

Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: Gene specific tissue distribution, sex differences, developmental programming, and estrogen regulation

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Abstract

In teleost fish, the predominant brain form of cytochrome P450 aromatase (P450aromB) is a neural marker of estrogen effect, and an entry point for studying the role of hormonal and environmental estrogens on neurodevelopment and neuroplasticity. As part of a project using zebrafish to investigate these issues, we developed and validated a rapid, sensitive, and reproducible real-time polymerase chain reaction (PCR) assay for quantifying and comparing P450aromB and P450aromA expression in unfertilized eggs, embryos/larvae, and dissected tissues of adult fish. Results confirm that P450aromB and -A predominate in brain and ovary, respectively, and further show that the degree of overlapping expression (ratio, B:A) is 100:1 in brain, 1:50 in ovary, 1:1 in eye, and 2:1 in testis. Sex differences were observed in eye only (female > male). When compared to whole ovaries, unfertilized eggs had similar levels of P450aromA but enrichment of P450aromB, which suggests preferential synthesis or accumulation in mature oocytes. Both of the maternally derived aromatase isoforms were rapidly degraded post-fertilization, but the onset of embryonic P450aromB expression (5 hpf) was much earlier than P450aromA (48 hpf), and reached higher maximum levels (e.g., 10-fold at 72 hpf). Consistent with earlier reports, P450aromB but not -A was estrogen-inducible, but the estrogen response system in embryos was far more robust than in adults (>100- vs. <4-fold maximal induction, respectively). Application of this real-time PCR assay to measurement of P450aromB and -A in zebrafish embryos has utility for routine screening of chemicals and environmental samples for estrogen-like bioactivity and neural effects.

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1. Introduction

Aromatization of androgen to estrogen, the key step in estrogen biosynthesis, is catalyzed by cytochrome P450 aromatase (P450arom), a product of the CYP19 gene (for review; Simpson and Davis, 2001; Simpson et al., 1994). Although estrogen is generally regarded as a circulating hormone derived from the gonads or placenta, P450arom is expressed in the brain and many other estrogen target tissues, where it is positioned to regulate the location, timing,

and quantity of estrogen available for activating genomic and non-genomic signaling pathways (for review; McEwen and Alves, 1999; McEwen et al., 1982; Naftolin et al., 1975). In mammals, it is well-established that aromatization in situ during neurodevelopment mediates brain sex differentiation and, in the peripubertal and adult brain, modulates neuroendocrine functions and sex behavior. Historically, researchers focused on estrogen biosynthesis and actions in reproductive control centers of the brain. Recent human and animal studies, however, indicate that estrogen is a general neurotrophic and neuroprotective factor in many brain regions and at different life stages (for review; Merchenthaler et al., 2003; Wise et al., 2005), but it is unclear whether brain-formed (neuro-) estrogen

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contributes significantly to support these processes (for review; Garcia-Segura et al., 2001; McCullough et al., 2003). Moreover, factors and mechanisms involved in the physiological regulation of neural P450arom expression are largely unknown (for review; Simpson and Davis, 2001). The zebrafish (*Danio rerio*) is an excellent experimental model to address these questions.

Adult fish have extraordinarily high levels of brain aromatase activity when compared to the gonads of the same species or to the brain of mammals (Pasmanik and Callard, 1985). Coincidentally, the teleostean brain continues to grow throughout life and retains a remarkable potential for neuroregeneration (Raymond and Easter, 1983; Stuermer, 1988; Yoon, 1975). Whereas CYP19 is found as a single copy in the haploid human genome, with multiple tissue-specific promoters and splicing mechanisms (Simpson et al., 1994), fish have two structurally and functionally distinct P450arom isoforms, which are encoded by separate gene loci (*cyp19a* and *-b*, or alternately *cyp19a1* and *-a2*) and are predominantly expressed in ovary and brain, respectively (Tchoudakova and Callard, 1998; Zhao et al., 2001). Conveniently, *cyp19* gene duplicates with subdivided expression domains allow recognition and experimental manipulation of predominant brain (-B) and ovarian (-A) expression, even in whole embryos (Kishida and Callard, 2001).

Superimposed on high constitutive P450aromB expression in teleost brain is a robust estrogen-inducible component, which can be demonstrated by administration of estrogens, xenoestrogens or aromatizable androgen to adult fish in vivo or to embryos in vitro (Gelinis et al., 1998; Kishida and Callard, 2001; Kishida et al., 2001; Pasmanik et al., 1988). In embryos the onset of P450aromB expression was previously reported as early as 12 h post-fertilization (hpf), and the onset of estrogen responsiveness at 24 hpf, when measured as induction of P450aromB (Kishida and Callard, 2001). Treatment effects are blocked by an estrogen receptor (ER) antagonist and consistent with the presence of multiple estrogen response elements (ERE) and half-sites in the regulatory region of the *cyp19b* gene, implying it is a direct target of estrogen/ER action (Kazeto et al., 2001, 2004; Tchoudakova et al., 2001). By contrast, the regulatory region of the *cyp19a* gene is lacking an ERE, nor is it estrogen-inducible. Whereas estrogen induction of brain aromatase expression in adult fish is thought to be a part of an autoregulatory feedback loop that drives seasonal reproductive recrudescence (Pasmanik and Callard, 1988), its functional significance in embryos is entirely unknown.

We and others are currently measuring P450aromB in several different fish species as a neural marker of estrogen effect in the natural environment, and as an entry point for understanding effects of endocrine disrupting chemicals (EDC) on neural and neuroendocrine processes (Blazquez and Piferrer, 2004; Gonzalez and Piferrer, 2003; Greytak et al., 2005; Halm et al., 2002; Kazeto et al., 2004; Kishida and Callard, 2001; Kishida et al., 2001). Additionally, as

part of a project to utilize zebrafish embryos for routine chemical screening (Burnam et al., 2005; Novillo and Callard, 2005; Sawyer et al., 2005b), we sought to develop and validate a real-time quantitative RT-PCR assay for P450aromA and -B mRNAs. Methods used in previous investigations (Northern analysis; semi-quantitative RT-PCR with/without Southern transfer/hybridization analysis) are unsuited to high throughput analysis of multiple samples, owing to the large amount of RNA required; a lack of sensitivity, specificity or precision; the time and labor necessary to obtain results; or a lack of standardization to allow comparison between genes, sample sets, experiments, and operators (for review; Bustin, 2002; Freeman et al., 1999; Ginzinger, 2002). Although Trant and co-workers (Goto-Kazeto et al., 2004; Trant et al., 2001) described a real-time PCR method using TaqMan probes for the two zebrafish P450arom mRNA's, their assay was not sufficiently sensitive to detect the early onset of expression we observed in previous studies using semi-quantitative RT-PCR followed by southern transfer (Kishida and Callard, 2001). Perhaps this is due to Trant and co-workers use of single embryos for gene expression analysis. Using procedures described here we have confirmed our previous studies of aromatase expression in embryos, and extend these findings to include tissue and sex specific differences in aromatase expression in adult fish with or without estrogen exposure.

2. Materials and methods

2.1. Zebrafish and treatments

Adult male and female zebrafish, *Danio rerio*, (wild-type, outbred) were obtained from a commercial supplier (Ekkwill, Gibsonton, FL). Groups of ~140 fish of mixed sex were maintained at 28 °C and a 14:10 light–dark (LD) cycle in 30gal (~114L) aquaria with recirculating deionized water conditioned with Aqualab I Conditioner (Mardel Laboratories, Glendale Heights, IL; pH 7.0) (Kishida and Callard, 2001). For adult treatment experiments, reproductively active fish (~9 month of age; 6 of each sex per experimental group) were transferred to closed 3gal (~11L) aquaria with aeration and maintained as described above. After 3 day acclimation, 17- β -estradiol (E2; final concentration, 1 nM; Sigma, St. Louis, MO) or vehicle alone (dimethylsulfoxide, DMSO; final concentration, 0.00005%) was added directly to aquarium water. This dose of E2 is within the range of levels found in sewage treatment effluents in the UK, US, and Germany (~0.3 nM) and in Israeli farm regions (~0.2–1.5 nM) (for review; Halm et al., 2002). One-half vol aquarium water with additive was replaced daily after feeding. After 2 or 5 days, fish were anesthetized (0.06% MS-222; Sigma) and decapitated. Brains, eyes, and gonads were collected, pooled by sex and treatment (3 fish per pool), quick-frozen on dry ice, and stored at –70 °C for RNA extraction.

Unfertilized eggs were collected by stripping gravid females. Fertilized eggs were collected after natural spawning, washed, and distributed into 20 × 100 mm culture plates (BD Biosciences, Bedford, MA). Embryos [120 embryos/50 ml egg water (Westerfield, 2000)] were allowed to develop at 28 °C on a 14L:10D cycle as previously described (Kishida and Callard, 2001). Where specified, E2 (final concentration, 0.1 or 1.0 μ M,) or vehicle alone (DMSO; final concentration, 0.00006%) was added directly to egg water and replaced daily. Embryos were collected after timed intervals (24, 48, 72, and 120 hpf), quick-frozen on dry ice, and stored at –70 °C until analysis.

2.2. RNA extraction and reverse transcription

Pooled frozen embryos or adult tissues were homogenized in Tri Reagent (Sigma) to extract total RNA as previously described (Tchoudakova et al., 2001). Following treatment with DNase I (Roche, Indianapolis, IN), an aliquot of each extract was used for gel electrophoresis and spectrophotometry to determine RNA quality and concentration. cDNA was synthesized from total RNA (5 µg; 20 µl final reaction volume) with oligo(dT) priming using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.3. Oligonucleotides

Gene-specific oligonucleotide primers were developed using Primer Express software (Applied Biosystems, Foster City, CA) and synthesized by Invitrogen. Nucleotide (nt) positions corresponded to GenBank entries #AF226619 (P450aromB) and #AF226620 (P450arom A). Forward (F) and reverse (R) primers for P450aromB were F 5'-AAAGAGTTACT AATAAAGATCCACCGGTAT-3' (nt 704–733), R—5'-TCCACAAG CTTTCCCATTTC-3' (nt 832–852). Forward and reverse primers for P450aromA were: F—5'-TCTGCTTCAGAAGATTCATAAATACTTT-3' (nt 703–730), R—5'-CCTGCAACTCCTGAGCATCTC-3' (nt 804–824). β -Actin primers were obtained from the Real-Time PCR Primer Databank (<http://medgen.ugent.be/rtprimerdb/>) and were as follows: F—5'-CGCG CAGGAGATGGGAACC-3'; R—5'-CAACGGAAACG CTCATTGC-3' (Keegan et al., 2002). All original primer designs have been submitted to the Real-Time PCR Primer Databank (ID numbers 3442 and 3443). Primer sets were tested for specificity by standard PCR under conditions previously described (Kishida and Callard, 2001) and by real-time PCR using as templates: (a) plasmids with authentic zebrafish P450aromA and -B cDNA inserts (Kishida and Callard, 2001); and (b) cDNA generated from zebrafish tissues.

2.4. Real-time PCR

Real-time PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems) with SYBR green fluorescent label. Samples (10 µl final vol) contained the following: 1× SYBR green master mix (Applied Biosystems), 3–5 pmol of each primer, and 0.25 µl of the RT reaction. Samples were run in triplicate in optically clear 384-well plates (Corning, NY). Cycling parameters were as follows: 50 °C × 2 min, 95 °C × 10 min, then 40 cycles of the following 95 °C × 15 s, 60 °C × 1 min. A melting temperature-determining dissociation step was performed at 95 °C × 15 s, 60 °C × 15 s, and 95 °C × 15 s at the end of the amplification phase.

2.5. Synthesis of P450aromB cRNA by in vitro transcription

For use in generating an external standard curve, single stranded P450aromB cRNA was produced by IVT from previously cloned P450aromB cDNA (Kishida and Callard, 2001). DNA template for the in vitro transcription (IVT) reactions was generated by PCR with T7 polymerase promoter conjugated P450aromB specific primers: F—5'-TAAT ACGA CTCACTATAGGGTGTGCCAAAGTCTGCAATG-3' (underlined nt 426–444) and R—5'-TCCACAAGCTTTCCCATTTC-3' (nt 832–852) (GenBank # AF226619). The PCR (50 µl final vol) contained 100 ng of plasmid DNA, 10 µM of each primer, *Taq* polymerase (Promega, Madison, WI), buffer, and dNTPs, as described by the manufacturer. Amplification conditions using a PE 9700 thermocycler (Perkin-Elmer, Foster City, CA) were 95 °C for 5 min followed by 35 cycles of 95 °C × 30 s, 58 °C × 30 s, and 72 °C × 1 min, followed by extension at 72 °C × 7 min. The 426 bp PCR product was gel purified using the Ultrafree-DA spin column (Millipore, Billerica, MA) and quantified spectrophotometrically. IVT was performed with 2 µg of cDNA template and 40 U of T7 polymerase (Fisher Scientific, Pittsburgh, PA) according to manufacturer's instructions. Resulting cRNA was purified using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA) and an aliquot was used for gel electrophoresis and spectrophotometry

to determine quality and concentration. A cRNA dilution series of known concentrations was reverse transcribed and analyzed by real-time PCR to generate a standard curve for use in determining the working range of the P450aromB assay (ng cRNA equivalents), and to standardize results in the experiment specified.

2.6. Data analysis

Data generated by real-time PCR were compiled and collected using SDS 2.2 software (Applied Biosystems). Routinely, analyses of data were conducted with *QGene*, a publicly available Excel script package (Muller et al., 2002). Cycle threshold (C_t) values were adjusted for differences in the amplification efficiency (E) of each primer set/gene target where $E = 10^{(-1/\text{slope})}$ as determined by linear regression analysis of a dilution series of reactions (expressed as percent input cDNA), and normalized to β -actin as an internal reference ($NE = (E_{\text{ref}})^{C_{t\text{ref}}} / (E_{\text{target}})^{C_{t\text{target}}}$). Acceptability for each set of triplicate reactions was set at <15% standard error of mean (SEM). Data from biological replicates were averaged and shown as normalized gene expression \pm SEM. Statistical analysis was performed using the Sigma-Stat 2.0 package (Aspire Software, Leesburg, VA). Data were first tested for normality, log transformed to normalize when necessary, and subsequently analyzed by one- or two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls (SNK) method for pairwise multiple comparisons. Data that could not be normalized by transformation were analyzed non-parametrically by Kruskal–Wallis ANOVA on Ranks followed by the SNK method for pairwise comparisons. Student's t test was used to compare expressed levels of P450aromA and -B mRNA where specified. Significance was set at $P < 0.05$.

3. Results

3.1. Development and validation of real-time PCR assays for P450aromB and -A analysis

To quantify P450aromB and P450aromA expression in whole zebrafish embryos and dissected tissues from adults, a real-time PCR assay was implemented in our laboratory. Gene-specific primer sets were designed to target nucleotides in adjacent exons to avoid genomic DNA artifacts. The specificity of each primer set was indicated by the presence of a single amplicon of the predicted size (148 and 121 bp for P450aromB and A, respectively) on ethidium bromide (EtBr) stained gels when plasmids with authentic P450aromB or -A cDNA inserts or cDNA from aromatase-expressing zebrafish tissues were used as template. No products were obtained when primer pairs and P450arom-containing plasmids were mismatched. Primer specificity and the absence of primer-dimer formation during real-time PCR analysis was indicated in each sample by the presence of a single peak in the dissociation (melt) curve at the end of the amplification program. Amplification efficiency, linearity and working range were determined by linear regression analysis of serial dilutions of cDNA [between 5 and 100% of standard cDNA input (0.25 µl/rxn)] from 96 hpf embryos. Analysis of results using *QGene* showed reaction efficiencies (E) of 1.9 for P450aromB, 2.0 for P450aromA, and 2.0 for β -actin, and correlation coefficients of 0.99, 0.97 and 0.98 for P450aromB, -A, and β -actin mRNA, respectively. An external RT-PCR standard curve generated from known amounts of P450aromB in vitro transcribed cRNA was

linear between 10^{-1} and 10^{-7} ng input cRNA, and had a correlation coefficient of 0.97 (data not shown).

To determine biological and methodological reproducibility of real-time RT-PCR across a range of expected values, P450aromB was analyzed in 3 or 4 independent embryo experiments by two different operators beginning at multiple assay starting points: RNA extraction, reverse transcription, and PCR amplification. As shown in Table 1, a compilation of data demonstrated an average difference of less than one C_t between operators and between embryo experiments, corresponding to a coefficient of variation of <5%. Percent variance was lowest at the highest level of P450aromB expression.

Additionally, internal (β -actin) and external (standard curve) methods of normalizing resultant P450aromB mRNA levels were compared. The difference between controls and low and high E2-treated groups was \sim 100-fold, whether C_t values were converted to nanogram of input cRNA from an external standard curve or normalized to β -actin as an internal standard. Due to the many advantages of using a single in-assay standard for comparing multiple target mRNAs (for review; Bustin, 2000; Freeman et al., 1999; Ginzinger, 2002), C_t values for P450aromB and -A mRNAs were routinely corrected for primer/target specific amplification efficiency and expressed relative to β -actin.

3.2. Tissue distribution of P450aromB and -A mRNAs in adult male and female zebrafish

To compare the two P450arom mRNA isoforms in different aromatase-expressing tissues of reproductively active male and female zebrafish, brain, eye, and gonad were analyzed by real-time PCR. Analysis of results by one-way ANOVA showed significant effects of tissue-type and sex: P450aromB ($F = 94.535$, $P = <0.001$); P450aromA ($F = 20.783$, $P = <0.001$); however, the two isoforms varied independently (Fig. 1). The brain was the major aromatase expressing tissue, and P450aromB was the predominant isoform in the brain (ratio B:A, \sim 100:1) in both males and females. By contrast, the level of expression of P450aromB, and the B:A ratio was much lower in the eye, another neural tissue, and sex differences were

observed with P450aromB only (female > male). The ovary was the predominant P450aromA expressing tissue (ratio A:B, \sim 50:1), but the testis expressed both isoforms to some extent (B:A, 2:1).

3.3. Differential expression of P450aromA and -B isoforms during development

Temporal changes in P450aromB and P450aromA expression during development were quantified by real-time PCR between 0 and 120 hpf. Fig. 2 depicts β -actin expression as actual C_t values (upper panel), and P450aromB and -A expression normalized to β -actin, (lower panel). Note that unfertilized eggs, when compared to whole ovaries, had similar levels of P450aromA, but a 10-fold enrichment of P450aromB (compare Figs. 1 and 2, note differences of scale in y-axes). Between fertilization and the onset of embryonic transcription (\sim 3–5 hpf) (Westerfield, 2000), both isoforms dramatically decreased ($t^{1/2} = \sim$ 1 h). Subsequently, each P450arom mRNA form had a qualitatively and quantitatively unique developmental expression pattern: (1) The onset of mRNA accumulation of P450aromB (5–7 hpf) occurred earlier than P450aromA (48 hpf); (2) P450aromB mRNA was 5–50 times higher than -A at all time points up to 96 hpf; (3) P450aromB and -A levels peaked at 72 hpf; and (4) the two isoforms were equivalent by 120 hpf. In marked contrast to changes in aromatase expression, β -actin was relatively constant during the period observed.

3.4. Cumulative effects of estrogen on P450aromB, but not P450aromA, during development

To determine the cumulative effects of estrogen on developmentally programmed P450aromA and -B expression, E2 (0.1 or 1.0 μ M, final concentration) was added to embryo medium beginning at 24 hpf, and embryos/larvae were collected at 48, 72, 96, and 120 hpf for real-time PCR. Analysis of results by ANOVA on Ranks showed significant effects of stage and treatment on P450aromB ($H = 32.168$; $P = <0.001$) but no dose-related differences on P450aromB expression; therefore, data from the two E2 treatment groups were combined (Fig. 3, upper panel). E2 robustly

Table 1
Intra- and interassay variance of real-time RT-PCR analysis of zebrafish P450aromB mRNA

	Control	0.1 μ M E2	1.0 μ M E2
Embryo experiments (<i>n</i>)	4	3	3
RT reactions (<i>n</i>)	7	5	4
PCR runs (<i>n</i>)	8	6	5
Mean C_t values \pm SEM	31 \pm 1.4	25 \pm 0.8	25 \pm 0.5
Coefficient of variation (%)	4.7	3.4	2.1
Mean ng cRNA equivalent	1 \times 10 ⁻⁷	1 \times 10 ⁻⁵	1 \times 10 ⁻⁵
Mean normalized expression \pm SEM	1.9 \pm 0.66 \times 10 ⁻⁴	2.9 \pm 0.60 \times 10 ⁻²	1.8 \pm 0.37 \times 10 ⁻²

In three or four experiments carried out independently by two different operators, embryos were obtained by natural spawning, and exposed to E2 (0.1 or 1.0 μ M) or vehicle alone (DMS0, 0.0006%) between 24 and 96 hpf. P450aromB expression was analyzed at 96 hpf. Each RNA extract was used for one or more RT reactions and PCR amplifications (each in triplicate). Data from all experiments were combined for analysis. Actual C_t values were normalized for β -actin expression and also converted to ng cRNA using an external standard curve method (see Section 2).

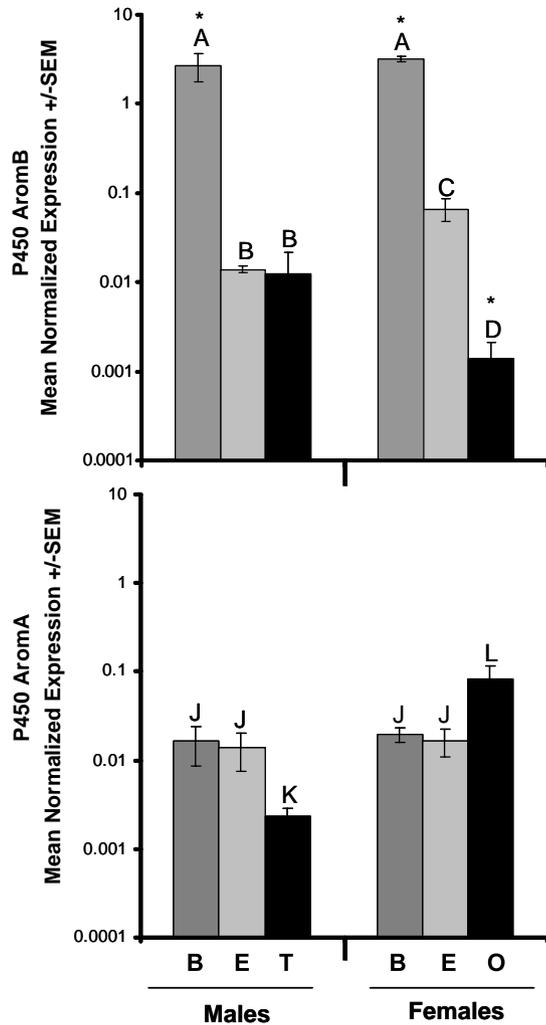


Fig. 1. Tissue-specific and sex differences in P450aromB and P450aromA expression in adult zebrafish. Brain (B), eye (E), and testis (T) or ovary (O) of reproductively active male and female zebrafish were collected for real-time PCR analysis of P450aromB (upper panel) and P450aromA (lower panel). Each bar represents the mean normalized expression (\pm SEM) of three or four independent tissue pools (3 fish per pool), and are plotted on a log scale. One-way ANOVA was used to analyze data by tissue type and sex separately for each marker, followed by SNK analysis to determine which means differed significantly ($P < 0.01$), as indicated by different letters (A–D and J–L for P450aromB and -A, respectively). Student's *t* test was used for pairwise comparisons of P450aromB and -A in a given tissue and sex, and significant differences ($P < 0.01$) are labeled with asterisks (*). Mean C_t values \pm SEM for β -actin did not differ significantly by tissue type or sex: 15.9 ± 0.3 , 16.0 ± 0.2 , and 15.9 ± 0.3 for brain, eye, and gonad of males; 16.0 ± 0.4 , 15.9 ± 0.2 , and 14.6 ± 0.5 for brain, eye, and gonad of females.

up-regulated P450aromB expression at all time points, when compared to matched controls, but the fold-induction and the mean level of E2-induced expression differed as a function of developmental stage and/or duration of E2 exposure: e.g., 24 h of E2 treatment up-regulated P450aromB expression \sim 20-fold in 48 hpf embryos, but continued exposure induced >100 -fold increases by 96 and 120 hpf. Interestingly, although the level of E2-inducible P450aromB expression did not differ between 72 and 120 hpf, 72 hpf was an estrogen hypo-responsive stage

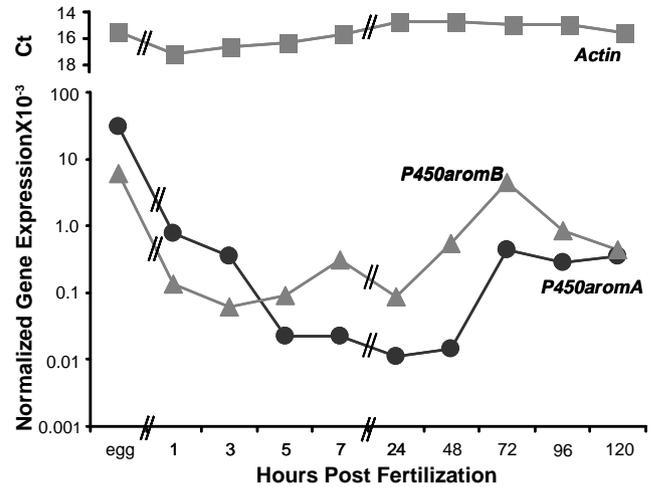


Fig. 2. Stage-related expression of P450aromB and P450aromA during development. Unfertilized eggs (0 hpf) and embryos/larvae at defined developmental stages (1–120 hpf) were analyzed by real-time PCR. Expressed levels of P450aromB (filled triangles) and P450aromA (filled circles) mRNA were obtained from C_t values normalized to β -actin, and are shown on a log scale (lower panel). Actual C_t values for β -actin (filled squares) are shown in the upper panel. Results are representative of three independent experiments. Note that the scale of the y-axis in Figs. 2 and 3 is 1×10^{-3} of that in Fig. 1.

(\sim 5-fold induction), which could be explained by a peak in constitutive expression at 72 hpf in controls. A peak in constitutive expression at 72 hpf was seen in additional embryo series (compare Figs. 2 and 3). In contrast to P450aromB, analysis of P450aromA by ANOVA on Ranks showed no effect of E2 treatment but a significant effect of stage ($H = 14.699$; $P = 0.002$). Similar to data in Fig. 2, P450aromA expression was significantly lower than P450aromB at each developmental stage. Mortality, motility, rate of development, and gross morphology of E2 treated embryos did not differ from controls (data not shown), nor was there an effect of E2 on β -actin expression (see legend to Fig. 3).

3.5. Differential effects of estrogen treatment on P450aromB and -A expression in the brains, eyes, and gonads of adult male and female zebrafish

To determine estrogen effects on aromatase expression in different tissues of reproductively active males and females, fish were exposed to E2 (1 nM) or vehicle in aquarium water, and tissues were collected 2 or 5 d later for real-time PCR. Two-way ANOVA of data in Fig. 4 showed significant effects of E2 treatment ($F = 387.934$; $P < 0.001$) but not time on brain P450aromB expression in males, and no effect of treatment or time on brain P450aromA in males, or on brain levels of P450aromB or -A in females. In brain of adult males, the effect of E2 on P450aromB (2-fold, after 2 or 5 days) was much weaker than that obtained with whole embryos (20- to >100 -fold after 1–4 days; compare Figs. 3 and 4).

When P450aromB was quantified in eye, a second neural tissue, two-way ANOVA revealed significant effects of E2

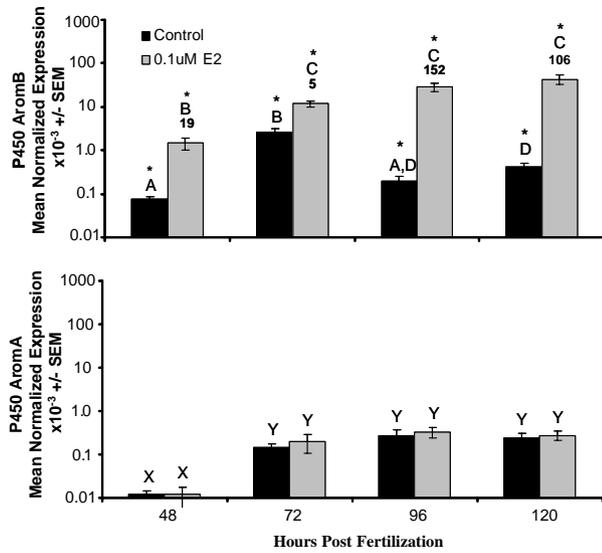


Fig. 3. Cumulative effects of estrogen on P450aromB and P450aromA expression during development. Beginning at 24 hpf, embryos were treated with 0.1 or 1.0 μ M E2 or vehicle alone. Real-time PCR analysis was performed on embryos collected at 48, 72, 96, and 120 hpf. Results from three independent experiments were combined and expressed on a log scale as mean normalized expression (\pm SEM) of P450aromB (upper panel) and P450aromA (lower panel). Data were analyzed by ANOVA on Ranks separately for each gene marker, followed by the SNK method for pairwise comparison. Effects of the two E2 concentrations did not differ significantly and therefore were combined. Means that differed significantly ($P < 0.01$) are indicated by different letters (A–D and X–Y for P450aromB and -A, respectively). Numbers above the bars show the approximate fold-change when compared to the respective control. P450aromA and -B mRNA were compared at each time point and treatment condition using Student's t test, and significant differences ($P < 0.01$) are indicated by asterisks (*). Mean C_t values for β -actin (15.2 ± 0.3) were not significantly affected by treatment or stage.

treatment ($F = 15.305$; $P = 0.002$) and an interaction between treatment and time ($F = 36.708$; $P < 0.001$) in males, but not in females, and no effect on P450aromA (Fig. 5). Similar to brain, E2 up-regulated P450aromB 2- to 3-fold in eyes of males, but only in the 2 day treatment group, and not in eyes of females at either time point. Worth noting here is the high degree of variance when P450aromB was measured in brain or eye of females, which may reflect differences in the reproductive status of the individuals tested.

In contrast to effects seen in neural tissues, E2 did not alter P450aromB expression in ovaries or testes of adult fish, nor did E2 have effects on testicular P450aromA expression (Fig. 6). However, ovarian P450aromA mRNA was suppressed significantly by E2 exposure ($F = 100.198$; $P < 0.001$) and the response was greater after 5 day than after 2 day ($F = 24.902$; $P < 0.001$).

An increase in P450aromB between day 2 and 5 in control fish was significant when P450aromB was measured in eyes of male fish, and a similar trend was seen in eyes from female fish. These changes in untreated fish suggest a stress or treatment artifact, perhaps due to transfer from a large to a small aquarium, or to a change in the male:female ratio (F > M vs. F = M in the breeding and treatment tanks, respectively).

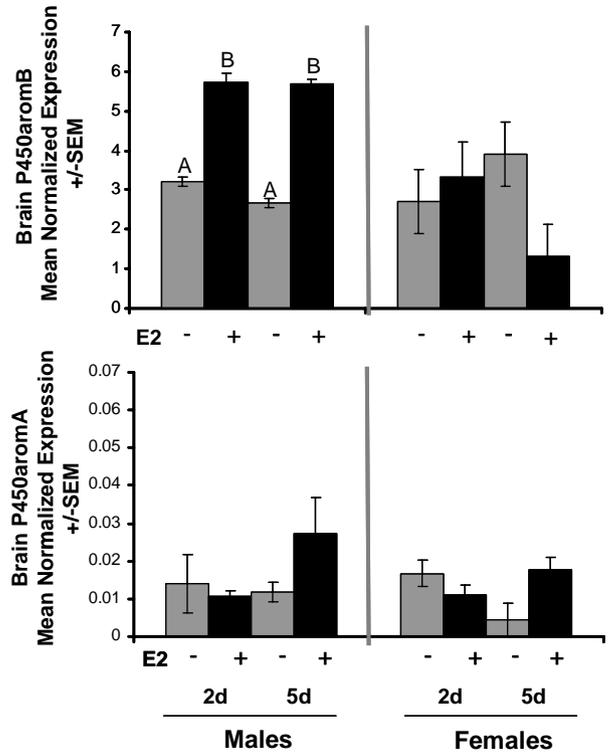


Fig. 4. Effects of estrogen on P450aromB and P450aromA expression in brains of mature male and female zebrafish. Reproductively active males and females were exposed to E2 (1 nM) or vehicle alone (DMSO) in aquarium water. After 2 or 5 days, P450aromB (upper panel) and P450aromA (lower panel) mRNA levels were determined by real-time PCR. Bars show mean normalized expression (\pm SEM) of four independent tissue pools (3 fish per pool). Note the 100-fold difference in scale in upper and lower panels. Two-way ANOVA was performed to determine effects of treatment and time separately for each sex and marker gene, followed by the SNK method for pairwise comparisons. Means that differed significantly ($P < 0.01$) are indicated by different letters (A–B, P450aromB in males). Where no letters are shown, means did not differ significantly by time or treatment (P450aromB in females, P450aromA in males and females). Mean C_t values for β -actin did not vary significantly by treatment, time or sex (15.9 ± 0.3).

4. Discussion

Using real-time RT-PCR, we have developed and validated a rapid, sensitive, gene-specific, and reproducible assay for quantifying and comparing P450aromB and P450aromA expression in unfertilized eggs, embryos/larvae, and dissected tissues of adult zebrafish. By normalizing data to the same internal reference mRNA (β -actin), and correcting for differences in amplification efficiency of the different primer sets, our assay reliably compares product formation from diverse mRNA targets in a single tissue extract under identical conditions. This approach is preferable to multiple idealized external standard curves, and avoids technical artifacts associated with the synthesis and handling of cRNA templates. Owing to its wide dynamic range (1×10^{-1} – 10^{-7} ng of P450aromB cRNA), the real-time PCR assay described here has much greater sensitivity, precision, and resolution than analytical methods previously used to measure zebrafish aromatase

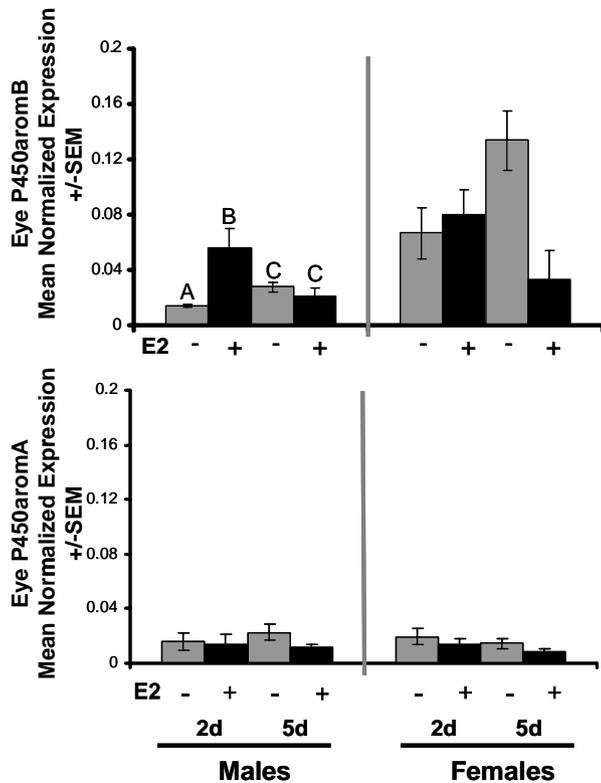


Fig. 5. Effects of estrogen on P450aromB and P450aromA expression in eyes of mature male and female zebrafish. Treatment and statistical analyses were as described in Fig. 4. Means that differed significantly ($P < 0.01$) are indicated by different letters (A–C, P450aromB in males). Where no letters are shown, means did not differ significantly by time or treatment (P450aromB in females, P450aromA in males and females). Mean C_t values for β -actin did not vary significantly by treatment, time or sex (15.9 ± 0.2).

expression, such as Northern blot analysis, semi-quantitative (sq) PCR with/without Southern transfer analysis, and real-time PCR employing TaqMan probes for amplification detection (Kazeto et al., 2004; Kishida and Callard, 2001; Kishida et al., 2001; Trant et al., 2001). Moreover, real-time PCR analysis overcomes technical drawbacks of other analytical methods, namely, the limited number of samples that can be processed simultaneously; the high degree of variability between replicates, assays, operators and laboratories; and the relative inefficiency of analyses in terms of time and labor. Currently in our laboratory, RNA extracts from up to 20 different treatment conditions in a single experiment can be analyzed in triplicate by a single technician in approximately 2–3 days. Although embryos and tissues were pooled to minimize biological variation during the development and validation phases of this research, the yield of total RNA obtained from a single embryo (e.g., 1 μ g at 72 hpf) is well within detection limits for P450aromB and -A using the procedures outlined above (e.g., 80 ng RNA input for RT/micro-well). In addition to the three genes targeted in the present study, we have found it practicable to analyze as many as 10 additional mRNAs of interest in aliquots of the same RNA extract (Burnam et al., 2005; Sawyer et al., 2005a,b).

Because even subtle changes in the expression of genes encoding members of the aromatase family of enzymes could have important implications for reproduction, development, adaptation, and species survival, it is significant that the assay described here has a low degree of intra- and interassay variability. This makes it possible to combine results from multiple biological replicates and independent experiments, thereby increasing the power of statistical analysis to recognize change.

To test the applicability of this assay under actual experimental conditions, we analyzed steady state levels of P450aromB and -A mRNA in brains, eyes, and gonads of reproductively active male and female fish. Results confirm our earlier work in teleosts, in which P450aromB was found to be the predominant brain isoform, while P450aromA was the predominant ovarian form (Gelinas et al., 1998; Kishida and Callard, 2001; Tchoudakova and Callard, 1998). In addition, our data show that the degree of overlapping expression (ratio, B:A) is 100:1 in brain but 1:50 in ovary and close to equivalence in eye (1:1). When measured as the sum of the two aromatase mRNAs, the order of expression in different tissues is brain \gg ovary $>$ eye (20:1:1). Consistent with earlier studies reporting that aromatase enzyme activity is \sim 10-times higher in goldfish brain than in ovaries or retina (Gelinas and Callard, 1993; Pasmanik and Callard, 1988), results of real-time PCR indicate that P450arom mRNA levels reliably predict the quantity of functional protein. Using a similar real-time PCR method to measure P450aromB and -A expression in wild-caught, adult killifish (*Fundulus heteroclitus*), Greytak et al. (2005) found essentially the same tissue levels and isoform ratios (Greytak et al., 2005). How tissue distribution translates to cell type and number remains to be determined.

Contrary to our earlier studies in goldfish or zebrafish, which failed to detect testicular P450arom mRNA by Northern or semi-quantitative PCR/Southern transfer techniques (Gelinas et al., 1998; Kishida and Callard, 2001; Tchoudakova and Callard, 1998), the sensitive real-time PCR method used here indicates that very low levels of both aromatase isoforms are present, and that the B:A ratio is opposite to that in ovaries (respectively, 2:1 vs. 1:50). Both P450aromA and -B were detected in zebrafish testis by Trant et al. (2001) using their real-time PCR assay, and by other methods in several fish species, including gobi, channel catfish, fathead minnow, sea bass, and tilapia (Blazquez and Piferrer, 2004; Halm et al., 2002; Kobayashi et al., 2004; Kwon et al., 2001; Trant et al., 1997). As defined by the ability to convert radiolabeled androgen to estrogen, functional aromatase enzyme has been definitively identified in testes of all major vertebrate groups (for review; Callard, 1996).

To further validate our real-time assay, we measured P450aromB and -A between 0 and 120 h of development. Consistent with results of semi-quantitative PCR/Southern transfer analysis (Kishida and Callard, 2001), we found both mRNA forms to be present in unfertilized

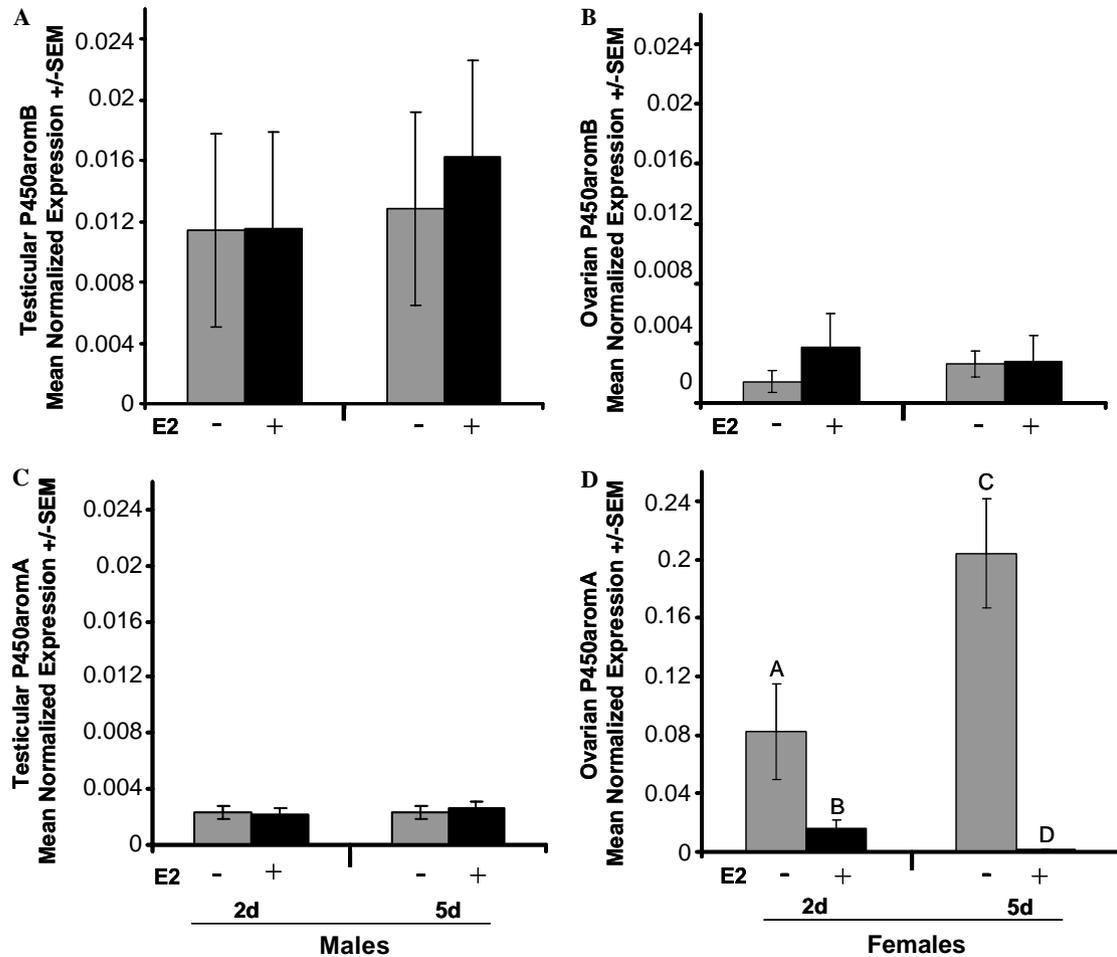


Fig. 6. Effects of estrogen on gonadal expression of P450aromB and P450aromA in zebrafish. Treatment and statistical analyses were as described in Fig. 4. Note that the scales of the y-axes in (A'–C') are one-tenth that in (D'). Means that differed significantly ($P < 0.01$) are indicated by different letters (A–D). Where no letters are shown, means did not differ significantly by time or treatment. Mean C_t values for β -actin did not vary significantly by treatment, time or sex (15.2 ± 0.5).

eggs and in embryos before the onset of zygotic transcription (~ 3 –5 hpf) (Westerfield, 2000), indicating a maternal derivation. Interestingly, although P450aromA mRNA is similar in unfertilized eggs and ovary P450aromB mRNA is enriched 10-fold in unfertilized eggs, which suggests preferential synthesis or accumulation in mature oocytes. Immediately post-fertilization, maternally derived B and A mRNAs are rapidly degraded, but the developmental program thereafter is gene-specific. As measured by an increase in mRNA accumulation, *cyp19b* is one of the earliest genes transcribed during development (5–7 hpf), and is substantially earlier in onset than *cyp19a* (48 hpf). Whether P450aromB mRNA is translated into functional enzyme, with a role in neurodevelopment, remains to be determined. Real-time PCR analysis also shows that P450aromB accumulates to >10 times higher levels than -A, but both peak at 72 hpf, just after hatching. Predictably, aromatase expression in the whole embryo is lower than levels in the predominant B and A expressing tissues of adult fish. By the time organogenesis is

complete (120 hpf) the two isoforms are equivalent. Although Trant et al. (2001) using real-time PCR with TaqMan probes, were unable to detect P450arom mRNA in unfertilized eggs or in embryos before hatching at 72 hpf, their study shows that differences in P450aromB expression levels later in development (24–40 days post fertilization) may be predictive of gonadal sex (Trant et al., 2001).

To investigate the utility of real-time PCR for detecting changes in aromatase mRNAs in response to estrogen-like environmental chemicals, embryos and adult fish were treated with authentic E2. Results confirm previous studies by showing that P450aromB, but not A, is estrogen-inducible. In addition, the data show that the estrogen response system in embryos and larvae is far more robust than previously measured by semi-quantitative PCR/Southern transfer (respectively, 20- vs. 2- to 5-fold induction at 48 hpf), and that the magnitude of the response increases with continued estrogen treatment (>100 -fold at 120 hpf). Interestingly, 72 hpf is an estrogen hyporesponsive period (5-fold induction), due to

already high levels of P450aromB in controls. One explanation is that endogenous estrogen is high during this period, perhaps as a consequence of final yolk reabsorption associated with hatching, or coincident with the onset of gonadal P450aromA expression.

Interpretation of P450aromB responses to estrogen exposure in adult zebrafish is far more complicated than in embryos, because the presence or absence, magnitude, and direction of an effect varies by sex, tissue-type, and treatment period. Moreover, when responses do occur, they are much more subtle (e.g., 2- to 3-fold) than in embryos. Although it can be argued that a higher dose of E2, or a different treatment regimen, would have been more effective, the fold-increase in P450aromB expression in adult male fish in our study is in the same range as that reported when zebrafish are treated for longer periods (Fenske and Segner, 2004; Trant et al., 2001) and also resembles responses reported in other fish species and E2 treatment paradigms: e.g., goldfish (Gelinias et al., 1998; Pasmanik et al., 1988); fathead minnows (Halm et al., 2002); sea bass (Blazquez and Piferrer, 2004); and killifish (Greytak and Callard, unpublished data). Significantly, changes in brain aromatase expression that correlate with seasonal reproductive cyclicity in male and female goldfish (Pasmanik and Callard, 1988) are of the same order of magnitude (2- to 6-fold) as in treatment studies, suggesting that this degree of responsiveness is physiological. It is worth noting that estrogen induction of P450aromB in adult fish is seen only in neural tissues, despite low constitutive expression in ovaries and testes. Perhaps only in neural tissues is there an appropriate cellular context for transcriptional activation by estrogen. A caveat to cross-tissue comparison of gene expression is tissue specific levels of the normalizer β -actin. In our hands C_t values for actin did not vary significantly and ranged from 15.9 ± 0.3 , 16.0 ± 0.2 , and 15.9 ± 0.3 for brain, eye, and gonad of males; 16.0 ± 0.4 , 15.9 ± 0.2 , and 14.6 ± 0.5 for brain, eye, and gonad of females.

Another interpretation of the response with E2 treatment of reproductively active fish is that effects can be exerted on brain or gonadal aromatase expression indirectly via the brain–pituitary–gonadal axis. Thus, negative feedback is a likely explanation for the potent suppressive effect of E2 on ovarian P450aromA mRNA in adult fish. Ample evidence for negative feedback effects of estrogen or aromatizable androgen in adult zebrafish includes a reduction in gonadosomatic index, histopathological changes in the gonads, impaired gametogenesis, and reduced sperm production (Fenske and Segner, 2004; Van den Belt et al., 2002; Weber et al., 2003).

In summary, we have developed and validated a real-time PCR assay for routine measurement of P450aromB and -A mRNAs in zebrafish. We are currently using this assay to measure changes in P450aromB expression in zebrafish embryos, as a whole organism screen for neuroactive, estrogen-like chemicals (Burnam et al.,

2005; Sawyer et al., 2005a,b), and for detecting estrogenic contaminants in water and sediments from polluted environments (Novillo and Callard, 2005). Although it was not a goal of the present study, real-time PCR analysis of P450aromB and -A expression in embryos is amenable to a higher degree of automation for the purposes of environmental testing. Additionally, in conjunction with methods such as microarray analysis for identifying estrogen target genes on a global scale, zebrafish embryos can contribute to basic estrogen biology by providing a convenient model for understanding functional estrogen response pathways in an *in vivo* context.

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