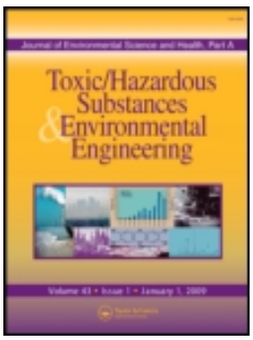

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Effects of anti-androgens cyproterone acetate, linuron, vinclozolin, and *p,p′*-DDE on the reproductive organs of the copepod *Acartia tonsa*

Burkard T. Watermann[a], Triantafyllos A. Albanis[b], Silvana Galassi[c], Katarina Gnass[a], Kresten O. Kusk[d], Vasilios A. Sakkas[b], and Leah Wollenberger[d]

[a]Laboratory for Aquatic Research and Comparative Pathology (LimnoMar), Hamburg, Germany; [b]Laboratory of Industrial Chemistry, Department of Chemistry, University of Ioannina, Ioannina, Greece; [c]Department of Biology, University of Milan, Milan, Italy; [d]Department of Environmental Engineering, Technical University of Denmark, Kgs. Lyngby, Denmark

ABSTRACT

The study was performed to detect the effects of anti-androgenic compounds on the reproduction. In this paper alterations observed in the marine calanoid copepod *Acartia tonsa* exposed to environmental concentrations of cyproterone acetate (CPA), linuron (LIN), vinclozolin (VIN), and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (*p,p′*-DDE) for 21 days covering a full life cycle are described. Histological alterations were studied with a focus on reproductive organs, gonad and accessory sexual glands. Exposure to $\geq$1.2 μg L^{-1} CPA caused degeneration of spermatocytes and deformation of the spermatophore in males. In a single male exposed to 33 μg L^{-1} CPA, an ovotestis was observed. In CPA exposed females, enhancement of oogenesis, increase in apoptosis and a decrease in proliferation occurred. Exposure of males to $\geq$12 μg L^{-1} LIN caused degenerative effects in spermatogonia, spermatocytes and spermatids, and at 4.7 μg L^{-1} LIN, the spermatophore wall displayed an irregular formation. In LIN exposed females, no such structural alterations were found; however, the proliferation index was reduced at 29 μg L^{-1} LIN. At an exposure concentration of $\geq$100 μg L^{-1} VIN, distinct areas in male gonad were stimulated, whereas others displayed a disturbed spermatogenesis and a deformed spermatophore wall. In VIN exposed female *A. tonsa*, no effects were observed. Male *A. tonsa* exposed to *p,p′*-DDE displayed an impairment of spermatogenesis in all stages with increased degrees of apoptosis. In *p,p′*-DDE-exposed females, a statistical significant increase of the proliferation index and an intensification of oogenesis were observed at 0.0088 μg L^{-1}.

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Comparative pathology; crustacea; endocrine disruption; histology; invertebrate

Introduction

Physiological processes in organisms at a higher level of organization are dependent on endocrine regulation. Growth, development, sexual differentiation and reproduction in crustaceans rely on ecdysteroids, terpenoids (juvenile hormones) and peptides, e.g. the androgenic gland hormone is responsible for development of male primary and secondary sexual characteristics.[1,2] Vertebrate-type steroids have been identified in crustacea; however, their origin and physiological role have not been clarified yet.[3,4] Studies with the calanoid copepod *Acartia tonsa* have shown that molting and time to sexual maturity responded very sensitively to compounds known to act as endocrine disrupters in vertebrates.[5–8] In a previous publication, we reported on alterations in the gonad and accessory sexual glands of *A. tonsa* exposed to compounds acting as androgens in vertebrates, namely methyltestosterone, letrozole, triphenyltin, and fenarimol, and gave a detailed description of the male and female reproductive systems of *A. tonsa*.[9]

The aim of this study was to investigate whether compounds with anti-androgenic potency in vertebrates would induce comparable effects in a crustacean. *A. tonsa* was therefore exposed to cyproterone acetate (CPA), linuron (LIN), vinclozolin (VIN), and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (*p,p′*-DDE).

CPA is pharmaceutical specifically synthesized to exert anti-androgenic effects through antagonistic interaction with androgen receptors and is primarily applied for the treatment of prostate tumors.[10,11]

The herbicide LIN was shown to be a weak competitive androgen receptor antagonist in rats, where it induced a positive response in the Hershberger assay and suppressed androgen-dependent gene expression.[12–14]

VIN is applied as non-systemic dicarboximide fungicide on fruit and vegetables to prevent spore germination and was one of the first endocrine disrupting chemicals reported to be an anti-androgen in vertebrates.[11,15] VIN is a potent androgen receptor antagonist *in vitro* and *in vivo* in mammals.[16]

p,p′-DDE is a metabolite of DDT, which is still used in some countries to control malaria-transmitting mosquitoes.[17] The anti-androgenic action of *p,p′*-DDE in vertebrates was first reported by Kelce et al.[18]

CONTACT Burkard T. Watermann watermann@limnomar.de Laboratory for Aquatic Research and Comparative Pathology (LimnoMar), Bei der Neuen Muenze 11, Hamburg D-22145, Germany.

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In arthropods, *p,p′*-DDE was shown to act as an anti-ecdysteroid *in vitro*.[19] In echinoderms, DDE and CPA interfered with the regulation of the reproductive and regenerative systems.[20,21]

Acartia tonsa was exposed to the four model anti-androgens for an entire life cycle, including periods of gonad development and reproduction. Histological investigations of reproductive organs, gonads and accessory sexual glands as well as the digestive tract, including the hindgut acting as respiratory organ, the nervous system and the musculature were performed subsequently to routine staining and immunolabeling for detection of apoptosis and proliferation activities.

Materials and methods

Chemicals

CPA (purity >98%) was obtained from Sigma-Aldrich (Steinheim, Germany). VIN (99.6%), *p,p'*-DDE (99.6%) and LIN (99.5%) were obtained as analytical standards from Riedel-de-Häen (Seelze-Hannover, Germany). Analytical grade chemicals for preparation of media were purchased from Merck KGaA, Darmstadt, Germany. LIN metabolite standards (>98%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and were used without further purification.

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.[22] Ethanol concentrations were the same at different exposure concentrations within each test and did not exceed 100 μL L^{-1} (79 mg L^{-1}) in any of the experiments, which is well below the LC10 of 390 mg L^{-1} we obtained in the adult toxicity test with *A. tonsa* according ISO 14669.[23] Additional solvent controls were included.

Exposures

Full life cycle exposures were conducted with *A. tonsa* starting with eggs and extending for 3 weeks to reproductively active adults following an Organization for Economic Co-operation and Development (OECD) guideline proposal.[24] Exposures were started by adding 600 *Acartia* eggs to 1000 mL vessels containing 800 mL of test solution. Three replicates were used for each concentration. The vessels were slightly aerated with sterile filtered air, and 80% of the medium was renewed 3 times a week. A fully defined synthetic saltwater medium with a salinity of 18‰ was used.[25] The unicellular alga *Rhodomonas salina* served as feed organism and was added to a density of 5×10^4 cells mL^{-1} subsequently to each medium renewal. Copepods were exposed for 21 days at 20 ± 0.5°C and a photoperiod of 16 h light followed by 8 h darkness was applied. Oxygen saturation and pH were monitored in fresh and old solutions each time the medium was renewed. The oxygen saturation in all experiments was >70% and the pH was within 8.3 ± 0.2; thus, the validity criteria specified in the draft OECD guideline proposal were met.

Exposure concentrations were selected in the range of environmental concentrations reported in the literature.[26–29] Exposure concentrations were as follows: (μg L^{-1} VIN) 40, 100, 240, 600, and 1480; (μg L^{-1} *p,p′*-DDE) 0.00055, 0.0014, 0.0035, 0.0088 and 0.022; (μg L^{-1} CPA) 1.2, 3.7, 11, 33 and 100; (μg L^{-1} LIN) 1.8, 4.7, 12, 29 and 72. For range-finding purposes, adult acute toxicity tests and early-life stage development tests were performed according to international guidelines.[23,25] As a general rule, the highest concentration to be tested was set to about 50% of the LC10 obtained in the 48 h adult acute toxicity test. For DDE and LIN, these levels correspond to environmentally relevant concentrations reported at levels of 0.001–100 ng L^{-1} DDE in Chinese coastal waters and LIN in the range of 0.06–1.83 μg L^{-1} in Canadian coastal waters.[30,31] Environmental concentrations of CPA were reported to reach levels up to 74 μg L^{-1}, and concentrations of 0.025 μg L^{-1} VIN were reported from agricultural areas.[27,28] Chemical analyses of test compounds in the exposure media were performed for *p,p′*-DDE and LIN. Concentrations refer to time-weighted means for *p,p′*-DDE and LIN and to nominal concentrations for VIN and CPA. Time-weighted mean concentrations were calculated as described in OECD Guideline 211.[32]

Chemical analyses

1,1-Dichloro-2,2-bis(p-chlorophenyl)ethylene

Water samples were extracted by liquid–liquid extraction adding *n*-hexane 1:10 directly to the glass bottles where the samples were stored. In this way, losses from adsorption onto the vessel walls were minimized. The two phases were stirred for 30 min, followed by two washings with small amounts of *n*-hexane for 15 min each. Hexane extracts were recovered after settling the phases by a separating funnel and concentrated to 1 mL.

Analyses were performed by injecting 1 μL extract in a gas chromatograph (Carlo Erba 8000 Top) equipped with a ^{63}Ni electron capture detector (ECD) at 300°C. The analyses were carried out under the following conditions—injection system: on-column (injection volume: 1 μL); column: CP-Sil-8 CB, 50 m × 0.25 mm ID, film thickness: 0.25 μm. Temperature program: 60–180°C, 20°C min^{-1}; 180–250°C, 4°C min^{-1}; 250–280°C, 2°C min^{-1}; followed by 280°C for 25 min. Helium was used as carrier gas (1 mL min^{-1}) and nitrogen as auxiliary gas (30 mL min^{-1}).

Quantification of *p,p′*-DDE was performed using an external reference standard solution (Dr. Ehrenstorfer, Germany) with a known concentration of *p,p′*-DDE (10.2 μg L^{-1}). The limit of quantification was 0.5 ng L^{-1}.[33]

Linuron

A solid-phase extraction (SPE) procedure for isolation and preconcentration of LIN was used. Empore™ extraction discs of 47 mm diameter containing 500 mg of styrene-divinylbenzene and 500 mg of C18 (octadecyl) polymer were purchased from 3 M (St. Paul, MN, USA). Filter Aid (FA 400) was also purchased from 3M. Methanol, dichloromethane, acetone, ethyl acetate, hexane, isooctane and toluene were trace analysis grade from Pestiscan (Labscan Ltd., Dublin, Ireland).

Prior to extraction, methanol modifier (0.25 mL) was added to 250 mL of water sample. A Chromabond® C18 Hydra column was placed in a conventional filtration apparatus and washed with 2 × 6 mL of solvent mixture, dichloromethane: acetone (1:1, v/v), under vacuum to remove residual bonding agents. It was then preconditioned with 6 mL of MeOH, with no vacuum applied for 3 min to ensure that the sorbent was soaked in MeOH. Furthermore, the cartridge was equilibrated with 6 mL of HPLC-grade water, and the sample was mixed well and percolated through the SPE column under vacuum at a flow rate of 5 mL min^{-1} by means of a peristaltic pump. After the sample had passed through the column, the sorbent bed was left to dry under vacuum for 5 min. Sorbed compounds were eluted using 2 × 6 mL of solvent mixture dichloromethane:acetone (1:1, v/v) as eluent. The eluate was evaporated to dryness under a gentle stream of nitrogen, and the dry residue was redissolved in a final volume of 0.05 mL acetonitrile:water (50:50) and vortex mixed for 15 s prior to LC analysis.

HPLC-UV/DAD: The HPLC system consisted of a Shimadzu (Kyoto, Japan) model LC-10ADVp pump associated with a 7725i Rheodyne six-port valve and a Shimadzu Model SPD-10AVp UV–vis diode array detector connected to a Shimadzu model Class-VP 5 integrator. The analytes were separated by means of a Discovery™ C18 (250 × 4.6 mm ID: 5 μm) analytical column from Supelco (Bellefonte, PA, USA) that was fitted with a guard column cartridge of the same composition. The detector was set at 250 nm. Gradient elution was performed by increasing the percentage of acetonitrile in water from 10% to 70% over the first 20 min and then to 100% over a 2-min period. This composition was maintained for 2 min, after which time the initial solvent conditions were restored using a linear ramp over a 3-min period. The column was equilibrated for an additional 5 min before the next sample injection. The flow rate was 1 mL min^{-1} and the volume injected was 20 μL. The oven temperature was set to 40°C. Linearity for LIN was studied by pre-concentrating exposure water samples spiked at different concentrations. Recoveries of LIN were over 83%, with relative standard deviations lower than 10% in all cases. Correlation coefficients were higher than 0.9994 yielding detection limits (S/N = 3) as low as 0.004 μg L^{-1}.

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, La Jolla, CA, 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.

Histology

Fixation

Twenty-one-day-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 (Micron). As it was not possible to orientate all *Acartia* specimens in parallel to the section level, the proportion of animals cut in longitudinal sections varied. Animals cut in more transversal sections were not suitable for further evaluation. Thus, the numbers of animals subjected to histological investigation and evaluated in stained sections differed between exposure groups (Table 1). To prepare histological sections of *A. tonsa* is a difficult task due to the small size of this species. The longitudinal sections were microscopically examined for pathological alterations of the digestive tract (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).

For a detailed description of the immunocytochemistry techniques, see the work by Watermann et al.[9]

Results

Chemical analysis

Nominal *p,p′*-DDE concentrations ranged from 0.002 to 0.2 μg L^{-1} with a factor of 2.5 between concentrations. Measured concentrations in freshly prepared solutions amounted to 26% of the nominal concentrations as an average. In samples taken just before media renewal (every second day), the average concentration was only 4.7% of the nominal. For *p,p′*-DDE, a time-weighted mean of 12% of the nominal concentrations was calculated.

Nominal LIN concentrations were in the range of 2–200 μg L^{-1} with a factor of 2.5 between concentrations. Measured concentrations in freshly prepared solutions amounted to 38% of the nominal concentrations as an average. In samples taken just before media renewal, an average of 34% of the nominal concentrations was found. Thus, a time-weighted mean of 36% of the nominal concentrations was calculated. The difference between the nominal concentration and the measured concentration was attributed to absorption and degradation processes which were not specified further.

Histology

The normal histology of *A. tonsa* is given in Figure 1a and b, which provides an overview of the respiratory organs, the digestive tract, the hepatopancreas, the muscular system and the reproductive organs of an adult male and female. Deviation from the normal structure of the internal organs except for the reproductive system was detected for none of the animals exposed to test compounds.

Cyproterone acetate

A total of 238 exposed specimens were subjected to histological evaluation, with 35 males and 177 females, whereas 44 females and 2 males from controls were evaluated (36 specimens could not be sexed) (Table 1).

In the gonad of males exposed to 1.2 and 33 μg L^{-1} CPA, 28% of primary spermatocytes displayed degenerative alterations. In the gonad of males exposed to 1.2 μg L^{-1} CPA, several

Table 1. The number of *A. tonsa* processed and evaluated for histological evaluation.

CPA (μg L^{-1})	Total number per concentration	Females	Males	Sex not determinable
Control	56	44	2	10
1.23	38	30	8	0
3.7	42	36	6	0
11.1	36	26	3	7
33.3	62	46	7	9
100.0	60	39	11	10
Total	294	221	37	36
LIN (μg L^{-1})	Total number per concentration	Females	Males	Sex not determinable
Control	56	28	9	19
1.80	26	17	6	3
4.68	33	15	6	12
11.52	66	33	10	23
28.80	48	27	10	11
72.00	35	20	12	3
Total	264	140	53	71
VIN (μg L^{-1})	Total number per concentration	Females	Males	Sex not determinable
Control	33	14	5	14
0.04	31	22	6	3
0.1	36	13	5	18
0.24	22	11	5	6
0.6	44	13	6	25
1.4	18	14	4	0
Total	184	87	31	66
p,p″-DDE (μg L^{-1})	Total number per concentration	Females	Males	Sex not determinable
Control	53	33	7	13
0.00055	25	21	4	0
0.00143	42	19	8	15
0.00352	42	21	5	16
0.0088	67	38	3	26
0.022	17	13	4	0
Total	246	145	31	70

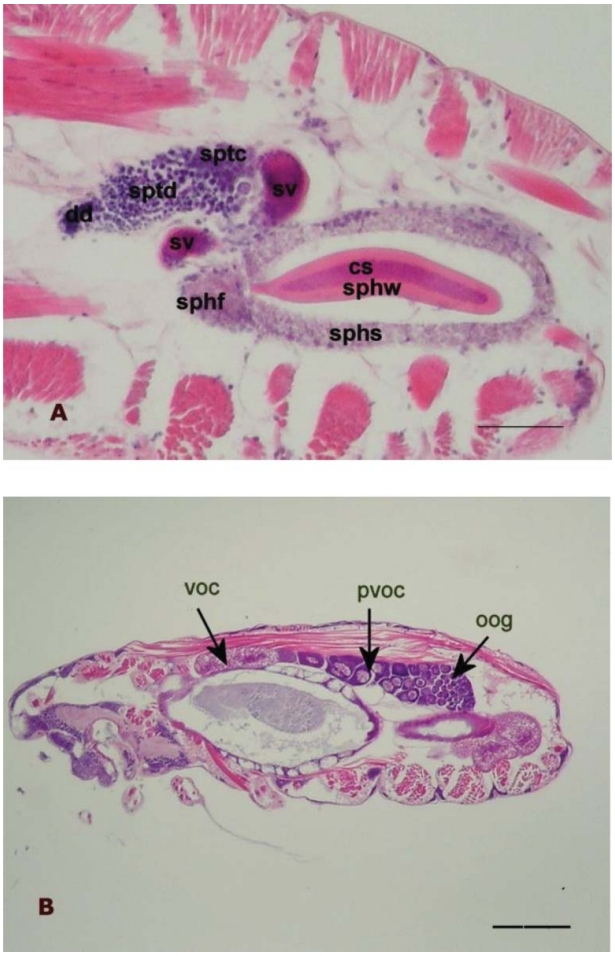


Figure 1. (a) Normal male reproductive system (sptc = spermatocytes, sptd = spermatids, dd = ductus deferens, sv = seminal vesicle, sphf = spermatophore former, sphw = spermatophore wall, sphs = spermatophore sac proper, cs = core secretions, H&E, bar = 50 μm). (b) Normal female reproductive system (oog = oogonia, pvoc = previtellogenic oocytes, voc = vitellogenic oocytes, H&E, bar = 100 μm).

spermatocytes displayed a weak positive apoptotic reaction. The spermatophore wall was of irregular shape and height (Fig. 2a).

At 33 μg L^{-1} CPA, in the testis of several males, numerous vacuolated spermatocytes with disturbed meiosis were present along with normal spermatogonia in active and undisturbed mitosis. In a single male exposed to 33 μg L^{-1}, an ovotestis with vitellogenic oocytes was present in an active testis containing spermatocytes and spermatids (Fig. 2b).

In all females exposed to CPA, maturation of oocytes was evident. At 1.2 μg L^{-1}, singular vitellogenic oocytes displayed irregular enlarged sites of yolk synthesis around the nucleus. At 33 μg L^{-1}, several oogonia underwent apoptosis, whereas vitellogenic oocytes were of normal structure (Fig. 2c). The proliferation index was slightly reduced at all concentrations tested; the reduction at 33 μg L^{-1} was statistically significant (Fig. 2d). The control animals of both sexes displayed active and undisturbed gametogenesis.

Linuron

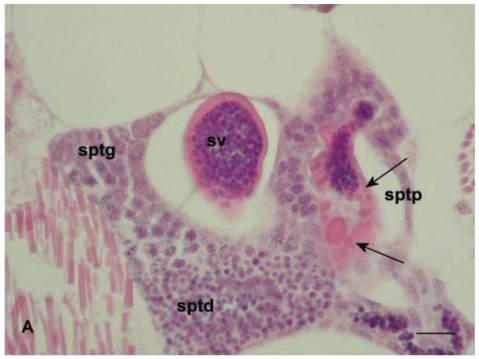
A total of 208 exposed specimens with 44 males and 112 females, with 28 females and 9 males as controls (71 specimens could not be sexed), were subjected to histological investigation (Table 1).

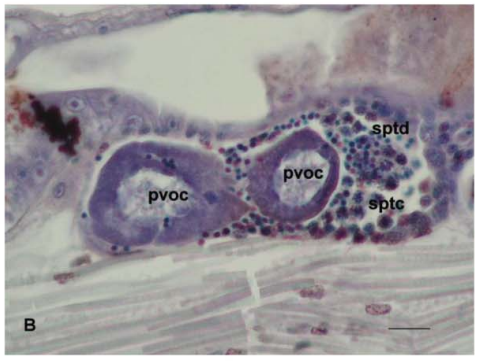
In the male gonad at 12 and 29 μg L^{-1}, spermatocytes in 60% of males were pale and contained a fading nucleus. The spermatids displayed a vacuolated cytoplasm and marginal nuclei in 30% of the individuals (Fig. 3a). At 4.7 μg L^{-1}, an irregular formation of the spermatophore wall was observed in 30% of the males. At a concentration of 72 μg L^{-1} LIN, the gonad of 36% males appeared unaffected; in 27% of testes, spermatogonia were absent. The spermatids displayed a translucent and vacuolated cytoplasm and clumped chromatin.

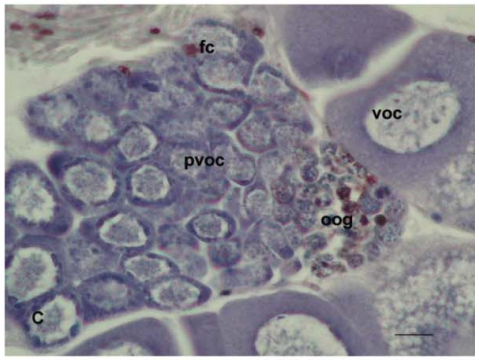
In the female gonad at a concentration of 29 μg L^{-1} LIN, a reduced proliferative activity was present in 30% of individuals. In parallel, the proliferation index was lowest at this concentration (Fig. 3b). The control animals of both sexes displayed active and undisturbed gametogenesis.

Vinclozolin

At 100–1480 μg L^{-1} VIN, a fairly high number of animals were dead before fixation and thus excluded from histological evaluation. As a consequence, the number of specimens evaluated was relatively low (184) varying from 18 to 33 in







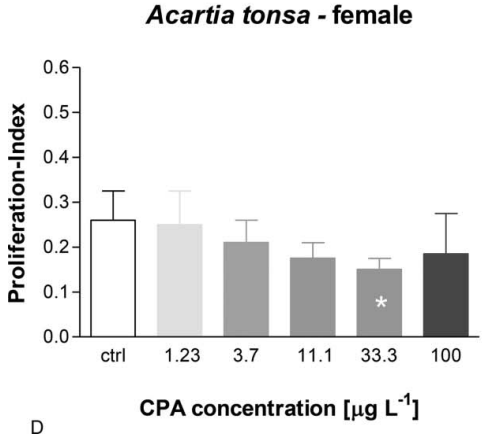
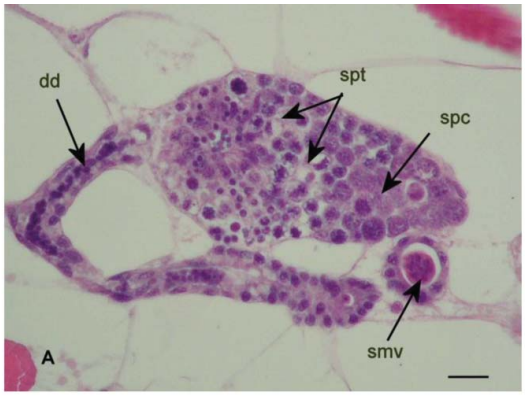


Figure 2. (a) Male gonad of *A. tonsa* exposed to 1.2 μg L^{-1} CPA with slightly vacuolized spermatocytes and disturbed formation of the spermatophore (sptp) wall (arrow). H&E, spct = spermatocytes, spt = spermatids, duc = vas deferens, bar = 10 μm. (b) Male gonad of *A. tonsa* exposed to 33 μg L^{-1} CPA with two pre-vitellogenic oocytes (pvoc), H&E, sptd = spermatids, sptc = spermatocytes, ss-DNA apostain, bar = 10 μm. (c) Female gonad of *A. tonsa* exposed to 33 μg L^{-1} CPA with untypical apoptotic oogonia and apoptotic follicle cells. Pvoc = pre-vitellogenic oocytes, voc = vitellogenic oocytes, fc = follicle cell, ss-DNA apostain, bar = 10 μm. (d) Female *A. tonsa* exposed to CPA, proliferation-index = number of proliferating oogonia/total number of oogonia. Statistics: Kruskal–Wallis test with Dunn's multiple comparison test, significant difference between the control and the exposure of 33 μg L^{-1}, level of significance $P < 0.05$, $n = 8$–10 per group*.



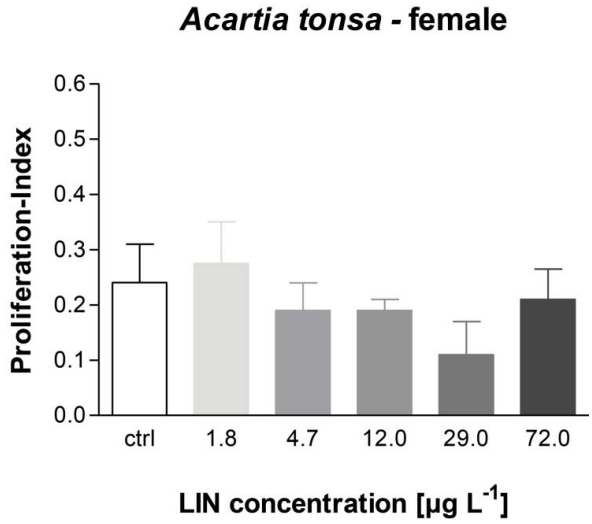


Figure 3. (a) Male gonad of *A. tonsa* exposed to 12 μg L^{-1} LIN with pale spermatocytes (arrows). sptc = spermatocytes, sptd = spermatids, dd = ductus deferens, sv = seminal vesicle, H&E, bar = 10 μm. (b) Female *A. tonsa* exposed to LIN, proliferation-index = number of proliferating oogonia/total number of oogonia. Statistics: Kruskal–Wallis test with Dunn's multiple comparison test, no significant difference, level of significance $P > 0.05$, $n = 8$–10 per group.

each group. 26 exposed males and 73 females with 14 control females and 5 males (in 66 specimens no gonads were present) were subjected to the histological evaluation (Table 1). The proliferation activity in most of the male gonads was fairly heterogeneous and varied from areas with very high activity to those with extremely low activity. Multiple spermatocytes exhibited a pale and slightly vacuolated cytoplasm at concentrations of ≥100 μg L^{-1}. At 240 μg L^{-1} VIN, some accessory cells in the male gonad showed apoptotic figures (Fig. 4a). In males exposed to 240 μg L^{-1} VIN, singular spermatocytes with vacuolated, pale cytoplasm were observed, and more with a higher percentage of such spermatocytes in the testis of males exposed to 1480 μg L^{-1} VIN. Most of the spermatogonia displayed a pale cytoplasm and were devoid of a distinct nucleus, along with decreased numbers of spermatids.

At 240 and 600 μg L^{-1} VIN, irregular secretions of the seminal wall and the spermatophore wall were observed, leading to spermatophores with an irregular outer surface.

In the female gonad, no alterations or pathological changes were observed. The proliferation index was insignificantly

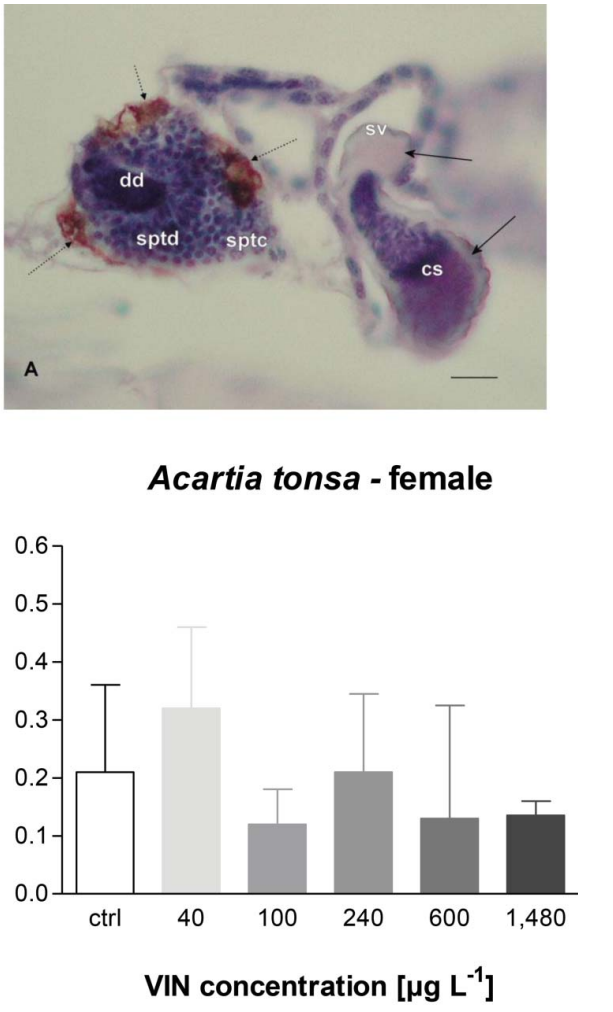


Figure 4. (a) Male gonad of *A. tonsa* exposed to 240 μg L^{-1} VIN with apoptotic accessory cells in the periphery of the gonad (dotted arrows) and a deformed spermatophore wall (arrows). sptc = spermatocytes, sptd = spermatids, dd = ductus deferens, sv = seminal vesicle, cs = core secretions, ss-DNA apostain, bar = 10 μm. (b) Female *A. tonsa* exposed to VIN, proliferation-index = number of proliferating oogonia/total number of oogonia. Statistics: Kruskal–Wallis test with Dunn's multiple comparison test, no significant difference, level of significance $P > 0.05$, no statistics with exposure of 1480 μg L^{-1} as n was too small, n = 6–10 per group.

higher at 40 μg L^{-1} compared to the control, and lower at 240 and 1480 μg L^{-1} VIN (Fig. 4b). The control animals of both sexes displayed active and undisturbed gametogenesis.

1,1-Dichloro-2,2-bis(p-chlorophenyl)ethylene

A total of 193 exposed specimens, with 24 males and 112 females with 33 females and 7 males as controls (70 specimens could not be sexed) exposed to *p,p′*-DDE, were subjected to histological evaluation (Table 1).

In the males exposed to 0.00055 μg L^{-1}, the structure of the testis was altered in one out of four males with the following features: primary and secondary spermatocytes displayed a pale cytoplasm and apoptotic cell death. The prevalence of meiotic figures decreased with increasing concentrations. Spermatocytes were the dominant cell type with intercellular spaces in the gonad (Fig. 5a). A quite similar effect was observed at 0.0014 μg L^{-1} where singular spermatocytes in apoptosis occurred and intercellular spaces appeared (Fig. 5b).

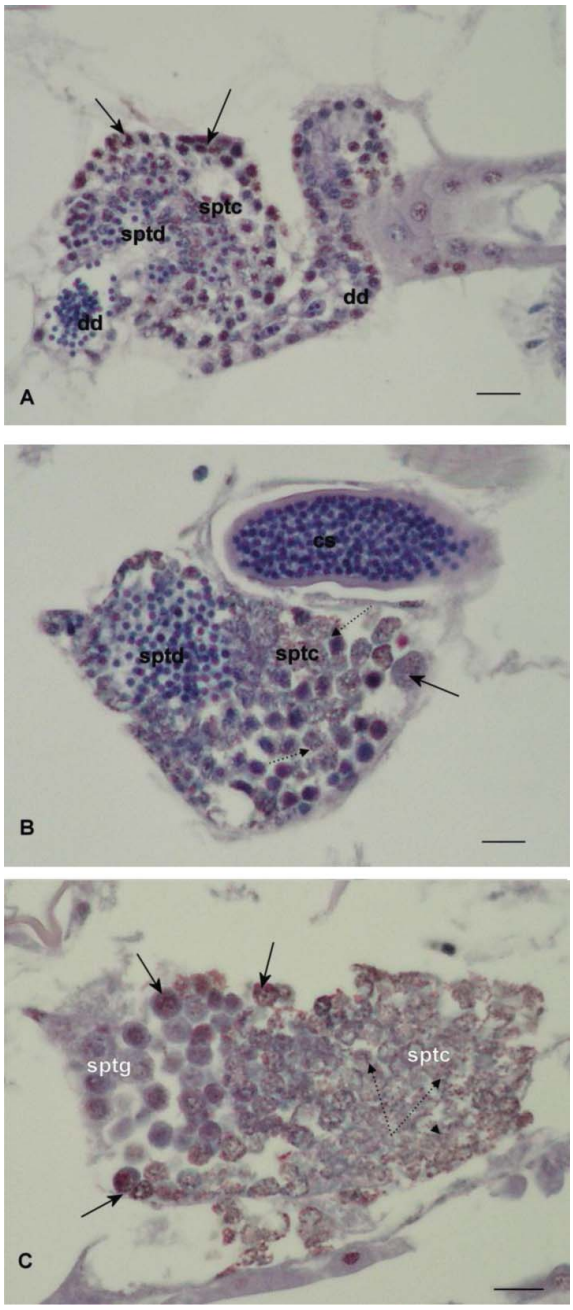


Figure 5. (a) Male gonad of *A. tonsa* exposed to 0.00055 μg L^{-1} DDE with disarrangement of gonadal structure and apoptotic spermatocytes (arrows). sptc = spermatocytes, sptd = spermatids, dd = ductus deferens, ss-DNA apostain, bar = 10 μm. (b) Male gonad of *A. tonsa* exposed to 0.0014 μg L^{-1} DDE with enlarged spermatogonia (arrow), degenerating spermatocytes (dotted arrows) and large intercellular spaces. sptc = spermatocytes, sptd = spermatids, cs = core secretions, ss-DNA apostain, bar = 10 μm. (c) Male gonad of *A. tonsa* exposed to 0.022 μg L^{-1} DDE with apoptotic spermatogonia (arrows) and necrotic unlabelled spermatocytes (dotted arrows). sptg = spermatogonia, sptc = spermatocytes, ss-DNA apostain, bar = 10 μm. (d) Spermatophore of male *A. tonsa* exposed to 0.0014 μg L^{-1} DDE with reduced core secretions arrows. cs = core secretions, sptp = spermatophore, sphs = spermatophore sac, ss-DNA apostain, bar = 10 μm. (e) Female gonad of *A. tonsa* exposed to 0.0014 μg L^{-1} DDE with numerous dark stained oogonia displaying apoptotic figures. Note the prominent perinuclear sites of yolk synthesis (arrows). oog = oogonia, poc = pre-vitellogenic oocytes, voc = vitellogenic oocytes, ss-DNA apostain, bar 10 μm. (f) Female *A. tonsa* exposed to DDE, proliferation-index = number of proliferating oogonia/total number of oogonia. Statistics: Kruskal–Wallis test with Dunn's multiple comparison test, significant difference between the control and the exposure of 0.0088 μg L^{-1}, level of significance $P < 0.01$, no statistics with exposure of 0.022 μg L^{-1} as n was too small, n = 7–23 per group.

This type of alteration was even stronger in males exposed to 0.0035 μg L^{-1} where most spermatogonia, spermatocytes and spermatids showed apoptotic figures. In the center of the gonad, a remarkable intercellular space occurred due to the lack of spermatocytes in late meiosis. Males exposed to the highest applied concentration of 0.022 μg L^{-1} *p,p′*-DDE exhibited a gonad dominated by spermatocytes and devoid of spermatogonia and spermatids. The spermatocytes either displayed apoptotic figures or were necrotic indicated by negative apostain labelling (Fig. 5c). The apoptotic index was elevated in relation to the control at 0.00055 and 0.0014 μg L^{-1} *p,p′*-DDE (Table 1).

At concentrations of 0.0014–0.0088 μg L^{-1} *p,p′*-DDE, in 30% of males the spermatophore wall was irregular in shape. Additionally, the epithelial cells of the vesicula seminalis and of the spermatophore sac proper contained a vacuolated cytoplasm. The number and density of core secretions were clearly reduced at 0.0014 μg L^{-1} *p,p′*-DDE (Fig. 5d). In the female gonad at *p,p′*-DDE concentrations of 0.0014–0.0088 μg L^{-1}, an increase in the number and size of pre-vitellogenic and vitellogenic oocytes as well as a prominent yolk synthesis was present. Moreover, in 15% of females exposed to 0.00055 μg L^{-1} *p,p'*-DDE, tightly packed oogonia and oocytes were encountered. The perinuclear sites of yolk synthesis were abundant and enlarged in relation to the control. A few oogonia displayed apoptotic figures at 0.00055 μg L^{-1}. Females exposed to 0.0014 μg L^{-1} showed a more pronounced yolk synthesis with extended perinuclear sites of yolk formation compared to those exposed to 0.00055 μg L^{-1}.

At 0.0014 μg L^{-1}, approximately a third of oogonia displayed apoptotic figures (Fig. 5e). At a concentration of 0.0035 μg L^{-1} *p,p′*-DDE, apoptosis in oogonia was even more frequent.

The proliferation index of the female gonad followed a u-shaped dose response by increasing in relation to the control after exposure to 0.00055 and 0.0035 μg L^{-1}, significantly elevating at 0.0088 μg L^{-1} and decreasing again at 0.0014 μg L^{-1} (Fig. 5f). No pathological alterations were observed in the central organs and tissues. The control animals of both sexes displayed active and undisturbed gametogenesis.

Discussion

Acartia tonsa has been cultured in our laboratory for more than 20 years. The adult sex ratio in stock cultures was determined at regular intervals and was at all times skewed toward a dominance of females (about 60% females). It is known that calanoid copepods, which require only one mating to stay fertile, have strongly female-skewed sex ratios in field populations.[34]

Control animals displayed the typical structure of *A. tonsa* with no pathological alterations in any of the tissues or organs (Fig. 1a and b). In singular male specimens of the control groups, stages of protozoan parasites were encountered in the gonad causing a destruction of the reproductive system. It is known that protozoan parasites and other agents may induce intersex in copepods, which may reach regional levels of ~20%.[35] On this background, specimens with parasites were excluded from further histological evaluation.

Cyproterone acetate

In *A. tonsa*, CPA caused effects in both sexes. In males, primarily spermatocytes were affected and displayed a vacuolated cytoplasm and meiosis arrest. In one male exposed to 33 μg L^{-1}, oocytes were encountered in the testis. In addition to the effects in the gonad, the formation of the spermatophore wall was disturbed at the lowest applied concentration. In female *Acartia*, CPA enhanced the maturation of oocytes by prominent yolk production, but exerted negative effects on oogonia development at 33 μg L^{-1}, which is also reflected in the proliferation index.

CPA is one of the most potent anti-androgenic steroids, which exerts also gestagenic effects in rats. It is suspected that CPA inhibits the binding of androgens to their specific receptors and impairs *in vivo* the metabolism of testosterone in the accessory sexual glands.[36] In the testis of rats, CPA inhibits sperm production by blocking meiosis and spermatid differentiation.[37]

In ecotoxicological studies, CPA is useful as a positive control for anti-androgenic action. In the gastropod species *Marisa cornuarietis* and *Nucella lapillus*, CPA caused intersex and virilization of females.[38] In *D. magna*, CPA inhibited growth and subsequently brood size in a specific, molt-independent way at concentrations ranging from 0.5 to 2 mg L^{-1}.[2]

In fish, CPA induced endocrine effects in combination with other endocrine compounds, but not alone.[28]

Linuron

LIN, a herbicide applied to control a variety of annual weeds, is produced in the EU in more than 1000 tons $year^{-1}$ with extended expiring time until 2016.[39] It has been used as a positive control for anti-androgenicity in vertebrate testing.[40]

In the gonad proper of *A. tonsa*, LIN affected spermatogonia and spermatocytes at concentrations of 12 and 29 μg L^{-1}. Disturbances in the formation of the spermatophore wall were present at 12 μg L^{-1}. These findings correspond to those in mammals where the testis and the accessory sexual glands were affected by LIN.[40] Slight but statistically not significant effects on the proliferation activity in the gonad of female *A. tonsa* were recorded.

LIN induced Leydig cell adenomas in rats via disruption of the hypothalamic–pituitary–testicular axis by sustained hypersecretion of luteinizing hormone.[12] In another study after administration of 40 mg kg^{-1} day^{-1} LIN, seminal vesicle and cauda epididymal weights were reduced.[41] In addition, numerous epididymal and testicular malformations (agenesis of the caput and/or corpora epididymides, testis atrophy, hypotrophy of androgen dependent tissues) were noted in LIN exposed rats.[41] After administration of 100 and 150 mg kg^{-1} day^{-1} LIN, epididymis and prostate weight decreased.[37] In fish, conflicting results of anti-androgenic effects were reported; in some studies, the effects were only observed in combination with other endocrine compounds.[42]

Vinclozolin

VIN is known as a potent anti-androgen in mammals.[15,43] Demasculinization effects of VIN were evident in male *A. tonsa*

in spermatogenesis disturbance. In the accessory sexual glands, deformation of the spermatophore, irregular formation of the spermatophore wall and reduced formation of core secretions were observed. In the female gonad, VIN did not induce any alterations apart from a slight increase in the proliferation index at 40 μg L^{-1}.

In the cladoceran *D. magna*, VIN reduced the number of neonate males at 1 mg L^{-1}.[44] Interestingly, this sex ratio modulation in a crustacean corresponds to the anti-androgenic action of VIN in vertebrates.[43]

p,p′- *DDE*

In *A. tonsa*, exposure to environmental relevant concentrations of *p,p′*-DDE reduced in the male gonad, the number of intact spermatogonia, spermatocytes and At 0.0014 μg L^{-1}, the columnar epithelial cells of the ductus deferens were swollen and the spermatophore displayed a massive reduction in core secretions. Lower concentrations induced apoptosis in male germ cells, whereas higher concentrations induced necrosis. *p,p'*-DDE has little affinity to the estrogen receptor but inhibits binding to the androgen receptor (AR), androgen-induced transcriptional activity as well as androgen action in developing, pubertal and adult male rats.[18] After administration of 100 mg kg^{-1} day^{-1} *p,p′*-DDE, Sprague–Dawley rats displayed hypospadias and increased numbers of retained nipples.[38] After administration of 750 and 1000 mg kg^{-1} day^{-1} *p,p′*-DDE, rats developed testicular changes, which were characterized by disorganization of the testis and loss of germ cells within a few, randomly distributed tubules.[37]

In females exposed to 0.0014 μg L^{-1} *p,p′*-DDE, a promotion of oogenesis was evident, i.e. an increase in the number and size of oocytes as well as enhanced yolk synthesis. By contrast, at lower concentrations, some oogonia displayed apoptosis reflected in an increased apoptotic index. However, the proliferation index of the female gonad was equal to or lower than in the control but significantly elevated at 0.0088 μg L^{-1}.

Summary of histological effects

The effects on histology of *A. tonsa* exposed to anti-androgenic compounds at low concentrations occurred exclusively in the reproductive system. Various malformations of the reproductive organs and strong effects on developing gametes in males and females were present in animals exposed to environmental relevant concentrations. Atrophic or hypertrophic alterations were seen rather than cell damages leading to cell death. Histological studies alone do not allow elucidating the mode of action of compounds under investigation. Mechanistic studies would be required to ascertain whether the observed effects are endocrine mediated. The responses to anti-androgens found on the tissue and cellular level in *A. tonsa* in this study revealed surprising similarities with alterations induced by the same anti-androgenic compounds in vertebrates. In contrast to the proven occurrence of steroids in higher crustaceans, the proof of the existence of AR proteins is rare and restricted to decapods species.[45] It remains unclear whether copepods possess AR and what mechanism has caused the reactions to the anti-androgens tested. From a comparative point of view, these findings are interesting because germ cell differentiation in vertebrates is controlled by androgens and estrogens.[46,47]

Regardless of the fundamental differences in the endocrine system, the vertebrate anti-androgens investigated in this study affected targets in male and female *A. tonsa* analogous to those typically controlled by sexual steroids in other phyla up to mammals. The same holds true for the sexual accessory glands in males, which are under control of the androgenic gland hormone. Furthermore, the results suggest that the sexual accessory glands are under different hormonal regulations than the gonad proper—another aspect that is well known in other phyla.

Conclusions

Exposure of the calanoid copepod *A. tonsa* to compounds acting as anti-androgens in vertebrates induced various alterations of the reproductive organs and strong effects on developing gametes in males and females. Summarizing the effects of CPA, LIN, VIN and DDE on *A. tonsa*, most detrimental effects were observed in males.

Similar effects of these anti-androgens are known for vertebrates. These findings deserve even more attention as compounds acting as androgens in vertebrates exerted stimulatory effects in males and inhibitory effects in females of *A. tonsa*.[9] The effects observed on the tissue and the cellular level indicate a compound- and dose-dependent action, but cannot elucidate the mode of action or interference with metabolic pathways. Pathological disorders were exclusively found in the reproductive system. No deviations from the normal structure were observed in other organs, indicating that the concentrations used in the exposure were below the level caused by general toxicity.

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