



Developmental expression and estrogen responses of endocrine genes in juvenile yellow perch (*Perca flavescens*)

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ABSTRACT

The present study examines the expression of growth-regulating genes (*gh*, *prl*, *smtl* and *igf1b*), the estrogen receptors (*esr1* and *esr2a*) and aromatase (*cyp19a1a*) in developing yellow perch. To gain an initial understanding into the endocrine control of growth preceding and involved with sexual size dimorphism (SSD), where females have been reported to grow faster and larger than males, young of the year fish were sampled for length, weight and tissues at several time points (102–421 days post-hatch (dph)). Positive growth was seen in both sexes over the sampling interval, but SSD was not manifested. Using real-time quantitative PCR, we found that pituitary growth hormone (*gh*) and liver insulin-like growth factor-1b (*igf1b*) mRNA levels were significantly affected by dph and levels were found to be correlated with growth in both sexes. Liver *cyp19a1a*, *esr1* and *esr2a* mRNA levels were significantly influenced by dph, whereas there was a significant dph * sex interaction on liver *esr2a* mRNA levels with males having higher levels than females at 379 and 421 dph. Ovarian *cyp19a1a* decreased with dph, but there were no changes in *esr1* or *esr2a* mRNA levels. Dietary treatment of juvenile (~300 dph) females with 20 mg/kg diet 17 β -estradiol resulted in significantly higher liver *esr1* mRNA levels and a sustained hepatosomatic index (I_H). Across all data sets liver *esr2a* mRNA levels showed the most significant positive correlation with liver *igf1b* mRNA levels. These findings show that growth is accompanied by increases in pituitary *gh*, liver *igf1b* and liver *esr1* and *esr2a* mRNAs in juvenile yellow perch.

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1. Introduction

The yellow perch is one of a group of economically and ecologically important fishes that exhibits sexual size dimorphism (SSD) in which females grow faster than males. Female-biased SSD in yellow perch has been demonstrated in the laboratory [52,53] and in wild populations [8,22,23], although this phenomenon has not been consistently seen in other laboratory and field studies [25,46,57]. Subsequent to these earlier studies, Malison et al. [32] found that low dosages of E₂ (2 and 20 μ g/g diet) significantly stimulated growth in yellow perch and the growth promoting effects were only noticeable in fish of 80–110 mm total length (TL) or greater. This critical size range is the same size that female-biased SSD begins to be manifested [52] and also appears to be the minimum body size for the onset of vitellogenesis and sper-

matogenesis in females and males [33], respectively. This observed estrogen responsiveness could point toward changes in the expression of estrogen receptors (ERs: *esr1*, *esr2a*, *esr2b*) on target tissues (liver, ovary or pituitary) and suggests a linkage between growth and estrogen physiology in this species.

In vertebrates, the pituitary gland plays a critical role in the regulation of aspects of growth and reproduction. Pituitary growth hormone (Gh) together with its anabolic intermediary, insulin-like growth factor-I (Igf-1), primarily produced in the liver, are considered to be the key components of the endocrine (somatotrophic) growth axis in teleosts [15]. There is increasing evidence that somatolactin (Smtl), another pituitary hormone found only in fish, is involved in metabolism, background adaptation, sexual maturation and reproduction [27,35,40,47,69]. Prolactin (PrI), another pituitary hormone, is well known for its involvement in fish osmoregulation and reproduction, but has also been shown to be associated with growth and metabolism in fish [51,55,56,67]. Estrogen receptors (ERs) show an ubiquitous distribution in many teleost tissues (including brain, pituitary, gonad, liver and scales) and are intricately involved in sexual determination and development [10,16,20,29,41,45,64]. Estrogen levels, however, are controlled

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primarily by P450 aromatase (*cyp19a1a*), the terminal enzyme in the conversion of androgens to estrogens. *Cyp19a1a* mRNA is expressed in many tissues associated with reproduction (gonad, brain and pituitary) [26,29] and growth (liver, brain and pituitary) [29,37], and its expression varies with sexual maturation [36].

The cDNAs for yellow perch *gh*, *prl*, *smtl*, *igf1b*, *esr1*, *esr2a* and *cyp19a1a* have been recently cloned and characterized [28,29,48] and qPCR assays to measure mRNA or expression levels of these genes have been developed [19,30]. While estrogen is believed to be involved in SSD it remains vague how SSD is manifested in yellow perch, so there is a need to understand the baseline sex-specific endocrine changes in juveniles of this species. As an initial step toward understanding the endocrine pathways involved with growth and development in yellow perch, this study examines the developmental and estrogen-dependent expression of these key endocrine genes in juvenile males and females of this important perciform teleost.

2. Materials and methods

2.1. Developmental samples

Yellow perch were hatched on April 17th, 2003 at The Ohio State University Aquaculture Research Center in Piketon, OH, and were sampled at several time points from July 2003 to June 2004, measured as days post-hatch (dph). Perch fry were hatched from egg ribbons in tanks and released into fertilized aquaculture ponds (0.10 ha, 2.13 m depth) for a period of 6 weeks. On June 1 (45 dph), ponds were seined and juvenile perch were moved to indoor flow through tanks (~1 L/min, ≤1 fish/4 L) on a pond:well water mix. While indoors, perch were kept at ambient light and constant aeration and fed a trout starter (50% protein, 17% fat) diet of Aquamax Starter (PMI Nutrition International, Inc., Brentwood, MO) at 7% body weight per day (BWD). From June 1 to June 25, perch were kept at 18–22 °C on a 50:50 pond:well water mix to optimize feed training. On June 25 (69 dph), fish were moved to new indoor tanks on a 25:75 pond:well water mix, which were maintained at a cooler temperature (16–18 °C). Previous experiments showed that the lower temperature prevents fungal contamination and maintains water quality. On July 25 (99 dph), yellow perch were transferred to an outside tank (2.13 m diameter, 0.91 m depth) and fed a new diet of Aquamax Grower 400 (PMI Nutrition International, Inc., Brentwood, MO). For the remainder of the study, perch were fed at 3% BWD and kept at ambient light and water temperature. Holding the perch outside at ambient temperature for a cold water period during winter was necessary to induce sexual maturation [24]. Water temperature was recorded daily and the temperature profile for the developmental period is shown in Fig. 1. Pond water temperature was measured 0.32 m from the pond bottom and both indoor and outdoor tank temperatures were measured at midpoint depth.

Fish were sampled at 102 (28 July), 152 (16 September), 195 (29 October), 236 (9 December), 282 (24 January), 330 (12 March), 379 (30 April) and 421 (11 June) dph. When sampled, animals were given a lethal dose of MS222, weighed (g) and measured for total length (mm) and then pituitary liver tissues were harvested. The animals were then sexed via gross examination of the gonads and ovary tissue was collected from females. Testes were collected from males only on 102 dph to verify sexing at that early time point. Tissues were immediately frozen and stored at –80 °C until analysis. All procedures were performed with the approval of both the University of Kentucky and The Ohio State University Committees on Animals Use and Care. Tissues were collected from animals on sample dates 102, 152, 195, 282, 379 and 421 dph. From this, a

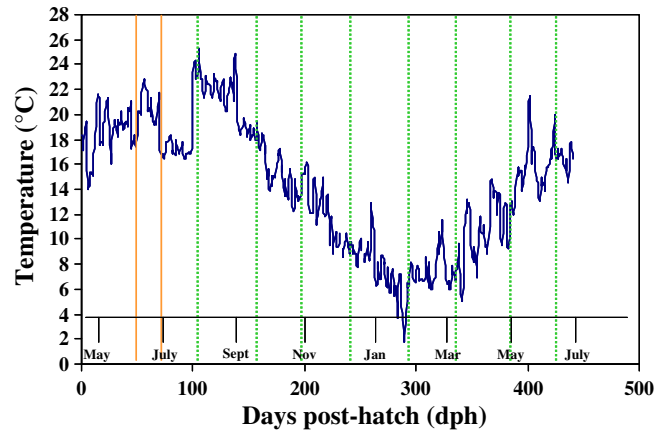


Fig. 1. Temperature (°C) profile for yellow perch during juvenile development. The left solid vertical line approximately indicates June 1 (45 dph) when perch were moved from the aquaculture pond to an indoor tank for feed training. The right solid vertical line approximately indicates June 25 (69 dph) when perch were moved to a cooler indoor holding tank. The fish were moved to an outdoor tank on July 25 (99 dph), 3 days prior to the first sampling. The dashed lines approximately indicate sampling dates at 102, 152, 195, 236, 282, 330, 379 and 421 dph.

subset of samples were randomly selected ($n = 6/\text{sex}$) for molecular analyses.

2.2. Dietary E_2 study

In a separate experiment, 10-month-old juvenile female yellow perch (~300 dph) were kept at the University of Wisconsin-Madison Lake Mills Aquaculture Facility in flow through tanks (~1 L/min) at ~21 °C with a 16:8 light:dark cycle and constant aeration. Perch were kept at a density of <1 fish/4 L and fed *ad libitum* with Aquamax Grower 400 (PMI Nutrition International, Inc., Brentwood, MO). Thirty perch (17 g ± 5.6 g) received a diet with 20 mg/kg 17 β -estradiol (E) while another 30 received untreated (U) food. Fifteen perch per treatment were sampled after 7 days and the remaining 15 perch/treatment were sampled after 28 days of exposure, providing four treatment groups (U7, E7, U28 and E28). When sampled, yellow perch were given a lethal dose of MS222, weighed (g) and sexed via gross examination of the gonads. Whole livers were removed and weighed from females and immediately frozen and stored at –80 °C until analysis. Hepatosomatic index (I_H) was later calculated as the percent liver weight of total body weight. All procedures were performed with the approval of both the University of Kentucky and University of Wisconsin Committees on Animals Use and Care.

2.3. Real-time qPCR

Total RNA was extracted from all samples with the GenElute™ Mammalian Total RNA Kit (Sigma, St. Louis, MO) and treated with amplification grade DNase I (Sigma, St. Louis, MO). Diluted RNA samples were quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), RNA quality verified by gel electrophoresis as previously described [28–30] and total RNA was reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). cDNA samples were quantified on a NanoDrop ND-1000 prior to quantitative PCR (see below).

Generally qPCR assays employ one of two standard procedures: relative or absolute quantification. Relative quantification compares expression of the target gene with three or more reference genes, however, recent studies have shown that expression levels of housekeeping (reference) genes can vary considerably within a sample set [3,14,54]. Given the limited number of reference genes

available in yellow perch at the time of this study and a lack of knowledge regarding their expression levels, we chose to utilize absolute qPCR [9,54]. Sequences for yellow perch *gh* (AY007303), *prl* (AY332491), *smtl* (AY332490), *igf1b* (AY332492), *esr1* (DQ984124), *esr2a* (DQ984125) and *cyp19a1a* (DQ984126) are available from the GenBank/EMBL/DDBJ nucleotide sequence database. Primer design, product identity verification, generation of standards and absolute qPCR are detailed in Lynn et al. [30]. Briefly, primers (Table 1) were designed and tested with appropriate cDNA templates and PCR products were visualized with electrophoresis, purified and sequenced for confirmation of identity.

Purified PCR products were serially diluted to generate six standards (10,000, 1000, 100, 10, 1 and 0.1 fg μl^{-1}). Real-time qPCRs were prepared in 0.2 ml thin-wall 96-well plates as detailed in Lynn et al. [30]. Amplification and detection of samples were performed with the Bio-Rad iCycler Thermal Cycler and Optical Module (Bio-Rad, Hercules, CA). Each 96-well plate had duplicate wells of standards, no template, RNA template and samples. Duplicate sample C_t values with a coefficient of variation (CV) >2% were re-run. When necessary, dilutions of template cDNAs were performed to ensure samples fell within the standard curve. All plates had standard curves with $R > 0.98$ and PCR efficiencies between 85% and 110% with the exception of IGF-1b which had PCR efficiencies up to 114%. Melt curves were performed following all runs to further verify primer template specificity. Real-time qPCR results were standardized to total (input) RNA [17,59] and log transformed before statistical analyses.

2.4. Statistics

I_H proportions were arcsine transformed (arcsine of square root for each value) [60] and tested for deviations from a normal distribution before any statistical analyses were performed. Measurements of weight, length, I_H and mRNA levels were initially examined for statistical outliers within each variable by performing a box plot with all data from a dataset and identifying points beyond three times the interquartile (IQ) range. For the developmental dataset, only five data points (one 195 dph F *prl*, one 195 *smtl* F SL, one 282 dph F *gh*, one 282 dph ovary *esr1* and one 282 dph ovary *esr2a* mRNA level) were identified as statistical outliers and removed from the dataset. For the dietary E_2 study, no outliers were identified.

For the developmental samples, a general linear model (GLM) was used to examine each variable (weight, length and *gh*, *prl*, *smtl*, *igf1b*, liver *esr1*, liver *esr2a* and liver *cyp19a1a* mRNA levels) for dph, sex and dph * sex interaction effects. For each GLM procedure

the dph * sex interaction effect was sliced by dph to identify significant differences between males and females. For variables in which only female data exists (ovary *esr1*, ovary *esr2a* and ovary *cyp19a1a* mRNA levels) a GLM was used to examine for dph effects only. Tukey's *post hoc* pairwise comparisons were performed on all variables showing a significant effect of dph.

For the E_2 dosing samples, a general linear model (GLM) was used to examine each variable (I_H and *igf1b*, liver *esr1*, liver *esr2a* and liver *cyp19a1a* mRNA levels) for treatment, day and treatment * day interaction effects. Differences among groups were further evaluated by two-way analysis of variance (GLM procedure) with treatment and day as independent variables. Pairwise comparisons between groups (e.g., E7, U28, etc.) were performed using Tukey's *post hoc* pairwise comparison for variables which showed a significant ($p \leq 0.05$) treatment, day or treatment * day effect. Relationships between variables were determined by generating a Pearson (r) correlation; significant differences from 0 were tested using Fisher's z transformation [68]. SYSTAT 13 for Windows (Systat, Chicago, IL) was used for all analyses and differences between groups and relationships between parameters were considered significant at $p \leq 0.05$.

3. Results

3.1. Developmental experiment

Average weights showed a significant ($p < 0.001$) effect of dph, but there were no effects of sex or dph * sex interaction on body weight and there were no significant differences between average male and female weights at any dph (Fig. 2a). Average weights, after pooling sexes, increased from 2.83 g at 102 dph to 22.45 g at 421 dph, but both sexes showed a decrease in growth rate from 200 to 400 dph which corresponded with seasonal decreases in temperature. Testes began to show milt production at 195 dph and maintained production through 379 dph until becoming clear and devoid of milt at 421 dph (S.G. Lynn, personal observation). Average length showed a significant ($p < 0.001$) effect of dph, but neither a sex nor a dph * sex interaction and there were no significant differences between average male and female length at any dph (Fig. 2b). Weight and length were highly correlated ($n = 148$; $r = 0.928$; $p < 0.001$).

Growth hormone (*gh*), prolactin (*prl*) and somatolactin (*smtl*) mRNA levels showed a significant ($p < 0.001$) effect of dph, but no sex or dph * sex interaction and there were no significant differences between average male and female levels at any dph (Fig. 3). Pituitary *gh* mRNA increased over the sampling interval and was

Table 1

Forward (F) and reverse (R) primer sequences and accession numbers used for gene specific qPCR assays.

Gene	Accession Nos.		Start	Primer sequence
<i>gh</i>	AY007303	F	224	CGG AGG AGC AGC GTC AAC
		R	370	CCC AGG ACT CGA CCA AAC G
<i>prl</i>	AY332491	F	304	ACC AGG CTC TTC AAG TAT CAG
		R	406	GTG TTA GCA GAG GTG GAG AG
<i>smtl</i>	AY332490	F	321	CTC CAA AGG TGA AAT CCA ACA G
		R	449	TCA GGA GCG GCA TCG TAG
<i>igf1b</i>	AY332492	F	539	CGC AGG GCA CAA AGT GGA C
		R	686	CCC AGT GTT GCC TCG ACT TG
<i>esr1</i>	DQ984124	F	1072	AGG TGC TGA TGA TCG GGC TC
		R	1165	TCG CCT ACG TTC CTG TCC AG
<i>esr2a</i>	DQ984125	F	1887	TCT GGA CGC TGT GAC GGA C
		R	1969	GGG CGA GGC GGG TGT AC
<i>cyp19a1a</i>	DQ984126	F	278	TCT GGG TTT GGG GCC ACT TC
		R	427	ACC GCT GAT GCT CTG CTG AG

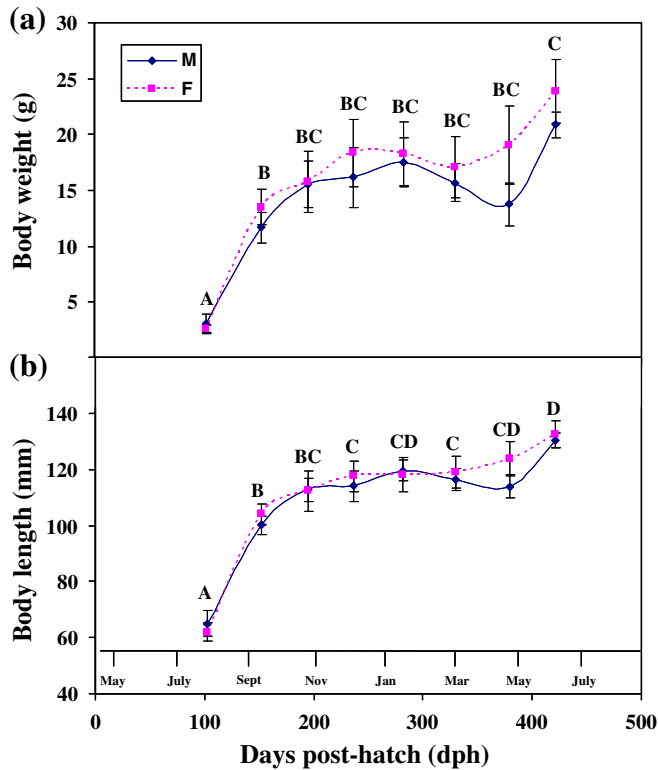


Fig. 2. Average weights and lengths of male (M) and female (F) yellow perch during juvenile development. Bars indicate positive and negative standard error and letters indicate significant differences between dph. Samples sizes by dph: 102 (M = 5, F = 7); 152 (M = 10, F = 9); 195 (M = 11, F = 8); 236 (M = 8, F = 12); 282 (M = 9, F = 11); 330 (M = 10, F = 10); 379 (M = 9, F = 10); 421 (M = 9, F = 10). The GLM for: (a) weight indicates a significant effect of dph ($F_{7,132} = 8.98$; $p < 0.001$) and no effect of sex ($F_{1,132} = 2.28$; $p = 0.13$) or dph * sex interaction ($F_{7,132} = 0.28$; $p = 0.96$). (b) Length indicates a significant effect of dph ($F_{7,132} = 28.58$; $p < 0.001$) and no effect of sex ($F_{1,132} = 0.86$; $p = 0.36$) or dph * sex interaction ($F_{7,132} = 0.37$; $p = 0.92$).

found to be significantly correlated with body length ($n = 71$; $r = 0.52$; $p < 0.001$) and body weight ($n = 71$; $r = 0.58$; $p < 0.001$) in both sexes. Pituitary *prl* mRNA showed very little variation over the sampling intervals except for a sharp increase in both sexes at the last sampling point (421 dph; June). Pituitary *smtl* mRNA levels were erratic over the sampling interval and showed no discernable seasonal or sex-specific patterns.

Liver *igf1b*, *esr1* and *cyp19a1a* mRNA levels showed a significant ($p < 0.001$) effect of dph, but no sex or a dph * sex interaction and there were no significant differences between average male and female levels at any dph (Fig. 4). Liver *igf1b* mRNA levels were significantly correlated with body weight ($n = 72$; $r = 0.52$; $p < 0.001$), body length ($n = 72$; $r = 0.67$; $p < 0.001$) and pituitary *gh* mRNA levels ($n = 71$; $r = 0.52$; $p < 0.001$) (Fig. 4a). Both liver *esr1* and *cyp19a1a* mRNA levels showed a significant effect of dph ($p < 0.001$) but no sex or dph * sex interaction (Fig. 4b and d). Liver *esr2a* mRNA levels showed a significant effect of dph ($p < 0.001$), sex ($p = 0.002$) and dph * sex interaction ($p < 0.001$), and at both 379 and 421 dph average male levels were significantly higher than average female levels (Fig. 4c). Additionally, liver *esr2a* mRNA levels showed the most significant positive correlation with liver *igf1b* mRNA levels ($n = 72$; $r = 0.84$; $p < 0.001$).

In the females sampled over this seasonal interval, neither ovary *esr1* nor ovary *esr2a* mRNA levels showed a significant effect of dph whereas ovarian *cyp19a1a* did show a significant ($p < 0.001$) effect of dph with levels decreasing over time (Fig. 5). At 102 dph there was no significant difference in gonadal *esr2a* mRNA levels between females (Fig. 5) and males ($\bar{X} = 8.21 \pm 0.19$; $n = 5$; $p = 0.093$) (male data not shown). However, male testes (data not

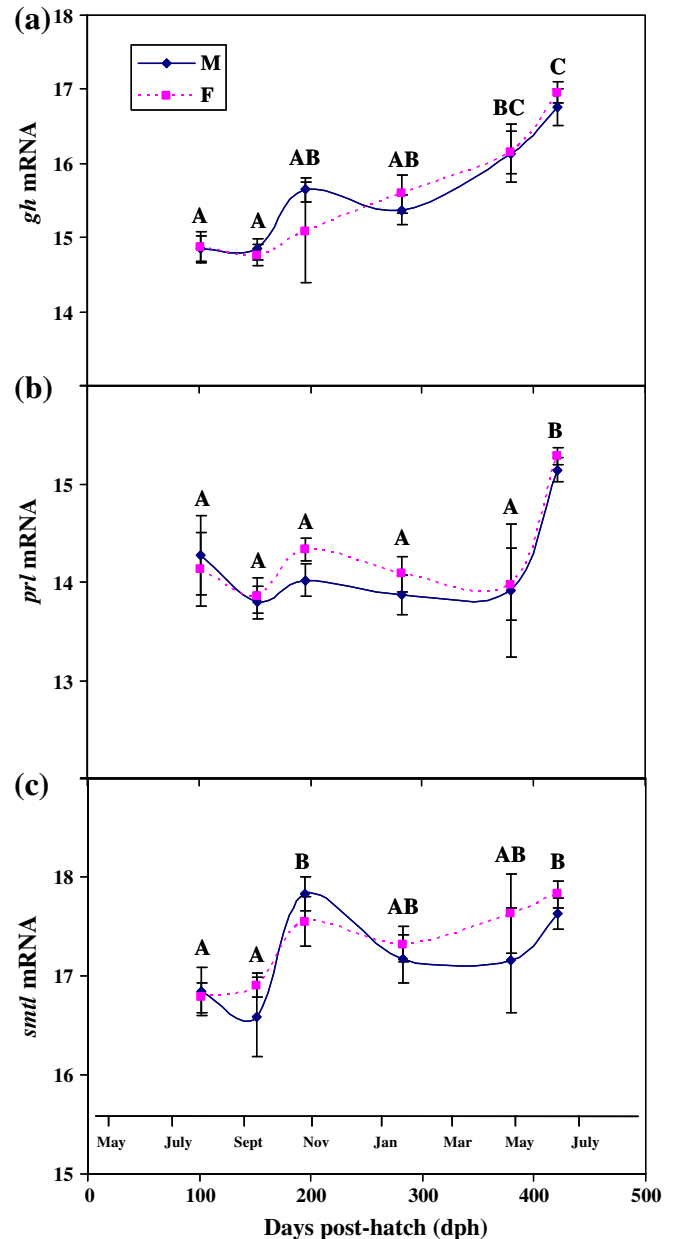


Fig. 3. Average pituitary *gh*, *prl* and *smtl* mRNA levels from male (M) and female (F) yellow perch during juvenile development. Units are expressed as $\ln(\text{ag of mRNA}/\mu\text{g of total RNA})$. Bars indicate positive and negative standard error and letters indicate significant differences between dph. Samples size for each sex-specific dph is $n = 6$ (102 dph F = 7, M = 5; 282 dph F = 5). The GLM for: (a) *gh* indicates a significant effect of dph ($F_{5,59} = 17.58$; $p < 0.001$) and no effect of sex ($F_{1,59} = 0.05$; $p = 0.83$) or dph * sex interaction ($F_{5,59} = 0.57$; $p = 0.72$). (b) *prl* indicates a significant effect of dph ($F_{5,59} = 6.63$; $p < 0.001$) and no effect of sex ($F_{1,59} = 0.44$; $p = 0.51$) or dph * sex interaction ($F_{5,59} = 0.15$; $p = 0.98$). (c) *smtl* indicates a significant effect of dph ($F_{5,59} = 5.46$; $p < 0.001$) and no effect of sex ($F_{1,59} = 0.82$; $p = 0.37$) or dph * sex interaction ($F_{5,59} = 0.56$; $p = 0.73$).

shown) at 102 dph had significantly lower *esr1* ($\bar{X} = 6.11 \pm 0.34$; $n = 5$; $p = 0.019$) and *cyp19a1a* ($\bar{X} = 4.66 \pm 0.03$; $n = 5$; $p < 0.001$) mRNA levels than female ovaries.

3.2. Dietary E_2 study

In juvenile female perch fed an estrogen-treated diet, there was a significant effect of sampling day ($p = 0.02$) on hepatosomatic index (I_H) but no effect of treatment or treatment * day interaction (Fig. 6a). Estrogen treatment prevented a decrease of I_H over the

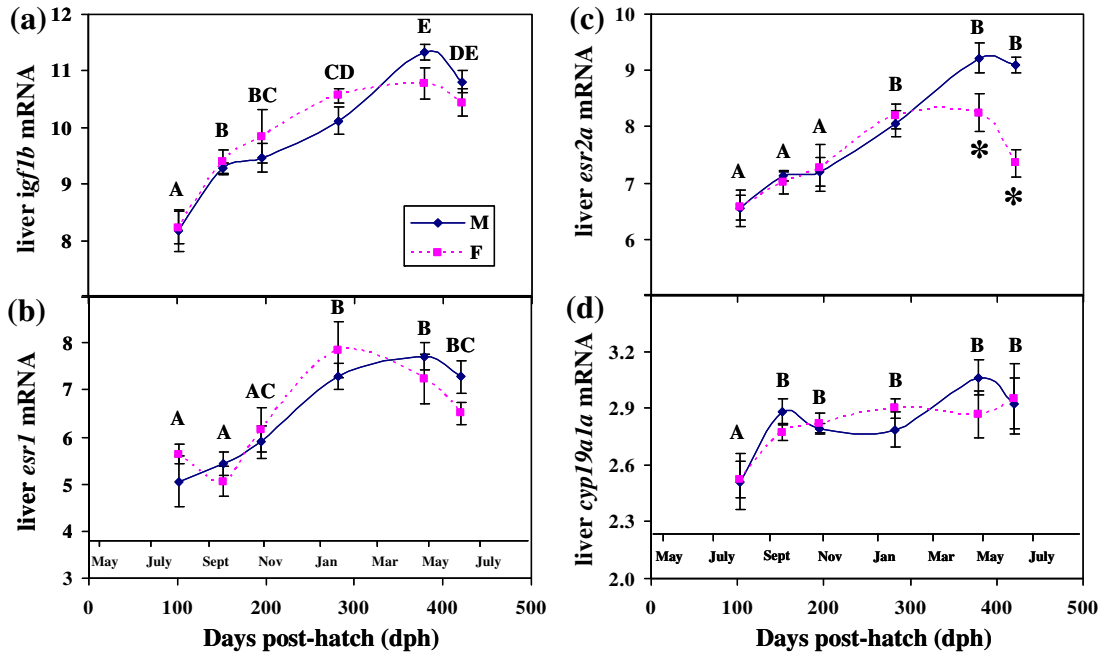


Fig. 4. Average liver *igf1b*, *esr1*, *esr2a* and *cyp19a1a* mRNA levels from male (M) and female (F) yellow perch during juvenile development. Units are expressed as $\ln(\text{ag of mRNA}/\mu\text{g of total RNA})$. Bars indicate positive and negative standard error and letters indicate significant differences between dph. Samples size for each sex-specific dph is $n = 6$ (102 dph $F = 7$, $M = 5$). The GLM for: (a) *igf1b* indicates a significant effect of dph ($F_{5,60} = 37.04$; $p < 0.001$) and no effect of sex ($F_{1,60} = 0.01$; $p = 0.94$) or dph * sex interaction ($F_{5,60} = 1.39$; $p = 0.24$). (b) *esr1* indicates a significant effect of dph ($F_{5,60} = 17.60$; $p < 0.001$) and no effect of sex ($F_{1,60} = 0.04$; $p = 0.85$) or dph * sex interaction ($F_{5,60} = 1.39$; $p = 0.24$). (c) *esr2a* indicates a significant effect of dph ($F_{5,60} = 24.65$; $p < 0.001$), sex ($F_{1,60} = 10.34$; $p = 0.002$) and dph * sex interaction ($F_{5,60} = 5.22$; $p < 0.001$). Asterisks indicate dphs (379 and 421) where male and female levels are significantly different at $p \leq 0.05$. (d) *cyp19a1a* indicates a significant effect of dph ($F_{5,60} = 5.78$; $p < 0.001$) and no effect of sex ($F_{1,60} = 0.15$; $p = 0.70$) or dph * sex interaction ($F_{5,60} = 0.71$; $p = 0.62$).

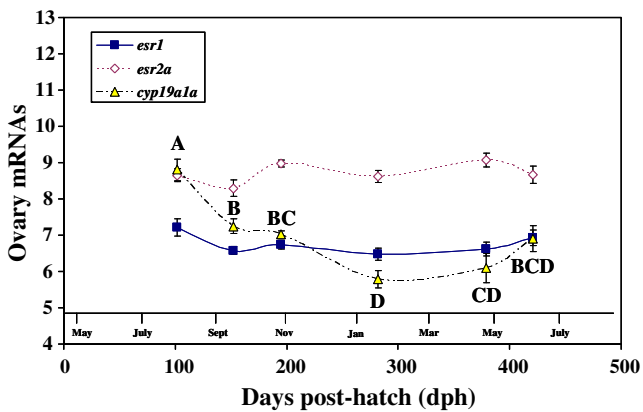


Fig. 5. Average ovary *esr1*, *esr2a* and *cyp19a1a* mRNA levels from female yellow perch during juvenile development. Units are expressed as $\ln(\text{ag of mRNA}/\mu\text{g of total RNA})$. Bars indicate positive and negative standard error and letters indicate significant differences between dph for ovary *cyp19a1a* only. Samples size for each dph is $n = 6$ (102 dph $n = 7$; 282 dph *esr1* $n = 5$; 282 dph *esr2a* $n = 5$). The GLMs indicate a significant effect of dph on *cyp19a1a* ($F_{5,31} = 17.32$; $p < 0.001$), but no significant effect on *esr1* ($F_{5,30} = 2.19$; $p = 0.06$) or *esr2a* ($F_{5,30} = 2.48$; $p = 0.054$).

experimental period and *post hoc* analysis reveals that untreated fish on day 7 (U7) had a significantly ($p = 0.04$) higher average I_H than untreated fish on day 28 (U28). Liver *esr1* mRNA levels showed a significant effect of day ($p = 0.02$), treatment ($p < 0.001$) and day * treatment interaction ($p = 0.001$) with levels increasing over time in the E_2 -treated group (Fig. 6b). *Post hoc* analysis reveals that treated fish on day 28 (E28) had a significantly ($p \leq 0.001$) higher average liver *esr1* mRNA level than any other group. Treated fish on day 7 (E7) had a significantly higher average liver *esr1* mRNA level than untreated fish on day 28 (U28) ($p = 0.045$). Liver *cyp19a1a* mRNA levels showed a significant effect of day

($F_{1,27} = 9.55$; $p = 0.005$) but no effect of treatment or day * treatment interaction (Fig. 6c). The *post hoc* analysis did not show any significant differences in average *cyp19a1a* mRNA levels for each specific treatment within sample day. Liver *igf1b* mRNA levels (data not shown) did not show a significant effect of day ($F_{1,27} = 0.00$; $p = 1.0$), treatment ($F_{1,27} = 0.55$; $p = 0.47$) or day * treatment interaction ($F_{1,27} = 1.13$; $p = 0.30$). Liver *esr2a* mRNA levels (data not shown) did not show any significant effect of day ($F_{1,27} = 0.007$; $p = 0.93$), treatment ($F_{1,27} = 0.59$; $p = 0.45$) or day * treatment interaction ($F_{1,27} = 0.28$; $p = 0.60$). Among all the variables measured in this dietary E_2 study, liver *igf1b* mRNA levels and liver *esr2a* mRNA levels (data not shown) showed the most significant positive correlation overall ($n = 31$; $r = 0.96$; $p < 0.001$) and within both the E_2 treated ($n = 17$; $r = 0.99$; $p < 0.001$) and untreated groups ($n = 14$; $r = 0.89$; $p < 0.001$).

4. Discussion

The present study provides an examination of key endocrine genes thought to be involved with growth and development in juvenile male and female yellow perch. While SSD was not manifested in this study, the data shows that the expression of pituitary growth hormone (*gh*) mRNA increases with age and size in juvenile yellow perch, that similar patterns exist for important endocrine genes expressed in the liver (estrogen receptors, insulin-like growth factor-I and aromatase) and that dietary estrogen increases liver estrogen receptor expression. Combined, the correlations between these genes during development and the sex-specific differences in estrogen receptor expression (developmental and dietary estrogen dosing) suggest that estrogen receptor gene expression may be involved with development and growth in yellow perch, although more work is needed to determine whether these genes are involved with the onset and occurrence of SSD in this species.

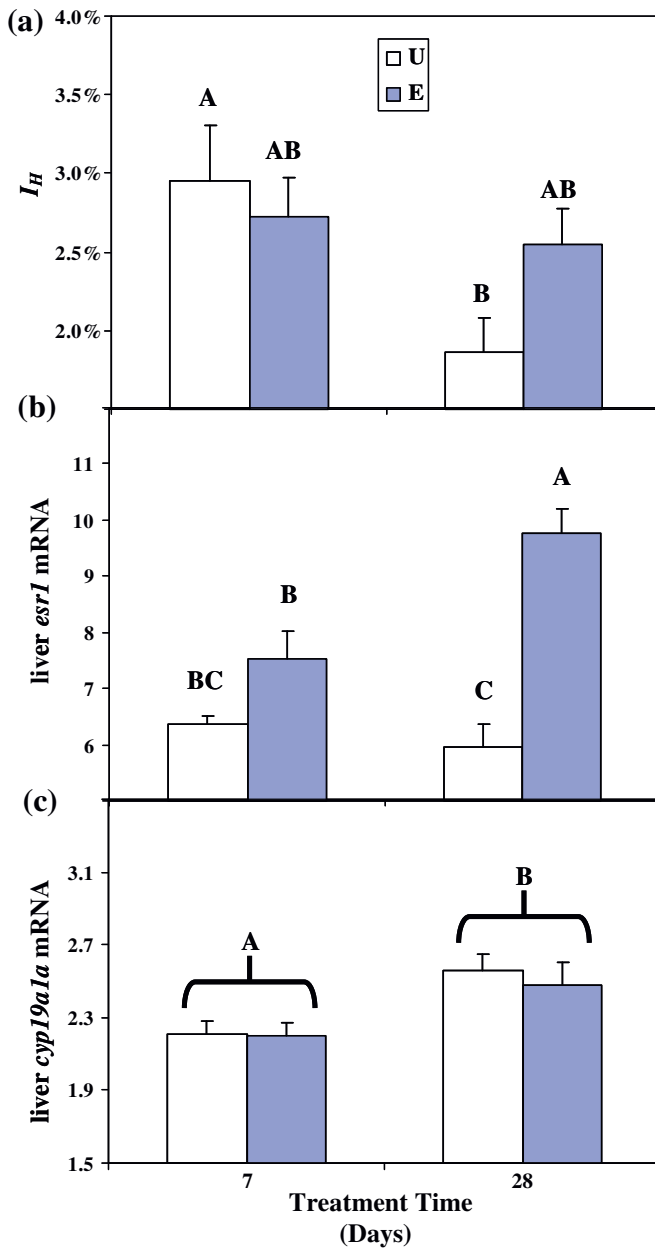


Fig. 6. Measurements from juvenile female yellow perch after 7 or 28 days fed an untreated diet (U) or a 20 mg/kg E_2 diet (E) *ad libitum*. Units for I_H (hepatosomatic index) are expressed as percent liver of body mass and units for liver *esr1* and liver *cyp19a1a* are expressed as $\ln(\text{ag of mRNA}/\mu\text{g of total RNA})$. Bars indicate standard errors and bold letters (A, B, etc.) designate statistical significance at $p \leq 0.05$. Sample sizes (n) are: U7 = 8; E7 = 7; U28 = 6; and E28 = 10. The GLM for: (a) I_H indicates a significant effect of day ($F_{1,27} = 5.90$; $p = 0.02$) and no effect of treatment ($F_{1,27} = 0.80$; $p = 0.38$) or day * treatment interaction ($F_{1,27} = 3.08$; $p = 0.09$). (b) Liver *esr1* indicates a significant effect of day ($F_{1,27} = 6.12$; $p = 0.02$), treatment ($F_{1,27} = 44.98$; $p < 0.001$) and day * treatment interaction ($F_{1,27} = 12.50$; $p = 0.001$). (c) Liver *cyp19a1a* indicates a significant effect of day ($F_{1,27} = 9.55$; $p = 0.005$), but no effect of treatment ($F_{1,27} = 0.19$; $p = 0.67$) or day * treatment interaction ($F_{1,27} = 0.15$; $p = 0.70$).

Many of the genes analyzed perceivably changed expression patterns after 195 dph when seasonal temperatures dropped below $\sim 15^\circ\text{C}$. While the ambient winter conditions may play a role in the expression patterns measured in this study, they should also more closely represent conditions (as opposed to thermostatic rearing conditions) that occur in wild populations of yellow perch, as well as perch raised in outdoor ponds where substantial variation in environmental parameters is seen. The juvenile male and

female perch from this study exhibited seasonal growth, which has been reported in aquaculture ponds [65,66] and is to be expected. Purchase et al. [46] give an average length at sexual maturation in yellow perch from 72 Ontario lakes at 81.5 mm for males and 141 mm for females. In our study, males reached an average length of 81.5 mm between 102 and 152 dph, just prior to the milt production noticed at 195 dph, while the maximum average female length of 132 mm at 421 dph falls short of the 141 mm cited above. While the females in this study may not have sexually matured by 421 dph, or have displayed SSD, the results of this study provide needed insight into potential endocrine pathways that could be involved in early development and sexual maturation in both sexes (specifically expression of liver estrogen receptors and insulin-like growth factor-1).

The literature is replete with examples of SSD occurring in laboratory and field studies of yellow perch [8,22,23,52,53]. While this phenomenon is known to occur, its absence in this study should not be surprising [25,46,57]. In the most comprehensive examination of SSD in yellow perch, Shewmon and co-workers [57] suggest that natural photoperiod and temperature are necessary for SSD to occur, however, such conditions did not ensure that SSD was sustained at the final sampling in their study. Additionally, in a growth study conducted by Jentoft et al. [25], wherein temperature and photoperiod were controlled (not natural), animals in some experiments showed SSD whereas others did not. Population/strain differences, onset of sexual maturation and/or endogenous steroid levels may be involved in the manifestation of SSD, although the actual conditions necessary are not clearly established and the precise mechanism(s) is unknown [57]. While SSD was not seen in the animals from this study, in the absence of any similar data in yellow perch, these findings are a significant and necessary foray into identifying potential endocrine pathways involved with sex-specific growth and development in juvenile perch exposed to seasonal cues.

Given the importance of the Gh, Prl, Smtl and Igf1 proteins in vertebrate growth and development, numerous studies have examined the early larval and seasonal expression of the genes for these proteins in several teleost species, but none have examined this through the first year of development or on a sex-specific basis in a fresh water perciform teleost. Although recent studies have begun evaluating the hormonal, sex-specific and seasonal regulation of *gh*, *prl*, *smtl* and *igf1b* mRNAs in adult yellow perch, comparison with animals from this study is difficult due to the differences in life-history stages [19,25,30,48]. As a consequence, the most comparable studies with which to compare our results in juvenile yellow perch are studies conducted in the economically important marine teleosts such as the gilthead sea bream (*Sparus aurata*) and the European sea bass (*Dicentrarchus labrax*) [13,39]. Since the European sea bass [50], like yellow perch, displays sexually-dimorphic growth we have chosen to compare our data with that of de Celis and co-workers [13] who examined plasma Gh, Smtl and Igf1 levels in male European sea bass over an annual cycle. In the sea bass, growth tended to be linear throughout the season, reached a plateau at 1300 g and plasma Igf1 levels were highly correlated with specific growth rate [13]. Although absolute sizes are different between yellow perch and sea bass, we observed similar trends in growth. In yellow perch from this study, we found that pituitary *gh* mRNA and liver *igf1b* mRNA levels increased over the sampling interval. In fact, both mRNAs were significantly correlated with body weight and length over the sampling time as well as there being a significant correlation between pituitary *gh* mRNA levels and liver *igf1b* mRNA levels. Igf1 has been shown to be Gh dependent in a number of salmonid and non-salmonid teleost species wherein a positive relationship between growth and Igf1 (and its mRNA, *igf1*) have also been demonstrated [4,13,18,58,61,62]. Our findings of a positive correlation between

igf1b and growth and *igf1b* and *gh* support the notion that *gh* and *igf1b* are involved with growth in yellow perch. While this information fills in an important knowledge gap in our understanding of the somatotrophic axis in yellow perch growth, the physiological control and effects of Gh and Igf1 in yellow perch remain poorly understood [25] and will require further study.

The developmental expression of pituitary *prl* mRNA levels was unremarkable except for a peak in pituitary *prl* mRNA expression levels which occurred at 421 dph (June) in both sexes. The influence of season on *smtl* mRNA levels is difficult to interpret but a recent study reported higher levels in adult perch sampled in spring versus autumn [30]. The results seen in adult wild perch [30] and cultured juvenile perch from this study underscore the need for a better understanding of how these genes (*prl* and *smtl*), and their products, are regulated and function at different life stages and between populations. By example, work in masu salmon has shown that there are significant changes in *gh*, *prl* and *smtl* mRNA levels at different stages of gonadal maturation [5,42] and the responses to E₂ in females were dependent on reproductive stage, with significant increases in *gh*, *prl* and *smtl* mRNA levels only in the pre-spawning stage and no effects before gonadal maturation or at the maturing stage [42].

Liver estrogen receptor α (*esr1*) mRNA levels showed significant changes over the sampling period and the elevated levels of liver *esr1* mRNA after 195 dph, in both sexes, indicate a potential shift in the estrogen responsiveness of the liver during development in yellow perch. Additionally, levels of liver estrogen receptor β (*esr2a*) mRNA became significantly elevated in juvenile male yellow perch (versus females) at 379 and 421 dph. While there are no previous reports of a developmental sex-specific liver estrogen receptor (ER) expression pattern in this species, there are related reports in other teleost species. A study by Filby and Tyler [16] showed a significant increase in liver *esr1* mRNA levels from 40 to 120 dpf and no changes in liver *esr2a* mRNA levels in both male and female fathead minnow. Liver *esr1* mRNA levels were significantly higher in female fathead minnow, but there were no differences in liver *esr2a* mRNA levels between the sexes and at 150 dpf there were no sex-specific differences in either liver *esr1* or *esr2a* mRNA levels. Although the time between fertilization and hatching in the fathead minnow is less than one week, the dpf sampling times used in Filby and Tyler [16] do not likely correspond well with the dph used in this study as time to sexual maturation in each species is quite different [46,63]. Davis and co-workers [12] reported that liver *esr2a* mRNA levels were higher in adult male tilapia and liver *esr1* mRNA levels were higher in female tilapia. A similar pattern was seen in adult European sea bass [21] and adult yellow perch [30]. While Halm et al. [21] and Blázquez et al. [6] examined mRNA levels of ERs in brain, pituitary and gonad tissues of juvenile (0–300 dph) European sea bass, there are few studies that examined the developmental ontogeny of estrogen receptors in the teleost liver and very little with which to compare the results of this study. As a consequence of a paucity of information in juvenile perch, the physiological significance of the relationship between sex and liver *esr2a* mRNA in yellow perch development and growth is unknown and will require further study.

In general, liver aromatase (*cyp19a1a*) mRNA levels oscillated over the sampling period and peaked at 379 and 421 dph for males and females, respectively (Fig. 4d). In this study, liver *cyp19a1a* expression increased from 102 to 152 dph which was followed by a significant increase in both liver ER mRNA levels from 195 to 282 dph. Though very little is known of the physiological significance of liver *cyp19a1a* in teleosts [44], these corresponding changes may suggest an autocrine (supplemental), *cyp19a1a*-dependent, E₂ production. Malison et al. [32] hypothesized that hepatic ERs were involved in growth and there was an upregulation

of these receptors at the critical size threshold for sexually related dimorphic growth. Though speculative and in need of further study, this autocrine E₂ production could drive increases in liver ER mRNA levels in a way that is preparatory for sex-specific growth in yellow perch.

In the ovary of developing perch, neither ER mRNA showed significant changes over the sample period, while ovarian *cyp19a1a* mRNA levels did show significant changes through the sample period (Fig. 5). At 379 dph (April) ovary *cyp19a1a* mRNA levels remained low but levels rebounded at 421 dph (June) which foreshadows the seasonal differences of ovary *cyp19a1a* mRNA levels seen in adult yellow perch [30]. Malison et al. [32,33] found that sexual differentiation of the gonads occurred around 16 mm TL and that a SSD began between 80 and 110 mm TL. In this study, there was a highly significant difference in gonadal *cyp19a1a* and *esr1* mRNA levels between males and females at 102 dph (60 mm). Although gonadal stage was not characterized in animals from this study, these changes in gonadal *cyp19a1a* and *esr1* mRNA may indicate a critical period for gonadal and sexual development in yellow perch.

A previous study found that the hepatosomatic index (*I_H*) in wild adult yellow perch (2.5–8 years in age) averages approximately 1.4% for females and 1.0% for males [30]. These adult yellow perch *I_H* levels were lower than levels seen in the dietary estrogen experiment in this study, which suggests that the *I_H* for yellow perch decreases as the fish matures (J.A. Malison, personal observation, Fig. 6a). The *I_H* data from the estrogen dosing experiment suggests that the fish were not completely mature and that E₂ dosing sustains an elevated juvenile *I_H*.

Liver *esr1* mRNA was the only parameter measured to show a significant effect of dietary estradiol treatment (Fig. 6b). Many studies have shown an increase in both female and male liver *esr1* mRNA levels in response to E₂ treatment [1,2,7,31,43,49] as the teleost *esr1* gene is known to have an estrogen response element (ERE) in the promoter [38]. Sabo-Attwood et al. [49] measured liver mRNA of all three ERs in male largemouth bass following injections of E₂ and found that liver *esr1* mRNA levels were much more sensitive than liver *esr2a* mRNA levels to estrogen exposure. In support of these findings, Goetz and co-workers [19] recently showed that long term (3 months) dietary estrogen treatment stimulated hepatic *esr1* mRNA levels with no effect on *esr2a* or *cyp19a1a* mRNA levels in male and female yellow perch. Liver *esr2a* mRNA levels have also been reported to remain unchanged or decrease in response to estrogen in tilapia [11,12], fathead minnow [16] and zebrafish [34,38], but the mechanism and significance of a differential regulation of liver ERs in response to estrogen in teleosts is not well understood.

In both the developmental and estrogen dosing studies, liver *igf1b* and *esr2a* mRNA levels showed a highly significant positive correlation with each other overall. A study on the seasonal mRNA levels in adult yellow perch [30] also revealed a significant positive correlation between liver *igf1b* and *esr2a* mRNA levels. While the correlation of the expression of these liver genes across different studies is striking, the mechanisms associated with these correlative relationships have yet to be adequately elucidated. Jentoft et al. [25] reported significant increases in growth of male and female yellow perch in response to 70 days of dietary estrogen treatment, but found no differences between treatments in serum Igf1 levels of animals at the end of the study. However, Goetz et al. [19] found that long-term (90 days) dietary E₂ treatment resulted in a significant increase in liver *esr1* mRNA levels in both male and female yellow perch but only females displayed significantly increased liver *igf1b* mRNA levels. Clearly, more work is needed to better define the relationships between estrogen and the endocrine growth (somatotrophic) axis in this species.

In summary, the measurement of *gh*, *prl*, *smtl*, *igf1b*, liver *esr1*, liver *esr2a*, liver *cyp19a1a*, ovary *esr1*, ovary *esr2a* and ovary *cyp19a1a* mRNA levels shows interesting gene-specific and sex-specific patterns through the first year of development in juvenile yellow perch. Also there is a distinct difference in male and female liver *esr2a* mRNA levels in the later sampling periods but both liver ERs showed a significant increase in mRNA levels after 195 dph or a total length of 120 mm. The correlation between liver *esr2a* and *igf1b* mRNA expression cannot be discounted until more work is done in understanding their relationship. These results indicate new avenues for research related to sex-specific and developmental expression of these genes in fish and also provide initial insight into yellow perch early growth and development.

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