Steroid Regulation of Postembryonic Development and Reproduction in *Drosophila*

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Ecdysteroids trigger major developmental transitions in Drosophila*, including larval molts and metamorphosis. Recent genetic studies strongly support a role for the Ecdysteroid receptor (EcR)/Ultraspiracle (USP) heterodimer as an ecdysteroid receptor at the onset of metamorphosis, functioning as both a transcriptional activator and repressor* in vivo*. Genetic analysis also indicates that USP, like its vertebrate homolog retinoid X receptor (RXR), might be involved in regulatory pathways independently of EcR. The ecdysteroid hierarchy was also shown recently to regulate* Drosophila *oogenesis and reproduction.*

Steroid hormones regulate a wide range of developmental and physiological responses in higher organisms, including reproduction, embryogenesis, postembryonic development and metamorphosis. *Drosophila melanogaster* provides an ideal model system for analysing the molecular mechanisms of steroid hormone action. In addition to its well-known advantages for molecular and genetic studies, a single steroid hormone, 20-hydroxyecdysone (20E), appears to be responsible for directing the major developmental transitions in this insect. The *Drosophila* life cycle begins with an embryonic stage at the end of which a motile and feeding first-instar larva hatches from the egg (Fig. 1). The larva grows and undergoes two molts during which a new cuticle is formed and the old cuticle is shed, along with the attached mouthhooks and anterior and posterior spiracles (which function as external openings for the larval respiratory system). The ecdysteroid titer increases before each larval molt and is required for triggering these developmental transitions. At the end of the third larval instar, a high titer peak of ecdysteroids triggers puparium formation and the onset of metamorphosis. Obsolete larval tissues are destroyed during metamorphosis and replaced by adult structures that develop from clusters of progenitor cells. Several pulses of ecdysteroids during metamorphosis are responsible for further differentiation of these adult structures. The ecdysteroid titer declines at the end of pupal development and is relatively low when the adult fly emerges from the pupal case (Fig. 1; reviewed in Ref. 1).

The molecular mechanisms of ecdysteroid action have been best characterized at the onset of metamorphosis when 20E, bound to its receptor, directly induces a small number of primary-response early genes, including the *Broad-complex* (*BR-C*), *E74* and *E75**. These early genes encode transcription factors that coordinate the induction of large sets of secondary-response late genes, leading to the appropriate stage- and tissue-specific biological responses[2–6].

Another hormone, the sesquiterpenoid juvenile hormone (JH), has been shown to modulate the action of ecdysteroids in various insect species[7,8] and is thought to regulate development and oogenesis in *Drosophila*[1,9]. By analogy with other species, it is likely that the balance between these two hormones affects the nature of developmental transitions, although this has yet to be established in *Drosophila*. Here, we discuss recent advances in our understanding of the mechanisms of ecdysteroid action during postembryonic development and reproduction, based on genetic studies in *Drosophila*.

• *Dare* is a Critical Component of Ecdysteroid Biosynthesis in *Drosophila*

Despite the biochemical and pharmacological characterization of several steroidogenic enzymes in various insect species[9–11], the biosynthetic pathway leading to the formation of ecdysteroids in *Drosophila* remains undefined. Recently however, a putative early component of the ecdysteroid biosynthetic pathway has been functionally characterized in *Drosophila*. This gene, designated *dare* (*defective in the avoidance of repellents*), encodes a close homolog of the vertebrate adrenodoxin reductase (AR), with 42% overall identity to the human AR and conserved binding sites for FAD and NADPH (Ref. 12). AR transfers electrons from NADPH to adrenodoxin protein which, in turn, donates them to the mitochondrial steroidogenic cytochrome P450 hydroxylases. AR is required for the synthesis of all vertebrate steroid hormones[13], implying that the *Drosophila* homolog may play a similar central role in steroidogenesis. More than 20 cytochrome P450 enzymes have been identified in *Drosophila*[14], and the expression of several of these is regulated by ecdysteroids[15,16]; however, the participation of these P450s in ecdysteroid biosynthesis has yet to be demonstrated.

Dare expression is greatly enriched in the larval ring gland, the endocrine organ that produces ecdysteroids, consistent with its role in steroid biosynthesis. Furthermore, strong *dare* mutants display defects in the second-to-third instar larval molt and pupariation[12]. Animals arrested at the larval molt exhibit two sets of mouthhooks and

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**BR-C*, *E74* and *E75* have been designated as *br*, *Ecdysone-induced protein 74EF* (*Eip74EF*) and *Ecdysone-induced protein 75B* (*Eip75B*), respectively, by FlyBase (http://flybase.bio.indiana.edu), but we use the original terms throughout this article.

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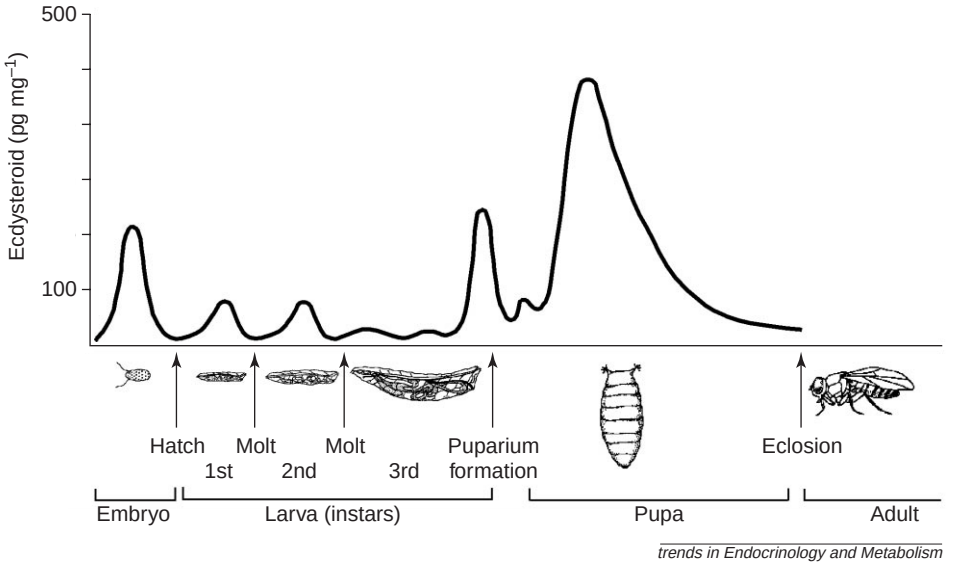

Figure 1. The ecdysteroid titer profile during *Drosophila* development. A composite ecdysteroid titer from whole-body homogenates is shown in 20E equivalents (adapted from Ref. 1). The stages of *Drosophila* development are depicted with arrows marking the major developmental transitions.

anterior spiracles, one resembling those of second instar larvae and the other pair resembling those of the third instar, demonstrating a failure in the molting process. Both molting and pupariation phenotypes can be efficiently rescued by feeding 20E to mutant larvae, convincingly demonstrating that an ecdysteroid deficiency is the cause of these mutant phenotypes[12]. An analysis of weak *dare* mutations also uncovered two intriguing phenotypes, abnormal behavioral responses to olfactory stimuli and degeneration of the adult nervous system, processes not previously known to be dependent on ecdysteroids.

- **The EcR/USP Heterodimer is Required for Ecdysteroid Signaling *in vivo***

Steroid hormones exert their effects on target tissues by activating their respective receptors, which are members of the nuclear receptor superfamily[17]. The *Drosophila* ecdysteroid receptor is a heterodimer of two such proteins: EcR (Ecdysteroid receptor, NR1H1) and the fly retinoid X receptor (RXR) homolog, USP (Ultraspiracle, NR2B4). Heterodimer formation is a prerequisite for DNA and ligand binding by the ecdysteroid receptor *in vitro*[18–20]. In addition, a recent study showed that, like vertebrate steroid receptors, the EcR/USP heterodimer only becomes active upon interaction with a molecular chaperone heterocomplex[21]. Not only does this add another level of previously unknown regulation, but this is also the first example of a requirement for chaperones in the activation of an RXR nuclear receptor heterodimer.

EcR encodes three protein isoforms with different amino-terminal A/B domains and identical DNA and ligand binding domains[22], all able to interact with USP and bind ecdysteroids with similar affinity (M.R. Koelle, PhD thesis, Stanford University, 1992). Functional studies of both *EcR* and *usp* have been reported recently[23–28]. A considerable proportion of *EcR* and *usp* mutants are arrested at the boundaries between larval stages, displaying additional sets of mouthhooks and spiracles[24,26], a phenotype reminiscent of *dare* mutants. Furthermore, most of the *EcR* and *usp* mutant phenotypes that were studied at the onset of metamorphosis are very similar, affecting both the destruction of larval tissues and the proliferation and morphogenesis of adult tissues, supporting the function of the EcR/USP heterodimer as an ecdysteroid receptor *in vivo*. Consistent with this observation, expression of ecdysteroid-inducible genes, including the *BR-C*, *E74* and *E75* early genes, is significantly reduced in both *EcR* and *usp* mutants at the onset of metamorphosis.[25,26]

The remarkable similarity between *EcR* and *usp* mutant phenotypes supports the proposal that USP functions as an obligate partner for all EcR isoforms (M.R. Koelle, op. cit.). However, the intriguing observation that *usp* mutants synthesize a supernumerary larval cuticle at the onset of metamorphosis[25], a phenotype not manifested in *EcR* mutants[23–26], strongly suggests that *usp* can also function independently of *EcR in vivo*. This may not be entirely surprising, as USP is a homolog of vertebrate RXR that is known to function as a homodimer or heterodimer with other nuclear receptors to coordinate multiple regulatory pathways (reviewed in Ref. 29). In fact, USP is known to heterodimerize with at least one other *Drosophila* nuclear receptor, DHR38 (NR4A4), the fly homolog of the vertebrate nerve growth factor indicible B nuclear (NGFI-B) receptors (NR4A1) (Ref. 30, reviewed in Ref. 31), paralleling the formation of NGFI-B/RXR heterodimers in vertebrates. However, the functional significance of this interaction in *Drosophila* is currently unknown.

The deposition of a supernumerary larval cuticle, as well as the induction of additional larval molts, can be produced by ectopic JH application in several insect species, but not in *Drosophila*[8,32,33]. This observation indicates that *usp* might be involved in JH-mediated events, integrating both JH and ecdysteroid signaling. Extensive efforts to demonstrate direct JH binding to USP or effects on its *trans*-activation function, however, have been unsuccessful[34] (D. Mangelsdorf, pers. comm.). A recent paper has shown that JH can cause USP to aggregate but the functional significance of this observation is unknown[35].

- **Unliganded EcR/USP Heterodimer can Interact with Cofactors and Repress Transcription *in vivo***

Unliganded EcR/USP heterodimer can act as a repressor in tissue culture cells[36,37], paralleling the mode of action of unliganded vertebrate nuclear receptors such as retinoic acid receptor

(RAR) and thyroid hormone receptor (TR) (reviewed in Ref. 38). These observations have recently been supported and extended by a series of studies showing that EcR/USP can interact with co-repressors and repress target gene transcription and developmental responses *in vivo*.

Two *Drosophila* co-repressors have been recently characterized: Alien and SMRTER (silencing mediator for RXR and TR-related ecdysteroid receptor interacting factor) (Refs 39, 40). These proteins can bind specifically to EcR, but not to USP, in the absence of ligand. *Drosophila* SMRTER is structurally divergent, but functionally similar, to vertebrate silencing mediator for RXR and TR (SMRT) and nuclear receptor co-repressor (N-CoR) in that it can also mediate repression by unliganded human RAR and TR (Ref. 40). In addition, SMRTER co-localizes with EcR/USP on salivary gland polytene chromosomes, suggesting that these proteins function together to repress target gene transcription *in vivo*. Like vertebrate SMRT and N-CoR, *Drosophila* SMRTER mediates repression by interaction with Sin3A, a repressor known to form a complex with the histone deacetylase Rpd3/HDAC (reviewed in Refs 38,41,42). Genetic interactions between *EcR* and *Sin3A* alleles strongly support a functional role for Sin3A in mediating repression *in vivo*[40].

Drosophila Alien represents another candidate co-repressor for the EcR/USP heterodimer belonging to a novel class of nuclear receptor co-repressors that are unrelated to SMRT or N-CoR. Alien is highly conserved in evolution, with 90% identity between the *Drosophila* and human proteins. Like SMRTER, *Drosophila* Alien mediates repression through the Sin3A pathway[39]. It will be interesting to determine whether SMRTER and Alien act synergistically *in vivo* or function independently of one another in a stage- or tissue-specific manner.

Recent functional studies of *usp* have indicated that the EcR/USP heterodimer can mediate repression *in vivo*. Some ecdysteroid-regulated genes that were examined in *usp* mutants, such as cuticle genes, are expressed at stages where they are normally silent, supporting a role for the EcR/USP receptor in repressing target gene expression[25]. In addition, clones of *usp* mutant cells in the progenitor of the adult wing show premature induction of the *BR-C* early gene as well as premature sensory neuron formation and axonal outgrowth[43], consistent with earlier data that *usp* suppresses cell differentiation in the progenitor of the adult eye[44]. Both hormone availability and the composition of the response elements in ecdysteroid-responsive genes were proposed to determine whether EcR/USP could function as an activator or repressor *in vivo*[43,45].

- **The Ecdysteroid Gene Hierarchy is Important for the Progression of *Drosophila* Oogenesis**

Studies of temperature-sensitive ecdysteroid deficient mutants, such as *l(3)ecd*^{1} and *L(3)3*^{DTS}, have indicated that ecdysteroids are likely to play a critical role in *Drosophila* oogenesis and fertility[46–48], paralleling the well-known role for steroids in vertebrate reproduction. A role for ecdysteroids in oogenesis and fertility has been recently investigated by several genetic studies[12,27,49]. An exciting result from this work is that many components of the ecdysteroid regulatory hierarchy, including *dare*, a subunit of the ecdysteroid receptor, and its downstream target genes are critical for the progression of oogenesis, representing a remarkable step forward in our understanding of the hormonal regulation of insect reproduction.

Oogenesis in *Drosophila* takes place in specialized structures called ovarioles, located within the ovaries (reviewed in Ref. 50). The germarium at the anterior end of each ovariole contains germ cells and somatic stem cells whose progeny are organized into egg chambers (Fig. 2). Egg chambers leave the germarium and move posteriorly to the vitellarium, where they continue to develop. Individual egg chambers contain three cell types: the oocyte, connected to the 15 nurse cells (both derived from the germline), all of which are surrounded by a monolayer of somatically-derived follicle cells. Each ovariole contains a linear progression of egg chambers, with the more mature eggs located closer to the posterior of the ovariole (Fig. 2). Oogenesis can be divided into 14 stages; yolk protein deposition in the oocyte is first evident at Stage 8, indicating the beginning of vitellogenesis. A major increase in oocyte size occurs between Stages 9 and 10 owing to the incorporation of yolk proteins. Both 20E and JH have been implicated in regulating vitellogenesis during these stages[1,51].

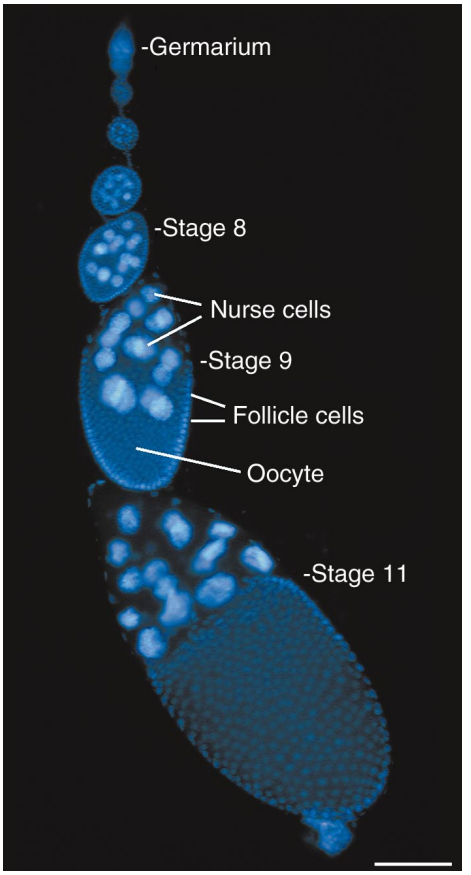

Figure 2. Structure of the *Drosophila* ovariole. A single ovariole is depicted with egg chambers between Stages 1 and 11. The germarium, 15 nurse cells, oocyte, and follicle cells are marked, along with Stage 8 and 9, and 11 egg chambers. This preparation was stained with DAPI in order to image the cell nuclei. Scale bar = 100 μm.

The ovary is the major ecdysteroidogenic tissue in adult *Drosophila* and, by analogy with other insects, it seemed probable that ecdysteroid biosynthesis occurred primarily in the follicle cells[1,52,53]. Consistent with these observations, expression of the *dare* gene is greatly enriched in the ovary. Interestingly, however, it is cell type-specific, appearing in the nurse cells at Stage 6

and reaching maximum expression at Stage 10 (Refs 12,27). The *E74* and *E75* early genes are coordinately induced in both germline-derived and somatic cells at Stage 8 and peak at Stage 10. BR-C protein(s) are upregulated in follicle cells at comparable stages, being undetectable, or at very low abundance, in nurse cells and the oocyte. These responses appear to be controlled by ecdysteroids because *E75* mRNA and BR-C protein(s) are significantly reduced in the ovaries of a temperature-sensitive *l(3)ecd[1]* mutant and, conversely, *E75* mRNA can be upregulated by exogenous 20E in cultured ovaries[27]. In contrast to the cell- and stage-specific expression of these primary-response genes, EcR and USP are present in both nurse and follicle cells at relatively constant levels throughout oogenesis[27,49,54]. These results clearly show that the ecdysteroid-triggered regulatory hierarchy is activated by the hormone during *Drosophila* oogenesis, and that this activation closely follows the induction of *dare*.

Most importantly, removal of either *dare*, *EcR* or *E75* function from the germline causes similar phenotypes, namely the arrest and degeneration of egg chambers during mid-oogenesis (around Stages 8–9), resulting in female sterility[27,49]. Nurse cells degenerate at Stage 9 in *E75* germline mutants, while the follicle cell layer remains remarkably intact. In contrast, *EcR* function appears to be required in both somatic and germline-derived cells[49].

Taken together, these genetic studies and the expression patterns of *dare*, *BR-C*, *E74* and *E75* suggest that ecdysteroids act in an autocrine or paracrine manner in the ovary. Ecdysteroids produced in the nurse cells appear to be promoting the survival of germline-derived cells and the stage-specific expression of ecdysteroid-inducible regulatory genes in the nurse and follicle cells. How this stage-specificity is achieved and the mechanism of ecdysteroid action in the ovary remains to be explored. It is known that the *Drosophila* ring gland produces relatively inactive precursors during larval development that are subsequently converted into 20E by peripheral tissues[9,11]. One interesting possibility is that both synthesis and conversion occur in the nurse cells in the ovary. Alternatively, the active ecdysteroids in the ovary may be different from 20E, or conversion to 20E may occur in the follicle cells or even outside the ovary. This latter model predicts that complex interactions between different cell types might be important for ecdysteroid signaling during oogenesis. Regardless, it is now clear that the ecdysteroid-induced gene cascade is critical for egg chamber progression beyond Stage 8, a control checkpoint known to depend on environmental, nutritional and hormonal cues[1,51]. It remains to be seen if these various factors converge or act in parallel to regulate proper progression through mid-oogenesis.

• Conclusions and Perspectives

The discovery and genetic characterization of *dare* provides a new opportunity to define the biosynthetic pathways that control ecdysteroid production. Moreover, the observation of both neurodegeneration and egg chamber degeneration in *dare* mutants suggests that ecdysteroid biosynthesis is essential for the development of these cell types. These observations raise the interesting possibility that *dare* may direct the production of local sources of ecdysteroids in the tissues outside the ring gland. Recent studies on the stage-specific expression and critical role for *dare* in oogenesis, as well as the participation of the ecdysteroid regulatory hierarchy in this pathway, also provide a new genetic system for understanding how steroids control female reproduction.

While extensive studies of vertebrate nuclear receptors have demonstrated roles for both cofactors and chaperones in modifying receptor activity, recent work in *Drosophila* has stressed the functional importance of these interactions. The essential role of chaperone heterocomplex in activation of the EcR/USP heterodimer indicates that, like classical steroid receptors, RXR heterodimers can undergo conformational changes to acquire their activity. Furthermore, the discovery of SMRTER and Alien indicate that, like its vertebrate counterparts, the *Drosophila* ecdysteroid receptor uses cofactors to modulate its activity. *Drosophila* USP can also mediate critical repressive functions in the absence of ligands, implicating an essential developmental role for these cofactors. It will be interesting to see what emerges from genetic characterization of these co-repressors, as well as how the discovery of other cofactors enhances our understanding of insect nuclear receptor function. In this regard, it is important to realize that *Drosophila* stands at the brink of the genomic era. The completion of the *Drosophila* genome sequence[55] holds the promise of providing access to all members of the *Drosophila* nuclear receptor superfamily, as well as an opportunity to identify more homologs of critical vertebrate regulators of hormone signaling. Combined with microarray analysis[16] and efforts to generate mutations in all protein coding regions of the genome[56], it seems probable that *Drosophila* will continue to provide new directions in our understanding of the molecular mechanisms of hormone action.

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