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ORIGINAL ARTICLE

Beyond imposex: other sexual alterations in a nassariid snail from Patagonia

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ABSTRACT

The occurrence of a penis in female gastropods has commonly been accepted as an indicator of tributyltin (TBT) pollution. During the surveys performed in Patagonian waters between 2002 and 2010, individuals of *Buccinanops deformis* with a short penis and an external vas deferens were initially considered as showing imposex. Doubt arose when different manifestations of the phenomenon were identified at the two sites with the densest populations of the species (San Antonio Bay, SAB, and Villarino). This led us to search at both locations for: (a) histological confirmation of sex, (b) butyltin measurements in sediments and snail tissues, and (c) incidence of trematode infestation. We found that both populations have individuals with a short penis. While all the inspected individuals from SAB were histologically identified as females (imposex), those from Villarino were identified as males. Trematodes were absent in individuals from SAB and showed very low prevalence in those from Villarino, with metacercariae found in the digestive gland. In snail tissues, butyltins were found mainly in the form of dibutyltin (DBT) in SAB and at negligible levels in Villarino. In sediments, butyltins were found only in the form of TBT, at higher levels in SAB than in Villarino. Thus, it became clear that our finding of a short penis is a manifestation of imposex on females from SAB related to the high TBT levels, whereas in Villarino it is a male trait, which is not related to TBT or a trematode infestation but may be a Dumpton syndrome-like abnormality.

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Abnormal penis; imposex; *Buccinanops deformis*; Patagonia

Introduction

Several environmental chemicals that affect hormonal systems have adverse health effects on wildlife and have also emerged as a major environmental issue. The effects of synthetic chemicals on sex hormone receptors and sex expression have attracted much attention, especially because they lead to reproductive failures in wildlife.

Neogastropod species have been repeatedly identified as suitable pollution indicators mainly related to organotin compounds (from paints used on commercial vessels and ports as biocides), which induce masculinization in females (imposex) (e.g. Bryan et al. 1988; Oehlmann et al. 1991; Nias et al. 1993; Shim et al. 2000; Muenpo et al. 2011). A common manifestation of imposex in females is the presence of a penis, whose length has been correlated with the concentrations of butyltins in the environment (Mensink et al. 1996; Tosten et al. 2013). However, this is not the only known reproductive alteration involving the development of a penis in gastropods. For instance, males of some species lose their penis after the breeding season, presumably as a way to optimize fertilization success and fitness and then recover the organ thereafter (Curtis & Barse 1990). Trematode infestation is another factor that may result in castration or decreased development of typical male sexual organs in marine snails (Gorbushin 1997; Rato et al. 2009; Averbuj & Cremonte 2010). Trematode infestation is also related to the phenomenon of gigantism (Mouritsen & Jensen 1994). Another curious phenomenon also manifested under tributyltin (TBT) contamination is the Dumpton syndrome, which is observed in the dogwhelk *Nucella lapillus* (Linnaeus, 1758), characterized by a broad range of phallic phenotypes, including the absence of a penis in males to the extent that they are incapable of copulation or sperm transfer (Huet et al. 1996; Barreiro et al. 1999; Gibbs 2005; Swennen & Horper 2008). Dumpton syndrome is caused by a recessive allele, possibly monogenic, that reveals its presence in gastropod populations subject to high TBT pollution (Gibbs 2005).

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The supplementary material for this article (Tables SI–SIII) is available at https://doi.org/10.1080/17451000.2016.1272700

Although many field studies have demonstrated the adverse effects of butyltins on female gastropods, the mechanism underlying the imposex phenomenon has not been fully elucidated. As quantification of TBT by chemical analysis in water or sediment is onerous, imposex has often been used as a tool to indicate contamination by organotin compounds (Cardoso et al. 2009; Titley-O'Neal et al. 2011). Several studies have assumed the occurrence of imposex, but lack TBT analyses and histological verifications of the sex. Thus, it is possible that, at least in some cases, alteration of penis size may be caused by a factor other than TBT.

Nassariid gastropods of the genus *Buccinanops* are conspicuous and common in Patagonian coastal zones both in sandy and rocky–pebble sites (Narvarte et al. 2008), but some important biological issues of these species are still not known. *Buccinanops deformis* (King, 1832) is the most variable in morphological traits across its extensive South Atlantic range. In fact, this species is characterized by great variation in several life-history features, including fecundity and growth rates, which have been identified for different subpopulations (Narvarte et al. 2008; Avaca et al. 2013a, 2013b). Although it can attain high densities on sandy bottoms like those of Playa Villarino and San Antonio Bay (Patagonia, Argentina) and several studies on its reproductive biology have been carried out (Avaca et al. 2012), some details related to the presence of different penis sizes in these two densest populations remain poorly understood. Nassariid species are very sensitive to contamination by TBT compounds (Titley-O'Neal et al. 2011). In particular, *B. deformis* has been mentioned as a suitable biological indicator of TBT pollution at moderate concentrations of this pollutant (Bigatti et al. 2009).

During several samplings of *B. deformis* carried out from 2002 to 2010, individuals with a short penis appeared with a relatively low frequency in samples from Villarino and San Antonio Bay (Narvarte et al. 2008; Avaca 2010). Considering the need to elucidate whether *B. deformis* can be unequivocally considered as an indicator of imposex (and then of TBT contamination), and to discriminate this from other sources causing short penis length, we carried out a study focused on the analysis of sampled individuals from a known TBT-contaminated site and one presumably less polluted. We analysed penis length of snails in relation to TBT and dibutyltin (DBT) concentrations in sediments and snail soft tissues. Also, we performed histological observations to confirm the sex of individuals and assess trematode prevalence, as another potential source of such variations.

Material and methods

The study was performed in San Matías Gulf (northern Patagonia, Argentina), where sites with dense populations of *Buccinanops deformis* were selected: (a) within the inner channel of San Antonio Bay (SAB, 40° 43.616′S, 64°56.789′W), in the low subtidal zone and (b) Villarino (40°50.300′S, 64°49.500′W), at a depth of 4 m at low tide (Figure 1).

Collection of sediments and animals

For the analysis of organotin compounds, sediment and snail samples were collected from both sites in April 2010. Snail samples were also taken for histological studies and analysed for the effects of parasite infestation. For the analysis of penis size in relation to shell length, data from the two sites obtained between 2002 and 2010 were pooled.

Biological bait (injured crabs, discarded fish and newly opened clams) was used to concentrate the individuals during samplings. Snails (586 individuals from SAB and 821 from Villarino) were collected by hand. One surface sediment sample (top 10 cm) per site was collected directly into a Teflon jar and then stored at −20°C until analysis (McFarland et al. 1995). Considering that butyltin concentrations may vary with the sediment grain size (Hoch et al. 2002), an additional sediment sample per site was collected for granulometry.

Shell length (distance from shell apex to the lip of the siphonal channel) was measured with Vernier callipers to the nearest 0.1 mm. At the same age distributions, shell length ranged from 10 to 27 mm in SAB, and from 20 to 56 mm in Villarino (according to the typical shell lengths found in each subpopulation,

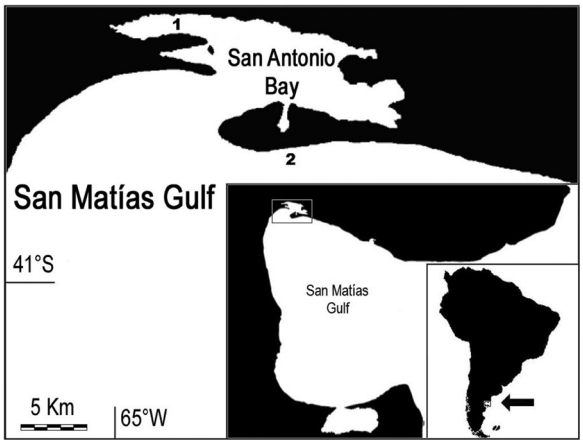

Figure 1. The two sampled sites (1, San Antonio Bay; 2, Villarino) in northern San Matías Gulf (Patagonia, Argentina).

Narvarte et al. 2008; Avaca 2010). Gender was first determined after shell removal by examination of the presence–absence of female accessory glands (capsule and albumen glands) and the colour of the gonads. We also recorded the presence or absence of a penis or penis bud in all the individuals sampled. The penis length of all individuals was measured to the nearest 0.1 mm and confirmed by a graduated eyepiece in a stereoscopic microscope, with an accuracy of 0.05 mm. From the sampling performed on April 2010, individuals with abnormal penis (short penis or bud) were separated and then reserved for further histological analysis ($n = 25$ for both sites) and determinations of TBT and DBT concentrations ($n = 25$ and 22 for SAB and Villarino, respectively). Also, a subsample of normal males from Villarino ($n = 16$) was kept for histological analysis to perform comparisons with individuals with an abnormal penis.

Histological techniques were used to confirm the sex of individuals and to determine, when present, the location of parasites (gonad or digestive gland). For that purpose, tissues of the posterior region were excised and fixed in Davidson solution for 24 h. Tissues were subsequently rinsed with water and stored in 70% ethanol until further analysis. Then, tissues were dehydrated using an ascending series of ethanol concentrations and embedded in paraffin. Sections were cut at 5–7 µm, stained with hematoxylin–eosin and observed under a light microscope. Histological sections of gonads (ovary and testis) were classified into immature and mature. Immature individuals were characterized by either no differentiation of gonadal tissue or a small gonad, containing primary or secondary spermatocytes and no spermatozoa in males and oogonia and primary oocytes but no vitellogenic or degenerating oocytes in females. Mature males showed abundant spermatozoa, while mature females might show either vitellogenic oocytes or few mature oocytes together with degenerating oocytes and other gametogenic remains (Avaca 2010). Micrographs were captured with a digital camera mounted on an Eclipse 200 Nikon light microscope. These slides were compared with slides of normal individuals (Avaca 2010). Trematode presence and location was determined in the same sections, according to Averbuj & Cremonte (2010).

Determination of DBT and TBT concentrations in sediments and snails

Sediment samples (20 g) were oven-dried at 105°C to constant weight. The method for extraction and derivatization of butyltins was that described in Delucchi et al. (2011; Table SI, supplementary material). The concentrations of TBT and DBT were determined using a Hewlett-Packard HP 6890 gas chromatograph coupled to an HP 5972 mass spectrometer (also according to Delucchi et al. 2011; Table SI, Table SII). The BT biodegradation index, which gives information about time since exposure, was calculated as (DBT + MBT)/TBT (Díez et al. 2002, 2006).

DBT and TBT concentrations were determined in the sample of 22–25 abnormal individuals suspected to be imposex females affected by DBT/TBT or to be males with atypical penis development. Muscle, gills, mantle and viscera (the remaining digestive gland) were used for TBT determination. Owing to the different size structure between populations including different frequency distributions and individual growth pattern (Narvarte et al. 2008), five replicates of 4–5 individuals each were used to prepare the extracts. The methods used for extraction from snail tissues and TBT analysis are those described by Delucchi et al. (2007), providing a detection limit of about 0.2 ng Sn g^{-1} dry weight.

Statistical analysis

The relationship between total shell length and penis length was studied by means of linear regression models. Mean penis lengths between normal males and individuals with abnormal penis development were compared by *t*-test and ANCOVA, using shell length as covariate. The Relative Penis Length Index (RPLI, Oehlmann et al. 1992) (broadly used to compare intensity and severity of imposex between populations; Barroso & Moreira 1998) was calculated as mean penis length of individuals with abnormal penis/mean penis length of normal males × 100.

Results

Granulometry of sediments and determination of DBT and TBT concentrations

The granulometric analysis of sediments from both sites showed that SAB sediment consisted mostly of clay and silt with measurable biogenic content and that Villarino has mostly sandy sediments (Table I). The concentrations of butyltin compounds in sediments from both SAB and Villarino (Figure 2a) showed detectable levels of TBT only, but the TBT level of SAB sediment was three times higher than that of the Villarino sediment. DBT levels were not measurable in sediments at either site, but the BT biodegradation index was near zero. In contrast, both DBT and TBT were measurable in snail tissues from both sites

Table I. Granulometric characteristics of sediments from both sites, San Antonio Bay and Villarino. D50 is the median of the grain size.

Level of selection	San Antonio Bay Moderate	Villarino High
D50 (μm)	6.4	148
Fine sands (%)	0	96
Silt (%)	69	0
Clay (%)	29	0
Biogenic remains (%)	2	<1

(Figure 2b), but in this case most of the butyltins were in the DBT form. TBT varied from 0.01 to 9.81 ng g^{-1} in snails from Villarino, and near the measurable level of 0.01 ng g^{-1} in snails from SAB.

Relationships between penis size and individual size

A total of 56 of the 586 snails from SAB and 189 of the 821 snails from Villarino showed a penis of relatively small size. Also, at both sites, a third of the abnormal individuals had buds instead of a penis. The relationships between penis length and shell length for abnormal and normal snails from SAB and Villarino are shown in Figure 3. Statistically significant linear dependence of the mean of penis length on shell length was detected in SAB (Figure 3A, $F(x) = 0.556x + 0.858$; $R^2 = 0.403$). In abnormal snails from Villarino, the penis length increased with shell size according to the linear function: $F(x) = 0.184x - 2.214$; $R^2 = 0.403$; $P < 0.001$, while in normal snails this increase was consistently higher $F(x) = 0.445x + 5.436$; $R^2 = 0.396$; $P < 0.001$ (Figure 3B). Regression slopes were significantly different (ANCOVA; $F_{(1,821)} = 29.832$; $P < 0.0001$). At both sites, the penis of abnormal snails was significantly smaller than that of normal ones (t Villarino = 12.561; $P < 0.001$; t SAB = 13.590; $P < 0.0001$). In Villarino, mean penis length of normal individuals was three-fold larger than that of abnormal ones, while in SAB, mean penis length of normal individuals was six times larger than that of abnormal ones (Figure 3). The RPLI in SAB was 14.9% and in Villarino 27.3%.

Besides shorter penis, abnormal individuals exhibited additional differences, such as a triangular basis of the penis, a small filamentous penis, and/or a conspicuous vas deferens (Figure 4A) compared to normal males (Figure 4B).

Gender identification and trematode-infestation analyses

All the individuals with short penis or bud and absence of accessory female glands from Villarino were identified as males, with normal appearance of spermatogenesis under a light microscope, similar to that of normal males from the same site (Figure 4C,D, respectively). Individuals identified as abnormal males ranged in size between 17.5 and 49.6 mm (mean ± SD: 31.3 ± 8.8, $n = 22$). A total of 61% of the males (of the 16 cases observed) used for histology showed gonads in a mature stage. The males with normal development of penis ranged in size between 20.3 and 54.6 mm (mean ± SD: 38.7 ± 9.5). Most of these individuals ($n = 13$) were sexually mature and three, the smallest ones, were immature. Male mature gonads were characterized by a large number of spermatozoa with visible tails closely packed in the lumen of each spermatogenic tubule. Tubules with evacuated gametes and other gametogenic remains were also observed. Larger precursor germ cells (spermatocytes) were located at the periphery while a thin layer of connective tissue served as separation between adjacent tubules (Figure 4D). In some slides, spermatic ducts full of spermatozoa were also observed.

All the individuals from SAB with a short penis were identified by histology as female (Figure 5B). Thus, imposex was confirmed at this site. The overall sample of imposexed females showed gonads in mature stages (Figure 5A). These females ranged in

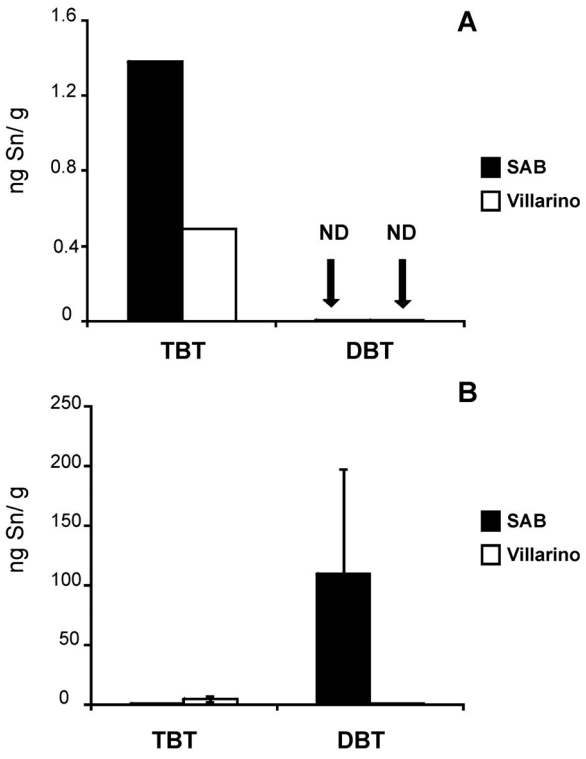

Figure 2. Tributyltin (TBT) and dibutyltin (DBT) concentration (ng Sn g^{-1} dry weight) in (A) sediments and (B) snail tissues, of San Antonio Bay (SAB) and Villarino. Bars on columns in (B) indicate standard deviations. ND, undetected.

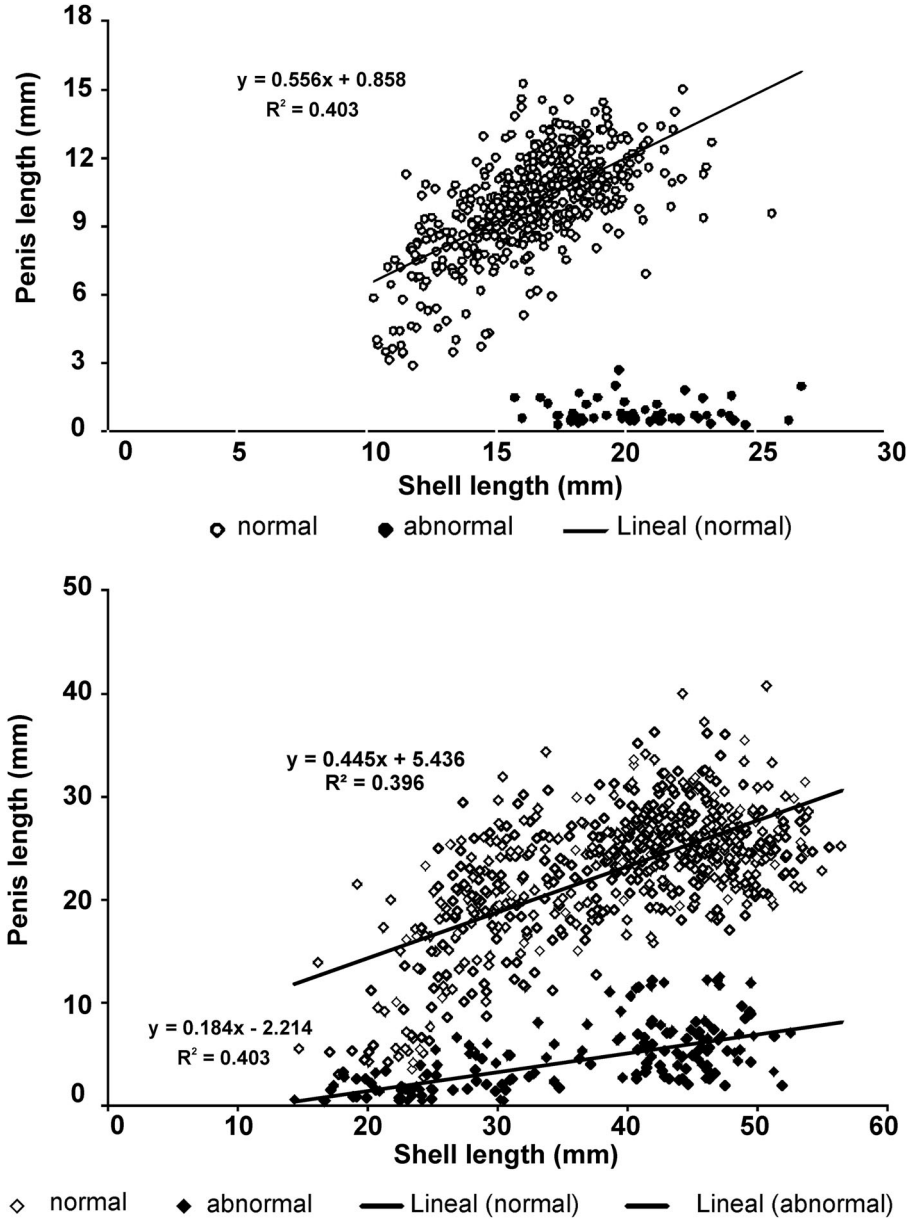

Figure 3. Penis length in relation to shell length in individuals with normal and abnormal penis of *Buccinanops deformis* from San Antonio Bay (top) and Villarino (bottom). In Villarino, combined data from specimens analysed at different samplings (240 in 2002–2003, 351 in 2006–2007 and 230 in April 2010) are shown. Aphallic individuals (those with only a bud instead a penis) were not considered for the graph or equation.

size between 16 and 25 mm. In this last case, the general appearance of the gonad of females with a penis was similar to that of normal females. Other features found in masculinized females in other studies, such as the formation of a conspicuous vas deferens, were also found here.

Trematode infestation was absent in the gonad samples from abnormal individuals ($n = 25$ from SAB and 22 from Villarino). Slide examination revealed the presence of larval parasitic stages only in the digestive gland of two individuals from Villarino. In the digestive gland, larval stages in sporocysts (of a non-determined digenean species) were found within the digestive tubules. One of these individuals was 19.4 mm in total shell length and showed no gonadal tissue and the other male was 30.0 mm long and showed a normal mature gonad.

Discussion

In this study, we demonstrate that, besides imposex, other sexual alterations not related to butyltins may arise in the nassariid *Buccinanops deformis*. We found that all the individuals with a short penis from SAB

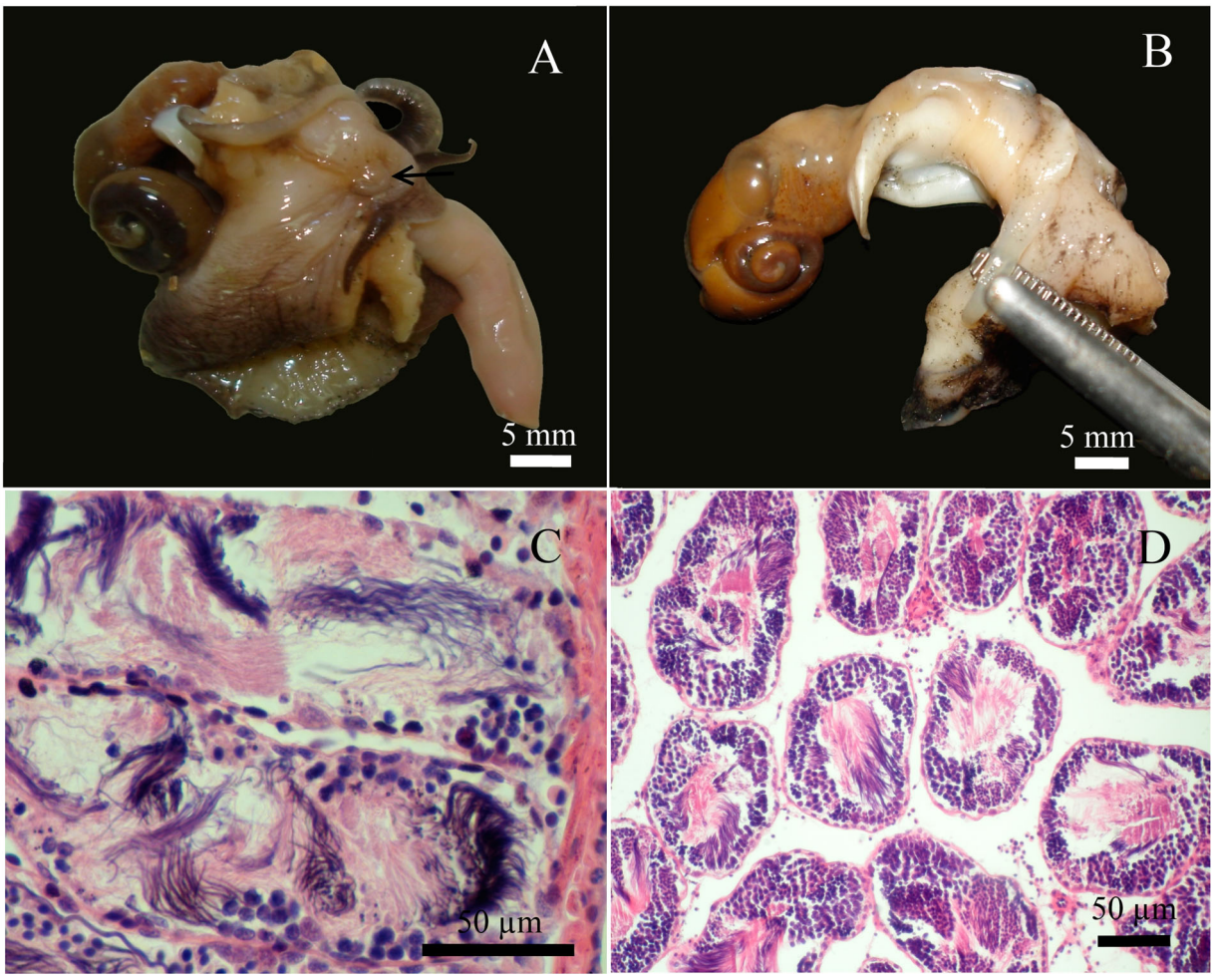

Figure 4. External and histological images of males from Villarino, with short penis (A and C) and males with normal penis (B and D). The short (abnormal) penis shown in A (arrow) is the type ending in a triangular bud.

were indeed imposexed females and that those individuals with a short penis from Villarino were all atypical males. Trematode infestation was not the cause of such malformations, as gonadal histological analyses showed that individuals with a short penis were not infested. Although spermatogenesis in males with

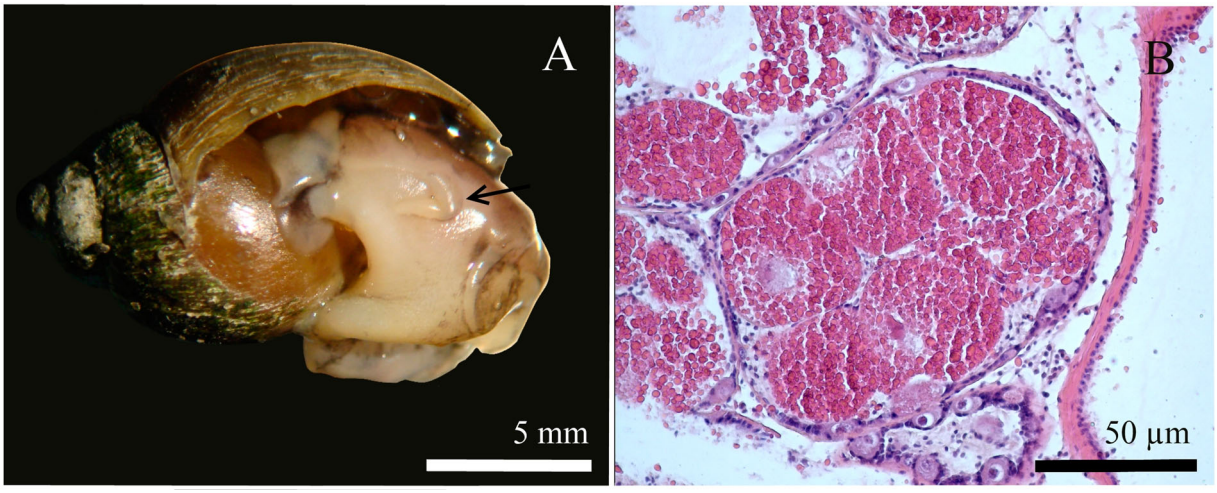

Figure 5. Females from San Antonio Bay; (A) image of the external appearance showing the penis (arrow) and vas deferens, and (B) a general histological image of the gonad.

atypical penis development seemed to take place normally, it poses the question of whether sperm transference could be successfully carried out. Regarding females showing imposex, the levels of butyltins in SAB seem not to limit their normal reproductive performance, as shown by the normal oviposition events observed in imposex females of that site (Avaca 2010; Avaca et al. 2012). Thus, if gender is accurately confirmed, *B. deformis* can be used as a biological indicator of imposex. In fact, this species has been proposed as an indicator of medium TBT contamination (Bigatti et al. 2009).

It is well known that males of some species such as *Tritia obsoleta* (Say, 1822) (Jenner & Chamberlain 1955; Curtis & Barse 1990) and some sea slugs (Lange et al. 2012; Milius 2013) normally lose the penis at the end of the reproductive season. If this were the case for *B. deformis* at Villarino, we would find males with variable penis sizes, irrespective of the individual size. However, because we found direct relationships between penis sizes and shell lengths, this phenomenon may not be the cause of the huge variations in penis sizes in the present study.

The RPLI is commonly used to compare imposex intensity and severity between populations in the same distributional area (Barroso & Moreira 1998; Titley-O'Neal et al. 2011). In fact, a positive relationship between tissue concentration of TBT and RPLI has been established for gastropod species of different families (Oehlmann et al. 1992; Solé et al. 1998; Titley-O'Neal et al. 2011). The RPLI found in the present study for SAB is similar to that established by Penchaszadeh et al. (2001) for zones with low maritime traffic (0–21%). In *Ocinebrina aciculata* (Lamarck, 1822), values of RPLI higher than 25% would indicate a snail population at decrease risk (Oehlmann et al. 1996). The RPLI does not apply for individuals from Villarino, as this index is valid only for imposexed females. The question of whether the environmental conditions which promote imposex in females also affect males has not been properly analysed. Bryan et al. (1988) noted that in sheltered locations with high TBT pollution males of *Nucella lapillus* show larger penis length. In relation to trematode infestations, Smith (1980) identified a reduced degree of penis expression among parasitized males of *T. obsoleta*. In fact, this finding, and also that performed in another *Buccinanops* species (*B. cochlidium* (Dillwyn, 1817); Averbuj & Cremonte 2010), led us to search for this infestation as a cause of the very variable penis sizes in our study, but our results were negative.

In the present study, both forms of butyltins, TBT and DBT, were detected in *B. deformis* from both sites analysed. Nevertheless, only TBT was detected in sediments at both sites, such that the concentration in SAB was higher (although also low compared to other studies; see Batista et al. 2016) than that in Villarino (only rarely at concentrations above the detection limit, and generally below the limits of European regulations: TBT > 1.3 ng g^{-1} for sediments). Furthermore, the prevalence of DBT over TBT in the tissues of *B. deformis* may reflect an efficient TBT degradation system or old TBT inputs (Phillip 2000; Wang et al. 2010; Batista et al. 2016). The presence of TBT and DBT in sediments suggests that the release of butyltins into the marine environment is related to shipping activity. Because the adsorption of TBT to sediments is reversible (and can vary with the pH of the water), TBT trapped in SAB sediments could be released back into the aquatic environment (Pinaert & Speleers 2004). The near-zero value of the butyltin biodegradation index indicates that there has been recent input of TBT into the marine environment. In fact, concentrations of TBT in SAB were threefold higher than those in Villarino, which may explain the frequency of imposexed females in that population. The fact that imposexed females have a size range similar to that of normal females would also indicate that the butyltin source is affecting females at present. This fact is important if we consider that, at the individual level, imposex is irreversible (Barroso et al. 2002).

Villarino and SAB are close to each other. However, Villarino is near an international regulated port, whereas SAB is near a regional inner port with less regulation applied in relation to paints with organotins for structures and commercial fishing vessels. The other reason explaining the differences in organotin levels between sites may be related to the percentage of organic matter in SAB, which is related to the fine fraction of sediments (Pinochet et al. 2009; Commendatore et al. 2015), which was much higher in SAB than in Villarino.

The finding of imposex in museum samples of gastropod individuals that lived before the TBT use (i.e. females with a penis and also males with two penes, Garaventa et al. 2006) challenges the TBT–imposex paradigm and is a clear demonstration that factors other than TBT could alter the endocrine control of sexual anomalies. For example, there are similarities between the phenomenon found in males of *B. deformis* inhabiting sediments of Villarino and the effects of Dumpton Syndrome found in males of *N. lapillus* of the northeastern Atlantic (Huet et al. 1996, Gibbs 2005). As is well known, Dumpton Syndrome demasculinizes both males and imposex females of *N. lapillus*, with the result that females

have much less severe imposex effects in the presence of TBT and males have varying degrees of penis reduction. Our study points out the need to be cautious when individuals with a short penis are qualified as imposex, as variable proportions of males develop a short penis, and then histology is needed to accurately sex them. Although in the butyltin analyses of *B deformis* we used females from SAB and males from Villarino, respectively, we do not think that the differences found in DBT levels between sexes is a gender-related response. The absence of significant differences in butyltin accumulation between sexes was also reported for other nassarid species (Couceiro et al. 2009; Batista et al. 2016; Cacciatore et al. 2016). Further studies on the effects of TBT, as well as of other environmental agents that may affect male expression and genetic approaches, are necessary to shed light on the cause of reproductive abnormalities such as those reported here.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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