell boundaries are labeled with anti‐Spectrin (green in a and b). Expression levels of Abd‐B and Spectrin are shown in magenta (c,d). Some SCs are outlined and shown as gray scale images in a'–d'. The magenta channel images (Abd‐B and Spectrin) are shown in c' and d'. Nuclei with or without Abd‐B expression are indicated by arrowheads (c') or open arrowheads (d'), respectively. Both vacuoles and Abd‐B expression are lost in reduced TOR signaling *dG30A > TOR[DN]* (b,d). Scale bar is 20 μm. (e,f) Postmating behavior of females mated with a control male (gray) or a male with reduced TOR (green) or insulin signaling (*dG30A > InR[DN]*, blue). The decrease in the size of SCs (*dG30A > TOR[DN]*) reduced egg production/female (e) and increased remating activity at 48 hr after the first mating (f). The number (*n*) of scored female flies is indicated.

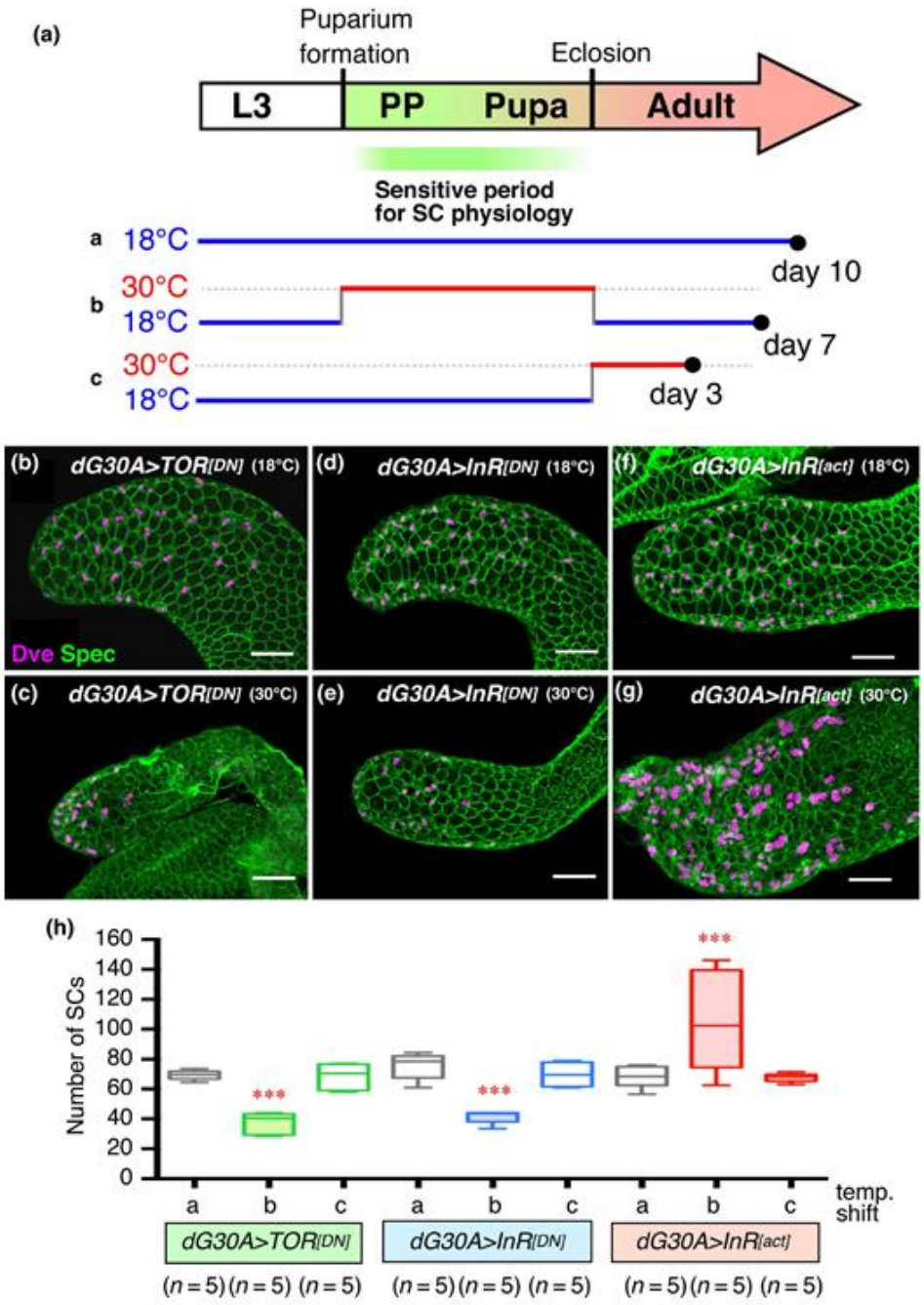


Fig 9. Nutrient conditions during the pupal stage are critical for secondary cell (SC) physiology.

(a) Schematic representation of temporally regulated changes in nutrient conditions: control (temperature shift pattern a), changes during the pupal and adult stages (shift pattern b and c, respectively). PP: prepupa. (b–g) Accessory glands of a control at 18˚ C (b, d and f; shift pattern a) and with changes in TOR or insulin signaling during the pupal stage at 30˚ C (c,e and g; shift pattern b). (b,c) *dG30A > TOR[DN] tubGAL80^{ts}*, (d,e) *dG30A > InR[DN] tub - GAL80^{ts}* and (f,g) *dG30A > InR[act] tub - GAL80^{ts}*. Cell boundaries are labeled with anti - Spectrin (green). Nuclei of SCs are labeled with anti - Dve (magenta). Scale bar is 50 μm. The reduction in the number of SCs was evident in reduced TOR or insulin signaling (c,e). The increase in the number of SCs was clearly observed in activated insulin signaling (g). (h) The number of SCs was only affected by changes in nutrient conditions during the pupal stage, but not in the adult stage. Differences between Figures 6 (g) and 9 (h) are due to enhanced GAL4 expression at 30˚ C. The number (*n*) of scored accessory glands is indicated

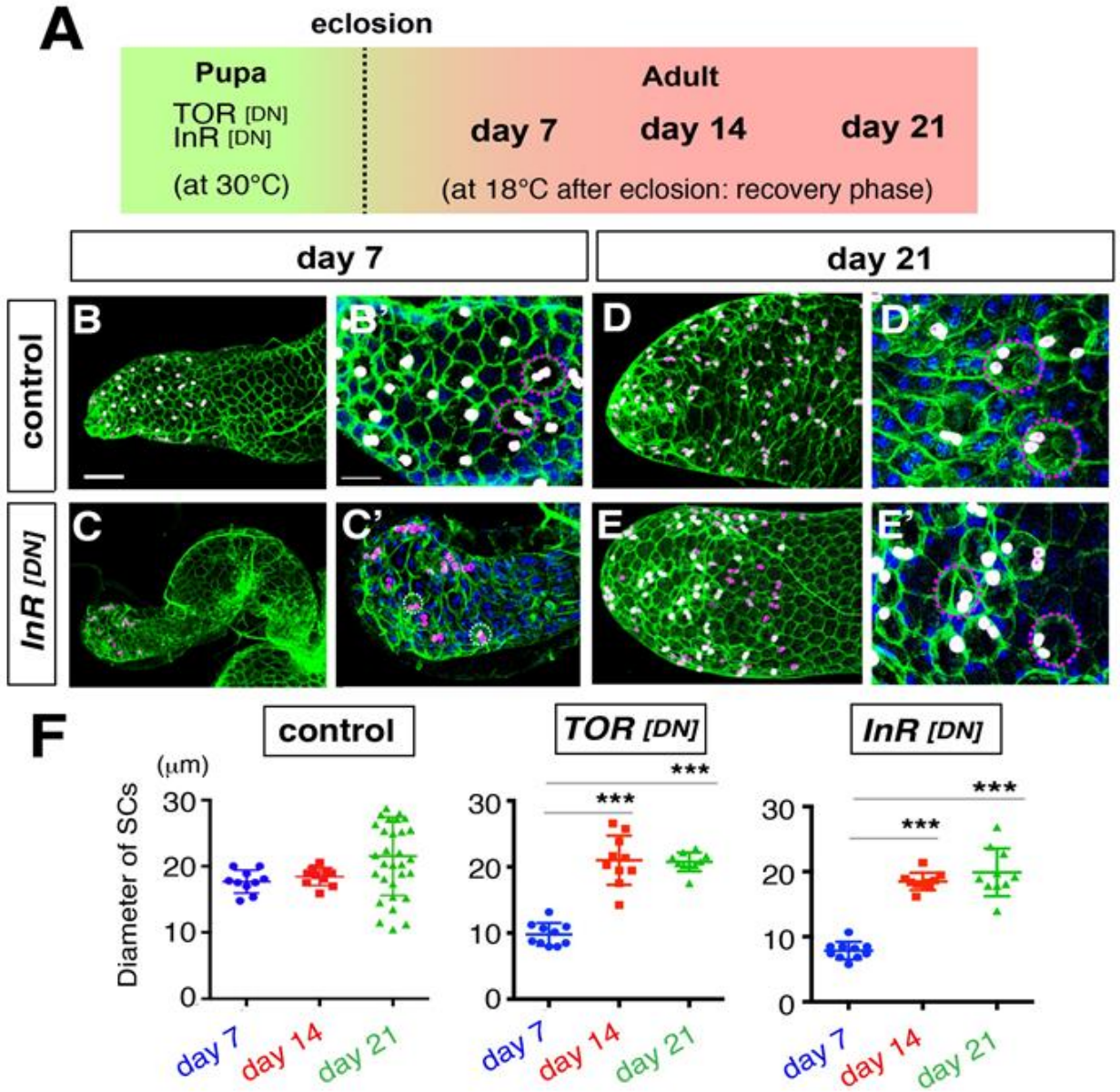


Fig 10. Plastic changes of SCs in the adult stage.

(A) Experimental design for pupal stage-specific inhibition of nutrient signaling. (B-E) AGs of flies (*w; tub-GAL80ts/UAS-InR[DN]; prd-GAL4/+*) reared at 18˚ C throughout development (B and D: control) or of flies reared at 30˚ C during the pupal stage (C and E: InR[DN]). Some SCs are outlined. Scale bar: 50 μm (B-E) and 20 μm (B'-E'). (F) The reduced size of SCs is evident at day 7 under normal nutrient conditions, while it is recovered at day 14 (about 7 days of recovery phase at 25˚ C).

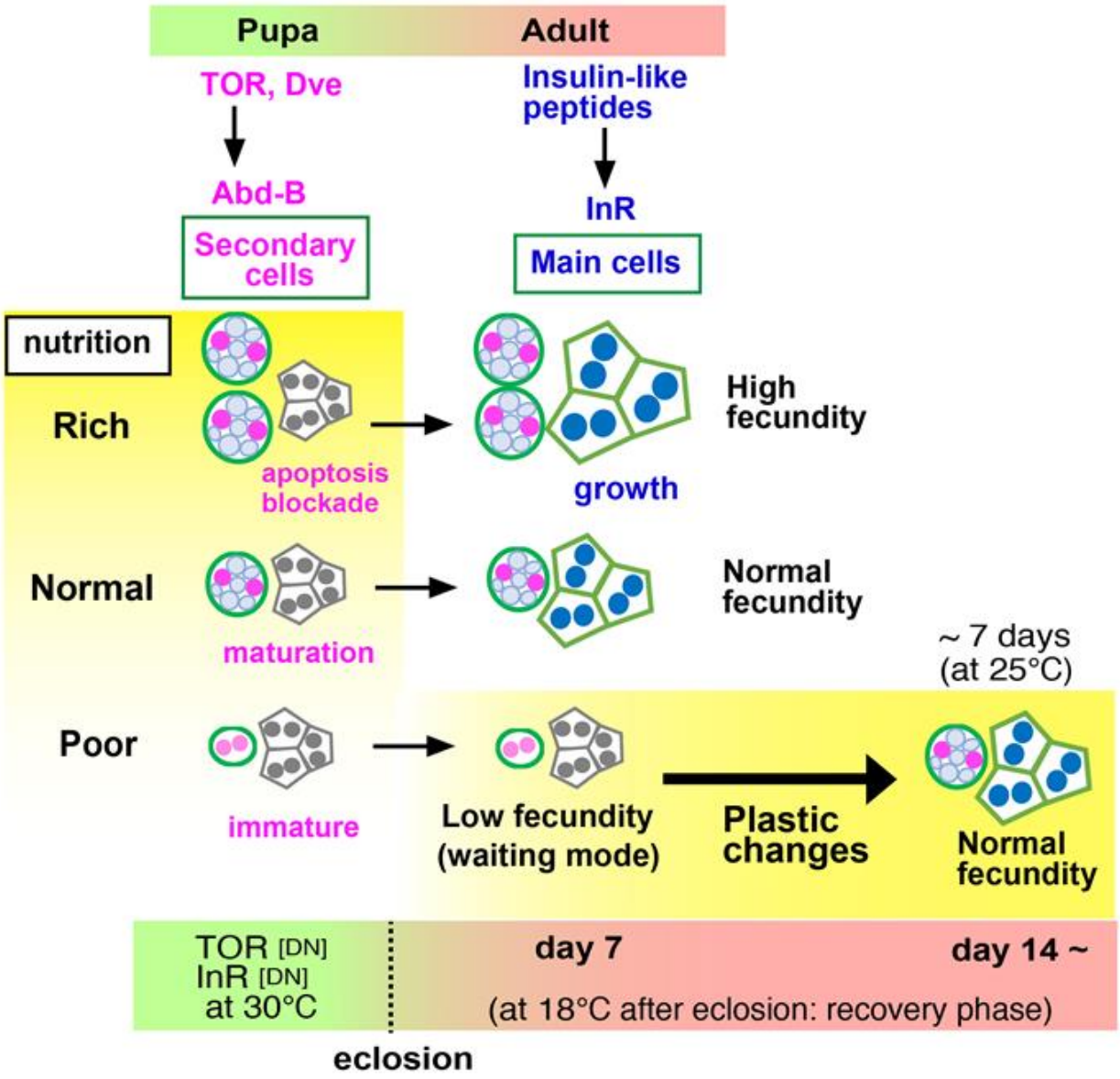


Fig 11. Nutrient conditions plastically optimize fecundity.

Nutrient conditions during development (pupal stage) mainly affect the size and the number of SCs by which changes of total volume are minimal. Nutrient availability in the adult stage promotes growth of main cells to enlarge the total volume for storing enough amount of seminal fluid. In addition, when poor nutrient conditions during development are improved in the adult stage, immature SCs can develop into mature SCs and acquire normal fecundity.

A

	Normal diet (ND)	Poor diet (PD)	High protein diet (HPD)	High sugar diet (HSD)	High fat diet (HFD)
H20 (mL)	300	300	226	242	210
Agar (g)	3	3	3	3	3
Glucose (g)	29	7.2	29	87	29
Cornmeal (g)	20	5	20	20	20
Wheat germ (g)	9	0	9	9	9
Dry yeast (g)	11	2.7	74	11	11
Coconut oil (g)	0	0	0	0	90
Propionic acid (mL)	0.9	0.9	0.9	0.9	0.9
15% Butyl parahydroxybenzoate (mL)	1	1	1	1	1

B

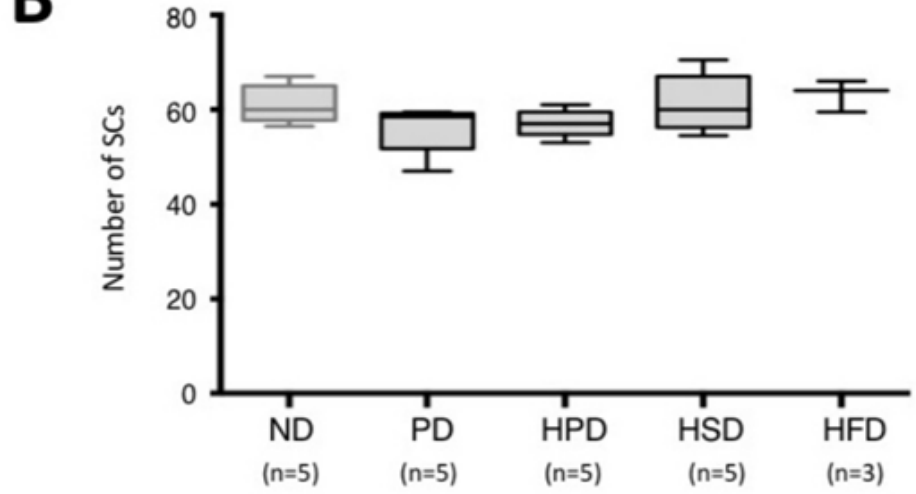


Fig S1. Effects of food components on morphology of the accessory gland.

(A) Diet composition.
(B) The number of SCs was unaffected with changes in diet composition.
The number (n) of scored accessory glands is indicated.

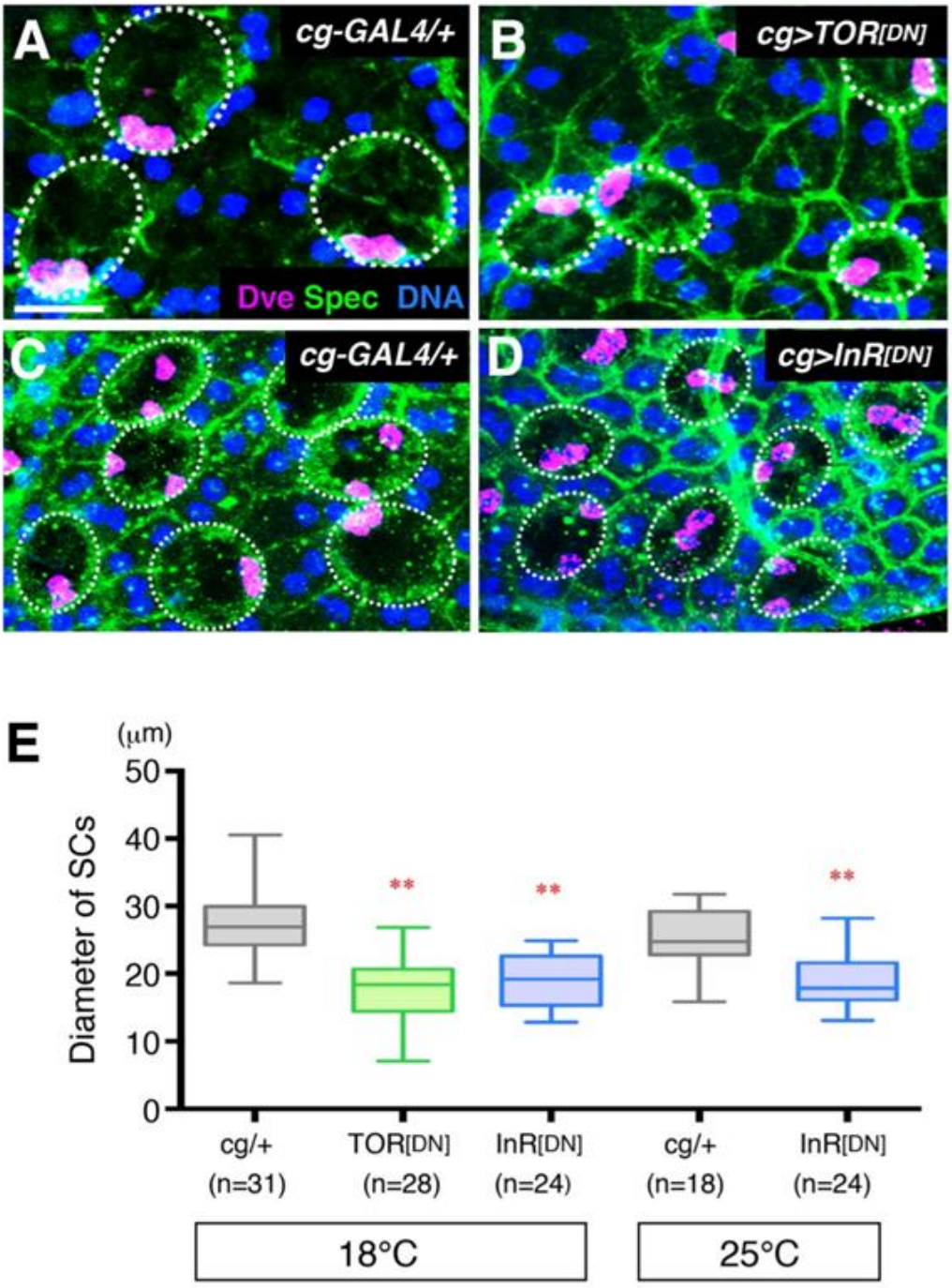


Fig S2. Accessory glands with reduced TOR or insulin signaling in the fat body.

(A, C) Accessory glands of control males (cg-GAL4/+) reared at 18˚ C (A) or 25˚ C (C). (B, D) Accessory glands with reduced TOR or insulin signaling in the fat body, cg>TOR[DN] at 18˚ C (B) and cg>InR[DN] at 25˚ C (D), slightly reduced the size of SCs. Progeny of cg>TOR[DN] never emerged at 21˚ C. Cell boundaries and nuclei are labeled with anti-Spectrin (green) and TOPRO-3 (blue), respectively. Nuclei of SCs are labeled with anti-Dve (magenta) and some cells are outlined. Scale bar: 20 μm. (E) The size of SCs was slightly reduced in conditions of cg>TOR[DN] and cg>InR[DN]. The number (n) of scored SCs is indicated.

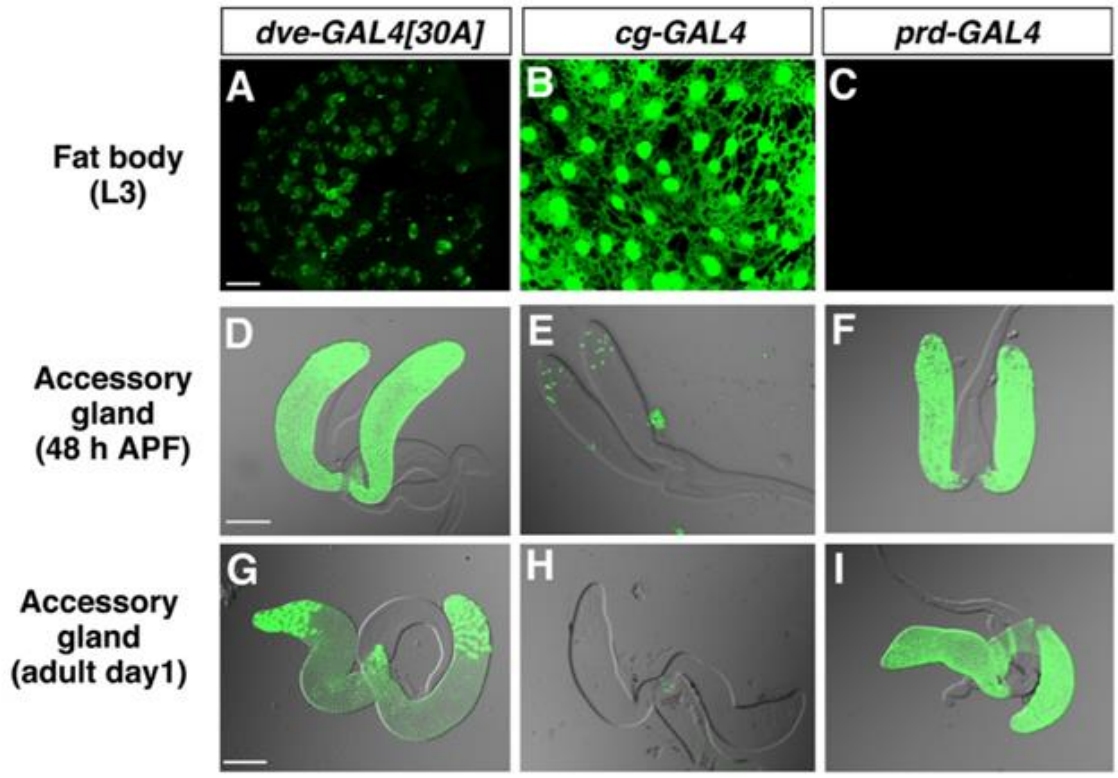


Figure S3. Comparison of GAL4 expression levels.

(A–C) Fat bodies in the late third larval instar (L3). (D–F) Accessory glands at 48 h after puparium formation (APF). (G–I) Accessory glands in 1-day-old adults. (A, D, G) dG30A>Stinger, (B, E, H) cg>Stinger, (C, F, I) prd>Stinger. Expression of nuclear-localized GFP (Stinger) is shown in green. (D–I) Phase contrast images are merged. Scale bar: 50 μm (A–C), 100 μm (D–F), and 200 μm (G–I).

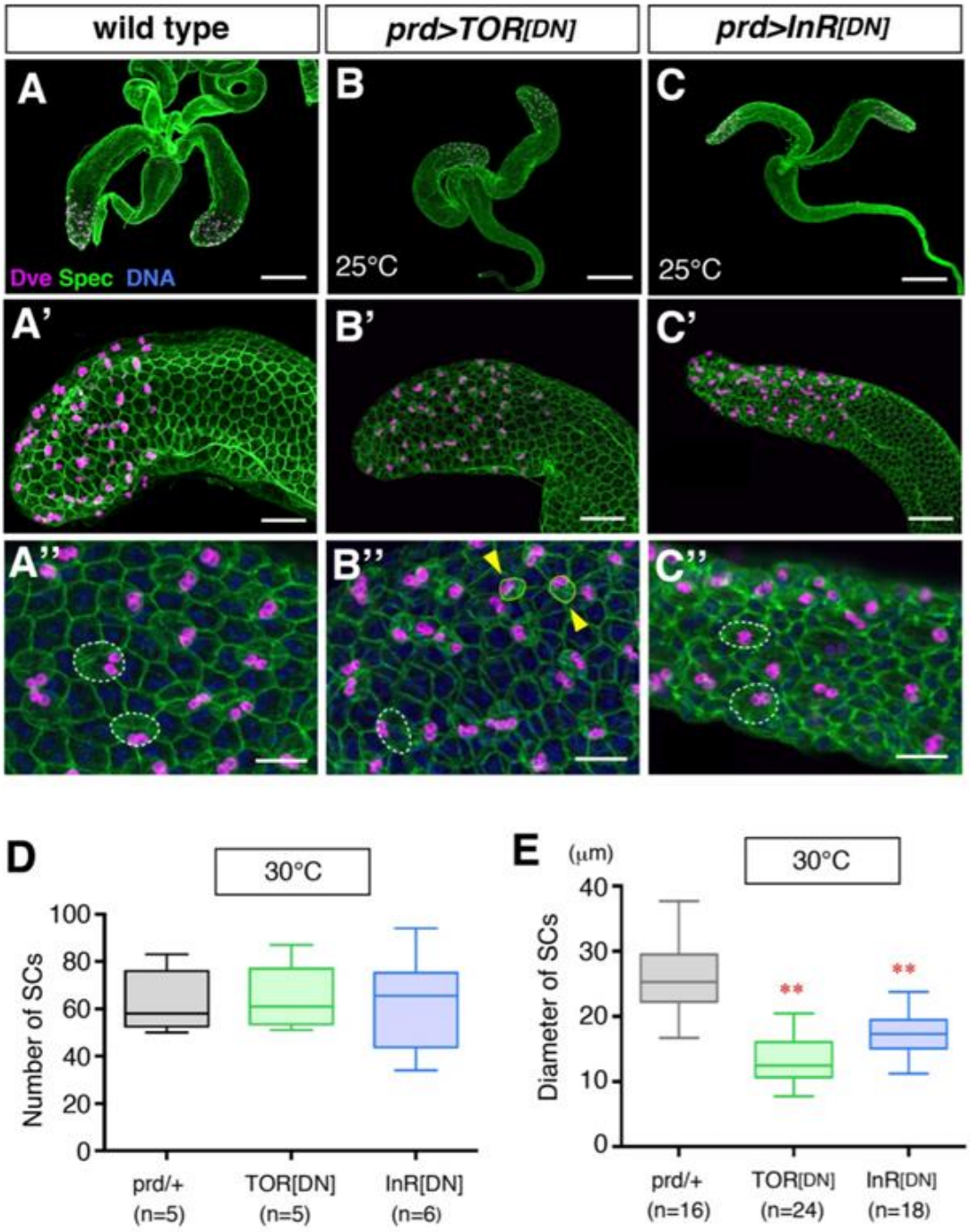


Fig S4. Nutrient signaling in the accessory gland affects morphology of both cell types.

(A–A") Wild-type control, (B–B") prd>TOR[DN] at 25° C, (C–C") prd>InR[DN] at 25° C. Cell boundaries and nuclei are labeled with anti-Spectrin (green) and TOPRO-3 (blue), respectively. Nuclei of secondary cells (SCs) are labeled with anti-Dve (magenta), and normal-sized cells are outlined with white-dotted lines. Arrowheads in (B") indicate SCs with reduced size. The size of main cells in (C–C") was greatly reduced. Scale bar: 200 μm (A–C), 50 μm (A'–C'), and 20 μm (A"–C"). (D) The number of SCs was unaffected even at 30° C during the pupal stage. (E) The size of SCs was clearly reduced in conditions of prd>TOR[DN] and prd>InR[DN] at 30° C during the pupal stage. The number (n) of scored accessory glands (D) or SCs (E) is indicated.

3. Ecdysone signaling regulates nutrient-dependent fecundity in *Drosophila* males

Abstract

Steroid hormones play a number of physiological roles including metabolism and reproduction. Steroid hormones in insects are ecdysteroids, and the major form in *Drosophila* is ecdysone. The ecdysone signaling is required for developmental transitions and metamorphosis. These events are closely correlated with nutrient conditions and are coordinated with systemic growth.

In *Drosophila* females, both nutrient conditions and ecdysone signaling affect oogenesis. In males, the accessory gland is responsive to nutrient-dependent regulation of fertility/fecundity, and ecdysone signaling is also crucial for male fecundity. However, the relationship between nutrition and ecdysone signaling for fecundity is still unclear.

The accessory gland is composed of two types of binucleated cells: a main cell and a secondary cell (SC). The transcription factors Defective proventriculus (Dve) and Abdominal-B (Abd-B) are strongly expressed in adult SCs. Experimental results show that ecdysone receptor (EcR) proteins are also highly expressed in adult SCs. Expression of two EcR isoforms (EcR.A and EcR.B1) in the pupal SCs depends on nutrient conditions, and is maintained by the Dve activity. Induction of Dve expression is also dependent on nutrient conditions, and it becomes nutrient-independent at later stage to determine the optimal number of SCs. Here, this research provide evidence that ecdysone signaling is crucial for male fecundity through nutrient-dependent regulation of SC maturation.

Introduction

Steroid hormones are commonly biosynthesized from cholesterol and have steroid skeleton. In mammals, the hormones are secreted from Adrenal gland, testis, ovary, and so on (For example, corticosteroid (Aldosterone, glucocorticoid), estrogen, progesterone, and androgen etc…). Steroid hormones are secreted toward specific receptors for various biological control by regulating target gene transcription regulation **(Tomkins, 1967; Patel 2018)**.

Female steroid hormones (ex. Estrogen and progesterone) are secreted from ovary. Estrogen promotes mammary gland cell proliferation and regulates ovulation from ovary, and progesterone induces phenotype of pregnancy; mammary gland growth and body temperature rising **(Yu et al., 2016; Luo et al., 2017; Rumi et al., 2017; Jones et al., 2017; Kayo et al., 2019)**. Androgen (testosterone, dihydrotestosterone etc…) is male steroid hormone. Testosterone is crucial for male secondary sex characteristic and promotes growth of testis and seminal vesicle. Testosterone is the source of estradiol, which is a kind of estrogen known as a female hormone for fertility **(Hess et al., 2018)**.

Drosophila melanogaster also secretes steroid hormone ecdysteroid (ecdysone). It is synthesized in prothoracic glands, and ecdysone is converted into the active form 20-hydroxyecdysone (20E), which acts as molting hormone for ecdysis and metamorphosis. It can bind to the hetero dimer of ecdysone receptor (EcR) isoforms (EcR.A, EcR.B1, and EcRB2). After binding to EcR, ecdysone signaling is transmitted and activates couple of transcription regulatory networks (ecdysone cascades), which is composed of various transcription factors (ex. Ftz-F1, Blimp-1) **(Agawa et al., 2007; Sultan et al., 2014; Akagi et al., 2016)**.

Ecdysone is also required for fertility/fecundity as well as mammalian steroid hormones. In adult females, ecdysone is expressed in the ovary, and is involved in oogenesis **(Carney et al., 2000; Ables et al., 2017; Uryu et al., 2015).** In adult males, ecdysone is expressed in the accessory gland, and the ecdysone signaling is required for male fertility **(Hentze et al., 2013; Sharma et al, 2017).** During development, ecdysone is synthesized in the prothoracic gland, and converted to the active metabolite 20-hydroxyecdysone (20E) in the fat body. In a juvenile growth period, nutrient conditions are also sensed in the fay body through the Insulin signaling pathway and the Target of rapamycin (TOR) pathway. Ecdysone signaling in the fat body inhibits juvenile body growth through production of Imaginal morphogenesis protein-Late 2 (Imp-L2), a *Drosophila* homolog of Insulin-like growth factor binding protein 7 (IGFBP7). Ecdysone-dependent growth suppression is due to attenuation of peripheral Insulin signaling and is independent of internal nutrient sensor in the fat body **(Lee et al.,**

2018). These ecdysone-mediated processes are required for developmental transitions and metamorphosis in *Drosophila* **(Rewitz et al., 2013; Faunes et al., 2016).**

Nutrient conditions also affect the reproductive potential and life span of many organisms through the insulin signaling pathway **(Tatar et al., 2003; Partridge et al., 2005).** The insulin signaling pathway is highly conserved during evolution and regulates various aspects of physiology including oocyte growth and maturation **(Das et al., 2017).** Under nutrient-deprivation conditions, *Drosophila* oogenesis is blocked by programmed cell death to reduce energy consumption **(Drummond-Barbosa et al., 2001).** In *Drosophila* males, nutrient-dependent regulation of fecundity is regulated in the accessory gland. The accessory gland secretes accessory gland proteins (Acps) into the seminal fluid, which are essential for male fertility/fecundity **(Chapman et al., 2004; Wolfner, 2002).** Each of the two lobes of the accessory gland is composed of two types of binucleated cells: about 1000 main cells and 40–60 secondary cells (SCs). Adult main cells are flat hexagonal cells and SCs are large spherical cells interspersed among the main cells at the distal tip of each accessory gland lobe. The transcription factors Defective proventriculus (Dve) and Abdominal-B (Abd-B) are strongly expressed in adult SCs; these transcription factors are essential for male fecundity **(Gligorov et al., 2013; Minami et al., 2012).** Nutrient conditions affect male fecundity by changing the number and the size of SCs through expression levels of Dve and Abd-B **(Fig 8 c-d).**

Here, this research provides evidence that ecdysone signaling and Dve cooperatively regulate the maturation of SCs in a nutrient-dependent manner. EcR expression regulated by parallel pathways of nutrient signaling and Dve makes it possible to optimize fecundity.

Results

Ecdysone signaling regulates the size and the number of secondary cells

In *Drosophila* females, ecdysone signaling is required for oogenesis **(Carney et al., 2000; Ables et al., 2017).** However, it remained unclear how ecdysone signaling is involved in male fertility/fecundity. Thus, I checked EcR expression in the male accessory gland. Expression of two isoforms EcR.A and EcR.B1 is clearly detected in Dve-positive secondary cells (SCs) **(Fig 12 A-C)**, whereas Ftz-F1 is expressed in both main cells and SCs **(Fig 12 D).** During development, EcR is broadly expressed in the genital disc of third-instar larva, while Dve is still undetectable **(Fig S5 A-C).** After 36 hr APF, expression patterns of Dve and EcR are nearly identical **(Fig S5 I-P).**

To examine the role of EcR in accessory gland development, the dominant-negative form of EcR isoforms, EcR.A [DN] or EcR.B1 [DN], was induced with *dve-GAL4[30A]* (*dG30A*). Total volume of these accessory glands (*dG30A>EcR.A[DN]* and *dG30A>EcR.B1[DN]*) was reduced in consistent with a previous report **(Sharma et al., 2018).** In addition, I found that the number and the size of SCs were markedly reduced, and that binucleation was also inhibited **(Fig 12 F, G, F', G', I, and J).** To further examine the effect of ecdysone signaling, the activity of the downstream effector Ftz-F1 was knocked down (*dG30A>ftz-F1-IR*). Although the number of SCs were unaffected, total volume and the size of SCs was reduced as well as those of EcR-inhibited conditions **(Fig 12 H-J).**

Fecundity of males having these accessory glands was examined. The amount of a major Acp, sex peptide (SP), was monitored as a fluorescent signal of SP::GFP fusion protein. According to the reduced volume of the accessory gland, levels of SP::GFP were reduced and leak of SP to the ejaculatory duct was also observed in EcR-inhibited conditions. Sperm motility was monitored as a fluorescent signal of protamineB (proB)::GFP fusion protein. Sperm are stored in spermatheca (spt) and seminal receptacle (src) after mating **(Fig 13 A-G, E'-G', and E"-G").** This sperm storage was greatly reduced in females mated with a male of EcR-inhibited condition. Storage of sperm derived from a *dve* mutant male ($dve^{E181/E144}$) was also reduced but it was highly variable **(Fig 13 H and I).** This may reflect that sperm motility largely depends on activities of Acps from main cells, and that ecdysone signaling in main cells is also crucial for these activities. As sperm storage is dependent on conformational changes of the uterus, the state of uterus conformation remained at earlier stages in females mated with a male of EcR-inhibited condition **(Fig 13 J and K).** In addition, female receptivity is greatly reduced for several days after mating. Thus, re-mating activity of mated female at 48 hr after first mating is very low, whereas the re-mating activity was clearly increased in females mated with a male of EcR-inhibited condition. Consistent with the previous result **(Sharma et al., 2017**), the re-mating rate of females mated with a *ftz-F1* knockdown (KD) male had only non-significant increase compared to the control females. Similar results were obtained with different GAL4 drivers *prd-GAL4* **(Figs S6 and S7)** and *NP3612* **(Fig S8).** Taken together, it has been shown that ecdysone signaling is required for accessory gland development, and for regulating the number and the size of SCs to acquire full fecundity.

Dve-dependent EcR signaling is crucial for Abd-B expression in secondary cell maturation

Expression patterns of Dve, EcR, and Abd-B are nearly identical after 36 hr APF. Abd-B expression depends on the activity of Dve and is required for SC maturation **(Fig S5 I-L and I').** Thus, I checked whether EcR expression is also dependent on the Dve activity. Although EcR expression precedes the induction of Dve expression in the accessory gland, both of EcR.A and EcR.B1 expression were abolished in *dve* mutant ($dve^{E181/E144}$) background **(Fig 14 A-F, A', and D').** This result indicates that EcR expression is maintained by the Dve activity, and that induction and maintenance of EcR expression are differently regulated. To check whether EcR functions are involved in Dve-dependent Abd-B expression, the GAL4 driver line NP3612 was used for SC-specific expression to minimize the effect on main cells after 36 hr APF **(Fig S8 A-D).** Inhibition of EcR (*NP3612>EcR.A[DN]* and *NP3612>EcR.B1[DN]*) did not affect Dve expression, whereas Abd-B expression was greatly reduced **(Fig 14 H and I).** This is also the case for inhibition of Ftz-F1 (*NP3612>ftz-F1-IR*), but expression of EcR.A and EcR.B1 was unaffected in *ftz-F1* KD conditions **(Fig 14 J-L).** These results suggest that ecdysone signaling activates Abd-B expression through the Ftz-F1 activity. To further examine the mechanism of EcR induction, the nutrient signaling pathway was inhibited with a dominant-negative form of Target of rapamycin (TOR) (*NP3612>TOR[DN]*). Under this condition, all of EcR.A, EcR.B1, and Abd-B expression were greatly reduced **(Fig 14 M-O)**, suggesting that EcR induction is dependent on nutrient conditions before puparium formation.

Dve induction during the early pupal stage is dependent on nutrient conditions

Maturation of SC is strictly regulated by nutrient conditions. Under poor diet conditions, the size of some SCs is reduced and Dve expression is also reduced in these small SCs **(Fig 6; Kubo; Matsuka et al., 2018).** This observation suggests that Dve expression is also nutrient-dependent. However, Inhibition of TOR using several GAL drivers (*dve-GAL4, prd-GAL4, NP3612*) did not affect Dve expression. This might be due to the timing for TOR inhibition and also for the lower threshold level for Dve induction. To test the nutrient-dependency of Dve expression, inhibition of TOR or InR was strongly induced in a temporally regulated manner in the earlier stage of development by *breathless* (*btl*)-*GAL4* **(Figs 15 A-B and S9 A-D).** Inhibition of TOR or InR during the prepupal stage (*btl-GAL4>TOR[DN] and btl-GAL4>InR[DN]*) abolished Dve expression, and expression of downstream genes EcR.A, EcR.B1, and Abd-B was also lost **(Figs 15 E-G, E' and S9 H-J, H')**. As expected, inhibition during 12-96 hr APF **(Fig 15 A-c)** did clearly reduced expression of these downstream genes without affecting Dve expression and the number of SCs **(Fig 15 H-J, H').** The number of SCs is determined by cell death of SC precursors in which the Dve activity is low **(Minami et al., 2012).** Thus, levels of Dve expression in response to nutrient conditions seem to be important to determine the optimal number of SCs **(see Discussion).**

EcR signaling is maintained by the Ftz-F1 activity

Expression of EcR is nutrient-dependent in the early stage, and then become Dve- and nutrient-dependent. Inhibition of TOR (*NP3612>TOR[DN]*) clearly reduced expression of EcR.A and EcR.B1 but did not affected Dve expression **(Fig 14 M and N).** At this time point, Dve expression becomes nutrient-independent, while EcR expression is still dependent on nutrient conditions. Thus, EcR expression is regulated by parallel pathways of nutrient signaling and the Dve activity. Inhibition of EcR from this time point (*NP3612>EcR.A[DN]* or *NP3612>EcR.B1[DN]*) only affected Abd-B expression, whereas the number of Dve-positive SCs was unaffected **(Fig 14 P).** When EcR expression was inhibited from earlier time points with *dve-GAL4* or *btl-GAL4*, the number of SCs was greatly reduced even in the presence of Dve activity **(Figs 12 I, 16 K and S9 P).** Thus, Dve- and nutrient-dependent EcR expression appears to be crucial to determine the optimal number of SCs. In this process, the downstream effector Ftz-F1 is also required for determination of the number of SCs **(Fig 16 K).** When *ftz-F1* KD was induced in the prepupal stage **(Fig 15 A-b)**, expression of EcR.A and EcR.B1 was also completely lost **(***btl-GAL4>ftz-F1-IR*, **Figs 16 H-J and S9 K-M).** This result indicates that there is a positive-feedback loop of EcR maintenance by the Ftz-F1 activity. This feedback loop might be transiently required, because *ftz-F1* KD at later stages did not affect EcR expression but impaired SC maturation with reduced Abd-B expression **(***NP3612>ftz-F1-IR*, **Figs 14 J-L).** Alternative explanation is that the positive-feedback loop of EcR-Ftz-F1 is redundantly required for EcR expression after establishment of nutrient-independent Dve maintenance.

Discussion

Nutrient-dependent differentiation of SCs is robustly regulated

Developmental transition and metamorphosis are regulated by ecdysone pulses, which occur just before molting and metamorphosis. The timing of these events is also dependent on nutrient conditions, because nutrient-poor conditions prolong the period of each larval stage **(Francis et al., 2010; Buhler et al.; Ahmad et al., 2018; Fig 17).** After puparium formation, pupae cannot get any nutrition by feeding. Thus, nutrient conditions just before puparium formation should be sensed precisely to achieve survival to the adulthood. It is assumed that the nutrient-dependent induction of EcR expression has a higher threshold level to ensure survival. During the pupal stage, nutrition in the hemolymph gradually declines, and Dve expression can also be induced in a nutrient-dependent manner with a lower threshold level until 24 hr APF throughout the accessory gland. Subsequently, the broad and high level of ecdysone pulse occurs at around 36 hr APF, and both of EcR and Dve expression in the male accessory gland are highly restricted into SCs at around 84 hr APF. This transition may imply that expression of EcR and Dve in main cells only depend on nutrient conditions, and that their expression in SCs have a different mechanism for their maintenance in a nutrient-independent manner. In fact, inhibition of the TOR signaling pathway with *dve-GAL4* (around 24 hr APF~) did not affect Dve expression **(Fig 6 e)**, and EcR expression is dependent on the Dve activity **(Figs 12 A-D and 14 A-F).** This transition for nutrient dependency seems to occur until 36 hr APF. If nutrient conditions are rich in the pupal stage, the size and the number of SCs are increased, whereas they are decreased under nutrient-poor conditions **(Fig 6 a-c).** The number of SCs is regulated by the Dve activity. Thus, transition of Dve expression mode from nutrient-dependent to -independent manners seems to respond to the nutrient conditions and to be crucial to determine the optimal number of SCs. A possible mechanism for this transition is that the state of nutrient-dependent Dve expression during a critical period is maintained at chromatin levels, as observed in homeotic gene expression during embryonic development **(Cavalli et al., 1998; Kassis et al., 2017).**

Taken together, nutrient-dependent differentiation of SCs is regulated by three steps. (1) nutrient-dependent induction of EcR and Dve expression, (2) nutrient-independent maintenance of Dve expression and Dve- and nutrient-dependent EcR expression, and (3) positive-feedback loop of EcR and Ftz-F1 expression **(see below).**

EcR-Ftz-F1 regulatory loop is required for SC maturation

Expression of Ftz-F1 depends on EcR signaling and regulates developmental transitions and metamorphosis **(Fig 5 B; White et al., 1997; Agawa et al., 2007; Ruaud et al., 2010; Sultan et al., 2014; Akagi et al., 2016).** In some cases (ex. neural metamorphosis), EcR expression is also regulated by the activity of Ftz-F1 **(Fig 5 C; Lavorgna et al., 1993; Lam et al., 2000; Boulanger et al., 2012; Yaniv et al., 2016).** During SC development in the accessory gland, *ftz-F1* KD also abolished EcR expression, indicating that the positive-feedback loop of EcR and Ftz-F1 is required for SC maturation.

Previous reports suggested that EcR signaling is required for accessory gland development, and that Ftz-F1 is not involved in this process **(Sharma et al., 2017; Leiblich et al., 2019).**

The discrepancy with my results may be due to the sensitivity of experimental methods. Consistent with the previous result, the re-mating rate of females at 48hr after mating with a *ftz-F1* KD male had only non-significant increase compared to the control females **(Figs 13 K, S7 K and S8 K).** However, *ftz-F1* KD with *btl-GAL4* resulted in severe reduction in the number and the size of SCs as observed with EcR inhibition, indicating that the Ftz-F1 activity is crucial as a downstream effector of ecdysone signaling **(Figs 16 and 17)**. In addition, there are some differences for requirement of EcR isoforms, presumably due to the different expression level of GAL4 driver lines, *prd-GAL4* and *dve-GAL4*. Expression patterns of *dve-GAL4* is nearly identical to that of Dve protein, and its expression level is very weak in the adult main cells but high in the pupal accessory gland cells **(Minami et al., 2012).** Expression level of *prd-GAL4* is high in adult main cells but relatively lower than that of *dve-GAL4* during pupal development. Inhibition of EcR.B1 by using *prd-GAL4* (*prd>EcR.B1[DN]*) had only slight reduction of the SC size **(Fig S6 F)**, whereas inhibition of EcR.B1 by using *dve-GAL4* had severe reduction in both the number and the size of SCs **(Fig 12 I and J).** Furthermore, previous reports have shown that either EcR isoform is more crucial for accessory gland development and maturation than the other **(Sharma et al., 2017; Leiblich et al., 2019)**. Although their results are consistent with my results, it seems likely that both isoforms are differently required. Thus, both EcR.A and EcR.B1 are required in the early stage of pupal development to determine the optimal number of SCs, and functions of EcR.A seem to be more important for the SC maturation. Though SC-specific inhibition of EcR (*NP3612>EcR.A[DN]* and *NP3612>EcR.B1[DN]*) also showed reduction only in the size but not in the number of SCs, small-population specific inhibition of EcR might show weaker effect than that of *dve-GAL4* condition (*dG30A>EcR.A[DN]* and *dG30A>EcR.B1[DN]*) **(Figs S8 I, J and 12 I, J).**

ftz-F1 KD with *dve-GAL4* or *prd-GAL4* had no effect on the number of SCs, while it had severe size reduction of SCs, suggesting that Ftz-F1-dependent maintenance of EcR expression mainly contributes to the maturation step of SCs. Because EcR expression is also maintained by the Dve activity, Ftz-F1 and Dve may act in parallel to ensure high level of EcR expression in SCs. Another possible explanation is that Dve and Ftz-F1 regulate different phases of EcR expression. Dve might upregulate EcR expression in presumptive SCs only during a critical period in response to nutrient conditions. Subsequently, this upregulated EcR expression might be maintained with the EcR-Ftz-F1 positive feedback loop for SC maturation **(See Fig S10).** Because EcR is highly expressed in adult SCs, this might also be required for plastic changes of the SC size. If flies were grown under nutrient-poor conditions, their SC size is reduced at eclosion, whereas nutrient-rich conditions in the adult stage clearly recover the size of SCs **(Fig 10; Matsuka et al., 2019).** Thus, food intake converts cholesterol to ecdysone and can activate the EcR signaling pathway. EcR-Ftz-F1 regulatory loop might amplify the ecdysone signaling to respond to these adult nutrient conditions.

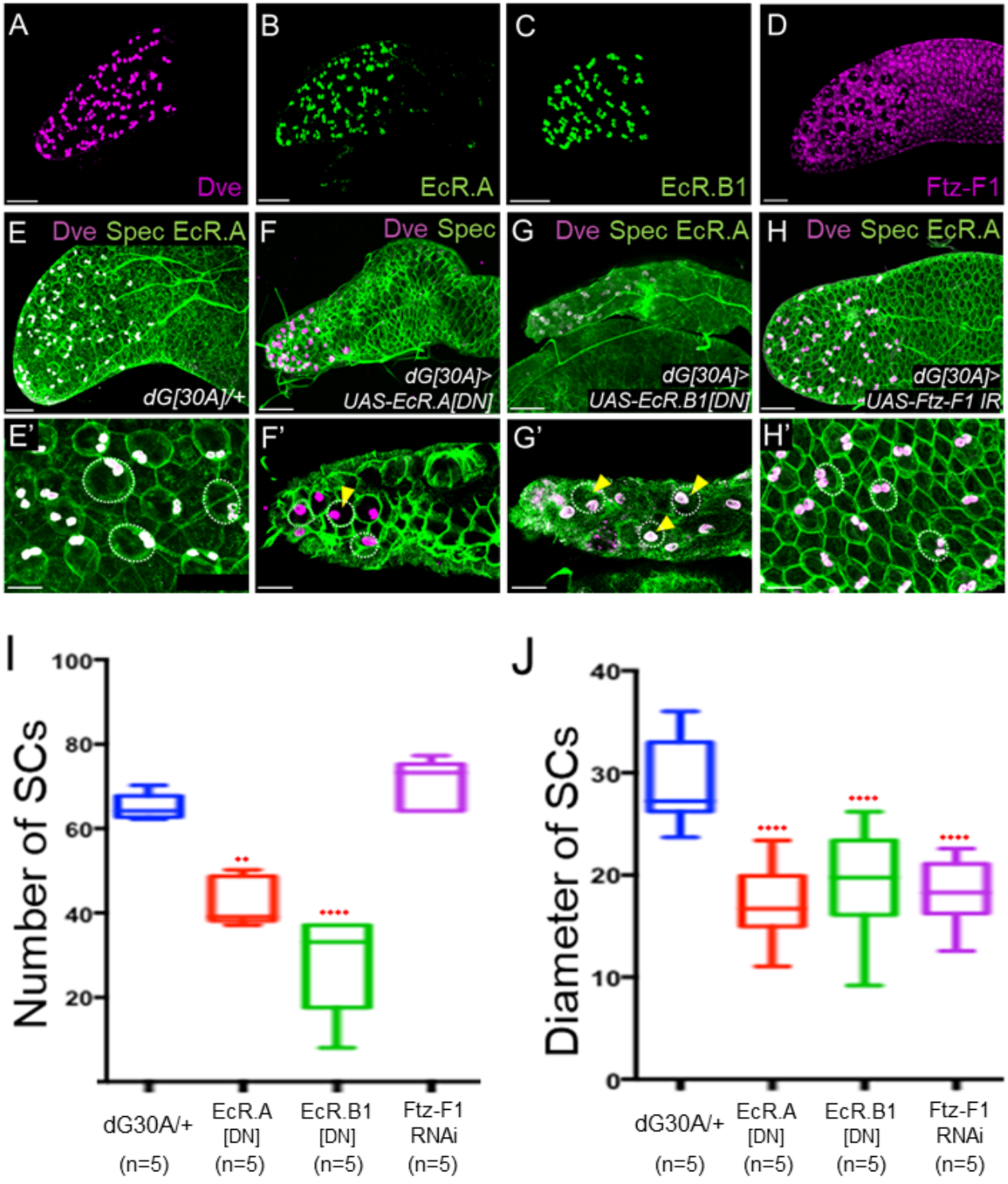


Fig 12. Accessory gland development requires ecdysone signaling

(A-D) Expression patterns of Dve (A), EcR.A (B), EcR.B (C), and Ftz-F1 (D) in the adult male accessory gland at 12 hr after eclosion. (E-J) Inhibition of ecdysone signaling impairs secondary cell (SC) development. Accessory glands of adult day4 males of *dve-GAL4[30A]/+* (*dG30A* control) (E), *dG30A>UAS-EcR.A[DN]*(F), *dG30A>UAS-EcR.B1[DN]* (G), and *dG30A>UAS-ftz-F1-IR* (H), respectively. Expression patterns of EcR.A and Spectrin (green), and Dve (magenta) are shown. Some SCs are outlined. Magnified views are shown in (E'-H'). Inhibition of EcR results in mononucleated SCs (yellow arrowheads in F' and G'). Scale bar: 50 μm (A-H), 20 μm (E'-H').

(I, J) Number (I) and diameter (J) of SCs in accessory glands of the indicated genotypes. The number (n) of scored accessory glands is indicated.

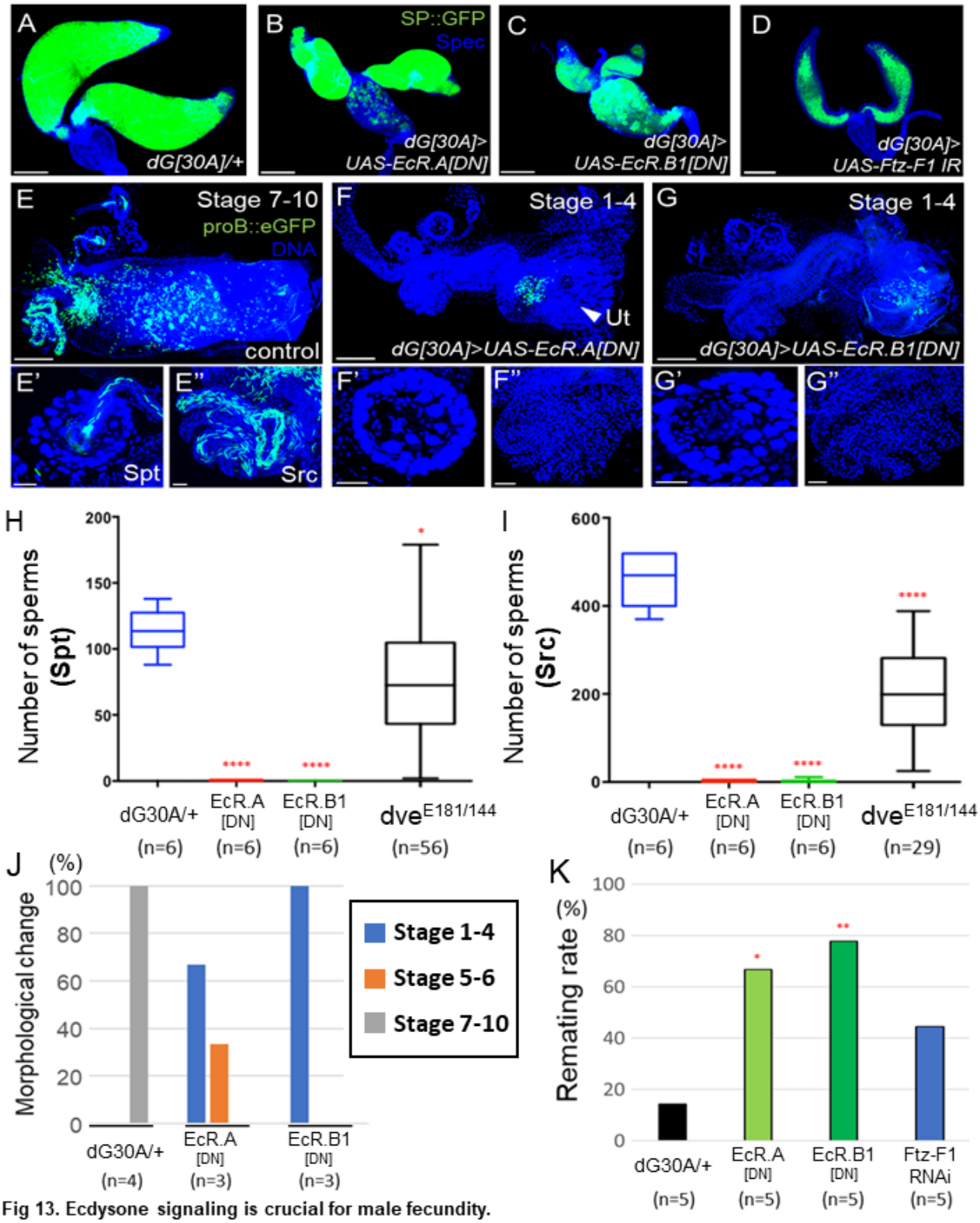


Fig 13. Ecdysone signaling is crucial for male fecundity.

(A-D) Accumulation of Sex Peptide (SP)::GFP fusion proteins (SP::GFP) in accessory glands of *dG30A* control (A), *dG30A>EcR.A[DN]*(B), *dG30A>EcR.B1[DN]* (C), and *dG30A>ftz-F1-IR* (D), respectively. (E-G) Female uterine at 30min after mating with a control male (E), a *dG30A>EcR.A[DN]* male (F), and a *dG30A>EcR.B1[DN]* male (G), respectively. Magnified views of sperm storage in spt (E'-G') and src (E''-G''). Sperm are labelled with protamine B::eGFP (green). DNA is labelled with TO-PRO3 (blue in A-G).

Scale bar: 200 μm (A-D), 100 μm (E-G), 20 μm (E'-G', E''-G'').

(H, I) Number of sperm stored in female spt (H) and src (I) at 30 min after mating with a male of the indicated genotypes. (J) Ratio of uterine conformation in females of (E-G). Stages 1-4 (blue), 5-6 (orange), and 7-10 (grey). (K) Re-mating rates of the day7 females at 48hr after first mating with a male of the indicated genotypes.

The number (n) of mated females is indicated.

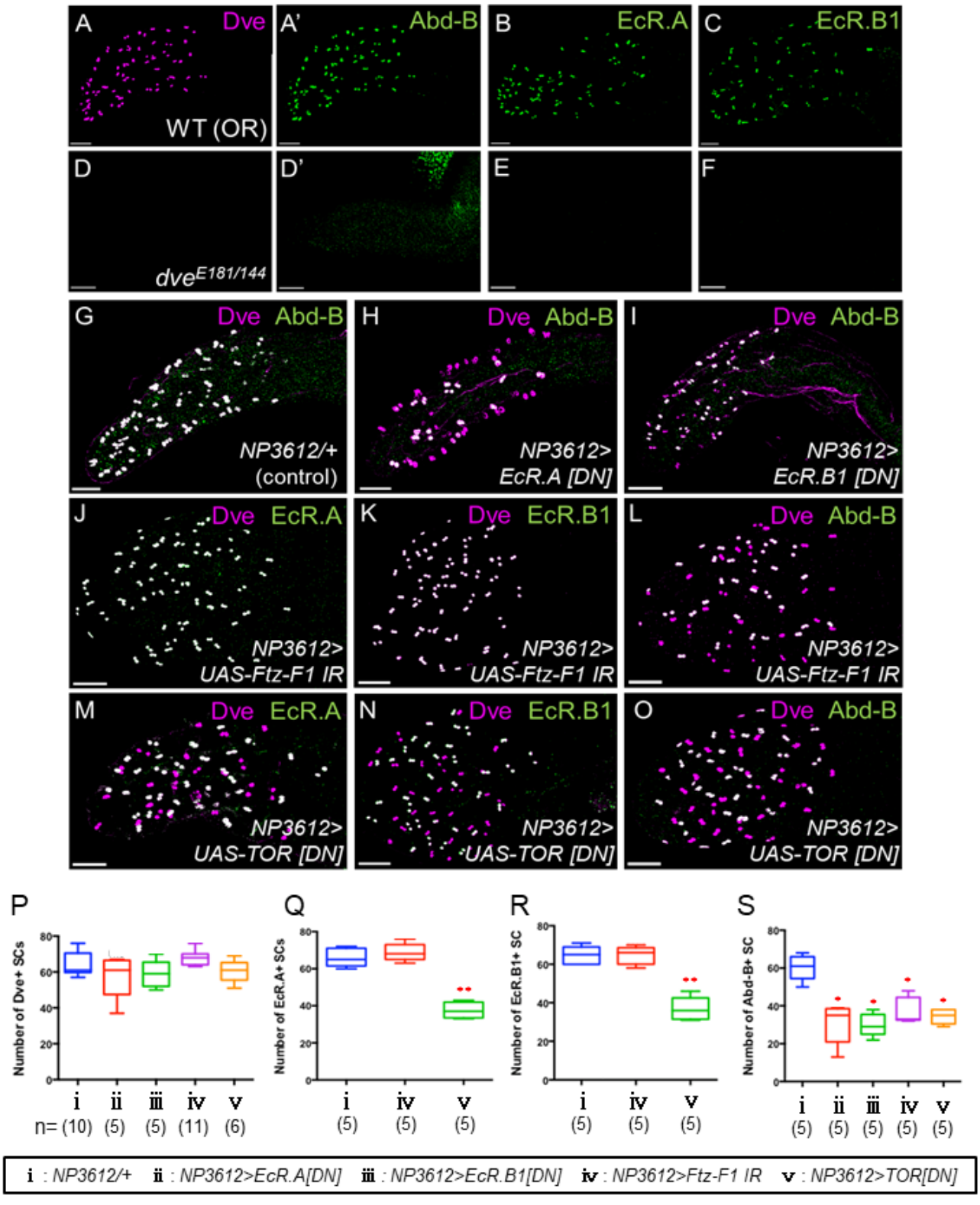


Fig 14. Abd-B expression requires Ecdysone signaling.
(A-C) Expression patterns of Dve (A), Abd-B (A'), EcR.A (B), and EcR.B (C) in wild-type male accessory glands at adult day4. (D-F) *dve* mutation (dve^{E181}/dve^{E144}) completely abolishes Dve expression (D) and also abolishes expression of Abd-B (D'), EcR.A (E), and EcR.B (F). (G-O) Secondary cell-specific inhibition of ecdysone signaling reduces Abd-B expression. Accessory glands of adult day4 males of *NP3612/+* (control) (G), *NP3612>EcR.A[DN]*(H), *NP3612>EcR.B1[DN]* (I), *NP3612>ftz-F1-IR* (J-L) and*NP3612>TOR[DN]* (M-O), respectively. Expression patterns of Dve (magenta, G-O), Abd-B (green in G-I, L, O), EcR.A1 (J, M), EcR.B1 (K, N) are shown. Scale bar: 50 μm (A-C, G), 30 μm (D-F, H-O). (P-S) Number of SCs expressing Dve (P), EcR.A (Q), EcR.B1 (R), and Abd-B (S) in accessory glands of the indicated genotypes. The number (n) of scored accessory glands is indicated.

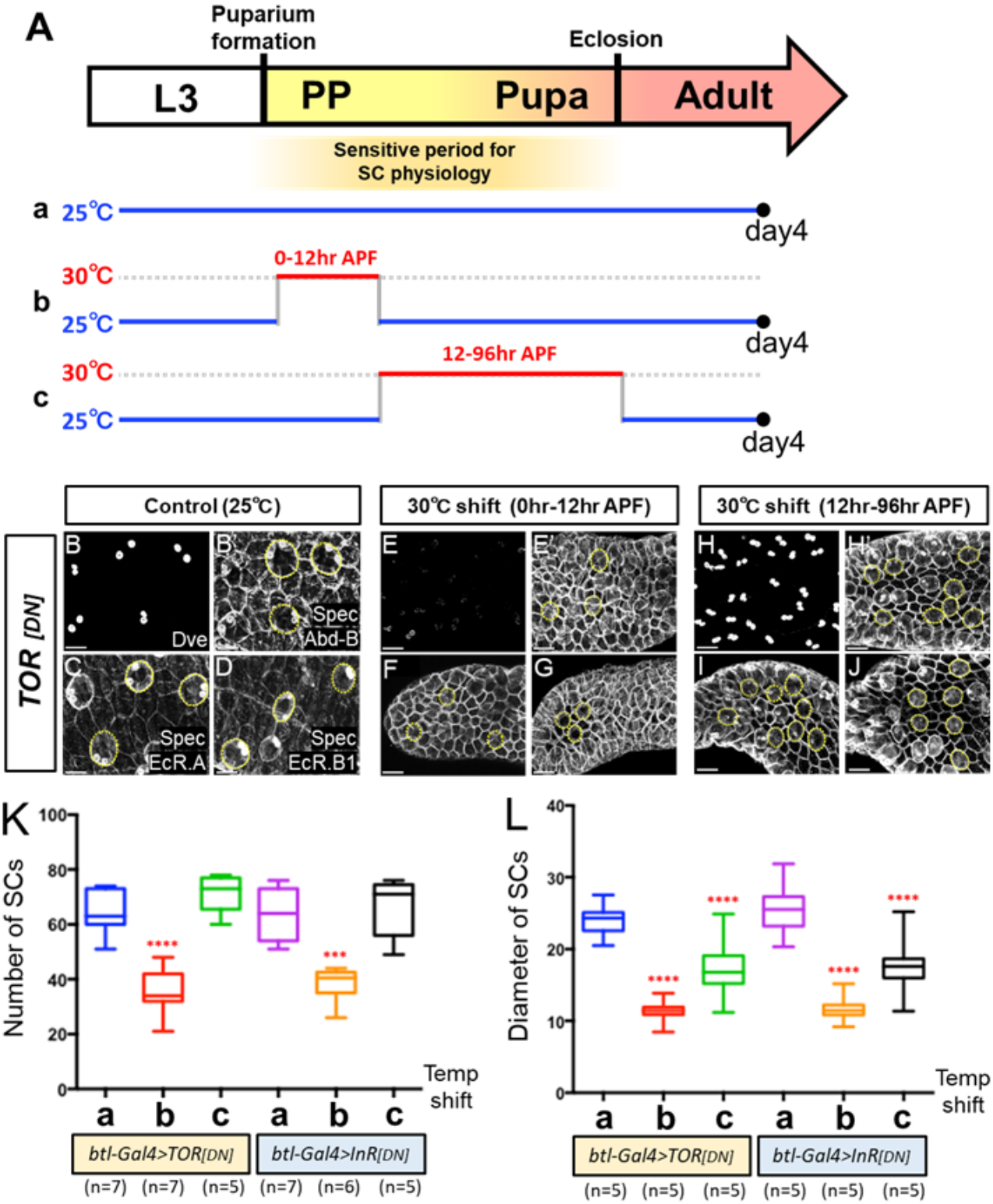


Fig 15. Nutrient signaling pathway during the prepupal stage is crucial for SC development
(A) Schematic representation of temporarily regulated changes in gene expression. Control (25°C constant, shift pattern a), changes during the prepupal and pupal stage (pattern b and c, respectively). L3: late third-instar larva, PP: prepupa. (B-J) Magnified views of SCs in *btl-Gal4>TOR[DN] tub-Gal80^{ts}*. Cell membrane is labelled with Spectrin, and some SCs are outlined. Expression patterns of Dve (B, E, H), Abd-B (B', E', H'), EcR.A (C, F, I), and EcR.B1 (D, G, J) are shown. Control (B-D), inhibition of TOR during the prepupal (E-G) and pupal (H-J) stages. Scale bar: 20 μm. (K) Number of SCs are reduced in the TOR- or InR-inhibited conditions during the prepupal stage (shift pattern b). (L) Diameter of SCs are reduced even in the TOR- or InR-inhibited conditions during the pupal stage (shift pattern c). The number (n) of scored accessory glands is indicated.

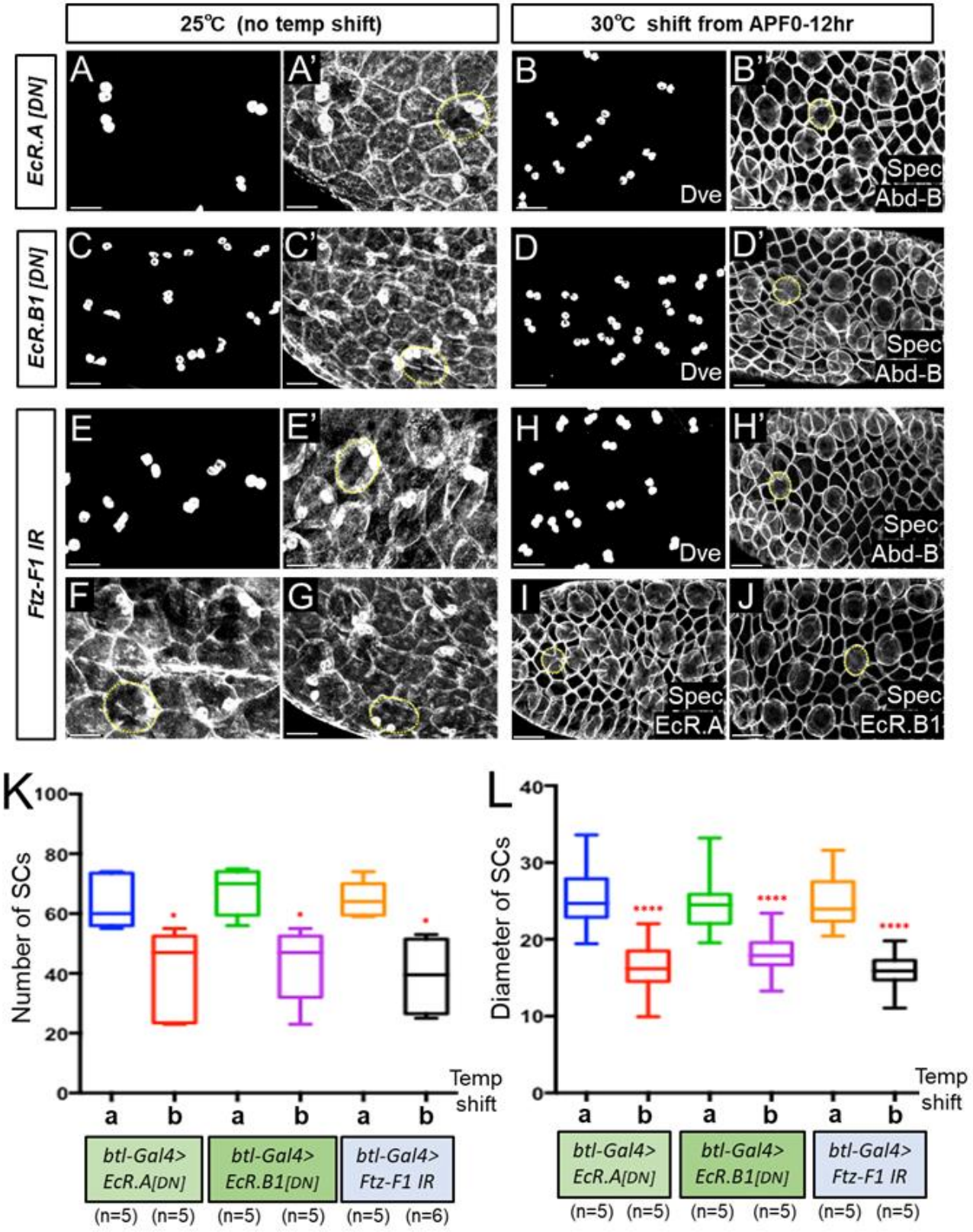


Fig 16. Ecdysone signaling during the prepupal stage is required for SC development

(A-J) Magnified views of SCs in *btl-Gal4>EcR.A[DN] tub-Gal80^{ts}* (A, B), *btl-Gal4>EcR.B1[DN] tub-Gal80^{ts}* (C, D), *btl-Gal4>ftz-F1-IR tub-Gal80^{ts}* (E-J). Control (A, C, E-G) and inhibition of ecdysone signaling during the prepupal stage (B, D, H-J). Cell membrane is labelled with Spectrin, and some SCs are outlined. Expression patterns of Dve (A-E, H), Abd-B (A'-E', H'), EcR.A (F, I), and EcR.B1 (G, J) are shown. Scale bar: 20 μm.

(K, L) Number (K) and diameter (L) of SCs are reduced in the ecdysone signal-inhibited conditions during the prepupal stage (shift pattern b in Fig.15A). The number (n) of scored accessory glands is indicated.

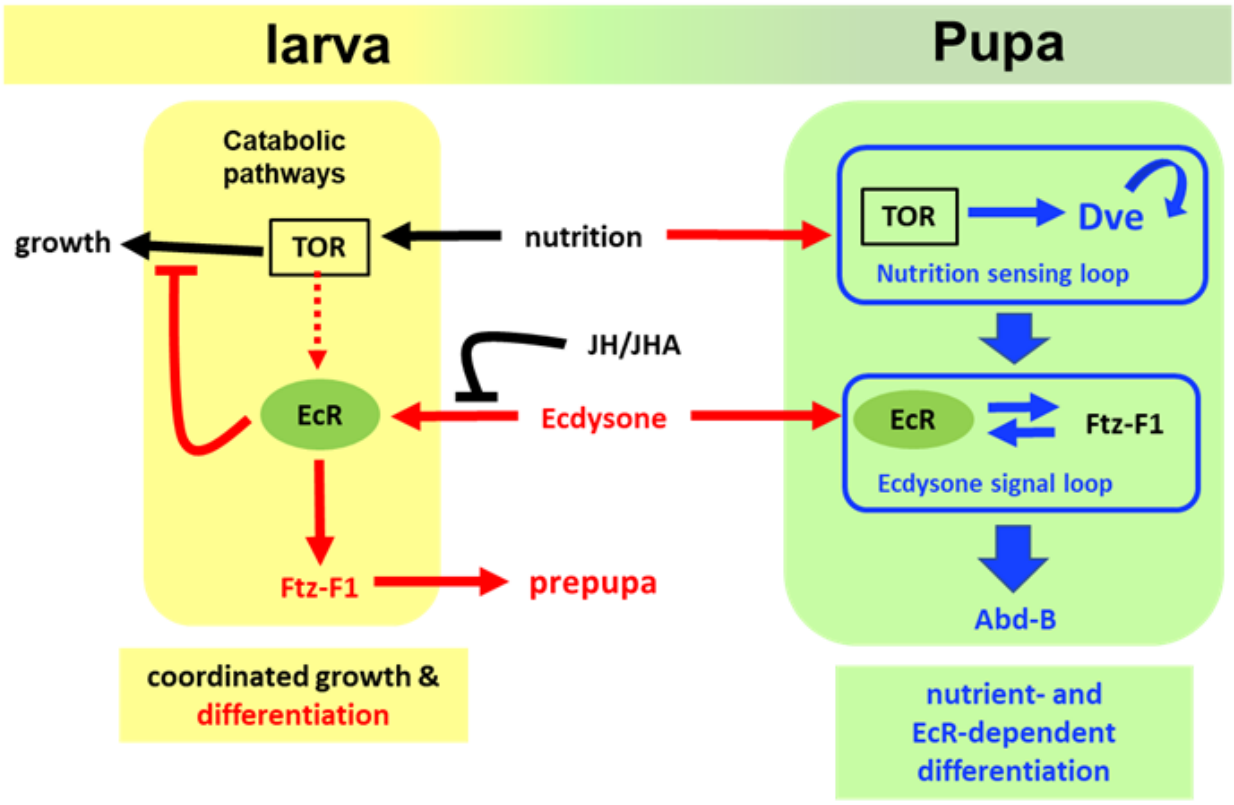


Fig 17. A schematic diagram of ecdysone signaling-dependent nutrition sensing during development.
In the larval stage, ecdysone signaling through EcR is induced under nutrient-rich conditions through TOR signaling to coordinate growth and differentiation. During the pupal stage, TOR-dependent Dve expression is induced and subsequently maintained in a nutrient-dependent manner (Nutrition-sensing loop). EcR expression is maintained in presumptive SCs by this Dve activity in response to the optimal number of SCs. EcR expression is also maintained by the positive-feedback loop through Ftz-F1 (Ecdysone signal loop), and both EcR and Ftz-F1 are required for Abd-B expression for SC maturation.

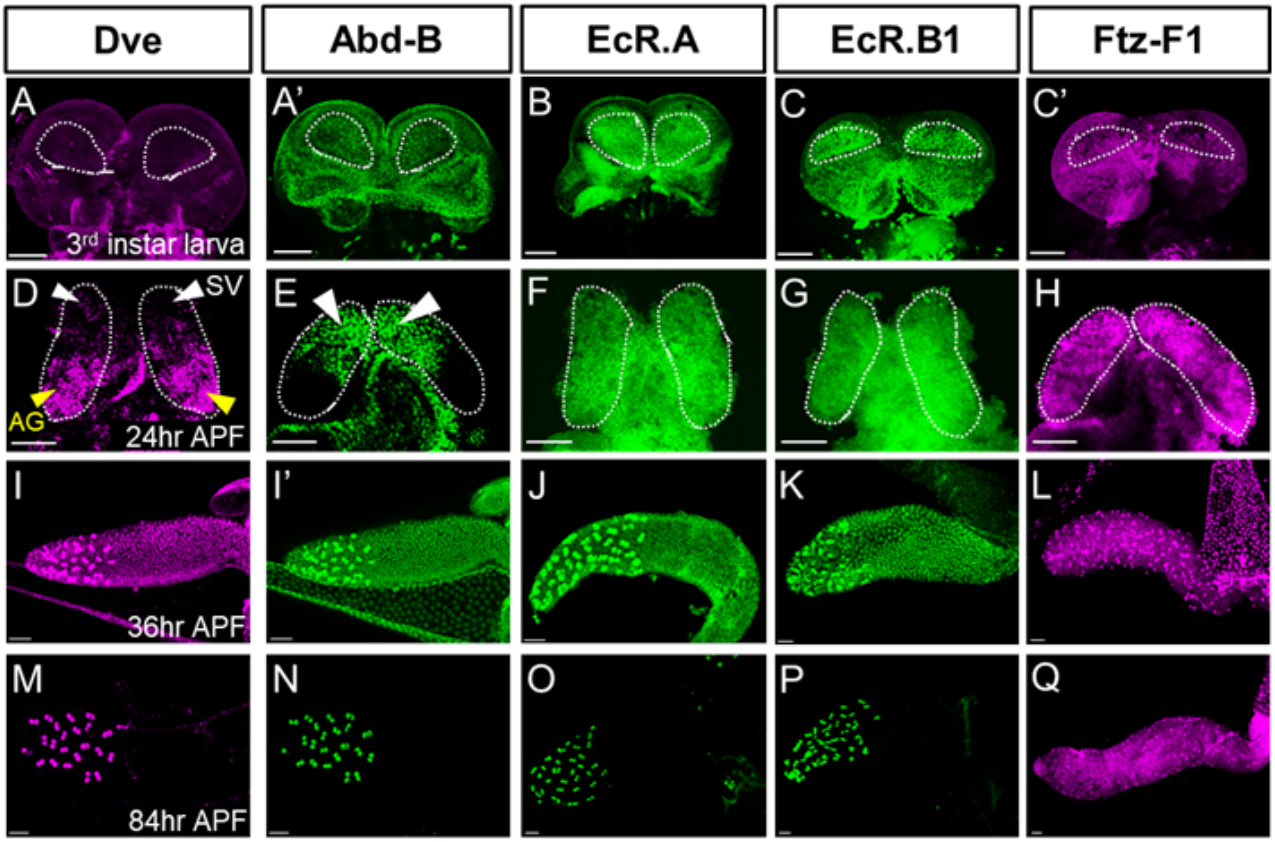


Fig S5. Dve and Ecdysone signal-related gene expression profiles of male genital disc and Accessory glands. (A-Q, A', C', I') Expression profiles of Morphogenesis-related genes (Dve and Abd-B) and Ecdysone-related genes (two EcR isoforms and Ftz-F1) in accessory glands. Male genital disc of late 3rd instar larva (A-C, A', C'). Early pupal accessory glands at 24hr APF (D-H); Seminal vesicle (SV) is pointed by white arrowhead and accessory gland (AG) are pointed by yellow arrowhead; Each accessory gland precursor region is circled by white dotted lines. Middle pupal Accessory glands at 36hr APF (I-L, I'). Late pupal Accessory glands at 84hr APF (M-Q).
Scale bar is 50 μm (A-H, A', C') and 20 μm (I-Q, I').

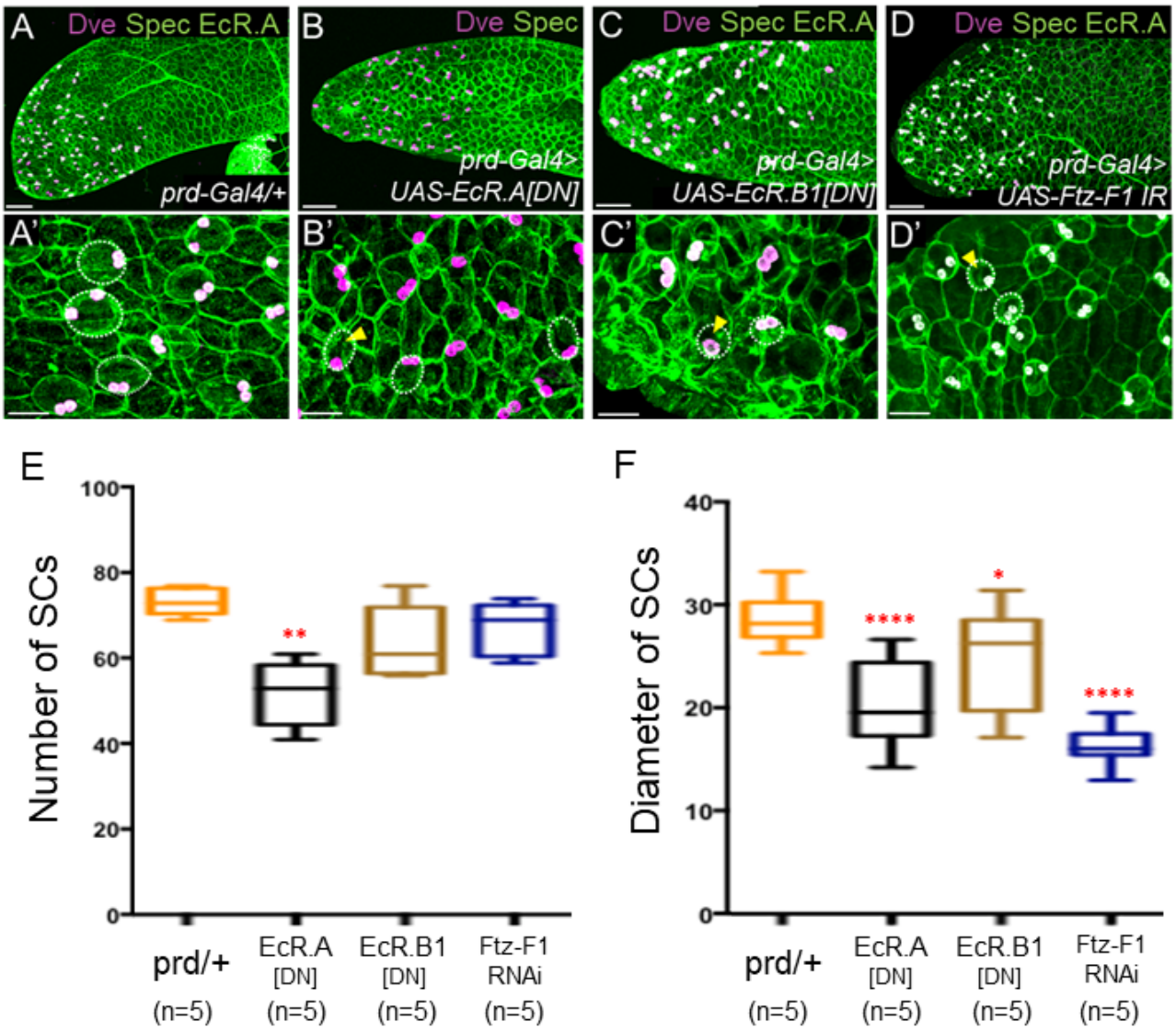


Fig S6. Effects of ecdysone signal blockade in late pupal to adult stages
(A-D) Accessory glands of adult day4 males of *prd-GAL4/+* (control) (A), *prd>EcR.A[DN]*(B), *prd>EcR.B1[DN]* (C), and *prd>ftz-F1-IR* (D), respectively. Expression patterns of EcR.A and Spectrin (green), and Dve (magenta) are shown. Magnified views are shown in (A'-D'). Mononucleated SCs are indicated by yellow arrowheads in (B'-D'). Scale bar: 50 μm (A-D), 20 μm (A'-D'). (E) Number of SCs are reduced in the EcR.A-inhibited condition. (F) Diameter of SCs are also reduced even in *ftz-F1* knockdown conditions. The number (n) of scored accessory glands is indicated.

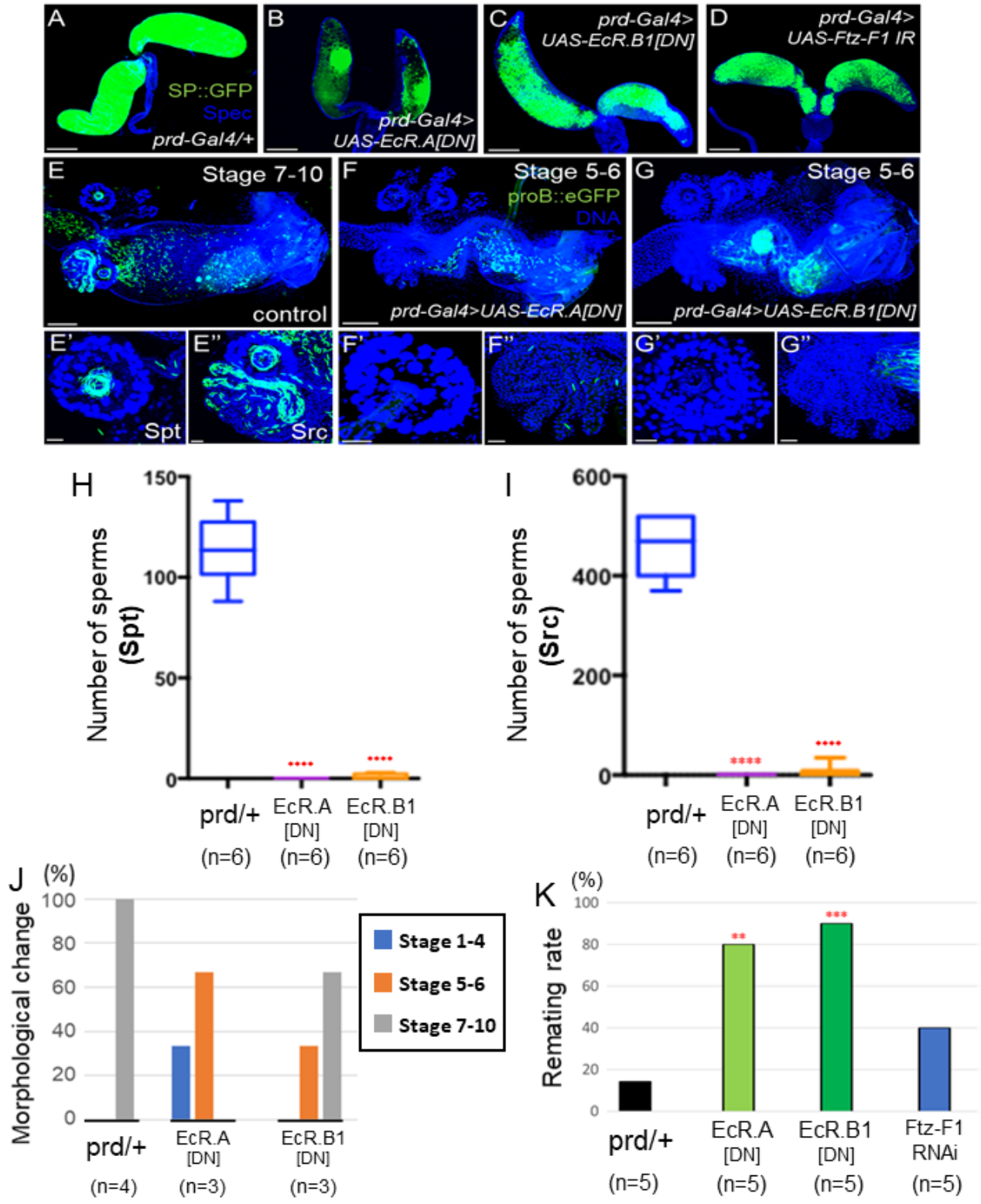


Fig S7. Male fecundity with ecdysone signal blockade in late pupal to adult stages

(A-D) Accumulation of Sex Peptide (SP)::GFP fusion proteins (SP::GFP) in accessory glands of *prd-GAL4* control (A), *prd>EcR.A[DN]*(B), *prd>EcR.B1[DN]* (C), and *prd>ftz-F1-IR* (D), respectively. (E-G) Female uterine at 30min after mating with a control male (E), a *dG30A>EcR.A[DN]* male (F), and a *dG30A>EcR.B1[DN]* male (G), respectively. Magnified views of sperm storage in spt (E'-G') and src (E''-G''). Sperm are labelled with protamine B::eGFP (green). DNA is labelled with TO-PRO3 (blue in A-G). Scale bar: 200 μm (A-D), 100 μm (E-G), 20 μm (E'-G', E''-G'').

(H, I) Number of sperm stored in female spt (H) and src (I) at 30 min after mating with a male of the indicated genotypes. The number (n) of mated females is indicated.

(J) Ratio of uterine conformation in females of (E-G). Stages 1-4 (blue), 5-6 (orange), and 7-10 (grey). (K) Re-mating rates of the day7 females at 48hr after first mating with a male of the indicated genotypes.

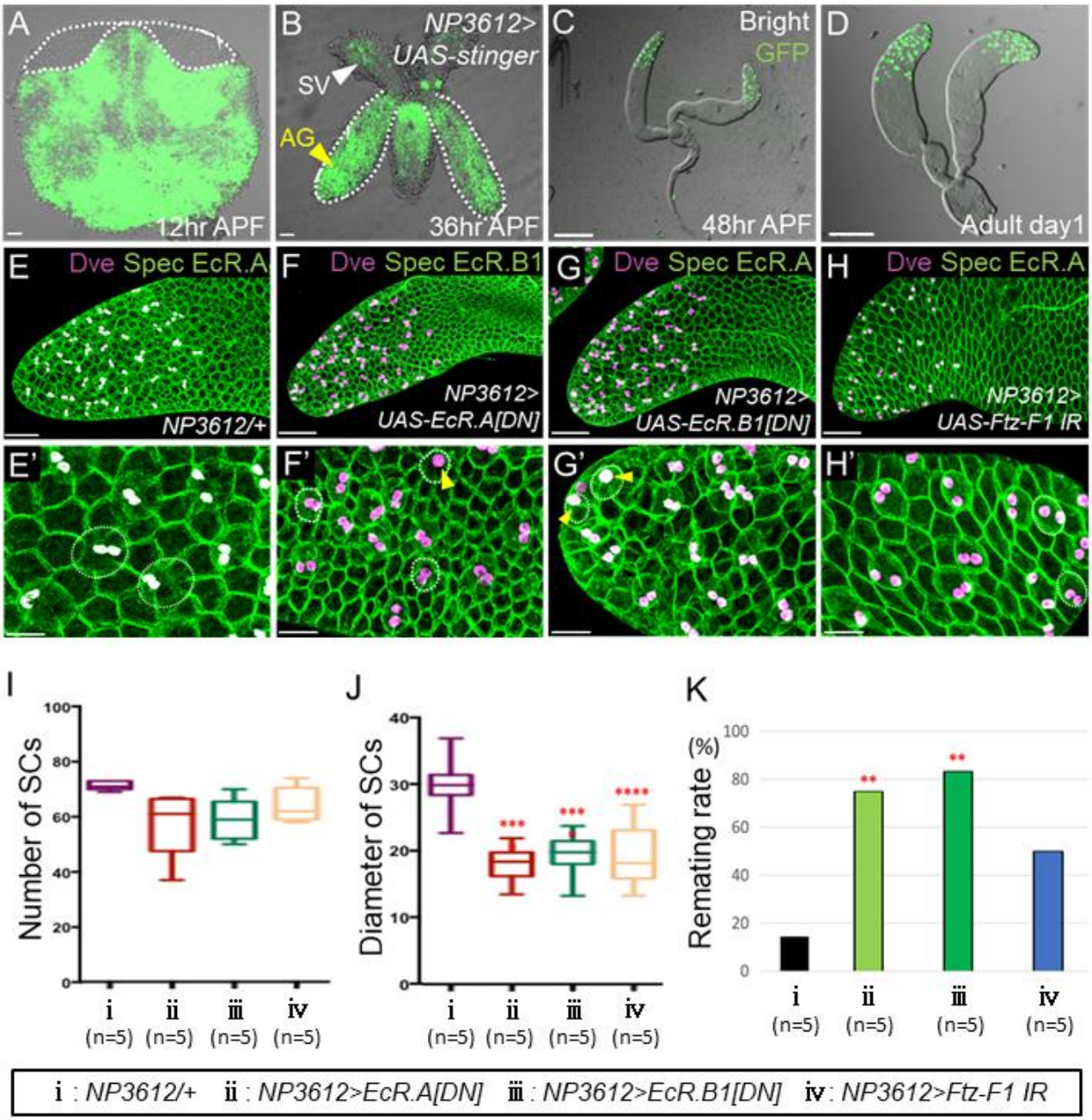


Fig S8. Effect of secondary cell-specific blockade of ecdysone signal after the mid-pupal stage.
(A-D) Expression patterns of *NP3612-GAL4>UAS-Stinger* in accessory gland primordia (outlined with white dot lines) at 12 hr APF (A) and 36 hr APF (B); white arrowheads indicate seminal vesicles (SV) and yellow arrowhead indicates accessory gland (AG). Accessory glands at 48 hr APF (C) and of an adult day1 male (D). (E-H) Accessory glands of adult day4 males of *NP3612/+* (*NP3612* control) (E), *NP3612>EcR.A[DN]*(F), *NP3612>EcR.B1[DN]* (G), and *NP3612>ftz-F1-IR* (H), respectively. Expression patterns of EcR.A and Spectrin (green), and Dve (magenta) are shown. Magnified views are shown in (E'-H'). Some SCs are outlined. Inhibition of EcR results in mononucleated SCs (yellow arrowheads in F' and G'). Scale bar: 20 μm (A, B, E'-H'), 200 μm (C, D), 50 μm (E-H). (I, J) Number (I) and diameter (J) of SCs in accessory glands of the indicated genotypes. (K) Re-mating rates of the day7 females at 48hr after first mating with a male of the indicated genotypes. The number (n) of scored accessory glands (I, J) and mated females (K) is indicated.

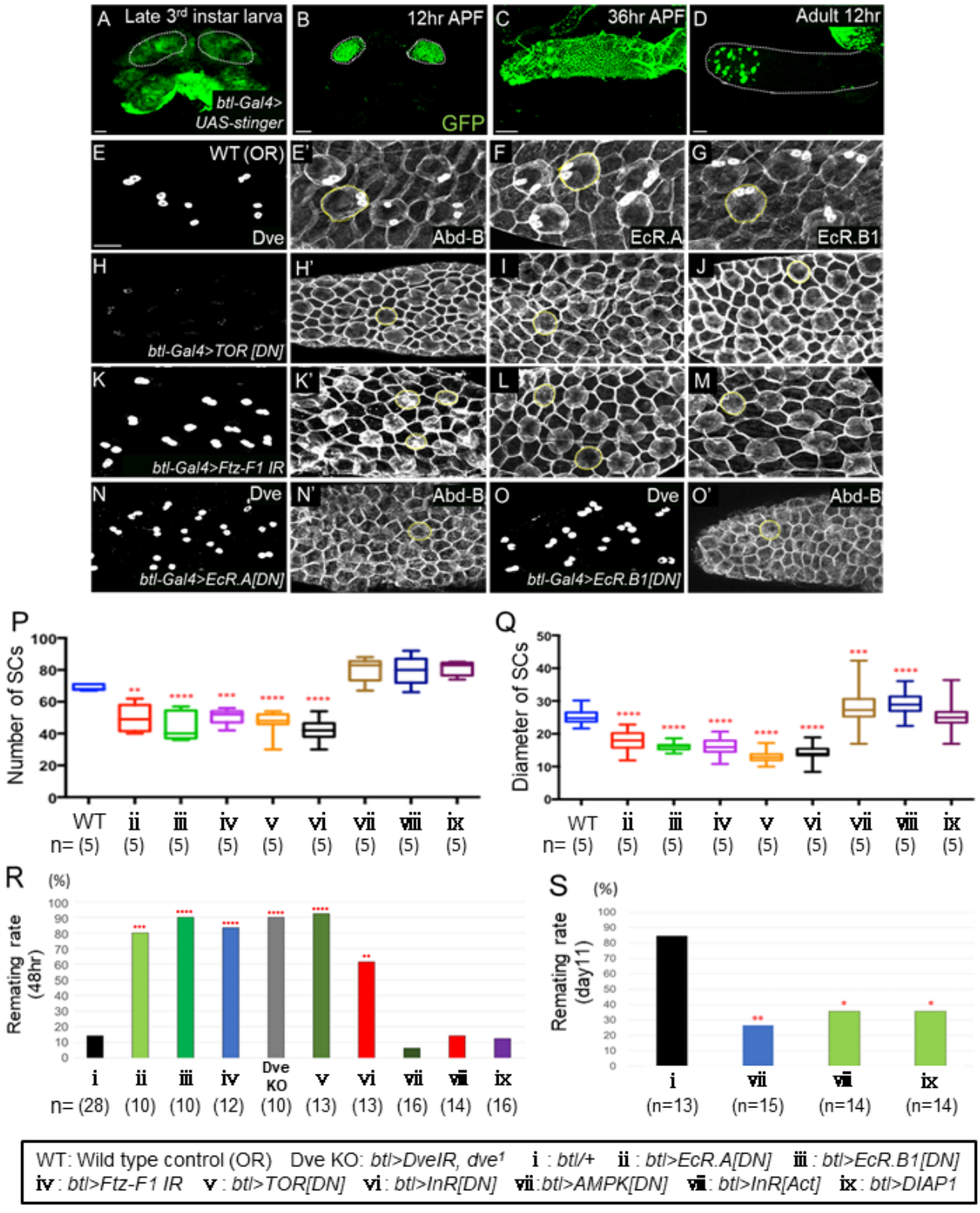


Fig S9. Effects of signal blockade from the prepupal stage

(A-D) Expression patterns of *btl-Gal4>UAS-stinger* in accessory gland primordia (outlined with white dot lines) in a male genital disc of third-instar larva (A) and at 12 hr APF (B). Accessory glands at 36 hr APF (C) and of an adult day1 male (D). (E-O) Accessory glands of adult day4 males of WT control (OR) (E-G), *btl-Gal4>TOR[DN]* (H-J), *btl-Gal4>ftz-F1-IR* (K-M), *btl-Gal4>EcR.A[DN]* (N), and *btl-Gal4>EcR.B1[DN]* (O), respectively. Expression patterns of Dve (E, H, K, N, O), Abd-B (E', H', K', N', O'), EcR.A (F. I, L), and EcR.B1 (G, J, M) are shown. Cell membrane is labelled with Spectrin. Some SCs are outlined. Scale bar: 50 μm (A, B), 20 μm (C-O).

(P, Q) Number (P) and diameter (Q) of SCs in accessory glands of the indicated genotypes. (R, S) Re-mating rates of the females at 48hr (R) and 11 days (S) after first mating with a male of the indicated genotypes, respectively. The number (n) of scored accessory glands (P, Q) and mated females (R, S) is indicated.

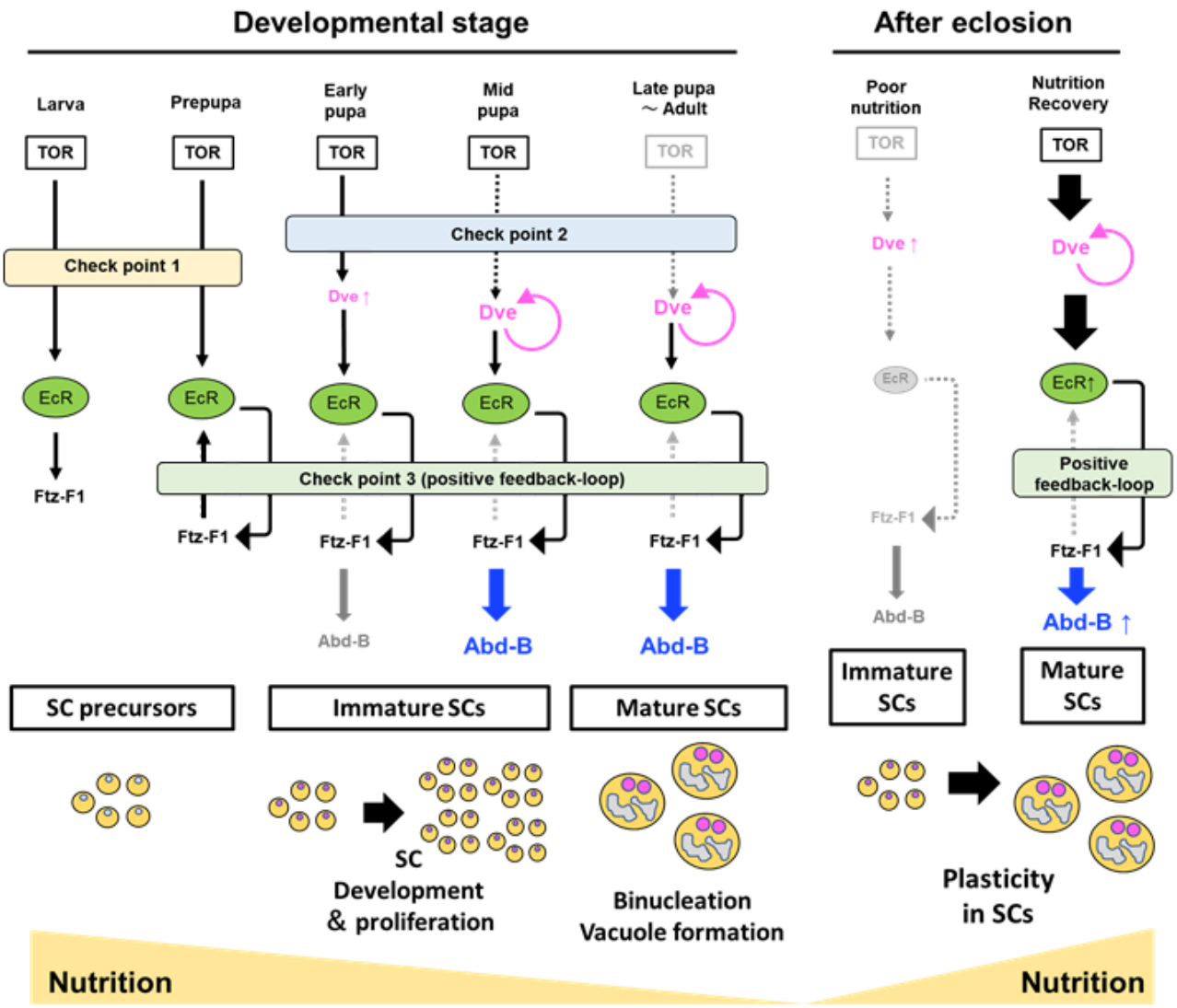


Fig S10. Nutrition sensing have several checkpoints to optimize fecundity

Nutrient-dependent differentiation of secondary cells (SCs) is regulated by three steps. (1) nutrient-dependent induction of EcR and Dve expression, (2) nutrient-independent maintenance of Dve expression and Dve- and nutrient-dependent EcR expression, and (3) positive-feedback loop of EcR and Ftz-F1 expression

Check point 1: Nutrient conditions just before puparium formation should be sensed precisely. EcR expression is induced in a nutrient-dependent manner to ensure survival to the adulthood. **Check point 2**: During the pupal stage, nutrition in the hemolymph gradually declines, and Dve expression can also be induced in a nutrient-dependent manner until 24 hr APF. The number of SCs expressing Dve is determined in response to nutrient conditions at this time point. Dve expression becomes independent of nutrient conditions, and Dve also maintains EcR expression. **Check point 3**: EcR expression is also maintained by Ftz-F1 through positive feedback loop.

4. Conclusion

Nutrient-dependent fecundity in *Drosophila* male accessory gland is regulated in multiple steps; nutrient-dependent induction of EcR and Dve, transition to nutrient-independent Dve maintenance, Dve- and nutrient-dependent EcR expression, and positive feedback loop of EcR and Ftz-F1. As secondary cells are small population in the accessory gland, they can sense nutrient conditions and effectively change their number and size with minimum volume change of total organ size to optimize fecundity. The molecular mechanism revealed in this work will provide insights into the regulatory mechanism of fertility/fecundity in mammals.

5. Materials and methods

Drosophila strains and husbandry

All flies were reared on a standard yeast and cornmeal-based diet at 25°C. The standard diet was made according to the following protocol: 3 g agar, 29 g glucose, 20 g cornmeal, 9 g wheat germ, 11 g dry yeast, 0.9 mL propionic acid, 1 mL of 15% butyl parahydroxy-benzoate, and 300 mL water **(See Fig S1A of Kubo; Matsuka et al., 2018).**

Oregon-R (OR) flies were used as wildtype controls. dve^{E181} and dve^{E144} are *dve-A*-specific mutant alleles that remove the first exon **(Nakagawa et al., 2011).** The following GFP marker strains were used: protamineB::GFP for sperm nuclei **(*Drosophila* Genetic Resource Center, DGRC) (Jayaramaiah et al., 2005)** and SP::GFP for expression of sex peptide **(Villella et al., 2006).**

The following GAL4/UAS lines were used: *dveGAL4[30A]* **(Minami et al. 2012)**, *prd-GAL4*, *NP3612* **(Secondary cell-specific expression in Accessory glands; Nakagawa et al., 2011)**, *btl-GAL4* **(Bloomington *Drosophila* Stock Center)**, *UAS-myc-DIAP1* **(Kindly gifted from Prof. Miura, Tokyo Univ.)**, *UAS-Ftz-F1 IR* **(kindly gifted from Prof. Ueda. H., Okayama Univ.)**, *UAS-TOR[DN](TED)*, *UAS-InR[DN](K1409A)*, *UAS-EcR.A[DN](W650A), UAS-EcRB1[DN](W650A),* and *tub-GAL80ts* **(Bloomington *Drosophila* Stock Center).**

Immunohistochemistry

Larvae, pupae, and adults were dissected in phosphate-buffered saline (PBS), fixed with 4% formaldehyde/PBS-0.3% Triton X-100 for 20 min, and washed several times with PBS-0.3% Triton X-100. The following primary antibodies were used: rabbit anti-Dve (1:500) **(Nakagoshi et al., 1998)**, rabbit anti-ftz-F1 (1:1000) (kindly gifted form Prof. Hitoshi Ueda), mouse anti-EcR.A (1:100), mouse anti-EcR.B1 (1:100), mouse anti-Abd-B (1:20), mouse anti-a-Spectrin (3A9) (1:100) (Developmental Studies Hybridoma Bank, DSHB). FITC-, Cy3- or Cy5-conjugated secondary antibodies (Jackson Immunoresearch) were used for detection. TO-PRO3 (Thermo Fisher Scientific, 1:500) was used to stain DNA. Confocal images of 0.2–1.22 μm sections were obtained with a confocal microscope (Olympus FV1200), and were processed using the Fluoview (Olympus) and Photoshop software (Adobe).

Behavioral assays

All flies were cultured at 25°C under 12 h light/dark cycles. Males and females were collected at eclosion and aged separately for 4–6 days in groups of 10–30 flies per vial. At circadian time 0:00 to 3:00, a pair of male and female flies was placed on a food medium with an observation chamber (30-mm diameter × 4-mm depth) for 1 h. A mated female was gently aspirated into a vial and used for the following assays.

Egg-laying assay: a mated female was transferred to a fresh vial every 24 h and eggs laid per day

were counted for 7 days or 14 days. The cumulative egg production per female was scored.

Remating assay: at 48 h after the first mating, mated females were individually paired with one new virgin male (4–6 days old) for 1 h. Several independent sets of remating assays were performed, and the cumulative percentage of remated females was scored.

To test the period of mating rejection, the remating assay was performed every 24 h during 7-14 days after the first mating.

Temporal Inhibition of Nutrient and Ecdysone Signaling

To change the levels of TOR, Insulin, and ecdysone signaling in a temporally regulated manner, the temperature-sensitive GAL80 ($GAL80^{ts}$) strain was used **(McGuire et al. 2003).** Flies were reared at the permissive temperature (25°C; R.T.), shifted to the restrictive temperature (30°C).

Quantification and Statistical Analysis

To visualize the gross morphology and cell size, half-section views of the AG were generated as projection images. Cell diameters were estimated from the circumference length using Image J software **(National Institute of Health).** Data are presented as boxplots and scatter plots. For each plot, the upper and bottom lines represent the first and third percentiles, and the middle line is the median. The significance of differences between the control and test progenies was analyzed using Prism6 **(GraphPad Software).** A one-way ANOVA with Dunnett's post hoc test was applied to compare the means of three or more groups for the number of SCs, the diameter of SCs and the cumulative egg production per female. Fisher's exact test was applied to compare the number of remated/rejected females between the control and each test group. The levels of significance are indicated by asterisks: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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