Use of transgenic zebrafish models to study the endocrine effects of natural and synthetic progestins

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Context

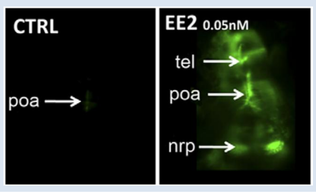
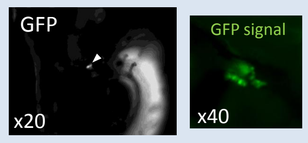
- Amongst **endocrine disruptors**, estrogenic compounds such as the estrogen 17α-ethinylestradiol (EE2) received much attention during the last decades regarding the risks they pose on **aquatic wildlife**.
- Conversely, far fewer studies addressed the environmental occurrence and the effects of natural and synthetic ligands of the **nuclear progesterone receptor (nPR)** on fish development and reproduction, resulting in a significant lack of data to assess the hazard and risk posed by these compounds on aquatic organisms.
- Yet, **progesterone** and its derivatives play key roles in development and reproduction in many vertebrates including fish (gametogenesis, ovulation, spermiation).
- **Progesterone** and **synthetic progestins** are widely used in contraception (often associated with estrogens like EE2) and other medical applications, and also widely used in livestock and cattle.
- Recent data showed that some progestins are present in the aquatic environment at low concentrations in the ng/L range, potentially impairing fish reproduction.
- Due to the potential risk of progestins to the environment, there is a need to understand the mechanisms of action and characterize the effects of these compounds towards the endocrine system of fish. This work is part of a national research project (ANR PROOFS) dedicated to environmental progestins and their effects in fish.

Objectives

To characterize the mechanisms of action and the biological effects of progestins on the endocrine system of fish with the use of complementary mechanism-based transgenic zebrafish bioassays.

Transgenic zebrafish larval models

24 nPR ligands (table 1) were selected to be tested on two **transgenic zebrafish lines** expressing Green Fluorescent Protein (GFP) under the control of promoters of steroidogenic genes.

	A model to study the estrogenic effects of compounds: the EASZY assay **Transgenic zebrafish line *cyp19a1b*-GFP**	**A model to study the effects of compounds on the glucocorticoids signaling pathway** **Transgenic zebrafish line *cyp11c1*-GFP**
Gene studied	*cyp19a1b*	*cyp11c1*
Enzyme encoded by the gene	P450 Aromatase B	11β-hydroxylase (11βH)
Localisation of the expression	Radial glial cells in brain	Interrenal cells and gonads
Role of the enzyme	Conversion of testosterone into estradiol	Cortisol and 11-ketotestosterone biosynthesis
Regulation of the gene expression	ER-regulated (Estrogen Receptor)	Potentially GR-regulated (Glucocorticoid Receptor)
Reporter gene	GFP	GFP
Bioassay	EASZY assay (Brion et al., 2012). Exposure from 0 to 4 days post-fertilization (dpf). *In vivo* imaging of 4 dpf old live transgenic *cyp19a1b*-GFP zebrafish embryos. Dorsal views (anterior to the top) of the telencephalon (tel), preoptic area (poa), and nucleus recessus posterioris (nrp) of the caudal hypothalamus. CTRL: solvent control, EE2: 17a-ethinylestradiol. 	Exposure from 4 to 6 dpf In vivo imaging of 6 dpf old live transgenic *cyp11c1*-GFP zebrafish embryos. Lateral views. 

Results

Screening of progesterone receptor ligands on the *cyp19a1b*-gfp larvae (EASZY assay)

- **All progestins derived from 19-nortestosterone induce GFP expression in a concentration-dependant manner in the EASZY assay (table 1 and figure 1).**
- **Progesterone, dydrogesterone, drospirenone and all progestins derived from 19-norprogesterone and 17α-hydroxyprogesterone show no effect on GFP expression in the EASZY assay (table 1).**

Table 1: Classification of some natural hormones and synthetic progestins. The effects on the EASZY assay and the calculated EC50 are shown. + : GFP induction, - : GFP inhibition, ne: no effect, nc: not calculated.

Classification		**Compound**	**Induction of GFP expression in the EASZY assay**	**EC50 (nM)**
Natural estrogen		17β-estradiol	+	1.7
Natural androgen		Testosterone	+	505
Natural progestin		Progesterone	ne	-
Retroprogesterone		Dydrogesterone	ne	-
Natural hormone		Pregnenolone (progesterone precursor)	+	1000
Synthetic estrogen		17α-ethinylestradiol	+	0.01
Progestins structurally related to progesterone	Derived from 17α-hydroxyprogesterone	Medroxyprogesterone	ne	-
		Medroxyprogesterone acetate	ne	-
		Megestrol acetate	ne	-
		Chlormadinone acetate	ne	-
	Derived from 19-norprogesterone	Cyproterone acetate	ne	-
		Promegestone	ne	-
		Nestorone	ne	-
		Nomegestrol acetate	ne	-
Progestins structurally related to testosterone: derived from 19-nortestosterone	Estranes	Ethisterone	+	40
		Ethynodiol diacetate	+	53
		Lynestrenol	+	16
		Norethindrone acetate	+	2.4
		Norethindrone	+	4.1
		Tibolone	+	0.3
	Gonanes	Desogestrel	+	nc
		Etonogestrel	+	39
		Gestodene	+	222
		Levonorgestrel	+	89
		Norgestimate	+	126
		Norgestrel	+	184
Progestin structurally related to spironolactone		Drospirenone	ne	-
PR antagonist		Mifepristone	+	nc

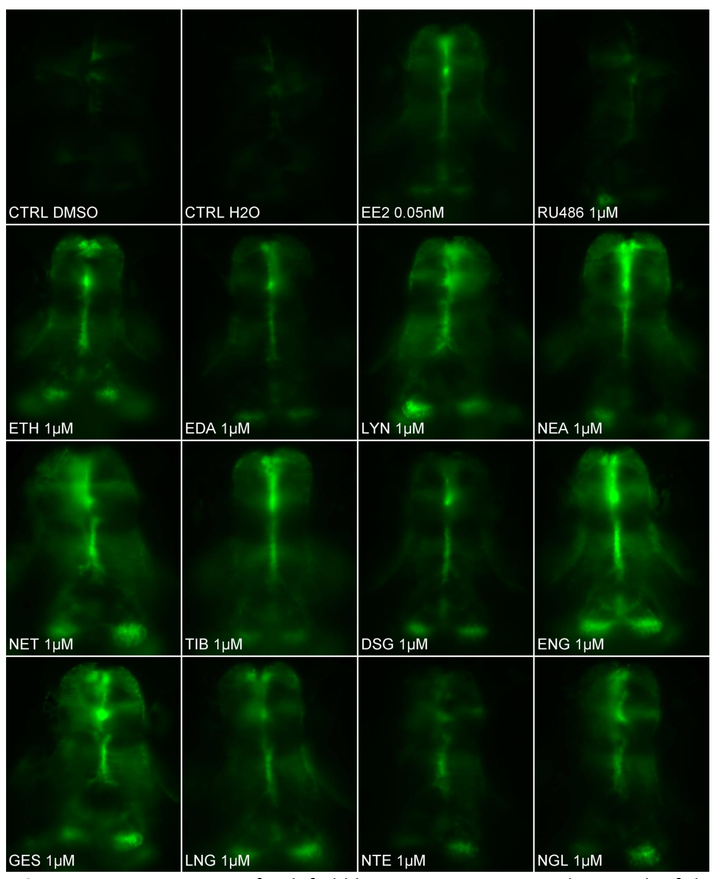


Figure 1: *In vivo* imaging of 4 dpf old live transgenic *cyp19a1b*-GFP zebrafish embryos exposed to progestins inducing GFP expression in radial glial progenitors. Dorsal views. For each chemical the concentration used is indicated. CTRL: solvent control, H2O: water control, EE2: 17α-ethinylestradiol, RU486: mifepristone, NET: norethindrone, TIB: tibolone, DSG: desogestrel, ENG: etonogestrel, ETH: ethisterone, EDA: ethynodiol diacetate, LYN: lynestrenol, NEA: norethindrone acetate, GES: gestodene, LNG: levonorgestrel, NTE: norgestimate, NGL: norgestrel.

Screening of progesterone receptor ligands on the *cyp11c1*-GFP larvae

Some of the pro-estrogenic progestins seem to down-regulate the expression of *cyp11c1* in the interrenal cells (figure 2).

Exposure of *cyp11c1*-GFP larvae to progestins from 4 to 6 dpf

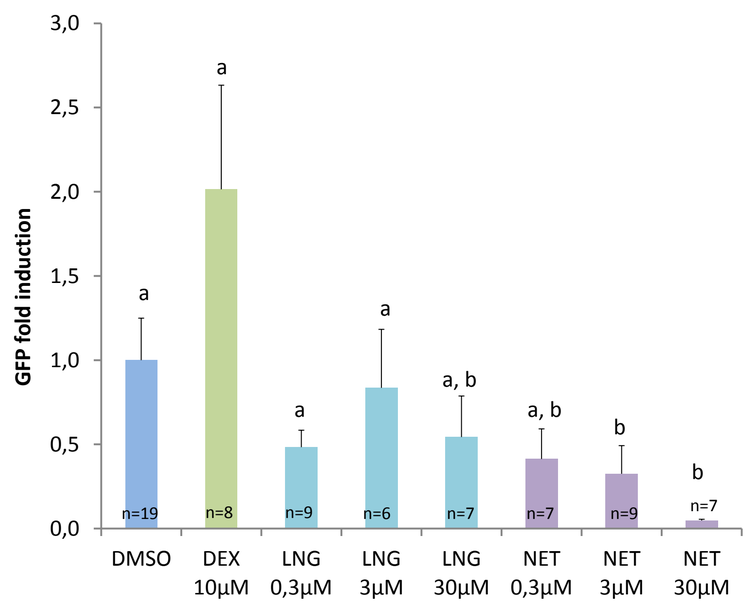


Figure 2: Induction of GFP expression in the interrenal cells of 6 dpf *cyp11c1*-GFP larvae compared to DMSO. DMSO: solvent control, DEX: dexamethasone, LNG: levonorgestrel, NET: norethindrone. Significant differences are shown by letters. Error bars represent sem.

- **Parallel experiments have shown that *cyp11c1* is up-regulated by dexamethasone, an agonist ligand of glucocorticoids receptor (GR).**
- **The results thus suggest that some progestins are capable to affect the expression of a GR-regulated gene.**

Conclusions

- Two transgenic zebrafish models have been successfully used to characterize the effects of progesterone and synthetic progestins.
- All the tested progestins derived from 19-nortestosterone induced the expression of Aromatase B, revealing their estrogenic activity *in vivo* and thus their ability to interfere with the expression of hormone-regulated genes in radial glial cells in developing embryos and larvae.
- Furthermore, some of the pro-estrogenic progestins were capable of down-regulating the expression of the GR-regulated gene *cyp11c1* coding for 11β-hydroxylase, thus showing that some progestins can disrupt expression of steroidogenic genes playing critical roles in the development of fish.
- The combination of zebrafish models allowed to demonstrate the capacity of emerging aquatic contaminants to disrupt the tissue-specific expression of steroidogenic genes involved in estrogen and corticosteroid synthesis.

Perspectives

- To complement this screening study, the mechanisms of action of progesterone and synthetic progestins will be further characterized on various human and zebrafish nuclear receptors using *in vitro* reporter gene assays. This approach will provide informations about the competitivity of hormones and potential species-specificity.
- To study the consequences of progestins interfering with the expression of ER- and GR-regulated genes, some active nPR ligands will be tested on adult zebrafish. This approach will provide data on the effects of these compounds on reproduction, notably by the quantification of transcripts levels of steroidogenic hormones in brain and gonads and plasma sex hormones levels.

References: *Hsu et al. 2003, Schindler et al. 2003, To et al. 2007, Liu et al. 2007, Alsop et al. 2008, Besse et al. 2009, Sun et al. 2010, Runnalls et al. 2010, Weger et al. 2012, Tomasicchio et al. 2013, Hinfray et al. 2014, Kumar et al. 2015, Fent 2015.*
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