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Penis Malformations in *Leucozonia nassa* (Gmelin, 1792) and *Leucozonia ocellata* (Gmelin, 1791) in a TBT Contaminated Region from Brazil

Mercia B. Costa (Corresponding author)

Biological Sciences Department, Federal University of Espirito Santo, Vitória, ES, Brazil

Center for Human and Natural Sciences, Postgraduate Program in Environmental Oceanography, Federal University of Espirito Santo, Vitória, ES, Brazil

E-mail: merciabc@gmail.com

Bruna S. Mardegan

Biological Sciences Department, Federal University of Espirito Santo, Vitória, ES, Brazil

E-mail: brunasmardegan@hotmail.com

Gabriela C. Zamprogno

Biological Sciences Department, Federal University of Espirito Santo, Vitória, ES, Brazil

Center for Human and Natural Sciences, Postgraduate Program in Environmental Oceanography, Federal University of Espirito Santo, Vitória, ES, Brazil

E-mail: gczamprogno@gmail.com

Felipe C. Pedruzzi

Biological Sciences Department, Federal University of Espirito Santo, Vitória, ES, Brazil

Institute of Oceanography, Postgraduate Program in Coastal Management, Federal University of Rio Grande, Rio Grande, RS, Brazil

E-mail: felipecorteletti@gmail.com

Letícia de Morais

Biological Sciences Department, Federal University of Espirito Santo, Vitória, ES, Brazil

E-mail: leticiademorais@gmail.com

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Gabriel Bautz Dalbem

Biological Sciences Department, Federal University of Espirito Santo, Vitória, ES, Brazil

E-mail: gbdalbem.26@gmail.com

Daniele Angeli da Silva

Biological Sciences Department, Federal University of Espirito Santo, Vitória, ES, Brazil

E-mail: daniangelisilva@gmail.com

Jones Bernardes Graceli

Department of Morphology, Federal University of Espirito Santo, Vitória, ES, Brazil,

Division of Pediatric Endocrinology, Johns Hopkins University, Baltimore, USA

E-mail: jbgraceli@gmail.com

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Abstract

Gastropod molluscs have potential for bioaccumulation of the organotin compounds (OTs) as tributyltin (TBT) and/or triphenyltin (TPT), used in antifouling paints applied to the hulls of ships to prevent biofouling. In gastropods, TBT may act as endocrine disruptors, and to induce imposex, impairing their reproductive capacity. The present study aimed to verify the occurrence of malformations, such as biphalic and triphalic penis in males and females with imposex in *Leucozonia nassa* and *L. ocellata* collected in Espírito Santo Bay between the years 2007 and 2011. During this period, 94.8% of female *L. nassa* and 24.5% of female *L. ocellata* exhibited imposex and 10 animals presented malformations. For *L. nassa*, malformations were found in seven females and one male, and for *L. ocellata* in two males. Most females with malformations presented VDSI stage ≥4 and these results indicate high levels of TBT contamination in the Espirito Santo Bay.

Keywords: Antifouling, Malformations, Penis, Imposex, *L. nassa*, *L. ocellata*

1. Introduction

Biofouling is the assemblage of phyto- and zoobenthos able to settle on natural or artificial hard substrata that are immersed in water (Dürr & Thomason, 2010) and can result in an undesirable accumulation of these organisms (Kotrikla, 2009).This accumulation can cause damage to the marine industry due to increased friction between the hull and water, which would require greater engine power and generate higher fuel consumption (Yebra et al., 2004). Furthermore, biofouling also facilitates the introduction of exotic species into aquatic environments.

Aiming to prevent biofouling, antifouling paints containing the organotin compounds (OTs) tributyltin (TBT) and triphenyltin (TPT) were introduced into the market in the 60s (Garaventa et al., 2006; Swennen et al., 2009). However, the use of these paints enables the gradual release of these compounds into the marine environment and affect non-target organisms (Alzieu, 1998). These substances are considered the most toxic man-made anddeliberately introduced into the environment (Goldberg, 1986; Stewart et al., 1992).

TBT has half-lives of days (Clark et al., 1988; Rudel, 2003), but in sediment the half-life of this compound can be very long, reaching to the years (Dowson et al., 1993, 1996). Tend to accumulate in the food chain and are stored in the tissues of mollusks, crustaceans, echinoderms, fish, birds and mammals (Duft et al., 2005; Guérin et al., 2007; Veltman et al., 2006; Choi et al., 2011). Thus, the toxic effects of these pollutants results in impacts that may cover one or more levels of the biological organization into an ecosystem (Ruiz et al., 1996).

In marine gastropods, TBT induces reproductive abnormalities of female animals. A first description of imposex was given by Blaber (1970) for *Nucella lapillus* when penis-like outgrowths were observed behind the right cephalic tentacle of females. This phenomenon, termed imposex by Smith et al. (1971) is characterized by the development of additional male sex organs (e.g., penis and/or vas deferens) on females. This syndrome is considered irreversible (Gibbs & Bryan, 1987) and in areas with high levels of TBT, females of gastropods may become sterile, due to the closure of the vulva, or die by necrosis in the capsule of eggs, leading to the decline or even extinction of a population (Gibbs & Bryan, 1994; Gibbs et al., 1997).

Gibbs et al. (1997) proposed that the magnitude of the development of male characters in females depends on the sensitivity of the species and concentration of TBT in the environment, according to a dose-response behavior. Therefore, imposex is considered the biological response most sensitive to TBT, being used as biomarker for different levels of contamination (Matthiessen & Gibbs, 1998).

The studies about the occurrence of OTs, particularly TBT, indicate a higher concentration of these substances in nearby ports and marinas, due to the activity of ships in these areas (Smith et al., 2006*;* Sousa et al., 2007). Due to the environmental effects of TBT, restrictions on the use on vessels less than 25m were imposed, first in France, then in the United Kingdom and in almost all developed countries during the eighties (Alzieu, 1991, 1996; Bosselmann, 1996; Champ, 2000; Yebra et al., 2004).

In 2001, the International Maritime Organization (IMO) adopted the 'International Convention on the Control of Harmful Anti-fouling Systems on ships' (AFS Convention), which prohibited the application of OTs as antifouling agents. This resolution called for a global prohibition on the application of OTs as biocides in antifouling paints on ships by the effective date of January 2003, and a complete banishment by January 2008 (Gipperth, 2009).

However, TBT pollution still persists in the marine environments of many countries (Murai et al., 2005; Shi et al., 2005; Cao et al., 2009; Strand et al., 2009; Gipperth, 2009). Due to the effectiveness of TBT-based paints, the absence of an equivalent substitute (Chambers et al., 2006) and also the persistence of these compounds in sediments (Fernandez et al., 2005; Godoi et al., 2003; Santos et al., 2009), a large amount of TBT containing waste is still being produced around ports (Kotrikla, 2009).

In Brazil, imposex has been studied primarily in *Stramonita haemastoma* (Castro et al., 2000, 2007a, b; Fernandez et al., 2002; 2005) and in *Stramonita rustica* (Lamarck, 1822) (Camillo et al., 2004; Castro et al., 2004, 2007a). Other species are also being used in biomonitoring programs, such as *Thais deltoidea* (Costa et al., 2008a), *Cymatium parthenopeumparthenopeum* (Costa et al., 2008b), *Nassarius vibex* (Cardoso et al., 2010) and species of the family Fasciolariidae, including *Leucozonia nassa* and *Leucozonia ocellata* (Costa et al., 2014).

Besides imposex, TBT contamination can cause malformations characterized by the appearance of a bifid and/or trifid penis. These malformations also occur in males and have been described recently for some species (Meirelles et al., 2007; Lahbib et al., 2008; Cardoso et al., 2009; Abidli et al., 2009) on studies in port areas.

Studies conducted in Espirito Santo Bay, Brazil, have shown the presence of high levels of contamination by TBT and its metabolites in the sediment in this area (Costa et al., 2014). The objective of this study was to verify the occurrence of malformation in males and imposexed females of the gastropods *L. nassa* and *L. ocellata* and its probable relationship to the occurrence of imposex in this region.

2. Materials and Methods

2.1 Study Area

Espirito Santo Bay, located in southeastern Brazil, is influenced by the presence of many ports like Port of Tubarão and Vitória Port Complex, marinas and intense maritime traffic, which are potential sources of OT pollution. Samples were collected at six sites in this bay (Figure 1): site 1- Point of Tubarão (369078, 7757961, site 2- Socó island (365753, 7756521), site 3 – Formosa beach (365531, 7755197), site 4 - Frade island beach (366347, 7754743), site 5 –Ribeiro beach (3671178, 7752228) and site 6 –Sossego beach (366973, 7752564). This area has been studied, and the occurrence of high levels of imposex confirms that pollution by TBT is producing deleterious effects on the local populations of gastropods (Costa et al., 2008a, b; Costa et al., 2014).

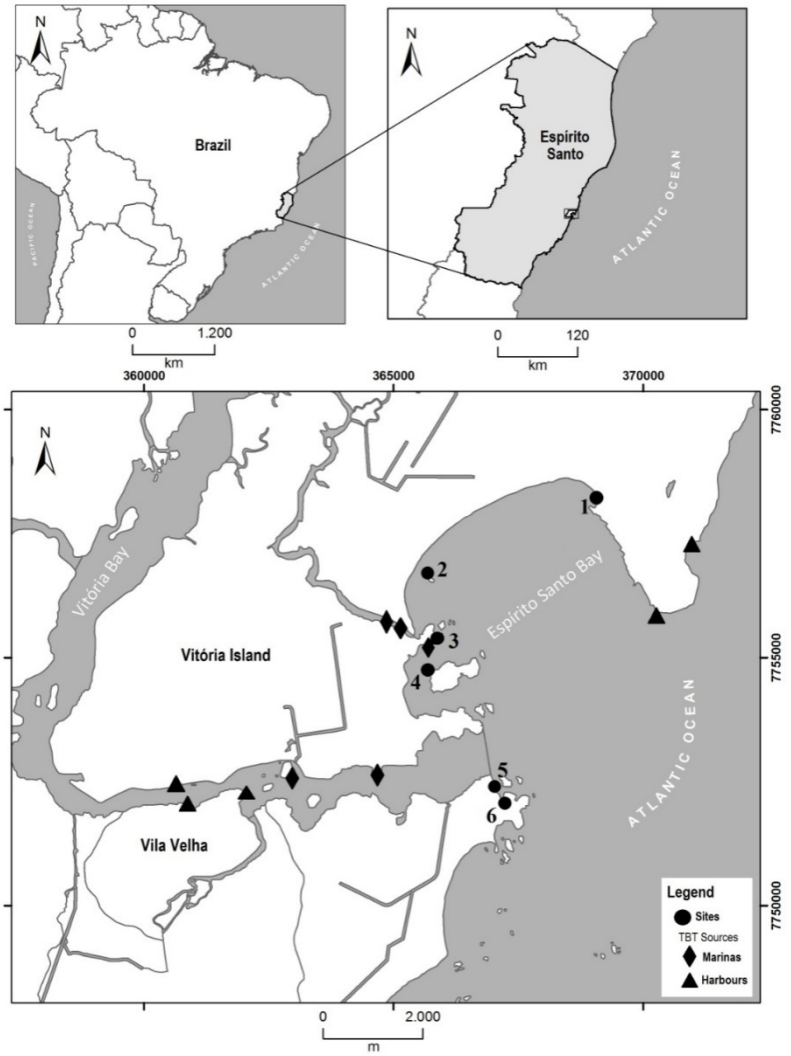

Figure 1. Espirito Santo Bay, ES, highlighting the collection points and the main sources of OTs

Notes: 1) Point of Tubarão, 2) Socó island, 3) Formosa beach, 4) Frade island beach, 5) Ribeiro beach and 6) Sossego beach.

2.2 Sampling and Imposex Analysis

This study was conducted between the years 2007-2011. Approximately 30 adult specimens of the species *L. nassa* and *L. ocellata* were collected during spring tides at each sampling site.

The collected samples were transported to the laboratory, where they remained in aquaria containing aerated seawater. For the analyses, the animals were anesthetized in a solution of 4% $MgCl_2$ in sea water and distilled water 1:1 and their shells were measured with calipers and broken with a vice. Individuals who had a capsule gland, seminal receptacle and vestibule were identified as females, and those who exhibited a penis and/or vas deferens in addition to these structures were identified as imposexed females. Individuals with a prostate,

penis and vas deferens were identified as males. Imposexed females and males who had a bifid and/or trifid penis were classified as having malformation (Figure 2).

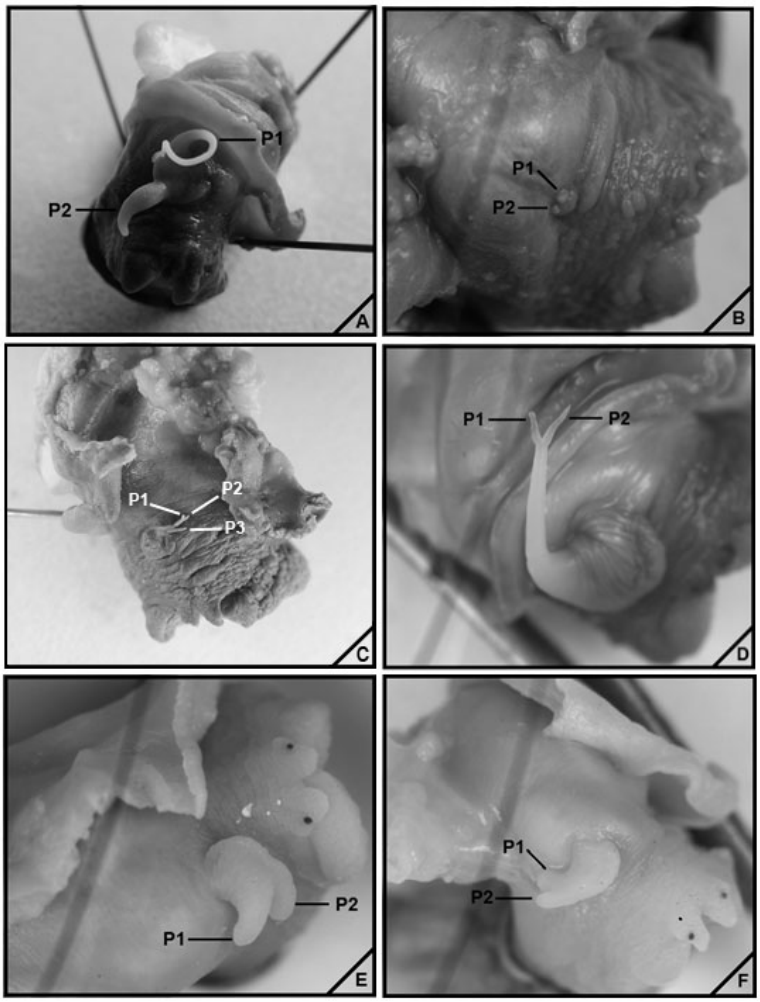

Figure 2. Individuals with malformation

Notes: A and B: *L. nassa* imposexed females with a bifid penis; C: *L. nassa* female with a trifid penis; D: *L. nassa* male with a bifid penis; E and F: *L. ocellata* male with a bifid penis. P1, P2 and P3: ramifications of the penis.

The length of the penis of males and imposexed females were measured with a caliper. To determine the levels of imposex were used three specific indexes: I% (percentage of imposex), RPLI (relative penis length index) and VDSI (vas deferens sequence index).The calculation of I% was made based on the number of imposexed females in relation to the total number of females. To determine the RPLI, the penis of males and imposexed females were measured and the values applied in the formula: (FPL/MPL) x 100, where FPL= average penis length of females and MPL = average penis length of the male, according to Gibbs and Bryan (1987). For the determination of the VDSI the scale proposed by Gibbs and Bryan (1994) was used. Correlations were observed between the rate of malformation and indices of

imposex (I%, RPLI and VDSI) by the Spearman test. Significance level threshold (α) for the latter test was set to 5%.

3. Results

From 2007 to 2011, 493 specimens of *L. nassa* were collected: 170 males and 323 females; of which 95% were affected by imposex. For *L. ocellata*, 58 individuals were collected: 29males and 29 females, with 24.5% of females exhibiting imposex.

At all sampling sites individuals with malformations were found. These malformations are represented in Figure 2 and included for example, the appearance of a bifid and/or trifid penis. Of the total individuals analyzed, 10 exhibited malformations, of those 7 were *L. nassa* females, 1 was *L. nassa* male and 2 were *L. ocellata* males.

The length of the penis of males and females, the VDSI stage and the type of malformation of the individuals are presented in Table 1. Individuals with malformation were found in all sampling dates. In 2008, one *L. nassa* female with the biggest penis length (16.9 mm) was found in the sampling site 5. From the same species, in 2011, one male with malformation and one female with trifid penis were registered in the sampling site 3.

Table 1. Analysis of individuals with malformation

Site	Year	Species	Sex	Penis lenght (mm)		VDSI	Malformation
				M	F		
1	2009	*L. nassa*	F		1.9	5	BP
1	2009	*L. nassa*	F		2.6	4	BP
2	2010	*L. nassa*	F		0.6	3	BP
3	2007	*L. nassa*	F		6.5	4	TP
4	2007	*L. nassa*	F		1.7	5	BP
4	2009	*L. nassa*	F		1.2	4	BP
4	2011	*L. nassa*	M	9			BP
5	2008	*L. nassa*	F		16.9	4	BP
6	2008	*L. ocellata*	M	3.3			BP
6	2008	*L. ocellata*	M	4			BP

Notes: M: Male; F: Female; VDSI: vas deferens sequence development; BP: bifid penis; TP: trifid penis. To measure the length of the penis was considered the higher branch.

The results of the indices (I%, RPLI and VDSI) and the percentage of malformations of all sample points in the four sampling years are shown in Table 2.The distribution of individuals did not follow a regular pattern, and in some points/years, specimens were not found. The percentage of imposex affected females for *L. nassa* were greater than 80% at all locations and years, while for *L. ocellata*, these indices were lower, ranging from 6 to 17%. RPLI values of *L. nassa* ranged between 3 and 37, and those of *L. ocellata* ranged from 18 to 23. In relation to VDSI, the indices ranged from 3.17 to 4.33 for *L. nassa* and from 3.00 to 3.44 for *L. ocellata*. For *L. nassa*, all points with the occurrence of malformation had 100%

imposexed females, except the Ribeiro beach with 83%, index also considered high. For *L. ocellata* malformations were found only in males. Although the malformation occurred at points with high levels of imposex, according to the Spearman test, was not significantly correlated between the rates of malformation and indexes (I%, RPLI, VDSI) ($p > 0.05$).

Table 2. Results obtained at the points where were found individuals of *L. nassa* and *L. ocellata* with malformation according to their years and collection sites

Species	Site	Year	N		N Malformation		%I	RPLI	VDSI	%Mr
			M	F	M	F				
		2007	16	24			100.00	18.18	4.08	
		2008	9	20			100.00	23.01	4.00	
L. nassa	1	2009	12	18		2	100.00	17.80	4.22	11.11
		2010	3	27			100.00	16.01	4.33	
		2011	5	25			100.00	19.06	4.12	
		2007	23	24			100.00	12.83	4.17	
		2008	12	18			100.00	12.09	4.00	
L. nassa	2	2009	13	17			82.35	3.65	3.80	
		2010	15	15		1	100.00	8.05	4.13	6.67
		2011	17	6			100.00	10.47	3.17	
L. nassa	3	2007	0	1		1	100.00		4.00	100.00
		2007	12	29		1	100.00	20.38	3.93	3.45
		2008	5	25			100.00	37.20	3.92	
L. nassa	4	2009	4	27		1	100.00	9.75	3.78	3.70
		2010	9	21			90.48	14.56	3.95	
		2011	10	20	1		90.00	9.48	3.89	10.00
L. nassa	5	2008	5	6		1	83.33	14.06	4.00	20.00
L. ocellata	6	2008	15	12	2		16.66	23.13	3.44	13.33
		2009	14	17			5.88	18.04	3.00	

Notes: N: number of specimens, M: male; F: female; I%: imposex percentage; RPLI: relative penis length index; VDSI: vas deferens sequence development; %Mr: malformation rate.

4. Discussion

The Espirito Santo Bay has intense maritime traffic due to the presence of ports and marinas in the region and has been monitored since 2007 with the species *Stramonita haemastoma* (unpublished data), *Thais deltoidea* (Costa et al., 2008a), *Cymatium parthenopeumparthenopeum* (Costa et al., 2008b), *Leucozonia nassa* and *L. ocellata* (Costa et al., 2014). This region has high levels of TBT and its metabolites were detected (Costa et al., 2014). So far there have been only records malformations in the genus *Leucozonia*.

According to the results of the present study, the values imposex indices were very high for *L. nassa* (95%) and lower for *L. ocellata* (24.5%). These results indicate a high contamination by TBT in the region, but also indicate a probable difference in response of these two species

to the pollutant, with *L. nassa* more sensitive than *L. ocellata*. This sensitivity difference to TBT between these species was evidenced by Costa et al. (2014). Interspecific differences to TBT were observed between other species, such as, *Murex brandaris* and *M. trunculus* (Lemghich & Benajiba, 2007) and *Thais rufotincta*, *T. distinguenda* and *T. bitubercularis* (Bech, 2002) and *Stramonita haemastoma* and *S. rustica* (Castro et al., 2007b).

For *L. nassa* the malformations occurred mainly in imposexed females while in *L. ocellata* only males presented malformations. The occurrence of malformations only in males of *L. ocellata* deserves further investigation, but suggests a difference in sensitivity between males and females of this species. The occurrence of malformations in both species may be more indicative of the deleterious effects of TBT on these organisms.

Most females with malformations presented VDSI stages equal to or greater than 4, which represents an advanced stage of imposex. Because all stations are located close to areas of naval activities, pollution by TBT was expected. Results obtained by Costa et al. (2014) confirm that this bay is contaminated by OTs, with values ranging from 383-7172 ng/g of TBT. The site 1 is greatly influenced by Tubarão Harbour, sites 2, 3 and 4 are under the influence of the Passage Channel, with many maintenance marinas for small boats and the Yacht Club, main leisure marina of Espirito Santo State, while sites 5 and 6 are influenced by the intense maritime traffic of large ships toward the Vitoria Port Complex.

Although the number of individuals that presented malformation is low, there is apparently sensitivity difference to TBT between the males and females of the species *L. nassa* and *L. ocellata,* once for *L. nassa* the malformations were present mainly in imposexed females while in *L. ocellata* only males presented malformations.

Malformations in *L. nassa* had already been reported by Meirelles et al. (2007), while malformations in *L. ocellata* are first reported in this study. Several authors have documented penis malformations in other species, such as Stroben et al. (1992) in *H. reticulata.* Terlizzi et al. (1998) observed penis bifurcation in imposexed females and males of *Hexaplex trunculus,* but with higher incidence in males. Vasconcelos et al. (2006) found males presenting penis excrescences and females exhibiting a rounded penis tip in *Hexaplex trunculus.* Similar malformations have also been reported by Lahbib et al. (2008) in males of *H. trunculus*. Cardoso et al. (2009) reported abnormalities in *Nassarius vibex*.

Although the mechanisms for the development of anomalies which are not well understood, some authors have implicated this malformations with TBT-polluted sites (Mensink et al., 2002; Lahbib et al., 2008).

The presence of imposex in *L. nassa* and *L. ocellata* and chemical analyses of the sediment (Costa et al., 2014) confirm the contamination by TBT in the study area and suggest a relationship between this contaminant and the incidence of malformations. The results also indicate a difference in sensitivity to TBT between the species and between the sexes. For *L. ocellata*, males are more susceptible than females, while *L. nassa* showed an inverse relationship.

5. Conclusions

Finally, reports on the imposex level and penis malformations in *L. nassa* and *L. ocellata* from a port area in Brazil are important for assessing the environmental consequences of the use of TBT-based antifouling paints in areas with boating activity. Further investigations on organotins in sediments and gastropod tissues are in progress. These results could be useful for verifying the effects of TBT-based antifouling and for supporting legislation to ban the use of TBT-based paints in Brazil.

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