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Laboratory Tests with Androgenic and Anti-Androgenic Pesticides – Comparative Studies on Endocrine Modulation in the Reproductive System of Invertebrates and Vertebrates

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1. Introduction

The acute toxicity and sublethal effects of pesticides are well established and published in a wealth of literature (Ecobichon, 2001). The endocrine potential of technical and agriculture biocides (pesticides) is less documented but gained considerable attention due to the fact that their impact on endocrine modulation was observed at much lower concentrations than observed for the induction of acute toxic effects. The first studies published on the endocrine potential of pesticides were *in vitro* or laboratory experiments with human breast cancer carcinoma cells (MCF-7) or hamster ovary cells (CHO K1) (Table 1 and 2). In ecotoxicology, terrestrial wildlife populations like birds or cats were primarily investigated. Aquatic species and invertebrates were largely ignored. Furthermore, the mode of action of pesticides at extremely low concentrations has not satisfactory be elucidated and validated. Regarding wildlife with numerous phyla and taxa, the mode of action has to be defined in each phylum as the receptors present in the mammalian kingdom may not be present in other phyla. Most of the results presented in this chapter have been gathered in the framework of the EU-Project COMPRENDO focussing on the understanding of the action androgenic and anti-androgenic compounds used as technical or agricultural biocides (Schulte-Oehlmann *et al.* 2006). Several compounds with these potentials have been selected to expose a broad spectrum of phyla from invertebrates to vertebrates.

Tributyltin compounds which were developed as molluscicides found their most wide spread application as antifouling biocide. Most authors link the androgenic potency of tributyltin oxide (TBT) to the inhibition of aromatase activity which was first detected in molluscs (Bettin *et al.* 1996). In addition, several other hypotheses of the mode of action of TBT can be found in the literature: inhibition of testosterone excretion, modulation of testosterone levels and effects on the release of neuropeptides (Oehlmann *et al.* 2007). Triphenyltin used as pesticides in potato culture can act as aromatase inhibitor (Schulte-Oehlmann *et al.*, 2000).

The systemic fungicide FEN is a potential androgen as it acts as aromatase inhibitor (Hirsch *et al.*1986, 1987), however, estrogenic activity was demonstrated as well (Andersen *et al.* 2002).

Compound	**Estrogenic**	**Anti-estrogenic**	**Active substance Compound/metabolites**
Captan		Androgen receptor binding	Compound
Chlordecon	Estrogen receptor binding	Androgen receptor binding	Compound
Chlorpyrifos	Estrogen receptor interaction		Compound
Deltamethrin	Estrogen receptor interaction		Compound
Dicofol	Estrogen receptor binding	Androgen receptor binding	Compound
Dieldrin	Estrogen receptor binding	Androgen receptor binding	Compound
Fenarimol	Receptor binding	Androgen receptor binding	Compound
Fenitothrion		Androgen receptor interaction	Compound
Iprodion	Aromatase stimulation		Compound
Methiocarb		Androgen receptor interaction	Compound
Methomyl	Aromatase stimulation		Compound
Methoxychlor	Estrogen receptor binding	Androgen receptor binding	Compound
Myclobutanil	Estrogen receptor binding	Androgen receptor binding	Compound
Nitrofen	Estrogen receptor binding	Receptor interaction	Compound
Primicarb	Aromatase stimulation		Compound
Prochloraz	Aromatase stimulation		Compound
Propamocarb	Aromatase stimulation		Compound
Tolclofos-methyl	Estrogen receptor interaction		Compound
Triadimefon		Receptor interaction	Compound
Tribenuron-methyl	Estrogen receptor interaction		Compound

Andersen *et al.* 2002, Okubo *et al*. 2004

Table 1. Mode of endocrine modulation *in vitro* of selected pesticides with of estrogenic or anti-estrogenic potential

Compound	**Androgenic**	**Anti-androgenic**	**Active substance Compound/metabolites**
DDE		Receptor Inhibition	Compound
Dichlorvos		Receptor Interaction	Compound
Endosulfan	Aromatase inhibition		Compound
Fenarimol (FEN)	Aromatase inhibition	Receptor Inhibition	Compound
Prochloraz	Aromatase inhibition		Compound
Tributyltin-oxide (TBT)	Aromatase inhibition		Compound
Triphenyltin-oxide (TPT)	Aromatase inhibtion		Compound
Vinclozolin (VIN)		Receptor Inhibition	M1/M2

Andersen *et al*. 2002, Körner *et al*. 2004, Okubo *et al.* 2004

Table 2. Mode of endocrine modulation *in vitro* of selected pesticides with androgenic or anti-androgenic potential

Vinclozolin is applied a as a non-systemic fungicide on fruit and vegetables where it prevents spore germination (US National Library of Medicine. 2006, Szeto *et al.* 1989) and was one of the first chemicals reported to be an anti-androgen (Gray *et al.* 1994). VIN itself has a poor affinity to the mammalian androgen receptor, however, in vivo it is hydrolyzed to two open-ringed metabolites, M1 (2-(3,5-dichlorophenyl)-carbamoyloxy-2-methyl-3-butenoic acid) and M2 (3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide) which act as androgen receptor antagonists by preventing transcription of androgen dependent genes (Kelce *et al.* 1994, Wong *et al*. 1995, Andersen *et al*. 2002).

The compound pp'-DDE (1,1-Dichloro-2,2-bis(4-chlorophenyl)ethylene) is the major metabolite of pp'-DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) which is still used in some African countries to control malaria transmitting mosquitoes (Nyarango *et al.* 2006). Pp'-DDE is even more persistent than DDT ($T_{½}$ = 2-20 years) and very bioaccumulative (log K_{ow} = 5.8). The anti-androgenic action of pp'-DDE was first reported by Kelce *et al.* (1995).

Methylurea-based compounds like diuron and linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) have been applied as herbicides to control a variety of annual weeds, was shown to be a weak competitive androgen receptor antagonist in vitro (Cook *et al.* 1993). They induced a positive response in the immature and adult rat Hershberger assay (Lambright *et al.* 2000), and suppressed androgen-dependent gene expression (McIntyre *et al.* 2000). It is relatively water soluble with a low potential for bioaccumulation (log K_{ow} 3.2) and listed as a possible human carcinogen (US National Library of Medicine 2006).

2. Endocrine modulation of pesticides with androgenic potential

2.1 Triphenyltin compounds

Laboratory experiments with TPT at concentrations of 100 – 500 ng/L revealed in two echinoderm species the potency to alter different reproductive parameters, such as gonad maturation and oocyte/egg development. Particularly, in both the sea urchin (*Paracentrotus lividus*) and the crinoid (*Antedon mediterranea*), TPT appeared to promote spermatogenesis and to inhibit oogenesis by stimulating the phagocytosis activity. In addition, TPT resulted to be an inhibitor of echinoderm oocyte development, as in both species cited it caused a significant size reduction. The androgenic activity of TPT observed on the reproductive endpoints was confirmed by the direct steroid level measurements carried out in parallel in the same exposed specimens. In fact, in both species the compound caused a significant increase of testosterone levels and a decrease of ethinylestradiol (Sugni *et al.* 2010).

TPT induced a concentration dependent decrease of P450-aromatase which was statistically significant at the highest TPT concentration tested (225 ng/L). Additionally, increased metabolism of testosterone to form dihydrotestosterone (DHT) and 5-androstane-3,17-diol was observed, suggesting increased 5-reductase activity in the gonads of TPT-exposed individuals (Lavado *et al.* 2006). At 100, 225 and 500 ng/L TPT females of *Paracentrotus lividus* displayed an increased percentage of oocytes with vacuolated ooplasm, up to 50% at the highest concentration. In parallel, the proliferation activity in the female gonad decreased (di Benedetto, 2003).

In crustacea the aromatase inhibitor TPT caused stimulating effects in the male reproductive system of *Acartia tonsa* at concentrations of 4.5 and 11 ng TPT-Sn/L, whereas at 28 ng TPT-Sn/L inhibiting effects were observed in the female gonad (Watermann *et al.* 2011a). In *A. tonsa,* adverse effects of TPT on oogenesis on the level number of oogonia, degeneration of previtellogenic and vitellogenic oocytes, yolk synthesis and maturation were evident at the two lowest exposure concentrations of 1.4 and 3.5 ng TPT-Sn/L. In contrast, at 22 ng TPT-Sn/L, the perinuclear sites of yolk formation were more prominent but irregular in shape. Thus, in males TPT exerted stimulation of the gonad at the lowest concentrations and an atrophic effect at the highest concentration. In *A. tonsa*, the apoptotic index of oogonia and oocytes was elevated compared to the control at all exposure concentrations. These observations indicate that degeneration and loss of oocytes were primarily due to apoptosis as it is known at deprivation of estrogens by aromatase inhibitors in mammals (Thiantanawat *et al.* 2003). In males of *A. tonsa* exposed to TPT, no disrupting effects on spermatogenesis were observed. In contrast, the proliferating activity of the gonad appeared more active in exposed groups than in the control. The latter was not quantifiable. However, in the accessory sexual glands like the spermatophore, the wall displayed irregular formation or hypertrophy along with reduced core secretions (Watermann *et al.* cit. op.).

The effects of TPT on molluscan species have been reported on a variety of species. In *Marisa cornuarietis* TPT induced several alterations in the female and male reproductive system. In males exposed to 30 ng/L TPT, in 40% of specimens the prostate was hypertrophic while the gonad was in a maturing or ripe stage. In males exposed to 250 ng/L TPT the gonad was ripe or spawned and no more spermatogenesis was present. In females exposed to 250 ng/L TPT the gonad contained in 50% of specimens singular atrophic oocytes. In 30% of specimens the albumen/capsule gland was transformed to a prostate. In females exposed to

500 ng/L TPT the size and number of follicles were reduced to 27.3% in relation to the control. Arrest of oogenesis was present in 72.7% of females. Oogonia were prematurely released from the follicle epithelium and floated in the follicle lumen. In 45% of females the albumen/capsule gland was transformed to a prostate gland. Morphological investigations in this species revealed after exposure to TPT concentrations in the range cited above the induction of imposex (Oehlmann *et al.* 2007).

In other molluscan species similar observations were published. In the females of the abalone *Haliotis gigantea*, it was a strong masculinizator agent, promoting spermatogenic processes within the ovary (Horiguchi *et al.* 2002), and in *Hinia reticulata* this compound induced ovary atrophy (Schulte-Oehlmann *et al.* 2000). In *Haliotis madama* a decrease in population size was registered following a displacement of male and female reproductive cycle in organisms exposed to TPT (Horiguchi *et al.* 2000). The interaction of TPT with the lipid metabolisms of the ramshorn snail *Marisa cornuarietis* at environmentally relevant concentrations of 30, 125, 500 ng/L as Sn in a semi-static water regime for 7 days was studied by Lyssimachou *et al.* (2009). Percentage of lipids and total fatty acid content decreased significantly in TPT-exposed females while the activity of peroxisomal acyl-CoA oxidase, involved in fatty acid catabolism, increased. In addition, fatty acid profiles (carbon chain length and unsaturation degree) were significantly altered in exposed females but not in males.

In fish laboratory experiments with a triazine pesticide revealed first indications of endocrine modulation on mature male Atlantic salmon (*Salmo salar*). Short term exposure to 2.0 – 20 µg/L atrazine impaired the mating abilities of male salmon (Moore & Waring, 1998). The most striking effect of TPT was observed in the female gonad of *Pimephales promelas*. The percentage of atretic oocytes in relation to the total number of oocytes was slightly decreased at 10, 32, and 100 ng/L, whereas at 1000 ng/L a significant increase could be observed (Figure 1). In female fish exposed to TPT a characteristic alteration of the shape of oocytes was encountered, described as pre-atretic oocytes progressing to atretic oocytes. The percentage of pre-atretic oocytes was significantly increased at 32 ng/L TPT in relation to the control. In the male liver the composition of stored material changed clearly in males into the direction of the dominant storage of fat instead of glycogen. Dependent on the selected concentration, TPT may act as an androgen and as an anti-androgen. In females of fathead minnow at concentrations of 10 – 100 ng/L TPT the percentage of atretic oocyte was decreased, at 1,000 ng/L it was significantly elevated (Figure 2). At all TPT concentrations elevated percentages of oocytes displayed indented chorion, indicating a pre-atretic stage, most pronounced at 32 ng/L. In parallel, the thickness of the chorion was very heterogenous leading to some oocytes with very thin chorion wall, most pronounced at 32 ng/L. On the other hand the percentage of postovulatory bodies was reduced at all TPT concentrations.

In another freshwater species *Rutilus rutilus* TPT induced in the female gonad an increased prevalence of atretic oocytes in relation to the control. Whereas the prevalence of atretic oocytes in the control was 10 – 12.5%, it increased to 33% at a concentration of 500ng/L (van Ballegoy & Watermann, unpublished).

Androgenic effects of TPT on amphibian species, e.g. the tadpole *Xenopus laevis* were observed in laboratory experiments. After exposure to concentrations of 0.04, 0.2, 0.4, 2.0, and 3.9 µg/L TPT males of this species exhibited a stimulation of spermatogenesis which was most pronounced in animals exposed to 0.04 µg/L TPT. The number of spermatocysts

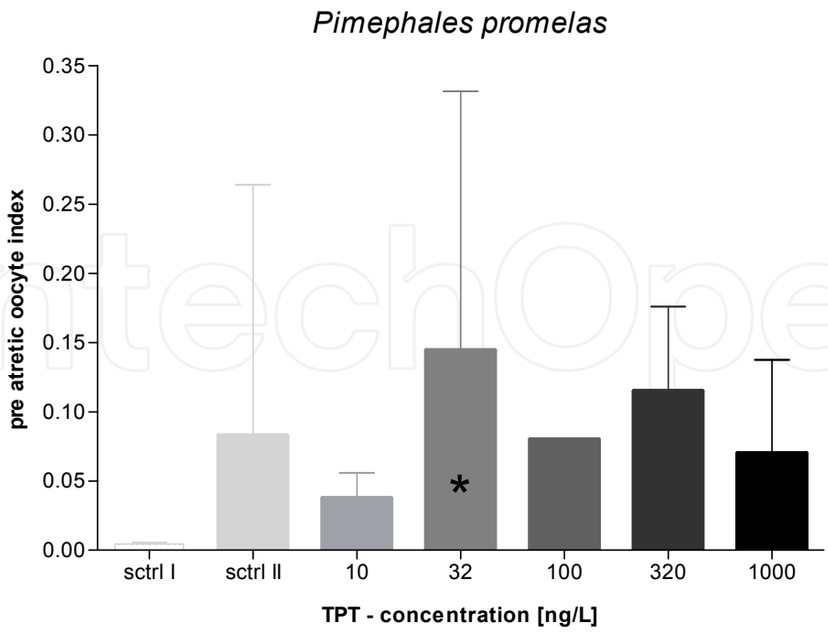


Fig. 1. *Pimephales promelas* exposed to triphenyltin, pre atreric index = number of pre atretic oocytes/total number of oocytes, 3 to 6 animals in one group, median & interquartile range, statistics: Kruskall Wallis with Dunn's Multiple Comparison Test, significant difference between solvent control I and exposure of 32 ng/L, level of significance P<0.05

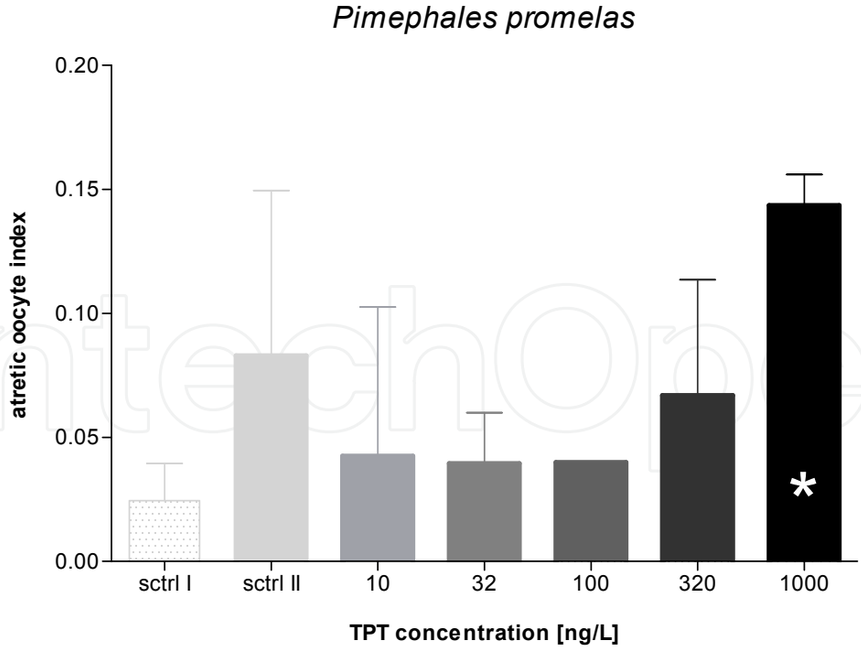


Fig. 2. *Pimephales promelas* exposed to triphenyltin, atretic index = number of tretic oocytes/total number of oocytes, 3 to 6 animals in one group, median & interquartile range, statistics: Kruskall Wallis with Dunn's Multiple Comparison Test, significant difference between solvent control I and exposure of 1000 ng/L, level of significance P<0.05

per gonad was elevated in relation to the control. In females exposed to the same range of concentrations of TPT, the number of follicles per animal was decreased in relation to the controls. The percentage of specimens with singular follicles was elevated with percentages of 12.5 - 25% at all concentrations. On the other hand, the size of the ovarian cavities increased with increasing concentrations (unpublished results).
In laboratory experiments with mammals, organotin compounds like TBT and TPT and pharmaceutical non-steroidal aromatase inhibitors like letrozole caused species-, developmental- and dose-dependent androgenic or anti-androgenic effects (Junker *et al.* 1994; Yu *et al.* 2004). In a recent laboratory study, TPT induced anti-androgenic effects in pubertal male rats. Administration of 2, 6, and 12 mg/kg/day decreased testis weight; epididymis and prostate weights were reduced at 6 and 12 mg/kg/day, and seminal vesicle weights at 6 mg/kg/day (Grote *et al.* 2004). In rats the histopathological effects of 2 or 6 mg TPT/kg b.w. on the reproductive tissue of female pubertal rats as part of a comprehensive pubertal assay. At both dose levels an increase in the number of all follicle stages was observed. Furthermore, exposure to 2 mg TPT/ kg b.w. led to a significant reduction in the diameter of tertiary follicles. A significant increase in the atretic index was observed in tertiary and pre-ovulatory follicles after exposure to 6 mg TPT (Watermann *et al.* 2009).

2.2 Tributlytin compounds

Publications on endocrine modulation by tributyltin compounds in echinoderms are rare. Probably the first report on endocrine effects of ingested TBT in echinoderms was the study of Mercier *et al.* (1994). Alterations in the female gonad of starfish (*Leptasterias polaris*) occurred at concentrations of TBT of 0.26 µg/g wet weight. Mature oocytes were smaller and the gonad possessed a thinner epithelium than in the control animals. Girard *et al.* (1997) reported on inhibition of sea urchin egg cleavage after exposure to TBT concentrations of 50 – 100 nM. In the Bay of Brest (France) the arrest and delay in embryonic development in sea urchin (*Sphaerechinus granularis*) was suspected to be linked with dumped pesticides and TBT (Quiniou *et al*. 1999).
In crustacea the first reports on endocrine effects of TBT were published by Johansen & Møhlenberg (1987). At concentrations of 10, 50 and 100 ng/L TBT the egg production of the copepod *Acartia tonsa* was reduced at 18, 19 and 37%. The authors pointed out that the selected concentrations were lower than found in Danish coastal waters. A study of Kusk & Petersen (1997) on acute and chronic toxicity of TBT in Acartia revealed inhibition of the developmental rate of larvae at 1 ng/L TBT.
The endocrine modulation of TBT in molluscs comprises a huge body of literature and was one of the first and intensively studied endocrine effects in invertebrates. More than 150 molluscan species worldwide were affected by the endocrine modulation induced by TBT. Deriving from laboratory experiments and in situ studies it was evident that the endocrine effects could be induced at extremely low concentration of 0.1 ng/L TBT (Oehlmann *et al.* 2007; Shi *et al*. 2005). In most species TBT is able to induce a phenomenon called imposex, the appearance of male sexual characteristics in females. In other species TBT can induce intersex phenomena which mean the presence of females with a female gonad and a progressive reduction of female sexual accessory organs, leading to a transformation into male accessory organs like prostate or rudimentary penis (Bauer *et al.* 1995, Watermann *et al.* 2008).

Molluscs can conjugate a variety of steroids to form fatty acid esters. The freshwater ramshorn snail *Marisa cornuarietis* was used to investigate sex differences in endogenous levels of esterified steroids. Testosterone and estradiol were mainly found in the esterified form in the digestive gland/gonad complex of *M. cornuarietis*, and males had higher levels of esterified steroids than females (4–10-fold). Exposure to TBT led to a decrease in both esterified testosterone (60–85%) and estradiol (16–53%) in females after 100 days exposure, but had no effect on the hormonal level in males (Janer et al. 2006). In contrast, histological investigations in male *Marisa cornuarietis* exposed to 60 and 250 ng/l TBT displayed disturbed spermatogenesis. Spermatogonia detached prematurely from the germinal epithelium in the tubules, spermatocytes and spermatids showed degenerative changes, whereas in singular tubules multinucleated giant cells of fused spermatids were visible. The prostate of males displayed at all concentrations enlarged, hypertrophied, and vacuolated gland cells. The surface of the penis sheath exhibited a low degree of invaginations and reduced numbers of mucous cells. In the gonad of females exposed to 30, 60, 125, 250 and 500 ng/L TBT several alterations in oogenesis were present. In 25 – 60% of females the oogonia detached prematurely of the follicle epithelium, floating in the lumen of the follicles. The albumen/capsule gland of up to 90% of females was transformed to prostate gland. The length of the vagina was reduced in 30 -50% of females in association with a reduced invagination of the vaginal epithelium.

In the amphibian species *Xenopus laevis* an advanced development and differentiation of the gonad was visible at a concentration of 33 ng TBT/L in relation to the control. At a concentration of 326 ng TBT/L approximately 30% of males straight tubules had increased in number and size compared to the control. In exposed females several indications of dedifferentiation and regression of the gonad could be observed. The percentage of animals with singular follicles increased slightly in relation to the control from a concentration of 160 – 3,255 ng/L TBT with 22.2% to 37.5%. The percentage of specimens with numerous follicles decreased from 80% at 16 ng TBT/L to 62.5% at 3,255 ng TBT/L. The size of the ovarian cavities decreased with increasing concentration of TBT. The resorption of oogonia was elevated at the lower concentrations between 3 and 33 ng TBT/L with prevalences of 37.5% and 31.6% respectively.

2.3 Fenarimol

A restricted number of laboratory studies were performed with echinoderms which elucidated a weak sensitivity to FEN. In females of *Paracentrotus lividus* at concentrations of 30 and 300 ng/L in the female gonad 50% and 18.2% respectively of animals exhibited enlarged, atrophic oocytes with vacuolated ooplasm. FEN was more effective on *A. mediterranea* specimens, altering both the maturative stage and reduced the oocyte size. In the crinoid species FEN seems to behave in agreement with its putative role of androgenic compounds, promoting male maturation, inhibiting oogenesis processes as well as inducing the production of smaller size oocytes. This FEN androgenic activity was partially confirmed by steroid measurement with an increase of testosterone in this specie. However in both the echinoderms also a marked estrogenic effect was detected, particularly in the crinoid, where an up to 10-fold E2 level increase was registered (Sugni *et al.* 2010).

In the crustacean copepod *Acartia tonsa* FEN caused disturbances of oogenesis on the level of oocyte differentiation and meiosis were observed at all concentrations (2.8 – 105 µg/L FEN).

However, the yolk production was not affected at 2.8 - 42 µg/L, but reduced at 105 µg/L. At 7.0 µg/L FEN. In exposed males, no alteration of spermatogenesis occurred, but the proliferation activity in the gonad was much more pronounced than in the control. In *Daphnia magna* FEN caused disturbances of the embryonic development and synergistic effects with testosterone (Mu and LeBlanc, 2005) as well as reduced fecundity (LeBlanc 2007).

In the molluscan species *Marisa cornuarietis,* males exposed to 100, 300 and 1000 ng/l FEN spermatogenesis was disturbed or even arrested in up to 75% of males. In numerous tubules aggregations of clumbed chromatin could be observed in spermatocytes and spermatids, indicating degenerating processes. The penis glands displayed degeneration of gland cells in association with an extreme dilatation and ramification of the gland ducts. In contrast, males exposed to 3000ng/l FEN displayed a normal gonad with active spermatogenesis. The female gonad displayed exposed to concentrations of 100, 300 and 1000 ng/L FEN disturbance and arrest in oogenesis with enlarged follicles. The follicle epithelium contained high amounts of lipo-pigments. In up to 75% of animals prematurely detached and degenerating oogonia were observed in the follicle lumen. The vagina of all specimens was reduced in length and the invagination of the vaginal epithelium was reduced. In contrast females exposed to 3000ng/l FEN displayed a normal gonad with active oogenesis. Exposure of Marisa cornuarietis lead to imposex induction (Oehlmann *et al.* 2007). Exposure to FEN and MT did not alter levels of esterified steroids in males or in females, although exposed females developed imposex after 150 days exposure.

In fish fenarimol induced in the female gonad of *Rutilus rutilus* elevated percentages of atresia in relation to the control (10 – 12%) with 16.7% (0.3 µg/L), 14.3% (1.7 µg/L) and 25% (3.3 µg/L). In the male gonad of this fish species a hypertrophy was observed with prevalence of 55.6% (1.7 µg/L) and 57.1% (3.3 µg/L).

Tadpoles exposed to concentrations of 3.312, 33.12, 165.6, 331.2 µg/L FEN showed a stimulation of spermatogenesis and disturbance of oogenesis. All males investigated displayed an early stage of gametogenesis with spermatocysts filled with spermatogonia, spermatocytes and early spermatids. In females the number of follicles decreased with increasing concentration of FEN. The percentage of singular follicles per specimen increased in relation to the control with 40% to 72.7 - 60% at concentrations of 165.6 - 331.2 µg/L FEN. In parallel the percentage of specimens with numerous follicles decreased in relation to the control with 60% down to 27 - 40% at concentrations of 165.6 - 331.2 µg/L FEN. The frequency of large ovarian cavities increased with increasing concentration compared to the control with a percentage of 20%, up to 63.6 - 50% at concentrations of 165.6 - 331.2 µg/L FEN. Correspondingly, the percentage of small ovarian follicles decreased with increasing concentrations.

In mammals FEN is acting as a potent inhibitor of the aromatase activity in the brain and ovary of rats, and in mammalian cell culture assays (Hirsch *et al.* 1987; Andersen *et al.* 2002). On the other hand, FEN binds both to the estrogen and androgen receptor in estrogen sensitive human breast cancer MCF-7 cells and is able to induce cell proliferation (Okubo *et al.* 2004). FEN prevents the increase of the number of nuclear estrogen receptors in the brain of male rats during the early postnatal period (Hirsch et al. 1987). This mechanism was supposed to cause a dose-dependent decrease in fertility in male rats (Hirsch *et al.* 1986).

3. Endocrine modulation of pesticides with anti-androgen potential

3.1 p,p'-DDE

In echinoderms several anti-androgenic effects were observed in a restricted number of species. In *Paracentrotus lividus* after exposure to 100, 500 and 2,500 ng/L DDE the density of ripe sperm in the tubules was reduced up to 67% of males at the highest concentration. DDE inhibited spermatogenesis and reduced the egg size in female *P. lividus* but enhanced the egg size in A*nterdon mediterranea* (Lavado *et al.* 2010). In experiments on echinoderm regenerative response DDE interfered with fundamental processes of developmental physiology via endocrine modulation of *A. mediterranea* (Sugni *et al.* 2008).

In the crustacean *A. tonsa* exposed to ≥0.55 ng/L p,p-DDE induced severe feminizing alterations in the male gonad and accessory organs. Spermatogenesis was impaired at all stages with increased rates of apoptosis. At 0.5 ng/L the gonad structure was altered in 20% of male *A. tonsa.* Primary and secondary spermatocytes displayed a pale cytoplasm and apoptotic cell death. The frequency of meiotic figures decreased. This type of alteration was even stronger in males exposed to 3.5 ng/L as the majority of visible spermatogonia, spermatocytes, and spermatids showed apoptotic figures. In the centre 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 22 ng/L p,p'-DDE exhibited a gonad with dominating spermatocytes devoid of spermatogonia and spermatids. The spermatocytes either displayed apoptotic figures or were necrotic. At concentrations of 0.0014 to 0.0088 µg/L p,p'-DDE the wall of the spermatophore was irregular in shape in 30% of males. Number and density of core secretions of the spermatophore were clearly reduced at a concentration of 0.0014 µg/L p,p'-DDE. In p,p-DDE-exposed females, an intensification of oogenesis with enhancement of oogonia proliferation and yolk synthesis was observed at ≥0.55 ng/L p,p-DDE.

In the female gonad at DDE concentrations of 1.4 – 8.8 ng/L, a striking feature was the increase in number and size of pre-vitellogenic and vitellogenic oocytes as well as a prominent yolk synthesis. Moreover, in 15% of females exposed to 0.5 ng/L DDE tightly packed oogonia and oocytes were encountered. The perinuclear sites of yolk synthesis were abundant and enlarged in relation to the control. Moreover, a few number of oogonia displayed apoptotic figures at 0.5 ng/L. Females exposed to 1.4 ng/L showed a more pronounced yolk synthesis with extended perinuclear sites of yolk formation compared to those exposed to 0.5 ng/L. The yolk masses were irregular formed in vitellogenic oocytes. The proliferation index of the female gonad increased in relation to the control after exposure to 0.5 and 3.5 ng/L, it was significantly elevated at 8.8 ng/L and decreased again at 1.4 ng/L (Figure 4) (Watermann *et al.* 2011b).

Apart of the modulating effects on the reproductive system, DDE was shown to act as an anti-ecdysteroid *in vitro* in crustaceans and aquatic insects (Dinan *et al.* 2001; Soin & Smagghe, 2007)) and is thus a potential endocrine disrupter in arthropods.

In the fish species *Pimephales promelas* disturbance in the spermatogenesis was noticed with increasing concentrations (10, 100, 1000, 10000 ng/L p,p'-DDE). Primarily, degenerative spermatocytes occurred to a higher degree in the tubules, leading to focal empty spaces in the tubule periphery. In the female gonad increasing DDE concentrations led to a heterogenous formation of the chorion leading to a certain percentage of oocytes with a thin chorion wall. In parallel the percentage of fat vacuoles in relation to yolk vacuoles increased with increasing concentration of DDE, right from 10 ng/L. The number of atretic oocytes was clearly elevated at 10,000 ng/L p,p'-DDE (Figure 3).

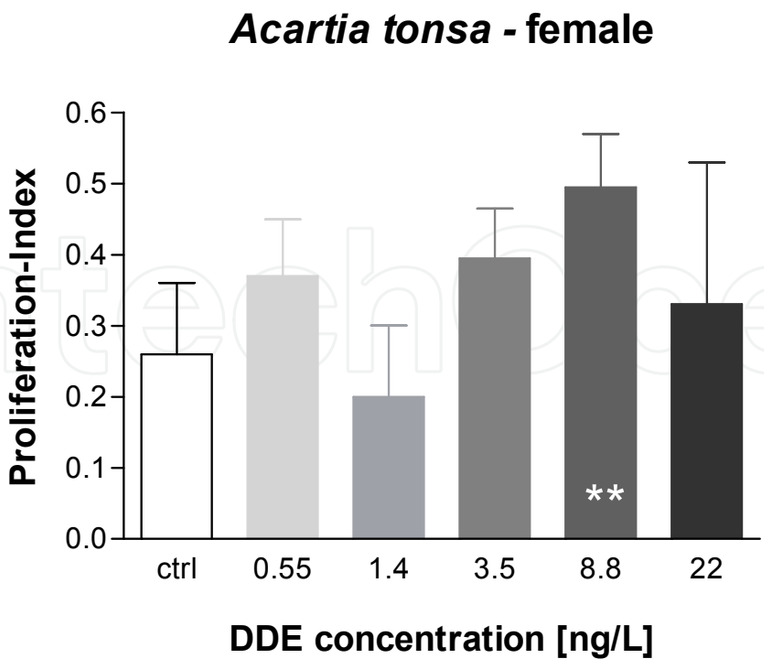


Fig. 3. *Acartia tonsa* exposed to DDE, Proliferation-Index = No. of proliferating oogonia / Total No. of oogonia, Statistics: Kruskall-Wallis test with Dunn's Multiple Comparison test, significant difference between control and exposure of 8.8 ng/L, level of significance $P<0.01$, no statistics with exposure of 22 ng/L as N was too small

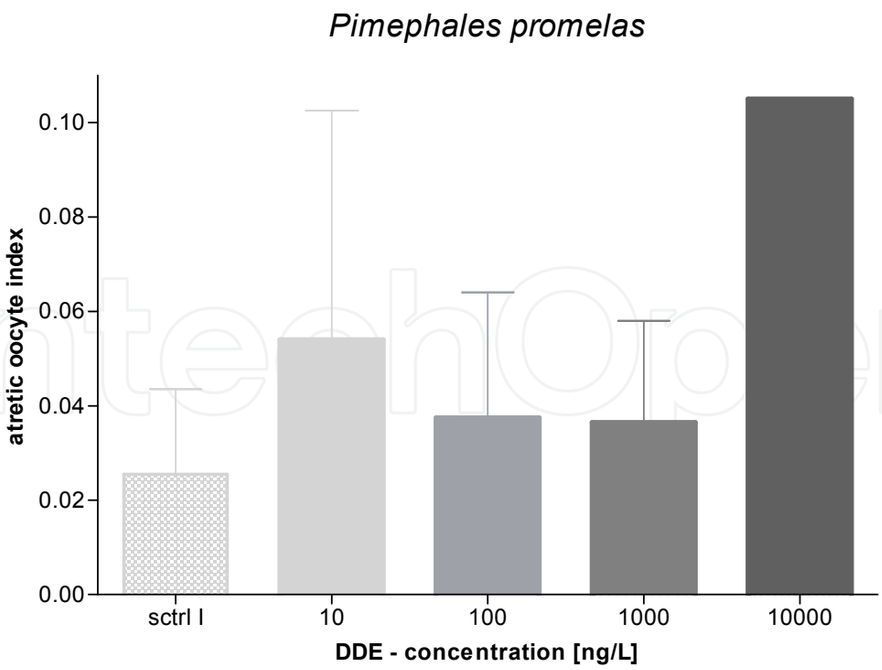


Fig. 4. Pimephales promelas exposed to DDE, atretic index = number of atretic oocytes/total number of oocytes, 2 to 5 animals in one group, median and interquartille range, no comparitive statistics, N too small

In males disturbance of spermatogenesis reflected in lack of cysts was noticed in all concentrations. The most pronouncecd effects are described for the testis displaying disturbance of spermatogenesis, but as elevated rates of atresia which cannot be sufficiently explained. From DDT-polluted rivers in the North of India combined with laboratory experiments Singh et al (2008) observed decreased sperm motility in catfish and *Heteropneustes fossilis*. In females of Pimephales a reduced chorion thickness, an elevated rate of atresia, and reduced percentage of postovulatory bodies was observed at 10,000 ng/L only.

In mammals p,p'-DDE has little ability to bind to the oestrogen receptor but inhibits androgen binding to the androgen receptor, androgen induced transcriptional activity, as well as androgen action in developing, pubertal and adult male rats (Kelce et al. 1995). After administration of 100 mg/kg/day DDE Sprague Dawley rats displayed hypospadiasis and increased numbers of retained nipples (Gray et al. 1999b). After administration of 750 and 1000 mg/kg/day DDE rats developed testicular changes, which were characterized by disorganization of the testis and loss of germ cells within a few, randomly distributed tubules. Effects on germ cells were more apparent in the epididymis, which contained increased numbers of sloughed, round germ cells within epididymal tubules of rats exposed 1000 mg/kg/day DDE (O'Connor et al. 2002). Shi et al. (2009) reported on apoptosis induced by p,p'-DDE in Sertoli cells of rat via a FasL-dependent pathway.

3.2 Vinclozolin

No investigations have been carried out with echinoderms up to now. In the crustacean copepod *Acartia tonsa* the response of the male gonad to ≥0.10 mg/L VIN exposure was heterogeneous; some areas in the gonad were stimulated, whereas others displayed a disturbed spermatogenesis. Multiple spermatocytes exhibited a diffuse and slightly vacuolated cyctoplasm at concentrations ≥0.10 mg/L. In addition, the spermatophore formation was affected leading to deformations. In female VIN exposed *A. tonsa* no statistically significant effects were observed (Watermann *et al.* 2011b).

In the cladoceran *Daphnia magna* VIN reduced the number of neonate males at 1 mg/L (Haeba *et al.* 2008). Interestingly, this sex ratio modulation in a crustacean corresponds to the anti-androgenic action of VIN in vertebrates.

In selected molluscan species (*Marias cornuarietis, Nucella lapillus* and *Hinia reticulate*a) exposure for 5 months with concentrations of vinclozolin between 30 ng/L and 1000 ng/L induced in males a reduction of the penis and prostate length (Tillmann *et al.* 2001; Oehlmann *et al.* 2007).

First reports on the anti-androgenic effects in fish were published by Makynen et al. (2000). They reported on slight increase of estradiol in the serum of male fish and gonad atrophy in female fish after exposure of 200 – 700 µg/L VIN. They suspected as active agents not the compound but the metabolites M1 and M2 (Makynen et al. 2000). Some years later Kiparissis et al. (2003) observed intersex in Japanese medaka after exposure of 5000 µg/L VIN. In *Rutilus rutilus* vinclozolin induced elevated levels of atresia in the female gonad. Whereas in the control group atresia was observed in 10 – 12% of females, the levels increased to 16.7% at 1.4 µg/L and to 28.6% at 2.86 µg/L. In the male gonad atrophy was present with prevalence of 50% at 1.4 µg/L and 44.4% at 2.86/L.

In mammals VIN has been found to induce significant feminization of male rats by altering the development of the reproductive tract, causing low sperm count, hypospadiasis, and other deformations (Kelce *et al.* 1994, Gray *et al.* 1999a). In laboratory experiments with rats

vinclozolin combined with five other anti-androgens markedly increased the frequencies of hypospadias in rat (Christiansen *et al.* 2008). In a similar study with a mixture of seven anti-androgens reproductive malformations in rats were more frequent than after exposure to vinclozolin alone (Rider et al. 2008).

4. Conclusions

Most data presented here were related to echinoderms, crustaceans, molluscs as invertebrates, and amphibian, fish as vertebrates. Comparison with data on studies with mammals show some striking similarities on the morphological and histological effect level. Focussing on the endocrine modulation of the reproductive system of the species under investigation, quite heterogenous structures of gonads, varying existence and structure of accessory organs, different regulatory pathways mediated by different hormone-types had to be taken into account. Nevertheless, some cell and tissue differentiation and reproduction features found in spermatogenesis and oogenesis display common structures and principles. It is not surprising that echinoderms may posses control mechanisms of physiological processes, in terms of molecules and actions, rather similar to those of vertebrates. Available data suggest that sex steroids (progestins, androgens and estrogens) have a role in regulating reproduction and other physiological processes in echinoderms. Past and recent investigations have identified vertebrate type steroids, i.e. progesterone, testosterone and 17b-estradiol in several echinoderm species (see for literature Sugni *et al.* 2007).
In crustaceans steroids with structural similarities to those of vertebrates could be identified (Fingerman *et al.* 1993, Lafont & Mathieu, 2007, LeBlanc, 2007).
Prosobranch molluscs provide strong evidence for EDC-related effects on development, fecundity and reproduction in invertebrates. The case of imposex as a result of exposure to TBT (and for some species after exposure to TPT) is the clearly dominant example of population-level EDC effects in wildlife. However, other xeno-androgens acting as aromatase inhibitors or AR agonists have been shown to cause almost identical effects at extremely low concentrations. These examples support the hypothesis that a modulation of vertebrate-type steroid levels in prosobranchs plays a key role in imposex development, although an involvement of neuropeptides cannot be ruled out. Furthermore, reproduction and sexual development of prosobranchs are also affected by xeno-estrogens such as Bisphenol A, Octylphenol, and estradiol at environmentally relevant exposure levels reflecting their high susceptibility to EDCs in general (Oehlmann *et al.* 2007).
Danish studies have indicated increased occurrence of cryptochordism in sons of female gardeners (Weidner et al. 1998) and reduced fecundability in female greenhouse workers (Abell *et al.* 2000). On the other hand surveillance studies of young men in Northern Europe showed that in relation to sperm counts from the 1940s with averages higher than 100mill/mL, this number went down to 40 mill/mL (Andersson *et al.* 2008). When this decline is continuing, the authors assume and increased number of infertile couples and lower fertility rates in the future. One of the most discussed factor is the multiple exposure to pesticides with dominant estrogenic or anti-androgenic properties. (Orton et al. 2011)

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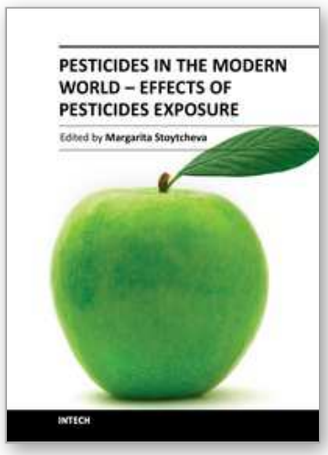
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Pesticides in the Modern World - Effects of Pesticides Exposure

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The introduction of the synthetic organochlorine, organophosphate, carbamate and pyrethroid pesticides by 1950â€™s marked the beginning of the modern pesticides era and a new stage in the agriculture development. Evolved from the chemicals designed originally as warfare agents, the synthetic pesticides demonstrated a high effectiveness in preventing, destroying or controlling any pest. Therefore, their application in the agriculture practices made it possible enhancing crops and livestockâ€™s yields and obtaining higher-quality products, to satisfy the food demand of the continuously rising worldâ€™s population. Nevertheless, the increase of the pesticide use estimated to 2.5 million tons annually worldwide since 1950., created a number of public and environment concerns. This book, organized in two sections, addresses the various aspects of the pesticides exposure and the related health effects. It offers a large amount of practical information to the professionals interested in pesticides issues.

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