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

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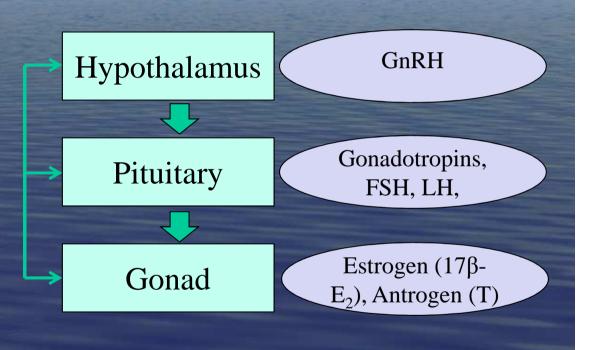
## Background

#### 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<sub>2</sub>) 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.

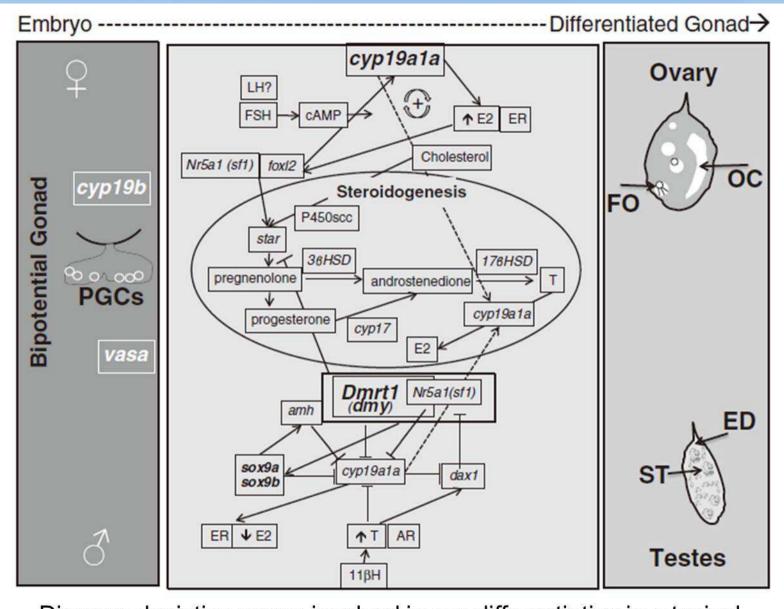


Diagram depicting genes involved in sex differentiation in a typical teleosts (Leet et al. (2011) *J. Appl. Toxicol.* 31:379-398)

## 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

Table 4					le 4 (continue	1)				
Reproductive effects, ranked from	m most common to least co	omme * in fish exposed in th	e laboratory to single	s	productive effe	et	EDC exposure	Species	References	
Reproductive effect	EDC exposure	Spec.	References		ersex gonads		estradiol	carp	Gimeno et al., 1998b	
Reduced egg production (or live young) <sup>1</sup>	estradiol	medaka	Patyna et al.	rt al., ?			ethynylestradiol	medaka	Metcalfe et al., 2001; Seki et al., 2002	
			2002				estrone	medaka	Metcalfe et al., 2001	
							estriol octylphenol	medaka medaka	Motealfe et al., 2001 Grav et al., 1999	
	ethynylestradiol	fathead minnow	H	Estradiol,			inchenol A	medaka	Metcalfe et al., 2001	
	emphylestraulo:	Illera					restosterone	medaka	Koger et al., 2000	
		tau.	methy	ltestosteror	ne.		methyltestosterone	zebrafish	Orn et al., 2003	
		sand goo,	-		-		estradiol	medaka	Kang et al., 2002	
	nonylphenol	fathead minne.	nonylphe	enol, bisphe	nolA,		ethynylestradiol	zebrafish	Segner et al., 2003	
	methoxychlor	fathead minner	~ 1	· •				fathead minnow	Pawlowski et al., 2004	
	bisphenol A		ethynyles	tradiol, p,p'	- DDE					
					Decrease characte					
Skeweć sex ratio <sup>1</sup>	<b>N</b> /				characte		Reduced egg production			
	Medaka					<b>1</b>	A Reduced egg production			
			98; Koger et al., 20				(1 1	<b>, •</b>		
	Fatha	Fathead minno			Males with a reduce	• 5	Skewed se	ex ratio		
	rameau miniov		w	holz and Gutzeit, 2000		~				
	Zabrafiah aarn net al. 2003		m et al., 2003	Reduced male GSI						
	Zebrafish, carp,									
	anna coldfich 8				• Т	Decreased	l cevual	hehavior		
guppies, goldfish,						L	Juliasu	i seruai		
					Alterations Intercov gonoda					
	Rainbow trout,				Intersex gonads					
Reduced male Gona Index (GSI)					Decreased • Reduced egg fertility					
	Sand goby				Decreased	• Ի	keduced e	egg Ierti	ltv	
	Dai	iu goby	, 19985		or hatch			00		
			.c al., 1996 .cr ct al., 1998						0	
	non,		Jobling et al., 1998		Physical deformu					
	octylphenoi		Jobling et al., 1996		Thysical determine	•	octvlphenol	medaka	Grav et al., 1999	
	pentylphenol	curp	Gimeno et al., 19985		Altered plasma st	eroid	nonylphenol	rainbow trout	Schwaiger et al., 2002	
	bisphenol A	fathead minnew	Sohoni et al., 2001		hormone conce		methoxychlor	fathead minnow	Ankley et al., 2001	
creased sexual behavior	estradiol	medaka	Oshima et al., 2003		Reduced female (	ionadosomati		fathead minnow	Schoni et al., 2001	
in males		goldfish	Bjerselius et al., 2001		Index (GSI)		vinclozolin		Makynen et al., 2000	
	ethynylestradiol	guppics zebratish	Bayley et al., 1999 Segner et al., 2003 Robinson et al., 2003 Gray et al., 1999		Females with mal	e sexual	methyltestosterone	fathead minnow	Ankley et al., 2001	
	carynyresuautor	sand goby			characteristics					
	octylphenol	medaka			Superscript indicates that two effects had identical ranking.					
	guppics		Bayley et al., 1999		<sup>a</sup> Ranking determined by summing the number of EDCs that caused the effect with the number of species exhibiting the effect.					
	bisphenol A	zebrafish	Segner et al., 2003							
	vinclozolin	guppies	Bayley et al., 2002							
	flutamide	guppies	Bayley et al., 2002							
itersex gonads	estradiol	medaka	Kang et al., 2002; Ke Metcalfe et al., 2001	ger et al., 2000;	Mills a	nd Ch	ichester (20	005) Sci. To	otal Environ. 343: 1-	

### 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

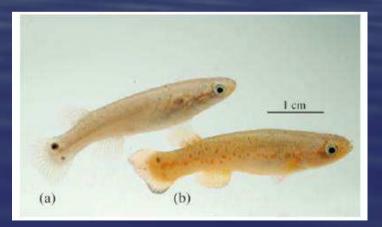
*EMSA* electrophoretic mobility shift assay, *GS-20* generation sequencing, *IHC* immunohistochemistry, *ISH* in situ hybridization, *qPCR* real-time quantitative PCR



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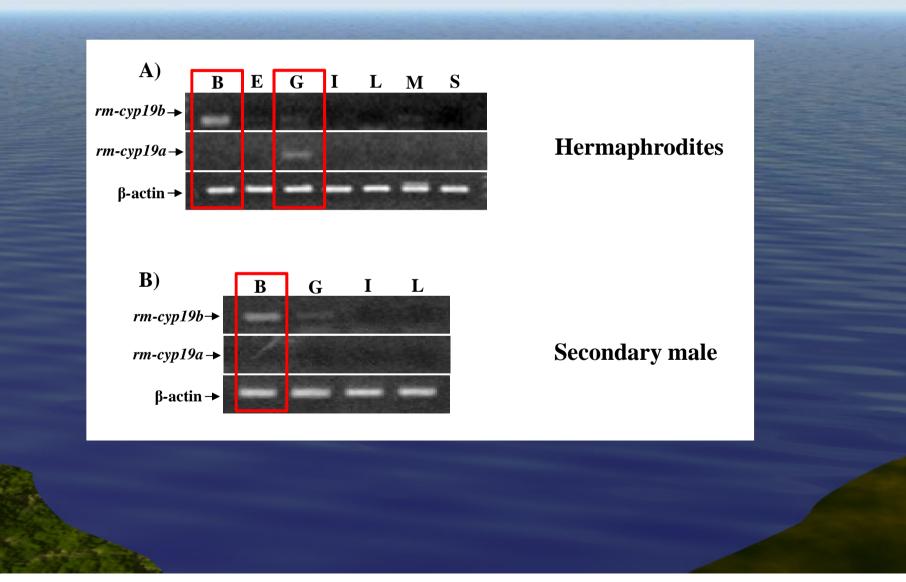
Using Roche 454, GS-20 sequencer, 59,732 transcripts in liver and 103, 526 transcripts in ovary were obtained.

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)

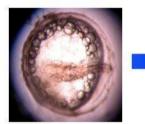
## Cyp19 gene

- 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



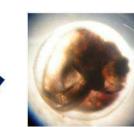
Stage 1 2-3d after fertilization



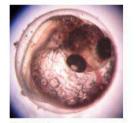
Stage 2 4-5d after fertilization



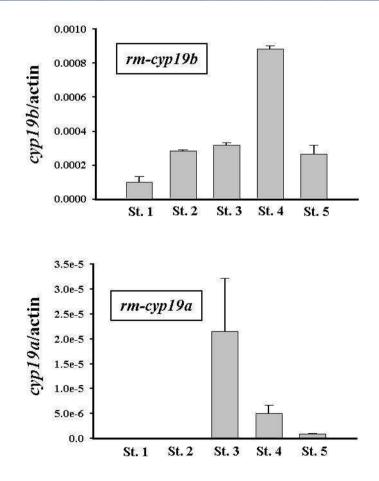
Stage 5 5h post-hatch



Stage 4 12-13d fertilization

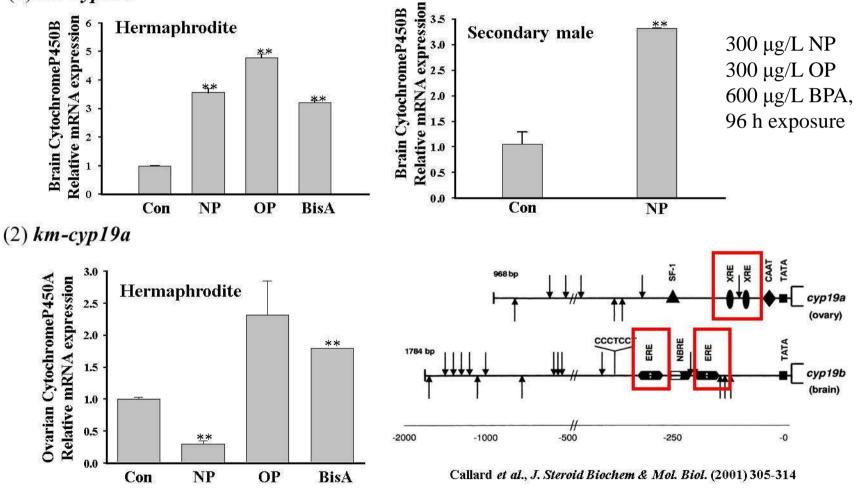


Stage 3 9-10d after fertilization



## Expression of *cyp19* genes after exposure to EDCs

(1) km-cyp19b

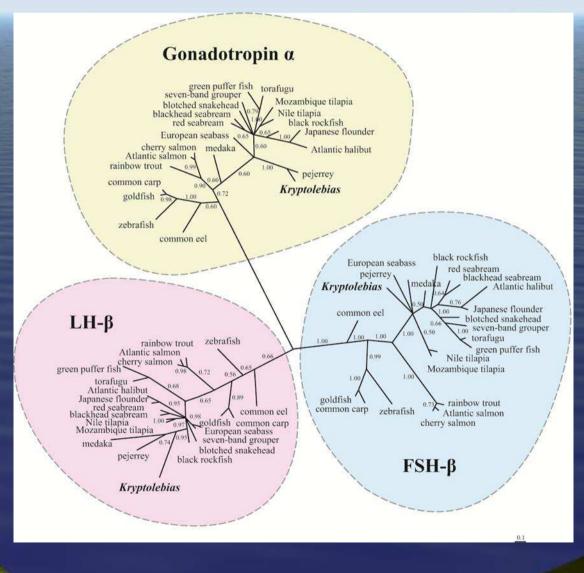


II. Bisphenol A modulates expression of gonadotropin subunit genes in the hermaphroditic fish, *Kryptolebias marmoratus* (Rhee *et al.*, 2010. *Comp. Biochem. Physiol.* C 152: 456-466)

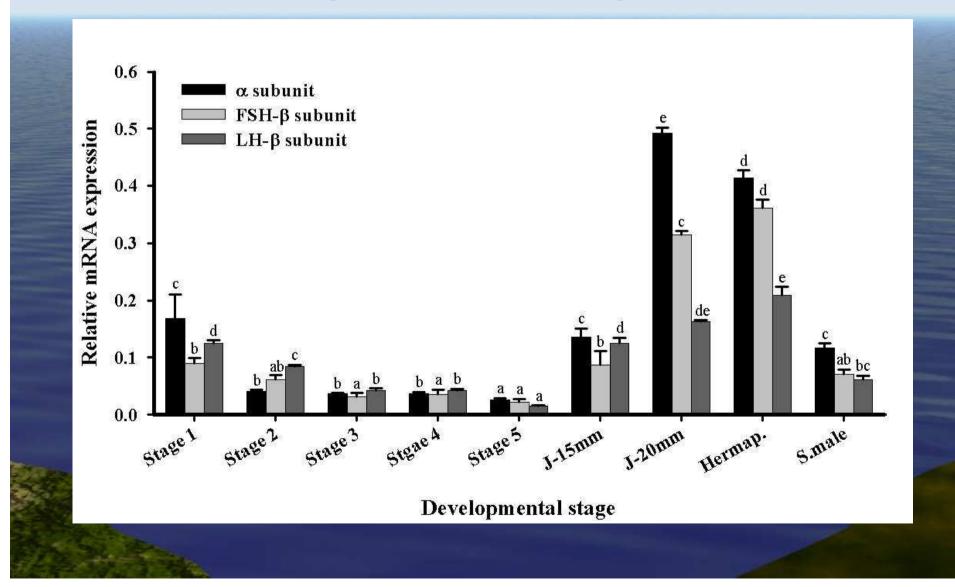
#### Gonadotropins

- 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.

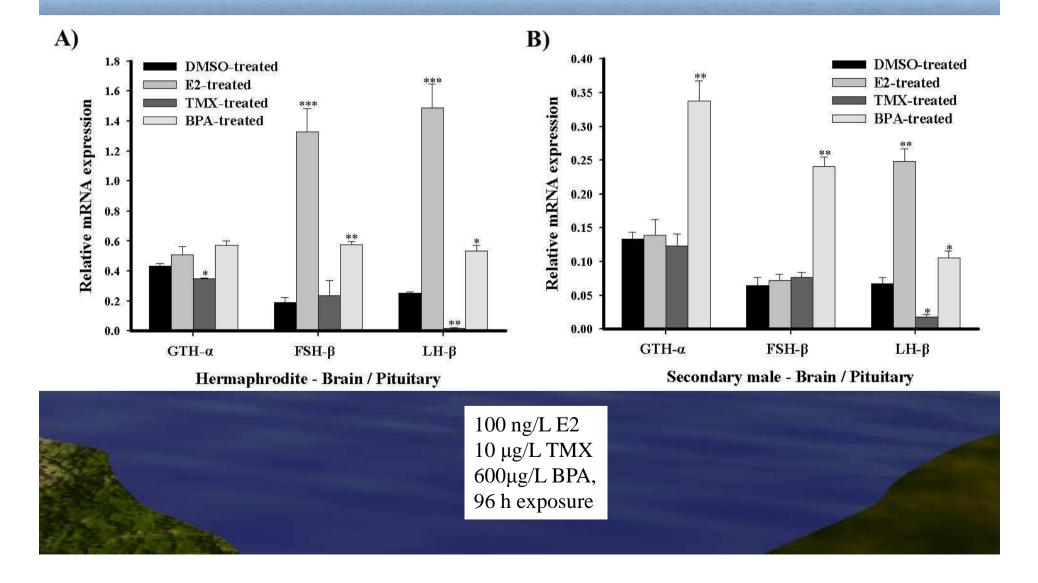
#### Phylogenetic relationship



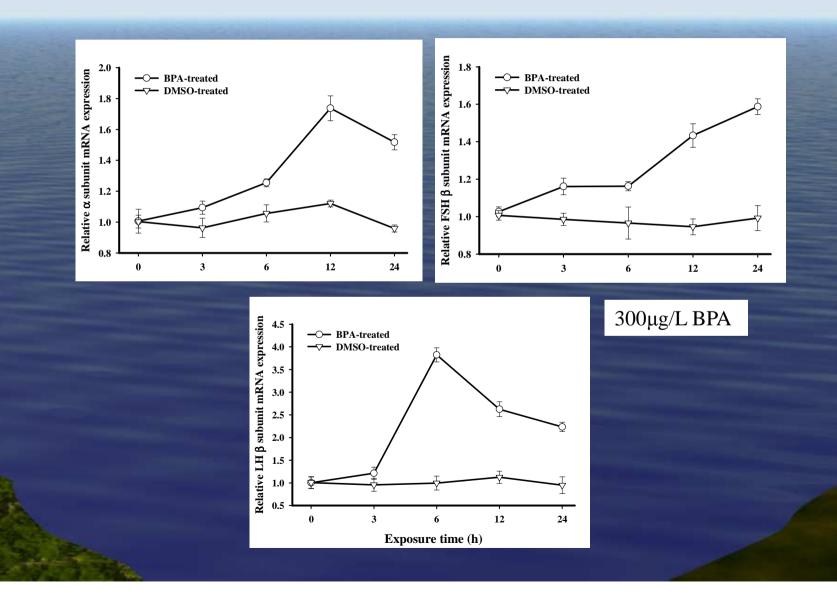
#### **Developmental expression**



## The effect of EDCs on the expression of gonadotropin genes

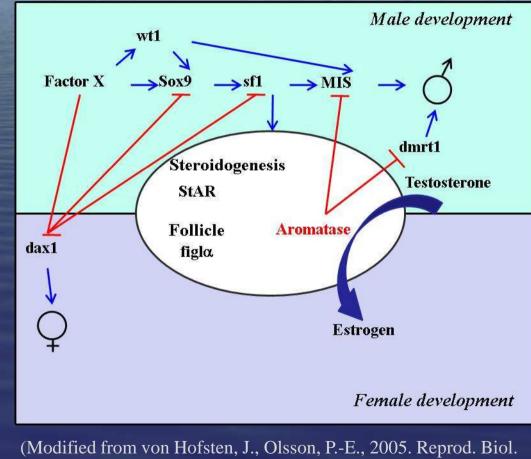


#### Time – course effect of BPA



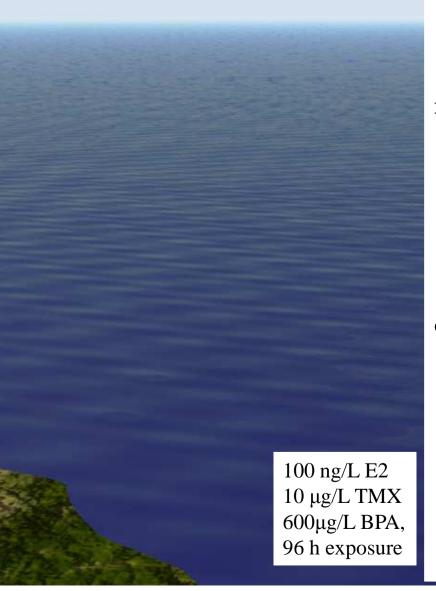
III. Bisphenol A modulates expression of sex differentiation genes in the self-fertilizing fish, *Kryptolebias marmoratus* (Rhee *et al.*, 2011. *Aquatic Toxicol.* 104: 218-229)

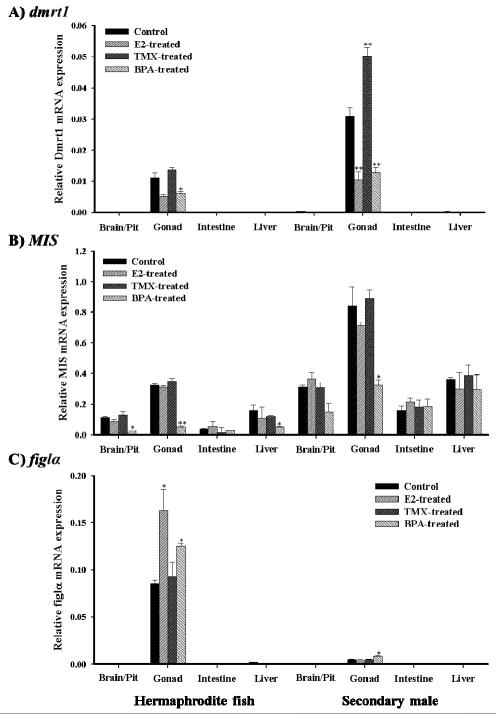
# Sex differentiation – related genes

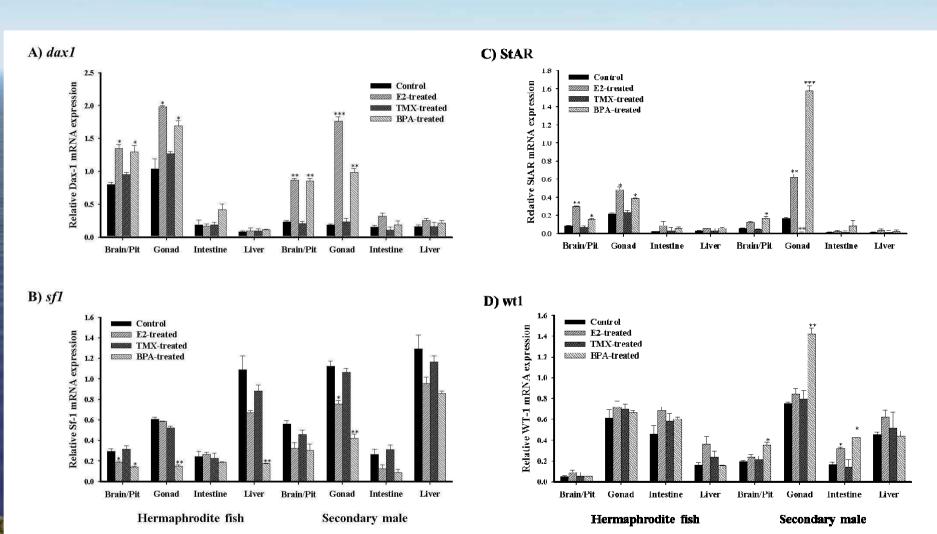


Endocrinol. 3, 63) (Rhee et al., 2012)

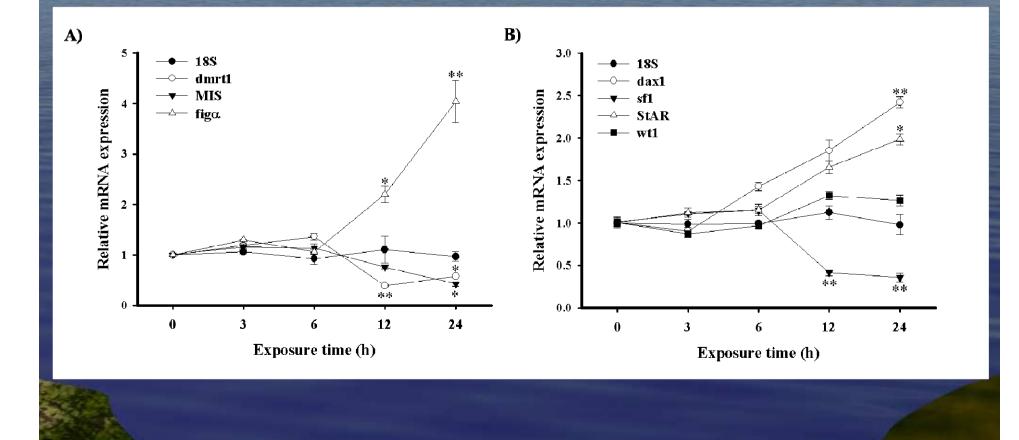
#### Effect of BPA



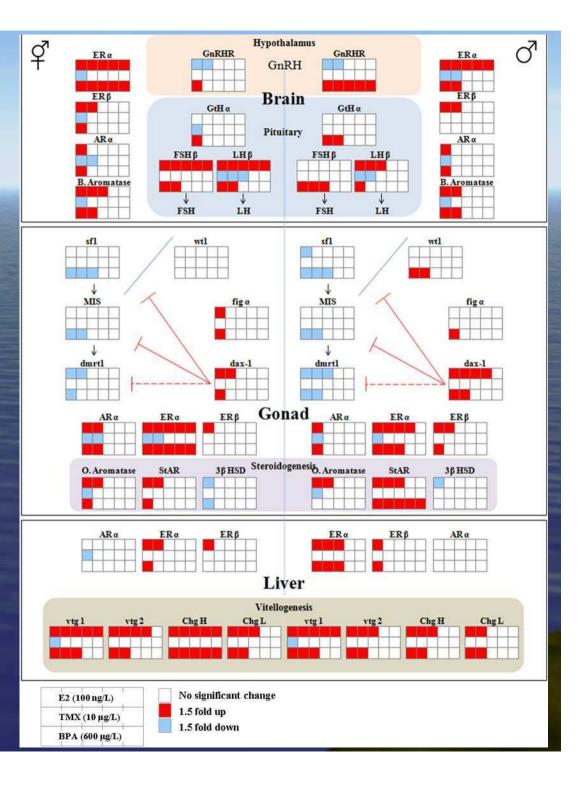




## Time-dependent effect of BPA in juvenile fish



## 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.