

Expression of aromatase in the embryonic brain of the olive ridley sea turtle (*Lepidochelys olivacea*), and the effect of bisphenol-A in sexually differentiated embryos

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ABSTRACT Brain aromatase participates in several biological processes, such as regulation of the reproductive-endocrine axis, memory, stress, sexual differentiation of the nervous system, male sexual behavior, and brain repair. Here we report the isolation and expression of brain aromatase in olive ridley sea turtle (*Lepidochelys olivacea*) embryos incubated at male- and female-promoting temperatures (MPT and FPT, respectively), at the thermosensitive period (TSP) and the sex-differentiated period. Also, aromatase expression was assessed in differentiated embryos exposed to bisphenol-A (BPA) during the TSP. BPA is a monomer of polycarbonate plastics and is considered an endocrine-disrupting compound. Normal aromatase expression was measured in both forebrain and hindbrain, showing higher expression levels in the forebrain of differentiated embryos at both incubation temperatures. Although no significant differences were detected in the hindbrain, expression was slightly higher at MPT. BPA did not affect aromatase expression neither in forebrains or hindbrains from embryos incubated at MPT, whereas at FPT an inverted U-shape curve was observed in forebrains with significant differences at lower concentrations, whereas in hindbrains a non-significant increment was observed at higher concentrations. Our data indicate that both incubation temperature and developmental stage are critical factors affecting aromatase expression in the forebrain. Because of the timing and location of aromatase expression in the brain, we suggest that brain aromatase may participate in the imprinting of sexual trends related to reproduction and sexual behavior at the onset of sex differentiation, and BPA exposure may impair aromatase function in the female forebrain.

KEY WORDS: *aromatase, gene expression, Lepidochelys olivacea, temperature-dependent sex determination, bisphenol-A*

Introduction

In vertebrates, sex is determined essentially by genetic and environmental factors. Many reptiles such as crocodylians, turtles and lizards display the latter mechanism, in which the temperature is the main factor acting on sex determination; therefore it is called temperature-dependent sex determination (TSD). In animals displaying TSD, the differentiation of the gonads into ovaries or testes depends on the incubation temperature of the eggs during a critical period of embryonic development known as the thermosensitive period (TSP).

Aromatase is a key enzyme responsible for the transformation

of androgens into estrogens, and it has been demonstrated that these are involved in gonadal differentiation in non-mammalian vertebrates (Kuntz *et al.*, 2004). Pieau and Dorizzi (2004) suggested that the gonads are the thermosensitive organ because they respond to temperature changes, and are the sites of expression and activity of aromatase. They also postulated that the incubation temperature during the TSP activates or represses the expression of the aromatase gene and that the production of estradiol is what

Abbreviations used in this paper: BPA, bisphenol-A; FPT, female-promoting temperature; MPT, male-promoting temperature; TSD, temperature-dependent sex determination; TSP, thermosensitive period.

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determines the sex of the embryo.

On the other hand, Willingham *et al.* (2000) suggested that the brain is the organ that initially perceives the temperature signal promoting the activation of aromatase at the beginning of the TSP, and then the steroidogenic activity is transferred to the gonads. In a previous study it was demonstrated that the gonads are innervated during early stages of development in the olive ridley *Lepidochelys olivacea* (Merchant-Larios *et al.*, 1989; Merchant-Larios and Díaz-Hernández, 2013), implicating the direct participation of neural factors on gonadal development.

Aromatase is expressed in both gonads and brain, but it has also been found in many other tissues such as liver, adipose tissue, kidney, digestive tract, skin and placenta (Simpson *et al.*, 2002). Brain aromatase has been implicated in several physiological and behavioral processes such as regulation of the reproductive-endocrine axis, memory and stress, sexual differentiation of the nervous system, male sexual behavior, and brain repair (Balthazart and Ball, 1998; García-Segura *et al.*, 1999). Fluctuations in brain aromatase activity may play an important role in the regulation of aggressive and social behavior. Some experimental studies suggest that brain aromatase activity also impacts cognitive function (García-Segura, 2008).

Neuronal metabolism of testosterone, particularly the synthesis of estradiol by aromatase, is important for male sexual behavior in many vertebrates. In the green anole lizard (*Anolis curvieri*), estradiol facilitates female receptivity and increases sexual motivation in males, suggesting that, as in other vertebrates, aromatization of androgens into estrogens in the brain may be important for the control of sex-specific reproductive behaviors (Cohen and Wade, 2011). Despite expression and activity of aromatase have been documented in the brain of reptiles, little is known about its distribution within this organ, especially in regions that control sexual behavior.

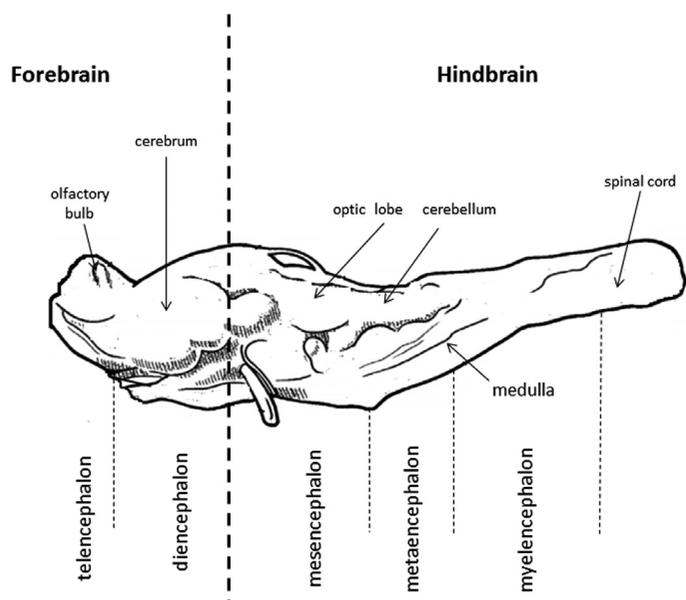


Fig. 1. Schematic representation of the sea turtle brain (adapted from Wyneken, 2001). The dashed line indicates the limit between forebrain and hindbrain.

In the brain of vertebrates, aromatase has been mainly localized in anterior brain areas related to reproduction control and sexual behavior (Lephart, 1996; Balthazart and Ball, 1998). Aromatase is expressed in the developing brain of alligators, leopard geckos, and red-eared slider turtles, and both activity and mRNA appear to be sexually monomorphic in these species (Milnes *et al.*, 2002; Endo *et al.*, 2008; Willingham *et al.*, 2000). Nevertheless, in diamondback terrapin it was found that the brain-specific transcript is expressed at higher levels at the beginning of the TSP in embryos incubated at FPT than at MPT. Aromatase expression in the male brain is low at the beginning of the TSP, but it becomes higher than in the female brain in the second half of the TSP (Jeyasuria and Place, 1998). Furthermore, brain aromatase activity has been shown to be sexually dimorphic in some mammals (Lephart, 1996), teleost fish, and the Japanese quail, in which males display higher activity than females in brain areas related to reproduction (Aste *et al.*, 1998; Balthazart *et al.*, 1990; González and Piferrer, 2002).

Bisphenol A (BPA) was first synthesized in 1891, and has been shown to act as a xenoestrogen since 1936 (von Saal *et al.*, 2007; Wolstenholme *et al.*, 2011). Final products in which BPA is used include food and beverage plastic storage and heating containers, dental fillings and sealants and the interior lacquer-coating of food cans, among others (Brotons *et al.*, 1994, Olea *et al.*, 1996; Biles *et al.*, 1997).

Effects of BPA exposure have been well documented, especially in mammalian model systems including effects on the male and female reproductive tract, immune system, metabolism and growth, and development of preneoplastic and neoplastic lesions of the mammary gland and the mammary gland's response to carcinogens (Vanderberg *et al.*, 2013).

In the brain, early exposures to BPA can lead to permanent changes in brain morphology, disruptions in behavior, and alterations in learning, memory, exploration, and emotional responsiveness (Wolstenholme *et al.*, 2011). Furthermore, BPA effects in the reproductive tract and the brain have been reported in non-mammalian systems, for instance, zebrafish embryos exposed to BPA presented high levels of brain P450aromB mRNA (Kishida *et al.*, 2001; Chung *et al.*, 2011), which could result in an abnormal estrogen production affecting the developing central nervous system and sexual behavior.

It has also been shown that *in ovo* exposure to BPA in the crocodilian *Caiman latirostris* causes sex reversal. When eggs were exposed to BPA during the critical time of sex determination, all resultant offspring were females regardless of the incubation temperature (Stoker *et al.*, 2003).

Regarding wildlife exposure effects, several studies have found endocrine disruption specially in the reproductive tract including alteration of sex determination and gonadal function from exposure both during and after gonadal organogenesis; induction of vitellogenin expression in males and juveniles, and other indicators of reproductive success such as reduction in spermatogenesis (for a review on BPA effects in wildlife see Crain *et al.*, 2007). To our knowledge, BPA effects in the brain have not been documented in wildlife.

Our goal was to investigate whether aromatase transcription in the brain is temperature-dependent, if the brain isoform is differentially expressed in anterior and posterior brain regions (Fig. 1) during development, and the potential effects of BPA exposure in the expression of aromatase at both incubation temperatures

and brain regions.

This study reports the isolation and expression of a brain aromatase transcript from olive ridley (*L. olivacea*) embryos incubated at MPT and FPT at the undifferentiated period (stage 24) and the differentiated period (stage 26-27). Aromatase expression was also analyzed in differentiated embryos exposed to BPA during the TSP.

Results and Discussion

Isolation of the brain aromatase transcript

The aromatase full coding sequence was isolated from the brain, and consisted of 1614 nucleotides (GenBank: KF411436), and showed 99% similarity with its gonad counterpart (GenBank: KF268021) and 98% similarity with aromatase from *Trachemys scripta* (GenBank: AF178949) and *Chrysemys picta* (GenBank: DQ383800).

The aromatase gene in *L. olivacea* contains nine exons as in humans. In the sea turtle, brain and gonad aromatase isoforms show almost the same coding sequence, except for point variations in few nucleotides. An alignment of *L. olivacea* brain and gonad aromatase amino acid sequences was performed using the ClustalW2 (EBI) (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) software (Fig. 2).

According to Murdock and Wibbels (2003), five functional domains (I- V) of aromatase have been found in the turtle *T. scripta*, as well as in other vertebrates such as alligator, chicken, bovine, human, frog, and tilapia. *L. olivacea* aromatase isoforms contained the functional domains characteristic of vertebrate aromatase molecules: I) membrane-spanning domain, II) helical domain, III) Ozols peptide domain, IV) aromatic domain, and V) heme-binding domain (Fig. 2). These functional domains (mainly domains IV and V) show high homology among vertebrate aromatase sequences (Murdock and Wibbels, 2003).

Aromatase expression in the brain

Expression differences between incubation temperatures and developmental stages

In forebrains, significant differences in expression levels were detected ($P=0.001$). The groups presenting statistical significant differences were: differentiated female (DF) vs undifferentiated female (UF) ($P=0.002$), differentiated female (DF) vs undifferentiated male (UM) ($P=0.013$), and differentiated male (DM) vs undifferentiated female (UF) ($P=0.042$) (Fig. 3A). Hindbrains presented lower levels of expression, and did not show significant differences between groups (Fig. 3B).

A two-way ANOVA revealed that variation in aromatase gene expression in the forebrain was due to the developmental stage ($P<0.001$), and that temperature was the factor affecting variation in gene expression in the hindbrain ($P=0.006$). Considering the whole brain, temperature was the main factor affecting aromatase expression ($P=0.048$), and the interaction between temperature and developmental stage was not significant ($P=0.989$).

The brain (unlike the gonad) does not undergo a complete morphological differentiation as develop-

ment progresses from the beginning to the end of the TSP. Nevertheless it is a sensorial organ and perceives environmental changes which in turn are transmitted to other organs through signaling events. For this reason, it is not surprising that temperature is the main factor affecting aromatase gene expression in the whole brain, and, because of the sex-related functions of the forebrain, it is also not surprising that both factors (temperature and developmental stage) affect aromatase expression in this region.

Research in *T. scripta*, *Chelydra serpentina*, and *Malaclemys terrapin* revealed that aromatase transcripts are found in the brains of embryos incubated at both temperatures, before and after the TSP, but they are more abundant at the beginning of this period in putative females. Moreover, the incubation temperature affects aromatase expression in the brain of *M. terrapin*, before differences become apparent in the gonads (Jeyasuria and Place, 1998). Later in embryogenesis, during the TSP, the levels of aromatase expression in the brains of putative males overcome expression levels of putative females, while aromatase in the gonads of putative females increases exponentially (Jeyasuria and Place, 1998; Place *et al.*, 2001; Ramsey and Crews, 2007).

These observations are not in complete agreement with the results obtained in the present study for *L. olivacea*, as both males and females at stage 27 (differentiated period) presented higher expression levels of brain aromatase, therefore the increase of expression levels are not limited to males. Nevertheless we divided the brain to measure gene expression, and the main site of aromatase expression was the forebrain. Results could vary when using the whole brain. Also, growth and development in freshwater

	I	
LoAromGonad	MILETLNLMHNYNITRVVPEVMPATATVPILLMLCFLLWNYEETSIPGGPFCMGIGPLIS	60
LoAromBrain	MILETLNLMHNYNITRVVPEVMPATATVPILLMLCFLLWNYEETSIPGGPFCMGIGPLIS	60

LoAromGonad	HGRFLWMGVGNACNYNMYGEMFRVWISGEETLIIKSSSIFHVMKHGQYISRFSGKLG	120
LoAromBrain	HGRFLWMGVGNACNYNMYGEMFRVWISGEETLIIKSSSIFHVMKHGQYISRFSGKLG	120

LoAromGonad	LQCI GMNENGIIFNNPALWKEIRPFFTKALSGPGLVRMIAICVESTKDHLDRLDVEDTAG	180
LoAromBrain	LQCI GMHENGIIIFNNPALWKEIRPFFTKALSGPGLVRMIAICVESTKDHLDRLDVEDTAG	180

LoAromGonad	LGNINVLNFMQRITLDASNTLFLGIPLDENAI VLKIQNYFDWQ TLLLPDIFFKISWLY	240
LoAromBrain	LGNINVLNFMQRITLDASNTLFLGIPLDENAI VLKIQNYFDWQ TLLLPDIFFKISWLY	240

LoAromGonad	KKYEKSAKDLKEAIEILIEQKRQLSTVEKLEDHMDPASQLIFAQSRGDLTGENVNQCVL	300
LoAromBrain	KKYEKSAKDLKEAIEILIEQKRQLSTVEKLEDHMDPASQLIFAQSRGDLTGENVNQCVL	300

LoAromGonad	EMMIAAPDLSVTLFFMLVLAIEHPKVEEDMMKEIQAIIGDRDVQSNMNSLNKVVFNFIN	360
LoAromBrain	EMMIAAPDLSVTLFFMLVLAIEHPKVEEDMMKEIQAIIGDRDVQSNMNSLNKVVFNFIN	360

LoAromGonad	ESMRYQPVVDLVMRKALQDDVIDGYPVKRGNTNII LNIGRMHKLFFPKPSEFSLKNFEKN	420
LoAromBrain	ESMRYQPVVDLVMRKALQDDVIDGYPVKRGNTNII LNIGRMHKLFFPKPSEFSLKNFEKN	420

LoAromGonad	VPSRYFPFGFGRGCVGKFIAMVMMKAILVTFLLRRYRVQTPKGRGLNNIQSNLDSMHP	480
LoAromBrain	VPSRYFPFGFGRGCVGKFIAMVMMKAILVTFLLRRYRVQTPKGRGLNNIQSNLDSMHP	480

LoAromGonad	NERQPFLEMFLMPPRRNIDKCQDD	503
LoAromBrain	NERQPFLEMFLMPPRRNIDKCQDD	503

Fig. 2. Amino acid sequences of *L. olivacea* gonad (LoAromGonad) and brain (LoAromBrain) aromatase isoforms. Aromatase gene domains are represented from I to V: I, membrane spanning region; II, helical region; III, Ozols peptide region; IV, aromatic domain; and V, heme-binding domain (adapted from Murdock and Wibbels, 2003).

turtles is faster than in sea turtles.

Previous studies point to the brain as a site of steroid hormone synthesis in response to temperature. Salame-Mendez *et al.*, (1998) measured E2 in the diencephalon/mesencephalon and telencephalon in *L. olivacea* demonstrating differences between incubation temperatures during the TSP. They found that the concentration of E2 was higher in the diencephalon/mesencephalon of putative females than in putative males during the TSP. These results agree with present findings, since higher aromatase expression in the female's forebrain was observed at the end of the TSP, although no significant differences were found between sexes ($P=0.331$).

Among reptiles, aromatase is expressed in the developing brain of alligators, leopard geckos, and turtles, and both expression and activity in the whole brain is apparently monomorphic (Endo *et al.*, 2008; Milnes *et al.*, 2002; Crews *et al.*, 2001; Cohen and Wade, 2012). For instance, in *Alligator mississippiensis*, the brain was divided in forebrain, hypothalamus, and hindbrain, and the highest aromatase expression was located in the hypothalamus at both male and female-promoting temperatures. There were no significant differences in aromatase expression in the brain between MPT and FPT, or between developmental stages (Gabriel *et al.*, 2001). These observations agree with present findings, since the results in *L. olivacea* suggest that expression of brain aromatase is monomorphic, showing higher expression levels in the forebrain.

Brain aromatase is also present during development of many other species such as Japanese quail (*Coturnix japonica*) (Schumacher and Balthazart, 1986; Aste *et al.*, 1998), mice and rats, in

which expression in the hypothalamus is higher in males than in females (Hutchison *et al.*, 1994). In sexually mature organisms, as in quails, immunohistochemistry and *in situ* hybridization studies have revealed that aromatase is specifically expressed in the dimorphic preoptic nucleus, showing higher expression in males (Schumacher and Balthazart, 1986; Aste *et al.*, 1998; Balthazart *et al.*, 1990; Foidart *et al.*, 1995; Voigt *et al.*, 2007), in which the activation of the copulatory behavior is controlled by an estrogen-androgen synergism, therefore, the action of testosterone along with aromatase and 5 α -reductase is necessary and sufficient for the activation of the male sexual behavior (Panzica *et al.*, 1996). Similar results have been found in rats (Roselli *et al.*, 1996).

Interestingly, in quail it has been demonstrated that sex differences in aromatase activity are not associated to expression levels. For instance, mRNA levels in the brain have been found to be higher in females than in males (Voigt *et al.*, 2007), but males have shown higher aromatase activity than females (Balthazart *et al.*, 2009). These results are consistent with previous studies in rats, where males show higher expression levels than females, but activity does not differ between sexes (Wagner and Morrell, 1996; 1997). Research in crocodiles indicates that there are no significant differences in aromatase expression levels between sexes or developmental stages, but an increase in enzymatic activity was observed during the TSP in both sexes, with higher enzymatic activity in putative females at stage 24 (Milnes *et al.*, 2002). These results indicate that mRNA expression is not always consistent to enzymatic activity. According to Voigt *et al.* (2011), these sex differences in enzymatic activity are probably the result of post-translational events. Possibly, these mechanisms modulate the activity of brain aromatase in quail without affecting mRNA and protein concentration. However, previous studies with *M. terrapin* demonstrated that the transcription of aromatase mRNA was consistent with enzymatic activity (Jeyasuria *et al.*, 1994; 1995; Jeyasuria and Place, 1998).

In teleost fish, research has revealed that aromatase expression and activity are detected in the brain, especially in forebrain (Forlano *et al.*, 2006). While semi-quantitative RT-PCR has shown that expression of *CYP19A2* was significantly higher in anterior brain areas in males; in posterior brain areas no differences were detected between sexes. In *L. olivacea* we found that the expression of aromatase occurs at lower levels in the brain than in the gonad (data not shown), nevertheless, like in fish, higher expression was found in the forebrain of sexually differentiated organisms (stage 27, Fig. 3A).

Whether the brain or gonad initially perceives the temperature signal is still unclear. Merchant-Larios *et al.* (1989) found innervation in the gonads during early stages of development in *L. olivacea*. This observation suggests that neural factors might mediate the influence of temperature in sex determination. Later, Willingham *et al.* (2000) supported this theory suggesting that the brain is the thermosensitive organ which initially perceives the temperature signal, promoting the activation of aromatase at the beginning of the TSP and then steroidogenic activity is transferred to the gonads. Epigenetic mechanisms might be involved in tissue-specific aromatase gene expression, since the environment often influences developmental gene expression by altering the epigenetic status in regulatory regions. Unraveling regulatory mechanisms in species displaying TSD will help to understand how the environment regulates gene expression during sex determination and differentiation.

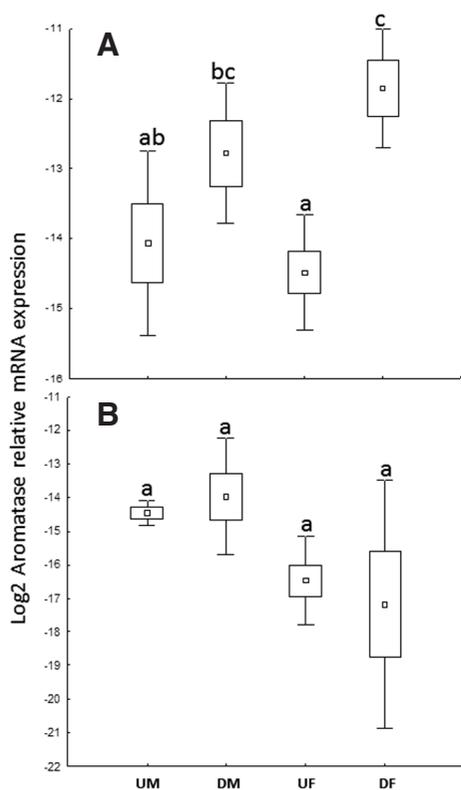


Fig. 3. Normal aromatase expression in forebrain (A) and hindbrain (B) of turtle embryos. UM, undifferentiated at MPT stage 24; DM, differentiated at MPT stage 27; UF, undifferentiated at FPT stage 24; DF, differentiated at FPT stage 27. Expression levels were normalized with log (base 2). Different letters indicate significant differences in expression levels.

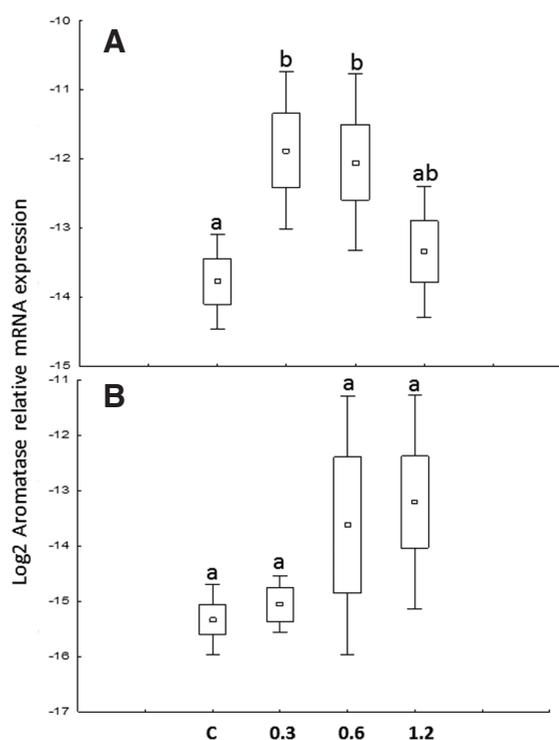


Fig. 4. Aromatase expression in the forebrain (A) and hindbrain (B) of bisphenol-A (BPA)-exposed embryos at female-promoting temperature-stage 26. C, ethanol control; 0.3, 0.6, and 1.2 indicate BPA concentrations in mg per egg. Expression levels were normalized with log (base 2). Different letters indicate significant differences in expression levels.

Since aromatase in the olive ridley's forebrain is expressed at lower levels during early stages of development (stage 24), increasing at later stages (stage 27), coinciding with the differentiation of the gonad into ovary, we suggest a forebrain-gonad communication system which may be related to sex differentiation.

Expression of brain aromatase in BPA-exposed embryos

Diverse environmental chemicals present endocrine disrupting activity by virtue of their ability to bind nuclear estrogen/androgen receptors (ER and AR respectively) or to mimic or block their biological responses; therefore these chemicals are known as endocrine-disrupting compounds (EDCs). Results obtained from BPA exposure experiments are consistent with previous studies showing that BPA induces expression of aromatase in the brain (Chung *et al.*, 2011). Recent work has revealed that BPA can lead to adverse effects in brain morphology and behavior (Chung *et al.*, 2011).

In *L. olivacea*, aromatase expression showed an inverted U-shape curve in BPA-exposed female forebrains, in which lower concentrations of BPA (0.3 and 0.6 mg per egg) were able to significantly induce aromatase expression ($P=0.015$ and $P=0.02$ respectively), whereas the highest concentration (1.2 mg per egg) did not show significant differences when compared to control animals ($P=0.546$) (Fig. 4A). This behavior is known as non-monotonic dose-response curve (NMDRC) in which the dose-response relationship is not linear; it has been observed in both *in vitro* and *in vivo* BPA exposure experiments, in which lower doses induce significant

differences in endpoints compared to controls, and higher doses do not (Vandenberg, *et al.*, 2013). Several mechanisms have been identified that demonstrate how hormones and EDCs produce non-monotonic responses in cells, tissues, and animals (for a review of NMDRC to EDCs see Vandenberg *et al.*, 2012). The simplest mechanism for NMDRCs explains that hormones can be acutely toxic at high doses but still elicit a response at low, physiologically relevant doses. Therefore, experiments working at concentrations that are cytotoxic are incapable of detecting responses that are mediated by ligand-binding interactions (Vandenberg *et al.*, 2012).

NMDRCs may also occur due to differences in receptor affinity at low versus high doses, for instance, at low doses, BPA almost exclusively binds to the estrogen receptor (ER, including the membrane-bound ER or mER), but at high doses it can also bind other hormone receptors, such as the androgen receptor (AR) (Vandenberg *et al.*, 2012). It has been shown that androgens regulate the expression of aromatase in the preoptic area and medial basal hypothalamus of adult rats (Abdelgadir *et al.*, 1994) and in the pituitary of the ricefield eel (Zhang *et al.*, 2012). Interestingly, BPA acts as an AR antagonist, blocking the action of androgens (Wolstenholme *et al.*, 2011). This antagonist effect of BPA could likely explain the aromatase down-regulation at the highest concentrations in *L. olivacea*'s forebrain, resulting in NMDRC. It is also well known that several hormones control or influence their own secretion using a feedback system, in this regard, it is also possible that the decreased expression of aromatase in the female forebrain at higher concentrations of BPA is the result of a negative feedback in the regulation of aromatase by estrogens.

In the female hindbrain, BPA exposure resulted in an increment of aromatase expression at higher concentrations; however, this increment was not significant ($P=0.15$) due probably to high individual variability (Fig. 4B). In the male brain, BPA did not produce any significant effect in the expression of aromatase ($P=0.705$ and $P=0.876$ for forebrain and hindbrain respectively), although individual variability was also high (Fig. 5).

Altogether, our results indicate that in the olive ridley, aromatase is expressed in the whole brain, showing higher expression levels in the forebrain, as in other vertebrates. Apparently, BPA exposure affects aromatase expression in the female forebrain of sexually differentiated embryos in a non-monotonic fashion, but expression does not seem to be significantly affected in the brain of male embryos. This sex-specific expression of aromatase may be due to the sex-specific differences of endogenous estrogens, since in females estrogenic effects from BPA may synergize with the production of endogenous estrogens (in gonads and brain), significantly increasing aromatase expression in the brain at lower concentrations of BPA, and then decreasing expression levels at higher concentrations as a negative feedback because of estrogen overproduction. In males, overall estrogen production is lower than in females; therefore, BPA did not show significant effects in aromatase expression, nevertheless individual variability was high, meaning that some embryos were more responsive than others.

Several studies have revealed that exposure to BPA disrupts development and reproduction in females, for instance, BPA affects ovulation in the female brown trout (Donaldson *et al.*, 1983), and estrous cycles in mammalian females (Porrini *et al.*, 2005; Rubin *et al.*, 2001). Kubo *et al.* (2003) reported that in rats, the exposure to a low dose of BPA during a critical period of development (fetal and suckling), disrupts the normal sexual behavior without any ef-

fect in the reproductive system. A different study revealed that like estrogen, BPA induces brain-specific expression of aromatase in early zebrafish embryos (Chung *et al.*, 2011). Zebrafish contains two aromatase genes due to genomic duplication during teleost evolution, *AroA* and *AroB*, expressed in the ovary and the brain respectively. The *AroA* gene is not induced by estrogen, whereas the *AroB* gene contains an estrogen-responsive element (ERE) (Boon *et al.*, 2010; Chung *et al.*, 2011). Other studies revealed that *AroB* is strongly expressed in the telencephalon, hypothalamus and preoptic areas, and that estrogen increases the levels of brain aromatase in these regions (Menuet *et al.*, 2005; Lassiter *et al.*, 2007). Interestingly, in mammalian (Lephart, 1996) and avian (Steimer and Hutchinson, 1981) brains, aromatase expression is also enhanced by estradiol (Boon *et al.*, 2010), but the mechanism by which estrogen regulates aromatase expression is still elusive since no EREs have been found in promoter regions. It is thought that epigenetic modifications might regulate this event (Kumar *et al.*, 2009). BPA may affect the epigenetic status of a gene altering its expression patterns either by increasing the expression of histones (Zhu *et al.*, 2009) or by changing the methylation status of cytosines (Dolinoy *et al.*, 2007) although the exact mechanism through which BPA affects epigenetically the expression of a gene is still unclear. Whether the brain aromatase gene from *L. olivacea* contains EREs or androgen-responsive elements (AREs) in the promoter region, or is epigenetically regulated has to be investigated.

Conclusions

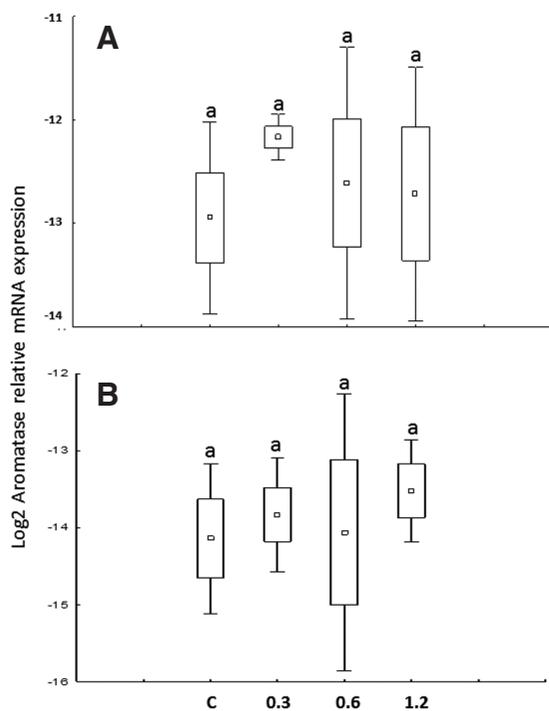


Fig. 5. Aromatase expression in the forebrain (A) and hindbrain (B) of BPA-exposed embryos at MPT-stage 26. c, ethanol control; 0.3, 0.6, and 1.2 indicate BPA concentrations in mg per egg. Expression levels were normalized with log (base 2). Different letters indicate significant differences in expression levels.

In the olive ridley sea turtle, brain aromatase is expressed in the brain, showing higher expression levels in the forebrain. The incubation temperature seemed to be the main factor affecting aromatase gene expression in the whole brain; nevertheless, the developmental stage also influences aromatase expression in the forebrain.

We suggest the existence of a forebrain-gonad communication system, in which production of estrogen increases (by aromatase activity) in male and female forebrains at the end of the TSP, coinciding with an increase of estrogen production in the female gonad and subsequent differentiation of the innervated ovary. Both incubation temperature and developmental stage are critical factors affecting aromatase expression in the forebrain. Since higher expression levels were detected in differentiated embryos, estrogen production in the embryonic forebrain may participate in the imprinting of sexual trends related to reproduction and sexual behavior at the onset of sex differentiation, time in which BPA exposure may impair aromatase function, particularly in female embryos.

Materials and Methods

Animals

Freshly laid eggs were obtained from La Escobilla beach (96° 27'16"W, 15° 40'36"N), Oaxaca, Mexico. 30 eggs were placed in covered plastic containers filled with moistened vermiculite into incubators at either 26°C (MPT) or 33°C (FPT) and few were periodically dissected to determine the degree of development. Temperature in both incubators was recorded every two hours with LogTag (www.datalogger.com.mx). For normal aromatase expression assays, brains of embryos corresponding to stage 24 (gonads morphologically undifferentiated) and stage 27 (gonads morphologically differentiated as ovary or testis) at both temperatures were obtained by microdissection.

It has been documented that 6–12 µg of 17β-estradiol (E2) per egg affect sex differentiation in *L. olivacea* (Merchant-Larios *et al.*, 1997). Since BPA is considered a weak xenoestrogen (Witorsch, 2002; Wolstenholme *et al.*, 2011), we chose three doses of BPA to perform exposure experiments: 1.2 mg per egg (100 times more concentrated than the highest E2 dose exerting an effect on *L. olivacea*), and two lower doses (0.6 and 0.3 mg per egg). Once the eggs entered the TSP (stages 23 and 24 for MPT and FPT respectively), they were subjected to a topical treatment with BPA using ethanol as vehicle. Ethanol alone was used as control.

Five to eight embryos incubated at both temperatures and the three concentrations of BPA were sampled at stage 26. All brains were divided into anterior (forebrain) and posterior (hindbrain) regions (Fig. 1) for expression analyses.

Cloning of *L. olivacea* brain aromatase transcript

Brain samples were taken from stage 27 embryos incubated at male- and female-promoting temperatures. After dissection, tissues were preserved in RNAlater (Ambion, UNIPARTS, Mexico) and stored at -20°C until use. Total RNA was isolated using Trizol reagent (Invitrogen, Accisolab, Mexico) according to the manufacturer's instructions, followed by a double digestion with DNase I to eliminate genomic DNA contamination. Reverse transcription was performed at 42°C using M-MLV RT (Promega, UNIPARTS, Mexico) and random primers. Reactions using RNA instead of cDNA were performed to confirm genomic DNA elimination.

Degenerate PCR primers (Arom-F: 5'-TTG GCATGMATG AAAATG G and Arom-R: 5'-CAT YAC CAT GGC DAT RWA YTT YCC) were designed from conserved regions of aromatase transcripts obtained from previously reported sequences in GenBank: *Xenopus laevis* (AB031278.1), *Rana pipiens* (DQ449025.1), *Trachemys scripta* (AF178949.1), *Alligator mississippiensis* (AY029233.1), and *Chrysemys picta* (FJ195593.1). PCR was performed using a high fidelity Taq polymerase (Advantage, Clontech,

UNIPARTS, Mexico), PCR conditions were: one cycle at 94°C 1 min; 40 cycles at 94°C 1 min, 48°C 1 min, and 68°C 1.5 min, and a final cycle at 68°C for 10 min, rendering a product of ~850 bp.

PCR fragments were ligated into a pGEM-T vector (Promega, UNIPARTS, Mexico) using T4 ligase according to manufacturer's protocol. Transformation was performed with *Escherichia coli* DH5 α competent cells (Invitrogen, Accsolab, Mexico). Positive clones were sequenced in Macrogen, Inc (South Korea). Sequences were manually edited using BioEdit and submitted to BLAST (Basic Local Alignment Search Tool) from NCBI (National Center for Biotechnology Information). Once the identity of this partial sequence was confirmed as aromatase, two new sets of primers were designed to obtain a larger sequence to the 5' and 3' ends (Arom5-F: 5'-GAT GAT ACT GGA AAC CCT GAA TC; Arom5-R: 5'-TCT TTC CAG AGT GCT GGG TT, and Arom3-F: 5'-CAT TCT GAA CAT TGG ACG TAT G; Arom3-R: 5'-AGA GTA TGC GTG ACA GCA CCT A, respectively). PCR was performed with Taq polymerase (Promega, UNIPARTS, Mexico) under the following conditions: one cycle at 94°C for 2 min, and 40 cycles at 94°C 1 min, 54°C 1 min, 72°C 1 min, rendering products of ~400 bp, which complemented the previous fragment producing the full coding sequence. Ligation, transformation, and sequencing were performed as above.

Real-time PCR

Gene-specific primers (Lo-Arom-F: 5'-TGG TCA TGC GCA AGG CTT TA and Lo-Arom-R: 5'-GGC CAA ATC CAA ATG GCT GA) were designed to perform real-time PCR using a CFX96 thermal cycler (Bio-Rad, Mexico) and Eva Green® Supermix 2X (Bio-Rad, Mexico). Five to nine individual samples per developmental stage and incubation temperature were analyzed in triplicate. Relative gene expression in brains was obtained through the Δ CT method (Livak and Schmittgen, 2001), using the geometric mean of two internal control genes, which proved to be equally stable according to Llera-Herrera *et al.*, (2012): 18S rRNA (Forward: 5'-CAC GGC CGG TAC AGT GA; Reverse: 5'-CCG GGT TGG TTT TGG TCT GA) and β -actin (Forward: 5'-ACA CAG GTG TGT AAG ATG GCT TTG G; Reverse: 5'-GAA GAC TCA GGT CCA GGA AAG GAA A) in order to normalize the aromatase raw CT data (Δ CT) (Vandesompele *et al.*, 2002). PCR conditions were as follows: one cycle at 95°C for 30 s; 40 cycles at 95°C 20 s, 60°C 20 s. PCR specificity was confirmed through melting curve analysis.

Statistical analysis

Δ CT values were converted to their linear form using the 2- Δ CT equation (Livak and Schmittgen, 2001). Gene expression results were log-transformed in order to reach normality and variance equality. One and two way ANOVAs coupled to the Holm-Sidak test for multiple comparisons were performed using SigmaPlot v11 and STATISTICA v7 in order to find statistical differences in aromatase relative expression levels ($P < 0.05$). Normalized data (log 2) were plotted according to Yamamoto *et al.*, (2013).

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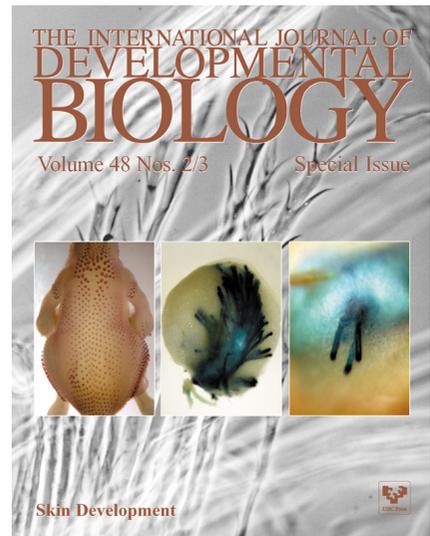
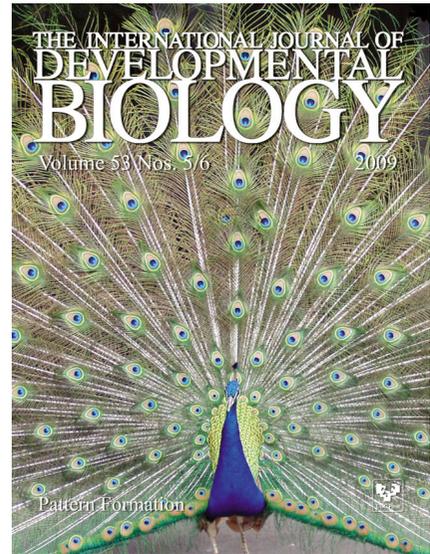
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