Journal of Steroid Biochemistry & Molecular Biology 127 (2011) 196–203

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Ecdysteroid metabolism in crustaceans☆

Donald L. Mykles*

Department of Biology, Colorado State University, Campus 1878, Fort Collins, CO 80523, USA

ARTICLE INFO

Article history:
Received 27 July 2010
Received in revised form 1 September 2010
Accepted 2 September 2010

Keywords:
Crustacean
Molting
Ecdysteroid biosynthesis
Ecdysteroid metabolism
Ecdysteroid excretion
Halloween genes
Review

ABSTRACT

The molting gland, or Y-organ (YO), is the primary site for ecdysteroid synthesis in decapod crustaceans. Ecdysteroid biosynthesis is divided into two stages: (1) conversion of cholesterol to 5β-diketol and (2) conversion of 5β-diketol to secreted products. Stage 1 involves the conversion of cholesterol to 7-dehydrocholesterol (7DC) by 7,8-dehydrogenase, the "Black Box" reactions involving 3-oxo-Δ^4 intermediates, and the conversion of Δ^4-diketol to 5β-diketol by 5β[H]-reductase. The stage 2 reactions generate four major products, depending on species: ecdysone, 3-dehydroecdysone (3DE), 25-deoxyecdysone (25dE), and 3-dehydro-25-deoxyecdysone (3D25dE). Peripheral tissues convert these compounds to the active hormones 20-hydroxyecdysone (20E) and ponasterone A (25-deoxy-20-hydroxyecdysone or 25d20E). The hydroxylations at C25, C22, C2, and C20 are catalyzed by cytochrome P-450 mono-oxygenases, which are encoded by the Halloween genes *Phantom*, *Disembodied*, *Shadow*, and *Shade*, respectively, in insects. Orthologs of these genes are present in the *Daphnia* genome and a cDNA encoding Phantom has been cloned from prawn. Inactivation involves conversion of ecdysteroids to polar metabolites and/or conjugates, which are eliminated in the urine and feces. The antennal gland is the major route for excretion of ecdysteroids synthesized by the YO. The hepatopancreas eliminates ingested ecdysteroids by forming apolar conjugates. The concentrations of ecdysteroids vary over the molt cycle and are determined by the combined effects biosynthesis, metabolism, and excretion.

Contents

1. Introduction

Ecdysteroids are polyhydroxylated C27 steroids that control molting in arthropods. As the research on crustaceans has lagged behind that on insects, it comes as no surprise that what we have learned from insects has provided insights and guided the work on crustaceans. This review is restricted to ecdysteroid biosynthesis in the crustacean molting gland, or Y-organ (YO), and ecdysteroid metabolism and excretion by peripheral tissues. It emphasizes research published over the last 20 years. The reader is referred to [1–7] for comprehensive reviews of the earlier work. As most of the work has focused on decapod crustaceans, little is known about ecdysteroid biosynthetic pathways in other crustacean groups. Data from non-decapod crustaceans are included where appropriate.

Due to space limitations, related topics are not covered. The reader is referred to reviews on the biochemistry of vertebrate-type steroids [8–10], endocrine disruptors [11–13], and the role of ecdysteroids on reproduction and larval development in crustaceans [9,10,12,14]. Moreover, recent reviews provide detailed treatment on the regulation of YO ecdysteroidogenesis by eyestalk neuropeptides [15–18].

☆ Article submitted for the special issue on Marine organisms.

* Tel.: +1 970 491 7616; fax: +1 970 491 0649.
E-mail address: don@lamar.colostate.edu.

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doi:10.1016/j.jsbmb.2010.09.001

2. Ecdysteroid synthesis by the Y-organ

Cholesterol is the precursor for ecdysteroid synthesis in the YO. The YOs are located in the cephalothorax, anterior to the branchial chamber. They are epithelioid tissues with cells containing numerous mitochondria and extensive endoplasmic reticulum, which is characteristic of steroidogenic tissues of vertebrates [1,2,19]. Arthropods cannot synthesize cholesterol *de novo*, and therefore acquire it from their diet [3,20,21]. Cholesterol binds to high-density lipoproteins in the hemolymph and is taken up by the YO [20–26]. The uptake of cholesterol is an important rate-limiting step in the synthesis of ecdysteroids and is regulated by eyestalk neuropeptides, such as molt-inhibiting hormone (MIH) [21–23,27,28].

Ecdysteroid biosynthesis in the molting glands of insects and crustaceans is similar, but the crustacean YO synthesizes a greater diversity of ecdysteroids [1,3,4,29,30]. The biosynthetic pathway in the crustacean YO is complex. For convenience, ecdysteroid biosynthesis can be divided into two stages: (1) conversion of cholesterol to 5β-diketol (Fig. 1) and (2) conversion of 5β-diketol to secreted products (Fig. 2). The first stage of ecdysteroid synthesis is common to all decapod species. The second stage has four pathways with branching points at 5β-diketol and 5β-ketodiol [1,31,32]. These pathways are named for the final ecdysteroid product secreted by the YO: ecdysone, 3-dehydroecdysone (3DE), 25-deoxyecdysone (25dE), and 3-dehydro-25-deoxyecdysone (3D25dE). YOs usually secrete two products, indicating that only two of the potential four pathways are operational at any given time in one species. This appears to be due to the relative activities of enzymes at the branch points, which directs metabolites along particular pathways.

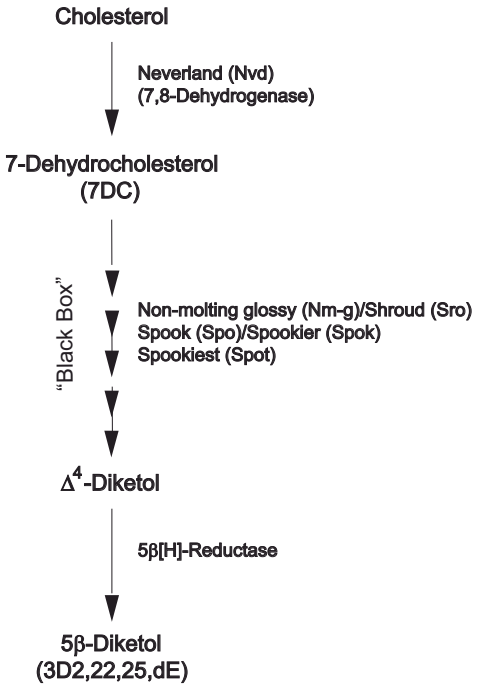

Fig. 1. Stage 1 of the ecdysteroid biosynthetic pathway in crustacean molting gland (Y-organ). In the first reaction, cholesterol is converted to 7-dehydrocholesterol (7DC) by 7,8-dehydrogenase, encoded by the *Neverland* (*nvd*) gene in insects. In a series of reactions involving enzymes encoded by *non-molting glossy*/*shroud*, *Spook*/*Spookier*, and *Spookiest*, termed the "Black Box", 7DC is converted to Δ^4-Diketol, which is converted to 5β-diketol by 5β[H]-reductase.
Modified from [1,29,31].

Much of what we know about the biosynthetic pathway in the YO comes from metabolic studies using radiolabelled precursors. The first step is the conversion of cholesterol to 7-dehydrocholesterol (7DC) by 7,8-dehydrogenase; the two H(β) at carbons #7 and #8 are removed, resulting in the formation of a double bond between those carbons (Fig. 1). Studies on a variety of decapod crustacean species have shown that radiolabelled cholesterol injected into animals is converted to ecdysone, 3DE, 20-hydroxyecdysone (20E), 25dE, 3-dehydro-20-hydroxyecdysone (3D20E), and/or ponasterone A (PA; 25-deoxy-20E or 25d20E) [21,26,33–35]. A [^{3}H]3β-hydroxy-Δ^4 compound is poorly metabolized by YO cells, indicating that the 3-oxo group is required for efficient 5β-reduction [36]. [^{3}H]25-hydroxycholesterol (25C) is converted to 7-dehydro-25C by the YOs of green crab (*Carcinus maenas*), shrimp (*Penaeus vannamei*), and crayfish (*Orconectes limosus*); the 7-dehydro-25C is converted to 3DE in *C. maenas* or to ecdysone and 3DE in *P. vannamei* and *O. limosus* [37]. The YOs from *Cancer antennarius* can also synthesize 3DE from 25C [38]. Although it is not a normal intermediate in the pathway, these results show that 25C can be a substrate for 7,8-dehydrogenase. The uptake of 25C is much faster than cholesterol, which makes it a useful tool for the study of the regulation of ecdysteroidogenesis [37,38].

The 7DC undergoes a series of reactions involving 3-oxo-Δ^4 intermediates [36], the so-called "Black Box", the details of which are becoming clearer [39]. YO cells from *C. maenas* convert a 25-deoxy-3-oxo-Δ^4 intermediate to 3DE and 25dE; no ecdysone was detected [36]. However, YO cells convert a 3-oxo-Δ^4 intermediate hydroxylated at C25 to 3DE and ecdysone [36]. This is consistent with studies showing that these ecdysteroids are synthesized from 25C as the precursor [32,37,38].

The part of the pathway after the "Black Box" reactions is better understood, particularly the hydroxylations at C2, C22, and C25, which increase the polarity of the secreted products. Δ^4-Diketol is first reduced at C5 by 5β[H]-reductase to form 5β-diketol (Fig. 2; 3-dehydro-2,22,25-deoxyecdysone or 3D2,22,25dE). 5β-Diketol is converted to 5β-ketodiol (2,22,25-deoxyecdysone or 2,22,25dE) by 3-dehydroecdysteroid-3β-reductase (Fig. 2) [31]. In *C. maenas*, [^{3}H]5β-diketol is ultimately converted to 3DE and 25dE by *C. maenas* YO cells [36]. [^{3}H]5β-ketodiol is hydroxylated at C2, C22, or C25 in *C. maenas* YOs to produce 22,25-deoxyecdysone (22,25dE), 2,25-deoxyecdysone (2,25dE), or 2,22-dideoxyecdysone (2,22dE), respectively [40–42]. 2,22dE and 2,25dE are converted to ecdysone and 25dE, respectively, and secreted by the YO [40,41]. 22,25dE is not processed further and is not secreted [40,41]. In the YOs of crayfish, [^{3}H]5β-diketol is converted to ecdysone and 3DE, whereas [^{3}H]5β-ketodiol is converted to ecdysone [31].

The major secreted products of the YO are ecdysone, 3DE, 3D25dE, and 25dE, but the relative amounts vary among species. Four patterns are observed in decapod crustaceans (Table 1). The

Table 1
Major ecdysteroid products secreted by the Y-organs of decapod crustaceans. The production of 25dE in *U. pugilator* is suggested by high hemolymph titers of ponasterone A in premolt animals [30,49].

Species	E[a]	3DE[a]	25dE[a]	3D25dE[a]
Pachygrapsus crassipes	•			
Cancer antennarius	•	•		
Orconectes limosus	•	•		
Procambarus clarkii	•	•		
Penaeus vannamei	•	•		
Macrobrachium rosenbergii	•	•		
Carcinus maenas	•		•	
Callinectes sapidus	•		•	
Uca pugilator	•		•	
Menippe mercenaria		•		•

[a] *Abbreviations*: E, ecdysone; 3DE, 3-dehydroecdysone; 25dE, 25-deoxyecdysone; 3D25dE, 3-dehydro-25-deoxyecdysone.

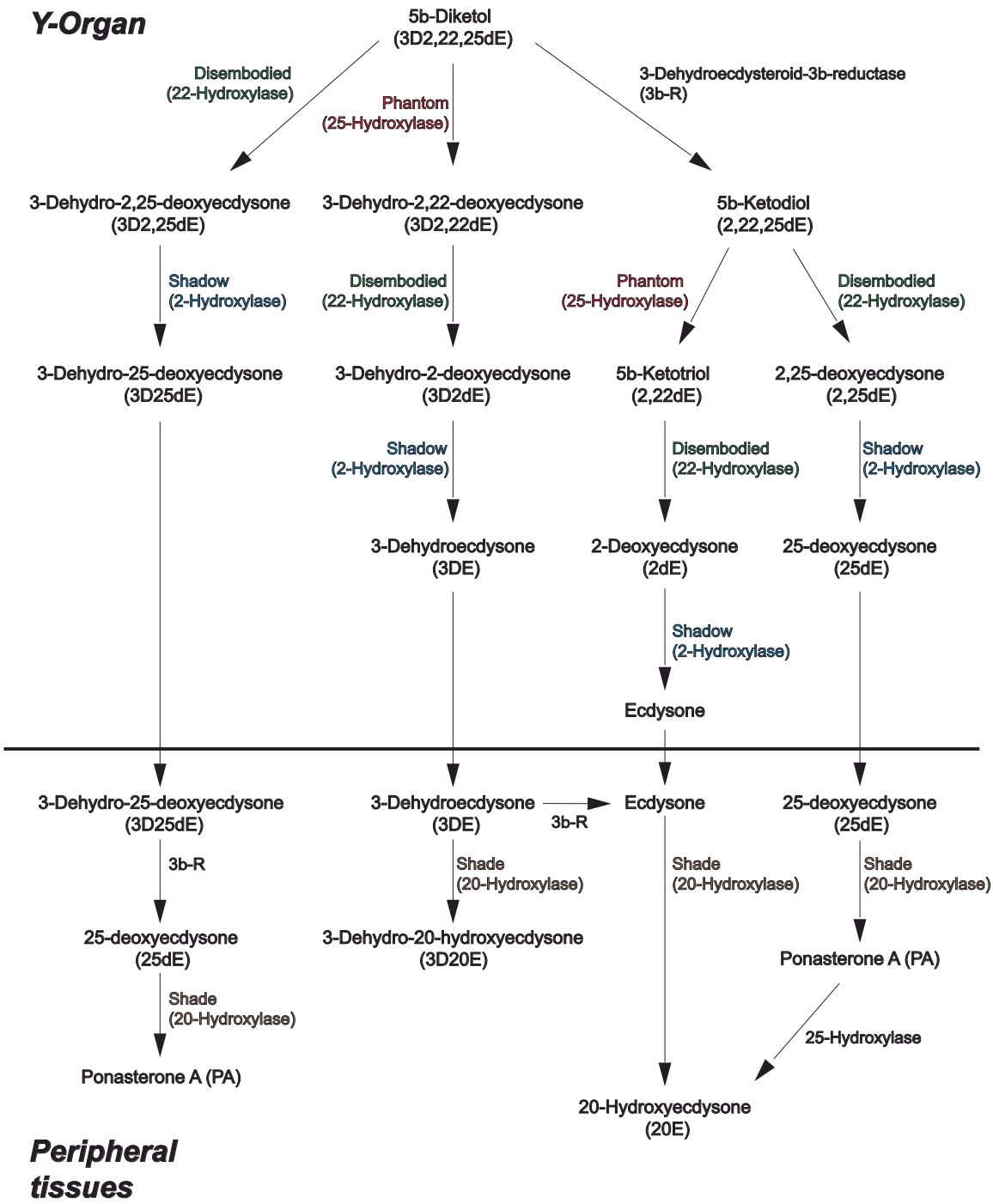

Fig. 2. Stage 2 of the ecdysteroid biosynthetic pathway in crustacean molting gland (Y-organ). Data indicate that there are two branching points, resulting in four pathways and four products secreted from the Y-organ. The products vary with species and molt stage, but no more than two major products are usually secreted at one time. 3β-Reductase and 20-hydroxylase activities in peripheral tissues convert the secreted products to the compounds circulating in the hemolymph. Therefore, the ecdysteroids in the hemolymph are determined by the compounds secreted by the YO and the activities of 3β-reductase and 20-hydroxylase. All the compounds metabolized by peripheral tissues are detected in hemolymph samples; the major ecdysteroids in hemolymph are 20E, PA, 3D20E, and 25d20E. In *M. mercenaria*, a potential metabolite, 3-dehydro-25-deoxy-20-hydroxyecdysone (3D25d20E), resulting from the 20-hydroxylation of 3D25dE has not been identified [32]. The terminal hydroxylations at carbons #25, #22, #2, and #20 are catalyzed by cytochrome P450 mono-oxygenases encoded by *Phantom* (*phm*), *Disembodied* (*dib*), *Shadow* (*sad*), and *Shade* (*shd*), respectively. The specificities of these enzymes are such that the hydroxylations occur in the same order: C25 → C22 → C2 → C20. Hence, hydroxylation at a specific carbon prevents hydroxylation by enzymes "upstream" of that step. For example, hydroxylation at C2 prevents hydroxylations at C25 and C22.
Modified from [1,29,31,32].

YOs in crayfish (*O. limosus* and *Procambarus clarkii*), shrimp (*P. vannamei* and *Macrobrachium rosenbergii*), and rock crab (*C. antennarius*) secrete ecdysone and 3DE [25,31,43–46], the YOs in green crab (*C. maenas*) and blue crab (*Callinectes sapidus*) secrete ecdysone and 25dE [34,41] (unpublished data on *C. sapidus* cited in [32]), the YOs in stone crab (*Menippe mercenaria*) secrete 3DE and 3D25dE [27,32], and the YOs in lined shore crab (*Pachygrapsus crassipes*) and fiddler crab (*Uca pugilator*) secrete ecdysone [47,48]. However, the high levels of PA in the hemoymph of premolt *U. pugilator* indicate that the YO of this species also secretes 25dE in addition to ecdysone

[49]. This suggests that the biosynthetic pathway has branching points at 5β-diketol and 5β-ketodiol, leading to four final ecdysteroid products (Fig. 2). At the first branching point 5β-diketol can be converted to 5β-ketodiol, 3-dehydro-2,22-deoxyecdysone (3D2,22dE), or 3-dehydro-2,25-deoxyecdysone (3D2,25dE) (Fig. 2). At the second branching point 5β-ketodiol can be converted to either 5β-ketotriol or 2,25-deoxyecdysone (2,25dE), which leads to the production of ecdysone or 25dE, respectively (Fig. 2). In the pathway leading to ecdysone, 5β-ketodiol is hydroxylated sequentially at C25, C22, and C2 [1]. At the first branching point, 5β-diketol is hydroxylated at C25 to 3D2,22dE; subsequent serial hydroxylations of 3D2,22dE at C22 and C2 produce 3DE [31]. [^{3}H]-3D2,22dE is converted to 3DE and ecdysone by *C. maenas* YO cells [36]. In *M. mercenaria*, 5β-diketol is apparently hydroxylated at C22 to 3D2,25dE; subsequent hydroxylation at C2 produces 3D25dE [32]. At the second branching point, 5β-ketodiol is hydroxylated at C22 to 2,25-deoxyecdysone (2,25dE); subsequent hydroxylation at C2 produces 25dE.

The branching points in the YO ecdsteroid synthetic pathway suggest that the P450 mono-oxygenases, encoded by the Halloween genes *Phantom* (*phm*), *Disembodied* (*dib*), *Shadow* (*sad*), and *Shade* (*shd*) can bind multiple substrates. Phm apparently can hydroxylate 5β-diketol or 5β-ketodiol at C25; Dib apparently can hydroxylate 5β-diketol, 3D2,22dE, 5β-ketodiol, or 5β-ketotriol at C22; Sad apparently can hydroxylate 3D2dE, 2,25dE, or 2dE at C2; and Shd apparently can hydroxylate 25dE, 3DE, or ecdysone at C20 (Fig. 2). However, the specificities of the Phm, Dib, Sad, and Shd enzymes are such that the C25 → C22 → C2 → C20 order of hydroxylations is maintained (Fig. 2) [3,29]. For example, compounds hydroxylated at C22 (2,25dE and 25dE) cannot be hydroxylated at C25 in the YO from *C. maenas* [40]. Hydroxylation at C2 (22,25dE) prevents hydroxylations at C22 and C25 [1,40].

Ecdysteroid biosynthetic reactions are catalyzed by reductases and P450 mono-oxygenases (CYPs). The reduction of cholesterol to 7DC (Fig. 1) is catalyzed by a 7,8-dehydrogenase, which is encoded by the *Neverland* (*nvd*) gene in insects and nematode [50]. The Nvd protein has two conserved domains involved in catalysis: a Rieske-like domain containing a 2Fe-2S binding motif and a C-terminal domain containing a non-heme Fe(II)-binding motif [50]. Halloween genes constitute a family of P450 mono-oxygenases, which have been well characterized in insects [29]. They are characterized by having five conserved structural motifs (Helix-C or WxxxR sequence, Helix-I or GxE/DDT/S sequence, Helix-K or ExxR sequence, PERF motif or PxxFxPE/DRF sequence, and heme-binding or PFxxGxRxCxG/A sequence), as well as six substrate recognition sites (SRS 1, 2/3, 4, 5, and 6)[51]. The "Black Box" reactions are catalyzed by *non-molting glossy* (*nm-g*)/*shroud* (*sro*) and several Halloween genes: *spook* (*spo*) and its paralog *spookier* (*spok*); and *spookiest* (*spot*) [51,52]. Spo and Phm belong to the CYP2 clade of P450 enzymes and are associated with the endoplasmic reticulum (ER), whereas Dib, Sad, and Shd are associated with the inner membrane of the mitochondrion [29,53–55]. *Nm-g*/*sro* encodes a short-chain NAD(P)H dehydrogenase/reductase, which appears to act upstream of Spo [52]. Nm-g/sro has an N-terminal NADP/NAD binding domain and a central catalytic domain and is expressed primarily in prothoracic gland and ovary [52].

Crustaceans appear to have the same enzymes for ecdysteroid biosynthesis as insects. An inhibitor of steroid 5α-reductase, L-645390, blocks the conversion of cholesterol to 7DC in the YO of *M. mercenaria* [22]. The 5β-reductase that converts Δ^4-diketol to 5β-diketol is a cytosolic enzyme in YO cells that requires NADPH for activity [36]. Orthologs of *nvd*, *nm-g*/*sro*, *spo*, *phm*, *dib*, *sad*, and *shd* have been identified in the *Daphnia pulex* genome [52,54,56], and a cDNA encoding *Phm* has been cloned from Kuruma prawn, *Marsupenaeus japonicus* [57]. *Nvd* and *spo* are located adjacent to each other in the *D. pulex* genome [54]. The *M. japonicus* Phm and *D. pulex* Phm have five conserved motifs present in insect Phm (WxxxR, GxE/DTT/S, ExxR, PxxFxPE/DRF, and PFxxGxRxCxG/A heme-binding motif), five of six substrate recognition sites (SRS 1, 2/3, 4, and 5), and N-terminal ER-targeting and Pro/Gly-rich sequences [57]. *Mj-Phm* is a target of eyestalk neuropeptides, as its expression in the YO is increased as much as 7-fold during premolt and is decreased about 2.5-fold by sinus gland extract and recombinant MIH [57]. None of the crustacean Halloween enzymes have been characterized biochemically.

A member of the clade 4 of cytochrome P450 enzymes has been cloned from *O. limosus* (CYP4C15) and *C. maenas* (CYP4C39) [58,59]. Both genes encode proteins with hydrophobic N-terminal ER-targeting and Pro/Gly-rich sequences characteristic of microsomal cytochrome P450 enzymes [58,59]. CYP4C15 is expressed in the YO and the protein is associated with the ER [58,60]. Moreover, CYP4C15 expression in the YO is increased during premolt and is decreased by MIH [58,60]. These data suggest that they are involved in ecdysteroid biosynthesis, but their precise role remains to be determined. It has been hypothesized that the gene catalyzes 7,8-dehydrogenation or 25-hydroxylation [58,60]. However, CYP4C15 and CYP4C39 have low sequence identity/similarity to *nvd* and *phm*, making it unlikely that these genes are involved in 7,8-dehydrogenation and 25-hydroxylation, respectively, in the YO. CYP4C39 is expressed in the hepatopancreas, which suggests it may have a role in ecdysteroid metabolism by peripheral tissues [59].

Ecdysteroids secreted by the YO are converted to 20-hydroxyecdysone (20E) and related compounds by peripheral tissues, such as gonad, hindgut, abdominal ganglia, eyestalk ganglia, hepatopancreas, antennal gland, and epidermis (Fig. 2) [38,61–66]. YOs cannot convert 3DE to ecdysone or ecdysone to 20E [31,43,61]. In peripheral tissues, ecdysone is hydroxylated at C20, probably by Shd, to produce 20E [38,40,41,62–64]. A 20-hydroxylase activity in the hepatopancreas of the blackback land crab (*Gecarcinus lateralis*) requires NADPH, is inhibited by metyrapone (an inhibitor of cytochrome P450 enzymes), and is associated with the mitochondrial fraction [62]. A 3β-reductase converts 3DE to ecdysone, which is hydroxylated at C20, to produce 20E [32,38,45,61,63]. In *M. mercenaria*, 3β-reductase in peripheral tissues converts 3D25dE to 25dE, which is then converted to PA [32]. In *C. maenas*, 25dE is converted to PA by a 20-hydroxylase in the testis [34,40,41]. PA can be hydroxylated at C25 or C26 to produce 20E or inokosterone, respectively [67]. In crayfish (*O. limosus*), abdominal ganglia convert 3DE to ecdysone, 20E, and 3D20E [63]. Antennal glands of crayfish express an aldoketoreductase, but its precise function is unknown; it may catalyze 3β-reduction or may have an osmoregulatory role by converting D-glucose to sorbitol [68]. These data indicate that peripheral tissues have 3β-reductase, 25-hydroxylase, and 20-hydroxylase activities, which can convert products secreted by the YO to 20E and related compounds.

3. Hemolymph ecdysteroids over the molt cycle

Total ecdysteroid titers in the hemolymph vary over the molt cycle. In general, ecdysteroids are low during intermolt and postmolt; during premolt, concentrations rise and reach a peak shortly before molting (ecdysis) in a variety of crustacean species [2,5–7,46,62,69–89]. A precipitous drop in hemolymph ecdysteroids triggers the shedding of the exoskeleton [90]. Eyestalk ablation, which removes the primary source of inhibitory neuropeptides (e.g., MIH), activates the YO and increases hemolymph ecdysteroid levels [1,2,21,23,70,79,86,91,92]. In addition to the peak in hemolymph titers at the end of the premolt stage, there are smaller peaks during postmolt and early premolt stages, which may have physiological functions [30,48,93–95]. For species with terminal molts, the YOs degenerate and ecdysteroids remain at

low concentrations in the hemolymph, even after eyestalk ablation [2,96].

The major ecdysteroids identified in the hemolymph of decapod crustaceans are ecdysone, 20E, 3D20E, and PA, but the specific compounds and their levels vary between species, developmental stage, molt stage, and season [32,44,48,49,61,64,66,67,70,78,81,82,87,93,97]. In stone crabs (*M. mercenaria*) that have been eyestalk ablated to activate the YO, 20E, 3D20E, and PA are present in about equal amounts; a fourth large peak eluting from a reverse-phase column was not identified [32]. In fiddler crab (*U. pugilator*) induced to molt by eyestalk ablation, there are two transitory peaks of hemolymph ecdysteroid that differ in the relative titers of ecdysteroids. Peak I, which occurs at early premolt, has a higher ratio of ecdysone to 20E than peak II, which occurs at late premolt [48]. Eyestalk ablation alters the composition of ecdysteroids circulating in the hemolymph, but the effect differs between species. In blue crabs (*C. sapidus*) at late premolt, PA is the major ecdysteroid in intact animals, whereas 20E is the major ecdysteroid in eyestalk-ablated animals [70]. In *U. pugilator*, the PA:20E ratio in the hemolymph is higher in animals induced to molt by eyestalk ablation than in animals induced to molt by multiple leg autotomy [49]. In Kuruma prawn (*Penaeus japonicus*), 20E and PA are the major ecdysteroids during the postmolt and intermolt stages, while 20E alone is the major ecdysteroid during early and late premolt stages [87]. In *C. maenas*, ecdysone and 20E are detected at low levels in the hemolymph of intermolt animals, whereas the PA level is higher than the 20E level in the hemolymph of premolt animals [34,67,78]. At late premolt (stage D_3), the titers of PA in the hemolymph decline more rapidly than those of ecdysone and 20E [78]. This corresponds to a change in the relative amounts of ecdysone and 25dE, a precursor to PA, secreted by the YO between intermolt and premolt animals; YOs from premolt animals secrete greater amounts of 25dE than ecdysone than YOs from intermolt animals [40,41]. This appears to depend on the activities of enzymes at the branching points (Fig. 2). For example, in rock crab and crayfish YOs, an increase in 3β-reductase activity would shift production and secretion from 3DE to ecdysone [31]. In green and fiddler crabs, an increase in 25-hydroxylase activity would shift secretion from 25dE to ecdysone [31].

Ecdysteroid composition can be affected by developmental stage. During the last three molts, blue crabs (*C. sapidus*) transition through juvenile, prepubertal, and adult stages. PA is the major hemolymph ecdysteroid at all three stages. However, the titers of PA in juveniles are significantly higher than and the titers of 20E significantly lower than the titers in prepubertal and adult stages [70]. In juvenile *C. sapidus*, eyestalk ablation increases hemolymph ecdysteroid titers, but there is no peak in ecdysteroids prior to ecdysis and titers during postmolt remain elevated compared to intact animals [70]. These data indicate that there are stage-specific differences in ecdysteroid metabolism, but the functional significance of these changes remains to be investigated.

4. Ecdysteroid metabolism and excretion

20E and PA are the major active ecdysteroids circulating in the hemolymph of decapod crustaceans. Inactivation involves conversion to more polar metabolites and/or formation of conjugates, which are excreted in the urine or eliminated in the feces. Thus, the two major organs responsible for removing ecdysteroids from the hemolymph are the antennal gland and hepatopancreas (midgut gland) in decapods. The diversity of inactivation pathways in crustaceans is reminiscent to those used by insects to detoxify ecdysteroids ingested from plants (see [98] for review). The primary site for excretion of ecdysteroids is the antennal gland. In lobster (*Homarus americanus*), between 81% and 96% of excreted ecdysteroids is in the urine, depending on the molt stage; the remainder is in the feces [66,97]. In crayfish (*O. limosus*), there is a preferential excretion of ecdysone over 20E. About two-thirds of the [^{3}H]ecdysone injected into the hemolymph appears in the water after 1 h [99]. In contrast, most of the [^{3}H]20E injected into the hemolymph remains in the hemolymph, with less than 5% of the [^{3}H]20E appearing in the water after 1 h [99]. This suggests that selective excretion of unmetabolized ecdysone by the antennal gland keeps hemolymph ecdysone titers low.

Conversion of ecdysteroids to more polar compounds involves two modifications of the side chain at the #26 carbon: hydroxylation and oxidation. In insects, 20E is inactivated by a cytochrome P450 26-hydroxylase [100,101]. In decapod crustaceans, a 26-hydroxylase activity in various tissues converts 20E to 20,26-dihydroxyecdysone (20,26E) and PA to inokosterone (25-deoxy-20,26-dihydroxyecdysone or 25d20,26E) [63,65–67,97]. Oxidation at the C26 forms ecdysonoic acids (EAs). For example, 20E and PA are metabolized to 20-hydroxyecdysonoic acid (20EA) and 25-deoxy-20-hydroxyecdysonoic acid (25d20EA), respectively, and are excreted in the urine [65–67,97,102]. Ecdysteroids can also be converted to their 3α-hydroxy epimers by peripheral tissues. In crayfish (*P. clarkii*), a 3α-reductase activity in the epidermis converts ecdysone and 20E to 3α-hydroxyecdysone and 3α,20-hydroxyecdysone, respectively, which are selectively excreted [61].

Various tissues can convert ecdysteroids and their metabolites to highly polar (HP) conjugates, which can be excreted in the urine [61,63,65,66,97]. The levels of HP conjugates vary over the molt cycle and can at times be the predominant ecdysteroid in the hemolymph [61,77,82,87,88,93]. In *H. americanus*, the rapid drop in ecdysteroids at the end of premolt is associated with an increase in HP metabolites in the hemolymph and excretion in the urine [93,97]. Using [^{3}H]ecdysone as a tracer, a greater proportion (about 75% after 72 h) is converted into HP conjugates in postmolt (stage A) and intermolt (stage C) lobsters than is converted in early premolt (stages D_0 and D_1) lobsters (about 25% after 72 h) [66]. The conversion by late premolt (stage D_2) lobsters increases after injection and reaches early premolt levels by 96 h as animals transition through the late premolt ecdysteroid peak [66]. In *C. maenas*, intermolt and premolt animals differ in the metabolism of [^{3}H]PA injected into the hemolymph. Forty-eight hours after injection into intermolt animals, the major metabolites were conjugates and 20EA, whereas 24 h after injection into premolt animals, the major metabolites were conjugates only [67]. Enzymatic hydrolysis with snail sulfatase or porcine liver esterase is used to analyze the composition of conjugates [66,93]. HP conjugates in the hemolymph contain various ecdysteroids and metabolites usually in proportion to their hemolymph concentrations [93,97]. This indicates that the modifications are generally nonspecific. However, HP conjugates in the urine contain relatively high proportions of polar metabolites, such as 20,26E, 3α-hydroxy compounds, and ecdysonoic acids [61,66,97]. In addition, excretion rates of *H. americanus* are higher in intermolt and late premolt (D_2) stages than early premolt (D_0 and D_1) stages [66]. These data suggest that the conversion of ecdysteroids to inactive polar metabolites and their preferential excretion by the antennal gland can determine the ecdysteroid titers in the hemolymph, especially at the end of premolt when titers drop precipitously.

The hepatopancreas is the primary site for the production of apolar conjugates (perhaps fatty acyl esters), which are then eliminated in the feces [65,66,99]. These conjugates can be formed from ecdysteroids in the hemolymph [63,65,66,84,97] or from ecdysteroids ingested with food [97]. When lobsters were fed [^{3}H]ecdysone, less than 0.4% of the radioactivity appeared in the hemolymph and urine; essentially all of the [^{3}H]ecdysone was in the form of apolar conjugates in the feces [97]. The amounts of apo-

lar conjugates in the hepatopancreas vary over the molt cycle. In *H. americanus*, apolar conjugates are highest in late premolt (stage D_3) and postmolt (stage A) animals, lower in early premolt (stage D_1), and lowest in intermolt (stage C_4) animals [65]. Although the hepatopancreas can excrete hemolymph ecdysteroids and their metabolites, it appears that it functions primarily in sequestering ecdysteroids obtained from the diet to prevent disruption of ecdysteroid-regulated processes.

5. Conclusions and summary

The concentrations of ecdysteroids circulating in the hemolymph are determined by the balance between production and removal. Production involves the synthesis of ecdysteroids by the YO and their conversion to active ecdysteroids (e.g., 20E and PA) by peripheral tissues. Removal involves conversion to inactive metabolites by peripheral tissues and excretion by the antennal gland. The increase in hemolymph ecdysteroid titers is largely due to increased biosynthesis and conversion to active ecdysteroids. The YOs from premolt animals synthesize and secrete ecdysteroids at increased rates [31,35,44–46,48,61,73,75]. In *H. americanus*, there are molt stage-specific differences in the levels of [^{3}H]20E converted from [^{3}H]ecdysone injected into the hemolymph. The levels of [^{3}H]20E in the hemolymph were higher (about 75% of the total ecdysteroids after 24 h) in early premolt animals than in postmolt and intermolt animals (<25% of the total ecdysteroids after 24 h) [66]. Interestingly, animals in late premolt (stage D_2) show an initial increase in [^{3}H]20E and then a decrease to intermolt levels by 96 h after injection as the animals transition through the late premolt ecdysteroid peak (stage D_3) [66]. An increase in ecdysone metabolism by the antennal gland may contribute to the inactivation and excretion of ecdysteroids in premolt animals [65].

An important question is: Do the levels and composition of circulating ecdysteroids have a function in regulating and coordinating the various physiological processes associated with molting? Other than 20E, very little is known about the biological activities of PA and other ecdysteroids in crustaceans (see [1,2,5–7,30] for reviews). Ecdysteroid action is mediated by the ecdysteroid receptor, which is a heterodimer composed of the products of the *EcR* and *RXR/USP* nuclear hormone genes in arthropods (see [30,103,104] for reviews). cDNAs encoding EcR and RXR have been cloned from several crustacean species [30,105,106]. Alternative mRNA splicing generates several isoforms of RXR, whereas one, or occasionally two, EcR isoforms occur in decapods [30]. These genes are widely expressed, indicating that most tissues can respond to hormone [107–110]. RXR variants differ in the hinge and ligand binding domains and these different isoforms determine the ligand and DNA binding properties of the receptor [103,108–113]. In blackback land crab (*G. lateralis*), differences in the expression of RXR isoforms and EcR in the claw and thoracic muscles may contribute to the differential sensitivity of the two muscles to ecdysteroid-stimulated muscle atrophy [79,108,114]. Molt cycle-dependent changes in EcR and/or RXR expression have been reported in YO, skeletal muscle, limb regenerates, hepatopancreas, epidermis, ovary, and testis [79,107,110,111,114,115]. The data suggest that the response of a tissue to hormone is determined by the composition of circulating ecdysteroids and the ecdysteroid receptor variants expressed in that tissue. Future work should now be directed at achieving a mechanistic understanding by linking *in vitro* analysis of receptor binding properties to the regulation of target genes.

Acknowledgements

The author recognizes the support from the National Science Foundation (IBN-0342982 and IOS-0745224) and thanks Dr. Ernest S. Chang, University of California-Davis Bodega Marine Lab, for helpful comments on the manuscript.

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