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The moulting arthropod: a complete genetic toolkit review

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ABSTRACT

Exoskeletons are a defining character of all arthropods that provide physical support for their segmented bodies and appendages as well as protection from the environment and predation. This ubiquitous yet evolutionarily variable feature has been instrumental in facilitating the adoption of a variety of lifestyles and the exploitation of ecological niches across all environments. Throughout the radiation that produced the more than one million described modern species, adaptability afforded by segmentation and exoskeletons has led to a diversity that is unrivalled amongst animals. However, because of the limited extensibility of exoskeleton chitin and cuticle components, they must be periodically shed and replaced with new larger ones, notably to accommodate the growing individuals encased within. Therefore, arthropods grow discontinuously by undergoing periodic moulting events, which follow a series of steps from the preparatory pre-moult phase to ecdysis itself and post-moult maturation of new exoskeletons. Each event represents a particularly vulnerable period in an arthropod's life cycle, so processes must be tightly regulated and meticulously executed to ensure successful transitions for normal growth and development. Decades of research in representative arthropods provide a foundation of understanding of the mechanisms involved. Building on this, studies continue to develop and test hypotheses on the presence and function of molecular components, including neuropeptides, hormones, and receptors, as well as the so-called early, late, and fate genes, across arthropod diversity. Here, we review the literature to develop a comprehensive overview of the status of accumulated knowledge of the genetic toolkit governing arthropod moulting. From biosynthesis and regulation of ecdysteroid and sesquiterpenoid hormones, to factors involved in hormonal stimulation responses and exoskeleton remodelling, we identify commonalities and differences, as well as highlighting major knowledge gaps, across arthropod groups. We examine the available evidence supporting current models of how components operate together to prepare for, execute, and recover from ecdysis, comparing reports from Chelicerata, Myriapoda, Crustacea, and Hexapoda. Evidence is generally highly taxonomically imbalanced, with most reports based on insect study systems. Biases are also evident in research on different moulting phases and processes, with the early triggers and late effectors generally being the least well explored. Our synthesis contrasts knowledge based on reported observations with reasonably plausible assumptions given current taxonomic sampling, and exposes weak assumptions or major gaps that need addressing. Encouragingly, advances in genomics are driving a diversification of tractable study systems by facilitating the cataloguing of putative genetic toolkits in previously under-explored taxa. Analysis of genome and transcriptome data supported by experimental investigations have validated the presence of an "ultra-conserved" core of arthropod genes involved in moulting processes. The molecular machinery has likely evolved with elaborations on this conserved pathway backbone, but more taxonomic exploration is needed to characterise lineage-specific changes and novelties. Furthermore, linking these to transformative innovations in moulting processes across Arthropoda remains hampered by knowledge gaps and hypotheses based on untested assumptions.

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Promisingly however, emerging from the synthesis is a framework that highlights research avenues from the underlying genetics to the dynamic molecular biology through to the complex physiology of moulting.

Key words: Arthropoda, development, ecdysis, evolution, exoskeleton, genes, hormones, mechanisms, pathways, regulation.

CONTENTS

I. INTRODUCTION

Arthropoda represents a striking example of evolutionary success and diversity within the animal kingdom, holding the record for the phylum with the largest number of species amongst Metazoa, with more than one million described species in the Catalogue of Life (Bánki *et al.*, 2023). The arthropods emerged more than 500 million years ago from their last common ancestor within the superphylum Ecdysozoa, which includes all animals that grow by ecdysis, or the moulting of their exoskeletons (Daley *et al.*, 2018; Giribet & Edgecombe, 2019). The emerging lineages formed what have come to be recognised, albeit with ongoing refinements, as four major extant arthropod groups (Fig. 1A): Chelicerata, Myriapoda, Crustacea, and Hexapoda, where hexapods and the paraphyletic crustaceans together form the monophyletic Pancrustacea (Giribet & Edgecombe, 2019). Throughout their evolutionary history, arthropods have developed a broad variety of lifestyles and have populated ecological niches in all environments, colonising freshwater, marine, terrestrial, and aerial habitats (Rota-Stabelli, Daley & Pisani, 2013; Belles, 2019). Thus they play key roles in virtually all ecosystems and greatly impact human well-being, generally through provision of ecosystem services and specifically as pollinators, agricultural pests, and disease vectors.

Despite their unrivalled diversity, arthropods all share a conserved, modular body plan composed of units repeated along the anterior–posterior axis (Chipman & Edgecombe, 2019). Importantly, this segmented body plan provides the opportunities for morphological specialisations that have produced the diversity of forms observed today. Another key shared feature is the presence of a chitinous exoskeleton. With its rigid composition of mainly chitin and cuticular proteins, the exoskeleton provides general support for the body and specialised body structures, as well as forming a protective barrier from the external environment and from predation (Roer, Abehsera & Sagi, 2015). While arthropod bodies develop and grow, chitin and cuticle proteins are progressively deposited by the epidermis to reinforce the exoskeleton, thereby leading to constraints on the size and morphology of the encased tissues within. To escape these physical constraints, arthropods must undergo moulting events during which the old exoskeleton is shed and replaced with a new, larger one. These exuviation events are prompted by signals from shifts in the balance of external and internal conditions, such as tissue and limb damage, feeding status, and body threshold size (Nijhout *et al.*, 2014; Das, 2015). While many moulting features are similar in other ecdysozoans, molecular moulting components underlying these processes remain elusive for most ecdysozoans beyond Arthropoda, even nematodes (Lažetić & Fay, 2017; De Oliveira, Calcino & Wanninger, 2019), we therefore focus our review on the four major extant arthropod groups.

Moulting *sensu lato* involves a preparatory pre-moult phase, characterised by apolysis, the enzymatic digestion of the

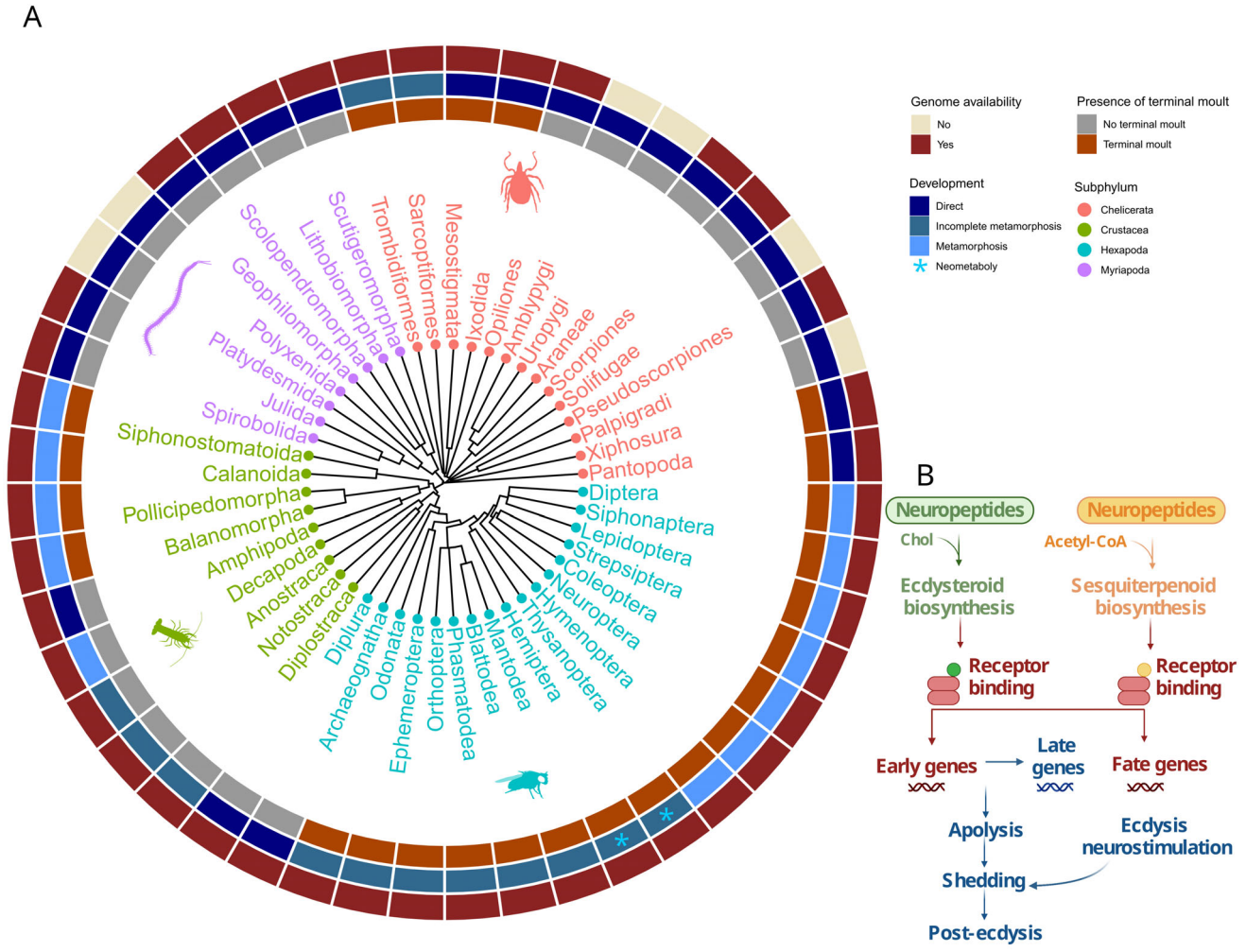

Fig. 1. The diversity of arthropod moulting and development modes is supported by a common molecular framework that governs moulting processes. (A) A subset of major arthropod orders according to the phylogeny from TimeTree.org (Kumar *et al.*, 2022) annotated with simplified categories of moulting and development modes, and genome data availability. The colour-coded legend shows categories as follows: taxonomy highlighting the four extant arthropod subphyla; presence of terminal moult indicating whether moulting events occur continuously throughout the entire life cycle; developmental traits grouped into four simplified classes; and genome availability indicating whether a genome assembly from at least one species from the group is available at the National Center for Biotechnology Information (NCBI) (05/09/2023) (Feron & Waterhouse, 2022*a*). Simplifications in developmental traits conceal known underlying complexity and variation, but aim to help clarify discussions of the framework of molecular components governing moulting processes in the context of arthropod development. Direct development refers to the absence of organismal transformation before the individual reaches adulthood, in contrast to metamorphosis where profound morphological, physiological, and ecological alterations are observed (Martin *et al.*, 2014; Ventura *et al.*, 2018; Jindra, 2019); where the profound transformations of the pupa into the adult form that define holometabolous insects are observed, it is termed "complete metamorphosis". Flexibility is allowed when several moults mediate subtle modifications gradually along the path to adulthood, as in the case of some crustacean anamorphic orders (Martin *et al.*, 2014); such groups are categorised as having "incomplete metamorphosis", based on hemimetabolous insects where nymphs indirectly develop as miniature adults, slowly maturing wing pads (Jindra, 2019; Truman, 2019; Suzuki *et al.*, 2021). Neometaboly refers to life cycles where feeding larvae become adults *via* non-feeding pupal stages and the appearance of external wing primordia (Suzuki *et al.*, 2021). (B) A schematic representation of the common molecular pathway components that facilitate the preparation for, execution of, and recovery from exuviation. Ecdysteroid and sesquiterpenoid synthesis is stimulated; hormone-receptor complexing triggers activation cascades of early, late, and fate determination genes; detachment of cuticle and the ecdysis motor is induced for exoskeleton shedding. Acetyl-CoA, acetyl coenzyme A; Chol, cholesterol. Details for each summarised phase are provided in colour-matched Figs 2–6. Silhouettes from phylopic.org: *Drosophila melanogaster* (Hexapoda), *Procambarus clarkii*, (Crustacea), *Strigamia maritima* (Myriapoda), *Ixodes scapularis* (Chelicerata).

inner layers of the existing cuticle to allow for the detachment of the old exoskeleton from a monolayer of epidermal cells that start actively producing a newly secreted soft casing (Roer *et al.*, 2015; Zhang *et al.*, 2021). Moulting *sensu stricto* refers to the ecdysis event itself, where the old exoskeleton is ruptured due to a combination of movement and increased haemolymph pressure within the body, allowing the animal to shed the exuvia and escape through sutural exit gapes (Daley & Drage, 2016). After emerging, the post-moult phase is characterised by cuticle tanning, which includes sclerotization, mineralisation, and pigmentation, which promote hardening by cross-linking of cuticle proteins and the deposition of minerals and pigments, eventually turning the pale and soft new outer casing into a mature exoskeleton.

While many key observable steps of the moulting process are consistently recognisable across diverse groups of arthropods, the specific behaviours and traits are adapted to accommodate differences in their morphologies, physiologies, and habitats. For instance, moulting modes vary according to the localisation of exuvial sutures, the presence of specific organs for secretion and absorption of biomolecules, and aquatic or terrestrial living conditions (Roer *et al.*, 2015; Daley & Drage, 2016; Ou *et al.*, 2016; Mykles, 2021). Moreover, different life histories and types of development (Fig. 1A) might comprise variable numbers of moulting events, and may or may not be characterised by a terminal moult which precedes adulthood and establishes an end to exoskeleton replacement (Tetlie, Brandt & Briggs, 2008; Belles, 2019; Truman, 2019; Moon & Tillinghast, 2020). Notably, the resemblance of moulted individuals to their previous inter-moult stage varies greatly, from essentially just an increase in size to the remarkable transformations of pupa to adult moults of holometabolous insects (Ventura *et al.*, 2018; Jindra, 2019).

Reaching a consensus on how to describe and define such transformations, that is processes of "arthropod metamorphosis", is extremely challenging, even among taxonomic groups of the same subphylum (Bishop *et al.*, 2006; Martin, Olesen & Høeg, 2014; Ventura *et al.*, 2018; Jindra, 2019). Defining metamorphosis is complicated because it is intimately connected to the criteria used for the identification of larval/nymphal stages and to the ability to distinguish the magnitude of changes that the juveniles undergo through moulting events (Martin *et al.*, 2014; Ventura *et al.*, 2018; Jindra, 2019). We therefore adopt a generalised framework where the acquisition of characteristics typical of a different developmental phase is referred to as metamorphosis when moulting results in profound morphological, physiological, and ecological alterations (Bishop *et al.*, 2006; Martin *et al.*, 2014; Ventura *et al.*, 2018; Jindra, 2019). For example, post-embryonic stages such as the highly variable crustacean larvae, which show adaptations typical of a planktonic, dispersal phase, undergo dramatic changes on the path to becoming benthic adults, and these profound alterations are referred to as metamorphosis (Martin *et al.*, 2014). The term "complete metamorphosis", however, is reserved for describing the profound transformations of the insect pupa into the adult form that characterise Holometabola, the hallmark feature after which this superorder is named. Metamorphosis in its broader sense can be applied to all arthropod subgroups when referring to changes that determine the loss of immature traits and substantially differentiate the organism from the previous stage. Metamorphosis does not strictly imply attaining of adulthood and sexual maturity nor a terminal moult (Fig. 1A) (Závodská, Sauman & Sehnal, 2003; Martin *et al.*, 2014; Miyakawa *et al.*, 2018; Ventura *et al.*, 2018). For example, crustaceans display a great variety of life histories, characterised by several morphological transformations before adulthood: multiple metamorphoses are possible and reproduction can alternate with moulting events, even after full maturity has been reached (Martin *et al.*, 2014; Miyakawa *et al.*, 2018; Ventura *et al.*, 2018).

Moulting processes are tightly regulated by hormonal control (Fig. 1B). The two main hormone groups are the ecdysteroids and the sesquiterpenoids (Cheong *et al.*, 2015; Qu *et al.*, 2018; Truman & Riddiford, 2019). Upon integration of multiple external and internal stimuli, neuropeptides transduce the signal for hormone biosynthesis from the nervous system to the secretory organs (Nijhout *et al.*, 2014; Ou *et al.*, 2016; Mykles, 2021). After several biochemical modifications, hormones are secreted into the haemolymph to reach their target tissues. As moulting proceeds, enzymes localised in the target tissues, or "peripheral" tissues, catalyse the production of a high concentration of ecdysteroid bioactive hormonal forms, which in turn act as ligands for nuclear receptors (Hill *et al.*, 2013; Qu *et al.*, 2018). Hormone-bound receptors then notably trigger the activation of transcription factors specific for the induction of respective downstream pathways, which in ecdysteroid signalling results in the stimulation of a hierarchical transcriptional cascade (Hyde, Elizur & Ventura, 2019*a*; Truman, 2019). Finally, the declining ecdysteroid titres, due to peripheral catabolism and declining biosynthesis, prompt neurosecretions that activate the "ecdysis motor", a stereotyped behaviour sequence that culminates with the exit from the exuvia followed by cuticle tanning (White & Ewer, 2014; Roer *et al.*, 2015; Zieger *et al.*, 2021).

These insights are the result of decades of scientific studies exploring the molecular mechanisms underlying arthropod moulting in selected representative species. In particular, insect model systems have provided a rich source of experimental evidence to characterise and understand the complexity of the main moulting pathways and their components. These studies provide the basis for a framework with which to develop and test hypotheses regarding the presence and function of molecular moulting components across the breadth of extant arthropod diversity. However, while our knowledge of moulting in insects has been continuously growing and maturing (Tettamanti & Casartelli, 2019; Tsang *et al.*, 2020; Xu *et al.*, 2020), investigations beyond insects remain relatively limited. The establishment of a broader variety of models is being assisted by modern genomics technologies that allow for the production of

high-quality genome resources for an increasing diversity of arthropods (Feron & Waterhouse, 2022*a*,*b*; Cheatle Jarvela & Wexler, 2023), although with non-insect groups still lacking representation (Fig. 1A). The best-studied non-insect group are the crustaceans, although even within this diverse and paraphyletic group the experimental focus has been restricted to only a few orders (Ventura *et al.*, 2018; Hyde *et al.*, 2019a; Knigge, LeBlanc & Ford, 2021; Zhang *et al.*, 2021). Beyond Pancrustacea, molecular experimental work on moulting in chelicerate and myriapod species remains extremely sparse (Chipman *et al.*, 2014; Honda *et al.*, 2017; Li *et al.*, 2017; Nicewicz *et al.*, 2021). Furthermore, research investigations that apply broad cross-taxa approaches to compare and contrast moulting mechanisms are scarce (Qu *et al.*, 2015; Schumann *et al.*, 2018; De Oliveira *et al.*, 2019; Zieger *et al.*, 2021).

These taxonomic imbalances mean that reference works reviewing the different aspects of moulting pathways and their components often form perspectives that are based on a small number of well-studied species (Stay & Tobe, 2007; Ventura *et al.*, 2018; Hyde *et al.*, 2019*a*; Santos, Humann & Hartfelder, 2019; Belles, 2019; Jindra, 2019; Tettamanti & Casartelli, 2019; Truman & Riddiford, 2019; Truman, 2019; Xu *et al.*, 2020; Knigge *et al.*, 2021). Our aim herein is to provide a broad overview of the current status of accumulated knowledge of molecular components implicated in moulting processes across Arthropoda. For this, we build on these reference works by identifying commonalities and key differences, as well as highlighting major gaps. We also incorporate recent advances that inform our understanding of moulting, with a focus on molecular mechanisms, across all extant arthropod groups. At each main phase of moulting we examine the sources of evidence from different taxa supporting the current models of how components operate together to prepare for, execute, and recover from exuviation. The key molecular factors involved include neuropeptides, hormonal biomolecules, hormonal biosynthetic enzymes, and receptors. We also review other genes in moulting, such as the regulatory networks of early genes and fate determination genes, and late genes. Our integrated overview aims to contrast knowledge based on reported observations, reasonably plausible assumptions given current taxonomic sampling, and weak assumptions or major gaps that need to be addressed to achieve a balanced perspective of moulting across Arthropoda.

II. ECDYSTEROID BIOSYNTHESIS AND REGULATION

Ecdysteroids represent the main regulators of the moulting process, since pulses of ecdysteroid titre finely orchestrate in time and space the molecular events specific to each moulting phase (Niwa & Niwa, 2016; Truman, 2019). After stimulation by neuropeptides, ecdysteroid biosynthesis takes place in secretory organs, where dietary sterol is the first substrate for a series of catalytic reactions that through different intermediates leads to release of hormones into the haemolymph. In peripheral tissues, enzymes then mediate a last step through the formation of the bioactive hormone derivative, whose peaking concentration is a strong moulting-inducing signal (Truman, 2019). This canonical pathway, with its key components and steps, has been described in detail in the fruit fly *Drosophila melanogaster* (Niwa & Niwa, 2016; Pan, Connacher & O'Connor, 2021). The elements identified in fruit flies have been systematically used as a benchmark to assess whether an arthropod is equipped with the genetic toolkit for ecdysteroid biosynthesis (De Oliveira *et al.*, 2019). In *D. melanogaster*, ecdysone and its bioactive form 20-hydroxyecdysone (20E) are the predominant hormones, however many further modified compounds have been detected in diverse arthropods (Song *et al.*, 2017). A notable example is that of ponasterone A (25-deoxy-20E, ponA), another ecdysone analogue, which has a moulting-inducing effect in some Chelicerata and Crustacea species (Song *et al.*, 2017; Li *et al.*, 2017; Yang *et al.*, 2021*b*; Legrand *et al.*, 2021). While different species probably have genes for yet to be discovered enzymes, as indicated by the heterogeneity of active biomolecules, the fruit fly reference pathway has served as the primary model guiding ecdysteroid research to date. Therefore, in this section, along with an overview of neuropeptides involved in the control of ecdysteroid production, we focus primarily on genes coding for the canonical (i.e. *Drosophila*) biosynthetic enzymes that have been explored in other arthropod subphyla to date.

(1) Ecdysteroid biosynthesis toolkits comprise stable and dynamic components with many unknowns

The synthesis of ecdysteroids occurs in insect prothoracic glands and ovaries, which have been established as a good model to study hormone synthesis and endocrine secretion (Niwa & Niwa, 2016; Ou *et al.*, 2016; Bian *et al.*, 2019). The pathway and its components (Fig. 2) have been thoroughly characterised in *D. melanogaster* and this system remains the cornerstone model for moulting steroid hormone studies in arthropods (Niwa & Niwa, 2016; Ou *et al.*, 2016; Schumann *et al.*, 2018). For this reason, although it is experimentally possible to detect a cocktail of differentially modified ecdysone derivatives in different lineages (Liu *et al.*, 2018; Yu, Han & Liu, 2020; Knigge *et al.*, 2021), attention has focused on studying the "canonical" enzymes as known from *D. melanogaster* and other model systems, such as the lepidopterans *Bombyx mori* and *Manduca sexta*, and the orthopteran *Locusta migratoria* (Niwa & Niwa, 2016; Ou *et al.*, 2016; Pan *et al.*, 2021).

The first enzyme component of the pathway is a Rieske mono-oxygenase encoded by the gene *neverland*, which produces 7-dehydrocholesterol from cholesterol (Pan *et al.*, 2021; Kamiyama & Niwa, 2022). Arthropods cannot synthesise cholesterol, and must therefore obtain it directly from their diets. Recently, the glutathione

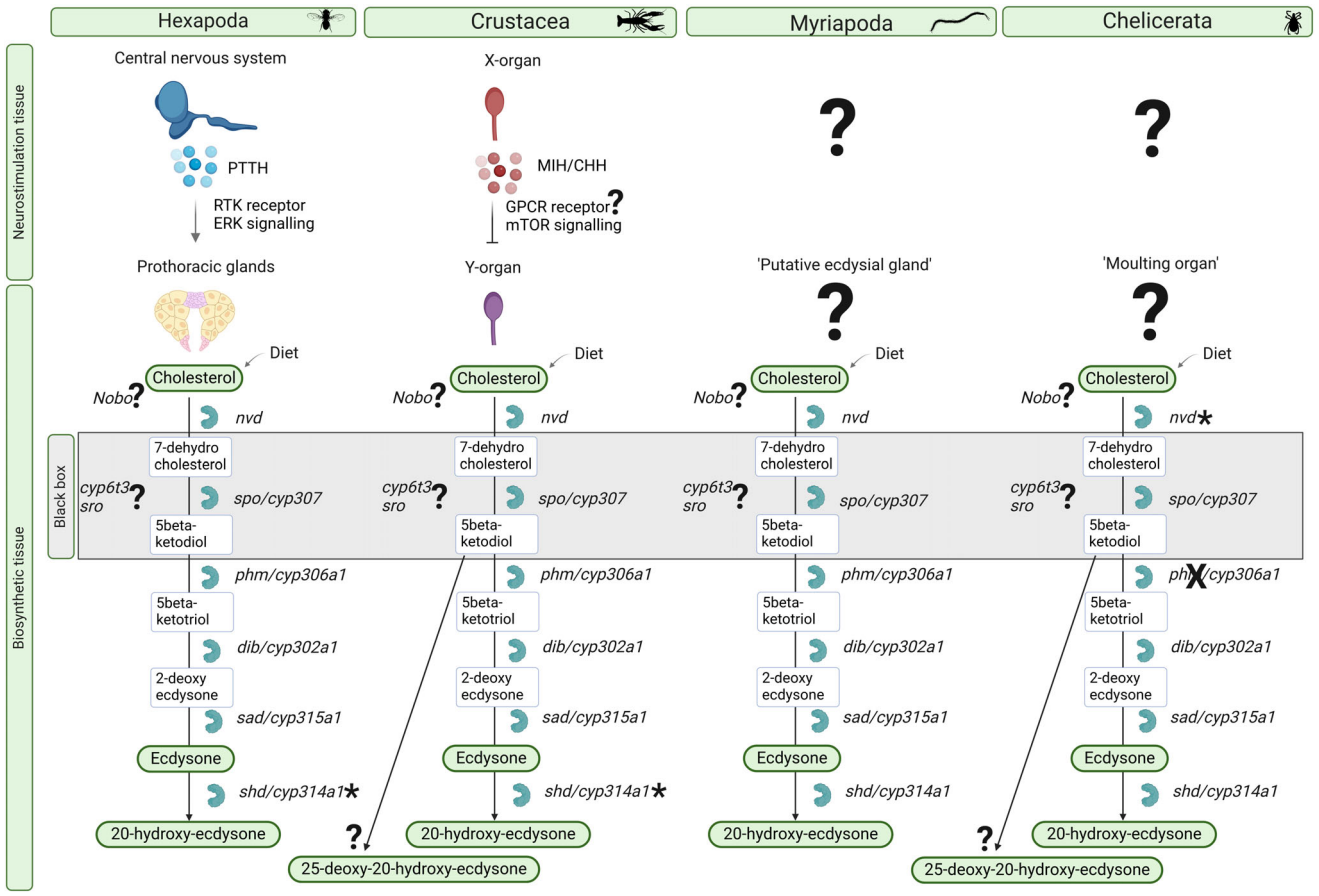

Fig. 2. Ecdysteroid biosynthetic pathways. In insects and crustaceans, initiation of the moulting process (top, neurostimulation tissue) relies, respectively, on positive stimulation of prothoracicotropic hormone (PTTH) and interruption of moulting inhibiting hormone (MIH) inhibition, targeting the prothoracic glands and the Y-organ, that is known specific secretory tissues. In myriapods and chelicerates, however, initiators remain largely elusive and proposed secretory tissues require further investigation. *Nobo*, *cyp6t3*, and *sro* have evidence only in a few insect model systems. Differentially modified ecdysone molecules, for example ponasterone A (ponA, 25-deoxy-20-hydroxy-ecdysone), might be the bioactive form in Crustacea and Chelicerata. *phm* has never been detected in Chelicerata. *shd* is missing in families from Hymenoptera and Decapoda and *nvd* from *Varroa* mite species. Only *spo*-like (*cyp307*) sequences and *sad* have been detected in barnacles. The four columns of pathway information refer to summary knowledge of the subphyla Hexapoda, Crustacea, Myriapoda, and Chelicerata. Question marks indicate that further clarifications in the group under consideration are needed with respect to assumed equivalent processes across the subphyla, while crosses indicate reported absences, and asterisks suggest additional lineage-specific observations. CHH, crustacean hyperglycemic hormone; cyp, cytochrome P450; dib, disembodied; GPCR, G-protein coupled receptor; MIH, moulting inhibiting hormone; mTOR, mammalian target of rapamycin; Nobo, noppera-bo; nvd, neverland; phm, phantom; PTTH, prothoracicotropic hormone; RTK, receptor tyrosine kinase; sad, shadow; shd, shade; spo, spook; sro, shroud.

S-transferase from the gene *noppera-bo* (*nobo*) was found to be required for moulting in *D. melanogaster*; it is involved in cholesterol intake, although its ligand has not yet been discovered. In *D. melanogaster*, it has been characterised even at the tertiary structure level (Koiwai *et al.*, 2020; Ebihara & Niwa, 2023), but information from other species is lacking, since it has been studied only in *Aedes aegypti*, *Anopheles gambiae*, *B. mori* and a few other lepidopterans (Enya *et al.*, 2015; Durand *et al.*, 2018; Pan *et al.*, 2021; Kamiyama & Niwa, 2022; Inaba *et al.*, 2022; Musdal *et al.*, 2023). Other ecdysteroid synthetic enzymes belong to the superfamily of cytochrome P450 mono-oxygenases and are transcribed from the so-called "Halloween genes", due to the ghostly phenotype of fruit fly lethal mutants (Kamiyama & Niwa, 2022). While this gene nomenclature is widely used, it conflicts with cytochrome P450 naming conventions so here for clarity we use both common and conventional gene names. The subsequent catalytic steps are aptly referred to as the "black box", due to the uncertainty, even in *D. melanogaster*, about the reactions that collectively catalyse formation of 5β-ketodiol. Black box genes include *shroud* (*sro*, also named *non-molting-glossy*), *cyp6t3*, *spook* (*spo*, cyp307a1), and *spookier* (*spok*, *cyp307a2*) (Pan *et al.*, 2021; Kamiyama & Niwa, 2022). *Shroud* has not received much attention beyond *Drosophila*

and other insects (Ogihara *et al.*, 2017; Knigge *et al.*, 2021; Kamiyama & Niwa, 2022); *cyp6t3* is considered drosophilid-specific (Miyakawa *et al.*, 2018; Kamiyama & Niwa, 2022), and recent CRISPR (clustered regularly interspaced short palindromic repeats) null mutants have shown that it is not required for normal development (Shimell & O'Connor, 2022). The enzymes from the genes *phantom* (*phm*, *phtm*, *cyp306a1*), *disembodied* (*dib*, *cyp302a1*), and *shadow* (*sad*, *cyp315a1*) mediate three additional hydroxylation reactions to complete ecdysone production and release into the circulatory system (Pan *et al.*, 2021; Kamiyama & Niwa, 2022). Importantly, when ecdysone reaches the periphery, it undergoes further modifications leading to the production of biologically active derivatives. The enzyme encoded by the gene *shade* (*shd*, *cyp314a1*) catalyses the formation of 20E, canonically considered the predominant and biologically most relevant ecdysteroid molecule (Liu *et al.*, 2018; Kamiyama & Niwa, 2022).

Efforts to reconstruct the ecdysteroid synthesis pathway in different arthropod lineages have been based on the components known in *D. melanogaster*. This effort has ignored non-insect hexapods and has only partially examined other subphyla. The *nvd* gene seems shared across most arthropod lineages, although experimental isolation has been performed mostly in Pancrustacea (Qu *et al.*, 2015; Sathapondecha, Panyim & Udomkit, 2017; Sandlund *et al.*, 2018; Schumann *et al.*, 2018; Miyakawa *et al.*, 2018; Perry, Scanlan & Robin, 2019; Dermauw, Van Leeuwen & Feyereisen, 2020; Legrand *et al.*, 2021). Multiple studies have failed to identify a candidate orthologue for the *nvd* gene in chelicerate species from the *Varroa* genus (Cabrera *et al.*, 2015; Techer *et al.*, 2019). Interestingly, their life cycle is synchronised with metabolism and hormonal fluctuations of the honeybee broods on which they feed, which suggests that some biosynthetic genes may be dispensable for this parasite (Cabrera *et al.*, 2015; Aurori *et al.*, 2021), so their loss might be genus-specific rather than representative of chelicerates.

Regarding the black box, recent investigations comparing several available arthropod genomes have suggested a highly dynamic evolutionary history of the CYP307 family, and experimental reports in crustaceans and chelicerates have started to provide support beyond simple genomic detection (Sin *et al.*, 2015; Qu *et al.*, 2015; Song *et al.*, 2017; Li *et al.*, 2017; Schumann *et al.*, 2018; Miyakawa *et al.*, 2018; Dermauw *et al.*, 2020; Legrand *et al.*, 2021): *spo* might have been present in the last common ancestor of Arthropoda, with candidate orthologues detected across all examined sublineages; on the other hand, *spok* arose from a duplication of *spo* and is restricted to drosophilids (Ono *et al.*, 2006; Schumann *et al.*, 2018; Dermauw *et al.*, 2020); a third gene, *spookiest*, might have originated in the last common ancestor of Pancrustacea, but remarkably has been lost in daphnids and drosophilids (Qu *et al.*, 2015; Schumann *et al.*, 2018; Dermauw *et al.*, 2020). Studies have repeatedly failed to detect genes related to *phm* (*cyp306a1*) beyond Mandibulata, with notably no orthologues in genomes of seven chelicerate species (Qu *et al.*, 2015; Schumann *et al.*, 2018; Dermauw *et al.*, 2020). Its absence has reinforced the hypothesis of ponA being the biologically active ecdysteroid molecule, supported by biochemical analysis and preliminary experimental characterisation of the ecdysteroid synthesis pathway in the chelicerates *Tetranychus urticae*, *Pardosa pseudoannulata*, and *Panonychus citri* (Li *et al.*, 2017, 2019*a*; Yang *et al.*, 2021*b*). *Phm* detection is uncertain in the genomes of the parasites *Lepeophtheirus salmonis* (a copepod) and *Varroa destructor* (a mite), but ecdysone and 20E and not ponA have been extracted from *Varroa* species (Sandlund *et al.*, 2018; Techer *et al.*, 2019; Humble *et al.*, 2019). Genomic presence of *dib* (*cyp302a1*) as well as *sad* (*cyp315a1*) was confirmed in all the four major arthropod groups, except for *Ixodes scapularis* (Qu *et al.*, 2015; Schumann *et al.*, 2018; Miyakawa *et al.*, 2018; Rotenberg *et al.*, 2020; Dermauw *et al.*, 2020; Qi *et al.*, 2023). Similarly, *shd* (*cyp314a1*) was found in most examined arthropods, and was even cloned from *Daphnia pulex* and *L. salmonis*, except for the decapod shrimps *Neocaridina denticulata*, *Litopenaeus vannamei*, *Penaeus monodon* and *Palaemon carinicauda* (Sumiya *et al.*, 2014; Qu *et al.*, 2015; Sandlund *et al.*, 2018; Ventura *et al.*, 2018; Dermauw *et al.*, 2020). Sequences related to the ecdysteroid synthetic cytochrome P450 *shade* have been proposed as candidates for being a lineage-restricted substitute of *shd* and thus named *shed*, in the genomes of the spiny lobster *Sagmariasus verreauxi* and of the crabs *Eriocheir sinensis* and *Gecarcinus lateralis* (Ventura *et al.*, 2017, 2018; Swall *et al.*, 2021), although functional assessments have been questioned and they might simply represent distant paralogues of the same, large P450 clan (Dermauw *et al.*, 2020). Another notable exception to the canonical machinery is that barnacles from the class Thecostraca appear to have an incomplete biosynthetic pathway, including only the *cyp307* duplication and *sad* (*cyp315a1*), although ecdysteroidogenesis must occur, since these species have ecdysteroid hormones (Dermauw *et al.*, 2020). Both 20E and ponA are commonly reported from crustacean species, therefore the existence of multiple enzymes that enrich the variety of lineage-specific hormones cannot be excluded. Surprisingly, *shd* (*cyp314a1*) is also missing in several fungus-farming ant species, despite being detected in many members of the same Myrmicinae subfamily (Dermauw *et al.*, 2020).

These cytochrome P450 mono-oxygenases are central to the study of arthropod moulting and their presence is considered to be a synapomorphy of Arthropoda (Dermauw *et al.*, 2020). The findings reviewed above point to a dynamic landscape of molecular components, for which a reconstruction of the broad evolutionary history of ecdysteroid synthesis would benefit from comparative studies with an extensive phylogenetic sampling to reveal lineage-specific peculiarities. Some of these variations could relate to diet (Trautenberg *et al.*, 2022), where the structural variety of acquired sterols may be reflected in variation in the transporters and enzymes needed for their uptake and transformation. Some of the apparent differences between Pancrustacea and the remaining Arthropoda might be a result of the disproportionate extent to which these two groups have been studied. Indeed,

almost all class-specific ecdysteroids have been found in Pancrustacea (Song *et al.*, 2017). On the other hand, variable detection of genes encoding for canonical enzymes and of ponA in the crustaceans and chelicerates supports the plausibility of alternative pathways, *via* a plurality of different undiscovered enzymes or by *ad hoc* flexible reshuffling of biochemical reactions. Thus it might be possible to draw a comparison with developmental systems drift, as genetic toolkits for hormone biosynthesis can turn over in evolution while the overall process remains conserved and homologous (True & Haag, 2001).

(2) Neuropeptides regulate ecdysteroid biosynthesis *via* stimulation or suppression

Neuropeptide hormones play an important role in the initiation of ecdysteroid biosynthesis, in concert with a wide range of signals. In insects, neurons from the central nervous system release prothoracicotropic hormone (PTTH) to regulate ecdysteroid synthesis and secretion from a specific secretory organ, the prothoracic glands (Nijhout *et al.*, 2014; Niwa & Niwa, 2016; Ou *et al.*, 2016; Pan *et al.*, 2021). The gene coding for PTTH is paralogous to the gene encoding Trunk, a signalling molecule involved in canonical *Drosophila* embryonic terminal patterning (Rewitz *et al.*, 2009; Duncan, Benton & Dearden, 2013; Jékely, 2013). Both Trunk and PTTH bind to the receptor tyrosine kinase (RTK) Torso, which activates the Ras/ERK (rat sarcoma/extracellular-signal-regulated kinase) signalling pathway (Rewitz *et al.*, 2009; Niwa & Niwa, 2016; Pan *et al.*, 2021; Kamiyama & Niwa, 2022). Trunk has an ancient origin, which may pre-date Bilateria, the origin of Torso is older than the divergence of lophotrochozoans and ecdysozoans, while PTTH was initially shown to be arthropod specific and according to a more recent investigation, it seems to be restricted to insects (Duncan *et al.*, 2013; Jékely, 2013; Skelly *et al.*, 2019; De Oliveira *et al.*, 2019). Timing of this stimulation is important, and studies in Diptera and Lepidoptera have observed that PTTH secretion has a circadian rhythmicity, but this remains to be fully elucidated (Niwa & Niwa, 2016; Bian *et al.*, 2019; Pan *et al.*, 2021; Kamiyama & Niwa, 2022; Qiu *et al.*, 2023). Indeed neurons for light sensing might signal to neurons expressing PTTH that the appropriate photoperiod gate is open, and thereby regulate eclosion timing (Nijhout *et al.*, 2014). This is evident at metamorphosis, where PTTH activates transcription of ecdysteroid-producing enzymes and induces light avoidance and the so-called "wandering behaviour" of feeding interruption to search for a suitable place for pupation (Nijhout *et al.*, 2014). However, despite PTTH being recognised as the primary stimulus for ecdysteroid synthesis, moulting is not abrogated in dipteran and lepidopteran larvae bearing PTTH mutant alleles or with ablated PTTH-producing neurons, implying that prothoracic glands may have autocrine secretion or express other RTKs able to transduce ERK signalling (Nijhout *et al.*, 2014; Niwa & Niwa, 2016; Pan *et al.*, 2021; Kamiyama & Niwa, 2022).

Studies exploiting fly and moth experimental advantages have provided evidence for the involvement of a wide range of signals in moulting initiation (Pan & O'Connor, 2021; Shimell & O'Connor, 2023). Nutritional status is one such factor, where the insulin/mTOR (mammalian target of rapamycin) pathway promotes cell growth and proliferation by releasing insulin-like peptides (ILPs) from brain cells, and where the fat body is an important integration system for macronutrient sensing (Nijhout *et al.*, 2014; Shimell & O'Connor, 2023). Indeed, when the critical body mass is reached, PTTH secretion is stimulated in *M. sexta* (Nijhout *et al.*, 2014). Remarkably, ILP-8 is able to delay moulting when tissue damage occurs in wing precursors and homeostatic organ growth is impaired (Nijhout *et al.*, 2014; Vallejo *et al.*, 2015). Mechanical triggers may also be involved, such as the activation of abdomen stretch receptors to initiate moulting in the hemipteran *Rhodnius prolixus*, and in *Oncopeltus fasciatus* and *Dipetalogaster maximus* that induce PTTH and subsequently ecdysone production (Nijhout *et al.*, 2014). Nevertheless, as observed in *D. melanogaster*, *B. mori*, and *M. sexta*, signalling integration upstream of PTTH is complex and far from completely understood, and is further complicated by the fact that PTTH production also responds to octopamine, serotonin, corazonin (Crz), and allatostatin A (AST-A)-releasing neurons (Ohhara *et al.*, 2015; Niwa & Niwa, 2016; Imura *et al.*, 2020; Pan *et al.*, 2021; Leyria *et al.*, 2022). Moverover, in *B. mori*, juvenile hormone (JH) suppresses the secretory activity of prothoracic glands and their ability to respond to PTTH (Sakurai, Okuda & Ohtaki, 1989) and there is evidence for prothoracicostatic peptides such as allatostatin B inhibiting PTTH-stimulated ecdysteroidogenesis in the prothoracic glands (Yamanaka *et al.*, 2010).

From what we know of decapods from the class Malacostraca, crustaceans have a different mechanism to regulate ecdysteroid biosynthesis. While in insects the neuronal secretion of PTTH, a positive inducer, stimulates ecdysteroidogenesis in the prothoracic glands, in decapods the ecdysteroid production site, the Y-organ, is permanently under repression by the moulting inhibiting hormone (MIH) during the intermoult phase (Ventura *et al.*, 2018; Knigge *et al.*, 2021; Mykles, 2021). MIH is released by the X-organ from the sinus gland complex located in the eyestalk (Ventura *et al.*, 2018; Knigge *et al.*, 2021; Mykles, 2021). Thus, it is the ceasing of this inhibitory signal that allows ecdysteroid synthesis and entry into the moult preparatory phase. These stimulatory and inhibitory hormonal regulation modes during moulting are accompanied by positive and negative feedback loops in which ecdysteroid concentrations act as feedback controllers (Techa & Chung, 2013, 2015). On the one hand, PTTH stimulates ecdysteroidogenesis and is under negative feedback regulation by ecdysteroids, and on the other hand, MIH inhibits ecdysteroid synthesis and appears to be under positive feedback regulation by ecdysteroids (Techa & Chung, 2013, 2015). The cues responsible for MIH decline are not well known and they are likely related to cholesterol intake and environmental

conditions such as light and larval settlement substrates (Ventura *et al.*, 2018; Zhang *et al.*, 2019*a*; Mykles, 2021; Kelly *et al.*, 2022). However, eyestalk ablation is an effective experimental procedure to trigger the moulting process and speed up organismal life cycles. This technique has made decapods experimentally highly tractable species for the study of moulting and it is a common practice in aquaculture (Hyde *et al.*, 2019*a*; Knigge *et al.*, 2021; Lemos & Weissman, 2021).

In decapods, moulting represents a process that allows them not only to grow, increasing in size and attaining maturity, but also to regenerate limbs even during adulthood, as limb autotomy requires ecdysis to make new appendages become fully functional. In this scenario, limb-precursors may produce moult-inducing factors to activate the Y-organ (Das, 2015; Mykles, 2021). Thus, similarities with the role of ILP-8 in postponing insect metamorphosis have been hypothesised (Mykles, 2021). Additionally, a window of reversibility exists during the early pre-moult, where the Y-organ remains sensitive to inhibitory signals to prevent moulting under disadvantageous conditions, for instance, in case of exceeding thermal limits (Mykles, 2021). In this initial phase, hormonal synthesis at transcriptional and translational levels is mTOR dependent. Later, transforming growth factor β (TGFβ) supports the Y-organ's full commitment to ecdysteroid production (Mykles, 2021). In decapods as well as insects, a broad variety of additional signals have been described that contribute to moulting initiation. The insulin receptor and other RTKs have been described upstream of mTOR during moulting, and corazonin, octopamine, and serotonin receptors are expressed by the Y-organ in *Carcinus maenas* and *G. lateralis* (Ventura *et al.*, 2018; Knigge *et al.*, 2021; Mykles, 2021).

The key player of the inhibitory mode is MIH, which belongs to the large gene family of crustacean hyperglycemic hormones (CHHs), together with the gonad-inhibiting hormone (GIH), the mandibular organ-inhibiting hormone (MOIH), and the ion transport peptide (ITP). ITP is the only component of this family found in (but not exclusive to) insects, where it regulates ion and water balance and may also be involved in regulation of ecdysis behaviour (Chen, Toullec & Lee, 2020; Knigge *et al.*, 2021; Mykles, 2021; Klöcklerová *et al.*, 2023). Peptides from the CHH family are highly similar in sequence and structure but they play different fundamental roles in many processes other than development, such as ionic and osmotic balance, cardiac activity control, and have even been recruited as venom toxins (Chen *et al.*, 2020). Many investigations have studied the CHH family to improve their functional characterisation, despite this being made experimentally more difficult by ubiquitous expression and presence of many different isoforms (Chen *et al.*, 2020; Mykles, 2021). Putative CHH and ITP receptors have been identified in the crustaceans *C. maenas* and *G. lateralis* based on sequence and structural similarities shared with the insect ITP G-protein coupled receptors (GPCRs) (Ventura *et al.*, 2018; Chen *et al.*, 2020; Knigge *et al.*, 2021; Mykles, 2021). Finally, MIH has been proposed to trigger cyclic secondary messengers and mediate mTOR activation, and GPCRs have been hypothesised as potential candidates for MIH binding. Interestingly, an ILP-8-like protein produced by limb buds to delay moulting has been suggested to inhibit ecdysteroidogenesis *via* MIH signalling, suggesting clear similarities with findings from insects (Mykles, 2021). Surprisingly however, in contrast to the well-established role of MIH in moulting inhibition, its receptor has not yet been identified (Tran *et al.*, 2019; Chen *et al.*, 2020; Mykles, 2021).

Little is known about crustacean neuropeptidic moulting regulation beyond Decapoda. The isopod *Armadillidium vulgare* genome comparative analysis highlighted more than 10 CHH-like sequences, while *Proasellus cavaticus* transcriptome assembly revealed putative sequences for CHH, MIH, ILPs, AST-A, but not Crz (Christie, 2017; Chebbi *et al.*, 2019). The branchiopod model species *Daphnia magna* lacks the Y-organ and endocrine ecdysteroid production has been suggested to take place in gut epithelial cells (Song *et al.*, 2017; Miyakawa *et al.*, 2018). In phyllopods, copepods, and remipedes, CHH family components other than ITP have not been detected (Chen *et al.*, 2020). Regarding the other arthropod subphyla, ecdysteroid-producing tissue has been labelled as a "putative ecdysial gland" in the myriapod *Lithobius forficatus* (Seifert & Bidmon, 1988); in the tick *Ornithodoros parkeri*, the adult integument has been identified as a source of ecdysteroid production (Zhu, Oliver & Dotson, 1991). On the other hand, the presence of a "moulting organ" (MO) formed by groups of cells in the lower part of the prosoma has been proposed as responsible for ecdysteroid production in several spider species, but its role has not been confirmed and follow-up studies are lacking (Sawadro, Bednarek & Babczyńska, 2017).

Some studies have approached the characterisation of non-toxic neuropeptides and hormones in chelicerate species, mainly exploiting transcriptomic and genomic data (Christie, 2015*a*; Christie & Chi, 2015; Veenstra, 2016*a*; Yu *et al.*, 2020; Waldman *et al.*, 2022; Down & Audsley, 2022). While PTTH was never detected, AST-A, CHH/ITP-like peptides, and a variable number of genes potentially coding for ILPs have been found in Acari, Araneae, and Scorpiones species, but their functions remain unknown (Christie, 2015*a*; Christie & Chi, 2015; Veenstra, 2016*a*; Yu *et al.*, 2020; Wegener & Chen, 2022; Waldman *et al.*, 2022; Down & Audsley, 2022; Lyu *et al.*, 2023). Crz seems to be present in ticks but missing in mites and scorpions and shows conflicting results in spiders (Christie, 2015*a*; Christie & Chi, 2015; Veenstra, 2016*a*; Yu *et al.*, 2020; Waldman *et al.*, 2022; Down & Audsley, 2022; Lyu *et al.*, 2023). Hopefully, such sequence analyses will provide useful resources for future experimental characterisation, since these, except for AST-A in *Parasteatoda tepidariorum*, are currently virtually non-existent (Sawadro *et al.*, 2017, 2019). In myriapods, the exploration of moulting-triggering neuropeptides has received even less attention. From the transcriptome of the symphylan *Symphylella vulgaris*, none of the aforementioned peptides were detected except for AST-A and ILP (Christie, 2015*b*). In

the genome assembly of the centipede *Strigamia maritima*, ITP, AST-A, Crz, and one ILP have been identified; although few other millipede and centipede species have been sequenced, comparative analyses aiming to investigate the neuropeptides regulating ecdysteroid biosynthesis are lacking (Chipman *et al.*, 2014; Qu *et al.*, 2020; So *et al.*, 2022).

In summary, several research efforts have established PTTH and MIH as important early neuroregulators of moulting and have started dissecting their pathways and signals that feed into these pathways, in Diptera, Lepidoptera, and Decapoda species. However, knowledge on neuropeptide regulation of ecdysteroid biosynthesis in chelicerates and especially myriapods should be considered preliminary at best, relying to date almost entirely on *in silico* analysis. From the currently incomplete picture it remains near-impossible to propose hypotheses on when and where across the arthropod phylogeny switches between mainly stimulatory or suppression-based regulation occurred, or even which was most likely the ancestral state.

III. SESQUITERPENOID BIOSYNTHESIS AND REGULATION

Similarly to ecdysteroids, biosynthesis of sesquiterpenoid hormones, such as JH and methyl farnesoate (MF), is controlled by the nervous system and takes place in specific endocrine organs. In insects, JH is primarily responsible for supporting proper development before adulthood, that is maintaining the "juvenile" form. Thus, in the presence of insect JH, ecdysteroids trigger moulting during which tissues and organs largely maintain their pre-moult phenotype. By contrast, when JH is absent, ecdysteroid exposure causes tissues and organs to change their commitment during moulting towards the adult stage (Jindra, Bellés & Shinoda, 2015; Qu *et al.*, 2018; Jindra, 2019; Tsang *et al.*, 2020). Sesquiterpenoids also regulate other processes, such as social differentiation, sex determination, and reproductive maturation, which are beyond the scope of this work but that have been reviewed elsewhere (De Loof & Schoofs, 2019; Miura, 2019; Santos *et al.*, 2019; Tsang *et al.*, 2020; He & Zhang, 2022). Much of the current understanding of sesquiterpenoid hormones is derived from studies on a few insect model systems, including *D. melanogaster* (Diptera), *Hyalophora cecropia*, *M. sexta*, *B. mori* (Lepidoptera), *R. prolixus* (Hemiptera), *Tribolium castaneum* (Coleoptera), *Apis mellifera* (Hymenoptera), *Schistocerca gregaria* (Orthoptera), and *Diploptera punctata* (Blattodea) (Riddiford, 2020; Tsang *et al.*, 2020). Genomic data are starting to extend our understanding of the sesquiterpenoid synthesis pathway beyond such insect models, enabling the discovery of genes encoding key enzymes and molecular components (Qu *et al.*, 2015). However, despite this progress the resources and information on the sesquiterpenoid hormone pathway mostly have been expanded to include some crustaceans, particularly Malacostraca, and are still skewed towards insects, while arthropods beyond Pancrustacea remain understudied (Jindra *et al.*, 2015; Qu *et al.*, 2018; Jindra, 2019; Tsang *et al.*, 2020). Indeed, knowledge pertaining to the regulation and localisation of sesquiterpenoid hormone biosynthesis in Myriapoda and Chelicerata remains rudimentary.

(1) Sesquiterpenoid production follows conserved steps with lineage-specific elaborations

The early catalytic steps of sesquiterpenoid hormone synthesis are generally referred to as the mevalonate branch (Fig. 3). This pathway is conserved throughout eukaryotes and is important in the production of essential isoprenoid compounds and key metabolites necessary for normal cell structure and metabolism (Hoshino & Gaucher, 2018; De Loof & Schoofs, 2019). Through several reactions, farnesyl pyrophosphate (Farnesyl-PP) is produced from acetyl-coenzyme A (acetyl-CoA) (Tsang *et al.*, 2020). Even though all arthropods use a common biosynthetic pathway for the formation of farnesoic acid (FA) from Farnesyl-PP, a variety of modified products have evolved in the downstream process of sesquiterpenoid production. This divergence in downstream steps is most likely underestimated due to limited exploration of different taxa, and is often simplified and presented as a hallmark feature that distinguishes insects from other arthropod groups. In Orthoptera, Blattodea, Hymenoptera, and Coleoptera, two downstream reactions lead to the formation of JH III: first FA is methylated by juvenile hormone acid methyltransferase (JHAMT) to produce MF, then the epoxidase CYP15A1 converts MF into JH III. However, in Lepidoptera the order of these two biosynthetic steps is reversed: firstly, a different epoxidase, CYP15C1, mediates the epoxidation step and secondly, JHAMT catalyses JH III formation (Tsang *et al.*, 2020). JH 0, I and II molecules, differing from JH III by the use of three, two, or one homomevalonates (respectively) for the synthesis of their (homo) sesquiterpenoid backbone, have also been found in Lepidoptera, while 2,3-epoxy-JH III is the only hormonal form in the Heteroptera suborder of Hemiptera (Tsang *et al.*, 2020; Matsumoto *et al.*, 2021; Yi *et al.*, 2023). Diptera represent another remarkable exception: cyp15a/c-like genes seem to be missing in the Cyclorrhapha suborder, instead the *cyp6g2* gene might be involved in the formation of the bisepoxy JH III (Jindra *et al.*, 2015; Dermauw *et al.*, 2020; Zhang, Li & Liu, 2022*b*; Smykal & Dolezel, 2023). In *D. melanogaster*, MF can act as a circulating hormone itself and *cyp15a1* null mutants in *A. aegypti* are able successfully to undergo the full life cycle (Wen *et al.*, 2015; Nouzova *et al.*, 2021). This diversity of potentially bioactive forms suggests possibilities for functional specialisations that remain to be characterised, especially in light of possible independent duplications of *JHAMT*-like genes in multiple insect orders (Smykal & Dolezel, 2023).

Epoxidation has only been found so far in insects and JH synthesis is therefore considered to be an insect innovation (Qu *et al.*, 2018; Nouzova *et al.*, 2021). However, non-insect hexapods have received little to no attention, missing

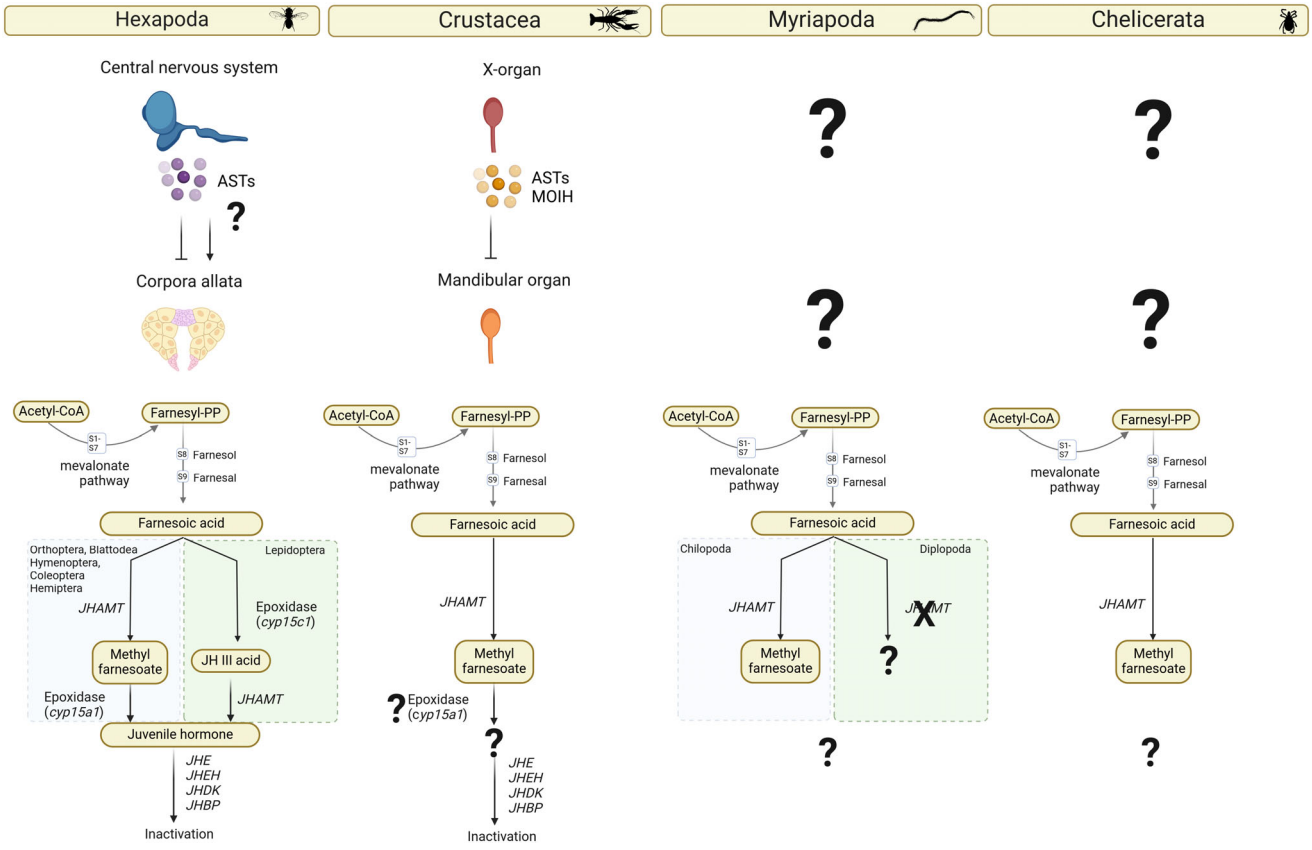

Fig. 3. Sesquiterpenoid biosynthetic pathways. The biosynthetic pathway of sesquiterpenoid hormones can be divided between the early mevalonate pathway and the reactions downstream farnesoic acid (FA) formation. Sesquiterpenoid hormones are secreted by the corpora allata and the mandibular organs, in insects and crustaceans, respectively. In Pancrustacea, allatostatins (ASTs) might regulate hormone production, along with mandibular organ inhibiting hormone (MOIH) in crustaceans. In insects, juvenile hormone methyl-transferase (JHAMT) and epoxidase (*cyp15a1*) lead to the formation of juvenile hormone (JH), whereas in other subphyla methyl farnesoate (MF) is synthesised from FA *via* JHAMT only. JHAMT has not been detected in diplopod millipedes, while *cyp15a1* homologues and enzymes for JH inactivation have been reported in some decapods (but epoxidase activity has not been demonstrated for these *cyp15a1* homologues). The four columns of pathway information refer to summary knowledge of the subphyla Hexapoda, Crustacea, Myriapoda, and Chelicerata. Question marks indicate that further clarifications in the group under consideration are needed with respect to assumed equivalent processes across the subphyla, while crosses indicate reported absences. Acetyl-CoA, acetyl coenzyme A; ASTs, allatostatins; JH III acid, juvenile hormone III acid; JHAMT, juvenile hormone methyl-transferase; JHBP, juvenile hormone binding protein; JHDK, juvenile hormone diol kinase; JHE, juvenile hormone esterase; JHEH, juvenile hormone epoxide hydrolase; MOIH, mandibular organ inhibiting hormone.

opportunities to bridge understanding of hormonal evolution between insects and non-hexapod pancrustacea. A rare exception is toxicological assays in *Folsomia candida* showing transgenerational effects of exposure to JH analogues, but this study did not investigate their mode of action (Campiche *et al.*, 2007).

The genomic and experimental identification of JHAMT in some non-insect arthropods spanning the subphyla and the demonstration of biological activity of MF in some crab species provided evidence that this part of the sesquiterpenoid hormone pathway is deeply conserved in Arthropoda and that MF likely plays an equivalent role to insect JH in moulting (Sin *et al.*, 2015; Miyakawa *et al.*, 2018; Qu *et al.*, 2018; Yang *et al.*, 2021*a*, 2022; Semchuchot *et al.*, 2023; Toyota *et al.*, 2023). However, conflicting reports on the presence and activity of key enzymes exist amongst different arthropod groups. While JHAMT has been generalised as a key enzyme in the production of MF in non-insect arthropods, experimental evidence of its presence in chelicerates such as spiders and ticks is still ambiguous and made more difficult due to detections of tens of JHAMT-like proteins (Zhu *et al.*, 2016; Nicewicz *et al.*, 2021; Yang *et al.*, 2021*a*, 2022; Smykal & Dolezel, 2023). Progress in characterisation of the tertiary structure of the substrate-binding pocket in different gene copies of JHAMT in the silkworm moth might offer valuable clues to establish whether candidate JHAMT-like orthologues have methyltransferase catalytic activity (Guo *et al.*, 2021; Zhang *et al.*, 2022*a*). A recent study further demonstrates the importance of newly sequenced genomes to reject poorly supported assumptions:

sequencing of five millipede species revealed that JHAMT is not present in millipedes, contrary to centipede genomes (So *et al.*, 2022). In the centipede *Thereuonema tuberculata*, FA titre is significantly higher than MF and it is able to elicit the upregulation of target genes. Conversely, these observations could not be reproduced in the millipede *Helicorthomorpha holstii*. This supports the hypothesis that in millipedes the bioactive sesquiterpenoid form is likely to be FA rather than MF (So *et al.*, 2022). Moreover, sequences belonging to the *cyp15a1* epoxidase P450 cytochrome group (the CYP2-clan) have been potentially identified in the decapods *Macrobrachium rosenbergii*, *S. verreauxi*, *Portunus trituberculatus*, *Chionoecetes opilio*, and *Scylla paramamosain*, exhibiting tissue-specific expression and raising further questions about our assumptions of processes such as epoxidation being specific to insects, but without supporting evidence (Ventura *et al.*, 2018; Tu *et al.*, 2022; Zhao *et al.*, 2022; Toyota *et al.*, 2023). A distant *cyp15a1* homologue has been suggested in *P. tepidariorum* but debated, and no candidates have been found in the spider *P. pseudoannulata* nor other chelicerate species (Yang *et al.*, 2022).

As well as biosynthesis, catabolic enzymes for sequestration and degradation of JH play key roles in controlling JH titres. Among these enzymes, juvenile hormone esterase (JHE) is the primary JH-specific degradation enzyme that serves as a negative regulator of JH titre, along with JH epoxide hydrolase (JHEH) and JH diol kinase (JHDK) (Kamita & Hammock, 2010; Tsang *et al.*, 2020; Borovsky *et al.*, 2022, 2023; Vasquez *et al.*, 2023). Their role has been characterised by early studies in Lepidoptera, followed by Coleoptera, and more recently in *D. melanogaster* and *A. aegypti* (Tsang *et al.*, 2020; Borovsky *et al.*, 2022, 2023; Vasquez *et al.*, 2023). JHE takes JH III as a substrate, converting it into JH III acid, which is then either available for returning to the pool of JH III, through JHAMT, or for degradation, being irreversibly transformed by JHEH into JH III diol acid and then inactivated by JHDK. In *A. aegypti*, double-stranded RNA interference (dsRNAi) against *jheh* did not impair normal development (Borovsky *et al.*, 2023). This is in contrast to JH accumulation causing lethality in *jheh*-knockdown nymphs of the hemipteran *Apolygus lucorum* (Tusun *et al.*, 2017). Existence of multiple *jheh* paralogues, for example up to three in Diptera, compared to the single copy found in lepidopterans, might hint at further functional specialisations in flies, indeed the so-called *jheh-2* gene is involved in microsomal and xenobiotic metabolism (Borovsky *et al.*, 2022). In *B. mori*, CRISPR-mediated JHE disruption resulted in extended late larval stages, upregulation of JHEH and JHDK and success in reaching adulthood, suggesting a compensatory mechanism (Zhang *et al.*, 2017*b*). In fact, combined knockdown of these enzymes in the beetles *Leptinotarsa decemlineata* and *Anthonomus grandis* impairs adult emergence and pupation (Fu *et al.*, 2015; Vasquez *et al.*, 2023). In JH regulation, the JH-binding protein (JHBP) might also play a role, since, by binding the hormone it not only allows its transportation from the secretory tissue, but it also protects it from degradation enzymes and it might prevent interactions with other components – interestingly a putative JHBP in *B. mori*, when overexpressed, causes repression of JH-responsive targets (Zhang *et al.*, 2023*a*).

In crustaceans, JHE and JHEH-like genes have been identified in several decapods but remain to be characterised in detail (Lee *et al.*, 2011; Sin *et al.*, 2015; Tao *et al.*, 2017; Zhao *et al.*, 2022; Semchuchot *et al.*, 2023). In *L. vannamei*, JHEH expression is induced by bacterial and viral infection and inhibition of JHE-like sequence transcription leads to strong downregulation of ecdysteroid-responsive genes and moulting failure (Zhang *et al.*, 2020*b*; Liu *et al.*, 2022*b*). JHE-like gene expression decreases during the developmental cycle and the hepatopancreas and the gonads appear to be the major sites for MF inactivation and MF esterase activity, as detected in several decapods (Lee *et al.*, 2011; Tao *et al.*, 2017; Zhang *et al.*, 2020*b*; Li *et al.*, 2021*d*; Liu *et al.*, 2022*b*). Other factors putatively involved in sesquiterpenoid turnover have been found beyond Pancrustacea, but they have not been extensively investigated (Qu *et al.*, 2015; So *et al.*, 2022).

The working model therefore implicates JH in insects while other arthropods rely on MF and FA (except Diplopoda with only FA). Nevertheless, the diversity of sesquiterpenoid hormones exhibiting JH-like activity implies possible undiscovered hormones, as well as methyltransferases and epoxidases. The identification of other enzymes outside the classical CYP15 clade remains as a future challenge, along with the exploration of components involved in MF degradation. In this direction, several JHBP genes have been preliminary characterised in *S. paramamosain*, some of them potentially binding MF and some JH (Zhao *et al.*, 2020). In summary, while key enzymes have been detected in some representative species of the four major arthropod groups, accumulating evidence repeatedly leads to reconsidering the validity of previous hypotheses or assumptions, both in terms of pushing back the origins of what we consider to be insect mechanisms and in terms of increasingly appreciating a greater heterogeneity of lineage-specific pathways, within and beyond insects, whose functions still remain to be elucidated in depth.

(2) Sesquiterpenoid biosynthesis is regulated by ancient pleiotropic neuropeptides

Sesquiterpenoid hormones regulate a range of processes, thus tight control of their concentrations and localisations is needed to ensure normal development. In insects, they are secreted by the corpora allata, located with the prothoracic glands inside the ring gland complex in *D. melanogaster* (Pesch *et al.*, 2019; Bendena *et al.*, 2020). Allatostatins (ASTs) are believed to be the principal neuropeptides that control sesquiterpenoid hormone production as neuromodulators (Stay & Tobe, 2007; Tsang *et al.*, 2020; Bendena *et al.*, 2020; Wegener & Chen, 2022). ASTs comprise an ancient pleiotropic family of peptides classified into three subfamilies, despite the lack of any homology amongst them: AST-A, AST-B, and AST-C, with AST-A, also known as FGLamide peptide, being the largest and most diverse (Stay & Tobe, 2007; Veenstra, 2016*b*,*c*; Bendena *et al.*, 2020; Wegener & Chen, 2022).

AST-A-like peptides are found across arthropods as well as in molluscs and platyhelminths, and AST-A signalling is phylogenetically related to kisspeptin and galanin signalling in Deuterostomia (Wegener & Chen, 2022). Arthropods harbour a single prepropeptide-encoding gene, notably missing in Coleoptera (Pandit *et al.*, 2019; Wegener & Chen, 2022). AST-A is involved in a plethora of homeostatic processes, such as regulation of food and water intake, sleep and locomotor activity, but negative regulation of JH production has been observed only in cockroaches, crickets, and termites (Wegener & Chen, 2022). AST-B-encoding genes have been detected across arthropods including in Pancrustacea species and in *S. maritima*, but may be missing in some Hymenoptera species. As for AST-A, suppression of the JH pathway by AST-B is limited to crickets (Bendena *et al.*, 2020). AST-C is homologous to the vertebrate somatostatin and in arthropods has undergone multiple rounds of gene duplication and loss. Of the species examined to date, most arthropods have three copies of the gene (AST-C, -CC, -CCC), while some insect orders have only AST-CC and AST-CCC (Veenstra, 2016*a*). However, Diptera, Lepidoptera, and Coleoptera have lost AST-CCC and retained AST-CC, along with AST-C, whose role in the JH pathway has been characterised in only a few insect species (Veenstra, 2016*a*; Pandit *et al.*, 2019). In *B. mori* and *M. sexta*, AST-C negatively regulates JH biosynthesis (Bendena *et al.*, 2020). Allatotropin (AT) also indirectly inhibits JH production by stimulating the short neuropeptide F (sNPF) (Fadda *et al.*, 2019; Bendena *et al.*, 2020). In *A. aegypti*, hormone synthesis relies on the rate of isoprenoid intermediate production in the mevalonate branch and thus is consequently dependent on regulation of those enzymes and components involved in those processes: AST-C limits the production of acetyl-CoA by blocking the citrate mitochondrial carrier (Nouzova *et al.*, 2011; Nouzova, Rivera-Perez & Noriega, 2015). AST-C inhibits JH synthesis also in beetles, however, contrasting results are found: unlike in *A. aegypti*, AT stimulates JH biosynthesis in the closely related mosquito species *Culex pipiens* (Bendena *et al.*, 2020; Sun *et al.*, 2022). Interestingly, MF production is upregulated in response to AT in *Daphnia*, while in the mud crab *S. paramamosain* both Ast-C and AST-CCC decrease expression of enzymes for MF biosynthesis (Liu *et al.*, 2021).

Aside from the ASTs, the MOIH from the abovementioned CHH superfamily (Section II.2) appears to have a similar role to the ecdysteroid-inhibiting function of the decapod MIH. It is secreted by the X-organ of the sinus gland complex in the eyestalk, as is MIH, and it inhibits MF synthesis by the mandibular organ, which is absent in daphnids (Miyakawa *et al.*, 2018; Ventura *et al.*, 2018). The mandibular organ is homologous to the insect corpora allata and it shows expression of sesquiterpenoid-synthesising enzymes (Xie *et al.*, 2016; Semchuchot *et al.*, 2023; Toyota *et al.*, 2023). Current knowledge is extremely limited, since MOIH has been studied only in a few brachyuran crab species, where it might operate as a lineage-specific paralogue with cyclic AMP (cAMP) as a secondary messenger (Chen *et al.*, 2020; Toyota *et al.*, 2023). Interestingly, synthetic ASTs based on the sequence of the cockroach *D. punctata* and applied to the mandibular organ of the crayfish *Procambarus clarkii* promoted MF synthesis (Kwok, Rui Zhang & Tobe, 2005).

While arthropods likely share a core set of ancient neuropeptides, for Myriapoda and Chelicerata this hypothesis mainly relies on analysis based on sequence similarity, as in the case of ecdysteroid synthesis neuroregulation, discussed in Section II.2 (Christie, 2015*a*; Christie & Chi, 2015; Veenstra, 2016*b*; Sawadro *et al.*, 2017; Yu *et al.*, 2020; Wegener & Chen, 2022; Waldman *et al.*, 2022; Down & Audsley, 2022). In *Ixodes* species and *P. tepidariorum* AST-C and sNPF have been successfully isolated from the synganglion and nervous system (Neupert *et al.*, 2009; Sawadro *et al.*, 2019; Medla *et al.*, 2023). However, the scarcity of functional validations of sesquiterpenoid synthesis and neuroregulation is unsurprising given that in contrast to the known insect corpora allata and the decapod mandibular organ, localisation of sesquiterpenoid hormone secretion remains unclear (Sawadro *et al.*, 2017). In the spider *P. pseudoannulata*, *jhamt-like* sequences have the highest expression in the abdomen compared to other tissues (Yang *et al.*, 2021*a*). In spiders generally, sesquiterpenoid hormone was previously hypothesised to be produced in the Schneider organ 2 (part of the stomatogastric ganglion), which was proposed to be homologous to the insect corpora allata (Sawadro *et al.*, 2017, 2019). However, a recent study in *P. tepidariorum* showed tissue-specific expression profiles of the distant *cyp15a1* homologue that suggest the main site of MF synthesis is in the integument and not the Schneider organ (Nicewicz *et al.*, 2021). In a previous study on the synganglion transcriptome of the ticks *Dermacentor variabilis*, *I. scapularis*, and *Ornithodoros turicata*, it was suggested that the mevalonate pathway is present only in the synganglion which is inclusive of the stomatogastric nervous system and the likely tick equivalent of the insect corpora allata (Zhu *et al.*, 2016). Meanwhile, its localisation in myriapods remains to be discovered, underscoring that much additional physiological research on these neuromodulators and their production sites across non-insect arthropod groups is needed.

The diversity of neuropeptides and modes of action involved in the JH pathway suggests that stimuli upstream of sesquiterpenoid synthesis in arthropods can be rich in interactions and variably regulated, but we are far from understanding the major regulator on/off switch mechanisms. While genomics can provide some clues about potential components, disentangling such complexity will require studies aiming to optimise specific protocols to attempt to overcome experimental limitations (Sawadro *et al.*, 2017), in order to dissect how neurosecretion is integrated from biosynthesis to target tissues for the control of sesquiterpenoid production.

IV. FACTORS INVOLVED IN HORMONAL STIMULATION RESPONSES

After their synthesis, ecdysteroid and sesquiterpenoid hormones bind to their respective receptors and trigger the transcription of early genes and fate determination genes to

regulate and drive tissue remodelling upon the expression of late genes. Understanding and characterisation of the factors involved in responses to moulting hormone stimulation have primarily relied on studies of well-known insect models. Insects including *D. melanogaster* (Diptera), *B. mori*, *M. sexta* (Lepidoptera), *T. castaneum* (Coleoptera), *Planococcus citri*, *Pyrrhocoris apterus*, *R. prolixus*, *O. fasciatus* (Hemiptera), and *Blattella germanica* (Blattodea) have well-established methods for experimental and genetic manipulation. Therefore, these species are the most frequently used in the studies reviewed here (Fig. 4), for the discovery and characterisation of genes acting downstream of hormonal receptor binding (Jindra *et al.*, 2015; Li, Jia & Li, 2019*b*; Jindra, 2019; Truman, 2019; Thompson, 2021; Kamiyama & Niwa, 2022). These genes in other hexapod groups remain generally poorly characterised, although work focusing on species such as *Catajapyx aquilonaris* (Diplura), *Thermobia domestica* (Zygentoma), and *Cloeon viridulum* and *Cloeon dipterum* (Ephemeroptera) has substantially advanced understanding of moulting in relation to the origin of insects, ametabolous development, and wing innovation (Truman & Riddiford, 2019; Truman, 2019; Kamsoi *et al.*, 2021). On the basis of evidence in insects, molecular aspects of moulting hormone responses have been studied in Crustacea but these investigations remain far from comprehensive. Despite the long history of *D. pulex* and *D. magna* (Branchiopoda) as model systems, reports on crabs (*E. sinensis*, *S. paramamosain*, *Callinectes sapidus*, *G. lateralis*, *C. maenas*), lobsters (*S. verreauxi*, *Homarus americanus*) and shrimps (*L. vannamei*, *Exopalaemon carinicauda*, *N. denticulata*, *Macrobrachium*

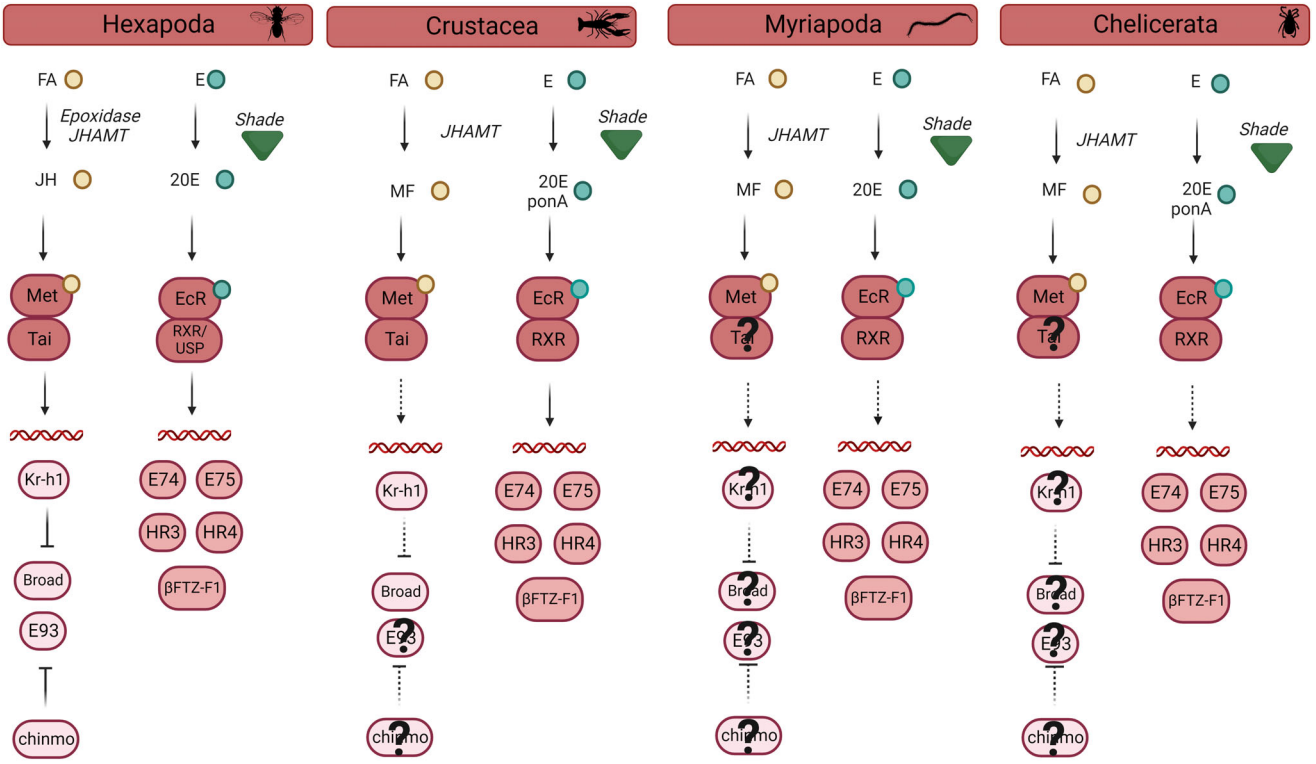

Fig. 4. Cascade responses upon moulting hormone stimulation. At the start of the cascade, farnesoic acid (FA) is converted to methyl farnesoate (MF) by juvenile hormone methyl-transferase (JHAMT) in all groups except in Insecta, where juvenile hormone (JH) production also requires an additional epoxidase-mediated catalytic step, and ecdysone is transformed to 20E by Shade enzymes and ponA can be found in Crustacea and Chelicerata. JH and MF bind to the Met receptor, during immature stages. In Insecta, Met and the coactivator Tai trigger the expression of Kr-h1, which translates the JH signal into repression of Broad and E93 and maintenance of the juvenile status. Chinmo is also able to determine juvenile phenotype, but it has been studied only in insects. In Crustacea, the adulthood gate-keeper gene E93 has not yet been detected and relationships between the receptor complex and Kr-h1, and between Kr-h1 and Broad are not clarified, while in the other subgroups little information about Broad, Tai, and Kr-h1 is available. Bioactive ecdysteroids bind to the nuclear heterodimer EcR/RXR (orthologous to USP in the insect orders Diptera and Lepidoptera) and turn on an early activation cascade of the transcription factors E74, E75, HR3, HR4, and βFTZ-F1. The four columns of pathway information refer to summary knowledge of the subphyla Hexapoda, Crustacea, Myriapoda, and Chelicerata. Question marks indicate that further clarifications in the group under consideration are needed with respect to assumed equivalent processes across the subphyla.20E, 20-deoxy-ecdysone; chinmo, chronologically inappropriate morphogenesis; E, ecdysone; E74/75/93, ecdysone-induced proteins 74/75/93; EcR, ecdysone receptor; FA, farnesoic acid; HR3/4, hormone receptor 3/4; JH, juvenile hormone; JHAMT, juvenile hormone methyl-transferase; Kr-h1, kruppel homolog 1; Met, methoprene-tolerant; MF, methyl farnesoate; ponA, ponasterone A; RXR, retinoid X receptor; ßFTZ-F1, beta fushi-tarazu transcription factor 1; Tai, taiman; USP, ultraspiracle.

nipponense, *Parhyale hawaiensis*, *P. monodon*, *P. clarkii*, *Gammarus fossarum*) have made Malacostraca the most intensively studied class (Fig. 4) (Chen *et al.*, 2017; Qu *et al.*, 2018; Hyde *et al.*, 2019*a*; Zhang *et al.*, 2019*a*). Unless otherwise specified, in surveying the current understanding of factors involved in hormonal stimulation responses we refer principally to these key species when discussing insects and crustaceans, to avoid redundancy and focus on less-studied arthropods and on differences across subgroups. Notably, insects have been intensively studied to understand the evolutionary history of the pupal stage of Holometabola in comparison with transitions during direct development of ametabolous wingless insects and incomplete metamorphosis of hemimetabolous insects. Knowledge of responses to hormonal stimulation, especially with respect to early and fate genes, is therefore inherently even more biased towards insects and "metamorphic moulting" than many other moulting processes. While acknowledging that these biases prejudice the information reviewed here, studying these genes through the lens of metamorphosis also informs understanding of moulting more generally, even if disentangling the two remains challenging.

(1) Bioactive molecules bind to hormone receptors

According to the canonical biosynthetic pathway in *D. melanogaster*, ecdysone is produced in secretory tissues and then released into the haemolymph to reach the body periphery where it is hydroxylated and converted into 20E, the generally accepted major ecdysteroid bioactive form. This reaction is catalysed by a cytoplasmic mono-oxygenase encoded by the *Shade/cyp314a1* gene (Fig. 2). While ecdysone itself can elicit a response in target tissues, such as epidermis apolysis during the period which precedes moulting *sensu stricto*, the ecdysone pulse is rapidly translated into a high 20E titre, which represents the signal that promotes the production of a new cuticle (Truman & Riddiford, 2019; Truman, 2019). In some species the preparatory phase can be prolonged by tissue damage signals, nutrition-dependent stimuli, and integration with the JH pathway, before the 20E peak and its subsequent decline trigger the irreversible ecdysis motor (Nijhout *et al.*, 2014; Pan *et al.*, 2021). Therefore, amongst factors involved in hormonal stimulation responses, 20-hydroxylation is a key step to control 20E quantity, and peripheral tissues constitute not only passive hormonal targets but are also involved in regulating the moulting process.

Ecdysteroids bind to the ecdysone receptor complex, which plays a central role in triggering the activation of sets of genes involved in moulting processes. Ecdysone receptor (EcR) belongs to group II of the superfamily of nuclear hormone receptors, with a canonical structure (Nuclear Receptors Nomenclature Committee, 1999). It is composed of an E domain for ligand binding, a DNA binding domain formed from two C4 zinc fingers located at the carboxy-terminal, and a transactivation domain, which is an A/B region at the amino-terminal. EcR is constitutively found in the nucleus, heterodimerised with retinoid X receptor [RXR, also called ultraspiracle (USP) in many insects] and bound to DNA (Hill *et al.*, 2013; Truman, 2019). The heterodimer is localised at so-called ecdysone responsive elements (EcR-Es) in a repressed state maintained by the EcR E-domain, while conformational changes upon ecdysteroid binding lead to the transition to the de-repressed state (Truman, 2019). Transactivation implies exchange of co-repressors with co-activators to mediate chromatin opening and recruitment of the transcriptional machinery (Robinson-Rechavi, Garcia & Laudet, 2003; Truman, 2019).

Species from different insect orders have been reported as mostly having two isoforms (EcR-A and EcR-B), generally differing in the A/B transactivating domain (Truman, 2019). Insect EcR genes also tend to have an additional F-domain, highly variable in sequence, whose structure and function remain unclear. *D. melanogaster* harbours three isoforms, the predominant is EcR-B1, which includes a ligand-independent activation domain absent in EcR-A (Truman & Riddiford, 2019; Truman, 2019). EcR-B1 is expressed in all tissues during the larval stage and drives neuronal remodelling from larva to adult, while EcR-A is specific to the wing imaginal discs and is upregulated when entering metamorphosis (Truman & Riddiford, 2019; Truman, 2019; Lai *et al.*, 2022). RXR exhibits the same domain composition as other members of the nuclear hormone receptor family and has undergone several changes during insect evolution. In the orders Hemiptera and Coleoptera, RXR lost the capacity to bind 9-cis-retinoic acids; in Diptera and Lepidoptera, the interface for dimerisation with EcR has expanded and binding of structural lipids has been enhanced, but EcR also lost homodimeric stability, resulting in EcR/USP coevolution as obligatory partners (Bonneton *et al.*, 2003; Hill *et al.*, 2013). In these two orders, the RXR orthologue is called *Ultraspiracle* (USP), although the use of "USP" to refer to the RXR gene in the whole group of Insecta is widespread.

While ecdysteroids are ligands for nuclear receptors, the receptor for sesquiterpenoids, methoprene-tolerant (Met), is an intracellular transcription factor member of the basic helix–loop–helix Per/Arnt/Sim (bHLH-PAS) family, where the two domains mediate DNA binding and dimerisation, respectively (Jindra *et al.*, 2015; Li *et al.*, 2019*b*; Jindra & Bittova, 2020). Studies of Met in *D. melanogaster* initially faced difficulties due to the paralogous *Gce* (germ cell-expressed) gene, which is active in the ovary and involved in vitellogenesis and reproduction (Jindra *et al.*, 2015). Met's binding partner is Taiman (Tai), another bHLH-PAS transcription factor, also known as steroid response coactivator (SRC). Upon JH binding, Met translocates into the nucleus and heterodimerizes with Tai, to bind the target JH response elements and activate transcription; a process requiring both their bHLH domains (Qu *et al.*, 2015; Jindra *et al.*, 2015; Santos *et al.*, 2019; Belles, 2019; Truman, 2019; Jindra & Bittova, 2020). Tai mutants show that Tai is required to mediate JH-induced response in different orders both in holometabolous and hemimetabolous insects (Jindra *et al.*, 2015; Santos *et al.*, 2019). Remarkably, some studies point to Tai promiscuity in dimerisation with the EcR/RXR

complex and recent evidence, showing Tai-dependent transcription of both JH- and ecdysone-responsive genes, suggests that Tai could represent a key factor in the cross-talk between the two main moulting regulation pathways (Jindra *et al.*, 2015; Xu *et al.*, 2019).

In contrast to the understanding of factors involved in hormonal stimulation responses from studies of insect species, much less has emerged from our literature survey about hormonal receptors in other arthropod lineages. Unlike the investigated insects, Crustacea generally have three or four EcR isoforms, and as many as eight different gene products in the shrimp *L. vannamei* (Dai *et al.*, 2016; Chen *et al.*, 2017). Splicing junctions are located in the E-domain and in the D region, which is a flexible hinge connecting the ligand-binding and DNA-binding domains (Chen *et al.*, 2017; Hyde *et al.*, 2019*a*). These findings are mostly restricted to the class Malacostraca and studies with a more diverse taxonomic sampling would help to confirm these trends of isoform numbers and differential splicing sites. In all examined insect and crustacean species, EcR isoforms show differential expression across tissues and fluctuations throughout the moulting cycle, supporting functional specificity in development (Chen *et al.*, 2017). Differences in RXR isoforms mostly correspond to insertions in the DNA-binding and ligand-binding domains for Crustacea, while in insects they are generally located in the amino-terminal activating region (Techa & Chung, 2013; Eichner *et al.*, 2015; Cheong *et al.*, 2015; Hyde *et al.*, 2019*a*). Over time, Met has been isolated in other insect model species as well as branchiopods and malacostracans (Jindra *et al.*, 2015; Hyde *et al.*, 2019*a*; Belles, 2019; Truman, 2019; Li *et al.*, 2021*b*), but efforts to characterise Met better in other arthropods remain rare. Of note, even though insects and crustaceans use different sesquiterpenoid molecules as the master moulting regulator, JH and MF respectively (Fig. 3), they are believed to target the same receptor. In insects a single substitution from threonine to valine in the Per-Arnt-Sim (PAS) domain appears to have enhanced affinity for JH binding (Miyakawa *et al.*, 2013; Qu *et al.*, 2015). With respect to Met's partner, SRC has to date only been reported in Daphniidae (Miyakawa *et al.*, 2013), so a conserved role for SRC across Pancrustacea cannot be assumed and investigations beyond branchiopods are required.

For Myriapoda too the picture remains far from complete, with EcR-RXR detected in a few centipede and millipede genomes (Chipman *et al.*, 2014; Qu *et al.*, 2015, 2020), but without follow-up experimental validations. Furthermore, our understanding of EcR and RXR in Chelicerata remains limited: while we have genomic evidence for the presence of both the heterodimer components for Acari (*T. urticae*, *Dermatophagoides farinae*, *I. scapularis*, *Panonychus citri*, *Neoseiulus cucumeris*), Araneae (*Stegodyphus mimosarum*), Scorpiones (*Mesobuthus martensii*), Xiphosura (*Limulus polyphemus*, *Carcinoscorpius rotundicauda*, *Tachypleus tridentatus*) (Qu *et al.*, 2015; Schumann *et al.*, 2018; Zhang *et al.*, 2019*b*), recent studies that experimentally characterised them are limited to few spider, mite, and tick species (Honda *et al.*, 2017; Li *et al.*, 2017; Nicewicz *et al.*, 2021; Lu *et al.*, 2021; Li *et al.*, 2022*a*; Yang *et al.*, 2023). Further analysis of EcR and RXR in these groups is required to assess if in chelicerates the predominant bioactive hormone is 20E or ponA (Fig. 2). To this end, results generated by widely used methods need to be carefully interpreted considering their limitations: injected bioforms are subject to differential metabolism that may mask intrinsic activity; expression analysis of genes coding for biosynthetic enzymes need to take into account all relevant genes; relationships between gene expression levels, active enzyme levels, and metabolite fluxes are complex. Quantification of circulating hormone levels gives direct evidence of hormonal forms and advancing mass spectrometry technologies might facilitate quantitative measurements. Experimental assays are needed to support evidence provided by alternative strategies involving structural modelling, such as for the EcR ligand-binding domain, as reported in the spider mite *Panonychus citri*, where ponA is shown to be structurally accommodated better than 20E (Li *et al.*, 2017).

The evidence confirming EcR and RXR presence in Myriapoda is the same that extends the presence of Met to Mandibulata (Chipman *et al.*, 2014; Qu *et al.*, 2015, 2020). Studies of chelicerates have produced conflicting results: Met appears to be absent from the genomes of the spider mite *T. urticae*, the dust mite *D. farinae*, and the scorpion *M. martensii*, whereas it has been identified in the tick *I. scapularis* and horseshoe crab genomes, and isolated at least in the spider *P. tepidariorum* (Qu *et al.*, 2015; Schumann *et al.*, 2018; Nicewicz *et al.*, 2021). Therefore, further characterisation of Met is required to confirm the hypothesis of its role as the main sesquiterpenoid receptor across the entire phylum. Additionally, although transcriptomics data are beginning to accumulate for some taxa, information at isoform level for Myriapoda and Chelicerata is currently almost non-existent (Honda *et al.*, 2017; Li *et al.*, 2017). The status of Met's partner is even less clear, since SRC has been detected in almost all of the few myriapod genomes available until now, but it remains largely unexplored to date, making investigations in other arthropod lineages a priority (Qu *et al.*, 2020; Nicewicz *et al.*, 2021; So *et al.*, 2022).

In summary, the widely held consensus on the presence and main functions of EcR, RXR, Met, and Tai in all major arthropod lineages is in fact based on weakly supported assumptions beyond Pancrustacea, despite these proteins playing a central role in moulting pathways. This is particularly relevant for the sesquiterpenoid receptors of the large and difficult to resolve bHLH-PAS family, whose ligands are variable, and whose roles in growth and development remain largely unclear.

(2) Early cascade genes regulate peripheral tissue remodelling

The gene activation cascade in response to ecdysone stimulation was first observed in the dipteran *Chironomus tentans* as a serial wave of "puffs" of unfolding chromatin on giant polytene chromosomes and then better quantified in

D. melanogaster salivary glands, leading to the description of a temporal sequence of gene transcription, the so-called "Ashburner cascade" (Ashburner *et al.*, 1974; Truman & Riddiford, 2019; Truman, 2019). Ecdysone-inducible genes 74 (E74) and 75 (E75) are named early genes as they are early direct targets of the EcR-RXR complex. Following E74 and E75, the transcription of hormone receptor 3 (HR3), hormone receptor 4 (HR4) and beta Fushi-tarazu transcription factor 1 (βFTZ-F1) indicates the time point for activation of late genes, coding for new exoskeleton components and enzymes (Truman & Riddiford, 2019; Truman, 2019). Apart from E74, which belongs to the E26 transformation-specific (ETS) family, all the aforementioned transcription factors are nuclear hormone receptors. Additional early transcription factors such as E63, E68, and E78 have been identified in insect models but their roles have not been fully elucidated (Liu & Finley, 2010; Swevers, 2019; Praggastis *et al.*, 2021).

These early and early-late genes define the core set of transcription factors orchestrating tissue remodelling, whose complex interactions are beginning to be appreciated thanks to investigations in well-studied insects. E75 and HR3 are localised in a chromatin loop for reciprocal repression (Niwa & Niwa, 2016; Song *et al.*, 2017). While the others are orphan receptors, E75 contains a heme prosthetic group, allowing binding of diatomic gases and regulation *via* nitric oxide of the interaction with HR3 (Hill *et al.*, 2013; Texada, Koyama & Rewitz, 2020). HR3 also negatively regulates HR4, which in turn inhibits βFTZ-F1 expression (Texada *et al.*, 2020). Recent studies in *Drosophila* keep revealing how extensive the spatio-temporal differential expression and chromatin activation is upon EcR binding: EcR both stimulates and represses specific sets of targets, both directly and *via* a set of coregulators (Uyehara, Leatham-Jensen & McKay, 2022; Krasnov *et al.*, 2023). Early genes contribute to sustain the increase of ecdysteroid concentrations by stimulation of hormonal biosynthesis genes, as well as to drive tissue remodelling, both through activation of chitin metabolism and deposition in the extracellular matrix and through intracellular responses, such as apoptosis and autophagy (Yao *et al.*, 2010; Niwa & Niwa, 2016; Zhao *et al.*, 2018; Tsang *et al.*, 2020). Cell death molecular mechanisms occur in response to early gene activation during complete metamorphosis in holometabolous insects (Tettamanti & Casartelli, 2019; Xu *et al.*, 2020). After the late-larval pulse and the prepupal ecdysteroid peak, activated E74, E75, βFTZ-F1, and the EcR/USP heterodimer promote transcription of proapoptotic genes such as *Reaper* (*rpr*), *Head involution defect* (*hid*), initiator caspase *Dronc*, and genes coding for autophagy-related proteins (Atgs), ultimately leading to degeneration of salivary glands, larval midgut, larval abdominal muscles, reshaping of fat body, and neuronal remodelling in the central nervous system (Tettamanti & Casartelli, 2019; Xu *et al.*, 2020). Experiments in *M. sexta*, *B. mori*, and *Helicoverpa armigera* indicate that similar molecular mechanisms act in metamorphic tissues and organs in Lepidoptera (Tettamanti & Casartelli, 2019; Xu *et al.*, 2020; Kang *et al.*, 2023). However, in both orders the cross-talk between apoptosis and autophagy components is complex, some results conversely show a pro-survival autophagic function and it is difficult to establish which pathway contributes most to tissue-specific cellular deletion (Tettamanti & Casartelli, 2019). In Lepidoptera, ecdysteroid stimulation additionally triggers silk and labial gland degradation and female-specific wing degeneration in some moth families (Xu *et al.*, 2020). In *B. germanica*, E75 and βFTZ-F1 regulate apoptotic activation in degenerating prothoracic glands (Tettamanti & Casartelli, 2019). Although rare, molecular investigations in other species show expression of apoptotic and autophagic proteins for both tissues with a physiological function and structures specifying morphological diversity. For instance, programmed cell death is involved in metamorphic remodelling of head projections in horned beetles (Tettamanti & Casartelli, 2019). Remarkably, salivary gland degeneration depends on EcR-induced caspase-dependent apoptosis in the tick *Rhipicephalus haemaphysaloides* (Lu *et al.*, 2021) and histolytic processes have been described in maturation of copulatory organs in male *P. tepidariorum* spiders (Quade *et al.*, 2019).

Beyond Insecta, the densest body of studies aiming to investigate ecdysone responsive genes is from daphnid and several decapod species: E74, E75, E78, HR3, HR4, and βFTZ-F1 are expressed in multiple tissues during moulting (Miyakawa *et al.*, 2018; Hyde *et al.*, 2019a; Brunet, Eichner & Male, 2021; Yuan *et al.*, 2021, 2022*a*; Legrand *et al.*, 2021). Three studies confirmed that E74, E75, and E78 are upregulated during moulting stages preceding ecdysis in *L. vannamei* (Qian *et al.*, 2014; Gao *et al.*, 2015, 2017). Despite an increasing number of whole-body and organ-specific transcriptomic data sets from decapod species, present works seem mainly to stop at functional enrichment analysis with limited experimental characterisation, as in *S. paramamosain* (Gao *et al.*, 2015; Ventura *et al.*, 2015; Lv *et al.*, 2017; Liu *et al.*, 2022*a*; Toyota *et al.*, 2023). A transcriptome analysis throughout the life cycle of the lobster *Panulirus ornatus* enabled annotation of all the nuclear receptors involved in moulting previously identified in insects, along with three new candidate receptors that likely emerged in Malacostraca and show widespread expression (Hyde *et al.*, 2019*b*). Nevertheless, even in crustaceans, reciprocal interactions amongst the core set of transcription factors remain to be explored, as well as moult-induced cell death processes.

Comparative approaches have helped to identify the set of hierarchical cascade genes across all major arthropod subgroups, including the horseshoe crab *L. polyphemus*, the spider *S. mimosarum*, and the centipede *S. maritima* (Fig. 4) (Schumann *et al.*, 2018). While these comparisons found only one cascade gene in *T. urticae*, which is a potential orthologue of βFTZ-F1, analysis of the draft genome had additionally reported putative orthologues of E75, HR3, and HR4 (Grbić *et al.*, 2011) and recent RNA sequencing found transcripts for these early genes in *T. urticae* deutonymphs (Li *et al.*, 2021*a*). Their presence in chelicerates is supported by the observation of fluctuating expression of E75, E78,

HR3, and βFTZ-F1 in *Panonychus citri* and *Agelena silvatica* (Fig. 4) (Honda *et al.*, 2017; Li *et al.*, 2017, 2020). Apart from *S. maritima*, no other myriapod species has been the object of such a study, so the putative repertoire of cascade genes is still largely based on assumptions of evolutionary conservation.

Genomic detection coupled with a small number of laboratory findings only provide a limited description of early cascade genes beyond insects and it highlights once again that Chelicerata and Myriapoda particularly suffer from underrepresentation in molecular studies of moulting components. Furthermore, in view of insect diversity, characterisation of early gene-driven tissue remodelling needs to be expanded beyond *D. melanogaster* and a restricted number of other insect model systems if a more complete picture is to emerge.

(3) Fate determination genes define metamorphic moulting

Insects have been intensively studied in order to understand the developmental mechanisms behind ametabolous, hemimetabolous, and holometabolous lifestyles (Jindra, 2019). Hypotheses and supporting evidence regarding these mechanisms have been thoroughly analysed in recent literature reviews and these highlight difficulties in drawing well-defined conclusions to answer this long-standing question (Jindra *et al.*, 2015; Belles, 2019; Truman & Riddiford, 2019; Truman, 2019). Nevertheless, investigating expression of fate determination genes has been a highly informative approach to compare the different stages from the three modes of insect development. Such an approach could also be helpful to delineate which molecular mechanisms underlie the richness of life strategies observed in other arthropod lineages. In hexapods, the outcome of the next moult depends on the simultaneous activation and repression of three key genes, tightly controlled by integration of the JH and ecdysone pathways (Belles, 2019; Jindra, 2019; Martín, Chafino & Franch-Marro, 2021; He & Zhang, 2022): Kruppel homologue 1 (Kr-h1), a zinc-finger transcription factor which represents the main player to translate the JH signal into the maintenance of immature stages; Broad, also belonging to the zinc finger transcription factor family, is considered to mediate the ecdysteroid-induced transition from the juvenile to the adult; and ecdysone-inducible protein 93F (E93), an HLH transcription factor with a chromatin remodelling function, as the gatekeeper for the establishment of the adulthood fate programme. Broad and E93 influence moulting also by supporting ecdysteroid titre towards peaking, through transcriptional stimulation of enzyme-coding genes (Tsang *et al.*, 2020; Kamiyama & Niwa, 2022). Met, Kr-h1, and E93 gave their names to the Met-Kr-h1-E93 (MEKRE93) pathway, but current understanding has established Broad as an essential component of this metamorphic gene network (Martín *et al.*, 2021; Truman & Riddiford, 2022). Here, we provide a brief overview of the overall mechanistics from findings in representative species of each metaboly mode.

Upon JH stimulation, the activated Met/Tai receptor dimer binds the so-called juvenile hormone response element located in the regulatory sequences of Kr-h1 (Jindra *et al.*, 2015; Li *et al.*, 2019*b*; Belles, 2019). Regardless of the species and the timing in the moulting phase, Kr-h1 expression always responds to JH stimulation. In the hemimetabolous *B. germanica* (Blattodea), Kr-h1 represses E93 and sustains expression of Broad, acting as a nymphal specifier during the entire nymphal period (Truman & Riddiford, 2019; Truman, 2019). At the last instar moult, JH is no longer present, E93 is upregulated by the ecdysteroid peak, it represses Broad and commits the organism to the adult fate (Truman & Riddiford, 2019; Truman, 2019). By contrast, during the larval instars in the holometabolous *T. castaneum* (Coleoptera) JH and Kr-h1 inhibit both Broad and E93, which can be expressed only when JH declines in the last instar (Truman & Riddiford, 2019; Truman, 2019). A small pre-pupal peak in JH titre conversely supports Broad against E93, to prevent a direct larva-to-adult transition and instead ensure a larva-to-pupa intermediate passage before adulthood. In fact, JH suppression in *T. castaneum* larvae results in a precocious adult phenotype and not in a block during pupation (Truman & Riddiford, 2019; Truman, 2019).

In *D. melanogaster* and *B. mori* (Diptera and Lepidoptera, respectively), unlike in *T. castaneum*, E93 is not expressed in the last instar but is found from the late pre-pupal stage, consistent with the observation that, in the absence of JH, Broad allows the development of pupae that are unable to emerge as adults (Truman & Riddiford, 2019; Truman, 2019). Endopterygota wings originate from the imaginal primordia, a monolayer of coiled-coil embryonic quiescent cells that are precursors of the imaginal discs, highly proliferating structures without secretory activity. In hemimetabolous nymphal instars, Broad is required for morphogenic growth in imaginal cells that invaginate and become committed and nutrient-independent (Truman & Riddiford, 2019; Truman, 2019). Similarly, Broad appears in the late larval instar, when the ecdysteroid peak induces morphogenic and metamorphosis commitment in imaginal discs (Truman & Riddiford, 2019). Time series analysis of chromatin accessibility and transcriptomics in *D. melanogaster* imaginal discs revealed extensive differential enhancer activation corresponding to an ecdysone-induced temporal cascade of transcription factors, highlighting the main role of E93 in controlling early and late-enhancer activity to define the adulthood programme (Uyehara *et al.*, 2017; Niederhuber & McKay, 2021).

Some evidence in insects that evolved neometaboly, a holometabolous-like lifestyle characterised by a variable number of feeding larvae and non-feeding pupal stages, seem to support the role of Kr-h1, Broad, and E93 as determinants of a juvenile status, pupal commitment, and adult fate, respectively. In *Frankliniella occidentalis* and *Halothrips brevitubus* of the order Thysanoptera, Kr-h1 and Broad are JH-responsive and their expression progressively decreases from the larval to the pro-pupal and pupal instars, while E93 has an opposite trend, making the overall expression profile similar to those in pre-pupal and pupal phases

(Truman, 2019; Suzuki *et al.*, 2021). *Planococcus kraunhiae* male individuals from the neometabolous family Pseudococcidae (Hemiptera) undergo metamorphosis, showing decreased Kr-h1 expression in the prepupal stage, while Broad appears and then is replaced by E93; in stark contrast, wingless females remain in a nymph-like state and do not express E93 (Truman, 2019). *Ericerus pela* from the family Coccidae have sex-specific transcriptomic profiles and developmental programs, as wing morphogenesis occurs only in non-neotenic males (Yang *et al.*, 2015). Similarly, in Strepsiptera (endoparasites of other insects), loss of complete metamorphosis is a sexually dimorphic trait: a neotenic larva-like, parasitic phenotype persists in females. Indeed, in stark contrast to metamorphic males that express Broad and E93, their expression in *Xenos vesparum* females is barely detectable (Truman, 2019). Despite a lack of studies on wingless species from early-branching Hexapoda lineages, there is evidence for Kr-h1, Broad, and E93 in the non-insect dipluran *C. aquilonaris* and in the ametabolous *T. domestica* (order Zygentoma), where they do not show significant differences in expression throughout post-embryonic development (Belles, 2019; Truman & Riddiford, 2019; Fernandez-Nicolas *et al.*, 2023).

Recent molecular studies of the hemimetabolous *C. dipterum*, from the order Ephemeroptera, investigated the unique development *via* the so-called subimago (Kamsoi *et al.*, 2021). Mayflies are the only extant species exhibiting initially thick, ciliated wings, of the subimago, which quickly moult into light, thin, wings (Belles, 2019; Truman & Riddiford, 2019). Kr-h1 and Broad expression decrease in the last nymphal stage, in contrast to E93, which is upregulated. As in other neopteran insects, applying a JH mimic results in a supernumerary nymph and E93 suppression (Kamsoi *et al.*, 2021). These observations indicate that when subimagos are formed, metamorphosis has already been determined and that the subimago should be considered as a first, adult instar and not a modified last nymphal stage. In Odonata, another hemimetabolous order closely related to Ephemeroptera, experiments in the dragonfly *Ischnura senegalensis* show that fate determination genes are expressed in a similar fashion as in mayflies and other hemimetabolous insects: Kr-h1 and Broad are expressed throughout the nymphal stages, whereas E93 is expressed during the late immature instar and downregulation of Kr-h1 results in upregulation of E93 and precocious metamorphosis (Okude *et al.*, 2022). Interestingly, downregulation of Broad showed, even without alteration in Kr-h1 and E93, variation of genes differentially expressed restricted to both the nymphal and adult fate and revealed a unique mosaic phenotype (Okude *et al.*, 2022).

Curiously, an adult moult has been found in neotenic females of the firefly *Lamprigera minor* (order Coleoptera), representing, along with mayflies, another apparent violation of the rule of the terminal moult in metamorphic insects (Kamsoi *et al.*, 2021). A supernumerary post-adult moult in four fully reproductive mature individuals was observed, likely caused by impeded oviposition, resulting in their deaths (Jeng *et al.*, 2021). Authors speculate that female dimorphism may imply not only strong morphological neotenic characters, but also physiological larval features, such as retainment of the prothoracic glands, that may cause aberrant Broad and E93 expression (Tettamanti & Casartelli, 2019; Jeng *et al.*, 2021).

Recent studies have identified additional putative key components of this fate determination regulatory network (Truman & Riddiford, 2022; Chafino *et al.*, 2023). Chronologically inappropriate morphogenesis (*chinmo*) gene, encoding a BTB/POZ (broad complex Tramtrack bric-a-brac/Pox virus and zinc finger) transcription factor like Broad, was proposed instead to play the role of a larval master gene. In *D. melanogaster*, it appears around mid-embryogenesis until the critical mass point is reached, determining larval development by repressing Broad, it then declines allowing Broad-dependent imaginal morphogenesis and when removed, larval and imaginal epithelium shows an altered metamorphic-like phenotype, along with Broad and E93 precocious expression (Truman & Riddiford, 2022; Chafino *et al.*, 2023). In *T. castaneum* chinmo acts cooperatively with the BTB transcription factor abrupt, where *chinmo-abrupt* double knockdown leads to precocious metamorphosis (Khong, Hattley & Suzuki, 2024). The expression pattern and knockdown experiments in *B. germanica* seem to confirm its role as a larval determinant also in hemimetabolous insects (Chafino *et al.*, 2023). In summary, insights from the study of fate determination genes, together with the accumulation of other morphological evidence beyond the scope of this work, corroborate the hypothesis that the holometabolous larva–pupa–adult phases correspond to the hemimetabolous pronymph–nymph–adult phases; in other words, the feeding larva is a cryptic modified embryo and progressive contraction of the number of nymphal instars led to the condensation into a pupal stage.

Despite analyses of the fate determination gene network having provided useful information for piecing together the changes in insect life-history transitions, how fate determination genes might have contributed to different life strategies is far from comparably explored in other arthropod subgroups. To the best of our knowledge, the role of E93 as an adulthood determinant has never been studied beyond Hexapoda and we did not find any published work investigating it in other arthropod groups of interest (Fig. 4). Chinmo has so far been studied beyond *D. melanogaster* only in *T. castaneum* (Khong *et al.*, 2024) and *B. germanica* (Chafino *et al.*, 2023). Kr-h1 has been isolated in *Daphnia* and in the crabs *S. paramamosain* and *E. sinensis* (Miyakawa *et al.*, 2018; Hyde *et al.*, 2019*a*; Li *et al.*, 2021*c*). In stark contrast to insect Kr-h1, which is the main target of the JH-activated Met receptor, in these species, Kr-h1 is not responsive to MF stimulation (Hyde *et al.*, 2019*a*). Additionally, Kr-h1 expression across different developmental stages has been described in the spider mite *Panonychus citri* as relatively stable with a peak upon adulthood (Li *et al.*, 2017). Broad has attracted particular attention: it was reported as missing in Myriapoda and Chelicerata, despite the low number of genomes studied

(Chipman *et al.*, 2014; Qu *et al.*, 2015). Its putative orthologue has been found as multi-copy genes in decapod species such as *P. monodon*, *N. denticulata*, *P. hawaiensis*, *M. nipponense*, and *L. vannamei*, the amphipod *G. fossarum*, and four barnacles, suggesting that Broad may be a pancrustacean innovation (Buaklin, Klinbunga & Mensveta, 2011; Buaklin *et al.*, 2013; Sin *et al.*, 2015; Jiang *et al.*, 2015; Qu *et al.*, 2015; Gouveia *et al.*, 2018; Hyde *et al.*, 2019*a*; Zhang *et al.*, 2019*a*; Gan *et al.*, 2020; Ip, Qiu & Chan, 2021).

Kr-h1 characterisation beyond Hexapoda remains poor and current studies have already pointed out potential differences in other arthropod species, thus further investigations are needed. If the role of Broad is further proved to be restricted to Pancrustacea and E93 and chinmo as restricted to Hexapoda, whether there exist equivalent master genes of fate determination in other arthropod lineages and their identification remain an open and unexplored field of investigation. These gaps mean that additional future research is needed to extend our knowledge of the roles of Broad, chinmo, Kr-h1, E93 and other putative clade- and stage-specific genes in relation to arthropod heterogeneity of life histories.

V. LATE GENES INVOLVED IN EXOSKELETON REMODELLING, FROM PRE- TO POST-ECDYSIS

The cuticle structures of arthropod exoskeletons and their mechanical properties are described by Bouligand's model of multiple helicoid layers of fibres, who first modelled it in crustaceans (Bouligand, 1965). The ultrastructural details and their implications for cuticle biology and arthropod morphology have been the subject of many studies, reviewed extensively elsewhere (Moussian, 2010; Minelli, 2011*a*,*b*; Roer *et al.*, 2015; Stamm, Saltin & Dirks, 2021; Politi *et al.*, 2021). Although the layer nomenclature may vary, the cuticle structure is highly conserved across arthropods and comprises two main functional regions covering the epidermis, the procuticle and the epicuticle (Moussian, 2010; Minelli, 2011*a*,*b*; Roer *et al.*, 2015; Politi *et al.*, 2021). The innermost procuticle, comprising the endocuticle and exocuticle, is a relatively thick region, made of a highly stratified organic matrix of parallel sheets formed by chitin and protein microfibres, with each sheet twisted by a small angle with respect to the one below resulting in the characteristic helicoidal arrangement. The much thinner outer region known as the epicuticle is a multi-stratified structure with an outermost layer (envelope, cement layer) composed of neutral lipids, wax esters, and proteins, which acts as a primary permeability barrier, and an ultrastructurally distinct layer beneath the envelope that also contains lipids and proteins and lacks chitin. Much of the protective strength of the cuticle is due to the helicoidal architecture of the procuticle, which derives from the arrangements of layers of chitin microfibrils (Neville & Luke, 1969) facilitated by a self-assembly process (Vaclaw *et al.*, 2018). Collectively, the properties of these layers with their different constituent components and arrangements provide protection from the environment and from compression and tension stresses applied by predators.

In insects, an increased titre of 20E initiates a response in the epithelial cells (Fig. 4) and triggers several concurrent processes (Fig. 1B), which include the synthesis of the new cuticle by chitin synthase (CHS), the digestion of the old one and the reabsorption of mineral and organic elements for reuse in the structural maturation of the new cuticle. In addition, specialised epithelial cells at the muscle–cuticle interface need to re-attach to the new cuticle. Early in moulting initiation, apolysis can be observed: the detachment of cells from the cuticular layers results in the formation of an apolysial space, where moulting enzymes encoded by late genes, such as chitinases (CHTs) and proteases are secreted (Bitsch & Bitsch, 2002; Song *et al.*, 2017). Neurostimulation prompts ecdysial escape and successful emergence from the old cuticle initiates post-ecdysial strengthening of the newly formed cuticular layers and restoration of pigmentation. This last section reviews our understanding of the late genes involved in structural aspects of exoskeleton remodelling, at the terminus of the ecdysteroid hormone stimulatory cascade.

(1) Enzymes and structural proteins mediate cuticular chitin synthesis, degradation, and organisation

Under the control of the premoulting surge of 20E and mediated by the late genes pathway, the storage carbohydrate trehalose is incorporated into chitin microfibrils in the new cuticle prior to moulting. The enzyme that catalyses the final of eight biochemical steps of chitin monomer synthesis from trehalose is generally referred to as chitin synthase (CHS) (Fig. 5) (Merzendorfer & Zimoch, 2003; Yao *et al.*, 2010; Zhang *et al.*, 2021). The number of *Chs* genes identified in an insect genome is usually limited to two genes, *Chs1* and *Chs2*. Whereas integument synthesis mostly depends on CHS1, CHS2 is involved in peritrophic membrane synthesis. Despite the limited number of *Chs* genes, they are reported to have numerous exons, for example, 24 in *Spodoptera exigua*, thus being capable of generating multiple alternative splicing variants (Liu, Zhang & Zhu, 2019*b*). In Crustacea, the number of *Chs* genes is variable, with cirripedes and copepods having two or more genes with unknown specialisation, while most decapods possess a single *Chs* gene (Zhang *et al.*, 2021; Xin *et al.*, 2021). Chelicerates also seem to harbour one unique copy (Zhang *et al.*, 2021; Xin *et al.*, 2021). Unfortunately, CHSs seem largely unexplored in spiders, compared to their preliminary explorations in ticks and mites, and information on these genes is also lacking for Myriapoda (Han *et al.*, 2005; Li *et al.*, 2015, 2021*e*,*f*; Liu *et al.*, 2019*a*; Becchimanzi *et al.*, 2020; Zhang *et al.*, 2021; Song *et al.*, 2021; Yuan *et al.*, 2022*b*; Chen *et al.*, 2023).

Degradation of the old cuticle by moulting fluid is initiated when the ecdysteroid titre drops shortly before ecdysis, allowing the exoskeleton to maintain its mechanical functions right

Phase	Pathway	Hexapoda	Crustacea	Myriapoda	Chelicerata
Pre-ecdysis and ecdysis	Chitin synthesis	CHS	CHS	?	CHS
	Chitin degradation	CHT CDA	CHT CDA	?	CHT CDA
	Cuticle maturation	knk, rtv, Obst-A, CDA	CPAP3 ?	?	?
	Muscle reattachment	Myospheroid, Fondue, Tiggrin, Talin, Thrombospondin	Thrombospondin ?	?	?
Post-ecdysis	Sclerotisation and melanisation	CP crosslinking: catechol quinone-based, TH, Ddc, tan, dat, b, e, MCO2, melanin	CP crosslinking: PO, hemocyanin ?	CP crosslinking: di-tyrosine ?	CP linking: transglutaminase-mediated on Glu and Lys ?
	Mineralisation	Rare calcification; HEBs	Calcification, HEBs	Calcification ? HEBs	? HEBs

Fig. 5. Summary of main late effector genes from pre-ecdysis, ecdysis, and post-ecdysis pathways. Late genes code for downstream effectors of exoskeleton remodelling processes throughout the whole moulting cycle, such as pre-ecdysis and ecdysis chitin synthesis and degradation, cuticle maturation, muscle reattachment, and post-ecdysis mineralisation and tanning (sclerotization and melanisation). Summary knowledge is presented for six pathways/processes for each of the four considered subphyla Hexapoda, Crustacea, Myriapoda, and Chelicerata. Question marks indicate that further clarifications in the group under consideration are needed with respect to assumed equivalent processes across the subphyla because the presence of the relevant gene(s) has not been confirmed. b, black; CDA, chitin deacetylase; CHS, chitin synthase; CHT, chitinase; CP, cuticle protein; CPAP3, proteins analogous to peritrophins 3; dat, dopamine N-acetyltransferase; Ddc, dopa decarboxylase; e, ebony; HEB, heavy element biomaterial; knk, Knickkopf; MCO2, multi-copper oxidase2 or laccase2; Obst-A, Obstructor-A; PO, phenoloxidase; rtv, retroactive; tan, tanning; TH, tyrosine hydroxylase.

up until shedding (Reynolds & Samuels, 1996). The chitin fibrils from the former exoskeleton are partially digested for resorption and the degradation of the old integument is mainly dependent on CHTs and chitin deacetylases (CDAs) (Fig. 5). These enzymes are responsible for renewing the pool of chitin monomers for new cuticle synthesis. All chitin-degrading enzymes belong to highly expanded multigene families and were identified for selected species from all arthropod subphyla (Han *et al.*, 2005; Li *et al.*, 2015, 2021*e*, *f*; Liu *et al.*, 2019*a*; Becchimanzi *et al.*, 2020; Zhang *et al.*, 2021; Song *et al.*, 2021; Yuan *et al.*, 2022*b*; Chen *et al.*, 2023). Enzymes identified in Insecta and Crustacea are further classified into several groups based on sequence diversity, but classifications might be refined as new genomes become available (Zhang *et al.*, 2021). Studies in a few representative species of all these subphyla have described the expression of enzymes involved in chitin metabolism across different tissues and developmental phases and have shown that suppression of CHSs and CDAs significantly impairs the moulting cycle (Moussian *et al.*, 2015; Ventura *et al.*, 2015; Ali *et al.*, 2020; Li *et al.*, 2021*e*; Zhang *et al.*, 2021; Xin *et al.*, 2021; Song *et al.*, 2021).

To initiate new cuticle synthesis, chitin bundles are secreted by epidermal cells, organised into microfibrils, and after binding with cuticle proteins (CPs) form a characteristic stereotypical structure (Moussian, 2010; Roer *et al.*, 2015; Politi *et al.*, 2021). CPs are grouped into 12 families based on their sequence motifs: the CPR family with the chitin-binding Rebers and Riddiford (R&R) consensus motif is the most abundant. It is widely present across all arthropod subphyla, with more than 150 proteins in *A. gambiae* and *L. vannamei* (Willis, 2010; Chipman *et al.*, 2014; Zhang *et al.*, 2019*a*; Li *et al.*, 2022*b*). Subclassifications of the R&R motif have been associated with properties of the cuticle, with the RR-1 motif associated with softer cuticle and RR-2 with harder cuticle. These R&R motif-containing CPs bind to chitin microfibrils, and are implicated in the natural self-assembly of cuticle macromolecular architectures (Vaclaw *et al.*, 2018), however, for the majority of the non-insect arthropod R&R CPs, only basic annotation studies have been conducted and experimental characterisations in recent years are scarce (Faircloth & Shafer, 2007; Willis, 2010; Moussian, 2010, 2013; Chipman *et al.*, 2014; Gao *et al.*, 2017; Vaclaw *et al.*, 2018; Li *et al.*, 2022*b*). Besides R&R CPs, several members of other CP families have proven physiological functions in protecting newly synthesised cuticle and in organising cuticular layers: in *D. melanogaster* and *T. castaneum*, the maturation of cuticle structure was reported

to be dependent on CPs, such as Knickkopf (knk), Retroactive (rtv) and Obstructor-A (Obst-A) (Fig. 5) (Moussian *et al.*, 2015; Pesch, Riedel & Behr, 2015; Pesch *et al.*, 2016, 2019). rtv participates in the trafficking of knk to the apical plasma membrane, which binds newly synthesised chitin fibrils and thus protects them from chitinase degradation; moreover, these two proteins are involved in the orientation of cuticular lamina (Chaudhari *et al.*, 2015). Obst-A and knk direct cuticle organisation together with the genes coding for CDA1 (*Serpentine*) and CDA2 (*Vermiform*) of Group I CDAs (Pesch *et al.*, 2015, 2016, 2019; Liu *et al.*, 2019*a*; Zhang *et al.*, 2023*b*). Obst-E is also necessary for control of the puparium shape and thus, for proper transition from larva to pupa, and it may be involved in the formation of the escape gap in the puparium (Tajiri *et al.*, 2017, 2023).

Beyond model systems, experimental manipulation studies of CPs with organisation roles are increasing in other insect species, while in Crustacea our knowledge is still rudimentary (Yang *et al.*, 2020). All members of the family of cuticle proteins analogous to peritrophins (CPAPs) related to Obst-A, E, and knk were annotated and functionally tested using RNAi experiments in the decapod *Cherax quadricarinatus* (Willis, 2010; Abehsera *et al.*, 2018*b*). Results revealed two cysteine-rich CP genes *CqCPAP3A* and *CqCPAP3E* that are probably involved in the three-dimensional organisation of the cuticle, similar to complexes described in insects (Fig. 5) (Abehsera *et al.*, 2018*b*). Moreover, homologues of CDA1, CDA2, and knk were annotated in the moulting cycle transcriptome of *C. quadricarinatus* cuticle, which suggests similar mechanisms of new cuticle maturation (Abehsera *et al.*, 2018*a*,*b*). In the spider *P. pseudoannulata*, a higher percentage of CPs were found expressed in the pre-moult phase than during the post-moulting period (Li *et al.*, 2022*b*). CPs are clearly present in myriapods, however, they still lack detailed exploration in relation to moulting (Chipman *et al.*, 2014; Kenny *et al.*, 2015).

After the formation of the new cuticle, muscles must be reattached to the new chitinous matrix to be able to escape successfully from the exuviae. Tendon cells are specialised epithelial cells that mediate the connection of muscles with the exoskeleton (Bitsch & Bitsch, 2002; Žnidaršič *et al.*, 2012; Mrak *et al.*, 2017; Muthukrishnan *et al.*, 2020). The apical part of the tendon cells forms fibrils that are anchored to the cuticle. During pre-moult, these fibrils form connections with both old and new cuticles, probably intensifying the pressure from muscle contraction during ecdysis. These specific epithelial cells were reported in *D. melanogaster* and a few species from other arthropod sublineages, such as in the crustacean orders Euphasiacea (*Euphasia superba*), Podocopida (*Bicornucythere bisanensis*), and Isopoda (*Eurydice pulchra* and *Porcellio scaber*), and the chelicerate orders Araneae (*Latrodectus mactans*), and Xiphorusa (*L. polyphemus*). No description is available for myriapods (Bitsch & Bitsch, 2002; Žnidaršič *et al.*, 2012; Mrak *et al.*, 2017; Muthukrishnan *et al.*, 2020). Nevertheless, even in insect species, the exact protein composition of the extracellular tendon fibrils – the microtubules inside the tendon cells – remains unknown. In *T. castaneum*, CDAs are necessary also in this process but several key players have been identified in insects, such as the clotting proteins Myospheroid, Fondue, Tiggrin, Talin, and Thrombospondin (Fig. 5) (Green *et al.*, 2016; Lv *et al.*, 2017; Muthukrishnan *et al.*, 2020; Mun *et al.*, 2022). Interestingly, Thrombospondin has been cloned in decapods: in the prawn *P. monodon*, it is widely expressed across tissues and developmental stages, while in the crab *E. sinensis* it has been found activated during pre-moult and is probably involved in the reattachment of tendon cells, as in insects (Zhou *et al.*, 2011; Tian & Jiao, 2019). As for Myriapoda, no information is available, and while proteomic analyses in the tarantula *Acanthoscurria geniculata* have suggested haemocyanin and clotting protein systems might be conserved in Chelicerata immunity, no link with fibril formation has been made (Sanggaard *et al.*, 2016).

In summary, enzymes involved in chitin metabolism, despite being activated only later during the moulting cascade, play a primary role in the success of moulting events. Compared to our understanding of the chitin synthesising and degrading proteins, characterisation of chitin-binding and chitin-organising cuticle proteins is highly unbalanced, since it is almost only detailed in *D. melanogaster* and *T. castaneum*.

(2) Neuropeptides regulate stereotypical ecdysis behaviours

After the formation of a new cuticle, arthropods need to shed the former exoskeleton and escape the exuviae, to complete the moulting process. An increase in internal pressure, due to air or water swallowing, allows the animal firstly to rupture the old cuticle along specific, ecdysial lines and then to push the body out, emerging from the exuvia and growing in size through expansion of the new, soft cuticle (White & Ewer, 2014). The entire process is mediated by stereotyped extension and expansion movements and abdominal and peristaltic muscle contractions along the anterior–posterior axis, and is generally referred to as ecdysis behaviour (White & Ewer, 2014). The proteins and neuropeptides implicated in regulating and executing these processes are generally identifiable across Arthropoda, for example the GPCR receptor for ecdysis triggering hormone, or the Bursicon neurohormone, but characterisations of their interactions and the networks or cascades in which they participate remain limited.

In insects, the initiation of the ecdysis behaviour pattern starts following a decline of 20E titre in the haemolymph, due in part to Cyp18a1, a cytochrome P450 enzyme with hydroxylase activity that inactivates 20E (Fig. 6) (Guittard *et al.*, 2011). It has been detected across the major arthropod sublineages (Schumann *et al.*, 2018; Qu *et al.*, 2018; Dermauw *et al.*, 2020). Interestingly, the *cyp18a1* gene is missing in the mite *T. urticae*, coherently with *phm* absence and ponA experimental identification, and is also absent from *Varroa* species, suggesting a loss in Acari. Moreover, *cyp18a1* is also

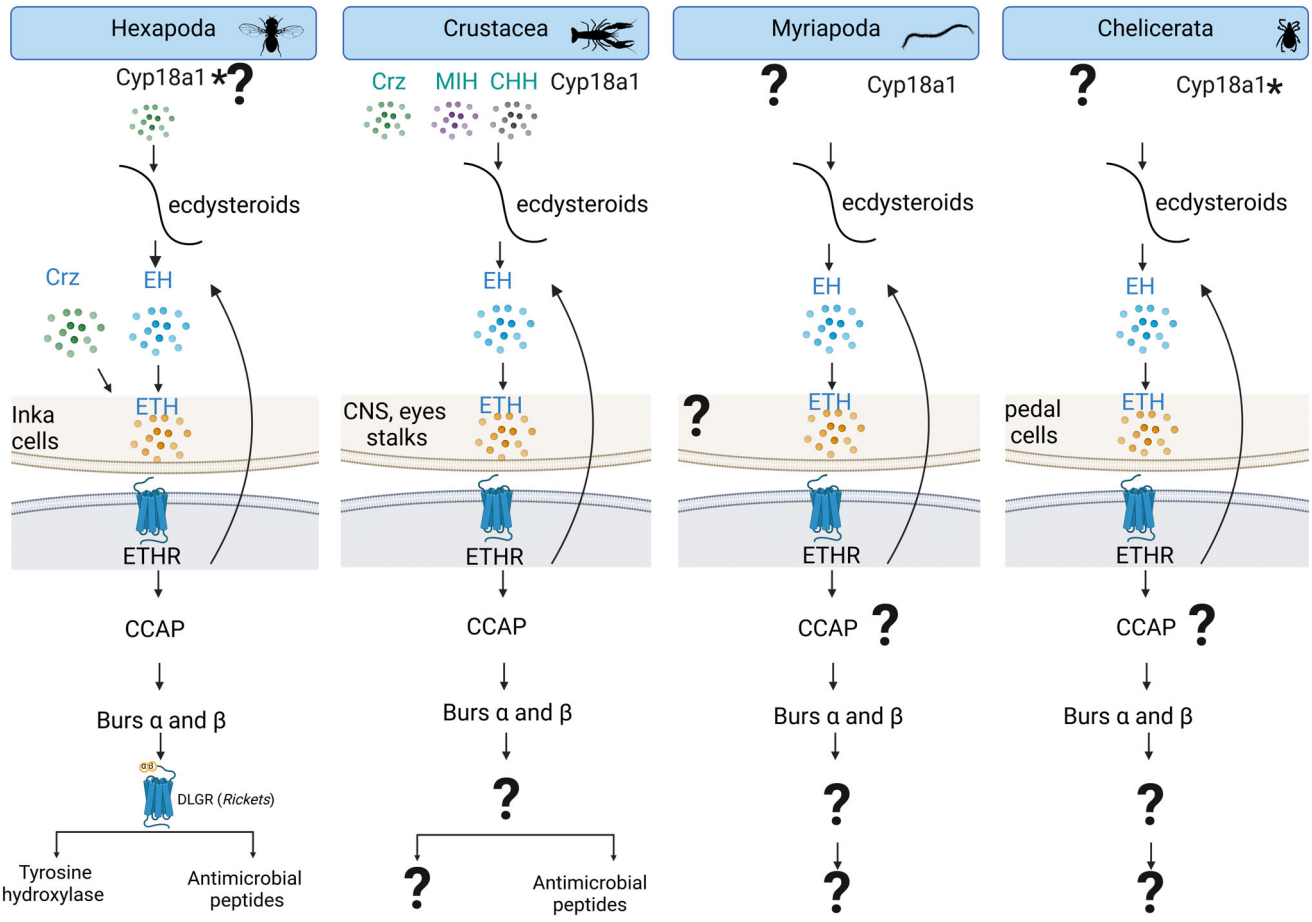

Fig. 6. Overview across Arthropoda of neuropeptide-associated pathways that lead to exuviae escape. In Pancrustacea, catabolism of ecdysteroids triggers the neuromotor cascade: release of eclosion hormone (EH) and ecdysis triggering hormone (ETH) neuropeptides, acting as a positive feedback loop, followed by crustacean cardioactive peptide (CCAP) and Bursicon (Burs), resulting in ecdysis and post-ecdysis responses, such as activation of sclerotization and tanning pathways and immune responses. Crz and the crustacean hyperglycemic hormone (CHH) family might also be involved. Cyp18a1 is missing in *Anopheles gambiae*, *Blattella germanica*, *Tetranychus urticae*, and *Varroa destructor*. The four columns of pathway information refer to summary knowledge of the subphyla Hexapoda, Crustacea, Myriapoda, and Chelicerata. Question marks indicate that further clarifications in the group under consideration are needed with respect to assumed equivalent processes across the subphyla.Burs, Bursicon; CCAP, crustacean cardioactive peptide; CHH, crustacean hyperglycemic hormone; CHH, crustacean hyperglycemic hormone; CNS, central nervous system; Crz, corazonin; DLGR, *Drosophila* leucine-rich repeat G protein-coupled receptor; EH, eclosion hormone; ETH, ecdysis triggering hormone; ETHR, ecdysis triggering hormone receptor; MIH, moulting inhibiting hormone.

reported as missing from *A. gambiae* and Blattellidae (Qu *et al.*, 2015; Techer *et al.*, 2019; Li *et al.*, 2019*a*; Dermauw *et al.*, 2020). These examples suggest the existence of alternative 20E catabolism and titre regulation, which may involve inactivating oxidation and epimerisation and/or conjugation processes. Studies from hemipteran, dipteran, lepidopteran, and hymenopteran insects show that 20E can be inactivated by oxidation and conjugation to phosphates, acetates, fatty acyl esters, or sulphonates (Lafont *et al.*, 2017), and that some members of the large ecdysteroid kinase-like gene family are known inactivators (Scanlan & Robin, 2024). The degradation of 20E plays a central role in the subsequent regulation of moulting, by triggering the coordinated release of the ecdysis triggering hormone (ETH) and eclosion hormone (EH) from the tracheal Inka cells and the central nervous system, respectively (Fig. 6). The ETH–EH system works as a positive feedback loop which reinforces itself and guides the irreversible stereotypical ecdysis behaviour (White & Ewer, 2014; Malhotra & Basu, 2023). The ecdysis motor was first discovered in the holometabolous *D. melanogaster*, *M. sexta*, *B. mori*, and *T. castaneum*, and in the hemimetabolous *S. gregaria*, which, after years of research, are by far the best characterised species (White & Ewer, 2014; Malhotra & Basu, 2023). Most insects possess a single gene that encodes EH, but the genomes of some species such as *Acyrthosiphon pisum*, *T. castaneum*, and *L. migratoria* have two or three putative paralogs of EH (Huybrechts *et al.*, 2010; White & Ewer, 2014; Veenstra, 2016*b*). From evidence in *M. sexta*

and more recently in *Bactrocera dorsalis*, an additional putative inducer of ETH release is corazonin, which might also be an inducer of ecdysteroid synthesis, as discussed in Section II.2 (Hou *et al.*, 2017; Malhotra & Basu, 2023). Notably, in *R. prolixus* corazonin is not required for moulting, while in *D. melanogaster* its role has been questioned (Song *et al.*, 2017; Sterkel *et al.*, 2022). Moreover, no immunoreactivity nor corazonin-coding gene and receptor were found in beetles (Hou *et al.*, 2017; Song *et al.*, 2017; Pandit *et al.*, 2019).

ETH seems conserved in insects beyond Pterygota, with some slight differences noted amongst insects: for the majority, the expression from a single *Eth* gene leads to two neuropeptide isoform variants, called ETH1 and ETH2, where ETH2 is called pre-ecdysis triggering hormone (PETH) in a few species of Lepidoptera, such as *M. sexta* and *B. mori* (Roller *et al.*, 2010; Derst *et al.*, 2016). These neuropeptides differ in length and activity: ETH1 is a longer peptide, while ETH2 (PETH) is shorter (White & Ewer, 2014; Malhotra & Basu, 2023). The activity of ETH2 (PETH) was shown specifically to initiate pre-ecdysis in *M. sexta* and *D. melanogaster*, while ETH1 alone is capable of initiating the whole sequence of ecdysis behaviour; by contrast, transcript variants of the *Eth* gene have interchangeable activity in *B. mori* and *A. aegypti* (Roller *et al.*, 2010). Only one type of ETH was confirmed for *A. mellifera*, *Nasonia vitripennis*, and *A. pisum*, which may suggest the role of ETH2 (PETH) is played by ETH1 on its own or by another component (Roller *et al.*, 2010). The activity of ETH is not limited to muscle contractions during ecdysis but includes an inducing effect on the frontal ganglion that initiates air-swallowing behaviour (Zilberstein, Ewer & Ayali, 2006; White & Ewer, 2014). The ETH signal is recognised by GPCRs that are transcribed from a single ecdysis triggering hormone receptor (ETHR) gene (*Ethr*) and transcripts go through alternative splicing to become alternative isoforms ETHR-A and ETHR-B. ETHRs are distributed both in neural and non-neural organs, but different ETHR isoforms might have different functional specialisations, as shown in *D. melanogaster*, *A. gambiae*, and the beetle *L. decemlineata* (Roller *et al.*, 2010; Diao *et al.*, 2016; Shen *et al.*, 2020; Jindal, Park & Kim, 2021; Daubnerová *et al.*, 2021).

The downstream pathway of EH is still unclear, but the evidence suggests that the signal is transmitted through two secondary messengers, cyclic GMP (cGMP) and Ca^{2+} followed by the release of ETH (White & Ewer, 2014; Song *et al.*, 2017; Malhotra & Basu, 2023). The activation of ETHR leads to Ca^{2+}-dependent pre-ecdysis excretion of neuropeptides, such as kinins, diuretic hormone and pigment dispersing factor, but the function of each particular hormone is still under investigation (White & Ewer, 2014; Song *et al.*, 2017; Malhotra & Basu, 2023). The release from the central nervous system of one of these, first identified in Crustacea and thus named crustacean cardioactive peptide (CCAP) despite being conserved across arthropods, is clearly induced by a surge in EH titre (White & Ewer, 2014). This represents the main signal to switch off the pre-ecdysial phase and initiate the irreversible pattern that finally leads to exuviae escape and wing expansion (White & Ewer, 2014). In *S. gregaria*, a prothoracicotropic inhibitory function on ecdysteroid synthesis has been reported for CCAP, strengthening its role as a "no-way-back" factor in the irreversible ecdysial motor (Verbakel *et al.*, 2021). Moreover, a combination of myoinhibitory peptides and sNPF is involved in the termination of ecdysis behaviour and the transition to post-moult functional specialisation of the cuticle (Daubnerová *et al.*, 2021).

Initiation of ecdysis behaviour in Crustacea involves the same hormones that regulate ecdysteroid synthesis – MIH and CHH. During pre-moult phases, CHH is expressed in the X-organ–sinus gland of the eyestalks and in gut endocrine cells. The main function of this neuropeptide is the regulation of water uptake and ion exchange that initiates body swelling. In addition, corazonin and its receptor might also be involved in ecdysis regulation through their influence on the Y-organ (Alexander *et al.*, 2017). Two copies of EH have been found in the genome of several crustaceans, such as *C. maenas*, *C. quadricarinatus*, *P. clarkii*, *L. vannamei*, and other decapods, while ETH and its receptor are each expressed from a single gene, both with a variable number of functional transcripts (Roller *et al.*, 2010; Veenstra, 2016*c*; Minh Nhut *et al.*, 2020). EH as well as ETH have differential expression levels within moult phases in the central nervous system and eyestalk, while in the case of other organs such an expression pattern is not obvious, at least in *C. maenas* (Oliphant *et al.*, 2018). However, it is still unclear whether crustacean EH/ETH trigger neuropeptide secretion as they do in insects (Minh Nhut *et al.*, 2020; Knigge *et al.*, 2021). EH in *E. carinicauda* is responsive to exogenous 20E and highly expressed during the pre-moult stage (Zhou *et al.*, 2017). RNAi against ETHR in *L. salmonis* does not cause any phenotypic modification in copepodids (Eichner *et al.*, 2014). By contrast, transcriptome analysis after RNAi-mediated ETH knockdown in *S. paramamosain* highlights differential expression of genes firstly involved in processes such as chitin binding and afterwards in cardiac muscle contraction (Chan *et al.*, 2022). In the gut of the crab *C. maenas*, CHH is strongly expressed just before the CCAP surge, since it also regulates water uptake it might be involved in the ecdysial neuromotor as well (Knigge *et al.*, 2021). Among the high variability of detected downstream neuropeptides in crustacean species, only a few have confirmed influence on muscle contractions and exuviae escaping, such as CCAP, proctolin, carcinine, and others (Oliphant *et al.*, 2018; Mykles, 2021). The elements of ecdysis behaviour in crabs, as representative of Crustacea, include rapid water accumulation, eye twitching, and visible peristaltic contractions of legs (Minh Nhut *et al.*, 2020). Thus while many key components have been identified in crustaceans, there is still only a limited understanding of how they interact to control stereotypical ecdysis behaviours.

The knowledge about the initiation of chelicerate ecdysis behaviour remains fragmentary and mostly limited to annotation reports of neuropeptides and their receptors, as

discussed in Section II.2. Three putative EH-coding genes have been found in *P. pseudoannulata* and *T. urticae*, each located on a different scaffold and their sequences are significantly different from the EH gene in insects (Veenstra, 2016*b*; Yu *et al.*, 2020). Five EH gene copies were found in the genome of horseshoe crab *C. rotundicauda*, prompting the hypothesis that this expansion relates to the provision of efficient hormonal support for moulting processes in such unusually large chelicerates (Shingate *et al.*, 2020). ETH and its receptor, are represented by single copies in *P. pseudoannulata*, *T. urticae*, and *C. rotundicauda*, while ETH1 and ETHR-B have been isolated in *Panonychus citri* but no representatives of EH and ETH genes were annotated in the spider *Latrodectus hesperus* (Christie, 2015*a*; Zhu *et al.*, 2019). Pedal cells of the ticks *Ixodes ricinus* and *Rhipicephalus appendiculatus* were identified as a synthesis site for ETH. Genomic cataloguing of ecdysis-related neuropeptides in 13 tick and spider species revealed the presence of ETH only in *I. scapularis* and *Rhipicephalus sanguineus*, the absence of ETH in *P. tepidariorum*, and the absence of CCAP from all of them, but they have been identified in other sequence comparison-based explorations (Roller *et al.*, 2010; Christie, 2015*a*; Christie & Chi, 2015; Veenstra, 2016*b*; Lyu *et al.*, 2023). Despite these contrasting results in chelicerates, comparative genomic investigations have traced the origin of these components beyond the arthropod ancestor, EH originated prior to the branching of Cnidaria and Bilateria while ETH and CCAP evolved in the ancestor of Bilateria (De Oliveira *et al.*, 2019; Zieger *et al.*, 2021). However, the set of downstream effector neuropeptides that control the main features of ecdysis behaviour remains unknown. The pattern of ecdysis behaviour of a representative chelicerate species *Aphonopelma chalcodes* includes rapid water accumulation and muscle contractions between the borders of prosoma and opisthosoma for suture formation, with further flexing of the legs to exit from the exuviae (Minch, 1977), suggesting common biology even if the molecular toolkits remain to be elucidated.

As in Chelicerata, the initiation of the ecdysis pattern in Myriapoda remains largely unknown. Analysis of the first sequenced myriapod genome of *S. maritima* revealed the duplication of genes involved in the EH–ETH loop and ETHR recognition receptor, but it is unknown whether the whole regulatory system remains similar or has become more elaborate (Chipman *et al.*, 2014). Similarly, two EH genes were identified in *Hanseniella* species, a symphylan, but none in the symphylan *S. vulgaris*, where ETH also is missing (Christie, 2015*b*; Derst *et al.*, 2016). The chilopod *L. forticatus* has both ETH and EH as single-copy genes (Derst *et al.*, 2016). Ecdysis behaviour of Myriapoda-representative species of *Lithobius* includes dorsoventral contractions of the head muscles as one of the main features of ecdysis (Minelli & Sombke, 2011; Christie, 2015*b*).

After initiation, CCAP in insects promotes the motor behaviour sequences that allow ecdysial emergence from the exuviae, as well as upregulation of the expression of the neuropeptide Bursicon (Burs), as the major regulator of the post-ecdysis phase. This initiates at least two key downstream processes, collectively referred to as tanning, comprising sclerotization, that is hardening of the expanded exoskeleton and wings, and pigmentation, that is mostly melanisation (Moussian, 2010; White & Ewer, 2014). It may also initiate the "plasticisation" of the new cuticle, enabling it to be more easily extracted from the exuvial sheath and then inflated under pressure (Reynolds, Taghert & Truman, 1979). Burs heterodimerizes with the protein encoded by the gene *Partner of Bursicon* (*Pburs*), thus they are also alternatively called, respectively, subunit α and β of the bursicon complex (Fig. 6). The heterodimeric complex is recognised by the transmembrane receptor Rickets, which belongs to the family of leucine-rich GPCRs, and activates protein kinase A *via* cAMP signalling (Song, 2012; Flaven-Pouchon *et al.*, 2020). The target of downstream phosphorylation is a tyrosine hydroxylase (TH) enzyme that catalyses formation of the dopamine precursor, a shared intermediate between the sclerotization and melanisation pathways (described in Section V.3) (Moussian, 2010; Flaven-Pouchon *et al.*, 2020). However, a recent study questioned the traditional model of Burs pathway activation, providing evidence that Rickets in the epidermis is expressed but not required for cuticle tanning, instead its expression in the ventral nervous system is necessary (Flaven-Pouchon *et al.*, 2020). Thus, additional elements might be involved in signal transmission from the site of Burs synthesis in the central nervous system to the effector tissue (Flaven-Pouchon *et al.*, 2020). In addition to GPCR-mediated TH activation by the Burs α-β complex, in *D. melanogaster*, *A. aegypti*, and *T. castaneum*, Burs homodimers activate immune responses and the expression of antimicrobial peptides that probably help prevent infections before cuticle hardening is complete (An *et al.*, 2012; Zhang *et al.*, 2017*a*; Li *et al.*, 2023). Beyond insects, Burs α and β have been detected in daphnid and several decapod species, mites, ticks, spiders, and myriapods but cloned and studied in relation to moulting only in a few crustaceans (Grbić *et al.*, 2011; Chung, Katayama & Dircksen, 2012; Chipman *et al.*, 2014; Christie, 2015*a*,*b*; Derst *et al.*, 2016; Veenstra, 2016*c*; Oliphant *et al.*, 2018; Tu *et al.*, 2021; Lyu *et al.*, 2023). Similar concurrent stimulation of antimicrobial peptides was described in the crayfish *P. clarkii* and the shrimp *Neocaridina heteropoda*, and even in the tick *Amblyomma americanum* (Li *et al.*, 2019*c*; Zhang *et al.*, 2020*a*; Lyu *et al.*, 2023). Therefore, the core components for initiating post-ecdysis processes are seemingly conserved, but many additional factors likely remain to be discovered.

Detection of the set of key neuromotor factors in Chelicerata and Myriapoda supports a conserved arthropod toolkit for ecdysis behaviour (De Oliveira *et al.*, 2019; Zieger *et al.*, 2021). These neuropeptides have been suggested to have originated in Ecdysozoa and even earlier, as coordinators of developmental transitions through the life cycle, and then to have been co-opted as ecdysis regulators (De Oliveira *et al.*, 2019; Zieger *et al.*, 2021). Nevertheless, we are far from comprehending how these neuropeptides

orchestrate the ecdysis event across arthropod diversity: detection in myriapods is limited to a handful of species and data about chelicerates might point to an incomplete set of ecdysis-related neuropeptides (Lyu *et al.*, 2023). Much experimental work needs to be done to dissect finely which particular role each of the components of the main neuropeptide ecdysial motor plays in the different sublineages, especially in terms of characterisation of downstream effectors and tissue specificity.

(3) Cuticle tanning and mineralisation complete the moulting process

Exoskeleton sclerotization and restoration of the protective cuticle colorations begin immediately after ecdysis, through the crosslinking of CPs, incorporation of mineral salts within cuticle layers, and the deposition of pigments.

The processes of cuticle sclerotization and melanisation are tightly connected through usage of the same intermediates and have been well characterised thanks to powerful genetic tools in *D. melanogaster* and *T. castaneum* (Moussian, 2010). Cuticle hardening is achieved by the formation of "bridge" links between CPs, intermediated by covalent binding to tanning agents with CP amino acid residues, resulting in tight connections between cuticular layers (Moussian, 2010). Thus, the level of cuticle rigidity is highly dependent on proteinaceous composition in a particular cuticle area (Moussian, 2010; Noh *et al.*, 2016). Synthesis of sclerotization agents initially starts in the cytoplasm of the epithelium below the cuticle, where tyrosine forms catecholamines through a two-step reaction, first dopa and then dopamine. These are positioned at the crossroads between the sclerotization and melanisation processes. Dopamine is converted into two sclerotization precursors, N-acetyldopamine (NADA) and N-β-alanyldopamine (NBAD) (Andersen, 2010; Moussian, 2010; Asano *et al.*, 2019; Sugumaran, 2022). The set of enzymes responsible for the whole conversion process includes TH (*pale* gene in *D. melanogaster*), dopa decarboxylase (*Ddc*), Tanning (*tan*), and dopamine N-acetyltransferase (*Dat*). An additional source of NBAD is L-aspartic acid, which is sequentially converted with two enzymes called *black* (*b*) and *ebony* (*e*) (Fig. 5). All the four intermediates (dopa, dopamine, NADA, and NBAD) are then oxidised, mainly by laccase-2 (from the *straw* locus in *D. melanogaster*), to form quinone compounds: the colourless quinones from dopa and dopamine are specific to the sclerotization pathways and play a direct role in CP crosslinking; the NADA and NBAD quinones are specific to the melanisation pathway and lead to formation of black melanin monomers, due to the enzyme from the gene *yellow* (*y*) (Andersen, 2010; Moussian, 2010; Asano *et al.*, 2019; Sugumaran, 2022). Separate monomers are organised into melanin granules and deposited within cuticular layers (Andersen, 2010; Moussian, 2010; Asano *et al.*, 2019; Sugumaran, 2022). Laccase2 is an insect-specific cluster of the multi-copper oxidases (3dMCOs) family, previously found even in bacteria but only as a single copy (Asano *et al.*, 2019). The insect laccase2/MCO2 has high sequence similarity amongst insects but it is absent from non-insect hexapods, non-insect Pancrustacea and myriapods, whereas chelicerates and the copepod *L. salmonis* seem to have lost MCO1 (Asano *et al.*, 2019). The lighter and stronger cuticle resulting from laccase-2-mediated cross-linking has been hypothesised to be a key innovation for the evolution of insect flight (Andersen, 2010). An alternative to quinone and laccase-2-mediated linking, in insects, CP crosslinking is realised through the formation of di-tyrosine and tri-tyrosine links, which is probably mediated by cuticular peroxidase. Crosslinks might form either between tyrosine-enriched CPs, such as resilin or between two resilins and free tyrosine amino acids (Sugumaran, 2022). While the biochemistry of these steps is largely well characterised, mechanisms controlling initiation, localisation, and intensity of cuticle sclerotization and melanisation remain to be fully explored.

Contrary to insects, in crustaceans sclerotization appears mainly to rely on phenoloxidase (PO) activity. PO has tyrosinase-like activity and is involved in defensive melanisation in the haemolymph of insects and crustaceans (Fig. 5) (Asano *et al.*, 2019). For cuticle sclerotization during the post-moult phase, PO is synthesised by haemocytes, as an inactive form, pro-phenoloxidase, whose involvement in post-moult and cuticle sclerotization was confirmed with expression pattern and RNAi experiments (Alvarez & Chung, 2015; Zhang *et al.*, 2019*a*). The unresolved question is what mechanism activates pro-phenoloxidase deposition from the haemocytes to the cuticle. Burs might be involved in this process, since experimental data confirm the correlation of Burs expression with haemocyte granulation and their aggregation underneath the hypodermis (Chung *et al.*, 2012). Conversion of pro-phenoloxidase to its active form occurs through serine proteinases, such as trypsin. Experimental studies confirmed cuticle localisation and a moult-related abundance pattern for trypsin in the decapods *Panulirus argus*, *C. sapidus*, and *Portunus pelagicus* (Kuballa & Elizur, 2008). Another putative player is a trypsin-like serine protease called pro-phenoloxidase-activating factor, which was identified in *C. sapidus* (Buda & Shafer, 2005). Active PO in the cuticle might be responsible for both melanin synthesis and crosslinking of CPs, with a possible involvement of glycosylation recognition of CPs through mannose-binding proteins (Kuballa & Elizur, 2008). The PO homologue possessing TH-like catalytic activity is haemocyanin, which is a blue-coloured haemolymph pigment, involved in gas exchange, hormone transportation, defensive reactions, gastrolith structuring, and others (Glazer *et al.*, 2013). After serine proteinase cleavage, haemocyanin acquires PO-like activity. The deposition of haemocyanin in the cuticle has been immunohistochemically confirmed. However, many questions remain unresolved, such as the mechanism of transportation into the cuticular layers, the activation mechanisms, and the enzymes involved (Adachi *et al.*, 2005).

The sclerotization of cuticle in myriapods from the class Chilopoda probably occurs independently of phenol compounds, because the putative "sclerotization agent" does

not dissolve in water or alcohols. It has been suggested that cuticle stabilisation might involve only the tyrosine residues of CPs or free tyrosine radicals through the formation of di-tyrosine crosslinks, as noted for the insect CP resilin, but the catalysation agent of such crosslinking has not been identified (Asano *et al.*, 2019). Transcriptome analysis of 39 Myriapoda species allowed researchers to identify enzyme transcripts associated with melanin synthesis, such as tan, TH, PO, Ddc, and several others. However, none of the 39 species examined has the whole set of genes required for the synthesis pathways known from insects. Remarkably, laccase2 transcripts were not identified for any of them (Wang *et al.*, 2021). Six types of pigments were identified in the cuticle of Myriapoda, and melanin is among them (Hopkin *et al.*, 1992). However, the whole enzyme network and melanin precursors are yet to be identified. The functional role of the melanin pathway was revealed only for Myriapoda haemolymph, based on PO and tyrosinase activity (Riciluca *et al.*, 2020). Additional studies are needed to confirm whether these enzymes are also involved in cuticle melanisation.

Spiders have functional PO and haemocyanin involved in immunity, but post-ecdysis cuticle hardening through catechol derivatives in chelicerates has not been confirmed in any available studies, although two strategies of CP crosslinking without tanning agents were suggested (Asano *et al.*, 2019; Cunningham *et al.*, 2020; Wang *et al.*, 2023). First, the chelicerate cuticle is enriched in sulphur residues, probably part of CPs, so hardening might be achieved through the formation of disulfide bonds between such proteins (Asano *et al.*, 2019). However, this hypothesis is in contrast with the amino acid composition of insect CPs, where the dominating CPR family of CPs has a very low percentage of sulphur-containing amino acids (Asano *et al.*, 2019). Another possible mechanism of CP crosslinking and the resulting cuticle rigidness was discovered in the horseshoe crab, as bond formation between glutamate and lysine residues of CPs with transglutaminase. Similarly, the organisation of the melanin synthesis pathway is still largely unknown in Chelicerata. For a long time, it was believed that chelicerates, and spiders in particular, do not have melanin pigments, and the absence of crucial synthesis pathway genes such as *e* and *y* in the genomes of two species supports this belief (Croucher *et al.*, 2013). Failed attempts to detect melanins from spiders chemically also supported this hypothesis until recently. A modification of standard Raman spectrometry finally allowed the dissolving and detection of spider melanins (Hsiung, Blackledge & Shawkey, 2015), however, the main players of melanin synthesis require further attention. The open question is which specific oxidising enzyme participates in the initial reaction, and whether it is a PO enzyme that comes from haemocytes as during wound melanisation, or haemocyanins, or a combination of both (Sawadro *et al.*, 2017; Bilandžija *et al.*, 2017; Cunningham *et al.*, 2020).

The abundance of calcium carbonate in marine habitats means that the process of cuticle hardening and stabilisation in crustaceans is mainly dependent on calcification, so CP crosslinking with dopamine derivatives is probably not a critical process for their development (Asano *et al.*, 2019). Calcification is the process of incorporating mineral salts into the structure formed by chitin fibrils and proteins (Giraud-Guille, Belamie & Mosser, 2004). Crystalline polymorphs of calcite, formed by calcium carbonate and calcium phosphate are mainly deposited, however, traces of higher solubility magnesian calcite are also found in crustaceans (Ulrich *et al.*, 2021). Calcification is a rare event in insects with just a few observed cases, such as ovipositor calcification in *Gabunia* wasps and puparium calcification in the fly *Musca autumnalis* (Roer *et al.*, 2015). A recent study suggests that cuticle calcification might serve as a compensatory mechanism for cuticle hardening, as observed in the pupal cuticle of a *B. dorsalis* mutant with sclerotization disruption (Rong *et al.*, 2019). However, the molecular machinery of calcification processes in insects remains unknown. In Crustacea, the main sources of the calcium salts include external water, food, and recycling of the digested cuticle; calcium is stored in tissues such as hepatopancreas, midgut, muscle, and haemolymph, so that it can be immediately used in the post-ecdysis period for cuticle calcification (Hecker *et al.*, 2004; Li & Cheng, 2012; Rupp, Walther & Ziegler, 2020). Biomechanical constraints mean that calcification cannot be uniform across the exoskeleton and thus the processes are permanently inhibited in appendage joints, in the lining of the branchial chamber, and in other soft epithelia-derived structures (Roer *et al.*, 2015). Currently, two main models are discussed, the main difference between the models is in what causes the initial inhibition of calcification, whether direct glycosylation of CPs, or binding of C-type lectin receptors to the CP to initiate the glycosylation (Kuballa & Elizur, 2008; Luquet, 2012). In any case, the complex of carbohydrate residues and C-type lectin receptors masks the CP sites of calcification during pre-ecdysis and ecdysis. After escape from the exuviae, an unknown mechanism causes the appearance of another player, a form of lectin known as mannose-binding proteins, which bind the receptor and glycosylation residues thus changing the conformation of CP so that calcification sites become exposed (Kuballa & Elizur, 2008; Luquet, 2012).

Remarkably, a characteristic feature of aquatic freshwater and terrestrial crustaceans is the formation of gastroliths as a form of temporary calcium storage, in the stomach columnar cell discs (Glazer *et al.*, 2015). The presence of gastroliths has not been confirmed in marine species (Li & Cheng, 2012). Calcium salts are bound by a set of proteins, the gastrolith matrix proteins (Dave *et al.*, 2021). In particular, gastrolith protein 65 (GAP65), homologous to CDA1, is necessary for mineral deposition and correct formation of crayfish gastroliths (Glazer *et al.*, 2015). Ecdysis initiates the release of calcium biphosphate from gastroliths (Abehsera *et al.*, 2018*a*). In the freshwater *C. quadricarinatus*, transportation of calcium salts to and from the gastrolith depends on two bicarbonate transporters from the solute carrier 4 (SLC4) family distributed in the cuticular epithelium and in the gastrolith discs with different activation times (Abehsera *et al.*, 2021). In this

crayfish and in the prawn *Penaeus japonicus*, the deposition of calcium salts within the cuticle is probably mediated through CPs with chitin-binding and calcium-binding abilities such as the calcification-associated proteins (Ikeya *et al.*, 2001; Roer *et al.*, 2015). Extended functional analysis has not yet been performed, although they are known to be mainly expressed during late developmental stages in *L. vannamei* and *S. verreauxi* (Ventura *et al.*, 2015; Gao *et al.*, 2017). In addition, a novel protein, named crustacean larval factor has been identified in crayfish larvae, which can bind calcium carbonate in an amorphous state. This novel factor shares domain similarities to an insect-specific follicle protein and two chelicerate sequences (Ventura *et al.*, 2019). By contrast, for the mineralisation protein necessary for the highly mineralised appendage of the mantis shrimp, no homology to any known protein was found, suggesting that tissue-specific processes can be mediated by totally unique and species-specific solutions (Amini *et al.*, 2019).

Although calcification is widely observed across Myriapoda, no annotation and experimental data about putative molecular players are available. Amongst the millipedes, a non-calcified exoskeleton is only observed in penicilate millipedes from the order Polyxenida, while all other reported species have a calcified and rigid cuticle throughout postembryonic development (Zapparoli, 2016). As for centipedes, Lithobiomorpha and Geophilomorpha are reported to have a soft cuticle with weak sclerotization (Zapparoli, 2016). It is unknown whether calcification mechanisms are inhibited or completely absent in these centipedes, as well as in species from the orders of Symphyla and Pauropoda (Zapparoli, 2016). The cuticle of Chelicerates generally is not mineralised with calcium salts, with a few exceptions among Opiliones and Acari (Mattei *et al.*, 2015; Gallant, Hochberg & Ada, 2016; Politi *et al.*, 2021). Calcification therefore represents a fascinating elaboration of the moulting machinery that warrants closer attention with further genomic and experimental cross-taxa comparisons.

In addition to calcium-containing compounds, the arthropod cuticle might be strengthened with heavier metal ions, such as Zn, Mn, Fe, Ni, and others. The cuticle that incorporates such metals is generally known as a heavy element biomaterial (HEB) (Fig. 5). Metal impregnation of cuticle is widely reported in early investigations in species from multiple lineages from all subphyla, as a part of ovipositors, jaws, and fangs in orthopterans, hemipterans, hymenopterans, coleopterans, isopods, decapods, amphipods, scorpions, pseudoscorpions, spiders, and diplopods (Zapparoli, 2016; Lehnert *et al.*, 2019; Schofield *et al.*, 2021; Okada *et al.*, 2022). However, the mechanisms and regulation of metal incorporation are largely unknown, for example in *C. sapidus* a zinc transporter activated during post-ecdysis has been hypothesised (Eagon & Zou, 2023). The study of mechanical properties of these body structures has shed light on the evolutionary advantages provided by stiffening the cuticle with heavy metals, rather than with calcium salts, such as more precise sharpening, higher stiffness, and better damage resistance (Lehnert *et al.*, 2019; Schofield *et al.*, 2021). The importance of HEBs across Arthropoda is likely underestimated and therefore understudied, leaving many questions unanswered even beyond their implications for moulting.

Overall, despite the exoskeleton being a relatively simple structure mostly made from only two types of components (chitin and cuticle proteins), the mechanisms behind its secretion and remodelling are complex. It is likely that each individual arthropod group has followed lineage-specific evolutionary paths strongly influenced by their physiology and ecology. Thus, it is particularly difficult to speculate which genetic toolkit might have been used by the arthropod ancestor, exposed to a different physico-chemical environment, since current knowledge in early branching species does not provide a basis for hypothesis formulation, except the exclusion of a strategy based on catalysis of catechol derivatives by laccase2. Indeed the acquisition of laccase2/MCO2 has been proposed as a critical factor that enabled the evolution of insect terrestrialisation and flight by building a lightweight cuticle without calcification (Asano, Hashimoto & Everroad, 2023).

VI. CONCLUSIONS

(1) A clear emerging trend is that a growing availability of genomic resources across the span of arthropod diversity is greatly facilitating the widespread testing of hypotheses formulated from knowledge acquired in a few well-studied insects. Although genomic detection of genes first characterised in powerful model systems is increasingly feasible, experimental investigations are reported in few species of other arthropod lineages. Nevertheless, independent analyses of sequencing data, supported by experimental reports, have validated the presence of what can be defined as an "ultra-conserved" core of arthropod genes involved in the moulting process since they were identified in all representative species of subphyla under investigation, and where the literature presented no exceptions or contradictions. These include the ecdysteroidogenic genes *disembodied* and *shadow*, the ecdysis-triggering gene *Bursicon*, as well as the transcription factors mediating hormone signalling: the ecdysone receptor complex with early genes and the sesquiterpenoid *Methoprene-tolerant* receptor gene. Interestingly, components that might be assumed to belong to such a core set of constrained genes may still offer surprises, as revealed through within-lineage explorations exemplified by the loss of *neverland* in *Varroa* mites and the controversial replacement hypothesis of *shade* in decapod shrimps.

(2) Membership of this "ultra-conserved" core, however, should be considered relative rather than absolute, as much more taxonomic exploration is needed to identify, confirm, and enumerate any exceptions. New sequencing technologies are increasingly producing high-quality and complete genome assemblies and annotations that allow for more confident assessments of gene presence or absence. Nevertheless, many lineages are still represented only with lower-quality

draft genomes and any inferences made based on these resources, especially with respect to gene losses, must be treated with caution. Although conclusions generated on a single representative species cannot be extended to an entire family, order, class, or subphylum, in many cases investigations using additional representatives are currently lacking. This has often been the case for the centipede *S. maritima*, which is essentially the only myriapod where early genes have been investigated. Where sampling is improving, tentative observations can start the transition towards evidence-supported conclusions, for example, examining millipede genomes has pointed to the loss of *juvenile hormone acid methyltransferase*, encoding a central enzyme for methyl farnesoate synthesis, and studies across several chelicerate orders now quite confidently reject the presence of *phantom* in this clade.

(3) As well as taxonomic imbalances, reviewing the literature also identified biases in the attention dedicated to research on different moulting phases and processes. Amongst all the pathways, the late genes involved in cuticle renewal and post-ecdysis represent one of the major gaps of knowledge. In non-insect arthropods particularly, investigations of these components merely scratch the surface of the potential diversity of cuticle-building mechanisms. In stark contrast, in canonical model species it has been described how chitin is synthesised, recycled, and organised with cuticle proteins that are then crosslinked, often at cellular and tissue-specific levels of granularity. Another large bias that gives rise to a sizable knowledge gap is the relative neglect to date in the characterisation of the neuropeptides upstream of hormone synthesis. In myriapods and chelicerates, almost nothing is known about the triggering stimuli nor the secretory tissues they target, for both ecdysteroids and sesquiterpenoids. Indeed similar gaps and uncertainties hinder progress towards a better understanding across the breadth of crustacean diversity. Here it is interesting to note how the two least studied phases of the moulting pathway, from a temporal perspective, lie at opposite ends of the whole process. As "input signals" and "output effectors", they likely represent the most variable parts of the pathway, based on the differences that are already emerging, such as the contrasting stimulatory or inhibitory mechanisms for hormone synthesis and the highly variable biochemistry underlying cuticle structures.

(4) Although true for all components, it is particularly relevant for lineage-specific elaborations to note that comparisons of genomic sequences can help catalogue the genetic toolkit that a species can potentially use, but not how this toolkit is used. For example, detection of neuropeptides well known for their pleiotropic functions in some species, such as allatostatins and crustacean hyperglycemic hormone, should not *a priori* lead to the assumption of their participation in moulting regulation in other species, but rather requires careful evaluation and dissection of their roles to reach evidence-based conclusions. Functional confirmation of the sequential switch-on of neuromotor components is also needed beyond Pancrustacea, especially to complement their fragmented detection in chelicerates. Additionally, functional studies would significantly improve our understanding of the biological activity of each hormonal form in relation to different lineages, for instance, to clarify whether putative homologues of the epoxidase *cyp15a1* play a true role in sesquiterpenoid synthesis in Crustacea. A consequence of the challenges facing functional approaches in non-model organisms is, again, the poor phylogenetic diversity of the experimentally tractable animals used. Despite being the most intensively studied after insects, the greatest part of all the knowledge we have about moulting in Crustacea is derived from decapods. This is indeed the only order where we have a relatively mature understanding of how the process works in its entirety during the different moulting phases, from the localisation of endocrine organs and regulation of their activity to the general mechanisms behind exoskeleton secretion and maturation.

(5) Implicating changes and novelties in the molecular machinery with transformative innovations in moulting processes across Arthropoda is hampered by knowledge gaps and hypotheses based on assumptions that remain to be tested. Nevertheless, reviewing the literature does point to a handful of potentially interesting candidates, such as how developing a better understanding of a single gene involved in cuticle sclerotization has the potential to support macroevolutionary hypotheses. The unique catalytic activity of Laccase2/MCO2 towards catecholamines might have played an important role during the terrestrialisation process: insect ancestors would have been able to take advantage of an environment rich in oxygen which favours Laccase2-mediated catalysis, setting them free from the necessity of a marine calcium-rich environment and resulting in a lighter cuticle structure, more suitable for the development of flight. While acknowledging gaps and biases in taxon representation, the accumulating genomic data are helping to map changes and novelties in the moulting molecular machinery that warrant follow-up experimental work. For example, further exploring seemingly lineage-restricted components such as the fate determination genes and less well-known parts of the hormonal pathways, such as *noppera-bo* and *shroud*, and *juvenile hormone esterase*, *JH epoxide hydrolase*, *JH diol kinase*, and *JH-binding protein*, for ecdysteroid and sesquiterpenoid metabolism, respectively.

(6) Overall, the molecular machinery, or genetic toolkit, of arthropod moulting processes has likely evolved with a flexibility of its conserved pathway backbone, at different levels. The four main gene sets (subpathways) functionally associated with each macro-temporal moulting phase have remained relatively constant. All arthropods still rely on the same classes of two main hormones to transduce *via* a network of transcription factors the signal of remodelling of an exoskeleton made of chitin and cuticle. In other words, no subphylum or subgroup has co-opted a different group of signalling molecules or set of cascades to trigger moulting, and no lineage has strikingly modified the building blocks of its exoskeleton. That said, moulting pathways have diverged with various elaborations throughout arthropod evolution. The literature highlights a set of principal mechanisms

through which lineages have altered the main developmental moulting backbone: copy number changes of biosynthetic and late genes; differences in the main bioactive ecdysteroid forms (20-hydroxyecdysone and ponasterone A); emergence of heterogeneous catalytic strategies for juvenile hormone insect innovation (juvenile hormone acid methyltransferase and epoxidase); variation of ecdysone receptor (EcR), retinoid X receptor (RXR), and methoprene-tolerant (Met) receptor specificity (EcR having isoform-specific roles, RXR being an obligatory partner of EcR, Met having higher affinity for juvenile hormone than methyl farnesoate); modification of the regulatory gene network towards the evolution of holometaboly (changes in inhibition relationships and expression pattern of *chinmo*, *Kr-h1*, *Broad*, *E93* genes); and a highly diversified equipment for exoskeleton maturation.

(7) Reviewing the current state of knowledge of the main molecular components implicated in moulting processes produced a thorough survey of reported genomic detection evidence and experimental assessments of the moulting genetic toolkit across Arthropoda, with a special focus on understudied lineages. This provides an integrated overview of major findings from hormonal biosynthesis and regulation to transcriptional responses and ecdysis itself, building a comprehensive picture of both the full set of pathways involved and a thorough sampling of investigated taxa across the phylum. The emerging framework points to routes from the underlying genetics and genomics to the dynamic biochemistry and molecular biology and eventually to the complex physiology and behaviour. Exploring these research avenues will require complementing phylogenetically extensive comparative analysis of all the genetic toolkits involved with increasingly detailed descriptions of life-history traits and moulting behaviour modes from a morphological macroevolutionary perspective.

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VIII. AUTHOR CONTRIBUTIONS

A.D.C., A.C.D., M.R.-R., and R.M.W. conceived the idea and provided supervision. G.C., O.V., K.K., and W.P.V. compiled the extensive list of published literature for review and drafted initial versions of texts and figures for Sections IV, V, III, and II, respectively. G.C. drafted initial versions of Fig. 1 and texts for Sections I and VI, expanded the list of literature for review, and extensively extended, revised, and harmonised texts from all sections and all figures. All authors contributed to the writing and figure preparation with constructive critical comments and suggestions for improvements, as well as to implementing revisions in response to reviewer comments. G.C. and R.M.W. edited and finalised the manuscript. Figures were created with BioRender.com under BioRender's Academic License Terms.

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