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Triphenyltin induces imposex in *Nucella lapillus* through an aphallic route

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ABSTRACT

Triphenyltin (TPT) was used until recently as a biocide in antifouling systems and nowadays is still applied as an agriculture pesticide in some countries. This compound is known to cause imposex (the imposition of male characters in females of gastropod molluscs) in a very limited number of species, when compared with tributyltin (TBT), the universally recognized imposex-causing agent. In this study, we tested if TPT could induce imposex in females of the dog-whelk *Nucella lapillus*. Experimental groups of 40 females were injected with a volume of 2 μL/g of soft tissue wet weight (ww) of one of the following treatments, using DMSO as a solvent carrier: DMSO (solvent control); 1 μg/g ww of TBT (positive control); 0.2, 1 and 5 μg/g ww of TPT and a non-injected group (negative control). Concentrations were confirmed in the organism tissues by means of chemical analyses of a pool of 10 specimens at T_0 and then after the imposex analysis at T_{56days}. After 8-week trial, results pointed out statistically significant differences between treatments, with both TPT and TBT positively inducing imposex. However, imposex development in TPT-injected females differed from that of TBT, since females that developed imposex presented an aphallic condition (no penis development) while the TBT-treated females developed standard imposex (with penis formation). These results suggest that TPT and TBT act differently in the sequential process of female masculinization, casting new insights about the hypothetical pathways underlying imposex development.

1. Introduction

Triphenyltin (TPT) is an organotin compound used until recent years as a fungicide in agriculture in United States and in some countries in Asia (Higley et al., 2013; Yi et al., 2012). For some decades it was also applied as a biocide in ship antifouling systems (AFS), together with the most common organotin biocide at the time—tributyltin (TBT). Both compounds were banned from ship AFS in 2008 through the AFS Convention implemented by the International Maritime Organisation. The leaching of TPT and TBT from ship hulls led to a wide dispersion of these compounds throughout the marine environment, reaching concentrations that could cause adverse effects in biota (Shim et al., 2000; Yi et al., 2012). One of most notable adverse effects is imposex, an endocrine disruption effect defined as the superimposition of male characteristics (penis and vas deferens) in mollusc gastropod females. This phenomenon has been already observed in more than 260 gastropod species worldwide (Titley-O'Neal et al., 2011) and is generally associated to TBT pollution. Although being known that the main causative agent of imposex is TBT, it has been shown in the laboratory that TPT can also induce imposex in the gastropods *Reishia clavigera* (Horiguchi et al., 1997), *Marisa cornuarietis* (Schulte-Oehlmann et al., 2000), *Nassarius reticulatus* (Barroso et al., 2002), *Bolinus brandaris* (Santos et al., 2006) and *Stramonita haemastoma* (Limaverde et al., 2007). However, in the dog-whelk *Nucella lapillus*, one of the most sensitive species to TBT pollution in Atlantic coasts, which has been recommended as a biomonitor by OSPAR (2009), no causative relationship has been obtained until now for TPT and imposex. Moreover, imposex development seems to occur through pathways that involve binding of the agents to nuclear receptors RXR (retinoid

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X receptor) and PPARγ (peroxisome proliferator-activated receptor gamma) (Castro et al., 2007; Nishikawa et al., 2004; Pascoal et al., 2013). Not only TBT, but also TPT has been shown to be able to bind with high affinity to these receptors (Grun et al., 2006; Kanayama et al., 2005; Nakanishi, 2008). Therefore, since TPT seems to be able to induce *in vitro* the pathways that may lead to imposex development, we have designed this experiment to understand if TPT is also able to induce imposex *in vivo* in *N. lapillus*.

2. Methods

Dog-whelks were collected from a previously monitored site (Espinho, NW Portugal, 41°00.44N; 8°38.71W), known to have very low imposex levels, with a vas deferens sequence index (VDSI) of 0.4. At the laboratory, specimens were narcotized for a period of 60 min with a solution of 7% $MgCl_2$ in distilled water and analysed under stereomicroscope by gently pulling the gastropod foot out of the shell, to make sex identification possible. Only females not showing signs of imposex were selected for experimentation (note that VDS stages 0 and 1 cannot be differentiated without cracking the shell of the animal; Gibbs et al., 1987). Selected females were left to recover in aquaria for one week, while the other organisms were returned to the environment. Experimental groups of 30 females were again narcotized and injected in the foot with a volume of 2 μL/g of soft tissue wet weight (ww) of one of the following treatments, using DMSO as a carrier solvent: DMSO (solvent control); 1 μg/g ww of TBT (positive control); three distinct experimental groups injected with 0.2, 1 and 5 μg/g ww of TPT, respectively, and a non-injected group (negative control). Each group was placed in six 750 mL flasks (5 organisms in each) to avoid possible cross contamination between treatments, and to avoid propagation of mortality (by fungus for instance). Animals were kept in constantly aerated artificial seawater at a temperature of 18 ± 1 °C. Additionally, ten individuals were injected with the same treatments as exposed animals, left to recover in artificial seawater and pooled to check for actual concentrations of injected TBT and TPT in tissues at the beginning of experiment (T_0). Tissues were freeze-dried and homogenized. The test had the duration of 56 days with water changed twice a week. During the bioassay the animals were not fed to avoid possible TBT contamination through diet, which could undermine the aim of this study since *N. lapillus* feeds on mussels, which are known to accumulate TBT (Barroso et al., 2004; Sousa et al., 2009). Field observations indicate that *N. lapillus* can be unfed for several days with no effect on mortality (Burrows and Hughes, 1991), and even periods of 4 months of starving have been reported in natural populations (Crothers, 1985). For the test to be valid, a mortality below 20% in control was accepted, as considered in other prosobranch bioassay guidelines (Ducrot et al., 2014; Duft et al., 2007). At the end of the experimental period, the imposex parameters, female penis length (FPL) and VDSI, were assessed and classified according to Gibbs et al. (1987). Since not all females followed the normal VDS development pathway we classified VDSI based on the vas deferens development as described by Sánchez-Marín et al. (2015). Briefly, females with VDS > 1 but no penis, were considered aphallic. A long vas deferens which only starts at the genital papilla is considered a VDS stage 2; an interrupted vas deferens which starts both from the genital papilla and the base of the right tentacle (where it should start the penis development) is considered a VDS stage 3; and a complete vas deferens from the genital papilla to the base of the right tentacle corresponds to VDS stage 4. After imposex examination, ten individuals of each treatment were pooled (T_{56}) for organotin quantification in tissues.

TPT, TBT and its metabolites dibutyltin (DBT) and monobutyltin (MBT) were extracted by ultrasonic extraction and determined by gas chromatography followed by mass spectrometry (GC–MS) in SRM mode. An appropriate amount of surrogate standard (TBT d_{27}) and 6 mL of a solvent mixture of acetic acid:methanol:water (1:1:1) was added to 0.5 g of sample (dw). The tubes were sonicated for 30 min in an ultrasonic bath and centrifuged to obtain a liquid/solid phase separation. Ten mL of acetate buffer solution (pH 5) and a fixed volume of internal standard were added to the liquid phase. The extraction solution was derivatized with $NaBPr_4$ solution and extracted in 2 mL of isooctane and the organic phase was transferred to a chromatography vial. Determination was carried out with a Thermo-Finnigan (Waltham, MA, USA) Trace GC chromatograph equipped with a Triplus autosampler, PTV injector and coupled to a triple quadrupole mass spectrometer (TSQ Quantum XLS). The analytical uncertainty was <20% and method quantification limit (MQL) was 0.011 μg/g ww for all substances. The analytical method was validated for MBT, DBT and TBT using ERM®-CE 477 Mussel Tissue. For TPT, the method was validated using spiked reference material, because there is no certified reference value in this material. Analytical recoveries of MBT, DBT, TBT and TPT were 50%, 80%, 107% and 90%, respectively.

Statistical analyses were performed with IBM SPSS Statistics 22 Software. Differences between treatments were evaluated through one-way ANOVA after normality and homogeneity were verified, followed by Dunnett's post hoc test. The critical significance level adopted was $p < 0.05$.

3. Results

The mortality during the test ranged between 17% in the control group, which validates the bioassay, and 50% in the highest TPT concentration. Even though the mortality in some experimental groups were high (for instance, 50%), we could still trust the test, because there is no induction of imposex in the control group. Moreover, at least a sample of 15 females per treatment by the end of the exposure period was analysed that guarantees an acceptable number of observations for the statistical analysis. The results presented in this manuscript are a repetition of a preliminary experiment in which the mortality in the control group did not meet the validity criterion of mortality <20%. Nevertheless, the organisms exposed to TPT analysed in that previous trial (unpublished data) shown an equal imposex induction, confirming our results.

The results showed a statistically significant increase in VDSI for all TPT concentrations and the TBT control (Fig. 1A). TPT seems to have the same effectiveness as TBT on imposex development, since VDSI is similar at the same concentration of 1 μg/g ww. However, clear differences are observed in FPL (Fig. 1B). While females injected with 1 μg/g ww of TBT developed a penis with variable length (from 0.62 to 1.30 mm), the majority of the specimens injected with TPT did not show any sign of penis development (Fig. 1C) but exhibited a conspicuous vas deferens reaching VDS values of 2–4 (Fig. 1D). Only 2 TPT-injected females exhibited penis, and this was at VDS advanced stages 4 and 5, while 27 imposex-affected females (VDSI ≥ 2) showed no penis being therefore considered to present an aphallic condition, as described in methods section. TBT-injected individuals developed a penis primordium already at VDS stage 2, which continues its development through the next stages (Fig. 1C), this being the normal described pathway in this species (Gibbs et al., 1987).

The results of the chemical analysis of tissues are shown in Table 1. At T_0, TBT and TPT concentrations in tissues were near nominal values, varying between 103% (TBT 1 μg/g) and 188% (TPT 0.2 μg/g) of nominal concentrations, while other non-injected compounds were at concentrations below the limit of quantification. At the end of the experiment (T_{56}), the concentrations of TBT and TPT were still high and different among treatments, although