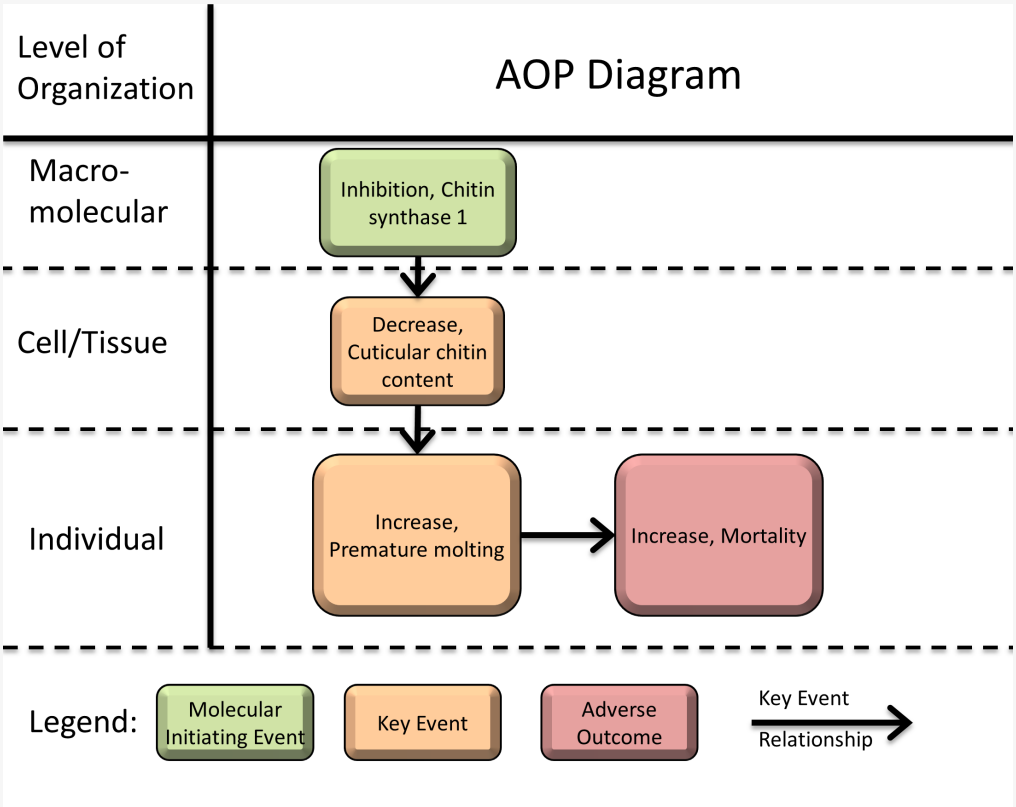
AOP ID and Title:

AOP 360: Chitin synthase 1 inhibition leading to mortality
Short Title: CHS-1 inhibition leading to mortality

Graphical Representation



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Status

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Abstract

In order to grow and develop, arthropods need to shed their exoskeleton (or cuticle) periodically and replace it with a new one in a process called molting. Successful molting, and therefore a successful development necessitates stability and integrity of the cuticle to support muscular contractions involved in the shedding of the old cuticle. The integrity of the cuticle is largely dependent on the *N*-acetylglucosamine (GlcNAc) polymer chitin. Therefore, arthropods heavily rely on chitin synthesis as chitin is one of the main constituents of the cuticle. The cuticular chitin synthase (CHS-1) is the key enzyme in the biosynthetic pathway and arthropods are therefore especially dependent on its proper function. The present AOP describes the effects of chemical inhibition of the cuticular chitin synthase (CHS-1) on the molting process leading to increased mortality in arthropods. Inhibition of CHS-1 is the molecular initiating event and leads to a decreased chitin content in the arthropod

cuticle which leaves the organism immature at the stage for ecdysis. This phenomenon can be described as premature molting. The organism eventually dies due to being stuck in the old cuticle or due to the consequences of a weak exoskeleton after ecdysis. The AOP is considered to be very consistent. Essentiality of key events was rated as high for every key event and the biological plausibility was rated as high for the whole AOP. However, there does not exist very much empirical evidence that allows to draw a representative conclusion on dose concordance along the AOP whereas time concordance can be supported by knockdown studies of CHS-1. Therefore, empirical evidence was considered to be moderate and the quantitative understanding was considered to be low. The overall confidence in the AOP was valued as moderate. The present AOP will guide assay development for further experimental studies by revealing data and knowledge gaps. One of its primary applications will also be providing guidance in screening strategies in order to identify chemicals directly interacting with CHS-1.

Background

Arthropods (including insects, crustaceans and arachnids) need to shed their exoskeleton in order to grow and reproduce. This process, also called molting or ecdysis, is mediated by behavioural mechanisms which involve the skeletal muscles (Ayali 2009; Song et al. 2017a). In order to properly shed its cuticle, the organism needs to possess a newly synthesized cuticle that possesses a certain integrity to support this process. Since chitin is a major constituent of the cuticle, it contributes substantially to its integrity (Cohen 2001; Vincent and Wegst 2004). Chitin is synthesized from uridine diphosphate-*N*-Acetylglucosamine (UDP-GlcNAc) in a polymerization reaction by the transmembrane enzyme chitin synthase isoform 1 (CHS-1). CHS-1 is localized on the apical side in the cuticular epithelium.
Since chitin and the process of chitin synthesis does not occur in vertebrates, it can and has been exploited for the design of pest controlling agents. Inhibitors of chitin synthesis may not only be of use for the control of unwanted arthropods and fungi, they may also pose a risk for beneficial arthropods such as insects and crustaceans. Disruption of chitin synthesis or the endocrine mechanisms controlling molting generally lead to a disruption of ecdysis (Merzendorfer et al. 2012; Song et al. 2017a; Song et al. 2017b). If the amount of chitin in the cuticle decreases, the affected organism may not be able to molt properly and will most probably die of starvation or suffocation (Camp et al. 2014; Song et al. 2017a). Alternatively, if molting is completed despite an immature cuticle, the organism may be deformed and die as a consequence of a weak cuticle.
Therefore, the present AOP should build the basis of a mechanistic approach for the systematic evaluation and the risk assessment of chemicals interfering with chitin synthesis by directly inhibiting CHS-1.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1522	Inhibition, Chitin synthase 1	Inhibition, CHS-1
2	KE	1523	Decrease, Cuticular chitin content	Decrease, Cuticular chitin content
3	KE	1524	Increase, Premature molting	Increase, Premature molting
4	AO	350	Increase, Mortality	Increase, Mortality

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Chitin synthase 1	adjacent	Decrease, Cuticular chitin content	Moderate	Low
Decrease, Cuticular chitin content	adjacent	Increase, Premature molting	Moderate	Low
Increase, Premature molting	adjacent	Increase, Mortality	Moderate	Low

Stressors

Name	Evidence
Polyoxin B	High
Polyoxin D	High
Nikkomycins	High
Captan	Moderate

Name	Evidence
Captafol	Moderate
Folpet	Moderate

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
larvae	High
Juvenile	High
Adult	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Pieris brassicae	Pieris brassicae	High	NCBI
Anopheles gambiae	Anopheles gambiae	High	NCBI
Lucilia cuprina	Lucilia cuprina	High	NCBI
Tribolium castaneum	Tribolium castaneum	High	NCBI
Bombyx mori	Bombyx mori	High	NCBI
Anopheles quadrimaculatus	Anopheles quadrimaculatus	High	NCBI
Trichoplusia ni	Trichoplusia ni	High	NCBI
Artemia salina	Artemia salina	High	NCBI
Daphnia magna	Daphnia magna	High	NCBI
Hyalophora cecropia	Hyalophora cecropia	High	NCBI
Ostrinia nubilalis	Ostrinia nubilalis	High	NCBI
Bradysia hygida	Bradysia hygida	Moderate	NCBI
Mamestra brassicae	Mamestra brassicae	Moderate	NCBI
Chilo suppressalis	Chilo suppressalis	Moderate	NCBI
Locusta migratoria	Locusta migratoria	Moderate	NCBI
Nilaparvata lugens	Nilaparvata lugens	Moderate	NCBI
Aphis glycines	Aphis glycines	Moderate	NCBI
Lepeophtheirus salmonis	Lepeophtheirus salmonis	Moderate	NCBI
Panonychus citri	Panonychus citri	Moderate	NCBI
Grapholita molesta	Grapholita molesta	Moderate	NCBI
Ectropis obliqua	Ectropis obliqua	Moderate	NCBI
Tigriopus japonicus	Tigriopus japonicus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Since the whole phylum of arthropods is dependent on the synthesis of chitin to molt successfully, it is extremely likely that the AOP is applicable to all arthropods. Effect data along the AOP exist from Dipteran, Lepidopteran and Coleopteran insect species as well as from Branchiopods and Anostracans of the crustacea. Although data is limited, KEs seem to be well conserved across taxa, as shown in available studies with specific stressors known to inhibit CHS and in studies where CHS-1 was knocked down by RNA interference. However, due to limited data availability, it was not possible to cover whole taxa but rather single species in the assessment of KEs. Alignment of amino acid residues in the catalytic center of CHS-1 using the Sequence Alignment to Predict Across Species Susceptibility tool (SeqAPASS, https://seqapass.epa.gov/seqapass, LaLone et al. 2016), confirmed structural and functional conservation in various insect, arachnid and crustacean species, strenghtening the evidence for the applicability domain to be the whole phylum of arthropods. However, taxonomic applicability may not only be defined by structural conservation of the protein sequence. So the evidence for the taxonomic applicability for

species with support only from sequence alignment was judged as moderate, whereas evidence for species with support from sequence alignment and effect data was judged as high.

Life stage: The AOP is applicable for organisms undergoing continuous molt cycles. As insects do not molt in their adulthood, the AOP is only applicable for larval and pupal stages of insects. Crustaceans and arachnids grow and molt throughout their lifetime (Passano 1961; Uhl et al. 2015), which makes the AOP applicable to all life stages, where juvenile life stages might be more susceptible to chemical perturbations due to higher growth rate and therefore more frequent molting.

Sex: The AOP is applicable to all sexes.

Chemical: Substances known to trigger the MIE and leading to the AO are of the family of pyrimidine nucleosides (e.g. polyoxin D, polyoxin B and nikkomycin Z) (Osada 2019). There also exists evidence for phthalimides (captan, captafol and folpet) to inhibit CHS-1 activity and to decrease the cuticular chitin content *in vitro* (Cohen and Casida 1982; Gelman and Borkovec 1986). However, as these substances are known to covalently bind to thiol groups in proteins (Lukens and Sisler 1958), it is not clear if the inhibition is due to specific CHS-1 inhibition or due to unspecific protein binding.

Essentiality of the Key Events

The essentiality of all key events was considered as high. Essentiality evaluations were mainly based on specifically designed studies demonstrating the expected effect pattern predicted by the AOP to occur after knockdown of CHS-1.

Inhibition, Chitin synthase 1 (High): Knockdown of the cuticular chitin synthase leads to the expected pattern of effects described in this AOP. It decreases the cuticular chitin content and leads to premature molting associated mortality in insects (Arakane et al. 2005; X. Zhang et al. 2010; Li et al. 2017; Zhai et al. 2017)**.** If the cuticular chitin content was not directly measured as endpoint, knockdown of the CHS-1 led directly to the occurrence of premature molting associated increase of mortality (Chen et al. 2008; X. Zhang et al. 2010; Wang et al. 2012; Yang et al. 2013; Shang et al. 2016; Mohammed et al. 2017; Wang et al. 2019; Ye et al. 2019; Ullah et al. 2020)

Decrease, Cuticular chitin content (High): Abolishment of the cuticular chitin synthesis through knockdown of CHS-1 leads to premature molting associated mortality (Arakane et al. 2005; X. Zhang et al. 2010; Li et al. 2017; Zhai et al. 2017). By knocking down the UDP-GlcNAc pyrophosphorylase (UAP), which catalyzes the last sugar conversion before the polymerization to chitin, it was shown that reduced chitin content leads to the same outcome as the knockdown of CHS-1. Namely premature molting and increased mortality (Arakane et al. 2011; Liu et al. 2013). Knockdown of trehalase genes, which constitutes the start of the chitin synthetic pathway and convert trehalose to glucose, leads to a similar pattern of effects, namely decreased cuticular chitin content and premature molting associated mortality (Chen et al. 2010; Shi et al. 2016).

Increase, Premature molting (High): Several studies show that premature molting is a direct consequence of decreased chitin synthesis and leads to increased mortality. The KE is consistently listed as cause for mortality when CHS-1 is knocked down throughout a number of studies (Arakane et al. 2005; Chen et al. 2008; J. Zhang et al. 2010; X. Zhang et al. 2010; Wang et al. 2012; Yang et al. 2013; Shang et al. 2016; Li et al. 2017; Mohammed et al. 2017; Zhai et al. 2017; Wang et al. 2019; Ye et al. 2019).

Increase, Mortality (High): Increased mortality was observed in all of the abovementioned studies.

Weight of Evidence Summary

Biological Plausibility: The biosynthesis of chitin is well characterized and is conserved among arthropods. Although the exact mode of action of chitin synthases remains elusive, it is widely accepted and well established that the chitin synthase is the key enzyme in the pathway, polymerizing chitin using UDP-*N*-Acetylglucosamine as substrate (Merzendorfer and Zimoch 2003).
Arthropod cuticles mostly consist of chitin embedded into a matrix of cuticular proteins. It is therefore widely accepted that chitin contributes crucially to the quality and function of the cuticle (Reynolds 1987; Muthukrishnan et al. 2012). The molting process requires the new cuticle to be strong enough to withstand the stresses of ecdysis.
During ecdysis, arthropods pause food intake and growth. If ecdysis is initiated before the new cuticle is strong enough, the organism likely dies of starvation or growth arrest (Song, Villeneuve, et al. 2017). It was also reported that certain arthropods pause respiration during ecdysis, which may lead to suffocation (Camp et al. 2014).
Based on the well-established biological knowledge on the processes this AOP bases on, the biological plausibility for all KER was rated as high.

Empirical Evidence: Empirical evidence assessment was conducted on the basis of *in vitro* and *in vivo* experiments performed with stressors affecting key events throughout the AOP. Studies showed that the key events are affected by model stressors such as Polyoxin D and Nikkomycin Z, which are able to competitively inhibit CHS1 (Endo et al. 1970). Several studies provide evidence that polyoxin B, polyoxin D and nikkomycin Z trigger the MIE in cell free systems of coleopteran, lepidopteran and dipteran insect species (Cohen 1982; Turnbull and Howells 1982; Kuwano and Cohen 1984; Cohen and Casida 1990; Zhang and Yan Zhu 2013). Also the cuticular chitin content was shown to be decreased by polyoxin D and nikkomycin Z in lepidopteran and dipteran species as well as in the crustacean *Artemia salina* (Gijswijt et al. 1979; Calcott and Fatig 1984; Gelman and Borkovec 1986; Zhuo et al. 2014). The AO is supported by in vivo studies with polyoxin D and nikkomycin Z in dipteran insects and *Daphnia magna* (Tellam et al. 2000; Tellam and Eisemann 2000; Zhu et al. 2007; Zhang and Yan Zhu 2013; New Zealand Environmental Protection Authority 2015). A major data gap constitutes the absence of data covering the KE “Increase, premature molting”. This KE is mentioned in some studies but never assessed as an individual endpoint (Gijswijt et al. 1979; Tellam et al. 2000). Another major data gap is the lacking quantitative data for KERs. As endpoints were only measured as individual endpoints and not in sequence, it makes it nearly impossible to evaluate the dose for the KEs and KERs. However, data from studies where CHS-1 was knocked down are able to support temporal concordance for all KERs. Knockdown of CHS-1 led to decreased chitin content and subsequently to premature molting associated mortality (Arakane et al., 2005; Li et al., 2017). Based on the major data gaps and therefore the lacking information on dose concordance as well as the given time concordance, empirical evidence was evaluated to be moderate for the whole AOP.

Overall confidence in the AOP: Both, essentiality of KEs and the biological plausibility of the whole AOP were considered to be high.

However, due to missing quantitative data and the lack of evidence for dose concordance, empirical evidence was judged to be moderate. Therefore the overall confidence in the AOP was evaluated as moderate.

Quantitative Consideration

Quantitative data are limited for all KER and therefore the whole AOP. Therefore, predictions on the occurrence of downstream KE and the AO on the basis of the occurrence of upstream KEs is not readily feasible. Quantitative understanding of the AOP was therefore considered to be low.

Considerations for Potential Applications of the AOP (optional)

Arthropods are responsible for many functions in terrestrial as well as aquatic ecosystems and are therefore jointly responsible for ecosystem health (Seastedt and Crossley 1984; Losey and Vaughan 2006; LeBlanc 2007). Therefore, it is important to develop AOPs which enhance the mechanistic knowledge on chemicals, such as chitin synthesis inhibitors, which may pose a risk to non-target arthropods. Those AOPs will contribute to the systematic use of mechanistic data to preserve beneficial arthropod populations and ecosystem health.
The present AOP will help to guide future experimental studies by identifying data gaps. This will lead to the identification and development suitable bioassays in order to populate the AOP with (quantitative) experimental data which may allow for predictions of regulatory relevant endpoints on the basis of the occurrence of the MIE.
The present AOP may also guide screening strategies in order to identify chemicals inhibiting CHS-1. The identified substances may then be prioritized and undergo a thorough hazard assessment.
As there already exist approaches to assess mixture toxicity using the AOP framework (Altenburger et al. 2012; Beyer et al. 2014), the present AOP could be employed for the effect assessment of mixtures of chemicals that share the same KEs (e.g. AOP #361, aopwiki.org/aops/361, AOP #358, aopwiki.org/aops/358, and AOP #359, aopwiki.org/aops/359).

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Appendix 1

List of MIEs in this AOP

Event: 1522: Inhibition, Chitin synthase 1

Short Name: Inhibition, CHS-1

Key Event Component

Process	Object	Action
chitin synthase activity		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:342 - S-adenosylmethionine depletion leading to population decline (1)	MolecularInitiatingEvent
Aop:360 - Chitin synthase 1 inhibition leading to mortality	MolecularInitiatingEvent

Stressors

Name
Polyoxin B
Polyoxin D
Nikkomycins
Captan

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

cuticle secreting cell

Organ term

Organ term

epithelium

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Stressors known to competitively inhibit CHS1 are polyoxin B, polyoxin D and Nikkomycin Z (Cohen and Casida 1982; Cohen and Casida 1990; Zhang and Yan Zhu 2013). There may also be stressors that inhibit CHS-1 in a non-competitive manner which may become apparent in further characterization efforts of this MIE. There is also a study that reports inhibition of CHS-1 by the phthalimide fungicide captan (Cohen and Casida 1982). However, it remains elusive if the observed inhibition is due to specific interaction with the enzyme or due to unspecific protein binding which is the predominant mode of action of phthalimides (Lukens and Sisler 1958).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Anopheles gambiae	Anopheles gambiae	High	NCBI
Tribolium castaneum	Tribolium castaneum	High	NCBI
Trichoplusia ni	Trichoplusia ni	High	NCBI
Hyalophora cecropia	Hyalophora cecropia	High	NCBI
Bradysia hygida	Bradysia hygida	Moderate	NCBI
Mamestra brassicae	Mamestra brassicae	Moderate	NCBI
Chilo suppressalis	Chilo suppressalis	Moderate	NCBI
Locusta migratoria	Locusta migratoria	Moderate	NCBI
Nilaparvata lugens	Nilaparvata lugens	Moderate	NCBI
Aphis glycines	Aphis glycines	Moderate	NCBI
Lepeophtheirus salmonis	Lepeophtheirus salmonis	Moderate	NCBI
Panonychus citri	Panonychus citri	Moderate	NCBI
Grapholita molesta	Grapholita molesta	Moderate	NCBI
Ectropis obliqua	Ectropis obliqua	Moderate	NCBI
Tigriopus japonicus	Tigriopus japonicus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
larvae	High
Juvenile	High
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Effect data for the occurrence of CHS1 inhibition exist from Dipteran, Lepidopteran and Coleopteran insect species. Sequence alignment of CHS1 protein sequences using the Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS, https://seqapass.epa.gov/seqapass) tool, yielded susceptibility predictions for various insect species, arachnids and crustacean taxa such as branchiopods, hexanauplia, malocostraca and merostomata. However, most of the protein sequences were not identified as CHS1. The alignment of amino acid residues believed to be critical for ligand binding were therefore carried out with sequences identified as CHS1. Evidence was rated as high for species with a susceptibility prediction and effect data. Evidence was rated as moderate when only alignment data were available. Although most of the sequences are not annotated as CHS1, all arthropods rely on the synthesis of cuticular chitin therefore it is extremely likely that this MIE is applicable to the whole phylum of arthropods.

Life stage: This MIE is applicable for organisms undergoing continuous molt cycles. Namely larval stages of insects and all life stages of crustaceans and arachnids.

Sex: The MIE is applicable to all sexes.

Chemical: Substances known to trigger inhibit CHS-1 are of the family of pyrimidine nucleosides (e.g. polyoxin D, polyoxin B and nikkomycin Z) (Cohen and Casida 1982; Kuwano and Cohen 1984; Cohen and Casida 1990; Zhang and Yan Zhu 2013; Osada 2019). There also exists evidence for the phthalimide captan to inhibit CHS-1 activity *in vitro* (Cohen and Casida 1982). However, as phthalimides are known to covalently bind to thiol groups in proteins (Lukens and Sisler 1958), it is not clear if the inhibition is due to specific CHS-1 inhibition or due to unspecific protein binding.

Key Event Description

Chitin synthases are essential enzymes for all organisms synthesizing chitin, for example arthropods and fungi (Latgé 2007; Merzendorfer 2011). Chitin synthases polymerize chitin and subsequently translocate chitin through the cell membrane (Merzendorfer 2006; Merzendorfer 2011). In arthropods, two isoforms of the chitin synthase are known, CHS1, which is responsible for the synthesis of cuticular chitin, and chitin synthase isoform 2, which synthesizes chitin in the midgut (Arakane et al. 2005). In this MIE, inhibition of CHS-1 is characterized. The biological state being measured is the activity of the enzyme. CHS-1 has an essential role in the cuticle biology, as it constitutes the last and most critical step in the chitin biosynthetic pathway by catalyzing the polymerization of UDP-GlcNAc to chitin (Merzendorfer and Zimoch 2003; Merzendorfer 2006).

How it is Measured or Detected

Since the purification or even recombinant production of CHS-1 has not been achieved yet, the most common way is to use crude enzyme preparations for CHS-1 activity assays. It is noteworthy that in crude enzyme preparations of whole organisms both CHS isoforms, CHS-1 and CHS-2, are present. However, the expression of CHS-1 was shown to be much higher than CHS-2 in *Anopheles gambiae* (Zhang et al. 2012), therefore the effect of CHS-2 may be regarded as negligible. Alternatively, the digestive tract of the respective organism could be removed before producing the enzyme preparation. Different ways exist to detect the activity of the enzyme. One can incubate the enzyme preparation with radioactively labelled chitin precursors (e.g. 14C-UDP-GlcNAc) and measure radioactivity in the formed chitin chains by scintillation counting (Cohen 1982; Cohen and Casida 1990). Chitin synthase activity can also be measured in a non-radioactive way after the addition of precursors to a crude enzyme extract. There, the detection of CHS-1 activity involves the binding of chitin chains to wheat germ agglutinin (WGA) which possesses specific chitin binding properties (Lucero et al. 2002; Zhang and Yan Zhu 2013). The assay builds on the principle of a sandwich-ELISA, where chitin binds to a layer of WGA. A second layer of WGA which is conjugated to horseradish peroxidase (HRP) is then added and subsequently incubated with a HRP substrate. The cleavage of the HRP substrate leads to color formation and the amount of chitin synthesized can be determined colorimetrically.

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List of Key Events in the AOP

Event: 1523: Decrease, Cuticular chitin content

Short Name: Decrease, Cuticular chitin content

Key Event Component

Process	Object	Action
cuticle development	cuticle	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:343 - S-adenosylmethionine depletion leading to population decline (2)	KeyEvent
Aop:342 - S-adenosylmethionine depletion leading to population decline (1)	KeyEvent
Aop:360 - Chitin synthase 1 inhibition leading to mortality	KeyEvent
Aop:361 - Sulfonylureareceptor binding leading to mortality	KeyEvent

Stressors

Name
Polyoxin D
Nikkomycins
Captan
Captafol
Folpet

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term

Original term

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Pieris brassicae	Pieris brassicae	High	NCBI
Lucilia cuprina	Lucilia cuprina	High	NCBI
Bombyx mori	Bombyx mori	High	NCBI
Artemia salina	Artemia salina	High	NCBI
Ostrinia nubilalis	Ostrinia nubilalis	High	NCBI

Life Stage Applicability

Life Stage	Evidence
larvae	High
Juvenile	High
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Effect data for the occurrence of this KE exist from *Pieris brassicae*, *Lucilia cuprina*, *Bombyx mori*, *Artemia salina* and *Ostrinia nubilalis*, defining its taxonomic applicability. Most likely, this KE is applicable to the whole phylum of arthropods, as they all rely on chitin as part of their exoskeleton.

Life stage: This KE is applicable for organisms synthesizing chitin in order to grow and develop, namely larval stages of insects and all life stages of crustaceans and arachnids.

Sex: This KE is applicable to all sexes.

Chemical: Substances known decrease the cuticular chitin content are of the family of pyrimidine nucleosides (e.g. polyoxin D and nikkomycin Z) (Gijswijt et al. 1979; Turnbull and Howells 1982; Calcott and Fatig 1984; Zhuo et al. 2014; Osada 2019). There also exists evidence for phthalimides (captan, captafol and folpet) to to decrease the cuticular chitin content *in vitro* (Gelman and Borkovec 1986). However, as these substances are known to covalently bind to thiol groups in proteins (Lukens and Sisler 1958), it is not clear if the inhibition is due to specific CHS-1 inhibition or due to unspecific protein binding.

Key Event Description

This key event describes the decrease in cuticular chitin content. Chitin is a major part of the arthropod cuticle and therefore also responsible for its integrity (Reynolds 1987; Muthukrishnan et al. 2012). The cuticle is the exoskeleton of arthropods and has manifold functions, it protects organisms from predators, loss of water, acts as a physical barrier against microbial pathogens and provides support for muscular function (Vincent and Wegst 2004). Hence, cuticular chitin is also indispensable for the development of arthropods, as an immaculate cuticle is required for proper molting and therefore also for the growth of an organism.

How it is Measured or Detected

Several ways to determine cuticular chitin are described in the literature. Some of them are based on the determination of amino sugars after digestion or hydrolysis of chitin. For example, after the digestion of chitin by a bacterial chitinase, the *N*-Acetylclucosamine (GlcNAc) amount can be determined colorimetrically by a modified Morgan-Elson assay (Reissig et al. 1955; Arakane et al. 2005). Alternatively, one can also quantify glucosamine colorimetrically after deacetylation and hydrolysis of chitin (Lehmann and White 1975; Zhang and Zhu 2006).
There also exists an approach based on the detection of fluorescence after staining with calcofluor white. In this assay, no treatment of the samples is necessary, the detection is carried out in homogenates of the respective organisms as calcofluor white directly binds to chitin (Henriques et al. 2020).
Chitin can also be quantified using radioactively labelled precursors (e.g. 14C-UDP-GlcNAc) which are incorporated into *in vitro* cultured integument pieces or into the cuticle of whole organisms (Gijswijt et al. 1979; Turnbull and Howells 1982; Calcott and Fatig 1984; Gelman and Borkovec 1986).
Another possibility is to use the non-radioactive assay developed to measure chitin synthase activity (Lucero et al. 2002; Zhang and Yan Zhu 2013). Instead of adding an enzyme extract and chitin precursors to the reaction, one could simply add homogenized chitin containing material to the reaction to quantify its chitin content.

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Event: 1524: Increase, Premature molting

Short Name: Increase, Premature molting

Key Event Component

Process	Object	Action
ecdysis, chitin-based cuticle		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:343 - S-adenosylmethionine depletion leading to population decline (2)	KeyEvent
Aop:342 - S-adenosylmethionine depletion leading to population decline (1)	KeyEvent
Aop:358 - Chitinase inhibition leading to mortality	KeyEvent
Aop:359 - Chitobiase inhibition leading to mortality	KeyEvent
Aop:360 - Chitin synthase 1 inhibition leading to mortality	KeyEvent
Aop:361 - Sulfonylureareceptor binding leading to mortality	KeyEvent

Stressors

Name
Polyoxin D
Nikkomycins

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Pieris brassicae	Pieris brassicae	High	NCBI
Lucilia cuprina	Lucilia cuprina	High	NCBI

Life Stage Applicability

Life Stage	Evidence
larvae	High
Juvenile	High
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Effect data for the occurrence of this KE exist from *Pieris brassicae* and *Lucilia cuprina*. However, all arthropods undergo molting, so it is highly likely that this KE is applicable to the whole phylum of arthropods.

Life stage: This KE is applicable for organisms that undergo molting in order to grow and develop, namely larval stages of insects and all life stages of crustaceans and arachnids.

Sex: This KE is applicable to all sexes.

Chemical: Substances known to induce premature molting are of the family of pyrimidine nucleosides (e.g. polyoxin D and nikkomycin Z) (Gijswijt et al. 1979; Tellam et al. 2000; Arakawa et al. 2008).

Key Event Description

This key event is measured on the level of the individual. In order to grow and develop, arthropods need to shed their exoskeleton periodically (molting) (Heming 2018). During molting, the newly secreted cuticle is subject to mechanical stress associated and therefore needs to possess enough structural and functional integrity. The ecdysis motor program, which constitutes the behavioral part of the cuticle shedding requires the newly secreted cuticle to possess a certain strength to support for muscular force in order to shed the old cuticle (Ewer 2005). Cuticular integrity is also important after ecdysis, as insects and crustaceans expand their new cuticle by increasing internal pressure by swallowing air and water, respectively. This happens in order to expand and provide stability to the new cuticle until it is hardened (tanned) (Clarke 1957; Lee 1961; Dall et al. 1978; deFur et al. 1985). If arthropods are not able to molt properly, the organism will eventually die. Premature molting describes the unsuccessful molting where the organism is not able to shed the old cuticle, but also other effects related to molting in an immature stage where the new cuticle is not mature enough for the molt, such as rupture of the new cuticle and associated desiccation, deformities, higher susceptibility to pathogens or impaired locomotion. Specific effects observed are animals stuck in their exuviae (Wang et al., 2019), and if molting can be completed despite an immature cuticle, animals might be smaller and die at subsequent molts (Arakawa et al., 2008; Chen et al., 2008; Mohammed et al., 2017).

How it is Measured or Detected

Premature molting can be determined by observation. No standardized tests for the endpoint of molting exist to date. However, during an OECD 202 *Daphnia* sp. Acute immobilization test (OECD 2004), the cumulative number of molts can be assessed as an additional endpoint. Molting can also be assessed during a OECD 211 *Daphnia* sp. Reproduction test (OECD 2012), as proposed previously (OECD 2003). One could even prolong the test to 96h to get a clearer result of this endpoint. Additionally, one could apply histopathological methods to monitor the maturity of the newly synthesized cuticle (e.g. thickness of procuticle).

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List of Adverse Outcomes in this AOP

Event: 350: Increase, Mortality

Short Name: Increase, Mortality

Key Event Component

Process	Object	Action
mortality		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:4 - Ecdysone receptor agonism leading to incomplete ecdysis associated mortality	KeyEvent
Aop:331 - Formation of DNA photoproducts leading to growth inhibition (1)	AdverseOutcome
Aop:327 - Excessive reactive oxygen species production leading to mortality (1)	AdverseOutcome
Aop:328 - Excessive reactive oxygen species production leading to mortality (2)	AdverseOutcome
Aop:329 - Excessive reactive oxygen species production leading to mortality (3)	AdverseOutcome
Aop:330 - Excessive reactive oxygen species production leading to mortality (4)	AdverseOutcome
Aop:343 - S-adenosylmethionine depletion leading to population decline (2)	AdverseOutcome
Aop:342 - S-adenosylmethionine depletion leading to population decline (1)	AdverseOutcome
Aop:358 - Chitinase inhibition leading to mortality	AdverseOutcome
Aop:359 - Chitobiase inhibition leading to mortality	AdverseOutcome
Aop:360 - Chitin synthase 1 inhibition leading to mortality	AdverseOutcome
Aop:361 - Sulfonylureareceptor binding leading to mortality	AdverseOutcome

Stressors

Name
Polyoxin D
Nikkomycins

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Lucilia cuprina	Lucilia cuprina	High	NCBI
Daphnia magna	Daphnia magna	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic: This AO is applicable to all living organisms.

Life stage: This AO is applicable to all life stages.

Sex: This AO is applicable to all sexes.

Chemical: Substances known to increase mortality in arthropods are of the family of pyrimidine nucleosides (e.g. polyoxin D and nikkomycin Z) (Gijswijt et al. 1979; Tellam et al. 2000; Arakawa et al. 2008).

Key Event Description

This key event is observed at the biological level of the individual and describes the increase of mortality of individuals upon exposure to a stressor.

How it is Measured or Detected

The AO can be detected by observation, for example by immobilization of the respective organisms. There exist guidelines for the characterization of this AO in arthropods. For example, the OECD 202 Daphnia sp. Acute immobilization test (OECD 2004) which can also be modified depending on the effect one expects.

Regulatory Significance of the AO

The Adverse Outcome is highly significant from a regulatory point of view. It is employed as regulatory endpoint in most studies assessing the toxicity of stressors.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1742: Inhibition, CHS-1 leads to Decrease, Cuticular chitin content

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
S-adenosylmethionine depletion leading to population decline (1)	adjacent		
Chitin synthase 1 inhibition leading to mortality	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
crustaceans	Daphnia magna	Moderate	NCBI
insects	insects	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
larvae	High
Juvenile	High
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Likely, this KER is likely applicable to the whole phylum of arthropods as they all depend on the synthesis of chitin.

Life stage: This KER is applicable for organisms synthesizing chitin in order to grow and develop, namely larval stages of insects and all life stages of crustaceans and arachnids.

Sex: This KER is applicable to all sexes.

Chemical: Substances inducing both, the inhibition of CHS-1 and the decrease in cuticular chitin content are of the family of pyrimidine nucleosides (e.g. polyoxin D, polyoxin B and nikkomycin Z) (Gijswijt et al. 1979; Cohen and Casida 1982; Turnbull and Howells 1982; Calcott and Fatig 1984; Kuwano and Cohen 1984; Cohen and Casida 1990; Zhang and Yan Zhu 2013; Zhuo et al. 2014; Osada 2019). The phthalimide captan was also shown to induce CHS-1 inhibition and a decrease in cuticular chitin content (Cohen and Casida 1982; Gelman and Borkovec 1986). However, studies assessing both endpoints in sequence are lacking.

Key Event Relationship Description

Chitin in the arthropod cuticle is synthesized by the chitin synthase isoform 1 (CHS-1) which spans the plasma membrane on the apical plasma membrane of epithelial cells (Locke and Huie 1979; Binnington 1985; Merzendorfer and Zimoch 2003; Merzendorfer 2006). Since CHS-1 is the enzyme to polymerize chitin from UDP-*N*-Acetylglucosamine (UDP-GlcNAc) (Merzendorfer 2006), it is solely responsible for the content of chitin in the exoskeleton. Consequently, the inhibition of CHS-1 leads to a decrease in chitin content in the arthropod cuticle.

Evidence Supporting this KER

Biological Plausibility

The process of chitin synthesis in arthropods is well characterized. Although the exact mechanism of the polymerization reaction remains elusive, CHS-1 is known to be the key enzyme in the biosynthesis of chitin and therefore, responsible for the cuticular chitin content (Merzendorfer and Zimoch 2003; Merzendorfer 2006). Therefore, the biological plausibility of this KER can be regarded as high.

Empirical Evidence

Empirical evidence for the occurrence of both KEs, the inhibition of CHS-1 and the decrease in cuticular chitin content exist. For example, the occurrence of chitin synthase inhibition was characterized using cell free crude enzyme preparations *in vitro* from coleopteran, lepidopteran and dipteran insect species upon treatment with polyoxin B, polyoxin D and nikkomycin Z (Cohen and Casida 1982; Kuwano and Cohen 1984; Cohen and Casida 1990; Zhang and Yan Zhu 2013). The cuticular chitin content was characterized *in vivo* in *Artemia salina* or using cultured integumental tissue from lepidopteran and dipteran species after exposure to polyoxin D and nikkomycin Z as well as the phthalimides captan, captafol, and folpet (Gijswijt et al. 1979; Turnbull and Howells 1982; Calcott and Fatig 1984; Gelman and Borkovec 1986; Zhuo et al. 2014). Data from studies with specific stressors assessing both endpoints and therefore supporting dose concordance of the KER are lacking. However, results from studies where CHS-1 was knocked down by RNA interference support temporal concordance of the KER (Arakane et al. 2005, Li et al. 2017, Zhang X. et al. 2010). Given the support for temporal concordance and the lack of studies showing dose concordance, the empirical evidence for this KER was judged as moderate.

Uncertainties and Inconsistencies

The major uncertainty in this KER is the absence of studies which assess both endpoints, the inhibition of the chitin synthase and the decrease in cuticular chitin content after exposure to specific stressors.

Quantitative Understanding of the Linkage

Response-response relationship

Due to the lack of studies linking the inhibition of CHS-1 to the decrease in cuticular chitin content, it is not possible to describe the nature of the response-response relationship.

Time-scale

Due to the lack of studies assessing the inhibition of CHS-1 and the decrease in cuticular chitin content, it is not possible to make a statement on the timescale of the relationship. However, the expression of CHS-1 peaks at the time of ecdysis (Ampasala et al. 2011; Wang et al. 2012), indicating the highest rate of chitin synthesis at this timepoint. Hence it can be assumed that a decrease in chitin content in the newly synthesized cuticle should become apparent shortly after. In studies where CHS-1 was knocked down, chitin contents were assessed after 3

and 7 days and found to be decreased (Arakane et al. 2005, Li et al. 2017, Zhang X. et al. 2010).

Known modulating factors

CHS is dependent on bivalent ions as cofactor such as Mg^{2+} or Mn^{2+} (Merzendorfer 2006). Both low and high levels of Mg^{2+} inhibited CHS activity *in vitro* (Zhang and Yan Zhu 2013).

Known Feedforward/Feedback loops influencing this KER

Upon knockdown of CHS-1 in the salmon louse *Lepeophtheirus salmonis*, upregulation of the UDP-GlcNAc pyrophosphorylase (UAP), which catalyzes the conversion of GlcNAc to UDP-GlcNAc, was observed (Braden et al. 2020). The knockdown of UAP also led to upregulation of CHS-1 demonstrating a clear dependence of the two enzymes. Most likely, the upregulation of UAP is a compensatory mechanism with the goal to restore homeostasis in absence of CHS-1. The exact regulation of the feedback, however, remains to be investigated.

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Relationship: 1743: Decrease, Cuticular chitin content leads to Increase, Premature molting

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
S-adenosylmethionine depletion leading to population decline (2)	adjacent		
S-adenosylmethionine depletion leading to population decline (1)	adjacent		
Chitin synthase 1 inhibition leading to mortality	adjacent	Moderate	Low
Sulfonylureareceptor binding leading to mortality	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
crustaceans	Daphnia magna	Moderate	NCBI
insects	insects	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
larvae	High
Juvenile	High
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: In all likelihood, this KER is applicable to the whole phylum of arthropods as they all depend on the synthesis of chitin and molting in order to develop.

Life stage: This KER is applicable for organisms synthesizing chitin and molting in order to grow and develop, namely larval stages of insects and all life stages of crustaceans and arachnids.

Sex: This KER is applicable to all sexes.

Chemical: Occurrence of a decrease in cticular chitin content as well as premature molting was observed after treatment with the pyrimidine nucleosides polyoxin D, polyoxin B and nikkomycin Z (Gijswijt et al. 1979; Turnbull and Howells 1982; Calcott and Fatig 1984; Gelman and Borkovec 1986; Tellam et al. 2000; Arakawa et al. 2008; Zhuo et al. 2014). However, studies causally linking both endpoints are lacking.

Key Event Relationship Description

As the arthropod cuticle is a central part in the molting process, its proper composition is indispensable for a proper molt. The ecdysis motor program, the behavioral part of ecdysis, constitutes a distinct motor pattern to split and shed the old cuticle (Ayali 2009). As the cuticle supports muscular function (Vincent and Wegst 2004), it needs to possess a certain integrity in order to successfully molt. The integrity of the cuticle is also important after ecdysis as arthropods, such as insects and crustaceans, expand the new cuticle by swallowing air or water in order to build up pressure to split the old and expand the new exoskeleton and provide stability to the soft new cuticle (Clarke 1957; Lee 1961; Dall et al. 1978; deFur et al. 1985). The arthropod cuticle mostly consists of chitin embedded in and crosslinked with a matrix of proteins (Muthukrishnan et al. 2012). If the chitin content is too low, the cuticle may not possess enough integrity to support muscular function or withstand the beforementioned stresses of ecdysis, which leads to the organism being stuck in the old cuticle or the rupture of the new cuticle.

Evidence Supporting this KER

Biological Plausibility

The ecdysis motor program, the behavioral part of ecdysis, constitutes a distinct motor pattern to split and shed the old cuticle (Ayali 2009). As the cuticle supports muscular function (Vincent and Wegst 2004), it needs to possess a certain integrity in order to successfully molt. The integrity of the cuticle is also important after ecdysis as arthropods, such as insects and crustaceans, expand the new cuticle by swallowing air or water in order to build up pressure to expand the new exoskeleton and provide stability to the soft new cuticle (Clarke 1957; Lee 1961; Dall et al. 1978; deFur et al. 1985). The arthropod cuticle mostly consists of chitin embedded in and crosslinked with a matrix of proteins (Muthukrishnan et al. 2012). Given the well biological understanding of the processes, the biological plausibility can be regarded as high.

Empirical Evidence

The cuticular chitin content was characterized *in vivo* in *Artemia salina* or using cultured integumental tissue from lepidopteran and dipteran insect species after exposure to polyoxin D and nikkomycin Z as well as the phthalimides captan, captafol, and folpet (Gijswijt et al. 1979; Turnbull and Howells 1982; Calcott and Fatig 1984; Gelman and Borkovec 1986; Zhuo et al. 2014). The event of premature molting was not assessed as endpoint in studies involving specific stressors rather than mentioned after exposure to polyoxin D, polyoxin B and nikkomycin Z (Gijswijt et al. 1979; Tellam et al. 2000; Arakawa et al. 2008). However, results from studies where CHS-1 was knocked down by RNA interference support temporal concordance of the KER (Arakane et al. 2005, Li et al. 2017, Zhang X. et al. 2010). Given the support for temporal concordance and the lack of studies showing dose concordance, the empirical evidence for this KER was judged as moderate.

Uncertainties and Inconsistencies

The absence of studies (quantitatively) assessing premature molting constitutes a major data gap. A further data gap is the absence of studies which assess both, the decrease in cuticular chitin content and the increase in premature molting.

Quantitative Understanding of the Linkage

Response-response relationship

Due to the lack of studies linking the decrease in cuticular chitin content with the increase in premature molting, it is not possible to describe the nature of the response-response relationship.

Time-scale

Due to the nature of the process, premature molting onsets at the time of ecdysis after the decrease in cuticular chitin content.

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Relationship: 1744: Increase, Premature molting leads to Increase, Mortality

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
S-adenosylmethionine depletion leading to population decline (2)	adjacent		
S-adenosylmethionine depletion leading to population decline (1)	adjacent		
Chitinase inhibition leading to mortality	adjacent	Moderate	Low
Chitobiase inhibition leading to mortality	adjacent	Moderate	Low
Chitin synthase 1 inhibition leading to mortality	adjacent	Moderate	Low
Sulfonylureareceptor binding leading to mortality	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
crustaceans	Daphnia magna	Moderate	NCBI
insects	insects	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
larvae	High
Juvenile	Moderate
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Likely, this KER is applicable to the whole phylum of arthropods as they all depend on molting in order to develop.

Life stage: This KER is applicable for organisms molting in order to grow and develop, namely larval stages of insects and all life stages of crustaceans and arachnids.

Sex: This KER is applicable to all sexes.

Chemical: Occurrence of premature molting and an increase in mortality observed after treatment with the pyrimidine nucleosides (e.g. polyoxin D, polyoxin B and nikkomycin Z) (Gijswijt et al. 1979; Tellam et al. 2000; Tellam and Eisemann 2000; Arakawa et al. 2008; New Zealand Environmental Protection Authority 2015). However, studies causally linking both endpoints are lacking.

Key Event Relationship Description

During molting, arthropods pause food uptake and in certain cases also respiration (Camp et al. 2014; Song et al. 2017a). If molting is disrupted and the organism is not able to shed the old exoskeleton, the organism may eventually die of starvation, suffocation or the rupture of the exoskeleton.

Evidence Supporting this KER

Biological Plausibility

In order to grow and develop, arthropods need to molt periodically (Heming 2018). Since molting is a determining point in arthropod development, the disruption of molting leads to increased mortality (Arakawa et al. 2008; Merzendorfer et al. 2012; Song et al. 2017a; Song et al. 2017b). During ecdysis, arthropods pause food intake and respiration (Camp et al. 2014; Song et al. 2017a). Therefore, if the molt cannot be completed, the organism may die of starvation or suffocation. Additionally, if the cuticle is immature, it may not withstand the stresses associated with ecdysis (Clarke 1957; Lee 1961; Dall et al. 1978; deFur et al. 1985), and the organism may die of desiccation or increased susceptibility to pathogens. Given the well understood biological processes, the biological plausibility of this KER was rated as high.

Empirical Evidence

The event of premature molting is not well characterized. It gets mentioned as cause of death in studies with *Pieris brassicae, Spodoptera litura*, *Bombyx mori* and *Lucilia cuprina* after treatment with polyoxin D, polyoxin B, polyoxin AL (a mixture of polyoxins) and nikkomycin Z (Gijswijt et al. 1979; Tellam et al. 2000; Arakawa et al. 2008). The increase in mortality was reported in studies with *Lucilia cuprina*, *Spodoptera litura* and *Bombyx mori* (Tellam et al. 2000; Tellam and Eisemann 2000; Arakawa et al. 2008). Evidence from studies which assess and link both endpoints, and therefore would support dose concordance, is lacking. However, results from studies where CHS-1 was knocked down by RNA interference support temporal concordance of the KER (Arakane et al. 2005, Li et al. 2017, Chen et al., 2008; Mohammed et al., 2017; Shang et al., 2016; Wang et al., 2012, 2019; Yang et al., 2013; Ye et al., 2019; Zhai et al., 2017; Zhang et al., 2010). Given the support for temporal concordance and the lack of studies showing dose concordance, the empirical evidence for this KER was judged as moderate.

Uncertainties and Inconsistencies

The absence of studies (quantitatively) assessing premature molting constitutes a major data gap. A further data gap is the absence of studies which assess both, increase in premature molting and the increase in mortality are lacking.

Quantitative Understanding of the Linkage

Response-response relationship

Due to the lack of studies linking the increase in premature molting with the increase in mortality, it is not possible to describe the nature of the response-response relationship.

Time-scale

Death occurs after premature molting. However, an exact time frame in which death occurs cannot be defined yet.

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