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Release of chitobiase as an indicator of potential molting disruption in juvenile *Daphnia magna* exposed to the ecdysone receptor agonist 20-hydroxyecdysone

You Song[*,a], Linn Mari Evenseth[b], Taisen Iguchi[c,d], Knut Erik Tollefsen[a,e]

[a]Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway

[b]Department of Medical Biology, Faculty of Health Sciences, University of Tromsø-The Arctic University of Norway, NO-9037 Tromsø, Norway

[c]Department of Basic Biology, Faculty of Life Science, SOKENDAI, (Graduate University for Advanced Studies), 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan

[d]Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology (NIBB), National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan

[e]Norwegian University of Life Sciences (NMBU), Faculty of Environmental Science and Technology, Department of Environmental Sciences (IMV), Centre for Environmental Radioactivity (CERAD). P.O. Box 5003, N-1432 Ås, Norway

*Corresponding author:

Name: You Song

Address: Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway

Phone: + 47 02348

Fax: + 47 22185200

E-mail address: you.song@niva.no

Abstract

During arthropod molting, the old exoskeleton is degraded and recycled by the molting fluid. Chitobiase, a major chitinolytic enzyme in the molting fluid, has been widely used as a biomarker to indicate endocrine disruption of molting in arthropods under environmental stress. Although the release of chitobiase has been extensively studied in organisms exposed to molting-inhibiting chemicals, its association with molting and the response of the molting hormone receptor, ecdysone receptor (EcR), is not well understood. The present study was therefore conducted to identify potential linkages between the release of chitobiase, molting frequency and EcR activation in a freshwater crustacean *Daphnia magna* after short-term (96h) exposure to the endogenous molting hormone 20-hydroxyecdysone (20E). A suite of bioassays was used for this purpose, including the chitobiase activity assay, molting frequency assay, viability assay and *in vitro* EcR activation assay. Effect concentrations were compared between different assays analyzed. The results clearly showed that exposure to 20E reduced the chitobiase release and molting frequency in a concentration-dependent manner. Exposure to as low as 250 nM 20E significantly reduced the release of chitobiase after 72h exposure, whereas adverse effects on molting frequency and incomplete molting-associated mortality required higher exposure concentrations of 20E to occur. The EcR reporter assay further showed that as low as 100 nM 20E may activate the EcR *in vitro*. The present study suggested that the release of chitobiase can be used as a sensitive indicator of potential molting disruption in crustaceans after exposure to EcR agonists such as 20E.

Key words

Endocrine disruption, *Daphnia magna*, Chitobiase, 20-Hydroxyecdysone, Ecdysone receptor, Molting

Introduction

Molting is an important biological process for growth, development and reproduction in arthropods and precisely regulated by complex neuroendocrine systems (Zitnan et al., 2007). During a molt cycle, a series of physiological and behavioral changes take place, such as formation of new cuticle, degradation and reuptake of old cuticle, shedding of old cuticle (ecdysis) and post-ecdysis cuticle tanning (Reynolds, 1987). The molting fluid is a cocktail of enzymes and inorganic ions involved in the degradation and recycling of old cuticles (Reynolds and Samuels, 1996). The molting fluid enzymes mainly include chitinolytic enzymes, such as chitinases and chitobiase (*N*-acetyl-β-glucosaminidase), and various types of proteases (Reynolds and Samuels, 1996). The chitinolytic enzymes are responsible for degradation of cuticle chitin to *N*-acetyl-β-glucosamine (NAG) monomers, whereas the proteases break down the cuticle proteins to peptides and amino acids (Reynolds and Samuels, 1996; Merzendorfer and Zimoch, 2003). Controlled expression and secretion of molting fluid enzymes are vital to successful molting in arthropods.

One of the chitinolytic enzymes, chitobiase, has been widely used as a biomarker to study the effects of environmental stressors on molting, growth, biomass and population dynamics in arthropods (Espie and Roff, 1995; Sastri and Roff, 2000a; Zou, 2005; Richards et al., 2008; Conley et al., 2009; Duchet et al., 2011; Qi et al., 2013; de Souza Machado et al., 2017).

Chitobiase is secreted in the epidermis and hepatopancreas (Spindler-Barth et al., 1990; Zou and Fingerman, 1999a) and released to the surrounding environment during molting (Espie and Roff, 1995). The expression of chitobiase is positively regulated by the endogenous molting hormone 20-hydroxyecdysone (20E) through transcriptional activation of the ecdysone receptor (EcR) (Zou and Fingerman, 1999b; Merzendorfer and Zimoch, 2003; Zheng et al., 2008), whereas its release to the environment is normally associated with the ecdysis behavior (Duchet et al., 2011). Therefore, both internal and ambient chitobiase activity may serve as indicators of the molting frequency and quality (Espie and Roff, 1995; Sastri and Roff, 2000b; Hanson and Lagadic, 2005).

Ecdysone receptor agonists are a group of chemicals that can activate the EcR and modulate molting signaling. Exposure to EcR agonists usually leads to delayed or precocious molting, most likely due to perturbation of ecdysis (Song et al., 2017). Based on this mode of action (MoA), various pesticides (e.g. tebufenozide, methoxyfenozide) have been developed against harmful arthropods (Nakagawa, 2005). In some cases, those EcR agonists may also affect nontarget species, such as aquatic crustaceans (e.g. shrimps, crabs and lobsters) which may have both ecological and economic values (De Wilde et al., 2013). Use of epidermal chitobiase mRNA expression and enzymatic activity has been proven to be reliable indicators of EcR activation by EcR agonists (Zou and Fingerman, 1999b; Meng and Zou, 2009). Since the release of chitobiase to the environment is usually associated with the cuticle shedding behavior, it may also become a non-invasive and integrated indicator of molting and disruption of this key step in arthropod development.

The next generation hazard assessment of chemicals requires enhanced predictability across

chemicals and taxa, better understanding and extrapolation between different biological levels of organization, increased use of alternative approaches such as *in vitro* screening and *in silico* modeling, and reduced animal tests (NRC, 2007). The introduction of the adverse outcome pathway (AOP) concept complies with these new strategies by developing a conceptual framework to aggregate, evaluate and visualize data relevant for given adverse effect. An AOP mechanistically links the molecular initiating event (MIE) of a chemical with its biological target, a series of key events (KEs) at multiple biological levels of organization and the adverse outcome (AO, adverse effect) by the causal relationships and weight of evidence (Ankley et al., 2010). An AOP of EcR agonism leading to incomplete ecdysis associated mortality has recently been developed for arthropods (Song et al., 2017). In this AOP, it is suggested that hyper-activation the EcR may inhibit the ecdysis (molting) behavior, thus causing failure in the separation of old and new cuticle and leading to mortality. Based on this mechanistic model, one may hypothesize that EcR activation can also lead to reduced release of chitobiase to the external environment as a result of failed molting. Although the chemically-mediated EcR agonism, internal and external chitobiase activity, and molting disruption have been individually studied, the causal linkages between these events are not clear. Effort to develop and evaluate multiple bioassay and provide causal relationships that support populating the AOP with relevant data is thus currently a key challenge to the development of the AOP itself and provision of tools that may assist Integrated Approaches to Testing and Assessment (IATA) for the hazard assessment of EDCs (Tollefsen et al., 2014).

The aim of the present study was therefore to characterize the quantitative relationships between the release of chitobiase, molting frequency and EcR activation in the Cladoceran

waterflea *Daphnia magna* after short-term (96h) exposure to the EcR agonist 20E. This freshwater crustacean has been used as a standard OECD (http://www.oecd.org/) toxicity testing species and demonstrated to be a good model for studying the effects of environmental endocrine disrupting chemicals (EDCs) on molting (Duchet et al., 2011; Sumiya et al., 2014; Song et al., 2016; Sumiya et al., 2016). Exposure to EcR agonists and chitin synthesis inhibitors have both been shown to inhibit molting in *D. magna* (Sumiya et al., 2014). Reduced release of chitobiase was also reported in *D. magna* exposed to the chitin synthesis inhibitor diflubenzuron (Duchet et al., 2011). Based on the knowledge, this study also aims to assess the sensitivity and suitability of ambient chitobiase activity as an indicator of molting disruption in *D. magna*.

Materials and Methods

Culture

Daphnia magna (DHI strain) were originally obtained from DHI Water Environment Health (Hørsholm, Denmark) and have been cultured in the laboratory of the Norwegian Institute for Water Research (NIVA) for more than 10 years. Briefly, 20 female *D. magna* were cultured in 1000 mL M7 medium (OECD Test Guideline 211) in each glass beaker (animal load: 50 mL medium/daphnid) under conditions of pH 7.8±0.2, temperature 20±1°C and photoperiod 16h:8h. The culture medium was renewed twice a week. Daphnids were daily fed with concentrated unicellular green algae *Pseudokirchneriella subcapitata*, corresponding to approximately 0.1 mg carbon daphnid^{-1} day^{-1}.

Exposure

20-Hydroxyecdysone (CAS 5289-74-7, purity >98%) was purchased from Abcam (Cambridge, UK) and dissolved in dimethyl sulfide (DMSO, Sigma-Aldrich, St. Louis, USA). Exposure was conducted based on the basic principles of the *Daphnia* sp. Acute Immobilisation Test (OECD Test Guideline 202). Briefly, neonatal (<24h old) *D. magna* were exposed to seven nominal concentrations (15.6, 31.2, 62.5, 125, 250, 500 and 1000 nM) of 20E and the solvent (DMSO) control for 96h. The solvent concentration was 0.01% in all groups. The test was run in quadruplicate (each containing 10 daphnids in 50 mL exposure medium in a 100 mL glass beaker). The exposure medium was renewed once after 48h. The test animals were fed daily with *P. subcapitata* to avoid potential stress from starvation. Survival, molting frequency (number of molts) and lethal incomplete ecdysis (old cuticle still attached to the new cuticle at death) were recorded daily. The exuvia and dead daphnids were removed after observation to avoid potential aggregation of microorganisms in the exposure medium. The exposure medium (1.5 mL) was sampled every 24h by filtering through a sterile 0.2 μm polycarbonate filter (Millipore Corp., Molsheim, France) using a sterile syringe to remove bacteria and algae, and stored at 4°C prior to chitobiase analysis.

Chitobiase analysis

The chitobiase activity in the exposure medium was determined according to the methods described previously (Sastri and Roff, 2000b; Hanson and Lagadic, 2005). Briefly, 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (MUF-NAG, Sigma-Aldrich) was used as a

substrate. This substrate can be cleaved into NAG and fluorescent 4-methylumbelliferone (MUF) by chitobiase and the fluorescent intensity of MUF is thus proportional to the activity of chitobiase. The stock solution of MUF-NAG (5 mM) was made by dissolving the chemical in methylcellosolve (Sigma-Aldrich). The medium samples (150 µL, N=4) were incubated with 0.31 mM MUF-NAG (diluted in 50 µL 0.15 M citrate phosphate buffer, pH 5.5) for 1h in Corning™ 96-well black polystyrene microplates (Fisher Scientific, Pittsburgh, USA). The reaction was run in technical triplicates and stopped by adding 50 µL 0.25 M NaOH. The fluorescence was measured immediately using Victor 3 fluorescent plate reader (Perkin Elmer, Waltham, USA) at excitation/emission wavelength of 360nm/450nm. Five concentrations (10, 30, 90, 270, 810 nM) of MUF (Sigma-Aldrich) were included in each plate for generating standard curves. The chitobiase activity was quantified based on the standard curve and expressed as nM MUF liberated per hour (nM/h). Relative chitobiase activity (%) was calculated by comparing the exposed groups to the control and the resulting data used for statistical analysis.

Two-hybrid reporter gene bioassay

A two-hybrid *in vitro* screening bioassay for detection of the EcR reporter gene activation in *D. magna* was performed according to Kato et al. (2007) with some modifications (Song et al., 2016). Briefly, Chinese hamster ovary (CHO-K1) cells (ECACC, Salisbury, UK) were cultured in Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific, Waltham, USA) containing 10% fetal bovine serum (Sigma-Aldrich) at 37°C and 5% CO_2. The cells were transferred to a 24-well plate (10000 cells/well) and transfected for 4h using FuGENE6 (Roche

Diagnostics, Basel, Switzerland) following the original protocol from the manufacturer. In the transfection mixture, 30 ng pBIND-dapEcR (LBD) vector, 30 ng pACT-dapUSP (LBD) vector, 100 ng aACT-droTaiman (LXXLL) vector, 300 ng pG5Luc vector and 100 μL fetal bovine serum were added. The vectors were initially provided by Prof. Taisen Iguchi (National Institute for Basic Biology, NIBB, Okazaki, Japan), cloned by transforming the chemically competent *Escherichia coli* (Invitrogen, Carlsbad, California, USA), and purified using GenElute™ HP Plasmid Midiprep Kit (Sigma-Aldrich), according to the manufacturer's instructions. The ecdysone steroid hormone ponasterone A (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used as the positive control at 1000 nM and DMSO used as the negative control. The concentration of 20E ranged from 0.1-1000 nM. The transfected cells were exposed to the hormone analogs for 40h at 37°C and the procedure was run in technical triplicates. The activity of the reporter gene was determined using Dual-Luciferase® Reporter Assay System Kit (Promega, Madison, USA). The luminescence intensity was measured using Victor 3 Multilabel Plate Reader (Perkin Elmer). The assay was repeated independently for three times. The median expression of the technical replicates in each independent study was calculated and used as biological replicates (N=3). Relative expression was scaled between 0 (negative control) and 100% (maximal induction, 1000 nM ponasterone A) prior to statistical analysis.

Statistical analysis

Data were checked for normality and equal variance prior to statistical analysis of differences between control and exposed animals using one-way analysis of variance (one-way ANOVA) followed by Tukey post-hoc test, or Kruskal-Wallis non-parametric test (no normality or equal

variance) followed by Dunn's posthoc test. The statistical analyses were performed in Graphpad Prism v6.01 (Graphpad Software Inc., La Jolla, USA) and a probability (p) value of 0.05 was applied to all analyses. The median lethal (LC50) and effect (EC50) concentrations were estimated based on a sigmoidal concentration-response curve (variable slope) calculated using Graphpad.

Results

The EcR reporter gene assay showed that exposure to as low as 100 nM 20E may marginally induce the reporter gene expression, whereas exposure to 1000 nM 20E caused approximately 25% activation of EcR *in vitro* compared to the positive control (Figure 1).

Results from the chitobiase analysis showed that the ambient chitobiase activity in the control media increased with the test duration, and coincided with the molting events that occurred in *D. magna* (Figure 2A). Exposure to 20E reduced the ambient chitobiase activity in a concentration-dependent manner, with exposure to 1000 nM 20E significantly reducing chitobiase activity after 24h (p=0.0008), 125 nM and higher concentrations of 20E significantly decreasing the chitobiase activity after 72h (p=0.0011), 250 nM and higher concentrations of 20E reducing chitobiase after 96h (p<0.0001). After 48h, slight but non-significant elevation of chitobiase activity was observed after exposure to 62.5 and 125 nM 20E, whereas a marginal non-significant reduction was observed at 1000 nM 20E. Based on the concentration-response curves, the estimated EC50s of 20E for chitobiase activity were 717.3 (24h), 446.7 (72h) and 397.2 (96h) nM.

The effect of 20E on the molting frequency of *D. magna* was also concentration-dependent (Figure 3A), with 1000 nM significantly reduced molting after 48h (p=0.01), 72h (p<0.0001) and 96h (p<0.0001). No significant reduction in molting frequency was observed after 24h exposure or at concentrations lower than 1000 nM, albeit slight, but non-significant decrease in molting frequency was apparently observed in daphnids exposed to the lowest (15.6 nM) concentration of 20E after 24, 48 and 96h.

Results from the 96h toxicity test showed that the lethal effect of 20E on the survival of *D. magna* was concentration-dependent, with exposure to 1000 nM 20E significantly reducing the survival after 48h (p<0.0001), 72h (p<0.0001) and 96h (p<0.0001). After 24h, the highest concentration of 20E slightly decreased the survival of *D. magna*, however, this reduction was not significant. Marginal and non-significant lethal effects were also observed in *D. magna* after 48, 72 and 96h exposure to 500 nM 20E. Exposure to lower concentrations of 20E did not lead to any lethality. Based on the concentration-response curves (Figure 3B), the estimated median lethal concentrations (LC50) of 20E for survival were 826.7 (72h) and 738.9 (96h) nM. A summary of estimated effect concentrations for different endpoints is shown in Table 1. In addition, lethal incomplete ecdysis was observed in daphnids after 72 and 96h exposure to 1000 nM 20E (Figure 3C).

Discussion

The present study mainly focused on using the release of chitobiase as an indicator of molting disruption in *D. magna* after short-term exposure to the endogenous EcR agonist 20E. The

chitobiase activities detected in the exposure medium were in the same order of magnitude as that reported for *D. magna* previously (Duchet et al., 2011; Qi et al., 2013), confirming the validity of the bioassay for determining exogenous enrichment of this molting indicator. Moreover, temporal changes of chitobiase activity in the controls (unexposed) were similar to that observed by Duchet et al. (2011) and Qi et al. (2013), who also used <24h old neonatal *D. magna* at the start of the exposure and found increased chitobiase release from 24 to 96h. In the present studies, a temporal increase in the release of chitobiase to the ambient medium was observed in unexposed daphnids, whereas a concentration-dependent decrease in the release of chitobiase was observed after exposure to 20E. Based on the concentration-response curves, as low as 250 nM 20E was found to significantly reduce the chitobiase release in *D. magna* after 72h, thus suggesting 72h to be optimal for studying the effect of 20E on chitobiase release. Although not tested specifically herein, chitobiase may be caused by other sources than the molting in *D. magna* such as microorganisms (Conley et al., 2009) and may potentially contribute to the chitobiase activity measured during the test. Since the exposure study described herein was conducted in a highly controlled manner (i.e. all groups under the same conditions), and only relative expression of chitobiase (i.e. exposed vs control) was used, it is considered that the influence from the microorganisms was minimized.

In comparison with molting, the temporal release of chitobiase was generally in accordance with the molting frequency in unexposed (control) animals. The activity of chitobiase in unexposed daphnids generally increased during the test period, possibly indicating that the growth (size) of the animal may also influence the ambient activity of chitobiase, as previously demonstrated (Conley et al., 2009; Duchet et al., 2011).

Results from the EcR reporter assay in the present study suggested that exposure to 1000 nM 20E *in vitro* significantly induce the EcR expression. This effect concentration was similar to that reported by Kato et al. (2007). Chitobiase is considered under direct regulation of EcR, as induction of chitobiase expression has been observed in both crustaceans and insects after exposure to EcR agonists (Reynolds and Samuels, 1996; Zou and Fingerman, 1999b; Merzendorfer and Zimoch, 2003; Zheng et al., 2008). It may be deduced from the *in vitro* data that the expression of chitobiase in *D. magna* was likely induced at similar concentrations in the present study. On the other hand, release of chitobiase to the environment may also be indicative of incomplete ecdysis as a downstream event of EcR-mediated disruption of normal molting behavior (Song et al., 2017), as the release of molting fluid is considered highly dependent on the separation of old and new cuticle at the end of a molt cycle (Reynolds and Samuels, 1996; Duchet et al., 2011). Recent effort to evaluate the causality between the hyper-activation of the EcR with triggering of key molecular and cellular events that eventually lead to molting interference and mortality, identify that processes relevant for chitobiase release are indeed key in an AOP that describe points of departure from normal endocrine function due to exposure to putative endocrine disruptors in arthropods (https://aopwiki.org/aops/4).

When comparing the chitobiase activity with the molting frequency in animals exposed to 20E, concordance was only found at 1000 nM 20E, where significant reduction in ambient chitobiase and molting were observed after 72 and 96h exposure. However, at this exposure concentration, severe lethal effects were also observed after 72 and 96h, thus making it difficult to judge whether these ED effects were consequences of lethality, or vice versa. It was likely that the lethal effect of 20E was attributed by impaired molting, as incomplete ecdysis was

observed in animals at death in the present study. Unsuccessful exuviation was observed in juvenile *D. magna* after 2d exposure to 1000 nM 20E (Bodar et al., 1990). Lethal incomplete ecdysis was previously documented in juvenile *D. magna* after exposure to 20E (260 nM) and ponasterone A (27 nM) in a 21d chronic test (Baldwin et al., 2001). Acute (48h) exposure to 500 nM 20E was also demonstrated to cause complete inhibition of molting in adult female *D. magna* (Sumiya et al., 2014). In addition, similar molting defects have been frequently reported in various insects after exposure to EcR agonists (Retnakaran et al., 2003; Reynolds et al., 2009; Gelbic et al., 2011). These evidences taken together suggest that the ED effects of 20E on molting were likely the causes of lethality.

This hypothesis was further supported by significant reduction in chitobiase release observed at sublethal concentrations of 20E (e.g. 250 and 500 nM after 72h, 500 nM after 96h). Interestingly, at these concentrations, no significant decrease in molting frequency was observed, indicating that physiological changes such as activation of EcR and chitobiase occurred at lower concentrations than those causing adverse effects on molting. The present study suggested that changes in chitobiase activity was likely not the only physiological change required to cause molting disturbance. This agree well with suggestions that activation of the EcR, inhibition of neuropeptide release, reduced neurotransmission and ecdysis motor program, and incomplete separation of old and new cuticles ultimately lead to molting disruption (Song et al., 2017). Although causal relationships between EcR activation, reduction in chitobiase and perturbations of normal molting is proposed herein, better synchronization of the test animals at the onset of studies may reduce variability and increase the sensitivity of the assays used. The duration of the molt cycle may serve as a sensitive endpoint for disturbance of normal

molting and thus be used to assess causality between molting quality and release of chitobiase, as in many cases, delayed molting may occur rather than complete inhibition of molting. Slight delay in molting may not influence the overall molting frequency when observations are made at time intervals longer than the molt cycle itself. This in particular applies to test with neonatal *D. magna* which normally perform the first and second juvenile molting at approximately 27.5 and 52h after birth (Mu and LeBlanc, 2004). It was clearly shown that the internal (tissue) activity of chitobiase negatively correlated with the duration of molt cycle in *D. magna* under food or temperature stress (Espie and Roff, 1995). The linkage between the release of chitobiase to the external environment and duration of molt cycles, however, has not been studied in detail yet.

Furthermore, when comparing the effect concentrations of 20E between different endpoints (Table 1), the release of chitobiase was identified to be the most sensitive endpoint in response to 20E, especially after 72 and 96h. The LOEC obtained from the current study suggested that chitobiase was the most sensitive indicator of changes to ecdysis. Induction of the EcR may likely occur at similar concentrations, although the experimental design of the *in vitro* EcR activation study did not allow sufficient discriminating power to assess this in detail. It is still not clear how reduction in chitobiase release correlates with the activation of the EcR *in vivo*, as both molting quality and other factors such as size of the animal may potentially influence the ambient chitobiase activity. Future studies should aim at determining the endogenous chitobiase mRNA and enzyme expression after exposure to EcR agonists, and causally as well as quantitatively link the responses to the duration of molt cycles and ambient chitobiase activities. In addition, the approaches described herein need to be further verified using other

environmental EcR agonists (e.g. pesticides) to evaluate the assays suitability for screen environmentally relevant EDCs. Knowledge generated from such studies may be used to develop the chitobiase assay as a screening tool for chemicals with EcR agonistic and molting disrupting activities, and by doing so assist screening and prioritization of chemicals, limiting experimental animal testing as part of IATA approaches in assessment of EDCs (Tollefsen et al., 2014).

Conclusions

The present study evaluated the applicability of using chitobiase release as an indicator of potential endocrine disruption of molting in juvenile *D. magna* after short-term exposure to the model EcR agonist 20E. The results clearly showed that the release of chitobiase was more sensitive than other relevant endpoints such as molting frequency and survival, and may together with activation of the EcR be used as a biomarker for endocrine disrupting chemicals that interfere with EcR-mediated toxicity pathways leading to molting disturbance. As a first attempt to identify linkages between EcR agonism, molting and chitobiase release, the present study provides an initial assessment of the suitability of these endpoints in ED studies focusing on molting and development in arthropods.

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Table 1. A summary of effect concentrations (nmol/L) of 20-hydroxyecdysone for different endpoints in juvenile *Daphnia magna*.

Endpoint	24h			48h			72h			96h			*in vitro*		
	NOEC[a]	LOEC[b]	EC50[c]	NOEC	LOEC	EC50	NOEC	LOEC	EC50	NOEC	LOEC	EC50	NOEC	LOEC	EC50
Survival	1000	>1000	1348[e]	500	1000	1520[e]	500	1000	826.7	500	1000	738.9	/	/	/
Molting	1000	>1000	2801[e]	500	1000	1489[e]	500	1000	1776[e]	500	1000	1478[e]	/	/	/
Chitobiase	500	1000	717.3	1000	>1000	3730[e]	125	250	446.7	250	500	397.2	/	/	/
EcR[d]	/	/	/	/	/	/	/	/	/	/	/	/	100	1000	2153[e]

a. NOEC: No Observed Effect Concentration

b. LOEC: Lowest Observed Effect Concentration

c. EC50: Median Effect Concentration

d. EcR: Ecdysone receptor activity

e. Predicted values, observed effect less than 50%

Figures:

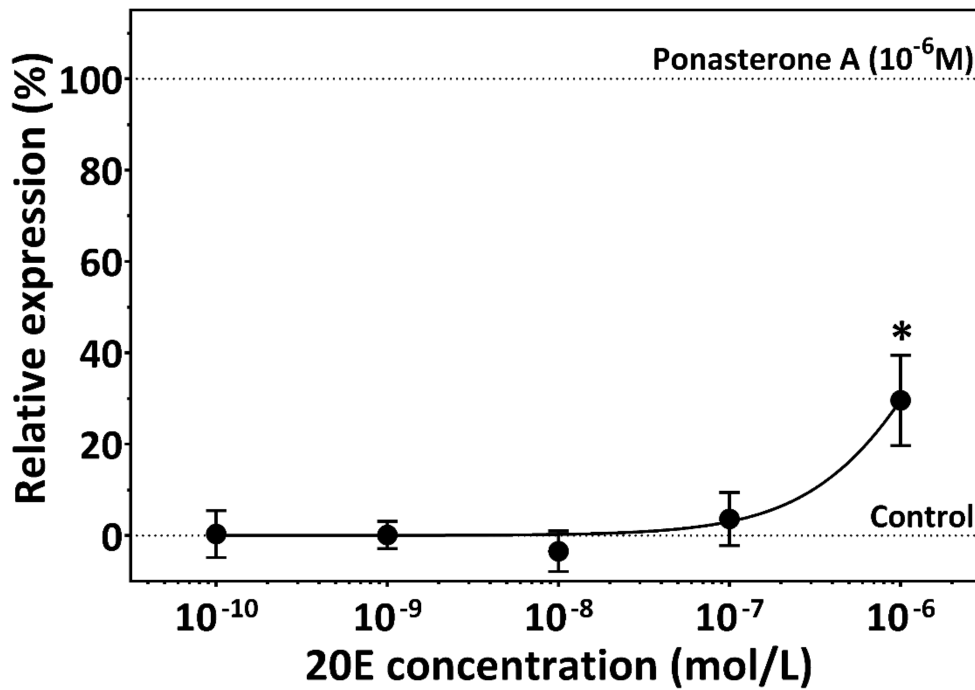

Figure 1. Relative expression of *Daphnia magna* ecdysone receptor (EcR) in transfected Chinese hamster ovary (CHO-K1) cells after 40h exposure to 0.1-1000 nM 20-hydroxyecdysone (20E). The relative expression was scaled between the expression of EcR in the control (DMSO, 0% induction) and positive control (1000 nM ponasterone A, 100% induction). Results are presented as mean±SEM, N=3. * denotes significant different from the control (p=0.0224).

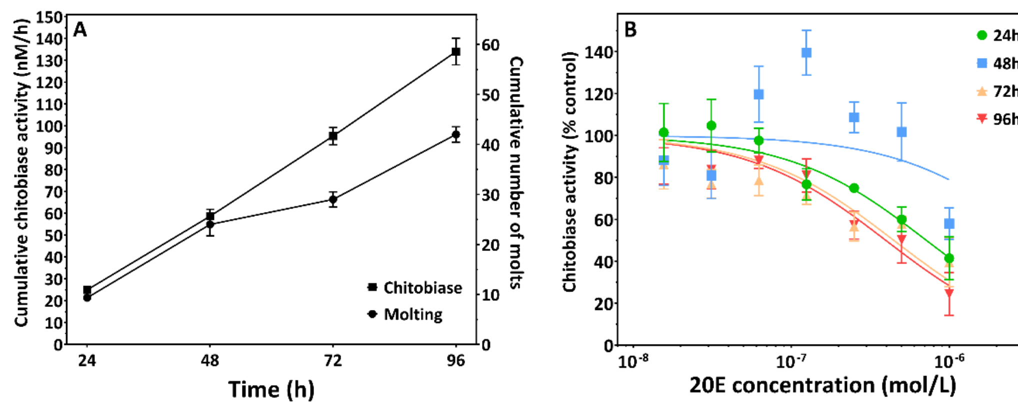

Figure 2. Temporal (24-96h) change of cumulative chitobiase activity and cumulative molting frequency in unexposed (control) juvenile *Daphnia magna* (A); Effects of 20-hydroxyecdysone on chitobiase release in *D. magna* after 24-96h exposure (B). Results are presented as mean±SEM, N=4 (10 individuals in each replicate).

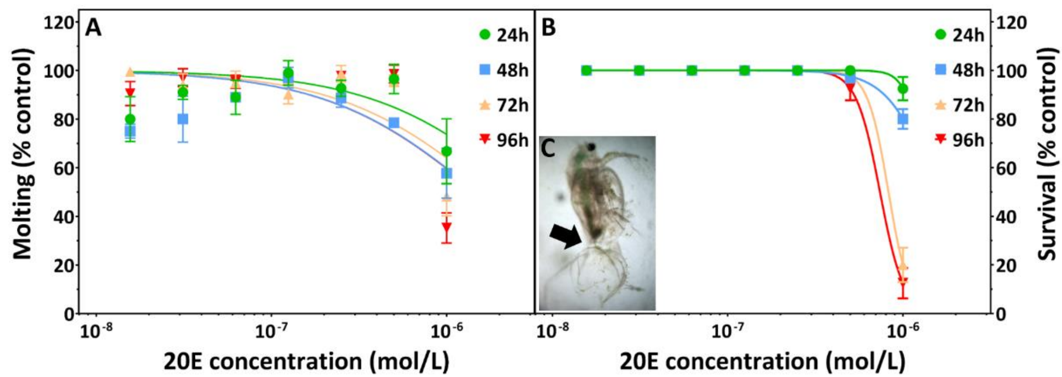

Figure 3. Molting frequency (A) and survival (B) in juvenile *Daphnia magna* after 24-96h exposure to 20-hydroxyecdysone (20E), results are shown in % compared to the control and presented as mean±SEM, N=4; Lethal incomplete ecdysis in *D. magna* after 72h exposure to 1000 nM 20E (C), arrow indicates incomplete shedding of old cuticle in *D. magna* at death.