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Additive effects of levonorgestrel and ethinylestradiol on brain aromatase (*cyp19a1b*) in zebrafish specific *in vitro* and *in vivo* bioassays

Hinfray N^{1*}, Tebby C², Garoche C¹, Piccini B¹, Bourgine G³, Aït-Aïssa S¹, Kah O³, Pakdel F⁴, Brion F¹.

¹ INERIS, Unité d'écotoxicologie *in vitro* et *in vivo*, Verneuil-en-Halatte, France

² INERIS, Unité Modèles pour l'Ecotoxicologie et la Toxicologie, Verneuil-en-Halatte, France

³ IRSET, équipe NEED, Université de Rennes 1, Rennes, France

⁴ IRSET, Inserm U1085, équipe TREC, Université de Rennes 1, Rennes, France

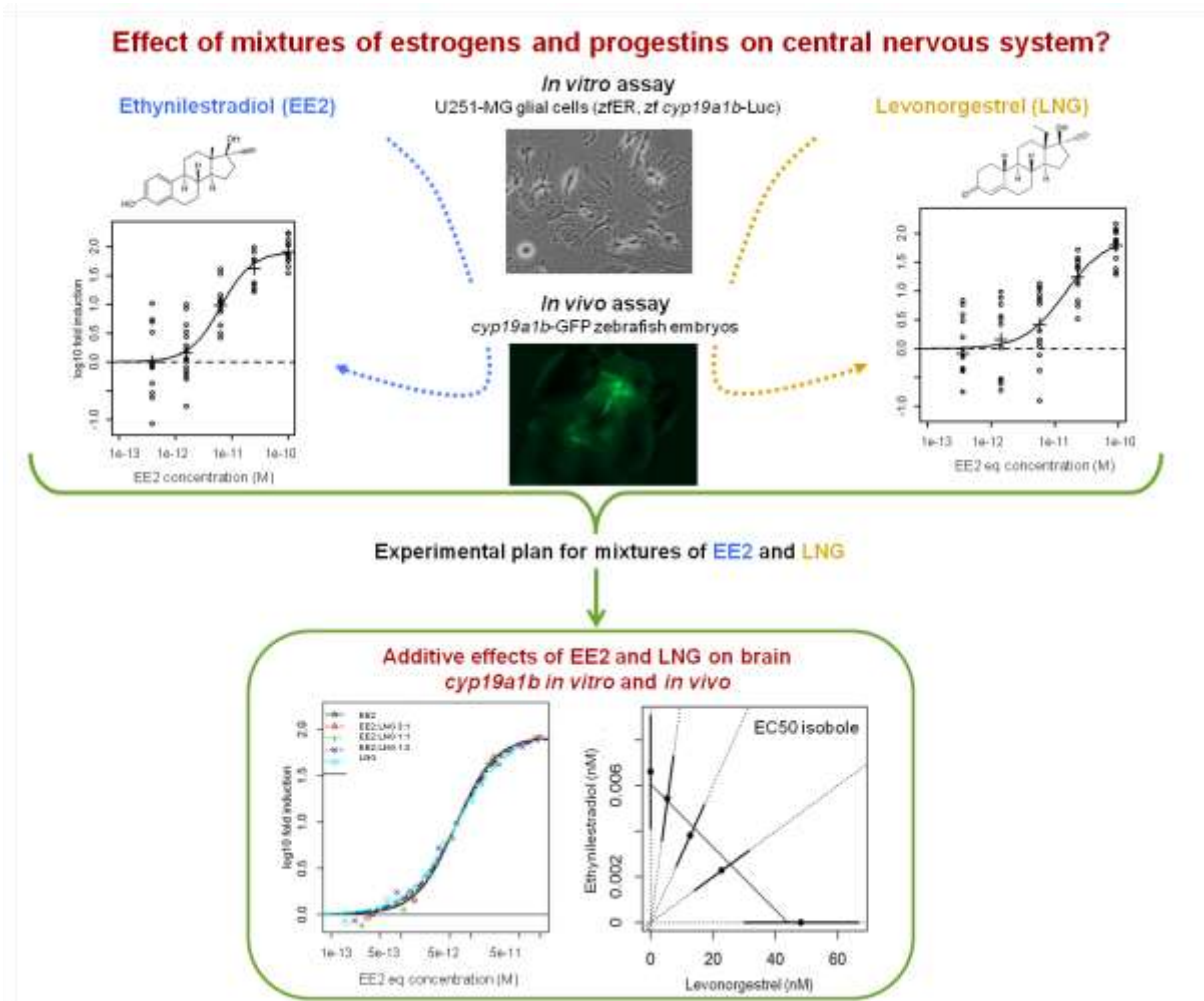
* Corresponding author: nathalie.hinfray@ineris.fr

ABSTRACT

Estrogens and progestins are widely used in combination in human medicine and both are present in aquatic environment. Despite the joint exposure of aquatic wildlife to estrogens and progestins, very little information is available on their combined effects. In the present study we investigated the effect of ethinylestradiol (EE2) and Levonorgestrel (LNG), alone and in mixtures, on the expression of the brain specific ER-regulated *cyp19a1b* gene. For that purpose, recently established zebrafish-derived tools were used: (i) an *in vitro* transient reporter gene assay in a human glial cell line (U251-MG) co-transfected with zebrafish estrogen receptors (zfERs) and the luciferase gene under the control of the zebrafish *cyp19a1b* gene promoter and (ii) an *in vivo* bioassay using a transgenic zebrafish expressing GFP under the control of the zebrafish *cyp19a1b* gene promoter (*cyp19a1b*-GFP). Concentration-response relationships for single chemicals were modeled and used to design the mixture experiments following a ray design. The results from mixture experiments were analyzed to predict joint effects according to concentration addition and statistical approaches were used to characterize the potential interactions between the components of the mixtures (synergism/antagonism). We confirmed that some progestins could elicit estrogenic effects in fish brain. In mixtures, EE2 and LNG exerted additive estrogenic effects both *in vitro* and *in vivo*, suggesting that some environmental progestin could exert effects that will add to those of environmental (xeno-)estrogens. Moreover, our zebrafish specific assays are valuable tools that could be used in risk assessment for both single chemicals and their mixtures.

KEYWORDS: levonorgestrel, ethinylestradiol, mixture, brain aromatase, transgenic zebrafish, U251-MG cells

GRAPHICAL ABSTRACT



HIGHLIGHTS

- Combined effects of EE2 and LNG were assessed on ER-dependent *cyp19a1b* expression
- EE2 and LNG alone induced brain aromatase in zebrafish specific bioassays
- Experimental ray design allowed complete concentration-response surfaces modeling
- EE2 and LNG exerted additive effects on brain aromatase in radial glial cells

INTRODUCTION

Endocrine disrupting chemicals (EDCs) have been extensively studied during the last decade due to their adverse effects on aquatic organism reproduction and development. To date, most attention on EDCs has been focused on compounds able to interact with the estrogen receptors (ERs). The occurrence, fate and effects of both natural and synthetic estrogens (estradiol (E2), estriol, estrone and ethinylestradiol (EE2)) and estrogen-like compounds are now well documented. As estrogens can be found in mixtures in the aquatic environment, a number of studies evaluated the effects of their combined exposure on estrogen signaling in aquatic organisms. Results from these studies demonstrate that binary or multi-component mixtures of ER agonists generally act in an additive manner on the expression of ER-regulated genes in both brain and liver (Thorpe *et al.* 2001, Rajapakse *et al.* 2004, Lin and Janz 2006, Kortenkamp 2007, Petersen and Tollefsen 2011, Brion *et al.* 2012, Petersen *et al.* 2013). In some cases, some deviations from additivity can be observed, especially with infra-additive effects, both in *in vitro* and *in vivo* experiments (Rajapakse *et al.* 2004, Lin and Janz 2006, Petersen and Tollefsen 2011, Petersen *et al.* 2013).

Estrogens and progestins are widely used in combination in human medicine, especially in oral contraceptives and hormone replacement therapy (Zeilinger *et al.* 2009). Their extensive use and poor removal by sewage treatment plants have led to contamination of the aquatic environment (Besse and Garric 2009, Liu *et al.* 2011). However, compared to estrogens, the occurrence, fate and effects of progestins were poorly studied. They are found in effluents and in surface waters (rivers, lakes, streams) and ground waters at concentrations up to tens of ng/L, but also in sediments from rivers at concentrations up to tens of ng/g (for review see (Besse and Garric 2009, Liu *et al.* 2011, Fent 2015). Moreover, progestins are potent developmental and reproductive toxicants for aquatic organisms (for review see

Zeilinger *et al.* 2009, Kumar *et al.* 2015). Among these progestins, levonorgestrel (LNG) is a synthetic progestin structurally related to testosterone (19-Nortestosterone derivative), used alone or in association with an estrogen such as ethinylestradiol for contraception purposes (emergency contraceptives or birth control pills). LNG has been detected in some effluents, sediments, ground water, tap water, but also in surface water of rivers at concentrations up to 38 ng/L (Vulliet *et al.* 2008, Besse and Garric 2009, Al-Odaini *et al.* 2010, Liu *et al.* 2011, Vulliet and Cren-Olivé 2011, Fent 2015). LNG exerts biological activities that differ from the natural progestin (progesterone) since it has progestagenic and androgenic activities (Besse and Garric 2009) and also estrogenic activities both *in vitro* and *in vivo* (Jeng *et al.* 1992, Brion *et al.* 2012, Zucchi *et al.* 2012, Creusot *et al.* 2014, Kroupova *et al.* 2014). However, despite the joint exposure of aquatic wildlife to estrogens and progestins, very little information is available on their combined toxicity (Runnalls *et al.* 2015, Säfholm *et al.* 2015).

This study aims at investigating the effects of single and combined exposure to EE2 and LNG on the expression of the zebrafish *cyp19a1b* gene both in *in vitro* and *in vivo* models. In zebrafish, the *cyp19a1b* gene encodes the brain form of aromatase (aromatase B) which is only expressed in radial glial cells that act as neuronal progenitors both in developing and adult brain (Pellegrini *et al.* 2007). The *cyp19a1b* gene is extremely sensitive to (xeno-)estrogens and this regulation is ER-dependent (Le Page *et al.* 2008, Brion *et al.* 2012). In the past few years, we developed both *in vitro* and *in vivo* bioassays, based on zebrafish *cyp19a1b* gene, that were used in this study: i) a human glial cell culture (U251-MG) co-transfected with zebrafish ER subtypes (zfER α , zfER β 1 and zfER β 2) and a luciferase gene under the control of the zebrafish *cyp19a1b* promoter (Le Page *et al.* 2006), ii) a transgenic zebrafish (*cyp19a1b*-GFP) line expressing GFP under the control of the zebrafish *cyp19a1b* promoter which is suitable to detect estrogenicity of chemicals alone and in mixtures (Brion *et al.* 2012,

Petersen *et al.* 2013). These *in vivo* and *in vitro* bioassays were used to assess the estrogenic responses of EE2 and LNG in mixtures; responses that were modeled using the concentration-addition (CA) prediction model. Deviations from this no-interaction model were characterized in terms of synergism or antagonism, modeled using Jonker's interaction terms (Jonker *et al.* 2005) and their significance was tested. By this approach, the present study reports additive estrogenic effects of EE2 and LNG in mixtures on the expression of an estrogen-regulated gene in a glial cell context.

MATERIAL AND METHODS

Chemicals

EE2 (purity \geq 98%, CAS number: 57-63-6; reference: E4876) and LNG (purity \geq 98%; CAS number: 797-63-7; reference: N2260) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Zebrafish maintenance and breeding

Animal culture, handling and experimentation were approved by the INERIS life science ethics committee and in accordance with French ethical laws. The *cyp19a1b*-GFP transgenic zebrafish (Tong *et al.* 2009) were held at the Institut National de l'Environnement Industriel et des Risques (INERIS, Verneuil-en-Halatte, France). They were maintained in 3.5 L aquaria in a recirculation system (Zebtec, Tecniplast, Buguggiate, Italy) on a 14 h light: 10 h dark cycle at a temperature of $25.1 \pm 1.0^\circ\text{C}$. They were allowed to reproduce (ratio of 2

males for 1 female). Fertilized eggs were harvested and disinfected 5 min in water supplemented with 0.1% of commercial bleach (2.6% of sodium hypochlorite).

Zebrafish exposure to EDCs

Fertilized *cyp19a1b*-GFP transgenic zebrafish eggs were exposed to chemicals (alone or in mixtures) or to solvent control (DMSO, 0.02% v/v) according to (Brion *et al.* 2012) with minor modifications. Briefly, for each experimental condition, 20 embryos were exposed in 100 ml of water. Embryos were kept at 28°C, under semi-static conditions. Exposures were performed from 0 days post fertilization (dpf) to 4 dpf without water renewal. At the end of the exposure period (96 hours), non-transgenic zebrafish were removed and 4-dpf old transgenic zebrafish were processed for fluorescence measurement by image analysis. Experiments were performed in accordance with European Union regulations concerning the protection of experimental animals (Directive 2010/63/UE).

In vivo imaging

In vivo fluorescence imaging was performed according to (Brion *et al.* 2012). Each live *cyp19a1b*-GFP transgenic embryo was photographed once in dorsal view using a Zeiss AxioImager Z1 fluorescence microscope equipped with an AxioCam Mrm camera (Zeiss GmbH, Göttingen, Germany). Each photograph was acquired under the same exposure conditions (X10 objective, 134 ms of fluorescent light exposure, maximal light intensity). Fluorescence quantification was performed using Image J software (available at: <http://rsb.info.nih.gov/ij/>). For each picture, the integrated density (IntDen) was measured, i.e.

the sum of the grey-values of all the pixels within the region of interest. All grey-values of 300 or less were defined as background values.

U251-MG cell bioassay

The ER-negative human glial cell line U251-MG (ECACC) culture, handling and the luciferase assay were performed according to (Le Page et al. 2006).

U251-MG cells were maintained at 37°C in 5% CO₂ atmosphere in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM-F12, Sigma-Aldrich, St Louis, MO, USA) supplemented with 8% fetal calf serum (FCS), 2 mM L-glutamine, 20 U/mL penicillin, 20 µg/mL streptomycin and 50 ng/mL amphotericin B.

For transfection experiments, U251-MG cells were plated in 24-well plates at a density of 0.2×10^5 cells/ml. After 24 hours, the medium was replaced with fresh phenol red-free DMEM containing 2% FCS. In each well, 25 ng of zfER expression vector (i.e. Topo-pcDNA3 expression vector containing the coding region of zfER α or zfER β 2 complementary DNA and the neomycin resistance gene (Menuet et al. 2002)), 25 ng of cytomegalovirus- β -galactosidase control plasmid and 150 ng of luciferase reporter construct (i.e. proximal promoter region of the zebrafish cyp19a1b gene coupled to the luciferase reporter gene (Menuet et al. 2005)) were transfected using JetPEITM reagent, as indicated by the manufacturer (Polyplus-transfection, France). After one night, medium was replaced with fresh DMEM-F12 containing 2% charcoal/dextran FCS with xeno-estrogens or vehicle (DMSO, 0.1% v/v). The luciferase activities were assayed after 48 h using the luciferase assay system (Promega). β -galactosidase activity was used to normalize transfection efficiency in all experiments. Results were expressed as fold induction relative to the solvent.

Data normalization

In the *in vivo* assay with *cyp19a1b*-GFP transgenic zebrafish, induction of GFP fluorescence was measured as IntDen and normalized by dividing by the geometric mean of the IntDen in the DMSO control group. In the preliminary single chemical experiments used for the design of mixture experiments, the concentration-response relationships were obtained in separate experiments. For that reason, the log-inductions were further normalized by the logarithm of the geometric mean of the positive controls (EE2; 0.05nM), which corresponds to a maximum response level.

In the *in vitro* assay with U251-MG cell cultures, the data were normalized by dividing by the geometric mean of the corresponding solvent control group. EE2 and levonorgestrel as single chemicals were tested on the same plate therefore no additional normalization was required.

Concentration-response modeling

The relationship between concentration and log-induction was modeled with a 3-parameter Hill model, where the basal level was set to 0:

$$\Phi(c) = \frac{Max}{1 + \left(\frac{c}{EC50}\right)^\beta} \quad (1)$$

where *Max* is the maximum level of induction, *c* is the concentration, *EC50* is the concentration producing 50% of the maximum induction, and β is the Hill slope. In both *in vivo* and *in vitro* single chemical experiments, common values of *Max* and β were estimated for EE2 and levonorgestrel. Lack-of-fit F-tests were performed to check the model fit compared to an analysis of variance, and to check that the model did not fit less well with

common values for both compounds. The parameters were estimated by least squares, using R 3.1.1 (R Core Team 2014) and package drc (Ritz and Streibig 2005).

Mixture experimental designs

Preliminary single chemical experiments were performed in order to construct the mixture experimental designs.

In the *in vivo* assay, three concentration-response datasets for EE2 and two concentration-response datasets for levonorgestrel were used. Each concentration-response was successfully modeled with the Hill model and common values for the maximum response level and the slope were successfully estimated. These models were used to build a ray design with single chemicals and three mixture ratios (3:1, 1:1, and 1:3 in terms of relative toxicity quantified by the EC50), with five concentrations along each ray, centered around the EC50 with 4-fold dilutions (range of EE2 concentrations from 9.77×10^{-8} μM (28.9 pg/L) to 1×10^{-4} μM (29.6 ng/L) and range of LNG concentrations from 0.293 nM (91 ng/L) to 0.3 μM (93 $\mu\text{g/L}$)) (Supplemental Table 1). In theory, the equimolar mixture ratio is the one where interactions are likely to be most visible.

In the *in vitro* assay, one experiment with EE2 and levonorgestrel on the same plate was used to calibrate the design. With levonorgestrel, no response saturation was observed in the range of concentrations used to test for ER receptor activation. The maximum response and Hill slope were therefore imputed from the EE2 dose-response. The data were successfully modeled with the 3-parameter Hill model. Given that the levonorgestrel ray and likely some of the mixture rays would not be modeled with the 3-parameters Hill model independently from the rest of the data because of the absence of response saturation, a factorial design for the mixture experiment was preferred over a ray design. This was build

with 3 levels of levonorgestrel (0, 1, and 10 μM) and 6 levels of EE2 (0, 0.001 nM to 10 nM with 10-fold dilutions).

Mixture concentration-response modeling

Concentration-response surfaces were modeled with the concentration addition (CA) model (Loewe 1953) using Berenbaum's general solution (Berenbaum 1985). This CA model is only defined when all mixture components produce the same maximum response and when the basal response is equal. In view to model mixture experiment data with the CA model, several adjustments were performed. Since the data for single compounds were obtained from several independent experiments, they were normalized (data were divided by the geometric mean of the non-treated group) to be able to compare the concentration-responses curves. The single compound data were modeled with a null basal response without detriment to the modeling (F-test, $p < 0.05$) and with no significant change of the EC50s. Then, common maximum responses were estimated. At present, there is still a discussion on the validity of the CA model when single compounds produce different slopes because this would suggest that the mode of action is different (Backhaus *et al.* 2004, Gennings *et al.* 2005): the concept of Toxic Equivalent Factor is a more restrictive version of CA (Webster 2013).. For that reason, when this was not detrimental to the model fit, a common slope was also estimated for single compounds. The only free remaining parameter was then the EC50. All these constraints do artificially slightly reduce the uncertainty round the EC50, but when they are not detrimental to the model fit, the estimation of the EC50 is not significantly affected by setting these three parameters (except for LNG in the U251-MG cells transfected with zfER β 2 where no saturation response was observed).

Interaction terms for simple antagonism/synergy (SA), dose-ratio dependent interactions (DR), and dose-level dependent interactions (DL) were subsequently added to the CA model (Jonker *et al.* 2005). Significance of the interactions was assessed using approximate F-tests on the residual sums of squares by considering that the models were nested. Acceptability of the concentration-response surface models was assessed with a lack-of-fit F-test compared to the analysis of variance model.

RESULTS

No effect due to chemical exposure was observed on lethality or time to hatch during any of the *in vivo* single chemical and mixture studies.

In vitro effects of single test compounds

The effect of EE2 and LNG alone was assessed in U251-MG glial cells co-transfected with zf-ERs and the *Zf-cyp19a1b* promoter-luciferase reporter (one exposure experiment for each compound and each ER, 3 measure replicates for each condition). EE2 treatment induced expression of luciferase activity in a concentration-dependent manner, and EC50 (median effective concentration) of 1.4×10^{-10} M for ER α and 6.63×10^{-12} M for ER β 2 were calculated (supplemental figure 1). LNG alone was poorly effective in inducing luciferase activity and no saturation of the response was observed. EC50s for LNG equal to 36.1×10^{-6} M for ER α and 12.6×10^{-6} M for ER β 2 were calculated in that *in vitro* model by assuming that the maximum response and the slope were equal to those of EE2.

In vitro effects of binary mixtures of EE2 and LNG

EC50 for EE2 and LNG were calculated from mixture experiments to confirm EC50 previously obtained in single test compounds experiments. EC50 for EE2 were 1.29×10^{-10} M and 1.19×10^{-11} M for U251-MG glial cells transfected with ER α and ER β 2 respectively. For LNG, EC50 were 22×10^{-6} M and 8.6×10^{-6} M for U251-MG glial cells transfected with ER α and ER β 2 respectively. All these EC50s are in the same range of order than those previously calculated in the single test compound experiments.

In vitro, mixtures of EE2 and LNG led to a concentration-dependent induction of luciferase activity in U251-MG cells transfected both with ER α and ER β 2 (Figure 1). The measured inductions of luciferase activity can be modeled by the CA model (lack-of-fit F-test compared to the analysis of variance model: $p = 0.548$ for ER α and $p = 0.164$ for ER β 2). None of the interactions added to the CA model improved the model fit significantly and using different slopes for both compounds in the CA model did not improve the model fit either (approximate F-test: $p = 0.44$ for ER α and $p = 0.68$ for ER β 2).

In vivo effects of single test compounds

Exposure of transgenic *cyp19a1b*-GFP zebrafish to EE2 for 96h led to a concentration-dependent induction of GFP expression, with an EC50 of $5.0 \times 10^{-12} \pm 1.2 \times 10^{-12}$ M (Supplemental figure 2; 3 independent experiments with 8-19 transgenic zebrafish per condition). This expression of GFP is localized to radial glial cells of the brain (Figure 2). LNG alone also led to a concentration-dependent induction of GFP expression in radial glial cells of the brain with an EC50 of $1.68 \times 10^{-8} \text{ M} \pm 0.6 \times 10^{-8} \text{ M}$ (Supplemental figure 3; 2 independent experiments with 8-19 transgenic zebrafish per condition).

In vivo effects of binary mixtures of EE2 and LNG

In mixture experiment with transgenic *cyp19a1b*-GFP zebrafish, EC50 were 6.18×10^{-12} M for EE2 and 4.55×10^{-8} M for LNG.

The mixtures of EE2 and LNG induced GFP expression in radial glial cells of the brain of transgenic *cyp19a1b*-GFP zebrafish in a concentration-dependent manner (Figure 3). Observed responses were compared to the concentration-response surfaces modeled with the CA model with identical slopes for each compound (Loewe 1953). Observed responses were in good agreement with the CA model (lack-of-fit F-test compared to the analysis of variance model: $p=1,00$) and no deviation of the EC50 isobole was observed (Figure 4). Interactions were then added to the CA model but none showed a significant improvement of the adjustment quality of the model (approximate F-tests: $p=0.43$ for SA, $p=0.82$ for DR and $p=0.92$ for DL), indicating that EE2 and LNG exerted additive effects in mixture. Using different slopes for both compounds in the CA model did not improve the model fit either (approximate F-test: $p=0.42$).

DISCUSSION

The synthetic estrogen EE2 showed a high estrogenic potency *in vivo* in transgenic *cyp19a1b*-GFP zebrafish with an EC50 of 6.18×10^{-12} M (1.84 ng/L) for the induction of GFP expression in the radial glial cells of the brain similar to previous studies using the same *in vivo* model (Brion *et al.* 2012, Petersen *et al.* 2013). In transgenic medaka stably expressing GFP under the control of the choriogenin gene promoter and in transgenic zebrafish stably expressing luciferase under the control of an ERE, EE2 was less potent (EC50s of 2.4×10^{-10} M (71.9 ng/L) and of 1×10^{-10} M (296.4 μ g/L) respectively) than in the *cyp19a1b*-GFP transgenic zebrafish, confirming the sensitivity of both the *cyp19a1b* gene and the *cyp19a1b*-GFP transgenic zebrafish line to synthetic estrogens (Legler *et al.* 2002, Brion *et al.* 2012,

Spirhanzlova *et al.* 2016). In U251-MG cells transfected with ER α or ER β 2, EC50s are similar to those obtained using the same *in vitro* models in a previous study (Le Page *et al.* 2006) and to those calculated from the zfERs transactivation assays using human embryonic kidney cells (293HEK) cells (Legler *et al.* 2002). Both the *in vitro* (U251-MG cells transfected with ZfERs) and the *in vivo* (*cyp19a1b*-GFP transgenic zebrafish) models proved to be suitable tools to detect estrogenic compounds as previously stated by (Le Page *et al.* 2006, Brion *et al.* 2012, Petersen *et al.* 2013).

Due to the potential risk LNG represents for aquatic organisms, numerous *in vivo* studies have been conducted the last few years to determine its negative impacts to non-target organisms, notably to fish and amphibian reproduction (for review see Kumar *et al.* 2015).

Studies on the effects of LNG in aquatic organisms focused essentially on peripheral reproductive organs, i.e. gonads and liver, while very few data are available on other target tissues such as the central nervous system. *In vivo*, in adult fish, LNG has clear androgenic effects since it induces spiggin production by the kidney in male and female sticklebacks (Svensson *et al.* 2013, Svensson *et al.* 2014) and also male secondary sexual characteristics in female fathead minnows (Zeilinger *et al.* 2009, Runnalls *et al.* 2013, Runnalls *et al.* 2015). On the other hand, information on the estrogenic effects of LNG in fish is scarce. For instance, effects of LNG on the expression of vitellogenin (*vtg*), a hepatic ER-regulated gene, are rather heterogeneous. While in adult female three-spined sticklebacks, an inhibition of *vtg* gene expression was observed in parallel to spiggin production induction after LNG exposure (Svensson *et al.* 2013), in male and female pubertal roach, LNG exposure led to an induction of *vtg* expression (Kroupova *et al.* 2014). The discrepancy between these studies may be attributed to the differences in LNG concentrations used (one order of magnitude higher in roach (3 μ g/L) as compared to three-spined stickleback), to the different developmental stages of the fish (adult three-spined stickleback vs pubertal roach), and to different

metabolization/biotransformation capabilities between the two species. Nevertheless, in our study, LNG induced GFP expression in the brain of *cyp19a1b*-GFP transgenic zebrafish after 4 days of exposure with an EC₅₀ of 4.55×10^{-8} M (14.2 µg/L), confirming our previous findings on the estrogenic potency of LNG (Brion *et al.* 2012, Cano-Nicolau *et al.*, 2016). In zebrafish, exposure to 231.95 ng/L of LNG from 4 hours post fertilization (hpf) to 144 hpf also led to a slight induction of *vtg* expression (1.2 fold), supporting this estrogenic effect of LNG in zebrafish larvae (Zucchi *et al.* 2012).

In vitro, in the U251-MG cells transfected with zfERs and the luciferase gene under the control of the promoter of the *cyp19a1b* gene, LNG stimulates luciferase activity both with zfER α (EC₅₀ of 22×10^{-6} M) and zfER β 2 (EC₅₀ of 8.6×10^{-6} M). Moreover, the estrogenic effect of LNG is mediated by zfERs since luciferase induction is blocked by the addition of ICI 182-780, a pure ER antagonist (Cano-Nicolau *et al.*, 2016). Only little information on the *in vitro* estrogenic potency of LNG is available in the literature. In the human breast cancer cells MCF-7 (expressing hER α), LNG stimulates the ER-dependent growth of the cells, and this effect is blocked by an ER antagonist, ICI 164-384, demonstrating this effect is mediated by ERs (Jeng *et al.* 1992). In MELN cells (cells derived from the MCF-7 stably transfected with the luciferase gene under the control of an ERE), LNG induces the expression of luciferase with an EC₂₀ of 6.4×10^{-8} M (Creusot *et al.* 2014) and an EC₅₀ of 2×10^{-7} M (personal communication from N. Creusot). In zebrafish liver cell lines co-transfected with zfERs and the luciferase gene under the control of an ERE (ZELH-zfER) (Cosnefroy *et al.* 2012), LNG exerted EC₅₀s of 4×10^{-7} M and 2×10^{-7} M for ER α and ER β 2 respectively (personal communication from S. Aït-Aïssa). The sensitivity differences between all these models clearly highlight the necessity of using fish specific models in risk assessment of chemicals for aquatic organisms.

In mammals, while LNG does not bind to the ERs, its metabolites do and they seem to be responsible for the estrogenic effects observed (Lemus *et al.* 1992, Santillán *et al.* 2001, García-Becerra *et al.* 2002). In human cells, the estrogenic activity of LNG was attributed to its metabolite 3 β ,5 α -tetrahydro-LNG and is mainly mediated by hER α (García-Becerra *et al.* 2002, Escande *et al.* 2006). LNG metabolization into 3 β ,5 α -tetrahydro-LNG also occurs in rat hypothalamus and pituitary (Larrea *et al.* 1987). Moreover, LNG does not bind to zebrafish ERs either (Cano-Nicolau *et al.*, 2016), suggesting its conversion into estrogenic metabolites, especially in the brain. The differences of sensitivity to LNG of the different biological models (*in vitro* human with hERs or zfERs, *in vitro* fish, *in vivo* fish) could thus rely on differences in LNG metabolization capabilities of these different cell lines/organisms and on differences in binding affinity of metabolites to hERs and zfERs. Also, the possibility that these differences of sensitivity could rely on an ability of LNG to increase the levels of ERs expression in the brain of fish could not be ruled out. Nevertheless, our results clearly suggest that LNG (or its metabolites) could interact both with zfER α and zfER β 2. Altogether, our results give interesting information on the ability of LNG to interact with the zebrafish estrogenic pathway.

Given that simultaneous exposure to synthetic progestins and estrogens occurs in aquatic wildlife, information on mixture effect of these compounds is relevant. In the present study, we investigated the combined estrogenic effects of EE2 and LNG on the expression of the *cyp19a1b* gene of zebrafish. The results show that EE2 and LNG exert additive effects on the expression of the zebrafish *cyp19a1b* gene both *in vivo* and *in vitro* but with a better sensitivity of the *cyp19a1b*-GFP transgenic zebrafish line compared to U251-MG cells. *In vitro*, in yeast cultures co-transfected with hER α and the β -galactosidase under the control of an ERE, EE2 and different metabolites of LNG showed additive effects on the β -galactosidase activity while LNG alone had no effect (Santillán *et al.* 2001), reinforcing the idea that LNG

estrogenicity is mediated by its metabolites. In fathead minnow, EE2 and LNG co-exposure reduced egg production in an additive manner while the increase of *vtg* expression was only explained by exposure to EE2 with no contribution of LNG (Runnalls *et al.* 2015). In the same way, in the juvenile African clawed frog, no estrogenic effect of LNG alone or in mixture with EE2 is observed on the ER-regulated expression of *vtg beta1* gene or on the sex ratio (Säfholm *et al.* 2015). All these studies show that additive mixture responses of EE2 and LNG do not occur on all ER-regulated endpoints in all tissues and, as previously stated by others, at all levels of biological organization (Runnalls *et al.* 2015, Säfholm *et al.* 2015).

In the present study, the effects of LNG alone or in combination are observed for quite high concentrations (high ng/L range to low µg/L range) as compared to environmental LNG concentrations (dozens of ng/L). Some endpoints were showed to be disturbed at lower concentrations such as reduced egg production in female fathead minnow after exposure to 0.8 ng/L for 21 days or masculinization of female zebrafish exposed to 10 ng/L LNG during the process of sexual differentiation (0-63 dpf) (Zeilinger *et al.* 2009, Hua *et al.* 2015). However, LNG was showed to strongly bioconcentrate in fish since exposure to a water concentration of 1 ng/L of LNG gave rise to ng/ml plasma concentrations in rainbow trout, exceeding the human therapeutic plasma concentration (Fick *et al.* 2010). Although the effective concentrations of LNG in the *cyp19a1b*-GFP transgenic zebrafish line are higher than in other assays, it should be kept in mind that this bioassay allows the detection of the estrogenic effects of LNG after only 96 h of exposure in a simple and reliable *in vivo* assay. Moreover, the flexibility and space-saving character of this assay allows the assessment of a great number of conditions at the same time, ideal for complete experimental design needed for mixture testing. Thus, the *cyp19a1b*-GFP transgenic zebrafish line clearly emerges as a relevant *in vivo* assay to assess the effect of mixtures of chemicals on the ER-signaling pathway at early critical developmental stages. And, although extrapolation from this assay to

environmental scenarios is challenging, studies using the *cyp19a1b*-GFP transgenic zebrafish line could possibly identify compounds/mixtures that will need further investigations in *in vivo* studies with more integrative endpoints.

In conclusion, in the present study we demonstrated (i) that our *in vivo* (*cyp19a1b*-GFP transgenic zebrafish) biological model is a valuable tool that can be used in risk assessment for both single chemicals and their mixtures, (ii) the interest of employing complete experiment design models such as ray design to allow a better characterization of the effects of mixtures, even if no synergistic/antagonistic interaction was highlighted in mixtures of EE2 and LNG in our study, (iii) that EE2 and LNG in mixtures exert additive estrogenic effects both *in vitro* and *in vivo*, suggesting that some environmental progestins could exert effects that will add to those of environmental (xeno-)estrogens. Furthermore, the additive effects we observed on the expression of *cyp19a1b* occurred at early life stages, for very short exposure duration time and located in radial glial cells that are progenitor cells of the brain. Our present work did not explore the potential adverse effects of these compounds alone and in mixture on the (neuro)development of zebrafish although these radial glial cells are known to play a crucial role in neurogenesis (Pellegrini *et al.* 2007, Diotel *et al.* 2013). Moreover, neurogenesis is affected by exposure to (xeno-)estrogens by means of a modulation of the neurogenic activity of these radial glial cells (Diotel *et al.* 2013). These considerations naturally argue in favor of further studies on the physiological consequences of these early perturbations of the *cyp19a1b* gene expression on fish brain development.

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FIGURE CAPTIONS

Figure 1: Concentration-response curves of luciferase activity in U251-MG cells transfected with ER α or ER β 2 after exposure to ethinylestradiol (EE2) alone or in combination with levonorgestrel (LNG) at different concentrations. All the data were modeled by fitting the concentration-addition model to the concentration-response surface. The points represent the triplicate of each measure.

Figure 2: *In vivo* imaging of 4-dpf old live transgenic *cyp19a1b*-GFP zebrafish embryos exposed to chemicals inducing GFP expression in radial glial cells. Dorsal view of the brain. For each chemical the concentration used is indicated. DMSO: dmsolvent control; EE2: ethinylestradiol; LNG: levonorgestrel.

Figure 3: Concentration-response curves of GFP in *cyp19a1b*-GFP transgenic zebrafish after exposure to ethinylestradiol (EE2) and levonorgestrel (LNG) alone or in combinations (3 different ratios of substances). These data originated from the first exposure experiment and are representative of all the experiments performed. All the data were modeled by the concentration-addition model. Open circle represents one measure of GFP realized in one transgenic fish brain (n= 8-19 fish per condition). In the last graphic (right, bottom) which gathers all the concentration-response curves, the points represent the means of the GFP experimentally measured.

Figure 4: Illustration of the EC50 for each ray of the ethinylestradiol + levonorgestrel mixtures. The points represent the EC50 and the bars represent the standard error. These data originated from the first exposure experiment with the *cyp19a1a*-GFP transgenic zebrafish line (described in figure 3) and are representative of all the experiments performed.

Supplemental figure 1: Concentration-response curves of luciferase activity in U251-MG cells transfected with ER α or ER β 2 after exposure to ethinylestradiol (EE2) or levonorgestrel (LNG) alone at different concentrations. All the data were modeled by the 4 parameters log-logistic model both without any constraint (black dotted line) and with constraints applied on the max, the min and the slope (red line). The points represent the triplicate of each measure. The grey surfaces represent the 95% confidence intervals.

Supplemental figure 2: Concentration-response curves of GFP in *cyp19a1b*-GFP transgenic zebrafish after exposure to ethinylestradiol (EE2) at different concentrations in three independent experiments. All the data were modeled by the 4 parameters log-logistic model both without any constraint (black dotted line) and with constraints applied on the max, the min and the slope (red line). Open circle represents one measure of GFP realized in one transgenic fish brain (n= 8-19 fish per condition). The grey surfaces represent the 95% confidence intervals.

Supplemental figure 3: Concentration-response curves of GFP in *cyp19a1b*-GFP transgenic zebrafish after exposure to Levonorgestrel (LNG) at different concentrations in two independent experiments. All the data were modeled by the 4 parameters log-logistic model both without any constraint (black dotted line) and with constraints applied on the max, the min and the slope (red line). Open circle represents one measure of GFP realized in one transgenic fish brain (n= 6-12 fish per condition). The grey surfaces represents the 95% confidence intervals.

Figure 1

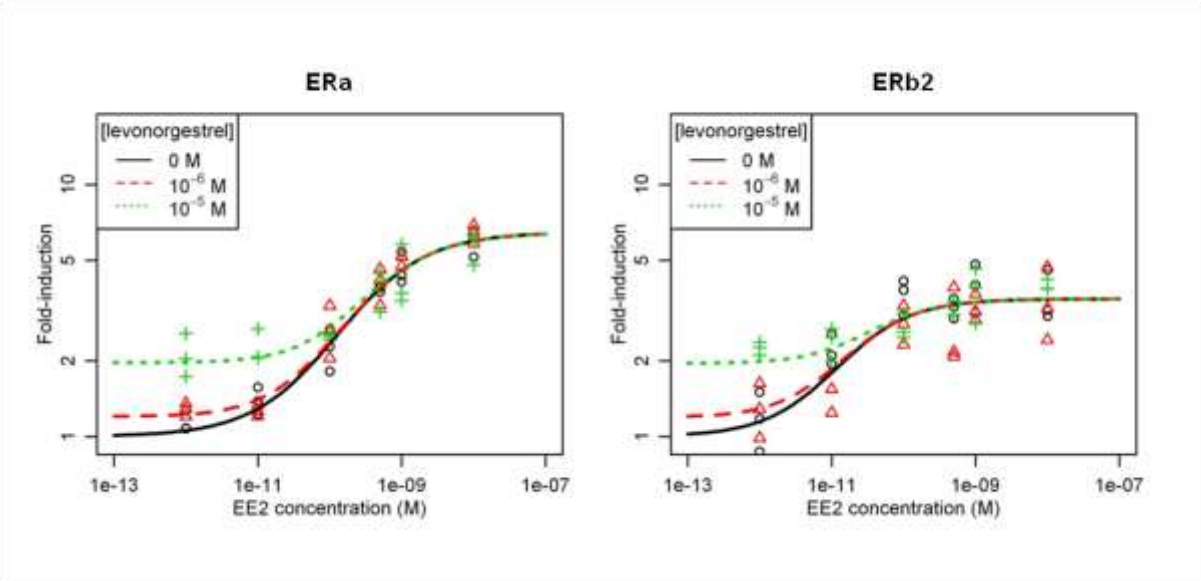


Figure 2

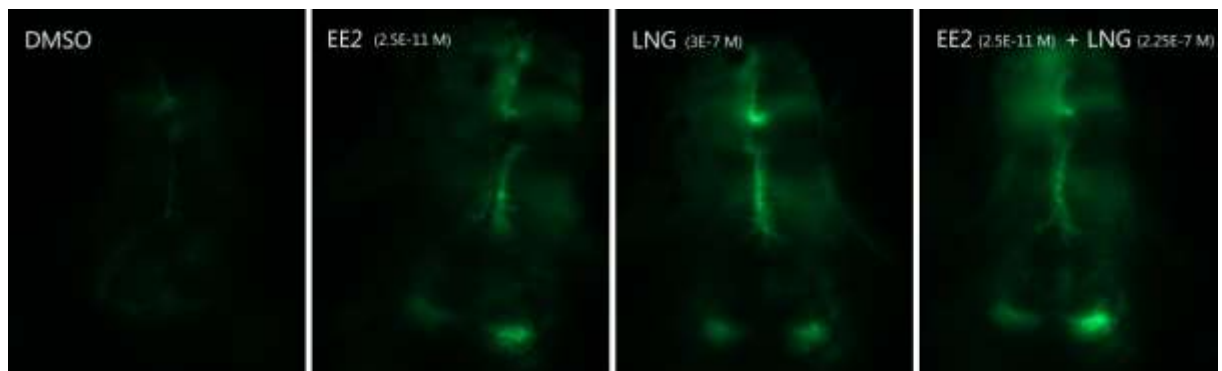


Figure 3

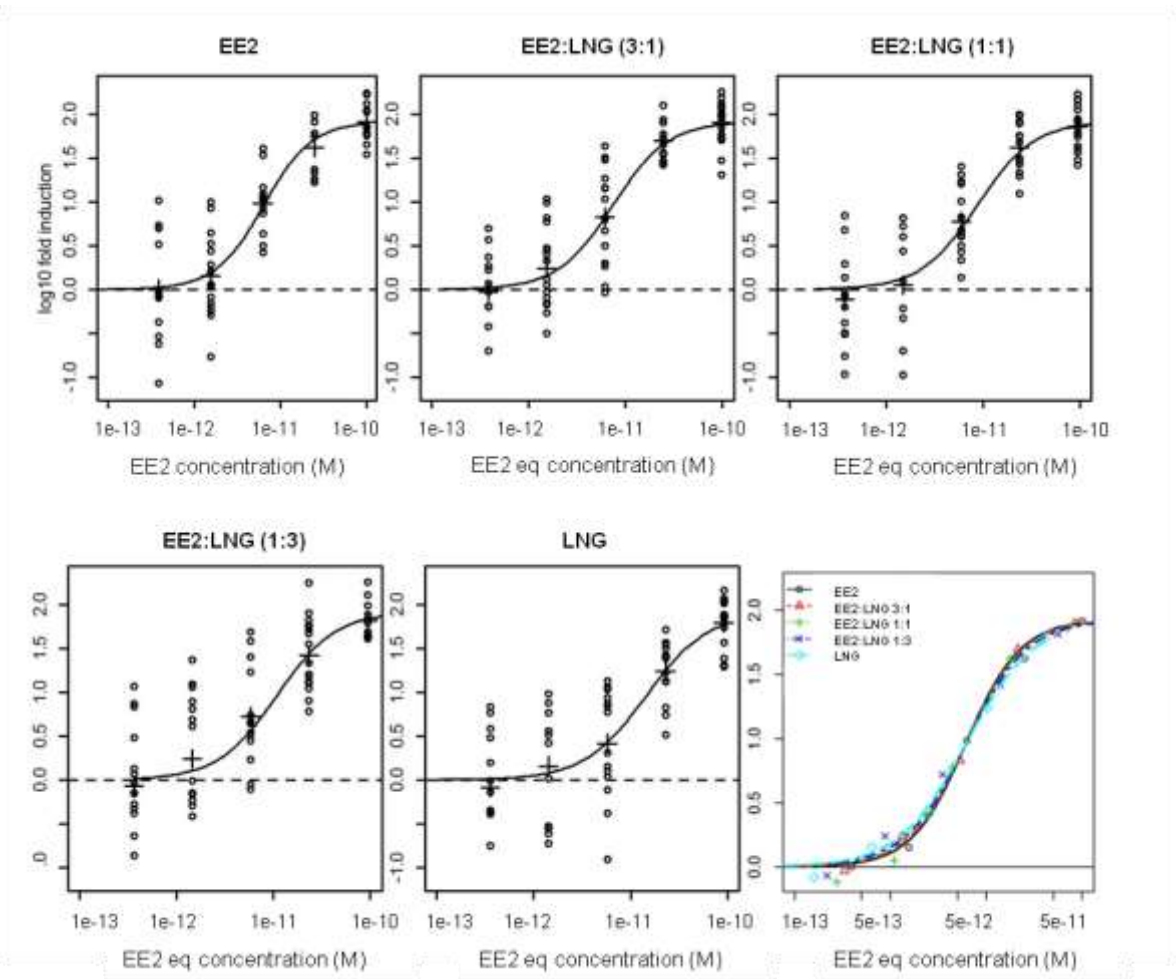
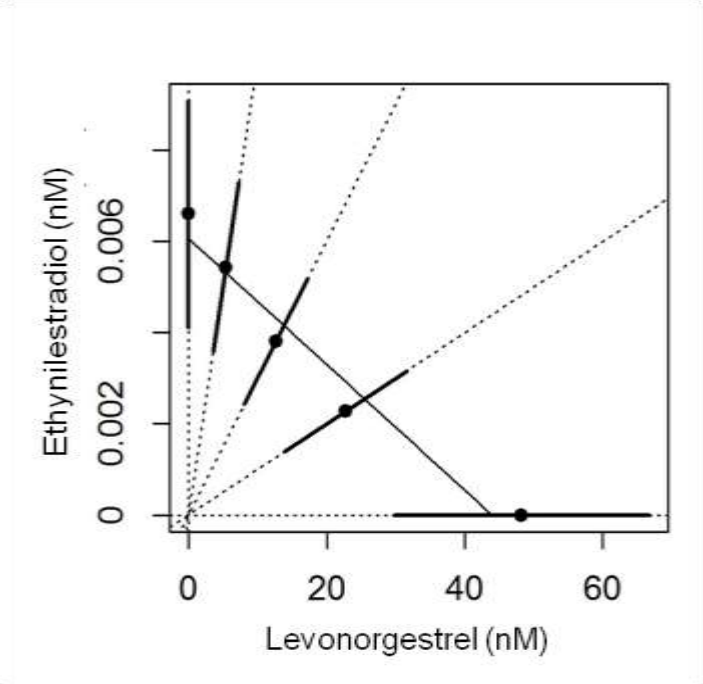
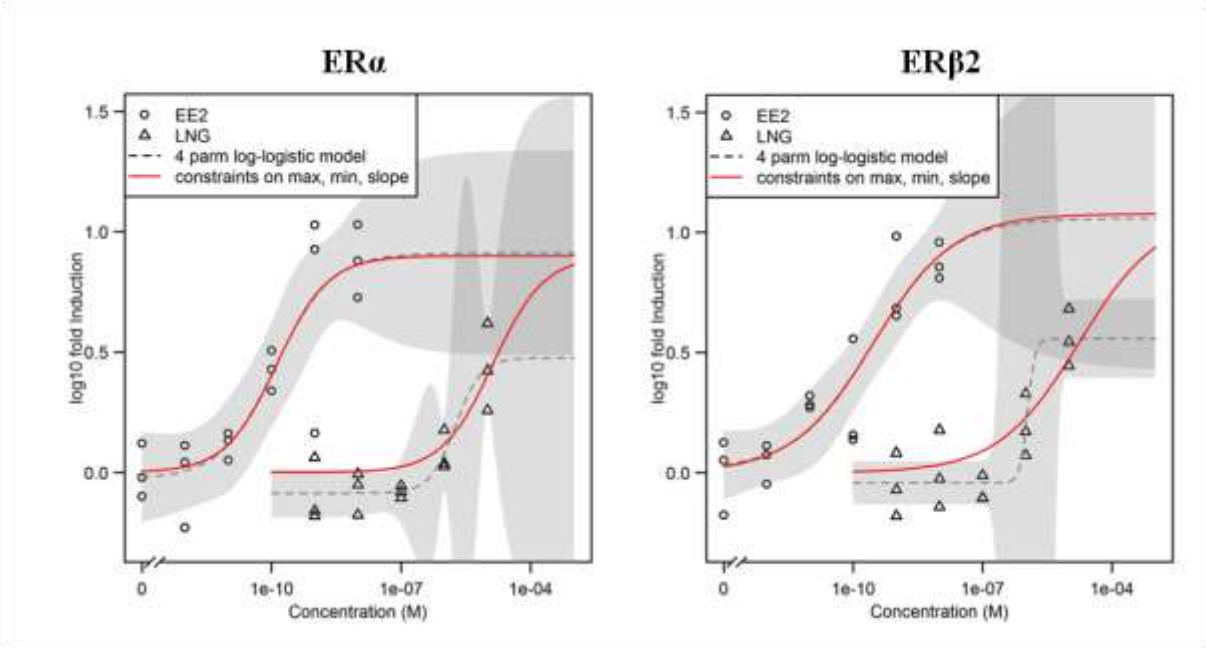


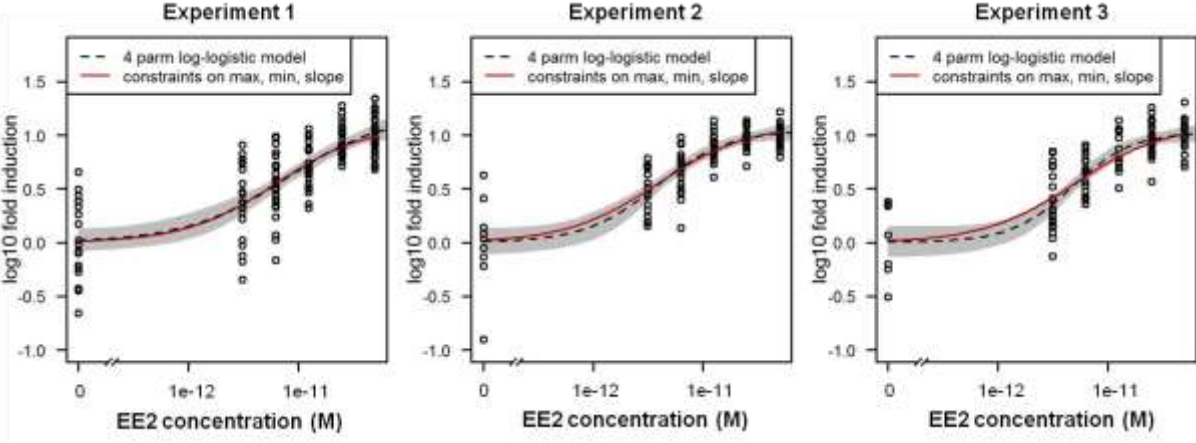
Figure 4



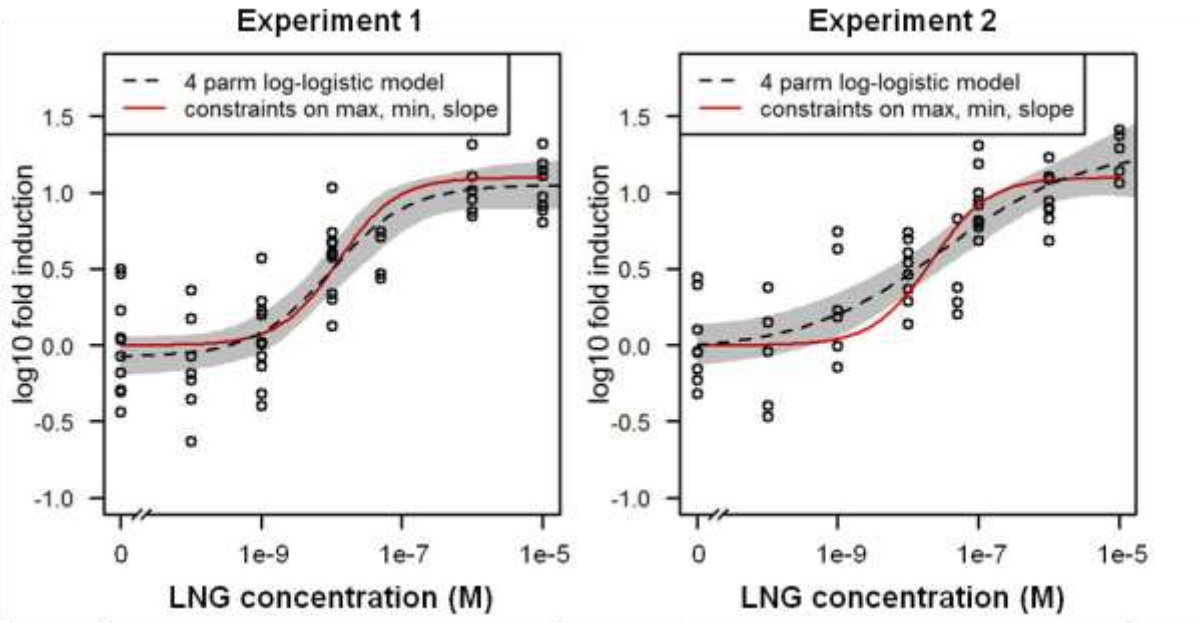
Supplemental figure 1



Supplemental figure 2



Supplemental figure 3



Supplemental Table 1: Experimental ray design for the assessment of the effects of ethinylestradiol (EE2) and levonorgestrel (LNG) alone and in mixtures on the expression of GFP in the brain of *cyp19a1b*-GFP transgenic zebrafish line.

Condition	[EE2] (μM)	[LNG] (μM)	Ray
1	0	0	/
2	1.00E-04	0	1:0
3	2.50E-05	0	1:0
4	6.25E-06	0	1:0
5	1.56E-06	0	1:0
6	3.91E-07	0	1:0
7	7.50E-05	0.075	3:1
8	1.88E-05	0.0188	3:1
9	4.69E-06	0.00469	3:1
10	1.17E-06	0.00117	3:1
11	2.93E-07	0.000293	3:1
12	5.00E-05	0.15	1:1
13	1.25E-05	0.0375	1:1
14	3.13E-06	0.00938	1:1
15	7.81E-07	0.00234	1:1
16	1.95E-07	0.000586	1:1
17	2.50E-05	0.225	1:3
18	6.25E-06	0.0563	1:3
19	1.56E-06	0.0141	1:3
20	3.91E-07	0.00352	1:3
21	9.77E-08	0.000879	1:3
22	0	0.3	0:1
23	0	0.075	0:1
24	0	0.0188	0:1
25	0	0.00469	0:1
26	0	0.00117	0:1