Detection of immunotoxic effects of estrogenic and androgenic endocrine disrupting compounds using splenic immune cells of the female three-spined stickleback, Gasterosteus aculeatus (L.)

Anne Bado-Nilles, Romy Techer, Jean-Marc Porcher, Alain Geffard, Béatrice Gagnaire, Stéphane Betoulle, Wilfried Sanchez

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Authors

A. Bado-Nilles[ab 1], R. Techer[b], J.M. Porcher[b], A. Geffard[a], B. Gagnaire[c], S. Betoulle[a] & W. Sanchez[b]

[a]Université Reims Champagne-Ardenne, EA 4689 Unité Interactions Animal-Environnement, Moulin de la Housse, B.P. 1039, 51687 Reims, France, Stephane.Betoulle@univ-reims.fr, Alain.Geffard@univ-reims.fr.

[b]Institut National de l'Environnement Industriel et des Risques (INERIS), Unité d'écotoxicologie in vitro et in vivo, B.P. 2, 60550 Verneuil-en-Halatte, France, Anne.Bado-Nilles@ineris.fr, Wilfried.Sanchez@ineris.fr, Jean-Marc.Porcher@ineris.fr.

[c]Institut de Radioprotection et de Sureté Nucléaire (IRSN), Laboratoire d'Ecotoxicologie des Radionucléides, Centre de Cadarache, Bât 186, B.P. 3, 13115 Saint-Paul-lez-Durance, France, beatrice.gagnaire@irsn.fr.

[*]Corresponding author: *Anne.Bado-Nilles@ineris.fr,* phone: +33-344-618-121; fax: +33-344-556-767.

Abstract

Today, the list of endocrine disrupting compounds (EDCs) in freshwater and marine environments that mimic or block endogenous hormones is expanding at an alarming rate. As immune and reproductive systems may interact in a bidirectional way, some authors proposed the immune capacities as attractive markers to evaluate the hormonal potential of environmental samples. Thus, the present work proposed to gain more knowledge on direct biological effects of natural and EDCs on female fish splenic leucocyte non-specific immune activities by using *ex vivo* assays. After determining the optimal required conditions to analyze splenic immune responses, seven different EDCs were tested *ex vivo* at 0.01, 1 and 100 nM over 12 h on the leucocyte functions of female three-spined stickleback, *Gasterosteus aculeatus*. In summary, we found that natural hormones acted as immunostimulants, whilst EDCs were immunosuppressive.

Keywords: Endocrine disruptors; Sex-steroid hormone; Respiratory burst; Necrosis; Apoptosis; Phagocytosis activity.

1. Introduction

An endocrine disruptor compound ((EDC) is defined as an exogenous substance that alters the functioning of the endocrine system and consequently causes adverse health effects on an intact organism, or its progeny, or (sub-)populations (IPCS, 2002). Due to their potential hazard on aquatic wildlife (Canesi et al., 2007; Casanova-Nakayama et al., 2011) and their expanding detection in freshwater (Peck et al., 2004; Pal et al., 2010) and marine (Braga et al., 2005; Pinto et al., 2005) environments, research on the effects of EDCs in the past few decades has grown rapidly. In this context, fish are considered as good indicators to assess the toxicity of endocrine disrupting chemicals (Jin et al., 2010). Also, numerous studies address the impact of EDCs on certain parameters of the reproductive system of fish at the molecular (e.g. brain aromatase gene expression), biochemical (e.g. biotransformation enzymes, plasma vitellogenin) and histological (e.g. sexual differentiation, gonad maturation, fecundity, fertility, intersex) levels. (Hinfray et al., 2006; Kallivretaki et al., 2006; Cheshenko et al., 2008).

Despite of the fact that immune and reproductive systems may interact in a bidirectional way (Ansar Ahmed, 2000; Engelsma et al., 2002), endocrine disruption effects on the fish immune system have received limited attention and more concern has been made on estrogenic compounds (Jin et al., 2010; Casanova-Nakayama et al., 2011). Recently, some studies demonstrated that environmental estrogens may also affect the immune system of aquatic wildlife (Canesi et al., 2007; Casanova-Nakayama et al., 2011). For example,

Liney et al. (2006) found that estrogen-active effluents changed the structure and function of the reproductive system of the roach, *Rutilus rutilus*, at higher concentration than those who impaired the immune function.

In fish, phagocytosis, from chemotaxism to bacterial destruction, as well as leucocyte mortality seem to be the main immune functions which are subject to hormonal impact. For instance, Slater and Schreck (1997) observed *in vitro* immunosuppressive effects of testosterone due to salmonid leucocytes death after direct hormonal action on leucocyte androgen receptors. On the contrary, in gilthead seabream (*Sparus aurata* L.), *ex vivo* exposure of head-kidney leucocytes to testosterone and 11-ketotestosterone (11KT) do not have any impact on head kidney leucocyte viability (Águila et al., 2013). This discrepancy underlines the toxic effect of androgens at higher concentrations (Slater and Schreck, 1997) and the destabilization of the immune function at lower concentrations (Águila et al., 2013). In fact, modification by both androgens of the expression of toll-like receptors in acidophilic granulocytes and in macrophages might regulate the sensitivity of phagocytes to pathogens, without leading to effects on leucocyte phagocytic and respiratory capacities (Águila et al., 2013). Concerning estrogen compounds, *in vivo* treatment with 17β–estradiol (E2) (Shelley et al., 2013) and *in vivo* and *in vitro* exposures to 17α–ethinylestradiol (EE2) (Cabas et al., 2012) alter the fish immune gene expression of pro-inflammatory molecules without leading to impacts on phagocytic capacity and respiratory burst. Moreover, as observed below, EE2 and E2 was able to alter in a dose-dependent manner the immune system of gilthead seabream (*Sparus aurata* L.) (Cabas et al., 2012) and goldfish (*Carassius auratus*) (Yin et al., 2007), respectively.

The aim of the present study was to evaluate the impacts of some estrogen and androgen on the immune function of female three-spined stickleback (*Gasterosteus aculeatus* L.). The stickleback is a well described model fish species used to assess the endocrine disruptor potential of EDCs due to the presence of both estrogenic (vitellogenin) and androgenic (spiggin) end-points (Katsiadaki et al., 2002; Hahlbeck et al., 2004; Jolly et al., 2006; Sanchez et al., 2008; Le Mer et al., 2013). Nevertheless, as showed by (Casanova-Nakayama et al., 2011), current data are too limited to inform conclusive ideas of the *in vivo* EDC impact mechanisms on fish immune function. Thus, after determining the optimal test conditions necessary to analyze fish splenic immune responses, we proposed here some *ex vivo* assays. These assays will increase knowledge on the direct biological effects of EDCs on fish non-specific immune activities, based on the phagocytic function (respiratory burst and phagocytosis activities) and cellular mortality (necrosis and apoptosis). Due to their roles in reproductive physiology, E2 and 11KT were chosen. E2 was the principal sex-steroid detected in the plasma of females whereas 11KT is usually more abundant in male fish (Gonçalves et al., 2010). In males, the 11KT tested concentrations are representative of plasmatic hormonal levels detected in the spawning period, with concentration ranging from 40 to 1,300 nM. Outside of the breeding season concentrations are orders of magnitude lower (< 7 nM) (Mayer et al., 1990; Páll et al., 2002; Ian et al., 2004). E2 plasmatic concentrations in females are clearly weakly modulated between sexual cycles. During the reproductive period, from May to July, the E2 concentration are around 3 nM, and are then lower hereafter (Björkblom et al., 2009). As female hormonal concentrations are weakly modulated, we chosen them as models for the assays The EE2, synthetic estrogen widely used as oral contraceptive, two industrial chemicals, bisphenol A (BPA) and 4-*n*-nonylphenol (NP) were studied due to their xenoestrogenic actions (Jones et al., 2000; Schultis and Metzger, 2004)

especially on fish (Olsen et al., 2005; Torres-Duarte et al., 2012). In the same manner, two synthetic androgens, trenbolone acetate (TB) (Davis et al., 2000) and 17α-methyltestosterone (MT) (Hahlbeck et al., 2004), were also tested. Moreover, the chosen concentrations were around current environmental concentrations of EDC detected in sewage-treatment plants effluents, rivers and seawater (Desbrow et al., 1998; OSPAR, 2001). The spleen tissue was chosen as it is an important target for steroids (Casanova-Nakayama et al., 2011; Milla et al., 2011).

2. Materials and methods

During this project, all experiments were conducted in accordance with the Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. Moreover, all experimental protocols were approved by the Ethical Committee of the French National Institute of Industrial Environment and Risks (INERIS).

2.1. Biological model

Ninety adult sticklebacks (6.2 ± 0.4 cm, 2.9 ± 0.7 g), from one spawn, were obtained in a home husbandry (INERIS, Verneuil-en-Halatte, France). Experiments were performed out of gametogenesis, in November 2012, to compensate immune modulation due to sex-steroid hormones. Prior to the experiments, fish were maintained for one month in a tank (50 L, 12 ± 1 °C, 350 µS/cm) with a 10/14-h light/dark cycle. During this period, sticklebacks were fed daily with frozen red mosquito larvae and brine shrimp (3 % of body weight/day; Europrix).

2.2. Leucocyte isolation

Each fish was sacrificed by decapitation, and then measured and weighed. To obtain a leucocyte suspension, spleen tissues were removed and gently pressed through sterilized nylon mesh (40 µm, Dutscher) with Leibovitz 15 (L15) medium (Sigma) containing heparin lithium

(10 U.mL^{-1}, Sigma), penicillin (500 U.mL^{-1}, Biochrom AG) and streptomycin (500 µg.mL^{-1}, Biochrom AG). Nevertheless, no foetal calf serum was used here due to their complex biochemical composition (i.e. cell activators; growth factors) which may be incompatible with immune toxicological and ecotoxicological objectives (Milla et al., 2011). In order to perform the analyses, the leucocyte concentration was adjusted to 10^6 cells.mL^{-1} with Malassez haemocytometer.

2.3. Exposure test development

The *ex vivo* exposure was initially optimised with 20 fish to reduce the drastic impacts of storage and solvent on the tested immune parameters. For this reason, 10 leucocyte suspensions were maintained during 0, 12, 24 and 48 h at two different temperatures (4 and 15 ± 1°C) and 10 others were spiked with different ratio of dimethylsulfoxide (DMSO): leucocyte suspension, from 0 to 1 % in L15 medium.

2.4. *Ex vivo* exposures

For the *ex vivo* exposures, some female fish were used with 8-10 leucocyte suspensions for each individual test concerning four estrogens (17 β-estradiol (E2), 17 α-ethinylestradiol (EE2), bisphenol A (BPA), 4-*n*-nonylphenol (NP)) and three androgens (trenbolone acetate (TB), 11-ketotestosterone (11KT), 17α-methyltestosterone (MT)) manufactured by Sigma Aldrich.

In order to allow the comparison between the tested chemicals, each EDC was tested at the same molar concentration, 0, 0.1, 1 and 100 nM. Each concentration was prepared daily by dilution in DMSO (Sigma, France) as the tested chemicals have low water solubilities. The ratio DMSO:leucocyte suspension did not exceed 0.2 % in order to avoid disturbance of the studied parameters.

Immediately after the pollutant dilution process, for each leucocyte suspension, each EDC at all concentrations, 1 mL of the leucocyte suspension were mixed with 10 µL of pollutant. In the same manner and in order to check the quality of the leucocyte suspension, 10 µL of DMSO at 20 % was mixed with 1 mL of leucocyte suspension to obtain a solvent control at 0.2 %. Prior to further analyses, all samples (controls and leucocyte suspension mixed with pollutant) were incubated at 4 °C for 12 h.

2.5. Innate immune biomarkers analysis

Analyses were carried out on whole leucocytes, using a Cyan^{TM} ADP flow cytometer (Beckman Coulter). For each leucocyte sample, 10,000 cells were counted.

Leucocyte distribution was obtained using FSC and SSC parameters for size and complexity, respectively. Cellular mortality was detected using a double markedly method without any inhibitory effect on cellular function (Idziorek et al., 1995). The YO-PRO®-1 (1 mM in DMSO, Invitrogen) and Propidium Iodide (PI, 1.5 mM in water, Invitrogen) were used in order to obtain cellular fluorescence parameters indicating the presence of apoptotic (FL1, green fluorescence) and necrotic (FL3, red fluorescence) leucocytes, respectively (Bado-Nilles et al., 2014). These two markers allow ultrasensitive detection of double-stranded nucleic acids. Nevertheless, activation of

P2X7 receptor in apoptotic cells enable penetration of YO-PRO®-1 (Baraldi et al., 2004) in contrary to PI, which is excluded from viable cells due to their membrane impermeant characteristics. Cell necrosis and apoptosis were detected after 10 min of incubation on ice with YO PRO®-1 (5 µM) and PI (7.5 µM) to limit potential dyes interference with cellular activities, membrane permeability and background staining.

Leucocyte respiratory burst, based on the technique described by Chilmonczyk and Monge (1999), was optimized for three-spined stickleback. Determination of reactive oxygen species (ROS) by unstimulated cell depends upon the cell incorporating 2'-7'-dichlorofluorescin diacetate (H_2DCF-DA, Sigma), which is a stable non-fluorescent molecule which is hydrolyzed to DCFH by cytosolic enzymes. When leucocytes are stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma), the more specific inductor of respiratory burst (Ambrozova et al., 2011; Chadzinska et al., 2012), H_2DCF-DA is also hydrolyzed by H_2O_2. Finally, the DCFH obtained is oxidised to the fluorescent dichlorofluorescein (DCF) to allow quantification by flow cytometry of unstimulated and stimulated cells. Stimulation index of respiratory burst was determined, after 30 min of incubation at room temperature, as the ratio of fluorescence of PMA stimulated cells (H_2DCF-DA at 60 µM plus PMA at 15 µM) to that of unstimulated cells (H_2DCF-DA at 60 µM). Results were expressed as mean fluorescence intensity (MFI) for ROS basal and activated levels.

The phagocytosis activity was measured using Fluorescent microsphere (2.7×10^{10} particles.mL^{-1}, Fluorospheres® carboxylate-modified microsphere, diameter 1 µm, Molecular Probes) as previously described (Bado-Nilles et al., 2009a). Cells were incubated with diluted

beads (ratio beads:cells 135:1) during 1 h at room temperature. Only the events showing a fluorescence of at least three beads were considered positive for phagocytic activity

2.6. Statistical analysis

All results were expressed as means ± standard error with n = 10 independent biological replicates. Verification of normality and of homogeneity of covariance matrices (homescedasticity) were conducted using respectively the Anderson–Darling test and the Bartlett test on XLStat 2008 (Addinsoft). To determine the effects of the solvent and storage and solvent on the parameters, a two- and a one-way analysis of variances (ANOVAs) were respectively made. Finally, a one-way ANOVA was used to assess the effect of each pollutant at each concentration in relation to mean DMSO control values. The Student Newman-Keuls's test was used for all multiple comparisons. All hypotheses were tested for statistical significance at the level of $p \leq 0.05$.

3. Results

3.1. Effects of storage on immune parameters

All storage results were shown on Table 1. At 4 °C, leucocyte distribution was not modified during the 48 h of storage ($p = 0.082$). On the opposite, a significant reduction of lymphocyte percentage during all the *ex vivo* maintenance periods was shown at 15 °C, the reduction corresponded to 8 % at 12 h and to 15 % at 48 h ($p < 0.0001$). In the same manner, at 12 h, a more important cellular mortality mainly due to apoptosis was detected at 15 °C (10.1 ± 0.8 % of total mortality; 7.8 ± 0.8 % of apoptosis) compared to 4 °C (6.3 ± 0.4 % of total mortality; 3.4 ± 0.4 % of apoptosis) which was close to initial values ($p \leq 0.001$). In fact, a significant increase of cellular mortality (8.2 ± 0.7 %) and apoptosis (6.0 ± 0.7 %) was shown only after the 24 h of maintenance at 4 °C and could be compared to those obtained after 12 h at 15 °C ($p < 0.0001$). Nevertheless, at the end of the storage time, the quantified leucocyte mortality (mean of 20.8 ± 0.5 %) and apoptosis (mean of 17.6 ± 0.2 %) were similar regardless of the temperature ($p = 0.854$). Necrosis percentages was not affected by the conditions ($p = 0.065$). Concerning the respiratory burst activity, only the ROS basal levels were modified by the different conditions. In fact, a significant increase of these values was shown after 48 h at 4 °C, from 13.0 ± 2.0 MFI at the beginning to 66.3 ± 18.0 MFI at the end, and after 24 h at 15 °C, from 20.0 ± 4.5 MFI at the beginning to 94.6 ± 23.3 MFI at the end ($p < 0.001$). As for other immune parameters, destabilisation was shown earlier at 15 °C than at 4 °C ($p < 0.0001$). The ROS activated levels ($p = 0.237$) and

stimulation data ($p = 0.965$) were very similar regardless the temperature and storage time. Finally, the same drastic decline of phagocytosis activity (8-fold) was detected after 48 h regardless the temperature ($p < 0.0001$).

3.2. Effects of DMSO solvent concentration on immune parameters

As observed on Table 2, except for leucocyte distribution ($p = 0.137$) and phagocytosis activity ($p = 0.241$), in comparison to control values, cellular mortality was drastically enhanced with 0.5 and 1 % of DMSO (1.6-fold higher for 0.5 % and of 3-fold for 1 %), in relationship with both apoptosis (1.6-fold higher for 0.5 % and of 2.1-fold for 1 %) and necrosis (1.5-fold higher for 0.5 % and of 10.5-fold for 1 %) percentages ($p \leq 0.033$). On the contrary, ROS basal levels were approximately 2.2-fold weaker when the DMSO concentration was 0.1 and 0.2 % ($p \leq 0.009$). No impact on ROS activated levels was induced for each tested DMSO concentration ($p = 0.813$). Nevertheless, the decrease of the ROS basal levels only have repercussions on the stimulation index of respiratory burst for the ratio of 0.1 % of DMSO:leucocyte suspension, with 3.2 ± 0.5 compared to 1.6 ± 0.1 for control value ($p = 0.012$).

3.3. Effects of EDCs on innate immune response

One estrogen, the 17β-estradiol (E2), and one androgen, the 11-ketotestosterone (11KT), have similar trend for each immune parameter tested. Despite of the absence of effects on leucocyte distribution (Fig. 1, $p \geq 0.144$), a significant increase of total leucocyte mortality (mean of 29.0 ± 4.1 %; $p < 0.0001$) was observed with 11KT compared to the DMSO control (15.3 ± 2.0 %). These results were certainly

due to a necrosis enhancement (8.4 ± 1.6 % for solvent control; mean of 19.3 ± 4.1 % for 11KT samples; $p = 0.001$) without any effect on apoptosis percentage (Table 3; $p = 0.054$). In the same manner, a similar tendency was detected after E2 treatment (Table 3). The major action of these natural hormones was on respiratory burst (Fig. 2) and phagocytosis (Fig. 3) activities. All concentrations of E2 and 11 KT, induced a significant increase of stimulation index of respiratory burst (4.0 ± 1.4 for DMSO control; mean of 12.7 ± 1.3 for E2 samples; mean of 16.3 ± 1.5 for 11KT samples; $p < 0.0001$). This was probably due to a significant increase of ROS activated levels (85.4 ± 21.9 MFI for DMSO control; mean of 281.5 ± 30.9 MFI for E2 samples; mean of 435.6 ± 42.4 MFI for 11KT samples; $p < 0.0001$) without any effect on the ROS basal levels (Fig. 2; $p \geq 0.281$). In the same manner, a significant enhancement of phagocytosis activity was detected at all tested concentrations only for the two natural hormones (mean of 31.6 ± 0.9 % for E2 samples; mean of 32.9 ± 1.1 % for 11KT samples) compared to solvent control (26.4 ± 1.3 %) (Fig. 3; $p < 0.001$).

For all EDCs, only trenbolone acetate (TB) at 1 nM destabilized leucocyte distribution by acting on the granulocyte-macrophage sub-population (from 29.8 ± 3.1 % for DMSO control to 17.2 ± 1.7 % with TB treatments; $p = 0.002$) (Fig. 1). Nevertheless, for this androgen, cellular mortality (mean of 13.6 ± 1.3 %; $p = 0.577$) was not affected by the treatment compared to the solvent control (15.3 ± 2.0 %). Only 4-*n*-nonylphenol (NP) had an impact on cellular mortality by increasing both total leucocyte mortality (mean of 20.2 ± 0.9 %; $p = 0.023$) and apoptosis (mean of 10.5 ± 0.8 %; $p = 0.001$) compared to DMSO control values (15.3 ± 2.0 % for total cellular mortality; 6.9 ± 0.9 % for apoptosis) (Table 3). In opposite to E2 and 11KT, all EDCs induced reduction of respiratory burst

activity (Fig. 2) and did not affect phagocytosis activity (Fig. 3; $p \geq 0.053$). More specifically for respiratory burst, a dose dependant increase of ROS basal levels (1.8- and 4.3-fold increase for estrogen and androgen, respectively; $p < 0.0001$) and a decrease of stimulation index of respiratory burst activity (2.5- and 3.4-fold decrease for estrogen and androgen, respectively, $p \leq 0.049$) were detected for each EDC. Nevertheless, the androgens presented more impact on this activity than estrogens. Only TB ($p = 0.006$) and 17α-methyltestosterone (MT; $p = 0.009$) still induced an increase of ROS activated levels at 100 nM (142.4 ± 11.2 MFI for TB; 202.5 ± 33.3 MFI for MT) compared to DMSO control data (85.4 ± 21.9 MFI).

4. Discussion

Over the long term, modulation of immune system capacities could induce important destabilization of fish populations by acting on individual health and fitness, mate selection during reproduction or population growth (Casanova-Nakayama et al., 2011; Bado-Nilles et al., 2014). Thus, it seems relevant to study the impacts of EDCs on the immune system of aquatic wildlife due to the bidirectional interaction between the immune and reproductive systems (Ansar Ahmed, 2000; Engelsma et al., 2002). Nevertheless, as few data are available on the EDC mechanisms on the fish immune function *in vivo*, we initiated the *ex vivo* experiments reported herein. Furthermore, as alternatives to *in vivo* experiments, many authors study the impact of pollutants on fish immune function by using *ex vivo* and/or *in vitro* assays on immune cells isolated from spleen or head-kidney. A large range of cell lines, incubation time, temperature, solvent and solvent concentrations have been used, as no standardized procedures are available. Since these factors have a significant influence on the outcome of the studies, comparisons of data between assays are difficult. In this context, we proposed here an optimized protocol to preserve splenic leucocyte characteristics from detrimental effects on leucocyte distribution, cellular mortality, respiratory burst and phagocytosis activity. First of all, the optimal temperature and incubation time were determined. For fish, 15°C and 4 °C were mainly used in *ex vivo* protocols (Sakai, 1992; O'Halloran et al., 1998; Bado-Nilles et al., 2009b). Nevertheless, as previously shown (Sakai, 1992; Bado-Nilles et al., 2009b), a storage temperature of 4 °C induced low immune destabilization throughout the incubation time. Moreover, at 4 °C, the major impact on the leucocyte immune system was detected at 48 h of incubation along with an important cellular

mortality starting from 24 h. In the manner, Misumi et al. (2005) showed an important decrease of cellular viability after 17 h especially on splenic leucocytes. As an incubation time of 12-16 h is enough to determine the direct effects of EDCs on cells (Yao et al., 2007; Águila et al., 2013), a maximum incubation time of 12 h at 4 °C was suggesting in order to limit immune destabilization caused by leucocyte death. Secondly, it is important to study the eventual effects of the solvent on immune parameter tested. Considering that many pollutants modify the percentage of viable cells in cultures (O'Halloran et al., 1998; Laville et al., 2004; Yao et al., 2007), it seems important to confirm that the solvent , often known to enhance cell membrane permeability, does not increase the percentage of cellular mortality. In this context, prior to the 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE) treatment, Misumi et al. (2005) incubated spleen and head-kidney leucocytes of chinook salmon (*Oncorhynchus tshawytscha*) with and without ethanol at 0.075 % [v/v] over 48 h of incubation. These authors demonstrated that the concentration of the solvent did not have an impact on leucocyte viability compared to control cells (Misumi et al., 2005). In this study, 0.2 % of DMSO [v/v] was enough to maintain natural leucocyte mortality and also limited impact on leucocyte distribution, respiratory burst and phagocytosis activity. Finally we proposed a standardized *ex vivo* protocol for fish splenic leucocyte which consists of a maximum exposure of 12 h at 4 °C with 0.2 % of DMSO [v/v]. Nevertheless, this protocol must be improved. Further studies should been conducted mainly on the reproducibility of the results, the reduction of inter-individual variability and the quantification of the relationships between the multiple factors.

After the development of the protocol, two hormones and five EDCs were tested to determine their impact on the female three-spined stickleback immune function. As reviewed by Milla et al. (2011), the effects of sex-steroid hormones, in both sexes on the immune function is highly dependent on the reproductive cycle. For this reason, our experiments were performed out of the gametogenesis period in order to limit interference with endogenous hormones which vary seasonally (Mayer et al., 1990; Páll et al., 2002; Ian et al., 2004; Björkblom et al., 2009). Moreover, the effects of a majority of hormones and EDC followed a non-monotone dose-response curve (Vandenberg et al., 2012; Beausoleil et al., 2013) explaining that this type of result was obtained even if the tested concentrations were quite different. Some results do not suggest that the mode of action of each EDC was similar regardless of the concentration. In fact, different intracellular factors led to a non-monotone dose-response curve, such as the saturation of metabolic pathways, alteration of signalling pathways, induction of detoxification pathways, competing pathways to receptors which change over a dose range (Beausoleil et al., 2013). Nevertheless, this study possessed only three doses covering a high concentration range. For this reason, we cannot really explain the response curve that we obtained.

Sex-steroid hormones induce important stimulation of respiratory burst and phagocytosis activity, of the female fish even if they were not in a reproductive stage. These results showed that immune cells seem to be very sensitive to natural hormones (E2, 11KT) which are probably due to their direct action on leucocyte activities through androgen (Slater et al., 1995) and estrogen (Liarte et al., 2011) receptors. In accordance with (Thilagam et al., 2009), a significant increase of the stimulation index of respiratory burst caused by the

potential enhancement of intracellular generation of ROS, highlighted by increase of ROS activated levels, was detected without destabilization of ROS basal levels. Moreover, a significant increase of phagocytosis activity after E2 and 11KT exposures also confirmed that these two hormones stimulated, in similar way, splenic leucocytes of female fish. As proposed by Filby et al. (2007), these results suggested that these hormones have an important impact on granulocytes and more particularly on macrophages, the more active phagocytic cells in fish (Esteban and Meseguer, 1997). This immunostimulatory activity could induce in a long term, cytotoxic effects which may be caused by the secretion of tumor necrosis factors by the activated macrophages (Secombes et al., 1996; Peddie et al., 2002). These results explained the important leucocyte mortality enhancement due to necrosis, without specific impact on one leucocyte population. This hypothesis was supported by other authors who showed sexual hormones induced an increase of different macrophage cytokines, which initiate or regulate inflammatory process (Jin et al., 2010; Sun et al., 2011). In another way, since an increase of ROS production could on a long term result in apoptotic cell death (Risso-de Faverney et al., 2001; Krumschnabel et al., 2005), the absence of apoptosis here seems to be due to stable ROS basal levels, which insinuates an absence of oxidative stress.

Concerning mimic-hormones, usually, the tested chemicals will not be equipotent and the relative estrogenic and androgenic potentials were relatively different depending on the identity of the tested EDC. For example, in rainbow trout (*Oncorhynchus mykiss*), NP presented a 2×10^{-4} relative binding activity (RBA) whereas BPA has a 2.9×10^{-3} RBA (Olsen et al., 2005). Furthermore, the hormonal potential was quite different depending on the species and the type of test (Tollefsen et al., 2002; Olsen et al., 2005; Jolly et al., 2006).

Nowadays, few data were available on the relative estrogenic and androgenic potential of chemicals on sticklebacks (Borg et al., 1993; Katsiadaki et al., 2002; Jolly et al., 2006) explained the difficulty to discuss results in terms of EDC potency. Even if the mode of action of EDC imitated endogenous hormones (Milla et al., 2011), very dissimilar immune effects were detected between natural and EDCs at each molar concentration. In our work, E2 and 11KT, two natural hormones, seem to preferentially immunostimulate defense capacities of female fish. On the opposite the other EDCs, have potentially immunosuppressive effects. This discrepancy could come from the sub-type of the receptor which was activated by EDCs. For example, Routledge et al. (2000) showed that dissimilar hormonal effects were detected on cells in depending on the type of estrogen receptor activated, such as an estrogen receptor α or β. Moreover, xenoestrogens could change the tertiary structure of receptors following ligand binding which induced various actions on cells (Routledge et al., 2000). In fish, some α and β androgen (Ikeuchi et al., 2001) and estrogen (Seo et al., 2006) receptors were detected in many tissues, suggesting the importance of further studies to search for alternative immune effect caused by the type of receptor bind. In the present study, each EDC induced a decrease of respiratory burst stimulatory index due to the increase of ROS basal levels. In the same manner, Yin et al. (2007) observed that *in vitro* exposure to BPA suppressed respiratory burst activity in fish macrophage and (Gong and Han, 2006) showed an increase of ROS production in rat Sertoli cells after NP *in vitro* exposure. This baseline ROS enhancement could come from a direct activation by hormones of protein kinase C (Wehling, 1997), a second messenger systems which leads to ROS production in fish (Betoulle et al., 2000). As previously discussed, an increase of this immune parameter could end by apoptotic cell death due to potential oxidative stress (Risso-de Faverney et al., 2001; Krumschnabel et al., 2005) and major inflammatory reaction (Yin et al., 2007).

Nevertheless, concomitant increase of ROS basal levels and leucocyte death was shown only with one estrogenic EDC: NP. These results suggest that cellular mortality could not only be attributed to an increase of ROS basal levels. In accordance with our results, during exposure of rat Sertoli cells to NP, an increase of ROS generation was not enough to lead cells to death (Gong and Han, 2006). Indeed, a different hypothesis of leucocyte death by apoptosis pathway could be proposed here; since NP reduces membrane fluidity on liver cells of rainbow trout, *Oncorhynchus mykiss* (Cakmak et al., 2006), we could suggest that leucocyte death by apoptosis could result in a change of membrane fluidity (Prasad et al., 2010; Tekpli et al., 2013). This effect may be linked with the influence of the nonspecific steroid on the lipid bilayer (Wehling, 1997). On the other hand, apoptosis could be enhanced by a Ca^{2+}-independent manner just like human osteoblast-like cells (Wang et al., 2005). After steroid receptor binding, increase in caspase-8 dependent processes in human Jurkat cells could also explain the rise of apoptosis caused by NP (Yao et al., 2007).

From the environmental perspective, adverse ecological effects compromise the immunocompetence of fish. In fact, a higher immunostimulation with natural hormones could over the long term induce potential reduction of the number of leucocyte number by necrosis which in turn may impact the immune capacities. In the same manner, each EDC caused a rapid reduction of the immune system capacities. These changes in immune system capacities could modify the susceptibility of fish to general and specific infectious agents and, ultimately, fish populations could also be impacted (e.g. individual fitness and health, mate selection during reproduction, population growth). Thus, as previously discussed (Casanova-Nakayama et al., 2011; Bado-Nilles et al., 2014), the use of immune parameters for the

determination of environmental risk assessment seems to be attractive due to *i)* its fundamental physiological role and *ii)* its function of early-warning indicator of destabilization of fish health and fish decline. Thus, we argue in favor of the deployment of these indicators in freshwater monitoring programs according to international recommendations for marine monitoring (ICES, 2011).

5. Conclusion

We successfully developed an *ex vivo* approach to assess impairment of the innate immune system of the female three-spined stickleback by chemicals. This assay highlights the immunotoxic effects of EDCs based on various mechanisms of action as reflected by the immunostimulant effects of E2 and 11KT and immunosuppression caused by the other tested EDCs. Now, further *ex vivo* studies are necessary in order to gain more knownledge on the effects of EDCs on immune responses, such as immune challenge, interaction with agonist and antagonist molecules and/or impact on leucocyte of different sex. In this context, the three-spined stickleback appears as a relevant model fish species to explore the relationship between endocrine disruption and immunotoxicity. Moreover, this study argues for a greater deployment of immune parameters in ecotoxicology studies due to their predictive understanding of fish population variation.

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6. References

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Table 1: Splenic immune leucocyte activities (leucocyte distribution, cellular mortality, respiratory burst and phagocytosis) of the three-spined stickleback (*Gasterosteus aculeatus* L.) were monitored in laboratory by flow cytometry after 0, 12, 24 and 48 h of storage at 4 ± 1 °C and 15 ± 1 °C.

		Leucocyte distribution (%)		**Cellular mortality (%)**			**Respiratory burst values (MFI)**			**Phagocytosis activity (%)**
		Lymphocytes	Granulocytes-macrophages	Total	Apoptosis	Necrosis	ROS basal level	ROS activated level	Stimulation index	
4 °C	T0	81.1 ± 1.2 ^{a}	18.9 ± 1.2 ^{a}	5.1 ± 0.6 ^{c}	1.7 ± 0.1 ^{c}	3.4 ± 0.6 ^{a}	13.0 ± 2.0 ^{b}	45.2 ± 7.2 ^{a}	3.8 ± 0.6 ^{a}	18.6 ± 1.3 ^{a}
	T12	79.7 ± 1.8 ^{a}	20.3 ± 1.8 ^{a}	6.3 ± 0.4 ^{bc}	3.4 ± 0.4 ^{c}	2.9 ± 0.4 ^{a}	8.3 ± 2.3 ^{b}	36.7 ± 9.3 ^{a}	4.6 ± 1.0 ^{a}	17.2 ± 0.5 ^{a}
	T24	77.2 ± 2.0 ^{a}	22.8 ± 2.0 ^{a}	8.2 ± 0.7 ^{b}	6.0 ± 0.7 ^{b}	2.2 ± 0.2 ^{a}	20.4 ± 4.2 ^{b}	37.8 ± 8.1 ^{a}	2.2 ± 0.4 ^{a}	16.5 ± 0.4 ^{a}
	T48	74.9 ± 1.9 ^{a}	25.1 ± 1.9 ^{a}	20.6 ± 1.4 ^{a}	17.0 ± 1.5 ^{a}	3.6 ± 0.4 ^{a}	66.3 ± 18.0 ^{a}	94.9 ± 23.8 ^{a}	2.5 ± 0.9 ^{a}	3.8 ± 0.3 ^{b}

15 °C	T0	81.8 ± 1.2 [a]	18.2 ± 1.2 [c]	4.8 ± 0.4 [c]	1.7 ± 0.2 [c]	3.1 ± 0.3 [a]	20.0 ± 4.5 [b]	50.4 ± 8.0 [a]	3.1 ± 0.5 [ab]	18.3 ± 2.1 [a]
	T12	74.1 ± 2.2 [b]	25.9 ± 2.2 [b]	10.1 ± 0.8 [b*]	7.8 ± 0.8 [b*]	2.3 ± 0.3 [ab]	25.0 ± 5.1 [b]	111.0 ± 38.6 [a]	5.0 ± 1.8 [a]	17.8 ± 1.1 [a]
	T24	72.2 ± 2.0 [b]	27.8 ± 2.0 [b]	11.5 ± 0.8 [b*]	9.6 ± 0.8 [b*]	1.9 ± 0.2 [b]	40.9 ± 8.2 [b*]	62.2 ± 18.5 [a]	1.8 ± 0.6 [bc]	16.2 ± 0.5 [a]
	T48	66.5 ± 2.1 [c*]	33.5 ± 2.1 [a*]	21.0 ± 1.5 [a]	18.2 ± 1.4 [a]	2.8 ± 0.3 [ab]	94.6 ± 23.3 [a]	95.3 ± 25.8 [a]	1.6 ± 0.7 [c]	2.9 ± 0.1 [b*]

Values correspond to means ± standard error (n = 10 independent biological replicates). Different letters denote for each parameter and temperature significant differences between incubation times for $p \leq 0.05$. * Concerned significant difference between temperatures for each date for $p \leq 0.05$.

Table 2: Splenic immune leucocyte activities (leucocyte distribution, cellular mortality, respiratory burst and phagocytosis) of the three-spined stickleback (*Gasterosteus aculeatus* L.) were monitored in laboratory by flow cytometry after 12 h of storage at 4 ± 1 °C with different concentrations of DMSO, the pollutant solvent.

	Leucocyte distribution (%)		**Cellular mortality (%)**			**Respiratory burst values (MFI)**			**Phagocytosis activity (%)**
	Lymphocytes	Granulocytes-macrophages	Total	Apoptosis	Necrosis	ROS basal level	ROS activated level	Stimulation index	
Control	70.7 ± 2.1	29.3 ± 2.1	13.6 ± 1.9	11.7 ± 1.8	1.8 ± 0.3	122.1 ± 23.0	198.9 ± 45.2	1.6 ± 0.1	13.4 ± 1.4
0.1 %	68.5 ± 2.5	31.5 ± 2.5	13.1 ± 1.5	10.8 ± 1.4	2.3 ± 0.3	52.0 ± 9.3 *	164.5 ± 37.3	3.2 ± 0.5 *	15.7 ± 0.7
0.2 %	70.9 ± 1.7	29.1 ± 1.7	12.9 ± 1.6	10.8 ± 1.4	2.2 ± 0.3	58.5 ± 6.3 *	146.1 ± 29.3	2.4 ± 0.4	14.5 ± 1.6

0.5 %	80.2 ± 2.8	19.8 ± 2.8	20.9 ± 3.2 *	18.2 ± 3.3 *	2.7 ± 0.4 *	92.8 ± 10.1	180.3 ± 52.8	1.9 ± 0.3	19.3 ± 1.9
1.0 %	64.1 ± 5.6	35.9 ± 5.6	41.9 ± 5.9 *	23.0 ± 2.7 *	18.9 ± 4.3 *	188.0 ± 73.4	159.2 ± 37.6	1.3 ± 0.4	15.1 ± 3.0

Values correspond to means ± standard error (n = 10 independent biological replicates). * Significant difference between DMSO concentration and control values for $p \leq 0.05$.

Table 3: Splenic leucocyte mortality of the three-spined stickleback (*Gasterosteus aculeatus* L.) was monitored out of gametogenesis in laboratory by flow cytometry following *ex vivo* exposure for 12 h at 4 ± 1 °C to DMSO control (0 nM) and seven endocrine disrupting compounds (0.01, 1, 100 nM).

		Total leucocyte mortality (%)				**Leucocyte apoptosis (%)**				**Leucocyte necrosis (%)**			
		0 nM	0.01 nM	1 nM	100 nM	0 nM	0.01 nM	1 nM	100 nM	0 nM	0.01 nM	1 nM	100 nM
ESTROGENS	*17 β-estradiol*	*15.3 ± 2.0 [a]*	*19.0 ± 1.2 [a]*	*19.8 ± 2.2 [a]*	*21.3 ± 1.6 [a]*	*6.9 ± 0.9 [a]*	*7.7 ± 0.6 [a]*	*8.3 ± 1.0 [a]*	*9.5 ± 0.9 [a]*	*8.4 ± 1.6 [a]*	*11.3 ± 1.1 [a]*	*11.5 ± 1.3 [a]*	*11.8 ± 1.0 [a]*
	17 α-ethinylestradiol	15.3 ± 2.0 [a]	9.6 ± 0.8 [a]	10.0 ± 0.9 [a]	12.2 ± 1.3 [a]	6.9 ± 0.9 [a]	5.0 ± 0.8 [a]	5.3 ± 0.9 [a]	6.3 ± 1.0 [a]	8.4 ± 1.6 [a]	4.6 ± 0.5 [a]	4.7 ± 0.5 [a]	5.9 ± 1.1 [a]
	Bisphenol A	15.3 ± 2.0 [a]	13.0 ± 1.1 [a]	12.7 ± 1.1 [a]	13.2 ± 1.0 [a]	6.9 ± 0.9 [a]	6.8 ± 0.7 [a]	6.8 ± 0.7 [a]	7.2 ± 0.8 [a]	8.4 ± 1.6 [a]	6.2 ± 0.6 [a]	5.9 ± 0.5 [a]	6.1 ± 0.5 [a]
	4-*n*-nonylphenol	15.3 ± 2.0 [b]	20.0 ± 1.0 [a]	19.3 ± 0.6 [a]	21.1 ± 1.1 [a]	6.9 ± 0.9 [b]	10.2 ± 0.7 [a]	9.9 ± 0.7 [a]	11.4 ± 1.0 [a]	8.4 ± 1.6 [a]	9.8 ± 0.7 [a]	9.5 ± 0.5 [a]	9.7 ± 0.9 [a]
ANDROGENS	*11-ketotestosterone*	*15.3 ± 2.0 [b]*	*23.7 ± 3.2 [a]*	*30.8 ± 4.4 [a]*	*32.4 ± 4.9 [a]*	*6.9 ± 0.9 [a]*	*9.1 ± 1.0 [a]*	*10.4 ± 0.8 [a]*	*9.4 ± 0.9 [a]*	*8.4 ± 1.6 [b]*	*14.5 ± 2.8 [ab]*	*20.4 ± 4.3 [a]*	*23.0 ± 5.2 [a]*
	Trenbolone acetate	15.3 ± 2.0 [a]	12.1 ± 1.3 [a]	14.6 ± 1.5 [a]	14.1 ± 1.3 [a]	6.9 ± 0.9 [a]	7.3 ± 1.1 [a]	8.9 ± 1.2 [a]	9.0 ± 1.1 [a]	8.4 ± 1.6 [a]	4.8 ± 0.5 [a]	5.7 ± 1.0 [a]	5.1 ± 0.8 [a]
	17α-methyltestosterone	15.3 ± 2.0 [a]	14.7 ± 1.0 [a]	15.5 ± 1.2 [a]	16.5 ± 1.5 [a]	6.9 ± 0.9 [a]	8.5 ± 0.9 [a]	8.8 ± 0.9 [a]	9.9 ± 1.1 [a]	8.4 ± 1.6 [a]	6.2 ± 0.5 [a]	6.6 ± 0.6 [a]	6.6 ± 0.6 [a]

Compounds *in italic* correspond to natural hormone whereas others concerned endocrine disrupting compounds. Values correspond to means ± standard error (n = 8-10 independent biological replicates). For each hormone, different letters denote statistically significant differences between groups with $p \leq 0.05$.

Fig. 1: Splenic immune leucocyte distribution of female three-spined stickleback (*Gasterosteus aculeatus* L.) was monitored out of gametogenesis following *ex vivo* exposure to oestrogenic (A) and androgenic (B) endocrine disrupting compounds. DMSO control values (0 nM) were represented by the boldface axis. Values correspond to means ± standard error (n = 8 - 10 independent biological replicates). For each compound, *asterisks* correspond to statistical difference between condition and DMSO control for $p \leq 0.05$.

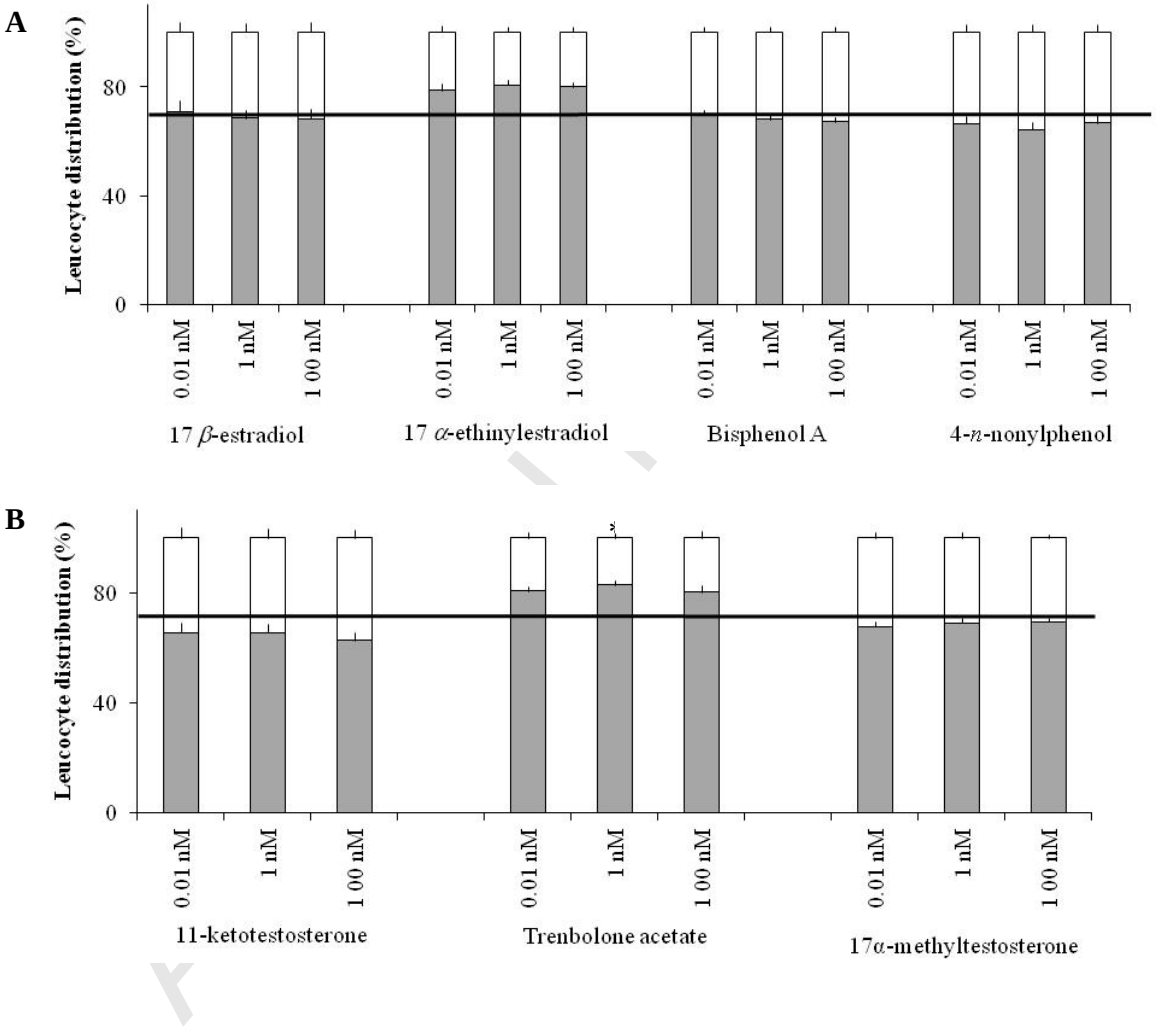


Fig. 2: Splenic immune respiratory burst of female three-spined stickleback (*Gasterosteus aculeatus* L.) was monitored out of gametogenesis following *ex vivo* exposure to oestrogenic (A) and androgenic (B) endocrine disrupting compounds. DMSO control values (0 nM) were represented by the boldface axis (ROS basal level and index of respiratory burst) and dotted line (ROS activated values). Values correspond to means ± standard error (n = 8 - 10 independent biological replicates). For each compound, *asterisks* correspond to statistical difference between condition and DMSO control for $p \leq 0.05$.

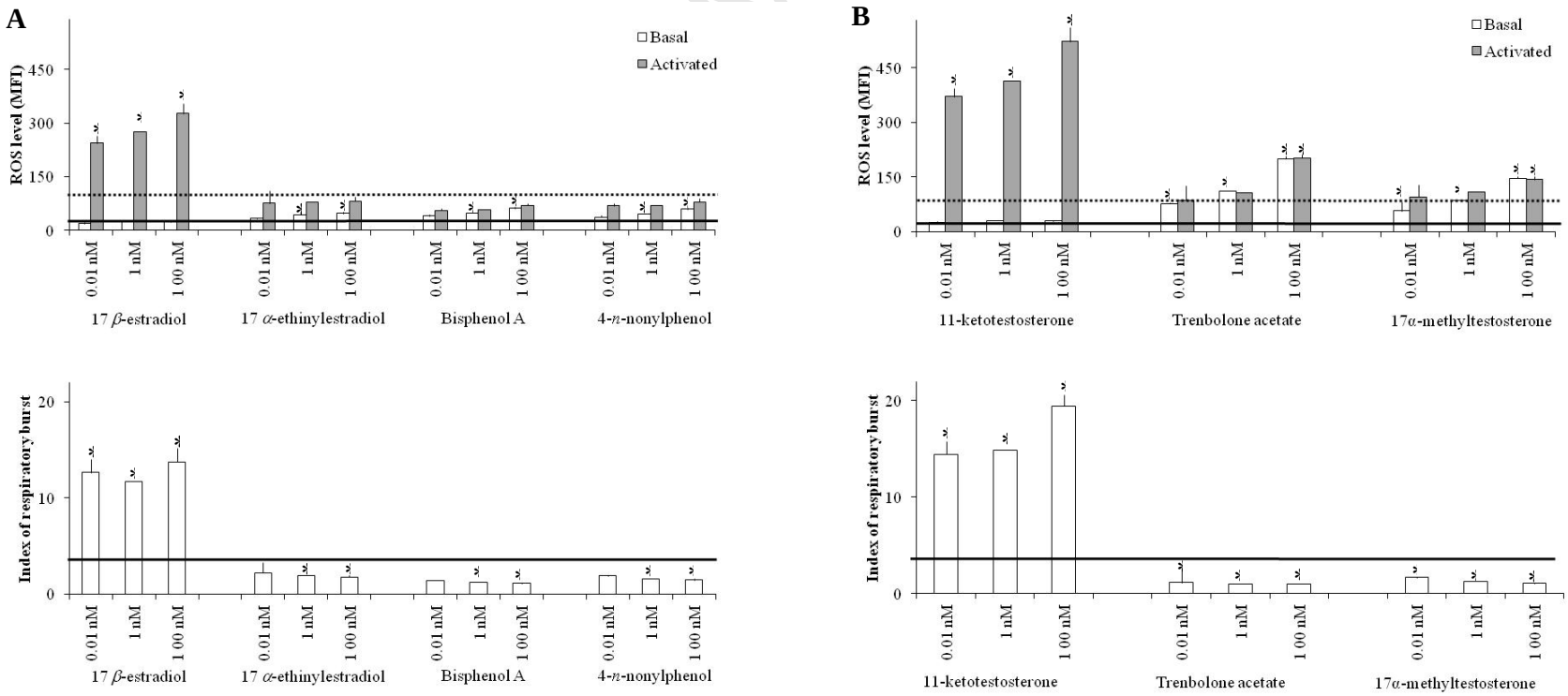
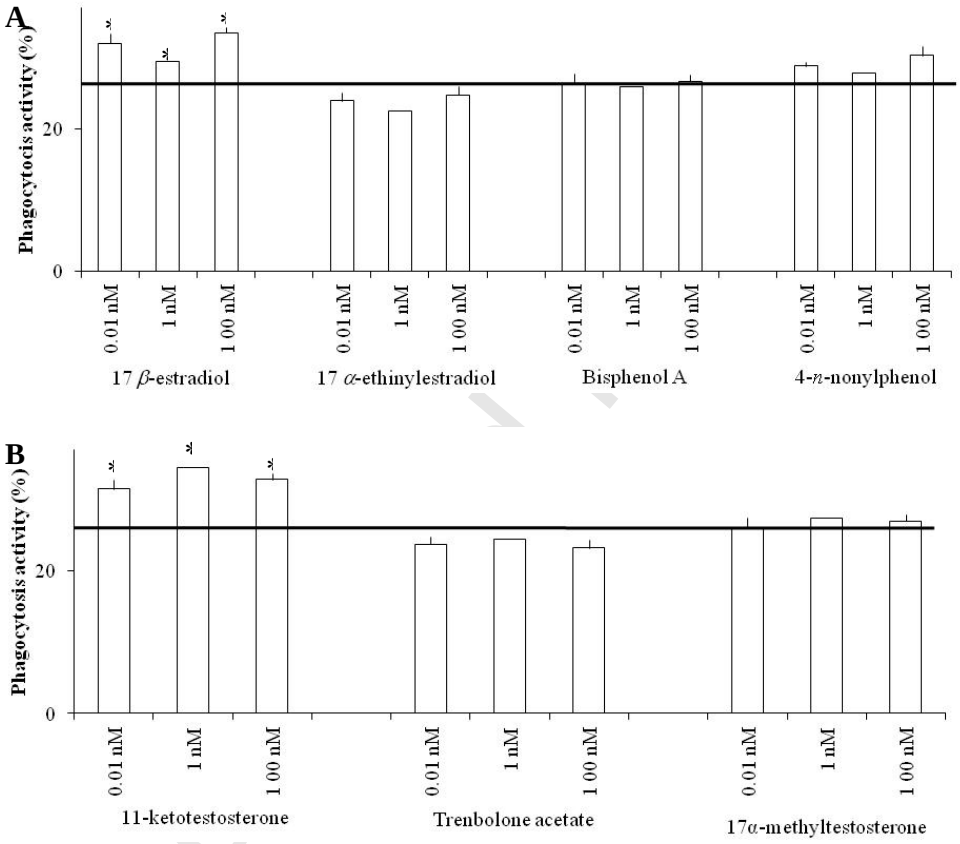


Fig. 3: Splenic immune phagocytosis activity of female three-spined stickleback (*Gasterosteus aculeatus* L.) was monitored out of gametogenesis following *ex vivo* exposure to oestrogenic (A) and androgenic (B) endocrine disrupting compounds. DMSO control values (0nM) were represented by the boldface axis. Values correspond to means ± standard error (n = 8 - 10 independent biological replicates). For each compound, *asterisks* correspond to statistical difference between condition and DMSO control for $p \leq 0.05$.



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Abbreviations: EDCs, endocrine disrupting compounds; 11KT, 11-ketotestosterone; E2, 17β–estradiol; EE2, 17α-ethinylestradiol; BPA, bisphenol A; NP, 4-*n*-nonylphenol; TB, trenbolone acetate, MT, 17α-methyltestosterone; L15, Leibovitz 15 medium; DMSO, dimethylsulfoxide; PI, Propidium Iodide; ROS, reactive oxygen species; H_2DCF-DA, 2'-7'-dichlorofluorescin diacetate; MFI, mean fluorescence intensity.

Highlights

► Understand *ex vivo* effects of hormones on fish non-specific immune activities ► Natural hormones were rather immunostimulant ► Mimic-hormones were rather immunosuppressive ► Stickleback is attractive to explore relationship between hormones and immunotoxicity