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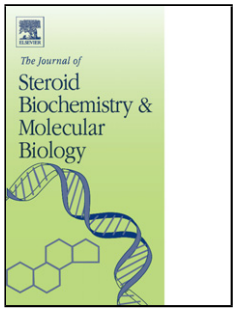

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CYP450s analysis across spiny lobster metamorphosis identifies a long sought missing link in crustacean development

Tomer Ventura[a,*], Utpal Bose[a], Quinn P. Fitzgibbon[b], Gregory G. Smith[b], P. Nicholas Shaw[c], Scott F. Cummins[a] and Abigail Elizur[a]

[a] GeneCology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, 4 Locked Bag, Maroochydore, Queensland 4558, Australia

[b] Institute for Marine and Antarctic Studies, University of Tasmania, Private Bag 49, Hobart, Tasmania 7001, Australia

[c] School of Pharmacy, the University of Queensland, St Lucia 4072, Australia

[*]Corresponding author:

Faculty of Science, Health, Education and Engineering, GeneCology Research Centre

University of the Sunshine Coast

4 Locked Bag, Maroochydore, Queensland, Australia 4558

Tel: +61-7-54565984

Email: tventura@usc.edu.au

Short title: **Spiny lobster metamorphosis CYPome**

Highlights:

- Missing link in crustacean ecdysone hydroxylation identified
- Metamorphic expression implicates un-annotated CYP450s in ecdysteroidogenesis
- CYPome of a spiny lobster enables annotation of known CYP450s based on insects

Abstract

Cytochrome P450s (CYP450s) are a rapidly evolving family of enzymes, making it difficult to identify *bona fide* orthologs with notable lineage-specific exceptions. In ecdysozoans, a small number of the most conserved orthologs include enzymes which metabolize ecdysteroids. Ecdysone pathway components were recently shown in a decapod crustacean but with a notable absence of shade, which is important for converting ecdysone to its active form, 20-hydroxyecdysone (20HE), suggesting that another CYP450 performs a similar function in crustaceans. A CYPome temporal expression analysis throughout metamorphosis performed in this research highlights several un-annotated CYP450s displaying differential expression and provides information into expression patterns of annotated CYP450s. Using the expression patterns in the Eastern spiny lobster *Sagmariasus verreauxi*, followed by 3D modelling and finally activity assays *in vitro*, we were able to conclude that a group of CYP450s, conserved across decapod crustaceans, function as the insect shade. To emphasize the fact that these genes share the function with shade but are phylogenetically distinct, we name this enzyme system Shed.

Keywords: CYP450; Ecdysone; Juvenile hormone; Decapoda; Molt; Metamorphosis

1. Introduction

1.1 Cytochrome P450 versatility

Cytochrome P450s (CYP450s) form an ancient family of enzymes with versatile roles [1]. In ecdysozoa, CYP450s are known to be involved in the metabolism of key compounds that regulate development, growth and reproduction [2-4]. CYP450s are also involved in detoxification; this may range from promiscuous enzymes, which can metabolize multiple substrates [5], to those specialized in metabolizing one or a few substrates [6, 7]. It is perhaps for these reasons that CYP450s form one of the most versatile enzyme families, where their rapid evolution enables the organism to cope with a changing environment on the one hand, yet on the other hand, it makes it hard to clearly define orthologs. An example for the high evolutionary rate of this family is evident from the observation that insect CYP450s alone range in number from 36 to 180 [8].

1.2 Conserved arthropod CYP450 orthologs: the Halloween genes

A small number of CYP450s are known to participate in the biosynthesis of the active form of the molt hormone, 20-hydroxyecdysone (20HE; (2β,3β,5β,22R)-2,3,14,20,22,25-hexahydroxycholest-7-en-6-one), one key factor which generates the active juvenile hormone (JH). In addition, several other CYP450s have been partially annotated based on phylogeny and their role deduced in one or more species [8]. In a sense, the CYP450 complement (also referred to as CYPome) of a species is a unique signature defined by its interaction with the environment and the mechanism by which it regulates development and reproduction.

A high evolutionary change rate might, in part, be associated with the need to cope with various toxins in the changing environment but, from a mechanistic point of view, it is also associated with the fact that the CYP450s tend to form clusters in the genome. Some duplicated gene clusters can be maintained over very long timescales. The head to tail pair of the close paralogs

CYP306A1 and CYP18A1 is conserved as a cluster in all insects studied so far (except in *Anopheles gambiae* that has lost the CYP18A1 gene), and is even found in the crustacean *Daphnia pulex*, thus dating this cluster to well over 500 MY [8]. Stable duplication events like this increase the evolutionary change rate, since chromosome rearrangement events might lead to exponential duplications [8]. While versatile and rapidly changing, all the protostome CYP genes identified to date can be assigned to one of four clans: CYP2, CYP3, CYP4 and the mitochondrial CYP clan [9]. While clan nomenclature is inferred by phylogeny [10], members of each clan can have various roles. The CYP4 clan in insects for instance, includes members associated with pheromone synthesis and breakdown [11], as well as cuticle hardening [12]. From a substrate perspective, several stages in the synthesis of a bio-active compound can involve CYP450s from different clans; such is the case of the CYP450s which synthesize 20HE.

The conserved arthropod CYP450 orthologs are those involved in 20HE biosynthesis and degradation as well as juvenile hormone biosynthesis. The primary source of ecdysteroid biosynthesis is the Y-organ in crustaceans, which is analogous to the insect prothoracic gland [13]. The synthesis of 20HE is negatively regulated in crustaceans by the molt inhibiting hormone (MIH), which acts through an as yet unidentified receptor on the YO membrane to block its function. MIH is produced predominantly in the X-organ, then transported to the sinus gland where it is stored until secretion [14]. This neuroendocrine complex, known as the X-organ-sinus gland complex (XO-SG), resides in the crustacean eyestalk. While in crustaceans 20HE is negatively regulated by MIH derived from the XO-SG, in insects 20HE synthesis is positively regulated by neurosecretory cells in the brain which produce the prothoracicotropic hormone (PTTH) [15]. In the biosynthesis pathway of 20HE, five CYP450s were discovered to be conserved in insects. They were named the Halloween genes due to the embryonic lethal effect of null mutations, resulting in disfigured flies, probably due to low titer of ecdysteroids

and inability to properly form a cuticle. Spook (CYP307A1) (and the diptera lineage specific paralogs spookier (CYP307A2) [16] and spookiest (CYP307B1) [17]) are expressed in the insect prothoracic gland in a stage-specific manner, regulating the first steps in 20HE synthesis [18, 19]. The *phantom* gene (CYP306A1) is also expressed predominantly in the insect prothoracic gland and the enzyme follows spook in the biosynthesis of 20HE [20]. These stages are followed by enzymatic reactions catalyzed by disembodied (CYP302A1), shadow (CYP315A1) [21] and shade (CYP314A1); the latter enzyme catalyzes the final step in the 20HE biosynthetic pathway in the target cells [6]. Degradation of 20HE is facilitated by CYP18A1 [22], which clusters with its paralog CYP306A1 in most insects studied as well as in *D. pulex* [23]. While spook, phantom and CYP18A1 are part of clan 2, the other three enzymes are part of the mitochondrial clan. A recent study has also identified orthologs of five out of the six genes (with the notable exception of shade) in the decapod cherry shrimp *Neocaridina denticulata* [24].

1.3 Metamorphosis in spiny lobsters

Metamorphosis in spiny lobsters is a dual phase process where an oceanic transparent, alien-like larva (phyllosoma) metamorphose into a nektonic miniature transparent version of the lobster (puerulus), manifesting massive restructuring of anatomy and physiology in a single step [35]. The puerulus swims towards the shore where it completes metamorphosis into the benthic juvenile form [38]. Our research to date shows that the phyllosoma-puerulus metamorphic transition in the Eastern spiny lobster *S. verreauxi* is accompanied by vast transcriptomic changes exceeding 25% of the transcriptome [35]. The lengthy transition, the large-sized larvae and their transparency enable clear molt staging by gut retraction. The availability of transcriptomic data for both the larval metamorphic transition at high resolution [35], alongside tissues of juveniles and adults from this species [44-49] enables thorough examination of expression patterns and correlation with spatial-temporal expression.

In this research we characterized the differential expression of CYP450s across the phyllosoma-puerulus transition in the Eastern spiny lobster *S. verreauxi*. We identified 43 putative CYP450s with clear phylogenetic annotation for eight of them. Expression throughout metamorphosis varied significantly for 11 out of the 43 CYP450s, with four predominant expression patterns. Inferred from expression pattern, 3D modelling, *in vitro* assay and cross-species analysis, we predict that a clan 4 CYP450, conserved in crustaceans, is the putative ecdysone to 20HE hydroxylase. By using ultra-high pressure liquid chromatography-quadrupole time of flight-mass spectrometry (UHPLC-QToF-MS), one enzyme from this group was shown to produce hydroxyecdysone (HE) *in vitro*. We thus conclude that this is the Shade ortholog in crustaceans and thus named it 'Shed'.

2. Materials and Methods

2.1 Bioinformatics analysis

The transcriptome of whole individuals sampled from five developmental stages throughout metamorphosis of *S. verreauxi* (in duplicates, including six phyllosoma and 4 puerulus) [35], was converted to amino acids (aa) of the most probable open reading frame (ORF) using OrfPredictor (proteomics.ysu.edu/tools/OrfPredictor.html). Where ORF was predicted to be partial, iterative tBLASTn searches using CLC Genomics Workbench (Qiagen, version 8.0.3), against the transcriptome of both developmental stages [35] and juvenile and mature tissues [44, 48], were performed in order to identify flanking regions. The predicted ORFs were searched for CYP450 domains using PFAM database in CLC Genomics Workbench. Mapping and quantification were previously performed using CLC Genomics Workbench and Partek Genomics Suit [35], and a fold-change ≥ 2 between the five sub-stages with P value ≤ 0.05 [with FDR; as previously calculated [35]] was considered as differential expression.

Multiple sequence alignments followed by phylogenetic trees constructed using Neighbor Joining (NJ) method (bootstrap = 1000) were performed using the CLC Genomics Workbench. The aligned sequences were tested using Maximum Likelihood and Maximum Parsimony (100 bootstraps for each) in MEGA 6.0 (Supplementary File S2).

Three dimensional modelling was performed using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) followed by rendering in CLC Genomics Workbench. Only models with C-score >0.75 were considered.

2.2 Cell Culture and Transient Transfection of Cells

Transient transfection and cell culture protocols were undertaken according to Aizen *et al.* [46]. Briefly, COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM

L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 100 U/mL nystatin (Life Technologies). Cells were grown at 37°C, with 5% CO_2 until 80% confluent, followed by transfection with either an empty pCDNA3.1+ vector (Promega), or a pCDNA3.1+ vector expressing Sv-Unigene1882 (Genscript), using TransIT®-LT1 Transfection Reagent (Mirus), according to the manufacturer's instructions. The cells were cultured for 8 h, then split into various groups in triplicates with or without the addition of 20 µg/mL ecdysone (Sigma).

2.3 Collection and isolation of samples for UHPLC-QtoF-MS analysis

Following 2 h of incubation with or without 20µg/mL ecdysone, COS-7 cells and culture medium were administered with an equivalent volume of methanol, vortexed thoroughly and then centrifuged at 16,000 xg for 10 min at 4°C. The supernatant was collected (cells removed) and subjected to freeze-drying, and the lyophilized samples stored at -80°C until subsequent analysis. Three biological replicates from two sample groups were used for LC-MS analysis.

2.4 Liquid chromatography-mass spectrometry analysis

Freeze-dried samples were resuspended to 15% of the original volume by adding 30 µL methanol and then 120 µL of MilliQ (Millipore) water to produce a 20:80 methanol:water solution. The extract solution was stored at -80°C until subsequent LC-MS analysis. Prior to LC-MS analysis, samples were thawed and kept at 4°C. The chromatographic separation of compounds and extracts was performed using Ultra High Performance Liquid Chromatography (UHPLC) on an Agilent 1290 series system (Agilent Technologies, USA). The UHPLC was coupled to an Agilent 6520 high-resolution accurate mass (HRAM) QToF mass spectrometer equipped with a multimode source (Agilent) and controlled using MassHunter acquisition software, (B. 02.01 SP3; Agilent). Separation was achieved using a 150 × 2.1 mm, 2.6 µm Kinetex Biphenyl column (Phenomenex, Australia). The chromatographic analysis was performed using 0.1% (v/v) aqueous formic acid (mobile phase A) and acetonitrile + 0.1 %

(v/v) formic acid (mobile phase B) at a flow rate of 0.20 mL/min. The column was pre-equilibrated for 15 min with 99.9% A and 0.1% B. After injection, the composition of mobile phase remained unchanged for 2 min. The composition was changed from 0.1% B to 25% B over a period of 6 min to 80% B by 25 min. Over the subsequent 15 min, the % of B changed from 80% B to 90% B, and in 1 min % B increased to 99.95% B and then held at 99.9% B for 2 min. subsequently, the mobile phase composition returned to the starting composition of 0.1% B over a period of 1 min and then re-equilibrated for 2 min prior to the next sample injection. The injection volume was 20 µL.

Mass spectrometry data were acquired in positive and negative ionization mode. A dual nebulizer electrospray source was used for continuous introduction of reference ions. In MS mode the instrument was set to scan from m/z 100 to 1700 for all samples at a scan rate of 3 cycles/sec. This mass range enabled the inclusion of two reference compounds, a lock mass solution including purine ($C_5H_4N_4$ at m/z 121.050873, 10 µmol/L) and hexakis (1H, 1H, 3H-tetrafluropentoxy)-phosphazene ($C_{18}H_{18}O_6N_3P_3F_{24}$ at *m/z* 922.009798, 2 µmol/L). Multimode (i.e. simultaneous Electrospray Ionisation [ESI] and Atmospheric Pressure Chemical Ionization [APCI]) was employed to ionize compounds optimally following their chromatographic separation.

2.5 Data processing and compound identification

Data processing was performed using Agilent MassHunter Qualitative software (Version B.05.00). The Molecular Feature Extractor (MFE) algorithm within MassHunter Qualitative analysis software was used to extract chemically qualified molecular features from the LC-QToF-MS data files. Data processing details are as those provided in Bose *et al.* [50].

In this study compound identification was performed by interrogating the in-house database using the *m/z* values of the mined compounds from accurate mass LC-MS through MFE and

Molecular Formula Generation. The search parameters implemented were as follows: mass tolerance (accurate mass) ≤ 5 ppm, maximum number of peaks to search when peaks are not specified graphically = 5, charge carriers (positive ions) = H^+, K^+, Na^+, negative ions = H loss and $HCOO^-$ and neutral loss = −H2O. The scoring algorithm for database searches uses not only accurate mass, but also isotope abundance and spacing. The mass position of the M+1 and M+2 isotopes were calculated based on the number and types of elements contributing to them, and the mass spacing from the M to the M+1 and M+2 isotopes were able to be measured with low- to sub-ppm accuracy and provide confidence for compound identification.

3. Results and Discussion

3.1 Spiny lobster CYPome characterization: expression pattern and annotation

We identified 43 putative CYP450s (11 partial, ranging in size from 311 to 441 aa and 32 complete, ranging in size from 485 to 562 aa) in the Eastern spiny lobster *S. verreauxi* transcriptome, assembled from RNA extracted from whole individuals sampled across metamorphosis [35] as well as from various tissues of juvenile and adult individuals [44, 48]. Phylogenetic analysis enabled the annotation of four out of the five Halloween CYP450s [not including shade (CYP314A1)], as well as CYP18A1, all predicted to be conserved in 20HE metabolism (Fig. 1).

In light of the absence of CYP314A1 (shade) in crustaceans and the overall lack of phylogenetic annotation for the majority of the CYP450s, we aimed to shortlist potential CYP450s candidates that play a role in molt and metamorphosis regulation. Taking advantage of the available libraries for different well-defined metamorphic stages [35], as well as juvenile

and adult tissues [44, 48], we assessed the CYP450s expression pattern during metamorphosis in *S. verreauxi*.

The putative CYP450s listed as Unigene47567, CL1826.Contig4, CL1826.Contig5, Unigene880 and CL2278.Contig2 all shared the same expression pattern. They were all expressed significantly higher in the intermolt phyllosoma stage compared with all later stages (Fig. 2). Unigene47567 and nine additional putative CYP450s from *S. verreauxi* clustered with insect CYP6A20 and CYP6G2 (Fig. 1). Unigene47567 was not expressed in any tissue examined post-metamorphosis, suggesting it is either a stage-specific CYP450 or specific to a tissue that was not examined in post-metamorphic samples. BLASTP of Unigene47567 against NCBI nr database gave high similarity [321 aa identical (60%) and 418 aa similar (77%) out of 537 aa] with the tiger shrimp *Penaeus monodon* thromboxane A synthase (GenBank Accession number AFJ11398) which regulates vasoconstriction and promotes thrombosis in vertebrates. Given the different mechanisms in place for this role in crustaceans, and given the stage-specific expression, it might be that the breakdown of prostaglandin E2 is relevant for the transition from phyllosoma to puerulus. CL1826.Contig4 and CL1826.Contig5 clustered with Unigene1882 discussed above and shared similar homology with spiny lobster CYP2L1 and crab CYP379A1. CL2278.Contig2 clustered tightly with crab CYP4C39, shrimp CYP4C15 and prawn CYPV20 (Fig. 1). Out of the three, CYP4C15 was found to be molt-stage specific in a crayfish and was thus hypothesized to be involved in 20HE metabolism [51].

CL5738.Contig3 and Unigene59184 shared similar expression patterns that included significantly lower expression in the post-molt puerulus and H-phase puerulus, compared with previous stages (Fig. 2). CL5738.Contig3 clustered with high bootstrap values together with nine other putative lobster CYP450s, as well as two insect CYP6A20s and two additional insect CYP6G2s (Fig. 1). In *Drosophila* CYP6G2 was found to be expressed in the *corpora allata* and was thus hypothesized to be involved in JH metabolism [36], while CYP6A20 was linked

with male aggressive behaviour [52, 53]. Interestingly, when considering the expression in the lobster tissues, CL5738.Contig3, as well as the two additional contigs CL5738.Contig1 and CL5738.Contig2, all show high expression in the brain, eyestalk, antennal gland and androgenic gland (Supplementary file 1), suggesting a correlation with CYP6A20 role. Unigene59184 clustered with Unigene1882 and perhaps is also involved in ecdysteroidogenesis as discussed above.

A single CYP450 transcript (Unigene44506) was found to be expressed predominantly in the early post-molt puerulus stage (Fig. 2). Since it tightly clustered with the crayfish CYP4 (Fig. 1), which was isolated from the Y-organ (GeneBank Accession number AAL56662), and based on its expression pattern, it is likely an ortholog of the insect CYP4G1. In insects, CYP4G1 has been ambiguously characterized as present in the prothoracic gland with a role in 20HE metabolism [54], as well as an oenocyte-specific P450 required for the regulation of triacylglycerol composition in the *Drosophila* starving larvae [55] and an insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis [12]. This ambiguity might be due to promiscuity of CYP4G1, perhaps enabling versatile roles required for transitional developmental phases. In the post-molt puerulus, a transitional phase with unique physiology, all three functions would be expected to be present specifically in this phase. It is therefore assumed that the *Drosophila* starving larval phase is the equivalent to the puerulus non-feeding phase.

Our previous analysis of expression pattern showed that Sv-CYP15A1 [ortholog of the insect CYP15A1, which degrades the active form of the juvenile hormone in crustaceans, MF [25, 31], into its crustacean inactive, yet insect active form – JH III [7]], is not expressed during the phyllosoma-puerulus transition and ramps up in expression prior to the puerulus-juvenile transition [35]. In the current study, we show that this expression pattern is shared with three additional CYP450s (Fig. 2). One of the three, Unigene44916 was annotated as Sv-CYP307

(the Halloween gene known as spook, which is upstream at the 'black box' stages of generating the active molt hormone 20HE [19, 56]; Fig. 1). This suggests spook might be the rate limiting factor in the reaction. The second CYP450 (Unigene1882) clustered tightly with three additional CYP450s (Unigene880, CL1826.Contig4 and CL1826.Contig5; Fig. 1). It is interesting to note that while bootstrap values are low (45), this cluster is placed next to the CYP307A1 cluster. This phylogenetic link and the shared expression pattern suggest Unigene1882 is involved in ecdysteroidogenesis. BLASTP against the NCBI nr database showed a high similarity between Unigene1882 [275 aa identical (69%) and 330 aa similar (88%) out of 396 aa] and the spiny lobster *Panulirus argus* hepatopancreas-expressed CYP2L1 [57]. Moderate similarity was observed between Unigene1882 [154 aa identical (39%) and 231 aa similar (58%) out of 394 aa] and crab CYP379A1, known to be molt stage specific and regulated by ecdysteroids and xenobiotics in the crab *Carcinus maenas* [58]. The third CYP450 (CL308.Contig3) clustered tightly with yet another two CYP450s (CL308.Contig2 and Unigene44506) and all three together formed a cluster with CYP4C15 or CYP4 from other crustaceans (Fig. 1). CYP4C15 was previously hypothesized to be involved in ecdysteroidogenesis in crustaceans based on changes in expression through the molt cycle [4, 51]. Other CYP4C15 from insects did not cluster with the crustacean CYP4C15, pointing to the high evolutionary change rate of CYP450s in this group. Given that shade was not identified in our database suggests that perhaps CYP4C15 is converging in terms of function and assumes the role of ecdysone hydroxylation into the active 20HE form in *S. verreauxi*.

3.2 Elucidation of the shade-like ('shed') group of genes that mediate ecdysone hydroxylation in crustaceans

Intriguingly, Unigene59184, CL1826.Contig4, CL1826.Contig5, Unigene880 and Unigene1882 clustered tightly together positioned between CYP307A1 (spook, which we found to be expressed primarily in the H-phase puerulus) cluster and CYP18A1 (which

degrades 20HE) cluster. Notwithstanding, all five show differential expressions which would potentially implicate them in ecdysteroidogenesis/metamorphosis.

All three-dimensional predictions of cytochrome P450s performed in this study (both from the spiny lobster and *Drosophila*) resulted with strictly cytochrome P450 proteins predicted as the list of 10 PDB hits that are structurally closest to the target, with the highest score of a ligand binding site resulting with the 'Heme B'. Three-dimensional predictions of Unigene1882, Unigene880, CL1826.Contig4 and Unigene59184 were all reliable based on the high model accuracy (C-score = 1.13 to 1.31; Estimated TM-score = 0.87±0.07 to 0.90±0.06; Estimated RMSD = 4.4±2.9Å to 4.6±3.0Å). When aligned, all gave high similarities to the *Drosophila* shade isoform C (Dm_CYP314A1; GenBank Accession number AAF49727; Fig. 3; C-score=1.05; Estimated TM-score = 0.86±0.07; Estimated RMSD = 4.8±3.1Å).

To test whether the CYP450 encoded by Unigene1882 functions as shade (due to its clustering with Spook and sharing the same expression pattern), an activity assay in transfected COS-7 cells was applied. The Unigene1882 transcript was expressed in COS-7 cells and the medium was supplemented with ecdysone. Following expression, metabolites were extracted and analysed by UHPLC-QToF-MS in order to identify ecdysone and its derivatives upon addition of Unigene1882. Only cells transfected with Unigene1882 (in triplicates) showed hydroxylated ecdysone derivatives when tested using metabolomics analysis (Fig. 4).

LC-MS analysis of an authentic reference standard for ecdysone extracted ion chromatogram (EIC) showed that the retention time is 17.92 min (Fig. 4A) and the accurate monoisotopic mass was *m/z* 477.3082 $(M+H-H_2O)^+$ (Fig. 4A′); ecdysone actual *m/z* in the database was *m/z* 464.3027 [M]. Positive ionisation mode LC-MS chromatograms for extracts of COS-7 cells supplemented with ecdysone showed that ecdysone eluted at 17.95 min (Fig. 4B), as confirmed by mass spectra with *m/z* 477.3087 $(M+H-H_2O)^+$ (Fig. 4B′). Matching of the retention times

for ecdysone (17.90 min for standard and 17.95 min for CYP-Shed transfected COS-7 cells extracted medium) and the respective mass spectra further strengthened the identification through the database search. Interestingly, upon addition of ecdysone to the medium of the COS-7 cells, we identified another compound produced in the sample extracted from the culture medium. The compound peak eluting at 10.95 min (Fig. 4C) was elucidated on the basis of its accurate mass, through an in-house database search, as HE; its monoisotopic mass was *m/z* 503.2944 $[M+Na]^+$ (Fig. 4C′) and that of HE in the database was 480.2987 [M]. This observation indicates that cells transfected with CYP_Shed catalyze the formation of HE from ecdysone in a single hydroxylation reaction. It has been reported that the C-20 hydroxylation of ecdysone to 20HE is an activation reaction, and this conversion occurs mainly by the fat body in *Manduca* sp. larval-pupal during their development [59]. It is possible that a signal produced during this conversion may trigger and coordinate the metamorphosis processes [60]. Given that CYP_Shed is up-regulated between the puerulus and juvenile stages of the lobster, it might explain in part the mechanism of 20HE surge required to complete metamorphosis.

We therefore conclude that the clade that includes Unigene1882 is the crustacean ortholog of the insect shade (sharing function with little phylogenetic relation) and we have thus named it *S. verreauxi Shed1* (*Sv-Shed1*). Accordingly, due to their high sequence and predicted structure similarity with *Sv-Shed1*, Unigene880, CL1826.Contig4, CL1826.Contig5 and Unigene59184 were named *Sv-Shed2*, *Sv-Shed3A*, *Sv-Shed3B* and *Sv-Shed4*, respectively.

This finding sheds light for the first time on the missing link in evolution of arthropods, bridging the gap between ancient malacostracan crustacean species and more advanced arthropods such as insects. We show here, for the first time, that very remotely-related CYP450s defined as different clans (mitochondrial in the case of insects' shade and clan 4 in the case of the crustaceans' shed) could serve similar functions, further emphasizing the versatility of this gene family.

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Author contribution statement

T.V. and U.B. wrote the main manuscript text, produced the results and prepared the figures. Q.P.F and G.G.S cultured the animals and provided the biological samples. Q.P.F., G.G.S., P.N.S., S.F.C. and A.E. reviewed the manuscript and added valuable comments.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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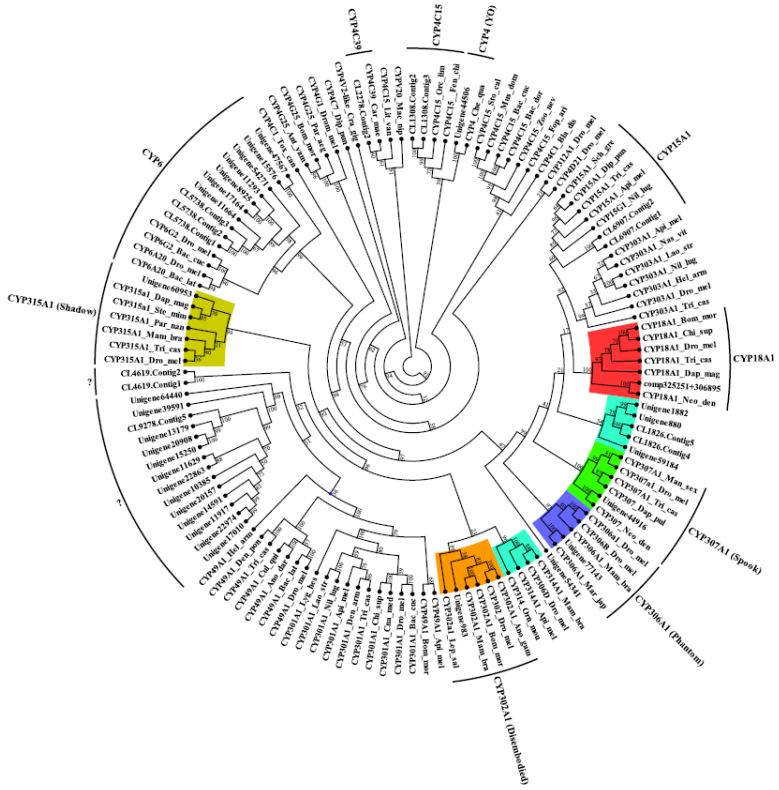
Figures and Figure captions

Figure 1

A



B

Sequence	Ortholog	Name
comp325251+306895	CYP18A1	20HE Degrading Enzyme
Unigene983	CYP302A1	Disembodied
Unigene77143	CYP306A1	Phantom
Unigene44916	CYP307A1	Spook
Unigene60953	CYP315A1	Shadow
Unigene1882	CYP314A1	Shade like (Shed)
Unigene880	CYP314A1	Shade like (Shed)
CL1826.Contig4	CYP314A1	Shade like (Shed)
CL1826.Contig5	CYP314A1	Shade like (Shed)
Unigene59184	CYP314A1	Shade like (Shed)

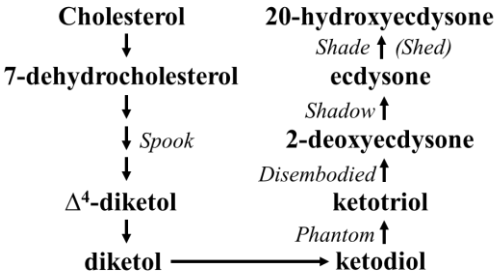


Figure 2: *S. verreauxi* CYP450-encoding transcripts expression throughout metamorphosis. Reads per kilobase per million reads in the library (RPKM; Y-Axis) are given for all *S. verreauxi* CYP450-encoding transcripts in 5 distinct stages at the transition from phyllosoma to puerulus. Transcripts discussed in the manuscript are denoted by name.

Figure 2

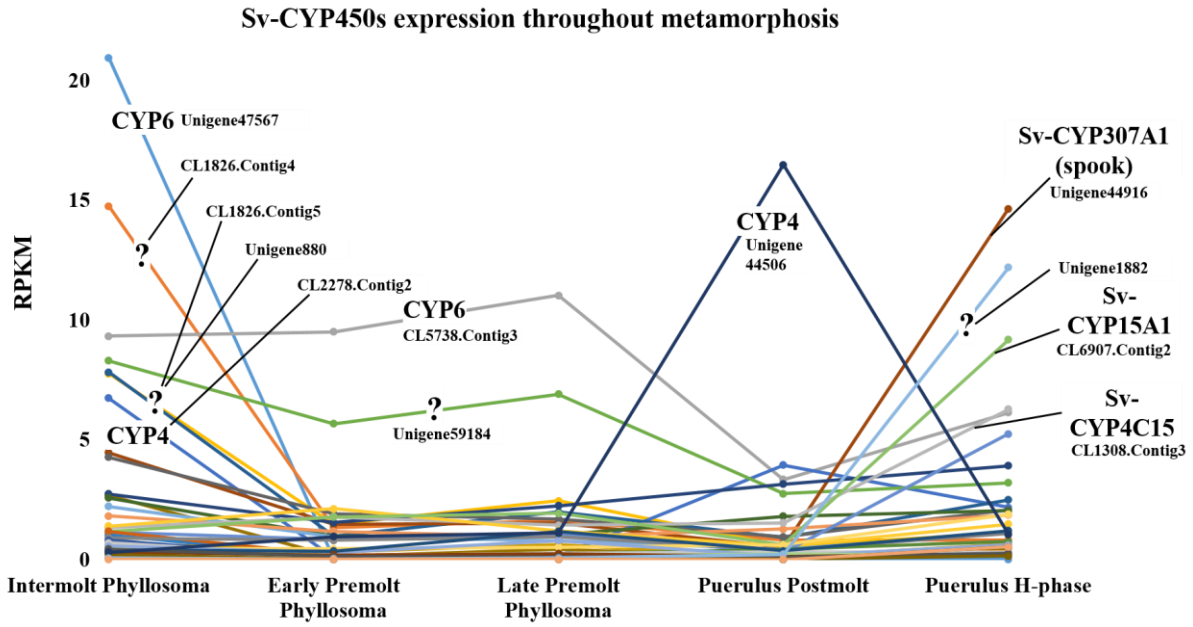


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Figure 3

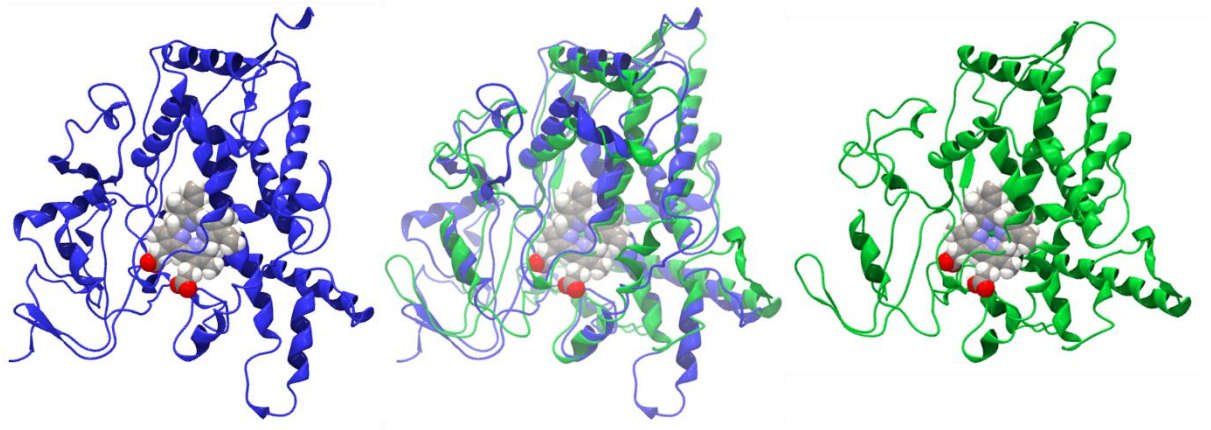

Figure 3: *S. verreauxi* CYP450-encoding transcript Unigene880 predicted 3D structure (left), alongside the closely similar *D. melanogaster* Dm_CYP314A1 predicted 3D structure (right) and their structure alignment (middle). The heme group is presented as a space-filled model.

Figure 4

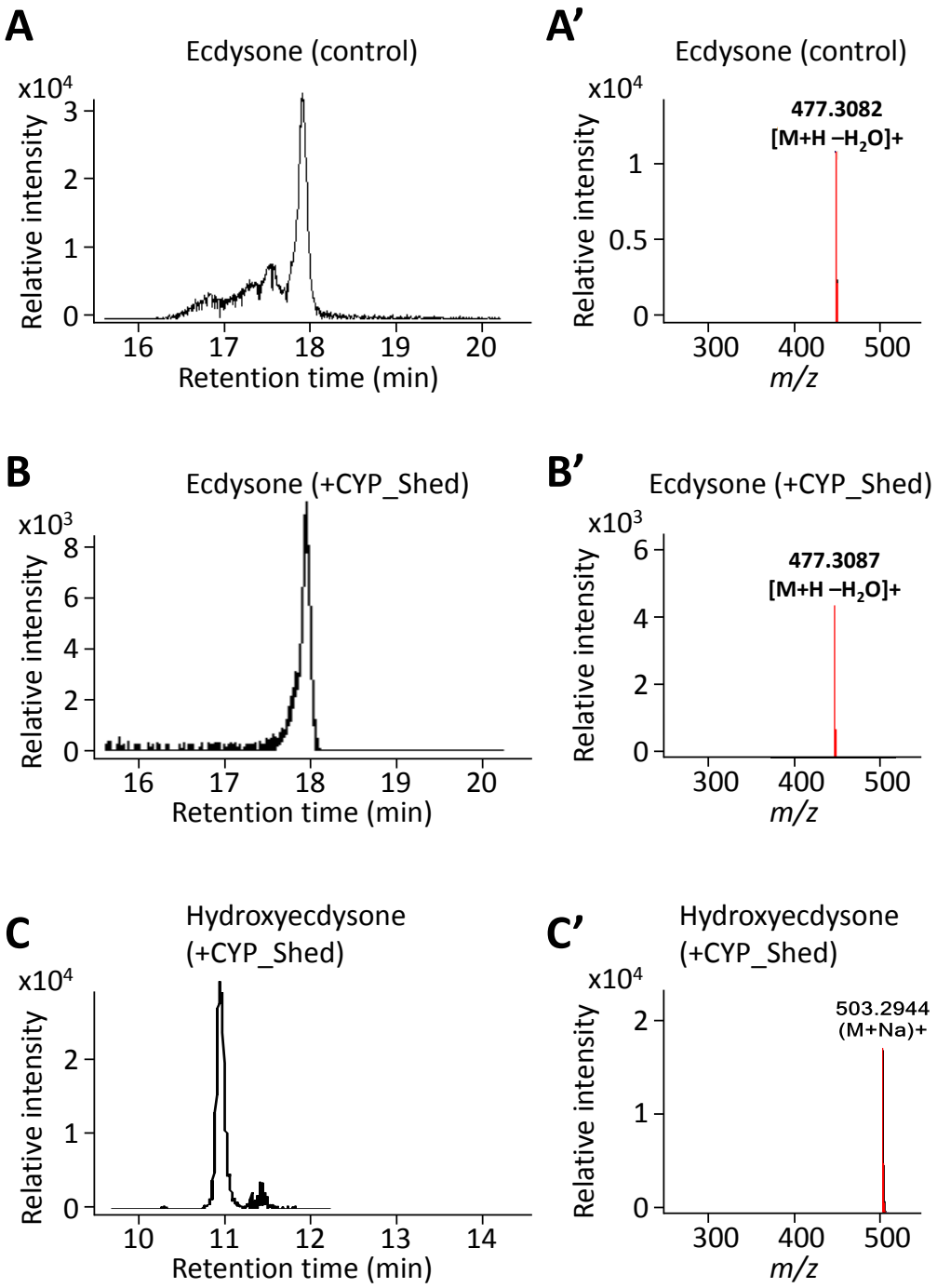

Figure 4: Identification of ecdysone and its metabolite by UHPLC-QToF-MS upon addition of CYP enzyme. (A) Extracted ion chromatogram (EIC) for ecdysone in the standard

(ecdysone-treated COS-7 cells). (A′) Mass spectrum for ecdysone in the standard *m/z* 477.3082 $(M+H-H_2O)^+$. (B) Extracted ion chromatogram for ecdysone (+CYP_shed). (B′) Mass spectrum for ecdysone (+CYP_shed) *m/z* 477.3087 $(M+H-H_2O)^+$ (C) Extracted ion chromatogram (EIC) for the metabolite HE (+CYP_shed). (C′) Mass spectrum for HE (+CYP_shed) *m/z* 503.2944 $(M+Na)^+$.