Compensatory response of fathead minnow larvae following a pulsed in-situ exposure to a seasonal agricultural runoff event


Highlights

- Fathead minnow larvae were maintained at the Elkhorn River Research Station for an in-situ exposure to a seasonally-occurring runoff.
- There was a 1.5- to 13-fold change in waterborne agrichemical contaminants including atrazine, acetochlor and metolachlor.
- Peaks in sediment contamination by agrichemicals was discordant with those of waterborne contaminants.
- Minnow larvae demonstrated compensation following reduction in size and androgenic gene expression by agrichemical exposure.

Abstract

Agriculturally-dominated waterways such as those found throughout the Midwestern United States often experience seasonal pulses of agrichemical contaminants which pose a potential hazard to aquatic organisms at varying life stages. The objective of this study was to characterize the developmental plasticity of fathead minnow larvae in a natural environment subject to a seasonal episodic perturbation in the form of a complex mixture of agricultural stressors. Fathead minnow larvae were maintained at the Elkhorn River Research Station for a 28-d in situ exposure to an agrichemical pulse event. Minnow larvae were sampled after 14 and 28 days to characterize developmental plasticity through growth measures and relative gene expression. Concentrations of agrichemical contaminants measured in water using polar organic chemical integrative samplers and composite sediment samples throughout the 28-d exposure were quantified using gas chromatography–mass spectrometry. Elevated concentrations of acetochlor, atrazine, and metolachlor were indicative of inputs from agricultural sources and were associated with reductions in body mass, condition factor, and androgenic gene expression in river exposed fathead minnow larvae. However, following a 14-d in situ depuration during the post-pulse period, river exposed larvae overcompensated in previously suppressed biological endpoints. These results indicate that fathead minnow larvae are capable of compensatory responses following episodic exposure to agrichemical stressors.

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1. Introduction

Successful development of any organism requires an accommodating environment. This is especially true for aquatic vertebrates such as fish that rely on a combination of environmental and genetic cues to regulate early life growth and ontogeny (Barioller et al. 2009; Pitman et al. 2013). Diel and seasonal oscillations in abiotic factors such as temperature, dissolved oxygen and suspended solids can profoundly alter growth, metabolism and survival of fish larvae (Pérez-Domínguez et al. 2006; Pérez-Domínguez and Holt 2006; Shrimpton et al. 2007; Villamizar et al. 2012; Armstrong et al. 2013). Beyond growth and development, several abiotic factors are also recognized to influence sexual determination and differentiation in a wide variety of species (as reviewed by Devlin and Nagahama 2002). For example, exposure of West African cichlid larvae (Pelvicachromis pulcher) to slight acidic conditions (pH 5.5) during early development results in a female-biased sex ratio (Reddon and Hurd 2013). Although fish acclimate to natural variation in their native environments, the presence of anthropogenic stressors presents novel challenges for larval and juvenile fish.

Exposure to agricultural runoff is one such example of a widespread anthropogenic stressor that can influence larval fish. In many Midwestern streams, agrichemical concentration is seasonal with the highest stressor like agricultural runoff. Furthermore, many of these studies only examine biological endpoints at a single time point after the exposure, an experimental design that fails to characterize how larval plasticity responds to intermittently polluted environments. The objective of this study is to characterize the developmental plasticity of fathead minnow larvae in a natural environment subject to a seasonal episodic perturbation in the form of a complex mixture of agricultural stressors. We hypothesized that 1) larval fish subjected to an agrichemical pulse under natural conditions would experience down regulations in endocrine function and growth immediately following exposure, and 2) the exposed larvae would show partial or complete compensation in endocrine function and growth following a recovery period in the field. To assess this, fathead minnow larvae were maintained at the Elkhorn River Research Station over the course of an agricultural runoff event. Larvae were assessed for changes in endocrine responsive gene markers and growth after 14 and 28 d following the start of the exposure and compared to controls maintained in clean water at the Elkhorn River Research Station.

2. Materials and methods

2.1. Animal production and maintenance

Fathead minnow larvae (Pimphales promelas) used for this experiment were obtained from the Animal Culture Unit at the University of Nebraska at Omaha, Omaha, NE. All procedures were conducted in compliance with protocols approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (Protocol #98-075-110). All fish were maintained in dechlorinated tap water at 25 ± 1.0 °C. Beginning on April 15, 2015, breeding triads of adult fathead minnows were established consisting of one male and two females. Fish were housed in 30 L aquaria, divided into two compartments by a plastic, porous divider with two triads in each aquarium. Breeding triads were provided with a breeding tile (12-cm-long sections of polyvinyl chloride tubing 8 cm in diameter split in half lengthwise) on which eggs were laid.

After the triads began to breed, the tiles with eggs were removed daily from the aquaria and transferred to 1 L aerated beakers. Unfertilized and fungus infected eggs were removed daily, and surviving embryos all hatched by 5 days post fertilization. Upon hatching, the larvae were transferred to 1 L beakers at a density of 100 larvae per liter. A daily static renewal of the water within these beakers was conducted replacing approximately 80% of the total volume. All larvae were fed daily with a mixture of newly hatched (~24 h old) Artemia nauplii (INVE Aquaculture, Salt Lake City, UT).

2.2. Exposure at the Elkhorn River Research Station

Exposure of all fathead minnow larvae was conducted at the Elkhorn River Research Station (ERRS) where previous field studies have identified biological impacts and changes in endocrine responsive gene expression following in situ exposure to agricultural runoff (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2016). The ERRS is an open-air facility located approximately 10 km upstream from the confluence of the Elkhorn and Platte Rivers, Nebraska, USA. The station
is equipped to pump water continuously from the Elkhorn River into stainless steel mesocosms capable of supporting aquatic organisms (Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2016).

The sampling regime for water, sediment and biological samples is outlined in Fig. 1A. Biological sampling consisted of a subset of fish taken 14 d after the start of the exposure (Exposure 1) to evaluate the immediate impact of the pulse, while the remaining fish were sampled 14 d after the pulse had subsided to evaluate their post-pulse recovery (Exposure 2). Passive samplers (POCIS) were deployed over three distinct 14-d windows (i.e. pre-pulse, pulse and post-pulse) (see Section 2.3 Water and sediment sampling) to evaluate changes in aqueous agrichemical contamination. Finally, weekly sediment samples (S) were collected across the larval fish exposure to quantify the overall contribution of sediment-associated agrichemicals to the in-situ exposure.

The start of the agrichemical pulse was determined using atrazine test strips (Abraxis, Warminster, PA) as described in previous studies within the Elkhorn River watershed (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015). Briefly, beginning April 15, 2015 atrazine test strips were used to test whether concentrations of atrazine in surface water in the Elkhorn River were above or below 3 μg·L⁻¹. These test strips have been demonstrated to accurately detect the presence of atrazine and other triazine herbicides at 3 μg·L⁻¹ in surface water (US EPA 2004), with further confirmation of there accuracy by previously collected field data (Knight et al. 2013; Ali and Kolok 2015). Tests were conducted every three to four days where the detection of three consecutive positive atrazine strips determined the start of the spring agrichemical pulse on May 8, 2015 (Fig. 1B). After the deployment of fathead minnow larvae atrazine strip tests were conducted weekly and following rainfall events.

Fathead minnow larvae, 5 days post-hatch (dph) were deployed from May 8 until June 5, 2015 and sampled at the end of Exposure 1 (19 dph) and Exposure 2 (33 dph) (Fig. 1A). During the exposure, larvae treatment groups were maintained at the ERRS in 3 L, half-crescent shaped, stainless steel chambers designed to fit inside the 10 L mesocosms (Ali et al. 2016). A portion of the 3 L chamber was made of 86 μm stainless steel mesh, allowing for river water to flow freely between the mesocosm and the larval chamber, without the loss of larvae. The 10 L mesocosms are maintained within a larger insulation tank (16.5 L) that circulates river water to maintain ambient water temperature of the Elkhorn River.

Two treatment groups, station control and river exposed, were maintained in separate mesocosms at the ERRS for a 28-day exposure (5–33 dph). One hundred larvae were transferred into each mesocosm at the start of the exposure. Station control larvae were transferred to a mesocosm at the ERRS that was supplied with dechlorinated tap water replacing the total volume by 80% daily. River exposed larvae were placed in a mesocosm at the ERRS that received continuous water flow directly from the Elkhorn River. Both treatment groups were fed, ad libitum, newly hatched A. nauplii once daily. Both station controls and river exposed mesocosms were placed within insulating tubes that circulated Elkhorn River water outside of the exposure chamber to maintain similar water temperatures. Additionally, both mesocosms were supplied with air-stones to control for differences in dissolved oxygen between treatment groups.

At the end of Exposure 1 and Exposure 2, larvae were transferred to aerated coolers containing water from their respective mesocosms and transported to the laboratory. Larvae from each treatment group at the end of Exposure 1 (n = 35–36) and Exposure 2 (n = 20) were euthanized using a lethal dose of MS-222. Body mass and length were collected from both age groups for morphometric analysis. Whole larvae were flash frozen in liquid nitrogen then stored at −80 °C for analysis of gene expression by RT-qPCR.

2.3. Water and sediment sampling

Water quality measurements including temperature (°C), pH, conductivity (μS·cm⁻¹), dissolved oxygen (mg·L⁻¹) and suspended solids (mg·L⁻¹) in both station control and river exposed mesocosms were taken daily. Sampling for aqueous pesticides was conducted using polar organic chemical integrative samplers (POCIS) (Environmental Sampling Technologies, St. Joseph, MO, USA). Prior to deployment, all POCIS were soaked in 2 L Nanopure water (19.7 MΩ) for 24 h. A single POCIS was deployed within the receiving 10-L mesocosm for each of the three sampling periods; a Pre-pulse (April 21–May 5), Pulse (May 8–22), and Post-pulse (May 22–June 5). A laboratory blank was maintained in dechlorinated laboratory tap water for 14 d to evaluate the laboratory water used for the station control mesocosm. At the end of deployment POCIS were stored separately at −20 °C until further analysis.

Suspended solids carried in the Elkhorn River accumulated as shoals of sediment in the bottom of the larval mesocosms over the course of the 28-d exposure. Sediment was collected weekly from these shoals for physical and chemical analysis. A composite sample was collected weekly using glass jars and frozen at −20 °C for pesticide analysis by the Water Sciences Laboratory at University of Nebraska Lincoln (described below). The remaining bulk sample was stored at room temperature for approximately 48 h allowing suspended solids to settle before removing excess water. The remaining sediment was dried at 100 °C until a constant mass was reached (12–24 h). Dry sediment samples were analyzed using standard methods to determine texture (Gee and Or 2002) and total organic carbon (TOC) (Islam and Weil, 1998).

2.4. Water and sediment chemistry

Sediment and POCIS samples were stored at −20 °C. Sediments were processed using microwave assisted solvent extraction (MASE) and extracts were analyzed using gas chromatography–mass spectrometry (GC/MS) Zhang et al. (2015). POCIS were extracted according to previously published protocols Sellin et al. (2009). Reference compounds
All extracts were analyzed on an Agilent 5973 GC/MS outfitted with aLeap CombiPAL autosampler with split-less injection using a Restek (Bellefonte, PA) RTX-1, 30 m × 0.25 mm ID and 0.25 μm film thickness capillary column. Oven temperature was programed to run at 80 °C for 0.75 min, ramp to 170 °C at 40 °C·min⁻¹, ramp to 236 °C at 2.5 °C·min⁻¹, ramp to 275 °C at 40 °C·min⁻¹ and hold for 9.62 min. The injection port temperature was 250 °C and the transfer line interface temperature was 280 °C. Retention times, quantifying ions, and instrument detection limits determined from repeated analysis of the lowest standard are included in Supplemental Table 1. 13C₃-atrazine was used as the internal standard for all compounds except for DEA and DIA which used their respective labelled analogues.

2.5. Relative gene expression analysis

Whole larvae bodies were used for reverse transcription quantita- tive polymerase chain reaction (RT-qPCR). RNA extraction utilized the SV Total RNA Isolation System (Promega, Sunnyvale, CA, USA) following manufacturer’s recommendations. RNA was resuspended and stored in nuclease-free water at −80 °C until analysis. Purity and concentration of RNA were assessed by Nanodrop (NanoDrop Technologies, Wilmington, DE, USA) based on optical densities at 260 nm/280 nm and 260 nm/230 nm. Total extracted RNA samples were diluted to 15 ng/μL in preparation for cDNA synthesis. First-strand cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA) per the manufacturer’s recommendations. All PCR reactions were performed on the CFX Connect Real-Time PCR Detection System using the iTaq Universal SYBR® Green Supermix, 2× concentration, (Bio-Rad) per the manufacturer’s protocol. Briefly, 2 μL of diluted cDNA template was added to 20 μL forward and reverse primers in a 15-μL volume containing iTaq Supermix. Target genes involved in ste- roid signaling and synthesis were selected for analysis (primer se- quences and their sources are provided in Supplemental Table 2), these included: androgen receptor (AR), insulin-like growth factor 1 (IGF1), 17β-hydroxysteroid dehydrogenase (HSD17B), gonadal aromatase (CYP19A), doublesex and mab-3 related transcription factor 1 (DMRT1) and estrogen receptor subtype 1 (ERα). The reference gene ribosomal protein (RPL8) was used to normalize gene expression (Kolok et al. 2007). All reaction efficiencies were between 85 and 110%.

2.6. Statistical analysis

Data were analyzed using JMP 11 software (SAS, Cary, NC, USA). Morphometric data for Exposure 1 (n = 35–36) and Exposure 2 (n = 35–36) larvae were compared between treatment groups using t-test. Comparison of relative gene expression between station control and river exposed larvae from Exposure 1 (n = 10–12) and Exposure 2 (n = 16–20) was also conducted using t-test. Welch’s t-test was used when the assumption of equal variances between treatment groups was not satisfied as determined by Bartlett’s test. Statistical significance was assumed at p < 0.05.

3. Results

3.1. Water quality and chemistry

The 2015 spring pulse, as determined by atrazine testing, began in early May and coincided with an approximately 2.5-fold increase in Elkhorn River discharge (Fig. 1). Over the course of the 28-d sampling period there was a major discharge event that peaked on May 8, follow- ed by two lesser peaks in river discharge on May 15 and May 26. There was considerable fluctuation in the levels of suspended solids being transported within the Elkhorn River which coincided with periods of elevated discharge.

Within the mesocosms, fathead minnow larvae experienced varia- tion in water quality parameters based on water source (i.e. laboratory
or river water). Average values (± SD) for water quality parameters in the river exposed mesocosm are summarized in Table 1. Station control larvae experienced less variation in water pH (7.52 ± 0.32), conductivity (457.1 ± 39.8 μS·cm⁻¹), and suspended solids (240.1 ± 184.4 mg·L⁻¹) than did the river exposed fish, but larvae in both groups were exposed to natural diel oscillations in water temperature (Fig. 2). Changes in dissolved oxygen levels of station control larvae decreased from 7.78 ± 0.15 mg·L⁻¹ over the pulse period to 5.86 ± 0.95 mg·L⁻¹ as temperature increased over the post-pulse period which were comparable to variations measured in the river exposed mesocosm (Table 1).

Analytical chemistry data collected from POCIS confirmed the elevated agrichemical concentrations during the 2015 pulse relative to pre- and post-pulse periods (Table 1). Relative to the pre- and post-pulse periods there was a >5-fold increase in total pesticide concentrations during the period between May 8 through May 22. Atrazine, acetochlor and metolachlor were consistently the most abundant herbicides measured in POCIS before, during and after the agrichemical pulse. Other pesticides analyzed but not detected in POCIS samplers included alachlor, butylate, chlorthalonil, cyazine, desisopropylatrazine (DIA), s-ethyl-dipropylthiocarbamate (EPTC), norflurazon, pendamethalin, permethrin, prometon, propachlor, propazine, simazine, tefluthrin, trifluralin.

3.2. Sediment characteristics and chemistry

Physical analysis of the suspended sediment deposited in the larval mesocosm showed greater accumulation of sediment following high flow events with a relatively consistent composition over the 28-d period (Table 2). Two to four times more sediment was accumulated in the river water mesocosm in the first two weeks relative to the second half of the exposure. As would be expected, during weeks with reduced river discharge there was a reduction in sand fraction and an increase in the silt fraction. The percentage of clay and TOC remained stable throughout the entire exposure.

The herbicides atrazine, acetochlor, deethylatrazine and metolachlor were detected in sediment samples collected over the course of the 28-d exposure (Table 2). The highest concentrations of sediment-associated herbicides occurred during the third sampling period (S3) following the pulse period. This increase in sediment associated agrichemicals was discordant with the peak in agrichemicals observed in the water chemistry during the two-week pulse period (Table 1). Other pesticides analyzed but not detected in sediment samples included alachlor, butylate, chlorthalonil, cyazine, DIA, dimethenamid, EPTC, metribuzin, pendamethalin, permethrin, prometon, propachlor, propazine, simazine, tefluthrin, trifluralin.

3.3. Biological effects

Immediately following exposure to the Elkhorn River agrichemical pulse there were differences in growth between station control and river exposed larvae at 19 dph that were reversed by 33 dph (Fig. 3). At the end of Exposure 1 (19 dph), river exposed larvae had a reduced body mass (t-test, df = 70; p < 0.001) and condition factor (t-test, df = 69; p < 0.001) relative to station controls. No significant difference in larval body length was detected at 19 dph. Conversely, by the end of Exposure 2 (33 dph) the river exposed larvae were significantly larger than station controls in terms of body mass (t-test, Welch's correction, df = 27.52; p = 0.011), length (t-test, df = 38; p = 0.038) and condition factor (t-test, df = 38; p = 0.001). By the end of the 28-d exposure there was 25% and 8% mortality of station control and river exposed larvae, respectively.

When compared to station controls, river exposed fathead minnow larvae showed significant changes in the whole-body expression of endocrine responsive genes following the pulse (Exposure 1) and post-pulse (Exposure 2) exposures (Fig. 4). At the end of Exposure 1 (5–19 dph), larvae had an upregulation of IGFl (t-test, Welch’s correction df = 22; p = 0.013) and a downregulation of AR (t-test, df = 21; p = 0.044) (Table 3). No difference was detected in the expression of CYP19A, DMRT1, ERx and HSD17B at the end of Exposure 1 (Table 3).

At the end of Exposure 2 (5–33 dph), river exposed larvae maintained the upregulation of IGFl relative to controls at 33 dph (Fig. 4; t-test, df = 34; p < 0.001). However, by the end of Exposure 2 the river exposed fathead minnow larvae experienced a significant upregulation of AR expression relative to station controls (t-test, df = 34; p < 0.0076). No differences were detected in the expression of the steroidogenic genes HSD17B and CYP19A, as well as genes DMRT1 and ERx (Table 3).

4. Discussion

The objective of this study was to characterize the developmental plasticity of fathead minnow larvae in a natural environment subject to a seasonal episodic perturbation in the form of a complex mixture of agricultural stressors. It was hypothesized that one of the short-term agricultural pulses would elicit a down regulation in endocrine gene expression and growth, and that post-pulse the larvae would compensate. Larvae exposed to a 14-d pulse showed suppressed endocrine gene expression and growth. Their response to the pulse induced suppression was an over compensation in both growth and endocrine function.

Table 1

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>April 21–May 5 (Pre-pulse) a,b</th>
<th>May 8–May 22 (Pulse)</th>
<th>May 22–June 5 (Post-pulse)</th>
<th>Fold change (Pre-pulse to pulse)</th>
<th>Fold change (Post-pulse to post-pulse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>16.70 (2.05)</td>
<td>20.00 (2.08)</td>
<td>–</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Dissolved O₂ (mg·L⁻¹)</td>
<td>8.42 (1.37)</td>
<td>5.75 (0.73)</td>
<td>–</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>8.23 (0.16)</td>
<td>8.32 (0.09)</td>
<td>–</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Conductivity (mkS·cm⁻¹)</td>
<td>75.4 (136.1)</td>
<td>20.9 (18.9)</td>
<td>1.8</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>Suspended Solids (mg·L⁻¹)</td>
<td>5426.7 (3376.2)</td>
<td>4460.7 (4178.5)</td>
<td>–</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>244.2</td>
<td>22.94</td>
<td>1517.4</td>
<td>339.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Dimethenamid</td>
<td>75.4</td>
<td>136.1</td>
<td>20.9</td>
<td>1.8</td>
<td>6.5</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>197.9</td>
<td>709.7</td>
<td>131.9</td>
<td>3.6</td>
<td>5.3</td>
</tr>
<tr>
<td>Deethylatrazine (DIA)</td>
<td>20.2</td>
<td>34.5</td>
<td>24.5</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Propazine</td>
<td>3.6</td>
<td>23.2</td>
<td>4.3</td>
<td>6.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Simazine</td>
<td>10.0</td>
<td>5.3</td>
<td>6.5</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Metribuzin</td>
<td>&lt; 5.0</td>
<td>14.3</td>
<td>&lt; 5.0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

a =–a indicates not measured.
b <5.0 = below estimated detection limits.
Although the sampling time periods for the POCIS were different than that for the sediment (Fig. 1), both illustrate short term changes in the chemical environment that larvae were exposed to. Changes in aqueous pesticide concentrations observed in this study are consistent with previously published differences in agrichemical concentrations during a discharge event relative to post-discharge. Knight et al. (2013) used POCIS samplers to compare agrichemical concentrations during and after a discharge event. The discharge event led to 1.6 to 28-fold increases in herbicide concentrations relative to post-discharge. Similarly, the present study observed increases (up to 13-fold) in atrazine, acetochlor and metolachlor (Table 2). These three herbicides represent a signature combination of pollutants found in runoff from corn and soybean production (LeRch et al. 2011a; LeRch et al. 2011b; Ali and Kolok 2015; Zhang et al. 2015; Fairbairn et al. 2016). The nearly identical concentration of herbicides before and after the discharge event highlights the ephemeral occurrence of waterborne agrichemicals in this watershed.

As seen in previous studies (Ali and Kolok 2015; Zhang et al. 2015), increases in river discharge were accompanied with increases in waterborne agrichemicals as well as the mobilization of sediments and other suspended solids carrying their own pesticide burden. Chemical analysis of accumulated suspended sediments revealed a mismatch between the peaks of pesticides in water and sediment over the 28-d sampling period (Table 2; Table 3). Specifically, the highest concentrations of waterborne pesticides coincided with the major discharge event whereas sediment-associated pesticide concentrations were the greatest during a lesser spike in river discharge during the post-pulse period, S3 (Fig. 1; Table 3). One plausible explanation for observed differences in aqueous and sediment-associated pesticide concentrations is that a fraction of dissolved pesticides entering the Elkhorn River during the pulse period partitioned into the sediment which was later mobilized as suspended solids during the lesser discharge event (S3). The role of sediments in the kinetics of agrichemicals in the Elkhorn River has been previously documented (Kolok et al. 2014; Zhang et al. 2015).

The aquatic environments of the pulse and post-pulse periods were distinct from one another not only in their pesticide profiles but in the fluctuations of several physicochemical parameters that can affect aquatic biota. This was evident by measured differences in conductance, dissolved oxygen levels, and accumulated mass of suspended solids between the pulse and post-pulse periods (Table 1; Table 2). All of these abiotic factors varied relative to river discharge (Kolok et al. 2014; Kjelland et al. 2015). Biological responses of fish living in this environment would be expected to be a function of these changing physicochemical parameters, as well as diurnal and seasonal (vernal) changes in temperature (Table 1) and photoperiod, (Clark et al. 2005; Blanco-Vives et al. 2011; Ali and Kolok 2015) regardless of the presence of any agrichemicals in the water. This underscores the importance of in situ studies for understanding the impacts of natural, composite exposures, as these are events that are not readily simulated under laboratory conditions.

4.2. Biological response of larvae to an episodic exposure

The present study documented an interesting dynamic between the expression of AR and IGF1 in fathead minnow larvae following exposure to a seasonal discharge event. Specifically, this is of interest due to their endocrine interactions as well as their responsiveness to environmental stimuli, including endocrine disrupting agrichemicals.

At the molecular level, peptide and steroid hormones facilitate the integration of environmental stimuli with the regulatory mechanisms of multiple physiological systems (Bradshaw 2007; Pittman et al. 2013). The peptide hormone, IGF1, has endocrine, paracrine and autocrine activity which is readily upregulated or downregulated in response to environmental factors including changes in salinity, nutritional status, temperature and photoperiod (reviewed by Reinecke 2010). As environmental conditions become optimal for survival and development of an organism, growth will occur. Warmer water temperatures stimulate growth in rainbow trout (Oncorhynchus
mykiss) through the increased release of growth hormone and subsequent stimulation of IGF1 (Gabillard et al. 2003). The strong association between IGF1 and growth in bony fish has led to the application of IGF1 mRNA as a molecular marker of growth under a variety of environmental manipulations (Vera Cruz et al. 2006; Montserrat et al. 2007; Vera Cruz and Brown, 2009; Reinecke 2010; Picha et al. 2014). However, it has been demonstrated that nuclear receptors also modulate the expression of IGF1 following activation by steroid hormones (Riley et al. 2004; Johns et al., 2011; Norbeck and Sheridan 2011; Cleveland and Weber 2015). Immature coho salmon (Oncorhynchus kisutch) injected with 11-ketotestosterone or testosterone had a significant increase of circulating IGF1 protein one to two weeks following treatment (Larsen et al. 2004). More recently, analysis of hepatic gene expression in female rainbow trout injected with either 17β-estradiol or dihydrotestosterone demonstrated that androgens stimulate IGF1 expression whereas estrogens suppressed IGF1 expression (Cleveland and Weber 2015).

The link between IGF1 regulation and sex steroids in fish leaves their early life growth and development susceptible to endocrine disruption by steroidogenic contaminants, such as agrochemicals; however, effects may be confounded by environmental conditions. Sustained in situ exposure of juvenile fathead minnows to agricultural ditch water containing a mixture of androgens, estrogens and pesticides lead to increased body size along with male-biased sex ratios at the end of a 6-week study (Leet et al. 2012). During a subsequent 45-day laboratory study, an increase in the body mass and length of fathead minnows when exposed to a simulated mixture of agrochemicals was observed, but
changes in steroid responsive genes at 20 dph or final sex ratio (Leet et al. 2015) was not detected. Leet et al. (2015) speculated that the disparity between outcomes from the in situ (Leet et al. 2012) and laboratory studies (Leet et al. 2015) was due to the absence of environmental factors such as spikes in temperature or sediment interactions which may contribute to the overall response of larval fish towards endocrine disrupting chemicals. In these studies, endpoint analysis demonstrated an effect of agrichemicals on growth and development, however they overlook the developmental response that occurs with the exposure.

The present study documents compensatory growth in fathead minnow larvae following exposure to a seasonal discharge event. Immediately after the major discharge event (Exposure 1; Fig. 1), river exposed larvae displayed suppressed growth and endocrine gene expression as determined by body mass and androgen receptor expression, respectively (Table 3; Figs. 3 and 4). This suppression of growth and endocrine function was reversed at the end of a depuration period following the main discharge event which allowed river exposed larvae to achieve greater growth and androgenic gene expression (i.e. masculinization), relative to station controls. Curiously, river exposed larvae maintained elevated IGF1 expression throughout the study which was discordant with the observed compensation in other biological endpoints. An explanation for this discrepancy is that induction of compensatory growth by the growth hormone-IGF1 axis is not directly mediated by IGF1 (Beckman 2011), rather the induction of compensatory growth is controlled by multiple endocrine and paracrine mechanisms (Won and Borski 2013). To our knowledge, this is the first study to characterize compensation of larval fish following an in situ episodic exposure to seasonally occurring agrichemical stressor.

Table 3
Summary of apical and molecular endpoints observed in river exposed larvae relative to their station control counterparts. Significant increases (↑) and decreases (↓) represented by arrows and no significant differences denoted by dash (−) (α = 0.05).

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Exposure 1 (Pulse, 19 dph)</th>
<th>Exposure 2 (Post-pulse, 33 dph)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass</td>
<td>↓↑</td>
<td></td>
</tr>
<tr>
<td>Body length</td>
<td>−↑</td>
<td></td>
</tr>
<tr>
<td>Condition factor (K)</td>
<td>↓↑</td>
<td></td>
</tr>
<tr>
<td>Molecular Endpoints</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androgen receptor (AR)</td>
<td>↓↑</td>
<td>↑</td>
</tr>
<tr>
<td>Insulin-like growth factor 1 (IGF1)</td>
<td>↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>Estrogen receptor α (ERα)</td>
<td>−−</td>
<td>−</td>
</tr>
<tr>
<td>Doublesex mab-3 related transcription factor 1 (DMRT1)</td>
<td>−−</td>
<td>−</td>
</tr>
<tr>
<td>Gonadal aromatase (CYP19A)</td>
<td>−−</td>
<td>−</td>
</tr>
<tr>
<td>17β-Hydroxysteroid dehydrogenase (15D217B)</td>
<td>−−</td>
<td>−</td>
</tr>
</tbody>
</table>

"dph" – days post-hatch.

an effect of agrichemicals on growth and development, however they overlook the developmental response that occurs with the exposure.
concentrations of agrochemicals, similar to the post-pulse concentrations seen in this study. In a subsequent study on the Elkhorn River, Zhang et al. (2015) found that agrochemical-laden sediments carried by pulse events were responsible for reductions in hepatic estrogen-androgen-receptor expression in adult fish. The downregulation of androgen receptor expression in adult fish (Zhang et al. 2015) is consistent with the response observed in the present study immediately following the pulse. It is likely that the absence of dememinization of minknow larvae in the present study is due to either environmental or biological factors. While Zhang et al. (2015) confirmed that sediment was a major source of agrochemical exposure for adult fish, this may not hold true for larvae whose interactions with sediment and water may differ from adult fish. A biological explanation for the absence of dememinization is that the expression profile of estrogen receptors in fathead minknow, namely receptor subtypes α, β and γ, has been shown to vary over the course of ontogeny which would determine what type of response larvae and juvenile fish might have towards an anti-estrogenic mixture (Filby and Tyler 2007; Johns et al. 2011; Leet et al. 2013). Therefore, while subtle differences were observed between the responses of larvae from the present study and those observed in adult fish from previous studies there is evidence for a consistent response of fish towards agrochemical pulses. Future studies are needed to investigate the impacts of similar exposures on multiple levels of endocrine activity and the mechanisms by which compensatory responses occur.

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Appendix A. Supplementary data

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References

