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Neurotoxicity and Immune Responses of Fish to Environmental Stressors

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Glossary

actb	Beta-actin
ADHD	Attention deficit and hyperactivity disorder
akt1	RAC serine/threonine-protein kinase/ V-akt murine thymoma viral oncogene homolog 1
ANOVA	Analysis of Variance
ASD	Autism spectrum disorder
ASTM	American Society for Testing and Materials
Ca^{2+}	Calcium
cacna1c	calcium channel, voltage-dependent, L type, alpha 1C subunit
cacna1sa	calcium channel, voltage-dependent, L type, alpha 1S subunit, a
camkk	Calcium calmodulin-dependent protein kinase kinase 1
CNS	central nervous system
creb	Cyclic-AMP response element-binding protein
dpf	days post fertilization
EDC	endocrine disrupting compounds
eef1a1	Elongation factor 1 alpha 1
eif4e	Eukaryotic translation initiation factor 4e
eif4e1a	Eukaryotic translation initiation factor 4e1a
eif4e1b	Eukaryotic translation initiation factor 4e1b
eif4e1c	Eukaryotic translation initiation factor 4e1c
eif4ebp1	eukaryotic translation initiation factor 4E binding protein 1
eif4g2a	Eukaryotic translation initiation factor 4, gamma 2a
eif4g2b	Eukaryotic translation initiation factor 4, gamma 2b
FCCL	Fish Conservation and Culture Laboratory
FR	Flame retardant
homer1b	Homer homolog 1b
hpf	hours post fertilization
Ich	Ichthyophthirius multifiliis
irs1	Insulin receptor substrate 1
LRT	likelihood ratio test
mapk1	Mitogen-activated protein kinase 1 (erk2)
mapk3	Mitogen-activated protein kinase 3 (erk1)
MDa	Megadalton
mlst8	mTOR associated protein/ Target of rapamycin complex subunit lst8-like

mtor	Mechanistic target of Rapamycin
NDD	Neurodevelopmental Disorder
PAMPs	Pathogen-associated molecular patterns
PCB	Polychlorinated Biphenyl
prkaa1	AMP-activated, alpha 1 catalytic subunit (ampk)
PRRs	Pathogen recognition receptors
psen1	Presenilin 1
psen2	Presenilin 2
rheb	Ras homolog enriched in brain
rictora	Rptor independent companion of mTOR, complex 2a
rpl7	60s ribosomal protein s7
rps6	40s ribosomal protein s6/ Ribosomal protein S6 kinase 1
rps6ka1	Ribosomal protein S6 kinase a, polypeptide 1
rps6kb1a	p70 ribosomal S6 kinase a
rps6kb1b	p70 ribosomal S6 kinase b
rptor	Rptor regulatory associated protein of MTOR
ryr1	Ryanodine receptor 1
ryr1a	Ryanodine receptor 1a
ryr1b	Ryanodine receptor 1b
ryr2	Ryanodine receptor 2
ryr2a	Ryanodine receptor 2a
ryr2b	Ryanodine receptor 2b
ryr3	Ryanodine receptor 3
stk11	serine/threonine kinase 11 (lkb1)
tgfb	Transforming growth factor beta-1
trdn	Triadin
tsc1a	Tuberous sclerosis 1a
tsc2	Tuberous sclerosis 2
U.S.	United States
USEPA	U.S. Environmental Protection Agency
wnt2ba	Wingless-type 2ba
wnt2bb	Wingless-type 2bb

Preface

This research includes four studies focusing on environmental stressors regularly found in aquatic habitats around the globe, all having the potential to cause adverse effects in fishes. Thereby one focus lies on environmental persistent chemicals, such as flame retardants and pesticides, which are also possessing risk of neurotoxic effects in fish and other vertebrate species, including humans. One experimental setup was specially designed and conducted to evaluate not only the effects in fish, but furthermore to make estimations of adverse effects in humans. This was achieved through investigation of fundamental pathways, conserved throughout the kingdom of eukaryotes and the use of a well-established model species for vertebrates (*Danio rerio*). The first chapter of this work contains the establishment of molecular tools to prepare the use of *D. rerio* as model species for this purpose. To further evaluate effects of a pesticide in a broader ecotoxicological context, the effects of a commonly and frequently used pesticide (bifenthrin) was investigated in the estuarine model species *Menidia beryllina*. A last study was incorporated in this research to estimate the molecular effects on stress and immune responses of a widespread freshwater fish parasite (*Ichthyophthirius multifiliis; Ich*), using delta smelt (*Hypomesus transpacificus*) as a model. *Ich* is a threat to wild fish populations as well as to aquaculture. The assessment of single stressors was the fundamental interest in all research chapters and was performed by investigation of molecular effects in conserved signaling mechanisms.

Summary

All Organisms are constantly challenged by different forms of abiotic and biotic stressors, either naturally occurring or originating from anthropogenic sources. A group of special concern within abiotic stressors is the rapidly growing number of synthesized chemical compounds, which have the tendency to accumulate in the environment. Especially agricultural chemicals like pesticides, are broadly applicated around the planet and are putting wildlife and humans in risk of exposure. The majority of applied pesticides is subsequently transported into aquatic ecosystems via runoff, finally accumulating in aquatic ecosystems or sediments. While standardized tests evaluate certain toxicity parameters of a chemical compound before it is allowed to be produced on large scale, the consequences of exposure to sublethal concentrations in non-target species is poorly known. In addition, there are also numerous other chemical compounds which degrade only slowly over time, such as flame retardants, which are permanently present in sublethal concentrations in dust, sediments or water bodies. Parasitic infections and resulting diseases are common biotic stressors, which can lead to severe population declines. Molecular methods offer new possibilities to evaluate the growing number of biotic and abiotic stressors by investigation of sensitive endpoints. This thesis describes the development of high-throughput *in-vivo* screening methods to assess effects of environmental persistent chemicals and immune responses following a parasitic infection, using different fish species as model. The core of all studies presented herein is the focus on well conserved signaling mechanisms, promising the transfer of methods to other fish species and the transferability of findings to other species, including other vertebrates. Fish were chosen as model species, because they are a) inhabitants of aquatic environments, which are mostly threatened by anthropogenic influences and b) have become an accepted model to predict adverse effects of chemicals in higher vertebrates, including humans.

Four distinct studies were conducted as part of this thesis:

1. The ontogenetic profile of the mechanistic target of rapamycin (mTOR) and ryanodine receptor (RyR) signaling pathways was evaluated in developing zebrafish (*Danio rerio*). Both pathways were selected because they regulate fundamental processes of neurodevelopment and are conserved throughout the eukaryotic kingdom. Therefore, the spatiotemporal expression of key transcripts in mTOR and RyR signaling pathways was evaluated at important time windows of larval development (24, 72 and 120 hours post fertilization; hpf). It was further determined if transcriptional profiles of genes in both pathways were altered by exposure to different concentrations of PCB 95 (2,2',3,5',6-pentachlorobiphenyl), a persistent environmental contaminant, causing developmental neurotoxicity

via RyR-dependent mechanisms in mammals. It could be shown that transcription generally increased with ongoing development, highlighting few robustly upregulated transcripts in both pathways, correlating with peak periods of synaptogenesis. Developmental exposure to PCB 95 significantly altered transcription of genes in both pathways and showed transcriptional changes comparable to those observed in rodent models. The detailed transcriptomic profiling serves a baseline to identify environmental stressors that modify normal spatiotemporal expression patterns of mTOR and RyR signaling pathways in the developing zebrafish, as demonstrated with PCB 95.

2. The effects of environmentally relevant concentrations of the pyrethroid insecticide bifenthrin were evaluated in developing wildtype zebrafish, with the goal to establish a sensitive high-throughput *in-vivo* method to identify neurotoxic chemicals. Bifenthrin has been increasingly employed by pest controllers in urban and agricultural areas and recent reports provide evidence that exposure to nanomolar (nM) concentrations of bifenthrin can cause alterations in calcium oscillations in rodent neurons, which are modulated via ryanodine receptor (RyR) activity, including the mTOR pathway. It was shown that environmentally realistic concentrations of bifenthrin alters transcription of genes involved in RyR and mTOR signaling pathways and that behavior was affected after a recovery period, which was measured via locomotor activity in response to external stimuli. These results demonstrate significant influences of developmental exposures to picomolar (ng/L) concentrations of bifenthrin on neurodevelopmental processes in zebrafish.

3. It was tested if the developed high-throughput *in-vivo* method is transferrable to other fish species. Therefore, inland silversides (*Menidia beryllina*), a small euryhaline fish species native to the North American east coast, were similarly exposed to picomolar (ng/L) concentrations of bifenthrin during early development. Experimental setups included evaluation of transcription in key members of RyR and mTOR signaling, as well as behavioral assessments during acute exposure and after a recovery period. It was measured that bifenthrin elicits significant non-monotonic transcriptional responses in the majority of genes examined during acute exposure and after a recovery period. Inland silversides showed no behavioral effects in response to visual stimuli, but significant altered behavior in response to olfactory stimuli after the recovery period, suggesting delayed or long-term effects of developmental exposures to bifenthrin. This study serves as example for the transfer of the high-throughput *in-vivo* screening method to other fish species, as well as the transferability of findings between teleosts.

4. The concept of measuring stressor caused effects in conserved signaling mechanisms was used to determine different severities of a parasitic infection with the ciliate *Ichthyophthirius multifiliis* (*Ich*) in

delta smelt (*Hypomesus transpacificus*). Since teleosts share a highly conserved response to pathogen infections, it was measured if different infection levels of the parasite cause tissue specific (gill, spleen and kidney) transcriptional responses in genes elementary for an innate immune response and a general stress response, with the goal to complement environmental monitoring programs with help of molecular markers. Significant differentiation of infection severity could be detected with a combination of molecular markers from immune and stress-related genes and was strongest in gill and kidney, however, most effective when combining the transcriptomic results from all tissues. These findings demonstrate how molecular markers targeting fundamental and conserved host responses associated with infections can be used to establish sensitive high-throughput *in-vivo* techniques to assess fish health in natural habitats and in aquaculture.

In conclusion, it was demonstrated that molecular biomarkers, originating from conserved signaling methods, can be used to measure sensitive effects of biotic and abiotic stressors in multiple areas of ecotoxicology via high-throughput *in-vivo* screening. In particular, this thesis describes the step by step development of an *in-vivo* approach to screen for sublethal effects of chemical compounds in zebrafish by investigating transcription of key genes in mTOR and RyR signaling. Furthermore, the transferability of the developed methods has been demonstrated with inland silversides, promising application in other fish species. Lastly, it has been shown that genes belonging to the innate immune and general stress response can be used to classify different severities of pathogen infections. The focus on conserved signaling mechanisms was successfully applied to establish molecular biomarkers, helpful to evaluate the detrimental impact of both abiotic and biotic stressors in teleost fishes. Overall, methods presented in this thesis have the potential to extend and complement current screening methods in neurotoxicological, ecotoxicological and environmental risk assessments.

Zusammenfassung

Alle Organismen sind kontinuierlich verschiedenen abiotischen oder biotischen Stressoren ausgesetzt, welche entweder natürlichen oder anthropogenen Ursprungs sind. Eine besonders bedenkliche Gruppe innerhalb der abiotischen Stressoren stellen die rasant wachsenden synthetisch-chemischen Substanzen dar, die die Tendenz haben sich in der Umwelt anzureichern. Vor allem landwirtschaftliche Chemikalien wie Pestizide werden weltweit breitflächig ausgebracht und stellen für Tierwelt und Mensch ein Expositionsrisiko dar. Der Großteil der ausgebrachten Pestizide landet letztendlich über Regenwasseroberflächenabfluss in aquatischen Ökosystemen und Sedimenten, in denen sie sich anreichern. Während mit standardisierten Verfahren bestimmte Toxizitätsparameter einer chemischen Substanz evaluiert werden bevor diese in großem Maßstab produziert wird, sind die Konsequenzen von subletalen Konzentrationen in Nichtzielarten kaum bekannt. Darüber hinaus gibt es noch zahlreiche weitere Substanzklassen wie Flammschutzmittel, die sich nur sehr langsam abbauen und somit dauerhaft in subletalen Konzentrationen in Staub, Sedimenten oder Gewässern vorhanden sind. Parasitäre Infektionen und daraus folgende Krankheiten sind weit verbreitete biotische Stressoren, welche zu schwerwiegenden Populationsrückgängen führen können. Molekulare Methoden bieten neue Möglichkeiten, um die wachsende Zahl von abiotischen und biotischen Stressoren mit Hilfe sensitiver Endpunkte zu untersuchen. In dieser Dissertation wird die Entwicklung von *in-vivo* Hochdurchsatzmethoden in verschiedenen Fischmodellen beschrieben, um Effekte von umweltbeständigen Chemikalien und Immunantworten einer parasitären Infektion zu erfassen. Der Kern aller aufgeführten Studien ist der Fokus auf konservierte Signalmechanismen, welche eine Übertragung der Methoden auf andere Fischarten, sowie den Transfer von Ergebnissen auf andere Arten inklusive dem Menschen, versprechen. Fische wurden als Modelart gewählt, da sie a) Bewohner aquatischer Ökosysteme sind, welche am meisten von anthropogenen Einflüssen bedroht sind und b) sich zu einem akzeptierten Modelorganismus entwickelt haben, um negative Effekte von Chemikalien in höheren Wirbeltieren, einschließlich dem Menschen, vorauszusagen.

Vier unterschiedliche Studien wurden als Teil dieser Dissertation durchgeführt:

1. Die ontogenetischen Profile des mechanistic target of rapamycin (mTOR; zu deutsch: mechanistisches Ziel des Rapamycins) und des Ryanodinrezeptor (RyR) Signalweges wurden in Zebrafischen (*Danio rerio*) evaluiert, die sich in der Entwicklung befanden. Beide Signalwege wurden ausgewählt, da sie fundamentale Prozesse der neuronalen Entwicklung regulieren und in Eukaryoten durchwegs konserviert sind. Deswegen wurde die räumlich-zeitliche Expression von Schlüssel-Transkripten des mTOR- und RyR-Signalweges während wichtiger Zeitfenster (24, 72, 120 Stunden

nach Befruchtung) der larvalen Entwicklung untersucht. Es wurde weiterhin ermittelt, ob Transkriptionsprofile von Genen aus beiden Signalwegen durch Exposition mit verschiedenen Konzentrationen von PCB 95 (2,2`,3,5`,6-Pentachlorbiphenyl), einem persistenter Umweltschadstoff der in Säugetiermodellen RyR abhängige Neurotoxizität hervorruft, verändert werden. Es konnte gezeigt werden, dass die Transkription mit fortschreitender Entwicklung generell hochreguliert wird, wobei einige Transkripte hervorzuheben sind, die korrelierend an elementaren Punkten der Synaptogenese besonders robust hochreguliert waren. Expositionen mit PCB 95 während der larvalen Entwicklung führten zu transkriptionellen Veränderungen in beiden Signalwegen, welche mit den Ergebnissen vergleichbar sind, die in Säugetiermodellen beobachtet wurden. Das detaillierte transkriptomische Profil dient als Basis um Umweltstressoren zu identifizieren, die das normale räumlich-zeitliche Zebrafisch-Expressionsprofil im mTOR- und RyR-Signalweg verändern, wie mit PCB 95 demonstriert wurde.

2. Die Auswirkungen umweltrelevanter Konzentrationen des Pyrethroid-Insektizids Bifenthrin wurden bei der Entwicklung von Wildtyp-Zebrafischen untersucht mit dem Ziel, eine empfindliche *in-vivo* Hochdurchsatzmethode zur Identifizierung neurotoxischer Chemikalien zu etablieren. Bifenthrin wird in zunehmendem Maße von Schädlingsbekämpfern in städtischen und landwirtschaftlichen Gebieten eingesetzt. Aktuelle Berichte belegen, dass die Exposition bei nanomolaren (nM) Konzentrationen von Bifenthrin zu Veränderungen der Kalziumoszillationen in Nagetierneuronen führen kann, welche über die Aktivität des Ryanodinrezeptors einschließlich mTOR-Signale moduliert werden. Es wurde gezeigt, dass umweltrelevante Konzentrationen von Bifenthrin die Transkription von Genen die an RyR- und mTOR-Signalwegen beteiligt sind beeinflussen, zudem gab es Verhaltensveränderungen nach einer Erholungsphase, die mittels Bewegungsaktivität in Reaktion auf externe Reize gemessen wurden. Diese Ergebnisse zeigen signifikante Einflüsse auf neurologische Entwicklungsprozesse in Zebrafischen, die durch Expositionen mit picomolaren (ng/L) Konzentrationen von Bifenthrin hervorgerufen werden.

3. Es wurde getestet, ob das entwickelte *in-vivo* Hochdurchsatzverfahren auf andere Fischarten übertragbar ist. Diesbezüglich wurden Inland silversides (*Menidia beryllina*), eine kleine euryhaline Fischart, die an der nordamerikanischen Ostküste beheimatet ist, während der frühen Entwicklungphase in ähnlicher Weise picomolaren Konzentrationen (ng / l) von Bifenthrin ausgesetzt. Der Versuchsaufbau beinhaltete die Auswertung der Transkription in Schlüsselgenen des RyR- und mTOR- Signalweges, sowie Verhaltensvesuche während einer akuten Expositionperiode und nach einer Erholungsphase. Es wurde gemessen, dass Bifenthrin während der akuten Expositionsperiode und nach einer Erholungsphase signifikante nicht-monotone transkriptionelle Antworten in der Mehrzahl der untersuchten Gene hervorruft. Inland silverides zeigten keine Verhaltenseffekte in

Reaktion auf visuelle Reize, jedoch wurde nach der Erholungsphase eine signifikante Verhaltensveränderung in Reaktion auf olfaktorische Reize gemessen, was auf verzögerte oder langfristige Auswirkungen schließen lässt, die in Folge einer Bifenthrin-Exposition wahrend der Entwicklungsphase entstehen. Diese Studie dient als Beispiel für die Übertragbarkeit des *in-vivo* Hochdurchsatzverfahrens auf andere Fischarten und belegt gleichzeitig die Übertragbarkeit von Ergebnissen zwischen Teleostei (Echte Knochenfische).

4. Das Konzept zur Messung von stressorbedingten Effekten in konservierten Signalmechanismen wurde weiterhin verwendet, um verschiedene Schweregrade einer parasitären Infektion mit dem Ciliaten Ichthy*ophthirius multifiliis* (*Ich*) in Delta smelt (*Hypomesus transpacificus*) zu bestimmen. Da Teleostei eine stark konservierte Infektionensreaktion nach Kontakt mit Pathogenen aufweisen wurde gemessen, ob unterschiedlich starke Infektionsraten mit dem Parasiten (*Ich*) zu gewebespezifischen (Kiemen, Milz und Nieren) Transkriptionsreaktionen in elementaren Genen der angeborenen Immunantwort und der allgemeinen Stressreaktion führen, mit dem Ziel Umweltmonitoring-Programme mit Hilfe molekularer Marker zu ergänzen. Eine signifikante Differenzierung des Infektionsschweregrads konnte mit einer Kombination molekularer Marker aus immun- und stressbedingten Genen nachgewiesen werden und war am stärksten in Kiemen- und Nierengewebe, jedoch am effektivsten, wenn die transkriptomischen Ergebnisse aller Gewebe kombiniert wurden. Diese Ergebnisse zeigen, wie molekulare Marker, die grundlegende und konservierte Wirtsreaktionen im Zusammenhang mit Infektionen messen, genutzt werden können um sensible *in-vivo* Hochdurchsatzmethoden zu etablieren, um die Gesundheit von Fischen in natürlichen Lebensräumen und Aquakultur zu beurteilen.

Zusammenfassend konnte gezeigt werden, dass molekulare Biomarker aus konservierten Signalwegen dazu geeignet sind, in verschiedenen Bereichen der Ökotoxikologie sensitive Effekte mit Hilfe von *in-vivo* Hochdurchsatzverfahren zu ermitteln, die durch biotische und abiotische Stressoren hervorgerufen werden. In dieser Arbeit wird insbesondere die schrittweise Entwicklung eines *in-vivo* Ansatzes zur Messung subletaler Wirkungen chemischer Substanzen im Zebrafischmodel beschrieben, indem die Transkription von Schlüsselgenen des mTOR- und RyR-Signalweges untersucht wird. Darüber hinaus wurde die Übertragbarkeit der entwickelten Methode in einer Studie mit Inland silversides demonstriert, was demnach eine Übertragung auf weitere Fischarten verspricht. Zuletzt wurde gezeigt, dass Gene der angeborenen Immunreaktion, sowie der allgemeinen Stressreaktion dazu verwendet werden können, um verschiedene Schweregrade von Pathogeninfektionen zu klassifizieren. Der Fokus auf konservierte Signalwege wurde erfolgreich angewendet, um molekulare Biomarker zu etablieren, die helfen können schädliche Auswirkungen abiotischer und biotischer

Stressoren in Teleostei zu messen. Insgesamt haben die Methoden, die in dieser Arbeit vorgestellt werden, das Potenzial derzeitige Messmethoden in neurotoxikologischen, ökotoxikologischen und ökologischen Risikobewertungen zu erweitern und zu ergänzen.

1. Introduction

Aquatic ecosystems and their biological diversity are increasingly threatened by a growing number of anthropogenic pollutants (Dudgeon et al., 2006; Geist, 2011). The scientific evaluation and characterization of these pollutants and their effects on ecosystem communities poses a constant challenge (Relyea and Hoverman, 2006). Toxicology has a long tradition in the classification of pollutants by conducting standardized tests, often with a focus on mortality or malformations in model organisms. However, since the majority of pollutants in ecosystem occur in low sublethal concentrations (Relyea, 2009), new experimental techniques including more sensitive endpoints are necessary to recognize impacts in natural communities. The application of sensitive techniques has also the potential to improve and complement evaluation strategies in current environmental risk assessments. Molecular methods have been successfully used to measure the health status of fishes and monitor aquatic ecosystems (Connon et al., 2012; Bourlat et al., 2013), promising to be a useful component in the assessment of pathogen infections and diseases.

1.1 Environmental Stressors

Organisms are constantly challenged by naturally occurring changes in environmental conditions, but also increasingly affected by anthropogenic influences. Both can result in specific organism responses, causing changes in behavior or physiology to acclimate to new environmental conditions (Schulte, 2014). Physiological acclimation can be assessed on different levels of biological organization such as the macro-molecular, cellular, organ or organism level, and used to predict adverse effects on organism or population level, which has been established to assess and predict the influences of chemicals to organisms (Ankley et al., 2010; Vinken, 2013; Villeneuve et al., 2014). External conditions are defined as either biotic or abiotic factors (Figure 1). Abiotic factors are all physicochemical properties shaping an ecosystem, while biotic factors are always related to or originate from other organisms and have influences on an ecosystem as well. While abiotic and biotic components are clearly defined, there exists discrepancy in literature in terms related to stress (stress, stressor, stress response), as they are used compatible (Schulte, 2014). The differing definitions originate from independently developed fields of research, such as stress assessments within biomedical science or in natural populations (Goldstein and Kopin, 2007; Romero et al., 2009; Homyack, 2010; Johnstone et al., 2012). In this work environmental stressors are referred to as either environmental persistent chemicals (abiotic factor) or ectoparasites (biotic factor), both eliciting adverse effects in freshwater fishes (Figure 1). The focus of this research is the evaluation of environmental induced stress responses on molecular and behavioral levels in freshwater fish species.

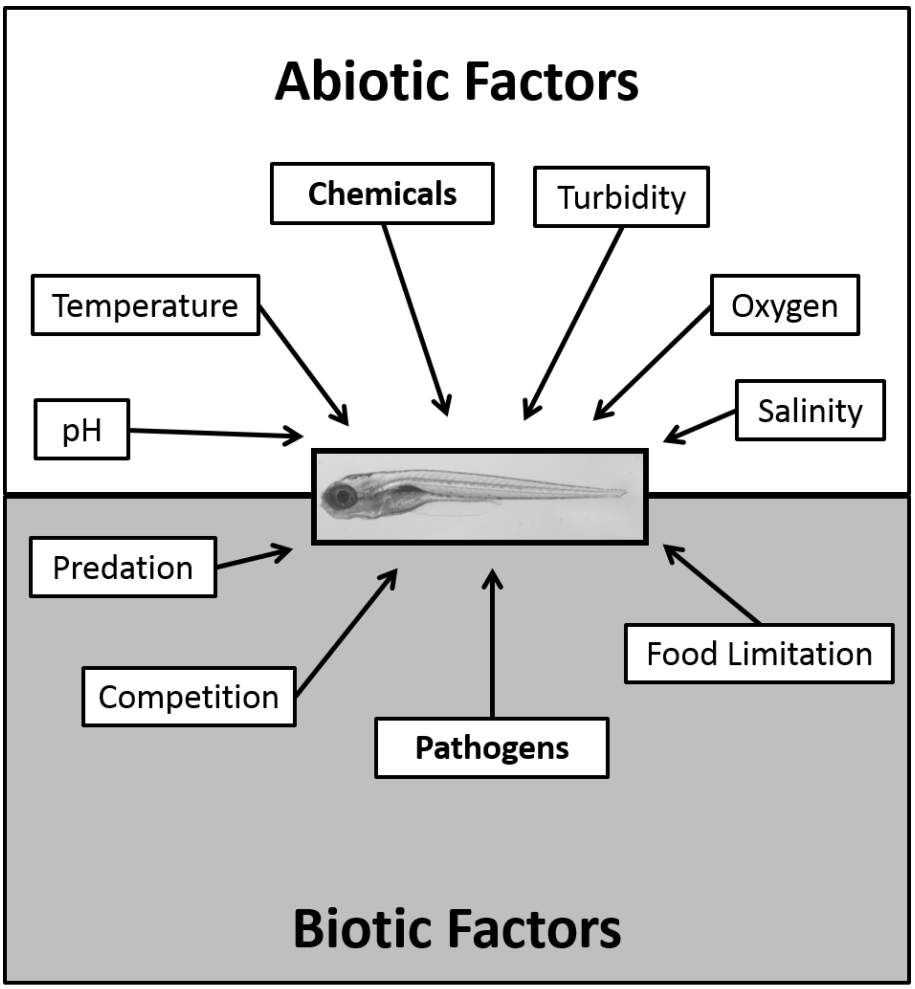

Figure 1. Examples of abiotic and biotic factors occurring in aquatic ecosystems. This thesis evaluates transcriptional and behavioral effects following exposure to chemicals (PCB95 and bifenthrin) and transcriptional effects after a pathogen infection (*Ichthyophthirius multifiliis*) in model and non-model fish species.

1.2 Chemicals – A group of rapid growing environmental contaminants

Synthetic chemical compounds are the fastest growing anthropogenic component within abiotic factors. The mass production of synthetic chemicals started during the industrial evolution in the 18th century (Derry and Williams, 1960). The enormous benefits for mankind have led to a steadily increasing chemical production and is currently counting for more than 133 million registered organic and inorganic substances and has therefore more than doubled within the past 5 years (www.cas.org).

Flame retardants (FR), a growing group of environmental persistent chemicals, are components within plastics, fabrics, electronics, cars and other materials to reduce their potential of catching fire (de Wit, 2002; Alaee et al., 2003). They can be generally grouped into halogenated organic, phosphorus-containing, nitrogen-containing or inorganic flame retardants (Birnbaum and Staskal,

2004; Law et al., 2006a). Due to their stable structure, they have the tendency to accumulate in the environment, and are regularly found in sediments (Alaee et al., 2003; Watanabe and Sakai, 2003), the atmosphere (Law et al., 2006b), water bodies and organisms (Hale et al., 2003).

Agricultural chemicals, such as pesticides, play a major role to secure the global production of food and crops and are another constantly growing group within chemical compounds (Popp et al., 2013). The production of organic synthesized pesticides has significantly grown since the early 1960s, when their large scale production started (Zhang et al., 2011). Until 2000 the growth rate in pesticide production followed a linear trend, increased > 750% and reached an amount > 3,75 megatons of annual produced pesticides in 2000 (Tilman et al., 2001). More recent data illustrates the economic dimension of pesticides, estimating a worldwide market value of 50 billion US dollar in 2008 (Stehle and Schulz, 2015). Latest predictions postulate a market value of 75 billion US dollar in 2017, with estimations of 90 billion US dollar in 2023 (https://www.techsciresearch.com/report/global-pesticides-market/1311.html). The rapid increase in pesticide production and their broad application in agricultural and urban areas contributes to environmental bioconcentration and accumulation (Barriada-Pereira et al., 2005), putting wildlife and humans in risk of exposure (Sharpe and Irvine, 2004; Khan et al., 2008). Aquatic systems are particularly in danger of pesticide contamination, since they are affected by runoff from agricultural and urban territories (Schriever and Liess, 2007; Weston and Lydy, 2012).

1.2.1 Polychlorinated Biphenyls

Polychlorinated Biphenyls (PCBs) are a group of halogenated flame retardants, widely used during the 20th century. PCBs have been synthesized for the first time in 1881 and were produced extensively between the 1930s until the 1970s (Cairns and Siegmund, 1981). During this period of time PCBs became the most abundant chlorinated aromatic pollutant in the environment (Cairns and Siegmund, 1981). After recognition of numerous toxic effects caused by PCBs, they were banned in the late 1970s, but because to their persistence and bioaccumulative properties, environmental concentrations of PCBs have not decreased significantly over the past decades (Martinez and Hornbuckle, 2011; Martinez et al., 2012). Therefore, PCBs remain widespread distributed and accumulated in natural environments and urban areas (Eljarrat and Barceló, 2009; Gdaniec-Pietryka et al., 2013; Vane et al., 2014; Herrick et al., 2016), putting wildlife, productive livestock and humans in risk of exposure (Humphrey et al., 2000; Costabeber et al., 2006; She et al., 2007; Asamoah et al., 2018).

A first case of acute human PCB toxicity on bigger scale occurred in 1968 in Japan, where patients consumed PCB contaminated rice oil and developed the so-called Yusho oil disease (Kuratsune et al., 1972). Over 1900 persons showed clinical symptoms including pigmentation of skin, nails, conjunctiva and mucosa; acneiform eruptions; increased discharge from the eyes and eyelid swellings; feet paresthesia; and numbness of extremities (Ikeda, 1996). About 500 people died from the poisoning and adverse effects on pregnancy of Yusho women were observable in the following 10 years (Tsukimori et al., 2008). This incident was one of the key events leading to the worldwide production decline of PCBs. About ten years later first studies were reporting adverse effects related to PCBs, by linking limited reproduction success in minks fed with PCB contaminated fish from the great lakes (Aulerich and Ringer, 1977), followed by similar observations and results in a study on Baltic seals (Jensen et al., 1979). Since then adverse effects of PCBs have been described in numerous studies around the globe and include for example endocrine disruption, developmental impairments and lately also neurotoxicology in humans and wildlife (Colborn et al., 1993; Longnecker et al., 1997; Tyler et al., 1998; Derraik, 2002; van der Oost et al., 2003; Polańska et al., 2013; Malisch and Kotz, 2014). Several studies have linked in utero or infancy exposures to PCBs with neurological deficits (Schantz et al., 2003; Korrick and Sagiv, 2008; Winneke, 2011; Sagiv et al., 2012; Berghuis et al., 2014; Berghuis et al., 2015). Therefore, PCBs have been named as potential risk factors for neurodevelopmental disorders (NDDs), including autism spectrum disorder (ASD), attention deficit and hyperactivity disorder (ADHD) and intellectual disability (Eubig et al., 2010; Neugebauer et al., 2015; Nowack et al., 2015; Caspersen et al., 2016; Lyall et al., 2016; Sealey et al., 2016).

Fish consumption has been repeatedly reported as source for PCB uptake in humans and fish feeding vertebrates (Svensson et al., 1994; Mendola et al., 1997; Smit et al., 1998; Persky et al., 2001), illustrating the widespread accumulation of PCBs in fish. It has been suggested predatory fish tend to higher concentrations of contaminants, such as PCBs, since they are at the end of the aquatic food web (Rasmussen et al., 1990).

In this research we investigated the effects of PCB 95 (2,2',3,5',6-pentachlorobiphenyl; Figure 2), known to cause developmental neurotoxicity in mouse models and altered Ca^{2+} signaling via RyR-dependent mechanisms (Pessah et al., 2010; Wayman et al., 2012b; Wayman et al., 2012a; Fritsch et al., 2015).

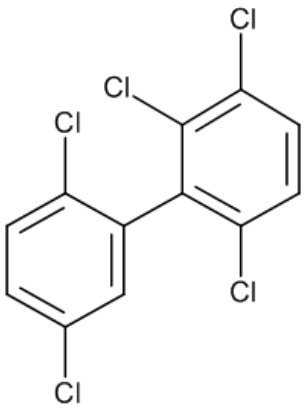

Figure 2. 2,2',3,5',6-pentachlorobiphenyl (PCB 95)

1.2.2 Bifenthrin

Bifenthrin (Figure 3) is a globally produced and used insecticide (Yadav et al., 2003; Chouaibou et al., 2006; Houndété et al., 2010; Li et al., 2017), belonging to the chemical class of pyrethroid insecticides. Pyrethroids are synthetic esters; chrysanthemic and pyrethric acid, which have insecticidal properties that were discovered in flowers of *Chrysanthemum cinerafolis* (Davies et al., 2007). Pyrethroids have gained popularity and importance since organophosphate pesticides were in the process of being phased out of production (Werner and Moran, 2008; Crago and Schlenk, 2015). Thirty eight percent of the global annual insecticide production did account for pyrethroid insecticides in 2015 (Crago and Schlenk, 2015; Frank et al., 2019), and bifenthrin is one of the most frequently detected pyrethroid insecticides in environmental samples (Nowell et al., 2013; Allinson et al., 2015).

Pyrethroids interact with voltage-gated sodium channels and can be classified in two groups: type I or type II. The two groups are chemically defined by possessing (type I) or missing (type II) an α-cyano-3-phenoxybenzyl moiety (Soderlund, 2012). The chemical differences also result in distinctive biological effects in their target: exposure to type I pyrethroids, results in tremors or convulsions, while exposure to type II pyrethroids leads predominantly to symptoms like choreoathetosis (Nasuti et al., 2003; Casida and Durkin, 2013). Bifenthrin belongs to the group of type I pyrethroids, known to bind temporarily to voltage-gated sodium channels in insects, causing constant action potentials.

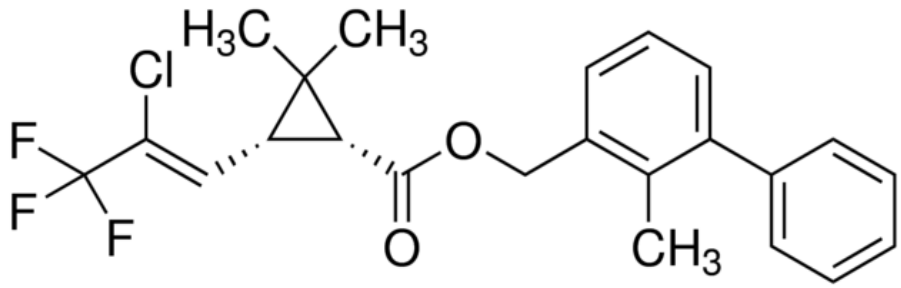

Figure 3. Cis-Bifenthrin

Despite predicted low-toxicity in mammals there is evidence that pyrethroids effect mammalian reproduction, such as altered sperm production in mouse models (Kumar et al., 2004). Pyrethroids have the potential to interact with voltage-gated sodium, chloride and calcium channels in mammals, possessing the risk of neurotoxic effects (Shafer and Meyer, 2004; Symington et al., 2008; Soderlund, 2012). It further has been shown that calcium uptake and depolarization-evoked neurotransmitter release in the brain of rats has been altered by several type I and II pyrethroids (Symington et al., 2007; Symington et al., 2008). Therefore, it is necessary to focus on potential neurotoxic effects of pyrethroid insecticides. Since pyrethroids are often the active substances in common household pesticides, putting infants in risk of exposure (Bennett et al., 2013). In fact, a recent case study linked the indoor application of a pyrethroid mixture, including bifenthrin, with facial paresthesia (manifesting as stinging, itching and numbness) in a toddler (Perkins et al., 2016). Paresthesia has been reported after dermal contact with pyrethroids, potentially resulting from local action in sensory neurons in the skin (Ray and Forshaw, 2000).

Although there have been adverse effects described in mammals, fish are even more vulnerable to pyrethroid exposure (Glickman and Lech, 1982). Fish have in general a higher disposition to accumulate pyrethroids (Tyler et al., 2002; Munaretto et al., 2013), and pyrethroids are found regularly in waterbodies and sediments (Nowell et al., 2013; Allinson et al., 2015). Even environmental realistic concentrations (nm/L) of pyrethroids, shown in experiments with bifenthrin, can impact the estrogen system and influence reproductive performance in fish (Brander et al., 2012b; DeGroot and Brander, 2014; Brander et al., 2016b), even over several generations (DeCourten and Brander, 2017).

1.3 Parasitic infection in fishes – *Ichthyophthirius multifiliis* as example

All parasites have by definition a negative effect on their host (Begon et al., 1986). Parasitic infections are mostly not limited by the utilization the hosts energy resources, but cause pathological and physiological effects in the host (Barber et al., 2000). Thus, parasitic infections can have serious impacts in populations of wild fish, including extremes like epidemics and mass mortalities (Wurtsbaugh and Alfaro Tapia, 1988; Pike and Wadsworth, 2000; Gozlan et al., 2005; Austin and Austin, 2007). Parasites can further evoke sublethal responses such as alterations in behavior, decreases in fertility or swimming performance, resulting in limited reproductive success or increased risk of predation (Barber et al., 2000; Frank et al., 2017b). Such effects can intensify by simultaneous exposure to multiple environmental stressors (Barber et al., 2000), for example exposure to chemicals. Parasites can be categorized by the specific tissue they infest in the host. They are referred to as either endoparasites, when living inside a host; or ectoparasites, when attached on the outer tissue of the host and therefore visible.

This research focused on the ciliate *Ichthyophthirius multifiliis* (*Ich*), a widespread endoparasite in freshwater fishes (Maki and Dickerson, 2003), infecting the outer tissues and therefore visible. *Ich* is known to cause adverse effects in fishes and can result in serious defects of skin and gill tissue (Matthews, 2005; Xu et al., 2005). Thus, *Ich* is recognized as one of the main pathogens in aquaculture (Dickerson and Findly, 2014; Christoffersen et al., 2017), and has caused serious losses in aquaculture facilities, as well as declines in wild fish populations (Jessop, 1995; Traxler et al., 1998; Martins et al., 2011).

Ich has a three-stage life cycle, named as theront (free-swimming, infecting stage), trophont (feeding stage) and tomont (reproductive stage) (Matthews, 2005). The 30 to 50 µm theront hatches from tomocysts and is able to move with help of cilia, driven by positive phototaxis (Wahli et al., 1991). Due to *Ich*s' swimming speed compared to velocities of water currents measured in its habitat, long range-chemotaxis is of minor importance for the ciliate (Haas et al., 1998). Whereas short range chemotaxis gains importance, when the theront is close to a potential host (Haas et al., 1999). The exact invasion mechanism when entering host tissue, remains unclear (Matthews, 2005). Once attached to the host, the theront is transforming into the trophont, which is residing inside a cyst, predominantly located in the basal lamnia of the skin (Ventura and Paperna, 1985). The trophont thrives and feeds mainly thrives in gill and skin epithelia, as well as in buccal and pharyngeal cavities, where a ciliates can be spotted as white dots with up to 1 mm in diameter (Ventura and Paperna, 1985; Lom and Dyková, 1992). Finally, the trophpont leaves the host in form the tomont, which is

transforming into the tomocyst within a time window of 15 minutes to 6 hours, starting to produce theronts (Ewing and Kocan, 1992).

1.4 Conserved signaling mechanisms in teleost fishes

Numerous cellular functions and their signaling mechanisms in vertebrates and eukaryotes have been conserved throughout evolutionary processes, particularly organogenesis, including neurodevelopment and neural connectivity (Louvi and Artavanis-Tsakonas, 2006; Gilbert, 2010; Costa-Mattioli and Monteggia, 2013), innate immune and general stress responses (Ausubel, 2005; Westerheide and Morimoto, 2005; Stein et al., 2007; Simmons et al., 2009). The evaluation of conserved signaling mechanisms in toxicological studies or environmental risk assessments promises transferability of methods to other fish species and also predictions of effects in other vertebrates, including humans (Perkins et al., 2013).

1.4.1 Ryanodine receptor (RyR) dependent Ca^{2+} signaling

RyRs are intracellular Calcium (Ca^{2+}) channels located in the sarcoplasmic and endoplasmic reticulum of cells (Lanner et al., 2010). RyRs are the channels responsible for Ca^{2+} release during excitation–contraction (EC) coupling in skeleton and cardiac muscle tissue (Takeshima et al., 1994; Lamb, 2000). Furthermore, RyRs are major components and regulators of neuronal development, synaptogenesis and function (Emptage et al., 1999; Berchtold et al., 2000; Hong et al., 2000; Mori et al., 2000; Fill and Copello, 2002; Bers, 2004; Berridge, 2006). An example are RyR regulated Ca^{2+} signals, which are regulating gene expression of wingless-type 2 (wnt2), a protein responsible for activity-dependent outgrowth of dendrites in neurons (Wayman et al., 2006; Wayman et al., 2012b). Increased RyR-dependent Ca^{2+} flows during development can increase dendritic arborization, cause alterations in synaptic plasticity and impair cognitive development (Yang et al., 2009a; Pessah et al., 2010; Wayman et al., 2012b; Wayman et al., 2012a). RyR signaling in neurons was significantly altered when exposed to PCB 95 (Wayman et al., 2012a) and Ca^{2+} homeostasis was impacted following exposure to the pyrethroid insecticide bifenthrin *in-vitro* (Cao et al., 2014). Thus, the investigation of RyR signaling following exposure to PCB 95 and bifenthrin became a point of major interest in this thesis.

With a size >2 megadalton (MDa), RyRs are the largest known ion channels and mammals exhibit three genes coding for RyRs (Ryr1–3) (Van Petegem, 2015). *Ryr1* is predominantly expressed in striated muscle, *ryr2* in cardiac and *ryr3* occurs at low levels in many tissues (Sutko and Airey, 1996). Zebrafish in contrast exhibit 5 paralogs of RyRs (Figure 4), with similar transcription sites in the organism (Wu et al., 2011): *Ryr1a* and *ryr1b* are mainly transcribed in slow and fast twitch muscles. During development

ryr2a and *ryr3* are transcribed predominantly in the central nervous system (CNS) and *ryr2b* in the heart (Thisse and Thisse, 2005; Wu et al., 2011; Frank et al., 2017a).

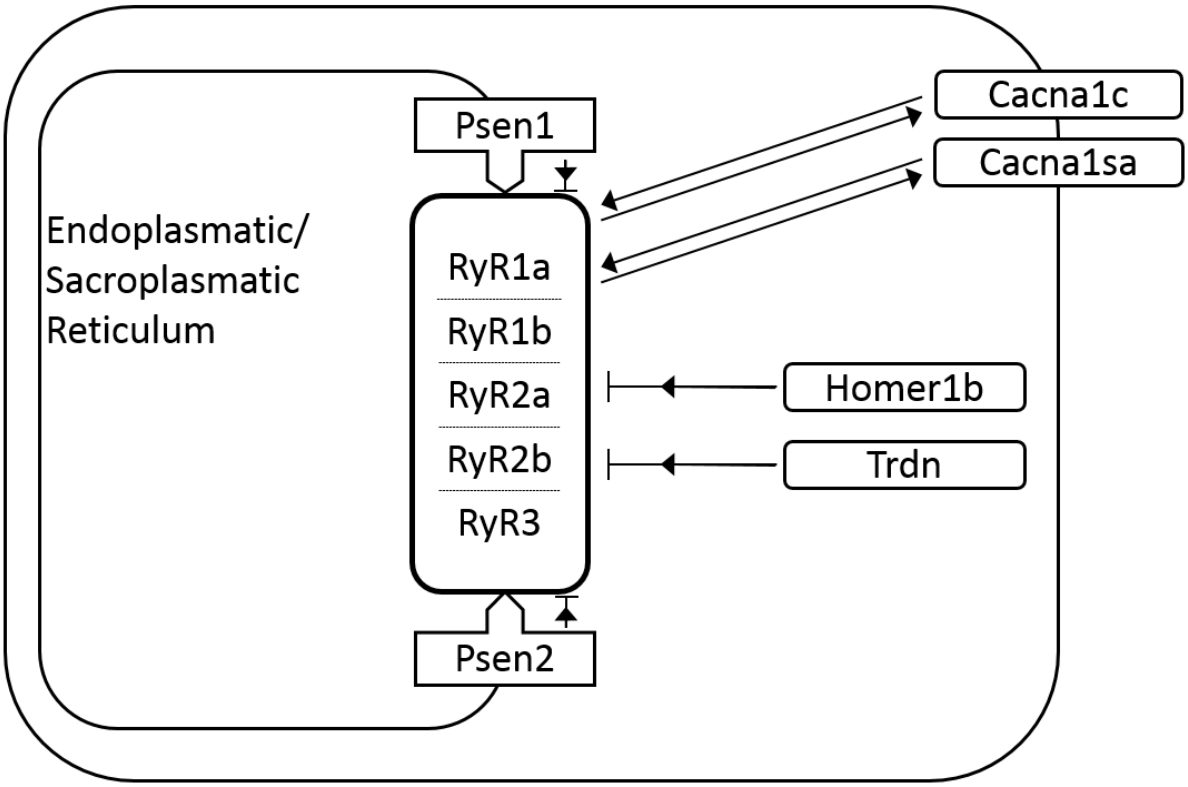

Figure 4. Schematic listing all the zebrafish ryanodine receptors (RyR) paralogs and regulatory proteins. Arrows highlight regulatory proteins that stabilize the RyR in its open configuration, which increases release of Ca^{2+} from internal stores; whereas blunt ends identify protein interactions that inhibit RyR activity.

1.4.2 mTOR (mechanistic target of rapamycin) signaling

MTOR signaling (Figure 5) is a fundamental pathway contributing to cell growth and proliferation by regulating anabolic processes, such as synthesis of proteins, organelles and lipids; as well as catabolic mechanisms like autophagy (Sarbassov et al., 2005a; Laplante and Sabatini, 2009; Laplante and Sabatini, 2012). Therefore, mTOR acts as controlling center of nutrient regulated signal transduction and dysfunction in mTOR signaling has been linked to metabolic diseases and cancer (Guertin and Sabatini, 2007; Zoncu et al., 2011). Moreover, mutations within negative regulators of the mTOR-signaling, resulting in increased mTOR activity, have been corelated to neurodevelopmental disorders (NDDs), such as Autism spectrum disorders (ASDs) (Ehninger and Silva, 2011; Tsai et al., 2012; Zhou and Parada, 2012; Costa-Mattioli and Monteggia, 2013; Tang et al., 2014). Thus, enhanced mTOR

activity can lead to alterations in neuronal structures. Furthermore, mTOR signaling is an elementary regulator of innate and adaptive immune homeostasis (Weichhart et al., 2008; Powell et al., 2012).

MTOR was named after the antiproliferative substance rapamycin, where mTOR has been originally identified as mediator of rapamycin's' toxic effects in yeast (Cafferkey et al., 1993; Kunz et al., 1993). Rapamycin, produced by the bacterium *Streptomyces hygroscopicus* and originally detected in soils of Rapa Nui (Costa-Mattioli and Monteggia, 2013), binds to the immunophilin FKBP12 (FK506 binding protein 12-kDa) (Laplante and Sabatini, 2012). FKBP12-rapamycin inhibits mTOR complex 1 (mTORc1), the central protein serine/threonine kinase of the signaling pathway via allosteric binding (Figure 5). Since then rapamycin has been used to identify different mechanistic functions of the mTOR signaling pathway.

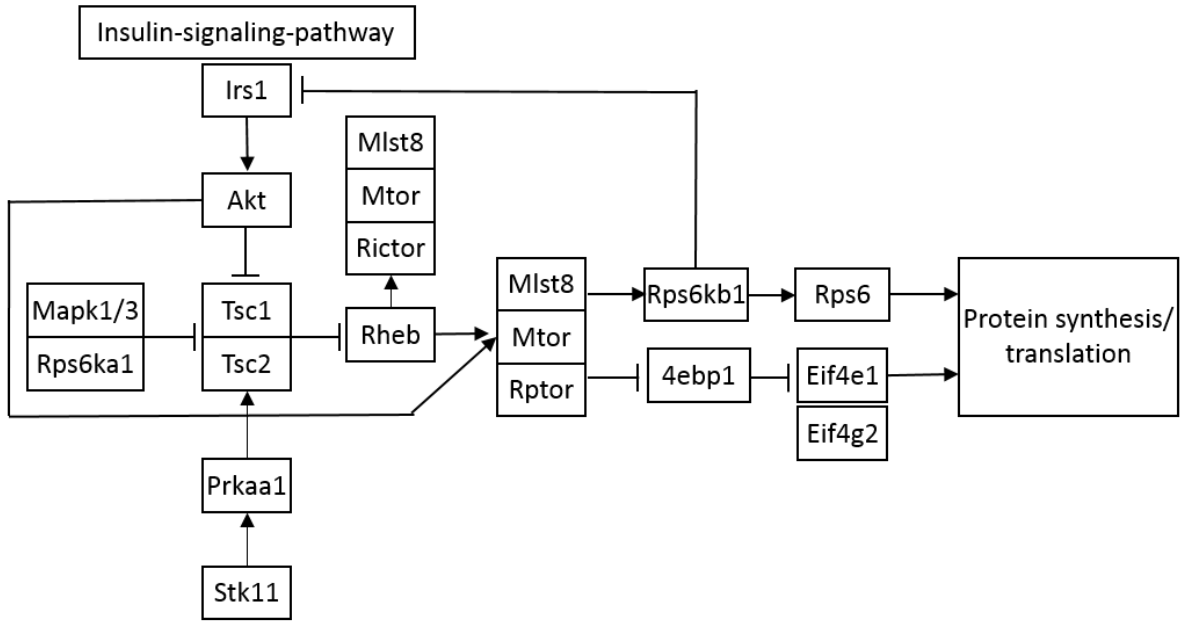

Figure 5. Schematic illustrating the functional relationship of the mTOR signaling molecules.

1.4.3 Innate immune and general stress response in fish

The immune response in vertebrates is initiated by immediate activation of innate (non-specific) and followed by an adaptive (specific) immune response (Magnadóttir, 2006; Alvarez-Pellitero, 2008). The functionality of an innate immune response is given by the recognition of molecular patterns which are associated with pathogens (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002). The detection of these patterns is carried out via pathogen recognition receptors. Once the receptors are bound to these patterns, they are recognized by macrophages and actively destroyed. Representatives of an activated innate immune system include members of the complement system, toll-like receptors, inflammation-inducing cytokines, immune cell migration-directing chemokines, anti-proteases, members of the mitogen activated pathway and major histocompatibility complex components (Dalmo et al., 1997; Dixon and Stet, 2001; Gasque, 2004; Sitjà-Bobadilla et al., 2006; Alvarez-Pellitero, 2008; Umasuthan et al., 2015). The activation of innate immunity normally correlates with a general stress response, which can be measured via heat shock proteins (HSPs) (Cho et al., 1997; Forsyth et al., 1997; Ackerman and Iwama, 2001). Both signaling mechanisms are highly conserved within teleosts (Westerheide and Morimoto, 2005; Stein et al., 2007; Simmons et al., 2009).

1.5 Model species

Different areas of biomedical research incorporate specific model species to answer field specific research questions. Neurotoxicology uses well-established model species to assess effects of stressors in individuals, aiming to predict mechanisms of action relevant for the human species (Segner, 2011). Ecotoxicology and environmental risk assessments evaluate the effects of stressors in populations, ecosystems and the biosphere (Balling, 2009; Newman, 2009), regularly by measuring effects in representative non-model species. This study incorporates model and non-model teleost fish species to evaluate effects of abiotic and biotic stressors, with the goal to establish connections between the different research areas.

1.5.1 Zebrafish (*Danio rerio*)

Zebrafish are freshwater fish, inhabiting small rivers and streams, but also stagnant or slow-moving water bodies near streams, like ponds or rice paddies (Metscher and Ahlberg, 1999; Daniels, 2002; Engeszer et al., 2007). Wild populations of zebrafish are mainly thriving in Ganges and Brahmaputra river basins in north-east India, Bangladesh and Nepal (Spence et al., 2008; Arunachalam et al., 2013), were they are often found in tight shoals (Engeszer et al., 2007).

Within the last fifty years zebrafish have become an important model species in the field of biomedical research. Several features of the zebrafish life cycle and living conditions have favored the rise as vertebrate model species. Since zebrafish prefer to form close shoals (Miller and Gerlai, 2007), they do not use as much space as other vertebrates in laboratory setups. In addition, zebrafish exhibit a fast ex-utero development, maturing during the first six months, combined with a long lifespan up to six years (Zhdanova et al., 2008). Within the first five days the transparent embryos develop all tissues and organs and transform into free-swimming larvae (Kimmel et al., 1995), allowing first behavioral experiments starting with observations at 2 days port fertilization (dpf).

First behavioral studies on zebrafish were published in the 1950s (Kalueff et al., 2014), followed by the development of first forward genetic techniques during the late 1960s at the university of Oregon (Grunwald and Eisen, 2002; Roeselers et al., 2011). The number of studies on zebrafish increased slowly until its real ascent as robust model species in biomedical research started in the early 1990s, (Kalueff et al., 2014; Shams et al., 2018), since then many tools have been developed to increase speed in genetic studies (Howe et al., 2013). The development of genetic tools includes amongst others the possibility of gene knockdown or overexpression, mutant-phenotype analysis and in-situ hybridization, making the zebrafish a favorable model in genetics (Amsterdam and Hopkins, 2006; Gerlai, 2010). Zebrafish have been increasingly used to investigate vertebrate gene functions, including genetic diseases in humans. The establishment as genetic model was climaxed by the complete sequencing of the zebrafish genome, revealing similar expression of >70 % homolog genes between humans and zebrafish (Howe et al., 2013). These features have established zebrafish as an alternative vertebrate model in neuroscience and its importance is still steadily growing (Kalueff et al., 2014). The major stages of neurodevelopment and the signaling molecules regulating neurodevelopmental processes are highly conserved between zebrafish and humans (Gilbert, 2010). In addition, larval zebrafish have proven to be a powerful model system to screen chemicals with potential neurotoxic and therapeutic adequacy (MacRae and Peterson, 2015; Brady et al., 2016; Garcia et al., 2016). Therefore, zebrafish appear as a well suited model organism to close the gap between ecotoxicological and neurotoxicological studies, when focusing on conserved pathways and mechanisms.

1.5.2 Inland silversides (*Menidia beryllina*)

The Inland silverside is an euryhaline fish species, native to the east coast of North America, but also inhabiting Californian water systems since its introduction in 1967 (Middaugh and Hemmer, 1992; Fluker et al., 2011). Inland silversides have been recently established as an alternative estuarine model and are part of the U.S. Environmental Protection Agency's (USEPAs) Whole Effluent Toxicity Testing

Program (USEPA, 2002; Brander et al., 2012a). The establishment as alternative model species in estuaries was favored, because M. *beryllina* belongs to the phylogenetic group of family Atherinidae, which are showing particular sensitiveness to toxicants and are important members of estuary food webs in the east and west of the North American continent (Clark et al., 1985). Furthermore, it has been demonstrated that inland silversides are affected by endocrine disrupting compounds (EDCs). Thus, a change in sex ratio was detected when exposed to estrogen (Duffy et al., 2009), biomarkers have been developed to assess the influences of EDCs (Brander et al., 2012a; Brander et al., 2012b) and environmental relevant concentrations of bifenthrin interfere with metabolic processes and endocrine signaling (Brander et al., 2016b). Furthermore, a number of transcriptomic assessments have been recently conducted in inland silversides, providing a suite of genetic sequences, elementary for molecular assessments in this species (Jeffries et al., 2015a; Brander et al., 2016b)

1.5.3 Delta smelt (*Hypomesus transpacificus*)

Another resident in estuaries of the North American Westcoast is the delta smelt (*Hypomesus transpacificus*), a pelagic fish endemic to the San Francisco Estuary and Sacramento-San Joaquin Delta (Frank et al., 2017b). Once the delta smelt has been one of the most widespread fish in its habitat (Erkkila et al., 1950; Radtke, 1966; Stevens and Miller, 1983), but has decreased significantly since the 1980s and even more rapid since the early 2000s (Moyle et al., 1992; Moyle, 2002; Feyrer et al., 2007; Sommer et al., 2007). Today *H. transpacificus* developed into a species of conservation concern, listed as threatened and endangered under the Federal and California Endangered Species Acts (CESA), respectively (USFWS, 1993; CDFW, 2017). Because of their continuous decrease, delta smelts are now representatives for the species decline in the Sacramento-San Joaquin Delta and used as an indicator for ecosystem health (Feyrer et al., 2007; Connon et al., 2011a). To support the conservation of delta smelt, specific culture techniques and a breeding program have been initiated to genetically manage and monitor a refuge population (Mager et al., 2003; Baskerville-Bridges B et al., 2005; Fisch et al., 2012). The breeding program of delta smelt further provides a supply of specimens to support research programs (Lindberg et al., 2013), with the goal to better understand species decline in strongly anthropogenic influenced habitats, such as deltas in general and in particular the San Francisco Estuary and Delta (Nichols et al., 1986; Lotze et al., 2006). Recent microarray studies have contributed to install delta smelt as model species for molecular assessments (Connon et al., 2009; Connon et al., 2011a; Connon et al., 2011b; Hasenbein et al., 2014; Jeffries et al., 2015b; Komoroske et al., 2015), with the goal to better understand effects of different abiotic and biotic factors in this vulnerable fish species.

1.6 Objectives and Hypotheses

A major challenge in environmental risk assessment is the evaluation of a growing numbers of abiotic and biotic stressors in representative model species. The overall goal of this study was therefore the establishment of new screening methods to evaluate chemical-induced adverse effects in fish models, as well as the development of a screening method to monitor fish health in aquaculture and the wild. The focus in all assessments presented herein is the use of conserved signaling mechanisms to a) provide experimental setups easily transferable to other fish species and b) to obtain results in fish models applicable to predict stressor induced adverse effects in other species, including humans.

1.6.1 Baseline transcription of mTOR and RyR-dependent calcium signaling in developing zebrafish

Undisrupted transcription in signaling molecules of mTOR and RyR-dependent Ca^{2+} signaling pathways in developing zebrafish was described in this chapter. Furthermore, adverse effects caused by developmental exposure to nanomolar concentrations of PCB 95 were assessed in both pathways.

Hypothesis 1.1: Exposure to PCB 95 alters similar transcripts in in zebrafish and rodent models.

1.6.2 Effects of developmental exposure to nanomolar concentrations of bifenthrin in zebrafish

This study evaluates neurotoxic effects caused by developmental exposure to environmental relevant concentrations of the pyrethroid bifenthrin in zebrafish, with a focus on long-term or delayed effects.

Hypothesis 2.1: Nanomolar concentrations will elicit adverse effects in signaling molecules of mTOR and RyR-dependent Ca^{2+} pathways in a dose dependent manner.

Hypothesis 2.2: Comparable dose-response results in transcriptomic and behavioral assessments, with higher sensitivity on molecular level.

1.6.3 Bifenthrin exposure effects early development in inland silversides

The transferability of the screening method developed in zebrafish to inland silverside was tested in this chapter.

Hypothesis 3.1: Effects in behavior will become most evident at higher exposure concentrations, but mechanistic effects at the molecular level will also be observed at lower concentrations.

Hypothesis 3.2: Results observed in inland silversides will be similar to effects described in the model species zebrafish

1.6.4 Molecular response after pathogen infestation in delta smelt

Molecular tools were developed to measure different infection severities of the ciliate *Ichthyophthirius multifiliis* in delta smelt (*Hypomesus transpacificus*), by examining different tissues.

Hypothesis 4.1: The evaluation of an *Ich*-infection will result in tissue-specific transcriptional responses that are dependent on the severity of infection.

2. Baseline transcription of mTOR and RyR-dependent calcium signaling in developing zebrafish

A similar version of this chapter was published:

Frank, D.F., Miller, G.W., Connon, R.E., Geist, J., Lein, P.J., 2017. Transcriptomic profiling of mTOR and ryanodine receptor signaling molecules in developing zebrafish in the absence and presence of PCB 95. PeerJ. 5, e4106.

Abstract

The mechanistic target of rapamycin (mTOR) and ryanodine receptor (RyR) signaling pathways regulate fundamental processes of neurodevelopment, and genetic mutations within these pathways have been linked to neurodevelopmental disorders. While previous studies have established that these signaling molecules are expressed in developing zebrafish, a detailed characterization of the ontogenetic profile of these signaling molecules is lacking. Thus, we evaluated the spatiotemporal expression of key transcripts in mTOR and RyR signaling pathways in wildtype zebrafish at 24, 72 and 120 hours post fertilization (hpf). We further determined whether transcriptional profiles of a subset of genes in both pathways were altered by exposure to PCB 95 (2,2',3,5',6-pentachlorobiphenyl), a pervasive environmental contaminant known to cause developmental neurotoxicity in mammalian systems via RyR-dependent mechanisms. Quantitative PCR revealed that transcription generally increased across development. Genes in the signaling pathway upstream of the mTORC1 complex, and the RyR-paralogs, ryr2a and ryr3, were robustly upregulated, and in situ hybridization of ryr3 coincided with a transcriptional shift from muscle to neuronal tissue after 24 hpf. Static waterborne exposure to PCB 95 beginning at 6 hpf significantly altered transcription of genes in both pathways. These changes were concentration- and time-dependent, and included downregulation of rptor, a member of the mTORC1 complex, at both 72 and 120 hpf, and increased transcript levels of the RyR paralog ryr2b and downstream target of RyR signaling, Wingless-type 2ba (wnt2ba) at 72 hpf. The detailed transcriptomic profiling of key genes within these two signaling pathways provides a baseline for identifying other environmental factors that modify normal spatiotemporal expression patterns of mTOR and RyR signaling pathways in the developing zebrafish, as illustrated here for PCB 95.

Introduction

Normal development of the nervous system requires the concomitant and coordinated ontogeny of specific signaling mechanisms in a temporally- and regionally-dependent manner, and perturbations of either the temporal or quantitative aspects of any of these signaling events have been associated with altered patterns of neuronal connectivity, which are thought to underlie many neurodevelopmental disorders (NDDs) (Stamou et al., 2013). There is currently a significant interest in identifying chemicals that interact with signaling pathways implicated in the pathogenesis of NDDs in order to identify potential environmental risk factors for NDDs (Lyall et al., 2016b; Stamou et al., 2013). Larval zebrafish (Danio rerio) may be a particularly useful model for this purpose since zebrafish express homologs for >70 % of human genes (Howe et al., 2013), and both the major stages of neurodevelopment and the signaling molecules that regulate neurodevelopment are highly conserved between zebrafish and humans (Gilbert, 2010). Moreover, larval zebrafish have proven to be a powerful model system for screening chemicals for potential neurotoxicity and therapeutic efficacy (Brady et al., 2016; Garcia et al., 2016; MacRae and Peterson, 2015).

Two intracellular signaling pathways known to be critically important in the development of the nervous system are the mechanistic target of rapamycin (mTOR)-dependent and ryanodine receptor (RyR)-regulated signaling systems (Costa-Mattioli and Monteggia, 2013; Pessah et al., 2010). mTOR, a serine-threonine kinase, is conserved throughout the eukaryotic kingdom, and mTOR-dependent signaling pathways are critically important for integrating and controlling diverse cellular functions throughout life (Sarbassov et al., 2005; Wullschleger et al., 2006). In the developing nervous system, mTOR signaling regulates early neurodevelopmental processes of cell growth and proliferation, as well as later stages of neurodevelopment, such as dendritic outgrowth and synaptogenesis (Kumar et al., 2005; Lee et al., 2011). RyRs, which are expressed in all eukaryotes (Mackrill, 2012), represent a family of calcium-induced calcium release channels located in the endoplasmic reticulum (ER) of neuronal cells, where they function to regulate the release of calcium from internal stores (Pessah et al., 2010). RyR activity is important in calcium-dependent signaling pathways that regulate neuronal development and function (Pessah et al., 2010). Genetic mutations in either mTOR (Costa-Mattioli and Monteggia, 2013; Wang and Doering, 2013) or RyR (Matsuo et al., 2009; Soueid et al., 2016; Stamou et al., 2013) signaling pathways have been linked to NDDs.

Polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants that pose a significant risk to human health. Despite being banned from production in the late 1970s, environmental levels have not decreased significantly over the past decade (Martinez and Hornbuckle, 2011; Martinez et

al., 2012). A primary endpoint of concern for human exposure to PCBs is developmental neurotoxicity. Multiple epidemiological studies have demonstrated an association between PCB exposures in utero or during infancy and neurological deficits in children (Berghuis et al., 2014; Berghuis et al., 2015; Korrick and Sagiv, 2008; Sagiv et al., 2012; Schantz et al., 2003; Winneke, 2011), and more recently, PCBs have been identified as possible risk factors for neurodevelopmental disorders (NDDs), such as autism spectrum disorder (ASD), attention deficit and hyperactivity disorder (ADHD) and intellectual disability (Caspersen et al., 2016; Eubig et al., 2010; Lyall et al., 2016a; Neugebauer et al., 2015; Nowack et al., 2015; Sealey et al., 2016). Non-dioxin-like PCB congeners are in particular associated with developmental neurotoxicity (Pessah et al., 2010; Stamou et al., 2013), and of these, PCB 95 (2,2',3,5',6-pentachlorobiphenyl) has been shown to disrupt normal patterns of neuronal connectivity in mammalian systems via RyR (Pessah et al., 2010; Wayman et al., 2012a; Wayman et al., 2012b; Yang et al., 2009) and mTOR-dependent mechanisms (Miller and Lein, personal communication).

The goal of this study was to characterize the ontogenetic expression of key genes within the mTOR signaling pathway (Figure 4), as well as RyR paralogs and selected genes involved in the regulation of RyR activity (Figure 5), such as the RyR inhibitor homer1b (Feng et al., 2008), and genes whose expression is regulated by RyR activity, such as Wingless-type 2 paralogs, wnt2ba and wnt2bb (Wayman et al., 2012b). We also determined transcription of ER transmembrane proteins presenilin 1 and 2 (psen1, psen2), which influence the probability and frequency of RyR opening, and triadin (trdn), which forms a complex with RyRs to modulate channel opening depending on luminal Ca2+ status (Györke et al., 2004; Payne et al., 2015). Finally, we investigated transcription of two types of voltage dependent L-type calcium channels (cacna1c, cacna1sa), which regulate entry of extracellular calcium, thereby activating RyR to trigger calcium release from internal stores (Lipscombe et al., 2013). Expression of these genes was examined at 24, 72 and 120 hours post fertilization (hpf), which correspond to periods of early and late neurodevelopment in the zebrafish nervous system. In addition, we selected a subset of genes in both pathways (identified in bold font in Tables 1 and 2) to examine transcription following exposure of developing zebrafish to varying concentrations of PCB 95.

Material & Methods

Chemicals

2,2',3,5',6-Pentachlorobiphenyl (PCB 95; 99.7 % purity) was purchased from AccuStandard (New Haven, CT, USA).

Fish husbandry and spawning

Fish husbandry, spawning and all research involving zebrafish were performed in accordance with UC Davis Institutional Animal Care and Use Committee (IACUC) protocol #17645. Adult wild-type, tropical 5D zebrafish (Danio rerio) were kept in 2 L tanks at a density of ten to fourteen fish at 28.5 ± 0.5°C under a 14 h light : 10 h dark cycle. Culture water pH was kept in the range of 7.2 to 7.8, and electric conductivity between 600 and 800 µS cm-1. Adult fish were fed twice a day with Artemia nauplii (INVE Aquaculture, Inc., Salt Lake City, UT, USA) and commercial flake (a combination of Zebrafish Select Diet, Aquaneering, San Diego, CA, USA and Golden Pearls, Artemia International LLC, Fairview, TX, USA). Embryos were obtained by spawning groups of eight to twelve fish in a 1:2 female/male ratio. Spawning time was coordinated using a barrier to separate male and female fish, which was removed in the morning after the lights turned on, thus producing age-matched fertilized eggs, which were collected within 20 min of spawning.

Quantitative polymerase chain reaction (qPCR)

For the initial studies of the normal ontogenetic profiles of transcription of mTOR and RyR signaling molecules, embryos were directly transferred into 100 x 20 mm polystyrene tissue culture dishes (Corning Inc.) containing 60 mL standardized Embryo Medium (Westerfield, 2000) and placed into an incubator at a constant temperature of 28.5 ± 1°C, with a 14 h light: 10 h dark photoperiod. Fish were maintained at a density of 50 individuals per petri dish, and RNA was extracted at three different time points (24, 72 and 120 hpf). Fish from three independent spawns were used to obtain three biological replicates at each time point. To minimize variability due to differences in spawning time, all embryos were collected within a 20 min spawning window. Fish within each spawn were pooled into batches of 350 or 500 to extract sufficient amounts of RNA for qPCR analyses. The first biological replicate represented a pooled sample of 500 embryos in order to collect a sufficient amount of RNA for method validation. Embryo numbers were chosen using a conservative approach of assessing average population transcription of target genes, rather than gene expression from single individuals. We

quantified baseline transcription of 36 genes, 23 associated with the mTOR signaling pathway (Table 1) and 13 associated with RyR-dependent Ca2+ signaling (Table 2).

Transcript levels of target genes were assessed by qPCR using gene-specific primers derived from mRNA sequences obtained from the Zebrafish Model Organism Database (http://zfin.org/). Primers were designed using NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and obtained from Integrated DNA Technology (Integrated DNA Technologies, Inc., Coralville, IA, USA).

RNA extractions were performed with a Qiagen Qiacube robotic workstation using RNeasy Mini Kit spin columns (Qiagen, Valencia, CA, USA) as per the manufacturer's directions. RNA concentration and quality were determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Samples were deemed to be of sufficient quality if 260/280 and 260/230 ratios ranged between 2.05 to 2.16 and 1.82 to 2.24, respectively. Total RNA integrity was verified using an RNA 6000 Nano Kit on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, 95051) (Appendix Figure S1). The RNA Integrity Ratio (RIN) scores were calculated, and RNA was deemed to be of good quality over a RIN score of 8 (Supplemental Table S1).

Complementary DNA (cDNA) was synthesized using 1 µg 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 1:5 ratio to produce acceptable concentrations for quantitative PCR evaluations. Success of the cDNA-synthesis was tested using beta-actin primers with a 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) visualized through gel electrophoresis.

qPCR was conducted using Power SYBR Green PCR Master Mix (Life-technologies, Carlsbad, CA, USA). Primer validation was performed using a seven point standard curve with three replicates; amplification efficiencies ranged between 90.1 and 108.7 %. 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 was calculated using the Log2−ΔΔCT method (Livak and Schmittgen, 2001) relative to the reference genes elongation factor 1 alpha (*eef1a1*) and beta actin 2 (*actb*) (Table 2), which sustained best scores in GeNorme (Vandesompele et al., 2002). All data were normalized to 24 hpf samples. To verify primer quality,

sequences obtained from NCBI were checked additionally in Ensembl genome browser (http://www.ensembl.org/index.html) to identify chromosome and exon location (Table 1 and 2).

Table 1. Genes from mTOR signaling selected for transcriptomic analysis. Transcripts indicated in bold font were quantified in the PCB 95 exposure studies.

Gene name	Gene code	Primer (5'->3')	Accession #	Efficiency %	Amplicon lengths	Exon	Chromosome
Target genes							
Insulin receptor substrate 1	*irs1*	**F:** GCTCAGTGCCTATGCCAGTA **R:** AAGCAGCGGCGGATTTTTAC	XM_682610	91.2	99	1-2	15
V-akt murine thymoma viral oncogene homolog 1	*akt1*	**F:** TAAGGAGCGACCGCAAGATG **R:** TGCAGGCAGCGTATGATGAA	NM_001281801	91.9	123	1-4	17
Tuberous sclerosis 1a	*tsc1a*	**F:** TCACGACACCCATGGGAAAG **R:** TGCAGGCACAAGACCTTTCAA	NM_200052	96.9	88	14-16	5
Tuberous sclerosis 2	*tsc2*	**F:** AGTATGACGTGGCTGGTTGG **R:** TCTTTGGTCTGTCGGGTGTG	XM_009294973	91.3	155	24-25	1
Ras homolog enriched in brain	*rheb*	**F:** TTGGACATGGTGGGGAAAGT **R:** TTCACAGCTGATCACTCGCT	NM_200729	95.9	87	1-3	24
mTOR associated protein	***mlst8***	**F:** ACATGCTCTGCTGACCAGAC **R:** CACAATCCCACATCCAGCCT	NM_199877	101.5	121	7	12
Mechanistic target of Rapamycin	***mtor***	**F:** ATGGTCACTGGCCTGAAGTG **R:** GTGCACGTGGCGTATCAATC	NM_001077211	102.1	117	41-42	8
Rptor regulatory associated protein of MTOR	***rptor***	**F:** TTCATCAAGCTGGCGGATCTC **R:** CATCTTCCTGGTGCGTGGA	XM_005157354	96.5	170	23-24	6
Rptor independent companion of mTOR, complex 2a	***rictora***	**F:** ATCTGATCCGTGACAGCAGC **R:** CCAATCGGAGGGCTTGAGTT	XM_009301197	102.2	123	5-6	5
eukaryotic translation initiation factor 4E binding protein 1	***eif4ebp1***	**F:** ACATGGGGGACGTTTTCACA **R:** GGAGTTGGATTTCCCCCACA	NM_199645	102.4	149	4	21
p70 ribosomal S6 kinase a (*p70s 6ka*)	rps6kb1a	**F:** ACAGCCCTGATGACACGAAG **R:** TTCTTGGGCTTCCCAGAACC	XM_685634	101.8	153	13-14	10
p70 ribosomal S6 kinase b (*p70s 6kb*)	rps6kb1b	**F:** TGACTGATTTCGGGCTGTGT **R:** CGATTGTGTCCGCTCCTCAT	NM_213076	94.9	115	8-9	15
Eukaryotic translation initiation factor 4e1a	*eif4e1a*	**F:** TCGTATTGCAGCTTGAGAATGT **R:** TGACATGAGGAATGTGGAACA	NM_131733	108.7	97	8	14
Eukaryotic translation initiation factor 4e1b	*eif4e1b*	**F:** GGTCAAAGAGTCATCATCTTGTATTG **R:** TCACAAAGTCTTGCATATTTCAAGTT	NM_131454	97.0	95	7	5
Eukaryotic translation initiation factor 4e1c	*eif4e1c*	**F:** TTCGGAGCCGCGAGGA **R:** AGATGAGACGCAGGTTTTCTGT	NM_001017851	91.9	170	1-3	13
Eukaryotic translation initiation factor 4, gamma 2a	*eif4g2a*	**F:** GAAAGAAGACATTACCCAGGAG **R:** CAGCCACATCAGCCACTG	NM_001014289	100.2	76	21-22	7
Eukaryotic translation initiation factor 4, gamma 2b	*eif4g2b*	**F:** GGTGCTTCTCGTTTCAGTGC **R:** TGCCGACAGTCTTGGGATA	NM_001013443	101.5	79	2-3	18
Ribosomal protein S6 kinase 1 (s6)	***rps6***	**F:** TCTGAGCCCTTACGCTTTCG **R:** TCTGTTGGGTGGGGAGATGAT	EF373681	100.9	99	1-2	1
AMP-activated, alpha 1 catalytic subunit (*ampk*)	prkaa1	**F:** AGAGCAGGCCGAATGACATC **R:** GACTGGGTTCTTCCTTCGCA	NM_001110286	90.6	113	8-9	5
serine/threonine kinase 11 (*lkb1*)	stk11	**F:** CACATGGATGCAGTTCCCGTA **R:** ACTGGAGATCCGGACATCTCT	NM_001017839	93.3	70	2	2
Mitogen-activated protein kinase 3 (*erk1*)	mapk3	**F:** TGACCGACTCCTCTTCGACT **R:** CGATTCCGCCATCGTTTCTG	NM_201507	102.7	158	1	3
Mitogen-activated protein kinase 1 (*erk2*)	mapk1	**F:** GCTTTTCGCCGTGTTTCACT **R:** CTGAAGCTGCCTTCACAAGC	NM_182888	90.1	92	1	5
Ribosomal protein S6 kinase a, polypeptide 1 (*rsk*)	rps6ka1	**F:** AATGCAGTTGTGGACCGGAT **R:** TCAGCCAATCAGCTCCCTTG	NM_001077775	92.4	166	8	17

Table 2. Genes from RyR signaling selected for transcriptomic analysis and in situ analysis. Transcripts indicated in bold font were quantified in the PCB 95 exposure studies.

Gene name	Gene code	Primer (5'->3')	Accession #	Efficiency %	Amplicon lengths	Exon	Chromosome
Reference genes							
Beta-actin	*actb*	**F:** AAGCAGGAGTACGATGAGTC **R:** TGGAGTCCTCAGATGCATTG	NM_181601	101.7	238	6	3
Elongation factor 1 alpha	*eef1a1*	**F:** GATGCACCACGAGTCTCTGA **R:** TGATGACCTGAGCGTTGAAG	NM_131263	99.1	158	6	19
Target genes							
Ryanodine receptor 1a	***ryr1a***	**F:** TCCTGCTACCGAATCATGTG **R:** GCCTCTCTGCATGTGGAGTT	XM_009305502	97.0	67	67-68	10
Ryanodine receptor 1b	***ryr1b***	**F:** AAGAAATCGGGCATCTGGCA **R:** TGAGTTGTTCGGAGGTGACG	NM_001102571	90.6	157	55	18
Ryanodine receptor 2a	***ryr2a***	**F:** AGGACTCAAGCCAAATCGAG **R:** TCACGACCATGTCCTTCTGA	XM_009300703	107.0	63	6-7	12
Ryanodine receptor 2b	***ryr2b***	**F:** TCCTTTAGTCATTTTTAAGCGAGA **R:** GTCCATCGAACTCCAGTTTACG	XM_017351708	97.9	59	93	17
Ryanodine receptor 3	***ryr3***	**F:** TCTTCGCTGCTCATTTGTTG **R:** AGACAGGATGGTCCTCAAGGT	AB355791	103.9	62	97	20
Homer homolog 1b	*homer1b*	**F:** GGAGGGAAAAACAACAGCAA **R:** GGGGTCTATCTGGAAGACGTG	XM_005157295	91.7	75	1-2	5
calcium channel, voltage-dependent, L type, alpha 1C subunit	*cacna1c*	**F:** ATATCTTCAGGCGGTCTGGTG **R:** GACTGTGGGAAGGAGACGTG	NM_131900	100.3	85	42-43	4
calcium channel, voltage-dependent, L type, alpha 1S subunit, a	*cacna1sa*	**F:** GATAGCAGAGCGGACAGGAC **R:** TGCATGCTGGGAAATGTGGT	NM_001146150	97.8	115	42-23	22
Presenilin 1	*psen1*	**F:** TTGACATCGGGAGCATCGTT **R:** TACAAAACACTCGCGTTTCCTT	NM_131024	90.6	180	11	17
Presenilin 2	*psen2*	**F:** TCAAATACGGCGCGAAACAC **R:** GTCCGCTCTTCTCGGTGTAG	NM_131514	91.4	111	3	1
Triadin	*trdn*	**F:** AAGGAAGCAGGCAGCCTTAG **R:** TTCTTCCGGCGCTACCAAAT	XM_009294909	94.8	135	3-4	20
Wingless-type 2ba	***wnt2ba***	**F:** AAGTACAACGCTGCTGTGGA **R:** GAACCTGCTGATCGGTCCAT	NM_182876	92.2	149	4-5	6
Wingless-type 2bb	*wnt2bb*	**F:** TTCGTCATCACCGCTGGAAT **R:** TCCACGTTTCTGTGGGTCAC	NM_001044344	92.3	191	2-3	8

Probe for *in situ* hybridization	Primer (5'->3')	Accession #	Amplicon lengths	Exon	Chromosome
Ryanodine receptor 3	**F:** CCTTGCTCTGCGTCTTTGGA **R: CATTAACCCTCACTAAAGGGAA**GCCACAACGGTGTAGAGGTAA	AB355791	601	92-97	20

Whole mount in-situ hybridization

Digoxigenin-labeled probes were prepared from 24 hpf embryonic cDNA for ryr3. We chose ryr3 because it experiences a highly significant increase in transcription between 24 and 72 hpf as well as between 24 and 120 hpf, and because of discrepancies in the published data, which describe ryr3 transcription in zebrafish prior to 24 hpf in skeletal muscle only, or predominantly in hindbrain regions at later time points (Thisse and Thisse, 2005; Wu et al., 2011). Specifically, we designed primers (Table 2) containing a T3 RNA polymerase promoter on the 5'-end of the reverse primer, thereby allowing antisense probe transcription. Procedures for embryo and larval preparation and in situ hybridization assay were performed as described previously (Thisse and Thisse, 2014), using BM purple (Roche, Basel, Switzerland) as labeling solution.

PCB 95 exposures

Embryos collected from spawning tanks were transferred to 100 x 20 mm polystyrene tissue culture dishes (Corning Inc., Corning, New York, USA) and maintained at a constant temperature of 28.5 ± 1°C. At 4 hpf, embryos were enzymatically dechorionated using 50 µL of 41 mg/ml pronase (Sigma-Aldrich, St. Louis, MO, USA) in 25 mL of culture water for a maximum of 6 min (Truong et al., 2011). Embryos were then allowed to recover for 2 h in culture water before being transferred 1 embryo per well into a 96-well plate (Falcon™, Corning Inc.) containing 100 µL of standardized Embryo Medium (Westerfield, 2007). At 6 hpf, 100 µL of 2X PCB 95 solution was added directly into each well to yield final concentrations of 0.1, 0.3, 1.0, 3.0 or 10.0 µM PCB 95; control embryos were exposed to vehicle (0.2 % DMSO). Plates were covered with Parafilm M (Bemis NA, Neenah, WI, USA) to reduce evaporation, and were then placed into an incubator at a constant temperature of 28.5 ± 1°C and a 14 h light: 10 h dark cycle until fish (n=16 per treatment in each plate) were harvested for transcriptomic assessments at 72 and 120 hpf. Embryos or larvae were pooled into batches of 12 to 16 individuals to generate sufficient RNA for qPCR analyses. Fish from three independent spawns were used to obtain three biological replicates at each time point.

Statistical Analysis

Significant differences in gene transcription at 72 and 120 hpf relative to transcription at 24 hpf were identified using one-way ANOVA with significance set at $P < 0.05$ followed by a Tukey's post hoc test. If data did not fit the ANOVA assumptions of normality, a Kruskal-Wallace test was applied ($P < 0.05$), followed by the Nemenyi-Damico-Wolfe-Dunn post hoc test. Shapiro-Wilk normality and Bartlett

tests were used to determine which algorithms are appropriate for determining significant differences between time points. R-packages “stats” (Team., 2014), “PMCMR”; pairwise multiple comparisons of mean rank sums (Pohlert, 2014) and “multcomp”; multiple components (Hothorn et al., 2014) were used to perform statistical analyses. Data from the PCB 95 exposure studies were similarly analyzed, with the different PCB exposures normalized to the solvent control.

Results

A critical first step in developing a zebrafish platform to screen for gene X environment interactions of relevance to NDDs is to establish the normal ontogenetic profile of NDD-relevant signaling molecules in the developing zebrafish. Therefore, we first characterized the ontogenetic expression profiles of 36 genes, 23 associated with the mTOR signaling pathway (Table 1) and 13 associated with RyR-dependent Ca2+ signaling (Table 2).

Ontogenetic profile of transcripts encoding mTOR signaling pathway genes

Overall, there was increased transcription of genes encoding signaling molecules upstream of mTOR between 24 and 120 hpf (Figure 6A, B). Ribosomal protein S6 kinase polypeptide 1 (rps6ka1) was significantly upregulated ($p<0.05$) at both 72 and 120 hpf relative to 24 hpf, while tuberous sclerosis 1a (tsc1a) and mitogen-activated protein kinase 1 (mapk1) were significantly upregulated ($p<0.05$) at 120 hpf relative to 24 hpf. Mitogen-activated protein kinase 3 (mapk3) and insulin receptor substrate 1 (irs1) remained constant across all time points, and V-akt murine thymoma viral oncogene homolog 1 (akt1) showed a declining trend in relative expression over time. All other upstream targets of the mTOR complex exhibited a general increased transcription from 24 to 120 hpf that was not statistically significant.

There were no significant differences in transcription of genes within the mTORC1 complex from 24 to 72 or 120 hpf (Figure 6C). However, relative to transcript levels at 24 hpf, transcription of raptor-independent companion of mTOR (rictora) decreased at 72 hpf but was significantly upregulated from 72 to 120 hpf. Transcription of genes encoding signaling molecules downstream of mTOR generally decreased with increasing hpf, but none of these changes were statistically significant (Figure 6D, E, F). The one exception was the p70 ribosomal S6 kinase a (rps6kb1a), which showed the tendency of increased transcription at 72 hpf, although this change was not statistically significant.

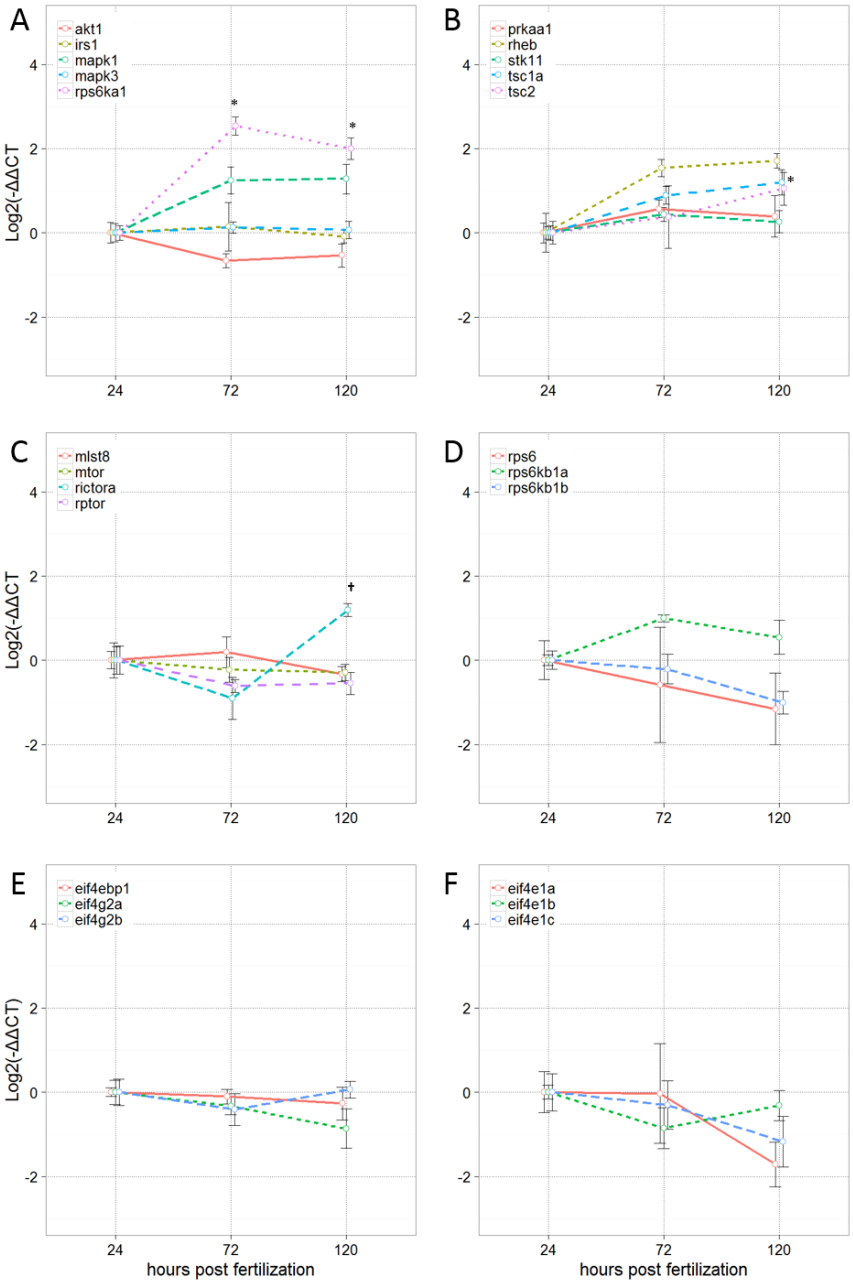

Figure 6. Relative expression of transcripts encoding mTOR signaling molecules at 24, 72 and 120 hpf. (A, B) Signaling molecules upstream of mTOR; (C) mTOR Complex 1 and 2; (D, E, F) signaling molecules downstream of mTOR. Data presented as the mean ± SE (n=3 independent biological replicates). Within each sample, values for the target transcript were normalized to the average of the values for the housekeeping genes, *actb* and *eef1a1*, within that same sample. *Significantly different from 24 hpf at P<0.05; †significantly different from 72 hpf at P<0.05 as determined by one-way ANOVA followed by Tukey's post hoc test, or if data did not meet the ANOVA assumptions, as determined by the Kruskal-Wallace test followed by a Nemenyi-Damico-Wolfe-Dunn post hoc test.

Baseline transcription of genes involved in RyR-dependent signaling

Zebrafish express five RyR-paralogs (Wu et al., 2011): ryr1a and ryr1b are transcribed predominantly in slow and fast twitch muscle tissue, respectively (Hirata et al., 2007); ryr2a is predominantly expressed in the CNS, ryr2b, in the heart of developing zebrafish (Wu et al., 2011); and ryr3 has been detected in zebrafish skeletal muscle prior to 24 hpf (Wu et al., 2011) but is reported to be expressed predominantly in hindbrain regions at later developmental stages (Thisse and Thisse, 2005). Transcripts of ryr2a and ryr3 were significantly elevated at 72 and 120 hpf, whereas relative expression of ryr1a, ryr1b and ryr2b did not change significantly over the 120 hpf assessment period (Figure 7A). Transcription of wnt2ba increased in a time-dependent manner with a significant increase noted at 120 hpf, whereas wnt2bb did not change significantly over the 120 hpf assessment (Figure 7B).

Significant increases in gene transcription were observed for Homer homolog 1b (homer 1b) and the alpha 1c subunit of the voltage-dependent L type calcium channel (cacna1c) at 72 and 120 hpf. Transcript levels of the alpha 1S subunit 1 of the voltage-dependent L type calcium channel (cacna1sa) were significantly increased at 72 hpf (Figure 7C). Transcription of psen1 and psen2 was elevated at 72 hpf, but this change was not significant (Figure 7C), whereas genes involved in regulating RyR-dependent signaling (like trdn) remained relatively consistent across the three time points evaluated in this study.

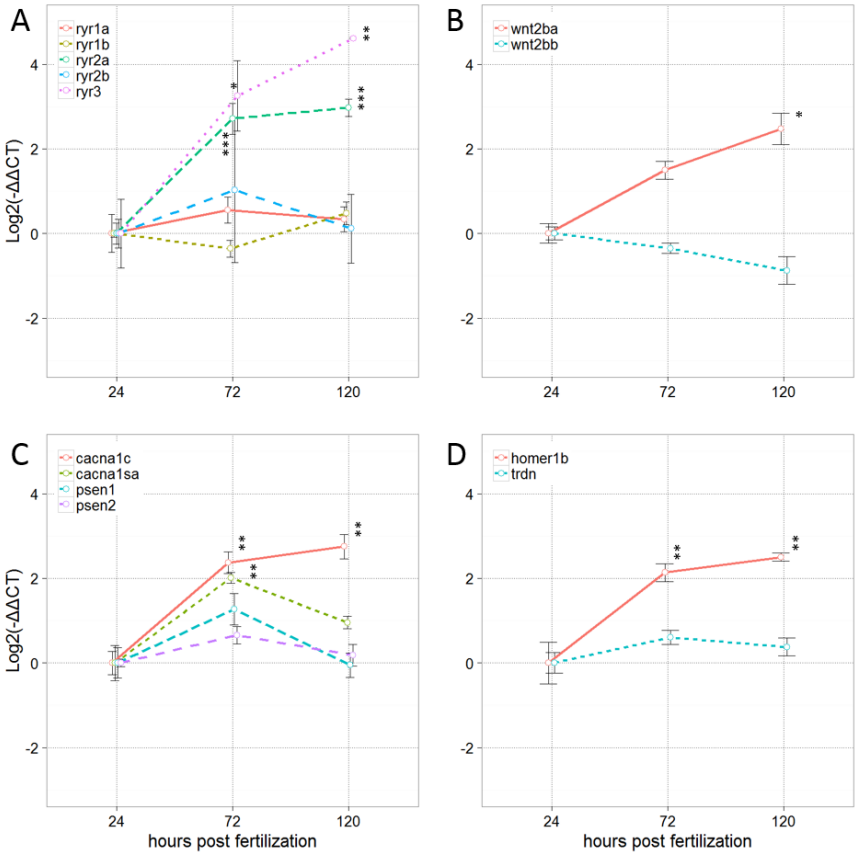

Figure 7. Relative expression of transcripts encoding ryanodine receptor (RyR) paralogs and regulatory molecules at 24, 72 and 120 hpf. (A) Zebrafish RyR paralogs; (B) Wnt2 orthologs; (C, D) RyR regulatory molecules. Data are shown as the mean ± SE (n=3 independent biological replicates). Within each sample, values for the target transcript were normalized to the average of the values for the housekeeping genes, *actb* and *eef1a1*, within that same sample. *Significantly different from 24 hpf at P<0.05, ** P<0.01, *** P<0.001, as determined by one-way ANOVA followed by Tukey's post-hoc test, or if data did not meet the ANOVA assumptions, as determined by the Kruskal-Wallace test followed by the Nemenyi-Damico-Wolfe-Dunn post-hoc test.

In-situ hybridization of ryr3

Since ryr3 is expressed in both the skeletal muscle and brain at different developmental stages (Thisse and Thisse, 2005; Wu et al., 2011), to determine whether increased transcripts of ryr3 detected by qPCR reflect ryr3 upregulation in the central nervous system (CNS), we examined its expression by in situ hybridization. At 24 hpf, ryr3 transcription was observed only in skeletal muscle (Figure 8A). However, by 26 hpf, ryr3 transcripts were detected in brain tissue (Figure 8B, C). By 72 hpf, ryr3 was abundantly expressed in the brain (Figure 8D).

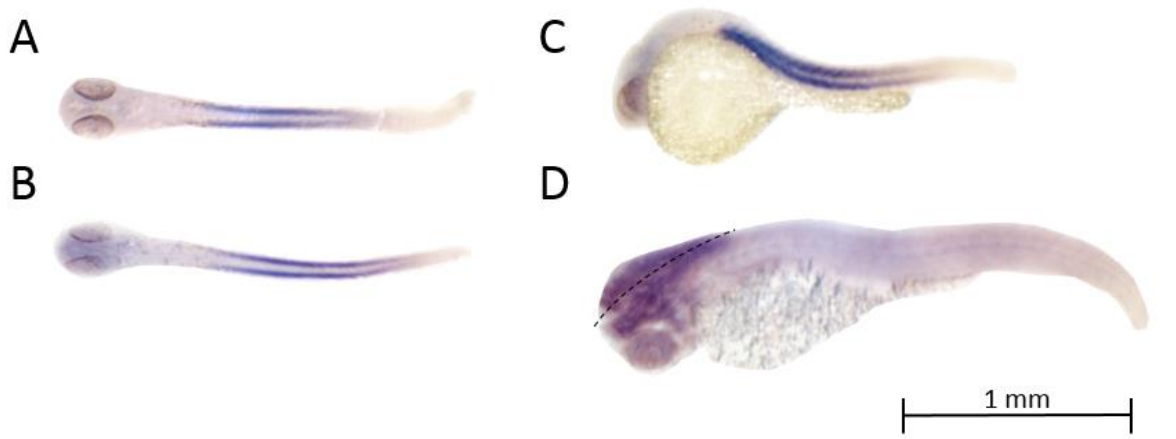

Figure 8. Spatial expression patterns of ryr3 transcripts as determined by in situ hybridization. To obtain dorsal views, the yolk sac was removed prior to imaging. (A) Dorsal view of ryr3 expression at 24 hpf; expression is predominantly in fast twitch muscles and somites. (B) Dorsal view of ryr3 expression at 26 hpf; in addition to expression in fast twitch muscles and somites; ryr3 mRNA is expressed in the hindbrain and telencephalon. (C) Lateral view of ryr3 expression at 26 hpf. Transcripts for ryr3 are present in the whole organism with higher intensity in the telencephalon and habenula. (D) Dorsolateral view of ryr3 expression at 72 hpf, to specially highlight expression in the brain. Transcripts for ryr3 are detected in fast twitch muscles and somites, but are more abundantly expressed in the hindbrain and telencephalon. The dotted line highlights the mid-line of the brain (from a dorsal view).

Transcriptional effects of PCB 95

The influence of developmental PCB 95 exposure on transcription of NDD-relevant genes was tested on 6 genes associated with the mTOR signaling pathway (Table 1; genes in bold font) and 6 associated with RyR-dependent Ca2+ signaling (Table 2; gene in bold font). The mTORC1 member, rptor, was significantly downregulated in PCB 95-exposed fish at both 72 and 120 hpf (Figure 9B). At both time points, the effect of PCB 95 was concentration-dependent. At 72 hpf, the fold-change progressively decreased with increasing concentrations of PCB 95, with statistical significance reached at the highest PCB 95 concentration of 10 µM. At 120 hpf, statistically significant downregulation was observed at 3.0 and 10.0 µM. The other five genes of the mTOR signaling pathway that we examined were not significantly altered at 72 or 120 hpf in fish exposed to PCB 95 at any of the concentrations tested in this study (Figure 9 A, C- F). Within the subset of genes relevant to RyR signaling that we examined (Figure 9 G-L), two were found to be significantly upregulated by PCB 95 exposure but interestingly, only at 72 hpf (Figure 9 K, L). Transcription of ryr2b and wnt2ba showed a concentration-dependent increase, which reached statistical significance at 10 µM PCB 95.

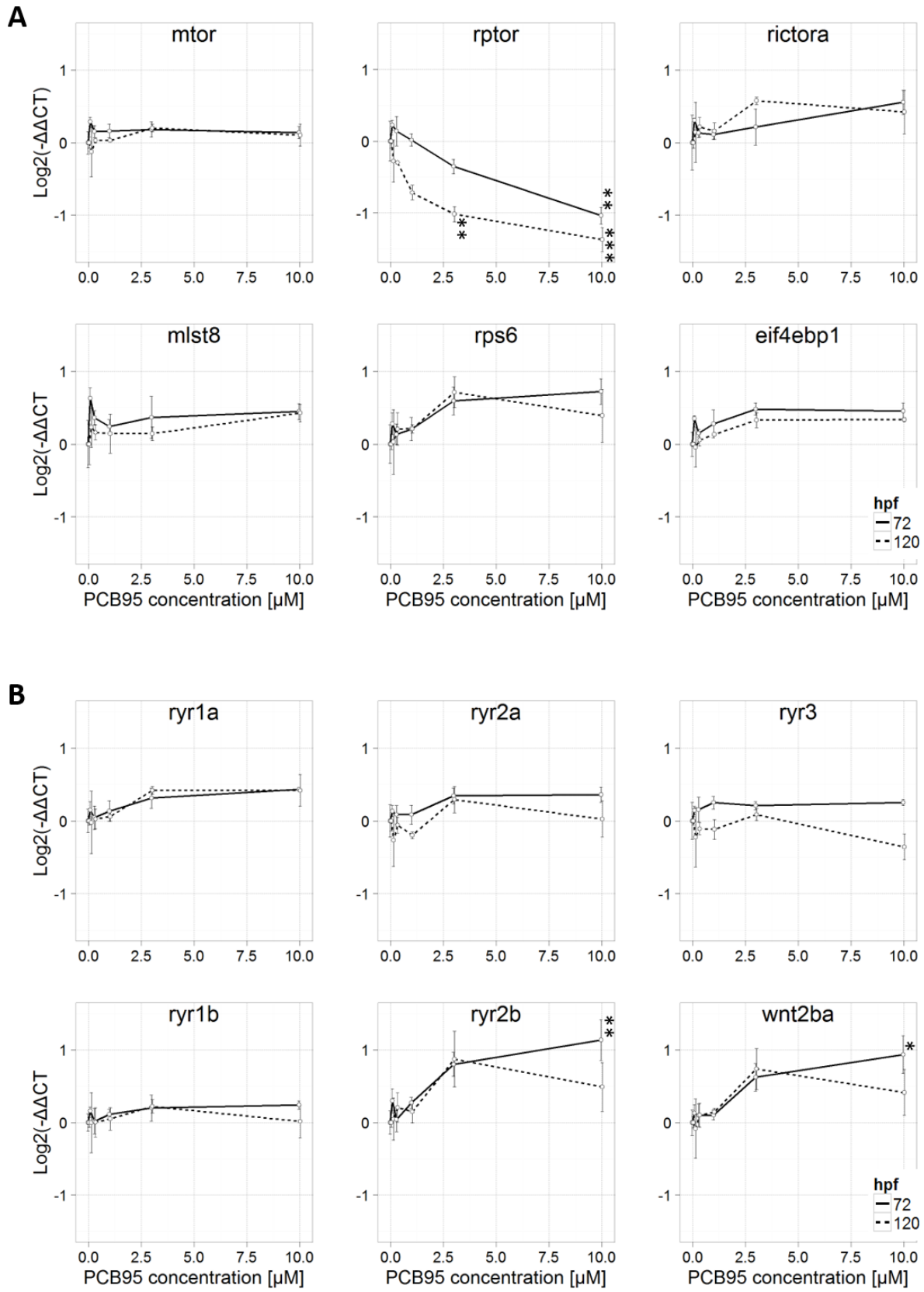

Figure 9. Concentration-dependent effects of PCB 95 on transcription of mTOR and ryanodine receptor (RyR) signaling pathways at 72 and 120 hpf. Fold-change in transcription of genes coding for (A-F) mTOR signaling molecules and (G-L) RyR paralogs and Wingless-type 2ba. The solid line depicts concentration-dependent effects of PCB 95 at 72 hpf; the dashed line,

120 hpf. Data are shown as the mean ± SE (n=3 independent biological replicates). Within each sample, values for the target transcript were normalized to the average of the values for the housekeeping genes, *actb* and *eef1a1*, within that same sample. Significant differences from vehicle controls at the same time point are identified by *P < 0.05, **P < 0.01, ***P < 0.001 as determined by one-way ANOVA followed by Tukey's post-hoc test, or if data did not meet the ANOVA assumptions, as determined by the Kruskal-Wallace test followed by the Nemenyi-Damico-Wolfe-Dunn post-hoc test.

Discussion

This study provides the most comprehensive characterization to date of the ontogenetic profile in developing zebrafish of 36 transcripts encoding molecules involved in mTOR and RyR signaling pathways. All 23 genes examined in the mTOR signaling cascade were detected at the mRNA level at all three developmental time points. However, only four were observed to be differentially regulated during the first 120 hpf: rps6ka1, mapk1 and the mTORC2 complex member rictora, all of which encode molecules that promote mTOR signaling (Anjum and Blenis, 2008; Guertin et al., 2006), and tsc1a, which encodes the tumor sclerosis complex protein, hamartin that inhibits mTOR signaling. All four genes were significantly upregulated, but at differing developmental stages: rps6ka1 and mapk1 were upregulated at 72 hpf; rictora and tsc1a, at 120 hpf. However, transcript levels for the majority of mTOR-related genes were relatively stable throughout the first five days of development, suggesting that chemical perturbation of transcription of these genes may have significant adverse impacts.

Consistent with previous studies (Wu et al., 2011), we observed that mRNA expression of the muscle-specific (ryr1a and ryr1b) and heart-specific (ryr2b) RyR homologs also remained largely unchanged throughout early zebrafish development. In contrast, transcript levels of ryr2a, the CNS-specific RyR paralog, and ryr3 were significantly elevated at 72 and 120 hpf, corresponding to the peak period of synaptogenesis (Brustein et al., 2003; Saint-Amant and Drapeau, 1998). As reported by others (Wu et al., 2011), we did not detect ryr3 in the brain prior to 24 hpf as determined by in situ hybridization. However, brain expression of ryr3 was apparent in the whole mount as early as 26 hpf, and by 72 hpf, ryr3 was strongly expressed throughout the brain, and to a lesser extent in the whole body. These in situ hybridization data suggest that increased ryr3 mRNA detected by qPCR reflects upregulation of ryr3 in the CNS.

Mammals have three RyR paralogs: ryr1, ryr2, and ryr3 (Pessah et al., 2010), and dynamic changes in the spatiotemporal expression of all three paralogs have been described in the developing mouse brain (Mori et al., 2000). Unlike zebrafish, ryr1 and ryr2 transcription in developing rodents

significantly increases in cardiac and muscle tissue during the first days after birth, and become more abundant with ongoing embryogenesis (Brillantes et al., 1994; Rosemblit et al., 1999). However, similar to zebrafish, developing mice exhibit reduced ryr3 expression in muscle and increased expression in the brain with ongoing development (Bertocchini et al., 1997; Mori et al., 2000; Takeshima et al., 1996).

Several genes that encode proteins important in the regulation of RyR activity were also found to be differentially expressed in developing zebrafish. Transcription of wnt2ba and cacna1c was upregulated at 72 and 120 hpf; whereas cacna1sa transcripts were significantly increased at 72 hpf but decreased at 120 hpf. Wnt2 transcription is linked to activity-dependent dendritic outgrowth in mammalian neurons (Wayman et al., 2006), cacna1c is described in rodents as the most abundant L-type Ca2+ channel in neurons, and cacna1sa is found in rodent muscle tissue. Mutations in all three genes have been linked to NDDs (Caracci et al., 2016; Kim and State, 2014; Kwan et al., 2016; Trevarrow et al., 1990). We also observed a significant increase in homer1b transcripts, which is consistent with published data demonstrating homer1b transcription in the myotome and nervous system during early stages of development that shifts towards the brain around 48 hpf (Thisse and Thisse, 2004). Similarly, developing exhibit predominant neural transcription of homer1b with low transcript levels in other organs (Shiraishi-Yamaguchi and Furuichi, 2007).

This study also demonstrated that developmental exposure to PCB 95 significantly altered transcription of a subset of mTOR and RyR signaling molecules in larval zebrafish. PCB 95 is a developmental neurotoxicant that promotes activity-dependent dendritic growth in primary rat neurons via RyR-dependent upregulation of wnt2 transcription (Wayman et al., 2012a; Wayman et al., 2012b) and activation of mTOR-dependent translational mechanisms (Miller and Lein, personal communication). PCB 95 caused a time- and concentration-dependent downregulation of rptor and upregulation of ryr2b and wnt2ba. These effects are not likely to be secondary consequences of decreased viability of fish exposed to PCB 95 because: (1) separate studies have observed no morbidity or teratogenic effects in zebrafish exposed to PCB 95 at 10 µM from 6 to 120 hpf (Miller and Lein, personal communication); and (2) the majority of genes (9 of 12) examined were not significantly altered by PCB 95 exposure. These findings are consistent with a recent report that ryr2 transcripts are upregulated in developing Atlantic killifish (Fundulus heteroclitus) from New Bedford Harbor that are exposed to high levels of non-dioxin-like PCBs (Fritsch et al., 2015). However, mtor transcripts were also found to be upregulated in the New Bedford Harbor killifish but not in our PCB 95-exposed zebrafish. This difference likely reflects species-dependent responses and/or differences in the exposures (complex environmental exposures vs. defined exposure to a single PCB congener).

The functional relevance of PCB 95 effects on transcription of mTOR and RyR signaling molecules in developing zebrafish remains to be determined. Experimental studies have shown that functional knockout of raptor significantly reduces dendritogenesis in the mammalian brain (Cloëtta et al., 2013; Urbanska et al., 2012), which is the opposite of PCB 95 effects on dendritic arborization. This suggests that PCB 95 downregulation of rptor is not causally related to PCB 95 effects on dendritic growth. Similarly, upregulation of ryr2b is likely not involved in the dendrite promoting activity of PCB 95 because the expression of this RyR paralog is limited to cardiac tissue (Wu et al., 2011). However, the observation that PCB 95 upregulated expression of wnt2ba in developing zebrafish is consistent with observations of increased levels of wnt2 transcripts in rat hippocampal neurons exposed to PCB 95, an effect that was causally linked to PCB 95 effects on dendritic arborization (Wayman et al., 2012b).

Conclusion

In conclusion, this study provides fundamental data regarding the transcriptional profiles of major components of the mTOR and RyR signaling pathways during the first five days of zebrafish development. Most genes differentially regulated during development were upregulated at times corresponding to peak synaptogenesis and formation of neuronal circuits, consistent with evidence implicating a major role for both pathways in normal neurodevelopment, and in the pathogenesis of NDDs. Developmental exposure to PCB 95 altered transcription of wnt2ba, a gene implicated PCB 95 developmental neurotoxicity in mammalian models (Stamou et al., 2013; Wayman et al., 2012b), demonstrating the feasibility of using the zebrafish to screen for chemicals that modulate expression of mTOR and RyR signaling pathways to identify potential environmental risk factors and/or therapeutics of relevance to NDDs.

3. Effects of developmental exposure to nanomolar concentrations of bifenthrin in zebrafish

A similar version of this chapter was published:

Frank, D.F., Miller, G.W., Harvey, D.J., Brander, S.M., Geist, J., Connon, R.E., Lein, P.J., 2018. Bifenthrin causes transcriptomic alterations in mTOR and ryanodine receptor-dependent signaling and delayed hyperactivity in developing zebrafish (Danio rerio). Aquatic Toxicology. 200, 50-61.

Abstract

Over the last few decades, the pyrethroid insecticide bifenthrin has been increasingly employed for pest control in urban and agricultural areas, putting humans and wildlife at increased risk of exposure. Exposures to nanomolar (nM) concentrations of bifenthrin have recently been reported to alter calcium oscillations in rodent neurons. Neuronal calcium oscillations are influenced by ryanodine receptor (RyR) activity, which modulates calcium-dependent signaling cascades, including the mechanistic target of rapamycin (mTOR) signaling pathway. RyR activity and mTOR signaling play critical roles in regulating neurodevelopmental processes. However, whether environmentally relevant levels of bifenthrin alter RyR or mTOR signaling pathways to influence neurodevelopment has not been addressed. Therefore, our main objectives in this study were to examine the transcriptomic responses of genes involved in RyR and mTOR signaling pathways in zebrafish (Danio rerio) exposed to low (ng/L) concentrations of bifenthrin, and to assess the potential functional consequences by measuring locomotor responses to external stimuli. Wildtype zebrafish were exposed for one, three and five days to 1, 10 and 50 ng/L bifenthrin, followed by a 14 d recovery period. Bifenthrin elicited significant concentration-dependent transcriptional responses in the majority of genes examined in both signaling cascades, and at all time points examined during the acute exposure period (1, 3, 5 days post fertilization; dpf), and at the post recovery assessment time point (19 dpf). Changes in locomotor behavior were not evident during the acute exposure period, but were observed at 19 dpf, with main effects (increased locomotor behavior) detected in fish exposed developmentally to the bifenthrin at 1 or 10 ng/L, but not 50 ng/L. These findings illustrate significant influences of developmental exposures to low (ng/L) concentrations of bifenthrin on neurodevelopmental processes in zebrafish.

Introduction

Since the production of photostable pyrethroids in the 1970s, pyrethroid insecticides have been increasingly employed for insect control over the last four decades (Schleier III and Peterson, 2011), and they now represent the fourth largest group of insecticides used worldwide (Kuivila et al., 2012b; Brander et al., 2016a). Moreover, in the past decade, bifenthrin, a broadly used pyrethroid, has been increasingly employed in urban areas (Jiang et al., 2012; Weston and Lydy, 2012; Saillenfait et al., 2015). As a result, there is the potential for high influx of bifenthrin into surface waters, particularly following heavy rains. Bifenthrin is commonly detected in surface waters in the low ng/L range (Weston and Lydy, 2012), although concentrations as high as 106 ng/L have been measured. Additionally, bifenthrin is the most frequently detected pyrethroid in river sediments in North America and Australia (Nowell et al., 2013; Allinson et al., 2015), and it has the potential to bioaccumulate in fish tissues (Munaretto et al., 2013). The latter is of particular concern because fish are considered more vulnerable to pyrethroid toxicity than mammals (Glickman and Lech, 1982).

Pyrethroids are classified as type I and type II, as defined by the absence or presence of an α-cyano-3-phenoxybenzyl moiety, respectively (Soderlund, 2012). This difference in chemical structure results in distinctive toxicologic effects: type I pyrethroids cause tremors or convulsions, while exposure to type II pyrethroids predominantly causes choreoathetosis (Casida and Durkin, 2013). Bifenthrin is a type I pyrethroid that binds to the voltage-gated sodium channels in insects to prolong the action potential in nerves; it also interacts with voltage-gated sodium channels of vertebrates (Mukherjee et al., 2010; Soderlund, 2012). More recently, pyrethroids have also been shown to affect calcium signaling via interactions with voltage-gated calcium ion channels (Soderlund, 2012), and exposure to nanomolar concentrations of bifenthrin has been shown to dysregulate Ca^{2+} homeostasis in neurons cultured from developing rodent brain (Cao et al., 2014). Calcium signaling regulates diverse neurodevelopmental processes (Berridge et al., 2000; Lohmann, 2009), and perturbation of calcium signaling in the developing brain has been linked to deficits in neurobehavior (Gargus, 2009; Stamou et al., 2013).

Whilst there is a rich literature addressing the role of intracellular calcium signaling in fish models, particularly in goldfish (Johnson and Chang, 2002; Sawisky and Chang, 2005), the effects of bifenthrin on calcium signaling in fish has not been previously addressed. Therefore, a main objective of this study was to assess the effects of bifenthrin on the transcriptional profile of calcium-dependent signaling molecules, in particular, ryanodine receptor (RyR) and mTOR-dependent signaling molecules, in developing fish. RyRs are calcium-dependent calcium release channels embedded in the endoplasmic reticulum that regulate calcium-dependent signaling in neurons, and their function is critical to normal

neurodevelopment (Pessah et al., 2010). The mTOR signaling pathway is also critical for normal neurodevelopment (Kumar et al., 2005; Lee et al., 2011; Bowling et al., 2014; Tang et al., 2014), and it is activated by increases in intracellular calcium (Zhang et al., 2012). Both RyRs (Mackrill, 2012) and mTOR signaling molecules (Hall, 2008) are conserved throughout the eukaryotic kingdom. We recently reported transcriptional alterations in key genes of both the RyR and mTOR signaling pathways in developing zebrafish exposed to µM concentrations of polychlorinated biphenyl (PCB) 95 (Frank et al., 2017a). PCB 95 is an environmental contaminant known to interfere with neurodevelopment in mammalian systems by modulating calcium-dependent signaling via RyR-dependent mechanisms (Wayman et al., 2012b; Wayman et al., 2012a). But whether classes of environmental contaminants other than PCBs that also alter calcium influx, such as bifenthrin, similarly alter the transcriptional profile of RyR and mTOR signaling molecules, and whether such transcriptional changes are associated with altered phenotypes in fish is not known.

To address these questions, we exposed wildtype zebrafish to low (ng/L) concentrations of bifenthrin during early development. Previous studies have examined both acute (Jin et al., 2009) and developmental toxicity (Shi et al., 2011; Tu et al., 2016) of bifenthrin in zebrafish, but there has been no study examining potential neurodevelopmental effects following exposure to picomolar concentrations of pyrethroids. Since the effects of developmental exposures to neurotoxic chemicals can manifest at later life stages (Levin et al., 2003), zebrafish were assessed for transcriptional and behavioral effects immediately following the cessation of bifenthrin exposure at 5 days post-fertilization (dpf), and at 19 dpf, 14 d after exposure ended.

Material & Methods

Chemicals

Bifenthrin (CAS# 82657-04- 3, purity > 98 %) was purchased from Chem Service (West Chester, PA, USA).

Fish husbandry and spawning

All research involving zebrafish, fish husbandry and spawning were performed in accordance with UC Davis Institutional Animal Care and Use Committee (IACUC) protocol #17645. Adult wild-type, Tropical 5D zebrafish (*Danio rerio*) were kept in 2.8 L tanks at a density of 5-7 fish per L at 28.5 ± 0.5°C on a 14 h light:10 h dark cycle in a recirculating system (ZS560 in Light Enclosure, Aquaneering, San Diego, CA, USA). Culture water pH and conductivity were continually monitored, and pH values ranged

between 7.2 and 7.8; electric conductivity between 600 and 800 µS cm^{-1}. Adult fish were fed twice a day with *Artemia nauplii* (INVE Aquaculture, Inc., Salt Lake City, UT, USA) and commercial flake (a combination of Zebrafish Select Diet, Aquaneering, San Diego, CA, USA and Golden Pearls, Artemia International LLC, Fairview, TX, USA). Embryos were obtained by naturally spawning groups of eight to twelve fish in a 1:2 female/male ratio. Spawning time was coordinated using a barrier to separate male and female fish to produce age-matched fertilized eggs. Fertilized eggs were collected within 60 min of spawning.

Bifenthrin exposures and recovery period

Bifenthrin concentrations were chosen based on similar studies that evaluated the impact of bifenthrin on endocrine disruption in fish (Brander et al., 2012b; DeGroot and Brander, 2014) and were within the range of concentrations present in aquatic habitats (Weston et al., 2009; Weston and Lydy, 2012; Weston et al., 2014; Weston et al., 2015a; Weston et al., 2015b). Fish from five independent spawns obtained on different days were used to obtain five biological replicates (n=5) for each treatment. Transcriptional and behavioral responses were evaluated across four time points – 1, 3, 5, and 19 dpf – in four experimental groups: three bifenthrin exposures – 1 , 10 and 50 ng/L bifenthrin pre-dissolved in methanol (MeOH) – and a vehicle (0.01 % MeOH v/v) control group (ASTM, 2014). Exposures were performed from 2 hpf to 5 dpf, and were followed by a 14 day recovery period to 19 dpf. A total of 480 embryos per spawn were split into groups of 30, directly transferred into 16 different glass petri dishes (100 x 20 mm; 30 embryos/dish; 4 replicates per concentration, one for every investigated time point) containing 60 mL standardized Embryo Medium (Westerfield, 2007), and placed into an incubator at a constant temperature of 28.1 ± 0.7°C, with a 14 h light: 10 h dark photoperiod. Embryos remained in the glass petri dishes throughout the exposure period, and were randomly sampled for transcriptomic and behavioral evaluations. All fish were examined to exclude individual fish with obvious abnormalities. Water changes (80 %) were performed daily and physicochemical parameters remained constant throughout the tests: pH 7.6 ± 0.2, dissolved oxygen 8.3 ± 0.3 mg/L and specific conductance of 2195 ± 85 µS cm^{-1}.

At 5 dpf, 30 fish from each group were transferred into 1.8 L glass tanks (Mason jars, Erie, PA, USA), containing 1.6 L of culture water from the recirculating system of the adult fish and maintained for a 14 d recovery period until 19 dpf. Fish were fed twice a day with commercial flake from 5 to 19 dpf. Feeding was supplemented with live *Artemia nauplii* starting at 10 dpf, and 80 % water changes were performed 60 min after the second feeding took place. To rule out the possibility that differences in transcriptome and behavioral readouts in bifenthrin-exposed vs. vehicle control fish were due to differences in food consumption during the 14 d recovery period, upon completion of the behavioral

assays at 19 dpf, all fish were euthanized on ice and transferred into an aluminum dish and placed overnight in a New Brunswick incubator E24 (Eppendorf, Germany) at 70°C. The mean dry weight of fish from each exposure group (n=5) was then measured using a Mettler Toledo AL104 precision scale (Mettler Toledo, Columbus, OH, USA, sensitivity of 0.0001g ± 0.0002g). The data from this experiment is presented in Figure S2. Physicochemical parameters remained constant throughout the recovery period: pH 7.5 ± 0.1, dissolved oxygen 7.9 ± 0.2 mg/L and specific conductance 875 ± 25 µS cm^{-1}.

Transcriptomic assessments

Embryos or larval fish were pooled at each sampling time point (1, 3, 5, 19 dpf) so as to obtain sufficient amounts of RNA for transcriptomic assessments. Specifically, 20 larvae were pooled for samples taken during the acute exposure period and four larvae per replicate were pooled after the recovery period. Prior to sampling, embryos and larvae were euthanized on ice and stored in RNALater (Thermo Fisher Scientific, Waltham, MA, USA) for subsequent RNA extraction.

Total RNA extractions were performed with a Qiagen QiaCube robotic workstation using RNeasy Mini Kit spin columns (Qiagen, Valencia, CA, USA) as per manufacturer's directions. RNA concentrations and extraction efficiency were determined using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Samples were deemed to be of acceptable quality, with 260/280 and 260/230 ratios ranging from 1.98 to 2.24 and 1.78 to 2.20, 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 manufacturer's protocol. 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 at a 1:5 ratio, to produce the required concentration for quantitative PCR analyses.

We quantified transcript levels of 20 genes, 6 associated with the mTOR signaling pathway and 14 associated with RyR-dependent Ca^{2+} signaling, at three different time points (1, 3 and 5 dpf) during the exposure period and at the end of the recovery period (19 dpf). Transcript levels of target genes were assessed by qPCR using previously designed and validated primers (Frank et al., 2017a) (Table 3).

Quantitative PCR was conducted using Power SYBR Green PCR Master Mix (Life-technologies, Carlsbad, CA, USA). 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 Biosystems). GeNorm (Vandesompele et al., 2002) was used to normalize gene expression relative to the reference genes *elongation factor 1 alpha (eef1a1),* beta-actin (*actb*) and beta-macroglobulin (β2m), which sustained best stability scores.

Table 3. Gene-specific primers for RyR- dependent Ca^{2+} signaling and the mTOR pathway.

Gene name	Gene code	Primer (5'->3')	Accession #	Amplification Efficiency %
Reference genes				
Beta-actin	*actb*	**F:** AAGCAGGAGTACGATGAGTC **R:** TGGAGTCCTCAGATGCATTG	NM_181601	101.7
Beta-2-Microglobulin	*b2m*	**F:** CCACTCCGAAAGTTCATGTGT **R:** ATCTCCTTTCTCTGGGGTGAA	NM_131163.2	93.7
Elongation factor 1 alpha 1	*eef1a1*	**F:** GATGCACCACGAGTCTCTGA **R:** TGATGACCTGAGCGTTGAAG	AY422992	99.1
Target genes				
Ryanodine receptor 1a	*ryr1a*	**F:** TCCTGCTACCGAATCATGTG **R:** GCCTCTCTGCATGTGGAGTT	XM_009305502	97.0
Ryanodine receptor 1b	*ryr1b*	**F:** AAGAAATCGGGCATCTGGCA **R:** TGAGTTGTTCGGAGGTGACG	NM_001102571	90.6
Ryanodine receptor 2a	*ryr2a*	**F:** AGGACTCAAGCCAAATCGAG **R:** TCACGACCATGTCCTTCTGA	XM_009300703	107.0
Ryanodine receptor 2b	*ryr2b*	**F:** TCCTTTAGTCATTTTTAAGCGAGA **R:** GTCCATCGAACTCCAGTTTACG	XM_017351708	97.9
Ryanodine receptor 3	*ryr3*	**F:** TCTTCGCTGCTCATTTGTTG **R:** AGACAGGATGGTCCTCAAGGT	AB355791	103.9
ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	*atp2a1*	**F:** TGAAGCTTTCCTTCCCAGTCA **R:** TCTGCGTGGAGTGCTGTTTTA	BC085636	95.1
ATPase, Ca++ transporting, cardiac muscle, slow twitch 2a	*atp2a2a*	**F:** TCTTGACTTTAGTGCATGTGTTGT **R:** TCGCTCCTAATGTTTGAGCTTCT	BC045327	90.4
calcium channel, voltage-dependent, L type, alpha 1C subunit	*cacna1c*	**F:** ATATCTTCAGGCGGTCTGGTG **R:** GACTGTGGGAAGGAGACGTG	NM_131900	100.3
calcium channel, voltage-dependent, L type, alpha 1S subunit, a	*cacna1sa*	**F:** GATAGCAGAGCGGACAGGAC **R:** TGCATGCTGGGAAATGTGGT	NM_001146150	98.2
calcium channel, voltage-dependent, L type, alpha 1S subunit, b	*cacna1sb*	**F:** GCATTCGGTCCGAGAAGCTA **R:** TGACCCGACCAGGATGATCT	NM_214726	93.4
Ras homolog enriched in brain	*rheb*	**F:** TTGGACATGGTGGGGAAAGT **R:** TTCACAGCTGATCACTCGCT	NM_200729	95.9
Mechanistic target of Rapamycin	*mtor*	**F:** ATGGTCACTGGCCTGAAGTG **R:** GTGCACGTGGCGTATCAATC	NM_001077211	102.1
Raptor regulatory associated protein of MTOR	*rptor*	**F:** TTCATCAAGCTGGCGGATCTC **R:** CATCTTCCTGGTGCGTGGA	XM_005157354	96.5
RPTOR independent companion of MTOR, complex 2a	*rictora*	**F:** ATCTGATCCGTGACAGCAGC **R:** CCAATCGGAGGGCTTGAGTT	XM_009301197	102.2
Ribosomal protein S6 kinase 1	*rps6*	**F:** TCTGAGCCCTTACGCTTTCG **R:** TCTGTTGGGTGGGGAGATGAT	EF373681	100.9
eukaryotic translation initiation factor 4E binding protein 1	*eif4ebp1*	**F:** ACATGGGGGACGTTTTCACA **R:** GGAGTTGGATTTCCCCCACA	NM_199645	102.4

Locomotor behavioral assessments

All behavioral assessments were performed using the DanioVision™ high-throughput behavior system (Noldus Information Technology, Inc., Netherlands). Locomotor activity was evaluated at 3 and 5 dpf during the exposure period and post-recovery at 19 dpf. A total of 30 larvae per group (n=5 corresponding to mean data from six larvae from each of five biological replicates) were examined individually at all time points. All behavioral experiments were conducted in the early afternoon to exclude any bias due to possible intraday-dependent locomotive variation in swimming behavior (MacPhail et al., 2009). Behavioral monitoring of 5 dpf larvae was conducted in a 96-well plate (Falcon™, Corning Inc., Corning, NY, USA), each well contained one larvae and 200 µl of exposure solution. Post recovery assessments were performed in 6-well plates (Falcon™, Corning Inc., Corning, NY, USA) containing 3 ml untreated culture water. Larval fish were transferred from the glass petri dish into polystyrene multi-well plates and allowed to acclimate for 30 min before they were placed into the DanioVision™ observation chamber.

During behavioral assessments, fish were exposed to alternating light/dark stimuli, which is an accepted method for tracking photomotor responses in larval zebrafish (Cario et al., 2011). Fish were initially exposed to light (75 % intensity of the system capacity; 1800-2000 Lux) for a 5 min acclimation period, after which behavioral assessments started with an additional 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 then finally a 10 minute dark period. Light/Dark preference in zebrafish depends on the stimuli used (Blaser and Peñalosa, 2011). The selected Light/Dark regime was determined after testing different combinations of dark and light periods, durations, and illumination intensities on larvae at 3, 5, and 19 dpf (results not shown). The same behavioral tracking settings were used during locomotive behavioral assessments for fish from the recovery tests, but conducted at reduced illumination (25 % intensity of the system capacity; 650-750 Lux) because higher light intensities resulted in permanent burst swimming in 19 dpf fish (data not shown).

Response to predator cue

At 19 dpf, zebrafish were challenged with a suspension of a homogenized tissue from an untreated adult fish, prepared in 10 mL culture water (referred to as a “predator-cue” hereafter) to test behavioral response to a predator cue. This approach ensures contact between the larval fish and the alarm compound, Schreckstoff, released from fish skin after injuries, which is detected by adjacent fish and causes predator avoidance behavior (Jesuthasan and Mathuru, 2008).

Thirty larval fish from each group (6 fish per replicate, n = 5 replicates) were used to assess predatory responses. Fish were placed in 6-well plates (one individual per well) and allowed to acclimate to the plate for 30 min before being transferred into the observation chamber. Once in the Daniovision™ chamber, they had an additional 5 min acclimation before locomotor tracking was initiated. Recording started with a 5-min free swimming evaluation, then 15 µL of the predator cue was added into each well and fish behavior was tracked for another 5 min. Acclimation and response to the predator cue was assessed during a continuous light period (25 % intensity of the system capacity; 650-750 Lux).

Statistical Analysis

Differences in gene transcription were tested using one-way ANOVA with significance set at $P < 0.05$, followed by a Tukey Honest Significant Difference (HSD) post-hoc test for pairwise difference. 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 (p-values are presented in the supplemental material (Table 3). Shapiro-Wilk normality and Bartlett tests were used to determine which algorithms were appropriate for determining significant differences between treatments.

Data were further analyzed using regression analyses to fit concentration-effect curves. Replicated regression approaches are recommended for evaluating concentration-effect relationships (versus pairwise comparisons) because of their greater statistical power (Isnard et al., 2001; Cottingham et al., 2005). Curve-fitting approaches also provides mechanistic information because they give a prediction of the direction of the responses. The OECD now recommends regression-based estimation procedures for the analysis of toxicity response data. A maximum likelihood estimate approach was used to evaluate whether non-monotonic curves were a better fit to the data than a null (intercept-only) model. Five different concentration-effect curves (linear regression, quadratic, sigmoidal, 5-parameter unimodal, and 6-parameter unimodal) were tested to fit responses of all three concentrations and the vehicle control (MeOH). A maximum likelihood ratio test was used to examine whether each curve provided a better fit than an intercept-only null model with a significance level of $P < 0.05$ (Bolker, 2008). All calculations for the concentration-effect curves were performed using fold-change values. In addition, heat maps of all transcriptomic data were prepared to show functional pathway correlations (supplementary material, Figure S3).

To assess locomotive behavior, distance moved by each larval zebrafish was recorded during each minute of the 30-min observation period. Using the trapezoidal rule, the area under the curve (AUC) was computed for each fish under 5 lighting conditions: Light 1, 1-5 min; Dark 1, 5-10 min; Light 2, 10-15 min; Dark 2, 15-20 min; and Free swim, 15-30 min (an extended period of Dark 2). Mixed effects

regression models, including zebrafish-specific random effects, were used to assess differences between groups defined by three concentrations of bifenthrin and the vehicle control group. Analytical variables were defined to capture differences in area under the curve between Light 1 and Dark 1, Dark 1 and Light 2, and Light 2 and Dark 2 as measures of changes in movement in the transition from light to dark or dark to light. Contrasts for differences between exposed groups and the solvent control group were specified for light and dark conditions separately to compare these transitions and the area under the curve during each of the lighting conditions. A natural logarithmic transformation was best suited to the area under the curve analysis, so as to stabilize the variance and meet the underlying assumptions of the mixed effects model. Due to zeroes, occurring from stagnating fish in the area under the curve, all values were shifted by 0.1 prior to transforming data to a natural logarithmic scale. Concentration (0.1 ng/L, 1 ng/L, 50 ng/L or MeOH), day (3, 5 and 19 dpf), and the transition variables were all compared in the models, including their interactions. Akaike information criterion was used for model selection and Wald tests for comparing groups were used, with a significance level of $p< 0.05$. All analyses were conducted using SAS university edition.

Area under the curve values were used to determine best fitting curves, in order to identify potential concentration-dependent behavioral responses. To allow comparison within endpoints, all datasets were rescaled into a range between 0.0 and 1.0 for graphical illustration using the normalization $x' = \frac{x-xmin}{xmax-xmin}$ at each time point (Figure 100-14). All untransformed datasets and corresponding graphs are presented as supplementary material (Figure S4).

Results

Molecular responses to bifenthrin

Exposure to low (ng/L) concentrations of bifenthrin resulted in time-dependent increases in transcription of both mTOR and RyR signaling pathway-associated genes during early development (at 1, 3, and 5dpf); however, there was an inverse relationship between bifenthrin and transcriptomic changes at 19 dpf following a 14 d recovery period (Figure 10-12). At 1 dpf, developmental exposure to bifenthrin caused a significant concentration-dependent increase in transcripts of *ryr1b*, the RyR paralog predominantly found in fast twitch muscles (Figure 10). Transcriptional responses of genes involved in the mTOR pathway were similarly increased. Data demonstrated either linear or quadratic (non-monotonic) responses, with increased transcription at 1 dpf of *mtor,* mTOR complex 2 (mTORc2) member *rictora,* or the upstream member *rheb* in fish exposed to bifenthrin at 50 ng/L. (Figure 11).

At 3 dpf, there was also a correlation between increased transcription of *ryr2a* and *ryr3,* the RyR-paralogs predominantly transcribed in the nervous system, and higher bifenthrin concentrations (Figure 10). Furthermore, strong concentration-dependent increases were observed in all calcium channel genes, with transcription of *cacna1c* and *cacna1sb* responding significantly. *Cacna1c* also showed significant upregulation in the 50ng/L exposure group in the ANOVA calculation, compared to all other treatments (Figure 12, Table S2). Another impact on calcium signaling systems was detected in the form of a significant quadratic response of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) *atp2a2a,* a paralog predominantly expressed in slow twitch muscle and heart tissue. This quadratic response shows decreased transcription of *atp2a2a* in larval fish from the lowest bifenthrin concentration and increased transcription in the 50 ng/L treatment, relative to vehicle control fish (Figure 12). Similar to observations at 1 dpf, *mtor* transcripts were significantly correlated with bifenthrin concentration at 3 dpf.

Larvae sampled on the last day of exposure (5 dpf) provided further evidence that bifenthrin altered transcription of signaling molecules in the calcium signaling network, with significant concentration-dependent increases in transcription of *atp2a1* and *cacna1c,* a calcium transporter and ion channel, respectively, and quadratic responses in *ryr2b* and *cacna1sa* (Figure 10 and 12). There were no significant changes in the transcription levels of RyR-paralogs at 5 dpf (Figure 10). As observed at 1 and 3 dpf, at 5 dpf there were significant transcriptional differences in *mtor*, as well as the downstream signaling member *eif4ebp1*, both of which correlated with bifenthrin concentration in a concentration-dependent manner (Figure 11).

Opposite to transcriptional changes observed during the exposure period, the transcription of most RyR paralogs decreased in a concentration-dependent manner in 19 dpf larvae. Transcripts of *ryr2b* were the only RyR genes that remained elevated during both exposure and recovery periods in fish exposed to bifenthrin at 50 ng/L (Figure 10). In contrast to measurements during the exposure period, there were no significant differences in transcription of genes coding for SERCA pumps and voltage-gated Ca^{2+} channels post recovery (Figure 12). There were concentration-dependent decreases in transcription at 19 dpf in all mTOR signaling pathway genes investigated in this study (Figure 11), with significant differences determined for *mtor*, as well as for the mTORc1 partner, *rptor,* and the downstream target, *eif4ebp1.*

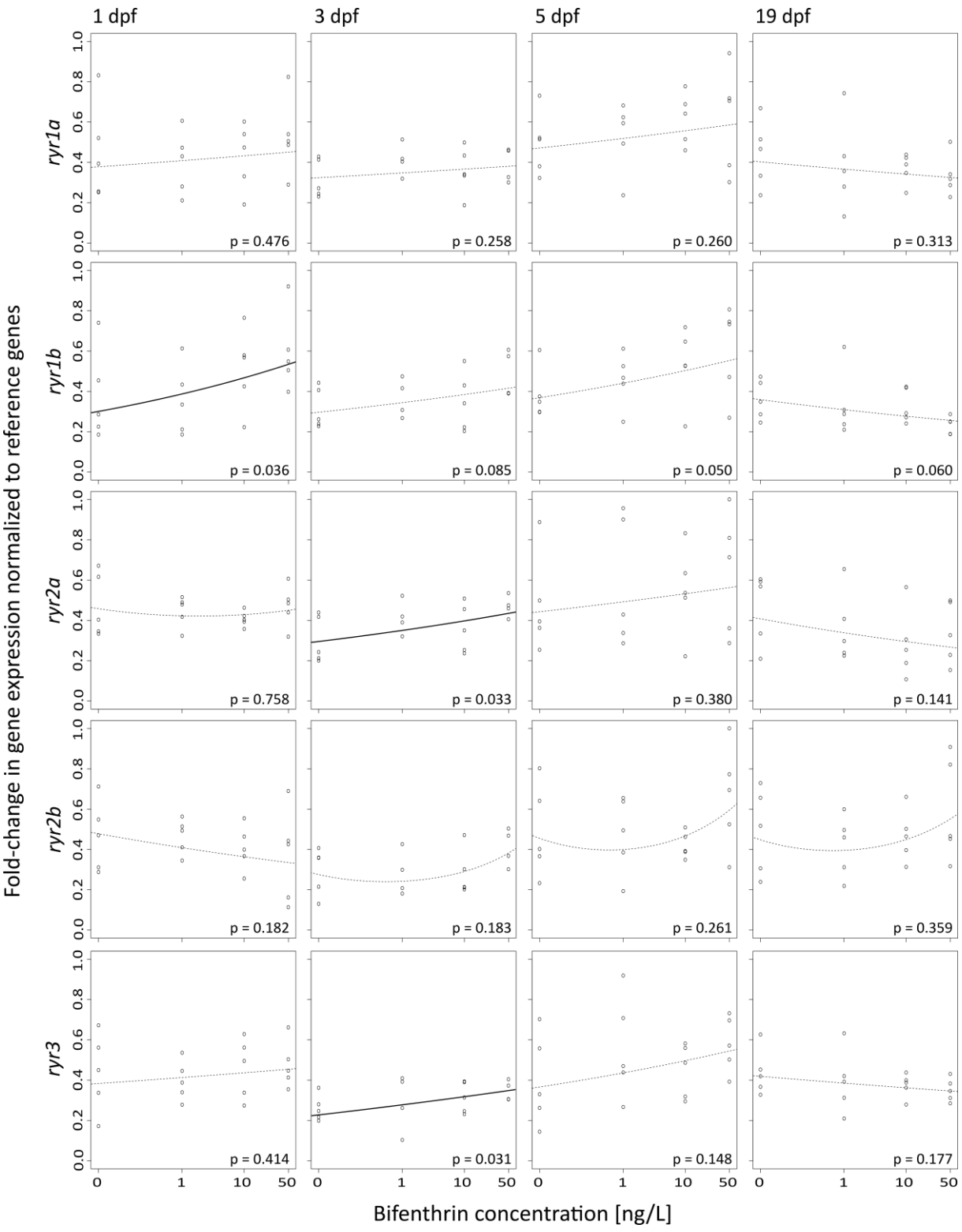

Figure 10. Transcriptional changes ryanodine receptor (RyR) paralogs in zebrafish larvae exposed to varying concentrations of bifenthrin from 1 to 5 dpf. Each dot represents the fold change value of a single biological replicate (n=5 biological replicates), normalized to the average of the reference genes *actb*, *b2m* and *eef1a1* within the same sample. Data are presented on a log10 X + 0.05 axis. For data in each panel, five curves (linear, unimodal 1, unimodal 2, sigmoidal and quadratic) were assessed for best fit using the maximum likelihood approach; the best fitting curve is shown in each panel. Curves shown 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 option (lowest p-value), but not significantly better than the null model. P-values for each fitting curves are shown in the panel. Fold-change values were rescaled between 0 and 1 with a normalization calculation for each time point, to facilitate comparison between genes. Graphs illustrating the actual values (Appendix, Figure S4) and heat maps of the data (Appendix, Figure S3) are provided in the supplementary material.

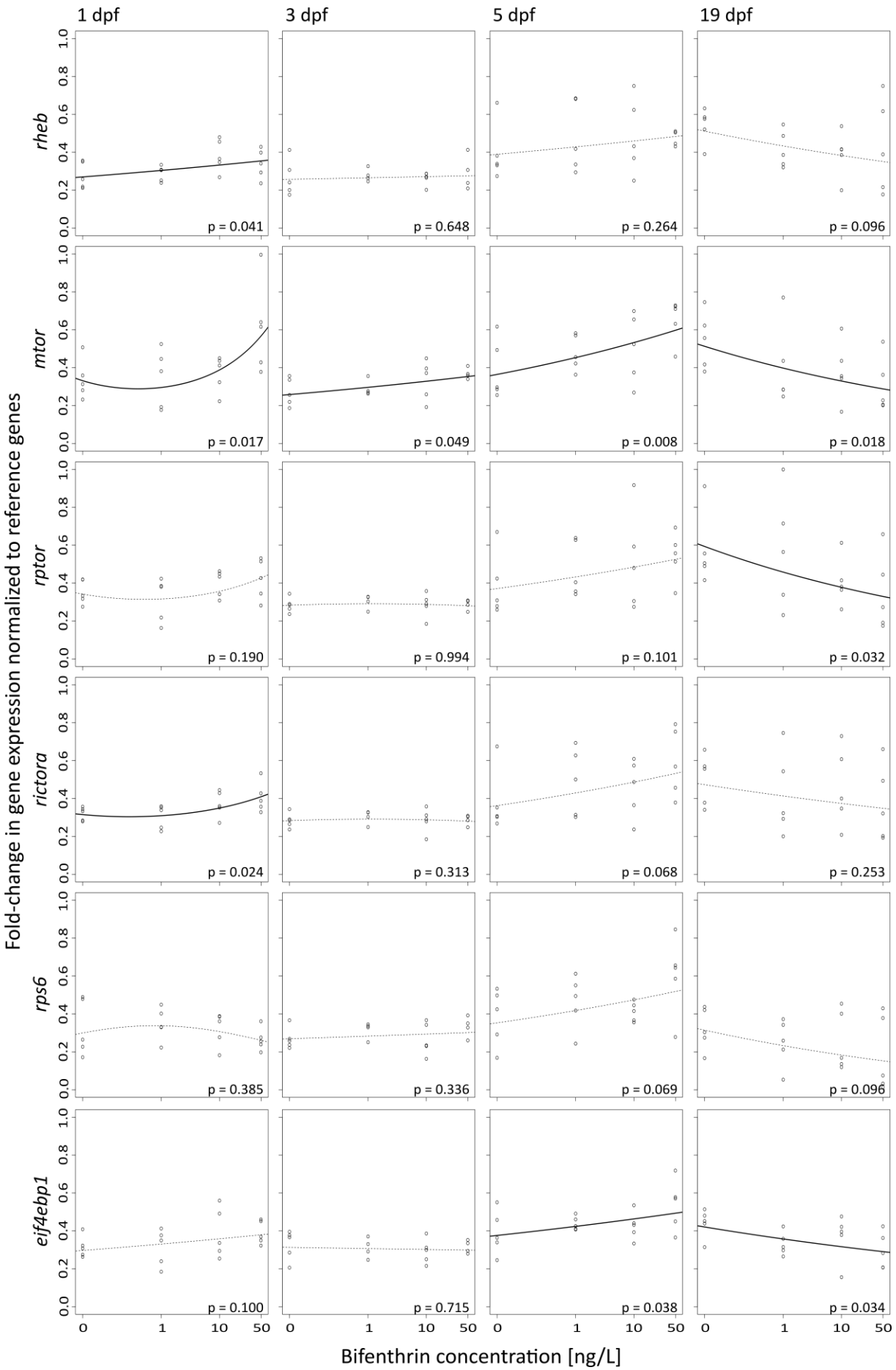
1 dpf
3 dpf
5 dpf
19 dpf
rheb
mtor
rptor
rictora
rps6
eif4ebp1
p = 0.041
p = 0.648
p = 0.264
p = 0.096
p = 0.017
p = 0.049
p = 0.008
p = 0.018
p = 0.190
p = 0.994
p = 0.101
p = 0.032
p = 0.024
p = 0.313
p = 0.068
p = 0.253
p = 0.385
p = 0.336
p = 0.069
p = 0.096
p = 0.100
p = 0.715
p = 0.038
p = 0.034
0.0
0.2
0.4
0.6
0.8
1.0
0
1
10
50
Fold-change in gene expression normalized to reference genes
Bifenthrin concentration [ng/L]

Figure 11. Transcriptional changes for mTOR signaling molecules in zebrafish larvae exposed to varying concentrations of bifenthrin from 1 to 5 dpf. Each dot represents the fold change value of a single biological replicate (n=5 biological replicates), normalized to the average of the reference genes *actb*, *b2m* and *eef1a1* within the same sample. Data are presented on a log10 X + 0.05 axis. For data in each panel, five curves (linear, unimodal 1, unimodal 2, sigmoidal and quadratic) were assessed for best fit using the maximum likelihood approach; the best fitting curve is shown in each panel. Curves shown 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 option (lowest p-value), but not significantly better than the null model. P-values for each fitting curves are shown in the panel. Fold-change values were rescaled between 0 and 1 with a normalization calculation for each time point, to facilitate comparison between genes. Graphs illustrating the actual values (Appendix, Figure S4) and heat maps of the data (Appendix, Figure S3) are provided in the supplementary material.

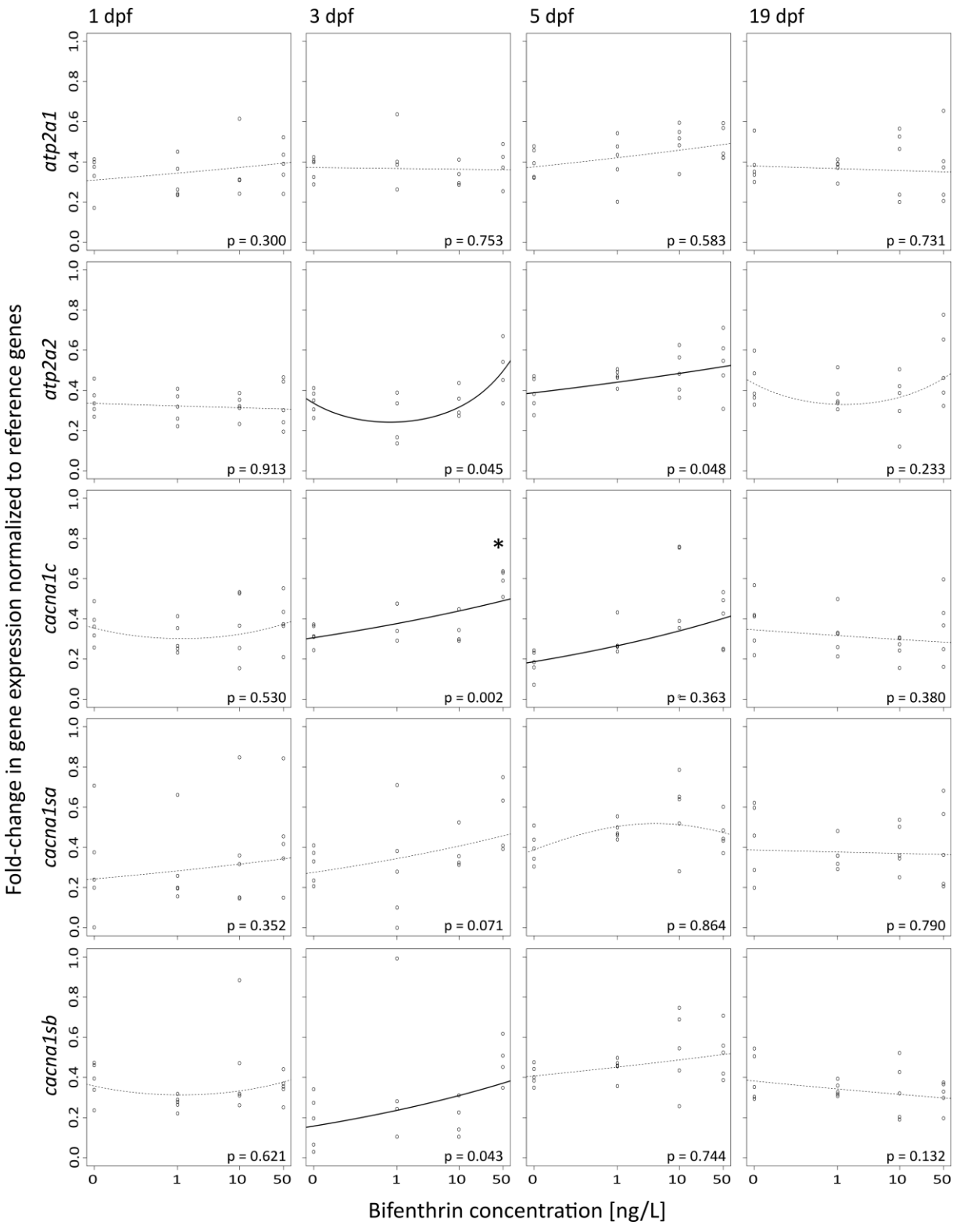

Figure 12. Transcriptional changes for SERCA pumps and voltage-gated Ca2+ channels in zebrafish larvae exposed to varying concentrations of bifenthrin from 1 to 5 dpf. Each dot represents the fold change value of a single biological replicate (n=5 biological replicates), normalized to the average of the reference genes *actb*, *b2m* and *eef1a1* within the same sample. Data are presented on a log10 X + 0.05 axis. For data in each panel, five curves (linear, unimodal 1, unimodal 2, sigmoidal and quadratic) were assessed for best fit using the maximum likelihood approach; the best fitting curve is shown in each panel. Curves shown 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 option (lowest p-value), but not significantly better than the null model. P-values for each fitting curves are shown in the panel. Fold-change values were rescaled between 0 and 1 with a normalization calculation for each time point, to facilitate comparison between genes. Graphs illustrating the actual values (Appendix, Figure S4) and heat maps of the data (Appendix, Figure S3) are provided in the supplementary material. * Significantly different from control, as identified using one-way ANOVA ($p < 0.05$).

Locomotor assessments

Freshly hatched larval fish (< 5 dpf) in all groups moved more during dark periods compared to light periods (illustrated for vehicle control fish, Figure 13A). This canonical photomotor behavior changed in all groups with ongoing development, such that by 19 dpf, fish swam greater distances during light periods (illustrated for vehicle control fish, Figure 13B).

Swimming performance was affected by bifenthrin exposure, but this was only significant in 19 dpf fish following the 14 d post-recovery period (Figure 13C, D). The majority of newly hatched larvae at 3 dpf showed little movement during light periods. Dark phases, however, triggered comparable swimming responses in all exposure groups, with occasional swimming bursts. At 5 dpf, larvae showed a tendency towards less movement with exposure to higher bifenthrin concentrations during both light phases and increased swim behavior in the Dark 1, Dark 2 and final Free swim period (Figure 13D). However, no statistically significant differences were detected between groups during the acute exposure period at 3 or 5 dpf. In contrast to the lack of bifenthrin effects on the photomotor response during acute exposure, there were significant bifenthrin-related differences in swimming performance in post-recovery larvae at 19 dpf (Figure 13D). Specifically, during a second light period challenge (Light 2), fish exposed to bifenthrin at 1 or 10 ng/L were significantly more mobile relative to either vehicle controls or the 50 ng/L bifenthrin groups. Similar patterns were observed in Dark 2 and the Free swim period, whereas no significant bifenthrin effect was observed during Light 1 and Dark 1.

The mixed model approach used to highlight differences between the single treatments integrates distance moved along with the transition between light and dark stimuli, providing a robust behavioral analysis. Calculations in the mixed model approach demonstrated increased movement for the larval fish from the low bifenthrin treatments (1 and 10 ng/L) during Dark 2 and Free swim on 19 dpf, showing significant differences when compared to the vehicle control (Figure 13). Furthermore, a significant decrease in movement was observed in fish from the 50 ng/L bifenthrin group relative to the solvent control group during Light 1 on 19 dpf.

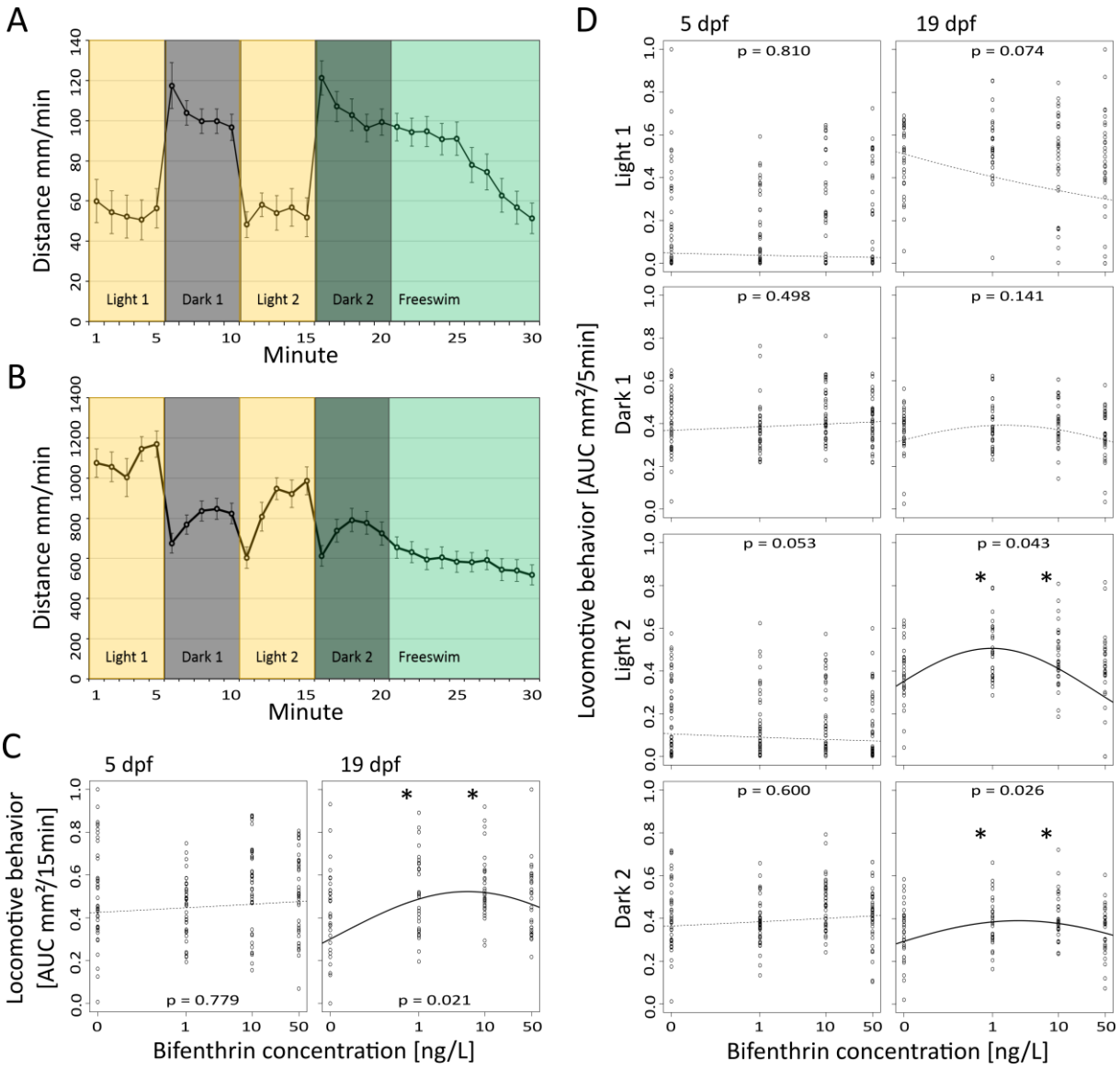

Figure 13. Developmental exposure to bifenthrin altered responses of zebrafish in the light-dark locomotor behavioral assay at 19 but not 5 dpf. Locomotor behavior of zebrafish in alternating periods of light and dark was assessed in zebrafish exposed to varying concentrations of bifenthrin from 1-5 dpf. Locomotor behavior in vehicle control fish during alternating light and dark periods at **(A)** 5 dpf and at **(B)** 19 dpf after a 14-day recovery period is shown as the mean distance in mm moved per minute ± SEM (n = 30 individual larval fish). **(C, D)** Locomotive behavior of 5 dpf (left) and 19 dpf (right) air larval zebrafish exposed to vehicle (0 ng/ml bifenthrin) or varying concentrations of bifenthrin with the distance moved presented as the area under the curve (AUC). Data are presented on a log10 X+ 0.05 axis, and each dot represents one larval fish (n=3o per treatment). **(C)** Data collected during an extended Dark 2 period (15 min "Free swim"). **(D)** Data collected during alternating light and dark periods, each lasting 5 min. AUC values were rescaled between 0 and 1 with a normalization calculation for each day (determined separately for the Free swim period) to facilitate comparison between light and dark periods at any given time point (graphs with raw values are presented in the supplementary material). Five concentration-effect curves (linear, unimodal 1, unimodal 2, sigmoidal, and quadratic) were fit using a maximum likelihood approach. 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 (all p-values for the fitted curves are also shown in the graphs representing one period). *Significantly different from control as identified

using a mixed model algorithm ($p < 0.05$). Graphs illustrating the actual values (Appendix, Figure S5) are provided in the supplementary material.

Response to a predator cue

The response to a predator cue was assessed at 19 dpf after a 14 d recovery period. There were no significant differences in swimming behavior during the initial 5 min acclimation period, but as noted in the light/dark locomotor tests, there was a tendency for increased movement in larvae exposed to 1 and 10 ng/L bifenthrin (Figure 14). Larvae from the control group responded with immediate immobilization (freezing) when challenged with a predator cue. Over the following 5 min they returned to normal (baseline) swimming activity. The majority of bifenthrin-exposed larvae also responded with initial freezing, but returned to baseline swimming behavior more rapidly than vehicle control individuals. Concentration-effect fitting curves demonstrated a significant non-monotonic, quadratic concentration-effect response, with increased movement in larvae exposed to 1 and 10 ng/L bifenthrin (Figure 14).

The increased movement of larval fish from the 1 ng/L treatment was further confirmed by the mixed model algorithm, which indicated significantly increased activity when compared to controls. While the 10 ng/L bifenthrin group showed increased activity as well, the data were not significantly different when assessed using the mixed model algorithm.

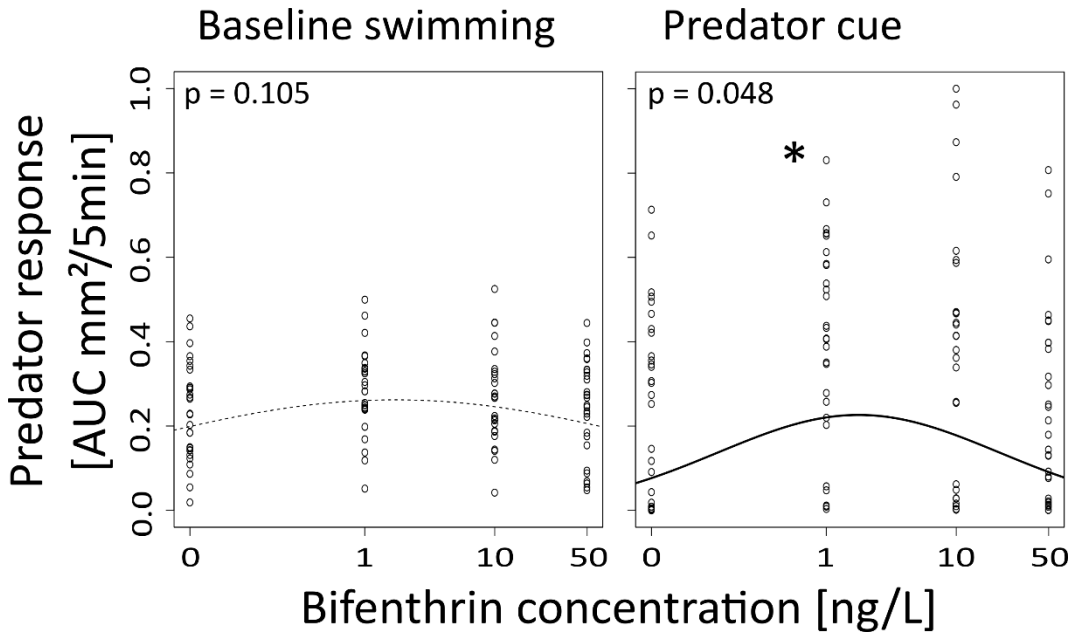

Figure 14. Response to an olfactory predator cue in 19 dpf zebrafish exposed to bifenthrin from 1 to 5 dpf. Swimming was tracked for 5 min during an acclimation period (baseline swimming), and after challenge with a predator cue. Each treatment (presented on a log10 X+ 0.05 axis) included n=30 larval 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 raw values are presented in the supplementary material). Five concentration-effect curves were fit using a maximum likelihood approach (linear, unimodal 1, unimodal 2, 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. *Significantly different from control, as identified using a mixed model algorithm ($p< 0.05$). Graphs illustrating the actual values (Appendix, Figure S6) are provided in the supplementary material.

Discussion

While prior studies have investigated micromolar bifenthrin concentrations exposure impacts on zebrafish development (Jin et al., 2009), to the best of our knowledge, this is the first study describing impacts of bifenthrin on neurodevelopmental processes in zebrafish exposed to low (ng/L) concentrations, which correspond to pM concentrations. The combined assessment of molecular and behavioral endpoints during chemical exposure from 2 to 5 dpf and after a recovery period reveals distinct responses at different levels of biological organization and illustrates delayed changes in behavior. Specifically, we observed that in the developing zebrafish, bifenthrin exposure during early development acutely upregulated transcription of mTOR and RyR-dependent signaling molecules and caused delayed downregulation of these transcripts and delayed behavioral deficits. The behavioral assessments conducted in this study, which included challenging fish with visual and olfactory stimuli, have been demonstrated to be strong indicators of neuronal dysfunction (Tierney, 2011). Perturbations of mTOR- and RyR-dependent Ca^{2+} signaling in the developing brain have previously been linked to adverse neurodevelopmental outcomes in rodent models (Mori et al., 2000; Bers, 2004; Kumar et al., 2005; Berridge, 2006; Lee et al., 2011; Bowling et al., 2014; Tang et al., 2014). Collectively, these data suggest a potential link between bifenthrin effects on transcription of mTOR and RyR-dependent signaling molecules and bifenthrin effects on behavior.

Specific gene transcription effects

Transcripts of RyR signaling molecules showed overall concentration-dependent increases during the exposure period, and subsequent decreases after the recovery period. A significant concentration-dependent response was observed at 3 dpf for *ryr2a*, a RyR-paralog found predominantly in nervous tissue during zebrafish development (Wu et al., 2011). A similar response was observed for *ryr3*, also known to be expressed in zebrafish nervous tissues (Darbandi and Franck, 2009). This is of particular interest since the developmental stage at which increased transcription of *ryr2a* and *ryr3* were observed corresponds to the timing of complex neuronal circuit formation and significant synaptogenesis in the developing zebrafish brain (Saint-Amant and Drapeau, 1998; Brustein et al., 2003b). Earlier studies demonstrated that chemical-induced increases in RyR-dependent Ca^{2+} signaling

during development were associated with enhanced dendritic outgrowth, increased synaptic density and impaired cognitive behavior (Yang et al., 2009a; Wayman et al., 2012b; Wayman et al., 2012a; Lesiak et al., 2014). Additional concentration-dependent impacts on Ca^{2+} homeostasis at 3 dpf included the upregulation of the SERCA pump *atp2a2a*, as well as voltage gated calcium channels *cacna1c* and *cansa1sb*, illustrating pervasive effects on calcium homeostasis (Figure 12). It is not clear how our findings relate to previous reports of decreased plasma calcium levels in freshwater catfish (*Heteropneustes fossilis*; 37-42g) following a 96 h exposure to 5.76 µg/L and 28 d exposure to 1.44 µg/L of the pyrethroid cypermethrin (Mishra et al., 2005). However, our findings are consistent with a previous study demonstrating that bifenthrin acutely increases calcium oscillations in cultured cortical rat neurons (Cao et al., 2014). Interestingly, bifenthrin effects in primary rat cultures were observed at nanomolar concentrations, whereas we observed bifenthrin effects on transcription of RyR-relevant genes in zebrafish at picomolar concentrations. This discrepancy in effective concentration levels is in contrast to the commonly held perception that *in vitro* model systems are more sensitive than *in vivo* model systems. While the biological explanation for this difference is unknown, it perhaps reflects the observation that fish are more vulnerable than mammals to pyrethroid toxicity (Glickman and Lech, 1982). Collectively, these observations support a generalized effect of pyrethroids on Ca^{2+} homeostasis and signaling *in vivo* as well as *in vitro*.

Concentration-dependent changes were observed in five of the six genes investigated within the mTOR pathway (Figure 11). The most extensive impacts where observed in the gene coding for mTOR, with significant concentration-dependent responses observed at all time points. At 1 dpf, the mTORC2 components, *mtor and rictora*, as well as the upstream signaling molecule, *rheb,* were upregulated in a concentration-dependent manner, indicating activation of the entire signaling cascade (Sarbassov et al., 2005b; Guertin et al., 2006; Bai et al., 2007). With ongoing exposure, a concentration-dependent significant increase in the downstream target, *eif4ebp1*, which encodes for a protein that inhibits the translational mechanisms of mTOR signaling (Ma and Blenis, 2009) was observed at 5 dpf. Upregulation of *eif4ebp1* has the potential to diminish pathway activation, since transcription of mTOR upstream members with activating function were consistently upregulated during earlier time points. Concentration-dependent decreases in transcription were observed for all mTOR pathway members after the recovery period, with the most robust changes observed for the mTORC2 members, *mtor* and *rictora*, and *eif4ebp1*. The most consistently decreased transcripts correlated with the higher bifenthrin concentrations.

Transcription vs. behavior

Here, we showed a biphasic transcriptomic response of genes involved in mTOR and RyR-dependent Ca^{2+} signaling pathways: transcription of these genes was generally increased during exposure with the most robust increases in transcription was detected in the fish exposed to the highest concentration of bifenthrin tested (50 ng/L). However, there was a clear trend of decreased transcription after a 14 day recovery from bifenthrin. Similar inverse relationships in transcriptomic responses have been observed after a recovery period in fathead minnow larvae, following short term (24 h) exposure to bifenthrin (Beggel et al., 2011). Given the extensive use of bifenthrin and its known neurotoxic effects, surprisingly few other studies have examined the effects of low (ng/L) concentrations in vertebrates. These include studies of the inland silverside (*Menidia beryllina*) (Brander et al., 2012b; DeGroot and Brander, 2014; Brander et al., 2016b), one of which described the integrated effects of a 14 d exposure to low levels of bifenthrin on transcription of genes representative of endocrine function and on reproduction (Brander et al., 2016b). Higher concentrations of bifenthrin have been shown to interfere with nervous system function. Thus, at 1.5 µg/L, bifenthrin interferes with dopaminergic signaling in juvenile rainbow trout (Crago and Schlenk, 2015), and at concentrations ranging from 75 ng/L to 4 µg/L, a 24 h exposure to bifenthrin decreased transcription of genes related to metabolism, growth, stress response, muscular and neuronal activity in larval fathead minnows (*Pimephales promelas*)(Beggel et al., 2011), suggesting broader impacts of bifenthrin exposure on organismal development at higher concentrations than those we tested here.

Bifenthrin did not cause significant behavioral changes at 5 dpf in our study. However, post-recovery behavioral assessment data demonstrate significant differences in both photomotor behavior and locomotor response to a predator cue at 19 dpf in zebrafish exposed to lower bifenthrin concentrations. It has previously been reported that the pyrethroid deltamethrin increased incidence of eye cataracts in adult Nile tilapia, *Oreochromis niloticus* (El-Sayed and Saad, 2008), suggesting the possibility that bifenthrin-induced behavioral deficits in response to light-dark stimuli are secondary to ocular toxicity. However, we think this is unlikely to be the case, because: (1) we did not observe any eye cataracts in any of the zebrafish in our studies, and (2) eye cataracts were observed in tilapia exposed to deltamethrin at 1.46 µg/L for 28 consecutive days, whereas we observed significant hyperreactivity in 19 dpf zebrafish exposed to bifenthrin at low (1 and 10 ng/L) but not high (50 ng/L) pM concentrations of bifenthrin for only 5 consecutive days with 14 days of “washout”. Moreover, the behavioral phenotype we observed in zebrafish exposed to bifenthrin is consistent with previous reports demonstrating hyperactivity associated with pyrethroid exposure in fish and rats (Jin et al., 2009; Richardson et al., 2015).

Another question is whether the delayed behavioral effects of bifenthrin reflect effects of bifenthrin on neurodevelopmental processes, or whether bifenthrin is still present in the brain at 19 dpf. In zebrafish larvae exposed to bifenthrin at 2 or 20 µg/L, the half-life of bifenthrin was found to be 15.9 or 38.5 hours, respectively (Tu et al., 2014). Assuming that bifenthrin is completely eliminated within 5 half-lives, it seems likely that behavioral deficits observed at 19 dpf, which is 14 d after exposure ended, are attributable to disruption of neurodevelopment. Interestingly, recent reports in a rodent model indicates that in the adult organism, bifenthrin elicits behavioral deficits during the exposure period, but not after a recovery period (Syed et al., 2018), suggesting differential effects of bifenthrin on the developing *versus* the mature brain.

When comparing results from molecular and behavioral assessments, the strongest transcriptional changes were correlated with higher chemical concentrations, whereas the greater impacts on behavior were observed in fish exposed to bifenthrin at 1 and 10 ng/L. These observations suggest several possibilities: (1) transcriptional changes in calcium-dependent signaling pathways are not causally related to behavior changes; (2) subtle differences observed at the molecular level contribute to, or manifest as, delayed effects as determined via behavioral assessments whereas robust increases in transcripts from mTOR and RyR-dependent Ca^{2+} signaling genes in the group exposed to 50 ng/L bifenthrin may be compensatory responses. The non-monotonic concentration-effect relationship for bifenthrin effects on behavioral endpoints was confirmed by curve fitting (which identified quadratic responses as the best fit) and by a mixed model algorithm. The biological reason(s) for the non-monotonic concentration-related effects of bifenthrin on behavior are not known. Non-monotonic concentration-effect relationships have been observed for bifenthrin effects on other endpoints. For example, low (ng/L) concentrations of bifenthrin have caused non-monotonic responses in gene transcription of endocrine-related genes in *inland silversides* (Brander et al., 2016b), with strongest effects observed with the lowest applied concentration of 0.5 ng/L. Similar non-monotonic concentration-effect relationships have been reported for PCB 95, a flame retardant that interferes with RyR dependent Ca^{2+} signaling to enhance dendritic growth and interfere with learning and memory at lower but not higher concentrations (Yang et al., 2009a; Wayman et al., 2012b; Wayman et al., 2012a).

Further research to assess protein levels of altered transcripts, as well as neurodevelopmental processes influenced by RyR- and mTOR-dependent signaling, such as axonal outgrowth, dendritic arborization and synapse stabilization in relevant brain regions, could help to identify the mechanistic link(s) between developmental exposure of bifenthrin, altered transcription of RyR- and mTOR signaling molecules, and delayed behavioral alterations.

Low (ng/L) concentrations and concentration-effect relationships

This study provides evidence for concentration-dependent neurobehavioral responses elicited by developmental exposures to low (ng/L) concentrations of bifenthrin. Environmental concentrations of chemical pollutants are generally below acutely toxic levels; however, potential sublethal toxic effects are of increasing concern (Sandahl et al., 2005; Geist et al., 2007; Connon et al., 2012). Persistent impacts on behavior following a developmental exposure to bifenthrin at 1 and 10 ng/L were identified in the present study. These findings illustrate the importance of including low concentrations in experiments that seek to evaluate the effects of environmentally relevant levels of persistent chemicals, and the need to focus on delayed effects. Given the small number of studies incorporating low (ng/L) concentrations of pyrethroid insecticides, but the strong effects measured following exposure of developing fish to these low levels, there are likely a larger number of sublethal effects resulting from exposures at these low concentrations that may adversely impact the overall health status of an organism that have yet to be determined.

Conclusion

In this study, RyR and mTOR signaling pathways were observed to be impacted by developmental exposures to bifenthrin in a concentration-dependent manner, although the direction of change varied depending on when it was measured. During the developmental exposure period, transcription was generally upregulated in the bifenthrin exposure groups, whereas following a recovery period, transcription was largely decreased, particularly in fish exposed developmentally to the higher bifenthrin concentrations. Collectively, these data confirm that low (ng/L) concentrations of bifenthrin alter transcription of key genes involved in neurodevelopment. Behavioral assessments provided evidence that developmental exposure to bifenthrin also altered neuronal function, evidenced as hyperactivity in 19 dpf fish exposed to the lower bifenthrin concentrations (1 ng/L and 10 ng/L). Our findings demonstrate the importance of conducting toxicological studies at low (ng/L) concentrations levels of environmental relevance. We further provided evidence that developmental exposures that do not cause detectable effects on behavior during acute exposure can cause delayed behavioral deficits in the older organism. Because neurodevelopment is highly conserved across species, and given the wide-spread exposure of humans, including children, to environmental levels of bifenthrin, these studies also identify bifenthrin as a potential environmental risk factor for NDDs, and in particular those characterized by hyperreactivity, such as attention deficit hyperactivity disorder.

4. Bifenthrin exposure effects early development in inland silversides

A similar version of this chapter was published:

Frank, D.F., Brander, S.M., Hasenbein, S., Harvey, D.J., Lein, P.J., Geist, J., Connon, R.E., 2019. 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). Aquatic Toxicology. 206, 1-13.

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 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 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.

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., 2012a; Weston and Lydy, 2012) . Bifenthrin enters watersheds primarily via runoff following agricultural and urban applications (Kuivila et al., 2012a), 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., 2009b; Velisek et al., 2009a). 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 (<50ng/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., 2016b; 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., 2016b). 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 regulatory approaches, 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 non-model 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., 2016b; DeCourten et al., In review), providing a

fundamental collection of RNA sequences towards the development of targeted molecular pathway assessments, such as that 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; Wayman et al., 2012a; 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).

Material & Methods

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), 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 hours, 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).

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 six 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., 2016b). 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 dph, for transcriptomic assessments, and post hatch, until sampled at 7dpf for both behavioral and transcriptomic assessments. One beaker from each treatment was used per time point, per spawn event (n=5).

At 7dpf 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 mg/L total ammonia.

Locomotor behavior

Locomotor behavior was assessed on the last day of the exposure period (7dpf) 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 (Figure 15) 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 80 m Night Vision IR Illuminator 60 Degree Waterproof Light CCTV Camera, Amazon web services Inc, Seattle, WA) were positioned to evenly illuminate the underside center of the plexiglass surface (Figure 15A (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 (Figure 15A (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, 15A/1800W, 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 58 mm 850nm Infrared IR Pass Filter, Neewer Technology ltd, Shenzen, China) was used to record fish locomotion (Figure 15A (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 hours, to minimize intraday-dependent locomotive variation in swimming behavior, which has previously been reported in fish (MacPhail et al., 2009).

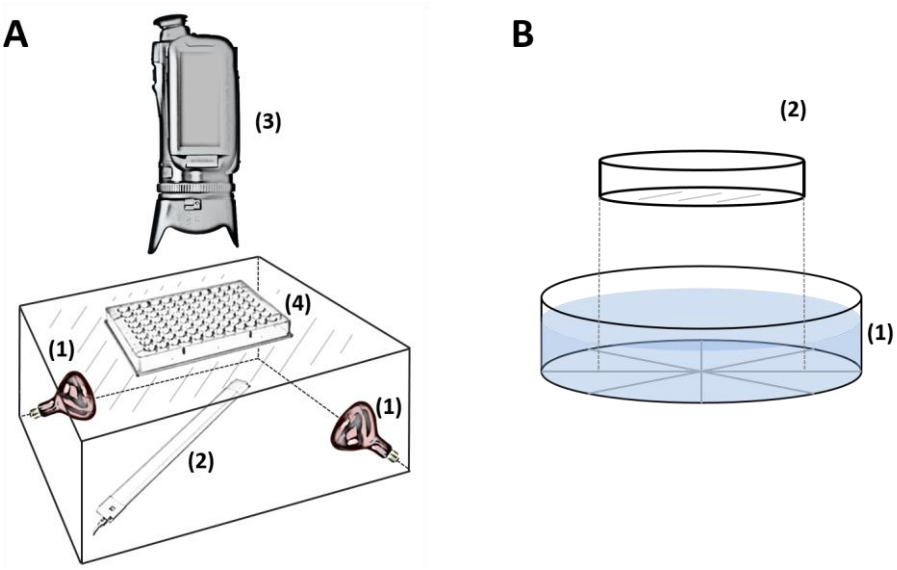

Figure 15. (A) Behavioral assessment lightbox setup. Two infrared light Emitting Diodes (LED) (1) placed within the corners of the light box were used for recording behavior under periods of darkness and a light bar (2) was placed diagonally across the base of the lightbox to illuminate the 96-well plate. 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.

Behavioral experiments at 7 dpf were executed in 96-well plates (Falcon™, Corning Inc., Corning, NY, USA) (Figure 15A (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.

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; Beggel et al., 2011). Experiments with 30 fish from each treatment were performed, including individuals from all 5 biological replicates. The racetrack (Figure 15B) was built with a 10.0 cm diameter petri dish (Figure 15B (1)), containing a centrally placed 7.5 cm diameter petri dish (Figure 15B (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 minute 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.

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 minutes to the multi-well plate, before being transferred into the observation light box, described above, where they were kept for a further 5 minutes before the tracking was initiated. Video recording (as used for locomotion studies) started with a 5-minute 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 minutes. 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 (21dpf) 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.

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 element-binding 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 lst8 (*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 40s 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., 2016b), 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 60s ribosomal protein s7 *(rpl7)* and beta actin (*actb*), which sustained best stability scores in GeNorm (Vandesompele et al., 2002).

Table 4. Genes selected for transcriptomic analysis in inland silversides.

Gene name	Gene Code	Primer (5'->3')	Efficiency %
Reference genes			
Beta-actin	*actb*	**F:** GCAATGAGAGGTTCCGTTGC **R:** CGCAGGACTCCATACCAAGG	98.1
60s 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
40s ribosomal protein s6	*rps6*	**F:** CAGCGTTCTCAACTTGGTCA **R:** GAAGAGTTTGCGGATCTTGC	96.1
Transforming growth factor beta-1	*tgfb*	**F:** CTCAGGAGGCCAAACAGAAG	94.9

		R: GTATCCACTTCCAGCCCAGA	
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

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 48h 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, 10ng) 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 30 m × 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.

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., 2016b; Goff et al., 2017). Therefore, per methods developed in Brander et al. (2016b) 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 minutes 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 minutes, Dark1: 5-10 minutes, Light2: 10-15 minutes, Dark2: 15-20 minutes, and Freeswim 15-30 minutes (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 S3).

Results

Response to the predator-cue

Baseline swimming (i.e., movement before the addition of the predator cue solution) of the post exposure recovery 21 dph larvae did not show differences amongst the bifenthrin treatments or the solvent control group (Figure 16A). 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 minute 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, $p = 0.048$; Figure 16A). 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 minutes following the addition of the blank sample, relative to those who were exposed to the predator cue (two-sample T-test, p = 0.05; Figure 16B).

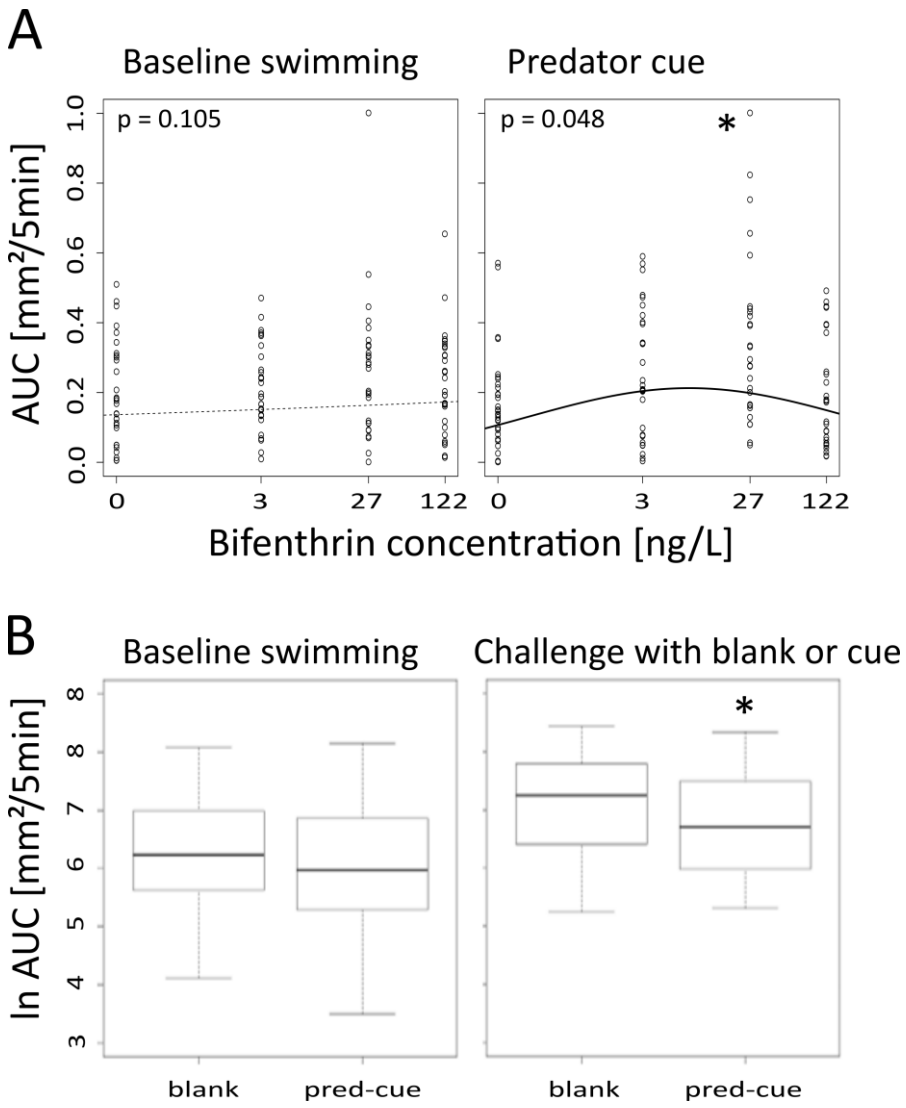

Figure 16. Predator cue responses: (A) 21 dpf inland silversides exposed to 0, 3, 27, 122 ng/L bifenthrin from 1 to 7 dpf were confronted with an olfactory predator cue. Baseline swimming was tracked for 5 min during an acclimation period before the cue was added, followed by a 5-minute evaluation after 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. S3). 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 ($p < 0.05$).

Locomotive behavioral responses

Locomotor behavior assessed at 7 and 21 dph, in response to alternating light and dark stimuli revealed consistent dose-response patterns, although they did not reach significance (Figure 17A; 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 (Figure 17A), 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 (Figure 17A; 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 (Figure 17C), 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 (Figure 17D).

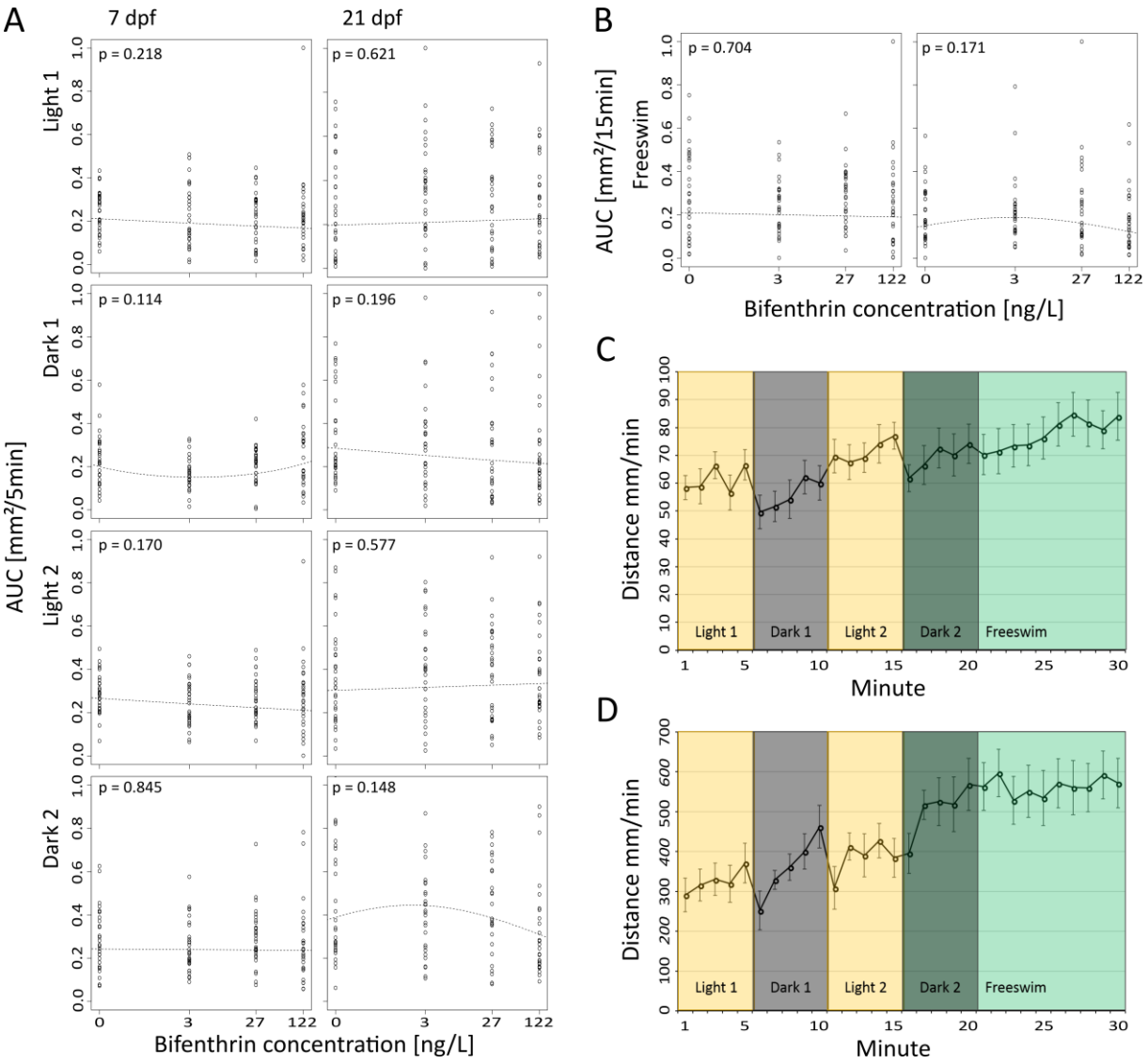

Figure 17. Locomotive responses of 7 and 21 dpf inland silversides to alternating light and dark periods, following developmental exposure (1 - 7 dpf) to different concentrations of bifenthrin (0, 3, 27, 122 ng/L). (A) Alternating 5 min light and dark periods. Each dot represents the distance covered 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 (Free swim). 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. S8) 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 minute ± SEM (n = 30 individual larval fish).

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 (Figure 18).

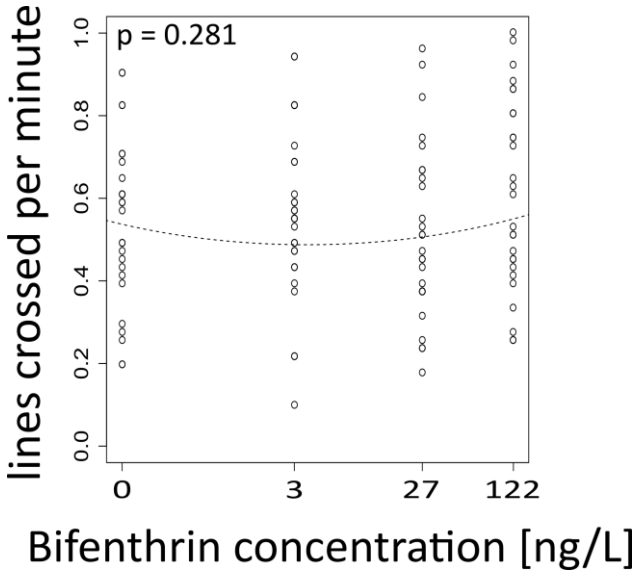

Figure 18. Swimming performance of 21 dpf inland silversides exposed to different concentrations of bifenthrin (0, 3, 27, 122 ng/L) during early development (1 to 7 dpf). Each dot represents the lines crossed during 1 minute in the racetrack arena. Values were rescaled between 0 and 1 with a normalization calculation; original values ranged from 39 to 90 lines crossed per minute. The dose-response calculation showed a p = 0.281.

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 values = 0.012, 0.038, 0.027; Figure 19), 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 values = 0.011, 0.018; Figure 20). 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*, r*yr2* 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 values = 0.020, 0.015, 0.038; Figure 20). *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 value = 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 value = 0.042).

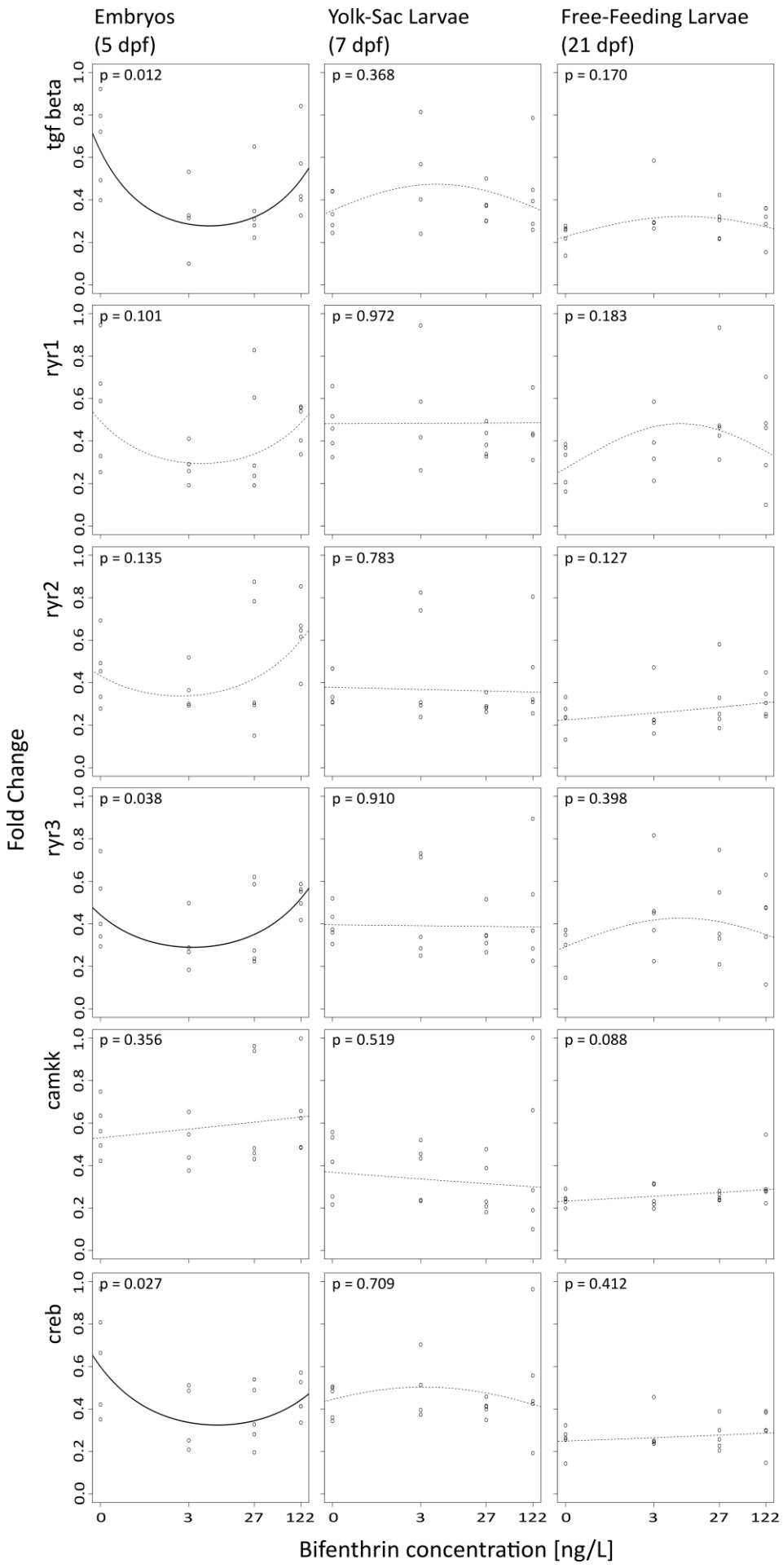
Embryos
(5 dpf)
Yolk-Sac Larvae
(7 dpf)
Free-Feeding Larvae
(21 dpf)
tgf beta
ryr1
ryr2
ryr3
camkk
creb
p = 0.012
p = 0.368
p = 0.170
p = 0.101
p = 0.972
p = 0.183
p = 0.135
p = 0.783
p = 0.127
p = 0.038
p = 0.910
p = 0.398
p = 0.356
p = 0.519
p = 0.088
p = 0.027
p = 0.709
p = 0.412
Fold Change
0.0
0.2
0.4
0.6
0.8
1.0
0
3
27
122
Bifenthrin concentration [ng/L]

Figure 19. Transcriptional changes in genes coding for members of RyR dependent Ca2+ 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 *actb* and rpl7; data are presented on a log10 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. S7A).

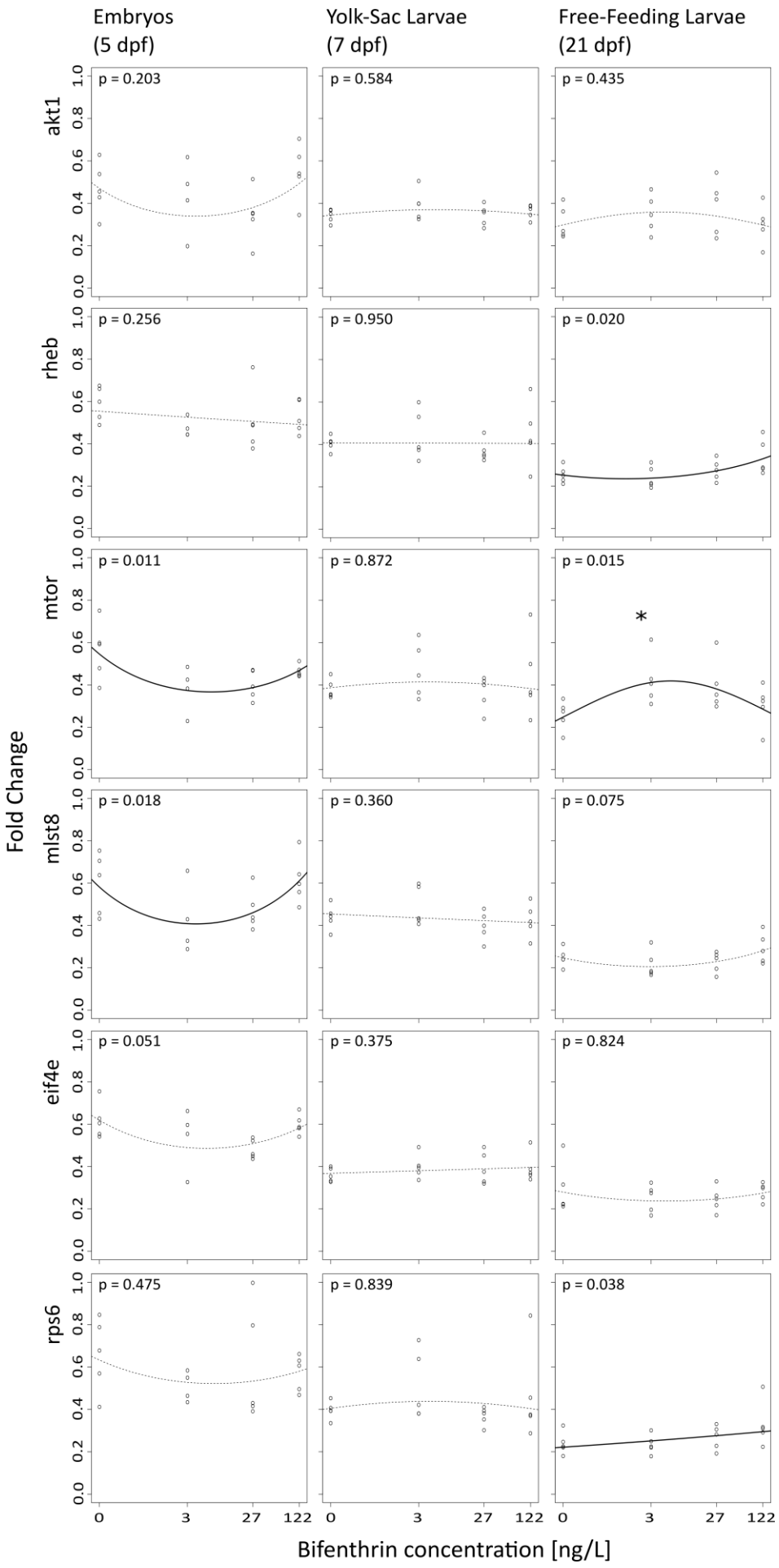
Embryos
(5 dpf)
Yolk-Sac Larvae
(7 dpf)
Free-Feeding Larvae
(21 dpf)
akt1
rheb
mtor
mlst8
eif4e
rps6
p = 0.203
p = 0.584
p = 0.435
p = 0.256
p = 0.950
p = 0.020
p = 0.011
p = 0.872
p = 0.015
*
p = 0.018
p = 0.360
p = 0.075
p = 0.051
p = 0.375
p = 0.824
p = 0.475
p = 0.839
p = 0.038
Fold Change
0.0
0.2
0.4
0.6
0.8
1.0
0
3
27
122
Bifenthrin concentration [ng/L]

Figure 20. 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 *actb* and *rpl7*; data are presented on a log10 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. S7B). * Significantly different from control, as identified using one-way ANOVA ($p< 0.05$).

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; Brander et al., 2016b; DeCourten and Brander, 2017; DeCourten et al., In review). 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; Wayman et al., 2012a) and *in vivo* (Lein et al., 2007; Yang et al., 2009b; 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 exposure to a 1000-fold higher concentration of bifenthrin during an acute exposure scenario, 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 could be 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 hour 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 5dpf 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 can only represent carryover effects since they are 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. Non-monotonic (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; Brander et al., 2016b). 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., 2016b). 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.

5. Molecular response after pathogen infestation in delta smelt

A similar version of this chapter was published:

Frank, D.F., Hasenbein, M., Eder, K., Jeffries, K.M., Geist, J., Fangue, N.A., Connon, R.E., 2017. Transcriptomic screening of the innate immune response in delta smelt during an Ichthyophthirius multifiliis infection. Aquaculture. 473, 80-88.

Abstract

Fish health can be affected by many environmental factors including infection with pathogens. Infectious disease can lead to high mortality in wild populations and in aquaculture, resulting in significant economic losses. Teleost fishes share a highly conserved response to pathogen infections, starting with an immediate activation of the innate immune response before shifting to an adaptive immune response. Fishes also show altered transcriptomic expression in stress-related genes during acute and chronic infections. The ciliate *Ichthyophthirius multifiliis* (*Ich*) is one of the most widely distributed ectoparasites for freshwater fish, providing the possibility to identify infection severity via phenotypical assessments. Molecular markers are increasingly incorporated in health assessments and to determine sublethal physiological responses to stressors. We evaluated the effects of an infection with *Ich* using the pelagic fish species delta smelt (*Hypomesus transpacificus*) as a model. We investigated transcriptional changes of ten genes belonging to the innate immune response and five heat shock genes related to thermal and general stress response. Molecular assessments were conducted in three tissues (gill, spleen and kidney), and collected from fish with different infection intensities. The combination of molecular markers, including immune and stress-related genes, resulted in significant differentiation between infection levels in all tissue types. The strongest results were observed in gill and kidney, however, the detection of infection severity was most effective when combining the transcriptomic results from all tissues. Chemokine *cxcb* was the most responsive gene in all tissues and provided significant upregulation in fish with visible *Ich*-infection. The identification of molecular markers targeting fundamental host responses associated with infections is contributing to the establishment of techniques to assess fish health in natural habitats and in aquaculture.

Introduction

Fish health can be affected by many environmental factors such as water quality, predation, food limitation, temperature stress, contaminants and pathogens (Baxter et al., 2008; Geist, 2011). Pathogens can contribute to mass mortalities both in wild fish populations and aquaculture, resulting in significant economic losses (Wurtsbaugh and Alfaro Tapia, 1988; Pike and Wadsworth, 2000; Gozlan et al., 2005; Austin and Austin, 2007). Pathogens further elicit sublethal responses that can alter behavior, decrease fertility or swimming performance, ultimately limiting reproductive success and increasing risk of predation (Barber et al., 2000). Such effects can be exacerbated by simultaneous exposure to multiple environmental stressors (Barber et al., 2000).

A first response of fishes to pathogens is the activation of the innate immune system, subsequently followed by a shift to an adaptive immune response (Magnadóttir, 2006; Alvarez-Pellitero, 2008). During the innate immune response, pathogens are detected via pathogen recognition receptors (PRRs) which elicit specific responses to various pathogen-associated molecular patterns (PAMPs) in the infected animal (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002). Indicators of an activated innate immune system as well as direct response mechanisms within an innate immune response include members of the complement system, toll-like receptors, inflammation-inducing cytokines, immune cell migration-directing chemokines, anti-proteases, members of the mitogen activated pathway and major histocompatibility complex components (Dalmo et al., 1997; Dixon and Stet, 2001; Gasque, 2004; Sitjà-Bobadilla et al., 2006; Alvarez-Pellitero, 2008; Umasuthan et al., 2015). Furthermore, heat shock proteins (HSPs) are also known to respond to a wide range of stressors, including pathogen infections (Cho et al., 1997; Forsyth et al., 1997; Ackerman and Iwama, 2001), and temperature is known to govern both the infection susceptibility as well as immune defense mechanisms in fishes (Bly and Clem, 1992; Tort et al., 2003; Matthews, 2005).

One of the most widespread ectoparasite for fishes is the ciliate *Ichthyophthirius multifiliis* (*Ich*) (Maki and Dickerson, 2003). The *Ich* life cycle comprises three life stages: a parasitic feeding stage, a reproductive stage, and a free-swimming stage. In the parasitic stage, the trophont lives in, and feeds on, the fish epithelia. It then leaves the host and initiates the reproductive tomont stage resulting in a tomocyst, releasing free swimming theronts which infect new hosts (Matthews, 2005). Growth and development rates of the parasite is positively correlated to temperature (Traxler et al., 1998). *Ich* is known to have adverse effects on fishes and can cause severe defects in gills and skin, where the feeding trophont resides (Matthews, 2005; Xu et al., 2005). Mortality due to *Ich* infection is high, and significant losses in fish farming as well as in wild populations have been reported (Jessop, 1995;

Traxler et al., 1998; Martins et al., 2011). Fish mortality is mainly attributed to extensive feeding of the parasite during the trophont stage prior to tomonts leaving the host, resulting in damage to the gill and skin epithelia of fish, and often in secondary infections (Ewing and Kocan, 1992; Athanassopoulou et al., 2004; Matthews, 2005).

Ich is known to affect a wide range of fish species, including the delta smelt (*Hypomesus transpacificus*). This pelagic fish is endemic to the San Francisco Estuary and Watershed (California, USA) and is listed as threatened and endangered under the Federal and California Endangered Species Acts (CESA), respectively (USFWS, 1993; CDFW, 2017). During a series of experiments conducted to investigate thermal stress responses in adult delta smelt, an *Ich* outbreak occurred. We opportunistically utilized this event to evaluate delta smelt transcriptomic response profiles at different severities of phenotypical *Ich* infection. We specifically investigated transcriptomic responses relating to the innate immune response as well as genes associated with thermal stress. Molecular biomarkers are becoming increasingly important tools to assess sublethal physiological responses to stressors (Miller et al., 2011; Connon et al., 2012), and have been specifically used to assess environmental stressors such as salinity, turbidity, contaminant exposure or stocking density (Terova et al., 2005; Valavanidis et al., 2006; Geist et al., 2007; Hasenbein et al., 2016; Komoroske et al., 2016). Recent studies have been highly successful in using transcriptomic approaches in diagnosing pathogen and parasites infection, as well as disease in fishes (Gonzalez et al., 2007a; Gonzalez et al., 2007b; Miller et al., 2011; Connon et al., 2012; Jeffries et al., 2014a). The potential of these markers as early disease indicators has, to the best of our knowledge, not yet been utilized to assess the health status of endangered fishes such as the delta smelt.

In this study we analyzed and compared gene transcription responses in *Ich*-infected delta smelt across three tissue types: gill, kidney and spleen. The gene expression patterns were linked with phenotypic levels of infection. We hypothesized tissue-specific responses dependent on the severity of infection. In addition, we compared these findings to earlier studies that described the effects of *Ich* on the innate immune response in other fish species.

Material & Methods

Test organisms, holding conditions and treatment

All fish were reared at the UC Davis Fish Conservation and Culture Laboratory (FCCL) in Byron, CA, USA, and transferred to the Putah Creek facility (Davis, CA) as adults (320 to 400 days post hatch; dph).

At UC Davis, fish were held in circular, flow through tanks (diameter 1.2m, depth 0.8m) with water inflow of 8 l minute^{-1}, at a density of 130 fish per tank. The system was supplied with non-chlorinated, air-equilibrated, temperature-controlled (12.5 ± 0.5 °C) well water. Tanks were covered with fine screen mesh to minimize illumination and disturbance.

The initial salinity while transferring them between the facilities was 5 PSU to minimize transport stress (Swanson et al., 1996), and reduced to 0.5 PSU at a constant rate over 10 days. After a 18 day adjustment period, fish were acclimated to three different temperatures (Low = 10.2 ± 0.5, Medium = 13.2 ± 0.5 and High = 18.1 ± 0.5 °C). At this time point all fish had the same background infection level or potential infection probability. Acclimation to the final temperatures was conducted within 7 days and fish were maintained at the final temperatures for 16 days. An observable *Ich* outbreak occurred in the two tanks with higher temperatures on day 11 after acclimation, at which time salinity was increased to 5 PSU in all tanks to assist in fish recovery and to control the *Ich* (Aihua and Buchmann, 2001). On day 16 all fish were euthanized, using a 50 mg/l dose of buffered (with $NaHCO_3$) MS-222 (Tricaine methanesulfonate, Finquel). At this time, 10 fish from each treatment were sampled and infection levels were quantified. Specimens were weighed to the nearest 0.1 g, measured (fork length to the nearest mm), photographed with a high resolution camera (Canon 5D MK II, fitted with a Canon EF Macro 100 mm f/2.8 lens), the sex of each fish was recorded, and tissues were sampled for molecular assessments (Table 5). Gill, kidney and spleen tissues were sampled from 30, 29 and 27 individuals (loss during sampling occurred), respectively, snap frozen in liquid nitrogen and subsequently stored at -80 °C. Phenotypical infection intensity was determined by counting visible trophonts on the whole left side of the fish body using high resolution images. Trophont size was determined using ImageJ (Version 1.48).

During the experiments delta smelt were fed a mixture of formulated 4/6 NRD diet (INVE Aquaculture, Dendermonde, Belgium) and 370 Hikari plankton food (By-Rite Pet Supply, Hayward, California) at a 2:1 volume ratio. Fish care was performed according to standard operating procedures established at FCCL (Lindberg et al., 2013). The pH was measured using a Beckman 240 pH meter and was recorded as 8.6 ± 0.1. Total ammonia concentration was measured with a HACH DR/890 Colorimeter and a HACH AmVerTM Low Range Ammonia Test 'N TubeTM Reagent Set 0–2.5 mg L^{-1} N (HACH Inc.) and ranged between 0 and 0.25 mg/L. Dissolved oxygen (DO) was quantified using a YSI 85 meter and was constant at 11.5 ± 0.8 mg/l before water temperature was altered. All procedures were performed in accordance with UC Davis Institutional Animal Care and Use Committee (IACUC protocol 16233).

Detection of molecular stress response

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Venlo, Limburg, The Netherlands) according to manufacturer's protocols. Extraction efficiency was confirmed using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Total RNA 260/280 and 260/230 ratios ranged from 1.93 to 2.14 and from 1.71 to 2.40, respectively. Complementary DNA (cDNA) was synthesized using 500 ng total RNA, with 50 units of Superscript III (Superscript III Reverse Transcriptase, Invitrogen, Carlsbad, CA, USA), 600 ng random primers, 10 units of RNaseOut, and 1 mM dNTPs (Invitrogen, Carlsbad, CA, USA) resulting in a final volume of 20 µL. Reactions were incubated for 50 min at 50 °C followed by a 5 min denaturation step at 95 °C. Samples were diluted with the addition of 100 µL nuclease-free water to a total volume of 120 µL for subsequent real-time qPCR assessments.

Targets for transcriptomic analysis were identified and selected using previously conducted microarray studies on delta smelt (Connon et al., 2009; Connon et al., 2011a; Connon et al., 2011b; Hasenbein et al., 2014; Jeffries et al., 2015b; Komoroske et al., 2015). Ten genes related to the complement system (*c1, c3, c5*), toll-like receptors (*tlr3*), mitogen-activated protein kinases (*mapk13*), immune cell migration-directing chemokines (*cxcb*), regulators of immune homeostasis (*tnfailp8L2*), anti-proteases (*a2m*), members of the mitogen activated pathway and major histocompatibility complex components (*mhc2, b2m*), as well as five heatshock genes (*hsp30, hsp70, hsp90a, hsp90b, hsc71*) were selected as molecular targets (Table 5).

Primers and probes were designed using Roche Universal Probe Library Assay Design Center (http://lifescience.roche.com/shop/en/us/overviews/brand/universal-probe-library). Fluorescent probes were supplied by Roche or Applied Biosystems and primers were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com). Universal PCR Mastermix was purchased from Applied Biosystems. An assay validation was performed using a six-point standard curve with three replicates, and efficiencies ranged from 93 and 107 % (Table 5). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C.

Quantitative polymerase chain reactions (qPCR) were conducted on the three tissue types to assess tissue-specific gene transcription. QPCR data were analyzed using the Sequence Detection Systems (SDS 2.4.1) Software (Applied Sciences). Gene expression was calculated using the $Log2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) relative to the reference genes 60S Ribosomal Protein L7 (RPL7) and Beta Actin (B-actin), which sustained best scores in GeNorme stability determination (Vandesompele et al.,

2002), and relative to the group which showed no phenotypical signs of infection (10°C). All changes in transcription are illustrated in the Log2 scale. Quantitative polymerase chain reaction (qPCR) and phenotypic responses were linked to evaluate fish condition.

Table 5 Primers and probes used in quantitative PCR analysis for delta smelt (*Hypomesus transpacificus*).

Gene name	Gene code	Primer 5'->3'	Primer 3'->5'	Roche probe #	Efficiency %
Reference genes					
60S Ribosomal Protein L7	*rpl7*	ccgtacagcccgcaaagtt	tgaagtcaatggccagtttgg	33	96
Beta Actin	*bactin*	tggatgccacaggactccata	ccatcggcaacgagaggtt	12	97
Immune response genes					
Mitogen-activated Protein Kinase 13	*mapk13*	tgcgagggcttctgtctga	gtaccttagtccacagagcatctgata	154	103
TNF-alpha induced protein 8-like protein 2	*tnfailp8L2*	aaggagccatgacagccattag	gacatcacggtcttgtcaaagg	39	97
Complement factor 1	*c1*	aggaggactccaggaccagac	gccactggatttggcttgg	95	106
Complement factor 3	*c3*	gcgaagccagacaggtgg	gacctttgtggttctggtgaca	2	99
Complement factor 5	*c5*	gctcagtggctccgtctacaa	ccgtcagagtcacgcagaact	136	96
Chemokine CXCb	*cxcb*	aagcttgtggtctcctgcttgt	gaactgggcatcgtagagctg	74	100
Alpha 2 Macroglobulin	*a2m*	gcctcctggtgaaggctga	tgctggacacagcaaccagt	112	93
Beta 2 Microglobulin	*b2m*	cacctgaccaagagtgtggactt	ggcgcactttgcagatgaa	101	99
Toll-like receptor 3	*tlr3*	gacttgcagcacaacgaggtt	aagattggcgcagcaacttag	100	105
Major histocompatibility complex class II	*mhc2*	caggccgatccaggatatga	taaagaggtgggggtgagc	73	100
General stress response genes					
Heat Shock Protein 30 kDa	*hsp30*	cccagaggacctgtgtgtcaa	cttctccgtcttcccactgact	4	95
Heat Shock Protein 70 kDa	*hsp70*	aatgatgccggtgatcgag	ctacaatctgctcagtgtccctgt	67	107
Heat Shock Protein 90 kDa Alpha	*hsp90a*	aggaggttgaccttgaagatgg	ttgtctttgtcctcagcggtag	63	93
Heat Shock Protein 90 kDa Beta	*hsp90b*	tgtctgaactgctgcgctac	cagtgagggaggtcatctcat	24	95
Heat Shock Cognate 71 kDa	*hsc71*	gcagaggcctacctgggaa	cgctgggagtcgttgaagtag	130	95

Statistical Analysis

Statistical analyses and Principal Component Analysis (PCA) were performed using R Version 3.3.1 (R-Core Team, 2016). Shapiro-Wilk normality and Bartlett tests ($P < 0.05$) were used to determine if the requirements for a one-factor Analysis of Variances (ANOVA) were fulfilled. Differences in gene transcription were assessed using a one-factor ANOVA followed by a Tukey post-hoc test ($P < 0.05$). If the data for did not fit the assumptions of an ANOVA, a Kruskal-Wallis test was used followed by a Dunn's post-hoc test ($P < 0.05$). PCA was conducted using transcription data, per tissue type and from all fish across the three treatments to illustrate differences in responses to infection. Therefore, PCA results were used to link transcriptomic and phenotypic responses, and statistical differences between groups were determined using a one-factor ANOVA followed by a Tukey post-hoc test ($P < 0.05$) on components PC1 and PC2, respectively.

Results

Infection severity

Low, medium and high temperatures corresponded with no, medium and high infection levels (Figure 21, Table 6) which differed significantly between treatment groups. In the 10.2 °C treatment no phenotypical signs of an infection were evident. Fish maintained at 13.2 °C were found to have a weak infection. Between 0 and 8 visible trophonts were identified for each individual fish on the left side of the body in this group (arithmetic mean 2 ± 2), with a maximum cyst size of 220 µm. Delta smelt held at 18.1 °C displayed extensive infection. Trophont numbers for individual fish in this group ranged from 13 to 137, with an arithmetic mean of 69 ± 46 trophonts and a maximum cyst size of 700 µm.

Male and female fish exhibited no differences in weight, length or trophont numbers (Table 6). No other statistical differences were identified among the three groups, nor were there any correlations between infection intensity and fish weight or length.

Figure 21. Different levels of delta smelt (*Hypomesus transcpacificus*) infection with the ciliate *Ichthyophthirius multifiliis*. A) fish from the 10.2 °C tank showed no phenotypical signs of an infection, B) fish held at 13.1 °C showed a light infection (1 trophonts visible) and C) fish from the 18.1 °C tank where strongly infected with *Ich* (137 trophonts visible)

Table 6. Fish data including sex, length, weight and infection intensity (visible trophonts on the left side of the body) of all fish used for phenotypical and molecular assessments.

Infection	Fish	sex	Length [mm]	Weight [g]	Trophonts
high	1	m	75	2.7	37
	2	m	62	1.8	95
	3	m	76	4.2	119
	4	f	73	2.4	60
	5	m	63	1.8	55
	6	m	73	3.1	137
	7	f	72	2.5	117
	8	m	75	2.4	23
	9	f	70	3.1	34
	10	f	85	5	13
medium	11	f	76	3.6	3
	12	f	83	6.2	4
	13	m	79	4.5	4
	14	f	80	4.4	8
	15	m	71	2.6	0
	16	f	83	5.6	0
	17	m	66	2.3	1
	18	m	77	3.1	1
	19	m	73	3.1	1
	20	m	64	1.9	1
low	21	f	77	3.6	0
	22	f	77	4.2	0
	23	f	74	3.5	0
	24	m	74	2.9	0
	25	m	76	4.1	0
	26	m	74	3.4	0
	27	m	82	4.5	0
	28	f	72	2.6	0
	29	m	85	4.3	0
	30	m	74	3.4	0

Tissue-specific transcriptomic responses

Transcriptional responses were specific for each tissue with this effect being most pronounced in the highest infected group. Gill tissue exhibited the greatest differences in transcription between infected fish (medium and high infection) and uninfected (low) fish (Figure 22A). Of the genes coding for the innate immune response, chemokine *cxcb* was significantly increased with infection. *Tnfaip8l2* was significantly increased with medium but not with high infection. Significant decreases were identified for *b2m*, *tlr3* and *mhc2* in fish with high infection levels. Interestingly, there was a decrease in transcription of HSPs (*hsp30*, *hsp70*, *hsp90a* and *hsc71)* in fish with heavy infection levels relative to fish maintained at lower temperatures.

Significant downregulation of *mapk13* was observed in kidney tissue along with an increase of Chemokine *cxcb* at both medium and high levels of infection (Figure 22B).

In spleen tissue, transcription of *mapk13* was decreased in fish with medium infection relative to non-infected and high infection levels, whereas transcription of *tnfaip8l2* and *cxcb* increased in fish from both infection groups. There were differences in stress responsive genes *hsp90a* and *hsc71* with decreased transcription in both infected groups (Figure 22C).

Most of the investigated genes responded with significant changes in transcription in at least one tissue. *Cxcb* transcript levels were constantly elevated in infected fish in all tissues and *cxcb* was the most responsive gene to the *Ich* infection. Complement factors showed tendencies for decreased transcription in all tissues of infected fish, however high variability in complement factor transcription was observed regardless of infection intensity or tissue. *Mapk13* transcription was significantly decreased in both spleen and kidney of infected fish.

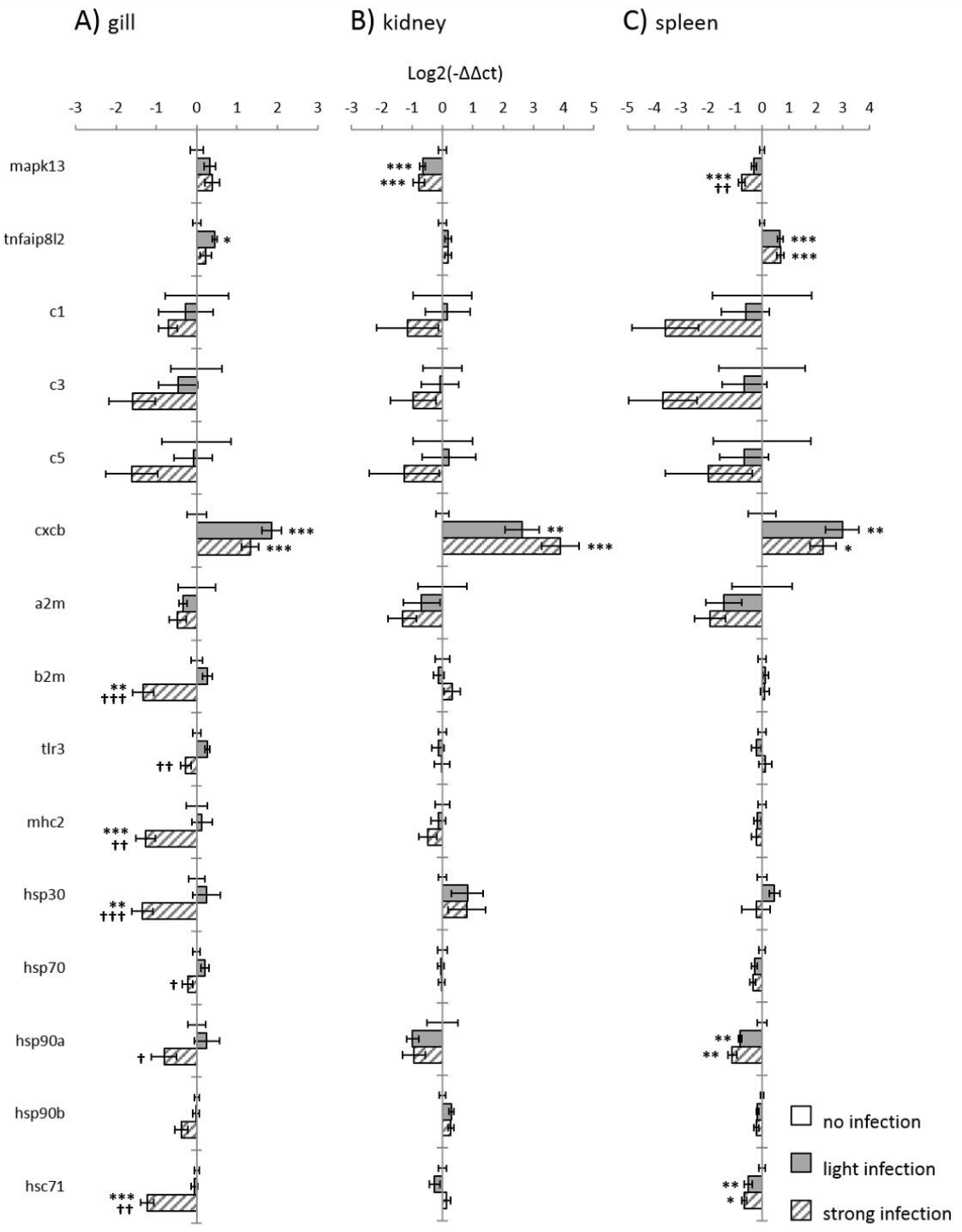

Figure 22. Transcriptional fold change of genes coding for heat shock proteins and important contributors to innate immune response in delta smelt (*Hypomesus transcpacificus*) in relation to the non-infected group. Significant differences between non-infected and lightly infected fish are highlighted by * and differences between lightly infected and strongly infected groups are represented by †. Statistical significance corresponds to * P<0.05, ** P<0.01, *** P<0.001 († respectively). A) gill tissue, B) kidney tissue and C) spleen tissue. All data are shown as mean ± SEM with 10 biological replicates. Genes were normalized to reference genes *rpl7* and *bactin*.

Multivariate differentiation of infection patterns

Principal Component Analysis characterized the transcriptomic data as cumulative percentages, ranging between 77.41 and 84.92. Transcriptomic responses in gill tissue were responsible for differentiating the highly infected fish from the other groups (Figure 23A), whereas responses in kidney tissue provided a distinction between the not infected group and both medium and high infection level groups (Figure 23B). While responses in spleen tissue differentiated between no infection and medium infection, a significant difference between fish from the medium infected and highly infected groups was not found. (Figure 23C). When combining transcriptomic data from all tissues into a single PCA, differentiation between not infected and infected groups were highlighted (Figure 23D), similar to that determined for kidney tissue.

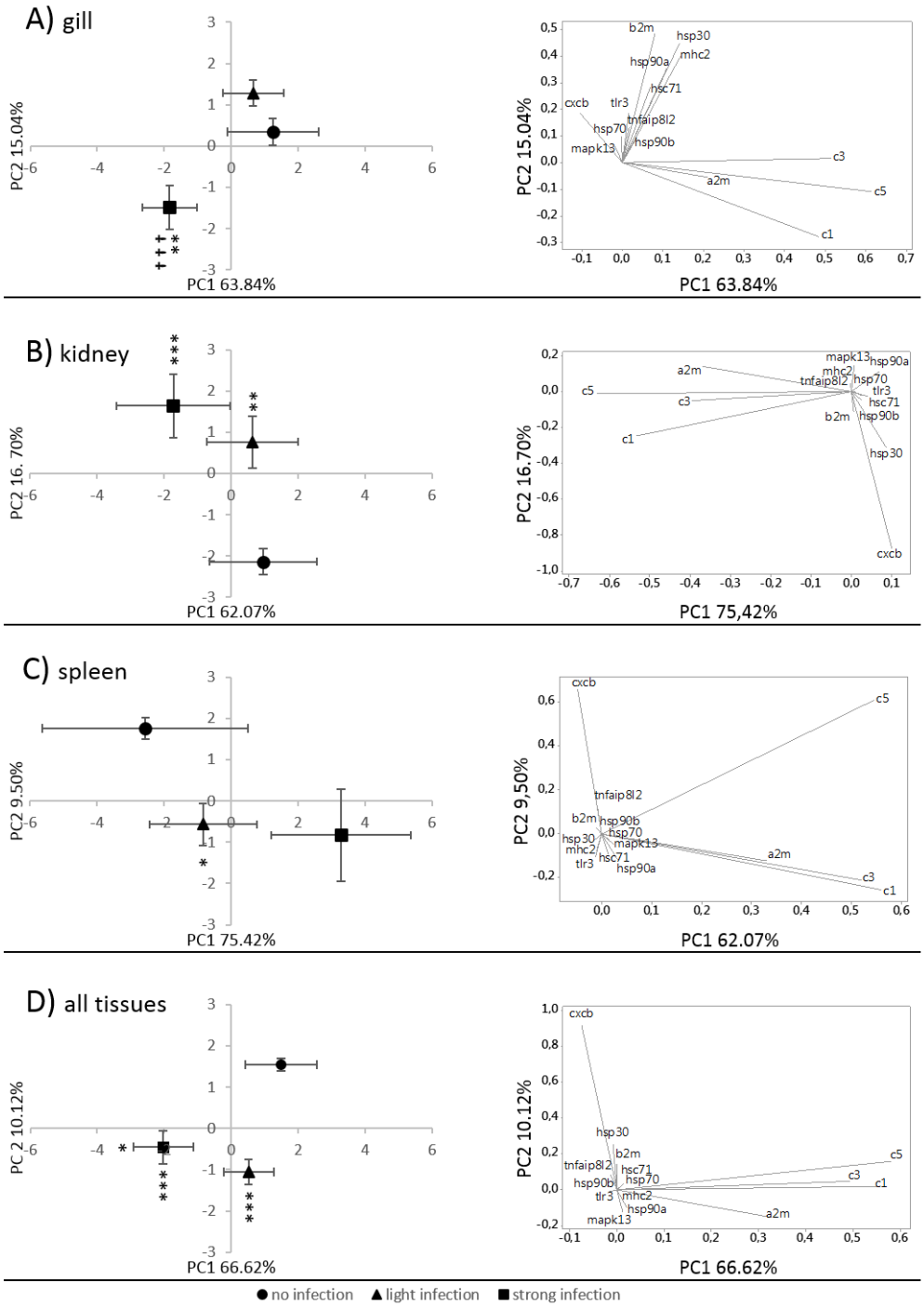

Figure 23. Principal component analysis on qPCR data, illustrating three levels of delta smelt (*Hypomesus transcpacificus*) infection with the ciliate *Ichthyophthirius multifiliis* in A) gill B) kidney C) spleen D) all (gill, spleen, kidney) tissues. Significant differences between not infected and light infected fish are highlighted by * and differences between light infected and strong infected groups are represented by †. Statistical significance corresponds to * P<0.05, ** P<0.01, *** P<0.001 and was determined by PC1 and PC2, respectively. All data are shown as mean ± SEM. Corresponding biplots are illustrating the impact of all genes.

Discussion

To the best of our knowledge, this is the first study using molecular biomarkers to assess severities of *Ichthyophthirius multifiliis* by linking changes in the transcriptome with phenotypical infection patterns using delta smelt as a model. Such characterization of transcriptional changes related to driving immune responses and of genes coding for general stress responses improves our mechanistic understanding of host defense against this common parasite, and can be an early warning system of infection. The combination of molecular targets, including general immune and stress responses, successfully detected different severities of infection and has the potential to be transferred to other fish species and other parasitic infections. Our study can serve as an example of gene transcription in host parasite interactions and can contribute to improving fish health diagnostics in both aquaculture and wild populations.

Chemokine *cxcb* was the most responsive gene, with significantly increased transcription in all investigated tissues. Chemokines take part in attracting immune effector cells to locations of infection, or injuries, and are key elements of translating innate responses into adaptive immune responses (Whyte, 2007). Tissues analyzed in this study were sampled on day six after visible *Ich* infection. The high level of transcription of *cxcb* at this late time point in infection progression highlights the importance of *cxcb* in the translation of innate to adaptive immune response, as well as confirms the physiological impact of the infection level.

Increased *mapk13* transcription has been described in earlier studies focusing on innate immune responses in the context of pro-inflammatory processes (Raingeaud et al., 1995; Cuadrado and Nebreda, 2010; Umasuthan et al., 2015), but was not observed in our assessment. Our study showed *mapk13* responding similarly in spleen and kidney with decreased transcription in both groups with visible infection. This may indicate that fish of our study did not react with inflammation to the parasitic infection, showed only local inflammation where cysts were present, or inflammatory response was already reduced at the time point of sampling. Increased transcription of *tnfailp8l2*, detected in gill and spleen, would support the last assumption. *Tnfailp8l2* has been identified as a negative regulator of both innate and adaptive immune responses to ensure immune homeostasis in vertebrates (Sun et al., 2008).

Beta-2-microgobulin and *mhc2*, both involved in the presentation of peptide antigens, had similar expression patterns with downregulations in gill tissue from highly infected fish, whereas no responses were determined for internal organs. The expression pattern of *b2m* is similar to that reported in a

study investigating *Ich* infection on *Cyprinus carpio L.,* which described an overall low expression without transcriptional changes in internal organs, but with decreased transcription in skin samples (Gonzalez et al., 2007b). An *Ich* infection study with rainbow trout [*Oncorhynchus mykiss* (Walbaum)], however, showed continuous upregulation for *mhc2* in skin and head-kidney, when investigating similar timepoints as in our study (Sigh et al., 2004). The same study also showed a decrease in transcription for *mhc2* in spleen tissue two days post-infection, and similarly to our findings, there were no significant responses at 6 days post-infection (Sigh et al., 2004).

Toll-like receptor 3 responded only in gill tissue with a decrease in transcription in the highly infected group compared to the no infection and medium infected groups. Studies conducted on Channel catfish (*Ictalurus punctatus*) highlight variability in *tlr3* responses at different time points following *Ich* infection (Zhao et al., 2013). Repeatedly detected responsiveness of *tlr3* after infection with ciliate parasites suggests its contribution in antigen presentation of different pathogens. Tlr3 function has been linked to recognition of double stranded viral RNA (Zou et al., 2010).

Surprisingly, we did not detect significant changes in members of the complement system among the different treatments, which has been described after *Ich* infection in earlier studies (Sigh et al., 2004; Gonzalez et al., 2007a). In fact, the complement system displayed the greatest transcriptional variability among individuals of the same infection groups.

Transcription of HSPs stayed stable or was significantly decreased in the highly infected group compared to both other groups in gill tissue, and it was also decreased in both infected groups for *hsp90a* and *hsc71* in spleen tissue. Similar observations were made in sea bream following vibriosis infection (Deane and Woo, 2005). Other studies described different transcriptional responses following bacterial and viral infections in teleosts, which resulted in transcriptional increases for *hsp70* and *hsp90* (Cho et al., 1997; Forsyth et al., 1997; Ackerman and Iwama, 2001). The discriminative power of HSP transcriptomic responses between infection groups was greatest when combined with immune-related genes in the PCA.

The principal component analysis highlights similarities in transcriptomic responses across all investigated tissue types and illustrates the differences between infection levels, similar to phenotypical assessments. While only two genes responded significantly in kidney tissue, PCA on all transcriptional data for this tissue significantly separated the non-infected from the infected groups. The PCA for gill tissue further highlighted that the transcriptomic responses from the highest infected group were significantly distinct from non-infected and medium infection groups, regarding PC1 and

PC2 for statistical assessment, respectively. This greater difference resulted in a grouping of non-infected with lightly infected individuals, suggesting that gills are highly suited for the diagnosis of infections that have reached a level sufficient to elicit immune-specific physiological stress, while internal organs such as kidney or spleen more broadly characterize different intensities of infection. This is not surprising because the gills present a large surface area that directly interacts with the external environment. Combining internal and external tissues for PCA provides a more holistic diagnosis of *Ich* infection in delta smelt.

The strong responses in gill tissue are of special interest for studies on endangered fish species where the possibility of a non-lethal sampling approach to investigate the health status using molecular biomarkers is desired. While non-lethal gill sampling in delta smelt is difficult due to its size and sensitivity, it is a possibility for larger fish such as salmonids, where a small non-lethal biopsy can be used to perform RNA-extraction and analysis on juveniles (Jeffries et al., 2014a). This method of sampling has also been successfully performed for larger adult fish (McCormick, 1993; Jeffries et al., 2014b) but has also been utilized on smaller, tolerant species such the fathead minnow (Cornwell et al., 2013).

Interpretation of responses measured in our study is somewhat complicated in that fish were maintained at different temperatures, thus responses related to temperature acclimation are potentially confounded with the immune responses. The occurrence of *Ich* is positively correlated with temperature, with more effective and rapid parasite development occurring at elevated temperatures (Noe and Dickerson, 1995; Aihua and Buchmann, 2001; Matthews, 2005). This might have led to the different infection levels of treatment groups. Because this was an opportunistic evaluation rather than a controlled infection, there are no true non-infected controls for each thermal treatment. Rather, this assessment presents a more realistic scenario resulting from thermally-induced immunosuppression, coinciding with favorable conditions for *Ich* development. Once the presence of *Ich* in our experimental tanks was discovered, we treated the fish by increasing the salinity to 5 PSU in all groups, including the non-infected fish. This would have undoubtedly altered both the level of infection and the response in fish, as *theront* numbers in newly developing *tomocysts* would be reduced and consequently, resulted in no further infection (Aihua and Buchmann, 2001). Regardless, our approach was sufficiently sensitive to detect ongoing, active immune responses in the infected populations.

It is known that increased thermal stress can alter the transcription of genes involved in the immune system (Pérez-Casanova et al., 2008). However, immune system responses directly relate to the

thermal tolerance of each species: complement activity was reportedly increased in salmonids and reduced in cyprinids at elevated temperatures after long-term acclimation (Collazos et al., 1994; Nikoskelainen et al., 2004). Thermal stress evaluations on delta smelt have demonstrated impacts on metabolism and immune system processes at acute elevated temperatures close to the critical thermal maximum of this species (Komoroske et al., 2015). The temperature treatments in the present study are within the temperature range of wild delta smelt (Jeffries et al., 2016), suggesting these temperatures are in a range that delta smelt routinely experience in the wild. This is supported by the lack of a classic heat shock response in larval delta smelt that were acutely exposed to 20 °C after acclimation to 14 °C (Jeffries et al., 2016). Therefore, significant differences in transcript levels at 18.1 °C implies either direct causation from infection, or elevated risk of infection at higher temperatures due to low thermally-induced immune system thresholds for this species. The decreased transcription for heat shock genes in strongly infected fish could be an indicator for impaired thermal tolerance caused by the parasitic infection (Bruneaux et al., 2016). Further investigations could help to evaluate the risk of pathogen infection and thermal stress, and resulting disease. This is particularly important for the delta smelt because climate change is predicted to result in rapid shifts in environmental conditions, causing earlier and shorter spawning windows for this species (Brown et al., 2016). These shifts in environmental conditions are already negatively impacting habitat suitability within the San Francisco Estuary and Watershed (Brown et al., 2016). This study highlights the importance of evaluating sublethal thresholds, to multiple environmental stressors, including pathogens in delta smelt. Our approach is directly transferable to monitoring and evaluation of a broad range of pathogens (Ribeiro et al., 2010; Miller, 2014). Recent years have seen the development of molecular technology to include environmental DNA and community metagenomics. Combining these tools with transcriptional assessments will allow for a broader evaluation of pathogen presence/absence, quantitation and specific disease-based responses, and severity of infection in fish species of interest. This is particularly important for the critically endangered delta smelt that is near extinction (Miller et al., 2012). Because the innate immune signaling pathways are highly conserved in teleosts (Stein et al., 2007), surrogate species could potentially be used to evaluate the risk of pathogen infections at locations where delta smelt are expected to reside.

Conclusion

This study demonstrated that molecular biomarkers can identify different severities of a parasitic *Ich* infection in delta smelt. We could successfully separate different infection levels in all investigated tissues, with the strongest results from gill and kidney tissue. The identical classification in phenotypic appearance and molecular approach, as well as tissue-specific responses following different intensities

of *Ich*-infection, confirmed our original hypothesis of tissue-specific responses dependent on the severity of infection. A focus on innate immune and stress related genes, linked to a general pathogen response in teleosts, suggests transferability to other fish species and different classes of pathogens or diseases. Identification of molecular markers targeting fundamental host responses associated with infections, such as immune and stress related genes, contributes to the establishment of new techniques to assess the health status of fish from natural habitats and in aquaculture.

6. General Discussion

This thesis describes the effects of abiotic (PCB 95 and bifenthrin) or biotic (ciliate parasite *Ichthyophthirius multifiliis*) stressors in model and non-model fish species, by combining behavioral experiments and evaluation of transcription in key genes of pathways elementary for neurodevelopment and immune responses.

The techniques applied in this thesis were mainly developed in the fields of neurotoxicology and ecotoxicology. Both fields use similar to identical tools to identify adverse effects of chemicals and stressors, including molecular methods, histopathological techniques or behavioral experiments. While both fields have developed next to each other, there is a gap between both, resulting from differing directions in their research focus. Neurotoxicology has the goal to assess effects of chemicals in individuals and use them to predict mechanisms of action relevant for the human species (Segner, 2011), ecotoxicology in contrast aims to evaluate the effects of contaminants in populations, ecosystems or the biosphere (Balling, 2009; Newman, 2009). Neurotoxicologists therefore use a repertoire of few well-established model species, such as mice (*Mus musculus*), rats (*Rattus norvegicus*), nematodes (*Caenorhabditis elegans*), or zebrafish (*Danio rerio*), the latter was also used as a model in chapter 2 and 3 in this thesis. Ecotoxicology in contrast, includes as well non-model species to answer ecosystem specific questions, such as inland silversides and the delta smelt, which were used as models in chapters 4 and 5 in this thesis. Recent ecotoxicological risk assessments started to incorporate the evaluation of different levels in biological organization (Ankley et al., 2010), which has the potential to become a useful tool in neurotoxicology. Similarly, the extensive information already described for well-established model species (like zebrafish), can be used in an ecotoxicological context, to evaluate complex molecular questions in detail. A fundamental objective in the studies presented herein, was the development of experimental designs to obtain findings valuable for both scientific fields and to show how neurotoxicology can profit from experimental setups, so far mainly used in ecotoxicological assessments. Therefore, chapters with components originating from both scientific branches (neuro and ecotoxicology) are included in this thesis.

The initial step to develop zebrafish as a high-throughput *in-vivo* model to investigate adverse effects in mTOR and RyR-dependent Ca^{2+} signaling, both pathways are important for neurodevelopment and are also highly conserved within eukaryotes (Hall, 2008; Mackrill, 2012), has been described in chapter 2. This study describes the establishment of molecular tools to assess transcriptomic responses in key genes of both pathways and their evaluation during undisturbed larval development. Additional to the baseline screening, transcriptomic alterations in both pathways were

measured following an exposure to PCB 95, which is known to promote dendrite growth and synaptic plasticity in mammalian models (Pessah et al., 2010; Wayman et al., 2012b; Wayman et al., 2012a). A validation of the established high-throughput *in-vivo* screening method is described in chapter 3, by evaluation of developmental exposures to picomolar (ng/L) concentrations of the pyrethroid insecticide bifenthrin in zebrafish. The application of molecular biomarkers evaluating mTOR and RyR-dependent signaling detected bifenthrin induced alterations in transcription in genes of both pathways. In addition, behavioral assessments were conducted to evaluate effects at another level of biological organization. Linking molecular responses with other levels of biological organization has been emphasized by (Ankley et al., 2010) within a framework designed to improve ecotoxicological risk assessment Therefore, not only effects during acute exposure scenarios but also after a recovery period are evaluated in chapter 3. Although zebrafish have become a popular model in both neuro- and ecotoxicology, the transferability of results to fish species from different ecosystems could be at question. The transferability was tested by measuring effects of developmental exposure to picomolar concentrations of bifenthrin in inland silversides (*Menidia beryllina*), a representative fish species of estuarine ecosystems recently developed as a molecular model (Jeffries et al., 2015a; Brander et al., 2016b; DeCourten and Brander, 2017). Results presented in chapters 2-4 illustrate the sensitivity of molecular markers by successfully detecting adverse effects in conserved pathways, which were caused by bifenthrin exposure. These results also serve as an example of interspecies transferability, since both model species were similarly affected by exposure to picomolar (ng/L) concentrations of bifenthrin. Molecular techniques were further used to assess fish health following confrontation with an abiotic stressor in chapter 5, by focusing on conserved signaling mechanisms. Therefore, the transcription of genes, coding for elementary members of the innate immune system and a general stress response, was assessed in delta smelt (*Hypomesus transpacificus*) infected with the ciliate parasite *Ichthyophthirius multifiliis*.

6.1 Establishment of a high-throughput *in-vivo* screening method in zebrafish

The rapidly growing number of new synthesized chemicals in addition to the huge amount of existing chemical compounds (133 million registered organic and inorganic substances; www.cas.org), increases the difficulty to evaluate effects induced by emerging pollutants and their metabolites (Thomaidis et al., 2012; Gavrilescu et al., 2015). One approach to face this challenge is the development and application of high-throughput modelling tools. High-throughput screenings started to gain popularity in the field of drug discovery during the 1990s (Wildey et al., 2017). The fast procedures are mainly accomplished with help of automatization and were broadly used to evaluate effects of drugs or small molecules in cell-lines, allowing rapid measurement of multiple contaminants (Wildey et al.,

2017). Another high-throughput method is the assessment of behavioral toxicity, which has been applied in zebrafish (MacRae and Peterson, 2015). There are also examples in literature, defining embryo tests in zebrafish as high-throughput methods that measure effects of chemicals until a developmental stage of 120 hpf (Truong et al., 2016). This thesis also evaluates the effects of chemical compounds during an exposure period lasting between five to seven days in developing fish larvae, but also incorporates "classical" high-throughput tools to measure behavioral effects. The method described in this thesis aims for a more comprehensive assessment of chemical induced effects, compared to high-throughput systems based on cell lines. To avoid confusion the applied method developed in this project is referred to as high-throughput *in-vivo* method. Mammalian models are well suited to investigate adverse effects and diseases in vertebrates and humans very detailed, because of high similarities in genomes, anatomy, cell biology and physiology (Lieschke and Currie, 2007), but lack the potential for high-throughput screening because of their slow development. Zebrafish in contrast develop rapidly and express over 70 % homologs of human genes (Howe et al., 2013), and have therefore become the only vertebrate model with the potential of high-throughput screenings in vivo (Garcia et al., 2016). The completely sequenced zebrafish genome offers access to an extensive pool of genetic information, resulting in a high number of potential molecular targets to screen for adverse effects. Therefore, the next step towards the development of high-throughput *in-vivo* screening tools is the selection of suitable pathways and signaling mechanisms. A promising approach to use zebrafish and even other fish species as human models, is the focus on well conserved pathways (Perkins et al., 2013). Chapter 2 and 3 describe the development of mTOR and RyR-dependent Ca^{2+} signaling as a high-throughput-screening tool, by evaluating chemical induced adverse effects in both pathways using zebrafish as a model.

Findings presented in chapter 2 demonstrate that exposure to PCB 95, a compound belonging to the neurotoxic group of non-dioxin-like PCB congeners (Pessah et al., 2010; Stamou et al., 2013), did alter transcription in a subset of genes belonging to mTOR and RyR-dependent Ca^{2+} signaling in developing zebrafish. The described high-throughput *in-vivo* screening method effectively identified transcriptomic alterations in signaling molecules of the RyR signaling pathway, which were also affected by PCB 95 exposures in mammalian models (Wayman et al., 2012b; Wayman et al., 2012a). Thus, this approach successfully determined adverse effects of PCB 95 exposures in the zebrafish model, suggesting zebrafish as reliable model to predict effects on RyR signaling in higher vertebrates. In addition to the validation of effects in RyR signaling, significant impacts were described for transcripts of the mTOR signaling pathway. To verify observed results, it would be a logical next step to assess transcription of mTOR signaling molecules in mammalian models exposed to PCB 95.

The evaluation of pesticides is in particular an area with urgent need for high-throughput *in-vivo* screening methods, since the production and synthesis of new compounds is constantly increasing (Tilman et al., 2001; Popp et al., 2013). Estimations predict that 99.9 % of all deployed pesticides are directly contaminating soil, air and water bodies including ground water (Pimentel and Levitan, 1986; Arias-Estévez et al., 2008), putting wildlife and humans in high risk of exposure. Therefore, the high-throughput *in-vivo* method developed to detect impacts in mTOR and RyR-dependent Ca^{2+} signaling was used to evaluate transcriptomic effects caused by developmental exposures to picomolar (ng/L) concentrations of bifenthrin. Findings presented in chapter 3 identified transcriptomic alterations in several genes of both pathways, during acute exposure and after a recovery period. *Ryr2a* and *ryr3* were significantly upregulated at 3 dpf, which correlates to the peak period of synaptogenesis (Saint-Amant and Drapeau, 1998; Brustein et al., 2003a). Both transcripts have been described as the CNS specific RyR paralogs in neuronal tissue and are particularly expressed in the developing zebrafish (Wu et al., 2011; Frank et al., 2017a). Thus, detected alterations within RyR signaling have the potential to elicit effects in other levels of biological organization. Findings in chapter 3 also report increased larval movement of fish exposed to bifenthrin when exposed to external stimuli, such as light or an olfactory predator-cue. While the direct link between alterations on transcriptomic and behavioral level are only suggestions at this stage, the observed effects complement each other and show a comprehensive picture how environmental relevant concentrations of bifenthrin cause adverse effects during acute exposure (measured with transcriptomic high-throughput *in-vivo* screening) and delayed hyperactivity (shown by behavioral experiments at the end of a 2-week recovery period). Since hyperactivity can increase the risk of predation, because of a higher likelihood to get recognized by predators (Zhou and Weis, 1998), nanomolar concentrations of bifenthrin can have severe consequences for fish in the wild.

The measured alterations in neurodevelopmental important pathways highlights the possibility, that bifenthrin might contribute to the occurrence of neurodevelopmental disorders (NDDs), which have increased significantly over several decades (Boyle et al., 2011; Baio, 2012; Landrigan et al., 2012). While mainly genetic factors are associated with increased risk of NDDs (Aldinger et al., 2011; Betancur, 2011; State and Levitt, 2011), for many NDDs there is incomplete penetrance of mutations and significant clinical heterogeneity, even within monozygotic twins (Vorstman and Ophoff, 2013). These observations suggest that individual risk for NDDs is influenced by a second insult, such as exposure to neurotoxic chemicals during sensitive windows of neurodevelopment (Landrigan et al., 2012; Stamou et al., 2013). Consistent with findings in chapter 3, pyrethroids have been recently implicated as an environmental risk factor for NDDs (Oulhote and Bouchard, 2013; Shelton et al., 2014; Viel et al., 2015; Wagner-Schuman et al., 2015).

The established high-throughput *in-vivo* method developed in zebrafish has effectively identified alterations in mTOR and RyR-dependent Ca^{2+} signaling following exposure to two chemical compounds, regularly found in aquatic ecosystems and sediments. Findings in chapter 2 validate the approach by detection of PCB 95 induced alterations in signaling molecules, also altered by PCB 95 exposures in mammalian models (Wayman et al., 2012b; Wayman et al., 2012a). Chapter 3 describes adverse effects caused by developmental exposure to the pyrethroid insecticide bifenthrin, identifying bifenthrin as a new neurotoxic compound in vertebrates. Both studies serve as example for high-throughput *in-vivo* screening assessments to detect chemical induced effects in mTOR and RyR-dependent Ca^{2+} signaling and serve as basis for further assessments with other chemical compounds.

6.2 Transfer of established methods: from model to non-model species

Since ecotoxicological assessments often focus on non-model species to assess ecosystem specific questions (Balling, 2009; Newman, 2009), the approach developed with zebrafish (chapter 3) was used to evaluate bifenthrin induced effects in developing inland silversides (chapter 4). Thus, transcriptional alterations were as well evaluated in mTOR and RyR-dependent Ca^{2+} signaling and linked to behavioral endpoints. The small euryhaline fish species, native in the east coast of North America and widespread in water systems of the west-coast after its introduction in the 1970s (Middaugh and Hemmer, 1992; Fluker et al., 2011), has been recently established as estuarine model species (USEPA, 2002; Brander et al., 2012a). Furthermore, recent microarray assessments provide a suite of genetic sequences, elementary for transcriptomic assessments in *M. beryllina* (Jeffries et al., 2015a; Brander et al., 2016b). This species was particularly selected to evaluate bifenthrin effects in an environment with lower temperature (23±1 ˚C) and higher salinity (15 ppt), compared to the zebrafish approach (27±1 ˚C; 0 ppt salinity).

The transcriptomic assessment of mTOR and RyR-dependent Ca^{2+} signaling detected alterations in both pathways. *Ryr3* transcription was altered during the embryonic stage on 5 dpf, which is similar to observations described in zebrafish at 3 dpf. For zebrafish the measured influence in *ryr3* transcription correlates with the peak phase of synaptogenesis (Saint-Amant and Drapeau, 1998; Brustein et al., 2003a). Regarding the slower development of inland silversides, it is highly likely that effects measured in transcripts coding for RyR-receptors, which have been described as CNS paralog in zebrafish (chapter2; Wu et al., 2011), occur in a sensitive period of neuronal connectivity as well. Further research, such as in-situ hybridization is needed to verify this assumption. Furthermore, the mTOR signaling molecule was altered at 5 dpf during acute exposure and after the recovery period on 21 dpf. Again, high similarities between both model species could be measured at the transcriptional level.

Similar in both model species was the behavioral hyperactivity measured in response to a predator cue, which is likely triggered via olfactory components, as described in chapter 4.

Overall the high-throughput *in-vivo* method developed in zebrafish was successfully transferred to inland silversides and serves as example for transferability of this screening method to other fish species. High similarities of effects induced by exposure to picomolar (ng/L) concentrations bifenthrin could be observed on transcriptomic level and in behavioral assessments in both species. Thus, serving as example how accurate the zebrafish model can predict results in other fish species, when focusing on conserved pathways like mTOR and RyR-dependent Ca^{2+} signaling. Similarly, the assessment with inland silversides demonstrates how alternative species may be of use to evaluate human and ecological risk assessment at the same time.

6.3 Utility of recovery periods in neurotoxicology

Recovery periods, as described in chapter 3 and 4, are regularly incorporated in toxicological risk assessments. While recovery periods used to evaluate toxicologic effects of pharmaceuticals in drug discovery underlie regulatory guidelines (Pandher et al., 2012; Perry et al., 2013), durations and designs of recovery periods in ecotoxicological studies strongly depend on the research question. The evaluation of continuing effects caused by pesticide exposure can include numerous parameters, such as hematological criteria (Adhikari et al., 2004), enzymatic activity (Morgan et al., 1990), or reports of transcriptomic and behavioral alterations (Beggel et al., 2011). Although recovery periods are regularly used in different toxicological assessments, their use is not common in neurotoxicological studies. This is surprising, since neurotoxic compounds have the potential to cause long-term or delayed effects, especially when organisms are exposed during periods of neuronal development, effects can manifest at later life stages (Levin et al., 2003). Findings in chapters 3 and 4 are an example of such delayed effects, caused by developmental exposure to nanomolar concentrations of bifenthrin. Both fish species did not show alterations in behavior during the exposure period, but at the end of a 14-day recovery period. Therefore, the integration of recovery periods has the potential to recognize delayed and long-term effects of neurotoxic compounds and the data collected in this thesis, suggests a broader application of recovery periods in neurotoxicological studies, especially when evaluating developmental exposures. Since studies with recovery periods are more time consuming, it would be helpful to first pre-select chemical compounds by evaluating potential effects on transcriptional level via high-throughput *in-vivo* screenings in representative signaling molecules important for neurodevelopmental processes, before a recovery period is initiated.

6.4 Conserved signaling mechanisms to monitor pathogens in teleosts

A common goal postulated half a century ago, is the reduction of animals in experimental setups including animal testing (Russell et al., 1959). The advantages of investigating signaling molecules of conserved pathways to detect adverse effects caused by chemical exposure has been discussed comprehensively above and the transferability of results to other fish species or vertebrates has been suggested in literature (Perkins et al., 2013) and was demonstrated with help of transcriptional high-throughput *in-vivo* screenings in neurodevelopmental important pathways in two fish species (chapter 3 and 4); thus, illustrating how molecular methods applied on conserved signaling mechanisms, have the potential to reduce animal testing. Environmental monitoring programs are another scientific area that are increasingly incorporating molecular methods, and recent studies successfully diagnosed pathogen and parasitic infections, or disease in fishes, with molecular tools (Gonzalez et al., 2007a; Gonzalez et al., 2007b; Miller et al., 2011; Connon et al., 2012; Jeffries et al., 2014a). Findings in chapter 5 describe the use of methods described in chapter 2-4, to evaluate different severities of an infection with the ciliate parasite *Ichthyophthirius multifiliis* in delta smelt. The core of the assessment was the transcriptomic evaluation of highly conserved signaling mechanisms in teleost fishes, such as signaling molecules of an innate immune response (Stein et al., 2007) and the general stress response via heat shock proteins (Westerheide and Morimoto, 2005; Simmons et al., 2009). Both signaling mechanisms are immediately activated after host contact with a non-specified pathogen, before the adaptive immune response activates a specific immune response (Magnadóttir, 2006; Alvarez-Pellitero, 2008). Transcriptomic responses were conducted in gill, kidney and spleen tissue originating from delta smelt grouped into three different phenotypical severities of *Ich* infection. Molecular markers could successfully separate different levels of infection in all investigated tissues. Therefore, this study presents a set of genes linked to a general pathogen response in teleosts, suggesting transferability to other fish species and different classes of pathogens or diseases. The established molecular markers offer an extension or complementation of environmental risk assessment by evaluating fish health in natural environments or in aquaculture.

6.5 Conclusion

The research presented in this thesis extends current molecular screening methods in the field of neurotoxicology, ecotoxicology and environmental risk assessment. The establishment of molecular markers belonging to key members of mTOR and RyR-dependent Ca^{2+} signaling in zebrafish provides a strong basis to measure chemical induced neurotoxicity in vertebrates. Furthermore, the integration of a recovery period (chapter 3 and 4) describes an approach to identify delayed or long-term effects

resulting from the developmental exposure to chemical compounds. A first application of this method could successfully identify identical signaling molecules affected by PCB 95 exposure in the zebrafish as described for rodent models (Stamou et al., 2013; Wayman et al., 2012b), which illustrates the power of using conserved signaling mechanisms as molecular markers. The methods presented in this thesis could further be used to identify neurotoxic characteristics of bifenthrin and results suggest bifenthrin as an environmental risk factor for NDDs (chapter 3). The transferability of the screening method to other fish species has been demonstrated with an assessment in inland silversides (chapter 4). Exposures to nanomolar concentrations of bifenthrin elicited comparable adverse effects in both fish species, including transcriptional alterations in of mTOR and RyR dependent Ca^{2+} signaling and similar responses when confronted with an olfactory predator cue at the end of a recovery period. Thus, highlighting the potential of using findings received from conserved signaling mechanisms in non-model species to predict effects in other vertebrates, including humans (Perkins et al., 2013). This thesis further describes the establishment of a second screening method relying on the same principle: conserved signaling mechanisms. The evaluation of an *Ichthyophthirius multifiliis* infection in the endangered delta smelt detected transcriptional alterations in key members of the innate immune and the general stress response. Different severities of infection could be successfully identified by analyzing molecular signaling molecules belonging to the innate immune and a general stress response. A focus on genes linked to a general pathogen response in teleosts suggests transferability to other fish species and different classes of pathogens. Overall, methods established in this thesis have the potential to extend and complement current screening methods in neurotoxicological, ecotoxicological and environmental risk assessments.

6.6 Outlook

The molecular methods described in chapter 2, 3 and 4 serve as example of a high-throughput *in-vivo* approach to evaluate neurotoxicity of chemical compounds via mTOR and RyR-dependent Ca^{2+} signaling mechanisms. The application of this screening method should be considered in future risk assessments, before evaluations of effects caused by exposures to chemicals are initiated in mammalian models. The methods and approaches described in this thesis illustrate the potential to reduce experiments with mammalian models.

Although the high-throughput *in-vivo* method identified similar effects caused by environmental realistic concentration of bifenthrin in two fish species, further research should examine if developmental exposure scenarios with bifenthrin cause delayed hyperactivity in rodent models and affect the mTOR and RyR-dependent Ca^{2+} signaling mechanisms in a similar way. Especially, since hyperactivity has been reported in offspring of mice exposed to deltamethrin (Richardson et al., 2015).

The focus on conserved signaling mechanisms linked to a general immune and stress response (chapter 5) suggests transferability of this method to other fish species and different diseases. Further studies evaluating transcriptomic effects of an *Ich*-infection in other fish species, or the evaluation of additional pathogens is needed, before the method has the potential to become included in risk assessments.

The research projects presented herein have extended the current knowledge of effects, caused by single stressors (bifenthrin, *Ich*-infection) in different fish species. Since both stressors are regularly found in freshwater ecosystems, further studies are needed to evaluate potential synergistic effects of both stressors. Synergistic effects have been described in chinook salmon exposed to esfenvalerate and infectious hematopoietic necrosis virus, when comparing to effects from exposure to the single stressors (Clifford et al., 2005; Eder et al., 2007; Eder et al., 2008). Of special interest in future applications is the evaluation of mTOR signaling, since this pathway is also a fundamental regulator of innate and adaptive immune homeostasis (Weichhart et al., 2008; Powell et al., 2012).

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Anna –

because every bird deserves
a dedication at some point!

8. Publication list

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Frank, D.F., Brander, S.M., Hasenbein, S., Harvey, D.J., Lein, P.J., Geist, J., Connon, R.E., 2019. 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). Aquatic Toxicology. 206, 1-13.

8.1 Oral and poster contributions related to this thesis

Oral presentations

Frank, D.F., Miller, G.W., Connon, R.E., Geist, J., Lein, P.J.: *Polychlorinated biphenyl (PCB)-induced neurodevelopmental impairments in zebrafish (Danio rerio).* Invited Talk, NORCAL SETAC, April 2016

Frank, D.F., Miller, G.W., Harvey, D.J., Brander, S.M., Geist, J., Connon, R.E., Lein, P.J.: *Toxicity of bifenthrin during early zebrafish (Danio rerio) development.* Invited Talk, SETAC Europe 27th Annual Meeting, Brussels, May 2017

Poster presentations

Frank, D.F., Hasenbein, M., Eder, K., Jeffries, K.M., Geist, J., Fangue, N.A., Connon, R.E.,: *Diagnosing disease state in delta smelt (Hypomesus transpacificus) following infection by Ichthyophthirius multifiliis.* Interagency Ecological Program Annual Workshop, Folsom, CA, March 2015

8.2 Author contributions to the chapters

Chapter 2

DFF, GWM, REC and PJL conceived of and designed the experiments; GWM provided technical advice and support; DFF conducted the experiments and analyzed the results. DFF wrote the initial draft of the manuscript; REC and PJL made extensive edits. All authors reviewed and approved of the final manuscript.

Chapter 3

DFF, REC, GWM, PJL and JG conceived the concept for the paper; GWM provided technical advice and support; DFF conducted the experiments and analyzed the results. Data analyses were conducted by DFF under guidance and with support by PJL, REC, GWM, DJH and SMB. The mixed model algorithm was written by DJH. DFF wrote the initial draft of the manuscript; REC, JG and PJL made extensive edits. All authors reviewed and approved of the final manuscript.

Chapter 4

DFF, REC, SMB, PJL and JG conceived the concept for the paper. DFF conducted the experiments and 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 of the final manuscript.

Chapter 5

DFF, RC and JG conceived the concept for the paper, and DF conducted the transcriptomic analyses based on fish exposures conducted by KE and MH under guidance by NF. Data analyses was primarily

conducted by DF under guidance and with support by RC and KJ. The paper was primarily written by DF with the support of RC and JG, as well as revisions by all authors. All authors have approved the publication of this research paper.

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10. Appendix

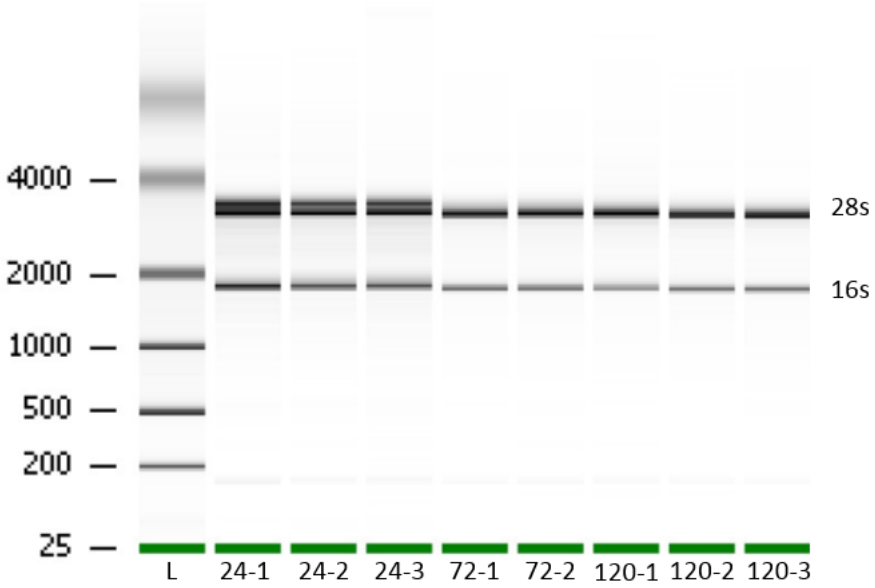


Figure S1. Electrophoresis run file summary. Bioanalyzer output corresponding to Table S1 RIN scores. Illustrated are all 24 hpf, 72 hpf and 120 hpf samples, with the exception of one 72hpf sample (72-3).

Table S1: Bioanalyzer results. RNA integrity ratio (RIN) scores above 8 were considered good quality.

Sample	RIN
24-1	8.50
24-2	8.70
24-3	8.50
72-1	10
72-2	9.90
120-1	9.80
120-2	10
120-3	10

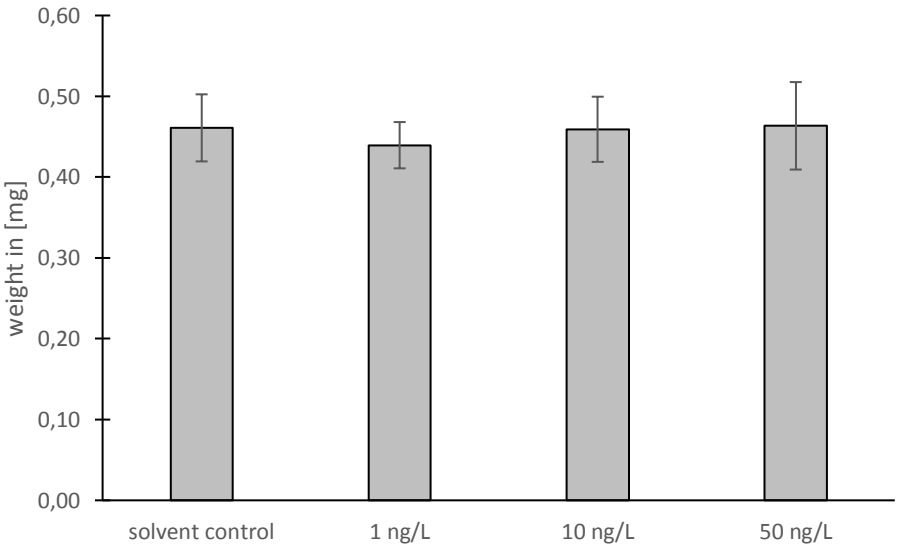


Figure S2. Dry weight of fish used for the behavioral assessments. After Fish were euthanized on ice they were transferred into aluminium dish (grouped as biological replicates) and stored in an incubator over night, to evaporate all water from the tissues. After all water was evaporated fish were weighed with a precission scale (accuracy 0.000 g).

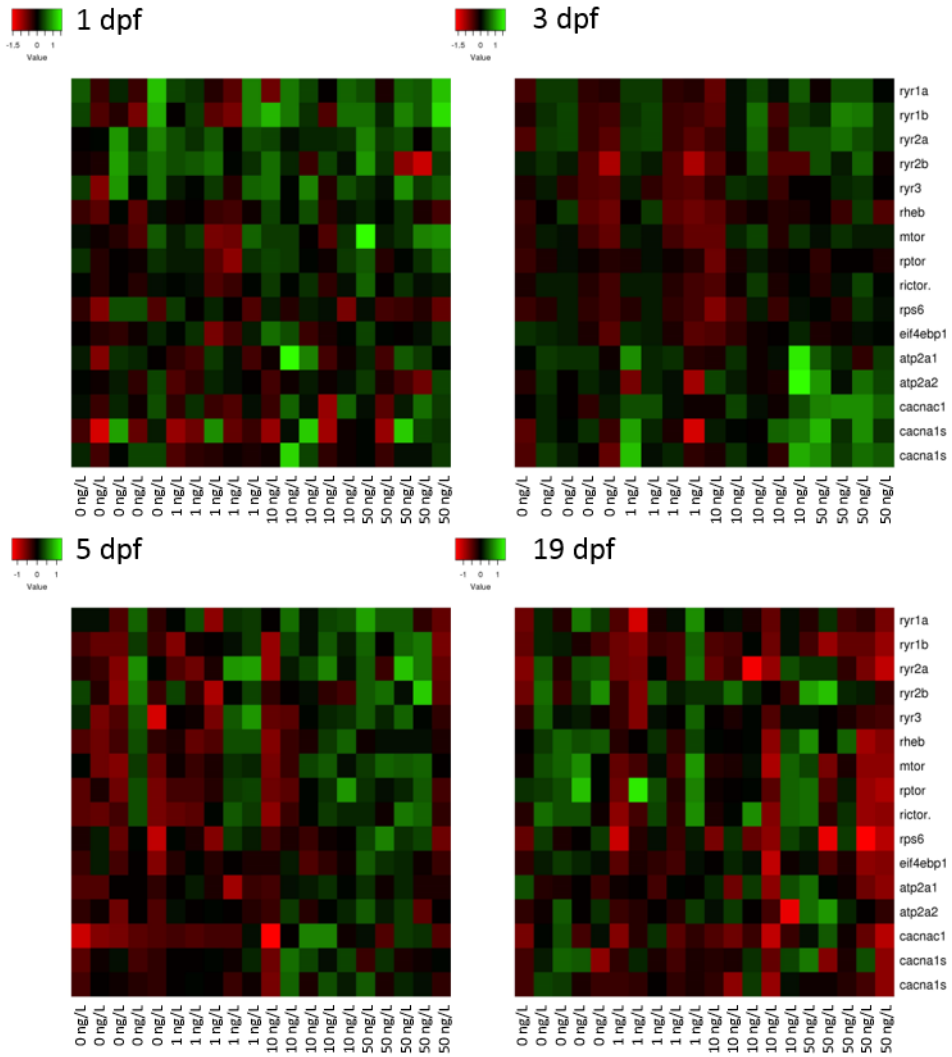
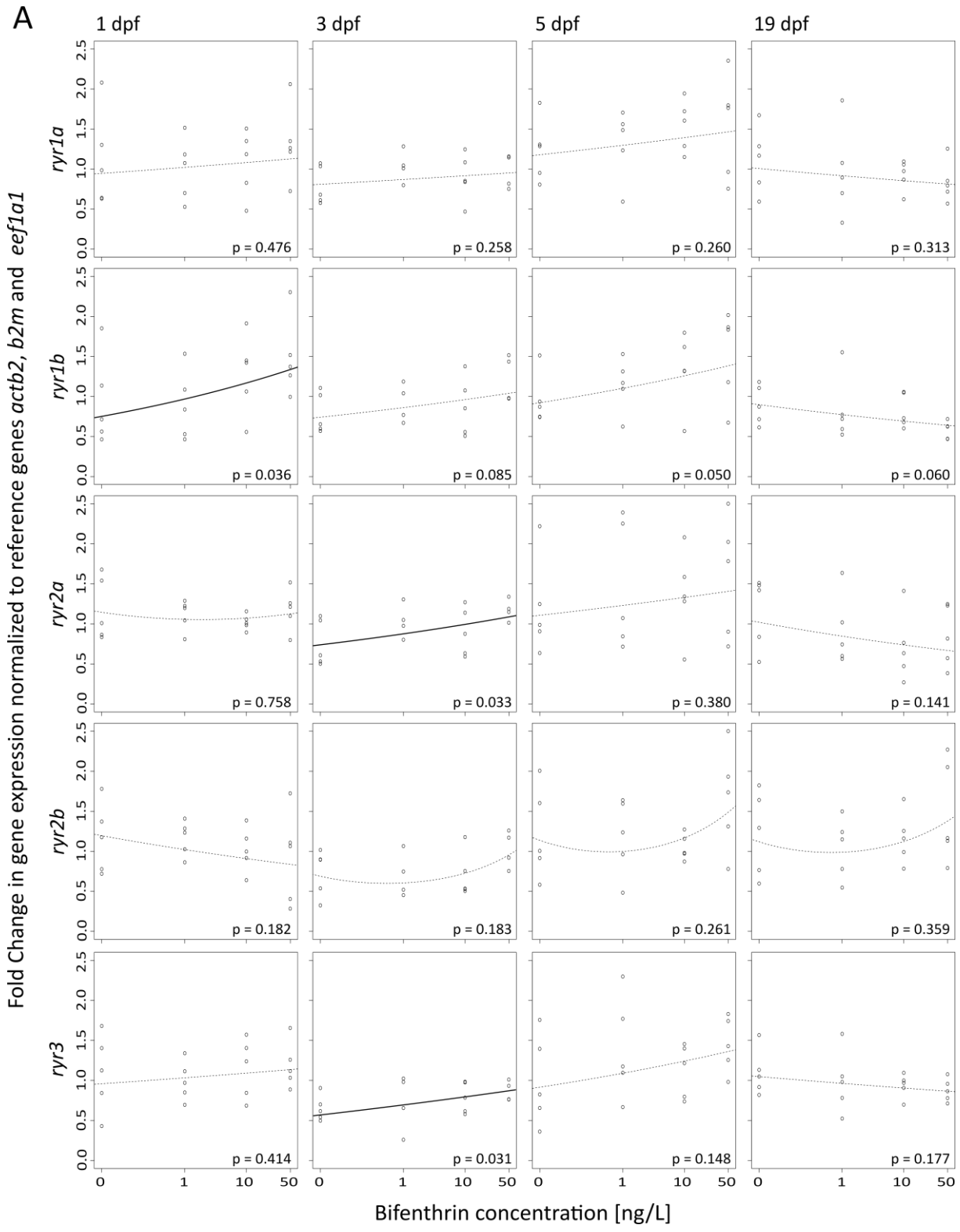
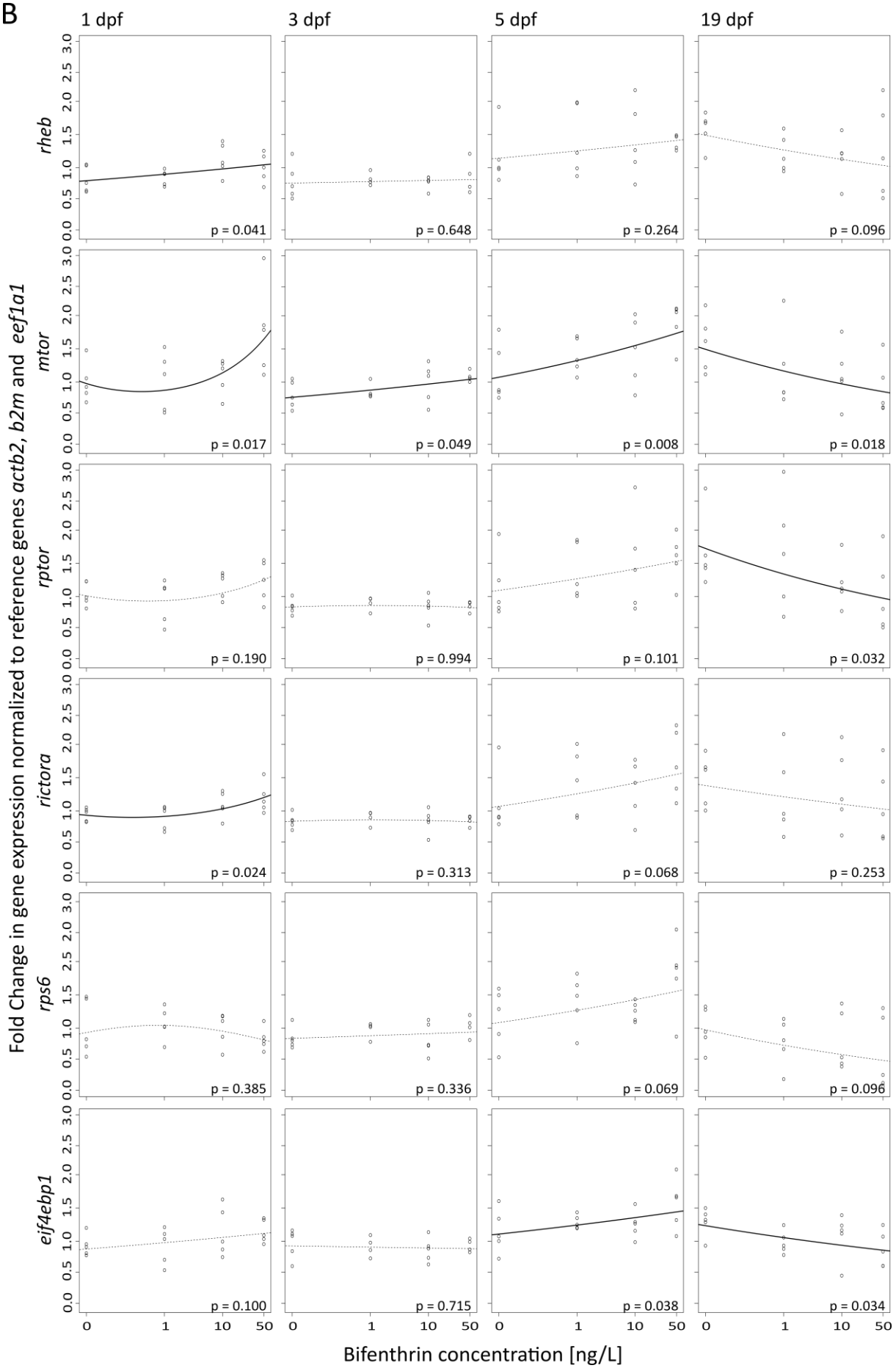


Figure S3. **Heat map of all investigated genes at 1,3,5, 19 dpf.** The color scale ranges from red for log ratios −1.5 (1 and 3dpf) or -1 (5, 19dpf) to green for log ratio 1 (1, 3, 5, 19dpf). Each gene is represented by a single row of colored boxes and each concentration (n=5 biological replicates) is represented by a single column. Gene expression was calculated using the Log2−ΔΔCT method (Livak et al., 2001), relative to the reference genes *actb*, *b2m* and *eef1a1*.


A
1 dpf
3 dpf
5 dpf
19 dpf
ryr1a
ryr1b
ryr2a
ryr2b
ryr3
p = 0.476
p = 0.258
p = 0.260
p = 0.313
p = 0.036
p = 0.085
p = 0.050
p = 0.060
p = 0.758
p = 0.033
p = 0.380
p = 0.141
p = 0.182
p = 0.183
p = 0.261
p = 0.359
p = 0.414
p = 0.031
p = 0.148
p = 0.177
0.0
0.5
1.0
1.5
2.0
2.5
0
1
10
50
Fold Change in gene expression normalized to reference genes actb2, b2m and eef1a1
Bifenthrin concentration [ng/L]


B
1 dpf
3 dpf
5 dpf
19 dpf
rheb
mtor
rptor
rictora
rps6
eif4ebp1
p = 0.041
p = 0.648
p = 0.264
p = 0.096
p = 0.017
p = 0.049
p = 0.008
p = 0.018
p = 0.190
p = 0.994
p = 0.101
p = 0.032
p = 0.024
p = 0.313
p = 0.068
p = 0.253
p = 0.385
p = 0.336
p = 0.069
p = 0.096
p = 0.100
p = 0.715
p = 0.038
p = 0.034
Fold Change in gene expression normalized to reference genes actb2, b2m and eef1a1
Bifenthrin concentration [ng/L]

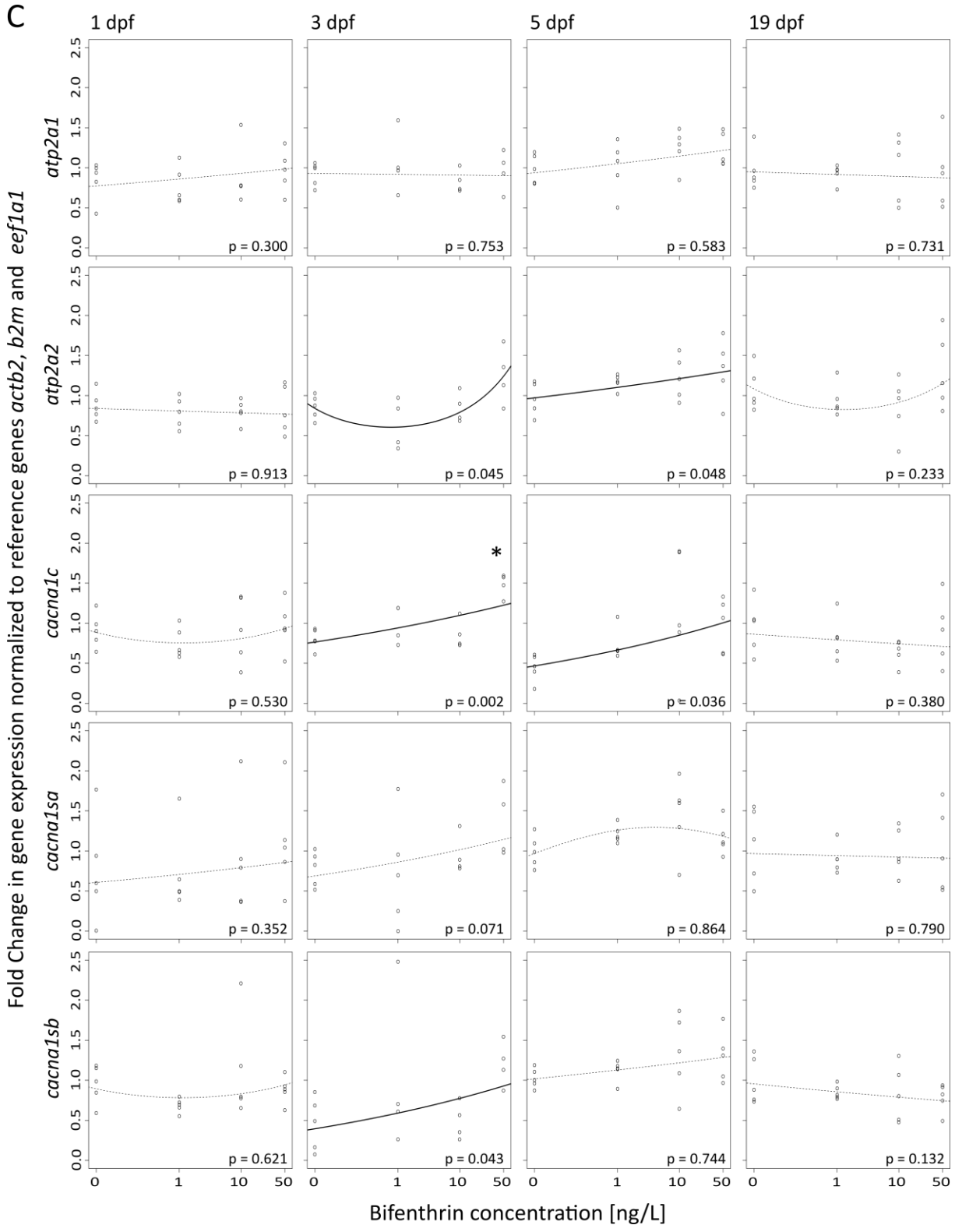


Figure S4. Transcriptional changes at 1, 3, 5 and 19 dpf in zebrafish larvae exposed to varying concentrations of bifenthrin from 1 to 5 dpf. Fold-change in transcript levels for **(A)** ryanodine receptor (RyR) paralogs, (**B**) mTOR signaling molecules, and (**C**) SERCA pumps and voltage-gated Ca^{2+} channels. Each dot represents the fold change value of a single biological replicate (n=5 biological replicates), normalized to the average of the reference genes *actb, b2m* and *eef1a1* within the same sample.

Data are presented on a $\log_{10} X + 0.05$ axis. For data in each panel, five curves (linear, unimodal 1, unimodal 2, sigmoidal and quadratic) were assessed for best fit using the maximum likelihood approach; the best fitting curve is shown in each panel. Curves shown 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 option (lowest p-value), but not significantly better than the null model. P-values for each fitting curves are shown in the panel.

Table S2. P-values calculated using one-way ANOVA with significance set at $P < 0.05$, followed by a Tukey Honest Significant Difference (HSD) post-hoc test for pairwise difference. 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 were appropriate for determining significant differences between treatments.

p-values Anova				
	1dpf	3dpf	5dpf	19dpf
ryr1a	0.744	0.772	0.7081	0.7738
ryr1b	0.1929	0.2241	0.3648	0.1996
ryr2a	0.8135	0.1893	0.8592	0.4251
ryr2b	0.5187	0.3727	0.3813	0.6393
ryr3	0.8438	0.1729	0.3724	0.6469
rheb	0.123	0.9629	0.7401	0.4417
mtor	0.06502	0.2779	0.07725	0.1737
rptor	0.2898	0.9671	0.5064	0.2266
rictor	0.08365	0.7307	0.3	0.6621
rps6	0.6657	0.4634	0.2316	0.4237
eif4ebp1	0.3721	0.9027	0.1731	0.1965
atp2a1	0.5072	0.9442	0.279	0.9855
atp2a2	0.9259	0.1242	0.2132	0.2759
cacnac1c	0.5072	0.001869	0.1842	0.4594
cacna1sa	0.7589	0.2063	0.2092	0.9684
cacna1sb	0.2557	0.2528	0.4687	0.582

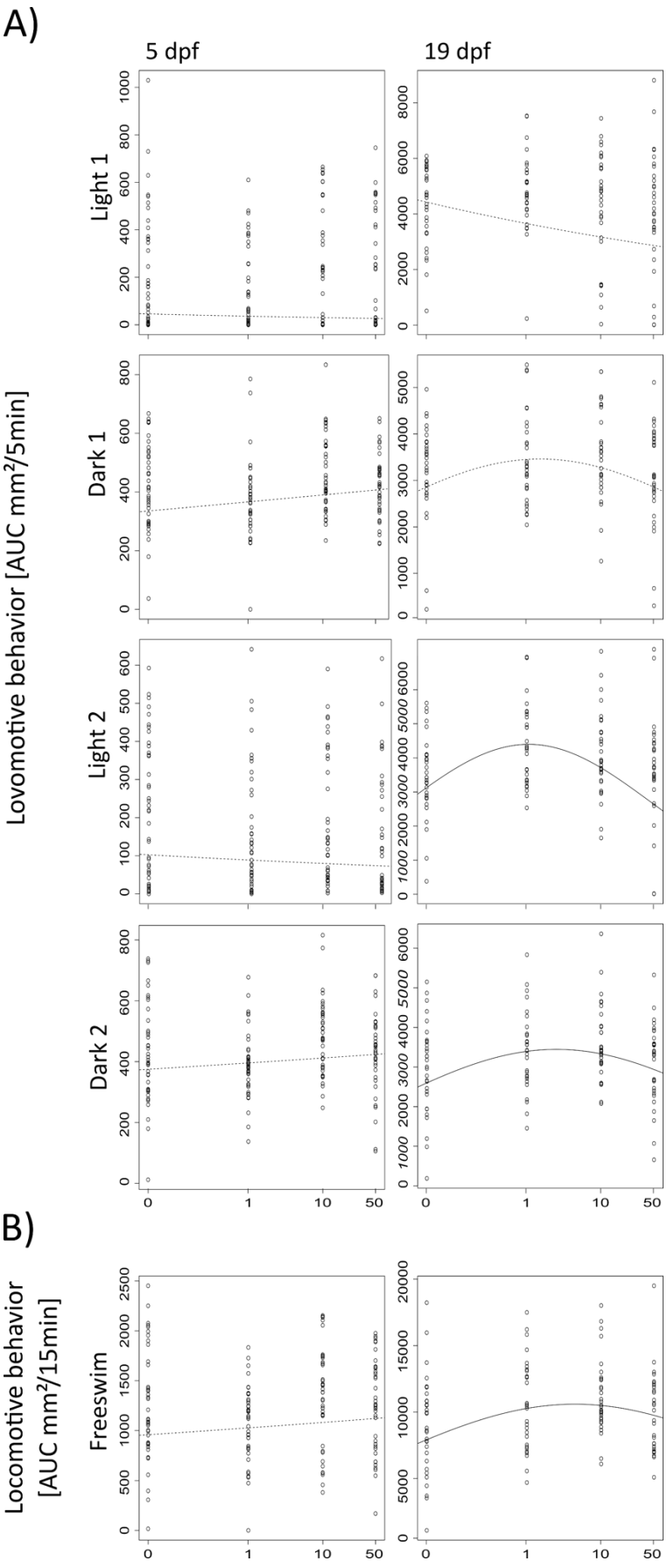

A)
5 dpf
19 dpf
Light 1
Dark 1
Light 2
Dark 2
Lovomotive behavior [AUC mm²/5min]
B)
Freeswim
Locomotive behavior [AUC mm²/15min]
0
1
10
50

Figure S5. **Developmental exposure to bifenthrin altered responses of zebrafish in the light-dark locomotor behavioral assay at 19 but not 5 dpf.** Locomotor behavior of zebrafish in alternating periods of light and dark was assessed in zebrafish exposed to varying concentrations of bifenthrin from 1-5 dpf.

Locomotive behavior of 5 dpf (left) and 19 dpf (right) air larval zebrafish exposed to vehicle (0 ng/ml bifenthrin) or varying concentrations of bifenthrin with the distance moved presented as the area under the curve (AUC). Data are presented on a log10 X+ 0.05 axis, and each dot represents one larval fish (n=3o per treatment). (**A**) Data collected during alternating light and dark periods, each lasting 5 min. (**B**) Data collected during an extended Dark 2 period (15 min "Free swim"). Five concentration-effect curves (linear, unimodal 1, unimodal 2, sigmoidal, and quadratic) were fit using a maximum likelihood approach. 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 (all p-values for the fitted curves are also shown in the graphs representing one period). *Significantly different from control as identified using a mixed model algorithm ($p< 0.05$).

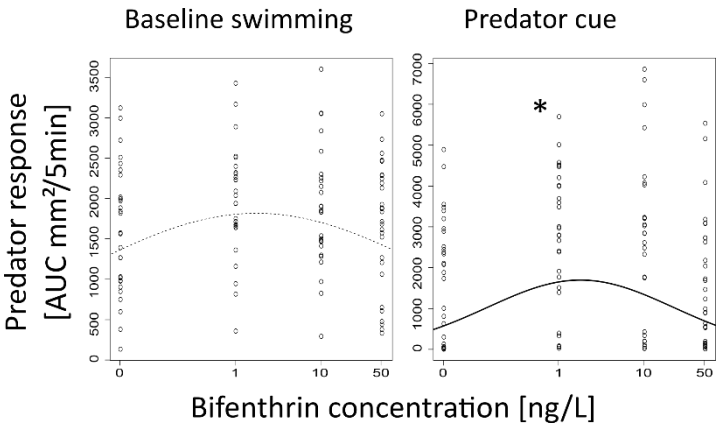
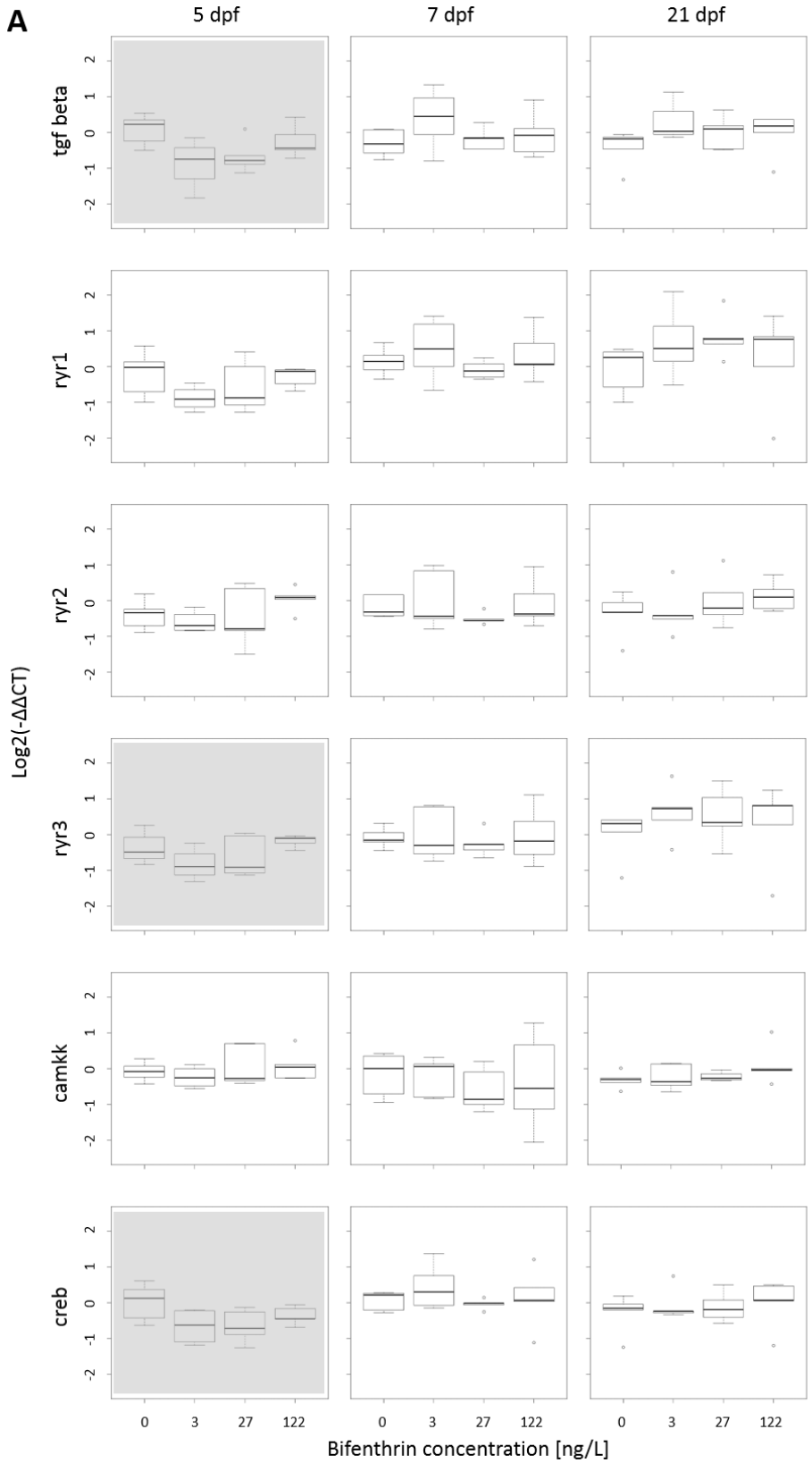


Figure S6. Exposed to bifenthrin from 1 to 5 dpf. Response to an olfactory predator cue in 19 dpf zebrafish. Swimming was tracked for 5 min during an acclimation period (baseline swimming), and after challenge with a predator cue. Each treatment (presented on a log10 X+ 0.05 axis) included n=30 larval 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. Five dose-response curves were fit using a maximum likelihood approach (linear, unimodal 1, unimodal 2, 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. *Significantly different from control, as identified using a mixed model algorithm ($p< 0.05$).


A
5 dpf
7 dpf
21 dpf
tgf beta
ryr1
ryr2
ryr3
camkk
creb
Log2(-ΔΔCT)
-2
-1
0
1
2
0
3
27
122
Bifenthrin concentration [ng/L]

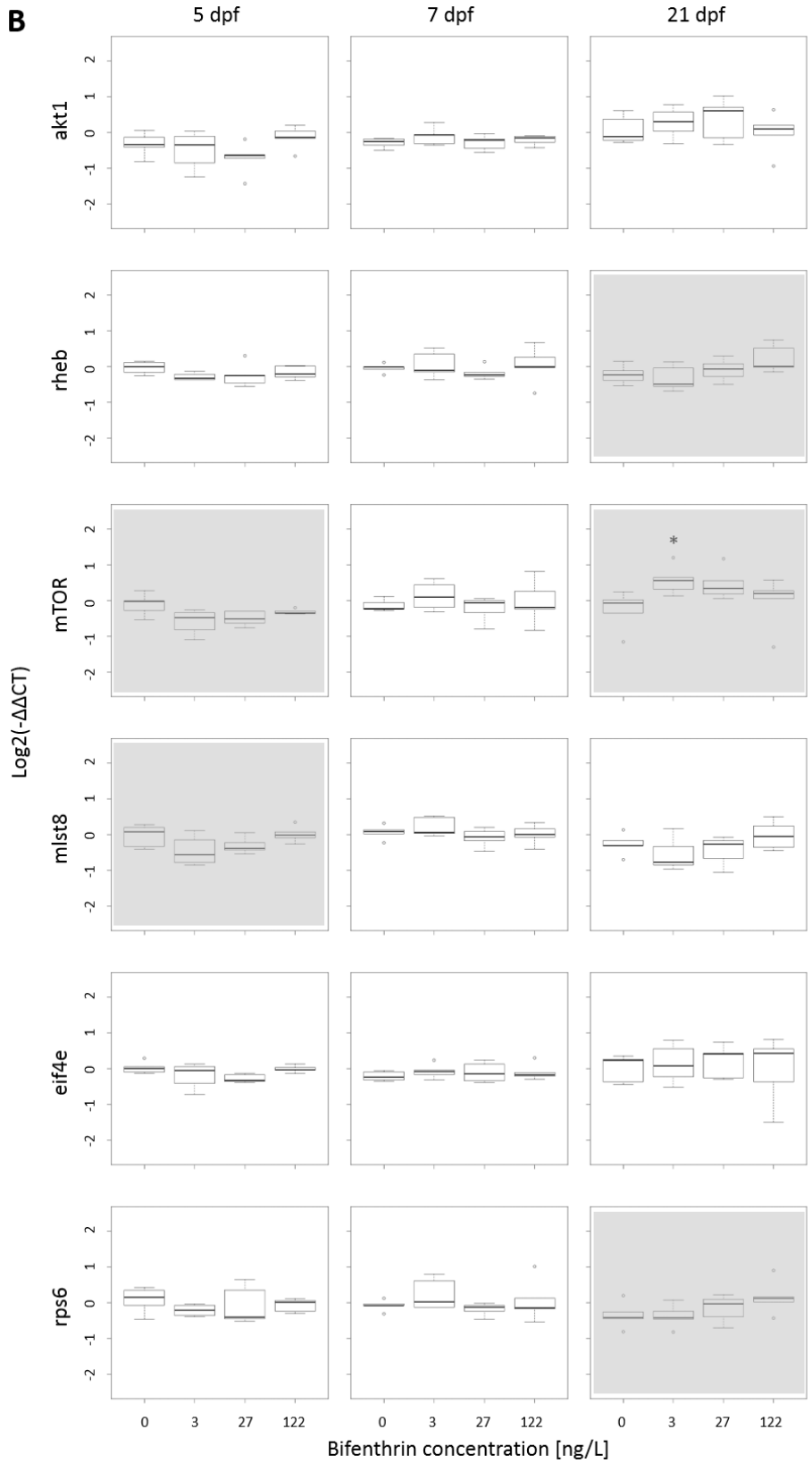


Figure S7. Transcriptional changes at 5, 7 and 21 dpf in inland silverside larvae exposed to varying concentrations of bifenthrin from 1 to 7 dpf. Log2(-ΔΔCT) transcript levels for **(A)** ryanodine receptor (RyR) paralogs, (**B**) mTOR signaling molecules. Each treatment includes n=5 biological replicates, normalized to the average of the reference genes *actb and*

rpl7). Genes shown with a grey background are significantly better fits than a null intercept-only model ($p < 0.05$) in the applied dose-response functions. * Significantly different from control, as identified using one-way ANOVA ($p< 0.05$).

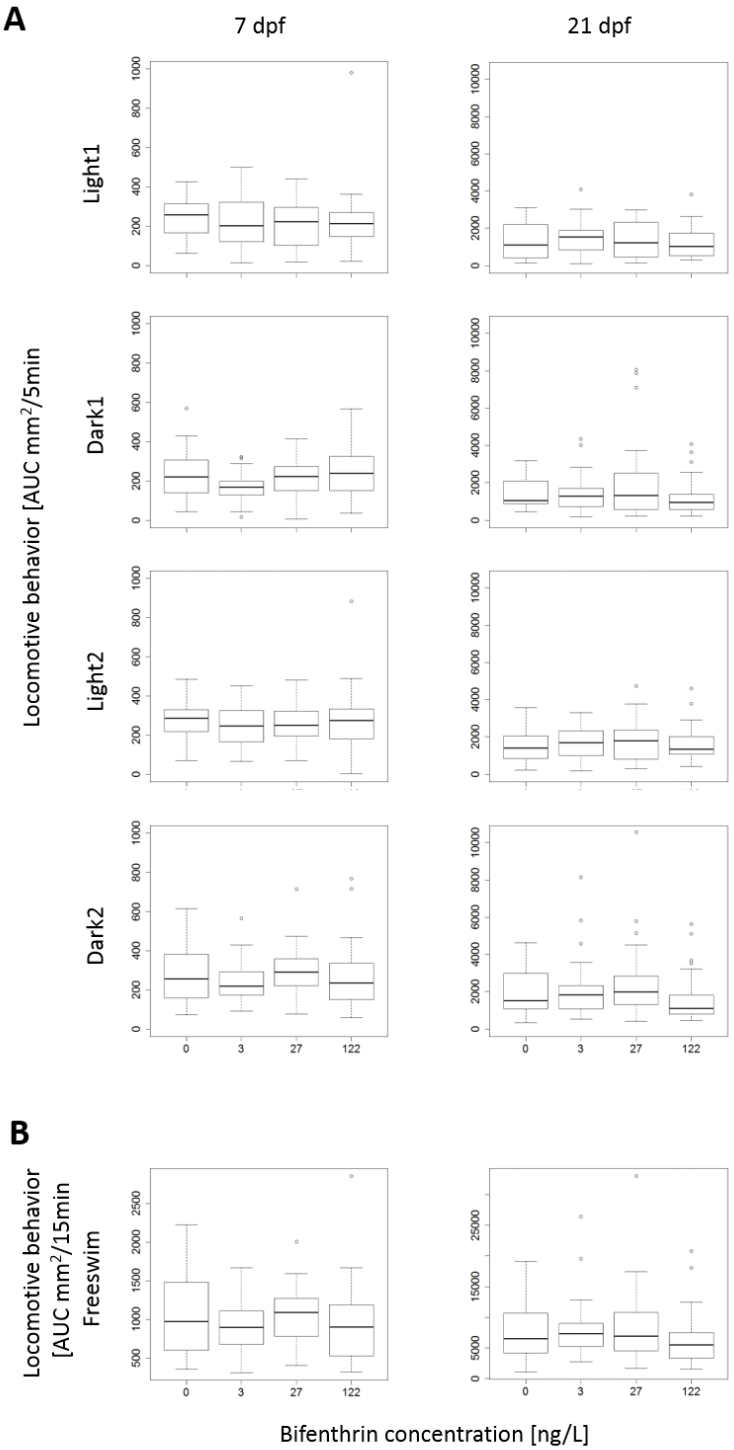


Figure S8. **Light-dark locomotor behavioral assay at 7 and 21 dpf in inland silverside larvae exposed to varying concentrations of bifenthrin from 1 to 7 dpf .** Locomotive behavior of 7 dpf (left) and 21 dpf (right) larval silversides exposed to vehicle (0 ng/ml bifenthrin) or varying concentrations of bifenthrin with the distance moved presented as the area under the curve (AUC). Every treatment includes n=30 individual fish. (**A**) Data collected during alternating light and dark periods, each lasting 5 min. (**B**) Data collected during an extended Dark2 period (15 min "Freeswim"). The mixed model approach ($p > 0.05$) and an ANOVA ($p > 0.05$) calculation, did not show different behavior between light and dark periods or between treatments.

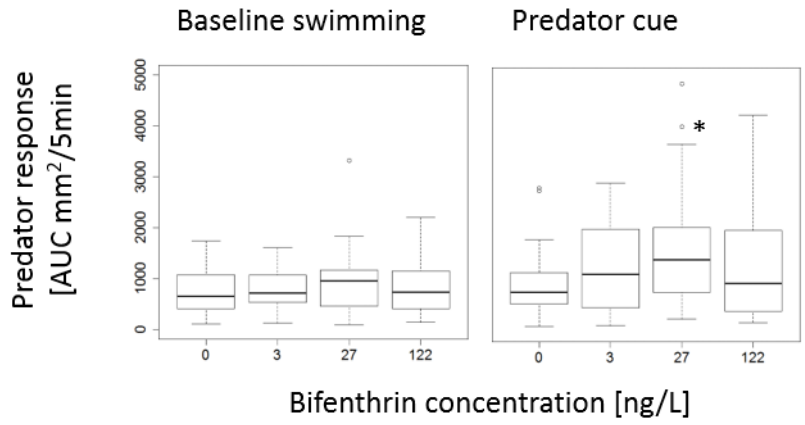


Figure S9. Response to an olfactory predator cue in 21 dpf inland silversides. Swimming was tracked for 5 min during an acclimation period (baseline swimming), and after challenge with a predator cue. Each treatment included n=30 larval fish. The distance moved is presented as the area under the curve (AUC) during a time interval of 5 min. *Significantly different from control, as identified using a mixed model algorithm ($p < 0.05$).

Table S3. P-values calculated for transcriptomic assessments in zebrafish for transcriptomic assessments in inland silversides using one-way ANOVA with significance set at $P < 0.05$, followed by a Tukey Honest Significant Difference (HSD) post-hoc test for pairwise difference. 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 were appropriate for determining significant differences between treatments.

ANOVA p values			
	5dpf	**7dpf**	**21 dpf**
akt1	0.1964	0.3875	0.6161
rheb	0.3262	0.3331	0.09171
mTOR	0.05374	0.5824	0.04184
mlst8	0.09119	0.3331	0.2186
eif4b	0.1053	0.7453	0.9337
eif4g	0.6256	0.443	0.7084
s6	0.676	0.5665	0.1652
eef1a1	0.1196	0.4383	0.6964
ryr1	0.2766	0.4712	0.413
ryr2	0.3185	0.3355	0.5241
ryr3	0.161	0.8936	0.6877
camkk	0.6298	0.8668	0.5784
creb	0.1116	0.5275	0.8725
tgfbeta	0.05477	0.2971	0.3222