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ORIGINAL ARTICLE

Transplant bioassay induces different imposex responses in two species of the genus *Stramonita*

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Abstract

A bioassay to study differential specific responses in imposex development due to marine organotin pollution was done by transplanting specimens of the gastropods *Stramonita haemastoma* and *Stramonita rustica* from an imposex-free area to a marina with high marine traffic inside a ship repair yard, a place where local populations of *S. haemastoma* were known to show high indices of imposex. Three hundred sexually mature, imposex-free specimens of each species were kept in cages for 120 days, and samples of 30 individuals were periodically analysed for imposex development. Shell length, penis length and vas deferens development were recorded and imposex development indices (% imposex, RPLI and VDSI) were calculated. Our results indicated that imposex induction in *S. haemastoma* is faster and more sensitive than in *S. rustica*. Imposex incidence in *S. haemastoma* could be a useful tool for monitoring marine pollution by organotin compounds in harbours along the Brazilian coast.

Key words: *Imposex, sensitivity,* Stramonita haemastoma, Stramonita rustica, *organotin, bioassay*

Introduction

Imposex (Smith 1971) is probably the most studied biological effect of organotin (OT) pollution. This endocrine disruption causes masculinization in female prosobranch gastropods, with affected females developing a penis and/or a vas deferens, which in some species led to sterilization and even death (Bryan et al. 1986; Gibbs & Bryan 1986).

Nowadays, imposex is a widespread problem, affecting more than 200 gastropod species (Shi et al. 2005; Sternberg et al. 2010). The occurrence and intensity of imposex is widely accepted as proportional to environmental levels of OT compounds, with a clear cause and effect relationship demonstrated (Matthiessen & Gibbs 1998; Horiguchi 2009; Sternberg et al. 2010). Hence, imposex has been widely used as an effective biomarker of OT pollution (Garaventa et al. 2006; Gravel et al. 2006; Vasconcelos et al. 2006; Galante-Oliveira et al. 2010; Mohamat-Yusuff et al. 2010). However, sensitivity may be different even among species from the same genus (Stroben 1996; Tan 1999), and a calibration of the intensity of imposex development is desirable for monitoring studies, making results comparable when more than one species is used (Gibbs et al. 1997).

Matthews (1968) reported the occurrence of six species belonging to the genus *Stramonita* on rocky shores along the Brazilian coast, of which *Stramonita haemastoma* (Linnaeus, 1767) and *Stramonita rustica* (Lamarck, 1822) have the largest spatial distribution. Moreover, *S. haemastoma* is the most abundant muricid species in Brazilian coastal environments, inhabiting mainly hard substrates in intertidal zones and eventually subtidal areas. Furthermore, this species is a key predator in rocky beach environments,

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feeding on barnacles, oysters and mussels (Gunter 1979). Additionally, some studies have reported that *S. haemastoma* is caught for human consumption by people along the Brazilian coast (Cavalcante-Braga et al. 2006). Both species showed imposex development and are good potential indicators of OT pollution in previous studies in Brazil (Fernandez et al. 2002; Castro et al. 2004), particularly since *S. haemastoma* is very common in tropical and subtropical areas, with populations spreading from southeastern Brazil to Florida, the Azores, eastern Atlantic and the Mediterranean (Spence et al. 1990; Rilov et al. 2000).

Imposex is widespread along the Brazilian coast (Castro et al. 2011b), indicating that antifouling pollution is probably continuing in many important coastal areas. Organotins have also been detected in sediments (Fernandez et al. 2005a; Santos et al. 2009; Oliveira et al. 2010) and animal tissues (Limaverde et al. 2007), mainly close to zones with high marine traffic zones such as the ports of Mucuripe, Rio de Janeiro, Paranaguá and Rio Grande (Castro et al. 2007a, 2007b). High imposex incidence have been observed in areas with high concentrations of OTs in sediments (Fernandez et al. 2005a), and a preliminary evaluation of possible human health effects of OTs ingestion through seafood was made in Brazil (Fernandez et al. 2005b). In Brazil, Resolution 357 of the Brazilian National Council of Environment (CONAMA 2005) recommends the maximum allowed concentrations of tributyltin (TBT) as 10 and 370 ng l^{-1}, according to the classes of saline waters (classes 1 and 2, respectively). In November 2007, the use of organotin-based antifouling paints was definitively banned in Brazil (NORMAM-23/DPC). However, the inspection and registration standards of all antifouling systems, as well as management of residues of these compounds, are not well controlled in Brazil. On the other hand, few OT monitoring studies were accomplished and the effectiveness of the restriction is poorly understood (Castro et al. 2011b). Even after the ban, some instances organotin pollution have been shown to continue (Toste et al. 2011).

The use of transplanted gastropods is useful for biomonitoring TBT pollution, as demonstrated with several species such as *Nucella lapillus* (Quintela et al. 2000; Smith et al. 2006), *Thais distinguenda* (Bech et al. 2002), *T. orbita* (Gibson & Wilson 2003) and *Hexaplex trunculus* (Lahbib et al. 2008). The present transplant experiment aimed to compare imposex responses between *S. haemastoma* and *S. rustica*, which are currently used as biomarkers for organotin pollution, along the coasts of Brazil.

Materials and methods

Stramonita haemastoma and *Stramonita rustica* with no signs of imposex were collected in September 2005 at Caponga Beach, Ceará state Brazil (Figure 1), prior to the TBT global ban in 2008. For each species, 300 adult specimens (shell length 20–40 mm) were collected, taken to the laboratory and kept in aerated aquaria. After a week of acclimation at the same salinity and temperature as the areas of origin, the animals were transferred to cages in the field. Cages were made of nylon netting, with five vertically arranged square wooden platforms measuring 45×45 cm. Animals were randomly distributed in two cages, separated by species. The cages, suspended from buoys, were settled in a small marina inside 'Industria Naval do Ceará' repair shipyard (see Figure 1). Animals were fed weekly with prey they would normally consume in the natural environment: *Crassostrea rhizophora* oysters (for *S. haemastoma*) and *Brachidontes* spp. mytilids (for *S. rustica*). These bivalves were collected from areas where the gastropods showed no imposex development. The imposex levels in the native *S. haemastoma* from the marina were checked every 30 days (positive control). Similarly, organisms of both species were collected at Caponga Beach every 60 days of the experiment to verify the imposex occurrence (negative control). Initially, 30 individuals of each species were sampled as $T = 0$, then similar samples were collected at $T = 15, 30, 45, 60, 75, 90, 105$ and 120 days. Animals collected at each sampling time were transported alive in aerated seawater to the laboratory.

In the laboratory, snail shell length was measured from the apex to the extremity of the siphonal canal with vernier calipers. Animals were then narcotized in 3.5% $MgCl_2$ (Huet et al. 1996) and soft parts extracted from the shells. Sexual identification was done by the presence of sperm ingesting gland, albumen and capsule glands in females and prostate in males. Penis length was measured to the nearest millimetre using calipers in both males and imposex-affected females. The imposex incidence and severity were assessed using the following indices:% of imposex-affected females (I%), Female Penis Length Index (FPLI = mean penis length of all females in the sample, including the zero values of aphalic females), Relative Penis Length Index (RPLI = [mean penis length in females/mean penis length in males] $\times$ 100)(Gibbs et al. 1987). Although animals were relaxed with $MgCl_2$, it can cause an increase penis measurements (Vasconcelos et al. 2011); all the measurements were done identically for all the individuals, so comparisons could be made in this experiment. Future works using the same species as in this work must take into account

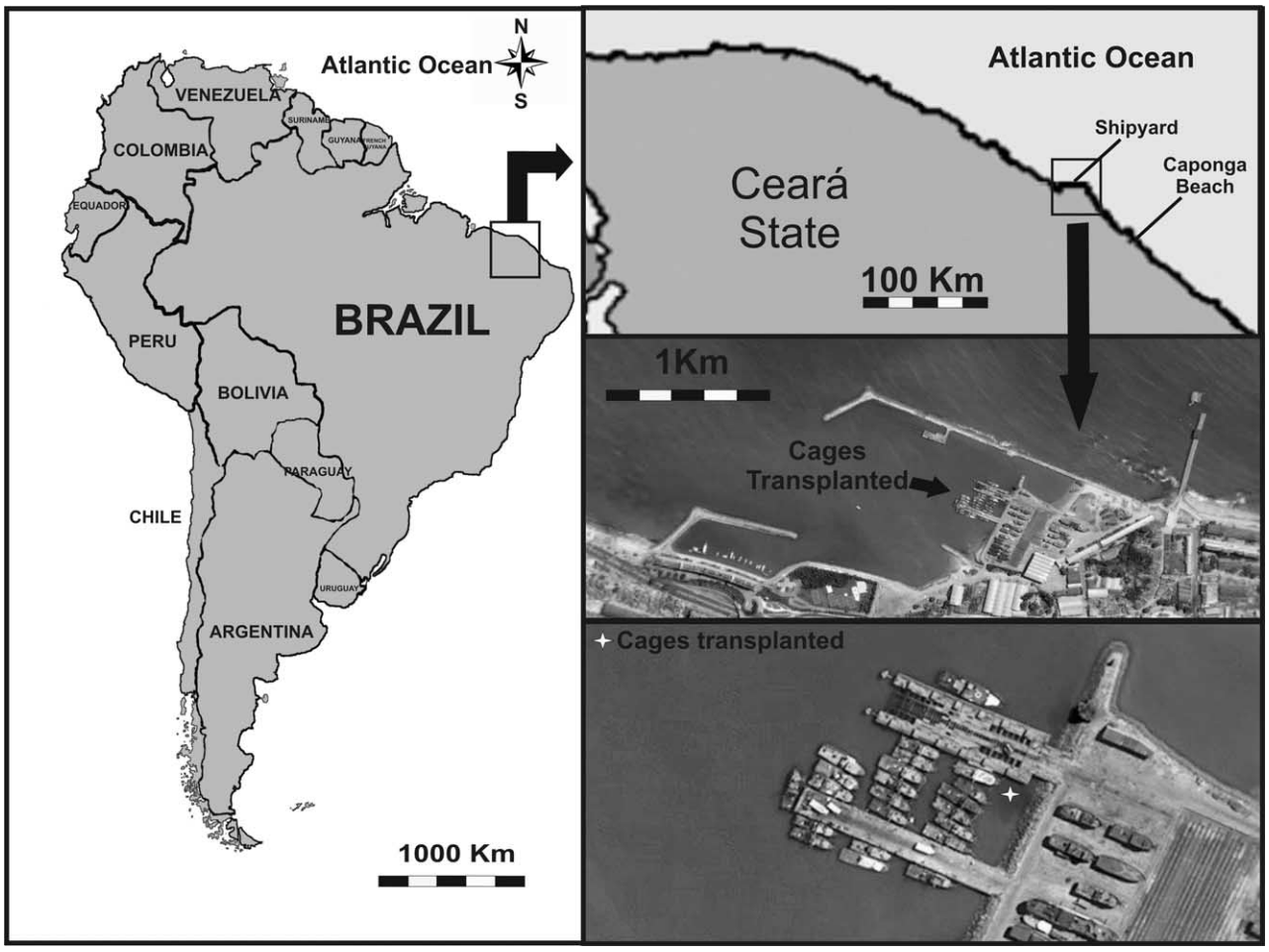

Figure 1. Sampling site (imposex-free) at Caponga beach and transplant site in a crowded shipyard.

the procedures made here for comparisons with our results. The Vas Deferens Sequence Index (VDSI), based on the development of male sexual characters (penis and/or vas deferens) in females, was calculated using a six-stage scale similar to the classic Gibbs scale for *Nucella lapillus* (Gibbs et al. 1987), adapted for *S. haemastoma* according to Fernandez et al. (2005a). In brief, the VDSI stages were determined as follows: (0) normal female, (I) beginning of penis or vas deferens formation, (II) penis developed [length < 2 mm], (III) penis developed [length > 2 mm], (IV) completely developed vas deferens, (V) vulva blocked by vas deferens growth, and (VI) dark mass of aborted eggs in the capsule gland.

Raw data were checked for normality using the Lilliefors test. Interspecific tests for FPLI and VDSI variation at Day 15 and Day 120 were done using the Mann–Whitney U-test. Correlation of the mean values of the imposex indices was inferred by the Pearson coefficient. In all statistical analyses, significance level was $p < 0.05$.

Results

Snails sampled at the control site did not show imposex incidence at 0, 60 and 120 days, while females of both transplanted species developed penises during the experiment (Figure 2). After 15 days of transplantation, *Stramonita haemastoma* was the only species presenting signs of imposex in 90% of the females (Figure 3). At this time, development of the vas deferens was registered in 78.6% of the females (Figure 4), while a penis could be measured in only one snail (3.5%). No females of *S. rustica* showed imposex at $T = 15$ days. However, at $T = 30$ days, 38.5% of females started to show imposex development, albeit lower than *S. haemastoma* with FPLI $= 0.4$ and VDS stage I. At this time, imposex development in *S. haemastoma* was much higher, with all females, presenting imposex (Figure 3). Faster imposex development in *S. haemastoma* was confirmed throughout the study period. At Day 90, imposex was induced in all females of both species. Female penises were much more developed in *S. haemastoma*, reaching values of RPLI comparable to

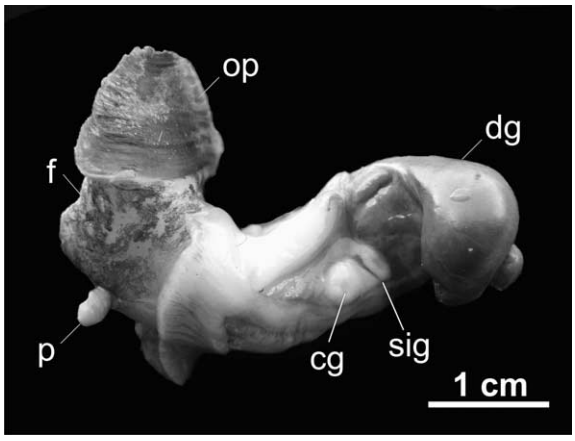

Figure 2. Imposex-affected *Stramonita haemastoma* female showing penis (p) and sperm ingesting gland (sig), operculum (op), digestive gland (dg), foot (f) and capsule gland (cg).

those found in snails sampled in the polluted area at the end of the 120 days (Figure 3). Vagina blocked by vas deferens tissues (VDSI $\geq$V) was observed in native organisms (9 – 17% of the samples). In transplanted *S. haemastoma*, genital blocking was detected after 75 days of transplantation, with percentage ranging between 11.1 and 28.6%, while in *S. rustica*, imposex development was much less pronounced than *S. haemasoma* at the end of the experiment (Figure 4).

All imposex indices showed a strong Pearson correlation (r^2) for the duration of the experiment, for *S. haemastoma* (FPLI = 0.96, VDSI = 0.91, RPLI = 0.96) and for *S. rustica* (FPLI = 0.91, VDSI = 0.93, RPLI = 0.94). Due to the initial imposex-free condition of females, comparisons were made from Day 15 to Day 120. As shown in Table I, females of both species showed significant penis development in this period. Imposex development, as indicated by FPLI and VDSI, was significantly different between species.

Discussion

Our results demonstrated that imposex is induced very quickly in both species, probably due to high sensitivity to TBT pollution as registered worldwide (Fent 2006; Bigatti et al. 2009; Santos et al. 2009; Castro et al. 2011a). The maximum time used in this experiment was only 120 days, but the rapid responses of the individuals treated (100% imposex at 30 days in *Stramonita haemastoma* and 90 days in *S. rustica*) allowed us to confirm the different

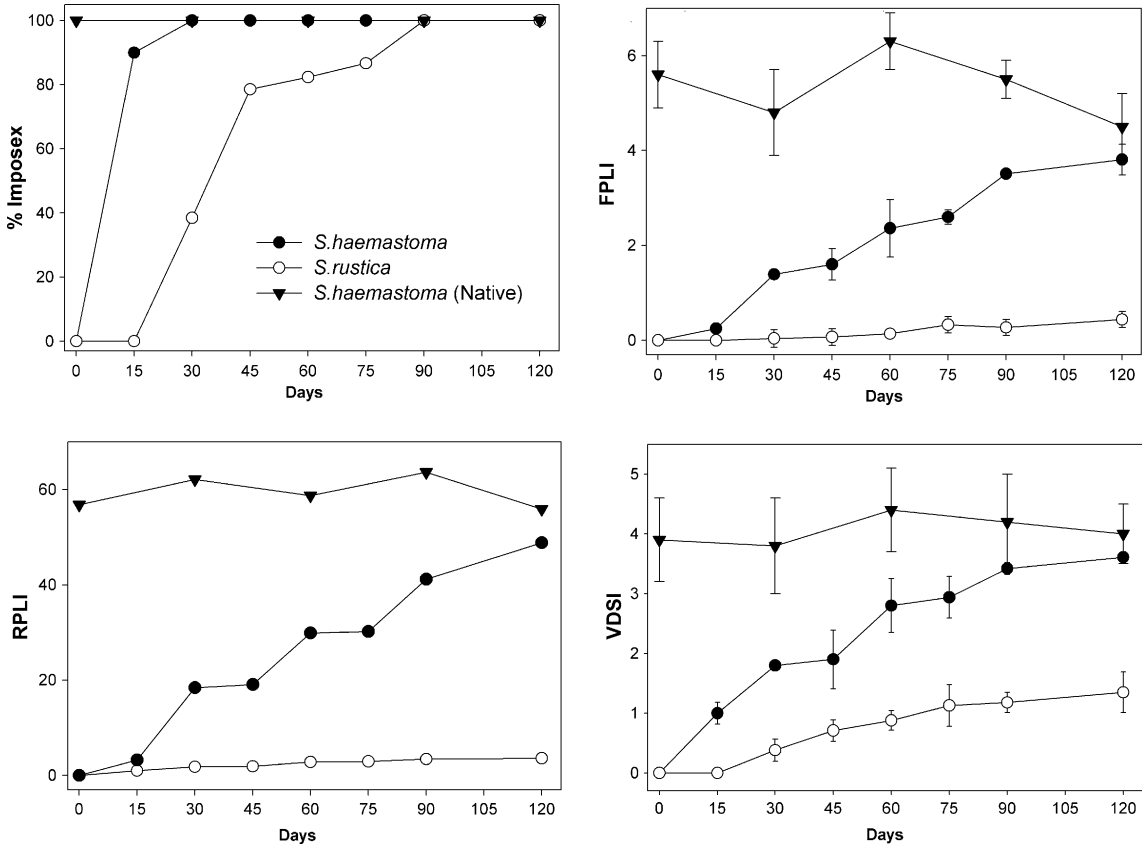

Figure 3. Variations in the imposex indices (I%, RPLI, FPLI and VDSI) of *Stramonita haemastoma* and *S. rustica* transplanted to a repair shipyard during 120 days. Bars indicate standard deviation.

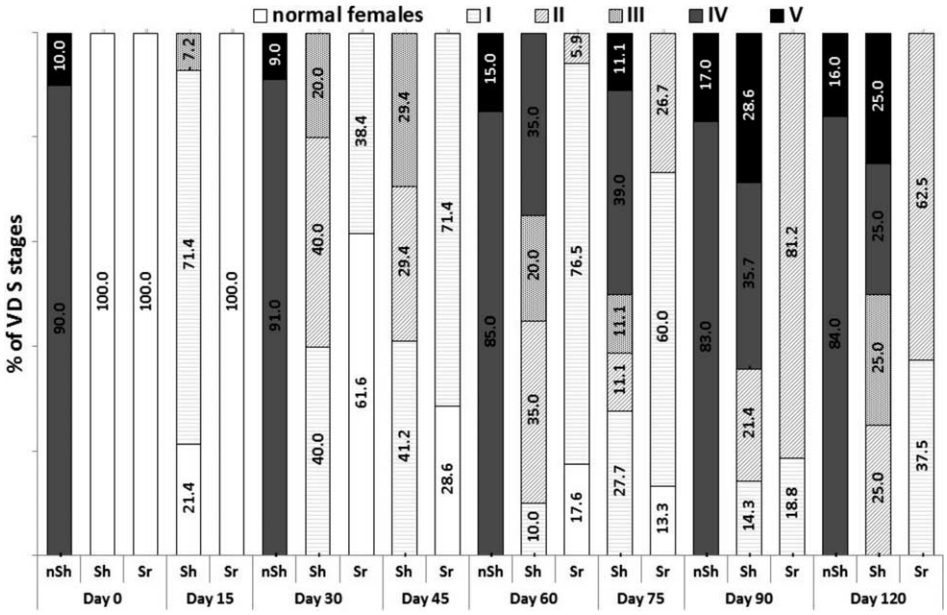

Figure 4. Frequency of VDS stages registered throughout the transplant experiment (native *Stramonita haemastoma* (nSh), *S. haemastoma* (Sh) and *S. rustica* (Sr) transplanted).

physiological responses of both species. Nevertheless, future experiments regarding specifically VDSI should be done over longer periods of time to reach the same condition as in native species. As biomonitoring studies are frequently hampered by the spatial distribution of bioindicator species (Liu et al. 1997), monitoring studies could provide relevant information about the imposex response of the bioindicator species (Bech et al. 2002; Lahbib et al. 2008). Several studies compared the sensitivity for imposex responses in different gastropod species (Bech 1999; Barroso et al. 2000; Tewari et al. 2002; Bigatti et al. 2009). However, even when species of the same genus are compared, specific differences in sensitivity may be apparent (see Stroben et al. 1995). These differences may be explained by differential physiological responses, bioaccumulation factors or excretion rates for xenobiotics, or even specific feeding habits (Gibbs et al. 1997). The results obtained by Huet et al. (1995) comparing VDSI and RPSI (Relative Penis Size index) development in *Ocenebra erinacea*, *Nucella lapillus* and *Hinia* (-*Nassarius*) *reticulata* support this observation.

In Brazil, *S. haemastoma* has been employed as a bioindicator species in many studies (Fernandez et al. 2005), as has *S. rustica* (Camillo et al. 2004; Castro et al. 2004), but both species seldom occur together. Near sites with high marine traffic as marinas, harbours and shipyards, sterile *S. haemastoma* were frequently detected in Brazilian coastal waters (Fernandez et al. 2005a). On the other hand, sterility was not observed in *S. rustica* populations in similar conditions. Another study with *S. haemastoma* showed that imposex development could be fast (in the order of 15–20 days), particularly when juvenile animals were exposed (Mensink et al. 2002). Those results are in accordance with our study that detected first signs of imposex at Day 30 and 100% imposex at Day 90 in both species. On the other hand, transplanted *S. haemastoma* showed an extremely fast response with 100% imposex at Day 30. In transplant

Table I. Mann–Whitney tests for intraspecific and interspecific comparisons of FPLI and VDSI in *Stramonita haemastoma* and *S. rustica*.

Tests			*p*-value
Intraspecific	*S. haemastoma*	FPLI (15 days) × (120 days)	0.000143
		VDSI (15 days) × (120 days)	0.000137
	S. rustica	FPLI (15 days) × (120 days)	0.000001
		VDSI (15 days) × (120 days)	0.000001
Interspecific	Initial FPLI (15 days) *S. haemastoma* × *S. rustica*		0.000254
	Final FPLI (120 days) *S. haemastoma* × *S. rustica*		0.000143
	Initial VDSI (15 days) *S. haemastoma* × *S. rustica*		0.000002
	Final VDSI (120 days) *S. haemastoma* × *S. rustica*		0.000235

experiments using other species, similar but slower responses were registered: Lahbib et al. (2008) reported 100% imposex in *Hexaplex trunculus* from Tunisia approximately 5 months after their release into a zone with high marine traffic. In the Australian *Thais orbita*, imposex incidence reached 73% after 9 weeks of transplantation (Gibson & Wilson 2003), while in *Thais distinguenda* imposex incidence reached 86.4% after 12 months of transplantation (Bech et al. 2002).

The present study clearly demonstrated that *S. haemastoma* is more sensitive to imposex induction than is *S. rustica*. The first species showed a faster imposex development and faster penis growth in females. It is remarkable that *S. haemastoma* starts to develop a vas deferens 15 days after transplantation. A field study performed in Bizerta channel (Tunisia) by Lahbib et al. (2010) demonstrated that *S. haemastoma* is less sensitive than *H. trunculus* to the same exposition to contaminated waters. Differences in species responses were also recorded by Bech (1998) for *T. distinguenda* from Phuket Island (Thailand) and by Bech (1999), that compared imposex development in the muricids *T. distinguenda*, *Thais bitubercularis* and *Morula musiva*. The last study indicated that *T. bitubercularis* was more sensitive, thus being a preferable bioindicator. Bigatti et al. (2009) also registered different imposex responses in two species of muricids from Patagonia exposed to the same TBT contamination in zones with high marine traffic. It is worth noting that while biomonitoring studies should always prefer the most sensitive species, these should also be abundant and broadly distributed to make the study viable; when these last conditions cannot be satisfied, it would be preferable to use a less sensitive but more abundant species (Forbes et al. 2006). In any case, comparative studies may allow verification of the differences among imposex responses when different species are sampled from the same areas (Stewart et al. 1992; Tan 1999; Tan & Liu 2001; Birchenough et al. 2002). In the case of *S. haemastoma*, it is distributed along almost all the Brazilian coast, and its abundance, high sensitivity and rapid imposex response make it a very good tool for monitoring marine pollution by OTs. Although TBT determinations were not performed in the studied area, the imposex incidence registered in this study could only be explained from contamination by antifouling paints. Although other harbour pollutants, such as hydrocarbons or heavy metals, are expected to be present (Commendatore et al. 2000; Bigatti et al. 2009), according to the current knowledge these classes of compounds do not induce the imposex response. In this sense, the degree of contamination of the soft tissues of *S. haemastoma* should be analysed as this species is consumed as food along the Brazilian coast.

Imposex development in *S. haemastoma* was extremely fast when compared to similar assays done in Thailand with *T. distinguenda* (Bech 2002a; Bech 2002b; Bech et al. 2002). Therefore, *S. haemastoma* is likely to be a useful bioindicator of OT pollution on rocky shores along approximately 9000 km of Brazilian coastline. However, the current study was performed before the global TBT ban issued in September 2008 by the International Maritime Organization. In this context, it would be interesting to continue sampling analyses in order to verify the effectiveness of the global TBT ban along the Brazilian coast. Knowing the differences in imposex induction between *S. haemastoma* and *S. rustica* is a useful tool for future monitoring programmes of TBT pollution in Brazilian coastal areas. As stated before (Fernandez & Pinheiro 2007), the very slow transition from TBT-based to TBT-free antifoulings in developing countries continues to make imposex a very useful tool to evaluate the extent and intensity of impact from antifoulants in coastal areas.

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