IDENTIFYING MOLECULAR BIOMARKERS OF GROWTH INHIBITION IN FATHEAD MINNOWS: ONTOGENETIC EXPRESSION PROFILES

by

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IDENTIFYING MOLECULAR BIOMARKERS OF GROWTH INHIBITION IN FATHEAD MINNOWS: ONTOGENETIC EXPRESSION PROFILES

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ABSTRACT

Measuring changes in gene expression has been established to effectively reflect contaminant-induced changes in physiological mechanisms. Prior research has found that growth-related genes are sensitive endpoints in toxicity tests that use larvae and have potential as endpoints for embryonic organisms. The purpose of this research was to identify the stages of development at which the expression of genetic endpoints that indicate reduced growth potential in fathead minnows is high enough to draw an accurate comparison with contaminant-induced decreases in expression. To accomplish this goal, we evaluated a suite of growth-related genes from embryos and larvae obtained at various 24-hour time points from spawn (Day 0) to Day 11 and quantitatively determined the time points at which expression of these genes was the highest. For the growth-related genes, expression levels of growth hormone (*gH*) were highest at Days 4-7 and 11, levels of growth hormone receptor (*gHR*) at Days 1-7 and 11, and levels of insulin-like growth factor (*igf1*) at Days 4-11. For the thyroid hormone receptors, thyroid hormone receptor-α (*TRα*) showed highest expression levels at Days 3-11 and thyroid hormone receptor-β (*TRβ*) showed highest levels at Days 2-5 and 9. For the deiodinase enzymes, deiodinase-1 (*Dio1*) expression levels were highest at Days 2-3 and 7-11, levels of deiodinase-2 (*Dio2*) were highest at Days 7-11, and levels of deiodinase-3 (*Dio3*) were highest at Days 1-5. The time points associated with highest expression levels amongst these genes may be the most effective points to measure alterations of expression in toxicity testing.

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INTRODUCTION

The United States employs various toxicity tests to assess acute and chronic toxicity associated with chemical effluents to protect environmental and human health. Often, these tests involve the use of early life-stage fathead minnows (*Pimephales promelas*). Two such tests are the larval growth and survival (LGS) test and the fish embryo toxicity (FET) test. These tests differ in the developmental stage of the organisms utilized in the metrics used to evaluate contaminant-induced physiological changes. The LGS test exposes larvae to chemicals or effluents for the first seven days post-hatch and assesses acute and chronic toxicity through the measurement of mortality and growth reduction (length and mass). The FET test exposes newly spawned embryos to the contaminant at test for 120 hours and measures mortality (determined via lack of somite formation, no detachment of the tail-bud from the yolk sac, etc.) during the experimental period, assessing only acute toxicity (Sellin Jeffries, 2015)

The metrics used in these toxicity testing methods present different advantages and disadvantages. Measuring mortality determines the lethality of the chemical/effluent. Because these toxicity test use at least five concentrations, evaluating mortality also aids in determining the LC50—median lethal concentration—of the contaminant at the end of the exposure period (Sellin Jeffries, 2014). However, using mortality as the only endpoint in toxicity testing provides evidence solely for acute toxicity and, therefore, does not provide information about sublethal effects on the organism. Assessing growth reduction addresses the need to identify adverse effects that are sublethal and provides a metric for chronic toxicity testing (Sellin Jeffries et al., 2015). Solely measuring growth reduction as a sublethal endpoint, however, does not fully explain contaminant-induced physiological effects. Additionally, growth reduction is a difficult endpoint to adopt for

toxicity tests that utilize embryonic organisms (i.e. the FET) due to measurement limitations. Assessing molecular biomarkers of contaminant-induced physiological alterations may address the shortcomings of mortality and growth as toxicity testing endpoints.

Utilizing changes in gene expression as a metric for toxicity testing may aid in the identification of sublethal adverse effects associated with different chemical and effluent exposures. Previous literature has identified that chemicals and effluents alter the expression of genes involved in growth- and thyroid-related pathways. Specifically, a study conducted by Jeffries et al. (2015) saw that both FET and LGS fathead minnows exposed to the mock effluent at test, a representation of municipal wastewater-treatment plant effluent, experienced a 1.6-fold decrease in expression of insulin-like growth factor 1 (*igf1*), a hormone crucial for growth and development (Johns et al., 2009). The organisms who showed these alterations also exhibited significant reductions in mass compared to their control counterparts (Sellin Jeffries et al., 2015). Additionally, results from Li et al. (2011) showed Chinese rare minnow (*Gobiocypris rarus*) larvae showed significant decreases in deiodinase-2 (*Dio2*) after 7 d of perchlorate exposure. Many studies have begun to involve growth- and thyroid-related genetic endpoints and have identified contaminant-induced decreased expression of these genes.

One challenge in using gene expression as a metric in toxicity testing is identifying the ideal developmental time point to measure expression of a given gene. If expression of a target gene is very low in an unexposed organism at a particular time point, then detecting statistically significant differences in growth- and thyroid-related gene expression in an exposed organism is unlikely. Ideally, growth and thyroid gene

expression would be measured at the time points at which normal expression of the gene is highest. This would allow for an enhanced likelihood of detecting statistically significant decreases in exposed organisms. Because of this, ontogenetic profiles of an organism's gene expression that identify these time points of elevated expression is necessary if gene expression is to be effectively used as an endpoint in toxicity tests utilizing early life stage organisms. However, the literature lacks growth and thyroid ontogenetic profiles for early life-stage fathead minnows at embryonic and early larval stages (≤11d), the developmental stages utilized in the FET and LGS tests.

The objective of this study was to provide ontogenetic expression profiles of an array of growth- and thyroid-related genes in fathead minnows and demonstrate the time point(s) at which the expression of these genesis maximal. To do so, whole embryos or larvae were collected at eight developmental time points from days 1 to 11 post-fertilization and the expression of growth- and thyroid-related genes was measured.

METHODS

Obtaining embryos

The study required the use of approximately 141 newly spawned fathead minnow embryos. The fathead minnow embryos were produced by the fathead minnow breeding colony at Texas Christian University. Upon embryo collection, the embryos were observed with a Leica DMI1 microscope (inverted microscope for tissue culture) for vitality and developmental stage before verified for use in the study. Embryos at a developmental stage of ≤32 cells were utilized in this experiment.

Test conditions

Groups of 45 embryos each were divided between 3 1 L aerated glass beakers that contained 700 mL of dechlorinated water, were covered with acrylic sheets, and were housed in a Panasonic Cooled Incubator at 27 ± 0.50 ºC with a 16:8-h light: dark photoperiod. The water was exchanged daily via 70% exchange. Prior to exchange, the renewal water was tested for the appropriate temperature, pH, conductivity, salinity, alkalinity, hardness, and ammonia concentration. Upon water/solution exchange, the existing water/solution of each replicate was tested for different measures of water quality, including temperature, pH, conductivity, and salinity. One replicate from each group was randomly selected for alkalinity, hardness, ammonia, nitrite, and nitrate testing. A feeding regime was for each replicate was begun at 2 d post-hatch of ~0.032 g newly hatched *Artemia nauplii* per 10 larvae in the beaker twice daily. Additionally, the aeration level for each replicate was reduced prior to expected hatch date. The total combined testing duration was 11 days.

Collecting tissue samples

Whole embryos or larvae were collected after initial embryo collection for gene expression analysis at eight different time periods. Three embryonic/larval samples were collected from each beaker at each of the time periods—24 hpf, 48 hpf, 72 hpf, 96 hpf, 120 hpf, 168 hpf, 216 hpf, and 264 hpf. For embryonic tissues (24-72 hpf), the tissues were collected and pooled in groups of 2. For larval tissues (96-264 hpf), the organisms were euthanized using tricaine methanesulfonate (MS-222) and evaluated for length and wet weight. All tissue samples were immediately flash frozen and stored at -80ºC for subsequent gene expression analysis.

Growth

Larval samples were assessed for two growth metrics—length and wet weight—post-euthanization and prior to storage. Length was measured in millimeters (mm) using a sterilized sliding Vernier caliper and wet weight was measured in milligrams (mg) using an analytical balance. Excess water was removed by blotting the samples with a Kimwipe prior to growth measurements. Length and wet weight was recorded for each larval tissue sample prior to flash freezing.

Gene expression analysis

To determine the expression of target genes at each time point, six samples were chosen for gene expression analysis. Samples selected for analysis were chosen based on similarities in mass and length, while ensuring that at least one sample from each replicate was included. Genes pertaining to growth, development, and stress were chosen for gene expression analysis, and the processed tissues were later analyzed for expression of these genes. The target genes, their primer sequences (in fathead minnows), and their annealing temperature are listed in Table 1. Primer sequences not obtained from the literature were created using Primer 3. (Thornton et al., 2016) (http://biotools.umassmed.edu/bioapps/primer3_www.cgi)

The tissue samples were homogenized using a QSonica Q125 Sonicator in 210 μL of homogenization solution (supplied from the manufacturer). The Total RNA was extracted from the homogenized tissues and purified for cDNA conversion and qPCR analysis using the Maxwell® Research System and Maxwell Simply RNA LEV kit. After RNA extraction, the RNA concentration and absorbance ratio (at 260 nm and 280 nm) were quantified using the NanoDrop to ensure that the RNA was appropriate for qPCR analysis (Sellin Jeffries et al., 2015).

The RNA was converted into complementary cDNA using the Bio-Rad iScript cDNA Synthesis kit and a TC-100 thermal cycler. The synthesis reactions included 2 µL iScript reaction mix, 0.5 µL reverse transcriptase, 0.1 µg of the sample's Total RNA diluted in 7.5 µL of nuclease-free water to eventually produce 10 µL cDNA. The thermal cycler modulated the synthesis with the following cycle: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. After the synthesis was completed, the cDNA was diluted with nuclease water (40 µL total).

In order to assess expression of the target genes in the fathead minnows used in the experimental component of the study, qPCR was performed with a CFX Connect real-time detection system and evaluated using Bio-Rad CFX Manager Software, Ver 3.0. with each of the samples' cDNA in triplicate for each gene being tested. The individual reactions occurred in the wells of Bio-Rad PCR plates, each consisting of 0.4 µL cDNA, 5 µL Bio-Rad iQ SYBR-Green supermix, nuclease free water, and 300 nM of both forward and reverse primers (generated by Eurofins MWG Operon) of the gene being tested, for a total of 10 µL. The plates containing these reactions underwent the following thermal cycling program: an activation step at 95°C for 3 min, 40 cycles of denaturing at 95°C for 10 seconds, and annealing for 45 seconds (according to the annealing temperatures listed in Table 1). Expression data was evaluated using a standard curve method that utilized standards of serially diluted cDNA samples (Sellin Jeffries et al., 2015).

Data Analysis

Growth and gene expression analysis was conducted via the JMP 11.0 statistical software package (SAS Institute), with the alpha set to 0.05 (Sellin Jeffries, 2015). Both

growth and gene expression data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparisons test. If the assumption of homogeneity of variance was not achieved, nonparametric comparisons using the Wilcoxon Method was then utilized. Analysis determined significant differences in growth and expression between embryonic and/or larval samples from different time points, which were demonstrated by connecting-letters reports. Expression for each gene was normalized using the samples' expression levels of a hallmark gene (elongation factor, *EF*).

Table 1.Genes included in ontogenetic expression profiles with corresponding primer sequences (both forward [F] and reverse [R]) and annealing temperature.

Target Gene	Primer Sequence	Annealing Temp. (°C)
Growth hormone (*gH1*)	F: AGATCACCGAGAAGCTGGAA R: GGCAGGGAGTCGTTATCATC	54.0
Growth hormone receptor (*gHR*)	F: GACCCTGCAGAGAAGAACGA R: ATCGTCGTCGCTTTTGAAAT	56.0
Insulin-like growth factor (*igf1*)	F: CAACGGCACACGGACATC R: CCTCGGCTTCGAGTTCTTCTG	57.0
Thyroid hormone receptor-α (*TRα*)	F: CCATCACACGAGTTGTGGAC R:TAAGGTCAGCGTCTCGCTCT	62.0
Thyroid hormone receptor-β (*TRβ*)	F: CAGTGAGTCCGTGGAAGACA R: CAGGTGATGCATCGGTAATG	61.0
Deiodenase-1 (*Dio1*)	F: ACGGACAGAAAACGAGCATT R: TGAGGAAATCTGCCACATCA	61.0
Deiodenase-2 (*Dio2*)	F: AATTTTCGGATGTGGCAGAC R: GCAGCAAACATCCTCTCCTC	61.0
Deiodenase-3 (*Dio3*)	F: TAATGAAGATGCGGGAAAGC R: CGCCGTTTTAAAGAAGTCCA	60.0

RESULTS

Growth

Average masses and lengths were calculated for each of the larval samples taken for gene expression at each sampling time point. Growth data was compared between the time points, as explained in Figure 1 (mass) and Figure 2 (length), to identify similar trends between physical growth changes and alterations in growth-related gene expression across time. Minimal variations in mass and length between replicates were maintained, since the larvae were chosen for gene expression based on their distance from the mean in growth measurements. Both larval mass and length exhibited similar

growth and mass observations for Day 4 and Day 5 samples, but then showed expected consistent increases in mass and length from Day 7 to Day 11.

Gene Expression

Expression of the target growth- and thyroid-related genes (*gH, gHR, igf1, TRα, TRβ, Dio1, Dio2, Dio3*) was evaluated at eight time points between 1 and 11 dpf. Statistical differences in expression levels across time points were illustrated via connecting letters reports.

The different genes involved in the growth pathway showed different patterns of gene expression changes over time. Growth hormone (*gH*) showed its lowest levels of expression until 4 dpf, the time point corresponding with hatch. Its expression showed significantly high levels up to 11 dpf, with the exception of a decrease in expression at 9 dpf. Expression of growth hormone receptor (*gHR*) only showed significant decreased expression at 9 dpf, with all other time points showing statistically similar levels of expression. Insulin-like growth factor (*igf1*) expression showed highest levels of expression at all time points ≥ 5 dpf. A summary of the relative gene expression for each gene in the growth pathway can be found in Figure 3.

For thyroid-related gene expression, expression profiles for two thyroid hormone receptors (*TRα* and *TRβ*) and three deiodinase enzymes (*Dio1, Dio2,* and *Dio3*) were developed. For thyroid hormone receptor-α (*TRα*), expression reached its significantly highest levels at 3-11 dpf. Expression of thyroid hormone receptor-β (*TRβ*) showed earlier levels of high expression, with its highest expression levels at 2-5 and 9 dpf. A summary of the relative gene expression of the thyroid hormone receptors can be found in Figure 4. For the deiodinase enzymes (Figure 5), deiodinase-1 (*Dio1*) showed

upregulations in expression at 2-3 dpf and again a 7-11 dpf. Deiodinase-2 (*Dio2*), showed steady increases in gene expression over the course of development, with highest levels at 7-11 dpf. Deiodeinase-3 (*Dio3*) demonstrated highest levels of expression at earlier time points, 1 and 3-5 dpf, and its lowest expression levels at 7-11 dpf.

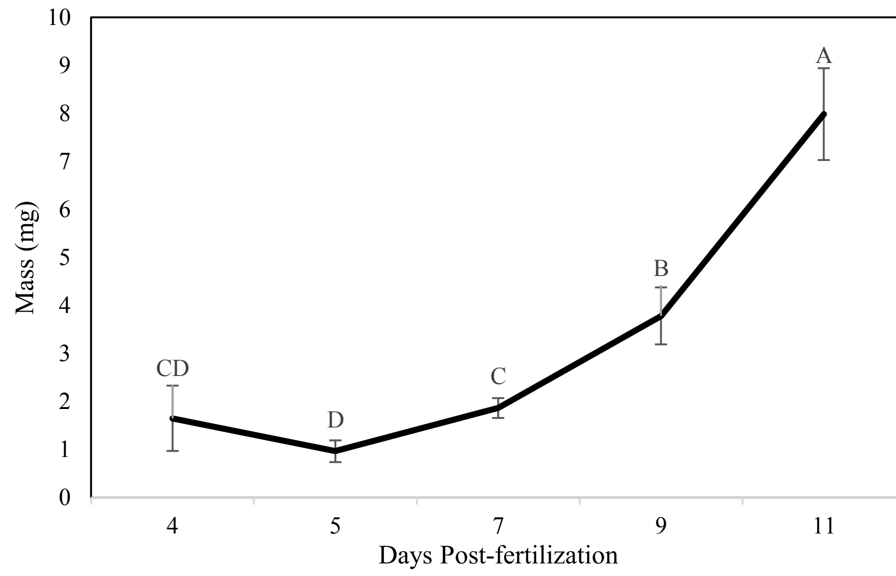

Figure 1. Mass of larval samples (Day 4-Day 11), with standard error indicated by error bars.

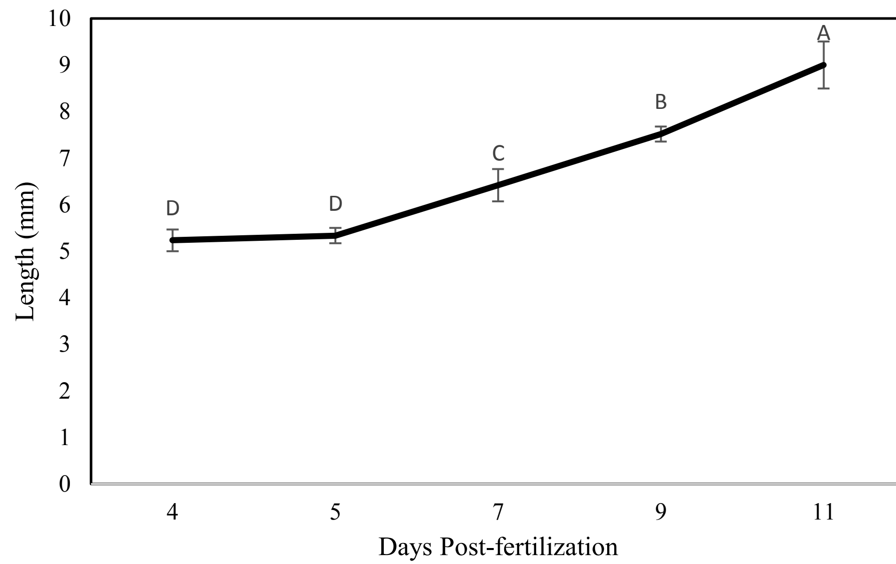

Figure 2. Length of larval samples (Day 4-Day 11), with standard error indicated by error bars.

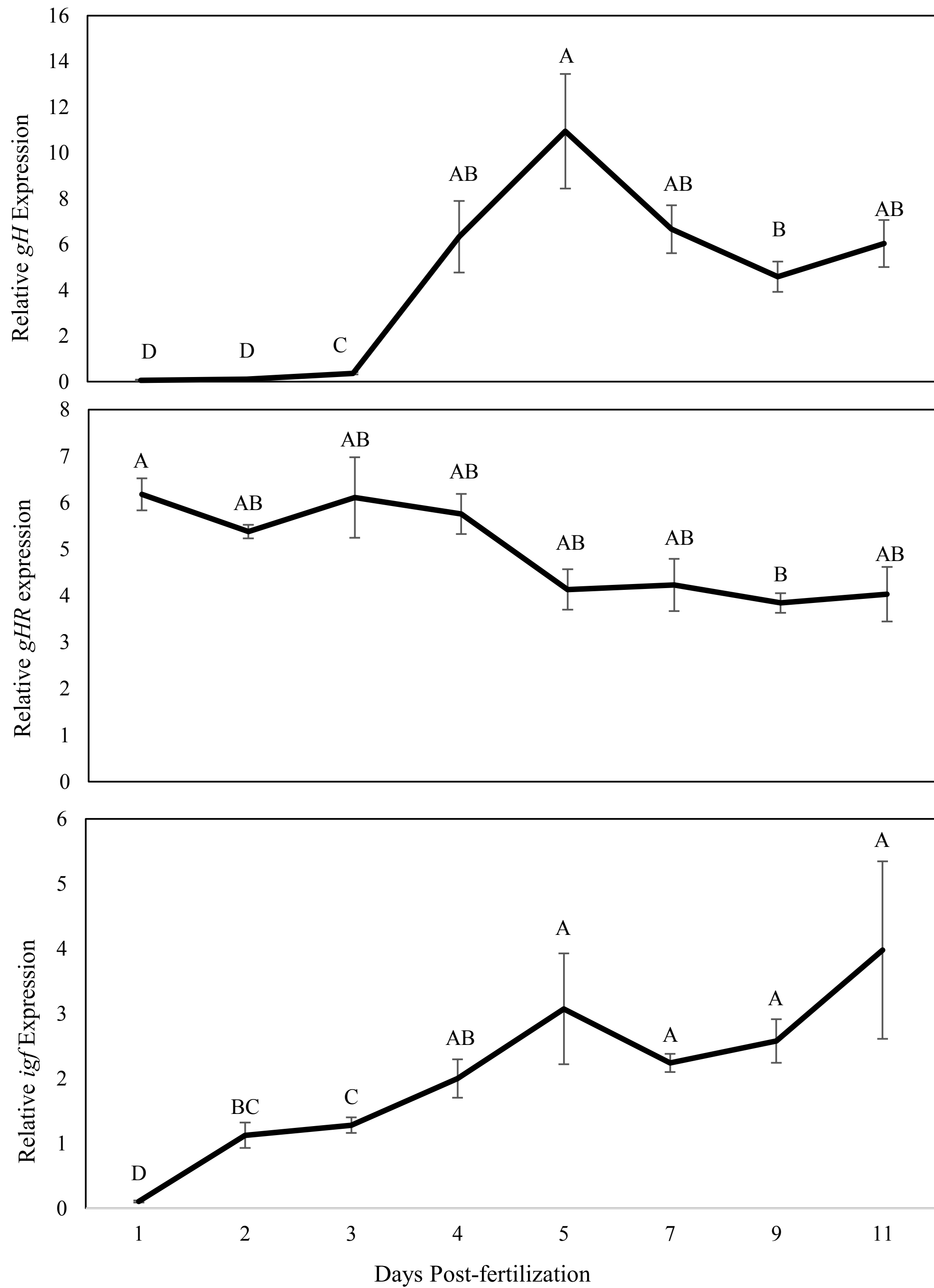

Figure 3. Relative expression of genes within the growth pathway, (A) *gH*, (B) *gHR*, and (C) *igf*, with the standard error indicated by error bars and statistical differences indicated by connecting letters.

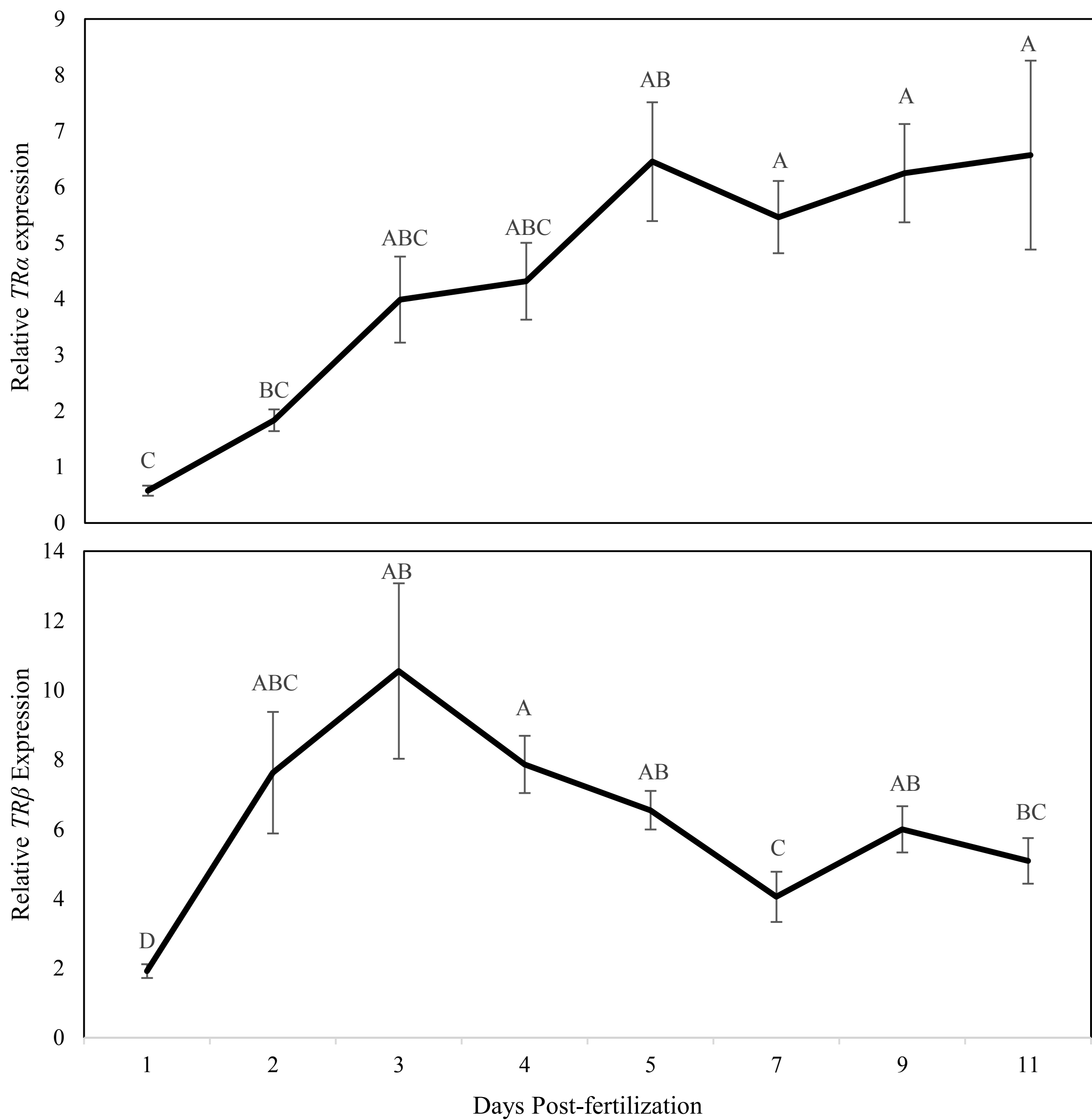

Figure 4. Relative expression of thyroid hormone receptor genes, (A) *TRα* and (B) *TRβ*, with the standard error indicated by error bars and statistical differences indicated by connecting letters.

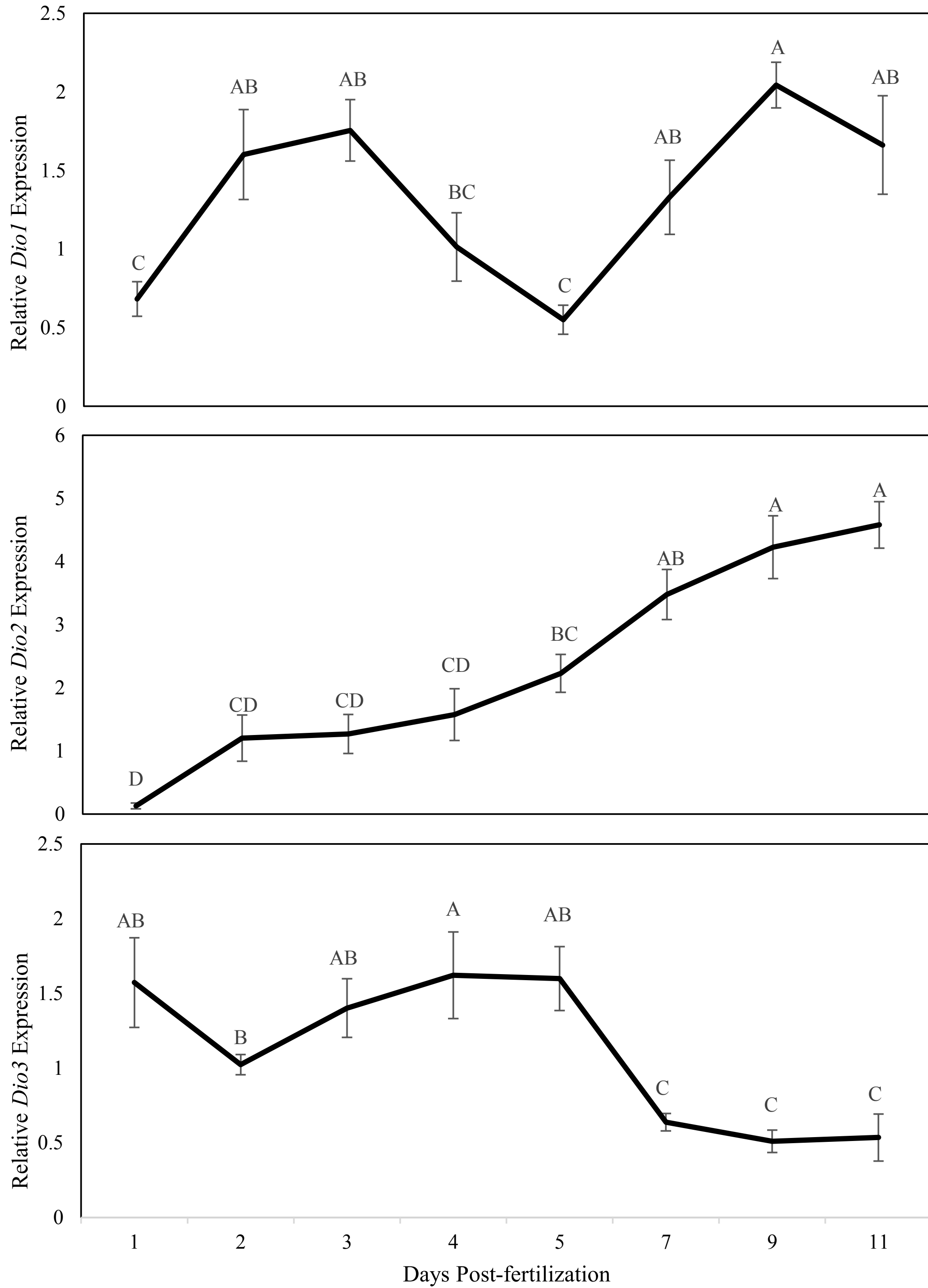

Figure 5. Relative expression of deiodinase enzymes, (A) *Dio1* and (B) *Dio2*, and (C) *Dio3* with the standard error indicated by error bars and statistical differences indicated by connecting letters.

DISCUSSION

The data from this study provides ontogenetic expression profiles for 24-264 hpf fathead minnow embryos and larvae for three growth-related genes, *gH, gHR, igf1*, and five thyroid-related genes, *TRα, TRβ, Dio1, Dio2, Dio3*. Studies like those by Filby, Tyler (2007) and Johns et al. (2009) have developed ontogenetic expression profiles of these genes in larval and juvenile fathead minnows, but the literature lacks expression data for embryonic expression of the genes and only provides data for certain larval time points.

Thyroid and growth-reated genes have a crucial role in growth, metabolism, differentiation, and other processes across fathead minnows and other vertebrate species (Le Gac et al., 1993). *gH* is released by the anterior pituitary and targets receptors (*gHR*) expressed in the liver. Upon *gH* binding, the liver releases *igf* that targets tissues throughout the organism to facilitate cell and systemic growth processes. Thyroid hormone receptors (*TRα, TRβ*) expressed in a variety of tissues throughout the organism bind thyroid hormones and engage its designated tissue in functions like development, heart rate, and digestion. The deiodinase enzymes alter the biological availability of thyroid hormones in the bloodstream, with *Dio1* and *Dio2* increasing its availability and *Dio3* decreasing its availability (Orozco, Valverde, 2005).

Growth and growth-related gene expression

The trends identified in the growth axis (*gH/gHR/igf*) were especially interesting because they seemed to coincide with logical developmental events across those time points. *gH* expression substantially increased at 4 dpf (hatch day), which could demonstrate the large involvement of growth-related processes needed in the embryo to larvae transition. It reached its highest expression at 5 dpf, and showed statistical

similarities in expression up to 11 dpf (with the exception of 9 dpf). This is comparable to Filby and Tyler's findings (2005) that found no significant differences in *gH* expression after 5 dpf. *gHR*, interestingly, showed steady expression over time with the only samples from 9 dpf showing significant decreases in expression compared to 1 dpf. Elevated expression of *gHR* at earlier time points was expected when comparing the data to that of *gH*; higher concentrations of the receptor are expressed in order to be sensitive to a lower concentration of its ligand. *Igf1* expression showed increases in expression going into larval stages, which aligns with the findings of Johns et al. (2009) that saw whole body expression of *igf* increase up to 15 dpf.

Since *gH, gHR,* and *igf1* are involved in the pathways that stimulate tissue and cell growth, it could be predicted that increases in larval length and mass over time would be explained by trends in expression of these genes. Larval samples experienced significant increases in mass from 7-9 dpf and 9-11 dpf and significant increases in length from 5-7 dpf, 7-9 dpf, and 9-11 dpf. These significant increases in larval growth/mass, interestingly, did not coincide with changes in expression of growth-related genes. No significant differences in expression occurred post-hatch (>4 dpf), with the exception of decreased *gH* and *gHR* expression at 9 dpf. These observations could be supported by the idea that most of the relative growth-related gene expression changes would occur upon transition from embryo to larvae (3 dpf to 4 dpf). This idea is verified by significant increases in both *gH* and *igf1* expression between the embryonic and larval samples (1-3 dpf and 4-11 dpf, respectively).

Thyroid hormone receptor gene expression

The different thyroid hormone receptor isoforms evaluated, *TRα* and *TRβ,* show different expression patterns. *TRα* shows high levels of expression from 3 dpf up to 11 dpf, while *TRβ* shows high levels of expression from 2-5 dpf and at 9 dpf, but shows significant decreases in expression at 7 and 11 dpf. Decreases in whole body *TRβ* were identified, but increases in *TRα* were observed, unlike the findings of Filby and Tyler (2005). Increased expression of TRs is attributed to the larval thyroid hormone surge (Crane et al., 2004). Their study also indicated higher overall expression of *TRα* over *TRβ* (<20d), from which they concluded could indicate a more general role of *TRα* in circulation and a more specific, localized role of *TRβ*. This could potentially explain my findings of increased *TRα* as opposed to *TRβ* in response to the larval thyroid hormone surge.

Deiodinase enzyme gene expression

Like the thyroid hormone receptors, the different deiodinase isoforms demonstrated different patterns of expression up to 11d in fathead minnows. Although both *Dio1* and *Dio2* are responsible for elevated thyroid hormone bioavailability, *Dio1* specifically increases hormone (T_3) levels in bloodstream circulation while *Dio2* is more involved in the production of thyroid hormone that will bind to local TRs (Galton, 2005; Becker et al., 1997). The present study showed steady increase of *Dio2* expression, particularly in the larval stages, which could be attributed to larval tissue development. Similar results were found by Li et al. (2011) in larval Chinese rare minnows with increases of *Dio2* expression from 12 hpf up to 7d. Walpita et al. (2007) also observed post-hatch increases in *Dio2* expression in zebrafish. This increase in *Dio2* could be a response to the thyroid hormone surge upon hatch and the need to convert T_4 to T_3, the

version of thyroid hormone that actively engages tissues in metabolism and development-related processes (Crane et al, 2004).

Dio3, however, is responsible for depressions of thyroid hormone (T_3) availability. Levels of *Dio3* expression contrasted the patterns of *Dio2* expression, with highest levels during embryonic time points up until 24 h post-hatch. It is expected that Dio3 expression would be lowest during the first days post-hatch, where the organism needs high concentrations of T_3 for metabolic processes. In a study by Crane et al. (2004), fathead minnows showed highest levels of T_3 at 9 dpf; the 9 dpf time point in the present study included significantly high levels of *Dio1* and *Dio2* expression and low levels of *Dio3* expression. This could be explained by the role of *Dio1/Dio2* in increasing T_3 availability and of *Dio3* in decreasing its availability.

Value of ontogenetic expression profiles in toxicity testing

Ontogenetic profiles are valuable tools for toxicity testing that seeks to utilize gene expression as an endpoint for gene expression. Understanding the normal levels of the target gene's expression (i.e. when the organism is not exposed to the contaminant) at a certain time point aids in predicting changes due to contaminant exposure when alterations in expression are identified. Knowing the time points of highest expression of growth- and thyroid-related genes gives those conducting toxicity tests insight as to when the organism will most likely exhibit contaminant-induced changes, which would be the ideal time point to evaluate that target gene. For example, 5 dpf larvae would more likely show exposure-related decreases in *gH* expression in comparison to 3 dpf embryos, since 1-3 dpf samples do not exhibit high enough baseline expression to be able to detect

effects due to a contaminant. Therefore a toxicity test that seeks to use growth hormone as an endpoint should evaluate this expression in 5 dpf samples.

Additionally, if a toxicity test seeks to determine gene expression changes at a certain time point, having ontogenetic profiles of different genes gives one an idea of which genes would most likely demonstrate exposure-related effects at that time point. If a toxicity test seeks to identify gene expression changes at 2 dpf, measuring changes in *Dio1* expression would be more valuable then measuring *Dio2* changes. This type of information becomes particularly valuable for early life-stage toxicity tests that strictly utilize organisms of a particular developmental stage, such as the FET and LGS tests.

Relevance to current toxicity testing models

The FET and LGS tests particular benefit from these ontogenetic expression profiles because they specifically utilize early life-stage organisms. This study provides growth- and thyroid-related gene expression data for five larval time points involved in the LGS test—4, 5, 7, 9, and 11 dpf. Ontogenetic profiles for these genes may aid in the utility of molecular biomarkers of growth inhibition as a sublethal endpoint in the LGS test. However, these profiles are particularly useful to the employment of the FET test, since the literature lacks ontogenetic expression profiles for embryonic fathead minnows. There has been a recent push to further the utility of the FET in the United States to reduce the harm and stress imposed on the animals involved in these testing methods. Because embryos undergo less stress than their larval, juvenile, and mature counterparts, the FET test is an ideal solution for these concerns. Providing embryonic ontogenetic expression data for growth- and thyroid-related genes provides information that could

contribute to the addition of gene expression as a FET test metric, which would offer a sublethal endpoint for the test and therefore increase the test’s utility.

Conclusions

By creating these ontogenetic expression profiles, the time points of the statistically highest levels of expression were identified for each gene (Figure 6). Identifying these alterations in expression across these genes not only contributes to toxicity testing efficiency and effectiveness, but can also predict, to some degree, events in fathead minnow physiological development across these time points. Future directions involve the use of common contaminants to identify changes in expression of these growth- and thyroid-related genes at the time points that correspond with highest levels of expression to assure that contaminant-induced expression changes can be identified. Other areas for future research include utilizing stress-related genes (heat-shock proteins, corticotropin releasing factor, etc.) in ontogenetic expression profiles due to their value as sublethal endpoints in toxicity testing.

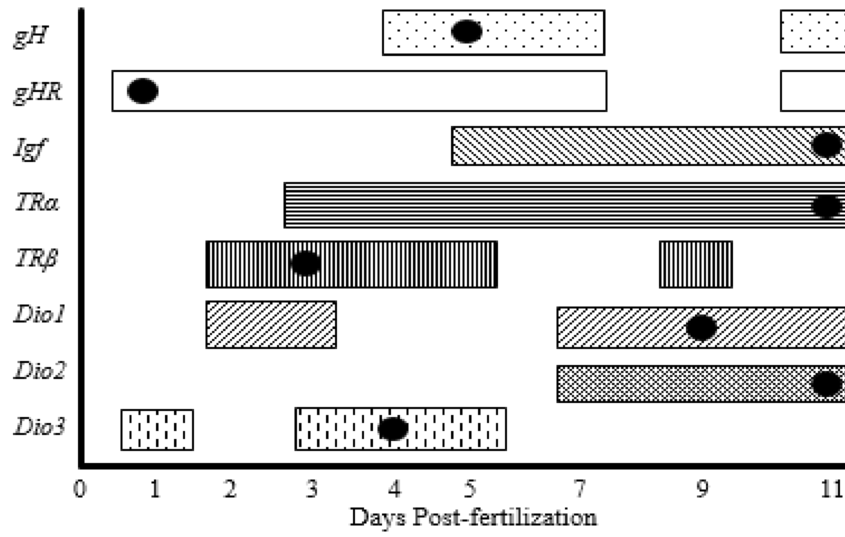

Figure 6. Time points of highest levels of gene expression per gene according to statistical significance; time points with maximal levels of gene expression for a gene are indicated by a black oval.

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