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Developmental exposure to environmentally relevant concentrations of bifenthrin alters transcription of mTOR and ryanodine receptor-dependent signaling molecules and impairs predator avoidance behavior across early life stages in inland silversides (*Menidia beryllina*)

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Abstract

Altered transcription of calcium-dependent signaling cascades involving the ryanodine receptor (RyR) and mechanistic target of rapamycin (mTOR) in response to environmental exposures have been described in model vertebrates, including zebrafish, while the relevance for wild fishes remains unknown. To address this knowledge gap, we exposed the euryhaline model species *Menidia beryllina* (inland silversides) to the insecticide bifenthrin, a known modulator of calcium signaling. The main objectives of this study were to determine: (1) whether exposure of developing silversides to environmentally relevant concentrations of bifenthrin alters their

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Author contributions

DFF, REC, SMB, PJL and JG conceived the concept for the paper. DFF conducted the experiments in the laboratories of SMB and REC, and DFF analyzed the results. Analytical Chemistry was performed by SH. Data analyses was primarily conducted by DFF under guidance and with support by REC, SMB, JG and DJH. The mixed model algorithm was written by DJH. DFF wrote the initial draft of the manuscript and all authors reviewed earlier versions and approved the final manuscript.

Conflict of interests

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2018.10.014.

behavior; and (2) whether behavioral changes correlate with altered expression of genes involved in RyR and mTOR-dependent signaling pathways. At six hours post fertilization (hpf), inland silversides were exposed to bifenthrin at 3, 27 and 122 ng/L until 7 days post fertilization (dpf, larvae hatched at 6dpf), followed by a 14-day recovery period in uncontaminated water. Transcriptional responses were measured at 5, 7 and 21 dpf; locomotor behavior following external stimuli and response to an olfactory predator cue were assessed at 7 and 21 dpf. Bifenthrin elicited significant non-monotonic transcriptional responses in the majority of genes examined at 5 dpf and at 21 dpf. Bifenthrin also significantly altered predator avoidance behavior via olfactory mechanisms with main effects identified for animals exposed to 3 and 27 ng/L. Behavioral effects were not detected in response to visual stimuli during acute exposure, but were significant in the predator-cue assessment following the recovery period, suggesting delayed and long-term effects of early developmental exposures to bifenthrin. Our findings demonstrate that at picomolar (pM) concentrations, which are often not represented in ecotoxicological studies, bifenthrin perturbs early development of inland silversides. These developmental impacts are manifested behaviorally at later life stages, specifically as altered patterns of predator avoidance behavior, which have been correlated with population decline. Collectively, these data suggest that bifenthrin may be negatively impacting wild fish populations.

Keywords

Ca2+-dependent signaling; Fish behavior; Insecticide; Neurodevelopment; Pesticide; Pyrethroid

1. Introduction

Accounting for approximately 38 % of the world's pesticide market in 2015, pyrethroid insecticides have been increasingly employed as a replacement for organophosphate pesticides (OPs) in response to pesticide regulations restricting OP use (Werner and Moran, 2008; Crago and Schlenk, 2015). Bifenthrin is a globally used pyrethroid that is extensively applied in the United States of America (Yadav et al., 2003; Chouaibou et al., 2006; Houndété et al., 2010; Li et al., 2017). Pyrethroid insecticides have been detected in sediments and in water from both urban and agricultural streams across the USA, with bifenthrin being the most frequently detected in recent times (Hladik and Kuivila, 2012). In the USA, bifenthrin is approved for application by professional pest controllers, and is marketed as a common-use household pesticide in various formulations (Kuivila et al., 2012; Weston and Lydy, 2012). Bifenthrin enters watersheds primarily via runoff following agricultural and urban applications (Kuivila et al., 2012), and concentrations up to 106 ng/L have been reported in Californian surface waters (Weston and Lydy, 2012).

Bifenthrin has the potential to alter biochemical, hematological and histopathological parameters, including plasma ammonia and glucose levels, erythrocyte properties, and hepatocyte degeneration at concentrations of 14.7 μg/L (*Oncorhynchus mykiss*) or 57.5 μg/L (*Cyprinus carpio*) of Talstar 10 EC pesticide preparation (active substance 100 g/L bifenthrin) (Velisek et al., 2009a, b). Zebrafish have been used to assess developmental toxicity following exposure to 50, 100 and 200 μg/L, which elicited impaired swimming performance in all exposure groups (Jin et al., 2009). Swimming capacity of fathead

minnows (*Pimephales promelas*) was also reduced following exposure to > 0.14 μg/L bifenthrin, while alterations in transcriptomic responses were determined at 0.07 μg/L of bifenthrin (Beggel et al., 2011). Together, adverse effects elicited by exposures to high concentrations of bifenthrin as detected in aquatic ecosystems, or well above detected levels, highlight that this pyrethroid pesticide may pose significant risks to wild fish populations, and that there is a need to investigate more environmentally realistic concentrations.

Studies that focus on effects of environmentally relevant, low exposure concentrations of bifenthrin (< 50 ng/L) on fishes are more limited. Bifenthrin was shown to elicit transcriptional responses in genes associated with Ca^{2+}-dependent signaling pathways in zebrafish (*Danio rerio*) exposed developmentally to 1, 10, and 50 ng/L, which corresponded functionally with increased locomotor behavior following an unexposed, recovery period of 14 days (Frank et al., 2018). Bioaccumulation of bifenthrin in tissues has also been demonstrated (Munaretto et al., 2013) potentially leading to long-term, or delayed effects resulting from developmental exposure. Endocrine disruption in response to bifenthrin (ng/L) has been repeatedly determined in inland silversides (*Menidia beryllina*), with significant impacts at concentrations ranging from 0.5 to 50 ng/L (Brander et al., 2012b; DeGroot and Brander, 2014; Brander et al., 2016; DeCourten and Brander, 2017). Interestingly, bifenthrin metabolites appear to contribute most to the estrogenic effects (DeGroot and Brander, 2014), and responses can be greater at lower concentrations. Studies investigating reproductive output with adult inland silversides determined a significant reduction when they were exposed to 0.5 ng/L for 21 days (Brander et al., 2016). The influences of 1 ng/L bifenthrin were further evaluated in a generation overlapping assay at both ambient and warmer temperatures, showing influences on sex ratios in the F1 generation and decreased viable offspring and deformities in F1 and F2 generation (DeCourten and Brander, 2017).

While it has been repeatedly demonstrated that environmentally relevant concentrations of bifenthrin act as endocrine disruptors (Brander et al., 2016), little is known about how these concentrations can impact neurological and behavior endpoints in fishes; a knowledge gap that needs to be addressed, since endocrine disruption has been linked to adverse neurodevelopmental outcomes in other vertebrates (Segner, 2009; Masuo and Ishido, 2011; Frye et al., 2012). It has been suggested that behavioral endpoints, including performance associated with predator avoidance, are more informative in the evaluation of ecological effects of toxicants than long-established endpoints used in pesticide regulation, such as growth and mortality. Recommendations include the assessment of visual and olfactory endpoints, specifically when evaluating the impact of chemicals that have the potential alter predator avoidance behavior (Sloman and McNeil, 2012). Bifenthrin has previously been shown to impact neurodevelopment in zebrafish, specifically interfering with ryanodine receptors (RyRs) and mechanistic target of rapamycin (mTOR) signaling pathways (Frank et al., 2018); pathways that are critically important in normal neurodevelopment (Pessah et al., 2010; Bowling et al., 2014; Fritsch et al., 2015), and thus directly relevant to the evaluation of contaminants impacts on fish behavior. Both signaling pathways have been directly linked to olfactory (Murmu et al., 2010; Skalecka et al., 2016) and visual functionality (Križaj, 2012; Ma et al., 2015). Thus, chemical exposures that impacts either have the potential to affect multiple sensory systems. These pathways, in particular, could therefore be developed

as biomarkers of effect, towards the evaluation of pesticide impacts on aquatic systems, utilizing representative model and nonmodel fishes.

The Inland silverside is a small euryhaline fish species, native to the East and Gulf coasts of North America, but introduced in 1967 and now invasive in California rivers and estuaries (Middaugh and Hemmer, 1992; Fluker et al., 2011). It is a well-suited species for ecotoxicological assessments representative of both freshwater and estuarine environments, for which it was developed as a model organism (Brander et al., 2012b). Several transcriptomic assessments have recently been conducted for this species (Jeffries et al., 2015a; Brander et al., 2016; DeCourten et al., 2018), providing a fundamental collection of RNA sequences towards the development of targeted molecular pathway assessments, such as the one presented herein.

The overall goal in this study was to evaluate effects during sensitive stages of development (embryo-hatching), which are predominantly associated with sediment. There are also seasonal fluctuations surrounding bifenthrin use and presence in the water column which would likely result in periods during which larval and juvenile fish may not be exposed. To encompass this, we exposed inland silversides to 3, 27, 122 ng/L bifenthrin, and evaluated responses at 5 and 7 days post fertilization (dpf), which correspond to embryonic and post-hatch yolk-sac larval stages, respectively, as well as following a two-week unexposed, recovery period (21 dpf). Our first objective was to evaluate bifenthrin-induced behavioral alterations in response to olfactory and visual stimuli, as well as swimming performance. Our second objective was to investigate whether expression of genes involved in RyR-dependent Ca2+ and the mTOR signaling pathways, during and after exposure, corresponded with observed behavioral responses. We focused on gene targets within these signaling pathways because altered expression and function of both RyR and mTOR signaling pathways are associated with perturbations of neurodevelopment (Pessah et al., 2010; Wayman et al., 2012b, a; Bowling et al., 2014). We hypothesized that effects on behavior would become most evident at higher exposure concentrations, but that mechanistic effects at the molecular level would also be observed at lower concentrations. We further hypothesized that results observed in inland silversides would be similar to effects observed in the model species zebrafish exposed to 1, 10 and 50 ng/L of bifenthrin (Frank et al., 2018).

2. Materials and methods

2.1. Fish husbandry and spawning

Fish husbandry, spawning and exposure experiments were performed at the University of North Carolina, Wilmington (UNCW) in accordance with UNCW Institutional Animal Care and Use Committee (IACUC) protocols #A1314-010 and #A1415-010. Adult Inland silverside broodstock, originally purchased as juveniles from Aquatic Biosystems (Ft. Collins, CO, USA), were kept in four aerated 150 L tanks, connected in a recirculating system, at a density of 40 fish per tank (sex ratio approximately 1:1) and with a 16:8 h light/dark photoperiod at the UNCW Center for Marine Sciences (Wilmington, NC). Water temperature was maintained at 23 ± 1 °C, with a salinity of 15 ± 1 ppt. Bleached and rinsed cotton yarn was used as spawning substrate (DeCourten and Brander, 2017). The yarn was

kept in the spawning tanks for 2 h, after which eggs were carefully separated from the substrate with fine scissors. Chorionic fibrils were removed from the embryos before exposure using a pair of precision scissors, following a standard procedure using Inland silversides as a toxicological model (Middaugh et al., 1994).

2.2. Bifenthrin exposures and recovery period

Static exposure to bifenthrin (purity 98.0 %, CAS 82657-04-3, Chem Service, West Chester, PA, USA) at one of three effective concentrations; 3, 27 and 122 ng/L was initiated at 6 hours post fertilization (hpf). This range of concentrations was chosen to reflect bifenthrin concentrations measured in Californian surface waters (Weston and Lydy, 2012), and earlier assessments of bifenthrin exposure with inland silversides (Brander et al., 2012b; DeGroot and Brander, 2014; Brander et al., 2016). Methanol was used as a solvent carrier for the bifenthrin treatments. All treatments, including solvent controls contained a final methanol concentration of 0.01% v/v in exposure water (ASTM, 2014). The stock solution was spiked into culture water (salinity 15.0 ± 1.0 ppt) and measured concentrations in the different exposure groups were determined as 3, 27 and 122 ng/L (measurement is described in detail under Analytical Chemistry, below).

Five independent spawn events were performed to maximize genetic variability of individuals used, and to obtain sufficient numbers to achieve five biological replicates for each time point; 5 dpf (as embryos), 7 dpf (hatched, yolk-sac larvae) and 21 dpf (free feeding larvae). Larvae began hatching at 6 dpf and fish larvae used in the remainder of the study had all hatched by 7 dpf; having been exposed as free swimming larvae for a further period of up to 24 h.

A total of 360 embryos per biological replicate were separated into groups of 30 individuals, transferred into 12 different 500 mL glass beakers (4 exposure treatments; 3 time points) containing 400 mL exposure solution, and placed into a water bath at a constant temperature of 25.0 ± 1.0 °C, with a 16:8 h light/dark photoperiod. Embryos remained in the glass exposure beakers until sampled, at 5 dpf, for transcriptomic assessments, and post hatch, until sampled at 7 dpf for both behavioral and transcriptomic assessments. One beaker from each treatment was used per time point, per spawn event (n = 5).

At 7 dpf a subset of 30 larval fish, per treatment and biological replicate, was transferred into aerated 1.4 L tanks, containing 1.2 L of culture water, and were allowed to recover from the exposure for 14 days, when final sampling occurred at 21 dpf. Following transfer to the recovery tanks, and upon yolk-sac absorption, fish were fed *ad libitum* twice a day with rotifer *Brachionus rotundiformis*, followed by a mixture of *B. rotundiformis* and *Artemia franciscana* (Brine Shrimp Direct, Ogden, UT, USA) between 10 and 21 dpf. During the recovery period, the proportion of *A. franciscana* was increased and *B. rotundiformis* equivalently decreased, to provide best maintenance for growing larval fish and to ensure the survival of smaller individuals.

Daily water changes were performed, changing 80 % of exposure solutions or culture water, and removing any debris. Water physicochemical parameters were measured daily with a YSI Professional Plus Quatro water quality meter and were as follows: 8.0 ± 0.2 pH, 7.0

± 0.2 mg/L dissolved oxygen, 25.3 ± 0.8 mS/cm specific conductance, salinity of 15 ± 1 ppt and < 0.001 mg/L total ammonia.

2.3. Locomotor behavior

Locomotor behavior was assessed on the last day of the exposure period (7 dpf) and at the end the recovery period (21 dpf), under alternating light and dark stimuli, using a lightbox developed specifically for behavioral assays with inland silversides. This lightbox (Fig. 1) was constructed by inserting a milk-plexiglas surface, originating from a Porta-trace lightbox (Gagne Inc., Johnson City, NY), on a wooden box, beneath which two infrared lights (Cisno AC 110v 96 LED 80m Night Vision IR Illuminator 60 ° Waterproof Light CCTV Camera, Amazon web services Inc, Seattle, WA) were positioned to evenly illuminate the underside center of the plexiglass surface (Fig. 1A (1)), where 96-well plates for the behavioral assessment were located. A single light bar (Utilitech Pro 12-in Plug-In Under Cabinet LED Light Bar 165 Lumen, Utilitech Lighting, West Lawn, PA) was also placed diagonally beneath the plexiglas surface as visible light source (Fig. 1A (2)). Both light systems were controlled with a digital timer (Enover 7-day Programmable Plug-in Digital Timer Switch with 3-prong Outlet for Lights and Appliances, 15 A/1800 W, Amazon web services inc, Seattle, WA) to ensure identical periods of light and dark stimuli among experimental runs. A Canon VIXIA HF G20 HD Camcorder (Canon Inc., Tokyo, Japan) with an 850 nm infrared filter (Neewer 58mm 850 nm Infrared IR Pass Filter, Neewer Technology ltd, Shenzen, China) was used to record fish locomotion (Fig. 1A (3)). A wooden frame was placed on the plexiglass surface to assure same positioning for multi-well plates, as well as to reduce light emission. The lightbox setup was located in a thick cardboard box, which was closed during behavioral tracking to ensure the light bar was the only light source during experimental runs. All behavioral experiments were conducted at 2 pm ± 2 h, to minimize intraday-dependent locomotive variation in swimming behavior, which has previously been reported in fish (MacPhail et al., 2009).

Behavioral experiments at 7 dpf were executed in 96-well plates (Falcon™, Corning Inc., Corning, NY, USA) (Fig. 1A (4)). A single well contained one larva and 200 µl of exposure medium. Due to the increased size of the fish at the end of the recovery period, assessments at 21 dpf were performed in 6-well plates (Falcon™, Corning Inc., Corning, NY, USA), each well containing 3 ml culture water and one larval fish. All behavioral data were assessed using a total of 30 fish per treatment, per spawn event (n = 5).

Fish were transferred from glass beakers (exposure beakers or recovery tanks) into polystyrene multi-well plates and allowed to acclimate for 30 min before they were placed into the light box. Behavioral tracking was conducted under alternating light/dark periods during a 30-min assay; adapted from successful behavioral tracking protocols for zebrafish (Cario et al., 2011). During dark phases, only infrared light was used to track the fish. Fish were initially exposed to the light conditions for 5 min as an acclimation period, tracking started with a 5 min light period (Light 1), followed by a 5 min dark period (Dark 1), a second 5 min light period (Light 2), a second 5 min dark period (Dark 2) and ended with a 10 min dark period. Identical behavioral tracking settings were used for 21 dpf fish on the

final day of the recovery period. Water temperature was checked before and after every run to ensure it was maintained at 25.0 ± 1.0 °C.

2.4. Swimming performance

Swimming performance was measured to assess maximum swimming capacity of larval fish at 21 dpf, the last day of the recovery period, using the circular “racetrack” method (Heath et al., 1993; Beggel et al., 2010, 2011). Experiments with 30 fish from each treatment were performed, including individuals from all 5 biological replicates. The racetrack (Fig. 1B) was built with a 10.0 cm diameter petri dish (Fig. 1B (1)), containing a centrally placed 7.5 cm diameter petri dish (Fig. 1B (2)). The bigger petri dish was filled with culture water to 1 cm height. The inner petri dish was also filled with water to keep it in position. An arena was placed on a background dividing the racetrack into 8 radial sectors. After an acclimation period of 1 min, distance moved was assessed by counting the lines a fish crossed during a 1 min period. If a fish stopped swimming, an escape response was provoked by gently touching its tail with help of a plastic rod. Larval fish from different treatments were examined randomly, assuring the experimenter had no knowledge from which treatment the fish came from to avoid subjectivity (Heath et al., 1993). Water changes were performed in between each fish test, and temperature was maintained in the range of 28.5 ± 0.5°C, which was confirmed before each new larval fish was placed into the arena.

2.5. Predatory response

Response to predator cues resulting from exposure to bifenthrin was evaluated following a 14 d recovery period; at 21 dpf. Fish were confronted with a suspension of homogenized tissue of a fish from the adult colony, diluted in 10 mL culture water (referred to as “predator cue” hereon). This approach assures contact between the larval fish and the compound Schreckstoff (Jesuthasan and Mathuru, 2008), an alarm pheromone present in the homogenate. Fish from the same species can detect this Schreckstoff via olfactory mechanisms and respond in the form of specialized behavioral patterns to avoid predation (Jesuthasan and Mathuru, 2008). To examine this response, 6 larval fish per treatment were placed in 6-well plates containing 3 ml culture water (1 fish per well). Fish were allowed to acclimate for 30 min to the multi-well plate, before being transferred into the observation light box, described above, where they were kept for a further 5 min before the tracking was initiated. Video recording (as used for locomotion studies) started with a 5-min free swimming evaluation, after which 20 µl of the predator cue suspension was added into each well and fish behavior was tracked for a further 5 min. This assessment, including acclimation time in the observation light box, was conducted during a continuous light period.

An identical setup was used to evaluate whether the predator response was also influenced by the addition of liquid in to the test chambers (handling effect); i.e. effects from disturbances in the waterbody at the predator cue was added. Therefore, we also compared the behavioral responses of 15 unexposed larval fish (21 dpf) challenged with 20 µl of a culture water sample (no cue) to the responses of 15 fish confronted with 20 µl of the predator-cue.

2.6. Transcriptomic assessments

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) in an automated QIAcube (Qiagen), following manufacturer's protocols. Fish per biological replicate and treatment were pooled into batches to obtain sufficient amount of RNA for the transcriptomic analysis (n = 5): 20 fish per treatment were pooled during the exposure period (5 and 7 dpf), and 4 specimens per treatment were pooled for sampling following the recovery period (21 dpf). Extraction efficiency and RNA quality was verified using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), total RNA 260/280 and 260/230 ratios ranged from 1.76 to 2.14 and from 1.88 to 2.18, respectively. Total RNA integrity was additionally visually verified by non-denaturing gel electrophoresis using a 1 % (w/v) agarose gel.

Complementary DNA (cDNA) was synthesized using 750 ng total RNA in a reaction with 4 μl Superscript Vilo Mastermix (Superscript® VILO™ MasterMix, Invitrogen, Carlsbad, CA, USA) according to the user's manual. Reactions were incubated for 10 min at 25 °C, 60 min at 42 °C followed by a 5 min denaturation step at 85 °C. Samples were then diluted with nuclease-free water in a 1:10 ratio. Successful cDNA-synthesis was verified by a following polymerase chain reaction (5 min at 95 °C; 30 s at 95 °C, 30 s at 60 °C, 45 s at 72 °C, in 35 cycles; 10 min at 72 °C).

We assessed the transcription of 12 genes, 6 associated with RyR-dependent Ca^{2+} signaling and 6 associated with mTOR signaling (Table 1). This included transcriptional analysis of all three RyR orthologs (*ryr1, ryr2, ryr3*) as well as Calcium calmodulin-dependent protein kinase kinase 1 (*camkk*) and Cyclic-AMP response elementbinding protein (*creb*), both members of the signaling pathway transmitting RyR-dependent Ca^{2+} signals important for dendrite development (Wayman et al., 2006). In addition, we screened transcription of cytokine Transforming growth factor beta-1 (*tgfb*), a transcriptional regulator of RyRs and an activator of Creb via phosphorylation (Giannini et al., 1992; Fukushima et al., 2007). Our approach further incorporated mTOR complex members: the Mechanistic target of rapamycin (*mTOR*) and Target of rapamycin complex subunit 1st8 (*mlst8*), its upstream members RAC serine/threonine-protein kinase (*akt1*) and Ras homolog enriched in brain (*rheb*), as well as downstream members. Eukaryotic translation initiation factor 4e (*eif4e*), which encodes for a protein that inhibits the translational mechanisms of mTOR signaling, and 40 s ribosomal protein s6 (*rps6*) a downstream target of the mTOR pathway, were also evaluated. Sequences were sourced from earlier *Menidia beryllina* whole transcriptome assessments conducted by our group (Jeffries et al., 2015a; Brander et al., 2016), and specific genes were selected based on prior research conducted towards evaluating impacts of bifenthrin exposure on Zebrafish (Frank et al., 2018). Primers were obtained from Integrated DNA Technology (Integrated DNA Technologies, Inc., Coralville, IA, USA).

Quantitative PCR was conducted using Power SYBR Green PCR Master Mix (Life-technologies, Carlsbad, CA, USA). Primer validation was performed using a seven-point standard curve in three replicates; efficiencies ranged between 92.3 and 103.6 % (Table 1). Cycling conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, followed by a thermal ramping stage for dissociation evaluation. Amplification data were analyzed using Sequence Detection Systems software (SDS v2.4.1,

Applied Biosciences). Relative gene expression data was normalized to the reference genes 60 s ribosomal protein s7 (rpl7) and beta actin (*actb*), which sustained best stability scores in GeNorm (Vandesompele et al., 2002).

2.7. Analytical chemistry

At test initiation, 1-L water samples for each treatment and the solvent control were collected in amber glass bottles and shipped overnight to University of California on ice for subsequent chemical analysis. Within 48 h of sample collection, samples were spiked with trans-permethrin (dimethyl D6, EQ Laboratories, Atlanta, GA, USA) as a recovery surrogate and extracted using solid phase extraction cartridges (Supelclean ENVI™ - C18, 500 mg, Sigma-Aldrich, St. Louis, MO, USA) following methods published in Hasenbein et al. (2015). Cartridges were pre-conditioned using 12 mL 1:1 ethyl acetate:hexane, 12 mL methanol, and 12 mL MilliQ water (Millipore). Samples were loaded on the cartridge and eluted with 10 mL 1:1 ethyl acetate: hexane and evaporated to 0.4 mL at 40 °C under a gentle stream of nitrogen. As an internal standard, 4-4′dibromo-octafluorobiphenyl (DBOFB, 10 ng) was spiked to all samples (Chem Service, West Chester, PA, USA). Extracts were analyzed using an GC-QTOF-MS (Agilent 7890B GC coupled to an Agilent QTOF/MS 7200B with a HP-5MS 30m × 0.25 mm, 0.25 μm column, Agilent Technologies, Inc.) operated in negative chemical ionization (NCI) mode using methane as collision gas (Moschet et al., 2016). Target quantification was conducted using *Agilent MassHunter Quantitative Analysis* software (B.07) with the main NCI fragment used as quantifier and two additional fragments used as qualifiers. For quality control, a procedural blank (extracted in ultrapure water) was used to ensure that no contamination occurred during sampling extraction and analysis. The surrogate trans-permethrin was added to each sample, including the blank, before extraction to monitor matrix effects and overall method performance. DBOFB was added to sample extracts before analysis in order to correct quantitative differences in extract volume as well as to monitor instrument conditions. Instrumental limit of detection was 0.1 ng/L and limit of quantification 0.4 ng/L. No bifenthrin was detected in the control or the method blank. All bifenthrin concentrations are herein reported as measured concentrations.

2.8. Statistical analysis

Differences in gene transcription were tested using one-way ANOVA and differences between the predator-cue predator-blank sample using a two sample t-test. Both approaches accepted significance at $p < 0.05$, One-way ANOVA tests were followed by a Tukey Honest Significant Difference (HSD) post-hoc test to evaluate pairwise differences. If data did not fit the ANOVA assumptions of normality, a Kruskal-Wallis test was applied ($p < 0.05$), followed by the Dunn's post-hoc test. Shapiro-Wilk normality and Bartlett tests were used to determine which algorithms are appropriate for determining significant differences between treatments.

We further tested a suite of potential dose-response functions among concentrations, because fitting curves better allows for insights into dose-response patterns and also provides increased statistical power compared to testing for pairwise differences between treatments with an ANOVA / Tukey post-hoc approach (Cottingham et al., 2005; Brander et al., 2016;

Goff et al., 2017). Therefore, per methods developed in Brander et al., 2016 and applied in Frank et al. (2018), five different dose-response curves (linear regression, quadratic, sigmoidal, 5-parameter unimodal, and 6-parameter uni-modal) were tested for the best-fit of observed responses at all three concentrations and the solvent control (MeOH). A likelihood ratio test (LRT) was used to examine whether each curve provided a better fit than an intercept-only null model (Bolker, 2008).

Distance covered by each larval fish was recorded during each minute of the 30 min observation on each sampling day. Therefore, the area under the curve (AUC) was computed for each fish under 4 lighting conditions, applying the trapezoidal rule (Boyce and DiPrima, 1988): Light1: 1–5 min, Dark1: 5–10 min, Light2: 10–15 min, Dark2: 15–20 min, and Freeswim 15–30 min (an extended Dark2 period). Mixed effects regression models were used to assess differences between groups defined by three concentrations of bifenthrin and the solvent control group. Analytical variables were defined to capture differences in the area under the curve between Light1 and Dark1, Dark1 and Light2, and Light2 and Dark2, as measures of changes in movement from light to dark or dark to light, respectively. Contrasts for differences between exposed groups and the solvent control group were specified to compare these changes and the area under the curve during each of the lighting conditions between the exposed groups and the solvent control group. Exploratory analysis indicated that a natural logarithmic transformation was needed for the area under the curve to stabilize the variance and meet the underlying assumptions of the mixed effect models. All values were therefore shifted by 0.1 prior to taking the natural logarithm, because zeros occurred in the area under the curve. Concentration (1 ng/L, 10 ng/L, 50 ng/L, MeOH), day (5, 7 and 21), and the transition variables were all of interest in the models, including their interactions. The Akaike information criterion was used for model selection and Wald tests for comparing groups were used (significance level $p < 0.05$). All analyses were conducted using SAS university edition.

To identify potential dose-response related behavior, AUC values were further used to determine best dose fitting curves, identical to the approach of the transcriptomic assessment. To allow better comparison, all datasets were rescaled to a scale between 0 and 1 for graphical illustration using a normalization $x' = \frac{x - xmin}{xmax - xmin}$ at each timepoint, respectively. All untransformed datasets and corresponding graphs can be found in the supplementary material, as well as p-Values (Table S1).

3. Results

3.1. Response to the predator-cue

Baseline swimming (i.e., movement before the addition of the predator cue solution) of the post exposure recovery 21 dpf larvae did not show differences amongst the bifenthrin treatments or the solvent control group (Fig. 2A). Initial challenge with the predator cue led to a sudden stop in swimming activity in fish from all solvent control and exposure groups. Larval fish remained without motion in this position, before starting to swim again within the next five min test period. Initial swimming movements were then quick and impulsive in all treatments, compared to those observed during baseline swimming in the acclimation

period. Fish from the 3 and 27 ng/L bifenthrin treatment groups, however, started to move earlier than fish from the solvent control group and the 122 ng/L bifenthrin exposure group. This resulted in a significant quadratic dose-response, demonstrating significant hyperactivity in larval fish from both 3 and 27 ng/L treatments (LRT, $p = 0.048$; Fig. 2A). The mixed-model algorithm used to analyze this dataset also provided evidence of increased movement in fish exposed to the 27 ng/L treatment, separating them significantly from fish in the control group (Wald test, $p = 0.047$), however, this conservative approach did not determine significant differences between the other groups.

In order to investigate potential responses to liquid handling resulting from the addition of the olfactory stimulus to the test chambers, an experiment was performed challenging an additional group of control fish (21 dpf) with either the predator cue or a culture water sample. During the acclimation period, before the predator cue solution or the blank samples were added, there were no differences in behavior between treatments. Fish stopped swimming immediately once the predator cue or the blank sample was added. However, fish challenged with the culture water sample were significantly more active in five min following the addition of the blank sample, relative to those who were exposed to the predator cue (two-sample *t*-test, $p = 0.05$; Fig. 2B).

3.2. Locomotive behavioral responses

Locomotor behavior assessed at 7 and 21 dpf, in response to alternating light and dark stimuli revealed consistent dose-response patterns, although they did not reach significance (Fig. 3A; 3B). During light periods, there was a non-significant pattern of decreased movement correlated with increased bifenthrin concentration at 7 dpf, whereas Dark1 resulted in reduced but non-significant swimming activity for the 3 and 27 ng/L treatments, compared to control and 122 ng/L treatments. Swimming activity following an exposure recovery period (21 dpf), however, resulted in non-significant dose-dependent patterns. Both light phases showed tendencies of increased movement correlated to increased bifenthrin concentrations (Fig. 3A), in contrast to Dark1, where fish showed a concentration related decreased non-significant pattern in their movement. Dark2 and the Free swim, however, showed increased movement for fish exposed to 3 and 27 ng/L bifenthrin concentrations compared to the 122 ng/L bifenthrin concentration and the solvent control group, resulting in a quadratic dose-response (Fig. 3A; 3B). There were also no significant differences between dark and light periods detected at either of the investigated time points. At 7 dpf, swimming activity was reduced from light to dark periods for fish from the solvent control group (Fig. 3C), but there was no difference in the overall activity. Similar observations were made for fish from the solvent control group at 21 dpf from Light1 to Dark1 and from Dark1 to Light 2 (Fig. 3D).

3.3. Swimming performance

Swimming performance as assessed in the racetrack arena on 21 dpf fish corresponded with a non-monotonic dose-response, which was also was non-significant. Results in the racetrack, however, suggest reduced swimming performance for both the 3 and the 27 ng/L bifenthrin treatment, compared to the solvent control and the 122 ng/L bifenthrin concentration applied (Fig. 4).

3.4. Transcriptomic analysis during acute bifenthrin exposure and after a recovery period

At 5 dpf, significant dose-responses were determined for *tgfb, ryr3* and *creb* (LRT, p = 0.012, 0.038, 0.027; Fig. 5), as well as for mTOR pathway members *mTOR* and *mlst8*, both coding for proteins involved in the pathways' central complex; mTOR complex 1 (LRT, p = 0.011, 0.018; Fig. 6). All transcripts were significantly decreased in abundance in the 3 and 27 ng/L treatment, in a quadratic dose-response manner. No significant dose-response patterns were observed for transcripts *ryr1*, *ryr2* and *camkk* of the RyR signaling pathway or for *akt1, eif4e, rps6* and *rheb* of the mTOR signaling pathway. The majority of these transcripts, however, showed non-significant patterns of decreased transcription in the 3 and 27 ng/L treatment, in the form of a quadratic dose-response. The only exceptions were *camkk* and *rheb*, which exhibited linear, non-significant patterns. At 7 dpf, no statistically significant fits were measured. At 21 dpf, however, *rheb, mTOR* and *rsp6* of the mTOR pathway showed significant dose-related responses (LRT, p = 0.020, 0.015, 0.038; Fig. 6). *Rheb* and *mTOR* responded in a quadratic dose-response manner. While *rheb* showed strongest transcription levels in larval fish from the 122 ng/L exposure treatment, *mTOR* significantly increased in transcription in the 3 and 27 ng/L treatment. Another significant dose-dependent increase in form of a linear dose response was observed for *rps6* (LRT, p = 0.038). Furthermore, the transcript coding for *mTOR* at 21 dpf was significantly different in the 3 ng/L exposure treatment, compared to the control (ANOVA, HSD, p = 0.042).

4. Discussion

Previous work has demonstrated that environmentally relevant levels of bifenthrin cause endocrine disruption and interfere with reproduction in inland silversides (Brander et al., 2012a, 2016; DeCourten and Brander, 2017; DeCourten et al., 2018). Here, we expand on these studies to demonstrate that bifenthrin levels in the picomolar (pM) concentration range have the potential to alter neurodevelopment, potentially impacting locomotion and behavior, thereby posing ecological consequences, such as increased risk of predation or impaired reproductive behavior and ability to obtain food. Specifically, exposure to 3 or 27 ng/L of bifenthrin during early development caused delayed effects in response to an olfactory predator cue, and this is correlated with both acute and delayed effects on transcriptomic profiles of RyR- and mTOR-dependent signaling.

The finding that behavioral differences among larval fish from different exposure groups were most distinct when exposed to an olfactory predator cue, specifically at later developmental stages (21 dpf; following a recovery period of 14 days) can likely be explained by altered connectivity in neural circuits important for olfaction as a result of developmental exposures to bifenthrin. Exposure to chemicals interacting with RyR-dependent Ca^{2+} signaling, for example polychlorinated biphenyl (PCB) 95, has been causally linked to altered dendritic outgrowth in rat models *in vitro* (Wayman et al., 2012b, a) and *in vivo* (Lein et al., 2007; Yang et al., 2009; Wayman et al., 2012a). While hyperactivity was most pronounced for the 27 ng/L exposure group, there was also a tendency of hyperactivity in the 3 ng/L exposed fish when confronted with the predator cue, while no differences were determined between the 127 ng/L exposure group and the solvent control. Similar behavioral results have been reported for zebrafish confronted with an

olfactory predator cue in a comparable developmental exposure scenario to bifenthrin concentrations of 1, 10 and 50 ng/L (Frank et al., 2018). This does not directly support our original hypotheses that behavioral differences would increase in a dose-dependent manner. In addition to hyperactive movements in the predator assessment, we previously reported increased locomotor activity in bifenthrin exposed zebrafish in response to light dark stimuli (Frank et al., 2018), which was not observed in this study. In general, silverside larvae did not respond to light dark stimuli in comparable developmental stages described for zebrafish; reduced motility during light periods (5 dpf) and increased motility during light periods at 19 dpf (Cario et al., 2011; Frank et al., 2018).

Increased motility, as observed in the response to predator cue experiments, has been repeatedly observed in fish and other vertebrate models, when exposed to pyrethroids. For example vigorous movements were detected in common carp following acute exposures to cypermethrin (Saha and Kaviraj, 2008); developing zebrafish showed hyperactivity in the form of increased swimming speed when exposed to 50, 100 or 200 μg of bifenthrin (Jin et al., 2009); and rats showed increased movement following developmental exposure to deltamethrin (Richardson et al., 2015). Interestingly, hyperactivity in fish was observed following an acute exposure to a 1000-fold higher concentration of bifenthrin, while the delayed effects in this study were detected in form of a non-monotonic response, showing no effects in the highest concentration evaluated. These differences can occur because acute exposures to chemical compounds have the potential to alter suppressive or excitatory signals on receptor level (Solomon and Kohn, 2014), which are not present after a period of recovery. The observed non-monotonic responses may have been induced because of concentration-dependent transcriptional regulation, receptor sensitivity, saturation levels or hormonal interactions, finally unfolding as increased or even antagonistic effects at different concentration levels of a chemical compound (Brodeur et al., 2013; Shuman-Goodier and Propper, 2016). Overall, different forms of hyperactivity have been most frequently described when evaluating behavioral responses following pyrethroid exposures to vertebrate model species. Our study demonstrates that developmental exposure to ng/L concentrations of bifenthrin can cause delayed effects, which are not evident during or immediately following exposure. Considering the high number of studies examining only the period of exposure, there may be a large number of underestimated long-term effects due to pyrethroid exposure.

Predator avoidance behavior can be triggered via visual, olfactory, tactile or auditory cues (Kelley and Magurran, 2003). Our assessment incorporated a combination of tactile, olfactory and potentially visual components, but was specifically designed to evaluate differences triggered via olfactory cues. An initial and immediate stop of swimming activity was observed regardless of whether larval fish were first confronted with the predator cue solution or the blank sample. Different durations in the motionless state between individuals from both tested groups can be therefore attributed to olfactory components. Altered olfactory abilities have been described for Atlantic salmon (*Salmo salar*) following exposure to < 4 ng/L cypermethrin, in response to F-type prostaglandin (*PGF2α*), a priming pheromone released by females before ovulation (Moore and Waring, 2001). Furthermore, exposure to cypermethrin has led to olfactory impairments in the mating behavior of brown trout (*Salmo trutta*) (Jaensson et al., 2007). To the best of our knowledge, this is the first

bifenthrin-related study to determine potential dysfunction in olfaction or olfactory signal transduction. Dysfunctionality of olfaction can have severe consequences for predator recognition and predator avoidance behavior, and can lead to increased mortality and subsequent population declines (Scott and Sloman, 2004; Pyle and Mirza, 2007; Dixson et al., 2010).

In contrast, we did not detect differences among bifenthrin treatments in the swimming performance assessment (racetrack). However, a non-significant pattern of decreased swimming performance in fish from the 3 and 27 ng/L treatments was noted, suggesting reduced swimming abilities in fish from both groups. Dose-dependent decreased swimming performance has been reported in juvenile (7 days post hatch) fathead minnows after a 24 h exposure to elevated concentrations of bifenthrin ranging from 0.75 to 4.00 μg/L, but fish seemingly recovered from exposure within 6 days (Beggel et al., 2011).

Alterations in transcriptional response profiles of mTOR pathway members were still evident following the exposure recovery period, whereas RyR-related genes did not show differences amongst treatments beyond 5 dpf. Significant responses of genes in the RyR-dependent Ca^{2+} signaling pathway, such as *ryr3* and *creb*, in 5 dpf exposed larvae, however, are potential indicators of altered neurodevelopment, as these genes code for key members of pathways important for dendrite growth in the developing brain (Wayman et al., 2012a), and could serve to explain carryover effects determined following the recovery period. While altered RyR signaling were not affected by exposure to ng/L concentrations of bifenthrin, there is a possibility that olfaction is correlated to a continuous alteration in gene transcription of *mTOR*, since the mTOR kinase is crucial for development and stabilization of dendritic arborization of olfactory bulb neurons (Skalecka et al., 2016).

A similar study to that presented herein, but utilizing zebrafish exposed to 1, 10 and 50 ng/L bifenthrin, resulted in transcriptional alterations in genes of the mTOR and RyR dependent-Ca^{2+} signaling, with the majority of genes responding in a linear manner (Frank et al., 2018). Further similarities were the transcriptional dose-dependent alterations of members in RyR-dependent-Ca^{2+} signaling during exposure but not after a 14 day recovery period, while mTOR pathway members still showed dose-dependent alterations after the recovery period in both species. Thus, these results provide further evidence that mTOR signaling in fish is strongly affected by exposure to picomolar concentrations of bifenthrin. The transcription of *mTOR* was altered throughout the developmental stages (1, 3, 5 and 19 dpf) tested in zebrafish (Frank et al., 2018), and was also affected at both the embryonic stage (5 dpf) and at the end of the recovery period, at 21 dpf in inland silversides. Both species, thus, show similarities at the transcriptomic level during exposure, as well as effects that are carried over into later life stages, as demonstrated by responses evaluated following a recovery period.

Significant dose-dependent responses measured herein were for the most part non-monotonic (quadratic), highlighting impacts at 3 and 27 ng/L; compared to both controls and the 122 ng/L treatments. Nonmonotonic (quadratic) appear to be more common than currently known, especially when evaluating mechanistic responses (Vandenberg et al., 2012), and have been reported for multiple contaminants including other pyrethroids like

permethrin (Jeffries et al., 2015b). Non-monotonic responses may occur as a result of a higher proportion of the pyrethroid being metabolized at these lower concentrations, compared to higher concentrations that would result in competition between metabolite and parent compound for binding sites at higher concentrations. This explanation assumes that metabolites are more biologically active than the parent compound itself, which has been demonstrated for bifenthrin (DeGroot and Brander, 2014).

Overall, this and earlier studies on bifenthrin have demonstrated that exposure at concentrations ranging from 0.5 to 30 ng/L can evoke significant transcriptomic, behavioral and reproductive alterations in inland silversides (Brander et al., 2012b, 2016). For example, studies on inland silversides reported transcription changes of genes with endocrine functions following exposure to comparable concentrations of bifenthrin (0.5, 5, 50 ng/L), and demonstrated significant alterations at the lowest exposure concentration (Brander et al., 2016). An evaluation of choriogenin (egg coat protein), another indicator of endocrine disruption, demonstrated increases at all exposure concentrations of bifenthrin (1, 10, 100 ng/L), with the greatest response at 1 ng/L, further supporting the non-monotonic responses observed in this study (Brander et al., 2012b). As such these lower, more ecologically relevant concentrations should receive more attention in future studies.

This study further confirms that bifenthrin uptake can occur through the chorion, as demonstrated by significant differences in transcriptomic responses at 5dpf. As previously indicated, bifenthrin is the most frequently detected pyrethroid insecticide in sediments and waters of both urban and agricultural streams (Hladik and Kuivila, 2012). Contaminated sediments are known to pose risks to benthic organisms (Kerambrun et al., 2012), as such sediment toxicity poses particular risks to fish embryos through direct contact.

Our results, therefore, further validate the use of fish embryo toxicity tests (FETs), which could also be used towards gaining greater ecological relevance in the assessment of sediment bound contaminants (Schreiber et al., 2018), and comply with the requirements for alternative test methods and strategies to reduce vertebrate animal testing (Embry et al., 2010). Furthermore, results from this and earlier studies evaluating picomolar effects of bifenthrin fall in line with conclusions made for multiple contaminants that lower concentrations do not necessarily mean less toxicity (Norman et al., 2015). Researchers and pesticide regulators thus need to re-think the principle that ‘dilution is the solution to pollution’, which underlies chemical management and mitigation (Stegemann, 2014).

In this study, we used the Inland silverside, an inhabitant of freshwater and estuarine systems in the East, Gulf, and West coasts of the USA (Middaugh and Hemmer, 1992; Fluker et al., 2011) as a representative species of aquatic systems where bifenthrin is the most frequently detected pyrethroid insecticide (Hladik and Kuivila, 2012). We detected exposure-dependent transcriptomic effects and delayed alterations in behavior. In contrast to our original hypothesis that behavioral differences would increase with in a dose dependent manner, we detected the strongest alterations for the 3 and 27 ng/L concentrations, but no differences between the control and the 122 ng/L bifenthrin concentration. These results complement a previous study using the vertebrate and human model species *Danio rerio* conducted by our research team (Frank et al., 2018); and emphasize the importance of evaluating dose-

response data for potential non-monotonic responses. This study with inland silversides not only validated behavioral and transcriptomic results obtained from zebrafish, but also extended the knowledge of developmental effects on olfactory impairments and verified results by evaluation of swimming performance. Both studies demonstrated consistent results across behavioral endpoints after a recovery period of 14 days, as well as impacts on highly conserved developmentally important calcium and mTOR signaling pathways in eukaryotes. The observed results are comprehensive between fish models from different ecosystems, and indicate the application of knowledge to other fish species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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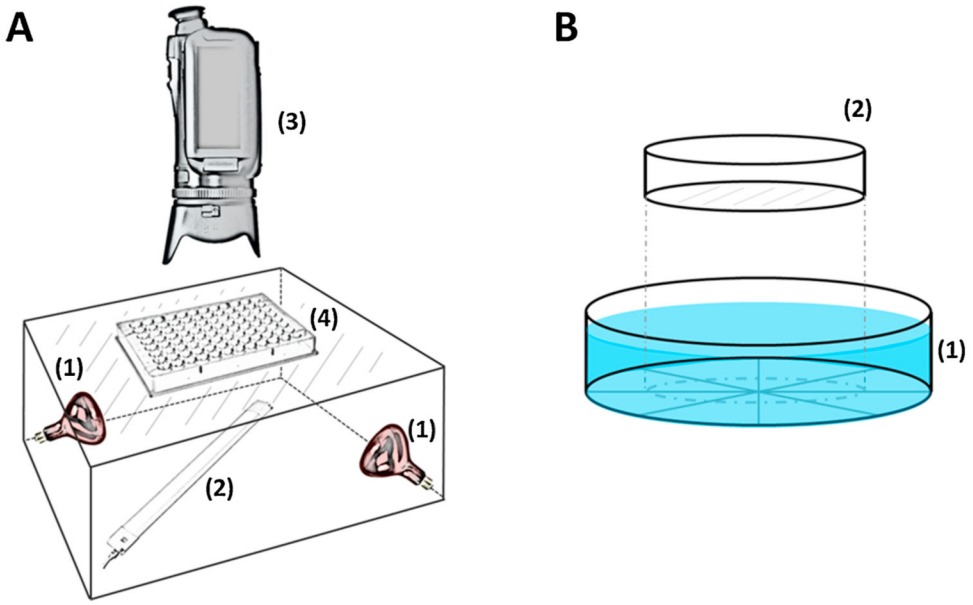

Fig. 1.

(A) Behavioral assessment lightbox setup. Two infrared light Emitting Diodes (LED) (1) placed within the corners of the light box were used for recording behavior under periods of darkness and a light bar (2) was placed diagonally across the base of the lightbox to illuminate the 96-well plate. A video camera (3) was used to record larval fish within a 96-well plate (4), which was placed on a light box with a milk-plexiglas surface for light diffusion purposes. (B) Racetrack Arena. A 10.0 cm diameter petri dish (1) was placed on an on a background dividing the racetrack into 8 radial sectors. A 7.5 cm diameter petri dish (2) was placed in the center to create the circular racetrack.

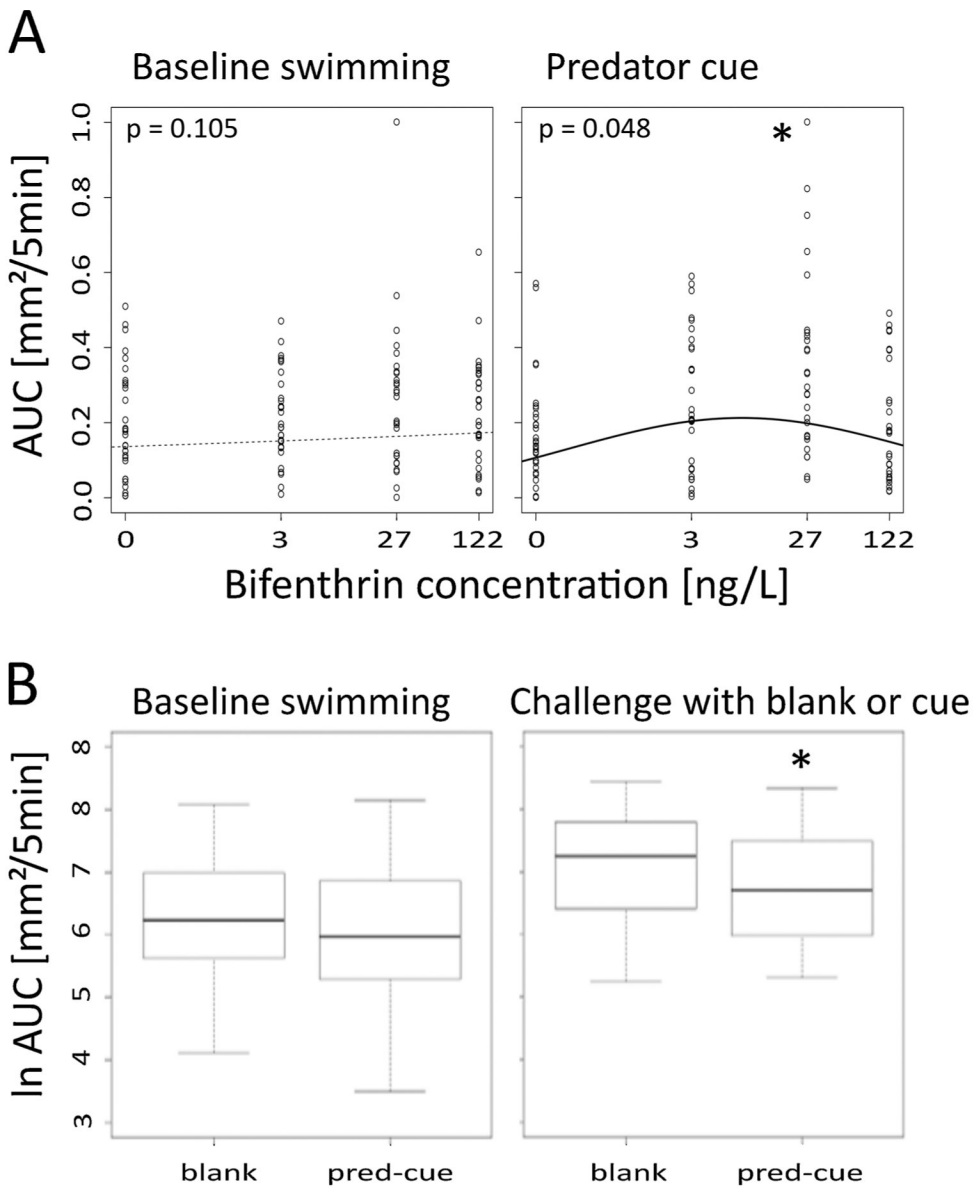

Fig. 2.

Predator cue responses: (A) 21 dpf inland silversides exposed to 0, 3, 27, 122 ng/L bifenthrin from 1 to 7 dpf were confronted with an olfactory predator cue. Baseline swimming was tracked for 5 min during an acclimation period before the cue was added, followed by a 5-min evaluation after confrontation with the predator cue. Each treatment (presented on a log10 X + 0.05 axis) included n = 30 larval fish, each represented by a single dot. The distance moved is presented as the area under the curve (AUC) during a time interval of 5 min. AUC values were rescaled between 0 and 1 using a normalization calculation (graphs with actual values are presented as boxplots in the supplementary section; Fig. S1). Five dose-response curves were fit using a maximum likelihood approach: linear, unimodal1, unimodal2, sigmoidal, and quadratic. Curves shown as a solid line are significantly better fits than a null intercept-only model ($p < 0.05$); curves represented by a

dashed line are the best-fit of the five curve options (lowest p-value), but not significantly better than the null model. P-values are represented for all dose-response curves. *Significantly different from control, as identified using a mixed model algorithm ($p < 0.05$). (**B**) Comparison of fish from the control group challenged with the predator cue (pred-cue) or with a blank water sample (n = 15 for blank or predator cue, respectively). *Significantly difference between the groups, identified using two-sample *t*-test ($p < 0.05$).

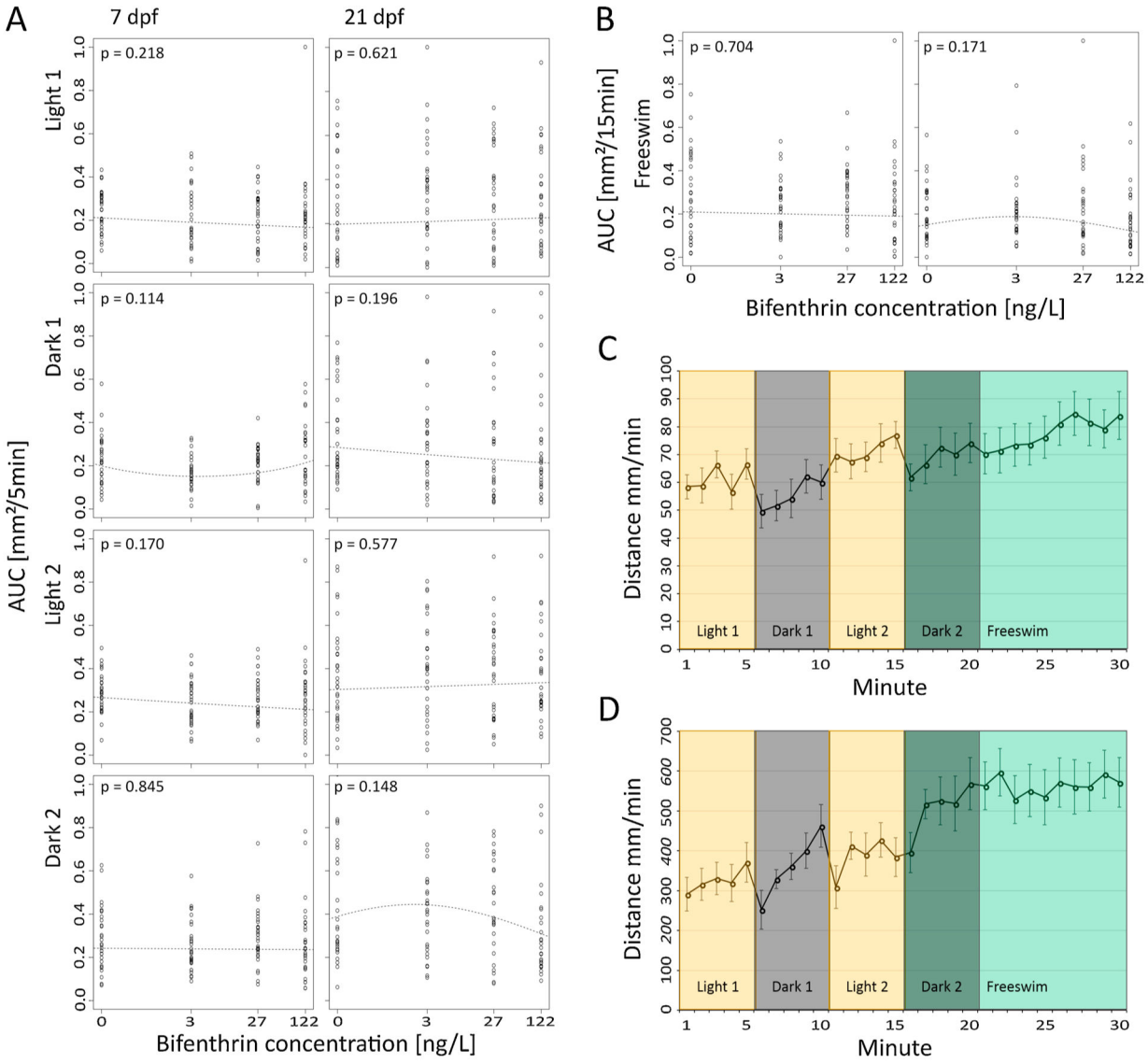

Fig. 3.

Locomotive responses of 7 and 21 dpf inland silversides to alternating light and dark periods, following developmental exposure (1–7 dpf) to different concentrations of bifenthrin (0, 3, 27, 122 ng/L). (**A**) Alternating 5 min light and dark periods. Each dot represents the distance covered by one larval fish during a 5 min period with n = 30 for each treatment (presented on a log10 X + 0.05 axis) and (**B**) an extended Dark2 period of 15 min (Freeswim). The distance is presented as area under the curve (AUC) during a time interval of 5 min (15 min for Freeswim). AUC values were rescaled between 0 and 1 with a normalization calculation for each day (determined separately for the Freeswim period) to facilitate comparison between light and dark periods at any given time point. Five dose-response curves were fit using a maximum likelihood approach: linear, unimodal1, unimodal2, sigmoidal, and quadratic. Curves shown as a solid line are significantly better fits than a null intercept-only model ($p < 0.05$); curves represented by a dashed line are the best-fit of the five curve options (lowest p-value), but not significantly better than the null

model. P-values are represented for all dose-response curves. Graphs illustrating the actual values (Fig. S2) are shown in the supplementary material as boxplots.

Locomotor behavior in vehicle control fish during alternating light and dark periods at **(C)** 5 dpf and at **(D)** 21 dpf after a 14-day recovery period is shown as the mean distance in mm moved per min ± SEM (n = 30 individual larval fish).

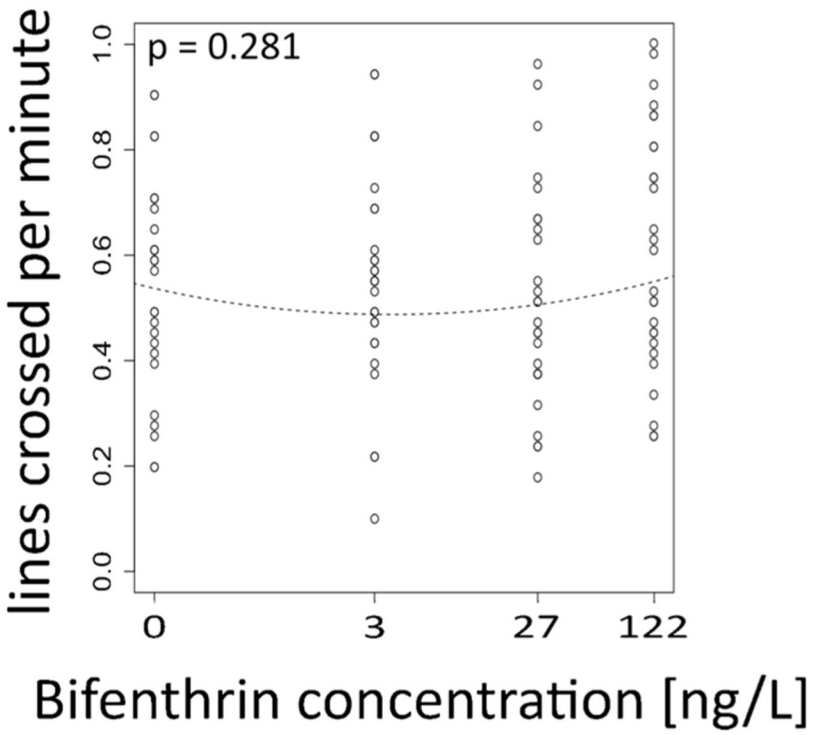

Fig. 4.

Swimming performance of 21 dpf inland silversides exposed to different concentrations of bifenthrin (0, 3, 27,122 ng/L) during early development (1 to 7 dpf). Each dot represents the lines crossed during 1 min in the racetrack arena (Fig. 1B). Values were rescaled between 0 and 1 with a normalization calculation; original values ranged from 39 to 90 lines crossed per min. The dose-response calculation showed a p = 0.281.

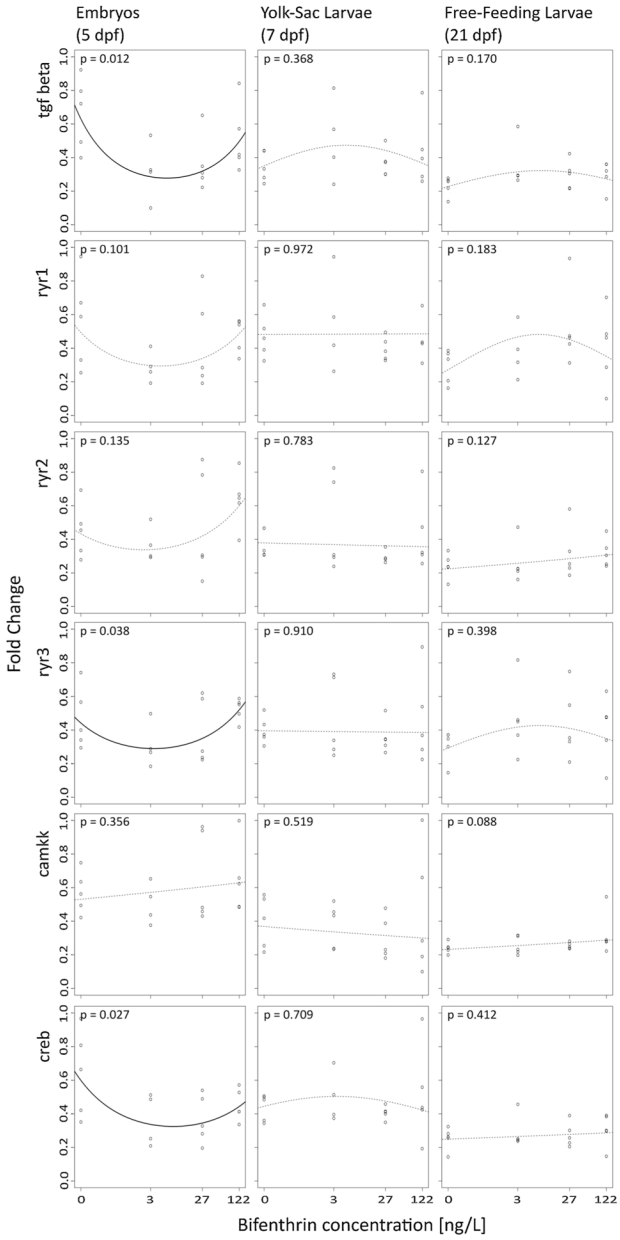

Fig. 5.

Transcriptional changes in genes coding for members of RyR dependent Ca^{2+} signaling in developing inland silversides, using qPCR. Larval fish were exposed to three concentrations of bifenthrin and a control group (0, 3, 27, 122 ng/L) from 1 to 7 dpf. The fold change value of a biological replicate (n = 5) is represented by a single dot and was normalized to reference genes *actb2* and *rpl7*; data are presented on a $\log_{10}$ X + 0.05 axis. For data in each panel, five curves – linear, unimodal1, unimodal2, sigmoidal and quadratic – were assessed for best fit using the maximum likelihood approach. Curves presented as a solid line are significantly better fits than a null intercept-only model ($p < 0.05$), curves shown as a dashed line are the best-fit of the five curve options (lowest p-value), but not significantly better than the null model. P-values are represented for all dose-response curves. Fold-change values

were rescaled between 0 and 1 with a normalization calculation for each time point, to allow comparison between genes (Graphs with the actual values are represented with help of boxplots in the supplementary section: Fig. S3 A).

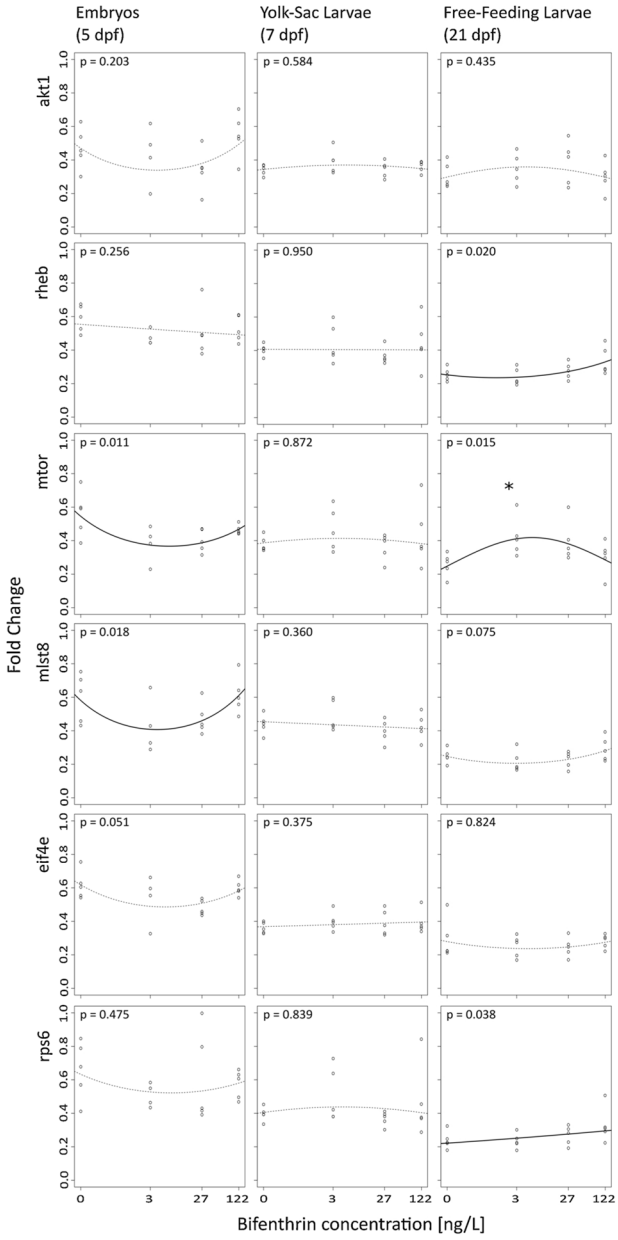

Fig. 6.

Transcriptional changes in genes coding for members of the mTOR signaling pathway in developing inland silversides, using qPCR. Larval fish were exposed to three concentrations of bifenthrin and a control group (0, 3, 27, 122 ng/L) from 1 to 7 dpf. The fold change value of a biological replicate (n = 5) is represented by a single dot and was normalized to reference genes *actb2* and *rpl7*; data are presented on a $\log_{10}$ X + 0.05 axis. For data in each panel, five curves – linear, unimodal1, unimodal2, sigmoidal and quadratic – were assessed for best fit using the maximum likelihood approach Curves presented as a solid line are significantly better fits than a null intercept-only model ($p < 0.05$), curves shown as a dashed line are the best-fit of the five curve options (lowest p-value), but not significantly better than the null model. P-values are represented for all dose-response curves. Fold-change values

were rescaled between 0 and 1 with a normalization calculation for each time point, to allow comparison between genes (Graphs with the actual values are represented in form of boxplots in the supplementary section: Fig. S3B). * Significantly different from control, as identified using one-way ANOVA ($p < 0.05$).

Table 1

Genes selected for transcriptomic analysis.

Gene name	Gene code	Primer (5'- > 3')	Efficiency %
Reference genes			
Beta-aetin	*actb*	F: GCAATGAGAGGTTCCGTTGC R: CGCAGGACTCCATACCAAGG	98.1
60 s ribosomal protein s7	*rpl7*	F: AACTTCTTGTGGCCGTTCAG R: TCGCCTCCCTCCACAAAGT	97.7
Target genes			
RAC serine/threonine-protein kinase	*akt1*	F: CAGAATGCCAGCTGATGAAA R: GTTCCTCGTCCTGCTGACTC	94.4
Ras homolog enriched in brain	*rheb*	F: ATACCGAAAGATCGCCGTTA R: AATTGCCCTTCCACAAACTG	97.7
Mechanistic target of rapamycin	*mtor*	F: TCATGCAGCTCTTTGGTTTG R: GATCACTGCGTAACGCTGAA	103.6
Target of rapamycin complex subunit lst8-like	*mlst8*	F: TCTGTTCACATCGACCCAGA R: TTCATCTCCCATTCCTCCAG	100.5
Eukaryotic translation initiation factor 4e	*eif4e*	F: ATACAGCAGCCCAGCAAACT R: ACACAAAAGCGTTTCCATCC	95.6
40 s ribosomal protein s6	*rps6*	F: CAGCGTTCTCAACTTGGTCA R: GAAGAGTTTGCGGATCTTGC	96.1
Transforming growth factor beta-1	*tgfb*	F: CTCAGGAGGCCAAACAGAAG R: GTATCCACTTCCAGCCCAGA	94.9
Ryanodine receptor 1	*ryr1*	F: TGGAGCTACAAGCCAAAGGT R: TCCGTAAGCAAATCGCTTCT	95.9
Ryanodine receptor 2	*ryr2*	F: GATGCAGTGGTTGGTTTCCT R: GGAAGATTCCACCAGCATGT	92.3
Ryanodine receptor 3	*ryr3*	F:CAGCAAGAGCAAAATGACCA R: ACGATCTCCCCGTTTCTTCT	102.7
Calcium calmodulin-dependent protein kinase kinase 1	*camkk*	F: TCTCCGCTGTGATTCTTGTG R: AGGTCCTCCAGCTCTCCTTC	102.5
Cyclic-AMP response element-binding protein	*creb*	F: GTGTTGATGGGCAAGAGGTT R: ACTCTCAGCGACGTCCACTT	99.3