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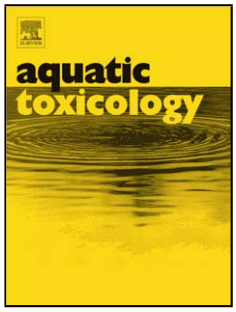

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Detection of the anti-androgenic effect of endocrine disrupting environmental contaminants using *in vivo* and *in vitro* assays in the three-spined stickleback.

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Abstract

We have previously developed a novel *in vitro* assay that utilises cultures of primed female stickleback kidney cells for the screening of potential androgenic and anti-androgenic environmental contaminants. Stickleback kidney cells are natural targets for steroid hormones and are able to produce a protein, spiggin, in response to androgenic stimulation. We undertook a combined *in vivo/in vitro* study where we used the magnitude of spiggin production as an endpoint to test the anti-androgenic properties of the pharmaceutical androgen antagonist flutamide and three environmental contaminants: the organophosphate insecticide fenitrothion, the urea-based herbicide linuron and the fungicide vinclozolin. *In vitro*, kidney cells were exposed to a range of concentrations [from 10^{-14}M (2.5pg/L) up to 10^{-6}M (280µg/L)] of the test compounds alone for determining agonist activities, or together with 10^{-8}M (3µg/L) dihydrotestosterone (DHT) for determining antagonist activities. An *in vivo* flow-trough aquarium-based study was carried out in parallel. Female sticklebacks were exposed to a range of concentrations of the same chemicals alone or in combination with DHT (5µg/L) for 21 days. All of the compounds significantly inhibited DHT-induced spiggin production in a concentration-dependent manner in both the *in vitro* (FN≥FL≥LN>VZ) and *in vivo* (FN>FL≥VZ>LN) assays. Fenitrothion and flutamide inhibited spiggin production *in vitro* at a concentration as low as 10^{-12}M ($P<0.05$), while linuron and vinclozolin inhibited DHT-induced spiggin production at concentrations of 10^{-10}M ($P<0.05$) and 10^{-6}M ($P<0.001$) respectively. Similarly, fenitrothion and flutamide were the most potent chemicals *in vivo* and significantly reduced DHT-induced spiggin production at a concentration of 10µg/L and 25µg/L respectively ($P<0.01$). Both linuron and vinclozolin induced a significant decrease in DHT-induced spiggin production at a concentration of 100µg/L when tested *in vivo*. In addition, kidney cell primary culture was used to test the (anti-)androgenic effects of the major environmental contaminants: oestradiol (E2), nonylphenol (NP) and bisphenol A (BPA) for the first time in teleosts. We observed that these compounds were able to significantly inhibit spiggin production at high doses (E2: 270µg/L; NP: 2.2µg/L; BPA: 2.3µg/L). When tested in the absence of DHT, none of the compounds showed a significant agonistic activity in either *in vivo* or *in vitro* assays. Overall, our data further demonstrate that kidney cell primary culture is a reliable and a sensitive screening tool for the detection of (anti-)androgenic compounds. In addition, our study represents the first attempt to develop a combined *in vivo/in vitro* screening strategy for assessing the effects of (anti-)androgenic endocrine disrupters.

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Key words: Stickleback, Anti-androgen, Spiggin, cell culture, *In vitro*, *In vivo*.

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Introduction

During the past decade an increasing incidence of reproductive disorders has been reported in several animal taxa including humans. An increasing number of field and laboratory studies have investigated the putative links between these disorders and the presence in the environment of anthropogenic chemicals that are able to interfere with the endocrine functions in both humans (Toppari *et al.*, 1996; Sharpe and Skakkebaek, 2003) and wildlife (Tyler *et al*., 1998; Vos *et al*., 2000).

Of particular concern are the chemicals that are able to mimic or block the responses typically induced by male sexual hormones, the androgens. Indeed, early studies reported that a number of environmental contaminants, including chemicals previously described as oestrogenic can also act as anti-androgens (Eil and Nisula, 1990; Hose an Guillette, 1995; Kelce *et al.,* 1995; Kelce and Wilson 1997; Sohoni and Sumpter, 1998). In particular, there are data available suggesting that several classes of pesticides are able to interfere with androgen functions and can cause severe impairment in male sexual development and reproduction (Kelce *et al*., 1995; Kelce *et al*., 1997; Makynen *et al*., 2000; Sohoni *et al*., 2001). Humans are potentially exposed to pesticides either directly, as workers in green-houses and in agriculture, or indirectly, via food consumption. In addition, it is likely that a significant amount of these pesticides and their metabolites reach rivers and estuaries via run-off from farmland thus creating localised "hot-spots" of chemicals discharges that are potentially toxic to wildlife. Despite these observations, most studies on aquatic species have focussed on the effects of oestrogenic environmental contaminants (Jobling *et al*., 1995; Toppari et al., 1996; Sumpter et al., 1996), while the potential effects of (anti-)androgenic chemicals on sexual development and maturation in both human and wildlife have been somewhat overlooked. In teleosts as in mammals, androgens are essential as they control male sexual differentiation and maturation (Borg, 1994). They act by binding to specific intracellular androgen receptors (AR) to activate

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or repress the expression of specific genes, notably those involved in the development of male primary and secondary sexual characters (reviewed by Delvin and Nagahama, 2002). It has been shown that most of the (anti-)androgenic environmental contaminants are able to interfere with the androgen signalling pathways by binding directly to the AR and act as agonists or antagonists (Kelce *et al*., 1997; Wong *et al*., 1995).

The screening and classification of endocrine disrupting chemicals (EDCs) are prerequisites for identifying their potential to cause adverse effects on whole organisms. For this reason we established a sensitive *in vitro* assay for the rapid screening of androgenic and anti-androgenic chemicals (Jolly *et al*., 2006). Our method uses a three-spined stickleback (*Gasterosteus aculeatus*) kidney cell primary culture and detects of spiggin production, an androgen induced protein. The ability of stickleback kidney cells to produce spiggin in response to androgenic stimulation has been extensively studied and is well documented. Under natural conditions, this protein is only secreted during the breeding season by male sticklebacks that use it as glue to build a nest (De Ruiter and Mein, 1982; Borg *et al*., 1993; Jakobsson *et al*., 1999; Jones *et al*., 2001). However, Katsiadaki *et al*., (2002; 2006) showed that spiggin can be artificially induced in female sticklebacks exposed to the androgens 17α-Methyltestosterone (MT) or 5α-Dihydrotestosterone (DHT) and that this induction was inhibited by flutamide, the synthetic AR antagonist used in prostate cancer treatment. The expression of this male secondary sexual character in female has been used as a biomarker for *in vivo* screening of (anti-)androgenic environmental contaminants (Katsiadaki *et al*, 2006). In a previous study, we demonstrated that similar results can be obtained *in vitro*: both DHT and the teleost specific androgen 11-ketotestosterone (11KT) induced spiggin production in kidney cells primary culture, a stimulating effect that was significantly inhibited by co-treatment with flutamide (Jolly *et al*., 2006).

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In the present study we tested the ability of three selected pesticides, fenitrothion, linuron and vinclozolin, to inhibit DHT-induced spiggin production in stickleback kidney cell primary culture. Fenitrothion, an organophosphate insecticide that represents one of the most widely used classes of pesticides and linuron, a selective urea-based herbicide, are used for crop protection. Vinclozolin is a fungicide widely used to control a variety of pathogens on fruit and vegetables and for crop protection. The pharmacokinetics of endocrine disrupting chemicals *in vivo* and their ability to interfere with multiple endocrine functions in a whole organism emphasises the need to demonstrate that the proposed mechanisms of action identified *in vitro* are also operative *in vivo*. In order to assess the degree to which results obtained with kidney cell primary culture are predictive of the sticklebacks' *in vivo* response, we exposed female adult fish to DHT (5µg/L) alone or in combination with a range of concentrations of the same test compounds (fenitrothion, linuron, vinclozolin or flutamide). Kidney cell primary culture was then used to assess, for the first time in teleosts, the (anti-)androgenic properties of two xenoestrogenic chemicals: nonylphenol (NP) and bisphenol A (BPA). It was the first time this procedure has been undertaken in teleosts. NP is one of the main degradation product of alkylphenol ethoxylate a class of chemicals used in several industrial, agricultural and domestic applications including detergent and pesticides (Jobling *et al*., 1996). BPA is a major component of consumer products such as polycarbonate plastics, epoxy resins, and flame-retardants (Hansen *et al*., 1998; Smeets *et al*., 1999). Several studies have demonstrated the ability of NP and BPA to inhibit androgen-induced process in mammalian *in vitro* models (Sohoni and Sumpter, 1998; Lee *et al*., 2003; Xu *et al*., 2005). For comparison purposes, we also tested *in vitro* the anti-androgenic of the natural oestrogen oestradiol (E_2).

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2. Material and Methods

2.1 Fish

Adult sticklebacks were caught by sand-net either in Oslo fjord (Drobak Marine Biological Station) or were obtained from a supplier (Moore & Moore Carp, Reading, UK) in 2004 and 2005. Sticklebacks from Oslo Fjord were held in 500L tanks supplied with through-flowing seawater (34‰) at 10°C at the University of Bergen. Specimens from this population were used in both the *in vivo* and *in vitro* experiments. Sticklebacks from the UK supplier were used for fenitrothion *in vivo* experiments only, and were kept in 1000L tanks supplied with constant flow of Windermere lake (UK) water at ambient temperature (5-12°C) at the Centre for Ecology and Hydrology (CEH, Lancaster UK). All fish were kept under a short photoperiod (8L: 16D). Earlier studies have shown that stickleback kept under this photo-thermal regime remain reproductively quiescent. The fish were fed daily with frozen red mosquito larvae (Aleds Akvarium AB, Sweden; Tropical Marine Centre, Hertfordshire, UK). Only female sticklebacks weighing more than 1g and showing no external signs of parasitic infections were used.

2.2 Chemicals

Fenitrothion, vinclozolin, linuron, bisphenol A and nonylphenol were purchased from Qmx Laboratories (Ausburg, UK). DHT, flutamide, and oestradiol were purchased from Sigma-Aldrich (Poole, UK). All chemicals were of analytical grade (>99% purity) and chemicals with the same batch number were used in all *in vitro* and *in vivo* studies. The chemical structures of the tested compounds are shown in Figure 1.

2.3 *In Vitro* experiments

Priming of female stickleback

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During previous validation of the *in vitro* assay using a primary culture of the stickleback kidney cells, it was determined that female sticklebacks needed to be primed by DHT exposure prior to tissue dissection (Jolly *et al*., 2006). For this, batches of females sticklebacks were transferred to a 40L glass aquarium under flow-through water conditions and kept under conditions of LD 12:12 photoperiod and 16°C. The androgen DHT was applied to the aquarium by means of a peristaltic pump, giving a final aquarium concentration of 5µg/L. A 10-day exposure to this DHT concentration resulted in an intermediate stage of kidney hypertrophy in female fish, which is required for culture (Jolly *et al.,* 2006).

Cell Dispersion

The protocol for cell culture experiments was previously described in detail (Jolly *et al*., 2006). Briefly, DHT-primed female sticklebacks were sacrificed by destruction of the brain and the kidneys quickly dissected and placed in ice-cold culture medium (M199, Gibco, France). For cell dispersion, kidney tissue samples were incubated at 25°C for 15 min, in a sterile solution of 0.8mg/ml porcine type II trypsin (Sigma) (prepared in Dulbecco's saline phosphate buffer without Ca2+, and Mg2+, with 100U/ml penicillin, 100µg/ml streptomycin, and 250ng/ml fungizone) (Gibco). This trypsin solution was then replaced by a solution of trypsin inhibitor (Sigma) and 1µg/ml DNAse (Sigma) in Dulbecco's buffer (DB) for 10 min. Kidney cells were gently dispersed in DB by repeated passages through a plastic transfer pipette (Falcon). The cell suspension was filtered through nylon mesh (30 µm pore size), and harvested by centrifugation at 800 rpm/min. Cells were then re-suspended in culture medium (CM): medium 199 with Earle's salts, sodium bicarbonate, 100U/ml penicillin, 100µg/ml streptomycin, and 250ng/ml Fungizone (Gibco).

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Culture system and assay procedure

Cells were cultured in 96 well tissue culture plates (Costar) coated with Poly-L-Lysine (Sigma) at a density of 125.000 cells/well. Plates were incubated at 18°C under 3% CO_2 and saturated humidity. After 24h of culture the medium was changed and the treatments started.

To determine whether any of the test chemicals possessed anti-androgenic activities in the assay system, the androgen DHT was added to the culture medium at a concentration of 3µg/L. The ability of FL, LN, FN, VZ, E2, NP and BPA to inhibit androgen-induced spiggin expression was then determined by adding a range of concentrations of the test compounds [from 2.2pg/L (10^{-14}M) to 280µg/L (10^{-6}M)] to the DHT treated cells. The agonistic activity of FL, LN, FN, VZ, E2, NP and BPA was also assessed by adding a range of concentrations (2.2pg/L to 280µg/L) of the test compounds alone.

For each test compound, a stock solution of 10mM was prepared in extra pure 96% ethanol (EtOH) and stored at -20°C. For each independent assay, working solutions of the compounds were prepared in culture medium on the day of use. Ethanol itself at 0.1% was applied to control wells (negative control). The effect of 0.1% of ethanol was evaluated by comparison to non-treated cells (medium only) in each independent experiment.

Spiggin extraction and ELISA

After 48h of incubation, cultures were stopped, the culture medium was removed and the cells were washed twice with sterile PBS (Gibco). The spiggin content of the cells was extracted by adding 100 µL of a denaturing buffer (100mM Tris-HCl, 10mM EDTA, 8M urea, 2% SDS and 200mM β-mercaptoethanol, pH 8.5) to each well, and by sequential freezing and thawing. The digests were collected and stored at -20°C until analysis.

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2.4 *In vivo* experiments

In vivo experiments were performed at the University of Bergen (Norway). Adult female sticklebacks were randomly selected from holding tanks. Although we took great care in selecting only female fish, some misidentifications were present as it is difficult to assign sex macroscopically in sticklebacks that are outside their breeding season. For this reason a total of 20 fish were selected (n=20 fish/aquarium) to ensure a minimum of 15 female sticklebacks per treatment. The experimental fish were transferred to 40L glass aquaria supplied with a constant flow of seawater (34‰; Flow rate of 100ml/min; FL, VZ, LN) or freshwater (FN) and kept under constant conditions of LD 12:12 and 15 ± 1°C. The water in all aquaria was aerated constantly, and temperature, oxygen, pH and salinity monitored every two days. The fish were fed daily with frozen mosquito larvae, and any accumulated waste products were siphoned off every other day. The water in each individual aquarium was constantly renewed (flow-through system) and chemicals were supplied by means of a microperistaltic pump (Watson-Marlow, UK) at a flow rate of 100µl/min in order to keep them at a constant concentration throughout the exposure time.

Treatments

All chemicals were dissolved in methanol, although the concentration of methanol in the tanks never exceeded 0.01%. The effect of 0.01% of methanol itself was evaluated by including a solvent control group in each independent experiment. A range of six concentrations of the test compounds were co-administered with 5µg/L DHT (equivalent to $1.7.10^{-8}$M). The nominal concentrations chosen for FL and LN were 2, 10, 25, 75, 100, and 250µg/L; for FN 2, 5, 25, 60, 120, and 240µg/L; and for VZ 0.25, 2.5, 25, 100, 250, 500µg/L) determined from an initial range-finding trial. In addition, each exposure experiment included

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a control (water only), a positive control (5µg/L DHT only), and a test compound control (test compound at the highest concentration tested in the absence of DHT).

A 21-day exposure was used, consistent with the OECD recommendations in the fish screening assays for EDCs (OECD 2004).

Organ sampling

After 21 days, the fish from all individual aquaria were terminally anesthetized in MS-222 (100mg/L), and snap frozen in liquid nitrogen. After weighing (±0.1g), the kidney was dissected out, weighed (±0.1mg), and stored at -80°C for future spiggin analysis. The sex of each individual fish was verified macroscopically and only female fish were used in the analyses (n=15 to 20).

Spiggin extraction and ELISA

200µL of denaturing buffer (as described above) was added to each kidney sample followed by heating for 30min, at 70°C in order to achieve complete digestion of the tissue. The digests were stored at -20°C until spiggin analysis. Spiggin level measurements were performed by ELISA (Katsiadaki *et al*., 2002) at the Cefas Weymouth laboratory. Spiggin protein levels were expressed as units of spiggin per gram of body weight.

2.5 Analytical chemistry

Water Sampling

In order to determine the actual concentration of the test compounds, chemical analysis of the tank water was performed during *in vivo* experiments. 1L of Water was collected from each

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tank every week on days 0, 7, 14 and 21. A volume of methanol equivalent to 0.1% of the sample volume was added to each sample. The water samples were then filtered through 0,45µm sterile filters (Acorcap filter units, PN 4482, Pall life science) and SPE cartridges (WAT023635, Waters Corporation, US) at a flow rate of 15ml/min. Prior to filtration, the SPE cartridges were conditioned and equilibrated by gently pushing 5mL of methanol followed by 5mL of distilled water. After filtration, the cartridges and filters were washed with 5mL distilled water and immediately stored at -20°C until analysis.

Sample analysis

The cartridges were washed through with 5ml methanol in order to elute the compounds. DHT was measured by radio-immunoassay (RIA) at the Cefas Weymouth laboratory employing the same procedure as for other teleosts steroids (Scott *et al*., 1984). Briefly, the methanol extracts were dried down under a stream of nitrogen gas at 45°C, reconstituted in 1 ml RIA buffer and stored frozen until required for assay. The recovery rate of DHT from the water was tested in three separate experiments and found to lie between 60% and 65%. These experiments involved "spiking" tank water samples with known amounts of DHT. Thus all measured levels of DHT were multiplied by a factor of 1.6 to correct for losses that occurred during extraction. Radiolabelled DHT (5α-Dihydro[1,2,4,5,6,7-^{3}H]testosterone ; product no. TRK 443) was purchased from GE Healthcare, Amersham, (UK) and kept in ethanol at -20°C. The standard was stored in a glass container at 4°C at a concentration of 0.5mg/ml in ethanol. The DHT antiserum was purchased from Biogenesis (Poole, UK, product no. 32500106).

Analytical chemistry was performed at the Cefas Weymouth laboratory using liquid chromatography-mass spectrometry (LC-MS) for flutamide and linuron, or gas chromatography-mass spectrometry (GC-MS) for vinclozolin and fenitrothion. TBBP-A

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(tetrabromobisphenol A) was used as an analytical internal standard for the LC-MS analysis of flutamide and linuron along the same lines as chlorobiphenyl (CB)#155 was deployed as an analytical internal standard for the GC-MS analysis of vinclozolin and fenitrothion. Data analysis, quantization and confirmation was achieved in Single Ion Monitoring (SIM) mode using ions with *m/z* of, 109 and 125 for fenitrothion, 105 and 159 for vinclozolin, and 360 and 362 for the internal standard CB#155, and 275.1±0.5, 247.4±1.0, and 543.1±1 for flutamide, linuron and TBBP-A respectively. SIM data were collected in a retention time scheduled event.

2.6 Statistical analysis

The effect of each compound was tested on two (*in vivo*) or three (*in vitro*) independent experiments (performed on different fish batches and cell preparations). Figures represent the results of one representative *in vivo* and *in vitro* experiment. Results are expressed as mean± SEM [spiggin units/body weight (n=15) *in vivo* or spiggin units/well (n=6) *in vitro*]. Data were tested for homogeneity of variance and normal distribution, and log-transformed when required. A one-way ANOVA was performed followed by pairwise comparisons between test and control group using a Tukey's test.

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3. Results

3.1 *In Vitro* experiments

3.1.1 Androgenic effects of dihydrotestosterone, flutamide, linuron, fenitrothion and vinclozolin on spiggin production.

Kidney cells treatment with a range of concentrations of DHT [10^{-14}M (3pg/L) to 10^{-6}M (300µg/L)] alone induced spiggin production in a concentration-dependent manner (figure 2). A concentration as low as 10^{-14}M (3pg/L), induced a significant increase in spiggin cell content (x1.4, $P<0.05$ as compared to control cell). The maximum effect was observed at the highest concentration tested; 10^{-6}M (300µg/L) DHT (x 1.7, $P< 0.001$ as compared to control cells).

The effects of FL, FN, LN or VZ on spiggin cell content *in vitro* are shown in figure 2. Applied alone to kidney cells, a range of concentrations of the test compounds [from 10^{-14}M (2.5pg/L) to 10^{-6}M (300µg/L)] had no significant effect on spiggin cell content.

In all exposures, the solvent alone had no effect on spiggin production as compared to non-treated cells (medium only) (data not shown).

3.1.2 Antiandrogenic effects of flutamide, fenitrothion, linuron and vinclozolin on DHT-induced spiggin production.

The reference agonist, DHT, was applied at a concentration of 10^{-8}M (3µg/L), and led to a significant increase in spiggin production in the positive control cells (from x1.6 to x1.7 according to the cells batches, $P<0.01$ as compared to control cells) (figure 3). Flutamide, Fenitrothion, Linuron and Vinclozolin, were able to significantly inhibit the stimulatory effect of DHT in a concentration-dependent manner as shown in figure 3 (A-D).

The inhibitory effect of flutamide is illustrated in figure 3A. Addition of 10^{-12}M (0.28ng/L) of flutamide reduced spiggin production in a significant manner (x0.82, $P<0.05$ as compared to

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positive control cells) resulting approximately in a half-maximal inhibition of the DHT stimulating effect. Spiggin production was further reduced at the higher tested concentrations [10^{-10}M (28ng/L), 10^{-8}M (2.8µg/L) and 10^{-6}M (280µg/L)] (x0.74, and x0.73, P<0.01).

Kidney cell treatment with 10^{-12}M (0.28ng/L) fenitrothion significantly inhibited DHT-induced spiggin production (x, 0.77, P<0.05) and led to a half-maximal inhibition of the DHT stimulating effect (figure 3B). The highest concentration tested [10^{-6}M L (280µg/)] induced a maximal inhibition of spiggin production (x0.65, P<0.01 as compared to positive control cells) resulting in a spiggin cell content similar to that of the negative control cells (x 1.06, NS).

Linuron induced a significant inhibition of spiggin production at a concentration of 10^{-10}M (25ng/L) (x 0.79, P<0.05 as compared to positive control cells) resulting in approximately half-maximal reduction of the DHT-induced spiggin production (figure 3C). Furthermore, this chemical caused a complete inhibition of DHT-induced spiggin production at a concentration of 10^{-6}M (250 µg/L) (x0.62, P<0.001) leading to a spiggin cell content identical to that of negative control cells (x 1, NS).

Vinclozolin induced a decrease in spiggin cell content only at the two highest concentrations tested, 10^{-8}M (2.8µg/L) and 10^{-6}M (280µg/L) (figure 3D). However only the highest concentration [10^{-6}M (280µg/L)] induced a spiggin cell content significantly different from that of positive control cells [10^{-8}M: x 0.89, NS; 10^{-6}M: x 0.68, P<0.001] and resulted in a spiggin cell content not significantly different from that of negative control cells (x 1.17, NS).

Results from the *in vitro* assays indicated that the four test compounds had the following order of anti-androgenic potency: fenitrothion ≥ flutamide ≥ linuron > vinclozolin.

3.1.3 Androgenic effects of DHT, E_2, NP and BPA

A range of concentrations of DHT [10^{-14}M (3pg/L) to 10^{-6}M (300µg/L)] were applied alone to cultured kidney cell (figure 4). DHT induced a significant concentration-dependent increase

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in spiggin production *in vitro* which was already observed at a concentration as low as 10^{-14}M (3pg/L) (x1.4, P<0.05 as compared to negative control cells). The maximal effect being observed at the highest concentration tested, 10^{-6}M (300µg/L) DHT (x 1.7, P< 0.001).

The agonist activity of E2 was tested by adding a range of concentrations of the hormone to kidney cells [from 10^{-14}M (2.7pg/L) to 10^{-6}M (270µg/L)] (figure 4). None of the concentrations tested were able to induce a statistically significant increase in spiggin cell content. Similarly, when tested alone, neither NP nor BPA were able to induce a significant increase in spiggin cell content at any of the concentrations tested [from 10^{-14}M (2.2pg/L and 2.3 pg/L respectively) to 10^{-6}M (228µg/L and 220µg/L respectively)].

3.1.4 Antiandrogenic effects of E_2, NP and BPA

In each individual experiment, kidney cells were treated with 10^{-8}M (3µg/L) DHT only (positive control) or with a combination of DHT (10^{-8}M) and a range of concentrations of the test compounds (figure 5). In all experiments, DHT treatment induced significant induction of spiggin production by kidney cells (from x1.5 to 1.6 according to the cells batches, P< 0.001 as compared to negative control cells).

E_2 induced a significant but incomplete inhibition of DHT-induced spiggin production only when applied at the highest concentration of 10^{-6}M (272µg/L) (x 0.79, P<0.01 as compared to positive control cells) (figure 5A). On the contrary, we observed that the two highest concentrations of NP were able to significantly inhibit DHT-induced spiggin production [10^{-8}M (2.2µg/L): x 0.82, P<0.01; 10^{-6}M (220µg/L): x0.66, P<0.01 as compared to positive control cells] (figure 5B). Notably, 10^{-6}M (220µg/L) of NP induced a complete inhibition of DHT-induced spiggin production resulting in spiggin cell content similar to that of the negative control cells (x 1.05, NS). BPA was able to induce a significant inhibition of DHT-

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induced spiggin production *in vitro* only at the highest dose tested, 10^{-6}M (228µg/L) (x 0.83, P<0.05, as compared to positive control cells) (figure 5C).

3.2 *In Vivo* experiments

3.2.2 Androgenic effects of dihydrotestosterone, flutamide, linuron, fenitrothion and vinclozolin on spiggin production

The results of *in vivo* assays for androgenic and anti-androgenic activity of flutamide, fenitrothion, linuron and vinclozolin are shown in figure 6 (A-D). In all exposures, the vehicle alone (solvent controls) had no effect on spiggin production, with kidney spiggin levels remaining at the basal level (10^1 to 10^2 spiggin units/g body weight, NS as compared to water control) (data not shown).

Exposure of female sticklebacks to 5µg/L DHT alone (positive control) for 21 days induced a significant increase in spiggin production leading to a spiggin kidney content of 10^4 to 10^5 units of spiggin/g of body weight according to the different experimental fish batches (from x100 to x1000 as compared to water control group; P<0.001) (figure 6 A-D).

Possible androgenic effects of FL, FN, LN and VZ, were assessed by applying the test compounds alone at the highest concentration tested. As shown in figure 6 (A-D), none of the test compounds induced spiggin production in female sticklebacks; kidney spiggin remaining at the basal level (10^1 to 10^2 spiggin units/g body weight): 250µg/L FL (x 0.8, NS, as compared to water control group); 250µg/L LN (x 1.2, NS); 240µg/L FN (x 0.44, NS); 500µg/L VZ (x 2, NS).

3.2.3 Antiandrogenic effects of flutamide, linuron, fenitrothion and vinclozolin on DHT-induced spiggin production

To differing degrees, FL, FN, LN, and VZ all inhibited DHT-induced spiggin production in females in a concentration-dependent manner (figure 6A-D).

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A 50% inhibition of spiggin production occurred approximately at 25µg/L FL (figure 6A). However, due to high inter-individual variability, this flutamide concentration led to a spiggin level in female stickleback not significantly different from that of the positive control group (x0.54, NS). Flutamide at 50µg/L induced a significant decrease in DHT-induced spiggin production (x0.16, $P<0.01$ as compared to the positive control group). The highest concentration of FL tested, 250µg/L, resulted in a complete inhibition of DHT stimulating effect and spiggin production in females (x0.002, $P<0.001$ as compared to the positive control group) leading to a spiggin kidney content not significantly different to that of the water control group (x 1.24, NS).

The effects of a range of concentrations of FN on spiggin production by DHT-stimulated female stickleback are shown in figure 6B. The lowest concentration tested (1.6µg/L) induced approximately 50% inhibition of DHT-induced spiggin production (x 0.46, NS as compared to positive control group). At that FN concentration some female fish were highly responsive while others produced spiggin. FN at 10µg/L induced a significant decrease in spiggin kidney content (x0.29, $P<0.01$, as compared to positive control group). We observed a complete inhibition of spiggin production at the highest concentration tested (240µg/L) (x 0.002, $P<0.001$ as compared to positive control group; x 1.11, NS as compared to the water control group). LN was able, significantly inhibit spiggin production content in fish exposed to DHT only at the two highest concentrations tested, 100µg/L and 250µg/L (x 0.4 and x 0.08, respectively, $P<0.05$; as compared to the positive control group) (figure 6C). None of the LN concentrations tested induced complete inhibition of androgen-induced spiggin production *in vivo*. Exposure of the fish to 100µg/L of VZ induced a significant inhibition of DHT-induced spiggin production in females (x 0.016, $P<0.001$ as compared to the positive control group). Inhibition of spiggin production was complete at the two highest VZ concentrations tested, namely 250 and 500µg/L (x0.0025 and x 0.0016 respectively, $P<0.001$ as compared to

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positive control group) leading to spiggin kidney content similar to that of the water control group (x 1.4, NS).

Results from the *in vivo* assays indicated that the four test compounds had the following order of anti-androgenic potency: fenitrothion > flutamide ≥ vinclozolin > linuron.

3.2.4 Water Chemistry

Mean measured concentrations for chemicals (DHT, flutamide, fenitrothion, linuron and vinclozolin) are presented in Table 1 (A-D). Measured concentrations of DHT as a proportion of nominal concentrations (percentage of recovery) ranged from 59.5% to 100%.

Mean measured levels of test compounds were below the nominal values: fenitrothion (from 64.7% to 74%), linuron (from 30.3% to 77%), flutamide (from 27.5% to 44.7%) and lower recovery rates for vinclozolin (from 6% to 11.4%).

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Discussion

Comparison *in vivo* / *in vitro* data

The aim of this work was, first, to assess whether kidney cell primary culture test is an accurate predictor of *in vivo* effects of EDCs. We observed that all the compounds tested were able to inhibit DHT-induced spiggin production in a concentration-dependent manner in both *in vivo* (FN>FL≥VZ>LN) and *in vitro* (FN≥FL≥LN>VZ) assays. This observation demonstrates that the mechanisms leading to spiggin production by androgens and inhibition by anti-androgens *in vivo* are conserved *in vitro*. Previous studies demonstrated that both kidney hypertrophy and spiggin production (Borg *et al*, 1993; Jakobsson *et al*., 1999) and spiggin mRNA expression (Olsson *et al*., 2005) *in vivo* are androgen dependent processes. In addition, we previously reported that spiggin induction by the teleost specific androgen, 11-ketotestosterone *in vitro* can be inhibited by the specific androgen receptor antagonist flutamide (Jolly *et al*., 2006). Taken together, these data strongly suggest that spiggin production by kidney cells is induced via a traditional AR-mediated pathway. Kidney cell primary cultures therefore appear to be a reliable and biologically relevant screening tool to predict the ability of environmental contaminants to interfere with androgen-induced signalling pathways. Our present study confirms that FL as well as FN, VZ, LN, BPA and NP are able to exert their anti-androgenic activity and block spiggin production via direct interaction with kidney cell physiological pathways.

Several rapid and cost effective *in vitro* bioassays have been developed for the screening of environmental (anti-)androgens. These cellular models are notably based either on mammalian cell lines (Paris *et al*., 2002; Térouanne *et al*., 2000) or yeast strain (Sohoni and Sumpter, 1998; Sanseverino *et al*., 2009) transfected with an androgen receptor. The overall sensitivity of these assays is very good and protocols used are relatively standardized and miniaturised. However cell transfection with receptor-reporter system may introduce

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variations that can lead to contradictory observations according to the bioassays selected. In addition, other parameters of the cellular environment, such as the presence of co-activators or co-repressors of AR transactivation, can significantly influence the (anti-)androgenic potencies of both natural hormones and xenobiotics (Simon and Mueller, 2006). For these reasons extrapolation from data obtained in heterologous/artificial models to responses elicited *in vivo* appears difficult. As natural targets of steroid hormones, stickleback kidney cells constitute a particularly powerful model to further investigate the molecular and cellular mechanisms by which man-made environmental contaminants exert their (anti-)androgenic effects.

In general, rapid and cost-effective *in vitro* assays are particularly suitable for primary screening experiments. Indeed, as compared to *in vivo* studies, *in vitro* systems use fewer experimental fish and they permit a significant reduction in the amount of harmful chemicals needing to be used. For example in the present study approximately 600 fish were sacrificed to test the anti-androgenic properties of six chemicals *in vivo* (five concentrations), while 50 fish only were needed to screen the same number of compounds *in vitro* (fives concentrations). This is of paramount importance in view of the thousand of man-made chemicals that are produced and released in the environment every year. Of particular concern are those chemicals possessing structural similarities to pharmaceutical anti-androgen flutamide, i.e. a phenol-ring (see figure 1), which appears to be a common feature of the natural or man-made compounds that are able to bind steroid hormone receptors. Based on this observation one can predict that a significant number of other chemicals, such as the insecticide parathion (Sohoni *et al.,* 2001), or the herbicide diuron (Bauer *et al*., 1998) could potentially act as (anti-)androgens.

The complexity of endocrine function in animals makes it difficult to rely on *in vitro* data alone to predict the endocrine disrupting properties of man-made chemicals in general. The

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use of the stickleback as model organism allows the development of comparative *in vivo/in vitro* studies for the first time in teleosts. This is particularly important to further understand transmission of the adverse effects that occur at the molecular and cellular levels at the organism and population level.

Analytical chemistry data showed that only low percentages of the nominal concentrations of flutamide and vinclozolin could be measured in the water (Table 1A and 1D). The reasons for such low recovery rates have not been clarified yet. However, comparable results were reported by Allen and co-workers (2008) in a stickleback intercalibrating *in vivo* study. Taken together these observations suggest that the true lowest observed effects values in the stickleback are potentially lower than those reported so far.

Comparison of stickleback versus mammalian models

Several studies reported that teleosts unlike mammals possess two AR (alpha and beta) subtypes that differ in their binding affinities for endogenous hormones and tissue distribution (Takeo and Yamashita, 1999; Todo *et al*, 1999; Ikeuchi *et al*, 1999). In the stickleback, an androgen receptor (ARβ) has been cloned from kidney mRNA and the molecular structure of the two splicing variants has been characterized (termed ARβ1 and ARβ2) (Olsson *et al*, 2005). These two variants show high similarities with the mammalian androgen receptor. Consistent with this finding we report a clear correlation between mammalian and stickleback *in vivo* and *in vitro* response to flutamide, fenitrothion, linuron and vinclozolin (summarized in Table 2A and 2B).

For example, Sohoni *et al*. (2001) showed that fenitrothion is able to antagonize the androgenic effect of DHT in recombinant yeast expressing the human receptor. Similarly, Tamura and co-workers (2001) reported that the potency of fenitrothion as a competitive AR antagonist was comparable to that of flutamide in human cell line transfected with the human

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AR as well as in the Hershberger assay. In the present study, fenitrothion was the most efficient chemical at inhibiting DHT-induced spiggin synthesis *in vivo* and appeared to possess a potency comparable to that of flutamide *in vitro*. Indeed, both chemicals induced a significant decrease in spiggin cell content at a concentration as low as 10^{-12}M (2.8ng/L). We observed that i*n vivo* fenitrothion significantly inhibited spiggin production at a nominal concentration as low as 10µg/L while flutamide and linuron were effective at 25µg/L and 100µg/L respectively. The ability of linuron to inhibit the binding of androgens to rat prostatic AR was reported in early competitive receptor binding studies (Cook *et al*., 1993; Bauer *et al*., 1998). Later Lambright *et al*. (2000) showed that linuron also has affinity for the human AR, using a transfected COS cell line assay, and that it induces a decrease in the weight of DHT-dependent tissues in the Hershberger assay. McIntyre *et al*, (2000) also observed that *in utero* exposure to linuron impairs androgen-mediated reproductive development in male rat. In the same study, the authors showed that in hepatoma cell line transfected with human AR linuron competitively antagonize AR DHT-induced transcriptional activity in a concentration-dependent manner. In the present study, linuron inhibited DHT-induced spiggin production *in vivo* and *in vitro* at a concentration of 100µg/L (nominal concentration) and 10^{-10}M (25ng/L), respectively. Linuron appeared to be the least potent of the chemicals tested *in vivo* while *in vitro* it appeared more potent than vinclozolin which induced a significant decrease in spiggin production only at the highest concentration tested (10^{-6}M or 280µg/L). *In vivo*, 100µg/L of vinclozolin (nominal concentration) proved to be sufficient to inhibit spiggin production in DHT-treated female sticklebacks. Gray and co-workers, (1994) reported for the first time the ability of this pesticide to inhibit sexual differentiation in an anti-androgenic manner in male rats.

It has been demonstrated that vinclozolin itself is a weak AR antagonist and that its anti-androgenic effects is mediated mainly via its two main degradation products, M1 and M2

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(Kelce *et al*., 1994). Wong *et al*., (1995) demonstrated that M1 and M2, are potent AR antagonists that are able to inhibit AR binding to androgen response element (ARE) sequence, thus inhibiting the transcription of AR-dependent genes in human cell line transfected with a recombinant human AR. In the present study, vinclozolin appeared as a weak anti-androgen *in vitro*.

Comparison of stickleback versus other teleost models

In addition to the stickleback, a number of teleosts are now promoted as model species in laboratory experiments to assess the adverse effect of man-made EDCs on aquatic wildlife. We observed a good correlation between our data and some of the results reported in the literature. For example exposure of juvenile guppies to vinclozolin or flutamide resulted in a reduction in the expression of male secondary sex characters, a reduced sperm count and finally skewed sex ratio towards female (Bayley *et al.,* 2002) and high vinclozolin concentration (2500µg/L) affected spermatogenesis in male Japanese medaka (*Oryzias latipes*) (Kiparissis *et al*., 2003). Panter and co-workers (2003 and 2004) reported that both flutamide and vinclozolin are able to block androgen induced processes in adult fish using a fathead minnow (*Pimephales promelas*) non-spawning assay. On the contrary, Makynen and colleagues (2000) reported that fathead minnow exposed to high concentrations of vinclozolin (90-1200µg/L) for 34 days through early embryonic and larval life stages developed normally in terms of growth and sexual differentiation. The authors also investigated through competitive radioligand binding experiments the affinity of vinclozolin and its metabolites for the fathead minnow androgen receptor and observed that neither vinclozolin nor M1 or M2 could compete for testosterone binding sites. Similarly, Wells and Van der Kraak (2000) reported that neither flutamide nor vinclozolin and its metabolistes possess affinity to ARs from the goldfish (*Crassius auratus*) or the rainbow trout (*Oncorhyncus mykiss*).

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It is likely that some of the discrepancies we report above and which are summarized in Table 2A and 2B, result from differences in the methodologies used to assess the endocrine activity of the test chemicals. However, the existence of significant differences in the regulation of the endocrine function among teleosts such as the occurrence of different AR subtypes with different affinities for both endogenous hormones and endocrine disrupting compounds cannot be ruled out. These differences should be accounted for especially when promoting new bio-indicator species. Our results suggest that the stickleback is potentially a suitable model to study the impact of EDCs on androgen mediated pathways in humans.

Oestrogens act as anti-androgens in stickleback kidney cell primary culture

After validation of our *in vitro* model with known anti-androgens we applied our assay to the screening of the natural steroid, E_2 and two estrogenic environmental chemicals (NP and BPA) that are able to interfere with the functioning of AR in mammals (Sohoni and Sumpter, 1998; Lee *et al*., 2003; Xu *et al*., 2005). Inhibition of DHT-induced spiggin production *in vitro* was observed at the highest concentrations tested only for all the three chemicals [i.e. E_2: 10^{-6}M (270µg/L); NP: 10^{-8} and 10^{-6}M (2.2 and 220µg/L); BPA: 10^{-8} and 10^{-6}M (2.3 and 230µg/L)]. Oguro (1957) reported for the first time the ability of E_2 to induce a regression of kidney hypertrophy in the stickleback *in vivo*. In the present study, we demonstrate the ability of E_2 to exert its anti-androgenic effect via direct interaction with kidney cells. The molecular mechanisms by which E_2 is able to interfere with spiggin production *in vitro* remain to be identified. Gaido *et al*., (1997) showed E_2 to be about $1/30^{th}$ the potency of DHT to bind the human AR, while Olsson and co-workers (2005) reported that E_2 is able to down regulate the expression of AR mRNA in the stickleback *in vivo*. These data suggest that E_2 is able to exert its anti-androgenic effect by interfering directly with AR expression and/or activity. However the steroid response in animals is highly complex and can include cross talk among steroids

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and steroids receptors. Therefore we cannot exclude that E_2 anti-androgenic effects involve receptors other than the AR.

Similarly, for the first time in teleosts, we report the anti-androgenic properties of two oestrogenic endocrine-disrupting compounds: BPA and NP. To our knowledge, the anti-androgenic properties of NP and BPA have been reported only in a recombinant *in vitro* system, using assays based on mammalian cell lines or yeast strain transfected with mammalian AR (Sohoni and Sumpter, 1998; Lee *et al*., 2003; Xu *et al*., 2005). Notably, Sohoni and Sumpter (1998) reported that BPA is able to antagonise androgens at concentrations above 10^{-6}M (228µg/L) in a yeast-based assay transfected with the human AR. Later, Lee *et al*., (2003) showed that BPA and NP have anti-androgenic activity at multiple steps of mouse AR activation and function *in vitro*. Both compounds were able to inhibit AR androgen binding, AR nuclear import and its subsequent trans-activation in a concentration-dependent manner. As for E_2, the molecular mechanisms involved in inhibition of androgen-induced spiggin production by BPA and NP *in vitro* remain to be clarified.

We have previously reported the anti-androgenic effect of ethinyl-oestradiol in an identical in vivo system (Katsiadaki et al., 2006) and we are currently analysing the data from in vivo exposures employing BPA, NP and E_2. Preliminary data suggest that the anti-androgenic effect of E_2 is apparent at lower concentrations than those detected by our *in vitro* system. Therefore our future *in vivo/in vitro* studies will aim at further deciphering the anti-androgenic properties and the mechanism of action of xenoestrogens in the stickleback.

Conclusion

The present study offers further validation of the *in vitro* kidney cell assay we have developed. Most importantly, we present here the first attempt to develop an integrated *in vivo/in vitro* screening strategy for assessing the effects of (anti-)androgenic environmental

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contaminants. These combined *in vivo/in vitro* assays are particularly needed to further understand the transmission at the organism and population level of adverse effects that occur at the molecular and cellular levels. In particular future studies will investigate the molecular mechanisms involved in inhibition of spiggin production by vinclozolin, linuron, fenitrothion, bisphenol A, nonylphenol and oestradiol in the three-spined stickleback.

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Figure legend

Figure 1.

Structural formulae of flutamide, fenitrothion, linuron, vinclozolin, nonylphenol and bisphenol A.

Figure 2.

Concentration-response curves of spiggin induction obtained with androgen-primed females kidney cells treated for 48h with solvent only (control) or a range of DHT concentrations (-○-) [from 10^{-14}M (3pg/L) to 10^{-6}M (300µg/L)], or Fenitrothion (-♦-), Flutamide (-⁎-), Vinclozolin (-■-) [from 10^{-14}M (2.8 pg/L) to 10^{-6}M (280µg/L)] and Linuron (-Δ-) [from 10^{-14}M (2.5pg/L) to 10^{-6}M (250µg/L)]. Means ± SEM (n=6) are given. Figure displays the results of one representative experiment from three independent experiments.

Figure 3.

Effect of flutamide (**A**), fenitrothion (**B**), linuron (**C**) and vinclozolin (**D**) on DHT-induced spiggin production by female kidney cells treated for 48h.

Diagonally hatched and grey columns show cells treated with DHT [10^{-8}M (3µg/L)] together with a range of concentrations of flutamide, fenitrothion and vinclozolin [from 10^{-14}M (2.8pg/L) to 10^{-6}M (280µg/L)] or linuron [from 10^{-14}M (2.5pg/L) to 10^{-6}M (250µg/L)]. Solvent control (open column): cells exposed to solvent only; Positive control (black column): cells treated with 10^{-8}M DHT (3µg/L) only. Mean values are given ± SEM shown. (n=6). Asterisks indicate groups that were significantly different from the positive control. Figure displays one representative experiment from three independent experiments.

Figure 4.

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Concentration-response curves of spiggin induction obtained with androgen-primed kidney cells of females treated for 48h with solvent only (control) or a range of DHT concentrations (-▲-) [from 10^{-14}M (3pg/L) to 10^{-6}M (300µg/L)], or oestradiol (-∗-) [from 10^{-14}M (2.7pg/L) to 10^{-6}M (270µg/L)], nonylphenol (-∘-) [from 10^{-14}M (2.2pg/L) to 10^{-6}M (200µg/L)], and bisphenol A (-♦-) [from 10^{-14}M (2.3pg/L) to 10^{-6}M (230µg/L)]. Means ± SEM (n=6) are given. Figure displays the results of one representative experiment from three independent experiments.

Figure 5.

Effects of oestradiol (**A**), nonylphenol (**B**), and bisphenol A (**C**) on DHT-induced spiggin production by female kidney cells treated for 48h.

Diagonally hatched and grey columns show cells treated with 10^{-8}M DHT (3µg/L) together with a range of oestradiol concentrations [from 10^{-14}M (2.7pg/L) to 10^{-6}M (270µg/L)], nonylphenol [from 10^{-14}M (2.2pg/L) to 10^{-6}M (220µg/L)] or bisphenol A [from 10^{-14}M (2.3pg/L) to 10^{-6}M (230µg/L)]. Solvent control (open column): cells exposed to solvent only; Positive control (black column): cells treated with 10^{-8}M DHT (3µg/L) only. Mean values are given ± SEM shown. (n=6). Asterisks indicate groups that were significantly different from the positive control. Figure displays one representative experiment from three independent experiments.

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Figure 6.

Effect of flutamide (**A**), linuron (**B**), fenitrothion (**C**) and vinclozolin (**D**), on spiggin production by DHT-stimulated females, during 21 days *in vivo* exposure. Diagonally hatched columns show a range of concentrations of the respective test compounds administered together with DHT (5 µg/L). Solvent control (open column): fish exposed to solvent only; Positive control (black column): treated with 5µg/L DHT only. Negative control (grey column): addition of the test compound alone, at the highest concentration used. Asterisks indicate groups that were significantly different from the positive control. Mean values are given ± SEM. (n=15 fish/tank).

Table 1 (A-D)

Measured and nominal concentrations of DHT and FL (A), FN (B), LN (C), and VZ (D) from each treatment. Values are the mean of four sampling occasions (days 0, 7, 14 and 21) during the exposure period. Concentrations are expressed as µg/L. nd= non detectable.

Table 2 (A and B)

Summarized results of androgenic and anti-androgenic activities of different chemicals in vivo (A) and in vitro (B). ↗ = agonist activity; ↘=antagonist activity; (-) = no effect; nd =no data available.

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Table 1-A

Flutamide	Nominal	**0**	**250**	**0**	**5**	**25**	**50**	**75**	**100**	**250**
	Measured (mean)	n.d.	69,0	n.d.	2,1	10,2	22,3	32,3	41,3	96,3
	sem	-	18,0	-	0,2	0,4	2,6	3,5	3,3	5,4
	% recovery		27,6		42,7	40,8	44,7	43,1	41,3	38,5
DHT	Nominal	**0,0**	**0,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**
	Measured (mean)	n.d.	n.d.	4,2	3,8	3,2	3,6	3,5	3,8	3,0
	sem	-	-	0,4	0,5	0,2	0,4	0,6	0,4	0,1
	% recovery			83,1	76,9	64,6	71,8	69,7	76,9	59,5

Table 1-B

Fenitrothion	Nominal	**0**	**240**	**0**	**2**	**10**	**25**	**60**	**120**	**240**
	Measured (mean)	n.d.	174,2	n.d.	1,1	6,5	16,8	41,2	88,0	177,6
	sem	-	9,0	-	0,0	0,3	0,6	1,4	2,5	7,2
	% recovery		72,6		70,6	64,7	67,1	68,7	73,3	74,0
DHT	Nominal	**0,0**	**0,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**
	Measured (mean)	n.d.	n.d.	4,9	4,9	5,1	4,3	5,1	4,5	3,9
	sem	-	-	0,3	0,3	0,2	0,2	0,3	0,4	0,3
	% recovery			97,2	98,5	102,2	86,8	102,2	89,8	78,8

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Table 1-C

Linuron	Nominal	**0**	**250**	**0**	**3**	**10**	**25**	**100**	**250**
	Measured (mean)	n.d.	121,5	n.d.	1,0	4,8	7,6	58,0	193,3
	sem	-	5,1	-	0,1	-	0,3	11,8	14,5
	% recovery		48,6		40,0	47,5	30,3	58,0	77,3
DHT	Nominal	**0,0**	**0,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**
	Measured (mean)	n.d.	n.d.	3,8	3,9	4,3	3,6	5,0	4,0
	sem	-	-	0,3	0,4	-	0,2	0,7	0,2
	% recovery			76,2	77,6	86,2	71,6	100,0	80,0

Table 1-D

Vinclozolin	Nominal	**0**	**500**	**0**	**0**	**3**	**25**	**100**	**250**	**500**
	Measured (mean)	n.d.	48,0	n.d.	n.d.	n.d.	2,0	11,4	15,1	41,5
	sem	-	6,2	-	-	-	0,5	5,1	3,1	5,8
	% recovery		9,6				7,9	11,4	6,0	8,3
DHT	Nominal	**0,0**	**0,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**	**5,0**
	Measured (mean)	n.d.	n.d.	4,3	4,9	4,5	4,4	4,1	4,9	4,5
	sem	-	-	0,2	0,4	0,2	0,5	0,7	0,6	0,7
	% recovery			86,4	97,0	90,0	87,6	82,0	98,4	89,2

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Table(s)

Table 2-A	**Stickleback** *(Gasterosteus aculeatus)*	**Medaka** *(Orysias latipes)*	**Guppy** *(Poecilia reticulata)*	**fathead minnow** *(Pimephales promelas)*	**rodent**	**human**
	(1)Oguro, 1957	*Kiparissis et al., 2003*	*Bayley et al., 2002*	*(1)Panter et al., 2004* *(2)Makynen et al., 2000*	*(1)Kang et al, 2004* *(2)Tamura et al., 2001* *(3)Kelce et al., 1994* *(4)Lambright et al., 2000* *(5)McIntyre et al., 2000* *(6)Sohoni et al., 2001* *(7) Sunami et al., 2000*	*McLeod et al., 1993* *McLeod, 1993*
Fenitrothion	↘↘↘↘	nd	nd	nd	↘↘↘(2)/-(6,7)	nd
Flutamide	↘↘↘	nd	↘	↘(1)	↘↘(1)	↘↘
Vinclozolin	↘↘	↘↘	↘	-/↘(2)	↘(1,3)	nd
Linuron	↘↘	nd	nd	nd	↘(1,4,5)	nd
Oestradiol	↘(1)	nd	nd	nd	nd	↘
Nonylphenol	nd	nd	nd	-	nd	nd
Bisphenol A	nd	nd	nd	nd	nd	nd

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Table(s)

Table 2-B	**Stickleback** *(Gasterosteus aculeatus)*	**Goldfish** *(Carassius auratus)*	**Rainbow trout** *(Oncorhynchus mykiss)*	**fathead minnow** *(Pimephales promelas)*	**rodent**	**human**
					(1)Monosson et al., 1999 *(2)Cook et al., 1993* *(3)Bauer et al., 1998* *(4)Lee et al., 2003*	*(1)Sohoni et al., 2001* *(2)Wong et al., 1995* *(3)Lambright et al., 2000* *(4)Sohoni & Sumpter, 1998* *(5) Paris et al., 2002* *(6)Sultan et al., 2001* *(7)Tamura et al., 2001* *(8) Gaido et al., 1997*
		Wells & Van der Kraak, 2000		*Makynen et al., 2000*		
Fenitrothion	↘↘↘	nd	nd	nd	nd	↘↘(1,7) / ↗(1)
Flutamide	↘↘	-	-	nd	nd	↘↘(2,4)
Vinclozolin	↘	-	-	-	↘(1)	↘(2,4) / ↗(4)
Linuron	↘↘	nd	nd	nd	↘(2,3)	↘(3)
Oestradiol	↘	nd	nd	nd	nd	↗↗(8,4)
Nonylphenol	↘	nd	nd	nd	↘(4)	↘(6) / ↗(4)
Bisphenol A	↘	nd	nd	nd	↘(4)	↘↘(4,5,6)

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Fig.1

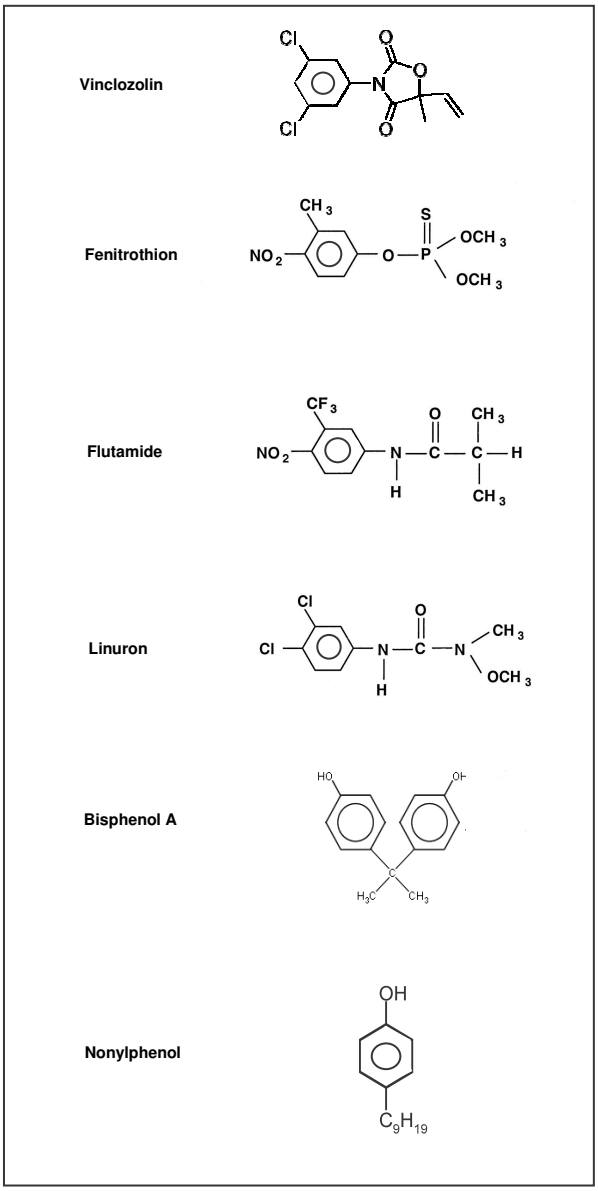

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Fig.2

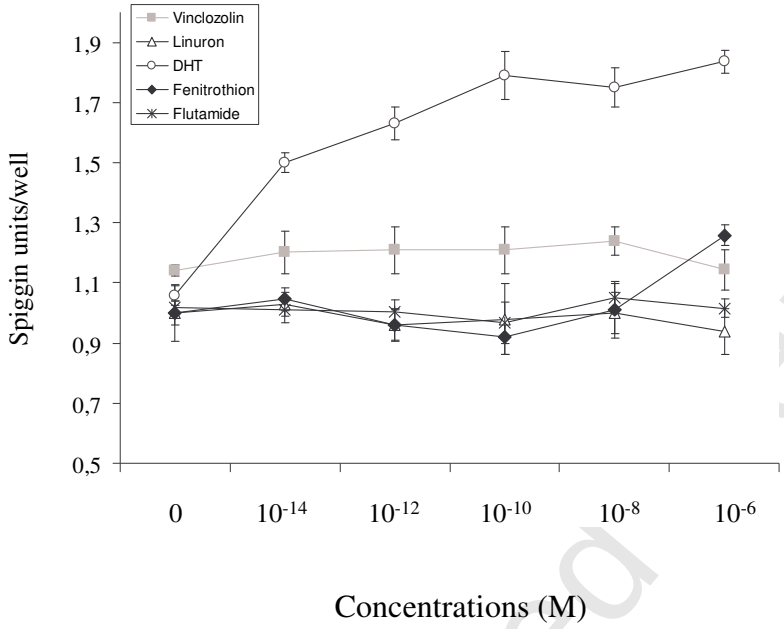

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Fig.3

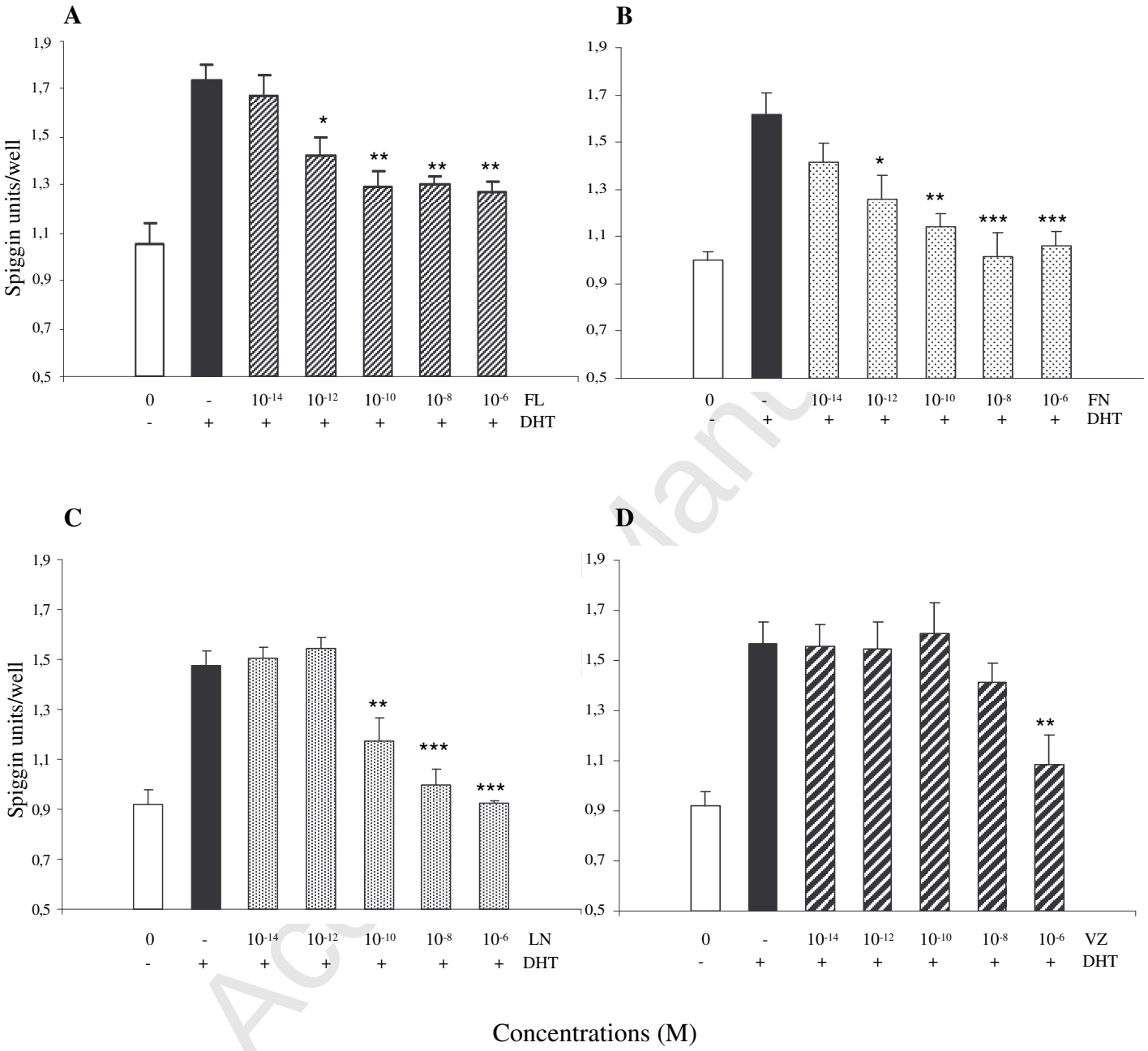

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Fig.4

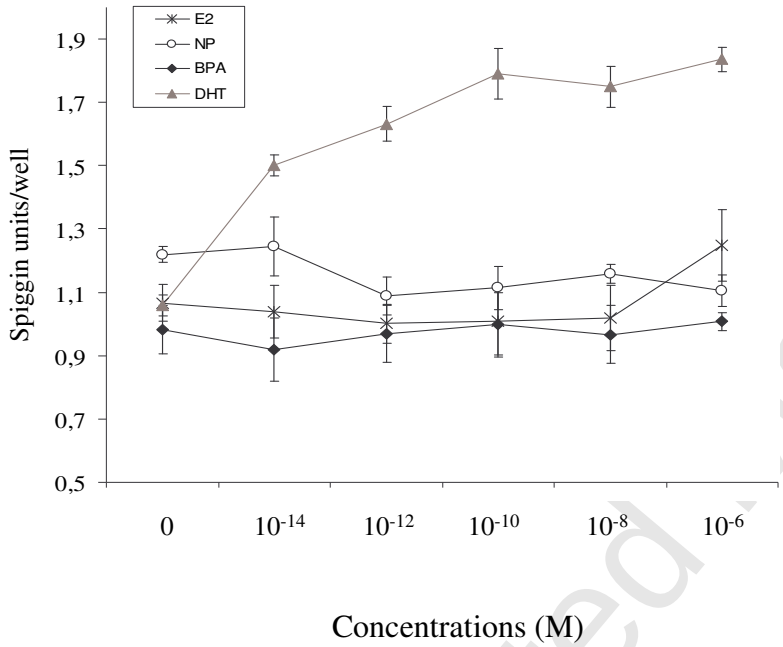

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Fig. 5

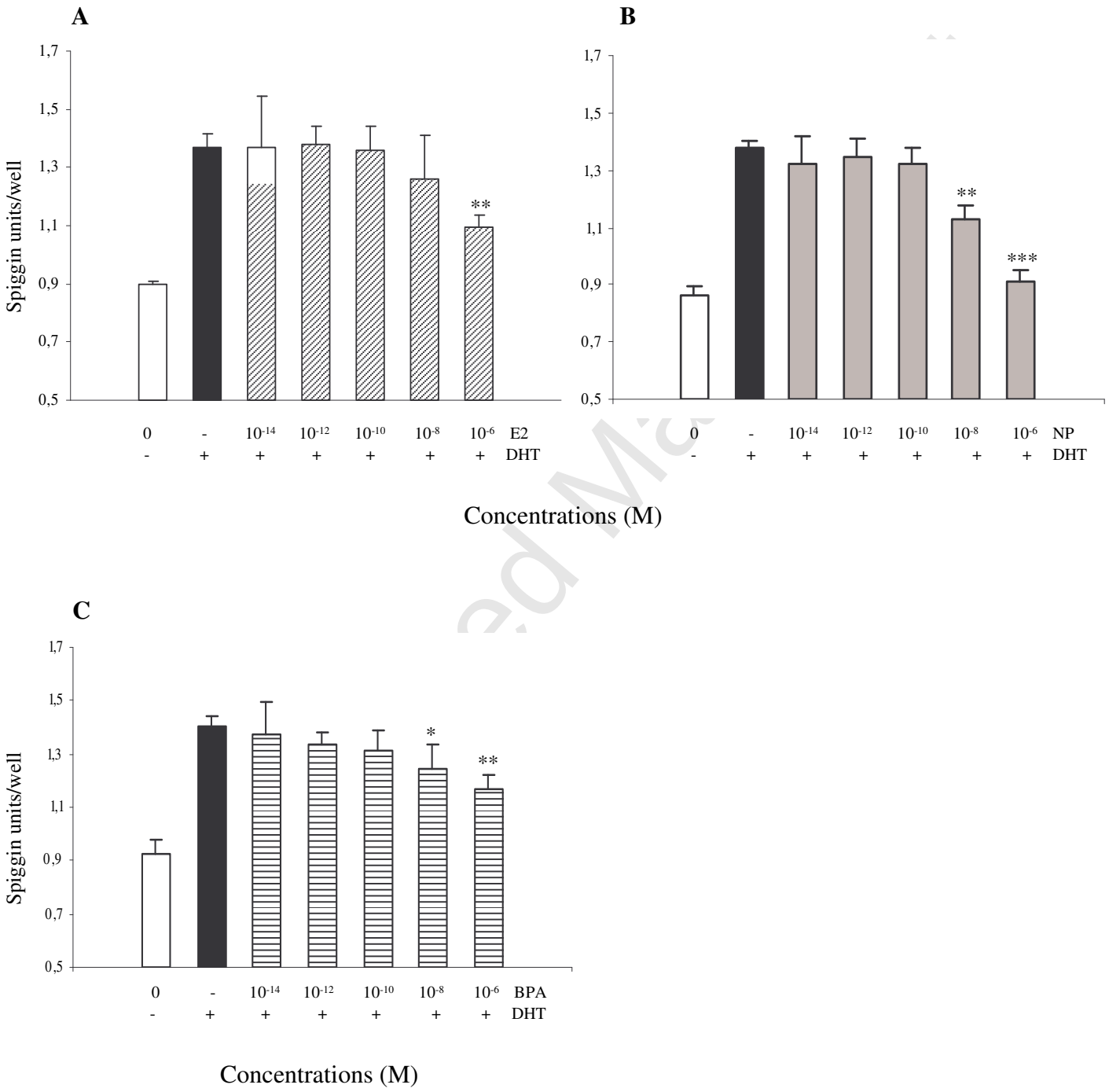

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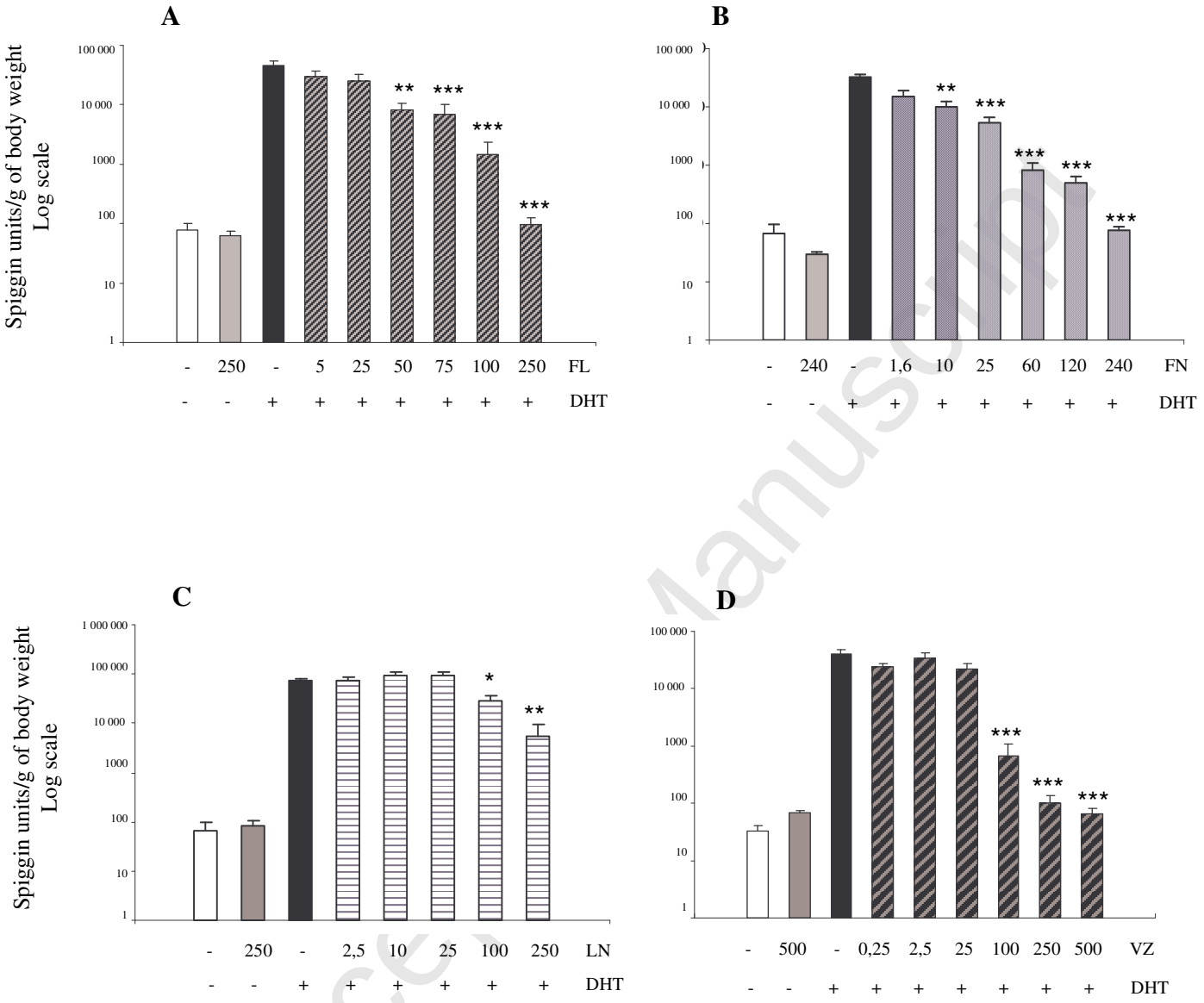
Fig. 6



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