Environmental Toxicology and Chemistry—Volume 40, Number 8—pp. 2112–2120, 2021
Received: 26 January 2021 | Revised: 26 February 2021 | Accepted: 19 March 2021

ET&C
PATHWAYS AND PREDICTIONS

Pathways and Predictions articles are summaries of multi-process biological responses to chemicals described by extensive datasets. Adverse outcome pathways (AOPs) are one example of this where comprehensive compilations of concepts and evidence comprising a given AOP can be obtained from an open-source AOP Wiki (aopwiki.org).

AOP Report: Inhibition of Chitin Synthase 1 Leading to Increased Mortality in Arthropods

Simon Schmid,[a,b] You Song,[a] and Knut Erik Tollefsen[a,b,c,*]

[a]Section of Ecotoxicology and Risk Assessment, Norwegian Institute for Water Research, Oslo, Norway
[b]Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, Ås, Norway
[c]Centre for Environmental Radioactivity, Norwegian University of Life Sciences, Ås, Norway

INTRODUCTION AND BACKGROUND:

Arthropods (including insects, crustaceans, and arachnids) rely on the synthesis of chitin to complete their life cycles (Merzendorfer 2011). The highly conserved chitin synthetic process and the absence of this process in vertebrates make it an exploitable target for pest management and veterinary medicines (Merzendorfer 2013; Junquera et al. 2019). Susceptible, nontarget organisms, such as insects and aquatic invertebrates, exposed to chitin synthesis inhibitors may suffer population declines, which may have a negative impact on ecosystems and associated services. Hence, it is important to properly identify, prioritize, and regulate relevant chemicals posing potential hazards to nontarget arthropods.

The need for a more cost-efficient and mechanistic approach in risk assessment has been clearly evident and triggered the development of the adverse outcome pathway (AOP) framework (Ankley et al. 2010). An AOP links a molecular initiating event (MIE) through key events (KEs) to an adverse outcome. The mechanistic understanding of the underlying toxicological processes leading to a regulation-relevant adverse outcome is necessary for the utilization of new approach methodologies (NAMs) and efficient coverage of wider chemical and taxonomic domains. In the last decade, the AOP framework has gained traction and expanded within the (eco)toxicological research community. However, there exists a lack of mature invertebrate AOPs describing molting defect–associated mortality triggered by direct inhibition of relevant enzymes in the chitin biosynthetic pathway (chitin synthesis inhibitors) or interference with associated endocrine systems by environmental chemicals (endocrine disruptors).

Arthropods undergo molting to grow and reproduce (Heming 2018). This process is comprised of the synthesis of a new exoskeleton, followed by the exuviation of the old exoskeleton (Reynolds 1987). The arthropod exoskeleton (cuticle) can be divided into 2 layers, the thin and nonchitinous epicuticle, which is the outermost layer of the cuticle, and the underlying chitinous procuticle. A single layer of epithelial cells is responsible for the synthesis and secretion of both cuticular layers (Neville 1975). The cuticle protects arthropods from predators and desiccation, acts as a physical barrier against pathogens, and allows for locomotion by providing support for muscular function (Vincent and Wegst 2004). Because the procuticle mainly consists of chitin microfibrils embedded in a matrix of cuticular proteins supplemented by lipids and minerals in insects (Muthukrishnan et al. 2012) and crustaceans (Cribb et al. 2009; Nagasawa 2012), chitin is a determinant factor for the appropriate composition of the cuticle and successful molting (Cohen 2001). A detailed overview of the endocrine

(Continued)

This article includes online-only Supplemental Data.

* Address correspondence to ket@niva.no
Published online 5 April 2021 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.5058

mechanisms regulating chitin synthesis is given in Supplemental Data, Figure S1. The shedding of the old exoskeleton in insects is mediated by a sequence of distinct muscular contractions, the ecdysis motor program (EMP; Ayali 2009; Song et al. 2017a). Like the expression of chitin synthase isoform 1 (CHS-1), the expression of peptide hormones regulating the EMP is also controlled by ecdysteroids (Antoniewski et al. 1993; Gagou et al. 2002; Ayali 2009).

Cuticular chitin is polymerized from uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) by the transmembrane enzyme CHS-1, which is localized in the epithelial plasma membrane in insects (Locke and Huie 1979; Binnington 1985; Merzendorfer and Zimoch 2003; Merzendorfer 2006). Because crustaceans are also dependent on the synthesis of chitin, the underlying mechanisms are believed to be similar, although less is known about different CHS isoforms and their localization (Rocha et al. 2012; Qian et al. 2014; Uddowla et al. 2014; Harðardóttir et al. 2019).

Disruption of either chitin synthesis or the upstream endocrine pathways can lead to lethal molting disruption (Arakawa et al. 2008; Merzendorfer et al. 2012; Song et al. 2017a, 2017b). In the case of chitin synthesis inhibition, molting disruption can be referred to as "premature molting." If ecdysis cannot be completed because of decreased chitin synthesis, the organism may not successfully molt. Even if ecdysis can be completed on inhibition of chitin synthesis, the organism may not survive because of the poor integrity of the new cuticle. These effects are observed in arthropods following molting, which fail to survive subsequent molts (Arakawa et al. 2008; Chen et al. 2008) or animals being stuck in their exuviae (Wang et al. 2019) and ultimately dying as a result of insufficient food or oxygen intake (Camp et al. 2014; Song et al. 2017a). The term "premature molting" is used to differentiate from the term "incomplete ecdysis," which describes inhibition of ecdysis on a behavioral level, namely through reduction of the EMP (Song et al. 2017a).

The present AOP describes molting-associated mortality through direct inhibition of the enzyme CHS-1. It expands the small but increasing number of invertebrate AOPs that have relevance to arthropods, the largest phylum within the animal kingdom (Bar-On et al. 2018). The development of this AOP will be useful in further research and regulatory initiatives related to assessment of CHS inhibitors and identification of critical knowledge gaps and may suggest new strategies for ecotoxicity testing efforts. *Environ Toxicol Chem* 2021;40:2112–2120. © 2021 *The Authors. Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Adverse outcome pathway; Invertebrate toxicology; Developmental toxicity; Mode of action; Toxic effects

AOP DESCRIPTION

The present AOP (Figure 1 and Textbox 1) describes the potential causal events initiated by inhibition of the enzyme CHS-1 (MIE, event 1522), leading to increased mortality (adverse outcome, event 350) via the KEs of decreased cuticular chitin content (event 1523) and increased premature molting (event 1524). The MIE constitutes inhibition of CHS-1, with the functional outcome of decreased CHS-1 activity, which leads directly to the decrease in cuticular chitin content. The decreased cuticular chitin content is a logical consequence of the inhibition of CHS-1, which polymerizes UDP-GlcNAc to chitin (Supplemental Data, Figure S1). Decreased cuticular chitin content is expected to lead directly to premature molting as the main cause of the adverse outcome increased mortality. The set of KEs is the minimal required series of events for the AOP. The event of decreased cuticular chitin synthesis (event 1523) is tightly associated with the inhibition of CHS-1. As discussed, the second KE, increased premature molting, describes molting disruptions associated with an immature cuticle at the time of ecdysis. Effects observed include the inability of arthropods to molt, the inability to grow, and the delayed disruption of molting at the subsequent molt because of a weak cuticle. The described effects do not occur chronologically but rather as separate events that are connected in the final KE (Increase, premature molting). These effects also have the same preceding and succeeding events, namely decreased cuticular chitin content and increased mortality, and were consequently combined in the event of premature molting (event 1524).

TEXTBOX 1: Adverse outcome pathway (AOP) identification

- Formal AOP title: Chitin synthase 1 inhibition leading to mortality
- AOP authors: Simon Schmid, You Song, Knut Erik Tollefsen
- AOP contributors: Simon Schmid, You Song, Knut Erik Tollefsen
- AOP number: 360
- Development status: Open for citation and comment.
- List of key events (KEs):
  - MIE: Inhibition, chitin synthase 1, event ID: 1522
  - KE: Decrease, cuticular chitin content, event ID: 1523
  - KE: Increase, premature molting, event ID: 1524
  - Adverse outcome: Increase, mortality, event ID: 350

The present AOP has been submitted to the AOP Wiki as AOP 360 (Society for the Advancement of Adverse Outcome Pathways 2016). This linear AOP is part of a larger AOP network describing inhibition of chitin synthesis and chitin degradation

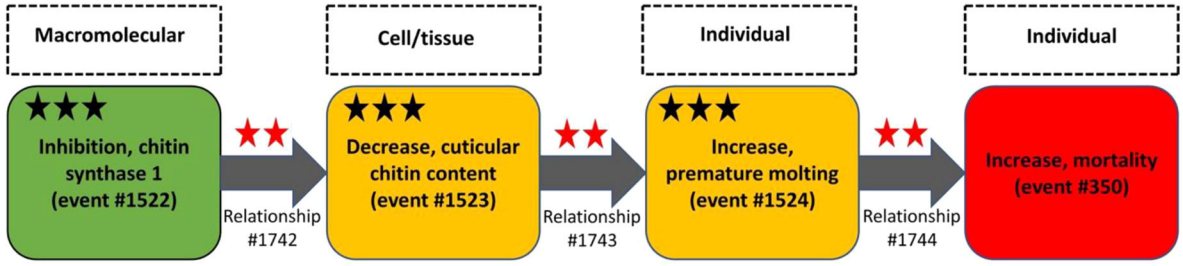

FIGURE 1: Graphical representation of adverse outcome pathway 360: chitin synthase 1 inhibition leading to mortality: green indicates molecular initiating event (MIE), yellow indicates key event (KE), and red indicates adverse outcome; stars indicate the weight of evidence, where 3 stars indicate high, 2 stars indicate moderate, and 1 star indicates low; red stars indicate the overall weight of evidence of KE relationships (biological plausibility and empirical evidence), and black stars indicate the weight of evidence of the essentiality of KEs. Information on the level of biological organization of the events is found in the dashed boxes above the MIE, KEs, and adverse outcome.

leading to premature molting associated mortality. In this report, however, we only describe the first linear AOP on CHS-1 inhibition, because of its central importance in the AOP network and the availability and quality of the supporting evidence. The rest of the AOPs in the network still require substantial efforts for further development and evaluation.

SCIENTIFIC EVIDENCE ASSESSMENT

Weight-of-evidence assessment was performed following the instructions in the *User's Handbook Supplement to the Guidance Document for Developing and Assessing AOPs* (Organisation for Economic Co-operation and Development 2018) according to the evolved Bradford-Hill considerations (Becker et al. 2015). The weight-of-evidence assessment criteria are organized into 3 categories: essentiality of KEs, biological plausibility of the KE relationships (KERs), and empirical evidence supporting the KERs. The essentiality of KEs has been rated as high for all KEs, and downstream KEs are dependent on the occurrence of upstream KEs (Table 1). For all KERs, the biological plausibility was rated as high because the processes of chitin synthesis and its importance for molting are well characterized (Table 2). Empirical evidence was judged as moderate because insufficient information on dose and incidence concordance are available. However, temporal concordance is given and was rated as high for all KERs, leaving empirical evidence at a moderate level for all KERs (Table 2). Based on these evaluations, the overall evidence in the AOP was judged as moderate.

TABLE 1: Summary of the assessment of essentiality for key events[a]

Key event	Event description	Level of evidence	Support for Essentiality
MIE	Inhibition, chitin synthase 1	Direct	High
KE1	Decrease, cuticular chitin content	Direct	High
KE2	Increase, premature molting	Direct	High
AO	Increase, mortality	—	—

[a]Detailed information on the studies used for the assessment of essentiality can be found in Supplemental Data, Table S1.
MIE = molecular initiating event; KE = key event; AO = adverse outcome.

Essentiality of KEs

One way to discern essentiality is through use of targeted chemical inhibitors. Chemicals such as the antifungal antibiotics polyoxin D and nikkomycin Z are known to competitively inhibit CHS and lead to decreased cuticular chitin content and increased mortality in lepidopteran, dipteran, and coleopteran insects (Cohen 1982; Turnbull and Howells 1982; Kuwano and Cohen 1984; Cohen and Casida 1990; Zhang and Zhu 2013; Zhuo et al. 2014).

Another way to demonstrate essentiality is through "knockdown/-out" experiments. Knockdown of CHS-1 by RNA interference has been reported to cause decreased cuticular chitin content and premature molting–associated mortality in insects (Arakane et al. 2005; Zhang et al. 2010; Li et al. 2017). In other studies where cuticular chitin content was not assessed as

TABLE 2: Summary of the overall weight-of-evidence considerations for all key event relationships[a]

KER no.	Upstream event	Relationship	Downstream event	Biological plausibility	Empirical evidence	Overall WoE	Quantitative understanding
1	Inhibition, chitin synthase 1	Directly leads to	Decrease, cuticular chitin content	High	Moderate	Moderate	Low
2	Decrease, cuticular chitin content	Directly leads to	Increase, premature molting	High	Moderate	Moderate	Low
3	Increase, premature molting	Directly leads to	Increase, mortality	High	Moderate	Moderate	Low

[a]Detailed information on the studies used for the assessment of empirical evidence can be found in Supplemental Data, Table S2.
KER = key event relationship; WoE = weight-of-evidence.

an endpoint, knockdown of CHS-1 has been shown to lead directly to the occurrence of premature molting and the associated increase of mortality (Chen et al. 2008; Mohammed et al. 2017; Wang et al. 2019). Based on these results, the essentiality of CHS-1 inhibition (MIE) was evaluated as high because knockdown of CHS-1 leads to the same outcome as chemical inhibition of the enzyme (summary in Table 1; detailed information in Supplemental Data, Table S1).

The cuticular chitin content was affected in experiments conducted with the CHS inhibitors polyoxin D and nikkomycin Z in lepidopteran insects and the crustacean *Artemia salina* (Gijswijt et al. 1979; Calcott and Fatig 1984; Gelman and Borkovec 1986; Zhuo et al. 2014). Essentiality of the decrease in cuticular chitin content was rated as high. The fact that knockdown of CHS-1 led to decreased cuticular chitin content which in turn led to premature molting–associated mortality in coleopteran, dipteran, hemipteran, and lepidopteran insects provides strong evidence for the essentiality of decreased cuticular chitin content (Arakane et al. 2005; Li et al. 2017; Zhai et al. 2017). Additional evidence for the essentiality of reduced cuticular chitin content in the AOP is provided by studies where knockdown of upstream enzymes of the chitin synthetic pathway affects the downstream events in a similar manner as knockdown of CHS-1. For example, knockdown of UDP-GlcNAc pyrophosphorylase, the enzyme that converts GlcNAc to UDP-GlcNAc, the substrate of CHS-1, led to decreased chitin content, premature molting, and increased mortality in coleopterans (Arakane et al. 2011). Knockdown of trehalase, which constitutes the start of the chitin synthetic pathway and converts trehalose to glucose, also caused decreased chitin content and premature molting–associated mortality in coleopteran and lepidopteran insects, similar to the consequences of CHS-1 knockdown (Chen et al. 2010; Shi et al. 2016; Supplemental Data, Table S1).

Essentiality of the increase in premature molting (event 1524) was rated as high (Table 1) because it is a direct consequence of decreased chitin synthesis and leads to increased mortality after knockdown of CHS-1 and upstream enzymes in the chitin biosynthetic pathway in all available studies (Supplemental Data, Table S1). Rescue studies, for example, by coinjection of CHS-1 mRNA, would strengthen the essentiality of the KE even more. In the assessment of the essentiality of KEs, no substantial knowledge gaps are apparent.

Biological plausibility of KERs

The biosynthetic pathway of chitin is well characterized, and although the exact mechanism by which CHS polymerizes to form chitin is unknown, it is well established that CHS is the critical enzyme in the biosynthesis of chitin from UDP-GlcNAc (Merzendorfer and Zimoch 2003). The arthropod cuticles mainly consist of chitin embedded into a protein matrix. Therefore, it is widely accepted that chitin contributes crucially to the integrity and function of the cuticle (Muthukrishnan et al. 2012; Reynolds 1987).

The newly secreted cuticle is subject to mechanical stress during ecdysis and hence needs to possess structural and functional integrity. The ecdysis motor program, which comprises the behavioral part of exoskeleton shedding, requires the new cuticle to possess a certain strength to transfer muscular force to the new cuticle to cast off the old cuticle (Ewer 2005; Ayali 2009). The integrity of the cuticle is important during and after ecdysis because insects and crustaceans split open the old and expand the new cuticle by increasing internal pressure with air and water to grow and provide structural stability to the new cuticle (Clarke 1957; Lee 1961; Dall and Smith 1978; deFur et al. 1985). This pressure persists until hardening and tanning (darkening) of the new exoskeleton is complete.

As a result of halted food intake and respiration during ecdysis in arthropods, starvation and suffocation can be consequences of an incomplete molting process because of immaturity of the organism at the time of molting (Camp et al. 2014; Song et al. 2017a). Another consequence of an immature cuticle is rupture of the new cuticle, loss of hemolymph, and subsequent osmotic stress due to desiccation, as well as imbalance in critical endogenous ions and malfunction of enzymes. The predominant cause of lethality associated with premature molting for affected arthropods is considered to be starvation after being unable to shed the old cuticle.

Because the underlying biological processes of the present AOP are well understood and there are no existing uncertainties or inconsistencies, confidence in biological plausibility for all KERs and the overall AOP was rated as high.

Empirical evidence of KERs

Although the essentiality of KEs and the biological plausibility of KERs of the present AOP are high, the availability of concentration (dose)–response data is very limited.

The inhibition of the CHS-1 (MIE) by polyoxin B, polyoxin D, and nikkomycin Z was assessed in several studies using cell-free systems of coleopteran, lepidopteran and dipteran insect species (Cohen 1982; Kuwano and Cohen 1984; Cohen and Casida 1990; Zhang and Zhu 2013). The inhibition of CHS activity is moderately well characterized in terms of concentration (dose)–response data. However, most studies only provide a half-maximal inhibitory concentration, rather than effects observed at different test concentrations, which limits evaluation of the dose dependence of responses.

The cuticular chitin content was decreased by polyoxin D and nikkomycin Z in lepidopteran and dipteran species as well as in the crustacean *A. salina* (Gijswijt et al. 1979; Turnbull and Howells 1982; Calcott and Fatig 1984; Gelman and Borkovec 1986; Zhuo et al. 2014). Polyoxin D also decreased the cuticular thickness in cultured integuments of *Chilo suppressalis*, which could ultimately be explained by a decreased chitin content (Nishioka et al. 1979). A single study reports in vitro effects on cuticular chitin content (event 1523) after incubation of *Ostrinia nubilalis* tissue with the fungicides captan, captafol, and folpet (Gelman and Borkovec 1986).

Increased mortality has been observed in dipterans and *Daphnia magna* following exposure to polyoxin D and nikkomycin Z, supporting the adverse outcome (Tellam et al. 2000; Tellam and Eisemann 2000; Zhu et al. 2007; Zhang and Zhu 2013; New Zealand Environmental Protection Authority 2015). Polyoxin D was also shown to induce both premature molting

and increased mortality, providing support for the KER between these KEs.

Assessment of empirical evidence is ideally based on studies that measure both KEs of a KER. In the present AOP, only propagated evidence (i.e., indirect linkage of KER between adjacent KEs) was found, where response–response relationships were assembled based on different stressor–response relationships. Hence, limitations in empirical evidence exist for all KERs. Further, premature molting is only mentioned as a potential cause of mortality in studies and not assessed specifically (Gijswijt et al. 1979; Tellam et al. 2000; Arakawa et al. 2008). Because of these limitations, it is difficult to draw a conclusion on the dose concordance between KEs. However, data from CHS-1 knockdown studies show temporal concordance for all KERs (Supplemental Data, Table S2). For example, knockdown of CHS-1 led to decreased cuticular chitin content with subsequent premature molting–associated mortality (Arakane et al. 2005; Li et al. 2017).

Overall, the empirical evidence supporting the KERs in the AOP is considered moderate. In addition, the absence of data for stressor–response and response–response relationships leaves a quantitative understanding of the overall AOP as low and as such limits the ability to develop a quantitative AOP (qAOP). A qAOP quantitatively links the KEs from MIE to adverse outcome and therefore allows predictions on the occurrence of the adverse outcome based on the occurrence of the MIE and intermediate events.

Chemical applicability domain

Several chemicals with the potential to trigger the MIE were identified (Supplemental Data, Table S3), among them pyrimidine nucleosides. Active compounds include polyoxins, several of which are produced by *Streptomyces cacaoi*, which comprise a family of 14 compounds (polyoxins A–L, N, O; Isono et al. 1969; Osada 2019). The most prominent substance from this group is polyoxin D (Figure 2), which is widely used as a fungicide in agriculture (Osada 2019). Another group of CHS inhibitors are the nikkomycins (or neopolyoxins), which share a core structure with the polyoxins (Figure 2; Dähn et al. 1976; Kobinata et al. 1980). Polyoxins structurally resemble the natural substrate UDP-GlcNAc, which allows them to act as competitive inhibitors of CHS in treated fungi (Endo et al. 1970), thus contributing to their wide use against fungal infestations (Copping and Duke 2007).

Another chemical group relevant for this AOP might be the phthalimide fungicides captan (Figure 2), captafol, and folpet. Phthalimides react with sulfhydryl groups (Lukens and Sisler 1958), which suggests that they might have a more nonspecific mode of action than polyoxins. However, captan, captafol, and folpet have been shown to specifically inhibit CHS in vitro in invertebrate systems (Cohen and Casida 1982; Gelman and Borkovec 1986). Because these chemicals have only been shown to affect chitin synthesis in vitro and no information exist on how they interact with CHS-1, evidence is still considered low.

Taxonomic applicability domain

The taxonomic applicability domain defines organisms or groups of organisms for which an AOP might be relevant. This is ideally done for each KE along the AOP. Because arthropods rely on the synthesis of chitin to molt successfully to develop, it can be assumed that the present AOP is applicable to the whole phylum of arthropods.

To strengthen this assumption, we focused on the MIE and assessed cross-species structural and, by inference, functional conservation of CHSs. Comparative analysis of the amino acid sequence of CHS in different taxa was determined using the Sequence Alignment to Predict Across Species Susceptibility tool (SeqAPASS [LaLone et al. 2016]), which employs the extensive National Center for Biotechnology Information protein sequence database to identify protein targets with similar structures in different species. The SeqAPASS tool runs sequence alignments on 3 different levels. In level 1, entire protein sequences are aligned; in level 2, conserved domains of a protein sequence are aligned; and level 3 performs alignment of amino acid residues which are involved in the binding of a specific stressor or ligand. The SeqAPASS tool was run using *Lucilia cuprina* CHS-1 as a query sequence because it was derived from experimentally determined gene sequences and the species is known to be susceptible to polyoxins (Turnbull and Howells 1982; Tellam et al. 2000; Tellam and Eisemann 2000). A more detailed description of the approach can be found in the Supplemental Data.

Alignment of the primary (entire) CHS-1 sequences (level 1; Supplemental Data, Table S4 and Figure S2A) as well as the C-terminal domain that contains the catalytic site which incorporates UDP-GlcNAc into chitin (conserved domain database: cd04190; level 2; Supplemental Data, Table S5 and Figure S2B) suggests high conservation among arthropod

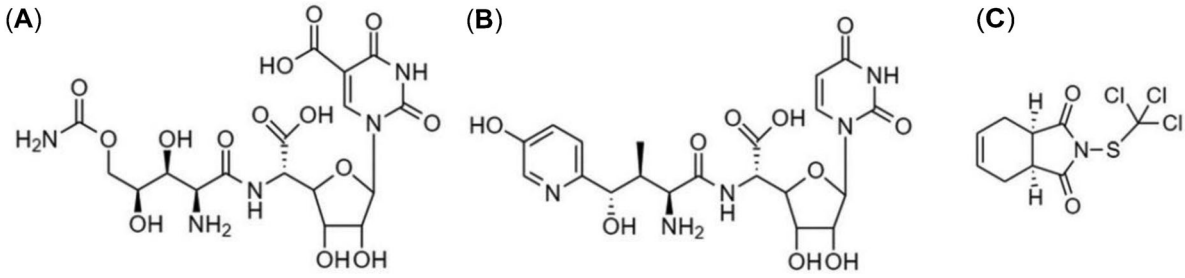

FIGURE 2: Molecular structure of different chemicals known to trigger the molecular initiating event of the present adverse outcome pathway: (**A**) polyoxin D, (**B**) nikkomycin Z, and (**C**) the phthalimide pesticide captan.

species. Alignment of amino acid residues of specific protein motifs was conducted on the basis of protein motifs believed to be involved in the catalysis of the polymerization reaction and the translocation of chitin (Kamst and Spaink 1999; Merzendorfer 2006). The amino acid alignment confirmed a high degree of structural and potential functional conservation between species (Supplemental Data, Table S6).

Moreover, experimental effect data for apical endpoints, which may support or expand the list of susceptible taxa and species, should also be considered. Effect data, although limited, exist for lepidopteran, dipteran, and coleopteran insect species as well as for crustaceans, confirming the taxonomic applicability of the AOP predicted in the sequence alignment.

Life stage and sex applicability domain

In terms of life stage applicability, it is known that insects, except mayflies, do not undergo molting in their adulthood (Maiorana 1979). Therefore, insects most likely affected by stressors relevant for this AOP are in larval, nymph, or pupal stages of their development. This is also supported by toxicity data, where all insects affected in experiments with chemicals relevant to the MIE were in their larval stages (Tellam et al. 2000; Tellam and Eisemann 2000). In contrast to insects, crustaceans and arachnids molt throughout their lifetime to grow and reproduce (Passano 1961; Uhl et al. 2015). Consequently, it is suggested that the present AOP is applicable to all life stages of crustaceans and arachnids. However, juveniles might be more susceptible than adults because of a faster growth rate and increased molting frequency (West and Costlow 1987). In terms of sex applicability, the AOP is considered relevant for all sexes.

APPLICATIONS OF THE AOP

Arthropods are indispensable for healthy ecosystems (Seastedt and Crossley 1984). Insects, for example, contribute to pollination and pest control and serve as food for other wildlife (Losey and Vaughan 2006), whereas different crustacean taxa play diverse critical roles in aquatic ecosystems such as critically contributing to the food web and the breakdown of organic matter (LeBlanc 2007; Szaniawska 2018). Nontarget arthropods, such as honeybees and aquatic crustaceans, may be affected when exposed to chitin synthesis inhibitors such as certain pesticides and veterinary medicines, thus supporting the need to develop AOPs that can increase the knowledge and assist developing pragmatic approaches to safeguard susceptible species. Because knowledge of chemicals directly inhibiting CHS-1 is limited, it is important to expand the knowledge on how and to what extent chemicals interact with this enzyme to characterize the environmental challenges they pose and support hazard assessment.

The present AOP can be used to guide the development of computational and in vitro methods for screening of chemicals, including the chemical inventories of the European Chemicals Agency (2008), ToxCast (US Environmental Protection Agency 2020), the NORMAN (2021) network, and IPCHEM (European Commission 2020) to identify and prioritize CHS inhibitors for more in-depth testing. Such methods may involve in silico approaches such as structural alerts, quantitative structure–activity relationships, or molecular docking methods (Evenseth et al. 2019; Mellor et al. 2020) and high-throughput screening using transfected cells or cellular extracts (Lucero

TEXTBOX 2: List of assays associated with the adverse outcome pathway

- Inhibition, chitin synthase 1 (event 1522): Inhibition of chitin synthase is measured with a chitin synthase activity assay using a crude enzyme preparation, similar to a sandwich enzyme-linked immunosorbent assay. The wells of a 96-well plate are coated with wheat germ agglutinin (WGA) and subsequently incubated with substrate, enzyme preparation, and inhibitors. Colorimetric quantification of synthesized chitin occurs through conversion of horseradish peroxidase substrate conjugated to WGA (Lucero et al. 2002; Zhang and Zhu 2013). An alternative is by incorporation of radioactively labeled precursors into chitin over time (Archer 1977; Zimoch et al. 2005).
- Decrease, cuticular chitin content (event 1523): To determine the decrease in cuticular chitin content, the chitin content of the respective model organism is measured. After homogenization of organisms, chitin is hydrolyzed to *N*-acetylglucosamine (GlcNAc) by addition of chitinase. After hydrolysis, GlcNAc concentrations are determined by a modified Morgan-Elson assay (Reissig et al. 1955; Arakane et al. 2005). Other possibilities exist for the determination of chitin, including direct quantification using calcofluor white, a fluorescent dye, binding to chitin (Henriques et al. 2020) or quantifying glucosamine after deacetylation and depolymerization of chitin (Lehmann and White 1975; Zhang and Zhu 2006).
- Increase, premature molting (event 1524): Premature molting can be assessed and quantified through observation of the molting frequency, ideally over several molt cycles. This endpoint is preferably assessed in the same experiment as the adverse outcome. Histopathological endpoints where the thickness of the newly synthesized cuticle is assessed could support observed changes in molting.
- Increase, mortality (event 350): The increase in mortality can be quantitatively assessed using standard Organisation for Economic Co-operation and Development (OECD) toxicity testing protocols for arthropods, for example, the OECD 202 *Daphnia* sp. acute immobilization test (Organisation for Economic Co-operation and Development 2004) or the OECD 235 *Chironomus* sp. acute immobilization test (Organisation for Economic Co-operation and Development 2011).

et al. 2002; Chan et al. 2019) for species within the taxonomic applicability domain.

The present AOP also provides a basis for the development and identification of assays and tests which can be used to quantify the KEs (Textbox 2). For example, there exists no standardized test for the endpoint of molting, and it has been proposed to incorporate this into the *Daphnia* sp. reproduction test (Organisation for Economic Co-operation and Development 2003). The present AOP will potentially assess the necessity of such a test (e.g., by screening chemical inventories), identify appropriate testing protocols (e.g., by KE, taxonomical, and gender specifications), and propose relevant chemicals to test (e.g., prioritization) to assist future hazard assessment of chemicals that interfere with the molting process. Such a tiered testing workflow aligns well with regulatory mandates that encourage the use of AOP-related data to support testing for specific endpoints (European Chemicals Agency 2017). Development of such AOP-informed testing approaches would in the longer perspective also support developing quantitative stressor–response and response–response relationships (e.g., qAOP development) and support risk assessment. This would ultimately also facilitate the protection of susceptible and endangered species where toxicity tests cannot be performed.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5058.

Acknowledgment—We thank the reviewer team (G. Ankley, M. Houde, and H. Poynton) for a very thorough review and numerous helpful suggestions on the manuscript. This project has received funding from the European Union's Horizon 2020 research and innovation program under Marie Skłodowska-Curie grant 859891 and was supported by the Norwegian Institute for Water Research's Computational Toxicology Program (www.niva.no/nctp).

AOP Editors and Reviewers—D. Knapen was the handling editor on this AOP Pathways and Predictions. H. Poynton, G. Ankley, and M. Houde reviewed the submission and supplied feedback.

Disclaimer—The authors declare no competing financial interest.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (ket@niva.no). All review reports can be accessed at https://aopwiki.org/aops/360/comments. The snapshot pdf can be accessed at https://aopwiki.org/aopwiki/snapshot/pdf_file/360-2021-02-26T08:13:55+00:00.pdf.

REFERENCES

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL. 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem* 29:730–741.

Antoniewski C, Laval M, Lepesant JA. 1993. Structural features critical to the activity of an ecdysone receptor binding site. *Insect Biochem Mol Biol* 23:105–114.

Arakane Y, Baguinon MC, Jasrapuria S, Chaudhari S, Doyungan A, Kramer KJ, Muthukrishnan S, Beeman RW. 2011. Both UDP *N*-acetylglucosamine pyrophosphorylases of *Tribolium castaneum* are critical for molting, survival and fecundity. *Insect Biochem Mol Biol* 41:42–50.

Arakane Y, Muthukrishnan S, Kramer KJ, Specht CA, Tomoyasu Y, Lorenzen MD, Kanost M, Beeman RW. 2005. The *Tribolium* chitin synthase genes TcCHS1 and TcCHS2 are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix. *Insect Mol Biol* 14:453–463.

Arakawa T, Yukuhiro F, Noda H. 2008. Insecticidal effect of a fungicide containing polyoxin B on the larvae of *Bombyx mori* (Lepidoptera: Bombycidae), *Mamestra brassicae*, *Mythimna separata*, and *Spodoptera litura* (Lepidoptera: Noctuidae). *Appl Entomol Zool* 43:173–181.

Archer DB. 1977. Chitin biosynthesis in protoplasts and subcellular fractions of *Aspergillus fumigatus*. *Biochem J* 164:653–658.

Ayali A. 2009. The role of the arthropod stomatogastric nervous system in moulting behaviour and ecdysis. *J Exp Biol* 212:453–459.

Bar-On YM, Phillips R, Milo R. 2018. The biomass distribution on Earth. *Proc Natl Acad Sci USA* 115:6506–6511.

Becker RA, Ankley GT, Edwards SW, Kennedy SW, Linkov I, Meek B, Sachana M, Segner H, Van Der Burg B, Villeneuve DL, Watanabe H, Barton-Maclaren TS. 2015. Increasing scientific confidence in adverse outcome pathways: Application of tailored Bradford-Hill considerations for evaluating weight of evidence. *Regul Toxicol Pharmacol* 72:514–537.

Binnington KC. 1985. Ultrastructural changes in the cuticle of the sheep blowfly, *Lucilia*, induced by certain insecticides and biological inhibitors. *Tissue Cell* 17:131–140.

Calcott PH, Fatig RO. 1984. Inhibition of chitin metabolism by avermectin in susceptible organisms. *J Antibiot (Tokyo)* 37:253–259.

Camp AA, Funk DH, Buchwalter DB. 2014. A stressful shortness of breath: Molting disrupts breathing in the mayfly *Cloeon dipterum*. *Freshw Sci* 33:695–699.

Chan YH, Chu KH, Chan KM. 2019. Ecdysteroid-mimicking compounds act as both agonists and antagonists to the crustacean ecdysone receptor. *Chemosphere* 237:124551.

Chen J, Tang B, Chen H, Yao Q, Huang X, Chen J, Zhang D, Zhang W. 2010. Different functions of the insect soluble and membrane-bound trehalase genes in chitin biosynthesis revealed by RNA interference. *PLoS One* 5:e0010133.

Chen X, Tian H, Zou L, Tang B, Hu J, Zhang W. 2008. Disruption of *Spodoptera exigua* larval development by silencing chitin synthase gene A with RNA interference. *Bull Entomol Res* 98:613–619.

Clarke KU. 1957. On the increase in linear size during growth in *Locusta migratoriaProceedings of the Royal Entomological Society of London Series A General Entomology* L. 32:35–39.

Cohen E. 2001. Chitin synthesis and inhibition: A revisit. *Pest Manag Sci* 57:946–950.

Cohen E. 1982. In vitro chitin synthesis in an insect: Formation and structure of microfibrils. *Eur J Cell Biol* 26:289–294.

Cohen E, Casida JE. 1990. Insect and fungal chitin synthetase activity: Specificity of lectins as enhancers and nucleoside peptides as inhibitors. *Pestic Biochem Physiol* 37:249–253.

Cohen E, Casida JE. 1982. Properties and inhibition of insect integumental chitin synthetase. *Pestic Biochem Physiol* 17:301–306.

Copping LG, Duke SO. 2007. Natural products that have been used commercially as crop protection agents. *Pest Manag Sci* 63:524–554.

Cribb BW, Rathmell A, Charters R, Rasch R, Huang H, Tibbetts IR. 2009. Structure, composition and properties of naturally occurring non-calcified crustacean cuticle. *Arthropod Struct Dev* 38:173–178.

Dähn U, Hagenmaier H, Höhne H, König WA, Wolf G, Zähner H, Dahn U, Hagenmaier H, Hohne H, Konig WA, Deutschland B. 1976. Stoffwechselprodukte von Mikroorganismen—154. Mitteilung. Nikkomycin, ein neuer Hemmstoff der Chitinsynthese bei Pilzen. *Arch Microbiol* 160:143–160.

Dall W, Smith DM. 1978. Water uptake at ecdysis in the western rock lobster. *J Exp Mar Biol Ecol* 35:165–176.

deFur PL, Mangum CP, McMahon BR. 1985. Cardiovascular and ventilatory changes during ecdysis in the blue crab *Callinectes sapidus* Rathbun. *J Crustac Biol* 5:207–215.

Endo A, Kakiki K, Misato T. 1970. Mechanism of action of the antifungal agent polyoxin D. *J Bacteriol* 104:189–196.

European Chemicals Agency. 2017. Guidance on information requirements and chemical safety assessment. Chapter R.7a: Endpoint specific guidance. Ver 6.0., Helsinki, Finland.

European Chemicals Agency. 2008. EC inventory. [cited 2021 February 22]. Available from: https://echa.europa.eu/de/information-on-chemicals/ec-inventory

European Commission. 2020. Information platform for chemical monitoring. [cited 2021 February 22]. Available from: https://ipchem.jrc.ec.europa.eu/#discovery

Evenseth LM, Kristiansen K, Song Y, Tollefsen KE, Sylte I. 2019. In silico site-directed mutagenesis of the *Daphnia magna* ecdysone receptor identifies critical amino acids for species-specific and inter-species differences in agonist binding. *Comput Toxicol* 12:100091.

Ewer J. 2005. How the ecdysozoan changed its coat. *PLoS Biol* 3:1696–1699.

Gagou ME, Kapsetaki M, Turberg A, Kafetzopoulos D. 2002. Stage-specific expression of the chitin synthase DmeChSA and DMeChSB genes during the onset of *Drosophila* metamorphosis. *Insect Biochem Mol Biol* 32:141–146

Gelman DB, Borkovec AB. 1986. The pharate adult clasper as a tool for measuring chitin synthesis and for identifying new chitin synthesis inhibitors. *Comp Biochem Physiol C Comp Pharmacol* 85:193–197.

Gijswijt MJ, Deul DH, de Jong BJ. 1979. Inhibition of chitin synthesis by benzoyl-phenylurea insecticides, III. Similarity in action in *Pieris brassicae* (L.) with polyoxin D. *Pestic Biochem Physiol* 12:87–94.

Harðardóttir HM, Male R, Nilsen F, Eichner C, Dondrup M, Dalvin S. 2019. Chitin synthesis and degradation in *Lepeophtheirus salmonis*: Molecular characterization and gene expression profile during synthesis of a new exoskeleton. *Comp Biochem Physiol A Mol Integr Physiol* 227:123–133.

Heming BS. 2018. *Insect Development and Evolution*. Cornell University Press, Ithaca, NY, USA.

Henriques BS, Garcia ES, Azambuja P, Genta FA. 2020. Determination of chitin content in insects: An alternate method based on calcofluor staining. *Front Physiol* 11:1–10.

Isono K, Asahi K, Suzuki S. 1969. Studies on polyoxins, antifungal antibiotics. XIII. The structure of polyoxins. *J Am Chem Soc* 91:7490–7505.

Junquera P, Hosking B, Gameiro M, MacDonald A. 2019. Benzoylphenyl ureas as veterinary antiparasitics. An overview and outlook with emphasis on efficacy, usage and resistance. *Parasite* 26:26.

Kamst E, Spaink PH. 1999. Functional domains in the chitin oligosaccharide synthase NodC and related β-polysaccharide synthases. *Trends Glycosci Glycotechnol* 11:187–199.

Kobinata K, Uramoto M, Nishii M, Kusakabe H, Nakamura G, Isono K. 1980. Neopolyoxins A, B, and C, new chitin synthetase inhibitors. *Agric Biol Chem* 44:1709–1711.

Kuwano E, Cohen E. 1984. The use of a *Tribolium* chitin synthetase assay in studying the effects of benzimidazoles with a terpene moiety and related compounds. *Agric Biol Chem* 48:1617–1620.

LaLone CA, Villeneuve DL, Lyons D, Helgen HW, Robinson SL, Swintek JA, Saari TW, Ankley GT. 2016. Sequence alignment to predict across species susceptibility (SeqAPASS): A web-based tool for addressing the challenges of cross-species extrapolation of chemical toxicity. *Toxicol Sci* 153:228–245.

LeBlanc GA. 2007. Crustacean endocrine toxicology: A review. *Ecotoxicology* 16:61–81.

Lee RM. 1961. The variation of blood volume with age in the desert locust (*Schistocerca gregaria* Forsk.). *J Insect Physiol* 6:36–51.

Lehmann PF, White LO. 1975. Chitin assay used to demonstrate renal localization and cortisone-enhanced growth of *Aspergillus fumigatus* mycelium in mice. *Infect Immun* 12:987–992.

Li T, Chen J, Fan X, Chen W, Zhang W. 2017. MicroRNA and dsRNA targeting chitin synthase A reveal a great potential for pest management of the hemipteran insect *Nilaparvata lugens*. *Pest Manag Sci* 73:1529–1537.

Locke M, Huie P. 1979. Apolysis and the turnover of plasmamembrane plaques during cuticle formation in an insect. *Tissue Cell* 11:277–291.

Losey JE, Vaughan M. 2006. The economic value of ecological services provided by insects. *Bioscience* 56:311–323.

Lucero HA, Kuranda MJ, Bulik DA. 2002. A nonradioactive, high throughput assay for chitin synthase activity. *Anal Biochem* 305:97–105.

Lukens RJ, Sisler HD. 1958. 2-Thiazolidinethione-4-carboxylic acid from the reaction of captan with cysteine. *Science* 127:650.

Maiorana VC. 1979. Why do adult insects not moult? *Biol J Linn Soc* 11:253–258.

Mellor CL, Tollefsen KE, LaLone C, Cronin MTD, Firman JW. 2020. In silico identification of chemicals capable of binding to the ecdysone receptor. *Environ Toxicol Chem* 39:1438–1450.

Merzendorfer H. 2013. Chitin synthesis inhibitors: Old molecules and new developments. *Insect Sci* 20:121–138.

Merzendorfer H. 2006. Insect chitin synthases: A review. *J Comp Physiol B* 176:1–15.

Merzendorfer H. 2011. The cellular basis of chitin synthesis in fungi and insects: Common principles and differences. *Eur J Cell Biol* 90:759–769.

Merzendorfer H, Kim HS, Chaudhari SS, Kumari M, Specht CA, Butcher S, Brown SJ, Robert Manak J, Beeman RW, Kramer KJ, Muthukrishnan S. 2012. Genomic and proteomic studies on the effects of the insect growth regulator diflubenzuron in the model beetle species *Tribolium castaneum*. *Insect Biochem Mol Biol* 42:264–276.

Merzendorfer H, Zimoch L. 2003. Chitin metabolism in insects: Structure, function and regulation of chitin synthases and chitinases. *J Exp Biol* 206:4393–4412.

Mohammed AMA, Diab MR, Abdelsattar M, Khalil SMS. 2017. Characterization and RNAi-mediated knockdown of chitin synthase A in the potato tuber moth, *Phthorimaea operculella*. *Sci Rep* 7:1–12.

Muthukrishnan S, Merzendorfer H, Arakane Y, Kramer KJ. 2012. Chitin metabolism in insects. In *Insect Molecular Biology and Biochemistry*. Elsevier, Amsterdam, The Netherlands, pp 193–235.

Nagasawa H. 2012. The crustacean cuticle: Structure, composition and mineralization. *Front Biosci (Elite Ed)* 4:711–720.

Neville AC. 1975. *Biology of the Arthropod Cuticle*. Springer, New York, NY, USA.

New Zealand Environmental Protection Authority. 2015. Application for approval to import ESTEEM for release. APP202334. Wellington, New Zealand.

Nishioka T, Fujita T, Nakajima M. 1979. Effect of chitin synthesis inhibitors on cuticle formation of the cultured integument of *Chilo suppressalis*. *J Pestic Sci* 4:367–374.

NORMAN. 2021. NORMAN Substance Database—NORMAN SusDat. [cited 2021 February 22]. Available from: https://www.norman-network.com/nds/susdat/susdatSearchShow.php

Organisation for Economic Co-operation and Development. 2011. Test No. 235: *ChironomusOECD Guidelines for the Testing of Chemicals* sp., acute immobilisation test. Paris, France.

Organisation for Economic Co-operation and Development. 2004. Test No. 202: *Daphnia spOECD Guidelines for the Testing of Chemicals*. acute immobilisation test. Paris, France.

Organisation for Economic Co-operation and Development. 2003. Proposal for an enhanced test guideline. *Daphnia magna* reproduction test. Draft 211 21. Paris, France.

Organisation for Economic Co-operation and Development. 2018. Users' handbook supplement to the guidance document for developing and assessing adverse outcome pathways. Paris, France.

Osada H. 2019. Discovery and applications of nucleoside antibiotics beyond polyoxin. *J Antibiot (Tokyo)* 72:855–864.

Passano LM. 1961. The regulation of crustacean metamorphosis. *Integr Comp Biol* 1:89–95.

Qian Z, He S, Liu T, Liu Y, Hou F, Liu Q, Wang X, Mi X, Wang P, Liu X. 2014. Identification of ecdysteroid signaling late-response genes from different tissues of the Pacific white shrimp, *Litopenaeus vannamei*. *Comp Biochem Physiol A Mol Integr Physiol* 172:10–30.

Reissig JL, Strominger JL, Leloir LF. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. *J Biol Chem* 217:959–966.

Reynolds SE. 1987. The cuticle, growth and moulting in insects: The essential background to the action of acylurea insecticides. *Pestic Sci* 20:131–146.

Rocha J, Garcia-Carreño FL, Muhlia-Almazán A, Peregrino-Uriarte AB, Yépiz-Plascencia G, Córdova-Murueta JH. 2012. Cuticular chitin synthase and chitinase mRNA of whiteleg shrimp *Litopenaeus vannamei* during the molting cycle. *Aquaculture* 330–333:111–115.

Seastedt TR, Crossley DA. 1984. Influence of on arthropods ecosystems. *Bioscience* 34:157–161.

Shi JF, Xu QY, Sun QK, Meng QW, Mu LL, Guo WC, Li GQ. 2016. Physiological roles of trehalose in *Leptinotarsa* larvae revealed by RNA interference of trehalose-6-phosphate synthase and trehalase genes. *Insect Biochem Mol Biol* 77:52–68.

Society for the Advancement of Adverse Outcome Pathways. 2016. Welcome to the Collaborative Adverse Outcome Pathway Wiki (AOP-Wiki). [cited 2021 February 18]. Available from: https://aopwiki.org/aops/360

Song Y, Evenseth LM, Iguchi T, Tollefsen KE. 2017a. Release of chitobiase as an indicator of potential molting disruption in juvenile *Daphnia magna* exposed to the ecdysone receptor agonist 20-hydroxyecdysone. *J Toxicol Environ Health A* 80:954–962.

Song Y, Villeneuve DL, Toyota K, Iguchi T, Tollefsen KE. 2017b. Ecdysone receptor agonism leading to lethal molting disruption in arthropods: Review and adverse outcome pathway development. *Environ Sci Technol* 51:4142–4157.

Szaniawska A. 2018. *Baltic Crustaceans*. Springer, Cham, Switzerland.

Tellam RL, Eisemann C. 2000. Chitin is only a minor component of the peritrophic matrix from larvae of *Lucilia cuprina*. *Insect Biochem Mol Biol* 30:1189–1201.

Tellam RL, Vuocolo T, Johnson SE, Jarmey J, Pearson RD. 2000. Insect chitin synthase. cDNA sequence, gene organization and expression. *Eur J Biochem* 267:6025–6043.

Turnbull IF, Howells AJ. 1982. Effects of several larvicidal compounds on chitin biosynthesis by isolated larval integuments of the sheep blowfly *Lucilia cuprina*. *Aust J Biol Sci* 35:491–504.

Uddowla MH, Kim AR, Park W-g, Kim H-W. 2014. cDNAs encoding chitin synthase from shrimp (*Pandalopsis japonica*): Molecular characterization and expression analysis. *J Aquac Res Dev* 6:1000298.

Uhl G, Zimmer SM, Renner D, Schneider JM. 2015. Exploiting a moment of weakness: Male spiders escape sexual cannibalism by copulating with moulting females. *Sci Rep* 5:16928.

US Environmental Protection Agency. 2020. TOXCAST: EPA ToxCast Screening Library. [cited 2021 February 22]. Available from: https://comptox.epa.gov/dashboard/chemical_lists/toxcast

Vincent JFV, Wegst UGK. 2004. Design and mechanical properties of insect cuticle. *Arthropod Struct Dev* 33:187–199.

Wang Z, Yang H, Zhou C, Yang WJ, Jin DC, Long GY. 2019. Molecular cloning, expression, and functional analysis of the chitin synthase 1 gene and its two alternative splicing variants in the white-backed planthopper, *Sogatella furcifera* (Hemiptera: Delphacidae). *Sci Rep* 9:1087.

West TL, Costlow JD. 1987. Size regulation in larvae of the crustacean *Balanus eburneus* (Cirripedia: Thoracica). *Mar Biol* 96:47–58.

Zhai Y, Fan X, Yin Z, Yue X, Men X, Zheng L, Zhang W. 2017. Identification and functional analysis of chitin synthase A in oriental armyworm, *Mythimna separata*. *Proteomics* 17:1700165.

Zhang J, Zhu KY. 2006. Characterization of a chitin synthase cDNA and its increased mRNA level associated with decreased chitin synthesis in *Anopheles quadrimaculatus* exposed to diflubenzuron. *Insect Biochem Mol Biol* 36:712–725.

Zhang X, Zhu KY. 2013. Biochemical characterization of chitin synthase activity and inhibition in the African malaria mosquito, *Anopheles gambiae*. *Insect Sci* 20:158–166.

Zhang X, Zhang J, Zhu KY. 2010. Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). *Insect Mol Biol* 19:683–693.

Zhu KY, Heise S, Zhang J, Anderson TD, Starkey SR. 2007. Comparative studies on effects of three chitin synthesis inhibitors on common malaria mosquito (Diptera: Culicidae). *J Med Entomol* 44:1047–1053.

Zhuo W, Fang Y, Kong L, Li X, Sima Y, Xu S. 2014. Chitin synthase A: A novel epidermal development regulation gene in the larvae of *Bombyx mori*. *Mol Biol Rep* 41:4177–4186.

Zimoch L, Hogenkamp DG, Kramer KJ, Muthukrishnan S, Merzendorfer H. 2005. Regulation of chitin synthesis in the larval midgut of *Manduca sexta*. *Insect Biochem Mol Biol* 35:515–527.

