Chemosphere 263 (2021) 127638

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

# Impacts of subchronic exposure to a commercial 2,4-D herbicide on developmental stages of multiple freshwater fish species

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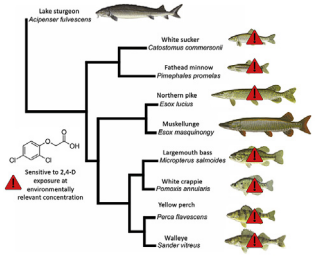
## HIGHLIGHTS

- Ecologically relevant concentrations of 2,4-D reduced survival in six fish species.
- 2,4-D reduced survival in species from 5 families and 3 orders.
- Sensitivity to 2,4-D exposure did not show correlation with phylogenetic proximity.
- Early developmental stages are more at risk to 2,4-D exposure than juvenile fish.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 15 April 2020
Received in revised form
1 July 2020
Accepted 6 July 2020
Available online 1 August 2020

Handling Editor: Jim Lazorchak

*Keywords:*
Larval fish
Herbicides
2,4-D
Developmental toxicology
Phylogeny
Ecotoxicology

## ABSTRACT

Invasive, nuisance aquatic species such as Eurasian watermilfoil (*Myriophyllum spicatum*) are rapidly spreading across the United States. One common active ingredient used to control this invasive species is 2,4-Dichlorophenoxyacetic acid (2,4-D). Application of 2,4-D to aquatic environments typically occurs while many freshwater fish are spawning and due to 2,4-D stability in aquatic environments, many non-target species experience prolonged exposure throughout embryogenesis and larval development. The impacts of 2,4-D exposure on phylogenetically distant fish species is poorly understood. Herein, we investigated the impacts of the 2,4-D commercial herbicide DMA4®IVM on nine freshwater fish species from six different families (four orders) at different points during ontogeny. Each species was exposed to ecologically relevant concentrations of a commercial 2,4-D herbicide (0.05, 0.50, and 2.00 ppm or mg/L 2,4-D a. e.), and effects on morphology, survival, and growth were evaluated. Our results demonstrate that exposure of embryonic and larval fish to ecologically relevant concentrations of a commercial 2,4-D herbicide reduced survival in early developmental stages of six freshwater species that spanned five phylogenetic families and three phylogenetic orders; however, sensitivity to 2,4-D exposure did not show correlation with phylogenetic proximity. Altogether, our results indicate that the use of 2,4-D herbicides in aquatic ecosystems at current recommended concentrations (≤2 ppm whole-lake treatment) could present risk to multiple freshwater fish species survival during early development.

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## 1. Introduction

Eurasian watermilfoil (*Myriophyllum spicatum*) (EWM) is one of

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https://doi.org/10.1016/j.chemosphere.2020.127638
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several invasive, non-native, aquatic plant species that are rapidly spreading across the United States [1,2]. EWM has invaded at least three Canadian Provinces and every state except Alaska and Hawaii (Pfingsten et al., 2019) and continues to invade new waterways (e.g. lakes and rivers) across the United States annually (Wisconsin Department of Natural Resources, 2007). As EWM colonizes new aquatic ecosystems, it outcompetes native monocots and dicots, decreases dissolved oxygen, creates habitats for disease-carrying insects, reduces water flow, reduces open areas along lake shores, and reduces shoreline property value (Eiswerth et al., 2000; Horsch and Lewis, 2009; Smith and Barko, 1990; Boylen et al., 1999). Approximately one hundred million dollars are invested annually in the US to control the spread of exotic, invasive aquatic plant species (Wagner et al., 2007; Pimentel et al., 2005; OTA, 1993).

One tool of aquatic plant management is chemical intervention, or a direct application of commercial herbicides to aquatic ecosystems. Chemical intervention has positively protected against the negative ecological, aesthetic, and recreational impacts of invasive infestations (Wagner et al., 2007; Glomski and Netherland, 2010), however, the indirect effects on non-target, aquatic organisms (e.g. fish, insects, zooplankton) are poorly understood. A common active ingredient used to effectively control and potentially eradicate *M. spicatum* is 2,4-Dichlorophenoxyacetic acid (2,4-D) (Glomski and Netherland, 2010). The active ingredient 2,4-D is designed to mimic the plant growth hormone auxin which causes uncontrolled cell growth and senescence that eventually leads to plant death (Song, 2014). Commercial 2,4-D herbicides can be directly applied to aquatic ecosystems (Nault et al., 2014) at an application rate up to 4 ppm (mg/L) spot treatment and up to 2 ppm whole-lake treatment, with a follow-up treatment 21 days after initial application (EPA, 2005). In the midwestern US, the highest concentrations of 2,4-D come from whole-lake treatments and commercial 2,4-D herbicides are commonly applied in the spring. The active ingredient 2,4-D can last in lakes for months, with a recorded half-life of >6 weeks in lakes that underwent whole-lake treatment (Nault et al., 2018). Since the majority of freshwater game species annually spawn in the spring, many freshwater species are exposed to 2,4-D throughout critical developmental life stages (Dehnert et al., 2018; Mohammed, 2013). Early developing life stages of aquatic organisms, including fish, have been shown to be more susceptible to toxicant exposure compared to their juvenile and adults counterparts (Mohammed, 2013; Sarikaya and Yilmaz, 2003; Laale and Lerner, 1981). Previous 2,4-D no-observable-effect-concentrations (NOEC) were recorded at 14.2–63.4 ppm acid and amine form for a variety of adult and juvenile fish species (EPA, 2005). However, recent peer-reviewed studies indicate that much lower concentrations of 2,4-D can impact the development and survival of fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*) (Dehnert et al., 2018, 2019a; DeQuattro and Karasov, 2016a; Gaaied et al., 2019) embryos and larvae.

For risk assessment, experimental results for a single species are commonly applied broadly to other species, but it can be challenging and sometimes inaccurate to predict toxicity data from tested species to untested species in the environment (Arapis, 2006). Such an extrapolative approach risks overlooking species- or taxon-specific sensitivities to individual chemicals (EPA, 2005; Klassen, 2008; Newman, 2015). Past literature has shown that one group of species can share a similar sensitivity to a toxicant (Ashauer et al., 2011; Relyea and Jones, 2009), while another group of species can vary by orders of magnitude (Ashauer et al., 2011; Jones et al., 2009; Cairns and Niederlehner, 1987; Cairns, 1986). Variation in a species sensitivity to a toxicant has been attributed to differences in physiology and biochemistry, such as absorption, biotransformation, distribution, and excretion of the toxicant (Newman, 2015), which may vary across species according to phylogenetic relationships (Guenard et al., 2014; Hammond et al., 2012). Fish ecotoxicology research commonly reports on two model species from the same phylogenetic family, Cyprinidae (e.g. *P. promelas* and *D. rerio*) (Dehnert et al., 2018; Gaaied et al., 2019; Ankley and Villeneuve, 2006; Stegeman et al., 2010). This might be considered an example of phylogenetic pseudoreplication, "which is caused by the presence of phylogenetic signal in the data (i.e., the degree of similarity in trait values between species due to their common ancestry)" (Hylton et al., 2018). Phylogenetic pseudoreplication could lead to over generalizing toxicity data from one phylogenetic group to other phylogenetically distant groups. Due to interspecies sensitivity differences, single model organism toxicity assessments or single phylogenetic family toxicity assessments should be interpreted and/or extrapolated with caution (Cairns, 1986). Therefore, to understand more comprehensively the impacts of 2,4-D on non-target fish species, toxicant exposure assessments are best performed on multiple, phylogenetically distant species.

Here, we expanded our knowledge of the impacts of 2,4-D herbicide exposure on multiple ontogenetic stages of Cyprinidae (e.g. *P. promelas* (Dehnert et al., 2018; DeQuattro and Karasov, 2016a) and *D. rerio* (Gaaied et al., 2019)) to other freshwater fish species in other phylogenetic families and orders. We hypothesized that only species closely related to the phylogenetic family Cyprinidae would be sensitive to environmentally relevant concentrations of 2,4-D exposure and thus there would be a strong correlation between phylogeny and sensitivity to 2,4-D exposure. To test this hypothesis, we studied the effects of commercial 2,4-D formulation DMA4®IVM (DMA4) exposure during embryonic, larval, and juvenile development on up to nine freshwater fish species spanning six families (four orders). We measured the impacts of ecologically relevant concentrations of DMA4 on survival, growth, and morphology of each developmental stage. Overall, the study achieves for fish a more comparative, phylogenetically informed assessment of the non-target impacts of 2,4-D exposure at environmentally relevant concentrations.

## 2. Methods

### 2.1. Chemicals and chemical analyses

DMA®4IVM (DMA4) (46.3% 2,4-D) commercial 2,4-D amine liquid herbicides was purchased from NewTech Bio (Howell, NJ, USA). In these experiments, we chose to use the commercial 2,4-D herbicide formulation rather than the active ingredient. Commercial formulations are commonly used in lakes, and commercial formulations have not shown any additional impacts on larval fish survival 30 days and younger compared to the active ingredient alone (Dehnert et al., 2018, 2019b). For embryo assays, concentrated stock solution of 20.00 ppm was diluted in Pyrex glass with distilled water to create 0.05, 0.50, and 2.00 ppm 2,4-D concentrations. Dilutions were made at the beginning of each set of embryo assays and stored in the dark at 8 °C for no more than 6 days. For larvae and juvenile assays, concentrated stock solutions were prepared such that their delivery to the dilution cells would result in target concentrations of 0.00 ppm (control), 0.05 ppm, 0.50 ppm, and 2.00 ppm of 2,4-D at the tank level in our system. The stock solutions for the exposure system were prepared in Pyrex glass by diluting parent herbicide with distilled water. The resulting mixture was aliquoted to 1-L amber glass bottles. Stock solutions were prepared every 6 days and stored in the dark at 8 °C for no more than 6 days. A new amber bottle for each treatment was placed into the exposure system daily.

Water samples for 2,4-D level analysis were taken before the bottle solutions were renewed for the day. Samples were

immediately frozen and stored at −20 °C. Analysis was performed by Wisconsin State Lab of Hygiene (Madison, WI) using a 2,4-D enzyme immunoassay (ELISA) kit (MDL = 1 ppb; Modern Water, New Castle, DE) (Dehnert et al., 2018, 2019b). The ELISA assay used has been validated (Lawruk et al., 1994; Hall et al., 1993) and used widely to verify water application of 2,4-D in the field (Glomski and Netherland, 2010; Nault et al., 2018; Chuang et al., 2005; Vdovenko et al., 2013). Treatment groups were named by their nominal concentration of 2,4-D. Embryo assay concentrations of 0.00, 0.05, 0.50, 2.00 ppm 2,4-D in DMA4 were confirmed by measuring samples on the day each batch of solutions was created and on the 4th day (Supporting Information Table S1). Larvae and juvenile assay concentrations of 0.00, 0.05, 0.50, 2.00 ppm 2,4-D in DMA4 were confirmed by measuring samples from two randomly selected tanks from each treatment every week (Supporting Information Table S1). The largest deviation from nominal concentrations was in the 0.50 ppm group, however, the results were consistent and repeatable for all three concentrations (0.05, 0.50, and 2.00) across all three life stages. Herbicide concentrations are reported here as 2,4-D a. e. Target concentrations (ppm) will be referred to from hereon.

### 2.2. Species exposed to DMA4

We chose nine different freshwater fish species native to the midwestern US that spanned four orders and six families, which were collected from various sites in Wisconsin, USA (Supporting Information Table S2). Water samples from each site were collected at the time of spawning harvest. All water samples collected from the various sites recorded a non-detect for 2,4-D. If there were other potential pollutants in the water or in the harvested embryos, we assumed we controlled for those putative effects by having a control to compare to in each experiment. For later phylogenetic comparisons, embryo and larval fathead minnow data were included from Dehnert et al. (2018). The selection of study species was designed to sample at least two exemplars of a given order, where possible. All species are freshwater species native to Wisconsin waterways where 2,4-D is directly applied or where 2,4-D enters from agriculture run-off. Each species, except *P. promelas*, annually spawns during the spring usually between early March to late June in Wisconsin.

### 2.3. Spawning and husbandry

Mature females were blotted dry, and eggs were hand-collected into a dry bowl. Mature males were blotted dry, and sperm was hand-collected into the same bowl. For species where males could not be hand spawned (i.e. white crappie (*Pomoxis annularis*) and largemouth bass (*Micropterus salmoides*)), males were euthanized, and testes were dissected and added to the same bowl. Water was added to the bowl to activate the sperm and gently mixed to ensure fertilization. The range of male-to-female ratios is specified in Supporting Information Table S2. After fertilization, embryos and larvae were raised at temperatures specified in Supporting Information Table S2. For embryo and larval assays, temperatures were chosen to be similar to *in situ* lake conditions when each individual species spawn. For juvenile assays, the temperature was chosen because it fell within the normal water temperature range of Wisconsin lakes in the summer months. Larval and juvenile diets are specified in Supporting Information Table S3. All exposures and laboratory practices were reviewed and approved by The University of Wisconsin−Madison under RARC protocol A005702.

### 2.4. Embryonic developmental assay

After fertilized embryos were collected and pooled, single embryos were randomly allocated into individual wells of a 24-well CELLTREAT® plastic microplate (one per well) and incubated in 2 mL of media (Arapis, 2006). Incubation media consisted of DMA4 at nominal concentrations of 0.00, 0.05, 0.50, or 2.00 ppm 2,4-D (n = 6 wells in each treatment per plate; 10−12 plates). Incubation media was exchanged daily. Developing embryos were observed daily under a dissecting microscope until hatch, and ranked as 1 = normal, 2 = deformed (i.e., signs of necrotic tissue, abnormal head development, abnormal tail development, scoliosis, slowed/erratic blood circulation or heart rate, pericardial edema), 3 = dead (DeQuattro and Karasov, 2016a). Embryos were defined as unaffected if they survived and did not have any deformities.

### 2.5. Larval and juvenile developmental assay

The present study used a flow-through exposure system (Dehnert et al., 2018; DeQuattro and Karasov, 2016a). Two four-channel, peristaltic pumps (model 07523−90, Cole Parmer) delivered the control (distilled water) and three 2,4-D stock solutions through tubing (size L/S 13 Masterflex® Norprene®, Cole Parmer) to individual glass dilution chambers at a rate of 0.5 mL/min. Heated, carbon-filtered City of Madison, WI water was added to the dilution chambers at a flow rate of 1.5 L/min, which diluted the DMA4 2,4-D stock solutions to the target 2,4-D concentrations of 0.00 ppm, 0.05 ppm, 0.50 ppm, and 2.00 ppm. The water then flowed from each dilution chamber to six replicate exposure tanks (15 L), twelve tanks per treatment. The flow rate into each tank was 250 mL/min. Each dilution chamber was constantly aerated. Throughout larval and juvenile developmental assays, dissolved oxygen (7.5−8.1 mg/L), pH (6.9−7.1), hardness (210−239 ppm), and ammonia (non-detectable (ND)) were measured weekly. For all larval assays, temperature was measured daily (±1 °C) and juvenile assays temperature was measured daily (24 ± 1 °C). All exposures and laboratory practices using native fish were reviewed and approved by The University of Wisconsin−Madison under RARC protocol A005702.

For the larval development assay, unexposed embryos were allowed to develop in McDonald jars. Within a 6−8 h period after hatching, larvae were allocated to twelve tanks within their respective treatments (n = 15−20 larvae per tank; Supporting Information Table S3). Larvae were raised to 30 days post-hatch (dph) in a 15 L tank. Larvae were fed three times a day *ad libitum* (Supporting Information Table S3). Larval survival per tank was determined weekly throughout the exposure through visual observation. Larval fish were exposed for 30 days (0.00, 0.05, 0.50, and 2.00 ppm), which conforms with the EPA's definition of chronic (long-term) test (cological Effects T, 2016). After 30 days of exposure, survivorship was quantified, larval fish were euthanized, and total length (±0.01 mm) and wet mass (±0.001 g) were measured (N = 12 tanks per each treatment).

For the juvenile development assay, unexposed juveniles (>60 dph) were randomly allocated to twelve 15 L-tanks and exposed to 0.00, 0.05, 0.50, and 2.00 ppm of 2,4-D for 90 days (n = 6−10 juveniles per tank; Supporting Information Table S3). Juveniles were fed three times a day *ad libitum* (Supporting Information Table S3) and survival per tank was measured weekly. After 90 days of exposure, survivorship was quantified, and juvenile fish were euthanized (N = 12 tanks). Following euthanasia, total length (±0.01 mm) and wet mass (±0.001 g) were measured (N = 5 fish from each tank; N = 60 fish per each treatment).

### 2.6. Endpoints

At the end of all the experiments, animals were euthanized with size-appropriate buffered concentrations of MS222. For embryo assays, newly hatched larvae were assayed for survival and presence of deformities. An unaffected embryo was defined as a living embryo exhibiting wild type morphology (i.e., no perceptible deformities). For larvae and juvenile assays, animals were gently blotted with Kim-wipes to remove excess fluid and wet mass was determined using an Ohaus analytical balance with ±0.001 g precision. Total length of animals was determined using a digital Mitutoyo absolute digimatic digital caliper with ±0.01 mm precision. Length measurements were made from the tip of the longest jaw or end of the snout to the longest caudal lobe (Kahn et al., 2004). Survival at various time points was determined by visual counting. Following the end of each experiment, fish were euthanized and preserved in −80 °C freezer for possible future analyses in the event that 2,4-D toxicity biomarkers are discovered in fish.

### 2.7. Data analysis

All data were analyzed using GraphPad Prism Software 8.2b (GraphPad Software Incorporated, La Jolla, Ca, USA). Prior to the use of parametric statistics, the assumption of normality was tested with a Shapiro–Wilk test, and the assumption of homoscedasticity was tested with Bartlett's test. When assumptions of normality and/or homoscedasticity were not met, appropriate data transformations were performed (e.g., log transformations). Parametric analyses were performed using a one-way-ANOVA followed by Dunnett's multiple comparison analysis (F statistic presented). Non-parametric analyses were performed using a Kruskal-Wallis test followed with a Dunn's multiple comparison analysis (X (Pimentel et al., 2005) statistic presented). Data are presented as means ± standard error of the mean (SEM; n = sample size). Significance was set at $p < 0.05$.

### 2.8. Phylogenetic reconstruction

Five loci were selected for analysis: 12S rRNA, 16S rRNA, cytochrome *b*, cytochrome *c* oxidase subunit I and rhodopsin. These loci were chosen for representation of genomic data of both mitochondrial and nuclear origin, as well as representation in publicly available repositories (NCBI GenBank). Sequence data were downloaded from GenBank, prioritizing selection of complete mitochondrial genomes, where available. Accession data for sequences used in this study are provided in Supporting Information Table S4. For each locus, de novo sequence alignment was performed using MUSCLE v.3.6 (Edgar et al., 2004), with trimming of overhanging ends to maximize dataset completeness. The final alignment consisted of 7.65% gaps or missing data.

Maximum likelihood (ML) analysis was conducted using RAxML ver. 8.2 (Stamatakis, 2014) for the five-locus dataset. A unique GTR model of sequence evolution with corrections for a discrete gamma distribution was specified for each data partition and 50 independent searches were implemented. Nodal support was estimated via the rapid bootstrap algorithm with 500 resampling replicates (Stamatakis et al., 2008).

Bayesian inference (BI) was conducted using MrBayes v.3.2 (Ronquist et al., 2012) implementing a unique GTR model of sequence evolution with corrections for a discrete gamma distribution and for invariant sites. Four runs, each with four chains and the default distribution of chain temperatures, were implemented for $2 \times 10^6$ generations, sampling every 10000th generation. Assessments of chain mixing and convergence diagnostics were performed using Tracer v.1.7.1 (Rambaut and Drummond, 2019) and inbuilt tools in MrBayes v.3.2. Subsequently, $2 \times 10^5$ generations (10%) were discarded as burn-in.

Ancestral state reconstruction of sensitivity to 2,4-D exposure was performed with discrete coding of character states. Sensitivity to 2,4-D exposure is defined as a negative impact on development and/or survival when exposed to ecologically relevant concentrations of 2,4-D. For discretization, sensitivity to 2,4-D exposure was coded as a binary character (effect present versus effect absent); the operational definition of a discernible effect of sensitivity to 2,4-D exposure was determined based on non-parametric analyses described above. Ancestral state reconstruction was performed on the Bayesian inference consensus tree in Mesquite v.3.51 (Maddison and Maddison, 2019), under an equal weights parsimony framework for character state transformations. To test for phylogenetic signal with sensitivity to 2,4-D exposure, a permutation test was performed on this tree, with reshuffling of character states 10,000 times to generate a null distribution. For the permutation tail probability (PTP) test, a p-value was approximated by integrating the proportion of the distribution less than or equal to the empirical value of the character on the tree.

## 3. Results

### 3.1. Molecular phylogenetics

We created a Bayesian inference tree that shows the taxonomic affiliations of all nine study species (Fig. 1). The Bayesian inference tree was used for the phylogenetic analyses in the present study. ML analysis recovered expected relationships of the nine study species, with maximal nodal support (100% bootstrap frequency) for all nodes ($\ln L = -25094.90$). Runs of MrBayes achieved convergence (as measured by split frequency value) within 80,000 generations, and the post-burn-in tree topology (mean $\ln L = -25040.24$; minimum ESS = 411) recovered the same relationships as the ML counterpart, also with maximal nodal support (posterior probability of 1.00). Both analyses recovered similar branch length distributions in the respective tree topologies, suggesting that their estimation is robust to selection of heuristic criteria.

### 3.2. Overall impacts

Six out of the eight species tested showed a significant negative impact on development and/or survival in at least one of the three ontogenetic stages when exposed to ecologically relevant concentrations of 2,4-D (Fig. 2; details follow below). However, we did not observe a correlation between phylogenetic distance and sensitivity to 2,4-D exposure. Ancestral state reconstruction on the Bayesian inference tree using discrete treatment of character states recovered no evidence for phylogenetic signal to the pattern of sensitivity to 2,4-D exposure across species (Fig. 2) ($p > 0.05$; PTP). Of the species tested at all three ontogenetic stages, one species, *Sander vitreus,* showed significant impacts when exposed to 2,4-D in DMA4 at two out of three stages, while the *Acipenser fulvescens* showed no significant impacts when exposed to 2,4-D in DMA4 at any of the three life stages. Altogether, we observed significant impacts to at least one of the three ontogenetic stages in three out of four orders, five out of six families, and seven out of the nine species (including *P. promelas* (Dehnert et al., 2018)) tested when exposed to 2,4-D in DMA4 at ecologically relevant concentrations (Fig. 2), but no correlation between phylogenetic distance and sensitivity to 2,4-D exposure.

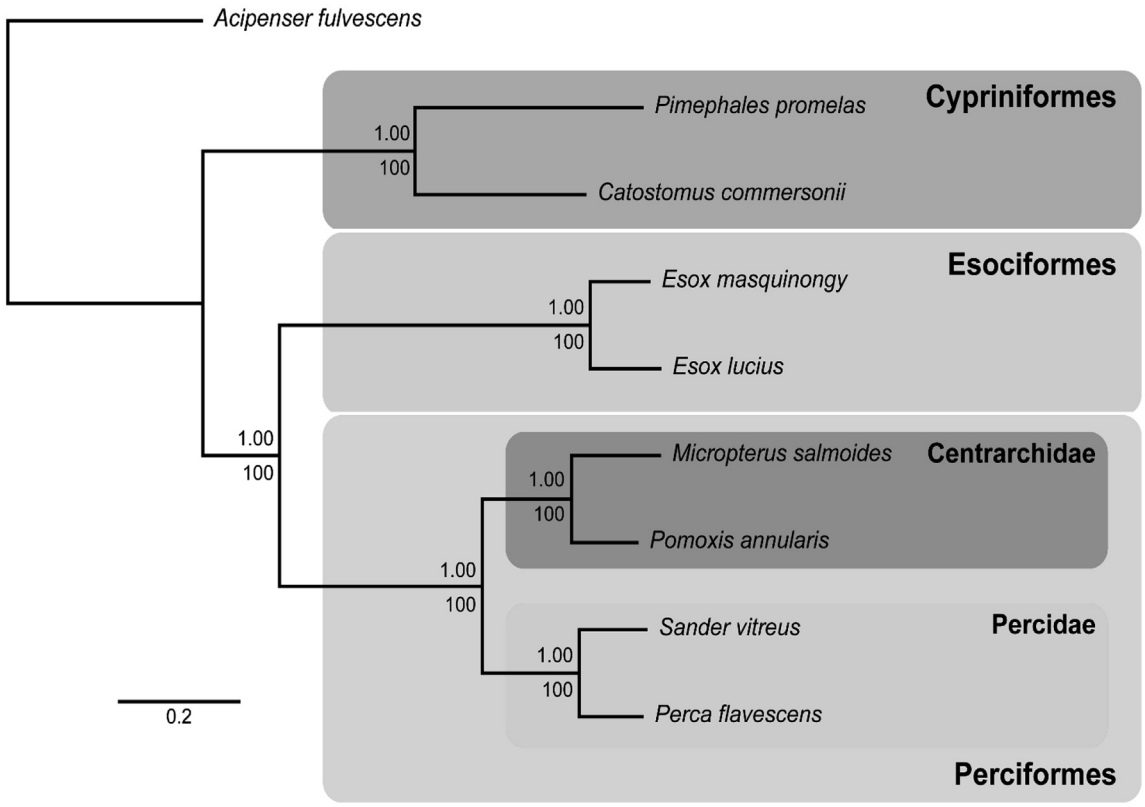


**Fig. 1.** Bayesian inference tree topology of five-locus phylogeny (ln$L = -25040.40$), showing taxonomic affiliations of study species. Numbers above nodes indicate posterior probabilities. Numbers below nodes indicate bootstrap resampling frequencies in maximum likelihood analysis (ln$L = -25094.90$). Branch length units are in units of substitutions per site.

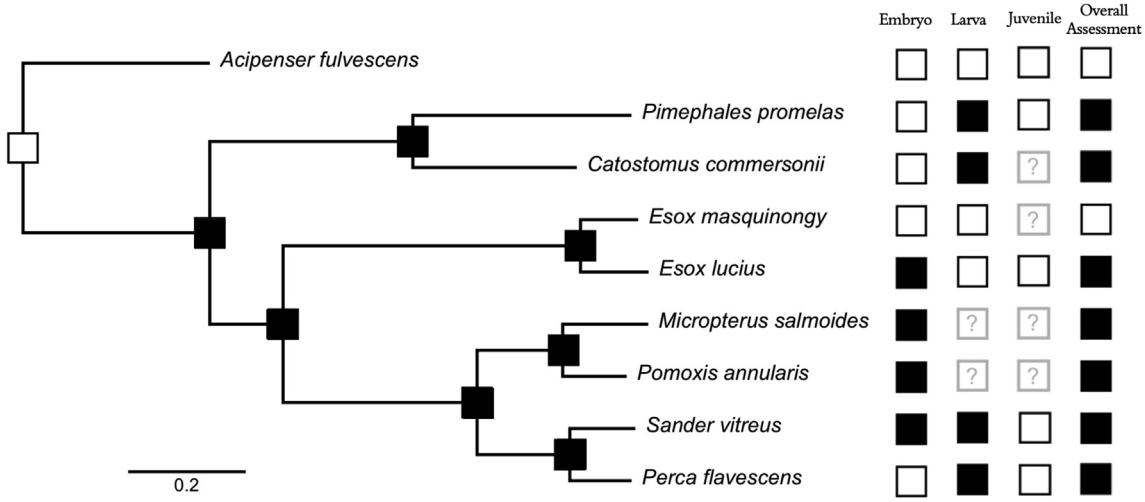


**Fig. 2.** Ancestral state reconstruction of sensitivity to 2,4-D exposure at the ontogenetic life stages tested under equal weights parsimony. Reconstructions on tree nodes correspond to the reconstruction of the overall assessment. Filled squares for embryo column indicate sensitivity, defined by at least one 2,4-D exposure concentration having a decreased percentage of unaffected embryos ($p \leq 0.05$) compared to controls by Dunn's multiple comparison test. Filled squares for larva column indicate sensitivity (i.e., decreased survivorship) determined by at least one concentration having a significant impact on larval survival ($p \leq 0.05$) as compared to control by Dunn's multiple comparison test. Filled squares for overall assessment column indicate sensitivity, defined by at least one 2,4-D exposure concentration having a significant negative impact on survival or development to at least one of the ontogenetic life stages tested ($p \leq 0.05$) as compared to controls by Dunn's multiple comparison test. Unfilled squares indicate non-response to 2,4-D. Squares containing ? indicates the species was not exposed to 2,4-D at this ontogenetic life stage. Branch length units are in units of substitutions per site.

### 3.3. Detailed analysis of embryonic data

Embryos of eight species (*Esox masquinongy, Esox lucius, Catostomus commersonii, M. salmoides, P. annularis, A. fulvescens, S. vitreus,* and *Perca flavescens*) were exposed to DMA4. Overall, four out of the eight species of embryos tested showed a decrease in the percentage of unaffected embryos (alive with no deformities) when exposed to ecologically relevant concentrations of 2,4-D in DMA4

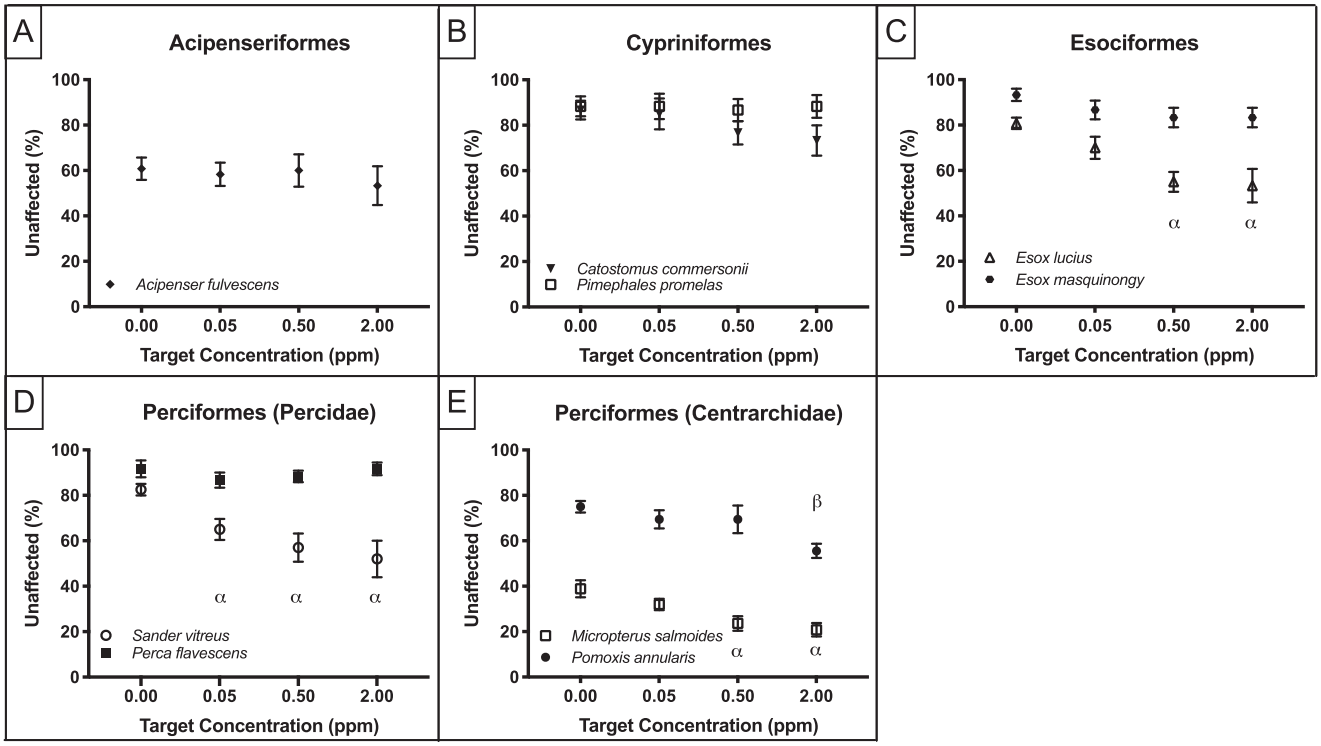


**Fig. 3.** Percent of unaffected embryos of *A. fulvescens* (A), *C. commersonii and P. promelas* (B), *E. masquinongy* and *E. lucius* (C), *S. vitreus* and *P. flavescens* (D), and *M. salmoides and P. annularis* (E) exposed to DMA4 (0.00, 0.05, 0,50, and 2.00 ppm 2,4-D) is shown at hatch. An unaffected embryo is defined as alive with no deformities. Data are mean ± SEM (n = 10–12 trays in each treatment, each with 6 embryos per treatment in each tray). α indicates significantly different value ($p \leq 0.05$) for *E. lucius*, *S. vitreus*, and *M. salmoides* as compared to their respective controls by Dunn's multiple comparison test. β indicates significantly different value ($p \leq 0.05$) for *P. annularis* as compared to control by Dunn's multiple comparison test. *P. promelas* embryo survival data are provided from Dehnert et al. (2018).

from fertilization to hatch. We observed a decrease in the percentage of unaffected embryos for *S. vitreus, E. lucius, M. salmoides, and P. annularis* (Fig. 3). Specifically, we observed significant decreases in the percentage of unaffected embryos exposed to 2,4-D at 0.05 ppm for *S. vitreus* ($\chi$ (Pimentel et al., 2005) = 19, df = 3, p = 0.0417), at 0.50 ppm for *S. vitreus*, *E. lucius,* and *M. salmoides* ($\chi$ (Pimentel et al., 2005) = 19, df = 3, p = 0.0015; $\chi$ (Pimentel et al., 2005) = 22.07, df = 3, p = 0.0002; and $\chi$ (Pimentel et al., 2005) = 15.09, df = 3, p = 0.0148, respectively), and at 2.00 ppm for *S. vitreus*, *E. lucius, M. salmoides*, and *P. annularis* ($\chi$ (Pimentel et al., 2005) = 19, df = 3, p = 0.0013; $\chi$ (Pimentel et al., 2005) = 22.07, df = 3, p = 0.0006; $\chi$ (Pimentel et al., 2005) = 15.09, df = 3, p = 0.0015; and $\chi$ (Pimentel et al., 2005) = 10.90, df = 3, p = 0.0044, respectively) as compared to controls. Individual species survival and malformation data are noted in Supporting Information Table S6. Altogether, we observed significant impacts to embryos in three out of four orders, three out of six families, and four out of the eight species tested (four out of nine species including *P. promelas* (Dehnert et al., 2018)) when exposed to 2,4-D in DMA4 at ecologically relevant concentrations (Fig. 2).

### 3.4. Detailed analysis of larval data

Larvae from six species (*E. masquinongy, E. lucius, C. commersonii, A. fulvescens, S. vitreus,* and *P. flavescens*) were exposed to DMA4. Overall, larval survival of three out of six species was negatively impacted by 2,4-D exposure at ecologically relevant concentrations from hatch to 30 dph. In the present study, we observed a significant decrease in larval survival for *C. commersonii, S. vitreus,* and *P. flavescens* (Fig. 4). Specifically, we observed significant decreases in larval survival when exposed to DMA4 at 0.50 ppm for *C. commersonii, S. vitreus,* and *P. flavescens* ($\chi$ (Pimentel et al., 2005) = 9.855, df = 3, p = 0.0233; $\chi$ (Pimentel et al., 2005) = 14.82, df = 3, p = 0.0403; and $\chi$ (Pimentel et al., 2005) = 34.57, df = 3, p = 0.0001, respectively), 2.00 ppm for *C. commersonii, S. vitreus,* and *P. flavescens* ($\chi$ (Pimentel et al., 2005) = 9.855, df = 3, p = 0.0228; $\chi$ (Pimentel et al., 2005) = 14.82, df = 3, p = 0.0028; and $\chi$ (Pimentel et al., 2005) = 34.57, df = 3, $p \leq 0.0001$) as compared to controls. Of the surviving larvae, we did not observe any decreases in growth parameters (e.g. total length and wet mass) on any of the species tested (Supporting Information Table S6). Altogether, we observed significant impacts to larval survival in three out of four orders, three out of five families, and three out of the six species tested (four out of seven species including *P. promelas* (Dehnert et al., 2018)) when exposed to 2,4-D in DMA4 at ecologically relevant concentrations.

### 3.5. Detailed analysis of data on juveniles

Juveniles from five species (*A. fulvescens, E. lucius, P. promelas, S. vitreus,* and *P. flavescens*) were exposed to DMA4. We did not observe any significant impacts on juvenile survival in any species tested when exposed to 2,4-D in DMA4 at any concentrations tested as compared to controls (Fig. 5). Surviving *A. fulvescens* juveniles exposed to 0.05 and 2.00 ppm DMA4 displayed a significant decrease in total length (total length: $F_{(3,236)} = 7.630$, p = 0.0267 and total length: $F_{(3,236)} = 7.630$, p = 0.0005; respectively) and surviving *P. flavescens* juveniles exposed to 0.50 ppm DMA4 displayed a significant decrease in wet mass (wet mass: $F_{(3,236)} = 3.616$, p = 0.0036) as compared to controls (Table 1).

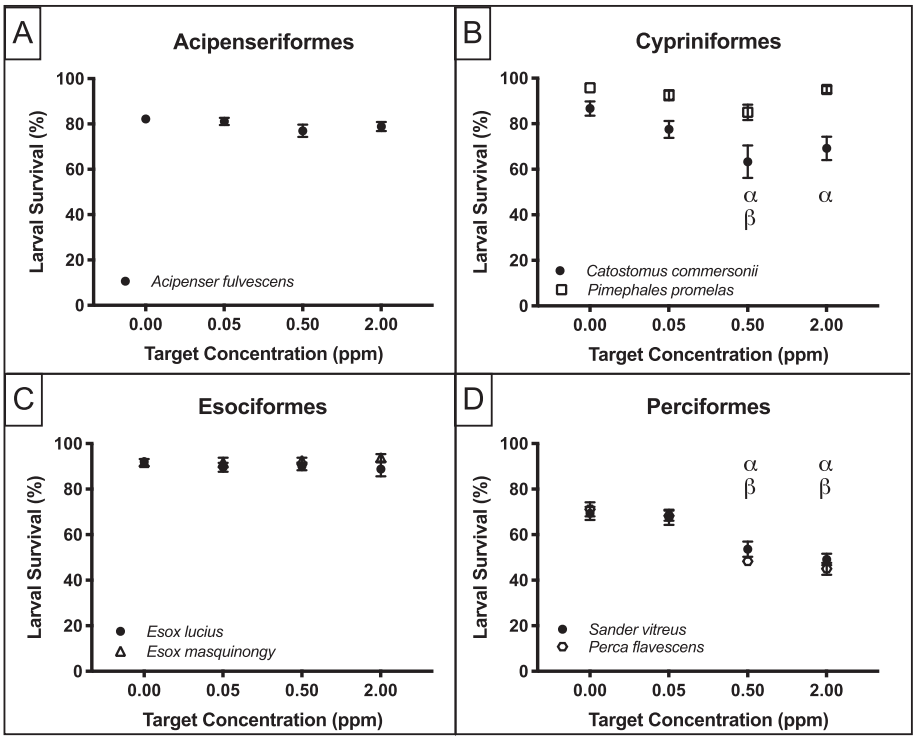


**Fig. 4.** Larval survival (%) for *A. fulvescens* (A), *C. commersonii* and *P. promelas* (B), *E. masquinongy* and *E. Lucius* (C), and *S. vitreus* and *P. flavescens* (D) exposed to DMA4 (0.00, 0.05, 0,50, and 2.00 ppm 2,4-D) is shown at 30 days post hatch. Data are mean ± SEM (n = 12 tanks in each treatment, each with 10–20 progeny). α indicates significantly different value ($p \leq 0.05$) for *S. vitreus* and *C. commersonii* as compared to their respective controls by Dunn's multiple comparison test. β indicates significantly different value ($p \leq 0.05$) for *P. flavescens* and *P. promelas* as compared to their respective controls by Dunn's multiple comparison test. *P. promelas* larval survival data were from Dehnert et al. (2018).

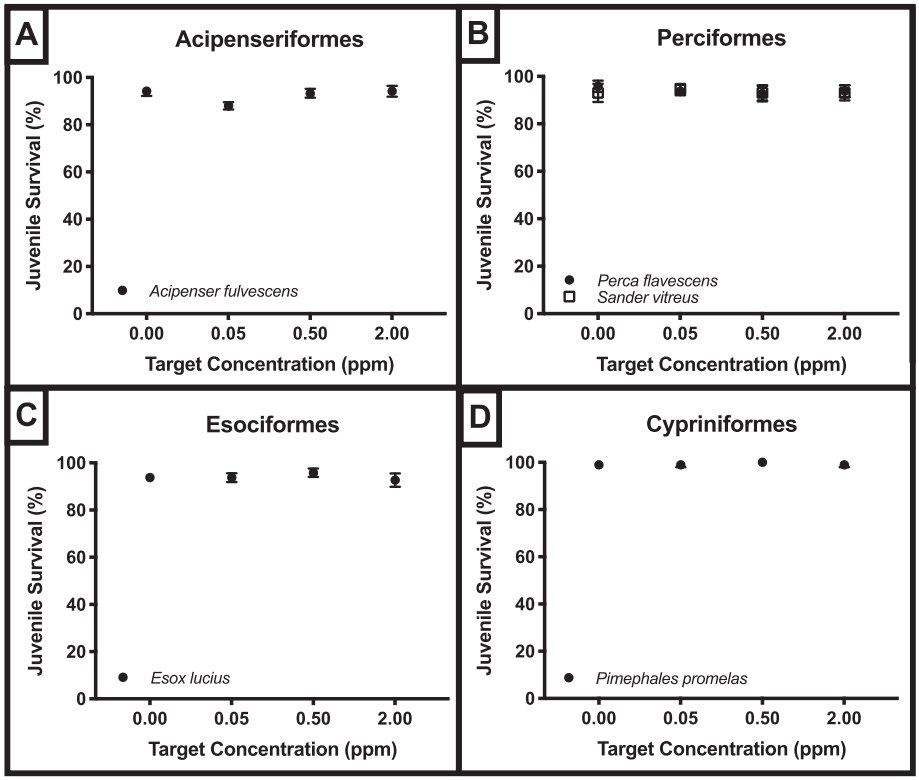


**Fig. 5.** Juvenile survival (%) for *A. fulvescens* (A), *S. vitreus* and *P. flavescens* (B), *E. Lucius* (C), and *P. promelas* (D) exposed to DMA4 (0.00, 0.05, 0,50, and 2.00 ppm 2,4-D is shown is shown at 90 days of exposure. Data are mean ± SEM (n = 12 tanks in each treatment, each with 6–10 progeny).

**Table 1**
Initial wet mass (g) and total length (cm) in juvenile fish. Survival (%), wet mass (g), and total length (cm) for *A. fulvescens*, *S. vitreus*, *P. flavescens*, *E. lucius*, and *P. promelas* is shown following 90 days exposure when exposed to three concentrations of DMA4®IVM (0.00, 0.05, 0.50, 2.00 ppm). Survival data are mean ± SEM (n = 12 tanks in each treatment, each with 6–10 progeny). Wet mass and total length data are mean ± SEM (5 fish from 12 tanks; N = 60 fish total per each treatment). Asterisk indicates significantly different value ($p \leq 0.05$) as compared to control by Dunnett's multiple comparison test.

| | *Acipenser fulvescens* | | | *Perca flavescens* | | | *Sander vitreus* | | | *Esox lucius* | | | *Pimephales promelas* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial Mass (g) ± SEM | 0.63 ± 0.01 | | Initial Mass (g) ± SEM | | 4.22 ± 0.03 | Initial Mass (g) ± SEM | | 1.47 ± 0.04 | Initial Mass (g) ± SEM | | 1.62 ± 0.084 | Initial Mass (g) ± SEM | | 0.57 ± 0.08 |
| | Initial Length (cm) ± SEM | 5.82 ± 0.04 | | Initial Length (cm) ± SEM | | 7.24 ± 0.03 | Initial Length (cm) ± SEM | | 5.92 ± 0.05 | Initial Length (cm) ± SEM | | 4.35 ± 0.11 | Initial Length (cm) ± SEM | | 2.08 ± 0.09 |
| | Survival (%) ± SEM | Mass (g) ± SEM | Length (cm) ± SEM | Survival (%) ± SEM | Mass (g) ± SEM | Length (cm) ± SEM | Survival (%) ± SEM | Mass (g) ± SEM | Length (cm) ± SEM | Survival (%) ± SEM | Mass (g) ± SEM | Length (cm) ± SEM | Survival (%) ± SEM | Mass (g) ± SEM | Length (cm) ± SEM |
| 0.00 ppm | 94.17 ± 1.93 | 2.202 ± 0.03 | 7.99 ± 0.05 | 95.83 ± 2.35 | 11.37 ± 0.46 | 10.08 ± 0.14 | 93.06 ± 3.82 | 4.72 ± 0.14 | 7.91 ± 0.10 | 93.75 ± 1.84 | 3.95 ± 0.18 | 7.12 ± 0.21 | 98.96 ± 1.04 | 2.14 ± 0.12 | 4.88 ± 0.08 |
| 0.05 ppm | 95.00 ± 1.51 | 2.306 ± 0.05 | 7.80 ± 0.05 * | 93.75 ± 1.88 | 10.34 ± 0.32 | 9.76 ± 0.46 | 94.44 ± 2.37 | 4.353 ± 0.12 | 7.61 ± 0.11 | 93.75 ± 1.84 | 4.09 ± 0.20 | 7.09 ± 0.25 | 98.96 ± 1.04 | 2.06 ± 0.14 | 4.69 ± 0.12 |
| 0.50 ppm | 93.33 ± 1.88 | 2.156 ± 0.03 | 8.00 ± 0.05 | 92.71 ± 3.25 | 9.43 ± 0.42 | 9.27 ± 0.22 * | 93.06 ± 3.22 | 4.587 ± 0.11 | 7.7 ± 0.10 | 95.83 ± 1.78 | 3.89 ± 0.25 | 7.37 ± 0.22 | 100.00 ± 0.00 | 2.11 ± 0.13 | 4.71 ± 0.07 |
| 2.00 ppm | 94.17 ± 2.29 | 2.257 ± 0.03 | 7.7 ± 0.06 * | 93.75 ± 2.43 | 10.58 ± 0.47 | 9.58 ± 0.20 | 93.06 ± 3.22 | 4.675 ± 0.13 | 7.90 ± 0.13 | 92.71 ± 2.86 | 3.97 ± 0.18 | 7.02 ± 0.17 | 98.96 ± 1.04 | 2.10 ± 0.11 | 4.81 ± 0.11 |

Overall, we observed no significant impacts on juvenile survival to all five species (4 different families and 4 different orders) when exposed to 2,4-D in DMA4 at ecologically relevant concentrations.

## 4. Discussion

### 4.1. Significance and overview

Our results demonstrate for the first time that exposure to environmentally relevant concentration of 2,4-D commercial herbicide formulation DMA4 reduces survival in early developmental stages of six freshwater species spanning five phylogenetic families, as well as decreases growth in two juvenile species. Six of the eight tested species that were exposed to DMA4 showed a decrease in survival of either embryos or larvae, however, we did not observe a correlation between phylogenetic distance and sensitivity to 2,4-D exposure. Additionally, we did not observe any impacts on juvenile survival in any of the species tested when the fish exposed to 2,4-D in DMA4. Altogether, these results demonstrate that in many freshwater fish species early developmental stages (e.g. embryo and larvae) are more at risk to 2,4-D exposure than juveniles and adults (Dehnert et al., 2018; DeQuattro and Karasov, 2016b), and results show that sensitivity to 2,4-D exposure is not restricted to a few closely related clades.

### 4.2. Early life stage sensitivity

Two out of three developmental stages tested showed significant reduction in survival when exposed to environmentally relevant concentrations of 2,4-D in DMA4. We observed a significant decrease in the percent of unaffected embryos for four species and a significant decrease in larval survival for three species (Figs. 3 and 4). Similarly, previous research showed that exposure to environmentally relevant concentrations of a 2,4-D commercial formulation decreased embryo survival in zebrafish (Gaaied et al., 2019), and that exposure to 2,4-D in DMA4 at environmentally relevant concentrations from fertilization to 14 dph significantly decreased larval survival in fathead minnows (Dehnert et al., 2018). However, present findings are the first published data to show a significant decrease in embryonic survival to any freshwater fish species. We did not observe any impacts on survival of juveniles (>90 d old), but did observe decreased growth in juveniles of two species. In agreement, previous literature observed no significant impacts on the survival of juvenile fish in a variety of species (e.g. rainbow trout (*Oncorhynchus mykiss*), bluegill (*Lepomis macrochirus*), striped bass (*Morone saxatilis*), banded killifish (*Fundulus diaphanous*), white perch (*Morone Americana*), and carp (*Cyprinus carpio*)) when exposed to the active ingredient 2,4-D (EPA, 2005), and no significant impacts on the survival of juvenile silver catfish but decreases in growth when exposed to another commercial 2,4-D formulation (U46D-Fluid) (Menezes et al., 2015). Our study using native species was not exhaustive and there may be other species, including endemic species, that are more sensitive and/or less sensitive to 2,4-D exposure than the species tested herein.

Early developmental stages may be more sensitive to 2,4-D than their older counterparts for a variety of reasons, such as underdeveloped homeostatic mechanisms to deal with toxicants, underdeveloped immune systems, and/or underdeveloped organs, e.g. liver and kidney (Mohammed, 2013; Mácová et al., 2008; Bentivegna and Piatkowski, 1998; McKim, 1977). As freshwater fish species mature, their organs become more developed and their skin becomes less permeable, and therefore potentially less vulnerable to 2,4-D exposure (Mohammed, 2013). Taken together, these data suggest that early developmental stages of fish (e.g. embryos and

larvae) are more sensitive to 2,4-D exposure as compared to their juvenile or adult counterparts (EPA, 2005; Dehnert et al., 2018, 2019b; DeQuattro and Karasov, 2016a; Gaaied et al., 2019; Menezes et al., 2015). Past literature suggests the mechanism of 2,4-D toxicity could be due to developmental and/or behavioral impacts to early life stages (Gaaied et al., 2019; Dehnert et al., 2019b), however, definitive conclusions cannot be made on the mechanisms of action and at this time there are no known biomarkers for 2,4-D toxicity in fish. The next step for investigating the impact of 2,4-D on freshwater fish ontogeny will be elucidating the molecular mechanisms underlying the observed morphogenetic defects, increased mortality, and reduced growth at different developmental life stages.

### 4.3. Phylogenetic aspects of species sensitivity

When evaluating aquatic environmental threats (e.g. 2,4-D), ecotoxicology studies commonly use two fish model species, i.e., *P. promelas* and *D. rerio* (Ankley and Villeneuve, 2006; Stegeman et al., 2010). This is arguably an instance of phylogenetic pseudoreplication (Hylton et al., 2018; Krull et al., 2013; Tincani et al., 2017) as both *P. promelas* and *D. rerio* belong to the same phylogenetic family, Cyprinidae. There are more than 1400 freshwater fish species in North America (Balian et al., 2008) which makes a comprehensive assessment of 2,4-D impacts on all native species unfeasible. In the present study, we did not observe significant impacts on survival and development in every species tested, but we did observe impacts in 5 of the 6 phylogenetic families tested. Thus, our study rejects the hypothesis that sensitivity to 2,4-D exposure is unique to Cyprinidae; perhaps a better null hypothesis for the future is that sensitivity to 2,4-D is relatively common across other freshwater fish families. Hence, the permitted use of commercial 2,4-D herbicide formulations to control invasive species in aquatic ecosystems, as per EPA regulations, could generally impact embryo and larval survival and development in multiple freshwater fish species.

For risk assessment, experimental results for a single species are commonly applied broadly to other species, but this predictive approach risks missing species-specific sensitivities to individual toxicants (EPA, 2005; Klassen, 2008; Newman, 2015). To protect non-target fish species, it is essential to understand interspecies sensitivity to toxicity and potential correlations between phylogeny, physiology, biochemistry, and toxicity (Klassen, 2008). Interspecies sensitivity to toxicity has been attributed to differences in physiology and biochemistry (EPA, 2003), such as uptake, biotransformation, distribution, and excretion of the toxicant (Newman, 2015). For instance, the differences in the embryo interspecies sensitivity to 2,4-D could be based on their rate of uptake of the chemical (Klassen, 2008; Newman, 2015). During embryo development, offspring are protected by a chorion, a thick lipophilic membrane that protects and isolates the mature egg from external environmental conditions (Cotelli et al., 1988; Bonsignorio et al., 1996). The chorion's structural complexity and macromolecular composition differs from species to species. Slight differences in thickness, surface configurations, number of layers, or composition could alter the lethal effect of 2,4-D by influencing the uptake of the active ingredient from the ecosystem (Stevanovic et al., 2017). Further research can unravel how these species chorions differ and if 2,4-D has distinctive permeability to individual species chorions.

In addition, phylogeny can be used as a tool to improve the prediction of interspecies sensitivity to a toxicant. However, we did not observe a significant correlation between sensitivity to 2,4-D exposure and phylogenetic distance. Additional phylogenetic comparative analyses that used individual life stages (e.g. embryo or larvae) and used the slope of survivorship vs. exposure concentration, a continuous variable as a measure of sensitivity, also showed no correlation between sensitivity to 2,4-D exposure and phylogenetic distances (Dehnert, 2020). If the results of our phylogenetic analyses are taken at face value, it would suggest that phylogeny alone may not be a strong predictor of sensitivity to 2,4-D exposure. The downstream implication of this inference is that risk assessments must sample a broad array of target species for off-target toxicant impacts, because data from one species may not reliably predict responses in taxonomically proximate species (e.g., congeneric or confamilial taxa). However, phylogeny should not be discarded for risk assessment because species that are more closely related have been documented to show similar impacts to some toxicants as compared to more distantly related species (Hylton et al., 2018). One possibility explaining why we did not detect evidence for phylogenetic inertia in the physiological response is because of low statistical power due to the limited number of species tested (Munkemuller et al., 2012; Brady et al., 2017; Freckleton et al., 2002; Freckleton and Rees, 2019). Additionally, our taxon selection was guided by the constraints of nativity to Wisconsin and significance to freshwater fisheries; the study species may have been too phylogenetically distant to observe a correlation with toxicant response (Brady et al., 2017). The next step for investigating the phylogenetic prediction of 2,4-D's impact on freshwater fish ontogeny survival will be exploring more comprehensive set of species that span a range of phylogenetic distances.

### 4.4. Ecological concerns

Chemical intervention by the application of a commercial herbicide is a commonly used strategy to control and prevent nuisance plants both in aquatic and terrestrial environments, despite the potential impacts the formulations could exert on non-target organisms (Ge et al., 2014). Currently, agriculture crops (e.g. corn, soybean, cotton, etc.) are being genetically modified to resist multiple active ingredients that are used to combat resistant weeds (Green et al., 2008) while simultaneously, the application of active ingredients has increased from 196 million to 516 million pounds between 1960 and 2008 in the United States (Fernandez-Cornejo et al., 2014) and will likely continue to grow globally (Tilman et al., 2001). Availability of genetically modified crops resistant to 2,4-D and other active ingredients (Frene et al., 2018), will likely lead to an increase in the use of products with the active ingredient 2,4-D (Islam et al., 2018). 2,4-D is one of the top five most used herbicides in the US (EPA, 2012) and as more 2,4-D is used on agricultural crops, a rise in non-target organisms exposure could be expected as 2,4-D is known to enter aquatic ecosystems from runoff, leaching, and spray drift (Islam et al., 2018; Carter, 2000). As multiple, phylogenetically distant fish species exposed to environmentally relevant concentrations of 2,4-D show significant reduction in survival, it is possible that 2,4-D entering aquatic ecosystems directly or indirectly could potentially impact larval survival, larval recruitment, species populations, and entire ecosystem dynamics. The known impacts of 2,4-D on survival combined with the impacts on other essential survival behaviors, (e.g. predator evasion and prey consumption (Dehnert et al., 2019b)), alongside other anthropogenic stressors, (e.g. temperature change, overfishing, pollution, habitat loss, etc.) could further lower larval recruitment and fitness of freshwater fish species, as fitness is influenced by the sum total of all stressors, which can act additively, antagonistically, and/or synergistically (Brady et al., 2017). Future studies on both direct toxicity and indirect impacts on essential survival behaviors of larval fish should be more extensively studied as commercial 2,4-D herbicide use will likely continue to grow in future.

## 5. Conclusion

Taken together, our results demonstrate that exposure to environmentally relevant concentrations of 2,4-D commercial herbicide formulation DMA4 reduces survival in early developmental stages of six freshwater species that span five phylogenetic families and three phylogenetic orders. However, sensitivity to 2,4-D exposure did not show correlation with phylogenetic proximity. Altogether, our results indicate that the use of 2,4-D herbicides in aquatic ecosystems at current recommended concentrations ($\leq$2 ppm whole-lake treatment) could present risk to multiple freshwater fish species survival during early development.

## Credit author statement

Gavin Dehnert: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Funding acquisition, Project administration Mariella Freitas: Methodology, Investigation, Writing - review & editing Prashant P. Sharma: Formal analysis, Data curation, Writing - review & editing Terry Barry: Resources, Supervision, Writing - review & editing William H. Karasov: Methodology, Project administration, Supervision, Funding acquisition, Writing - review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

This work was supported by the Wisconin Department of Natural Resources (AIRD-104-15) and the National Council for Science and Technology Development (CNPq) (scholarship to M. Freitas). The authors would like to thank Evan Routhier, Analise Lindborg, Brian Anton, and Paul Hoppe for animal care and husbandry and members of the Karasov labs for feedback on the experiments and analyses performed in this study.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127638.

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