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2,4-Dichlorophenoxyacetic acid containing herbicide impairs essential visually guided behaviors of larval fish

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ABSTRACT

Aquatic herbicides are used worldwide to eradicate nuisance and invasive plants despite limited knowledge of their toxicity to non-target organisms. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a common active ingredient in commercial herbicide formulations, which triggers plant cell death by mimicking the plant-specific hormone auxin. Application practices of 2,4-D commercial herbicides typically coincide with yearly freshwater fish spawning periods. This practice exposes fish to xenobiotics at their vulnerable larval stages. The full impacts of 2,4-D on larval fish remains poorly understood, and hence, whether it may alter larval survival, larval behavior, fish populations, and ecosystem dynamics. In the present study, we exposed embryonic and larval zebrafish (*Danio rerio*) to the active ingredient 2,4-D (pure 2,4-D) or a 2,4-D containing commercial herbicide DMA4®IVM (DMA4) and evaluated morphology, survival, behavior, and nervous system function. At 2,4-D concentrations producing no overt morphological defects during embryonic or early larval stages, we observed reduced survival throughout a 21-day larval assay (4–8 ppm DMA4 and 0.75–4 ppm pure 2,4-D). Notably, prey capture, a behavior essential to survival, was reduced in 2,4-D-exposed larval zebrafish (4–8 ppm DMA4 and 0.75–4 ppm pure 2,4-D) and yellow perch (*Perca flavescens*) (4–20 ppm DMA4). In zebrafish, 8 ppm DMA4 exposure reduced prey capture when exposure was restricted to the period of visual system development. Consistent with these results, larval zebrafish exposed to 8 ppm DMA4 showed reduced neural activity within the optic tectum following prey exposure. Together, our results suggest that 2,4-D alters the development and function of neural circuits underlying vision of larval fish, and thereby reduces visually guided behaviors required for survival.

1. Introduction

Herbicides are used worldwide to control nuisance and invasive plant species in freshwater waterways and agricultural regions (Baumgartner et al., 2017; Baharuddin et al., 2011; Harrahy et al., 2014). 2,4-Dichlorophenoxyacetic acid (2,4-D) is the active ingredient in many commonly used herbicide formulations (Pohanish, 2015). 2,4-D is designed to mimic auxin, a plant specific growth hormone, and thereby kill plants by overstimulating cell division (Grossmann, 2003; Song, 2014). 2,4-D-containing herbicides are applied to both terrestrial and aquatic environments, and have the potential to spread beyond the application site and contaminate surface and groundwater for prolonged periods (Glomski and Netherland, 2010; Nault et al., 2014). The half-life of 2,4-D is stated as 15 days in aerobic water systems, but can vary (to over 300 days) due to multiple water quality parameters (EPA, 2005; Nault et al., 2014). Thus, 2,4-D may have unintended consequences to non-target organisms and, subsequently, ecosystem dynamics. To determine whether 2,4-D containing herbicides represent a significant ecological concern, it is critical to evaluate the influence of 2,4-D on non-target organisms, especially fish.

Application practices and permitted concentrations of 2,4-D-containing herbicides have largely been defined by 2,4-D's chemical profile (EPA, 2005). The US Environmental Protection Agency (EPA) permits aquatic 2,4-D amine applications up to 4 ppm for spot treatments and up to 2 ppm for whole-lake treatments with an allowance for follow-up treatment 21 days after the initial application (EPA, 2005). To evaluate potential effects on non-target aquatic organisms, acute toxicity tests have been performed on juvenile or adult stage fish, including rainbow trout (*Oncorhynchus mykiss*), bluegill (*Lepomis macrochirus*), striped bass (*Morone saxatilis*), banded killfish (*Fundulus diaphanous*), white perch (*Morone Americana*), eel (*Anguilliformes rostrate)*, and carp (*Cyprinus carpio*). These studies report no observable effect concentrations of 2,4-D ranging from 14.2 to 63.4 ppm (EPA, 2005). Application of 2,4-D containing herbicides typically coincides with annual fish spawning

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periods and this practice exposes fish at their sensitive early embryonic and larval stages (Dehnert et al., 2018; Harrahy et al., 2014; Li et al., 2017; Mohammed, 2013). Therefore, it is essential to evaluate 2,4-D's influence on larval fish behavior to determine whether 2,4-D has an undesired impact on larval fish survival, fish populations, and ecosystem dynamics. Indeed, recent work showed that 2,4-D exposure reduced survival of larval fathead minnows, even at below-permitted concentrations (Dehnert et al., 2018; DeQuattro and Karasov, 2016). However, it remains unclear whether 2,4-D reduces survival of other fish species at the larval stage and if 2,4-D impacts behaviors essential to larval survival.

This study explores whether 2,4-D can impair essential behaviors in larval fish, including prey capture and predator evasion. We exposed embryonic and larval stage zebrafish (*Danio rerio*) to a 2,4-D commercial herbicide formulation DMA4®IVM (DMA4) or the active ingredient 2,4-D and evaluated their survival, morphology, behavior, and nervous system function. Zebrafish provide an ideal model system for toxicological analyses due to their well characterized and highly stereotyped developmental timing through embryonic and larval stages (Gahtan and Baier, 2004; Kimmel et al., 1995). Moreover, larval zebrafish are amenable to high throughput, semi-automated behavioral assays, which can define distinctly affected behaviors (Burgess et al., 2010; Burgess and Granato, 2007a; Gahtan and Baier, 2004; Kalueff et al., 2013; Wolman et al., 2011). Finally, an expansive toolkit is available to evaluate toxin effects on cellular makeup and neural activity in zebrafish. To understand if these impacts translated from a model organism to a freshwater game species, we exposed Yellow perch (*Perca flavescens*) to DMA4 and evaluated their survival, morphology, and behavior. Yellow perch are exposed to DMA4 during the spawning season and have a well characterized and highly stereotyped developmental timing through embryonic and larval stages (Brown et al., 2009; Brandt et al., 1987; Mansueti, 1964). The present study was designed to understand the impacts of 2,4-D on essential fish behaviors and their underlying neural substrates.

2. Methods

2.1. Chemicals and chemical analyses

Pure technical grade 2,4-Dichlorophenoxyacetic acid (2,4-D; purity > 95%) and DMA®4IVM, a commercial 2,4-D amine liquid herbicide formulation (DMA4; 46.3% 2,4-D), were purchased from Grainger Lab Supplies and Forestry Suppliers Inc. (Jackson, MS, USA), respectively. From here on, we will refer to the pure technical grade 2,4-D as "pure 2,4-D" and to the DMA4®IVM formulation as "DMA4." Concentrated stock (100 ppm) solutions were prepared in Pyrex glass by diluting DMA4 or pure 2,4-D in E3 embryo medium for zebrafish (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH adjusted to 6.8–6.9 with $NaHCO_3$) and system water for yellow perch (pH = 7.0, hardness = 250 ppm, ammonia = non-detectable, and 13 °C). This stock was further diluted with either E3 or system water to achieve target concentrations of 2.00, 4.00, 8.00, 10.00, 16.00, 20.00, 32.00 ppm for DMA4 and 0.10, 0.75, 2.00, 4.00, 6.00 ppm for pure 2,4-D. For DMA4, we refer to the concentration of 2,4-D (ppm) within the DMA4 formulation. These working solutions were aliquoted to one-liter amber glass bottles. Stock and working solutions were prepared every 6 days, stored in the dark at 4 °C, and kept for no more than 6 days.

To validate 2,4-D concentrations in the above working solutions, we collected samples at one and six days after the solutions were prepared. Samples were immediately frozen and stored at −20 °C until testing. Actual 2,4-D concentrations were measured by Wisconsin State Lab of Hygiene using a 2,4-D enzyme immunoassay (ELISA) kit (MDL = 1 ppb; Modern Water, New Castle, DE). Tables 1and 2 show actual concentrations compared to target concentrations as defined above for DMA4 (Table 1) and pure 2,4-D (Table 2). Target concentrations (ppm) will be referred to from here on.

Table 1
Measured 2,4-D concentrations for DMA4: mean ± S.E.M. (N = 16 samples for each treatment). ND = Non-detectable.

Target 2,4-D concentration (ppm)	Measured 2,4-D concentration (ppm) Mean ± SEM
0.00	ND
2.00	1.99 ± 0.193
4.00	4.55 ± 0.458
8.00	8.98 ± 1.00
10.00	10.57 ± 1.03
16.00	17.4 ± 1.12
20.00	21.3 ± 1.46
32.00	35.3 ± 2.26

Table 2
Measured 2,4-D concentrations for Pure 2,4-D: mean ± S.E.M. (N = 16 samples for each treatment). ND = Non-detectable.

Target 2,4-D concentration (ppm)	Measured 2,4-D concentration (ppm) Mean ± SEM
0.00	ND
0.10	0.0993 ± 0.00559
0.75	0.801 ± 0.0716
2.00	2.41 ± 0.377
4.00	4.56 ± 0.304
6.00	6.77 ± 0.379

2.2. Fish husbandry

Adult zebrafish (*Danio rerio*) of the AB wild type strain, between 6–12 months of age, were used to produce embryos and larvae for this study. Adults were maintained on a 14:10 h light:dark cycle and housed in groups within 10 L tanks (2–3 fish L^{-1}) in a recirculating system (Pentair Aquatic Ecosystems). System water was maintained at pH = 7.1, conductivity = 725 μS, and 28 °C, and 10–20% of the system water was replaced daily. Embryos/larvae were produced by pairwise crosses of adult zebrafish and raised as previously described (Kimmel et al., 1995; Gyda et al., 2012) in an E3 medium on a 14:10 h light:dark cycle at 28 °C.

Yellow perch (*Perca flavescens*) embryos were obtained from Coolwater Farms, LLC (Cambridge, WI). Embryos were produced using a ratio of two adult males to one adult female, and fertilized embryos/larvae were raised in system water on a 14:10 h light:dark cycle. All exposures and laboratory practices using zebrafish and yellow perch were reviewed and approved by The University of Wisconsin-Madison Research Animal Resource Center under protocols A005702 and L00547.

2.3. Survival assays

Zebrafish embryos were collected at 2 h post fertilization and pooled from a minimum of 10 independently derived clutches (˜1500–2000 total embryos). Embryos were verified for fertilization and sorted into 100 mm-wide Petri dishes at a density of 60 larvae/dish in 25 mL of E3. Replicate dishes (3–5) were prepared for each treatment group (0, 2, 4, 8, 16, 32 ppm DMA4 or 0, 0.10, 0.75, 2, 4, 6 ppm pure 2,4-D) per experiment. Toxicity curves were generated by exposing zebrafish embryos to 2,4-D concentrations of 0, 2, 4, 8, 16, 32 ppm DMA4 and, 0, 0.10, 0.75, 2, 4, 6 ppm pure 2,4-D. A range of concentration were chosen at or around ecologically relevant concentrations (EPA, 2005). Larvae were bathed in E3 media containing either DMA4 or pure 2,4-D beginning at 4 h post fertilization (hpf), and their respective media was changed daily. Deceased or malformed embryos were removed, and survival and deformities were recorded daily. At 3 and 6 days post fertilization (dpf), larvae were photographed for morphological assessment (Nikon SMZ18-CoolSNAP DYNO). On 6 dpf, larval fish were

counted to determine larval survival. This experiment was repeated 5 times.

At 6 dpf, larvae were transitioned from the 100 mm Petri dishes to 3 L tanks containing 500 mL of E3 with the same DMA4 or pure 2,4-D concentration in which the larvae had been previously exposed since 4 hpf. Larvae were fed paramecia (35 mL of 100–250 paramecia/mL) once daily. At 21 dpf, larval fish were counted to determine larval survival. This experiment was repeated 3 times.

Yellow perch embryos were hand fertilized. Two ribbons were pooled together; each ribbon was fertilized 1:2, female:male. Embryos were verified for fertilization and sorted into 100 mm-wide Petri dishes at a density of 60 larvae/dish in 25 mL of system water. 5 dishes were prepared for each treatment group (0, 2, 4, 10, 20 ppm DMA4). Toxicity curves were generated by exposing yellow perch embryos to 2,4-D concentrations of 0, 2, 4, 20 ppm DMA4. A range of concentration were chosen at or around ecologically relevant concentrations (EPA, 2005) Larvae were raised in DMA4 beginning at 4 hpf and their water was exchanged daily. Deceased or malformed embryos were removed, and survival was recorded daily. At hatch, 8 dpf, larvae were transitioned from the 100 mm Petri dishes to 3 L tanks containing 500 mL of system water with the same DMA4 concentration in which the larvae had been previously exposed since 4 hpf. Larvae were fed paramecia (35 mL of 100–250 paramecia/mL) once daily. At 21 days post hatch (dph), larval fish were counted to determine larval survival.

2.4. Behavioral assays and analyses

For zebrafish, prey capture analyses were performed with 6 dpf larvae similar to as previously described (Gahtan and Paul Baier, 2005). Briefly, 50 paramecia were added to each well of a 12 well plate in 0.75 mL of E3 medium. One larva was added to each well and allowed to actively hunt in lighted conditions at 28 °C for 4.5 h. After 4.5 h, larvae were removed, and the remaining paramecia were counted to determine the percentage of captured paramecia. Prey capture was defined as ((1 − (# of paramecia remaining/50))*100). Each 12 well plate included 4 treatment groups (3 DMA4 or 3 pure 2,4-D concentrations plus a control (0 ppm E3 only) and 3 larvae per treatment group. For yellow perch, we performed the identical assay, but on 3 dph larvae.

We evaluated spontaneous movement, phototaxis, acoustic startle responses, and prey capture behaviors of 6 dpf zebrafish larvae using an automated behavioral platform as previously described (Burgess et al., 2010; Burgess and Granato, 2007a, 2007b; Hao le et al., 2017; Miller et al., 2018; Wolman et al., 2011). Larval behavior was captured with an IDT MotionPro Y4 video camera and analyzed with the FLOTE software package (Burgess and Granato, 2007b, 2007a). FLOTE provides an experimenter independent, automated tracking analysis of larval zebrafish behavior. FLOTE tracks larval position frame by frame and characterizes locomotor maneuvers (e.g. turn, swim, C-bend, etc.) according to predefined kinematic parameters that distinguish these maneuvers (Burgess and Granato, 2007b, 2007a). On the day of behavioral testing, 6 dpf larvae were held in 60 mm-wide Petri dishes with 20 larvae in 10 mL E3, kept on a white light box for at least 1 h, and then acclimated them to testing conditions as described below.

To evaluate spontaneous, gross movement over time, larvae were transferred from their 60 mm Petri dish into individual wells of a 4 × 4 grid (Wolman et al., 2011) and given 5 min to acclimate to the grid and illumination conditions (25 μW/cm^2). Their position was then continuously recorded at 100 frames per second for 180 s and the distance of their movement was tracked by FLOTE. To evaluate spontaneous initiations of swims, turn maneuvers, and swimming performance, 20 larvae from the same treatment were grouped in a 60 mm Petri dish and 3 dishes were tested per treatment. Larvae were given 5 min to acclimate to the illumination conditions (25 μW/cm^2). We then captured 1 s recordings at 1000 frames per second and at 5 s intervals for a total of 30 trials. For each 1 s recording, FLOTE determined if each larva initiated a turn, swim, or remained stationary; only the first movement type in each recording was counted. This analysis was used to determine the mean frequency of a turn or swim maneuver initiation. To evaluate swimming performance, we used FLOTE to analyze kinematic parameters of half swim cycles, swim cycle magnitude, and distance swam per bout. A half swim cycle was defined as each leftward or rightward tail undulation.

To evaluate positive phototaxis behavior, 20 larvae were added to 7 mL of E3 in 60 mm Petri dishes for testing. Each dish of larvae was acclimated to an illuminated testing stage (25 μW/cm^2) for 5 min. To elicit phototaxis, the uniform illumination was removed, revealing a small target light (10 μW/cm^2) on one side of the arena for 30 s. Larvae initially positioned on the same side (half) of the dish as the target light were excluded from the analysis. Larvae on the side of the dish opposite of the target light were analyzed for phototaxis success, as defined by entry into target light area within the 30 s trial. Using FLOTE, larvae orientated perpendicular to target line (facing 75–105° to target light area) were analyzed for turn directionality and larvae facing within 30° of the target light were analyzed for swims towards the target. Each dish of larvae was tested for phototaxis 3 times, with 5-minutes of uniform illumination (25 μW/cm^2) between trials. Each treatment group was tested in three separate dishes.

To evaluate escape responses to acoustic stimuli, larvae were transferred from the 60 mm Petri dishes into individual wells of a 4 × 4 grid (Wolman et al., 2011) and allowed to acclimate to the grid and illumination conditions (25 μW/cm^2) for 3 min. Larvae were then exposed to 10 acoustic stimuli, with each stimulus separated by a 20 s interstimulus interval. Acoustic stimuli were of 3 ms duration, with 1000-Hz wave forms, and an intensity of 25 decibels (Wolman et al., 2011). FLOTE was used to track and define short-latency C-bend (SLC) escape maneuvers (Burgess and Granato, 2007a). Escape response initiation frequency was determined by calculating the percentage of stimuli in which each larva responded by performing a SLC escape maneuver. To evaluate SLC escape maneuver performance, we used FLOTE to analyze kinematic parameters of the SLCs, including the latency to initiate a C-bend, the head turning angle during a C-bend, and the distance moved as a result of initiating and executing a SLC.

To evaluate prey capture, larvae were transferred from their 60 mm Petri dish into individual wells of a 4 × 4 grid (Wolman et al., 2011) and given 1 min to acclimate to the grid and illumination conditions (25 μW/cm^2). Larvae were exposed to E3 media containing ˜100 paramecia per mL. Their position was then continuously recorded at 500 frames per second for 90 s. FLOTE was used to track and define prey capture maneuvers, including the J-Bend turns and burst swims (Borla et al., 2002; Budick and O'Malley, 2000; McElligott and O'Malley, 2005).

To evaluate larval survival in the presence of a predator, we followed a recently described zebrafish predation protocol. Adult female zebrafish (AB strain, 8–12 months old) were used as predators. To initiate the trial, one adult was placed into a 1 L tank ˜16 h prior to the onset of the predation trial. 3 h prior to the trial an opaque divider was inserted into the tank, which divided the tank into equally sized, side-by-side compartments. Then, fifty 6 dpf larvae, which had been raised in either E3 alone or 8 ppm DMA4, were added to the tank on the opposite side of the divider from the adult. At the end of an hour, we determined the percentage of larvae in the top, middle, and bottom third of the water column. The predation trial was then initiated by removing the tank divider, which allowed both the adult and larvae access to the entire tank. After 5 min, the adult was removed using a coarse net and the remaining larvae were counted to determine the percentage of captured larvae.

2.5. Tectal activity measurement

Neural activity in the optic tectum was performed as previously described (Randlett et al., 2015). Larvae were raised in either E3 alone

or 8 ppm DMA4. Individual larvae from each of the treatment groups were transferred to a single well in a 12 well plate with 0.75 mL of E3 and then provided with either 50 or 0 paramecia in 0.25 mL of E3. Larvae were allowed to actively hunt for 20 min and then fixed in 4% paraformaldehyde containing 4% sucrose for 1 h at room temperature. Fixed larvae were rinsed in PBS and their brains were isolated by dissection. Isolated brains were rinsed in PBS and then blocked with 5% normal goat serum, 1% DMSO, 0.3% triton-X, and 2 mg/mL bovine serum albumin in PBS for 1 h at room temperature. Brains were incubated with the primary antibodies anti-total ERK (tERK) (1:100, #4696, Cell Signaling Technologies) and anti-phosphorylated ERK (pERK) (1:100, #4370, Cell Signaling Technologies) overnight at 4 °C in blocking solution, washed out, and then detected by addition of AlexaFluor488 and AlexaFluor594 conjugated secondary antibodies (1:500, Life Technologies). Samples were mounted in Vectashield (Vector Labs). Z-stacks were acquired and processed with an Olympus IX81 laser scanning confocal (20× oil objective) using Olympus Fluoview software FV10-ASW. Imaging parameters (laser power, gain, offset) were held constant and images were acquired within a 48-hour period to ensure near identical imaging conditions. To determine the relative fluorescent intensity of pERK and tERK immunostaining, raw integrated densities were measured from regions of interest and background in summation projections (40 focal planes spanning 62 µm) using ImageJ software (Schindelin et al., 2012). Regions of interest were drawn around the cell bodies in the tectum (see Fig. 4). To quantify a potential change in tectal cells' pERK:tERK ratio due to paramecia exposure, we divided the mean pERK:tERK ratio measured from individual larvae exposed to paramecia by the mean pERK:tERK ratio measured from individual of larvae that were not exposed to paramecia. This normalization was performed independently for untreated (0 ppm DMA4) and 8 ppm DMA4 treated larvae to account for slight differences in the baseline pERK:tERK ratios measured from larvae not exposed to paramecia.

2.6. Statistics

All data were analyzed using GraphPad Prism Software 7.0b (GraphPad Software Incorporated, La Jolla, Ca, USA). Prior to use of parametric statistics, the assumption of normality was tested using Brown-Forsythe test and 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 either an unpaired *t*-test with Welch's correction (t statistic presented) or one-way-ANOVA followed by Tukey's multiple comparison analysis (F statistic presented). For non-parametric analyses, we performed a Kruskal-Wallis test followed with a Dunn's multiple comparison analysis (X^2 statistic presented). Data are presented as means ± standard error of the mean (SEM; n = sample size). Significance was set at $p \leq 0.05$. Sample size of N for each experiment is detailed in the results and/or figure legends.

3. Results

3.1. DMA4 and pure 2,4-D exposure reduced survival of larval zebrafish

To investigate whether 2,4-D exposure was toxic to embryonic and larval zebrafish, we exposed embryos beginning at 4 h post fertilization (hpf) to DMA4 and quantified survival rates at 21 days post fertilization (dpf). Compared to control larvae raised in E3, exposure to DMA4 reduced survival by 11% at 2 ppm, 42% at 4 ppm, 47% at 8 ppm, 61% at 16 ppm, and 100% at 32 ppm ($F_{5,42} = 378.8$; $p < 0.0001$) (Fig. 1A). The commercial 2,4-D herbicide formulation, DMA4, is a combination of the active ingredient and supplemented with inert ingredients (DMA4®IVM: ~47% 2,4-D and 53% inert ingredients). To determine if the reduced survival following DMA4 exposure was due to the active ingredient 2,4-D or the formulation DMA4, we repeated the 21-day survival assay with pure 2,4-D. Compared to control larvae raised in E3, exposure to pure 2,4-D reduced survival by 8% at 0.1 ppm, 21% at 0.75 ppm, 26% at 2 ppm, 42% at 4 ppm, and 100% at 6 ppm ($F_{5,42} = 400.8$; $p < 0.0001$) (Fig. 1B). These results indicate that pure 2,4-D alone can reduce larval survival.

Next, we aimed to define the critical time period in which 2,4-D exposure reduced larval survival. To address this, we asked whether DMA4 and pure 2,4-D caused mortality between early embryogenesis (4 hpf) and an early larval stage (6 dpf). Compared to control larvae raised in E3, exposure to DMA4 from 4 hpf to 6 dpf only reduced survival at concentrations ≥ 16 ppm ($X^2(6, N = 96) = 81.16$; $p < 0.0001$) (Fig. 1C). Exposure to pure 2,4-D during this period only reduced survival at concentrations ≥6.00 ppm ($X^2(6, N = 72) = 52.72$; $p < 0.0001$) (Fig. 1D). Notably, concentrations of DMA4 (2–8 ppm) and pure 2,4-D (0.75–4 ppm), which strongly reduced larval survival at 21 dpf, had no effect on survival by 6 dpf. Next, we examined the possibility that DMA4 or pure 2,4-D concentrations that strongly reduced survival at 21 dpf had caused overt morphological defects by 6 dpf (but not yet death), which would later manifest as death between 6 and 21 dpf. To address this, we evaluated the morphology of embryos at 3 dpf and larvae at 6 dpf, which had been treated with DMA4 or pure 2,4-D since 4 hpf (Fig. 1E). At these stages, we observed no overt morphological defects at ≤8 ppm DMA4 or ≤4 ppm pure 2,4-D (Fig. 1E). However, beginning at 16 ppm DMA4 and 6 ppm pure 2,4-D we observed an increase in the percentage of larvae exhibiting various morphological defects, which included cardiac edema, underinflated swim bladder, and curved anterior-posterior axis development (Fig. 1F–G).

3.2. DMA4 and pure 2,4-D exposure reduced prey capture by zebrafish larvae

One essential behavior for larval survival is their ability to forage. For zebrafish, 6 dpf marks the time point at which larvae have exhausted their yolk's supply of nutrients and must actively forage for sustenance. We hypothesized that 2,4-D exposure may have impaired larvae's ability to capture paramecia, which was used to feed zebrafish larvae from 6 to 21 dpf in the survival assays (Fig. 1A and B). To test this hypothesis, we exposed zebrafish to DMA4 or pure 2,4-D from 4 hpf to 6 dpf and assessed their ability to capture paramecia at 6 dpf. Compared to control larvae raised in E3, exposure to DMA4 reduced prey capture by 4% at 2 ppm, 11% at 4 ppm, 18% at 8 ppm, 24% at 16 ppm (Fig. 2A) ($X^2(5, N = 186) = 133.2$; $p < 0.0001$). Likewise, compared to control larvae raised in E3, exposure to pure 2,4-D reduced prey capture by 4% at 0.1 ppm, 11% at 0.75 ppm, 14% at 2 ppm, 20% at 4 ppm (Fig. 2B) ($F_{4,175} = 53.89$; $p < 0.0001$).

To characterize the prey capture impairment caused by 2,4-D exposure, we evaluated the stereotyped maneuvers that larvae employ to capture prey (Bianco et al., 2011; Borla et al., 2002). Larvae perform J-turn maneuvers to orient themselves in line with prey and then perform an explosive burst swim bout to rapidly approach their prey (Bianco et al., 2011; Borla et al., 2002; McElligott and O'Malley, 2005). Compared to larvae without paramecium in their media, we observed an increased frequency of J turns and burst swims initiated by untreated larvae with paramecium in their media (Fig. 2C and D) (J-Turn: $t = 13.89$, $df = 43.96$, $p < 0.0001$; Burst swim: $t = 13.87$, $df = 44.03$; $p < 0.0001$). This increase in prey capture maneuvers was attenuated in larvae that were raised in 8 ppm DMA4 from 4 hpf to 6 dpf (Fig. 2C–D) (J-Turn: $t = 8.541$, $df = 78.49$, $p < 0.0001$; Burst swim: $t = 8.979$, $df = 76.31$, $p < 0.0001$). These data indicate that exposure to 2,4-D decreases larval zebrafish prey capture maneuvers and success.

3.3. DMA4 exposure reduced visually guided behavior, but not general locomotion

The maneuvers used by zebrafish larvae to capture paramecia are

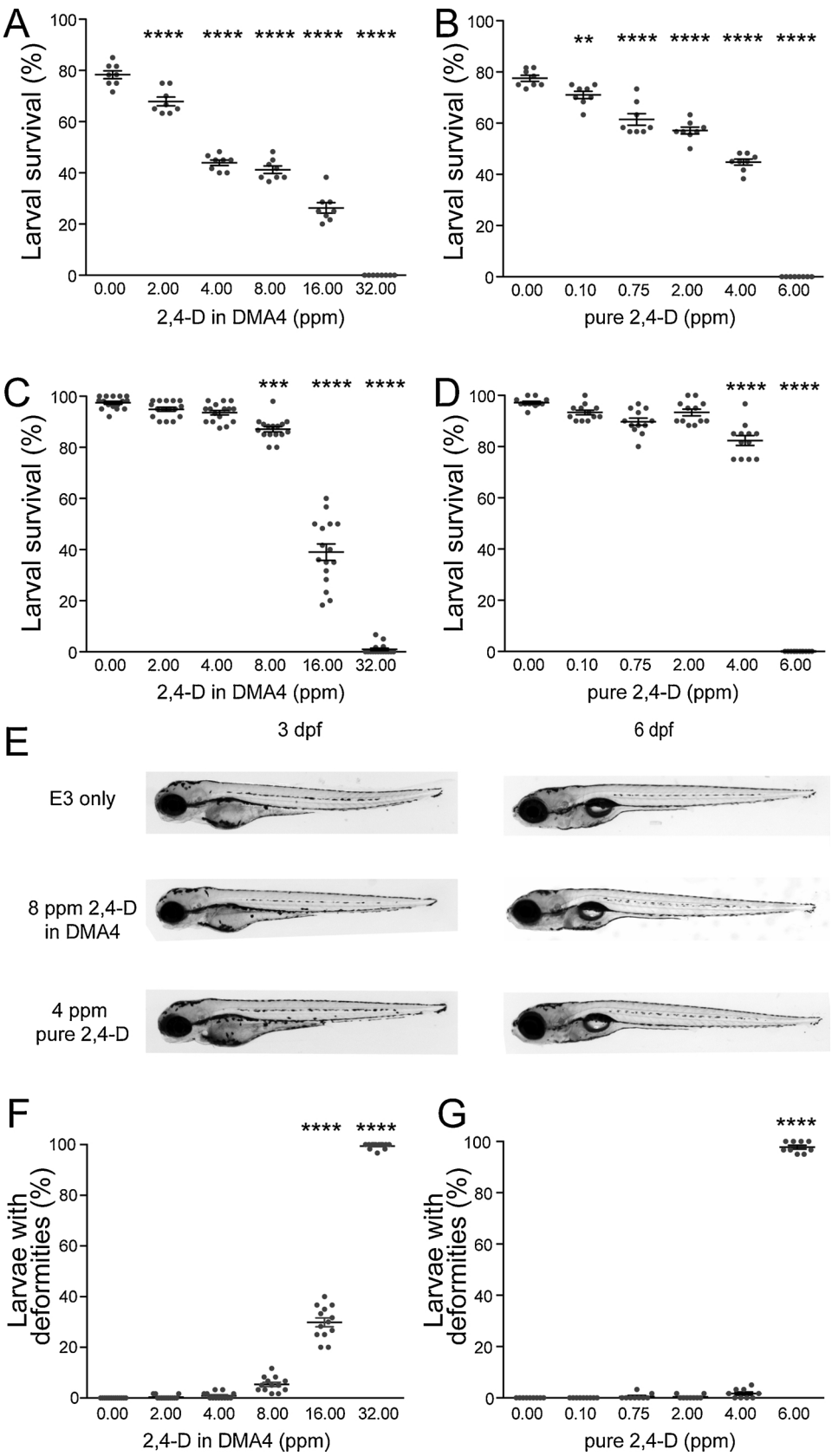

Fig. 1. DMA4 and pure 2,4-D reduce survival of larval zebrafish.
(A–D) Mean percentage of surviving larvae at 21 (A–B) or 6 dpf (C–D), following exposure to DMA4 or pure 2,4-D beginning at 4 hpf. (F–G) Mean percentage of larval with morphological deformities (cardiac edema, curved anterior-posterior axis, underinflated swim bladder) at 6 dpf. Each dot indicates survival/deformity percentage of a group (N) of 60 larvae. **p < 0.01, ***p < 0.001, ****p < 0.0001 versus untreated (0 ppm) larvae; one-way ANOVA with Tukeys multiple comparison analysis (A–B) or Kruskal-Wallis with Dunn's multiple comparison test (C–D, F–G). All error bars represent SEM. (E) Lateral views of 3 or 6 dpf zebrafish that were untreated (E3), exposed to 8 ppm 2,4-D in DMA4, or 4 ppm pure 2,4-D.

guided by visual cues (Gahtan and Baier, 2004; Muto and Kawakami, 2013). We hypothesized that if 2,4-D caused deficits in vision and/or locomotor capabilities, then these outcomes could contribute to the reduced initiation of prey capture maneuvers and thus, prey capture success. To determine whether DMA4 caused motility deficits, we evaluated gross movement and the initiation of routine turning and swimming maneuvers in 6 dpf larvae that were exposed to DMA4 from 4 hpf to 6 dpf. At DMA4 concentrations that reduced prey capture

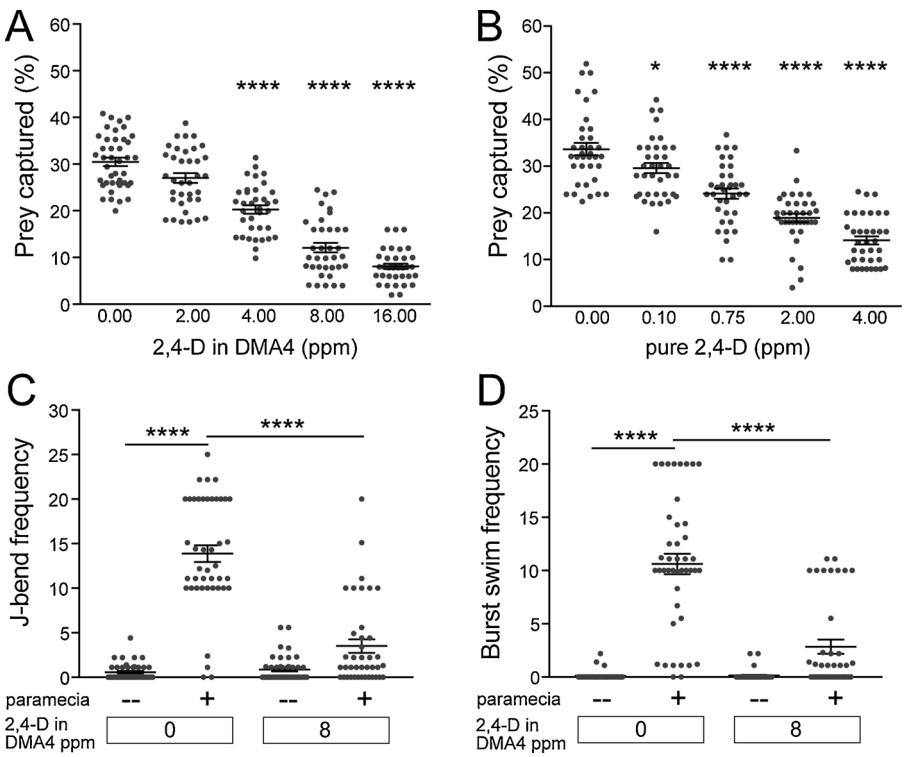

Fig. 2. DMA4 and pure 2,4-D reduce prey capture success and behaviors of larval zebrafish.
(A–B) Mean percentage of prey (paramecia) captured by 6 dpf larvae exposed from 4 hpf to 6 dpf to DMA4 (A) or pure 2,4-D (B). Each dot represents percentage of prey captured by an individual larva (N). *p < 0.05, ****p < 0.0001 versus untreated (0 ppm) larvae group; Kruskal-Wallis with Dunn's multiple comparison test (A) or one-way ANOVA with Tukeys multiple comparison analysis (B). (C-D) Mean frequency of initiating a J-bend turn (C) or a burst swim maneuver (D) per 5 second recording period. Each maneuver was quantified for untreated (0) or 8 ppm DMA4 exposed larvae that were in the presence (+) or absence (-) of paramecium. Each dot represents an individual larva (N). ****p < 0.001 unpaired T-test with Welch's correction (C–D). All error bars represent SEM.

(4–8 ppm), we observed a similar amount of gross movement ($F_{3,139}$ = 0.1403; p = 0.9357) (Fig. 3A) and initiation frequency of turn and swim maneuvers as compared to untreated larvae (Turn, $F_{3,8}$ = 0.5409, p = 0.6676; Swim, $F_{3,8}$ = 1.013, p = 0.4362) (Fig. 3B–C). Moreover, larvae exposed to DMA4 performed swim maneuvers with similar kinematic parameters as compared to untreated larvae (Distance swam per bout: X^2(4,N = 706) = 2.669; p = 0.4456; Half swim cycles: X^2(4,N = 706) = 10.81; p = 0.013; Swim cycle magnitude: X^2(4,N = 706) = 5.895; p = 0.1169) (Fig. 3D–F). Together, these results suggest that locomotor impairment is not the likely reason for prey capture deficits caused by DMA4 exposure.

Prey detection relies on a larva's ability to detect and interpret contrast in their visual field (Muto and Kawakami, 2013; Randlett et al., 2015). We hypothesized that the prey capture deficiency in larvae exposed to DMA4 was due to an impaired ability to detect contrast. To test this hypothesis, we evaluated contrast-mediated navigation to a light target (phototaxis behavior) in 6 dpf larvae exposed to DMA4 from 4 hpf to 6 dpf. Larvae were first acclimated to a uniformly illuminated arena. Subsequently the light was removed leaving a dark arena except for a small target light on one side. Under these conditions larvae stereotypically initiate a turn away from the darkened side of the dish and then initiate a series of swim bouts toward the target light until they reach the small illuminated area (Burgess et al., 2010). At DMA4 concentrations that reduced prey capture (4–8 ppm), we observed a reduction in the success of larvae to navigate to the target light within 30 s ($F_{3,211}$ = 5.518, p = 0.0018) (Fig. 3G). Within the 30 s phototaxis trials, DMA4 treated larvae exhibited deficits in initiating the dark and light-evoked maneuvers used for navigation based on a larva's orientation to darkened and illuminated regions of the arena (Swims toward light, $F_{3,8}$ = 25.02, p = 0.0002; Turns away from darkness, $F_{3,8}$ = 15.53, p = 0.0011) (Fig. 3H–I). These results, together with the assessment of DMA4 on locomotor ability (Fig. 3A–F), suggest that 2,4-D impairs the detection of stimuli that evoke visually guided behaviors, like prey capture.

3.4. 2,4-D reduced tectal neuronal activity during prey capture

Prey detection is mediated by neural circuits in the retina, optic tectum, and hindbrain, which define prey characteristics, including size, movement, and contrast (Bianco et al., 2011; Borla et al., 2002; McElligott and O'Malley, 2005; Muto and Kawakami, 2013; Randlett et al., 2015). During prey capture, neurons in the optic tectum have been shown to display increased activity (Fig. 4A) (Randlett et al., 2015). This activity can be measured by an increase in the ratio of phosphorylated ERK (pERK) to total ERK (tERK) using immunofluorescent antibodies against these substrates (Randlett et al., 2015). We hypothesized that if 2,4-D impairs the neural substrates mediating detection of prey, then the activity of these tectal neurons would be reduced in DMA4 exposed larvae presented with paramecia. To test this hypothesis, we compared the pERK:tERK immunofluorescent ratio in untreated and DMA4 treated 6 dpf larvae, which were either exposed to paramecia or not. After 20 min in E3 alone or with paramecia, the larvae were immediately fixed and then immunolabeled for pERK and tERK (Fig. 4B–E). For untreated and DMA4 treated larvae, pERK:tERK values of paramecia exposed larvae were normalized to pERK:tERK values of larvae that were not exposed to paramecia (see Methods). Untreated larvae exposed to paramecia showed a 2.34 fold increase in pERK:tERK, indicating an increase in tectal activity in the presence of paramecia (Fig. 4F). For larvae exposed to DMA4, paramecia exposure only yielded a 1.30 fold increase in pERK:tERK (t = 6.083, df = 16.22, p < 0.0001) (Fig. 4F). This difference suggests that the activity of neural circuits underlying prey detection is reduced in DMA4 treated larvae.

3.5. DMA4 exposure during visual system development reduced prey capture

For zebrafish larvae to have the ability to capture prey, its visual system must rapidly develop within the first 5 dpf (Gahtan and Paul Baier, 2005; Gahtan and Baier, 2004; Muto and Kawakami, 2013). We were curious to determine whether DMA4 exposure reduced prey capture by either altering visual system development or by acutely

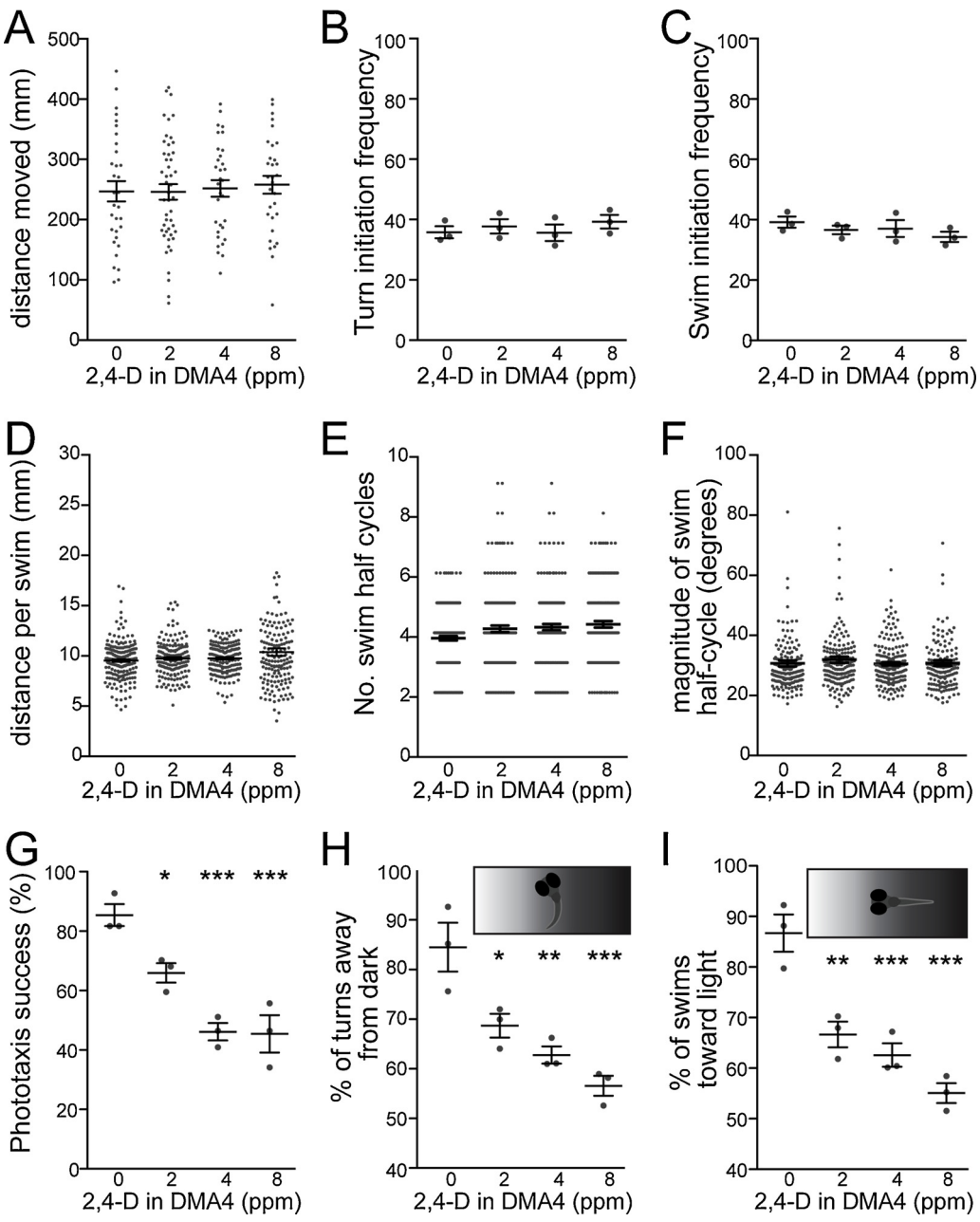

Fig. 3. DMA4 exposure reduces phototaxis behavior, but not locomotor ability, in larval zebrafish.
(A) Mean distance moved over 180 seconds as a result of non-evoked, spontaneous locomotion. Each dot represents an individual larva (N). (B–C) Mean initiation frequency of swim (B) or turn (C) maneuvers. Each dot represents movement initiation frequency of a group of 20 larvae (N = 3 groups of 20 larvae/group). (D–F) Kinematic parameters of swim performance, including mean distance moved per swim bout (D), mean number of half-swim cycles per swim bout (E), and mean magnitude of half-swim cycles (F). Each dot represents a measurement from a single swim bout (N). (G) Mean percentage of larvae reaching phototactic light target. Each dot represents percentage of larvae among a group of 20 (N = 3 groups of 20 larvae/group). (H–I) To perform phototaxis, larvae positioned perpendicular to a light gradient stereotypically turn away from darkness (H) and once positioned toward the light, a larva will initiate swim bouts (I) to approach the light target. (H) Mean percentage of turns initiated away from darkness by larvae positioned between 75–105° to the light-dark gradient. Each dot represents mean percentage of turns away from darkness amongst a group of 20 larvae during a 30 second-long phototaxis trial. Per trial, 19–38 turn events were analyzed per group (dot). (I) Mean percentage of swim maneuvers towards the light target by larvae facing +/− 30 degrees of the light stimulus. Each dot represents the mean percentage of swims toward the light amongst a group of 20 larvae during a 30 s-long phototaxis trial. Per trial, 35–77 swim events were analyzed per group (dot). All error bars represent SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus untreated (0 ppm) larvae; one-way ANOVA with Tukeys multiple comparison analysis (A–C, G–I) or Kruskal-Wallis with Dunn's multiple comparison test (D-F).

disrupting the system's post-developmental functionality. We hypothesized that if DMA4 impaired post-developmental visual system function alone, then exposure to 8 ppm DMA4 beginning on or after 5 dpf would be sufficient to reduce prey capture by 6 dpf larvae. Instead, we found that DMA4 exposure beginning at 5 dpf, ~13 h prior to and during the prey capture assay, only minimally reduced prey capture success as compared to untreated larvae ($F_{3,44} = 25.16$; $p = 0.1542$) (Fig. 5). Next, we next hypothesized that if DMA4 impaired visual system development, then exposure to 8 ppm DMA4 between 4 hpf and 5 dpf and then removal of DMA4 from the larvae's media would be sufficient to reduce prey capture by 6 dpf larvae. Indeed, this period of exposure reduced prey capture by 23% compared to untreated larvae ($F_{3,44} = 25.16$; $p < 0.0001$) (Fig. 5), and to a similar degree as DMA4 exposure from 4 hpf to 6 dpf.

3.6. DMA4 exposure increased larval capture by adult predators

Given the reduced visual circuit activity and behavior following DMA4 exposure, we were curious as to how DMA4 might influence other essential visually guided behaviors. To survive, larvae must detect and escape predators with reliance on their visual system (Fuiman et al., 2006). To determine whether DMA4 exposed larvae showed deficiencies in escaping predators, we introduced 6 dpf larvae that were either untreated or exposed to 8 ppm DMA4 from 4 hpf to 6 dpf to an adult zebrafish, which will prey on its larval conspecifics (Howe et al., 2018). We hypothesized that if DMA4 exposure reduced escape from predation, then more DMA4 exposed larvae would be captured compared to untreated larvae. Indeed, DMA4 exposure increased the number of larvae captured by a predator ($t = 3.949$, $df = 37.62$; $p = 0.0003$) (Fig. 6A). This increase in capture of DMA4 exposed larvae could be attributed their visual deficits (Figs. 3G–H, 4). DMA4 exposure

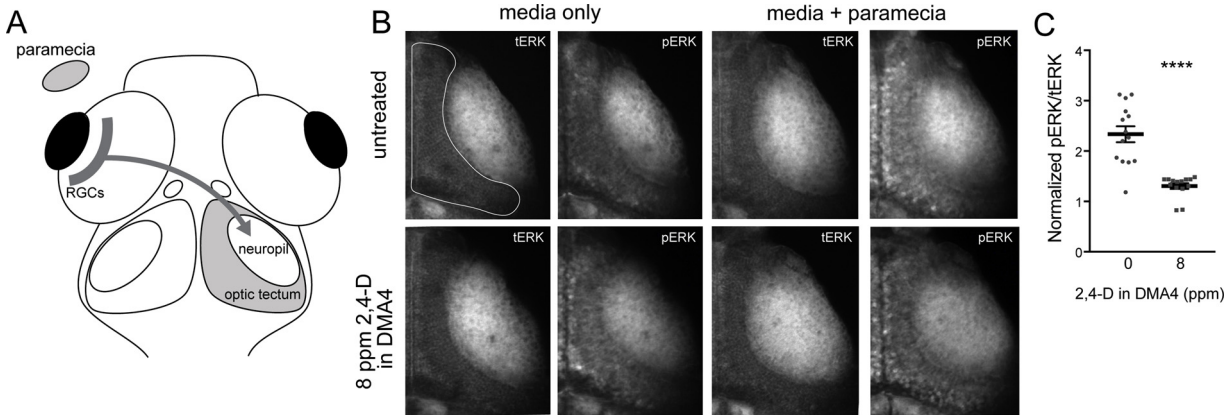

Fig. 4. DMA4 exposure reduces activity of tectal neurons during prey capture.
(A) Schematic representation of optic tectum of larval zebrafish, which receives input from contralaterally positioned retina. Shaded area marks region of tectal neurons active when paramecia is detected by contralateral retina. (B) Representative images of anti-pERK and anti-tERK immunolabeled tecta of untreated (top) and 8 ppm 2,4-D in DMA4 treated (bottom) 6 dpf larvae that were either swimming in E3 media alone or E3 with paramecia. Outline marks region of interest for pERK:tERK immunofluorescent signal measurements. (C) Immunofluorescent ratio of pERK:tERK. Each dot shows ratio of an individual larva (N). All error bars represent SEM. ****p < 0.0001; unpaired T-test with Welch's correction.

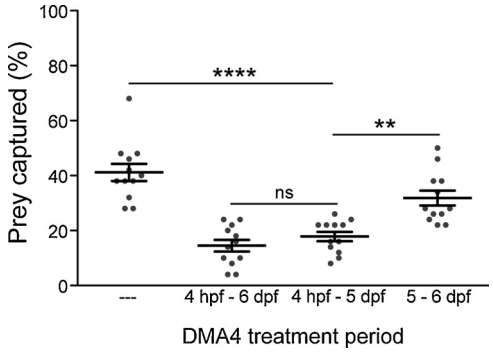

Fig. 5. Exposure to DMA4 during visual system development reduces prey capture behavior.
Percentage of prey (paramecia) captured by 6 dpf larvae which were untreated (0 ppm) or exposed to DMA4 at different time periods. Each dot shows prey capture by an individual larva (N). Bars represent means +/− SEM. **p < 0.01, ****p < 0.0001; one-way ANOVA with Tukey's multiple comparison test.

may have also altered the larvae's positional distribution within the water column, and therefore increased their susceptibility to capture. However, we observed no difference in water column distribution between untreated and DMA4 exposed larvae ($F_{3,28}$ = 2.143; p = 0.39) (Fig. 6B).

Next, we tested the possibility that DMA4 exposure could reduce a larva's ability to initiate and execute an escape response, which would likely yield an increased probability of capture. To assess escape ability, we evaluated the initiation of escape responses triggered by acoustic stimuli. We observed no defects in escape probability in DMA4 treated larvae as compared to untreated larvae ($F_{4,145}$ = 0.8471; p = 0.372) (Fig. 6C). Moreover, larvae exposed to DMA4 performed escape responses with identical kinematic parameters as untreated larvae (Latency, $F_{3,566}$ = 0. 9608, p = 0.9622; C-bend turn angle: $F_{3,566}$ = 0.661; p = 0.5765; Distance moved: $F_{3,566}$ = 1.837, p = 0.8393) (Fig. 6D–F). These results suggest that DMA4 exposure does not disrupt the acoustic, central, and motor circuits that mediate a larva's ability to initiate and execute an escape maneuver to acoustic stimuli. Rather, the visual circuits affected by DMA4 exposure (Figs. 3G–H, 4) are more likely to contribute to exposed larvae's increased capture.

3.7. 2,4-D exposure reduced larval survival and prey capture of yellow perch

Given the reduced prey capture in zebrafish, we hypothesized that fish species that populate waters where 2,4-D herbicide formulations are applied would be similarly affected at equivalent life stages. To address this, we exposed yellow perch to DMA4 from 4 hpf to 21 dph and assessed larval survival. Compared to untreated larvae, exposure to DMA4 reduced survival by 16% at 4 ppm, 27% at 10 ppm, and 58% at 20 ppm ($F_{4,115}$ = 82.86, p < 0.0001). Additionally, we exposed yellow perch to DMA4 and assessed their ability to capture paramecia. Like 6 dpf for zebrafish, 3 dph yellow perch represent the stage when larvae have extinguished their yolk's supply of nutrients and must begin to forage. Compared to untreated larvae, exposure to DMA4 from 4hpf to 3 dph (11dpf) reduced prey capture by 2% at 2 ppm, 11% at 4 ppm, 17% at 10 ppm, and 39% at 20 ppm ($F_{4,115}$ = 82.86, p < 0.0001) (Fig. 7). These results indicate that 2,4-D reduces larval survival and prey capture success for a game species native to waterways where 2,4-D is applied.

4. Discussion

4.1. Overview and significance

Results of this study demonstrate that exposure of larval fish to 2,4-D impairs essential visually guided behaviors and reduces survival. Both outcomes occur without overt anatomical malformations at the fish's embryonic or larval stages. Through behavioral and neural activity assays in zebrafish, our results indicate that 2,4-D exposure during embryonic development impairs visual system function. Taken together, these results reveal that 2,4-D exposure can have detrimental impacts on the nervous system and survival of non-target aquatic organisms, such as larval fish.

4.2. Pure 2,4-D and DMA4 formulation show differences in potency

Our results demonstrate that exposure of zebrafish larvae to DMA4 or pure 2,4-D reduces larval survival and prey capture, indicating that the active ingredient is causative of these outcomes. Larvae exposed to DMA4 or pure 2,4-D show a typical dose response: larval survival decreases as 2,4-D concentration increases (Fig. 1A–B). 100% mortality was observed at 6 ppm for pure 2,4-D at both 6 dpf and 21 dpf, whereas lethal concentrations were at 32 ppm for DMA4 at both those ages,

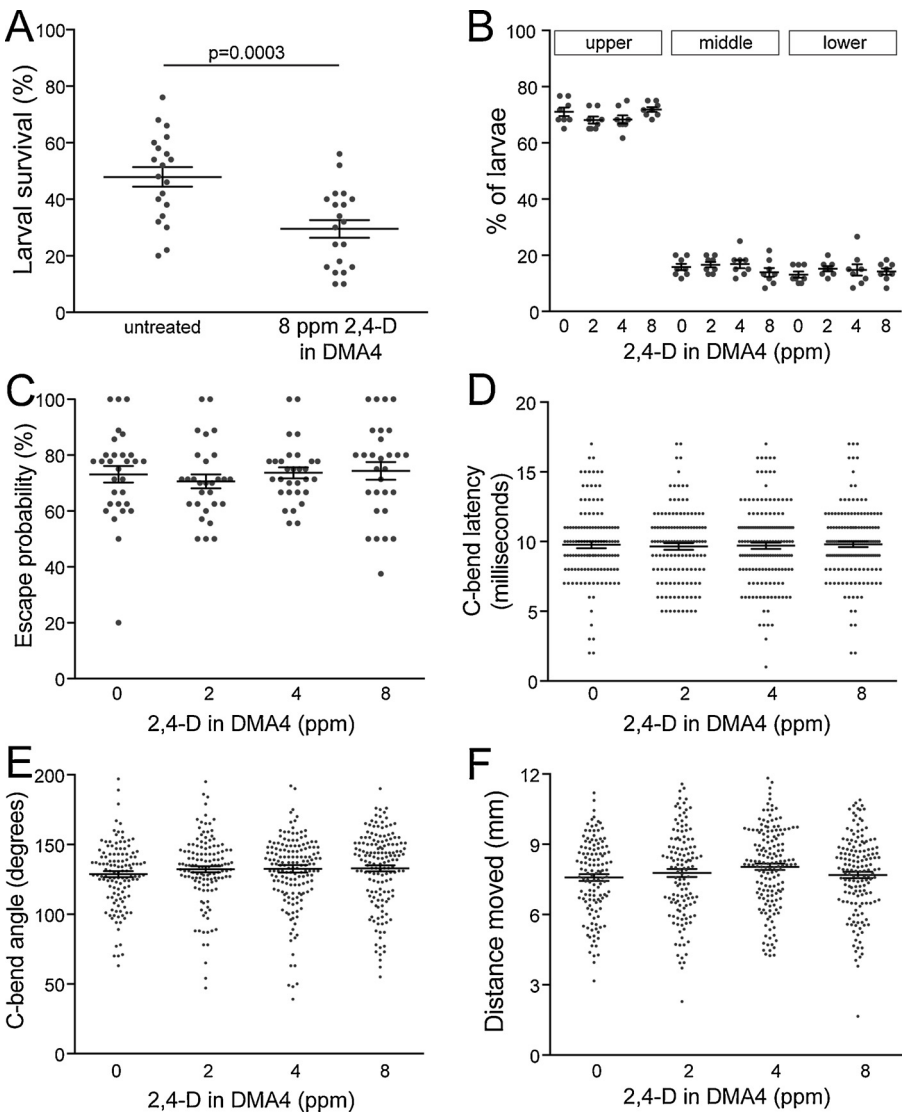

Fig. 6. DMA4 exposure reduces predator avoidance by larval zebrafish.
(A) Mean percentage of 6 dpf larvae remaining in prey capture arena after 5-min-long exposure to adult predator. Each dot indicates outcome of single trial with a predator (N). p = 0.0003; unpaired *t*-test with Welch's correction. (B) Mean percentage of larvae present in the upper, middle, or lower third of the prey capture arena after 1-h acclimation. Dots indicate outcomes for tanks (N) of 50 larvae. (C) Mean probability of 6 dpf larvae to initiate a short-latency C-bend escape maneuver (SLC escape) when exposed to an acoustic stimulus. Each dot represents an individual larva (N). (D–F) Kinematic parameters of SLC escape responses. Each dot represents measure from an individual SLC escape response. All bars represent SEM. One-way ANOVA with Tukeys multiple comparison analysis determined no significance between DMA4 treated larvae and untreated (0 ppm) larvae (B–F).

indicating the pure form was more potent. The potency difference is likely due to the inert ingredients in DMA4. Inert ingredients in commercial formulations, like DMA4, can consist of fragrances, solvents, surfactants, and preservatives (Cox and Surgan, 2006; EPA, 2005). Each ingredient can alter 2,4-D's toxicity by affecting its exposure or solubility (Cox and Surgan, 2006; Dehnert et al., 2018; EPA, 2005; Pérez et al., 2011; Stehr et al., 2009). For example, in vitro studies have shown that inert ingredients in a 2,4-D formulation, Tordon75®, can increase the toxicity of 2,4-D and even be toxic themselves (Oakes and Pollak, 2000, 1999). Based on our analyses of morphology, prey capture, and larval survival in zebrafish, the inert ingredients in DMA4 appeared to decrease the toxicity of the active ingredient, 2,4-D. This discrepancy may be due to differences in the composition of the inert ingredients in DMA4 and Tordon75 or in the susceptibility of the organisms tested.

4.3. 2,4-D impairs essential visually guided behaviors

Upon the exhaustion of the yolk sac's supply of nutrients, larval fish must be able to forage for sustenance (D´Abramo, 2002; Kimmel et al.,

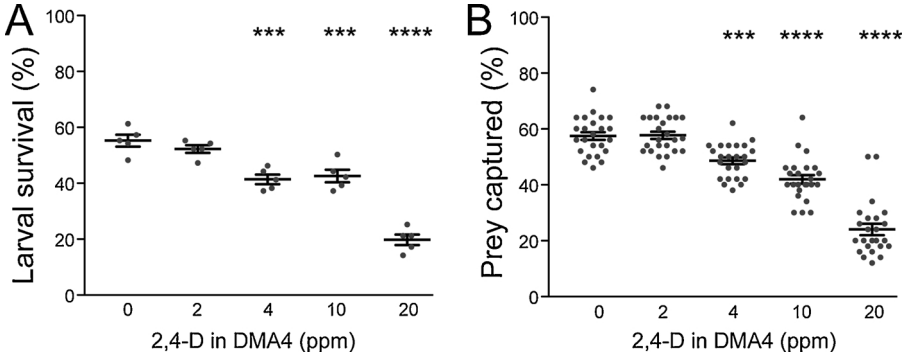

Fig. 7. DMA4 reduces survival and prey capture success of larval yellow perch.
(A) Mean percentage of surviving larvae at 21 dph following exposure to DMA4 beginning at 4 hpf. Each dot indicates survival percentage of a group (N) of 50 larvae. (B) Mean percentage of prey (paramecia) captured by 3 dph larvae exposed to DMA4 beginning at 4 hpf. Each dot shows the percentage of prey captured by an individual larva (N) (B). All bars represent SEM. ***p < 0.001, ****p < 0.0001 versus untreated (0 ppm) larvae; one-way ANOVA with Tukeys multiple comparison analysis.

1995; Lawrence, 2007; Muto and Kawakami, 2013). For zebrafish, the ability to capture prey is highly dependent on the rapid development of the visual system (Bianco et al., 2011; D´Abramo, 2002; Muto and Kawakami, 2013). Instinctual visually guided behaviors, e.g. prey capture and predator evasion, are essential to larval survival (Brandt et al., 1987; Fuiman et al., 2006; Muto and Kawakami, 2013). Here, we observed that larvae exposed to 2,4-D showed reduced success and reduced initiation of stereotyped prey capture maneuvers that larval zebrafish perform to capture paramecia. Prey capture behavior is controlled by neural circuits that mediate sensory perception and detection, followed by interpretation and decision making about the prey, which triggers a motor output (McElligott and O'Malley, 2005; Muto and Kawakami, 2013). Any deficits at any point in this circuitry cascade would reduce prey capture ability (Patterson et al., 2013). Our observations of spontaneous and stimulus evoked behaviors indicated that 2,4-D exposed larvae exposed to 2,4-D exhibit normal motor function (Figs. 3 and 6). These results suggest that 2,4-D exposure did not alter the functionality of the larvae's motor circuits. It is more likely that 2,4-D affects prey capture circuitry at the level of the visual system. This possibility is supported by several lines of evidence. First, in addition to a reduction in prey capture, 2,4-D exposed larvae exhibited deficits in other visually guided behaviors (Fig. 3G–I). Second, when offered paramecia, 2,4-D exposed larvae showed reduced activity of the tectal neurons that receive sensory input from the retina detecting paramecia (Fig. 4) (Gahtan and Paul Baier, 2005; Gahtan and Baier, 2004; Randlett et al., 2015). Third, 2,4-D exposed larvae showed a decrease in their ability to avoid predation (Fig. 6A), which is a behavior triggered by the visual stimulus of the shadow of a looming predator (Bianco et al., 2011; Nair et al., 2017; Quist and Guy, 2004). Together, these behavioral outcomes, coupled with our understanding of the neural circuits underlying visually guided behaviors, suggest that 2,4-D disrupts the visual rather than motor circuits.

Herbicides have been shown to negatively impact predation and prey consumption in aquatic organisms (Browne and Moore, 2014; Chollett et al., 2014; Morgan and Kiceniuk, 1990). In particular, 2,4-D has been shown to impact sensory organs used for foraging and prey consumption in crayfish (Browne and Moore, 2014). Moreover, chronic exposure of rats to 2,4-D can impair the visual system (Charles et al., 1996a, 1996b; Mattsson et al., 1997). It is unclear why sensory systems may be more vulnerable compared to motor systems when exposed to 2,4-D. One possibility is the increase accessibility of sensory organs to toxins, but at this time we cannot make definitive conclusions. We may have a good understanding of the lethal effects herbicides have on organisms, but we have a weak understanding of how herbicides can affect visual systems involved in predator-prey interactions (Fuiman et al., 2006; Hanlon and Relyea, 2013; Schulz and Dabrowski, 2001).

4.4. 2,4-D disrupts neural circuits mediating vision

Larval fish have a strong pressure to rapidly develop their visual system as is it needed to capture prey and avoid predation. In zebrafish, eye morphogenesis begins at 12 hpf and the retina is populated with its essential cell types by 3 dpf (Burrill and Ester, 1995; Hu and Ester, 1999; Schmitt and Dowling, 1994). From 2–5 dpf the retina's cells differentiate and form intraretinal connections and retinal ganglion cells extend axons to the tectum where they will form synaptic connections (Schmitt and Dowling, 1999). This rapid development produces a functional visual system by 5 dpf (Biehlmaier et al., 2003). Our results indicate that 2,4-D exposure during development (4hpf to 5dpf) reduces prey capture, whereas acute post-developmental exposure (5-6dpf) does not (Fig. 5). The reduced tectal neuron activity in 2,4-D exposed larvae suggests a developmental defect in the visual system is most likely to occur in the tectal neurons, the retinotectal connections, or in the retina. We did not observe any aberrations in eye morphology in 6 dpf larvae zebrafish exposed to concentrations of DMA4 or pure 2,4-D that altered visual functions (Fig. 1E). Moreover, histological analyses of retinas of 8 ppm DMA4 exposed larvae failed to show retinal malformations, such as disrupted cellular lamination or loss of retinal cells (data not shown). ERK immunostaining did not indicate any gross disruption of the tectal neuropil or cellular layers (Fig. 4). Therefore, it remains unclear where 2,4-D disrupts visual circuit development and function.

In mammals, 2,4-D has been shown to disrupt the integrity of neural circuits' cellular makeup. Rats chronically exposed to 2,4-D exhibit defects in their cerebral granule cells (Bongiovanni et al., 2007; Rosso, 2000), serotonergic neurons, and astroglial cells (Garcia et al., 2001) (Evangelista de Duffard et al., 1995a; Brusco et al., 1997), and show reduced myelination (Duffard et al.,1996). It has been proposed that 2,4-D negatively impacts the nervous system through its stimulation of neurotoxic free radicals (Bongiovanni et al., 2007) and oxidative stress (Atamaniuk et al., 2013). 2,4-D has also been shown to affect circuit function through its influence on synaptic transmission (Bernard et al., 1985; da Fonseca et al., 2008). For example, da Fonseca et al. (2008) showed that 2,4-D exposure reduced acetylcholinesterase (AChE) in both the brain and muscle of piava, a freshwater fish (*Leporinus obtusidens*). Thus, in the visual system, 2,4-D may disrupt the formation of critical circuit cellular components that mediate the transmission of neural signals coding visual input. Future studies with more sensitive measurements of neural circuit integrity and function will be required to pinpoint the affected locus in the visual system.

4.5. Potential ecological impacts of 2,4-D

Recent work has shown that 2,4-D negatively impacts various fish species, even at previously defined no observable effect concentrations (Dehnert et al., 2018; DeQuattro and Karasov, 2016; Li et al., 2017; Menezes et al., 2015; Ruiz de Arcaute et al., 2018, 2016). 2,4-D herbicide formulations are commonly applied during spawning seasons of freshwater species that spawn once per year (Brown et al., 2009; Harrahy et al., 2014; Nault et al., 2014; Starzynski and Lauer, 2015). This application process therefore exposes fish at their larval stage; a time when they are highly sensitive to toxicant exposure (Dehnert et al., 2018; Fairchild et al., 2009; Mohammed, 2013). Therefore, any negative impact on the ability of larval fish to consume prey and avoid predation may directly suppress their survival and natural recruitment. This outcome could eventually lead to an impact on the overall fitness of a species (Quist and Guy, 2004; Scott and Sloman, 2004). 2,4-D's impact on fish larvae together with other stressors – temperature change, overfishing, additional pollution, habitat loss – could lower the fitness of critical game species in freshwater lakes and ecosystem dynamics. Future studies on both direct toxicity and indirect impacts on essential survival behaviors of larval fish should be understood before commercial herbicides are applied in aquatic environments.

5. Conclusion

In summary, this study reveals that 2,4-D exposure reduces survival and essential visually guided behaviors of larval fish at concentrations that did not cause overt morphological deformities. Additionally, we provide evidence that 2,4-D exposure during embryonic development can alter neural activity in the visual system. These results suggest that usage of 2,4-D herbicide formulations in aquatic environments warrants caution and a reevaluation is needed for application practices.

Competing interests

The authors declare that they have no competing interest.

Acknowledgments

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