Transcriptional expression profiles of sex differentiation genes in the hermaphroditic fish *Kryotolebias marmoratus* exposed to endocrine disrupting chemicals

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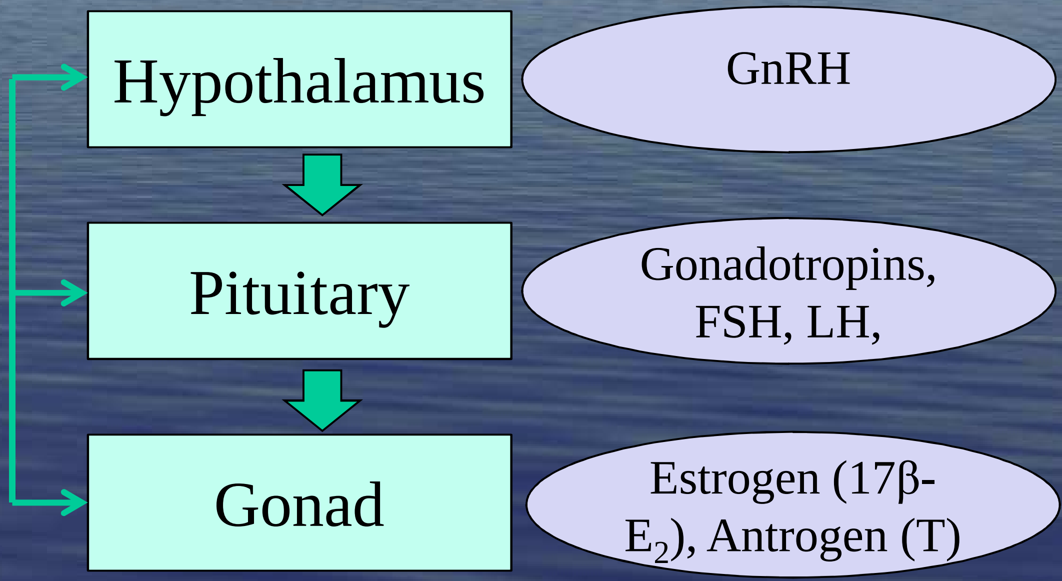
Background

Sex-determining/ -differentiation

- In fish, the sex-determining mechanisms are diverse and often different even in closely related species (Tanaka et al., 2007)
  - Sex-determining gene: *dmy* gene in medaka
- However, the genes involved in fish sex differentiation are quite conserved throughout the species and even across the different groups of vertebrates (Munger et al., 2009; Piferrer, 2011)

In teleosts, reproductive process

- is mainly regulated by the hypothalamus–pituitary–gonad (***HPG***) axis.



Gonadotropins

- FSH (Follicle stimulating hormone) is involved in the initiation of gametogenesis and the regulation of gonadal growth. The level in the blood increase during early oocyte development and stimulate the synthesis of T, which is then aromatized into E2 (Cyr and Eales, 1996)
- LH (Luteinizing hormone) regulates gonadal maturation and spermiation/ovulation (Mateos et al., 2002).

Steroid hormones

- 17β-estradiol(E_2) and testosterone influence reproduction and development, including gonadal sex determination, sex differentiation, and sexual behavior.
- They are involved in the feedback control of the reproductive cycle in the HPG axis.
- Steroid hormone are synthesized via steroidogenesis.

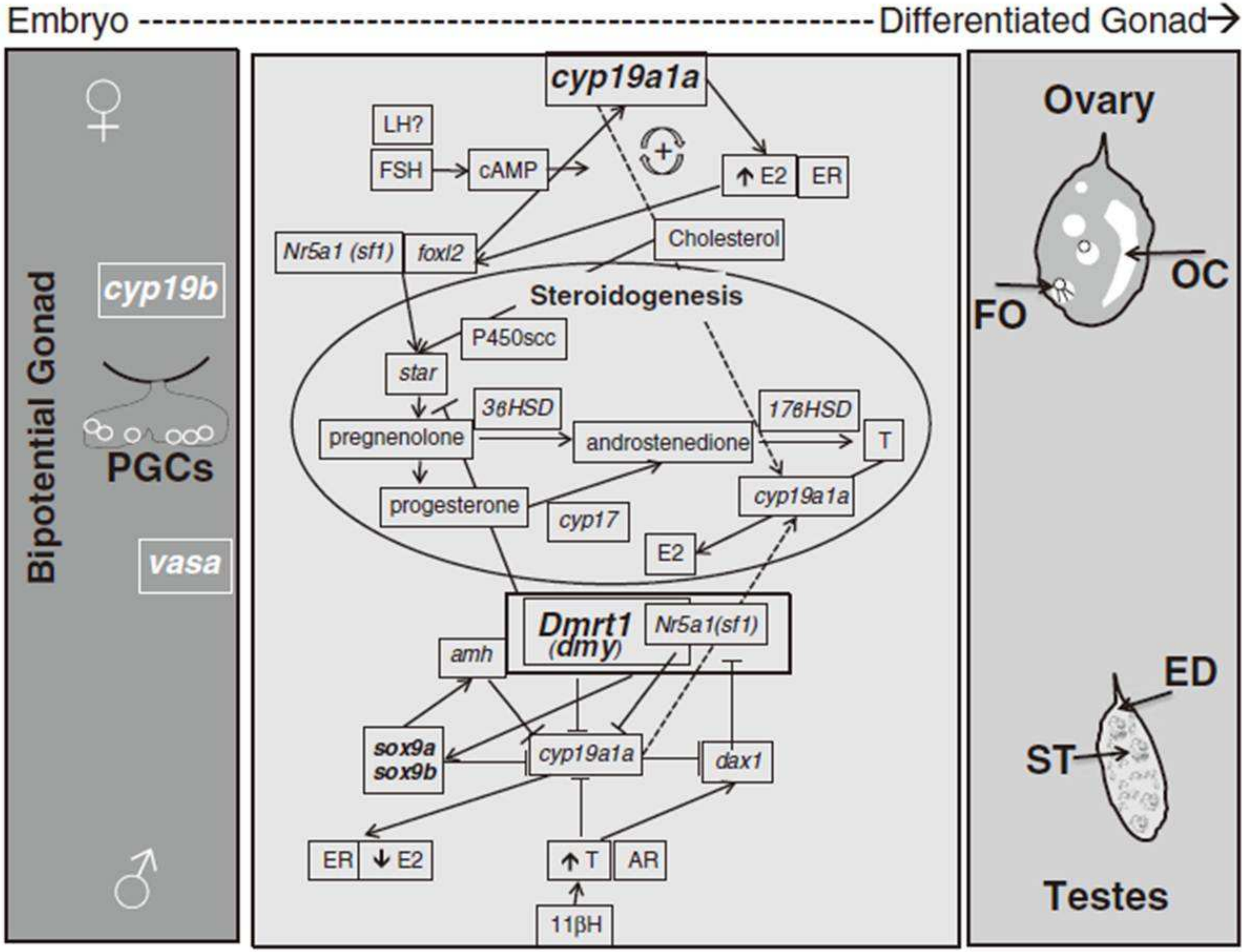


Diagram depicting genes involved in sex differentiation in a typical teleosts (Leet et al. (2011) *J. Appl. Toxicol.* 31:379-398)

Endocrine disrupting chemicals (EDCs)

- The effect of EDCs have been an emerging issue in environmental pollution.
  - An exogenous substance of mixture that alters functions of the endocrine system and consequently causes adverse effects in an intact organisms, or its progeny, or subpopulations (WHO, 2002)

Mechanisms of Action of EDCs

- In most teleosts, gonads retain bipotentiality even after gonadal differentiation (Devlin and Nagahama, 2002).
- Thus, exogenous hormones have the potential to cause sex reversal and disrupt reproductive processes even after sex differentiation has occurred.
- The biochemical pathway underlying fish reproduction, in particular, the key enzymes involved in steroidogenesis would be a potential target for EDCs

Reproductive effect of EDCs

Estradiol,
methyltestosterone,
nonylphenol, bisphenolA,
ethynylestradiol, *p,p'*- DDE

Medaka
Fathead minnow
Zebrafish, carp,
guppies, goldfish,
Rainbow trout,
Sand goby

- Reduced egg production
- Skewed sex ratio
- Reduced male GSI
- Decreased sexual behavior
- Intersex gonads
- Reduced egg fertility

Mills and Chichester (2005) *Sci. Total Environ*. 343: 1-34

Genomic approaches

Table 1 Summary of some studies on fish gonadal development using genomic approaches

Species common name	Monosex	Stage	Method	Verification	References
Guppy	No	Adults	454 titanium	PCR and RNA-seq	Fraser et al. (2011)
Largemouth bass	No	Adults	GS-20 and microarray	qPCR	García-Reyero et al. (2008)
Rainbow trout	Yes	Juvenile	Macroarray, microarray	qPCR	Baron et al. (2007, 2008)
Senegalese sole	No	Adult female	Microarray	ISH and qPCR	Tingaud-Sequeira et al. (2009)
Sturgeon	No	Juvenile and adults	454 GS		Hale et al. (2009)
Sturgeon	No	Adults	454 GS and titanium	qPCR	Hale et al. (2010)
Nile tilapia	Yes	Larvae and fry	qPCR		Ijiri et al. (2008)
Nile tilapia	Yes	Adult	Cell transfections, transgenics	EMSA, ISH	Wang et al. (2010)
Pejerrey	No	Juveniles	Microarray	qPCR	Fernandino et al. (2011)
Platyfish	No	Adults	454 titanium	qPCR	Zhang et al. (2011)
Zebrafish	Transgenic	Fry	Transgenics	IHC, histology	Wang et al. (2007)
Zebrafish	No	Adults	Microarrays	ISH and qPCR	Sreenivasan et al. (2008)
Zebrafish	No	Adults	Microarrays	qPCR	Small et al. (2009)

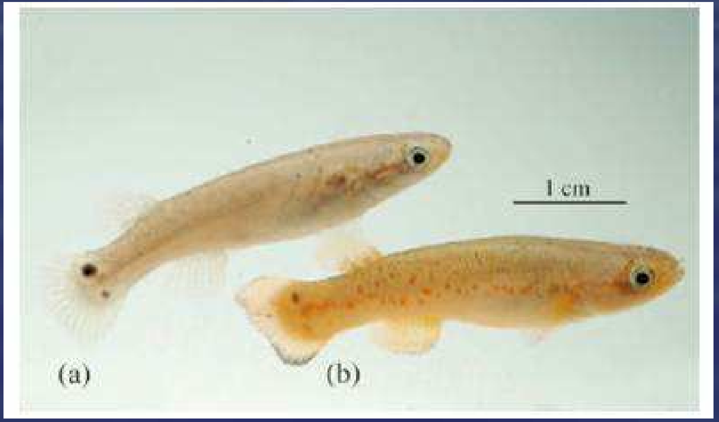
454 titanium and 454 GS are commercial products from Roche

EMSA electrophoretic mobility shift assay, *GS-20* generation sequencing, *IHC* immunohistochemistry, *ISH* in situ hybridization, *qPCR* real-time quantitative PCR

Piferrer *et al*. (2012) *Mar. Biotechnol*. 14:591-604

Model species
Kryptolebias marmoratus

- The mangrove killifish; synonym *Rivulus marmoratus* (Poey, 1880), order Cyprinodontiformes, family Rivulidae
- The only known internally self-fertilizing, protogynous hermaphroditic vertebrate in the world



a) Hermophrodite
b) Secondary male

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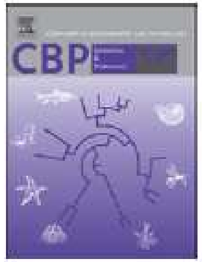

Analysis of expressed sequence tags from the liver and ovary of the euryhaline hermaphroditic fish, *Kryptolebias marmoratus*

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Using Roche 454, GS-20 sequencer,
59,732 transcripts in liver and 103, 526 transcripts in ovary were obtained.

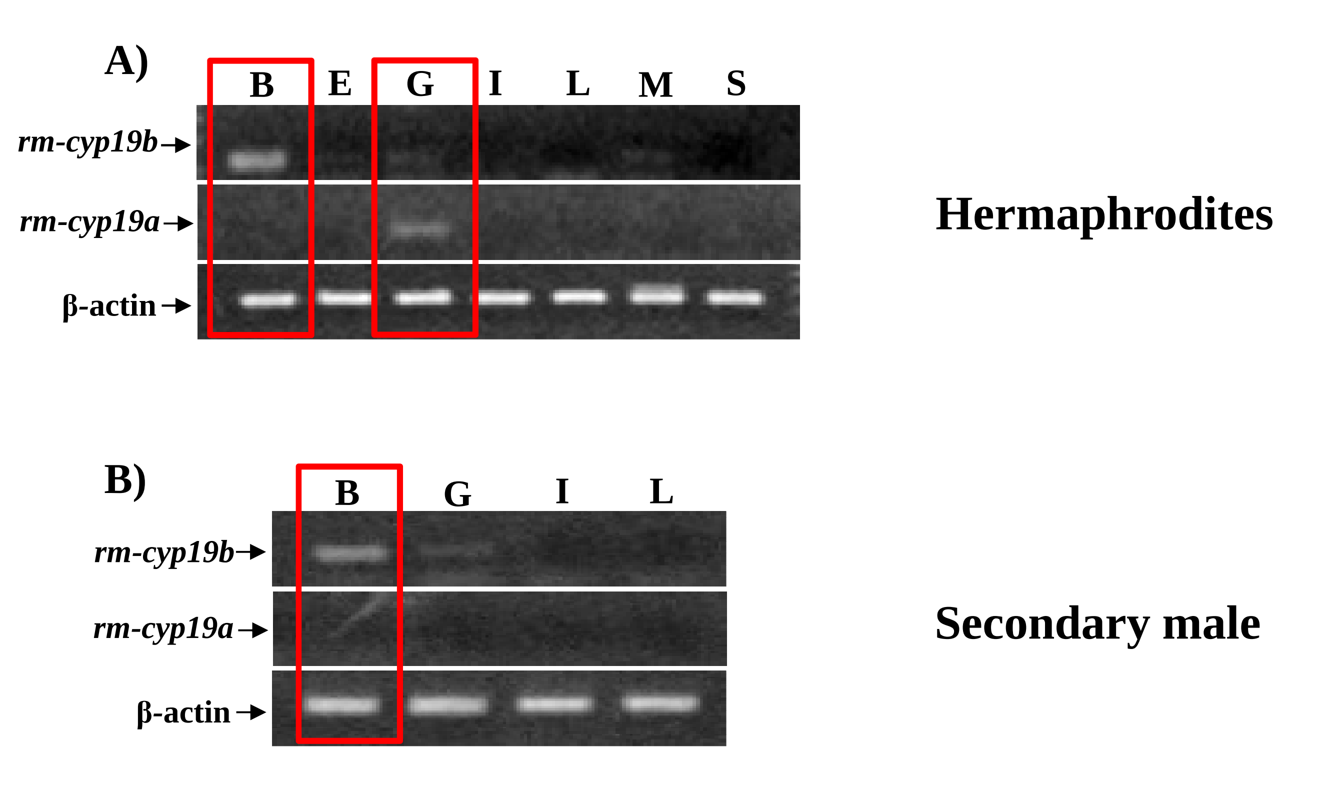
I. EDCs modulate expression of two distinct cytochrome P450 aromatase genes differently in gender types of the hermaphroditic fish *Kryptolebias marmoratus*

(Lee *et al*., 2006. *BBRC* 345: 894-903)

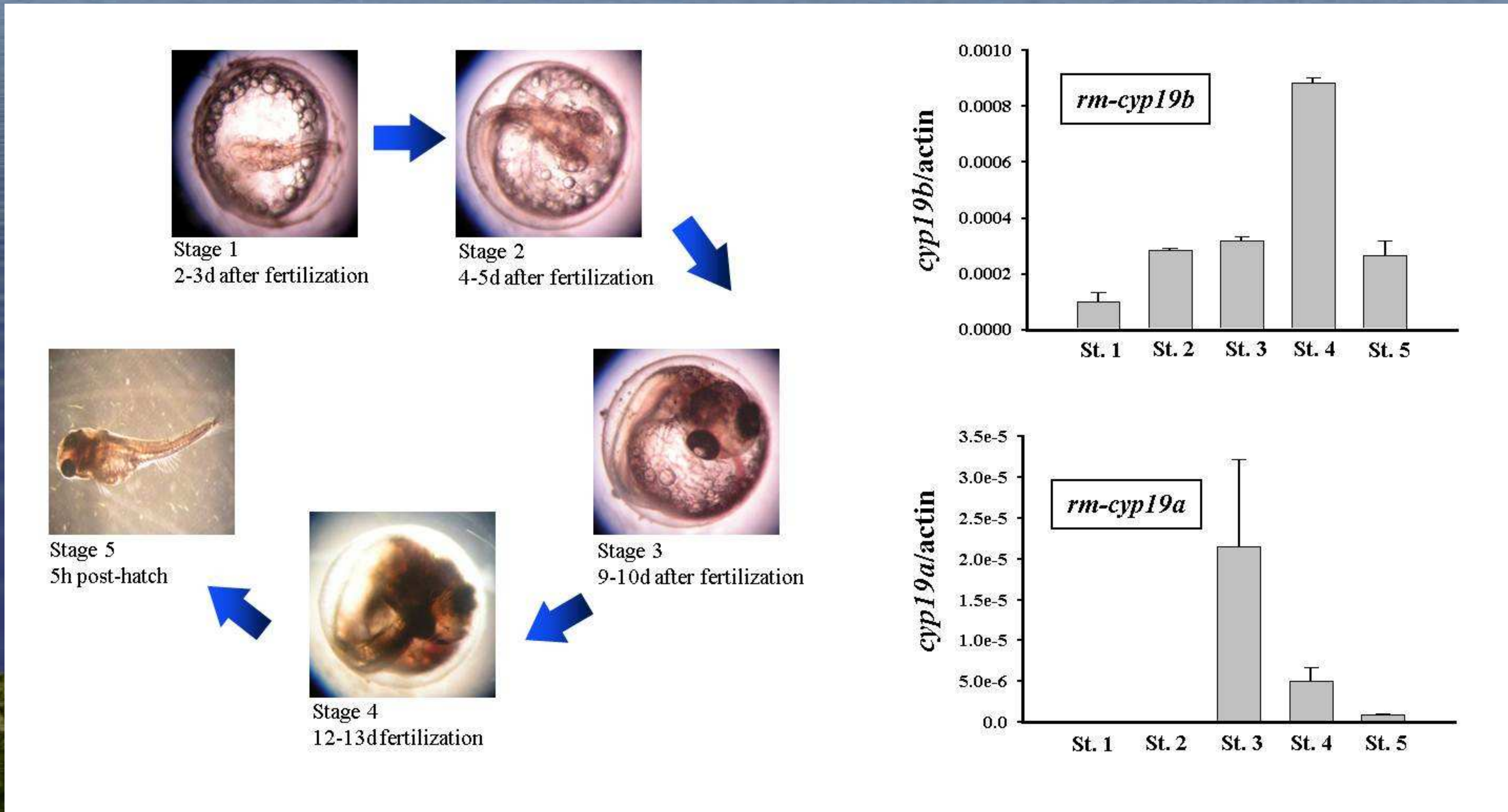
Cyp19 gene

- The *cyp19* gene is highly conserved throughout the vertebrate phylum, human, mouse, rat, cow, birds like chicken, zebra finch, reptile and fishes
- In mammals, with the exception of pig, there is a single *cyp19* gene with multiple tissue-specific promoters.
- In teleost fishes, at least two separate and distinct *cyp19* loci: *cyp19a* (predominantly gonad form) and *cyp19b* (predominantly brain form) encode structurally and functionally different aromatase isoforms.
- CYP19b is involved in the neural differentiation, survival, morphology and sexual behavior, while CYP19a is involved in sexual differentiation and oocyte growth.

Tissue distribution

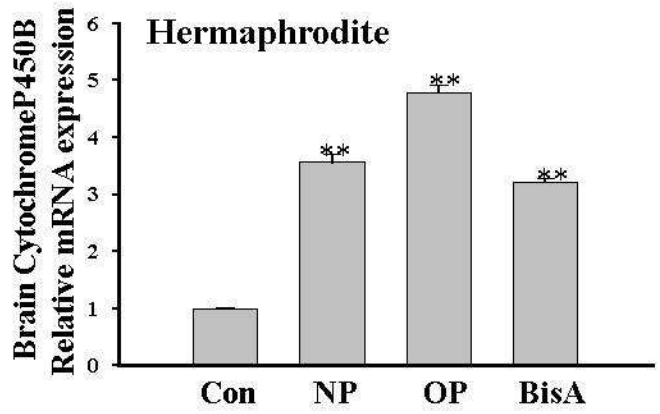


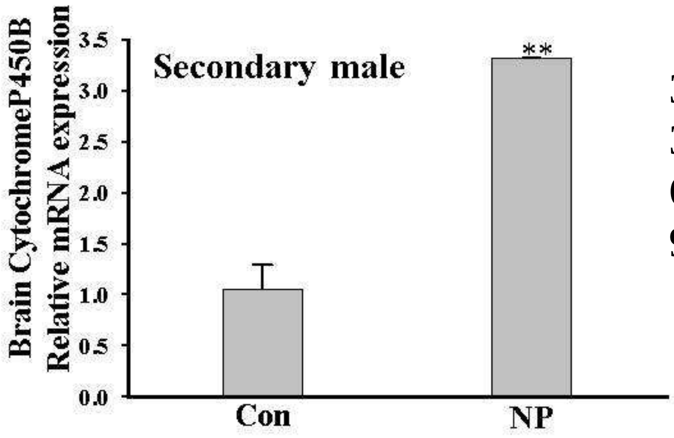
Developmental expression of *cyp19* genes



Expression of *cyp19* genes after exposure to EDCs

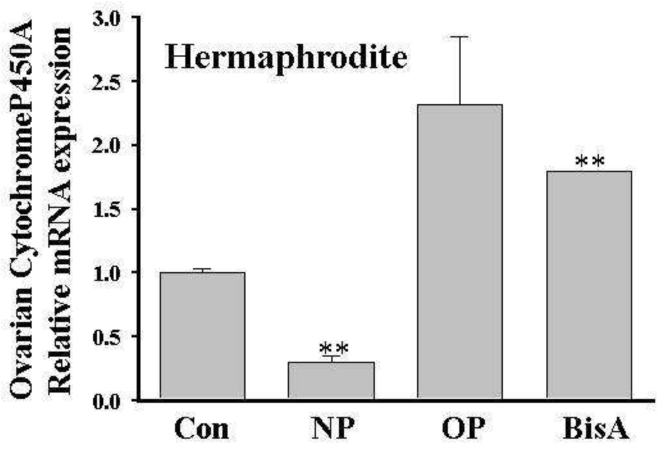
(1) ***km-cyp19b***

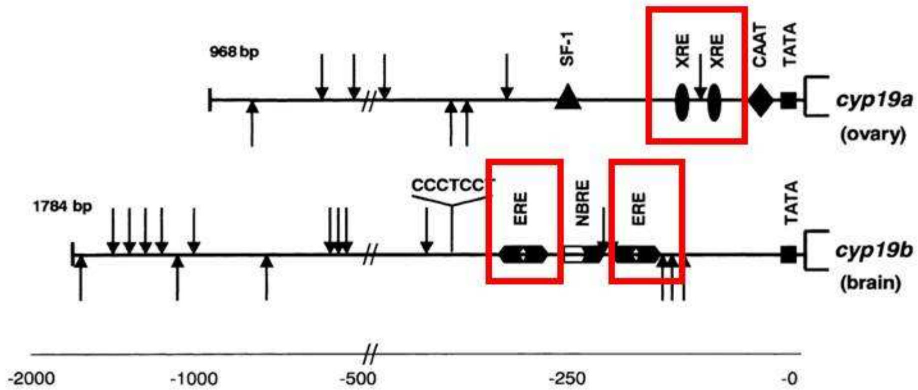




300 µg/L NP
300 µg/L OP
600 µg/L BPA,
96 h exposure

(2) ***km-cyp19a***





Callard *et al., J. Steroid Biochem & Mol. Biol.* (2001) 305-314

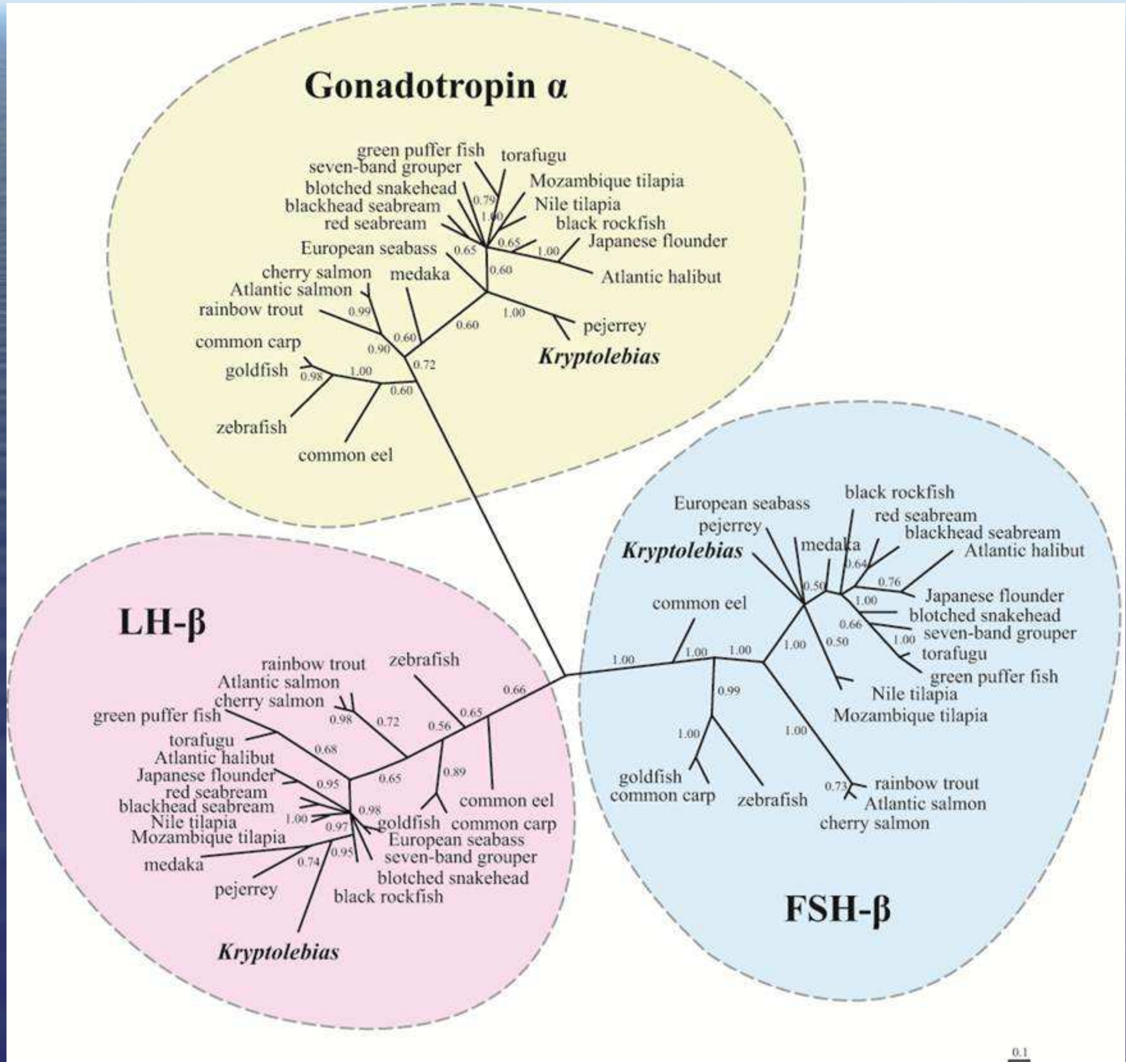
II. Bisphenol A modulates expression of gonadotropin subunit genes in the hermaphroditic fish, *Kryptolebias marmoratus*

(Rhee *et al*., 2010. *Comp. Biochem. Physiol*. C 152: 456-466)

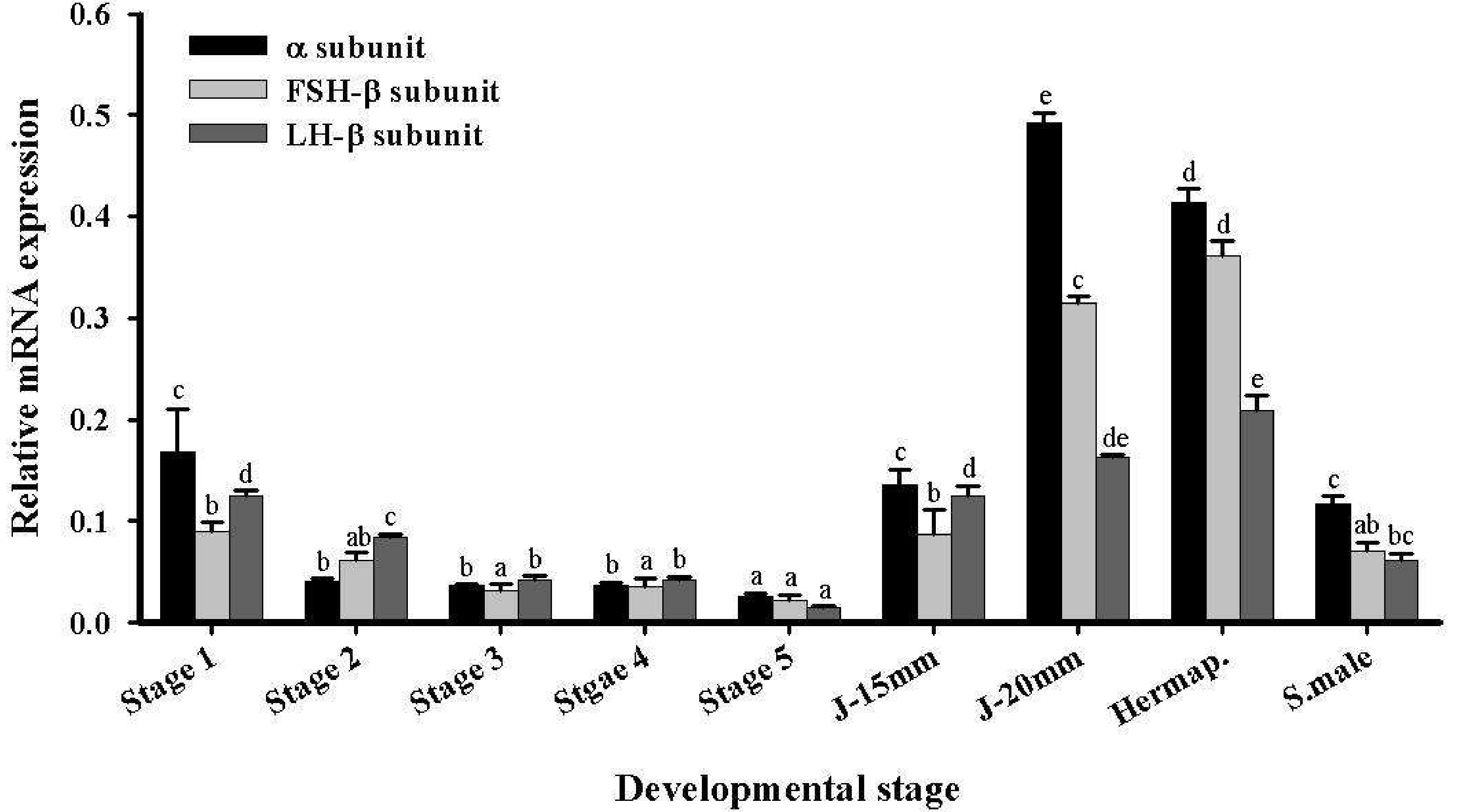
Gonadotropins

- Gonadotropins (GTHs) constitute a α:β heterodimer consisting of a common α subunit and the follicle-stimulating hormone β (FSH-β, GTHI) and luteinizing hormone β (LH-β, GTHII).
- They belong to the heterodimeric glycoprotein hormone family and are secreted in pituitary during reproductive development.

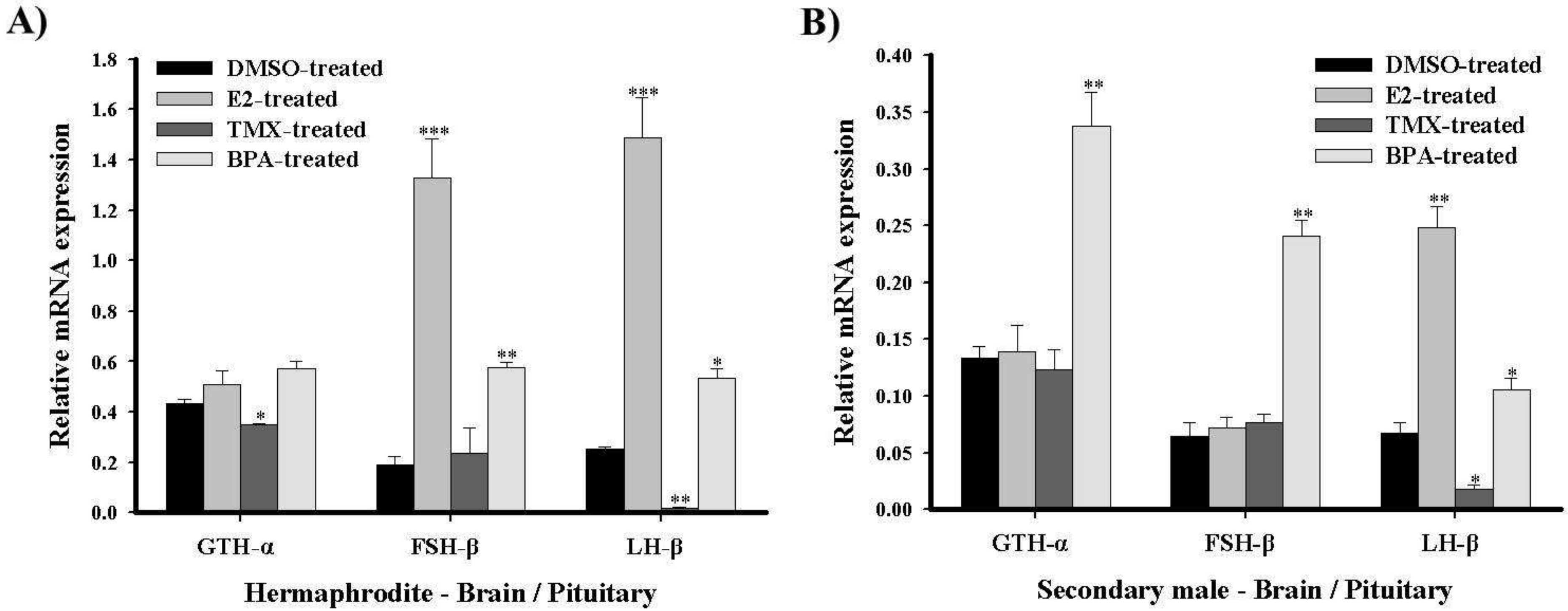
Phylogenetic relationship



Developmental expression

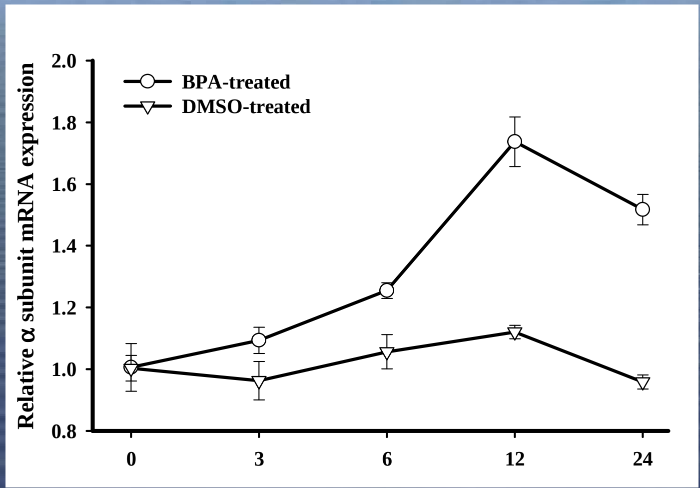


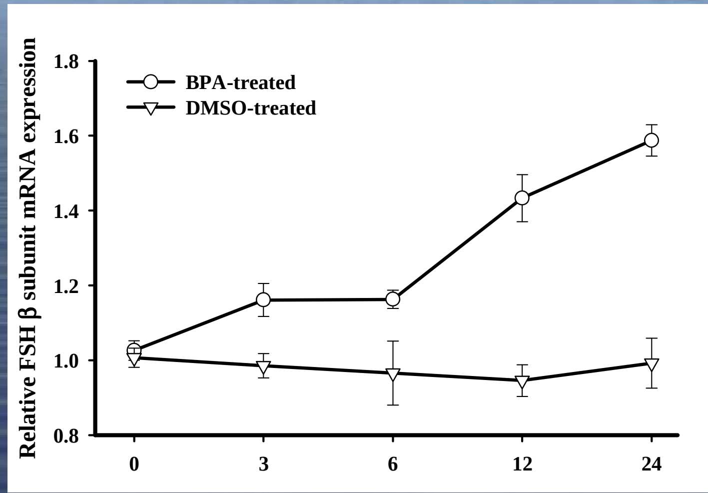
The effect of EDCs on the expression of gonadotropin genes

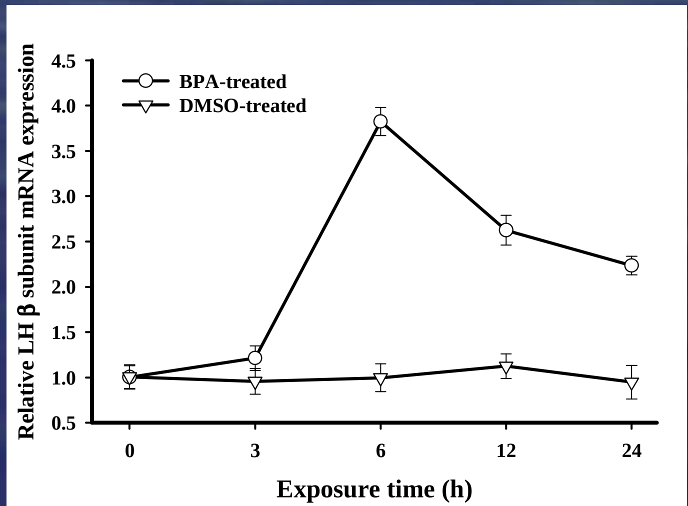


100 ng/L E2
10 μg/L TMX
600μg/L BPA,
96 h exposure

Time – course effect of BPA





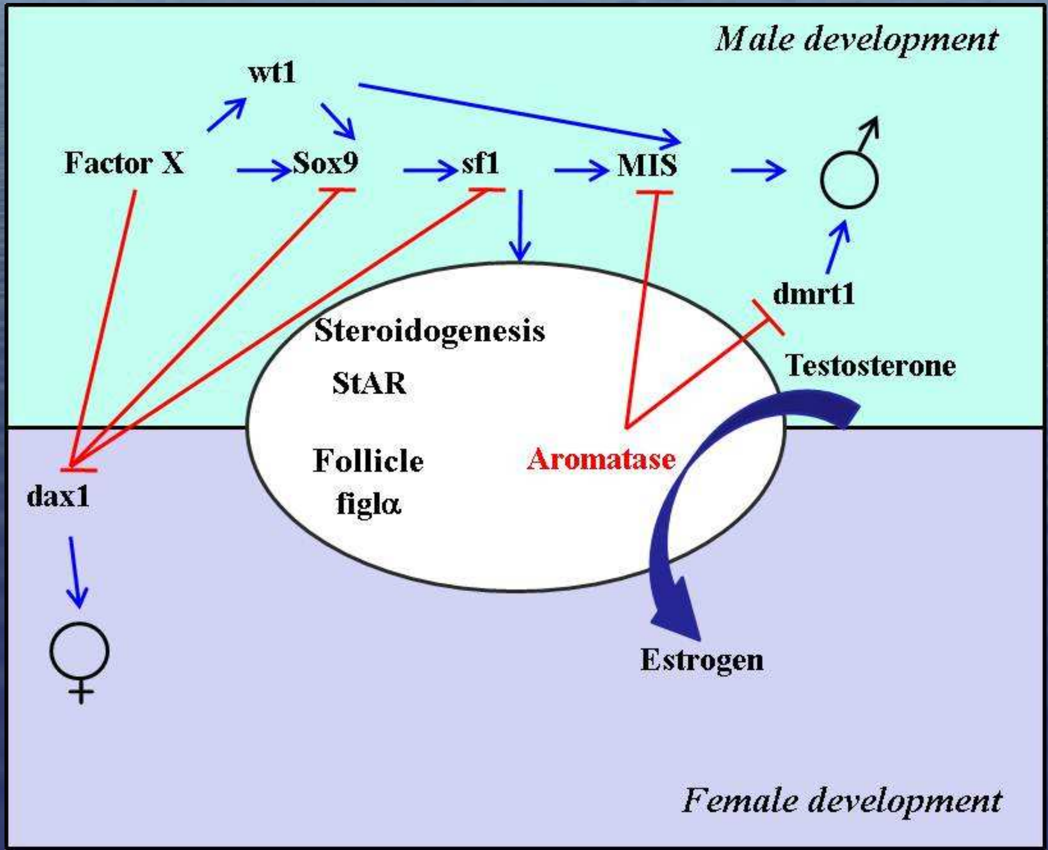


300μg/L BPA

III. Bisphenol A modulates expression of sex differentiation genes in the self-fertilizing fish, *Kryptolebias marmoratus*

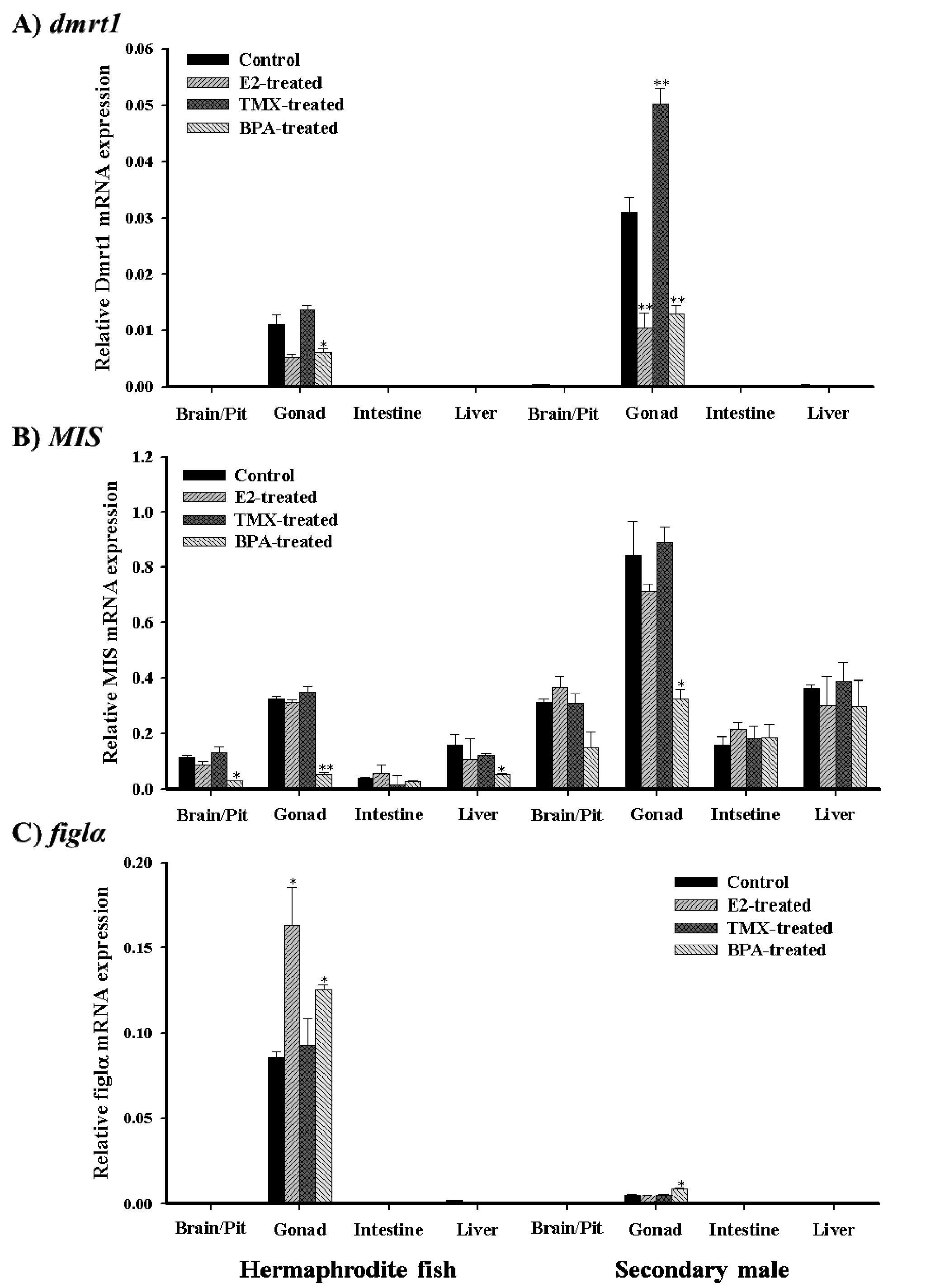
(Rhee *et al*., 2011. *Aquatic Toxicol*. 104: 218-229)

Sex differentiation – related genes

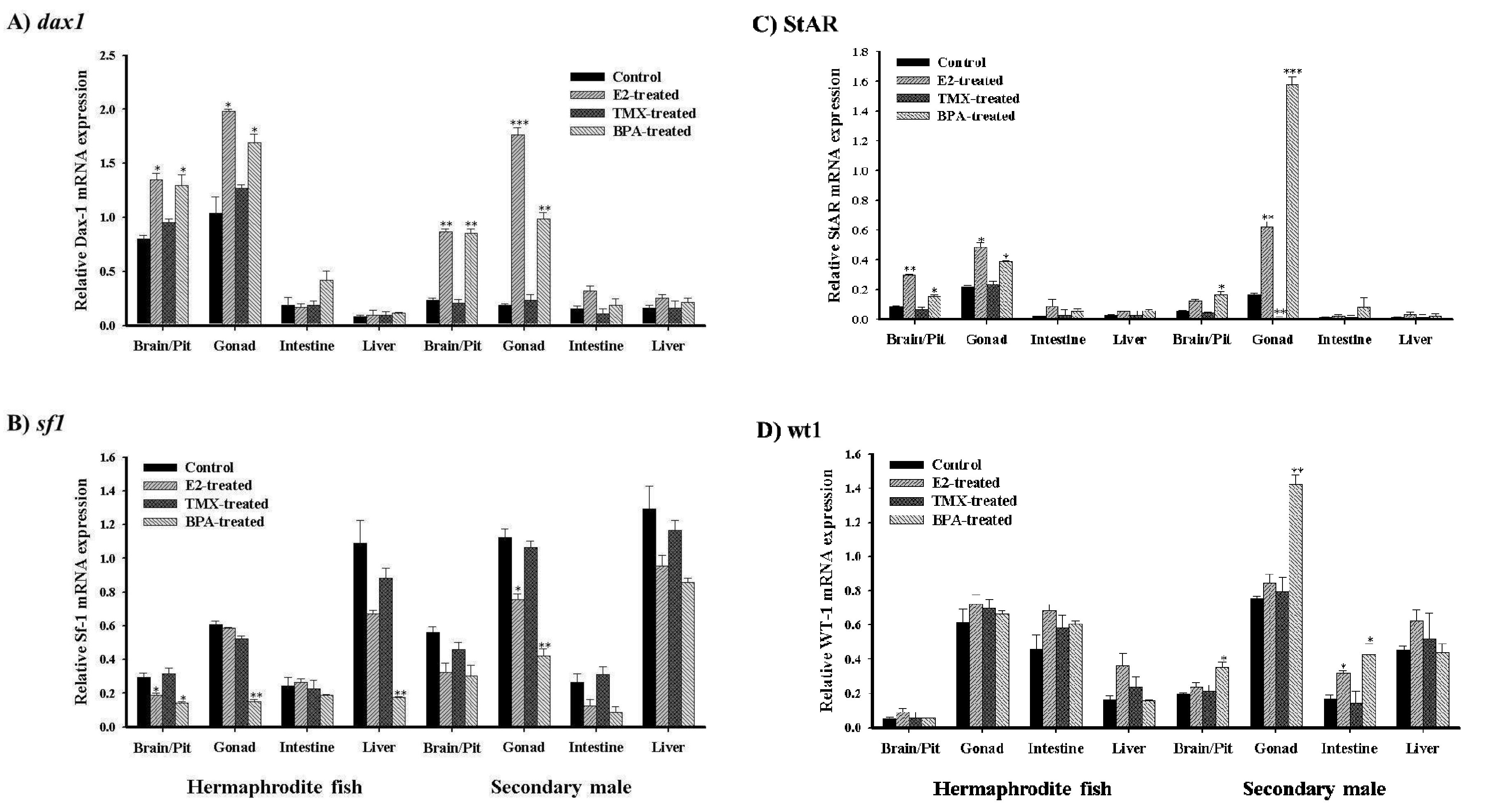


(Modified from von Hofsten, J., Olsson, P.-E., 2005. Reprod. Biol. Endocrinol. 3, 63) (Rhee et al., 2012)

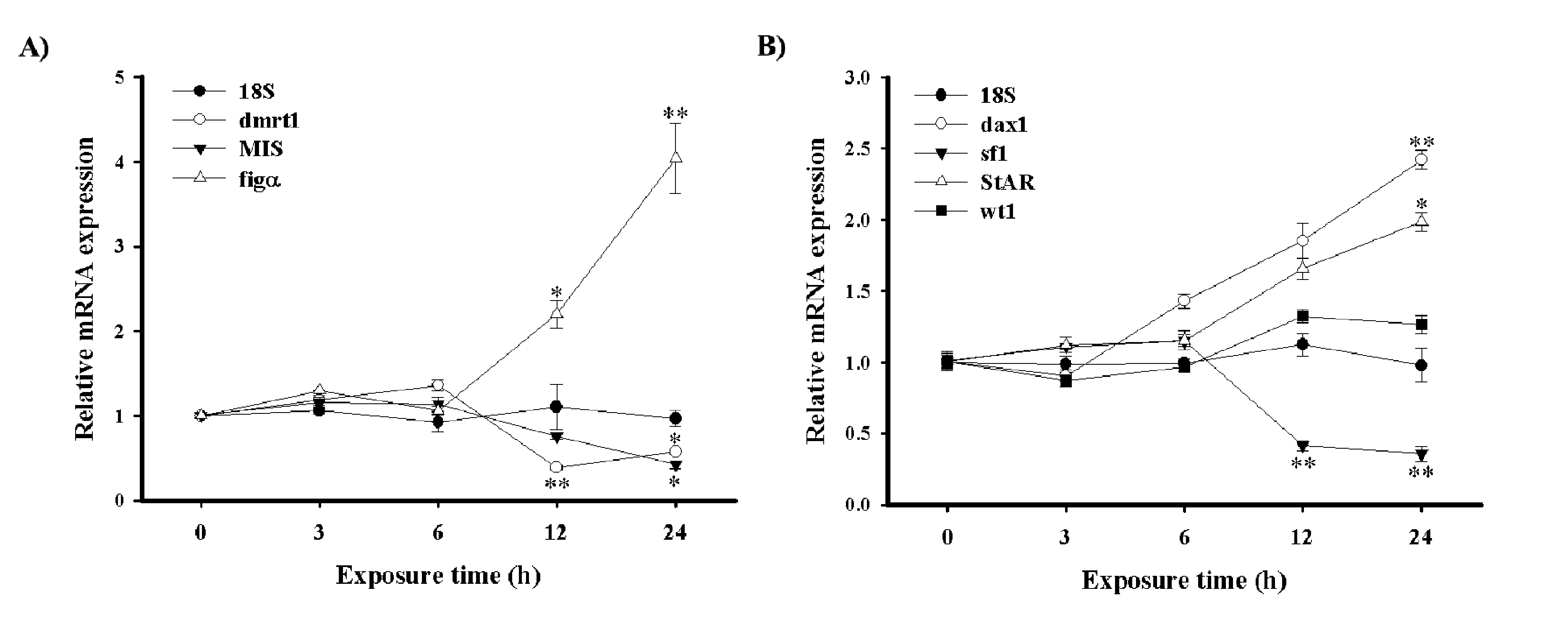
Effect of BPA



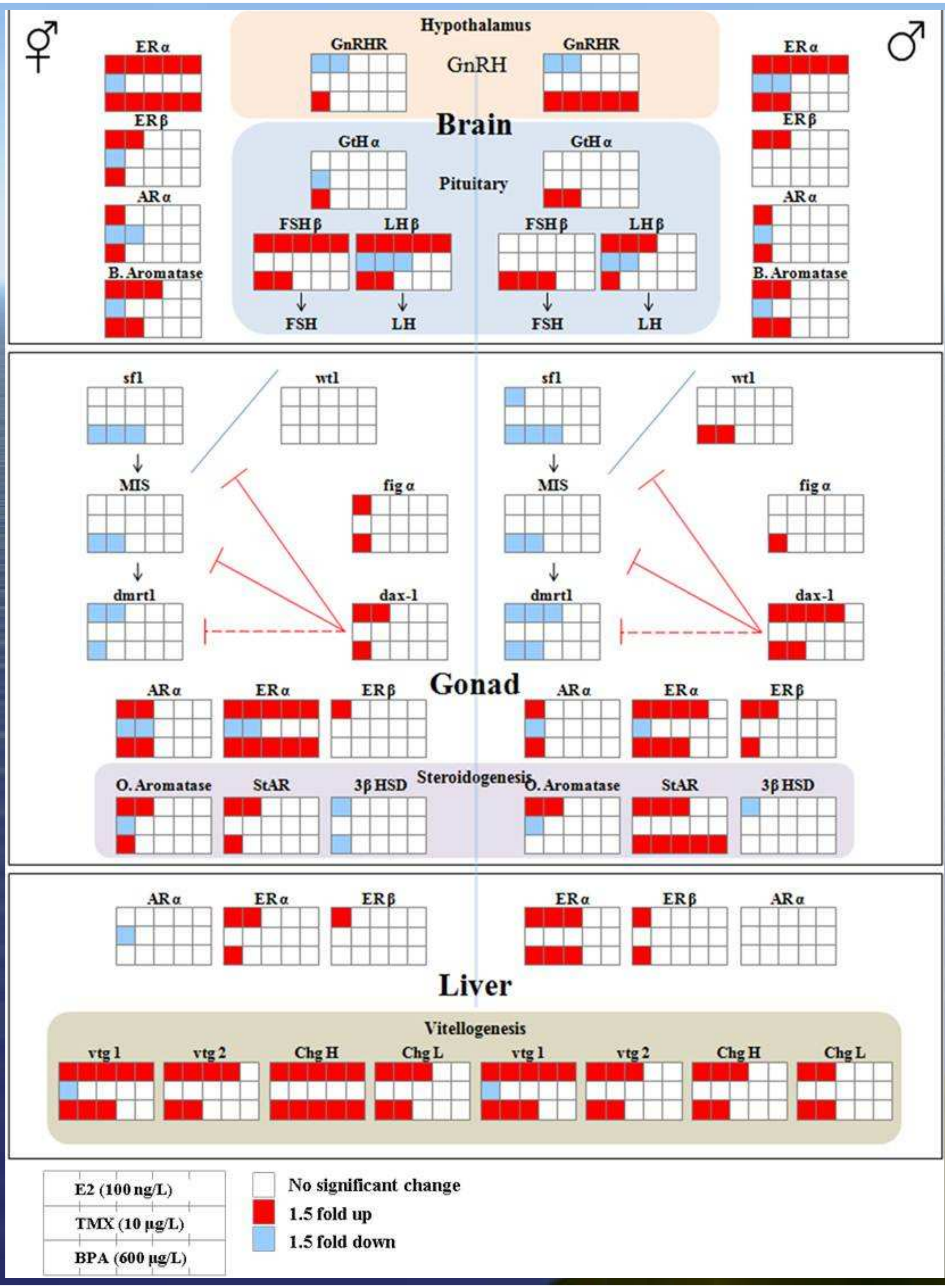
100 ng/L E2
10 μg/L TMX
600μg/L BPA,
96 h exposure


A) dax1
Relative Dax-1 mRNA expression
B) sf1
Relative Sf-1 mRNA expression
C) StAR
Relative StAR mRNA expression
D) wt1
Relative WT-1 mRNA expression
Control
E2-treated
TMX-treated
BPA-treated
Brain/Pit
Gonad
Intestine
Liver
Hermaphrodite fish
Secondary male

Time-dependent effect of BPA in juvenile fish



Real time PCR array



Summary

- BPA may modulates the expression of sex differentiation and steroidogenesis pathway genes.
- Differentially expressed genes in response to BPA may be potential biomarkers for risk assessment of EDCs exposure in aquatic environment.
- This study would provide a better understanding on molecular mechanisms of BPA exposure in the hermaphroditic fish, *K. marmoratus*.