2006

CLONING AND EXPRESSION OF KEY ENDOCRINE GENES IN A STUDY ON ESTROGEN STIMULATED SEXUAL SIZE DIMORPHISM (SSD) IN YELLOW PERCH

Scott George Lynn
University of Kentucky, sglynn@uky.edu

Right click to open a feedback form in a new tab to let us know how this document benefits you.

ABSTRACT OF DISSERTATION

Scott George Lynn

CLONING AND EXPRESSION OF KEY ENDOCRINE GENES
IN A STUDY ON ESTROGEN STIMULATED
SEXUAL SIZE DIMORPHISM (SSD) IN YELLOW PERCH

_____

ABSTRACT OF DISSERTATION
_____

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By
Scott George Lynn

Lexington, Kentucky

Director: Dr. David French Westneat, Professor of Biology

Lexington, Kentucky

2006

ABSTRACT OF DISSERTATION

CLONING AND EXPRESSION OF KEY ENDOCRINE GENES
IN A STUDY ON ESTROGEN STIMULATED
SEXUAL SIZE DIMORPHISM (SSD) IN YELLOW PERCH

Yellow perch (*Perca flavescens*) exhibit an estrogen stimulated sexual size dimorphism (SSD) wherein females grow faster and larger than males. In an effort to gain better understanding of this phenomenon, several genes associated with sexual development, reproduction and growth were cloned, including prolactin (PRL), somatolactin (SL), insulin-like growth factor-I (IGF-Ib), the estrogen receptors (ERα and ERβa) and ovarian aromatase (CYP19A1). Real-time quantitative PCR (qPCR) assays for all the genes listed above, plus growth hormone (GH), were developed to measure mRNA levels in pituitary, liver and ovary.

Adult fish were collected from Lake Erie in the spring (May) and autumn (October) over two years and tissue mRNA levels, body weight, age, gonadasomatic index (GSI) and hepatasomatic index (HSI) were determined. Sex-specific differences included females having higher body weights, HSI and liver ERα mRNA levels than males and males having higher liver ERβa and liver CYP19A1 mRNA levels than females. Season had a significant effect on growth factors (GH and IGF-Ib), with higher mRNA levels in spring, which corresponded with higher liver CYP19A1 mRNA levels. Ovary CYP19A1 mRNA levels, which were higher in autumn, had a significant negative correlation with GH and IGF-Ib mRNA levels and liver ERβa mRNA levels had a significant positive correlation with IGF-Ib mRNA levels.

A brood of juvenile yellow perch was sampled through the first year of development up to 421 days post-hatching (dph). There was a significant effect of dph on body weight, GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa and ovary CYP19A1 mRNA levels. Only liver ERβa mRNA had a significant effect of sex and exhibited significant differences between males and females at 379 and 421 days post-hatching (dph). This work on yellow perch can provide predictive capabilities for estrogen-dependent physiological processes in other species, especially teleosts, and can also make yellow perch an exciting option for future ecotoxicogenomic studies.

Key words: estrogen, growth, gene expression, yellow perch, sex

Scott George Lynn

October 13th, 2006

CLONING AND EXPRESSION OF KEY ENDOCRINE GENES
IN A STUDY ON ESTROGEN STIMULATED
SEXUAL SIZE DIMORPHISM (SSD) IN YELLOW PERCH

By

Scott George Lynn

Dr. David F. Westneat
Director of Dissertation

Dr. Brian Rymond
Director of Graduate Studies

October 13th, 2006

RULES FOR THE USE OF DISSERTATIONS

Name   Date

_____

_____

_____

_____

_____

_____

_____

_____

DISSERTATION

Scott George Lynn

The Graduate School

University of Kentucky

2006

CLONING AND EXPRESSION OF KEY ENDOCRINE GENES
IN A STUDY ON ESTROGEN STIMULATED
SEXUAL SIZE DIMORPHISM (SSD) IN YELLOW PERCH

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By
Scott George Lynn

Lexington, Kentucky

Director: Dr. David French Westneat, Professor of Biology

Lexington, Kentucky

2006

DEDICATION

I dedicate this dissertation to Emily Blair Morrison:

my fiancée, my love, my inspiration and my life.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FILES

ScottLynnPhDdissertation.pdf

Chapter 1: Introduction

1.1 Yellow Perch

Yellow perch are an economically, ecologically and recreationally important teleost species [1], especially in the Great Lakes, and have historically comprised the largest inland fishery in North America [2]. Yellow perch commercial fisheries harvest >10 million lb yr^{-1} in the Great Lakes, with most of that coming from Lake Erie, however these harvests are approximately 6-8 fold less than levels seen only a few decades ago [1]. Lake Michigan has seen an 80% decrease in yellow perch harvests since the early 90s [1], and in recent years females have constituted only 20% of the population [3, 4]. A recent study by Heyer *et al.* [5] indicated that maternal effects on larval traits may be substantially influencing the recruitment of Lake Michigan yellow perch. These maternal effects are most probably tempered by the maternal endocrine status, which can be influenced by a number of biotic (age, size, gonadosomatic index) and abiotic (photoperiod, temperature, salinity) [6] factors.

Yellow perch exhibit an interesting female biased sexual size dimorphism (SSD) wherein females grow faster than males [7]. The female biased SSD in yellow perch was demonstrated in laboratory [8, 9] and wild populations [10-12] many years ago, but it was not until the mid 1980s [7, 13, 14] that studies identified 17β-estradiol (E$_2$) as a growth stimulator in yellow perch SSD. In a series of experiments using E$_2$, 17α-methyltestosterone (MT), triiodothyronine (T$_3$) and zeranol, with different size classes of juvenile perch, Malison *et al.* [13] found that only the low dosages of E$_2$ (2 and 20 μg/g diet) significantly stimulated growth. Further, higher levels of E$_2$ (50 μg/g diet) depressed growth and the growth promoting effects of E$_2$ were only noticeable in fish of 80-110 mm total length (TL) or greater [13]. In a separate study, Malison *et al.* [14] found that sexual differentiation occurs around 16 mm TL in yellow perch and E$_2$ treatment of male perch initially 90-110 mm TL did not result in sex reversal. These findings together indicate that the influence E$_2$ has on the growth of male yellow perch 80+ mm TL is not simply the result of a feminizing effect, however the possibility that feminizing male perch may increase their growth rate cannot be excluded. This critical size range of 80-110 mm TL is also the same size at which females normally begin to outgrow males [8] and female-biased SSD begins to be manifested. This critical period

is also the specific minimum body size for the onset of vitellogenesis and spermatogenesis in females and males [14], respectively, pointing towards an upregulation of E_2 receptors (ERs) on target tissues (ovary, liver or pituitary) and a coinciding increase in tissue expression of growth factors. Malison *et al.* [7] reported that in addition to a growth response, E_2 treatment stimulated feed consumption, and in a different study [15] growth rate and growth efficiency of female yellow perch exceeded those of males two-fold in animals fed untreated food without restriction. These observations suggest that the growth-promoting effects of estrogen may work in part through appetite centers of the central nervous system and could involve pituitary hormones. They also point out a clear linkage of growth and reproductive development in this species.

1.2 Pituitary Genes

In vertebrates, the pituitary gland is the "master gland" and as such at least partially regulates aspects of reproduction, growth and sexual development. The pituitary hormone, growth hormone (GH), and its intermediates, the insulin-like growth factors (IGF-I and IGF-II), are key players in all of the above processes [16]. GH is produced and secreted from the pituitary gland and studies have shown that GH stimulates production and release of IGF-I in the liver (Figure 1.1). The IGF system plays an important role in controlling animal development and growth. There is strong evidence for the role of IGFs as mediators for the somatogenic action of GH [16-18] and recently, the importance of IGF in the development of juvenile and larval teleosts has become apparent [19-21]. These studies imply a developmental pattern of expression not only for steroid hormones and receptors, but also for metabolic hormones which have a definitive effect on pituitary function. Presently there is contradictory evidence that steroid hormones (estrogen (E_2) and testosterone (T)) directly regulate or modulate GH transcription or secretion [22, 23]. A couple *in vivo* studies have found that E_2 upregulated GH mRNA expression [24, 25], however E_2 exposure to cultured pituitary cells failed to stimulate *in vitro* GH mRNA levels [26, 27]. A possible explanation for these differences are that gonadotropin-releasing hormone (GnRH), which stimulates gonadotropin release, may act as an intermediate to E_2 induced GH release and

transcription. Parhar and Iwata [28] found that GnRH neurons project to GH cells in Steelhead trout (*Oncorhynchus mykiss*) providing direct morphological evidence of the association of GnRH and GH and, by proxy, E_2 and GH.

There is increasing evidence that somatolactin (SL) (Figures 1.1 and 1.2), another pituitary hormone found only in fish, is involved in sexual maturation and reproductive cycle regulation [29-31]. Although the precise function of SL remains unclear, studies have shown a seasonal, possibly growth related, rhythm in gilthead sea bream (*Sparus aurata*) [32] and rainbow trout (*Oncorhynchus mykiss*) [33]. Recent studies have shown a pattern opposite to that of GH, indicating SL as an anti-obesity hormone that helps to expedite growth and/or reproductive processes [34, 35]. The first SL receptor (SLR) was cloned from the liver of chum salmon (*Oncorhynchus masou*) in 2005 [36] and, to date, only one other SLR clone has been produced from the medaka (*Oryzias latipes*) [37]. Fukada *et al.* [36] found the highest levels of SLR expression in liver and visercal fat with moderate levels of expression in muscle and gonad of both sexes.

Prolactin (PRL) (Figures 1.1 and 1.2) has long been suspected of involvement in fish reproduction because of its well known role in mammalian reproduction [38] (Figure 1.1). Weber *et al.* [39] found that GnRH is a potent stimulator of PRL release in tilapia (*Oreochromis mossambicus*) cultured pituitaries, which can be enhanced 3-fold by sex steroid hormones (E_2 and T). However Brinca *et al.* [40] found seasonally dependent results with *in vitro* PRL secretion in response to E_2 treatment in sea bream (*Sparus aurata*). Winter sea bream pituitaries increased PRL secretion in response to E_2, while spring pituitaries decreased PRL secretion. These results support a theory that there may be a shift in control of PRL secretion with changes in the reproductive state of the fish. Growth-promoting actions of PRL have been reported in higher vertebrates, but are less well established in teleosts. Recently though, ghrelin (Ghr), a new peptide which specifically stimulates growth hormone (GH) release from the pituitary, was shown, for the first time in teleosts, to significantly stimulate PRL release in tilapia [41]. The ability of tilapia PRL ($tPRL_{177}$) to elevate IGF-I mRNA levels in the liver [42] indicates that PRL must possess somatotropic actions similar to GH. GH, SL and PRL are associated with parr-smolt transformation (smoltification) of Atlantic salmon (*Salmo salar*) [43] and chum salmon (*Oncorhynchus keta*) [44, 45] further linking them to sexual maturation and

3

reproduction. These pituitary hormones all show sexually-dimorphic secretory patterns during development [46-48] and studies point to the importance of steroids (estrogen and testosterone) in the regulation of pituitary hormone release and gene expression in mature animals [49-52].

1.3 Estrogen Related Genes

Reproduction and sexual maturation are significantly influenced by sex steroids, but these steroidal effects are ultimately modulated by the distribution and expression of the steroid receptors. The estrogen receptors (ERα and β) (Figure 1.2) are distributed on many tissues in teleosts (gonad, liver, brain and pituitaries) [53, 54] and are intricately involved in sexual determination and development [55]. Mosconi *et al.* [56] found that the liver in the seabream was resistant, with regard to vitellogenin (vtg) production, to either GH or E_2 during the postspawning period. They also report that ER levels were higher in sea bream liver at the prespawning stage compared with those at the spawning or postspawning stages, which points toward an upregulation of ER as an important component of estrogen-mediated hepatic responses in this teleost. Estrogen levels, however, are controlled primarily by P450 aromatase or CYP19A (Figure 1.2), the terminal enzyme in the conversion of testosterone to estrogen, and blocking this enzyme, via an aromatase inhibitor (e.g. fadrozole), causes sex reversal in several species of teleosts [57-59]. Aromatase mRNA is expressed in many tissues associated with reproduction (gonad, brain, and pituitary) [60] and growth (liver, brain, and pituitary) [61], and varies with sexual maturation [62], age and season [63].

While these findings indicate the presence of an intricate, sex-specific (steroid), endocrine regulatory relationship between the pituitary, liver and gonad in teleosts, it is still unclear how estrogen stimulated SSD in yellow perch is achieved. Regulation of the somatic growth axis by sex steroids is poorly understood as studies have reported contradictory evidence on the process. Kamegai *et al.* [64] assert that estrogen targets growth hormone releasing hormone (GHRH) neurons as a mechanism for modulating GH release in male rats; however, Scanlan and Skinner [65] recently found that estrogen does not act directly on GHRH neurons to influence GH secretion in the female sheep. These conflicting results could be the result of a species-specific difference or perhaps a sex-

specific regulation of GH by E_2. Contreras and Talamantes [66] showed that GH and E_2 synergistically upregulated growth hormone receptor (GHR), growth hormone binding protein (GHBP) and ER expression in mouse hepatocytes. Both ERα and ERβ were detected in growth plate chondrocytes of rats before sexual maturation, implying a role for estrogen in juvenile growth [67]. Interestingly, when animals were given ovariectomies, there was an increase in body weight gain, tibial length and growth plate width but not a corresponding increase in growth plate IGF-I mRNA expression [67]. Does the estrogen stimulated SSD in yellow perch, which occurs after sexual determination, involve the GH-IGF growth axis and how many of the endocrine genes described here play a role? Comprehensive endocrine studies (sex-specific, seasonal and developmental gene expression levels) on yellow perch will provide insight into how estrogen promotes growth in yellow perch. Such studies could also yield predictive tools in the fisheries management and aquaculture of the species, as well as provide robust, predictive capabilities to other species as a "general" template for E_2-dependent physiological processes.

1.4 Conclusions

My PhD work focused on determining the endocrine mechanisms of estrogen stimulated SSD in yellow perch and using the yellow perch as an experimental model to study the endogenous endocrine pathways associated with sexual maturation and growth. My PhD project entailed cloning several endocrine genes of interest (GH, PRL, SL, IGF-I, ERα, ERβ, aromatase (CYP19A1) and β-actin) in yellow perch (*Perca flavescens*) and developing real-time quantitative PCR (qPCR) assays to measure mRNA levels. Sex-specific tissue (pituitary, liver and gonad) mRNA levels were measured at two times of the year in adult yellow perch from a natural population in Lake Erie. Newly hatched yellow perch were sampled during the first year of development and sex-specific tissue mRNA levels were determined. My aim was to establish a comprehensive endocrine gene expression profile for adult and juvenile yellow perch to gain insight into the manner in which E_2 contributes to SSD in yellow perch.
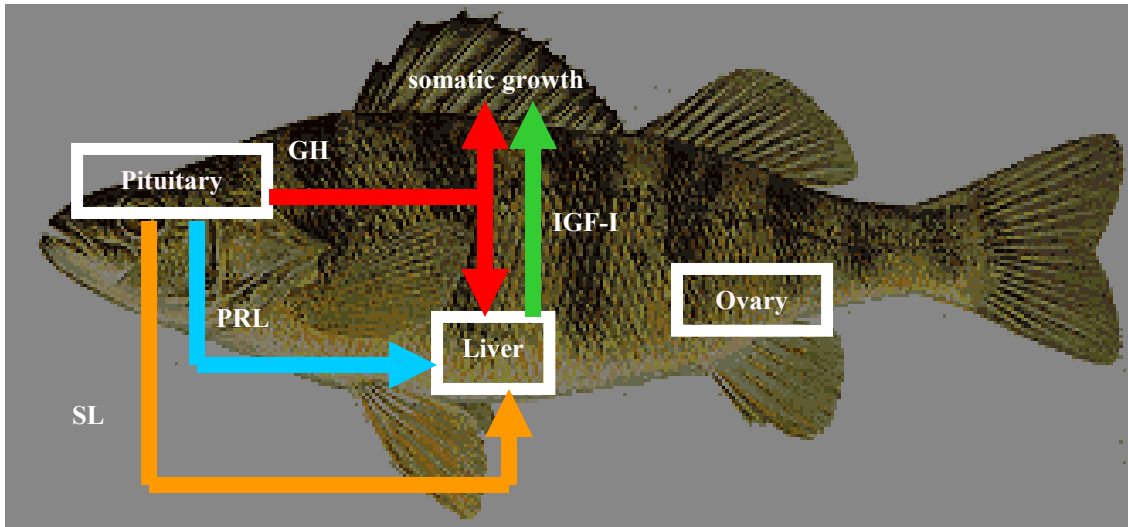
Figure 1.1 Simplified representation of the vertebrate growth axis.
Pituitary, liver and ovary tissues are represented with white boxes. Red lines indicate growth hormone (GH), blue lines indicate prolactin (PRL), orange lines indicate somatolactin (SL) and green lines indicate insulin-like growth factor I (IGF-I) pathways.
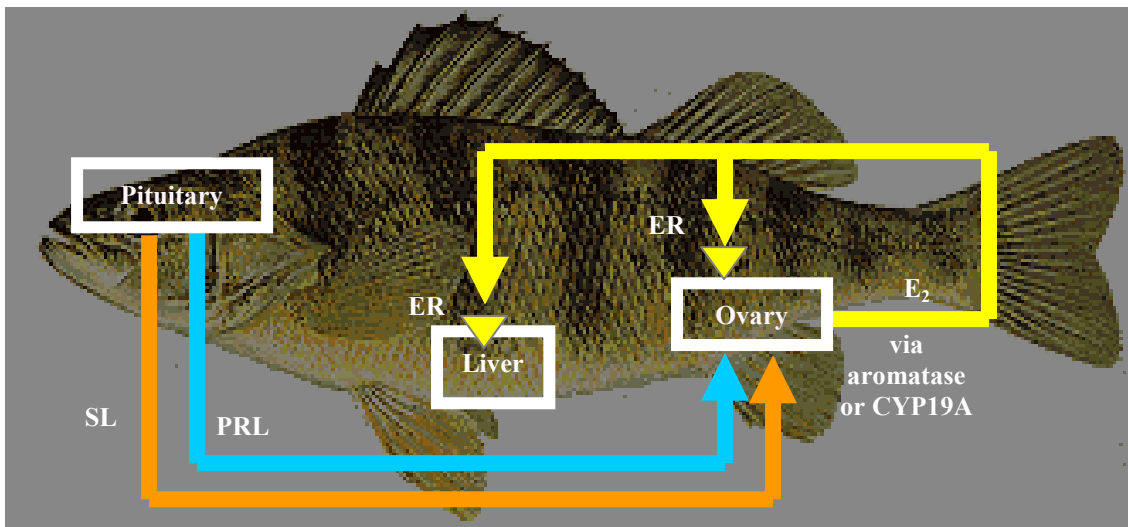
Figure 1.2 Simplified representation of the vertebrate estrogen axis.
Pituitary, liver and ovary tissues are represented with white boxes. Blue lines indicate prolactin (PRL), orange lines indicate somatolactin (SL) and yellow lines indicate estrogen (E_2) pathways. Yellow triangles indicate the presence of estrogen receptors (ER) in the tissue.

Chapter 2: Sequence and sex-specific tissue expression of prolactin, somatolactin and insulin-like growth factor-I cDNAs in yellow perch

2.1 Introduction

Prolactin (PRL) is a protein hormone in the growth hormone (GH), somatolactin (SL) and placental lactogen (PL) family which is thought to have resulted from gene duplication some 400 million years ago [68]. The primary site of PRL production in all known vertebrates is the anterior pituitary gland but recent work has highlighted extrapituitary PRL production [69]. In teleosts, the seabream (*Sparus aurata*) showed PRL expression in the intestine, liver, ovary and testes [70]. In the goldfish (*Carassius auratus*), PRL expression was detected in the brain, gill, kidney, spleen, liver, muscle, ovary and testis [71]. And in the orange-spotted grouper (*Epinephelus coioides*), PRL expression was detected in brain, gill, spleen, adipose and ovary tissues [72]. The effects of PRL are mediated by the PRL receptor (PRLR) which in teleosts is expressed primarily in the gill, kidney, intestine and gonads [73-76]. Other studies have also found PRLR expression in brain [74, 77, 78], pituitary [74, 79], skin [77-79], liver [74, 79] and spleen [77, 78] and only Santos *et al.* [79] detected PRLR mRNA in heart, muscle and bone.

The distribution of PRLR in teleosts is consistent with its primary function as a freshwater adapting hormone in euryhaline fish, however the full spectrum of PRL functions, in teleosts, humans or otherwise, is not completely understood. PRL reportedly affects more physiological processes than all other pituitary hormones combined, with over 300 separate functions identified in vertebrates [80]. And while there is little evidence of PRLR expression in teleost liver, Shepherd *et al.* [42] found PRL_{177} elevated IGF I mRNA in tilapia (*Oreochromis mossambicus*), presumably by acting as a competitive ligand for GH receptors. Ghrelin (Ghr), a peptide which specifically stimulates GH release from the pituitary, was shown for the first time in teleosts to significantly stimulate PRL release in tilapia [41]. These findings indicate at least a peripheral somatotrophic action of PRL in teleosts. PRL has also shown evidence of somatotrophic actions in rats by increasing body weight [81] and inducing expression of growth-related genes in the liver [82]. More specifically, ovine PRL

increased IGF I mRNA abundance in the livers of hypophysectomized rats 12 h after injection [83]. PRL has also been tied to lipid metabolism in rats [84] and pigeons [85]. In teleosts, Leena *et al.* [86] found PRL inhibited several enzymes involved in biosynthesis of fatty acids in climbing perch (*Anabas testudineus*) liver and PRL treatment in juvenile coho salmon (*Onchorhynchus kisutch*) resulted in pronounced lipid depletion [87].

PRL originally received its name from its well known effect of promoting lactation in mammals and it has also been identified as a juvenile hormone in amphibian metamorphosis. In mammals, E_2 is known to stimulate PRL release from the pituitary [38], however PRLs role in fish reproduction and sexual maturation and the relationship between PRL and E_2 still remains to be clarified as there are numerous conflicting reports. Pituitary PRL expression levels in gilthead seabream (*S. aurata*) were significantly reduced by E_2 treatment in adults for experiments done in November in Portugal by Cavaco *et al.* [88]. In contrast, Brinca *et al.* [40] found seasonally dependent results with *in vitro* PRL secretion in response to E_2 treatment in gilthead sea bream (*S. aurata*). While winter PRL secretion was significantly higher than spring levels, E_2 increased PRL secretion in winter (February, Portugal) and decreased PRL secretion in spring. Galas and Epler [89] found that PRL had a significant suppressive effect on E_2 secretion in cultured rainbow trout ovary follicular cells indicating that PRL may play a role in fish reproduction. Weber *et al.* [39] found that GnRH stimulation of PRL release in tilapia (*O. mossambicus*) cultured pituitaries, was enhanced 3-fold by sex steroid hormones (estrogen and testosterone). And in a later work, Weber and Grau [90] showed significantly higher levels of PRL in brooding females than in non-brooding females.

In juveniles, Cavaco *et al.* [88] found significantly increased levels of PRL expression in response to E_2 which is contrasted by a lack of response in PRL expression to E_2 in immature rainbow trout (*Oncorhynchus mykiss*) [91]. Furthermore, gonadal transcripts of PRLR were significantly increased by E_2 in adult gonads but reduced 24-fold in juvenile gonads [88]. Onuma *et al.* [92] measured PRL mRNA expression in response to E_2 in masu salmon at four time points representing different maturational and reproductive stages. While they did not find differences in PRL

mRNA expression between the different stages, there were significant differences in the responsiveness of E_2 between the sexes and between stages. Female salmon showed no response to E_2 until reaching sexual maturity when E_2 significantly increased PRL mRNA expression, while males showed no response to E_2 in PRL mRNA expression at any maturational stage. And in adult pre-spawning salmon, E_2 significantly decreased PRL mRNA expression in females, but males showed no response to E_2 [92]. All of these studies indicate sex specific and maturational differences in the expression and function of PRL in teleosts, particularly in response to estrogen.

Somatolactin (SL) is the newest member of the GH/PRL/SL/PL family of hormones first cloned from pituitaries of flounder (*Paralichthys olivaceus*) in 1990 [93]. SL has only been found in fish and does not appear to have a homologue in tetrapods, but some fish have been found to have more than one distinct somatolactin subtype [94-96]. SL is primarily produced in the par intermedia of the pituitary gland but at least one study has found extrapituitary expression in brain, gill, heart, kidney, liver, skeletal muscle, spleen, ovary and testis [97]. The first SL receptor (SLR) was cloned from the liver of chum salmon (*Oncorhynchus masou*) in 2005 [36] and, to date, only one other SLR clone has been produced from the medaka (*Oryzias latipes*) [37]. Fukada *et al.* [36] found the highest levels of SLR expression in liver and visercal fat with moderate levels of expression in muscle and gonad of both sexes.

Since its discovery in 1990, SL has been tied to a number of physiological processes which include smoltification [98], stress response [99], pigmentation [100, 101], immune function [102], acid base balance [103], elemental metabolism [104-106], gonadal steroid biosynthesis [107] and gonadal maturation [31]. However, SLR expression patterns support the notion that a major function of SL is regulation of lipid metabolism [36]. The SL deficient mutant (*ci*) medaka had significantly higher hepato-somatic index (HSI), muscle triglyerides, liver triglycerides and lower plasma cortisol levels [37] than the wildtype medaka. And a "cobalt" variant of rainbow trout which lacks most of the pars intermedia and is essentially SL deficient, showed similar patterns in tryglycerides and cortisol levels [108]. These studies on SL mutants and other studies on physiologically normal fish [34, 35, 109] give clear evidence that SL is involved in energy homeostasis, lipid metabolism and possibly growth. Moreover, SL

was able to stimulate hepatic insulin growth factor I (IGF-I) expression *in vivo* in coho salmon. Thus, SL could also promote growth in the earlier development stages through their involvement in the IGF-I/GH regulation [17].

There is also evidence that SL is involved in sexual maturation and gonadal development. Rand-Weaver [31] found high plasma SL levels during the period of gonadal growth and maturation in coho salmon (*O. kisutch*) and these levels were correlated with estradiol levels in females and 11-ketotestosterone levels in males. Parhar and Iwata [28] found that gonadotropin releasing hormone (GnRH) neurons project to somatolactin cells in the steelhead trout (*O. mykiss*) providing a direct link between steroids and SL. Peyon *et al.* [110] found that SL cells were sensitive to leptin and neuropeptide Y only in prepubertal and pubertal stages of European sea bass (*Dicentrarchus labrax*), indicating a potential role of SL in the nutritional control of the onset of puberty. Onuma *et al.* [44] found that in prespawning chum salmon (*Oncorhynchu keta*), SL elevates with final maturation regardless of osmotic environment and the study by Taniyama *et al.* [45] suggests that expression of the SL gene is elevated with sexual maturation in chum salmon. All of these studies indicate a steroidal link with SL expression and function in teleosts involving gender and sexual maturation.

Insulin-like growth factors (IGFs) are mitogenic peptide hormones produced by all known vertebrates, mainly in the liver. Two separate IGFs (IGF-I and IGF-II) have been identified in bony fish and have been shown to mediate the growth-promoting actions of pituitary growth hormone (GH). Multiple IGF-I mRNA transcripts have been identified in several species of fish including chinook salmon (*Oncorhynchus tshawytscha*) [111], coho salmon (*O. kisutch*) [112], Thai catfish (*Clarias macrocephalus*) [113], Atlantic salmon (*Salmo salar*) [114], tilapia (*O. mossambicus*) [115], carp (*Cyprinus carpio*) [116], Japanese flounder (*Paralichthys olivaceus*) [117], zebrafish (*Danio rerio*) [118] barramundi (*Lates calcarifer*) [119] and Eurasian perch (*Perca fluviatilis*) [120]. For some of these species, the multiple transcripts have been shown to be derived from multiple genes while others involve alternative splicing of mRNA. While the liver is the primary site of IGF-I expression in teleosts, numerous studies have found substantial nonhepatic expression in brain [112, 118, 120-122],

pituitary [123], gill [112, 118, 121-123], heart [120, 121, 124, 125], stomach [118], spleen [118, 120, 121, 123, 124], kidney [120-123, 125], muscle [112, 118, 121, 125, 126], testis [121] and ovary [112, 122, 126]. IGF activity can be further regulated by the distribution of IGF receptors and the activities of the IGF binding proteins (for review see Wood *et al.* [127]).

There is abundant evidence supporting the role of IGF-I in teleost growth and indicating that GH is the primary regulator of IGF-I synthesis and secretion [128-131]. Dosing experiments with coho salmon (*O. kisutch*) showed that exogenous treatment with mammalian IGF-I can stimulate growth [132]. Iwatani *et al.* [133] obtained results indicating that the enhanced growth of juvenile four-spine sculpin (*Cottus kazika*) transferred from freshwater to saltwater is related to increases in liver IGF-I mRNA expression. Both recombinant trout and human IGF-Is stimulated cell proliferation in rainbow trout muscle preparations [134] and IGF-I receptor levels correlated with increased bone density in Atlantic salmon (*S. salar*) [135]. The use of the cartilage sulfation ($^{35}SO_4$) assay to show that IGF-I stimulates cartilage or skeletal growth has been used effectively in numerous teleosts including the long-jawed mudsucker (*Gillichthys nirabilis*) [136], tilapia (*O. mossambicus*) [137], coho salmon (*O. kisutch*) [138], goldfish (*C. auratus*) [139], carp (*C. carpio*) [140] and rainbow trout (*O. mykiss*) [141]. And IGF-I stimulated ^{14}C glycine incorporation into protein in a dose-dependent manner in the muscle of gulf killifish (*Fundulus grandis*) [142].

Few fish studies have examined the effects of estrogen on sex-specific hepatic IGF-I expression or plasma levels, but there has been work done on IGF-I expression in gonads, particularly its role in developing ovarian follicles and gonadal steroidogenesis. Studies have shown that IGF-I, and in some cases IGF-II, are key factors in the early stages of oocyte maturation in Nile tilapia (*Oreochromis niloticus*) [143], Mozambique tilapia (*O. mossambicus*) [115, 144], red seabream (*Pagrus major*) [145-148], brown trout (*Salmo trutta*) [149], common carp (*C. carpio*) [150, 151], killifish (*Fundulus heteroclitus*) [152], coho salmon (*O. kisutch*) [153], gilthead seabream (*S. aurata*) [154] and white bass (*Morone chrysops*) [155]. And in gilthead sea bream (*S. aurata*), E_2 stimulated ovary IGF-I mRNA but only in reproductive fish, as IGF-I mRNA in

prereproductive ovaries was decreased. IGF-II mRNA also was upregulated in reproductive ovaries by E_2, but showed no differences in prereproductive ovaries [156].

While gonadal, particularly ovarian, IGF seems important for oocyte and perhaps even ovarian development, there is evidence that liver IGF-I mRNA expression is inhibited by E_2 at least in a few species. Tilapia [157] and coho salmon [158] males grow larger than females and steroids (i.e. androgens) play a key role in this sexually dimorphic growth pattern. Two studies indicate that treatment with E_2 lowers liver IGF-I mRNA expression levels in Mozambique tilapia (*O. mossambicus*) [157, 159]. And in the marine gilthead seabream (*S. aurata*) both IGF-I and -II liver mRNA levels were decreased in response to E_2 treatment [129]. Also, exposure to 17β-estradiol did not cause a significant effect on plasma IGF-I levels in Atlantic salmon (*S. salar*) at any of the three parr-smolt transformation stages investigated [160]. However, in rats E_2 upregulated hepatic IGF-I through a GHR independent mechanism [161] and an estrogenic isoflavone significantly elevated IGF-I production of osteoclast cultures [162]. While these studies are limited and somewhat contradictory, they all indicate a direct regulatory relationship between IGF and steroids. Also, IGF-I stimulated production and release of gonadotropins (I and II) in the pituitaries of coho salmon (*O. kisutch*) [163], rainbow trout (*O. mykiss*) [164] and eel (*Anguilla anguilla*) [165] demonstrate this relationship to be bidirectional.

The yellow perch is one of a group of important fishes which exhibit a sexual size dimorphism (SSD) in which females grow faster than males [7]. Other fishes in this group include sea bass [166, 167], halibut [168], eel [169], plaice, walleye, and tench [170, 171]. The female biased SSD in yellow perch was demonstrated in laboratory [8, 9] and wild populations [10-12] many years ago, but it was not until the mid 1980s [7, 13, 14] that studies identified 17β-estradiol (E_2) as a growth stimulator in yellow perch SSD. Further, the growth promoting effects of E_2 were only noticeable in fish of 80-110 mm total length (TL) or greater [13] and this critical size range is also the same size at which females normally begin to outgrow males [8] and a female-biased SSD begins to be manifested. This critical period is also the specific minimum body size for the onset of vitellogenesis and spermatogenesis in females and males, respectively [14], pointing towards an upregulation of E_2 receptors (ERs) on target

tissues (ovary, liver or pituitary) and a coinciding increase in tissue expression of growth factors. Malison *et al.* [7] reported that in addition to a growth response, E_2 treatment stimulated food consumption, and in another study [15] growth rate and growth efficiency of female yellow perch exceeded those of males two-fold in animals fed without restriction. These observations suggest that the growth-promoting effects of estrogen may work in part through appetite centers of the central nervous system and could involve pituitary hormones. They also point out a clear linkage of growth and reproductive development in this species. As a first step in investigating the roles of PRL, SL and the IGFs in the estrogen stimulated SSD of yellow perch, the full length cDNAs for these genes were cloned and sequenced and sex specific tissue expression was examined.

2.2 Methods

Cloning

Pituitary and liver tissues were collected from fish brought into the Pannuzzo Fish Co. (Lorain, OH) by recreational fishermen for cleaning. Only fish that had been caught within 12 hours and kept on ice were used. Tissues were harvested from the offal, immediately frozen on dry ice, transported back to the University of Kentucky and stored at -80°C until total RNA was extracted with the GenElute™ Mammalian Total RNA Kit (Sigma, St. Louis, MO). RNA samples were treated with amplification grade DNase I (Sigma, St. Louis, MO) and quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). First-strand cDNA with ligated 5' and 3' anchor primers was generated from 5 μg total RNA using the GeneRacer™ Kit (Invitrogen, Carlsbad, CA).

GenBank was searched for neoteleost PRL, SL, IGF-I and IGF-II cDNAs and several sequences were aligned using Vector NTI Suite 7.0 (Informax, Inc., Frederick, MD) and GeneDoc (http://www.psc.edu/biomed/genedoc/) [172]. Consensus sequences, or fragments thereof, were put into Primer3, a web-based primer design program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [173] and general neoteleost primers for genes of interest were generated. Generated primers were used exclusively or in combination with the GeneRacer™ primers (5' and 3') (Table 2.1) provided in the

GeneRacer™ Kit (Invitrogen, Carlsbad, CA) to obtain initial PCR products. A 50 µl total volume PCR mixture using a MasterTaq Kit (Eppendorf Scientific Inc., Westbury, NY) was subjected to a touch down PCR amplification (Table 2.2) with either pituitary (PRL and SL) or liver (IGF-I) tissue as a template. PCR products were electrophoresed in 1% agarose gels with a 1 kb DNA ladder (Gibco/BRL, Gaithersburg, MD) and visualized by ethidium bromide staining. PCR products of expected size were electrophoresed in 1% low melt agarose gels, excised and purified using GenElute™ Minus EtBr Spin Columns (Sigma, St. Louis, MO). Purified PCR products were ligated into a pCR®4-TOPO® vector and transformed into TOP10 chemically competent cells using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA). The plasmid DNA was then extracted from the bacterial cells using the GenElute™ Plasmid Miniprep Kit (Sigma, Sigma, St. Louis, MO). Plasmid samples were quantified and up to 600 ng of plasmid DNA was put into a sequencing PCR using BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing PCR consisted of 35 cycles of 30 s at 96 °C, 15 s at 50 °C and 4:00 min at 60 °C. After the PCR, each sample received 27 µl ddH$_2$O, 60 µl 100% EtOH and 3 µl 3 M NaOAc. This mixture was transferred to a 1.5 mL microcentrifuge tube, vortexed and left overnight to precipitate. The next day, samples were spun at maximum speed at 4 °C for $\geq$ 30 min, decanted and washed with 250 µl of 70% EtOH, spun again, decanted and allowed to air dry. Samples were kept at -20 °C until they were transported to the University of Kentucky Advanced Genetic Technologies Center (http://www.uky.edu/Centers/AGTC/) for sequencing. Species-specific PRL, SL and IGF-I primers were developed based on the sequences generated and the PCR, cloning, and sequencing procedure was repeated as necessary (with 5' and/or 3' GeneRacer™ primers) to achieve full length (5'UTR+CDS+3'UTR) sequences. BLASTN v. 2.2.14 [174] searches were used to determine similarities with other teleosts and to verify gene identity.

Species-specific primers were designed and synthesized to amplify the full coding region of yellow perch GH (Accession #AY007303) [175], PRL, SL and IGF-I cDNAs (Table 2.3). These primers were tested using 1 µl of cDNA template. Total RNA (750ng) extracted from pituitary (PRL and SL) or liver (IGF-I) tissues was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA) to generate

cDNA templates. A 50 µl total volume PCR mixture was subjected to a touch down PCR amplification (Table 2.4). PCR products were electrophoresed in 1% low-melt agarose gels with a 1 kb DNA ladder (Gibco/BRL, Gaithersburg, MD) and stained with ethidium brromide. The resulting bands were excised, purified, cloned and sequenced, as described above, to verify sequence data and primer-gene specificity. Transcript sizes of pituitary mRNAs were also verified with northern blotting. GeneDoc [172] was used to generate amino acid sequence alignments of PRL, SL and IGF-I from teleost sequences given by a BLASTP v. 2.2.14 search [174]. Alignments were produced in Clustal X1.81 [176] and used to generate phylogenetic tree data using the Neighbor Joining tree method [177] with 1000 bootstrap trials and TreeView v. 1.61 was used to create a visual phylogenetic tree.

Sex-specific tissue expression

Tissues were collected from adult yellow perch kept in IACUC (#00251L2001) approved aquaculture facilities at the University of Kentucky, Lexington, KY. Perch were kept in a flow through tank (~1L/min) chilled to ~17 °C, a 14:10 light:dark cycle and constant aeration. Fish were kept at a density of < 1 fish/gallon and fed three times per week to satiation with Aquamax Grower 400 (PMI Nutrition International, Inc., Brentwood, MO) for a period of one year before sampling. Fish were sampled in spring (March) and euthanized with MS 222 before tissues were harvested. Both male and female brain, pituitary, gill, heart, liver, stomach, spleen, kidney, skeletal muscle and gonad tissues were collected along with post-vitellogenic oocyte tissue [6]. Tissues were collected and flash frozen at -80 °C until analysis. Total RNA was extracted, treated with amplification grade DNase, quantified and up to 750 ng was reverse transcribed to cDNA and quantified. PCR primers and protocol are listed in Tables 2.3 and 2.4, respectively, and 900 ng of cDNA template was used for all tissues. Products were electrophoresed in 1% agarose gels and visualized using ethidium bromide staining. cDNA template quality was verified by analyzing for β-actin mRNA levels using real-time quantitative PCR (qPCR) (data not shown).

2.3 Results

PRL

From the sequences generated, yellow perch PRL cDNA (Accession #AY332491) was found to consist of 2306 bp containing an open reading frame of 633 bp, a 62 bp 5'-UTR and a 1,608 bp 3'-UTR (Figure 2.1). The consensus AATAAA polyadenylation signal was located 23 bases upstream of the poly (A) tail. The open reading frame encoded a protein of 211 amino acids, which included a putative signal peptide of 24 amino acids and a mature protein of 187 amino acids. The isoelectric point and molecular mass of the mature PRL peptide are 6.31 and 20,640.56 Da, respectively, as calculated by Vector NTI BioPlot. Five cysteine residues were present, with only four in the mature peptide and one in the signal peptide. Northern blots verified a single transcript of approximately 2,300 bp in length from total pituitary RNA (data not shown).

Comparison of the deduced amino acid sequence of the yellow perch prePRL with other known teleost prePRLs is shown in Figure 2.2. A BLASTP v. 2.2.14 search [174] reveals that yellow perch prePRL has the following homologies: 85% to orange spotted grouper; 81% to silver seabream and four-spine sculpin; 80% to European sea bass, red seabream and bluefin tuna; 79% to gilthead seabream and black seabream; 78% to three spot gourami; 75% to Mozambique tilapia, Nile tilapia and bastard halibut; 69% to pufferfish; 65% to Arctic cisco, Atlantic salmon and rainbow trout; 64% to chum salmon, and chinook salmon; 62% to stinging catfish; 61% channel catfish; 58% to noble carp; 57% to common carp, zebrafish, goldfish, and Japanese eel; 56% to European eel; 40% to lungfish; 38% to bullfrog, streamside salamander, African clawed frog and green sea turtle; 37% to horse and rabbit; 36% to human, common quail and chicken; 35% to crocodile, cat, American alligator, mouse and bovine; and 34% to dog and pig. The four cysteine residues in the mature peptide of the yellow perch PRL were conserved throughout the teleost lineage and have been shown to be involved in the formation of disulfide bonds. Yellow perch was unique in comparison to all other teleosts examined as it was missing an amino acid at position 66 in the comparison sequence (Figure 2.2) which corresponds to position 63 in the yellow perch sequence (Figure 2.1).

A phylogenetic tree of mature PRL proteins in teleosts and sturgeon (Figure 2.3) was constructed from alignment results. The tree indicates an early divergence of

Neoteleostei. The tree also indicates that Salmoniformes (salmon and trout) share a common ancestor with Anguilliformes (eels), Siluriformes (catfish) and Cypriniformes (goldfish, etc.), but they split into their own clade before those other groups diverge. Within Neoteleostei, the order Tetraodontiformes (pufferfish) splits first, then the families Paralichthyidae (bastard halibut) and Cichlidae (tiliapia) are grouped in the next split, despite the former being in the order Pleuronectiformes and latter being Perciformes. The families Osphronemidae (three spot gourami), Scombridae (bluefin tuna), Moronidae (European sea bass) and Cottidae (four spine sculpin) split as a group, but subdivide in the above order. All of those families are in the order Perciformes with the exception of Cottidae which is Scorpaeniformes. Next Serranidae (orange spotted grouper) and Percidae (yellow perch) split before the emergence of Sparidae (seabreams).
SL

Yellow perch SL cDNA (Accession #AY332490) was found to consist of 1589 bp containing an open reading frame of 693 bp, a 27 bp 5'-UTR and a 866 bp 3'-UTR (Figure 2.4). The consensus AATAAA polyadenylation signal was located 15 bases upstream of the poly(A) tail. The open reading frame encoded a protein of 231 amino acids, which included a putative signal peptide of 24 amino acids and a mature protein of 207 amino acids. The isoelectric point and molecular mass of the mature SL peptide are 5.49 and 23,896.39 Da, respectively, as calculated by Vector NTI BioPlot. Seven cysteine residues were present in the mature peptide and a potential N-glycosylation site was located (Asn_{145}-Lys-Thr_{147}). Northern blots verified a single transcript of approximately 1,600 bp in length from total pituitary RNA (data not shown).

Comparison of the deduced amino acid sequence of the yellow perch preSL with other known teleost preSLs is shown in Figure 2.5. The conserved amino acid residues of fish SLs cluster in four domains designated A, B, C and D [178]. A BLASTP v. 2.2.14 search [174] reveals that yellow perch preSL has the following homologies: 92% to orange spotted grouper and bluefin tuna; 90% to bastard halibut; 87% to red seabream; 86% red drum, Atlantic halibut, lumpfish, black sea bream; 85% Japanese medaka; 84% gilthead seabream; 83% rabbitfish; 82% sole; 76% chum salmon; 73% Atlantic cod; 71% spotted green pufferfish; 66% white sturgeon, zebrafish α; 62% marbled lungfish; 61% European eel; 58% pufferfish; 57% channel catfish; and 48% goldfish and zebrafish β.

Of the seven cysteine residues in the mature peptide of the yellow perch SL, five were conserved throughout the teleost lineage and two were conserved in all but three fish (spotted green pufferfish, goldfish and channel catfish). And the potential N-glycosylation site (Asn-Lys-Thr) at alignment positions 150-152 exists in all species except spotted green pufferfish, chum salmon, rainbow trout, goldfish, zebrafish α and channel catfish.

A phylogenetic tree of mature SL proteins in teleosts and sturgeon (Figure 2.6) was constructed from alignment results. The tree indicates an early divergence of the orders Anguilliformes (eel), Siluriformes (catfish) and Cypriniformes (goldfish etc.). The order Salmoniformes (salmon and trout) splits before the emergence of Neoteleostei. Gadiformes (cod) is the first order within Neoteleostei to emerge followed by Tetraodontiformes (pufferfish) and Pleuronectiformes (sole and halibut). Most of the rest of the fish are in the order Perciformes with the exception of Japanese medaka (Adrianichthyidae) in Beloniformes and lumpfish (Cyclopteridae) in Scorpaeniformes. These two species, along with the families Percidae (yellow perch) and Serranidae (orange-spotted grouper), both of which are in Perciformes, emerge from the remainder of the Perciformes. Next Scombridae (bluefin tuna) and Sciaenidae (red drum) split before Siganidae (rabbitfish) emerges from Sparidae (seabreams). Interestingly, zebrafish SLβ and rainbow trout SLP are grouped with European eel, channel catfish and goldfish.

IGF-Ib

Yellow perch IGF-Ib cDNA (Accession #AY332492) was found to consist of 814 bp containing an open reading frame of 558 bp, a 147 bp 5'-UTR and a 106 bp 3'-UTR (Figure 2.7). The open reading frame encoded a protein of 186 amino acids, which included a putative signal peptide of 44 amino acids, an E domain of 74 amino acids and a mature protein of 68 amino acids, consisting of a B domain (29 aa), C domain (10 aa), A domain (21) and a D domain (8 aa). The isoelectric point and molecular mass of the mature IGF-Ib peptide are 8.31 and 7,642.13 Da, respectively, as calculated by Vector NTI BioPlot. Six conserved cysteine residues, six conserved residues for binding receptors and seven conserved residues for binding IGFBPs were found in the A and B domains of the mature peptide.

Comparison of the deduced amino acid sequence of the yellow perch preIGF-Ib with other known teleost preIGF-Is is shown in Figure 2.8. A BLASTP v. 2.2.14 search [174] reveals that yellow perch preIGF-Ib has the following homologies: 100% to Eurasian perch; 99% bastard halibut; 98% European sea bass, shi drum, yellowfin seabream and orange-spotted grouper; 97% black seabream and gilthead seabream; 95% rabbitfish and four-spine sculpin; 94% flathead mullet; 93% short-horned sculpin; 88% Mozambique tilapia; 86% chinook salmon; 85% chum salmon; 82% rainbow trout and coho salmon; 68% common carp, goldfish and giant danio; 67% triangular bream, zebrafish, channel catfish and mud carp; 66% bluntsnout bream and barbodes; 65% scale-less car; 64% fathead minnow; 60% starlet; 58% African clawed frog, turkey and chicken; 56% pig, dog, horse, rabbit, bovine and sheep; 55% humans. Of the six cysteine residues in the mature peptide of the yellow perch IGF-Ib, all were conserved throughout the teleost lineage. Of the six potential receptor binding sites, only the residue at position 124 of the consensus showed any differences and only the orange-spotted grouper had an asparagine instead of a tyrosine. However, all seven of the potential binding protein binding sites were conserved in all known teleosts.

A phylogenetic tree of mature IGF-I proteins in teleosts and sterlet (Figure 2.9) was constructed from alignment results. The tree splits tilapia into its own clade before the divergence of Neoteleostei. After Neoteleostei emerge from the other Teleostei, the flathead mullet (Mugilidae) splits before the emergence of the order Perciformes. The tree inserts both sculpins (Scorpaeniformes) and the bastard halibut and turbot (Pleuronectiformes) into Perciformes. Within Perciformes, the tree groups the seabreams (Sparidae) together and also groups the perch (Percidae) together. After the emergence of Neoteleostei, Salmonidae splits and then the channel catfish (Silurifomes) splits from the rest of Teleostei. The fathead minnow (Pimephales) is the first split in the Cyprinidae family and goldfish (Carassius) is the second. The tree grouped the giant danio in with the megalobrama instead of with the zebrafish of the same genus, *Danio*.

IGF-II

The partial yellow perch IGF-II cDNA (Accession #DQ984123) sequence generated consisted of 335 bp corresponding to bps 75-409 in the open reading frame

(Figure 2.10). These bases encode for amino acid residues 26-136 as estimated from alignment with other teleost IGF-II sequences (not shown).

Tissue expression

The primers in Table 2.3 were used to generate full length coding region PCR products which are visualized in Figure 2.11. The GH product was 678 bp in length and corresponded with the published GH EMBL/GenBank sequence (Accession: AY007303). The PRL, SL and IGF-Ib products were 694, 767 and 620 bps in length and verified the sequences presented in Figures 2.1, 2.4 and 2.7, respectively. These primers were then used to determine sex-specific tissue expression (Figure 2.12). GH, PRL, and SL all showed the highest level of expression in pituitary tissue in both sexes. Male gill tissue had low levels of GH expression, but otherwise there was no extrapituitary expression of GH. Male gill tissue showed substantial PRL expression and male brain tissue showed low PRL expression. No female tissues, besides pituitary, showed any expression of PRL, however post-vitellogenic oocyte tissue did show PRL expression, presumably from maternally derived transcripts. Male brain, stomach, spleen and kidney tissues showed expression of SL, while female gill, liver and stomach tissues showed SL expression. In the sex-specific tissue expression experiment, the IGF-I primers generated two PCR products. The first product was 620 bp in length (IGF-Ib), corresponding to the PCR product seen in Figure 2.11, while the second product was slightly smaller at ~540 bp in length (IGF-Ia). While the highest expression in both sexes was of the IGF-Ib product in liver tissue, the two products showed tissue and sex-specific differences in expression. Male brain, pituitary, gill, liver, spleen and kidney tissues showed moderate expression of both IGF-I products. Male stomach tissue showed low levels of expression of both IGF-Is and male testis tissue only showed the IGF-Ia product. Female brain, liver, spleen and kidney tissues also showed moderate expression of both IGF-I products. However, female pituitary, gill, heart and stomach tissues showed moderate expression of only the IGF-Ib product and female ovary tissue showed low expression of only the IGF-Ib product.

2.4 Discussion

The full length PRL cDNA of the yellow perch was cloned by directional RACE procedures and encodes a protein of 211 amino acids. Based on the sequence homology analysis, the mature PRL protein contains 187 amino acids, which is similar to the size of most fish PRLs. This mature PRL, however, is one amino acid residue smaller than the six closest neoteleost species as it lacks an amino acid at position 66 of the alignment (Figure 2.2) which, in 23 of the 26 known teleost species, is a methionine. The significance of this absent amino acid residue remains unknown, as protein activity and function cannot be determined until the natural peptide is isolated and characterized. It does seem unlikely though that this minor difference in structure would produce dramatic effects in function. The mature peptide maintains the four conserved cysteines that are responsible for the two disulfide bonds that result in protein folding [38]. Amino acid alignment and phylogenetic analysis indicate that yellow perch PRL shares the highest degree of homology with other members of the order Perciformes, such as the orange-spotted grouper and the four seabream species (silver, gilthead, black and red).

PCR detected a single PRL transcript when amplifying the coding sequence of cDNA and several sequencing efforts produced a single nucleotide sequence. These results suggest perhaps only one form of PRL is present in the pituitary of yellow perch as is the case with most teleosts. The most notable exceptions are the Nile tilapia (*O. niloticus*) [179, 180] and Mozambique tilapia (*O. mossambicus*) [181, 182] which have two distinct forms of PRL. The first is $tPRL_{188}$ which is very similar to other teleost PRLs (~70%) and the second is $tPRL_{177}$ which is 11 amino acids shorter and has much lower homology to other teleost PRLs (~55%) [182]. Both are functional prolactins with different activities, at least in regards to osmoregulation [183]. There was no N-glycosylation site found in the yellow perch PRL, whereas one N-glycosylation site was found in the seabream PRL, resulting in a higher molecular weight compared to other teleost PRLs [70].

The full length SL cDNA of the yellow perch was cloned by directional RACE procedures and encodes a protein of 231 amino acids. Based on the sequence homology analysis, the mature SL protein contains 207 amino acids, which is similar to the size of most fish SLs. The mature yellow perch SL contains seven cysteines, six of which are

required for the three intra-peptide disulfide bonds [184]. The cysteines at consensus positions 31 and 41 form the first bond and the last four cysteines are responsible for the other two bonds. Only the spotted green pufferfish lacks one of these important structural cysteines by having an alanine (Ala) at consensus position 31 and is one of the six species listed in Figure 2.5 that does not contain the conserved N-glycosylation site at consensus position 150-152. Some species such as the rainbow trout [97] may only have a non-glycosylated form of SL, however both glycosylated and non-glycosylated forms of SL were secreted from *in vitro* cultures of pituitaries from gilthead seabream and sole [185].

The low homology of the published goldfish SL sequence [96] to yellow perch SL (48%) and most other teleost SLs, could perhaps indicate the existence of a second form of SL (β) in this species, as was recently discovered in zebrafish [94] and is reportedly being investigated in common carp [178]. Interestingly, rainbow trout SLP [95] grouped with zebrafish β, goldfish and channel catfish instead of with rainbow trout and chum salmon SL (Figure 2.6). Apparently neither zebrafish SL sequence (α or β) contains a potential N-glycosylation site [94], but rainbow trout somatolactin-like protein (SLP), and not rainbow trout SL, does contain the conserved N-glycosylation site present in almost all teleosts (Figure 2.5). The presence of SL paralogues (α and β) in some fish species raises some interesting questions on evolution in the Actinopterygia (ray-finned fish). While zbSLβ, rtSLP, goldfish SL and channel catfish SL seem to be orthologs of each other and paralogues of the αSLs [94] (Figure 2.6), it remains unknown if more species have a second SL. With the βSL form showing up in two separate families of fish (i.e. Cyprinidae and Salmonidae), it might be expected that more fish species will have a second SL. The phylogenetic trees of PRL (Figure 2.3) and IGF-I (Figure 2.9) both indicate an early split of Neoteleostei from the other teleosts, while the SL phylogenetic tree (Figure 2.6) shows the more ancestral species "peeling off" before the emergence of Neoteleostei. There are very few known SL sequences for non-Neoteleostei fish and more data might reveal an evolutionary pattern for SL similar to PRL and IGF-I and may reveal more species with a βSL gene.

The full length IGF-I cDNA of the yellow perch was cloned by directional RACE procedures and encodes a protein of 186 amino acids. Based on the sequence homology

analysis, the mature IGF-I protein contains 68 amino acids, which is similar in size to most fish IGF-Is. The families Cyprinidae, Ictaluridae and Salmonidae possess histidine (His) and asparagine (Asn) residues at consensus positions 103 and 104, respectively (Figure 2.8), while all Neoteleostei lack these residues. The mature yellow perch IGF-I has six conserved cysteine residues with two in the B domain and four in the A domain. These cysteines are conserved in the vertebrate genome [186] and are involved in generating the tertiary structure of the mature IGF-I protein. In the mature yellow perch IGF-I peptide the putative disulfide bonds would be: Cys_6 - Cys_{46}; Cys_{18} - Cys_{59}; and Cys_{45} - Cys_{50} [187]. The greatest conservation in amino acid residues occurs in the B and A domains (86-97%), while considerably less conservation is observed in the C and D domains. The importance of the high sequence identity in the A and B domains lies not only with the maintenance of tertiary structure by cysteine disulfide bonds but also in the binding of IGF-I with its receptor and IGF-binding proteins (IGFBPs) (Figure 2.8). Studies done in mammalian systems show residues involved with binding of mature IGF-I with its receptor include Arg_{21} and the Phe_{23}-Tyr_{24}-Phe_{25} motif in the B domain and Arg_{54} and Tyr_{58} in the A domain [188, 189], while residues involved in IGF-I binding with IGFBP include Glu_3, Thr_4, Glu_9, Gln_{15} and Phe_{16} in the B domain and Phe_{47} and Ser_{49} in the A domain [190, 191]. Conserved residues in the C and D domains have also been reported to be important in hIGF1R binding, such as C domain Tyr_{31} and Arg_{36}-Arg_{37} residues and D domain Lys_{63} and Lys_{66} residues [188, 192].

Sex-specific tissue expression analysis in yellow perch reveals that pituitary gland tissue is the primary expression site for GH, PRL and SL (Figure 2.12). Male gill tissue showed low levels of GH and moderate levels of PRL expression which is consistent with their roles in osmoregulation [193]. However female gill tissue did not show GH or PRL expression, but instead showed moderate levels of SL expression. While other studies have found SL expression in gill tissue [97], it is unclear what functional role gill SL could play or why there would be a sex-specific expression pattern in yellow perch. Both male and female gill tissues showed moderately high levels of SLR expression (equivalent with pituitary tissue) in coho salmon, which raises the possibility that gill SL expression could have an autocrine function separate from SLs pituitary endocrine function [36]. Another example of a sexually dimorphic tissue expression pattern in

yellow perch is the PRL and SL expression in brain tissue. Male brain tissue shows low expression levels of both PRL and SL while female brain tissue does not show any expression. SL also exhibited low expression levels in male stomach, spleen and kidney tissues and female liver and stomach tissues. In rainbow trout, GH, PRL and SL were all detected in mature oocytes presumably as maternal messages [194]. IGF-I has similarly been detected in mature ooctyes of Mozambique tilapia [115, 144] but only PRL, and not GH, SL or IGF-I, was expressed in yellow perch post-vitellogenic oocyte tissue. These oocytes were collected in March and are post-vitellogenic but pre-final maturation as described in Dabrowski *et al.* [195] and Ciereszko *et al.* [6]. The PRL messages detected are presumably maternal messages deposited during vitellogenesis indicating that PRL is involved and perhaps necessary for the final maturation of the oocyte.

IGF-I shows a very global expression pattern in both male and female yellow perch and tissue expression analysis reveals two IGF-I bands, corresponding to ~620bp (IGF-Ib) and ~540bp (IGF-Ia). These results indicate the presence of two separate yellow perch IGF-I mRNA transcripts which correspond directly with Eurasian perch IGF-Ib and IGF-Ia [120]. The two transcripts of IGF-I in Eurasian perch are generated by alternative splicing of the pre-mRNA and Eurasian perch IGF-Ib has over 99% homology with yellow perch IGF-Ib (Figure 2.8). Eurasian perch IGF-Ia is missing 81 nucleotides, corresponding to 27 amino acids from the E domain starting with Val_{129} and ending with Lys_{155} (Figure 2.7). The Eurasian perch IGF-Ia and IGF-Ib also correspond to the two IGF-I transcripts identified in Japanese flounder [117] and barramundi [119]. The E domain of IGF-I is highly variable and four different forms have been found in several salmonids which are designated IGF-I Ea-1, Ea-2, Ea-3, and Ea-4 [112, 196]. The four transcripts are derived from two separate duplicated IGF-I genes [197] with two alternatively spliced transcripts from each duplicated gene [111]. Yellow perch IGF-Ib shows high homology to coho salmon Ea-4 (Figure 2.8) and Eurasian perch IGF-Ia and the second yellow perch transcript (IGF-Ia) correspond with salmon Ea-2 [112]. Interestingly though, salmon Ea-2 was not detected in liver and only liver Ea-1 and Ea-3, not Ea-4, responded to GH treatment and most non-hepatic tissues expressed only the Ea-4 transcript. There was a clear sex-specific expression pattern with IGF-I in yellow perch non-hepatic tissues. All male yellow perch tissues that expressed IGF-I expressed

both transcripts, with the exception of testis tissue, but female yellow perch tissues in many cases expressed only IGF-Ib. However, yellow perch testis tissue only expressed the putative IGF-Ia transcript and yellow perch ovary tissue had very low levels of expression of only IGF-Ib (Figure 2.12). Several studies have shown that the E-peptides of pro-IGF-I posses novel biological activities controlling malignant properties of cancer cells *in vitro* [198], stimulation of mitogenic activity in several cell lines [199] and regulation of cell growth and differentiation [200]. Consequentially, the difference in sex-specific tissue expression of the two IGF-I transcripts may suggest that the E peptides in this species also possess biological activities perhaps even related to sexual function.

In conclusion, this study provides the full length nucleotide sequences for yellow perch PRL, SL and IGF-I cDNAs and shows that there is a high degree of similarity with other fish species at the amino acid level. Yellow perch GH, PRL and SL all showed only one transcript and significantly higher levels were observed in the pituitary as compared with other tissues. IGF-I however showed two transcripts (designated IGF-Ib and IGF-Ia) which showed distinct sex-specific tissue expression differences. These results indicate new avenues for research related to extra-pituitary expression of these genes and the sex-specific tissue expression of IGF-I transcripts. The production and publication of these yellow perch sequences provides molecular tools to investigate growth and other characteristics of yellow perch.

Cincinnati, helped considerably with the generation and interpretation of the phylogenetic trees.

Table 2.1 Primer pair sequences used to generate initial PCR products for PRL, SL, IGF-I and IGF-II.

Start numbers indicate the forward (5' end) or reverse (3' end) primer nucleotide start. Forward and reverse primer start numbers are calculated beginning with the initiation methionine (see Figures 2.1, 2.4 and 2.7).

Gene	Start	Primer Sequence
PRL	481-	GeneRacer™ 5' primer AGG GCA GTG AGG AGA TGG CCT GAG C
SL	631-	GeneRacer™ 5' primer GGG CGT CTT TCT TGA AGC AGC TGA G
IGF-I	446-	GeneRacer™ 5' primer TCT GGC TGC TGT GCT GTC CTA CG
IGF-II	75- 409-	GGT CAA GAA GAT GTC TTC GTC CAG TCG CCT GTT TTA ATG CGG GCA TCA CG

Table 2.2 Touch down PCR protocol used to generate initial products for PRL, SL, IGF-I and IGF-I cloning.

Temp (°C)	Time (mm:ss)	Cycles
94	2:00	1
94	0:30	
72	0:30	5
70	1:00	
94	0:30	
70	0:30	5
70	1:00	
94	0:30	
68	0:30	25
70	1:00	
70	10:00	1

Table 2.3 Primer pair sequences used to generate full length cDNA coding region PCR products for GH, PRL, SL and IGF-I.

Start numbers indicate the forward (5' end) or reverse (3' end) primer nucleotide start. Forward and reverse primer start numbers are calculated beginning with the initiation methionine (see Figures 2.1, 2.4 and 2.7).

Gene	Start #	Primer Sequence	Size (bp)
GH	(-28)-	CTC AAC CAG AAC TCA ACC AGA ACC AG	
	651-	GAT GGA GGG GCA GGG CTA CAT GAT	678
PRL	(-31)-	AGG CCA ACA AAC AGG AGG CAG AGA A	
	664-	AAG AAA ATC ATG CCG GCC GCC TCA C	694
SL	(-26)-	GAC AGA ATG CAC ATG GTG ACA G	
	742-	AGT GCA CGG CTA CCA ACC TTA GGA A	767
IGF-I	(-29)-	CGG GCT TTG TCT TGC GGA GAC C	
	592-	GTC GCT GGG CAT TTG TCC ATT CCT T	620

Table 2.4 Touch down PCR protocol used to generate full cDNA coding region products for GH, PRL, SL and IGF-I.

Temp (°C)	Time (mm:ss)	Cycles
94	2:00	1
94	0:30	
72	0:30	5
72	1:00	
94	0:30	
68	0:30	5
72	1:00	
94	0:30	
64	0:30	30
72	1:00	
72	10:00	1

```
 -62   -AAGAGACAGAAGCGCCGAGATAAAGAAGCACAGGCCAACAAACAGGAGGCAGA

                M   A   H   R   K   T   N   G   S   K   I   F   M   T   V    15
  -9   GAAAGAGAGATGGCTCACAGAAAAACCAATGGAAGCAAAATCTTCATGACAGTG

        L   Y   M   V   A   A   C   R   A   V   A   I   N   D   L   L   D   R    33
  46   CTGTATATGGTGGCAGCGTGCAGGGCCGTCGCCATCAACGACCTGCTGGACCGA

        A   S   E   R   S   D   I   M   H   S   L   S   T   T   L   S   H   D    51
 100   GCCTCTGAGCGCTCCGACATAATGCACTCCCTCAGCACCACCCTCAGCCATGAC

        L   D   S   Q   F   P   P   I   G   R   V   M   P   R   P   S   M   C    69
 154   CTGGATTCTCAATTCCCTCCTATAGGCCGGGTGATGCCCCGCCCCTCAATGTGC

        H   T   A   S   L   Q   T   P   I   D   K   Y   Q   A   L   Q   V   S    87
 208   CACACCGCCTCTCTGCAGACACCCATTGACAAGTACCAGGCTCTTCAAGTATCA

        E   S   D   L   L   S   L   A   R   S   L   L   Q   A   W   E   D   P   105
 262   GAGTCAGACCTGTTGTCACTGGCTCGCTCCTTGCTCCAAGCCTGGGAAGACCCC

        L   A   V   L   S   T   S   A   N   T   L   P   H   P   A   Q   S   S   123
 316   CTGGCGGTCCTCTCCACCTCTGCTAACACCCTGCCTCACCCGGCCCAGAGCAGC

        I   S   N   K   I   Q   E   L   Q   E   H   S   K   N   L   G   D   G   141
 370   ATATCCAACAAGATCCAGGAGCTGCAGGAGCACTCCAAGAACCTGGGAGACGGC

        L   D   I   L   S   G   K   M   G   P   A   A   Q   T   I   A   S   L   159
 424   CTGGACATCCTATCTGGCAAGATGGGTCCGGCGGCTCAGACCATCGCCTCGCTG

        P   Y   R   G   G   N   D   I   G   E   D   R   I   S   K   L   I   N   177
 478   CCCTACAGAGGAGGCAACGACATCGGCGAGGACAGGATTTCCAAATTGATCAAC

        F   N   F   L   L   S   C   F   R   R   D   S   H   K   I   D   S   F   195
 532   TTCAATTTCCTGTTGTCCTGCTTCCGCCGCGACTCCCACAAGATCGACAGCTTC

        L   K   I   L   R   C   R   A   A   K   M   Q   P   E   M   C   *       211
 586   CTGAAAATCCTGCGCTGCCGGGCGGCAAAAATGCAACCAGAGATGTGCTGAAGA

 640   GTGAGGCGGCCGGCATGATTTTCTTTCTCAAAACTTGTATTGACCTACCAACTA
 694   TATTAACATTAGCACGCTTTGAATCTGCTGTAATATCCTAATTACTAGTTGGCA
 748   TTCTGAGCTTCCAGAAGGTGTAGAGAAGGAGGAGTTACATGGCCCACACAGGAA
 802   ATCCTATTTTGAAACACTTTTTTAGTCATATAATGGCGTTTTAGCTCTTATTGA
 856   TTTTGATAGAAACATTTCCCTTGTATATTTGCAAAGAAGGGCAAAAGTTGTACT
 910   TCCTAAACAACTTCCTTTCAGACTTAAAAGGTCAAAAGGTAAAATCAGGTTAAG
 964   ACATTTCAGGTTGTTCATTCAACAGCTTCTAATGTCCAGTCTTGCTAATCTGGA
1018   CAAAGCATGGCTCAGACACAGATGTTTCCAGCTGGTCTGGAATTTTGGACTGAC
1072   CTTATTGTGGTCACAAACGACACATTCTCACTCCCCAGCGTGTCAAAAAAGCA
1126   ACGCTTGGTCAGGTGCCTTTGGCGTTCGTTACTGACGCAAAAAGTCTTTTTAGC
1180   GTCTAGCATTTGGCGTCATTTCTAGATGCGCTTGGTCGGGACTCTGGCGTCACT
1234   TTTTGACGCTCTGGGCGGGACGTACGTTTAACTGGCGTGCGGCACAAGAACGGG
1288   ACGGCTAGGTTTAGGAAAAGATCGTGGGTGGGGTTATTAAATGTACGTTTCTGT
1342   GAGGACGAGAACGGGACCCAAACCTCCGGTCTCCTGGGTGAAAGTCCTGTGCTT
```

29

```
1396   GTTTGACCCATCCACCACCCCAACCAACTTCCTTAAGCCCAATTTATGCTTTTG
1450   CGTTGAATCCACGCAGTAGGTGCGTACGTAGATATGTACGTAGATACGGACCCT
1504   ACAGAGCCAAATCCCTGTCACTCTCTCTCACTCCAACACACACTCCCCACGCAT
1558   GCACACACACACACACACACACACACACACACACACATGCTGGCCCTGCTACTCTC
1612   TTAAAAGAGATACGCTAGAACAACGCAGACACCAGCGCACAAGCATAAACTTCA
1666   GGCCACTTACGTAGGCTACATTGTAAGCGTGGAGCCCCCGCAGAAGCATAAATG
1720   CAGCTTTACGCGGATTTTCGTACTACTCGCTATCGTCGTCGCTCTTAATGCTAC
1774   GTTATCTTACAGTGCTGCGGTCGAGCTACTGCTATGCCCGGTGTGTTCCAAACA
1828   GACGCTAAAGGGTGATATCTGAGTCGGTACGTGACGCTGAGAGCCACTGACCAA
1882   GCGTCCGCATTTGATGAGTTGGGAAAGAGAACAGGCTGCACAAACTGTGCTATA
1936   CTGGCGTGTTGTCTCTCCTGTGACTCCTTCTTGTCCTTCTCCCGAAGCTCTGTG
1990   TGTGTGGTGACAGGCAGGCTGTGACATATGAAATCCCTCCGGCAGCCTCTCTTC
2044   AAACCATTCTTCACACAGGCTTCAATGGAGAGTAATTTTCTCTTCCCGACTAAC
2098   GCTTGACTGATTGCACTTTAGATTTAAATACACTTTCTTGAAAGCCTTTTGGAA
2152   GCGACGCTTATCAATTTCAATGTTTATTTCAAATCTGCACATTACTA**AATAAA**A
2206   ACAAATCATCTCTTGCACAGTAAAAAAAAAAAAAAAAAAA
```

Figure 2.1 Nucleotide and deduced amino acid sequences of yellow perch PRL.

The nucleotides (lower row) are numbered on the left and the amino acids (upper row)
are numbered on the right and both begin with the initiation methionine. The signal
peptide is underlined and the four cysteine residues in the mature protein are shaded. The
putative polyadenylation signal is in bold and double underlined. Yellow perch PRL
cDNA sequence is available from the EMBL/GenBank database with accession
#AY332491.

```
                                          *         20        *         40
yellow_perch          : MAHR-KTNGSKIFMTVL-YMVAACRAVAINDLLDRASERS :  38
orange_spotted_grouper: ..Q.-HSD.N.LL....-......S..P.........Q.. :  38
silver_seabream       : ....-E.....L.II..-C.....G..P.........Q.. :  38
gilthead_seabream     : ....-E.....L.I...-C.....S..P.........Q.. :  38
black_seabream        : ..R.-E.....L.I...-C.....G..P.S.......Q.. :  38
red_seabream          : ....-G.......I...-CI....S..P.........Q.. :  38
European_sea_bass     : ..Q.-......L..M..-......S.IP.S.......Q.. :  38
four-spine_sculpin    : ...CGG.....F.IA.M-......GGIP.S.......Q.. :  39
three_spot_gourami    : ..Q.-G.....V.VA..-..A.V.G..HVS.......Q.. :  38
pufferfish            : ..D.-RPSSIVL....WGVALTV.GG.STG.......... :  39
Mozambique_tilapia    : ..Q.-R.S.TNL......-CV..M....P..E.FE...QH. :  38
Nile_tilapia          : .RQ.-R.S.TNL......-CV..M....P..E.FE...QH. :  38
bastard_halibut       : .T..-R.KLFMMAAV.S-.VMTS.G..P.........Q.. :  38
bluefin_tuna          : ....-G.....L.....-.T.T...S.IP.S.......QH. :  38
goldfish              : .T---QGSRLYFAVA..MCGFVSING.GL....E...QL. :  37
common_carp           : .T---QGSRLYFAV...MCAFVSING.GL....E...QL. :  37
noble_carp            : ..---EGSRLYFAV...MCAFVSING.GL....E...QL. :  37
zebrafish             : ..---QGSRQYFAVAI.MCAFVSING.GL........QL. :  37
Arctic_cisco          : ..R.SQGTK---LHLAVLCL.VS.H.IGLS..ME...Q.. :  37
chinook_salmon        : ..R.SQGTK---LHLAVLCL.VS.H.IGLS..ME...Q.. :  37
chum_salmon           : ..R.SQGTK---LHLAVLCL.VS.H.IGLS..ME...Q.. :  37
Atlantic_salmon       : ..R.SQGTK---LHLAVLCL.VS.H.IGLS..ME...Q.. :  37
rainbow_trout         : ..R.SQGTK---LHLAVLCL.VS.H.IGLS..ME...Q.. :  37
stinging_catfish      : ..RCC.CPRLHLAV...ACVLVFTEG.NL........QL. :  40
channel_catfish       : ..RCC.CPRLHLAV...ACVLVFTEG.NL........QL. :  40
Japanese_eel          : ..Q.F.GSN--L.L.A.LCLASQGH..GLG.M.E...QL. :  38
European_eel          : ..Q.F.GRS--L.L.A.LCLASQGY..GLG.M.E...QL. :  38


                                          *         60        *         80
yellow_perch          : DIMHSLSTTLSHDLDSQFPPIGRVM-PRPSMCHTASLQTP :  77
orange_spotted_grouper: .RL.............FN......MAM.......S..... :  78
silver_seabream       : .TL.......TK..SNHV..V.WTVM....L...S..... :  78
gilthead_seabream     : .ML.......TK..SNHV..V.WT.M....PL...S..... :  78
black_seabream        : .TL.......TQ..NNHI..V.WM.M....T...S..... :  78
red_seabream          : .TL.......TQ..NNHV....WM.M....L...S..... :  78
European_sea_bass     : .TL.......TQ....H...M...IT........S..H.. :  78
four-spine_sculpin    : .KL.......TQ....H.......IM..T.....S..... :  79
three_spot_gourami    : .KL.......TQEM..H...LS.AFM........S..M.. :  78
pufferfish            : .LI.....V..Q....HL.SL...HV........S..... :  79
Mozambique_tilapia    : .KL.......TQE...H.......IM...A....S..... :  78
Nile_tilapia          : .KL....S..TQE...H.......IM...A....S..... :  78
bastard_halibut       : .QL.......QE...H......IM........SA.... :  78
bluefin_tuna          : .KL.....I.TQE...H.......IT...A....S..E.. :  78
goldfish              : .KL.....S.TN....H...V...M........S...I. :  77
common_carp           : .KL.....S.TN....H...V...M........S...I. :  77
noble_carp            : .KL.....S.TN....H...V...M........S...I. :  77
zebrafish             : .KL.Y...S.TN....H......G.M....L...S...I. :  77
Arctic_cisco          : .KL.....S.TK....H...M...M.........S..... :  77
chinook_salmon        : .KL.....S.NK....H...M...M.........S...I. :  77
chum_salmon           : .KL.....S.TK....H...M...M.........S..... :  77
Atlantic_salmon       : .KL.....S.TK....H...M...M.........S..... :  77
rainbow_trout         : .KL.....S.TK....H...M...M.........S..... :  77
stinging_catfish      : .K......S.TN....H.SSV.GKLM-......S...I. :  79
channel_catfish       : .K......S.TN....H.SSV.GKLM-......S...I. :  79
Japanese_eel          : .KL.....S.TN...TH...M.KILM............. :  78
European_eel          : .KL.....S.TN...TH...M.KILM............. :  78
```

```
                                     *         100         *         120
yellow_perch            : IDKYQALQVSESDLLSLARSLLQAWEDPLAVLSTSANTLP : 117
orange_spotted_grouper: S..E.....................A...V.......... : 118
silver_seabream         : N..A....L.....M..........R....D..N...S.L : 118
gilthead_seabream       : N..E....L.....M..........Q...VD..N...S.L : 118
black_seabream          : N..E....L................Q...V...N...S.V : 118
red_seabream            : N..E.T..L................Q...VD......S.L : 118
European_sea_bass       : ...E......A.....V.......R...VI.......... : 118
four-spine_sculpin      : N..E............V...........M.......... : 119
three_spot_gourami      : N..E................S.V...L...V...S.. : 118
pufferfish              : M..E......K..............A...LF..N.VS... : 119
Mozambique_tilapia      : ...D........M..........S...V...S..S... : 118
Nile_tilapia            : ...D........M..........S...V...S..S... : 118
bastard_halibut         : N..T........E............A...SA..S..FS.. : 118
bluefin_tuna            : N..E......N..............A...V...A...S.. : 118
goldfish                : N..D...K.P.DE.........L..S....L..SE.SS.A : 117
common_carp             : N..D...KIP.DE.........L..S....L..SE.SS.A : 117
noble_carp              : N..D...K.P.DE.........L..S....L..SE.SS.A : 117
zebrafish               : N..D..MK.P.DE.........L..S....L..SE.SS.A : 117
Arctic_cisco            : K..E...R...NE.I.......L..N...LL..SE.P... : 117
chinook_salmon          : K..E...R...NE.I.......L..N...LL..SE.P... : 117
chum_salmon             : K..E...K...NE.I.......L..N...LL..SE.P... : 117
Atlantic_salmon         : K..E...K...NE.I.......L..N...LL..SE.P... : 117
rainbow_trout           : K..E...K...NE.I.......L..N...LL..SE.P... : 117
stinging_catfish        : N..D...S.P.GE....V....M..S....L..SE.TS.. : 119
channel_catfish         : N..D...S.P.GE....V....M..S....L..SE.TS.. : 119
Japanese_eel            : H..D...R.P..E......A..LS.N...LL.TSE.P..S : 118
European_eel            : H..D...R.P..E...I..A..LS.N...LL.ASE.P..S : 118


                                     *         140         *         160
yellow_perch            : HPAQSSISNKIQELQEHSKNLGDGLDILSGKMGPAAQTIA : 157
orange_spotted_grouper: .....T...........T.S.................L.S : 158
silver_seabream         : ..S........R.......S.................S : 158
gilthead_seabream       : ..S........R.......S...........V.....A.S : 158
black_seabream          : ..S........R.......S...........E..A.S : 158
red_seabream            : ..S.T......R.................R......A.S : 158
European_sea_bass       : ....N...T.V...L..T.S............F.....S.S : 158
four-spine_sculpin      : .....A.............Y.S....VV..D..A......S : 159
three_spot_gourami      : ..................T.D...............E..A.S : 158
pufferfish              : QT...NA.......KD...I......V..AR..Q...A.S : 159
Mozambique_tilapia      : .....T.F.....M.QY..S.K....V..S...SP..A.T : 158
Nile_tilapia            : .....T.F.....M.QY..S.K....V..S...SP..A.T : 158
bastard_halibut         : .......F..VR.M...................E...ALS : 158
bluefin_tuna            : ....N.....V...............N......E.M....S : 158
goldfish                : ..ERNT.DS.TK...DNINS..A..EHVFN..DSTSDNLS : 157
common_carp             : ..ERNT.DS.TK...DNINS..A..EHVFQ...SSSDNLS : 157
noble_carp              : ..ERNT.NS.TK...DNINS..A..ERVVH...SSSDNLS : 157
zebrafish               : ..ERNT.NS.TK...DNINS..A..EHVVH...SSSDNLS : 157
Arctic_cisco            : ..SNGD..S..R...DY..S.......VN....SS.Y.S : 157
chinook_salmon          : ..SNGD..S..R...DY..S.......VN....SS.Y.S : 157
chum_salmon             : ..SNGD..S..R...DY..S.....MVN....SS.Y.S : 157
Atlantic_salmon         : ..SNGD..S..R...DY..S.....MVN....SS.Y.S : 157
rainbow_trout           : ..SNGD..S..R...DY..S.....MVN....SS.Y.S : 157
stinging_catfish        : ..ERN..NT.TR...D.TST..A...R.VR...SSSESLS : 159
channel_catfish         : ..ERN..NT.TR...D.TNS..A..ER.GR...SSPESLS : 159
Japanese_eel            : ..QNGV.YS.TR...DQ.NS.SS...R.IH.I.SSSKSLS : 158
European_eel            : ..QNGA.YS.TR...DQ.NS.SS...R.IH.I.SSSKALS : 158
```

32

```
                                       *         180         *         200
yellow_perch          : SLPYRGGNDIGEDRISKLINFNFLLSCFRRDSHKIDSFLK : 197
orange_spotted_grouper: ...........Q......V................... : 198
silver_seabream       : .....AS......N....T..H................ : 198
gilthead_seabream     : ......S......N....T..H................ : 198
black_seabream        : ......S......S....T..H...............N : 198
red_seabream          : .........G...Q.T..H................... : 198
European_sea_bass     : .........SQ...R.T..H..M............... : 198
four-spine_sculpin    : L...S......Q.T..Q..................... : 199
three_spot_gourami    : M.........Q...T..A..H................. : 198
pufferfish            : ....T....L.Q.KL...V..H................ : 199
Mozambique_tilapia    : .......TNL.H.K.T..........L........... : 198
Nile_tilapia          : .......TNL.H.K.T..........L........... : 198
bastard_halibut       : ...F..-..V.Q.........H................ : 197
bluefin_tuna          : ......-.N..Q.E........................ : 197
goldfish              : ...FDI-.SL.Q.KT.R.V..H................ : 196
common_carp           : ...FYT-SSL.Q.KT.R.V..H................ : 196
noble_carp            : ...FYS-.SL.Q.KT.R.V..H................ : 196
zebrafish             : T..FN-.NL.Q.KT.R.V..H................. : 196
Arctic_cisco          : .I.FK.-G.L.N.KT.R....H..M............. : 196
chinook_salmon        : LI.FK.-G.L.N.KT.R....H..M............. : 196
chum_salmon           : .I.FK.-G.L.N.KT.R....H..M............. : 196
Atlantic_salmon       : .I.FK.-G.L.N.KT.R....H..M............. : 196
rainbow_trout         : .I.FK.-G.L.N.KT.R....H..M............. : 196
stinging_catfish      : ...FN.-..L.Q.NV.R.V..H................ : 198
channel_catfish       : ...FNS-..L.Q.N..R.V..H................ : 198
Japanese_eel          : P..FQ-G.L.S.KN.R....Y............N... : 197
European_eel          : P..LQ-G.L.S.KN.R....Y............N... : 197


                                   *
yellow_perch          : ILRCRAAKMQPEMC- : 211
orange_spotted_grouper: V.......LR...- : 212
silver_seabream       : V.......LR...- : 212
gilthead_seabream     : V.......V....- : 212
black_seabream        : V.......L....- : 212
red_seabream          : V.......V....- : 212
European_sea_bass     : V.......L....- : 212
four-spine_sculpin    : ........Q.L...- : 213
three_spot_gourami    : V.......I...L.- : 212
pufferfish            : V....M.N.L...- : 213
Mozambique_tilapia    : V............- : 212
Nile_tilapia          : V............- : 212
bastard_halibut       : V......NT....- : 211
bluefin_tuna          : V.........QL.- : 211
goldfish              : V......KR....- : 210
common_carp           : V......KR....- : 210
noble_carp            : V......KR....- : 210
zebrafish             : V......KR.D..- : 210
Arctic_cisco          : V....T..R..T.- : 210
chinook_salmon        : V....T..R..T.- : 210
chum_salmon           : V....T.IR..T.- : 210
Atlantic_salmon       : V....T..R..T.- : 210
rainbow_trout         : V....T..R..A.- : 210
stinging_catfish      : V.......L.D..- : 212
channel_catfish       : V...D....L....- : 212
Japanese_eel          : L......Q--DR.- : 209
European_eel          : L......Q--DR.- : 209
```

Figure 2.2 Alignment of yellow perch prePRL deduced amino acid sequence with other teleost prePRLs.

Conserved amino acid residues are indicated with a (.), inserted gaps are indicated with a (-), and conserved cysteine residues in the mature peptide are shaded. Sequences were downloaded from the EMBL/GenBank database with the following accession numbers: orange spotted grouper (*Epinephelus coioides*) AAO11695; silver seabream (*Rhabdosargus sarba*) ABB17072; gilthead seabream (*Sparus aurata*) CAD52820; black seabream (*Acanthopagrus schlegelii*) AAX21764; red seabream (*Pagrus major*) BAE43854; European sea bass (*Dicentrarchus labrax*) CAA55369; four-spine sculpin (*Cottus kazika*) BAE19673; three spot gourami (*Trichogaster trichopterus*) AAX09323; pufferfish (*Tetraodon nigroviridis*) AAR25696; Mozambique tilapia (*Oreochromis mossambicus*) CAA63124; Nile tilapia (*Oreochromis niloticus*) AAA53281; bastard halibut (*Paralichthys olivaceus*) AAD15746; bluefin tuna (*Thunnus thynnus*) BAE45636; goldfish (*Carassius auratus*) AAT74865; common carp (*Cyprinus carpio*) CAA37063; noble carp (*Hypophthalmichthys nobilis*) CAA43383; zebrafish (*Danio rerio*) AAH92358; Arctic cisco (*Coregonus autumnalis*) CAA80660; chinook salmon (*Oncorhynchus tshawytscha*) AAB28216; chum salmon (*Oncorhynchus keta*) CAA45407; Atlantic salmon (*Salmo salar*) CAA59258; rainbow trout (*Oncorhynchus mykiss*) AAA49611; stinging catfish (*Heteropneustes fossilis*) AAK53436; channel catfish (*Ictalurus punctatus*) AAF82287; Japanese eel (*Anguilla japonica*) AAO17792; European eel (*Anguilla anguilla*) CAA48902.
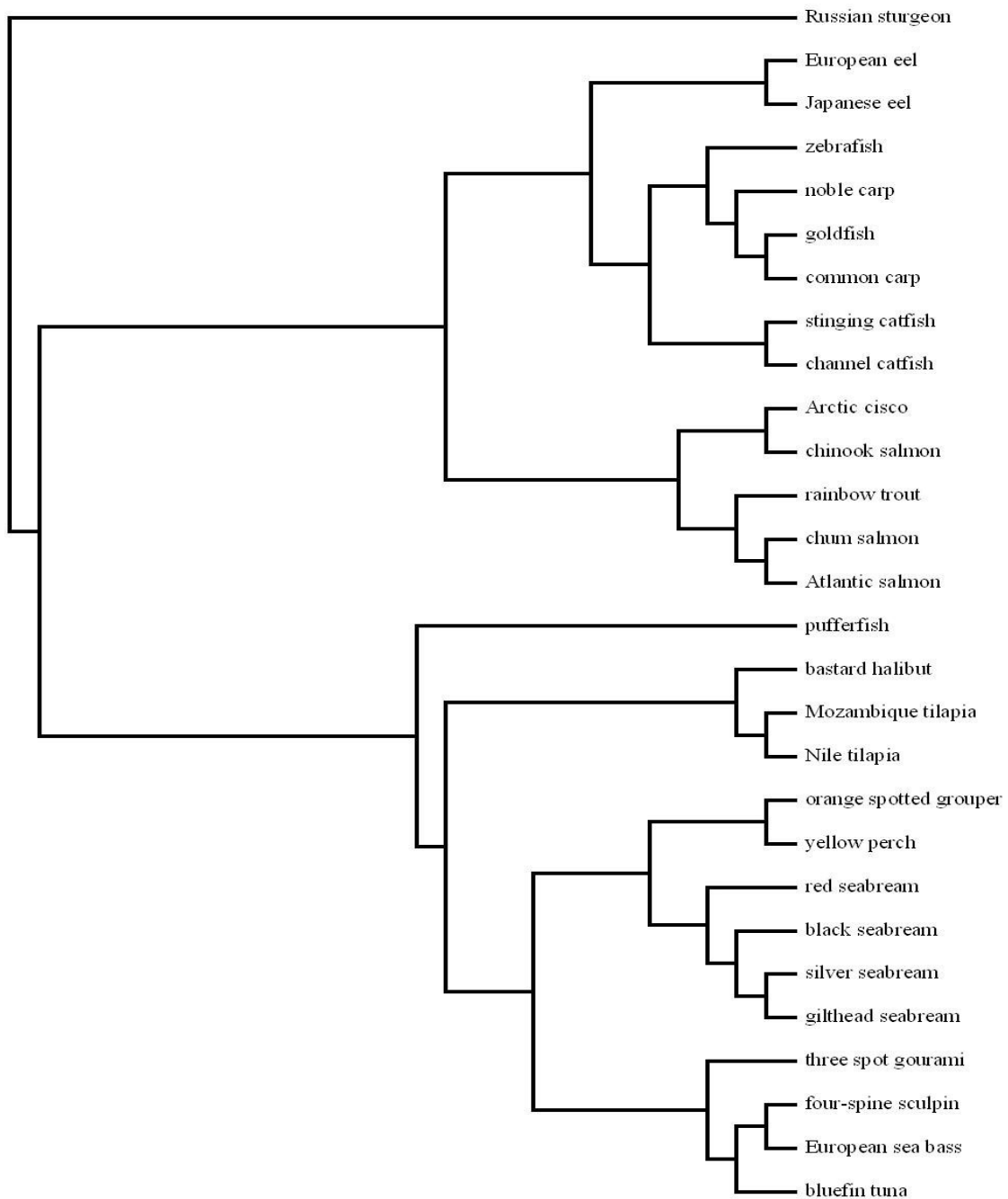
Figure 2.3 Phylogenetic tree of mature PRL amino acid sequences.

Tree was produced using a Clustal X (1.81) alignment and TreeView (Win 32) v. 1.6.6. The tree was rooted with Russian sturgeon (*Acipenser gueldenstaedtii*) PRL (Accession #AAB28396) as an outgroup representing the closest available species not in the group Teleostei. See Figure 2.2 for EMBI/GenBank accession numbers for the other sequences used.

```
                                        M   H   M   V   T   V   M   Q   Q      9
  -27  AAGAAGGACTTGAAGACTACTGACAGAATGCACATGGTGACAGTCATGCAGCAG

         G   V   W   A   V   L   L   L   P   Y   L   L   T   V   S   I   P   L     27
   28  GGTGTATGGGCTGTCTTGCTCTTGCCCTATCTTCTTACCGTAAGCATCCCGCTA

         D   C   R   E   E   Q   G   S   L   S   R   C   P   S   I   S   Q   E     45
   82  GACTGTAGGGAGGAGCAGGGTAGCCTCTCCCGCTGCCCTTCCATCTCCCAAGAG

         K   L   L   D   R   V   I   Q   H   A   E   L   I   Y   R   V   S   E     63
  136  AAACTCCTAGACCGAGTCATCCAGCATGCTGAGCTAATCTACCGTGTCTCAGAA

         E   S   C   S   L   F   E   Q   M   F   I   P   F   P   L   Q   L   Q     81
  190  GAATCATGCTCTTTGTTTGAGCAGATGTTTATCCCGTTCCCATTGCAGCTCCAG

         R   N   Q   A   G   Y   A   C   I   T   K   A   L   P   I   P   S   S     99
  244  AGGAACCAAGCAGGCTATGCATGCATCACAAAAGCTTTACCCATCCCCAGCTCC

         K   G   E   I   Q   Q   I   S   D   K   W   L   L   H   S   V   L   M    117
  298  AAAGGTGAAATCCAACAGATATCTGACAAATGGTTGCTCCACTCTGTGTTGATG

         L   V   Q   S   W   I   E   P   L   V   Y   L   Q   T   S   L   D   R    135
  352  CTCGTCCAGTCGTGGATCGAGCCTTTGGTCTACCTGCAGACCTCACTGGATCGC

         Y   D   A   A   P   D   M   L   L   N   K   T   K   W   V   S   E   K    153
  406  TACGATGCCGCTCCTGACATGCTGCTCAACAAGACCAAGTGGGTGTCTGAGAAA

         L   I   S   L   E   Q   G   V   V   V   L   I   K   K   M   L   D   E    171
  460  CTGATCAGTCTGGAGCAAGGCGTTGTGGTCCTCATCAAGAAGATGTTGGATGAG

         G   M   L   T   A   T   R   S   E   Q   G   L   F   Q   Y   D   V   Q    189
  514  GGAATGTTGACCGCAACCCGCAGTGAACAGGGCCTATTCCAGTATGATGTGCAG

         P   E   M   L   E   S   V   M   R   D   Y   T   L   L   S   C   F   K    207
  568  CCAGAGATGCTGGAATCTGTGATGAGAGACTATACCTTACTCAGCTGCTTCAAG

         K   D   A   H   K   M   E   T   F   L   K   L   L   K   C   R   Q   T    225
  622  AAAGATGCCCATAAGATGGAGACTTTCCTAAAGCTACTGAAATGTCGGCAAACT

         D   L   Y   N   C   A   *                                             231
  676  GACTTATACAACTGTGCATAAGACATGAAGCGAAATGTTTAAATAATACAGCTT

  730  TAAATGAATTCCTAAGGTTGGTAGCCGTGCACTTAAAGACATGACCATGCCTTA
  784  GGCGATTCAGCCTTGCTTGAATTGCAGTACATTCTTTATTGATTGTTTTGGAAC
  838  ACCTTCACACAAACCTAGTAGGTGTAATGCTGTGCCCTTTCTACAACACTGCAT
  892  TTTATATTTCCCTTCTCACTTGTTTTTTAACCTGGCAAAGGCAACAGAGGGCAA
  946  ACTCCCAAAAGATTAGTTGCGTGTCGAGCTGTCAAAAAAATCTGCATATCCTAC
 1000  GATTGATTTCCATTTCCTTTGTTCTTAACTGGAGTTTGTATTCCTCGCTGGCTC
 1054  TTGCAGTGTTTTGATTATTCCCACGGCCCCCAGAGAATTCAGTGAGACGGTTTC
 1108  ACTTCTGCATTAGTGAAATGAAACACTTTCACCGGAGATGGGAGTCAAGCAGAG
 1162  AGCAATCACTACTTTAAATAAGACACACATTTTGATTGGTGTAAGAGAGTGTGA
```

36

```
1216    GGGAAACAGTGAGAGGGAACAAATAACAGTTAAAATGAGGGGTAATGCTTATTT
1270    GGAGTTTAAGGTTGGCGCTCATGACAGAATCAGGAATATTCCCATGCCTAAATA
1324    TGCCTAATTTAACCTAAATATAATATAATAACTTATTCACTTGAAATGATAAAA
1378    GGAGATACATCTTAGATTTGAATTGTTAGATGTTATGAATATGTAATGCAGAAA
1432    TCAAACTATTTTTGCAATCTTTTGTGTAAATAGAAGAACCCCAATGTACAGTGG
1486    AAACCTTTTTAATGGACTGCTGATTTAAGGCACT**AATAAA**GCAAATTGTAGAAT
1540    AAAAAAAAAAAAAAAAAAAAAAA
```

Figure 2.4 Nucleotide and deduced amino acid sequences of yellow perch SL cDNA.
The nucleotides (lower row) are numbered on the left and the amino acids (upper row)
are numbered on the right and both begin with the initiation methionine. The signal
peptide is underlined and the seven cysteine residues in the mature protein are shaded.
The putative polyadenylation signal is in bold and double underlined and a putative
glycosylation site is in bold starting at nucleotide 433. Yellow perch SL cDNA sequence
is available from the EMBL/GenBank database with accession # AY332490.

```
                                         *        20        *        40
yellow_perch           : MHMVTVMQQ-GV-WAVLLLPYLLTVSIPLDCREEQGSLSR :  38
orange-spotted grouper:  ........R-..-.PL..W.F.R........Q.....P.. :  38
lumpfish               : ..L.S.I.R-..-.....W.N..AS.V............I... :  38
bluefin_tuna           : .N.M......-.I-...S.W...........K........ :  38
red_drum               : .Y.M.AL.R-..-..S..W....I.I......K........ :  38
rabbitfish             : .L.F.AI.R-..-.VA..W.H...A.M........N.N... :  38
red_seabream           : ...MRAIK.-.Q-.....W....AI.......D...G... :  38
black_seabream         : ---MRAIK.-.Q-.....W....IT.......D...G... :  35
gilthead_seabream      : .R.MRAIK.-.Q-..I..W.....T.......D...G... :  38
sole                   : --.M.AVK.S..-.....W....A...Q....D...NM.. :  37
Atlantic_halibut       : .N.M..K.--..-..A..W....AA......KD....F.A :  37
bastard_halibut        : .N.M..K...-..-..A..W....A......K........ :  38
Japanese_medaka        : --.H.KVL.Q.L-..L..W.H.F...V....DD.A..A. :  37
pufferfish             : ---MAAL.E--.LL....W.V.V.I.N.IN.GD..S...N :  35
spotted_green_puffer   : ---M.AL..SLL--....W.V.F...N.INAG...ST..N :  35
Atlantic_cod           : ..TLAAVVLQ.C..AV.W.CPP.H.S.V......AGS.Q :  40
chum_salmon            : .N.MQ...SV-.-.....W.C.VSLGV..E.KD....IIL :  38
rainbow_trout          : .N.MQ...SV-.-.....W.C.VSLGV.VE.KD....IIL :  38
European_eel           : --.FSIRMNKVLQGF.C.MLTHRI.GY.M..K.D.DG-T. :  37
goldfish               : ---MKKTTVLQ.CMVFVVCSLQAVIGS.V..PDQDTAGVS :  37
zebrafish α            : --.N..KVLQVCVCVLI.GQF.VSGAV....KDDA..--. :  36
channel_catfish        : ---MIKTKVLQAWMGIW.CAVNGLLGSDQ..SDRDPTG.. :  37

                                         *        60        *        80
yellow_perch           : CPSISQEKLLDR---VIQHAELIYRVSEESCSLFEQMFIP :  75
orange-spotted grouper:  ............---...............K......E.... :  75
lumpfish               : ............---..E.............Y.D.... :  75
bluefin_tuna           : ............---...............E..V. :  75
red_drum               : ............---...............E..V. :  75
rabbitfish             : ..T.........---...............E..V. :  75
red_seabream           : ............---...............E.... :  75
black_seabream         : ............---...............E.... :  72
gilthead_seabream      : ............---...............E.... :  75
sole                   : ..F.........---I.......S.I.......EL.V. :  74
Atlantic_halibut       : ............---.............M..E..V. :  74
bastard_halibut        : ............---.............M..E..V. :  75
Japanese_medaka        : ............---..H..............E.... :  74
pufferfish             : .L...E......ESFSTLSSSIVFLKNHVL.LRRCLSHSQ :  75
spotted_green_puffer   : .L...E......---...............A.... :  72
Atlantic_cod           : ..T.........---....T..........M..D..V. :  77
chum_salmon            : .A...K......---..............T...E..V. :  75
rainbow_trout          : .A...K......---..............T...E..V. :  75
European_eel           : .....LD.....---I..............T...E.Y.. :  74
goldfish               : .-I..L.....E.---AV......HHIA...KL..DE.L.S :  73
zebrafish α            : .A..........---..............C.T...D..V. :  73
channel_catfish        : .-...V......---A.........I.D.ART...E.... :  73
                         ▶-------------A_SL-------------◀
```

```
                                        *     100       *      120
yellow_perch         : FPLQLQRNQAGYACITKALPIPSSKGEIQQISDKWLLHSV : 115
orange-spotted grouper: .Q..............L....S.............. : 115
lumpfish             : --..F....V......T..V....N.............. : 113
bluefin_tuna         : ...R........T.........S................ : 115
red_drum             : .S.................F....S.............. : 115
rabbitfish           : .........FT......A......S.............. : 115
red_seabream         : ..........P............S.............. : 115
black_seabream       : ..........P............S.............. : 112
gilthead_seabream    : ..........P............S.............. : 115
sole                 : ...R....TV...........S.............Q.. : 114
Atlantic_halibut     : ...R..................S......T....... : 114
bastard_halibut      : ...R..................S......T...... : 115
Japanese_medaka      : L..R..S...G......M.......S....L....... : 114
pufferfish           : YSSRQA.QE--N..M..V.......S.....T......A. : 113
spotted_green_puffer : --V..PT....N..M..G.......S.....T...... : 110
Atlantic_cod         : ..VR.......NT....DF...T..N.L.....T..... : 117
chum_salmon          : ..MRS.......T.A...F...G..S............. : 115
rainbow_trout        : ..MRS.......T.A...F...G..S............. : 115
European_eel         : SSIRA.LSRG.N..S.RSV..---Q.R............T : 111
goldfish             : .GVVNLHISE.TM.SP.TVSV.M..T............. : 113
zebrafish α          : Y..HVLI....NT.HS.HI...T..S.......S...... : 113
channel_catfish      : LLIPAHQVHG.NS.TSNLVRV.I..L.............I : 113
                                            ▶--------------B_SL---
```

```
                                        *     140       *      160
yellow_perch         : LMLVQSWIEPLVYLQTSLDRYDAAPDMLLNKTKWVSEKLI : 155
orange-spotted grouper: ..............P........................ : 155
lumpfish             : ....................N...E.............. : 153
bluefin_tuna         : ................T.....D................ : 155
red_drum             : ........G..A...NTM.H..G................ : 155
rabbitfish           : ...............NT.....G................ : 155
red_seabream         : ................T.....GV............... : 155
black_seabream       : ...............TMN...GV...............M : 152
gilthead_seabream    : ...............T.N...GV...........D..M : 155
sole                 : .T.............T.....N...V...........V : 154
Atlantic_halibut     : .L......D......T.....N.SE.........D... : 154
bastard_halibut      : ...............T.....N...........D... : 155
Japanese_medaka      : ...............MT.....H................ : 154
pufferfish           : .......K......TMV...Y.S.V.........L.... : 153
spotted_green_puffer : .......K...H...TMVH..D.S.V....I...L.... : 150
Atlantic_cod         : ...............T.....DV..V.......M..... : 157
chum_salmon          : .I.............T.....D...T..K.........L : 155
rainbow_trout        : .I.............T.....D...T..K.........L : 155
European_eel         : .VVI...TG..QS..ITM.L..N...G......M.T..M : 151
goldfish             : .I...F..N...DV.A..MN.QN..SA.VDRS.LM.T.IT : 153
zebrafish α          : .F.....M...L....T.....D..NA..S......D..L : 153
channel_catfish      : SI...V.....AD..D...M..NV.SS.IS..R.M.T..M : 153
                       ----------◄           ▶-------------
```

```
                                  *      180         *     200
yellow_perch          : SLEQGVVVLIKKMLDEG-MLTATRSEQGLFQYDVQ-PEML : 193
orange-spotted grouper: ..................-....NH...........-.... : 193
lumpfish              : ..................-...INH.....L.NG..-.Q.. : 191
bluefin_tuna          : ...P..............-...T.N...........-.... : 193
red_drum              : ..................-I..T.Y.........E..-.D.. : 193
rabbitfish            : ...............-.A.TAYN..S...D.A.-.D.. : 193
red_seabream          : ................-.M.T.Y...S...D.G.-.... : 193
black_seabream        : ................-.M.T.YN......D.G.-.... : 190
gilthead_seabream     : ......A.........-LM.T.Y......D.G.-.... : 193
sole                  : .........R......-T..T.YN..D.L....L-.D.. : 192
Atlantic_halibut      : .........R......-....YN.........L-.D.. : 192
bastard_halibut       : .........R......-....YN........A.-.D.. : 193
Japanese_medaka       : ...............-AM.T.Y....A.......-L... : 192
pufferfish            : .......I....I.N.A-VM.T.V..LD..PT.L.-.DI. : 191
spotted_green_puffer  : .............N.A-VT.T.V..LD.LPT.L.-LDI. : 188
Atlantic_cod          : .........R....GA-I.NSSYN.YSAV.L...-..V. : 195
chum_salmon           : .........R....DD-...TSYY...VAP.AL.-..V. : 193
rainbow_trout         : .........R....DD-...NSYY...VAP.AL.-..V. : 193
European_eel          : N.....T...R...N.D--ILVSDPS.N.THFAT.-.N.V : 188
goldfish              : .....IL...RQI.G..--GLVVEGPEDTSDHF.S-SDTF : 190
zebrafish α           : .........R......I.ASS.IFDHTQSP..G.F..V. : 193
channel_catfish       : N.K...L...MS......--SVELENNESMLRHI.A-.A.A : 190
                        --C_SL--------◄
```

```
                                  *      220         *     240
yellow_perch          : ESVMRDYTLLSCFKKDAHKMETFLKLLKCRQTDLYNCA-- : 231
orange-spotted grouper: D........F..............A............K...-- : 231
lumpfish              : .....................A.............R...S-- : 229
bluefin_tuna          : ......................L..........I....-- : 231
red_drum              : ....K..N..............IL.........I...P-- : 231
rabbitfish            : ......................IL.......N.I.S..-- : 231
red_seabream          : ......................IL.......N.I.S..-- : 231
black_seabream        : .Y....................IL.......N.IHS..-- : 228
gilthead_seabream     : .Y....................IL.......N.IHS..-- : 231
sole                  : .......................I..........KF...-- : 230
Atlantic_halibut      : .......................I..........K...P-- : 230
bastard_halibut       : .......................I..........K...-- : 231
Japanese_medaka       : .Y........T.L...................K...-- : 230
pufferfish            : ....N..S..........R.I.IL.......RN.M...-- : 229
spotted_green_puffer  : ..I.N.HN..............IL.......RN.M...-- : 226
Atlantic_cod          : ..IL...NV.C.........I..I.......I.K....LY : 235
chum_salmon           : ...L..........................K.S.FLH : 233
rainbow_trout         : ...L..........................K.S.FLH : 233
European_eel          : ...LT.....T..R....RV..........S.RLS.FLY : 228
goldfish              : .T.R...SVIY..R.....IQ.L.......I.KE..SLF : 230
zebrafish α           : ...I...H..T.....T.............SNKLS.LPQ : 233
channel_catfish       : .H.L...AV.....................R.....NPT.SLF : 230
                        ►-----------------D_SL----------------◄
```

Figure 2.5 Alignment of yellow perch preSL deduced amino acid sequence with other teleost preSLs.

Conserved amino acid residues are indicated with a (.) and inserted gaps are indicated with a (-). The seven mature peptide cysteines and the potential glycosylation site are

highlighted. Four conserved domains (A_{SL}, B_{SL}, C_{SL}, D_{SL}) identified by Company *et al.* 2000, are indicated at the bottom of the alignment in bold. Sequences were downloaded from the EMBL/GenBank database with the following accession numbers: orange-spotted grouper (*Epinephelus coioides*) AAN18040; lumpfish (*Cyclopterus lumpus*) AAC38004; bluefin tuna (*Thunnus thynnus*) BAE45637; red drum (*Sciaenops ocellatus*) AAD17534; rabbitfish (*Siganus guttatus*) BAA83467; red seabream (*Pagrus major*) BAE43855; black seabream (*Acanthopagrus schlegelii*) AAU43768; gilthead seabream (*Sparus aurata*) CAA72031; sole (*Solea senegalensis*) AAA61873; Atlantic halibut (*Hippoglossus hippoglossus*) AAC38003; bastard halibut (*Paralichthys olivaceus*) AAA49444; Japanese medaka (*Oryzias latipes*) AAT58046; pufferfish (*Tetraodon miurus*) AAF64522; spotted green puffer (*Tetraodon nigroviridis*) AAR25695; Atlantic cod (*Gadus morhua*) BAA01486; chum salmon (*Oncorhynchus keta*) BAA01485; rainbow trout (*Oncorhynchus mykiss*) Yang *et al.* 1997; European eel (*Anguilla anguilla*) AAB53035; goldfish (*Carassius auratus*) AAC60098; zebrafish α (*Danio rerio*) AAR25212; channel catfish (*Ictalurus punctatus*) AAF78945.
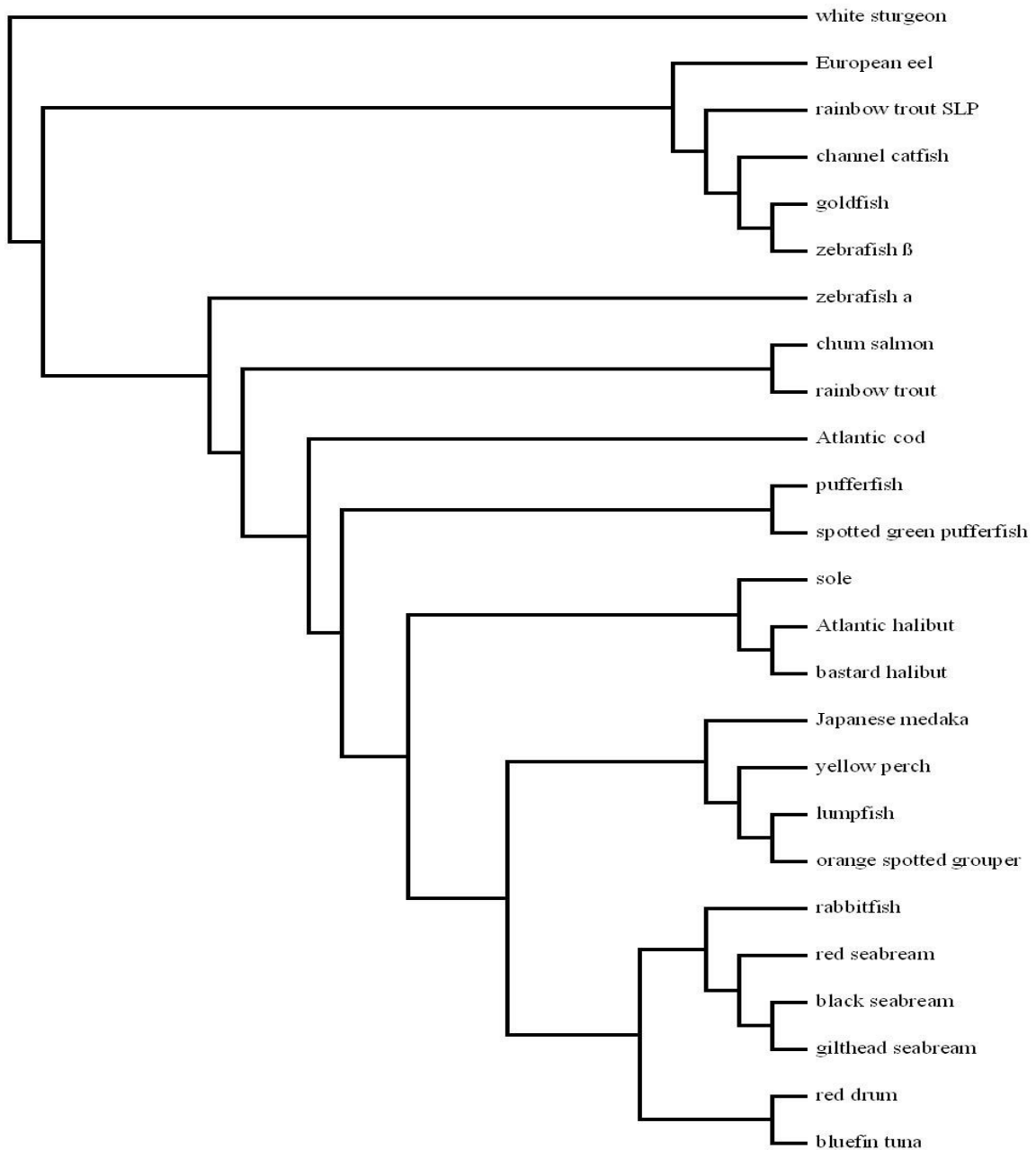
Figure 2.6 Phylogenetic tree of mature SL amino acid sequences.

Tree was produced using a Clustal X (1.81) alignment and TreeView (Win 32) v. 1.6.6. The tree was rooted with white sturgeon (*Acipenser transmontanus*) SL (Accession: BAA32608) as an outgroup representing the closest available species not in the group Teleostei. See Figure 2.5 for EMBI/GenBank accession numbers except: zebrafish β (*Danio rerio*) NP_001032763; rainbow trout SLP (*Oncorhynchus mykiss*) Yang and Chen 2003 [95].

```
-147  -CACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTCGGAATATTGAGATGT
 -94  GACATTGCCCGCATCTCATCCTCTTTCTCCCCGTTTTAATGACTTCAAACAAGT

                                              ►Signal peptide
                                               M   S   S   A   L      5
 -40  TCATTTTCGCCGGGCTTTGTCTTGCGGAGACCCGTGGGGATGTCTAGCGCTCTT

      (1-132)
       S   F   Q   W   H   L   C   D   V   F   K   S   A   M   C   C   I   S    23
  16  TCCTTTCAGTGGCATTTATGTGATGTCTTTAAGAGTGCGATGTGCTGTATCTCC

       C   S   H   T   L   S   L   L   L   C   V   L   T   L   T   P   T   A    41
  70  TGTAGCCACACCCTCTCACTACTGCTGTGCGTCCTCACCCTGACTCCGACGGCA

              ►B domain  (133-219)
       T   G   A   G   P   E   T   L   C   G   A   E   L   V   D   T   L   Q    59
 124  ACAGGGGCGGGCCCAGAGACCCTGTGCGGGGCGGAGCTGGTCGACACGCTGCAG

                                              ►C domain
       F   V   C   G   E   R   G   F   Y   F   S   K   P   T   G   Y   G   P    77
 178  TTTGTGTGTGGAGAGAGAGGCTTTTATTTCAGTAAACCAACAGGCTATGGCCCC

      (220-249)              ►A domain  (250-312)
       N   A   R   R   S   R   G   I   V   D   E   C   C   F   Q   S   C   E    95
 232  AATGCACGGCGGTCACGTGGCATTGTGGACGAGTGCTGCTTCCAAAGCTGTGAG

                             ►D domain  (313-336)          ►E
       L   R   R   L   E   M   Y   C   A   P   A   K   T   S   K   A   A   R   113
 286  CTGCGGCGCCTGGAGATGTACTGTGCACCTGCCAAGACTAGCAAGGCTGCTCGC

      domain  (337-558)
       S   V   R   A   Q   R   H   T   D   M   P   R   A   P   K   V   S   T   131
 340  TCTGTGCGTGCACAGCGCCACACAGATATGCCGAGAGCACCTAAGGTTAGTACC

       A   G   H   K   V   D   K   G   T   E   R   R   T   A   Q   Q   P   D   149
 394  GCAGGGCACAAAGTGGACAAGGGCACAGAGCGTAGGACAGCACAGCAGCCAGAC

       K   T   K   N   K   K   R   P   L   P   G   H   S   H   S   S   F   K   167
 448  AAGACAAAAAACAAGAAGAGACCTTTACCTGGACATAGTCATTCATCCTTCAAG

       E   V   H   Q   K   N   S   S   R   G   N   T   G   G   R   N   Y   R   185
 502  GAAGTTCATCAGAAAAACTCAAGTCGAGGCAACACTGGGGGCAGAAATTACCGA

       M   ***                                                             186
 556  ATGTAGGGAAAGAAGGAATGGACAAATGCCCAGCGACTTGGGAAGAGAGAAGGG
 610  AGTGGCCTTACCTGGTACCCCTGTGGAATGGTTCACTGTAAAAAAAAAAAAAAA
 664  AAAA
```

Figure 2.7 Nucleotide and deduced amino acid sequences of yellow perch IGF-Ib cDNA.

The nucleotides (lower row) are numbered on the left and the amino acids (upper row) are numbered on the right and both begin with the initiation methionine. The signal peptide and the five structural domains (B, C, A, D & E) are indicated above the amino acids with the respective nucleotide ranges in parentheses. In the A and B domains of the mature peptide the conserved cysteines are shaded, the conserved residues for binding IGF-I receptors are underlined and the conserved residues for binding IGFBP are in bold. Yellow perch IGF-Ib cDNA sequence is available from the EMBL/GenBank database with accession #AY332492.

```
                                         *         20         *         40
yellow_perch           : ----------------MSSALSFQWHLCDVFKSAMCCIS :   23
Eurasian_perch         : ----------------....................... :   23
bastard_halibut        : ----------------....................... :   23
shi_drum               : ----------------....................... :   23
black_sea_bream        : ----------------....................... :   23
gilthead_seabream      : ----------------....................... :   23
yellowfin_seabream     : ----------------....................... :   23
four-spine_sculpin     : ----------------....................... :   23
short-horned_sculpin   : ----------------....................... :   23
orange-spotted_grouper: ----------------....................... :   23
rabbitfish             : ----------------....................... :   23
European_sea_bass      : ----------------...............G...... :   23
flathead_mullet        : ----------------....................... :   23
Mozambique_tilapia     : ----------------....................... :   23
Chinook_salmon         : ----------------...GHF...............V. :   23
chum_salmon            : ----------------...GHF...............V. :   23
rainbow_trout          : ----------------...GHF...............V. :   23
coho_salmon            : ----------------...GHL................. :   23
fathead_minnow         : ----------------...GHF..G.W..A..CT.R.L. :   23
goldfish               : ----------------...IHF..G.W.....CT.R.L. :   23
triangular_bream       : ----------------...GHF..G.W.....CT.R.L. :   23
giant_danio            : ----------------...GHF.LG.W......T.R.L. :   23
bluntsnout_bream       : ----------------...GHF..G.W.....CT.R.L. :   23
zebrafish              : ----------------...GHF..G.W.....CT.R.LP :   23
scale-less_car         : MTSNKFFFAGLLLETQG...GHF..G.W.....CT.H.L. :   40
mud_carp               : ----------------...GHF..G.W.....CT.R.LP :   23
common_carp            : ----------------...GHF..G.W.....CT.R.L. :   23
barbodes               : ----------------...GHF..G.W.....RT.H.L. :   23
channel_catfish        : ----------------..RGHL-----G..L.WT.R.V. :   18
```
▶**Signal peptide**

45

```
                                      *         60         *         80
yellow_perch          : ---CSHTLSLLLCVLTLTPTATGAGPETLCGAELVDTLQF :  60
Eurasian_perch        : ---.....................G..............  :  60
bastard_halibut       : ---.....................G...........P.. :  60
shi_drum              : ---.....................G..............  :  60
black_sea_bream       : ---.....................S..............  :  60
gilthead_seabream     : ---.....................S..............  :  60
yellowfin_seabream    : ---.....................S..............  :  60
four-spine_sculpin    : ---.................R...G..............  :  60
short-horned_sculpin  : ---..............SL...R.G..............  :  60
orange-spotted_grouper: ---.....................G..............  :  60
rabbitfish            : ---..........I..........G..............  :  60
European_sea_bass     : ---.....................G..............  :  60
flathead_mullet       : ---.....................G..............  :  60
Mozambique_tilapia    : ---.....................G..............  :  60
Chinook_salmon        : ---.T.............SA....G..............  :  60
chum_salmon           : ---.T.............SA....G..............  :  60
rainbow_trout         : ---.T.............SA....G..............  :  60
coho_salmon           : ---.T.............SA....G..............  :  60
fathead_minnow        : ---.T.....V....A...ATLE.G..............  :  60
goldfish              : ---.T..........A...ATLE.G..............  :  60
triangular_bream      : ---.T.....V....A...ATLE.G..............  :  60
giant_danio           : ---.T.....V....A...ATLE.G..............  :  60
bluntsnout_bream      : ---.T.....V.F..A...ATLE.G..............  :  60
zebrafish             : ---ST.....V....A...ATLE.G..............  :  60
scale-less_car        : ---.T.....V....A...ATLE.G..............  :  77
mud_carp              : ---.T.....V....A...ATLE.G..............  :  60
common_carp           : ---.T.....V....A...ATLE.G..............  :  60
barbodes              : ---ST.....V....A...ATLE.G..............  :  60
channel_catfish       : RGRALAR.L....A.A...V.AR.G..............  :  58
                                             ▶B domain
```

46

```
                                    *        100        *       120
yellow_perch        : VCGERGFYFSKPTGYGPNARRS--RGIVDECCFQSCELRR :  98
Eurasian_perch      : ...................................--.................... :  98
bastard_halibut     : ...................................--.................... :  98
shi_drum            : ...................................--.................... :  98
black_sea_bream     : ..............-...............--.................... :  97
gilthead_seabream   : ..............-...............--.................... :  97
yellowfin_seabream  : ..............-...............--.................... :  97
four-spine_sculpin  : ..............-...............--.................... :  97
short-horned_sculpin: ...............G...............--.................... :  98
orange-spotted_grouper: ................V...--.................... :  98
rabbitfish          : ................S..P--.................... :  98
European_sea_bass   : ...............................--.................... :  98
flathead_mullet     : ....D..........................--.................... :  98
Mozambique_tilapia  : ..........N......S.....--..............Q. :  98
Chinook_salmon      : .................SS...HN................. : 100
chum_salmon         : .................SS...HN................. : 100
rainbow_trout       : .................SS...HN................. : 100
coho_salmon         : .................SS...HN................. : 100
fathead_minnow      : ....D.....N..A...S.S...NNY................. : 100
goldfish            : ....D.........S...HN................. : 100
triangular_bream    : ....D.........SS...HN................. : 100
giant_danio         : ....D.........SS...HN................. : 100
bluntsnout_bream    : ....D.........SS...HN................. : 100
zebrafish           : ....D.........SS...HN................. : 100
scale-less_car      : ....D.........SS...HN................. : 117
mud_carp            : ....D.........SS...HN................. : 100
common_carp         : ....D.........SS...HN................. : 100
barbodes            : ....D.........SS...HN................. : 100
channel_catfish     : ....D.........S..LHN...............K. :  98
                                          ►C domain   ►A domain
```

47

```
                                        *         140          *          160
yellow_perch          : LEMYCAPAKTSKAARSVRAQRHTDMPRAPKVSTAGHKVDK : 138
Eurasian_perch        : ......................................... : 138
bastard_halibut       : ......................................... : 138
shi_drum              : ............................E.......GN. : 138
black_sea_bream       : ......................................... : 137
gilthead_seabream     : ......................................... : 137
yellowfin_seabream    : ......................................... : 137
four-spine_sculpin    : ......P...............--......S....... : 135
short-horned_sculpin  : ......P...............--......A....... : 136
orange-spotted_grouper: ...N..................................... : 138
rabbitfish            : ........................T....A..Q.... : 138
European_sea_bass     : ........G................................ : 138
flathead_mullet       : .......N.SV....S........T............. : 138
Mozambique_tilapia    : ...V..P.IS....S...........SR---AN. : 135
Chinook_salmon        : ..V.SG...............T......VQN..R : 140
chum_salmon           : ..V.SG...............T...I...VQN..R : 140
rainbow_trout         : ..V.SG...............T......VQS..R : 140
coho_salmon           : ..V.SG...............T......VQN..R : 140
fathead_minnow        : ..V..G.TP...L.......IT.TA.---------- : 130
goldfish              : ..V.PG.TP...L.......GT.TA.---------- : 130
triangular_bream      : ..V..G.TP...L.......IT.TA.---------- : 130
giant_danio           : ..V..G.TP...L.......I..TA.---------- : 130
bluntsnout_bream      : ..V..G.TP...L.......IT.TA.---------- : 130
zebrafish             : ..V..G.SP...L.......I..T..---------- : 130
scale-less_car        : ..V.PG.SP...L.......S..TA.---------- : 147
mud_carp              : ..V.PG.TP...I.......S.KTA.---------- : 130
common_carp           : ..V.PG.TP...........S..TA.---------- : 130
barbodes              : ..V.PG.TP...L.......S..TA.---------- : 130
channel_catfish       : ..V.SG..P....E.....T.KT..---------- : 128
                        ▶D dom. ▶E domain
```

48

```
                                                    *         180          *          200
yellow_perch           : GTERRTAQQPDKTKNKKRPLPGHSHSSFKEVHQKNSSRGN : 178
Eurasian_perch         : ....................................... : 178
bastard_halibut        : ....................................... : 178
shi_drum               : ....................................... : 178
black_sea_bream        : ...............................P....... : 177
gilthead_seabream      : ...............................P....... : 177
yellowfin_seabream     : ....................................... : 177
four-spine_sculpin     : S.........................D............ : 175
short-horned_sculpin   : S........G................D.........T : 176
orange-spotted_grouper: ....................................... : 178
rabbitfish             : .............S.....S................... : 178
European_sea_bass      : ....................................... : 178
flathead_mullet        : .A................IS.................S : 178
Mozambique_tilapia     : .......P..H........S....-............S : 174
Chinook_salmon         : ........H.....T..K..S.N..T.C.......... : 180
chum_salmon            : ........H.....P..K..S.N..T.C.......... : 180
rainbow_trout          : ........H.....P..------------......... : 168
coho_salmon            : ........H.....P..------------......... : 168
fathead_minnow         : ----------------K.IS..R...C..........I : 153
goldfish               : ----------------K.IC.....C........... : 153
triangular_bream       : ----------------K.IS......C.......... : 153
giant_danio            : ----------------K.IS......C.......... : 153
bluntsnout_bream       : ----------------K.IS......C.......... : 153
zebrafish              : ----------------K.IS......C.......... : 153
scale-less_car         : ----------------K.IS.AG...C.......... : 170
mud_carp               : ----------------K.VS......C.......... : 153
common_carp            : ----------------K....Q....Y.......... : 153
barbodes               : ----------------K.IS......C.......... : 153
channel_catfish        : ----------------K.IS....T.C.......... : 151
```

```
yellow_perch          : TGGRNYRM : 186
Eurasian_perch        : ........ : 186
bastard_halibut       : ........ : 186
shi_drum              : ........ : 186
black_sea_bream       : A....... : 185
gilthead_seabream     : A....... : 185
yellowfin_seabream    : ........ : 185
four-spine_sculpin    : M....... : 183
short-horned_sculpin  : M....... : 184
orange-spotted_groupe : ........ : 186
rabbitfish            : ........ : 186
European_sea_bass     : ........ : 186
flathead_mullet       : ........ : 186
Mozambique_tilapia    : S....... : 182
Chinook_salmon        : ........ : 188
chum_salmon           : ........ : 188
rainbow_trout         : ........ : 176
coho_salmon           : ........ : 176
fathead_minnow        : ........ : 161
goldfish              : ........ : 161
triangular_bream      : .......I : 161
giant_danio           : ........ : 161
bluntsnout_bream      : .......I : 161
zebrafish             : ........ : 161
scale-less_car        : .......I : 178
mud_carp              : ...S...I : 161
common_carp           : .......I : 161
barbodes              : .......I : 161
channel_catfish       : ........ : 159
```

Figure 2.8 Alignment of yellow perch preIGF-Ib deduced amino acid sequence with other teleost preIGF-Is.

Conserved amino acid residues are indicated with a (.), inserted gaps are indicated with a (-) and the six conserved cysteine residues in the mature peptide are shaded. Structural domain start positions (B, C, A, D, E) are identified with ☐ and at the bottom of the alignment in bold. Sequences were downloaded from the EMBL/GenBank database with the following accession numbers: Eurasian perch (*Perca fluviatilis*) CAE52915; bastard halibut (*Paralichthys olivaceus*) CAA09268; shi drum (*Umbrina cirrosa*) AAY21628; black seabream (*Acanthopagrus schlegelii*) AAD01917; gilthead seabream (*Sparus aurata*) AAY46225; yellowfin seabream (*Acanthopagrus latus*) AAT35826; four-spine sculpin (*Cottus kazika*) BAC07249; short-horned sculpin (*Myoxocephalus scorpius*) CAA73162; orange-spotted grouper (*Epinephelus coioides*) AAS01183; rabbitfish (*Siganus guttatus*) AAO47742; European sea bass (*Dicentrarchus labrax*) AAV67967; flathead mullet (*Mugil cephalus*) AAR06903; Mozambique tilapia (*Oreochromis mossambicus*) AAC17494; chinook salmon (*Oncorhynchus tshawytscha*) AAA67263;

chum salmon (*Oncorhynchus keta*) AAC18833; rainbow trout (*Oncorhynchus mykiss*) AAA49412; coho salmon (*Oncorhynchus kisutch*) AAA49410; fathead minnow (*Pimephales promelas*) AAT02176; goldfish (*Carassius auratus*) AAC83443; triangular bream (*Megalobrama terminalis*) AAO89239; giant danio (*Danio aequipinnatus*) ABB05519; bluntsnout bream (*Megalobrama amblycephala*) AAK16727; zebrafish (*Danio rerio*) AAI14263; scale-less car (*Gymnocypris przewalskii*) AAX21106; mud carp (*Cirrhinus molitorella*) AAY21902; common carp (*Cyprinus carpio*) BAA11878; barbodes (*Spinibarbus sinensis*) ABE03747; channel catfish (*Ictalurus punctatus*) AAZ28918.
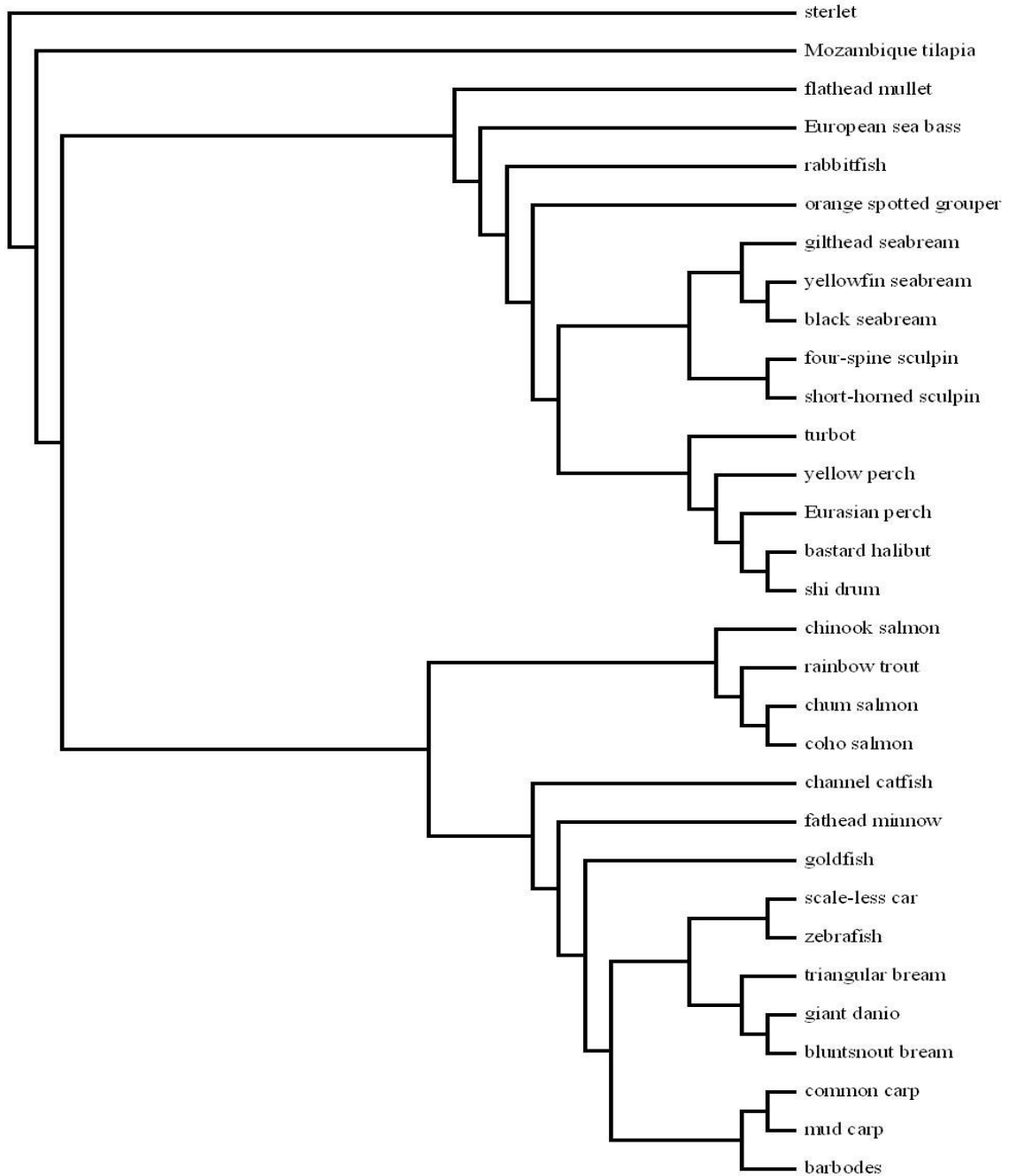
Figure 2.9 Phylogenetic tree of mature IGF-I amino acid sequences.

Tree produced using a Clustal X (1.81) alignment and TreeView (Win 32) v. 1.6.6. The tree was rooted with sterlet (*Acipenser ruthenus*) IGF-I (Accession: ABC54785) as an outgroup representing the closest available species not in the group Teleostei. See Figure 2.8 for EMBI/GenBank accession numbers except: turbot (*Scophthalmus maximus*) Duval *et al.* 2002 [186].

```
          *  V  K  K  M  S  S  S  R  A  L  L  F  A  L  S  L     42
  75   --GGTCAAGAAGATGTCTTCGTCCAGTCGCGCGCTGCTGTTTGCACTGTCCCTC


          A  L  Y  A  V  E  I  A  S  A  E  T  L  C  G  G  E  L     60
 127   GCGCTCTACGCTGTGGAGATAGCCTCGGCGGAGACGCTGTGTGGGGGAGAGCTG


          V  D  A  L  Q  F  V  C  E  D  R  G  F  Y  F  S  R  P     78
 181   GTGGATGCGCTGCAGTTTGTCTGTGAAGACAGAGGCTTCTATTTCAGTAGGCCA


          T  S  R  G  N  N  R  R  N  Q  N  R  G  I  V  E  E  C     96
 235   ACCAGCAGGGGTAACAACCGGCGCAACCAGAACCGTGGGATCGTAGAGGAGTGT


          C  F  R  S  C  D  L  N  L  L  E  Q  Y  C  A  K  P  A    114
 289   TGTTTCCGTAGCTGTGACCTCAACCTGCTGGAGCAGTACTGTGCCAAACCCGCC


          K  S  E  R  D  V  S  A  T  S  L  Q  V  I  P  V  M  P    132
 343   AAGTCCGAAAGGGACGTGTCGGCCACCTCTCTGCAGGTCATACCCGTGATGCCC


          A  L  K  Q  *                                          136
 397   GCATTAAAACAGG----------------------------------------
```

Figure 2.10 Partial nucleotide and deduced amino acid sequence of yellow perch IGF-II.

Numbers for nucleotides (left) and amino acids (right) are from the initiation methionine based on alignment with related teleost sequences. Yellow perch partial IGF-II cDNA sequence will be available from the EMBL/GenBank database with accession # DQ984123 on or before October 27, 2007.
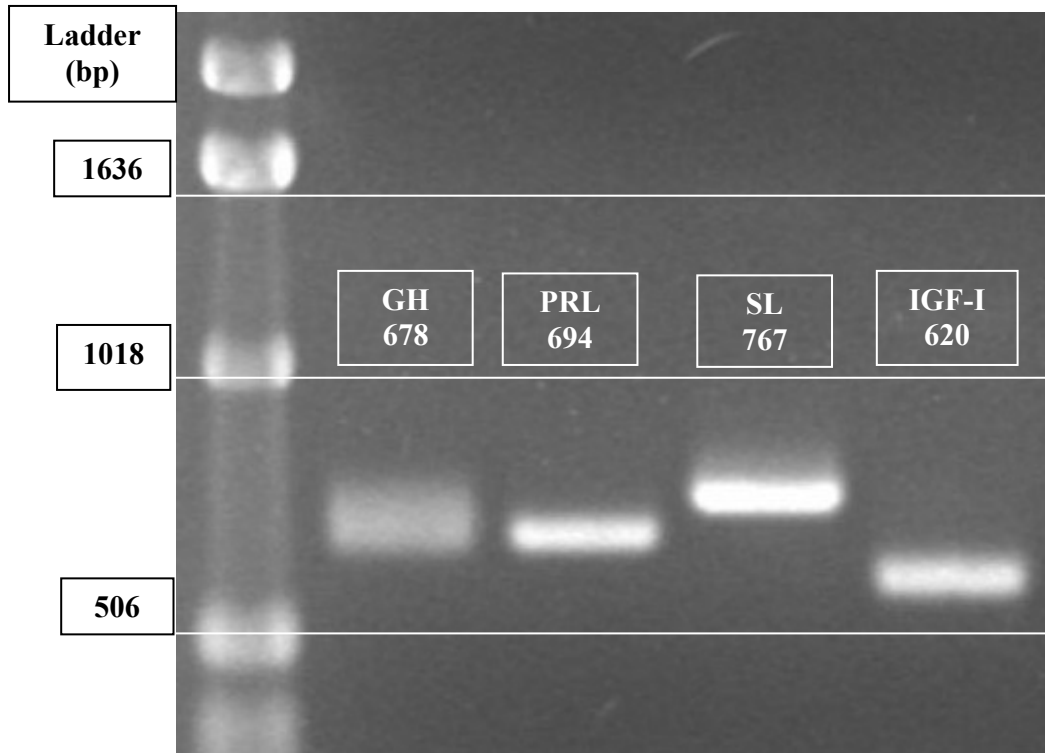
Figure 2.11 Full cDNA coding region PCR products for GH, PRL, SL and IGF-I.
For PCR primers used see Table 2.3 and PCR protocol used see Table 2.4. Template used was 1 µl of cDNA generated from adult yellow perch pituitary (GH, PRL and SL) or liver (IGF-Ib) mRNA. PCR products were then run on a 1% low melt agarose gel with a 1 KB ladder and visualized using ethidium bromide staining.
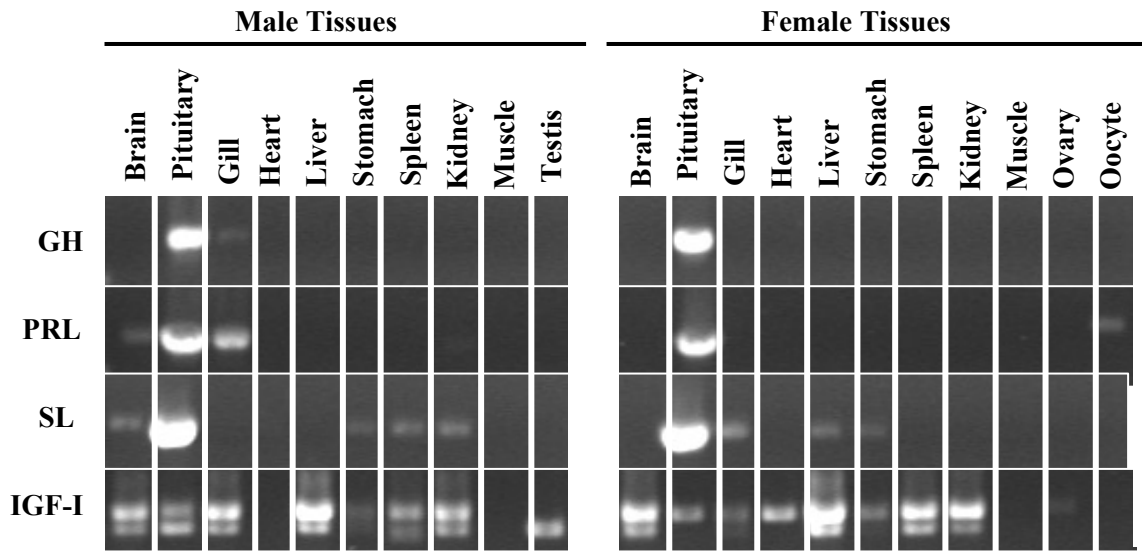
Figure 2.12 Sex-specific tissue expression for GH, PRL, SL and IGF-I.

For PCR primers used see Table 2.3 and PCR protocol used see Table 2.4. Template used was 900 ng of cDNA generated from adult yellow perch mRNA from each sex-specific tissue. PCR products were then run on a 1% agarose gel and visualized using ethidium bromide staining. cDNA template quality was verified by analyzing for β-actin mRNA levels using real-time quantitative PCR (qPCR).

Chapter 3: Sequence and sex-specific tissue expression of estrogen receptor α, estrogen receptor βa and ovarian aromatase (CYP19A1) cDNAs in yellow perch

3.1 Introduction

Steroid hormone receptors are members of a large family of ligand-activated nuclear transcription factors that are critical to the reproduction, differentiation and development of vertebrates. All steroid receptors are believed to have derived from an ancient estrogen receptor (ER) through the process of two large-scale genome expansions, one before the advent of the jawed vertebrates and the second after [201]. ERs are members of a family of nuclear transcription factors including receptors for sex steroids, thyroid hormone, retinoids as well as many "orphan" receptors for which no ligands have been identified [202]. All members of this family share a modular structure that consists of a variable trans-activation domain (A/B), a highly conserved DNA binding domain (C or DBD), a variable hinge region (D), a well-conserved ligand binding domain (E or LBD), and a variable C-terminal region (F) [202-204]. It was believed that there was only one type of ER in vertebrates, now called ERα, up until the discovery of a second ER subtype, ERβ, in 1996 [205]. ERα and ERβ are the products of separate genes [205] and have distinct, yet partially overlapping, distributions in estrogen target tissues [53, 206-208] and also have different ligand binding affinities [208]. In fish, the ERβ has two subtypes designated ERβa and ERβb (sometimes called ERβ1 and ERβ2, or ERγ and ERβ) [203, 209-213] which arose from the duplication of an ancestral ERβ gene early in the teleost lineage after the split of the tetrapods and fish [212, 214, 215]. Now, in addition to nuclear ERs there is evidence for membrane localization of ERs [216, 217] and novel ERs in brain tissues [218].

The primary physiological ligand for ERs is the sex steroid estradiol-17β or simply estradiol. Estradiol, the most common and physiologically active form of estrogen (E_2), is synthesized mainly in the ovary as an endocrine factor and locally in several tissues as a paracrine or autocrine factor. In teleosts, estrogen receptors are expressed during sexual differentiation [55, 210] indicating that the role of estrogen receptors can be of paramount importance for main events during sexual development, sexual maturation or reproduction [219]. Halm *et al.* [210] found significant changes in the expression of all

three ER subtypes in pituitary, brain and gonad tissues over the first year of development in juvenile sea bass (*D. labrax*). Male juvenile catfish had high levels of ERβ expression and almost no ERα in the liver while female juvenile catfish had moderate expression levels of both ERs in liver [206]. Teves *et al.* [220] found that the relative ERα expression levels gradually increased in African catfish pituitaries during pubertal development. In addition, rainbow trout ovary had increasing levels of ERα expression corresponding with increasing ovarian follicle diameter [221]. At 120 dpf (days post fertilization) in fathead minnow there were significant differences between male and female liver expression in both ERα and ERβb, with females having higher expression [222]. Also at 120 dpf, females had higher gonad expression levels of ERβb than males, but males had higher gonad expression levels of ERα than females.

In adult sea bass, ERα was predominantly expressed in liver and pituitary with significantly higher expression levels in females than males while ERβ1 expression levels were generally higher in male tissues than female tissues, particularly liver, brain and pituitary [210]. In adult gilthead seabream, females showed ERα expression in liver, pituitary, heart and muscle while males showed expression in liver, pituitary and testis. ERβa expression was markedly different with females having the highest expression levels in ovary, pituitary and skin and males had high expression levels in testis, liver, pituitary and kidney [214]. In goldfish, females showed the highest ERα levels in pituitary with moderate levels in ovary, brain, pituitary and liver. Males also had high ERα levels in pituitary, but had higher relative levels in liver and testis than the other tissues as compared to females. ERβ1 expression was highest in the gonads of both sexes with moderate levels in all other tissues measured [53]. Zebrafish showed ERα expression, from highest to lowest, in pituitary, liver and testis and ERβ1 expression in liver, testis, intestine, brain, pituitary and ovary [211]. Largemouth bass females had significantly higher levels of ERβ than ERα in ovary and pituitary tissues while liver tissue had equivalent levels of ERβ and ERα expression [223]. These studies highlight the sex-specific, tissue-specific and maturational differences in the expression of the different ER subtypes in fish.

P450 aromatase (P450$_{arom}$, CYP19), a CYP19 gene product, is a member of the cytochrome P450 superfamily. Aromatase is the terminal steroidogenic enzyme in the

biosynthesis pathway of E_2 by catalyzing the formation of aromatic C_{18} estrogen from C_{19} androgren [224]. Aromatase was originally cloned from the human placenta in 1988 [225] and since then CYP19 genes have been identified in all classes of vertebrates examined [226]. However, in teleost fish two CYP19 loci coding for two distinct isoforms that are structurally and functionally different have been identified: CYP19A1 and CYP19A2 [227] (sometimes incorrectly termed CYP19a and CYP19b). These forms are preferentially expressed in the ovary (CYP19A1) and brain (CYP19A2) of bony fish. Species in which two forms have been found are: Atlantic halibut (*Hippoglossus hippoglossus*) [228]; pejerry fish (*Odontesthes bonariensis*) [229]; Nile tilapia (*Oreochromis niloticus*) [230, 231]; channel catfish (*Ictalurus punctatus*) [232]; European sea bass (*Dicentrarchus labrax*) [233]; orange-spotted grouper (*Epinephelus coiodes*) [234]; goldfish (*Carrasius araurata*) [235]; medaka (*Oryzias latipes*) [236]; zebrafish (*Danio rerio*) [237]; rainbow trout (*Oncorhynchus mykiss*) [238]; and gobiid fish (*Trimma okinawae*) [239]. A phylogenetic analysis shows that the brain aromatase forms share higher homology between species than with their respective ovarian aromatase [233].

Ovarian aromatase (CYP19A1) is primarily expressed in the ovary but also shows considerable expression in other tissues, most notably brain and spleen. In mammals, the major sources of circulating E_2 are ovary tissue and/or placenta while peripheral tissues, such as adipose, bone and brain synthesize E_2 to function in a paracrine or autocrine manner [224]. Adult southern flounder showed highest expression of CYP19A1 in ovary and spleen tissues with much lower levels present in brain, testis, gill and liver and no expression detected in muscle, heart, intestine or kidney [240]. Conversely, Atlantic halibut adults showed CYP19A1 expression in brain, heart, gonad, pituitary and spleen tissues with no expression in gill, intestine or liver tissues [228]. In the wrasse, CYP19A1 expression was detected in high levels in the ovary, moderate levels in the kidney, and low levels in the brain, liver, testis, gill, spleen, muscle, and heart; no expression was detected in the intestine [226]. Nile tilapia showed CYP19A1 expression in brain, heart, gills, muscle, blood, kidney, intestine, spleen and testis tissues [230] in one study but in another study showed high expression in ovary tissue, moderate expression in brain, spleen and testis tissues and could not be detected in eye, kidney or

liver tissues [231]. The black porgy CYP19A1 was highly expressed in the ovary and weakly in the brain and testis with no expression detected in heart, liver, kidney, intestine and muscle [241] and the orange-spotted grouper showed a similar CYP19A1 expression pattern with high levels in ovary, pituitary, spleen, and gill tissues but low levels in brain tissue and blood cells [234]. The expression of CYP19A1 was undetectable in the heart, liver, kidney, muscle and adipose tissues of the adult orange-spotted grouper. The gobiid fish, *Trimma okinawae*, had high CYP19A1 expression in ovary tissue and moderate expression in brain, spleen and testis tissues [239].

Not only does CYP19A1 expression show tissue-specific differences but there is evidence that there are tissue sex-specific differences outside of gonadal expression. In a recent study, Atlantic halibut males showed similar CYP19A1 expression levels to females in brain, pituitary, gill, and spleen tissues however males had substantially higher kidney, stomach and intestine expression than females [242]. Zebrafish showed sex-specific differences in CYP19A1 expression in eye tissue, with females having higher levels of expression than males [243]. Juvenile southern flounder showed a sex-specific expression difference in CYP19A1 levels in gonads with a clear divergence beginning around 65mm TL, becoming more pronounced by 80mm TL, and being maintained throughout the size range that defines the period of histological sex differentiation. Specifically, high CYP19A1 expression was always correlated with signs of ovarian development (e.g. ovarian cavity and oocytes) and low expression with testicular development (e.g. seminal lobules and spermatogenesis) [240, 244]. Atlantic halibut begin gonadal differentiation at 32mm TL and Matsuoka *et al.* [242] contend that the ratio of CYP19A1/CYP19A2 should show sex-specific levels with females higher than males, as females increase expression of CYP19A1 during gonadal development but males do not. In Nile tilapia, CYP19A1 expression was detected in gonads after 15 days of development, but only in females and not males [230]. These studies highlight the sex-specific, tissue-specific and maturational differences in the expression of CYP19A1 in fish.

The yellow perch is one of a group of important fishes which exhibit a sexual size dimorphism (SSD) in which females grow faster than males [7]. Other fishes in this group include sea bass [166, 167], halibut [168], eel [169], plaice, walleye, and

tench [170, 171].   The female biased SSD in yellow perch was demonstrated in laboratory [8, 9] and wild populations [10-12] many years ago, but it was not until the mid 1980s [7, 13, 14] that studies identified 17β-estradiol (E_2) as a growth stimulator in yellow perch SSD. Further, the growth promoting effects of E_2 were only noticeable in fish of 80-110 mm total length (TL) or greater [13] and this critical size range is also the same size at which females normally begin to outgrow males [8] and a female-biased SSD begins to be manifested. This critical period is also the specific minimum body size for the onset of vitellogenesis and spermatogenesis in females and males, respectively [14], pointing towards an upregulation of E_2 receptors (ERs) on target tissues (ovary, liver or pituitary) and a coinciding increase in tissue expression of growth factors. Malison *et al.* [7] reported that in addition to a growth response, E_2 treatment stimulated feed consumption, and growth rate and growth efficiency of female yellow perch exceeded those of males two-fold in animals fed without restriction [15]. These observations suggest that the growth-promoting effects of estrogen may work in part through appetite centers of the central nervous system and could involve pituitary hormones. They also point out a clear linkage of growth and reproductive development in this species. As a first step in investigating estrogen stimulated SSD of yellow perch, the full length cDNAs for ERα, ERβ, aromatase (CYP19A1) and β-actin were cloned and sequenced and sex specific tissue expression was examined.

3.2 Methods

Cloning

Gravid ovary tissues were collected from the offal of fish brought into the Pannuzzo Fish Co. (Lorain, OH) by recreational fishermen for cleaning. Only fish that had been caught within 12 hours and kept on ice were used. Tissues were harvested, immediately frozen on dry ice, transported back to the laboratory at the University of Kentucky and stored at -80 °C until total RNA was extracted with the GenElute™ Mammalian Total RNA Kit (Sigma, St. Louis, MO). RNA samples were treated with amplification grade DNase I (Sigma, St. Louis, MO) and quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). First-strand cDNA with ligated 5' and

3' anchor primers was generated from 5 μg total RNA using the GeneRacer™ Kit (Invitrogen, Carlsbad, CA).

GenBank was searched for neoteleost ERα, ERβ, aromatase (CYP19A1) and β-actin cDNAs and several sequences were aligned using Vector NTI Suite 7.0 (Informax, Inc., Frederick, MD) and GeneDoc (http://www.psc.edu/biomed/genedoc/) [172]. Consensus sequences, or fragments thereof, were put into Primer3, a web-based primer design program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [173] and general neoteleost primers for genes of interest were generated. Generated primers were used exclusively or in combination with the GeneRacer™ primers (5' and 3') (Table 3.1) provided in the GeneRacer™ Kit (Invitrogen, Carlsbad, CA) to obtain initial PCR products. A 50 μl total volume PCR mixture using a MasterTaq Kit (Eppendorf Scientific Inc., Westbury, NY) was subjected to a touch down PCR amplification (Table 3.2) with gravid ovary (ERα, ERβ and CYP19A1) or liver (β-actin) tissues as a template. PCR products were electrophoresed in 1% agarose gels with a 1 kb DNA ladder (Gibco/BRL, Gaithersburg, MD) and visualized by ethidium bromide staining. PCR products of expected size were electrophoresed in 1% low melt agarose gels, excised and purified using GenElute™ Minus EtBr Spin Columns (Sigma, St. Louis, MO). Purified PCR products were ligated into a pCR®4-TOPO® vector and transformed into TOP10 chemically competent cells using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA). The plasmid DNA was then extracted from the bacterial cells using the GenElute™ Plasmid Miniprep Kit (Sigma, Sigma, St. Louis, MO). Plasmid samples were quantified and up to 600 ng of plasmid DNA was put into a sequencing PCR using BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing PCR consisted of 35 cycles of 30 s at 96 °C, 15 s at 50 °C and 4:00 min at 60 °C. After the PCR, each sample received 27 μl ddH$_2$O, 60 μl 100% EtOH and 3 μl 3 M NaOAc. This mixture was transferred to a 1.5 mL microcentrifuge tube, vortexed and left overnight to precipitate. The next day, samples were spun at maximum speed at 4 °C for ≥ 30 min, decanted and washed with 250 μl of 70% EtOH, spun again, decanted and allowed to air dry. Samples were kept at -20 °C until they were transported to the University of Kentucky Advanced Genetic Technologies Center (http://www.uky.edu/Centers/AGTC/) for sequencing. Species-

specific ERα, ERβ, CYP19A1 and β-actin primers were developed based on the sequences generated and the PCR, cloning, and sequencing procedure was repeated as necessary (with 5' and/or 3' GeneRacer™ primers) to achieve full length (5'UTR+CDS+3'UTR) sequences. BLASTN v. 2.2.14 [174] searches were used to determine similarities with other teleosts and to verify gene identity.

Species-specific primers were designed and synthesized to amplify the full coding region of yellow perch ERα, ERβ, CYP19A1 and β-actin cDNAs (Table 3.3). These primers were tested using 1 µl of cDNA template. Total RNA (750 ng) extracted from gravid ovary (ERα, ERβ and CYP19A1) or liver (β-actin) tissues was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA) to generate cDNA templates. A 50 µl total volume PCR mixture was subjected to a touch down PCR amplification (Table 3.4). The resulting bands were excised, purified, cloned and sequenced as described above to verify sequence data and primer-gene specificity. GeneDoc [172] was used to generate amino acid sequence alignments of ERα, ERβ, CYP19A1 and β-actin from teleost sequences given by a BLASTP v. 2.2.14 search [174]. Alignments were produced in Clustal X1.81 [176] and used to generate phylogenetic tree data using the Neighbor Joining tree method [177] with 1000 bootstrap trials and TreeView v. 1.61 was used to create a visual phylogenetic tree.

Sex-specific tissue expression

Tissues were collected from adult yellow perch maintained in IACUC (#00251L2001) approved aquaculture facilities at the University of Kentucky, Lexington, KY. Perch were kept in a flow through tank (~1L/min) chilled to ~17 °C, a 14:10 light:dark cycle and constant aeration. Fish were held at a density of < 1 fish/gallon and fed three times per week to satiation with Aquamax Grower 400 (PMI Nutrition International, Inc., Brentwood, MO) for a period of one year before sampling. Fish were sampled in spring (March) and euthanized with MS 222 before tissues were harvested. Both male and female brain, pituitary, gill, heart, liver, stomach, spleen, kidney, skeletal muscle and gonad tissues were collected along with post-vitellogenic oocyte tissue [6]. Tissues were collected and flash frozen at -80 °C until analysis. Total RNA was extracted, treated with amplification grade DNase, quantified and up to 750 ng was reverse transcribed to cDNA and quantified. PCR primers and cycling conditions are

listed in Tables 3.3 and 3.4, respectively, and 900 ng of cDNA template was used for all tissues. Products were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining.

3.3 Results

ERα

Yellow perch ERα cDNA (Accession #DQ984124) was found to consist of 3052 bp containing an open reading frame (ORF) of 1731 bp, a 41 bp 5'-UTR and a 1,280 bp 3'-UTR (Figure 3.1). The open reading frame encoded a protein of 576 amino acids, which included an N terminal A/B domain, a highly conserved C domain also termed the DNA binding domain (DBD) or zinc finger motif (ZFM), a highly variable D domain, a conserved E domain also termed the ligand-binding domain (LBD) and an F domain. The C domain (DBD) has nine conserved cysteine residues common to all nuclear receptors and the first eight (minus Cys_{204}) are key to the formation of the two zinc finger motifs [245, 246]. The C domain (DBD) also contains the ER conserved P-box (PATNQ) and D-box (EGCKA) that have been recognized to be involved in binding to estrogen response element (ERE) sequences along with the conserved residues of Lys_{165} to Gln_{173} [245, 247]. The E domain (LBD) in the human ERα has nine amino acids recognized to be involved with E_2 binding that are conserved in the yellow perch ERα [248]. However Ekena *et al.* [249] reported that a mutation of human ERα Met_{528}, corresponding to yellow perch ERα Ile_{486}, to an Ala residue caused an 11-fold reduction in E_2-induced transcription, although all known teleost ERα sequences have an Ile in place of the human Met at that position (Figure 3.2). The other three residues shown to be important to E_2 binding in human ERα remain constant in all fish including the yellow perch (Gly_{490}, His_{493} and Leu_{494}). In the estrogen receptor two transcriptional activation functions (TAFs) have been defined using transient transfection experiments [250]. TAF-1 site is in the N-terminal domain and the TAF-2 site is in the E domain (LBD) but their activities can depend upon the responsive promoter and cell type. Unlike sea bass and goldfish ERα [210], yellow perch ERα does not appear to contain the TAF-1, however the eight residues associated with the TAF-2 localized in the LBD are completely conserved from the human and mouse ERαs [250]. The two putative

polyadenylation signals were located 15 and 130 bases upstream of the poly(A) tail and the calculated isoelectric point and molecular mass of the ERα peptide are 8.62 and 63,386.52 Da, respectively, as calculated by Vector NTI BioPlot.

Comparison of the deduced amino acid sequence of the yellow perch ERα with other known teleost ERαs is shown in Figure 3.2. A BLASTP v. 2.2.14 search [174] reveals that yellow perch ERα has the following homologies with other ERαs: 85% European sea bass, gilthead seabream and red seabream; 84% largemouth bass, black seabream; 82% eelpout; 80% wrasse; 79% bastard halibut, Nile tilapia, bamboo grass wrasse and Javanese ricefish; 78% African cichlid, spotted green pufferfish and killifish; 77% Japanese medaka; 75% blue tilapia; 70% rainbow trout and Atlantic salmon; 67% cherry salmon; 64% zebrafish, roach and Taiwan minnow; 63% fathead minnow; 62% goldfish; 61% North African catfish; 60% Taiwan shoveljaw carp and channel catfish; 53% mouse; 52% golden hamster, rat, zebrafinch, spectacled caiman, Nile crocodile, American alligator and African clawed frog; 51% Japanese quail, cat and chicken; 50% pig; 49% dog, horse and bovine; 48% human.

A phylogenetic tree of ERα proteins in teleosts and African clawed frog (Figure 3.3) was constructed from alignment results. The tree indicates an early split of Neoteleostei from other Teleostei (Cypriniformes and Siluriformes) with the exception of Salmoniformes (salmon and trout) which splits from Neoteleostei later. Cypriniformes splits from Siluriformes (catfish) before goldfish and Taiwan shoveljaw carp split from the rest of the family Cyprinidae. The order Salmoniformes splits from Neoteleostei and within that grouping Tetraodontiformes (pufferfish) splits. The next split takes the family Cichlidae (tilapia and cichlid), in the order Perciformes, and splits it off with the orders Cyprinodontiformes (killifish) and Beloniformes (ricefish and medaka). After these groups split from the rest of Neoteleostei, the family Cichlidae splits from the others before the split of Fundulidae (killifish) from Adrianichthyidae (ricefish and medaka). Next Pleuronectiformes (bastard halibut) splits from the remaining Perciformes and then Labridae (wrasses) splits followed by Percidae (yellow perch) and Zoarcidae (eelpout). Centrarchidae (largemouth bass) and then Moronidae (European sea bass) then sequentially split from Sparidae (seabreams).

ERβa

In fish, the ERβ has two subtypes designated ERβa and ERβb and a BLASTN v. 2.2.14 [174] search revealed the sequence for yellow perch ERβ generated here has a higher homology to the ERβa group of ERβs and therefore is designated as yellow perch ERβa. Yellow perch ERβa cDNA (Accession #DQ984125) was found to consist of 2462 bp containing an ORF of 1,668 bp, a 546 bp 5'-UTR and a 248 bp 3'-UTR (Figure 3.4). The designated open reading frame encoded a protein of 555 amino acids, which included an N terminal A/B domain, a highly conserved C domain also termed the DNA binding domain (DBD) or zinc finger motif (ZFM), a highly variable D domain, a conserved E domain also termed the ligand-binding domain (LBD) and an F domain. The C domain (DBD) has ten cysteine residues, nine of which are common to all nuclear receptors and the first eight have been shown to be key to the formation of the two zinc finger motifs [245, 246]. The C domain (DBD) also contains the ER conserved P-box (PATNQ) and D-box (EGCKA) which have been recognized to be involved in binding to estrogen response element (ERE) sequences along with the conserved residues of Lys_{184} to Gln_{192} [245, 247]. The E domain (LBD) in the human ERα has nine amino acids recognized to be involved with E_2 binding which are conserved in the yellow perch ERβa [248], as are all four key resides described by Ekena *et al.* [249].

The 5'-UTR had 9 supplemental ATG initiation codons starting at bases -41, -44, -105, -190, -200, -280, -356, -365 and -368. These were all in a different reading frame from the yellow perch ERβa protein with the exception of the ATG beginning at base -356 (Figure 3.4, in bold and underlined). This short ORF was in the same reading frame as the ERβ protein and was 168 bases long coding for 55 amino acids before ending with a TGA stop codon. Multiple potential translation initiation sites in the 5'-UTR were reported for several other fish and mammalian ERs [213, 214, 251-255]. In the estrogen receptor two transcriptional activation functions (TAFs) have been defined using transient transfection experiments [250]. TAF-1 site is in the N-terminal domain and the TAF-2 site is in the E domain (LBD) but their activities can depend upon the responsive promoter and cell type. Yellow perch ERβa does not appear to contain the TAF-1, however the eight residues associated with the TAF-2 localized in the LBD are completely conserved from the human and mouse ERs [250]. No polyadenylation signals

were located and the calculated isoelectric point and molecular mass of the ERβa peptide are 8.47 and 61,864.83 Da, respectively, as calculated by Vector NTI BioPlot.

Comparison of the deduced amino acid sequence of the yellow perch ERβa with other teleost ERβs from the same group is shown in Figure 3.5. A BLASTP v. 2.2.14 search [174] reveals that yellow perch ERβa has the following homologies: 83% bastard halibut ERβ; 82% largemouth bass ERγ; 80% black seabream ERβ and Nile tilapia ERβ; 79% gilthead seabream ERβ; 77% killifish ERβA and Javanese ricefish ERβ; 76% Atlantic croaker ERγ and Japanese medaka ERβ; 70% Taiwan shoveljaw carp ERβ and common carp ERβ; 69% Atlantic salmon ERβ and spiny barbed minnow ERβ; 67% rainbow trout ERβ and goldfish ERβ1; 66% zebrafish ERβ2; 61% Japanese eel ERβ and conger eel ERβ; 59% sheep ERβ; 58% European sea bass ERβ2, rat ERβ, human ERβ and bovine ERβ; 57% goldfish ERβ2, Taiwan minnow ER(βb), Taiwan shoveljaw carp ER(βb), roach ERβ, fathead minnow ERβ,, spiny dogfish ERβ, pig ERβ, mouse ERβ, Japanese quail ERβ and silurana tropicalis ERβ; 56% largemouth bass ERβ, gilthead seabream ERβb and Nile tilapia ERβ2; 55% killifish ERβB, Atlantic croaker ERβ, wrasse ERβ and channel catfish ERβ; 54% zebrafish ERβ1; 53% bamboo grass wrasse ERβ.

A phylogenetic tree of ERβ proteins in teleosts and spiny dogfish shark (Figure 3.6) was constructed from alignment results. The tree indicates that a very distinct split between the two types of fish ERβs occurs very early in the teleost lineage [256] with the bottom group termed ERβa and the top group termed ERβb [203]. The ERβa group shows the order Anguilliformes (eels) splits first, followed by Cypriniformes (goldfish, carp etc.). Strangely, rainbow trout (Salmoniformes) is grouped in with the Cypriniformes, but the other Salmoniformes (Atlantic salmon) splits before the divergence of Neoteleostei. Cyprinodontiformes (killifish) and Beloniformes (ricefish and medaka) split as a group before the emergence of the order Perciformes. Cichlidae (tilapia) is the first split within the order Perciformes and then Percidae (yellow perch) splits. However, yellow perch is grouped with bastard halibut (Paralichthyidae), which is not in the order Perciformes, but rather Pleuronectiformes. Centrarchidae (largemouth bass) and Sciaenidae (Atlantic croaker) then split respectively before Sparidae (seabreams).

The ERβb group shows a clear and early split between Neoteleostei and the other teleosts. The order Siluriformes (catfish) splits off from Cypriniformes (goldfish, carp etc.) which then split into various groups and subgroups. Cyprinodontiformes (killifish) is grouped with the Nile tilapia (Cichlidae), which split together from the remaining fish in the order Perciformes. Within the order Perciformes, the family Labridae (wrasses) splits, then Sparidae (gilthead seabream), Moronidae (European sea bass) and Sciaenidae (Atlantic croaker) leaving Centrarchidae (largemouth bass).

Aromatase (CYP19A1)

It is now well known that teleost fish have at least two CYP19 loci encoding for two different P450 aromatase enzymes: the ovary-derived or ovarian type, CYP19A1 and the brain-derived or brain type, CYP19A2. A BLASTN v. 2.2.14 [174] search revealed the generated yellow perch aromatase cDNA sequence to be a P450arom with a high homology to other fish ovarian aromatases (CYP19A1). Yellow perch ovarian aromatase (CYP19A1) cDNA (Accession #DQ984126) was found to consist of 1859 bp containing an ORF of 1,557 bp, a 68 bp 5'-UTR and a 234 bp 3'-UTR (Figure 3.7). The designated open reading frame encoded a protein of 518 amino acids. Two in-frame initiation codons were found and a putative N-glycosylation site was identified from Asn_{29}-Val_{33}. Yellow perch CYP19A1 contains four conserved regions present in all steroidogenic cytochrome P-450s [257]: I helix region Ala_{305}-Gln_{336}; Ozol's peptide Cys_{362}-Asp_{383}; aromatase specific conserved region Asp_{392}-Arg_{415}; and heme-binding region Phe_{442}-Ala_{455}. A putative polyadenylation signal was located 20 bp from the poly (A+) tail and the calculated isoelectric point and molecular mass of the aromatase peptide are 8.51 and 58,535.77 Da, respectively, as calculated by Vector NTI BioPlot.

Comparison of the deduced amino acid sequence of the yellow perch ovarian aromatase (CYP19A1) with other known teleost ovarian aromatases is shown in Figure 3.8. A BLASTP v. 2.2.14 search [174] reveals that yellow perch aromatase has the following homologies: 90% orange-spotted grouper, red-spotted grouper and barramundi perch; 89% European sea bass; 88% Atlantic croaker and red seabream; 87% black seabream; 86% gilthead seabream, humpback grouper and flathead mullet; 85% bastard halibut; 84% bamboo grass wrasse and rice field eel; 83% Atlantic halibut, Japanese medaka and killifish; 82% wrasse; 81% Mozambique tilapia and blue tilapia; 80% Nile

tilapia; 76% rainbow trout; 71% broad barred goby; 70% goldfish; 68% yellow catfish; 66% zebrafish and roach; 64% Japanese eel, southern catfish and channel catfish; 57% Japanese firebelly newt and wrinkled frog; 56% Atlantic stingray and African clawed frog; 54% pig and chicken; 52% zebrafinch, American alligator, mouse, dog, goat, sheep, bovine and rabbit; 51% horse and human.

A phylogenetic tree of ovarian aromatase (CYP19A1) proteins in teleosts and Atlantic stingray (Figure 3.9) was constructed from alignment results. The tree indicates an evolutionary "peeling off" of groups. The first bony fish is the Japanese eel, followed by the majority of the non-Neoteleostean fishes. The Cypriniformes (goldfish, etc.) and Siluriformes (catfish) split off together but then later diverge into their own clades. Interestingly, the next split is the broad barred goby which is not only a Neoteleostei, but also in the order Perciformes. The non-Neoteleostei rainbow trout (Salmonidae) splits next, followed by Cyprinodontiformes (killifish) and Beloniformes (Japanese medaka). The family Labridae (wrasses), also in the order Perciformes, then split followed by a grouping with families Cichlidae (tilapia) and Synbranchidae (rice field eel). But while tilapia are in the order Perciformes, the rice field eel is not and is in Synbranchiformes. Sparidae (seabreams) splits off next, followed by the flathead mullet which also is not in the order Perciformes, but Mugilomorpha. The halibuts (Atlantic and bastard) split next along with Moronidae (European sea bass) which is the order Perciformes but the halibuts are Pleuronectiformes (the families Pleuronectidae and Paralichthydiae, respectively). Sciaenidae (Atlantic croaker) splits, then Percidae (yellow perch), Serranidae (groupers) and Latidae (barramundi perch), all of which are in the order Perciformes.

β-actin

The yellow perch β-actin cDNA (Accession #AY332493) was found to consist of 1900 bp containing an ORF of 1,128 bp, an 82 bp 5'-UTR and a 690 bp 3'-UTR (Figure 3.10). The putative polyadenylation signal was located 19 bases upstream of the poly (A) tail and the calculated isoelectric point and molecular mass of the β-actin peptide are 5.30 and 41,749.52 Da, respectively, as calculated by Vector NTI BioPlot.

Tissue expression

The primers in Table 3.3 were used to generate full length coding region PCR products which are visualized in Figure 3.11. The β-actin, ERα, ERβa and CYP19A1 products were 1,176, 1,781, 1,718 and 1,605 bps in length and sequencing these products verified the data presented in Figures 3.1, 3.4, 3.7 and 3.10, respectively. These primers were then used to determine sex-specific tissue expression (Figure 3.12). Both ERα and ERβa showed the highest expression in female tissues, particularly liver and ovary. Female liver and ovary tissues showed substantial expression of ERα and the only other female tissue to show ERα expression was the spleen tissue. Female liver and ovary tissues also showed substantial expression of ERβa with low expression in stomach, kidney and post-vitellogenic oocyte tissues. Male spleen, liver and stomach tissues showed moderate to low expression of ERα and male kidney, liver, and spleen tissues showed moderate expression of ERβa. ERβa expression was low in male stomach, heart, gill and brain tissues. CYP19A1 showed a very global expression pattern both male and female yellow perch tissues. Female spleen and liver along with post-vitellogenic oocyte tissues showed high expression of CYP19A1, while female brain, gill, stomach and ovary tissues all showed moderate to low expression of CYP19A1. Male pituitary, gill and spleen tissues showed moderate to high expression of CYP19A1 while male heart, stomach and kidney tissues showed only low expression. Female ovary tissue had the highest expression of β-actin of all the tissues tested in females, while male brain and gill tissues had the highest expression in males. All tissue cDNA samples were tested for quality by measuring the presence of β-actin transcripts using real-time quantitative PCR (qPCR) (unpublished data).

3.4 Discussion

The full length ERα cDNA of the yellow perch was cloned by directional RACE procedures and was shown to encode a protein of 576 amino acids. The yellow perch ERα retained the conserved cysteines in the C domain responsible for the formation of the zinc fingers, the P and D box regions associated with DNA binding and the nine residues and AF2 region associated with E_2 binding [245-249]. Alignment of yellow perch ERα protein with other teleost ERαs reveals the A/B domain to have 141 residues,

the C domain to have 72 residues, the D domain to have 56 residues, the E domain to have 240 residues and the F domain to have 67 residues (Figures 3.1 and 3.2). The C and E domains are the most conserved regions within the teleost lineage having similarities of almost 100% and ~80%, respectively. There are several amino acid substitutions that are unique to the yellow perch, such as the Tyr_{155} in the C domain where all other known fish have a His residue. In the E domain there are 5 such residues unique to yellow perch, Ala_{335}, Ser_{351}, Val_{366}, Val_{373}, Tyr_{419} substitute Val, Ile, Asp, Glu and Ser, respectively (Figure 3.2). Although, several other species show similar unique substitutions such as bamboo grass wrasse Ala_{247}, goldfish Lys_{346}, black seabream Phe_{363}, etc., so the significance is probably minimal. The most interesting unique substitution is the wrasse Thr_{308} substitution for Ala, which is one of the key residues in the E domain responsible for E_2 binding [248]. Amino acid and phylogenetic analysis indicate that yellow perch ERα shares the highest degree of homology with eelpout (*Zoarces viviparous*).

The recent discovery of additional ERβ forms in fish [212] has made ERβ receptor nomenclature ambiguous, as is clearly indicated by Figures 3.5 and 3.6. Recently however, Hawkins and Thomas [203] proposed to adopt the zebrafish official nomenclature [258, 259] that designates the two fish paralogs as ERβa and ERβb. A BLASTN 2.2.14 [174] search revealed the cDNA sequence generated for yellow perch ERβ has a higher homology with the ERβa group, and thus the present manuscript follows this proposed nomenclature by designating this sequence ERβa. The full length ERβa cDNA of the yellow perch was cloned by directional RACE procedures and encodes a protein of 555 amino acids. The yellow perch ERβa sequence showed that the 546 bp 5'-UTR contained a total of nine supplemental ATG initiation codons and the single in-frame supplemental codon had a short ORF of 55 amino acids. Small ORFs with uncharacterized functions have also been identified in the 5'-UTR of ER cDNAs from many species of fish [210, 213, 214, 251, 254, 260-262]. However, in human and mouse ERαs the small upstream ORFs are involved in the regulation of the levels of translation of the ER protein, providing the potential to regulate expression [263].

The yellow perch ERβa retained the conserved cysteines in the C domain responsible for the formation of the zinc fingers, the P and D box regions associated with DNA binding and the nine residues and AF2 region associated with E_2 binding [245-

249]. Alignment of yellow perch ERβa protein with other teleost ERβas reveals the A/B domain to have 160 residues, the C domain to have 75 residues, the D domain to have 75 residues, the E domain to have 210 residues and the F domain to have 35 residues (Figures 3.1 and 3.2). The C and E domains are the most conserved regions within the teleost lineage as these are the regions associated with ligand and DNA binding. There are several amino acid substitutions that are unique to the yellow perch, the most notable being Cys_{224} in the C domain where all other known fish have a Tyr residue with the exception of an Asn in largemouth bass. The only other C domain substitution in yellow perch is the His_{169} replacing a Tyr. The E domain only contains three unique substitutions in yellow perch with yellow perch Asn_{384}, Val_{431} and Ala_{487} replacing Ser, Ser and Val or Leu, respectively (Figure 3.5). Surprisingly, amino acid and phylogenetic analysis indicate that yellow perch ERβa shares the highest degree of homology with bastard halibut (*Paralichthys olivaceus*) despite bastard halibut being in the order Pleuronectiformes and yellow perch being in Perciformes.

The presence of two ERβ subtypes in at least 10 fish species (Figure 3.6: fathead minnow and European sea bass partial ERβa sequences not included) spanning a number of families (Sparidae, Sciaenidae, Moronidae [210], Centrarchidae, Cichlidae, Fundulidae, Cyprinidae) raises the distinct, and almost inevitable, possibility that yellow perch (Percidae) has a third, yet undiscovered, ER sequence (ERβb). All known mammalian ERα subtypes are around 600 aa in length whereas ERβ subtypes are considerably shorter and range from 485 to 530 aa. Most fish ERαs range from 560 to 620 aa (Figure 3.2) with an average size around 590 aa and most fish ERβas range from 555 to 570 aa (Figure 3.4) with an average size around 564 aa. Fish ERβbs, as a general rule though, are larger than the ERβa orthologs from the same species (e.g. Atlantic croaker ERβ/ERγ {673 vs 565 aa}; zebrafish ERβ1/ERβ2 {592 vs 554 aa}; goldfish ERβ2/ERβ1 {611 vs 568 aa}; largemouth bass ERβ/ERγ {670 vs 556 aa}) with a range from 575 to 678 aa and an average size around 634 aa (data not shown). This indicates that the putative yellow perch ERβb should be larger than either of the two ER sequences (α or βa) presented here. The variability in size of the F and, particularly, A/B domain is the major contributing factor to overall size differences among different ERs. In at least two species, two transcript sizes have been identified that are determined by the length of

the A/B domain. In channel catfish, the long ERα variant is produced by an additional 503 bp segment at the 5'-end, whose in-frame start codon adds 36 aa to the protein [264]. In rainbow trout, the long ERα variant (rtER$_L$) is produced by alternative splicing involving exon I, intron I and exon II [265]. In rainbow trout, the two variants show different tissue expression [266] and data suggest that the additional residues in the A/B domain modify the hormone-independent *trans*-activation function of the receptor [265].

The C domain (DBD) is so homologous between the ERα and ERβ subtypes (Figures 3.2 and 3.5) that one would expect them to bind equally to the same type of DNA estrogen response element (ERE) especially given the conserved regions of the P- and D-boxes. Nevertheless, it has been reported that mouse ERβ has slightly lower affinities than mouse ERα for an ERE [267] and Huang *et al.* [268] found that E$_2$ enhanced the ERE binding of human ERα but not that of ERβ. In zebrafish, ERβ2(a) and ERα, in contrast to ER β1(b), were able to significantly stimulate the expression of the ERα gene in the presence of E$_2$ through the ERα ERE [269]. The E domain (LBD), also maintains a high level of homology, however even slight changes in amino acid sequence can alter relative ligand binding affinity. Atlantic croaker ERβa showed higher relative binding affinities for estradiol, estriol and RU486 and lower relative binding affinities for synthetic estrogens and antiestrogens then the other ERs (ERα and ERβb) [203]. Mouse ERα has a slightly higher affinity than mouse ERβ for E$_2$ [267] and rat ERα has a five fold higher affinity than rat ERβ for 17α-estradiol [208]. Channel catfish ERα binds E$_2$ with an order of magnitude lower affinity than catfish ERβb [206]. And lastly, liver ERα, but not ERβ, mediated regulation of the IGF-I gene by antiestrogens in a human hepatocyte cell line [270]. These results on differential DNA and ligand binding properties suggest diverging functions of the orthologous ERs in controlling estrogen responses within a tissue.

Studies have also shown tissue expression differences between ERs in the same species. In European sea bass, ERα expression in females was much greater than males in liver and pituitary tissues, while ERβa expression was higher in males than females in most tissues [210]. In both gilthead seabream and goldfish, ERβa was the only ER to show high levels of expression in ovary tissue, while ERβb was the only ER to show moderate expression in the digestive system [53, 214]. In immature channel catfish,

males had substantially lower liver ERα than ERβb expression and in adults head kidney tissue had only ERα expression in both sexes [206]. In zebrafish intestine tissue, both ERβs had substantial expression with no ERα expression [211] and in fathead minnow ERβa had higher expression in gonad, pituitary, muscle, brain, and intestine tissues in both sexes than either ERα or ERβb [222]. In this study, female liver and ovary tissues had greater expression of ERα and ERβa than any other tissue, supporting their role as E_2 target tissues associated with reproduction. Male liver tissue showed low to moderate expression of both ERs, but testis tissue did not, despite expression of all three ERs being found in testis of gilthead seabream [214], channel catfish [206], zebrafish [211], goldfish [53], fathead minnow [222] and Eurpean sea bass [210]. Interestingly though, in this study pituitary tissue did not show expression of either ER in males or females despite being a known estrogen responsive tissue, however there could be sensitivity issues regarding both pituitary and testis ER expression. ERβa showed a much more global expression than ERα in both males and females with noteworthy expression in brain, stomach, kidney and post-vitellogenic oocyte tissues. While the highest levels of both ERs in females were clearly seen in liver and ovary tissues, the highest ERα and ERβa expression in males occurred in spleen and kidney tissues, respectively. The steroidogenic potential of the spleen will be discussed later (under CYP19A1), but the kidney tissue used here was not separated from the corticosteroidogenic cells associated with the head kidney. Unlike mammals, teleost fish lack a discrete adrenal gland and corticosteroidogenesis is located in the interrenal cells distributed around the postcardinal vein in the anterior part of the kidney. These cells secrete cortisol, the primary corticosteroid in bony fish [271, 272]. Cortisol biosynthesis involves a series of steps beginning with the transport of cholesterol from outer to inner mitochondrial membrane via the enzyme steroidogenic acute regulatory protein (StAR). The next step is conversion of cholesterol to pregnenolone via the enzyme CYP11A and these are also the initial steps of steroidogenesis or the production of testosterone and estrogen. The rat StAR gene promoter has an estrogen receptor half-site [273] providing a possible mechanism for estrogenic modulation of corticosteroidogenesis and an explanation for ER expression in head kidney tissue.

In regards to estrogen synthesis, bony fish were found to be unique from the rest of the vertebrates in the late 1990s [235] when it was determined they had a second enzymatically active [235, 237, 239] brain-type of aromatase designated CYP19A2 [227]. All fish CYP19A1s reported to date have been in a range of 517-523 amino acids (Figure 3.7) and CYP19A2s have been in the a range of 488-508 amino acids [226]. The full length CYP19A1 cDNA of the yellow perch was cloned by directional RACE procedures and was shown to encode a protein of 518 amino acids. Two potential initiation ATG sites are located at 1 bp and 31 bp in Figure 3.7, however neither of these two initiation codons represents the optimal sequence for initiation as described by Kozak [274], so there is some uncertainty to the true initiation site. A similar situation has been identified in the CYP19A cDNAs in several other teleost species [229, 233, 235, 236, 275-277]. Most RNAs contain a polyadenylation signal of either AATAAA or ATTAAA, but in yellow perch only a putative (AGTAAA) [278] polyadenylation signal was found 20 bp upstream of the poly (A)+ tail (Figure 3.7). Similar putative polyadenylation signals (ATAAA) were found in Japanese medaka CYP19A1 [236] and fathead minnow CYP19A2 [279].

Yellow perch CYP19A1 contains four conserved regions present in all steroidogenic cytochrome P-450s [257] with the region of highest homology being the heme-binding region (Figure 3.8). This region, along with the I-helix region, is believed to form the substrate binding pocket [224, 257]. The heme disc is embedded between the proximal (heme-binding region) and distal (I helix region) helices. Several residues in the heme-binding region have been indicated as being important for the binding of the heme group [280] and almost all of these are conserved throughout the known fishes. For example, Cys_{470} (Figure 3.8) is believed to provide the thiolate linkage for the heme prosthethic group and Arg_{468} may be in position to hydrogen bond with one of the heme propionates. The two glycines of the heme binding region, Gly_{464} and Gly_{466}, are reported to be invariant, but it is prudent to note at this point that humpback grouper CYP19A1 does not subscribe to this structure (Figure 3.8). In fact, Phe_{463}, another reported invariant residue, is supposed to bracket the Cys_{470} and, along with Lys_{473} and the human Ala_{438} (corresponding to Val_{471} in Figure 3.8), may form a pocket for the thiolate ligand [280]. Replacement of the human Ala_{438} with a Val residue occurs in all

known fish (Figure 3.8: including Atlantic stingray), birds (zebra finch [281], chicken [282], Japanese quail [283]) and reptiles (red-eared slider turtle [284], American alligator [285], leopard gecko [286]) but not in any mammals or amphibians (African clawed frog {Accession #BAA90529}, Silurana tropicalis {BAE93232}, northern leopard frog {ABE03744}, Iberian ribbed newt [287], wrinkled frog [288], Japanese firebelly newt {BAD12119}), resulting in a very curious evolutionary pattern.

In the I helix region, the consensus residues Glu_{314}, Pro_{320}, Asp_{321}, Thr_{322} and Ser_{324} are highly conserved throughout known cytochrome P450s and mutations of these residues cause major changes in the catalytic function of the aromatase enzyme [257, 280, 289]. A mutation of the consensus residue Cys_{311} to an Ala resulted in low enzyme activity that suggests a conformation change, making it a sixth I helix residue that is an important part of the active site [257, 289]. In the Ozol's peptide region, the substitution of Arg_{377} to an Ala or Lys resulted in a complete inactivity of the enzyme [280]. It is believed that the C and D rings of the steroid interact with the Ozol's peptide and aromatase specific conserved region. A conserved domain not noted on Figures 3.7 and 3.8 is found from around yellow perch Ser_{131} to Asn_{152} and reportedly interacts with the A ring of the steroid [280].

While there appears to be a great amount of amino acid homology among fish CYP19A1 sequences, especially in the labeled conserved domains (Figure 3.8), one exception seems to stand out. Humpback grouper, a protogynous fish, CYP19A1 cDNA [290] has substitutions for several key residues listed above in the heme-binding domain that are not shared even with the other groupers (Figure 3.8), while the wrasse, also a protogynous fish [226], seems to have a very conserved sequence. Also, it is interesting to note that the broad barred goby CYP19A1 sequence, also published in Gardner *et al.* [290] along with the barramundi perch CYP19A1 sequence, is unique enough that a phylogenetic analysis classified it in its own clade near seemingly unrelated fish (Figure 3.9). Despite these unique aspects of their sequences, Gardner *et al.* [290] surprisingly state that comparative analyses "displayed negligible differences" with previous gonochoristic findings. One problem with identifying conserved structures of CYP19A1 cDNAs is that there seems to be more interest focused on CYP19A2 (or CYP19B as it

has been called) and the role it plays in neuronal development and sexual determination [229, 232, 233, 291-294].

The expression level of CYP19 mRNA in tissue is correlated with aromatase activity [295] and aromatase activity is considered as an indicator of estrogen synthesis [296], so expression of CYP19 mRNA in tissue should (assuming adequate substrate (i.e. testosterone)) indicate estrogen production. Tissue expression of yellow perch CYP19A1 was most predominant in male and female spleen, female liver and post-vitellogenic oocyte tissues. Moderate expression was found in male and female gill, male pituitary and female brain tissues, while low expression levels were seen in male and female stomach, male heart, male kidney and ovary tissues (Figure 3.12). Few studies have investigated tissue expression levels of CYP19A1 on a sex-specific basis (excluding gonads), however in this study several tissues (brain, pituitary, heart, liver, and kidney) showed distinct sexually dimorphic patterns of expression. As a rare exception, a study on Atlantic halibut by van Nes *et al.* [228] examined sex-specific expression in brain, gill, heart, intestine, kidney, liver, pituitary, spleen and gonad. They reported sex-specific differences in gonad and kidney expression, with female ovary and male kidney tissues showing expression. This study shows similar results in yellow perch (Figure 3.12), but while most studies showed high expression levels of CYP19A1 in ovary tissue this study only found low to moderate levels. It is conjectured that levels of CYP19A1 expression in ovary, oocytes and pituitary are very sensitive to seasonal and reproductive status, possibly giving some explanation to conflicting results [226, 242, 279]. To highlight this, Matsuoka *et al.* [242] had conflicting results to van Nes *et al.* [228] on expression of CYP19A1 in gill, gonad, intestine, heart and liver of the same species, Atlantic halibut. They contend that, while the method of detection was different between the two studies, the life stage of the fish (immature vs. mature, reproductive status, etc.) is likely a more significant factor.

The lack of CYP19A1 expression in male brain tissue and the low expression in female brain tissue in yellow perch gives an indication that while this study did not produce a brain-type CYP19 it is probable that one exists, as the teleost brain is generally characterized by exceptional high levels of aromatase activity [297]. The pituitary is known to be an estrogen sensitive organ, but while females have relatively high levels of

ovarian produced E_2 in circulation, males generally do not, possibly making locally produced E_2 in the male pituitary a significant form of gonadatropin regulation. The expression of CYP19A1 in the gill tissue is supported by similar findings in rainbow trout [238], orange-spotted grouper [234], Atlantic halibut [242], southern flounder [240] Nile tilapia [230] and wrasse [226] gill tissue, although interestingly no transformation of testosterone to estrogen was demonstrated in rainbow trout gill epithelial cells [298]. Only a few recent studies have found expression of CYP19A1 in fish heart tissue [226, 228, 230] but Harada *et al.* [299] demonstrated expression of aromatase in adult human arterial smooth muscle cells. Aromatase activity has been well documented in the mammalian liver [224] but until recently [226, 240] there had been no record of CYP19A1 expression in fish liver. Some researchers went so far as to state that teleost liver was unlikely to have estrogenic activity [231], a rather short-sighted contention given the role the liver plays in important estrogen-driven processes such as vitellogenesis.

Other studies have found high expression levels of aromatase in spleen tissue [226, 228, 231, 234, 239, 240, 242] and even whole blood [234], as the spleen is involved in blood cell (both red and white) production. A function of splenic aromatase is the autocrine synthesis of E_2 releasing cytokines IL-2 and IL-6 from the splenic T lymphocytes which maintains an immune response after trauma-hemorrhage [300]. This raises the possibility of an artificial spike in splenic CYP19A1 transcription derived from the sampling methodology. In this study, spleens were probably removed within 15 min of the first incision, however transcription levels have been shown to respond to a stimulus in under one hour. Whether the levels of expression in spleen tissue are increased from the sampling methodology or not, the prevalence of fish species showing transcription of CYP19A1 in spleen tissue gives an indication of a role between immune function and steroidogenesis. Again, only recent studies have shown CYP19A1 expression in fish kidney tissue [226, 228, 230, 242] and, as mentioned above, van Nes *et al.* [228] also reported male specific expression.

Lastly, yellow perch showed high levels of CYP19A1 expression in oocyte tissue and studies in rainbow trout [301] and Nile tilapia [275] have shown detectable expression in pre-vitellogenic oocytes, increasing toward mid-vitellogenesis, after which

it declines sharply to non-detectable levels in post-vitellogenic oocytes. Ciereszko *et al.* [6] found that yellow perch have synchronous oocyte growth during autumn and winter and by February oocytes have completed vitellogenesis. While Kolkovski and Dabrowski [302] demonstrated that yellow perch can be manipulated to spawn out of season, they did so by completely inverting the photothermal conditions, while an earlier study indicated that yellow perch ovulation and spawning are rather recalcitrant to minor changes in photothermal conditions [6]. The yellow perch used in this study for tissue expression analyses were kept at temperatures which fluctuated slightly (1-2 °C) with season (summer higher than winter) and on a constant light:dark (14:10) cycle and spawned normally in spring in prior years. Therefore it seems responsible to assume to that the oocytes sampled in this study taken from yellow perch in aquaculture are in the post-vitellogenic stage which would be normal for the time period. It remains unknown how or if the results of this study can be reconciled with the studies previously mentioned above [275, 301].

In conclusion, this study provides the full length nucleotide sequences for yellow perch ERα, ERβa, CYP19A1 and β-actin cDNAs and shows that there is a high degree of similarity with other fish species at the amino acid level. Yellow perch ERα, ERβa, CYP19A1 and β-actin all showed only one transcript in all of the tissues examined. Both ERs had the highest expression levels in female estrogen-sensitive tissues (liver and ovary) while CYP19A1 had a more global but still a sex-specific expression pattern. These results indicate new avenues for research related to sex-specific tissue expression of these genes. The production and publication of these yellow perch sequences provides molecular tools to investigate estrogen stimulated SSD and other estrogen actions in yellow perch.

Table 3.1 Primer pair sequences used to generate initial PCR products for ERα, ERβa, CYP19A1 and β-actin.

Start numbers indicate the forward (5' end) or reverse (3' end) primer nucleotide start. Forward and reverse primer start numbers are calculated beginning with the initiation methionine (see Figures 3.1, 3.4, 3.7 and 3.10).

Gene	Start	Primer Sequence
ERα	611-	GCT ACG AAG TGG GCA TGA TGA AAG GA
	1504-	TCT CCA GCA GCA GGT CGT ACA GAG G
ERβ	528-	CGT CTG GTC GTG TGA GGG GTG TAA G
	1286-	AGG CAC ATG TTG GAG TTG AGG AGG A
CYP19A1	290-	AGT ACG GAG ACA TTG TCA GAG TCT
	1463-	TTG TTG GTC TGT GGG AGG CAG TC
β-actin	340-	GCC AAC AGG GAG AAG ATG ACA CAG ATC A
	3'UTR-	GeneRacer™ 3' nested primer

Table 3.2 Touch down PCR protocol used to generate initial products for ERα, ERβa, CYP19A1 and β-actin cloning.

Temp (°C)	Time (mm:ss)	Cycles
94	2:00	1
94	0:30	
72	0:30	5
70	1:00	
94	0:30	
70	0:30	5
70	1:00	
94	0:30	
68	0:30	25
70	1:00	
70	10:00	1

Table 3.3 Primer pair sequences used to generate full length cDNA coding region PCR products for ERα, ERβa, CYP19A1 and β-actin.

Start numbers indicate the forward (5' end) or reverse (3' end) primer nucleotide start. Forward and reverse primer start numbers are calculated beginning with the initiation methionine (see Figures 3.1, 3.4, 3.7 and 3.10).

Gene	Start #	Primer Sequence	Size (bp)
ERα	(-23)-	CAC CGT GTG CCC TCT CCA GTG AC	
	1759-	CTT CAC ATG TTG CTT GCC GTG CTC	1781
ERβa	(-26)-	CGA TAC TGA CAC CGT CTG TAG TTG C	
	1693-	GCT GTG GTC CAC CTC CAG AGT GCT	1718
CYP19A1	(-42)-	GTT TGT GCA GTC GTG TGC AGG TTG T	
	1564-	CAG AGT CTC AGA GTT TTT GCC AGC TTC C	1605
β-actin	(-26)-	GCC GAT AAC CCT CCC TCA AAC AGA A	
	1151-	GGG GGA GAG GAG GAA CAG TCT	1176

Table 3.4 Touch down PCR protocol used to generate full cDNA coding region products for ERα, ERβa, CYP19A1 and β-actin.

Temp (°C)	Time (mm:ss)	Cycles
94	2:00	1
94	0:30	
72	0:30	5
72	1:00	
94	0:30	
68	0:30	5
72	1:00	
94	0:30	
64	0:30	30
72	1:00	
72	10:00	1

```
                                             ►A/B domain
              M   Y   H   E   4
-41  -AGACAGAGCCCTCACCTCCACCGTGTGCCCTCTCCAGTGACATGTACCACGAA

       E   S   R   G   S   G   G   V   A   T   V   D   F   L   E   G   T   Y   22
 13  GAGAGTCGGGGGTCTGGAGGGGTAGCCACTGTGGACTTCCTGGAAGGGACATAC

       D   Y   A   A   S   T   P   A   P   I   P   P   Y   S   H   F   T   T   40
 67  GACTATGCTGCCTCCACCCCTGCTCCGATTCCTCCTTACAGCCACTTTACCACT

       G   Y   Y   S   A   P   L   D   A   H   G   P   P   S   D   G   S   Q   58
121  GGCTACTACTCTGCTCCTTTAGACGCCCACGGACCACCCTCAGATGGCAGCCAG

       S   L   G   S   G   P   T   S   P   L   V   F   V   P   S   S   P   R   76
175  TCCCTGGGTAGTGGGCCTACCAGTCCTCTTGTGTTTGTGCCCTCCAGCCCCCGA

       F   S   R   F   M   H   P   P   S   H   H   H   L   E   T   S   S   T   94
229  TTCAGCCGCTTTATGCACCCACCCAGCCACCACCATTTGGAAACCTCCTCAACA

       P   V   Y   R   S   S   V   P   S   S   Q   Q   S   V   S   M   E   D   112
283  CCCGTCTATAGGTCCAGTGTCCCATCCAGTCAGCAGTCAGTCTCCATGGAGGAC

       Q   Y   N   T   S   D   E   S   Y   S   V   G   E   S   G   A   G   A   130
337  CAGTACAACACCAGTGACGAGTCATACAGTGTGGGGGAGTCAGGAGCTGGAGCC

                                             ►C domain (DBD or ZFM)
       G   A   R   G   F   E   I   V   K   G   T   R   F   C   A   V   C   S   148
391  GGGGCCAGGGGGTTTGAGATTGTCAAAGGGACGCGTTTCTGTGCCGTGTGCAGT

       D   Y   A   S   G   Y   Y   Y   Y   G   V   W   S   C   E   G   C   K   A   166
445  GACTATGCCTCTGGGTACTACTACGGGGTGTGGTCCTGTGAGGGCTGCAAAGCC

       F   F   K   R   S   I   Q   G   H   N   D   Y   M   C   P   A   T   N   184
499  TTCTTTAAGAGGAGCATCCAGGGTCACAATGACTATATGTGCCCAGCAACCAAT

       Q   C   T   I   D   R   N   R   R   K   S   C   Q   A   C   R   L   R   202
553  CAGTGTACTATTGACAGGAATCGGAGGAAGAGCTGCCAGGCTTGCCGTCTTAGG

                                             ►D domain
       K   C   Y   E   V   G   M   M   K   G   G   V   R   K   D   R   G   R   220
607  AAGTGTTACGAAGTTGGCATGATGAAAGGAGGTGTGCGCAAGGACCGCGGGCGT

       V   L   R   R   D   K   R   R   T   G   T   N   D   R   D   K   A   S   238
661  GTTTTGCGGCGTGACAAACGACGGACTGGCACCAATGACAGAGACAAGGCGTCA

       K   D   L   A   Q   R   T   V   P   P   Q   D   G   R   K   R   S   S   256
715  AAGGACCTGGCGCAAAGGACAGTGCCCCCTCAGGATGGGAGGAAACGCAGTAGT

                                             ►E domain (LBD)
       A   C   G   G   G   G   K   L   L   L   T   G   M   P   P   D   Q   V   274
769  GCTTGTGGTGGAGGTGGAAAATTATTGTTGACTGGCATGCCTCCTGACCAGGTG
```

```
       L  L  L  L  Q  G  A  E  P  P  I  L  C  S  R  Q  K  L  292
823  CTCCTCCTTCTCCAGGGTGCTGAGCCCCCAATACTGTGCTCCCGTCAGAAGCTA

       N  R  P  Y  T  E  V  T  M  M  T  L  L  T  S  M  **A**  D  310
877  AACCGACCCTATACTGAGGTCACCATGATGACCCTGCTCACCAGCATG**GCC**GAT

       K  **E**  L  V  H  M  I  A  W  A  K  K  L  P  G  F  L  Q  328
931  AAG**GAG**CTGGTCCACATGATTGCATGGGCCAAGAAGCTTCCAGGTTTCCTGCAG

       L  G  L  H  D  Q  A  Q  L  L  E  S  S  W  L  E  V  **L**  346
985  CTGGGCCTCCATGACCAGGCGCAGCTGCTGGAGAGCTCATGGCTGGAGGTG**CTG**

       M  I  G  L  S  W  **R**  S  I  H  C  P  G  K  L  I  **F**  A  364
1039 ATGATCGGGCTCAGCTGG**AGG**TCCATCCACTGCCCCGGCAAACTCATC**TTC**GCA

       Q  V  L  I  L  D  R  N  V  G  D  C  V  E  G  M  A  E  382
1093 CAGGTCCTCATACTGGACAGGAACGTAGGCGATTGTGTCGAAGGCATGGCTGAG

       **I**  F  D  M  L  L  A  T  A  S  R  F  R  L  L  K  L  K  400
1147 **ATC**TTCGACATGCTGCTAGCCACTGCTTCCCGCTTCCGCTTGCTCAAACTGAAG

       P  E  E  F  V  C  L  K  A  I  I  L  L  N  S  G  A  F  418
1201 CCCGAGGAGTTTGTCTGTCTCAAAGCTATTATCTTGCTCAACTCTGGTGCCTTC

       Y  F  C  T  G  T  M  E  P  L  H  D  T  A  A  V  Q  N  436
1255 TATTTCTGCACCGGTACAATGGAGCCCCTCCATGACACTGCGGCAGTGCAGAAC

       M  L  D  T  I  T  D  A  L  I  H  H  I  S  Q  S  G  C  454
1309 ATGCTGGACACCATCACAGACGCTCTCATACATCACATCAGCCAATCAGGATGC

       S  V  Q  Q  Q  S  R  R  Q  A  Q  L  L  L  L  L  S  H  472
1363 TCAGTTCAGCAGCAGTCGAGACGGCAAGCACAGCTGCTCCTCCTGCTCTCCCAC

       I  R  H  M  S  N  K  **G**  M  E  **H**  **L**  Y  S  I  K  C  K  490
1417 ATCAGGCACATGAGCAACAAA**GGC**ATGGAG**CATCTC**TACAGCATTAAGTGCAAG

       N  K  V  P  L  Y  D̲  L̲  L̲  L̲  E̲  M̲  L̲  D̲  A  H  H  V  508
1471 AACAAAGTGCCTCTGTAC̲G̲A̲C̲C̲T̲G̲C̲T̲G̲C̲T̲G̲G̲A̲G̲A̲T̲G̲C̲T̲G̲G̲A̲C̲GCTCACCACGTC

       ►F domain
       Q  R  P  D  R  P  D  Q  P  W  S  Q  A  D  G  E  L  P  526
1525 CAGCGGCCAGACAGACCAGATCAGCCCTGGTCCCAGGCTGATGGAGAGCTTCCC

       S  T  I  S  N  S  S  N  S  S  G  G  G  S  S  S  G  G  544
1579 TCCACCATCAGCAACAGCAGCAACAGCAGCGGTGGTGGCTCCTCTTCAGGTGGA

       S  N  S  G  P  R  V  S  R  E  S  P  S  R  A  P  T  V  562
1633 TCCAATTCAGGACCCCGAGTGAGCCGTGAGAGCCCGAGCAGAGCCCCCACAGTC

       L  Q  Y  A  E  S  R  S  D  C  T  R  I  L  ***          576
1687 CTGCAGTACGCAGAGTCCCGCTCTGACTGCACCCGCATCCTATGAGACCGAGCA
```

83

```
1741   CGGCAAGCAACATGTGAAGGTCAAAAGTCATTTTTATGGGTGATGTGTGTAGTA
1795   CAGGAGGAGAAAGGTTTGGGAATAAAATCCAAAGGTTGTTTTTGATTGATTTCA
1849   TGAGATAATAATTTATAAATTAATTGAATTTAGGGAGTTTTTCTTTGCACTACT
1903   GCAATTCACTACAATCTGAGCTTTAATGCAGTTACTCTTCTATGCTGTCTTCTA
1957   TGTGATTTTGAGTCTCTAACAGCTTTACAGTACAGCTAATTATTAAATAATATT
2011   TACAAAACGGCAGGTGTTAGGTCATATTGTGGCACTCTATTAGCTTTGAAATGA
2065   CGAGCAGCTAATTTTTATTTTTATTCGCCTCGACCAAAGTGCACTTCCTCCGGG
2119   GTTTAAGGGTCTACAGGCACATTTTTTTCTTTGACATATTATGGATGATTACAC
2173   ACTAAACTATGATAACACTGGTTCAAATGAATGTGTAATTTATTTTGTTTGAAT
2227   TCCAACATTAAGGACTTAACATCAGGTATTGTGTTTTCTGCACACACACAAGTG
2281   TAAGCTTAATATATTTGGGAAGAAAATTATTTTAATGATGAAATATATGAGGAT
2335   ATATCTGCTGAATTTTGCAGCTTTTTGTGTGTAGTCGGTTTGTTCTTTATGCTT
2389   ACTTGAACTCAAGTTTTCTGAAGTGTGTGTGTCTGTGTGTCTGATTTTTATTTT
2443   ATTTTTTCTCTTGATGCAGTTGTTCAACCACTTCAAAGCTACTACCGGTTATTG
2497   TTCATCCAGTAATTCAGTGTTTACATGTTGAGTTTGGCTGGATTTAGACACCTC
2551   CAAAAACCTAATTATCATAATGCATTTGCACATATTCCATTGTTGTTATTTACT
2605   TACTTATTCTCGTTTCTGTACGTTCATGAAACAGATCTATAAGCCCTGGGAATG
2659   GCGCAAATATGTCGCTCAATTCGCACTCGCCTCAAACATTTGTTTCTCATTCTG
2713   TCTGAAAGGTCAGAATTGAGAAGTTGCAACCAGGGACTATTTGGCGTTTTTACT
2767   TGAAAAATGACTAACTGCTGGTCCAAATAGTTGTGGATTGTTTTCCTGTCGGTT
2821   AACTAACCAATTAATTGACTAATCGTTTCTGTACTTAAAA**AATAAA**GGCAATTA
2875   AAATGTATGAAAGCCTCCATACAGGATACAATAGGATTTGGCCACTTGAGCTG
2929   AAAGTCCAGTCAAACCTGAACTGACTGTCATTTGTTTACTGTGCATAAA**AATAA**
2983   **A**AGCATTAATTGAGAAAAAAAAAAAAAAA
```

Figure 3.1 Nucleotide and deduced amino acid sequences of yellow perch ERα cDNA.

The nucleotides (lower row) are numbered on the left and the amino acids (upper row) are numbered on the right and both begin with the initiation methionine. The six functionally independent domain (A, B, C, D, E & F) putative start points are indicated with black arrows above the amino acids. Domain C is also referred to as the DNA binding domain (DBD) or the zinc finger motif (ZFM) and domain E is also referred to as the ligand binding domain (LBD). The nine conserved cysteines of the C domain are shaded and the ten amino acid residues corresponding to the P- and D-boxes are boxed. The nine amino acids of the E (LBD) domain recognized to be involved in E_2 binding are in bold and the eight amino acid region corresponding to AF2 is underlined. Two putative polyadenylation signals are in bold and double underlined. Yellow perch ERα cDNA sequence will be available from the EMBL/GenBank database with accession #DQ984124 on or before October 27, 2007.

```
                                        *        20       *       40
yellow_perch         : ---------------------------------------- :   -
eelpout              : ---------------------------------------- :   -
largemouth_bass      : -MCKRQSPAQSKQPCGTVLRPRIG-PAFTELETLSPQHPS : 38
European_sea_bass    : -MCKRQSPAQSRQHCGPVVRPRISPAGFTELETLSPQRPS : 39
red_seabream         : ---------------------------------------- :   -
black_seabream       : ---------------------------------------- :   -
gilthead_seabream    : ---------------------------------------- :   -
wrasse               : ---------------------------------------- :   -
bamboo_grass_wrasse  : ---------------------------------------- :   -
bastard_halibut      : ---------------------------------------- :   -
African_cichlid      : ---------------------------------------- :   -
blue_tilapia         : ---------------------------------------- :   -
Nile_tilapia         : ---------------------------------------- :   -
killifish            : -MYKGQNPVQSKEAFGPALRPRLSPASS-ELETLSPPRLP : 38
Javanese_ricefish    : -MLLRQSSVQIRQLFGPALRSRISPASS-ELETLSPPRL- : 37
Japanese_medaka      : -MSKRQSSVQIRQLFGPALRSRISPASS-ELETLSPPRL- : 37
spotted_green_puffer : MSSSKQSPVQSRQTRGPEVKARITAAFT-ELETLSPQHPS : 39
Atlantic_salmon      : -MLVRQSHTQISKPLGAPLR---SRTTLESHAISPPKLSP : 36
cherry_salmon        : -MLVRQSHTQISKPLGAPLR---SRTTLESHVISPTKLSP : 36
rainbow_trout        : ---------------------------------------- :   -
goldfish             : ---------------------------------------- :   -
Taiwan_shoveljaw_carp: ---------------------------------------- :   -
zebrafish            : ---------------------------------------- :   -
Taiwan_minnow        : ---------------------------------------- :   -
fathead_minnow       : ---------------------------------------- :   -
roach                : ---------------------------------------- :   -
North_African_catfish: ------------MSGEQTRTEALAGAKQRRRSELEGYSA : 27
channel_catfish      : ------------MSEEQARAEAPAGARQRRRSELEGYSV : 27
```

▶ **A/B domain**

85

```
                                    *         60        *        80
yellow_perch          : ---------MYHEESRGSGG--VATVDFLEGTYDYAASTP :  29
eelpout               : ---------..P.........--.T...............P.. :  29
largemouth_bass       : PPLRAPLSD..P.........--G.............V.P.. :  76
European_sea_bass     : PPLRAPLSD..P.........--A.............P.. :  77
red_seabream          : ---------..P.D.......--.............P.. :  29
black_seabream        : ---------..P.D..V...--.............P.. :  29
gilthead_seabream     : ---------..P.D..V...--.............P.. :  29
wrasse                : ---------..P.........--.G...........T.P.. :  29
bamboo_grass_wrasse   : ---------..P.....---------------------- :   8
bastard_halibut       : ---------..P.........--A.............P.. :  29
African_cichlid       : ---------..P.........--.............P.. :  29
blue_tilapia          : ---------..P.........--........LMTMT.P.. :  29
Nile_tilapia          : ---------..P.........--.............P.. :  29
killifish             : PSPRASLGD..P.........--..A...........TP.. :  76
Javanese_ricefish     : -SPRDPLGD..P.........--..A...........PN. :  74
Japanese_medaka       : -SPRDPLGD..P.........--..A...........PN. :  74
spotted_green_puffer  : PPLRALLTD..P......R.--..N......P.....P.A :  77
Atlantic_salmon       : QQPTTPNSN..P....RG..--A.AF...D.G...T.P.Q :  74
cherry_salmon         : QQPTTPNSN..P.....G..--A.AF...D.G...T.PAQ :  74
rainbow_trout         : ---------..P.....G..--A.AF...D.G...T.PAQ :  29
goldfish              : ---------..PK.E-H.T.GISS.....D.A...SNL.E :  30
Taiwan_shoveljaw_carp : ---------..PK.E-H.A.GISS.....D.A.E.PNP.Q :  30
zebrafish             : ---------..PK.E-H.A.GISS.....D.A.E.PNP.Q :  30
Taiwan_minnow         : ---------..PK.E-H.A.GISS.....D.A.E.PDPIQ :  30
fathead_minnow        : ---------..PK.E-H.AEGISS.....D.A.E.PDP.Q :  30
roach                 : ---------..P..E-H.A.GTSS.....D.A.E.PDP.Q :  30
North_African_catfish : SLASLKLSP..P..EQQAT.GMSS.AH..D......---- :  63
channel_catfish       : SLASLKLSP..P..EQR.T.GISS.AH..D.....T---- :  63
```

```
                                                 *         100          *          120
yellow_perch           : A--PIPPYSHFTTGYYSAPLD--AHGPPSDGS-QSLGS-G :  63
eelpout                : .--.T.L...S.P.........--S.R......L......-. :  64
largemouth_bass        : V--.T.L...S--...........--.Q......L......-. : 109
European_sea_bass      : .--.T.L...S.P..........--.........L......-. : 112
red_seabream           : .--.T.L...S.P..........--.........L......-. :  64
black_seabream         : .--.T.L...S.P..........--.........L......-. :  64
gilthead_seabream      : .--.T.L...S.P..........--.........L......-. :  64
wrasse                 : .--.T-L..LS.Q.....A..--T..Q...S.I......-. :  63
bamboo_grass_wrasse    : -----------ARVCTM.AAR--EE.EVLHSKTRHHHD-. :  34
bastard_halibut        : .--QT.L...S...........--.........RH....-. :  64
African_cichlid        : .--.T.L...S...C.......--.........L......-. :  64
blue_tilapia           : .--.T.L...S...C.......--....L...L......-. :  64
Nile_tilapia           : .--.T.L...S...C.......--....L...L......-. :  64
killifish              : .--.T.L...S...........--.Q.......LH....-. : 111
Javanese_ricefish      : .--AT.L..QSG.........E--TN....E..L......-. : 109
Japanese_medaka        : .--TT.L..QS..........E--TN....E..L......-. : 109
spotted_green_puffer   : .--TT.L...S...SC......--.........LH....-. : 112
Atlantic_salmon        : G--.A.L.------.S.T.Q.--.........M......-S : 103
cherry_salmon          : G--.A.L.------.S.T.Q.--.........M......-S : 103
rainbow_trout          : G--.A.L.------.S.T.Q.--.........M......-S :  58
goldfish               : TFGTSS.TEPA.F...P..P.--P.A..AEEHL.N.-GT. :  67
Taiwan_shoveljaw_carp  : TYGTSS.TEPA.V...P..P.--P.A..AEEHL...-GT. :  67
zebrafish              : .FGTSS.AEPA.V...P..P.--P.----EEHL...-GG. :  63
Taiwan_minnow          : TYGTSS.AEPA.V...P..T.--P.ES.IEEHL...-GA. :  67
fathead_minnow         : TYGTTS.AEPL.V...L..T.--H.A..VEEHM..F-.GE :  67
roach                  : TYGTTS.AEPA.V...P..T.--P.A..VEEHL...-GGE :  67
North_African_catfish  : ---SN.--PNP.VD....AP.LQVAPE.QEENL.P..AN. :  98
channel_catfish        : ---TN.DA.NS.VD...------VAPE.QEENL.P.-PN. :  93
```

87

```
                                        *          140          *          160
yellow_perch           : PTSPLVFVPSSP----RFSRFM-HPPSHH--------HLE :  90
eelpout                : ............----.L.P..-....--------Y.. :  91
largemouth_bass        : ............----.L.P..-....--------Y.. : 136
European_sea_bass      : ............----.L.P..-....--------Y.. : 139
red_seabream           : .N..........----.L.P..-....--------Y.. :  91
black_seabream         : .N..........----.L.P..-....--------Y.. :  91
gilthead_seabream      : .N..........----HL.P..-Q.AN.--------Y.. :  91
wrasse                 : ............----.L.P..-.L....--------Y.. :  90
bamboo_grass_wrasse    : AG.----.N..---------------------------- :  41
bastard_halibut        : ....H.......----.L.P..-....--------Y.. :  91
African_cichlid        : T...........----.L.P..-....--------Y.. :  91
blue_tilapia           : ............----.L.P..-....--------Y.. :  91
Nile_tilapia           : ............----.L.P..-....--------Y.. :  91
killifish              : ............----.L.L..-.A..Q.--------Y.. : 138
Javanese_ricefish      : ............----.L.P..-...N..--------Y.. : 136
Japanese_medaka        : ............----.L.P..-....--------Y.. : 136
spotted_green_puffer   : ............----.L.P..-....Q.--------Y.. : 139
Atlantic_salmon        : ..G.....S...----QL.P..-...G..GL-PSQSYY.. : 137
cherry_salmon          : ..G.....S...QLSPQL.P..-......GL-PSQSYY.. : 141
rainbow_trout          : ..G.....S...QLSPQL.P..-......GL-PSQSYY.. :  96
goldfish               : .G......A....----QL.P..N.HGG..ST-HQVSYY.D : 102
Taiwan_shoveljaw_carp  : SG......A....----QL....N.HGG..ST-HQVSYY.D : 102
zebrafish              : S.......A....----QL.P..S.HGG..TTPHQVSYY.D :  99
Taiwan_minnow          : S.......A....----QL.P..S.HGG..ST-HQVSYY.D : 102
fathead_minnow         : S.......A....----QL.P..S.HGG..ST-HQVSYY.D : 102
roach                  : S.......S....----QL.P..S.HGG..ST-HQVSYY.D : 102
North_African_catfish  : SA.......S...----QL.P..S...AGQHVAQQVPYY.. : 134
channel_catfish        : S...P.......----QL.P..G...AGQHTAQQVPYY.. : 129
```

88

```
                               *        180         *         200
yellow_perch         : TSSTPVYRSSVPSSQQSVSM--EDQYNTSDESYSVGESGA : 128
eelpout              : ....H..--..........R--...CG........V.... : 127
largemouth_bass      : ...........L....P.PR--...CA......C...... : 174
European_sea_bass    : .................P..R--..PCG...D........ : 177
red_seabream         : ...................R--...CG...D......... : 129
black_seabream       : ...............H.A.R--...CG...D......... : 129
gilthead_seabream    : .......--......H...R--...CG...D......... : 127
wrasse               : ...........S.......R--.EHCG............. : 128
bamboo_grass_wrasse  : EG.........S.......R--.EHCG............. :  79
bastard_halibut      : ..A.S...----....P..R--..HCGPR.......... : 125
African_cichlid      : .........----..H.P.PR--D..CG.R..A.G...L.. : 125
blue_tilapia         : .........----..H.P.PR--...CG.R..A.....L.. : 125
Nile_tilapia         : .........----..H.P.PR--...CG.R..A.....L.. : 125
killifish            : .A......----..H.PA.R--...C..R..AC....L.. : 172
Javanese_ricefish    : .........----..H.GA.R--.E.CG.RE..CG...L.. : 170
Japanese_medaka      : .........----..H.GA.R--...CG.RED.C....L.. : 170
spotted_green_puffer : ..L......A.T.G.DA..R--...CG...G..GG..AVV : 177
Atlantic_salmon      : ...........V.N.L.A.E--.KLCI...RQQ.YAAA.S : 175
cherry_salmon        : ...........V.N.L.A.E--.KLCIA..RQQ.YSAA.S : 179
rainbow_trout        : ...........V.N.L.A.E--.KLCIA..RQQ.YSAA.S : 134
goldfish             : .P..T......VC...AGVGLC.ELCSAA.RQELFSG.R. : 142
Taiwan_shoveljaw_carp: .P..T......VC...AGVGLC..LCSA..RQELYSG.R. : 142
zebrafish            : ....T......V....AAVGLC.ELCSA..RQELYTG.R. : 139
Taiwan_minnow        : ....T......V....GVGLC.VLCSA..RQELYTG.R. : 142
fathead_minnow       : ....T......V....AGVGLC.VLCSA..RQEMYTG.R. : 142
roach                : ....T......L....AGVGLC.VLCSA..RQEMYP-AR. : 141
North_African_catfish: P.G........LA.AG.RV----ELCSA.SRQDVYTAV.. : 170
channel_catfish      : P.G.S......LA.AG.RV----ELCSAPGRQDVYTAV.. : 165
```

```
                                        *         220         *         240
yellow_perch        : GAGAR------GFEIVKGTRFCAVCSDYASGYYYGVWSCE : 162
eelpout             : ....G------....A.E.............H........  : 161
largemouth_bass     : .--.G------....A.EM............H........  : 206
European_sea_bass   : ..R.G------....A.DM............H........  : 211
red_seabream        : ..L.A------....A.EM............H........  : 163
black_seabream      : ....A------....A.EM............H........  : 163
gilthead_seabream   : ....A------....A.EM............H........  : 161
wrasse              : ..A.G------C...A.EM............H........  : 162
bamboo_grass_wrasse : ..A.G------C...A.EM............H........  : 113
bastard_halibut     : A...E------....A.E.............H........  : 159
African_cichlid     : .--.G------....T.E.............H........  : 157
blue_tilapia        : .--.G------....T.N.............H........  : 157
Nile_tilapia        : .--.G------....T.D.............H........  : 157
killifish           : ....GAA--AG....A.E.............H........  : 210
Javanese_ricefish   : ....G------....A.D.............H........  : 204
Japanese_medaka     : ....G------....A.D.............H........  : 204
spotted_green_puffer: ...PA------S.....EM............H........  : 211
Atlantic_salmon     : .VR--------V...ANE............H........  : 207
cherry_salmon       : .VR--------V...ANE............H........  : 211
rainbow_trout       : .VR--------V...ANE............H........  : 166
goldfish            : AG---------..DSE.E............H........  : 173
Taiwan_shoveljaw_carp : AG---------..DSE.E............H........  : 173
zebrafish           : AG---------..DSG.E............H........  : 170
Taiwan_minnow       : AG---------.LDSG.E............H........  : 173
fathead_minnow      : AG---------..DSE.E............H........  : 173
roach               : AG---------..DSE.E............H........  : 172
North_African_catfish : SRPSGASGTSGAIG...EI...........H........  : 210
channel_catfish     : SGPSGASGPSGAIG...EI...S........H........  : 205
                                      ► C domain (DBD or ZFM)
```

90

```
                                    *       260        *       280
yellow_perch       : GCKAFFKRSIQGHNDYMCPATNQCTIDRNRRKSCQACRLR : 202
eelpout            : ...................C......C.........C..C... : 201
largemouth_bass    : ...................C......C.........C..C... : 246
European_sea_bass  : ...................C......C.........C..C... : 251
red_seabream       : ...................C......C.........C..C... : 203
black_seabream     : ...................C......C.........C..C... : 203
gilthead_seabream  : ...................C......C.........C..C... : 201
wrasse             : ...................C......C.........C..C... : 202
bamboo_grass_wrasse: ...................C......C.........C..C... : 153
bastard_halibut    : ...................C......C.........C..C... : 199
African_cichlid    : ...................C......C.........C..C... : 197
blue_tilapia       : ...................C......C.........C..C... : 197
Nile_tilapia       : ...................C......C.........C..C... : 197
killifish          : ...................C......C.........C..C... : 250
Javanese_ricefish  : ...................C......C.........C.YC... : 244
Japanese_medaka    : ...................C......C.........C..C... : 244
spotted_green_puffer: ..................C......C.........C..C... : 251
Atlantic_salmon    : ...................C......C.........C..C... : 247
cherry_salmon      : ...................C......C.........C..C... : 251
rainbow_trout      : ...................C......C.........C..C... : 206
goldfish           : ...................C......C.........C..C... : 213
Taiwan_shoveljaw_carp : ................C......C.........C..C... : 213
zebrafish          : ...................C......C.........C..C... : 210
Taiwan_minnow      : ...................C......C.........C..C... : 213
fathead_minnow     : ...................C......C.........C..C... : 213
roach              : ...................C......C.........C..C... : 212
North_African_catfish : ................C......C.........C..C... : 250
channel_catfish    : ...................C......C.........C..C... : 245
```

```
                                      *         300         *         320
yellow_perch          : KCYEVGMMKGGVRKDRG-RVLRRDKRRTG-TNDRDKASKD : 240
eelpout               : ..................-H.........D-LS........ : 239
largemouth_bass       : ..................-.........A.-.......... : 284
European_sea_bass     : ..................-.......R-AG..E..... : 289
red_seabream          : ..................-......Q...-.S.......G : 241
black_seabream        : ..................-.......-.S.......G : 241
gilthead_seabream     : ..................-.......-.S.......G : 239
wrasse                : ..................-.......-.S...NG... : 240
bamboo_grass_wrasse   : ..................-.......-.S....G... : 191
bastard_halibut       : ................S-H.......A.-.......... : 237
African_cichlid       : ..................-.....E...---AY....PA.. : 233
blue_tilapia          : ..................-.....E.HG---PAQ.-QT.Q. : 232
Nile_tilapia          : ..................-.....E...---AC....PA.. : 233
killifish             : ..............E..-.........A-IS..E..V.G : 288
Javanese_ricefish     : ................V-.........-VG.G..VV.G : 282
Japanese_medaka       : ................I-.........-VG.G..VV.G : 282
spotted_green_puffer  : ................I-.......P.-NSG.E.S... : 289
Atlantic_salmon       : ................G.......YC.PAG..E.PYG. : 287
cherry_salmon         : ................G.......YC.PAG..E.PYG. : 291
rainbow_trout         : ................G.......YC.PAG..E.PYG. : 246
goldfish              : ..............E..G.A.........-NE..V.SYSE : 252
Taiwan_shoveljaw_carp : ................G.T.......A-NE..V.CYSE : 252
zebrafish             : ................G.S...E....S-NE....S.S. : 249
Taiwan_minnow         : ................G.....E....S-NE..V.SYSE : 252
fathead_minnow        : ................G.A...E....D-NE....SYSE : 252
roach                 : ................G.A...E.....-NE....SYSE : 251
North_African_catfish : ..........Y..E..G.A..HS..HG.-LKE.E.GYNE : 289
channel_catfish       : ..........F..E..G....H...P..-LKE.E.GYSK : 284
                                ► D domain
```

92

```
                               *         340         *         360
yellow_perch            : LAQ----RTVPPQDGRKRS--SAC-----GGGGKLLLTGM : 269
eelpout                 : .EH----....S..R....SI.SAC-----V...S...S. : 270
largemouth_bass         : .EY----.......R..H.SS..G-----.....SS.... : 315
European_sea_bass       : .EH----.......R..H.SS.SSSSSAV.....SS.I.. : 325
red_seabream            : .EH----..A....R..HISS..G-----.....SS.IS. : 272
black_seabream          : .EH----..A....R..HISS..A-----.....SS.IS. : 272
gilthead_seabream       : .EH----..A....R..HISS..G-----.....SS.IS. : 270
wrasse                  : RE.----......GR..HGSSVGG--------..SP.IS. : 268
bamboo_grass_wrasse     : REH----......GR..HGSSVGG--------..SP.IS. : 219
bastard_halibut         : QDH----....L.....S.SS..G--------..SS..A. : 265
African_cichlid         : .PH----TKA..H....HATS.SSTS---.....SS.NS. : 266
blue_tilapia            : .PT----HKAS.......AMS.SSTS---.P...SS.NN. : 265
Nile_tilapia            : .PH----TRAS.......AMS.SSTS---.....SS.NN. : 266
killifish               : .EP----..S.H..K...GSA--------L..D.SS.AS. : 316
Javanese_ricefish       : QEH----....HY......SS.GGGGGGG......S..S. : 318
Japanese_medaka         : QEH----...-HY......ST---GGGGG......S..S. : 314
spotted_green_puffer    : .E.-----..A....R..HHSS.GDGS----..E.CA.PE. : 321
Atlantic_salmon         : .EH----..A.....G.N.SS.SLSG---...WCGPRIT. : 320
cherry_salmon           : .EH----..A.....V.N.SS.-LNG---...W.GPRIT. : 323
rainbow_trout           : .EH----..A.....G.N.SS.-LNG---...W.GPRIT. : 278
goldfish                : QSNRTGL..A....R..S.-----------ST.V.STLC. : 281
Taiwan_shoveljaw_carp   : QSSRNCL..A.L..R...-----------SS.V.SALC. : 281
zebrafish               : QCSRAGV..TG...K...R-----------S..V.STLC. : 278
Taiwan_minnow           : QSSRAAV..A....K...-----------SS.MARALC. : 281
fathead_minnow          : QSSRVGL..P--..K...-----------SAEV.SALC. : 279
roach                   : .STRVGL..A--..K...-----------S..M.SALC. : 278
North_African_catfish   : AQSGSDA.EA......ST.GI---------.SAVAG.VC. : 320
channel_catfish         : AQSGSDV.EAL....QSS.GI---------...VAD.VC. : 315
```

```
                                      *         380         *         400
yellow_perch        : PPDQVLLLLQGAEPPILCSRQKLNRPYTEVTMMTLLTSMA : 309
eelpout             : .........C...............S.............. : 310
largemouth_bass     : S........................S.............. : 355
European_sea_bass   : ..E......................S.............. : 365
red_seabream        : ........................................ : 312
black_seabream      : ........................................ : 312
gilthead_seabream   : .........R............................. : 310
wrasse              : .........................S............[T] : 308
bamboo_grass_wrasse : .........................S...A.......... : 259
bastard_halibut     : L.......................Q............... : 305
African_cichlid     : ...............T.......Q................ : 306
blue_tilapia        : .........................S.............. : 305
Nile_tilapia        : ...............S.....S.................. : 306
killifish           : .SE.....F................S.............. : 356
Javanese_ricefish   : ..E......................S.............. : 358
Japanese_medaka     : ..E......................S.............. : 354
spotted_green_puffer: ..E...FQ.................S.....A.......N.. : 361
Atlantic_salmon     : ..E...F........A.......A................ : 360
cherry_salmon       : ..E...F........A.......A................ : 363
rainbow_trout       : ..E...F........A.......A................ : 318
goldfish            : ........L.....A......HS.............N.. : 321
Taiwan_shoveljaw_carp : ........L.....A......HS.............N.. : 321
zebrafish           : ........L.....A......HS.............N.. : 318
Taiwan_minnow       : ........L.....A......HS.............N.. : 321
fathead_minnow      : ........L.....A......HSP............N.. : 319
roach               : .S......L.....A......HSP............N.. : 318
North_African_catfish : A.E......LR....T.S.....S...........N.. : 360
channel_catfish     : S.E......LR....T......HS...........N.. : 355
                    ► E domain (LBD)
```

94

```
                                    *        420         *        440
yellow_perch          : DKELVHMIAWAKKLPGFLQLGLHDQAQLLESSWLEVLMIG : 349
eelpout               : ...........................V............ : 350
largemouth_bass       : ........T...........S....V............. : 395
European_sea_bass     : ....................S....V............. : 405
red_seabream          : ....................S....V............. : 352
black_seabream        : ....................S....V............. : 352
gilthead_seabream     : ....................S....V............. : 350
wrasse                : ....................T....V............. : 348
bamboo_grass_wrasse   : ....................T....V............. : 299
bastard_halibut       : ....................S....V............. : 345
African_cichlid       : ....................S....VL............ : 346
blue_tilapia          : ........T...........S....VL............ : 345
Nile_tilapia          : ........T...........S....VL............ : 346
killifish             : ....................A....VL............ : 396
Javanese_ricefish     : ....................S....VL............ : 398
Japanese_medaka       : ....................S....VL............ : 394
spotted_green_puffer  : ....................S....VK............ : 401
Atlantic_salmon       : ................Q..S....V............. : 400
cherry_salmon         : ................Q..S....V............. : 403
rainbow_trout         : ................Q..S....V............. : 358
goldfish              : ................QD.S...KV............. : 361
Taiwan_shoveljaw_carp : ................QD.S....V............. : 361
zebrafish             : ................QD.S....V............. : 358
Taiwan_minnow         : ................QD.S....V............. : 361
fathead_minnow        : ................QD.S....V............. : 359
roach                 : ................QD.S....V............. : 358
North_African_catfish : ................QD.S....V............. : 400
channel_catfish       : ................QD.S....V............. : 395
```

95

```
                                          *         460         *         480
yellow_perch         : LSWRSIHCPGKLIFAQVLILDRNVGDCVEGMAEIFDMLLA : 389
eelpout              : .I..............D......E................ : 390
largemouth_bass      : .I..............D......E.......FV........ : 435
European_sea_bass    : .I..............D.....SE................ : 445
red_seabream         : .I..............D.....SE................ : 392
black_seabream       : .I........F....DF....SE................ : 392
gilthead_seabream    : .I..............D.....SE................ : 390
wrasse               : .I..............D.....SE................ : 388
bamboo_grass_wrasse  : .I..............D.....SE...............S : 339
bastard_halibut      : .I..............D......E................ : 385
African_cichlid      : .I..............D.....TE.T.............. : 386
blue_tilapia         : .I....Q.......E.D......E.T.............. : 385
Nile_tilapia         : .I....Q.........D......E.T.............. : 386
killifish            : .I..............D......E.......T........ : 436
Javanese_ricefish    : .I...T..........D......E.......T........ : 438
Japanese_medaka      : .I..............D......E.......T........ : 434
spotted_green_puffer : .I.....Y.......PD......E................ : 441
Atlantic_salmon      : .I..............D.....SE................ : 440
cherry_salmon        : .I..............D.....SE................ : 443
rainbow_trout        : .I..............D.....SE................ : 398
goldfish             : .I.....S........D......E.E.............. : 401
Taiwan_shoveljaw_carp: .I.....S........D.....SE.E.............. : 401
zebrafish            : .I.....S........D.....SE.E.............. : 398
Taiwan_minnow        : .I.....S........D.....SE.E.............. : 401
fathead_minnow       : .I.....S........D......E.E.............. : 399
roach                : .I.....S........D......E.E.............. : 398
North_African_catfish: .I.....S........D.....TE.E.............. : 440
channel_catfish      : .I....YT........D.....SE.E.............. : 435
```

```
                                       *         500         *         520
yellow_perch          : TASRFRLLKLKPEEFVCLKAIILLNSGAFYFCTGTMEPLH : 429
eelpout               : .T............................S...A...... : 430
largemouth_bass       : ..............................S.......... : 475
European_sea_bass     : ..............................S.......... : 485
red_seabream          : ..............................S.......... : 432
black_seabream        : ..............................S.......... : 432
gilthead_seabream     : ..............................S.......... : 430
wrasse                : .T............................S.......... : 428
bamboo_grass_wrasse   : ..............................S.......... : 379
bastard_halibut       : .........S...F............S.S.......... : 425
African_cichlid       : ..............................S.......... : 426
blue_tilapia          : .V-...........................S.......... : 424
Nile_tilapia          : ..............................S.......... : 426
killifish             : ..............................S.......... : 476
Javanese_ricefish     : ..............................S.......... : 478
Japanese_medaka       : ..............................S.......... : 474
spotted_green_puffer  : .T......Q..............F.....S.......... : 481
Atlantic_salmon       : .V............................S...N...S.. : 480
cherry_salmon         : .V............................S...N...S.. : 483
rainbow_trout         : .V............................S...N...S.. : 438
goldfish              : .V....S....L..................S...SP....M : 441
Taiwan_shoveljaw_carp : .VA...S....L..................S...SP....M : 441
zebrafish             : .VA...S....L..................S...SP....M : 438
Taiwan_minnow         : .VA...S....L..................S...SP....M : 441
fathead_minnow        : .VA.L.S....L..................S...SP....M : 439
roach                 : .VA...S....L..................S...SP....M : 438
North_African_catfish : .VA...A....S...............S..S.Y.SP....R : 480
channel_catfish       : .VA...T....S..................S...SP....R : 475
```

```
                                      *         540         *         560
yellow_perch         : DTAAVQNMLDTITDALIHHISQSGCSVQQQSRRQAQLLLL : 469
eelpout              : ......H.......T.....G................... : 470
largemouth_bass      : ..VE.H.....................A............ : 515
European_sea_bass    : ..........................A............ : 525
red_seabream         : .G.................N.....A............. : 472
black_seabream       : .G.................N.....A............. : 472
gilthead_seabream    : ..................N.....A............. : 470
wrasse               : .NE.......I...............AH........... : 468
bamboo_grass_wrasse  : ..........I.................H.......... : 419
bastard_halibut      : .........E...............P....W........ : 465
African_cichlid      : ......H..........F....L...A.H.......... : 466
blue_tilapia         : ..V...H..........F...HF...A............ : 464
Nile_tilapia         : ......H..........F...HL...A............ : 466
killifish            : ..V......................F.....A........ : 516
Javanese_ricefish    : ......S..................YLA...A....R.... : 518
Japanese_medaka      : ......S...........Y.....YLA...A......... : 514
spotted_green_puffer : ..........N.M...........F.A...F......... : 521
Atlantic_salmon      : ..S...S...N.........H..A.....P......... : 520
cherry_salmon        : ..S...S...N.........H..A.....P......... : 523
rainbow_trout        : ..S...S...N.........H..A.....P......... : 478
goldfish             : ..FM..C...N....F.YG..K..A...L.......... : 481
Taiwan_shoveljaw_carp: ..FM..C...N...G..YC..K..A...L.A........ : 481
zebrafish            : .NFM..C...N......YC..K..A...L.......... : 478
Taiwan_minnow        : ..FT..C...N......YC..K..A...L.......... : 481
fathead_minnow       : ..FM..C...N......YG..K..A...L.......... : 479
roach                : ..FM..C...N......YG..K..A...L.......... : 478
North_African_catfish: .GFM..C...N.......Y.....IP..L.......... : 520
channel_catfish      : .GFM..C...N......YY.....I...L.......... : 515
```

```
                                           *       580          *       600
yellow_perch         : LSHIRHMSNKGMEHLYSIKCKNKVPLYDLLLEMLDAHHVQ : 509
eelpout              : .......................................R.H : 510
largemouth_bass      : .......................................R.H : 555
European_sea_bass    : .......................................R.. : 565
red_seabream         : .......................................R.H : 512
black_seabream       : .......................................R.H : 512
gilthead_seabream    : .......................................R.H : 510
wrasse               : .......................................R.H : 508
bamboo_grass_wrasse  : .......................................R.H : 459
bastard_halibut      : .......................................C.H : 505
African_cichlid      : ......................................QR.H : 506
blue_tilapia         : .......................................R.H : 504
Nile_tilapia         : .......................................R.H : 506
killifish            : .......................................R-H : 555
Javanese_ricefish    : .......................................R.H : 558
Japanese_medaka      : .......................................R.H : 554
spotted_green_puffer : .....Q................................QR.. : 561
Atlantic_salmon      : .....................................G.R.. : 560
cherry_salmon        : .....................................G.R.. : 563
rainbow_trout        : .....................................G.R.. : 518
goldfish             : ...............R....................QRFH : 521
Taiwan_shoveljaw_carp : ...............R....................QRFH : 521
zebrafish            : ...............R....................QRF. : 518
Taiwan_minnow        : ...............H....................QRF. : 521
fathead_minnow       : ...............H....................QRF. : 519
roach                : ...............H....................QRF. : 518
North_African_catfish : ........Y.......N..................R.R : 560
channel_catfish      : ........Y.........................R.R : 555
```

99

```
                                           *         620         *          640
yellow_perch            : RPDRPDQPWSQADGELPSTISNSSNSS----GGGSSSGGS : 545
eelpout                 : .....G.S.FP....TLC.T..N----------I..... : 539
largemouth_bass         : .....A.F.......P.FITV.NC...--SN..V...V.. : 593
European_sea_bass       : .....A.S.......P.F...TNN.NNNISG..S...A.. : 605
red_seabream            : .A...A.T.....R.P.F.SR-N.SGGGGGG......A.. : 551
black_seabream          : .....H.T.....R.P.F.SR-NNRG.--GG......A.. : 549
gilthead_seabream       : .....A.T.....R.PLF.SR-N.S..SGGG......A.. : 549
wrasse                  : .....A.S.Y.T.R.PAY.S.A..TND-------N..SSP : 541
bamboo_grass_wrasse     : .....A.S...T...PAYITNTN..N.--------...SS. : 492
bastard_halibut         : ..A...A.S.L...R.PSAAGN.NN...S-IIIS.GG.SSA : 544
African_cichlid         : ..V...S.S...G.RDS.---NT..SGGGGSDDE....A.. : 543
blue_tilapia            : ..V...S.S...G.RDS..AS.T..RGGGGGDDE.A..A.. : 544
Nile_tilapia            : ..V..F.S...G.RDS..AS.T..SGGGGGDDE.A..A.. : 546
killifish               : H.V..----..DGKSP...S.FGAGCE-----.....A.. : 586
Javanese_ricefish       : ..V.AP.SL..STETP.LPAAAGAELL-----PLFEAESR : 593
Japanese_medaka         : H.V.AP.SL..V.RDP...S.GGGGIA-----P..I.ASR : 589
spotted_green_puffer    : ..E------------------------------------- : 564
Atlantic_salmon         : S.G.VA.AGE.TE.PST..T.S.G-----------..I.P : 589
cherry_salmon           : S.G.VA.AGE.TE.PST..T.S.G-----------..I.P : 592
rainbow_trout           : S.G.VA.AGE.TE.PST..T.S.G-----------..I.P : 547
goldfish                : SSG.VQRL.A.SEKDP.-----------------..PT. : 543
Taiwan_shoveljaw_carp   : SSE.VQRA.A.SEKDPQ-----------------..PT. : 543
zebrafish               : SSG.VQRV...SEKNP.-----------------..PT. : 540
Taiwan_minnow           : T.G.VQRL.A.SEKDP.-----------------..PT. : 543
fathead_minnow          : S.GEVQRLGA.SEKDP.-----------------..P-- : 539
roach                   : T.GEVQRLRA.SEKDP.-----------------..P-- : 538
North_African_catfish   : PLG.VSKS.ADRVSN..V.S.L.QTA---------..TT. : 591
channel_catfish         : PLG.VPRI.ADRVSSS...TAT.PT.N-------...TT. : 588
                          ▶ F domain
```

100

```
                                      *        660         *        680
yellow_perch           : NSGPRVSRESP-------------------SRAPTVLQYA : 566
eelpout                : G......HD..---------------------.........G : 560
largemouth_bass        : S......H...S----------------RGPTG.G....G : 617
European_sea_bass      : S......H...S----------------RGPTC.G....G : 629
red_seabream           : T......H..------------------PTS.G....G : 571
black_seabream         : T..T...L.N------------------PTG.G....G : 569
gilthead_seabream      : T...Q.NL.-------------------PTG.G...LR : 569
wrasse                 : -A.S.A.Q...N----------------RPPTGH.....G : 564
bamboo_grass_wrasse    : SA.....L...N----------------KTPIGQG....G : 516
bastard_halibut        : S..H.G.Q...S----------------RA.TG.....HG : 568
African_cichlid        : S...QGNH...RCE-----------NLSRAPTG.G....R : 572
blue_tilapia           : S...QG.H...RRE-----------NLSRAP.G.G....R : 573
Nile_tilapia           : S...QG.H...RRE-----------NLSRAPTG.G....R : 575
killifish              : S....G.GD----------------NLMRIPS..G....G : 610
Javanese_ricefish      : VRVEAPPPPASFSMEGRVLTAPRPFKTERTVQG.VF.F.G : 633
Japanese_medaka        : GRIESP.--------------------RGPF.......G : 608
spotted_green_puffer   : ----------------------------------------- :   -
Atlantic_salmon        : MR.SQD.HIR-------------------.PGSG....G : 609
cherry_salmon          : MR.SQD.HIR-------------------.PGVLQYGSP : 612
rainbow_trout          : MR.SQD.HIR-------------------.PGSG....G : 567
goldfish               : S.-----------------------------.G.GT.LPN : 554
Taiwan_shoveljaw_carp  : S.SS------------------------P..G.GA.LPN : 558
zebrafish              : S.SSSNNS--------------------PRGGAAA..SN : 559
Taiwan_minnow          : S.SSS-----------------------P..G.GA..PN : 559
fathead_minnow         : ----------------------------P..G.GAT.PN : 550
roach                  : ----------------------------S..G.GA..PN : 549
North_African_catfish  : T.TNQQ.S--------------------APPC.AD.PSN : 610
channel_catfish        : T.THHP.N--------------------G.TC.AD.PSN : 607
```

101

```
                                           *
yellow_perch             : ESRSDCTRIL---- : 576
eelpout                  : G.I....H..---- : 570
largemouth_bass          : G......H..---- : 627
European_sea_bass        : G......H..---- : 639
red_seabream             : G...E..H..---- : 581
black_seabream           : R.APSAPHP.KPTE : 583
gilthead_seabream        : ----VHPHP.KPTE : 579
wrasse                   : G......H..---- : 574
bamboo_grass_wrasse      : G..P...H..---- : 526
bastard_halibut          : G..P...H..---- : 578
African_cichlid          : G.H....P..---- : 582
blue_tilapia             : G.H......P---- : 583
Nile_tilapia             : G.H......P---- : 585
killifish                : G.....AQ..---- : 620
Javanese_ricefish        : G..VQVGTS----- : 642
Japanese_medaka          : G..P...PA.QD-- : 620
spotted_green_puffer     : ------------- :    -
Atlantic_salmon          : SPS..QMP.PLEQK : 623
cherry_salmon            : S.DQMPIP------ : 620
rainbow_trout            : SPS..QMP.P---- : 577
goldfish                 : IACHEQ.PDP---- : 564
Taiwan_shoveljaw_carp    : IACH.Q.PDP---- : 568
zebrafish                : GACHSH.PDP---- : 569
Taiwan_minnow            : TAC----PDP---- : 565
fathead_minnow           : TGCLSQ.PDP---- : 560
roach                    : TACLSQ.PEP---- : 559
North_African_catfish    : PPC..Q.PSP---- : 620
channel_catfish          : PPGPGQ.PSP---- : 617
```

Figure 3.2 Alignment of yellow perch ERα deduced amino acid sequence with other teleost ERαs.

Conserved amino acid residues are indicated with (.), inserted gaps are indicated with (-) and structural domains are identified at the bottom of the alignment in bold. The nine conserved cysteines of the C domain are shaded, the ten amino acid residues corresponding to the P- and D-boxes are boxed and the eight amino acid region corresponding to AF2 is underlined. Sequences were downloaded from the EMBL/GenBank database with the following accession numbers: eelpout (*Zoarces viviparous*) AAO66473; largemouth bass (*Micropterus salmoides*) AAG44622; European sea bass (*Dicentrarchus labrax*) CAD43599; red seabream (*Pagrus major*) BAA22517; black seabream (*Acanthopagrus schlegelii*) AAL82743; gilthead seabream (*Sparus aurata*) CAB51479; wrasse (*Halichoeres tenuispinis*) AAP72178; bamboo grass wrasse (*Pseudolabrus japonicus*) ABB96483; bastard halibut (*Paralichthys olivaceus*) BAB85622; African cichlid (*Astatotilapia burtoni*) AAR82891; blue tilapia

(*Oreochromis aureus*) CAA63774; Nile tilapia (*Oreochromis niloticus*) AAD00245; killifish (*Fundulus heteroclitus*) BAC76957; Javanese ricefish (*Oryzias javanicus*) AAX13999; Japanese medaka (*Oryzias latipes*) BAA86925; spotted green puffer (*Tetraodon nigroviridis*) CAG03596; Atlantic salmon (*Salmo salar*) AAY25396; cherry salmon (*Oncorhynchus masou*) AAS92970; rainbow trout (*Oncorhynchus mykiss*) CAB45140; goldfish (*Carassius auratus*) AAL12298; Taiwan shoveljaw carp (*Onychostoma barbatula*) CAD67996; zebrafish (*Danio rerio*) BAB16893; Taiwan minnow (*Candidia barbatus*) CAD32175; fathead minnow (*Pimephales promelas*) AAU87498; roach (*Rutilus rutilus*) BAD91035; North African catfish (*Clarias gariepinus*) CAC37560; channel catfish (*Ictalurus punctatus*) AAG24543.
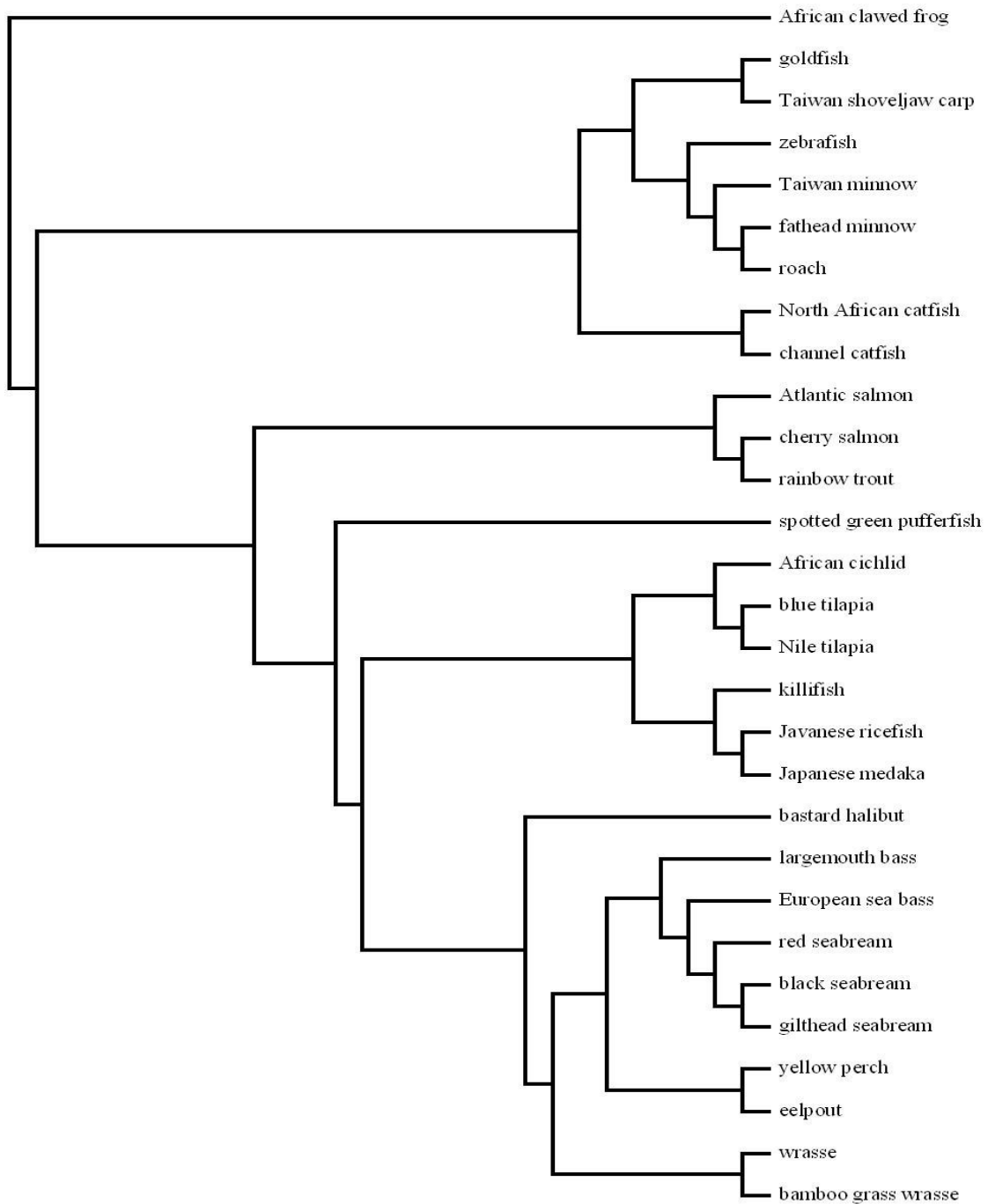
Figure 3.3 Phylogenetic tree of ERα amino acid sequences.

Tree produced using a Clustal X (1.81) alignment and TreeView (Win 32) v. 1.6.6. The tree was rooted with African clawed frog (*Xenopus laevis*) ERα (Accession: AAQ84782) as an outgroup representing the closest available species not in the group Teleostei. See Figure 3.2 for EMBI/GenBank accession numbers for the other sequences used.

```
-546  ACTCTGTGTTTGGCCTTCCTGCAGCACATAACAGCTCCCCAAACTATTGATTCC
-492  ACACTTTACCAGCGTTTGACACCGCAGCGGGACTTCATATTGTCAGACTGTGAC
-438  TGCGCTTGTTGTTTGTGGAAGAAGGGGTGGAAAGGCCCGGCACACGTCTTACTC
-384  AAAGTGTTATCACTTAATGATGTGAAACTCATGGCTGGACTGATCACTGAACAT
-330  ATTCCCTGCTGTGTTCACTATCCTCTGGATCATTTCACATCTCCGACTCTATGT
-276  GCTCACAGTGAGCACAGTATAAATCACAGTCAGCAACAGGTTGGATTGGAGTCA
-222  TCCGTCACCCTGGGAATTTGCAATGTGAGCTCATGAGTAACATTAAACCTCCCT
-168  CGGCCTGTGTAGGTAGTTGTGCTGGCCTTTGCTTTTGTTTTTCAGGGCCAGTAC
-114  TCGTAGATAATGATATTATCTCAGTAAACTCGACACCAGGAGAGAACTCTACCC
-60   TAGGCTCGTATTCTTTATGATGTGGTAAATCTTGCGATACTGACACCGTCTGTA
```

▶A/B domain

```
          M   A   A   A   S   S   L   E   K   N   Q   P   L   L   Q   L    16
-6   GTTGCGATGGCTGCTGCTTCCTCTTTAGAGAAGAATCAGCCCCTCCTCCAGCTC

      Q   E   V   D   S   S   R   V   D   S   R   V   L   A   P   V   L   S    34
49   CAGGAGGTGGACTCCAGTCGAGTTGACAGTCGCGTCCTCGCTCCGGTCCTCAGC

      S   S   S   P   G   L   S   L   E   T   S   Q   P   I   C   I   A   S    52
103  TCCTCCTCTCCTGGCCTGTCCCTGGAAACCAGCCAACCCATCTGCATCGCTTCC

      P   Y   T   E   F   G   H   D   F   T   T   I   P   F   Y   S   P   A    70
157  CCTTACACTGAATTTGGCCACGACTTCACCACCATACCTTTCTACAGTCCAGCT

      I   F   S   Y   A   S   P   G   I   P   D   G   P   S   V   H   Q   S    88
211  ATCTTCAGTTATGCCAGTCCGGGCATTCCAGATGGCCCCTCTGTCCATCAGTCA

      L   S   P   S   I   F   W   P   S   H   G   R   V   G   P   P   V   P    106
265  CTAAGCCCCTCCATATTCTGGCCCAGCCACGGCCGCGTGGGGCCACCCGTACCC

      L   H   H   S   P   A   R   T   Q   H   G   Q   P   I   Q   S   P   W    124
319  CTGCACCACTCCCCGGCTCGGACTCAGCACGGGCAGCCAATCCAGAGTCCATGG

      V   E   L   S   P   R   D   T   V   F   T   T   S   K   S   V   K   R    142
373  GTGGAGCTGTCGCCGCGGGACACTGTCTTTACAACCAGTAAGAGTGTGAAGAGG

      R   S   Q   E   S   E   E   A   V   V   S   S   G   G   K   V   D   L    160
427  CGTTCTCAGGAGAGCGAGGAGGCGGTGGTGTCATCCGGCGGGAAGGTGGACCTC
```

▶ C domain (DBD or ZFM)

```
      H   Y   C   A   V   C   N   D   H   A   S   G   Y   H   Y   G   V   W    178
481  CACTACTGTGCTGTGTGTAACGACCACGCCTCGGGCTACCACTACGGCGTCTGG

      S   C   E   G   C   K   A   F   F   K   R   S   I   Q   G   H   N   D    196
535  TCGTGTGAGGGGTGTAAGGCCTTCTTCAAGAGGAGCATCCAAGGACACAATGAC

      Y   I   C   P   A   T   N   Q   C   T   I   D   K   N   R   R   K   S    214
589  TACATCTGCCCCGCAACCAATCAATGCACTATAGACAAGAATCGCCGTAAGAGC
```

```
        C  Q  A  C  R  L  R  K  C  C  E  V  G  M  T  K  C  G  232
643  TGCCAGGCGTGCCGCCTTCGCAAATGCTGCGAAGTTGGTATGACCAAGTGTGGT
```

▶D domain

```
        M  R  K  E  R  G  N  Y  R  N  P  Q  T  R  R  V  T  R  250
697  ATGCGAAAGGAGCGTGGAAACTACAGGAACCCCCAGACGAGGCGAGTGACCCGT

        L  S  S  Q  G  R  T  N  R  P  K  A  L  S  G  P  T  V  268
751  CTGTCCTCACAGGGCAGGACCAACAGACCGAAGGCGTTAAGCGGACCAACGGTG

        G  L  L  P  A  P  H  P  P  A  L  T  P  E  Q  L  I  G  286
805  GGTTTGTTACCCGCACCCCACCCTCCTGCACTGACCCCAGAGCAGCTGATTGGG

        Q  I  M  E  A  E  P  P  E  I  Y  L  M  K  D  M  M  R  304
859  CAGATAATGGAGGCGGAGCCGCCGGAGATCTACCTCATGAAGGACATGATGAGG
```

▶E domain (LBD)

```
        P  L  T  E  A  N  V  M  M  S  L  T  N  L  A  D  R  E  322
913  CCGCTGACTGAAGCCAACGTCATGATGTCGCTCACCAACCTGGCTGATAGGGAG

        L  V  H  M  I  S  W  A  K  K  I  P  G  F  V  E  L  S  340
967  CTGGTTCACATGATCAGCTGGGCCAAGAAGATTCCAGGGTTTGTAGAGCTCAGC

        L  L  D  Q  V  H  L  L  E  C  C  W  L  E  V  L  M  I  358
1021  CTCTTGGACCAGGTGCACCTGCTGGAGTGCTGCTGGCTGGAGGTGCTGATGATC

        G  L  M  W  R  S  V  D  H  P  G  K  L  I  F  S  P  D  376
1075  GGACTGATGTGGAGATCAGTGGACCATCCAGGGAAACTTATCTTCTCCCCTGAC

        L  G  L  S  R  E  E  G  N  C  V  Q  G  F  S  E  I  F  394
1129  CTCGGCCTGAGCAGAGAAGAGGGGAACTGTGTCCAGGGCTTCTCAGAAATCTTT

        D  M  L  L  A  A  T  S  R  V  R  E  L  K  L  Q  K  E  412
1183  GATATGCTGTTGGCTGCTACGTCCAGGGTGAGAGAACTCAAGCTCCAGAAGGAG

        E  Y  I  C  L  K  A  M  I  L  L  N  S  N  M  C  L  S  430
1237  GAGTATATCTGCCTCAAGGCCATGATCCTCCTTAACTCCAACATGTGCCTCAGC

        V  T  E  G  S  E  E  L  Q  S  R  S  K  L  L  R  L  L  448
1291  GTGACCGAGGGCAGCGAGGAGCTGCAGAGTCGCTCCAAGCTCCTACGACTTCTG

        D  A  V  T  D  A  L  V  W  A  I  A  K  T  G  L  T  F  466
1345  GACGCTGTGACGGACGCTCTGGTGTGGGCCATCGCCAAAACCGGCCTCACCTTT

        R  Q  Q  Y  T  R  L  A  H  L  L  M  L  L  S  H  I  R  484
1399  CGCCAACAGTACACCCGCCTCGCCCACCTGCTCATGCTGCTCTCGCACATCCGC

        H  A  S  N  K  G  M  D  H  L  H  C  M  K  M  K  N  V  502
1453  CATGCCAGTAACAAAGGCATGGACCACCTCCACTGCATGAAAATGAAGAACGTG

        V  P  L  Y  D  L  L  L  E  M  L  D  A  H  I  M  H  S  520
1507  GTGCCTTTGTATGACCTGTTGCTGGAGATGTTGGATGCCCACATCATGCACAGC
```

106

```
      ►F domain
       S   R   L   P   R   Q   P   M   Q   Q   E   S   G   D   Q   T   G   V  538
1561  TCCCGTCTGCCTCGCCAGCCTATGCAGCAGGAGTCCGGGGACCAGACGGGGGTT

       P   A   R   P   L   S   S   H   S   G   P   S   N   T   W   T   P  *** 555
1615  CCTGCTCGGCCGCTCAGCTCTCATAGCGGACCCTCAAACACCTGGACTCCATAG

1669  CAGCACTCTGGAGGTGGACCACAGCAGTCGAATGCAATGAATTGTCACCGCTTT
1723  GCACAAAACTAGTTCTGACGAGACGTTTTACTGGAACATTCTGCTGAATTTTGT
1777  GAACCATCATATGTCGCTCAAGCTTTGACACACTGCGCACTTATTTTAGTGAAC
1831  TTCTGGATGTTAAGACATCACTCTGCAGACGGACCGAACTTGCAGTATTCTGTG
1885  CCTTATCATCCCCACAAAAAAAAAAAAAAAAA
```

Figure 3.4 Nucleotide and deduced amino acid sequences of yellow perch ERβa cDNA.

The nucleotides (lower row) are numbered on the left and the amino acids (upper row) are numbered on the right and both begin with the initiation methionine. The six functionally independent domain (A, B, C, D, E & F) putative start points are indicated with black arrows above the amino acids. Domain C is also referred to as the DNA binding domain (DBD) or the zinc finger motif (ZFM) and domain E is also referred to as the ligand binding domain (LBD). The nine conserved cysteines of the C domain are shaded and the ten amino acid residues corresponding to the P- and D-boxes are boxed. The nine amino acids of the E (LBD) domain recognized to be involved in E_2 binding are in bold and the eight amino acid region corresponding to AF2 is underlined. The nine supplemental ATG initiation codons in the 5'-UTR are double underlined and the single in-frame ORF beginning at base -356 is underlined in bold. Yellow perch ERβa cDNA sequence will be available from the EMBL/GenBank database with accession #DQ984125 on or before October 27, 2007.

```
                                           *        20         *        40
yellow_perch_ERBa      : ------------------MAAASSLEKN-QPLLQLQEVDS :  21
bastard_halibut_ERB    : ------------------M..A.A...-........... :  20
largemouth_bass_ERy    : ------------------..G.C.....-.......K... :  21
Atlantic_croaker_ERy   : ------------------..V...P...-.......K... :  21
black_seabream_ERB     : ------------------..V.C.P...-.S.....K... :  21
gilthead_seabream_ERB  : ------------------..V.C.P...-.S.....K... :  21
Nile_tilapia_ERB       : ----------------M......P..----......... :  19
killifish_ERBA         : ------------------......P..H-.......... :  21
Javanese_ricefish_ERB  : ------------------.GTTLDA..H-.......... :  21
Japanese_medaka_ERB    : ------------------.GTTLDS..H-.......... :  21
Atlantic_salmon_ERB    : MSQYRRLPGLPSELPQSP...SPLP...SAT..K.....P :  40
zebrafish_ERB2         : --------------------MSEYP.G.-S......... :  19
rainbow_trout_ERB      : -MHQQSPVDDVTALNSSA.TMSEYP.GE-S.-....D... :  37
goldfish_ERB1          : ----------MTALNSYAF.MSEYA.G.-SS........ :  29
common_carp_ERB        : --------------------MSVYP.G.-S......... :  19
Taiwan_shoveljaw_carp  : --------------------MSEYP.G.-S......... :  19
Spiny barb minnow_ERB  : ----------MTALNSSDFTMSEYP.G.-S........ :  29
Japanese_eel_ERB       : ------------------M.G.PGNE-L.......... :  19
conger_eel_ERB         : ---------------MMAKMTG.PGNE-L......... :  23
                       ▶ A/B domain

                                           *        60         *        80
yellow_perch_ERBa      : SRVD-----SRVLAPVLSSSSPGLSLETSQPICIASPYTE :  56
bastard_halibut_ERB    : ...R-----.C..S.............DG......P..... :  55
largemouth_bass_ERy    : ...G-----....S...N.P-----.........T....D :  51
Atlantic_croaker_ERy   : ...G-----GQ..S.T....-----.........T....D :  51
black_seabream_ERB     : ...--------..S.....P-----...N.....P....D :  48
gilthead_seabream_ERB  : ...--------..S.....P-----...N.....P....D :  48
Nile_tilapia_ERB       : ..AG-----....S...G.......H........R....D :  54
killifish_ERBA         : ...G-----....S.G..N----------.A...PA...D :  46
Javanese_ricefish_ERB  : ..TG-----.C..S.N.N.......HD.......PA..AD :  56
Japanese_medaka_ERB    : ..AG-----.C..S.N.........H.......PA..AD :  56
Atlantic_salmon_ERB    : ...GR---GG...S..F.AP..A.P..A-H....P....D :  76
zebrafish_ERB2         : G..G-----GH..S..FN....S.P..N-H....P....D :  53
rainbow_trout_ERB      : ...G-----GH..S..FN....S.P...-H....Q....D :  71
goldfish_ERB1          : ...G-----GH..S.TFN....S.P...-H....P....D :  63
common_carp_ERB        : ...G-----GH..S.AFN....S.P...-H....P....D :  53
Taiwan_shoveljaw_carp  : ...G-----GH..S..FN....S.P...-H....P....D :  53
Spiny barb minnow_ERB  : ...G-----GH..S..FN...SS.P.DR-HL...P....D :  63
Japanese_eel_ERB       : ...GESGGS.G..PT.YNGAL.A.....-HA...P....D :  58
conger_eel_ERB         : ...GENGGSAG..PS.YNGAL.T....N-HA...P....D :  62
```

```
                               *         100         *         120
yellow_perch_ERBa       : FGHDFTTIPFYSPAIFSYAS-PGIPDGPSVHQSLSPSIFW :  95
bastard_halibut_ERB     : L....A.....G.T.....A-.S...C............. :  94
largemouth_bass_ERy     : HS.........N.T......-...S.R............. :  90
Atlantic_croaker_ERy    : L....P.......T.....G-.S.S.CT......N..... :  90
black_seabream_ERB      : R....PA......TN....NP.S.S.R........S.... :  88
gilthead_seabream_ERB   : R....P......ATN....NP.A.S.R............. :  88
Nile_tilapia_ERB        : L............T....GG-.S.SECS.......A.... :  93
killifish_ERBA          : L.....P.....T....SG-Q..SEC.........A.... :  85
Javanese_ricefish_ERB   : LS.........N.T.....N-.S.SEC........A.... :  95
Japanese_medaka_ERB     : LS.........N.T.....-.S.SEC........A.... :  95
Atlantic_salmon_ERB     : I....NP.S....T.L...G-.A...C..A.......... : 115
zebrafish_ERB2          : L..............LG.S.-SP.S.CS..R......... :  92
rainbow_trout_ERB       : L..............LG.G.-SP.SECS..R......... : 110
goldfish_ERB1           : L............S.LG.G.-SP.S.C...R......... : 102
common_carp_ERB         : LS.............LG.GA-AP.S.C...R......... :  92
Taiwan_shoveljaw_carp   : L..............LG.GA-SP.S.C...R......... :  92
Spiny barb minnow_ERB   : L..............LG.G.-SP.S.C...R......... : 102
Japanese_eel_ERB        : SS...AA.T....P.L.HGG-.A..ES.AAR......... :  97
conger_eel_ERB          : S.......T..R.P.LGH.G-.A..E..D..P........ : 101


                               *         140         *         160
yellow_perch_ERBa       : PSHGRVGPPVPL-HHSPARTQHGQPIQSPWVELSPRDTVF : 134
bastard_halibut_ERB     : ....H.....T.-.R.QG...Q........G......G.L : 133
largemouth_bass_ERy     : ....H..S....-...QP.P.YR....N..A....LE.TL : 129
Atlantic_croaker_ERy    : ..R.H..S....-...----............L.N.L : 125
black_seabream_ERB      : ....H..TT...-.LQ..P.............L.N.L : 127
gilthead_seabream_ERB   : ....H..TT...-.LQ..P....A.........L.N.L : 127
Nile_tilapia_ERB        : .......T..T.-.CPQG...Q..SA....------...I : 126
killifish_ERBA          : ..R.H..AA...-.R.Q..A..T.AA.N..------E..L : 118
Javanese_ricefish_ERB   : Q..SH...T...-.R.Q..A..........------.G.L : 128
Japanese_medaka_ERB     : Q...H...T...-.R.Q..A..........------.G.L : 128
Atlantic_salmon_ERB     : .PQAH.....S.H.RPQS.P.Q...TRVS.A.P---HA.S : 152
zebrafish_ERB2          : .P.SH.SS-.T.-QQQ-S.L.QNHATSGT.T.H..H.H.E : 129
rainbow_trout_ERB       : .P.SQ.SS-.A.-.QQHT.L.QNH.TGGT.T....H.HSE : 148
goldfish_ERB1           : .P.SH.SS-.A.-.QQQT.L.PNH.TGGT.A....H.HGE : 140
common_carp_ERB         : .P.NH.SS-.V.-.QQQT.L.QNH.TGGS.A....H.HSE : 130
Taiwan_shoveljaw_carp   : .P.SH.SS-.A.-.QQQT.L.QNH.AGGT.A....H.HSE : 130
Spiny barb minnow_ERB   : .P.SH.SS-.T.-.QQQT.L.QNH.AGGT.A....H.HGE : 140
Japanese_eel_ERB        : .A..HH.HVS..AL.FQQPLVYR..AH...A.PK.LEHGQ : 137
conger_eel_ERB          : ....HH.HVPQ.AL.FQQPLLYR..PH...GDPK.LEQGH : 141
```

```
                                                 *        180         *        200
yellow_perch_ERBa     : TTS---------------KSVKRRSQESEEAVVSSG---- : 155
bastard_halibut_ERB   : AN.---------------.G.........DG.....---- : 154
largemouth_bass_ERy   : ...---------------.............S....---- : 150
Atlantic_croaker_ERy  : K.KQDGASLPLAVVPVRH..A................---- : 161
black_seabream_ERB    : ...---------------..A.........GE....---- : 148
gilthead_seabream_ERB : ...---------------..A.....N..GE....---- : 148
Nile_tilapia_ERB      : ...---------------..A.........S.....---- : 147
killifish_ERBA        : A..---------------..A..L............---- : 139
Javanese_ricefish_ERB : ...---------------.G.................---- : 149
Japanese_medaka_ERB   : ...---------------.G.................---- : 149
Atlantic_salmon_ERB   : E..---------------P...C......T...LE---- : 173
zebrafish_ERB2        : EEN--------------S.P.V..VAD...TS..LR---- : 151
rainbow_trout_ERB     : EEY--------------R.P.V..VADA..TST.LR---- : 170
goldfish_ERB1         : EEN--------------C.P.S..VAVA..TST.LR---- : 162
common_carp_ERB       : EEN--------------C.P.A..VADA..TSA.LR---- : 152
Taiwan_shoveljaw_carp : EEN--------------C.P.A..VADA..TSTPLR---- : 152
Spiny barb minnow_ERB : EEN--------------C.P.V..VVDA..IST.LR---- : 162
Japanese_eel_ERB      : AQ.S--------------.LAG..MA....GTS.V.GCFA : 163
conger_eel_ERB        : AQ.S--------------.LT-..VA....GAS.G.GCFA : 166


                                                 *        220         *        240
yellow_perch_ERBa     : GKVDLHYCAVCNDHASGYHYGVWSCEGCKAFFKRSIQGHN : 195
bastard_halibut_ERB   : ..S.........H.Y......................... : 194
largemouth_bass_ERy   : ..A.........Q.Y.....................R.. : 190
Atlantic_croaker_ERy  : ..T.........H.Y.....................RD. : 201
black_seabream_ERB    : ..A.........H.Y.....................R.. : 188
gilthead_seabream_ERB : ..A.........H.Y.....................R.. : 188
Nile_tilapia_ERB      : ..A.........H.Y......................... : 187
killifish_ERBA        : ..A.........H.Y......................... : 179
Javanese_ricefish_ERB : ..SE........H.Y.....................T : 189
Japanese_medaka_ERB   : ..SE........H.Y.....................T : 189
Atlantic_salmon_ERB   : ..AE........H.Y......................... : 213
zebrafish_ERB2        : ..A.........S.Y......................... : 191
rainbow_trout_ERB     : ..A.........S.Y......................... : 210
goldfish_ERB1         : ..A.........S.Y......................... : 202
common_carp_ERB       : ..A.........S.Y......................... : 192
Taiwan_shoveljaw_carp : ..A.........G.Y......................... : 192
Spiny barb minnow_ERB : ..A.........S.Y......................... : 202
Japanese_eel_ERB      : ..G.........H.Y......................... : 203
conger_eel_ERB        : ..G.........H.Y......................... : 206
                        ► C domain (DBD or ZFM)
```

110

```
                                    *         260        *         280
yellow_perch_ERBa     : DYICPATNQCTIDKNRRKSCQACRLRKCCEVGMTKCGMRK : 235
bastard_halibut_ERB   : ...........................Y............ : 234
largemouth_bass_ERy   : ..........................N......F.... : 230
Atlantic_croaker_ERy  : E..........................Y............ : 241
black_seabream_ERB    : .......................H..YN........... : 228
gilthead_seabream_ERB : .......................H..YN........... : 228
Nile_tilapia_ERB      : ..........................Y............ : 227
killifish_ERBA        : ..........................Y............ : 219
Javanese_ricefish_ERB : ..........................Y............ : 229
Japanese_medaka_ERB   : ..........................Y............ : 229
Atlantic_salmon_ERB   : ..........................Y............ : 253
zebrafish_ERB2        : ..........................Y....M...... : 231
rainbow_trout_ERB     : ..........................Y....M...... : 250
goldfish_ERB1         : ..........................Y....M...... : 242
common_carp_ERB       : ..........................Y....M...... : 232
Taiwan_shoveljaw_carp : ..........................Y....M...... : 232
Spiny barb minnow_ERB : ..........................Y....M...... : 242
Japanese_eel_ERB      : G.........................Y....M...... : 243
conger_eel_ERB        : ..........................Y....M...... : 246


                                    *         300        *         320
yellow_perch_ERBa     : ERGNY--RNPQTRRVTRLSSQGRTNRPKAL---SGPTVGL : 270
bastard_halibut_ERB   : DH.S.--...K.............ASG....--...V.A. : 269
largemouth_bass_ERy   : ....C--....M................AV.--...AA.S : 265
Atlantic_croaker_ERy  : .....--.S..M...............SSSV.--..SA.VS : 276
black_seabream_ERB    : .....--....M..............---------N..A..P : 257
gilthead_seabream_ERB : .....--....M..............SG.SV.---N..A..P : 263
Nile_tilapia_ERB      : .....--..S.A..............AE..G.---...AE.S : 262
killifish_ERBA        : ....C--.STLV.......A...L....V.---.T.AESS : 254
Javanese_ricefish_ERB : ...S.--.SAPA.........S.ISG..VS--...KESS : 264
Japanese_medaka_ERB   : ..SS.--.SAPA..AG.......M.G..VS--...KESS : 264
Atlantic_salmon_ERB   : D.SS.--.GH.P...G.FF.R.TASG..R.---LAEGGEP : 288
zebrafish_ERB2        : D.SS.QQ.GA.Q...V.F.GRM.MTG..SQEIK.I.RPLS : 271
rainbow_trout_ERB     : D..S.QQ.GA.Q...A.F.GRM..SG..SQEMK.V.CPLS : 290
goldfish_ERB1         : D.SS.QQ.GA.QN....F.GRM..SG..SQEIK.VQRPLS : 282
common_carp_ERB       : D..S.QQ.GARQ...A.F.GRM..CG..SQEIK.V.RPLG : 272
Taiwan_shoveljaw_carp : D..S.QQ.GA.Q...A.F.GRM..CG..SQEIQ.V.CPLG : 272
Spiny barb minnow_ERB : D..S.QQ.GA.Q...V.F.GRM..SG..SQEIK.V.RPLS : 282
Japanese_eel_ERB      : ..CT.--.GARH...PH.RELAG.GGGARTQRRGEGV.PQ : 281
conger_eel_ERB        : ..CT.--.GARH...PQ.RDLAG.GGGARAHRRGEGPATQ : 284
                        ► D domain
```

111

```
                                        *         340          *          360
yellow_perch_ERBa     : LPAPHPPALTPEQLIGQIMEAEPPEIYLMKDMMRPLTEAN : 310
bastard_halibut_ERB   : .NELQ..........ER.......V.......SG...... : 309
largemouth_bass_ERy   : .IV..S.........ER..D......D.....R....... : 305
Atlantic_croaker_ERy  : .N..Q.S...S....ER................K......K : 316
black_seabream_ERB    : .I..Q.....SK...ER................R...... : 297
gilthead_seabream_ERB : .NT.Q.....SK...ER................R...... : 303
Nile_tilapia_ERB      : .NK.EK........ER.............AK......S : 302
killifish_ERBA        : SSE.L..G.......ER.......D.......K...... : 294
Javanese_ricefish_ERB : GSESSSLH.......ER.......D......TKT.....V : 304
Japanese_medaka_ERB   : GNEQSSLH.......AR.......D......TK......V : 304
Atlantic_salmon_ERB   : .KELR.TV........R..........Q...R....... : 328
zebrafish_ERB2        : GNEVVRIS.......SR...........K..F.... : 311
rainbow_trout_ERB     : GNEVVNM........AR..D........K..F.... : 330
goldfish_ERB1         : GNKVVTM........AR..D........K..F.... : 322
common_carp_ERB       : GNKVVSI........AR..D......N..K..F.... : 312
Taiwan_shoveljaw_carp : GNKVVSI........AR..D........K..F.... : 312
Spiny barb minnow_ERB : GNKVVTM........SR..D........KN.F.... : 322
Japanese_eel_ERB      : TQEAQSS........NR.............E.K..F..DS : 321
conger_eel_ERB        : ---V.TS........HR.............E.K..F..DS : 321


                                        *         380          *          400
yellow_perch_ERBa     : VMMSLTNLADRELVHMISWAKKIPGFVELSLLDQVHLLEC : 350
bastard_halibut_ERB   : ......H.......................G.......... : 349
largemouth_bass_ERy   : ..............................G.......... : 345
Atlantic_croaker_ERy  : ..............................G.......... : 356
black_seabream_ERB    : ..............................G.......... : 337
gilthead_seabream_ERB : ..............................G.......... : 343
Nile_tilapia_ERB      : ...L..................................... : 342
killifish_ERBA        : ......................................... : 334
Javanese_ricefish_ERB : ......................................... : 344
Japanese_medaka_ERB   : ......................................... : 344
Atlantic_salmon_ERB   : ...........................D.C.F........ : 368
zebrafish_ERB2        : ...............................F........ : 351
rainbow_trout_ERB     : ...............................F........ : 370
goldfish_ERB1         : .............................G.F........ : 362
common_carp_ERB       : ...............................F........ : 352
Taiwan_shoveljaw_carp : ...............................F........ : 352
Spiny barb minnow_ERB : ...............................F........ : 362
Japanese_eel_ERB      : ...............L..............D.S........ : 361
conger_eel_ERB        : ..R...........L..............D.S........ : 361
                            ► E domain (LBD)
```

```
                                 *         420         *         440
yellow_perch_ERBa     : CWLEVLMIGLMWRSVDHPGKLIFSPDLGLSREEGNCVQGF : 390
bastard_halibut_ERB   : ............................S......S..... : 389
largemouth_bass_ERy   : ...........................R..S......S..... : 385
Atlantic_croaker_ERy  : ............................S......S..... : 396
black_seabream_ERB    : ............................S......S..... : 377
gilthead_seabream_ERB : ............................S......S..... : 383
Nile_tilapia_ERB      : ........................C...S......S..... : 382
killifish_ERBA        : ........................C......S..... : 374
Javanese_ricefish_ERB : ...................G........S......S..... : 384
Japanese_medaka_ERB   : ...................G........S......S..... : 384
Atlantic_salmon_ERB   : ...................G...........S.N....S..... : 408
zebrafish_ERB2        : ........................S...D.SS....L : 391
rainbow_trout_ERB     : ........................S...D..S..... : 410
goldfish_ERB1         : ........................S...D..S..... : 402
common_carp_ERB       : ........................S...D..S..... : 392
Taiwan_shoveljaw_carp : ........................S...D..S..... : 392
Spiny barb minnow_ERB : ........................S...D..S..... : 402
Japanese_eel_ERB      : ....................K.N.D..S....I : 401
conger_eel_ERB        : ....................K.N.D..S..D.I : 401


                                 *         460         *         480
yellow_perch_ERBa     : SEIFDMLLAATSRVRELKLQKEEYICLKAMILLNSNMCLS : 430
bastard_halibut_ERB   : ....................................... : 429
largemouth_bass_ERy   : A...................................... : 425
Atlantic_croaker_ERy  : A...................................... : 436
black_seabream_ERB    : L...................................... : 417
gilthead_seabream_ERB : L...................................... : 423
Nile_tilapia_ERB      : V...................................... : 422
killifish_ERBA        : A...................T.................. : 414
Javanese_ricefish_ERB : V...................................... : 424
Japanese_medaka_ERB   : V...................N.................. : 424
Atlantic_salmon_ERB   : VD.........F.......................... : 448
zebrafish_ERB2        : V.........F.........................G : 431
rainbow_trout_ERB     : V.........F.......................... : 450
goldfish_ERB1         : A.........F.........A................ : 442
common_carp_ERB       : V.........F.........A................ : 432
Taiwan_shoveljaw_carp : V.........F.......................... : 432
Spiny barb minnow_ERB : V.........F.......................... : 442
Japanese_eel_ERB      : L.........F.................P...T. : 441
conger_eel_ERB        : L.........F.................P...SA : 441
```

```
                                           *        500         *        520
yellow_perch_ERBa      : VT-EGSEELQSRSKLLRLLDAVTDALVWAIAKTGLTFRQQ : 469
bastard_halibut_ERB    : S.-......H......L...................... : 468
largemouth_bass_ERy    : S.-................................... : 464
Atlantic_croaker_ERy   : S.-.S.------.................S..G..V...... : 469
black_seabream_ERB     : S.-.............H.................... : 456
gilthead_seabream_ERB  : S.-................................... : 462
Nile_tilapia_ERB       : S.-DC..D..................L..G........ : 461
killifish_ERBA         : S.-................................... : 453
Javanese_ricefish_ERB  : S.-..G..............T.........G....... : 463
Japanese_medaka_ERB    : S.-..GG..H......C...........G....N.... : 463
Atlantic_salmon_ERB    : S.-...............................Q.. : 487
zebrafish_ERB2         : S.-..G.D........C...S........S......Q.R : 470
rainbow_trout_ERB      : S.-..G....R.....C...S........S......Q.R : 489
goldfish_ERB1          : SA-..G.........C...S........S......Q.R : 481
common_carp_ERB        : SA-..G.........C...S........S......Q.R : 471
Taiwan_shoveljaw_carp  : S.-..G.........C...S........S......Q.R : 471
Spiny barb minnow_ERB  : S.-..G.........PC...S........S......Q.R : 481
Japanese_eel_ERB       : S.-.NR......N...H...S.......T...K....Q.. : 480
conger_eel_ERB         : SATDNR..T.......H...........T...R....Q.. : 481


                                           *        540         *        560
yellow_perch_ERBa      : YTRLAHLLMLLSHIRHASNKGMDHLHCMKMKNVVPLYDLL : 509
bastard_halibut_ERB    : .................V................... : 508
largemouth_bass_ERy    : .................V................... : 504
Atlantic_croaker_ERy   : .................V................... : 509
black_seabream_ERB     : .................V........G.......... : 496
gilthead_seabream_ERB  : .................V........G.......... : 502
Nile_tilapia_ERB       : .................V................... : 501
killifish_ERBA         : .................V................... : 493
Javanese_ricefish_ERB  : .................V................... : 503
Japanese_medaka_ERB    : .................V................... : 503
Atlantic_salmon_ERB    : SA...............V................... : 527
zebrafish_ERB2         : S................V............K....... : 510
rainbow_trout_ERB      : S................L............K....... : 529
goldfish_ERB1          : S................V.........S....K....... : 521
common_carp_ERB        : S................V............K....... : 511
Taiwan_shoveljaw_carp  : S.....P..........L............K....... : 511
Spiny barb minnow_ERB  : S................L.........N..K....... : 521
Japanese_eel_ERB       : SA.........A....L.....E..SN..R.......... : 520
conger_eel_ERB         : SA.........A....V.....E..SN..R.......... : 521
```

114

```
                                          *         580         *         600
yellow_perch_ERBa     : LEMLDAHIMHSSRLPRQP------MQQESGDQTGVPARPL : 543
bastard_halibut_ERB   : ................HHAS-----P.P.FT..GE.....G : 543
largemouth_bass_ERy   : ...............SH..------T..DAE..REA....H : 538
Atlantic_croaker_ERy  : ...............RS------P...PE..ADA..P.H : 543
black_seabream_ERB    : ...............RS------P.......CDG....H : 530
gilthead_seabream_ERB : ...............RS------P....VE.CDA....H : 536
Nile_tilapia_ERB      : .............C..H..------P..D.K...E..-A.. : 534
killifish_ERBA        : ................R.------A..D.A...EAAPDQA : 527
Javanese_ricefish_ERB : .................------P..DAA.P.ENS-AQG : 536
Japanese_medaka_ERB   : .................------PP.DAA.P.ETS-AQG : 536
Atlantic_salmon_ERB   : ..........P...H.ANSAGPCP.VSHPQP.TSA.A.A : 567
zebrafish_ERB2        : ............SHSGP--------------RA..AHK : 536
rainbow_trout_ERB     : ..........G...SHSG----------------PA.K : 551
goldfish_ERB1         : ..........G...SHSGP-------------QADPV.K : 547
common_carp_ERB       : ............SHSGP-------------RAAPA.K : 537
Taiwan_shoveljaw_carp : ............SHSGP-------------RAAPV.K : 537
Spiny_barb_minnow_ERB : ............SHSGP-------------R.APA.K : 547
Japanese_eel_ERB      : ......NT......SASYSS------.P.PWSQAAQSQ.G : 554
conger_eel_ERB        : ......NT..G...SESYS-------.PPPWPPTAQSQ.S : 554
                                      ► F domain

                                          *         620         *         640
yellow_perch_ERBa     : SSH-SGPSNTWTP-------------------------- : 555
bastard_halibut_ERB   : ..G-N.S......STSGDGGEPQ---------------- : 565
largemouth_bass_ERy   : ..G-.C.......----GGGEPQ---------------- : 556
Atlantic_croaker_ERy  : ..G-....Y....SSSEGAGEPQ---------------- : 565
black_seabream_ERB    : .PGP.........SSTGGRGEPQ---------------- : 553
gilthead_seabream_ERB : .PGT.........SCTGGRGEPQ---------------- : 559
Nile_tilapia_ERB      : H.SAG........SSARAGGESQ---------------- : 557
killifish_ERBA        : HCSAA........SGAGVAGEAQCSD------------- : 553
Javanese_ricefish_ERB : QRSCW-V.KA...SSPGAAGAAQKSDQN----------- : 563
Japanese_medaka_ERB   : QRSCCDV.KA..TSSAGTAEEPQKSD------------- : 562
Atlantic_salmon_ERB   : RHGPPAAEA.LNSRSHWTAGTPVERQW------------ : 594
zebrafish_ERB2        : DNKSVQEAFPC.SQHGP--------------------- : 553
rainbow_trout_ERB     : E.TGVQEATLSVLKNDL--------------------- : 568
goldfish_ERB1         : E.NCVQE.F.C.SQHGGTLRP----------------- : 568
common_carp_ERB       : E.KGVQEAL.R.SQSGGTLAGP---------------- : 559
Taiwan_shoveljaw_carp : E.KAVQEAFAC.SQHGGT-SGP---------------- : 558
Spiny_barb_minnow_ERB : E.KAIQEAFAC.SQHGP--------------------- : 564
Japanese_eel_ERB      : PPPSCSGECPCP.KESSTI------------------- : 573
conger_eel_ERB        : PQPSCSGEGPCP.KESITAAVFGHGEDRVIPGLHTGTTSR : 594
```

```
yellow_perch_ERBa      : -- :    -
bastard_halibut_ERB    : -- :    -
largemouth_bass_ERy    : -- :    -
Atlantic_croaker_ERy   : -- :    -
black_seabream_ERB     : -- :    -
gilthead_seabream_ERB  : -- :    -
Nile_tilapia_ERB       : -- :    -
killifish_ERBA         : -- :    -
Javanese_ricefish_ERB  : -- :    -
Japanese_medaka_ERB    : -- :    -
Atlantic_salmon_ERB    : -- :    -
zebrafish_ERB2         : -- :    -
rainbow_trout_ERB      : -- :    -
goldfish_ERB1          : -- :    -
common_carp_ERB        : -- :    -
Taiwan_shoveljaw_carp  : -- :    -
Spiny_barb_minnow_ERB  : -- :    -
Japanese_eel_ERB       : -- :    -
conger_eel_ERB         : RD : 596
```

Figure 3.5 Alignment of yellow perch ERβa deduced amino acid sequence with other teleost ERβs in the same group.

Conserved amino acid residues are indicated with (.), inserted gaps are indicated with (-) and structural domains are identified at the bottom of the alignment in bold. The nine conserved cysteines of the C domain are shaded, the ten amino acid residues corresponding to the P- and D-boxes are boxed and the eight amino acid region corresponding to AF2 is underlined. Sequences were downloaded from the EMBL/GenBank database with the following accession numbers: bastard halibut ERβ (*Paralichthys olivaceus*) BAB85623; largemouth bass ERγ (*Micropterus salmoides*) AAO39211; Atlantic croaker ERγ (*Micropogonias undulates*) AAG16712; black seabream ERβ (*Acanthopagrus schlegelii*) AAL82742; gilthead seabream ERβ (*Sparus aurata*) AAD31033; Nile tilapia ERβ (*Oreochromis niloticus*) AAD00246; killifish ERβA (*Fundulus heteroclitus*) AAU44352; Javanese ricefish ERβ (*Oryzias javanicus*) AAX14000; Japanese medaka ERβ (*Oryzias latipes*) BAB79705; Atlantic salmon ERβ (*Salmo salar*) AAR92486; zebrafish ERβ2 (*Danio rerio*) CAC93849; rainbow trout ERβ (*Oncorhynchus mykiss*) CAC06714; goldfish ERβ1 (*Carassius auratus*) AAD26921; common carp ERβ (*Cyprinus carpio*) BAB91218; Taiwan shoveljaw carp ERβ (*Onychostoma barbatula*) CAD67997; Spiny barbed minnow ERβ (*Spinibarbus denticulatus*) ABF56052; Japanese eel ERβ (*Anguilla japonica*) BAA19851; conger eel ERβ (*Conger myriaster*) BAD02929.

spiny dogfish ERB

channel catfish ERB
zebrafish ERB1
goldfish ERB2
Taiwan shoveljaw carp ER Bb
Taiwan minnow ER Bb
fathead minnow ERB
roach ERB
gilthead seabream ERBb
European sea bass ERB2
Atlantic croaker ERB
largemouth bass ERB
wrasse ERB
bamboo grass wrasse ERB
Nile tilapia ERB2
killifish ERBB

ERβb

Japanese eel ERB
conger eel ERB
zebrafish ERB2
rainbow trout ERB
goldfish ERB1
common carp ERB
Taiwan shoveljaw carp ERB
spiny barbed minnow ERB
Atlantic salmon ERB
killifish ERBA
Javanese ricefish ERB
Japanese medaka ERB
Nile tilapia ERB
largemouth bass ERy
Atlantic croaker ERy
black seabream ERB
gilthead seabream ERB
bastard halibut ERB
yellow perch ERBa

ERβa

Figure 3.6 Phylogenetic tree of ERβ amino acid sequences.

Tree produced using a Clustal X (1.81) alignment and TreeView (Win 32) v. 1.6.6. The tree was rooted with spiny dogfish shark (*Squalus acanthias*) ERβ (Accession: AAK57823) as an outgroup representing the closest available species not in the group Teleostei. See Figure 3.5 for EMBI/GenBank accession numbers except: largemouth bass ERβ (*Micropterus salmoides*) AAO39210; Atlantic croaker ERβ (*Micropogonias undulates*) AAG16711; Nile tilapia ERβ2 (*Oreochromis niloticus*) ABE73151; gilthead seabream ERβ2 (*Sparus aurata*) CAE30470; European sea bass ERβ2 (*Dicentrarchus labrax*) CAD33852; wrasse ERβ (*Halichoeres tenuispinis*) AAP72179; killifish ERβB (*Fundulus heteroclitus*) AAU44353; bamboo grass wrasse ERβ (*Pseudolabrus japonicus*) ABB96484; channel catfish ERβ (*Ictalurus punctatus*) AAF63157; zebrafish ERβ1 (*Danio rerio*) CAC93848; Taiwan shoveljaw carp ER(β2) (*Onychostoma barbatula*) CAC85366; Taiwan minnow ER(β2) (*Candidia barbatus*) CAC85356; goldfish ERβ2 (*Carassius auratus*) AAF35170; roach ERβ, (*Rutilus rutilus*) BAD91036; fathead minnow ERβ, (*Pimephales promelas*) AAT45195.

```
-68   -CTCTCGTCACTCGGACAGTTTGTCGTGTTTGTGCAGTCGTGTGCAGGTTGTTT

                      M  D  L  I  S  T  C  E  R  A  M  T  P   13
-15   TAAATCTGCCTTCTTATGGATCTGATCTCTACTTGTGAAAGGGCGATGACTCCT

      V  G  L  G  A  K  V  A  D  L  I  P  M  S  P  N  A  T   31
 40   GTAGGTTTGGGTGCCAAAGTGGCAGACCTGATCCCCATGTCCCCGAACGCCACT

      A  V  G  S  P  G  I  S  M  V  T  R  T  L  I  L  L  G   49
 94   GCAGTTGGATCTCCGGGTATCTCTATGGTAACCAGAACCCTTATCCTGCTTGGC

      C  L  L  L  F  A  W  S  H  K  D  K  K  T  V  P  G  P   67
148   TGTCTGCTCCTGTTTGCTTGGAGTCACAAAGACAAGAAAACTGTGCCAGGTCCC

      S  F  C  L  G  L  G  P  L  L  S  Y  V  R  F  I  W  T   85
202   TCTTTCTGTCTGGGTTTGGGGCCACTTCTGTCATATGTGAGATTCATCTGGACC

      G  I  G  T  A  S  N  Y  Y  N  N  K  Y  G  D  I  V  R  103
256   GGTATAGGCACAGCCAGCAACTACTACAACAACAAGTACGGAGACATTGTCAGA

      V  W  I  N  G  E  E  T  L  I  L  S  R  A  S  A  V  H  121
310   GTCTGGATCAATGGAGAGGAGACCCTCATACTCAGCAGAGCATCAGCGGTGCAC

      H  V  L  K  N  R  H  Y  T  S  R  F  G  S  K  Q  G  L  139
364   CATGTACTGAAGAACAGACATTATACTTCACGTTTTGGGAGCAAGCAGGGACTC

      S  C  M  G  M  N  E  R  G  I  I  F  N  S  N  I  P  L  157
418   AGCTGCATGGGCATGAATGAGAGAGGCATCATATTTAACAGCAACATACCTCTG

      W  K  T  I  R  T  Y  F  T  K  A  L  T  G  P  G  L  Q  175
472   TGGAAGACAATACGCACCTACTTCACCAAAGCGCTGACAGGTCCGGGCTTGCAG

      Q  T  V  E  V  S  V  S  S  T  Q  T  H  L  D  D  L  E  193
526   CAGACAGTTGAGGTTAGCGTCTCCTCCACACAGACTCACCTGGACGACCTGGAG

      S  L  D  H  V  D  V  L  S  L  L  R  C  T  V  V  D  I  211
580   AGTTTGGACCATGTGGATGTTCTCAGTTTGCTGCGCTGCACCGTGGTCGACATC

      S  N  R  L  F  L  G  V  P  V  N  E  K  E  L  L  L  K  229
634   TCAAACAGACTCTTCCTGGGTGTACCTGTCAATGAAAAAGAGCTGCTGCTGAAG

      I  Q  K  Y  F  D  T  W  Q  T  V  L  I  K  P  D  L  Y  247
688   ATTCAGAAGTATTTTGACACGTGGCAAACTGTGCTAATCAAACCAGACCTTTAC

      F  K  F  G  W  I  H  Q  R  H  K  T  A  A  R  E  L  Q  265
742   TTCAAATTTGGCTGGATTCACCAGAGGCACAAGACAGCAGCCCGGGAGTTGCAA

      D  A  I  E  S  L  V  E  Q  K  R  R  A  M  E  Q  A  D  283
796   GATGCAATAGAAAGCCTTGTAGAACAGAAGAGGAGAGCTATGGAGCAAGCAGAT

      K  L  D  N  I  N  F  T  A  E  L  I  F  A  Q  N  H  G  301
850   AAACTGGACAACATCAACTTCACCGCGGAGCTCATATTTGCACAGAACCATGGT
```

119

```
                ▶                     I helix region
        E  L  S  A  E  N  V  R  Q  C  V  L  E  M  V  I  A  A      319
   904  GAACTGTCTGCGGAGAATGTGAGGCAGTGCGTGCTGGAGATGGTGATCGCAGCA

                                                           ◀
        P  D  T  L  S  I  S  L  F  F  M  L  L  L  K  Q  N         337
   958  CCAGACACTCTGTCCATCAGCCTCTTCTTCATGCTGCTGCTCCTCAAACAGAAT

        P  D  V  E  L  Q  L  L  Q  G  I  D  T  V  V  C  D  R      355
  1012  CCAGATGTGGAGCTGCAGCTGCTTCAAGGGATAGACACTGTTGTATGTGATAGA

                              ▶           Ozol's peptide
        Q  L  Q  N  G  D  C  Q  K  L  Q  V  L  E  S  S  I  N      373
  1066  CAGCTTCAGAACGGGGACTGTCAGAAGTTGCAGGTGCTGGAAAGCTCCATCAAC

                                  ◀
        E  C  L  R  F  H  P  V  V  D  F  T  M  R  R  A  L  S      391
  1120  GAATGCTTGCGCTTCCACCCTGTGGTGGACTTCACCATGCGTCGAGCCCTGTCT

              ▶         aromatase specific conserved region
        D  D  I  I  D  G  Y  R  V  P  K  G  T  N  I  I  L  N      409
  1174  GATGACATCATAGATGGCTACAGGGTACCGAAGGGCACAAATATAATTCTGAAC

                              ◀
        V  G  H  M  H  R  T  E  F  F  F  K  P  N  K  F  S  L      427
  1228  GTCGGCCACATGCACCGGACAGAGTTTTTCTTCAAACCCAATAAATTCAGTCTG

                                                ▶
        E  N  F  G  K  N  V  P  R  R  Y  F  Q  P  F  G  S  G      445
  1282  GAAAACTTTGGAAAAAATGTTCCTCGTCGTTACTTCCAGCCGTTCGGTTCGGGC

          heme-binding region                   ◀
        P  R  A  C  V  G  K  H  I  A  M  V  M  M  K  S  I  L      463
  1336  CCTCGTGCCTGCGTTGGCAAGCACATCGCCATGGTGATGATGAAGTCCATCCTA

        V  T  L  L  S  Q  Y  S  V  C  P  H  E  G  L  T  L  D      481
  1390  GTGACATTGCTTTCCCAGTACTCTGTTTGCCCCCATGAGGGCTTGACCTTGGAC

        C  L  P  Q  T  N  N  L  S  Q  Q  P  V  E  H  Q  Q  E      499
  1444  TGCCTCCCACAGACCAACAACCTCTCCCAGCAGCCAGTTGAGCATCAGCAGGAG

        A  E  Q  L  S  M  R  F  L  P  R  Q  R  G  S  W  Q  K      517
  1498  GCCGAACAGCTCAGCATGAGATTCTTACCCAGACAGAGAGGAAGCTGGCAAAAA

        L  ***                                                    518
  1552  CTCTGAGACTCTGAACTTTAGCTGAACCTATACATTTATACAGAATATATACAT

  1606  ATATATGATCTCCATTACTTCATTTATATTATCTCATGACTGTACAAAGCTGTT
  1660  TTATATTTTGGTATGTATTAAAAAATTACTGTTTTTCTACTTGAACTATTATGC
  1714  ATTATTAGAAATGTAAGTTTAGACATGCTAATACTTA**AGTAAA**TGTAAATTATT
  1768  GTGCAAATAAAAAAAAAAAAAAAAA
```

120

Figure 3.7 Nucleotide and deduced amino acid sequences of yellow perch ovarian aromatase (CYP19A1) cDNA.

The nucleotides (lower row) are numbered on the left and the amino acids (upper row) are numbered on the right and both begin with the initiation methionine. The two in-frame initiation codons are in bold and a possible polyadenylation signal is in bold and double underlined. A putative N-glycosylation site is underlined and the following conserved regions of all steroidogenic cytochrome P-450s are shaded and labeled in bold with arrows above the amino acid sequence: I helix region, Ozol's peptide, aromatase specific conserved region and the heme-binding region. Yellow perch CYP19A1 cDNA sequence will be available from the EMBL/GenBank database with accession #DQ984126 on or before October 27, 2007.

```
                                        *         20        *        40
yellow_perch          : M-DLIS-TCERAMTPVGLGAKVADLIP-MSPNATAVGSPG :  37
red-spotted_grouper   : .-....-A..........DDM....AT-............ :  37
orange-spotted_grouper: .-....-A..........DDM....AT-............ :  37
humpback_grouper      : .-....-A..........DDM....AT-....T....... :  37
barramundi_perch      : .-....-A..........DDM....AT-....T....... :  37
Atlantic_croaker      : .-....-A..G..D..R.D.M...E..S-.P......... :  37
European_sea_bass     : .-....-A.........DTI.....S-T............ :  37
Atlantic_halibut      : .-.R.P-A.DM.........A.G...S-T........... :  37
bastard_halibut       : .-.R.P-A.DL.........A.G...S-T.......R... :  37
flathead_mullet       : .-....-A..Q.....A.D.M.....S-.......DE... :  37
red_seabream          : .-....-A..LV.PQ...DTA.....S-..H......... :  37
black_seabream        : .-....-A...V.PQ...DTT......-..H......A.. :  37
gilthead_seabream     : .-....-A...V.PQ...DTTA.....-..H......A.. :  37
rice_field_eel        : .-...P-A.....V....D.AA...DS-..S.......A. :  37
Mozambique_tilapia    : .-....-A..Q..N....D.V....SV-T.N---..Q.H. :  34
blue_tilapia          : .-....-A..Q.......D.V....SV-T.N---..Q.H. :  34
Nile_tilapia          : .-....-A..Q.......D.V..RS.C-DLK---CHPID. :  34
wrasse                : .-....-A...T......D.E.G..GY-..Q...V.VLQ. :  37
bamboo_grass_wrasse   : .-....-A...T......D.E.R...Y-..Q...V..LL. :  37
killifish             : .-....-..GGT....D.DGV.E.R.S-.AS.V.VSL... :  37
Japanese_medaka       : .-...P-A.D.T..---SSCL..E..S-.A..T.VGLPS. :  34
broad_barred_goby     : ---------------.S.DDV.L.CRS-Y.ATGLL.P.-. :  23
rainbow_trout         : .-....PV.G.V.AV.C.DTV.....VSE.R....TR.E. :  39
goldfish              : .AGE.LQP.G--.KQ.H..EA.LE..MQGAH.S.YGAQDN :  38
zebrafish             : .AGD.LQP.G--.K..R..EA.V...IQRAH.G.ERAQDN :  38
roach                 : .AGD.LQP.G--.K..R..EAAV.F.VEGAH.G.EGAQDN :  38
southern_catfish      : .AAH.FQM...G.K..RFSEN.ME..LHE.R.G.NPEPEN :  40
channel_catfish       : .AAH.FPM...TRK..HFSET.ME..LREAR.G.DPRYEN :  40
yellow_catfish        : ------------.K..LFSET.ME..LHKAQ.G.NPRYEN :  28
Japanese_eel          : .-KH.EEIVME..M.ASRN.TQTAGRV-----V.GATAAL :  34
```

122

```
                                      *         60        *         80
yellow_perch          : ISMVTRTLILL-GCLLLFAWSHKDKKT----VPGPSFCLG :  72
red-spotted_grouper   : ...A..L....-I....A....T....---......... :  72
orange-spotted_grouper: ...A..L....-I....A....T....---......... :  72
humpback_grouper      : ...A..L....-I....A....T....---......... :  72
barramundi_perch      : ...A..L....-I....A....T....---......... :  72
Atlantic_croaker      : ...A......-T....V....T....---......... :  72
European_sea_bass     : ...A.I.....-V....V....T..N.---......... :  72
Atlantic_halibut      : ...A.......-V....V....T....---.....P.... :  72
bastard_halibut       : ...A.......-V....V....T....---.....P.... :  72
flathead_mullet       : ...A.......-V....V....TE..D---......... :  72
red_seabream          : ...A.......-V....V..N.TE...---......... :  72
black_seabream        : ...........-I....V..NS.E...---......... :  72
gilthead_seabream     : .......F...-I....V..NSME...---......... :  72
rice_field_eel        : ...A..A....-V....VT.N.TE..H---......... :  72
Mozambique_tilapia    : ...A.......-V....V....T...I---......... :  69
blue_tilapia          : ...A.......-V....V....T...I---......... :  69
Nile_tilapia          : ...A.......-V....V....T...I---......... :  69
wrasse                : ..TA.......-F....A....TE...---......... :  72
bamboo_grass_wrasse   : ...A.....P.-FF...A..N......---......... :  72
killifish             : .P.A.......-V....V....TE.NA---......... :  72
Japanese_medaka       : .P.A.......-V....MV...SE...---......... :  69
broad_barred_goby     : ...AI.A.A..-L.........ME..KRTAD....W.... :  62
rainbow_trout         : ...A.G.....-L....A..R.T.NN.----...F.... :  74
goldfish              : .CGAMA.....LL....AIRH.WTE.DH---...C.L.. :  75
zebrafish             : ACGA.A.....LL....AIRH..PH..H---......FF. :  75
roach                 : .CGA.A.....LL....AIRH..PEE.H---...C.F.. :  75
southern_catfish      : P.G-ITLF...CLV...TV.NCFE..NS---...R.... :  76
channel_catfish       : PRG-ITL....CLV...TV.NRHE..CS---......... :  76
yellow_catfish        : PRG-ITL....CLV...AV.NRN....C---......... :  64
Japanese_eel          : ..GA.AA....-L.A..A...RS..S.----....P.Y.. :  69
```

```
                                      *         100         *         120
yellow_perch          : LGPLLSYVRFIWTGIGTASNYYNNKYGDIVRVWIN----G : 108
red-spotted_grouper   : ......................................----. : 108
orange-spotted_grouper: ......................................----. : 108
humpback_grouper      : ......................................----. : 108
barramundi_perch      : ......................................----. : 108
Atlantic_croaker      : ......................................----. : 108
European_sea_bass     : ......................................----. : 108
Atlantic_halibut      : ........................K.............----. : 108
bastard_halibut       : ..................C....K.............----. : 108
flathead_mullet       : ......................................----. : 108
red_seabream          : ........................KT............----. : 108
black_seabream        : ..........S.............K.............----. : 108
gilthead_seabream     : ..........S.............K.............----. : 108
rice_field_eel        : ......................................----. : 108
Mozambique_tilapia    : ......................................----. : 105
blue_tilapia          : ......................................----. : 105
Nile_tilapia          : ......................................----. : 105
wrasse                : ......................ST.............----Y. : 108
bamboo_grass_wrasse   : ......................ST.............VWIY. : 112
killifish             : ........................K.............----. : 108
Japanese_medaka       : ......................................----. : 105
broad_barred_goby     : .......................Q.............----. :  98
rainbow_trout         : ...............................S.....----. : 110
goldfish              : .......C.......................S.....----. : 111
zebrafish             : .......C.......................S.....----. : 111
roach                 : ...F...C...R...................S.....----. : 111
southern_catfish      : .......C....M..........E..........S----. : 112
channel_catfish       : .......C....M..........E..........S----. : 112
yellow_catfish        : .......C....M...A......E..........S----. : 100
Japanese_eel          : .......F...............E.............----. : 105
```

124

```
                                                   *         140         *         160
yellow_perch          : EETLILSRASAVHHVLKNRHYTSRFGSKQGLSCMGMNERG : 148
red-spotted_grouper   : ..................GN..................... : 148
orange-spotted_grouper: ..................GN...............K.. : 148
humpback_grouper      : ..................GN.........R.......... : 148
barramundi_perch      : ..................GN..................... : 148
Atlantic_croaker      : ...................S................S. : 148
European_sea_bass     : .........V......G................Y... : 148
Atlantic_halibut      : ...........Y.....G................Y... : 148
bastard_halibut       : ...........Y.....G................Y... : 148
flathead_mullet       : ..................EQ..................... : 148
red_seabream          : ..................GQ..................... : 148
black_seabream        : ..................AQ..................... : 148
gilthead_seabream     : .................SGQ..................... : 148
rice_field_eel        : ........S.........G..................... : 148
Mozambique_tilapia    : ........S.........GN.....I............. : 145
blue_tilapia          : ........S.........GN.....I............. : 145
Nile_tilapia          : ........S.........GN.....I....Y....... : 145
wrasse                : .................SSS.................G.. : 148
bamboo_grass_wrasse   : .........S.......SG..................... : 152
killifish             : ...........N.....GN.........K.......... : 148
Japanese_medaka       : ...................K..................... : 145
broad_barred_goby     : .................SSN.......L..GY...H... : 138
rainbow_trout         : ...F...SS........QGR..................... : 150
goldfish              : ........S...Y....KSL.......L..Q....H.Q. : 151
zebrafish             : ........S...Y....KSL.......L..Q....H.Q. : 151
roach                 : ........S...C....RSL.......L..Q....H.Q. : 151
southern_catfish      : ........P...Y....HSQ.......L..Q....H.Q. : 152
channel_catfish       : ........P...Y....HSQ.......L..Q....H.Q. : 152
yellow_catfish        : ........P...Y....HSQ.......L..Q......Q. : 140
Japanese_eel          : ........S...YQ...KPQ...........R....H... : 145
```

```
                                            *         180           *          200
yellow_perch           : IIFNSNIPLWKTIRTYFTKALTGPGLQQTVEVSVSSTQTH : 188
red-spotted_grouper    : ....N..E...K.........................A.... : 188
orange-spotted_grouper: ....N..E...K.........................A.... : 188
humpback_grouper       : ....N..E...K.........................A.... : 188
barramundi_perch       : ....N..E...K.........................A.... : 188
Atlantic_croaker       : ....N..T...K....................T..C....... : 188
European_sea_bass      : ....N..T...Q..N.................C....... : 188
Atlantic_halibut       : ....N..S...KT..H............K....C....... : 188
bastard_halibut        : ....N..S...K...H............K....C....... : 188
flathead_mullet        : ....N..A...K.............S..K....CA...... : 188
red_seabream           : ....N..T...K.............S.......C....... : 188
black_seabream         : ....N..N...K.............S.......C.....A. : 188
gilthead_seabream      : ....N..T...K.............S.......C....... : 188
rice_field_eel         : ....N..A...K..M..I...............C....... : 188
Mozambique_tilapia     : ....N..T...K.....A......N....AD.C...I.A. : 185
blue_tilapia           : ....N..T...K.....A......N.....D.C...I.A. : 185
Nile_tilapia           : ....N..T...K.....A......N.....D.C...I.A. : 185
wrasse                 : ....N......K.....................C....... : 188
bamboo_grass_wrasse    : ....N......K.....................C....... : 192
killifish              : ....N..A...K.....A......S.......C....... : 188
Japanese_medaka        : ....N..A...K..........N.........C....... : 185
broad_barred_goby      : ....N..D...K..A.................H..D.C.....R. : 178
rainbow_trout          : .......A...KT....A..........K..D.C....... : 190
goldfish               : .......A...K.....A..........R....C....N.. : 191
zebrafish              : .......A...K..A..A..........R....CT...N.. : 191
roach                  : .......V...K.....A..........R....CT...T.. : 191
southern_catfish       : .......T...K..V.............R....CT..AN.. : 192
channel_catfish        : .......T...K.....A..........R....CTM..N.. : 192
yellow_catfish         : .......T...K..LH.A..........R....CT...N.. : 180
Japanese_eel           : ....N..E...K.....A..........R..A.C.A..D.. : 185
```

126

```
                                              *        220        *        240
yellow_perch          : LDDLES--------LDHVDVLSLLRCTVVDISNRLFLGVP : 220
red-spotted_grouper   : ....DG--------.G.....................D.. : 220
orange-spotted_grouper: ....DG--------.G.....................D.. : 220
humpback_grouper      : ....DG--------.G.................F.....D.. : 220
barramundi_perch      : ....DG--------.G.....................D.. : 220
Atlantic_croaker      : ....D.--------......................... : 220
European_sea_bass     : ....DK--------..N...................... : 220
Atlantic_halibut      : ....D.--------A.....................D.. : 220
bastard_halibut       : ....DG--------.G.....................D.. : 220
flathead_mullet       : .....--------A......................... : 220
red_seabream          : ....D.--------......................... : 220
black_seabream        : ....DG--------..Q....................T. : 220
gilthead_seabream     : ....DV--------..Q...................DT. : 220
rice_field_eel        : ....DN--------.G.................F........ : 220
Mozambique_tilapia    : ..H.D.--------.G.....N...............D.. : 217
blue_tilapia          : ..H.D.--------.G.....N...............N.. : 217
Nile_tilapia          : ..H.D.--------.G.....N...............N.. : 217
wrasse                : ....D.--------..Q..........A.........D.. : 220
bamboo_grass_wrasse   : ....D.--------..D...................D.. : 224
killifish             : ....D.--------AQ....................D.. : 220
Japanese_medaka       : ....S.--------SY....GF.................. : 217
broad_barred_goby     : .EE.KVGTTGPQQP.G.........G............D.. : 218
rainbow_trout         : ..A..GPD---GLMGGQ....................... : 227
goldfish              : ....SHLM----DARGQ....N....I............. : 227
zebrafish             : ....SQLT----DAQGQ....N....I............. : 227
roach                 : ....SHLT----DARGQ....N....I............. : 227
southern_catfish      : ..H.SRLT----DTQG.....N....I.........D.. : 228
channel_catfish       : ..G.SRLT----DAQG.....N....I.........D.. : 228
yellow_catfish        : ....SQLT----DAQG.....N....I.........D.. : 216
Japanese_eel          : ..Q..ELT----DLSGQ....N...........Q...R.. : 221
```

```
                                           *        260         *         280
yellow_perch          : VNEKELLLKIQKYFDTWQTVLIKPDLYFKFG-WIHQRHKT : 259
red-spotted_grouper   : ..........F....................D-........ : 259
orange-spotted_grouper: .S........L....................D-........ : 259
humpback_grouper      : ..........S-V.................D-........ : 258
barramundi_perch      : ..........L....................D-........ : 259
Atlantic_croaker      : .............................CA-........ : 259
European_sea_bass     : ..............................D-........ : 259
Atlantic_halibut      : ..........L..................LD-.......A : 259
bastard_halibut       : ..........L...................D-.......A : 259
flathead_mullet       : ..............................D-........ : 259
red_seabream          : ...............................-........ : 259
black_seabream        : ...............................-.......A : 259
gilthead_seabream     : ...............................-.......A : 259
rice_field_eel        : ..........H...E...C............-..YK...A : 259
Mozambique_tilapia    : .............H...D............-...H.... : 256
blue_tilapia          : .............H...D............-...H.... : 256
Nile_tilapia          : .............H...D............-...H.... : 256
wrasse                : ..........H..................LD-..Q....M : 259
bamboo_grass_wrasse   : .............................LD-........ : 263
killifish             : ..........H..................LS-........ : 259
Japanese_medaka       : .......Q..H...................S-........ : 256
broad_barred_goby     : ....D..K..HL..E..............MN-...K.... : 257
rainbow_trout         : .......Q.....................LD-.......R : 266
goldfish              : ...HD..Q..H..................LAW...R...R : 267
zebrafish             : ...HD..Q..H..................LD-...K...R : 266
roach                 : ....D..Q.....................LD-...K...R : 266
southern_catfish      : ...EK..S..HQ.................LK-..QD...N : 267
channel_catfish       : ...QN..F..H...E.........F...LK-...D...N : 267
yellow_catfish        : ...QN..S..H..................LK-...N...N : 255
Japanese_eel          : ..............EA.........FL...E-..YKE..E : 260
```

128

```
                                              *         300         *          320
yellow_perch          : AARELQDAIESLVEQKRRAMEQADKLDNINFTAELIFAQN : 299
red-spotted_grouper   : ..Q...............D..................... : 299
orange-spotted_grouper: ..Q...............D..................... : 299
humpback_grouper      : ..QS..............D......-....E.......-  : 296
barramundi_perch      : ..Q...............D..................... : 299
Atlantic_croaker      : ..Q.....K.........D...................RH : 299
European_sea_bass     : ..Q...............D......-.....D........ : 298
Atlantic_halibut      : .V...H...GD.......D.............TG...... : 299
bastard_halibut       : .VQ..H...GD.......D.............TG...... : 299
flathead_mullet       : ..Q...............D....E................ : 299
red_seabream          : ..Q...............D..................... : 299
black_seabream        : ..Q...............D..................... : 299
gilthead_seabream     : ..Q...............D..................... : 299
rice_field_eel        : ...G..............D..................... : 299
Mozambique_tilapia    : .TQ......KR..D....N.....T............... : 296
blue_tilapia          : .TQ......KR..D....N..................... : 296
Nile_tilapia          : .TQ......KR..D....N..................... : 296
wrasse                : ..Q...G...........E....E...............S : 299
bamboo_grass_wrasse   : .TQ...............E....E...............S : 303
killifish             : ..Q..R....G.......Q......-.....D...... : 298
Japanese_medaka       : ..Q...........R...E....E..............G : 296
broad_barred_goby     : ..Q.......E...V...V.........H.....D...... : 297
rainbow_trout         : ..Q.........D....G........H.....D.....S : 306
goldfish              : D.Q......AA......VQ.TR.E.F.Q..........S : 307
zebrafish             : D.Q......TA......VQ.VH.E...H..........S : 306
roach                 : E.Q......TA......VQ.TH.E...Q..........S : 306
southern_catfish      : .....H....E......SE....E........E....S.S : 307
channel_catfish       : ..Q..H....D......TE....E........E......S : 307
yellow_catfish        : T.Q..H....A......TE....E...H....E....S.S : 295
Japanese_eel          : ..H..HE...I...K........E....A..ATD...... : 300
```

129

```
                                      *         340         *         360
yellow_perch          : HGELSAENVRQCVLEMVIAAPDTLSISLFFMLLLLKQNPD : 339
red-spotted_grouper   : ............................................. : 339
orange-spotted_grouper: ............................................. : 339
humpback_grouper      : ICD.......S................................. : 336
barramundi_perch      : N........................................... : 339
Atlantic_croaker      : ...........S...........................H.. : 339
European_sea_bass     : R........................................... : 338
Atlantic_halibut      : ...........VHG.............................. : 339
bastard_halibut       : ...........V................................ : 339
flathead_mullet       : ..........................................H : 339
red_seabream          : ............................................. : 339
black_seabream        : ......................................H.. : 339
gilthead_seabream     : ......................................H.. : 339
rice_field_eel        : ...........T................................ : 339
Mozambique_tilapia    : ...........T...........................H : 336
blue_tilapia          : ...........T..A........................Y : 336
Nile_tilapia          : ...........T...........................H : 336
wrasse                : ...........Y..........................H.. : 339
bamboo_grass_wrasse   : ......................................H.. : 343
killifish             : ...........T...........................H : 338
Japanese_medaka       : ........................................H : 336
broad_barred_goby     : ..........................L........H.L : 337
rainbow_trout         : ............................................. : 346
goldfish              : .....T...................................... : 347
zebrafish             : ............................................. : 346
roach                 : ............................................. : 346
southern_catfish      : ......................................AE : 347
channel_catfish       : ......................................AE : 347
yellow_catfish        : ...........S.........................AE : 335
Japanese_eel          : ...........Q.........................E : 340
                        ►              I helix region          ◄
```

```
                                      *         380          *          400
yellow_perch          : VELQLLQGIDTVVCDRQLQNGDCQKLQVLESSINECLRFH : 379
red-spotted_grouper   : .......E.....GE.....A.L........F........ : 379
orange-spotted_grouper: .............GE.......L........F........ : 379
humpback_grouper      : .K.....E.....GE.......L........F........ : 376
barramundi_perch      : .......E.....GE.......L................. : 379
Atlantic_croaker      : .......E.....GE.......L........F........ : 379
European_sea_bass     : .......E.....GE.......L........F........ : 378
Atlantic_halibut      : ......RE.....GE.......L........F........ : 379
bastard_halibut       : ......RE.....GE.......L........F........ : 379
flathead_mullet       : .......E.....GE..P....L...P....F........ : 379
red_seabream          : .......E.....G......R.P...H....F........ : 379
black_seabream        : .......E.....GE.......P...H....F........ : 379
gilthead_seabream     : .......E.....GE.......P...H....F........ : 379
rice_field_eel        : .......E.....GE.....E.LP......F........ : 379
Mozambique_tilapia    : ..P....E..A..GE.....Q.LH.......F.Y.M.... : 376
blue_tilapia          : ..P....E..A..GE.....Q.LH.......F.Y...... : 376
Nile_tilapia          : ..P....E..A..GE.....Q.LH.......F.Y...... : 376
wrasse                : .......E.....G.........LH..P....F........ : 379
bamboo_grass_wrasse   : .......E....GEG......L...P....F........ : 383
killifish             : .......E..K..G..........L........F........ : 378
Japanese_medaka       : .......E.....G.S....Q.L........F........ : 376
broad_barred_goby     : .......E.....GE.....S.LPQ.....F...S.... : 377
rainbow_trout         : .......E...A.G....H.S.L.N.R....F...S.... : 386
goldfish              : ...K...E..A..AG.S..HSHLSGFH....F...S.... : 387
zebrafish             : ...K...E.....AGQS..HSHLS.......F...S.... : 386
roach                 : ...K...E.E...AG.S..HSHLS..N...RF...S.... : 386
southern_catfish      : ..KR..TE.H...GES...HSHLPQ.R...CF...A.... : 387
channel_catfish       : ..RR..TE.H...G.T...HSHLSQ.H...CF...A.... : 387
yellow_catfish        : ..RR..TE.H...GEA...HSHLSQ.H...CF...S.... : 375
Japanese_eel          : ..Q...KE.....G..KA.S.L.H.I....F...S.... : 380
                                            ►    Ozol's peptide
```

```
                                     *        420          *         440
yellow_perch         : PVVDFTMRRALSDDIIDGYRVPKGTNIILNVGHMHRTEFF : 419
red-spotted_grouper  : ...................S........T.R....... : 419
orange-spotted_grouper: ........................T.R....... : 419
humpback_grouper     : ........................T.R....... : 416
barramundi_perch     : ........................T.R....... : 419
Atlantic_croaker     : ...............G.........T.Q....... : 419
European_sea_bass    : ........................T......... : 418
Atlantic_halibut     : ....................M....T.R....... : 419
bastard_halibut      : ........................T.R....... : 419
flathead_mullet      : ..................G......T.R....... : 419
red_seabream         : ........................T......... : 419
black_seabream       : ...................S....T......... : 419
gilthead_seabream    : ........................T......... : 419
rice_field_eel       : ........Q...............T.R....... : 419
Mozambique_tilapia   : ...............E....S...T.R....... : 416
blue_tilapia         : ...G...........E....S...T.R....... : 416
Nile_tilapia         : ...............E....S...T.R....... : 416
wrasse               : ...............E........T......S. : 419
bamboo_grass_wrasse  : ...............E........T.R....... : 423
killifish            : ........................T.R....... : 418
Japanese_medaka      : ..........F......H..Q...T.R....... : 416
broad_barred_goby    : ..........A......S......T.R....... : 417
rainbow_trout        : ..............S.........R......... : 426
goldfish             : ........D....E....K.....R......... : 427
zebrafish            : ........D....E..N.K.....R......... : 426
roach                : ........D....E....K.....R....D.. : 426
southern_catfish     : ......PD....E...........R......... : 427
channel_catfish      : ....Y....D....E.........R......... : 427
yellow_catfish       : ........D....E....L.....R......... : 415
Japanese_eel         : ......S.E....E....F.........R...C... : 420
                       ◄        ►    aromatase specific   ◄
                                     conserved region
```

132

```
                              *         460       *        480
yellow_perch           : FKPNKFSLENFGKNVPRRYFQPFGSGPRACVGKHIAMVMM : 459
red-spotted_grouper    : L.A.E......E..A..............S.......... : 459
orange-spotted_grouper : L.A.E......E..A..............S.......... : 459
humpback_grouper       : L.A.E......E..A...LLPAVRL..SS........... : 456
barramundi_perch       : L.A.E......E..A..............S.......... : 459
Atlantic_croaker       : L...E......Q..A..............S.......... : 459
European_sea_bass      : L...E.N.D..K..P......................... : 458
Atlantic_halibut       : C...E.R.S..E..A..............S.......... : 459
bastard_halibut        : C...E.R.D..E.TA..............S......A... : 459
flathead_mullet        : L...E....H.KSHA......................... : 459
red_seabream           : N...E.R....E..A..............S.......... : 459
black_seabream         : N...E.R....E..A..............S.......... : 459
gilthead_seabream      : N...E.R....E.TA..............S.......... : 459
rice_field_eel         : L...E.N....E..A..............S.......... : 459
Mozambique_tilapia     : L.A.Q.N..H.EN........................... : 456
blue_tilapia           : L.G.Q.N..H.EN........................... : 456
Nile_tilapia           : L.G.Q.N..H.EN........................... : 456
wrasse                 : H...D.N.K..E..A..............S.......... : 459
bamboo_grass_wrasse    : L...D......E..A......................... : 463
killifish              : H.A.E......Q..T......................... : 458
Japanese_medaka        : H.A.E......Q..T......................... : 456
broad_barred_goby      : H...E...D..E..T.............N........... : 457
rainbow_trout          : L...E...D..E....N............S.......... : 466
goldfish               : P...E...D..Q....S............S.......... : 467
zebrafish              : S...Q...D..Q....S............S.......... : 466
roach                  : P...E...D..Q....S............S.......... : 466
southern_catfish       : P..AE...N..T.P..S............S.......... : 467
channel_catfish        : P..AD...D..N.P..S............S.......... : 467
yellow_catfish         : P..TE...D..N.P..S............S.......... : 455
Japanese_eel           : S...E......E.T..N............S......S.... : 460
                                             ▶heme-binding◀
                                                 region
```

133

```
                                       *         500          *          520
yellow_perch          : KSILVTLLSQYSVCPHEGLTLDCLPQTNNLSQQPVEHQQE : 499
red-spotted_grouper   : ...............T........................ : 499
orange-spotted_grouper: ........................................ : 499
humpback_grouper      : ........................................ : 496
barramundi_perch      : ........................................ : 499
Atlantic_croaker      : ...............K.........I.............. : 499
European_sea_bass     : ...............K........................ : 498
Atlantic_halibut      : ...............K...A...............P.    : 499
bastard_halibut       : ........................................ : 499
flathead_mullet       : ...................EG...............R.   : 499
red_seabream          : ........................................ : 499
black_seabream        : ........................................ : 499
gilthead_seabream     : ........................................ : 499
rice_field_eel        : ...............K........................ : 499
Mozambique_tilapia    : ..............T...PI...................A : 496
blue_tilapia          : ..............T...PI...................A : 496
Nile_tilapia          : ..............T...PI...................A : 496
wrasse                : ...............K.....G.T............H.K  : 499
bamboo_grass_wrasse   : .........R.............G............H..  : 503
killifish             : ....A..............................H..  : 498
Japanese_medaka       : ...................................H..  : 496
broad_barred_goby     : ...................EL...............V... : 497
rainbow_trout         : .........R.........................E.G. : 506
goldfish              : .........R.....VK.C...S............EPSS : 507
zebrafish             : .....A...R.....MKAC..EN...........EPSS : 506
roach                 : .........R.....VKDC..ES............DPSS : 506
southern_catfish      : .A....G..R..G..E.SC..EN.AH..........DKHT : 507
channel_catfish       : .A...M...R.....E.SC..EN.AH..........DKHT : 507
yellow_catfish        : .A...M...R.....E.SC..EN.AH..........DKHT : 495
Japanese_eel          : .A..A....R.....RD.R...N.RK.......LA.---K : 497
```

```
                                     *         540         *         560
yellow_perch          : AE-QLSMRFLPRQRGSWQKL-------------------- : 518
red-spotted_grouper   : ..-H.............KT.-------------------- : 518
orange-spotted_grouper: .D-H.............T.--------------------- : 518
humpback_grouper      : .D-H.............T.--------------------- : 515
barramundi_perch      : .D-H.............T.--------------------- : 518
Atlantic_croaker      : ..-H.........N..T.--------------------- : 518
European_sea_bass     : ..-H......S.....KT.-------------------- : 517
Atlantic_halibut      : .P-.............T.PDSDL--------------- : 523
bastard_halibut       : .P-H.N..........T.--------------------- : 518
flathead_mullet       : T.-H.R.........RT.TDSDL--------------- : 523
red_seabream          : ..SH..........S...T.------------------- : 519
black_seabream        : ..SH.............T.-------------------- : 519
gilthead_seabream     : ..SH.............T.-------------------- : 519
rice_field_eel        : .--N........HS..C.T.------------------- : 517
Mozambique_tilapia    : ETEH.H........S.C.T.RDPNL------------- : 521
blue_tilapia          : ETEH.H.......GS.C.T.KDPNL------------- : 521
Nile_tilapia          : ETEH.H.......GS.C.T.KDPNL------------- : 521
wrasse                : N.-P.G...........N.-------------------- : 518
bamboo_grass_wrasse   : N.-P.G...........T.-------------------- : 522
killifish             : ..-..............TP-------------------- : 517
Japanese_medaka       : .D-H...T.......I..SPSPF---------------- : 518
broad_barred_goby     : P---...T....S.A.T..ERRQGQEGHPNKESGLNLGDS : 534
rainbow_trout         : PH---........HQARK.S------------------- : 522
goldfish              : LS----.QL.L.NAL----------------------- : 518
zebrafish             : LS----.QL.L.NTL----------------------- : 517
roach                 : LS----.QL.L.NTL----------------------- : 517
southern_catfish      : LS----......NTH.QTHTPTHTHTHTHTHTTETKK--- : 540
channel_catfish       : LS----......NTH.RN.KA------------------ : 524
yellow_catfish        : LS----......NTH.TN.NHI----------------- : 513
Japanese_eel          : DSE--LTMMFTPR.RQ---------------------- : 511
```

135

```
yellow_perch           : --- :    -
red-spotted_grouper    : --- :    -
orange-spotted_groupe  : --- :    -
humpback_grouper       : --- :    -
barramundi_perch       : --- :    -
Atlantic_croaker       : --- :    -
European_sea_bass      : --- :    -
Atlantic_halibut       : --- :    -
bastard_halibut        : --- :    -
flathead_mullet        : --- :    -
red_seabream           : --- :    -
black_seabream         : --- :    -
gilthead_seabream      : --- :    -
rice_field_eel         : --- :    -
Mozambique_tilapia     : --- :    -
blue_tilapia           : --- :    -
Nile_tilapia           : --- :    -
wrasse                 : --- :    -
bamboo_grass_wrasse    : --- :    -
killifish              : --- :    -
Japanese_medaka        : --- :    -
broad_barred_goby      : EEQ :  537
rainbow_trout          : --- :    -
goldfish               : --- :    -
zebrafish              : --- :    -
roach                  : --- :    -
southern_catfish       : --- :    -
channel_catfish        : --- :    -
yellow_catfish         : --- :    -
Japanese_eel           : --- :    -
```

Figure 3.8 Alignment of yellow perch ovarian aromatase (CYP19A1) deduced amino acid sequence with other teleost ovarian aromatases.

Conserved amino acid residues are indicated with (.), inserted gaps are indicated with (-). Structural domains conserved in all steroidogenic cytochrome P-450s are identified at the bottom of the alignment in bold. Sequences were downloaded from the EMBL/GenBank database with the following accession numbers: red-spotted grouper (*Epinephelus akaara*) AAS58448; orange-spotted grouper (*Epinephelus coioides*) AAR97601; humpback grouper (*Cromileptes altivelis*) AAV91178; barramundi perch (*Lates calcarifer*) AAV91179; Atlantic croaker (*Micropogonias undulatus*) ABA26927; European sea bass (*Dicentrarchus labrax*) CAC21712; Atlantic halibut (*Hippoglossus hippoglossus*) CAC36394; bastard halibut (*Paralichthys olivaceus*) BAA74777; flathead mullet (*Mugil cephalus*) AAW72732; red seabream (*Pagrus major*) BAB82524; black seabream (*Acanthopagrus schlegelii*) AAP23236; gilthead seabream (*Sparus aurata*) AAL27699; rice field eel (*Monopterus albus*) AAS94314; Mozambique tilapia

(*Oreochromis mossambicus*) AAD31031; blue tilapia (*Oreochromis aureus*) ABB89869; Nile tilapia (*Oreochromis niloticus*) AAO62625; wrasse (*Halichoeres tenuispinis*) AAR37048; bamboo grass wrasse (*Pseudolabrus japonicus*) ABB96485; killifish (*Fundulus heteroclitus*) AAR97268; Japanese medaka (*Oryzias latipes*) BAA11657; broad barred goby (*Gobiodon histrio*) AAV91177; rainbow trout (*Oncorhynchus mykiss*) 1806325A; goldfish (*Carassius auratus*) AAC14013; zebrafish (*Danio rerio*) AAK00643; roach (*Rutilus rutilus*) BAD91037; southern catfish (*Silurus meridionalis*) AAP83133; channel catfish (*Ictalurus punctatus*) AAB32613; yellow catfish (*Pseudobagrus fulvidraco*) AAW65999; Japanese eel (*Anguilla japonica*) AAS47028.
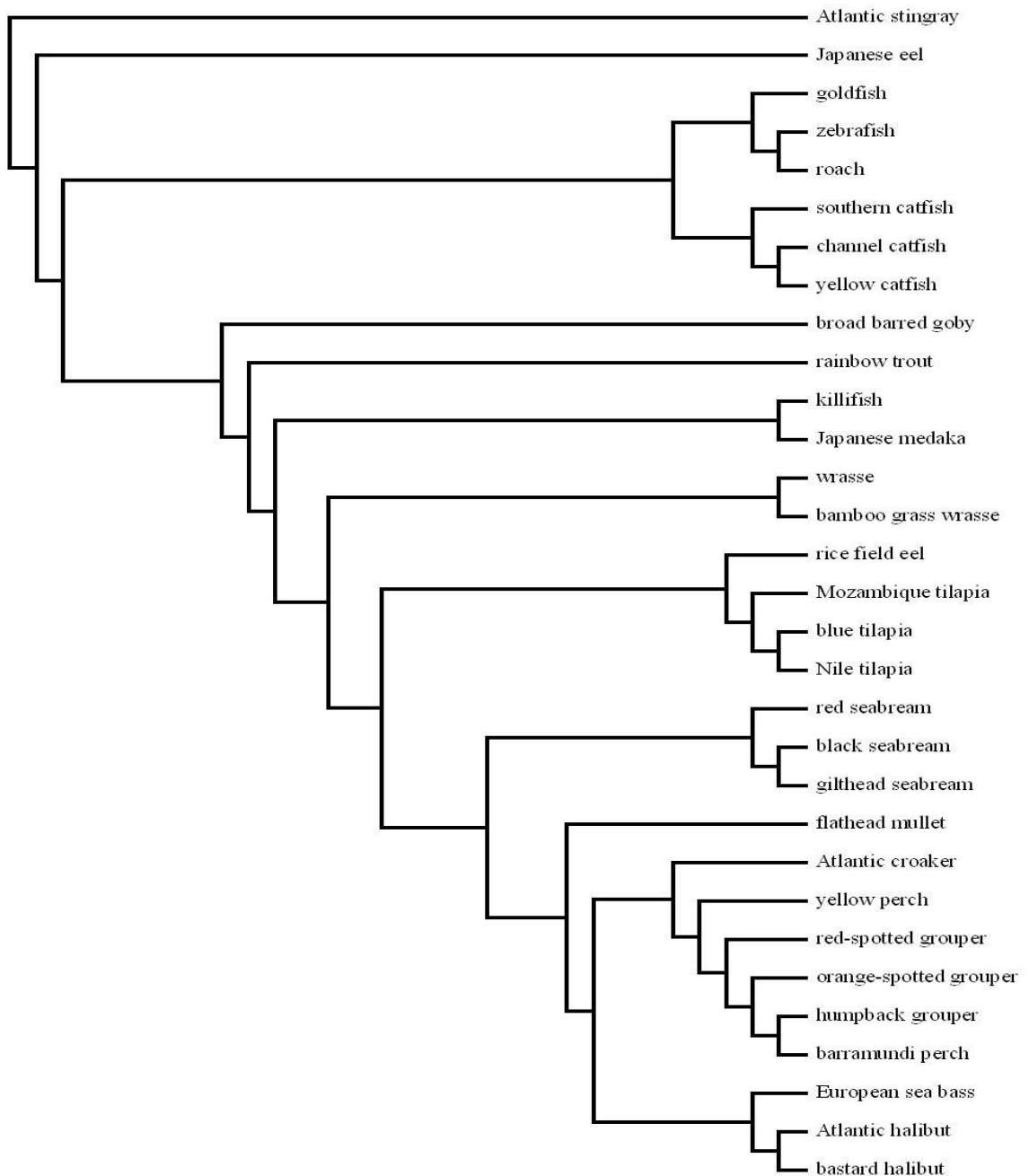
Figure 3.9 Phylogenetic tree of ovarian aromatase (CYP19A1) amino acid sequences.

Tree produced using a Clustal X (1.81) alignment and TreeView (Win 32) v. 1.6.6. The tree was rooted with Atlantic stingray (*Dasyatis sabina*) aromatase (Accession: AAF04617) as an outgroup representing the closest available species not in the group Teleostei. See Figure 3.8 for EMBI/GenBank accession numbers for the other sequences used.

```
-82    --GCAGTCAAGCTCAGACATCCTACCGGAGTGTAGCGTTCAGTCACAGCCCTTT

                                  M  D  D  E  I  A  A  L     8
-30    GTAAGCCGATAACCCTCCCTCAAACAGAAAATGGATGACGAAATCGCCGCCCTC

         V  V  D  N  G  S  G  M  C  K  A  G  F  A  G  D  D  A  26
 25    GTTGTTGACAACGGATCCGGTATGTGCAAAGCTGGCTTTGCAGGAGATGATGCT

         P  R  A  V  F  P  S  I  V  G  R  P  R  H  Q  G  V  M  44
 79    CCGCGTGCTGTGTTCCCCTCCATTGTTGGACGTCCAAGACATCAGGGTGTGATG

         V  G  M  G  Q  K  D  S  Y  V  G  D  E  A  Q  S  K  R  62
133    GTTGGCATGGGCCAGAAAGATAGCTATGTTGGTGATGAGGCACAGAGCAAAAGG

         G  I  L  T  L  K  Y  P  I  E  H  G  I  V  T  N  W  D  80
187    GGTATCCTGACCCTGAAGTACCCCATTGAGCATGGTATCGTCACCAACTGGGAC

         D  M  E  K  I  W  H  H  T  F  Y  N  E  L  R  V  A  P  98
241    GACATGGAGAAGATCTGGCATCACACCTTCTACAACGAGCTGAGAGTTGCGCCC

         E  E  H  P  V  L  L  T  E  A  P  L  N  P  K  A  N  R  116
295    GAGGAGCACCCCGTCCTGCTCACAGAGGCTCCCCTGAACCCCAAGGCCAACAGG

         E  K  M  T  Q  I  M  F  E  T  F  N  T  P  A  M  Y  V  134
349    GAAAAGATGACCCAGATCATGTTCGAGACCTTCAACACCCCTGCCATGTACGTT

         A  I  Q  A  V  L  S  L  Y  A  S  G  R  T  T  G  I  V  152
403    GCCATCCAGGCCGTGCTGTCCCTGTATGCCTCTGGTCGTACCACTGGTATCGTC

         M  D  S  G  D  G  V  T  H  T  V  P  I  Y  E  G  Y  A  170
457    ATGGACTCCGGTGATGGTGTGACCCACACAGTGCCCATCTATGAGGGCTATGCC

         L  P  H  A  I  L  R  L  D  L  A  G  R  D  L  T  D  Y  188
511    CTGCCCCACGCCATCCTGCGTCTGGACTTGGCTGGCCGTGACCTCACAGACTAC

         L  M  K  I  L  T  E  R  G  Y  S  F  T  T  T  A  E  R  206
565    CTCATGAAGATCCTGACAGAGCGTGGGTACTCATTCACCACCACAGCTGAGAGG

         E  I  V  R  D  I  K  E  K  L  C  Y  V  A  L  D  F  E  224
619    GAAATCGTGCGTGACATCAAGGAGAAGCTGTGCTATGTCGCCCTGGACTTCGAG

         Q  E  M  G  T  A  A  S  S  S  S  L  E  K  S  Y  E  L  242
673    CAGGAGATGGGCACTGCTGCCTCCTCTTCCTCCCTGGAGAAGAGCTACGAGCTG

         P  D  G  Q  V  I  T  I  G  N  E  R  F  R  C  P  E  A  260
727    CCCGACGGACAGGTCATCACCATCGGCAATGAGAGGTTCCGTTGCCCAGAGGCC

         L  F  Q  P  S  F  L  G  M  E  S  C  G  I  H  E  T  T  278
781    CTCTTCCAGCCTTCCTTCCTCGGTATGGAGTCCTGCGGAATCCATGAGACCACC

         Y  N  S  I  M  K  C  D  V  D  I  R  K  D  L  Y  A  N  296
835    TACAACAGCATTATGAAGTGTGATGTCGACATCCGTAAGGACCTGTACGCCAAC
```

```
      T   V   L   S   G   G   T   T   M   Y   P   G   I   A   D   R   M   Q   314
  889 ACCGTGCTGTCTGGAGGTACCACCATGTACCCCGGCATTGCCGACAGGATGCAG

      K   E   I   T   A   L   A   P   S   T   M   K   I   K   I   I   A   P   332
  943 AAGGAGATCACAGCCCTGGCCCCATCCACCATGAAAATCAAGATTATTGCCCCA

      P   E   R   K   Y   S   V   W   I   G   G   S   I   L   A   S   L   S   350
  997 CCAGAGCGTAAATACTCTGTCTGGATCGGAGGCTCCATCCTGGCCTCTCTGTCC

      T   F   Q   Q   M   W   I   S   K   Q   E   Y   D   E   S   G   P   S   368
 1051 ACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTACGATGAGTCCGGCCCCTCC

      I   V   H   R   K   C   F ***                                       375
 1105 ATCGTCCACCGTAAATGCTTCTAAACAGACTGTTCCTCCTCTCCCCCCCTCCCC

 1159 AACCAACGCCCAACAACTTCAGCTCTGTGCAAAACAACCACACACACTACATTT
 1213 CTCATACACACTCAGGCGCAGAGCCTAGACAACCAACTCATTGGCATGGCTTCA
 1267 GTTATTTTTGGCGCTTGACTCAGGATTTAAAAAAAACTGGAACGATGAAGGAGA
 1321 ACGTAATGTTTTGGCTAGGTTATAAAAACAAAAGCACCCCAGCATTTTGCAGTT
 1375 GCATCTGGGGACCTAAAAATGTACATTTTGTTTTCTTTTGAGTCATTCCAAATG
 1429 TTTGTTAACTGCATTGTTCAGACACATGATTCCAAATGTTAACTGCATTGTTCA
 1483 GACACCGTATTCGCCTCTATGAAGGCTGCCCAGTGGTTGGCGCATACTTAAACA
 1537 TGGTTGTAGTATCGCTTGTATGTAAATTATGTCTGGGTTTTTTGTACTTTCAGC
 1591 CTTAAAAATATCTTGGTCCTGTTTAATTTTTTTGTTTTTGTTATGCAAAACCCA
 1645 ATCGTGACCTCTTCTTCCCCCTATTGGAGGTTTCCATCCCCTGGGTGGTGGGGC
 1699 AAGGGGTCTCAAAGTGATGGGGTAACATGGGGTGCCAGACCGGTGGGGCCAACA
 1753 TGTACACTGACTAAACAATCCC**<u><u>AATAAA</u></u>**GTGCACATGTGTTCCGACAAAAAAAA
 1807 AAAAAAAAAAA
```

Figure 3.10 Complete nucleotide and deduced amino acid sequence of yellow perch β-actin.

Numbers for nucleotides (left) and amino acids (right) are from the initiation methionine based on alignment with related teleost sequences. The putative polyadenylation signal is in bold and double underlined. Yellow perch β-actin cDNA sequence has been deposited in the EMBL/GenBank database with accession #AY332493.

140
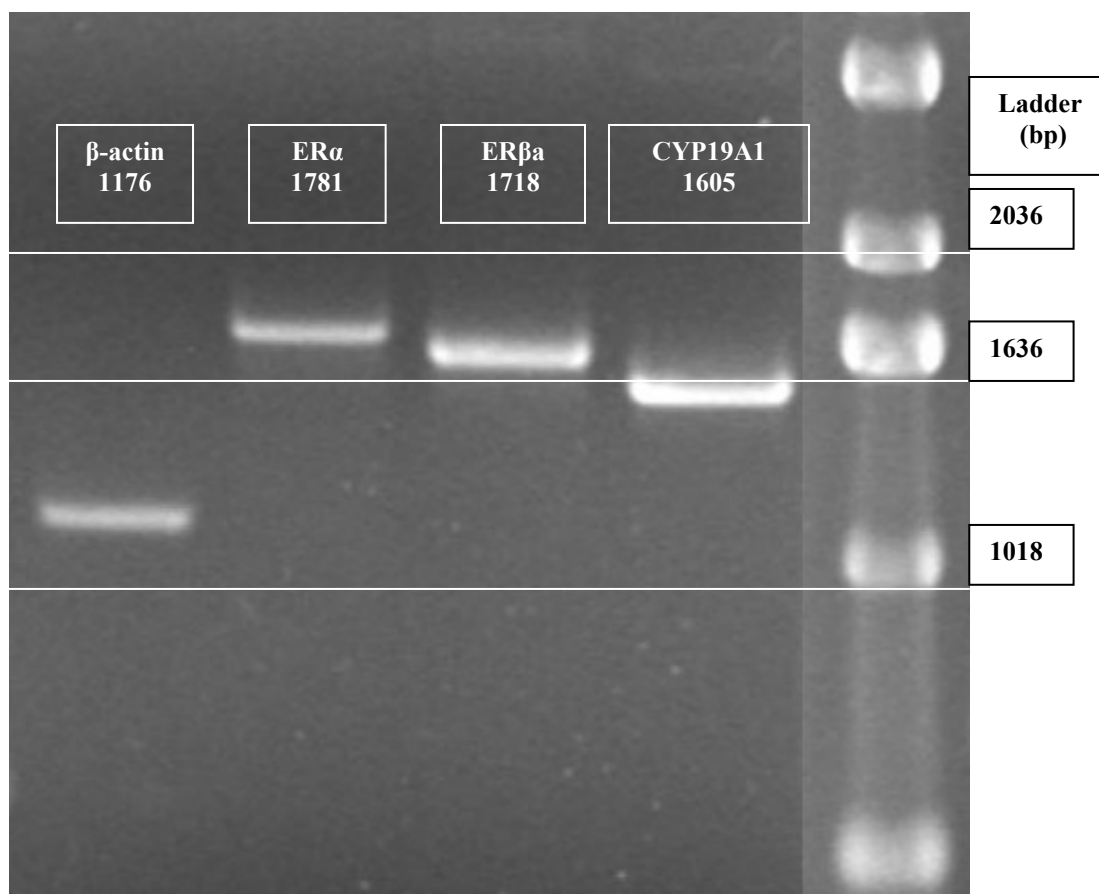
Figure 3.11 Full cDNA coding region PCR products for β-actin, ERα, ERβa and CYP19A1.

For PCR primers used see Table 3.3 and PCR protocol used see Table 3.4. Template used was 1 µl of cDNA generated from adult yellow perch gravid ovary mRNA. PCR products were then run on a 1% low melt agarose gel with a 1 KB ladder and visualized using ethidium bromide staining.
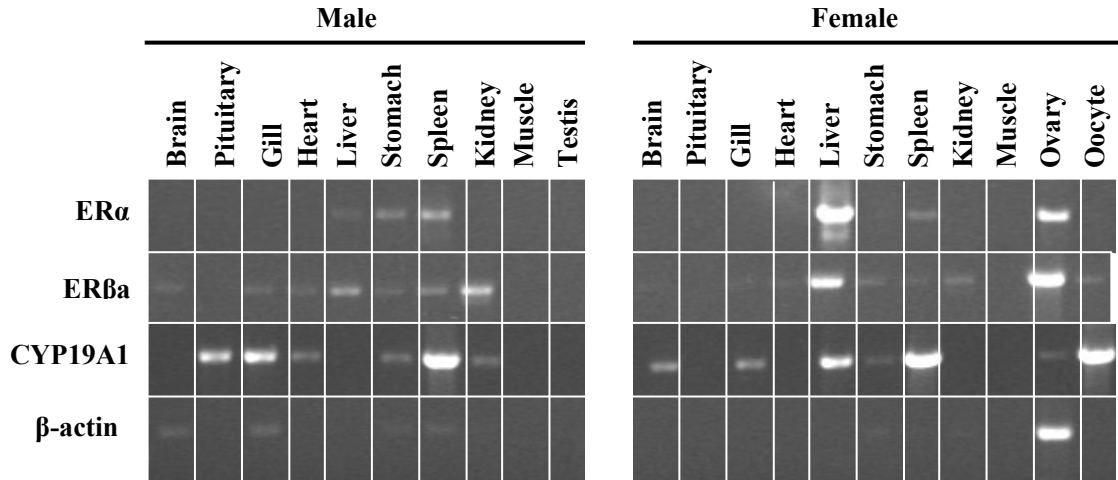
Figure 3.12 Sex-specific tissue expression for ERα, ERβa, CYP19A1 and β-actin.
For PCR primers used see Table 3.3 and PCR protocol used see Table 3.4. Template used was 900 ng of cDNA generated from adult yellow perch mRNA from each sex-specific tissue. PCR products were then run on a 1% agarose gel and visualized using ethidium bromide staining. cDNA template quality was verified by analyzing for β-actin mRNA levels using real-time quantitative PCR (qPCR).

Chapter 4: Sex-specific and seasonal mRNA levels of key endocrine genes in adult yellow perch from Lake Erie

4.1 Introduction

Yellow perch (*Perca flavescens*) are an economically, ecologically and recreationally important teleost species [1], especially in the Great Lakes, and have historically comprised the largest inland fishery in North America [2]. Yellow perch commercial fisheries harvest >10 million Ib yr^{-1} in the Great Lakes, with most of that coming from Lake Erie, however these harvests are approximately 6-8 fold less than levels seen only a few decades ago [1]. Lake Michigan has seen an 80% decrease in yellow perch harvests since the early 90s [1], and in the late 90s females constituted only 20% of the population [3, 4]. A study by Heyer *et al.* [5] indicated that maternal effects on larval traits may be substantially influencing the recruitment of Lake Michigan yellow perch. These maternal effects are most probably tempered by the maternal endocrine status, which can be influenced by a number of biotic (age, size, gonadosomatic index) and abiotic (photoperiod, temperature, salinity) [6] factors.

The yellow perch is one of a group of important fishes which exhibit a sexual size dimorphism (SSD) in which females grow faster than males [7]. Other fishes in this group include sea bass [166, 167], halibut [168], eel [169], plaice, walleye, and tench [170, 171]. The female biased SSD in yellow perch was demonstrated in laboratory [8, 9] and wild populations [10-12] many years ago, but it was not until the mid 1980s [7, 13, 14] that studies identified 17β-estradiol (E_2) as a growth stimulator in yellow perch SSD. In a series of experiments using E_2, MT, triiodothyronine (T_3) and zeranol, with different size classes of juvenile perch, Malison *et al.* [13] found that only the low dosages of E_2 (2 and 20 μg/g diet) significantly stimulated growth. Further, higher levels of E_2 (50 μg/g diet) depressed growth and the growth promoting effects of E_2 were only noticeable in fish of 80-110 mm total length (TL) or greater [13]. E2 enhanced growth of both sexes of perch but did not eliminate the normal pattern of SSD when treatment was initiated in fish that were 90-110 mm TL [13]. In a separate study, Malison *et al.* [14] found that sexual differentiation occurs around 16 mm TL in yellow perch and E_2 treatment of male perch initially 90-110 mm TL did not result in sex

reversal. These findings together indicate that the influence E_2 has on the growth of male yellow perch 80+ mm TL is not simply the result of a feminizing effect, however the possibility that feminizing male perch may increase their growth rate cannot be excluded. This critical size range of 80-110 mm TL is also the same size at which females normally begin to outgrow males [8] and when female-biased SSD begins to be manifested. This critical period is also the specific minimum body size for the onset of vitellogenesis and spermatogenesis in females and males [14], respectively, pointing towards an upregulation of E_2 receptors (ERs) on target tissues (ovary, liver or pituitary) and a coinciding increase in tissue expression of growth factors. Malison *et al.* [7] reported that in addition to a growth response, E_2 treatment stimulated feed consumption, and in a different study [15] growth rate and growth efficiency of female yellow perch exceeded those of males two-fold in animals fed without restriction. These observations suggest that the growth-promoting effects of estrogen may work in part through appetite centers of the central nervous system and could involve pituitary hormones. They also point out a clear linkage of growth and reproductive development in this species.

It is of interest in the pursuit of understanding estrogen stimulated SSD in yellow perch to identify and quantify endocrine genes involved both in growth and sexual function. Endocrine genes associated with growth and reproduction in fish include the pituitary hormones (growth hormone (GH), somatolactin (SL) and prolactin (PRL)), the growth hormone intermediate insulin-like growth factor I (IGF-I), the estrogen receptors (ERs) and aromatase. In vertebrates, the pituitary gland is the "master gland" and as such at least partially regulates aspects of growth and reproduction. Pituitary GH together with IGF-I and IGF-II are considered to be THE key players in the growth process [16]. Administering bovine GH to striped bass hybrids resulted in an increase in specific growth rate and food conversion efficiency [303]. Many studies have shown a causative effect of GH on the release of IGF-I [129, 304, 305] and it is believed that much of the effect GH has on growth is mediated through liver IGF-I [123, 125]. There is increasing evidence that somatolactin (SL), another pituitary hormone found only in fish, is involved in metabolism, sexual maturation and reproductive cycle regulation [29-31]. The first somatolactin receptor

144

(SLR) was recently characterized [36] and the highest SLR levels in masu salmon (*Oncorhynchus masou*) were found in liver and fat. Although the precise function of SL still remains unclear, studies have shown a seasonal, possibly growth related, rhythm in gilthead sea bream (*Sparus aurata*) [32] and rainbow trout (*Oncorhynchus mykiss*) [33]. Recent studies indicate a metabolic role with activity opposite to that of GH, suggesting SL is an anti-obesity hormone that helps to expedite growth and/or reproductive processes [34, 35, 109, 110]. Prolactin, another pituitary hormone, has long been suspected of involvement in fish reproduction because of its well known role in mammalian reproduction [38]. Weber *et al.* [39] found that gonadotropin releasing hormone (GnRH) is a potent stimulator of PRL release in tilapia (*Oreochromis mossambicus*) cultured pituitaries, which can be enhanced 3-fold by sex steroid hormones (estrogen and testosterone). However Brinca *et al.* [40] found seasonally dependent results with *in vitro* PRL secretion in response to E_2 treatment in sea bream (*Sparus aurata*). Winter sea bream pituitaries showed an increased PRL secretion in response to E_2, while spring pituitaries showed a decreased PRL secretion. These results support a theory that there may be a shift in control of PRL secretion with changes in the reproductive state of the fish. Growth-promoting actions of PRL have been reported in higher vertebrates, but are less well established in teleosts. Ghrelin (Ghr), a newly discovered peptide which specifically stimulates GH release from the pituitary, was shown for the first time in teleosts to significantly stimulate PRL release in tilapia [41]. The ability of tilapia PRL ($tPRL_{177}$) to elevate IGF-I mRNA levels in the liver [42] indicates that PRL may possess somatotropic actions similar to GH.

Reproduction and sexual development, or maturation, are significantly influenced, if not controlled, by sex steroids, but these steroidal effects are ultimately modulated by the distribution and expression of the steroid receptors. Estrogen receptors are distributed in many tissues in teleosts (gonad, liver, brain and pituitaries) [53, 54] and are intricately involved in sexual determination and development [55]. Mosconi *et al.* [56] found that the liver of seabream was resistant, with regard to vitellogenin (vtg) production, to either GH or E_2 during the postspawning period. They also report that ER levels were higher in sea bream liver at the prespawning stage compared with those at the spawning or postspawning stages, which points toward an

upregulation of ER as an important component of estrogen-mediated hepatic responses in this teleost. Estrogen levels, however, are controlled primarily by P450 aromatase, the terminal enzyme in the conversion of testosterone to estrogen, and blocking this enzyme, via an aromatase inhibitor (i.e. fadrozole), causes sex reversal in several species of teleosts [57-59] . Aromatase mRNA is expressed in many tissues associated with reproduction (gonad, brain, and pituitary) [60] and growth (liver, brain, and pituitary) [61], and varies with sexual maturation [62], age and season [63]. All these findings indicate the presence of an intricate, sex-specific, endocrine regulatory relationship between the pituitary, liver and gonad in teleosts, but it remains unclear how estrogen-stimulated growth in yellow perch is achieved.

In an effort to investigate estrogen stimulated SSD in yellow perch, male and female fish were collected twice per year, just after spawning (May) and during the autumnal active growth phase of ova (October), over a two year period from Lake Erie. The cDNAs for yellow perch GH, PRL, SL, IGF-I, ERα, ERβa and CYP19A1 (aromatase) are published (Chapter 2 and Chapter 3), and in this study real-time quantitative PCR assays (qPCR) were developed for these genes to measure sex-specific tissue expression. Seasonal expression levels of these key endocrine genes in both male and female yellow perch will provide a better understanding of their sex-specific regulation and may provide important information towards their role in estrogen stimulated SSD.

4.2 Methods

Samples

Adult yellow perch from Lake Erie were sampled on May 26th and October 20th for two years. Trap nets designed to exclude fish < 6" were placed and maintained just north of Sandusky, OH by a commercial fisheries company, Swartz Fisheries (Port Clinton, OH). Nets were pulled and up to 30 adult fish were randomly placed into 5 gallon aerated buckets (5/bucket) with 1 g of MS222 and 2 g of $NaHCO_3$. Fish were anesthetized with MS222 to prevent a stress response as both plasma GH and PRL have been shown to increase in response to confinement [306]. Once on shore, the fish were given a lethal dose of MS222, weighed, sexed and pituitary, liver and ovary tissues were

collected. Liver and ovary tissues were weighed to determine hepato-somatic index (HSI) and gonado-somatic index (GSI), respectively. Tissues were immediately frozen, transported to the University of Kentucky and stored at -80 °C until analysis. Otiliths were removed, dried and fractured to count annual rings for age determination. Age was not determined for all fish as in some cases otiliths were unrecoverable or age could not be determined using the above method. For each sampling time point (4: twice/year for two years), 6 male and 6 female sets of samples were randomly chosen for further analysis. For these samples, total RNA was extracted with the GenElute™ Mammalian Total RNA Kit (Sigma, St. Louis, MO). RNA samples were treated with amplification grade DNase I (Sigma, St. Louis, MO) and quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Up to 750 ng of RNA was reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA) and quantified.

Real-time qPCR

Sequences for yellow perch GH (Accession #AY007303), PRL (Accession #AY332491), SL (Accession #AY332490), IGF-Ib (Accession #AY332492), ERα (Accession #DQ984124), ERβa (Accession #DQ984125) and CYP19A1 (Accession #DQ984126) either presently are available or will be by Oct 2007 from the GenBank/EMBL/DDBJ nucleotide sequence database. Primers (Table 4.1) were designed for real-time quantitative PCR (qPCR) using Beacon Designer v 3.0 (PREMIER Biosoft International, Palo Alto, CA). Designed primers were tested with a 25 μl total volume PCR mixture using a MasterTaq Kit (Eppendorf Scientific Inc., Westbury, NY) and 1 μl of cDNA template. GH, PRL and SL used pituitary tissue as a template, IGF-Ib used liver tissue as a template and ERα, ERβa, and CYP19A1 used gravid ovary tissue as a template. Real-time qPCR consisted of 3:00 min at 94 °C follow by 45 cycles of 45 s at 94 °C and 45 s at 60 °C. PCR products were electrophoresed in 1% low melt agarose/2% nuseive gels with a 100 bp DNA ladder (Takara Shuzo Co., Otsu, Japan) and visualized by ethidium bromide staining for size verification. PCR products were then purified using Amicon Centrifugal Ultrafiltration Devices (Millipore, Billerica, MA) and quantified. Purified PCR products were ligated into a pCR®4-TOPO® vector and transformed into TOP10 chemically competent cells using the TOPO TA Cloning® Kit

for Sequencing (Invitrogen, Carlsbad, CA). The plasmid DNA was then extracted from the bacterial cells using the GenElute™ Plasmid Miniprep Kit (Sigma, Sigma, St. Louis, MO). Plasmid samples were quantified and up to 600 ng of plasmid DNA was put into a sequencing PCR using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing PCR consisted of 35 cycles of 30 s at 96 °C, 15 s at 50 °C and 4:00 min at 60 °C. After the PCR, each sample received 27 µl ddH$_2$O, 60µl 100% EtOH and 3 µl 3 M NaOAc. This mixture was transferred to a 1.5 mL microcentrifuge tube, vortexed and left overnight to precipitate. The next day, samples were spun at maximum speed at 4 °C for ≥ 30 min, decanted and washed with 250 µl of 70% EtOH, spun again, decanted and allowed to air dry. Samples were kept at -20 °C until they were transported to the University of Kentucky Advanced Genetic Technologies Center (http://www.uky.edu/Centers/AGTC/) for sequencing. Sequencing products were compared to known template sequences using Vector NTI Suite 7.0 (Informax, Inc., Frederick, MD) and GeneDoc (http://www.psc.edu/biomed/genedoc/) [172] to verify primer specificity.

Purified PCR products were then serially diluted to generate 6 standards ranging from 10 pg/µl to 100 ag/µl. Real-time qPCR reactions (25 µl) were prepared in 0.2 ml thin-wall 96 well plates (BioRad, Hercules, CA) each containing the following components: 12.5 µl iQ™ SYBR® Green Supermix (BioRad, Hercules, CA), 0.75 µl (15 ng) each forward and reverse primers, 1 µl template and 10 µl ddH$_2$0. After pipetting, the plates were sealed with Bio-Rad iCycler iQ Optical Tape and spun at 2200 rcf for 1 min. Amplification and detection of samples were performed with the BioRad iCycler Thermal Cycler and Optical Module (BioRad, Hercules, CA). Each 96 well plate had duplicate wells of standards (6), no template (1), RNA template (1) and samples (40). Duplicate sample Ct values with a coefficient of variation >2% were rerun. When necessary, dilutions of template cDNAs were performed to ensure samples fell within the standard curve. All plates had standard curves of R>0.98 and PCR efficiencies between 85-110% with the exception of IGF-Ib which had PCR efficiencies up to 115%. Real-time qPCR results (ag/µl) were standardized to total cDNA (µg) in the template and log transformed before statistical analyses.

Statistics

Statistical outliers within each variable were identified using a box plot as data points beyond 3 times the interquartile (IQ) range. Only one data point, an autumn male GH mRNA measurement (12.10), was defined as an outlier and removed from all analyses. A general linear model (GLM) was then used to determine year effects for each variable and if a year effect existed then the data was divided into sex-specific seasonal groups for further clarification of year effect. Then a GLM was used to examine variables (wt, age, HSI and GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa and liver CYP19A1 mRNA levels) for sex, season and sex*season interaction effects. For variables in which only female data exist (GSI and ovary ERα, ovary ERβa and ovary CYP19A1 mRNA levels) a t-test was used to examine for season effects only. Relationships between 13 measured variables were determined by generating a Pearson (r) correlation matrix; significant differences from 0 were tested using Fisher's z. SYSTAT Grad Pack v. 10.0 was used for all analyses and differences between groups and relationships between parameters were considered significant at $p \leq 0.05$.

4.3 Results

Year effects

Only HSI showed a significant effect of year (n=48; F=21.361; p≤0.001) but when the data were separated into sex-specific seasonal groups (e.g. autumn males, spring females, etc.) only the autumn groups (autumn males and autumn females) showed significant differences between years for HSI.

Sex and season effects

Sex had a significant effect on body weight with female yellow perch having a significantly higher average body weight than male yellow perch (Figure 4.1). Season had a significant effect on age with spring yellow perch having a significantly higher average age than autumn yellow perch (Figure 4.2). Average male age in spring was higher than any other average sex-specific seasonal age (Figure 4.2). Season had a significant effect on GH mRNA levels with spring yellow perch having a significantly higher average GH mRNA level than autumn yellow perch (Figure 4.3). Average female GH mRNA level in spring was significantly higher than the average male GH mRNA

level in autumn.   Neither season nor sex had a significant effect on PRL (Figure 4.4) or SL (Figure 4.5) mRNA levels, nor was there a significant effect of the sex*season interaction.

Sex had a significant effect on HSI with female yellow perch having a significantly higher average HSI than male yellow perch (Figure 4.6). Average female HSI in autumn was significantly higher than the average male HSIs in both autumn and spring. Both sex and season had a significant effect on IGF-Ib mRNA levels with male yellow perch having significantly higher IGF-Ib mRNA levels than female yellow perch and spring yellow perch having significantly higher IGF-Ib mRNA levels than autumn yellow perch (Figure 4.7). The average autumn female IGF-Ib mRNA level was lower than any other sex-specific seasonal IGF-Ib mRNA level.

Both sex and season had a significant effect on liver ERα mRNA levels with female yellow perch having significantly higher liver ERα mRNA levels than male yellow perch and autumn yellow perch having significantly higher liver ERα mRNA levels than spring yellow perch (Figure 4.8). The average autumn female liver ERα mRNA level was higher than any other sex-specific seasonal liver ERα mRNA level and the spring female liver ERα mRNA level was higher than either male seasonal liver ERα mRNA level. Sex had a significant effect on liver ERβa mRNA levels with male yellow perch having a significantly higher average liver ERβa mRNA level than female yellow perch (Figure 4.9). Average male liver ERβa mRNA level in autumn was higher than the average female liver ERβa mRNA level in autumn. Both sex and season had a significant effect on liver CYP19A1 mRNA levels with male yellow perch having significantly higher liver CYP19A1 mRNA levels than female yellow perch and spring yellow perch having significantly higher liver CYP19A1 mRNA levels than autumn yellow perch (Figure 4.10). Average female autumn liver CYP19A1 mRNA levels were lower than either average male or female spring liver CYP19A1 mRNA levels.

Season did not have a significant effect on ovary ERα or ERβa mRNA levels, however season did have a significant effect on ovary CYP19A1 mRNA levels with autumn ovaries having a significantly higher average CYP19A1 mRNA level than spring ovaries (Figure 4.11).   GSI in the autumn (4.4%; n=12), with ovaries containing previtellogenic oocytes, was significantly higher (F=66.558; p<0.001) than GSI in the

spring (1.7%; n=12), with post-spawning ovaries that were deplete of all forms of gametes (S Lynn, pers. obs.).

Correlations

Body weight had significant negative correlations with IGF-Ib and liver CYP19A1 mRNA levels (Table 4.2). GH mRNA levels had significant positive correlations with PRL and SL mRNA levels and significant negative correlations with GSI and ovary CYP19A1 mRNA levels (Table 4.2). PRL mRNA levels only showed a significant relationship with GH mRNA levels (Table 4.2). SL mRNA levels had a significant positive correlation with GH and liver CYP19A1 mRNA levels and a significant negative correlation with liver ERβa mRNA levels (Table 4.2). HSI had a significant positive correlation with liver ERα mRNA levels and significant negative correlations with IGF-Ib and liver ERβa mRNA levels (Table 4.2). IGF-Ib mRNA levels had significant positive correlations with liver ERβa and liver CYP19A1 mRNA levels and significant negative correlations with weight, HSI, GSI and liver ERα and ovary CYP19A1 mRNA levels (Table 4.2). Liver ERα mRNA levels had significant positive correlations with HSI, GSI and ovary CYP19A1 mRNA levels and significant negative correlations with IGF-Ib and liver CYP19A1 mRNA levels (Table 4.2). Liver ERβa had a significant positive correlation with IGF-Ib mRNA levels and significant negative correlations with HSI and SL mRNA levels (Table 4.2). GSI had significant positive correlations with liver ERα and ovary CYP19A1 mRNA levels and had significant negative correlations with GH, IGF-Ib and liver CYP19A1 mRNA levels (Table 4.2). Ovary ERα mRNA levels had only one significant relationship which was a positive correlation with ovary ERβa mRNA levels (Table 4.2). And finally, ovary CYP19A1 mRNA levels had significant positive correlations with GSI and liver ERα mRNA levels and significant negative correlations with GH, IGF-Ib and liver CYP19A1 mRNA levels (Table 4.2).

4.4 Discussion

This study is the first to comprehensively measure the mRNA levels of these key endocrine genes in a natural adult population of yellow perch. Several sex and seasonal differences have been found along with significant relationships that not only give a

greater understanding to yellow perch endocrinology, but also could be applicable to a number of fish species. Since there is little potential for measurement error in HSI, the year effect on sex-specific autumn HSIs indicates that, while the sampling took place on the same day of the year, some environmental aspect was significantly different between the years (e.g. temperature, light, water quality, etc.). There are even biological factors for yellow perch that might influence their perceived season, which might be an interesting topic for future study.

Female yellow perch sampled from Lake Erie for this study were on average larger than males (Figure 4.1) which is consistent with female biased SSD in yellow perch [307]. Correlation analyses suggest that small yellow perch would have high liver CYP19A1 and IGF-Ib mRNA levels, while large yellow perch would have low liver CYP19A1 and IGF-Ib mRNA levels. This could possibly be an indication that the smaller yellow perch are growing faster and hence have higher expression of those liver genes. There was a significant effect of season, with fish sampled in the spring being older than fish sampled in autumn, and sex*season interaction on average age of yellow perch sampled (Figure 4.2), and males caught in the spring had the highest average age. These observations could indicate that older males are more likely to spawn, and ultimately be caught in the spring, than younger males. The lowest age for both males and females was 2.5 years which is either at or above the average age of sexual maturation (males≈1.9 years; females≈2.4 years) found in Lake Erie populations from four 1 year studies [308]. Also, the weights of the fish in this study correspond to lengths that are well above the 80 – 150 mm TL [13, 308] generally perceived to be the threshold for reproductive adulthood in yellow perch. Therefore, the analyses performed in this study were carried out under the assumption that all yellow perch sampled were adults.

GH mRNA levels followed a pattern of higher levels in females and higher levels in spring (Figure 4.3). Thus females in the spring had the highest average GH mRNA levels and males in the autumn had the lowest average GH mRNA levels. A study which measured GH protein in yellow perch pituitaries showed a significant increase in GH during the month of May [175] with about a 10 fold difference compared to October. In salmonids, evidence has shown that increases in both daylength and temperature elevate GH levels, particularly those in the parr-smolt phase (for review see Björnsson 1997

[309]). Melamed *et al.* [22] reported that plasma GH levels increase at ovulation and spawning as the somatotrophs become more active. Both gilthead seabream [310] and carp [311] showed seasonal differences in GH expression with high levels in spring, but Meiri *et al.* [310] did not find significant differences between males and females in seasonal GH expression. However, Degani *et al.* [312] found that different levels of GH transcription could explain the growth rate differences between male and female European eels. All of these studies support the seasonal and sex-specific patterns observed in GH mRNA levels of yellow perch in this study.

Females showed a significant negative correlation between GH and ovary CYP19A1 mRNA levels, which theoretically should represent circulating E_2 levels as the ovary is the primary site of global E_2 synthesis. The implication is that when E_2 levels in females are elevated in autumn, along with liver ERα mRNA levels, GH mRNA levels are depressed. Also in support of this are the highly significant positive correlations between GSI and ovary CYP19A1 and liver ERα mRNA levels (Table 4.2) and the highly significant negative correlation between GSI and IGF-Ib mRNA levels. GSI also had a significant negative correlation with GH mRNA levels, although this correlation was not nearly as strong. On top of that, liver CYP19A1 mRNA levels had a significant negative correlation with GSI and liver ERα and ovary CYP19A1 mRNA levels. All of this data indicates that when ovaries are largest (in autumn) there are higher ovary CYP19A1 mRNA levels and therefore more E_2 production. This in turn leads to lower liver CYP19A1 mRNA levels and higher liver ERα mRNA levels, ultimately producing lower mRNA levels of growth factors (i.e. GH and IGF-Ib), which contradicts the theory of an estrogen sensitive SSD in yellow perch. While these correlations are significant, the true nature of the relationships between these parameters is speculative, however studies have shown a connection between steroids and growth factors in fish. In orange-spotted grouper both salmon and mammalian GnRH (gonadotropin releasing hormone) increased GH mRNA levels and release in the pituitary [313], and one of the primary roles of GnRH is to ultimately stimulate the production and release of steroids, or E_2 in females. Estradiol increased pituitary content or plasma levels of GH in immature rainbow trout [314], Nile tilapia [315], rainbow trout [316], goldfish [317] and most importantly, yellow perch fed an E_2-treated diet showed a significantly higher GH protein

level in pituitaries than control fish [175]. Interestingly, the majority of studies of E_2 effects on IGF-I indicate that E_2 decreases hepatic content and plasma levels of IGF-I in gilthead seabream [129], Mozambique tilapia [157, 159] and Atlantic salmon [160].

Several studies in gilthead seabream and European sea bass have shown expression patterns and activity of SL opposite to that of GH [32, 34, 35, 109, 110] however the results here actually show a significant positive correlation between GH and SL expression (Table 4.2). GH mRNA levels also showed a significant positive correlation with PRL mRNA levels, which is also slightly surprising given their contrary actions in regard to osmoregulation [193]. Another surprising result is the non-significant positive correlation between GH and IGF-Ib mRNA levels as many studies have shown that GH stimulates the transcription and release of IGF-I from the liver [128-131]. However, both yellow perch [318] and the closely related Eurasian perch [120] showed a lack of responsiveness in hepatic IGF-I mRNA levels to exogenous GH treatment. This unresponsiveness to growth hormone treatment is not specific to perch as barramundi also showed no response to GH treatment in IGF-I mRNA levels [119]. These findings indicate that the lack of responsiveness in hepatic IGF-I expression to GH in yellow perch may prevent a significant positive correlation between the two parameters.

Studies have also shown that IGF-I not only inhibits GH release, as part of a negative feedback loop, but that it also is a direct stimulator of PRL release [319-321]. To that end, PRL mRNA levels did not show a significant correlation with any other parameter (with the exception of GH mRNA levels mentioned above), even ovary CYP19A1 mRNA levels (Table 4.2), which could be considered as an indicator of E_2 production. In mammals, E_2 is known to stimulate PRL release from the pituitary [38] but the relationship in teleosts is a little more tenuous [39, 40, 92, 322]. Rainbow trout pituitaries exposed to E_2 *in vivo* increased synthesis and secretion of PRL in a concentration-dependent manner [316]. However, E_2 administration at several different doses did not alter pituitary PRL mRNA levels in silver eels [322] while Brinca *et al.* [40] found that fish treated with E_2 showed seasonal responses in PRL secretion *in vitro* with winter gilthead seabream pituitaries having increased release and summer pituitaries having decreased release. Onuma *et al.* [92] showed similar results in regard to

reproductive state with E_2 increasing the pituitary PRL mRNA levels in the pre-spawning stage of masu salmon, but halving PRL mRNA levels in the spawning stage of females. PRL mRNA levels did show some non-significant trends with females generally having higher levels than males and autumn levels being higher than spring levels (Figure 4.4). Brinca et al. [40] found that winter (February) gildthead seabream pituitaries secreted significantly more PRL in vitro than spring (April) pituitaries. Tang et al. [323] found seasonal differences in pituitary PRL mRNA levels in channel catfish with the highest levels occurring in April and July and the lowest in December. An analysis of seasonal pituitary PRL mRNA levels in carp revealed the highest levels in summer-acclimatized carp and the lowest levels in winter-acclimatized carp [324]. Weber and Grau [90] found no differences in pituitary content of PRL_{188} in female Nile tilapia during the brooding phase of the reproductive cycle whereas more recently Tacon et al. [325] did find significant differences in pituitary content of PRL_{188} in female Nile tilapia during the brooding phase of the reproductive cycle. Bhandari et al. [49] showed pituitary PRL mRNA levels increased dramatically during the spring (Mar-May) in both male and female masu salmon.

SL mRNA levels did not show any significant effect of sex or season (Figure 4.5) and outside of GH mRNA levels, the most significant relationship seemed to be a positive correlation with liver CYP19A1 mRNA levels (Table 4.2). While the results were not significant, SL mRNA levels seemed to be higher in spring than in autumn for each sex, but studies on seasonal SL expression have produced rather conflicting results. In channel catfish [323], SL mRNA levels in the largest adult class sampled were higher in April than in December. However, in rainbow trout [33] the highest levels of plasma SL in both mature male and mature female fish were in late summer (August) with a secondary peak in spring (April). In masu salmon [49] during the second year of development, the highest SL mRNA levels were in late summer (August) but in gilthead seabream [32] the highest levels of plasma SL were very clearly in the winter (December). Perhaps seasonal SL expression levels are species dependent and related to the reproductive strategy of the fish, as there are several studies that have shown SL to both be stimulated by GnRH and to stimulate steroid production [30, 31, 107, 326].

There were significant differences between male and female HSI with females having larger HSIs (>1%) than males (≤1%) (Figure 4.6), yet there was no effect of season on HSI. Both sex and season had a significant effect on liver IGF-Ib mRNA levels, with females in the autumn having lower levels than the other sex-specific seasonal groups (Figure 4.7). Unfortunately, there are few studies that have examined seasonal expression of liver IGF-I and within those studies there is somewhat conflicting evidence. Measurements of IGF-I mRNA levels in coho salmon over the course of a year showed the highest levels around May [327], and measurement of plasma IGF-I levels in chinook salmon from the months of Jan to May showed the highest levels in late March and early May [328]. More recently though, measurements of plasma IGF-I levels in juvenile rainbow trout [329] and juvenile coho salmon [330] showed peaks in the late September time frame.

There also are few studies that have examined the sex-specific expression of IGF-I, however Riley *et al.* [159] investigated the effects of steroids (E_2 and 5α-dihydrotestosterone (DHT)) on IGF-I expression in male and female Mozambique tilapia hepatocyte cultures. They found that DHT significantly increased IGF-I mRNA levels in male hepatocytes and significantly decreased IGF-I mRNA levels in female hepatocytes and E_2 significantly decreased IGF-I mRNA levels in both male and female hepatocytes. They contend that the different growth patterns in tilapia (males grow larger than females) likely result from a difference in the sensitivity of male and female hepatocytes to gonadal steroid hormones. Since yellow perch are reported to have an opposite sexually dimorphic growth rate from tilapia, one might assume that the reverse would be true in regard to steroidogenic effects on IGF-I mRNA levels. This assumption would imply that E_2 levels in females are lower during the autumn and higher during the spring, however monthly plasma concentrations in female yellow perch from October to April show estradiol levels are high in autumn and low in spring, while testosterone levels show an inverse pattern (low in autumn, high in spring) [6, 331]. This coincides with the significantly higher ovarian CYP19A1 mRNA levels in autumn as compared to spring (Figure 4.11), but it also implies that IGF-Ib expression in female yellow perch is downregulated by high levels of E_2. This chain of events would not support estrogen

stimulated SSD in yellow perch but does explain the significant negative correlation between IGF-Ib and ovary CYP19A1 mRNA levels (Table 4.2).

Both liver ERα and liver ERβa mRNA levels had significant effects of sex and sex*season interaction but only liver ERα mRNA levels had a significant effect of season (Figures 4.8 and 4.9). Females clearly had higher liver ERα mRNA levels than males and males had higher liver ERβa mRNA levels than females. Liver ERα showed the highest mRNA levels in autumn females while liver ERβa showed the highest mRNA levels in autumn males. While female liver ERα mRNA levels were significantly higher in autumn than spring, there was no difference in male liver ERα mRNA levels between seasons. Outside of GSI and ovary CYP19A1 mRNA levels, liver ERα mRNA levels had the strongest relationship with IGF-Ib mRNA levels with a significant negative correlation (r=-0.456; Table 4.2). However, the relationship between liver ERα and ovary CYP19A1 mRNA levels was a highly significant positive correlation (Table 4.2). This relationship is supported in the literature as the liver ERα gene is known to have an estrogen response element (ERE) that upregulates it in response to increased E_2 levels [332-335]. Bowman *et al.* [333] showed that the expression window for liver ERα defines the primary response to E_2 in largemouth bass and other genes (vitellogenin) show a delayed primary response. Menuet *et al.* [269] found that in zebrafish both liver ERα and liver ERβ2 (ERβa), but not liver ERβ1, induced the zfERα gene promoter in the presence of E_2, indicating there is a differential activation of genes by the different ERs. They also found that expression of liver ERβ1 was strongly downregulated by *in vivo* E_2 treatment which suggests diverging functions for ERs in the liver. This implies that if the expression levels of ERs differ between sexes, then the effects of E_2 could lead to different levels of gene expression or perhaps even the expression of entirely different genes. Unlike liver ERα mRNA levels, liver ERβa mRNA levels were not significantly correlated with ovary CYP19A1 mRNA levels (Table 4.2), and the strongest relationship for liver ERβa mRNA levels was a highly significant positive correlation with IGF-Ib mRNA levels.

Some of the most interesting results of this study involve liver ER and liver CYP19A1 mRNA levels, unfortunately though, there are few comparative studies in the literature. Surprisingly, little work had been done on seasonal expression levels of liver

ERs in fish despite their obvious role in reproduction and only recently have studies really begun to examine the sex-specific expression levels of the various ERs in liver. Gilthead seabream had significantly higher ER protein levels in liver at the pre-spawning stage compared to the spawning and post-spawning stages [56]. Halm *et al.* [210] found very similar results to this study, with females having much higher liver ERα mRNA levels than males and males having higher liver ERβ1 mRNA levels than females. Sabo-Attwood *et al.* [223] observed significantly higher ERα mRNA levels than ERγ (ERβa) mRNA levels in livers from female largemouth bass in December. Direct comparisons between liver ER mRNA levels have been avoided in this study as unidentified differences in PCR efficiency and template amplification may make this inappropriate. In contrast to these studies though, Choi and Habibi [53] measured ER mRNA levels in immature goldfish liver and found significantly higher levels of ERα mRNA in males than females. They also found higher levels of ERβ1 mRNA in female liver than male liver, however it remains to be seen if this is a species difference or a maturational difference, as they used immature fish. The livers of female spotted seatrout showed increased levels of ER mRNA (presumably ERα) during the summer months along with a corresponding increase in GSI [336]. Only Sabo-Attwood *et al.* [223] have examined seasonal expression levels of all three ER mRNAs, which was done in female largemouth bass livers from October to March. In their study, liver ERα mRNA levels in females peaked in the late winter (February – March) and liver ERγ (ERβa) mRNA levels changed very little over the 6 month sampling period with a slight increase during February to March. In this study, liver ERβa mRNA levels in females increased in the spring, but liver ERα mRNA levels in females decreased significantly from autumn to spring.

Both sex and season had significant effects on liver CYP19A1 mRNA levels (Figure 4.10) with males being higher than females and spring being higher than autumn. The highest level of liver CYP19A1 mRNA was in spring males while the lowest level of liver CYP19A1 mRNA was in autumn females. Few studies in fish have examined liver CYP19A1 mRNA levels and at least one publication contended that the liver was a non-steroidogenic tissue, so there were no studies with which to compare these results. This is unfortunate, as liver CYP19A1 showed some very interesting relationships with other

variables. Liver CYP19A1 mRNA levels showed significant negative correlations with body weight and liver ERα mRNA levels, but showed a significant positive correlation with SL mRNA levels and a highly significant positive correlation with IGF-Ib mRNA levels (Table 4.2). The relationship between liver CYP19A1 and ovary CYP19A1 mRNA levels was significant with a negative correlation (Table 4.2) which indicates that liver CYP19A1 mRNA levels are tied to circulating estrogen levels and the results previously discussed (relationships with weight and IGF-Ib and liver ERα mRNA levels) indicate that it could function as an intermediate between the ovarian estrogen and liver growth axes.

Neither ovarian ERα nor ERβa mRNA levels showed significant differences between seasons (Figure 4.11). Again, only Sabo-Attwood *et al.* [223] examined seasonal levels of all three ER mRNAs in female largemouth bass ovaries from October to March. In their study, ovary ERα mRNA levels showed a minor peak in the late autumn (October - November) and liver ERγ (ERβa) mRNA levels showed a highly significant drop from autumn (October) to spring (March-April). The differences seen between this study and the largemouth bass study could be attributed to differences between the reproductive physiology of the species or the climate of the sampling location. Regardless, it seems quite apparent that the seasonal expression and role of gonadal and even hepatic ERs in fish is a topic that is desperately in need of future study. Ovarian ERα mRNA levels only showed a significant relationship with ovarian ERβa mRNA levels, and vica versa, with a positive correlation (Table 4.2). Unlike ovarian ERs, yellow perch ovarian CYP19A1 mRNA levels did show a significant effect of season with autumn levels being significantly higher than spring levels (Figure 4.11). Also ovarian CYP19A1 mRNA levels showed significant negative correlations with both growth factors (GH and IGF-Ib mRNA levels) and liver CYP19A1 mRNA levels and showed highly significant positive correlations with liver ERα mRNA levels and GSI (Table 4.2). And unlike ovarian ERs or liver CYP19A1 mRNA levels, there are a reasonable number of studies on ovarian CYP19A1 mRNA levels. A study in adult killifish by Patel *et al.* [337], found no differences between female ovary CYP19A1 mRNA levels between winter and summer exposure conditions. However, a separate study on adult killifish [338] did find significant differences in ovarian CYP19A1 mRNA

levels with regard to season or reproductive activity. They found that reproductively active females (May-July) had higher ovarian CYP19A1 mRNA levels than reproductively inactive females (August-September). Another study on fathead minnow found similar results with higher ovarian CYP19A1 mRNA levels in reproductively active females than non-reproductively active females [339]. Choi *et al.* [226] found steadily increasing levels of CYP19A1 mRNA in ovaries of wrasse from May to August with levels peaking during spawning period in July and August. In addition, ovarian follicles in channel catfish showed higher levels of CYP19A1 mRNA corresponding with vitellogensis during the winter months (February) [60]. As mentioned earlier, monthly plasma concentrations in female yellow perch from October to April showed higher plasma estradiol levels in autumn than spring, while testosterone levels show an inverse pattern (low in autumn, high in spring) [6, 331]. Also, yellow perch ovarian follicles showed significantly higher production of estradiol in October than any other month in the experimental period (October to April) [195] These results coincide with the significantly higher ovarian CYP19A1 mRNA levels in autumn as compared to spring.

In summary, the measurement of GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa, liver CYP19A1, ovary ERα, ovary ERβa and ovary CYP19A1 mRNA levels displayed significant sex-specific and seasonal differences in adult yellow perch from Lake Erie. These results indicate that both male and female yellow perch have increased mRNA levels of growth regulating hormones (GH and IGF-Ib) in spring as opposed to autumn. Also there is a distinct difference in male and female liver ER (ERα and ERβa) mRNA levels with both isotypes showing significant effects of sex and liver ERα having a significant effect of season. Lastly, ovarian CYP19A1 mRNA levels (a possible indicator of plasma E_2 levels) showed a significant negative correlation with GH, IGF-Ib and liver CYP19A1 mRNA levels and a significant positive correlation with liver ERα mRNA levels and GSI in females. These results indicate new avenues for research related to sex-specific and seasonal expression of these genes in fish and also provide new insights into estrogen stimulated SSD and other estrogen actions in yellow perch.

Acknowledgements

Table 4.1 Target gene Accession numbers and primer sequences for qPCR.

Target Gene	GenBank Accession #	Start[a]	Primer Sequence
GH	AY007303	F161-	CGG AGG AGC AGC GTC AAC
		R307-	CCC AGG ACT CGA CCA AAC G
PRL	AY332491	F242-	ACC AGG CTC TTC AAG TAT CAG
		R344-	GTG TTA GCA GAG GTG GAG AG
SL	AY332490	F294-	CTC CAA AGG TGA AAT CCA ACA G
		R422-	TCA GGA GCG GCA TCG TAG
IGF-Ib	AY332492	F393-	CGC AGG GCA CAA AGT GGA C
		R540-	CCC AGT GTT GCC TCG ACT TG
ERα	DQ984124	F1031-	AGG TGC TGA TGA TCG GGC TC
		R1124-	TCG CCT ACG TTC CTG TCC AG
ERβa	DQ984125	F1341-	TCT GGA CGC TGT GAC GGA C
		R1423-	GGG CGA GGC GGG TGT AC
CYP19A1	DQ984126	F210-	TCT GGG TTT GGG GCC ACT TC
		R359-	ACC GCT GAT GCT CTG CTG AG

[a]Forward (F) and reverse (R) primer start numbers are calculated beginning with the initiation methionine.

Table 4.2 Correlations between 13 measured variables from adult Lake Erie yellow perch.

n=48		Wt	GH[a]	PRL	SL	HSI	IGF-Ib	liver ERα	liver ERβa	liver CYP19	GSI[b]	ovary ERα[b]	ovary ERβa[b]
GH[a]	r	-0.090											
	p	0.547											
PRL	r	-0.043	0.447										
	p	0.772	0.002										
SL	r	-0.036	0.599	0.264									
	p	0.810	<0.001	0.070									
HSI	r	n/a	-0.082	-0.075	-0.026								
	p		0.585	0.612	0.859								
IGF-Ib	r	-0.328	0.125	-0.222	-0.013	-0.337							
	p	0.023	0.401	0.129	0.932	0.019							
liver ERα	r	0.132	-0.134	0.213	0.056	0.413	-0.456						
	p	0.369	0.369	0.147	0.707	0.004	0.001						
liver ERβa	r	-0.253	-0.268	-0.166	-0.312	-0.341	0.633	-0.165					
	p	0.083	0.069	0.258	0.031	0.018	<0.001	0.262					
liver CYP19A1	r	-0.339	0.162	-0.076	0.349	-0.273	0.502	-0.350	0.144				
	p	0.019	0.277	0.610	0.015	0.060	<0.001	0.015	0.327				
GSI[b]	r	n/a	-0.405	0.180	0.026	0.362	-0.795	0.757	-0.402	-0.453			
	p		0.050	0.401	0.904	0.082	<0.001	<0.001	0.051	0.026			
ovary ERα[b]	r	-0.103	0.112	-0.047	0.074	0.073	0.148	-0.339	-0.176	0.342	-0.149		
	p	0.633	0.602	0.829	0.731	0.735	0.490	0.105	0.411	0.102	0.486		
ovary ERβa[b]	r	-0.014	-0.017	0.038	-0.113	-0.098	-0.012	-0.126	-0.116	0.123	-0.045	0.786	
	p	0.949	0.937	0.861	0.600	0.649	0.956	0.557	0.589	0.567	0.834	<0.001	
ovary CYP19A1[b]	r	0.063	-0.486	0.176	-0.168	0.173	-0.591	0.719	-0.253	-0.491	0.729	0.232	0.387
	p	0.770	0.016	0.410	0.434	0.419	0.002	<0.001	0.233	0.015	<0.001	0.275	0.062

[a]single outlier (male autumn) was removed n=47; [b]female specific variables n=24.

163
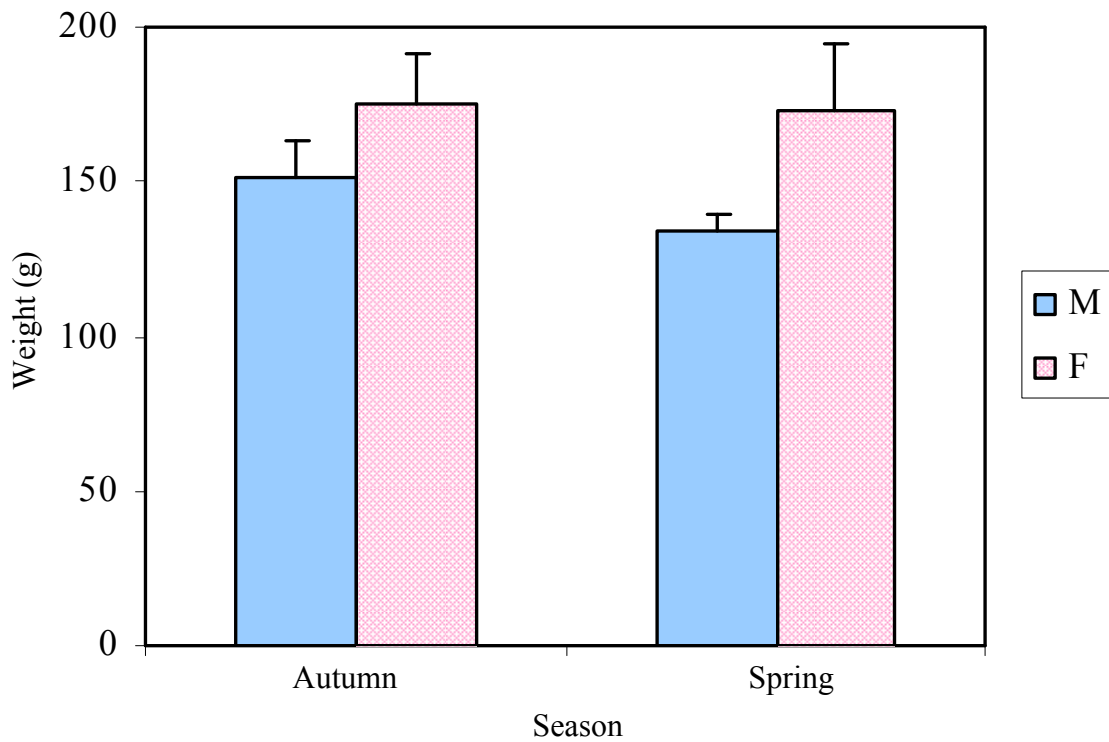
Figure 4.1 Average body weights of male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Bars indicate standard error and sample size for each sex-specific seasonal group is n=12. The GLM indicates a significant effect of sex ($F_{1,44}$=5.054; p=0.031), with females being higher than males, and no effect of season ($F_{1,44}$=0.464; p=0.499) or sex*season interaction ($F_{1,44}$=0.308; p=0.582).
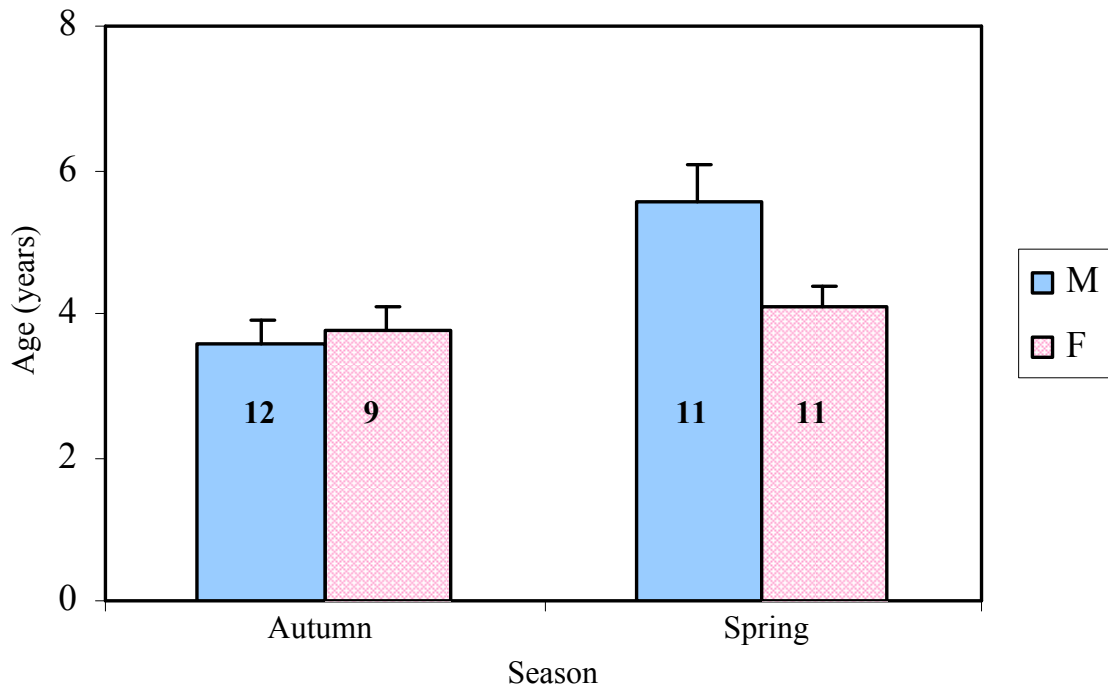
Figure 4.2 Average ages of male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Bars indicate standard error and bold numbers indicate sample sizes (n) of each sex-specific seasonal group.   The GLM indicates a significant effect of season ($F_{1,39}$=10.825; p=0.002), with spring being higher than autumn, and sex*season interaction ($F_{1,39}$=5.646; p=0.023) but no effect of sex ($F_{1,39}$=3.356; p=0.075).
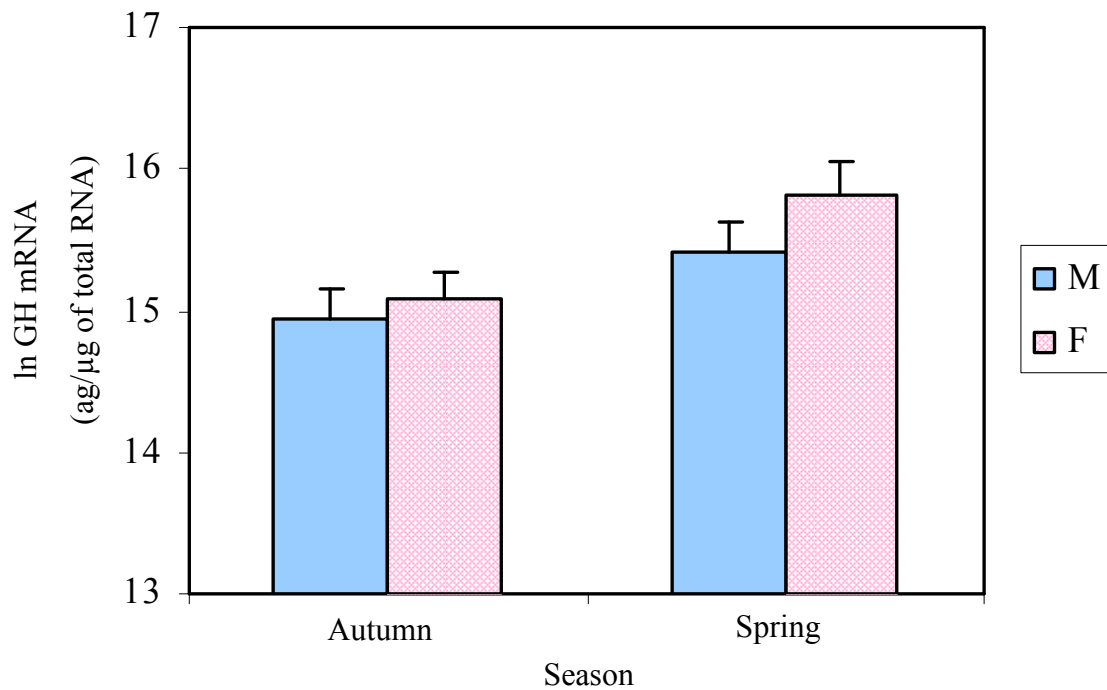
Figure 4.3 Average pituitary GH mRNA levels in male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Units are expressed as ln (ag of mRNA/μg of total RNA). Bars indicate standard error and sample size for each sex-specific seasonal group is n=12 (with the exception of autumn males where n=11). The GLM indicates a significant effect of season ($F_{1,43}$=9.001; p=0.004) with spring being higher than autumn and no effect of sex ($F_{1,43}$=2.003; p=0.164) or sex*season interaction ($F_{1,43}$=0.468; p=0.498).
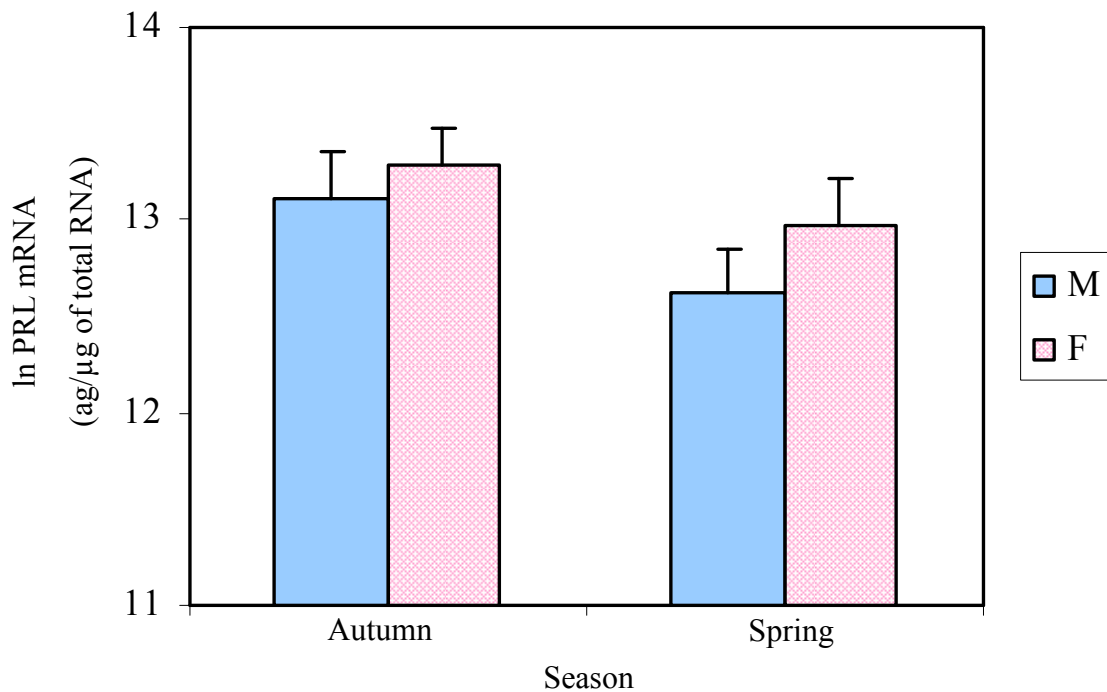
Figure 4.4 Average pituitary PRL mRNA levels in male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Units are expressed as ln (ag of mRNA/µg of total RNA). Bars indicate standard error and sample size for each sex-specific seasonal group is n=12. The GLM indicates no significant effect of sex ($F_{1,44}$=1.528; p=0.223), season ($F_{1,44}$=3.485; p=0.069) or sex*season interaction ($F_{1,44}$=0.169; p=0.683).
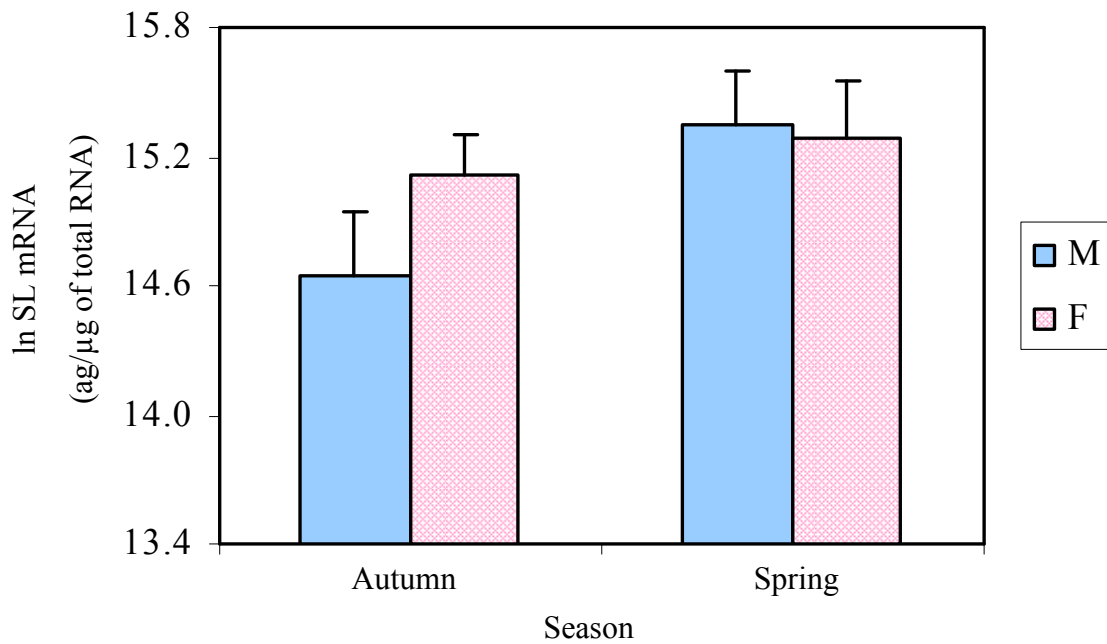
Figure 4.5 Average pituitary SL mRNA levels in male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Units are expressed as ln (ag of mRNA/µg of total RNA). Bars indicate standard error and sample size for each sex-specific seasonal group is n=12. The GLM indicates no significant effect of sex ($F_{1,44}$=0.678; p=0.415), season ($F_{1,44}$=3.188; p=0.081) or sex*season interaction ($F_{1,44}$=1.218; p=0.276).
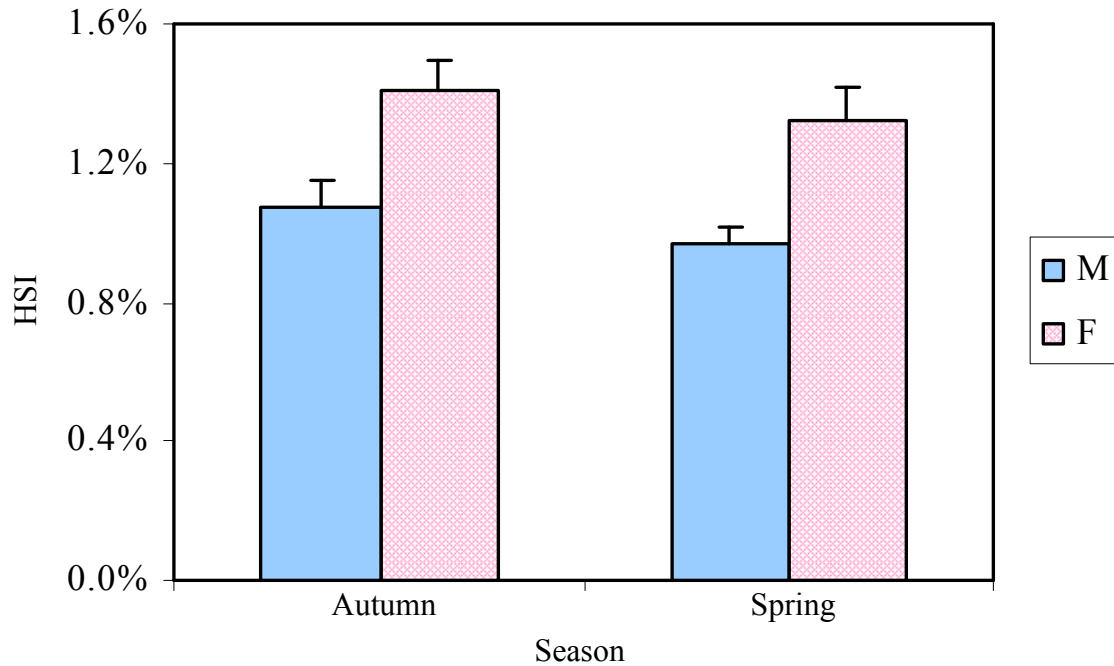
Figure 4.6 Average HSIs of male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Units are expressed as a percentage of liver weight divided by body weight. Bars indicate standard error and sample size for each sex-specific seasonal group is n=12. The GLM indicates a significant effect of sex ($F_{1,44}$=21.130; p<0.001), with females being higher than males, and no effect of season ($F_{1,44}$=1.487; p=0.229) or sex*season interaction ($F_{1,44}$=0.016; p=0.900).
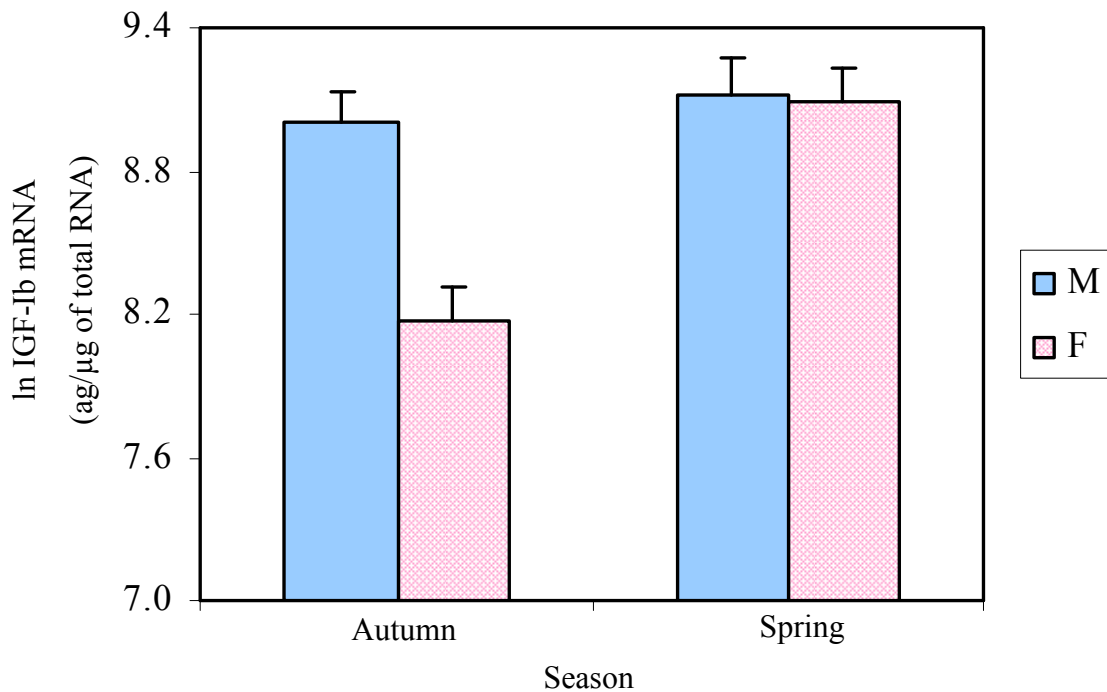
Figure 4.7 Liver IGF-Ib mRNA levels in male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Units are expressed as ln (ag of mRNA/μg of total RNA). Bars indicate standard error and sample size for each sex-specific seasonal group is n=12. The GLM indicates a significant effect of sex ($F_{1,44}$=9.549; p=0.003), with males being higher than females, season ($F_{1,44}$=14.403; p<0.001), with spring being higher than autumn, and sex*season interaction ($F_{1,44}$=8.697; p=0.005).
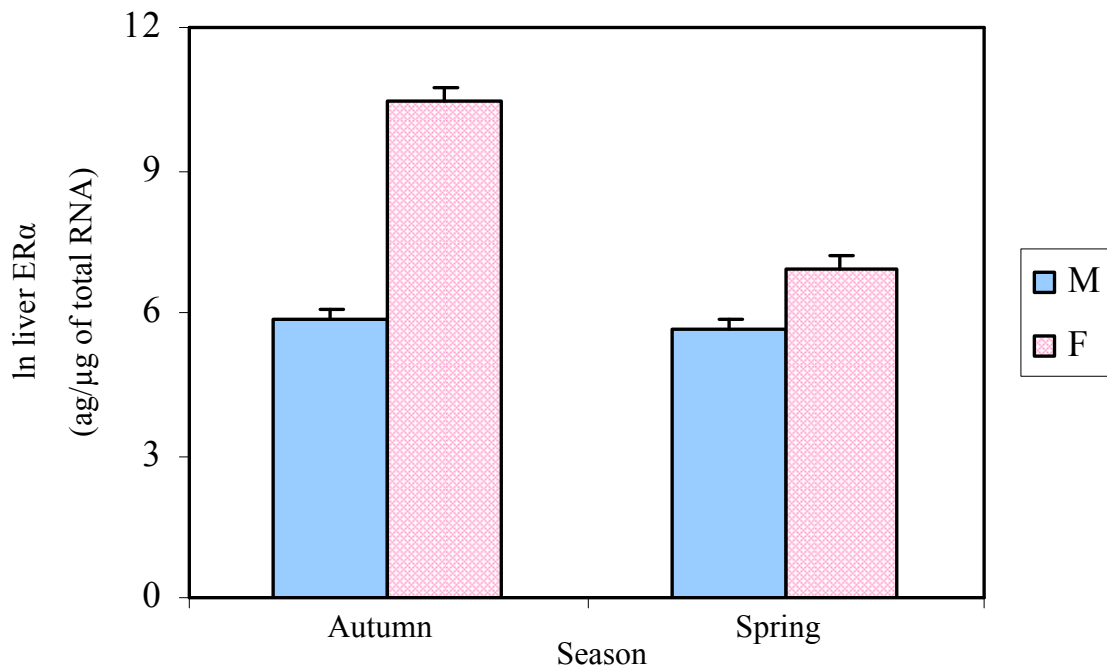
Figure 4.8 Average liver ERα mRNA levels in male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Units are expressed as ln (ag of mRNA/μg of total RNA). Bars indicate standard error and sample size for each sex-specific seasonal group is n=12. The GLM indicates a significant effect of sex ($F_{1,44}$=179.011; $p<0.001$), with females being higher than males, season ($F_{1,44}$=69.517; $p<0.001$), with autumn being higher than spring, and sex*season interaction ($F_{1,44}$=58.482; $p<0.001$).
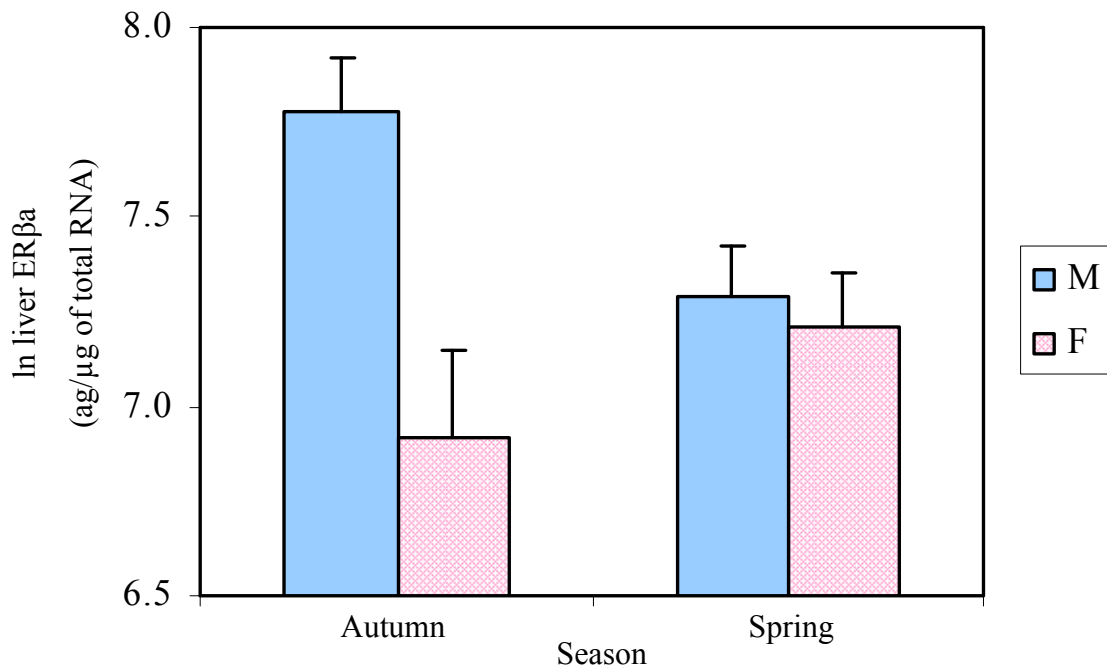
Figure 4.9 Average liver ERβa mRNA levels in male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Units are expressed as ln (ag of mRNA/μg of total RNA). Bars indicate standard error and sample size for each sex-specific seasonal group is n=12. The GLM indicates a significant effect of sex ($F_{1,44}$=8.725; p=0.005), with males being higher than females, and sex*season interaction ($F_{1,44}$=6.188; p=0.017) but no effect of season ($F_{1,44}$=0.382; p=0.540).
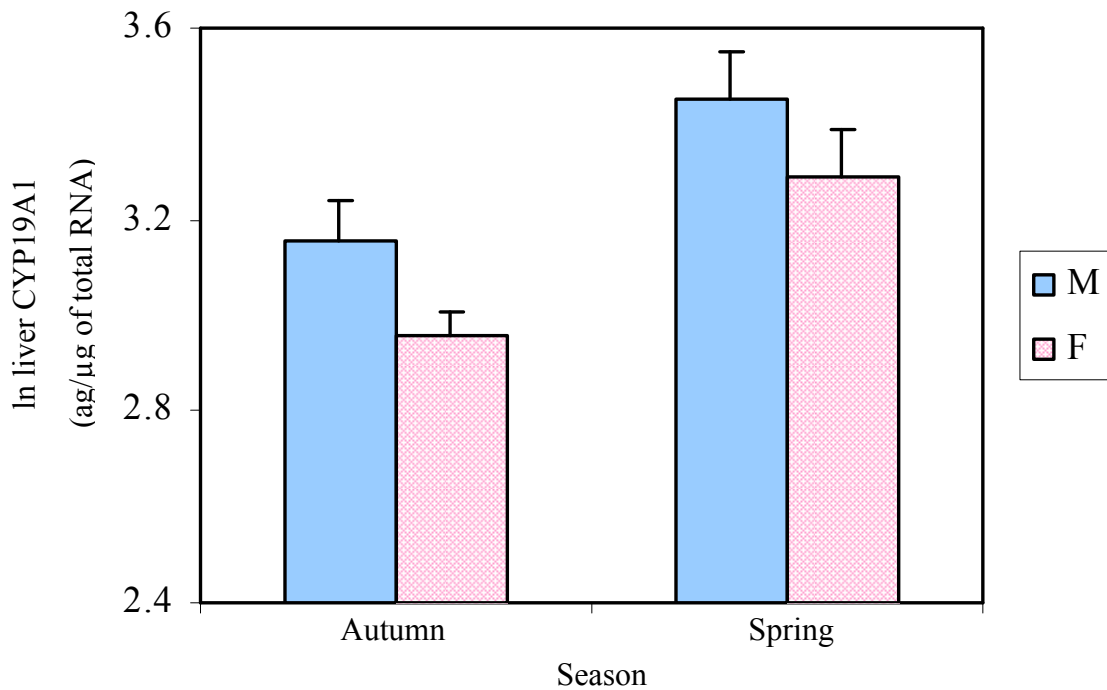
Figure 4.10 Average liver CYP19A1 mRNA levels in male (M) and female (F) yellow perch from Lake Erie at two times of the year.

Units are expressed as ln (ag of mRNA/μg of total RNA). Bars indicate standard error and sample size for each sex-specific seasonal group is n=12. The GLM indicates a significant effect of sex ($F_{1,44}$=5.077; p=0.029), with males being higher than females, season ($F_{1,44}$=15.046; p<0.001), with spring being higher than autumn, but no effect of sex*season interaction ($F_{1,44}$=0.061; p=0.806).
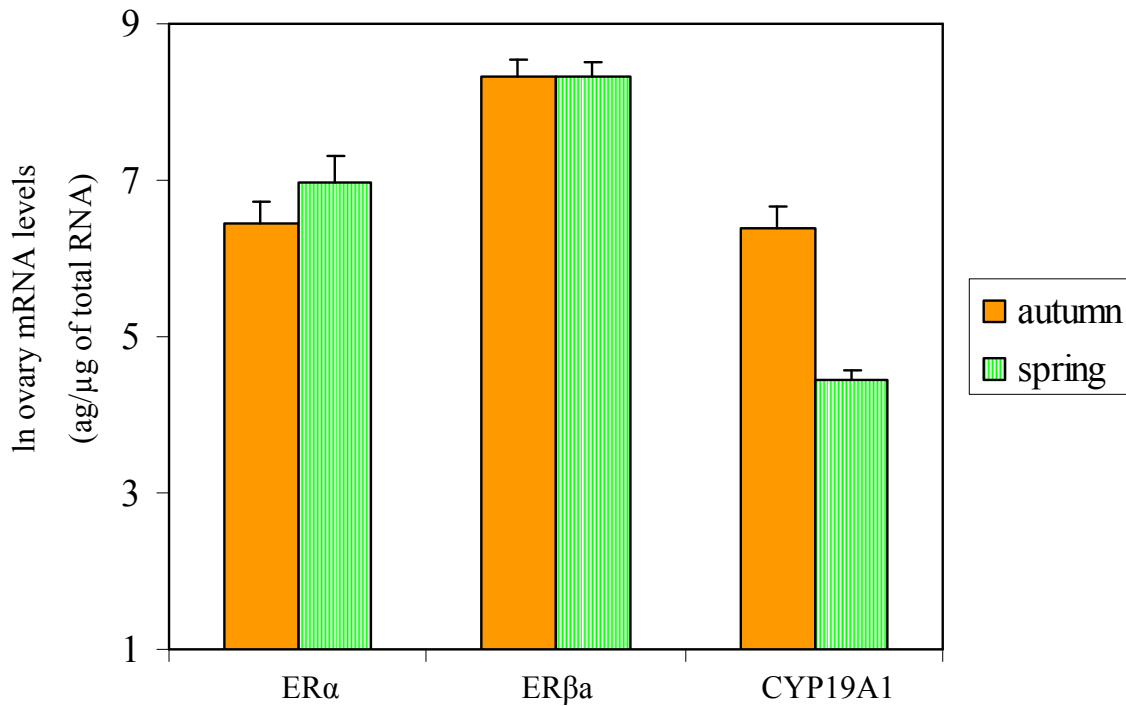
Figure 4.11 Average ovary mRNA levels of ERα, ERβa and CYP19A1 in female yellow perch from Lake Erie at two times of the year.

Units are expressed as ln (ag of mRNA/μg of total RNA). Bars indicate standard error and sample size for each gene-specific seasonal group is n=12. The t-tests indicate no effect of season on ERα (t=-1.168; p=0.255) or ERβa (t=-0.045; p=0.965) but a significant effect of season on CYP19A1 (t=6.790; p<0.001).

Chapter 5: Developmental mRNA levels of key endocrine genes in male and female juvenile yellow perch

5.1 Introduction

Sexual size dimorphism (SSD) is a widespread phenomenon found in all vertebrate classes with mammals and birds generally having larger males, however there are both male and female biased examples of SSD in fish [159, 168, 169, 340, 341], amphibians [342, 343], reptiles [344-347], birds [348-350] and mammals [351-355]. The yellow perch is one of a group of important fishes which exhibits a SSD in which females grow faster than males [7]. Other fishes in this group include sea bass [166, 167], halibut [168], eel [169], plaice, walleye, and tench [170, 171]. The female biased SSD in yellow perch was demonstrated in laboratory [8, 9] and wild populations [10-12] many years ago, but it was not until the mid 1980s [7, 13, 14] that studies identified 17β-estradiol (E_2) as a growth stimulator in yellow perch SSD. In a series of experiments using E_2, 17α-methyltestosterone (MT), triiodothyronine (T_3) and zeranol, with different size classes of juvenile perch, Malison *et al.* [13] found that only the low dosages of E_2 (2 and 20 μg/g diet) significantly stimulated growth. Further, higher levels of E_2 (50 μg/g diet) depressed growth and the growth promoting effects of E_2 were only noticeable in fish of 80-110 mm total length (TL) or greater [13]. In a separate study, Malison *et al.* [14] found that sexual differentiation occurs around 16 mm TL in yellow perch and E_2 treatment of male perch initially 90-110 mm TL did not result in sex reversal. These findings together indicate that the influence E_2 has on the growth of male yellow perch 80+ mm TL is not simply the result of a feminizing effect, however the possibility that feminizing male perch may increase their growth rate cannot be excluded. This critical size range of 80-110 mm TL is also the same size at which females normally begin to outgrow males [8] and female-biased SSD begins to be manifested. This critical period is also the specific minimum body size for the onset of vitellogenesis and spermatogenesis in females and males [14], respectively, pointing towards an upregulation of E_2 receptors (ERs) on target tissues (ovary, liver or pituitary) and a coinciding increase in tissue expression of growth factors. Malison *et al.* [7] reported that in addition to a growth response, E_2 treatment stimulated feed

consumption, and in a different study [15], growth rate and growth efficiency of female yellow perch exceeded those of males two-fold in animals fed without restriction. These observations suggest that the growth-promoting effects of estrogen may work in part through appetite centers of the central nervous system and could involve pituitary hormones. They also point out a clear linkage of growth and reproductive development in this species.

Yellow perch have recreational, economic and ecological importance, particularly in the Great Lakes region [1], deriving from their function as a sport fish [356], an aquaculture species [1, 357] and a commercial fishery [4]. All of these factors contribute to creating an interest in better understanding yellow perch SSD. Endocrine genes associated with growth and reproduction in fish include the pituitary hormones (growth hormone (GH), somatolactin (SL) and prolactin (PRL)), the growth hormone intermediate insulin-like growth factor I (IGF-I), the estrogen receptors (ERs) and aromatase. In vertebrates, the pituitary gland is the "master gland" and as such at least partially regulates aspects of growth and reproduction. Pituitary GH together with IGF-I and IGF-II are considered to be THE key players in the growth process [16]. Administering bovine GH to striped bass hybrids resulted in an increase in specific growth rate and food conversion efficiency [303]. Many studies have shown a causative effect of GH on the production and release of hepatic IGF-I [129, 304, 305] and it is believed that much of the effect GH has on growth is mediated through liver IGF-I [123, 125]. There is increasing evidence that somatolactin (SL), another pituitary hormone found only in fish, is involved in metabolism, sexual maturation and reproductive cycle regulation [29-31]. The first somatolactin receptor (SLR) was very recently characterized [36] and the highest SLR levels in masu salmon (*Oncorhynchus masou*) were found in liver, fat and gonad. Although the precise function of SL still remains unclear, studies have shown a seasonal, possibly growth related, rhythm in gilthead sea bream (*Sparus aurata*) [32] and rainbow trout (*Oncorhynchus mykiss*) [33]. There is also evidence that SL is involved in sexual maturation and gonadal development. Rand-Weaver [31] found high plasma SL levels during the period of gonadal growth and maturation in coho salmon (*O. kisutch*) and these levels were correlated with estradiol levels in females and 11-ketotestosterone levels in males.

Parhar and Iwata [28] found that gonadotropin releasing hormone (GnRH) neurons project to somatolactin cells in the steelhead trout (*O. mykiss*) providing a direct link between steroids and SL regulation. Peyon *et al.* [110] found that SL cells were sensitive to leptin and neuropeptide Y only in prepubertal and pubertal stages of European sea bass (*Dicentrarchus labrax*), indicating a potential role of SL in the nutritional control of the onset of puberty. And the study by Taniyama *et al.* [45] suggests that expression of the SL gene is elevated with sexual maturation in chum salmon (*Oncorhynchu keta*). All of these studies indicate a steroidal link with SL expression and function in teleosts involving gender and sexual maturation.

Prolactin, another pituitary hormone, has long been suspected of involvement in fish reproduction because of its well known role in mammalian reproduction [38]. In juveniles, Cavaco *et al.* [88] found significantly increased levels of PRL expression in response to E_2 which is contrasted by a lack of response in PRL expression to E_2 in immature rainbow trout (*Oncorhynchus mykiss*) [91]. Furthermore, gonadal transcripts of PRL receptor (PRLR) were significantly increased by E_2 in adult gonads but reduced 24-fold in juvenile gonads [88]. Onuma *et al.* [92] measured PRL mRNA expression in response to E_2 in masu salmon at four time points representing different maturational and reproductive stages. While they did not find differences in PRL mRNA expression between the different stages, there were significant differences in the responsiveness of E_2 between the sexes and between stages. Female salmon showed no response to E_2 until reaching sexual maturity when E_2 significantly increased PRL mRNA expression, while males showed no response to E_2 in PRL mRNA expression at any maturational stage. GH, SL and PRL are associated with parr-smolt transformation (smoltification) of Atlantic salmon (*Salmo salar*) [43] and chum salmon (*Oncorhynchus keta*) [44, 45] further linking them to sexual maturation and reproduction. These pituitary hormones all show sexually-dimorphic secretory patterns during development [46-48] and studies point to the importance of steroids (estrogen and testosterone) in the regulation of pituitary hormone release and gene expression in mature animals [49-52].

Reproduction and sexual development, or maturation, are significantly influenced, if not controlled, by sex steroids, but these steroidal effects are ultimately modulated by the distribution and expression of the steroid receptors. ERβ mRNA and

protein levels in rat brain showed not only a difference in age, with post-natal levels being higher than adult levels, but also showed a difference in sex, with females having higher levels than males [358]. Estrogen receptors are distributed in many tissues in teleosts (gonad, liver, brain and pituitaries) [53, 54] and are intricately involved in sexual determination and development [55]. Mosconi *et al.* [56] found that the liver of seabream was resistant, with regard to vitellogenin (vtg) production, to either GH or E_2 during the postspawning period. They also report that ER levels were higher in sea bream liver at the prespawning stage compared with those at the spawning or postspawning stages, which points toward an upregulation of ER as an important component of estrogen-mediated hepatic responses in this teleost. Estrogen levels, however, are controlled primarily by P450 aromatase, the terminal enzyme in the conversion of testosterone to estrogen, and blocking this enzyme, via an aromatase inhibitor (i.e. fadrozole), causes sex reversal in several species of teleosts [57-59]. Aromatase mRNA is expressed in many tissues associated with reproduction (gonad, brain, and pituitary) [60] and growth (liver, brain, and pituitary) [61], and varies with sexual maturation [62], age and season [63]. All these findings indicate the presence of an intricate, sex-specific, endocrine regulatory relationship between the pituitary, liver and gonad in teleosts, but it remains unclear how estrogen stimulated SSD in yellow perch is achieved.

In an effort to investigate estrogen stimulated SSD in yellow perch, male and female fish were collected every six or twelve weeks during the critical first year of development. The cDNAs for yellow perch GH, PRL, SL, IGF-Ib, ERα, ERβa and CYP19A1 (aromatase) are published (Chapter 2 and Chapter 3), and in this study real-time quantitative PCR assays (qPCR) were used to measure sex-specific tissue expression levels for these genes. Developmental expression levels of these key endocrine genes in both male and female yellow perch may provide important information and a better understanding of their sex-specific regulation in regard to developmental status and their role in estrogen stimulated SSD.

5.2 Methods

Samples

Yellow perch, hatched on April 18th, 2003 at The Ohio State University Aquaculture Extension Office in Piketon, OH were sampled at several time points, measured as days post-hatch (dph), from July 2003 to June 2004. Perch fry were hatched from egg scanes in tanks and released into aquaculture ponds for a period of 6 weeks. After six weeks, ponds were seined and juvenile perch were kept in flow through tanks (~1 L/min, ≤1 fish/gallon) with an ambient light cycle and constant aeration. During the first month in tanks, perch were kept inside at 20-22 °C and fed a trout starter diet (45% protein, 11% fat). Temperature was then decreased to ~12 °C and perch were fed a maintenance diet of 2% body weight per day of Aquamax Grower 400 (PMI Nutrition International, Inc., Brentwood, MO). At 218 days post-hatching (dph) (18 November) yellow perch juveniles were transferred to an outside tank at ambient water temperature to "cold shock" them or induce sexual maturation.

Up to 30 fish were sampled at 102 (29 July), 152 (16 September), 195 (28 October), 282 (23 January), 379 (29 April) and 421 (10 June) dph. When sampled, yellow perch were given a lethal dose of MS222, weighed, measured and sexed and pituitary, liver and ovary tissues were collected. Tissues were immediately frozen, transported to the University of Kentucky and stored at -80 °C until analysis. For each sampling time point, 6 male and 6 female sets of samples were randomly chosen for further analysis. The fish sampled at 102 dph were visually unidentifiable for gender, so 12 sets of samples were randomly chosen. Total RNA was extracted with the GenElute™ Mammalian Total RNA Kit (Sigma, St. Louis, MO). RNA samples were treated with amplification grade DNase I (Sigma, St. Louis, MO) and quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Up to 750 ng of RNA was reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA) and quantified.

Real-time qPCR

Sequences for yellow perch GH (Accession #AY007303), PRL (Accession #AY332491), SL (Accession #AY332490), IGF-Ib (Accession #AY332492), ERα (Accession #DQ984124), ERβa (Accession #DQ984125) and CYP19A1 (Accession

#DQ984126) either presently are or will be available by Oct 2007 from the GenBank/EMBL/DDBJ nucleotide sequence database. Primers (Table 5.1) were designed for real-time quantitative PCR (qPCR) using Beacon Designer v. 3.0 (PREMIER Biosoft International, Palo Alto, CA). Designed primers were tested with a 25 µl total volume PCR mixture using a MasterTaq Kit (Eppendorf Scientific Inc., Westbury, NY) and 1 µl of cDNA template. GH, PRL and SL used pituitary tissue as a template, IGF-Ib used liver tissue as a template and ERα, ERβa, and CYP19A1 used gravid ovary tissue as a template. Real-time qPCR consisted of 3:00 min at 94 °C follow by 45 cycles of 45 s at 94 °C and 45 s at 60 °C. PCR products were electrophoresed in 1% low melt agarose/2% nuseive gels with a 100 bp DNA ladder (Takara Shuzo Co., Otsu, Japan) and visualized by ethidium bromide staining for size verification. PCR products were then purified using Amicon Centrifugal Ultrafiltration Devices (Millipore, Billerica, MA) and quantified. Purified PCR products were ligated into a pCR®4-TOPO® vector and transformed into TOP10 chemically competent cells using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA). The plasmid DNA was then extracted from the bacterial cells using the GenElute™ Plasmid Miniprep Kit (Sigma, Sigma, St. Louis, MO). Plasmid samples were quantified and up to 600 ng of plasmid DNA was put into a sequencing PCR using BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing PCR consisted of 35 cycles of 30 s at 96 °C, 15 s at 50 °C and 4:00 min at 60 °C. After the PCR, each sample received 27 µl ddH$_2$O, 60 µl 100% EtOH and 3 µl 3M NaOAc. This mixture was transferred to a 1.5 mL microcentrifuge tube, vortexed and left overnight to precipitate. The next day, samples were spun at maximum speed at 4 °C for ≥ 30 min, decanted and washed with 250 µl of 70% EtOH, spun again, decanted and allowed to air dry. Samples were kept at -20 °C until they were transported to the University of Kentucky Advanced Genetic Technologies Center (http://www.uky.edu/Centers/AGTC/) for sequencing. Sequencing products were compared to known template sequences using Vector NTI Suite v. 7.0 (Informax, Inc., Frederick, MD) and GeneDoc (http://www.psc.edu/biomed/genedoc/) [172] to verify primer specificity.

Purified PCR products were then serially diluted to generate 6 standards ranging from 10 pg/µl to 100 ag/µl. Real-time qPCR reactions (25 µl) were prepared in 0.2 ml

thin-wall 96 well plates (BioRad, Hercules, CA) each containing the following components: 12.5 µl iQ™ SYBR® Green Supermix (BioRad, Hercules, CA), 0.75 µl (15 ng) each forward and reverse primers, 1 µl template and 10 µl ddH$_2$0. After pipetting, the plates were sealed with Bio-Rad iCycler iQ Optical Tape and spun at 2200 rcf for 1 min. Amplification and detection of samples were performed with the BioRad iCycler Thermal Cycler and Optical Module (BioRad, Hercules, CA). Each 96 well plate had duplicate wells of standards (6), no template (1), RNA template (1) and samples (40). Duplicate sample Ct values with a coefficient of variation >2% were rerun. When necessary, dilutions of template cDNAs were performed to ensure samples fell within the standard curve. All plates had standard curves of R>0.98 and PCR efficiencies between 85-110% with the exception of IGF-Ib which had PCR efficiencies up to 115%. Real-time qPCR results (ag/µl) were standardized to total cDNA (µg) in the template and log transformed before statistical analyses.

Statistics

A general linear model (GLM) was used to examine each variable (wt, length and GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa and liver CYP19A1 mRNA levels) for dph (using only 152, 195, 282, 379 and 421 dph), sex and dph*sex interaction effects. For each GLM procedure the dph*sex interaction effect was sliced by dph to identify significant differences between males and females. For variables in which only female data exists (ovary ERα, ovary ERβa and ovary CYP19A1 mRNA levels) a GLM was used to examine for dph (using only 152, 195, 282, 379 and 421 dph) effects only. Measurements of mRNA levels were examined for statistical outliers within each variable by performing a box plot with all data and identifying points beyond 3 times the interquartile (IQ) range. Only four data points (one 282 dph F GH, one 195 dph F PRL, one 195 dph F SL and one 282 dph ovary ERα mRNA level) were identified as potential statistical outliers using this method and these data points were then removed from the data set and all relevant analyses were rerun. While the removal of the outliers changed the level of significance in all cases, it did not change the presence of significance in any instance and it was decided that the analyses including all the data points would be presented. SYSTAT Grad Pack v. 10.0 was used for all analyses and differences between groups and relationships between parameters were considered significant at $p \leq 0.05$.

5.3 Results

Average weights, after pooling sexes, increased from 2.83 g at 102 dph to 22.83 g at 421 dph. Weight showed a significant effect of dph, but not sex or dph*sex interaction and there were no significant differences between average male and female weight at any dph (Figure 5.1). Average male weight increased dramatically until 195 dph, which is the sampling period just prior to the fish being moved outside. Average male weight then remained constant from 195 to 282 dph and decreased slightly at 379 dph before finally reaching a high at 421 dph. Testes began to show milt production at 195 dph and maintained production through 379 dph until becoming clear and devoid of milt at 421 dph (S Lynn, pers. obs.). Average female weight increased consistently over the sampling period but did not show linear growth. Average length showed a significant effect of dph, but not sex or dph*sex interaction and there were no significant differences between average male and female weight at any dph (Figure 5.2). Average length was also highly correlated with average weight for each sex and followed the same patterns.

GH mRNA levels showed a significant effect of dph, but not sex or dph*sex interaction and there were no significant differences between average male and female weight at any dph (Figure 5.3). Both sexes showed significant increases in GH mRNA levels until 195 dph when male GH mRNA levels seemed to plateau and female levels dropped slightly at 282 dph. These pattern changes in GH mRNA levels correspond with the transfer to outside tanks on 218 dph. After 282 dph, GH mRNA levels increased consistently and reached a high on 421 dph. PRL mRNA levels also showed a significant effect of dph, but not sex or dph*sex interaction and there were no significant differences between average male and female weight at any dph (Figure 5.4). There was a similar leveling off of PRL mRNA levels at 195 dph, however after 195 dph average PRL mRNA levels did not noticeably increase for females until 421 dph. Average male PRL mRNA levels actually dropped slightly at 379 dph, as compared to earlier sampling dates, before recovering to a comparable level with female PRL mRNA levels at 421 dph. SL mRNA levels showed a significant effect of dph, but not sex or dph*sex interaction and there were no significant differences between average male and female weight at any dph (Figure 5.5). Average male SL mRNA levels increased until 195 dph and then dropped

for the next two sampling periods before rebounding at 421 dph. Average female SL mRNA levels showed a constant pattern of increase, with a lower slope of increase after 195 dph.

IGF-Ib mRNA levels showed a significant effect of dph, but not sex or dph*sex interaction and there were no significant differences between average male and female weight at any dph (Figure 5.6). There was little difference between average male and female IGF mRNA levels during the sampling period, with the exception of a slightly higher average female mRNA level at 282 dph and slightly higher average male mRNA level at 379 and 421 dph. Liver ERα mRNA levels showed patterns very similar to IGF-Ib mRNA levels (Figure 5.7) with a significant effect of dph, but not sex or dph*sex interaction and there were no significant differences between average male and female weight at any dph. Average female mRNA levels surpassed average male mRNA levels at 282 dph, reaching their peak and then declining for the next two sampling periods. Average male mRNA levels reached a peak at 379 dph and were slightly higher than average female mRNA levels at both 379 and 421 dph. Liver ERβa mRNA levels showed a significant effect of dph, sex and dph*sex interaction and there were significant differences between average male and female weight at both 379 and 421 dph (Figure 5.8). Average male liver ERβa mRNA levels were significantly higher than average female mRNA levels at both 379 and 421 dph, with both sexes peaking at 379 dph. Liver CYP19A1 mRNA levels showed no significant effect of dph, sex or dph*sex interaction and there were no significant differences between average male and female weight at any dph (Figure 5.9). Average liver CYP19A1 mRNA levels increased from 102 to 152 dph and then fluctuated only slightly over the remainder of the sampling period with the highest levels in males recorded at 379 dph and the highest female levels recorded at 421 dph. Neither ovary ERα nor ovary ERβa mRNA levels showed a significant effect of dph while ovary CYP19A1 did show a significant effect of dph (Figure 5.10).

5.4 Discussion

The present study demonstrates for the first time that there are sex specific juvenile gene expression patterns in yellow perch which could ultimately be tied to their SSD. Also, there was a clear increase in mRNA levels of many of the genes analyzed

throughout the first year of development along with the increase in body weight. Many of the genes analyzed perceivably changed expression patterns after the fish were transferred to outside tanks on 218 dph. While the transfer to ambient winter conditions may play a role in the expression patterns measured in this study, it also should more closely represent the natural development period of wild yellow perch.

Average body weights began around ~2.8 gr at 102 dph and increased to ~22.8 gr at 421 dph, while average body weights for adult perch collected from Lake Erie ranged between ~134.4 to ~175.1 depending on season and sex (Chapter 4). Male yellow perch showed a very different, albeit not significant, pattern of developmental weight gain from female yellow perch (Figure 5.1) with a distinct drop in weight at 379 dph. Males began to show milt in the testis at 195 dph while females never showed full maturation of oocytes. Purchase *et al.* [308] lists the age at maturation of yellow perch in almost 72 Ontario lakes as ~1.8 years for males, which is noticeably lower than females (~2.9 years). While the yellow perch in this study were only within the first year of development (≤1 year), several yellow perch aquaculture studies [13, 14, 307, 357] have shown that sexual maturation can be sped up dramatically under optimal growth conditions that don't occur in the field. Perhaps a better indicator for sexual maturation is length [13] and Purchase *et al.* [308] gives an average length at sexual maturation from the 72 Ontario lakes of 81.5 mm for males and 141 mm for females. In this study, males reached an average length of 81.5 mm between 102 and 152 dph, just prior to the milt production noticed at 195 dph, while the maximum average female length of 132 mm at 421 dph falls short of the 141 mm cited above.

GH mRNA levels increased significantly over the approximately 300 day sampling period (Figure 5.3), but juvenile GH mRNA levels were lower than adult female yellow perch levels (Chapter 4) until 421 dph. However as early as 195 dph, juvenile GH mRNA levels were comparable to adult GH mRNA levels (Chapter 4), indicating that GH expression reaches adult levels as early as 200 dph. Several studies have examined the early larval expression of GH in fish [19, 359-361], but none have examined this through the first year of development or on a sex-specific basis. Li *et al.* [359] examined the developmental GH mRNA levels in orange-spotted grouper but only from 1 to 14 dph. Deane *et al.* [19] performed similar measurements in the silver sea

bream, but their sampling only went from 1 to 46 dph. The GH mRNA levels of milkfish were determined by semiquantitative RT-PCR for 1 to 10 dph by de Jesus *et al.* [360] and lastly, Funkenstein and Cohen [361] studied GH mRNA levels in gilthead sea bream from 1 to 16 dph. All of these studies found increasing levels of GH mRNA through the developmental sampling periods. In a study on masu salmon, Onuma *et al.* [92] found that there were significant changes in GH, PRL and SL mRNA levels at different stages of gonadal maturation. Also, the responses to E_2 in females were dependent on reproductive stage, with significant increases in GH, PRL and SL mRNA levels only in the prespawning stage and no effects before gonadal maturation or at the maturing stage.

PRL mRNA levels showed significant increases over the sampling period (Figure 5.4), but by 152 dph levels were comparable to adult yellow perch PRL mRNA levels (Chapter 4). GH and PRL have been shown to increase in response to confinement [306] which could be a confounding factor within this study, as the fish spent unequal amounts of time in the sampling buckets. A recent minireview on the developmental ontogeny of prolactin and its receptor in fish provided new data on gilthead seabream PRL mRNA levels from 1 to 89 dph and there was little change in expression during this sampling period [362]. Zhang *et al.* [72] found PRL mRNA levels in orange-spotted grouper embryos increased dramatically after 9 h post-fertilization and remained high until the end of the sampling period at 45 dph. Santos *et al.* [363] also measured PRL mRNA levels in gilthead sea bream from 1 to 46 dph. Interestingly, PRL mRNA levels at 1, 2, 3, and 4 dph were not significantly different from levels at 8, 10, 15, 24 or 46 dph even though pituitary lactotroph formation doesn't occur until approximately 4 dph [364]. Yang *et al.* [194] found similar results in rainbow trout with PRL and SL expression occurring before embryonic day (ED) 14 when ontogenesis of the pituitary gland takes place. Interestingly though, while PRL expression started before ED 14 in rainbow trout, an analysis of the posterior body of ED 47 embryos only indicated the presence of SL mRNAs, implying that the pre-pituitary prolactin expression is generated in an anterior tissue. Lastly, Cavaco *et al.* [88] not only found distinct differences between gilthead seabream PRL mRNA levels in juveniles and adults (adults > juveniles), but also found a differential response of PRL mRNA levels to E_2. Juvenile gilthead seabream dosed with E_2 had significantly higher pituitary PRL mRNA levels and two year old adult seabream

dosed with the same E_2 level had significantly lower pituitary PRL mRNA levels than their respective controls [88]. A similar effect was seen by Onuma *et al.* [92] in masu salmon where E_2 increased the amounts of PRL and SL mRNAs in pre-spawning stage fish and halved PRL mRNA levels in spawning stage fish. Unfortunately neither study provides the age of the juvenile fish used and no studies were found that examined PRL expression in juveniles past 85 dph.

SL mRNA levels showed significant increases over the sampling period (Figure 5.5), with 102 dph levels being significantly lower than all other sampling time points. Juvenile yellow perch SL mRNA levels were higher than adult levels after 152 dph (Chapter 4) and the dynamic changes in juvenile SL mRNA levels closely mirror the changes in weight. In a study on masu salmon, Bhandari *et al.* [49] found that regardless of sex and GnRHa-implantation, the SL mRNA levels increased during sexual maturation and tended to decrease toward spawning period. Peyon *et al.* [110] found that SL cells were sensitive to leptin and neuropeptide Y only in prepubertal and pubertal stages of European sea bass (*Dicentrarchus labrax*), indicating a potential role of SL in the nutritional control of the onset of puberty. Onuma *et al.* [44] found that in prespawning chum salmon (*Oncorhynchu keta*) SL elevates with final maturation regardless of osmotic environment and the study by Taniyama *et al.* [45] suggests that expression of the SL gene is elevated with sexual maturation in chum salmon. The exact role of SL in fish remains to be elucidated but there is a strong possibility that SL has several functions including playing a role in juvenile growth and sexual maturation.

IGF-Ib mRNA levels showed significant increases over the sampling period (Figure 5.6), with 102 dph levels being lower than all other sampling time points. Juvenile yellow perch IGF-Ib mRNA levels were higher than adult levels after 195 dph (Chapter 4) but in contrast, Shamblott and Chen [196] found that adult rainbow trout had significantly higher liver IGF mRNA levels than juveniles. Deane *et al.* [19] measured IGF-I mRNA levels in larval silver seabream and found a dramatic increase at 35 dph, but they only sampled until 46 dph. Ayson *et al.* [20] determined IGF-I mRNA levels during early larval development in a rabbitfish and found no significant changes in the first 5 days after hatching. And lastly, Duguay *et al.* [365] found little change in IGF-I mRNA levels in gilthead seabream from 1 to 16 dph. It seems likely that IGF-Ib in

yellow perch is involved in developmental growth and perhaps even sexual maturation as IGF-I has been identified in a number of fish to play a role in developmental growth, oocyte maturation and steroidogensis [143, 145, 150, 152].

Liver ERα mRNA levels showed significant changes over the sampling period with female levels peaking at 282 dph and male levels peaking at 379 dph (Figure 5.7). Male juvenile liver ERα mRNA levels exceeded adult male liver ERα mRNA levels after 195 dph and remained higher through the sampling period. Female juvenile liver ERα mRNA levels exceeded adult female liver ERα mRNA levels in the spring after 195 dph and remained higher through the sampling period. However, female juvenile liver ERα mRNA levels were always lower than the average adult female liver ERα mRNA level in autumn. There were, however, no significant differences between sexes in liver ERα mRNA levels during the developmental period sampled. The high levels of liver ERα mRNA after 195 dph indicate a shift in the estrogen responsiveness of liver during the process of sexual development in yellow perch.

Liver ERβa mRNA levels showed significant increases over the sampling period and liver ERβa mRNA was the only variable measured to show significant differences between males and females during juvenile development (Figure 5.8). At both 379 (n=12; $F_{1,10}$=7.86; p=0.007) and 421 (n=12; $F_{1,10}$=26.60; p<0.001) dph, average juvenile male liver ERβa mRNA levels were significantly higher than female levels. At 282 dph liver ERβa mRNA levels for both male and female juvenile yellow perch showed a considerable increase from 195 dph levels, but while female levels plateaued and ultimately decreased at 421 dph, male levels went significantly higher. Juvenile male liver ERβa mRNA levels at 379 and 421 dph were not only significantly higher than corresponding female levels, but they were higher than any adult yellow perch level, including the highest levels found in adult males in autumn (Chapter 4). A fairly recent study by Filby and Tyler [222] examined mRNA levels of all three ERs in liver and gonad of male and female fathead minnow up to 120 days post fertilization (dpf). Both male and female fathead minnow showed a significant increase in liver esr1 (ERα) mRNA levels from 40 to 120 dpf but neither showed any differences in liver esr2a (ERβa) mRNA levels between the different samplings. And liver ers1 (ERα) mRNA levels were significantly higher in females than males, while there were no differences in

liver ers2a (ERβa) mRNA levels between the sexes. Although the time between fertilization and hatching in the fathead minnow is less than one week, the dpf sampling times used in Filby and Tyler [222] probably don't correspond well with the dph used in this study as time to sexual maturation in each species is quite different [308, 366]. Halm *et al.* [210] examined mRNA levels of all three ERs in European sea bass at 50 day intervals from 150 to 300 dph, but unfortunately only examined brain, pituitary and gonad but not liver expression. Surprisingly, there are only a few studies that examined the developmental ontogeny of estrogen receptors in fish liver however it seems clear from this study that maturation of the liver ERs correspond with increases in pituitary and liver growth factors. And the study of adult yellow perch (Chapter 4) indicates that the relationship between liver ERβa and IGF-Ib mRNA levels is particularly striking.

Liver CYP19A1 mRNA levels did not significantly change over the sampling period (Figure 5.9), but levels increased by 152 dph and increased only slightly after that. Both male and female liver CYP19A1 mRNA levels oscillated over the sampling period and male mRNA levels reached the highest levels at 379 dph while female mRNA levels reached the highest levels at 421 dph. Juvenile liver CYP19A1 mRNA levels were lower than adult yellow perch mRNA levels with the exception of female levels in autumn (Chapter 4). Only the highest juvenile liver CYP19A1 mRNA levels, at 379 and 421 dph for males and females respectively, were not lower than the male mRNA levels in autumn. It is interesting to note that the average length at 152, 195 and 282 dph were 103, 120 and 125 mm, respectively, while the range of 80-110 mm is where Malison *et al.* [13] contend that estrogen sensitive sexually dimorphic growth begins. Malison *et al.* [13] further speculated that hepatic ERs were involved in growth and there was a maturation of these receptors at the critical size threshold for sexually related dimorphic growth. This data supports that theory as liver CYP19A1 expression increases significantly from 102 to 152 dph followed by a significant increase in both liver ER mRNA levels from 195 to 282 dph, implying that autocrine liver E_2 production and liver ER mRNA levels are important components in estrogen sensitive sex-specific growth in yellow perch.

Neither ovary ERα nor ERβa mRNA levels show significant changes through the sample period, but ovary CYP19A1 mRNA levels did show significant changes through

the sample period. Ovary CYP19A1 mRNA levels were somewhat elevated at 152 and 195 dph, but fell significantly by 282 dph and then increased at 375 and again at 421 dph (Figure 5.10). Also, ovarian ER mRNA levels in juveniles were not different from adult ovarian ER mRNA levels, but there were differences between juvenile and adult ovarian CYP19A1 mRNA levels (Chapter 4). All juvenile ovary CYP19A1 mRNA levels were higher than adult ovary CYP19A1 mRNA levels from spring. Initial juvenile mRNA levels are comparable to adult mRNA levels from autumn until 282 dph (January) when juvenile levels decreased. At 379 dph (April) ovary CYP19A1 mRNA levels remained low but levels rebounded at 421 dph (June) which foreshadows the significant seasonal differences of ovary CYP19A1 mRNA levels seen in adult yellow perch (Chapter 4). Guiguen *et al.* [55] measured ERα and CYP19A1 mRNA levels in developing rainbow trout gonads from 55 to 127 dpf and found no significant changes during the sampling period but did find a highly significant difference between male and female CYP19A1 levels at the very beginning of the sampling kinetic (55 dpf) before a histological difference. Other studies [367] have also shown that a sex specific difference in gonadal CYP19A1 mRNA levels is observed at a stage when no difference can be observed in the histological features between gonads of male and female populations. In this study, because the gonads of the 102 dph fish were visually indistinct from each other, 12 fish were randomly sampled and pooled without sexual identification. Reviewing the data reveals a distinct bimodal distribution in the gonad CYP19A1 mRNA levels at 102 dph with 5 individuals ("males") having low mRNA levels ranging from 4.33 to 4.46 and 7 individuals ("females") having high mRNA levels ranging from 7.09 to 9.39. There is a significant difference (p≤0.001) between these two groups, with the 'females' having higher ovarian CYP19A1 mRNA levels than the 'males'. Furthermore, there is also a significant difference in the gonadal ERα mRNA levels of these two groups (p=0.012), again with the 'females' having a higher level than the 'males'. Interestingly though, Guiguen *et al.* [55] did not find any significant differences between male and female rainbow trout gonad ERα mRNA levels during their sampling period (55 to 127 dpf). Halm *et al.* [210] found significant differences in gonad mRNA levels of all three ERs over their sampling period of 150 to 300 dph, however each data point represents 7 individuals sampled from a mixed population whose sex ratio was only determined after

the experiment, making it likely that the male/female ratio at each of the sampling points (150, 200, 250 and 300 dph) was not consistent. And Filby and Tyler [222] found significant differences in gonadal mRNA levels of all three ERs between male and female fathead minnow at 80 and 120 dpf. Several studies have measured CYP19A1 mRNA levels of fish during development, but some of these measured whole body expression levels [228, 231]. Kwon *et al.* [231] used quantitative RT-PCR to measure whole body CYP19A1 mRNA levels in Nile tilapia and found after 15 dpf that females had significantly higher levels than males. And in a more recent study, Chang *et al.* [230] also found different levels of CYP19A1 mRNA levels in Nile tilapia gonads from 15 to 35 dph. Luckenbach *et al.* [240] contend that gonadal CYP19A1 expression segregates into female and male patterns in southern flounder when fish reach approximately 65 mm TL. While none of the other parameters measured show significant differences between the 102 dph 'male' and 'female' groups it seems very likely the significant differences in gonad ERα and CYP19A1 mRNA levels at 102 dph are real differences between juvenile male and female yellow perch gonads.

All of the data presented in this study support the observations of estrogen stimulated SSD in yellow perch. Malison *et al.* [14] found that sexual differentiation of the gonads was occurring around 16 mm TL and Malison *et al.* [13] found that a SSD began between 80 – 110 mm TL. The significant differences between the 'male' and 'female' yellow perch gonadal CYP19A1 and ERα mRNA levels at ~63 mm TL in this study indicates sexual differentiation has already occurred and the higher ovarian CYP19A1 mRNA levels in juveniles than adults indicates that ovarian E_2 production is a key aspect of sexual maturation. Also, the lack of significant changes in ovarian ER mRNA levels through the first year of development and the lack of differences between juvenile and adult ovarian ER mRNA levels implies that ovarian function has already reached basal adult levels as early as 63 mm TL despite a lack of reproductive ability. The finding of significant upregulation of GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa and liver CYP19A1 mRNA levels between 102 and 421 dph indicates the role of these genes in yellow perch development. The two liver ER mRNAs, responsible for mediating the effects of E_2 on liver function, measured in this study showed significant increases at 195 dph when the fish had an average 119 mm TL, close to the size range

mentioned above for the onset of yellow perch SSD. Also, SL and IGF-Ib mRNAs had higher levels in juvenile than adult yellow perch indicating these genes have a distinct role in juvenile growth and development.

In summary, the measurement of GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa, liver CYP19A1, ovary ERα, ovary ERβa and ovary CYP19A1 mRNA levels shows interesting gene-specific and sex-specific patterns through the first of year of development in juvenile yellow perch. The results indicate that both male and female yellow perch show significant changes in mRNA levels of GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa and liver CYP19A1 through the first year of development with a significant upregulation. Also there is a distinct difference in male and female liver ERβa mRNA levels in the later sampling periods but both liver ERs showed a significant increase in mRNA levels after 195 dph or a total length of 120 mm. These results indicate new avenues for research related to sex-specific and developmental expression of these genes in fish and also provide new insights into estrogen stimulated SSD in yellow perch.

Table 5.1 Target gene Accession numbers and primer sequences for qPCR.

Target Gene	GenBank Accession #	Start[a]	Primer Sequence
GH	AY007303	F161-	CGG AGG AGC AGC GTC AAC
		R307-	CCC AGG ACT CGA CCA AAC G
PRL	AY332491	F242-	ACC AGG CTC TTC AAG TAT CAG
		R344-	GTG TTA GCA GAG GTG GAG AG
SL	AY332490	F294-	CTC CAA AGG TGA AAT CCA ACA G
		R422-	TCA GGA GCG GCA TCG TAG
IGF-Ib	AY332492	F393-	CGC AGG GCA CAA AGT GGA C
		R540-	CCC AGT GTT GCC TCG ACT TG
ERα	DQ984124	F1031-	AGG TGC TGA TGA TCG GGC TC
		R1124-	TCG CCT ACG TTC CTG TCC AG
ERβa	DQ984125	F1341-	TCT GGA CGC TGT GAC GGA C
		R1423-	GGG CGA GGC GGG TGT AC
CYP19A1	DQ984126	F210-	TCT GGG TTT GGG GCC ACT TC
		R359-	ACC GCT GAT GCT CTG CTG AG

[a]Forward (F) and reverse (R) primer start numbers are calculated beginning with the initiation methionine.

Figure 5.1 Average weights of male (M) and female (F) yellow perch during juvenile development.

All fish sampled at 102 dph (days post-hatching) (n=12) were labeled as undetermined (UN) since sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error and sample size for each sex-specific dph is n=6. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLM indicates a significant effect of dph ($F_{4,50}$=2.983; p=0.028) and no effect of sex ($F_{1,50}$=0.640; p=0.395) or dph*sex interaction ($F_{4,50}$=1.041; p=0.395).
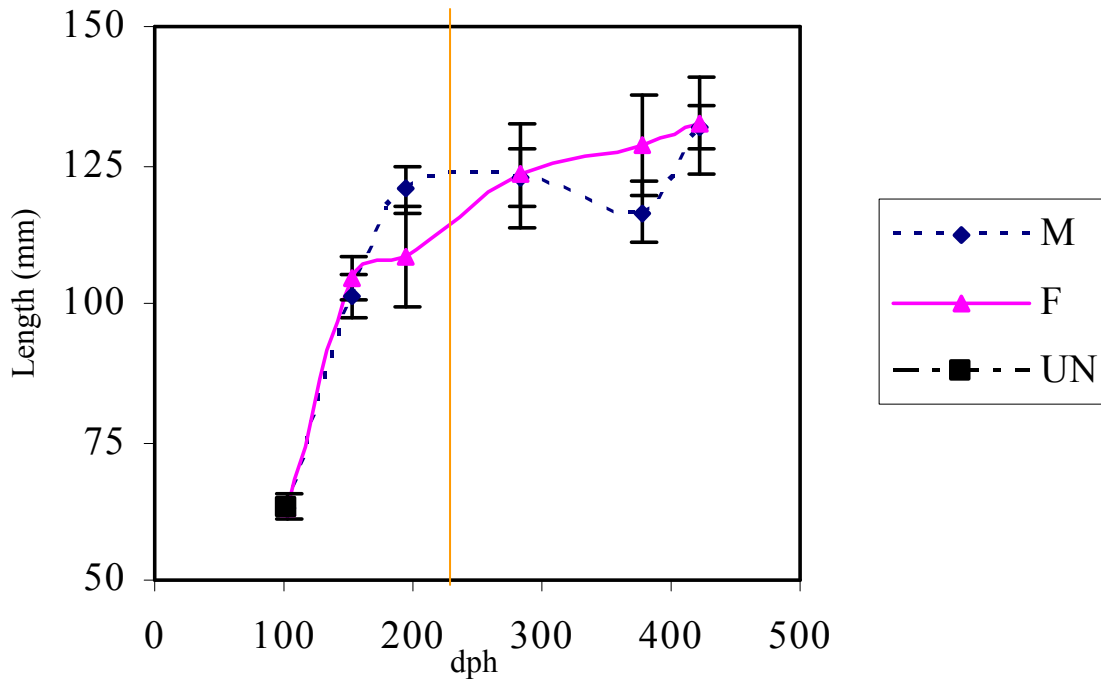
Figure 5.2 Average lengths of male (M) and female (F) yellow perch during juvenile development.

All fish sampled at 102 dph (days post-hatching) (n=12) were labeled as undetermined (UN) since sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error and sample size for each sex-specific dph is n=6. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLM indicates a significant effect of dph ($F_{4,50}$=6.510; p<0.001) and no effect of sex ($F_{1,50}$=0.034; p=0.853) or dph*sex interaction ($F_{4,50}$=1.032; p=0.400).
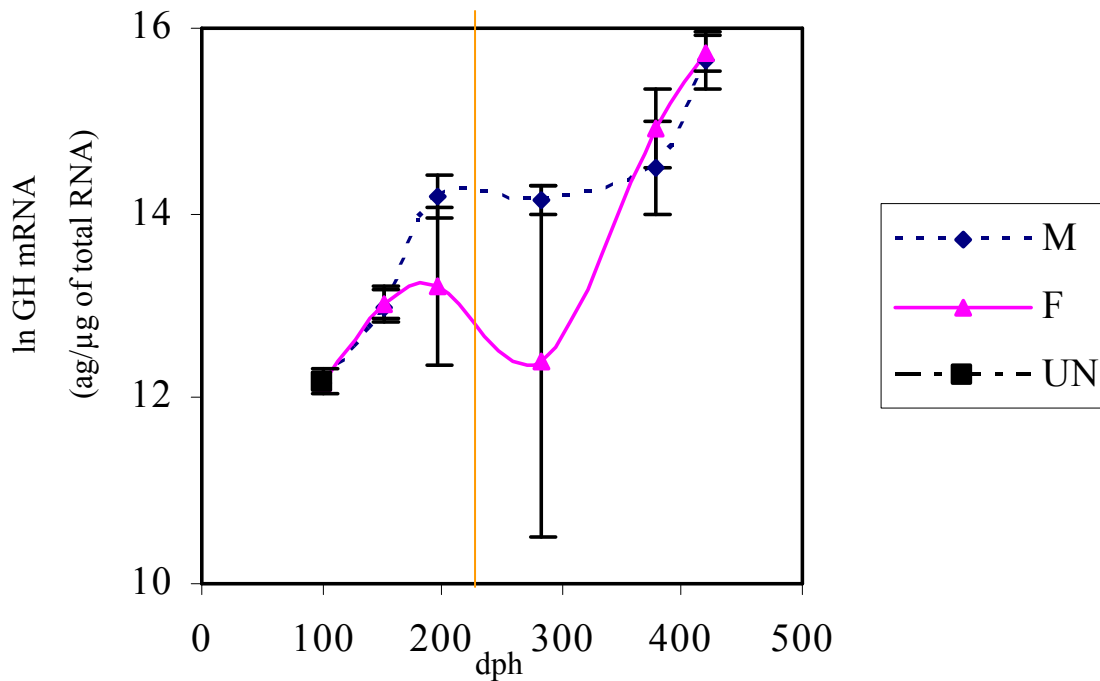
Figure 5.3 Average GH mRNA levels from male (M) and female (F) yellow perch during juvenile development.

Units are expressed as ln (ag of mRNA/μg of total RNA). All fish sampled at 102 dph (days post-hatching) (n=12) were labeled as undetermined (UN) since sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error and sample size for each sex-specific dph is n=6. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLM indicates a significant effect of dph ($F_{4,50}$=5.822; p=0.001) and no effect of sex ($F_{1,50}$=1.090; p=0.302) or dph*sex interaction ($F_{4,50}$=0.968; p=0.433).
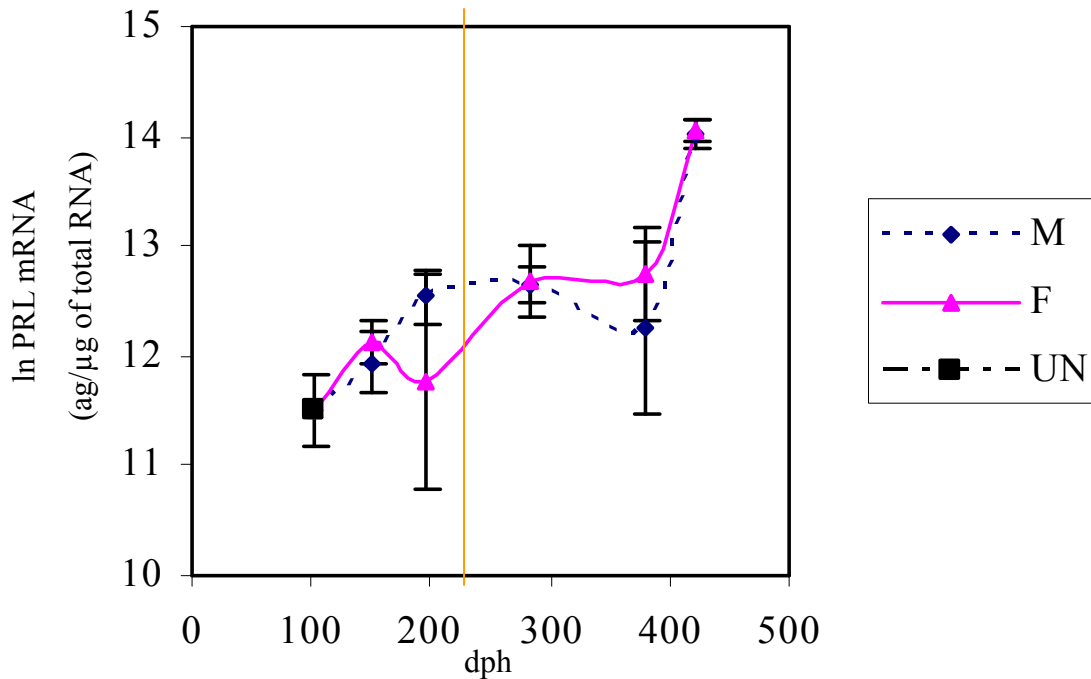
Figure 5.4 Average PRL mRNA levels from male (M) and female (F) yellow perch during juvenile development.

Units are expressed as ln (ag of mRNA/µg of total RNA). All fish sampled at 102 dph (days post-hatching) (n=12) were labeled as undetermined (UN) since sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error and sample size for each sex-specific dph is n=6. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLM indicates a significant effect of dph ($F_{4,50}$=7.442; p<0.001) and no effect of sex ($F_{1,50}$=0.001; p=0.980) or dph*sex interaction ($F_{4,50}$=0.632; p=0.642).
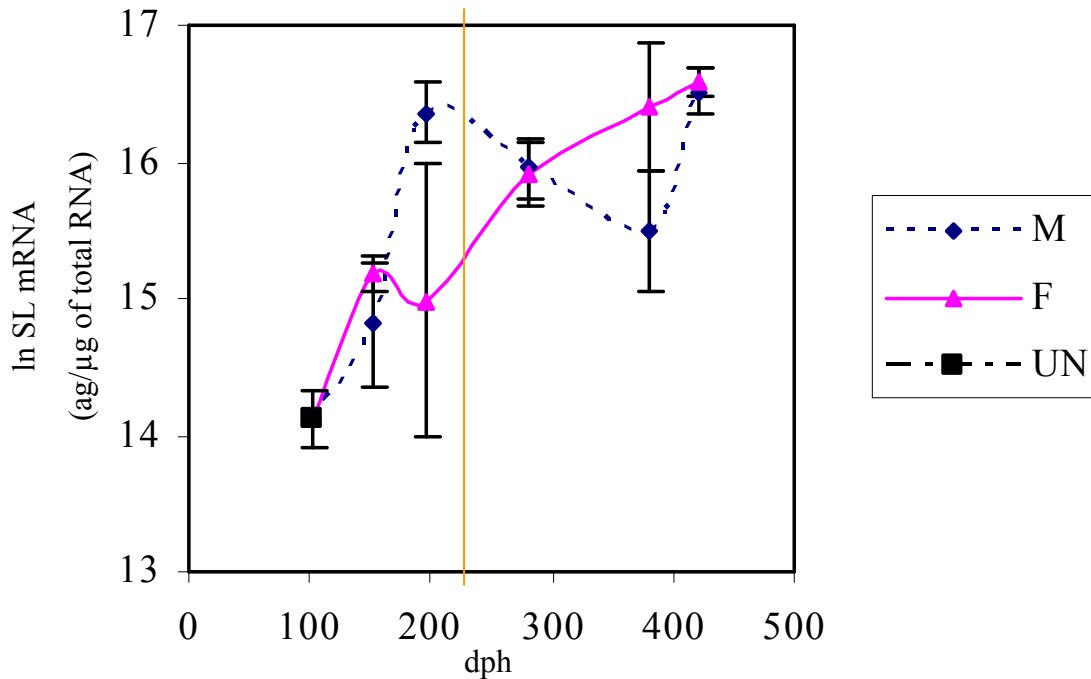
Figure 5.5 Average SL mRNA levels from male (M) and female (F) yellow perch during juvenile development.

Units are expressed as ln (ag of mRNA/μg of total RNA). All fish sampled at 102 dph (days post-hatching) (n=12) were labeled as undetermined (UN) since sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error and sample size for each sex-specific dph is n=6. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLM indicates a significant effect of dph ($F_{4,50}$=4.158; p=0.006) and no effect of sex ($F_{1,50}$=0.003; p=0.956) or dph*sex interaction ($F_{4,50}$=2.333; p=0.068).
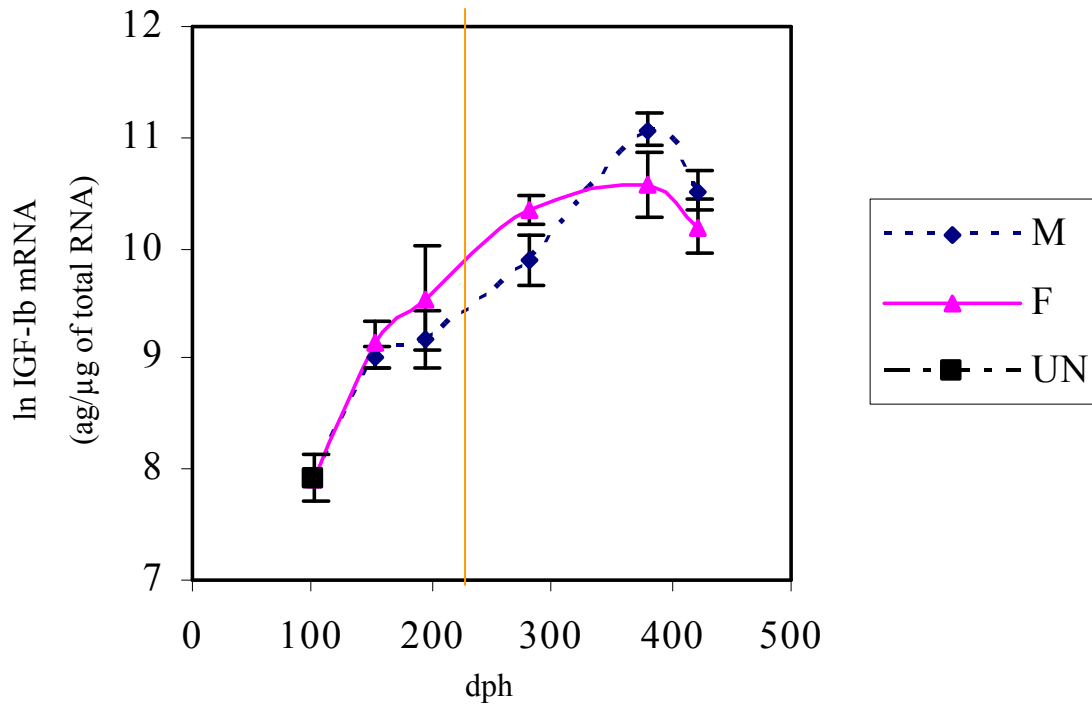
Figure 5.6 Average IGF-Ib mRNA levels from male (M) and female (F) yellow perch during juvenile development.

Units are expressed as ln (ag of mRNA/μg of total RNA). All fish sampled at 102 dph (days post-hatching) (n=12) were labeled as undetermined (UN) since sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error and sample size for each sex-specific dph is n=6. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLM indicates a significant effect of dph ($F_{4,50}$=20.607; p<0.001) and no effect of sex ($F_{1,50}$=0.026; p=0.872) or dph*sex interaction ($F_{4,50}$=1.760; p=0.152).
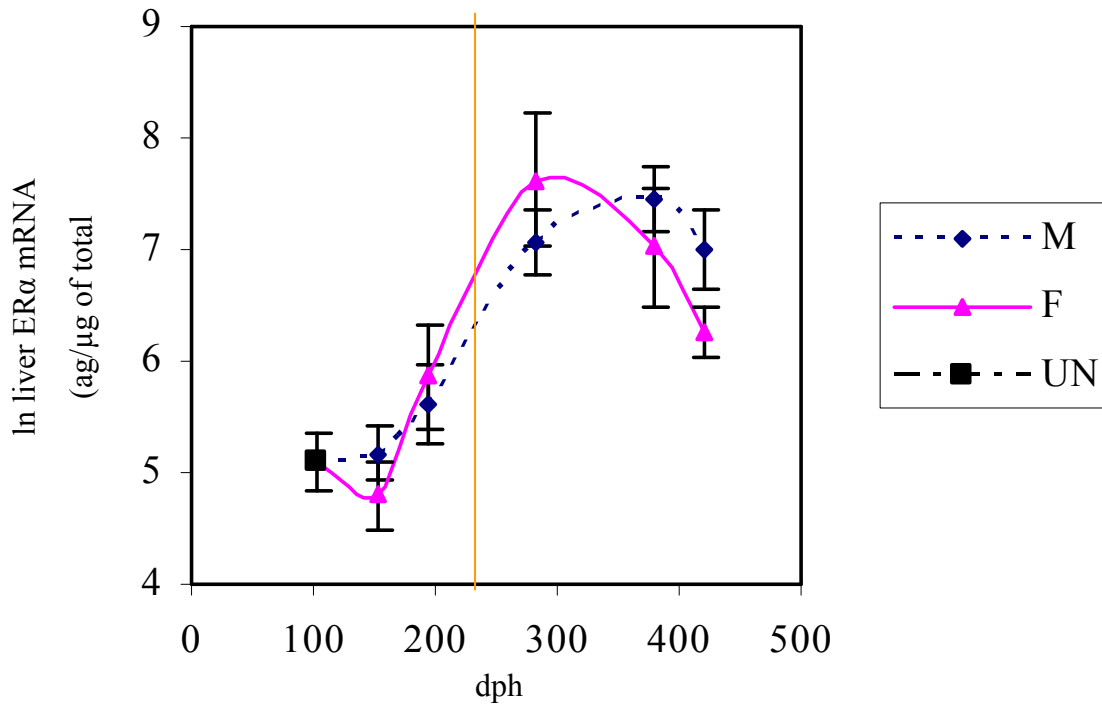
Figure 5.7 Average liver ERα mRNA levels from male (M) and female (F) yellow perch during juvenile development.

Units are expressed as ln (ag of mRNA/μg of total RNA). All fish sampled at 102 dph (days post-hatching) (n=12) were labeled as undetermined (UN) since sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error and sample size for each sex-specific dph is n=6. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLM indicates a significant effect of dph ($F_{4,50}$=16.599; p<0.001) and no effect of sex ($F_{1,50}$=0.429; p=0.515) or dph*sex interaction ($F_{4,50}$=1.162; p=0.339).
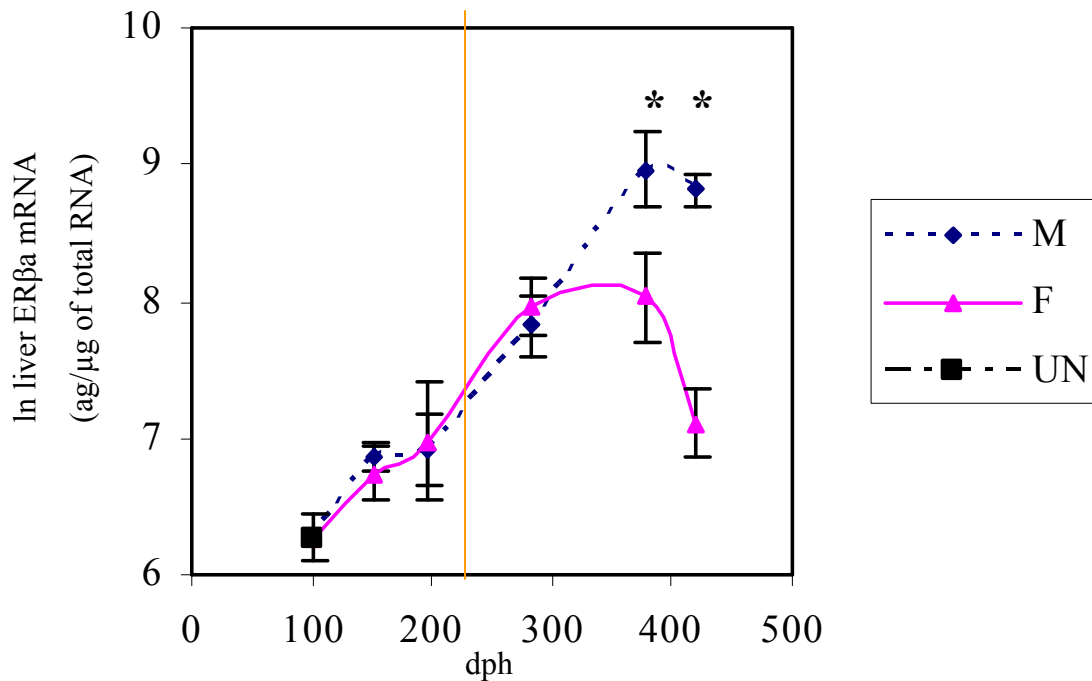
Figure 5.8 Average liver ERβa mRNA levels from male (M) and female (F) yellow perch during juvenile development.

Units are expressed as ln (ag of mRNA/µg of total RNA). All fish sampled at 102 dph (days post-hatching) (n=12) were labeled as undetermined (UN) since sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error, sample size for each sex-specific dph is n=6 and asterisks indicate sample times where male and female levels are significantly different at $p \leq 0.05$. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLM indicates a significant effect of dph ($F_{4,50}$=19.108; p<0.001), sex ($F_{1,50}$=11.776; p=0.001) and dph*sex interaction ($F_{4,50}$=5.742; p=0.001).
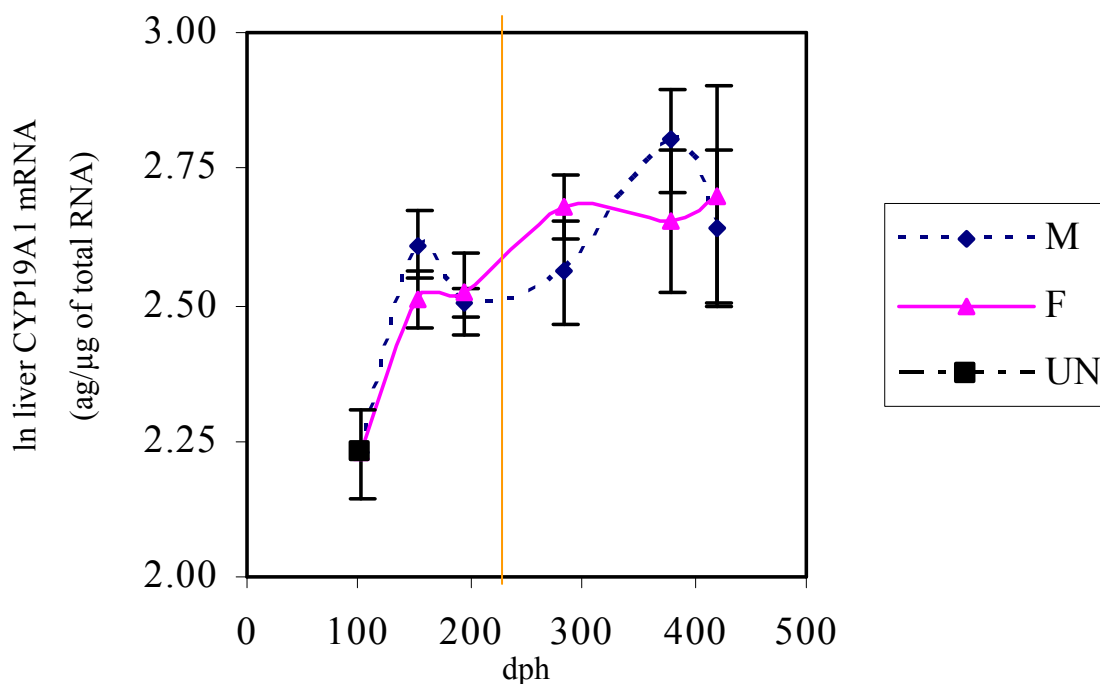
Figure 5.9 Average liver CYP19A1 mRNA levels from male (M) and female (F) yellow perch during juvenile development.

Units are expressed as ln (ag of mRNA/μg of total RNA). All fish sampled at 102 dph (days post-hatching) (n=12) were labeled as undetermined (UN) since sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error and sample size for each sex-specific dph is n=6. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLM indicates no significant effect of dph ($F_{4,50}$=1.597; p=0.190), sex ($F_{1,50}$=0.029; p=0.865) and dph*sex interaction ($F_{4,50}$=0.670; p=0.616).
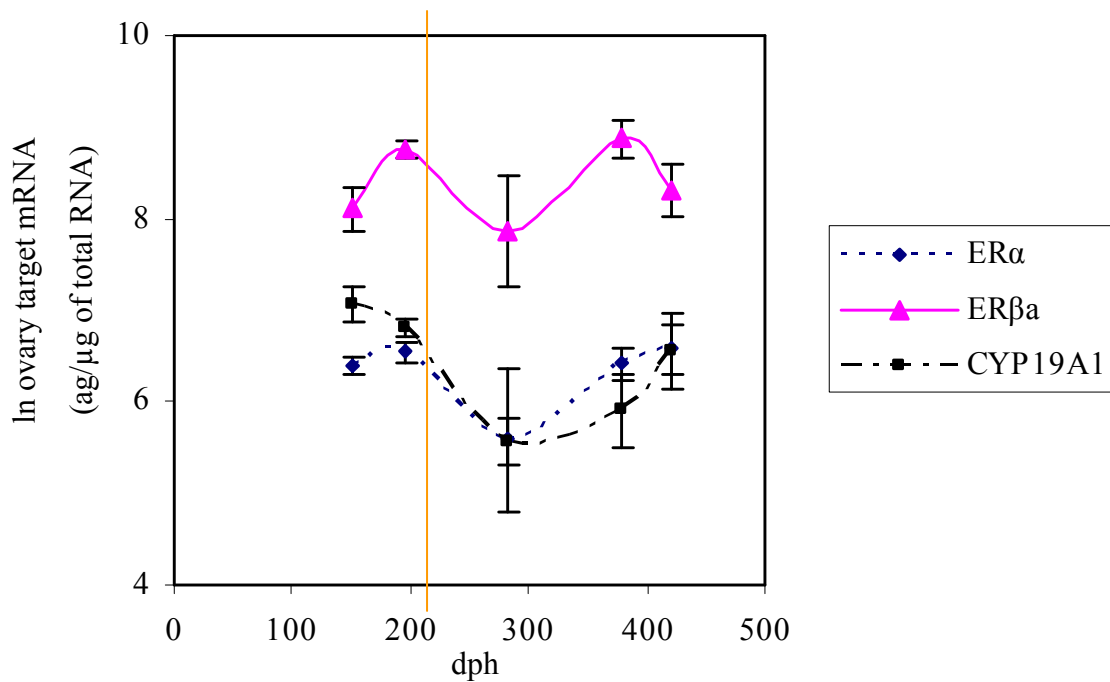
Figure 5.10 Average ovary mRNA levels of ERα, ERβa and CYP19A1 in female yellow perch during juvenile development.

Units are expressed as ln (ag of mRNA/μg of total RNA). Fish sampled at 102 dph (days post-hatching) (n=12) are not included as sex could not be identified by examination of gross anatomy. Bars indicate positive and negative standard error and sample size for each dph is n=6. The orange vertical line approximately indicates when the fish were moved to an outdoor tank at 218 dph. The GLMs indicate no effect of dph on ERα ($F_{4,25}$=1.397; p=0.264) or ERβa ($F_{4,25}$=1.949; p=0.134), but a significant effect of dph on CYP19A1 ($F_{4,25}$=5.409; p=0.003).

Chapter 6: Conclusions

6.1 Sexual Size Dimorphism (SSD) in Fish

Sexual size dimorphism (SSD) is a widespread phenomenon found in all vertebrate classes with mammals and birds generally having larger males, however there are both male and female biased examples of SSD in fish [159, 168, 169, 340, 341], amphibians [342, 343], reptiles [344-347], birds [348-350] and mammals [351-355]. There are a number of fish species that show evidence of SSD, with more examples of female biased [167-169, 308, 340, 368-381] than male biased [159, 340, 382-387] species in the literature. It can be difficult sometimes to identify SSD in species as different populations of the same species can display opposite patterns (male biased vs female biased) [340, 341]. Tilapia have long been considered to exhibit male-biased SSD [157, 159], but a study by Schreiber *et al.* [388] presents evidence to the contrary and suggests that the results of other studies are based on behavior factors as opposed to physiological ones. Adding further confusion to the issue, Melamed *et al.* [389] demonstrated that E_2 acted directly at the level of the pituitary to enhance GH release in tilapia.

There are a number of evolutionary factors that can play into SSD for a species, including natural and sexual selection, but for many species with SSD there is an inherent difference in growth rate (e.g. [368]). These instances imply an intrinsic difference between the sexes of a species, most probably in the levels of steroid hormones, the responsiveness to steroid hormones or both. A number of studies in fish have been conducted on growth responses to exogenous sex steroids, in particular for aquaculture purposes, and Donaldson *et al.* [170] presents a very comprehensive review of the earlier studies. In most of the studies cited, which are primarily with salmonids and tilapia, exposure to androgens increased anabolic growth with the notable exceptions of guppy and platyfish, which express a female-biased SSD. In one study [390], goldfish showed a significant weight gain in response to the dietary dosing of 17α-methyltestosterone (MT), a commonly used synthetic steroid, but no response to estrogen. Although, subsequent *in vivo* studies on goldfish indicate that testosterone enhances growth through its aromatization to estrogen [391] and estrogen implants increased plasma GH levels [317, 392]. In a recent study [393], MT was shown to be

aromatized to 17α-methylestradiol (ME2), an estrogen receptor (ER) agonist [394], and produced estrogenic effects in fathead minnows. This presents a problem in deciphering the results of studies which examined the effects of testosterone on fish growth, as a significant number of studies utilized MT as a representative androgen [170, 395-400].

6.2 SSD in Yellow Perch

The female biased SSD in yellow perch was demonstrated in laboratory [8, 9] and wild populations [10-12] many years ago and recent studies in laboratory [318, 357] and wild populations [308] continue to support this. It was in the mid 1980s [7, 13, 14] that studies identified 17β-estradiol (E_2) as a growth stimulator in yellow perch SSD. In a series of experiments using E_2, MT, triiodothyronine (T_3) and zeranol, with different size classes of juvenile perch, Malison *et al.* [13] found that only the low dosages of E_2 (2 and 20 μg/g diet) significantly stimulated growth. Further, higher levels of E_2 (50 μg/g diet) depressed growth and the growth promoting effects of E_2 were only noticeable in fish of 80-110 mm total length (TL) or greater [13]. In a separate study, Malison *et al.* [14] found that sexual differentiation occurs around 16 mm TL in yellow perch and E_2 treatment of male perch initially 90-110 mm TL did not result in sex reversal. These findings together indicate that the influence E_2 has on the growth of male yellow perch 80+ mm TL is not simply the result of a feminizing effect, however the possibility that feminizing male perch may increase their growth rate cannot be excluded. This critical size range of 80-110 mm TL is also the same size at which females normally begin to outgrow males [8] and female-biased SSD begins to be manifested. This critical period is also the specific minimum body size for the onset of vitellogenesis and spermatogenesis in females and males [14], respectively, pointing towards an upregulation of E_2 receptors (ERs) on target tissues (ovary, liver or pituitary) and a coinciding increase in tissue expression of growth factors. Malison *et al.* [7] reported that in addition to a growth response, E_2 treatment stimulated feed consumption, and in a different study [15] growth rate and growth efficiency of female yellow perch exceeded those of males two-fold in animals fed without restriction. Ko and Malison [401] examined the possibility of using genistein as an estrogenic growth

stimulating alternative but they observed only marginal success. Roberts *et al.* [175] showed the first mechanistic link between E_2 treatment and growth after first cloning yellow perch GH cDNA and isolating and purifying the yellow perch GH protein. They then measured pituitary GH content after oral treatment of a diet with 20 mg/kg of E_2 and observed significantly higher levels than the control treatment.

Chapter 2 of this study provided the full length nucleotide sequences for yellow perch prolactin (PRL), somatolactin (SL) and insulin-like growth factor (IGF-I) cDNAs and it also examines the sex-specific tissue expression of all those genes, plus growth hormone (GH). The expression studies in yellow perch showed the three pituitary hormones to be predominantly expressed in pituitary tissue and it also showed two IGF-I transcripts that displayed both sex and tissue-specific expression with more male tissues expressing the putative IGF-Ia transcript. Yellow perch IGF-Ib amino acid sequence showed a high homology with IGF-Is from other closely related teleost, but particularly with Eurasian perch [120] (100%) which also exhibits a female-biased SSD [395]. However a study by Mandiki *et al.* [402] found no clear evidence for E_2 action on sexual growth dimorphism in Eurasian perch, but showed that testosterone may decrease growth in males by decreasing food intake.

Chapter 3 of this study provided the full length nucleotide sequences for yellow perch ERα, ERβa and CYP19A1 cDNAs, also examining sex-specific tissue expression. All three genes showed only one transcript in all of the tissues examined while both ERs displayed the highest expression levels in female estrogen-sensitive tissues (liver and ovary) and CYP19A1 had a more global but still a sex-specific expression pattern. CYP19A1 showed a very high level of expression in unfertilized post-vitellogenic oocyte tissue and a distinct sex-specific expression difference in brain and pituitary tissues implying a gender difference in estrogen function at the neuronal or neuroendocrine level. The relatively low levels of CYP19A1 expression in brain tissue would indicate that, like most teleosts, yellow perch also has a brain-derived aromatase, or CYP19A2, that has yet to be identified.

Chapter 4 of this study measured GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa, liver CYP19A1, ovary ERα, ovary ERβa and ovary CYP19A1 average mRNA levels in autumn and spring from both male and female Lake Erie yellow perch. Adult female

yellow perch from Lake Erie had significantly higher average weights than adult male perch supporting a female-biased SSD. While sex did not have a signficant effect on GH mRNA levels there was a signficant effect of season and sex*season interaction, with spring female levels being highest and male autumn levels being lowest. Other sex-specific differences included females having higher HSI and liver ERα mRNA levels and males having higher liver IGF-Ib, liver ERβa and liver CYP19A1 mRNA levels. Ovary CYP19A1 mRNA levels (a possible indicator of plasma E_2 levels) showed a significant negative correlation with GH, IGF-Ib and liver CYP19A1 mRNA levels and a significant positive correlation with liver ERα mRNA levels and GSI in females. Overall these data do not support an endogenous estrogen-derived SSD in yellow perch raising the question of how exogenous estrogen stimulates anabolic growth.

Chapter 5 of this study measured GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa, liver CYP19A1, ovary ERα, ovary ERβa and ovary CYP19A1 average mRNA levels through the first of year of development in female and male juvenile yellow perch. A brood of fry (young of the year) were sampled at several time points, measured as days post-hatch (dph), through the first year of development. All genes analyzed showed a significant effect of dph ($p \leq 0.05$) over the sampling period (152 to 421 dph) with the exception of the ovarian ERs and liver CYP19A1 mRNA levels. Both body weight and SL showed similar non-significant differences in patterns of mRNA levels between sexes indicating the importance of SL in juvenile growth. Only liver ERβa mRNA levels showed a significant effect of sex ($p=0.001$) and dph*sex interaction ($p=0.001$). Similarly, only liver ERβa mRNA levels showed a significant difference between sexes ($p \leq 0.05$) within a specific dph, with males having higher levels than females at 379 and 421 dph. The two liver ER mRNAs, responsible for mediating the effects of E_2 on liver function, measured in this study showed increases at 195 dph when the fish had an average 119 mm TL, close to the size range mentioned above for the onset of yellow perch SSD. The results from Chapter 5 support previous findings in regards to sexual differentiation occurring before a SSD becomes apparent [14] and that there is an increase in liver ER mRNA levels [13] concurrent with the size range (80-110 mm TL) at which a female-biased SSD becomes apparent.

Lastly, an unpublished study, not included here, measured GH, PRL, SL, IGF-Ib, liver ERα, liver ERβa, liver CYP19A1, ovary ERα, ovary ERβa and ovary CYP19A1 average mRNA levels in juvenile female yellow perch fed untreated or 20 mg/kg E_2 treated food. Fish were sampled after 7 and 28 days, and only liver ERα showed a significant difference between treatments at either sampling time. The lack of a significant GH mRNA response to E_2 treatment in that study raises some interesting questions about estrogen stimulated growth in yellow perch. While the GH mRNA levels of E_2 treated yellow perch were higher than untreated fish, they were not significantly higher. The difference in the length of the unpublished study and the Roberts *et al.* [175] study (28 vs. 46 days) could contribute to the discrepancies in pituitary content of GH protein and mRNA levels, but other studies have also shown discrepancies between those two parameters. Chan *et al.* [403] found that growth hormone secretagogue stimulation of GH secretion was independent of GH gene transcription in black seabream. And Zou *et al.* [392] found that while *in vivo* E_2 dosing increased pituitary GH protein levels and plasma GH levels there were no associated changes in steady-state pituitary GH mRNA levels. In rats estrogen has been shown to decrease apoptosis [404] and increase mitotic activity [405] in the anterior pituitary, raising the possibility that this leads to a proliferation of growth hormone producing somatotrophs and ultimately more pituitary GH content. But because the measurements in this study were normalized to total RNA, that ratio would not necessarily change with cell proliferation leading to a disparity between pituitary GH content and GH mRNA levels.

In addition, Jentoft *et al.* [318] observed increased growth in response to E_2 treatment in yellow perch, but there were no significant increases in growth in response to either bovine GH or recombinant yellow perch GH. The lack of a response to growth hormone treatment in yellow perch is quite perplexing, but Eurasian perch injected with bovine GH also showed no growth enhancement [120]. While southern black bream GH stimulated growth in Eurasian perch, neither the black bream nor bovine GH treatments affected liver IGF-I mRNA levels [120], although a non-responsiveness to GH treatment by IGF-I is not unique [119, 406]. Taken together, these results suggest that treatment with exogenous E_2 stimulates pituitary GH

production and may stimulate GH secretion but its effect on overall growth may work through a mechanism outside of the traditional growth axis (GH-IGF).

6.3 Estrogen and Growth

The most parsimonious explanation for E_2 stimulated growth is through the GH-IGF axis. But exogenous E_2 administration has been shown in several species, both fish and mammal alike, to inhibit IGF-I [129, 159, 160, 407-409] and the effects on GH have been confusing [22, 23, 410]. In regards to mammals, Borski *et al.* [411] found that estrogen implants into ovariectomized rats decreased plasma IGF-I, liver IGF-I mRNA and pituitary GH content, but increased plasma GH levels. And Yan *et al.* [412] found that E_2 replacement in aromatase knockout (ArKO) mice resulted in increased GH mRNA levels. Leung *et al.* [410] provides a fairly comprehensive review of estrogen regulation of growth hormone action in mammals and they surmise that the route of administration of estrogen (oral vs non-oral) determines the effect on GH and subsequently IGF-I. This is a very interesting contention as studies in fish on estrogen exposure utilize both food treatment [175, 318] and i.p. injections or implants [314, 392] which brings to issue their comparability.

In tilapia, estrogen acted directly at the level of the pituitary to enhance GH release [389] but the mechanism for this is unknown. The GH gene, with promoter region, has been characterized in 15 teleost species [413] and in several of these an estrogen response element (ERE) has been identified [414-416]. While there are few studies in teleosts on GH promoter activity, there is evidence that estrogen binding to the ER plays a direct role in GH gene expression at the transcriptional level [417]. However the possibility remains open that ER might influence the GH gene transcription through protein-protein interaction, which has recently been shown in the prolactin gene promoter [418, 419]. In a very recent paper Wong *et al.* [420] proposed a novel intrapituitary feedback loop regulating GH release and GH gene expression involving luteinizing hormone (LH) release from neighboring gonadotrophs. Gonadal steroids are able to modulate GH secretion by affecting either the hypothalamic releasing hormones, or receptor or post-receptor mediated mechanisms at the level of the pituitary [22]. If estrogen is acting directly on the pituitary to control GH, it is most

likely binding to ERs within the pituitary. While Salbert *et al.* [421] did not find a difference in pituitary ER levels between control and E_2 treated fish, Luo *et al.* [422] found that estrogen did control pituitary ER mRNA levels dependent on season or reproductive state. However, it is possible that estrogen is not working through the ERs but possibly through another pituitary receptor. Holloway and Leatherland [314] found that E_2 implantation resulted in significantly decreased triiodothyronine (T_3) levels and DiPippo *et al.* [423] proposed the possibility that estrogens may be able to activate GH transcription through unoccupied T_3 receptors. This was observed in rats with low levels of endogenous T_3 but whether or not the E_2 induced decrease in T_3, coupled with the hyperphysiological levels of plasma E_2 following E_2 dosing, is enough to give E_2 the competitive advantage in binding with the T_3 receptors is unknown.

As a possible mechanism for E_2 effects on GH, gonadotropin-releasing hormone (GnRH) may act as an intermediate to E_2 induced GH release and transcription. Parhar and Iwata [28] found that GnRH neurons project to GH cells in steelhead trout (*Oncorhynchus mykiss*), providing direct morphological evidence of the association of GnRH and GH and, by proxy, GH and E_2. This evidence opens the possibility of E_2 affecting GH and/or growth through neuronal mechanisms associated with the brain. Indeed, many species show a sexually dimorphic expression and even regulation of estrogen receptors and aromatase in neuronal tissue [62, 358, 424]. Melo and Ramsdell [62] found that there was a sexually dimorphic difference in spatial aromatase activity in the medaka brain with males having a more rostral expression pattern than females, however after a 10 day exposure at 250 µg/L of E_2, male medaka had a more caudal (i.e. female) pattern of aromatase activity. And Crews *et al.* [424] found not only a sexual dimorphism in ER expression in whiptail lizards, but also found that there was sexual dimorphism in the response of ER expression to steroid treatments. There are, quite literally, a dozen or perhaps even more neuroendocrine factors that either inhibit or stimulate growth hormone secretion (for reviews see Wong *et al.* [420], Holloway and Leatherland [23] and Peng and Peter [425]). Several of these have been shown to be responsive to estrogen such as somatostatin [426], N-methyl-D,L-aspartate (NMA) [427], dopamine [391], and gonadotropin releasing hormone (GnRH) [428] just to name a few. So it seems necessary that any future studies examining estrogen

stimulated SSD in yellow perch should address the possibility of neuroendocrine control of the GH axis and its modulation via E_2. Cloning of these genes, along with the brain-like form of aromatase (CYP19A2) and the putative third ER (ERβb) would be a first step in understanding their role in yellow perch SSD.

Every one of those neuroendocrine factors would still produce its effect through the GH-IGF axis, however myostatin (MSTN) is a recently discovered gene that inhibits muscle growth at the level of the myoblast cell [429]. In mammals, MSTN controls myoblast cell cycle progression, and reduction in MSTN expression results in increased cell proliferation, with both hyperplastic and hypertrophic muscle growth [430] although it is only speculation that MSTN functions to negatively regulate muscle growth in fish. Kocabas *et al.* [431] measured MSTN mRNA levels in 12 different tissues from channel catfish and found that levels in the muscle were significantly elevated as compared to levels in any other tissue. While a partial cDNA sequence of yellow perch MSTN was generated by Roberts and Goetz [432] the possibility of MSTN playing a role in estrogen stimulated SSD has not been examined. While it remains to be seen if estrogen can regulate MSTN expression in yellow perch, comparison of fish MSTN sequences reveals that MSTN is extremely well conserved through evolution [433]. Only two promoter sequences for fish MSTN have been generated, both in brook trout [434], but there are several mammalian promoter sequences available [435]. While none of the promoter sequences generated to date contain an ERE, most contain an androgen response element (ARE) [434-436]. This corroborates a link between MSTN expression in muscle and the levels of circulating steroid hormones. Is it possible that chronic exposure to oral estrogen (minimum of 28 days for a growth response [7, 13, 14]) in yellow perch skews the steroidal balance to the point of changing MSTN regulation in the myoblast? Along these lines, the two brook trout promoter sequences were generated from brain/muscle and ovary and while many of the regions were conserved between the two there were some unique differences [434]. Only the ovarian MSTN promoter sequence contained a steroidogenic factor-1 (SF-1) site again tying the reproductive axis to the regulation and function of MSTN. SF-1 (also known as adrenal 4-binding protein, Ad4BP) was originally identified as a transcription factor that binds to DNA regulatory elements in

the proximal promoter regions of steroidogenic enzyme genes [437]. In fact, SF-1 is considered a master regulator of reproduction, because its targets include genes at every level of the hypothalamic-pituitary-gonadal axis, as well as most genes involved in gonadal and adrenal steroidogenesis [438]. In most cases, SF-1 functions cooperatively with other transcription factors to modulate the timing and level of gene expression such as ER [439] and SF-1 itself is regulated by the gonadotropic axis [440]. While Chapter 3 showed that there was little to no ER expression in muscle tissue, which is evidence against direct regulation of muscle MSTN by estrogen, the interaction of the gonadotropic axis and MSTN cannot be ignored in future studies.

In conclusion, this work has produced a number of cDNA sequences in yellow perch that are related to growth and sexual or reproductive function. This sequence data was then used to develop real-time quantitative PCR (qPCR) assays to measure tissue-specific mRNA levels of these genes under different life history stages and environmental circumstances. A number of interesting results have been generated relating to sex-specific expression of key endocrine genes on a seasonal and developmental basis and several points have been illuminated that deserve future study. And while the results of an estrogen dosing experiment were equivocal the molecular tools generated from this research will surely benefit future endeavors investigating estrogen sensitive SSD in yellow perch.

References

1.   Malison, J.A., A white paper on the status and needs of yellow perch aquaculture in the North Central Region. 1999, North Central Regional Aquaculture Center: Madison, Wisconsin.

2.   Troutman, M.B., The Fishes of Ohio. 1980, Columbus: Ohio State University Press and the Ohio Sea Grant Program and Center for Lake Erie Area Research.

3.   Karl, J., Yellow Perch Update. 1998, University of Wisconsin Sea Grant College Program: Madison, Wisconsin. p. 2.

4.   Coomber, S. 1997. Where have all the yellow perch gone? University of Wisconsin Sea Grant College Program.

5.   Heyer, C.J., T.J. Miller, F.P. Binkowski, E.M. Caldarone, and J.A. Rice. Maternal effects as a recruitment mechanism in Lake Michigan yellow perch (*Perca flavescens*). Can. J. Fish. Aquat. Sci. 2001. 58:1477-1487.

6.   Ciereszko, R., K. Dabrowski, A. Ciereszko, J. Ebeling, and J. Ottobre. Effects of temperature and photoperiod on reproduction of female yellow perch *Perca flavescens*: Plasma concentrations of steriod hormones, spontaneous and induced ovulation and quality of eggs. J World Aquacult Soc. 1997. 28:344-356.

7.   Malison, J., T. Kayes, B. Wentworth, and C. Amundson. Growth and feeding responses of male versus female yellow perch (*Perca flavescens*) treated with estradiol 17β. Can J Fish Aquat Sci. 1988. 45:1942-1948.

8.   Schott, E., T. Kayes, and H. Calbert. Compartive growth of male versus female yellow perch fingerlings under controlled environmental conditions. Am Fish Soc Spec Publ. 1978. 11:181-186.

9.   Schott, E. 1980. Sexually dimorphic growth in young-of-the-year yellow perch (*Perca flavescens*) under controlled environmental conditions. MS thesis. University of Wisconsin-Madison, Madison, WI. pp.

10.   Hile, R. and F. Jobes. Age and growth of the yellow perch, *Perca flavescens* (Mitchell), in Wisconsin waters of Green Bay and northern Lake Michigan. Pap Mich Acad Sci Arts Lett. 1942. 27:241-266.

11.   Hasler, A. Observations on the winter perch population of Lake Mendota. Ecology. 1945. 26:90-94.

12.   Brazo, D., P. Tack, and C. Liston. Age, growth, and fecundity of yellow perch, *Perca flavescens*, in Lake Michigan near Ludington, Michigan. Trans Am Fish Soc. 1975. 104:726-730.

13. Malison, J., C. Best, T. Kayes, and C. Amundson. Hormonal growth promotion and evidence for size-related differences in response to estradiol-17β in yellow perch (*Perca flavescens*). Can J Fish Aquat Sci. 1985. 42:1627-1633.

14. Malison, J., T. Kayes, C. Best, and C. Amundson. Sexual differentiation and use of hormones to control sex in yellow perch (*Perca flavescens*). Can J Fish Aquat Sci. 1986. 43:26-35.

15. Hayward, R.S. and N. Wang. Failure to induce over-compensation of growth in maturing yellow perch. J. Fish Biol. 2001. 59:126-140.

16. Duan, C. The insulin-like growth factor system and its biological actions in fish. Amer Zool. 1997. 37:491-503.

17. Duan, C., S.J. Duguay, and E.M. Plisetskaya. Insulin-like growth factor I (IGF-I) mRNA expression in coho salmon, *Oncorhynchus kisutch*: tissue distribution and effects of growth hormone/prolactin family proteins. Fish Physiol Biochem. 1993. 11:371-379.

18. Duan, C., N. Hanzawa, Y. Takeuchi, E. Hamada, S. Miyachi, and T. Hirano. Use of primary cultures of salmon hepatocytes for the study of hormonal regulation of insulin-like growth factor I expression *in vitro*. Zool Sci. 1993. 10:473-480.

19. Deane, E., S. Kelly, P. Collins, and N. Woo. Larval development of silver sea bream (*Sparus sarba*): Ontogeny of RNA-DNA ratio, GH, IGF-I, and Na$^+$-K$^+$-ATPase. Mar Biotechnol. 2003. 5:79-91.

20. Ayson, F., E. de Jesus, S. Moriyama, S. Hyodo, B. Funkenstein, A. Gertler, and H. Kawauchi. Differential expression of insulin-like growth factoer I and II mRNAs during embryogenesis and early larval development in rabbitfish, *Siganus guttatus*. Gen Comp Endocrinol. 2002. 126:165-174.

21. Chen, J., J. Chen, C. Chang, S. Shen, M. Chen, and J. Wu. Expression of recombinant tilapia insulin-like growth factor-I and stimulation of juvenile tilapia growth by injection of recombinant IGFs polypeptides. Aquaculture. 2000. 181:

22. Melamed, P., H. Rosenfeld, A. Elizur, and Z. Yaron. Endocrine regulation of gonadotropin and growth hormone gene transcription in fish. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 1998. 119:325-338.

23. Holloway, A.C. and J.F. Leatherland. Neuroendocrine regulation of growth hormone secretion in teleost fishes with emphasis on the involvement of gonadal sex steroids. Rev. Fish. Biol. Fisher. 1998. 8:409-429.

24. Yadetie, F. and R. Male. Effects of 4-nonylphenol on gene expression of pituitary hormones in juvenile Atlantic salmon (*Salmo salar*). Aquat. Tox. 2002. 58:113-129.

25. Holloway, A., M. Sheridan, and J. Leatherland. Estradiol inhibits plasma somatostatin 14 (SRIF-14) levels and inhibits the response of somatotrophic cells to SRIF-14 challenge *in vitro* in rainbow trout, *Oncorhychus mykiss*. Gen Comp Endocrinol. 1997. 106:407-414.

26. Farchi-Pisanty, O., J. Hackett, P.B., and B. Moav. Regulation of fish growth hormone transcription. Mol. Mar. Biol. Biotechnol. 1995. 4:215-223.

27. Xiong, F., D. Liu, Y. Le Dréan, H.P. Elsholtz, and C.L. Hew. Differential recruitment of steroid hormone response elements may dictate the expression of the pituitary gonadotropin IIbeta subunit gene during salmon maturation. Mol. Endocrinol. 1994. 8:782-793.

28. Parhar, I. and M. Iwata. Gonadotropin releasing hormone (GnRH) neurons project to growth hormone and somatolactin cells in Steelhead trout. Histochemistry. 1994. 102:195-203.

29. Mousa, M.A. and S.A. Mousa. Implication of somatolactin in the regulation of sexual maturation and spawning of Mugil cephalus. Journal of Experimental Zoology. 2000. 287:62-73.

30. Mayer, I., M. Rand-Weaver, and B. Borg. Effects of gonadectomy and steroids on plasma and pituitary levels of somatolactin in Atlantic salmon, *Salmo salar*. Gen Comp Endcorinol. 1998. 109:223-231.

31. Rand-Weaver, M., P. Swanson, H. Kawauchi, and W. Dickhoff. Somatolactin, a novel pituitary protein: purification and plasma levels during reproductive maturation of coho salmon. J Endocrinol. 1992. 133:393-403.

32. Mingarro, M., S. Vega-Rubín de Celis, A. Astola, C. Pendón, M. Valdivia, and J. Pérez-Sánchez. Endocrine mediators of growth and feeding behaviour in gilthead sea bream (*Sparus aurata*): growth hormone and somatolactin paradigm. Gen Comp Endocrinol. 2002. 128:102-111.

33. Rand-Weaver, M., T. Pottinger, and J. Sumpter. Pronounced seasonal rhythms in plasma somatolactin levels in rainbow trout. J Endocrinol. 1995. 146:113-119.

34. Vega-Rubín de Celis, S., P. Gómez, J. Calduch-Giner, F. Médale, and J. Pérez-Sánchez. Expression and characterization of European sea bass (*Dicentrarchus labrax*) somatolactin: Assessment of *in vivo* metabolic effects. Mar Biotechnol. 2003. 5:92-101.

35. Company, R., A. Astola, C. Pendón, M. Valdivia, and J. Pérez-Sánchez. Somatotropic regulation of fish growth and adiposity: growth hormone (GH) and somatolactin (SL) relationship. Comp Biochem Physiol C Toxicol Pharmacol. 2001. 130:435-445.

36. Fukada, H., Y. Ozaki, A. Pierce, S. Adachi, K. Yamauchi, A. Hara, P. Swanson, and W. Dickhoff. Identification of the salmon somatolactin receptor, a new member of the cytokine receptor family. Endocrinol. 2005. 146:2354-2361.

37. Fukamachi, S., T. Yada, and H. Mitani. Medaka receptors for somatolactin and growth hormone: Phylogenetic paradox among fish growth hormone receptors. Genetics. 2005. 171:1875-1883.

38. Freeman, M.E., B. Kanyicska, A. Lerant, and G. Nagy. Prolactin: Structure, function, and regulation of secretion. Physiol. Rev. 2000. 80:1523-1631.

39. Weber, G., J. Powell, M. Park, W. Fischer, A. Craig, J. Rivier, U. Nanakorn, I. Parhar, S. Ngamvongchon, E. Grau, and N. Sherwood. Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. J Endocrinol. 1997. 155:121-132.

40. Brinca, L., J. Fuentes, and D. Power. The regulatory action of estrogen and vasoactive intestinal peptide on prolactin secretion in sea bream (*Sparus aurata*, L.). Gen Comp Endcorinol. 2003. 131:117-125.

41. Riley, L., T. Hirano, and E. Grau. Rat ghrelin stimulates growth hormone and prolactin release in the tilapia, *Oreochromis mossambicus*. Zool Sci. 2002. 19:797-800.

42. Shepherd, B.S., T. Sakamoto, R.S. Nishioka, N.H. Richman, I. Mori, S.S. Madsen, T.T. Chen, T. Hirano, H.A. Bern, and E.G. Grau. Somatotropic actions of the homologous growth hormone and prolactins in the euryhaline teleost, the tilapia, *Oreochromis mossambicus*. Proc Natl Acad Sci USA. 1997. 94:2068-2072.

43. Ágústsson, T., K. Sundell, T. Sakamoto, M. Ando, and B.T. Björnsson. Pituitary gene expression of somatolactin, prolactin, and growth hormone during Atlantic salmon parr-smolt transformation. Aquaculture. 2003. 222:229-238.

44. Onuma, T., T. Kitahashi, S. Taniyama, D. Saito, H. Ando, and A. Urano. Changes in expression of genes encoding gonadotropin subunits and growth hormone/prolactin/somatolactin family hormones during final maturation and freshwater adaptation in prespawning chum salmon. Endocrine. 2003. 20:23-33.

45. Taniyama, S., T. Kitahashi, H. Ando, M. Ban, H. Ueda, and A. Urano. Changes in the levels of mRNAs for GH/prolalctin/somatolactin family and Pit-1/GHF-1 in the pituitaries of pre-spawning chum salmon. J Mol Endocrinol. 1999. 23:189-198.

46. Gonzalez-Parra, S., J. Argente, L.M. Garcia-Segura, and J.A. Chowen. Cellular composition of the adult rat anterior pituitary is influenced by the neonatal sex steriod environment. Neuroendocrinology. 1998. 68:152-162.

47. Harvey, S. and W.H. Daughaday, Growth hormone release: profiles, in Growth Hormone, Harvey, S., C.G. Scanes, and W.H. Daughaday, Editors. 1995, CRC Press: London. p. 193-223.

48. Takahashi, S. and S. Kawashima. Age-related changes in prolactin cell percentage and serum prolactin levels in intact and neonatally gonadectomized male and female rats. Acta Anat. 1982. 113:211-217.

49. Bhandari, R., S. Taniyama, T. Kitahashi, H. Ando, K. Yamauchi, Y. Zohar, H. Ueda, and A. Urano. Seasonal changes of responses to gonadotropin-releasing hormone analog in expression of growth hormone/prolactin/somatolactin genes in the pituitary of masu salmon. Gen Comp Endcorinol. 2003. 130:55-63.

50. Kakizawa, S., T. Kaneko, T. Ogasawara, and T. Hirano. Changes in plasma somatolactin levels during spawning migration of chum salmon (*Oncorhynchus keta*). Fish Physiol. Biochem. 1995. 14:93-101.

51. Borski, R.J., L.M.H. Helms, N.H. Richman, and E.G. Grau. Cortisol rapidly reduces prolactin release and cAMP and $^{45}CA^{2+}$ accumulation in the cichlid fish pituitary *in vitro*. Proc. Natl. Acad. Sci. USA. 1991. 88:2758-2762.

52. Sumpter, J.P., R.F. Lincoln, V.J. Bye, J.F. Carragher, and P.Y. Le Bail. Plasma growth hormone levels during sexual maturation in diploid and triploid rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 1991. 83:103-110.

53. Choi, C. and H. Habibi. Molecular cloning of estrogen receptor α and expression pattern of estrogen receptor subtypes in male and female goldfish. Mol Cell Endocrinol. 2003. 204:

54. Vetillard, A., C. Atteke, C. Saligaut, P. Jego, and T. Bailhache. Differential regulation of tyrosine hydroxylase and estradiol receptor expression in the rainbow trout brain. Mol. Cell Endocrinol. 2003. 199:37-47.

55. Guiguen, Y., J. Baroiller, M. Ricordel, K. Iseki, O. McMeel, S. Martin, and A. Fostier. Involvement of estrogens in the process of sex differentiation in two fish species: The rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). Mol Reprod Dev. 1999. 54:154-162.

56. Mosconi, G., O. Carnevali, H. Habibi, R. Sanyal, and A. Polzonetti-Magni. Hormonal mechanisms regulating hepatic vitellogenin synthesis in the gilthead sea bream, *Sparus aurata*. Am J Physiol Cell Physiol. 2002. 283:C673-8.

57. Lee, Y., W. Yueh, J. Du, L. Sun, and C. Chang. Aromatase inhibitors block natural sex change and induce male function in the protandrous black porgy, *Acanthopagrus schlegeli* Bleeker: Possible mechanism of natural sex change. Biol. Reprod. 2002. 66:1749-1754.

58. Afonso, L.O.B., G.J. Wassermann, and R.T. De Oliveira. Sex reversal in Nile tilapia (*Oreochromis niloticus*) using a nonsteroidal aromatase inhibitor. J. Exper. Zool. 2001. 290:

59. Kitano, T., K. Takamune, Y. Nagahama, and S. Abe. Aromatase inhibitor and 17 alpha-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). Mol. Reprod. Develop. 2000. 56:1-5.

60. Kumar, R., S. Ijiri, and J. Trant. Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish (*Ictalurus punctatus*) ovary throughout a reproductive cycle. Biol Reprod. 2000. 63:1676-1682.

61. Menuet, A., I. Anglade, R. Le Guevel, E. Pellegrini, F. Pakdel, and O. Kah. Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: Comparison with estrogen receptor alpha. J. Comp. Neurol. 2003. 462:180-193.

62. Melo, A. and J. Ramsdell. Sexual dimorphism of brain aromatase activity in medaka: Induction of a female phenotype by estradiol. Environ Health Perspect. 2001. 109:257-264.

63. González, A. and F. Piferrer. Aromatase activity in the European sea bass (*Dicentrarchus labrax* L.) brain. Distribution and changes in relation to age, sex, and the annual reproductive cycle. Gen. Comp. Endcorinol. 2003. 132:223-230.

64. Kamegai, J., H. Tamura, T. Shimizu, A. Ishii, H. Sugihara, and I. Wakabayashi. Estrogen receptor (ER)alpha, but not ERbeta, gene is expressed in growth hormone-releasing hormone neurons of the male rat hypothalamus. Endocrinology. 2001. 142:538-43.

65. Scanlan, N. and D.C. Skinner. Estradiol modulation of growth hormone secretion in the ewe; no growth hormone-releasing hormone neurons and few somatotropes express estradiol receptor alpha. Biol. Reprod. 2002. 66:1267-73.

66. Contreras, B. and F. Talamantes. Growth hormone (GH) and 17beta-estradiol regulation of the expression of mouse GH receptor and GH-binding protein in cultured mouse hepatocytes. Endocrinology. 1999. 140:4725-31.

67. van der Eerden, B.C.J., J. Emons, S. Ahmed, H.W. van Essen, C.W.G.M. Lowik, J.M. Wit, and M. Karperien. Evidence for genomic and nongenomic actions of estrogen in growth plate regulation in female and male rats at the onset of sexual maturation. J. Endocrinol. 2002. 175:277-288.

68. Miller, W. and N. Eberhardt. Structure and evolution of the growth hormone gene family. Endo. Rev. 1983. 4:97-130.

69.   Ben-Jonathan, N., J. Mershon, D. Allen, and R. Steinmetz. Extrapituitary prolactin: Distribution, regulation, functions and clinical aspects. Endo. Rev. 1996. 17:639-669.

70.   Santos, C., L. Brinca, P. Ingleton, and D. Power. Cloning, expression, and tissue localisation of prolactin in adult sea bream (*Sparus aurata*). Gen Comp Endocrinol. 1999. 114:57-66.

71.   Imaoka, T., M. Matsuda, and T. Mori. Extrapituitary expression of the prolactin gene in the goldfish, African clawed frog and mouse. Zool. Sci. 2000. 17:791-796.

72.   Zhang, W., J. Tian, L. Zhang, Y. Zhang, X. Li, and H. Lin. cDNA sequence and spatio-temporal expression of prolactin in the orange-spotted grouper, *Epinephelus coioides*. . Gen Comp Endocrinol. 2004. 136:134-142.

73.   Lee, K., T. Kaneko, and K. Aida. Prolactin and prolactin receptor expressions in a marine teleost, pufferfish *Takifugu rubripes*. Gen. Comp. Endcorinol. 2006. In press:

74.   San Martín, R., P. Cáceres, R. Azócar, M. Alvarez, A. Molina, M. Vera, and M. Krauskopf. Seasonal environmental changes modulate the prolactin receptor expression in an eurythermal fish. J Cell Biochem. 2004. 92:42-52.

75.   Higashimoto, Y., N. Nakao, T. Ohkubo, M. Tanaka, and K. Nakashima. Structure and tissue distribution of prolactin receptor mRNA in Japanese flounder (Paralichtys olivaceus): Conserved and preferential expression in osmoregulatory organs. Gen. Comp. Endcorinol. 2001. 123:170-179.

76.   Tse, D., B. Chow, C. Chan, L. Lee, and C. Cheng. Molecular cloning and expression studies of a prolactin receptor in goldfish (*Carassius auratus*). Life Sci. 2000. 66:593-605.

77.   Prunet, P., O. Sandra, P. Rouzic, O. Marchand, and V. Laudet. Molecular characterization of the prolactin receptor in two fish species, tilapia *Oreochromis niloticus* and rainbow trout, *Oncorhynchus mykiss*: A comparative approach. Can. J. Physiol. Pharmacol. 2000. 78:1086-1096.

78.   Sandra, O., P. Rouzic, C. Cauty, M. Edery, and P. Prunet. Expression of the prolactin receptor (tiPRL-R) gene in tilapia Oreochromis niloticus: tissue distribution and cellular localization in osmoregulatory organs. J Mol Endo. 2000. 24:215-224.

79.   Santos, C., P. Ingleton, J. Cavaco, P. Kelly, M. Edery, and D. Power. Cloning, characterization, and tissue distribution of prolactin receptor in the sea bream (*Sparus aurata*). Gen Comp Endocrinol. 2001. 121:32-47.

80.   Bole-Feysot, C., V. Goffin, M. Edery, N. Binart, and P. Kelley. Prolactin (PRL) and its receptor: Actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endo. Rev. 1998. 19:225-268.

81.   Pérez-Villamil, B., E. Bordiú, and M. Puente-Cueva. Involvement of physiological prolactin levels in growth and prolactin receptor content of prostate glands and testes in developing male rats. J. Endocrinol. 1992. 132:449-459.

82.   Crowe, P., A. Buckley, N. Zorn, and H. Rui. Prolactin activates protein kinase C and stimulates growth-related gene expression in rat liver. Mol. Cell Endocrinol. 1991. 79:29-35.

83.   Murphy, L.J., K. Tachibana, and H.G. Friesen. Stimulation of hepatic insulin-like growth factor-I gene expression by ovine prolactin: Evidence for intrinsic somatogenic activity in the rat. Endocrinology. 1988. 122:2027-2033.

84.   Machida, T., M. Taga, and H. Minaguchi. Effect of prolactin (PRL) on lipoprotein lipase (LPL) activity in the rat fetal liver. Asia Oceania J Obstet Gynaecol. 1990. 16:261-265.

85.   Garrison, M. and R. Scow. Effect of prolactin on lipoprotein lipase in crop sac and adipose tissue of pigeons. Am J Physio. 1975. 228:1542-1544.

86.   Leena, S., B. Shameena, and O. Oommen. In vivo and in vitro effects of prolactin and growth hormone on lipid metabolism in a teleost, *Anabas testudineus* (Bloch). Comp Biochem Physiol Part B. 2001. 128:761-766.

87.   Sheridan, M.A. Effects of thyroxin, cortisol, growth hormone, and prolactin on lipid metabolism of coho salmon, *Oncorhynchus kisutch*, during smoltification. Gen. Comp. Endocrinol. 1986. 64:220-238.

88.   Cavaco, J., C. Santos, P. Ingleton, A. Canario, and D. Power. Quantification of prolactin (PRL) and PRL receptor messenger RNA in gilthead seabream (*Sparus aurata*) after treatment with estradiol-17β. Biol Reprod. 2003. 68:588-594.

89.   Galas, J. and P. Epler. Does prolactin affect steroid secretion by isolated rainbow trout ovarian cells? Comp Biochem Physiol B Biochem Mol Biol. 2002. 132:287-297.

90.   Weber, G. and E. Grau. Changes in serum concentrations and pituitary content of the two prolactins and growth hormone during the reproductive cycle in female tilapia, *Oreochromis mossambicus*, compared with changes during fasting. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 1999. 124:323-335.

91.   Le Goff, P., G. Salbert, P. Prunet, C. Saligaut, B. Bjornsson, C. Haux, and Y. Valotaire. Absence of direct regulation of prolactin cells by estradiol-17β in rainbow trout (*Oncorhynchus mykiss*). Mol Cell Endocrinol. 1992. 90:133-139.

92. Onuma, T., H. Ando, N. Koide, H. Okada, and A. Urano. Effects of salmon GnRH and sex steroid hormones on expression of genes encoding growth hormone/prolactin/somatolactin family hormones and a pituitary-specific transcription factor in masu salmon pituitary cells in vitro. Gen Comp Endocrinol. 2005. 143:129-141.

93. Ono, M., Y. Takayama, M. Rand-Weaver, S. Sakata, T. Yasunaga, T. Noso, and H. Kawauchi. cDNA cloning of somatolactin, a pituitary protein related to growth hormone and prolactin. Proc Natl Acad Sci. 1990. 87:4330-4334.

94. Zhu, Y., J. Stiller, M. Shaner, A. Baldini, J.-L. Scemama, and A. Capehart. Cloning of somatolactin α and β cDNAs in zebrafish and phylogenetic analysis of two distinct somatolactin subtypes in fish. J Endocrinol. 2004. 182:509-518.

95. Yang, B.-Y. and T. Chen. Identification of a new growth hormone family protein, somatolactin-like protein, in the rainbow trout (*Oncorhyncus mykiss*) pituitary gland. Endocrinol. 2003. 144:850-857.

96. Cheng, K.-W., Y.-H. Chan, Y.-D. Chen, K.-L. Yu, and K. Chan. Sequence of a cDNA clone encoding a novel somatolactin in goldfish, *Carassius auratus*. Biochem Biophys Res Commun. 1997. 232:282-287.

97. Yang, B.-Y., M. Arab, and T. Chen. Cloning and characterization of rainbow trout (*Oncorhynchus mykiss*) somatolactin cDNA and its expression in pituitary and nonpituitary tissues. Gen Comp Endocrinol. 1997. 106:271-280.

98. Rand-Weaver, M. and P. Swanson. Plasma somatolactin levels in coho salmon (*Oncorhynchus kisutch*) during smolitification and sexual maturation. Fish Physiol Biochem. 1993. 11:175-182.

99. Rand-Weaver, M., T. Pottinger, and J. Sumpter. Plasma somatolactin concentrations in salmonid fish are elevated by stress. J Endocrinol. 1993. 138:509-515.

100. Fukamachi, S., M. Sugimoto, H. Mitani, and A. Shima. Somatolactin selectively regulates proliferation and morphogenesis of neural-crest derived pigment cells in medaka. Proc Natl Acad Sci. 2004. 101:10661-10666.

101. Zhu, T. and P. Thomas. Elevations of somatolactin in plasma and pituitaries and increased alpha-MSH cell activity in red drum exposed to black background and decreased illumination. Gen Comp Endocrinol. 1996. 101:21-31.

102. Calduch-Giner, J., C. Pendón, M. Valdivia, and J. Pérez-Sánchez. Recombinant somatolactin as a stable and bioactive protein in a cell culture bioassay: development and validation of a sensitive and reproducible radioimmunoassay. J Endocrinol. 1998. 156:441-447.

103. Kakizawa, S., A. Ishimatsu, T. Takeda, T. Kaneko, and T. Hirano. Possible involvement of somatolactin in the regulation of plasma bicarbonate for the compensation of acidosis in rainbow trout. J Exp Biol. 1997. 200:2675-2683.

104. Lu, M., P. Swanson, and J. Renfro. Effect of somatolactin and related hormones on phosphate transport by flounder renal tubule primary cultures. Am J Physiol Regul Integr Comp Physiol. 1995. 268:577-582.

105. Zhu, Y. and P. Thomas. Red drum somatolactin: Development of a homologous radioimmunoassay and plasma levels after exposure to stressors or various backgrounds. Gen Comp Endocrinol. 1995. 99:275-288.

106. Kakizawa, S., T. Kaneko, S. Hasegawa, and T. Hirano. Effects of feeding, fasting, background adaptation, acute stress and exhaustive exercise on plasma somatolactin concentrations in rainbow trout. Gen Comp Endocrinol. 1995. 98:137-146.

107. Planas, J., P. Swanson, M. Rand-Weaver, and W. Dickhoff. Somatolactin stimulates *in vitro* gonadal steroidogenesis in coho salmon, *Oncorhynchus kisutch*. Gen Comp Endocrinol. 1992. 87:1-5.

108. Yada, T., S. Moriyama, Y. Suzuki, T. Azuma, A. Takahashi, S. Hirose, and N. Naito. Relationships between obesity and metabolic hormones in the "cobalt" variant of rainbow trout. Gen Comp Endocrinol. 2002. 128:36-43.

109. Vega-Rubín de Celis, S., P. Rojas, P. Gómez-Requeni, A. Albalat, J. Gutiérrez, F. Médale, S. Kaushik, I. Navarro, and J. Pérez-Sánchez. Nutritional assessment of somatolactin function in gilthead sea bream (*Sparus aurata*): concurrent changes in somatotropic axis and pancreatic hormones. Comp Biochem Physiol Part A. 2004. 138:533-542.

110. Peyon, P., S. Vega-Rubín de Celis, P. Gómez-Requeni, S. Zanuy, J. Pérez-Sánchez, and M. Carrillo. *In vitro* effect of leptin on somatolactin release in the European sea bass (*Dicentrarchus labrax*): dependence on the reproductive status and interaction with NPY and GnRH. Gen Comp Endocrinol. 2003. 132:284-292.

111. Wallis, A. and R. Devlin. Duplicate insulin-like growth factor I genes in salmon display alternative splicing pathways. Mol Endocrinol. 1993. 7:409-422.

112. Duguay, S.J., P. Swanson, and W.W. Dickhoff. Differential expression and hormonal regulation of alternatively spliced IGF-I mRNA transcripts in salmon. J Mol Endocrinol. 1994. 12:25-37.

113. McRory, J. and N. Sherwood. Catfish express two forms of insulin-like growth factor-I (IGF-I) in the brain. J Biol Chem. 1994. 269:18588-18592.

114. Duguay, S.J., L.K. Park, M. Samadpour, and W.W. Dickhoff. Nucleotide sequence and tissue distribution of three insulin-like growth factor I prohormones in salmon. Mol Endocrinol. 1992. 6:1202-1210.

115. Schmid, A., E. Näf, W. Kloas, and M. Reinecke. Insulin-like growth factor-I and -II in the ovary of a bony fish, *Oreochromis mossambicus*, the tilapia: in situ hybridisation, immunohistochemical localisation, Northern blot and cDNA sequences. Mol Cell Endocrinol. 1999. 156:141-149.

116. Hashimoto, H., S. Mikawa, E. Takayama, Y. Yokoyama, H. Toyohara, and M. Sakaguchi. Molecular cloning and growth hormone-regulated gene expression of carp insulin-like growth factor-I. Biochem Mol Biol Int. 1997. 41:877-886.

117. Tanaka, M., T. Taniguchi, I. Yamamoto, K. Sakaguchi, H. Yoshizato, T. Ohkubo, and K. Nakashima. Gene and cDNA structures of flounder insulin-like growth factor-I (IGF-I): Multiple mRNA species encode a single short mature IGF-I. DNA Cell Biol. 1998. 17:859-868.

118. Chen, M.-C., G.-H. Lin, H.-Y. Gong, C.-F. Weng, C.-Y. Chang, and J.-L. Wu. The characterization of prepro-insulin-like growth factor-1 Ea-2 expression and insulin-like growth factor-1 genes (devoid 81 bp) in the zebrafish (*Danio rerio*). Gene. 2001. 268:67-75.

119. Ståhlbom, A., V. Sara, and P. Hoeben. Insulin-like growth factor mRNA in Barramundi (*Lates calcarifer*): Alternative splicing and nonresponsiveness to growth hormone. Biochem Genet. 1999. 37:69-93.

120. Jentoft, S., A. Aastveit, and Ø. Andersen. Molecular cloning and expression of insulin-like growth factor-I (IGF-I) in Eurasian perch (*Perca fluviatilis*): lack of responsiveness to growth hormone treatment. Fish Physiol Biochem. 2004. 30:67-76.

121. Caelers, A., G. Berishvili, M. Meli, E. Eppler, and M. Reinecke. Establishment of a real-time RT-PCR for the determination of absolute amounts of IGF-I and IGF-II gene expression in liver and extrahepatic sites of the tilapia. Gen Comp Endocrinol. 2004. 137:196-204.

122. Reinecke, M., A. Schmid, R. Ermatinger, and D. Loffing-Cueni. Insulin-like growth factor I in the teleost *Oreochromis mossambicus*, the tilapia: Gene sequence, tissue expression, and cellular localization. Endocrinology. 1997. 138:3613-3619.

123. Pierce, A., J. Dickey, D. Larsen, H. Fukada, P. Swanson, and W. Dickhoff. A quantitative real-time RT-PCR assay for salmon IGF-I mRNA, and its application in the study of GH regulation of IGF-I gene expression in primary culture of salmon hepatocytes. Gen Comp Endocrinol. 2004. 135:401-411.

124. Inoue, K., H. Iwatani, and Y. Takei. Growth hormone and insulin-like growth factor I of a Euryhaline fish *Cottus kazika*: cDNA cloning and expression after seawater acclimation. Gen Comp Endocrinol. 2003. 131:77-84.

125. Vong, Q., K. Chan, and C. Cheng. Quantification of common carp (*Cyprinus carpio*) IGF-I and IGF-II mRNA by real-time PCR: differential regulation of expression by GH. J Endocrinol. 2003. 178:513-521.

126. Shi, F., W. Li, C. Bai, and H. Lin. IGF-I of orange spotted grouper *Epinephelus coioides*: cDNA cloning, sequencing and expression in *Escherichia coli*. Fish Physiol Biochem. 2002. 27:147-156.

127. Wood, A., C. Duan, and H. Bern. Insulin-like growth factor signaling in fish. Int Rev Cytol. 2005. 243:215-285.

128. Biga, P., B. Peterson, G. Schelling, R. Hardy, K. Cain, K. Overturf, and T. Ott. Bovine growth hormone treatment increased IGF-I in circulation and induced the production of a specific immune response in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 2005. 246:437-445.

129. Carnevali, O., M. Cardinali, F. Maradonna, M. Parisi, I. Olivotto, A. Polzonetti-Magni, G. Mosconi, and B. Funkenstein. Hormonal regulation of hepatic IGF-I and IGF-II gene expression in the marine teleost *Sparus aurata*. Mol Reprod Dev. 2005. 71:12-18.

130. Kajimura, S., K. Uchida, T. Yada, L. Riley, J. Byatt, R. Collier, K. Aida, T. Hirano, and E. Grau. Stimulation of insulin-like growth factor-I production by recombinant bovine growth hormone in Mozambique tilapia, *Oreochromis mossambicus*. Fish Physiol Biochem. 2001. 25:221-230.

131. Funkenstein, B., A. Silbergeld, B. Cavari, and Z. Laron. Growth hormone increases plasma levels of insulin-like growth factor (IGF-I) in a teleost, the gilthead seabream (*Sparus aurata*). J Endocrinol. 1989. 120:R19-R21.

132. McCormick, S., K. Kelley, G. Young, R. Nishioka, and H. Bern. Stimulation of coho salmon growth by insulin-like growth factor-I. Gen Comp Endocrinol. 1992. 86:398-406.

133. Iwatani, H., K. Inoue, and Y. Takei. Expression of insulin-like growth factor I gene is involved in enhanced growth of juvenile four-spine sculpin *Cottus kazika* in seawater. Fish Sci. 2005. 71:621-626.

134. Castillo, J., M. Codina, M. Martínez, I. Navarro, and J. Gutiérrez. Metabolic and mitogenic effects of IGF-I and insulin on muscle cells of rainbow trout. Am J Physiol Regul Integr Comp Physiol. 2004. 286:R935-R941.

135. Wargelius, A., P.-G. Fjelldal, S. Benedet, T. Hansen, B. Björnsson, and U. Nordgarden. A peak in *gh-receptor* expression is associated with growth

activation in Atlantic salmon vertebrae, while upregulation of *igf-I receptor* expression is related to increased bone density. Gen Comp Endocrinol. 2005. 142:163-168.

136. Gray, E. and K. Kelley. Growth regulation in the gobiid teleost, *Gillichthys mirabilis*: roles of growth hormone, hepatic growth hormone receptors and insulin-like growth factor-I. J Endocrinol. 1991. 131:57-66.

137. Ng, K., J. Datuin, and H. Bern. Effects of estrogens *in vitro* and *in vivo* on cartilage growth in the tilapia (*Oreochromis mossambicus*). Gen Comp Endocrinol. 2001. 121:295-304.

138. Tsai, P., S. Madsen, S. McCormick, and H. Bern. Endocrine control of cartilage growth in coho salmon: GH influence *in vivo* on the response to IGF-I *in vitro*. Zool Sci. 1994. 11:299-303.

139. Marchant, T. and B. Moroz. Hormonal influences on *in vitro* [^{35}S]-sulfate uptake by gill arches from the goldfish (*Carassius auratus* L.). Fish Physiol Biochem. 1993. 11:393-399.

140. Cheng, C. and T. Chen. Synergism of growth hormone (GH) and insulin-like growth factor-I (IGF-I) in stimulation of sulphate uptake by teleostean branchial cartilage *in vitro*. J Endocrinol. 1995. 147:67-73.

141. Takagi, Y. and B. Björnsson. Regulation of cartilage glycosaminoglycan synthesis in the rainbow trout, *Oncorhynchus mykiss*, by 3,3',5-tri-iodo-L-thyronine and IGF-I. J Endocrinol. 1996. 149:357-365.

142. Negatu, Z. and A. Meier. *In vitro* incorporation of [^{14}C]glycine into muscle protein of Gulf killifish (*Fundulus grandis*) in response to insulin-like growth factor-I. Gen Comp Endocrinol. 1995. 98:193-201.

143. Berishvili, G., H. D'Cotta, J.-F. Baroiller, H. Segner, and M. Reinecke. Differential expression of IGF-I mRNA and peptide in the male and female gonad during early development of a bony fish, the tilapia *Oreochromis niloticus*. Gen Comp Endocrinol. 2006. 146:204-210.

144. Kajimura, S., N. Kawaguchi, T. Kaneko, I. Kawazoe, T. Hirano, N. Visitacion, E. Grau, and K. Aida. Identification of the growth hormone receptor in an advanced teleost, the tilapia (*Oreochromis mossambicus*) with special reference to its distinct expression pattern in the ovary. J Endocrinol. 2004. 181:65-76.

145. Kagawa, H., K. Gen, K. Okuzawa, and H. Tanaka. Effects of luteinizing hormone and follicle-stimulating hormone and insulin-like growth factor-I on aromatase activity and P450 aromatase gene expression in the ovarian follicles of red seabream, *Pagrus major*. Biol Reprod. 2003. 68:1562-1568.

146. Patiño, R. and H. Kagawa. Regulation of gap junctions and oocyte maturational competence by gonadotropin and insulin-like growth factor-I in ovarian follicles of red seabream. Gen Comp Endocrinol. 1999. 115:454-462.

147. Kagawa, H., S. Moriyama, and H. Kawauchi. Immunocytochemical localization of IGF-I in the ovary of the red seabream, *Pagrus major*. Gen Comp Endocrinol. 1995. 99:307-315.

148. Kagawa, H., M. Kobayashi, Y. Hasegawa, and K. Aida. Insulin and insulin-like growth factors I and II induce final maturation of oocytes of red seabream, *Pagrus major*, *in vitro*. Gen Comp Endocrinol. 1994. 95:293-300.

149. Maestro, M., E. Méndez, J. Planas, and J. Gutiérrez. Dynamics of insulin and insulin-like growth factor-I (IGF-I) ovarian receptors during maturation in the brown trout (*Salmo trutta*). Fish Physiol Biochem. 1999. 20:341-349.

150. Behl, R. and R. Pandey. Effect of insulin-like growth factor-1 on steroidogenesis in cultured carp ovarian follicles: Interactions with estradiol. Indian J Exp Biol. 1999. 37:138-142.

151. Maestro, M., E. Méndez, M. Párrizas, and J. Gutiérrez. Characterization of insulin and insulin-like growth factor-I ovarian receptors during the reproductive cycle of carp (*Cyprinus carpio*). Biol Reprod. 1997. 56:1126-1132.

152. Negatu, Z., S. Hsiao, and R. Wallace. Effects of insulin-like growth factor-I on final oocyte maturation and steroid production in *Fundulus heteroclitus*. Fish Physiol Biochem. 1998. 19:13-21.

153. Maestro, M., J. Planas, S. Moriyama, J. Gutiérrez, J. Planas, and P. Swanson. Ovarian receptors for insulin and insulin-like growth factor I (IGF-I) and effects of IGF-I on steroid production by isolated follicular layers of the preovulatory coho salmon ovarian follicle. Gen Comp Endocrinol. 1997. 106:189-201.

154. Perrot, V., E. Moiseeva, Y. Gozes, S. Chan, and B. Funkenstein. Insulin-like growth factor receptors and their ligands in gonads of a hermaphroditic species, the gilthead seabream (*Sparus aurata*): Expression and cellular localization. Biol Reprod. 2000. 63:229-241.

155. Weber, G. and C. Sullivan. Insulin-like growth factor-I induces oocyte maturational competence but not meiotic resumption in white bass (*Morone chrysops*) follicles *in vitro*: evidence for rapid evolution of insulin-like growth factor action. Biol Reprod. 2005. 72:1177-1186.

156. Gioacchini, G., M. Cardinali, F. Maradonna, B. Funkenstein, G. Mosconi, and O. Carnevali. Hormonal control of the IGF system in the sea bream ovary. Ann NY Acad Sci. 2005. 1040:320-322.

157.   Riley, L., T. Hirano, and E. Grau. Disparate effects of gonadal steroid hormones on plasma and liver mRNA levels of insulin-like growth factor-I and vitellogenin in the tilapia, *Oreochromis mossambicus*. Fish Physiol Biochem. 2002. 26:223-230.

158.   Larsen, D., M. Shimizu, K. Cooper, P. Swanson, and W. Dickhoff. Androgen effects on plasma GH, IGF-I, and 41-kDa IGFBP in coho salmon (*Oncorhynchus kisutch*). Gen Comp Endocrinol. 2004. 139:29-37.

159.   Riley, L., T. Hirano, and E. Grau. Estradiol-17β and dihydrotestosterone differentially regulate vitellogenin and insulin-like growth factor-I production in primary hepatocytes of the tilapia *Oreochromis mossambicus*. Comp Biochem Physiol Part C. 2004. 138:177-186.

160.   Arsenault, J., W. Fairchild, D. MacLatchy, L. Burridge, K. Haya, and S. Brown. Effects of water-borne 4-nonylphenol and 17β-estradiol exposures during parr-smolt transformation on growth and plasma IGF-I of Atlantic salmon (*Salmo salar* L.). Aquat Tox. 2004. 66:255-265.

161.   Venken, K., F. Schuit, L. Van Lommel, K. Tsukamoto, J. Kopchick, K. Coschigano, C. Ohlsson, S. Movérare, S. Boonen, R. Bouillon, and D. Vanderschueren. Growth without growth hormone receptor: Estradiol is a major growth hormone-independent regulator of hepatic IGF-I synthesis. J Bone Miner Res. 2005. 20:2138-2149.

162.   Joo, S.-S., T.-J. Won, H.-C. Kang, and D.-I. Lee. Isoflavones extracted from *Sophorae fructus* upregulate IGF-I and TGF-β and inhibit osteoclastogenesis in rat bone marrow cells. Arch Pharm Res. 2004. 27:99-105.

163.   Baker, D., B. Davies, W. Dickhoff, and P. Swanson. Insulin-like growth factor I increases follicle-stimulating hormone (FSH) content and gonadotropin-releasing hormone-stimulated FSH release from coho salmon pituitary cells *in vitro*. Biol Reprod. 2000. 63:865-871.

164.   Weil, C., F. Carré, O. Blaise, B. Breton, and P.-Y. Le Bail. Differential effect of insulin-like growth factor I on *in vitro* gonadotropin (I and II) and growth hormone secretions in rainbow trout (*Oncorhynchus mykiss*) at different stages of the reproductive cycle. Endocrinology. 1999. 140:2054-2062.

165.   Huang, Y., K. Rousseau, N. Le Belle, B. Vidal, E. Burzawa-Gerard, J. Marchelidon, and S. Dufour. Insulin-like growth factor-I stimulates gonadotrophin production from eel pituitary cells: a possible metabolic signal for induction of puberty. J Endocrinol. 1998. 159:43-52.

166.   Gorshov, S., G. Gorshova, B. Colorini, and H. Gordin. Effects of natural estradiol-17β and synthetic 17α-ethenylestradiol on direct feminization of European sea bass *Dicentrarchus labrax*. J World Aquacult Soc. 2004. 35:

167.   Papadaki, M., F. Piferrer, S. Zanuy, E. Maingot, P. Divanach, and C.C. Mylonas. Growth, sex differentiation and gonad and plasma levels of sex steroids in male- and female-dominant populations of *Dicentrarchus labrax* obtained through repeated size grading. J Fish Biol. 2005. 66:938-956.

168.   Weltzien, F.-A., Ø. Karlson, and B. Norberg. Growth patterns and plasma levels of testosterone, 11-ketotestosterone, and IGF-I in male Atlantic halibut (*Hippoglossus hippoglossus*) from juvenile stages throughtout sexual development. Fish Physiol Biochem. 2003. 28:227-228.

169.   Tzchori, I., G. Degani, R. Elisha, R. Eliyahu, A. Hurvitz, J. Vaya, and B. Moav. The influence of phytoestrogens and oestradiol-17β on growth and sex determination in the European eel (*Anguilla anguilla*). Aquacult Res. 2004. 35:1213-1219.

170.   Donaldson, E., U. Fagerlund, D. Higgs, and J. McBride, Hormonal Enhancement of Growth in Fish, in Fish Physiology: Bioenergetics and Growth, Hoar, W., D. Randall, and J. Brett, Editors. 1979, Academic Press, Inc.: New York. p. 456-597.

171.   Kocour, M., O. Linhart, D. Gela, and M. Rodina. Growth performance of all-female and mixed-sex common carp *Cyprinus carpio* L. populations in the central Europe climatic conditions. J World Aquacult Soc. 2005. 36:103-113.

172.   Nicholas, K.B., H.B. Nicholas, and D.W.I. Deerfield. GeneDoc: Analysis and visualization of genetic variation. EMBNEW.NEWS. 1997. 4:14.

173.   Rozen, S. and H.J. Skaletsky, eds. Primer3 on the WWW for general users and for biologist programmers. Bioinformatics Methods and Protocols: Methods in Molecular Biology, ed. Krawetz, S. and S. Misener. 2000, Humana Press: Totowa, NJ USA. 365-386.

174.   Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997. 25:3389-3402.

175.   Roberts, S., T. Barry, J. Malison, and F. Goetz. Production of a recombinantly derived growth hormone antibody and the characterization of growth hormone levels in yellow perch. Aquaculture. 2004. 232:591-602.

176.   Thompson, J.D., D.G. Higgins, and T.J. Gibson. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994. 22:4673-4680.

177.   Saitou, N. and M. Nei. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987. 4:406-425.

178. Company, R., J. Calduch-Giner, M. Mingarro, and J. Pérez-Sánchez. cDNA cloning and sequence of European sea bass (*Dicentrarchus labrax*) somatolactin. Comp Biochem Physiol Part B. 2000. 127:183-192.

179. Rentier-Delrue, F., D. Swennen, P. Prunet, M. Lion, and J. Martial. Tilapia prolactin: molecular cloning of two cDNAs and expression in *Escherichia coli*. DNA. 1989. 8:261-270.

180. Swennen, D., F. Rentier-Delrue, B. Auperin, P. Prunet, G. Flik, S. Wendelaar Bonga, M. Lion, and J. Martial. Production and purification of biologically active recombinant tilapia (*Oreochromis niloticus*) prolactins. J. Endocrinol. 1991. 131:219-227.

181. Specker, J., D. King, R. Nishioka, K. Shirahata, K. Yamaguchi, and H. Bern. Isolation and partial characterization of a pair of prolactins released *in vitro* by the pituitary of cichlid fish, *Oreochromis niloticus*. Proc Natl Acad Sci USA. 1985. 82:7490-7494.

182. Yamaguchi, K., J. Specker, D. King, Y. Yokoo, R. Nishioka, T. Hirano, and H. Bern. Complete amino acid sequences of a pair of fish (tilapia) prolactins, $tPRL_{177}$ and $tPRL_{188}$. J. Biol. Chem. 1988. 263:9113-9121.

183. Auperin, B., F. Rentier-Delrue, J. Martial, and P. Prunet. Evidence that two tilapia (*Oreochromis niloticus*) prolactins have different osmoregulatory functions during adaptation to a hyperosmotic environment. J. Mol. Endocrinol. 1994. 12:13-24.

184. Rand-Weaver, M., T. Noso, K. Muramoto, and H. Kawauchi. Isolation and characterization of somatolactin, a new protein related to growth hormone and prolactin from Atlantic cod (*Gadus morhua*) pituitary glands. Biochemistry. 1991. 30:1509-1515.

185. Pendon, C., A. Astola, J. Pérez-Sánchez, and M. Valdivia. Release of glycosylated and non-glycosylated forms of somatolactin by fish pituitary culture *in vitro*. Ann N Y Acad Sci. 1998. 839:478-479.

186. Duval, H., K. Rousseau, G. Eliès, P. Le Bail, S. Dufour, G. Boeuf, and D. Boujard. Cloning, characterization, and comparative activity of turbot IGF-I and IGF-II. Gen Comp Endocrinol. 2002. 126:269-278.

187. Narhi, L., Q. Hua, T. Arakawa, G. Fox, L. Tsai, R. Rosenfield, P. Holst, J. Miller, and M. Weiss. Role of native disulfide bonds in the structure and activity of insulin-like growth factor 1: genetic models of protein-folding intermediates. Biochemisty. 1993. 32:

188. Bayne, M., J. Applebaum, G. Chicchi, R. Miller, and M. Cascieri. The roles of tyosines 24, 31, and 60 in the high affinity binding of insulin-like growth factor-I

to the type 1 insulin-like growth factor receptor. J Biol Chem. 1990. 265:15648-15652.

189.   Cascieri, M., G. Chicchi, J. Applebaum, N. Hayes, B. Green, and M. Bayne. Mutants of human insulin-like growth factor I with reduced affinity for the type 1 insulin-like growth factor receptor. Biochemistry. 1988. 27:3229-3233.

190.   Clemmons, D., M. Dehoff, W. Busby, M. Bayne, and M. Cascieri. Competition for binding to insulin-like growth factor (IGF) binding protein-2, 3, 4 and 5 by the IGFs and IGF analogs. Endocrinology. 1992. 131:890-895.

191.   Magee, B., G. Shooter, J. Wallace, and G. Francis. Insulin-like growth factor I and its binding proteins: a study of the binding interface using B-domain analogues. Biochemistry. 1999. 38:15863-15870.

192.   Zhang, W., T. Gustafson, W. Rutter, and J. Johnson. Positively charged side chains in the insulin-like growth factor-1 C- and D-regions determine receptor binding specificity. J Biol Chem. 1994. 269:10609-10613.

193.   Sakamoto, T. and S. McCormick. Prolactin and growth hormone in fish osmoregulation. Gen Comp Endocrinol. 2006. 147:24-30.

194.   Yang, B.-Y., M. Greene, and T. Chen. Early embryonic expression of the growth hormone family protein genes in the developing rainbow trout, *Oncorhynchus mykiss*. Mol Reprod Dev. 1999. 53:127-134.

195.   Dabrowski, K., R. Ciereszko, A. Ciereszko, and J. Ottobre. *In vitro* production of ovarian steroids in yellow perch (*Perca flavescens*): effects of photothermal manipulation, gonadotropin and phorbol ester. Reprod Biol. 2002. 2:163-186.

196.   Shamblott, M. and T. Chen. Age-related and tissue-specific levels of five forms of insulin-like growth factor mRNA in a teleost. Mol Mar Biol Biotechnol. 1993. 2:351-361.

197.   Kavsan, V., V. Grebenjuk, A. Koval, A. Skorokhod, C. Roberts Jr., and D. LeRoith. Isolation of a second nonallalic insulin-like growth factor I gene from the salmon genome. DNA Cell Biol. 1994. 13:555-559.

198.   Chen, M., Y.-H. Kuo, X. Tian, and T. Chen. Novel biological activities of the fish pro-IGF-I E-peptides: studies on effects of fish pro-IGF-I E-peptide on morphological change, anchorage-dependent cell division, and invasiveness in tumor cells. Gen Comp Endocrinol. 2002. 126:342-351.

199.   Tian, X., M. Chen, A. Pantschenko, T. Yang, and T. Chen. Recombinant E-peptides of pro-IGF-I have mitogenic activity. Endocrinology. 1999. 140:3387-3390.

200. Kuo, Y.-H. and T. Chen. Novel activities of pro-IGF-I E peptides: regulation of morphological differentiation and anchorage-independent growth in human neuroblastoma cells. Exp Cell Res. 2002. 280:75-89.

201. Thorton, J. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proc Natl Acad Sci USA. 2001. 98:5671-5676.

202. Hewitt, S. and K. Korach. Estrogen receptors: structure, mechanisms and function. Rev Endocr Metab Disord. 2002. 3:193-200.

203. Hawkins, M. and P. Thomas. The unusual binding properties of the thrid distinct teleost estrogen receptor subtype ERβa are accompanied by highly conserved amino acid changes in the ligand binding domain. Endocrinology. 2004. 145:2968-2977.

204. Tsai, M.-J. and B. O'Malley. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem. 1994. 63:451-486.

205. Kuiper, G., E. Enmark, M. Pelto-Huikko, S. Nilsson, and J.-Å. Gustafsson. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA. 1996. 93:5925-5930.

206. Xia, Z., W. Gale, X. Chang, D. Langenau, R. Patiño, A. Maul, and L. Densmore. Phylogenetic sequence analysis, recombinant expression, and tissue distribution of a channel catfish estrogen receptor β. Gen Comp Endocrinol. 2000. 118:139-149.

207. Xia, Z., R. Patiño, W. Gale, A. Maule, and L. Densmore. Cloning, *in vitro* expression, novel phylogenetic classification of a channel catfish estrogen receptor. Gen Comp Endocrinol. 1999. 113:360-368.

208. Kuiper, G., B. Carlson, K. Grandien, E. Enmark, J. Häggblad, S. Nilsson, and J.-Å. Gustafsson. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology. 1997. 138:863-870.

209. Hawkins, M., J. Godwin, D. Crews, and P. Thomas. The distributions of the duplicate oestrogen receptors ER-βa and ER-βb in the forebrain of the Atlantic croaker (*Micropogonias undulatus*): evidence for subfunctionalization after gene duplication. Proc R Soc B. 2005. 272:633-641.

210. Halm, S., G. Martínez-Rodríguez, L. Rodríguez, F. Prat, C. Mylonas, M. Carrillo, and S. Zanuy. Cloning, chraracterisation and expression of three oestrogen receptors (ERα, ERβ1 and ERβ2) in the European sea bass, *Dicentrarchus labrax*. Mol Cell Endocrinol. 2004. 223:63-75.

211. Menuet, A., E. Pellegrini, I. Anglade, O. Blaise, V. Laudet, O. Kah, and F. Pakdel. Molecular characterization of three estrogen receptor forms in zebrafish:

binding characteristics, transactivation properties, and tissue distributions. Biol Reprod. 2002. 66:1881-1892.

212.   Hawkins, M., J. Thorton, D. Crews, J. Skipper, A. Dotte, and P. Thomas. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc Natl Acad Sci USA. 2000. 97:10751-10756.

213.   Ma, C., K. Dong, and K. Yu. cDNA cloning and expression of a novel estrogen receptor β-subtype in goldfish (*Carassius auratus*). Biochim Biophys Acta. 2000. 1490:145-152.

214.   Pinto, P., A. Passos, R. Martins, D. Power, and A. Canário. Characterization of estrogen receptor βb in sea bream (*Sparus auratus*): phylogeny, ligand-binding, and comparative analysis of expression. Gen Comp Endocrinol. 2006. 145:197-207.

215.   Wilson, V., M. Cardon, J. Thorton, J. Korte, G. Ankley, J. Welch, L. Gray Jr., and P. Hartig. Cloning and *in vitro* expression and characterization of the androgen receptor and isolation of estrogen receptor α from the fathead minnow (*Pimephales promelas*). Environ Sci Technol. 2004. 38:6314-6321.

216.   Thomas, P., G. Dressing, Y. Pang, H. Berg, C. Tubbs, A. Benninghoff, and K. Doughty. Progestin, estrogen and androgen G-protein coupled receptors in fish gonads. Steroids. 2006. 71:310-316.

217.   Thomas, P., Y. Pang, E. Filardo, and J. Dong. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology. 2005. 146:624-632.

218.   Toran-Allerand, C. Minireview: A plethora of estrogen receptors in the brain: Where will it end? Endocrinology. 2004. 145:1069-1074.

219.   Cavaco, J., C. Vilrokx, V. Trudeau, R. Schulz, and H. Goos. Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). Am J Physiol. 1998. 275:R1793-802.

220.   Teves, A., J. Granneman, W. van Dijk, and J. Bogerd. Cloning and expression of a functional estrogen receptor-α from African catfish (*Clarias gariepinus*) pituitary. J Mol Endocrinol. 2003. 30:173-185.

221.   Nagler, J., M. Krisfalusi, and D. Cyr. Quantification of rainbow trout (Oncorhynchus mykiss) estrogen receptor-α messenger RNA and its expression in the ovary during the reproductive cycle. J Mol Endocrinol. 2000. 25:243-251.

222.   Filby, A. and C. Tyler. Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). Biol Reprod. 2005. 73:648-662.

223. Sabo-Attwood, T., K. Kroll, and N. Denslow. Differential expression of largemouth bass (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. Mol Cell Endocrinol. 2004. 218:107-118.

224. Simpson, E., M. Mahendroo, G. Means, M. Kilgore, M. Hinshelwood, S. Graham-Lorence, B. Amarneh, Y. Ito, C. Fisher, M. Michael, C. Mendelson, and S. Bulun. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endo. Rev. 1994. 15:342-355.

225. Corbin, C., L. Graham, M. McPhaul, J. Mason, C. Mendelson, and E. Simpson. Isolation of a full-length cDNA insert encoding human aromatase system cytochrome P-450 and its expression in nonsteroidogenic cells. Proc Natl Acad Sci USA. 1988. 85:8948-8952.

226. Choi, J., J. Park, H. Jeong, Y. Lee, A. Takemura, and S. Kim. Molecular cloning of cytochrome P450 aromatases in the protogynous wrasse, *Halichoeres tenuispins*. Comp Biochem Physiol B Biochem Mol Biol. 2005. 141:49-59.

227. Kazeto, Y., S. Ijiri, A. Place, Y. Zohar, and J. Trant. The 5'-flanking regions of CYP19A1 and CYP19A2 in zebrafish. Biochem Biophys Res Comm. 2001. 288:503-508.

228. van Nes, S., M. Moe, and Ø. Andersen. Molecular characterization and expression of two *cyp*19 (P450 aromatase) genes in embryos, larvae, and adults of Atlantic halibut (*Hippoglossus hippoglossus*). Mol Reprod Dev. 2005. 72:437-449.

229. Strobl-Mazzulla, P., N. Moncaut, G. López, L. Miranda, A. Canario, and G. Somoza. Brain aromatase from pejerrey fish (*Odontesthes bonariensis*): cDNA cloning, tissue expression, and immunohistochemical localization. Gen Comp Endocrinol. 2005. 143:21-32.

230. Chang, X., T. Kobayashi, B. Senthilkumaran, H. Kobayashi-Kajura, C. Sudhakumari, and Y. Nagahama. Two types of aromatase with different encoding genes, tissue distribution and development expression in Nile tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol. 2005. 141:101-115.

231. Kwon, J., B. McAndrew, and D. Penman. Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia *Oreochromis niloticus*. Mol Reprod Dev. 2001. 59:359-370.

232. Kazeto, Y. and J. Trant. Molecular biology of channel catfish brain cytochrome P450 aromatase (CYP19A2): cloning, preovulatory induction of gene expression, hormonal gene regulation and analysis of promoter region. J Mol Endocrinol. 2005. 35:571-583.

233. Blázquez, M. and F. Piferrer. Cloning, sequence analysis, tissue distribution, and sex-specific expression of the neural form of P450 aromatase in juvenile sea bass (*Dicentrarchus labrax*). Mol Cell Endocrinol. 2004. 219:83-94.

234. Zhang, Y., W. Zhang, L. Zhang, T. Zhu, J. Tian, X. Li, and H. Lin. Two distinct cytochrome P450 aromatases in the orange-spotted grouper (*Epinephelus coioides*): cDNA cloning and differential mRNA expression. J Steroid Biochem Mol Biol. 2004. 92:39-50.

235. Tchoudakova, A. and G. Callard. Identification of multiple CYP19 genes encoding different cytochrome P450 aromatase isozymes in brain and ovary. Endocrinology. 1998. 139:2179-2189.

236. Fukada, S., M. Tanaka, M. Matsuyama, D. Kobayashi, and Y. Nagahama. Isolation, characterization, and expression of cDNAs encoding the medaka (*Oryzias latipes*) ovarian follicle cytochrome P-450 aromatase. Mol Reprod Dev. 1996. 45:285-290.

237. Chiang, E., Y. Yan, Y. Guiguen, J. Postlethwait, and B. Chung. Two cyp19 (P450 aromatase) gene on duplicated zebrafish chromosomes are expressed in ovary or brain. Mol Biol Evol. 2001. 18:542-550.

238. Dalla Valle, L., A. Ramina, S. Vianello, P. Belvedere, and L. Colombo. Cloning of two mRNA variants of brain aromatase cytochrome P450 in rainbow trout (*Oncorhynchus mykiss* Walbaum). J Steroid Biochem Mol Biol. 2002. 82:19-32.

239. Kobayashi, Y., T. Kobayashi, M. Nakamura, T. Sunobe, C. Morrey, N. Suzuki, and Y. Nagahama. Characterization of two types of cytochrome P450 aromatase in the serial-sex changing gobiid fish, *Trimma okinawae.* Zool Sci. 2004. 21:417-425.

240. Luckenbach, J., L. Early, A. Rowe, R. Borski, H. Daniels, and J. Godwin. Aromatase cytochrome P450: cloning, intron variation, and ontogeny of gene expression in southern flounder (*Paralichthys lethostigma*). J Exp Zool. 2005. 303A:643-656.

241. Liu, X., B. Liang, and S. Zhang. Sequence and expression of cytochrome P450 aromatase and FTZ-F1 genes in the protandrous black porgy (*Acanthopagrus schlegeli*). Gen Comp Endocrinol. 2004. 138:247-254.

242. Matsuoka, M., S. van Nes, Ø. Andersen, T. Benfey, and M. Reith. Real-time PCR analysis of ovary- and brain-type aromatase gene expression during Atlantic halibut (*Hippoglossus hippoglossus*) development. Comp Biochem Physiol B Biochem Mol Biol. 2006. 144:128-135.

243. Sawyer, S., K. Gerstner, and G. Callard. Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: Gene specific tissue distribution, sex

differences, developmental programming, and estrogen regulation. Gen Comp Endocrinol. 2006. 147:108-117.

244. Luckenbach, J., J. Godwin, H. Daniels, and R. Borski. Gonadal differentiation and effects of temperature on sex determination in southern flounder (*Paralichthys lethostigma*). . Aquaculture. 2003. 216:315-327.

245. Härd, T. and J.-Å. Gustafsson. Structure and function of the DNA-binding domain of the glucocorticoid receptor and other members of the nuclear receptor supergene family. Acc Chem Res. 1993. 26:644-650.

246. Schwabe, J., D. Neuhaus, and D. Rhodes. Solution structure of the DNA-binding domain of the oestrogen receptor. Nature. 1990. 348:458-461.

247. Koike, S., M. Sakai, and M. Muramatsu. Molecular cloning and characterization of rat estrogen receptor cDNA. Nucleic Acids Res. 1987. 15:2499-2513.

248. Brzozowski, A., A. Pike, Z. Dauter, R. Hubbard, T. Bonn, O. Engström, L. Öhman, G. Greene, J.-Å. Gustafsson, and M. Carlquist. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature. 1997. 16:753-758.

249. Ekena, K., K. Weis, J. Katzenellenbogen, and B. Katzenellenbogen. Identification of amino acids in the hormone binding domain of the human estrogen receptor important in the estrogen binding. J Biol Chem. 1996. 271:20053-20059.

250. Danielian, P., R. White, J. Lees, and M. Parker. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. (Published erratum appears in *EMBO Journal* 1992 11 2366.). EMBO J. 1992. 11:1025-1033.

251. Tchoudakova, A., S. Pathak, and G. Callard. Molecular cloning of an estrogen receptor β subtype from the goldfish, *Carassius auratus*. Gen Comp Endocrinol. 1999. 113:388-400.

252. Wilson, M., R. Price Jr, and R. Handa. Estrogen receptor-β messenger ribonucleic acid expression in the pituitary gland. Endocrinology. 1998. 139:5151-5156.

253. Shughrue, P., P. Scrimo, M. Lane, R. Askew, and I. Merchenthaler. The distribution of estrogen receptor-beta mRNA in forebrain regions of the estrogen receptor-alpha knockout mouse. Endocrinology. 1997. 138:5649-5652.

254. Todo, T., S. Adachi, and K. Yamauchi. Molecular cloning and characterization of Japanese eel estrogen receptor cDNA. Mol Cell Endocrinol. 1996. 119:37-45.

255. Green, S., P. Walter, V. Kumar, A. Krust, J. Bornert, P. Argos, and P. Chambon. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature. 1986. 320:124-139.

256. Robinson-Rechavi, M., O. Marchand, H. Escriva, P.-L. Bardet, D. Zelus, S. Hughes, and V. Laudet. Euteleost fish genomes are characterized by expansion of gene families. Genome Res. 2001. 11:781-788.

257. Graham-Lorence, S., M. Khalil, M. Lorence, C. Mendelson, and E. Simpson. Structure-function relationships of human aromatase cytochrome P-450 using molecular modeling and site-directed mutagenesis. J Biol Chem. 1991. 266:11939-11946.

258. Sprague, J., D. Clements, T. Conlin, P. Edwards, K. Frazer, K. Schaper, E. Segerdell, P. Song, B. Sprunger, and M. Westerfield. The Zebrafish Information Network (ZFIN): the zebrafish model organism database. Nucleic Acids Res. 2003. 31:241-243.

259. Sprague, J., L. Bayraktaroglu, D. Clements, T. Conlin, D. Fashena, K. Frazer, M. Haendel, D. Howe, P. Mani, S. Ramachandran, K. Schaper, E. Segerdell, P. Song, B. Sprunger, S. Taylor, C. Van Slyke, and M. Westerfield. The Zebrafish Information Network: the zebrafish model organism database. Nucleic Acids Res. 2006. 34:D581-585.

260. Muñoz-Cueto, J., E. Burzawa-Gérard, O. Hah, Y. Valotaire, and F. Pakdel. Cloning and sequencing of the gilthead sea bream estrogen receptor cDNA. DNA Seq. 1999. 10:75-84.

261. Tan, N., T. Lam, and J. Ding. The first contiguous estrogen receptor gene from a fish, *Oreochromis aureus*: evidence for multiple transcripts (Erratum in: Mol Cell Endocrinol 1996 123(1):107-10). Mol Cell Endocrinol. 1996. 120:177-192.

262. Pakdel, F., F. Le Gac, P. Le Goff, and Y. Valotaire. Full-length sequence and *in vitro* expression of rainbow trout estrogen receptor cDNA. Mol Cell Endocrinol. 1990. 71:195-204.

263. Koš, M., S. Denger, G. Reid, and F. Gannon. Upstream open reading frames regulate the translation of the multiple mRNA variants of the estrogen receptor α. J Biol Chem. 2002. 40:37131-37138.

264. Patiño, R., Z. Xia, W. Gale, C. Wu, A. Maul, and X. Chang. Novel transcripts of the estrogen receptor α gene in channel catfish. Gen Comp Endocrinol. 2000. 120:314-325.

265. Pakdel, F., R. Métivier, G. Flouriot, and Y. Valotaire. Two estrogen receptor (ER) isoforms with different estrogen dependencies are generated from the trout ER gene. Endocrinology. 2000. 141:571-580.

266. Menuet, A., I. Anglade, G. Flouriot, F. Pakdel, and O. Kah. Tissue-specific expression of two structurally different estrogen receptor alpha isoforms along the female reproductive axis of an oviparous species, the rainbow trout. Biol Reprod. 2001. 65:1548-1557.

267. Tremblay, G., A. Tremblay, N. Copeland, D. Gilbert, N. Jenkins, F. Labrie, and V. Giguére. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol. 1997. 11:353-365.

268. Huang, J., X. Li, C. Maguire, R. Hilf, R. Bambara, and M. Muyan. Binding of estrogen receptor β to estrogen response element *in situ* is independent of estradiol and impaired by its amino terminus. Mol Endocrinol. 2005. 19:2696-2712.

269. Menuet, A., Y. Le Page, O. Torres, L. Kern, O. Kah, and F. Pakdel. Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERα, ERβ1 and ERβ2. J Mol Endocrinol. 2004. 32:975-986.

270. Fournier, B., S. Gutzwiller, T. Dittmar, G. Matthias, P. Steenbergh, and P. Matthias. Estrogen receptor (ER)-α, but not ER-β, mediates regulation of the insulin-like growth factor I gene by antiestrogens. J Biol Chem. 2001. 276:35444-35449.

271. Takei, Y. and C. Loretz, Endocrinology, in The Physiology of Fishes, Evans, D. and J. Claiborne, Editors. 2006, CRC Press: Boca Raton, FL USA. p. 271-318.

272. Iwama, G., L. Afonso, and M. Vijayan, Stress in Fish, in The Physiology of Fishes, Evans, D. and J. Claiborne, Editors. 2006, CRC Press: Boca Raton, FL USA. p. 319-342.

273. Sandhoff, T., D. Hales, K. Hales, and M. McLean. Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1. Endocrinology. 1998. 139:4820-4831.

274. Kozak, M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell. 1986. 44:283-292.

275. Chang, X., T. Kobayashi, H. Kajimura, M. Nakamura, and Y. Nagahama. Isolation and characterization of the cDNA encoding the tilapia (*Oreochromis niloticus*) cytochrome P450 aromatase (P450arom): changes in P450arom mRNA, protein and enzyme activity in ovarian follicles during oogenesis. J Mol Endocrinol. 1997. 18:57-66.

276. Dalla Valle, L., L. Lunardi, L. Colombo, and P. Belvedere. European sea bass (*Dicentrarchus labrax* L.) cytochrome P450arom: cDNA cloning, expression and genomic organization. J Steroid Biochem Mol Biol. 2002. 80:25-34.

277. Trant, J.M. Isolation and characterization of the cDNA-encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome P450Arom. Gen. Comp. Endcorinol. 1994. 95:155-168.

278. Sheets, M., S. Ogg, and M. Wickens. Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation *in vitro*. Nucleic Acids Res. 1990. 18:5799-5805.

279. Halm, S., M. Rand-Weaver, J. Sumpter, and C. Tyler. Cloning and molecular characterization of an ovarian-derived (brain-like) P450 aromatase cDNA and development of a competitive RT-PCR assay to quantify its expression in the fathead minnow (*Pimephales promelas*) Fish Physiol Biochem. 2001. 24:49-62.

280. Chen, S. and D. Zhou. Functional domains of aromatase cytochrome P450 inferred from comparative analyses of amino acid sequences and substantiated by site-directed mutagenesis experiments (Erratum in: J Biol Chem. 1994. 269(2):1564.). J Biol Chem. 1992. 267:22587-22594.

281. Shen, P., C. Campagnoni, K. Kampf, B. Schlinger, A. Arnold, and A. Campagnoni. Isolation and characterization of a zebra finch aromatase cDNA: *in situ* hybridization reveals high aromatase expression in brain. Brain Res Mol Brain Res. 1994. 24:227-237.

282. McPhaul, M., J. Noble, E. Simpson, C. Mendelson, and J. Wilson. The expression of a functional cDNA encoding the chicken cytochrome P-450arom (aromatase) that catalyzes the formation of estrogen from androgen. J Biol Chem. 1988. 263:16358-16363.

283. Balthazart, J., M. Baillien, T. Charlier, and G. Ball. Calcium-dependent phosphorylation processes control brain aromatase in quail. Eur J Neurosci. 2003. 17:1591-1606.

284. Murdock, C. and T. Wibbels. Cloning and expression of aromatase in a turtle with temperature-dependent sex determination. Gen Comp Endocrinol. 2003. 130:109-119.

285. Gabriel, W., B. Blumberg, S. Sutton, A. Place, and V. Lance. Alligator aromatase cDNA sequence and its expression in embryos at male and female incubation temperatures. J Exp Zool. 2001. 290:439448.

286. Endo, D. and M. Park. Molecular cloning of P450 aromatase from the leopard gecko and its expression in the ovary. J Steroid Biochem Mol Biol. 2005. 96:131-140.

287. Kuntz, S., A. Chesnel, M. Duterque-Coquillaud, I. Grillier-Vuissoz, M. Callier, C. Dournon, S. Flament, and D. Chardard. Differential expression of P450 aromatase during gonadal sex differentiation and sex reversal of the newt *Pleurodeles waltl.* J Steroid Biochem Mol Biol. 2003. 84:89-100.

288. Kato, T., K. Matsui, M. Takase, M. Kobayashi, and M. Nakamura. Expression of P450 aromatase protein in developing and in sex-reversed gonads of the XX/XY type of the frog *Rana rugosa*. Gen Comp Endocrinol. 2004. 137:227-236.

289. Zhou, D., K. Korzekwa, T. Poulos, and S. Chen. A site-directed mutagenesis study of human placental aromatase. J Biol Chem. 1992. 267:762-768.

290. Gardner, L., T. Anderson, A. Place, B. Dixon, and A. Elizur. Sex change strategy and the aromatase genes. J Steroid Biochem Mol Biol. 2005. 94:395-404.

291. Hallgren, S., M. Linderoth, and K. Olsén. Inhibition of cytochrome p450 brain aromatase reduces two male specific sexual behaviours in the male Endler guppy (*Poecilia reticulata*). Gen Comp Endocrinol. 2006. 147:323-328.

292. Kuhl, A., S. Manning, and M. Brouwer. Brain aromatase in Japanese medaka (*Oryzias latipes*): Molecular characterization and role in xenoestrogen-induced sex reversal. J Steroid Biochem Mol Biol. 2005. 96:67-77.

293. Pellegrini, E., A. Menuet, C. Lethimonier, F. Adrio, M.-M. Gueguen, C. Tascon, I. Anglade, F. Pakdel, and O. Kah. Relationships between aromatase and estrogen receptors in the brain of teleost fish. Gen Comp Endocrinol. 2005. 142:60-66.

294. Menuet, A., E. Pellegrini, F. Brion, M.-M. Gueguen, I. Anglade, F. Pakdel, and O. Kah. Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. J Comp Neurol. 2005. 485:304-320.

295. Gelinas, D., G. Pitoc, and G. Callard. Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the seasonal cycle and after steroid treatment. Mol Cell Endocrinol. 1998. 138:81-93.

296. Norris, D., Vertebrate Endocrinology. 1997, London: Academic Press. 634.

297. Callard, G., B. Schlinger, M. Pasmanik, and K. Corina. Nonmammalian vertebrate models in studies of brain - steroid interactions. J Exp Zool Suppl. 1990. 4:6-16.

298. Leguen, I., C. Carlsson, E. Perdu-Durand, P. Prunet, P. Pärt, and J. Cravedi. Xenobiotic and steroid biotransformation activities in rainbow trout gill epithelial cells in culture. Aquat Toxicol. 2000. 48:165-176.

299. Harada, N., H. Sasano, H. Murakami, T. Ohkuma, H. Nagura, and Y. Takagi. Localized expression of aromatase in human vascular tissues. Circ Res. 1999. 84:1285-1291.

300. Samy, T., R. Zheng, T. Matsutani, L. Rue III, K. Bland, and I. Chaudry. Mechanism for normal splenic T lymphocyte functions in proestrus females after trauma: enhanced local synthesis of 17β-estradiol. Am J Physiol Cell Physiol. 2003. 285:C139-C149.

301. Tanaka, M., T. Telecky, S. Fukada, S. Adachi, S. Chen, and Y. Nagahama. Cloning and sequence analysis of the cDNA encoding P-450 aromatase (P450arom) from a rainbow trout (*Oncorhynchus mykiss*) ovary; relationship

between the amount of P450arom mRNA and the production of oestradiol-17β in the ovary. J Mol Endocrinol. 1992. 8:53-61.

302. Kolkovski, S. and K. Dabrowski. Off-season spawning of yellow perch. Prog Fish-Cult. 1998. 60:133-136.

303. Farmanfarmaian, A. and L. Sun. Growth hormone effects on essential amino acid absorption, muscle amino acid profile, N-retention and nutritional requirements of striped bass hybrids. Genet Anal. 1999. 15:107-113.

304. Silverstein, J.T., W.R. Wolters, M. Shimizu, and W.W. Dickhoff. Bovine growth hormone treatment of channel catfish: strain and temperature effects on growth, plasma IGF-I levels, feed intake and efficiency and body composition. Aquaculture. 2000. 190:77-88.

305. Shamblott, M.J., C.M. Cheng, D. Bolt, and T.T. Chen. Appearance of insulin-like growth factor mRNA in the liver pyloric ceca of a teleost in response to exogenous growth hormone. Proc. Natl. Acad. Sci. USA. 1995. 92:6943-6946.

306. Auperin, B., J.F. Baroiller, M.J. Ricordel, A. Fostier, and P. Prunet. Effect of confinement stress on circulating levels of growth hormone and two prolactins in freshwater-adapted tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol. 1997. 108:35-44.

307. Best, C. 1981. Initiation of artifical feeding and the control of sex differentiation in yellow perch, *Perca flavescens*. MS thesis. University of Wisconsin-Madison, Madison, WI. pp. 121.

308. Purchase, C.F., N.C. Collins, G.E. Morgan, and B.J. Shuter. Sex-specific covariation among life-history traits of yellow perch (*Perca flavescens*). Evol Ecol Res. 2005. 7:549-566.

309. Björnsson, B. The biology of salmon growth hormone: from daylight to dominance. Fish Physiol Biochem. 1997. 17:9-24.

310. Meiri, I., W. Knibb, Y. Zohar, and A. Elizur. Temporal profile of β follicle-stimulating hormone, β luteinizing hormone, and growth hormone gene expression in the protandrous hermaphrodite, gilthead seabream, *Sparus aurata*. Gen Comp Endocrinol. 2004. 137:288-299.

311. Figueroa, J., R. San Martín, C. Flores, H. Grothusen, and G. Kausel. Seasonal modulation of growth hormone mRNA and protein levels in carp pituitary: evidence for two expressed genes. J Comp Physiol B. 2005. 175:185-192.

312. Degani, G., I. Tzchori, S. Yom-Din, D. Goldberg, and K. Jackson. Growth differences and growth hormone expression in male and female European eels [*Anguilla anguilla* (L.)]. Gen Comp Endocrinol. 2003. 134:88-93.

313. Ran, X.-Q., W.-S. Li, and H.-R. Lin. Stimulatory effects of gonadotropin-releasing hormone and dopamine on growth hormone release and growth hormone mRNA expression in *Epinephelus coioides*. Sheng Li Xue Bao. 2004. 56:644-650.

314. Holloway, A. and J. Leatherland. Effect of gonadal steroid hormones on plasma growth hormone concentrations in sexually immature rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol. 1997. 105:246-254.

315. Poh, L., A. Munro, and C. Tan. The effects of oestradiol on the prolactin and growth hormone content of the pituitary of the tilapia, *Oreochromis mossambicus*, with observations on the incidence of black males. Zool Sci. 1997. 14:979-986.

316. Elango, A., B. Shepherd, and T.T. Chen. Effects of endocrine disrupters on the expression of growth hormone and prolactin mRNA in the rainbow trout pituitary. Gen Comp Endcorinol. 2006. 145:116-127.

317. Trudeau, V., G. Somoza, C. Nahorniak, and R. Peter. Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrinology. 1992. 56:483-490.

318. Jentoft, S., N. Topp, M. Seeliger, J.A. Malison, T.B. Barry, J.A. Held, S. Roberts, and F. Goetz. Lack of growth enhancement by exogenous growth hormone treatment in yellow perch (*Perca flavescens*) in four separate experiments. Aquaculture. 2005. 250:471-479.

319. Fruchtman, S., B. Gift, B. Howes, and R. Borski. Insulin-like growth factor-I augments prolactin and inhibits hormone release through distinct as well as overlapping cellular signaling pathways. Comp Biochem Physiol B Biochem Mol Biol. 2001. 129:237-242.

320. Kajimura, S., K. Uchida, T. Yada, T. Hirano, K. Aida, and E. Grau. Effects of insulin-like growth factors (IGF-I and -II) on growth hormone and prolactin release and gene expression in euryhaline tilapia, *Oreochromis mossambicus*. Gen Comp Endocrinol. 2002. 127:223-231.

321. Fruchtman, S., L. Jackson, and R. Borski. Insulin-like growth factor I disparately regulates prolactin and growth hormone synthesis and secretion: studies using the teleost pituitary model. Endocrinology. 2000. 141:2886-2894.

322. Quérat, B., B. Cardinaud, A. Hardy, B. Vidal, and G. D'Angelo. Sequence and regulation of European eel prolactin mRNA. Mol Cell Endocrinol. 1994. 102:151-160.

323. Tang, Y., B. Shepherd, A. Nichols, R. Dunham, and T. Chen. Influence of environmental salinity on mRNA levels of growth hormone, prolactin and

somatolactin in the pituitary of the channel catfish, *Ictalurus punctatus*. Mar Biotechnol. 2001. 3:205-217.

324. Figueroa, J., A. Molina, M. Alvarez, J. Villanueva, A. Reyes, G. León, and M. Krauskopf. Prolactin gene expression and changes of prolactin pituitary level during the seasonal acclimatization of the carp. Comp Biochem Physiol Biochem Mol Biol. 1994. 108:551-560.

325. Tacon, P., J. Baroiller, P. Le Bail, P. Prunet, and B. Jalabert. Effect of egg deprivation on sex steroids, gonadotropin, prolactin, and growth hormone profiles during the reproductive cycle of the mouthbrooding cichlid fish *Oreochromis niloticus*. Gen Comp Endocrinol. 2000. 117:54-65.

326. Taniyama, S., T. Kitahashi, H. Ando, M. Kaeriyama, Y. Zohar, H. Ueda, and A. Urano. Effects of gonadotropin-releasing hormone analog on expression of genes encoding the growth hormone/prolactin/somatolactin family and a pituitary-specific transcription factor in the pituitaries of prespawning sockeye salmon. Gen Comp Endcorinol. 2000. 118:418-424.

327. Duan, C., E.M. Plisetskaya, and W.W. Dickhoff. Expression of insulin-like growth factor I in normally and abnormally developing coho salmon (*Oncorhynchus kisutch*). Endocrinology. 1995. 136:446-452.

328. Beckman, B., D. Larsen, S. Moriyama, B. Lee-Pawlak, and W. Dickhoff. Insulin-like growth factor-I and environmental modulation of growth during smoltification of spring chinook salmon (*Oncorhynchus tshawytscha*). Gen Comp Endcorinol. 1998. 109:325-335.

329. Taylor, J., H. Migaud, M. Porter, and N. Bromage. Photoperiod influences growth rate and plasma insulin-like growth factor-I levels in juvenile rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endcorinol. 2005. 142:169-185.

330. Beckman, B., M. Shimizu, B. Gadberry, and K. Cooper. Response of the somatotropic axis of juvenile coho salmon to alterations in plane of nutrition with an analysis of the relationships among growth rate and circulating IGF-I and 41 kDa IGFBP. Gen Comp Endcorinol. 2004. 135:334-344.

331. Dabrowski, K., R. Ciereszko, A. Ciereszko, G. Toth, S. Christ, D. El-Saidy, and J. Ottobre. Reproductive physiology of yellow perch (*Perca flavescens*): environmental and endocrinological cues. J Appl Ichthyol. 1996. 12:139-148.

332. Andreassen, T., K. Skjoedt, and B. Korsgaard. Upregulation of estrogen receptor α and vitellogenin in eelpout (*Zoarces viviparus*) by waterborne exposure to 4-*tert*-octylphenol and 17β-estradiol. Comp Biochem Physiol C Toxicol Pharmacol. 2005. 140:340-346.

333. Bowman, C., K. Kroll, T. Gross, and N. Denslow. Estradiol-induced gene expression in largemouth bass (*Micropterus salmoides*). Mol Cell Endocrinol. 2002. 196:67-77.

334. Pakdel, F., F. Delaunay, B. Ducouret, G. Flouriot, L. Kern, G. Lazennec, Y. Le Dréan, F. Petit, G. Salbert, D. Saligaut, M. Tujague, and Y. Valotaire. Regulation of gene expression and biological activity of rainbow trout estrogen receptor. Fish Physiol Biochem. 1997. 17:123-133.

335. MacKay, M., J. Raelson, and C. Lazier. Up-regulation of estrogen receptor mRNA and estrogen receptor activity by estradiol in liver of rainbow trout and other teleostean fish. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 1996. 115:201-209.

336. Smith, J. and P. Thomas. Changes in hepatic estrogen-receptor concentrations during the annual reproductive and ovarian cycles of a marine teleost, the spotted seatrout, *Cynoscion nebulosus*. Gen Comp Endcorinol. 1991. 81:234-245.

337. Patel, M., B. Scheffler, L. Wang, and K. Willett. Effects of benzo(*a*)pyrene exposure on killifish (*Fundulus heteroclitus*) aromatase activities and mRNA. Aquat Toxicol. 2006. 77:267-278.

338. Greytak, S., D. Champlin, and G. Callard. Isolation and characterization of two cytochrome P450 aromatase forms in killifish (*Fundulus heteroclitus*): Differential expression in fish from polluted and unpolluted environments. Aquat Tox. 2005. 71:371-389.

339. Villeneuve, D., I. Knoebl, M. Kahl, K. Jensen, D. Hammermeister, K. Greene, L. Blake, and G. Ankley. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (*Pimephales promelas*). Aquat Tox. 2006. 76:353-368.

340. Young, K.A. Life-history variation and allometry for sexual size dimorphism in Pacific salmon and trout. Proc R Soc B. 2005. 272:167-172.

341. Tamate, T. and K. Maekawa. Latitudinal variation in sexual size dimorphism of sea-run masu salmon, *Oncorhynchus masou*. Evolution Int J Org Evolution. 2006. 60:196-201.

342. Kupfer, A., A. Kramer, and W. Himstedt. Sex-related growth patterns in a caecilian amphibian (genus Ichthyophis): evidence from laboratory data. J Zool. 2004. 262:173-178.

343. Sinsch, U., I. Di Tada, and A. Martino. Longevity, demography and sex-specific growth of the Pampa de Achala Toad, *Bufo achalensis* CEI, 1972. Stud Neotrop Fauna Environ. 2001. 36:95-104.

344. Taylor, E. and D. Denardo. Sexual size dimorphism and growth plasticity in snakes: an experiment on the Western Diamond-backed Rattlesnake (*Crotalus atrox*). J Exp Zoolog A Comp Exp Biol. 2005. 303:598-607.

345. Hailey, A. and I. Coulson. The growth pattern of the African tortoise *Geochelone paradalis* and other chelonians. Can J Zool/Rev Can Zool. 1999. 77:181-193.

346. Pearson, D., R. Shine, and A. Williams. Geographic variation in sexual size dimorphism within a single snake species (*Morelia spilota*, Pythonidae). Oecologia. 2002. 131:418-426.

347. Ewert, M., R. Hatcher, and J. Goode. Sex determination and ontogeny in *Malacochersus tornieri*, the Pancake Tortoise. J Herpetol. 2004. 38:291-295.

348. Fairbairn, J. and R. Shine. Patterns of sexual size dimorphism in seabirds of the southern-hemisphere. Oikos. 1993. 68:139-145.

349. Andersson, M. Evolution of reversed sex-roles, sexual size dimorphism, and mating system in coucals (Centropodidae, Aves). Biol J Linn Soc. 1995. 54:173-181.

350. Badyaev, A., L. Whittingham, and G. Hill. The evolution of sexual size dimorphism in the house finch. III. Developmental basis. Evolution. 2001. 55:176-189.

351. Isaac, J. Potential causes and life-history consequences of sexual size dimorphism in mammals. Mamm Rev. 2005. 35:101-115.

352. Schulte-Hostedde, A., J. Millar, and H. Gibbs. Female-biased sexual size dimorphism in the yellow-pine chipmunk (*Tamias amoenus*): sex-specific patterns of annual reproductive success and survival. Evolution Int J Org Evolution. 2002. 56:2519-2529.

353. Garel, M., E. Solberg, B. Saether, I. Herfindal, and K. Hogda. The length of growing season and adult sex ratio affect sexual size dimorphism in moose. Ecology. 2006. 87:745-758.

354. Krasnov, B., S. Morand, H. Hawlena, I. Khokhlova, and G. Shenbrot. Sex-biased parasitism, seasonality and sexual size dimorphism in desert rodents. Oecologia. 2005. 146:209-217.

355. Gustafsson, A. and P. Lindenfors. Human size evolution: no evolutionary allometric relationship between male and female stature. J Hum Evol. 2004. 47:253-266.

356. VanDeValk, A., C. Adams, L. Rudstam, J. Forney, T. Brooking, M. Gerken, B. Young, and J. Hooper. Comparison of angler and cormorant harvest of walleye

and yellow perch in Oneida Lake, New York. Trans Am Fish Soc. 2002. 131:27-39.

357.   Wallat, G.K., L.G. Tiu, H.P. Wang, D. Rapp, and C. Leighfield. The effects of size grading on production efficiency and growth performance of yellow perch in earthern ponds. N Am J Aquacult. 2005. 67:34-41.

358.   Ikeda, Y., A. Nagai, M.-A. Ikeda, and S. Hayashi. Sexually dimorphic and estrogen-dependent expression of estrogen receptor β in the ventromedial hypothalamus during rat postnatal development. Endocrinology. 2003. 144:5098-5104.

359.   Li, W., D. Chen, A. Wong, and H. Lin. Molecular cloning, tissue distribution, and ontogeny of mRNA expression of growth hormone in orange-spotted grouper (*Epinephelus coioides*). Gen Comp Endcorinol. 2005. 144:78-89.

360.   de Jesus, E., F. Ayson, Y. Amemiya, S. Moriyama, S. Hyodo, T. Hirano, and H. Kawauchi. Milkfish (Chanos chanos) growth hormone cDNA cloning and mRNA expression in embryos and early larval stages. Aquaculture. 2002. 208:177-188.

361.   Funkenstein, B. and I. Cohen. Ontogeny of growth hormone protein and mRNA in the gilthead sea bream *Sparus aurata*. Growth Regul. 1996. 6:16-21.

362.   Power, D. Developmental ontogeny of prolactin and its receptor in fish. Gen Comp Endocrinol. 2005. 142:25-33.

363.   Santos, C., J. Cavaco, P. Ingleton, and D. Power. Developmental ontogeny of prolactin and prolactin receptor in the sea bream (*Sparus aurata*). Gen Comp Endocrinol. 2003. 132:304-314.

364.   Power, D. and A. Canario. Immunocytochemistry of somatotrophs, gonadotrophs, prolactin and adrenocorticotropin cells in larval sea bream (*Sparus auratus*) pituitaries. Cell Tissue Res. 1992. 269:341-346.

365.   Duguay, S.J., J. Lai-Zhang, D.F. Steiner, B. Funkenstein, and S.J. Chan. Developmental and tissue-regulated expression of IGF-I and IGF-II mRNAs in *Sparus aurata*. J Mol Endocrinol. 1996. 16:123-132.

366.   van Aerle, R., T. Runnalls, and C. Tyler. Ontogeny of gonadal sex development relative to growth in fathead minnow. J Fish Biol. 2004. 64:355-369.

367.   Kobayashi, T., H. Kajiura-Kobayashi, and Y. Nagahama. Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, tilapia. Cytogenet Genome Res. 2003. 101:289-294.

368.   Osmundson, D.B. Proximate causes of sexual size dimorphism in Colorado pikeminnow, a long-lived cyprinid. J Fish Biol. 2006. 68:1563-1588.

369. Shaw, F.R. and D.R. Gunderson. Life history traits of the greenstriped rockfish, *Sebastes elongatus.* Cal Fish Game. 2006. 92:1-23.

370. Lopes, M., A.G. Murta, and H.N. Cabral. Discrimination of snipefish *Macroramphosus species* and boarfish *Capros aper* morphotypes through multivariate analysis of body shape. Helgol Meersunters. 2006. 60:18-24.

371. Prokes, M., P. Sovcik, M. Penaz, V. Barus, P. Spurny, and L. Vilizzi. Growth of barbel, *Barbus barbus*, in the River Jihlava following major habitat alteration and estimated by two methods. Folia Zool. 2006. 55:86-96.

372. Marriott, P., P.L. Horn, and P. McMillan. Species identification and age estimation for the ridge-scaled Macrourid (*Macrourus whitsoni*) from the Ross Sea. CCAMLR Sci. 2003. 10:37-51.

373. Henderson, B.A., N. Collins, G.E. Morgan, and A. Vaillancourt. Sexual size dimorphism of walleye (*Stizostedion vitreum vitreum*). Can J Fish Aquat Sci. 2003. 60:1345-1352.

374. Mallen-Cooper, M. and I.G. Stuart. Age, growth and non-flood recruitment of two potamodromous fishes in a large semi-arid/temperate river system. River Res Appl. 2003. 19:697-719.

375. Chakroun-Marzouk, N. and M.H. Ktari. The brown meagre from Tunisian coasts, *Sciaena umbra* (Sciaenidae): sexual cycle, age and growth. Cybium. 2003. 27:211-225.

376. Saillant, E., A. Fostier, B. Menu, P. Haffray, and B. Chatain. Sexual growth dimorphism in sea bass *Dicentrarchus labrax.* Aquaculture. 2001. 202:371-387.

377. Juell, J.E. and O.I. Lekang. The effect of feed supply rate on growth of juvenile perch (*Perca fluviatilis*). Aquacult Res. 2001. 32:459-464.

378. Swain, D.P. and M.J. Morgan. Sex-specific temperature distribution in four populations of American plaice *Hippoglossoides platessoides.* Mar Ecol Prog Ser. 2001. 212:233-246.

379. Pongthana, N., D.J. Penman, P. Baoprasertkul, M.G. Hussain, M.S. Islam, S.F. Powell, and B.J. McAndrew. Monosex female production in the silver barb (*Puntius gonionotus* Bleeker). Aquaculture. 1999. 173:247-256.

380. Sheehan, R.J., S.P. Shasteen, A.V. Suresh, A.R. Kapuscinski, and J.E. Seeb. Better growth in all-female diploid and triploid rainbow trout. Trans Am Fish Soc. 1999. 128:491-498.

381. Imsland, A.K., A. Folkvard, G.L. Grung, S.O. Stefansson, and G.L. Taranger. Sexual dimorphism in growth and maturation of turbot *Scophthalmus maximus* (Rafinesque, 1810). Aquacult Res. 1997. 28:101-114.

382.	Takahashi, E., R.M. Connolly, and S.Y. Lee. Growth and reproduction of double-ended pipefish, *Syngnathoides biaculeatus*, in Moreton Bay, Queensland, Australia. Environ Biol Fish. 2003. 67:23-33.

383.	Rezk, M.A., E.A. Kamel, A.A. Ramadan, and R.A. Dunham. Comparative growth of Egyptian tilapias in response to declining water temperature. Aquaculture. 2002. 207:239-247.

384.	Haffray, P., C. Vauchez, M. Vandeputte, and O. Linhart. Different growth and processing traits in males and females of European catfish, *Silurus glanis*. Aquat Living Resour. 1998. 11:341-345.

385.	Katano, O. Growth of dark chub, *Zacco temmincki* (Cyprinidae), with a discussion of sexual size differences. Environ Biol Fish. 1998. 52:305-312.

386.	Gunther, J. Growth of the fish *Cichlasoma managuense* (Pisces: Cichlidae) under intense cultivation in land ponds. Rev Biol Trop. 1996. 44:813-818.

387.	Heins, D.C. and M.D. Machado. Spawning season, clutch characteristics, sexual dimorphism and sex-ratio in the redfin darter *Etheostoma-whipplei*. Am Midl Nat. 1993. 129:161-171.

388.	Schreiber, S., U. Focken, and K. Becker. Individually reared female Nile tilapia (*Oreochromis niloticus*) can grow faster than males. J Appl Ichthyol. 1998. 14:43-47.

389.	Melamed, P., N. Eliahu, M. Ofir, B. Levavi-Sivan, J. Smal, F. Rentier-Delrue, and Z. Yaron. The effects of gonadal development and sex steroids on growth hormone secretion in the male tilapia hybrid (*Oreochromis niloticus* x *O. aureus*). Fish Physiol Biochem. 1995. 14:267-277.

390.	Yamazaki, F. Application of hormones in fish culture. J Fish Res Bd Can. 1976. 33:948-958.

391.	Trudeau, V.L., B.D. Sloley, A.O.L. Wong, and R.E. Peter. Interactions of gonadal steroids with brain dopamine and gonadotropin-releasing hormone in the control of gonadotropin-II secretion in the goldfish. Gen Comp Endcorinol. 1993. 89:39-50.

392.	Zou, J., V. Trudeau, Z. Cui, J. Brechin, K. Mackenzie, Z. Zhu, D. Houlihan, and R. Peter. Estradiol stimulates growth hormone production in female goldfish. Gen Comp Endocrinol. 1997. 106:102-112.

393.	Hornung, M.W., K.M. Jensen, J.J. Korte, M.D. Kahl, E.J. Durhan, J.S. Denny, T.R. Henry, and G.T. Ankley. Mechanistic basis for estrogenic effects in fathead minnow (*Pimephales promelas*) following exposure to the androgen 17α-methyltestosterone: conversion of 17α-methyltestosterone to 17α-methylestradiol. Aquat Tox. 2004. 66:15-23.

394.   Feenstra, A., W. Vaalburg, G.M. Nolten, S. Reiffers, A.G. Talma, T. Wiegman, H.D. van der Molen, and M.G. Woldring. Estrogen receptor binding radiopharmaceuticals: II. Tissue distribution of 17α-methylestradiol in normal and tumor-bearing rats. J Nucl Med. 1983. 24:522-528.

395.   Mandiki, S.N.M., M. Houbart, I. Babiak, E. Vandeloise, J.N. Gardeur, and P. Kestemont. Are sex steroids involved in the sexual growth dimorphism in Eurasian perch juveniles? Physiol Behav. 2004. 80:603-609.

396.   Davis, K.B. and G.M. Ludwig. Hormonal effects on sex differentiation and growth in sunshine bass *Morone chrysops* x *Morone saxatilis*. Aquaculture. 2004. 231:587-596.

397.   Sparks, R.T., B.S. Shepherd, B. Ron, N.H. Richman, L.G. Riley, G.K. Iwama, T. Hirano, and E.G. Grau. Effects of environmental salinity and 17α-methyltestosterone on growth and oxygen consumption in the tilapia, *Oreochromis mossambicus*. Comp Biochem Physiol B Biochem Mol Biol. 2003. 136:657-665.

398.   Rinchard, J., K. Dabrowski, M.A. Garcia-Abiado, and J. Ottobre. Uptake and depletion of plasma 17α-methyltesterone during induction of masculinization in muskellunge, *Esox masquinongy*: Effect on plasma steroids and sex reversal. Steroids. 1999. 64:518-525.

399.   Shepherd, B.S., B. Ron, A. Burch, R. Sparks, N.H. Richman, S.K. Shimoda, M.H. Stetson, C. Lim, and E.G. Grau. Effects of salinity, dietary level of protein and 17α-methyltestosterone on growth hormone (GH) and prolactin ($tPRL_{177}$ and $tPRL_{188}$) levels in the tilapia, *Oreochromis mossambicus*. Fish Physiol Biochem. 1997. 17:279-288.

400.   DemskaZakes, K. and Z. Zakes. Effect of 17 α-methyltestosterone on gonadal differentiation in pikeperch, *Stizostedion luciopeca* L. Aquacult Res. 1997. 28:59-63.

401.   Ko, K. and J.A. Malison. Effect of genistein on the growth and reproductive function of male and female yellow perch *Perca flavescens*. J World Aquacult Soc. 1999. 30:73-79.

402.   Mandiki, S.N.M., I. Babiak, J.M. Bopopi, F. Leprieur, and P. Kestemont. Effects of sex steroids and their inhibitors on endocrine parameters and gender growth differences in Eurasian perch (*Perca fluviatilis*) juveniles. Steroids. 2005. 70:85-94.

403.   Chan, C., C. Fung, W. Fung, M. Tse, and C. Cheng. Stimulation of growth hormone secretion from seabream pituitary cells in primary culture by growth hormone secretagogues is independent of growth hormone transcription. Comp Biochem Physiol C Toxicol Pharmacol. 2004. 139:77-85.

404. Caporali, S., M. Imai, L. Altucci, M. Cancemi, S. Caristi, L. Cicatiello, F. Matarese, R. Penta, D.K. Sarkar, F. Bresciani, and A. Weisz. Distinct signaling pathways mediate stimulation of cell cycle progression and prevention and apoptotic cell death by estrogen in rat pituitary tumor PR1 cells. Mol Biol Cell. 2003. 14:5051-5059.

405. Nolan, L.A. and A. Levy. The effects of testosterone and oestrogen on gonadectomised and intact male rat anterior pituitary mitotic and apoptotic activity. J Endocrinol. 2006. 188:387-396.

406. Duan, C., S.J. Duguay, P. Swanson, W.W. Dickhoff, and E.M. Plisetskaya. Tissue specific expression of insulin-like growth factor-I messenger ribonucleic acids in salmonids: development, hormonal and nutritional regulation. in Perspectives in Comparative Endocrinology. 1994. Ottawa: National Research Council of Canada.

407. Pucarelli, I., M. Segni, M. Ortore, A. Moretti, R. Iannaccone, and A. Pasquino. Combined therapy with GnRH analog plus growth hormone in central precocious puberty. J Pediatr Endocrinol Metab. 2000. 13:811-820.

408. Garnero, P., Y. Tsouderos, I. Marton, C. Pelissier, C. Varin, and P. Delmas. Effects of intranasal 17β-estradiol on bone turnover and serum insulin-like growth factor I in postmenopausal women. J Clin Endocrinol Metab. 1999. 84:2390-2397.

409. Wilson, M. Effects of estradiol and exogenous insulin-like growth factor I (IGF-I) on the IGF-I axis during growth hormone inhibition and antagonism. J Clin Endocrinol Metab. 1998. 83:4013-4021.

410. Leung, K.-C., G. Johannsson, G. Leong, and K. Ho. Estrogen regulation of growth hormone action. Endocr Rev. 2004. 25:693-721.

411. Borski, R., W. Tsai, R. DeMott-Freiberg, and A. Barkan. Regulation of somatic growth and the somatotropic axis by gonadal steroids: primary effect on insulin-like growth factor I gene expression and secretion. Endocrinology. 1996. 137:3253-3259.

412. Yan, M., M. Jones, M. Hernandez, D. Liu, E. Simpson, and C. Chen. Functional modification of pituitary somatotropes in the aromatase knockout mouse and the effect of estrogen replacement. Endocrinology. 2004. 145:604-612.

413. Moriyama, S., M. Oda, A. Takahashi, S. Sower, and H. Kawauchi. Genomic structure of the sea lamprey growth hormone-encoding gene. Gen Comp Endcorinol. 2006. 148:33-40.

414. Almuly, R., B. Cavari, H. Ferstman, O. Kolodny, and B. Funkenstein. Genomic structure and sequence of the gilthead seabream (Sparus aurata) growth hormone-

encoding gene: identification of minisatellite polymorphism in intron I. Genome. 2000. 43:836-845.

415. Yang, B.-Y., K.-M. Chan, C.-M. Lin, and T.T. Chen. Characterization of rainbow trout (*Oncorhynchus mykiss*) growth hormone 1 and the promoter region of growth hormone 2 gene. Arch Biochem Biophys. 1997. 340:359-368.

416. Zhu, Z., H. Ling, and T.T. Chen. Primary structure and evolutionary analyses of the growth hormone gene from grass carp (*Ctenopharyngodon idellus*). Eur J Biochem. 1992. 207:643-648.

417. Iwasaki, Y., M. Morishita, M. Asai, A. Onishi, M. Yoshida, Y. Oiso, and K. Inoue. Effects of hormones targeting nuclear receptors on transcriptional regulation of the growth hormone gene in the MtT/S rat somatotrope cell line. Neuroendocrinol. 2004. 79:229-236.

418. Ying, C. and D.H. Lin. Estrogen-modulated estrogen receptor-Pit-1 protein complex formation and prolactin gene activation require novel protein synthesis. J Biol Chem. 2000. 275:15407-15412.

419. Chuang, F.M., B.L. West, J.D. Baxter, and F. Schaufele. Activities in Pit-1 determine whether receptor interacting protein 140 activates or inhibits Pit-1/nuclear receptor transcriptional synergy. Mol Endocrinol. 1997. 11:1332-1341.

420. Wong, A.O.L., H. Zhou, Y. Jiang, and W.K.W. Ko. Feedback regulation of growth hormone synthesis and secretion in fish and the emerging concept of intrapituitary feedback loop. Comp Biochem Physiol A Mol Integr Physiol. 2006. 144:284-305.

421. Salbert, G., C. Atteke, G. Bonnec, and P. Jego. Differential regulation of the estrogen receptor mRNA by estradiol in the trout hypothalamus and pituitary. Mol Cell Endocrinol. 1993. 96:177-182.

422. Luo, Q., M. Ban, H. Ando, T. Kitahashi, R. Bhandari, S. McCormick, and A. Urano. Distinct effects of 4-nonylphenol and estrogen-17β on expression of estrogen receptor α gene in smolting sockeye salmon. Comp Biochem Physiol C Toxicol Pharmacol. 2005. 140:123-130.

423. DiPippo, V.A. and C.A. Powers. Estrogen induction of growth hormone in the thyroidectomized rat. Endocrinology. 1991. 129:1696-1700.

424. Crews, D., C. Gill, and K. Wennstrom. Sexually dimorphic regulation of estrogen receptor α mRNA in the ventromedial hypothalamus of adult whiptail lizards is testosterone dependent. Brain Res. 2004. 1004:136-141.

425. Peng, C. and R. Peter. Neuroendocrine regulation of GH secretion and growth in fish. Zool Stud. 1997. 36:79-89.

426. Holloway, A.C., G.T. Melroe, M.M. Ehrman, P.K. Reddy, J.F. Leatherland, and M.A. Sheridan. Effect of 17β-estradiol on the expression of somatostatin genes in rainbow trout (*Oncorhynchus mykiss*). Am J Physiol Regul Integr Comp Physiol. 2000. 279:R389-R393.

427. Holloway, A. and J. Leatherland. The influence of reproductive status on the stimulatory action of N-methyl-D,L-aspartate on growth hormone secretion, *in vitro* in rainbow trout, *Oncorhynchus mykiss*. Fish Physiol Biochem. 1997. 16:411-418.

428. Melamed, P., G. Gur, A. Elizur, H. Rosenfeld, B. Sivan, F. Rentier-Delrue, and Z. Yaron. Differential effects of gonadotropin-releasing hormone, dopamine and somatostatin and their second messengers on the mRNA levels of gonadotropin IIβ subunit and growth hormone in the teleost fish, tilapia. Neuroendocrinology. 1996. 64:320-328.

429. McPherron, A.C., A.M. Lawler, and S.J. Lee. Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature. 1997. 387:83-90.

430. Thomas, M., B. Langley, C. Berry, M. Sharma, S. Kirk, J. Bass, and R. Kambadur. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J Biol Chem. 2000. 275:40235-40243.

431. Kocabas, A.M., H. Kucuktas, R.A. Dunham, and Z. Liu. Molecular characterization and differential expression of the myostatin gene in channel catfish (*Ictalurus punctatus*). Biochim Biophys Acta. 2002. 1575:99-107.

432. Roberts, S.B. and F.W. Goetz. Differential skeletal muscle expression of myostatin across teleost species, and the isolation of multiple myostatin isoforms. FEBS Letters. 2001. 491:212-216.

433. Rodgers, B.D. and G.M. Weber. Sequence conservation among fish myostatin orthologues and the characterization of two additional cDNA clones from *Morone saxatilis* and *Morone americana*. Comp Biochem Physiol B Biochem Mol Biol. 2001. 129:597-603.

434. Roberts, S.B. and F.W. Goetz. Myostatin protein and mRNA transcript levels in adult and developing brook trout. Mol Cell Endocrinol. 2003. 210:9-20.

435. Du, R., Y.F. Chen, X.R. An, X.Y. Yang, Y. Ma, L. Zhang, X.L. Yuan, L.M. Chen, and J. Qin. Cloning and seqence analysis of myostatin promoter in sheep. DNA Seq. 2005. 16:412-417.

436. Ma, K., C. Mallidis, J. Artaza, W. Taylor, N. Gonzalez-Cadavid, and S. Bhasin. Characterization of 5'-regulatory region of human myostatin gene: regulation by dexamethasone *in vitro*. Am J Physiol Endocrinol Metab. 2001. 281:E1128-E1136.

437.   Parker, K.L., D.A. Rice, D.S. Lala, Y. Ikeda, X. Luo, M. Wong, M. Bakke, L. Zhao, C. Frigeri, N.A. Hanley, N. Stallings, and B.P. Schimmer. Steroidogenic factor 1: an essential mediator of endocrine development. Recent Prog Horm Res. 2002. 57:19-36.

438.   Jameson, J.L. Editorial: Of Mice and Men: the tale of steroidogenic factor-1. J Clin Endocrinol Metab. 2004. 89:5927-5929.

439.   Drean, Y.L., D. Liu, A.O. Wong, F. Xiong, and C.L. Hew. Steroidogenic factor 1 and estradiol receptor act in synergism to regulate the expression of the salmon gonadotropin II beta subunit gene. Mol Endocrinol. 1996. 10:217-229.

440.   Haisenleder, D.J., M. Yasin, A.C. Dalkin, J. Gilrain, and J.C. Marshall. GnRH regulates steroidogenic factor-1 (SF-1) gene expression in the rat pituitary. Endocrinology. 1996. 137:5719-5722.

<center>**Vita**</center>

Birth date and location: September 16th, 1968 in Pittsburgh, PA

Professional Preparation
PhD in Biology, expected December 2006
University of Kentucky, Lexington, Kentucky
Disseration title: Cloning and expression of key endocrine genes in a study of estrogen
        stimulated sexual size dimorphism in yellow perch (*Perca flavescens*)
MS in Bioscience and Biotechnology, September 1998
Drexel University, Philadelphia, Pennsylvania
Thesis title: Effects of nutrient availability on the biochemical and elemental
        stoichiometry of a freshwater diatom *Stephanodiscus minutulus*
        (Bacillariophyceae)
BS in Environmental Resource Management, May 1993
The Pennsylvania State University, University Park, Pennsylvania

Appointments

Department of Biology, University of Kentucky	Aug 98 to Aug 06
Lexington, KY 40506	
Teaching assistant in biology	
Student Support Services, University of Kentucky	Jan 01 to May 02
Lexington, KY 40506	
Tutor for disadvantaged undergraduate students	
United States Environmental Protection Agency	Jun 00 to Aug 00
Cincinnati, OH 45268	
Summer intern working on isolation of *Helicobactor pylori*	
Department of Bioscience, Drexel University	Apr 95 to Jun 98
Philadelphia, PA 19104	
Research assistant on NSF grant examining algal food quality for zooplankton	
Department of Bioscience, Drexel University	Apr 96 to Mar 98
Philadelphia, PA 19104	
Teaching assistant in biology	
CH2M Hill Environmental Consulting	Nov 94 to Jan 95
Philadelphia, PA 19103	
Field and laboratory assistant on Paoli, PA EPA Superfund Site	
Department of Entomology, Penn State University	Apr 94 to Aug 94
University Park PA 16802	
Research technician on project studying european corn borer ecology	
ERRI, Penn State University	Aug 93 to Mar 94
University Park PA 16802	
Staff research technician in water analysis laboratory	
Department of Entomology, Penn State University	Apr 93 to Aug 93
University Park PA 16802	
Research technician on project studying pear thrips ecology	
Department of Biology, Penn State University	Aug 92 to Mar 93

<center>252</center>

University Park PA 16802
Research technician on project studying remediation of acid mine drainage

Publications
- **Lynn SG**, DJ Price, WJ Birge and SS Kilham. 2006. Effect of nutrient availability on the uptake of PCB congener 2,2',6,6'-Tetrachlorobiphenyl by a diatom (*Stephanodiscus minutulus*) and transfer to a zooplankton (*Daphnia pulicaria*). Environmental Toxicology and Chemistry. In submission.
- **Lynn SG** and Lindle C. 2002. The effect of anthropogenic habitat modification on habitat use by *Afrana angolensis* along the Dodwe River, Tanzania. African Journal of Herpetology. 51(1):69-73.
- JS Rehage, **SG Lynn**, JI Hammond, BD Palmer and A Sih. 2002. Effects of larval exposure to triphenyltin on the survival, growth and behavior of larval and juvenile *Ambystoma barbouri* salamanders. Environmental Toxicology and Chemistry. 21(4):807-815.
- **Lynn SG**, SS Kilham, DA Kreeger and SJ Interlandi. 2000. Effects of nutrient availability on the biochemical and elemental stoichiometry of the freshwater diatom *Stephanodiscus minutulus* (Bacillariophyceae). Journal of Phycology. 36(3):510-522.
- Kilham SS, DA Kreeger, **SG Lynn**, CE Goulden and L Herrera. 1998. COMBO: A defined freshwater culture medium for algae and zooplankton. Hydrobiologia. 377:147-159.
- Kreeger DA, CE Goulden, SS Kilham, **SG Lynn**, S Datta and SJ Interlandi. 1997. Seasonal changes in the biochemistry of lake seston. Freshwater Biology. 38(3):539-554.
- Kilham SS, DA Kreeger, CE Goulden and **SG Lynn**. 1997. Effects of nutrient limitation on biochemical constituents of *Ankistrodesmus falcatus*. Freshwater Biology. 38(3):591-596.
- Kilham SS, DA Kreeger, CE Goulden and **SG Lynn**. 1997. Effects of algal food quality on fecundity and population growth rates of *Daphnia*. Freshwater Biology. 38(3):639-647.

Published GenBank Sequences
- **Lynn SG** and BS Shepherd. 2006. Cloning of ovarian aromatase (CYP19A1) from yellow perch (*Perca flavescens*). DQ984126. Scheduled to be released Oct. 27th, 2007
- **Lynn SG** and BS Shepherd. 2006. Cloning of estrogen receptor βa from yellow perch (*Perca flavescens*). DQ984125. Scheduled to be released Oct. 27th, 2007
- **Lynn SG**, WJ Birge and BS Shepherd. 2006. Cloning of estrogen receptor α from yellow perch (*Perca flavescens*). DQ984124. Scheduled to be released Oct. 27th, 2007
- **Lynn SG** and BS Shepherd. 2006. Cloning of insulin-like growth factor II from yellow perch (*Perca flavescens*). DQ984123. Scheduled to be released Oct. 27th, 2007
- **Lynn SG**, AR Scholik, HY Yan and BS Shepherd. 2004. Cloning of cDNA for HSP70 from fathead minnow (*Pimephales promelas*). AY538777

- Struewing IT, **SG Lynn** and BS Shepherd. 2003. Cloning of growth hormone from streamside salamander (*Ambystoma barbouri*). AY333114
- **Lynn SG**, IT Struewing and BS Shepherd. 2003. Cloning of prolactin from streamside salamander (*Ambystoma barbouri*). AY332494
- **Lynn SG**, JJ Arambasik and BS Shepherd. 2003. Cloning of beta-actin from yellow perch (*Perca flavescens*). AY332493
- **Lynn SG** and BS Shepherd. 2003. Cloning of insulin-like growth factor I from yellow perch (*Perca flavescens*). AY332492
- **Lynn SG** and BS Shepherd. 2003. Cloning of prolactin from yellow perch (*Perca flavescens*). AY332491
- **Lynn SG** and BS Shepherd. 2003. Cloning of somatolactin from yellow perch (*Perca flavescens*). AY332490

Grants, Fellowships and Awards

- **Elsevier/Society for Environmental Toxicology and Chemistry Best Student Platform Presentation Award, Co-Winner**. Awarded to best student presentation at the national meeting. $163. November 2005.
- **Ohio Valley Chapter (OVC) Society for Environmental Toxicology and Chemistry Student Travel Award**. Encourages participation of student members in annual meeting. $125. November 2005.
- **Society for Environmental Toxicology and Chemistry Student Travel Award**. Encourages participation of student members in annual meeting. $500. November 2005.
- **University of Kentucky Graduate School Commonwealth Research Award**. Supports outstanding graduate students engaged in cutting-edge research to present at the premier conference affliated with the student's discipline. $1,000. Spring 2004.
- **University of Kentucky Graduate School Dissertation Enhancement Award**. Supports post-qualifying examination graduate student doctoral research projects which require that the research be pursued at a site distant from the campus. $2,960. July to December 2003.
- **American Museum of Natural History Lerner-Gray Fund for Marine Research Award**. Analysis of Leatherback sea turtle (*Dermochelys coriacea*) eggs for potential endocrine disrupting compounds (EDCs), including pesticides, PCBs, PBBs and PBDEs. $1,170. August 2002 to July 2003.
- **University of Kentucky Graduate School Graduate Student Incentive Program Fellowship**. Awards graduate student recipients of nationally competitive grants. $1,000/year. June 2002 to May 2005.
- **National Estuarine Research Reserve Graduate Research Fellowship**. Atrazine body burdens and endocrine correlates in channel catfish (*Ictalurus punctatus*) and yellow perch (*Perca flavescens*) from Old Woman Creek (OWC) National Estuarine Research Reserve and Lake Erie. $25,000/year. June 2002 to May 2005.
- **University of Kentucky Department of Biology Ribble Award**. Sponsored recipient for a Tropical Biology Association field ecology course in Tanzania. $1,300. September 1999.

- **University of Kentucky Graduate School Academic Year Fellowship**. Supplemental stipend award based on academic and professional qualifications. $3,000. August 1998 to May 1999.

Presentations and Abstracts

- **SG Lynn***, JA Malison, WJ Birge and BS Shepherd. **14th International Conference on Environmental Bioindicators** at Baltimore, Maryland USA. April 24-26, 2006. Invited platform presentation: Developing molecular tools in yellow perch: Effects of 17β-estradiol on key endocrine genes.
- **SG Lynn***, JA Malison, BS Shepherd and WJ Birge. **Society of Environmental Toxicology and Chemistry (SETAC) 26th Annual Meeting** at Baltimore, Maryland USA. November 13-17, 2005. Platform presentation: Cloning, tissue-specific expression and responses to 17β-estradiol of key endocrine genes in yellow perch (*Perca flavescens*).
- **SG Lynn***. **American Society of Ichthyology and Herpetology (ASIH) 85th Annual Meeting** at Tampa, Florida USA. July 6-11, 2005. Platform presentation: Seasonal expression of key endocrine genes in Lake Erie yellow perch (*Perca flavescens*).
- **SG Lynn***, DJ Price, WJ Birge and SS Kilham. **Society of Environmental Toxicology and Chemistry (SETAC) Europe 14th Annual Meeting** at Prague, Czech. April 18-23, 2004. Poster presentation: Uptake of PCB Congener 2,2',6,6'-Tetrachlorobiphenyl in Nutrient Controlled Diatoms (*Stephanodiscus minutulus*) and Transfer to a Zooplankton (*Daphnia pulicaria*).
- **SG Lynn*** and BS Shepherd. **World Aquaculture Society (WAS) Triennial Meeting** at Honolulu, Hawaii USA. March 1-5, 2004. Invited platform presentation: Cloning and expression studies of pituitary endocrine genes in yellow perch (*Perca flavescens*).
- **SG Lynn***, GM Weber and BS Shepherd. **Kentucky Academy of Science (KAS) 88th Annual Meeting** at Highland Heights, Kentucky USA. November 7-9, 2002. Invited platform presentation: Complementary DNA cloning and expression studies of the pituitary hormones growth hormone, prolactin, and somatolactin in the yellow perch (*Perca flavescens*).
- **SG Lynn*** and BS Shepherd. **Ohio Valley Chapter (OVC) of the Society of Environmental Toxicology and Chemistry (SETAC) 19th Annual Meeting** at Louisville, Kentucky USA. May 16-17, 2002. Poster presentation: Herbicide body burdens and endocrine profiles of yellow perch in Old Woman Creek Estuarine Research Reserve and Lake Erie.
- **SG Lynn*** and C Lindle. **American Society of Ichthyology and Herpetology (ASIH) 81st Annual Meeting** at University Park, Pennsylvania USA. July 5-10, 2001. Poster presentation: Habitat use by *Rana angolensis* along the Dodwe River in the East Usambara Mountains, Tanzania.
- AE McDaniels* and **SG Lynn**. **American Society for Microbiology (ASM) 101st Annual Meeting** at Orlando, Florida USA. May 20-24, 2001. Poster presentation: Recovery of *Helicobacter pylori* from water by immunomagnetic capture.
- **SG Lynn***, JS Rehage, A Sih and BD Palmer. **Society of Environmental Toxicology and Chemistry (SETAC) 21st Annual Meeting** at Nashville, Tennesse USA.

November 12-16, 2000. Platform presentation: Effects of triphenyltin on growth and development in the salamander *Ambystoma barbouri*.

- **SG Lynn*** and JS Spromberg. **American Society of Ichthyology and Herpetology (ASIH) 79th Annual Meeting** at University Park, Pennsylvania USA. June 24-30, 1999. Poster presentation: The bioaccumulation of organic xenobiotics in frogs: A physiologically-based toxicokinetic (PBTK) model.
- **SG Lynn***, SS Kilham, DA Kreeger, and CE Goulden. **Pocono Comparative Lakes Program Annual Meeting** at Lake Ariel, Pennsylvania USA. October 18-19, 1996. Platform presentation: Effects of algal food quality on zooplankton fecundity and population growth rates.
- **SG Lynn***, SS Kilham, DA Kreeger, and CE Goulden. **Pocono Comparative Lakes Program Annual Meeting** at Lake Ariel, Pennsylvania USA. October 6-7, 1995. Platform presentation: Effects of nutrient limitation on biochemical constituents of *Ankistrodesmus falcatus*.

Memberships
Society for Environmental Toxicology and Chemistry: 1997-2006
Organization for Tropical Studies: 2000-2005
World Aquaculture Society: 2002-2004
American Society of Ichthyology and Herpetology: 1999, 2002, 2005

Volunteer/Community Service and Other Synergistic Activities
Volunteer Judge for the 3rd Annual Kentucky Science and Engineering Fair in Richmond, KY. April 2, 2005
Volunteer Judge for the 1st Annual Kentucky Science and Engineering Fair in Richmond, KY. March 27, 2004
Volunteer Judge for the Glendover Elementary School Science Fair in Lexington, KY. January 22, 2004
Volunteer Judge for the 53rd International Science and Engineering Fair in Louisville, KY. May 12-18, 2002
PADI open water SCUBA certification, October 1999

Scott George Lynn

October 13th, 2006