

Imposex in One of the World's Busiest Shipping Zones

Carter Li and Rachel Collin

ABSTRACT. Tributyltin pollution from antifouling paint is well known to disrupt the endocrine system in female marine gastropods. The masculinization of females, including the aberrant growth of a penis and vas deferens and occlusion of the capsule gland, has been reported primarily in neogastropods and is particularly well documented in muricids. Compared to temperate areas, few studies of imposex have been undertaken in the tropics, and there are few studies in general on non-neogastropods. Here we report a high frequency of imposex near the Pacific mouth of the Panama Canal in two species of muricids and two species of calyptraeids. The frequency of imposex declined rapidly with distance away from the canal, and several species appeared to be mostly normal less than 10 km from the entrance. This is the first report of imposex in *Acanthais brevidentata*, *Thaisella kiosquiformis*, *Bostrycapulus calyptraeformis*, *Crepidula* cf. *nivea*, and *Anachis fluctuata*. Because imposex has not previously been reported for the Calyptraeidae, a family of protandrous gastropods, a laboratory study was conducted to verify that imposex was not simply retention of the penis after sex change. The 2007 ratification of the International Maritime Organization's convention on antifouling systems should reduce the levels of TBT worldwide, but the persistence of this compound in sediments suggests that imposex may continue to be a problem at the mouth of the canal as routine dredging and large tides frequently resuspend sediment.

INTRODUCTION

Tributyltin (TBT) is well known to be a highly effective antifouling agent, used primarily on ship hulls, but it has numerous detrimental effects on a wide variety of non-target taxa. Despite having demonstrable effects on molluscan shell growth (Alzieu et al., 1981), embryological development of fish and marine invertebrates (Hano et al., 2007; Inoue et al., 2006), neurulation in ascidians (Dolcemascolo et al., 2005), and testosterone metabolism in mysids (Verslycke et al., 2003), the most well studied and widespread effect is the disruption of the endocrine system in marine gastropods. Exposure to very low levels (as little as 0.5 ng/L) of TBT causes the masculinization of females, including the aberrant growth of a penis and occlusion of the capsule gland (Gibbs and Bryan, 1996). This condition is referred to as imposex, and severe cases can lead to reproductive failure. For example, an extreme case of population decline as a result of imposex has been demonstrated for *Nucella lapillus* in southwest England (Bryan et al., 1986; Gibbs and Bryan, 1986).

Carter Li, Department of Biology, McGill University (current address: Department of Surgery, Montreal General Hospital). Rachel Collin, Smithsonian Tropical Research Institute, MRC 0580-08, Unit 9100, Box 0948, DPO AA 34002, USA. Corresponding author: R. Collin (collin@si.edu). Manuscript received 21 July 2008; accepted 20 April 2009.

In a recent review, Shi et al. (2005) reported that imposex has been recorded in 170 species of gastropods from 28 families. The vast majority, 134 species, are neogastropods. Among the non-neogastropod, caenogastropod families, ampullarids, rissoids, cypraeids, cymatids, and tonnids all contain several species for which imposex has been reported (Shi et al., 2005). Although the taxonomic coverage is wide, much of the basic information on imposex in relation to TBT pollution is centered on muricids, buccinids, and conids (Fioroni et al., 1991; Shi et al., 2005). On a worldwide scale, it is necessary to extend the scope of studies to include more tropical forms and locations (Ellis and Pattisina, 1990) to get a global picture of the effects of TBT pollution on gastropods.

The Panama Canal is one of the world's busiest shipping zones, and commercial transport through the canal represents about 5% of world trade. About 14,000 vessels pass through the Canal annually (statistics available from the Autoridad del Canal de Panama web site <http://www.panacanal.com/>), and the most common shipping route is between the east coast of North America and Asia. Most of the shipping traffic is composed of large, oceangoing vessels, which have not previously been subject to restrictions on the use of tributyltin antifouling paint. The entrance to the Canal, on the Pacific coast adjacent to Panama City, was the site of Rodman Naval Base (1943–1999), and is currently the site of the container port of Balboa and a shipyard. The anchorage for the canal commonly has more than 30 vessels waiting to transit the Canal. The substrate in this area is primarily a mix of rocky debris and sandy mud in the intertidal and fine mud in the subtidal. With the consistently high levels of shipping traffic, frequent dredging, and muddy substrate (which is known to retain TBT for years, as reviewed in de Mora, 1996), the local levels of TBT and, therefore, imposex are expected to be higher around the entrance to the Canal than they are along the open coast. We conducted a survey of four common intertidal gastropod species around the mouth of the canal to document the levels and geographic extent of imposex in this area.

MATERIALS AND METHODS

Gastropods were collected between February and April 2005 from four sites along the Pacific coast of Panama at varying distances from the mouth of the Panama Canal (Figure 1). The site closest to the mouth of

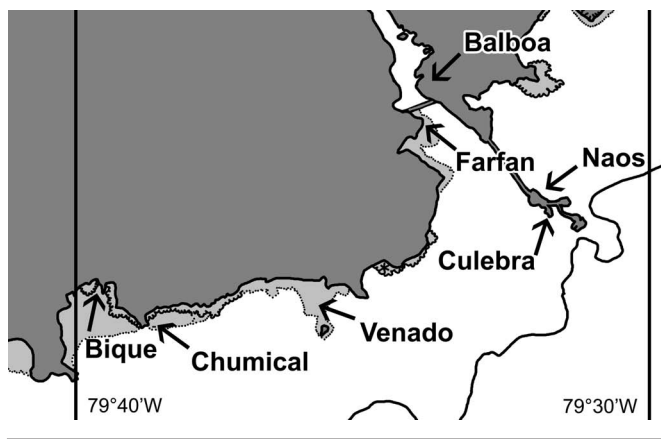


FIGURE 1. Map of the study area at the entrance to the Panama Canal. Arrows indicate the sites of sample collection and locations mentioned in the text.

the canal consisted of rocky outcrops near Farfan beach (8.93°N, 79.58°W) and the Bridge of the Americas. Progressively further away to the west were Isla Venado (8.91°N, 79.63°W), Chumical (8.5°N, 79.66°W), and Bique (8.90°N, 79.66°W). In November 2007 additional samples were collected from Punta Culebra (8.91°N, 79.53°W), which faces the entrance to the Canal and is at the edge of the Canal anchorage.

We collected four species, which were clearly identifiable and abundant at two or more of the sites. Efforts were made to collect the same species from all sites, but because of the habitat heterogeneity in the area, we were not able to collect sufficient numbers of females for statistical analyses for several sites. Adequate samples were collected for the muricids *Acanthais brevidentata* (Wood, 1828) from Farfan and Chumical and *Thaisella kiosquiformis* (Duclos, 1832) from Farfan and Bique, and the calyptraeids *Bostrycapulus calyptraeformis* (Deshayes, 1830) and *Crepidula* cf. *nivea* from Farfan, Venado, and Chumical (Table 1).

Shell length was measured with vernier calipers, and live snails were extracted from their shells. The reproductive system was immediately examined under a stereomicroscope, and the sex was determined based on characteristics of the gonad and presence or absence of seminal receptacles and seminal vesicles. If the sex was not easily identified, sex was verified by examining gametes from a smear of gonad. The length of the penis (if present) was measured using an ocular micrometer on a stereomicroscope.

TABLE 1. Frequency of imposex in four gastropod species at sites arranged here from nearest to furthest from the Panama Canal entrance. Frequency at each site was compared to that at Farfan (the site at the entrance to the Canal) using a Fisher's exact test. A one-tailed test was used, but two-tailed results did not differ; * $P = 0.001$; ** $P = 0.0001$; *** $P = < 0.0001$.

Species	Site				
	Farfan	Culebra	Venado	Chumical	Bique
Muricids					
<i>Thaisella kiosquiformis</i>	29/53	–	–	–	13/52*
<i>Acanthais brevidentata</i>	8/32	–	–	0/57**	–
Calyptreaids					
<i>Bostrycapulus calyptraeformis</i>	60/63	22/43 ^{a***}	2/79 ^{***}	1/122 ^{***}	–
<i>Crepidula cf. nivea</i>	87/90	19/22	–	0/99 ^{***}	–

^a Significantly different from Venado and Chumical < 0.0001 .

Significant differences in the frequency of imposex between the entrance to the Canal and more distant sites were tested for using Fisher's exact test. Analysis of covariance (ANCOVA) was used to examine the relationship between penis length in male and imposex females, with shell length as a covariate for samples collected from Venado and Farfan. Because samples from Culebra were preserved in ethanol before examination, the penis length from these samples could not be directly compared to the others that were measured fresh.

Experiments to determine if imposex develops in adult snails after exposure to ambient water levels of TBT were conducted at STRIP's Naos Marine Laboratories, only a few hundred meters from the Culebra site. *Anachis fluctuata* (Sowerby, 1832) and *Bostrycapulus calyptraeformis* were both collected from Isla Venado, an area with low levels of imposex, and maintained in the laboratory. Sixty adult *Anachis fluctuata* were kept in a 100 L fiberglass tank in the outside seawater system and fed frozen commercial clams once a week. After five months the animals were killed and levels of imposex were determined as already described. *Bostrycapulus calyptraeformis* were collected as small males. They were maintained in pairs in the laboratory in 350 mL plastic cups. The water was changed every other day and the animals were fed 10 mL *Isochrysis galbana* culture every day. Animals were measured every four weeks, and their sexual state was recorded on the basis of external features. The experiment was terminated after 400 days. Both species were cultured using the same source of seawater (from the side of Isla Naos away from the Canal entrance), and neither was exposed to local sediment other than that which settled out of the seawater.

RESULTS

FIELD COLLECTIONS

Imposex was detected in all four species. In the two muricids, the imposex was almost always in the early stages with limited penis development and no indication of any occlusion of the capsule gland. We never observed imposex that was so far advanced that the females were found to retain eggs or that an obvious vas deferens had developed. Imposex in the calyptraeids was more developed; penes were large in many specimens and could easily be confused with a normal male penis. Several imposex females of *Bostrycapulus calyptraeformis* and *Crepidula cf. nivea* were observed brooding egg capsules, showing that imposex females were not sterile. Near the entrance of the Canal the frequency of imposex ranged from 25% to 50% in muricids and was greater than 80% in calyptraeids. The number of females collected for each species at each site and the frequency of imposex are given in Table 1. In all cases the frequency of imposex was significantly higher near the entrance to the Canal than at farther sites (Table 1).

Acanthais brevidentata: Because there were no imposex individuals in Bique and because animals from that site were significantly larger (mean = 28.9 mm) than from Farfan (mean = 26.9 mm; $P < 0.001$), comparisons of imposex females with normal males and females were conducted for data collected from Farfan only. Imposex females were significantly larger (length = 30.1 mm) than non-imposex females (length = 26.4 mm; $P < 0.02$). ANCOVA showed that there were significant effects of shell length ($P = 0.01$) and imposex ($P < 0.001$) on

penis length as well as a significant interaction effect ($P < 0.04$). Penes of imposex females were smaller than those of normal males, and male penis length increased with shell length, although imposex penis length was not associated with shell length (Figure 2).

Thaisella kiosquiformis: Animals from Bique and Farfan did not differ in size, nor did the sexes differ in size. Imposex females were also the same size as non-imposex females. The average size for all categories was 26–27 mm. Data

from Bique and Farfan were combined for the analysis. ANCOVA showed that there were significant effects of shell length ($P < 0.0001$) and imposex ($P < 0.0001$) on penis length as well as a significant interaction effect ($P = 0.003$). Penes of imposex females were smaller than those of normal males, and penis length increased with shell length in both sexes (Figure 2). There was a significant incidence of imposex at Bique, despite it being the site furthest from the Canal. We attribute this relatively high frequency of imposex to this site's prox-

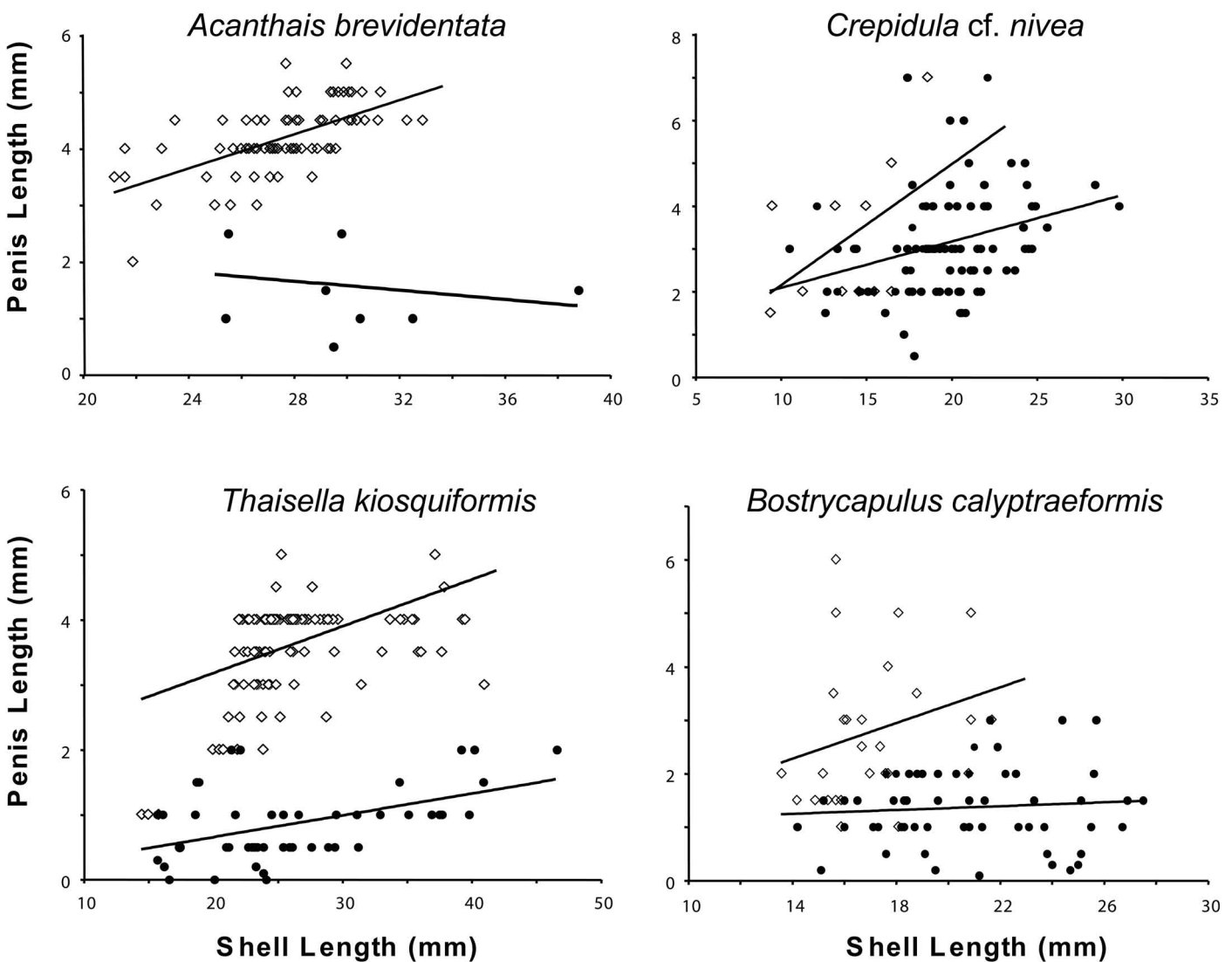


FIGURE 2. Relationship between shell length and penis length for males (white diamonds) and imposex females (black dots) of *Acanthais brevidentata*, *Thaisella kiosquiformis*, *Crepidula cf. nivea*, and *Bostrycapulus calyptreaformis*.

imity to a dry dock facility slightly further to the west in Vacamonte.

Crepidula cf. nivea: Calyptraeids are protandrous hermaphrodites (Collin, 2006) and the small animals are almost always males; therefore, we did not make as much effort to collect the smallest animals as we did in the other species. The size of females differed significantly between the sites (16.1 mm for Chumical vs. 19.6 mm for Farfan; $P < 0.0001$). Imposex females were larger than non-imposex females. Because all females at Chumical were normal and virtually all in Farfan had imposex, this size difference cannot be distinguished from a site effect on size. An ANCOVA showed that shell length had a significant effect on penis length ($P = 0.03$), that imposex females and males did not differ in penis length as there was considerable penis growth in the imposex females, and that there was no significant interaction between imposex status and shell length (see Figure 2). Although there were significant levels of imposex in samples from Culebra, in all these cases the penis was very small; they were not much more developed than a small bump at the base of the tentacle, whereas those from Farfan were often as long as or longer than the tentacles.

Bostrycapulus calyptraeformis: The average size of females differed significantly among the three sites (17.5 mm at Chumical; 16.5 mm at Venado; 20.6 at Farfan; $P < 0.01$). Again, because nearly all the females in Farfan had imposex but no imposex was detected in the other locations, the larger size of imposex females may have been a site effect. ANCOVA analysis of animals from Farfan showed that there was a significant effect of imposex on penis length ($P < 0.001$), and imposex females had smaller penes than males. Shell length and the interaction between shell length and imposex had no significant effect on penis length. The level of imposex in animals from Culebra was again very rudi-

mentary, with penes little more than a nub at the base of the tentacle.

In summary, all four species showed significant higher rates of imposex near the entrance of the canal. By 20 km away, rates were generally of the order of 1%–2%. In the two muricids and one of the calyptraeids, the penes of imposex females were smaller than those of similar-sized males. In the two muricids and the other calyptraeid, shell length was a significant covariate of penis length, and in two species the penis length of imposex females increased with shell length.

LABORATORY EXPERIMENTS

After five months in the laboratory, 2 of 29 female *Anachis fluctuata* had developed penes, indicating that this species can develop imposex. However, this was not statistically significantly different from the frequency of imposex in the field in Venado ($P = 0.09$; Table 2). No comparisons to the entrance to the Canal could be made because this species could not be found there.

Of the 60 *Bostrycapulus calyptraeformis* that were raised in the laboratory, the largest animals in 6 of the 30 cups retained penes throughout the experiment and did not change to become female. In the remaining 24 cups, the larger of the 2 animals lost the penis, indicating sex change from male to female. Of these 24 animals, 10 lost the penis and then subsequently regained it 1 to 3 months after sex change. In many cases the penis was not as long or thick as a normal male penis, but they were fairly large, and casual observers would be likely to categorize such animals as males (Figure 3). The largest animals in the remaining 14 cups underwent transition to normal females and did not develop imposex before the end of the experiment. The smaller of the 2 animals in each cup was not examined, as they usually remain male in the presence of the larger animals (Collin et al., 2005).

TABLE 2. Frequency of imposex from field-collected and laboratory-reared snails.

Species	Laboratory	Venado	Chumical	Fisher's exact test, P value
<i>Anachis fluctuata</i>	2/29	1/133	–	$P = 0.091$
<i>Bostrycapulus calyptraeformis</i>	10/24	2/79	1/122	$P < 0.0001, < 0.0001$

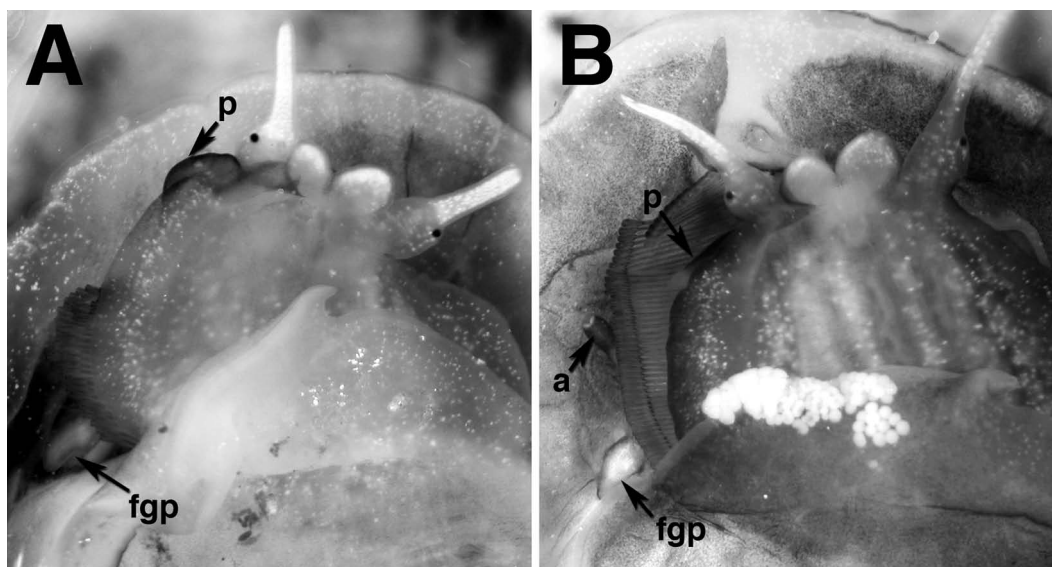


FIGURE 3. A, B. Photographs of two individuals with well-developed imposex in *Crepidula cf. nivea* from Farfan, with penis (p) and female genital papilla (fgp) indicated in each image. B. The female genital papilla can be confused with the anus (a), which is usually obscured by the gills; brooded eggs are visible as the light mass in this image.

DISCUSSION

Imposex was found in all snail species examined from the mouth of the Panama Canal, and in all cases the frequency and severity of imposex declined with distance away from the Canal. The frequency of imposex differed among the species examined, with calyptraeids more likely to display imposex than the muricids. Calyptraeids showed higher frequencies of imposex at the mouth of the Canal, and the penes of imposex females were much more fully developed than we ever observed in muricids. Species differences in sensitivity both to TBT (Wilson et al., 1993; Tan, 1999) and to its bioaccumulation (Liu et al., 1997) have been demonstrated in other surveys. Liu et al. (1997) found that imposex was much more severe in *Thais* species than *Morula*, despite similar organotin burdens, and suggested a genus-specific susceptibility to organotin pollution with the ranking order of *Nucella*, *Thais*, and *Morula*. The differences in habitat (high on the intertidal versus low on the intertidal), diet, and physiology have been suggested as causes of interspecific differences in imposex (Tan, 1999). If TBT were primarily waterborne either in solution as *bis*(tributyltin) oxide or adsorbed by suspended solids (de Mora, 1996) at our study sites, it is possible that filter-feeding calyptraeids would be exposed to more TBT, by filtering large volumes

of water, than would other gastropods. Suspended particles may have TBT adhered to them and may be captured in the mucous net and ingested during filter feeding, thus increasing the exposure of calyptraeids relative to the muricids. These scenarios are not in agreement with a number of laboratory studies (Bryan et al., 1989; see Gibbs and Bryan, 1996, for review) that show that TBT accumulates more rapidly from the diet than from the ambient water and which suggest that carnivores could accumulate more TBT from their diet than would herbivores. However, controlled experimental comparisons of bioaccumulation between carnivorous and suspension-feeding gastropods have not been made, and the effects of suspended solids have not been examined.

Another factor that can influence the expression of imposex is age. Because extended exposure to TBT is necessary to elicit imposex, those species that are longer lived or slower growing may be more likely to have high levels of TBT and thus exhibit imposex. Studies have also shown that juvenile snails are more sensitive to TBT than are adults (Gibbs and Bryan, 1996). Our data for *Acanthis brevidentata*, showing that females with imposex are larger than normal females, are consistent with either increase in imposex development with long-term exposure or recent reductions in TBT levels. However, *Thaisella kiosquiformis* did not show this pattern. Few data on the

age or lifespans of tropical gastropods are available and so this possibility is difficult to evaluate. However, Panamanian calyptraeids grown in the laboratory generally reach maturity at sizes similar to animals that matured in the field, in less than a year (Collin et al., 2005, and personal observation), and it seems unlikely that TBT in the sediment, which has a half-life of years, would have changed drastically in such a short interval.

Imposex has not been previously reported in calyptraeid gastropods. Because animals normally change from males to females and transitional animals may sometimes retain a penis while also showing well-developed female reproductive structures, it is possible that imposex individuals have previously been misidentified as undergoing the normal transition between the male and females phases. Here we found, in sites with low expected TBT exposure, that there are virtually no individuals that display both male and female characteristics at the same time. In addition, our laboratory studies show that during sex change the penis can be reabsorbed and that the penes of imposex individuals can grow following this reabsorption. These results show that the abundant large females with penes collected at the entrance to the Canal are indeed imposex females and not transitional individuals that have yet to lose the penis.

Numerous studies have shown a tight relationship between levels of TBT in the environment, levels of TBT in gastropod tissues, and the frequency of imposex within species (Gibbs et al., 1987; Horiguchi et al., 1994; Minchin et al., 1997; Ruiz et al., 1998). However, the relationships between sites, species, and the different types of triorganotins are not always simple (Ide et al., 1997). Imposex has also been shown to be a more sensitive way to detect TBT than many chemical detection methods, and imposex has been used as a bio-indicator when TBT levels are too low for easy analytical detection (Gibbs and Bryan, 1996). Despite an extensive literature on the relationship between TBT and imposex, one study (Nias et al., 1993) indicates imposex could result from exposure to paint matrix or copper. However, this result has not been pursued or elaborated. Although we could not measure levels of TBT directly at the sites around the Canal, it can, in the light of this literature, be inferred with some level of confidence that the exposure of animals to TBT is higher at the entrance to the Canal than it is in the surrounding areas. Despite the high levels of shipping and presumably high levels of TBT leaching into the surrounding water, the development of imposex was not so severe as has been reported for areas with high shipping traffic in Europe and Asia, and TBT does not have an extreme impact on reproduction by occluding the pallial oviduct

or splitting the bursa copulatrix and capsule gland, as has been reported from these regions (Oehlmann et al., 1996; Shi et al., 2005). Less obvious effects on reproduction were not directly evaluated in this study. The high amount of flushing in the area, from large volumes of discharge from the Canal and the 6 m tides, may help to prevent local buildup of high concentrations of TBT in this partially enclosed area.

In 2002 the International Maritime Organization adopted a Convention on Antifouling Systems (AFS) that called for a global prohibition of the application of organotin compounds as biocides in antifouling systems on ships by 1 January 2003 and a complete prohibition by 1 January 2008. However, the prohibition was only to be implemented 12 months after 25 states representing 25% of the world's merchant shipping tonnage ratified it. In September 2007 this quota was met when Panama ratified the convention, and therefore these regulations went into effect in September 2008. As the AFS convention applies to ships flagged in, operated by, or docking in states that have ratified it, the convention should significantly reduce the exposure of Panama's marine habitats to TBT pollution in the coming years. This regulation is especially important because the planned expansion of the Canal in 2014 will significantly increase shipping traffic along both the Pacific and Caribbean coasts of Panama.

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