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Marisa cornuarietis (Gastropoda, Prosobranchia): a potential TBT bioindicator for freshwater environments

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The ramshorn snail *Marisa cornuarietis* (L., 1767) exhibits imposex (occurrence of male parts in addition to the female genital duct) under the influence of TBT (tributyltin) in laboratory experiments and accumulates this biocide in a time- and concentration-dependent manner. A comparison of BCF (bioconcentration factors) demonstrates that this limnic species accumulates more TBT than marine species. Evidence is given that TBT causes hormonal disorders which are responsible for imposex development. The testosterone/oestradiol quotient reflects the imposex development which can be described by a classification scheme differentiating four stages in *Marisa cornuarietis*. In the background of the constantly increasing organotin concentration in the limnic environment *Marisa cornuarietis* could be a well-suited bioindicator for TBT pollution. No other limnic species with a lower threshold concentration for measurable TBT effects is reported within the literature.

Keywords: Imposex; TBT pollution; biomonitoring; reproduction; *Marisa cornuarietis*.

Introduction

Many investigations in recent years have shown clearly that tributyltin (TBT) pollution in the marine environment can be assessed by the intensity of imposex. Imposex (Smith 1971) or pseudohermaphroditism (PSH) (Jenner 1979) is the superimposition of male sex characteristics on females in prosobranch species. Today the use of organotins is restricted in many applications but these compounds are still employed as biocides, catalysts and stabilizers in various formulations. Solution effects have led to an increasing contamination of freshwaters where a potential TBT bioindicator has been missing until now. Females of the ramshorn snail *Marisa cornuarietis* (L., 1767) already exhibit a very low degree of imposex naturally. Therefore, this ampullariid species which lives in stagnant and slow-running freshwaters in South America is appropriate to demonstrate that a freshwater gastropod reacts to TBT contamination in the same manner as marine snails, i.e. *Nucella lapillus* (Gibbs *et al*. 1987, Oehlmann *et al*. 1991), *Hinia reticulata* (Stroben *et al*. 1992b, c), *Trivia arctica* and *Trivia monacha* (Stroben *et al*. 1992a).

It has been shown that TBT pollution causes hormonal disorders in marine species (Spooner *et al*. 1991, Stroben *et al*. 1991). Marine prosobranchs exhibit a significantly

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enhanced testosterone titre when they are exposed to TBT. This increase of androgens is responsible for imposex development (Oehlmann *et al.* 1992a) in marine gastropods. Consequently, it should be examined whether there are similar effects in *M. cornuarietis*.

Methods

All the results are based on laboratory experiments with more than 700 analysed specimens of *M. cornuarietis*. Ramshorn snails were kept in 120 l glass aquaria with a 48 h static renewal system and provided with Eheim power filters. The water temperature was approximately 25 °C. The snails were exposed to different nominal aqueous TBT concentrations (50 and 200 $ng\,l^{-1}$ TBT as Sn (TBT-Sn), solution in ethanol) for 6 months. The specimens of the solvent control group received only ethanol. The animals were analysed at monthly intervals (sample size 30 individuals) and narcotized in 2% $MgCl_2$ solution in distilled water. Before cracking the shell with a vice, the shell and aperture height were measured and after cracking the shell the external properties of the genital tract were measured to the nearest 0.1 mm. For imposex measurements the snails were sexed, the penis sheath measured and the imposex stage (according to Oehlmann *et al.* (1991) and Stroben *et al.* (1992b)) determined (Fig. 1). As imposex indices the VDS (vas deferens sequence = average imposex stage of a population with values of 0–3 in *M. cornuarietis*) (Gibbs *et al.* 1987, Fioroni *et al.* 1991) and the mean female penis sheath length (PSL) of each sample were calculated.

The determination of TBT compounds was based on Stroben *et al.* (1992b). The analysis includes an extraction of TBT and DBT compounds with hexane, the elimination of DBT (dibutyltin) by washing the hexane extract with NaOH and quantification using atomic absorption spectroscopy (Perkin-Elmer HGA-500 attached to a Perkin-Elmer 5000 AAS with background correction; 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, delivered by the National Research Council of Canada) was analysed additionally. Our results were within the standard deviation of the certified values for the CRM.

Endogenous steroids of the snails were measured after extraction by radioimmunoassays with ^{125}J-labelled tracers using the Clinical Assay™ (Sorin Biomedica, Italy) for the determination of 17β-oestradiol and the Double Antibody Assay (Diagnostic Products Corporation, USA) for the assessment of testosterone (Bettin *et al.* 1994). Specimens were homogenized in ethanol and frozen for at least 24 h. Homogenates were extracted with diethyl ether and in two further extractions with diethyl ether : ethanol (4 : 1, v/v). The solvent extracts were combined and evaporated under a stream of compressed air at 37 °C. After redissolving in 80% methanol the residues were washed with petroleum ether to remove the lipid fraction. The washed methanol fractions were evaporated to dryness and redissolved in borate buffer for radioimmunological determination. Calculated recovery values were 62.2 ± 5.04% for testosterone ($n = 9$) and 84.3 ± 5.84% for 17β-oestradiol ($n = 9$). For scanning electron microscopy (SEM) the specimens were fixed in Bouin's fluid and then preserved in 70% ethanol, dehydrated via graded ethanol series, critical-point dried, coated with gold and examined with a Hitachi SEM S-530.

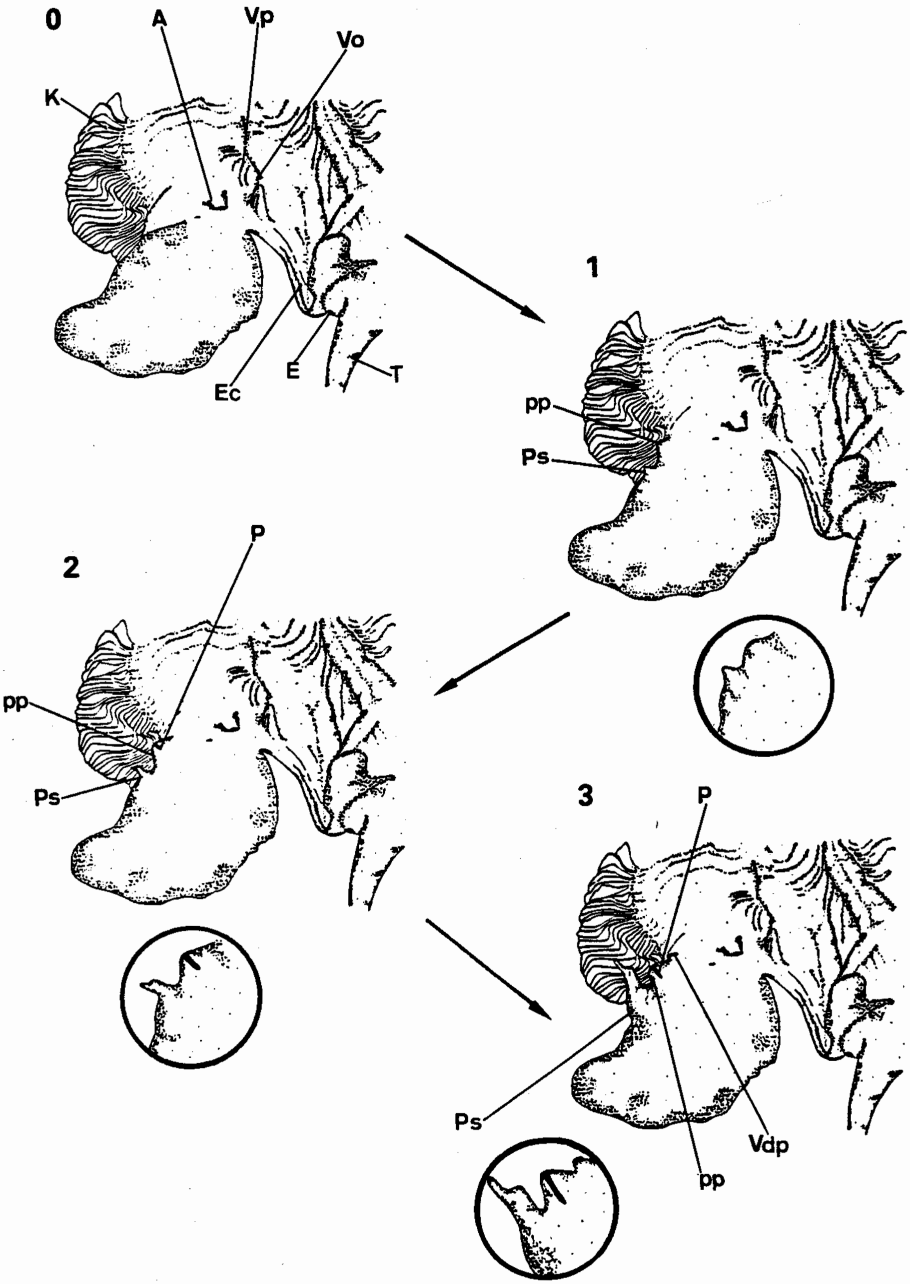

Fig. 1. Imposex development scheme (stages 0–3) in *M. cornuarietis*. A, anus; E, eye; Ec, egg channel; K, ctenidium; P, penis; pp, penis pouch; Ps, penis sheath; Vdp, vas deferens papilla; Vo, vaginal opening; Vp, vaginal papilla.

Results

Morphological aspects of imposex development

Imposex development in female *M. cornuarietis* as a result of TBT exposure can be described by using a classification scheme with four different stages. This represents a successive increase of masculinization in females. The anatomy and histology of the normal female (stage 0) and of the male genital system are described by Mello (1988) and Schulte-Oehlmann *et al*. (1994). The rare stage 0 is a female without any parts of the male genital system (0.62% of females in the control group) (Figs 1 and 2a). Stage 1 represents the level of imposex, most female *M. cornuarietis* show naturally (99.38% of females in the control group). Two small swellings on the top of the inner side of the mantle cavity present the primordia of the male penis sheath and the penis pouch (Figs 1 and 2b). Stages 2 (3.08% of females exposed to 50 ng l^{-1} TBT-Sn and 21.21% of females exposed to 200 ng l^{-1} TBT-Sn and 3 (1.54% of females exposed to 50 ng l^{-1} TBT-Sn and 32.58% of females exposed to 200 ng l^{-1} TBT-Sn) do not occur naturally but as a result of TBT exposure. Both, the penis sheath and pouch grow gradually and

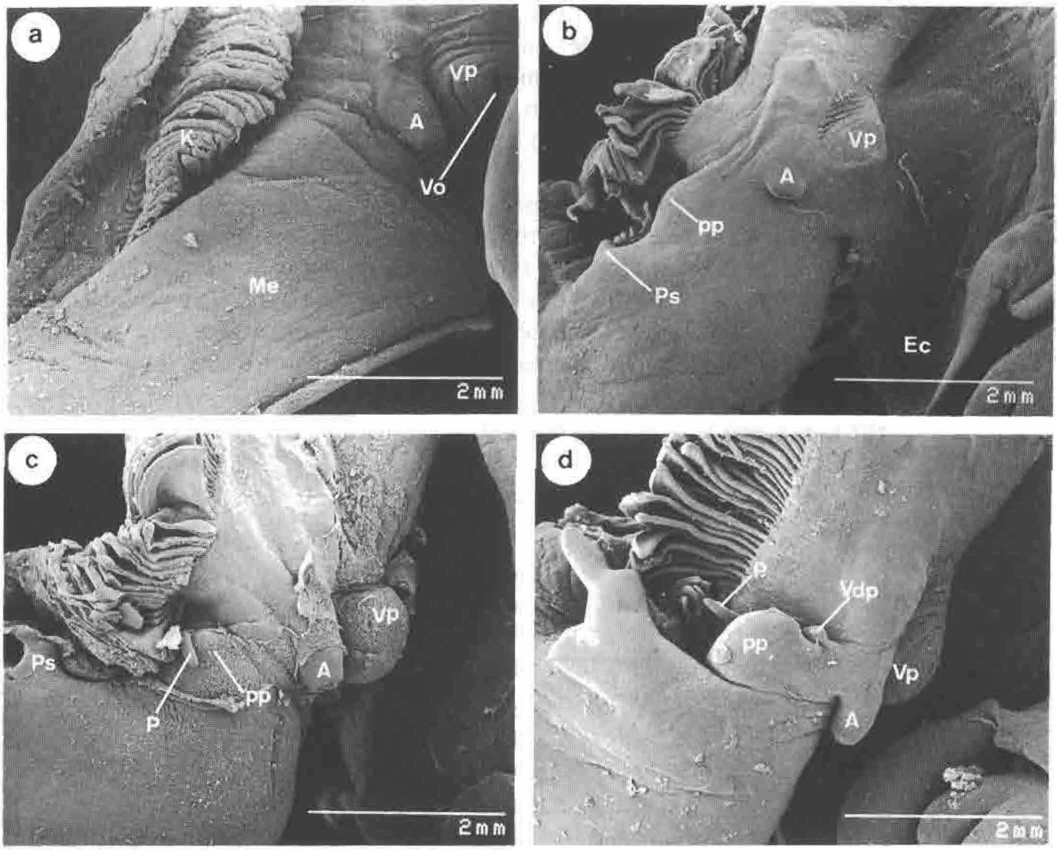

Fig. 2. SEM photographs of imposex stages in *M. cornuarietis*. (a) Stage 0 (pure female), (b) stage 1, (c) stage 2, (d) stage 3. me, mantle epithelium (for the remaining abbreviations see Fig. 1).

a small penis appears as a new formation in stage 2 (Figs 1 and 2c). Additionally, stage 3 is characterized by a vas deferens papilla and enlarged male sex organs (Figs 1 and 2d).

TBT accumulation

Figure 3 shows that *M. cornuarietis* accumulates TBT in a time- and concentration-dependent manner under laboratory conditions. The TBT uptake is equilibrated after 3 (exposure to 50 ng l^{-1} TBT-Sn) or 4 months (contamination with 200 ng l^{-1} TBT-Sn). Snails exposed to 200 ng l^{-1} TBT-Sn had the highest biocide concentrations in their tissues during the entire experiment but a comparison of bioconcentration factors (BCF) reveals that ramshorn snails contaminated with 50 ng l^{-1} TBT-Sn ($BCF = 1.68 \times 10^4$) accumulated comparatively more than those specimens exposed to 200 ng l^{-1} TBT-Sn ($BCF = 9.10 \times 10^3$). Females accumulate significantly more TBT (3.7%) than comparable males (TBT concentration of females = 1.037 × TBT concentration of males; Students *t*-test: $n = 18$, $r = 0.999$, $p < 0.0005$).

Indices for TBT biomonitoring

To determine the sensitivity of a potential bioindicator reliable and valid parameters which correspond with specific biocide concentrations have to be found. The VDS index (Fig. 4a) (Gibbs *et al*. 1987, Fioroni *et al*. 1991) is well established for TBT biomonitoring in marine prosobranchs. In particular, for the ampullariids the PSL (mean female penis sheath length) (Fig. 4b) is another useful index system.

After 2 months of TBT exposure the VDS index of the specimens exposed to 200 ng l^{-1} TBT-Sn had increased continuously from stage 1 to stage 3 by the end of the experiment (Fig. 4a). For the test group with the lower biocide concentration as well as for the control group the VDS index rests more or less constant at 1.0. At the end of the experiment a slight increase in the VDS index in the 50 ng l^{-1} TBT-Sn group can be detected. With regard to the PSL, the preceding results are comparable (Fig. 4b). The mean female penis sheath length increases from 0.02 to 1.6 mm only in the

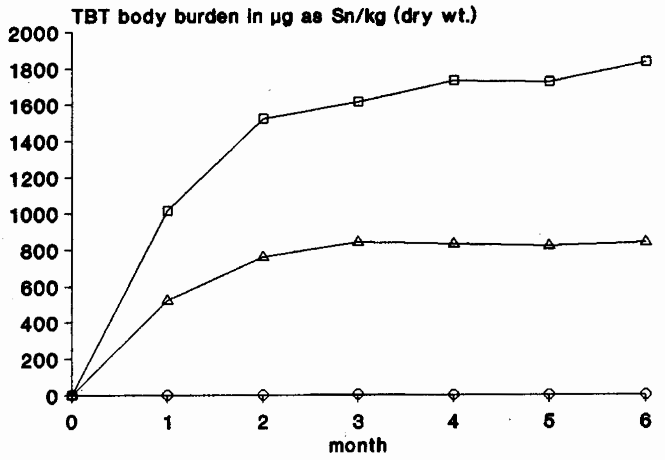

Fig. 3. TBT body burden in *M. cornuarietis* in laboratory experiments. Test groups: (○) solvent control group, (△) 50 ng^{-1} TBT-Sn, (□) 200 ng^{-1} TBT-Sn.

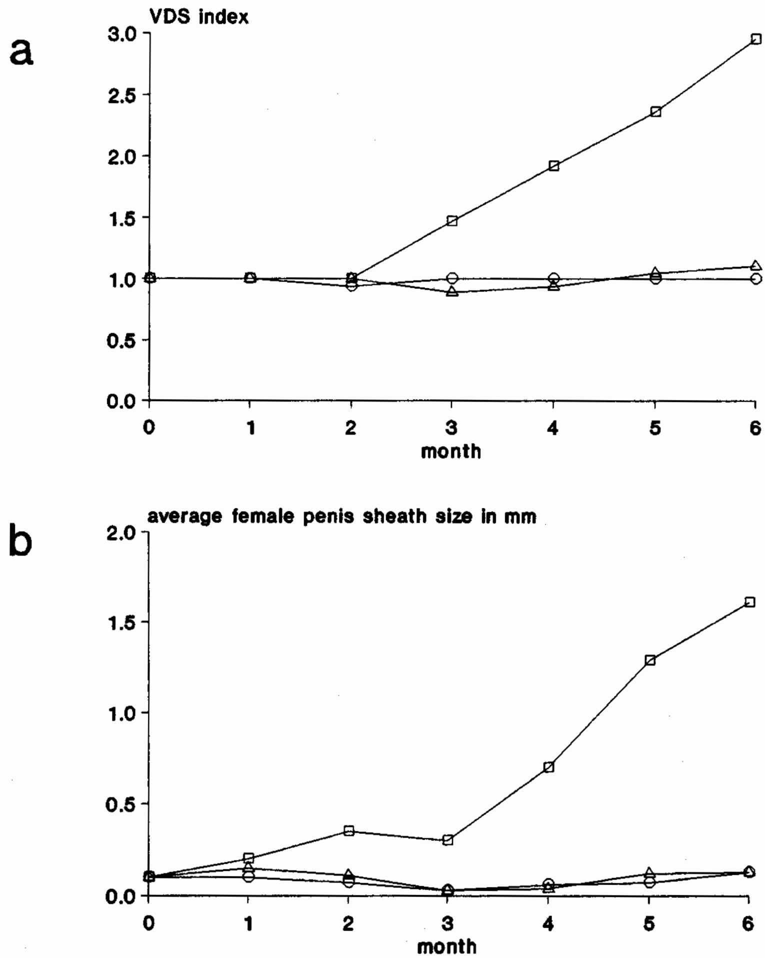

Fig. 4. Development of (a) the VDS and (b) the PSL index in *M. cornuarietis* in laboratory experiments. Test groups: (○) solvent control group, (△) 50 ng^{-1} TBT-Sn, (□) 200 ng^{-1} TBT-Sn.

experimental group exposed to 200 $ng\,l^{-1}$ TBT-Sn. The most important difference between the two parameters, the VDS and the PSL, is that the latter exhibits a higher sensitivity, i.e. the PSL values had already increased after 4 weeks of exposure in the experimental group contaminated with 200 $ng\,l^{-1}$ TBT-Sn whereas the VDS index increases only after 8 weeks of exposure.

The higher sensitivity of the PSL can be verified by the highly significant correlations between the TBT body burden of *M. cornuarietis* and the VDS index and PSL, respectively (Fig. 5a and b). Below 1600 μg TBT-Sn kg^{-1} (dry weight) in the tissues, the VDS index remains constant at the natural level of 1.0 whereas the threshold body burden for an increase of the PSL is only 800 μg TBT-Sn kg^{-1} (dry weight). Beyond these limits both imposex parameters increase rapidly.

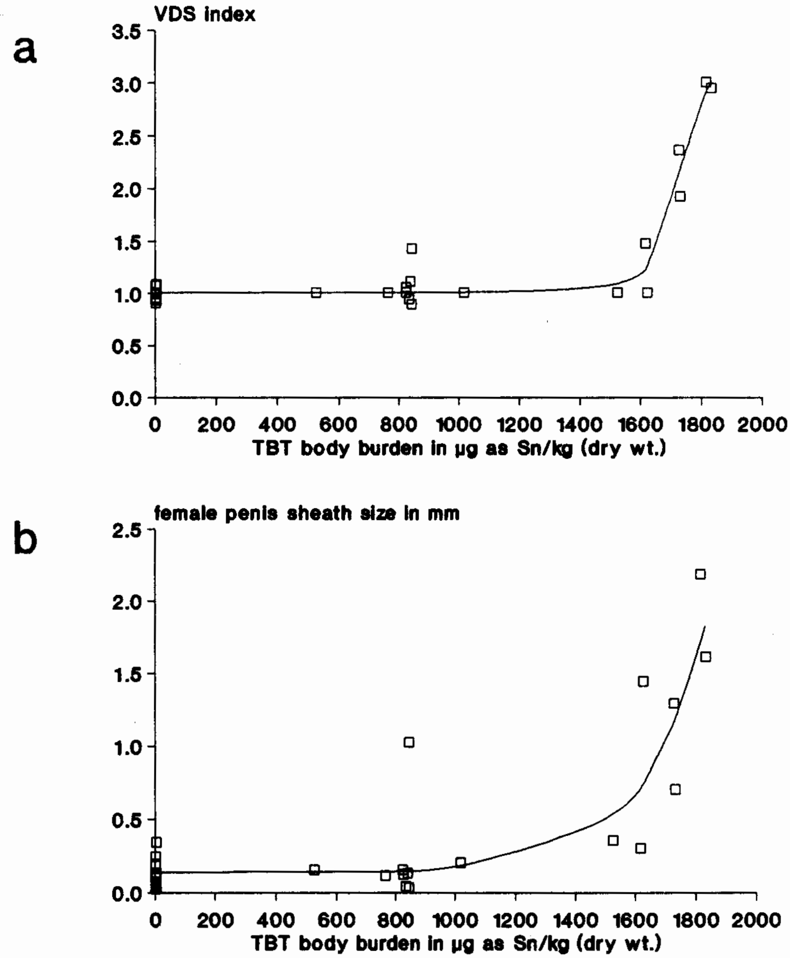

Fig. 5. Correlation of TBT body burden of female *Marisa cornuarietis* to the VDS (a) and to the PSL (b) index with calculated regressions ($y = -2.21 \div (1 + e^{0.0221(x-1724)}) + 3.22$; $n = 33$, $r = 0.971$, $p < 0.0005$ for the VDS; ($y = -770 \div (1 + e^{0.0059(x-2046)}) + 7.84$; $n = 33$, $r = 0.881$, $p < 0.0005$ for the PSL).

TBT effects on the steroid metabolism

Figure 6a and b shows that the steroid titres of all test groups in the course of the experiment decline in general. The decrease is continuous for the 17β-oestradiol titre and can be detected for the testosterone titre of TBT-exposed test groups after 3 months. This fact might be due to the beginning of sexual repose and corresponds with different steroid production within the reproductive cycle.

In the first 3 months the testosterone titres of TBT-contaminated snails increase continuously (Fig. 6a). The androgen content in specimens exposed to the highest aqueous biocide concentration is significantly enhanced compared to the 50 ng l^{-1} TBT-Sn-exposed group. Both are significantly higher than the androgen content in the

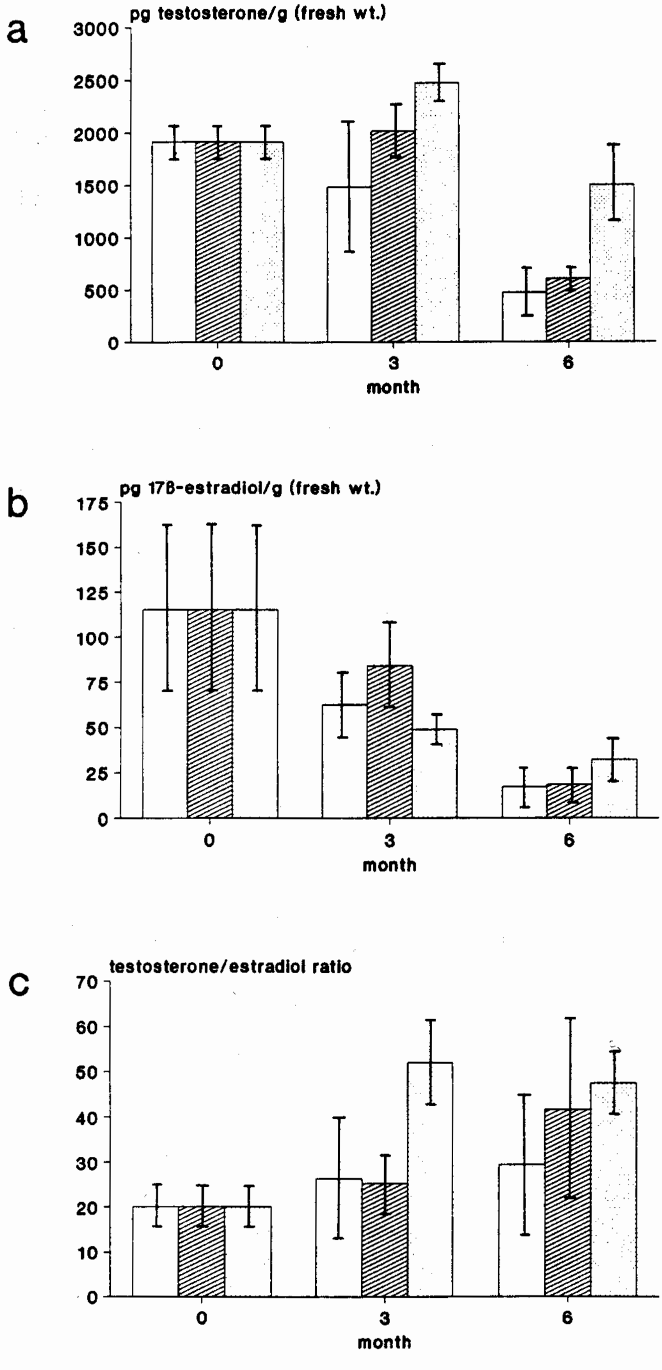

Fig. 6. Differences in (a) testosterone and (b) 17β-oestradiol titres and (c) the resulting testosterone : oestradiol ratio in female *M. cornuarietis* in laboratory experiments. Presented are the mean values with standard deviations. White bar, solvent control group; hatched bar, 50 ng^{-1} TBT-Sn; dotted bar, 200 ng^{-1} TBT-Sn.

tissue of the control group (Students *t*-test: $p < 0.0005$ for animals exposed to 200 ng l^{-1} TBT-Sn and $p < 0.01$ for individuals exposed to 50 ng l^{-1} TBT-Sn).

In the second period of the experiment the testosterone titre of the control and both organotin-contaminated groups falls off. The difference in the testosterone concentration between the control group and the test group with 200 ng l^{-1} TBT-Sn is significant through to the end of the experiment. According to Stroben *et al*. (1991), Oehlmann *et al*. (1992a) and Bettin *et al*. (1994) TBT inhibits the aromatization of androgens to oestrogens. Therefore, an increase in testosterone concentrations compared to the controls could be expected in the biocide-contaminated specimens. Consequently, this increase should also provoke changes in the 17β-oestradiol titres.

In the first period of the experiment a significant decrease in the 17β-oestradiol titre compared to the control group could only be demonstrated in the group receiving 200 ng l^{-1} TBT-Sn (Fig. 6b). In the second period, no significant difference between either of the biocide-contaminated groups and the control group was detectable.

Figure 6c reflects the development of the testosterone : oestradiol ratio. The test group exposed to the higher TBT concentration had the most marked ascent in their quotient during the first 3 months whereas the ratio of the 50 ng l^{-1} TBT-Sn group exhibited no significant differences to the control in the same period of time.

The quotient of the individuals contaminated with 200 ng l^{-1} TBT-Sn falls off slightly during the last 3 months. The ratio in the group exposed to the lower aqueous TBT concentration increased rapidly and is also significantly different to the control group after 6 months exposure.

The testosterone : oestradiol ratio in the tissue of *M. cornuarietis* reflects the development of the imposex parameters (VDS index and PSL) during the experiment in a better way than the testosterone or 17β-oestradiol levels alone.

Discussion

The phenomenon of imposex has been analysed in marine prosobranchs with increasing intensity since 1970 by different research groups (e.g. Bryan *et al*. 1986, Gibbs and Bryan 1986, Fioroni *et al*. 1991, Oehlmann *et al*. 1991, 1992b, Stroben *et al*. 1992a, b, c). In these species imposex is induced by the contamination of the coastal environment with the biocide TBT as a component of antifouling paints. As already mentioned the overwhelming majority of female *M. cornuarietis* exhibit a very low stage of imposex irrespective of TBT exposure. Therefore, this phenomenon should be considered as 'natural imposex'.

As a consequence the early stage of the imposex situation in the female genital system of *M. cornuarietis* is not induced by TBT but this biocide does cause a marked increase in the degree (stages 1–3) of masculinization in females. This can be detected by an increase of imposex intensities during the experiments.

The biocide accumulates in a time- and concentration-dependent manner with an equilibration of TBT concentrations in the tissue after 3–4 months. These results are in good accordance with results for the marine prosobranchs *Nucella lapillus* and *Hinia reticulata* which also attained a plateau in TBT body burden after 4 months of exposure (Stroben *et al*. 1992c). A comparison of the BCF of *M. cornuarietis* (1.68×10^4 at 50 ng l^{-1} TBT-Sn and 9.10×10^3 at 200 ng l^{-1} TBT-Sn), *N. lapillus* (1.00×10^4 at 50 ng l^{-1} TBT-Sn) and *H. reticulata* (8.91×10^3 at 50 ng l^{-1} TBT-Sn and 1.11×10^4 at

200 ng l^{-1} TBT-Sn (Stroben *et al*. 1992c) is possible because the tested TBT concentrations and the employed experimental set-up were identical. This comparison shows that *M. cornuarietis* accumulates approximately 200% more TBT than *H. reticulata* and approximately 60% more TBT than *N. lapillus*. These data and the investigation of Tsuda *et al*. (1990) corroborate that freshwater specimens accumulate more TBT than specimens in the marine environment, probably due to differences in the MFO system (Tsuda *et al*. 1990).

The fact that female ramshorn snails accumulate significantly more TBT than males is in good accordance with observations made for the mesogastropods *Trivia arctica* and *T. monacha* (Stroben *et al*. 1992a) and the neogastropods *N. lapillus* (Bryan *et al*. 1987) and *H. reticulata* (Stroben *et al*. 1992b). These sex-related differences might be due to the fact that the TBT uptake from ambient water (and additionally in the field from a TBT-contaminated diet) is higher in female snails or that TBT degradation in female snails is reduced compared to their male counterparts. Because of high lipophilicity of organotin compounds it is also conceivable that the higher lipid contents in the tissues of female gastropods could be responsible for this. Further investigations will have to elucidate this problem.

Marisa cornuarietis is well suited as a bioindicator for TBT contamination not only because of high BCF for this biocide but also because of the easily quantifiable and recognizable morphological characteristics of imposex development. Furthermore imposex parameters exhibit a positive correlation with the TBT body burden. An exposure to relatively low concentrations of 50 and 200 ng l^{-1} TBT-Sn for 3 months leads to an increase of both imposex parameters (PSL and VDS index). These facts allow the use of *M. cornuarietis* as a bioindicator in its natural subtropical environment or for a limited period in transplantation experiments in summer-warm freshwaters of moderate latitudes. Because ramshorn snails can be sexed easily (Schulte-Oehlmann *et al*. 1994) females can be used exclusively in such experiments to prevent an undesirable spread of this species.

In the background of the laboratory experiments basing results on only two different TBT concentrations it is impossible to make a decision in favour of the attitude of one of the imposex parameters. The advantage of the PSL is its higher sensitivity, i.e. values increase 1 month earlier compared with the values of the VDS index at lower TBT body burdens. In addition, the determination of the different imposex stages as the basis of the VDS index requires experience because a recognition of the female penis requires a dissection of the filigran penis pouch. On the other hand, statistical data on the correlation of the TBT body burden and imposex parameters (Figs 5a,b) give preference to the VDS ($r = 0.971$ and $r = 0.881$ for the PSL). Finally, the differences must be seen in close relation to the comparatively rapid growth of the penis sheath, whereas the growth of the other imposex features acting as the basis of the VDS index lasts longer.

For TBT biomonitoring purposes in the marine environment the VDS index and the mean female penis sheath length are the best and most well-established parameters (Fioroni *et al*. 1991; Oehlmann *et al*. 1991, 1992b, Stroben *et al.* 1992a, b, c). The main advantage of the VDS index is its general applicability for all imposex-affected species including freshwater prosobranchs. This enables interspecific comparisons and has demonstrated species-specific TBT sensitivities (Oehlmann *et al*. 1992b, Stroben *et al*. 1992a, c).

The calculation of the TBT BCF values in the experiments (1.68×10^4 at $50\,ng\,l^{-1}$ TBT-Sn, 9.10×10^3 at $200\,ng\,l^{-1}$ TBT-Sn) and the estimation of threshold body burdens of 800 (PSL) and 1600 μg TBT-Sn kg^{-1} (dry weight) (VDS index) (Fig. 5a and b) allows the determination of ambient TBT threshold concentrations for an increase of imposex parameters in *M. cornuarietis* under laboratory conditions. In this background the threshold concentration for *M. cornuarietis* is expected to be in the range of 50–85 $ng\,l^{-1}$ TBT-Sn. The calculation demonstrates that the critical concentration of the biocide in the limnic environment of this species is higher than the corresponding values of 0.5–1.5 $ng\,l^{-1}$ TBT-Sn in seawater for marine prosobranchs (Bryan *et al.* 1987, Oehlmann *et al.* 1991, 1992b, Stroben *et al.* 1992a, b). Furthermore, this offers a good explanation for the very low degree of imposex development in the experimental group exposed to 50 $ng\,l^{-1}$ TBT-Sn. Regarding the high TBT concentrations which were analysed in different freshwater environments, e.g. municipal wastewater (Fent and Müller 1991, Fent *et al.* 1991), limnic marinas and lakes (Kalbfus *et al.* 1991, Becker *et al.* 1992) the TBT sensitivity of the ramshorn snail is sufficient to monitor these levels of pollution.

As in many other prosobranch species *M. cornuarietis* exhibits a seasonal reproductive cycle with a maximum of breeding activity in spring and summer. This seasonal behaviour was reflected by steroid concentrations in the tissues which were higher in April, i.e. at the beginning of the experiment compared to June and October at the end of the experiment. While the absolute testosterone and 17β-oestradiol levels were characterized by great fluctuations according to the reproductive cycle, the testosterone : oestradiol ratio was quite stable in the control group, during the entire experiment. Contrary to the decrease in testosterone titres in the control group the TBT-contaminated snails showed increasing androgen levels during the experimental period in a biocide concentration-dependent manner. Although the values decreased after 3 months they were still significantly higher than in the control group at the same time. The inconsistent development of the 17β-oestradiol titres in both TBT-exposed groups demonstrates that TBT is not able to block the aromatization of androgens to oestrogens completely (cf. Stroben *et al.* 1991, Oehlmann *et al.* 1992a); it seems that TBT acts as a competitive inhibitor of the aromatization system (Bettin *et al.* 1994).

The testosterone : oestradiol ratio is more likely to be responsible for the induction of imposex enhancement in *M. cornuarietis* than the absolute content of testosterone as in marine prosobranchs (Spooner *et al.* 1991, Stroben *et al.* 1991, Oehlmann *et al.* 1992a). A careful interpretation of the results shows that an increase of the quotient above a critical threshold value of 30–40 (Fig. 6c) induces an increase in the imposex intensity. Further experiments are needed to elucidate the detailed mechanism of imposex development in the ramshorn snail.

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