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Regulation of elements of the thyroid hormone and corticosteroid systems by stress, hormone treatment, and atrazine during ontogeny of red drum (*Sciaenops ocellatus*)

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Regulation of elements of the thyroid hormone and corticosteroid systems by stress, hormone treatment, and atrazine during ontogeny of red drum (*Sciaenops ocellatus*)

by

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Regulation of elements of the thyroid hormone and corticosteroid systems by stress, hormone treatment, and atrazine during ontogeny of red drum (*Sciaenops ocellatus*)

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The University of Texas at Austin, 2008

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Abstract

Survival of teleost larvae requires growth and development which depend upon endocrine processes. In this dissertation I have examined the ontogeny of elements of the thyroid hormone (TH) and corticosteroid (CS) systems in red drum (*Sciaenops ocellatus*). Basal cortisol production was first detected 3 days post-hatch (DPH) and a cortisol stress response was present from 6 DPH forward. Changes in steroidogenic enzyme mRNA (*CYP11B* and *CYP21*) levels did not correlate with these events. The time necessary to reach peak cortisol levels as well as return to basal levels declined as larvae developed. A second set of studies examined ontogenetic patterns in levels of mRNAs encoding thyroid (*soTRα* and *soTRβ*) and corticosteroid (*soGR*) hormone receptors and assessed the

regulation of these mRNAs by exogenous triiodothyronine (T3) and cortisol. *soTRα*, *soTRβ* and *soGR* were expressed in all stages of red drum examined. *soTRα* levels increased during the time when surging TH levels have been reported. *soTRβ* levels did not differ significantly during development. *soGR* levels were strongly correlated with those of *soTRα*. T3 up-regulated *soTRα* and *soTRβ* levels in 7 DPH, but not older larvae. Cross-regulation of receptor mRNAs by exogenous treatment with T3 or cortisol was not observed. Finally, I assessed the influence of a common herbicide, atrazine, on receptor mRNA, TH levels and growth of red drum. In two experiments, red drum exposed to environmentally relevant levels of atrazine did not alter hormone receptor mRNA levels, or TH content. However, atrazine did depress growth in some instances. In conclusion, the expression patterns of hormone receptor mRNA in embryos suggests receptor proteins could be activated by maternal hormones prior to the onset of endogenous hormone production. A correlation between *soTRα* and *soGR* mRNA levels suggests coordinated function of TH and CS systems, although regulatory interactions between these systems were not evident under the conditions in this study. Patterns in *soTRα* and *soTRβ* mRNA levels support an important role for TH in the larval to juvenile transformation of red drum larvae. The results also support growing evidence indicating atrazine exposure effects larval growth and may impact their survival in the wild.

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Chapter 1: The onset of cortisol production and the cortisol stress response in larval red drum (*Sciaenops ocellatus*) are independent of changes in *CYP11B* and *CYP21* mRNA levels

Abstract

Although cortisol plays an important role in teleost development, the onset of cortisol production and the initiation of the cortisol stress response in teleosts are poorly understood. In the present study we have measured basal cortisol levels and the development of cortisol stress response in larval red drum (*Sciaenops ocellatus*). We isolated partial nucleic acid sequences encoding two key corticosteroidogenic enzymes, *CYP11B* and *CYP21* and assessed ontogenetic patterns of their mRNA levels in relation to basal and stress-induced cortisol production. Cortisol (0.65 ± 0.19 ng/g), presumably of maternal origin, was measured in red drum embryos collected 1 h post-fertilization (HPF). Embryonic cortisol declined rapidly until it was low or undetectable by 11 h. In larvae, basal cortisol was first detected 3 days post-hatch (DPH) and reached a maximum (1.72 ± 0.23 ng/g) at 9 DPH. Cortisol did not increase in response to an acute stressor prior to 6 DPH. From 6 DPH forward, stress caused significant increases in larval cortisol content. Stress-induced cortisol levels in 6 to 9 DPH larvae were highest 1 h application of the stressor. In larvae 11 DPH and older, the highest cortisol measurements occurred 0.5 h post-stress. The time necessary to return to basal levels also changed during ontogeny. Elevated cortisol was still evident after 3 h in 6 DPH larvae. From 11 DPH onward, basal cortisol levels were reestablished in larvae by 1 h post-stress. The *CYP11B*

and *CYP21* nucleotide sequences isolated for this study were highly similar to the homologous sequences from other teleosts and were expressed in classical steroidogenic tissues including head kidney, ovary and testis. Both transcripts were detected in red drum 12 h prior to hatching as well as in all post-hatch larvae examined. Changes in *CYP11B* and *CYP21* mRNA levels did not occur in association with the ontogenetic appearance of cortisol, or the onset of the stress response. However, the steady-state levels of *CYP11B* and *CYP21* were positively correlated with one another. As larvae developed, the dynamics of the cortisol stress response matured from a low magnitude, slow recovery response, to a response similar to that observed in juvenile and adult fish.

Introduction

An immense amount of information exists on the physiological effects of stress in fishes (Wendelaar Bonga 1997; Barton 2002) including a substantial emphasis on the primary teleost corticosteroid cortisol and its role in the adaptive response to stress (Mommsen et al. 1999). In teleosts, as in other vertebrates, the physiological effects of corticosteroids are numerous and include influences metabolism, immune status, growth and reproduction (Wendelaar Bonga 1997; Mommsen et al. 1999). Cortisol also fulfills a mineralocorticoid function in teleosts and is therefore critical to ion homeostasis (Wendelaar Bonga 1997; McCormick 2001). The abundant studies of cortisol in teleosts report primarily on adult fishes. An important but largely overlooked area of investigation is the ontogeny of cortisol production and the development of the stress response, as well as the corresponding function of cortisol during early life.

In addition to their role in stress and ion homeostasis, corticosteroids have unique functions during the early life of vertebrates. In mammals an increase in fetal adrenocortical activity occurs which is necessary for maturation of the fetal organs and initiation of parturition (Liggins 1976; Thorburn and Challis 1979). Several studies implicate functions of cortisol during the early life of teleosts as well. Maternal cortisol (Eriksen et al. 2006; McCormick 2006) and exogenous cortisol treatment (de Jesus et al. 1991; Brown and Kim 1995; Kim and Brown 1997) have been shown to influence larval growth, development and survival. Some of these effects may be mediated through a synergistic interaction with thyroid hormones (de Jesus et al. 1990). A clear understanding of when and how cortisol production is initiated in developing fish is valuable for maximizing rearing success in captivity and mitigating anthropogenic impacts on early life stages in nature.

A number of studies have investigated the ontogeny of basal cortisol and the cortisol stress response in teleosts. *De novo* cortisol synthesis generally begins shortly after hatching (Hwang et al. 1992; Barry et al. 1995a; Sampath-Kumar et al. 1995; Pérez-Domínguez et al. 1999; Jentoft et al. 2002; Deane and Woo 2003; Szisch et al. 2005) although it commences prior to hatching in some species (Stouthart et al. 1998). A cortisol stress response becomes evident days to weeks later, depending on the species. While these temporal patterns have been described, the mechanism by which these events are regulated and the level of biological organization at which regulation occurs is unresolved and largely unexplored.

The corticosteroid component of the vertebrate stress response is mediated via the hypothalamic-pituitary-interrenal (HPI) axis. Perceived stressors stimulate the hypothalamus to secrete corticotropin-releasing hormone (CRH) which then induces the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the plasma. Circulating ACTH subsequently acts on the interrenal cells of the head kidney to stimulate cortisol synthesis. Initiation and regulation of the mature CS stress response necessitates that each of these tissues be sufficiently developed to synthesize their respective endocrine/neuroendocrine signals, as well as receive and transduce signals from other elements of the HPI axis. Few studies have sought to establish the developmental events that account for the ontogenetic onset of cortisol synthesis and the stress response.

Both CRH and ACTH appear to be present early in teleost development. While CRH mRNA can be found in several areas of the developing *Danio rerio* brain, the transcript is first detected within 25 hours post-fertilization (HPF) in the developing hypothalamus (Chandrasekar et al. 2007). In *Oreochromis mossambicus*, CRH immunoreactivity in the hypothalamus and ACTH immunoreactivity in the pars distalis were evident at 2 DPH, the earliest age examined in the study (Pepels and Balm 2004). However, the presence of ACTH and mature corticotropes is generally detected at or prior to hatching (Saga et al. 1993; Stouthart et al. 1998; Villaplana et al. 2002).

Experimental investigations of HPI axis integration during development are also scarce. *Oncorhynchus mykiss* interrenal cells are sensitive to ACTH before and after hatching *in vitro*, although no stress response occurs *in vivo* at this stage (Barry et al.

1995a,b), suggesting that maturation of the stress response occurs at the level of the brain. In contrast, constant levels of brain CRH during the cortisol stress response of tilapia larvae may suggest that the response is regulated independent of hypothalamic CRH at this stage (Pepels and Balm 2004). The lack of a clear regulatory site upstream of the interrenal cells in the HPI axis suggests that there may be local control of these processes. It is conceivable that the ontogenetic onset of cortisol production and the initiation of the stress response may be dependent in part or entirely upon patterns of steroidogenic enzyme expression in emergent interrenal cells.

In teleosts, cortisol synthesis occurs in interrenal cells located in the anterior portion of the kidney where steroidogenic enzymes, including multiple cytochromes P450 (CYPs) and hydroxysteroid dehydrogenases, sequentially convert cholesterol to various steroid forms. CYP11B and CYP21 catalyze the final steps in the production of cortisol and serve in sex steroid production. Studying the pattern of *CYP11B* and *CYP21* mRNA levels during embryonic and larval development may provide an indication of the mechanisms underlying the onset of cortisol synthesis and the development of the stress response.

In the present study we isolated portions of cDNAs that putatively encode red drum (*Sciaenops ocellatus*) CYP11B and CYP21. Levels of these mRNAs were quantified during red drum ontogeny. We also measured embryonic and larval cortisol content and experimentally assessed the development of the corticosteroid stress response. Comparison of the cortisol stress response at several developmental stages reveals ontogenetic changes in stress response dynamics. Finally, we addressed the

hypothesis that the onset of basal cortisol production and the stress response are associated with changes in the levels of *CYP21* and *CYP11B* mRNA.

Materials and Methods

Experimental animals

Red drum eggs were obtained from broodstock induced to spawn by temperature and photoperiod control (Arnold 1988) at the Fisheries and Mariculture Laboratory of the University of Texas at Austin, Marine Science Institute in Port Aransas, Texas and the Texas Parks and Wildlife Department hatchery in Flour Bluff, Texas. Eggs were disinfected in seawater containing 10 ppm formalin for 20 min before being rinsed with clean seawater. Eggs were then transferred to 1-L beakers of filtered seawater at a density of 1000 eggs/L. Embryos hatched approximately 24 h later (1 day post-hatch, DPH) and larvae were moved to 600-L cylindrical tanks with internal biological filters at a density of 5000 per tank. An airstone at the bottom of the tank provided water circulation and maintained dissolved oxygen near saturation levels. Salinity and temperature were maintained at approximately 27 °C and 30 psu respectively, and photoperiod was 12:12 hour (light:dark).

Exogenous feeding begins 3 DPH at which time larvae were co-fed live rotifers (*Brachionus plicatilis*) at 2-5 rotifers/mL along with Otohime microparticulate diet (Reed Mariculture, Campbell, CA). Prior to use, rotifers were enriched overnight with *Isochrysis galbana* and Algamac 2000 (Biomarine, Inc. Hawthorne, CA). Microparticulate diet was administered by automatic feeders throughout the day.

Beginning at 10 DPH larvae were fed enriched *Artemia* nauplii and Otohime microparticulate diet. By 14 DPH, larvae were fed microparticulate diet exclusively.

Cortisol content of fertilized eggs/developing embryos

Reproductively active red drum broodstock commence spawning shortly after lights are turned off (2100 h in the present study). The buoyant eggs accumulate in egg collectors shortly thereafter. Fertilized eggs were removed from egg collectors beginning approximately 1 HPF, and at 2-h intervals thereafter. Egg samples were frozen immediately at -80 °C until cortisol determination.

Ontogeny of basal cortisol and the corticosteroid stress response

Stress experiments were conducted beginning at 0900 on 1, 3, 5, 6, 9, 11, 18, 25, 33 DPH. Groups of larvae were transferred from rearing tanks to 1-L beakers of filtered seawater on the evening prior to stress trials. At 0900 the following day larvae were exposed to a stressor. Larvae 15 DPH and older were collected in a fine mesh aquarium net and the net with the fish held out of water for 30 s, followed by an additional 30 s on ice. Larvae 11 DPH and younger were too delicate to survive aerial exposure and were therefore stressed by stirring with a glass rod at 2 revolutions per second for 1 min. Treated larvae were then transferred to clean seawater in 1-L beakers. At 0.5, 1, and 3 h post-stress all larvae from one beaker were collected and frozen at -80 °C. Corresponding control larvae were collected from adjacent tanks at each sampling interval. Samples were transferred to pre-weighed micro-centrifuge tubes, frozen on dry ice and stored at -

80 °C until determination of wet tissue weight and preparation for cortisol radioimmunoassay. On the day of each stress test, 15 larvae were selected from the rearing tank and measured for standard length (SL).

Cortisol extraction and radioimmunoassay

Cortisol extraction and radioimmunoassay (RIA) were conducted using methods previously validated for red drum larvae (Pérez-Domínguez and Holt 2006). Briefly, frozen red drum larvae were homogenized in phosphate-buffered saline (0.01 M, 0.15 M NaCl, pH 7.4), and twice extracted in diethyl ether. Ether extracts were evaporated under nitrogen, and resuspended in carbon tetrachloride into which excess lipid was partitioned after the addition of PBS-gelatin (PBS, 0.1% gelatin). After centrifugation, the aqueous fraction was analyzed in duplicate for cortisol content by RIA.

Cloning, sequencing and tissue distribution of CYP11B and CYP21

Partial *CYP11B* and *CYP21* sequences were amplified from cDNA libraries reverse transcribed from red drum interrenal total RNA. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA was DNase (Promega, Madison, WI) treated and repurified using TRIzol. The resulting pellet was dissolved in nuclease-free water and the concentration determined spectrophotometrically. First-strand cDNA was synthesized in a 10-µl reverse transcription (RT) reaction which included 1 µg of interrenal total RNA and 100 ng of random hexamers. RT reactions were conducted with SuperScript III reverse

transcriptase (Invitrogen) according to the manufacturer's protocol. Primer pairs (Table 1.1) amplifying 150 and 904 bp fragments of *CYP11B* and *CYP21*, respectively in the closely related sciaenid, Atlantic croaker (*Micropogonias undulatus*) (GenBank accession numbers EU673091 and EU673090, respectively) were used to amplify similar sequences from red drum RT template by polymerase chain reaction (PCR). Amplicons of approximately the predicted size (as determined by agarose gel electrophoresis) were ligated into the pDrive cloning vector (Qiagen, Valencia, CA) and sequenced.

The distribution of *CYP11B* and *CYP21* transcripts among adult red drum tissues was assessed by RT-PCR. Adult red drum were sacrificed and excised tissues were immediately frozen in liquid nitrogen. Total RNA, isolated and DNase-treated as above, was used as template in RT-PCR reactions only after confirmation of the absence of genomic contamination. Each 15-µl RT-PCR reaction contained 1.5 µl template, 1 µM of each primer, 1.5 µl 10X reaction buffer, 1 mM $MgCl_2$, 200 µM dNTPs and 0.5 U Amplitaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). Fragments of *CYP11B* (141 bp), *CYP21* (711 bp) and actin (240 bp) were amplified with primers described in Table 1.1. Thermal parameters for reactions were 95 °C for 8 min, followed by 35 cycles of 95 °C for 30 s, 65 °C (actin, 60 °C) for 30 s, and 72 °C for 60 s. Products were separated by electrophoresis in a 1.5 % agarose gel in Tris–borate (90 mM), EDTA (2 mM) buffer (TBE).

Ontogeny of CYP11B and CYP21 mRNA levels

Three replicate pools of larval red drum (approximately 50 mg tissue) were collected from a single batch of fish at ages ranging from 12 h prior to hatching, through 33 DPH for gene expression analysis. Tissue was immediately frozen on dry ice and stored at -80 °C until RNA extraction. An additional 15 larvae were collected from the rearing tanks on each sample date for determination of SL. Total RNA was extracted and DNase treated as described above. RT-negative reactions were used as template for PCR with actin primers to confirm the absence of genomic DNA. DNA-free RNA (1 µg) was used in 20 µl RT reactions with SSIII reverse transcriptase (Invitrogen) and subsequently diluted to a total volume of 60 µl with DNase free water. Relative expression of *CYP21* and *CYP11B* mRNAs were determined in real-time quantitative reverse transcriptase polymerase chain reaction (qPCR) assays using the Brilliant II SYBR QPCR Master Mix (Stratagene, La Jolla, CA). Each 25-µl reaction mixture contained 5 µl cDNA template and 400 µM forward and reverse primers (Table 1.1). Reactions were cycled and the resulting fluorescence detected with an Eppendorf RealCycler (Eppendorf, Westbury, NY) under the following cycling parameters: 95 °C for 10 min; and then 40 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min. The size of the amplified product was verified for each reaction by agarose gel electrophoresis. Levels of *CYP11B* and *CYP21* mRNA were normalized to that of ribosomal *18S* RNA determined in separate qPCRs using specific primers (Table 1.1) and a hexachlorofluorescein (HEX) labeled TaqMan probe (Sigma Genosys, The Woodlands, TX). Thermal parameters for the *18S* reactions were: 95 °C for 10 min; and 40 cycles of 95 °C for 30 s, 60 °C for 1 min. All samples

were run in duplicate. *CYP11B* and *CYP21* mRNA levels were assessed relative to *18S* as determined by the method of Fink et al. (1998).

Statistical analyses

In stress response experiments, statistical differences in cortisol content between control and stressed larvae were assessed using two-way analysis of variance (ANOVA) of log transformed data. One-way ANOVA was used to test for ontogenetic changes in basal cortisol and mRNA levels. Where significant main effects were found, Fisher's Least Significant Difference test was used to determine which groups differed significantly. The Spearman correlation test was used to determine whether a relationship existed between *CYP11B* and *CYP21* mRNA levels. Statistical analyses were conducted with SYSTAT 10.0 (Systat Software, Inc., San Jose, CA) and Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

Results

Ontogeny of basal cortisol and the corticosteroid stress response

Eggs collected within 1 h of fertilization contained 0.21 ± 0.19 ng/g cortisol (mean ± S.D., Fig. 1.1). Cortisol content declined steadily over the following 11 h to low or undetectable levels in embryo samples. Basal cortisol was first detected 3 DPH (Fig. 1.2). Cortisol levels increased significantly between 5 and 9 DPH to a maximum of 1.72 ± 0.23 ng/g. Cortisol levels remained elevated through 11 DPH and possibly longer. By

18 DPH cortisol levels had declined, and remained between 0.5 and 1.0 ng/g for the rest of the study period.

A stress-induced elevation of whole body cortisol was first observed 6 DPH and occurred on all dates tested thereafter (Fig. 1.3). In stress response experiments, the highest whole body cortisol levels measured in larvae 6 and 9 DPH occurred at 1 h. Beyond 9 DPH, the speed of this response increased and the highest measured levels were attained 0.5 h post-stress. The dynamics of the stress response also changed with age and growth of larvae. While cortisol in larvae 6 DPH was still significantly elevated at 3 h post-stress, cortisol in larvae 9 DPH had returned to basal levels between 1 and 3 h post-stress. In fish 11 DPH and older, peak cortisol measurements occurred at 0.5 h and had returned to basal levels by 1 h.

Sequence and tissue distribution of CYP11B and CYP21

CYP11B and *CYP21* partial cDNAs were successfully isolated and the sequences can be found in Appendix 1. The 150 bp red drum *CYP11B* sequence shared 98 % identity with the same region in *M. undulatus*. The 907 bp fragment of red drum *CYP21* shared 97 % identity with the same region in *M. undulatus*. Results of a BLAST search (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for sequences similar to red drum *CYP11B* comprise exclusively teleost *CYP11B* sequences (Table 1.2). Similarly, a BLAST search for sequences similar to red drum *CYP21* returned sequences known or predicted to be *CYP21* for teleosts (Table 1.2) and the anuran *Xenopus laevis*. Using RT-PCR the expression of *CYP11B* was detected in testis,

ovary and interrenal cells. *CYP21* expression was detected in interrenal cells and testis (Fig 1.4).

Ontogeny of CYP11B and CYP21 mRNA levels

Expression of *CYP11B* was detected in all ages of embryos and larvae examined (Fig. 1.2). No significant differences in *CYP11B* mRNA levels were observed relative to 1 DPH. *CYP21* expression was also detected in all developmental stages; however, a significant increase in *CYP21* mRNA relative to 1 DPH was observed in samples 15 to 25 DPH ($P < 0.05$). Levels of *CYP11B* and *CYP21* mRNA were positively correlated (Spearman r = 0.5242, $P < 0.001$). Changes in *CYP11B* and *CYP21* mRNA levels did not occur in association with the ontogenetic appearance of cortisol or the onset of the stress response.

Larvae used in determining the ontogeny of *CYP11B* and *CYP21* mRNA levels had a slightly, but significantly, lower growth rate (ANOVA, $P = 0.045$) than those used for stress response experiments. Development of fish larvae tends to be more closely associated with changes in length than age (Fuiman 1994; Fuiman et al. 1998); results are therefore presented with respect to both age and size (SL).

Discussion

The ontogenetic pattern of basal cortisol for red drum was typical of that observed in other teleosts. While the stress response was apparent within the first week of life, the dynamics of the response changed dramatically during larval development. The content

of *CYP11B* mRNA was recently described for part of the larval period of *Dicentrarchus labrax* (Socorro et al. 2007) and *D. rerio* (Alsop and Vijayan 2008). Our results extended these studies by examining levels of *CYP11B* mRNA in red drum over a longer period of development (embryos, and continuing through most of the larval period). We also report the pattern of *CYP21* mRNA levels which, to our knowledge, has not been documented in teleost larvae. Our results indicate that the onset of cortisol production and the corticosteroid stress response are independent of changes in the abundance of these transcripts.

The presence of cortisol (de Jesus et al. 1991; de Jesus and Hirano 1992; Hwang et al. 1992) and other steroids (de Jesus and Hirano 1992) in teleost eggs is well established and is presumably the result of maternal transfer during oogenesis. We found that cortisol is present in recently fertilized red drum eggs. The mean cortisol content in eggs 1 h post-fertilization (0.21 ng/g) is lower than reported for several other freshwater and marine fish, including *Oncorhynchus keta* (20 ng/g, de Jesus and Hirano 1992) and *Paralichthys olivaceus* (2.5 ng/g, de Jesus et al. 1991). Red drum was most similar to *Sparus auratus* (0.83 ng/g, Szisch et al. 2005) and *Lates calcarifer* (0.6-2.25 ng/g, Sampath-Kumar et al. 1995) both of which are warm-water species with short embryonic periods comparable to red drum.

Cortisol content declined during embryonic development until it reached low to undetectable levels approximately 11 h after fertilization. These results agree with earlier observations in red drum (Pérez-Domínguez and Holt 2006) in which cortisol was not detected in embryos collected the morning after fertilization. This pattern of declining

embryonic cortisol content appears to be characteristic of oviparous teleosts, having been reported for several marine and freshwater species (de Jesus et al. 1991; de Jesus and Hirano 1992; Hwang et al. 1992; Barry et al. 1995a; Sampath-Kumar et al. 1995; Stouthart et al. 1998; Jentoft et al. 2002).

At present it is unknown whether these hormones have a role in teleost embryonic development although several studies suggest they have effects on the developing larvae (McCormick 1998, 1999, 2006; Eriksen et al. 2006). However, glucocorticoid receptor mRNA has been detected in *Oreochromis mossambicus* (Tagawa et al 1997), *D. rerio* (Alsop and Vijayan 2007) and red drum (Applebaum et al, unpublished data) embryos, perhaps providing a means for cortisol signaling in embryonic fishes. To our knowledge, it has not yet been established whether glucocorticoid receptor protein is present during early development. However, the identification of cortisol binding sites in *O. mykiss* embryos (Pillai and Terner 1974) suggests this may be the case.

Larval samples collected prior to treatment in the stress response experiments (Time 0 in Fig. 1.3) illustrate the ontogeny of basal cortisol levels. The ontogenetic profile of red drum whole body cortisol has several characteristics in common with those reported for other species. Cortisol production began after hatching, between 1 and 3 DPH. This is in agreement with an earlier study (Pérez-Domínguez and Holt 2006), in which cortisol was first detected 2 DPH. The timing of the onset of cortisol production relative to hatching appears to be species specific. Most species have the ability to produce cortisol shortly after hatching (de Jesus et al. 1991; Hwang et al. 1992; Barry et al. 1995a; Sampath-Kumar et al. 1995; Jentoft et al. 2002; Szisch et al. 2005). However,

increases in cortisol content have been reported prior to hatching for *O. keta* (de Jesus and Hirano 1992) and *Cyprinus carpio* (Stouthart et al. 1998). It is important to consider that hatching is not an ontogenetic event and various species hatch at different percentage of ontogenetic development. In the future it would be more appropriate to compare the relative to ontogenetic stage at which the cortisol production begins.

In red drum, the onset of cortisol production occurs near the transition from endogenous to exogenous feeding. Similar patterns are evident in other marine teleosts (Pérez-Domínguez et al. 1999; Deane and Woo 2003; Szisch et al. 2005). Activation of cortisol biosynthesis may be important for adapting to different sources of dietary protein and carbohydrate as the transition from endogenous to exogenous nutrition occurs. Prior to exogenous feeding the glucocorticoid actions of cortisol may have a deleterious effect on larvae by diverting the limited energetic stores of the yolk away from the critical development of somatic tissues. With the initiation of endogenous feeding the utilization of portions of exogenously supplied nutrients for both energetic and growth processes may be dependent upon regulation by cortisol.

A surge in thyroid hormones associated with the larval to juvenile transformation is typical of fishes with a larval period (de Jesus et al. 1991; Pérez-Domínguez et al. 1999; Deane and Woo 2003; Crane et al. 2004; Szisch et al. 2005; Pérez-Domínguez and Holt 2006; Yamano et al. 2007; Kawakami et al. 2008; Klaren et al. 2008). A rise in cortisol production slightly before, or coincident with increased thyroid hormone has been reported for several species (de Jesus et al. 1991; Pérez-Domínguez et al. 1999; Deane and Woo 2003; Szisch et al. 2005). While thyroid hormones were not measured in

the present study, comparison to an earlier study shows that the elevated basal cortisol levels occur slightly prior to the age (between 10 and 16 DPH) and size (4.8-5 mm SL) at which thyroid hormones increased significantly in red drum (Pérez-Domínguez and Holt 2006).

While not addressed here, patterns in basal cortisol levels may also be associated with the development of larval ionoregulatory function. Ontogenetic changes in the ability of larvae to regulate ion and water balance as well as developmental changes in the tissues and organs associated with these processes may be dependent on the mineralocorticoid functions of cortisol (Varsamos et al. 2005).

Despite the general similarities, there was a notable difference between our results for the ontogeny of basal cortisol in red drum and those of Pérez-Domínguez and Holt (Pérez-Domínguez and Holt 2006). The earlier study reported a sharp increase in cortisol beginning 2 DPH, and peaking at > 7 ng/g at 4 DPH. We did not observe this peak even though we used similar techniques (Pérez-Domínguez, personal communication). Our results, however, are consistent with patterns reported for other fishes. I therefore believe that our results accurately represent the typical ontogeny of basal cortisol in red drum.

Although basal cortisol was detected by 3 DPH, stress did not elicit an increase in whole body cortisol content in red drum larvae 1 to 5 DPH. It is unlikely that the stressor was insufficient, as the same treatment elicited a response in larvae 9 and 11 DPH. Further, in a pilot study, stressor treatment was sufficient to cause a low level of mortality (<10%) of larvae, indicating that the treatment was in fact stressful to larvae. Several possible explanations exist for the absence of a stress response, despite the capacity for

cortisol production in the early larvae. Lack of a stress response could be due to a yet undeveloped element of the HPI axis. Alternatively, a well developed and integrated HPI axis may be suppressed at this stage of development. Possibly more interesting than the mechanism themselves is determining whether the delayed onset of the stress response has an adaptive role. Fish larvae have limited energetic reserves before and after the transition to exogenous nutrition. It is possible that the absence of a stress response may protect early larvae from elevated metabolic demands caused by increased cortisol levels. The developmental mechanisms and possible functions of the non-responsive need further investigation.

While not directly addressed in our experiments, ontogenetic changes in the capacity for synthesis and degradation of cortisol were evident in the dynamics of the stress response. The relatively low magnitude of the cortisol response observed at 6 DPH and persistent elevation over basal levels for at least 3 h suggest that the enzymatic capacity for cortisol synthesis and degradation is limited. While the change in stressors used for stress tests at 11 DPH precludes a direct comparison of the magnitude of the stress response, peak stress-induced cortisol concentrations were substantially higher in larvae 9 DPH and older than in younger larvae. When examined with respect to size, elevated levels of *CYP21* mRNA occur at nearly the same SL as the increase in peak cortisol, suggesting increased synthetic capacity; although a reduction in degradation rates cannot be ruled out. Further, the time necessary to return to basal levels is reduced (1 h or less at 21 DPH), indicating increased capacity for degradation of steroids and/or changes in the negative feedback regulation of cortisol synthesis. The recent report that

the onset of negative feedback regulation of cortisol synthesis occurs between 7 and 21 DPH in *Paralichthys dentatus* (Veillette et al. 2007) supports this conclusion.

As the capacity for synthesis, degradation, and feedback regulation develop, a maturation occurs from a low magnitude, slow recovery stress response, observed in larvae 6 DPH to one remarkably similar to that observed in the plasma of juvenile red drum (Robertson et al. 1987, 1988; Thomas and Robertson 1991). Whole body cortisol measurements have been demonstrated to be a useful indicator of stress-induced cortisol increases in the plasma, although the dynamics of circulating cortisol may not be identical to whole body measurements (Pottinger and Mosuwe 1994; King and Berlinsky 2006).

We isolated cDNAs putatively encoding portions of red drum *CYP11B* and *CYP21*. These sequences were highly similar (> 97 %) to known *CYP11B* and *CYP21* sequences in a closely related species, *M. undulatus*. Both transcripts were detected exclusively in classical steroidogenic tissues, including testis and interrenal tissue. *CYP11B,* but not *CYP21* was also detected in ovarian tissue. Ovarian CYP21 catalyzes the production of 11-deoxycortisol, the precursor to sciaenid maturation-inducing steroid (MIS), 17α,20β,21-hydroxy-4-pregnen-3-one (20β-S) (Trant et al. 1986; Thomas and Trant 1989; Trant and Thomas 1989b, 1989a; Patino and Thomas 1990). In seasonally spawning species, ovarian MIS concentrations are typically low until the final stages of reproduction when MIS concentrations increase sharply (Trant et al. 1986; Nagahama 1994, 1997). Ovarian *CYP21* mRNA expression is therefore likely to be transient, occurring for brief periods during the seasonal spawning cycle. Ovaries used in this study

were from three different adult females that were not reproductively active. The absence of *CYP21* in these ovaries may therefore be expected. Together, the high similarity of red drum cDNA sequences to those from Atlantic croaker and other teleosts, coupled with an appropriate tissue distribution strongly support the conclusion that we isolated partial *CYP11B* and *CYP21* cDNAs.

Teleost larvae lack a differentiated and steroidogenically active gonad. Expression of *CYP11B* and *CYP21* during this period should therefore be attributed to interrenal cells. This assertion is supported by results from *L. calcarifer* larvae where CYP21 immunoreactivity was observed only in the region of the developing head kidney (Sampath-Kumar et al. 1996). Changes in *CYP11B* and *CYP21* expression in larvae should therefore be associated with corticosteroid production and regulation of the HPI axis.

Using histochemical stains, red drum interrenal cells were first identified 7 DPH, 5 days after cortisol production began (Pérez-Domínguez and Holt 2006). In the present study, both CYP transcripts were detected prior to hatching suggesting that although they may not be distinguishable using histochemical stains, interrenal cells are differentiated much earlier in development. Production of cortisol on or before 3 DPH demonstrates that functional proteins are being translated from these transcripts. The limitations of histological techniques are also evident in a study of *L. calcarifer* where adrenodoxin and CYP21 immunoreactivity was identifiable in the head kidney as early as 1 DPH, despite the inability to distinguish these cells until 5 DPH (Sampath-Kumar et al. 1996) histologically.

Considering their close functional relationship and sequential action in the production of corticosteroids, a substantial degree of co-variation should be expected in the abundance of *CYP11B* and *CYP21* mRNAs and the proteins they encode. This view is supported by the strongly correlated abundance of these transcripts in red drum larvae. Ontogenetic patterns of *CYP11B* and *CYP21* mRNA content were not associated with the onset of cortisol production or the stress response. It is therefore unlikely that the expression of these transcripts is a major determinant of the initiation of these processes.

A significant increase in *CYP11B* and steroidogenic acute regulatory enzyme (*StAR*) occurs in *Danio rerio* shortly after hatching, but preceding increased cortisol production (Alsop and Vijayan 2008). Such a pattern was not evident for *CYP11B* or *CYP21* in red drum. In the present study, embryos (0 DPH) were sampled 8-10 h prior to hatching. It is possible that had we sampled earlier in the embryonic period, levels would have been lower and we would have seen a difference from post-hatch levels.

Conclusions and future perspective

Our results did not support the hypothesis that ontogenetic changes in red drum cortisol production and the stress response are related to changes in *CYP11B* and *CYP21* mRNA levels. This suggests that the onset of these events is determined elsewhere in the HPI axis. However, our results do not preclude a role of for these steroidogenic proteins in regulating stress response ontogeny. We only addressed changes in transcript abundance, and post-transcriptional/translational processes may be involved and should be addressed in the future. The limited studies available on the cortisol stress response

during the early life have emphasized steroidogenic processes. Our observation of changing dynamics of the stress response in developing larvae suggests that equal investigative weight should be given to factors involved in the degradation and removal of bioactive steroids from larval circulation (i.e., reductases, sulfotransferases, and glucoronidases). Finally, while the mechanisms underlying the development of cortisol production and the stress response must still be elucidated, new research efforts should also address the potential biological significance of the latency in cortisol production in yolk-sac larvae, and the quiescent period in the stress response. These studies should include assessments of the sensitivity of early larvae to elevated cortisol and the possible role of cortisol in the transition to exogenous feeding and the development of ionoregulatory function.

Table 1.1. C*YP11B* and *CYP21* forward (**F**) and reverse primers (**R**), and dual-labeled fluorescent probe (**Probe**) combinations used in isolation of sequences, determination of tissue distribution and quantification of mRNA levels by qPCR in red drum. *18S* probe was labeled with 5' hexachlorofluorescein (HEX) and 3' Black Hole Quencher (BHQ1).

Gene of interest	**Application**	**Primer sequences** (5'→3')
CYP11B	*Sequence isolation*	**F:** GCAACACACAGAGAGACACGCCAGCA **R:** CCTCGCCACGTCGTCAAGCAG
	qPCR and tissue distribution	**F:** ACAGAGAGACACGCCAGCA **R:** TCGCCACGTCGTCAAGCA
CYP21	*Sequence isolation*	**F:** AATGGCAATAGAAATGTCAGTGATCAG **R:** CGATCCAGTGATGGCGTCCACATT
	qPCR	**F:** AGGTCCTCCCAGCCTCATC **R:** GTCCACATTTAAGGCGGTA
	Tissue distribution	F: GCTGCTGTTAATAGTGCTGATTTGC R: GAGTCCAGAGCAGAGATCCAAGTGG
Actin	*Tissue distribution*	**F:** TCGTCATGGACTCTGGTGATGG **R:** CTCCTGCTCAAAGTCCAGTGCAAC
18S	*qPCR*	**F:** GTTAATTCCGATAACGAACGAGACTC **R:** ACAGACCTGTTATTGCTCAATCTCGTG **Probe:** TTCTTAGAGGGACAAGTGGCGTT

Table 1.2. Lowest scores returned from BLAST analysis (discontinuous megablast) of putative red drum *CYP11B* and *CYP21* nucleotide sequences. *E* represents the number of hits expected to be returned by chance when querying a database containing the current number of sequences. Values of *E* approaching zero, such as those listed here, indicate matches are very unlikely to occur simply by chance, and provide substantial evidence that sequences are closely related.

Search	***Species***	***Accession No.***	***E***
CYP11B	*Dicentrarchus labrax CYP11B*	AF449173.2	2×10^{-43}
	Oncorhynchus mykiss CYP11B	AF217273.1	3×10^{-33}
	Oncorhynchus mykiss CYP11B	AF179894.1	1×10^{-31}
	Acanthopagrus schlegelii CYP11B	EF423618.1	2×10^{-30}
	Oryzias latipes CYP11B	EF025509.1	9×10^{-28}
CYP21	*Anguilla japonica CYP21*	AB095111.1	9×10^{-133}
	Oryzias latipes CYP21	EU159457.1	6×10^{-97}
	Predicted*: Danio rerio CYP21* (3 results returned)	XM_001333627.1	3×10^{-88}
	Oncorhynchus mykiss CYP21	EU246942.1	5×10^{-67}
	Takifugu rubripes CYP21	AJ506968.1	7×10^{-33}

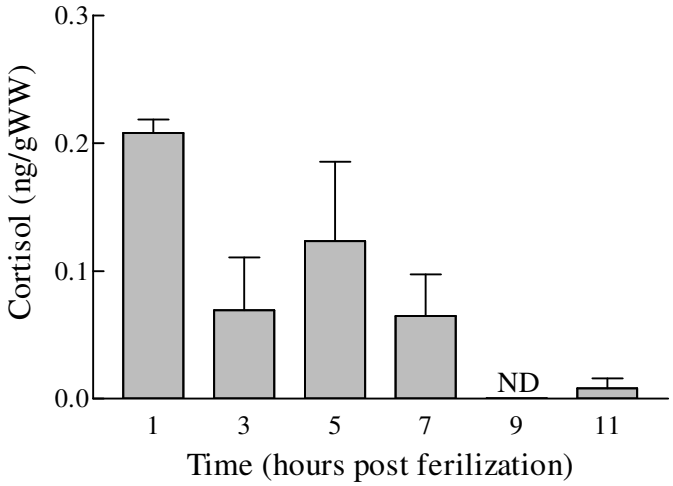


Figure 1.1. Cortisol content of red drum fertilized eggs and developing embryos from 1 through 11 HPF. Values are means ± SE (n = 3). ND denotes no cortisol detected.

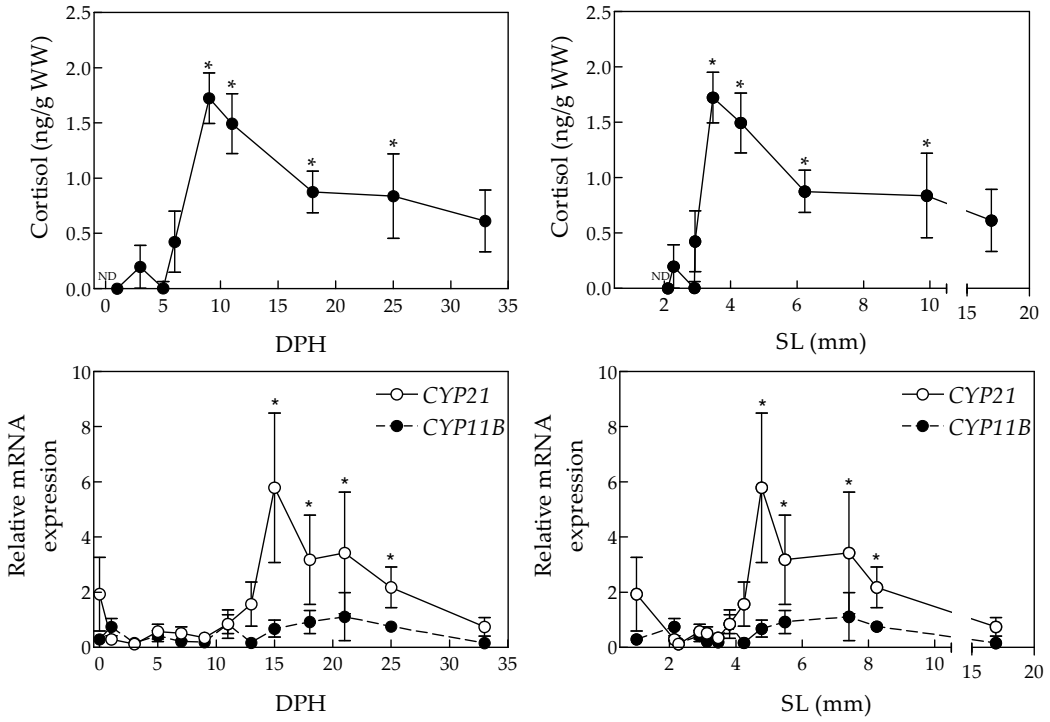


Figure 1.2. A) Whole body cortisol content and B) *CYP11B* and *CYP21* mRNA levels during red drum ontogeny. Values are means ± SE (n = 3). Asterisks denote a significant difference from 1 DPH ($P < 0.05$). ND denotes no cortisol detected.

Figure 1.3. Cortisol stress response in red drum larvae at multiple ages from 1 through 33 DPH. Values are mean ± SE (n = 3). Filled symbols denote fish exposed to stress; open symbols denote controls. Note the change in scale of y-axes in each row. Asterisks denote significant differences from respective controls ($P < 0.05$). Mean standard length on each date is listed in the upper right corner.

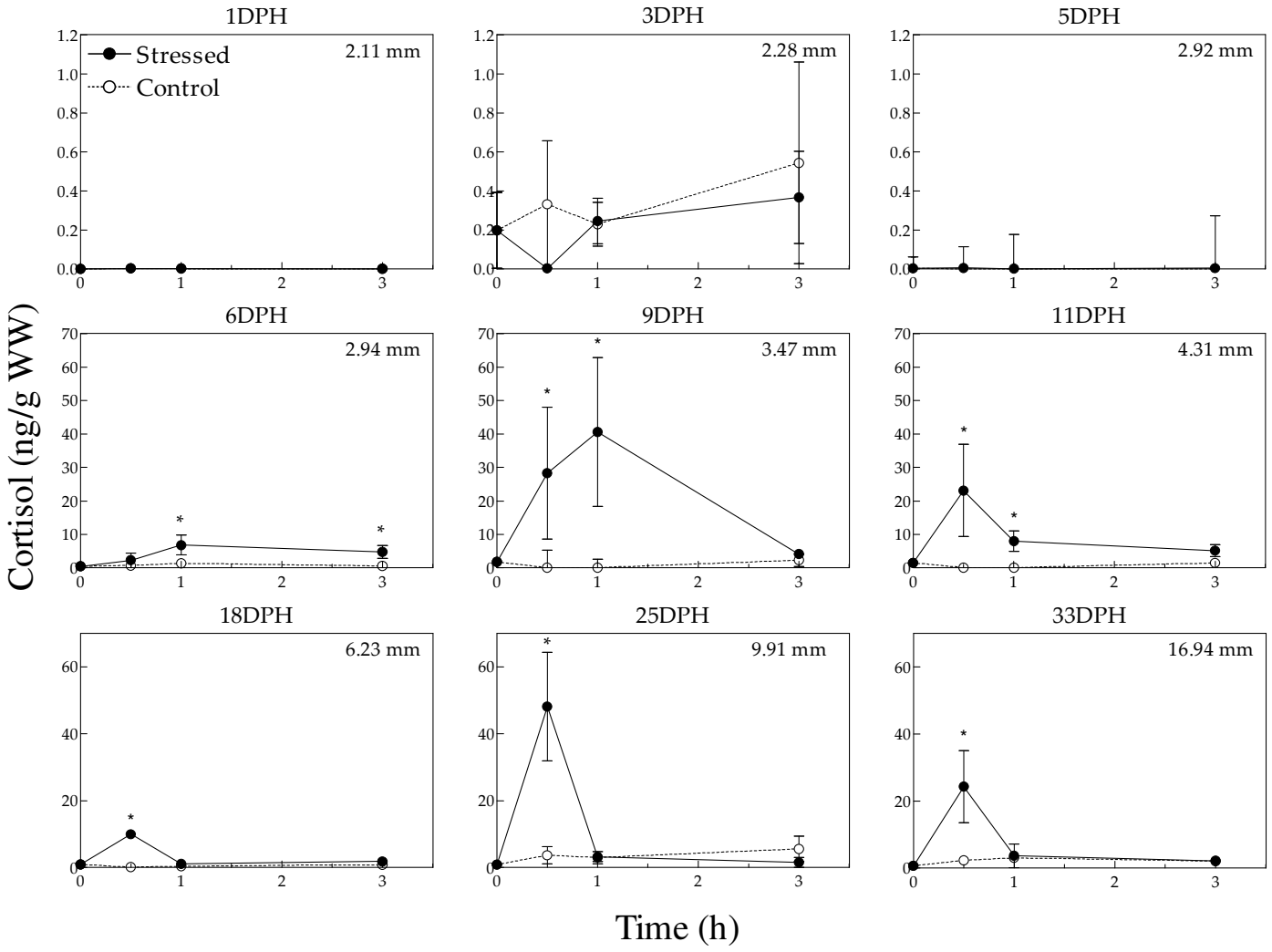

1DPH
3DPH
5DPH
6DPH
9DPH
11DPH
18DPH
25DPH
33DPH
Stressed
Control
2.11 mm
2.28 mm
2.92 mm
2.94 mm
3.47 mm
4.31 mm
6.23 mm
9.91 mm
16.94 mm
Cortisol (ng/g WW)
Time (h)

Figure 1.3

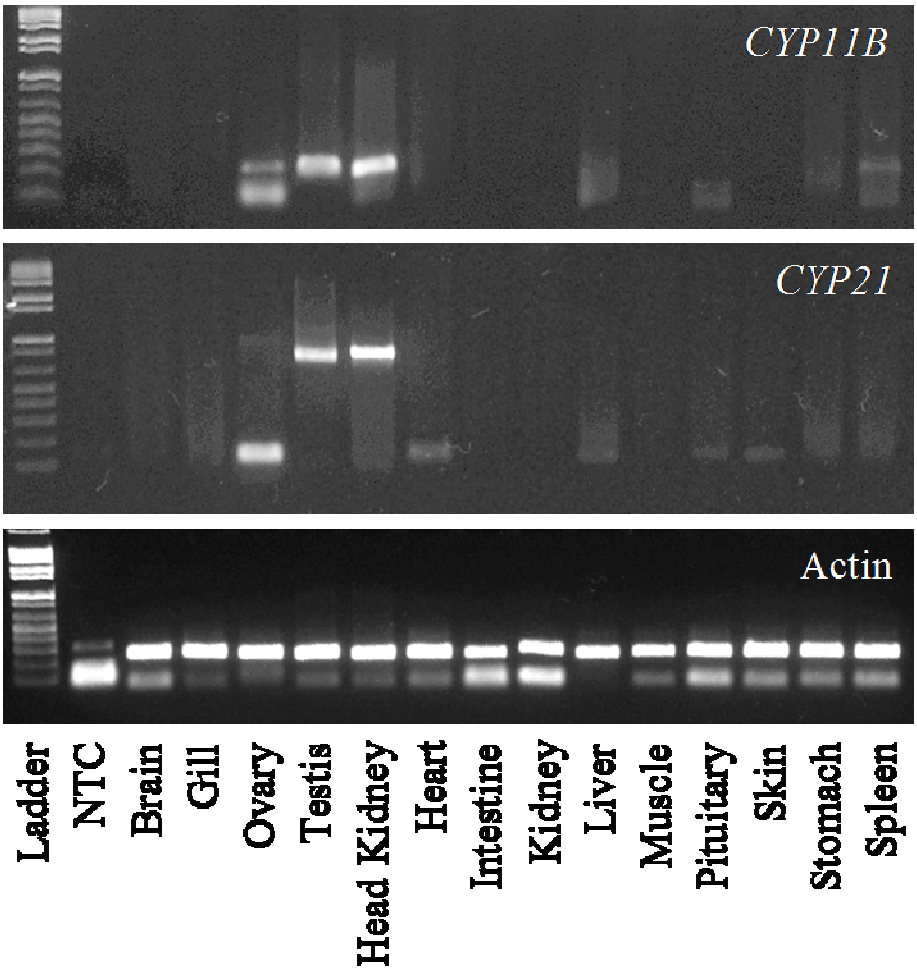


Figure 1.4. Representative tissue distribution of *CYP11B* and *CYP21* in male and female adult red drum as determined by RT-PCR. Actin was used as an internal positive control to insure template quality.

Chapter 2: Ontogeny of whole body thyroid hormone and glucocorticoid receptor mRNA levels and their hormonal regulation of during larval development of red drum (*Sciaenops ocellatus*)

Abstract

The survival of marine teleost larvae is intimately tied to their growth and development, both of which are strongly dependent on thyroid hormones (TH) and corticosteroids (CS). I examined ontogenetic patterns of thyroid (*soTRα* and *soTRβ*) and corticosteroid (*soGR*) hormone receptor mRNA levels, as well as hormonal auto-induction and cross-regulation of receptor mRNA levels in red drum (*Sciaenops ocellatus*) larvae. *soTRα* and *soTRβ* complementary DNAs expressed in green monkey kidney (COS-1) cells were able to trans-activate a thyroid response element reporter construct indicating they encoded functional thyroid receptors. A low level of constitutive trans-activation was also evident in *soTRα* transected cells. A cDNA encoding red drum *soGR* was also isolated. The ontogenetic pattern of *soTRα*, *soTRβ* and *soGR* levels revealed that mRNAs were present in all larval stages examined from 12 h post-fertilization (HPF) through 33 day post-hatch (DPH). Relative to 0 DPH, *soTRα* was significantly increased at 18-25 DPH, while *soGR* was increased at 7 and 11-25 DPH. *soTRβ* did not change significantly during development. The level of *soTRα* were significantly correlated with that of *soTRβ* mRNA during ontogeny as was the level of *soTRα* with *soGR*. To assess the regulation of these transcripts by exogenous TH or CS, red drum were exposed to seawater containing triiodothyronine (T3) or cortisol. T3

elicited significant increases in levels of *soTRα* and *soTRβ* mRNAs in early (7 DPH), but not in mid- (21 DPH) or late (35 DPH) larvae. T3 did not affect *soGR* mRNA levels at any age, nor did cortisol alter levels of *soTRα* or *soTRβ* at any age. Cortisol-treated larvae 35 DPH had significantly lower levels of *soGR* after 24 h. Embryonic expression of *soTRα*, *soTRβ* and *soGR* suggested that maternal hormones found in embryos and early larvae could activate receptors prior to the onset of endogenous hormone production. The correlation between levels of *soTRα* and *soGR* may reflect coordinated function of the TH and CS systems despite a lack of evidence of regulatory interactions between the thyroid and corticosteroid hormone concentrations and receptor mRNA under the conditions in this study. These results support an important role for TH in the larval to juvenile transformation of red drum larvae.

Introduction

Many marine teleosts produce small eggs and larvae that upon hatching are poorly developed. Mortality during this period is far higher than in subsequent life stages, often exceeding 99% (Houde 1987). Following rapid growth and extensive developmental change the larval period terminates with the transformation of a larva to a juvenile. Growth and development, both of which are strongly dependent on endocrine regulation, are therefore intimately tied to larval survival.

The thyroid and corticosteroid hormone axes are common to all vertebrates and have important and mutable roles throughout life. In vertebrates, thyroid hormones (TH) regulate metabolic rate and growth (Hulbert 2000). In addition, TH control the

physiological and morphological events which comprise the developmental transformation from larva to juvenile in many amphibian (Shi 1994; Shi et al. 1996) and teleost species (de Jesus et al. 1993; Solbakken et al. 1999; Power et al. 2001). Corticosteroids (CS), such as the primary teleost CS cortisol, have prominent roles in the stress response (Wendelaar Bonga 1997; Mommsen et al. 1999; Charmandari et al. 2005) and hydromineral balance (Wendelaar Bonga 1997; Bonvalet 1998; McCormick 2001), but also function in development (Liggins 1976; Thorburn and Challis 1979; Wada 2008). Results from teleosts and several other vertebrate groups suggest that, in addition to their independent activities, the TH and CS systems may have synergistic, antagonistic, or co-regulatory interactions with one another at various life stages including during larval development (Kuhn et al. 1998; Wada 2008).

Most actions of TH are mediated through the thyroid hormone receptors (TR), *TRα* and *TRβ*, which are members of the nuclear receptor superfamily (Mangelsdorf et al. 1995). These ligand-dependent nuclear transcription factors can enhance or inhibit transcription by binding specific nucleotide sequences (thyroid response elements, TRE) in the regulatory regions of TH-sensitive genes (Wu and Koenig 2000). In contrast to mammals that have one gene each for *TRα* and *TRβ*, teleosts typically have two different *TRα* genes (Yamano et al. 1994; Essner et al. 1997; Liu et al. 2000; Marchand et al. 2001; Galay-Burgos et al. 2008). In some cases two *TRβ* genes are present as well (Kawakami et al. 2003a). As in amphibian metamorphosis (Yaoita and Brown 1990; Krain and Denver 2004; Brown and Cai 2007) the expression of one or multiple thyroid receptor

forms is correlated with surging TH concentrations during the teleost transformation process (Yamano and Miwa 1998; Marchand et al. 2004; Galay-Burgos et al. 2008).

Like TH, the effects of cortisol are mediated though specific nuclear receptors (glucocorticoid receptor; GR) that subsequently act on CS-responsive genes to change their transcription rates. At present, information is not available on the ontogenetic expression of these receptors in marine teleost larvae. Cortisol has been shown to promote growth and development in teleosts (Mathiyalagan et al. 1996). Further, several studies indicate that cortisol can act synergistically with TH (de Jesus et al. 1990; Brown and Kim 1995; Kim and Brown 1997) although the mechanism of action is unknown. An increase in cortisol coincident with, or slightly preceding the TH surge is evident in several species, further supporting a relationship between these systems (de Jesus et al. 1991; Pérez-Domínguez et al. 1999; Deane and Woo 2003; Szisch et al. 2005).

In this study, I isolated cDNAs that encode TRα, TRβ and GR from red drum (*Sciaenops ocellatus*) and determined their ontogenetic pattern of expression in laboratory-reared larvae. Both the thyroid and corticosteroid axes involve complex regulatory pathways that integrate neural, endocrine and peripheral tissues. The nuclear receptors and their respective ligands represent the proximate site of action for these pathways and are therefore strong candidates for the mediation of thyroid/corticosteroid system interactions. At present, the ontogeny of hormonal auto-induction and cross-regulation of TR and GR mRNA have not been examined. To address the hypothesis that stage-dependent interactions between the thyroid and corticosteroid axes occur at the level of hormone production and receptor expression, I examined the response of these

mRNAs to exogenous hormone treatment in early (7 day post-hatch; DPH), mid- (21 DPH) and late (35 DPH) larvae.

While our results do not provide evidence of hormonal cross-regulation of these endocrine systems, a correlation between the levels of *TRα* and *GR* mRNAs suggests that the systems have a coordinated function in development. Further, we observed ontogenetic changes in the auto-regulation of both *TRα* and *TRβ* mRNA levels that may support an important role of thyroid hormones and receptors in the larval to juvenile transformation of red drum.

Methods

Rearing of experimental animals

Fertilized red drum eggs were obtained from broodstock induced to spawn by temperature and photoperiod control (Arnold 1988) at the Fisheries and Mariculture Laboratory of the University of Texas at Austin, Marine Science Institute in Port Aransas, Texas and the Texas Parks and Wildlife Department Hatchery in Flour Bluff, Texas. Eggs were disinfected in seawater containing 10 ppm formalin for 20 min before being rinsed with clean seawater. Eggs were then transferred to 1-L beakers of filtered seawater at a density of 1000 eggs/L. Embryos hatched approximately 24 h later (1 DPH) and larvae were moved to 600-L cylindrical tanks with internal biological filters at a density of 8000 per tank. An airstone at the bottom of the tank provided additional water circulation and maintained dissolved oxygen near saturation levels. Salinity and

temperature were maintained at approximately 27 °C and 30 psu respectively, and photoperiod was 12:12 h (light:dark).

Exogenous feeding begins 3 DPH at which time larvae were co-fed live rotifers (*Brachionus plicatilis*) at 2-5 rotifers/mL along with Otohime microparticulate diet (Reed Mariculture, Campbell, CA). Prior to use, rotifers were enriched overnight with *Isochrysis galbana* and Algamac 2000 (Biomarine, Inc. Hawthorne, CA). Microparticulate diet was administered by automatic feeders throughout the day. Beginning at 10 DPH larvae were fed enriched *Artemia* nauplii and Otohime microparticulate diet. By 14 DPH, larvae were fed microparticulate diet exclusively.

Isolation of TRα and TRβ, and GR cDNAs

Receptor sequences were amplified from cDNA libraries reverse transcribed from whole, 33 DPH red drum larvae RNA. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, DNase (Promega, Madison, WI) treated and repurified using TRIzol. The resulting pellet was dissolved in nuclease-free water and the concentration determined spectrophotometrically by absorbance at 260 nm. First-strand cDNA was synthesized in a 10 µl reverse transcription (RT) reaction which included 1 µg of RNA and 100 ng of random hexamers. RT reactions were conducted with SuperScript III reverse transcriptase (SSIII; Invitrogen) according to the manufacturer's protocol. RT products were used as template for the amplification of receptor transcripts by polymerase chain reaction (PCR). Initial fragments of both *soTRα* and *soTRβ* were amplified using degenerate primers previously reported to amplify

teleosts thyroid receptors (Marchand et al. 2001). Amplification of an initial fragment of *soGR* was accomplished using a forward primer based upon the consensus sequence for the start of the protein coding region in aligned *GR* sequences from *Astatotilapia burtoni* (AF263278, AY495372, AY263739, AF263740), *Dicentrarchus labrax* (AY549305, AY619996), *Paralichthys olivaceus* (AB013444), *Sparus aurata* (DQ486890). The reverse primer was derived from a partial *GR* sequence of a close relative of red drum, spotted seatrout *Cynoscion nebulosus* (Drs. J. Bergeron and P. Thomas, unpublished). Primer pairs are listed in Table 2.1. PCR reactions were conducted using AmpliTaq Gold polymerase system (Applied Biosystems, Foster City, CA). Cycling conditions were as follows: initial denaturation at 94 °C for 8 min, followed by 40 cycles of denaturation for 1 min at 94 °C, annealing for 30 s at 40-60 °C, and 1 min extension at 72 °C. Amplicons of approximately the expected size (as determined by agarose gel electrophoresis) were ligated into the pDrive cloning vector (Qiagen, Valencia, CA) and sequenced.

The 5' and 3' flanking regions of the *TRα* and *TRβ*, and the 3' end of *GR* were isolated by rapid amplification of cDNA ends (RACE). RACE-ready cDNA libraries were prepared using the Clontech SMART-RACE system (Takara Bio Inc. Japan) according to the manufacturers' protocol. Primer pairs and cycling conditions used to amplify flanking regions can be found in Table 2.1. Amplicons were ligated into the pDrive cloning vector and sequenced.

Heterologous expression of TRα and TRβ in COS-1 cells

The coding region of each receptor was amplified and ligated into an expression vector containing the mammalian CMV promoter (*TRα:* pCDNA3.1, *TRβ*: pCR3.1). The insertion and proper orientation of the desired receptor sequences into expression constructs was verified by sequencing. COS-1 (green monkey kidney) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomyocin. The day prior to transfection, cells were plated in 12-well plates at a density sufficient to attain 50-80% confluency the following day. Each well was transfected with 0.45 µg of receptor expression construct, 0.45 µg pGL2-xDR4 reporter, and 0.1 µg pCMV β-gal vector using the TransIT-COS transfection kit (Mirus Bio Corp., Madison, WI) according to the manufacturer's instructions. Control cells were transfected with empty expression vector in place of those containing receptor sequences. After 24 h cells received fresh DMEM prepared with charcoal-stripped fetal bovine serum to which was added 1 µL/mL triiodothyronine (T3) stock in ethanol at concentrations sufficient to produce 10 or 100 nM final T3 concentrations. Control groups received ethanol only. Cells were treated for 24 h and then lysed for determination of β-galactosidase and luciferase activity. Each treatment was conducted in duplicate for each of three independent transfections per receptor. Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency, and results were expressed as percent of control cell luciferase activity in the absence of T3. The pGL2-xDR4 reporter construct (Marchand et al. 2004) was donated by Drs. Laure Bernard and Vincent Laudet.

Ontogeny of TRα, TRβ and GR expression

Pools of larvae comprising approximately 50 mg of wet tissue were collected from spawns of red drum at times between 12 h post-fertilization (HPF) and 33 DPH. Three replicates of this ontogenetic series were collected from a single batch of fish. Fifteen larvae were also collected from the rearing tank for determination of standard length (SL) on each sample date. Tissue was immediately frozen on dry ice and stored at -80 °C until RNA extraction and determination of mRNA levels.

Hormonal regulation of receptor mRNA levels and hormone concentrations

Experiments were conducted with 7, 21 and 35 DPH larvae to determine the response of *TRα*, *TRβ* and *GR* mRNA levels to exogenous thyroid or corticosteroid treatment. Mean length of larvae in rearing tanks was determined on each test date. On the day of experiments, groups of larvae were transferred to 1-L beakers of filtered seawater containing one the following treatments: T3 (50 nM), cortisol (250 nM), control (vehicle only). T3 (50 mM) and cortisol (250 mM) stocks were prepared in NaOH (0.5 N) and DMSO vehicles respectively. Vehicle controls were applied such that all groups of larvae received equal amounts of NaOH and DMSO. In experimental treatments of 7, 21, and 35 DPH larvae, 80, 30 or 10 larvae, respectively, were placed in each enclosure. A single replicate of the experiment included four groups of fish exposed to each treatment (12 beakers). At 4, 8, 12 and 24 h, one group of each treatment was collected, rinsed in deionized water and frozen at -80 °C for determination of mRNA levels and hormone concentration. Samples were collected from source tanks prior to initiation of

treatments (time 0) to determine the initial status of larvae. Approximately 1.5 ml of water was also collected from 24-h beakers to determine effective hormone concentrations. The experiment was replicated with larvae from three separate spawns.

Quantitative reverse-transcription polymerase chain reaction assays

Total RNA was extracted and DNase-treated as described above for cloning and sequencing. RT-negative reactions were used as template for PCR with actin primers to confirm the absence of genomic DNA. DNA-free RNA (1 µg) was used in 20 µl RT reactions with SSIII reverse transcriptase (Invitrogen) and subsequently diluted to a total volume of 60 µl with DNase-free water. Steady-state levels of *TRα*, *TRβ* and GR mRNA were determined in real-time quantitative reverse transcriptase polymerase chain reaction (qPCR) assays using Brilliant II QPCR Master Mix (Stratagene, La Jolla, CA). Each 25 µl reaction mixture contained 5 µl cDNA template, 400 µM of both forward and reverse primers (Table 2.1), and 200 µM TaqMan probe (Sigma Genosys, The Woodlands, TX). Reactions were cycled and the resulting fluorescence detected with an Eppendorf RealCycler (Eppendorf, Westbury, NY) using the primer and probe combinations and reaction conditions described in Table 2.2. Receptor mRNA levels were normalized to ribosomal *18S* RNA levels determined in separate qPCR reactions. In the ontogenetic study, relative mRNA levels were calculated according to the method of Fink et al. (1998). In hormonal regulation experiments, the number of copies per sample was determined by reference to a standard curve created using dilutions of linearized plasmids containing a calculated number of copies of the amplified region of the target gene.

Standard curves were included in each 96-well qPCR assay. All qPCR samples were run in duplicate.

Cortisol and thyroid hormone assays

Extractions were performed according to methods previously validated for red drum larvae (Pérez-Domínguez et al. 1999). Quantification of whole body cortisol was performed by a radioimmunoassay method modified from de Jesus et al. (1991). Cortisol antibody was purchased from Fitzgerald Industries International, Inc. (cat. no. CR-50, Concord, MA). Assays were conducted in borosilicate tubes and contained 50 µL of extracted sample, 100 µL of cortisol antibody diluted 1:3200 in EDTA-PBS (0.01M PBS, 40mM EDTA, pH 7.0) and 10,000 dpm of radiolabeled cortisol (cat. no. Net-396, Perkin–Elmer, Boston, MA,) in 50 µL assay buffer (0.01M PBS, 0.1% gelatin, pH 7.4). Tubes were covered, vortexed briefly, and incubated overnight at 4 °C. After incubation, 150 µL of 0.25% dextran-coated charcoal in assay buffer was added to tubes. Tubes were vortexed three times for 1 s, incubated at 4 °C for 15 min, and then shaken at 100 RPM for 15 min before centrifugation at 5000 RPM (10 min, 4 °C). Finally, 250 µL of each supernatant was transferred to a scintillation vial for counting.

Serial dilutions of red drum pooled larval homogenates produced binding curves parallel (slopes were not significantly different, $P = 0.81$) to that of purified cortisol standards, verifying the assay's suitability for measuring red drum whole body cortisol (Fig. 2.5).

Thyroid hormones, T3 and T4 were measured in separate enzyme immunoassays using methods previously validated for red drum larvae (Pérez-Domínguez 2004) with the following changes. Horseradish peroxidase-conjugated T3 and T4 tracers were purchased from Fitzgerald Antibody, Inc. (Concord, MA, product nos. 65-IT50 and 65-IT35) and used at 0.2 U/mL and 0.4 U/mL respectively. Antibody dilutions for plate coating were 1:8000 and 1:1500 for T3 and T4, respectively.

Statistical analyses

Phylogenetic trees were prepared by the Neighbor Joining Method using *MEGA* version 4 software (Tamura et al. 2007). Heterologous expression experiments were assessed by two-way ANOVA followed by the Bonferroni post-hoc test to determine treatments that differed significantly. Ontogenetic changes in receptor mRNAs levels were assessed by one-way ANOVA. Relationships in the pattern of receptor mRNA levels during ontogeny were assessed by Pearson correlation of log-transformed values. In hormonal regulation experiments, changes in receptor mRNA levels and hormone content were assessed by a completely randomized block design two-way ANOVA. The spawn from which larvae were obtained was used as the blocking factor. Where appropriate, Tukey's post-hoc test was used to determine treatment groups that differed significantly. The less conservative Fisher's Least Significant difference test was used for pairwise comparisons in several instances where Tukey's test did not detect differences despite a significant ANOVA main effect. Where necessary, data were log-transformed prior to ANOVA to attain equality of variance. In two instances where equality of

variance could not be obtained a comparison of treatments within time was analyzed with the non-parametric Kruskal-Wallis test, using the Bonferroni correction to adjust the alpha values for the number of tests conducted. One-way and two-way ANOVA were conducted using SYSTAT 10 (Systat Software, Inc., San Jose, CA). Correlations and the slope test were conducted using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). In all statistical tests, results were accepted as statistically significant if $P < 0.05$.

Results

Isolation of TRα, TRβ and GR cDNAs

Red drum *TRα*, *TRβ* and *GR* (hereafter referred to as *soTRα*, *soTRβ* and *soGR,* respectively) cDNAs were isolated using a combination of RT-PCR and RACE. Most, if not all, of thyroid receptor mRNA sequences were obtained, including 5' and 3'-untranslated (UTR) regions. The *soTRα* cDNA (1378 bp) included an open reading frame (1248 bp) that encoded a deduced protein of 415 amino acids (AA). The *soTRβ* cDNA (2545 bp) included an open reading frame (1185 bp) that encoded a deduced protein of 394 AA. The *soGR* cDNA (3155 bp) included an open reading frame (2388 bp) that encoded a deduced protein that is similar in size (795 AA) to other GR proteins. In addition, three individual clones contained 3'-UTR with poly-A tracts at the 3' terminus (not included in transcript lengths). Multiple attempts at isolating *soGR* 5'-UTR sequence were unsuccessful. The forward primer for the *GR* (Table 2.1, primer *GR* F) overlapped the predicted start site, so the exact identity of the first 22 bp is not known with certainty. Raw sequences can be found in Appendix 1.

In a phylogenetic analysis the amino acid sequences deduced from *soTRα* and *soTRβ* grouped with their respective orthologs from teleosts, amphibians and mammals (Fig. 2.1). The deduced soTRα protein was most closely related to that of *Acanthropagrus schlegelii,* while the deduced soTRβ protein was mostly closely related to the *S. aurata* and *A. schlegelii* TRβ proteins. The deduced soGR protein grouped with GR proteins of other teleosts and specifically with the GR1 subgroup (Fig. 2.2). Of the sequences included in this analysis, soGR was most similar to *D.labrax* GR1 and *P. olivaceus* GR.

Heterologous expression of soTRα and soTRβ in COS-1 cells

Relative to control cells, significantly greater luciferase activity was measured in COS-1 cells transected with *soTRα*, even in the absence of T3 (Fig. 2.3A). A dose-dependent increase in luciferase activity was observed in COS-1 cells transfected with *soTRα*, but not in cells transfected with empty expression plasmid. A statistically significant difference was evident between untreated *soTRα* transfected cells (ethanol only) and those treated with 100 nM T3. In the absence of T3, luciferase activity in cells transfected with *soTRβ* did not differ from that of control cells, but there was significantly greater luciferase activity in *soTRβ* transfected cells treated with 10 or 100 nM T3 relative to treated control cells (Fig.2.3B).

Ontogenetic expression of soTRα, soTRβ and soGR

soTRα, soTRβ and *soGR* were expressed beginning at the earliest sampling time (12 HPF, 0 DPH, Figure 2.4). Steady-state levels of *soTRα* increased during ontogeny the level of *soTRα* was significantly higher at 18-25 DPH than 0 DPH. Steady-state levels of *soGR* mRNA also increased during most of the larval period. *soGR* mRNA levels were significantly greater than 0 DPH on 7 DPH, and 11 through 25 DPH. Both *soGR* and *soTRα* steady-state mRNA levels declined steeply by the end of the larval period (35 DPH). *soTRβ* mRNA levels were highly variable on several dates and mean levels did not change significantly during larval development. There were significant correlations between levels of *soTRα* and *soGR* ($r = 0.61$, $P < 0.0001$ and between *soTRα* and *soTRβ* levels ($r = 0.43$, $P < 0.05$). *soGR* and *soTRβ* levels were not significantly correlated ($r = 0.23$, $P = 0.19$).

Hormonal regulation of receptor mRNA levels and hormone concentrations

Steady-state levels of *soTRα*, *soTRβ* did not change significantly in response to cortisol treatment at 7, 21 or 35 DPH (Fig. 2.6). Cortisol did not affect *soGR* levels in 7 or 21 DPH larvae. However, after 24 h of cortisol treatment the level of *soGR* mRNA was significantly less in 35 DPH larvae than untreated controls (ANOVA, $P < 0.01$). T3 treatment significantly increased *soTRα* mRNA levels in 7 DPH larvae (ANOVA, $P < 0.001$) 4, 8 and 12 h after treatment was initiated. No change in *soTRα* mRNA occurred in response to T3 in larvae 21 or 35 DPH. T3 treatment significantly increased *soTRβ* mRNA in 7 DPH larvae (ANOVA, $P < 0.001$) at all times examined. T3 did not increase

soTRβ mRNA in 21 or 35 DPH larvae. The levels of *soGR* mRNA did not change significantly in response to T3 treatment at 7, 21, or 35 DPH.

Exogenous cortisol treatment did not significantly change cortisol, T3 or T4 levels in red drum larvae (Fig. 2.7). A significant increase in T3 (ANOVA, $P < 0.001$) was detected at 4 and 8 h in 21 DPH T3 treated larvae. Cortisol was not significantly different from controls in T3 treated larvae. In T3 treated 35 DPH larvae, T4 was significantly greater than controls after 8 h (ANOVA, $P < 0.01$). Nominal levels of T3 and cortisol in seawater sampled from exogenous T3 or cortisol treatments at the end of 24 h were 55.9 ± 5.2 nM and 100.8 ± 12.7 nM respectively. The SL of larvae used in regulation experiments is summarized in Table 2.3.

Discussion

The ontogenetic pattern of *soTRα* and *soTRβ* mRNA levels is similar to that of other teleost larvae and supports a role for thyroid hormones in the larval-to-juvenile transformation of red drum. During the early larval period *soTRα* and *soTRβ* mRNA levels were auto-regulated by their primary ligand, T3. A change in receptor regulation occurs by the mid-larval period and TR mRNAs are no longer auto-induced. I did not observe interactive regulation of receptors or hormones in response to exogenous hormone treatments. However, a correlation between *soTRα* and *soGR* mRNA levels over the ontogenetic period examined may reflect cooperative activity or a common regulatory mechanism of the TH and CS systems.

Vertebrates possess two TR genes, *TRα* and *TRβ*, each capable of producing a number of subforms by differential splicing (Lazar 1993). In addition, two distinct *TRα* genes are present in several teleosts (Yamano et al. 1994; Essner et al. 1997; Liu et al. 2000). The identification of cDNAs similar to each *TRα* form in a number of additional species suggests that two *TRα* genes may be characteristic of the group (Jones et al. 2002; Kawakami et al. 2003b; Galay-Burgos et al. 2008). Further, a second *TRβ* gene has been identified in the conger eel (Kawakami et al. 2003a). In the present study, one *TRα* and one *TRβ* transcript were successfully isolated from red drum. In phylogenetic analysis, TRs group by *α* or *β* form across vertebrate groups reflecting the genesis of these isoforms in a gene duplication event early in vertebrate evolution (Laudet et al. 1992; Laudet 1997). Within both the *TRα* and *TRβ* branches, teleost TR proteins are most closely related, forming a distinct subgroup. A subsequent gene duplication within the teleost lineage is believed to be the source of the second *TRα* gene found in most teleosts examined (Robinson-Rechavi et al. 2001). I did not identify a second *soTRα*; however, it is probable that additional variants of the isolated *soTRα* transcript, as well as an additional *soTRα* gene exist.

The *soTRβ* sequence contained a 27-nucleotide (nine-amino acid) insertion unique to teleosts and located in the beginning of the ligand-binding domain. This insertion, which is likely the product of an additional exon (Marchand et al. 2001) has been found in all teleost *TRβ* sequences examined to date, although it is not included in all splice variants (Yamano and Inui 1995; Marchand et al. 2001). The impact of the presence/absence of this insertion on receptor function is unknown.

When expressed in COS-1 cells, both *soTRα* and *soTRβ* trans-activated a luciferase reporter in response to T3. T3 (10 or 100 nM) significantly elevated luciferase activity of *soTRα* and *soTRβ* transfected cells over that of control cells. The magnitude of luciferase production as a result of TRE trans-activation in response to T3 was similar for the two receptors. Typically, unliganded TRs, acting as dimers with cis-retinoic acid receptors repress trans-activation of TH-inducible genes (Graupner et al. 1989; Horlein et al. 1995). Nonetheless, significant luciferase activity was evident in *soTRα* transfected COS-1 cells in the absence of TH. In this experiment, transfected cells were grown in media prepared with charcoal-stripped serum, removing the media as a source of TH. In addition, constitutive activity was not apparent in cells transfected with *soTRβ.* A similar pattern of unliganded trans-activation activity in heterologous transfection has been reported for *D. rerio TRα* and *TRβ* (Liu et al. 2000). The results of these transfection experiments further indicate that the isolated cDNAs encode functional red drum TRs.

Two phylogenetically distinct GR genes have been identified in teleosts and are believed to be present throughout the group (Bridgham et al. 2006; Stolte et al. 2006). The genes are hypothesized to have arisen from a teleost genome duplication which occurred 335-404 million years ago (Hoegg et al. 2004; Bury and Sturm 2007). The amino acid sequence deduced from *soGR* is clearly associated with teleost GR type 1 subgroup in the phylogenetic analysis. Further, the red drum GR includes a nine-amino acid insertion which typifies a GR1 variant found in a number of other teleosts (Ducouret et al. 1995; Takeo et al. 1996; Greenwood et al. 2003; Stolte et al. 2006). This insertion, located in the DNA binding domain, is encoded by an additional exon (Lethimonier et al.

2002; Stolte et al. 2006) and appears to be unique to teleosts. Although only one so*GR* was identified in this study, it is likely that splice variants of *soGR* as well as an additional so*GR* gene remain to be identified in red drum. However, at least one teleost (*Danio rerio*) has only one *GR*, presumably having lost the second form subsequent to the teleost genome duplication (Alsop and Vijayan 2008).

The presence of thyroid hormones (Tagawa et al. 1990) and cortisol (de Jesus et al. 1991; de Jesus and Hirano 1992; Hwang et al. 1992) in teleost eggs is well established and is presumably the result of maternal transfer during oogenesis. However, the function these hormones play in embryonic development is relatively unknown, as are temporal patterns of mRNA and protein expression, and accessibility of receptors to maternal ligand. In this study, the expression of *soTRα* and *soTRβ* was detected in the earliest developmental stage examined, (12 HPF). This suggests that an active signaling mechanism for TH action exists in embryonic red drum. Previous reports of TR expression in teleost embryos come primarily from *D. rerio* (Essner et al. 1997; Liu et al. 2000; Liu and Chan 2002), although the presence of *TRβ* has also been shown in embryos of at least one marine species, *Sparus aurata* (Nowell et al. 2001). A functional role for TH and TRs in the embryonic-to-larval transition is indicated by a study of *D. rerio* in which embryos co-treated with an anti-goitrogen and TR antagonist displayed severe developmental abnormalities and reduced survival after hatching. These detrimental effects were rescued by T4 treatment (Liu and Chan 2002). Increasing tissue T3 levels by exogenous supplementation can also alter development and gene expression, A low dose of exogenous T3 (5 nM) accelerated the development of pigmentation in *D. rerio* and up-

regulated *TRα* expression while type II deiodinase expression was depressed (Walpita et al. 2007). Studies of other species are necessary to determine the general actions of TH in teleost embryos and whether the effects are mediated by TRs.

Relative to TH, far less is known about the role of cortisol in embryonic teleosts. Several studies suggest that embryonic cortisol variation influences larval growth, survival and development (McCormick 1998, 1999, 2006; Eriksen et al. 2006). Glucocorticoid receptor mRNA has been detected in embryos of freshwater teleosts including *Oreochromis mossambicus* (Tagawa et al 1997) and *D. rerio* (Alsop and Vijayan 2007). Embryonic expression of *soGR* is in agreement with these studies and indicates that nuclear CS signaling can occur in red drum embryos. To my knowledge, it has not yet been established whether glucocorticoid receptor protein is present during early development. However, the identification of cortisol binding sites in *Oncorhynchus mykiss* embryos (Pillai and Terner 1974) suggests this is the case.

The teleost larval period terminates with a period of morphological and physiological change to attain the juvenile form. This process is most dramatic in flatfishes (Pleuronectiformes) which metamorphose from bilaterally symmetrical larvae living in the water column, to asymmetrical, benthic forms. Extensive study of this process has shown that increased T3 and/or T4 levels (de Jesus et al. 1991; Yamano and Miwa 1998; Hotta et al. 2001; Galay-Burgos et al. 2008; Klaren et al. 2008) and a corresponding increase in levels mRNA encoding of one or multiple forms of TR (Yamano and Miwa 1998; Power et al. 2001; Marchand et al. 2004; Galay-Burgos et al. 2008) coincide with the transformation process. Further, thyroid hormone actions are

necessary for proper development in these fishes (de Jesus et al. 1993; Schreiber and Specker 1998; Solbakken et al. 1999).

Although non-pleuronectiform teleost larvae lack the dramatic change in bauplan, critical changes in morphology and physiology occur as they transform from larva to juvenile. A surge in thyroid hormone content during the transformation period is evident in numerous non-flatfish teleosts (Pérez-Domínguez et al. 1999; Deane and Woo 2003; Kawakami et al. 2003b; Crane et al. 2004; Szisch et al. 2005; Pérez-Domínguez and Holt 2006; Yamano et al. 2007; Kawakami et al. 2008; Klaren et al. 2008) including red drum (Pérez-Domínguez and Holt 2006). The concurrent increase in TR mRNA is also reported in instances where TR cDNA have been isolated and examined during ontogeny (Kawakami et al. 2003b, 2008). Further, a dependence of the transformation process on TH and/or TRs has been demonstrated in diverse non-flatfish teleost species including *Megalops cyprinoides* (Shiao and Hwang 2006), *Plectropomus leopardus* (Trijuno et al. 2002), *Epinephelus coioides* (de Jesus et al. 1998) and *D. rerio* (Brown 1997). The pattern of *soTRα* expression presented here supports a functional role for TH and thyroid receptors in larval to juvenile transformation of red drum. Although the ontogenetic patterns of larval T3 and T4 content were not measured in the current study, the increase in *soTRα* expression around 18 DPH corresponds well with the timing of larval transformation and increased TH levels in developing red drum (Pérez-Domínguez and Holt 2006). While *TRβ* did not change significantly during ontogeny, a moderate correlation with *soTRα* expression was evident. With the exception of highly variable mRNA levels during early larval development (3 and 9 DPH), mean *soTRβ* mRNA levels

were highest on 18, 21, and 25 DPH, the same days on which significant elevations of *soTRα* were observed. This similarity in the expression pattern to that of *soTRα* suggests that *soTRβ* may also involved in the transformation process. Possibly, *soTRβ* plays a lesser role in this process, or is found in a limited number of tissues.

The patterns of TR mRNA levels in red drum agree well with those from other teleosts. An increase in one or multiple forms of TR during transformation is typical of teleosts, although the specific patterns of TR isoform levels are far from uniform. Transformation-associated increases may occur for *TRα* only, *TRβ* only, or for both isoforms. For example, a strong increase in *TRα* levels, with little or no change in *TRβ*, occurs during transformation in *Scopthalmus maximus* (Marchand et al. 2004). Conversely, *TRβ* forms were up-regulated during transformation in *Conger myriaster*, while *TRα* forms showed little to no change (Kawakami et al. 2003b). Finally, one or multiple forms of both *TRα* and *TRβ* may be up-regulated during transformation and coincident with surging TH levels, as in *Paralichthys olivaceus*, *Hippoglossus hippoglossus* and *Thunnus orientalis* (Yamano and Miwa 1998; Galay-Burgos et al. 2008; Kawakami et al. 2008). The variation in these patterns seems to indicate that the function of individual TR forms during larval development differs substantially among species.

In contrast to TRs, levels of GR have not been well characterized during teleost ontogeny. Whole body levels of *D. rerio GR* have only been described within embryonic and early larval development, during which transcripts declined through 25 HPF but increased significantly by 49 HPF, or approximately 12 h post-hatch (Alsop and Vijayan

2008). Levels of *GR* in freshwater *Pimephales promelas* larvae did not differ significantly between 1 and 40 days post fertilization (Filby and Tyler 2007) although only four widely spaced sample dates were analyzed within the larval period.

Whole larvae levels of *soGR* increased significantly from low pre-hatching levels by 7 DPH, corresponding with the increase in red drum basal cortisol production between 5 and 9 DPH, and the onset of the cortisol stress response at 6 DPH (refer to Chapter 1). This pattern suggests a shift in the role of red drum GR from transducer of developmental cortisol signals to a mediator of the stress response. Possibly, early larvae express *soGR* in a limited number of tissues whose development is sensitive to cortisol; as the stress response emerges, *soGR* expression becomes prominent in a wider array of tissues to facilitate the well established role of the receptor in mediating the stress response. Levels of *soGR* were strongly correlated with that of *TRα*, peaking during larval transformation and finally plummeting very low levels near the end of the larval period. While this is not evidence of a mechanistic linkage or interaction between CS and TH systems, it does indicate coordinated action of these systems in developing red drum which could result from interaction.

It should be considered that measurements reported here reflect the combined levels of receptor mRNA in whole larvae and tissue-specific patterns of expression may differ markedly from the overall pattern. Further, the abundance of receptor proteins may not directly correlate to the abundance of the transcripts encoding them.

The levels of exogenous hormone used to treat larvae in this study were not always effective in increasing tissue concentrations of that hormone. Cortisol treatment

did not lead to a statistically significant elevation of larval cortisol on any date. Although not statistically significant, higher means were observed particularly in younger/smaller fish although variability was exceptionally high. Cortisol levels were often high in control groups, suggesting that larvae may have been stressed in the experimental enclosures or the rearing tanks from which they were collected prior to experiments. Similar to cortisol, T3 treatment appeared to be more effective at increasing tissue levels of hormone in younger/smaller larvae. Significant increases in T3 content occurred in T3-treated larvae 21 DPH. Mean levels of TH were increased 4 and 8 h after initiation of treatment in larvae 7 DPH, although not by statistically significant amounts. In this case, high and heteroscedastic variability required the use of less powerful non-parametric statistic methods.

Overall, tissue levels of the treatment hormones were highly variable. Statistically significant increases in hormone by exogenous hormone treatment were not uniformly observed. However, trends in the means suggest that exogenous hormone accumulated more readily in younger/smaller larvae which may be partly due to the greater ration of surface area to volume in smaller organisms. The inconsistent accumulation of exogenous hormone in larvae is somewhat surprising. The lipophilic character of CS and TH presumably allows these hormones to passively diffuse through biological membranes and into larval tissue. The variability in accumulation of hormone in this study suggests that larvae have a substantial capacity for degrading, inactivating or regulating the transport of hormones. T3 immersion effectively increased tissue T3 in the early embryonic stages of *D. rerio* (Walpita et al. 2007) and *O. mykiss* (Raine et al. 2004)

although in both cases levels increased and then decreased, suggesting that regulatory mechanisms are present very early in teleosts. The results of the present study also indicate that these processes change dramatically over larval development and vary between groups of larvae of similar age/size.

A significant increase in T4 relative to control was observed at 8 h in the 35 DPH larvae. This may seem counterintuitive as increased T3 levels typically decrease T4 production via negative feedback. However, it has been shown in *D. rerio* larvae that exogenous T3 can down-regulate the expression of type II deiodinase (D2) which catalyzes the conversion of T4 to T3 (Walpita et al. 2007). If negative feedback on T4 secretion is slower than a change in D2 activity, a transient increase in T4 could result.

Negative auto-regulation of *GR* mRNA levels is typical in mammalian systems (Yudt and Cidlowski 2002), and cortisol treatment of 35 DPH larvae significantly reduced levels of *soGR* after 24 h. However, the regulation of *GR* in teleosts may differ depending upon the duration of exposure. In agreement with results presented here, acute cortisol treatment of *O. mossambicus* reduced *GR* 24 h after cortisol injection. In contrast, chronic cortisol exposure has been shown to up-regulate GR mRNA in *O. mossambicus and O. mykiss* (Vijayan et al. 2003; Takahashi et al. 2006) while concurrently decreasing protein abundance in *O. mykiss* (Vijayan et al. 2003).

This study shows that so*TRα* and so*TRβ* are auto-induced by their ligand during the early larval development of red drum, suggesting the presence of thyroid hormone response element (TRE) in the promoter regions of these genes. TREs have been identified in the promoter of TR genes in *Xenopus laevis* (Kanamori and Brown 1992;

Machuca et al. 1995) for which auto-induction of the *TRα* and *TRβ* has also been demonstrated (Yaoita and Brown 1990). Levels of *soTRα* and *soTRβ* were auto-induced in 7 DPH red drum, but this response was not apparent in 21 or 35 DPH larvae. Presumably, increased endogenous T3 levels which occur during larval transformation (Pérez-Domínguez 2004) have already induced maximal levels of TR transcripts in 21 and 35 DPH larvae. However, it is possible that a period of competency for TR induction occurs beyond which the responsiveness of the receptor mRNA levels to TH stimulation is eliminated. A test for auto-induction of TRs in juveniles where TH levels have declined would be useful for answering this question.

Basal levels as well as the magnitude of auto-induction differed between *soTRα* and *soTRβ*. Basal (control, time 0) levels of *soTRβ* were 3 to 10.4-fold greater than that of *soTR*α. Basal *soTRα* rose 2.5-fold between 7 and 35 DPH, while *soTRβ* increased 5.9-fold. In response to T3 *soTRα* was significantly induced by 8 h and attained a maximum of 3-fold induction at 12 h. The response of *soTRβ* was more rapid, increasing as early as 4 h after the initiation of treatment and attaining a maximum of 4-fold induction at 12 h. Studies of the tissue-specific distribution of these transcripts will be helpful for determining whether differences in levels are due to variation in the density of transcripts within a tissue, or the extent of their distribution among tissues.

The exogenous hormone treatments used in this study did not cross-regulate whole body levels of *soGR*, *soTRα* or *soTRβ*. However, changes in hormone dose, time of treatment, and application of inhibitors and examination of tissue specific responses may still reveal interactions. While potentially informative, experiments employing exogenous

hormone treatments have inherent limitations which must be recognized. Specifically, under conditions where endogenous hormones are sufficient to elicit maximal responses, exogenous hormone treatments will not show effects even if target tissues are potentially sensitive to the ligand. Studies employing inhibitors of hormone production or antagonists of receptors are important compliments to hormone supplementation approaches and should be used in continuing assessments of receptor regulation. Although the current study did not reveal regulatory interactions between these systems, both up- and down-regulation of receptor transcripts may have occurred on a tissue-specific basis. Such changes could potentially offset one another and therefore go undetected by the whole body mRNA measurements used in this study.

Conclusions and future perspective

Ontogenetic patterns in TR mRNA levels as well as ontogenetic changes in auto-induction support a role for TH in mediating red drum transformation similar to that observed in flatfish and amphibians. Evidence of regulatory interactions between the TH and CS system of developing red drum were not observed. However, interactions cannot be precluded by these results. Continued study should investigate the response of receptor mRNA levels on a tissue-specific basis in response to exogenous hormones as well as inhibitors of hormone production and action. Interactions between TH and CS systems may also occur at levels of the hypothalamic-pituitary-thyroid and hypothalamic pituitary-interrenal axes other than that of hormones and their receptors. The strong relationship between *soTRα* and *soGR* expression supports a relationship between these

endocrine systems and may be indicative of a common regulatory factor at the neural end of the regulatory chain.

Table 2.1. Sequence of forward (**F**) and reverse (**R**) primers used to isolate red drum *soTRα*, *soTRβ* and *soGR* cDNAs. References are provided where published primers were used.

Gene of interest	***Primer sequences*** *(5'→3')*	***Reference***
TRα	***TRα* DEG F1:** AC(CT)TG(CT)GAGGGITG(CT)AAGGG ***TRα* DEG F2:** (CA)GIACIAT(CT)CA(GA)AA(GA)AACCT ***TRα* DEG R1:** ATGGTGIAG(GA)AAGC(GA)(GA)CTGGC ***TRα* DEG R2:** AG(CG)AG(CT)TT(GC)GGCCAGAAGTG ***TRα* 3' RACE:** CTGCTGCATGGAGATCATGTCGC ***TRα* 5' RACE**: CGCAGGTCCGTCACCTTCATCGA	Marchand et al. 2001
TRβ	***TRβ* DEG F1:** ACITG(CT)GA(AG)GGITG(CT)AAGGG ***TRβ* DEG F2:** (AT)(CG)(CT)TA(CT)(GT)C(CT)TG(CT)AA(AG)TA(TC)GA ***TRβ* DEG R1:** GG(AG)CACTCCAC(CT)TTCATGTG ***TRβ* DEG R2:** AG(GA)TCIGT(CG)AC(CT)TTCAT ***TRβ* 3' RACE1**: AATGGAGGCTTGGGCGTAGTCTCGGACG ***TRβ* 3' RACE2**: GCTGCCTTGTGAAGACCAGATCATCCTG **5' RACE**: CCGCTGCACTCAGGAATTTCCGCT	Marchand et al. 2001
GR	***GR* F:** ATGGATCAGGGTGGACTGAAGC ***GR* R:** TGTTGATAGGCTGCGATGATCCAGGG ***GR* RACE 3'** TGTCCTTCAGTCTTGGGTGGAGGTCATACG	

Table 2.2. Sequence, thermocycling conditions, amplification efficiency, and amplicon size of forward primer (**F**), reverse primer (**R**), and dual-labeled fluorescent probe (**P**) combinations used to quantify mRNA levels in qPCR assays. *soTRα*, *soTRβ* and *soGR* probes were dual-labeled with 5' carboxyfluorescein (FAM) and 3' Black Hole Quencher (BHQ1). The *18S* probe was labeled with 5' hexachlorofluorescein (HEX) and 3' BHQ1.

Gene of interest	***Primer sequences*** *(5'→3')*	***Thermocycling parameters***	***Efficiency*** *(%)*	***Amplicon size*** *(bp)*
soTRα	**F:** ATGTCGAGCAAGCAGGATAGCAACTCA **R:** ACGCACGGCTCATCCTTCTCCAAGTA **P:** ACATGCTCTTCACCGAACACTGGCTGTTC	95 °C, 10 min 40 cycles: 95 °C, 30 s 62 °C, 1 min	68.6 ± 1.2	170
soTRβ	**F:** GAGGGAGGAACTCCAGAAGACGGTGT **R:** CCGCTGCACTCAGGAATTTCCGCT **P:** CGACTGGAGCCCACCCAGGAGGAGT	95 °C, 10 min 40 cycles: 95 °C, 30 s 62 °C, 1 min	69.7 ± 2.9	149
soGR	**F:** TGTCCTTCAGTCTTGGGTGGAGGTCAT **R:** CAGCATCTGCTCGCACTGGTCAGTC **P:** CCTGATCTTGTCATCAACGAGGAGCGTATG	95 °C, 10 min 40 cycles: 95 °C, 30 s 62 °C, 1 min	96.8 ± 2.4	131
18S	**F:** GTTAATTCCGATAACGAACGAGACTC **R:** ACAGACCTGTTATTGCTCAATCTCGTG **P:** TTCTTAGAGGGACAAGTGGCGTT	95 °C, 10 min 40 cycles: 95 °C, 30 s 60 °C, 1 min	82.0 ± 2.9	127

Table 2.3. Mean SL ± SE (mm) of fish in source tanks used to stock hormone regulation trials. Within DPH, trials that do not share a common superscript letter differed significantly ($P < 0.001$). Trials were conducted with three different spawns for each experiment although the same three spawns were not necessarily used for 7, 21, and 35 DPH.

DPH	***Trial 1***	***Trial 2***	***Trial 3***
7	3.12 ± 0.05^{a}	3.07 ± 0.03^{a}	2.73 ± 0.03^{b}
21	6.98 ± 0.21^{a}	6.96 ± 0.13^{a}	7.15 ± 0.09^{a}
35	15.39 ± 0.53^{a}	18.65 ± 0.29^{b}	18.42 ± 0.22^{b}

Figure 2.1. Phylogenetic tree of *Sciaenops ocellatus* and other vertebrate thyroid receptor (TR) deduced amino acid sequences. Tree was rooted with *Mus musculus* cis-retinoic acid receptor (RXR). Bootstrap values listed at nodes are for 2000 iterations. GenBank accession numbers for nucleotide sequences used to deduce amino acid sequences are listed in parentheses.

Figure 2.1

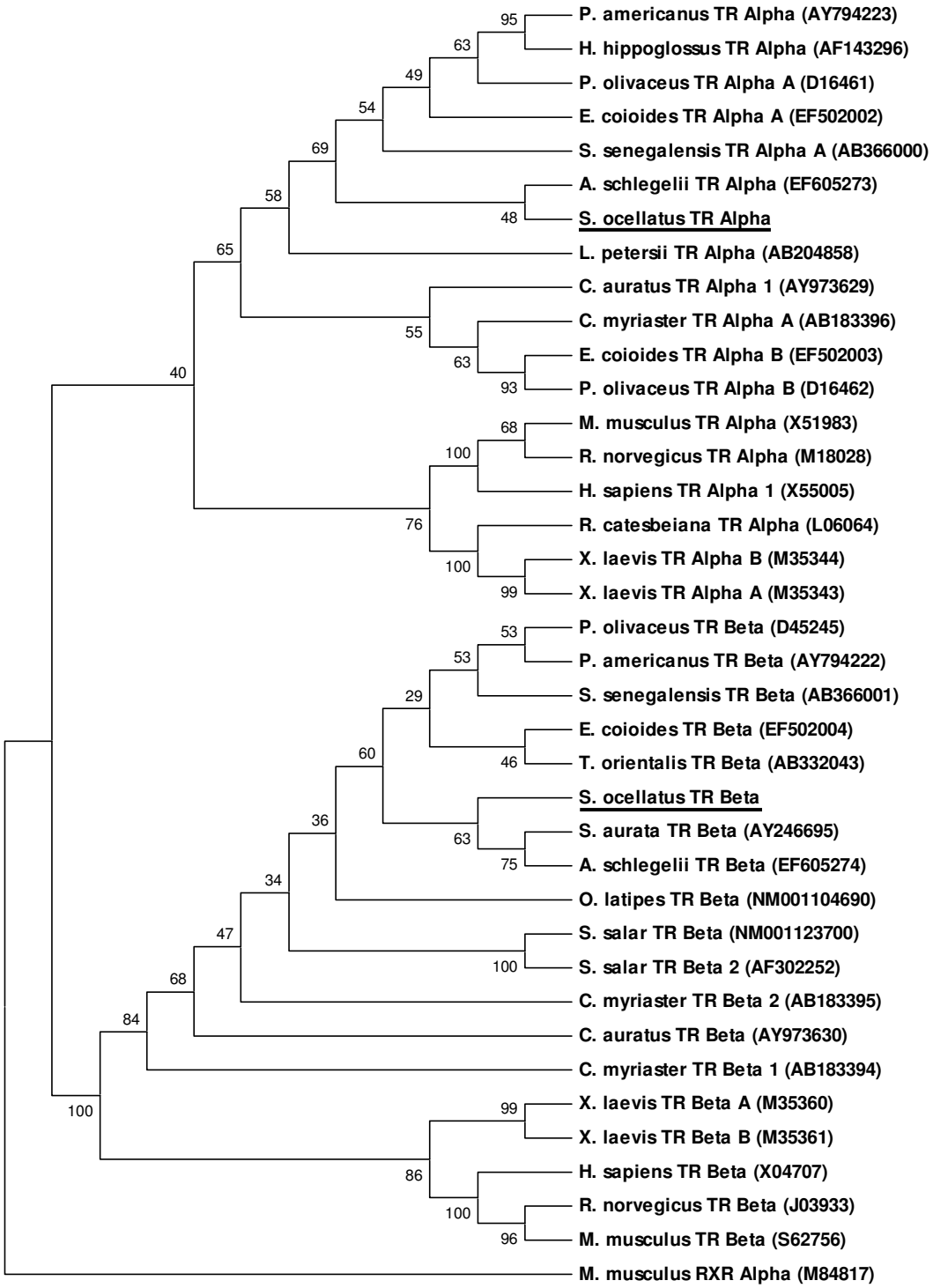
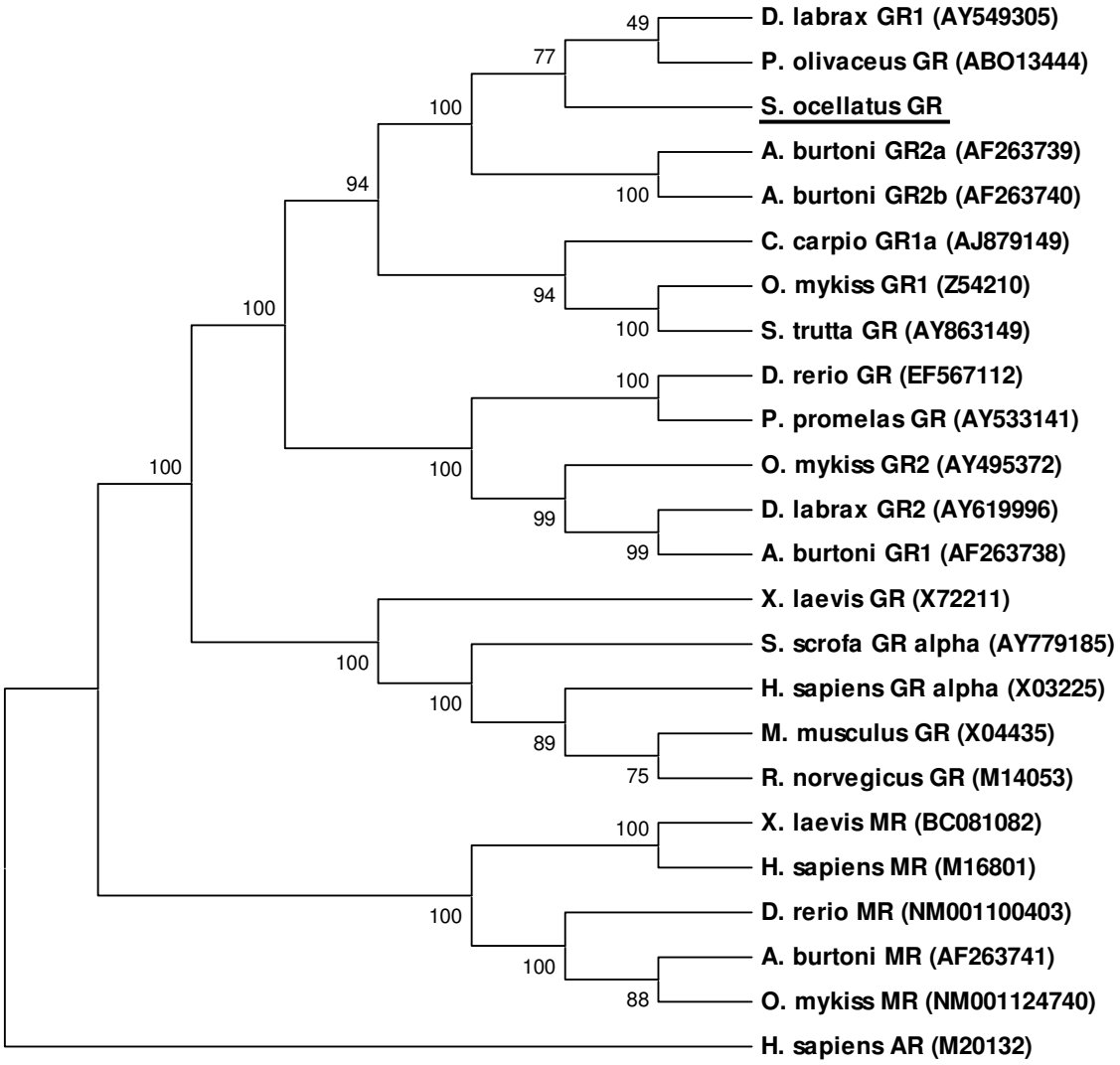

P. americanus TR Alpha (AY794223)
H. hippoglossus TR Alpha (AF143296)
P. olivaceus TR Alpha A (D16461)
E. coioides TR Alpha A (EF502002)
S. senegalensis TR Alpha A (AB366000)
A. schlegelii TR Alpha (EF605273)
S. ocellatus TR Alpha
L. petersii TR Alpha (AB204858)
C. auratus TR Alpha 1 (AY973629)
C. myriaster TR Alpha A (AB183396)
E. coioides TR Alpha B (EF502003)
P. olivaceus TR Alpha B (D16462)
M. musculus TR Alpha (X51983)
R. norvegicus TR Alpha (M18028)
H. sapiens TR Alpha 1 (X55005)
R. catesbeiana TR Alpha (L06064)
X. laevis TR Alpha B (M35344)
X. laevis TR Alpha A (M35343)
P. olivaceus TR Beta (D45245)
P. americanus TR Beta (AY794222)
S. senegalensis TR Beta (AB366001)
E. coioides TR Beta (EF502004)
T. orientalis TR Beta (AB332043)
S. ocellatus TR Beta
S. aurata TR Beta (AY246695)
A. schlegelii TR Beta (EF605274)
O. latipes TR Beta (NM001104690)
S. salar TR Beta (NM001123700)
S. salar TR Beta 2 (AF302252)
C. myriaster TR Beta 2 (AB183395)
C. auratus TR Beta (AY973630)
C. myriaster TR Beta 1 (AB183394)
X. laevis TR Beta A (M35360)
X. laevis TR Beta B (M35361)
H. sapiens TR Beta (X04707)
R. norvegicus TR Beta (J03933)
M. musculus TR Beta (S62756)
M. musculus RXR Alpha (M84817)

Figure 2.2. Phylogenetic tree of *Sciaenops ocellatus* glucocorticoid receptor (GR) and other vertebrate GR and mineralocorticoid (MR) receptor deduced amino acid sequences. Bootstrap values listed at nodes are for 2000 iterations. GenBank accession numbers for nucleotide sequences used to deduce amino acid sequences are listed in parentheses. Tree was rooted with the *Homo sapiens* androgen receptor (AR) cis-retinoic acid receptor. **Note:** *A. burtoni* GR sequences have been previously identified as being improperly numbered (Stolte et al. 2006; Alsop and Vijayan 2008); i.e. GR labeled as type 1 are actually type 2, and vice versa.

Figure 2.2


D. labrax GR1 (AY549305)
P. olivaceus GR (ABO13444)
S. ocellatus GR
A. burtoni GR2a (AF263739)
A. burtoni GR2b (AF263740)
C. carpio GR1a (AJ879149)
O. mykiss GR1 (Z54210)
S. trutta GR (AY863149)
D. rerio GR (EF567112)
P. promelas GR (AY533141)
O. mykiss GR2 (AY495372)
D. labrax GR2 (AY619996)
A. burtoni GR1 (AF263738)
X. laevis GR (X72211)
S. scrofa GR alpha (AY779185)
H. sapiens GR alpha (X03225)
M. musculus GR (X04435)
R. norvegicus GR (M14053)
X. laevis MR (BC081082)
H. sapiens MR (M16801)
D. rerio MR (NM001100403)
A. burtoni MR (AF263741)
O. mykiss MR (NM001124740)
H. sapiens AR (M20132)
49
77
100
94
100
94
100
100
100
100
99
99
100
100
100
89
75
100
100
100
88

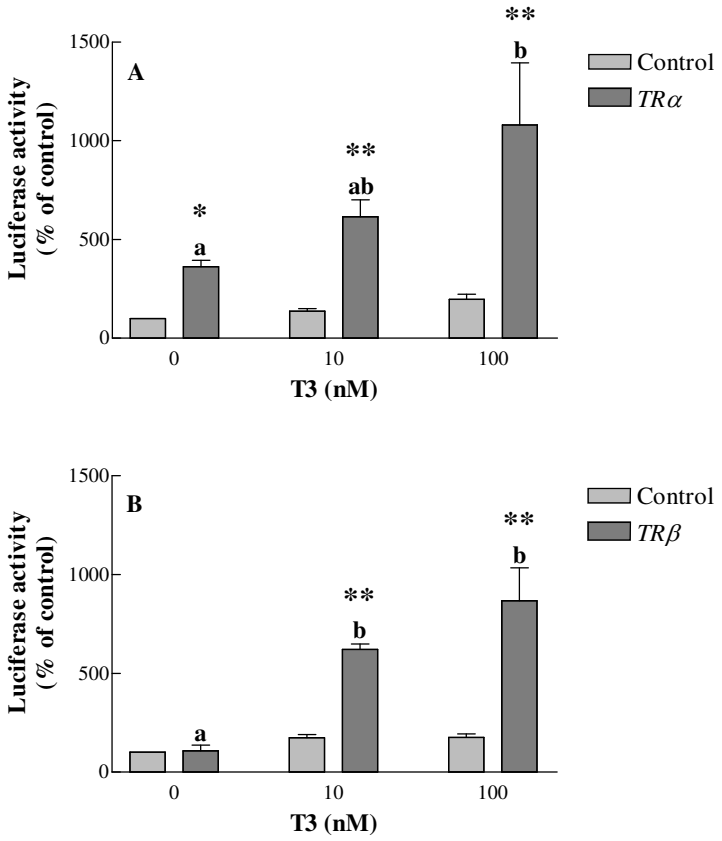
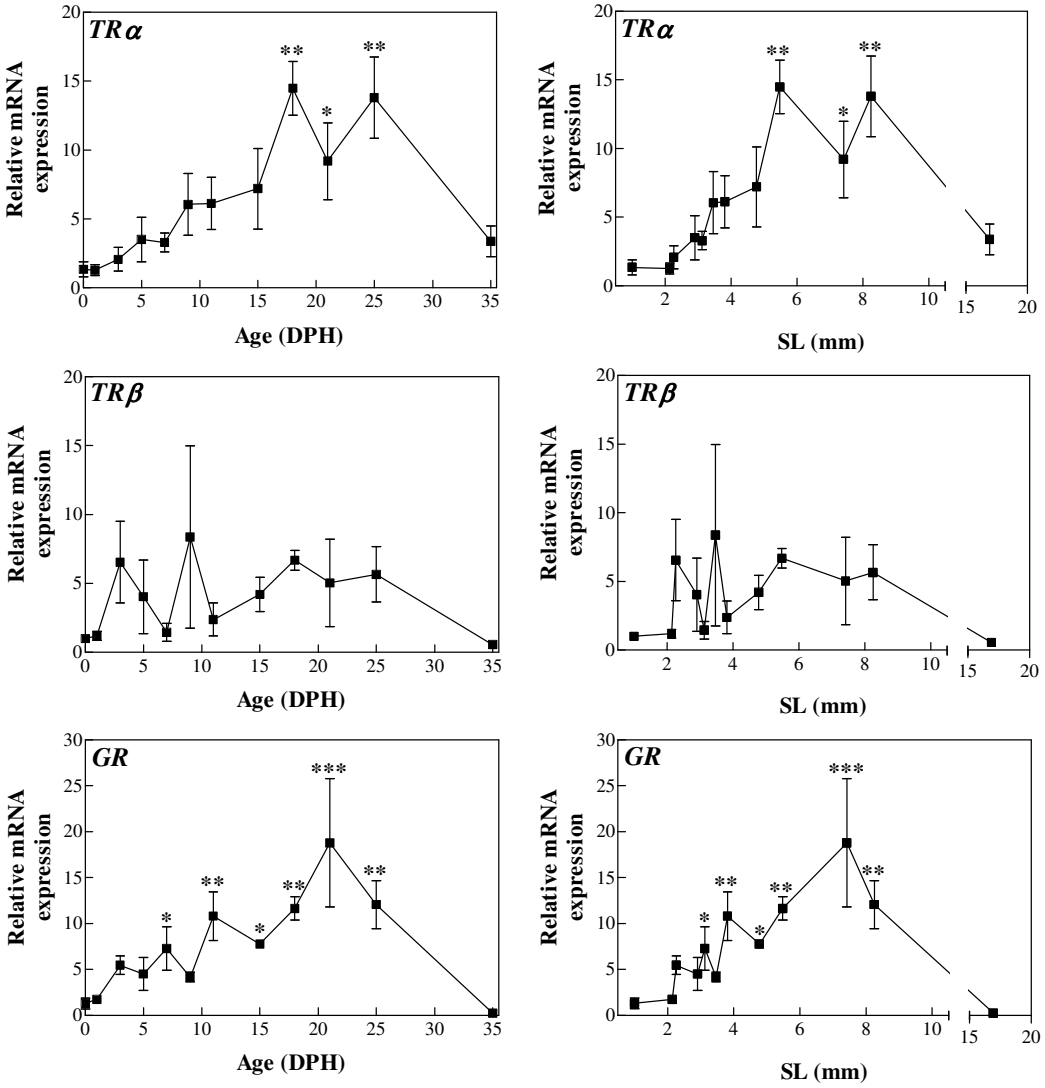


Figure 2.3. Luciferase activity in T3-treated COS-1 cells co-transfected with *S. ocellatus* A) *soTRα* or B) *soTRβ* expression constructs and the pGL2-xDR4 reporter plasmid and pCMV-β-gal plasmid. Luciferase activity was normalized to β-galactosidase activity and expressed as percent of the control cells in absence of T3. Asterisks denote a significant increase in luciferase activity relative to control cells (* $P < 0.005$, ** $P < 0.001$). Significant differences in the luciferase activity of receptor-transfected cells treated with different doses of T3 are indicated by letters ($P < 0.05$). Those groups that do not share a letter were significantly different. No significant differences were observed between control cells in response to T3 in either experiment. Values are means ± SE (n = 3).

Figure 2.4. Relative expression of whole body *soTRα*, *soTRβ* and *soGR* during red drum ontogeny relative to standard length (SL) and age (DPH). Values are means ± SE (n = 3). Asterisks denote a significant difference from 0 DPH (*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$).

Figure 2.4



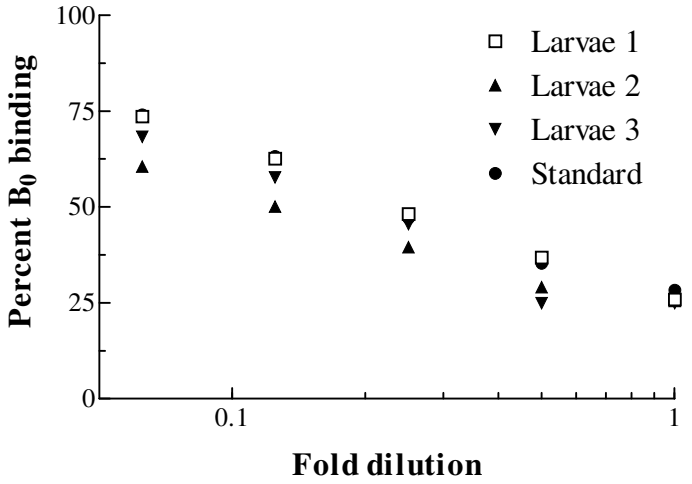


Figure 2.5. Validation of the suitability of the cortisol RIA for use in extracts of whole red drum larvae. Binding of radiolabeled cortisol was inhibited by serial dilutions of extracts from three independent pools of red drum larvae in parallel with a standard curve prepared with purified cortisol.

Figure 2.6. Hormonal regulation of *soTRα*, *soTRβ* and *soGR* expression by exogenous T3 or cortisol treatment. Samples taken at 12 and 24 h were not successfully assayed and are therefore not included in the results. Values are means ± SE (n = 3). Asterisks denote significant differences of treated larvae from their respective control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Figure 2.6

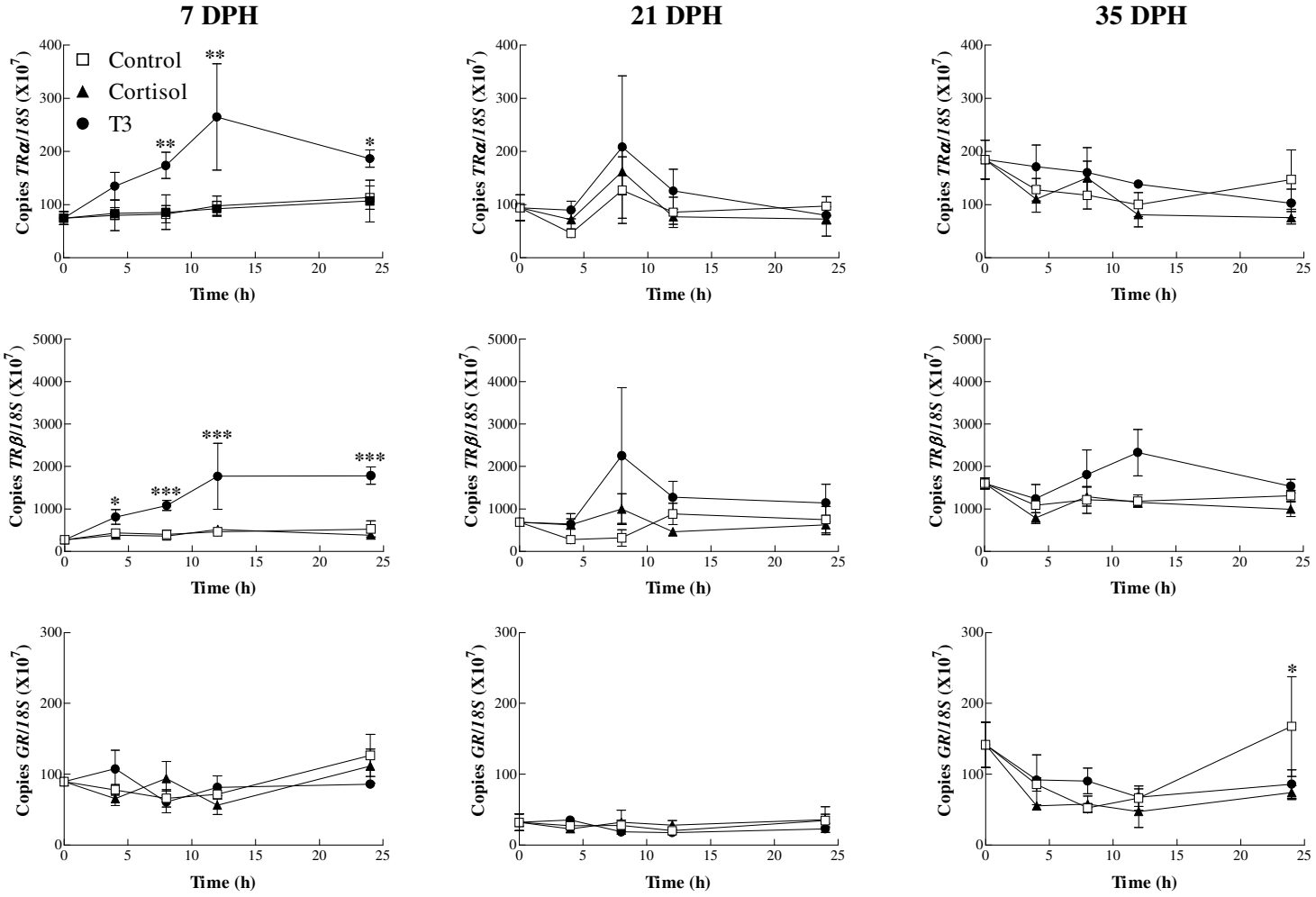
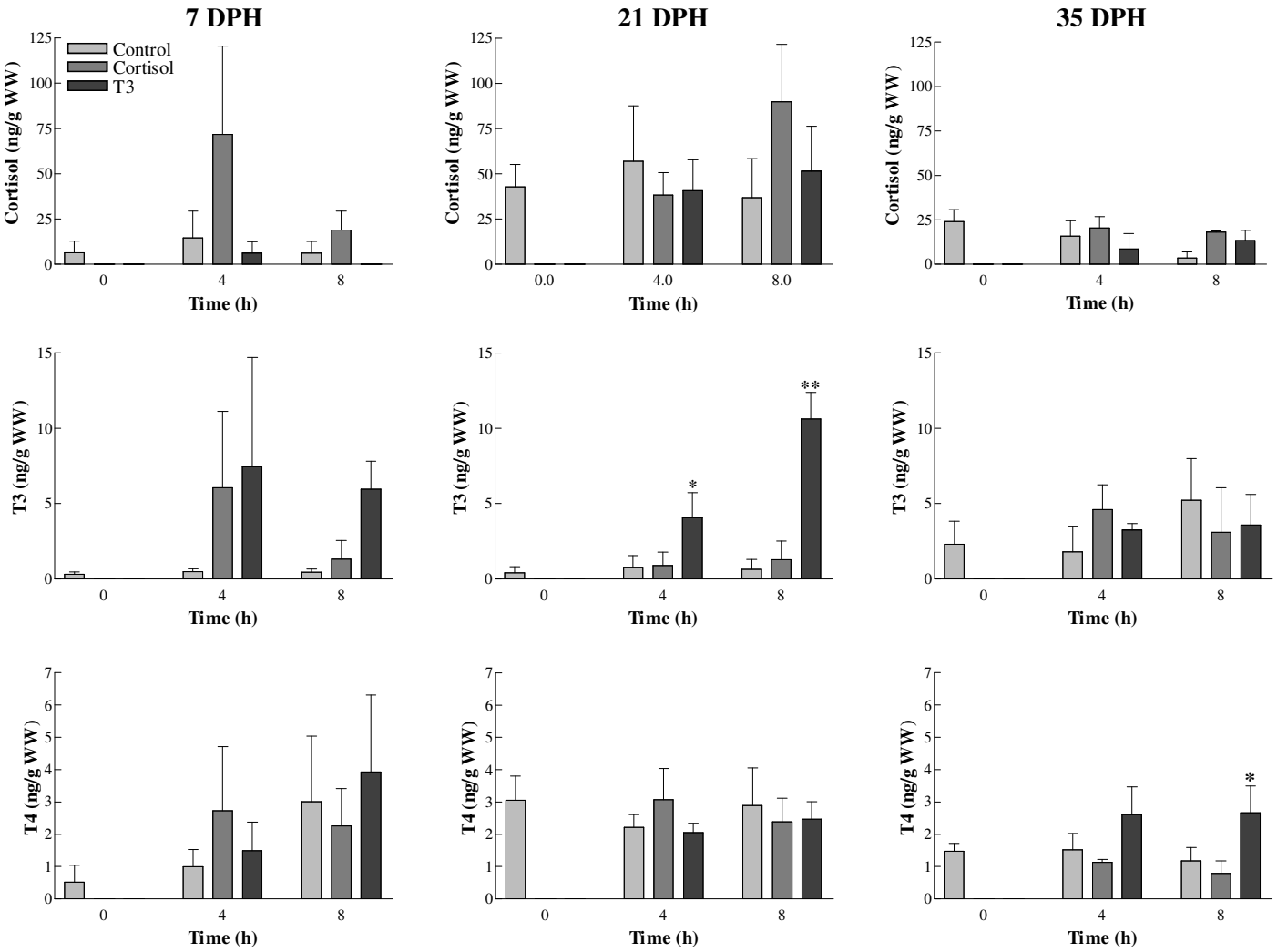

7 DPH
21 DPH
35 DPH
Control
Cortisol
T3
Copies TRα/18S (X10^7)
Copies TRβ/18S (X10^7)
Copies GR/18S (X10^7)
Time (h)
*
**
***

Figure 2.7. Cortisol, T3 and T4 contents of control, 50 nM T3 treated, or 250 nM cortisol treated red drum larvae. Values are means ± SE (n = 3). Asterisks denote significant differences of treated larvae from their respective control (*$P < 0.05$, **$P < 0.001$).

Figure 2.7


7 DPH
21 DPH
35 DPH
Control
Cortisol
T3
Cortisol (ng/g WW)
T3 (ng/g WW)
T4 (ng/g WW)
Time (h)
0
4
8
0.0
4.0
8.0
*
**

Chapter 3: Influence of atrazine exposure on glucocorticoid and thyroid hormone receptor mRNA levels, thyroid hormone content and growth in red drum (*Sciaenops ocellatus*) larvae

Abstract

The herbicide atrazine is a prevalent contaminant of ground and surface waters including coastal estuaries. Fish larvae in estuarine habitats may be exposed to atrazine contamination and are at risk for possible toxic effects. In two experiments, red drum (*Sciaenops ocellatus*) larvae were exposed to environmentally relevant levels of atrazine to determine its effect on whole body levels of mRNAs that encode two thyroid receptors (*soTRα*; *soTRβ*) and a glucocorticoid receptor (*soGR*), thyroid hormone content (triiodothyronine, T3; thyroxine, T4) and growth. In Experiment 1, larvae were exposed to 20 or 80 ppb atrazine for 20 days beginning at 15 days post-hatch (approximate age at which larvae settle in estuarine nursery habitat). Samples collected every 96 h showed no significant difference in the steady-state levels of *soGR*, *soTRα* or *soTRβ* mRNA. Further, T3 and T4 levels and growth rates showed no significant difference between treatments. In Experiment 2 larvae were exposed to 20 ppb or 80 ppb atrazine for 15 days beginning at 15 days post-hatch. Samples were collected for determination of *soTRα*, *soTRβ* and *soGR*, levels, T3 and T4 content, and SL only at the end of the experiment. A significant difference from controls was evident in SL for larvae in the 20-ppb but not 80-ppb atrazine treatment. These results support an increasing body of evidence that atrazine

exposure has subtle but important effects on red drum growth which have the potential to impact their survival in the wild.

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*S*-triazine) is one of the most commonly used herbicides in the United States and throughout the world (U.S. Environmental Protection Agency 1994; Solomon et al. 1996). As a result of its low soil adsorption and relatively high solubility in water, substantial amounts of atrazine are transported from application sites by irrigation and precipitation runoff to groundwater, lakes, rivers, and coastal marine habitats. Consequently, atrazine is the most common herbicide found in ground and surface waters in Texas (Land and Brown 1996) and throughout the US (U.S. Environmental Protection Agency 2001). Aquatic organisms including fishes are therefore regularly exposed to atrazine-contaminated waters and at risk for possible toxic effects.

Environmentally relevant concentrations of atrazine have been shown to impinge on several aspects of fish biology. Atrazine decreased the capacity for pheromonal detection and reduced milt expression and steroid levels in male *Salmo salar* (Moore and Waring 1998; Moore and Lower 2001), likely decreasing the reproductive success of these fish. *Carassius auratus* showed changes in swimming activity and decreased grouping behaviors (Saglio and Trijasse 1998) and substrate preference in *Danio rerio* was altered (Steinberg et al. 1995) when exposed to atrazine, suggesting atrazine affects neural areas involved in locomotion, sensory acquisition, and behavior. Atrazine also has

the potential to induce genetic disorders and disturb physiological homeostasis, as genotoxicity has been reported in erythrocytes of atrazine-exposed *Oreochromis niloticus* (de Campos Ventura et al. 2008).

A number of studies indicate that atrazine can alter the activity of thyroid and corticosteroid hormone systems in teleosts (Waring and Moore 2004; Nieves-Puigdoller et al. 2007) and amphibians (Larson et al. 1998). Thyroid hormones (TH) are important regulators of the developmental transition from larva to juvenile in teleosts (Inui and Miwa 1985; Brown 1997; de Jesus et al. 1998). The corticosteroid (CS) cortisol has prominent roles in the stress response (Wendelaar Bonga 1997; Mommsen et al. 1999) and in maintaining hydromineral balance (Wendelaar Bonga 1997; McCormick 2001), but also influence development, possibly by interacting with TH (de Jesus et al. 1990; Brown and Kim 1995; Kim and Brown 1997). Early life stages of aquatic organisms are typically the most susceptible to environmental pollutants/endocrine disruption (Weis and Weis 1987). Consequently, the vulnerability of the TH/CS systems to disruption by atrazine contamination may peak during this period, increasing the potential for disturbance of growth, development, and ultimately survival.

Red drum (*Sciaenops ocellatus*) is an ecologically and economically important species found in the Gulf of Mexico and along the southeastern coast of the United States. Along the Texas coast, red drum spawn from approximately late summer through early fall (Holt et al. 1985). After a planktonic period in coastal waters (Peters and McMichael 1987) red drum larvae are transported into estuaries and settle in seagrass beds where they transform into juveniles (Holt et al. 1983; Rooker et al. 1998; Herzka et

al. 2002). These events occur during maximum annual precipitation and freshwater inflow to Texas estuaries (Ward 1997). Consequently, this is also the time when atrazine is mostly likely to be eluted from nearby agricultural plots into estuaries. Measurements of atrazine levels in Texas estuaries are limited, but one study found an average concentration of 3.9 ppb, with pulses reaching 62.5 ppb (Pennington et al. 2001).

Results of previous studies in which larval red drum were exposed to environmentally relevant levels of atrazine indicate decreased growth (Alvarez and Fuiman 2005; McCarthy and Fuiman 2008), increased routine swimming activity and increased metabolic rate (Alvarez and Fuiman 2005). Atrazine also decreased the efficiency of protein retention in red drum larvae (McCarthy and Fuiman 2008), further emphasizing an energetic cost of atrazine exposure. However, it is unknown whether atrazine alters endocrine function in red drum, possibly contributing to these observations. We investigated the effects of environmentally relevant concentrations of atrazine on whole body levels of mRNAs that encode two thyroid hormone receptors (*soTRα*; *soTRβ*) and a glucocorticoid receptor (*soGR*), thyroid hormone content (triiodothyronine, T3; thyroxine, T4) and larval growth during the early life history of red drum. This information is critical for evaluating whether atrazine contamination of coastal waters is a threat to estuarine-dependent life stages of fishes. This study indicates that atrazine exposure does not influence TH levels or steady-state levels of TR or GR mRNA in red drum larvae, but can reduce growth.

Methods

Experimental animals

Fertilized red drum eggs were obtained from broodstock induced to spawn by temperature and photoperiod control (Arnold 1988) at the Fisheries and Mariculture Laboratory of the University of Texas at Austin, Marine Science Institute in Port Aransas, Texas and the Texas Parks and Wildlife Department Hatchery in Corpus Christi, Texas. Eggs were disinfected in seawater containing 10 ppm formalin for 20 min before being rinsed with clean seawater. Eggs were then transferred to 1-L beakers of filtered seawater at approximately 1000 eggs/L. Hatching occurred the following morning and larvae were moved to 600-L cylindrical tanks with internal biological filters at a density of 5000 per tank. An airstone at the bottom of the tank provided water circulation and maintained dissolved oxygen near saturation levels. Salinity and temperature were maintained at approximately 27 °C and 30 psu respectively, and photoperiod was 12:12 hour (light:dark). Beginning at 3 DPH (onset of exogenous feeding) larvae were fed live rotifers (*Brachionus plicatilis*) at 2-5 rotifers/mL. Prior to feeding, rotifers were enriched overnight with *Isochrysis galbana* and Algamac 2000 (Biomarine, Inc. Hawthorne, CA). Beginning at 10 DPH larvae were fed *Artemia* nauplii that were freshly hatched or enriched overnight with Algamac 2000.

Experiment 1

Atrazine exposure experiments were conducted in 14-L cylindrical, light blue plastic buckets. Each bucket was filled to 12 L with clean seawater and aerated to maintain oxygen concentrations at or near saturation. Buckets were placed in a

circulating water bath to maintain constant temperature (27 °C) across all experimental enclosures. On the morning of 14 DPH, 140 larvae were transferred to each of 6 buckets containing 12 L clean seawater. Mean standard length (SL) of larvae used to stock experimental enclosures was 4.28 ± 0.17 mm. Atrazine treatments were initiated the following morning by addition of 120 µl (10 µl atrazine stock/L seawater) of the appropriate atrazine stock in acetone to experimental enclosures to achieve 20 or 80 ppb final concentration. Control groups received acetone only. Atrazine (98.1% purity) was purchased from Chem Service, Inc. (West Chester, PA). Stock solutions were prepared in acetone at concentrations of 8000 and 2000 mg/L. Larvae were fed twice daily with either freshly hatched or enriched *Artemia* nauplii *ad libitum*. Every 96 h treatments received a 100 percent water change to remove metabolic wastes and renew the appropriate atrazine dose. During water changes two samples of 3-5 larvae per treatment were transferred to microcentrifuge tubes and frozen immediately at -80 °C until preparation for thyroid hormone and mRNA expression analyses. An additional 10 larvae were preserved in 10% buffered formalin for determination of standard length (SL). Experiments continued through 35 DPH. The experiment was replicated with larvae reared from three different spawns.

Experiment 2

Experiment 2 was conducted in the same manner as Experiment 1 with the following modifications. Larvae were stocked at an initial density of 55 larvae per bucket. Sampling was conducted only at 30 DPH. Two samples of three larvae each were

collected in microcentrifuge tubes and frozen immediately at -80 °C until preparation for thyroid hormone and mRNA expression analyses. All remaining larvae were preserved in 10% buffered formalin for determination of SL and morphometric analysis (see Appendix 2).

Quantitative reverse-transcription polymerase chain reaction assays

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, DNase (Promega, Madison, WI) treated, and again purified in TRIzol. The resulting pellet was resuspended in nuclease-free water and the concentration determined spectrophotometrically. The absence of genomic contamination of RNA samples was confirmed by preparing reverse transcription (RT) reactions lacking reverse transcriptase and using the product as template for polymerase chain reaction.

First-strand cDNAs were produced in 20 µl RT reactions with 2.5 µg of DNA-free total RNA. Reaction mixtures were prepared with AffinityScript qPCR cDNA Synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Reactions were incubated at 25°C for 5 min, 55°C for 30 min, and 95°C for 5 minutes. Terminated reactions were diluted to a final volume of 60 µl with sterile water.

Steady-state mRNA levels of two red drum thyroid receptors (*soTRα* and *soTRβ*), the glucocorticoid receptor (*soGR*) and *18S* RNA were determined by real time quantitative reverse transcription-polymerase chain reaction (qPCR) assays. Assays were conducted using Brilliant II qPCR Master Mix (Stratagene) according to manufacturer's protocol. Each 25 µl reaction mixture contained 5 µl of cDNA template, 200 µM dual-

labeled probe and 400 µM each of forward and reverse primers (Table 3.1). Reactions were carried out on, and resulting fluorescence detected with an Eppendorf RealCycler (Eppendorf, Westbury, NY) as defined in Table 3.1.

The number of copies per sample was determined by reference to a standard curve created using dilutions of linearized plasmids containing a calculated number of copies of the amplified region of the target gene. Standard curves were included in each 96-well qPCR assay. Target gene copy number was normalized to levels of ribosomal *18S* RNA determined in separate qPCR reactions. All samples were run in duplicate.

Thyroid hormone EIA

Thyroid hormones, T3 and T4 were measured in separate enzyme immunoassays using methods previously validated for red drum larvae (Pérez-Domínguez 2004) with the following changes. Horseradish peroxidase-conjugated T3 and T4 tracers were purchased from Fitzgerald Antibody, Inc. (Concord, MA, product nos. 65-IT50 and 65-IT35) and used in assays at 0.2 U/mL and 0.4 U/mL respectively. Antibody dilutions for plate coating were 1:8000 and 1:1500 for T3 and T4 respectively.

Growth

Images of preserved larvae were captured via dissecting scope equipped with a digital camera. ImageJ software (http://rsb.info.nih.gov/ij) was used to measure SL. In Experiment 1, instantaneous growth rates G (d^{-1}) were calculated using an exponential growth model:

$$SL_t = SL_{tpr}\, e^{Gt}$$

where SL_t is mean SL at time, t (days) and SL_{tpr} is mean SL at the previous sampling date.

Statistical Analyses

The effects of atrazine treatment on mRNA levels, hormone content, growth coefficients (Experiment 1) and SL (Experiment 2) were assessed by completely randomized block design two-way ANOVA and one-way ANOVA for Experiments 1 and 2, respectively. The spawn from which larvae were obtained was used as the blocking factor. Where necessary, data were log-transformed prior to ANOVA. Differences in slopes were assessed by ANCOVA using day of experiment as the covariate. Growth coefficients were compared between Experiment 1 and 2 using the Kruskal-Wallis test. In all cases, results were accepted as significantly different if $P < 0.05$. Where significant main effects were identified, Tukey's post-hoc test was used to determine which groups differed. Statistical analyses were conducted using SYSTAT 10 (Systat Software, Inc., San Jose, CA).

Results

Experiment 1

Levels of *soTRα*, *soTRβ* and *soGR* in red drum larvae treated with 20 or 80 ppb atrazine did not differ significantly from control larvae on any of the sampling days (Fig. 3.1). Further, T3 and T4 did not differ between atrazine treated larvae and control larvae

on any of the sampling days (Fig. 3.2). Growth rates of control (0.058 ± 0.004 d^{-1}), 20 (0.053 ± 0.009 d^{-1}), and 80 ppb (0.057 ± 0.007 d^{-1}) treatments did not differ significantly (Fig. 3.3). In several cases, treatment-independent larval survival in low atrazine treatment (see discussion) limited the number of larvae available for preserved samples.

Experiment 2:

Expression of *soTRα*, *soTRβ* and *soGR* in red drum larvae treated with 20 or 80 ppb atrazine was not significantly different from control larvae (Fig. 3.4). T3 and T4 did not differ between atrazine-treated larvae and control larvae (Fig. 3.5). A significant effect of treatment on SL (ANOVA, $P = 0.019$) was detected. Pairwise comparisons indicate a significant reduction in SL at 20 ppb atrazine but not at 80 ppb relative to control (Fig. 3.6). Growth coefficients were significantly greater in Experiment 2 than in Experiment 1 (Kruskal-Wallis, $P = 0.004$). Morphometric analyses of larvae indicate differences in the shape of atrazine treated larvae (Appendix 2).

Discussion

Changes in hormone concentrations are a common endpoint used in the assessment of pollutants effects on organisms. A number of studies of fish and amphibians have examined the influence of atrazine on cortisol and TH under various conditions. However, the influence of potential endocrine disruptors on the expression of the nuclear receptors through which these ligands act has not been examined. This study demonstrates that exposure to environmentally relevant concentrations of atrazine can

reduce growth in red drum larvae. However, the observed reduction in growth does not appear to be mediated by changes in the levels of T3, T4, or TR and GR mRNAs.

Along the Texas coast, red drum spawn in nearshore areas during late summer and fall (Holt 2008). After a planktonic period of 2-3 weeks, larvae, aided by ocean currents, enter estuaries and settle in seagrass beds, at SL between 4 and 11 (Herzka et al. 2002). In experiments, treatments were initiated at 15 DPH to coincide with the earliest time and size at which red drum are expected to settle in estuarine habitats where they may be exposed to high levels of atrazine. In both Experiment 1 and 2 the whole body levels of *soTRα*, *soTRβ* and *GR* mRNA, as well as T3 and T4 content were unchanged by continuous rearing of larvae in seawater containing environmentally relevant levels of atrazine. There is no information on the effect of atrazine on expression of TR and GR mRNA at present. While the results presented here indicate that whole body levels of mRNA are not affected by atrazine, it is possible that tissue-specific alterations in receptor expression occur but are not detectable using this approach.

We are not aware of studies of atrazine's influence on TH in teleost larvae. However, a few studies have examined the effect of atrazine in *S. salar* smolts. Atrazine did not affect smolt T3 or T4 in freshwater, but reduced T3 and T4 of previously exposed smolts after a 24-h seawater challenge (Nieves-Puigdoller et al. 2007). In another study, a 7-d atrazine treatment did not alter T4, but seawater challenge of previously exposed smolts resulted in high mortality, and elevated T4 in the surviving smolts (Waring and Moore 2004). These studies suggest that while we did not observe a change in basal hormone levels of atrazine-treated red drum, it is possible that early life exposure may

alter endocrine responsiveness of these fish later in life, especially under conditions of fluctuating environmental salinity.

Detection of environmental contaminant effects on thyroid hormone status is complicated by the large number sites at which a toxicants may act on the thyroid system as well as the inherent ability of this system to compensate for fluctuations in hormone availability (Eales et al. 1999; Eales and Brown 2005). The choice of the endpoints suitable for detecting disturbance of the thyroid axis is difficult, and it may be that only by measuring and interpreting several endpoints simultaneously that disruption of thyroid status may be assessed (Eales et al. 1999). Potential sites of disruption include the uptake and incorporation of elemental iodine, synthesis of the TH precursor thyroglobulin, TH secretion and the regulation thereof, peripheral deiodination of TH and receptor mediation of TH actions. The choice of endpoints for detecting disruption of thyroid hormone system depends on the suspected target of contaminant. In the absence of this information, it may be necessary to simultaneously assess endpoints indicative of hormone production, conversion to active forms, and mechanisms of hormone action. In the present study a measurement of the activity or mRNA levels of the deiodinase responsible for conversion of T4 to T3 would be a valuable complement to the measurements of TH levels and TR mRNA levels.

In Experiment 1, no significant difference in growth rate was detected between control larvae and atrazine treated larvae. The number of larvae available for measurement of growth rate was limited in some trials by unexpectedly high levels of mortality that was not caused by atrazine exposure. Mortality occurred immediately after

the transfer of larvae from rearing tanks to experimental tanks, and prior to the application of atrazine. Persistent cannibalism was also observed in Experiment 1, further contributing to the reduced number of larvae available for growth measurements. As size disparity within a batch of red drum larvae increases during captive rearing, cannibalism becomes increasingly common, as has also been observed in other species (Francis and Bengtson 1999; Correa and Cerqueira 2007). Typically, one or several larvae outgrew and began to consume smaller individuals. In the small experimental enclosures used for these experiments a few fast growing larvae could potentially kill a substantial proportion of the larvae in a given treatment. The reduced number of larvae for growth assessment due to mortality associated with transfer and cannibalism reduced the statistical power to detect differences in growth rates among treatments. For this reason an additional experiment (Experiment 2) was performed to further investigate the response of larval growth to atrazine treatment.

Experiment 2 was designed to increase the number of fish remaining for growth analysis at the end of the experiment while reducing the total number of fishes in the experimental tanks. This was accomplished by collecting larval samples at the end of the 15 day experiment only. Additionally, one to three oversized fish were removed from each treatment tank over the course of the 15-d experiment to reduce the occurrence of cannibalism. Removal of the oversized fish and the lower number of fish per tank may also have reduced social growth suppression as growth rates were significantly greater in Experiment 2 than Experiment 1.

It is perplexing that atrazine significantly reduced growth rates of red drum in Experiment 2 at the lower but not the higher dose. This result contrasts with an earlier study which found a significant depression of growth in larval red drum at the higher (80 ppb), but not the lower (40 ppb) atrazine dose (Alvarez and Fuiman 2005). However, in a recent study of exposure of red drum to either 40 ppb or 80 ppb atrazine, length was unchanged while wet weight and protein content decreased (McCarthy and Fuiman 2008). This suggests that although SL of 80 ppb treated larvae was unaffected in this study, a reduction in growth may still have occurred. Outside of red drum, the effects of atrazine on larval growth have only been examined in *Fundulus heteroclitus*, which did not show an effect in terms of length or condition factor.

Conclusions and future perspective

Despite differences between existing studies in the doses at which effects are observed, as well the method of growth measurement, a reduction in growth appears to be a consistent effect of atrazine treatment on red drum larvae. This finding is important as reduced growth rates will prolong the duration of the larval stage. Individuals that grow less rapidly will have a diminished capacity to avoid predation relative to larvae with unimpaired growth (Bailey and Houde 1989; Fuiman and Magurran 1994) thereby diminishing survival of larvae to the juvenile stage.

Future experiments should compare food intake rates of treated and untreated larvae, as a reduction in food intake has been reported in atrazine-treated *S. salar* smolts (Nieves-Puigdoller et al. 2007). A similar effect of atrazine on red drum might account

for observations of diminished growth. Second, an increase in the size of experimental tanks may be useful in maximizing growth potential under experimental conditions, and allowing greater numbers of samples from which to determine growth rates. Growth should be assessed not only in terms of length, but by other metrics including weight or protein content as well. Finally, an investigation of the expression of receptor expression on a tissue specific basis would be valuable for confirming or refuting the constancy of TR and GR mRNA levels in atrazine-treated larvae.

Table 3.1. Sequence, thermocycling conditions, and amplicon size of forward primer (**F**), reverse primer (**R**), and dual-labeled fluorescent probe (**Probe**) combinations used to quantify mRNA levels by qPCR. *TRα*, *TRβ* and *GR* probes were dual-labeled with 5' carboxyfluorescein (FAM) and 3' Black Hole Quencher (BHQ1). *18S* probe was labeled with 5' hexachlorofluorescein (HEX) and 3' BHQ1.

Gene of interest	***Primer sequences*** *(5'→3')*	***Thermocycling parameters***	***Amplicon size*** *(bp)*
TRα	**F:** ATGTCGAGCAAGCAGGATAGCAACTCA **R:** ACGCACGGCTCATCCTTCTCCAAGTA **Probe:** ACATGCTCTTCACCGAACACTGGCTGTTC	95 °C, 10 min 40 cycles: 95 °C, 30 s 62 °C, 1 min	170
TRβ	**F:** GAGGGAGGAACTCCAGAAGACGGTGT **R:** CCGCTGCACTCAGGAATTTCCGCT **Probe:** CGACTGGAGCCCACCCAGGAGGAGT	95 °C, 10 min 40 cycles: 95 °C, 30 s 62 °C, 1 min	149
GR	**F:** TGTCCTTCAGTCTTGGGTGGAGGTCAT **R:** CAGCATCTGCTCGCACTGGTCAGTC **Probe:** CCTGATCTTGTCATCAACGAGGAGCGTATG	95 °C, 10 min 40 cycles: 95 °C, 30 s 62 °C, 1 min	131
18S	**F:** GTTAATTCCGATAACGAACGAGACTC **R:** ACAGACCTGTTATTGCTCAATCTCGTG **Probe:** TTCTTAGAGGGACAAGTGGCGTT	95 °C, 10 min 40 cycles: 95 °C, 30 s 60 °C, 1 min	127

Figure 3.1. Whole body steady state levels of *soGR*, *soTRα* and *soTRβ* over a 20-day period in red drum larvae control groups, or treated continuously with 20 ppb or 80 ppb atrazine from 15 to 35 DPH. Values are means of three experimental trials ± SE.

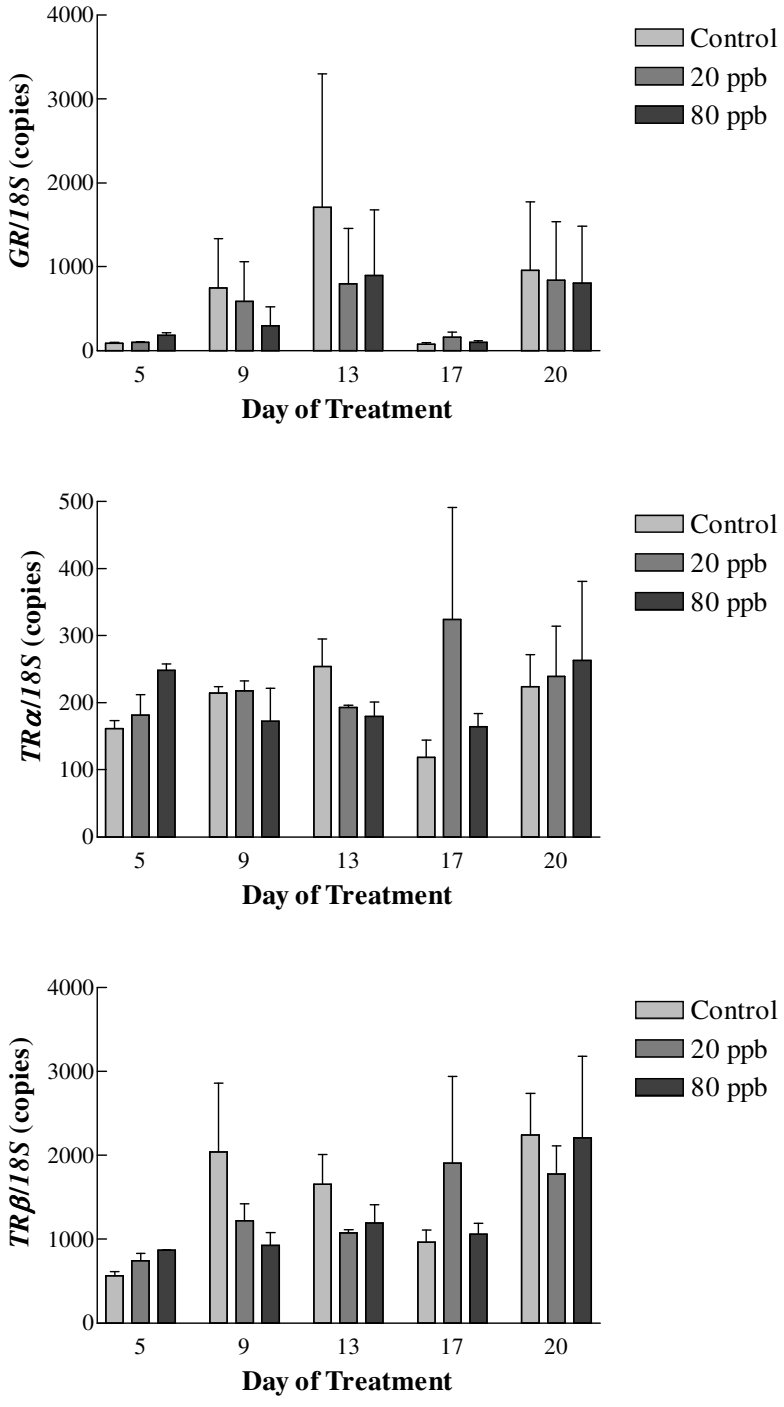

4000
3000
2000
1000
0
GR/18S (copies)
5
9
13
17
20
Day of Treatment
Control
20 ppb
80 ppb
500
400
300
200
100
0
TRα/18S (copies)
5
9
13
17
20
Day of Treatment
Control
20 ppb
80 ppb
4000
3000
2000
1000
0
TRβ/18S (copies)
5
9
13
17
20
Day of Treatment
Control
20 ppb
80 ppb

Figure 3.1.

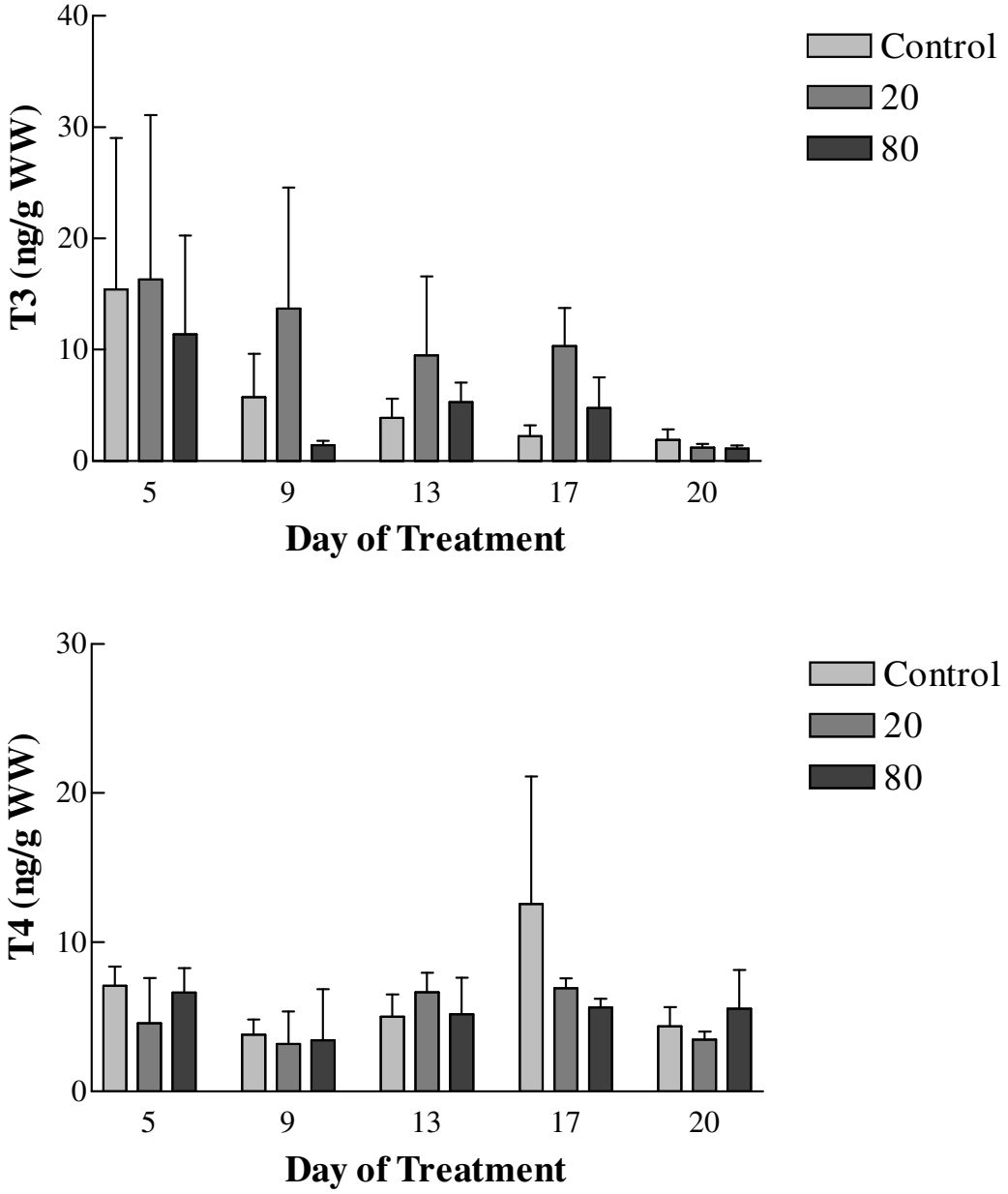


Figure 3.2. Whole body T3 and T4 content over a 20-day period of red drum larvae control groups, or treated continuously with 20 ppb or 80 ppb atrazine. Values are means of three experimental trials ± SE.

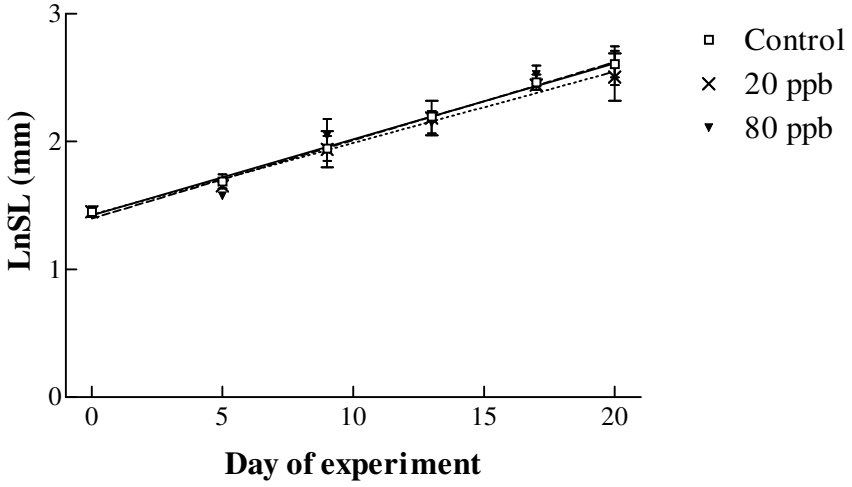


Figure 3.3. Growth of red drum larvae over a 20-day period for control, 20 or 80 ppb atrazine treatments. Lines represent linear regressions fit to each group (control: solid line, 20 ppb: dotted line, 80 ppb: dashed line).

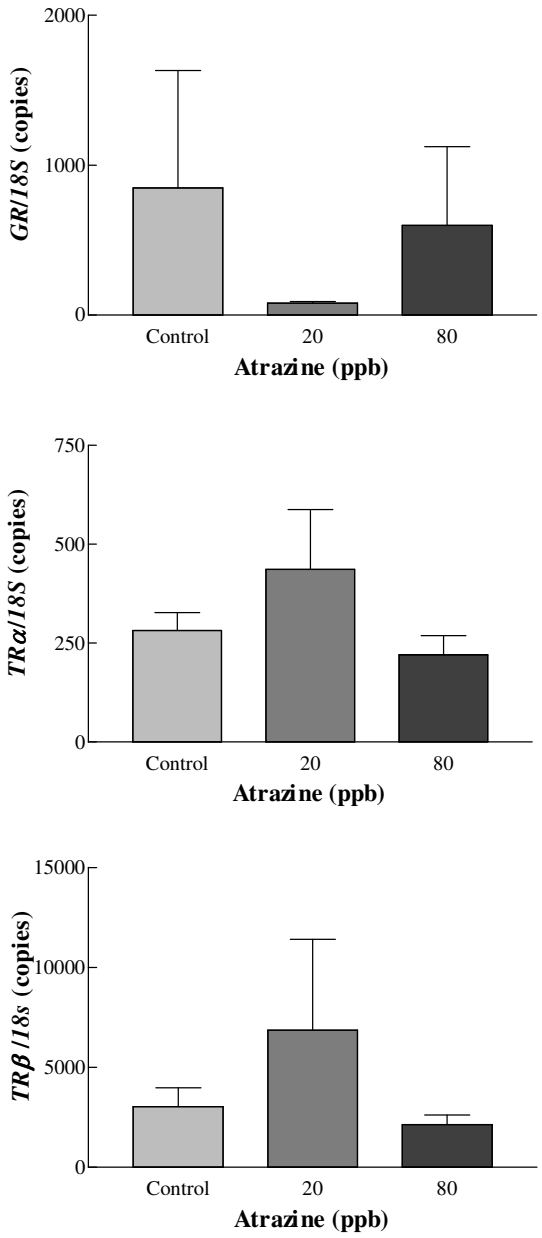


Figure 3.4. Whole body steady-state levels of *soGR*, *soTRα* and *soTRβ* of red drum larvae after a 15-day control, 20 ppb or 80 ppb atrazine treatment. Values are means of three experimental trials ± SE.

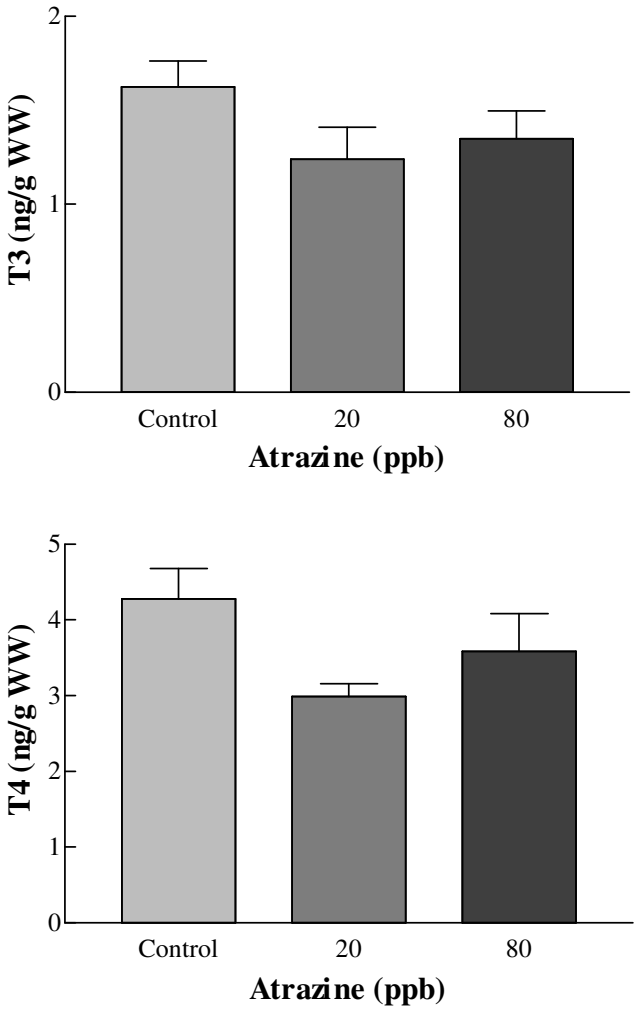


Figure 3.5. Whole body T3 and T4 content of red drum larvae after a 15-day control, 20 ppb or 80 ppb atrazine treatment. Values are means of three experimental trials ± SE.

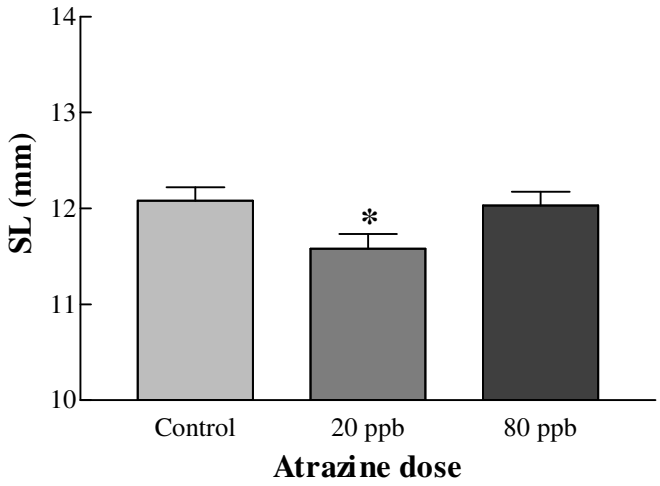


Figure 3.6. Standard length (SL) of red drum larvae after a 15-day control, 20 ppb or 80 ppb atrazine treatment. Values are means of three experimental trials ± SE. (*$P < 0.05$).

Appendix 1. Nucleotide sequences

Sciaenops ocellatus CYP11B

CAACACACAGAGAGACACGCCAGCACAGCAAGGGGGTCTTCCTCAAGGACG
GTGATGAATGGCGTGCTGACCGTCTGCTTCTTAACAAGGAGGTGATGATGAG
TTCGGCCGTGAAGCGCTTTCTCCCCCTGCTTGACGACGTGGCGAGGA

Sciaenops ocellatus CYP21

TAATGGCAATAGAAATGTCAGTGATCAGTGTGGGAGCTGTGTTCCTAGTTGT
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TTATAAGAAAACATCTCAACAATTACAAGCAGTCACAAGACAAAATAAATGT
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GAACATGGAGTGCTTCTGACAGATATTCATGTTCACATGGCCACTGTGGACCT
TCTCATCGGGGGAACCTGAGACCACTGCAGCCTGGCTGAACTGACTTGCTCT
CCAT

Sciaenops ocellatus thyroid hormone receptor alpha (*soTRα*)

TAAGCAGTGGTATCAACGCAGAGTACGCGGGTGCTGAGCAGGCTGGCGAGGT
CGAGGTCCGCCTCATTGTCCTGTGATTGGCTGCCTGGCGGGACTGAGGCCAC
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ACCAAGATCATGACCCCCGCCATCACACGTGTCGTCGACTTCGCCAAGAAAC
TGCCCATGTTCTCAGAGCTGCCTTGTGAAGACCAGATCATCTTGCTGAAGGGC
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AAAAAAAAA

Sciaenops ocellatus thyroid hormone receptor alpha (*soTRβ*)

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AACAATACCTTTAAACTTGTTTGTAATCCTCAACACGCGCAAGATAATTTTGT
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Sciaenops ocellatus glucocorticoid receptor (*soGR*)

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Appendix 2. Morphometric analysis of control and atrazine-treated larvae

Method

Landmark-based thin plate spline relative warp morphometric analyses were performed on preserved larval samples from Chapter 3, Experiment 2. Digital images of larvae were analyzed using tpsDig2 (Version 2.10), tpsRelw (Version 1.45) and tpsSplin (Version 1.20) software (http://life.bio.sunysb.edu/morph/) to place landmarks in digital images, perform relative warp analysis, and visualize differences in the distribution of landmarks, respectively. Nine landmarks (Fig. A2.1) were placed in digital images of 19 randomly selected fish from each of three treatment replicates using tpsDig2. The effect of atrazine treatment and spawn were analyzed for the first three relative warps (RW) using nested-ANOVA (spawn nested within treatment). Differences between treatments were assessed by ANCOVA with SL as the covariate.

Results

RW2 and 3 did not differ significantly between treatments. RW1 did not differ among spawns and therefore fish from different spawns were pooled within treatments. Their was no significant interaction between SL and RW1 ($P < 0.642$) and this facto was therefore excluded from the analysis. There was a significant effect of treatment on RW1 (Figure, A2.2, ANCOVA, $P < 0.001$).). Warp scores indicated that the differences in RW1 were primarily associated with changes in landmarks 1, 5, and 6 as illustrated in Figure A2.3.

Table A2.1. Definition of location of landmarks used in relative warp analysis.

Landmark	***Description***
1	Tip of snout
2	Anterior terminus of dorsal fin base
3	Posterior terminus of dorsal fin base
4	Posterior margin of hypural plate
5	Posterior terminus of pelvic fin base
6	Anterior terminus of pelvic fin base
7	Anterior terminus of pectoral fin base
8	Hinge of jaw
9	Center of eye

Table A2.2. Results of ANCOVA analysis of RW1 values from relative warp analysis of red drum larvae treated with 0, 20 or 80 ppb atrazine.

	sum of square	***Df***	***mean square***	***F-ratio***	***P***
treatment	0.011	2	0.006	19.387	0.000
SL	0.001	1	0.001	4.222	0.041
Error	0.054	186	0.000		

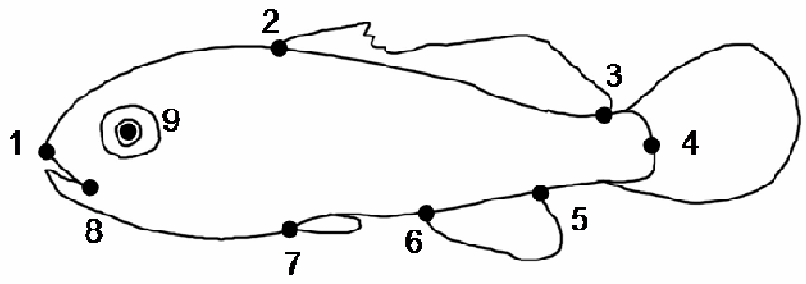

Figure A2.1. Location of nine landmarks used in relative warp analysis. Drawing courtesy of S. Nakayama.

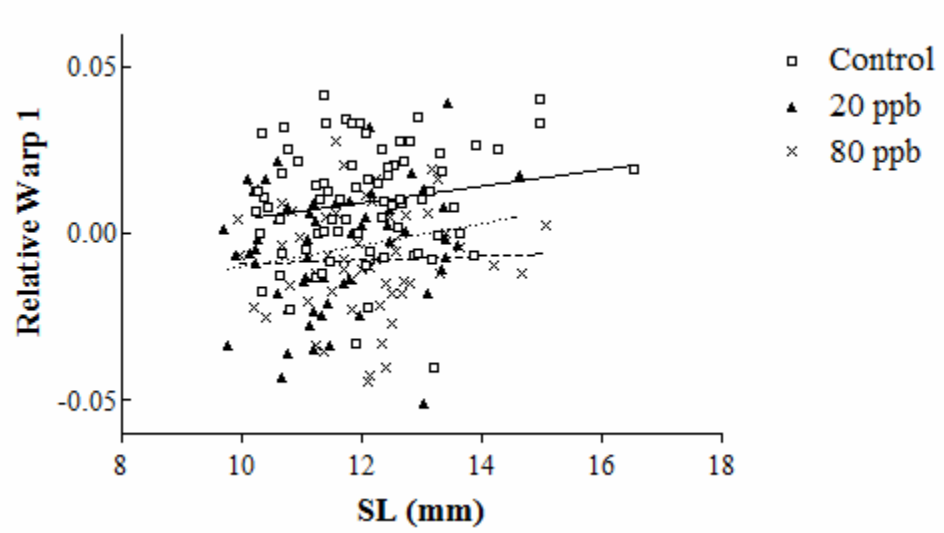

Figure A2.2. RW1 scores versus standard length for red drum larvae after 15 days of control, 20 ppb, or 80 ppb atrazine treatment. Lines represent linear regressions fit to each group (control: solid line, 20 ppb: dotted line, 80 ppb: dashed line).

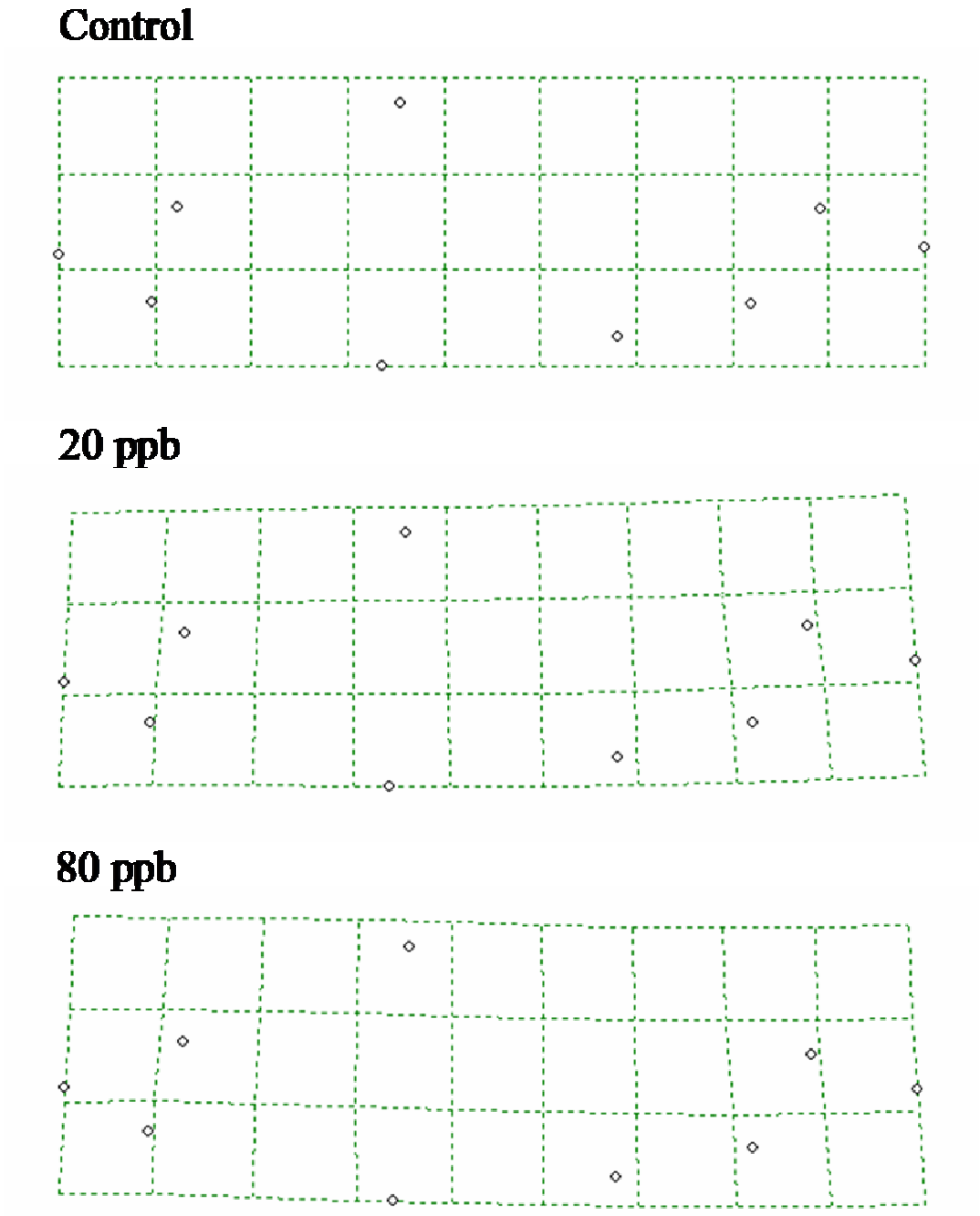

Figure A2.3. Relative warps of red drum shape landmarks projected as thin plate splines for control, 20 ppb and 80 ppb treated larvae after 15 days of atrazine treatment. The head of each fish is oriented to the left.

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