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LECTURE

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Tributyltin biomonitoring using prosobranchs as sentinel organisms

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Abstract Tributyltin (TBT) compounds, some of the most toxic xenobiotics, produce a variety of pathological reactions in animals. A reliable biomonitoring method to assess the degree of environmental TBT pollution has been described based on investigations of virilization phenomena in prosobranch snails (Mollusca: Gastropoda). Examples are the imposex phenomenon in marine and freshwater species, the intersex reaction in littorinids and the reduction of female sexual glands and offspring numbers in further species resulting mainly in a sterilization of females. The degree of imposex or intersex in populations is determined by different biomonitoring indices which allow to assess the TBT pollution of the environment at low costs with high precision. The effectiveness of TBT legislations is analysed by extensive surveys in France and Ireland indicating that there is still a continuing threat to sensitive marine organisms. TBT disturbs the biosynthesis of steroid hormones on the level of estrogen biosynthesis. The observed virilization phenomena seem due to an inhibition of the cytochrome *P*-450 dependent aromatase by this organotin compound.

Introduction

Tributyltin (TBT) compounds used as biocides in antifouling paints and in various other formulations are known to produce a variety of malformations in aquatic animals with molluscs as one of the most TBT-sensitive groups of invertebrates (for review [1, 2]). As the impact of TBT on nontarget organisms became apparent in the early 1980s, France was the first European country to draw up regulations to control TBT emission and banned the use of TBT antifoulings on small boats (length < 25 m) in 1982. But up to now the TBT pollution of coastal waters in many regions is still high and further controls are necessary [3, 4]. Because the analysis of organotin compounds in environmental samples is rather difficult, time consuming and expensive, a biomonitoring system based on easily detectable morphological parameters would give useful results.

The imposex phenomenon of prosobranchs, i.e. the formation of a penis and/or vas deferens on females of gonochoristic species, has been successfully used as a biomonitoring system to assess TBT pollution in the marine environment [2, 3, 5–8]. But the established European imposex species for TBT biomonitoring (e.g. *Nucella lapillus*, *Hinia reticulata*, *Ocenebra erinacea*) are absent on the German North Sea coast or can only be found in restricted areas. The periwinkle *Littorina littorea* is the only prosobranch which is very common and can be sampled in sufficient numbers. In this species TBT induces intersex, i.e. pathological alterations in the female genital system. The potential of the intersex phenomenon in *L. littorea* for TBT biomonitoring is evaluated. In contrast to the situation in the marine environment where a sufficient number of biomonitoring species for the quantification of TBT contamination is available there is a lack of biomonitoring species in freshwater environments in spite of the fact that here increasing organotin pollution is a cause for concern [9, 10]. Imposex development in the ramshorn snail *Marisa cornuarietis* and reduction of embryo numbers in the brood pouch of *Potamopyrgus antipodarum* are described as first approaches for a TBT biomonitoring in limnic ecosystems.

Although imposex has been known since years the detailed biochemical mechanisms of its induction have remained obscure. The clearing up of these mechanisms can help to answer the question whether or not the

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described virilization phenomena are a specific effect of TBT exposure or can also be induced by other xenobiotics.

Experimental

Since March 1988 the external topography of more than 40,000 specimens belonging to 30 different prosobranch species was examined. Before the shell was cracked with a vice, marine snails were narcotized using 7% $MgCl_2$ and freshwater snails using 2% $MgCl_2$ in distilled water. Shell and aperture height and the external dimensions of the genital tract including vas deferens extension and penis length were measured to the nearest 0.1 mm. For all populations (sample size $\geqslant$ 30 specimens) the female penis length (FPL) and the vas deferens sequence (VDS) index were calculated (for details see [2]). VDS values above 4.0 indicate that at least a portion of the females in the population is sterilized. A value of 0 demonstrates that no imposex affected females can be found in the sample.

All laboratory experiments with marine snails were conducted with *Nucella lapillus* (L., 1758) and *Hinia reticulata* (L., 1758), but because of limited space only the results for *N. lapillus* are communicated here. No significant differences between the results for both species were found. A 24 to 48 h static renewal system in 80 or 120 l glass aquaria provided with an Eheim power filter and artificial sea-water (salinity 35‰.) was used. The natural temperature fluctuations at Roscoff (Brittany, France; 8.5 °C in winter, 16 °C in summer) were simulated. Two hundred to 250 snails of each species were kept in two connected aquaria and a tide simulation system was employed. The analyses were conducted at monthly intervals (sample size 30 specimens) with an additional sample two weeks after starting the experiments.

The determination of TBT in water was performed as described in [7]. Water samples of 0.5 or 1.0 l were taken in polycarbonate bottles at a depth of 0.5 m below water surface, acidified with 5 or 10 ml of concentrated HCl (Merck "suprapur") and extracted with 5 or 10 ml of hexane (pesticide grade) for 30 min on an automatic shaker. TBT as Sn (TBT-Sn) was determined in the hexane extract after shaking with 1.5 or 3 ml 1 mol/l NaOH for 3 min using a Perkin-Elmer HGA-500 attached to a Perkin-Elmer 5000 AAS (wavelength 224.6 nm; slit 0.7 nm; injection volume 25 μl). Internal standardization (standard addition with spiked samples) was employed. Certified reference material (CRM: PACS-1, National Research Council of Canada) was analyzed additionally. Our results were within the standard deviation of the certified values for the CRM. Recovery factors were 91.4 $\pm$ 8.4%. The detection limit (3σ) was 1.5 ng TBT-Sn/l.

For steroid analyses all solvents used for extraction and purification were p.a. grade, obtained from Merck, FRG, or J.T. Baker, Netherlands. Single specimens were homogenised with 2 ml ethanol in stoppered tubes and than frozen for 24 h at -20 °C. Homogenates were extracted three times with 10 ml diethyl ether: ethanol (4:1) and extracts evaporated under nitrogen. After redissolving in 2 ml 80% methanol, the solutions were washed two times with 5 ml petroleum ether to remove lipids. The washed methanol extracts, suitable for radioimmunoassay (RIA), were evaporated under nitrogen and than redissolved in borate buffer (pH 7.8).

RIA-kits were obtained from Biermann Diagnostica, FRG, (testosterone-RIA) and Sorin Biomedica, Italy, (estradiol-RIA). Calculated recovery values for the extraction were 62.2 $\pm$ 5.04% for testosterone and 84.3 $\pm$ 5.84% for estradiol. In every sample steroid levels were determined in six males and six females of each species.

Results and discussion

Molluscs are known as the most sensitive group of possible TBT biomonitors from the animal kingdom [1]. These species do not only accumulate the organometallic compound with measurable bioconcentration factors of up to $3.0 \cdot 10^5$ in *Ocinebrina aciculata*, $1.5 \cdot 10^5$ in *Nucella lapillus* and $9.0 \cdot 10^4$ in *Hinia reticulata* but furthermore they show specific effects following a TBT exposure which can be used for a very sensitive effect monitoring.

Imposex in marine snails

Pseudohermaphroditism or imposex, the superimposition of male sex characters on females, normally a penis and/or vas deferens, was first described in 1970 and is known today for more than 120 species belonging to more than 60 genera [2]. The gradual virilization of imposex affected females can be described by a development scheme with 6 stages, furthermore divided in 3 different types (a–c) (Fig. 1). It has the advantage to be applicable to all imposex-affected species described up to now. Stage 0 is a normal female without any male sex characters. In most species imposex development is initiated by the formation of a primordial penis (stage 1a) or a short distal vas deferens section behind the right ocular tentacle (stage 1b). In a few muricids (*Nucella lapillus*, *Nucella canaliculata*, *Nucella emarginata*, *Nucella lamellosa*, *Ocenebra lurida*, *Searlesia dira*) a short vas deferens section infolds at the bottom of the mantle cavity close to the genital papilla (stage 1c). The dogwhelk *N. lapillus* is the only species which is characterized by both centres of vas deferens development. From the stages 1a–c to stage 4, the extension of vas deferens and/or penis is increasing. Stage 4 with its penis and a vas deferens reaching up to the vulva is the last still fertile stage of imposex development. In stage 4^+ the vas deferens passes the vaginal opening and runs into the ventral channel of the capsule gland.

TBT-induced sterilization (stages 5 and 6) occurs generally in muricid gastropods. Sterilization is either caused by a blockade of the pallial oviduct (stages 5a, b) as in *Nucella lapillus* at ambient TBT concentrations above 2.0 ng as Sn/l or by a split bursa copulatrix and capsule gland (stage 5c) as in *Ocenebra erinacea* (threshold concentration 8 ng TBT as Sn/l). The first possibility prevents the deposition of egg capsules resulting in an accumulation of abortive capsular material in the pallial oviduct (stages 6a, b); the second prevents copulation and capsule formation. In young and sexual immature specimens of some muricid species a protogyne sex-change can be induced by TBT concentrations above 10 ng as Sn/l, e.g. in *N. lapillus* [1, 2].

The classification in 6 different, clearly defined stages is the basis of the VDS (vas deferens sequence) index, calculated as the mean imposex stage of a population. This parameter allows the assessment of imposex intensities in natural populations and laboratory groups. Furthermore, it allows the interspecific comparison of

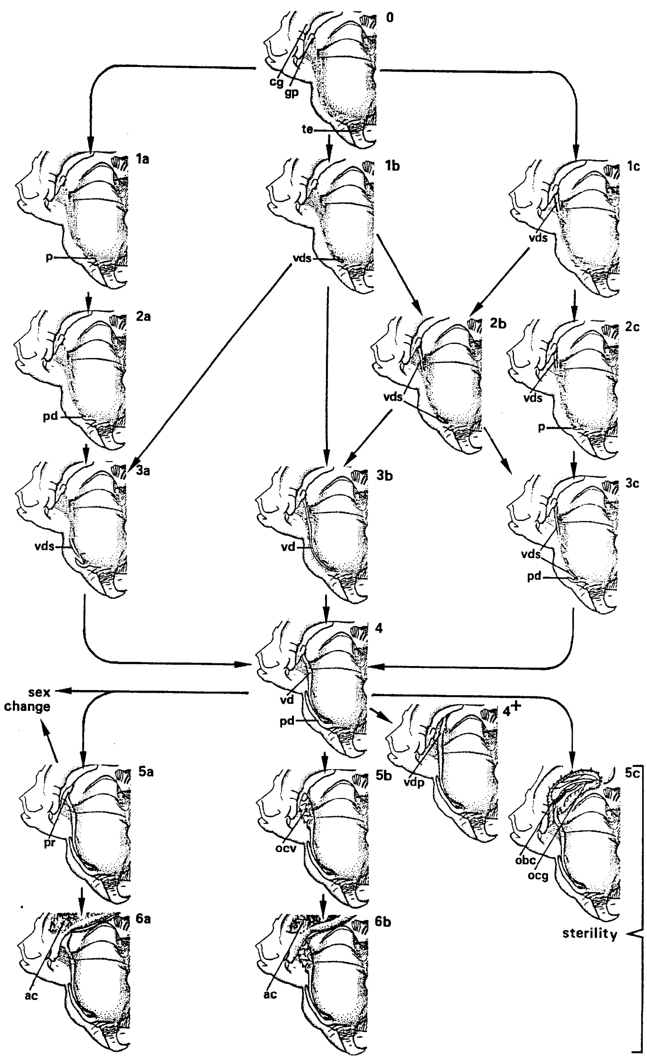

Fig. 1 General scheme of imposex development in prosobranchs. With the stages 0 (normal female), the imposex stages *1–6* (divided in the types *a–c*) and sex change. ac: abortive capsules, *cg*: capsule gland, *gp*: genital papilla, *obc*: open bursa copulatrix, *ocg*: open capsule gland, *ocv*: occlusion of the vulva, *p*: penis without duct, *pd*: penis with duct, *pr*: prostate, *te*: tentacle, *vd*: vas deferens, *vdp*: vas deferens passage into the capsule gland, *vds*: vas deferens section

the TBT sensitivity of different species and informs about the reproductive capability of a population.

For all imposex-affected prosobranchs the spatial distribution of imposex in relation to boating activity suggests that these species have potentials as bioindicators of TBT contamination. The imposex phenomenon has been successfully used in TBT biomonitoring studies in Scotland [11–13], England [14,15], Ireland [4,16], France [3] and outside Europe, e.g. in the United States [17,18], Canada [19], southeast Asia [20], New Zealand [21,22] and Australia [23,24].

Close to sources of TBT, like harbours and marinas, the VDS values are high. Significant correlations ($p < 0.001$) between the TBT concentration in sea water and the VDS indices in *Nucella lapillus*, *Ocenebra erinacea*, *Hinia reticulata*, *Hinia incrassata*, *Trivia arctica* and *Trivia monacha* were found (Fig. 2).

Comparative analyses gave evidence for systematic trends within the Prosobranchia concerning imposex expression and TBT sensitivity. Carnivorous mesogastropods like *Trivia* species exhibit a high TBT sensitivity but no sterilization effects [6]. The Buccinidae also show no sterilization but great interspecific differences of TBT sensitivity. Although in buccinids and mesogastropods, obvious imposex characteristics can be observed at very low environmental TBT concentrations, the muricid species exhibit the greatest TBT sensitivity and most marked species-specific threshold concentrations for the occurrence of sterilized females. The fact that at many sampling stations imposex affected species can be found together in the same habitat allows a direct comparison of their TBT sensitivity on the base of imposex development. It has been shown that *Ocinebrina aciculata* exhibits the greatest TBT sensitivity [25] followed by *Nucella lapillus*, *Ocenebra erinacea*, *Trivia arctica* and *Trivia monacha*, *Hinia reticulata* and at last

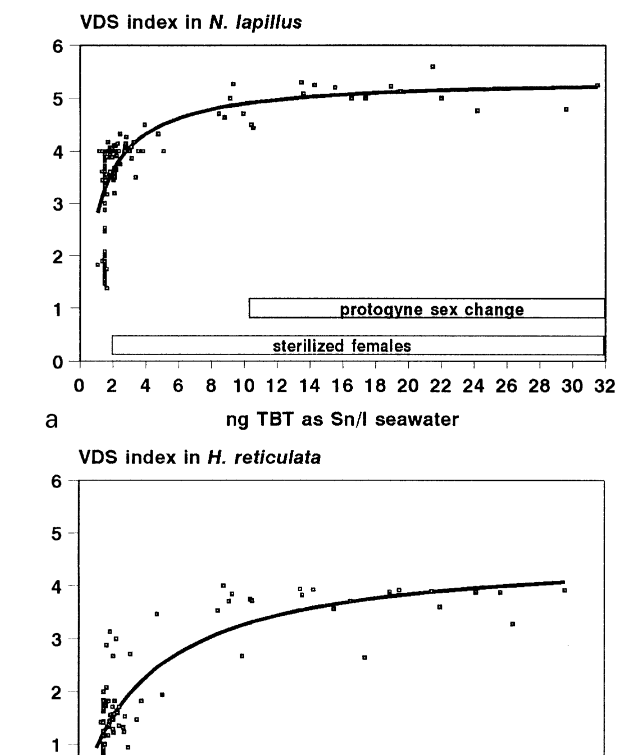

Fig. 2a, b Relationship between TBT concentrations in sea water and VDS indices of *Nucella lapillus* (**a**) and *Hinia reticulata* (**b**) with calculated regressions. **a** $y = (5.38 \cdot x) \div (0.974 + x)$; $n = 131$; $r = 0.678$; $p < 0.0005$. **b** $y = (4.66 \cdot x) \div (4.28 + x)$; $n = 87$; $r = 0.856$; $p < 0.0005$

Hinia incrassata. The determination of imposex intensities in natural populations allows an exact assessment of coastal TBT pollution on a wide geographical scale. Even in regions where established sentinel species, e.g. the dogwhelk *N. lapillus*, are exterminated due to their high TBT sensitivity, imposex surveys with further prosobranch species offer the possibility of effect monitoring approaches.

France was the first European country to draw up regulations to control TBT emission and banned the use of TBT antifoulings on small boats (length < 25 m) in 1982, i.e. five years before in Ireland and in the United Kingdom and seven years before in the rest of the European Union identical legislative controls came into force. In spite of recent reports that TBT concentrations in coastal waters and imposex intensities in the UK are generally in decline [26] evidence was found that at least in areas adjacent to harbours in France [3], Ireland [4] and Germany [27] organotin concentrations are still high enough to endanger sensitive species. Consequently, further controls are necessary.

TBT causes not only a virilization of females by inducing imposex but also by a reduction of female glands which are responsible for the storage and the resorption of sperm, the nourishing of oocytes within the capsules and the production of the egg capsule itself. Evidence was found that in all species analyzed in detail the extension of these female glands, e.g. albumen, ingestion and capsule glands of neogastropod species is decreasing with increasing environmental TBT concentrations. At highly polluted sites the length of the glands is reduced by up to 25% and its volume by up to 59% compared to slightly polluted populations [25]. It seems highly improbable that the reproductive performance of female prosobranchs is unaffected by this diminution of the pallial oviduct. Smaller glands will produce smaller egg capsules filled with fewer oocytes and supplied with a smaller amount of intracapsular fluid. As a consequence the number of offspring in the progeny is also reduced.

Intersex in *Littorina littorea*

In German coastal waters the established TBT biomonitoring species are absent. The periwinkle *Littorina littorea* does not develop imposex but especially in direct proximity to harbours and marinas malformations of the female genital tract were found which were termed as intersex [28]. The female specimens affected by intersex were either characterized by the development of male features on the female pallial organs (inhibition of the ontogenetic closure of the pallial oviduct) or female sex organs were supplanted by the corresponding male formations. The intersex phenomenon of *Littorina littorea* is a gradual transformation of the female pallial tract which can be described by an evolutive scheme with four stages [28].

Intersex development causes restrictions of the reproductive capability of females. In stage 1 a loss of sperm during copulation is possible and consequently the reproductive success is reduced. Females in stages 2–4 are definitively sterile because the capsular material is spilled into the mantle cavity (stage 2) or the glands responsible for the formation of the egg capsule are missing (stages 3 and 4). Due to female sterility, populations of *Littorina littorea* can be in decline but are not likely to become extinct because of the planktonic veliger larvae of the species. Veligers produced by populations with lower intersex intensities can guarantee a minimum abundance of periwinkles even at sites suffering from high TBT contamination and reproductive failure.

The assessment of intersex intensities in periwinkle populations bases on the same principle as described for the VDS index during imposex development. The intersex index (ISI) is the average intersex stage in a population. A value of 0.0 indicates that only normal females (stage 0) occur and no restrictions of the reproductive capability have to be expected. ISI values above 0 show that intersex affected females can be found and that reproductive success may be reduced.

In 1993 the range of TBT body burden in populations of *Littorina littorea* from the German North Sea coast was between 150.9 (Harlesiel) and 1289.5 μg TBT as Sn/kg dry wt. (Dornumersiel) [28]. In a reference from Roscoff harbour (Brittany, France; mean TBT concentration in sea water between 1989 and 1992: 15.4 ± 5.84 ng TBT as Sn/l, $n = 19$) a TBT body burden of 406.6 to 534.5 μg TBT as Sn/kg dry wt. was determined. This shows that at least at some sites of the German North Sea coast aquatic TBT concentrations exceed the values measured at Roscoff harbour considerably.

TBT biomonitoring approaches in freshwater environments

The wide application of organotin compounds is also responsible for an increasing pollution of freshwater environments with these compounds. Especially high TBT concentration in raw sewage, sewage sludge and even in effluents from sewage treatment plants are a cause for concern [9, 10]. First results show that prosobranchs can also be used in limnic ecosystems for tributyltin biomonitoring purposes.

The ramshorn snail *Marisa cornuarietis* exhibits as other species from the family Ampullariidae a vestigial copulatory apparatus also in the female sex. In laboratory experiments the low natural degree of imposex or pseudohermaphroditism increased markedly above a threshold TBT body burden of 1500 μg as Sn/kg (dry wt.) which is equivalent to an aqueous TBT exposure of 80 ng as Sn/l [29] (Fig. 3a).

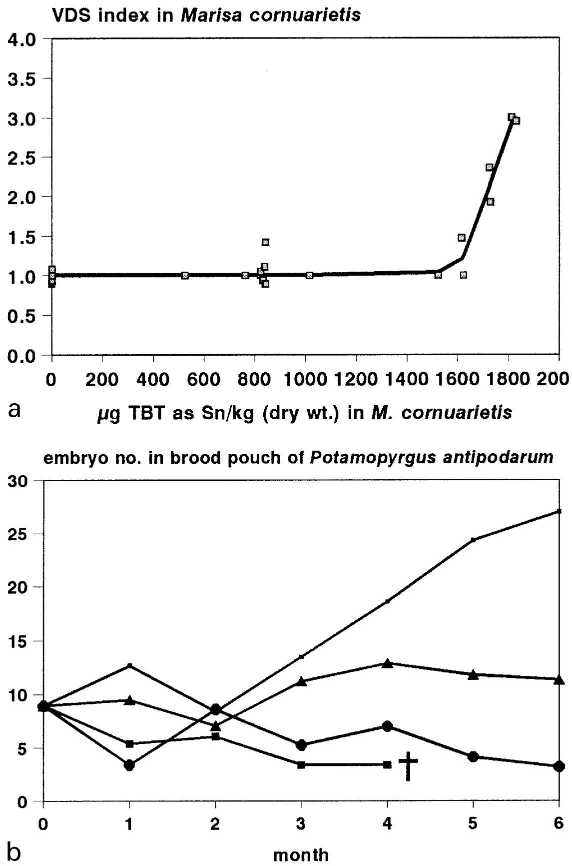

Fig. 3 a Relationship between TBT body burden and VDS indices of *Marisa cornuarietis* in laboratory experiments with calculated regressions: $y = -2.21 \div (1 + e^{0.021\cdot(x-1724)}) + 3.22$; $n = 33$; $r = 0.971$; $p < 0.0005$. **b** *Potamopyrgus antipodarum*. Development of average number of embryos in the brood pouch during a TBT exposure experiment in the laboratory. (·) control, (■) 100 ng TBT as Sn/l, (●) 200 ng TBT as Sn/l, (▲) 400 ng TBT as Sn/l (100% mortality after 4 months)

The hydrobiid snail *Potamopyrgus antipodarum* is the only parthenogenetic prosobranch species in central Europe and consequently populations consist exclusively of females. In several series of laboratory experiments it was shown that the average number of embryos in the brood pouch of females was reduced in a time and concentration dependent manner during TBT exposure (Fig. 3b). Consequently, also the number of offspring in the progeny will be reduced and finally this diminution of offspring number will result in reproductive failure.

Physiological causes for the virilization phenomena

All phenomena described, imposex and intersex development in marine and limnic species, reduction of female sex glands in oviparous and of offspring number in (ovo)viviparous species can be interpreted as a virilization of females. To elucidate the biochemical and physiological causes for these effects field investigations and laboratory experiments were carried out with *Nucella lapillus* and *Hinia reticulata*. It could be demonstrated that higher imposex stages exhibited higher testosterone levels in the tissue compared to pure females (imposex stage 0). This positive correlation between TBT exposure and testosterone content of the tissue led to further experiments. Dogwhelks were exposed to either TBT or testosterone. It was found that not only TBT, but also testosterone in the absence of TBT induces imposex development. At a concentration of 500 ng testosterone/l, imposex development is faster and more intense compared to TBT at concentrations from 5 to 100 ng TBT as Sn/l in the experiments (Fig. 4a).

During the TBT exposure experiments steroid concentrations in males and females were determined. In the control group average testosterone titres of females were in the range of 800–1000 pg/g (fresh wt.) while in the three TBT exposure groups (5, 50, and 100 ng TBT as Sn/l) testosterone titres in the tissue increased with increasing organotin concentrations and duration of

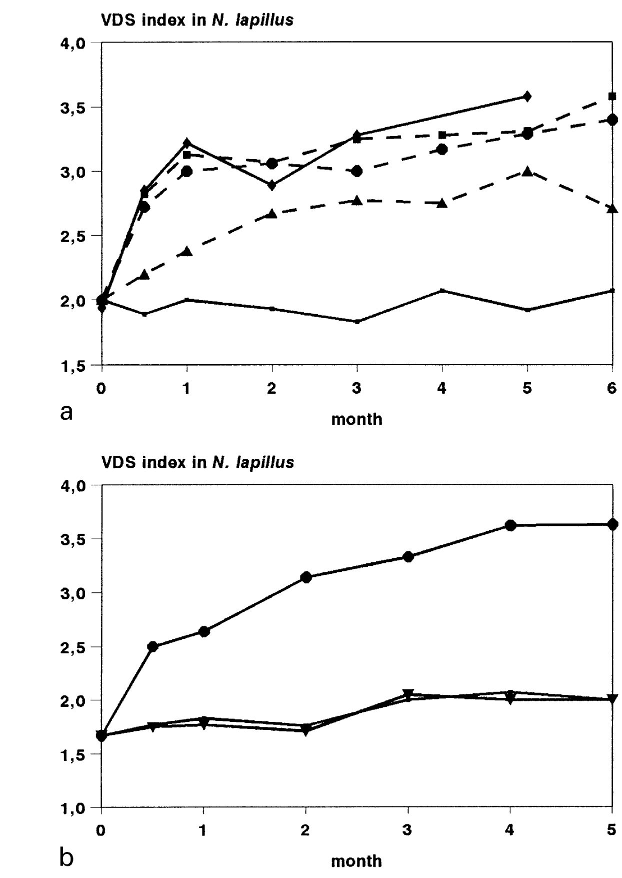

Fig. 4a, b *Nucella lapillus*. **a** Effects of TBT and testosterone exposure on imposex intensities. **b** Effects of TBT and the antiandrogen cyproterone acetate plus TBT on imposex intensities. (·) control, (▲) 5 ng TBT as Sn/l, (●) 50 ng TBT as Sn/l, (■) 100 ng TBT as Sn/l, (◆) 500 ng testosterone/l, (▼) 50 ng TBT as Sn/l plus 1.25 mg cyproterone acetate/l

TBT exposure. After six months, snails from all three experiments exhibited significant higher testosterone concentrations compared to the control: 1380 pg/g, 1550 pg/g and 1900 pg/g in the 5, 50 and 100 ng TBT as Sn/l exposure groups, respectively. Similar results were obtained by [30].

Evidence for the responsibility of testosterone in promoting imposex was also found in another experiment. Animals were simultaneously exposed to high TBT concentrations and to the antiandrogen cyproterone acetate at a concentration of 1.25 mg/1. This is a competitive inhibitor of androgen receptors. Further imposex development in the females of *Nucella lapillus* was completely suppressed (Fig. 4b). This experiment proves that TBT does not induce imposex development directly but mediated by androgens.

TBT disturbs the biosynthesis of steroid hormones on the level of estrogen biosynthesis. In molluscs, the multifunctional oxygenase system (MFO) catalyzes the aromatization of androgens to estrogens [31]. The same MFO system is believed to be responsible for the debutylation of TBT [32]. It can be presumed that TBT leads either to a competitive inhibition of the estradiol aromatization or to a suppression of the MFO system activity by binding to active sites of the enzyme system. The consequence is an increase of the testosterone level in the tissue for both alternatives.

If TBT inhibits the aromatization of androgens, specific aromatization inhibitors should also be able to induce imposex. In a further experiment, the effects of a specific steroidal aromatase inhibitor (SH 489 = 1-Methyl-1,4-androstadien-3,17-dion) were compared to a solvent control and a TBT exposure group with 50 ng TBT as Sn/l. Imposex intensities, measured as the VDS index increased and average female penis length increased and were significantly greater than in the control group.

These results show that also other xenobiotics with direct (e.g. synthetic androgens) or indirect androgenic effects (e.g. aromatase inhibitors) might be able to induce imposex and the other virilization phenomena described.

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