TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Aquatische Systembiologie

Integrative Assessment of Stressor Effects in Aquatic Ecosystems: Bridging the Gap between 'Omics' and Behavioral Responses

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzende: Univ.-Prof. Dr. I. Kögel-Knabner

Prüfer der Dissertation:

1. Univ.-Prof. Dr. J. P. Geist

2. Univ.-Prof. Dr. A. Melzer

Die Dissertation wurde am 12.12.2012 bei der Technischen Universität München eingereicht und durch die Fakultät für Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 20.02.2013 angenommen.

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GLOSSARY

AI	Active Ingredient
ANOVA	Analysis Of Variance
AOP	Adverse Outcome Pathway
Aspa	Aspartoacylase
B2m	Beta2-microglobulin
Ca^{2+}	Calcium ion
CAS	Chemical Abstracts Service
cDNA	Complementary Desoxy Ribonucleic Acid
Ck	Creatine kinase
Cl^-	Chloride
Cu^{2+}	Copper
CWA	Clean Water Act
Cyp1a	Cytochrome P450 1A
Cyp3a	Cytochrome P450 3A126
DDT	Dichlordiphenyltrichlorethan
DNA	Desoxy Ribonucleic Acid
DOM	Dissolved Organic Matter
Ecac	Epithelial calcium channel
EC_x	Effect Concentration causing x% effect
Ef1a	Elongation factor 1 alpha
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GABA	Gamma-aminobutyric acid
G6pd	Glucose-6-phosphate dehydrogenase
Gcr	Glucocorticoid receptor
Gh	Growth hormone
Gst	Glutathione S-transferase
Hsp90	Heat shock protein90
Igf	Insulin-like growth factor
LC_x	Lethal Concentration (x % mortality within test population)
LOEC	Lowest observed effect concentration

MOA	Mode Of Action
mRNA	Messenger Ribonuclei Acid
Mt	Metallothionein
Mx	Mx protein
Na^+	Sodium
NOAEL	No observed adverse effect level
NOEC	No observed effect concentration
OECD	Organisation for Economic Co-operation and Development
PBO	Piperonyl butoxide
PCA	Principal Component Analysis
POD	Pelagic Organism Decline
Pvalb	Parvalbumin
QPCR	Quantitative reverse transcriptase polymerase chain reaction
REACH	EU regulation for Registration, Evaluation, Authorisation and Restriction of Chemical substances
SE	Standard error
SD	Standard deviation
Tgfb	Transforming growth factor beta
TNF	Tumor necrosis factor
USA	United States of America
USEPA	United States Environmental Protection Agency
Vtg	Vitellogenin precursor
Zp3	Zona pellucida glycoprotein 3

ZUSAMMENFASSUNG

Welweit stellt die Beeinträchtigung von Süßwassersystemen durch industrielle oder natürliche chemische Verbindungen eine der größten Umwelproblematiken menschlicher Gesellschaften dar. Ein Großteil dieser Verbindungen liegt dabei in relativ niedrigen Konzentrationen vor, was angemessene und senstive Bewertungsmethoden erfordert, um mögliche schädliche toxikologische Effekte innerhalb dieser Systeme und deren Organismen zu erheben. In der vorliegendenden Arbeit wird die Effektcharakterisierung durch Insektizide verursachter Stresseffekte in larvalen Fischen anhand eines integrativen Ansatzes vorgestellt. Das übergordnete Ziel der Arbeit ist die Verknüpfung von Effekten auf molekularer und Individuen-/Populationsebene zur Interpretation zellulärer Effekte in deren ökologischem Kontext. Den Schwerpunkt bildet dabei die quantitative und kausale Untersuchung subletaler Stressantworten auf der Transkriptomebene bis zu subletalen Verhaltensänderungen im Verhältnis zu artspezifischen Mortalitäts-Endpunkten. Integrative Untersuchungsansätze sind notwendig und erleichtern die Einschätzung wie sich Änderungen auf molekularer Ebene auf Individuen, Gemeinschaften, Populationen oder sogar Ökosysteme auswirken. Molekulare Biomarker stellen dabei eine schnelle, kosteneffektive und sensitive Diagnosemethode zur Messung physiologischer Veränderungen dar. Am Beispiel der Expositionen larvaler Dickkopfelritzen (*Pimephales promelas*) wird die Sensitivität molekularer und verhaltensspezifischer Effekte verdeutlicht. Dies erfolgte in den vorliegenden Untersuchungen in erster Linie anhand der Wirkung zweier gebräuchlicher Insektizide, dem synthetische Pyrethroid Bifenthrin und dem Phenylpyrazol Fipronil sowie ihrer kommerziell erhältlichen Formulierungen.

In Kapitel I und II wird das Schwimmverhalten als toxikologischer Endpunkt herangezogen um subletale Auswirkungen des synthetischen Pyrethroids Bifenthrin und des Phenylpyrazols Fipronil sowie der kommerziell erhältlichen Formulierungen Talstar® und Termidor® zu charakterisieren. Verhaltenspezifische Endpunkte sind von höchster Relevanz bei der Effektbewertung, sind jedoch mitunter komplex in ihrer Erhebung. Im Falle aquatischer Organismen und speziell bei Fischen ermöglicht die Untersuchung des Schwimmverhaltens Rückschlüsse auf den individuellen Performancestatus. Insbesondere der Zusammenhang der meisten bedeutenden „life-history“ Variablen, Respiration, Fraßverhalten, Räuber-Beute Interaktionen und sozialer Interaktionen wie „Werbung“ und Laichgeschäft, mit dem Schwimmverhalten verdeutlichen dessen Bedeutung für Überleben, Wachstum und

Reproduktion und damit den evolutionären Erfolg. Kapitel I beinhaltet den Vergleich zweier Testsysteme die eine adequate Sensitivität zur Evaluierung der Schwimmleistung aufweisen und die Erhebung quantitativer Daten erlauben. Zum einen die Messung der geschwommenen Distanz mittels Videoanalyse und zum anderen die Schwimmleistung als Reaktion auf einen externen Stimulus. Dies wurde exemplarisch mit dem auf Bifenthrin basierenden Insektizid Talstar® und dem antibakteriellen Wirkstoff Triclosan untersucht. Die Ergebnisse bestätigen die Aussagekraft von Schwimmleistungs- und Verhaltenstests bei der Bewertung toxischer Effekte und konnten als geeignete Methoden zur quantitativen Erhebung subletaler Stressantworten in larvalen Fischen identifiziert werden.

Dieser Ansatz wird in Kapitel II erweitert durch gleichzeitige Betrachtung des Wachstums und zusätzlicher Messzeitpunkte innerhalb einer sechstätigen Erholungsphase nach der jeweiligen Exposition. Ziel dieser Teilstudie war der direkte Vergleich zwischen den reinen aktiven Inhaltstoffen und den formulierten Produkten von Bifenthrin und Fipronil. Die subletalen Belastungsstufen wurden im prozentualen Verhältnis zu akuten LC_{10} Werten gewählt, um die erhobenen Effekte in Relation zu „klassischen“ Mortalitätsdaten zu setzen. Es konnte festgestellt werden, dass die Schwimmleistung signifikant beeinträchtigt wird ab Konzentrationen die 20% des LC_{10} Wertes von Bifenthrin (0,14 $\mu g.L^{-1}$) bzw. 10% des LC_{10} Wertes von Talstar® (0,03 $\mu g.L^{-1}$) betragen. Im Vergleich von Fipronil und Termidor® wurde eine signifikante Beeinträchtigung der Schwimmleistung bei 142 $\mu g.L^{-1}$, bzw. 148 $\mu g.L^{-1}$ gemessen, mit einer stärkeren Ausprägung des Effekts im Falle der Formulierung. Verändertes Wachstum wurde ab 10% des LC_{10} Wertes (53 $\mu g.L^{-1}$) von Fipronil festgestellt.

Die in Kapitel II erhobenen Datensätze wurden in Kapitel III für einen direkten Vergleich mit Änderungen der Transkription ausgewählter Markergene herangezogen, hier mit Fokus auf den aktiven Wirkstoff Bifenthrin. Die komplementäre Betrachtung verschiedener biologischer Organisationsebenen erlaubte die Charakterisierung potenzieller Verknüpfungen der erhobenen Endpunkte. Die Markergene wurden anhand ihrer Stellung in zellulären Pathways ausgewählt, unter anderem Wachstum (insuline-like growth factor, *igf*; growth hormone, *gh*), Energiemetabolismus (glucose-6-phosphate dehydrogenase, *g6pd*; creatine kinase, *ck*), muskuläre und neuronale Funktion (parvalbumin, *pvalb*; aspartoacylase, *aspa*) sowie generelle Stressantworten und Detoxifikationsmechanismen (cytochrome p450 1a, *cyp1a*; glutathione S-transferase rho, *gst*; heat shock protein 90, *hsp90*; metallothionein, *mt*). Signifikante Änderungen der Transkription wurden dabei ab 0,07 $\mu g.L^{-1}$ Bifenthrin festgestellt. Die transkriptionalen Änderungen folgten mehrfach einem bi-phasischen und

keinem linearen Dosis-Wirkungsverhältnis in der ansteigenden Konzentrationsreihe. Die Muster der Transkription von detoxifikationsrelevanten sowie in neuromuskuläre Funktion und Energiemetabolismus involvierten Genen zeigten dabei einen Zusammenhang mit der Beeinträchtigung des Schwimmverhaltens ab 0,14 $\mu g.L^{-1}$ Bifenthrin. Mit ansteigender Konzentration wurde eine signifikante Abregulierung für die Marker Cyp3a, Aspartoacylase und Creatine kinase gemessen, wogegen Metallothionein aufreguliert wurde. Endokrine Reaktion auf die Bifenthrin Belastung wurden deutlich anhand signifikanter Induktion von Vitellogenin und einer Abregulierung des Insuline-like growth factor. Eine generelle Erholung wurde nach sechs Tagen in Abhängigkeit von der anfänglichen Intensität des Stressors beobachtet.

In Kapitel IV wurden in einem vergleichbaren Ansatz die Effekte des aktiven Wirkstoffs Fipronil charakterisiert, der Schwerpunkt in dieser Studie lag jedoch in der zeitlichen Abfolge. Die Notwendigkeit zur Betrachtung multipler Zeitpunkte in Studien zur Transkriptomantwort wurde besonders deutlich anhand der Transkriptionsprofile nach Belastung mit Fipronil. Nach der 24-stündigen Expositionsdauer konnten hochsignifikante Änderungen der Gentranskription ab einer Konzentration von $\geq 31\ \mu g.L^{-1}$ für Aspartoacylase, Metallothionein, Glucocorticoid receptor, Cyp3a und Vitellogenin detektiert werden. Im zeitlichen Verlauf des Experiments wurden dabei unterschiedliche Toxizitätsmechanismen deutlich, mit eindeutig neurotoxischen Effekten als akute Wirkung. Dagegen waren nach der 6-tägigen Erholungsphase vorwiegend endokrine Effekte feststellbar, mit einer 90-fachen Überexprimierung von Vitellogenin bei 61 $\mu g.L^{-1}$ Fipronil. Entgegen der bei Bifenthrin beobachteten Erholungseffekte resultierte die Fipronil Belastung in einer verstärkten Änderung der Genregulierung im zeitlichen Verlauf.

In Kapitel V wurde der vorgestellte integrative Ansatz bei einer Nicht-Model Spezies, der Fischart Delta Smelt (*Hypomesus transpacificus*), zur Effektcharakterisierung nach Kupfer-Exposition durchgeführt. Anhand eines für diese Art entwickelten Microarrays konnten dabei potentielle Biomarker identifiziert und mittels quantitativer PCR validiert werden. Die funktionale Klassifizierung der Transkriptomantworten zeigten Effekte auf neuromuskuläre Funktion, Verdauung und Immunsystem in den Kupfer-exponierten Fischen. Zusätzlich zeigte sich eine unterschiedliche Sensitivät zwischen juvenilen und larvalen Lebensstadien (96 h-LC_{50} juvenil und larval, 25,2 und 80,4 $\mu g.L^{-1}$ Cu^{2+}). Ein messbarer Rückgang der Schwimmaktivität stand im direkten Verhältnis zur Kupferbelastung ($r = -0.911$, $p < 0.05$)

und konnte funktional mit der Gentranskription von Aspartoacylase, Hemopexin, α-actin, und Calcium regulierenden Proteinen erklärt werden.

Die im Rahmen dieser Arbeit vorgestellten Ergebnisse verdeutlichen die Schadwirkung selbst geringer Insektizidkonzentrationen auf sensitive Lebensstadien von Fischen. Integrative Ansätze, welche die Schadstoffinduzierten Effekte auf mehr als einer Ebene biologischer Organisation erfassen und diese als Funktion sowohl der Konzentration als auch der Zeit beschreiben sind notwendig um Effektschwellenwerte zu erstellen und zudem geeignete Biomarker zu etablieren. Dies ermöglicht die Erkennung von geeigneten Schwellenwerten zur Effektbewertung und die Vorhersage und Einschätzung transkriptionaler Reaktionen hinsichtlich ihrer phänotypischen Ausprägung. Der vorgestellte Ansatz verdeutlicht die Relevanz funktionaler Charakterisierung von Biomarkern und bildet die Grundlage für deren Anwendung zur Bewertung von subletalen Schadstoffbelastungen in Feldstudien und Monitoringprogrammen.

SUMMARY

Freshwater systems worldwide are influenced by industrial and natural chemical compounds representing one of the key environmental problems for human societies. A high proportion of these compounds are present at low concentrations, which demands suitable and sensitive tools for the assessment of possible harmful toxicological effects in the respective systems and the organisms therein. This thesis contributes novel aspects about stressor effects in aquatic organisms using the example of sublethal effects of insecticidal chemicals in larval fish. An integrative approach, utilizing molecular and whole-organism endpoints was chosen, in order to enhance the understanding of transcriptomic responses related to adverse outcomes on higher levels of biological organization. With this approach the work contributes several aspects to the ongoing and still challenging field of ecotoxicogenomics.

The overall goal was aimed at linking effects at the molecular and whole-organism levels for the interpretation of cellular effects in an ecological context. Integrative approaches are needed to facilitate the prediction of consequences of molecular alterations to individuals, communities, populations or even ecosystems. Molecular biomarkers have thereby the potential to be rapid, cost-effective and sensitive diagnostic tools to assess physiological impacts on organism. Using the established model species fathead minnow (*Pimephales promelas*) the sensitivity of molecular and behavioral effects could be underlined. The investigations focused therby on two common use insecticides, the synthetic pyrethroid bifenthrin and the phenylpyrazole fipronil, and their respective commercial formulations.

In chapters I and II swimming behavior was used to characterize sublethal impacts of the synthetic pyrethroid bifenthrin and the phenylpyrazole fipronil as well as their commercially available formulations Talstar® and Termidor®. Behavioral endpoints are most relevant for whole organism testing, but can be rather complex to assess. However, in case of aquatic organisms such as fish, swimming behavior is an excellent individual endpoint that highly reflects the status of individual performance. A number of life-history variables are dependent on swimming ability, including respiration, feeding, predator–prey interactions, and social interactions such as courtship and spawning, which are fundamental to survival, growth, and reproduction, the most important traits in evolutionary success.

In chapter I two different test setups are compared that provide adequate sensitivity for swimming performance evaluation and yield quantitative data, the measurement of swimming distance by video analysis of non-provoked swimming vs. swimming performance in a

forced-swim test. This was exemplarily done with the bifenthrin-based insecticide Talstar® and the antibacterial agent triclosan. Obtained results were used to support the use of swimming performance and behavior to assess toxicant effects, and to demonstrate adequate methods for the assessment indicators of sublethal stress in larval fish.

In chapter II this approach was expanded by the addition of growth as an endpoint and the incorporation of additional time points during a 6 day recovery time period after exposure. The main objective thereby was the direct comparison between the active ingredients and the formulated products of the insecticides bifenthrin and fipronil. Swimming performance was significantly impaired at 20% of the LC_{10} (0.14 $\mu g.L^{-1}$) of bifenthrin and 10% of the LC_{10} of Talstar® (0.03 $\mu g.L^{-1}$). Fipronil and Termidor® led to a significant impairment of swimming performance at 142 $\mu g.L^{-1}$ and 148 $\mu g.L^{-1}$, respectively, with more pronounced effects for the formulation. Effects on growth were observed at 10% of the LC_{10} (53 $\mu g.L^{-1}$) fipronil.

Data evaluated in chapter II was incorporated in a direct comparison between gene transcription changes of selected molecular markers, called biomarkers, and whole organism performance in chapter III with focus on the active ingredient of bifenthrin. The complementary assessment of endpoints at different levels of biological organization allowed the characterization of possible linkages between these endpoints. Biomarker genes were selected from cellular pathways involved in organism growth (insuline-like growth factor, *igf*; growth hormone, *gh*), energy metabolism (glucose-6-phosphate dehydrogenase, *g6pd*; creatine kinase, *ck*), muscular and neuronal function (parvalbumin, *pvalb*; aspartoacylase, *aspa*), as well as general stress responses (cytochrome p450 1a, *cyp1a*; gluthione S-transferase rho, *gst*; heat shock protein 90, *hsp90*; metallothionein, *mt*). Significant transcriptomic responses were observed at 0.07 $\mu g.L^{-1}$ bifenthrin but mostly followed a biphasic rather than a linear dose–response with increasing concentration. Transcript patterns for genes involved in detoxification, neuromuscular function and energy metabolism were thereby linked to the impairment of swimming performance at concentrations $\geq$0.14 $\mu g.L^{-1}$ bifenthrin. With increasing treatment concentration, a significant down-regulation was observed for genes coding for cyp3a, aspartoacylase, and creatine kinase, whereas metallothionein was up-regulated. Additionally, bifenthrin induced endocrine responses as evident from a significant up-regulation of vitellogenin and down-regulation of insuline-like growth factor transcripts. Recovery occurred after 6 days and was dependent on the magnitude of the initial stress. During the recovery period, down-regulation of vitellogenin

was observed at lowest exposure concentrations, indicating endocrine disruptive properties of the tested pyrethroid.

A similar approach was used in chapter IV with focus on the active ingredient of fipronil, but the emphasis here was laid on trajectories of effects over time. The importance of including multiple time points in transcriptomic studies was clarified by the observed response profiles to fipronil exposure. Immediately after 24 h exposure to concentrations of $\geq$ 31 $\mu g.L^{-1}$, highly significant changes in gene transcription were observed for aspartoacylase, metallothionein, glucocorticoid receptor, cyp3a and vitellogenin. Different mechanisms of toxicity were apparent over the course of the experiment, with short-term responses indicating acute neurotoxic effects. After 6 d of recovery, endocrine effects were observed with vitellogenin being up-regulated 90-fold at 61 $\mu g.L^{-1}$ fipronil. Multivariate analysis demonstrated a significant increase in gene transcription changes over time and during the recovery period. In conclusion, multiple mechanisms of action were observed in response to fipronil exposure, and unknown delayed effects would have been missed if transcriptomic responses had only been measured at a single time-point.

In chapter V the approach was expanded by combining the assessment molecular and whole-organism responses in a non-model species, the delta smelt (*Hypomesus transpacificus*), for the effect characterization of the model contaminant copper. Target genes were identified as potential biomarkers utilizing a custom microarray and subsequently validated by quantitative PCR. The functional classification of the transcriptomic responses indicated effects upon neuromuscular function, digestion and immune system in copper-exposed fish. Additionally, a differential sensitivity in larval and juvenile life-stages became obvious (96 h-LC_{50} juvenile and larval, 25.2 and 80.4 $\mu g.L^{-1}$ Cu^{2+}, respectively). The measured decrease in swimming activity showed to be in a direct relationship with the copper-exposure ($r = -0.911$, $p < 0.05$) and could be functionally explained by the transcription of aspartoacylase, hemopexin, α-actin, and calcium regulation proteins.

The overall results presented in this work confirm that even low insecticide concentrations cause harmful effects towards sensitive life stages of fish. Integrative approaches, describing toxicant induced effects on different levels of biological organization and as a function of both, concentration and time, are necessary for the detection of thresholds for effect assessment and biomarker development. Additionally, more than one time point is important in effect assessment, especially if responses observed on the transcription level are used to predict or estimate adverse effect on higher levels of biological organization. The presented

approach highlights the suitability of functionally characterized molecular biomarkers to assess effects of sublethal contaminant exposure in field scenarios and monitoring programs.

INTRODUCTION

Background

Freshwater ecosystems are among the most threatened systems worldwide and a strong loss in biodiversity over the last decades is recently recognized, driving increasing research effort to evaluate possible causes (Moyle 2011, Geist 2011, Burkhardt-Holm et al. 2005). Among the various threats to freshwater ecosystems caused by anthropogenic activities, like nutrient shifts, acidification, habitat loss or fragmentation, exploitation and biological invasions, chemical contamination is a significant driver of environmental stress. This is of special importance, as most of these threats are presumably increasing due to global alterations of ecosystems caused by climate change (Stevenson and Sabater 2010, Kattwinkel et al. 2011).

Undoubtedly, chemicals in general are essential for human societies in many aspects, for the production of consumer goods, protection or restoration of health or the optimization of food production. The production, application, use and trade of chemicals are therefore significant aspects of economic wealth in developed countries worldwide (World Bank Group, Environment Strategy 2010). According to database entries, the number of new developed or discovered chemical compounds is steadily increasing, now being over 60 million inorganic and organic substances (Chemical Abstract Service, CAS: www.cas.org).The occurrence of man-made chemicals, so called xenobiotics, in the environment is of concern, when adverse effects are caused that affect components, processes and functions within the respective ecosystems. However, the characterization of these effects is sophisticated, caused by the complexity in the biological structure of ecosystems, the interrelationships within components, and the high variety in pathways of distribution of xenobiotics in the environment. Most industrialized countries possess a regulatory organ, which is responsible for the authorization and registration of chemicals, such as the REACH regulation (EC 1907/2006) or the Directive 98/8/EC on biocides within the European Union. Similar approaches are used in the USA based on the Clean Water Act (CWA), regulating the pollution discharge and water quality standards, and the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), regulating pesticide distribution, sale and use. One main goal is to promote the decrease in acute and excessive pollution by chemicals and highly toxic and environmentally persistent substances such as the insecticide DDT as the most commonly known example.

Responsible for the scientific basis that underlies regulatory decisions is ecotoxicological research. Ecotoxicology focuses on the effects of chemicals in the biotic environment and emerged within the 1960s as a scientific area. It was first defined by Truhaut in the year 1969 as a discipline studying "the toxic effects, caused by natural or synthesized pollutants, to the constituents of ecosystems, animals (including human), vegetable and microbial, in an integral context" (Truhaut 1977). Ecotoxicology is the combination of concepts and principals from different scientific disciplines, such as environmental chemistry, ecology and toxicology. Most recently, molecular biology and bioinformatics can be added to this list, which proves that ecotoxicology itself is a highly dynamic discipline.

Basic principles

There are three basic frameworks which have to be considered in ecotoxicology to provide a profound risk assessment of chemicals in the environment, being (i) the behavior and transport in the environment, (ii) the routes of exposure and bioavailability and (iii) the specific toxicity at different levels of biological organization (Fent 2003). Each of these frameworks can be seen as sub-disciplines in ecotoxicology and are typically divided into exposure assessment and effect assessment. A brief introduction with focus on insecticides is provided subsequently to highlight the most relevant aspects for the objectives of this thesis.

(i) In aquatic ecosystems transport routes of xenobiotics are either direct emission by point sources, such as wastewater effluents or accidental spills, or indirect emission by various diffuse source pathways, such as surface runoff or atmospheric deposition. However, not all chemicals are released into the environment or have chemical inert properties that do not cause negative effects. Pesticides form a special role in this context, as they are intentionally applied into the environment and toxic effects are desired for respective target organisms. As summarized by Cooper and Dobson (2007), pesticides are defined generally by their use for the control of pests and diseases and their respective vectors, the control of human and livestock disease vectors and nuisance organisms, and the prevention or control of organisms that harm other human activities and structures. Despite accidential or intentional direct discharges, the main transport route of insecticides into aquatic ecosystems is discharge through surface runoff from areas with with intensive agricultural activity (Schulz 2004, TDC-Environmental 2008, Werner et al. 2004) as well as urban areas (Budd et al. 2007, Sandahl et al. 2007). The

translocation of insecticide residuals is thereby determined by the specific characteristics of near stream environments, such as topography, precipitation patterns or irrigation regimes as well as soil type (mainly determined by soil organic carbon content) and vegetation coverage (Kattwinkel et al. 2011). In urban areas, with a high percentage of sealed surfaces, runoff or leaching has been identified as the main pathway in insecticide translocation (Brady et al. 2006, Weston et al. 2005, Weston 2012). The appearance of insecticide concentrations is therby detectable up to several weeks after application in the effluent at potentially hazardous concentrations for aquatic organisms (Greenberg et al. 2010). For instance, the synthetic pyrethroid bifenthrin has been frequently found at concentrations acutely toxic to sensitive aquatic organisms in California urban creeks during both summer irrigation and winter rain events (Budd et al. 2007, Weston et al. 2009, Weston et al. 2005). Likewise, the phenypyrazole fipronil has recently been found in urban surface streams in several states, including California.

(ii) For aquatic organisms exposure routes are immediate as they are immersed in water with direct physiological susceptibility to aquaeous contaminants. Gill and skin epithelia as well as the gastro-intestinal system represent direct interfaces with the surrounding water and are the main routes of exposure. Exposure routes and the bioavailability are highly depended on the chemical properties of the respective substance, for example by its distribution or partitioning in the respective environmental medium. The uptake is further dependant on the physiological and biochemical properties of the target organism and the specific physico-chemical properties of the water. The bioavailability of chemicals in aquatic systems is best described by the specific hydrophobicity. This property is classified through the octanol-water partitioning coefficient (K_{ow}), calculated from the equilibrium ratio of the solubility in *n*-octanol to water. Under environmental conditions, high coefficients result in the partitioning of the chemical upon surfaces of relevant abiotic compounds such as detritus, dissolved organic matter (DOM), sediment or humic substances (Liu et al. 2004, Tipping 1994). Therefore the determination of aquatic toxicity is dependant on this property. Additional environmental factors that influence bioavailability and therefore toxicity include particulate organic matter, dissolved organic carbon, pH and temperature (Maul et al. 2008, Yang et al. 2007). Bioavailability and toxicity are highly related to concentrations of dissolved organic matter in case of synthetic pyrethroids

(Yang 2007). An additional important aspect in this context is the organism specific uptake dependent on feeding type, which was demonstrated by Palmquist et al. (2008) for invertebrate functional feeding groups. Interactions with abiotic environmental parameters highlight that the extent to which organisms are exposed to the chemical is variable and how crucial the determination of these processes are for risk estimation (Di Toro et al. 2001, DiToro et al. 1991, Tipping 1994, in Spurgeon 2010).

(iii) A major part in ecotoxicological research is the empirical characterization of adverse effects at different levels of biological organization from whole ecosystems, populations and the individual organism down to cells and tissues and intracellular molecular pathways (Connon et al. 2012). Complexity of the system as well as the environmental relevance are increasing with increasing organizational level, whereas the response to a certain perturbation is more pronounced at lower levels, due to greater sensitivity (Figure 1). In regulatory toxicology approaches for effect assessment of environmental chemicals to aquatic organisms typically rely on standardized laboratory toxicity tests to describe deleterious effects to a test population, where organism mortality is the classical endpoint (USEPA 2002, OECD 1992). Standard toxicity testing protocols are available for several model organisms from different taxa and trophic levels. Most organism tests are specified to a single species and toxicants are used either as single chemicals or as effluents before or after discharge into the receiving environment. Typical freshwater fish model organisms are zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*) medaka (*Oryzias latipes*), carp (*Cyprinus carpio*) or rainbow trout (*Oncorhynchus mykiss*). Test populations are maintained under controlled, standardized conditions for a defined time period in a defined range of exposure concentrations in addition to a control treatment. Test durations range from 24 h to 96 h in acute tests up to several weeks in chronic tests. The test setups are designed to provide dose-response information, generally expressed as the effect concentration causing mortality in 50% of the test population (LC_{50}) within the prescribed period of time (e.g. 24-96 h in acute test setups). Additional endpoints are expressed as effect concentrations (EC_x values), for instance growth, behavior or reproduction in the test population related to exposure concentration. Other important values in this context include the level or concentration at which no effects occur within the test duration (NOAEL and NOEC). More environmentally realistic are sublethal effects, which can

be determined by effect concentrations utilizing sublethal endpoints that can cover a wide range of physiological processes.

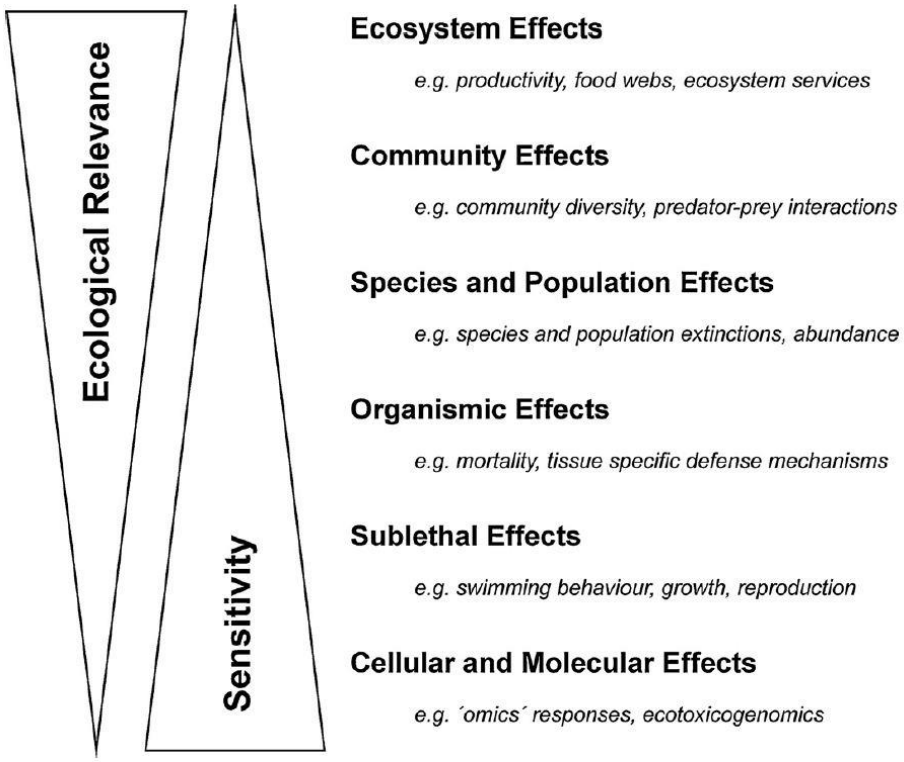

Figure 1: Toxic effects on different levels of biological organization, considering ecological relevance and sensitivity of response (from: Geist 2011).

Effect Assessment

High effect concentrations of insecticides that cause mortality are generally unlike to appear in the environment, although accidental releases of toxic substances are always possible to occur. Environmentally realistic exposure scenarios are most likely short-term and in a sublethal range of concentrations, for example as result from stormwater or irrigation runoff caused translocation of insecticide residues. Adequate endpoints have to be chosen to detect possible impacts on aquatic ecosystems and to enable the interpretation of causal relationships within these systems. Integrative approaches include endpoints from different levels of biological organization to combine indicators with high environmental relevance with those of high sensitivity. In particular, transcriptomic responses are considered highly sensitive indicators of stress with rapid responses, as the initial interaction takes place on the molecular

level and builds the mechanistic basis for subsequent phenotypic outcomes (Schirmer et al. 2010).

So called “omics” technologies allow investigation of chemical modes of action from a mechanistic point of view (Ankley et al. 2006, Fedorenkova et al. 2010, Schirmer et al. 2010). “Omics” is the short term that includes the measurement of mRNA transcripts (transcriptomics), the translation into proteins (proteomics) as well as primary and secondary metabolites (metabolomics). Available tools like microarrays or quantitative Polymerase Chain Reaction (qPCR) and most recently next-generation sequencing are used to determine if a specific transcript is differentially regulated in response to perturbations caused by environmental factors, such as exposure to a chemical stressor. These responses to various types of external stressors can be associated with changes in normal patterns of gene transcription and subsequent expression, creating a specific transcriptomic profile depending on dose, exposure time and mode of action (MOA) of the stressor.

In case of chemical stressors these responses can be either direct or indirect. Examples for a direct response would be the modulation (increase or decrease) in transcription of a specific gene initiated by the binding of steroid hormones or analogs to a receptor or transcription factor. The induction of hepatic vitellogenin after exposure to estrogenic or xenestrogenic compounds is one of the best studied examples. Such compounds taken up by the organism exhibit a high binding affinity to estrogen receptors and form together an estrogen response element which serves as a transcription factor to iniate mRNA transcription coding for the vitellogenin protein (DiGiulio and Hinton 2008). Various insecticides are suspected to possess endocrine disrupting properties, but effects might be underestimated or neglected, as the primary neurotoxic mode of action appears more hazardous. Other responses can be considered compensatory responses to adjust to or compensate for the disturbance. Depending on the magnitude of the stressor – in time or in dose - these responses enable the physiological restitution. Beyond a certain threshold the activation of defense mechanisms, such as antioxidant defense, biotransformation and detoxification can be accompanied by signs of reduced vital activity. If the magnitude of initial stress exceeds a certain level or persists over an extended time-period, the organism will be irreversibly damaged ultimately leading to death. Figure 2 illustrates the relationship between time and stressor intensity and the transcriptional responses, highlighting the varying profiles that can be observed (Denslow et al. 2007). It is assumed that the initial stages, adaptation or compensation are reversible, and could be used to predict adverse outcomes (Denslow et al. 2007). Thus, gene transcription

data can provide useful mechanistic insight regarding responses to toxicants and the health status of an organism (Connon et al. 2008). Integrative approaches are of particular importance for the interpretation of cellular effects in an ecological context. Adversity is detected at higher level of biological organization and can theoretically be related to the observed transcriptional changes.

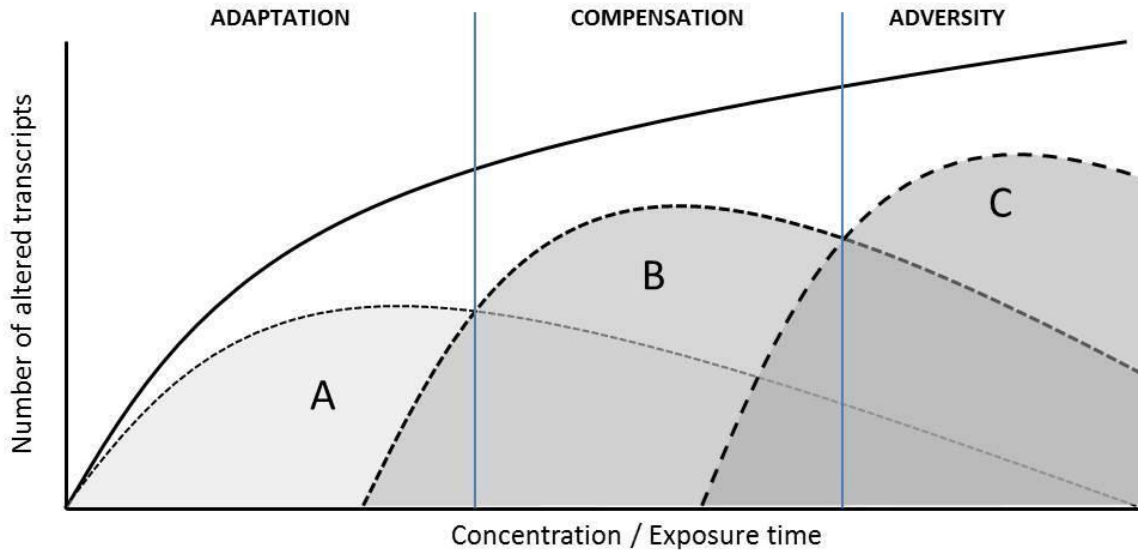

Figure 2: Schematic overview of gene transcript regulation changes over different stages of toxicity (Modified after Denslow et al. 2007): Different stages of toxicity (Adaptation, compensation and toxicity) result in distinct gene transcript responses associated with each the respective phase. A: Adaptive Responses; B: Compensatory Responses; C: Terminal toxicity.

Test Organism

Basic research is thereby often dependant on the use of model species with well described life-history traits and available toxicological and genomic information. The fathead minnow (*Pimephales promelas*) is one vertebrate model species that is commonly used in ecotoxicological studies in the United States. Historically, this fish species was chosen as a suitable model as it has a ubiquitous distribution, providing a general environmental relevance in comparison to specialized species only occurying in distinct habitats. Suitable handling and maintenance in laboratory experiments and the continuous reproduction without a distinct spawning period of this species are characteristic practical aspects. Furthermore, adult specimens exhibit a distinct sexual dimorphism, which facilitates the analysis of endocrine disrupting effects. Recently, the amount of genomic information is steadily increasing and available for the scientific community via international databases provided by the National Center for Biotechnology Information (GenBank: ncbi.nlm.nih.gov/genbank), the European

Molecular Biology Laboratory (EMBL: embl.de), or the DNA Data Base of Japan (DDBJ: http://www.ddbj.nig.ac.jp).

The use of model species therefore facilitates the application of different analytical approaches and the comparability with other studies using the same model. To discover basic relationships in stress response for example genomic vs. whole-organism, model species are suitable for the reasons mentioned above. For true environmental relevance, however, it is necessary to relate the effects measured in the model to non-model species.

Test substances

Industrial chemicals and products, such as solvents, petrochemicals, lubricants and flame retardants (e.g. benzenes, xylenes, phthalates, PCBs, polybrominated diphenylethers) consumer products, such as detergents, pharmaceuticals, hormones and personal-care products (e.g. nonylphenol ethoxylates, antibiotics, ethinyl estradiol, UV-filters), biocides, including pesticides (e.g. organochlorines, carbamates, pyrethroids, neonicotinoids) and nonagricultural biocides (e.g. tributyltin, triclosan) as well as natural or geogenic chemicals, such as heavy metals, inorganics and cyanotoxins (e.g. cadmium, mercury, copper, arsenic, fluoride, uranium, microcystins) as well as transformation products of all above are ubiquitously present in many aquatic environments (Schwarzenbach et al. 2006). A geographical area where most of the mentioned stressors occur in combination is the Central Valley and the San Francisco Estuary in the state of California, USA, resulting in a shift in fish species assemblages and major decline in pelagic fish species, described as the pelagic organism decline - POD (Sommer et al. 2007).

Frequently detected are thereby insecticides, caused by the widespread use in agricultural and urban areas, which are transported to surface aquatic systems via irrigation- and stormwater-induced runoff. The increasing use of pyrethroid insecticides in structural and landscape maintenance in California was recently reported by Greenberg et al. (2010) for the time period between 1997 and 2007. The use of the synthetic pyrethroid bifenthrin (Figure 3) increased from 40 to 22025 kg. Bifenthrin is one of the most frequently detected insecticides (Weston and Lydy 2010), and runoff from residential areas contained bifenthrin at concentrations of 0.12 $\mu g.L^{-1}$ to 6.12 $\mu g.L^{-1}$ (Gan et al. 2012), effluent of wastewater treatment plants contained up to 0.02 $\mu g.L^{-1}$ bifenthrin (Weston and Lydy 2010). Like all pyrethroids, bifenthrin is highly toxic to aquatic organisms. The physical properties are summarized in Table 1. The main

mode of action is the interference with voltage-gated ion channels and ATPase enzymes (Burr and Ray 2004) causing hyperexcitability, tremors, convulsions and ultimately death (Bradbury and Coats 1989). The 24 h LC_{50} and 96 h LC_{50} of bifenthrin for larval fathead minnow are reported to be 1.90 $\mu g.L^{-1}$ (Beggel et al. 2010) and 0.26 $\mu g.L^{-1}$ (Werner and Moran 2008), respectively. In a study by Jin et al. (2009) it was demonstrated that exposure concentrations of bifenthrin above 50 $\mu g.L^{-1}$ significantly affect swimming activity in zebrafish (*Danio rerio*) as well as the induction of vitellogenin, a validated biomarker of endocrine disruption.

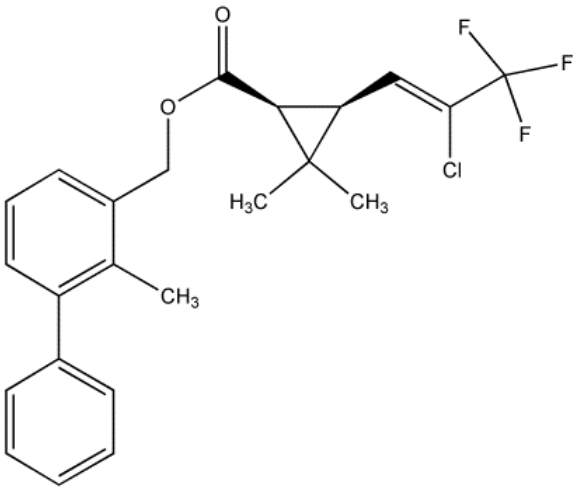

Figure 3: Chemical structure of bifenthrin (2-Methyl{1,1'-biphenyl}-3-yl)methyl 3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopropanecarboxylate) (PAN Pesticide Database: http://www.pesticideinfo.org)

Table 1: Physical properties of the pyrethroid-class insecticide bifenthrin (Chemical Services Abstract Number (CAS) 82657-04-3) (Product information, CDPR 1999, Kegley et al. 2008).

Physical properties	Value
Molecular weight	422.9 $g.mol^{-1}$
Water solubility (25°C)	0.1 $mg.L^{-1}$
Vapor pressure (25°C)	1.81 x 10^{-7} $mm.Hg^{-1}$
Henry's constant (pH 7, 25°C)	7.20 x 10^{-3} $atm.m^3.mol^{-1}$
Hydrolysis half-life (pH 6.7, 25°C)	Stable
Octanol-water coefficient (K_{ow})	1.0 x 10^6
Anaerobic half-life	97-156 d
Aerobic half-life	65-125 d
Specific gravity (25°C)	1.212 g.mL
Soil adsorption coefficient (K_{oc})	1.31 - 3.02x10^5
Photolysis	276-416 d

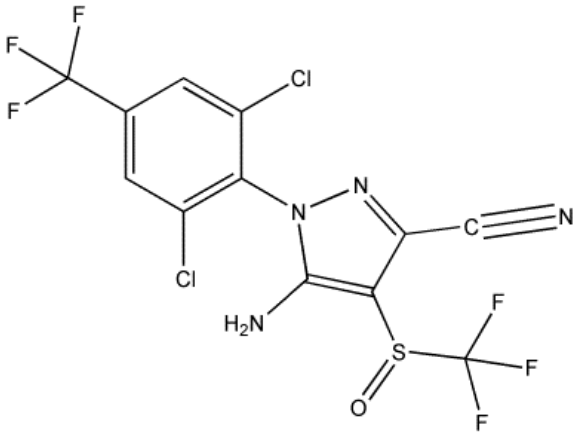

Figure 4: Chemical structure fipronil (5-Amino-1-(2,6-dichloro-4-(trifluoromethyl)phenyl)-4-(1,R,S)-(trifluoromethyl)sulfinyl)-1H-pyrazole-3-carbonitrile) (PAN Pesticide Database: http://www.pesticideinfo.org).

Table 2: Physical properties of the phenypyrazole-class insecticide fipronil (Chemical Services Abstract Number (CAS) 120068-37-3) (Product information, Gunasekara et al. 2007, Kegley et al. 2008).

Physical properties	Value
Molecular weight	473.2 $g.mol^{-1}$
Water solubility (pH 5)	1.9 $mg.L^{-1}$
Vapor pressure (calculated)	2.8 x 10^{-9} mmHg
Henry's constant (pH 7, 25°C)	7.20 x 10^{-3} $atm.m^3.mol^{-1}$
Hydrolysis half-life (pH 6.7, 25°C)	30 d
Octanol-water coefficient (K_{ow})	1.0 x 10^4
Anaerobic half-life	123 d
Aerobic half-life	366 d
Specific gravity (20°C)	1.55 $g.cm^3$
Soil adsorption coefficient (K_{oc})	749.0
Photolysis	0.33 d

The use of alternative substances, such as the phenylpyrazole fipronil, a highly efficient insecticide that is preferably used against ants and termites, has increased from 0 to 29374 kg per year within 10 years after the registration in California. Fipronil (Figure 4) is a "new generation" phenylpyrazole insecticide, whose mode of action differs from organophosphates and pyrethroids, to which numerous insects have developed resistance (Bloomquist 2003, Soderlund 2008). The physical properties are summarized in Table 2. The main mode of action of phenylpyrazoles is the interference with the function of γ-aminobutyric acid (GABA)-gated Cl^-channels (Cole et al. 1993). In insects and mammals, the behavioral effects of GABA antagonists include hyperactivity, hyperexcitability, and convulsions, which are correlated with increased spontaneous nerve activity (Gunasekara et al. 2007). Fish LC_{50} values have been reported for sheepshead minnow (130 $\mu g.L^{-1}$, 96 h LC_{50}), bluegill sunfish (83 $\mu g.L^{-1}$, 96 h LC_{50}) and rainbow trout (100 $\mu g.L^{-1}$, 96 h LC_{50}) (Gunasekara et al. 2007,

Kegley et al. 2008). Concentrations measured in irrigation runoff from residential areas ranged from 0.122 to 10.0 $\mu g.L^{-1}$ (Gan et al. 2012), and ≤ 9 $\mu g.L^{-1}$ in surface waters downstream of treated rice fields (Schlenk et al. 2001).

The major objectives of this thesis were

(i) The establishment of swimming performance test systems for the characterization of sublethal effects in larval fathead minnow (*Pimephales promelas*) after short term insecticide exposure.

(ii) The comparison of the sensitivity of different endpoints on the individual level (mortality, swimming performance, growth) with changes in gene transcription of multiple biomarkers assessed by quantitative PCR.

(iii) The evaluation of interrelationships between transcriptomic changes and higher level outcomes in a time series including recovery (24h exposure, 6 d recovery).

(iv) The application of an integrative effect assessment for a non-model species, the endangered delta smelt (*Hypomesus transpacificus*).

Chapter I -Measuring swimming performance as a sublethal stress-response in larval fathead minnow (*Pimephales promelas*): A method comparison.

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This chapter is currently in preparation for publication.

Abstract

Swimming behavior and performance are often used to evaluate sublethal stress responses in aquatic species, especially fish. Here, we compare the results obtained from a manual swim tests and from video-based tracking software with regard to method specific sensitivity. We exposed larval fathead minnow (*Pimephales promelas*) to 2 chemicals, the insecticide Talstar®, a pesticide formulation containing the pyrethroid bifenthrin as active ingredient and the antibacterial agent triclosan. Following exposure, individual fish underwent video monitoring, as well as manual tests to determine non-provoked or forced swimming performance, respectively. Exposure to both chemicals led to a dose-dependent decrease in distance traveled in manual swim tests and a dose-dependent decrease in swimming distance recorded by video tracking. These impacts were observed at sublethal concentrations. Talstar® caused a stronger decline on forced swimming than on non-provoked swimming. This difference was not significant for triclosan exposed fish. There was a significant relationship between forced swimming and non-provoked swimming distance for both Talstar® ($r=0.52$, $p<0.01$) and triclosan ($r=0.62$, $p<0.01$) exposed fish. Together these results support the use of swimming performance and behavior to assess toxicant effects, and demonstrate that both methods are adequate indicators of sublethal stress in larval fish. Furthermore, these results show that normal swimming activity, as well as, forced swimming performance are correlated and are affected by chemical contaminants with diverse mechanisms of action.

Introduction

Approaches to measure the toxicity of chemicals to aquatic organisms typically rely on standardized laboratory toxicity tests (USEPA 2002), where endpoints and effect thresholds are dominantly expressed as in relation to mortality. However, the toxic effects of contaminants are often subtle, and behavioral changes can occur at exposure levels far below the concentrations that cause lethality. Behavioral endpoints can be useful tools indicative of ecological fitness, but should be chosen carefully based on their significance to a given species and the feasibility of the method. In aquatic organisms such as fish, swimming is associated with many important ecologically relevant traits including foraging, capturing prey and evading predators, maintaining position within a water body or against a current, and reproduction or social behaviors (Little and Finger 1990, Scott and Sloman 2004). Impaired swimming can have severe consequences for an organism and numerous studies have utilized swimming behavior and performance to evaluate chemical and physical stressors (reviewed by Scott and Sloman 2004). Historically, tests designed to assess swimming typically utilized manual tests acquired through experimenter - controlled apparatus or hand - scoring from captured video (Kane et al. 2004). These protocols have provided valuable information regarding swimming performance, predator prey responses (Floyd et al. 2007), endurance (Fisher and Leis 2010), and reproductive behaviors or social interactions (Scott and Sloman 2004). An increasing number of protocols have introduced automated tracking systems as a way to assess swimming without potential bias of the experimenter (Kane et al. 2004, Mathur et al. 2011). In addition to the forced swimming parameters gained from manual tests, automated systems are useful when assessing normal swimming activity and non-apparent sublethal stress responses, such as pattern development or lack of mobility (Kane et al. 2004).

There are several aspects which test to use in an experimental setup and depending on the initial hypothesis and the kind of stressor it might be crucial to choose the adequate test system. For instance, when studying social interactions, dominance hierarchies or mating behavior in fish, a simple measurement of swimming distance might not reveal possible disruption of these behaviors. However, in many cases it might be just a matter of cost and benefit, when a reliable test system is chosen that is ready available and have high costs.

Here we compare the sensitivity of manual and automated tracking protocols of swimming behavior as measures of sublethal responses to chemicals in larval fish. The manual approach used in this study was designed by Heath et al. (1993) as a way to assess burst swimming and endurance in larval fish exposed to contaminants. This method was also shown to be a

suitable and sensitive tool for measuring the sublethal impacts of two insecticides, bifenthrin and fipronil on larval fathead minnow (Beggel et al. 2010). An automated, video based system, Ethovision XT Software (Noldus Information Technology Inc. Leesburg, VA), also used in the present study, was designed as an automated tracking system that can be run without direct influence of the experimenter. Along with measuring overall swimming distance, this system can generate a large amount of swim pattern data that can be useful when assessing the effects of chemical perturbations.

The present study utilizes the two methods to assess sublethal stress responses in larval fish exposed to two chemicals with different modes of action (MOA). One is a pesticide formulation containing the synthetic pyrethroid insecticide, bifenthrin, which is frequently been detected in sediments and surface waters in California, USA (Weston et al. 2009). Bifenthrin is highly toxic to fish, interfering with Na^+ channel gatings and other ion-channels such as Cl^- and Ca^{2+} channels in the nerve cell endings (Burr and Ray 2004). This leads to continuous neurotransmission, causing hyperexcitability, tremors, convulsions and ultimately death (Bradbury and Coats 1989). The second substance used in this study is the antibacterial agent, triclosan, added to a great number of personal care products, which has been detected in surface waters (Kolpin et al. 2002), sediment (Chalew and Halden 2009) and tissue of wild fish (Adolfsson-Erici et al. 2002). There is currently no defined MOA for triclosan but recent studies have shown that the phenolic compound alters Ca^{2+} signaling in muscle cells (Ahn et al. 2008), acts as an endocrine disrupting compound (Foran et al. 2002, Ishibashi et al. 2004) and can alter xenobiotic metabolism (Wang et al. 2004, Morisseau et al. 2009) in human liver fractions.

Swimming of fish is dependent on several physiological processes which can be affected by chemical contaminants. In this study we describe the influence of bifenthrin, as formulated in the commercial insecticide Talstar® (hereafter referred to as bifenthrin) and triclosan on swimming ability in larval fathead minnow (*Pimephales promelas*). We assess swimming behavior and performance utilizing two protocols: i) manual experiments designed to assess burst speed and endurances ii) automated tracking systems designed to assess non-provoked swimming activity.

Here we compare the results and interpretations of swimming impairment detected by both methods as well as their sensitivity for the detection of sublethal stress in fish.

Material and Methods

Model Organism: Fathead minnow larvae were obtained from Aquatox Inc. (Hot Springs, AR, USA) at 7 d post-hatch on the day of arrival. For all tests, control and dilution water consisted of deionized water, modified to USEPA moderately hard standards (USEPA 2002a). Upon arrival, fish were acclimated for at least 6 hours in control water held at 25˚C with aeration. During this time mortality and behavior were monitored and the larval fish were fed an excess of *artemia nauplii*.

Chemical exposure: Bifenthrin formulation of Talstar® (USEPA Reg.No. 279-3155, 7.9% active ingredient; FMC Corporation, Philadelphia, PA, USA) and Triclosan (CAS Number 3380-34-5; 97% purity; Fluka Chemical Corporation, St.Paul Milwaukee, WI, USA) were purchased commercially. All exposures were conducted at the Aquatic Toxicology Laboratory, School of Veterinary Medicine, University of California Davis, USA.

Concentrations used for both bifenthrin and triclosan experiments were within a sublethal range, based on preliminary exposure experiments (data not shown). Tests were conducted at 16:8 light:dark photoperiod and all fish were not fed during testing. Effects of bifenthrin were determined after a 24 h exposure period to nominal bifenthrin concentrations of 0.17, 0.33, 0.56 or 0.83 $\mu g.L^{-1}$ (AI in the formulation Talstar®) and compared to the DIEPAMH control. Each treatment consisted of 3 replicates of 10 fish placed in 250 mL of exposure solution. Overall, 15 fish were used for swimming tests per treatment, 5 from each replicate. Effects of triclosan were determined after a 2 h exposure period to DIEPAMH control, a solvent control (0.01% Methanol, Fisher Scientific, Pittsburgh, PA) and nominal triclosan concentrations of 150, 300 and 450 $\mu g.L^{-1}$ in 0.01% methanol. Each treatment consisted of 4 replicates of 10 fish placed in 250 mL of exposure solution. After 2 hours, survival was recorded and 12 fish per treatment were used for swimming tests, 3 from each replicate.

For both tests water temperature (T), pH and dissolved oxygen (DO) were averaged across replicates per treatment. For bifenthrin, the mean values (±SD) were T: 22.5 °C (±0.2), pH: 7.7 (±0.1) and DO: 7.7 $mg.L^{-1}$ (± 0.3). For triclosan, the mean values (±SD) were T: 22.4 °C (±0.1), pH: 7.7 (±0.1) and DO: 8.5 $mg.L^{-1}$ (±0.1).

Swimming tests: After exposure to the respective chemicals, fish swimming behavior was assessed in two stages: (a) spontaneous swimming activity as non-provoked swimming

monitored by overhead video equipment and b) forced swimming behavior to assess predator avoidance and swimming performance.

Spontaneous swimming activity was recorded in MPEG-2 format, using a Panasonic black and white CCTV Camera (12V DC) connected to a portable laptop-computer via a USB Frame grabber (Model WinTV-HVR 950 by Hauppauge, NY) with 30 frames per second. Fish were randomly chosen from each replicate and transferred individually into an 85 mm culturing dish filled with approximately 100 mm of DIEPAMH. Before the actual test, fish remained undisturbed for acclimation of at least 1.0 min. After acclimation, fish were recorded for 2.0 min. Videos were analyzed in Ethovision XT 6.1 Software (Noldus Information Technology Inc. Leesburg, VA) to compute total distance traveled within 1.5 min.

Following video tracking, fish were immediately transferred to a different test vessel for manual swimming tests to ensure direct comparability. Here, forced swimming performance was measured using a circular 'racetrack' (Heath and Cech 1993) consisting of a 130 mm diameter Petri dish with an upside-down 80 mm diameter Petri dish in the center. The track was divided into 8 sectors by radiating lines drawn on the bottom of the testing dish, and the dish was filled to a depth of 100 mm with the respective control water. Before testing, fish were allowed to acclimate to the race track for 1.0 min. A plastic rod was then used to trigger the fish's escape response by gentlytouching the tail fin every time the fish stopped moving. The number of lines or sectors crossed by the fish within 1.0 min was recorded and used as a measure of swimming performance. Total swimming distance was calculated by multiplying the number of sectors crossed times the mean distance between two lines along the middle circumference of the testing device (factor 4.12).

Statistical analysis: Univariate Analysis of Variance and Correlation Analysis (ANOVA) followed by a Dunnett's means separation were utilized to evaluate differences in swimming performance data for both non-provoked and forced swimming performance tests. Pearson Correlation was used to test the relationship between the different swimming protocols as well as the relationship between swimming performance measures and treatment concentration. All statistical applications were performed utilizing SPSS 11.0 Statistical Software.

Results

Bifenthrin: Exposure of larval fathead minnow to bifenthrin led to a significant decrease in swimming performance after 24 h, observed in both, the manually performed racetrack and the automated video tracking system. There was a strong negative correlation between the calculated forced swimming distance with bifenthrin concentrations ($r = -0.76$, $p < 0.01$; Table 3). Similarly, total distance traveled during non-provoked swimming measured by videotracking, was negatively correlated with bifenthrin concentration levels (Pearson correlation $r = -0.56$, $p < 0.01$; Table 3).

Significant effects on swimming occurred at ≥ 0.55 $\mu g.L^{-1}$ bifenthrin. This relationship was consistent for both methods but was more pronounced in the forced swimming test (non-provoked swimming $p < 0.01$, forced swimming $p < 0.001$). Estimated distance traveled in the forced swimming test was greater than distance traveled during non-provoked swimming. Even at the highest exposure concentration, 0.83 $\mu g.L^{-1}$ bifenthrin, fish traveled a greater distance in the racetrack (Figure 5A). Swimming distance data obtained from the two methods showed a significant positive correlation ($r = 0.49$, $p < 0.01$).

Triclosan: Triclosan had a negative impact on both forced and non-provoked swimming performance after just two hours of exposure. A significant decrease was observed at 300 $\mu g.L^{-1}$ (non-provoked swimming, $p < 0.01$; forced swimming $p < 0.001$) and 450 $\mu g.L^{-1}$ (non-provoked swimming, $p < 0.001$; forced swimming $p < 0.001$). Exposed fish experienced a dose-dependent decrease in swimming distance in both forced and non-provoked swimming trials. Swimming distance within the racetrack was negatively correlated with triclosan concentration ($r = -0.81$, $p < 0.01$; Table 3). Similarly, a significant negative correlation was observed for the total non-provoked swimming distance assessed by videotracking and triclosan concentration ($r = -0.67$, $p < 0.01$; Table 3).

Table 3: Pearson Correlations between treatment concentrations and swimming distances evaluated by manual racetrack test and automatic Ethovision test. (**) indicates correlation is significant at the 0.01 level. Swimming distances were both negatively correlated with increasing treatment concentration for both tested substances. Distances for swimming from both test systems were significantly correlated for each substance.

	Bifenthrin/Talstar®		**Triclosan**	
	Racetrack	Ethovision	Racetrack	Ethovision
Racetrack				
Pearson Correlation	1	0.49**	1	0.62**
Sig. (2-tailed)		< 0.001		< 0.001
N	78	78	44	44
Treatment Conc.				
Pearson Correlation	-0.76**	-0.56**	-0.81**	-0.67**
Sig. (2-tailed)	< 0.001	< 0.001	< 0.001	< 0.001
N	78	78	44	44

The observed patterns of the decrease in swimming distance show that the external stimulus in the racetrack test results in a higher distance measurement as in the non-provoked test for the control treatments and at 150 $\mu g.L^{-1}$ triclosan (Figure 5). At the higher concentration levels of 300 $\mu g.L^{-1}$ and 450 $\mu g.L^{-1}$ triclosan it seems that fish lacked to respond to the external stimulus, as total swimming distance covered by fish were almost identical in racetrack tests and non-provoked swimming assessments. This observation is contrary to those from exposure experiments with bifenthrin, in which swimming distances measurements showed larger differences between the two methods (Figure 5 A). Pearson's correlation between the measures of distance evaluated by the different test protocols showed a significant linear relationship ($r = 0.62$, $p < 0.01$), again supporting the suitability of both methods to assess sublethal stress response.

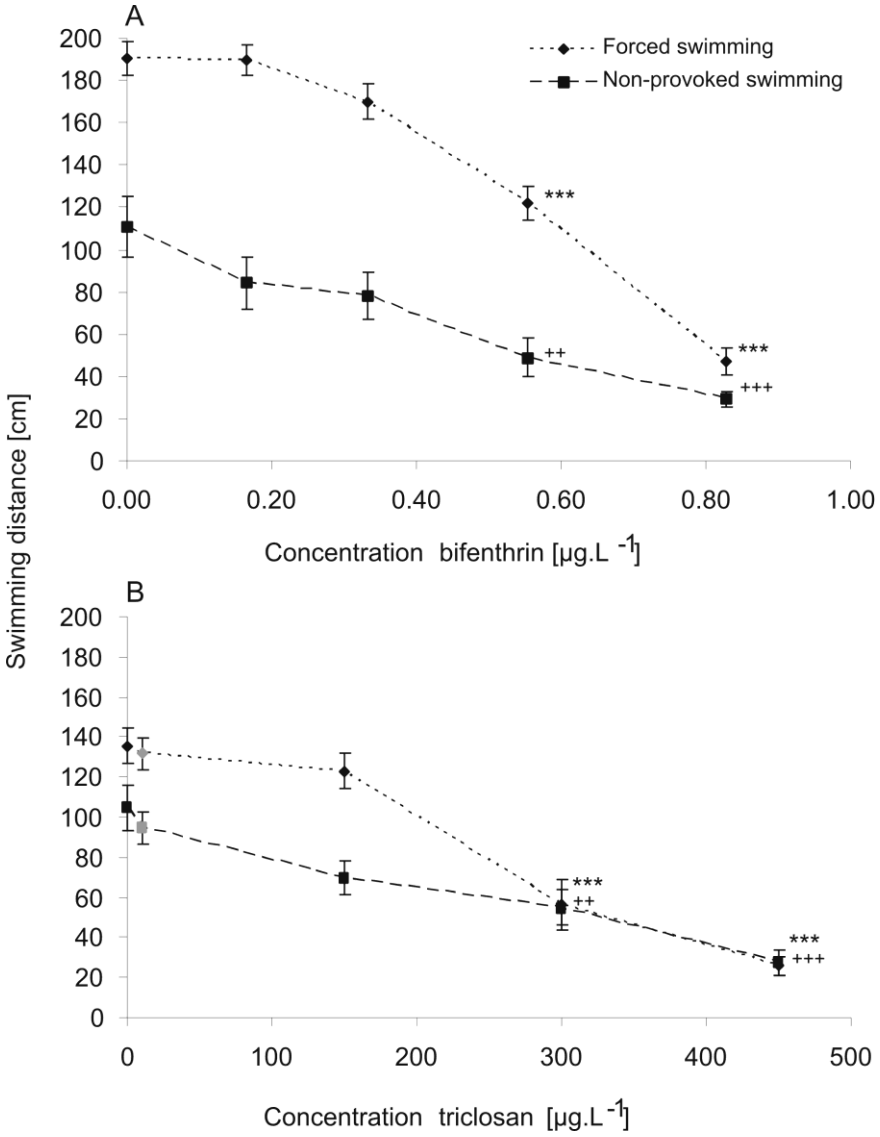

Figure 5: Comparison in swimming distance evaluated by manual, forced swim-test (Racetrack) and automated, non-provoked swim-test (Ethovision) procedures. A: Bifenthrin/Talstar®. B: Triclosan, datapoints for solvent control shifted for visibility (grey). Data is presented as arithmetic mean ± standard error. Significant differences to control (p <0.05) are indicated with (*) and (+).

Discussion

In the present study we compared two methods of swimming tests to evaluate sublethal stress responses in larval fish exposed to two chemicals with differing toxicological MOA. Results show that both, the forced swimming test system (racetrack), which was designed to assess predator avoidance responses and endurance, as well as the non-provoked swimming performance, measured using an automated videotracking system, reveal highly significant, negative correlations with increasing chemical exposure concentrations. Both tests were highly sensitive and variability was comparable between the two test methods. Overall, the assumption that forced swimming tests - due to the added external stimulus - would be more sensitive predictors of stress was only supported by those experiments conducted with bifenthrin, a neurotoxic insecticide, which caused a more radical change in forced swimming distance, or predator avoidance, than in non-provoked swimming behavior. As a consequence, the correlation between the two methods was higher for triclosan than for the pyrethroid bifenthrin. These results are important, as they highlight the importance of differentiating the impacts of aquatic pollutants based on mode of action. The greater negative effect of bifenthrin on forced swimming, i.e. burst swim speed or endurance, is likely due to the insecticides known effect on Na^+ channels and neuromuscular cell function. As summarized by Bradbury and Coats (1989), the exposure of fish to increasing synthetic pyrethroid concentrations results in initial hyperactivity and hyperexcitability prior to a loss in buoyancy and inactivity of the fish, matching the results of this study.

In contrast, the antibacterial agent, triclosan, doesn't affect the organism's neuromuscular control by disturbing nerve signal transmission, and therefore would not lead to differences in normal swimming and burst swimming caused by hyperactivity. It was previously shown that triclosan is negatively affecting Ca^{2+}-signaling by targeting ryanodine receptors and therefore triggers Ca^{2+}-release from the sarcoplasmatic reticulum during skeletal muscle contraction (Ahn et al. 2008). This effect results in reduced muscle function, as shown here by the general reduction in swimming distance. The impairment of proper muscle function by triclosan could be explanatory for the observed effect that fish did not respond to the external stimulus in the forced swim test for concentrations $\geq 300\ \mu g.L^{-1}$, resulting in almost identical measurements of swimming distance for both test methods. However, these relationships with MOA still need to be fully assessed, but our results suggest overall, that sublethal swimming responses are valid indicators of stress for chemicals with different MOA.

We found that both methods, the forced as well as the non-provoked swimming protocols can be utilized to assess neuromuscular effects in larval fish as stress response. Each method has advantages and disadvantages that may prove important when designing an experiment. Most notably, manual experiments can be easily performed without the need for extra laboratory space or costly equipment. Similarly, forced swimming techniques may provide information on additional differences in swimming behavior by triggering sensitive responses, such as escape responses, that might not be captured by an automated system. However, manual methods add an extra source of error and bias by introducing an external stimulus to the experiment, and results may vary due to experimenter-specific bias and subtle differences in experimental design. On the other hand, software based systems are designed to reduce stress to the organism and reduce the amount of error and bias in an experiment. The automated systems can theoretically run for an unlimited time-period without harming or handling animals. Advantages of automated systems are the options to track an organism's overall pattern of movement leading to assessments of total distance, velocity, levels of immobility, and even calculated trajectories or body rotations (http://www.noldus.com). The additional capacity of automated systems may be necessary to examine other stress responses than total distance, which may not be the most sensitive endpoint when determining altered behavior. However, in this study, other parameters measured by the software such as the duration of movement or the general mobility yielded no information that was more sensitive in comparison to the distance parameter.

In conclusion, both test methods were well-suited to evaluate disruption of swimming ability after contaminant exposure. Sensitivity of the tests was similar, but the forced swimming test may reveal additional effects on predator avoidance that non-provoked swimming behavior could not. Therefore, the two methods are valuable individually for elucidating the effects of sublethal stress in fish, but it may be wise to utilize both protocols when studying the effects of neurotoxic compounds as these appear to affect the predator avoidance response more strongly than normal swimming behavior. Environmental regulations, however, often do not consider altered behavioral patterns as toxicological endpoints, focusing instead on growth endpoints or lethality (USEPA 2002a), which are often too insensitive to detect sublethal effects caused by contaminants. Sublethal effects such as swimming behavior can be vitally important for successful predator avoidance, forage and even reproductive success (Weis et al. 2001, Floyd et al. 2008). We compared two established methods that generate quantitative behavioral data and are sensitive indicators of sublethal chemical stress without being destructive to the test species. In the future, this information can be combined with molecular

or cellular biomarker data to develop model-based analysis tools for use in a field setting. These models would be valuable when determining detrimental responses, other than survival, to environmentally relevant concentrations of single chemicals or complex mixtures.

Chapter II - Sublethal toxicity of commercial insecticide formulations and their active ingredients to larval Fathead Minnow (*Pimephales promelas*)

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A similar version of this chapter was published in: Science of the Total Environment 408 (2010) 3169–3175

Abstract

Toxic effect concentrations of insecticides are generally determined using the technical grade or pure active ingredient. Commercial pesticide formulations, however, contain a significant proportion (>90%) of so-called inert ingredients, which may alter the toxicity of the active ingredient(s). This study compares the sublethal toxicity of two insecticides, the pyrethroid bifenthrin, and the phenylpyrazole fipronil, to their commercial formulations, Talstar® and Termidor®. Both insecticides are used for mosquito control, landscape treatment and structural pest control, and can be transported into surface water bodies via stormwater and irrigation runoff. We used larval fathead minnow (*Pimephales promelas*), to determine effects on growth and swimming performance after short-term (24 h) exposure to sublethal concentrations of pure insecticides and the respective formulations. Effects on growth were observed at 10% of the LC_{10} (53 $\mu g.L^{-1}$) fipronil. Swimming performance was significantly impaired at 20% of the LC_{10} (0.14 $\mu g.L^{-1}$) of bifenthrin and 10% of the LC_{10} of Talstar® (0.03 $\mu g.L^{-1}$). Fipronil and Termidor® led to a significant impairment of swimming performance at 142 $\mu g.L^{-1}$ and 148 $\mu g.L^{-1}$ respectively, with more pronounced effects for the formulation. Our data shows that both formulations were more toxic than the pure active ingredients, suggesting that increased toxicity due to inert ingredients should be considered in risk assessments and regulation of insecticides.

Introduction

Insecticides are contaminating surface water bodies of agricultural areas in California, USA, and elsewhere (Schulz 2004, TDC-Environmental 2008, Werner et al. 2004). It is, however, a misconception that attributes insecticide use to agricultural activities alone. Insecticides are also heavily used in urban areas where application by homeowners and professionals for mosquito control, landscape treatment and structural pest control results in an extensive source of contamination (Budd et al. 2007, Sandahl et al. 2007). Even if not applied in the vicinity of surface water bodies, insecticides can be transported via irrigation runoff and stormwater into urban streams and waterways (Brady et al. 2006, Weston et al. 2005). Aquatic invertebrates and fish thus become targets of toxic substances at potentially hazardous concentrations. This is of special concern if sensitive larval and developmental stages are affected. Toxicity of insecticides to fish and other aquatic species is generally determined via threshold concentrations such as LC/EC_{50} for the pure active ingredient (AI) of commercial products (Cox and Surgan 2006, USEPA 2007a). However, commercial products contain the AI mixed with non-insecticidal ingredients, so-called “inert” or “other” ingredients, which in some cases comprise more than 90% in volume of insecticide formulations (Cox and Surgan 2006). They need not be identified on the product label, unless classified as highly toxic (USEPA 2007b), and act as adjuvants, solvents, emulsifiers, surfactants and/or preservatives. Numerous commercial formulations often exist for each AI, and it is known that availability and toxicity of the AI may be substantially altered by inert ingredients (Schmuck et al. 1994). Studies have shown that in many cases the toxicity of commercial formulations is higher than that of the active ingredient, but this is not always the case. Mayer and Ellersieck (1986) compared the toxicity of 161 technical grade pesticides to their formulations and showed that overall toxicity was not affected in 57%, decreased in 11% and increased in 32% of the cases. In a more recent study (Schmuck et al. 1994), 95% of 273 herbicide, fungicide and insecticide formulations were more toxic to fish than the respective pure AI The study presented here aims to contribute information about the comparative toxicity of pure bifenthrin and fipronil and two of their formulation products focusing on sublethal endpoints in larval fish. To our knowledge no such information is currently available for these substances. Both bifenthrin and fipronil are widely used in structural pest control and other urban and agricultural applications (Oros and Werner 2005, TDC-Environmental 2008). The pyrethroid, bifenthrin, is one of the most frequently detected contaminants in surface water bodies of areas with urban and agricultural land use (Budd et al. 2007). Similarly, the phenylpyrazole, fipronil, was found to be present in runoff from

metropolitan areas throughout the United States (Sprague and Nowell 2008). Both insecticides are commercially available in a large number of formulated products, generally containing <10% AI The bifenthrin formulation; Talstar®, contains 7.9% AI as microcapsules (as indicated on product label, 2008), where the insecticide is encased in a coat of "inert" ingredients to ensure its slow release and stabilization (Tsuji 2001). Termidor®, a fipronil formulation, contains 9.1% AI in the form of crystalline particles forming a liquid suspension concentrate (as indicated on product label, 2008). Like all pyrethroids, bifenthrin is highly toxic to fish, interfering with Na^+ channel gating in the nerve cell endings, but other ion-channels such as Cl^- and Ca^{2+} channels can be targeted as well (Burr and Ray 2004). This leads to continuous neurotransmission, causing hyperexcitability, tremors, convulsions and ultimately death (Bradbury and Coats 1989, Haya 1989). Reported LC_{50} values of bifenthrin for fish range from 0.15 μg L^{-1} (rainbow trout, 96 h LC_{50}) to 17.5 μg L^{-1} (sheepshead minnow, 96 h LC_{50}) (Kegley et al. 2008, Werner and Moran 2008). Runoff from residential areas contained bifenthrin at concentrations of 0.12 μg L^{-1} to 6.12 μg L^{-1}, measured at storm water drainage outflows (Gan et al. 2012). Fipronil is a "new generation" phenylpyrazole insecticide, whose mode of action differs from organophosphates and pyrethroids, to which numerous insects have developed resistance (Bloomquist 2003, Soderlund 2008). Phenylpyrazoles interfere with the function of γ-aminobutyric acid (GABA)-gated Cl^-channels (Cole et al. 1993). In insects and mammals, the behavioral effects of GABA antagonists include hyperactivity, hyperexcitability, and convulsions, which are correlated with increased spontaneous nerve activity (Gunasekara et al. 2007). Fish LC_{50} values have been reported for sheepshead minnow (130 μg L^{-1}, 96 h LC_{50}), bluegill sunfish (83 μg L^{-1}, 96 h LC_{50}) and rainbow trout (100 μg L^{-1}, 96 h LC_{50}) (Gunasekara et al. 2007, Kegley et al. 2008). Concentrations measured in irrigation runoff from residential areas ranged from 0.122 to 10.0 μg L^{-1} (Gan et al. 2012), and ≤9 μg L^{-1} in surface waters downstream of treated rice fields (Schlenk et al. 2001). Here we tested the hypothesis that the toxicity of the pure active ingredients, bifenthrin and fipronil, differs from the toxicity of their respective formulations, Talstar® and Termidor®.We used swimming performance and growth as toxicological endpoints in larval fathead minnow (*Pimephales promelas* Rafinesque), and a short exposure period (24 h), to mimic runoff-related pulse exposures (Pick et al. 1984, Werner et al. 2004). Sublethal exposure concentrations were based on previously determined acute LC_{10} values.

Material and methods

Fish source, acclimation and quality assurance

Fathead minnow larvae were obtained from Aquatox Inc. (Hot Springs, AR, USA) at 7 d post-hatch on the day of arrival. Control water consisted of deionized water, modified with salts to meet USEPA specifications (electric conductivity (EC): 265–293 μS.cm^{-1}; hardness: 80–100 as mg CaCO3 L^{-1}; alkalinity: 57–64 as mg $CaCO_3$ L^{-1} (USEPA 2002a)). Fish were acclimated for a minimum period of 4 h in control water at a temperature of 25 °C. During the acclimation period <1% mortality was observed, and the fish fed and swam normally. During the project period, routine monthly reference toxicant tests were performed using NaCl to ascertain whether organism response fell within the acceptable range according to USEPA requirements (USEPA 2002a). Each test consists of a dilution series (5 test concentrations) and a control. All test organisms responded normally (within 95% confidence interval of running mean) and sensitivity was considered typical.

Insecticide exposure

Reference standard grade bifenthrin [[1α3α(2)]-(±)(2-methyl [1,1′-biphenyl]-3-yl)methyl 3-(2-chloro-3,3,3, trifluoro-1-propenyl)- 2,2-dimethylcyclopropanecarboxylate], 99% purity (CAS number 82657-04-3), and fipronil (5-amino-1 [2,6-dichloro-4-(triflouromethyl) phenyl]-4 [(triflouromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile), 98.5% purity (CAS number 120068-37-3) were obtained from ChemService inc. (West Chester, PA, USA). Commercial insecticide formulations Talstar® (US EPA Reg. No. 279-3155; 7.9% bifenthrin per volume; FMC Corporation, Philadelphia, PA, USA) and Termidor® (US EPA Reg. No. 7969-210; 9.1% fipronil per volume; BASF Corporation, Research Triangle Park, NC, USA) were purchased commercially. Bifenthrin consists of 97% cis-isomer both in the pure compound and the formulated product. Pure fipronil is a 50:50 racemic mixture, just like its formulation product. All insecticide exposure experiments were conducted at the Aquatic Toxicology Laboratory, School of Veterinary Medicine, University of California Davis. To determine acute toxic effects on survival, 7 d old larval fish were exposed for 24 h to the following nominal concentrations: 0.75, 1.0, 1.5, 2.0, 3.0 and 4.0 μg L^{-1} bifenthrin, 3.0, 4.0, 4.5, 5.0 and 6.0 μg L^{-1} bifenthrin as amount AI in Talstar®, 150, 200, 250, 300, 350 and 400 μg L^{-1} fipronil, and 150, 200, 350, 400 and 450 μg L^{-1} of fipronil as amount AI in Termidor®. The exposure concentrations used to determine acute toxicity refer to AI concentrations (pure chemical or respective formulation) to ensure direct comparability. For the pure substances

we used 1 $mL.L^{-1}$ methanol (MeOH) as the solvent carrier and one treatment group containing the same MeOH concentration in control water was added as a solvent control. No solvent carrier was required for the formulations as they are designed to mix with water. Stock solutions were prepared in MeOH for pure insecticides (2000 $mg.L^{-1}$) and used for both, 24 h LC_{50} determination and sublethal exposure experiments. Exposure concentrations used for the swimming performance and growth test series were calculated as percentages of the nominal LC_{10} values derived from the acute toxicity tests (Table 4). For each chemical, treatments consisted of a control, solvent control (pure chemicals only), and 10%, 20%, 33% and 50% of the nominal LC_{10}. Each treatment consisted of 13 replicate 600 ml Pyrex beakers containing 250 ml test solution and 10 fish larvae. Subsequently, we used 9 replicates to determine swimming performance at three different time points and 4 replicates to determine growth. At test initiation, 10 larvae were transferred from the acclimatization tank to each beaker and exposed for 24 h at a water temperature of 25 °C and a 16:8 light–dark ratio. Test vessels were then manually distributed in a random manner, within the exposure water bath. Fish were not fed during the exposure period.

For the sublethal concentrations, sub-samples of each test solution (1 L) were preserved with dichloromethane (Fisher Scientific, USA) at test initiation, shipped overnight to the California Department of Fish and Game Water Pollution Laboratory (Rancho Cordova, CA, USA), extracted within 24 h of arrival, and analyzed using gas chromatography with mass spectrometry and ion-trap detection. Reporting limits for detection of bifenthrin and fipronil were 0.002 $\mu g\ L^{-1}$ (recovery 88.3%) and 0.2 $\mu g\ L^{-1}$ (recovery 83.1%), respectively. Talstar® samples were filtered through 0.45 µm glass fiber filter to separate microcapsules from the water phase, and "particulate" and dissolved bifenthrin concentrations were determined. Concentrations for Talstar® are presented as the dissolved fraction. Measured and nominal insecticide concentrations are shown in Table 5.

7 d growth

Following the 24 h insecticide exposure, fish were transferred to control water and maintained for 6 days at 25 °C and a 16:8 light:dark photoperiod. During transfer, fish were gently rinsed in control water, using a fine-meshed sieve and moved to vessels containing control water. From days 2 to 7, approximately 80% of the water was exchanged daily and the number of surviving fish was recorded. Physicochemical variables (pH, dissolved oxygen, temperature, EC) were measured per treatment before and after each water exchange and at test termination. Measurements were conducted on pooled replicates of each treatment. After each

water renewal the test beakers were manually distributed in a random manner, throughout the exposure waterbath. Fish were fed *ad libitum* twice a day with newly hatched *Artemia* nauplii (ranging from 30 to 50 individuals). At test termination, surviving fish were euthanized with MS-222 (Tricaine Methanesulfonate, Sigma, St. Louis, MO, USA), then transferred to pre-weighed aluminium weigh boats and dried for 24 h at 100 °C. Dry weight per fish (±0.001 mg) was calculated by measuring whole dry weight divided by the number of fish in each replicate.

Swimming performance ("one minute racetrack")

Swimming performance was measured at three different time points: (1) Immediately after the 24 h insecticide exposure; (2) after a total of 48 h (24 h recovery in control water), and (3) after a total of 7 days (6 d recovery in control water). At each time point, seven fish per replicate from three replicate beakers per treatment were tested using a circular "racetrack" method (Heath et al. 1993). This racetrack consisted of a 13 cm diameter Petri dish with an upsidedown 8 cm diameter Petri dish centrally placed, divided into 8 sectors by radiating lines drawn on the bottom of the testing dish, and filled with control water to a depth of 1 cm. Fish from pre-selected beakers were transferred individually into the testing device and allowed to acclimate for 1 min. A plastic rod was then used to trigger the fish's escape response by gently touching the tail fin every time the fish stopped moving. Due to possible bias in experimental technique, groups of fish were tested in a random manner, without the experimenter's knowledge of exposure concentration following Heath et al. (1993). The number of lines or sectors crossed by the fish within 1 min was recorded and used as a measure of swimming performance. Water in the testing device was renewed after testing 7 fish from individual replicates.

Statistical analysis

We used the Comprehensive Environmental Toxicity Information System (CETIS) by Tidepool Scientific Software (McKinleyville, CA, USA) to calculate nominal effect concentrations for 24 h survival (NOEC, LC_{50}, LC_{10}) based on AI Statistical analyses of sublethal endpoints utilized the measured dissolved AI concentrations. The Shapiro–Wilk normality test was used to evaluate whether quantitative data met the assumptions of the parametric ANOVA. For multiple comparisons the JMP 7.0 Software by SAS Institute Inc. was used. To evaluate differences between treatments in swimming performance and growth data we used one-way ANOVA and Dunett's multiple comparison *post hoc* test to compare

insecticide treatments to controls and solvent controls. Assumptions of normality and homogeneity of variances were met, except for the highest concentrations, but due to the large differences in swimming performance, the ANOVA is considered to be robust (Underwood 1997), particularly since the distribution of residuals was unimodal.

Results

Water chemistry

Physicochemical parameters measured at the start and end of the 24 h exposure period were within the acceptable range for the test organism (USEPA 2002 a,b) for all experiments and treatments. The measured mean values (± SD) were pH: 7.51 (± 0.19), dissolved oxygen 7. 2 (± 0.5) $mg.L^{-1}$, temperature: 23.1 (± 0.3) °C, and EC: 278 (± 6) $\mu S.cm^{-1}$.

Table 4: Acute nominal effect concentrations (mortality) for 7 d old fathead minnow after 24 h exposure to bifenthrin, fipronil and their formulations, Talstar® and Termidor®. Effective concentrations, LC_{50} and LC_{10}. Values in parenthesis represent 95% confidence intervals determined via probit analysis.

Substance	NOEC [µg.L^{-1}]	LOEC [µg.L^{-1}]	24h LC_{50} [µg.L^{-1}]	24h LC_{10} [µg.L^{-1}]
Fipronil, pure	300	350	398.29 (376.27 - 438.79)	305.57 (275.56 - 324.12)
Fipronil formulation	200	350	379.47 (355.13 - 405.48)	233.01 (201.99 - 307.94)
Bifenthrin, pure	0.5	1	1.9 (1.69 - 2.12)	0.92 (0.72 - 1.09)
Bifenthrin formulation	< 3	3	4.85 (4.47 - 5.34)	2.99 (2.36 -3.39)

Sublethal effects

Individual effects were observed for each substance at concentrations below 50% of the LC_{10}. Concentration levels in the following sections refer to the measured dissolved fractions of AI, or to percentages of the nominal LC_{10} values determined by initial acute toxicity tests (Table 5).

Table 5: Nominal and measured concentrations for 24 h exposure of 7 d old fathead minnow to bifenthrin, Talstar®, fipronil and Termidor®. Treatment concentrations used for swimming performance and growth tests, calculated as percentages of the LC_{10}-value (10%, 20%, 33% and 50% LC_{10}).

Substance	Concentration [µg.L^{-1}]	10% LC_{10}	20% LC_{10}	33% LC_{10}	50% LC_{10}
bifenthrin pure AI	**measured**	**0.07**	**0.14**	**0.24**	**0.35**
	nominal	0.09	0.18	0.31	0.46
Talstar®	**measured -dissolved**	**0.03**	**0.05**	**0.08**	**0.16**
	measured -particulate	0.19	0.39	0.57	0.81
	nominal	0.29	0.59	0.99	1.49
Fipronil pure AI	**measured**	**53**	**142**	**333**	**365**
	nominal	31	61	102	153
Termidor®	**measured**	**28**	**128**	**148**	**192**
	nominal	23	47	78	117

Swimming performance

Bifenthrin. Immediately following the 24 h exposure to pure bifenthrin, the swimming performance of fish from the lowest concentration treatment (0.07 µg.L^{-1} or 10% LC_{10}) showed no statistical difference to control or solvent control treatments (Figure 6). Swimming performance of fish exposed to concentrations ≥0.14 µg.L^{-1} (20% LC_{10}, $p < 0.001$) was significantly decreased compared to solvent controls. In comparison, exposure to the commercial formulation Talstar® led to decreased swimming performance at ≥0.03 µg.L^{-1} bifenthrin (10% LC_{10}, $p < 0.001$). After transfer to control water for a 24 h recovery period, swimming performance of exposed fish improved in most insecticide treatments. Fish exposed to bifenthrin concentrations of 0.07–0.14 µg.L^{-1} as pure chemical (Figure 6A), and 0.03–0.05 µg L^{-1} as Talstar® (Figure 6B) recovered completely. After a recovery period of 6 days, no statistically significant differences between treatments were observed. When comparing dissolved bifenthrin concentrations between pure bifenthrin and Talstar®, the formulation was approximately 5 times more toxic than the pure active ingredient.

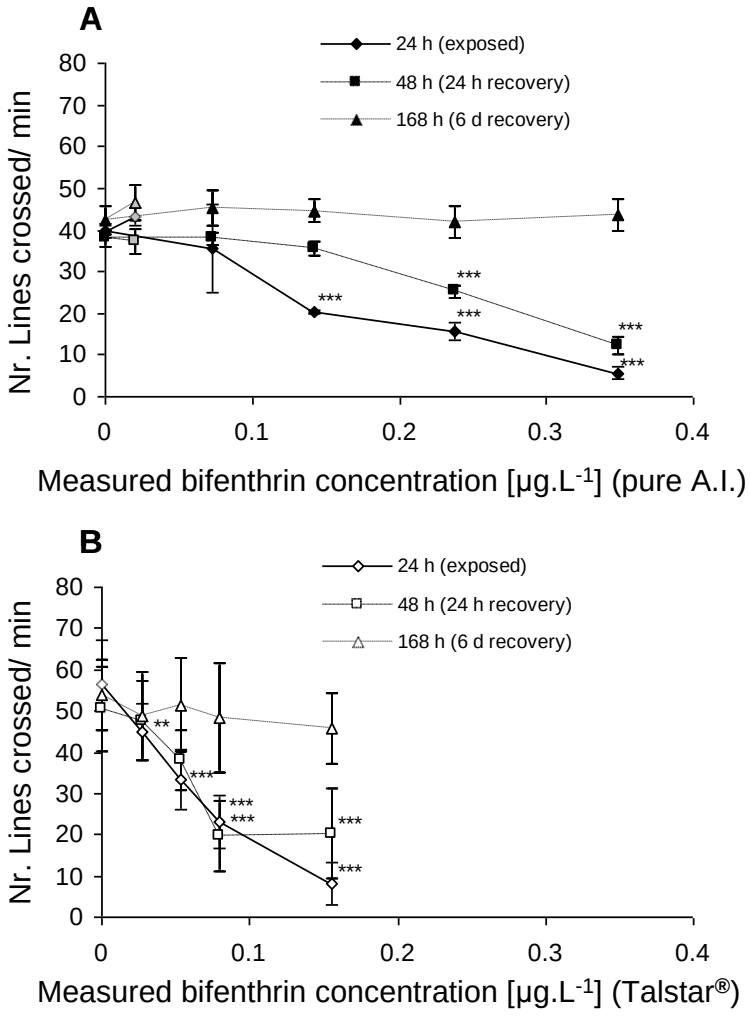

Figure 6: Swimming performance of larval fathead minnow after 24 h exposure to bifenthrin and Talstar®, 24 h recovery and 6 d recovery. Asterisks indicate significant differences in treatments compared to control/solvent control (*: p<0.05. **: p<0.01. ***: p<0.001). Data shown as arithmetic mean±SD; n=7. A: pure bifenthrin, control group shifted to x=0.02 for visibility (grey); B: Talstar®.

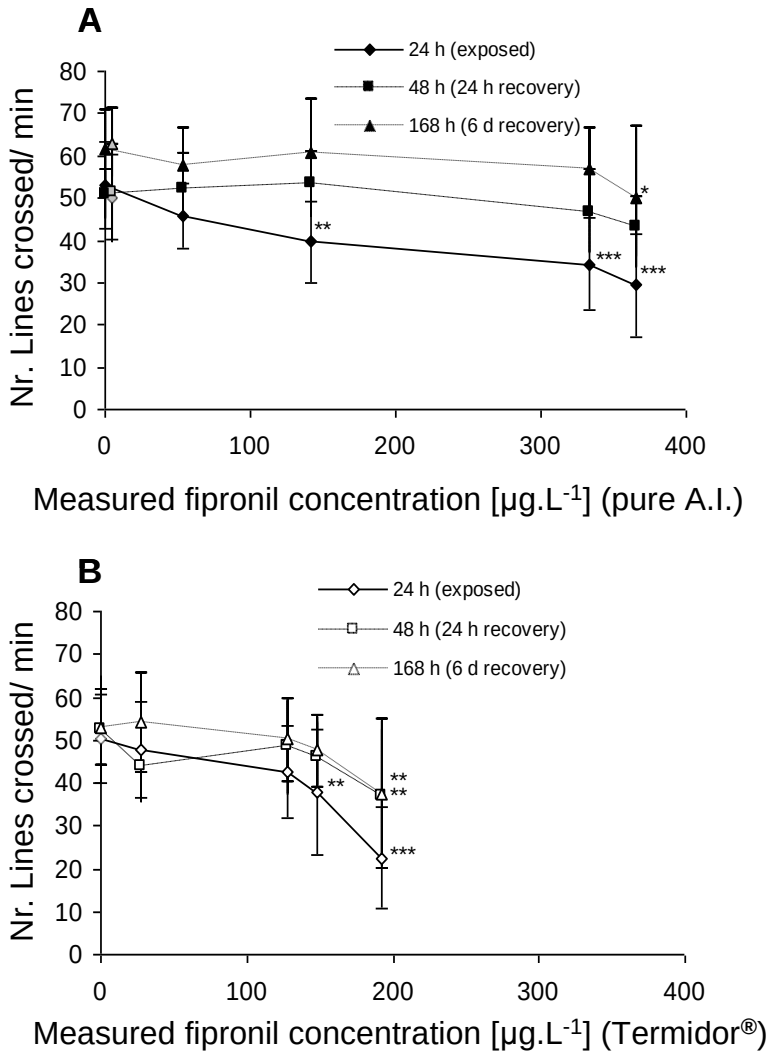

Figure 7: Swimming performance of larval fathead minnow after 24 h exposure to fipronil and Termidor®, 24 h recovery and 6 d recovery. Asterisks indicate significant differences in treatments compared to control/solvent control (*: p<0.05. **: p<0.01. ***: p<0.001). Data shown as arithmetic mean±SD; n=7. A: pure fipronil, control group shifted to x=5 for visibility (grey); B: Termidor®.

Fipronil. Swimming performance after 24 h was significantly decreased in fish exposed to concentrations ≥142 µg.L^{-1} pure fipronil (20% LC_{10}, $p < 0.001$) and ≥148 µg.L^{-1} Termidor® (33% LC_{10}, $p < 0.01$) (Figure 7). Although the measured concentrations at this time point are in a similar range, the formulation had a stronger negative impact on swimming at higher concentrations. Fish exposed to 192 µg.L^{-1} Termidor® (50% LC_{10}) exhibited statistically significant lower swimming activity than fish exposed to 333 µg.L^{-1} fipronil treatment (33% LC_{10}). After 24 h recovery in control water no significant effects on swimming performance were observed in fish exposed to pure fipronil, but after the 6 d recovery period, there was a statistically significant negative effect ($p < 0.01$, Figure 7A). In contrast to the pure fipronil treatments, swimming performance of fish exposed to 192 µg.L^{-1} Termidor® (50% LC_{10}) remained suppressed after the 24 h recovery period. This effect persisted throughout the test, and no recovery of swimming performance was observed after 6 days (Figure 7B).

7 d growth

Bifenthrin. Neither pure bifenthrin (maximum test concentrations: 0.35 µg.L^{-1}, 50% LC_{10},) nor Talstar® (maximum test concentration 0.16 µg.L^{-1} AI, 50% LC_{10}) caused any growth effects in larval fathead minnow (Figure 8).

Fipronil. Fish exposed to pure fipronil at all concentrations tested grew significantly more than fish exposed to the solvent alone (53 µg.L^{-1}, $p < 0.05$; 333 µg.L^{-1}, $p < 0.01$; 365 µg.L^{-1}, $p < 0.01$, Figure 9). Exposure to Termidor® did not result in negative or positive effects on growth. In addition to the observed effects on 7 d growth, fish exposed to both pure fipronil and Termidor® showed deformities of the spine (data not presented). Four to five days after the 24 h insecticide exposure, several fish showed scoliosis and in some cases both scoliosis and lordosis. At test termination 5 of the fish exposed to 365 µg.L^{-1} and 1 of the fish exposed to 333 µg.L^{-1} pure fipronil had developmental abnormalities. The same effect was visible for 4 of the fish exposed to 192 µg.L^{-1} and 1 of the fish exposed to 148 µg.L^{-1} Termidor®. No such effects were observed in any of the other treatments.

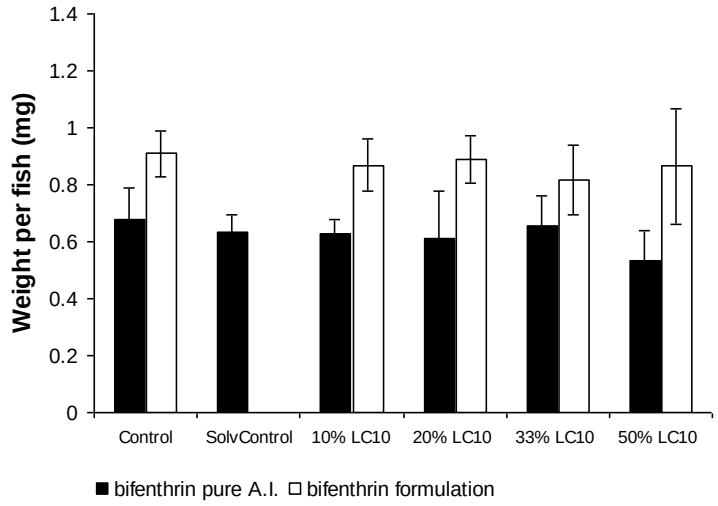

Figure 8: Average dry weight per fish after 24 h exposure to bifenthrin and Talstar® and 6 d recovery of larval fathead minnows at concentrations as low as 10% of the LC_{10} for the bifenthrin formulation Talstar® and 20% of the LC_{10} for pure bifenthrin

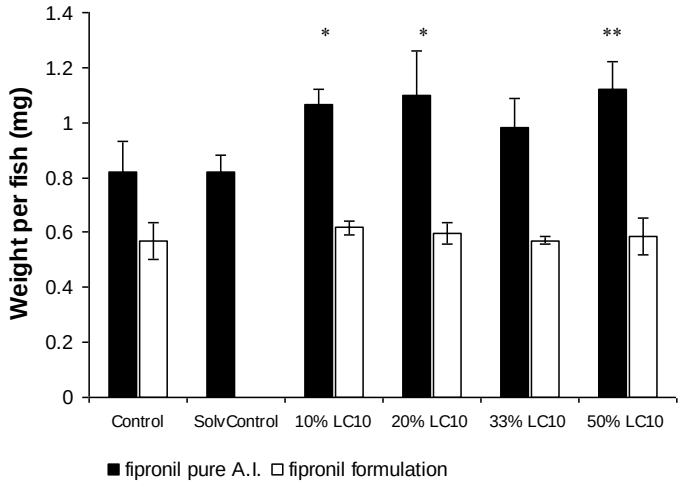

Figure 9: Average dry weight per fish after 24 h exposure to fipronil and Termidor® and 6 d recovery. Fish exposed to pure fipronil had significantly higher average weight than fish in control treatments (*: p<0.05. **: p<0.01).

Discussion

This study provides new information on the sublethal toxicity of two pure insecticides and two of their commercial formulations to larval stage fathead minnow. Results demonstrate that short-term (24 h) exposures to sublethal concentrations of pure and formulated bifenthrin and fipronil significantly impaired swimming performance. Bifenthrin and Talstar® concentrations that affected swimming performance (0.14 $\mu g.L^{-1}$ and 0.03 $\mu g.L^{-1}$, respectively) were in the range of environmental relevance, however, environmental factors such as particulate or dissolved organic matter can reduce bioavailability (Yang et al. 2006) and complicate an ecotoxicological assessment. Sublethal effect concentrations of fipronil and Termidor® ($\geq$142 $\mu g.L^{-1}$ and 148 $\mu g.L^{-1}$, respectively) were higher than known environmental levels. Swimming performance is a highly suitable endpoint for estimating individual level effects of environmental contaminants on fish, as it integrates biochemical and physiological processes (Geist et al. 2007, Kane et al. 2005). Especially insecticides with neurotoxic modes of action have been shown to negatively affect swimming ability and predator avoidance (Floyd et al. 2008, Heath et al. 1993, Little and Finger 1990). We used a simple and easy to perform test to assess swimming behavior. It simulates predatory chase and integrates both neural and metabolic aspects of fish, since swimming involves nerve cell transmissions and muscle activity (Heath et al. 1993) which is particularly affected by neurotoxicants (Jin et al. 2009). This is of special ecological importance during early life stages when fish are highly vulnerable to predation. Inability to swim properly after a brief exposure to insecticides therefore negatively affects individual fitness and survival, with potential consequences at the population level (Little and Finger 1990). As demonstrated in this study fish can recover, but in a field situation, not being able to feed or evade predators for a certain period of time, will likely lead to negative impacts on individual survival and population dynamics. In this study, growth was not a sensitive endpoint for measuring the effects of bifenthrin. While other pyrethroids have been shown to cause a reduction in growth of fathead minnow and other fish species (Haya 1989, Jarvinen and Tanner 1982), we did not observe this effect after bifenthrin and Talstar® exposures. This may be due to the low concentrations used in our experiments ($\leq$50% of the LC_{10}). Floyd et al. (2008) reported significantly reduced 7 d growth in larval fathead minnow after short-term (4 h) exposure to the pyrethroid esfenvalerate, however, effect concentrations were $\geq$22% of the LC_{50}. The relatively long recovery period (6 d after 24 h exposure) from pyrethroid poisoning may have enabled the fish to compensate for any initial impairment. We did not rigorously quantify food uptake in this study, but during daily water renewal, remaining food quantity was observed to be greater in treatments with

decreased swimming performance than in control treatments up to 2 d after insecticide exposure. Exposure to pure fipronil enhanced growth of larval fathead minnow, while its formulated form, Termidor® did not produce this effect. Enhanced growth following exposure to fipronil has not been previously reported and causative factors should be investigated in more detail, but were beyond the scope of this investigation. A limited number of studies found fipronil to be altering normal thyroid function and thyroid hormone levels in rats (Hurley et al. 1998, Leghait et al. 2009) and chicken (Russ 2005). As thyroid hormones also play a role in larval and juvenile development of fish (Power et al. 2001) the observed growth abnormalities may be related to this effect.

Developmental effects such as those observed in this study for a small number of the fish exposed to ≥148 $\mu g.L^{-1}$ Termidor® and ≥333 $\mu g.L^{-1}$ pure fipronil, were also reported by Stehr et al. (2006), in particular notochord degeneration and shortening along the rostral-caudal body axis in zebrafish (*Danio rerio*) embryos after continuous exposure to fipronil at nominal concentrations at or above 0.7 mM (333 $mg.L^{-1}$). These authors also observed ineffective tail flips and uncoordinated muscle contractions in response to touch. Although most concentrations used in our study were below that range, similar behavioral abnormalities were observed and resulted in a measurable decrease of swimming performance. We found strong differences in toxicity between pure and formulated insecticides. Both formulated products were more toxic than the respective AI, based on measured dissolved concentrations. Talstar® impaired fathead minnow swimming performance at approximately one fifth of the effect concentration of pure bifenthrin. However, when adding the concentration of bifenthrin measured in the particulate fraction of Talstar®, the total concentration that caused negative effects on swimming was approximately 2 times higher for Talstar® than for pure bifenthrin (Table 5). Microcapsules may have been ingested by the larval fish, thus adding a dietary exposure route to the aqueous exposure to dissolved bifenthrin, which could account for the higher toxicity of the formulated product based on dissolved concentrations.

In addition, it is possible that the presence of 0.1 methanol added as a carrier increased bioavailability and toxicity of the pure insecticides, however, we found no difference in swimming performance or growth between control and solvent control treatments. For pure fipronil and Termidor®, effect concentrations for swimming performance were similar, but impairment was more persistent in fish exposed to the formulated product. Insecticide formulations can act as mixtures and environmental risks cannot be determined by assessing the toxicity of the AI alone. The relevance of these findings is obvious as pure insecticides are

never applied in the environment. Extrapolating our laboratory results to a field exposure scenario is, however, beyond the scope of this study. For determination of toxicity under environmental conditions many additional factors have to be taken into account. Sediment particles, dissolved organic carbon, water pH and temperature can change the bioavailability and therefore toxicity of pesticides (Maul et al. 2008, Yang et al. 2007). Despite that, the consideration of short-term exposures, delayed effects and sublethal toxicity is of importance as exposure of aquatic organisms to insecticides is most likely to be of short duration and below lethal levels. For example, Brady et al. (2006) demonstrated that the majority of insecticide runoff of two insecticides, diazinon and esfenvalerate, occurred within the first hour of a simulated rain event.

Information on inert ingredients is largely treated as trade secret, but these chemicals have been shown to exert additive or synergistic toxicity, due to either their mechanismof action or through increasing the bioavailability of the AI. Emulsifiable formulations of pyrethroids were found to be 2.2 to 8.5 times more lethal to fish than the pure substance (Haya 1989) as a consequence of enhanced uptake via the gill epithelium. In other products, enzyme altering synergists like piperonyl butoxide (PBO) are added (Amweg and Weston, 2007) to enhance toxicity of the AI The solvent propylene glycol is part of the Talstar® formulation, but its toxicity to fish is low (*Pimephales promelas* 48 h LC_{50}: 790,000 µg.L^{-1} (Kegley et al. 2008, TDC-Environmental 2008)), and it was found to not significantly modify the toxicity of bifenthrin to cultured human cells (Skandrani et al. 2006). Chemicals used in pesticide formulations may also increase mobility of the AI thus facilitating pesticide movement into aquatic environments.

Suspension liquids such as Termidor® or microencapsulated products like Talstar® are designed to not immediately bind to porous surfaces, and are therefore more susceptible to runoff or leaching. For example, Armbrust and Peeler (2002) reported that the concentration of the insecticide imidacloprid was higher in runoff from turf that was treated with granules than turf treated with wettable powder. Similar formulation effects were observed for herbicide runoff from container plant nurseries (Briggs et al. 2002). Kenimer et al. (1997) reported higher surface runoff of alachlor microencapsulated formulation compared to alachlor emulsifiable concentrate formulation, as microcapsule movement was similar to that of eroded sediment. Talstar® is formulated as a so-called microencapsulation of bifenthrin, resulting in µm-sized particles, where the AI forms a core that is coated by an outer wall consisting of “inert” ingredients (Scher et al. 1998, Tsuji 2001). The toxicity of this

formulation is therefore dependent on how fast and how much of the active ingredient is released through the capsule (Jarvinen and Tanner 1982). As this formulation is designed to be more persistent at the site of application, the release is probably slow. This explains why measured concentrations of dissolved bifenthrin were lower in the Talstar® experiment than in the exposures to pure bifenthrin. The use of such controlled-release insecticides may lead to lower exposure concentrations but increased exposure time of non-target organisms. Future investigations on these types of products should therefore consider a long-term exposure scenario to lower concentrations.

Conclusions

Our study demonstrated that formulated products of two widely used insecticides, the pyrethroid bifenthrin and the phenylpyrazole, fipronil, were approximately 5 and 2 times more toxic to larval fathead minnow than the active ingredients alone. Growth was not a sensitive toxicity endpoint, but the fish's ability to swim normally was impaired at Talstar® (bifenthrin) and Termidor® (fipronil) concentrations 10 and 3 times lower, respectively, than the 24 h LC_{10}. Results suggest that these neurotoxic insecticides can decrease ecological fitness of sensitive aquatic species at concentrations far below the lethal level. We have demonstrated that behavioral endpoints such as swimming are valuable tools to detect sublethal effects of neurotoxic chemicals. Future risk assessments should include information on sublethal endpoints such as swimming behavior, and additional safety factors to account for the greater toxicity of formulated pesticide products.

Chapter III - Changes in gene transcription and whole organism responses in larval fathead minnow (*Pimephales promelas*) following short-term exposure to the synthetic pyrethroid bifenthrin

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A similar version of this chapter was published in Aquatic Toxicology 105 (2011), 180 – 188.

Abstract

The combination of molecular and whole-organism endpoints in ecotoxicology provides valuable information about the ecological relevance of sublethal stressor effects in aquatic ecosystems such as those caused by the use of insecticides and translocation of their residues into surface waters. This study contributes knowledge about the sublethal effects of a common use insecticide, the synthetic pyrethroid bifenthrin, on larval fathead minnow (*Pimephales promelas*). Transcriptomic responses, assessed by quantitative real-time PCR, combined with individual effects on swimming performance were used to estimate the ecological relevance of insecticide impacts. Significant transcriptomic responses were observed at 0.07 $\mu g.L^{-1}$ bifenthrin (lowest observed effect concentration, LOEC) but mostly followed a biphasic rather than a linear dose–response with increasing concentration. Transcript patterns for genes involved in detoxification, neuromuscular function and energy metabolism were linked to an impairment of swimming performance at $\geq$0.14 $\mu g.L^{-1}$ bifenthrin. With increasing treatment concentration, a significant down-regulation was observed for genes coding for cyp3a, aspartoacylase, and creatine kinase, whereas metallothionein was up-regulated. Additionally, bifenthrin induced endocrine responses as evident from a significant up-regulation of vitellogenin and down-regulation of insuline-like growth factor transcripts. Recovery occurred after 6 days and was dependent on the magnitude of the initial stress. During the recovery period, down-regulation of vitellogenin was observed at lowest exposure concentrations. The data presented here emphasize that links can be made between gene transcription changes and behavioral responses which is of great value for the evaluation and interpretation of biomarker responses.

Introduction

The use of pyrethroid insecticides in agricultural and urban areas poses a threat to aquatic ecosystems and non-target organisms, as residues get translocated into surface waters via irrigation or stormwater runoff (Amweg et al. 2006, Greenberg et al. 2010, Werner et al. 2010). Organism survival is the classical endpoint used in bioassays with fish and aquatic invertebrates for regulatory toxicology and risk assessment to describe deleterious effects caused by environmental chemicals; yet sublethal responses are likely to be of higher ecological relevance as exposures are most likely short-term and in a low concentration range. Adequate methods are needed, which are sensitive enough to detect these responses, such as molecular approaches which are rapidly becoming validated towards their use in environmental risk assessment in the field of ecotoxicological research. So called "omics" technologies allow investigation of chemical modes of action from a mechanistic point of view (Ankley et al. 2006, Fedorenkova et al. 2010, Schirmer et al. 2010). These techniques are suitable to describe the effects of environmental stressors, including chemical contaminants, on organisms at concentration levels far below the lethal range.

Molecular biomarkers have the potential to be rapid, cost-effective and sensitive diagnostic tools to assess physiological impacts on organism. Thus, gene transcription data can provide useful mechanistic insight regarding responses to toxicants and the health status of an organism (Connon et al. 2008). However, there is a need for better interpretation and understanding of molecular biomarkers in an ecological context (Forbes et al. 2006). It is often unclear if molecular responses are indicating exposure and therefore act as early warning signals for environmental stress, or if they have predictive value for deleterious effects at higher levels of biological organization. Recent studies have indicated that the transcriptomic and whole-organism levels (behavior, growth) can be linked on a mechanistic basis (Connon et al. 2011, Geist 2011, Heckmann et al. 2008). However, the extrapolation of effects seen at the transcriptome level to the individual or population level remains challenging. To date, most ecotoxicogenomic studies focus on the molecular responses, discovering and describing pathways or mechanistically explaining effects, but do not relate these findings to higher-level effects.

The Central Valley of California, USA, is a geographic region where insecticides have frequently been detected in surface waters and sediments, and where aquatic invertebrates and fish are consequently exposed to toxic substances at potentially hazardous concentrations. The synthetic pyrethroid bifenthrin is one of the most frequently detected insecticides (Weston and

Lydy 2010), and runoff from residential areas contained bifenthrin at concentrations of 0.12–6.12 $\mu g.L^{-1}$ (Gan et al. 2012), whilst effluent of wastewater treatment plants contained up to 0.02 $\mu g.L^{-1}$ bifenthrin (Weston and Lydy 2010). Like all pyrethroids, bifenthrin is highly toxic to aquatic organisms. The main mode of action is the interference with voltage-gated ion channels and ATPase enzymes (Burr and Ray 2004) causing hyperexcitability, tremors, convulsions and ultimately death (Bradbury and Coats 1989). The 24 h LC_{50} and 96-h LC_{50} of bifenthrin for larval fathead minnow are reported to be 1.90 $\mu g.L^{-1}$ (Beggel et al. 2010) and 0.26 $\mu g.L^{-1}$ (Werner and Moran 2008), respectively. Wang et al. (2007) reported the estrogenic potential of bifenthrin leading to significant VTG induction in medaka (*Oryzias latipes*) liver after 10 d exposure to 10 $\mu g.L^{-1}$. Additionally, Jin et al. (2009) demonstrated that high exposure concentrations of bifenthrin above 50 $\mu g.L^{-1}$ significantly affected swimming activity in zebrafish (*Danio rerio*) as well as the induction of vitellogenin.

The present study aims at linking effects at the molecular and whole-organism levels to facilitate interpretation of cellular effects in an ecological context. It provides new information on the transcription of selected genes in response to short-term exposure to sublethal concentrations of the synthetic pyrethroid bifenthrin, and quantitatively compares gene transcription to higher-level organismal effects. We used a common model organism, the fathead minnow (*Pimephales promelas*) to facilitate the production of primers for gene transcription analyses, and the measurement of responses at higher levels of biological organization, following standard protocols. Biomarker genes were selected from cellular pathways involved in organism growth (insuline-like growth factor, *igf*; growth hormone, *gh*), energy metabolism (glucose-6-phosphate dehydrogenase, *g6pd*; creatine kinase, *ck*), muscular and neuronal function (parvalbumin, *pvalb*; aspartoacylase, *aspa*), as well as general stress responses (cytochrome p450 1a, *cyp1a*; gluthione S-transferase rho, *gst*; heat shock protein 90, *hsp90*; metallothionein, *mt*). We used a short-term, 24 h exposure, because the relatively hydrophobic pyrethroids are generally expected to quickly bind to particles and sediments after entering a surface water body (Beggel et al. 2010, Brady et al. 2006, USEPA 2002). Sublethal exposure concentrations were calculated as percentages of the acute 24 h LC_{10} value determined by Beggel et al. (2010). In order to observe possible delayed toxic effects, a six day recovery period, during which the fish were kept in clean water, was included in the experiments to determine if recovery occurred, and if gene transcription reflected recovery at the organism level.

Material and Methods

Fish source, acclimation and quality assurance: The exposure design in this study followed the standard protocols of previous ecotoxicological tests with this species, described in chapter II (Beggel et al. 2010). Fathead minnow larvae were obtained from Aquatox Inc. (Hot Springs, AR, USA) at 7 d post-hatch on the day of arrival. Fish were acclimated to control water at a temperature of 25 °C. Control water consisted of deionized water, modified with salts to meet USEPA specifications (specific conductivity: 265–293 $\mu S.cm^{-1}$; hardness: 80–100 as mg $CaCO_3$ L^{-1}; alkalinity: 57–64 as mg $CaCO_3$ L^{-1} (USEPA 2002a). During the acclimation period <1% mortality was observed, and the fish fed and swam normally. During the project period, routine monthly reference toxicant tests were performed using NaCl to ascertain that organism response fell within the acceptable range according to USEPA requirements (USEPA 2002a). Each test consisted of a dilution series (5 test concentrations and a control). All test organisms responded normally (within 95% confidence interval of running mean) and sensitivity was considered typical.

Insecticide exposure: Reference standard grade bifenthrin [[1_3_(2)]-(±)(2-methyl[1,1_-biphenyl]-3-yl)methyl 3-(2-chloro-3,3,3, trifluoro-1-propenyl)-2,2-dimethylcyclopropane-carboxylate], 99% purity (CAS number 82657-04-3) was obtained from ChemService Inc. (West Chester, PA, USA). Bifenthrin consists of 97% cis-isomer. All insecticide exposure experiments were conducted at the Aquatic Toxicology Laboratory, School of Veterinary Medicine, University of California Davis, USA.

To determine acute toxic effects on survival, 7 d old larval fish were exposed for 24 h to the following nominal concentrations: 0.75, 1.0, 1.5, 2.0, 3.0 and 4.0 $\mu g.L^{-1}$ bifenthrin. We used 1 $mL.L^{-1}$ methanol (MeOH) as solvent carrier and one treatment group containing the same MeOH concentration in control water was added as a solvent control. Stock solutions were prepared in MeOH (2000 $mg.L^{-1}$) and used for both, 24 h LC_{50} determination and sublethal exposure experiments.

Exposure concentrations used for assessment of sublethal stress responses were calculated as percentages of nominal LC_{10} values derived from the acute toxicity tests. Treatments consisted of a control, solvent control and 10%, 20%, 33% and 50% of the nominal 24 h LC_{10}. Each treatment consisted of 9 replicate 600 mL Pyrex beakers containing 250 mL test solution and 10 fish larvae. At test initiation, 10 larvae were transferred from the acclimatization tank to each beaker and exposed to test solutions for 24 h at a water temperature of 25 °C and a

16:8 light–dark ratio. Test vessels were manually distributed in a random manner, within the exposure water bath. Fish were not fed during the exposure period.

For molecular analysis, three time points were sampled per treatment (i) after 24 h, (ii) after 48 h, including a 24 h recovery period in control water, and (iii) after 7 d, including a 6 d recovery period. For the recovery period the fish were transferred to control water and maintained according to standard US EPA protocols. During recovery period fish were fed *ad libitum* with *Artemia nauplii* 2 times each day.

At each of the three time points, 30 fish from 3 replicate vessels (10 fish per vessel) were sampled per treatment. Fish were euthanized in tricaine methanesulfonate (MS-222), individually transferred into 1.5 mL microcentrifuge tubes and immediately flash-frozen in liquid nitrogen. Samples were stored at −80 °C until processing. The exposure scenario described here is identical to previously reported studies to evaluate effects on swimming performance and growth. Experiments were performed parallel in order to provide direct comparability. Swimming performance testing was performed according to a method by Heath et al. (1993), described in detail in chapter II (Beggel et al. 2010). Briefly, fish were touched at the tail fin in order to trigger escape responses for the duration of 1 min. The distance traveled by the fish was recorded and used as a measure for swimming performance.

Water chemistry: For measuring exposure concentrations, sub-samples of each test solution (1 L) were preserved with dichloromethane (Fisher Scientific, USA) at test initiation, shipped overnight to the California Department of Fish and Game Water Pollution Laboratory (Rancho Cordova, CA, USA), extracted within 24 h of arrival, and analyzed using gas chromatography with mass spectrometry and ion-trap detection. Reporting limit for detection of bifenthrin was 0.002 $\mu g.L^{-1}$ (recovery 88.3 %).

Sample processing and primer probe design: For RNA extraction we used 5 fish from each treatment replicate, resulting in 15 biological replicates per treatment and time-point. Total RNA was extracted from whole individual organisms, using QIAGEN RNeasy MiniKit according to manufacturer's instructions. RNA concentration was estimated from absorbance at 260 nm (NanoDrop ND 1000) and RNA purity was verified by A260/A280 ratios, RNA integrity was verified by electrophoresis on ethidium bromide-stained 1% agarose gels. One microgram of total RNA was used for cDNA synthesis. Primer and probes for qPCR analyses were designed using Roche Universal Probe Library Assay Design Center (https://www.roche-applied-science.com) and are detailed in Table 4.1. Designed primers

were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com), and TaqMan® probes were supplied by Roche.

Quantitative PCR: Complementary DNA was synthesized using 1.0 µg total RNA, with random primers and SuperScript® III reverse transcriptase (200 U.µL-1; Invitrogen, Carlsbad, CA, USA), and diluted to a total of 150 µl with nuclease free water to generate sufficient template for qPCR analysis. TaqMan® Universal PCR Mastermix (Applied Biosystems, Carlsbad, CA, USA) was used in qPCR amplifications in a reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625U AmpliTaq Gold DNA polymerase per reaction, 0.25U AmpErase UNG per reaction and 5 µL of cDNA sample in a final volume of 12 µL.

Samples were placed in 384 well plates, and targeted gene fragments were amplified in an automated fluorometer (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, Carlsbad, CA, USA). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60s at 60°C. Fluorescence of samples was measured every 7 s and signals were considered positive if fluorescence intensity exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle, CT). SDS 2.2.1 software (Applied Biosystems, Carlsbad, CA, USA) was used to quantify transcription.

Cloning and sequencing: Sequence information for aspartoacylase (*aspa*) and transforming growth factor beta (*tgfb*) for the fathead minnow was not available from the database. Degenerate primers were designed to match conserved regions of these two sequences in other teleost species (Vector NTI software, Invitrogen, Carlsbad, CA, USA and primer3 software (Rozen and Skaletsky 2000) for *aspa* (Forward: 5' gayctycacaacaccacrkcyaaca 3'; reverse: 5' avmacwccwtgrggytgmggyccvacctc 3'), or taken from the literature for *tgfb* (Forward: 5' gacttccgsaaggacctsggctggaagtgg 3'; reverse: 5' cacttrcargarcgcacratcat 3'; Hardie et al. 1998). Degenerate primers were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com). The PCR (34 cycles, denaturation 95°C, annealing 55°C, elongation 72°C) was performed using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, USA) and the products were separated on a 1% agarose gel. Amplified DNA products with the expected product size were subsequently extracted from agarose gel by centrifugation using Ultrafree-DA tubes (Millipore, Billerica, MA, USA). Cloning reactions were performed using One Shot TOP10 Chemically Competent *E.coli* provided by TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

Plasmid purification to isolate target DNA was performed using the Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA, USA). Plasmid DNA products were sequenced using standard M13 primers at the UC Davis DNA sequencing facility (http://www.dnaseq.ucdavis.edu/services.html). Sequence information was confirmed using BLASTn (http//www.ncbi.nlm.nih.gov, Altschul et al. 1997), resulting in expect values 5e-60 for *aspa* and 5e-87 for *tgfb*. Maximum identities were observed for aligned sequence information for the same protein from other teleost species.

Table 6: Primer pairs and TaqMan® probes used in qPCR assessment of molecular biomarkers.

Gene name	Gene symbol	qPCR primer sequences	Roche library probe No.	Accession No.
CYP3A126	*cyp3a*	F caacccagaggccatgaaga R gggccttatttgggaaggtct	63	EU332792
Parvalbumin	*pvalb*	F gctctgtctgctgacaacgtg R cagcaaaaccccttcagcacaa	63	-
Epithelial calcium channel	*ecac*	F gagaggccagaaggattcttga R cagattccacttcagccttacga	109	EF628374
Glucose-6-phosphate dehydrogenase	*g6pd*	F tgagggaagcctggagaatct R ggtggcgtcttctctttctcg	35	EF628372
Cytochrome P4501A	*cyp1a*	F tctaacggttgtcccgatcct R gaggcgcattagcagatacaca	9	-
Insulin-like growth factor-1	*igf*	F ggcaaaactccacgatcccta R atgtccagatataggtttctttgctg	9	AY533140
Beta2-microglobulin	*b2m*	F aactgctgaaagatggagtggtt R ccctttttcgaaggccaggt	3	-
Mx protein	*mx*	F gaaatggcatgggagaatcag R cctgggcttcacgaatctttt	3	AY751300
Vitellogenin precursor	*vtg*	F ccatttgttcctgccactaagc R cttgatgggaatctgaagctga	106	AF130354
Elongation factor 1-alpha	*ef1a*	F ctctttctgttacctggcaaagg R tcccatgattgattagtttcaggat	66	AY643400
Glucocorticoid receptor	*gcr*	F caagaagggacgtcaaacagg R ggaaagattgcgctctggaat	66	AY533141
Heat shock protein 90	*hsp90*	F ctggtcatcctcctgttcgag R tgtgtctgaggatcgtccaatg	56	-
Metallothionein	*mt*	F ctgccagtgtacaacatgcaaga R gcacatttgctgcaaccaga	56	-
Creatine kinase	*ck*	F ggaaatatgccacccccttcatt R ccttgaacacctcataggtctcttc	5	-
Growth hormone	*gh*	F gtggtcctggttagtttgttggt R tgactgcgttgttgaagagcc	103	AY643399
Zona pellucida glycoprotein 3	*zp3*	F atcatgggtgctttgtggatg R gcttgtgaccgaggcatga	11	AF192407
Glutathione S-transferase	*gst*	F ccggcaagagcttcaccat R agtgaagtcgtgggaaataggc	48	EF628373
Aspartoacylase	*aspa*	F tctggtaatggatgtcccgatt R gacctctatggaaaagccatgc	94	FR856858*
Transforming Growth Factor beta 3	*tgfb*	F tgagcctgtacaacacgctga R gtcccacgtagtagaggatggtg	1	JF903043*

Note: "*" indicates sequences identified in this study; "-" indicates sequences supplied by Prof. Denslow, UFL.

Statistical analysis: We used the geNorm algorithm to estimate the variability of reference gene transcription, and to determine an optimal gene for normalization of the data (Vandesompele et al. 2002). Quantitative PCR data was analyzed using the relative quantification $2^{(-\text{Delta Delta CT})}$ method (Livak and Schmittgen 2001). Transcription levels were

calculated relative to elongation factor 1-alpha (*ef1a*) determined by geNorm as the least variable gene in this study, whose suitability as a reference gene was also confirmed by other studies with fish (Jorgensen at al. 2006).

Statistical analyses were conducted using SPSS 11 for windows statistical software (SPSS Inc.). Differences in transcription levels of single genes were evaluated using univariate Analysis of Variance (ANOVA) with Tukey's multiple comparison *post hoc* test to compare between treatments, and each gene was treated as a separate experiment. Data was analyzed in the log2-scale, representing the log2-fold change in gene transcription relative to the reference gene. With the log2 dataset the assumptions of ANOVA for normal distribution and homogeneity of variances were met. Correlations between treatment concentrations and transcription levels were evaluated using Pearson's Correlation with a two-tailed test for significance.

For analysis of gene transcription data from recovery time-points, each dataset was adjusted to the control by dividing each data point with the corresponding control data point prior to statistical analysis. This procedure created a ratio or adjusted values that allow direct comparison between the different time-points and treatments. Adjusted values were used to generate a timeline trajectory plot. Control values were tested with ANOVA and showed no differences between plates and time-points. Averaged control values (± SE) for each time-point were included into trajectory plot for visualization of recovery effect.

Principal Component Analysis (PCA) was applied to yield information about principal factors underlying the variation in the dataset. We used Varimax with Kaiser Normalization as Rotation method and the Kaiser-Meyer-Olkin (KMO) measure verified the sampling adequacy for the analysis of the gene transcription data an acceptable limit of KMO = 0.5 (Sharma 1996). Bartlett's Test of Sphericity was applied to control if correlations between items were sufficiently large for PCA. Factor scores from Principal Components were used to generate a geometric trajectory plot in order to visualize variation of transcription levels over time (Keun et al. 2004).

Results

Water chemistry, physicochemical parameters and sublethal exposure: Physicochemical parameters measured at the beginning and end of the 24 h exposure period were within the acceptable range for the test organism (USEPA 2002a) for all experiments and treatments. The measured mean values (± standard deviation) were pH: 7.5 (± 0.2), dissolved oxygen 7.2 (± 0.5) $mg.L^{-1}$, temperature: 23.1 (± 0.3) ˚C, and EC: 278 (± 6) $\mu S.cm^{-1}$.

Gene transcription of target genes involved in stress responses, neuromuscular function, and endocrine function was determined, and insecticide-exposed fish were compared with untreated (control) fish. Individual effects were observed for each substance at concentrations below 50% of the LC_{10}. Concentration levels in the following sections refer to the measured dissolved fractions of bifenthrin or to percentages of the nominal LC_{10} values determined by initial acute toxicity tests, respectively (Table 7).

Table 7 : Nominal and measured effect concentrations in [µg.L^{-1}] of bifenthrin for larval fathead minnow.

Bifenthrin concentration [µg.L^{-1}]	10% LC_{10}	20% LC_{10}	33% LC_{10}	50% LC_{10}
nominal	0.09	0.18	0.31	0.46
measured	0.07	0.14	0.24	0.35

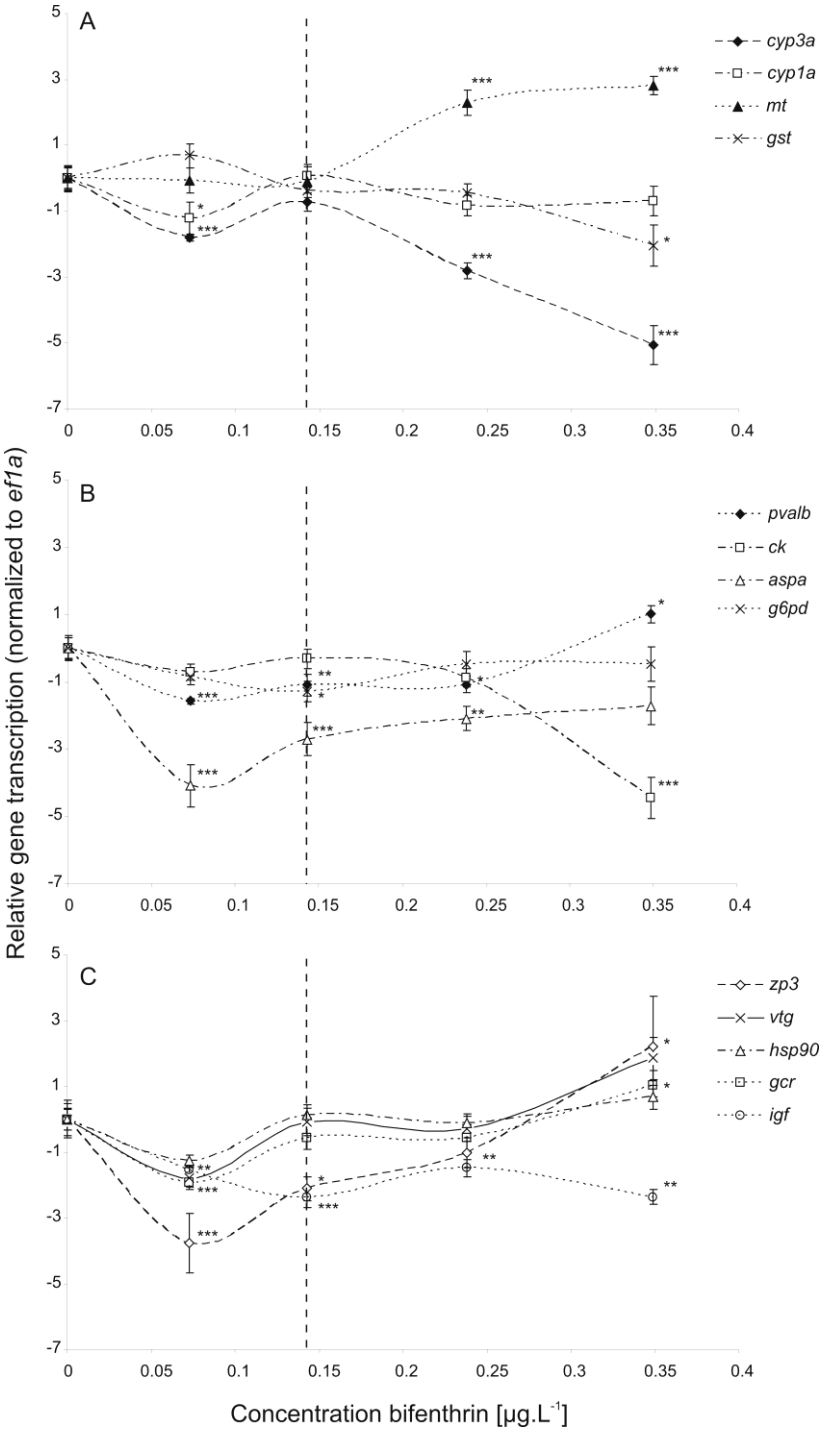

Figure 10: Changes in gene transcription in response to bifenthrin exposure assessed by quantitative PCR; (A) detoxification, general stress response, (B) neuromuscular function, energy, (C) endocrine function. Significant changes occurred at 0.07 µg.L^{-1} bifenthrin as initial shift in homeostasis. Measured concentration levels ≥ 0.14 µg.L^{-1} resulted in negative effects on swimming performance (lowest observed effect threshold for swimming indicated by vertical dotted line). Asterisks indicate significant differences in treatment compared to control (p< 0.05). Strongest differences to control treatment were observed for genes involved in detoxification, energy and neuromuscular function. Data is presented as mean value ± standard error (n=15). (*Mt*= metallothionein; *gst*= glutathione S-transferase; pvalb= parvalbumin; *ck*= creatine kinase; aspa= aspartoacylase; *g6pd*= glucose-6-phosphate dehydrogenase; *zp3*= zona pellucida glycoprotein3; *vtg*= vitellogenin precursor; *hsp90*= heat shock protein 90; *gcr*= glucocorticoid receptor; *igf*= Insuline-like growth factor).

*General effects on gene transcription:*Significant gene transcription responses to bifenthrin exposures were already observed at the lowest tested concentration of 0.07 $\mu g.L^{-1}$, equal to 10% of the 24 h LC_{10} (Figure 10). With increasing treatment concentration, the response was often biphasic, and genes that were down-regulated at the lowest exposure concentration were generally up-regulated at higher concentrations and vice versa. Beyond a certain threshold concentration, transcription of most genes increased or decreased in a dose-dependent manner. This threshold concentration of performance of the fish (Figure 11). In a few cases, transcription levels remained unchanged at higher exposure concentrations after initial up- or down-regulation. These general response patterns are also reflected by plotting Principal Component factor scores against treatment concentration (Figure 12). Principal Component Analysis (PCA) was conducted on 13 of the genes tested in this study and showed three factors being responsible for variation in the dataset. Transcription data for glutathione S-transferase rho (*gst*), metallothionein (*mt*), mx protein (*mx*), microglobulin (*b2m*), growth hormone (*gh*), insuline-like growth factor (*igf*) and transforming growth factor beta (*tgfb*) had to be excluded from the analysis in order to meet the requirements for the PCA. Three components had eigenvalues above Kaiser's criterion of 1 and in combination explained 66.4% of the total variance. Genes that were clustered in the three components were not necessarily functionally related, but can be interpreted as grouped together by similar transcription patterns with increasing treatment concentration. Genes that clustered in component 1 (PC1) were aspartoacylase (*aspa*), parvalbumin (*pvalb*), heat shock protein 90 (*hsp90*), vitellogenin (*vtg*), glucocorticoid receptor (*gcr*), epithelial Ca^{2+} channel (*ecac*) and zona pellucida glycoprotein (*zp3*). Factor scores of PC1 were positively correlated with treatment concentration ($r = 0.40$, $p < 0.01$) and negatively correlated with swimming performance ($r = -0.39$, $p < 0.01$). Genes clustered in component 2 (PC2) were creatine kinase (*ck*), *cyp3a* and *cyp1a*. PC2 was negatively correlated with treatment concentration ($r = -0.67$, $p < 0.01$) and positively correlated with swimming performance ($r = 0.36$, $p < 0.01$). Component 3 (PC3) showed no concentration dependent patterns (Figure 12).

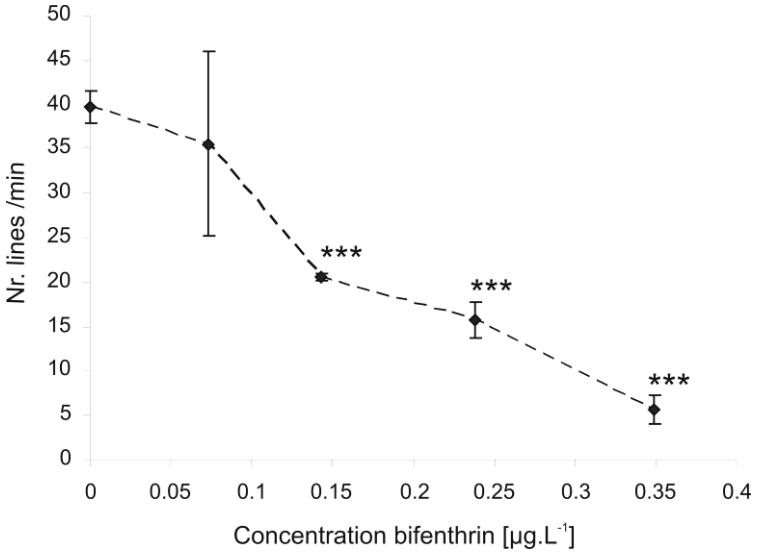

Figure 11: Swimming performance of larval fathead minnow after 24 h exposure to bifenthrin (measured by the number of lines crossed within 1 min). ***Refers to highly significant ($p < 0.001$) differences of mean distance measurements to control. Data are presented as arithmetic mean± standard deviation (n = 3 replicates × 7 fish).

Individual responses: Significant gene responses to bifenthrin exposure at the lowest test concentration of 0.07 $\mu g.L^{-1}$ (10% LC_{10}) were measured for genes involved in endocrine function and growth: *Zp3* ($p < 0.001$), *gcr* ($p < 0.001$), *igf* ($p < 0.01$) and *hsp90* ($p < 0.001$). Significant responses to bifenthrin were also observed for genes involved in nerve repair, neuromuscular function and energetic status. *Aspa* was significantly down-regulated at all exposure concentrationsexcept the highest treatment (0.07 $\mu g.L^{-1}$, $p < 0.001$; 0.14 $\mu g.L^{-1}$, $p < 0.01$; 0.24 $\mu g.L^{-1}$, $p < 0.05$) and *pvalb* was significantly down-regulated up to 1.5-fold at 0.07 $\mu g.L^{-1}$ ($p < 0.001$), 0.14 $\mu g.L^{-1}$ ($p < 0.01$) and 0.24 $\mu g.L^{-1}$ ($p < 0.05$) but twice up-regulated at 0.35 $\mu g.L^{-1}$ ($p < 0.05$). *Ck* as an indicator of energy status was significantly ($p < 0.001$) down-regulated up to 4-fold in fishes exposed to 0.35 $\mu g.L^{-1}$ bifenthrin. Transcripts of *mt* were significantly up-regulated in fish exposed to 0.24 $\mu g.L^{-1}$ ($p < 0.001$) and 0.35 $\mu g.L^{-1}$ ($p < 0.001$) bifenthrin. The 24 h exposure led to a significant down-regulation of Cytochrome *cyp3a* at concentrations of 0.24 $\mu g.L^{-1}$ ($p < 0.001$) and 0.35 $\mu g.L^{-1}$ ($p < 0.001$). *Gst* was significantly down-regulated up to2-fold at the highest exposure concentration ($p < 0.05$).

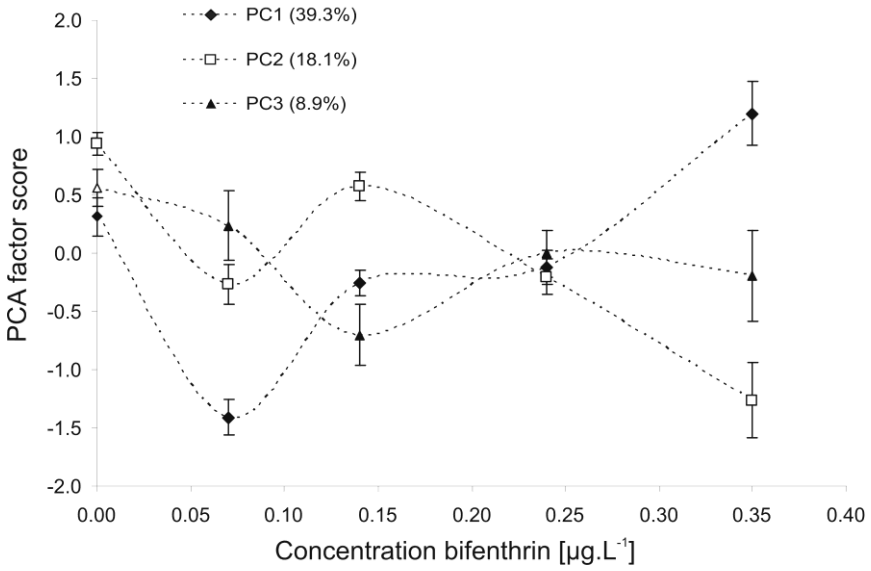

Figure 12: Gene transcription patterns as explained by Principal Component Analysis.

Recovery time series: After the 24 h exposure to bifenthrin fish were transferred into control media and samples were taken at (i) 48 h (including a 24 h recovery period in control medium) and after 7 d (including a 6 d recovery period). Quantitative PCR analysis was performed for a subset of genes for the two treatments with the lower concentrations (0.07 µg.L^{-1} and 0.14 µg.L^{-1} bifenthrin) due to their higher ecological relevance. Only genes that responded significantly at the 24 h time-point in most of the treatments were used in the time series experiment, i.e. *cyp3a*, *pvalb*, *vtg*, *hsp90*, *ck*, *gst* and *aspa* (Table 8). At these concentration levels no differences to control could be seen in swimming performance after the 24 h recovery period (see also Beggel et al. 2010).

At the lowest concentration level of 0.07 µg.L^{-1}, a significant down-regulation for *cyp3a* was observed in samples at the 24 h time-point. The following measurement at the 48 h time-point (including a 24 h recovery period) showed a slight up-regulation compared to control without being significant. *Cyp3a* remained slightly up-regulated after 6 days of recovery, but without statistical differences to the control. Comparison of the measurements at the recovery time-points to the 24 h time-point however, showed significant differences ($p < 0.001$) within this treatment. The *cyp3a* transcription data for 0.14 µg.L^{-1} bifenthrin showed the same general pattern as observed at the lower concentration, a down-regulation directly after the exposure and a slight up-regulation at the following recovery time-points, but less pronounced and without statistical significance.

For *gst*, no statistical significant differences were evident at concentration levels of 0.07 and 0.14 µg.L^{-1} bifenthrin. The observed temporal pattern began with a slight up-regulation after 24 h. Transcription levels remained elevated at 48 h and was down-regulated after 7 d. *Ck* was slightly down-regulated after 24 h in both treatments tested and stayed slightly up-regulated at the recovery time-points for the 0.14 µg.L^{-1} treatment. *Aspa* was significantly down-regulated after 24 h (up to 4-fold for treatment concentration 0.07 µg.L^{-1} and 3-fold for treatment concentration 0.14 µg.L^{-1}). There were no statistically significant differences in aspa transcription of between treatments and controls at the two recovery time-points.

Table 8: Changes in mean gene transcription levels and standard errors (SE) of control, 0.07 µg.L^{-1} and 0.14 µg.L^{-1} bifenthrin exposed fathead minnows, expressed as n-fold changes, normalized to *ef1a* gene transcription at three time-points: (i) 24 h (exposure period); (ii) 48 h (24 h exposure, 24 h recovery); (iii) 7 d (24 h exposure, 6 d recovery).

Time point	*cyp3a*	*pvalb*	*vtg*	*hsp90*	*ck*	*gst*	*aspa*
24 h							
Control mean	-0.1	0.1	0.1	-0.1	-0.3	-0.2	0.0
SE	0.4	0.3	0.5	0.3	0.3	0.4	0.4
0.07 µg.L^{-1}	-1.9***	-1.5***	-1.7**	-1.3***	-1.0	0.5	-4.1***
SE	0.1	0.1	0.4	0.2	0.2	0.4	0.6
0.14 µg.L^{-1}	-0.8	-1.0**	0.0	0.1	-0.6	-0.6	-2.7***
SE	0.3	0.3	0.4	0.3	0.3	0.3	0.5
48 h							
Control mean	0.2	0.2	-0.6	-0.6	0.3	-0.1	0.0
SE	0.3	0.4	0.3	0.4	0.4	0.6	0.4
0.07 µg.L^{-1}	0.6	1.6***	-8.0***	0.4	0.9	1.1	-0.3
SE	0.3	0.1	1.0	0.3	0.4	0.4	0.4
0.14 µg.L^{-1}	0.3	1.6***	-7.4***	0.7	1.5*	0.4	0.2
SE	0.4	0.1	0.9	0.3	0.1	0.3	0.3
7 d							
Control mean	-0.1	0.0	0.0	0.0	0.1	0.0	0.0
SE	0.1	0.1	0.2	0.1	0.1	0.2	0.2
0.07 µg.L^{-1}	0.1	0.0	0.0	0.0	0.4	-0.2	0.0
SE	0.1	0.2	0.2	0.1	0.1	0.2	0.1
0.14 µg.L^{-1}	-0.1	-0.5	-0.8	-0.4*	0.5*	-0.5	0.0
SE	0.1	0.1	0.4	0.1	0.2	0.2	0.2

Note: *, ** and *** refer to significant differences of mean transcription level values to control at p-values of < 0.05, < 0.01 and < 0.001, respectively.

At the 48 h time-point, transcription levels for *vtg* were reduced by a factor of 7 for both concentrations tested, and statistical differences were significant when compared to controls as well as to other time-points ($p < 0.001$). For treatment concentration 0.07 µg.L^{-1} *hsp90* was significantly down-regulated at the 24 h time-point. Expression was slightly higher after 48 h

and similar to controls after 7 d. For 0.14 µg.L^{-1} bifenthrin, there was a significant up-regulation in *hsp90* at the 48 h time-point.

Principal Component Analysis identified 2 factors responsible for the variation in the recovery time-course dataset, with factor 2 (PC2) consisting solely of *vtg* data. Factor scores (PC1 and PC2) plotted in a trajectory plot (Figure 13) show that for 0.07 µg.L^{-1} at 24 h only changes in PC1 describe the deviation from zero (=equalized controls). At the concentration of 0.14µg.L^{-1} the deviation is described by both factors in the order PC1>PC2, indicating higher intensity of stress at this concentration compared to 0.07 µg.L^{-1}. After a recovery period of 24 h, the shift in coordinates is almost identical for both concentrations, but still deviates from zero, although for most genes no statistical difference was evident if compared to treatment control. At the 7 d time-point, factor scores tended to return to control levels for the 0.07 µg.L^{-1} treatment but not for 0.14 µg.L^{-1}, with mostly PC2 being responsible for the shift. In contrast to these findings, we previously reported a complete recovery in swimming performance of fish at this time-point.

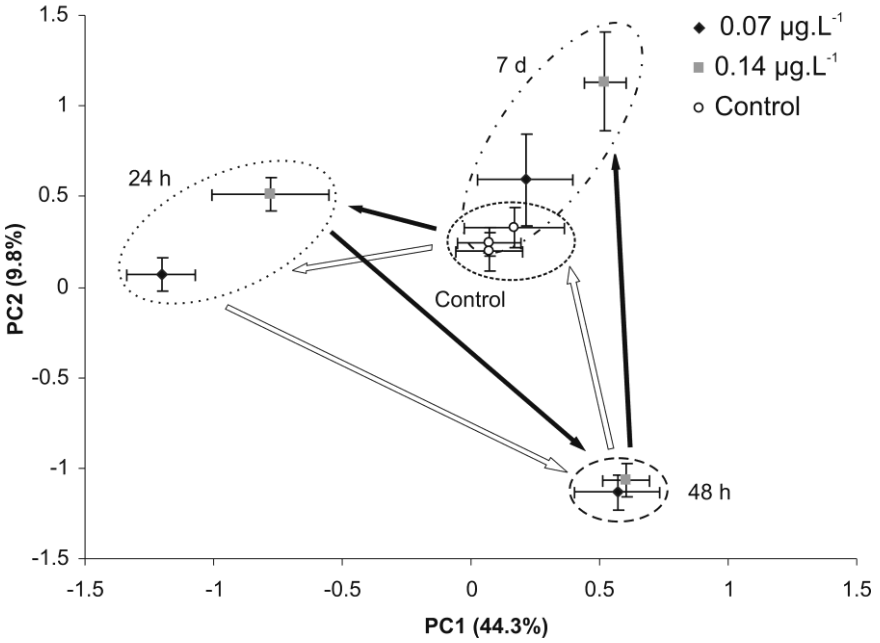

Figure 13: Trajectory plot of gene transcription responses over the 6 day recovery time series after 24 h treatment with 0.07 µg.L^{-1} (♦) and 0.14 µg.L^{-1} (■) bifenthrin. Error bars represent the standard error for each time point average of principal component factor scores. Unequalized controls (○) for each time-point are included for visualization of recovery process.

Discussion

An integrative assessment of multiple endpoints at different levels of biological organization is essential to understanding and interpreting data from transcriptome studies. Our study demonstrated that changes in gene transcription occurred at the lowest exposure concentration used, indicating great sensitivity of the molecular response to bifenthrin exposure. Recovery was observed both at the molecular level and at the level of swimming performance suggesting that gene transcription may be a valuable tool to identify physiological impairment in fish due to insecticides.

Several genes responded strongly to bifenthrin and are of special functional significance due to their importance in neuromuscular processes. Aspartoacylase (*aspa*), an enzyme that catalyzes hydrolysis of N-acetyl-L-aspartate (NAA) to aspartate and acetate in the vertebrate brain, was significantly down-regulated in bifenthrin exposed fish. Deficiency in *aspa* activity leads to degeneration of the myelin sheet of nerve cell axons. Our data corroborate findings by Connon et al. (2009), who observed significant down-regulation of aspa in the delta smelt (*Hypomesus transpacificus*), after exposure to sublethal concentrations of another pyrethroid insecticide, esfenvalerate. Aspa may therefore be a useful molecular marker for neurological damage in fish species, as we can support here by successful application of this marker in the fathead minnow. Another gene involved in neuromuscular processes, which was down-regulated following bifenthrin exposure, is creatine kinase. Creatine kinase as part of the creatine/phosphocreatine/creatine kinase system plays an important role in the cellular energy metabolism and energy homeostasis in the vertebrate central nervous system by the regeneration of ATP, as it catalyzes the transfer of the phosphoryl group from phosphocreatine to ADP (Ames 2000, Béard and Braissant 2010, Wallimann et al. 1992). Reduction in *ck* activity was found to be associated with decreased energetic status and oxidative stress in humans with neurodegenerative diseases (Béard and Braissant 2010, Schulze 2003) and rats after spinal chord injury (Aksenova et al. 2002). Despite that, ck associated with the sarcoplasmatic reticulum is involved in muscle contraction by regulation of ion fluxes, especially Ca^{2+} uptake and ATP/ADP ratios (Rossi et al. 1990). Of interest in this context is also the up-regulation of *pvalb*, an intercellular Ca^{2+}-binding protein most abundant in fast-contracting muscle fibers of fish and other vertebrates (Arif 2009). Ca^{2+} is the main regulatory ion in muscle fibers and contractibility depends on proteins for cellular Ca^{2+} binding and transport. One of the main functions of *pvalb* is the facilitation of muscle relaxation by regulating Ca^{2+} exchange between the sarcoplasmatic reticulum and the

myofibrils and is therefore involved in the cellular processes to maintain correct muscle performance (Coughlin et al. 2007). Increased *pvalb* transcript levels at the higher exposure concentrations can be interpreted as response to pyrethroid-induced hyperexcitability and loss of muscle control. Parvalbumins are also found in GABA-ergic interneurons in brain tissue and are involved in modulation of neuronal excitability and activity to protect neurons from excessive Ca^{2+} accumulation associated with high neuronal activity. In combination, the regulations of the latter genes indicate a strong link between Ca^{2+} regulation and energy in the context of proper muscle function and the decline in measured swimming performance in these fish after exposure to bifenthrin. Although neuromuscular responses within this study were observed in a relatively small number of genes, in combination, they indicate effects upon fish motility and can be considered part of the underlying mechanisms for the observed impairment in swimming ability.

Gene transcription of *mt* was significantly up-regulated in fish exposed to bifenthrin, contrary to findings by Geist et al. (2007), who observed no induction of *mt* following esfenvalerate exposure. *Mt* is commonly used as a biomarker for metal-induced stress (Ghedira et al. 2010, Roesijadi 1992), but it is also known to be involved in a variety of other cellular processes, including protection against oxidative stress (Baumann et al. 1991), neuroprotection and regeneration after CNS injuries (West et al. 2008). These latter functions would support its biomarker value in neuronal damage caused by pyrethroid poisoning, however, this example demonstrates the importance of studying a larger number of gene responses.

Other genes coding for enzymes involved in detoxification and defense against oxidative stress were also differentially expressed following bifenthrin exposure. Cytochrome *cyp3a*, which plays an important role in the detoxification of xenobiotics in fish (Christen et al., 2010), was down-regulated, as was *gst*. Down-regulation of these gene-transcripts has the potential to negatively affect the organism's detoxification capacity, thus compromising the ability to effectively respond to additional chemical or disease stressors.

Changes in gene transcription also suggest effects on the endocrine system of bifenthrin exposed fish. The egg yolk precursor *vtg*, a well-known biomarker of exposure to estrogenic compounds, was significantly up-regulated. Along with the down-regulation of *cyp3a* this may be indicative of endocrine effects of bifenthrin. For example, suppressed *cyp3a* protein expression was accompanied by increased vitellogenin serum protein levels in male adult medaka (*Oryzias latipes*) after 17β-estradiol exposure (Kashiwada et al. 2007). Pyrethroid degradation products have been shown to trigger endocrine activity (Tyler et al. 2000), and

bifenthrin, in particular, had estrogenic effects in larval zebrafish (*Danio rerio*) (Jin et al. 2009) measured as significant induction of *vtg*. Changes in *vtg* transcription in our study using immature fish were seen directly during bifenthrin exposure as well as after the recovery period. The changes in vtg transcription due to bifenthrin exposure might influence important developmental processes in immature fish, as it is differentially expressed during ontogenesis. In a study by Johns et al. (2009) the gene transcription of vitellogenin (*vtg1*), among others, was measured during the first month of fathead minnow embryonic development, showing a significant up-regulation at 7 and 10 days post fertilization, but not at later time-points. In our test setup, the age of fish after the 24 h bifenthrin exposure was 8 days post-hatch (approximately 12 days post fertilization), which is within the onset-phase of female gonadal sex development (Van Aerle et al. 2004).

From gene transcription to higher level effects: Observed responses in gene transcription demonstrate exposure-related cellular effects in fathead minnow larvae. It might be expected that these energy demanding responses result in measurable negative outcomes on the whole-organism level, i.e. behavior, growth and reproduction. We recently demonstrated significant impacts of sublethal bifenthrin concentrations on swimming performance and behavior in larval fathead minnow (Beggel et al. 2010). Swimming performance was significantly affected following 24 h exposure to ≥ 0.14 $\mu g.L^{-1}$ bifenthrin. This threshold corresponds to the onset of the second phase responses at the molecular level, which tended to be dose-dependent. Further assessment of swimming performance after 48 h and 6 d in control water showed that fish are able to recover from initial negative effects. The neuromuscular impairment after short bifenthrin exposure was reversible. However, even reversibly altered swimming behavior due to pyrethroid exposure was shown to result in increased predation risk in larval fathead minnow (Floyd et al. 2008).

Time series recovery in the trajectory plot also showed that at recovery day 6 the data point coordinates shift to zero for the 0.07 $\mu g.L^{-1}$ treatment. In case of the higher treatment tested in this time series there was no complete recovery, but this may be independent from neuromuscular impairments, as the PCA factor score attributable for the position consisted of *vtg* alone.

Dose-dependent response patterns: Our results demonstrate that the gene transcription profiles of low dose exposures differ from higher doses and generally show a biphasic

response with increasing treatment concentration. This can be considered a typical hormesis response, which is, according to Calabrese and Baldwin (2002), defined as "an adaptive response characterized by biphasic dose responses of generally similar quantitative features with respect to amplitude and range of the stimulatory response that are either directly induced or the result of compensatory biological processes following an initial disruption in homeostasis.". This pattern of response is often controversially discussed in the literature but seems to be a principal response in biological systems (Chapman 2002, Kefford et al. 2008). A linear dose-dependant response is often typical if relatively high exposure concentrations are used and mortality is used as an endpoint. In studies where concentrations at a sublethal, and oftentimes more environmentally realistic, level are utilized, the response curve shows a biphasic pattern (Connon et al. 2008). In the present study we could also see that, with increasing concentration, transcription profiles change towards a dose-dependent relationship which corresponds with negative effects measurable on the whole-organism level. This trend in transcription was not only measured for most of the genes, it was also reflected in general transcript patterns evident from the results of the PCA. Considering the threshold point at 0.14 $\mu g.L^{-1}$ that is coincident with the onset of change in transcription could contribute valuable information with regard to the application of biomarkers in field studies or monitoring programs. It is of great importance to distinguish between normal adaptive responses in gene transcription and those that are associated with damage of the organism (Steinberg et al., 2008). This can only accomplished by a combination of different endpoints, such as those used here. A dose-related biomarker response can serve as an indicator of exposure, but with the hormetic change in biomarker transcription that leads to detrimental effects on the whole-organism level a more valuable prediction of environmental relevance is evident.

Conclusions

This study showed that the use of gene transcription changes in a small set of molecular markers is a highly sensitive tool to detect chemical stress in larval fish. Changes in gene transcription after short-term and sublethal exposure to bifenthrin were measured at concentrations as low as 10% of the respective 24 h LC_{10}, being half the concentration at which higher level of biological organization responses, like swimming performance, could be measured. Carefully selected markers can deliver sensitive signals about perturbations in the organism of interest.

Yet, interpretations about specific changes in gene transcription require caution, as initial hormetic responses are early warning signals, but may not allow for prediction of stressor effects at higher concentration levels. Therefore we strongly recommend the combination of molecular and whole organism endpoints for investigations of biomarker suitability. It could be shown that adverse neuromuscular effects of bifenthrin are reversible at low concentrations and the organism is able to recover from the resulting behavioral impairment given the time and environmental condition to do so. However, our results indicate that even short exposure times can result in significant effects on the transcription of endocrine-related genes.

Chapter IV - Impacts of the phenylpyrazole insecticide fipronil on larval fish: Time-series gene transcription responses in fathead minnow (*Pimephales promelas*) following short-term exposure

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A similar version of this chapter was published in Science of the Total Environment 426 (2012), 160-165.

Abstract

The utilization of molecular endpoints in ecotoxicology can provide rapid and valuable information on immediate organismal responses to chemical stressors and is increasingly used for mechanistic interpretation of effects at higher levels of biological organization. This study contributes knowledge on the sublethal effects of a commonly used insecticide, the phenylpyrazole fipronil, on larval fathead minnow (*Pimephales promelas*), utilizing a quantitative transcriptomic approach. Immediately after 24 h of exposure to fipronil concentrations of ≥ 31 $\mu g.L^{-1}$, highly significant changes in gene transcription were observed for aspartoacylase, metallothionein, glucocorticoid receptor, cytochrome P450 3A126 and vitellogenin. Different mechanisms of toxicity were apparent over the course of the experiment, with short-term responses indicating neurotoxic effects. After 6 days of recovery, endocrine effects were observed with vitellogenin being up-regulated 90-fold at 61 $\mu g.L^{-1}$ fipronil. Principal component analysis demonstrated a significant increase in gene transcription changes over time and during the recovery period. In conclusion, multiple mechanisms of action were observed in response to fipronil exposure, and unknown delayed effects would have been missed if transcriptomic responses had only been measured at a single time-point. These challenges can be overcome by the inclusion of multiple endpoints and delayed effects in experimental designs.

Introduction

Freshwater ecosystems are among the most threatened worldwide and suffer from a strong loss of biodiversity in recent decades (Burkhardt-Holm et al. 2005, Geist 2011). The various threats to freshwater ecosystems include climate change, nutrient shifts, acidification, habitat loss, exploitation and biological invasions. In addition, chemical contamination is also a significant factor. An important source of chemical stressors is constituted by the application of insecticides in urban and agricultural areas that can be hazardous to sensitive organisms in aquatic ecosystems (Schäfer et al. 2011).

The phenylpyrazole insecticide fipronil is commonly applied both in urban areas and in agriculture. The typical application in urban areas is for landscape maintenance and structural pest control, and fipronil is widely used by licensed professionals and homeowners alike (Jiang et al. 2010, Sandahl et al. 2007). Several studies confirm that insecticides can be transported via irrigation runoff and stormwater into urban streams and waterways, even if not applied in the vicinity of surface water bodies (Gunasekara et al. 2007 and Jiang et al. 2010). Fipronil has frequently been detected in surface waters and sediments in metropolitan areas (Sprague and Nowell 2008) and measured concentrations in irrigation runoff from residential areas were in a range of 0.13 to 12.6 $\mu g.L^{-1}$ (Gan et al. 2012). In agriculture, fipronil is commonly applied in rice cultivation with values $\geq$ 9 $\mu g.L^{-1}$ measured in surface waters downstream of treated fields (Schlenk et al. 2001). Fipronil is a potent disrupter of the insect central nervous system via interference with the γ-aminobutyric acid (GABA)-gated Cl^- channel function (Cole et al. 1993, Hainzl and Casida 1996). GABA is a major inhibitory neurotransmitter in the vertebrate central nervous system. In insects and mammals, the behavioral effects of GABA antagonists include hyperactivity, hyperexcitability, and convulsions, which are correlated with increased spontaneous nerve activity (Gunasekara et al. 2007). Binding affinity of fipronil is known to be higher for invertebrate GABA receptor subunits, but binding to vertebrate GABA receptors is possible (Grant et al. 1990, Hainzl and Casida 1996), resulting in a neurotoxic effect also in vertebrates. Generally, aquatic invertebrates and early life stages of fish are most sensitive to insecticides (Kegley et al. 2008).

Nowadays, high effect concentrations of insecticides that cause acute toxicity or mortality are less likely to occur in the environment. Therefore, the effects in a sublethal range of concentration are of higher environmental relevance and adequate endpoints have to be chosen to detect possible impacts on aquatic ecosystems, such as behavioral alterations or

endocrine disrupting effects. For characterizing sublethal chemical effects the usefulness of integrative approaches linking molecular endpoints such as mRNA transcription with endpoints at higher levels of biological organization such as behavioral alterations or population growth has been demonstrated (Beggel et al. 2011, Connon et al. 2009, Heckmann et al. 2008, Miller et al. 2007, Swain et al. 2010). However, the establishment of linkages between alterations at the molecular and biochemical level to effects on individuals or populations still remains a major challenge.

In particular, transcriptomic responses are typically rapid and thus considered highly sensitive indicators of stress, as the initial interaction takes place on the molecular level and builds the mechanistic basis for subsequent consequences at higher levels of biological organization (Schirmer et al. 2010). However, the responses measured on the transcript level are heavily influenced by concentration, exposure duration and uptake route, mechanism-of-action, or the type of tissue sampled. Especially when utilizing sublethal concentrations and several time points, biphasic or even multiphasic responses can be observed (Ankley et al. 2009, Beggel et al. 2011, Heckmann et al. 2008).

We investigated sublethal effects of fipronil on larval fathead minnow (*Pimephales promelas* Raffinesque) and assessed resulting gene transcription responses to gain insight into possible causative effects of previously reported impacts of fipronil on swimming performance and growth in larval fathead minnow at nominal concentrations $\geq 31\ \mu g.L^{-1}$ (Beggel et al. 2010). There is little information available from the literature on how fipronil affects gene transcription in larval fish and how it mechanistically affects larval development.

The core objective of this study was to assess the sensitivity of transcriptomic responses to fipronil exposure in a time course experiment, after 24 h short-term exposure and during a subsequent 6-day recovery period. We employed a suite of target genes that cover different physiological functions, selected from cellular pathways involved in endocrine function, including molecular biomarkers for endocrine disruption and somatic growth (vitellogenin, *vtg*; insulin-like growth factor, *igf*; growth hormone, *gh*; zona pellucida glycoprotein 3, *zp3;* glucocorticoid receptor, *gcr*), energy metabolism and muscular and neuronal functions (glucose-6-phosphate dehydrogenase, *g6pd*; creatine kinase, *ck*, parvalbumin, *pvalb*, aspartoacylase, *aspa*), as well as detoxification and general stress responses (cytochromes P450 1A and 3A126, *cyp1a* and *cyp3a*; glutathione S-transferase rho, *gst*; heat shock protein 90, *hsp90* and metallothionein, *mt*).

Materials and Methods

Fish source, acclimation and quality assurance:

Fathead minnow larvae were obtained from Aquatox Inc. (Hot Springs, AR, USA) at 7 days post-hatch on the day of arrival. Control water consisted of deionized water, modified with salts to meet US EPA specifications (specific conductivity: 265–293 $\mu S.cm^{-1}$; hardness: 80–100 as mg $CaCO_3$ L^{-1}; alkalinity: 57–64 as mg $CaCO_3$ L^{-1} (USEPA, 2002a)). Fish were acclimated for a minimum period of 4 h in control water at a temperature of 25 °C. During the acclimation period < 1% mortality was observed, and the fish fed and swam normally.

During the project period, routine monthly reference toxicant tests were performed using NaCl to ascertain whether organism response fell within the acceptable range according to USEPA requirements (USEPA, 2002a). Each test consisted of a dilution series (5 test concentrations) and a control. All test organisms responded normally (within 95% confidence interval of running mean) and sensitivity was considered typical.

Insecticide exposure:

Reference standard grade fipronil (5-amino-1 [2,6-dichloro-4-(triflouromethyl) phenyl]-4 [(triflouromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile), with 98.5% purity (CAS number 120068-37-3) was obtained from ChemService Inc. (West Chester, PA, USA). Pure fipronil is a 50:50 racemic mixture. All insecticide exposure experiments were conducted at the Aquatic Toxicology Laboratory, School of Veterinary Medicine, University of California Davis, USA.

Exposure concentrations used for the assessment of sublethal stress responses were calculated as percentages of the nominal LC_{10} values derived from acute toxicity tests as reported previously (Beggel et al., 2010). Treatments consisted of a control, solvent control and 10%, 20%, 33% and 50% of the nominal LC10 for fipronil corresponding to concentrations of 31, 61, 102 and 153 $\mu g.L^{-1}$. Sub-samples of each test solution (1 L) were analyzed using gas chromatography with mass spectrometry and ion-trap detection, at the California Department of Fish and Game Water Pollution Laboratory (Rancho Cordova, CA, USA), with a limit of detection of 0.2 $\mu g.L^{-1}$ (reporting limit; recovery 83.1%). Measured concentrations were 53, 142, 333 and 365 $\mu g.L^{-1}$ and thus on average 2.4 times higher than nominal concentrations. We were unable to determine the reason for this discrepancy which had previously also occurred with the same chemical in other experiments using independent preparation of stock

solutions. Since the ratio of measured to nominal concentration varied from 1.7 to 3.3 for different concentrations, an error in analytical chemistry seems more likely than in medium preparation. Consequently, the more plausible nominal concentrations are referred to throughout the manuscript.

Each experimental treatment consisted of 9 replicate 600-mL Pyrex beakers containing 250 mL test solution and 10 fish larvae. Fish were exposed to the test solutions for 24 h at a water temperature of 25 °C and a 16:8 light–dark ratio and were not fed during exposure. We used 5 fish samples from each of the 3 replicates per treatment for gene transcription analysis at each of three time points: 24 h, 48 h which included 24 h of recovery time and 168 h which included 144 h of recovery time. For recovery, fish were transferred to control water after the 24 h exposure and maintained according to standard USEPA protocols. During the recovery period, fish were fed *ad libitum* with *Artemia* nauplii 2 times each day. At each time point, a subset of three replicate exposure containers per treatment was sampled for gene transcription analysis. Fish were euthanized in tricane methanesulfonate (MS-222), individually transferred into DNase/RNase-free 1.5-mL tubes (Eppendorf, Germany) and immediately flash-frozen in liquid nitrogen. Samples were stored at − 80 °C until processing.

Sample processing:

Total RNA was extracted from whole fish larvae using the QIAGEN RNeasy MiniKit, with on-column DNase digestion, according to the manufacturer's instructions. Total RNA concentration was determined from absorbance at 260 nm (NanoDrop ND 1000) and RNA quality was verified by electrophoresis on ethidium bromide-stained 1.5% agarose gels and A260/A280 ratios.

Quantitative PCR:

Primer and probes for qPCR analyses were designed using Roche Universal Probe Library Assay Design Center (https://www.roche-applied-science.com). Designed primers were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com) and TaqMan probes were supplied by Roche. Transcription of the following target genes were assessed at the 24-h time point: vitellogenin (*vtg*) as well as the zona pellucida glycoprotein 3 (*zp3*) were used as indicators of estrogenic and xenestrogenic exposure (Genovese et al. 2011, Sumpter and Jobling 1995). Insulin-like growth factor (*igf*), growth hormone (*gh*) and glucocorticoid receptor (*gcr*) regulate several physiological functions, such as growth and development (Filby and Tyler 2007, Leatherland et al. 2010). Impacts on energy metabolism and muscular

and neuronal functions were assessed by glucose-6-phosphate dehydrogenase (*g6pd*), creatine kinase (*ck*), parvalbumin (*pvalb*) and epithelial calcium channel (*ecac*), genes involved in energy status, muscle contraction and Ca^{2+} signaling (Coughlin et al. 2007, Rossi et al. 1990) and aspartoacylase (*aspa*), for example involved in myelin sheet integrity in nerve cells (Connon et al. 2009). Target genes also included biomarkers for xenobiotic detoxification and general stress response (cytochromes P450 1A and 3A126, *cyp1a* and *cyp3a*; glutathione S-transferase rho, *gst*; heat shock protein 90, *hsp90* and metallothionein, *mt*) (Christen et al. 2010, Bauman et al. 1991, Hodgson and Rose 2008). Elongation factor 1-alpha (*ef1a*) was used for normalization. Primers and probes for investigated biomarkers are listed in detail in Beggel et al. (2011). Transcripts analyzed in the time series experiment were *aspa*, *ck*, *pvalb*, *cyp3a*, *gst*, *vtg* and *hsp90* and responses are summarized in Table 9.

Complementary DNA (cDNA) was synthesized using 1.0 µg total RNA, with random primers and SuperScript® III reverse transcriptase (Invitrogen, Carlsbad, CA). A dilution to a total volume of 150 µl was carried out with nuclease free water to generate sufficient template for qPCR analysis. TaqMan® Universal PCR Mastermix (Applied Biosystems, Carlsbad, CA) was used in qPCR amplifications in a reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625U AmpliTaq Gold DNA polymerase per reaction, 0.25U AmpErase UNG per reaction and 5 µL of cDNA sample in a final volume of 12 µL.

Samples were placed in 384 well plates, and cDNA was amplified in an automated fluorometer (ABI PRISM 7900 Sequence Detection System, Applied Biosystems). 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. Fluorescence of samples was measured every 7 s and signals were considered positive if fluorescence intensity exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle, CT). SDS 2.2.1 software (Applied Biosystems, Carlsbad, CA) was used to quantify transcription.

Data analysis:

We used the geNorm algorithm to estimate the variability of reference gene expression, and to determine an optimal gene for normalization of the data (Vandesompele et al. 2002). Quantitative PCR data were analyzed using the relative quantification $2^{(-\text{ Delta Delta CT})}$ method (Livak and Schmittgen 2001). Transcription levels were calculated relative to elongation factor 1-alpha (*ef1a*) determined by geNorm as the least variable transcript in this study,

whose suitability as a reference gene was also confirmed in previous studies with fish (Jorgensen et al. 2006). Statistical analyses were conducted using SPSS 11 for Windows statistical software (SPSS Inc.). Differences in transcription levels of single genes were evaluated using univariate analysis of variance (ANOVA) with Tukey's multiple comparison post hoc tests to compare between treatments, treating each gene separately. Data were analyzed in the log2-scale, in order to meet the assumptions of ANOVA for normal distribution and homogeneity of variances were met. Data are presented in the non-transformed scale, representing the n-fold change in gene transcription relative to the reference gene *ef1a*. For analysis of gene transcription, data from recovery time points, each dataset was adjusted to the control by dividing each data point with the corresponding control data point at each time point prior to statistical analysis. This procedure created a ratio of adjusted values that allow direct comparison between the different time points and treatments. Control values were tested with ANOVA and showed no differences between plates and time points. Averaged control values (± SE) for each time point were included into a trajectory plot for visualization of recovery effect. Principal component analysis (PCA) was applied to identify principal factors underlying the variation in the dataset. We used Varimax with Kaiser Normalization as Rotation method and the Kaiser–Meyer–Olkin (KMO) measure verified the sampling adequacy for the analysis of the gene transcription data at an acceptable limit of KMO = 0.5 (Sharma 1996). Bartlett's test of sphericity was applied to control if correlations between items were sufficiently large for PCA. Factor scores from principal components were used to generate a geometric trajectory plot in order to visualize variation of transcription levels over time (Keun et al. 2004).

Results

Single gene transcript responses

Effects resulting in differential transcription of individual genes were observed at all test concentrations below 50% of the nominal LC_{10} and significant responses in gene transcription were observed starting at the lowest exposure concentration of 31 $\mu g.L^{-1}$ fipronil (10% LC_{10}, nominal concentration). The typical patterns of single gene transcript responses did not follow linear dose–response relationships. Within the concentration range used here, the responses were often multiphasic, i.e. genes that were up-regulated at the lowest exposure concentration were down-regulated with increasing treatment concentration and again up-regulated at highest concentrations (Figure 14).

At the 24-h time point, significant up-regulation was observed in the transcription of *ecac* ($p = 0.002$), *vtg* ($p < 0.05$) and mt ($p = 0.027$) at the lowest exposure concentration (31 $\mu g.L^{-1}$ fipronil, nominal concentration, 10% LC_{10}). However, strongest responses were observed at the highest exposure concentrations of fipronil. A significant up-regulation was evident for *cyp3a* ($p = 0.002$) and *ecac* ($p = 0.001$) at the highest fipronil concentration (153 $\mu g.L^{-1}$, nominal concentration, 50% LC_{10}). This was even more pronounced for *mt*, where significant up-regulation was seen at the two highest exposure concentrations (102 $\mu g.L^{-1}$, nominal concentration, 33% LC_{10}, $p = 0.011$ and 153 $\mu g.L^{-1}$, nominal concentration, 50% LC_{10}, $p < 0.001$). Fipronil exposure also caused down-regulation of *aspa* at 61 $\mu g.L^{-1}$ ($p < 0.001$) and an up-regulation of this gene at 153 $\mu g.L^{-1}$ ($p = 0.001$). The glucocorticoid receptor (*gcr*) which mediates the effects of glucocorticoids, mainly cortisol in fish, was up-regulated at 153 $\mu g.L^{-1}$ fipronil ($p < 0.001$). We observed significant up-regulation of igf at 102 $\mu g.L^{-1}$ fipronil ($p = 0.008$) and down-regulation of *zp3* at 61 $\mu g.L^{-1}$ fipronil ($p = 0.002$), but this was not measured at other treatment concentrations. Other tested genes, such as *hsp90*, *gh*, *g6pd*, *pvalb* and *gst*, were not significantly affected by fipronil exposure at the 24 h time point, regardless of treatment concentration.

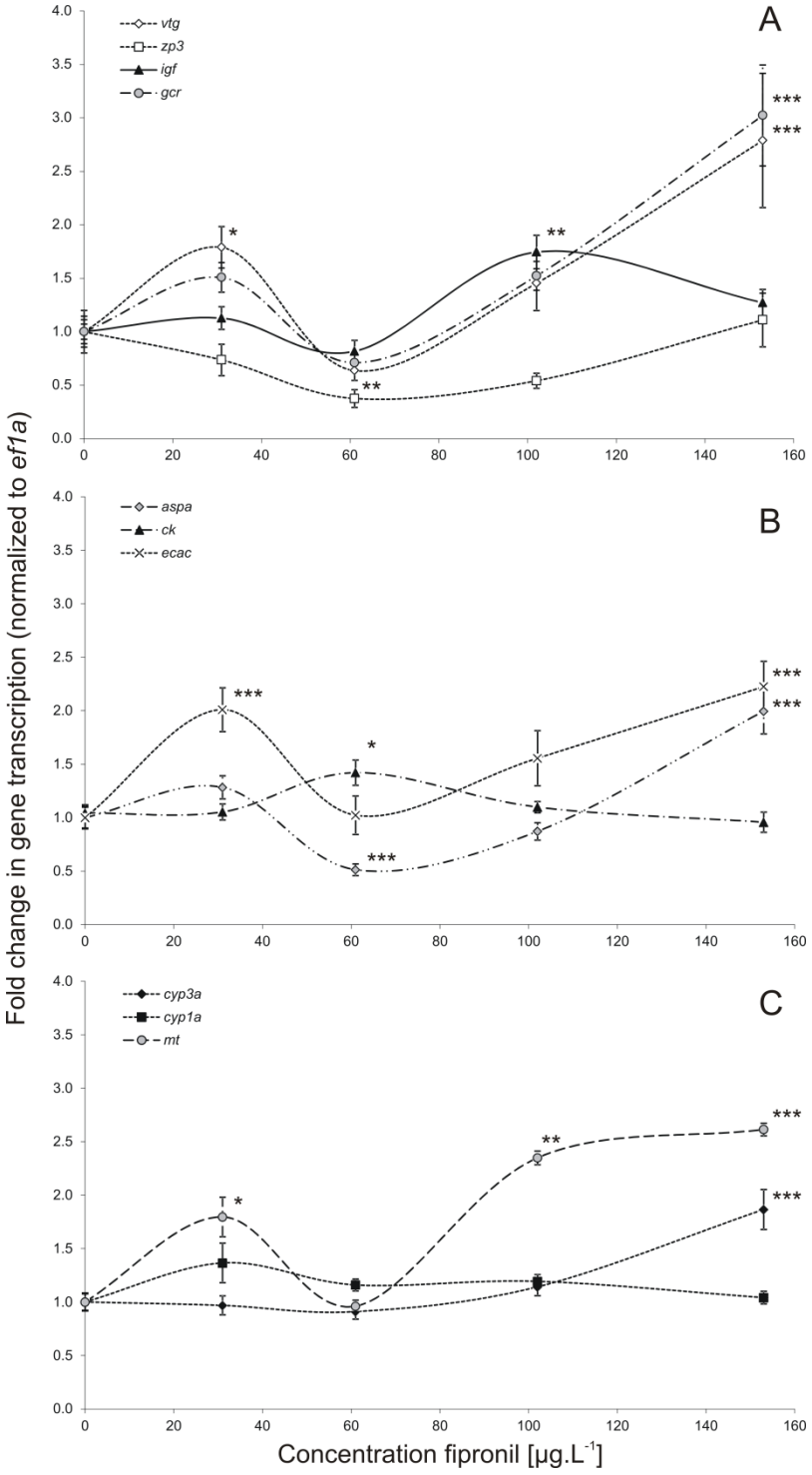

Figure 14: Changes in gene transcription in response to fipronil exposure assessed by quantitative PCR; (A) endocrine function, (B) neuromuscular function, energy, (C) detoxification, general stress response. Significant changes occurred at 31 µg.L^{-1} fipronil. Asterisks indicate significant differences in treatment compared to control (*: p<0.05; **: p< 0.01; ***:p< 0.001). Strongest differences to control treatment were observed at 153µg.L^{-1}. Data is presented as mean value ± standard error (n=15). (*vtg*= vitellogenin precursor; *zp3*= zona pellucida glycoprotein3; *igf*= Insuline-like growth factor; *gcr*= glucocorticoid receptor; *aspa*= aspartoacylase; *ck*= creatine kinase; *ecac*= epithelial calcium channel; *mt*= metallothionein).

Table 9: Changes in mean gene transcription levels and standard errors (SE) of control, 31 µg.L-1 and 61 µg.L-1 fipronil exposed fathead minnows, expressed as n-fold changes, normalized to *ef1a* gene transcription at three time points: (i) 24 h (exposure period); (ii) 48 h (24 h exposure, 24 h recovery); (iii) 7 d (24 h exposure, 6 d recovery).

Time point	*cyp3a*	*pvalb*	*vtg*	*hsp90*	*ck*	*gst*	*aspa*
24 h							
Control mean	-0.3	0.5	0.1	-0.1	0.2	0.5	-0.1
SE	0.3	0.4	0.5	0.3	0.3	0.4	0.4
31 µg.L^{-1}	-0.4	0.9	2.0	0.4	0.1	-0.6	0.9
SE	0.2	0.4	0.2	0.3	0.3	0.5	0.3
61 µg.L^{-1}	-0.3	0.9	-0.7	0.8	1.0	0.2	**-2.0****
SE	0.3	0.3	0.4	0.3	0.3	0.4	0.2
48 h							
Control mean	0.1	0.1	-0.6	0.1	-0.1	-0.1	0.2
SE	0.3	0.4	0.4	0.3	0.3	0.4	0.3
31 µg.L^{-1}	0.7	1.4	-1.2	0.9	**-0.4*****	1.1	1.0
SE	0.3	0.1	0.4	0.3	0.4	0.3	0.3
61 µg.L^{-1}	0.4	0.9	-1.8	1.2	**1.0*****	0.0	-0.1
SE	0.3	0.3	0.1	0.2	0.3	0.4	0.3
7 d							
Control mean	-0.2	0.3	-0.8	-0.1	-0.1	0.2	-0.1
SE	0.4	0.4	0.5	0.4	0.4	0.5	0.3
31 µg.L^{-1}	-0.5	0.2	-0.8	0.5	**-2.3*****	**2.7*****	0.3
SE	0.4	0.4	0.3	0.3	0.2	0.4	0.4
61 µg.L^{-1}	**-1.6*****	-1.2	**90.6*****	-0.8	**-1.7*****	1.3	-1.0
SE	0.1	0.3	5.2	0.3	0.1	0.4	0.3

Note: Bold numbers indicate significant differences of mean transcription level values to control at p-values of < 0.05 (*), < 0.01 (**) and < 0.001 (***), respectively.

After 24 h of exposure to fipronil, fish were transferred into control water. Quantitative PCR analysis was performed as a time series for a subset of genes using fish samples from the two lowest exposure concentrations (nominal fipronil concentrations of 31 µg.L^{-1} and 61 µg.L^{-1} fipronil) and two additional time points during the recovery phase. Important elements of the cellular energy metabolism, represented by *ck* in this study, showed significant differential transcription at the 24-h time point in the 61 µg.L^{-1} treatment, becoming more pronounced during the recovery phase of the experiment. Creatine kinase transcripts were significantly up-regulated at 61 µg.L^{-1} ($p = 0.025$) fipronil after 48 h, but significantly down-regulated at all exposure concentrations after 6 days of recovery (31 µg.L^{-1}, $p < 0.001$ and 61 µg.L^{-1}, $p = 0.001$; Table 7). Although not affected directly after exposure, a significant up-regulation of gst was detected after 6 days of recovery (31 µg.L^{-1}, $p < 0.001$). The strongest overall increase in gene transcription was observed for *vtg*, which was significantly ($p < 0.05$) up-

regulated 2-fold 24 h after exposure to 31 µg.L^{-1} fipronil, and as high as 90-fold ($p < 0.001$) after 6 days of recovery following exposure to 61 µg.L^{-1} fipronil.

Timeline trajectory of multivariate gene transcript response analysis

The general trend of increasing responses over time was more apparent when comprehensively considering the transcriptional changes of all investigated genes using multivariate analysis. Recovery time series were analyzed for the lowest two concentrations, 31 and 61 µg.L^{-1} fipronil, and the trajectory of factor scores derived from principal component analysis visualized the responses after exposure and during recovery period (Figure 15).

Principal component analysis yielded 2 factors being responsible for 56.6% of the variation in the dataset. These patterns show that deviations from the control range increase over time for both exposure concentrations and are similar in the direction of response. One-way ANOVA conducted on the factor score showed no differences between the controls and the data at the 24 h time point, but indicated significant differences to the 48 h time point (31 µg.L^{-1}, $p = 0.050$; 61 µg.L^{-1}, $p = 0.014$). Highly significant differences occurred at the 7 day time point and were mostly attributable to PC2 (31 µg.L^{-1}, $p < 0.001$, 61 µg.L^{-1}, $p < 0.001$). In general, these effects were most pronounced for the 61 µg.L^{-1} treatment. These patterns are indicative of delayed effects caused by fipronil exposure that shift from acute effects on neuromuscular function and detoxification to changes in energy metabolism and endocrine signaling during the recovery period (see also Table 9).

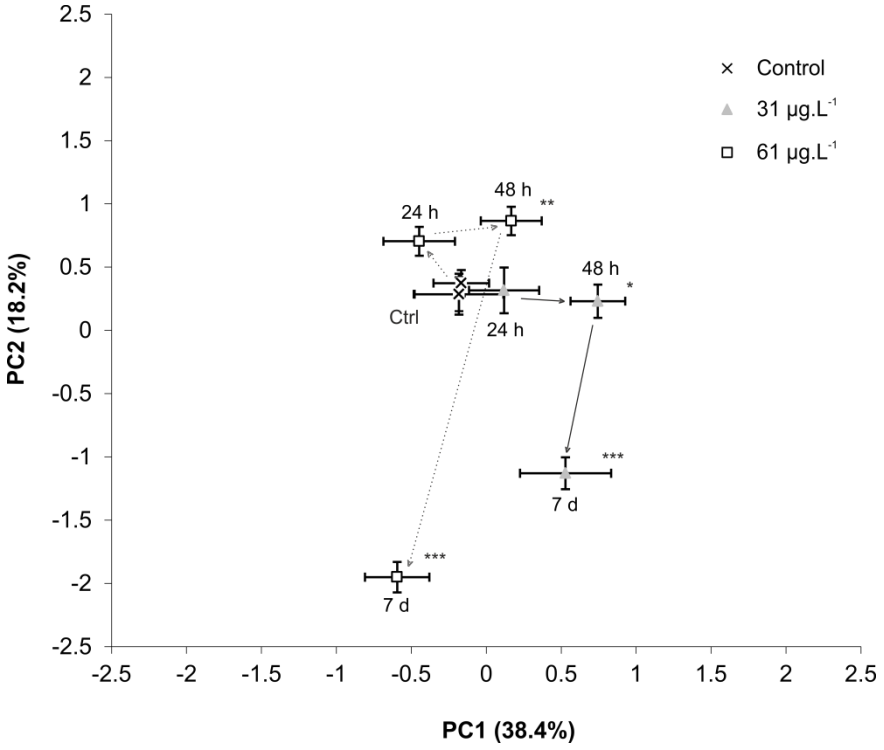

Figure 15: Trajectory plot of gene transcription responses over the 6 day recovery time series after 24 h treatment with 31 µg.L^{-1} (▲) and 61 µg.L^{-1} (□) fipronil and controls (x). Error bars represent the standard error for each time point average of principal component factor scores. Asterisks indicate significant differences between each time point and time point control based on Principal Component factors scores (*: p<0.05; **: p< 0.01; ***:p< 0.001).

Discussion

This study reports on sublethal transcriptomic responses to short-term fipronil exposure and delayed effects in larval fathead minnow. Differential transcript regulation affected target genes involved in pathways that include responses to xenobiotic stimulus, general cellular metabolism, hormone metabolism, oxidation–reduction processes and lipid metabolism. Surprisingly, the most pronounced effects were not observed directly after the 24 h exposure, but after 6 days of recovery in control medium. Multivariate analysis proved to be most useful for the characterization of the described general response patterns in transcription of target genes over time.

The initial expectation that fipronil would exert mostly neurotoxic effects in larval fish was not confirmed. Such effects were far less pronounced than in previous experiments with the synthetic pyrethroid bifenthrin, where neuromuscular impairment was detected by reduced swimming performance, as well as by the differential expression of genes involved in neuromuscular function such as *aspa* and *ck* (Beggel et al. 2011). Beggel et al. (2010) showed

that the physiological effects of fipronil indicated developmental alterations in the form of increased average weight and deformities such as scoliosis rather than neurotoxicity at nominal concentrations above 31 $\mu g.L^{-1}$. However, the concentration of 31 $\mu g.L^{-1}$ used in this experiment is about 3-fold above reported values from the environment (Gan et al. 2012, Schlenk et al. 2001). Developmental effects were not observed after the initial short-term exposure but only after 6 days of recovery. These observations suggest that unexpected delayed effects of short-term exposure to fipronil should be considered for a complete assessment of its ecotoxicological effects.

We were able to confirm the neuro-muscular toxicity of fipronil in fathead minnow in a recent study by measuring reduced swimming performance after exposure to nominal fipronil concentrations ≥ 61 $\mu g.L^{-1}$ (Beggel et al. 2010). Transcriptomic responses of genes linked to neuromuscular function analyzed here did not show a clear dose-dependent response, however, most genes that were up-regulated at the highest exposure concentration are associated with detoxification, stress response and neurological function, such as *mt*, *cyp3a*, *gcr* and *aspa*. Differential transcription of these genes also suggests that cellular xenobiotic detoxification processes were initiated and maintenance of ion homeostasis was affected. Especially gene transcription of mt was significantly up-regulated at most of the exposure concentrations. It is known to be involved not only in a variety of cellular processes, including metal detoxification, protection against oxidative stress (Bauman et al. 1991), but also in neuroprotection and regeneration after CNS injuries (West et al. 2008). Similar effects were previously also reported for the synthetic pyrethroid bifenthrin (Beggel et al. 2011).

Alterations in endocrine signaling can affect important physiological parameters such as organism growth and development (Filby et al. 2006). Beggel et al. (2010) demonstrated that fipronil at concentrations ≥ 31 $\mu g.L^{-1}$ affected growth of larval fathead minnow. Such effects could be linked to alterations in endocrine signaling as observed in this study. Similar physiological effects after exposure to a weak estrogenic compound on fathead minnow early life stages were reported by Johns et al. 2009a and Johns et al. 2009b, where exposure to the mycotoxin zearalone (ZEAR) resulted in increased body size in larval fathead minnow along with an up-regulation in insulin-like growth factor (*igf*). In fish (and higher vertebrates) several interrelationships exist within and between tissues that mediate endocrine control of reproduction, growth, development and other physiological processes (Filby et al. 2006). The effects of environmental estrogens were shown to affect endocrine-mediated processes including somatic growth, glucocorticoid-mediated stress response, as well as embryonic

development, but the mechanistic details behind these effects are not yet fully understood (Filby et al. 2006 and references therein). The significant up-regulation of *vtg* suggests endocrine, in particular estrogenic, disruption caused by fipronil. We observed a significant up-regulation of the egg-yolk precursor *vtg* and – to a lower extend – of *igf* immediately after the 24 h exposure at high fipronil concentrations. At lower concentrations, the same effect appeared during the recovery period in case of *vtg*, suggesting strong interference of fipronil with ontogenetic development. Vitellogenin is normally only expressed in adult females, which makes it an ideal marker for the detection of estrogenic effects in males and juveniles, but several studies also confirmed its expression in larval fish during development (Johns et al. 2009a, Johns et al. 2009b). Differential regulation in *vtg* transcription observed herein occurred during the recovery phase between 8 and 15 days post-hatch. This might be associated with pathways not investigated in this study. In fathead minnows, ovarian differentiation is initiated between 10 and 25 days post-hatch, while male germ cells do not develop until 90 days post-hatch (Van Aerle et al. 2004). Thus, it is likely that exposure to fipronil concentrations > 61 $\mu g.L^{-1}$ affects gonadal development in larval fathead minnows.

Surprisingly, we did not detect significant changes in *hsp90* transcription, which would be assumed to be involved in steroid receptor activation. Similar observations were reported for adult killifish (*Fundulus heteroclitus*), where ethinylestradiol exposure did not lead to an increase in hepatic *hsp90* transcripts (Hogan et al. 2010). The absence of significant transcriptomic changes in *hsp90* in both studies could be explained by the involvement of other molecular chaperones not measured in either study, or by the main response occurring on the protein level. Additionally, gene transcript activation of vitellogenin could be initiated via non-genomic second messenger systems that do not involve *hsp90* induction (Thomas 2008).

Transcriptomic responses are usually considered relatively quick responses compared to endpoints at higher levels of biological organization (Schirmer et al. 2010) and are detectable within hours after exposure. As evident from this study, the timing of analysis is crucial since data obtained from a single time point are often insufficient to capture the extremes in transcriptomic regulation, and because delayed effects during the recovery period can be important. The relatively short exposure to low doses of fipronil resulted in transcriptomic responses reflecting adaptation or acclimatory adjustments to maintain cellular homeostasis that usually do not cause irreversible damage in the organism. With increasing treatment concentration outside the range of homeostasis, no linear relation between exposure

concentration and transcriptomic response can be expected since the activation of different pathways that are rather compensatory than adaptive or acclimatory prevails under such conditions (Denslow et al. 2007). The relevance of selecting the ideal time point for transcriptomic analyses is particularly evident for *vtg* transcription where the strongest response was only detected after 6 days of recovery in control medium.

Conclusions

This study demonstrated that fipronil may negatively affect the neuromuscular and endocrine systems of larval fish. The delayed transcriptomic responses in certain genes, following short-term exposure and subsequent recovery, question the representativeness of single time point analyses in such experiments. Instead, the simultaneous inclusion of multiple genes and several time points, extending beyond the actual exposure time, provides a more comprehensive understanding of response patterns. Multivariate analyses can be particularly useful in revealing the temporal patterns of transcriptomic responses, and to link these with effects at higher levels of biological organization.

Chapter V - Linking molecular biomarkers with higher level condition indicators to identify effects of copper exposures on the endangered delta smelt (*Hypomesus transpacificus*)

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A similar version of this chapter was published in Environmental Toxicology and Chemistry 30(2) (2011), 290-300.

Abstract

The delta smelt (*Hypomesus transpacificus*) is an endangered pelagic fish species endemic to the Sacramento–San Joaquin estuary (CA, USA), and considered an indicator of ecosystem health. Copper is a contaminant of concern in Californian waterways that may affect the development and survival of this endangered species. The experimental combination of molecular biomarkers with higher level effects may allow for interpretation of responses in a functional context that can be used to predict detrimental outcomes caused by exposure. A delta smelt microarray was developed and applied to screen for candidate molecular biomarkers that may be used in monitoring programs. Functional classifications of microarray responses were used along with quantitative polymerase chain reaction determining effects upon neuromuscular, digestive, and immune responses in Cu-exposed delta smelt. Differences in sensitivity were measured between juveniles and larvae (median lethal concentration=25.2 and 80.4 $\mu g.L^{-1}$ Cu^{2+}, respectively). Swimming velocity declined with higher exposure concentrations in a dose-dependent manner ($r = -0.911$, $p < 0.05$), though was not statistically significant to controls. Genes encoding for aspartoacylase, hemopexin, α-actin, and calcium regulation proteins were significantly affected by exposure and were functionally interpreted with measured swimming responses. Effects on digestion were measured by up-regulation of chitinase and down-regulation of amylase, whereas down-regulation of tumor necrosis factor indicated a probable compromised immune system. Results from this study, and many others, support the use of functionally characterized molecular biomarkers to assess effects of contaminants in field scenarios. We thus propose that to attribute environmental relevance to molecular biomarkers, research should concentrate on their application in field studies with the aim of assisting monitoring programs.

Introduction

The application and significance of biomarkers in an environmental context has been criticized due to the lack of linkage between biomarker response in individual organisms and effects (Handy et al. 2003), such as reproductive fitness or population decline, at higher levels of organization (e.g., populations and ecosystems). The experimental combination of biomarkers with indicators of population or ecosystem condition, allows for the evaluation of effects upon an individual and subsequent extrapolation of endpoints such as population effects. Recent studies have hypothesized, identified, and demonstrated links between gene expression and higher levels of organization (Geist et al. 2007, Heckmann et al. 2008, Connon et al. 2009). The success behind the use of biomarkers as early and sensitive warning tools thus lies in interpreting biomarker responses in individuals in the context of affected cellular pathways, integrated with extensive life history knowledge of the species in question. This requirement is especially true when assessing effects on non-model species, or organisms living in ecological systems where sensitivity to stressors could greatly differ from model organisms. In fish, not only swimming behavior, but maintenance of optimal swimming performance is of particular importance for optimal fitness. A number of life history variables are dependent on swimming ability, including respiration, feeding, predator–prey interactions, and social interactions such as courtship and spawning, which are fundamental to survival, growth, and reproduction, the most important traits in evolutionary success. Contaminant exposures that predominantly affect neuromuscular structure and activity may translate to swimming impairments; however, other maintenance aspects, such as immune system and acquisition of adequate nutrients, may also play a role. Furthermore, exposure may affect olfactory senses and related behavioral responses, such as contaminant avoidance and homing, that will further affect individuals' chances of survival and reproduction, and as such have direct effects on population dynamics. Contaminant avoidance is, in itself, generally seen as a beneficial response; however, should the avoidance coincide with homing and identification of limited spawning sites, it would impinge on reproduction and consequently population dynamics (Scholz et al. 2000).

The delta smelt (*Hypomesus transpacificus*) is an endangered pelagic fish species endemic to the Sacramento–San Joaquin estuary, California (USA), whose abundance has dramatically declined since the 1980s, and more precipitously in recent years (Sommer et al. 2007). A number of complex factors, such as freshwater export and habitat destruction, have been attributed to the decline of delta smelt in its native environment, contaminants being another

key issue due to intense agriculture, urban runoff, and other anthropogenic activities (Geist et al. 2007). A recent steep decline has prompted considerable efforts to understand the causative factors of reduced population size (Sommer et al. 2007), especially because several other pelagic species have shown similar population trends. Delta smelt are restricted to the Sacramento–San Joaquin estuary, spawning in late winter and early spring, at limited freshwater sites both known and speculated, based on sediment type and other physicochemical properties such as slope, vegetation, depth, temperature, and salinity (Swanson et al. 2000). A major site of delta smelt spawning is in the Lower Sacramento River, from which hatched larvae are transported with the flow to brackish waters downstream, where they mature, during a short-lived one-year cycle (Moyle et al. 2002). This spawning site is located within agricultural areas and downstream from the Sacramento municipal wastewater treatment plant. Thus, emerging larvae are potentially exposed to contaminant mixtures, coinciding with pesticide applications that mix with urban effluent contaminants.

Copper is a contaminant of concern in Californian waterways. It is not only common in urban storm water runoff, and transport off old mining sites, but it is regularly used as a pesticide and fungicide in agricultural areas (Geist et al. 2007). Seasonally fluctuating dissolved Cu concentrations in the Sacramento River have been reported approximating 2 $\mu g.L^{-1}$, however, concentrations in tributaries (e.g. Arcade Creek) have been measured above 6 $\mu g.L^{-1}$ (Domagalski et al. 1998) and have been reported to exceed 500 $\mu g.L^{-1}$ in rice field effluents following application (Huang and Guy 1998). Copper, although essential for multiple cellular proteins, can be toxic to many aquatic organisms, including fish. The mode of action of Cu in several fish species has been reported to involve inhibition of Na^+ channels in gill epithelium, although other mechanisms are likely to be important as well (Handy et al. 2002). Knowledge of the effects of Cu on model organisms is extensive, making this an ideal contaminant to utilize in this biomarker assessment proof-of-concept study, where our aim is to link molecular responses with higher level condition indicators—in this study, swimming performance. In the present study, we describe the effects of Cu on delta smelt at sensitive larval and juvenile developmental stages, the development of molecular biomarkers, and their link with swimming performance. The need for inclusion of molecular biomarkers in monitoring programs is emphasized to understand mechanisms by which contaminants exert effects upon endangered organisms.

Materials and Methods

Fish exposures and swimming assessments

Delta smelt were obtained from the Fish Conservation and Culture Laboratory (FCCL), University of California (UC) Davis (CA, USA) and maintained for a minimum of 24 h in experimental conditions prior to test initiation. Two separate tests were conducted with juveniles and larvae, and used to assess gene expression through microarray and quantitative polymerase chain reaction (qPCR) applications, respectively. Swimming behavior of larval fish was investigated and compared to qPCR responses as detailed below. All experiments and use of test organisms were approved by the UC Davis Institutional Animal Care and Use Committee (Animal Use Protocol for Animal Care and Use 13361). This institution is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare, assurance number A3433-01.

Exposures used for microarray analysis

Juvenile delta smelt (90-d-old) were exposed for 7 d to 5, 10, 25, and 50 $\mu g.L^{-1}$ total Cu (nominal), sourced from $CuCl_2$–dihydrate (American Chemical Society reagent purity ≥99.0%; Sigma-Aldrich). Controls were maintained in diluted well-water; salinity <1 ppt, adjusted to a specific conductance of 450 $\mu S.cm^{-1}$, with deionized water, and a pH of 8.45, using hydrochloric acid. Total water hardness, expressed as $CaCO_3$ was 92 $mg.L^{-1}$, and temperature was maintained at 21°C±1. Juveniles were acclimated to control water for 24 h prior to test initiation. Replicate experimental treatments (n=4) were initiated with 10 juveniles in 7 L of water at 20°C. Fish were fed twice daily with <48-h-old *Artemia franciscana* (Argent Chemical Laboratories). The light:dark cycle was 16:8 h. Approximately 80% of the water in each replicate was renewed at test initiation and on days 2, 4, and 6. At test end, surviving fish were euthanized with MS-222 (tricaine methanesulfonate; Sigma), and snap-frozen in liquid nitrogen and stored at -80°C for subsequent analyses. Comprehensive Environmental Toxicity Information System (CETIS) by Tidepool Scientific Software was used to calculate nominal lethal concentrations. Surviving juveniles from 50.0 $\mu g.L^{-1}$ Cu^{2+} were assessed against controls, utilizing the developed microarrays (see below). Due to mortality at this concentration, three of the four replicates were used, and four surviving fish from each replicate were pooled for this assessment.

Exposures used for qPCR and swimming analyses

Larval delta smelt (47-d-old) were exposed for 4 days to 27, 53, 106, 213 $\mu g.L^{-1}$ total Cu (nominal), sourced from $CuCl_2$–dihydrate (American Chemical Society reagent purity ≥99.0%; Sigma-Aldrich). Controls were maintained in hatchery water with specific conductance of 930 $\mu S.cm^{-1}$ using Instant Ocean (Aquarium Systems), salinity <1 ppt, and pH of 7.9. Total water hardness, expressed as $CaCO_3$ was 100 $mg.L^{-1}$, and temperature was maintained at 17°C±1. Larvae were acclimated to control water for 24 h prior to test initiation. Antibiotics (Maracyn and Maracyn-2, Virbac Animal Health) were added during the acclimation period at concentrations of 5.3 $mg.L^{-1}$ Maracyn (erythromycin) and 0.26 $mg.L^{-1}$ Maracyn-2 (minocycline), to eliminate any gram-positive and gram-negative bacteria, respectively. Fish were fed twice daily with *A. franciscana* (<48-h-old). The light:dark cycle was 16 h:8 h. Approximately 80% of the water in each replicate was renewed at test initiation and on the second exposure day. At test end, a subset of fish were used for swimming assessments, and remaining fish were snap-frozen and stored at -80°C for subsequent biomarker analyses. The CETIS software was used to calculate nominal lethal concentrations. Swimming assessments were performed at test takedown. Fish were placed in rectangular tanks (12 x 6 x 9 cm) containing control water, and allowed to acclimate for 5 min. Threeminute video imaging, recorded in MPEG-2 format, was performed at 30 frames per second using a black and white Panasonic CCTV camera (12V-DC) connected to a laptop computer via a USB framegrabber (Model WinTV-HVR 950). Video analysis was carried out using Ethovision XT (Ver 6.1.326, Noldus Information Technology). Average velocity was determined for each fish by analyzing a total of 72 s per video test. One-way analysis of variance (ANOVA), with Dunnett's multiple comparison *post hoc* tests was used to compare swimming data of exposed treatments to controls. The ANOVA assumptions were verified using F_{max}. Prism 4.0 (Graphpad Software) was used to perform swimming data ANOVA and dose–response correlation analyses.

Experimental physicochemistry

For all exposure tests, water temperature, pH, and DO were measured daily. Water hardness was measured at test initiation, and total ammonia concentrations were measured prior to each water renewal and at test termination. Dissolved Cu analyses were carried out by the Department of Fish and Game—Water Pollution Laboratory (Rancho Cordova, CA, USA).

Microarray application

Development of the delta smelt microarray was described in Connon et al. (2009); briefly, we have constructed a cDNA microarray with 8,448 expressed sequence tags which were pinprinted in duplicate onto epoxysilane-coated glass slides. Total RNA was extracted from whole, individual organisms, using Trizol Reagent (Invitrogen) as per manufacturer's guidelines. Fifteen micrograms of total RNA were used for cDNA synthesis, spiked with *Arabidopsis thaliana* control RNA (SpotReport TM genes: Chlorophyll A-B binding protein [CAB], rubisco activase [RCA], ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit [RBCL], and lipid transfer protein 4 [Ltp4]; Stratagene) and labeled with Alexa fluor dyes, using SuperScriptTM Plus Indirect cDNA Labeling System (Invitrogen). Microarray assessments were carried out using three replicate treatments. Each experimental sample or control was combined with a reference pool cDNA prior to hybridization using an automated Tecan HS4800 hybridization station. Slides were scanned using a GenePix 4000B scanner (Axon Instruments). Microarray data are available for download through the Gene Expression Omnibus repository (http:// www.ncbi.nlm.nih.gov) accession number GSE23238. Normalization and analytical methods are described in Connon et al. (2009) and Loguinov et al. (2004). In brief, print tip normalization was carried out within slides, and sequential single-slide data analysis was carried out as an alternative to between-slide normalization. Anα-outlier-generating model was used to identify differentially expressed genes by applying the following decision rule for multiple-slide data analysis: A given gene was selected as a candidate if it was consistently detected as up- or down-regulated in three of three replicates (raw p value = 0.0625 using exact binomial test and considering outcomes as Bernoulli trials). The approach did not use scale estimation for statistical inference and, due to limited replication, between-slide normalizations were not performed (Loguinov et al. 2004). A higher than usual cut-off point of 0.0625, due to normal microarray normalization stringencies, was used because the purpose of this investigation was to identify genes that could be assessed as probable qPCR–based molecular biomarkers for future monitoring programs (see below). Sequencing was performed at the College of Agriculture and Environmental Sciences' Genomic Facility (UC Davis). Using the Basic Local Alignment Search Tool, translated nucleotide (BLASTx) searches were performed on specific fragments that responded significantly to the exposure treatments. Only genes that were differentially expressed following exposure were sequenced. Sequences were annotated according to homologies to protein database searches using translated nucleotide sequences and direct nucleotide queries (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). Sequences were only annotated if

found to have a BLASTx match with an expectation value smaller than 1 x 10^{-5} and a score above 50.

Functional classifications

Differentially expressed genes were classified according to the Kyoto Encyclopedia of Genes and Genomes (KEGG; http:// www.genome.jp/kegg/kegg2.html) and gene ontology (http:// www.uniprot.org/uniprot), and information gathered from literature, into functional groups. Classification was carried out based on gene expression changes in control and exposed organisms, regardless of whether these were up- or down-regulated. Specific genes of interest were selected for further investigation using qPCR (see below).

Biomarker development

Genes were selected according to level of expression significance, knowledge base from literature, and functional classification. Primer and probes for qPCR analyses were designed using Roche Universal Probe Library Assay Design Center (http://www.roche-applied-science.com). Designed primers were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com), and TaqMan® probes were supplied by Roche. Sequences for all genes assessed by qPCR analyses have been submitted to GenBank (http://www.ncbi.nlm.nih.gov). Primers and probes for investigated biomarkers are detailed in Table 10.

Quantitative PCR

Complementary cDNA was synthesized using 1.0 µg total RNA, with random primers and SuperScript® III reverse transcriptase (Invitrogen), and diluted to a total of 120 µl with nuclease-free water to generate sufficient template for qPCR analysis. TaqMan® Universal PCR Mastermix (Applied Biosystems) was used in qPCR amplifications in a reaction containing 10mM Tris–HCl (pH 8.3), 50mM KCl, 5mM $MgCl_2$, 2.5mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 µl of cDNA sample in a final volume of 12 µl. The samples were placed in 384 well plates and cDNA was amplified in an automated fluorometer (ABI PRISM1 7900 Sequence Detection System; Applied Biosystems). 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. Fluorescence of samples was measured every 7 s, and signals were considered positive if fluorescence intensity exceeded 10 times the standard deviation of the baseline fluorescence (threshold

cycle, CT). The SDS 2.2.1 software (Applied Biosystems) was used to quantify transcription. The geNorm algorithm (Vandesomele et al. 2002) was used to estimate the variability of the reference genes, and to determine an optimal normalization gene. Quantitative PCR data was analyzed using the relative quantification $2^{(-\text{Delta Delta CT})}$ method (Livak and Schmittgen 2002). Expression was calculated relative to β-actin determined by geNorm as the least variable gene in this study. Quantitative PCR data were not normally distributed, therefore, significant differences in gene expression, relative to the unexposed controls, were assessed using two-tailed Mann–Whitney U test, single comparison alpha = 0.05, with Bonferroni's correction experiment-wide alpha = 0.15, treating each gene as a separate experiment. Prism 4.0 (Graphpad Software) was used to perform this analysis along with correlation tests with swimming performance.

Table 10: Molecular biomarkers: Primer and probe sequences used for quantitative-polymerase chain reaction (qPCR) analyses of gene expression in *Hypomesus transpacificus*

Gene	GenBank accesion No.		qPCR primer sequences	Roche probe No.
Myozenin	FJ711583	F	ccaatgtcgtgctggtacacc	106
		R	ctgccagacattgatgtagcca	
Creatine kinase	FJ711584	F	cgatcggcgttggagatg	163
		R	gccaagttcaacgagattctgg	
Sarcoendoplasmatic reticulum Ca ATPase	GU564437	F	catgatcattgggggagca	148
		R	tgctgtgatgacaacgaggac	
α-Actin	GU564441	F	cctgcctcgtcgtactcctg	11
		R	catcctggcttccctgtcc	
m-Calpain	GU564439	F	ccctccgacatgggaagagt	30
		R	accaactatgccttgcccaa	
Aspartoacylase	FJ711577	F	ggaggcacacatgggaatg	109
		R	cttcctctgaatctctgttccattatc	
Hemopexin	FJ711576	F	catgcactacgaggacgacaag	143
		R	tggtagtagctgaacaccttgctg	
Chitinase	GU564440	F	tgtgatcaagttcctccgtcagt	147
		R	ccggggtattcccagtcaat	
α-Amylase	GU564441	F	gatcaccatgttcttgatctgacg	99
		R	ccatcaatcctgaccaaacctg	
Transforming growth factor-β	GU564442	F	caacggcatagtgcatgtgg	76
		R	gaatgtgtgcacgttgttggt	
Tumor necrosis factor-decoy receptor	GU564443	F	ctttttccgctgttccatgttc	2
		R	gttaccagcatacgcagtgtcc	
β-Actin[a]	GU564444	F	tgccacaggactccatacc	12
		R	catcggcaacgagaggtt	

[a] Reference gene

Results and Discussion

Experimental water physicochemistry

Water physicochemical parameters for all tests remained stable throughout the exposures (Table 11), except for a lower ammonia concentration in the 50.0 $\mu g.L^{-1}$ Cu^{2+} 7-d test used for microarray analysis, which was attributed to a lower number of remaining fish due to high mortality. Discrepancy in ammonia linking molecular biomarkers with swimming performance concentrations between exposure sets are attributed to development stage, age, and size differences between juvenile and larval delta smelt. Toxicity of ammonia is influenced by pH, temperature, and salinity, and is known to affect ion transport and membrane permeability, especially important in gills. The present study did not investigate interactions of ammonia with Cu. Water hardness is known to influence Cu toxicity; the California Toxic Rules Criteria and U.S. Environmental Protection Agency National Ambient Water Quality Criteria (NAWQC) for protection of aquatic life (http://www.epa.gov/waterscience) set 9.0 $\mu g.L^{-1}$ Cu^{2+} at 100 $mg.L^{-1}$ $CaCO_3$, thus the concentration chosen for microarray assessments was over four times higher than this criteria. Concentrations presented throughout the remaining sections represent measured dissolved Cu.

Fish exposures and swimming assessments

Exposures used for microarray analyses. Juvenile delta smelt (90-d-old) were highly sensitive to Cu exposure (Table 3), resulting in an estimated 7-d median lethal concentration ($LC_{50\text{-}7d}$) of 17.8 $\mu g.L^{-1}$ Cu^{2+}. In test controls, there was no mortality. Microarray assessments were carried out at 42 $\mu g.L$ Cu^{2+} ; a higher than ambient concentration was chosen to ensure that Cu-specific responses were elicited in exposed juveniles towards biomarker development.

Exposures used for qPCR and swimming video-analyses. Exposure of 47-d-old larval delta smelt to Cu for 4 d resulted in an $LC_{50\text{-}96h}$ = 80.4 $\mu g.L^{-1}$ Cu^{2+} (Table 10). Differences in Cu sensitivity between juvenile and larval exposures were attributed not only to age and size, but also to temperature and conductivity, which are known to affect metal uptake and toxicity (Sorensen 1991). In test controls, there was 93% survival. Due to high mortality resulting at the highest Cu concentration, surviving fish numbers were not sufficient for use in qPCR tests and thus were discarded from further analysis. Video-analysis of larval swimming performance (Figure 16) has indicated an overall effect of Cu exposure correlating with declining velocity in a dose-dependent manner ($r = -0.911$, $p < 0.05$). However, swimming velocity was not statistically different from controls ($p = 0.439$).

Table 11: Physicochemical parameters of control and test waters from 4- and 7-d copper exposures

Treatment	Measured Cu^{2+} ($\mu g.L^{-1}$)	Temp (°C ±1)	pH	EC ($\mu S.cm^{-1}$)	DO ($mg.L^{-1}$)	NH_4/NH_3 ($mg.L^{-1}$)	NH_3 ($mg.L^{-1}$)	Hardness ($CaCO_3$ $mg.L^{-1}$)
Exposures used for microarray analysis (90-d-old juvenile, 7-d exposure)								
Control water	NA	21	8.40	431	8.8	0.28	0.025	92.0
5$\mu g.L^{-1}$ Cu^{2+}	NA	21	8.49	456	8.7	0.24	0.026	NA
10$\mu g.L^{-1}$ Cu^{2+}	NA	21	8.48	461	9	0.23	0.024	NA
25 $\mu g.L^{-1}$ Cu^{2+}	NA	21	8.46	455	8.8	0.37	0.037	NA
50 $\mu g.L^{-1}$ Cu^{2+}	42.0	21	8.39	457	8.9	0.14	0.012	NA
Experiment mean		21	8.44	452	8.84	0.25	0.025	
Exposures used for quantitative polymerase chain reaction and swimming ability analyses (47-d-old larvae, 4-d exposure)								
Control water	1.60	17	7.86	931	9.1	0.025	0	100.0
27 $\mu g.L^{-1}$ Cu^{2+}	24.00	17	7.84	926	9.3	0.037	0.001	NA
53 $\mu g.L^{-1}$ Cu^{2+}	52.40	17	7.89	927	9.4	0.057	0.001	NA
106 $\mu g.L^{-1}$ Cu^{2+}	105.00	17	7.93	931	9.4	0.047	0.001	NA
213 $\mu g.L^{-1}$ Cu^{2+}	213.00	17	7.89	931	9.5	0.033	0.001	NA
Experiment mean		17	7.88	929	9.34	0.039	0.001	

[a] EC = electric conductance; DO = dissolved oxygen; NA = data not available

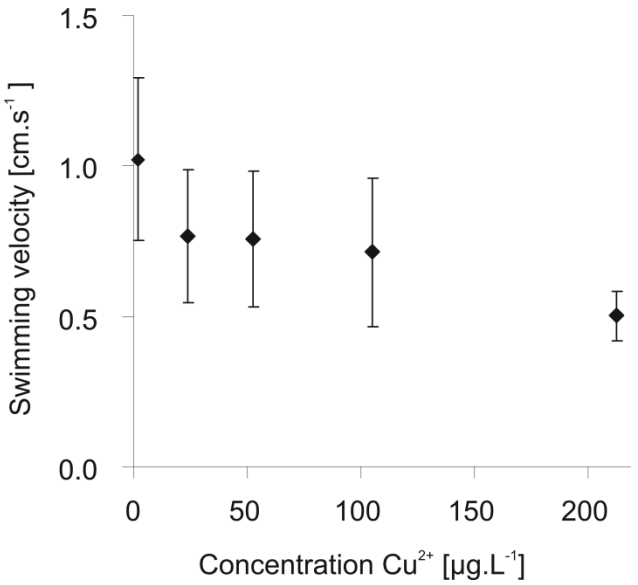

Figure 16: Swimming performance of 47-d-old larval delta smelt exposed for 96 h to 0, 27, 53, 106, and 213 µg.L^{-1} total copper (nominal).

Comparative toxicological data. Differences in the above reported toxicity between juvenile and larval delta smelt (Table 12) are due to exposure duration discrepancies and developmental stage, but variations in experimental conditions, such as differences in water conductivity, pH, and temperature, also contributed to the higher Cu toxicity to juveniles. Furthermore, the larval exposure tests were carried out using antibiotic treatments. Though juvenile delta smelt appear more sensitive to Cu exposure than do larvae, acute toxicity results are thus inconclusive; however, genomic responses have been analyzed successfully in a comparative manner (see below).

Table 12: Acute toxicity data from copper-exposed 90-d-old juvenile and 47-d-old larval *Hypomesus transpacificus* (µg.L^{-1} dissolved copper)

	Endpoint		Juvenile exposure		Larval exposure	
	96-h	95% CI	7-d	95% CI	96-h	95% CI
Control survival	100%	-	100%	-	93%	-
NOEC	8.4	-	8.4	-	53.0	-
LOEC	21	-	21	-	106	-
LC_{10}	9.6	4.2 – 11.4	9	4.2 – 10.3	9.3	27.0 – 77.8
LC_{25}	13.4	10.6 – 18.0	11.7	9.7 – 13.0	44.8	27.0 – 83.1
LC_{50}	25.2	16.4 – 35.4	17.8	14.4 – 22.4	80.4	48.7 – 227.2

[a] NOEC = no-observed-effect concentration; LOEC = lowest-observed-effect concentration; LC_{10}, LC_{25}, and LC_{50} = estimated lethal concentrations percentages; CI = confidence interval; - = data not available.

Microarray responses

Differentially expressed genes resulting from exposure to 42.0 µg.L^{-1} Cu^{2+} are presented in Table 13. A functional classification based on KEGG and gene ontology of up- and down-regulated genes responding to Cu exposure are presented in Figure 17. Copper exposure impacted on neuromuscular activity, affecting muscle integrity and contraction activity (e.g., creatine kinase, myozenin, sarcoendoplasmic reticulum calcium ATPase [SER Ca], titin a), neurological effects resulting in Ca and phosphate signaling (e.g., m-calpain, cyclophilin-a), and nerve maintenance (hemopexin and aspartoacylase [ASPA]). Copper is reported to inhibit iron storage through interaction with peroxidases causing oxidative stress which leads to disruption in Ca homeostasis (Viarengo and Nicotera 1991). Digestion was also affected by Cu exposure, including genes encoding a number of proteins involved in glycolysis, cholesterol efflux, lipid transport, chymotrypsin activity, and proteolysis (e.g., amylase-3, gastric chitinase). Other responses indicate compromised immunity (e.g., tumor necrosis factor [TNF], transforming growth factor- beta [TGF-β]), and cellular homeostasis and tumor malignancy (e.g., vitronectin); changes in expression of these proteins have been implicated in a variety of diseases. Gene classification from KEGG orthology analyses also indicate effects on expression of genes encoding proteins involved in the peroxisome proliferator-activated receptor pathway, receptors that function as transcription factors regulating gene expression, playing an essential role in the regulation of cellular differentiation, development, and metabolism of carbohydrates, lipids, and proteins, and tumorgenesis. This pathway integrates the majority of genes classified into digestion and metabolism.

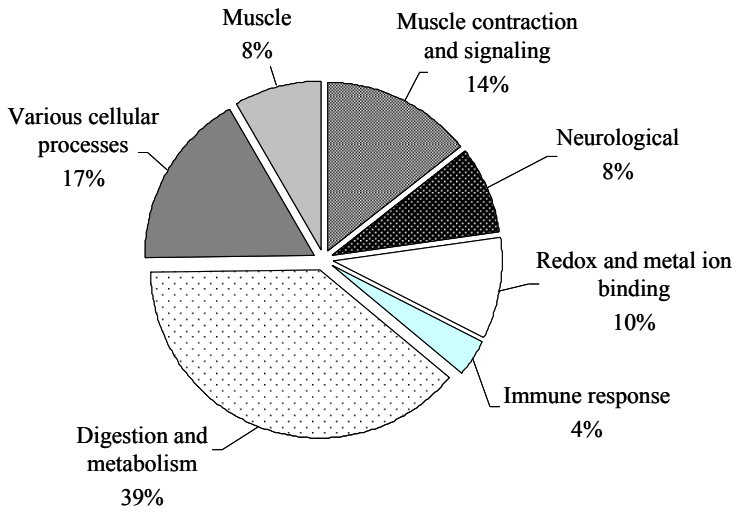

Figure 17: Microarray responses: Systematic analysis of Kyoto Encyclopedia for Genes and Genomes (KEGG) orthology and gene onthology-based functional classification of delta smelt genes significantly differing in juveniles exposed to copper (42.0 µg.L^{-1} Cu^{2+}) for 7 d.

Molecular biomarker responses

Genes were selected according to level of expression significance and to represent the identified functional classifications. Thus, genes involved in muscular, neurological, digestive, and immune system functional groups were further investigated, using qPCR, and assessed as probable biomarkers of copper exposure in *H. transpacificus*. Results confirm microarray identified functional responses.

Quantitative PCR verified copper elicited responses in neuromuscular, digestive, and immune system functions (Figure 18 A-D), with significant differences in expression of muscle a-actin, ASPA, hemopexin, chitinase, and TNF ($p < 0.05$). Remaining assessed genes displayed dose–response relationships, and/or differences in expression trends, but were not statistically significant compared to controls. Although not statistically significant in their expression level, responses from these genes directly facilitate the interpretation of functionally affected systems and are thus interpreted with biological significance. Muscular structure and activity. Muscular structure and integrity appears to have been affected by Cu exposure (Figure 18A), as indicated by effects on contractile muscle systems, α-actin and myozenin. Skeletal a-actin, was significantly down-regulated at all concentrations, correlating with swimming performance ($r = 0.957$). Alpha actin is reported to induce expression of a number of other myogenic genes essential for muscle formation (Gunning et al. 2001), thus it may serve as holistic muscle integrity and functioning effect biomarker. Myozenin is a Z-line, a-actinin- and g-filamin-binding protein expressed predominantly in skeletal muscle, and has been suggested as a biomarker for muscular dystrophy and other neuromusculardisorders Takada et al. 2001).

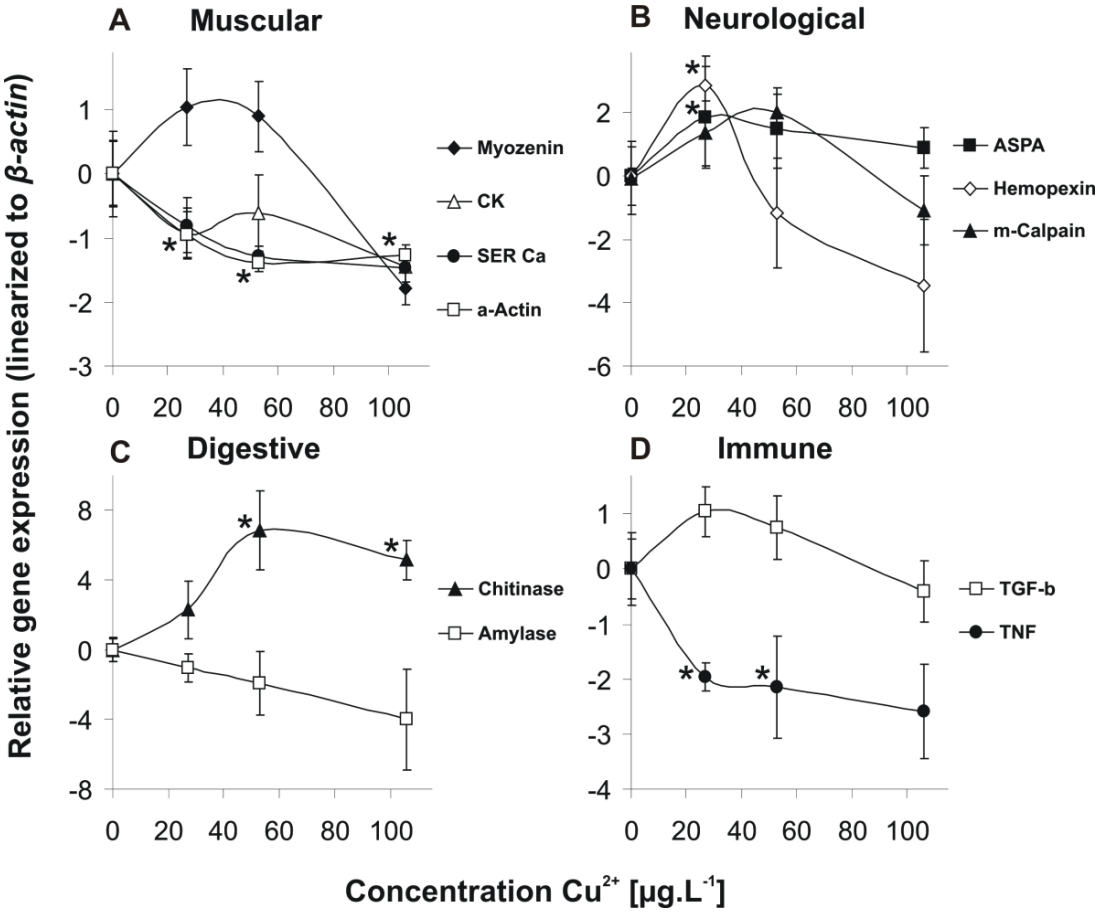

Figure 18: (A-D) Quantitative polymerase chain reaction expression assessments of selected delta smelt genes responding to copper exposed larvae. CK= Creatine kinase; SER Ca=sarcoendoplasmatic reticulum Ca ATPase; ASPA=Aspartoacylase; TGF=transforming growth factor; TNF=tumor necrosis factor. The asterisk (*) indicates tests' significant differences; $p<0.05$ at n=9, 9, 8 and 5 for concentrations 1.6, 24.0, 52.4 and 105.0 µg.L^{-1}, respectively. (Data points have been connected to aid visiualization).

Table 13: Annotation, gene ontology (GO), and regulation of gene expression, in juvenile *Hypomesus transpacificus* exposed to copper (42µg.L^{-1} Cu^{2+})[a,b]

Gene most similar to	Species match	Accesion No.	E-value	Score	GO	Fold-change
Pancreatic protein with two somatomedin B domains	*Paralichthys olivaceus*	BAA88246	2.00E-95	352	GO:0005179	7.54
Cell division cycle 14 homolog A	*Danio rerio*	CAP09233	3.00E-19	99	GO:0004725	5.76
Corticotropin-lipotropin A precurser	*Oncorhynchus mykiss*	Q04617	7.00E-63	244	GO:0005179	5.20
Elastase 2-like protein	*Sparus aurata*	AAT45251	2.00E-89	332	GO:0006508	5.08
Actin alpha 2, skeletal muscle[a]	*Pagrus major*	BAF80060	1.00E-94	384	GO:0003774	4.88
Phoshoglucose isomerase-2	*Plecoglossus altivelis*	BAF91566	1.00E-120	435	GO:0006096	4.86
Apolipoprotein A-I-2 precursor	*Oncorhynchus mykiss*	O57524	4.00E-71	271	GO:0033344	4.81
Pepsinogen A form IIa	*Pseudopleuronectes americanus*	AAD56283	1.00E-105	384	GO:0004194	4.65
Arachidonate 12-lipoxygenase	*Danio rerio*	NP_955912	4.00E-71	112	GO:0004052	4.42
Chitinase 1	*Paralichthys olivaceus*	BAD15059	1.00E-127	458	GO:0004568	4.25
Lipoxygenase 12 R (Predicted: similar to)	*Ornithorhynchus anatinus*	XP_001518171	8.00E-06	55	GO:0016165	4.17
Apolipoprotein Eb	*Danio rerio*	NP_571173	2.00E-38	162	GO:0033344	4.16
SPARC: secreted protein, acidic, rich in cysteine	*Danio rerio*	AAT012113	2.00E-31	139	GO:0006816	4.14
Pepsin A2	*Trematomus bernacchii*	CAD80096	2.00E-88	253	GO:0004194	4.05
Apolipoprotein A-I-1 precursor (Apo-AI-1)	*Oncorhynchus mykiss*	O57523	8.00E-76	286	GO:0033344	3.99
Chymotypsinogen 2-like protein	*Sparus aurata*	AAT45254	1.00E-20	101	GO:0004263	3.93
Myozenin 1[a]	*Danio rerio*	NP_991241	2.00E-25	119	GO:0030346	3.91
NADH dehydrogenase subunit 5	*Osmerus mordax*	ABI35911	1.00E-107	390	GO:0008137	3.88
Astacin-like metallo-protease	*Oryzias latipes*	NP_001098207	2.00E-83	311	GO:0008533	3.87
Hect domain and RLD 4 (Predicted: similar to)	*Danio rerio*	XP_685685	7.00E-76	286	GO:0006512	3.76
Actin, alpha 2, smooth muscle, aorta	*Danio rerio*	AAH75896	1.00E-107	391	GO:0003774	3.75
Chitinase[a]	*Oncorhynchus mykiss*	CAD59687	9.00E-68	260	GO:0004568	3.75
F-type lectin	*Morone saxatilis*	ABB29997	1.00E-46	188	GO:0016467	3.73
Pgk1(phosphoglycerate kinase 1) protein	*Danio rerio*	AAH65888	9.00E-84	313	GO:0006096	3.67
Aldolase a, fructose-bisphosphate	*Danio rerio*	NP_919358	1.00E-124	447	GO:0006096	3.47
NADH dehydrogenase subunit 5	*Osmerus mordax*	ABI35911	5.00E-94	308	GO:0008137	3.47

(Table 13 continued)

Gene most similar to	*Species match*	Accesion No.	E-value	Score	GO	Fold-change
Pepsinogen C (progastricsin)	*Salvelinus fontinalis*	AAG35646	1.00E-107	390	GO:0004194	3.41
Amylase-3 protein	*Tetraodon nigroviridis*	CAC87127	3.00E-54	213	GO:0004556	3.36
Simple type II keratin K8b (S2)	*Oncorhynchus mykiss*	CAA63300	3.00E-74	281	GO:0005882	3.26
Glutamate dehydrogenase 1	*Danio rerio*	NP_955839	1.00E-107	392	GO:0004352	3.24
α-Amylase[a]	*Pseudopleuronectes americanus*	AAF65827	1.00E-144	513	GO:0004556	3.06
Pepsinogen	*Paralichthys olivaceus*	BAC87742	3.00E-77	291	GO:0004194	3.04
NADH dehydrogenase subunit 6	*Salangichthys microdon*	NP_795843	1.00E-107	392	GO:0008137	3.03
Hemopexin[a]	*Danio rerio*	NP_001104617	1.00E-59	233	GO:0046872	3.02
Gamma2-synuclein	*Takifugu rubripes*	NP_001029019	2.00E-41	172	GO:0030424	2.93
Actin, alpha, cardiac muscle 1-like	*Danio rerio*	NP_001001409	1.00E-141	503	GO:0003774	2.92
Cardiac muscle ATP synthase, alpha 1	*Danio rerio*	NP_001070823	7.00E-62	240	GO:0015662	2.91
Selenoprotein P, 1a	*Danio rerio*	NP_840082	1.00E-53	213	GO:0001887	2.86
Intestinal fatty acid-binding protein	*Danio rerio*	AAF00925	3.00E-56	221	GO:0008289	2.82
L-arginine:glycine amidinotransferase	*Danio rerio*	NP_955825	5.00E-83	310	GO:0016740	2.76
Apolipoprotein A-IV	*Danio rerio*	AAH93239	1.00E-73	279	GO:0006869	2.72
Peptidylprolyl isomerase A (cyclophilin)	*Danio rerio*	AAQ91263	2.00E-74	282	GO:0003755	2.66
Histone methyltransferase SmyD1b	*Danio rerio*	ABC54714	1.00E-108	394	GO:0030239	2.62
Sarcoendoplasmatic reticulum calcium ATPase	*Silurus lanzhouensis*	ABG90496	8.00E+79	297	GO:0006937	2.47
1-Acylglycerol-3-phosphate O-acyltransferase 3	*Danio rerio*	NP_998590	4.00E-68	261	GO:0003841	2.36
Chitin-binding peritrophin-A domain	*Danio rerio*	AAH45331	4.00E-69	264	GO:0016490	2.34
Apolipoprotein A-I	*Danio rerio*	NP_571203	1.00E-81	306	GO:0033344	2.28
Calpain 1 protein	*Danio rerio*	AAH91999	2.00E-68	262	GO:0005509	2.27
Apolipoprotein B	*Salmo salar*	CAA57449	3.00E-24	115	GO:0030301	2.23
Sarcoendoplasmatic reticulum calcium ATPase [a]	*Makaira nigricans*	AAB08097	1.00E-83	313	GO:0006937	2.22
Muscle creatine kinase [a]	*Danio rerio*	CAM16434	1.00E-112	406	GO:0004111	2.21
Transmembrane protein 38A	*Danio rerio*	NP_957194	8.00E-81	303	GO:0005267	2.21

(Table 13 continued)

Gene most similar to	*Species match*	Accesion No.	E-value	Score	GO	Fold-change
Tripartite motif-containing 45	*Xenopus tropicalis*	NP_001011026	3.00E-27	125	GO:0046872	2.2
Titin a	*Danio rerio*	ABG48500	3.00E-88	328	GO:0031432	2.19
C1q-like protein	*Dissostichus mawsoni*	ABN45966	3.00E-38	162	GO:0006817	2.17
Apolipoprotein CII	*Oncorhynchus mykiss*	AAG11410	1.00E-19	100	GO:0006869	2.02
Guanine nucleotide-binding protein (G-protein), beta 1	*Danio rerio*	NP_997774	1.00E-117	424	GO:0003924	1.88
Alpha tubulin (protein LOC573122)	*Danio rerio*	NP_001098596	1.00E-120	434	GO:0007018	1.86
DAZAP2-like protein (deleted in azoospermia-associated)	*Takifugu rubripes*	NP_001072102	5.00E-59	230	GO:0030154	1.86
Aacyl-CoA synthetase long-chain family member 5	*Tetraodon nigroviridis*	CAG06540	1.00E-102	375	GO:0004467	1.83
Carboxypeptidase H	*Paralichthys olivaceus*	AAO92752	1.00E-82	309	GO:0004467	1.83
Apolipoprotein	*Tetraodon nigroviridis*	CAG03661	1.00E-38	78	GO:0030301	1.8
Transforming growth factor, beta-induced [a]	*Danio rerio*	NP_878282	3.00E-21	105	GO:0008083	1.59
Neurotransmitter transporter, glycine, member 9 (SLC6A9)	*Danio rerio*	CAM14205	1.00E-100	367	GO:0006836	0.65
Calcium-binding protein 39	*Danio rerio*	NP_998666	1.00E-76	290	GO:0019855	0.63
Cytochrome P450, family 46, subfamily A, polypeptide 1	*Danio rerio*	NP_001018358	2.00E-65	252	GO:0004497	0.63
E3 ubiquitin-protein ligase MARCH2	*Danio rerio*	Q1LVZ2	2.00E-87	325	GO:0006512	0.6
Calcitonin receptor-like receptor	*Oncorhynchus gorbuscha*	CAD48406	5.00E-56	221	GO:0004948	0.59
Dopachrome tautomerase	*Salmo salar*	ABD73808	1.00E-85	318	GO:0016491	0.56
Tetraspanin 7b	*Danio rerio*	NP_001005581	1.00E-110	400	GO:0022857	0.54
Isocitrate dehydrogenase 3 (NAD+) gamma	*Danio rerio*	NP_001017713	2.00E-14	83	GO:0016616	0.53
Cofilin 2 (muscle)	*Danio rerio*	NP_991263	5.00E-84	314	GO:0003779	0.5
m-Calpain [a]	*Oncorhynchus mykiss*	BAD77825	1.00E-108	396	GO:0005509	0.5
Zona pellucida protein X	*Sparus aurata*	AAY21008	1.00E-68	263	GO:0032190	0.5
Suppressor of ypt1	*Danio rerio*	NP_878281	1.00E-122	442	GO:0016192	0.47

(Table 13 continued)

Gene most similar to	*Species match*	Accesion No.	E-value	Score	GO	Fold-change
Thioredoxin-like 1	*Danio rerio*	NP_957432	1.00E-107	391	GO:0045454	0.44
Lactase-phlorizin hydrolase (Predicted: similar to)	*Danio rerio*	XP_001336765	1.00E-110	401	GO:0005975	0.43
Potassium channel tetramerization domain containing 5	*Danio rerio*	NP_996932	2.00E-76	288	GO:0005249	0.39
Zinc finger protein 503	*Danio rerio*	NP_942137	3.00E-63	245	GO:0003676	0.39
Ornithine decarboxylase	*Paralichtyes olivaceus*	AAO92750	9.00E-67	256	GO:0006596	0.35
Proteasome subunit alpha type 7	*Danio rerio*	NP_998331	1.00E-112	409	GO:0030163	0.35
Proteasome (prosome, macropain) 26S subunit, ATPase, 4	*Danio rerio*	AAI53480	1.00E-109	396	GO:0030163	0.34
TNF (tumor necrosis factor) decoy receptor [a]	*Oncorhynchus mykiss*	AAK91758	5.00E-67	257	GO:0004872	0.26
APEX nuclease (apurinic/apyrimidinic endonuclease) 2	*Xenopus tropicalis*	NP_001006804	6.00E-25	118	GO:0006281	0.22

[a]Genes selected for biomarker development.
[b]NAD = nicotinamide adenine dinucleotide; NADH = reduced form of NAD.

Muscle activity was also affected by Cu exposure, altering Ca^{2+} homeostasis, denoted by SER Ca ATPase and creatine kinase-altered expression. Sarcoendoplasmic reticulum Ca ATPase is a muscle calcium ATPase pump responsible for the transfer of calcium from the cytoplasm into the SER after muscle activity (deMeis et al. 2005). It was down-regulated by Cu exposure in a dose-dependent manner ($r = -0.976$) and correlated with resulting swimming performance ($r = 0.965$). This is suggestive of a potential biomarker of muscular activity, indicative of mobility impairments, and likely apoptotic responses. Downregulation of SER Ca ATPase mRNA signifies a decrease in enzyme synthesis, likely causing a decrease in Ca^{2+} in the SER lumen. Disruption of Ca^{2+} homeostasis within the SER has been postulated as an early warning sign of apoptosis, thus inhibition of SER Ca ATPase could lead to cell death (Verkhratsky and Toescu 2003). A further biomarker assessed to measure muscular effects of Cu exposure was creatine kinase. Protein concentrations are used as a diagnosis of diseases like cardiac infarction and skeletal muscle necrosis (Jokusch et al. 1990). It is specifically bound to sarcoendoplasmic reticulum, and regulates Ca uptake and ATP/ ADP ratios (Rossi et al. 1990), thus is directly linked with SER Ca ATPase and involved in muscle activity. Though not statistically significant, creatine kinase expression was also reduced by increasing Cu concentration, in a similar manner displayed by SER Ca and α-actin, suggesting a decline in Ca regulation and overall energetic activity. Neurological activity. Copper exposure is known to affect the nervous system through the formation of reactive radicals (Viarengo and Nicotera 1991), an effect that was sustained in this study (Figure 18B), through expression of hemopexin, which was significantly up-regulated at lower concentrations. Hemopexin is synthesized by Schwann cells following nerve injury, has been reported in the peripheral nervous system, and is specifically regulated during repair (Swerts et al. 1992). The measured downregulation at higher concentrations could imply inhibition of repair mechanisms. Wallerian degeneration generally occurs following axonal injury and is critical for its repair. This is characterized by axonal and myelin degeneration, thus ASPA, a gene identified in a previous study (Connon et al. 2009), was investigated because it is expressed in myelin sheaths and involved in their maintenance. It was chosen to further assess neurological damage, because of its functional proximity to hemopexin, however, ASPA did not respond significantly to Cu exposure but displayed an increase at lower concentrations, supporting measured differences in hemopexin transcription. Both hemopexin and ASPA expression were elevated at lower concentrations and reduced at higher concentrations. Effects of Cu exposure on Ca availability were further corroborated by changes in m-calpain expression (Fig. 18B). m-Calpain is a Ca-dependent cysteine protease, known to co-localize with a

calcium-sensing receptor, where calcium not only activates the m-calpain enzyme, but also causes it to undergo autolysis through subunit dissociation (Kifor et al. 2003). The physiological roles of calpains are still poorly understood; however, they have been shown to control cell fusions in myoblasts, playing an important role during myogenesis and thus muscle regeneration (Raynaud et al. 2004). Interestingly, activation of m-calpain in the peripheral nervous system has been also reported to be involved in Wallerian degeneration, with increased expression being initiated following nerve injury (Glass et al. 2002), which was also indicated by changes in ASPA expression levels (see above). Thus, m-calpain is a potential biomarker of neuromuscular activity and, as such, clustered into both muscular and neurological responses. However, due to high variability in control subjects, and the low number of replicates available in this study, changes in expression were not significantly different in this test. Digestion. Copper exposure resulted in significant responses in transcription of genes involved in delta smelt digestion (Figure 18C). Alpha-amylase is a digestive enzyme that hydrolyzes starch into maltose (Englyst and Cummings 1985). It was down-regulated, significantly correlating ($r = 0.978$) with increasing Cu concentration. Downregulation of α-amylase transcription has previously been associated with Cu exposure (Lewis and Keller 2009). Chitinase, an enzyme required in the digestion of chitin structures in the exoskeletons of crustaceans and many insects, was significantly up-regulated on exposure to Cu. Kurokawa et al. (2004) have demonstrated that fish express chitinase in their guts, thus hypothesized to be involved in arthropod digestion, and also in the defense against Gram-positive bacteria and fungal pathogens. Immune responses. Microarray analysis also identified effects upon the immune system that were confirmed through qPCR (Figure 18D). Tumor necrosis factor, a proinflammatory cytokine, was significantly down-regulated by copper exposure, indicating a compromised immune system. Produced in many cell types, TNF plays an important role in immunity and inflammation, and in the control of cell proliferation, differentiation, and apoptosis; its downregulation has been implicated with various diseases in humans, such as Crohn's, arthritis, multiple sclerosis, and Alzheimer's (Balkwill et al. 2000). Balkwill et al. (2000) indicate that TNF knock-out causes autoreactive T cells' regulation, resulting epitope spreading, thus leading to a state of disease. Transforming growth factor-β is an anti-inflammatory cytokine expressed functionally during development, and in tissue maintenance and homeostasis, regulating proliferation and differentiation, cell survival, and apoptosis (Bottner et al. 2000). Up-regulation of TGF-β has been linked with neurodegenerative diseases and ischemic injuries (Lippa et al. 1995); interestingly, it is reported to induce muscle α-actin expression (Desmouliere et al. 1993), which was down-

regulated by Cu exposure. Transforming growth factor-β displayed an upregulation at lower levels of Cu exposure, in similar trends observed in ASPA and hemopexin, further suggesting immune responses resulting from probable neurological or signaling impairments. Molecular biomarkers in monitoring programs. Molecular biomarkers have, for many years, successfully been used in human medicine as diagnostic tools, for example, the assessment of prostate specific antigen gene expression as an indicator of prostate cancer (Dixon et al. 2001), and are increasingly being used in the pharmaceutical arena to assess the mode of action, safety, efficiency, and targeted effects of novel and developed drugs (Amir-Aslani and Mangematin 2009). Research in ecotoxicology has been concentrating on attributing or defining an ecological relevance to responses measured using biomarkers, through measuring responses at the individual level and attempting to extrapolate results to population responses. This work has been the subject of extensive critical discussion that has successfully strengthened biomarker research, but has concurrently discouraged widespread application in field studies. However, a limited number of researchers have successfully applied molecular biomarkers in field studies, identifying contaminant stress-induced responses (McClain et al. 2003) and metal-contamination effects (Perceval et al. 2004) in various field-collected aquatic organisms. In most aquatic organisms, and particularly in fish, a number of life-cycle characteristics are dependent on swimming behavior, from respiration to reproduction, thus swimming performance in fish is likely the single most significant environmentally relevant parameter that can be measured because it is indicative of effects upon behavior. Thus, effects upon individual fish, however minimal, will reduce reproductive success of a population. Neuromuscular alterations will likely have significant effects on swimming performance. In this study, we utilize a suite of molecular biomarkers, designed specifically to address neuromuscular disturbances, in an attempt to indicate, allow interpretation of, and correlate stressor modes of action that may impinge on swimming ability. The use of biomarkers to specifically address key health parameters have extensively been investigated, through proteomics, a global genomics approach, or with the application of molecular probes, such as neuromuscular activity, digestion, and immune responses, as in this study, or with the addition of developmental assessments, such as links with endocrine responses, growth, and sexual development. In the search for biomarkers of effect and exposure, it is traditional to ascertain as useful only those whose functional responses correlate with exposure concentration. However, hormetic or biphasic dose responses appear to be indicative of changes in homeostasis (Calabrese 2008), and as such should be treated as an invaluable technique to identify concentrations at which organisms can no longer compensate adequately

to exposure. It is our contention that biphasic responses can therefore be more informative than dose-responsive biomarkers, which are solely indicative of exposures, without identifying lowest concentrations at which detrimental effects may occur. Thus, a suite of biomarkers, both biphasic and dose-respondent, should be utilized in conjunction to elucidate effects upon an organism. Previously, we have demonstrated the ability to link neurological effects from pyrethroid exposures in the delta smelt, with changes in gene expression that correlated with swimming behavior (Connon et al. 2009). To attribute environmental relevance to molecular biomarkers, research should concentrate on their application in field studies with the aim of developing monitoring programs. Only through the inclusion of these techniques in monitoring programs, can biomarkers truly be evaluated.

Molecular biomarkers in the delta smelt.

Delta smelt are highly sensitive to handling and extremely difficult to work with under laboratory conditions, and these studies are not necessarily informative of what the organisms are exposed to in their habitat. Thus, reliable biomarkers for determining the health status and exposure history of delta smelt in field-based studies are essential. Molecular biomarkers such as those identified in this study, could therefore be coupled to, and carried out in collaboration with, the Californian Department of Fish and Game annual townet surveys and monitoring programs (http://www.dfg.ca.gov/delta), thus creating an informative database of genomic responses indicative of delta smelt health status, aiding the process of toxicity identification and evaluation through the identification of contaminant-specific responses within complex chemical mixtures. Baseline gene expression, for comparative purposes, could be generated from assessing hatchery-raised larval, juvenile, and adult delta smelt, alongside temporal, site-specific variations in townet-surveyed wild fish.

Recommendations for field-based monitoring studies.

A number of delta smelt genes, identified in this and previous studies (Connon et al. 2009), have been demonstrated as specific and informative biomarkers to warrant their application in field studies. Utilizing the molecular biomarkers identified this far, we are able to identify and further understand neuromuscular effects resulting from copper and pesticide exposures. Combining gene expression changes in aspartoacylase and hemopexin, for example, has proven successful in the identification of neurological insults, suggesting degradation of axon myelin sheaths and nerve repair (Connon et al. 2009). Biphasic changes in expression seemingly differentiate homeostatic responses, allowing repair mechanisms, from

concentrations that are chronically detrimental, as implied by aspartoacylase and hemopexin responses in this study. Effects of Cu on muscular activity and integrity can also be ascertained through the resulting downregulation of SER Ca ATPase and α-actin, respectively. Furthermore, the health status of an organism can be assessed in terms of digestion capacity and immune system functioning. Additional to the qPCR–based biomarkers already described, a number of prospective genes have been identified with the microarray application and will be isolated from future studies, to expand genomic information towards a suite of functionally classified biomarkers to be included in monitoring programs, and identify classes of contaminants present in the delta smelt habitat range that may be responsible for toxicity. Thus, the presently assessed suite of biomarkers could be applied to field studies, comprising site-specific collected water-sample exposures within laboratory conditions, as well as upon wild specimens caught during townet surveys.

Linking molecular responses to swimming performance.

The short-term Cu exposure (4 d) in this study resulted in an overall decrease in swimming velocity in larval delta smelt with increasing concentration. Though not statistically significant to controls, the reduction in swimming activity could possibly be explained by alterations measured by all neuromuscular molecular biomarkers, because these indicate the mechanisms of action of Cu upon the delta smelt. SER Ca ATPase was a particularly informative gene, because the measured downregulation supports indications of interference of Cu with Ca^{2+} homeostasis, neurological signaling, and muscle activity. The strong downregulation of α-actin, at all concentrations, sustains reported effects of Cu on contractile muscle proteins (Kurokawa et al. 2004), further supported by increases in m-calpain expression, involved in muscle regeneration (Raynaud et al. 2004). Food consumption was not measured in this test; thus it is not clear from this study whether the measured digestive changes are directly due to Cu exposure acting upon specific enzymes, or whether this is a result of altered swimming performance impinging on prey capture and ingestion. Under unexposed conditions, elevated levels of chitinase would likely signify higher levels of ingestion. It is unlikely that Cu-exposed fish had a higher consumption level; thus our contention is that Cu directly acts upon digestion, either impeding the proteolytic process through synthesis inhibition, enzyme degradation, or mRNA expression inhibition, as indicated through amylase expression. Further responses to Cu exposure indicate a compromised immune system, with specific links to the central nervous system, as suggested by the measured upregulation of TGF-β, reported to be linked to neurodegenerative diseases

(Lippa et al. 1995); its involvement in the induction of contractile muscle protein (Desmouliere et al. 1993) further supports neuromuscular damage.

Conclusions

Copper concentrations used in this study, though high, are not uncommon in Californian surface waters (Desmouliere et al. 1993), especially the lowest concentration investigated. Results from this study are indicative of short-term exposure responses. Bioaccumulation properties of heavy metals are well researched (Poynton et al. 2008), and we extrapolate that longer-term exposures to lower levels of Cu are likely to have detrimental effects on swimming performance and alter the overall chances of delta smelt survival in the wild. Indisputably, the primary reason for the decline in number of pelagic organisms in the Sacramento–San Joaquin Delta is directly related to water exports (Sommer et al. 2007). However, organisms that manage to survive this habitat destruction are exposed to elevated concentrations of contaminant resulting from industrial, agricultural, and urban pollution, and lower water flows combined with a lesser dilution rate. Management systems to monitor the extent of change resulting from anthropogenic loads are essential, and this study enhances the argument for the use of a suite of molecular biomarkers as a successful approach towards identifying effects and causal factors of species decline.

DISCUSSION

Freshwater systems worldwide are influenced by industrial and natural chemical compounds representing one of the key environmental problems for human societies (Schwarzenbach et al. 2006). A high proportion of these compounds are present at low concentrations, which demands suitable and sensitive tools for the assessment of possible harmful toxicological effects in the respective systems and the organisms therein. With the results presented in chapters I to IV, it could be demonstrated that sublethal stress responses in larval fish can be measured at different levels of biological organization with high sensitivity. Sublethal insecticide exposures resulted in molecular, physiological and behavioral alterations that can be interpreted as mostly compensatory or adaptive mechanisms that allow the maintenance of homeostasis after short exposure times. It could also be show that long-term consequences of these immediate alterations can only be interpreted in time-series experiments. It is a general assumption in ecotoxicology that the magnitude of the exposure concentration, or dose, is in a tight relationship with the subsequent consequences for example for an individual organism (Fent 2003). Substances that might be beneficial at low concentrations or even essential for biological function, such as nutrients or trace metals, can exhibit negative effects for a specific organisms if the substance- and organism-specific threshold concentration is exceeded. In a more global context, the adversity of a disturbance is dependant on the systems' ability to react to altered conditions and recover or adapt. Disturbances become deleterious if no recovery is possible and the system is irreversibly damaged. Such conditions are typically measured in acute exposure experiments, with high stressor intensity or in chronic tests, when the exposure to the stressor is long-term or permanent. In contrast, obvious negative outcomes in experiments that mainly focus on mortality as an endpoint, are unlikely observed in sublethal exposure scenarios. Especially genomic endpoints, such as the measurement of gene transcript alterations, need to be interpreted regarding their biological meaning by the identification of adverse stress responses at higher levels of biological organization.

As summarized in Forbes and Calow (1997), the definition of stress is either referring to the factors that cause stress or the referring to the effects caused by it, dependent on the point of view. According to Sibly and Calow (1989) stress is the "environmental condition that, when first applied, impairs Darwinian fitness". Hofmann and Parsons (1991) defined stress as "an

environmental factor causing a change in a biological system which is potentially injurious.". A more specific definition was given by Bayne (1975), defining stress as "a measureable alteration of a physiological (or behavioral, biochemical or cytological) steady state which is induced by an environmental change, and which renders the individual (or the population, or the community) more vulnerable to further environmental change." All these definitions are more or less precise, depending on the overall scope. The definition provided by Bayne (1975) can be seen as the most appropriate one regarding the type of experiments that are presented within this thesis. The steady-state of a certain system, regardless of cellular, organismal or higher-level, is under constant change, however, for the proper functioning it is important that the system itself is able to maintain this status. For true risk assessment of "stress" caused by chemical impact it is important to determine at which magnitude of the stressor the resilience is exceeded. The results presented in this thesis confirm that the complementary assessment of sublethal endpoints on behavior and gene transcription is suitable for stress threshold determination .

In chapter I and II it was demonstrated how the exposure to insecticides results in acute negative effects on swimming behavior in larval fish being negatively correlated to exposure concentration for all tested chemicals. Thus the assessment of behavioral endpoints provides highly relevant information for contaminant effects, as alterations in behavior directly reflect outcomes at the individual level of biological organization being a result or even a determinant of molecular, physiological, and ecological aspects of toxicology (Scott and Sloman 2004). There is a broad spectrum of behavioral traits that can be considered, ranging from basic reactions that are easily measured to more complex behaviors. Most commonly measured behaviors include swimming activity, avoidance reactions - either to a chemical gradient or to a predator-, feeding activity, foraging behavior, reproductive and social behavior (Garcia-Reyero 2011, Floyd et al. 2008, Weis et al. 1999, Little and Finger 1990). In comparison to molecular or physiological endpoints, there are several often stated advantages of behavioral endpoints: (i) behavioral responses are an integrated effect of the underlying biochemical and physiological disturbances, (ii) behavioral responses are more sensitive than other approaches, and (iii) certain behavioral responses can be energetically linked to higher level responses (Little and Finger 1990). Despite this, the integration of behavioral indicators of toxicity with those of other levels allows a better interpretation of toxicological effects in natural systems. Especially insecticides with neurotoxic modes of action have been shown to negatively affect swimming ability and predator avoidance in fish (Floyd et al. 2008, Heath et al. 1993b, Little and Finger, 1990). This is of special ecological importance during early life

stages when fish are highly vulnerable to predation. Inability to swim properly after a brief exposure to insecticides therefore negatively affects individual fitness and survival, with potential consequences at the population level (Little and Finger, 1990). The magnitude of the initial neurotoxic impact of the tested substances bifenthrin and fipronil became obvious by using individual swimming performance as an endpoint. This response was generally supported by the transcriptomic regulation of genes that can be functionally related to neuromuscular processes. Neuromuscular responses, studied in a selected number of genes, indicated effects on fish motility and can be considered part of the underlying mechanisms for the observed impairment in swimming ability. The comparison of the different substance classes of insecticides thereby showed a more pronounced effect for the pyrethroid bifenthrin after the 24 h exposure period in the tested fish species. The gene coding for the aspartoacylase (*aspa*) enzyme in the vertebrate brain was thereby confirmed as a suitable marker for neurotoxicity. These findings are of special importance, since differential regulation of *aspa* is also described for other fish species. Connon et al. (2009) observed significant down-regulation of *aspa* in the delta smelt (*Hypomesus transpacificus*) after exposure to sublethal concentrations of the pyrethroid insecticide esfenvalerate. Another gene, creatine kinase *(ck)*, as part of the creatine/phosphocreatine/creatine kinase system plays an important role in the cellular energy metabolism and energy homeostasis in the vertebrate central nervous system. It is also associated with the sarcoplasmatic reticulum and involved in muscle contraction by regulation of ion fluxes, especially Ca^{2+} uptake and ATP/ADP ratios (Rossi et al., 1990). In combination, the regulation of the genes named above amongst others involved in the Ca^{2+} signaling pathway indicate a strong link between Ca^{2+} regulation and energy in the context of proper muscle function and the decline in measured swimming performance after exposure to neurotoxicants.

However, despite this ecologically important reduction in individual fitness of the tested fish, it could also be demonstrated that allowing a certain recovery time, the impairment of swimming performance decreased over time, especially in case of the pyrethroid bifenthrin. Results from the fipronil exposed fish showed less pronounced alterations on the transcriptome level, although the impairment of swimming performance appeared to be similar to the pyrethroid and occurred at a comparable concentration level in relation to mortality (Beggel et al. 2010). The consideration of genomic responses in contrast provides more detailed insight in the different modes of action of toxicity, which can be of critical importance for the estimation of long-term consequences such as developmental alterations.

As demonstrated in the chapters above fish can recover, but in a field situation, not being able to feed or evade predators for a certain period of time, will likely lead to negative impacts on individual survival and population dynamics. In contrast to these reversible effects on individual performance, developmental alterations can have even more ecologically relevant impacts at the population level. Exposure to pure fipronil enhanced growth of larval fathead minnow, while its formulated form, Termidor® did not produce this effect. Enhanced growth following exposure to fipronil has not been previously reported and causative factors could be explained by endocrine disruptive properties which are supported by the significant up-regulation of *vtg*. We observed a significant up-regulation of the egg-yolk precursor *vtg* and - to a lower extend - of *igf* immediately after the 24 h exposure at high fipronil concentrations. At lower concentrations, the same effect appeared during the recovery period, suggesting strong interference of fipronil with ontogenetic development. These observations clearly confirm that acute exposures can induce chronic effects even at low concentrations.

A linear dose-dependent response is often typical if relatively high exposure concentrations are used and mortality is used as an endpoint. In studies where concentrations at a sublethal, and oftentimes more environmentally realistic, level are utilized, the response curve shows a biphasic pattern (Connon et al. 2008). Results from the studies presented here and from other work from the literature confirms that with transcriptomic approaches a linear dose response relationship is not generally occurring (e.g. Heckmann et al. 2008). Typically, transcription profiles of low dose exposures differ from higher doses which is, however depending on the concentration range tested, often described as a biphasic or even multiphasic responses with increasing treatment concentration. This can be considered a typical hormesis response as discussed in more detail in chapter III. It was already stated that this pattern of response is controversially discussed in the literature, however it seems to be a principal response in biological systems (Chapman 2002, Kefford et al. 2008). Moreover, toxicity is also a process in time (Heckmann 2010) and has to be considered for the interpretation of biomarkers. It is of great importance to distinguish between normal adaptive responses in gene transcription and those that are associated with damage of the organism (Steinberg et al. 2008). This can only accomplished by a combination of different endpoints, such as those used here. A dose-related biomarker response can serve as an indicator of exposure, but with the hormetic change in gene transcript regulation that leads to detrimental effects on the whole-organism level a more valuable prediction of environmental relevance is evident. As shown in chapter III and IV, biphasic responses can be more informative than dose-responsive biomarkers, as those are solely indicative of exposures, without identifying lowest concentrations at which

detrimental effects may occur. The success behind the use of molecular biomarkers as early and sensitive warning tools lies in interpreting biomarker responses in individuals in the context of affected cellular pathways, integrated with extensive life history knowledge of the respective organism. This requirement is especially true when assessing effects on non-model species, or organisms living in ecological systems where sensitivity to stressors could greatly differ from model organisms. To attribute environmental relevance to molecular biomarkers, research should concentrate on their application in field studies with the aim of developing adaptive monitoring programs (Connon et al. 2011).

Gene transcription profiling showed to be a very sensitive and useful method to detect sublethal responses in organisms after chemical exposure, here exemplarily demonstrated for larval fish. In recent years there is an increasing research focus on utilizing integrative approaches in order to characterize sublethal chemical effects with complementary investigations of endpoints at different levels of biological organization (Beggel et al. 2011, Connon et al. 2009, Heckmann et al. 2008, Miller et al. 2006, Swain et al. 2010). The application of such methods can be most suitable for the prediction or determination of the actual status in environmental health, as real-time information is provided in opposite to more retrospective information of species composition analysis (Klaper and Thomas 2004). For regulatory purposes the use of transcriptomic techniques will also become necessary to have the best available science as the foundation for policy making (Hook 2010). The main goal in the application of transcriptomic techniques for risk assessment is the identification of transcript changes that are associated with the fitness, or performance in the organism of interest. The measurement of homeostatic adjustments does not possess the predictive power of the relationship with possible negative consequences on higher levels of organization. Therefore the link to adverse outcomes has to be established for a confound risk assessment. One approach that addresses the mechanistic link between gene transcript responses and relevant higher level effects was suggested by Ankley et al. (2010). The “Adverse Outcome Pathway” (AOP) approach represents a conceptual framework that displays the linkage or relationship between a molecular initiating event and an adverse outcome. Relationships among levels of biological organization may be causal, mechanistic, interferential or correlative. The AOP mainly focuses on the estrogen receptor which regulates known biochemical pathways that are consequently leading to negative effects on loss of fitness and reproduction. This approach has already been successfully applied for the relatively well understood pathways of endocrine signaling. However, for chemicals affecting other, less

well studied, metabolic pathways there is still a high demand in the identification of key components. Furthermore, the application in field monitoring studies is relatively scarce.

Conclusions

The presented results demonstrate that the use of gene transcription changes in a small set of molecular markers is a fast and highly sensitive tool to detect chemical stress in larval fish. Changes in gene transcription after short-term and sublethal exposure to the studied insecticidal chemicals were measured at concentrations as low as 10% of the respective 24 h LC_{10}, being half the concentration at which higher level of biological organization responses, like swimming performance, could be measured. Results suggest that these neurotoxic insecticides can decrease ecological fitness of sensitive aquatic species at concentrations far below the lethal level. Carefully selected markers can deliver sensitive signals about perturbations in the organism of interest. Most appropriate analysis for effect characterization in this study was a multivariate approach to reveal temporal patterns of transcriptomic responses that corresponded with developmental alterations on the whole-organism level. Yet, interpretations about specific changes in gene transcription require caution, as initial hormetic responses are early warning signals, but may not allow the prediction of stressor effects at higher concentration levels. Therefore the combination of molecular and whole organism endpoints for investigations of biomarker suitability is highly recommended. It could be shown that adverse neuromuscular effects of insecticides are reversible at low concentrations and the organism is able to recover from the resulting behavioral impairment given the time and environmental condition to do so. However, the results indicate that even short acute exposures can result in significant effects on the transcription of endocrine-related genes and induce chronic effects. Depending on the respective signaling pathway, specific effects might not immediately be captured by the assessment of transcriptomic responses at a single time point. More than one time point is important in effect assessment, especially if responses observed on the transcription level are used to predict or estimate adverse effect on higher levels of biological organization. In a field context, endangered organisms often inhabit habitats that are influenced by various natural, physical or chemical such as elevated concentrations of contaminant resulting from industrial, agricultural, and urban pollution. Management systems to monitor the extent of impacts on aquatic organisms resulting from anthropogenic loads in the environment are essential. Future risk assessments should include additional safety factors to account for the greater toxicity of formulated pesticide products. Furthermore, the studies presented here enhance the argument for the use of a suite of molecular biomarkers as a successful approach towards identifying effects and causal factors of species decline.

Outlook, further research

The determination of thresholds is still a major concept in ecotoxicology and forms the basis for legislation, which concentration can be seen as still tolerable in natural environments. This threshold determination however has already been questioned back in 1974 by Woddwell, who wrote: “is it reasonable to assume that thresholds for effect of disturbance exist in natural ecosystems or are all disturbances effective, cumulative, and detrimental to the normal functioning of natural ecosystems?” As stated earlier, the detection of changes in homeostasis alone is not providing information about negative consequences. Stress responses act as evolutionary mechanisms for compensation or even adaptation to environmental changes. Ecological concepts like homeostasis or even allostasis describe the dynamic reactions in a biological system in response to biotic and abiotic alterations of environmental conditions. Diurnal, seasonal or annual adjustments to changes in temperature, oxygen, turbidity or salinity are often essential for an organism’s proper function and survival. As pointed out in the chapters above, measurements of biomarker responses have to be considered regarding their ecological relevance to avoid either under- or overestimation of toxicity. Therefore, future ecotoxicological research has to incorporate the mechanistic bases of stress responses into a broader ecological context for the understanding how disturbances affect populations and ecosystem functioning. This has to include the effects of energetic and dominance status of an individual, as well as the influence of pathogens, parasites and even predator cues. Future challenges will also lie in the analysis of chemical mixture effects including transformation products, representing the most likely and nowadays most environmentally realistic scenario. In this special case the application of genomic tools such as the generation of transcriptomic profiles as stressor specific fingerprints can be included in the integrative approach presented here. The holistic understanding of the mechanisms that drive adverse outcomes is most essential for a full functional ecological risk assessment.

Acknowledgements

The work was conducted under supervision by Prof. Dr. Jürgen Geist, head of the Aquatic Systems Biology Chair, Department of Ecology and Ecosystems Management at the Wissenschaftszentrum Weihenstephan, Technische Universität München, Germany. Major parts of this work were conducted under supervision by Dr. Inge Werner and Dr. Richard Connon at the Aquatic Toxicology Laboratory, School of Veterinary Medicine, Department of Anatomy, Physiology and Cell Biology at the University of California, Davis, USA. All experiments and use of test organisms were approved by the UC Davis Institutional Animal Care and Use Committee (Animal Use Protocol for Animal Care and Use 13361).

The work has been funded by a postgraduate scholarship to Sebastian Beggel (Contract No. DOK-82-07 to J. Geist) by the Bayerische Forschungsstiftung and the Interagency Ecological Program, Sacramento, California (Contract No. 4600008070 to I. Werner).

This work would not have been possible without the help and support of several people that deserve my highest appreciation. I would like to thank my committee, Prof. Dr. Jürgen Geist and Prof. Dr. Arnulf Melzer and Prof. Dr. Ingrid Kögel-Knabner for the evaluation of this thesis. Special Thanks deserve Dr. Inge Werner and Dr. Richard Connon for hosting, all their experience and support during my learning phases and also, if not of most importance, for being good friends. From the Aquatic Toxicology Laboratory my special thanks deserve Linda Deanovic, Marie Stillway, Joy Kamphangan, Nathan Offer, Dan Markiewitz and the rest of this amazing team, and of course, Leandro D'Abronzo, which always provided a helping hand. I also would like to thank Prof. Josef J. Cech and Dr. Robert Kaufman, who had significant influence on my choice of swimming behavior as individual endpoint. Thanks also to Prof. Nancy Denslow, University of Florida, for her advice and provision of *Pimephales promelas* RNA sequence information that was not available from the database. I would like to thank Beeka Hudson-Davies, Petra Kunz, Janice Pfeiff and Eric Durieux for being awesome colleagues, roommates and friends. Most of all I thank my mother Alice, my father Jürgen, my sister Simone, my girlfriend Ingrid and the rest of all those I consider family and friends for their support and love during the time I stayed abroad.

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