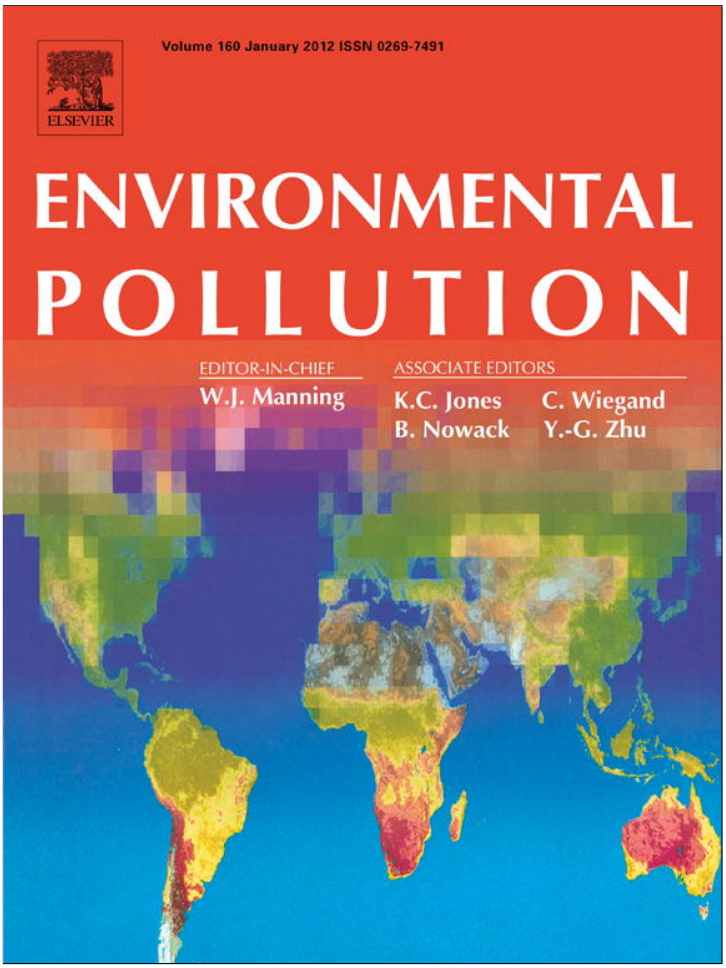

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Evidence of small modulation of ethinylestradiol induced effects by concurrent exposure to trenbolone in male eelpout *Zoarces viviparus*

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ABSTRACT

The interaction of xenobiotics is common in aquatic ecosystems; therefore, we wanted to evaluate if trenbolone (TB) modulates the effects of 17α-ethinylestradiol (EE2). Male eelpout (*Zoarces viviparus*) were exposed to 5 ng L^{-1} EE2 continuously for 19 d (EE2-C) or discontinuously (11 d, EE2-D) alone or in combination with low (50 ng L^{-1}, TBL) or high (500 ng L^{-1}, TBH) concentrations of TB (19 d). Exposure to EE2 caused reduced gonadosomatic index, increased plasma vitellogenin concentrations, up-regulated *vtg* and *era* mRNA expression and severe alterations in gonadal histology. TBL and TBH did not affect plasma vitellogenin, *era* or *vtg* mRNA expression. TBL and TBH did not counteract the EE2-induced increase in plasma vitellogenin and reduction in 11-ketotestosterone whereas TBH counteracted the EE2 induced increase in *vtg* and *era* mRNA expression. Exposure to TBH and EE2-C + TBH lead to severe gonadal histology alterations. TBL and EE2-D + TBH exposed fish showed less histopathological alterations.

1. Introduction

The worldwide use of human and veterinary pharmaceuticals and their occurrence in aquatic ecosystems have become a great concern due to their potential as endocrine disrupters (EDCs) (Kolpin et al., 2002; Snyder et al., 2003). Traces of pharmaceuticals have been detected in effluents, surface waters, municipal and hospital wastewaters, groundwater, and sewage treatment plants (Fent et al., 2006; Christen et al., 2010).

The most common pharmaceuticals found in the environment are lipid-regulators, anti-inflammatories, natural and synthetic steroids. 17α-ethinylestradiol (EE2), a synthetic contraceptive, is one of the most frequently detected at ng L^{-1} concentration (reviewed by Langston et al., 2005). EE2 has a strong estrogenic effect in fish even at concentrations lower than 5 ng/L (Fenske et al., 2005; Bjerregaard et al., 2008; Körner et al., 2008; Hashimoto et al., 2009).

Anabolic implants, in a single or combined form, have been used in beef production for more than 50 years due to their ability to increase the body protein content, growth efficiency and carcass weight (Montgomery et al., 2001). Estrogens (*e.g.* estradiol), progestins (*e.g.* progesterone) and androgens (*e.g.* trenbolone acetate – TbA) are the main ingredients of those implants.

The environmental relevance of synthetic androgens like TbA is a consequence of its massive use as growth promoter in farm animals in countries like the United States and Canada (doses higher than 100 mg) and also to its illegal use for bodybuilding practices in humans (WADA, 2011). TbA is hydrolysed into 17α- and 17β-trenbolone (TB) which metabolites are stable in animal wastes with potent androgenic effect in fish and mammals (Schiffer et al., 2001; Galbraith, 2002; Durhan et al., 2005). The interest in determining the mechanism of action of TB in fish and its health implications has increased during the last years (Ankley et al., 2003; Orlando et al., 2004; Larsen and Baatrup, 2010; Morthorst et al., 2010; Velasco-Santamaría et al., 2010a).

Mixture exposure experiments with the specific purpose to identify the interaction of multiple compounds and potential antagonistic or agonistic effects are of acknowledged relevance. Nevertheless, few studies have been done to evaluate the interaction of natural or synthetic oestrogens with androgenic compounds which is a common situation in the environment (Korsgaard, 2006; Ankley et al., 2010; Velasco-Santamaría et al., 2010a,b). In male fathead minnow (*Pimephales promelas*), lack of modulation in vitellogenin levels was observed when fish were exposed to EE2 in

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combination with 400 ng L^{-1} TB (Ankley et al., 2010). In contrast, in male eelpout (*Zoarces viviparus*) high concentrations of methyltestosterone restored the GSI after 17β-estradiol exposure for 10 days (Korsgaard, 2006).

The presence of intersex and reduced gonadosomatic index are the most reported fish gonadal alterations over the last years due to the presence of endocrine disruptors (Scholz and Klüver, 2009). In some species, however, the presence of intersexuality is well known, a reason why it has been proposed that some degree of intersex in gonochoristic fish species could be a natural phenomenon (Gercken and Sordyl, 2002).

Eelpout (*Zoarces viviparus* L.) is a viviparous fish species with unrestricted spermatogonial testis which has been proposed as a bioindicator of contamination of aquatic environments of Northern Europe regions showing a good response to estrogenic, androgenic and antiestrogenic compounds (Christiansen et al., 1998; Andreassen and Korsgaard, 2000; Rasmussen et al., 2005; Korsgaard, 2006; Velasco-Santamaría et al., 2010a) and also in studies of embryonic and larval abnormalities (Vetemaa et al., 1997; Larsson et al., 2000; Strand et al., 2004), among others.

A recent study of eelpout *Zoarces viviparus* starting spermatogenesis showed that TB up to 16 ng L^{-1} was unable to counteract the EE2 estrogenic effects (Velasco-Santamaría et al., 2010a,b); however, we proposed that higher but still environmental relevant TB concentrations may counteract the EE2 effects and also that TB in such concentrations could disrupt the spermatogenesis. Therefore, the aim of this study was to investigate if TB at low (50 ng L^{-1}) or high (500 ng L^{-1}) nominal concentrations is able to offset the EE2 effects using plasma vitellogenin (VTG), steroids hormones, gene expression and gonadal histology as a biomarkers.

2. Materials and methods

2.1. Fish

Sexually mature male eelpout (*Zoarces viviparus* L.) with 80 ± 2.6 g body mass and length 26 ± 0.3 cm were caught in the sea south of Funen, Denmark and kept in polyethylene tanks under laboratory conditions in 8 indoor tanks (125 L), supplied with aerated fresh-flow through seawater taken from the Great Belt of Kerteminde, Denmark and cleared in a sedimentation tank before entering to the experimental aquaria. The tanks were prepared by running seawater, few weeks before the experiments started. The fish were acclimatized under the above conditions during 8 days at 12 h:12 h light:dark photoperiod.

Food was not offered during the acclimatization and the experimental phase to avoid water contamination and possible interaction with the substances used. Mortality among the fish was recorded daily and dead fish removed from the tanks. The experiment was carried out at the Marine Biological Research Centre (Kerteminde), Denmark during May to June 2010.

2.2. Chemicals

17α-ethinylestradiol (EE2) was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) and diluted in 2-propanol (Sigma–Aldrich) to prepare a 1 μg μl^{-1} stock solution. 17β-trenbolone (4, 9, 11-estratrien-17β-ol-3-one, TB) was provided by Steraloids Inc. (Newport, Rhode Island, USA). The 11-ketotestosterone (11-KT) kit was purchased from Cayman Chemical (Ann Arbour, MI, USA).

2.3. Exposure

A continuous flow-through seawater system was used consisting of 8 tanks individually connected to a stock solution pump adapted to a multi-channel water pump in order to keep the nominal concentrations in each tank constant. The water flow rate was 200 L per 24 h and the stock solution flow rate was 72 mL per 24 h (3 mL h^{-1}). The water was aerated and submerged Secchi pumps secured maximum mixing with an average water temperature of 11 °C; the salinity of the Great Belt water may vary between 10 and 30‰ depending on prevailing wind and currents, with an average in the spring season around 15‰.

Sexually mature males were exposed in the following groups: Control (exposed to the solvent 2-propanol, $n = 5$); 5 ng L^{-1} EE2 (EE2, $n = 6$); 50 ng L^{-1} TB (TBL, $n = 7$); 500 ng L^{-1} TB (TBH, $n = 6$). In addition the following combinations were used to investigate possible reversible effects of EE2 during the TB-exposure: 5 ng L^{-1} EE2 + 50 ng L^{-1} TB (EE2-C + TBL, $n = 7$); 5 ng L^{-1} EE2 + 500 ng L^{-1} TB (EE2-C + TBH, $n = 9$). All of these groups were exposed continuously to the EE2 and/or TB treatment during all 19 days of experiment. To further elucidate if the effect of EE2 could be abolished, weakened or reversed by TB, 2 additional groups were included: 5 ng L^{-1} EE2 + 50 ng L^{-1} TB (EE2-D + TBL, $n = 7$) and 5 ng L^{-1} EE2 + 500 ng L^{-1} TB (EE2-D + TBH, $n = 5$). The fish in these 2 latter groups were exposed continuously to the EE2 and TB for the first 11 days after which the EE2 exposure was discontinued and the fish only exposed to the two different concentrations of TB for the remaining 8 days of the experiment.

2.4. LC-MS/MS quantification

For each treatment, water samples were collected once per week in a 500 mL plastic bottle and keep at −20 °C degrees until further quantification using HPLC-MS-MS (1200 Series HPLC and a 6410 Triple Quad LC/MS, Agilent Technologies, Delaware, USA). Several studies have been done previously in the laboratory proving that neither EE2 nor TB are present in plastic bottles supporting this by the fact that EE2 and trenbolone were not detected in the blanks. Similarly, a recovery close to 100% have been observed in positive controls when using plastic and glass bottles meaning that these recipients material are appropriate to store and collect water for further measurements.

The measurement of the actual EE2 and TB concentrations were performed using solid phase extraction (SPE) with a column Strata-X 8B-S100-ECH Polymeric RP sorbent (100 mg/6 ml, Phenomenex, Torrance, CA, USA). The re-solution was done with 50% MeOH and 0.2% HCOOH. After SPE, the eluent was injected in the HPLC-MS-MS following the method described by Velasco-Santamaría et al. (2010b) with slight modifications. The modifications included: isocratic step with 0.1% Formic acid and acetonitril in a proportion 60:40 and stoptime 4 min. The other LC-MS-MS variables were kept the same as the previous study.

Methyltestosterone (MT) was used as an internal standard using a precursor ion 303.3, quantifier ion 97.2, dwell 200, fragmentor 120 and collision energy 40. The retention time for EE2, TB and MT was 2.328, 1.524 and 3.160, respectively. The standards were prepared using 17β-trenbolone (E3170-000 Steraloids) and 17α-ethinylestradiol (Sigma–Aldrich cat. no. E4876) and diluted in 50% MeOH and 0.2% HCOOH. Detection limits were approximately 1 ng TB L^{-1} and 2 ng EE2 L^{-1} which included the sample preparation step in SPE.

2.5. Sampling

After 19 days of exposure, the males were anaesthetised in 2‰ 2-phenoxyethanol (Sigma–Aldrich) and body mass (g) and total length (cm) were measured. Blood samples were collected from the caudal vessels using a heparinized syringe, stored in heparinized Eppendorf tubes for further centrifugation at 20,000 *g* for 5 min to obtain the plasma. Plasma was stored at −80 °C for later determination of vitellogenin (VTG) and 11-ketotestosterone concentrations. Subsequently, the fish were sacrificed by decapitation and the liver and testis were excised and weighed to determine the hepatosomatic (HSI) and gonadosomatic indexes (GSI) calculated as: (tissue weight/body weight) × 100. The condition factor (*Kn*) was calculated as: (body weight × 100)/length^3. Liver and one part of the testis were kept in liquid N_2 and stored at −80 °C for further quantification of gene expression by RT-qPCR. The other part of the testes was fixed in Bouin's solution for subsequent histology.

2.6. ELISA

2.6.1. Plasma vitellogenin (VTG) concentration

A direct non-competitive sandwich ELISA using a homologous antibody was used to determine plasma VTG concentration as reported by Korsgaard and Pedersen (1998) with modifications as described by Velasco-Santamaría et al. (2010b).

2.6.2. Plasma 11-ketotestosterone (11-KT) concentration

The plasma 11-KT quantification was performed according to the methodology described by Velasco-Santamaría et al. (2011). Briefly, four times volume ethyl acetate/hexane (1:1) was added to pure plasma samples and vigorously mixed. The layers were allowed to separate and the organic phase was carefully collected and partially evaporated at 30 °C (TurboVap LV Caliper Life Sciences). This procedure was repeated twice to guarantee a complete steroid extraction. The extracted sample was dissolved in EIA buffer depending on the initial volume. A Cayman 11-KT EIA kit (Cayman Chemical, Ann Arbour, MI, USA) was used and the reading procedure was done according to the manufactured instructions. The 11-KT recovery was 106 ± 9%, the intra assay variation was 5.3% and the inter assay variation 8.1%.

2.7. Gene expression

2.7.1. RNA purification and reverse transcription

RNA isolation and reverse transcription were carried out as described in Velasco-Santamaría et al. (2010b). Total RNA isolation was done from eelpout testis sections using 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers protocol. The RNA concentration and purity were assessed using a Nano Drop spectrophotometer at 230, 260 and 280 nm. A DNAse treatment was

done prior to the reverse transcription according to the manufacturers protocol (Promega Corporation, Madison, USA). Reverse transcription from 1000 ng of total RNA was carried out using M-MLV reverse transcriptase (Finnzymes DyNAmo™, Espoo, Finland) following the manufacturers protocol. The cDNA samples were kept at −20 °C until the next step.

2.7.2. Real-time qPCR

Primers for α-estrogen receptor (*era*), *vtg* mRNA and *β-actin* species-specific were designed according to the sequences published in the National Center of Biotechnology Information (NCBI) and developed using the Primer3 program (Rozen and Skaletsky, 1999). The oligo sequence of forward and reverse primers, amplicon size and the GenBank accession number are summarized in Velasco-Santamaría et al. (2010b).

The real-time qPCR analysis was run in a Stratagene Mx3000p instrument (Stratagene, La Jolla, CA, USA) using SYBR Green detection. The PCR reactions were done with 1 μl cDNA (40 ng RNA), 250 nM for forward and reverse mRNA primer, 2× SYBR Green JumpStart (Sigma–Aldrich, St Louis, MO, USA). *β-actin* was used as a normalization gene previously verifying that no differences between treatments were observed. The PCR reaction was carried out under the following conditions: pre-amplification segment at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 1 min and final segment with 95 °C for 1 min. At the end of each reaction, a melting curve analysis was routinely carried out to verify the presence of the desired amplicon in each reaction and to check the absence of primer–dimer formation. Potential contamination was never detected in no-template controls (NTC).

Amplification efficiencies were determined in duplicate with a series dilution of pooled liver cDNA. The slopes and copy number efficiency obtained in each cycle were −3.435 and 1.92, −3.351 and 1.98, and −3.458 and 1.9 for *era*, *vtg* and *β-actin* mRNA, respectively. The relative copy numbers for each target gene were calculated using the equation $RC = 10^{(Ct/slope)}$, where Ct is the threshold cycle number and the slope is the data obtained from the efficiency curve. The mRNA expression was calculated as relative copies number of each target gene/β-actin relative copy numbers.

2.8. Gonadal histology

After fixation, dehydration and clearing in graded ethanol/Tissue Clear series, the testes were embedded in paraffin wax for posterior serial longitudinal and transversal sections of 5 μm thickness. Two sections from each testis were stained with Mayer haematoxylin and eosin Y (H&E) and examined using light microscopy. In each testis two different sections were analysed with an interval of 30 μm each.

The gonadal staging and histological alterations were done following the criteria reported by Rasmussen et al. (2005) and Velasco-Santamaría et al. (2010a): testes without evident histological alterations were considered as normal (0); those with apparently normal tubular structure but with mild interstitial fibrosis were classified as mildly affected (1), those with apparently normal tubular structure but with moderate interstitial fibrosis were classified as moderately affected (2), and testes severely affected included testis with evident alterations in the tubular structure and severe interstitial fibrosis (3). In addition, the presence of germinal cells was qualitatively scored based on the cell abundance as absent (0), low (1), medium (2) and high (3). The presence of intratubular germ cell syncytia was observed during the histological evaluation, therefore its presence was classified as absent (0), mild (less than 5 clusters), moderate (between 6 and 14 clusters) and severe (with more than 15 clusters) under 20× magnification. The length of the tubules was qualitatively determined as short, medium and long, the latter representing the normal and expected length.

2.9. Statistics

All values are expressed as mean ± S.E.M. Data normality distribution and homogeneity of variance were checked using Kolmogorov–Smirnov and Bartlett's tests, respectively. In addition, the data was analysed considering the plot residual distributions. Log10 or square root transformations were done if the criteria of normality and homogeneity of variance failed. Data were analysed by one-way analysis of variance (ANOVA) followed by a Tukey test ($p < 0.05$) to detect differences between treatments. Dunnett test was done to detect differences compared to the control group. In addition, a similarity test between the treatments, a Bray–Curtis analysis and MANOVA analysis were done to analyze the histopathological alterations using Primer6 (version 6.1.10 – 2007). The chi-square (χ^2) test was used to evaluate overall significant differences in the testis histopathological alterations between control and treatment groups. All statistical analysis was conducted using SAS system for Windows software version 9.2 (2002–2008 by SAS Institute, Cary, NC, USA).

3. Results

3.1. Water concentrations, body weight and somatic index

The actual concentrations of TB and EE2 are shown in Table 1. The EE2 concentration varied from 23 to 45% lower than the nominal concentrations in three of the five treatments with EE2. TB concentrations were higher (8–47%) than the nominal concentration. Due to the use of the stock solution pump and the continuous flow-through seawater system, the EE2 and TB concentrations were kept constant through all experiment since the three different samplings showed similar values.

Table 1
Water concentration of 17β-trenbolone (TB) and 17α-ethinylestradiol (EE2) in tanks with *Zoarces viviparus* males exposed for 19 days ($n = 3$). The EE2-C corresponds to continuous exposure to EE2 and TB during 19 days. EE2-D corresponds to continuous exposure to EE2 and TB for the first 11 days after which the fish were only exposed to TB for the remaining 8 days.

Treatment	Nominal concentration (ng L^{-1})		EE2 (ng L^{-1})	17β-Trenbolone (ng L^{-1})
	EE2	TB		
Solvent control	0	0	<2	<1
EE2	5	0	3.6 ± 0.5	<1
TBL	0	50	<2	59.3 ± 7.3
TBH	0	500	<2	684 ± 148
EE2-C + TBL	5	50	2.7 ± 0.2	61 ± 6.8
EE2-D + TBL	5	50	4.9 ± 3.0	53.8 ± 5.6
EE2-C + TBH	5	500	3.8 ± 0.7	733 ± 57
EE2-D + TBH	5	500	6.1 ± 4.9	649 ± 108

The values with the sign < indicates a concentration lower than the detection limit.

Body mass, total length and somatic indices are shown in Table 2. The overall body mass and total length were 79.7 ± 2.6 g and 26.2 ± 0.3 cm, respectively, with no significant effect in any treatment (Table 2, $p > 0.05$). The overall condition factor (*Kn*) was 0.44 ± 0.005 without any significant difference between treatments ($p > 0.05$). Likewise, no significant effect in the HSI was observed during the exposure in any treatment (Table 2, $p > 0.05$). However, a severe reduction in the GSI when compared to controls was observed in males exposed to EE2 and those exposed continuously or discontinuously to EE2 in combination with TBL (Table 2, $p < 0.0001$).

3.2. Plasma vitellogenin concentration

Plasma vitellogenin concentrations in males exposed to TBL or TBH concentration were similar to control fish (0.035 ± 0.009 μg mL^{-1}, $p > 0.05$) (Fig. 1). All fish exposed to EE2 alone or in combination with TB had a significant increase (more than 1019-fold) in plasma VTG levels (>35 μg mL^{-1}) compared to control fish or those exposed only to trenbolone ($p < 0.05$, Fig. 1). The induction of plasma VTG after EE2 exposure was not modulated by the TB co-exposure in any treatment.

Table 2
Final body mass, total length and somatic indices of male eelpout (*Zoarces viviparus*) exposed continuously (EE2-C) or discontinuously (EE2-D) to 5 ng L^{-1} EE2 and to low (TBL) or high (TBH) TB concentration, separately or in combination. Values are expressed as mean ± SEM. Values in parentheses show the number of fish.

Treatment (ng L^{-1})	Body mass (g)	Total length (cm)	HSI (%)	GSI (%)
Solvent control (5)	79.8 ± 2.3[a]	26.6 ± 0.1[a]	1.51 ± 0.19[a]	3.02 ± 0.18[a]
EE2 (6)	82.5 ± 7.1[a]	26.4 ± 0.5[a]	1.47 ± 0.17[a]	0.44 ± 0.06[b] *
TBL (7)	91.4 ± 10.2[a]	27.4 ± 1.2[a]	1.56 ± 0.15[a]	2.99 ± 0.33[a]
TBH (6)	71.9 ± 5.6[a]	25.7 ± 0.5[a]	1.45 ± 0.18[a]	2.47 ± 0.13[a]
EE2-C + TBL (6)	74.3 ± 2.7[a]	25.7 ± 0.3[a]	1.39 ± 0.16[a]	0.90 ± 0.24[b] *
EE2-D + TBL (7)	87.5 ± 8.9[a]	26.9 ± 0.9[a]	1.56 ± 0.17[a]	0.76 ± 0.17[b] *
EE2-C + TBH (9)	73.1 ± 5.8[a]	25.2 ± 0.6[a]	1.60 ± 0.15[a]	2.26 ± 0.13[a]
EE2-D + TBH (5)	73.1 ± 6.8[a]	25.7 ± 0.8[a]	1.43 ± 0.18[a]	2.09 ± 0.32[a]

[a,b] Between treatments different letters show significant differences (Tukey test, $p < 0.05$).
* Indicated significant difference compared to the control group (Dunnett test, $p < 0.05$).

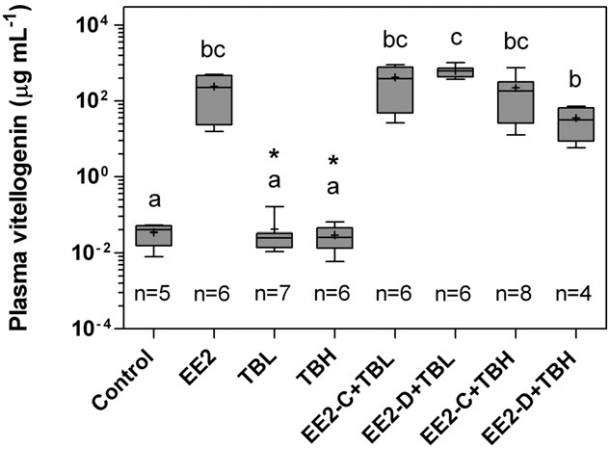

Fig. 1. Plasma vitellogenin concentration (μg mL^{-1}) of male eelpout (*Zoarces viviparus*) exposed continuously (EE2-C) or discontinuously (EE2-D) to 5 ng L^{-1} EE2 and to low (TBL) or high (TBH) TB concentration, separately or in combination. Values are expressed as mean (+), median (central line), and 25th and 75th percentiles values. The whiskers show the 90th and 10th percentiles. The number of fish per treatment is shown in parenthesis. Bars with different letters indicate significant differences (Log transformed data, ANOVA, $p < 0.05$). * Bars with asterisk indicate difference compared to the control group (Dunnett's test, $p < 0.05$).

3.3. 11-KT concentration

The 11-KT concentration in fish exposed continuously to EE2 in combination with TBL or TBH were 87% and 93% lower than controls, respectively, (Fig. 2, $p < 0.05$); however, no significant differences between control and EE2 groups were observed as one control fish had low concentration (161.5 pg mL^{-1}) compared to the rest (1823 ± 431 pg mL^{-1}). A significant reduction in 11-KT was observed in all fish exposed continuously to EE2 alone or in combination with TB ($p < 0.001$) when compared to fish exposed to the respective TB concentrations. Fish exposed to TBL or TBH concentration had similar 11-KT levels as control fish ($p > 0.05$). 11-KT concentrations were higher after discontinuous than after continuous EE2 exposure in both TBL and TBH groups.

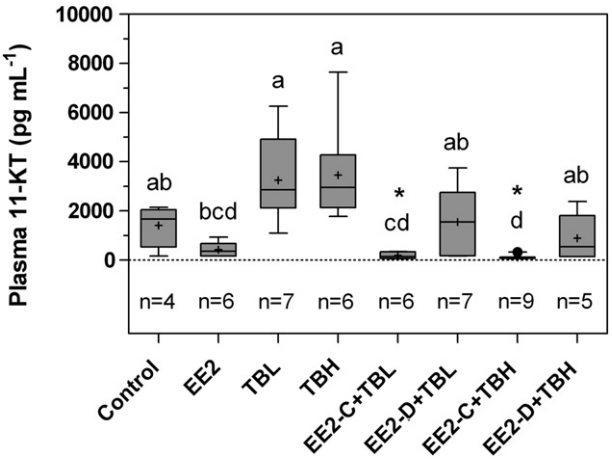

Fig. 2. Plasma 11-KT concentration (pg mL^{-1}) of male eelpout (*Zoarces viviparus*) exposed continuously (EE2-C) or discontinuously (EE2-D) to 5 ng L^{-1} EE2 and to low (TBL) or high (TBH) TB concentration, separately or in combination. Values are expressed as mean (+), median (central line), and 25th and 75th percentiles values. The whiskers show the 90th and 10th percentiles. The number of fish per treatment is shown in parenthesis. Bars with different letters indicate significant differences (Log transformed data, ANOVA, $p < 0.05$). * Bars with asterisk indicate difference compared to the control group (Dunnett's test, $p < 0.05$). Black dots show outlier.

3.4. Hepatic gene expression

A significant up-regulation in the hepatic *vtg* mRNA (>50 arbitrary units) was observed in males exposed to 5 ng L^{-1} EE2 or exposed continuously to EE2 in combination with TBL when compared to controls (0.2 arbitrary units, $p < 0.05$, Fig. 3); however, males exposed discontinuously to EE2 in combination with TBL had similar *vtg* mRNA levels as control males ($p > 0.05$). Interestingly, those males exposed continuously or discontinuously to EE2 in combination with TBH concentration did not show any significant differences in *vtg* mRNA expression when compared to control group or the 2 trenbolone groups ($p > 0.05$). Fish exposed to both concentrations of TB had similar *vtg* mRNA expression as the control group ($p > 0.05$).

Similar to the liver *vtg* mRNA expression, only males exposed to 5 ng L^{-1} EE2 or those continuously exposed to EE2 in combination with TBL concentration showed an up-regulation in the hepatic *era* mRNA (>31 arbitrary units) when compared to controls (0.9 arbitrary units) and to those exposed to TB (0.31 − 1.0 arbitrary units) (Fig. 4, $p < 0.05$). Males exposed discontinuously to EE2 in combination with both TB concentrations had statistical similar *era* mRNA expression as control males ($p > 0.05$). Males exposed to either TB concentration showed similar *era* mRNA expression as control group ($p > 0.05$).

3.5. Gonadal histology

A random presence of primary oocytes was observed in one fish exposed to TBL, two fish exposed to EE2-C + TBL and two fish of EE2-D + TBL group.

Immature testicular stages were observed in more than 75% of the males exposed to EE2 alone, EE2-C + TBL or EE2-D + TBL and also in males exposed to TBH when compared to control males (Table 3, Fig. 5, χ^2 test, Likelihood ratio > 16, $p < 0.001$). Absence or low proportion of spermatogonia and spermatocytes were observed only in two groups: TBH alone or in combination with continuous EE2 (Table 3, χ^2 test, Likelihood ratio > 14, $p < 0.01$). Interestingly, only fish exposed discontinuously to EE2 in combination with TBH had medium and high proportion of spermatids

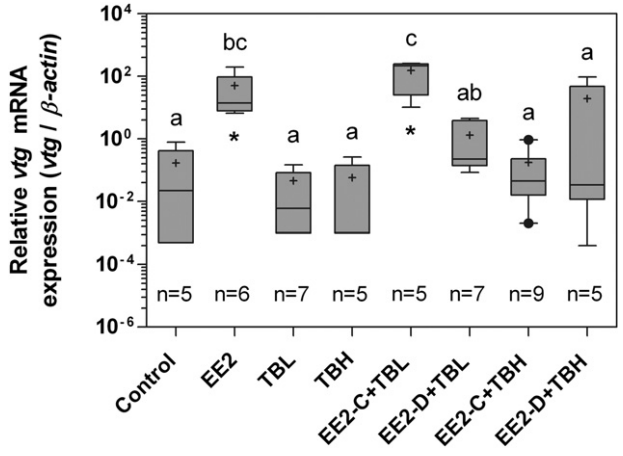

Fig. 3. Box plots of transcript relative liver *vtg* abundances (normalised to β-actin transcript abundance in the same samples) in male eelpout (*Zoarces viviparus*) exposed continuously (EE2-C) or discontinuously (EE2-D) to 5 ng L^{-1} EE2 and to low (TBL) or high (TBH) TB concentration, separately or in combination. Values are expressed as mean (+), median (central line), and 25th and 75th percentiles values. The whiskers show the 90th and 10th percentiles. The number of fish per treatment is shown in parenthesis. Bars with different letters indicate significant differences (Log transformed data, ANOVA, $p < 0.05$). * Bars with asterisk indicate difference compared to the control group (Dunnett's test, $p < 0.05$). Black dots show outlier.

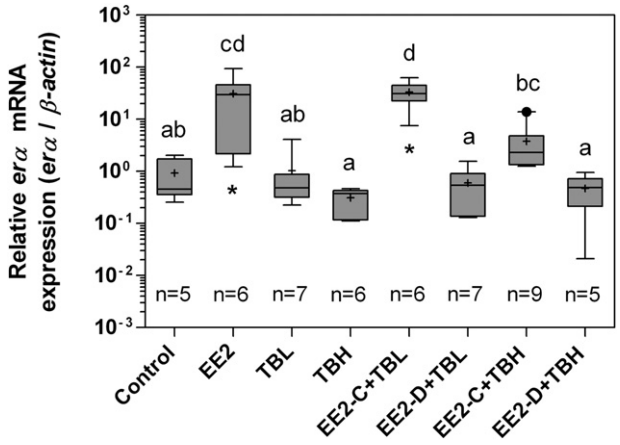

Fig. 4. Box plots of transcript relative liver *erα* abundances (normalised to *β-actin* transcript abundance in the same samples) in male eelpout (*Zoarces viviparus*) exposed continuously (EE2-C) or discontinuously (EE2-D) to 5 ng L^{-1} EE2 and to low (TBL) or high (TBH) TB concentration, separately or in combination. Values are expressed as mean (+), median (central line), and 25th and 75th percentiles values. The whiskers show the 90th and 10th percentiles. Bars with different letters indicate significant differences (Log transformed data, ANOVA, $p < 0.05$). * Bars with asterisk indicate difference compared to the control group (Dunnett's test, $p < 0.05$). Black dots show outlier.

being similar to control (Table 3, χ^2 test, Likelihood ratio > 0.9, $p > 0.05$). Absence or low proportion of spermatozoa (more than 92%) was observed in males exposed to EE2 alone or EE2 exposed continuously in combination with TBL (χ^2 test, Likelihood ratio > 11, $p < 0.05$). In contrast, males exposed to TBH had a high proportion of spermatozoa compared to control (Table 3, χ^2 test, Likelihood ratio $= 16$, $p < 0.01$).

All treatments had a significantly shorter or medium tubule length compared to controls (Table 4, χ^2 test, Likelihood ratio > 10, $p < 0.05$). Severe interstitial fibrosis was observed in all treatments except in those males exposed to TBL or exposed to EE2 in combination with TBH (Table 4, Fig. 5, χ^2 test, Likelihood ratio $= 13–30$, $p < 0.01$). Finally, moderate and severe germ cell syncytia were observed in all treatments except the control group (Table 4, Fig. 5, χ^2 test, Likelihood ratio $= 12–36$, $p < 0.05$). In all treatments with TBH, the interstitial fibrosis was accompanied with severe degeneration of germ cells (Fig. 5).

Considered all variables analysed in the testicular histology, the Bray–Curtis similarity test and MANOVA test showed more than 77% of similarity within groups, the control group being the most homogeneous (91%). Interestingly, only two treatments showed higher similarity with the control: the TBL and EE2-D + TBH with 85 and 78% similarity, respectively. The most dissimilar treatments as compared with the control group were EE2 and TBH (58 and 54%, respectively). The treatments more similar to EE2 were EE2-C + TBL (84%), EE2-D + TBL (78%) and TBH (78%) and the group showing higher similarity to TBH was EE2-C + TBH (81%) (Fig. 6).

4. Discussion

The results of GSI, gene expression, gonadal histology and 11-KT levels suggest that exposures to relatively high concentrations of TB can only modify some of the EE2 induced effects. In addition, this study demonstrated that high TB concentration exerts a deleterious effect on the gonad structure of male eelpout even if gene expression and steroid hormone levels were not affected. We also observed that a low percentage of intersex in some males did not relate to the treatments.

One of the strongest biomarkers in fish to detect estrogenic exposure is vitellogenin, a yolk precursor protein synthesized in the liver during the process of vitellogenesis. In the present study, the protein induction in all males exposed to EE2 was more than 1000 times higher than control males confirming the strong ability of EE2 to bind the hepatic *er* and induce VTG production (Fig. 1). The fact that no decrease in VTG levels was observed in males exposed discontinuously to EE2 suggests that the VTG clearance in eelpout is longer than the 8 days subsequent to the discontinuation of the EE2 exposure. In juvenile zebrafish (*Danio rerio*) exposed for 28 days to 15 ng L^{-1} EE2 it was observed that even 46 days post hatching the levels of VTG were significantly higher than in control fish, with a calculated half-life of 2.4 days (Andersen et al., 2003). Similarly, the VTG levels were close to the basal levels in approximately 48 days after EE2 injection in rainbow trout (*O. mykiss*), with a half-life varying from 1.8 to 6 days (Schultz et al., 2001). In fathead minnow (*P. promelas*) the VTG half-life varied from 2.2 to 21.3 days depending on the compartment model phases (Schmid et al., 2002). Although we expected to observe decreased levels of plasma VTG in males exposed to TB after discontinuous EE2 exposure, the above results suggest that in those males the plasma

Table 3
Testicular stage and germ cells proportion from male eelpout (*Zoarces viviparus*) exposed continuously (EE2-C) or discontinuously (EE2-D) to 5 ng L^{-1} EE2 and to low (TBL) or high (TBH) TB concentration, separately or in combination. Values are expressed as percentages.

Germinal cells	Score	Control	EE2	TBL	TBH	EE2-C + TBL	EE2-D + TBL	EE2-C + TBH	EE2-D + TBH
Stage	1 (immature)	0	100 ***	0	83.3 ***	83.3 ***	75 ***	22.2	20
	2 (mature)	100	0	100	16.7	16.7	25	77.8	80
Gonia	1 (low)	10	25	7.1	91.7 ***	0	0	88.9 ***	20
	2 (medium)	90	75	92.9	8.3	83.3	66.7	11.1	80
	3 (high)	0	0	0	0	16.7	33.3	0	0
Spermatocyte	0 (absent)	0	0	0	0	0	0	5.6	0
	1 (low)	0	0	0	75 ***	0	0	16.7 **	0
	2 (medium)	10	50	14.3	25	25	0	55.6	20
	3 (high)	90	50	85.7	0	75	100	22.2	80
Spermatid	0 (absent)	0	75	0	0	83.3	66.7	5.6	0
	1 (low)	10	25	14.3	83.3	16.7	16.7	44.4	20
	2 (medium)	40	0	85.7	16.7	0	16.7	44.4	50
	3 (high)	50	0 ***	0 **	0 **	0 ***	0 **	5.6 *	30
Spermatozoa	0 (absent)	0	50 *	0	0	25 *	8.3	11.1	0
	1 (low)	50	41.7	57.1	0	75	66.7	22.2	20
	2 (medium)	50	8.3	42.9	41.7	0	25	50	40
	3 (high)	0	0	0	58.3 **	0	0	16.7	40

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

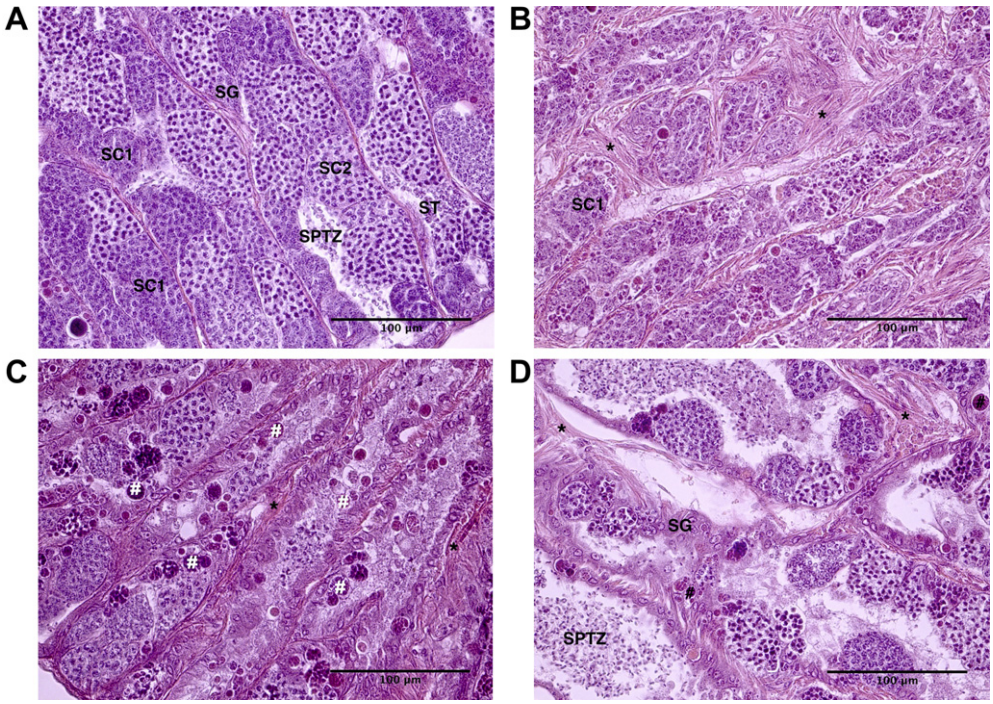

Fig. 5. Testis morphology of male eelpout (*Zoarces viviparus*) after 19 days exposure to A, control; B, continuously exposed to 5 ng L^{-1} EE2 (EE2-C); C, high TB concentration (TBH); D, exposed continuously to 5 ng L^{-1} EE2 in combination to TBH (EE2-C + TBH); E, exposed discontinuously to 5 ng L^{-1} EE2 in combination to TBH (EE2-D + TBH). A, Testis of control fish showed normal histology in a mature stage with cysts in spermatogenesis. SG: Spermatogonia, SC1: primary spermatocytes, SC2: secondary spermatocytes, ST: spermatids, SPTZ: spermatozoa. B, Testis with severe interstitial fibrosis (*), some degree of germ cell syncytia, cellular necrosis (arrows) and abnormal germ cells structure. Note the presence of brownish hue (circles) compatible with phagocytic material or vitellogenin aggregation. C, testis from fish exposed to high 17β-trenbolone concentration showed moderate interstitial fibrosis (*), severe germ cell syncytia (#), degeneration of germ cells and cellular necrosis. D, testis with severe interstitial fibrosis (*), cellular necrosis (arrows), degeneration of germ cells (#) and increase in the proportion of spermatogonia (SG). Note the presence of brownish hue (circles) compatible with phagocytic material or vitellogenin aggregation. E, Moderate interstitial fibrosis (*) and germ cell syncytia (#), SPTZ: spermatozoa. Note that tubules tubules have apparent normal stages of germ cells. Sections were 5 µm thickness and stained with H&E. Bar 100 µm.

circulating VTG is a consequence of the initial EE2 exposure for 11 days and not for the remaining 8 days of EE2 depuration.

The relatively long half-life of VTG makes this protein a useful marker to detect previous estrogenic exposure even after several weeks (Bjerregaard et al., 2008); however, it is difficult to determine if a compound like trenbolone is able to modulate the estrogenic effects using only plasma VTG as biomarker due to the long VTG half-life which can mask the response. Therefore, the quantification of the hepatic *era* and *vtg* mRNA expression and also protein expression could help to elucidate this possible effect owing to the rapid induction of the *vtg* mRNA expression in response to EE2 exposure (Schmid et al., 2002).

Several studies have shown that the *er*-gene is more sensitive to the estrogenic hormones than the *vtg*-gene, making the *er*-gene the early candidate in a time-course study (Flouriot et al., 1996). To identify an estrogenic response via the *er* mediated pathway, the potential ligand must have an affinity for the estrogen receptor. Binding, however, to the *er* is not sufficient to positively identifying

Table 4
Tubule length and testicular alterations from male eelpout (*Zoarces viviparus*) exposed continuously (EE2-C) or discontinuously (EE2-D) to 5 ng L^{-1} EE2 and to low (TBL) or high (TBH) TB concentration, separately or in combination. Values are expressed as percentages.

Lesion	Score	Control	EE2	TBL	TBH	EE2-C + TBL	EE2-D + TBL	EE2-C + TBH	EE2-D + TBH
Tubule Length	1 (short)	0	83.3 ***	14.3 *	66.7 ***	41.7 *	41.7 *	33.3 ***	20 **
	2 (medium)	10	16.7	50	33.3	41.7	25	66.7	70
	3 (long)	90	0	35.7	0	16.7	33.3	0	10
Affected	0 (absent)	100	0	50	0	8.3	33.3	0	10
	1 (minimal)	0	0	35.7	0	8.3	8.3	38.9	60
	2 (moderate)	0	41.7	14.3	33.3	66.7	8.3	38.9	10
	3 (severe)	0	58.3 ***	0 *	66.7 ***	16.7 ***	50 *	22.2 ***	20 **
Fibrosis	0 (absent)	40	0	7.1	0	8.3	8.3	0	10
	1 (minimal)	60	0	57.1	0	8.3	25	5.6	40
	2 (moderate)	0	41.7	35.7	41.7	41.7	25	72.2	30
	3 (severe)	0	58.3 ***	0 *	58.3 ***	41.7 **	41.7 **	22.2 ***	20
Germ cell syncytia	0 (absent)	40	0	0	0	0	0	0	0
	1 (minimal)	60	33.3	42.9	0	16.7	58.3	0	0
	2 (moderate)	0	0	21.4	16.7	41.7	25	27.8	40
	3 (severe)	0	66.7 **	35.7 **	83.3 ***	41.7 ***	16.7 *	72.2 ***	60 ***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

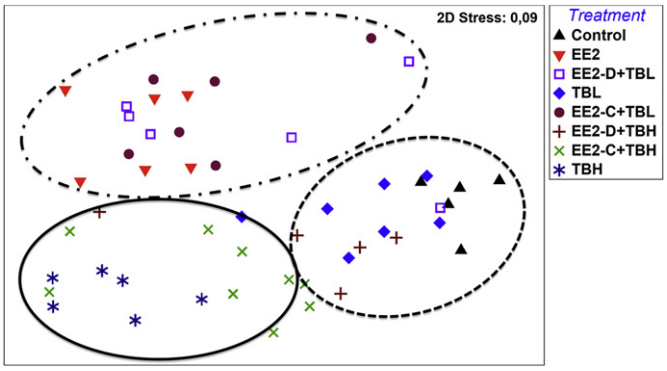

Fig. 6. Bray–Curtis similarity plot (MDS – Multi-Dimensional Scaling) based on the gonadal histology analysis from males exposed continuously (EE2-C) or discontinuously (EE2-D) to 5 ng L^{-1} EE2 and to low (TBL) or high (TBH) TB concentration, separately or in combination. Each ellipse shows those treatments with some degree of similarity considering all histological variables.

estrogenic compounds, as estrogenic antagonists may also have very high affinity to the *er* without activating the *er* and the transcriptional process.

Andreassen and Korsgaard (2000) observed a correlation between cytosolic liver *er* and VTG in plasma of estradiol-treated eelpout showing that vitellogenin was found in the plasma when the concentration of the binding sites exceeded a certain threshold. This indicates that when high cytosolic *er* concentrations are present, the induction of VTG follows in the presence of estradiol. The observations also indicated that priming of the liver makes the liver more susceptible to repeated or continuous xenoestrogenic exposure by the so-called memory effect phenomenon.

Not many studies have been done in fish trying to evaluate the interaction of TB and EE2. In male eelpout exposed simultaneously to EE2 and TB, no clear modulating effect could be observed, presumably due to the low TB concentrations used (lower than 16 ng L^{-1}) (Velasco-Santamaría et al., 2010b). However, the authors did not exclude at that time that higher TB concentrations could be able to counteract the EE2 effects. The fact that in the present study an up-regulation in the *vtg* and *era* mRNA levels was observed only in males exposed to EE2 alone or exposed continuously to EE2 in combination with TBL, could suggest that TB at higher doses is able to modulate the EE2 effects. However, this suggestion must be confirmed with future studies exposing fish only to EE2 discontinuously with the expecting results of up-regulation in *vtg* and *era* mRNA levels and alterations in protein levels.

In male fathead minnow no modulation of plasma VTG levels was observed after TB co-exposure even at a concentration higher than 400 ng L^{-1} (Ankley et al., 2010). In contrast, a modulating effect of TB with concentrations higher than 50 ng L^{-1} has been observed in female fathead minnow with a drastic decrease in plasma VTG (Ankley et al., 2003). It is well known that VTG synthesis is regulated by endogenous estrogens or xenoestrogens having a strong affinity to the *er* to subsequently induce the protein synthesis. Therefore, chemicals disrupting the steroidogenesis may result in decreased levels of *vtg* mRNA or vitellogenin measured by ELISA.

Within the steroids, T and 11-KT are the most important androgens in fish and its quantification helps to identify the general status of the gonadal steroidogenesis (Schulz et al., 2010). In our study, the significant reduction in the plasma 11-KT in males exposed continuously to EE2 in combination with high TB concentration could to some extent exert a negative feedback on the hypothalamus–pituitary–gonad axis and thus inhibit the steroid synthesis which could lead to down-regulation in the *era* and subsequently in the *vtg* mRNA levels as observed in the present study (cf. Figs. 3 and 4). Even if lower 11-KT concentration was observed also in males exposed continuously to EE2 in combination with low TB, it is still possible that EE2 exerts a stronger stimulation than 11-KT on the endocrine axis. This could explain why the low TB concentration was unable to counteract EE2 as also indicated by the up-regulated levels of *vtg* and *era* mRNA.

In male eelpout the estrogen receptor has been identified not only in the liver but also in the pituitary and testicular tissues (Andreassen et al., 2003). Estrogen, therefore, should be expected to be active at very low levels in those organs in males. The existence of a role for estrogen in the testis function of fish may imply that estrogens not only may be available to the testis but also that high levels of estrogen or estrogenic compounds in the circulation would induce synthesis of liver vitellogenin and impair other physiological functions in the male fish as it has been observed by Christiansen et al. (1998) in the eelpout. Estrogenic compounds may act directly on the gonadotropic cells of the pituitary which have been shown to express the *er* in eelpout (Andreassen et al., 2003). The vast distribution of *er* in the reproductive regulatory axis of fish indicate that xenobiotic compounds such as EE2 and trenbolone may have the potential to disrupt several aspects of the normal reproductive function also in male fish. The hypothesis regarding the stronger effect on the gonadal development by EE2 alone or in continuous co-exposure with low TB is supported by the severe gonadal histological alterations not only associated with the low GSI, but also with those lesions observed and grouped in the Bray–Curtis plot (cf. Fig. 6). Velasco-Santamaría et al. (2010b) suggested that EE2 could mimic the negative feedback of the endogenous estradiol leading to alterations in the testicular histological structure including disruption in the spermatogenesis and subsequently reduced GSI.

Few studies in fish have evaluated the histological effect of trenbolone on the gonad structure. Recently, Morthorst et al. (2010) demonstrated that environmental TB concentration causes an irreversible masculinization effect in zebrafish (*Danio rerio*). Moderate interstitial fibrosis and decreased tubule diameter were observed in eelpout (*Zoarces viviparus*) exposed to 10.6 ng L^{-1} TB (Velasco-Santamaría et al., 2010a). The short tubule length, severe interstitial fibrosis and germ cell syncytia as observed in the present study reflected testicular degeneration in those males exposed to EE2 alone or co-exposed to TB and also in males exposed to high TB. However, males exposed discontinuously to EE2 with high TB showed less severity in the gonadal alterations. Interestingly, in the present study greater amounts of spermatozoa was observed in males exposed to 684 ng L^{-1} TB, which is in agreement with the findings observed in medaka and zebrafish exposed to 50 ng L^{-1} TB (Örn et al., 2006). These authors attributed the observed results to stimulation of the spermatogenesis, however, in our study low proportion of gonias, spermatocytes and spermatids were observed in fish exposed to the high TB concentration suggesting an arrest in the spermatogenesis. The presence of oocytes in the testis of eelpout suggests that the fish caught for the present experiments may have been exposed to endocrine disrupting compounds generating a mild degree of intersex. In a German study it was observed that eelpout caught from natural environments exhibited high intersex prevalence (up to 27%) together with severe histological alterations (Gercken and Sordyl, 2002).

In conclusion, high concentrations of TB cause severe effect on the spermatogenesis similar to those caused by environmental EE2 concentration. We also observed that high TB concentrations could be able to modulate the EE2 effects in male eelpout under discontinuous exposure.

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