Ecdysone Receptor Agonism Leading to Lethal Molting Disruption in Arthropods: Review and Adverse Outcome Pathway Development

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ABSTRACT

Molting is critical for growth, development, reproduction and survival in arthropods. Complex neuroendocrine pathways are involved in the regulation of molting and may potentially become targets of environmental endocrine disrupting chemicals (EDCs). Based on several known ED mechanisms, a wide range of pesticides has been developed to combat unwanted organisms in food production activities such as agriculture and aquaculture. Meanwhile, these chemicals may also pose hazards to nontarget species by causing molting defects, and thus potentially affecting the health of the ecosystems. The present review summarizes the available knowledge on molting-related endocrine regulation and chemically-mediated disruption in arthropods (with special focus on insects and crustaceans), to identify research gaps and develop mechanistic model for assessing environmental hazards of these compounds. Based on the review, multiple targets of EDCs in the molting processes were identified and the link between mode of action (MoA) and adverse effects characterized to inform future studies. An adverse outcome pathway (AOP) describing ecdysone receptor agonism leading to incomplete ecdysis associated mortality was developed according to the OECD guideline and subjected to weight of evidence considerations by evolved Bradford Hill Criteria. This review proposes the first invertebrate ED AOP and may serve as a knowledge foundation for future environmental studies and AOP development.

KEY WORDS

Adverse outcome pathway, Molting, Endocrine Disruption, Arthropod, Weight of evidence, Ecdysone receptor

■ INTRODUCTION

Molting is a natural biological process in arthropods. During molting, the animal generates a new exoskeleton and sheds the old one in order to grow and develop. A complete molt cycle includes detachment of the old cuticle (apolysis), generation of the new cuticle, degradation and re-uptake of the old cuticle, shedding of the old cuticle (ecdysis) and tanning of the new cuticle.[1, 2] Successful molting is key to survival, development and reproduction.[3] Over half a century research on arthropod endocrinology reveals that molting is precisely controlled by a complex multi-hormone system, with 20-hydroxyecdysone (20E) being the key hormone mediating various physiological and behavioral changes that are essential for molting.[4] The hormonal action of 20E is exerted through activation (agonism) of the ecdysone receptor (EcR), an invertebrate nuclear receptor responsible for

transcriptional regulation of molting. Based on this mode of action (MoA), endocrine disrupting chemicals (EDCs) targeting the EcR have been developed as insect growth regulators (IGRs), pesticides and anti-parasite pharmaceuticals for control of "harmful" arthropods. However, due to highly conserved endocrine systems in arthropods,[5-9] the environmental residues of these EDCs may also affect ecologically and economically important nontarget species, such as aquatic crustaceans (e.g. crabs and lobsters). Substantial efforts are therefore needed to assess the environmental hazards and risks of EDCs to nontarget arthropod species.

The high number (over a million described) of species in the phylum of Arthropoda (*Animalia, Eumetazoa*, *Ecdysozoa*) and wide range of EDCs make it impossible to conduct toxicity tests for every species or chemical. Potential solutions may include developing ecotoxicological model species (forecaster species) that are phylogenetically related, and identifying chemicals with the most relevant properties by computational (*in silico*) approaches such as quantitative structure-activity relationships (QSAR) and structural alerts along with identifying relevant bioactivities. The adverse outcome pathway (AOP) framework[10] fits this purpose well. Employing the AOP framework, causal relationships linking initial perturbation of a biological system resulting from chemical interaction with a target biomolecule (termed molecular initiating event) to adverse outcomes (AOs) considered relevant from a risk assessment/regulatory perspective via a series of measurable biological events spanning multiple levels of biological organization are defined. These relationships are supported by fundamental understanding of the structural and functional relationships between the measurable key events (i.e., biological plausibility) as well as evidence that associates a change in an upstream event with a consequent change in a downstream event. Thus, the quality and robustness of an AOP can be evaluated based on a weight of evidence (WoE) approach, according to the General Assessment Factors (GAFs) for assessing the quality of individual scientific publications and Bradford Hill considerations for assessing causality.[11-14] Since an AOP is not species- or chemical-specific, extraction and synthesis of consensus information from different taxa and chemical domains for construction of models with broad applicability is facilitated.[15, 16] The discovery, development and application of AOP may be further expanded using advanced *in sillico* prediction, bioinformatics, broad content OMICS approaches, high-throughput laboratory screening bioassays for identification of MIE and KEs at the molecular/cellular level across taxa and stressors. The successful anchoring of data along the AOP continuum can potentially inform regulatory processes by directing the use of testing resources; perform screening and prioritization of chemicals, limiting experimental animal testing, supporting Integrated Approaches to Testing and Assessment (IATA).[17]

As an initial effort in invertebrate AOP development, the current review focuses on EcR agonism-mediated molting disruption and subsequent lethality in arthropods. The aim of the work was to review available knowledge, primarily for insects and crustaceans, in order to assemble an AOP and identify critical research gaps to address in future studies. An extensive literature survey was conducted to provide an overview of neuroendocrine regulation of molting and molting related ED effects. Based on the review, an AOP is proposed and evaluated for WoE and applicability. The proposed AOP provides a foundation for the development and applications of a high throughput EcR assay, as well as in silico structure-based approaches for predicting EcR interactions, as efficient and cost effective tools for screening large inventories of chemicals for their potential to cause endocrine disruption and subsequent lethality in a diverse phylum of organisms occupying a broad range of ecological niches and involved in important ecological functions.

■ NEUROENDOCRINE REGULATION OF MOLTING

Regulation of Ecdysteroid Titer. Ecdysteroids (Ec) are well-known molting hormones in arthropods.[18] Recent molecular phylogenetic investigations of arthropods have revealed that Hexapoda (insects) and Crustacea form Pancrustacea, and extant lineages of Crustacea could be categorized into three major groups; Oligostraca (e.g., ostracods), Multicrustacea (e.g., malacostracans such as crabs and shrimps), and Allotriocarida (e.g., branchiopods such as water fleas and brine shrimps).[19-21] In insects, the biosynthesis of ecdysteroids utilizing dietary cholesterol takes place in the prothoracic gland (PG).[3] In crustaceans, the molting hormone systems of malacostracans and branchiopods have received much more attention than those in other crustaceans. Currently, the Y-organ is considered an endocrine organ of Ec in malacostracan crustaceans.[22, 23] Recent studies on the water flea *Daphnia magna* (Crustacea,

Branchipoda, Cladocera, Daphniidae) also suggested that Ec may be alternatively synthesized in the gut epithelial cells, as the Y-organ has not been identified in this species.[24, 25] The synthetic pathway of Ec (Fig. 1A) is highly conserved in arthropods.[26] The process starts with the conversion of cholesterol to 7-dehydrocholesterol (7-dc) by 7,8-dehydrogenase (encoded by *neverland*/*Nvd*), followed by unknown steps that convert 7-dc to 5β-ketodiol and two known enzymatic steps to convert 5β-ketodiol to ecdysone.[27, 28] Several Halloween family genes, such as *spook* (*Spo*/*cytochrome p450 307a1), spookier* (*Spok/Cyp307a2*), *Cyp6t1, phantom* (*Phm*/*Cyp306a1*), *disembodied* (*Dib*/*Cyp302a1*) and *shadow* (*Sad*/*Cyp315a1*), and a non-Halloween gene, *shroud* (*Sro*), are responsible for these enzymatic conversions[27, 28] and found to be highly conserved in arthropods.[29] Ecdysone is ultimately converted to its effective form 20E in the epidermis cells by 20-hydroxylase (encoded by *shade*/*Shd*) and released into the hemolymph as a direct ligand for EcR binding.[27, 28] In addition to 20E, ponasterone A (PoA; 25-deoxy-20E) is considered a major form of Ec in chelicerate species (e.g., mites, ticks, and scorpions) due to a lack of *Phm/Cyp306a1* orthologs in their genome.[26] Moreover, decapod (malacostracan) crustaceans also synthesize PoA along with 20E,[30] and both forms have physiological activity. Although PoA is detected as a major molecule of Ec and then 20E as the second form in the hemolymph during the pre-molt stage of shore crabs (*Callinectes sapidus* and *Carcinus maenas*),[31, 32] the physiological role of PoA is still not well-understood in crustaceans.[27] The degradation of 20E through 26-hydroxylation and further oxidation into 26-carboxylic acids are catalyzed by an enzyme encoded by the EcR-responsive *Cyp18a1* gene in insects,[33-35] and both malacostracan and branchiopod crustaceans.[24, 25, 27]

Precise regulation of the endogenous 20E titer is pivotal to successful molting. Supporting evidence from both insects and crustaceans consistently suggests that a pulse (rise and decline) of the 20E titer is necessary for a complete molt cycle.[4, 24, 25, 36, 37] The synthesis and secretion of 20E are fine-tuned by neuropeptides secreted from the central nervous system (CNS). In insects, a circadian clock controls the rhythmic release of the prothoracicotropic hormone (Ptth) in the brain to trigger the biosynthesis and secretion of Ec.[38] The Ptth induces genes involved in the ecdysteroidogenesis through binding to the Torso receptor and activation of downstream signal transduction pathways, including Ras signaling, Raf signaling and extracellular signal-regulated kinase (ERK) signaling (reviewed in Niwa and Niwa [28]). In contrast, two inhibitory neuropeptides secreted by the sinus gland/X-organ, namely molt-inhibiting hormone (Mih) and crustacean hyperglycemic hormone (Chh) are responsible for regulating the Ec synthesis in decapod malacostracan crustaceans.[39] These neuropeptides bind to the G-protein-coupled receptors and act through cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) mediated secondary messenger signaling to suppress the activity of Ec synthesis and secretion.[40-42] The role of the circadian clock in the regulation of Mih/Chh has not been well-understood in decapod crustaceans. In addition, the ecdysone titer may also provide feedbacks to the CNS and influence the level of neuropeptides,[43] possibly through an EcR signaling pathway.

Ecdysone Receptor. The arthropod EcRs are ligand-dependent transcription factors and belong to the nuclear receptor (NR) subfamily.[44, 45] A typical EcR is comprised of a ligand-binding domain (LBD) and a DNA-binding domain (DBD).[46] For effective ligand binding, the EcR dimerizes with the ultraspiracle protein (Usp), a homolog of the vertebrate retinoid X receptor (RXR) to form a functional heterodimer (Fig. 1A).[45, 47-49] The LBD of EcR/Usp shares a common architecture with other nuclear receptors, which contains a generally folded 3D structure comprised of a three-layered, antiparallel, α-helical sandwich and a β-sheet.[50] Agonism of EcR leads to the formation of a hydrophobic cleft through canonical active conformation and allows the binding of co-activators.[46] The EcRs and Usps have been cloned and characterized in a wide range of arthropod species, including insects, crustaceans, chelicerates and myriapods (reviewed in Nakagawa and Henrich [45]). The primary sequences of EcRs and Usps are found to be highly conserved across taxa.[51, 52]

Ecdysone Receptor Signaling. The heterodimer of EcR/Usp binds to the ecdysone response element (EcRE) of a target gene to achieve transactivation and transcriptional regulation.[53, 54] Among the directly responsive genes, *Broad-complex* (*Br-c*), transcription factor *E74* and *E75* are identified as early-response genes, which are normally up-regulated by EcR at the onset of a molt cycle (Fig. 1D).[55, 56] These genes act as key upstream transcriptional regulators in molting and metamorphosis.[57] Null mutations and RNA interference (RNAi)-aided silencing of these genes lead to lethal molting and developmental defects in *Drosophila*, [58-65] thus confirming their roles in molting and metamorphosis.

When the 20E titer increases to peak levels, several early-late genes are expressed, such as *hormone receptor 3* (*Hr3*), *Hr4* and *Hr38* (Fig. 1D).[56, 66, 67] Silencing of *Hr3* and *Hr4* by RNAi both resulted in delayed and incomplete molting in insects.[68, 69] Null mutants of *Hr38* displayed reduced cuticle gene expression, fragility and rupturing of cuticle, and adult lethality in *Drosophila*.[70, 71] When the 20E titer declines to a low level shortly before ecdysis, genes that are initially suppressed by high 20E titer become expressed, including *Fushi tarazu factor-1* (*Ftz-f1*) and *Dopa decarboxylase* (*Ddc*). [56, 72-74] The mid-prepupal competence factor *Ftz-f1* also plays a central role in the coordination of different molting processes. Silencing of *Ftz-f1* in the nymphs of German cockroach (*Blattella germanica*) resulted in failed ecdysis, developmental arrest and morphological abnormalities.[75] In *Drosophila* larva, silenced beta *Ftz-f1* (*βFtz-f1*) caused double structures of mouthparts, lack of ecdysis behavior and failed shedding of the existing cuticle.[76] The regulation of *Ftz-f1* is mainly through a combination of actions mediated by *Hr3* and *E75*, with *Hr3* directly activating *Ftz-f1* at low 20E titer and *E75b* dimerizing with *Hr3* to suppress the expression of *Ftz-f1* at high 20E levels (Fig. 1D).[73, 75, 77] The dimerized *Hr3-E75* can be dissociated by the action of nitric oxide (NO), as demonstrated in *Drosophila*.[78] The *Hr4* gene, which is normally suppressed by *Hr3*, may also be involved in the inhibition of *Ftz-f1* at relatively high 20E levels.[56, 79, 80] In addition, *Ftz-f1*, *Hr3* and *Hr4* may reverse the regulate of ecdysteroidogenesis, as shown by RNAi. [81, 82] The Ddc gene, which contains an EcRE, is positively regulated by EcR and *Br-c*, and suppressed by *E75b* and *Hr4* (Fig. 1E). [72, 79] Silencing of *Ddc* has been shown to cause incomplete or failed pupation.[83] The sequential activation of genes allows sufficient physiological controls, precisely-regulated behavioral execution of molting and fine-tuned transition between developmental stages.

Regulation of Apolysis. The term "apolysis" describes the detachment of the epidermis layer at the onset of a molt cycle in arthropods.[84] During apolysis, the epidermis cells separate from the old cuticle and form an apolysial space.[1] Apolysis is triggered by rising 20E titer and considered as an initial sign of molting.[85-87] The regulation of apolysis has not been well-studied, but is likely under the control of EcR signaling.

Regulation of New Cuticle Secretion. Immediately after apolysis, the epidermal cells proliferate and the new cuticle is secreted to the apolysial space.[1] Typical cuticles of insects and crustaceans mainly consist of chitin, cuticle proteins and multiple minor components such as lipids and minerals.[3, 88, 89] The cuticle chitin is synthesized from uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) by chitin synthases (encoded by *Chs-1*, *Chs-2* and *krotzkopf verkehrt/Kkv*), which have been identified in a number of insects (reviewed in Merzendorfer and Zimoch [90]) and crustaceans.[91-95] Analysis of *Drosophila Chs* sequences shows that the EcRE is present, suggesting direct transcriptional regulation by EcR.[96, 97] Experimental studies further support that the expression of *Chs* is positively regulated by increased 20E titer during early inter-molt period.[98] Diverse types of cuticular proteins (Cp) have been found in insects[3, 99] and crustaceans.[100, 101] Some of the genes encoding CPs are directly regulated by ecdysone responsive genes, such as *Ftz-f1*, *Br-c* and *Hr38*[102], suggesting that the expression of Cps is highly dependent on the 20E titer (Fig. 1B).

Regulation of Old Cuticle Degradation. Following the initiation of apolysis, the molting fluid is secreted into the apolysial space by the epidermis.[1] In general, the synthesis and secretion of molting fluid are induced by increased 20E titer, whereas its full activation requires a decline of the 20E level. [103] The molting fluid mainly contains two types of chitinolytic enzymes, namely chitinases and chitobiase (Fig. 1C), and various proteases for old cuticle digestion.[103] Chitinase hydrolyses the cuticle chitin to oligomers and trimers, while chitobiase further hydrolyses the oligomers and trimers to *N*-acetyl-D-glucosamine (GlcNAc/NAG) and monomers.[90] The chitinolytic genes and enzymes have been identified in a wide range of insects[104-118] and crustacean species, except for *Allotriocarida*.[119-137] The activities of chitinolytic enzymes positively rely on the rising 20E titer, with chitobiase being induced earlier at relatively lower 20E level and chitinases being induced when the 20E level peaks.[106, 138] The EcREs have not been identified in the sequences of genes encoding the chitinolytic enzymes,[90] indicating the possibility of indirect transcriptional regulation by EcR. However, it has been shown that protein synthesis is not required for induction of chitinolytic genes by 20E, suggesting that the regulation of these genes is likely through direct genomic actions downstream of EcR,[139] possibly by early EcR-

responsive nuclear receptors. Silencing of genes encoding chitinolytic enzymes led to lethal molting defect in various insect species,[104, 116, 140-146] thus suggesting an active role in degradation of the cuticle.

Multiple types of molting fluid proteases (Mfp, Fig. 1C) have been identified in insects and crustaceans, such as trypsin-like serine proteases, cysteine proteases, carboxypeptidases and metalloproteases.[147-161] The Mfps are responsible for digestion of Cps by cleavage of the peptide bonds. More importantly, they may function as proteolytic activators of chitinase precursors.[103, 106, 149] The major serine proteases characterized in the molting fluid were found to be negatively regulated by the 20E titer, such as serine protease meta fission product-1 (Mfp-1) in the tobacco hornworm (*Manduca sexta*)[103, 162] and trypsin-like protease 2 (Tlp2) in the cotton bollworm *Helicoverpa armigera*.[152] Other types of MFPs such as carboxypeptidase A (Cpa)[155, 163] and cathepsin L (CL) [164], however, were found to be constantly up-regulated by the 20E titer, possibly due to their universal roles for digestion of dietary proteins in the guts and cuticle proteins in the integuments. The expression of CPA was shown to be positively regulated by a cascade of *EcR-b*, transcription factor forkhead box O (*FoxO*) and broad-complex isoform 7 (*Br-cz7*),[163] while the regulation of CL by EcR is thought to be through an intermediate transcription factor Relish (*Rel*) in *H. armigera*.[164] Silencing of genes encoding MFPs results in severe molting defects and associated mortality, including endoplasmic reticulum type I signal peptidase complex (Spc1)[165] and trypsin-like serine protease (Tsp)[156] in the Oriental migratory locust (*Locusta migratoria manilensis*), chymotrypsin-like peptidases (Ctlp5c and Ctlp6c)[148] and Cpa[166] in the red flour beetle (*Tribolium castaneum*), CL in *H. armigera*[151, 167] and the pea aphid (*Acyrthosiphon pisum*)[168, 169], and signal peptidase complex member 12 (Space12) in *Drosophila*.[161]

In addition, to avoid the degradation of newly secreted procuticle by molting fluid, a thin nonchitinous layer of epicuticle (or cuticulin) is deposited by the epidermal cells, as shown in both insects and crustaceans.[3, 170] A more recent study with *T. castaneum* also suggested that the actions of chitinases may be protected by Knickkopf (KnK), a protein that is highly conserved in insects, crustaceans and nematodes.[171] Knockout and suppression of the *KnK* gene leads to chitinase-dependent degradation of chitin in the new cuticle, molting defect, development arrest and lethality.[172] However, the transcriptional regulation of *KnK* has not been characterized.

Regulation of Ecdysis. Shedding of old cuticle (ecdysis) is a visible behavioral action of molting and a milestone of developmental transition in arthropods.[173, 174] The shedding behavior is achieved through the ecdysis motor program (EMP), in which a series of repetitive behavioral actions are rhythmically conducted in skeletal muscles, such as air swallowing and water uptake, body stretch and muscle contraction.[3, 175] The EMP is activated by multiple neuropeptides through their actions on the CNS and peripheral synaptic transmission.[4] Among these neuropeptides, the ecdysis triggering hormone (Eth) plays a central role in the regulation of ecdysis (Fig. 1D). Two types of ETHs, pre-ecdysis triggering hormone (Peth or Eth1) and Eth2 have been identified in a number of arthropods and found to be highly conserved across species, with a common peptide sequence.[176] Null mutations of the ETH genes resulted in the absence of the cuticle-shedding behavior, incomplete molting and lethality in larval *Drosophila*, whereas injection of synthetic Eth1 rescued all deficits.[177] The regulation of the ecdysis behavior by Eth is achieved through activation of Eth receptors (EthR)[178] and tightly controlled by the 20E pulse mainly through two steps.[2] First, the expression and synthesis and Eths are directly induced by EcR at high 20E levels, as EcREs are present in the promotor region of the *Eth* genes.[4] Two other factors, cryptocephal (*Crc*) and dimmed (*Dimm*) are thought to participate in the co-regulation of Eth.[179, 180] Second, the release of Eth by the peripheral endocrine cells (Inka cells) into the hemolymph is suppressed at high 20E levels,[181] but promoted when the 20E titer declines.[2] It was demonstrated more than three decades ago that high ecdysteroid titer may cause delay in ecdysis behavior.[182-184] A more recent study showed that injection of 20E in larval *M. sexta* resulted in dose-dependent delay of Eth release and ecdysis.[185] Two neuropeptides have been identified as the activators for Eth release, corazonin (Crz) and eclosion hormone (Eh, Fig. 1D).[186, 187] The initial release of Eth is likely triggered by low levels of Crz through activation of its G-protein-coupled corazonin receptor (CrzR) in *M. sexta*,[187] albeit this mechanism has not been verified in insects such as *Drosophila*.[188] Low levels of Eths then activate EthR-A to promote the release of Eh by the ventromedial (VM) cells in insects.[189-191] The Eths are massively released by low levels of Eh and further eliminated when levels of EH are high.[186, 192] Eclosion hormone induces the secretion of Eth through binding to its receptor, guanylyl cyclase (Gc), and activation of the cyclic guanosine monophosphate (cGMP) signaling pathway, for which calcium signaling may also play a

role.[186, 193] Although Eth can promote the release of Eth, a study in *Drosophila* suggested that Eth was not necessary for Eth release.[188] Since no EcRE has been identified in the *Crz* or *Eh* gene, it is not clear how these neuropeptides are transcriptionally regulated in response to EcR. Besides the activators, it has also been suggested that the secretory competence of Inka cells is a prerequisite for Eth release.[4] Clear evidence for this phenomenon was shown in the pharate pupae of *M. sexta* where Inka cells were not competent for Eth release until the ecdysteroid titer decreased to a sufficiently low level ($\leq 0.1\mu g/mL$) shortly before ecdysis.[181] In addition, injection of Crz or Eh alone failed to induce premature Eth release in *Drosophila*, confirming that the acquisition of the secretory competence in the endocrine cells is necessary for the stimulation of Eth release by neuropeptides.[4, 188] Interestingly, the expression of the competence factor *Ftz-f1* consistently coincides with the decline of 20E titer and lack of this gene resulted in the absence of the ecdysis behavior, as shown in several insect species.[4, 74, 80, 194-196] A recent RNAi study on *Drosophila* clearly revealed that silencing of *βFtz-f1* suppressed the release of Eth in Inca cells, caused phenotypic effects such as double mouthparts, absence of ecdysis and failed molting in the larva, and led to various developmental defects in mid-prepupal and adult stages.[76] The same study also demonstrated that the arrested larva could be rescued by Eth injection or Inca cell-targeted *βFtz-f1* expression, indicating a key role of *βFtz-f1* in the stimulation of the secretory competence for ETH release in insects. In addition to Eth, Eh and crustacean cardioactive peptide (Ccap) may also be involved in the regulation of ecdysis via CNS-mediated processes.[173, 197, 198] Eclosion hormone may activate the EMP independently through cGMP activation and/or induces the expression of Ccap for direct regulation of ecdysis.[4, 189, 197, 199, 200] The ecdysis sequence of different insect species has been extensively reviewed and the universal models for regulation of the ecdysis behavior has been proposed by Zitnan and Adams.[2]

The neuropeptides and their receptors involved in the regulation of insect ecdysis have also been recently predicted and identified in crustaceans, such as waterflea,[201-205] copepods,[206-208] crayfish,[209] lobster,[210] shrimp and prawn.[211] However, the full functions of these neuropeptides in crustacean ecdysis still need to be verified.

Regulation of Cuticle Tanning. Tanning occurs following the secretion of new cuticle and is mainly comprised of two processes, sclerotization (hardening) and melanization (darkening).[212] As described by Kramer and co-workers,[213] the new cuticle tanning takes place both before (pre-ecdysis) and after ecdysis (post-ecdysis). The post-ecdysis tanning is better characterized than pre-ecdysis tanning, as dramatic changes are observable following the shedding of old cuticle.[103] For melanization, dopamine melanin is utilized by most insects as a darkening agent.[214] Dopamine melanin is a metabolic product of the amino acid tyrosine. In this metabolic process, tyrosine is first hydroxylated to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (Th, encoded by *Ple*), then decarboxylated to dopamine by DDC (encoded by *Ddc*). After a few more reactions catalyzed by di-phenoloxidase, dopamine is finally converted to dopamine melanin.[214] For sclerotization, the *N*-acetyltransferase catalyzes the *N*-acylatation of dopamine to the tanning agents *N*-acetyldopamine (NADA) and *N*-β-alanyldopamine (NBAD). The tanning agents are then secreted by the epidermis to the cuticle for cross-linking proteins and chitin. As a result, the cuticle becomes hardened and hydrophobic.[212]

The regulation of cuticle tanning in arthropods is thought to be mediated by a neuropeptide hormone, bursicon (Burs), which is thought to play a central role in the regulation of post-ecdysis tanning. Alignment of Burs peptide sequences shows that this hormone is highly conserved in insects and crustaceans.[215, 216] It is generally accepted that Burs is induced by Ccap and released to the hemolymph by Ccap-expression neurons during post-ecdysis regulation.[216] The hormonal action of Burs is exerted through activation of its G-protein-coupled receptor LGR2 (encoded by *Rickets/Rk*).[217] Knockout and knockdown of *Burs* or its receptor gene *Rk* leads to defects in tanning of new cuticle and developmental abnormalities.[198, 218-222] Bursicon regulates the cuticle tanning by activation of protein kinase A (Pka) via cAMP signaling (Fig. 1E). Protein kinase A then triggers the phosphorylation of Th into its active form for hydroxylation of tyrosine.[212] Another enzyme in this metabolic pathway, Ddc, is likely under direct control of EcR and *Br-c*,[72, 223] as previously discussed (Fig. 1E).

■ ADVERSE EFFECTS OF ECDYSONE RECEPTOR AGONISTS

EcR Agonists. A wide range of chemicals can disrupt the molting processes in arthropods through various MoAs. Among these, the EcR agonists are a group of chemicals that act as direct ligands for binding and activation (agonism) of EcR thus causing molting-associated endocrine disruption. Ecdysone receptor agonists include the endogenous invertebrate molting hormones such as ecdysone and 20E, steroidal hormones such as ponasterone A (PoA), muristerone A, makisterone A, cyasterone and inokosterone, and non-steroidal pesticides such as RH-5849, tebufenozide (TEB or RH-5992), methoxyfenozide (RH-2485), halofenozide (RH-0345) and chromafenozide (ANS-118).[18, 224] The non-steroidal EcR agonists are of special environmental concern, as they have been developed as insecticides and anti-parasitic agents and are widely used in agriculture and aquaculture against harmful arthropods.[225]

Incomplete ecdysis. The adverse effects of EcR agonists on molting have not been universally defined. However, a few observed phenomena such as "precocious molting", "incomplete ecdysis" and "premature molting" have been frequently reported. These terms refer to the same phenotypic effect characterized by an animal failing to completely shed its old cuticle during a molt cycle, while the new cuticle is generated (i.e. presence of a double-layer cuticle, Fig. 2). This molting defect is usually lethal, possibly due to growth arrest and/or lack of feeding. In insects, the 5th instar larva of the spruce budworm (*Choristoneura fumiferana*) fed or injected with 100 ng TEB for 48h failed to separate the old cuticle from the new.[225] Oral administering of 0.00001-10 ppm TEB to the African cotton leafworm (*Spodoptera littoralis*) resulted in incomplete molting of the old larval cuticle and death.[226] Dietary exposure of the larval tobacco hornworm *M. sexta* to RH-5849 failed to shed the 6th-stage larval cuticle and died as pharate pupae. [227] In crustaceans, acute (48h) exposure of female *D. magna* to 500 nM 20E led to complete molting inhibition in 66% of the test animals.[24] The histological analysis in the same study further revealed that both old and new cuticles were present in the treated animals. Lack of old cuticle dissociation and subsequent death were also found in juvenile *D. magna* after 5d exposure to 1000 nM 20E,[228] and after 48h and 8d exposure to 20E and ecdysone, respectively.[229] Chronic (21d) exposure to as low as 260 nM 20E and 27 nM PoA caused 100% and 70% lethal incomplete ecdysis in *D. magna*, respectively.[230] After exposure to 10 mg/L RH-5849, the zoeae of a crab (*Rhithropanopeus harrisii*) successfully underwent apolysis, but failed to execute ecdysis and subsequently died.[231] Collectively, the body of evidence strongly suggests that exposure to EcR agonists can cause molting failure and death by disruption of normal ecdysis.

■ ADVERSE OUTCOME PATHWAY DEVELOPMENT

Identification of the Conceptual AOP for EcR Mediated Endocrine Disruption. As discussed earlier, ecdysis is under direct control of Eth and associated Ec-EcR signaling in insects, and likely in crustaceans in general. Therefore, the endocrine regulation of Eth is a critical factor determining whether an arthropod can undergo normal ecdysis. A number of studies on insects showed that dysregulation of hemolymph Eth levels resulted in incomplete ecdysis which is often lethal. The expression of Eth is positively regulated by the ecdysteroid titer through EcR, and the secretion of Eth into the hemolymph is negatively regulated. The intermediate processes between the activation of EcR and inhibition of Eth had not been well-understood until recently when a RNAi study published by Cho and coworkers demonstrated that the expression of the *βFtz-f1* gene played a key role in determining the release of Eth.[76] These authors further showed that selective silencing of *βFtz-f1* in endocrine Inka cells prevented Eth release and ultimately caused developmental arrest at all stages in *Drosophila*. It has also been suggested that *βFtz-f1* is down-regulated by the EcR early-responsive gene *E75b* at high ecdysteroid titer, whereas up-regulated by *Hr3* when the 20E titer declines in the end of a molt cycle. Based on this knowledge, the causal relationships between the activation of EcR by agonists, leading to induction of *E75b*, suppression of *βFtz-f1*, inhibition of ETH release and reduced muscle contraction, and incomplete ecdysis can be established and described using an AOP framework.

AOP Assembly. Based on the knowledge from arthropod endocrinology and experimental evidences from ED studies, a conceptual AOP describing "ecdsyone receptor agonism leading to incomplete ecdysis associated mortality" was constructed and submitted to the AOP-Wiki (https://aopwiki.org/aops/4), a publicly accessible and internationally harmonized source of AOP

information. This AOP starts with direct activation of EcR by agonists as the MIE, followed by sequential occurrence of 8 KEs at different organismal levels leading to mortality as the AO (Fig. 3).

Assessment of the AOP. ***Criteria.*** The weight of evidence assessment is based on the Evolved Bradford Hill considerations[232] implemented in OECD's guidance document for developing and assessing AOPs.[233] The main criteria include: support for biological plausibility of the key event relationships (KERs), support for essentiality of KEs, empirical support for KERs (dose-response and temporal concordance; taxonomic, species and stressor consistency) and quantitative understanding of the KERs. The confidence for each criterion is assessed as high (strong), moderate or low (weak) based on a set of guiding questions outlined in the Users' handbook supplement to the guidance document for developing and assessing AOPs.[233] These confidence "calls" represent the subjective evaluation of the AOP developer(s) based on familiarity and detailed evaluation of the supporting evidence critically. However, the scientific support on which the AOP is based is transparently assembled in the AOP-Wiki, allowing any potential user of the AOP to evaluate the technical quality and robustness of the relationships and decide on the appropriate application(s) of the knowledge.

Essentiality of Key Events. Essentiality of the KEs is one of the primary considerations in assessing the confidence in the causal relationships between the various KEs included in an AOP construct. The guiding question for evaluating essentiality is whether there is evidence that downstream KEs are prevented if an upstream KE is blocked or prevented.[233] For example, studies showing that knock-out of the gene coding for *Ftz-f1* (KE-2) results in an absence of ecdysis behavior (KE-8) provides support for the essentiality of this KE in the pathway (Fig. 3). Similarly, data showing that null mutation of the *Eth* gene in *Drosophila* leads to lethal incomplete molting and that injection of synthetic Eth1 rescues the deficit[177] provide strong support for the essentiality of KE-3 in the AOP (Fig. 3). Based on the criteria presented in the OECD guidance,[233] overall support for essentiality of the KEs in this AOP was judged to be high, as there were multiple KEs for which direct evidence of essentiality was present in the literature (Table 1). Strongest support for essentiality was observed for KEs (1, 3, 4, 5, 8), while weaker support was available for KEs (2, 6, 7). Based on the proposed analysis, this AOP may be strengthened through further experimentation such as transcriptional analysis, immunoenzymatic detection of neuropeptides, electrophysiological recording of neurotransmission and behavioral analysis of organisms exposed to EcR agonists.

Weight of Evidence Assessment of Key Event Relationships. Key event relationships describe the scientifically-credible basis for the ability to extrapolate along the AOP and infer the likely state of a downstream KE, based on a measurement of an upstream KE. Support for the KERs are based on evaluation of their biological plausibility (i.e., known structural or functional relationships between the biological entities being measured) and evidence showing that when changes in the upstream event occur, changes in the downstream event also occur, in a manner consistent with a causal relationship between the events.

In terms of evaluating the biological plausibility of the KERs in the AOP linking EcR agonism to molting failure-related mortality, the guiding question is whether there is a known mechanistic (structural or functional) relationship between the two KEs that is consistent with current biological understanding. In the case of this AOP, biological plausibility was judged to be strong for all KERs represented in the pathway (Table 2). Studies focused on establishing the fundamental biological relationships between these KE pairs would be useful.

Empirical support for the KERs was considered on the basis of whether observations of the two KEs in various studies was consistent with the expected patterns of concordance, or whether deviations were explainable[224]. Based on those considerations, empirical support for the KERs was generally judged to be moderate. KERs 1, 2, 9 have the strongest empirical support. Empirical evidence was not quite as robust for KERs 3-8, based on lack of dose-response data. However, the temporal concordance of these KERs is verified in most studies, therefore the empirical support is considered to be moderate.

Finally, KERs were assessed with regard to quantitative understanding of how much change in the upstream KE is needed to evoke some unit of change in the downstream KE, and the extent to which it is understood how other variables such as genetic background, diet, environmental variables, may influence that relationship.[233] Except for KER-9, which has a reported quantitative relationship between the KEs, the quantitative understanding for other KERs in this AOP is considered to be weak. The full

list of literature support for WoE assessment of the EcR AOP can be found in Supporting Information (SI, Table S1 and S2).

Applicability of the AOP. Another aspect of AOP evaluation involves defining its domain of applicability. This includes defining the chemical/stressor space for which it is known to be relevant. It also includes defining the biological domain of applicability in terms of taxa for which the AOP is expected to apply, as well as life stage and sex.

Chemical domain. The chemical domain of this AOP includes both steroidal and non-steroidal EcR agonists. Known steroidal agonists include ecdysone, 20E, PoA, muristerone A, makisterone A, cyasterone and inokosterone. Non-steroidal agonists include RH-5849, tebufenozide (RH-5992), methoxyfenozide (RH-2485), halofenozide (RH-0345) and chromafenozide (ANS-118). Known non-steroidal agonists mainly belong to groups of chemicals with similar structures, such as dibenzoylhydrazine (DBH), aclaminoketone (AAK) and tetrahydroquinoline (THQ). Chemicals within these groups are likely to be part of the chemical applicability domain of this AOP. The experimentally verified EcR agonists in insects and crustaceans are summarized in Supporting Information (SI, Table S3).

Taxonomic domain. The current AOP is fully supported by studies on insects, such as *Drosophila* (*Diptera*), *M. sexta* (*Lepidoptera*), *Bombyx mori* (*Lepidoptera*), *T. castaneum* (*Coleoptera*). The AOP also draws upon multiple studies with crustaceans, although crustacean-based evidence for certain elements of the pathway is sparse. The EcR itself is thought to be well conserved among all arthropods, as is the role of Eth in stimulating muscle contraction behavior required for completing ecdysis. Certain elements such as the involvement of *E75b* expression and the role of *Ftz-f1* have not been characterized in as broad a range of species, but again, based on evaluation of known sequence conservation and phylogenetic relationships, it is expected that this AOP may be applied broadly to most arthropods, although differences in the exact nature of quantitative relationships between some of the KEs may vary among taxa.

Sex and life stages. This AOP is potentially applicable for all life stages and sexes. Strong supporting evidence has been obtained from studies on prepupal and pharate pupal stages of insects.

■ FUTURE DIRECTIONS

Development of AOPs are considered an active process where new data and information are used to expand the AOP itself, strengthen the supporting data and WoE considerations, and introduce new AOPs that share common MIE (i.e. the EcR), KEs or AO and thus support the development of an AOP network.[15, 234] All of these avenues for further development seems highly relevant for this AOP. For example, there are still gaps in the intermediate KEs of the AOP (e.g. the transition from KE-3 to KE-4), there are several KERs with weak WoE considerations, particularly with regard to empirical support and quantitative understand, and the role of EcR-mediated pathways involving other KE leading to molting disturbances than those proposed are still unresolved. Although a number of EcR agonists have been characterized already, the highly diverse chemical universe contains thousands (e.g. typical of US TOXCAST, ECOTOX and REACH dossier data sets) to millions registered chemicals (https://www.cas.org/) will likely lead to expansion of the chemical applicability domain as computational and experimental efforts screen large numbers of novel chemicals for their ability to interact with the EcR in arthropods. Expansion of the taxonomic applicability domain by a combination of *in silico* and experimental approaches are highly warranted. For example, use of sequence alignment approaches (e.g. SeqAPASS; https://seqapass.epa.gov/seqapass/) to identify conserved biological targets in combination with *in vitro* and *in vivo* experimental approaches to verify these targets along the AOP continuum in arthropods can be potential options. Verification of the current AOP in different arthropod species using suites of *in silico* tools for identifying the taxonomic and chemical applicability, *in vitro* screening of novel EcR ligands and targeted *in vivo* studies to characterize the KE and AO of the AOP is expected to expand our knowledge of this AOP and develop suites of tools to support IATA approaches in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information (Excel file) is available free of charge on the ACS Publications website at DOI:

Table S1: Essentiality of KEs

Table S2: WoE assessment of KERs

Table S3: List of EcR agonists

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Notes

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■ LIST OF ABBREVIATIONS

20E	20-hydroxyecdysone
7-dc	7-dehydrocholesterol
AAK	Aclaminoketone
ANS-118	Chromafenozide
AO	Adverse outcome
AOP	Adverse outcome pathway
Br-c	Broad-complex
Br-cz7	Broad-complex isoform 7
Burs	Bursicon
cAMP	Cyclic adenosine monophosphate
Ccap	Crustacean cardioactive peptide
cGMP	Cyclic guanosine monophosphate
Chh	Crustacean hyperglycemic hormone
Chs	Chitin synthase
Cht	Chitinase
CL	Cathepsin L
CNS	Central nervous system
Cp	Cuticular protein
Cpa	Carboxypeptidase A
Crc	Cryptocephal
Crz	Corazonin
CrzR	Corazonin receptor
Ctbs	Chitobiase

Ctlp	Chymotrypsin-like peptidase
Cyp18a1	Cytochrome p450 18a1
Cyp6t1	Cytochrome p450 6t1
DBH	Dibenzoylhydrazine
Ddc	Dopa decarboxylase
Dib	Disembodied/Cytochrome p450 p302a1
Dimm	Dimmed
DOPA	Dihydroxyphenylalanine
E	Ecdysone
E74	Nuclear recpetor E74
E75b	Nuclear receptor E75B
E78	Nuclear recpetor E78
Ec	Ecdysteroid
ECOTOX	US EPA ECOTOX Knowledgebase
EcR	Ecdysone receptor
EcRE	Ecdysone response element
ED	Endocrine disruption
EDC	Endocrine disrupting chemical
Eh	Eclosion hormone
EMP	Ecdysis motor program
ERK	Extracellular signal-regulated kinase
Eth	Ecdysis triggering hormone
EthR	Ecdysis triggering hormone receptor
FoxO	Transcription factor forkhead box O
Ftz-f1	Fushi tarazu factor-1
GAF	General assessment factor
Gc	Guanylyl cyclase
GlcNAc/NAG	N-acetyl-D-glucosamine
Hr3	Hormone recpetor 3
Hr38	Hormone receptor 38
Hr4	Hormone recpetor 4
IATA	Integrated Approaches to Testing and Assessment
IGR	Insect growth regulator
KE	Key event
KER	Key event relationship
Kkv	Krotzkopf verkehrt
KnK	Knickkopf
LBD	Ligand-binding domain
Mfp	Molting fluid protease
Mfp-1	Serine protease meta fission product-1
MIE	Molecular initiating event
Mih	Molt-inhibiting hormone
MoA	Mode of action
NADA	N-acetyldopamine
NBAD	N-β-alanyldopamine
NO	Nitric oxide
NR	Nuclear receptor
Nvd	Neverland
OECD	Organization for Economic Co-operation and Development
OMICS	Genomics, transcriptomics, proteomics, metabolomics

Peth	Pre-ecdysis triggering hormone
PG	Prothoracic gland
Phm	Phantom/Cytochrome p450 306a1
Pka	Protein kinase A
PoA	Ponasterone A
ppm	Parts per million
Ptth	Prothoracicotropic hormone
QSAR	Quantitative structure-activity relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
Rel	Relish
RH-0345	Halofenozide
RH-2485	Methoxyfenozide
Rickets/*Rk*	G-protein-coupled receptor LGR2
RNAi	RNA interference
RXR	Retinoid X receptor
Sad	Shadow/Cytochrome p450 p315a1
SeqAPASS	Sequence Alignment to Predict Across Species Susceptibility
Shade	Shd/20-hydroxylase
Space12	Signal peptidase complex member 12
Spc1	Endoplasmic reticulum type I signal peptidase complex
Spo	Spook/Cytochrome p450 307a1
Spok	Spookier/Cytochrome p450 307a2
Sro	Shroud
TEB/RH-5992	Tebufenozide
Th	Tyrosine hydroxylase
THQ	Tetrahydroquinoline
Tlp2	Trypsin-like protease 2
TOXCAST	US EPA Toxicity ForeCaster (ToxCast™) Data
Tsp	Trypsin-like serine protease
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
Usp	Ultraspiracle protein
VM	Ventromedial
WoE	Weight of evidence

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Tables

Table 1. Support for Essentiality of Key Events (KEs).

KE#	Event description	Support for essentiality	Detection method	Target for detection
MIE	EcR, Activation		In vitro EcR binding assay; Transcriptional analysis	EcR transfected mammalian cells; mRNA from cell, tissue and whole organism
KE-1	*E75B* gene, Induction	Strong	Transcriptional analysis	mRNA from cell, tissue and whole organism
KE-2	*Ftz-f1* gene, Suppression	Moderate	Transcriptional analysis	mRNA from cell, tissue and whole organism
KE-3	Release of circulating ETH, Reduction	Strong	Enzyme immunoassay; Immunohistochemical staining	Hemolymph; Isolated endocrine tissue
KE-4	Release of circulating CCAP, Reduction	Strong	Enzyme immunoassay; Immunohistochemical staining	Hemolymph; Isolated endocrine tissue
KE-5	Ecdysis motoneuron bursts, Reduction	Strong	Electrophysiological recording	Isolated CNS, abdominal ganglion
KE-6	Excitatory postsynaptic potential, Reduction	Moderate	Electrophysiological recording; FM1-43 fluorescent labeling	Skeletal muscles
KE-7	Abdominal muscle contraction, Reduction	Moderate	Electrophysiological recording; Behavioral (Air/water swallowing) assays	Skeletal muscles; Whole organism
KE-8	Incomplete ecdysis, Induction	Strong	Light microscope, histopathology	Cuticle; Whole organism
AO	Mortality, Increased		Survival test	Whole organism

Table 2. Weight of Evidence (WoE) Assessment of Key Event Relationships (KERs).

KER#	Upstream event	Relationship	Downstream event	Biological plausibility	Empirical support	Overall WoE	Quantitative understanding
KER-1	EcR, Activation	Directly leads to	*E75B* gene, Induction	Strong	Strong	Strong	Weak
KER-2	*E75B* gene, Induction	Directly leads to	*Ftz-f1* gene, Suppression	Strong	Strong	Strong	Weak
KER-3	*Ftz-f1* gene, Suppression	Directly leads to	Release of circulating ETH, Reduction	Strong	Moderate	Moderate	Weak
KER-4	Release of circulating ETH, Reduction	Indirectly leads to	Release of circulating CCAP, Reduction	Strong	Moderate	Moderate	Weak
KER-5	Release of circulating CCAP, Reduction	Directly leads to	Ecdysis motoneuron bursts, Reduction	Strong	Moderate	Moderate	Weak
KER-6	Ecdysis motoneuron bursts, Reduction	Directly leads to	Excitatory postsynaptic potential, Reduction	Strong	Moderate	Moderate	Weak
KER-7	Excitatory postsynaptic potential, Reduction	Directly leads to	Abdominal muscle contraction, Reduction	Strong	Moderate	Moderate	Weak
KER-8	Abdominal muscle contraction, Reduction	Directly leads to	Incomplete ecdysis, Induction	Strong	Moderate	Moderate	Weak
KER-9	Incomplete ecdysis, Induction	Directly leads to	Mortality, Increased	Strong	Strong	Strong	Strong

Figures

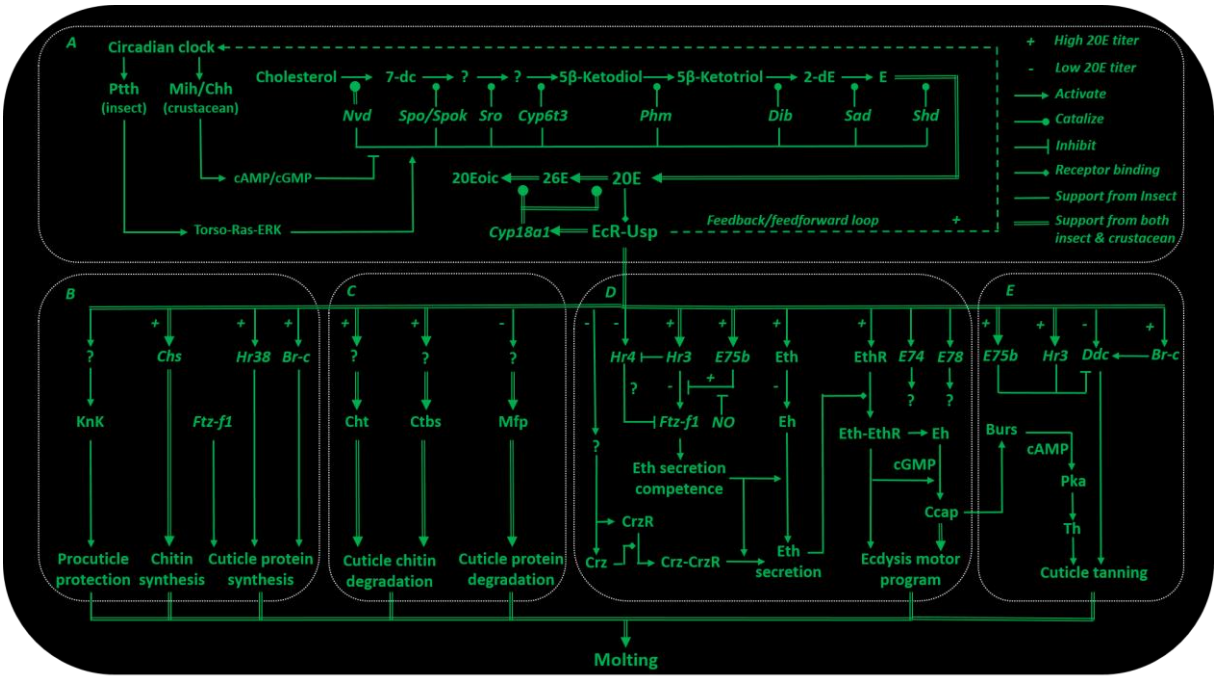

Fig. 1 Putative model illustrating the neuroendocrine regulation of molting in arthropods. A: Regulation of ecdysteroid titer; B: Regulation of new cuticle secretion; C: Regulation of old cuticle degradation; D: Regulation of ecdysis behavior; E: Regulation of cuticle tanning. Ptth: prothoracicotropic hormone; Mih: Molt-inhibiting hormone; Chh: crustacean hyperglycemic hormone; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; Torso: Ptth receptor; Ras: Ras signaling; ERK: extracellular signal-regulated kinase; 7-dc: 7-dehydrocholesterol; E: ecdysone; 2-dE: 2-deoxyecdysone; 20E: 20-hydroxyecdysone; 20, 26E: 20, 26-dihydroxyecdysone; 20Eoic: 20-hydroxyecdysonoic acid; *Nvd*: *Neverland* (7, 8-dehydrogenase); *Spo*: *spook/Cyp307a1*; *Spok*: *spookier/Cyp307a2*; *Sro*: shroud; *Cyp6t3*: cytochrome p450 6t3; *Phm*: *phantom/Cyp306a1* (25-hydroxylase); *Dib*: *disembodied/Cyp302a1* (22-hydroxylase); *Sad*: *shadow/Cyp315a1* (2-hydroxylase); *Shd*: *shade/Cyp314a1* (20-hydroxylase); EcR: ecdysone receptor; Usp: ultraspiracle protein; *Cyp18a1*: cytochrome p450 18a1; *KnK*: Knickkopf; *Chs*: chitin synthase; Cht: chitinase; Ctbs: chitobiase; Mfp: molting fluid protease; *Hr38*: hormone receptor 38; *Br-c*: broad-complex; *Ftz-f1*: Fushi tarazu factor-1; NO: nitric oxide; *Hr4*: hormone receptor 4; *Hr3*: hormone receptor 3; *E75b*: nuclear receptor E75B; *E74*: nuclear receptor E74; *E78*: nuclear receptor E78; Crz: corazonin; CrzR: corazonin receptor; Eth: ecdysis triggering hormone; EthR: ecdysis triggering hormone receptor; Eh: eclosion hormone; Ccap: crustacean cardioactive peptide; *Ddc*: dopa decarboxylase; Burs: bursicon; Pka: protein kinase A; Th: tyrosine hydroxylase.

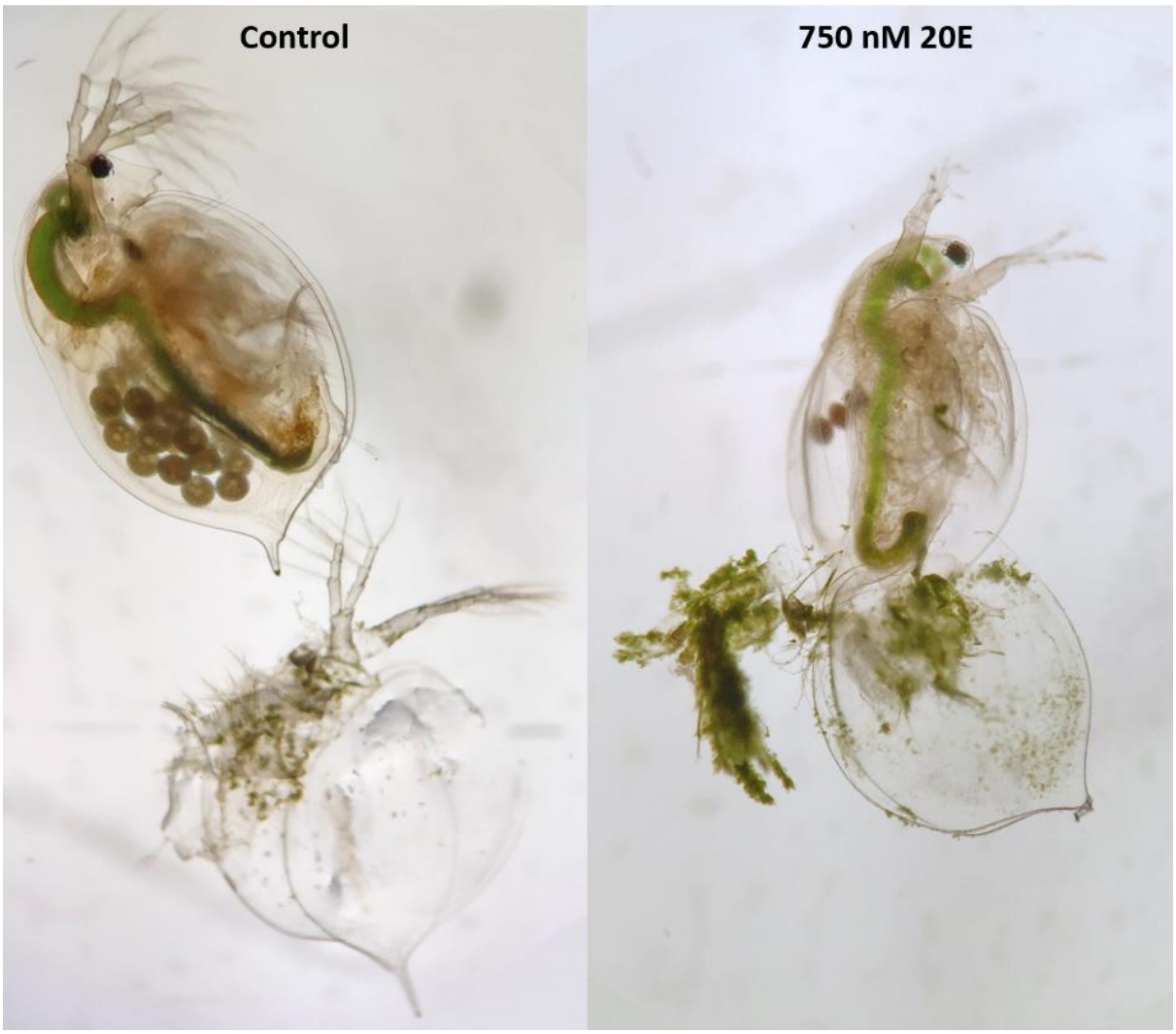

Fig. 2 Incomplete ecdysis in adult female *Daphnia magna* after 96h exposure to 750 nmol/L of the endogenous ecdysone receptor agonist 20-hydroxyecdysone (20E).

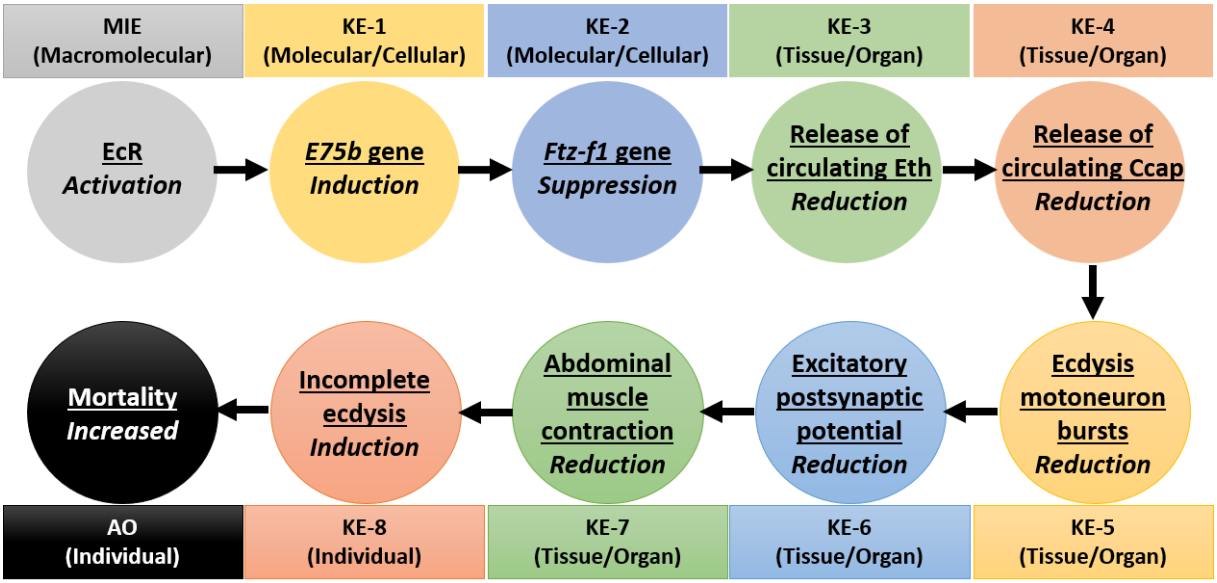

Fig. 3 Adverse outcome pathway of ecdysone receptor agonism leading to incomplete ecdysis associated mortality. MIE: Molecular initiating event; KE: Key event; AO: Adverse outcome; EcR: ecdysone receptor; *E75b*: nuclear receptor E75B; *Ftz-f1*: Fushi tarazu factor-1; Eth: ecdysis triggering hormone; Ccap: crustacean cardioactive peptide; Solid line with arrow: directly triggers; Dashed line with arrow: Indirectly triggers.

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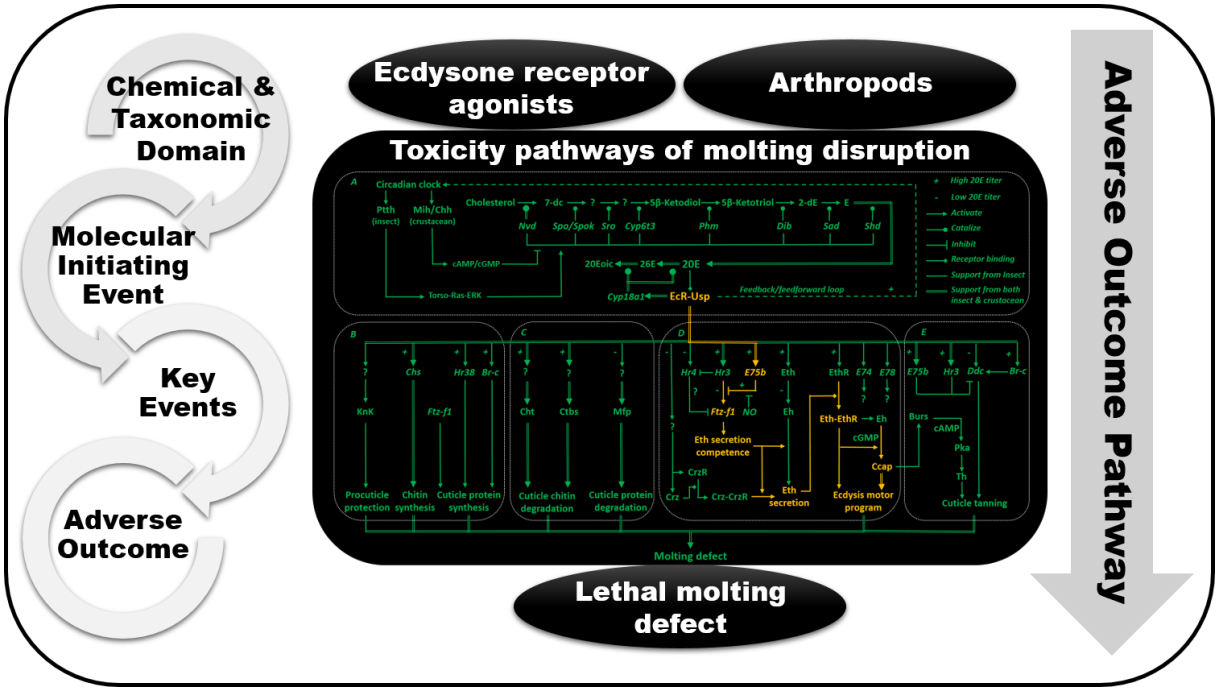

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