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Effects of methyltestosterone, letrozole, triphenyltin and fenarimol on histology of reproductive organs of the copepod *Acartia tonsa*

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HIGHLIGHTS

- Compounds acting as androgen in vertebrates affected reproductive organs in a copepod.
- These effects were specific for the reproductive system.
- Gonads and accessory glands were altered.

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ABSTRACT

The marine calanoid copepod *Acartia tonsa* was exposed to methyltestosterone (MET, 1.6–126 μg L^{-1}), letrozole (LET, 10–1000 μg L^{-1}), triphenyltin chloride (TPT, 0.0014–0.0088 μg L^{-1} TPT-Sn) and fenarimol (FEN, 2.8–105 μg L^{-1}) for 21 d covering a full life-cycle. All four compounds investigated are known to act as androgens in vertebrates. The digestive tract, musculature, nervous system, reproductive organs, gonad and accessory sexual glands were examined by light microscopy after routine staining and immune-labelling for detection of apoptosis and determination of proliferation activities. MET induced an inhibition of oogenesis, oocyte maturation and yolk formation, respectively, which was most pronounced at the lowest concentrations tested. In LET exposed males, spermatogenesis was enhanced with very prominent gamete stages; in some stages apoptosis occurred. The spermatophore was hypertrophied and displayed deformations. In females, LET induced a disorder of oogenesis and disturbances in yolk synthesis. TPT stimulated the male reproductive system at 0.0014 and 0.0035 μg TPT-Sn L^{-1}, whereas inhibiting effects were observed in the female gonad at 0.0088 μg TPT-Sn L^{-1}. In FEN exposed females proliferation of gametes was reduced and yolk formation showed irregular features at 2.8–105 μg L^{-1}. In FEN exposed males an elevated proliferation activity was observed. No pathological alterations in other organ systems, e.g. the digestive tract including the hindgut acting as respiratory organ, the nervous system, or the musculature were seen. This indicates that the effects on gonads might be caused rather by disturbance of endocrine signalling or interference with hormone metabolism than by general toxicity.

1. Introduction

Sexual differentiation and gonad development in crustacea depends on the presence or absence of a protein produced by the androgenic gland, i.e. the androgenic gland hormone, which induces male morphogenesis, development of testis, and male sexual characteristics (Hasegawa et al., 1993). Vertebrate-type steroid hormones, e.g. 17β-estradiol, estrone, progesterone and testosterone have been identified in crustaceans, however, their specific role for sexual differentiation and gametogenesis is unclear, and *de novo* synthesis remains questionable (Fingerman et al., 1993; Fingerman, 1997; Lafont and Mathieu, 2007; LeBlanc, 2007). Several studies revealed a notable capability of crustaceans, e.g. the cladoceran *Daphnia magna*, the mysid *Neomysis integer* and the amphipod *Hyalella azteca*, to metabolize testosterone (Baldwin and LeBlanc, 1994a,b; LeBlanc et al., 1999; Verslycke et al., 2002, 2003; Janer et al., 2005). Exogenous testosterone stimulated

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spermatogenesis in the prawn *Parapaeopsis hardwickii* (Nagabushanam and Kulkarni, 1981), induced conversion of ovaries to testis in the crab *Ocypoda platytarsis* (Sarojini, 1963) and caused hypertrophy/hyperplasia in the testis of the penaeid shrimp *Parapenaeopsis hardwickii* (Nagabushanam and Kulkarni, 1981) whereas testosterone did not affect larval development of *A. tonsa* (Andersen et al., 2001). Testosterone has been shown to interact with the endocrine system of crustaceans although the mode of action remains unclear. Janer and Porte (2007) reviewed the presence and metabolism of sex steroids in several invertebrate species including crustaceans and suggested mechanisms of endocrine disruption other than the interaction with nuclear receptors such as the interference with steroid metabolism and with non-genomic signalling cascades. Nevertheless, all of these studies have not lead to any final conclusion on the hormonal function of vertebrate-type sex steroids in crustaceans. To date, only preliminary evidence on the presence of testosterone and estrogen receptors in the amphipod *Hyalella azteca* has been provided (Köhler et al., 2007).

The majority of studies on endocrine disruption in crustaceans has focussed on female reproductive endpoints or growth/moulting related endpoints, which are easy to observe in routine toxicity testing (Ford, 2008). Surprisingly little is known about the impact of endocrine active compounds on male crustaceans.

The calanoid copepod *A. tonsa* was selected as a the test organism for several reasons: it is an ecologically important species; a standardised acute test method exists (ISO, 1998); it has a short reproduction cycle; it is easy to culture and handle in the laboratory, it is sexually dimorphic, and the morphology and the histology have been described earlier for the genus (Heberer, 1932), for the male (Blades and Youngbluth, 1981; Blades-Eckelbarger and Youngbluth, 1982) and for the female (Blades-Eckelbarger and Youngbluth, 1984). Besides, several studies with *Acartia tonsa* have shown that moulting (Kusk and Petersen, 1997; Andersen et al., 2001; Wollenberger et al., 2005; Kusk et al., 2011) and time to sexual maturity (Andersen et al., 1999) are very sensitive to many chemicals including compounds known to act as endocrine disrupters in vertebrates. Consequently, an early life-stage test with *A. tonsa* was recently proposed as an ISO standard for the assessment of sub-chronic toxicity of chemicals (ISO, 2012). As a supplement to routine tests, histological investigations may provide important information about effects on the cellular level, effects on tissue, and information on comparative aspects of chemical mode of action. However, histological investigations cannot entirely replace mechanistic studies as they cannot confidently attribute certain responses as being endocrine disruption specific.

The aim of the present study was to investigate whether chemical substances with androgenic effects on vertebrates and molluscs also cause histological alterations in a crustacean species. Therefore, *A. tonsa* was exposed to the model androgens methyltestosterone (MET), letrozole (LET), triphenyltin (TPT) and fenarimol (FEN) for an entire life-cycle including periods of gonad development and reproduction. Histological investigations of reproductive organs, gonads, and accessory sexual glands were performed subsequently to routine staining and immunolabelling for detection of apoptosis and determination of proliferation activities. Also the digestive tract including the hindgut, which acts as respiratory organ, the nervous system and the musculature were investigated. The synthetic androgen receptor agonist MET is a potent synthetic androgen receptor agonist and is widely used for sex reversal of fish in aquaculture (Goetz et al., 1979; Körner et al., 2004). Therefore, most publications on hormonal effects of MET refer to fish (see Pawlowski et al., 2004) where administration of low doses exert androgenic and estrogenic effects. The aromatase inhibitor LET has been applied as a reference compound in endocrine disrupter studies with fish (Sun et al., 2009). The compound is primarily used as a pharmaceutical to treat estrogen dependent breast cancer and infertility caused by ovulation problems (Harper-Wynne et al., 2002; Thiantanawat et al., 2003).

The androgenic potency of tributyltin (TBT) and TPT in molluscs was causally linked to the inhibition of aromatase activity, i.e. to a blocking of the transformation of testosterone into estradiol, which in female snails leads to the development of a pseudopenis – a phenomenon known as imposex (Bettin et al., 1996; Schulte-Oehlmann et al., 2000).

No information on the effects of MET, FEN and LET on *A. tonsa* or other copepods has been published so far, and histological alterations in crustaceans caused by chemicals have been studied only to a very limited extent.

2. Materials and methods

2.1. Chemicals

MET (purity 99.4%) and FEN (purity 99.9%) were obtained as analytical standards from Riedel-de-Häen (Seelze-Hannover, Germany). TPT (purity > 98%) was obtained as analytical standard from Merck-Schuchard (Hohenbrunn, Germany) and LET (purity 99.9%) was obtained from United States Pharmacopeia (USP), (Rockville, USA). Analytical grade chemicals for preparation of media were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Test solutions

Ethanol stock solutions were prepared and stored at 4 °C in the dark. Working test solutions were prepared just before use in ultrasonic bath by diluting ethanol stock solutions in 18‰ synthetic seawater medium (Kusk and Wollenberger, 1999). Ethanol concentrations in the experiments did not exceed 100 μL L^{-1} (79 mg L^{-1}). Additional solvent controls were included.

2.3. Exposures

Full life-cycle exposures were conducted with *A. tonsa*, starting with eggs and extending for 3 weeks to the point where the adults become reproductively active, following an OECD guideline proposal (Kusk and Wollenberger, 2007). For range finding purposes, adult acute toxicity tests and early-life stage development tests were performed as described by Wollenberger et al. (2003). Exposures were started by adding 600 *Acartia* eggs to 1000-mL vessels containing 800 mL test solution. The vessels were slightly aerated with sterile filtered air and 80% of the medium was renewed three times a week. The unicellular alga *Rhodomonas salina* served as feed and was added at a density of 5×10^4 cells mL^{-1} subsequent to each medium renewal. Copepods were exposed for 21 d at 20 ± 0.5 °C and a photoperiod of 16 h light followed by 8 h darkness.

2.4. Chemical analyses

Chemical analyses of test compounds in the exposure media were performed for MET, TPT and FEN. Exposure concentrations provided here refer to time-weighted means for MET, TPT and FEN and to nominal concentrations for LET. Time-weighted mean concentrations were calculated as described in OECD Guideline 211 (OECD, 1998). The following concentration series were applied: 1.6, 4.7, 14, 42 and 126 μg L^{-1} MET; 12, 37, 110, 330, and 1000 μg L^{-1} LET (nominal concentration); 0.0014, 0.0035, 0.0088 μg L^{-1} TPT-Sn; and, 2.8, 7.0, 17, 42 and 105 μg L^{-1} FEN.

2.4.1. Methyltestosterone

MET was concentrated by SPE on Oasis HLB cartridges (Waters, France), which were conditioned by passing 5 mL of diethyether, 5 mL of methanol and 5 mL of HPLC water through successively. 100 mL of medium was percolated through the cartridge at 4 mL min^{-1}. Two cleaning steps were performed to remove interfering organic and inorganic compounds. After drying for 30 min, compounds were eluted with a mixture of diethylether/methanol 90/10 (2 × 5 mL). The extracts were evaporated to 1 mL under gentle nitrogen stream before analysis.

The chromatographic system was composed of a Thermo Finnigan autosampler (ASE 2000) and a Thermofinnigan HPLC pump (Surveyor MS). LC–MS/MS analyses were performed by using a DECA XP + Thermo-Finnigan ion trap instrument equipped with an Atmospheric Pressure Chemical Ionisation source (APCI). The reversed phase column was an Omnispher C-18 100 ∗ 2 mm i.d. and 3 μm particle size (Varian-Chrompack, Les Ulis, France). A gradient of mobile phases constituted of methanol and water at 0.2 mL min^{-1} was used in order to separate the steroids and 20 μL of sample was injected in the column.

MET and the internal standard Testosterone D3 were detected in positive APCI mode as their protonated molecular ions $(M + H)^+$ at m z^{-1} 303 and 292, respectively. These ions were isolated and fragmented in the MS/MS mode and quantification was based upon the transitions to the product ions at m z^{-1} 285, 267 and m z^{-1} 274, 256, respectively, after losing one and two water molecules. Under these operating conditions, the MET quantification limit was 0.015 μg L^{-1}.

2.4.2. Triphenyltin

GC/MS/MS analyses were performed using a Thermofinnigan (*Les Ulis, France*) system consisting of a Trace GC 2000 gas chromatograph equipped with a PTV split-splitless temperature injector, an AS 2000 autosampler and a POLARIS Q ion-trap mass spectrometer. For data processing, Excalibur software from Thermofinnigan was used. The injector was equipped with a 12 cm × 2 mm i.d. Silcoseeve liner (Thermofinnigan). 60 μL of sample was injected onto the PTV injector in constant flow mode set at 1 mL min^{-1} and with an injection rate of 1 μL s^{-1}. The split flow was set at 50 mL min^{-1}. The temperature of the injector was initially set at 70 °C then increased to 90 °C at 5 °C s^{-1} (evaporation phase) and to 280 °C at 10 °C s^{-1} where it was maintained for 7 min. The PTV split/splitless valve was operating in splitless mode until a temperature of 280 °C was achieved. Once the temperature stabilised, it was maintained for a period of 1.5 min then changed to split mode.

Organotin compounds were separated on a 30 m × 0.25 mm i.d. column, coated with 0.25 μm of 65% dimethyl–35% phenyl polysiloxane phase (BPX-35, SGE, Courtaboeuf, France). The temperature of the column was initially set at 100 °C for a period of 2.5 min, after which it increased to 280 °C. Helium was used as carrier gas at a constant flow of 1 mL min^{-1}. The transfer line was set at 300 °C with the external ion source at 280 °C. The ions in EI for the target compounds were selected and fragmented with helium gas CID in the ion trap. The mass spectra resulting from these fragments were scanned in successive segments from m z^{-1} ion 50 to the mass of the selected ions plus 10 (m z^{-1}).

The concentrations were calculated using the calibration curves established for each compound in internal standardisation mode with tripropyltin and diheptyltin as internal standards.

100 mL of medium samples were mixed with 1 mL of tetraethylborate (NaBEt4) (2%) at pH 4.8 (100 mL of acetate buffer 1.2 M). After eliptical stirring for 30 min, organotin species were recovered in 5 mL of 2,2,4-trimethylpentane and evaporated to 1 mL under gentle nitrogen flow, before analysis by GC–MS/MS. The limits of quantification were 0.002 μg L^{-1}-Sn for TPT and diphenyltin and 0.010 μg L^{-1} -Sn for monophenyltin.

2.4.3. Fenarimol

Analytical grade standard (99.9%) was used without further purification. The exposure water samples were adjusted to pH 3 before extraction of FEN. SDB discs (47 mm diameter containing 500 mg of styrene–divinylbenzene and 500 mg of C18 (octadecyl) polymer) (3 M Saint Paul, MN, USA) were activated by wetting with 6 mL of acetone after which they were washed with 2 × 5 mL of dichloromethane/ethyl acetate (50:50 v/v) and vacuum dried. Methanol (5 mL) was then percolated through the discs and without letting the disc become dry. A 200-mL water sample was applied to the disc at a flow rate of 20 mL min^{-1} using a vacuum pump. Next the discs were dried under vacuum for 10 min. The analyte was eluted in the opposite way to the sample application (backflush desorption) with 2 × 5 mL of dichloromethane/ethyl acetate mixture (50:50 v/v). The extract was dried over anhydrous sodium sulphate. Extracts were concentrated to a final volume of 0.2 mL with solvent change to toluene under a gentle stream of nitrogen and were then analysed by GC-ECD.

GC-ECD chromatographic analysis was performed using a Shimadzu 14A capillary gas chromatograph equipped with a ^{63}Ni electron capture detector (ECD) at 300 °C. A DB-1 column (J & W. Scientific, Folsom, CA, USA) was used, 30 m × 0.32 mm i.d., containing dimethylpolysiloxane with a phase thickness of 0.25 μm. The temperature program used for the analysis was: from 55 °C (2 min) to 210 °C (15 min) at 5 °C min^{-1} and to 270 °C at 10 °C min^{-1}. The injector was set to 240 °C in the splitless mode. Helium was used as carrier at 1.5 mL min^{-1} and nitrogen was used as the make-up gas at 35 mL min^{-1} according to the optimisation results of the instrument given by the manufacturer. Identification of peaks was based on the comparison of the retention times of compounds in the standard solutions. Quantification of the analysed compounds was performed using the method of the internal standard.

With this method a quantification limit of 0.030 μg L^{-1} was reached.

2.5. Histology

2.5.1. Fixation

21 d old animals were fixed in Bouin's solution for 24 h and then transferred to 80% ethanol. After dehydration (Shandon Hypercenter®), the fixed animals were embedded in paraffin. 50–60 animals from each control and exposure group were embedded in cassettes and continual serial sections of 2–3 μm were cut using a rotating microtome (Microm). As it was not possible to align all *Acartia* specimens in parallel to the section level, the proportion of animals cut in longitudinal sections was variable. Animals cut in more transversal sections were not suitable for further evaluation. Thus, numbers of animals subjected to histological investigation and evaluated in stained sections differed between exposure groups. The longitudinal sections were microscopically examined for pathological alterations of the digestive tract (including foregut, midgut and hindgut for respiratory function), the nervous system, the musculature, and the reproductive organs. For routine histology, sections were stained with haematoxylin–eosin (H&E).

2.5.2. Immunocytochemistry

2.5.2.1. Proliferation. Immunocytochemistry to detect proliferation was performed using Anti-Proliferating Cell Nuclear Antigen (PCNA) (DAKO Cytomation; No. M 0879) and DAKO EnVision+, Peroxidase, Mouse (DAKO Cytomation; No. K4000). Staining of formalin fixed paraffin embedded tissue sections was carried out.

3–4 μm sections from paraffin blocks, attached to superfrost slides (silane-coated) were heated in oven at 56–60 °C for 30 min.

Tissue sections were deparaffinised in xylene for 15 min, washed sequentially in 100%, 90%, 70%, 50%, 30% ethanol (2 min each) and distilled water for 5 min.

Heat-induced epitope retrieval was performed by transfer of the slides into a jar containing citrate buffer (Sørensen's I) with a pH of 6 on a hot water bath (96 °C to 99 °C) for 30 min. After 20 min cooling at room temperature, the slides were washed with PBS (Dulbecco's Phosphate Buffered Saline) and distilled water. Endogenous peroxidase was quenched in 3% hydrogen peroxide for 5 min with subsequent rinsing in PBS.

100 μL of the monoclonal mouse anti PCNA (PCNA clone PC 10; DAKO No. M 0879) was applied to the slides which were incubated at room temperature in a humidity chamber for 30 min and rinsed in PBS (concentration PCNA $\geqslant$1:200 in PBS). 100 μL of EnVision+(Peroxidase, Mouse, Ready-to-use; DAKO No. K4000) was applied followed by incubation for 30 min in a humidity chamber, and rinsing with PBS. Application of chromagen solution NovaRED and incubation in a humidity chamber for 15 min was performed. Washing in distilled water for 5 min (NovaRED Subtrate Kit – for Peroxidase, Vector No. SK-4800) and counterstaining with haematoxylin were the final steps of the treatment.

The proliferation index was calculated by dividing the total number of counted gametes by the number of marked gametes in the female gonad.

2.5.2.2. Apoptosis. Sections of 3–4 μm were attached to Superfrost silane-coated slides and oven heated at 56–60 °C for 30 min. Tissue sections were deparaffinised in xylene for 15 min, washed sequentially in 100%, 90%, 70%, 50%, 30% ethanol (2 min each) and PBS for 5 min (Dulbecco's PBS). Slides were incubated with saponin Merck No. 159665 blank (0.1 mg mL^{-1} PBS) at room temperature for precisely 20 min, washed in PBS and then incubated in Proteinase K (20 μg mL^{-1} PBS for 20 min at room temperature; Proteinase K; Roth No. 7528.1) and washed three times in distilled water. The slides were transferred into a jar with 50% formamide (Sigma Chemicals No. F-7503, v/v distilled water) which were preheated in water bath to 56–60 °C followed and the slides were incubated with formamide solution for 20 min in the water bath.

After heating the slides were transferred into ice-cold PBS for 5 min. This was followed by quenching of endogenous peroxidase in 3% hydrogen peroxide for 5 min. To block non-specific antibody binding, sections were treated with 3% non-fat (or low-fat) dry milk for 15 min and rinsed in PBS.

100 μL of monoclonal antibody F7-26 (Alexis No. APO-20A-078-L001) was applied to the slides which were then incubated at room temperature in a humidity chamber for 30 min and rinsed in PBS. Application of 100 μL of peroxidase-conjugated anti-mouse IgM, incubation for 30 min in a humidity chamber and rinsing with PBS followed. The working concentration of the second antibody, peroxidase-conjugated rat monoclonal anti-mouse IgM (Zytomed No. 04 6820) was 1:100 in PBS.

A chromagen solution NovaRED was applied followed by incubation in a humidity chamber for 15 min. After washing in distilled water for 5 min the slides were counterstained with haematoxylin.

The apoptotic index was calculated by dividing the total number of specimens without visible apoptotic cells by the number of specimens with apoptotic cells in the gonad and genital tract. This was performed separately for males and females.

2.6. Statistical analysis

The proliferation index was calculated by dividing the number of proliferating oogonia by the total number of oogonia. The statistical evaluation was performed using GraphPad Prism version 4 for Windows, GraphPad Software, USA. Kruskal–Wallis test with Dunn's multiple comparison test was applied. For statistical confirmation a probability error of 5% ($p < 0.05$) was defined.

3. Results

3.1. Chemical analysis

Concentrations were calculated as time-weighted means (OECD, 1998) and amounted for MET to 25.2%, for TPT to 23.7%, and for FEN to 70.0% of the nominal concentrations.

3.2. Histology

Control animals displayed the typical structure of *Acartia tonsa* with no pathological alterations in any of the tissues or organs (Fig. 1A and B). In singular male specimens of the control group, stages of protozoan parasites were encountered in the gonad. These parasites caused a destruction of the whole reproductive organs including the gonad and the reproductive tract. The afflicted specimens were excluded from further evaluation.

3.2.1. Alterations in non-reproduction organs

Pathological disorders were exclusively found in the reproductive system. In other organs, no deviations from the normal structure were observed. Special emphasis was placed on associated or concomitant alterations of the digestive tract (foregut, midgut and hindgut acting as respiratory organ), musculature, and the nervous system.

3.2.2. Methyltestosterone

24 males and 53 females of *A. tonsa* were investigated for histological alterations. In MET-exposed males no pathological alterations in the gonad were observed at any of the tested concentrations, except of 42 μg L^{-1} MET. Here, in a single specimen where spermatogonia and primary spermatocytes were present in increased number and volume in the anterior part of the male gonad.

In females, 30% of all specimens exposed to concentrations of 1.6, 4.7, and 14 μg L^{-1} MET, showed reduced formation of yolk around the nucleus. The sites of yolk synthesis around the nuclear membrane of pre-vitellogenic and vitellogenic oocytes (*nuages*), were fairly thin and pale. The composition of the yolk was clearly different from that of the controls as the normal staining reaction toward an eosinophilic colouration with increasing yolk content failed (Fig. 1C). In addition, the nuclear membrane persisted in singular ripe vitellogenic oocytes.

Clumped chromatin occurred in oogonia of females exposed to 1.6 μg L^{-1} MET in the late meiosis stage. These oogonia reacted positively to apoptotic labelling (Fig. 1D). At 14 μg L^{-1} MET, 30% of the females displayed degenerating oogonia with vacuolated cytoplasm.

The apoptotic index of oogonia and oocytes was highest (0.17) at 1.6 μg L^{-1} MET and the proliferation index was significantly reduced (0.15) at 4.7 μg L^{-1} MET (Table 1 and Fig. 1E).

3.2.3. Letrozole

216 females and 74 males were subjected to histological investigations. In males all stages of gametes were altered in shape and size at concentrations of 12 and 37 μg L^{-1}. The spermatogonia were enlarged displaying large nuclei with scattered chromatin masses or perinuclear chromatin masses and a prominent nucleolus. The cytoplasm stain was atypically eosinophilic (Fig. 2A). The spermatocytes displayed prominent meiotic figures, sometimes in eccentric orientation of the chromatin masses. Their translucent cytoplasm was highly vacuolized building a halo around the

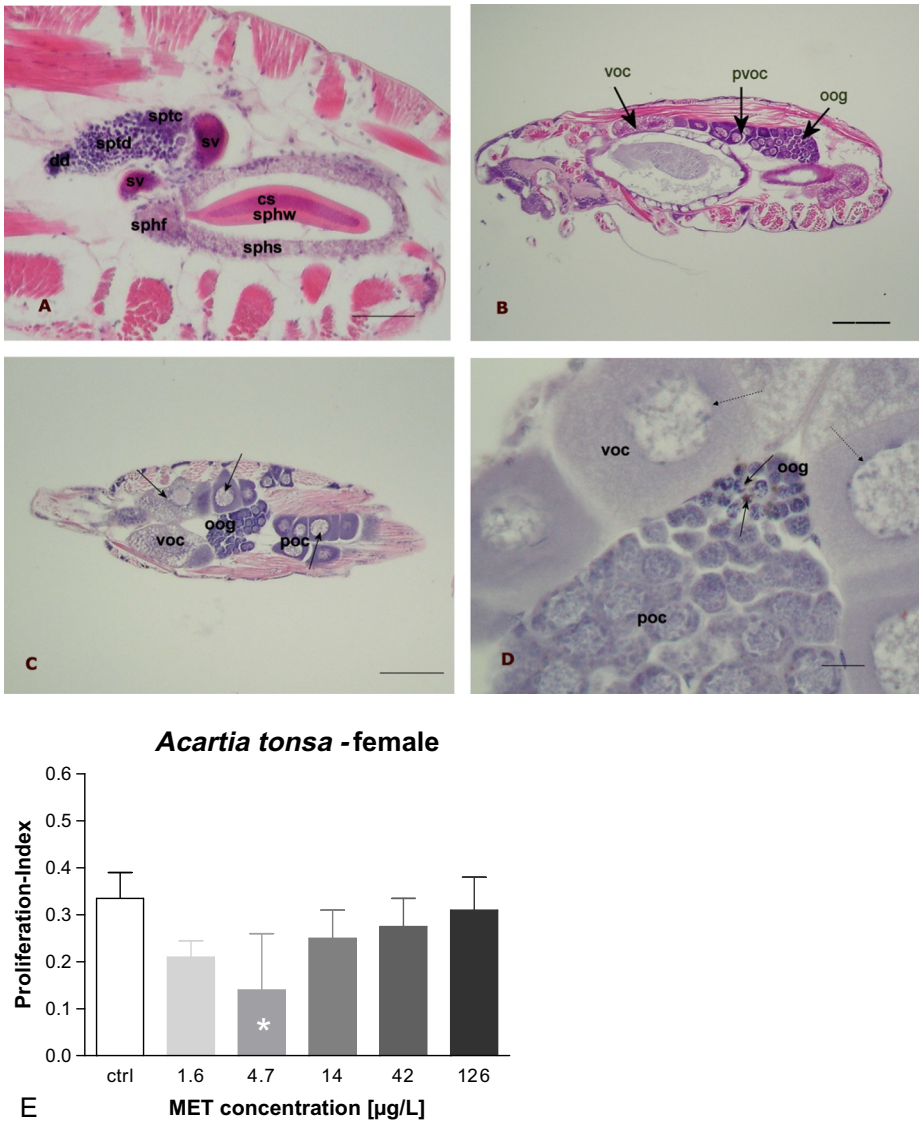

Fig. 1. (A) Normal male reproductive system (sptc = spermatocytes, sptd = spermatids, dd = ductus deferens, sv = seminal vesicle, sphf = spermatophore former, sphw spermatophore wall, sphs = spermatophore sac proper, cs = core secretions, H&E, bar = 50 µm). (B) Normal female reproductive system (oog = oogonia, pvoc = pre-vitellogenic oocytes, voc = vitellogenic oocytes, H&E, bar = 100 µm). (C) Female *Acartia* exposed to 4.7 µg L^{-1} MET with decreased formation of yolk around the nucleus (arrows) and basophilic vitellogenic oocytes (voc). oog = oogonia, poc = pre-vitellogenic oocytes, H&E, bar 100 µm. (D) Female gonad of *Acartia* exposed to 1.6 µg L^{-1} MET with apoptotic oogonia (arrows) and basophilic vitellogenic oocytes with persisting nuclear membrane (dotted arrows). oog = oogonia, poc = pre-vitellogenic oocytes, ss-DNA apostain, bar = 10 µm. (E) *Acartia tonsa* exposed to MET. Proliferation Index, * = significant difference between control and exposure of 4.7 µg L^{-1}, MET (no statistics with exposure of 126 µg L^{-1}, *N* too small).

Table 1
Apoptotic index in females of *Acartia* exposed to MET.

Concentration (µg L^{-1})	Apoptotic index
0	0.10
1.6	0.17
4.7	0
14	0.08
42	0
126	0

nucleus. Singular spermatocytes in apoptosis were present. The number of visible spermatids was fairly low. In all sections of the gonad, only one or two mitotic figures per specimen were detected. At 1000 µg L^{-1} LET, numerous spermatocytes displayed aberrant meiotic figures (Fig. 2B). The apoptotic index of male gametes was elevated at 12 µg L^{-1} LET and decreased to 0.0 at 330 µg L^{-1} and 0.2 at 1000 µg L^{-1} LET (Table 2).

At 12 µg L^{-1} LET, the majority of males displayed deformations of the genital tract including the ductus deferens and the seminal vesicle. At 330 µg L^{-1} LET the epithelial cells of the ductus deferens and the seminal vesicle were atrophic and their height was reduced. The epithelial wall of the spermatophore was reduced in height compared to controls, and the production of core secretions was scarce. In the spermatophore former, only some droplets of wall material were visible. In parallel, the epithelial cells of the spermatophore sac proper built a layer with varying height due to irregular, extensive vacuolation of the cytoplasm (Fig. 2C). Male specimens exposed to 110, 330 and 1000 µg L^{-1} LET displayed disturbed core secretions of the spermatophore with a prevalence of 30%, 25.5% and 14%, respectively (Table 1 in Supplementary material). In single males, the deformation of the wall appeared as an additional rudimentary spermatophore formation (Fig. 2D).

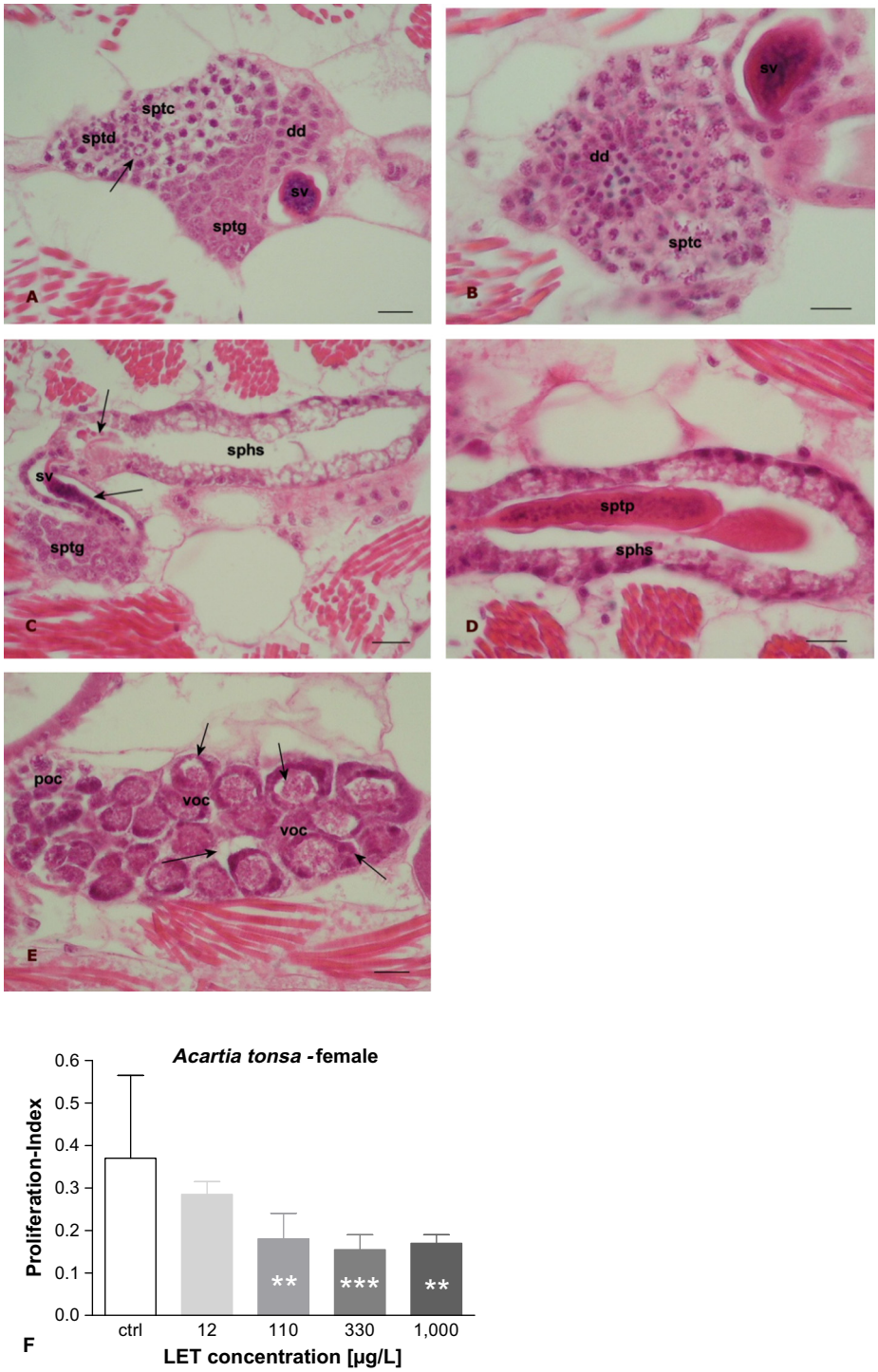

Fig. 2. (A) Male gonad of *Acartia* exposed to 12 µg L^{-1} LET with atypically enlarged spermatogonia, and some spermatocytes with apoptotic figures (arrow) and vacuolized spermatids. sptg = spermatogonia, sptc = spermatocytes, sptd = spermatids, dd = ductus deferens, sv = seminal vesicle, H&E, bar = 10 µm. (B) Male gonad of *Acartia* exposed to 1000 µg L^{-1} LET with heavily vacuolated spermatocytes displaying aberrant meiotic figures. sptc = spermatocytes, dd = ductus deferens, sv = seminal vesicle, H&E, bar = 10 µm. (C) Spermatophore sac of *Acartia* exposed to 330 µg L^{-1} LET, left spermatophore former, right spermatophore sac proper with irregular vacuolated epithelial cells and deformed spermatophore (arrows). sptg = spermatogonia, sv = seminal vesicle, sphs = spermatophore sac, H&E, bar = 10 µm. (D) Spermatophore of *Acartia* exposed to 330 µg L^{-1} LET with irregular wall formation (left) and a second rudimentary spermatophore (right). Spermatophore sac proper with irregular vacuolated epithelial cells. sptp = spermatophore, sphs = spermatophore sac, H&E, bar = 15 µm. (E) Gonad of female *Acartia* exposed to 330 µg L^{-1} LET with atypical pre-vitellogenic oocytes (poc) and vitellogenic oocytes displaying nuclei irregular dispersed chromatin and vacuolized cytoplasm (voc). H&E, bar = 10 µm. (F) *Acartia tonsa* exposed to LET, Proliferation Index, * = significant difference between control and exposure of 110, 330 and 1000 µg L^{-1} LET.

In females exposed to LET, several disorders in the gonad were encountered. At all concentrations tested, oogenesis was disturbed which was reflected in degenerating oogonia and primary oocytes. The chromatin was clumped or irregularly dispersed and the nucleus displayed vacuoles at the periphery. An additional prominent feature was a disturbance in yolk synthesis manifested by irregular, focal accumulation of perinuclear sites of yolk synthesis (Fig. 2E).

At 330 and 1000 µg L^{-1} respectively 5% and 21% of all females displayed a lack of primary oocytes. This disturbance in oogenesis

Table 2
Apoptotic index in the gonad of female and male *Acartia* exposed to LET.

Exposure (μg L^{-1})	Apoptotic index females	Apoptotic index Males
Control	0	0
12	0.08	0.4
37	0	0.17
110	0.03	0.08
330	0.02	0
1000	0.05	0.20

was manifested in the proliferation index, which was significantly reduced at concentrations ranging from 110 to 1000 μg L^{-1} LET (Fig. 2F). The apoptotic index of oogonia and oocytes in the gonad of LET exposed specimens was fairly low and varied between 0 and 0.08 (Table 2).

3.2.4. Triphenyltin

78 females and 51 males were investigated for histological alterations. In the gonad of TPT exposed males, no alterations in the gametogenesis were observed. Immunohistostaining with PCNA showed a very intense labelling. Apoptosis of male gametes could hardly be detected.

Deformations of the spermatophore wall occurred in 12% of males exposed to 0.0014 μg TPT-Sn L^{-1} and in 27% exposed to 0.0088 μg TPT-Sn L^{-1}. These deformations were accompanied by reduced and irregular core secretions. In one specimen, unilateral hypertrophy of the spermatophore wall was observed along with pale and sparse, irregular core secretions. In addition, the height of the epithelium of the spermatophore sac was reduced (Fig. 3A).

In the female gonad the most striking effects of TPT at all concentrations tested were seen in developing gametes. Effects were observed in follicles and gametes. The prevalence of oogonia present in the gonad dropped in a concentration-dependent manner from 100% in the control to 45.5% at 0.0088 μg TPT-Sn L^{-1}. No degenerating oogonia were observed in the controls; the prevalence of degenerated oogonia in exposed females was 20% at 1.4, 12.5% at 0.0035, and 0% at 0.0088 μg TPT-Sn L^{-1} (Table 3).

The proliferation index of oogonia was slightly (but not statistically significant) reduced at all concentrations in relation to the control (Fig. 3B).

The prevalence of degenerating pre-vitellogenic oocytes was zero in the control, and 60% (at 0.0014 μg L^{-1} and 0.0035 μg TPT-Sn L^{-1}), and 91% (at 0.0088 μg TPT-Sn L^{-1}) in exposed females (Table 3). At 0.0088 μg L^{-1} TPT-Sn the pre-vitellogenic oocytes displayed a pale vacuolated cytoplasm and polymorphic nuclei with prominent, irregular sites of yolk formation (Fig. 3C).

The percentage of degenerating vitellogenic oocytes varied between 25% and 40% compared to 0% in the control. In contrast to the controls, the cytoplasm of the vitellogenic oocytes of individuals exposed to 0.0014 and 0.0035 μg L^{-1} TPT-Sn, stained basophilic instead of eosinophilic and contained only minuscule amounts of yolk. In addition, the breakdown of the nuclear envelope did not occur in ripe vitellogenic oocytes.

The apoptotic index of female gametes was 0.09 in the control and clearly elevated at 0.0014, 0.0035, and 0.0088 μg L^{-1} TPT-Sn (Table 3). Immunolabelling revealed that the degenerating oocytes as well as the degenerating follicle cells underwent apoptosis (Fig. 3D). Along with degenerating germ cells, elevated numbers of degenerated follicle cells were encountered in specimens exposed to 0.0014 and 0.0035 μg L^{-1} TPT-Sn.

3.2.5. Fenarimol

A total of 17 males and 32 females were subjected to histological investigations. In males, no pathological changes were observed in the gonad. The histological sections immunostained with PCNA showed a very intense labelling indicating an elevated proliferation activity. Apoptotic gametes in the male gonad occurred in singular cases.

In female *Acartia* exposed to FEN, oogenesis was disturbed at all tested concentrations. The synthesis of yolk was irregular and focally very prominent at 2.8 μg L^{-1} FEN (Fig. 4A). At 42 μg L^{-1} FEN, pre-vitellogenic and vitellogenic oocytes displayed a translucent cytoplasm and large nuclei with condensed chromatin and focally very intense yolk synthesis at the perinuclear sites of synthesis. The synthesis of yolk started even in very small-sized pre-vitellogenic oocytes, which do not normally display vitellogenesis.

At a concentration of 105 μg L^{-1} FEN singular pre-vitellogenic oocytes underwent apoptosis. The perinuclear areas of yolk formation in vitellogenic oocytes were clearly reduced and less prominent compared to animals exposed to lower concentrations and to the control (Fig. 4B). The proliferation index was reduced at all concentrations except for 7 μg L^{-1} FEN. However, there was no significant deviation from the control (Fig. 4C).

4. Discussion

The histological structure of the male and female gonad of *Acartia tonsa* is consistent with descriptions of the general feature of reproductive organs of copepods (Blades and Youngbluth, 1981; Blades-Eckelbarger and Youngbluth, 1982, 1984). None of the specimens investigated in the present study displayed signs of intersexuality, which supports the findings of other authors characterising *Acartia* as a gonochoristic species (Boxshall, 1992). The observed protozoan stages resemble sporozoan parasites, which are well known parasites in crustaceans, especially in the copepod gonad. Some of them have castrating effects; others are regarded as commensales (Myers, 1990). The occurrence of sporozoa is of high importance as there is increasing evidence that they can induce intersexuality in amphipods (Kelly et al., 2004). In copepods, intersexuality has been described e.g. in harpacticoids at polluted sites of the Firth of Forth, Scotland (Moore and Stevenson, 1991; Sillett and Stemberger, 1998). However, so far there are no reports available on intersexuality due to parasitic infestation in copepods. As all parasitic stages are very distinct and could clearly be discriminated in the paraffin sections, infected organisms were identified and rejected from further evaluation. Thus, interferences with effects of the compounds under investigation can be excluded.

4.1.1. Effects on males

A striking feature in male *A. tonsa* exposed to LET was the occurrence of enlarged spermatogonia, spermatocytes and vacuolated spermatids. Along with these alterations, regular mitotic activities have been observed in the male gonad. Probably, spermatogenesis was promoted at different levels. In the spermatophore both the production of core secretions and wall formation were reduced.

No disrupting effects on spermatogenesis were observed in *A. tonsa*. In contrast, the proliferating activity of the gonad was stimulated. However, in the spermatophore, the wall displayed irregular formation or hypertrophy along with reduced core secretions. Deformations of the spermatophore wall occurred in 12% of males exposed to 0.0014 μg TPT-Sn L^{-1} and in 27% exposed to 0.0088 μg TPT-Sn L^{-1}. It may be worth to mention that alterations of secondary sexual organs like urosome deformities were observed by Bang et al. (2009) in *Tigriopus janaonicus* following exposure to benz(a)-pyrene, which acts as an antiandrogen in vertebrates. The urosome of copepods is different in males and females. These secondary sex characteristics are probably under influence of the endocrine sys-

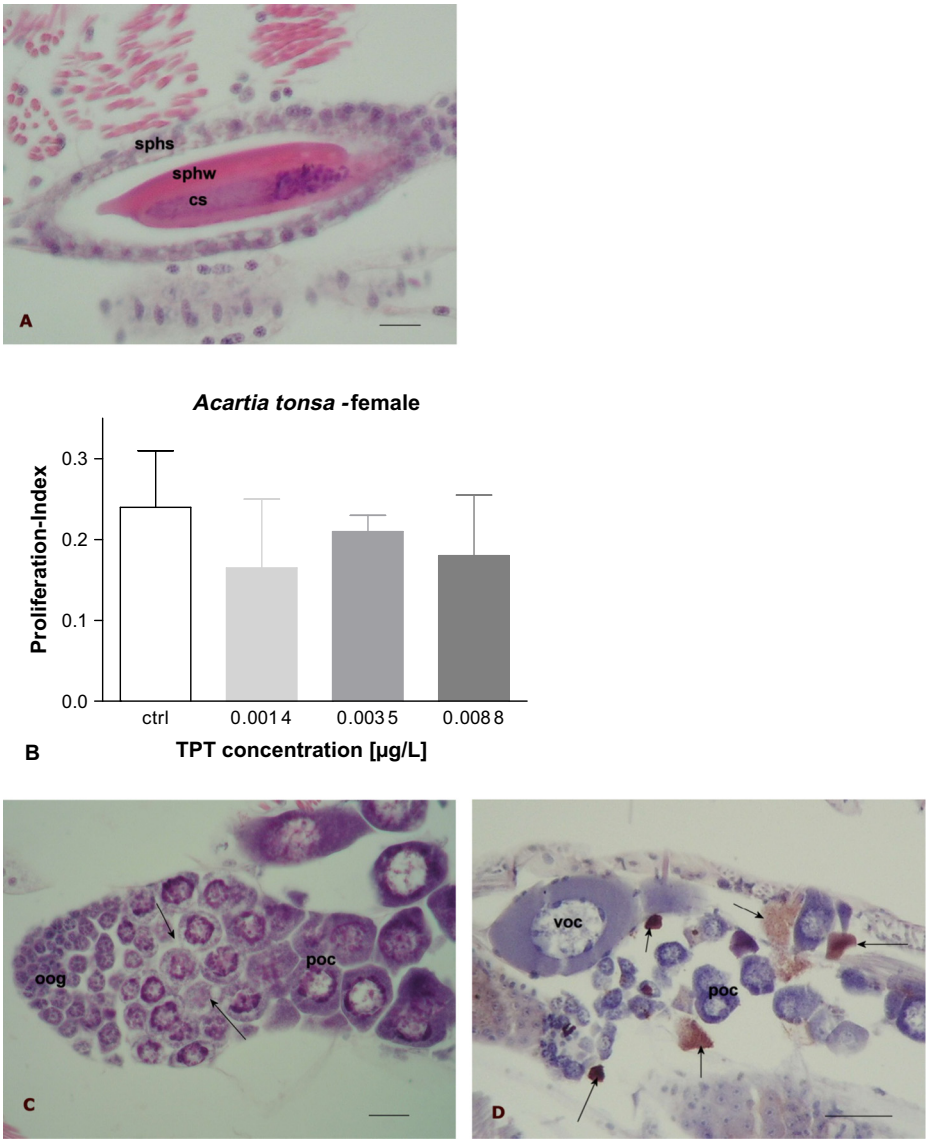

Fig. 3. (A) Spermatophore in male reproductive system of *Acartia* exposed to 0.0035 µg L^{-1} TPT, with unilateral hypertrophied spermatophore wall (sphw) and pale core secretions. sphs = spermatophore sac proper, cs = core secretions, H&E, bar = 10 µm. (B) *Acartia tonsa* exposed to TPT, Proliferation Index, no significant difference, level of significance $P > 0.05$, no statistics with exposure of 0.0014 and 0.0088 µg L^{-1}, N too small. (C) Female gonad of *Acartia* exposed to 0.0088 µg L^{-1} TPT with degenerating pre-vitellogenic oocytes with pale staining cytoplasm (arrows) and nuclei with prominent and irregular sites of yolk formation. poc = pre-vitellogenic oocytes, oog = oogonia, H&E, bar 10 µm. (D) Female gonad of *Acartia* exposed to 0.0035 µg L^{-1} TPT with numerous apoptotic pre-vitellogenic oocytes (arrows). poc = pre-vitellogenic oocytes, voc = vitellogenic oocytes, ss-DNA apostain, bar = 50 µm.

Table 3
Occurrence of disturbance in oogenesis in the gonad of female *Acartia* exposed to three different concentrations of TPT (concentrations in measured Sn).

Exposure concentr.	Oogonia not present (%)	Oogonia degen. (%)	Previtell. Oocytes degen. (%)	Vitell. Oocytes degen. (%)	Follicle cells degen. (%)	Apoptotic index
Control	0	0	0	0	0	0.09
0.0014 µg L^{-1} TPT-Sn ($n = 15$)	33.3	20	60	40	26.7	0.4
0.0035 µg L^{-1} TPT-Sn ($n = 8$)	37.5	12.5	62.5	25	0	0.22
0.0088 µg L^{-1} TPT-Sn ($n = 11$)	54.5	0	91	36.4	36.4	0.5

tem controlling molting and/or the sexual development. The mode of action of benz(a)pyrene in the cited study may possess similarities to the mode of action in vertebrates. Gaertner et al. (2012) have identified an ecdysone receptor in the harpacticoid copepod *Amphiascus tenuiremis*. Moreover they demonstrated that an endocrine active pesticide induced the expression of the ecdysone receptor. Although the structure of the ecdysone receptor may be different, the mode of action with binding to the receptor pos-

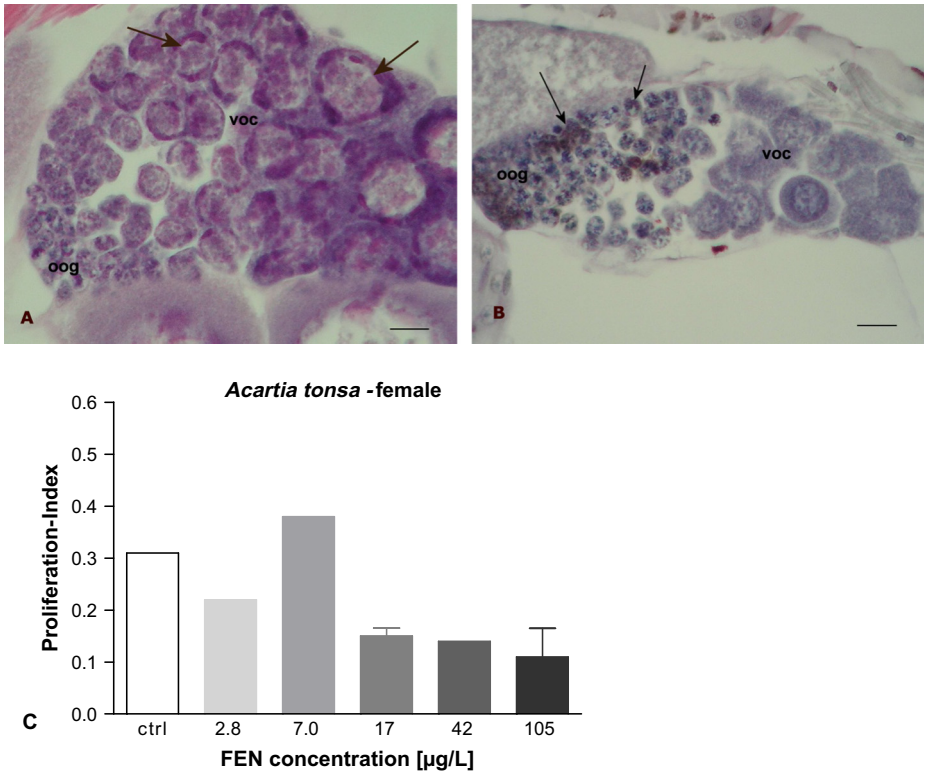

Fig. 4. (A) Gonad of female *Acartia* exposed to 2.8 µg L^{-1} FEN with vacuolized oogonia and irregular, intense yolk synthesis of vitellogenic oocytes. oog = oogonia, voc = vitellogenic oocytes, H&E, bar = 10 µm. (B) Gonad of *Acartia* exposed to 105 µg L^{-1} FEN with apoptotic pre-vitellogenic oocytes with atypical meiotic figures (arrows). oog = oogonia, voc = vitellogenic oocytes, ss-DNA apostain, bar = 10 µm. (C) *Acartia tonsa* exposed to FEN. Proliferation Index, no statistics, *N* too small.

sesses strong similarities to the mode of action of compounds acting as androgens and anti-androgens in vertebrates.

4.1.2. Effects on females

In females exposed to MET, the production of yolk was clearly reduced at all concentrations tested, i.e. 1.6–126 µg L^{-1}. An atypical basophilic staining reaction indicated that the composition of yolk was altered. Furthermore, the lowest concentration of 1.6 µg L^{-1} induced apoptosis in oogonia corresponding to the highest apoptotic index. The proliferation index of oogonia and oocytes was significantly reduced at 4.7 µg L^{-1}. No effect of MET exposure was observed in males.

In females exposed to LET, degeneration of oogonia and oocytes was observed at all exposure concentrations, which was reflected in significantly reduced proliferation indices. The perinuclear areas of yolk production appeared focally enlarged but irregular and were visible in females exposed to high concentrations. These results are similar to the effects of TPT, the other aromatase inhibitor tested in the present study.

The effects of organotin compounds in female *A. tonsa* were first recorded for TBT, which was shown to cause impaired egg production at concentrations below those measured near Danish marinas (Johansen and Møhlenberg, 1987). Kusk and Petersen (1997) demonstrated acute and subchronic effects of TBT towards *A. tonsa* at environmentally relevant concentrations, i.e. reduced survival of larvae at 15–20 ng L^{-1} TBT and inhibition of early life stage development (moulting) at 1 ng L^{-1} TBT.

In female *A. tonsa,* exposed to TPT, adverse effects were evident on oogenesis, on the level number of oogonia, on the pre-vitellogenic and vitellogenic oocytes, on yolk synthesis and on maturation at the two lowest exposure concentrations of 0.0014 and 0.0035 µg TPT-Sn L^{-1}. In contrast, at 0.0088 µg TPT-Sn L^{-1}, the perinuclear sites of yolk formation were more prominent but irregular in shape. In female *A. tonsa* exposed to TPT, the apoptotic index of oogonia and oocytes was elevated compared to the control at all exposure concentrations, indicating that degeneration and loss of oocytes was primarily due to apoptosis as it is seen in deprivation of estrogens by aromatase inhibitors in mammals (Thiantanawat et al., 2003). On the other hand, compounds with estrogenic effects in mammals stimulated the maturation of oocytes in *A. tonsa* (Andersen et al., 1999).

It is worth noting that the histopathological alterations reported here occurred at environmentally relevant TPT concentrations (0.0014–0.0088 µg TPT-Sn L^{-1}). The test concentrations are below levels measured in coastal waters; e.g. 0.353 µg TPT-Sn L^{-1} at Sharm el Sheikh harbour, Egypt (Arnold et al., 1998), 0.007 µg TPT-Sn L^{-1} at Barcelona harbour, Spain, (Alzieu et al., 1991) and <0.0003–0.0097 µg TPT-Sn L^{-1} at the French Mediterranean coast (Tolosa et al., 1996).

In FEN exposed *Acartia* females, disturbances of oogenesis on the level of oocyte differentiation and meiosis were observed at all concentrations. The yolk production was not affected at 2.8–42 µg L^{-1}, but was reduced at 105 µg L^{-1}. At a concentration of 7.0 µg L^{-1} FEN, the proliferation index in female *Acartia* was elevated compared to the control, while it was reduced at the other concentrations. However, deviations from controls were not statistically significant.

4.1. Summary of histological effects

Effects on the histology of *A. tonsa* exposed to MET, LET, TPT, and FEN at low concentrations occurred exclusively in the reproductive system and were not accompanied by pathological alterations in other organs. All compounds induced various malformations of the reproductive organs and strong effects on

developing gametes in males and females. Atrophic or hypertrophic alterations of the gonad and the sexual accessory glands were the dominant features. Apoptotic processes in gametes as found in the present study can be caused by general toxic action or by withdrawel of hormonal stimuli. Associated necrotic processes, which would indicate general toxicity, were not observed.

In vertebrates germ cell differentiation is controlled by steroidal androgens and estrogens (Billig et al., 1993; Nakanishi and Shiratsuchi, 2004) whereas in crustacean the hormonal regulation is partly effected by non-steroidal compounds and by the steroid ecdysone. An ecdysone receptor has recently been identified in another copepod *Amphiascus tenuiremis* (Gaertner et al., 2012). Binding to this receptor may be the reason that compounds like TPT, FEN and MET, which act androgenically in vertebrates and some invertebrate phyla such as echinoderms and molluscs (Lavado et al., 2006; Duft et al., 2007) are also able to interfere with the hormonal systems of calanoid copepods like *Acartia tonsa*.

In summary the stimulatory and inhibitory effects of the tested compounds on the male gonad were as follows: In male copepods spermatogenesis was normal except for males exposed to LET. The proliferation index was elevated by all compounds except for MET. The apoptotic index was unaffected.

In females oogenesis was inhibited by all compounds except MET. Proliferation index and yolk formation were reduced. No formation of ovotestis was observed. The apoptotic index increased except after exposure to LET. TPT and FEN down-regulated the female gonad and increased the apoptotic index whereas males remained mostly unaffected.

Since the sixties it is well known that organochlorine compounds such as lindane, DDT, PCBs, and PCDFs may interfere with moulting in higher crustaceans, e.g. decapods (Weis and Mantel, 1976; Fingerman and Fingerman, 1977; Reddy and Rao, 1987). In more recent publications Baldwin et al. (1995) and Zou and Fingerman (1997) could demonstrate that estrogens affect the moulting process in *D. magna*, possibly by interacting with the ecdysone receptor. Wollenberger (2005) summarised these findings, and demonstrated that larval development in *A. tonsa* is more sensitive than moulting in *D. magna*. Ecdysteroids similar to vertebrate steroid hormones play a major role in the control of growth, development and reproduction in crustaceans including copepods (Gaertner et al., 2012). Therefore, these processes might be vulnerable to endocrine disrupters.

For amphipods it could be shown by Hyne (2011) that the ovarian and reproduction cycles of female gammaridean amphipods are closely correlated with the moult cycle, which is under direct control by the steroid hormone 20-hydroxyecdysone. Xenobiotics such as the fungicide fenarimol have been shown to elicit endocrine disruption in some crustaceans by acting as an agonist of 20-hydroxyecdysone at the ecdysone receptor or by inhibiting the synthesis of 20-hydroxyecdysone, respectively, resulting in disruption of moulting and reproduction. Thus, there are several indications that the histological alterations in the reproductive organs observed here are a consequence of an interaction of test chemicals with the ecdysone receptor in the same way as they interact with the sex hormone receptor in vertebrates.

5. Conclusion

Regardless fundamental differences in the endocrine system of arthropods and other phyla, the present study revealed that compounds with an androgenic potential in vertebrates are capable of interfering with the hormonal regulation in male and female *A. tonsa*.

In the present study, histopathological alterations in the calanoid copepod *A. tonsa* were found following exposure to MET, LET and FEN at low concentrations (1.6–1000 $\mu g\ L^{-1}$), and TPT at environmentally relevant concentrations (0.0014–0.0088 $\mu g\ L^{-1}$ TPT-Sn). Stimulating or inhibiting effects on developing gametes in male and female *A. tonsa* were found following exposure to the four compounds acting as androgenic in vertebrates. These effects were reflected in atrophic or hypertrophic processes rather than in cell damages leading to cell death. No associated necrotic processes, which would indicate general toxicity, occurred. Moreover, effects on histology were exclusively found in the reproductive system and were not accompanied by any pathological alterations in other organs. The dominant inhibitory effects in females deserve further attention. It would require mechanistic studies to attribute certain responses confidently as being endocrine-mediated. The observed effects on *A. tonsa* may be associated with the strong interaction of reproduction and molting in crustaceans possibly by interference with the ecdysone receptor leading to histological as well as morphological alterations as described in the cited literature.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.03.053.

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