Whole-organism transcriptomic analysis provides mechanistic insight into the acute toxicity of emamectin benzoate in *Daphnia magna*

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ABSTRACT

Emamectin benzoate (EMB) is an anti-sea lice chemical widely used in the aquaculture that may also unintentionally affect non-target crustaceans in the environment. Although the adverse effects of this compound are well documented in various species, the full modes of action (MoAs) are still not well characterized. The current study was therefore conducted to characterize the MoAs of EMB and link perturbations of key toxicological pathways to adverse effects in the model freshwater crustacean *Daphnia magna*. Effects on molting and survival were determined after 48h exposure to EMB, whereas global transcriptional changes and the ecdysone receptor (EcR) binding potency was determined to characterize the MoA. The results showed that the molting frequency and survival of *D. magna* decreased in a concentration-dependent manner, and the observed changes could not be attributed to direct interactions with the EcR. Major MoAs such as activation of glutamate-gated chloride channels and gamma-aminobutyric acid signaling, disruption of neuroendocrine regulation of molting, perturbation of energy homeostasis, suppression of DNA repair and induction of programmed cell death were observed by transcriptional analysis and successfully linked to the adverse effects. This study has demonstrated that acute exposure to intermediate and high pM levels of EMB may pose hazards to non-target crustaceans in the aquatic environment.

KEYWORDS: Emamectin benzoate, *Daphnia magna*, mode of action, molting, adverse outcome pathway

■ INTRODUCTION

Sea lice are a family of copepods (C*rustacea, Arthropoda*) living as parasites in the mucus, epidermal tissues and blood of the host fish and severely affecting the fitness of farmed fish, thus causing great economic loss.[1] These external parasites may cause skin ulcerations in the neck regions and lead to dysfunction of

osmoregulation and secondary bacterial infections in fish.[2] Anti-sea lice treatments such as using avermetin-derived medicines have proven to be highly efficient for controlling the life cycles of both juvenile and adult sea lice with minimal hazards to fish and human,[3, 4] albeit these treatments are costly (over €300 million/year).[5] Emamectin benzoate (EMB) is the main ingredient of the anti-sea lice treatment SLICE® (Merck Animal Health) and heavily used in major regions of salmonid aquaculture worldwide, including Norway, Canada, Scotland, Ireland and Chile.[2] Treatment with EMB is dependent on the type of sea louse and the infected host fish. For example, An oral dosage of 50 μg/kg EMB daily for seven consecutive days has been commonly used to treat infected Atlantic salmon (*Salmo salar*) against the salmon louse *Lepeophtheirus salmonis*.[2] The use of EMB tends to increase in most of these countries,[6-8] due to elevated resistance in sea lice to EMB or reversion to EMB from alternative treatments such as hydrogen peroxide, azamethiphos and pyrethroids, upon development of resistance also to these compounds.[9] Although EMB is mainly used against copepods, it may affect other aquatic crustaceans such as the American lobsters (*Homarus americanus*) and the spot prawn (*Pandalus platyceros*) living in the vicinity of salmon farms as these animals also ingest EMB-medicated salmon feed.[10-16] This raises the concern that non-target aquatic organisms, especially invertebrates, may potentially be impacted by use of anti-sea lice treatments in areas with intense fish aquaculture activities.

Avermectins (AMs) such as EMB are a class of endectocides (antiparasitic drugs) and generally identified as positive allosteric modulators of ligand-gated chloride channels.[17] Some researchers propose that the main mode of action (MoA) of AMs in invertebrates is to activate glutamate-gated chloride channels (GluCls), while others suggest that AMs may bind to and activate the ionotropic gamma-aminobutyric acid receptors (GABAR).[18, 19] Both MoAs may increase the membrane permeability of the chloride ions and inhibit the somatic neurotransmission, thus causing paralysis and associated mortality.[19] Other effects of EMB in crustaceans such as interference with molting[11] and induction of apoptosis[16] have also been reported. However, the toxic mechanisms leading to these effects have not been well characterized.

The water flea *Daphnia magna* has become a model crustacean species in ecotoxicological studies. Being used as a standard OECD (http://www.oecd.org/) toxicity test species, *D. magna* possesses a number of advantages, such as short asexual reproduction cycle, transparent body, well-studied genome, easy to maintain under laboratory conditions and sensitive to environmental contamination. The benefits of using whole-organism *D. magna* for studying the GABAergic effects and neuro-endocrine disruption have been widely recognized.[20] To understand the toxic effects and mechanisms of EMB on non-target crustaceans, the current study used *D. magna* as the test model, with the main objectives to: 1) identify short-term (48h) effects of EMB on survival and molting; 2) confirm the known MoAs and characterize potential novel MoAs of EMB based on transcriptional analyses and an *in vitro* assay for interaction with the EcR; and 3) link toxic mechanisms and adverse effects of EMB for future hazard assessment.

■ MATERIALS AND METHODS

Daphnia culture and exposure. *Daphnia magna* (DHI strain) was cultured in M7 medium (pH 7.8±0.2, 20±1°C) under a photoperiod of 16h:8h (Supporting Information, SI). Juvenile *D. magna* (<24h old) were used in all tests. Culture and exposure conditions were according to the OECD test guideline 202 (http://www.oecd-ilibrary.org/). The exposure concentrations were chosen based on a range-finding toxicity test (48h). A time-course exposure was performed first to determine the optimal sampling time point for transcriptional analysis. The optimal sampling time was chosen based on the responses of genes involved in a

few known MoAs of EMB, such as glutamate and GABA receptor signaling pathways. In the temporal study, neonatal *D. magna* were exposed to 2000 pM EMB for 48h. Twenty individuals were sampled at 0, 12, 24, and 48h post exposure, pooled for each time point (N=1) in RNALater (Qiagen, Hilden, Germany) and stored in -80°C until later use. The global gene expression of exposed group was compared to the corresponding control (DMSO) at each time point. In the second study, 5 nominal exposure concentrations of EMB, 7.8, 31.2, 125, 500 and 2000 pM were tested. Neonatal *D. magna* in each independent exposure unit (N=9) containing 50 mL of exposure medium were exposed to EMB or the solvent control. After 12h exposure (as determined to be optimal by the time-course study), 5 biological replicates (each contained 8 pooled individuals) were sampled in RNALater for transcriptional analysis (microarray and qPCR) and stored in -80°C until use. The 4 remaining replicates (each contained 5 individuals) continued exposure until 48 h. Molting frequency and immobilization were recorded after 12, 24, and 48h exposure. Exposure variables such as pH, dissolved oxygen and temperature were recorded throughout the experiment. The exposure medium in the lowest and highest EMB exposure groups were sampled at 0, 12 and 48h post exposure for chemical analysis of EMB using HPLC/HRMS (SI).

Microarray design and transcriptional analysis. The high density custom *D. magna* oligonucleotide microarrays were designed based on *de novo* assembly of *D. magna* sequences from RNA sequencing and manufactured by Agilent Technologies (Santa Clara, CA, USA). The microarray platform can be accessed at Gene Expression Omnibus (GEO, accession No.: GPL22095). Total RNA was isolated using ZR Tissue & Insect RNA MicroPrep™ kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's protocol (SI). The RNA purity (260/280>1.8, yield>50 ng/μL) and integrity (clear peaks of RNA and flat baseline) were checked using Nanodrop® ND-1000 (Nanodrop Technologies, Wilminton, Delaware, USA) and Bioanalyzer (Agilent), respectively. The one-color microarray analysis (N=4 biological replicates out of five sampled) with 50 ng of input RNA was performed according to Agilent's standard protocol with modifications.[21] Raw microarray data was quality assessed and normalized (quantile method) using Gene Spring v10.7 (Agilent), and deposited in GEO (accession No.: GSE83859). Differentially expressed genes (DEGs) were determined using one-way ANOVA followed by Benjamini and Hochberg false discovery rate correction (corrected $p<0.05$) and enriched for Gene Ontology (GO) functions as previously described.[21] Pathway analysis was performed using mapped homolog DEGs of *D. magna* towards curated *D. melanogaster* pathways in Reactome (http://www.reactome.org/). Quantitative real-time RT-PCR analysis (N=5 biological replicates) was performed on the same RNA sample to verify the microarray analysis for a selection of DEGs (14 target and 2 reference genes) and to characterize the transcriptional responses of relevant biomarker genes for a selection of potential MoAs (SI).

Two-hybrid EcR reporter assay. An *in vitro* screening assay for EcR binding and reporter gene activation was performed as described previously by Kato and colleagues,[22] with modifications (SI).

■ RESULTS AND DISCUSSION

Exposure Verification. The measured pH before and after 48h exposure to EMB was in the optimal range (7.8±0.2) for *D. magna*. Slight increase of pH (e.g. from 7.70±0.02 to 7.78±0.02) was observed in all groups after 48h exposure to EMB, but considered to be within normal variance in such tests. The exposure concentrations of EMB in the test media decreased in a time-dependent manner, with the lowest (7.8 pM)

exposure group decreased to approx. 41% and 22% of the nominal concentrations after 12 h and 48 h, respectively, and the highest (2000 pM) exposure group decreased to approx. 31% and 20% of the nominal concentrations after 12 h and 48 h, respectively (SI, Figure S1). Reduction of EMB concentrations was also observed in another *D. magna* study in which the measured EMB concentrations were 70% lower than nominal after 48h exposure.[23] The instability of EMB in the exposure system has been proposed to be due to its rapid photodegradation in aqueous solution.[24]

Adverse Effects and Ecdysone Receptor Activity. After 48 h exposure, increase in mortality of *D. magna* were observed at concentrations as low as 31.2 pM and with 100% mortality occurring at 2000 pM EMB (Figure 1). No-observed-effect-concentration (NOEC) and half maximal effective concentration (EC50) were determined to be 7.8 pM and 143.3 pM, respectively, and were several orders of magnitude lower than previously reported values for EMB in other crustaceans.[19] The fairly large differences in effect concentrations may partly be due differences in exposure conditions and susceptibility of *D. magna* strains to the toxicant.[25, 26] but mortality observed herein seems to correspond well to that of sea lice and several other non-target marine crustaceans, such as *Acartia clausi*, *Pseudocalanus elongatus*, *Temora longicornis*, *Oithona similis* and the spot prawn.[16, 19, 27]

The molting frequency of *D. magna* was slightly increased after exposure to 7.8 and 31.2 pM EMB, and decreased after 24h exposure to 500 and 2000 pM of EMB (Figure 1). Clear concentration-dependent decrease of molting frequency was observed after 48h exposure (EC50=165.1 pM). The highest concentration of EMB did not inhibit molting completely after 48h, however. High correlation (R^2=0.98, p=0.0001) between lethal effects and molting inhibition was found, indicating a connection between increased lethality and molting (Figure 1). However, it is interesting to note that after 24h, exposure to 500 pM EMB did not result in any mortality but still caused substantial reduction of molting frequency, indicating that at lower concentration(s), inhibition of molting may occur independent of lethal effects or even contribute to lethality. The molting inhibition effect of EMB has been recently reported for *D. magna* with a 24h lowest-observed-effect-concentration (LOEC) of 600 pM,[23] compared to the 48h LOEC of 125 pM found in this study. The effects of EMB on molting were slightly different in other crustacean species, where premature molting and associated mortality were repeatedly documented in the American lobsters after long-term (>14d) oral exposure to 0.05-1 μg/g body weight^{-1} of EMB.[10-14] No effect on molting, but significant mortality was reported for the spot prawn after 8d sediment exposure to 0.1-0.8 mg/kg sediment of EMB.[16]

The EcR *in vitro* reporter assay did not identify EMB to be a ligand for the *D. magna* EcR (one-way ANOVA, p=0.94). In contrast, the potent EcR agonist ponansterone A led to concentration-dependent induction of the EcR reporter gene with an EC50 of 6294 pM (Figure 1).

Global Transcriptional Responses and Functional Analysis. The preliminary time-course gene expression study showed that dramatic transcriptional responses occurred in *D. magna* after 12h exposure to 2000 pM EMB, including several highly up-regulated genes associated with the primary MoA of EMB, such as sodium-chloride GABA transporter, cuticle protein 3A and EcR A2 (SI, Figure S2), while less than 10% mortality was observed. Therefore, 12h was considered as an optimal time point studying the effects of EMB on gene expression. In the second (concentration-response) study, massive transcriptional changes were also identified in *D. magna* after 12h exposure to 31.2 (2880 DEGs), 500 (4541 DEGs) and 2000 pM EMB (5759 DEGs), whereas relatively marginal responses were found in animals exposed to the lowest (7.8 pM, 210 DEGs) and intermediate (125 pM, 119 DEGs) concentrations of EMB (SI, Figure S3). The

no-observed-transcriptional-effect-level (NOTEL) was likely below 7.8 pM. Interestingly, exposure to 125 pM EMB resulted in more than 50% mortality of *D. magna* after 48h, but only caused marginal transcriptional responses after 12h. This was likely due to a transition from sublethal to lethal toxic mechanisms at this intermediate exposure concentration of EMB. It has been currently recognized that environmental stressors such as toxins and ionizing radiation may produce hormetic responses (i.e. stimulation at low dose, inhibition at high dose), especially when the research focus is on the early stress responses at the molecular levels (e.g. gene expression, protein synthesis etc.).[28] This may also be the case for EMB, which likely activated massive transcriptional responses in *D. magna* at low concentrations to maintain homeostasis. When the accumulated damage exceeded the capacity of the defense mechanisms, other mechanisms may be activated. Nevertheless, the hermetic responses are rather complicated and further investigations are needed. Based on k-means clustering analysis, 4 major patterns of global transcriptional responses were identified, with two of them displaying concentration-dependent up-regulation and down-regulation of DEGs (SI, Figure S4).

Functional analysis of DEGs showed that a total of 276 (31.2 pM), 113 (500 pM) and 98 (2000 pM) GO functions were overrepresented, with the majority of GOs being concentration-specific (SI, Figure S5). Briefly, exposure to 31.2 pM EMB specifically regulated DEGs involved in the immune responses, calcium homeostasis, neurogenesis and DNA repair. Exposure to 500 pM EMB uniquely affected DEGs associated with neural tube formation and protein ubiquitination, whereas 2000 pM EMB resulted in differential regulation of genes related to cell cycle regulation and DNA repair, redox reactions, respiratory system development and neurotransmitter transportation. Functions such as ion transport, neuron development, oxidoreductase activities were also found to be commonly regulated by exposure to 31.2 and 500 pM EMB.

Ortholog mapping of DEGs showed that approximately 92% (7.8 pM), 84% (31.2 pM), 89% (125 pM), 85% (500 pM) and 89% (2000 pm) of the *D. magna* DEGs were identified to be potential *Drosophila melanogaster* orthologs. The pathway enrichment analysis showed that a total of 12 (7.8 pM), 107 (31.2 pM), 1 (125 pM), 109 (500 pM) and 65 (2000 pM) *D. melanogaster* Reactome pathways were affected by EMB (SI, Figure S6). Pathways into gene expression (7.8 and 2000 pM), signal transduction (31.2 and 500 pM) and transmembrane transport of small molecules (125 pM) were identified by functional grouping to be the top categories with the most supporting pathways (SI, Figure S7). Cell-cell communication (31.2 pM), programmed cell death (500 pM), DNA repair (2000 pM) and organelle biogenesis and maintenance (2000 pM) were found to be only affected by specific concentrations of EMB. A Venn diagram analysis further identified common and unique pathways that were affected by exposure to EMB (Figure 2). Based on the results of transcriptional analysis, toxicologically relevant pathways and supporting DEGs representative of potential MoAs of EMB were summarized (SI, Table S2).

Toxic Mechanisms. ***Activation of ligand-gated chloride channels.*** In line with the previously proposed MoA of EMB in invertebrates, the present study identified DEGs related to both GluCl and GABAR signaling. The ionotropic glutamate receptor (*iGluR/CG3822*) was significantly up-regulated in *D. magna* after exposure to 2000 pM EMB, while the ligand-gated chloride channel homolog 3 (*Lcch3*), an ortholog of the arthropod ionotropic $GABA_A$ receptor,[29] was significantly up-regulated after exposure to 500 pM (microarray) and 2000 pM (qPCR) EMB (Figure 3). The GABA type b receptor subunit 2 (*GABA-B-R2*) was also found to be

significantly up-regulated (2000 pM EMB) by qPCR, albeit the microarray analysis suggested that EMB suppressed the expression of this gene. The discrepancies of results generated might be due to probe design for the microarray is more susceptible to errors than the primer design for qPCR due to the larger sequence used for microarray probes. Overall, the results obtained from microarray were in agreement with the qPCR, as the patterns of transcriptional changes were similar for most of the genes tested using the two techniques. The $GABA_B$ receptors are metabotropic transmembrane receptors operating the potassium channels and often targeted by therapeutic drugs such as baclofen.[30] These results suggested that besides activation of the ionotropic receptors, EMB may also interfere with the metabotropic receptors, thus modulating the transmembrane conductance and neurotransmission. Recent studies on the sea lice (*Lpeophtheirus salmonis*) and the silkworm (*Bombyx mori*) both showed that exposure to AMs activated both the GABA and iGluR, and thus verify that the MoA of EMB is similar to that seen in other arthropods.[31, 32]

Results from the current study also suggested that exposure to EMB may affect the GABA metabolic processes in *D. magna*. The pathway of GABA synthesis, release, reuptake and degradation was significantly enriched by DEGs. The up-regulation of glutamic acid decarboxylase 1 (gad1, 31.2 and 500 pM EMB), which regulates the decarboxylation of glutamate to synthesize GABA,[33] indicating potential activation of GABA synthesis. Mitochondrial GABA transaminase (*Abat*), which regulates the degradation of GABA into succinate semialdehyde,[34] was marginally induced by 31.2 and 500 pM EMB. Succinic semialdehyde dehydrogenase (*Ssadh*), which is responsible for the downstream metabolism of GABA transaminase,[34] however, was down-regulated by exposure to 31.2 and 500 pM EMB. These results suggested that the homeostasis of GABA was likely affected by exposure to EMB. Additional evidences on increased GABA actions were supported by the repression of DEGs related to GABA transport, such as vesicular GABA transporter (*VGAT*) and sodium-chloride-dependent GABA transporter (*Gat*), which all were down-regulated by exposure to 500 and 2000 pM EMB, respectively. Both transporters are involved in the GABA re-uptake processes in the synaptic cleft responsible for eliminating the GABA actions and identified as targets of various drugs and toxicants.[33]

Disruption of molting signaling. The ecdysone signaling, which regulates molting, developmental and reproductive processes in *D. magna* through the EcR signaling pathway,[35] was potentially disrupted by exposure to EMB. Although not being identified as DEGs by the microarray, the EcR A1-beta (*EcR-a1b*) and *EcR-b* were both significantly up-regulated by exposure to 31.2, 500 and 2000 pM EMB by qPCR. A few EcR-responsive transcription factors, such as broad (*br*)[36] and Fushi tarazu factor-1 (*ftz-f1*)[37] were up-regulated in a concentration-dependent manner, with significant up-regulation after exposure to 2000 pM EMB (Figure 3). Genes involved in the new cuticle formation, such as the chitin synthase (*kkv*), and old cuticle degradation, such as chitinases (*cht4*, *cht7* and *cht8*) were also highly induced (microarray), confirming that EMB perturbed EcR-mediated physiological preparations of molting in a similar way as reported for *L. salmonis*.[31]

Although the EcR genes were induced, results from the *EcR* reporter assay did not show significant activation of EcR by EMB, thus suggesting that the activation of EcR signaling may be a downstream response to EMB exposure. It is likely that exposure to EMB affected the ecdysteroid synthesis, as ecdysone 20-monooxygenase/shade (*shd*), which converts ecdysone to the EcR ligand 20-hydroxyecdysone (20E),[38] was up-regulated at 31.2 pM (qPCR) and 2000 pM (microarray and qPCR) EMB. The synthetic pathway of

ecdysteroids in crustaceans is mainly regulated by the neuropeptide molt inhibiting hormone (MIH), which suppresses ecdysteroidogenesis.[39] The hormonal action of MIH is exerted through binding to its G-protein coupled receptor (MIH-R), induction of adenylyl cyclase (Ac) and activation a cascade of cyclic adenosine monophosphate/cyclic guanosine monophosphate (cAMP/cGMP) signaling pathways.[40] A previous study with the American lobster hypothesized that exposure to EMB may inhibit the activity of MIH, thus promoting 20E synthesis and EcR signaling, which lead to premature molting,[11] as activation of GABA signaling was shown to inhibit the release of several other neuropeptides in crustaceans.[41] This hypothetical mechanism was supported by the current study, as genes involved in the upstream signaling of the MIH pathway, such as adenylyl cyclase 76E (*Ac76E*) and protein kinase cAMP-dependent regulatory subunit type 1 (*Pka-R1*), and downstream signaling, such as guanylyl cyclase beta-subunit at 100B (*Gycβ100B*) and cGMP-dependent protein kinase isozyme 2 (*Pkg2*) were suppressed by exposure to EMB.

Exposure to low concentrations of EMB (7.8 and 31.2 pM) slightly increased the molting frequency of *D. magna* after 24 and 48h, whereas exposure to higher concentrations (500 and 2000 pM) consistently inhibited molting in a concentration-dependent manner. The molting inhibition was likely caused by high level of 20E resulting from elevated ecdysteroidogenesis, as a pulse (rise and decline) of the 20E level is required for successful molting in *D. magna*.[35] Studies on arthropod molting suggested that a decline of 20E titer is necessary for triggering the execution of the cuticle shedding (ecdysis) through appropriate body contractions.[42, 43] Interestingly, cytochrome P450 18A1 (*cyp18a1*), which regulates the degradation of 20E, was up-regulated in a similar manner as the EcRs, possibly in response to increased 20E in the hemolymph.[44] It was also likely that GABA-induced paralysis may affect the muscle contractions thus hampering the ecdysis behavior.

Perturbation of energy homeostasis. Short-term exposure to EMB potentially affected the energy homeostasis in *D. magna*. This MoA was initially supported by the concentration-dependent induction of the 5' adenosine monophosphate-activated protein kinase α subunit (*AMPKα*) gene (Figure 3), which serves a sensor of cellular ATP expenditure.[45] Further evidences for perturbed energy homeostasis was shown by DEGs involved in the mechanistic target of rapamycin (mTOR) signaling, which is another indicator of energy imbalance downstream of the AMPK signaling.[45] Down-regulation of DEGs in the mTOR signaling, such as target of rapamycin (*Tor*), mTOR complex subunit lst8 (*Lst8/GβL*), ribosomal protein S6 kinase II (*Sk6 II*) and eukaryotic initiation factor 4B (*eIF-4b*), and up-regulation of genes which are normally suppressed by mTOR, such as autophagy-related 8a (*Atg8a*), eukaryotic translation initiation factor 4E binding (*Thor/4ebp1*) and programmed cell death 4 (*Pdcd4*)[45] supported the potential inhibition of mTOR signaling and energy deficiency in *D. magna* after exposure to EMB.

The potential perturbation of energy homeostasis by EMB may also be reflected by the induction of genes involved in the mitochondrial electron transport chain (ETC), such as NADH dehydrogenase 20 subunit-like (*ND-20L*) in Complex I, succinate dehydrogenase subunit A (*Sdha*) in Complex II, cytochrome c oxidase subunit 6A (*Cox6al*) in Complex IV and ATP synthase ε subunit-like (*ATPsynεL*) in Complex V. The mitochondrial ETC is a key component in the production of ATP by oxidative phosphorylation (OXPHOS) and has been identified as a major target of environmental toxicants.[46] The elevated ETC activity in response to higher demand for ATP may be a compensatory mechanism for potential energy crisis caused by EMB. Previous studies also suggested that abnormal calcium influx may affect the mitochondrial respiration, thus

causing loss of ATP production.[47] Interestingly, activation of the $GABA_B$ receptor signaling was shown to cause opening of the voltage-dependent calcium channels and increased calcium influx to the cells,[48] thus providing support for potential linkages between the primary MoA involving activation of GABA signaling and perturbation of energy homeostasis by EMB.

Lack of sufficient supply of energy may result in increased metabolism of lipids. There is growing evidence that inhibition of mTOR signaling facilitates the stimulation of lipolysis.[45] Interestingly, the current Reactome analysis showed that 500 pM EMB led to enrichment of DEGs related to membrane-based cellular metabolic and signaling processes of digestion of dietary lipids, mobilization and transport (e.g. lipolysis and sphingolipid metabolism).[49] Exposure to 2000 oM EMB affected the digestion of dietary lipid and synthesis of ketone bodies, which are involved in the routine lipolysis for energy, and peroxisomal lipid metabolism, which regulates the fatty acids oxidation,[50] Reduction of lipid storage representative of increased lipid metabolism has recently been documented for *D. magna* after 24 h exposure to 30-300 pM EMB,[23] indicating potential demand for energy supply.

Inhibition of DNA repair. DNA damage has been previously documented in arthropods such as the silkworm *Bombyx mori* and higher organisms such as rat after exposure to avermectins.[51, 52] A study on the fall armyworm *Spodoptera frugiperda* Sf-9 cell line has also shown that exposure to EMB induced both single-strand and double-strand DNA breaks *in vitro*.[53] In the present study, a number of DEGs and pathways related to cell cycle regulation and DNA repair were found to be affected by exposure to EMB. The pathway of cell cycle checkpoints was found to be highly enriched, suggesting that responses to potential DNA damage were already stimulated after exposure to as low as 31.2 pM EMB. Higher enrichment of the cell cycle checkpoints pathway with more supporting DEGs was identified in *D. magna* after exposure to 500 pM EMB. Exposure to 2000 pM of EMB affected both cell cycle regulation and DNA repair pathways. Interestingly, most of the supporting DEGs in these pathways were found to be repressed, such as DNA excision repair protein ERCC-1 (Ercc1), double-strand break repair protein MRE11 (Mre11) and 8-oxoguanine DNA glycosylase (Ogg1), albeit these genes were normally up-regulated in arthropods after exposure to genotoxic agents. [54-58] Suppression of DNA repair may be caused by different mechanisms. One possible explanation may be the unbalanced expression between the proliferating cell nuclear antigen (PCNA) and cyclin-dependent kinase inhibitor 1 (P21) genes which are upstream regulators of DNA repair signaling.[59, 60] Interestingly, the PCNA gene was down-regulated in a concentration-dependent manner, whereas the double-strand-break repair protein rad21 homolog (Vtd) gene were slightly up-regulated after exposure to 500 pM EMB and marginally down-regulated by exposure to 2000 pM EMB in this study, suggesting that suppression of DNA repair signaling may likely be a consequence of abnormal expression of these genes in *D. magna* after exposure to EMB.

Induction of programmed cell death. Programmed cell death (PCD) such as apoptosis and autophagy was likely induced in *D. magna* by exposure to EMB. The up-regulation of death related ICE-like caspase (Ice/Drice), an effector caspase well-known in *D. malanogaster* and homolog of mammalian caspase 3,[61, 62] and several other DEGs involved in the apoptotic signaling such as BCL2/adenovirus E1B 19 kDa

protein-interacting protein 3 (Bnip3),[63] programmed cell death 4 ortholog (Pdcd4)[64] and death related ced-3/Nedd2-like caspase (Dredd)[65] were indicative of potential activation of apoptosis in *D. magna* after exposure to as low as 125 pM EMB. Exposure to EMB has recently been reported to induce apoptosis in the fall armyworm sf-9 cells [53]. Induction of apoptosis by exposure to avermectins has also been documented in higher organisms, such as the king pigeon *Columba livia* [66-68] and Wistar rats *Rattus norvegicus* [69]. Apoptosis is usually a consequence of oxidative stress, DNA damage and mitochondrial dysfunction [70]. In this study, one of the antioxidant genes, catalase (Cat), was found to be up-regulated after exposure to 31.2 and 500 pM EMB by microarray analysis. This result, however, was not confirmed by qPCR, in which the Cat gene was marginally up-regulated, but the change was not significant. Since no clear evidences were found to support the induction of oxidative stressor based on DEGs. The induction of apoptotic signaling may possibly be a downstream effect of mitochondrial ETC disturbance or suppression of DNA repair. Autophagy as another type of PCD might also be induced, as the autophagy-related 1 (Atg1), 2 (Atg2), 8a (Atg8a) and 17 (Atg17) genes were found to be up-regulated by EMB in a concentration-dependent manner. The autophagy-related 7 (Atg7) gene was also up-regulated by exposure to 500 pM EMB. Autophagy is usually activated in response to lack of nutrients and hormonal actions to eliminate unnecessary or dysfunctional cellular components [71], and suppressed by activation of mTOR signaling [45]. Autophagy has not been well documented in arthropods after exposure to avermetins, however, avermectin-induced autophagy has been observed in higher organisms such as pigeons [72, 73].

Model Construction and Adverse Outcome Pathway Development. On basis of the current findings, a putative network of toxicity pathways causing molting inhibition and mortality is proposed for *D. magna* and closely related species (Figure 4). Briefly, exposure to EMB may activate the GluCl and GABA signaling, thus increasing the chloride channel conductance and inhibiting the neurotransmission (paralysis). Severe paralysis at high concentrations (e.g. 500-2000 pM) of EMB may directly lead to lethality. Although the molting inhibition may likely be associated with mortality at lethal concentrations of EMB, 12h gene expression analysis and 24h toxicity data in the current study showed that molting suppression may also be caused by endocrine-related mechanisms, suggesting multiple mechanisms may exist and contribute to molting failure in *D. magna*. As discussed earlier, activation of GABA signaling may suppress the molting inhibiting neuropeptide MIH expression through the cGMP signaling pathway and stimulate the synthesis of ecdysteroids, leading to increased ecdysteroid titer. Although increased ecdysteroid titer positively regulates the physiological preparations of molting (e.g. new cuticle generation, old cuticle degradation), it blocks the ecdysis behavior (i.e. shedding the old cuticle through somatic muscle contraction), as decline of ecdysone titer in the end of a molt cycle is necessary for successful molting in arthropods. Lack of molting behavior may also be due to GABA activation-associated paralysis. It is currently not clear which mechanism is the predominant one causing molting inhibition in *D. magna* at low exposure concentrations of EMB. Incomplete molting may cause mortality. In addition, activation of GABA signaling may increase the calcium influx, thus causing mitochondrial dysfunction and reduced ATP supply. Reduction of energy can be rapid sensed by the AMPK signaling, which then inhibits the mTOR signaling pathway to reduce the use of energy for protein and lipid synthesis and elevates the activity of mitochondrial ETC to produce more ATP. Influx of calcium into

the cytosol may also activate the mitochondrial OXPHOS. Increased activity of ETC may result in excessive ROS formation and potentially cause oxidative stress. Suppressed mTOR signaling may activate autophagy and lipid metabolism. Exposure to EMB may potentially suppress DNA repair, leading to accumulation of damaged or incorrectly repaired DNA. This together with mitochondrial dysfunction may induce apoptosis, which in combination with autophagy abandon the damaged cells, lead to potential loss of tissue functions and ultimately contribute to death.

As part of this mechanistic model, several conceptual Adverse Outcome Pathways (AOPs) were developed. Assembling and portraying potential causal relationships between molecular initiating event, key events at the molecular, cellular and organ level and adverse outcomes at the individual or population level as AOPs is becoming increasingly important in predictive ecotoxicology and future regulatory toxicology.[74] One of the putative AOPs, entitled "Ionotropic GABAR activation mediated neurotransmission inhibition leading to mortality" has been submitted to the AOP repository database AOP-Wiki (https://aopwiki.org/aops/Aop:160).

Environmental Relevance. The current study used the freshwater crustacean *D. magna* as a model to understand the potential hazard of EMB to non-target species. The major MoAs of EMB characterized herein may potentially be extrapolated to other crustaceans such as lobsters, crabs and shrimps, as a number of genes and proteins in *Daphnia* are phylogenetically conserved across the phylum of *Arthropoda* or even in mammalian species.[75] Attempts were made to link 12h transcriptional responses to 48h adverse effects of EMB, as molecular alterations usually occur ahead of measurable phenotypic changes and adaptation,[76] and which require sufficient time for accumulation of damage at higher organismal levels.[77] Moreover, short-term exposure may reflect the real exposure scenarios of planktonic crustaceans to anti-sea lice chemicals, as residue EMB from salmon food can be rapidly adsorbed by the sediments (half-life: 164-175 d) and mainly affect the benthic species in a long term after treatment.[2, 78] In Norway, for example, the water concentration of EMB was estimated to be less than 1.0 ng/L (approx. 1.1 pM) after 2 months of anti-sea lice treatment, which was close to the UK environmental quality standard (EQS) of 0.22 ng/L (approx. 0.2 pM), whereas the sediment concentrations of EMB exceeded the UK EQS of 0.763 ng/g (dw) in 5 occasions.[79, 80] The measured concentrations of EMB near the salmon aquaculture sites in Canada exceeded the UK EQSs, with up to 209 pg/L (approx. 0.2 pM) and 35 ng/g detected in the seawater and sediment, respectively.[81] In the freshwater system, <0.01–2.5 mg/kg EMB was detected in the sediment close to the effluent outfall from four aquaculture areas in Atlantic Canada.[82] Although not directly comparable with the current experimental studies, these monitoring results together with the current study suggested that pM level of EMB may be present in the aquatic environment and potentially pose hazards to both planktonic and benthic crustaceans.

■ ASSOCIATED CONTENT

Supporting Information

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Figures

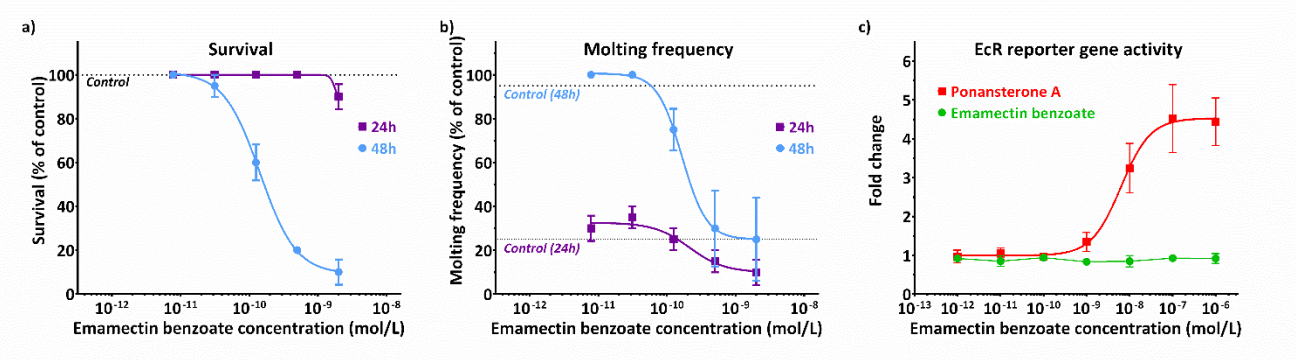

Fig.1. Concentration-response relationships of emamectin benzoate (EMB) on different toxicological endpoints. a): Survival (determined as % survived animals of the total animals) of *Daphnia magna* (N=4) after 24 and 48h exposure to EMB; b): Molting frequency (determined as % molted animals of the total animals) of *D. magna* (N=4) after 24 and 48 h exposure to EMB. c): Expression of the ecdysone receptor (EcR) reporter gene in Chinese hamster ovary cells (N=3) after 40h exposure to EMB and ponansterone A.

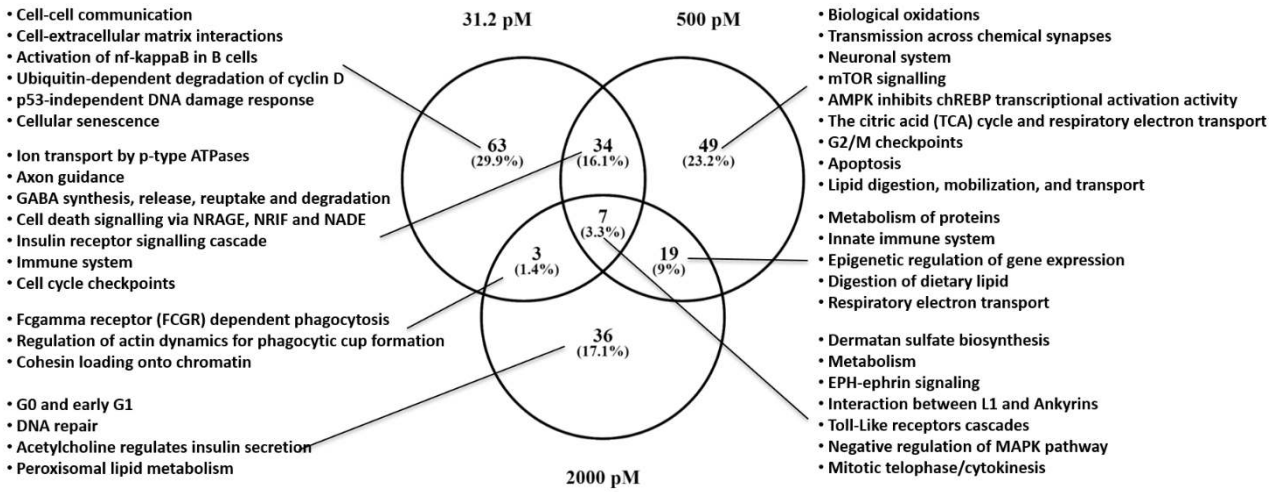

Fig.2. Venn diagram analysis of Reactome pathways that were enriched by differentially expressed genes in *Daphnia magna* (N=4) after 12h exposure to 31.2-2000 pM emamectin benzoate. Examples of toxicologically relevant pathways were presented as insertions.

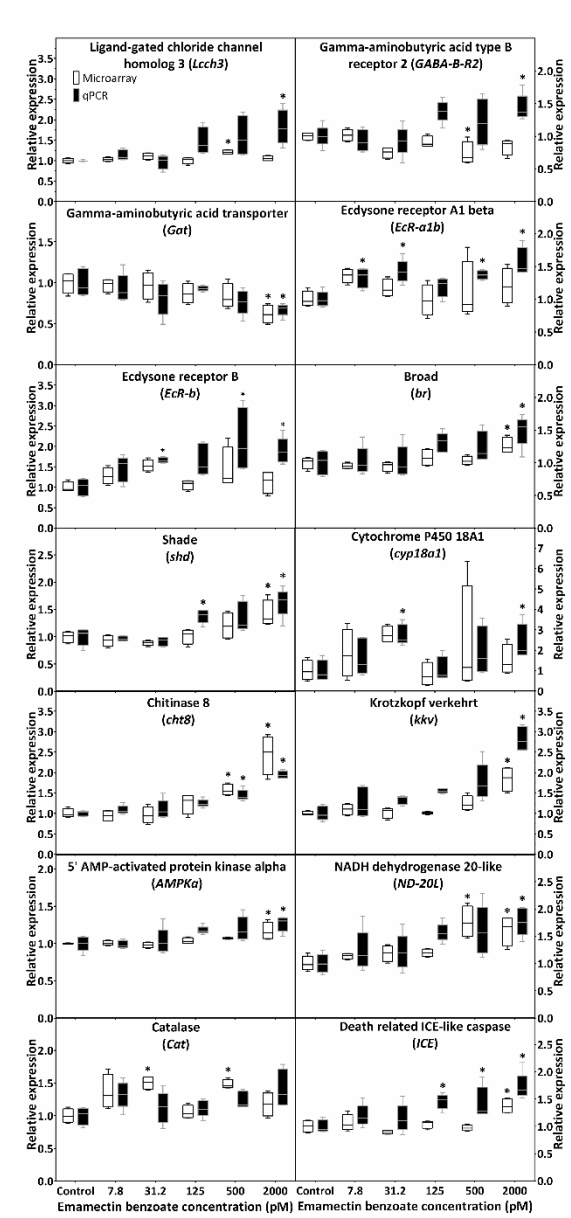

Fig.3. Biomarker gene responses in *Daphnia magna* after 12h exposure to 7.8-2000 pM nominal concentrations of emamectin benzoate determined by quantitative real-time reverse transcription polymerase chain reaction (qPCR). The results were compared between qPCR (white box, N=5) and microarray (black box, N=4). * denotes significant difference ($p<0.05$) from the corresponding control (DMSO).

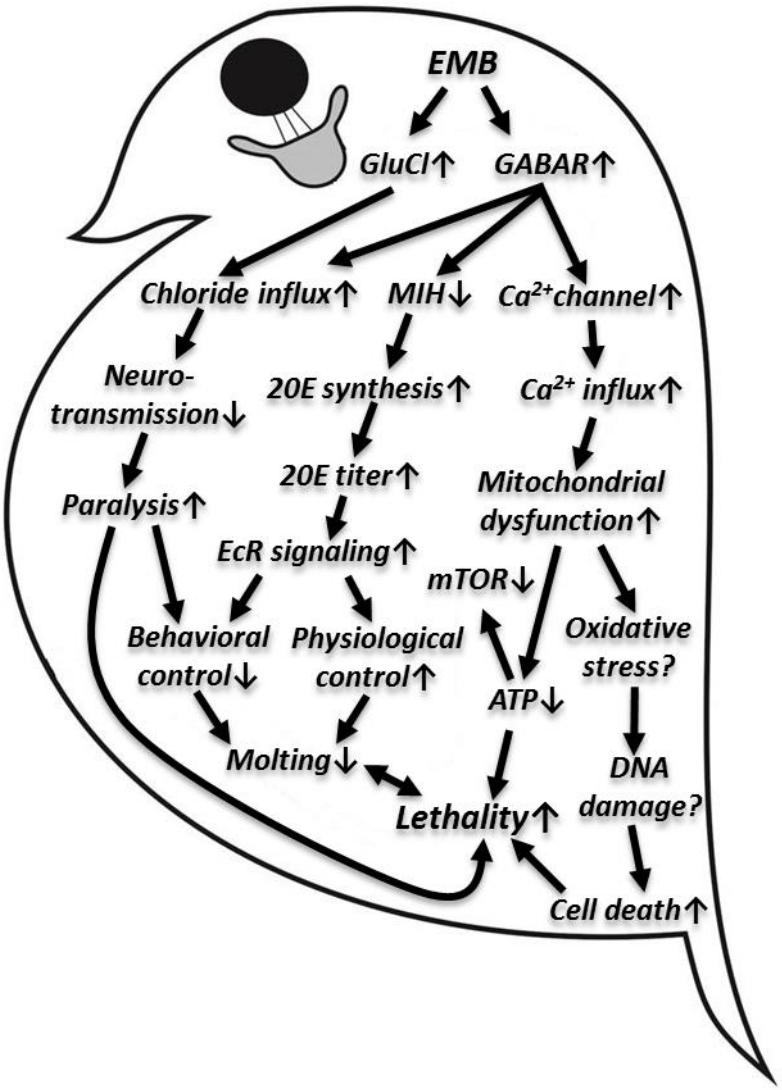

Fig.4. A putative model illustrating potential toxicity pathways leading to molting inhibition and lethality in *Daphnia magna* after short-term exposure to emamectin benzoate (EMB). GluCl: glutamate-gated chloride channels; GABAR: gamma-aminobutyric acid receptors; 20E: 20-hydroxyecdysone; EcR: ecdysone receptor; mTOR: mechanistic target of rapamycin. ↑: Activation; ↓: Inhibition.