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

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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)
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).

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 pathways and has 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. Envir 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

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
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.

**Essentiality of KEs**

One way to discern essentiality is through use of targeted chemical inhibitors. Chemicals such as the antifungal antibiotics polyoxin D and nikkormycin 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 mRNA 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

![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 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.](image-url)

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**TABLE 1: Summary of the assessment of essentiality for key events**

| 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 | —     | —   |

*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.

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

| 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 |

*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 caecaI, 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; Kobilina 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
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 Spink 1999; Merender 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 tests and 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.

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**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/aop/360/360/comments. The snapshot.pdf can be accessed at https://aopwiki.org/aopwiki/snapshot/pdf_file/360-2021-02-26T08:13:55+00:00.pdf.

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