Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity. II. A focus on growth impairment in fish

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Review

Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: II. A focus on growth impairment in fish

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HIGHLIGHTS

- Development of AOPs for chronic toxicity helps identify alternative tests.
- Interference of chemicals with behavior can cause growth impairment in fish.
- Assessment of locomotion may be used to identify chemicals that may affect growth.
- Reallocation of energy resources induced by chemicals can cause growth impairment.
- Metabolic activity measures may be used to identify chemicals that may affect growth.

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ABSTRACT

Adverse outcome pathways (AOPs) organize knowledge on the progression of toxicity through levels of biological organization. By determining the linkages between toxicity events at different levels, AOPs lay the foundation for mechanism-based alternative testing approaches to hazard assessment. Here, we focus on growth impairment in fish to illustrate the initial stages in the process of AOP development for chronic toxicity outcomes. Growth is an apical endpoint commonly assessed in chronic toxicity tests for which a replacement is desirable. Based on several criteria, we identified reduction in food intake to be a suitable key event for initiation of middle-out AOP development. To start exploring the upstream and downstream links of this key event, we developed three AOP case studies, for pyrethroids, selective serotonin reuptake inhibitors (SSRIs) and cadmium. Our analysis showed that the effect of pyrethroids and SSRIs on food intake is strongly linked to growth impairment, while cadmium causes a reduction in growth due to increased metabolic demands rather than changes in food intake. Locomotion impairment by pyrethroids is strongly linked to their effects on food intake and growth, while for SSRIs their direct influence on appetite may play a more important role. We further discuss which alternative tests could be used to inform on the predictive key events identified in the case studies. In conclusion, our work demonstrates how the AOP concept can be used in practice to assess critically the knowledge available for specific chronic toxicity cases and to identify existing knowledge gaps and potential alternative tests.

Abbreviations: AO, adverse outcome; AOP, adverse outcome pathway; Cd, cadmium; KE, key event; MIE, molecular initiating event; SSRI, selective serotonin reuptake inhibitor.

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Contents

1. Introduction

In the preceding paper, we discussed how the adverse outcome pathway (AOP) concept can be used to improve understanding and prediction of chronic toxicity and what are the potential venues for extension of the AOP framework to incorporate additional information required for chemical- and site-specific risk assessment (Groh et al., in press). In the present paper, we focus on growth impairment as an outcome of chronic toxicity in fish and develop three selected AOP case studies with which we illustrate (i) how the AOP concept can be used to guide collection and assessment of available knowledge on specific toxicity cases, (ii) which criteria can be applied to select specific AOPs or AOP case studies to be developed, (iii) how AOP case studies can be used to identify knowledge gaps to guide further research to support development of generalized AOPs, (iv) what additional aspects should be considered during chemical- and site-specific risk assessment and (v) how AOP case studies can be used to identify potential alternative tests.

2. Justification for focus on fish growth

Impairment of fish growth in response to exposure to diverse chemicals is frequently observed. Therefore, growth is commonly assessed as an apical endpoint in fish chronic toxicity tests used to inform risk assessment for aquatic environments. This individual-based parameter is plausibly linked to population-level effects, because many population-relevant processes are size-dependent. For example, body size affects vulnerability to predation (Law et al., 2009; Pettorelli et al., 2011), overwinter survival rates (Quinn and Peterson, 1996) and reproductive success (Jawad and Busneina, 2000; Rideout and Morgan, 2010). Growth-related metrics can be relatively easily incorporated into population models (Crowder et al., 1992; Weitz and Levin, 2006; Murphy et al., 2008; Baldwin et al., 2009; Huebert and Peck, 2014).

One drawback of the currently used tests for chronic toxicity to fish is that they are extremely resource- and labor-intensive, and typically take weeks to months to complete. This makes it impractical to perform fish chronic toxicity tests for all chemicals that may require such testing. Moreover, such tests typically provide very limited information, which is mostly descriptive with little mechanistic insights. This severely limits the usefulness of data derived from these resource-intensive tests for extrapolation across other chemicals and species. Furthermore, not only economical, but also ethical concerns underlie the urgent demand for development of alternative toxicity assessment methods that could refine or replace the current chronic toxicity tests using large numbers of fish. Certainly, more mechanistic understanding of chemical effects on growth in fish would be beneficial, as this would both increase the value of information obtained in chronic toxicity tests as well as support the identification and development of potential alternative tests. Such mechanistic insights can be gained through developing AOPs that cover diverse facets of chemical impacts on fish growth. Moreover, in the longer term the knowledge integrated through AOPs can help to understand better the consequences of growth impairment in individuals for population fitness and support the extrapolation from laboratory to the field as well as across species.

3. AOPs for growth impairment: development strategy and selection of case studies

In the following subsections we will explain our choice of AOP development strategy and describe the criteria we used for our selection of AOP case studies.

3.1. AOPs and AOP development strategies

An AOP depicts the progression of toxicity across biological organization scales from a molecular initiating event (MIE) through subsequent key events (KEs) to an adverse outcome (AO). MIE is a direct chemical-induced perturbation of a molecular target and as such essentially represents a "special case" of first KE in the AOP sequence. KEs are toxicity responses at molecular, cellular, suborganismal or organism levels that are measurable and necessary for an AO to occur. AO is a toxic effect relevant for regulatory risk assessment. Typical examples of AOs are impacts on survival, growth or reproduction in individuals, or population-level

effects. The linkages between MIE, KE and AO are described by key event relationships, which can be either qualitative or quantitative, depending on the maturity of the AOP and the availability of information. Thus, by visualizing the pathways and identifying the linkages between different events, the AOP provides a basis for prediction of toxicity effects across levels of biological organization (Ankley et al., 2010).

Three approaches to AOP development can be distinguished: (i) bottom-up, where one starts at an MIE and develops the AOP forward to an AO, (ii) top-down, where one starts at an AO and develops the AOP backwards to an MIE and (iii) middle-out, where one starts at a KE at an intermediate level and develops the AOP in both directions, to an MIE and to an AO. By definition, AOPs are non-chemical-specific entities that describe generalized motifs of biological response to a specific perturbation (i.e., MIE) that results in an AO through a series of linked events (Villeneuve et al., in press). In contrast, an AOP case study aims to construct a putative AOP based on the data obtained from empirical studies with specific chemicals or chemical groups. Thus, AOP case studies are chemical-specific. Once enough evidence is collected through such AOP case studies to support the occurrence of downstream KEs and AOs that follow a particular MIE, an AOP can be generalized and consequently becomes non-chemical-specific. In this way, AOP case studies serve to support the initial development of a generalized AOP.

Growth is regulated by a broad array of factors acting at molecular, cellular and physiological levels. There are many growth regulation pathways that are susceptible to perturbation by chemicals. Therefore, developing growth impairment AOPs by bottom-up or top-down approaches might prove daunting due to the difficulty of prioritizing the most important MIEs or pathways on which to focus first. In this context, the middle-out AOP development approach can be more efficient. This is because intermediate-level KEs, while still providing plausible links to an AO in question, at the same time allow narrowing down the scope of potential MIEs (Villeneuve et al., 2014). Moreover, specific criteria can be defined to select the KEs most suitable for particular purposes of AOP development. Therefore, a middle-out AOP development strategy was employed in the current study.

3.2. Criteria for selection of KEs to focus on for middle-out AOP development

The middle-out AOP development approach has been previously used to develop AOPs in support of alternative testing strategies. These strategies aim to prioritize or replace the lengthy fish early life stage tests for chronic toxicity with the short-term fish embryo toxicity tests (Villeneuve et al., 2014). In this study, the selection of KEs was guided by the criteria that the KE should be related to sublethal morphological endpoints that are easily observed in embryos and can be plausibly linked, at least theoretically, to adverse outcomes such as reduced survival or growth impairment past the embryonic stage. Other potentially important aspects, such as the environmental relevance of effective concentrations, received much less attention during the KE selection in this study (Villeneuve et al., 2014).

In our case, we wanted to ensure that the growth impairment AOPs, which we select for priority development, would describe effects occurring at environmentally relevant chemical concentrations and would be applicable across chemicals and species as broadly as possible. Therefore, we decided to select the KEs according to the following four criteria: (i) importance of a particular process associated with this KE for growth regulation, (ii) conservation of associated molecular pathway or physiological response across species, (iii) frequency of occurrence of a certain disruption (e.g. how many chemicals are suspected to interfere with the KE in question) and (iv) environmental relevance of chemical-induced effects (e.g. concentrations at which the effects are observed).

We did not want to limit our KE choices by predefining the test system where associated processes can be assessed. Instead, we considered all pathways known to be involved in the regulation of growth at molecular, cellular and physiological levels, as well as all chemicals reported to perturb relevant processes. Our main focus was on somatic growth in fish. However, we also collected information on conservation of identified pathways and processes in invertebrates, paying specific attention to similarities between the effects induced by particular chemicals in different phyla. With this analysis, several growth regulation pathways susceptible to interference by chemicals were identified, with KEs located from the subcellular through to organism levels. Each of these pathways can potentially be perturbed and thus can constitute an AOP or a set of AOPs, but discussing all of them is beyond the scope of this work. Based on the criteria outlined above, we prioritized one particular KE to focus on for middle-out AOP development in the current study: reduction in food intake.

3.3. KE "reduction in food intake": choice justification and exploration of potential underlying mechanisms

An efficient acquisition of food is an important prerequisite to ensure normal physiological growth. The ability to find and acquire food can be negatively impacted by chemical exposure in a number of ways. For example, diverse morphological deformities, such as vertebral column curvature, non-inflation of swim bladder and craniofacial malformations, can directly impair the ability to catch or handle prey. Many such morphological defects can already be detected or predicted by performing toxicity tests in embryos, thus offering a plausible venue for development of respective AOPs and alternative testing approaches, as has been suggested previously (Villeneuve et al., 2014). However, apart from a few cases of specifically acting toxicants, such as the aryl hydrocarbon receptor-active compounds (Yoshioka et al., 2011; King-Heiden et al., 2012), severe morphological deformities often manifest only at relatively high concentrations of little environmental relevance (Carlsson et al., 2013; Ali et al., 2014).

Chemicals are also known to influence behavior in diverse ways, which can negatively impact the amount and/or quality of acquired food. Diverse behavioral alterations have been reported to occur in response to a wide range of toxicants, frequently at concentrations much lower than those that induce any visible defects or mortality (Scott and Sloman, 2004; Sloman and McNeil, 2012; Melvin and Wilson, 2013). For example, certain toxicants are known to impair olfaction, interfering with the ability to recognize prey or causing aversion from particular food sources (Langer-Jaesrich et al., 2010; Tierney et al., 2010), while others can affect appetite (Baker et al., 1996; Gaworecki and Klaine, 2008; Mennigen et al., 2009). Yet other chemicals are reported to impair cognitive functions (Weis, 2009; Sledge et al., 2011). However, the most prominent and best-researched type of behavioral alterations that can be plausibly linked to reduction in food intake is impairment of locomotion, broadly understood as a failure to maintain normal performance levels of vital locomotory behavior components, such as activity patterns, speed of movements and orientation in space. Such effects have been reported to occur in response to chemicals belonging to many diverse categories, including heavy metals, industrial chemicals, insecticides and pharmaceuticals (Scott and Sloman, 2004; Tsai and Liao, 2006; Jordaan et al., 2013; Selderslaghs et al., 2013; Leon-Olea et al., 2014). Concentrations that affect locomotory behavior are often similar to those causing growth impairment in the longer-term (Little and Finger, 1990; Melvin and Wilson, 2013). Detrimental effects of chemicals

on locomotory behavior have also been reported for various invertebrate species (Salanki, 2000; Tu et al., 2010; Hellou, 2011; Pekar, 2012; Oliveira et al., 2013; Pereira et al., 2013; Fong and Ford, 2014) and in some studies directly shown to coincide with reduction in food intake as well (Das and Khangarot, 2011; Agatz et al., 2012; Nyman et al., 2013). This suggests that the role in growth impairment of both KEs, impairment of locomotion and reduction in food intake, may be conserved in a broad spectra of taxa.

3.4. Selection of AOP case studies

As discussed above, reduction in food intake is frequently observed across different species in response to a broad variety of chemicals, often at environmentally relevant concentrations. Since the links between reduction in food intake, growth impairment (downstream) and impairment of locomotory behavior (upstream) are plausible, we decided to develop several AOP case studies with which we aimed to evaluate (i) whether the reduction in food intake is sufficient to explain growth impairment in all cases, (ii) whether impairment of locomotory behavior is necessary and sufficient for the effects on food intake and growth to occur and thus could be used as an endpoint to establish alternative testing approaches to predict these downstream effects, and (iii) what other mechanisms, apart from chemical effects on locomotory behavior, may play a role in each case. It has to be noted that some chemicals have been reported to cause sustained stimulatory effects on locomotory behavior, leading to an increase in feeding rate, as for example in European perch (*Perca fluviatilis*) exposed to a benzodiazepine anxiolytic drug, oxazepam (Brodin et al., 2013). However, as our primary focus here was on the pathways that cause reduction in food intake, we did not aim to cover such compounds within the AOP case studies chosen.

For AOP case studies, we chose to focus on growth impairment in juvenile fish caused by pyrethroids, selective serotonin reuptake inhibitors (SSRIs) and cadmium. Pyrethroids were selected as an example of a broadly studied group of chemicals for which their mode of action (interference with neurotransmission) is relatively well understood. Selective serotonin reuptake inhibitors were selected because these less-well studied chemicals are also known to interfere with central nervous system functions and have been shown to cause both locomotion impairment and reduction in food intake. However, the underlying mechanisms might differ from those playing a role in the case of pyrethroids. Cadmium was selected as an example of a chemical for which the effects on growth are well documented, but, although the effects on feeding and locomotion have been reported as well, they do not appear to be the primary mechanism underlying growth impairment in this case.

In all three case studies (pyrethroids, SSRIs and cadmium), we linked the individual-level AO "growth impairment" to population-level AO "reduced young-of-year survival", because reduction in growth of juveniles has been shown to negatively affect their long-term survival (Quinn and Peterson, 1996; Baldwin et al., 2009; Law et al., 2009; Pettorelli et al., 2011). However, discussing the details behind these links to the population level is beyond the scope of this manuscript. Apart from evaluating the upstream links of the KE "reduction in food intake", we also use these three AOP case studies to illustrate in support of the preceding paper (Groh et al., in press) additional aspects that might need to be considered in each case during chemical- and site-specific ecotoxicological risk assessment. In the next sections, the three AOP case studies will be presented and discussed.

4. AOP case study for growth impairment by pyrethroids

Pyrethroids are widely used neurotoxic insecticides frequently found in aquatic systems worldwide (Jorgenson et al., 2013) and shown to persist in sediments (Amweg et al., 2005; Weston et al., 2013). Pyrethroids are highly toxic to non-target organisms, including aquatic invertebrates and fish (Werner and Moran, 2008).

4.1. Description of AOP for growth impairment by pyrethroids

The proposed AOP for growth impairment caused by pyrethroids is shown in Fig. 1. The detrimental effects of pyrethroids on growth have been observed in many different fish species, including sheepshead minnow (*Cyprinodon variegatus*) (Hansen et al., 1983), steelhead trout (*Salmo gairdneri*) (Curtis et al., 1985), fathead minnow (*Pimephales promelas*) (Jarvinen et al., 1988; Floyd et al., 2008), bluegill sunfish (*Lepomis macrochirus*) (Little et al., 1993; Tanner and Knuth, 1996) and freshwater catfish (*Heteropneustes fossilis*) (Saha and Kaviraj, 2013).

Both natural and synthetic pyrethroids are known to interact with voltage-gated sodium and potassium channels, leading to "delayed closure" or "prolonged opening" of individual channels. This leads to membrane depolarization, repetitive discharges (repetitive firing) and consequently synaptic disturbances, which contribute to the hyper-excitatory symptoms of poisoning

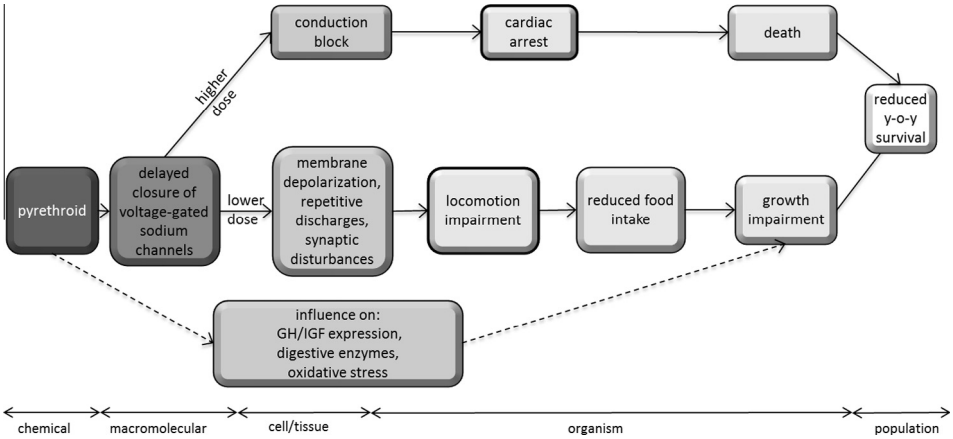

Fig. 1. AOP case study for growth impairment by pyrethroids. The boxes describing specific events are aligned along the increasing levels of biological organization shown in the bottom panel. Solid arrows denote postulated key event relationships. Dashed arrows indicate that the evidence for hypothesized relationship is currently insufficient. *Abbreviations:* y-o-y, young-of-year; GH/IGF, growth hormone/insulin-like growth factor.

(Bradbury and Coats, 1989; Soderlund et al., 2002; Shafer and Meyer, 2004).

At higher concentrations, a complete conduction block occurs, which likely leads to cardiac arrest (Haverinen and Vornanen, 2014), resulting in mortality. Acute mortality following exposure to pyrethroids has been shown for sheepshead minnow (Clark et al., 1985), bluegill sunfish (Fairchild et al., 1992), fathead minnow (Lozano et al., 1992; Werner et al., 2002; Denton et al., 2003), guppy (*Poecilia reticulata*) (Mittal et al., 1994; Baser et al., 2003), Chinook salmon (*Oncorhynchus tshawytscha*) (Eder et al., 2004), Sacramento splittail (*Pogonichthys macrolepidotus*) (Werner et al., 2002), tilapia (*Oreochromis mossambicus*) (Vijayavel and Balasubramanian, 2007; Prasnanth et al., 2011), red drum (*Sciaenops ocellatus*) (Parent et al., 2011) and common edible carp (*Labeo rohita*) (Tiwari et al., 2012). These studies suggest that susceptibility to pyrethroid toxicity is well conserved among fish. This is further supported by a high degree of sequence homology exhibited by the molecular target of pyrethroids (the voltage-gated sodium channel) across different species (LaLone et al., 2013).

Exposure to lower pyrethroid concentrations typically results in subtle behavioral alterations manifesting as head and body shaking (tremors), initial hyperactivity followed by hypoactivity and lethargy as well as loss of equilibrium. Such abnormalities of locomotory behavior have been documented in bluegill (Little et al., 1993), Sacramento splittail (Teh et al., 2005), striped bass (*Morone saxatilis*) (Geist et al., 2007), fathead minnow (Floyd et al., 2008; Beggel et al., 2010, 2011), Nile tilapia (*Oreochromis niloticus*) (El-Sayed and Saad, 2008), Delta smelt (*Hypomesus transpacificus*) (Connon et al., 2009), zebrafish (*Danio rerio*) (Jin et al., 2009) and rainbow trout (*Oncorhynchus mykiss*) (Goulding et al., 2013). Pyrethroid concentrations that affect behavior are similar to those causing growth impairment. Compared to acute LC_{50} values, they are around one order of magnitude lower, with deviations depending on the species and particular chemical.

Pyrethroid-caused impairment of locomotion can be expected to reduce the ability of fish to catch prey, leading to a reduction in food intake and a subsequent growth impairment. Indeed, in esfenvalerate-exposed larval fathead minnow, impaired swimming was shown to result in a reduction of feeding on a live prey and was associated with growth impairment (Floyd et al., 2008). Behavioral abnormalities coinciding with inhibition of growth by pyrethroids have also been reported for bluegill (Little et al., 1993) and Sacramento splittail (Teh et al., 2005). Reduction in post-exposure feeding rates on live prey has also been observed in guppy, however, growth was not assessed in that study (Moreira et al., 2010). Although only a few studies simultaneously assessed the effects of pyrethroids on locomotion, food intake and growth in fish within the same experiment, the large body of evidence obtained for effects of pyrethroids on locomotion and growth individually suggests a strong link between locomotion impairment and growth impairment by pyrethroids, mediated through reduced foraging abilities. This AOP may appear to be particularly relevant for predatory fish, while for herbivorous fish we could not find any studies that directly assessed pyrethroid effects on feeding. However, decreased feeding rate associated with growth reduction following pyrethroid exposure has also been reported in steelhead trout (Curtis et al., 1985) and tilapia (Vijayavel and Balasubramanian, 2007) fed with formulated diet. Thus, it is possible that neurotoxic effects of pyrethroids can influence not only the predatory abilities, but also lead to a disruption of normal behavioral functions, such as feeding, in general. Interestingly, in invertebrates, pyrethroids were shown to impair locomotion in the common prawn (*Palaemon serratus*) (Oliveira et al., 2012) and to reduce growth (Pieters et al., 2005) as well as both locomotion and feeding efficiency (Christensen et al., 2005) in *Daphnia magna*. This indicates that the AOP for growth impairment by pyrethroids mediated via effects on locomotory behavior and food intake may be conserved across species of a broad taxonomic origin.

It has to be noted that biochemical and gene expression analyses have also pointed to other potential mechanisms of pyrethroid action that could be related to their negative effects on growth. These include perturbation of several enzymes involved in digestion following continuous exposure (Vijayavel and Balasubramanian, 2007; Connon et al., 2009), as well as transient (Beggel et al., 2011) or even persistent (Aksakal et al., 2010) down-regulation of insulin-like growth factor (IGF) expression following short-term exposure. Moreover, the influence of pyrethroids on various other molecular, biochemical and hematological parameters has been documented, most prominently for biomarkers of oxidative stress (Kaviraj and Gupta, 2014). However, at present, the extent of these factors' contribution to growth reduction, compared to the consequences of impaired swimming for food intake, is not yet clear and might need further investigation.

4.2. Additional considerations

Application of the proposed AOP(s) in quantitative risk assessment would require more detailed quantitative definition of key event relationships as well as the integration of information on environmental exposure conditions. This is needed in order to distinguish between pyrethroid concentrations likely to cause acute (direct mortality) and sublethal (impairment of locomotion) effects (Fig. 1). An important exogenous parameter to consider in this case is water temperature, as lower values are known to result in higher pyrethroid toxicity (Narahashi et al., 1998; Talent, 2005; Satpute et al., 2007). This effect of temperature can be explained by both reduced biotransformation of parent compound and increased nerve sensitivity at lower temperatures (Harwood et al., 2009). Another important thing to consider is the frequency and duration of exposure to pyrethroids, as well as the presence of organic matter, which could reduce the bioavailability of pyrethroids (Thomas et al., 2008). In the environment, repeated exposure pulses of short duration are most likely to occur, because poorly soluble pyrethroids are usually quickly adsorbed to organic particles and thus become less bioavailable (Laskowski, 2002; He et al., 2008). Most of the above-cited studies on sublethal effects of pyrethroids in fish have been designed following the peak exposure scenario and thus the obtained information on sublethal toxicity of pyrethroids is environmentally relevant. Nonetheless, several data gaps may require further clarification.

Since the effects of pyrethroids on growth can occur at a later time point than the exposure itself, this may represent a case of delayed toxicity. For example, in fathead minnow growth impairment was observed following a very short (4 h) exposure to a pyrethroid followed by 7 d of rearing in clean water, even though behavioral responses have recovered within 3 d after the treatment, probably coinciding with compound elimination from the body (Floyd et al., 2008). This reduction in growth observed 7 d after a peak exposure could be a consequence of severely impaired locomotion and feeding shortly after the exposure event and insufficient time for compensatory growth to occur afterwards. However, another explanation could be that other factors, such as a lasting interference with growth hormone (GH)/IGFs system (Aksakal et al., 2010), could play a role in persistent effects of pyrethroids on growth. In this regard, it might be informative to examine whether pyrethroids can specifically affect the epigenetic regulation of respective genes. In addition, the shortest exposure durations able to induce subsequent growth reduction, the degree of persistence or reversibility of observed effects as well as the time needed for organisms to completely recover from exposure event need to be defined in more detail.

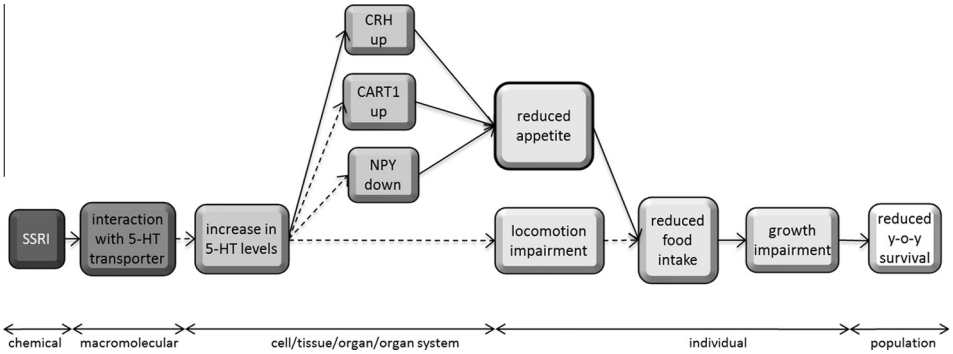

Fig. 2. AOP case study for growth impairment by selective serotonin reuptake inhibitors. The boxes describing specific events are aligned along the increasing levels of biological organization shown in the bottom panel. Solid arrows denote postulated key event relationships. Dashed arrows indicate that the evidence for hypothesized relationship is currently insufficient. *Abbreviations:* SSRI, selective serotonin reuptake inhibitor; y-o-y, young-of-year; 5-HT, 5-hydroxytryptamine (serotonin); CRH, corticotropin releasing hormone; CART1, cocaine and amphetamine-regulated transcript 1; NPY, neuropeptide Y.

Chemical-specific quantitative risk assessment of pyrethroids would need to take into account differences in the toxicokinetic and toxicodynamic characteristics of different compounds. For example, compared to type I pyrethroids, type II pyrethroids, which are characterized by the presence of an α-cyano group, are generally more potent in inducing toxicity effects (Narahashi et al., 2007), in particular on swimming performance (Goulding et al., 2013). This can be explained by the fact that type II pyrethroids have longer half-lives because they are biotransformed at a slower rate due to α-cyano group physically blocking the hydrolysis of the ester linkage (Muir et al., 1994). In addition, type II pyrethroids induce a much longer sodium type current, leading to a faster membrane depolarization (Vijverberg and van den Bercken, 1990). Moreover, more than 50-fold differences in toxic potency have been reported for some pyrethroid enantiomers (Ma et al., 2009; Zhao et al., 2010). Careful consideration of toxicokinetic aspects may also be relevant for assessment of mixture effects with compounds other than pyrethroids. For example, co-exposure with organophosphate pesticides has been shown to synergistically increase the toxicity of pyrethroids (Denton et al., 2003; Belden and Lydy, 2006), explained by the fact that organophosphates inhibit esterases and thus diminish the biotransformation capacities needed to detoxify pyrethroids.

5. AOP case study for growth impairment by selective serotonin reuptake inhibitors

Selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine and sertraline, are widely prescribed antidepressants (Wong et al., 1995). Fluoxetine and its equipotent metabolite norfluoxetine (Hiemke and Haertter, 2000) are fairly resistant to hydrolysis and photolysis (Kwon and Armbrust, 2006; Styrishave et al., 2011) and thus are frequently found at concentrations up to low μg L^{-1} in the aquatic systems (Vasskog et al., 2008; Metcalfe et al., 2010) and up to low μg kg^{-1} in fish tissues (Chu and Metcalfe, 2007).

5.1. Description of AOP for growth impairment by SSRIs

The proposed AOP for growth impairment by SSRIs is shown in Fig. 2. Growth reduction has been documented for goldfish (*Carassius auratus*) in response to repeated fluoxetine injections (Mennigen et al., 2009) and for goldfish (Mennigen et al., 2010), fathead minnow (Stanley et al., 2007; Painter et al., 2009) and hybrid striped bass (*M. saxatilis* × *M. chrysops*) (Gaworecki and Klaine, 2008) following waterborne fluoxetine exposure. In most of these studies, an associated reduction in food intake has also been observed. Exposure to fluoxetine and sertraline has also been shown to decrease feeding in fathead minnow (Weinberger and Klaper, 2014) and European perch (Hedgespeth et al., 2014), respectively, but the fish growth was not assessed in these studies. SSRI effects on growth associated with reduced food intake have also been observed in amphibians *Rana pipiens* (Foster et al., 2010) and *Xenopus laevis* (Conners et al., 2009). Compared to acute LC_{50} values, SSRI concentrations that affect feeding and growth are around two orders of magnitude lower. Based on the presented evidence, the reduction in food intake can be seen as the primary reason for growth impairment in response to SSRIs.

Considering the potential causes of food intake reduction, the effects of SSRIs on locomotion may offer a plausible explanation at a first glance. Exposure to SSRIs is known to affect behavior in diverse species, generally eliciting hypoactive responses. For example, SSRI exposure slowed predator avoidance behaviors in fathead minnows (Painter et al., 2009; Weinberger and Klaper, 2014) and Arabian killifish (*Aphanius dispar*) (Barry, 2013), reduced anxiety-like behaviors in zebrafish (Wong et al., 2013), increased lethargy in western mosquitofish (*Gambusia affinis*) (Henry and Black, 2008) and reduced swimming activity in Arabian killifish (Barry, 2013) and sheepshead minnow (Winder et al., 2012). SSRI effects on locomotory behavior have also been observed in several invertebrate species (Fong and Ford, 2014; Hazelton et al., 2014). However, although SSRIs were also reported to reduce the ability of hybrid striped bass to capture prey possibly due to decreased locomotion (Gaworecki and Klaine, 2008; Bisesi et al., 2014), there is currently insufficient evidence to support the notion that reduced food intake caused by SSRIs is mediated through its effects specifically on locomotory abilities.

Another important SSRI effect that can be linked to the reduction in feeding is the interference with the abundance of appetite-controlling neuropeptides in the brain, resulting in decreased appetite and consequently less food intake. In humans, fluoxetine administration was found to result in weight loss (Halford et al., 2007) and key feeding circuits are known to be conserved between fish and mammals (Volkoff et al., 2005; Polakof et al., 2007). Indeed, the anorexigenic neuropeptides, corticotropin releasing hormone (CRH) (De Pedro et al., 1993) and cocaine and amphetamine-regulated transcript (CART1) (Volkoff and Peter, 2000), were found to increase in the goldfish brain following fluoxetine treatment, while the orexigenic neuropeptide Y (NPY) decreased (Mennigen et al., 2009, 2010). These responses in fish are similar to those reported for mammals (Baker et al., 1996). The decrease in NPY levels was also associated with the decrease in circulating plasma glucose levels (Mennigen et al., 2010). Although the direct neuropeptide-mediated influence of SSRIs on

appetite would be biologically plausible (Halford et al., 2007), also considering the major role in appetite regulation played by serotonin in general (Overli et al., 1998; Lam and Heisler, 2007), more research needs to be done to confirm the postulated linkages. For example, similar investigations need to be carried out in more fish species and also the conflicting results of opposing neuropeptide levels found in different brain regions (Mennigen et al., 2009) need to be resolved.

Another issue that still requires further research is determination of the exact influence of SSRI exposure on serotonin levels in fish. SSRIs act to specifically block the presynaptic membrane serotonin transporter (SLC6A4), thereby inhibiting synaptic reuptake and recycling of serotonin (Wong et al., 1995). Compared to mammalian SLC6A4, fish SLC6A4 was found to have more conserved residues involved in SSRI binding than even birds, with very similar reuptake inhibition constants found in fish and rat (Gould et al., 2007). The serotonergic system in general is also highly conserved between fish and mammals (Kreke and Dietrich, 2008; Rico et al., 2011). In mammals, the consensus is that SSRI administration leads to increase in brain serotonin levels, which is one of the main actions responsible for their effectiveness in treating depression (Baker et al., 1996). However, contradicting evidence was reported for fish. While an increase in brain serotonin levels was found in goldfish after repeated fluoxetine injections (Mennigen et al., 2009), chronic waterborne exposure to fluoxetine was reported to cause reduction of serotonin concentrations in the brain of goldfish (Mennigen et al., 2010) and hybrid striped bass (Gaworecki et al., 2012; Bisesi et al., 2014). One proposed explanation is that, while serotonergic endpoints may still be affected due to target conservation across fish and mammals, the exact nature of the modulation itself may differ (Mennigen et al., 2011). Alternatively, it could be that higher serotonin levels occur only in certain brain parts or only at certain critical time points during the exposure (Beyer and Cremers, 2008) and therefore they could be missed at the time points and brain regions selected for analysis in fish studies. Moreover, continuous exposure to fluoxetine in water could lead to feedback inhibition with time, resulting in apparent suppression of serotonin levels at the end of the exposure when they were analyzed.

5.2. Additional considerations

As discussed in the preceding section, the process of assembling a putative AOP for SSRI effects on fish growth presented in Fig. 2 has revealed several large data gaps that still need to be filled. More research is needed to elucidate molecular mechanisms operating in different species and to establish the quantitative relationships between molecular responses to SSRI exposure and subsequent outcomes on the organism level. In particular, exposure routes, concentrations and duration that result in specific effects need to be defined in more detail. A recently published critical commentary highlighted the widely differing potencies of SSRIs reported in different studies. It was suggested that some of these studies, particularly those reporting effects at very low SSRI concentrations, had considerable limitations. These included lack of concentration–response relationships, insufficient statistical power and the use of non-standard endpoints with poorly characterized baselines (Sumpter et al., 2014).

In regard to variable effective concentrations of SSRIs reported by different studies, it might be important to consider some intrinsic chemical properties of these compounds (Brooks, 2014). SSRIs are ionizable compounds and thus their toxicity and bioaccumulative potential may differ significantly depending on pH (Valenti et al., 2009; Rendal et al., 2011). For example, in Japanese medaka (*Oryzias latipes*), 96-h LC50 values were 5.5, 1.3 and 0.20 mg L^{-1} and bioconcentration factors for liver were calculated to be 330, 580 and 3100 at pH 7, 8 and 9, respectively (Nakamura et al., 2008). Moreover, most SSRIs are chiral compounds present as racemic mixtures often in uncharacterized proportions. Different enantiomers are known to have different potency. For example, 9.4-fold higher toxicity that caused growth inhibition in fathead minnows was reported for S-fluoxetine compared to respective R-enantiomer (Stanley et al., 2007). Therefore, some of the differences in reported effective concentrations could potentially be explained by variability in these aspects across studies.

Nonetheless, considerable research gaps remain an obstacle to understanding the causes of the very high sensitivity to SSRIs reported for some species; many results do not reconcile with the read-across hypothesis (Sumpter and Margiotta-Casaluci, 2014). This hypothesis predicts that, provided that the pharmaceutical target is the same (highly conserved serotonin transporter in the case of SSRIs), similar (behavioral) effects would be expected to occur in fish or invertebrates at the blood concentrations comparable to human therapeutic concentrations (Hugget et al., 2003; Rand-Weaver et al., 2013; Sumpter and Margiotta-Casaluci, 2014). In this regard, it should be noted that in fish the metabolism of fluoxetine was found to be slower (Smith et al., 2010) and its persistence longer (Paterson and Metcalfe, 2008) than in mammals. Such differences in metabolism could partially explain the observed discrepancies.

6. AOP case study for growth impairment by cadmium

Cadmium (Cd) is a heavy metal pollutant present in terrestrial and aquatic environments due to natural emissions as well as anthropogenic activities such as mining and industrial processes. In European rivers, Cd concentrations around 1 ppb or lower are typically reported (Pan et al., 2010), while higher values (up to 1 ppm) can occur in developing countries with rapidly growing industries (Yabe et al., 2010; Anetor, 2012). Cd is known to exert genotoxic and also carcinogenic activity (Waisberg et al., 2003; Bertin and Averbeck, 2006) and thus could possibly contribute to promotion of tumor formation in the aquatic organisms. However, Cd effects on individual fitness, such as impacts on growth, may be more ecologically relevant and have higher prevalence in nature.

6.1. Description of AOP for growth impairment by cadmium

The proposed AOP for growth impairment by Cd is shown in Fig. 3. Exposure to Cd was documented to cause growth impairment in a variety of fish species, including brook trout (*Salvelinus fontinalis*) (Eaton et al., 1978), Atlantic salmon (*Salmo salar*) (Rombaugh and Garside, 1982; Peterson et al., 1983), rainbow trout (Woodworth and Pascoe, 1982; Ricard et al., 1998; Heydarnejad et al., 2013), white sucker (*Catostomus comersoni*) and common shiner (*Notropis cornutus*) (Borgmann and Ralph, 1986), guppy (Miliou et al., 1998), bull trout (*Salvelinus confluentus*) (Hansen et al., 2002b), topsmelt (*Atherinops affinis*) (Rose et al., 2005, 2006), common carp (*Cyprinus carpio*) (Reynders et al., 2006), brown trout (*Salmo trutta*) (Brinkman and Hansen, 2007), European eel (*Anguilla anguilla*) (Pierron et al., 2007), silver catfish (*Rhamdia quelen*) (Benaduce et al., 2008), red sea bream (*Pagrus major*) (Cao et al., 2009), Japanese flounder (*Paralichthys olivaceus*) (Cao et al., 2010) and ide (*Leuciscus idus*) (Witeska et al., 2014). In *Aphanius fasciatus* collected in the field, a high accumulation of Cd associated with a decreased growth rate and condition index was observed (Kessabi et al., 2013). Compared to acute LC_{50} values, Cd concentrations that affect growth are 1–3 orders of magnitude lower, depending on the species and exposure conditions.

In larval topsmelt exposed to Cd, reduction in food intake was associated with diminished growth (Rose et al., 2006). Association

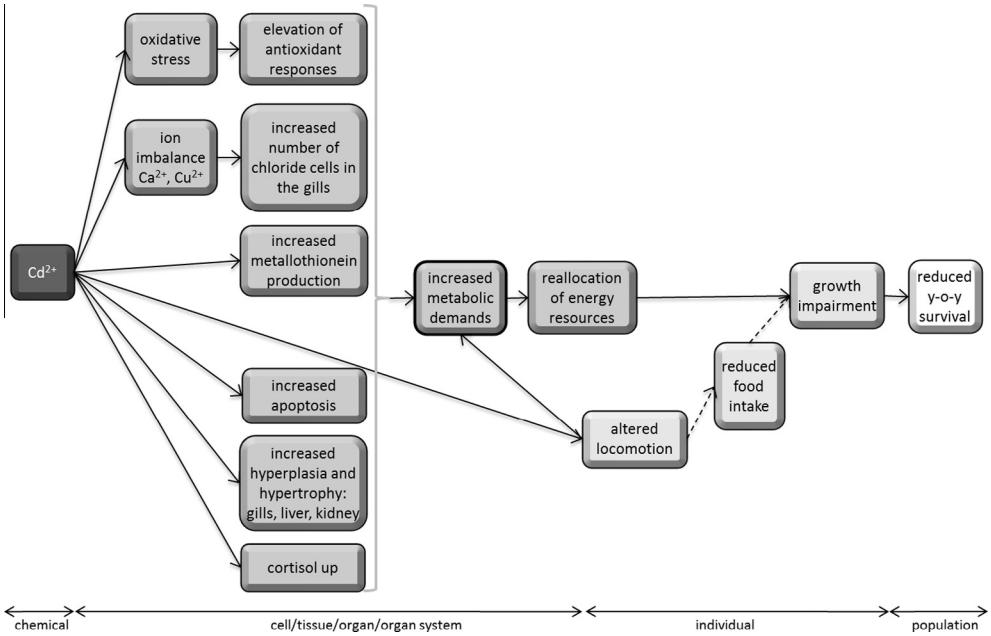

Fig. 3. AOP case study for growth impairment by cadmium. The boxes describing specific events are aligned along the increasing levels of biological organization shown in the bottom panel. Solid arrows denote postulated key event relationships. Dashed arrows indicate that the evidence for hypothesized relationship is currently insufficient. *Abbreviations:* y-o-y, young-of-year.

between growth impairment and reduction in food intake was also observed in Cd-exposed common carp (Ferrari et al., 2011) and rainbow trout (Heydarnejad et al., 2013). However, a reduced food intake but no observable effects on growth were noted after Cd exposure in rainbow trout (McGeer et al., 2000) and Nile tilapia (Almeida et al., 2002), while in white sucker and common shiner Cd reduced growth but had no observable effects on feeding (Borgmann and Ralph, 1986). In the study with topsmelt, food intake was positively correlated with the final weight in Cd-exposed fish. This indicated that, although reduction in food intake may have slightly contributed to the observed growth impairment, factors other than food consumption have higher significance for this outcome (Rose et al., 2006).

The available evidence regarding the effects of Cd on locomotory behavior as potential cause of reduction in food intake is rather inconclusive. Hypoactivity in response to Cd exposure has been observed in common carp (Eissa et al., 2006) and in *Astyanax altoparanae*, a fish native to Argentina (Eissa et al., 2010). However, induction of hyperactivity by Cd has also been reported, for example, for bluegill sunfish (Ellgaard et al., 1978), rainbow trout (Majewski and Giles, 1981) and Atlantic salmon (Peterson et al., 1983). It is possible that the observed discrepancy in Cd effects on locomotory behavior could partially be due to the use of different parameters for final assessment of the hypoactive or hyperactive nature of behavioral alterations. For example, while activity index decreased in Cd-exposed *A. facetum*, an increase in swimming velocity was observed at the same time (Eissa et al., 2010).

Do alternative explanations exist for extensively documented effects of Cd on growth in fish? Exposure to Cd is known to invoke diverse energy-consuming responses, including hyperactive behavior as well as diverse compensatory and defense pathways such as ion imbalance compensation, metallothionein production, oxidative stress defense, increased cell proliferation and induction of apoptosis. Therefore, we propose that a "generic" KE of increase in metabolic demands during exposure to Cd causes a reallocation of the energy that would have otherwise been directed towards growth. This in turn results in growth impairment. The supporting scientific evidence will be discussed in the next paragraphs.

Cd is known to disrupt ion balance through interference with Ca influx in the gills of freshwater fish (Verbost et al., 1987; Reynders et al., 2006) as well as with Ca uptake in the kidney and intestine of marine fish (Schoenmakers et al., 1992) and also by inhibiting Na^+/K^+-ATPase activity (Pratap and Wendelaar Bonga, 1993; Lionetto et al., 2000; Garcia-Santos et al., 2011). Fish exposed to metals often compensate for the experienced disruption of ion regulation by increasing the number of chloride cells in the gills, a process that implies additional energetic demands (Verbost et al., 1987; Lee et al., 1996; Wong and Wong, 2000). Moreover, additional energy expenditures may be needed to cope with Cd-caused gill damage. For example, Cd exposure was shown to result in hypertrophy of gill filaments, hyperplasia and necrosis of the gill lamellae and increased mucus secretion in the exposed fish (Evans, 1987; Verbost et al., 1987; Ferrari et al., 2005).

In addition to gills, other organs are known to respond to Cd exposure with hyperplasia and hypertrophy. For example, liver size was found to increase after long-term exposure to Cd in rainbow trout (Lowe-Jinde and Niimi, 1984) and exposure of gilthead sea bream (*Sparus aurata*) to Cd caused an increase in liver size and upregulation of a cell proliferation marker (proliferating cell nuclear antigen) in liver and kidney (Garcia-Santos et al., 2011). Similarly, increased cell proliferation was found in the liver and kidney of *Puntius gonionotus* exposed to Cd through the diet. This effect was assumed to be a response aiming to compensate for concomitantly occurring cell necrosis (Rangsayatorn et al., 2004).

Exposure to Cd, similar to many other metals, is also known to induce a variety of stress responses. An increase in plasma cortisol levels has been noted in fish exposed to Cd, including tilapia (Fu et al., 1990; Pratap and Wendelaar Bonga, 1990; Ricard et al., 1998), hybrid tilapia (*Oreochromis* sp.) (Wu et al., 2007) and gilthead sea bream (Garcia-Santos et al., 2011). The production of metallothioneins, the proteins that serve to protect the organism by sequestering metals (Olsson, 1993), is frequently induced by Cd exposure, as was shown in tilapia (Fu et al., 1990), red sea bream (Kuroshima et al., 1993), turbot (*Scophthalmus maximus*) (George et al., 1996), common carp (De Smet and Blust, 2001), Atlantic salmon (Berntssen et al., 2001) and yellow catfish

(*Pelteobagrus fulvidraco*) (Kim et al., 2012). Furthermore, increased levels of several ABC transporters, potentially involved in Cd excretion as one of the cellular detoxification mechanisms, have been observed in Cd-exposed Antarctic fish *Trematomus bernacchi* exposed to elevated Cd levels (Zucchi et al., 2010).

Induction of various oxidative stress biomarkers by Cd has also been frequently measured, for example, in Nile tilapia (Almeida et al., 2002; Atli and Canli, 2007), Japanese flounder (Cao et al., 2010), silver catfish (Pretto et al., 2010), marine fish *Salaria basilisca* (Messaoudi et al., 2009) and gilthead sea bream (Souid et al., 2013). An increase in apoptosis, an energetically costly process of programmed cell death, frequently induced by ROS signaling (Robertson and Orrenius, 2000), has also been observed. For example, Cd induced apoptotic DNA fragmentation or apoptotic cell death in dub (*Limanda limanda*) (Piechotta et al., 1999), Atlantic salmon (Berntssen et al., 2001) and topsmelt (Rose et al., 2006).

Obviously, induction of all these processes increases the demands on cellular metabolism to provide the required precursor metabolites as well as energy required to synthesize various proteins and other molecules involved in stress response and defense pathways. Indeed, an association between high levels of metallothioneins and reduction in energetic reserves and/or growth has been found in Cd-exposed topsmelt (Rose et al., 2006), tilapia (Wu et al., 2000) and sea bass (*Dicentrarchus labrax*) (Cattani et al., 1996).

Cd exposure has been frequently observed to result in increased oxygen consumption rates (Suresh et al., 1993; Espina et al., 2000; Rose et al., 2006; Ferrari et al., 2011). This can be assumed to be a compensatory response reflecting an increased need for oxidative metabolism and ATP production required to cope with Cd-induced stress. An alternative explanation suggested by some authors is that the increase in oxygen consumption rates could be due to the hyperactivity induced by Cd exposure. However, Cd-induced hyperactivity has mostly been reported for shorter-term exposures, while during chronic exposure, increased aerobic metabolism appears to be a more plausible explanation for the rise in oxygen consumption. Despite the attempt of the organism to respond to the rising energetic demands by increasing the energy production, it is likely that all the surplus energy produced, as well as a significant proportion of basal energy reserves, would be allocated to cover the additional metabolic costs arising due to Cd. In this way, the energy would be diverted from growth, which would result in growth impairment. Thus, as depicted in Fig. 3, the "generic" KEs of increased metabolic demands and toxicant-induced reallocation of energy resources appear to be the main reason for the growth impairment caused by Cd, while effects on locomotion and their potential association with reduced food intake seem to play only a minor role.

The AOP shown in Fig. 3 is somewhat unique in a sense that it depicts several upstream KEs that all converge at a single common KE (increased metabolic demands), as opposed to a conventional practice of representing individual AOPs as a linear sequence of single KEs. While each of the paths that lead to the KE of increased metabolic demands represents a potential contribution, any one of these individual paths alone may not be sufficient to induce a perturbation of energy fluxes that is strong enough to result in an AO. Thus, the effect of Cd appears to be a net result of multiple pathway activation that need to be considered in the context of AOP networks rather than as a single linear AOPs. The quantitative consideration of how many of these various upstream pathways would need to be simultaneously impacted in order to result in growth impairment can be embedded in the key event relationships between upstream KEs and KE of increased metabolic demands. Alternatively, the quantitative understanding of the magnitude of metabolic perturbation that would lead to impairment of growth could be embedded in the key event relationship between this KE and AO.

6.2. Additional considerations

In environmental risk assessment of Cd effects, it is necessary to take into account the influence of water composition and other external exposure conditions (Peakall and Burger, 2003). For example, Cd toxicity was shown to decrease in media with higher hardness (Hansen et al., 2002a,b; Brinkman and Hansen, 2007; Benaduce et al., 2008) and increase under hypoxic conditions (Hattlink et al., 2005). In regard to toxicity outcomes, environmentally relevant factors, such as food limitation, have been shown to affect the degree of Cd toxicity (Rose et al., 2005).

Significant differences in sensitivity to Cd have been reported across species (Eaton et al., 1978; Hansen et al., 2002a; Tan et al., 2008; Wang et al., 2013). Various factors can account for this. These include variations in metabolic rate (Kolath et al., 2006; Eya et al., 2012; Fuentes et al., 2013) and differential capacities to induce protective responses such as metallothionein production (Kalman et al., 2010) or oxidative stress defense (Hauser-Davis et al., 2012; Srikanth et al., 2013), as well as differences in Cd uptake and bioaccumulation. In regard to the latter, significant cross-species differences in Cd uptake rates have been reported (Niyogi and Wood, 2004; Wang and Rainbow, 2008) and fishes from higher trophic levels were shown to accumulate higher levels of some metals, with a positive relationship between species body weight and metal levels observed (Burger et al., 2002).

Sensitivity differences across life stages are also known. Interestingly, post-swim-up fry are often reported to be more sensitive to Cd compared to embryos and early larvae. Such is the case in Atlantic salmon (Peterson et al., 1983) and brown trout (Brinkman and Hansen, 2007). However, a detailed time-resolved analysis of Cd toxicity during the first day of development showed that the earliest life stages of Japanese medaka (up to morula) were the most sensitive to Cd, with sensitivity rapidly decreasing if exposure was started at later time points (Michibata et al., 1987). In juvenile fish, lower body size was shown to be associated with higher sensitivity to Cd. This was attributed to higher Cd accumulation levels resulting in greater damage occurring before the upregulation of protective responses (Kuroshima et al., 1993).

The proposed AOP for Cd-caused growth impairment may be broadly applicable to other metals as many of them are known to induce similar energetically costly cellular responses (Monserrat et al., 2007; Yoon et al., 2008; Zhou et al., 2008; Chen et al., 2012; Hauser-Davis et al., 2012). Apart from metals, many other compounds were also shown to cause elevation of oxygen consumption rates associated with diminishment of growth, as was the case for example for dieldrin exposure in juvenile largemouth bass (*Micropterus salmoides*) (Beyers et al., 1999). Clearly, energy reallocation can be viewed as a significant "generic" KE occurring in response to many toxicants. Interestingly, even for the compounds where a relatively straightforward explanation of the mode of action appears to be established, as is the case for pyrethroids' effects on locomotion through interference with neurotransmission (see Section 4), an alternative explanation for the observed decrease in locomotion has been suggested to be the disrupted energy allocation, since exposure to pyrethroids could divert the energy for detoxification and antioxidant protection instead of swimming (Oliveira et al., 2012). Theoretically, exposure to pyrethroids could also result in diversion of energy from growth itself. Recently, it has been shown that dietary supplementation of ascorbic acid counteracted the detrimental effects of cypermethrin on growth in the freshwater catfish (Saha and Kaviraj, 2013), but the exact mechanisms behind this action of ascorbic acid have not been established yet. In the future, systematic approaches need

to be developed that allow "ranking" the relative contribution of energy disruption KEs, compared to those associated with more specific modes of action, to the emergence of the observed AOs. Integration of AOP-derived knowledge with computational approaches, such as dynamic energy budget (DEB) models, used to characterize toxicant-induced disruption of energy fluxes (Kooijman and Bedaux, 1996; Jager et al., 2006), may help in establishing and evaluating the quantitative relationships between different events.

7. Potential alternative methods for prediction of effects on fish growth identified through AOP case studies

The AOP case studies presented above highlighted several important KEs plausibly linked to the AO growth impairment, for which we would now like to discuss the potential venues for development of alternative assays.

7.1. KEs "locomotion impairment" and "reduction in food intake"

The KE "reduction in food intake" appeared to be strongly linked to the AO "growth impairment" for two out of the three AOP case studies examined: pyrethroids and SSRIs. Indeed, the notion that a sufficient reduction in food intake would likely lead to growth impairment is biologically plausible. Therefore, once this relationship is understood quantitatively, the measurement of chemical effects on food intake could substitute direct measurement of growth. However, measuring food intake in aquatic organisms is challenging because of the need for longer experiment duration (days to weeks) and the relatively high numbers of animals needed to account for individual variability and control for confounding factors. This makes assessing food intake not much more efficient than measuring growth directly. Therefore, it would be extremely beneficial if an additional upstream KE could be established that can predict probable impacts on food intake and that could be assessed in a more straightforward manner.

The predictive utility of KE "locomotion impairment" for KE "reduction in food intake" appeared to be strong for pyrethroids. For SSRIs, a direct influence on appetite through interference with neuropeptides in the brain appeared to play a more significant role in reduction of feeding, with more research needed to detail these linkages. However, many other insecticides apart from pyrethroids, for example organochlorines, organophosphates and carbamates, are known to interfere with neurotransmission and thus could potentially influence locomotory and foraging abilities of the animals. Therefore, the proposed AOP for growth impairment by pyrethroids mediated through effects on locomotion may prove useful for several other classes of compounds. Furthermore, behavioral alterations in fish larvae can be linked to other apical outcomes, such as survival, and computational modeling approaches, such as individual-based models (IBM), can be used to predict the effects on populations (Murphy et al., 2008). All this may justify an investment into further research on development of locomotion assays with fish early life stages to be used for prioritization or even potential replacement of chronic toxicity tests assessing chemical effects on growth. Automated systems for high-throughput examination of locomotory responses in young fish already exist and recently developed computer-assisted platforms can even be used to study in fish larvae not only locomotion *per se* but also more complex behaviors such as prey capture (Bianco et al., 2011). So far, the most systematic work on fish larvae behavior has been performed with zebrafish (Brustein et al., 2003; Gerlai, 2010; Padilla et al., 2011; Tierney, 2011; Schnoerr et al., 2012; Ahmad and Richardson, 2013; Kalueff et al., 2013; Selderslaghs et al., 2013). Further research on fish larvae locomotion as an endpoint for prediction of prey capture ability and thus potential effects on growth should focus on (i) characterization of robustness and persistence of locomotory responses in fish larvae, (ii) evaluation of predictive capacity of behavioral changes assessed in short-term assays with larvae for longer-term effects on locomotory behavior and prey catching abilities in older animals, (iii) elucidation of quantitative aspects to support such extrapolation and (iv) evaluation of comparability of fish larvae behavioral responses across several different species.

Another question to consider is whether it would be worthwhile to invest in further development of the embryo model with the goal to substitute direct behavioral observations in later stages by embryo-based behavioral or molecular tests. If successful, this "non-animal" model could replace testing with animal life stages that are protected under animal welfare legislation in Europe (EU, 2010). Indeed, assessment of movement can be done in embryos and it was recently suggested as a potential assay for developmental neurotoxicity testing (Selderslaghs et al., 2010, 2013). However, in many cases the patterns of responses as well as sensitivity to certain toxicants significantly differ between embryos and later stages (Airhart et al., 2007; Jin et al., 2009; Lange et al., 2012; Sloman and McNeil, 2012), and one particular disadvantage of embryonic stages is that spontaneous swimming activity is not yet established.

Theoretically, the embryos could also be used to assess certain molecular or biochemical responses related to functioning of nervous system. For example, one might attempt to examine the correlation between the changes in the levels of certain neuropeptides in the embryos and effects on appetite observed later on. Similarly, molecular markers related to performance and control of movements could be assessed. However, for prediction of effects on complex physiological responses such as behavior or appetite, multiple potential molecular mechanisms of disruption would likely need to be tested in the embryo. Even then, the evidence for later occurrence of adverse effects on locomotory or feeding behavior may still remain inconclusive due to the insufficient knowledge on the crosstalk and compensatory circuits among the different pathways. Furthermore, certain molecular players may simply be absent during the embryonic stage due to the yet incomplete maturation of the nervous system. In addition, toxicokinetic aspects such as differences in uptake and biotransformation, as well as the absence of exogenous feeding, may further contribute to discrepancies between behavioral responses observed in embryos and larvae.

Therefore, instead of using the embryos to carry out the inconclusive evaluation of movement patterns or multiple molecular pathways that could later manifest in behavioral alterations, a much more efficient strategy to assess the effects of chemicals on locomotory behavior and prey catching ability might be to use the phenotypic screens during the earliest life stage that would already exhibit such responses physiologically. Early fish larvae that already feed exogenously are known to exhibit several robust locomotory behavior patterns reminiscent of those in juveniles or adult fish. Moreover, even for certain molecular investigations, such as studies of appetite-controlling neuropeptides, the use of exogenously feeding larvae instead of embryos may prove to be a much more realistic test setup, providing data useful for further extrapolation to later stages. At the same time, similar to embryos, the experiments with early larvae still require rather modest space and resource investments. Therefore, although the use of fish larvae falls within the scope of animal experimentation laws (e.g. EU, 2010), modification of current practices for chronic toxicity assessment from prolonged tests with juvenile or adult fish to targeted assessment of relevant physiological responses in the larvae would still offer a significant improvement in terms of animal welfare, namely refinement.

Another research direction could focus on a closer evaluation of locomotion impairment assessment in invertebrates in regard to its capacity to predict similar effects in vertebrates. In particular the mechanisms of neurotransmission are known to be well conserved across these taxa (Narahashi et al., 1998; Salanki, 2000; Francis et al., 2003). However, both higher CNS functions as well as toxicokinetic processes are known to differ widely, which may complicate the establishment of quantitative prediction methods.

7.2. KE "increased metabolic demands"

As discussed above, the organisms exposed to Cd attempt to compensate for increased metabolic demands by increasing their energy production. ATP is most efficiently produced aerobically in actively respiring mitochondria through the mechanism of oxidative phosphorylation. Since this process requires oxygen, oxygen consumption can be used as an indirect measure of metabolic activity and thus may constitute a valuable assay for the KE "increased metabolic demands". Indeed, exposure to Cd has been shown to result in higher oxygen consumption rates in a number of cases (Suresh et al., 1993; Espina et al., 2000; Rose et al., 2006; Ferrari et al., 2011). Another effective measure of mitochondrial activity is the ATP/ADP ratio, which is high when mitochondria are actively respiring and low when ATP production occurs mainly through anaerobic mechanisms such as glycolysis. Furthermore, mitochondrial function in fish can be evaluated by traditional biochemical methods involving isolation of mitochondria or by newer methods, which use fluorescent dyes such as Mitotracker to measure mitochondrial function *in vivo* in cells or even in whole organisms, such as zebrafish embryos (Lin et al., 2006). In addition, a transgenic zebrafish with fluorescent mitochondria has been created, which could be useful for live imaging of mitochondrial effects (Kim et al., 2008). Thus, the described alternative assays can be used to obtain information on mitochondrial activity and its modulation by chemical exposure, allowing to screen for chemicals that could act primarily through induction of cellular toxicity responses resulting in increased metabolic demands and reallocation of organism energy reserves.

8. Conclusion

In conclusion, the AOPs offer a powerful approach to organize and assess the available knowledge, as we have demonstrated by applying the middle-out AOP development strategy on the example of growth impairment as an outcome of chronic toxicity in fish. For selection of KEs to focus on, we suggest to consider (i) importance of a particular process for outcomes related to the endpoint in question, (ii) pathway conservation across species, (iii) frequency of occurrence of a certain disruption and (iv) environmental relevance of chemical-induced effects. Our analysis of AOP case studies for growth impairment by pyrethroids, SSRIs and Cd demonstrated that the reduction in food intake is an important KE strongly linked to growth impairment in case of pyrethroids and SSRIs. The involvement of locomotion impairment in effects on feeding and growth was found to be strong for pyrethroids. For SSRI-induced reduction in food intake, their direct effects on appetite may play a role more important than their impacts on locomotion. In the case of Cd, not the reduction in food acquisition, but the drastically increased metabolic demands appear to best explain the observed growth reduction. This points to a reallocation of energy resources as the main cause of growth impairment by Cd. With these examples we demonstrate that thinking in terms of AOPs allows one to critically review the existing experimental evidence concerning the linkages between toxicity events at different levels of organization. This in turn supports identifying important knowledge gaps as well as potential alternative tests that could be developed for practical risk assessment purposes. The presented AOP case studies also illustrate the difficulties associated with development of single linear AOPs. This emphasizes the need to understand and quantitatively characterize the interplay between multiple pathways potentially contributing to an AO in question. In the future, development of AOP networks should allow the consideration of the net effects of chemicals and chemical mixtures in the context of multiple interlinked AOPs.

The work presented above has been initiated at an international expert workshop "Advancing AOPs for integrated toxicology and regulatory applications" that took place on March 2–7th, 2014, in Somma Lombardo, Italy. Many more aspects related to development and applications of AOPs for risk assessment are highlighted in several other papers originating from the same workshop, which can be found at https://aopkb.org/saop/. In particular, these manuscripts provide a detailed guidance on strategical approaches to AOP development and discuss weight-of-evidence evaluation of AOPs, regulatory acceptance of AOPs and use of AOPs in guiding integrated approaches to testing and assessment.

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