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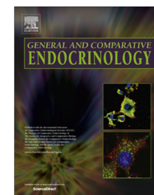

Identification of putative ecdysteroid and juvenile hormone pathway genes in the shrimp *Neocaridina denticulata*

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ABSTRACT

Although the sesquiterpenoid juvenile hormone (JH) and the steroidal ecdysteroids are of vital importance to the development and reproduction of insects, our understanding of the evolution of these crucial hormonal regulators in other arthropods is limited. To better understand arthropod hormone evolution and regulation, here we describe the hormonal pathway genes (e.g. those involved in hormone biosynthesis, degradation, regulation and signal transduction) of a new decapod model, the shrimp *Neocaridina denticulata*. The majority of known insect sesquiterpenoid and ecdysteroid pathway genes and their regulators are contained in the *N. denticulata* genome. In the sesquiterpenoid pathway, these include biosynthetic pathway components: juvenile hormone acid methyltransferase (JHAMT); hormone binding protein: juvenile hormone binding protein (JHBP); and degradation pathway components: juvenile hormone esterase (JHE), juvenile hormone esterase binding protein (JHEBP) and juvenile hormone epoxide hydrolase (JHEH), with the JHBP, JHEBP and JHEH genes being discovered in a crustacean for the first time here. Ecdysteroid biosynthetic pathway genes identified include *spook*, *phantom*, *disembodied*, *shadow* and *CYP18*. Potential hormonal regulators and signal transducers such as allatostatins (ASTs), Methoprene-tolerant (Met), Retinoid X receptor (RXR), Ecdysone receptor (EcR), calponin-like protein Chd64, FK509-binding protein (FKBP39), Broad-complex (Br-c), and crustacean hyperglycemic hormone/molt-inhibiting hormone/gonad-inhibiting hormone (CHH/MIH/GIH) genes are all present in the shrimp *N. denticulata*. To our knowledge, this is the first report of these hormonal pathways and their regulatory genes together in a single decapod, providing a vital resource for further research into development, reproduction, endocrinology and evolution of crustaceans, and arthropods in general.

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1. Introduction

Recent investigations into arthropod phylogeny have nested the Insecta within the Crustacea, forming a clade known as the Pancrustacea (Glenner et al., 2006; Regier et al., 2005). This has led to renewed interest in the ancestral state of a range of characters previously regarded as insect-specific, whose origin may in fact lie within crustaceans. One prominent example of such an under-investigated trait is the endocrine system that controls molting and reproduction, which is regulated primarily by sesquiterpene hormones and ecdysteroids.

Sesquiterpene hormone production in crustaceans and insects shares a bilaterian-conserved mevalonate biosynthetic pathway (Kenny et al., 2013; Tobe and Bendena, 1999). However, different final products are generated in different animal groups; for example, juvenile hormone (JH) is produced in the corpora allata (CA) of insects, and methyl farnesoate (MF) and farnesoic acid (FA) in the mandibular organ of crustaceans (Fig. 1) (Laufer and Biggers, 2001). Both hormones are believed to serve similar functions in development, growth, molting and reproduction (Homola and Chang, 1997b). As yet, JH has not been conclusively identified in

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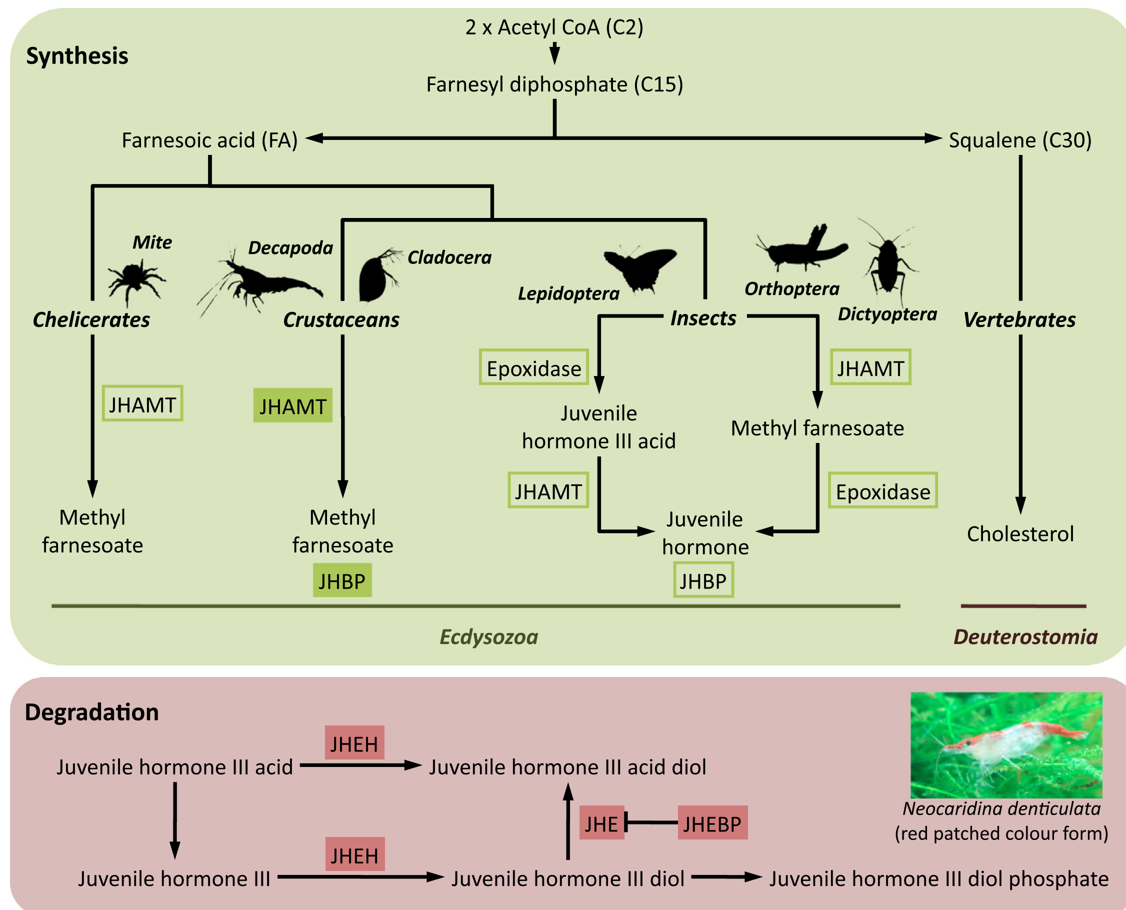

Fig. 1. Juvenile hormone pathway overview (biosynthesis and degradation). Comparative endocrinology of the mevalonate pathway in the Bilateria modified from Bellés et al. (2005), Hui et al. (2013), and Tobe and Bendena (1999). Genes identified in *Neocaridina denticulata* are shown in boxes with darker coloration (i.e. JHAMT, JHBP, JHEH, JHE and JHEBP). Note that the crustacean water flea *Daphnia pulex* (Hui et al., 2010), shrimp *Neocaridina denticulata* (this study), and chelicerate spider mite *Tetranychus urticae* (Grbić et al., 2011) contain JHAMT proteins in their genomes, but may not produce JH. For details, please refer to text.

crustaceans, and MF is generally thought to be the crustacean equivalent of JH, with JH historically considered an evolutionary derivative of MF unique to insects. However, in the early embryonic life of *Diploptera punctata*, more MF is biosynthesized and released than JH (Stay et al., 2002). The ability of the embryonic CA to convert MF to JH is acquired gradually in approximately 18–30 day old embryos. MF production in insects by the early embryonic CA suggests that MF as the final product of the sesquiterpenoid pathway could represent an ancestrally shared trait in arthropods.

In the last decades, one key rate-determining step in the biosynthesis of the juvenoid hormones has been thought to be the final conversion to JH or MF through an *S*-adenosyl-methyltransferase (SAM)-dependent methylation (Tobe and Bendena, 1999; Hui et al., 2010, 2013). In insects, juvenile hormone acid methyltransferase (JHAMT) is responsible for the methylation which converts juvenile hormone acid (JHA) to JH in the Lepidoptera, and farnesoic acid (FA) to MF in other insects (Fig. 1) (Defelipe et al., 2011). However, all insect JHAMTs are able to recognize both FA and JHA as substrates (Defelipe et al., 2011), and thus have the ability to convert FA to MF. The identification of *JHAMT* in the water flea *Daphnia pulex* (Hui et al., 2010) raises many questions as to the role and function of JHAMT in crustacean endocrinology, and the possible wider presence of the JH pathway across the crustaceans and other arthropods (Hui et al., 2010).

In both crustaceans and insects, development and reproduction are regulated by ecdysteroids or molting hormones, which are synthesized from dietary cholesterol by steroidogenic enzymes of the cytochrome P450 (CYP) family (Chávez et al., 2000; Mykles, 2011; Niwa et al., 2004; Petryk et al., 2003; Warren et al., 2002, 2004). The molting hormone 20-hydroxyecdysone (20E) is highly conserved in insects (Fig. 2) (Rewitz et al., 2007). The steroidogenic CYPs are encoded by the Halloween genes *phantom* (*CYP306A1*), *disembodied* (*CYP302A1*), *shadow* (*CYP315A1*) and *shade* (*CYP314A1*), which are collectively responsible for the last four hydroxylations in the pathway leading to 20E. Each of the Halloween enzymes is believed to mediate one specific enzymatic step in the biosynthesis of 20E, as mutations in these genes result in low ecdysteroid levels and embryonic death. Another CYP enzyme, *spook* (*CYP307A1*), is believed to mediate an uncharacterized step in the biosynthesis of 20E, as its mutation will also result in low 20E production (Rewitz et al., 2007). In crustaceans, orthologues of the Halloween genes have only been identified in a branchiopod, the water flea *D. pulex* (Rewitz and Gilbert, 2008), and the biosynthetic pathway of ecdysteroids remains largely undescribed in crustaceans outside the Insecta.

These gaps in our understanding can however now be addressed. Recent advances in genomic research have revolutionized comparative biology and allowed the investigation of trait evolution at the molecular level (Kenny et al., 2013), providing a

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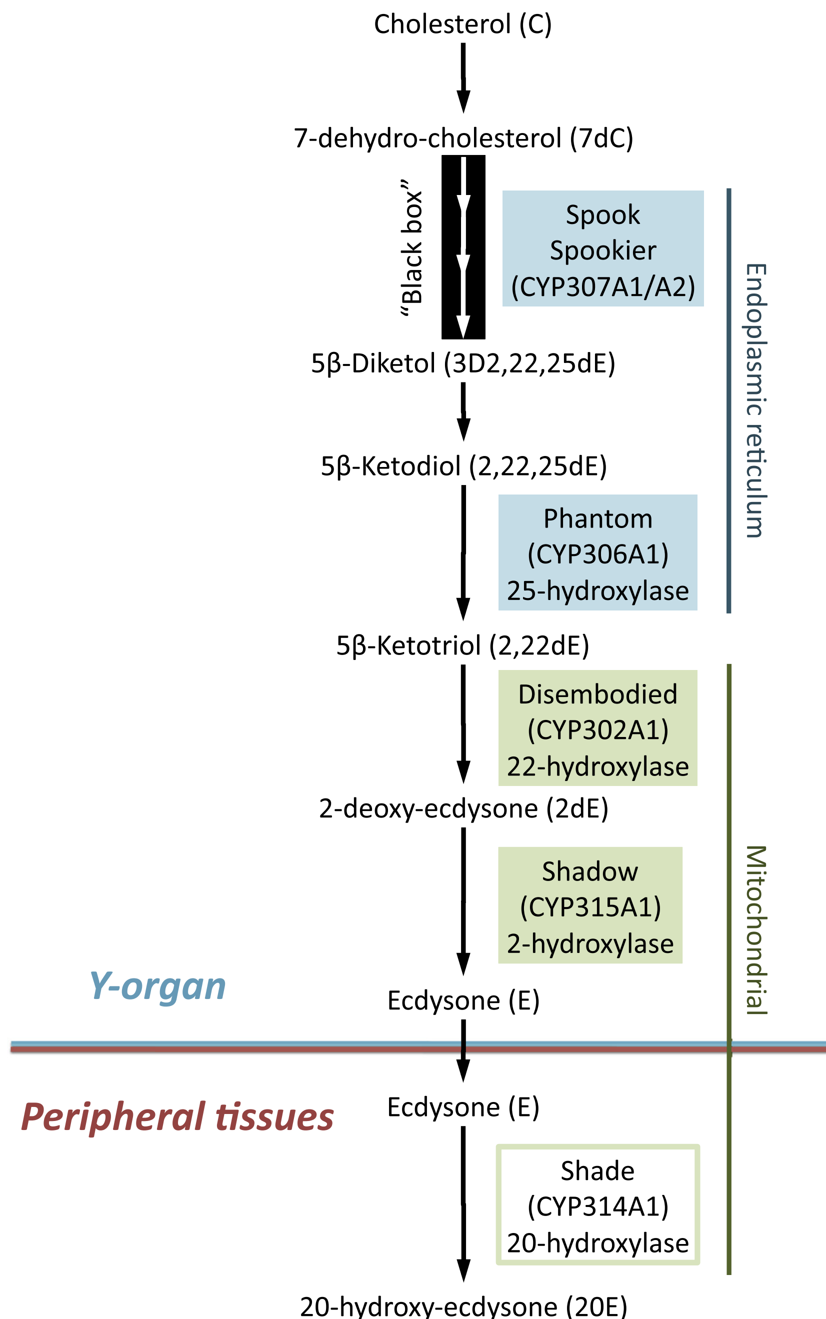

Fig. 2. Ecdysone hormone pathway overview (biosynthesis). The ecdysteroid biosynthetic pathway, showing the steroidogenic CYP enzymes mediating steps in the conversion of cholesterol to ecdysone in the crustacean molting gland (Y-organ), and the conversion of ecdysone to 20-hydroxyecdysone (20E) in the peripheral tissues. The "Black Box" indicates an uncharacterized series of oxidative modifications converting 7-dehydrocholesterol (7dC) to 5β-diketol (3-dehydro-2,22,25-deoxyecdysone or 3D2,22,25dE). The terminal hydroxylations at carbons #25, #22, #2, and #20 are catalyzed by enzymes encoded by *Phantom*, *Disembodied*, *Shadow*, and *Shade*, respectively. Genes identified in *Neocaridina denticulata* are shown in boxes with darker coloration (i.e. *spook*, *phantom*, *disembodied*, and *shadow*). The figure is modified from Rewitz and Gilbert (2008) and Mykles (2011).

new window into the evolution of hormonal pathways regulating arthropod development and reproduction. In this study, we identify genes involved in the biosynthesis and degradation of sesquiterpernoids, and biosynthesis of ecdysteroids in the newly sequenced cherry shrimp *Neocaridina denticulata* (Kenny et al., 2014). This information will provide an understanding of the evolution of hormonal systems across the Pancrustacea and Arthropoda as a whole.

2. Materials and methods

2.1. Gene identification in N. denticulata genome resources

Gene sequences were identified in the *N. denticulata* genome (Kenny et al., 2014) using TBLASTN (Altschul et al., 1990) searches. Gene sequences of known identity acquired from the NCBI non-redundant (nr) database were used as queries to identify

homologous sequences in *N. denticulata*. Putatively identified genes were then reciprocally compared against the NCBI nr database using BLASTX (Altschul et al., 1990) to further confirm their identities. Where identity was uncertain, characteristic domains were used to confirm homology, followed by phylogenetic analysis (see Section 2.2).

2.2. Phylogenetic analysis

Gene sequences were translated into amino acid sequences and aligned to sequences of known homology using MUSCLE (Edgar, 2004). Bayesian inference was performed on the consensus alignments using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The model jumping command in MrBayes was implemented, which selects substitution models in proportion to their posterior probability. A Markov chain Monte Carlo (MCMC) search was initiated with random trees and run for 2,000,000 generations, sampling every 100 generations. Convergence was indicated when the average standard deviation of split frequencies was less than 0.01 (Ronquist et al., 2005). We also checked for convergence by plotting the likelihood scores against generations and discarded the first 25% of the generations as "burn-in". Trees were visualized in FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) for display.

2.3. RNA isolation, RT-PCR, subcloning and sequencing

To check if contigs containing different exons of some genes could be merged, RNA was isolated from several individuals of different life stages using TRIzol reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. cDNA synthesis was performed using a reverse transcription kit (PrimeScript RT Master Mix; Takara Bio Inc, Shiga, Japan) following the manufacturer's protocol.

Primers for PCR amplification of identified target genes were designed based on exonic regions, with sequences as found in Supp. File 3. To amplify transcribed genes from *N. denticulata*, PCR was performed in a 20-μl reaction mix that contained 10–30 ng of cDNA, 0.5 μM of each primer, 200 μM of each dNTP (Takara Bio Inc, Shiga, Japan), 1× PCR buffer, 3 mM $MgCl_2$, and 2 units Taq (Roche, Germany). The PCR cycle began with incubation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, annealing temperature (as seen in Supp. File 3) for 30 s, and 72 °C for 1 min/1000 bp according to amplicon length, ending with an extension step at 72 °C for 10 min. The PCR products of expected size were gel purified and cloned. Sequences of cloned genes were obtained by direct DNA sequencing on bacterial cultures (BGI, Hong Kong), using dideoxynucleotide chain termination and an automated DNA Sequencer (ABI3730, Applied Biosystem, Foster City, CA, USA). Nucleotide sequences were analyzed using CodonCode Aligner 3.7.1 (CodonCode, Dedham, MA, USA) and were compared with known sequences using the NCBI BLASTX searches to confirm their identity. The partial cDNA sequences from *N. denticulata* were assigned GenBank accession numbers: KJ200310-KJ200321, KJ579126-KJ579131 and KJ956470-KJ956473 (Tables 1 and 2).

3. Results and discussion

Evidence presented here for the existence of sesquiterpenoid and ecdysteroid biosynthetic pathway genes in the shrimp *N. denticulata* supports the conjecture that hormonal pathways previously thought to be unique to insects are found more generally throughout the Pancrustacea. Whereas previous genomic-level studies in the Crustacea have been limited to the branchiopod *D. pulex*, the newly sequenced crustacean decapod model *N. denticulata* belongs to the order Decapoda, which includes a variety of economically important species, including crabs, crayfish, lobsters, and shrimps. The identification of these genes in this study thus also provides baseline information for future hormonal and aquacultural research in economically important crustaceans. In the following sections, genes validated by reciprocal blast, subcloning, sequence alignment (Supp. File 1) and Bayesian phylogenetic analyses (Supp. File 2) are shown and discussed, including genes involved in biosynthesis and degradation of JH (Fig. 1 and Table 1), biosynthesis of ecdysteroid (Fig. 2 and Table 2), and hormonal regulation and signal transduction (Fig. 3, Tables 1 and 2).

3.1. Juvenoid hormone biosynthesis

In insects, JHAMT plays a major role in the biosynthesis of JHs (Shinoda and Itoyama, 2003), and an orthologue is found in the *N. denticulata* genome (Supp. File 2, Fig. S25). In insects, the last two steps of JH biosynthesis differ depending on the insect group (Fig. 1). In the Lepidoptera, epoxidation by an epoxidase converts FA to JH III acid and its homologues, which are subsequently methylated by JHAMT, whereas in most other insects epoxidation follows methylation of FA to MF by JHAMT. This methylation is the last reaction of the sesquiterpenoid pathway in crustaceans as MF is the final product in crustaceans, rather than JH as in insects (Defelipe et al., 2011). JHAMT has only been reported in the crustacean water flea *D. pulex* to date (Hui et al., 2010), but the discovery of JHAMT in *N. denticulata* (Supp. File 1 Fig. S1 and Supp. File 2 Fig. S25) suggests that JHAMT is more generally present across crustaceans and may play an important role in the biosynthesis of MF in crustaceans. The presence of JHAMT in the sesquiterpenoid biosynthetic pathway of crustaceans and insects indicates the JH biosynthetic pathway found in insects may have already been present in the Pancrustacean as shown in Fig. 1. In Fig. 4 we show the expression pattern of the *JHAMT* gene in embryonic, juvenile and adult samples, showing at least weak transcription is ubiquitous across the body of this species, albeit very weak in posterior regions of juveniles and adults. This suggests that JHAMT may be active around the body, rather than limited in action to the MO of this species.

Furthermore, an orthologue of *cytosolic juvenile hormone binding protein (JHBP)*, which has been proposed to be the cytoplasmic receptor of JH in insects (Chang et al., 1980), can also be identified in the *N. denticulata* genome (Supp. File 1 Fig. S3). JHBP has a folded structure, consisting of a long α-helix wrapped in a highly curved antiparallel β-sheet, which is used for binding JH (Kolodziejczyk et al., 2008). To our knowledge, this is the first discovery of a JHBP orthologue in crustaceans. Earlier studies have shown that methyl farnesoate binding protein (MFBP) is present in crustaceans (King et al., 1995; Prestwich et al., 1990; Takáč et al., 1998; Tamone et al., 1997), and the hemolymph of the crab *Libinia emarginata* contained moderate MF binding affinity ($KD = 4.5 \times 10^{-6}$ M) (Li and Borst, 1991). Whether this JHBP orthologue in *N. denticulata* (Supp. File 1 Fig. S3) plays a similar role in binding MF remains to be elucidated.

3.2. Juvenoid hormone degradation

In addition to biosynthesis, orthologues of genes involved in the JH degradation pathway, including *juvenile hormone epoxide hydrolase (JHEH), juvenile hormone esterase (JHE)* and *juvenile hormone esterase binding protein (JHEBP)* can also be identified in the shrimp *N. denticulata* genome (Fig. 1, Table 1, Supp. File 1 Fig. S2, S4 and S5).

In some insects, JHEH (Share and Roe, 1988) and JHE (Hammock and Sparks, 1977) are enzymes that are involved in the degradation of JH to maintain its titre at appropriate levels. For example, JHEH

Table 1
Juvenile hormone pathway genes.

Gene name and GenBank accession number	Abbreviation	Function	*N. denticulata*	Found in Crustacea?	Found in Insecta?	Other arthropods?
Biosynthetic pathway components						
Juvenile hormone acid methyltransferase (KJ200310)	JHAMT	Synthesis of JH or MF from FA	Present	*Daphnia pulex* Hui et al. (2010)	Yes	Spider mite Grbić et al. (2011)
Cytosolic juvenile hormone binding protein (KJ200315)	JHBP	Carrier of JH to transport it from the synthesis site to target tissues	Present	No	Yes	
Degradation pathway components						
Juvenile hormone esterase (KJ956473)	JHE	JH degradation	Present	*Pandalopsis japonica* Lee et al. (2011)	Yes	
Juvenile hormone esterase binding protein (KJ200316)	JHEBP	Transport and degradation of JHE	Present	No	Yes Shanmugavelu et al. (2000), Liu et al. (2007) and Hao et al. (2013)	
Juvenile hormone epoxide hydrolase (KJ200311)	JHEH	JH degradation	Present	No	Yes; multiple taxa	
Canonical regulatory components as shown in other arthropods						
Allatostatin-A, -B, -C (KJ200313, KJ579126, KJ200314)	Ast-A, Ast-B, Ast-C	Inhibition of JH synthesis	Present	Ast-A *Carcinus maenas* Duve et al. (1997) *Orconectes limosus* Dircksen et al. (1999) *Machrobrachium rosenbergii* Yin et al. (2006) *Penaeus monodon* Duve et al. (2002) Ast-B *Cancer borealis* and *Cancer productus* Fu et al. (2007) Ast-C *Homarus americanus* Dickinson et al. (2009) *Cancer borealis* Ma et al. (2009)	Yes; multiple taxa (reviewed in Stay and Tobe (2007))	
Allatostatin-A, -B, -C receptor (KJ956470, KJ956471, KJ956472)	Ast-A-R, Ast-B-R, Ast-C-R	Receptors for allatostatins	Present	*Penaeus monodon* Panchan et al. (2003)	Yes; multiple taxa	
Methoprene-tolerant (KJ579130)	Met	Met and steroid receptor coactivator proteins form a heterodimer important for JH reception	Present	*Daphnia pulex* and *D. magna* Miyakawa et al. (2013)	Yes; multiple taxa	
Other potential regulatory components						
Broad-complex (KJ579129)	BR-C	Target of Met	Present	Giant tiger shrimp *Penaeus monodon* Buaklin et al. (2013)	Yes; multiple taxa	
Chd64 (KJ200321)	Chd64	JH response element binding protein	Present	No	*Drosophila melanogaster* Li et al. (2007)	
FKBP39 [A]	FKBP39	JH response element binding protein	Present	No	*Drosophila melanogaster* Li et al. (2007)	
Hexamerin	Hex	Regulator of JH	Not found (hexapod specific)	No	Yes; multiple taxa	
Crustacean hyperglycemic hormone/ Molt-inhibiting hormone/Gonad-inhibiting hormone (KJ200320)	CHH/MIH/GIH	Potential regulator of MF	Present	*Litopenaeus vannamei* Tiu et al. (2007)	Yes; multiple taxa	

[A] No GenBank accession number for FKBP39 since GenBank does not accept sequences shorter than 200 bp. The sequence is provided in Supplementary 4.

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Table 2
Ecdysteroid pathway genes.

Gene name	Abbreviation	GenBank accession number	Function	*N. denticulata*?	Found in Crustacea?	Found in Insecta?	Other arthropods?
spook (*CYP307A1*)	*spo*	KJ200319	Synthesize 20-hydroxyecdysone (20E) from cholesterol	Present	*Daphnia pulex* Rewitz and Gilbert (2008)	Yes	Spider mite (Grbić et al. 2011)
phantom (*CYP306A1*)	*phm*	KJ200318	Synthesize 20E from cholesterol	Present	*Daphnia pulex* Rewitz and Gilbert (2008); *Marsupenaeus japonicus* Asazuma et al. (2009)	Yes	
disembodied (*CYP302A1*)	*dib*	KJ200317	Synthesize 20E from cholesterol	Present	*Daphnia pulex* Rewitz and Gilbert (2008)	Yes	Spider mite (Grbić et al. 2011)
shadow (*CYP315A1*)	*sad*	KJ579127	Synthesize 20E from cholesterol	Present	*Daphnia pulex* Rewitz and Gilbert (2008)	Yes	Spider mite (Grbić et al. 2011)
shade (*CYP314A1*)	*shd*		Synthesize 20E from cholesterol	Not found	*Daphnia pulex* Rewitz and Gilbert (2008)	Yes	Spider mite (Grbić et al. 2011)
CYP18A1		KJ579128	Paralog of *CYP306*	Present	*Daphnia pulex* Rewitz and Gilbert (2008)	Yes	
Canonical regulatory components as shown in other arthropods							
Ecdysone receptor	EcR	KJ579131	Ecdysteroids regulate molting by activating a heterodimer formed by the ecdysone receptor (EcR) and retinoid X receptor (RXR)	Present	Intertidal copepod *Tigriopus japonicus* Hwang et al. (2010) Kuruma prawn, *Marsupenaeus japonicus* Asazuma et al. (2007) Fiddler crab *Celuca pugilator* Chung et al. (1998) *Uca pugilator* Durica and Hopkins (1996))	Yes	
Retinoid X receptor/ ultraspiracle	RXR /USP	KJ200312	Ecdysteroids regulate molting by activating a heterodimer formed by the ecdysone receptor (EcR) and retinoid X receptor (RXR)	Present	*Daphnia magna* Kato et al. (2007) *Uca pugilator* Durica and Hopkins (1996); Wu et al. (2004) *Homarus americanus* Tarrant et al. (2011) mysid shrimp *Americamysis bahia* Yokota et al. (2011) brown shrimp *Crangon crangon* Verhaegen et al. (2011) sand shrimp *Metapenaeus ensis* Cui et al. (2013)	Yes; multiple taxa	Centipede Lithobius peregrinus Bortolin et al. (2011)

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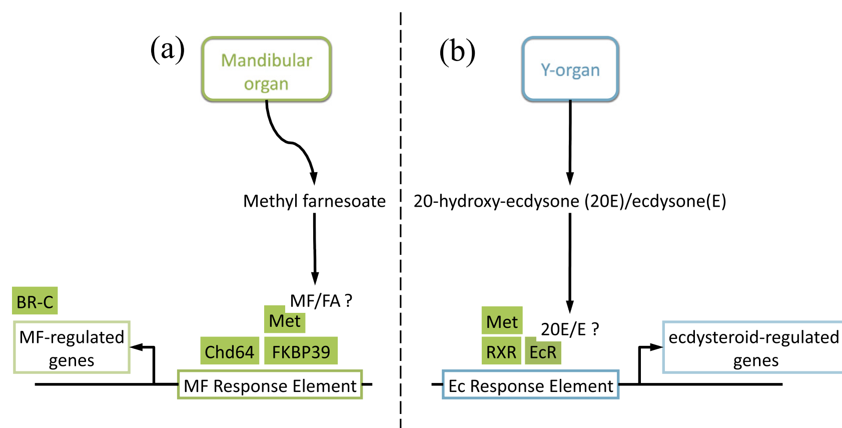

Fig. 3. Expression of JH/MF and ecdysteroid pathway genes (signal transduction and target-gene regulation). Model of the signal transduction pathway for (a) JH/MF-regulated genes and (b) ecdysteroid-regulated genes in crustaceans. MF response element mediation of MF action is modified from the proposed signal transduction pathway for JH-regulated genes (Li et al., 2007). Met is the putative receptor of MF. Chd64 and FKBP39 could mediate the action of MF-Met on the MF response element. The heterodimeric nuclear receptor comprising EcR and RxR mediates ecdysteroid action and regulates ecdysteroid-response genes. For details, please refer to text. Genes identified in *Neocaridina denticulata* are shown in boxes with darker coloration (i.e. *Met, RXR, EcR, Chd64, FKBP39* and *Br-c*).

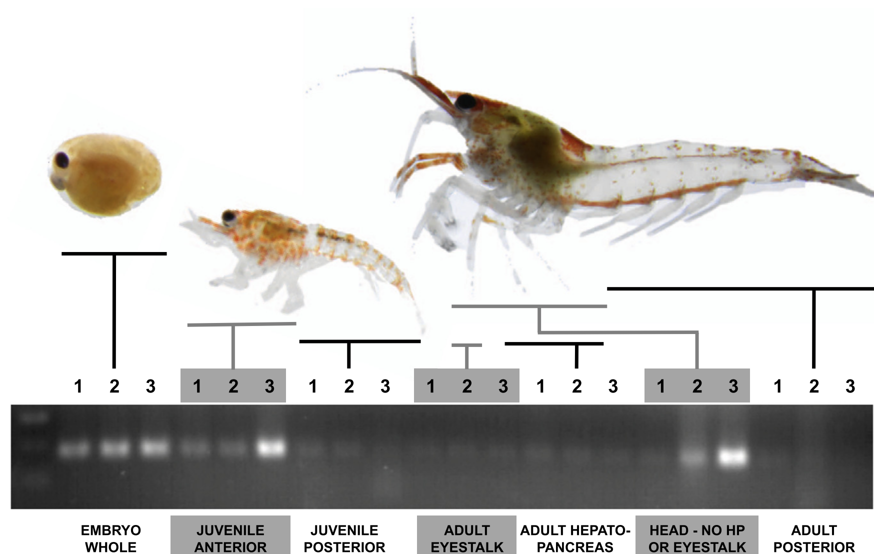

Fig. 4. Expression of JHAMT in *N. denticulata* embryos, juveniles and adults. Expression of JHAMT in embryonic, juvenile and adult *N. denticulata* samples as noted, determined using PCR on cDNA made from equivalent quantities of total RNA. Three independent replicate individual shrimp samples are shown for each life stage and sample. Note strongest expression is found in embryos and juvenile anterior, with relatively strong expression in adult head and weak expression in all other samples, especially in adult posterior, where some samples show only very weak expression. Lane at far left is Invitrogen 1 kb+ ladder, with 100 bp, 200 bp and 300 bp bands shown from lowermost – topmost. Primers used detailed in Supp. File 3 Table S2.

can degrade JH through hydration of the epoxide moiety at C10–C11 to form JH diol, whereas JHE in the hemolymph can degrade JH by hydrolysis of its methyl ester at C1 to form the JH acid metabolite (Fig. 1) (Anand et al., 2008). As the catabolism of JH by both JHEH and JHE generates inactive JH acid diol that has no biological activity (Share and Roe, 1988), metamorphosis can thus be induced. Although the extent of JH degrading activity by JHEH and JHE depends on the insect species and stages in their life cycle (Keiser et al., 2002; Khlebodarova et al., 1996; Lassiter et al., 1995), both enzymes seem to be important in JH degradation in insects. As in insects, specific carboxylesterases are responsible for the ester hydrolysis of MF to FA in crustaceans (rather than using JH as the substrate in insects). The hepatopancreas and the gonads are the major tissues for MF metabolism (Homola and Chang, 1997a). However, as there is currently no evidence that MF will be degraded by JHEH and JHE, the actual roles played by these orthologues in crustaceans remains largely unexplored.

JHEBP, on the other hand, functions in JHE transportation and degradation (Hao et al., 2013), and thus controls JH titer indirectly through the hydrolysis of JHE. To date, an orthologue of JHE has only previously been identified in the shrimp *Pandalopsis japonica* (Lee et al., 2011), while JHEH and JHEBP have to our knowledge never before been identified in crustaceans. The identification of genes of the JH degradation pathway in *N. denticulata* indicates that the mechanism of hormonal regulation of JH levels in insects could be a potential conserved mechanism across the Pancrustacea. However, the exact roles and regulatory mechanisms of these genes in crustaceans remains to be investigated.

3.3. Juvenoid hormone regulation

Allatostatins (ASTs) are well known neuropeptides that inhibit the biosynthesis of JH by the CA in insects (Stay and Tobe, 2007), and several allatostatin-like peptides were identified in the

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N. denticulata genome (Supp. File 1 Fig. S6–S8). In insects, there are three major types of ASTs: A-type, muscle-inhibiting peptide (MIP) type, and C-type.

FGLamide or A-type ASTs were peptides first isolated from the cockroach *D. punctata,* and possess the repeated, conserved pentapeptide C-terminal sequence Y/F-X-F-G-L/I amide (Woodhead et al., 1989, 1994). The A-type AST precursor of *N. denticulata* contains 36 AST-like sequences with the conserved pentapeptide sequence, which are similar to the FGLamides reported in other crustaceans (Yin et al., 2006; Yasuda-Kamatani and Yasuda, 2006; Martinez-Perez et al., 2011), as can readily be identified in Fig. S6 (Supp. File 1).

MIPs (which also previously known as AST B-type) were first isolated in the cricket, *Gryllus bimaculatus*, and are also termed $W(X)_6$Wamides given these C-terminally amidated peptides contain tryptophan in the second and ninth positions (Lorenz et al., 1995; Wang et al., 2004). The MIP identified in *N. denticulata* also possesses amino acid sequence with tryptophan in the second and ninth positions, as can be seen in Fig. S7 (Supp. File 1).

C-type ASTs were first identified in the tobacco hornworm *Manduca sexta* (Kramer et al., 1991), and have also been named "PISCF" as they contain 15-amino-acid peptides with the nonamidated C-terminal pentapeptide P-I-S-C-F. This C- terminal pentapeptide P-I-S-C-F is also possessed by the C-type AST identified in *N. denticulata,* as shown in Fig. S8 (Supp. File 1).

Treatment using *D. punctata* FGLamide AST on the MO of the crayfish *Procambarus clarkii in vitro* showed a stimulatory effect on MF synthesis in adults (Kwok et al., 2005), similar to the stimulatory effect found in early *D. punctata* embryos (Stay et al., 2002). These previous results, together with the presence of all three types of ASTs in *N. denticulata* suggest that allatostatin-like peptides could share established roles across the Pancrustacea, for example, they may have been the regulators of sesquiterpene biosynthesis or muscle contraction before the emergence of insects.

3.4. Ecdysteroid production

Many physiological events throughout the life cycle of insects are regulated by the combined action of sesquiterpenoids and the ecdysteroid 20E (Gelman et al., 2007). In crustaceans, ecdysteroid is synthesized in the Y-organ (YO), which is located in the cephalothorax, anterior to the branchial chamber. The YO takes up dietary cholesterol from the hemolymph and converts it to 5β-diketol (3D2, 22, 25, dE) by a series of reactions involving enzymes from the "Halloween gene" family, which form a variety of secreted products (Chávez et al., 2000; Mykles, 2011; Niwa et al., 2004; Petryk et al., 2003; Warren et al., 2002, 2004). The Halloween genes identified to date include *spook*, *phantom*, *disembodied*, and *shadow,* as well as *CYP18*, which has only been previously identified in *D. pulex* within the Crustacea *sensu stricto* (Rewitz and Gilbert, 2008), whereas *phantom* has also been cloned from the kuruma prawn *Marsupenaeus japonicus* (Asazuma et al., 2009). The ecdysteroid products (e.g. ecdysone and 3-Dehydro-25-deoxyecdysone) secreted by the YO are converted to 20E and related compounds by peripheral tissues, such as the hepatopancreas, epidermis, gonad, hindgut and eyestalk ganglia (Chang and O'Connor, 1978; Spaziani et al., 1997). In peripheral tissues, products secreted by the YO are converted to 20E and related compounds, most likely by *Shade*. Ecdysteroid biosynthesis in insects and crustaceans is therefore broadly similar, although the crustacean YO synthesizes a greater diversity of ecdysteroids than is found in insects (Mykles, 2011).

In *N. denticulata,* the Halloween family of genes encoding the steroidogenic CYPs that produce the molting hormone 20E in insects are all found to be present with the exception of *shade* (Fig. 2, Table 2, Supp. File 1 Fig. S18–22 and Supp. File 2 Fig. S24). The absence of *shade* from our dataset could perhaps be due to poor recovery of this genomic locus, because 20E (a product from ecdysone by the action of Shade) has been found in crustaceans (Subramoniam, 2000). Furthermore, the identification of Halloween genes in the spider mite *Tetranychus urticae* (Grbić et al., 2011), in the subphylum Chelicerata, suggests that the origin of the ecdysteroid biosynthetic pathway is deeply rooted in the evolutionary history of arthropods, before the divergence of the first pancrustacean from the Arthropoda stem lineage.

3.5. Regulators of both juvenoid hormones and ecdysteroids

Genes encoding regulatory components that mediate the molecular action of JH and 20E (Fig. 1, Tables 1 and 2), including *Methoprene-tolerant* (*Met*) (Supp. File 1 Fig. S10), *Retinoid X receptor* (*RXR*; also called *Ultraspiracle* [*Usp*] in some insects) (Supp. File 1 Fig. S12), *Ecdysone receptor* (*EcR*) (Supp. File 1 Fig. S11), *calponin-like protein* (*Chd64*) (Supp. File 1 Fig. S15), *FK509-binding protein* (*FKBP39*) (Supp. File 1 Fig. S16), *Broad-complex* (*Br-c*) (Supp. File 1 Fig. S14), and the neuropeptides *crustacean hyperglycemic hormone* (*CHH*)/*molt-inhibiting hormone* (*MIH*)/*gonad-inhibiting hormone* (*GIH*) (Supp. File 1 Fig. S17 and Supp. File 2 Fig. S23) are also present in the *N. denticulata* genome.

In arthropods, 20E regulates molting by activating a heterodimer formed by EcR and RXR. This binding of 20E activates the dimer and regulates transcription of target genes through ecdysteroid response elements (Durica and Hopkins, 1996; Thomas et al., 1993). Met is the putative JH receptor that binds JH with high affinity (Miura et al., 2005), acts as an important transducer of the JH signal (Konopova et al., 2011), and can also interact with Chd64 and FKBP39, which bind to putative JH response elements (JHRE) in the genome (Li et al., 2007). In crustaceans, MF (and perhaps FA) acts as the innate ligand rather than JH III as in insects, and the substitution of a single amino acid within *Daphnia* Met enhances its responsiveness to JH III, suggesting that a single mutation could contribute to the evolution of a JH III receptor system in insects (Miyakawa et al., 2013). Met can also interact with EcR and RXR in their binding to ecdysone response elements, so 20E could drive molting and metamorphosis. Chd64 and FKBP39 can interact with EcR-RXR-Met to varying degrees as well, depending on the level of JH (Li et al., 2007). Further, *Br-c* is the early ecdysteroid responsive gene encoding a family of zinc-finger transcription factors, which coordinate changes in gene expression during embryogenesis and metamorphosis (Chen et al., 2004; Konopova and Jindra, 2008; Piulachs et al., 2010). Therefore, Met, Chd64 and FKBP39 probably play important roles in allowing cross-talk between JH and ecdysteroids, but their precise interactions remain to be investigated in crustaceans. Considering that ecdysteroid mediated molting has been demonstrated to be present in other non-arthropods such as the chelicerate spider mite (Grbić et al., 2011), the existence of this full regulatory complement in the *N. denticulata* genome suggests that the ecdysteroid pathway, similarly to the JH cassette, is fully extant in this species and could have evolved early in the Arthropoda radiation.

4. Conclusions

The identification and cataloguing of gene orthologues for the sesquiterpene biosynthetic and degradation pathways, ecdysteroid biosynthetic pathway, and their regulatory components in shrimp *N. denticulata* strongly suggest that the complete JH and ecdysteroid pathways were present in the insect-crustacean common ancestor, and these regulatory systems could possibly also date back to the arthropod ancestor. Many questions remain, for example, could the JH and ecdysteroid systems have evolved concur-

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rently with the emergence of the exoskeleton in arthropod radiation? How are these different pathways components regulated *in vivo* in different arthropods? Detailed survey of the presence of their genes in other non-insect classes of the Arthropoda, and investigations of their regulation and functions in different arthropods, including the crustaceans, would be of immense importance for research on aquaculture, development, endocrinology, and pest management, and would also provide useful information for understanding the evolution and functions of the JH and ecdysteroid regulatory systems across this diverse and important Phylum.

Availability of supporting data

The hormonal pathway gene sequences reported here have been submitted to the NCBI GenBank resource with accession numbers KJ200310-KJ200321, KJ579126-KJ579131 and KJ956470-KJ956473. The genome of *N. denticulata* has been published as a research article in *Marine Drugs* (Kenny et al., 2014), and is available for download from that source.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ygcen.2014.07.018.

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