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Local expression of CYP19A1 and CYP19A2 in developing and adult killifish (*Fundulus heteroclitus*)

Wu Dong and Kristine L. Willett*

Pharmacology and Environmental Toxicology Research Program, University of Mississippi, School of Pharmacy, University, MS

Abstract

P450 aromatase (CYP19) is the terminal enzyme in the steroidogenic pathway and catalyzes the conversion of androgens to estrogens. *Fundulus heteroclitus* like other teleosts, express two CYP19 genes, CYP19A1 and CYP19A2. The expression of CYP19s in *Fundulus* was measured by *in situ* hybridization throughout development. In 90 dpf (day post-fertilization) fish and adult fish, CYP19A1 was expressed in the ooplasm of early stage I oocytes (primary growth stage). Expression of CYP19A1 was localized in the follicle cell layer of late stage I (previtellogenic stage) and stage II (vitellogenic stage) follicles, but by stage III (early maturational follicles) CYP19A1 expression was localized in the vitelline envelope. Overall, CYP19A1 oocyte membrane expression gradually declined from highest expression at late stage I to nondetectable levels by stage IV. Highest expression of CYP19A2 was detected in the brain including the hypothalamus from 4, 6, 8, 10, 14 dpf embryos, 90 dpf fry fish and adult fish brain. In females compared to males, there was higher CYP19A2 expression in olfactory bulb. In addition to the brain, there was strong CYP19A2 signal in adrenal/kidney cells in 6-14 dpf embryos. This work establishes the localization and constitutive expression of CYP19s in *Fundulus* which can then be compared with potential disruption of CYP19A1 and CYP19A2 expression and physiological consequences caused by environmental contaminants.

Keywords

Aromatase; *Fundulus*; CYP19; oocyte; brain

Introduction

Interference with steroid hormone biosynthesis is an important contributing mechanism that could impact proper growth, development, behavior, reproduction, and/or lead to endocrine associated cancers (Sanderson 2006). An important enzyme in regulating the balance of androgens and estrogens is the P450 enzyme aromatase or CYP19. Proper control of circulating and local estrogen concentrations are critical for diverse physiological functions including germ cell maturation in both sexes, bone, brain, and adipose physiology (reviewed by (Meyer 1999; Simpson et al., 2002)). The human CYP19 gene is a single gene for which tissue specific

*To whom correspondence should be addressed: Department of Pharmacology and Environmental Toxicology Research Program, School of Pharmacy, The University of Mississippi, 315 Faser Hall, Box 1848, University, MS 38677. PH: 662-915-6691. FX: 662-915-5148. E-mail: kwillett@olemiss.edu

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expression is controlled by alternate promoter splicing (Meinhardt and Mullis 2002). In contrast, in fish it is well established that there are two distinct CYP19 genes, a predominantly ovarian form (CYP19A1 or CYP19a) and a predominantly brain form (CYP19A2 or CYP19b). However, analyses at the gene sequence level suggest little functional divergence among vertebrate aromatases though transcription of these genes can be highly variable (Wilson et al., 2005). To expand our understanding of the potential physiological significance and developmental roles of CYP19A1 and CYP19A2 we have investigated the tissue specific expression of these genes in *Fundulus heteroclitus* (Atlantic killifish or mummichog) by *in situ* hybridization.

The primarily ovarian aromatase gene, CYP19A1, has been cloned from a number of fish species including those with diverse reproductive strategies including daily spawners like zebrafish and medaka, biweekly spawners (*Fundulus*), annual spawners (catfish, rainbow trout and goldfish) and fish capable of sex change (black porgy, wrasse, goby). Promoter analysis of fish CYP19A1s has identified cAMP-response elements, aryl hydrocarbon response elements, steroidogenic factor 1 (SF-1), TATA box, estrogen receptor recognition half sites, GATA-3, progesterone receptor response elements, Ad4BP binding motif and SRY/SOX motifs (Kazeto et al., 2001; Nocillado et al., 2007; Tanaka et al., 1995; Tchoudakova et al., 2001; Tong and Chung 2003). Using quantitative RT-PCR, unfertilized zebrafish eggs had similar levels of CYP19A1 expression as whole ovaries however these levels decreased during development until 48 hpf when CYP19A1 mRNA began to accumulate and peak at 72 hpf. In addition, the developmentally expressed CYP19A1 mRNA was not inducible by estradiol exposure (Sawyer et al., 2006). In adult ovaries of Atlantic croaker and rainbow trout, levels of CYP19A1 message were detected early in ovarian development and vitellogenesis with dramatically decreased expression later (Nakamura et al., 2005; Nunez and Applebaum 2006). The CYP19A1 mRNA expression correlated with plasma estradiol levels in both channel catfish and rainbow trout (Kumar et al., 2000; Nakamura et al., 2005). Relatively few studies have investigated CYP19A1 expression during embryonic or ovarian development by *in situ* hybridization or immunohistochemistry. Highest CYP19A1 mRNA expression was found in Stage III B zebrafish ovarian vitellogenic follicles (Goto-Kazeto et al., 2004; Rodriguez-Mari et al., 2005). Kobayashi and colleagues found CYP19A2 mRNA expression in goby thecal cells of previtellogenic follicles while the expression was more abundant in granulosa cells of vitellogenic follicles (Kobayashi et al., 2004). However, aromatase immunoreactivity was found in both cell types of female goby (Sunobe et al., 2005).

In contrast to CYP19A1, the neuronal CYP19A2 is inducible by estrogenic compounds and its cellular expression has been more thoroughly defined particularly in the plainfin midshipman, rainbow trout, zebrafish, goldfish, bluehead wrasse and pejerrey (Forlano et al., 2005; Forlano et al., 2001; Gelinias and Callard 1997; Goto-Kazeto et al., 2004; Marsh et al., 2006; Menuet et al., 2003; Menuet et al., 2005; Pellegrini et al., 2007; Strobl-Mazzulla et al., 2005). Aromatase expression is found in glial cells predominantly in the olfactory bulbs and hypothalamus, with expression also in the pituitary, telecephalon, and diencephalon. The unusually high capacity to synthesize estrogen in the fish brain, and the radial glial cells specifically, has been suggested as a mechanism involved in the continuous neurogenesis found in fish (Pellegrini et al., 2007). Other potential roles of neuronal aromatase include reproduction-related vocalizations in midshipman (Forlano et al., 2001) and sex determination in sea bass, medaka, pejerrey and wrasse (Blazquez and Pieferrer 2004; Marsh et al., 2006; Melo and Ramsdell 2001; Strobl-Mazzulla et al., 2005). Developmental expression of CYP19A2 has been largely investigated in zebrafish (Menuet et al., 2005; Sawyer et al., 2006; Trant et al., 2001) and more recently *Fundulus* (see below). In comparison to CYP19A1, in embryos CYP19A2 expression occurs sooner and reaches higher maximum levels and is estrogen inducible (Sawyer et al., 2006). Estrogen responsiveness has been associated with

estrogen response elements (EREs) and ERE half-sites in the fish CYP19A2 promoter region (Kuhl et al., 2005; Tchoudakova et al., 2001).

Both CYP19 genes have been previously cloned in *Fundulus* (Greytak et al., 2005; Patel et al., 2006). *Fundulus* have been used as an environmentally relevant toxicology model organism to study endocrine disruption (Boudreau et al., 2005; Dube and MacLachy 2001; Greytak and Callard 2007; Kelly and Di Giulio 2000), environmental carcinogenesis and polycyclic aromatic hydrocarbon (PAH) toxicity (Billiard et al., 2006; Vogelbein et al., 1990; Wang et al., 2006), and chemically mediated changes in gene expression (Meyer et al., 2005; Paschall et al., 2004; Powell et al., 2000). The embryonic developmental stages of *Fundulus* have been described (Armstrong and Child 1965). Furthermore, in both field and laboratory conditions, the follicular cycle of *Fundulus* is consistently reproduced at 2 week intervals year around leading to the proposal that *Fundulus* could be used as a general model organism for cyclic reproductive activity (Hsiao et al., 1996). The three phases of *Fundulus* ovarian development (recruitment, maturation and ovulation) have been well characterized with respect to timing, vitellogenesis, and steroid responsiveness (Cerde et al., 1996; Cerde et al., 1998; Petrino et al., 1990; Petrino et al., 1989a; Petrino et al., 1989b; Selman and Wallace 1983; Subhedar et al., 1997; Wallace and Selman 1985). Less work has characterized the roles of the CYP19s specifically in *Fundulus*. *In vitro*, *Fundulus* ovarian follicle cells were capable of 17 α -hydroxy-20 β -dihydroprogesterone, testosterone and estrogen production when *Fundulus* pituitary extract was added, however theca and surface epithelium preparations were incapable of estrogen production (Petrino et al., 1989a) suggesting that aromatase activity was present in follicle but not thecal cell layers. In laboratory control *Fundulus* brain CYP19A2 mRNA expression was not different between males and females although females had higher enzyme activity (Patel et al., 2006). In whole homogenized brain, CYP19A2 expression was about 100-fold higher than CYP19A1 which was consistent with other fish species. Adult females also had ~700-times more CYP19A1 in their gonads compared to males (Patel et al., 2006). Furthermore, as measured by quantitative PCR, whole embryos also had about 5-fold higher CYP19A2 compared to CYP19A1. Greytak and coworkers have studied CYP19 expression in wild fish collected from polluted and non-polluted sites and found CYP19A2 expression 2-fold higher in polluted fish compared to non-polluted fish, but no site differences were found in CYP19A1 expression. Both CYP19A1 and CYP19A2 were lower in reproductively inactive *Fundulus* compared to reproductively active fish collected in June and July (Greytak et al., 2005). To add insight into when during development and in what tissue types the CYP19s are expressed, we have used *in situ* hybridization to measure CYP19 expression throughout *Fundulus* development.

Materials and methods

Fundulus eggs

A parental population of *F. heteroclitus* collected from an uncontaminated site at the New River inlet near Beaufort, North Carolina was raised under the University Institutional Animal Care and Use Committee (IACUC) approved conditions. Sexually mature fish were bred and kept in salt water (20-25 ppt). The fish were maintained at a 14:10 light-dark cycle in summer and 10:14 light-dark cycle in winter. Adult fish were fed twice daily with tropical flake fish food (Tetramin, Tetra Werke, Germany) and live brine shrimp. First generation offspring, from wild parents, were used for the studies described here.

Whole mount *in situ* hybridization

1-Phenyl-2-thiourea (0.003%) was included the embryos' water from 5 dpf to prevent pigmentation of *Fundulus* larva. *Fundulus* embryos were fixed in 4% (w/v) paraformaldehyde in phosphate saline solution (pH 7.4) overnight and stored in -20°C freezer after removal of

the membrane by watchmaker's forceps. Whole mount *in situ* hybridization was carried out as described elsewhere (Dong et al., 2002). Probes were generated by PCR amplification from *Fundulus* CYP19A1 or CYP 19A2 plasmids (originally cloned by Patel et al., 2006) AY713118 or AY494837). The CYP19A1 corresponded to nucleotides 1-1660, while the CYP19A2 was 717 bp and corresponded to nucleotides 183-1000. Probes were purified and subcloned in pGEM-TEasy Vector (Promega, Madison, USA). Antisense and sense digoxigenin probes were synthesized by *in vitro* transcription using T7 and SP6 polymerase on template DNA linearized with *Apa* I or *Spe* I, respectively. Following hybridization overnight at 65°C, embryos were washed with 2x SSC and 0.2x SSC twice for 30 min, respectively. After blocking with 2% blocking reagent (Roche), embryos were incubated overnight with 4000x diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) at 4°C. The color reaction was carried out by incubation with BM-purple substrate (Roche).

Paraffin sectioning and *in situ* hybridization

Fundulus tissues were fixed in 4% (w/v) paraformaldehyde in phosphate saline solution (pH 7.4) overnight, followed by dehydration in increasing gradients of ethanol (70%, 80%, 90%, 95%, 100% I and 100% II). After cleared in Clearify™ (American Master Tech Scientific, Inc), tissues were embedded in molten paraffin (Paraplast embedding media paraplast X-tra, Sigma). Sections of 7 µm thickness were cut using a microtome (OLYMPUS CUT 4055, Olympus American Inc). The sections were cleared in gradient of ethanol (100%-70%) and hybridized with the antisense probe of *Fundulus* CYP19A1 as described above. Following hybridization overnight at 50 °C, sections were washed with 2x SSC and 0.2x SSC twice for 20 min, respectively. After blocking with 2% blocking reagent (Roche), embryos were incubated overnight with 4000x diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) at 4°C. The color reaction was carried out by incubation with BM-purple substrate (Roche).

Expression quantification and statistics

For measuring the intensity of CYP19A1 or CYP19A2 expression, pictures were obtained with digital camera (OPTRONICS) with a light microscope (BX40, Olympus). To quantitate, regions of interest (ROIs) were traced and mean pixel intensity was analyzed by means of KODAK 1D image Analysis Software. Results are presented as mean ± SE. Significant differences between means were determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test ($p < 0.05$). For analysis of adult brain CYP19A2 expression, a two-way ANOVA was done to measure potential interaction between brain region and sex.

Results

Expression of CYP19A1 in developing *Fundulus*

To investigate the distribution of CYP19A1 gene, 2, 3, 4, 6, 8, 10, 14, 30 and 90 dpf *Fundulus* were fixed, and *in situ* hybridization using antisense 1660 base pair probe of CYP19A1 was carried out. No signal was detected at 2, 3, 4, 6, 8, 10, and 14 dpf in *Fundulus* (data not shown). As shown in Fig. 1. A, B, C, expression of CYP19A1 was first detected (blue precipitate) in the ooplasm of early stage I oocytes (primary growth stage, n=5) as strong and evenly distributed signals in the ovary of 30 and 90 dpf old *Fundulus*. No CYP19A1 signal was detected in brain or other tissues (data not shown) or when the sense probe was used (Fig. 1D). Hematoxylin-eosin-stain (H&E stain) was used to show histological features and to compare with CYP19A1 expression by *in situ* hybridization (Fig. 1. E and F).

Expression of CYP19A1 in the ovary of adult *Fundulus*

For analysis of CYP19A1 expression in the oocytes, developing oocytes were divided into stage I (previtellogenic up to 0.6 mm diameter), stage II (vitellogenesis 0.6 - 1.3 mm), stage

III (maturation 1.3 - 1.9 mm with slight protein accumulation) and stage IV (maturation without protein accumulation) as defined by (Wallace and Selman 1985). We have further divided the stage I follicles into three size based categories: early stage I (stage Ia, 0 - 0.15 mm), mid stage I (stage Ib, 0.15 - 0.25 mm), and late stage I (stage Ic, 0.25 - 0.6 mm).

Similar to ovary of 90 dpf *Fundulus*, expression of CYP19A1 mRNA was detected in the ooplasm of stage Ia oocytes as strong and evenly distributed signals in the ovary of adult *Fundulus* (Fig. 2 E, M). In the stage Ib oocytes, the signal gradually declined at both the ooplasm and membrane of oocytes and became barely detectable (Fig. 2 F, G, N and O). From stage Ic, the CYP19A1 expression was found only in the outer region of oocytes (Fig. 2.H and P). From stage Ia to stage Ib, mean intensity of CYP19A1 expression in the ooplasm was measured and expression significantly decreased between the stage Ia and stage Ib (Fig. 3).

Figure 4 provides further details of the follicular expression of CYP19A1 in adults. In stage Ic oocytes, the CYP19A1 expression was isolated to the follicular cell layer and expression was wavy possibly associated with outer cell membranes of individual follicle cells (for electron micrograph see (Selman and Wallace 1983)). From stage II to III expression appeared to transfer to the acellular vitelline envelope, and by stage IV there was no apparent CYP19A1 expression.

Compared to ovary, weak CYP19A1 expression was found in the adult testis (interstitial cells, data not shown), but positive CYP19A1 expression was not detected in brain of adult *Fundulus* by either whole mount or section *in situ* hybridization (data not shown).

Expression of CYP19A2 in developing *Fundulus*

Temporal changes in CYP19A2 expression during development were measured by *in situ* hybridization after fertilization at 2, 3, 4, 6, 8, 10, and 14 dpf. CYP19A2 expression was not detected in 2 dpf embryos (data not shown) but was first observed at 3 dpf in the brain including the midbrain. In the 4 dpf embryos, the CYP19A2 signal was found in the hypothalamus and ventral telencephalon (Fig 5). Similar to 4 dpf, CYP19A2 expression also was detected at 6, 8, 10 and 14 dpf. In addition to brain expression, CYP19A2 showed very intense staining in the adrenal tissue of the head kidney at 6, 8, 10 and 14 dpf (Fig 6).

In the 90 dpf *Fundulus* brain, CYP19A2 expression by whole mount *in situ* hybridization was found in the hypothalamus and ventral telencephalon, but was not found in the pituitary except where it combines with the hypothalamus (HY) or the olfactory bulb. Magnified images of the pituitary are shown in the insert photos (Fig. 7, A, B and C).

Expression of CYP19A2 in the brain of adult *Fundulus*

To investigate the distribution of CYP19A2 expression in adult *Fundulus*, brains were obtained from male and female fish at similar season (February). Highest CYP19A2 mRNA expression was detected in the pituitary and hypothalamus. The ventral telencephalon and olfactory bulb had moderate and lower levels expression, respectively by whole mount *in situ* hybridization (Fig. 8, A and B). The distribution of CYP19A2 expression was confirmed by sectioning after whole-mount *in situ* hybridization (Fig. 8 C, D, E and F). The intensity of CYP19A2 within a given brain region in males and females was not significantly different except for in the olfactory bulb where female expression was higher than that in males ($p < 0.05$, Fig. 9). In addition, very weak expression of CYP19A2 also was found in the ovary in the stage I to stage III follicles (data not shown).

Discussion

In this study we have used *in situ* hybridization to define the oocyte expression of CYP19A1 and the brain regions in which CYP19A2 is expressed during *Fundulus* development. Previous studies by our lab and others have measured general ovarian and brain CYP19 mRNA and aromatase activity in whole homogenized tissues (Greytak et al., 2005; Patel et al., 2006) however the cellular localization (without amplification) has not been previously reported in *Fundulus*. For example, while both CYP19A1 and CYP19A2 mRNA expression was detectable in *Fundulus* and zebrafish embryos by sensitive qRT-RT PCR methods (Kishida and Callard 2002; Patel et al., 2006), expression of CYP19A1 and CYP19A2 expression was not detected until 30 and 3 dpf, respectively, by *in situ* hybridization. Disruptions of CYP19A2 expression, therefore, may be more significant early in *Fundulus* development while variant CYP19A1 expression may impact reproductive potential of adults.

As described in the introduction *Fundulus* reproduction and oocyte maturation has been extensively studied particularly by the Selman and Wallace laboratories. *Fundulus* are asynchronous spawners with semilunar spawning. Ovaries from reproductively immature female fish (30 and 90 dpf in this study) contained many small previtellogenic oocytes that stained strongly for CYP19A1 mRNA. Similarly in adult ovaries the small previtellogenic follicles stained strongly. However, in later stage I and stage II (vitellogenic) follicles, CYP19A1 expression was isolated to the follicular cell layer and was wispy in appearance. By stage III (early maturation) there was a more solid CYP19A1 expression in the vitelline envelop which was not seen in mature stage IV follicles (Fig 4). These results are consistent with work done by Petrino and coworkers where *Fundulus* follicle cell preparations but not denuded oocytes or theca/surface epithelium preparations were capable of estrogen production when stimulated by pituitary extracts (Petrino et al., 1989a). CYP19A1 expression has also been recently described in the follicular layer of zebrafish vitellogenic oocytes (Goto-Kazeto et al., 2004). These results suggest that aromatase and local estrogen synthesis may be physiologically important in both early oocyte growth and during vitellogenesis.

We found very weak CYP19A1 staining in the interstitial cells of the adult testis. Our previous PCR results suggested that there was approximately 700 times less CYP19A1 expression in male gonads compared to females, and there was actually more CYP19A2 in testis than CYP19A1 (Patel et al., 2006). CYP19A1 expression was also reported in goby testis but the regulatory mechanisms or the physiological significance of aromatase in the male gonad is currently unknown (Kobayashi et al., 2004).

To our knowledge, a unique finding of this study was the CYP19A2 expression detected in what we believe are adrenal like cells in the head kidney in *Fundulus* embryos from 6 - 14 dpf (Fig. 6). In sections of 90 dpf and adults, we did not see similar expression, however, it could have been weak and missed in the whole sections of the fish torso. Male *Fundulus* adrenocortical tissue possesses enzyme activities associated with steroid synthesis, specifically 3 β -, 3 α -, 11 β -, and 17 β -hydroxysteroid dehydrogenase activities (Bara 1968). Similarly, male African catfish adrenal (interrenal) tissue was capable of synthesis of at least 15 different steroids including predominantly cortisol and androstenediones (Vermeulen et al., 1995). Head kidneys from adult European sea bass have very low but detectable aromatase activity (González and Piferrer 2003). CYP19A2 RNA expression has been detected by PCR in adult fish kidneys of several species (see review by Piferrer and Blazquez, 2005) including pejerrey (Strobl-Mazzulla et al., 2005), Nile tilapia (Kwon et al, 2001) and by Northern blot in orange spotted grouper (Zhang et al, 2004). In their study of tissue specific expression of CYP19s in *Fundulus* by PCR, Greytak and coworkers did not test kidney for CYP19 RNA expression (Greytak et al., 2005). However, enzymatically active aromatase has been reported in newborn pig adrenal glands whereas it is relatively absent in mature pigs (Conley et al., 1996). These

data suggest that adrenal estrogen synthesis may be important in *Fundulus* development and further study is warranted.

The earliest expression of CYP19A2 was detected at 3 dpf in the midbrain, and by 4 dpf expression was clearly localized in the hypothalamus and telencephalon. Compared to adult CYP19A2 expression, relatively less is known about the significance of early developmental expression of brain aromatase. In control zebrafish CYP19A2 was not detected by *in situ* hybridization at 24, 36, and 48 hpf (Menuet et al., 2005), however 10^{-8} M estradiol induced CYP19A2 by 36 hpf in the preoptic area and hypothalamus and by 48 hpf in the telencephalon also. For comparison, a 29 hpf zebrafish is developmentally similar to a *Fundulus* at 3 dpf with respect to mid and hind brain development. Therefore, *Fundulus* may have earlier and higher CYP19A2 expression compared to zebrafish, or there were experimental differences that increased the sensitivity of the *Fundulus in situ* hybridization.

By 90 dpf of *Fundulus* development, the CYP19A2 expression was still predominantly in hypothalamus and telencephalon, but in adults expression was also found in the pituitary and olfactory bulb. The regions of expression were similar to those reported by *in situ* hybridization and immunohistochemistry in adult zebrafish (Goto-Kazeto et al., 2004; Menuet et al., 2005) with the exception that there were no sex differences in CYP19A2 RNA expression in zebrafish. In our study, we found that adult female *Fundulus* had significantly higher CYP19A2 expression in the olfactory bulb region. In contrast to *Fundulus*, adult female sea bass had significantly lower brain aromatase activities in the telencephalon, hypothalamus, pituitary and optic bulb, but not in the olfactory bulb (González and Piferrer 2003). Sexual dimorphism in brain aromatase activity has also been reported in sections along the longitudinal axis of the Japanese medaka brain. Males had higher aromatase activity in sections containing the preoptic hypothalamic nuclei and the suprachiasmatic nucleus whereas highest activities in females were more caudal containing the periventricular dorsalis, ventralis, caudalis, tuberi anteriores, and posteriores nuclei (Melo and Ramsdell 2001).

The exact physiological significance of brain CYP19A2 expression has not been determined, but recent work has suggested its role in estrogen dependent neurogenesis (which continuous through adulthood in fish) (Forlano et al., 2001; Pellegrini et al., 2005, 2007), sex change and behavior (Marsh et al., 2006), and regulation of the gonadal-pituitary-hypothalamic feedback loop (Melo and Ramsdell 2001; Villeneuve et al., 2007). Additional studies with estrogenic inducers and aromatase inhibitors together with measurement of CYP19A2 expression and phenotypic effects (for example (Greytak et al., 2005; Kishida et al., 2002)) will further refine the physiological significance of aromatase in fish.

In summary, both CYP19A1 and CYP19A2 expression were cell type and developmental stage dependent. CYP19A1 was diffusely expressed in early follicles and in larger follicles expression was localized to the follicular cell layer then the vitelline envelop. CYP19A2 expression was detected in adrenal/kidney cells during early *Fundulus* development and in brain by 3 dpf. Regional expression of CYP19A2 in the brain differed by age and expression was higher in female adult olfactory bulb compared to males. This work establishes the constitutive expression of CYP19s which can then be compared with potential disruption of CYP19A1 and CYP19A2 expression by environmental contaminants such as benzo(a)pyrene and ethinylestradiol.

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Figure 1.

Expression of CYP19A1 in 3 month old (90 dpf) oocytes visualized by *in situ* hybridization using antisense (A-C) and sense (D) CYP19A1 probes and H&E staining (E and F). Expression of CYP19A1 mRNA was detected in the ooplasm of stage I oocytes (primary growth stage) as strong and evenly distributed signals in 3 month old fish. B is magnification of A; C and F are magnification of B and E, respectively. n=5.

Figure 2.

Expression of CYP19A1 in adult oocytes. Adult ovaries were sectioned and visualized by *in situ* hybridization using CYP19A1 antisense probe (E, F, G, H, M, N, O and P) or H&E staining (A, B, C, D, I, J, K and L). Expression of CYP19A1 mRNA was detected in the ooplasm of stage Ia oocytes (primary growth stage, E and M) as strong and evenly distributed signals in adult fish. Signal gradually decreased and became barely detectable by stage Ib oocytes (previtellogenic follicle stage, F, G, N, and O). Oocytes were characterized by the presence of yolk granules in ooplasm. In the stage Ic, expression was detected in the follicular cell layer (H and P). I, J, K and L are higher magnification of A, B, C and D; M, N, O and P are higher magnification of E, F, G and H.

Figure 3. Intensity of CYP19A1 expression at early stage I (stage Ia) to mid stage I (Stage Ib) in the oocytes. Expressed regions were analyzed by means of KODAK 1D image Analysis Software (Creating regions of interest, ROIs). Bars with different letters are statistically different ($p < 0.05$). Each bar represents the mean $\pm$ S.E. from 3-5 oocytes per developmental stage (stage Ia and Ib) from 5 fish.

Figure 4.

Expression of CYP19A1 in the membrane of adult oocytes. Expression of CYP19A1 mRNA was detected in the membrane in the stage Ib (previtellogenic follicle stage, A); stage Ic (B); stage II (vitellogenic, C and D); stage III (early maturational follicles, E) oocytes. No signal was detected in stage IV (maturational follicles, F) oocytes. G and H are H&E stain of C and F, respectively. Arrow indicates expression in follicular cell layer and/or vitelline envelop in A-E. Bar=100 µm.

Figure 5. CYP19A2 expression in the brain at 3, 4 and 10 dpf. Embryos were fixed by 4% PFA, and expression of CYP19A2 mRNA in the brain was assessed by whole-mount *in situ* hybridization. Representative embryos are shown Laterally (A, B, and C) and Ventrally (D, E and F). Arrow indicates CYP19A2 expression in the hypothalamus (HY) and ventral telencephalon (VT), (n=10-12 embryos/age).

Figure 6.

Expression of CYP 19A2 in the adrenal /kidney tissue by whole-mount *in situ* hybridization. The representative embryos (14 dpf) are shown in Lateral (A) Ventral (B) views and C and D are cross sections of B. D is a higher magnification of C. Arrow indicates expression in the adrenal cells in A, B, C and D (n=10-12 embryos).

Figure 7.

Expression of CYP19A2 in 90 dpf *Fundulus* brain by whole-mount *in situ* hybridization. The large image is a ventral view of the brain. Inset graphs show the Ventral (A and B) and Lateral (C) views of the pituitary. A is a magnification of the rectangular area, B and C are magnification of pituitary after dissection from the brain (n= 8 embryos).

Figure 8.

Expression of CYP19A2 in adult *Fundulus* brain. The CYP19A2 transcripts were found at the hypothalamus (HY), pituitary (HP), ventral telencephalon (VT), and extending to the olfactory bulb (OB). Ventral views. A, Antisense probe; B, Sense probe. C and D are sections of A at the black line. E and F are higher magnification of C and D, respectively. C and E are H&E stain. Arrows indicate CYP19A2 expression in the pituitary.

Figure 9. Intensity of CYP19A2 expression within a given part in the adult brain of each sex. HY: hypothalamus; VT: ventral telencephalon; OB: olfactory bulb; HP: pituitary. Asterisk indicates a significant difference between the male and female ($p < 0.05$). Each bar represents the mean $\pm$ S.E. of 5 fish per sex.