

EDA-EMERGE, 30 January, Verneuil-en-Halatte, France



Endocrine Disrupting Chemicals (EDCs) in fish

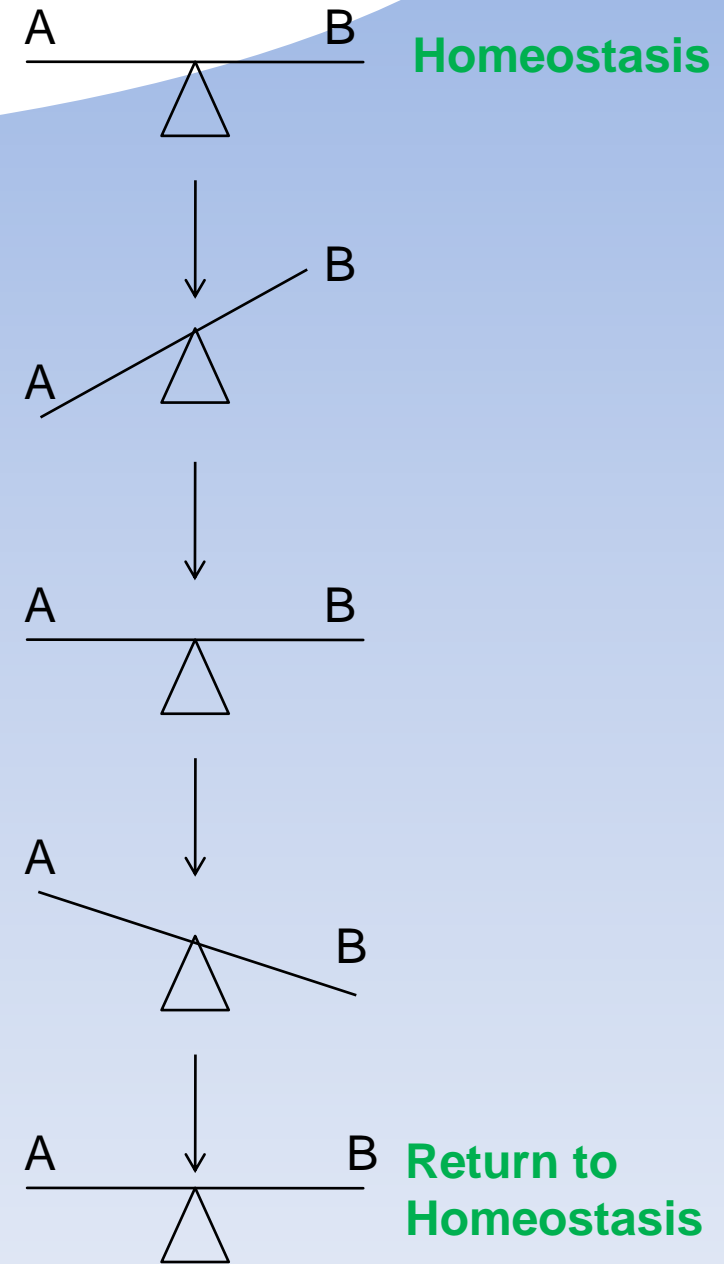
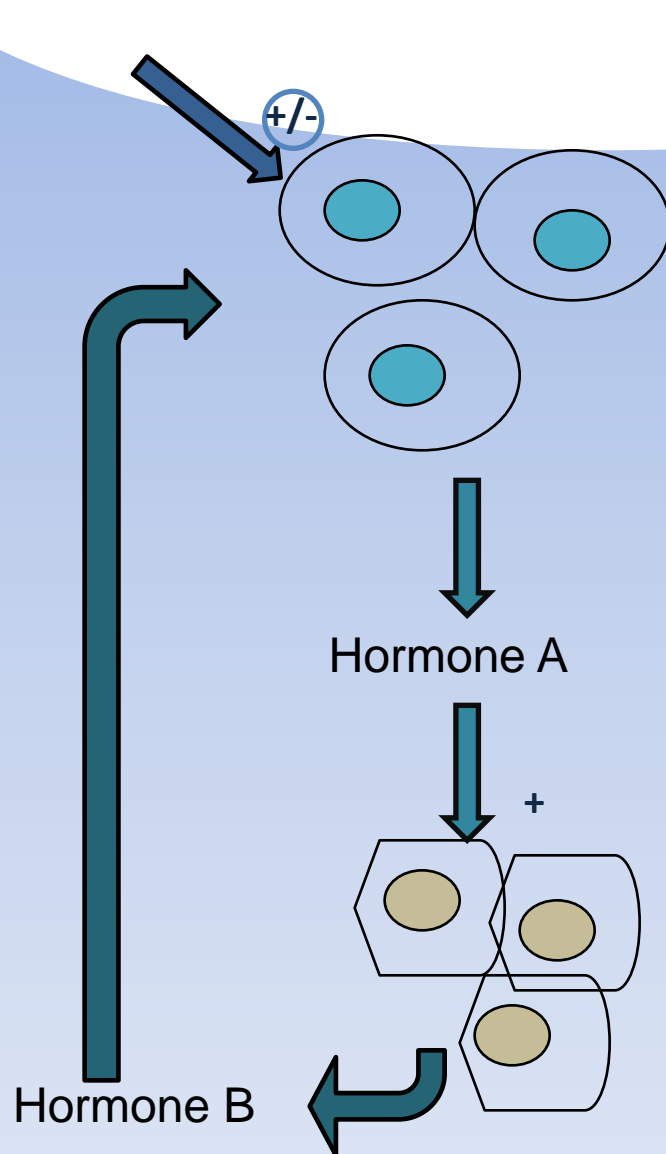
François BRION

INERIS, Unité d'écotoxicologie in vitro et in vivo

INERIS

- Complex system composed of numerous glands
- Control important physiological functions
 - Immunity
 - development and growth
 - Reproduction
- The endocrine system is **regulated**

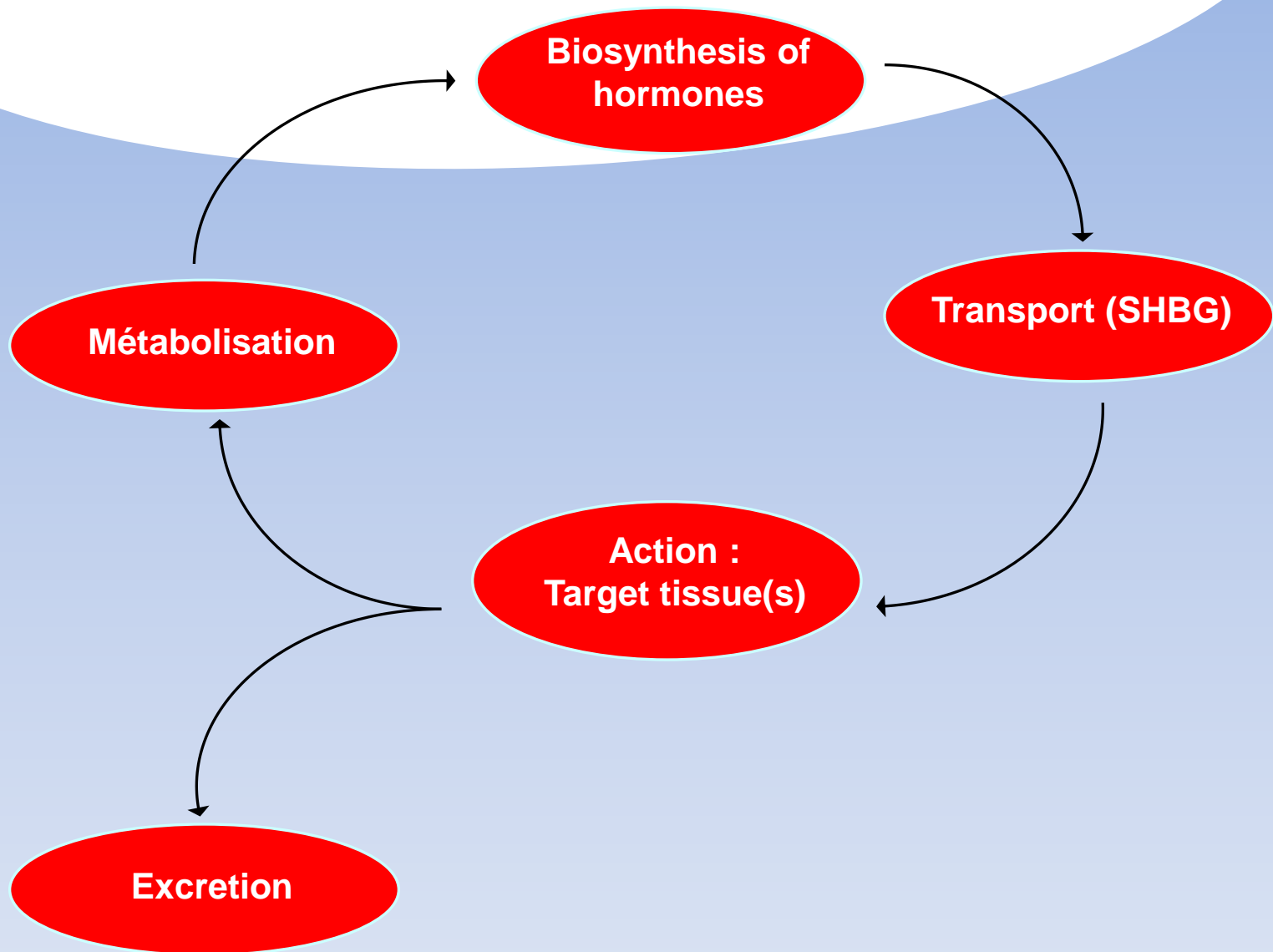
Some Generals about the endocrine system



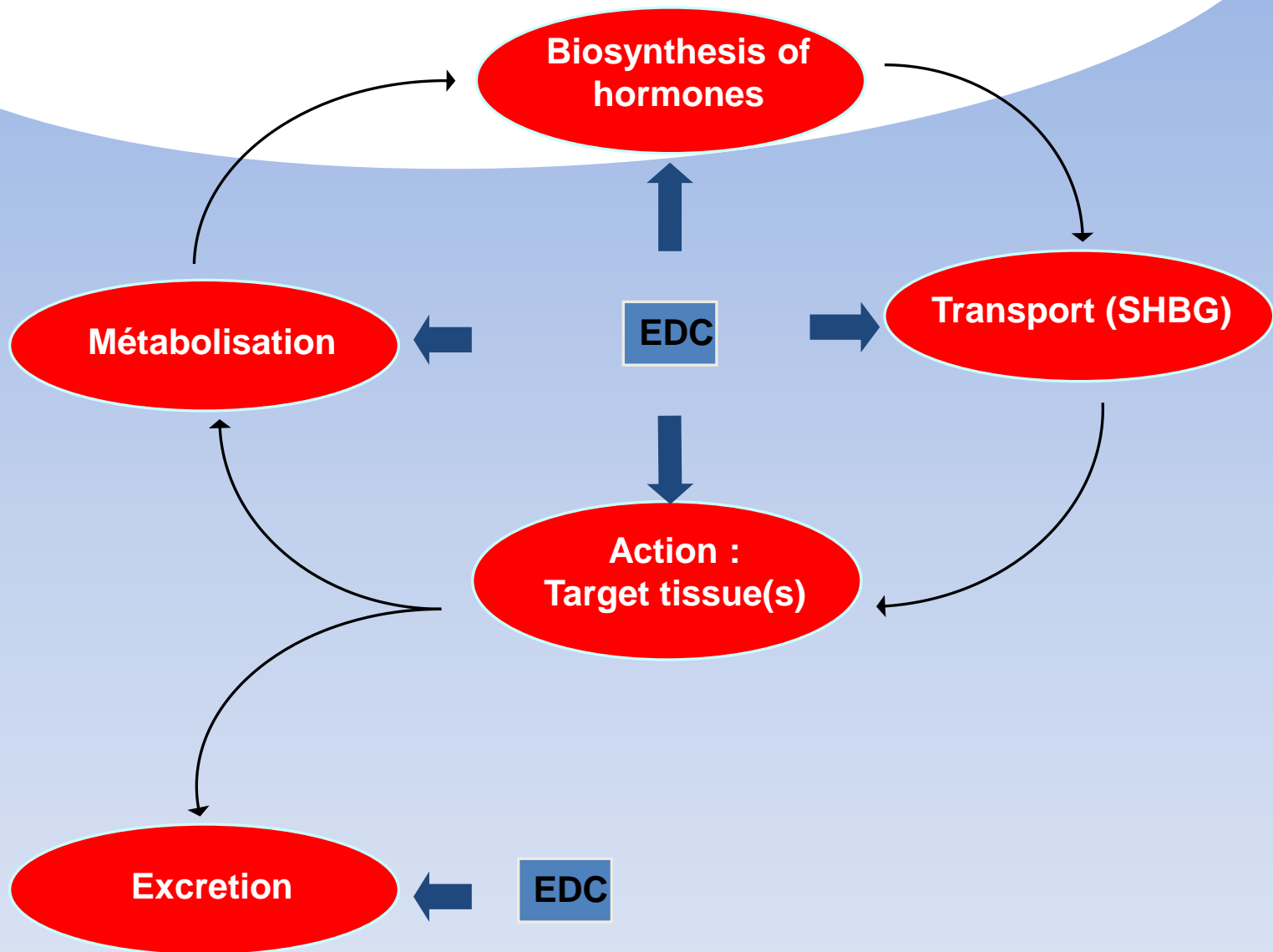
EDCs are

“...exogenous agents that interfere with the **production, release, transport, metabolism, binding, action or elimination** of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental processes.” (Kavlock et al., 1996)

Endocrine System and hormonal dynamic



Endocrine System and hormonal dynamic



Various mode of action through which EDCs may interfere with the hormonal dynamics

EDCs are

“...exogenous agents that interfere with the **production, release, transport, metabolism, binding, action or elimination** of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental processes.” (Kavlock et al., 1996)

“...an exogenous substance that causes adverse health effect in an intact **organism, or its progeny**, secondary (consequent) to **changes in endocrine function** and consequently causes **adverse health effects** in an intact **organism, or its progeny, or (sub)populations**” (EU, 1997).

Detergents
(alkylphénols)

Plasticizers
phthalates)

Natural hormones
(endogenous
hormones, phyto-
œstrogens)

Synthetic hormones
(contraceptive pills)

EDCs in aquatic environment:
Which compounds?

PAHs, PCBs

Pesticides

Emerging pollutants:
Personal care products
(UV filters;),
pharmaceuticals,
...

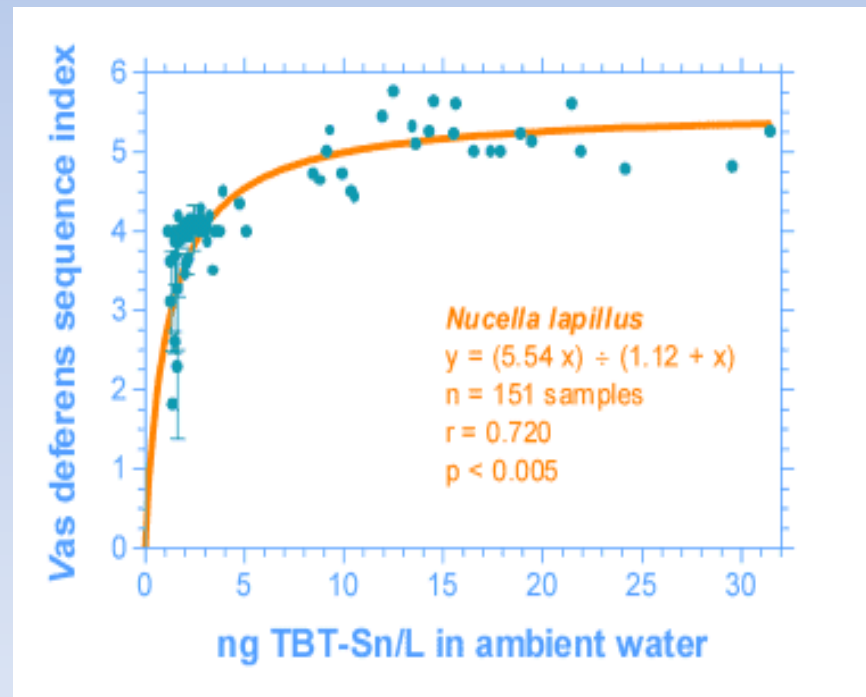
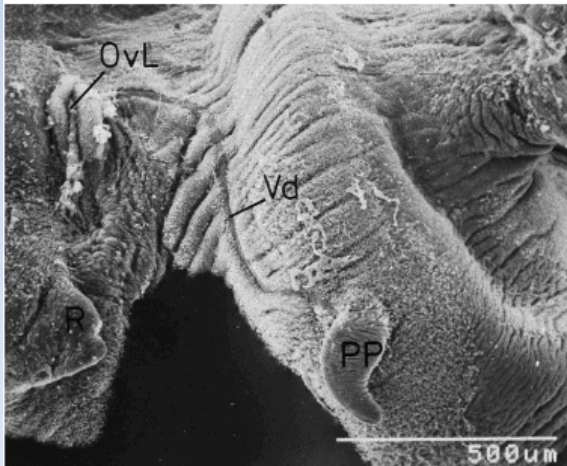
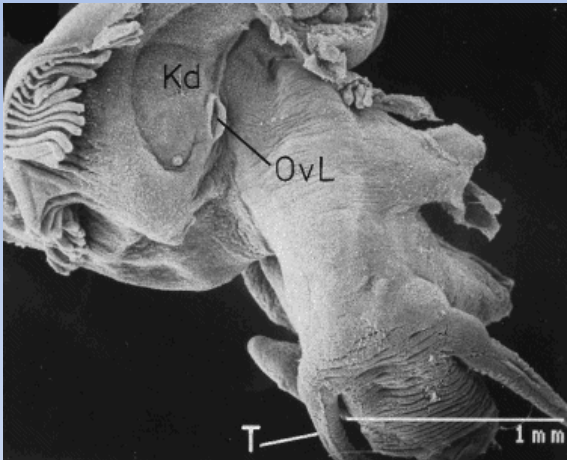
Numerous compounds belonging to diverse chemical families



Prosobranch gastropods in marin environment

Tributyltin (TBT) anti-fouling agent

Imposex: male sex organ (penis, vas deferens) in female



Link between TBT exposure and imposex in sea snails

E2, EE2, APs,...

Exposure to Xeno-
estrogens

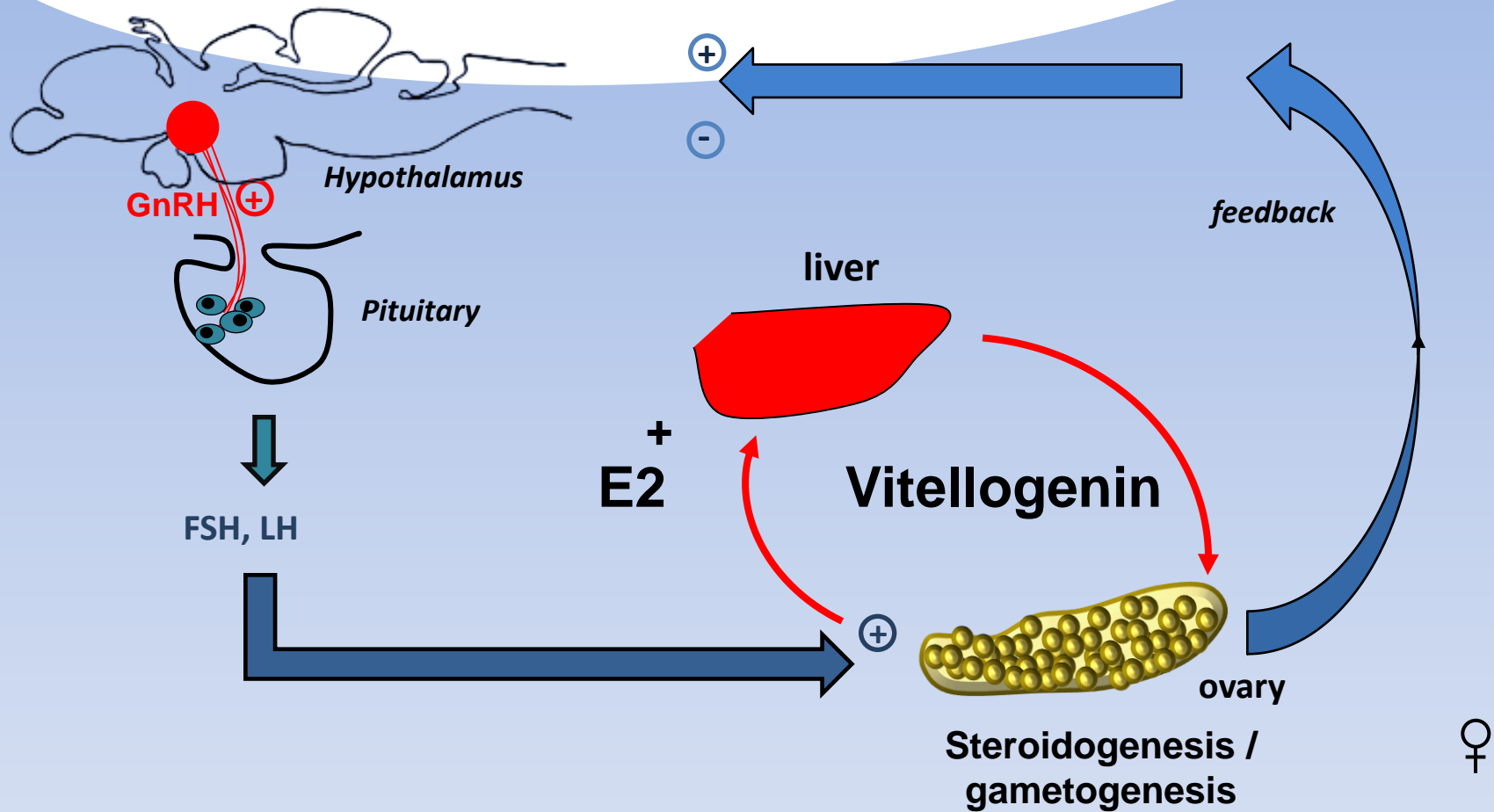


Feminized fish

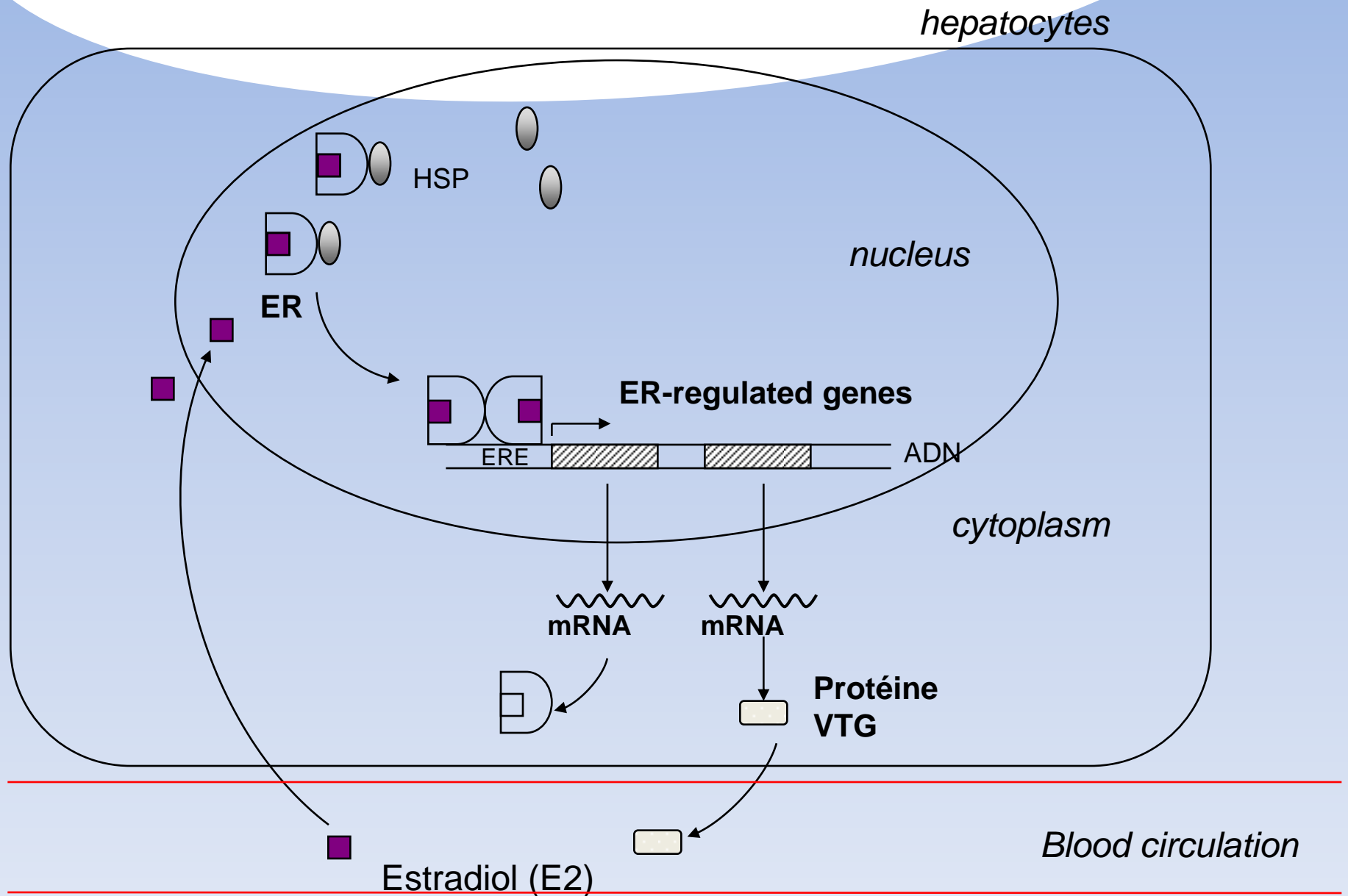


**Abnormal Vitellgenin
synthesis in male**

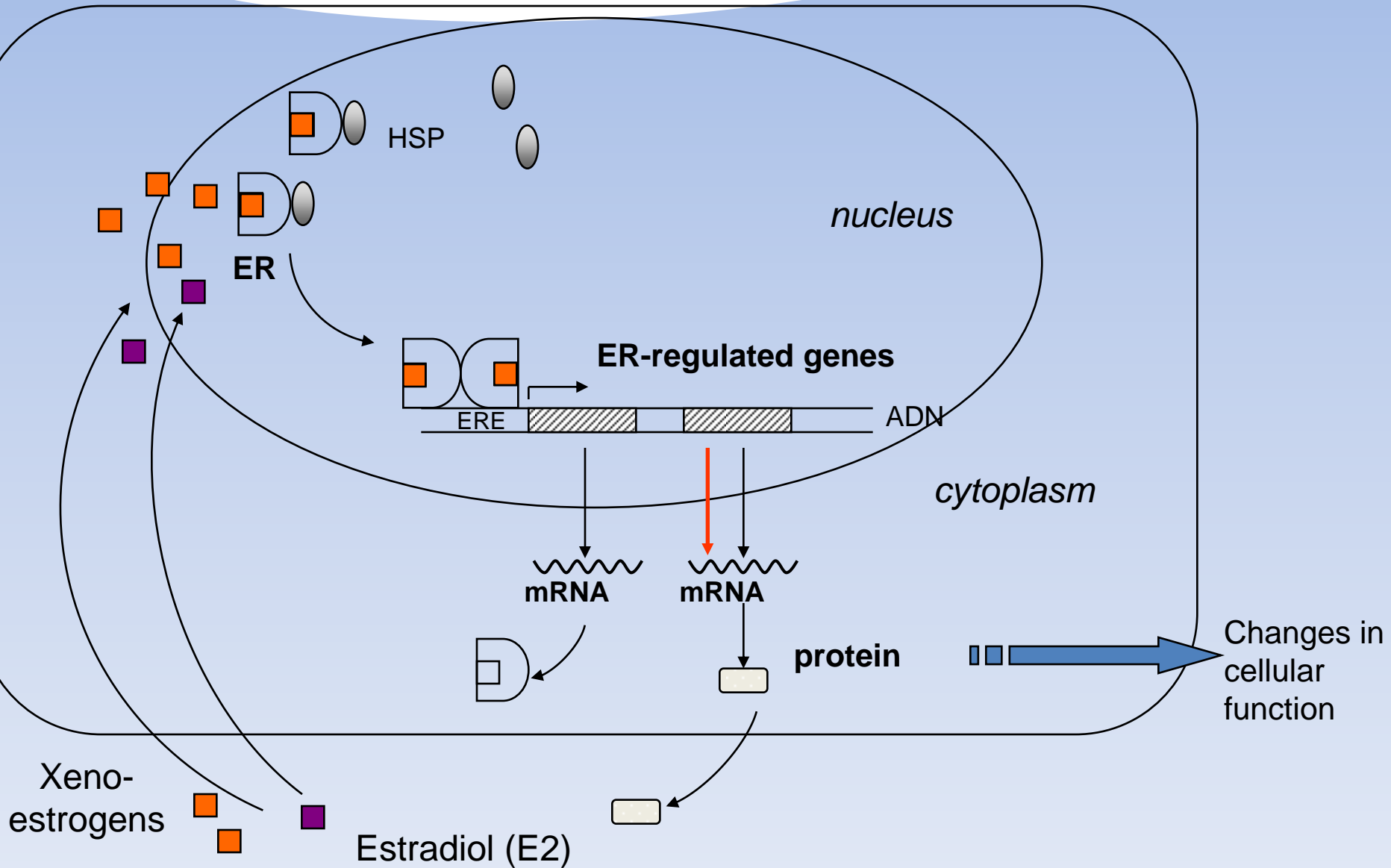
Vitellogenesis in fish



ER-regulated genes in the liver



Effect of xeno-estrogens on liver ER-regulated genes



E2, EE2, APs,...

Exposure to Xeno-
estrogens

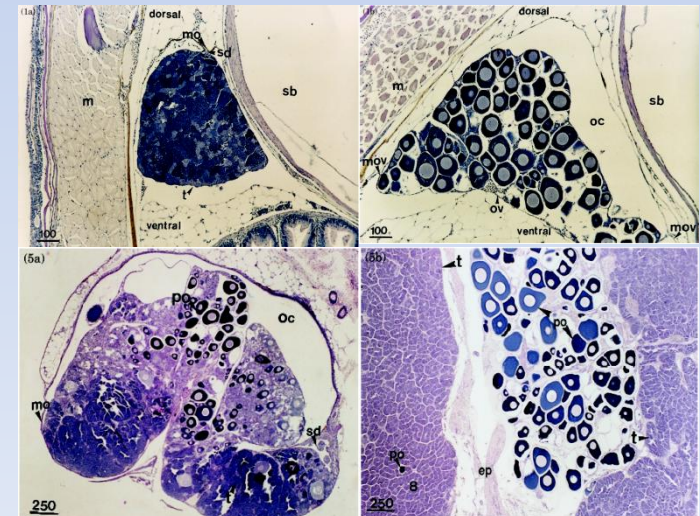


Feminized fish



Intersex fish

**Abnormal Vitellgenin
synthesis in male**



Nolan et al., *J. Fish Biol* 2001

Exposure to EE2

Population dynamics

Individual fitness

Cellular / biological functions

Induction of Vtg

Biochemical pathways

Molecular pathways

Change in the structure of the fish population

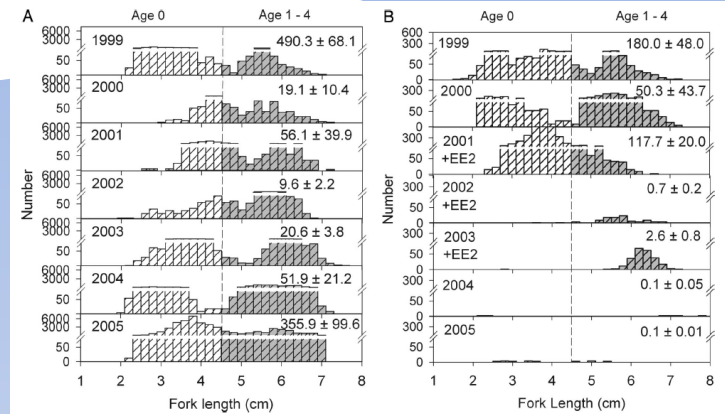
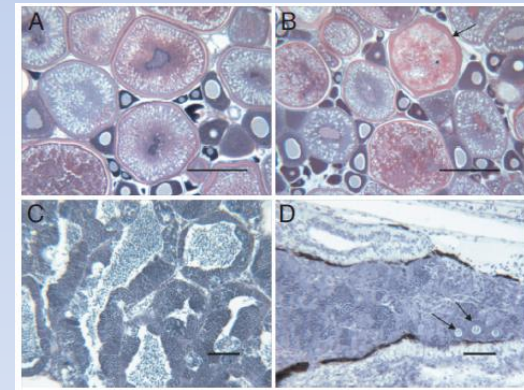
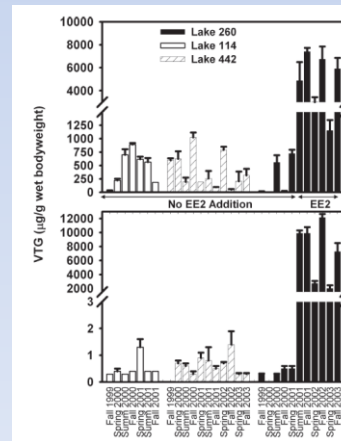


Fig. 3. Length frequency distributions of fathead minnow captured in trap nets in reference Lake 442 (A) and Lake 260 (B) (amended with 5–6 ng·L⁻¹ of EE2 in 2001–2003) during the fall of 1999–2005. Distributions for each fall have been standardized to 100 trap-net days. Mean ± SE daily trap-net CPUE data for adults and juveniles for the fall catches are shown in the panels.

Intersex in male fish

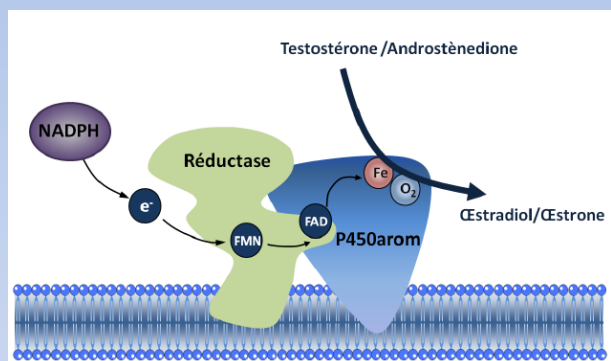
Kidd et al., *PNAS* 2007

EDCs can act at multiple levels of biological organization

- Vtg as a sensitive marker to xeno-estrogens
- Numerous studies reported abnormal Vtg synthesis in wild population of fish inhabiting polluted rivers, estuaries
- Decreased Gonado-somatic index (GSI)
- Occurrence of intersex fish
- No mechanistic link between expression of an ER-regulated protein in the liver and differentiation of the gonads
- EDCs can act on the endocrine system through other mechanisms of action
 - Other Nuclear Receptors (Patrick Balaguer's presentation)
 - Non NR-mediated effect

Effect of EDCs on gonadal steroidogenesis in fish

- Pharmaceutical compound, persistent & hydrophobe
- Inhibitor of various P450 enzyme activities in vitro
- Inhibition of aromatase activities in rainbow trout ovarian microsomes (e.g., Hinfray et al., CBPc 2006)

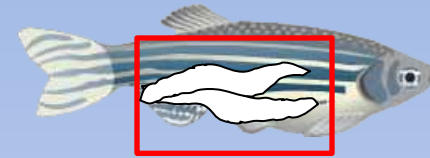
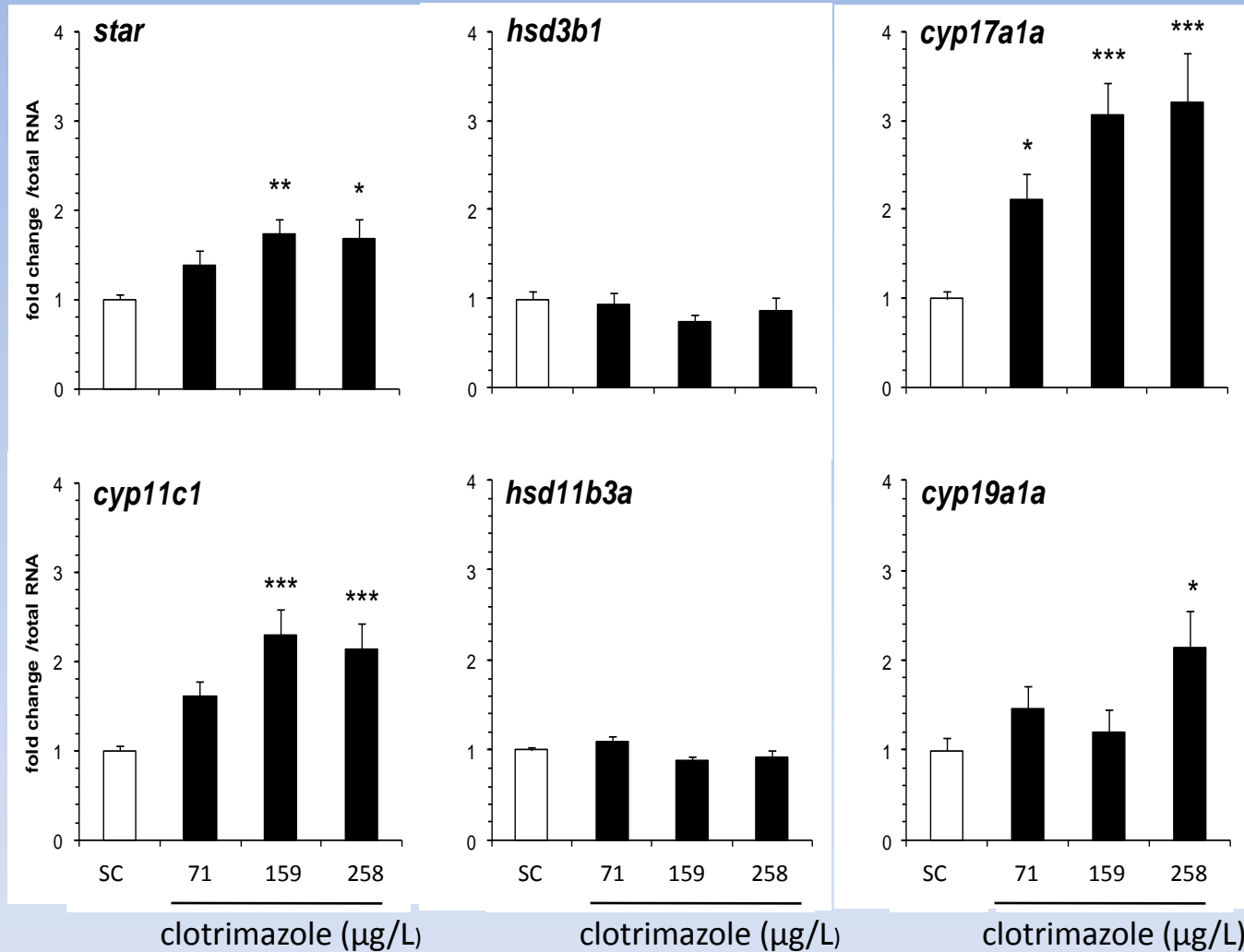


- Inhibition of P450 c17 17α -hydroxylase & $17,20$ lyase activities in rat testis microsomes (Ayub et al., 1987)



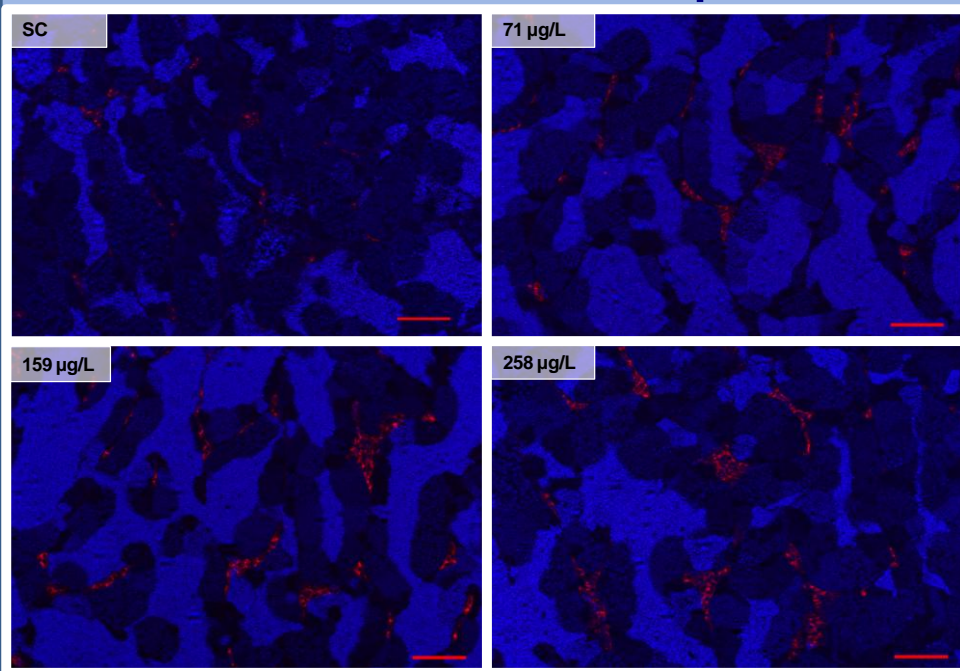
Effet on testicular steroidogenesis,
biosynthesis of 11 KT & spermatogenesis?

Effect of *in vivo* exposure to clotrimazole for 7 days

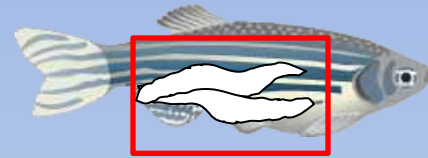


Concentration-dependent inductions of steroidogenesis-related genes in testicular tissue

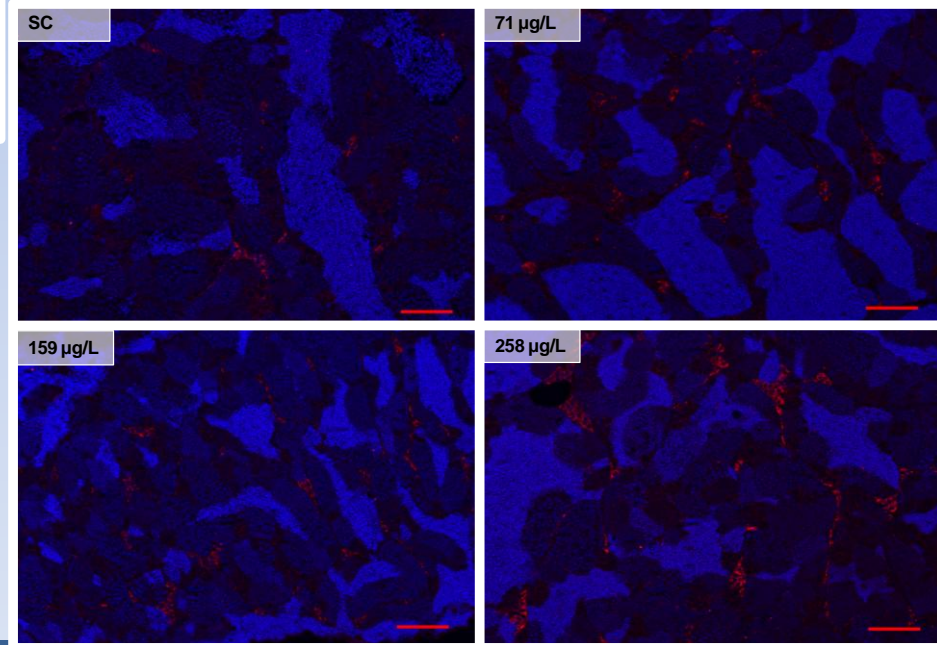
Effect of *in vivo* exposure to clotrimazole for 7 days



CYP 17a1

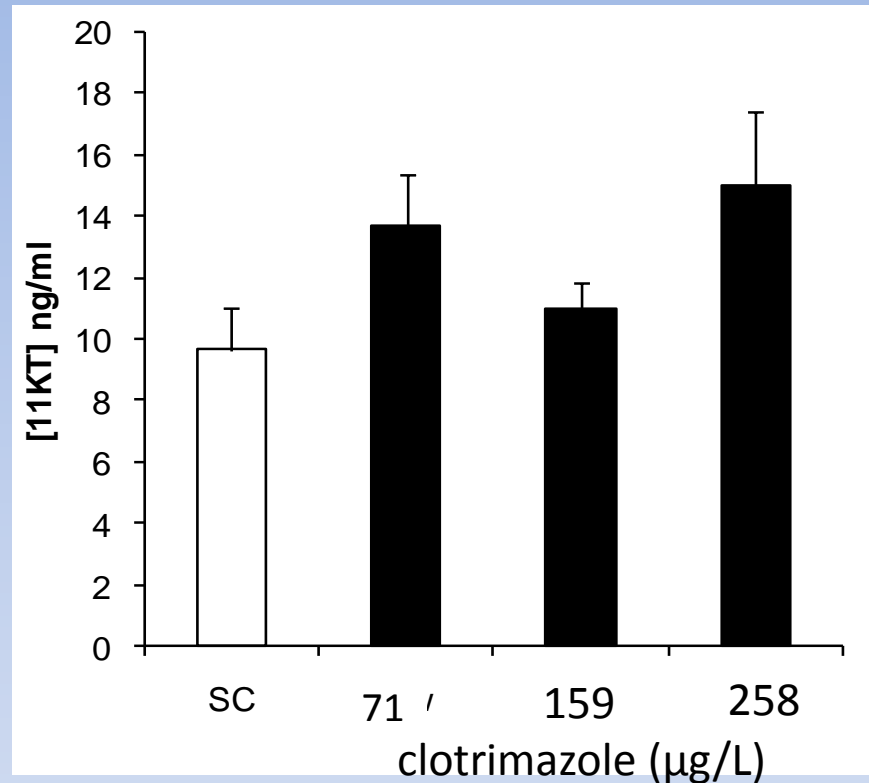


CYP 11c1



Clotrimazole exposure up-regulate CYP17a1 and CYP11c1 protein expression in Leydig cells

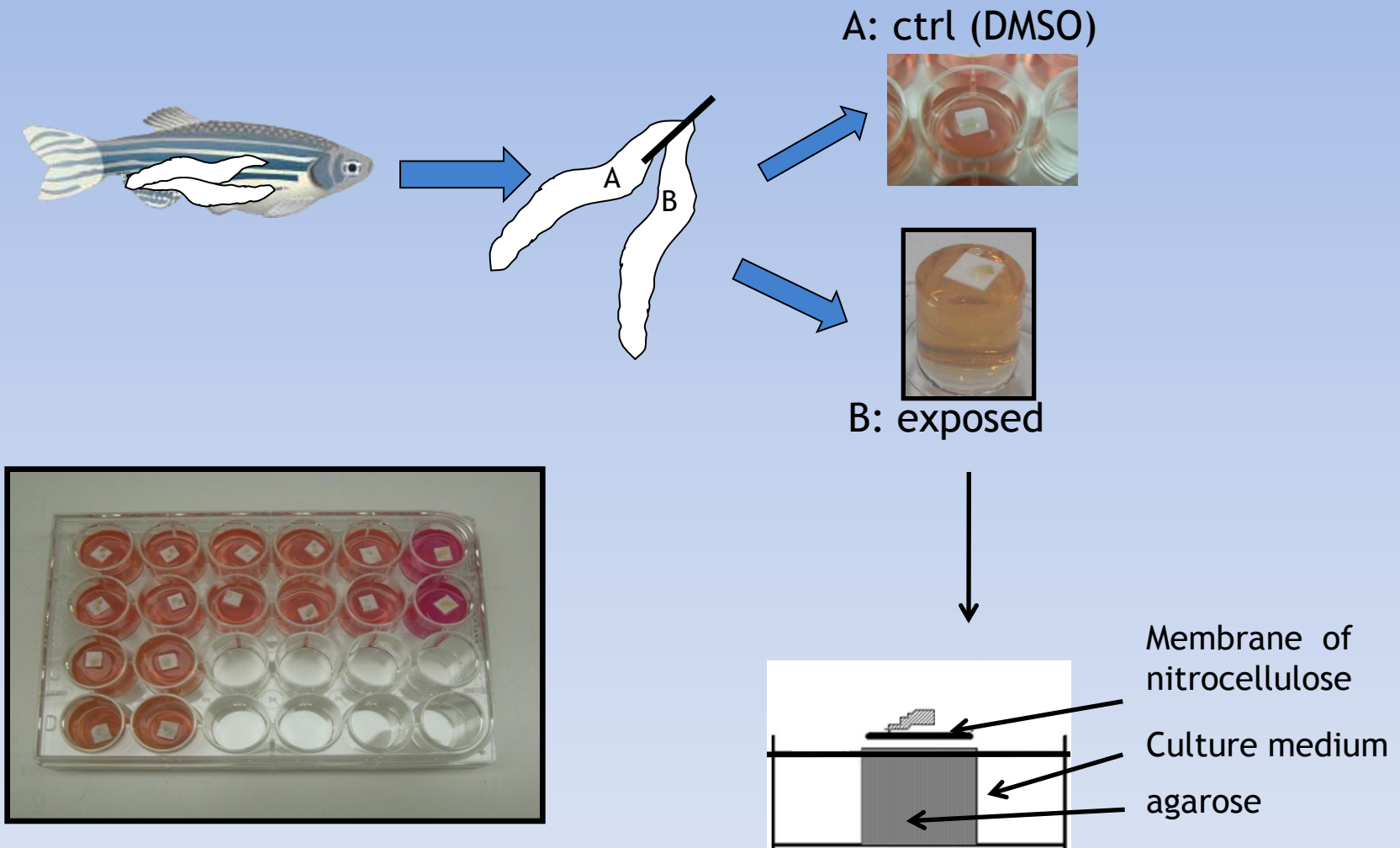
Effect on circulating concentrations of 11-KT



Mean \pm SEM
n=11-16/cond.

No significant effect on circulating 11KT concentrations
Mode of action of clotrimazole on zebrafish testis?

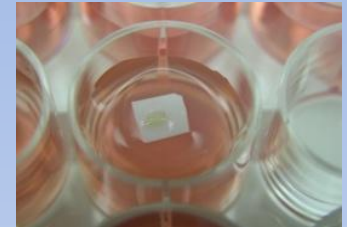
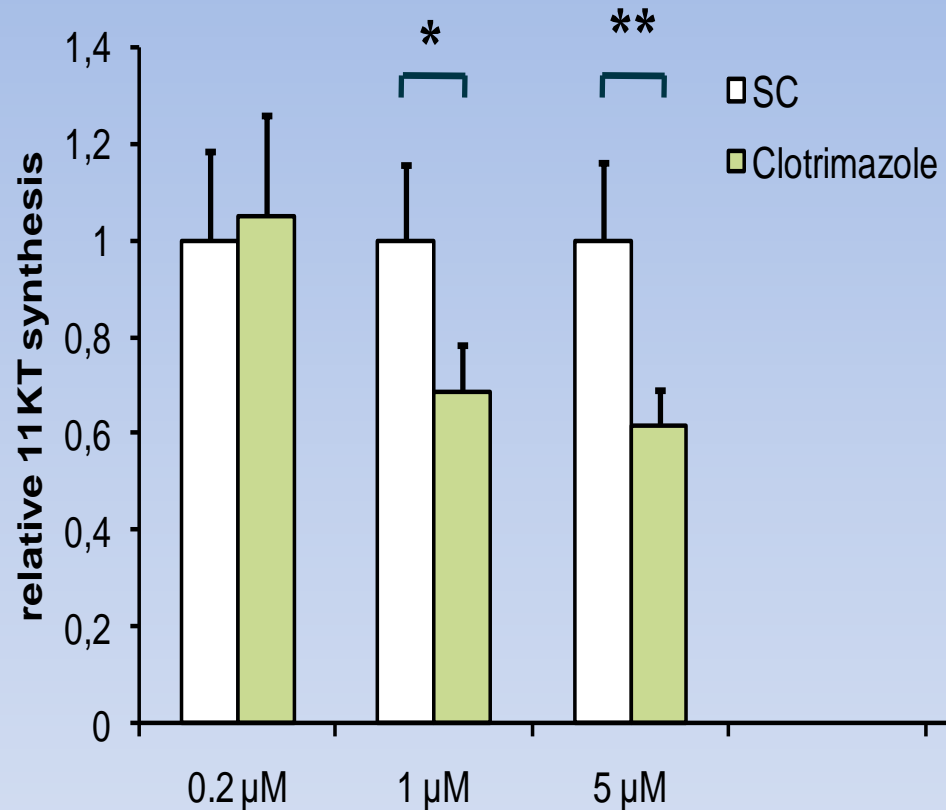
Zebrafish testicular explant culture system



Leal et al., 2009

Measurements of 11KT in the culture medium at day 6

In vitro, clotrimazole inhibits 11KT release in culture medium after 6 days of exposure

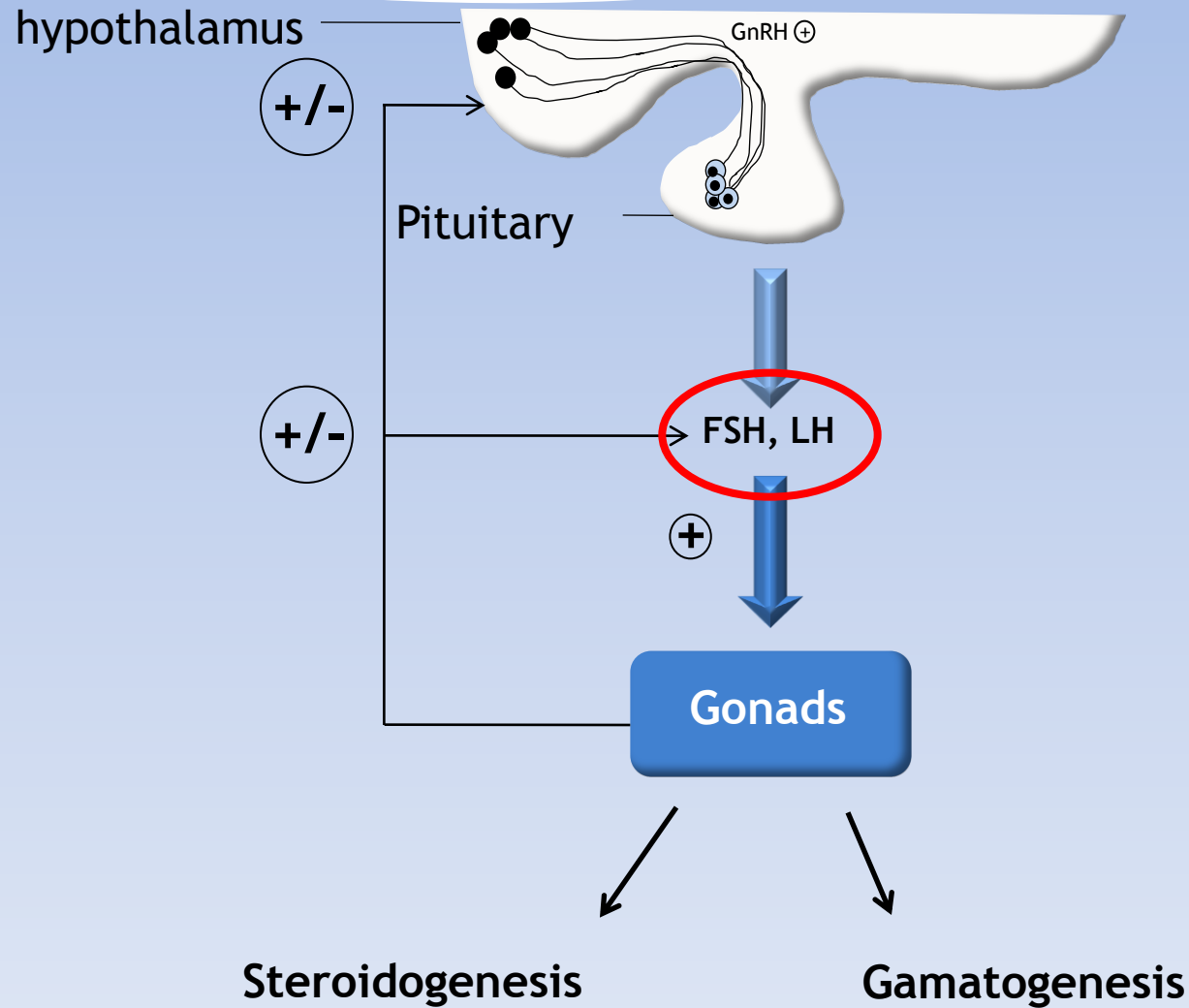


Hinfray et al., GCE 2011

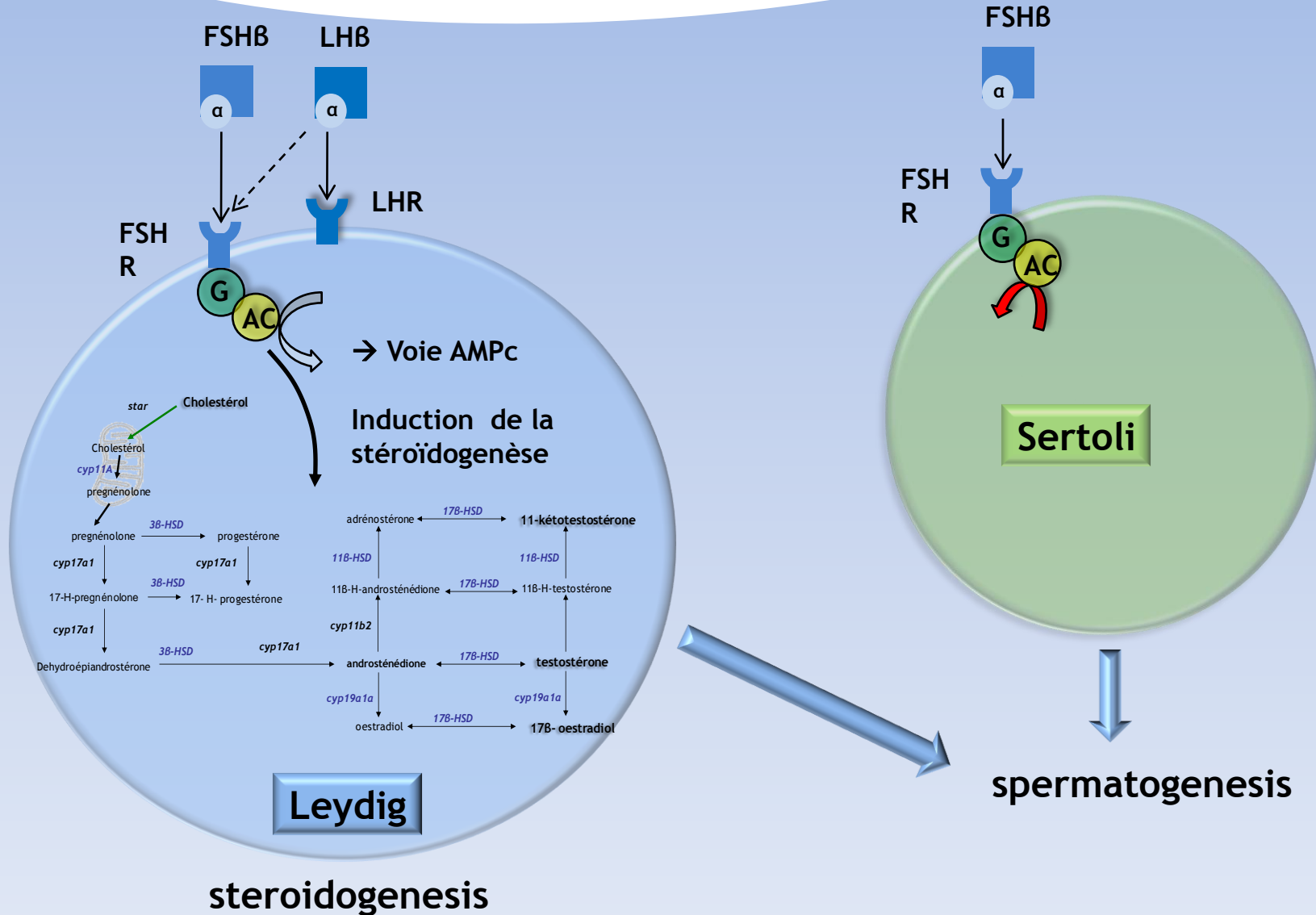
Clotrimazole does not act directly on testis to regulate steroidogenesis.

Mechanism responsible for induction of steroidogenesis in vivo?

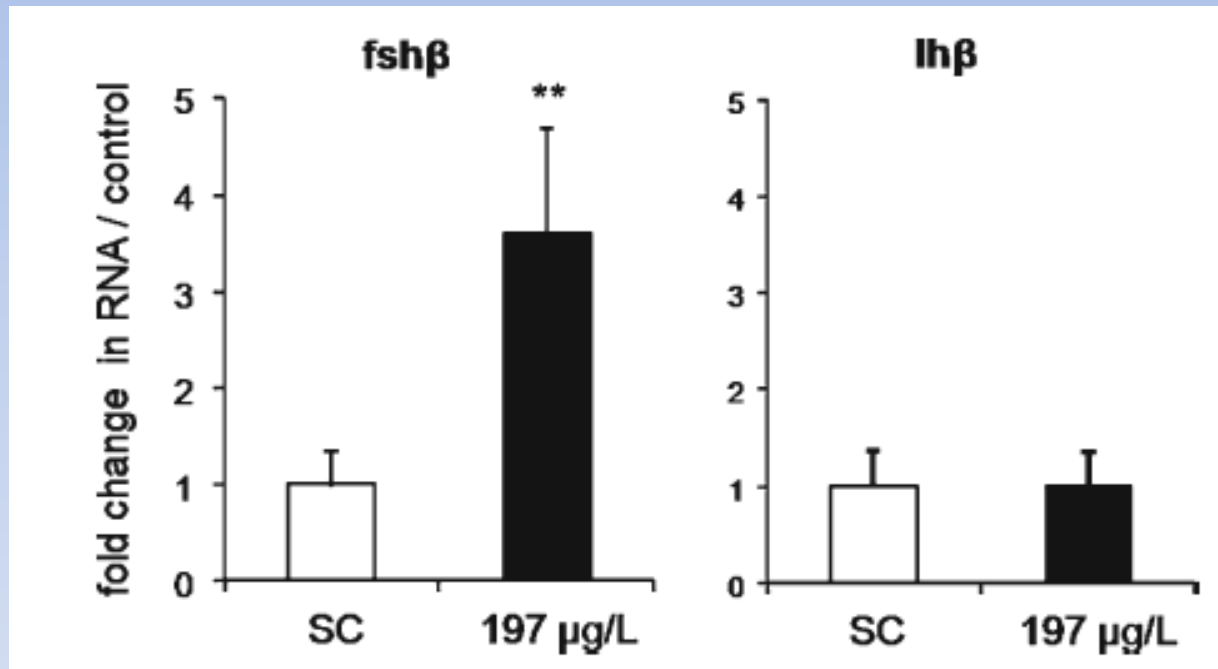
Hypothalamus-Pituitary-Gonad (HPG) axis



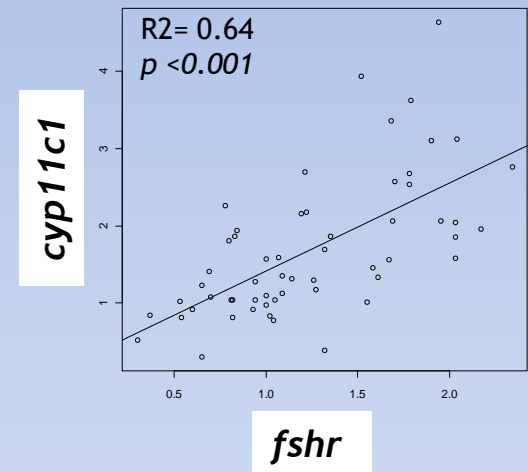
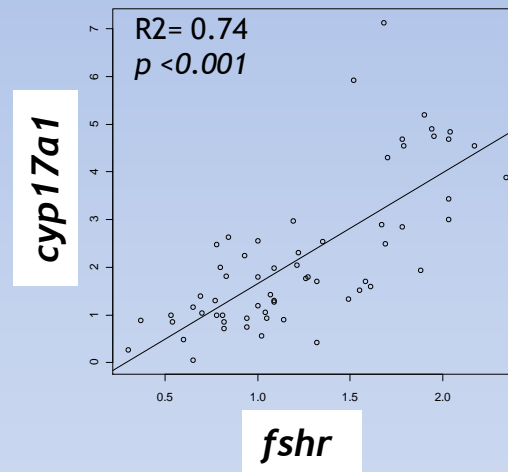
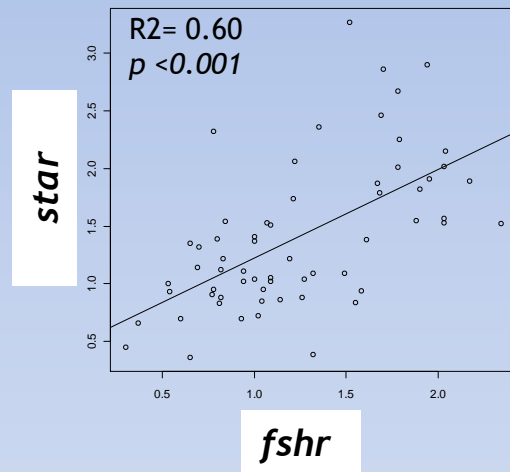
Gonadotropins in fish and their receptors



Clotrimazole exposure affect the pituitary expression of Fsh β but not Lh β



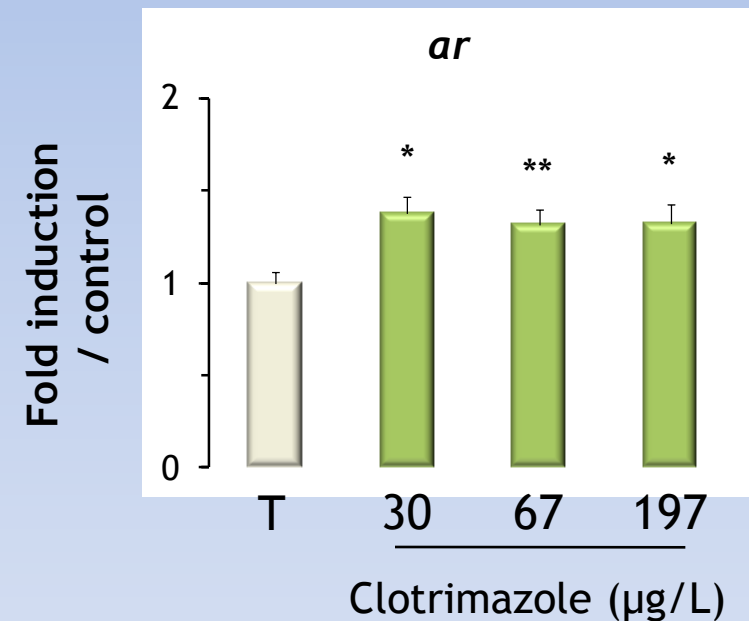
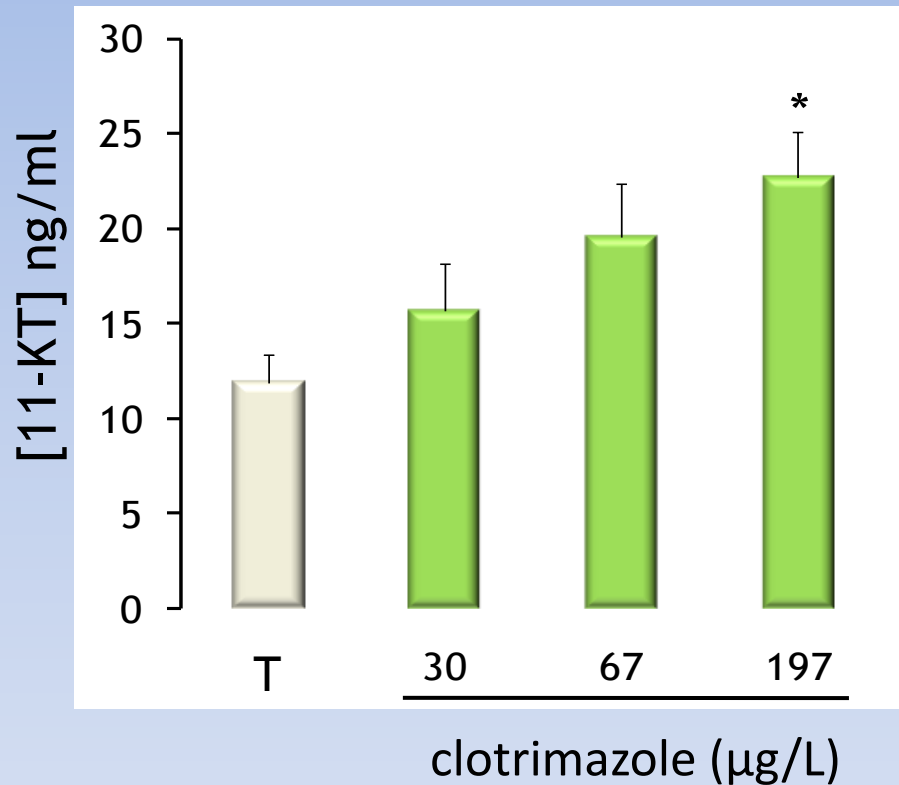
Clotrimazole exposure affect FSHR expression in testis



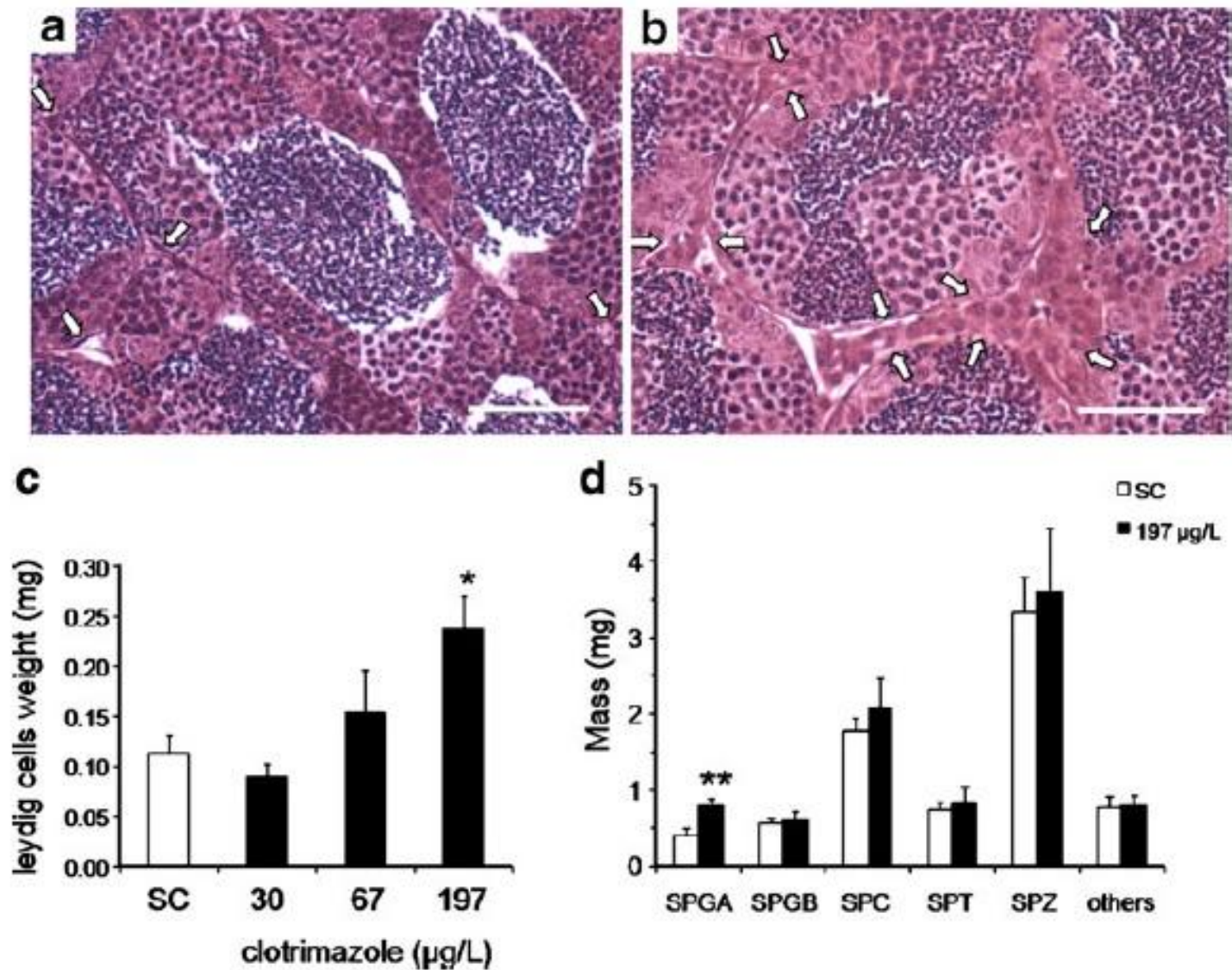
Baudiffier et al., Toxicology 2012

Strong correlation between *fshr* and induced-steroidogenic gene expressions

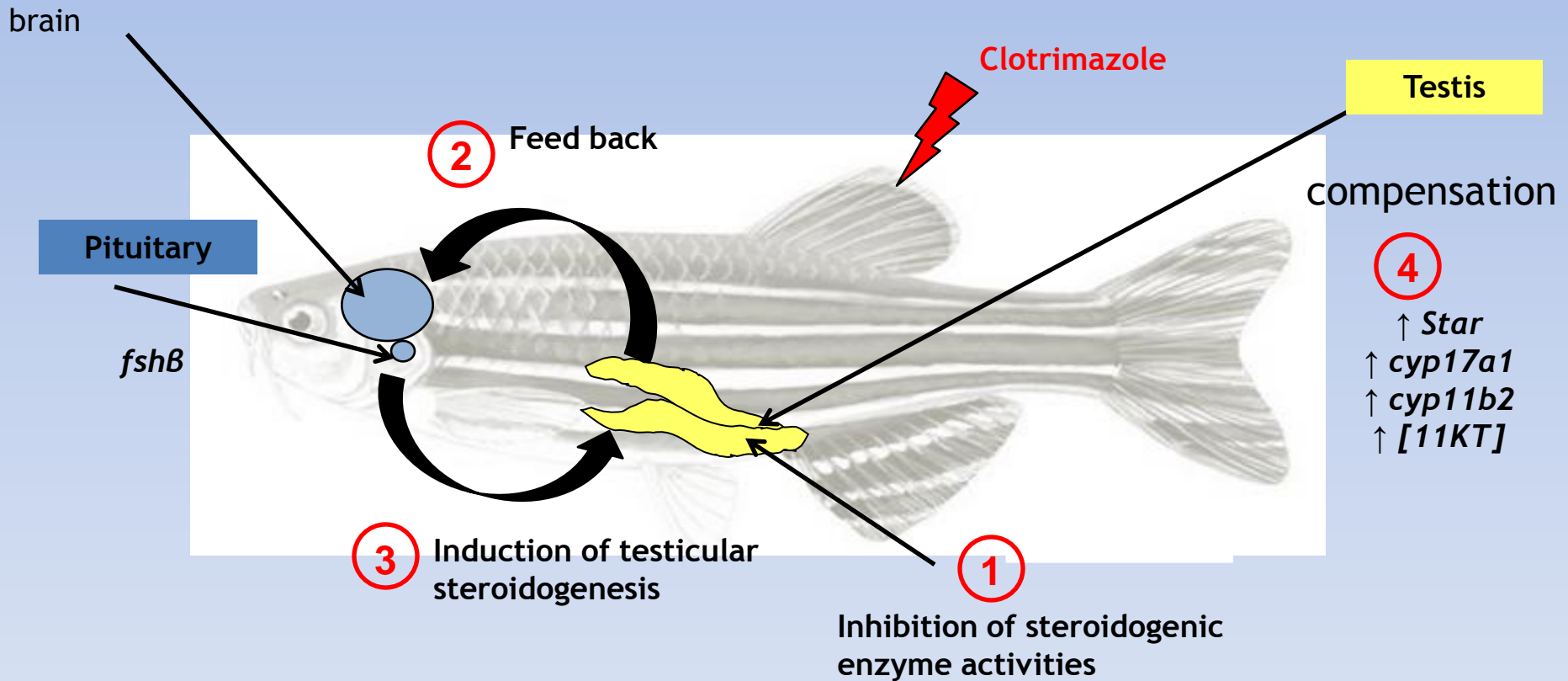
After 21 days of exposure, clotrimazole affects circulating concentrations of 11KT and AR expression



Clotrimazole exposure affects the testis tissue



Mechanism of action of clotrimazole

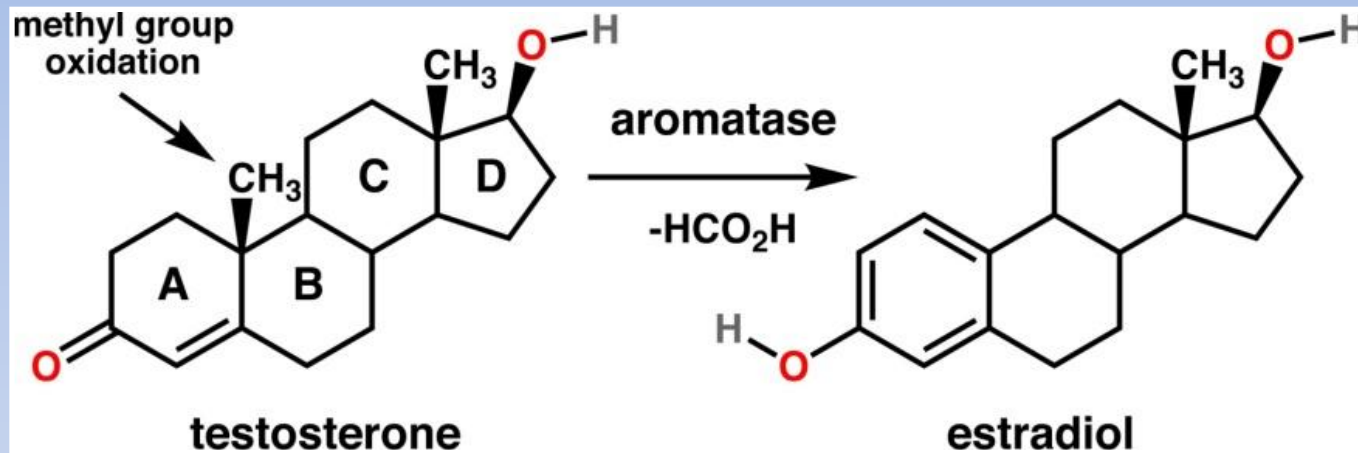


Effect of clotrimazole on steroidogenesis and spermatogenesis

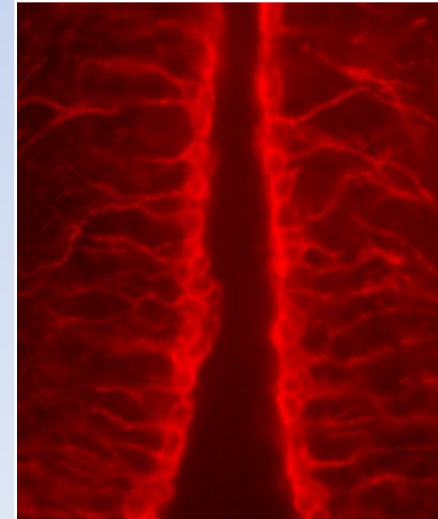
- In vitro assays (microsomes, testicular explants): inhibitory effect of clotrimazole on steroidogenic enzyme activities
- In vivo exposure: disruption of the HPG axis, leading to a compensation of the inhibitory effect of clotrimazole on steroidogenic enzyme activities
- Relevance of studying a network of functional genes along the HPG axis in parallel with histological analysis

Effect of EDCs on brain steroidogenesis in fish

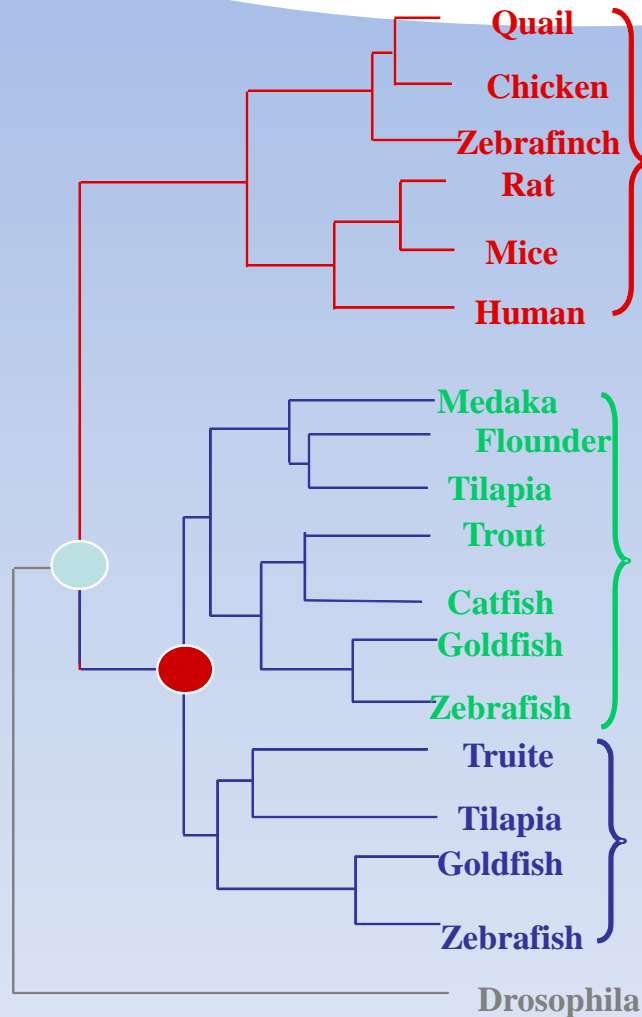
- Aromatase is the only enzyme responsible for the irreversible conversion of androgens into estrogens



- High aromatase activity in adult fish: 100 to 1000 times higher as compared to other vertebrates
- Aromatase is expressed in radial glial cells
- RGCs play a critical role in neurogenesis



Aromatase in fish



1 aromatase gene
Gonads
Brain



2 aromatase genes

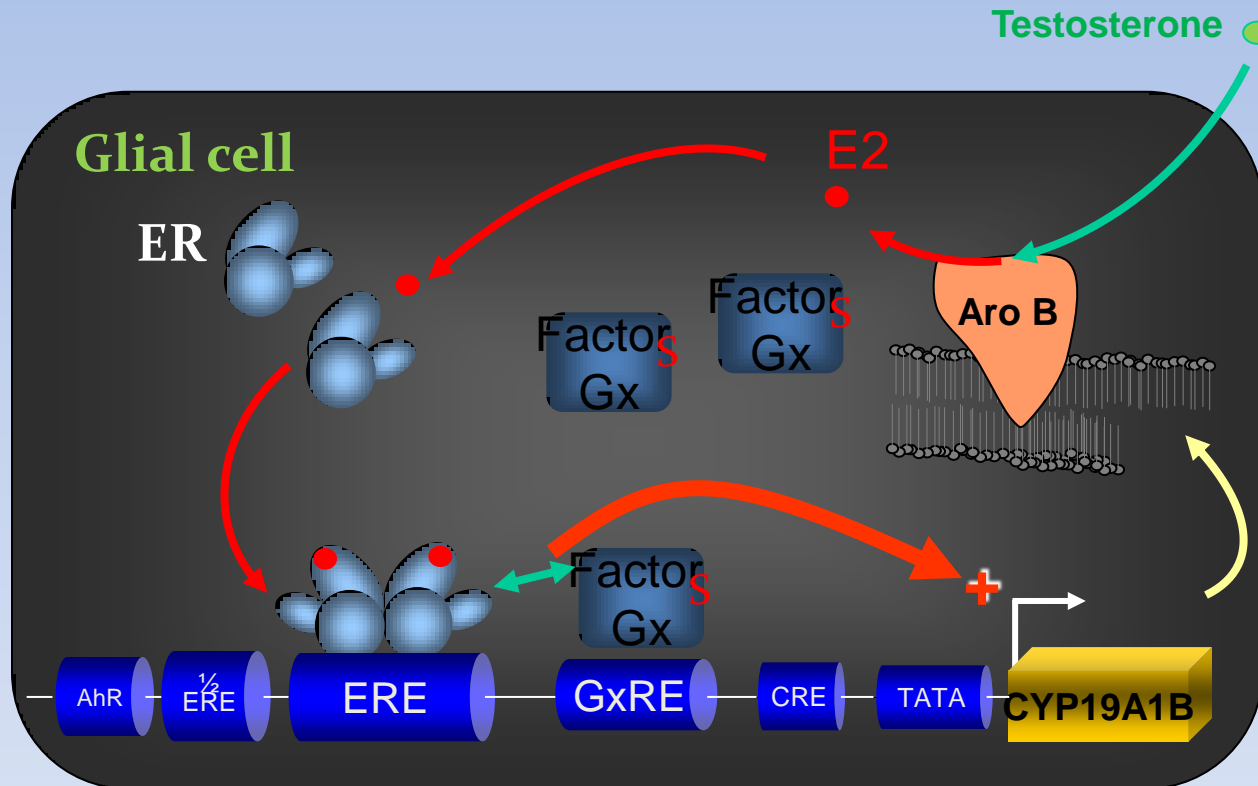
cyp19a1a
Gonads

cyp19a1b
Brain



Brain Aromatase: regulation

- The *cyp19a1b* gene is regulated by estrogens



The *cyp19a1b*-GFP transgenic zebrafish line

TECHNOLOGY REPORT

A *cyp19a1b*-GFP (Aromatase B) Transgenic Zebrafish Line That Expresses GFP in Radial Glial Cells

Sok-Keng Tong,^{1,2} Karen Mouriec,³ Ming-Wei Kuo,¹ Elisabeth Pellegrini,³ Marie-Madeleine Gueguen,³ François Brion,⁴ Olivier Kah,^{3*} and Bon-chu Chung^{1,2*}

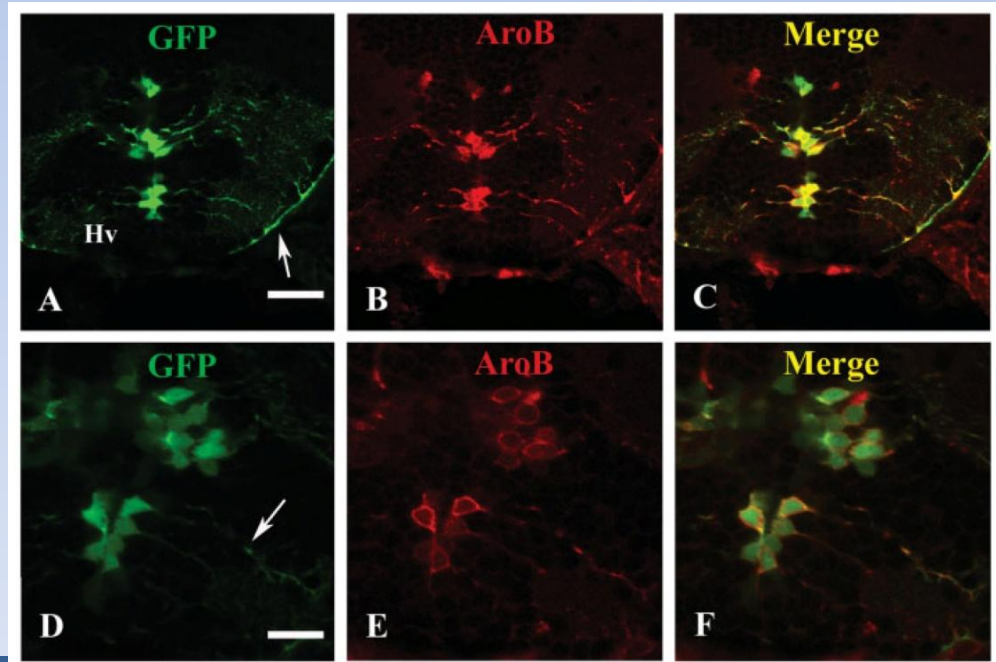
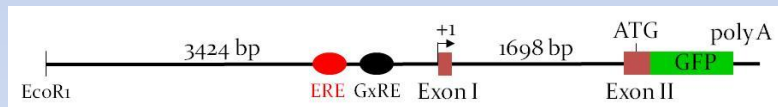
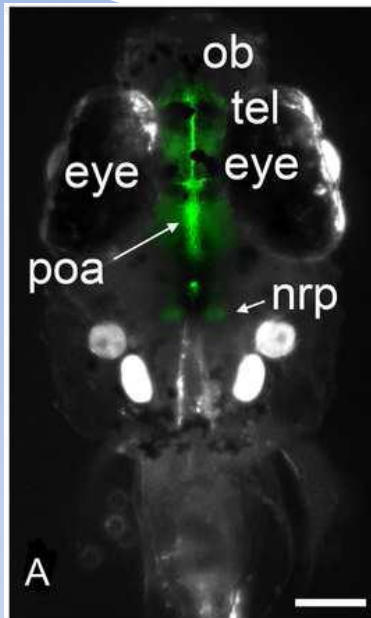
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Received 28 August 2008; Revised 26 September 2008; Accepted 28 September 2008



Tong *et al.*, Genesis (2009)

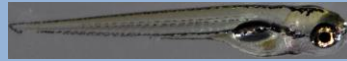
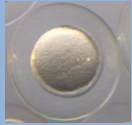
Perfect co-expression of GFP with the endogenous brain aromatase

Development of an *in vivo* assay to detect Endocrine
Active Substances, acting through ER, on transgenic
cyp19a1b-GFP Zebrafish embrYos

(**EASZY** assay)

Principle of the EASZY assay

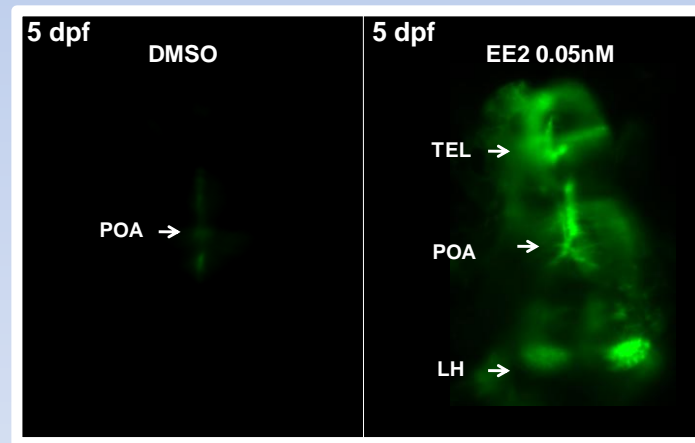
Semi-static exposure



In vivo
Fluorescence
measurement

20-30 embryos, 100ml of water, 28°C, 14:12 D/L
DMSO (0.01% v/v)

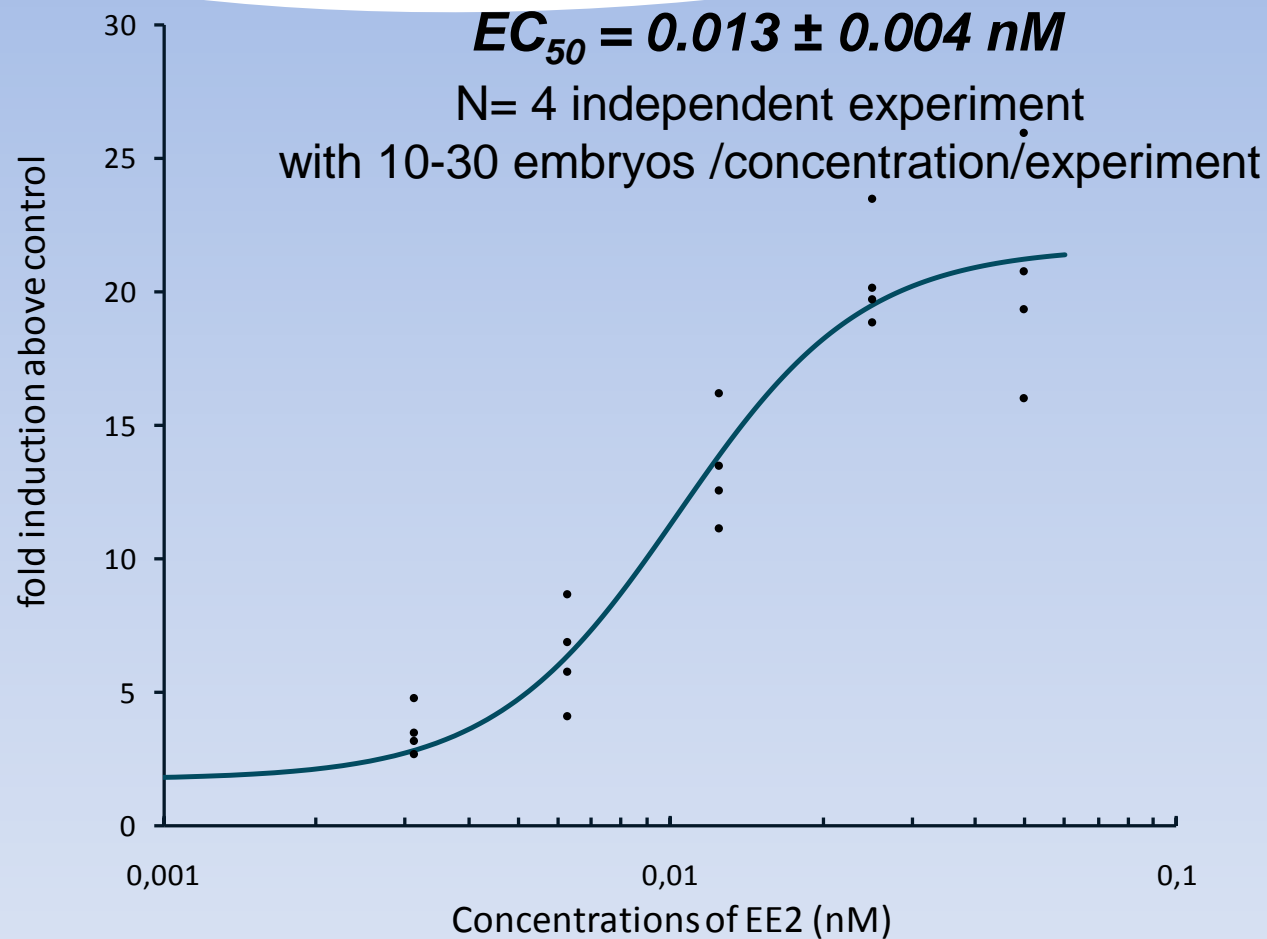
Image
&
Statistical
analysis



GFP expression in control and
EE2-exposed zebrafish

Brion et al., PlosOne 2012

Response of cyp19a1b-GFP embryos to 17 α ethinylestradiol



Concentration-dependent induction of GFP, **extreme sensitivity to EE2**, good reproducibility between experiments

Fluorescence intensity reflects the physiological response of the *cyp19a1b* gene

Compound	Method	EC ₅₀ (nM)
E2	<i>cyp19a1b</i> mRNA	2.8
	GFP mRNA	4.1
	Image analysis	1.5
EE2	<i>cyp19a1b</i> mRNA	0.04
	GFP mRNA	0.02
	Image analysis	0.01
E1	<i>cyp19a1b</i> mRNA	2.3
	GFP mRNA	1.3
	Image analysis	2.4
Genistein	<i>cyp19a1b</i> mRNA	3545
	GFP mRNA	2466
	Image analysis	2166

Very good agreement between image analysis and the endogenous gene expression

EASZY assay: Screening of various compounds belonging to different chemical families

Natural and synthetic estrogens:

Phyto and myco-estrogens:

Pesticides

Alkylphenols

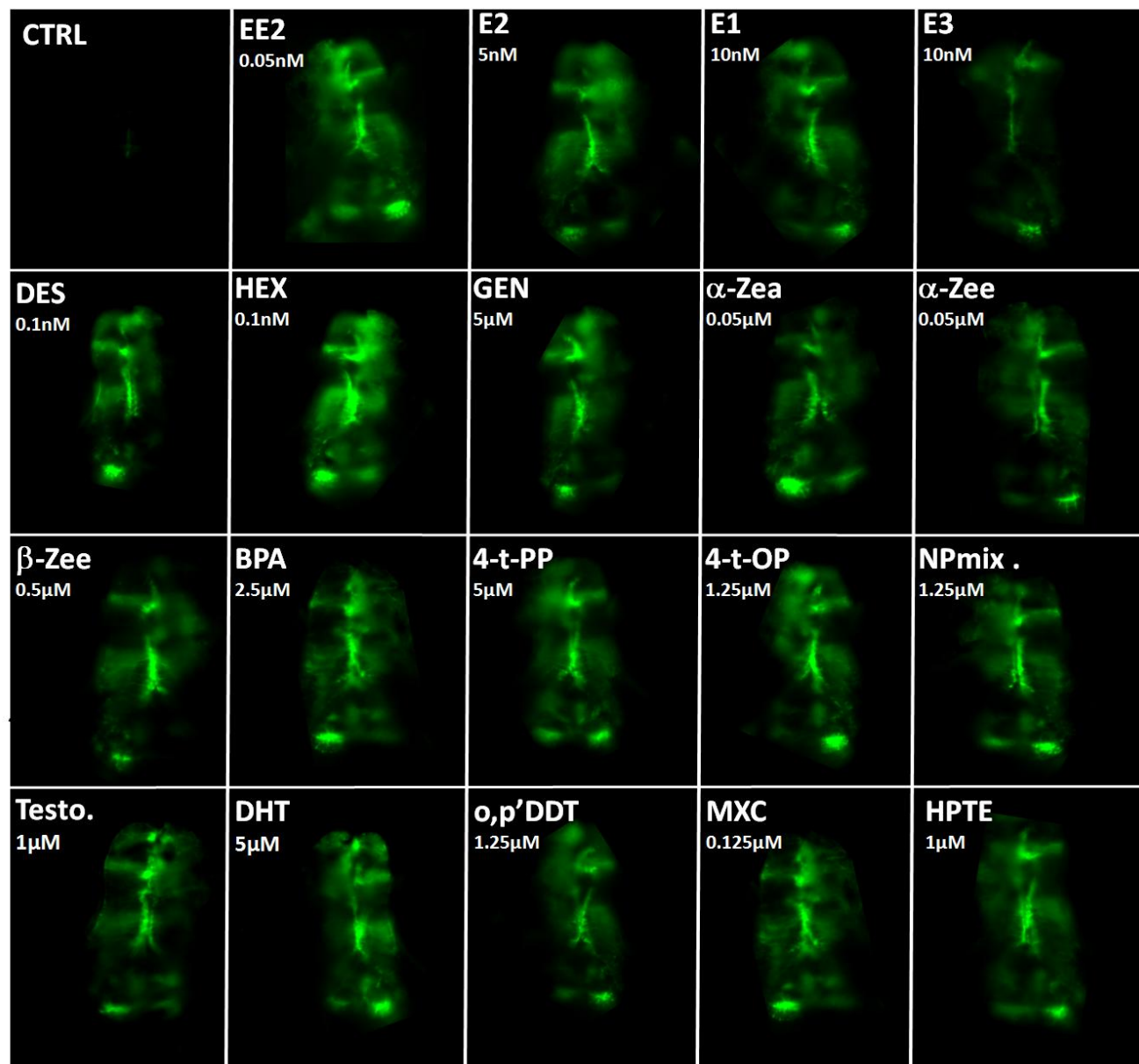
Androgens

Progestins

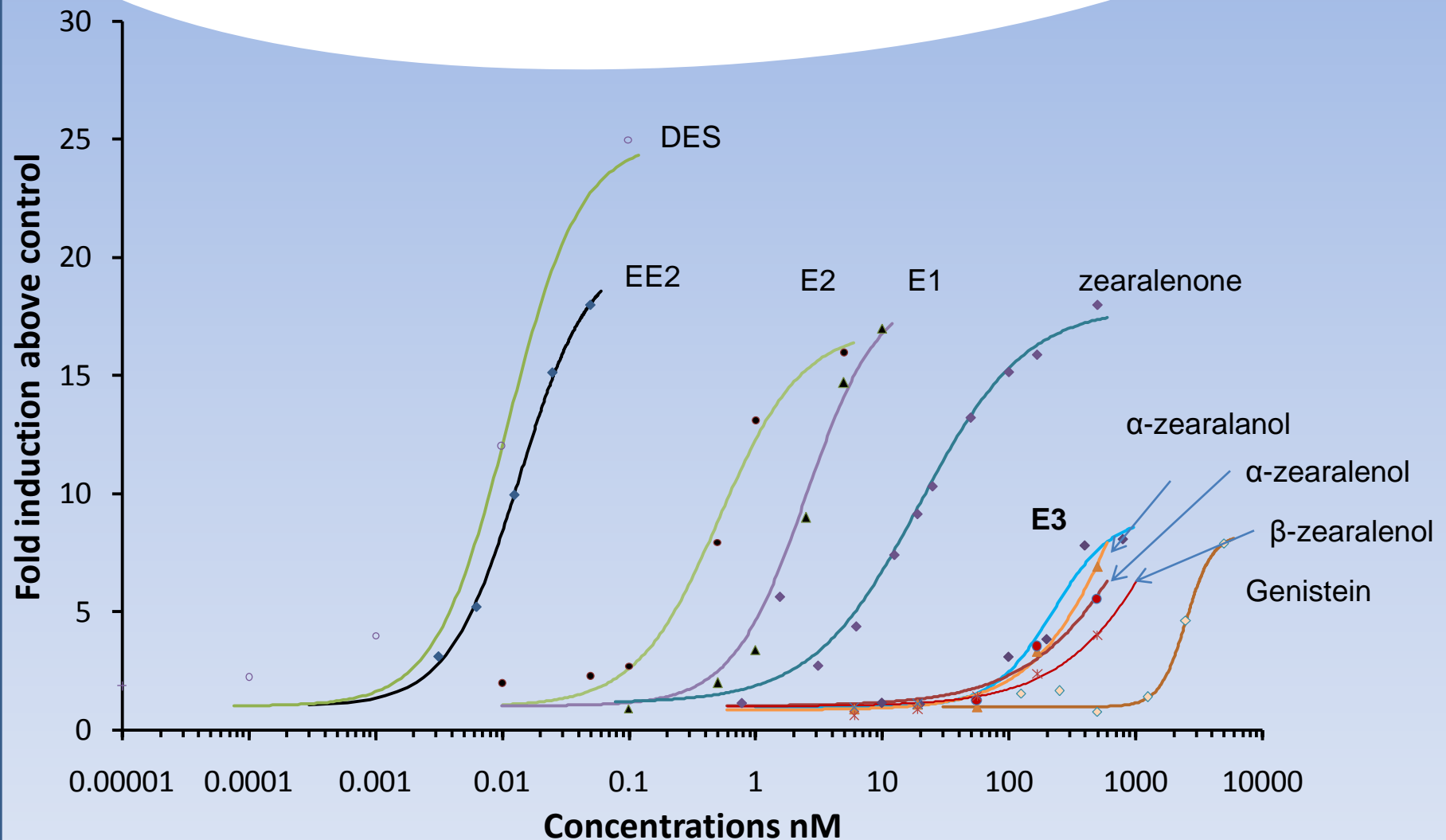
Corticosteroids

Dioxine and PAHs

Negative compounds

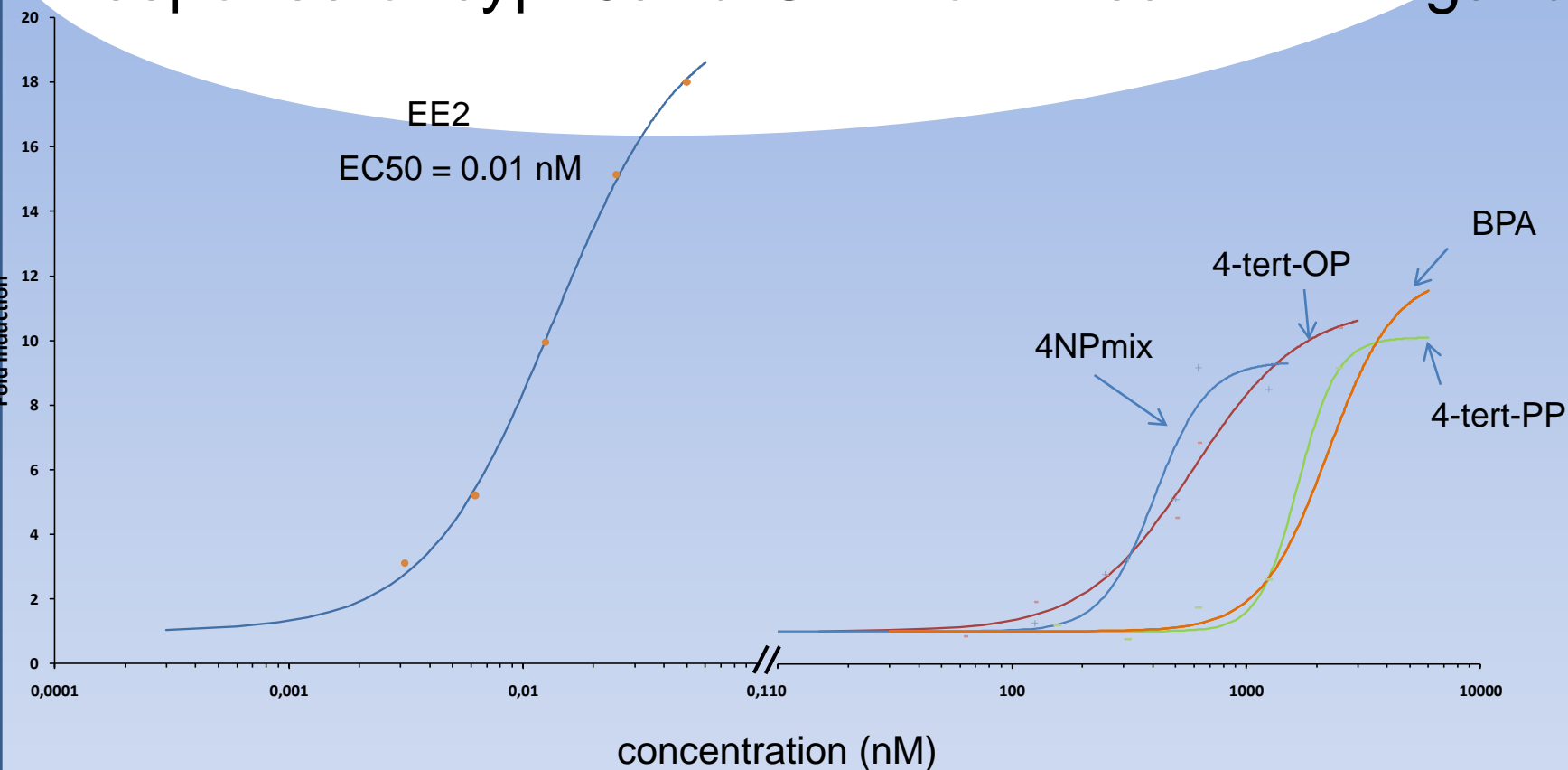


Responses of cyp19a1b-GFP to ER ligands



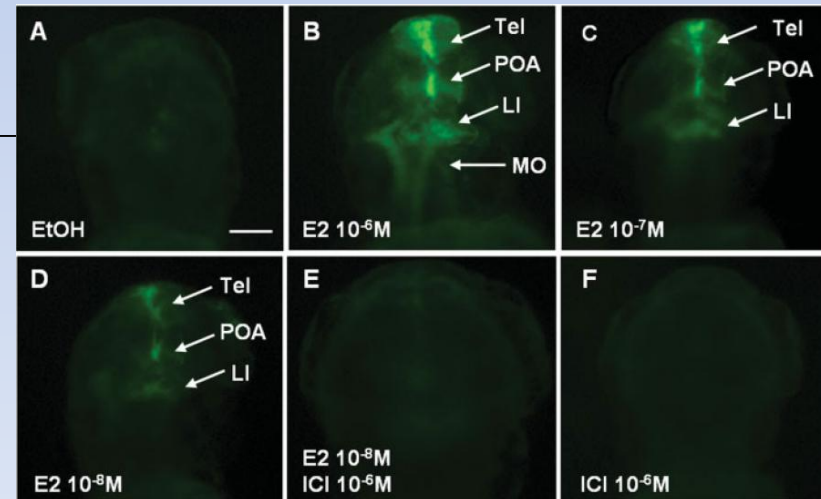
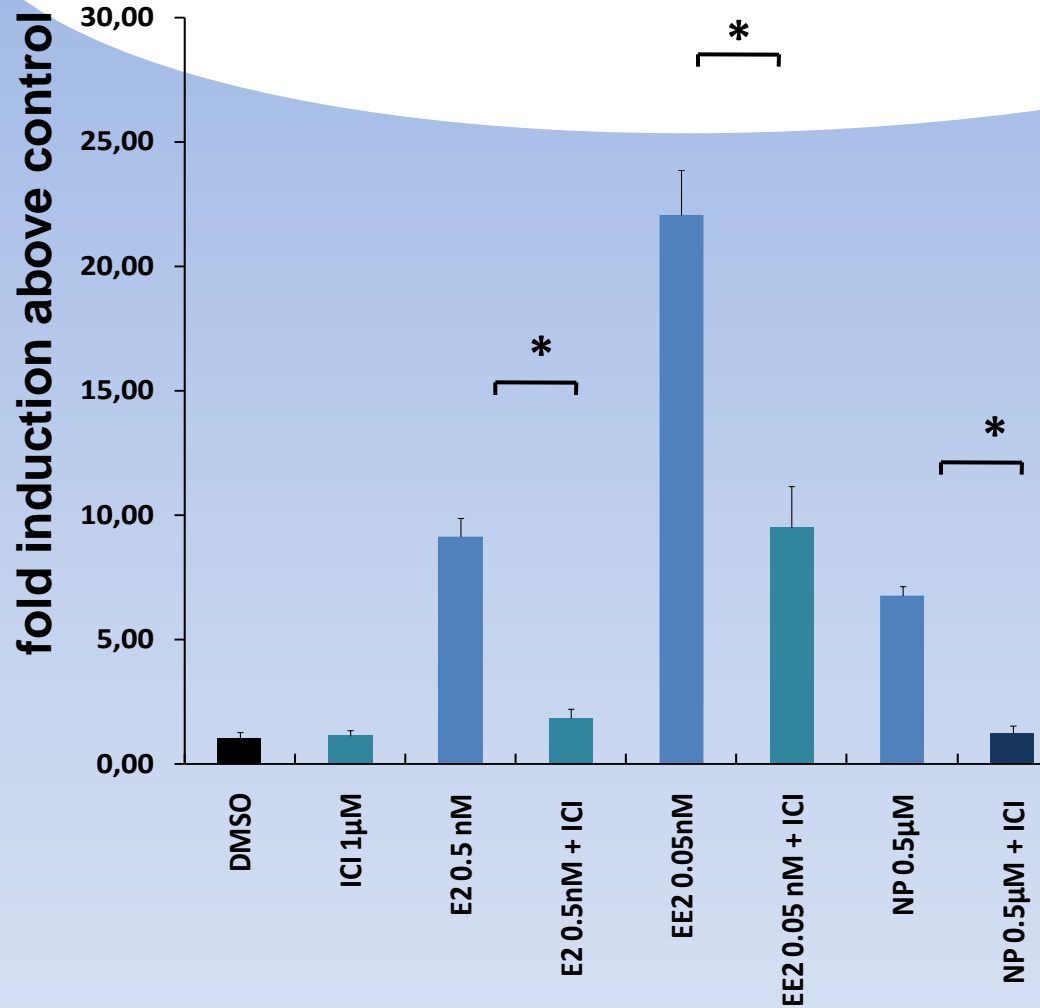
Concentration-dependent induction of GFP to steroidal estrogens

Response of cyp19a1b-GFP to « weak » ER ligands



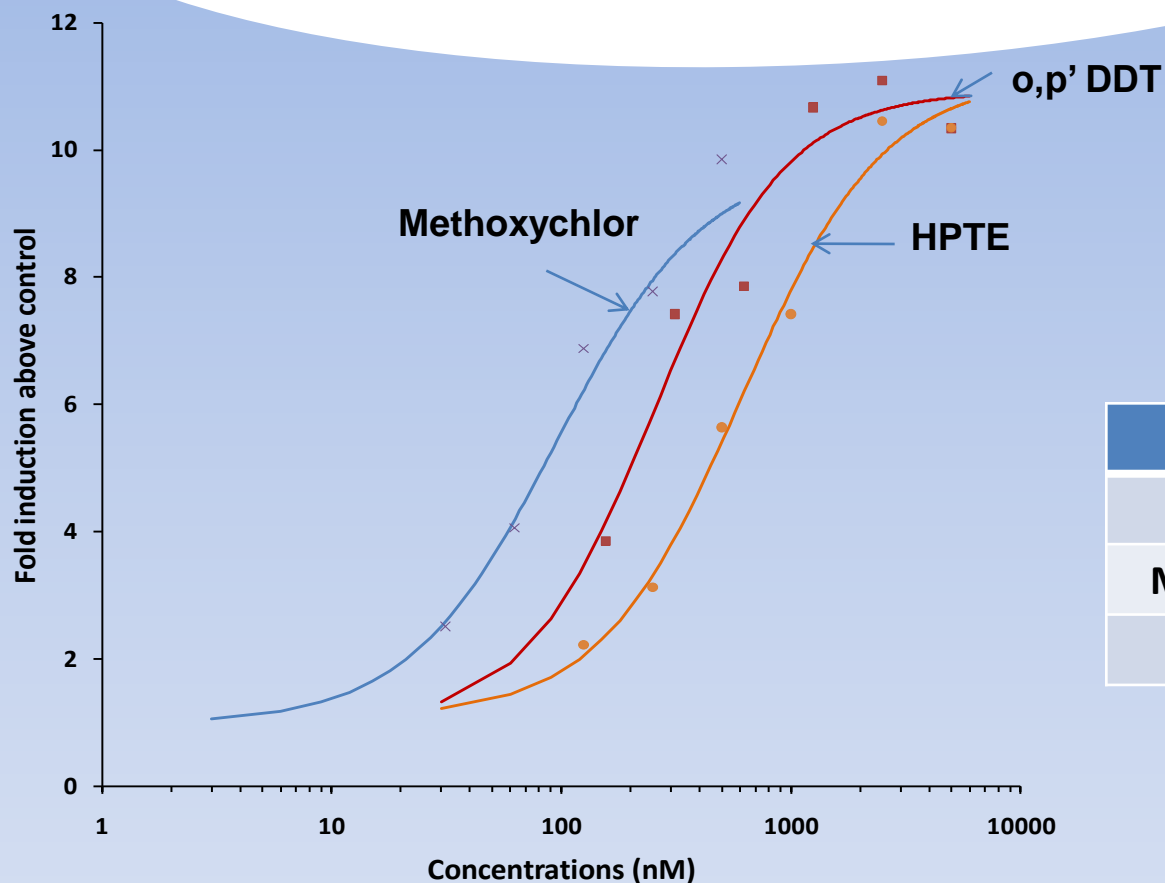
Compounds	EC 50 nM
4-n-NP	no effect
NPmix	406
4-tert-OP	595
4-tert-PP	2541
BPA	3303

GFP induction are blocked by ICI 182 780



the GFP inductions are specific of an estrogenic effect

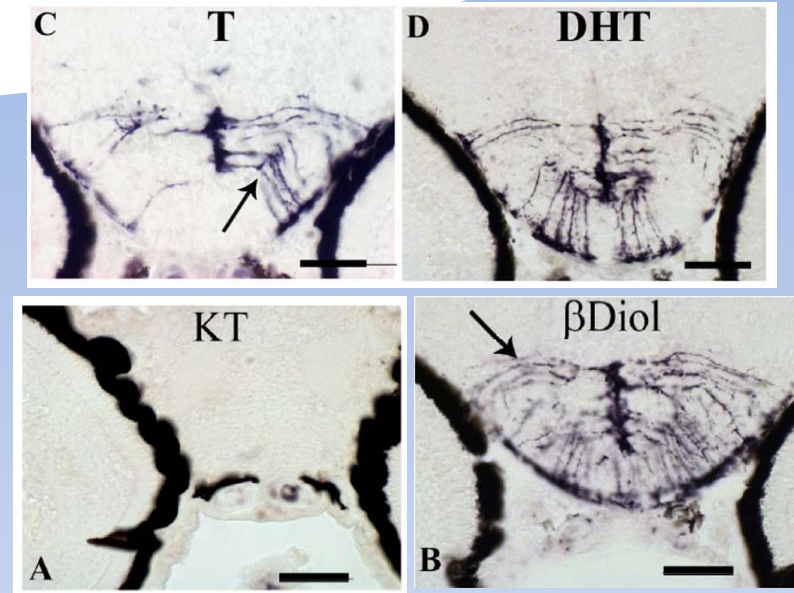
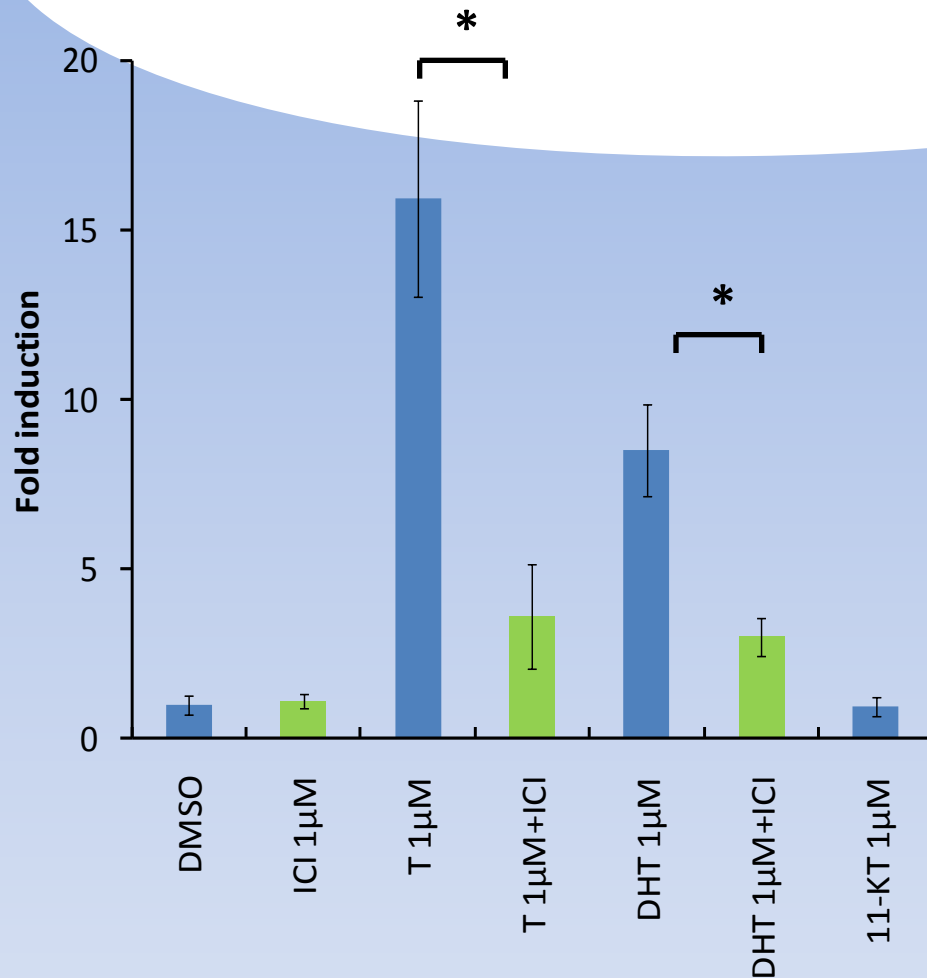
Responses of *cyp19a1b*-GFP to DDT-Related Compounds and their metabolites



Compounds	EC 50 nM
op'DDT	257
Methoxychlor	85
HPTE	477

the *cyp19a1b*-GFP assay detect indirect estrogens that required metabolic activation

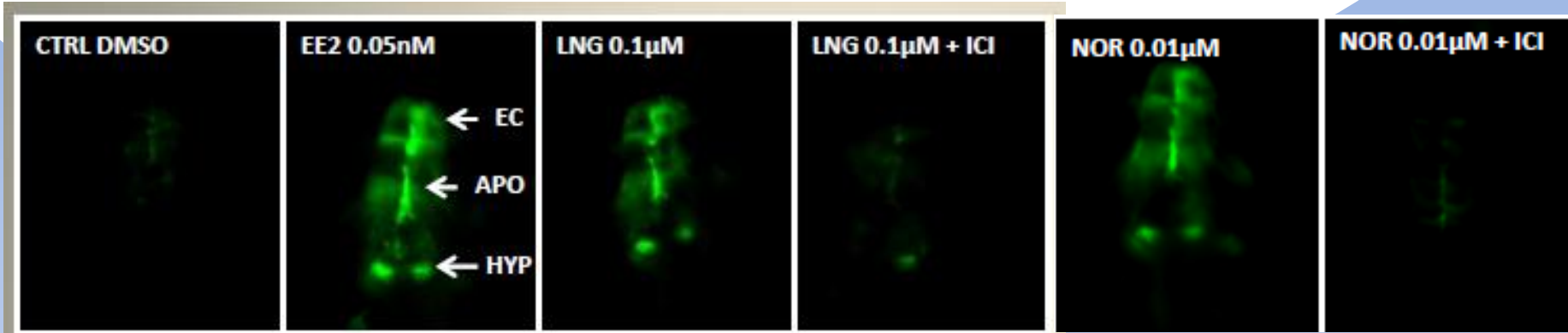
Response of *cyp19a1b*-GFP to androgens



Mouriec et al., 2010

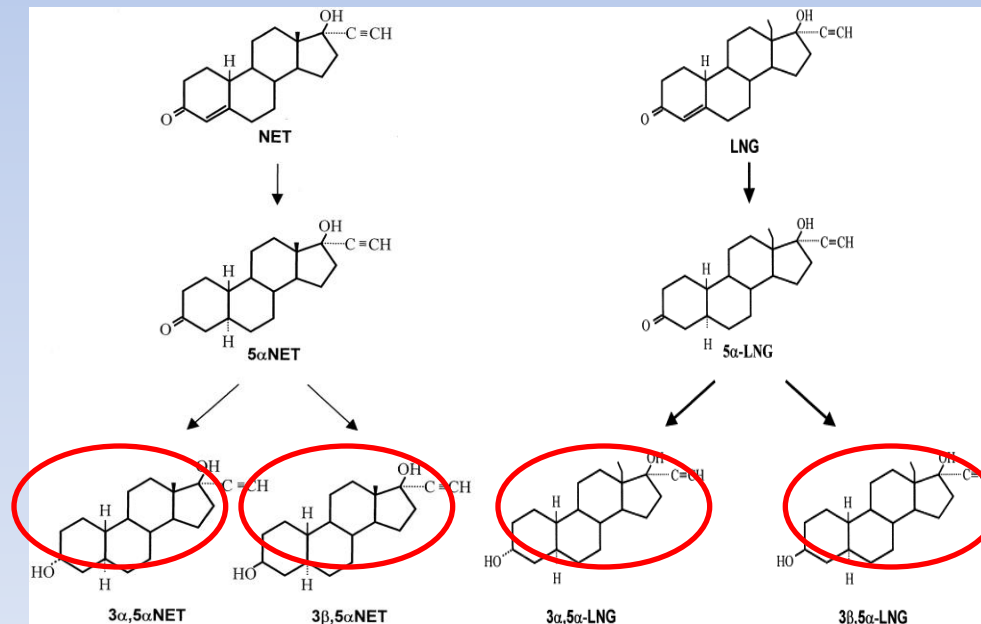
Metabolisation of DHT to 5 alpha-androstan-3 beta, 17 beta-diol (β diol), a known estrogenic androgen derivative that activates ERs

Response of *cyp19a1b*-GFP to Progestins



NET= Norethindrone

LGN= Levonorgestrel



3α,5α- and 3β,5α-tetrahydro derivatives of NET and LNG are estrogens in mammalian models Larrera et al., 2001 , Garcia-Becerra et al., 2002

The *cyp19a1b*-GFP is a useful model for rapid *in vivo* screening of the endocrine disrupting potency of chemicals

- Simple
- Fast
- Reliable
- Sensitive
- Specific , Mechanism –based response
- True physiological brain-specific response
- Critical life stage of development

- EDCs are of high environmental concerns
- Numerous compounds can act on the endocrine system
- Complex mode of action
 - Hormono-regulated genes in brain, pituitary, liver, gonads
 - Disruption of HPG axis
- The development of mechanism-based bio-assays (either in vitro or in vivo) is relevant
- In vivo assays in fish embryos (eg., zf tg cyp19a1b-GFP embryo assay) could allow to take into account this complexity and be useful for environmental monitoring