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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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 g.L⁻¹, highly significant changes in gene transcription were observed for aspartoacylase, metallothionein, glutathione peroxidase, 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 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.

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1. 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; 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 g.L⁻¹ (Gan et al., 2012). In agriculture, fipronil is commonly applied in rice cultivation with values ≥39 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 affinities of fipronil are 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; Rafflesiaque) 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 ≥31 μg.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 (zpg3), 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 metallothionine, mt).

2. Material and methods

2.1. 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 (electric conductivity (EC): 265–293 μS cm−1; hardness: 80–100 as mg CaCO3 L−1; alkalinity: 57–64 as mg CaCO3 L−1 (USEPA, 2002)). 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, 2002). 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.

2.2. Insecticide exposure

Reference standard grade fipronil (5-amino-1 [2,6-dichloro-4-(trifluoromethyl) phenyl]-4 [(trifluoromethyl)sulfonyl]-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 LC10 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 μg.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 μg.L−1 (reporting limit; recovery 83.1%). Measured concentrations were 53, 142, 333 and 365 μg.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 tricaine 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.

2.3. 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.

2.4. 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 (zpg3) were used as indicators of estrogenic and xenobiotic stress (Genovese et al., 2011; Sumpter and Jobling, 1995). Insulin-like growth factor (igf), growth hormone

(gr) and glucocorticoid receptor (ger) regulate several physiological functions, such as growth and development (Filby and Tyler, 2007; Leitherland 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 Ca2+ signaling (Coughlin et al., 2007; Rossi et al., 1990) and aspartoacylase (aspa), for example involved in myelin sheath integrity in nerve cells (Connon et al., 2009). Target genes also included biomarkers for xenobiotic detoxification and general stress response (cytochromes P450 1A and 3A12, 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 (e1a) 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 1.

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 MgCl2, 2.5 mM deoxynucleotide triphosphates, 0.625U AmpliTaq Gold DNA polymerase per reaction, 0.25U AmpliTaq Gold DNA polymerase per reaction, 0.25U AmpliTrip 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.

### 2.5. Data analysis

We used the geNorm algorithm to estimate the variability of 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−ΔΔCT method (Livak and Schmittgen, 2001). Transcription levels were calculated relative to elongation factor 1-alpha (e1a) 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 e1a. 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 (± standard error) 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).

### 3. Results

#### 3.1. Single gene transcript responses (24 h)

Effects resulting in differential transcription of individual genes were observed at all test concentrations below 50% of the nominal LC10 and significant responses in gene transcription were observed starting at the lowest exposure concentration of 31 μg.L−1 fipronil (10% LC10, 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 (Fig. 1).

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 μg.L−1 fipronil, nominal concentration, 10% LC10). 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 μg.L−1, nominal concentration, 50% LC10). This was even more pronounced for mt, where significant up-regulation was seen at the two highest exposure concentrations (102 μg.L−1, nominal concentration, 33% LC10, p = 0.011 and 153 μg.L−1, nominal concentration, 50% LC10, p<0.001). Fipronil exposure also caused down-

### Table 1

Changes in mean gene transcription levels and standard errors (S.E., n = 15) of fathead minnows exposed to control water, 31 μg.L−1 and 61 μg.L−1 fipronil for 24 h then allowed to recover in control water. Values are expressed as n-fold changes, normalized to e1a gene transcription at three time points: 24 h (exposure period); 48 h (24 h exposure, 24 h recovery); 7 days (24 h exposure, 6 days of recovery).

<table>
<thead>
<tr>
<th>Time point</th>
<th>cyp3a</th>
<th>pvalb</th>
<th>vtg</th>
<th>hsp90</th>
<th>ck</th>
<th>gst</th>
<th>aspa</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>1.21</td>
<td>1.21</td>
<td>1.02</td>
<td>1.04</td>
<td>1.17</td>
<td>1.05</td>
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<td>0.06</td>
<td>0.08</td>
<td>0.13</td>
<td>0.10</td>
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<tr>
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<td>1.42</td>
<td>2.00</td>
<td>1.18</td>
<td>1.05</td>
<td>1.00</td>
<td>1.34</td>
</tr>
<tr>
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<td>0.21</td>
<td>0.19</td>
<td>0.09</td>
<td>0.08</td>
<td>0.17</td>
<td>0.11</td>
</tr>
<tr>
<td>61 μg.L−1</td>
<td>0.95</td>
<td>1.31</td>
<td>0.84</td>
<td>1.23</td>
<td>1.42</td>
<td>1.23</td>
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<td>0.09</td>
<td>0.12</td>
<td>0.14</td>
<td>0.05</td>
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<tr>
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<td>0.89</td>
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<td>1.01</td>
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<td>0.05</td>
<td>0.09</td>
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<tr>
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<td>0.10</td>
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<td>0.07</td>
<td>0.07</td>
<td>0.10</td>
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<tr>
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<td>0.16</td>
<td>0.07</td>
<td>0.05</td>
<td>0.24</td>
<td>0.07</td>
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</tbody>
</table>

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.
regulation of aspa at 61 μg.L⁻¹ (p<0.001) and an up-regulation of this gene at 153 μg.L⁻¹ (p=0.001). The glucocorticoid receptor (gcr) which mediates the effects of glucocorticoids, mainly cortisol in fish, was up-regulated at 153 μg.L⁻¹ fipronil (p<0.001). We observed significant up-regulation of igf at 102 μg.L⁻¹ fipronil (p=0.008) and down-regulation of zp3 at 61 μg.L⁻¹ fipronil (p=0.002), but this was not measured at other treatment concentrations. Other tested genes, such as lsp90, gh, g6pd, pvalb and gst, were not significantly affected by fipronil exposure at the 24-h time point, regardless of treatment concentration.

After 24 h of exposure to fipronil, fish were transferred into control water. QPCR 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=0.0025) 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 1). 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.

3.2. Timeline trajectory of multivariate gene transcript responses

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 (Fig. 2).

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=0.05; 61 μg.L⁻¹, p=0.14). 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 1).

4. 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 μg L\(^{-1}\) \(\text{μg} \text{L}^{-1}\). However, the concentration of 31 μg L\(^{-1}\) \(\mu\text{g} \text{L}^{-1}\) 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 \(\geq 61 \mu\text{g} \text{L}^{-1}\) \(61 \mu\text{g} \text{L}^{-1}\) (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 neuronal 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 \(\geq 31 \mu\text{g} \text{L}^{-1}\) \(31 \mu\text{g} \text{L}^{-1}\) 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, 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 ontogenic 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, 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 μg L\(^{-1}\) \(\mu\text{g} \text{L}^{-1}\) 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 acclamatory 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.

5. 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.
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