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Review Paper

Crustacean metamorphosis: an omics perspective

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Abstract Metamorphosis involves a complex network of genes that orchestrate a perfectly timed reorganization of one body form to another. The molecular pathways that start to unravel for an increasing number of species show that there exists great diversity among different species, as would be expected by their wide range of life histories and transformation strategies. The metamorphosis process could account for a considerably high percentile of transcribed sequences over a short period of time, with the genome encoding for different life forms. Such important changes in expression patterns for a high number of genes pose a challenge for accurately assign each gene to a function. Several key conserved factors are consistently expressed and can be placed at the center of metamorphosis, including the mechanisms involving the molt hormone, 20 Hydroxy-Ecdysone, and the juvenile hormone. Yet, many additional factors are not characterized, remain unannotated, or do not have a function assigned. This manuscript provides several examples of how an integrated omics approach can develop further insights into crustacean metamorphosis and eventually lead to discovery of key factors for metamorphosis.

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Introduction

Crustacean metamorphosis is as diverse as the subphylum itself. In some organisms, free larval stages outside the embryo are brief or non-existing, whereas in others the larval phase is prolonged and includes a series of molts with either gradual or dramatic morphological changes (Ventura et al., 2015). Ecdysis (also referred to as molting) is the cyclic process through which ecdysozoans grow, by replacing a hardened old exoskeleton with another and then hardening the newly formed, larger exoskeleton. It is a means to increase in size, regenerate lost limbs and in some species it is required for enabling reproduction either in a cyclic manner (Raviv et al., 2008), or through a pubertal molt (Hopkins & Fingerman, 1989). Metamorphosis can be regarded as a special molt event. The basic characterization of crustacean metamorphosis remains contentious and is complicated by the sheer diversity of larval development strategies. In this review, we adhere to the model defined by Klaus Anger from his seminal 2001 text where metamorphosis was defined as "a sudden and dramatic change in the morphology of two subsequent stages which is usually accompanied by changes in behaviour, feeding, ecology and physiology and are typical of transitions between different phases of development" (Anger, 2001). In this sense, crustacean metamorphosis is not restricted to the transition between the final larvae to first juvenile stage and implies that species can pass through none, one, or more metamorphosis during development. Much of what is known regarding the molecular basis of decapod crustacean metamorphosis is informed by research into model organisms like *Drosophila melanogaster* (Meigen, 1830) (Chang & Mykles, 2011). Additionally, since metamorphosis can be considered as a special ecdysis, much of what we know regarding metamorphosis relies on the molting mechanism that was defined in post-metamorphic stages and informed earlier stages. An important consideration and possible limitation of the holometabolous insects model to describe crustacean metamorphosis is that insect metamorphosis involves the transition between larvae to adults, whereas there can be a great diversity in the timing of crustacean metamorphosis which can involve transitions between larvae to larvae and juvenile stages. This manuscript reviews some of the key findings on the molecular

mechanisms that govern crustacean metamorphosis and follows on to highlight the role of omics in developing new understanding of the complex pathways behind this process.

The molt hormone

The hormone acting as a key hallmark of molting in Pancrustacea is a lipid metabolite, predominantly 20 Hydroxy-Ecdysone (20HE). Variations in the enzymatic pathway that generates 20HE leads to the derivative Ponasterone A being the active molt hormone in chelicerates (Qu et al., 2015). The ecdysone, immediate precursor molecule of 20HE, is produced and secreted by a specific endocrine gland, known as the Y organ (YO) in crustaceans or the prothoracic gland in insects (Chang & Mykles, 2011; Mykles, 2011). Further to different endocrine glands, the upstream regulator of the molting process is entirely different between decapod crustaceans and insects. In decapod crustaceans, a continual suppressive signal is transmitted in the form of a small proteinaceous neuro-hormone known as the molt inhibiting hormone (MIH). The primary location of production and secretion of this hormone is the X organ-Sinus gland complex (XO-SG) which resides at the crustacean eyestalk. Molt inhibiting hormone is part of a family of neuropeptides known as the crustacean hyperglycemic hormones (CHHs). These include several isoforms per species and can be structurally divided into two subclasses: CHH-like and MIH/GIH-like. Their roles bifurcate and overlap, making it hard to assign roles without functional assays (Webster et al., 2012). While there is much debate regarding the accuracy of transcriptomic data and its relevance to the actual levels of the processed, secreted, circulating, and functionally active protein, at least in the case of transcription, there is high correlation between recent work done in crustaceans and previously established literature (Ventura et al., 2014). As an example, Fig. 1 shows a word cloud describing the prevalence of neuropeptides expression in a spiny lobster eyestalk as compared with brain and thoracic ganglia, the three main sources of neuropeptide production in crustaceans. The CHH family peptides expression is highly represented in the eyestalk, while very little is expressed in the brain and thoracic ganglia.
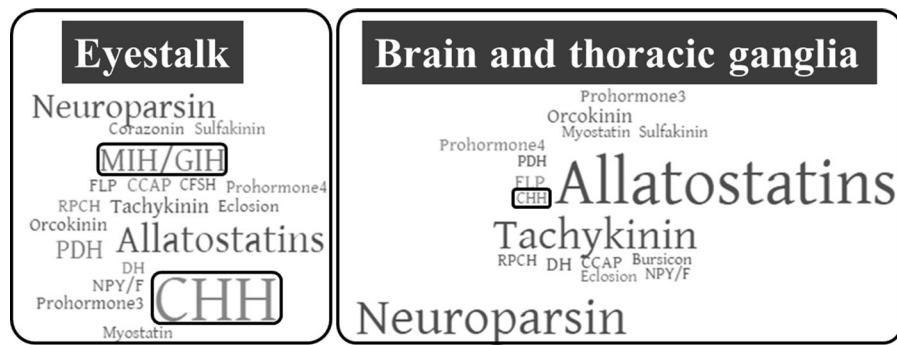
Fig. 1 Word cloud depiction of neuropeptides prevalence in transcriptomic libraries of the Eastern spiny lobster *Sagmariasus verreauxi* eyestalk and brain and thoracic ganglia. Font size represents relative expression (based on RNA-seq quantitative analysis) of each neuropeptide group. The inhibitory CHH family peptides (marked) are more prevalent in the eyestalk. The word cloud is based on digitally computed values presented by Ventura et al. (2014)

The MIH signal is inhibitory and is predominantly produced and secreted from the eyestalk. As a consequence, most of early physiological studies relied on the fact that molting is being promoted simply by removal of the eyestalk and the suppressive signal along with it (Gabe, 1953; Echalier, 1959). Since these early studies, MIH was identified and functionally characterized in a multitude of decapod crustacean species. The MIH is predicted to suppress ecdysone production by the YO through binding a G protein coupled receptor (GPCR). Such GPCR is yet to be identified, although candidate receptors were identified in transcriptomic libraries from some decapod crustaceans (Buckley et al., 2016), based on phylogenetic similarity with GPCRs which bind ion transport peptide—a CHH-derived hormone in the silk moth (Nagai et al., 2014). In insects, on the other hand, the upstream neuroendocrine signal that governs ecdysone production is stimulatory and includes a larger neuro-hormone named prothoracicotropic hormone, which is produced in the brain (Gilbert & Rybczynski, 2008). While upstream, there is much difference between crustaceans and insects, downstream of 20HE there is more similarity between the subphyla at the molecular level of the molting process; in both decapod crustaceans and insects, the secreted ecdysone diffuses through the hemolymph and it is converted to the active, hydroxylated 20HE once it reaches target tissues. This active form then binds to nuclear receptors that upon binding, dimerize with partner promiscuous receptors (e.g., ultraspiracle in *D. melanogaster*) and together, this heterodimer enables a chain of transcriptomic activations that lead to molting (King-Jones & Thummel, 2005). The retinoid X receptor (RxR) is perhaps the common thread throughout evolution of metamorphosing animals, as it is the single most conserved component featuring in metamorphosis from cnidarians (Fuchs et al., 2014) to higher vertebrata (Brown & Cai, 2007; Laudet, 2011). Both 20HE receptor and RxR were characterized in decapod crustaceans (Chung et al., 1998; Durica et al., 2002; Nagaraju et al., 2011; Techa & Chung, 2013; Girish et al., 2015; Gong et al., 2015), with 20HE receptor implicated in reproduction regulation (Durica et al., 2002; Nagaraju et al., 2011; Gong et al., 2015). The mechanism of ecdysis is thoroughly studied at the biochemical and more recently the transcriptome levels using the YO as a model tissue to develop a better understanding of how an upstream regulatory pathway affects the ecdysone production and secretion (Das et al., 2016). Annotation of genes relevant for the process rely predominantly on information gathered in insects with the earliest studies performed on polytene chromosomes isolated from *D. melanogaster* salivary glands of pre-pupal 3rd instar larvae (Daneholt, 1975). At this stage, the salivary glands include unique chromosomes that are generated by multiple replication events in the absence of cell divisions. This leads to enlarged cells that include large chromosomes, each including a bundle of many hundreds of chromatids aligned and packed with histones to form a specific banding pattern that correlates with the active genes (euchromatin; genomic regions that are not densely packed and thus appear as bright when stained) and the non-active genes (heterochromatin; densely packed DNA that

appear as dark bands). The banding pattern was found to be highly repetitive among individual larvae and allowed for establishing the role of 20HE in promoting the wide network of gene activation required for metamorphosis. When a gene is actively transcribed in polytene chromosomes, the chromatids loosen to enable the binding of the transcription factors, forming what was visually defined as a puff (Daneholt, 1975). In order to define the role of 20HE at the molecular level, salivary glands were isolated and incubated with varying amounts of 20HE (Ashburner, 1973). The genes transcribed following 20HE incubation over several time points were then visualized and later sequenced (Dworniczak et al., 1983; Guay & Guild, 1991). This methodology clearly showed that 20HE promotes the transcription of a small group of genes known as the early genes, as they are all activated within minutes from exposure of the salivary glands to 20HE. Then, when these genes were sequenced, it became evident that they include additional nuclear receptors as well as transcription factors (Guay & Guild, 1991). This means that the broad effects mediated by 20HE are amplified by recruiting additional transcription regulatory mechanisms that act downstream 20HE. Later it became evident that there are several isoforms for 20HE receptor that show tissue and stage-specific patterns of expression (Talbot et al., 1993), further explaining the complex nature of the molting process that enables the unfolding of a wide array of genes in an accurately orchestrated spatial–temporal manner. In several decapod crustaceans, multi-genic studies have identified key components important for the molt process, downstream of 20HE (Bauer et al., 2013; Powell et al., 2015), including studies of life stages in various taxa which rely on transcriptomics (Qian et al., 2014; Wei et al., 2014; Li et al., 2015). Parallel to establishing this mechanism, genes that are involved in the metabolism of 20HE from its initial precursor cholesterol started to be identified. These included the Halloween genes, named after the disfigured individuals that lacked them (null mutations) (Rewitz et al., 2006a, b). These disfigurations were owing to the absence of 20HE required to enable properly timed molting (Rewitz et al., 2007). Several stages in the process of converting cholesterol to 20HE are better defined than others. A review of what is known in crustaceans was given by Mykles (2011). The Halloween genes are a group of Cytochrome P450 (CYP450) genes that are conserved across ecdysozoans (Rewitz et al., 2007). In arthropods, these genes, as well as CYP18A1 which converts 20HE into an inactive metabolite (Guittard et al., 2011), were identified as the most conserved enzymes from the exceptionally rapidly evolving CYP450 group (Feyereisen, 2011). In decapod crustaceans, there was until recently a clear omission of a shade ortholog (Sin et al., 2015). Shade (CYP314A1) is responsible in insects for the final stage of converting ecdysone to the active 20HE (Petryk et al., 2003). Since 20HE was found to be the active hormone in decapod crustaceans (Chang and Mykles 2011), it raised the question of whether there is another CYP450 in decapod crustaceans that fills this roll. As later discussed in this article, an integrated omics approach enabled the identification of such a group of enzymes. Since unique to crustaceans and apparently different to the shade group of genes, this group is named 'shed' (Ventura et al., 2017).

Juvenile hormone

The juvenile hormone (JH) is the key factor correlated with the metamorphic transition in insects (Tobe & Bendena, 1999). It is important to highlight that in holometabolous insects, with which much comparative research has been performed, metamorphosis is part of the transition from larval to adult, whereas in crustaceans, there is great diversity in metamorphosis mechanisms and life strategies in decapods, further restricting the comparisons that can be drawn, even within decapods. Like ecdysone, it is also a lipid metabolite, resembling the retinoic acid that modulates transcription by binding RxR. Indeed, RxR was considered as a plausible nuclear receptor that can regulate transcription upon binding JH, although recently a more likely candidate named that the methoprene-tolerant (Met) gene was identified and characterized (Charles et al., 2011; Miyakawa et al., 2013; Zhao et al., 2014). The insect JH was found to include several active forms with species-specific variation in presence and activity. With many exceptions and complex fluctuations of both 20HE and JH, it is generally accepted that the presence of JH prevents metamorphic molts. Therefore, molts initiated by 20HE in the presence of JH lead to a larva-to-larva transition. When JH is cleared from the hemolymph and target cells, 20HE-induced ecdysis eventuates in

metamorphosis (Tobe & Bendena, 1999; Daimon et al., 2012). In crustaceans, there is no clear role for JH and even the presence of JH is debated (Laufer et al., 1987). Moreover, it is not clear if there exists a CYP450 enzyme that converts the precursor methyl farnesoate (MF) to JH (known in insects as CYP15A1) (Helvig et al., 2004; Sin et al., 2015). Methyl farnesoate was found to be the active compound in decapod crustaceans (Laufer et al., 1987) and it is generally accepted that MF serves as the decapod equivalent of the insects JHs, which similar to JH also assumes a role in promoting reproduction later in development (Laufer & Biggers, 2001). However, the role of MF in crustacean metamorphosis is less understood particularly for metamorphic transitions which do not coincide with juvenile development. Similar to the 20HE precursor produced by the YO, MF's precursor, farnesoic acid (FA) is produced in crustaceans by a defined endocrine gland known as the mandibular organ (MO). The equivalent organ in insects is the corpus allatum. Just like the YO, the MO is under the suppressive regulation of a hormone from the CHH family of neuropeptides that is quite similar to MIH and is known as MO inhibiting hormone (MOIH). It is important to note that the MIH/MOIH are functionally defined only in a handful of species and are so remarkably variable between species that it is hard to define with no functional assay which plays what role. Additionally, all the CHH group of neuropeptides are considered to be pleotropic and synergistic in effect making it even harder to distinguish and assign clear roles for each isoform (Liu et al., 1997). Downstream from the MO, the secreted FA is being converted to the active MF in target tissues. Methyl farnesoate then binds to a nuclear receptor Met as in insects (Charles et al., 2011; Zhao et al., 2014) and as the MF binding receptor in *Daphnia magna* (Straus, 1820) and *Daphnia pulex* (Linnaeus, 1758) (Miyakawa et al., 2013), where one amino acid change skews the receptor's affinity from JH towards MF. In *D. melanogaster*, it was also shown that not only JH derivatives, but also MF can bind Met to suppress metamorphosis (Wen et al., 2015). The interaction pathway between 20HE, RxR, and Met (Bitra & Palli, 2009) is still lacking and more research is required to enable a clear understanding of how larval forms are maintained in the presence of MF/JH and how metamorphosis is enabled at its absence.

An illustration of the endocrine pathway which regulates ecdysis and metamorphosis in crustaceans is presented in Fig. 2. CHH family members produced primarily by the eyestalk XO-SG inhibit the synthesis of ecdysteroids by the YO and also inhibit the synthesis of farnesoic acid by the MO. When the inhibitory signal is removed, YO synthesizes and
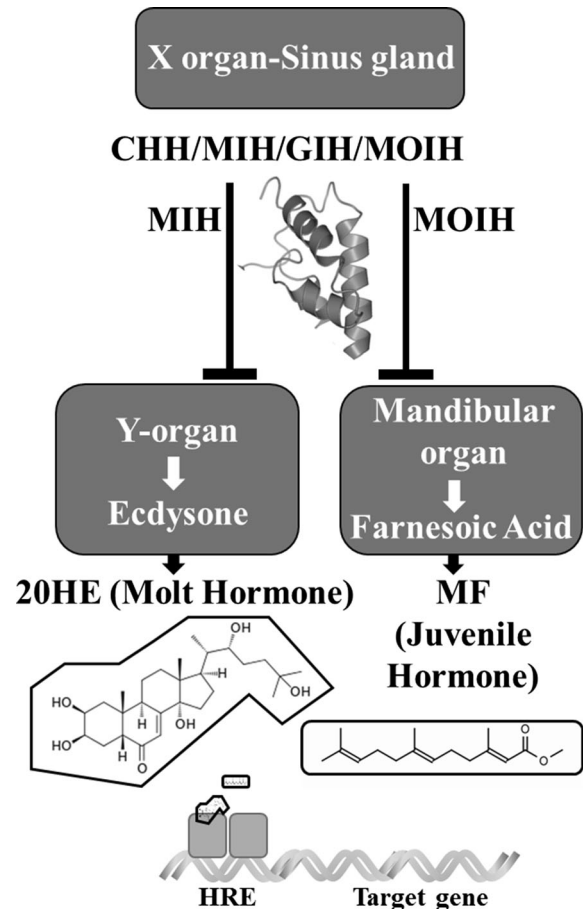
Fig. 2 Illustration of the endocrine pathway which regulates ecdysis and metamorphosis in crustaceans. The X Organ-Sinus Gland-borne inhibitory signals include members of the Crustacean Hyperglycemic Hormone (CHH) family. More specifically, Molting Inhibiting Hormone (MIH) inhibits the synthesis of ecdysteroids by the Y Organ (YO) and Mandibular Organ Inhibiting Hormone (MOIH) inhibits synthesis of farnesoic acid (FA) by the mandibular organ. When the inhibitory signal is removed, YO synthesizes and secretes ecdysone that is converted to 20HE in epithelia across the animal. Similarly, FA is converted to Methyl Farnesoate (MF) and both act through nuclear receptors that dimerize with different partners upon binding of the hormone the hormone-bound dimer binds to regulatory regions upstream target genes (hormone responsive element; HRE) and activates their transcription

secretes ecdysone which is converted to 20HE in epithelia across the animal and similarly Farnesoic acid is converted to MF. Both 20HE and MF act through nuclear receptors that dimerize with different partners like RxR and upon binding of the hormone, the hormone-bound dimer binds to regulatory regions upstream target genes and activates their transcription. This simplified illustration requires revisiting to accommodate the vast differences in molecular mechanisms that must accompany the different life strategies exhibited by various groups of decapod crustaceans. Following expression patterns of key molecules at defined sub-stages of molting towards metamorphosis could lead to a better resolution.

Diversity in mechanisms

As an example for how diverse decapod crustacean life histories leading to metamorphosis are, the freshwater crayfishes do not exhibit any larval stages outside the embryo, whereas spiny lobsters have an extended larval phase lasting as much as 24 months, composed of up to 24 molts, with slight morphological changes and size increment between these stages (Booth & Phillips, 1994; Palero et al., 2014). These culminate to the metamorphic ecdysis process that transforms a planktonic larva into the benthic juvenile stage. Since juveniles of some spiny lobsters require benthic coastal habitat, the extended planktonic larval phase poses a significant spatial and energetic cost due to their wide dispersal with oceanic currents (Fitzgibbon et al., 2014). To meet this cost, spiny lobster larval development incorporates a mechanism for navigating back to the continental shelf. This is accomplished by biphasic larval to juvenile metamorphosis; instead of a direct juvenile metamorphosis as encountered in palaemonid prawns for instance (Ventura et al., 2013). The leaf-shaped, flat, and planktonic spiny lobster larvae (termed phyllosoma) metamorphose into an intermediary, nektonic, miniature adult-like larval phase, called puerulus. The puerulus migrates towards the shore while supporting energy demands with lipid reserves accumulated during the phyllosoma stages (Jeffs et al., 2001). Once reaching suitable benthic habitat, it settles and completes its metamorphosis by molting to a spinous, pigmented juvenile that then can commence feeding again (Jeffs et al., 2005). The large size (up to 40 mm), slow

development (20–30 days), and transparency of the spiny lobsters final larval stages, enable precise identification of molt phases throughout metamorphosis. Using transcriptomic libraries of various stages of the Eastern spiny lobster *Sagmariasus verreauxi* (H. Milne Edwards, 1851), the expression pattern of many genes was followed throughout metamorphosis (Ventura et al., 2015). As generally the case in decapod crustacean transcriptomes, the level of uncertainty in annotation is high and reaches up to 70% unannotated transcripts. The lack of publicly available genomes adds to the misidentification of genes and even the annotated 30% could be misidentified and not adequately annotated when guided by BLAST results rather than phylogenetic analysis. Only after meticulous phylogenetic analysis can it be identified that BLAST results tend to be very misleading, with the exception of highly similar sequences of genes that encode a clear homolog of previously defined genes.

Omics-derived insights

Metamorphosis accounts for significant restructuring of body parts, including apoptosis of larval cells and generation of new appendages. Much research has been done into the salivary glands in *D. melanogaster*, which undergo apoptosis during metamorphosis in response to ecdysone (Denton et al., 2013), providing a visual model for transcription as it occurs (Dworniczak et al., 1983; Guay & Guild, 1991), as well as the emergence of wing disks (Garcia-Bellido, 1975). Metamorphic changes account for a large proportion of the genome being differentially transcribed within a short timeframe. Therefore, a broader understanding of the transcription regulation network is required. Recently, more and more approaches combine computerized and high-throughput in vivo methodologies to detect these networks using knowledge of the transcription factors and regulatory elements they bind on the genome, as well as a suite of tools for gene editing/expression regulation (Martín et al., 2016). The lack of fully annotated genomes and high-throughput in vivo tools in malacostracan crustaceans [with the exceptions of the widely used gene silencing approach (Sagi et al., 2013) and recent use of CRISPR technology (Martin et al., 2016)], hinders the progress of comparative analysis of metamorphosis and the knowledge gained in insect research is expected to

continue driving new discoveries in other taxa. Nevertheless, crustaceans include diverse life forms with varying modes of metamorphosis, providing a fertile ground for comparative research (Scholtz, 2004). This versatility could enable a more comprehensive view of metamorphosis compared to what is learned through inspection of just a few ecdysozoan model organisms.

Ecdysone and JH/MH are the two mechanisms most identified with metamorphosis in arthropods. As lipid compounds, they may translate a nutritional cue into a developmental increment. Like metamorphosis, reproduction is also an energy-demanding process and it is thus not surprising to find that both ecdysone and JH/MF play a role in ovarian maturation regulation (Comas et al., 2001; Laufer & Biggers, 2001; Rotllant et al., 2001; Nagaraju et al., 2003; Parthasarathy et al., 2010). Less studied in crustaceans though, are additional energetic threshold translating mechanisms. These include the insulin-like peptide (ILP) and nutrient-regulated pathways that regulate yolk accumulation in mosquito eggs, alongside ecdysteroids and terpenoids (Hansen et al., 2014). Although ILPs in decapod crustaceans were identified long ago, they were considered until recently to involve a singular case of masculinizing hormone secreted from a male-specific endocrine gland (Ventura et al., 2011). With recent identification of additional, non-masculinizing ILPs (Chandler et al., 2015), as well as ILP receptors (Sharabi et al., 2015; Aizen et al., 2016), it is expected that a more complete picture of the regulatory mechanisms that govern metamorphosis will be gained in crustaceans through utilizing the ever-increasing number of developmental stages transcriptomes. In species like *S. verreauxi*, where knowledge regarding the identity of the molecular components that facilitate metamorphosis was absent, a thorough transcriptomic investigation highlights all the components known to be involved in metamorphosis in other crustaceans (Ventura et al., 2015). As an example, sequences encoding for peptides of the CHH family can be rapidly identified. Expression pattern analysis might then lead to speculation of which CHH is the MOIH, as it is expected to fluctuate through metamorphosis (Ventura et al., 2015). This approach, while speculative, narrows down the search and facilitates a shortlist of candidate hormones that could serve for validation using in vitro or in vivo assays. Additionally, since a vast percentage of the transcripts are differentially expressed throughout metamorphosis and are vastly unknown sequences (Ventura et al., 2015), a computerized approach could serve to highlight key components that are novel and have features of potential hormones based on structure and expression pattern, as was done in the moon jellyfish *Aurelia aurita* (Linneus, 1758) (Fuchs et al., 2014).

Moreover, there is near a complete knowledge gap in the molecular pathways that regulate the influence on environmental cues in crustacean metamorphosis. It is well established that physical or biological cues can influence the timing of settlement and metamorphosis of the final larvae stage of marine invertebrates, including crustaceans (Pechenik, 1990; Anger, 2001). For benthic marine species with planktonic larval stages, flexibility in the timing of settlement and juvenile transition is advantageous because it can enhance the probability of finding suitable habitat for juvenile development. In the absence of appropriate cues, larvae can delay metamorphosis until a temporal or nutritional threshold is reached, beyond which metamorphosis can occur spontaneously (Pechenik, 2006). When settlement and juvenile molting is delayed, it can result in reduced post-larval fitness. For example, delayed metamorphosis in the absence of settlement cues results in reduced survival and body size of 1st instar juvenile crabs (Gebauer et al., 1998). Numerous cues have been identified that trigger metamorphosis in crustaceans with most related to physical, chemical, or biological properties of favorable settlement substrates or habitat, including substrate type, vegetation, habitat-associated sound, and chemical substances released by conspecifics (Anger et al., 2006; Stanley et al., 2015; Pine et al., 2016). Similar to what has been demonstrated with other marine invertebrate taxa, the application of modern omic techniques will be crucial to trace molecular pathways that trigger larval settlement and the progression of crustacean metamorphosis (Thiyagarajan, 2010).

Potential identification of CYP15A1

Most CYP450s, apart from the Halloween family, are not involved in metamorphosis, except for CYP15A1—the enzyme that converts MF to JH, and thus regulates JH levels in insects (Helvig et al., 2004; Daimon & Shinoda, 2013). Since MF is the active

juvenoid compound in crustaceans, it aligns with the fact that CYP15A1 could not be identified in most crustaceans (Sin et al., 2015). Two transcriptomic studies have identified CYP15A1 orthologs in crustaceans; the first was identified in the giant freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879) (Ventura et al., 2013) and the next in the Eastern spiny lobster *S. verreauxi* (Ventura et al., 2015), although their function is yet to be proven. In *S. verreauxi*, CYP15A1 was found to be specific to the antennal gland (which is linked with the mandibular gland in this species) and was therefore assumed to be involved in breakdown of MF into JH and clearance through the antennal gland pore. This was supported by increased expression of *CYP15A1* during late metamorphosis (Ventura et al., 2015). A support for the putatively conserved function of the crustacean CYP15A1 homologs in converting MF to JH is given by a recent study in *M. rosenbergii* that identified the genes encoding the enzymes that play a role in the JH production pathway at the transcriptome level and the actual JH at the metabolome level. Since the antennal gland also secretes pheromones, it was hypothesized that JH could serve as a pheromone in *M. rosenbergii* females, as it was identified immediately following a reproductive molt (Bose et al., 2017). Yet, it is not clear if there is a circulating JH in crustaceans.

Identification of shed

As in the case of CYP15A1, explaining how MF is the active juvenoid in crustaceans, another missing link that could not be so readily explained is the enzyme which hydroxylates ecdysone into 20HE. The active ecdysis hormone is 20HE in both crustaceans and insects and it is thus expected that both taxa will have a mechanism in place to facilitate a well-regulated hydroxylation of ecdysone. Still, while the Halloween gene named 'shade' was found to be highly conserved in insects, no clear ortholog could be assigned in crustaceans (Mykles, 2011; Sin et al., 2015). A phylogenetic examination of the repertoire of CYP450 transcripts identified a group of five unannotated CYP450s in the Eastern spiny lobster *S. verreauxi*, including one differentially expressed through metamorphosis in a pattern that suggests it is implicated in metamorphosis. Additionally, these five unannotated CYP450s formed a cluster between

Fig. 3 Phylogenetic analysis of crustacean and insect Halloween genes highlighting the convergent evolution of the crustacean *shed* clade and the insect *shade* clade. Full species names and sequences used can be found in Supplementary File 1. Molecular Phylogenetic analysis was performed by Maximum Likelihood based on the Le and Gascuel model (Le & Gascuel, 2008). The tree with the highest log likelihood (− 49740.4739) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.0745)). The rate variation model allowed for some sites to be evolutionarily invariable ([+ I], 1.0614% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 68 amino acid sequences. There were a total of 721 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The crustacean 'shed' clade (blue) includes enzymes that are structurally similar to the insect 'shade' clade (green). This can be seen by the superimposed predicted 3-dimensional representatives (models were modified from (Ventura et al., 2017)). In vitro assay proved that a 'shed' clade representative converts ecdysone to 20HE, similar to 'shade' (Ventura et al., 2017)

'spook' and CYP18A1, which are involved in early stages of ecdysteroidogenesis and 20HE degradation, respectively. While different in primary sequence from the insects 'shade' gene, they showed high structural similarity when their predicted 3D structure was superimposed on the *D. melanogaster* 'shade' protein [Fig. 1; modified from (Ventura et al., 2017)]. Finally, using an in vitro assay, it was shown that a gene from this cluster transfected into mammalian cells facilitated the hydroxylation of ecdysone (Ventura et al., 2017). Since this cluster of enzymes is distinct from the insects 'shade' and since crustaceans shed their exoskeleton well before insects evolved, this enzyme system was named 'shed' (Ventura et al., 2017). In this manuscript, a molecular phylogenetic tree depicts additional 'shed' clade members gathered from publicly available databases, as well as additional Halloween genes from insects and crustaceans (Fig. 3). As can be seen, this is a well-conserved CYP450 clade in crustaceans. Of note, two isoforms of 'spook' were identified in the black back land crab *Gecarcinus lateralis* (Freminville, 1835), as evident in several insects. The characterization of Shed sequences within the recently obtained genome of
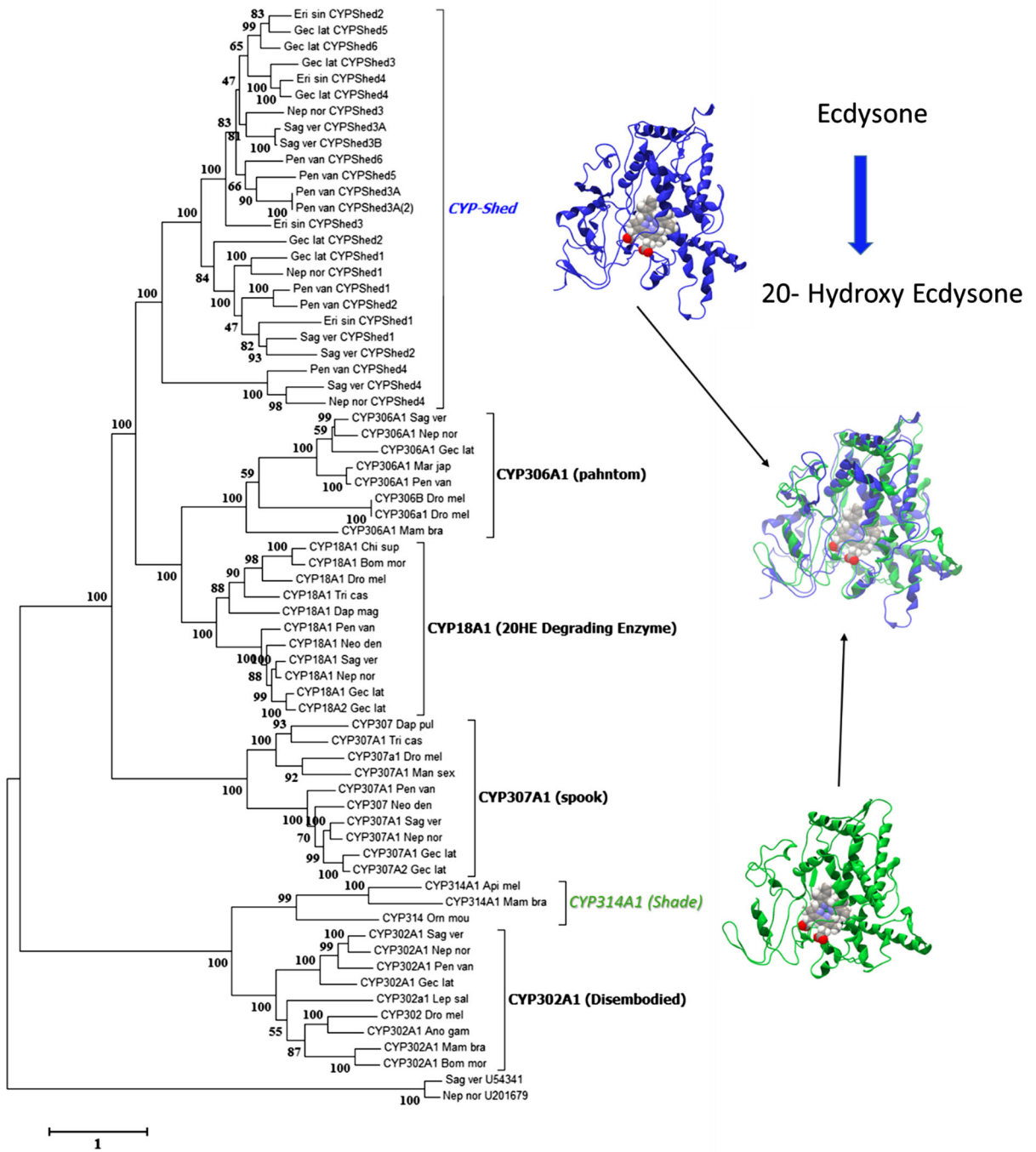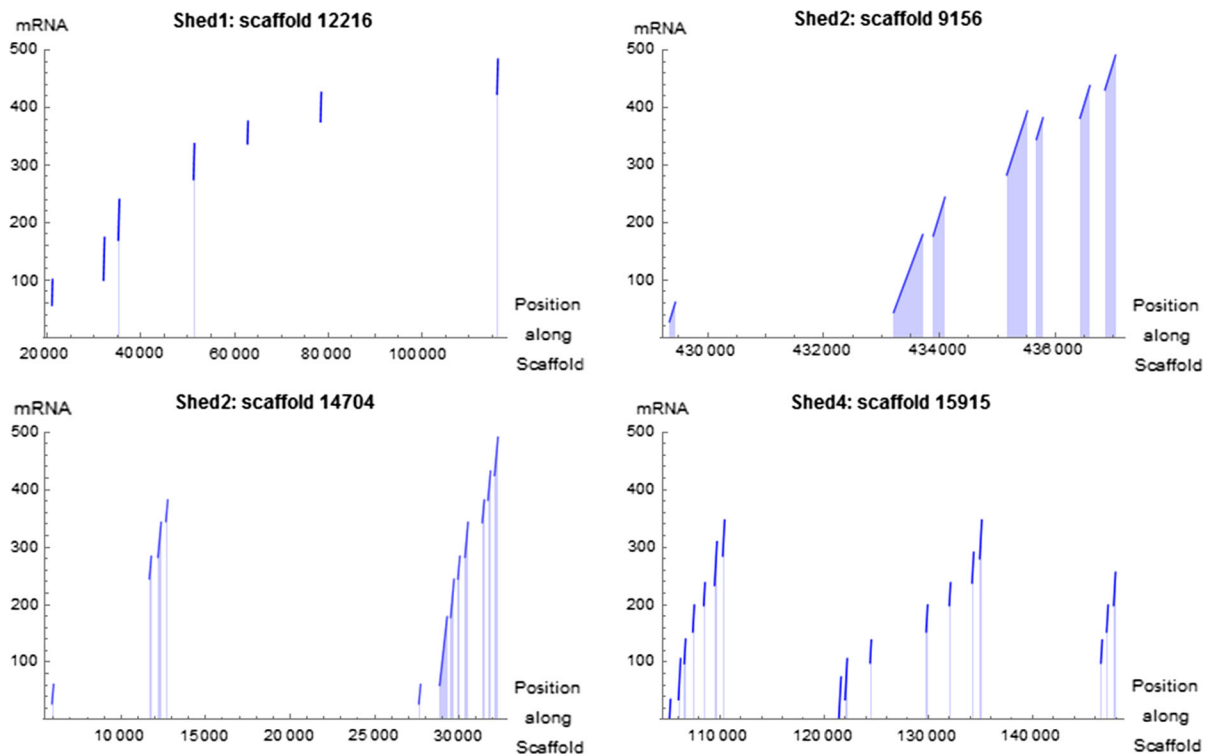
Fig. 4 Genomic architecture of several Shed genes in the Chinese mitten crab. The sequences of several Shed genes obtained from transcriptomic data were blasted against the recently assembled genome of *Eriocheir sinensis* and allowed us to identify exonic regions (blue lines) and intronic regions (gaps between consecutive lines). The relative position of the exons along the genomic scaffold is indicated along the X axis. Multiple copies appeared in tandem (bottom figures) and for those cases (scaffolds 14704 and 15915) each *Shed* gene copy is located about 10–20 kb apart

the Chinese mitten crab *Eriocheir sinensis* (H. Milne Edwards, 1853), allowed us to identify the regions encoding for Shed1, Shed2, and Shed4 in genomic scaffolds and therefore to characterize their exon–intron architecture. Interestingly, Shed1 was found to span around 100 kb, including an extremely large intron about 40 kb in length, Shed2 was found in several scaffolds and Shed4 was present in multiple copies located in tandem about 10-20 kb apart within the same scaffold (Fig. 4).

Conclusions

Omics technologies have considerably paced up the rate at which molecular mechanisms which regulate metamorphosis are discovered in crustaceans. Critically, the immense information gathered is predominantly relying on previous research performed either in insects or crustaceans. High-throughput methods for examining new hypotheses either in vitro or in vivo, in conjunction with computerized methodologies to screen unannotated sequences, will truly transform the field of research.

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