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New Imposex Development Index (IDI) for *Stramonita haemastoma* (Mollusca: Muricidae): A Transplantation Experiment in the Brazilian Northeast

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ABSTRACT

A total of 600 *Stramonita haemastoma* (Mollusca: Muricidae) individuals, free of imposex, were transplanted into a marina with an intense ship flow. Thirty individuals were collected periodically until 210 days and analyzed according to RPLI (Relative Penis Length Index), RPSI (Relative Penis Size Index), IDI (Imposex Development Index, developed in this study), and imposex percentage. The proposed Imposex Development Index (IDI) presented three developmental paths, being two of them aphallic. The incidence of aphally was quite high along the experiment. An underdevelopment of the male characters in imposexed females was clearly observed. At first sample (15 days) all females showed evidence of imposex. RPLI and RPSI had a strong correlation with time along the transplantation. Correlation of imposex development (IDI) with time was also significant. This index increased gradually, reaching a stage IV at day 210, with all females showing a complete *vas deferens*.

Key words: aphally, Brazilian northeast coast, imposex, organotin, ship flow, *Stramonita haemastoma*, transplantation, tributiltin.

RESUMO

Novo Índice de Desenvolvimento de Imposex (IDI) para *Stramonita haemastoma* (Mollusca: Muricidae): um experimento de transplante no Nordeste brasileiro

Foram transplantados 600 espécimes de *Stramonita haemastoma* (Mollusca: Muricidae) sem imposex para uma marina com fluxo intenso de embarcações. Trinta indivíduos foram coletados periodicamente até 210 dias e analisados quanto aos índices RPLI (Relative Penis Length Index), RPSI (Relative Penis Size Index), IDI (Imposex Development Index, desenvolvido neste estudo) e porcentagem de imposex. O IDI apresentou três rotas de desenvolvimento de imposex, sendo duas afálicas. A incidência de afalia foi consideravelmente alta ao longo do experimento. Um subdesenvolvimento dos caracteres sexuais masculinos em fêmeas imposexadas foi bastante evidente. Indícios de imposex foram observados com apenas 15 dias de experimento. O RPLI e RPSI apresentaram forte correlação com o tempo de experimento. A correlação entre o desenvolvimento de imposex (IDI) e o tempo foi também significativamente alta. Este índice cresceu gradualmente, alcançando um valor máximo de IV em 210 dias, com todas as fêmeas apresentando vaso deferente completo.

Palavras-chave: afalia, fluxo de embarcações, imposex, Nordeste brasileiro, organoestânicos, *Stramonita haemastoma,* transplante, tributilestanho.

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INTRODUCTION

Imposex is the best-known effect of the contamination by organotins, where tributiltin (TBT) is considered the most toxic pollutant deliberately introduced by man into marine environment (Goldberg, 1986; Terlizzi *et al.*, 2001). Imposex consists of the superimposition of male sexual characters onto females of gastropods. As a result of enzymatic inhibition of specific metabolic sites, imposex is proportional to the concentration of these compounds, with a clear cause/effect relationship between pollution by organotins and the occurrence of imposex (Matthiessen & Gibbs, 1998).

Generally, gastropods with imposex have been recorded in areas with a heavy flux of ships (Smith & McVeagh, 1991) or close to ports (Evans, 1999), therefore the use of imposex-affected gastropods species has been widely used to assess organotin pollution. Thus, several imposex quantification indexes were developed. Gibbs *et al*. (1987) were the first to develop an imposex quantification scale, the Vas Deferens Sequence Index (VDSI).

After them, Stroben *et al*. (1995) created another index demonstrating alternative paths for the development of the penis and *vas deferens* in *Trivia arctica, Trivia monacha, Nucella lapillus, Ocenebra erinacea, Hinia incrassata* and *Hinia reticulata.*

For *Stramonita haemastoma,* Fernandez *et al*. (2005) developed a VDSI scale based essentially in penis length and, whenever possible, in *vas deferens*. However, imposex has recently been observed with underdevelopment, or non-development (aphally), of the penis (Gibbs, 2005).

This study aimed to create an imposex quantification index for *S. haemastoma*, since VDSI scale adapted for Fernandez *et al*. (2005) did not fit in aphallic imposex cases, and to relate the appearance of imposex to the time of exposure in healthy females transplanted to a port area.

MATERIALS AND METHODS

At first, samplings were performed in areas where an imposex-free population of *S. haemastoma* was identified (Lima *et al.*, 2006). A total of 630 adult *S. haemastoma* individuals were collected at Caponga Beach, in the eastern coast of Ceara, and 600 were put in plastic cages and transferred to a marina of a shipyard (Figure 1) with very high marine traffic, in Fortaleza, Ceara State. The remaining 30 individuals were previous analyzed for the presence of imposex. No indications of imposex were found in any of the animals examined.

The caged animals were fed every two weeks with *Crassostrea rhizophorae* oysters from the transplantation site. Samples of 30 individuals were removed from the cages at days 15, 30, 45, 60, 75, 90, 120, 150, 180 and 210 after transplantation. The animals were anesthetized with a 3.5% magnesium chloride saline solution and the shells removed.

The females were analyzed for the presence of imposex, according to four indexes: RPLI [(mean length of female penis)/(mean length of male penis) × 100], RPSI [(mean length of female penis)^3/(mean length of male penis)^3 × 100] and the percentage of imposex. The forth index, IDI (Imposex Development Index) a modified VDSI, was proposed in the present study since the indexes found in the literature do not include a developmental path found in this study (aphallic imposex).

Due to the occurrence of many females without a penis, only with a *vas deferens*, RPLI and RPSI were calculated using only the penis sizes of the others females.

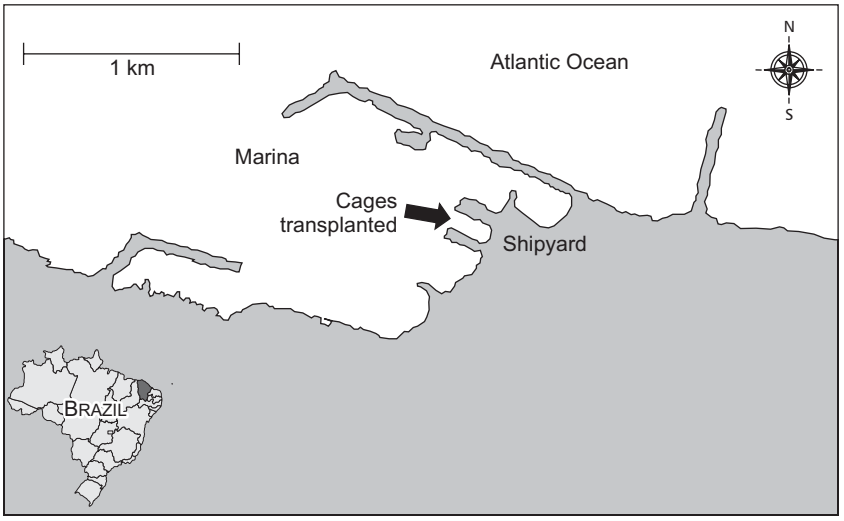

Figure 1 – Geographic region of transplantation site in a marina on Northeast of Brazil.

A Pearson parametric test was used to assess the correlation of RPSI, RPLI and IDI with time of exposure ($p < 0.05$); these statistical treatments were done using software *Statistica* v6.0. The Kruskal-Wallis test was applied to compare the IDI averages of each sampling, followed by the Dunn multiple comparisons test ($p < 0.05$); these treatments were done using the software *GraphPad Instat* 3.01.

RESULTS

Imposex Development Index (IDI)

Three different developmental sequence of male characters were observed (Figure 2) corresponding to three imposex paths.

In the "a" path, the females developed the same kind of imposex usually reported in the literature (Spence *et al.,* 1990), with the development of a penis and a *vas deferens*; in the remaining two paths the development of a vas deference always occurred, but in path "b" the penis did not develop, staying just as a papilla behind the right ocular tentacle; in path "c" the females were totally aphallic. Each of these paths was divided in four (I-IV) stages.

At the Ia stage, the papilla appears close to the right tentacle; in stage IIa, a *vas deferens* is developed, with a size up to 1/3 of its usual full length close to the penis, which is shorter than 2 mm; in stage IIIa, the penis is longer than 2 mm and the *vas deferens* reaches 2/3 of its full length; finally, in stage IVa the *vas deferens* is complete, linked to the capsule's gland (Figure 2).

For paths "b" and "c", in which no penis is observed, the characterization of the different stages was based only on the length of the *vas deferens*, where stage I represents a papilla or initial *vas deferens*; in stage II a *vas deferens* with 1/3 of its full length is observed; in stage III it reaches 2/3 of its total length; and in stage IV the *vas deferens* is complete.

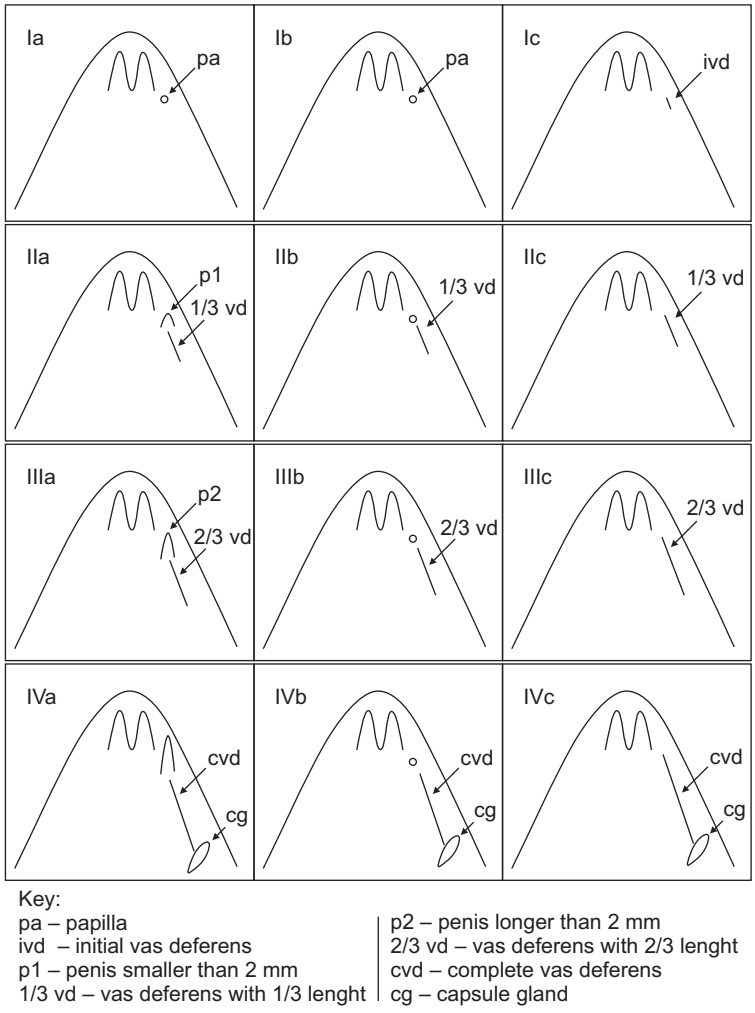

Figure 2 – Imposex Development Index (IDI) proposal for the species *Stramonita haemastoma*, detailing the alternatives paths of the penises development.

Imposex levels

The occurrence of imposex was observed already at the first sampling (day 15) in 100% of the analyzed females. In this sampling, imposex indexes showed the following values: RPLI = 1.34; RPSI = 0.0002, with all females corresponding to stage I of the IDI scale.

The RPLI showed a strong correlation with time ($r = 0.81$; $p < 0.01$), reaching a peak of 76.16 at day 120. In the last sampling (day 210), this index reached a value of 53.68 (Figure 3). The RPSI correlation with time along the experiment was extremely significant ($r = 0.86$; $p < 0.01$). Its highest value was reached at the last sampling (day 210) with a value of 15.47 (Figure 4). The correlation between imposex development (IDI) and time showed to be the most significant index ($r = 0.96$; $p < 0.01$) (Figure 5).

According to the statistical tests, the IDI means' variation throughout the experiment was considered significantly higher than a random result ($p < 0.01$). In the multiple comparisons test, the IDI means showed a significant difference between the initial and final sampling months (Table 1).

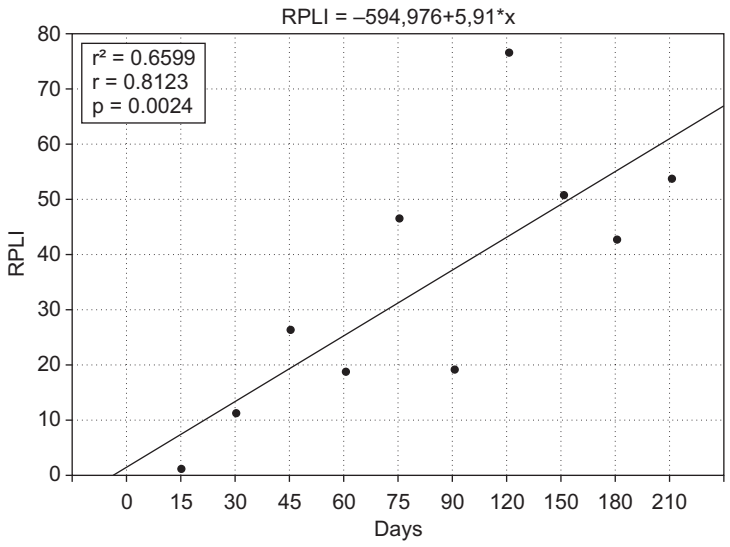

Figure 3 – Correlation of RPLI with experiment time.

Table 1 – Months considered significantly different in agreement with the multiple comparison test for the IDI means.

Days after transplantation compared	Significance level	p value
30 x 120	***	$p < 0.001$
30 x 150	***	$p < 0.001$
30 x 180	***	$p < 0.001$
45 x 120	**	$p < 0.01$
45 x 150	***	$p < 0.001$
45 x 180	**	$p < 0.01$
60 x 120	**	$p < 0.01$
60 x 150	***	$p < 0.001$
60 x 180	***	$p < 0.001$
90 x 150	*	$p < 0.05$

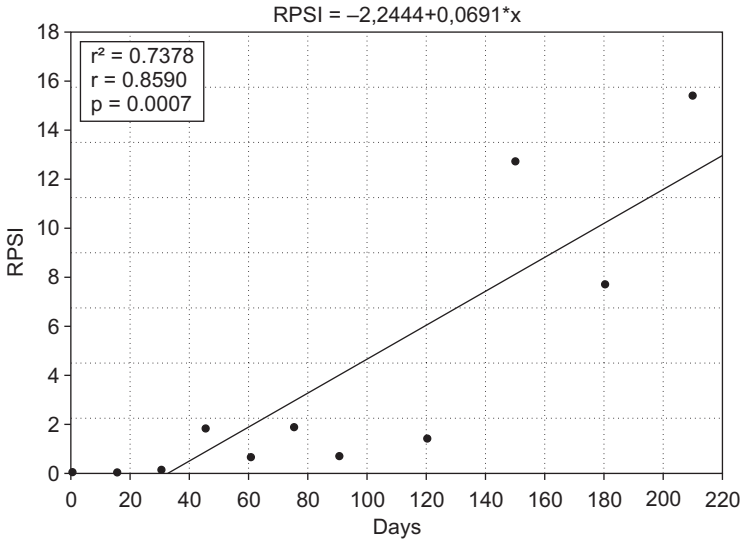

Figure 4 – Correlation of RPSI with experiment time.

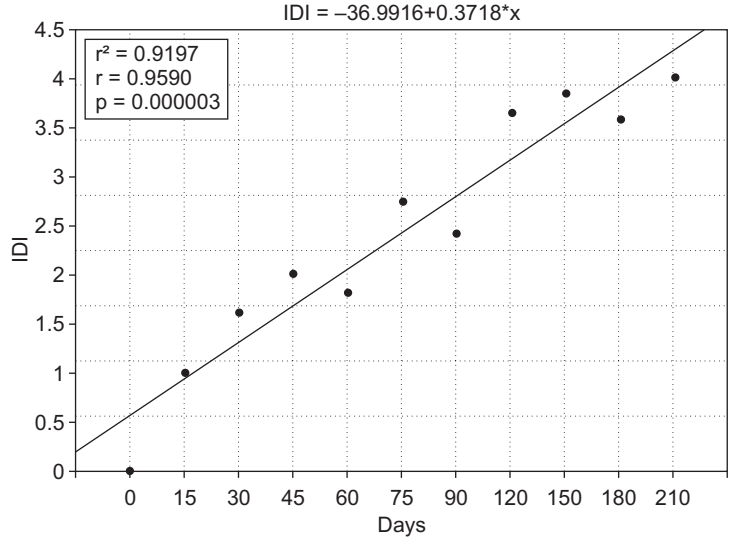

Figure 5 – Correlation of IDI with experiment time.

Aphallic paths

The development of alternative paths was observed only after 60 days from the start of the experiment, since during the first samplings it was impossible to check which imposex developmental path each individual was following.

Females were considered aphallic when no penis was present or when they showed just a papilla. The percentage of aphally was high, especially at days 150 and 180, representing 46.2% and 41.7% of the imposexed females, respectively.

Most females followed the traditional imposex developmental path ("a" path), with a well-developed penis. From day 60, individuals following the IDI "b" path were found. The "c" path was only observed at day 150 and day 180 (Figure 6). This path was less common along the experiment, yet showed a higher frequency at those two samplings.

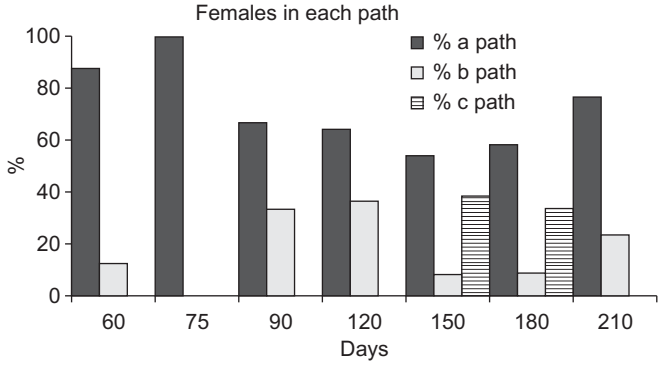

Figure 6 – Percentage of females in each IDI path. The "a" path – females with imposex usually reported; the "b" path – the penis just as a papilla; the "c" path – females totally aphallic.

DISCUSSION

Imposex Development Index (IDI)

Expression of imposex has been reported in different ways for many gastropod species, resulting in different imposex quantification indexes. The first one, developed by Gibbs *et al*. (1987) for *Nucella lapillus,* was the VDSI, which was adapted for other species (Fioroni *et al.*, 1991; Gibbs *et al.*, 1991; Stroben *et al.*, 1992, 1995; Axiak *et al.*, 1995; Barreiro *et al.*, 1999; Terlizzi *et al*., 1999; Mensink *et al.*, 2002).

Although a VDSI scale was developed for *Stramonita haemastoma* by Fernandez *et al*. (2005), it was not adequate for the aphallic paths found in this study. The occurrence of a high number of aphallic females made this index hard to use, since it is based mainly on the size of the penis. However, in the present study a new expression of imposex was observed, in which the females developed just a papilla without a complete development of a penis, plus a complete *vas deferens* ("b" path of the IDI). This new developmental path was not yet included in any VDSI scale, hence, the establishment of IDI will be helpful to assure the accuracy of imposex quantification.

No sterile females were observed in this study (VDSI > IV, scale of Gibbs *et al*. (1987)). Likewise, muricids with high TBT tissue concentrations in the coast of Taiwan, such as *Thais tuberosa, Thais clavigera* and *Morula granulata*, did not present any blocking of the vulva or abortive capsules, which would indicate a VDSI stage V or VI, respectively (Liu & Suen, 1996; Liu *et al*., 1997). Working with *S. haemastoma* populations in the Azores, Spence *et al*. (1990) also did not find any of these stages. In Brazilian coast, studies on *S. haemastoma* showed a low occurrence of VDSI stage V females (Fernandez *et al*., 2002; Castro, 2005; Lima *et al*., 2006).

Such differential sensitivity to TBT was also observed between different neogastropod species (Huet *et al.*, 1995; Stroben *et al.*, 1995; Gibbs *et al.*, 1997; Bech, 1999a), which can be classified according to their maximum level of masculinization. Level I would be common in nassarids and buccinids, in which only a penis and *vas deferens* are formed, without any sterility. Levels II and III are common in muricids, in which sterilization and sex change occur, respectively (Gibbs *et al*., 1997). Although *S. haemastoma* belongs to the Muricidae family, the imposex manifestation seems to be more related to the masculinization Level I. Even if stages V and VI are not included in the development scheme proposed by this study, such stages can be added if the characteristics described by Gibbs *et al*. (1987) are found in future studies.

Imposex quantification

Imposex induction has been observed in several field (Shim *et al.*, 2000; Bech *et al.*, 2002) and laboratory (Gibbs *et al.*, 1987; Liu & Suen, 1996; Horiguchi *et al.*, 1997; Lima *et al.*, 2006) experiments. In this transplantation experiment, imposex induction in *S. haemastoma* with a 100% incidence was observed in just 15 days of exposure. A similar transplantation study was carried out in Korea with *Thais clavigera*, where the females showed a significant growth of the penis after 32 days of the beginning of the study (Shim *et al*., 2000). Bech (1999b) observed in Taiwan the appearance of imposex in *T. distinguenda* after 80 days of exposure, but in a low percentage of individuals (18.5%). After this, Bech *et al*. (2002) verified 45% of incidence of imposex, yet only after five months.

The fast expression in *S. haemastoma* could be reflecting the high sensitivity of this species to organotin contamination, but measurements of TBT concentrations in the study area must be performed to confirm this. Castro (2005) observed, in a similar experiment with *S. haemastoma*, that this species developed high imposex levels (90%) only 15 days after transplantation, while *Stramonita rustica* showed evidence of imposex after 30 days in 38.46% of the subjects in the same area.

A significant correlation was observed between the imposex indexes (IDI, RPLI and RPSI) and time. These results clearly show that *S. haemastoma* develops imposex as a function

of the time of exposure to polluted waters. Such results are in agreement with other transplantation experiments with the same species (Castro, 2005) and with *Thais distinguenda* (Bech *et al*., 2002).

Aphallic paths

Females that did not present a penis or that developed only a papilla were considered aphallic. The incidence of aphally was quite high during most of the study, reaching its highest level (46.1%) at day 150. Other studies with *S. haemastoma* reported aphallic individuals in the Brazilian northeast (Lima *et al.*, 2006), in the latter case with 5% of aphallic females.

In the last years, a growing number of studies have reported aphallic animals (Gibbs, 1993, 2005; Huet *et al.*, 1995; Ruiz *et al.*, 1998; Quintela *et al.*, 2002; Ribeiro, 2002; Santos *et al.*, 2002; Castro, 2005; Lima *et al.*, 2006). Oehlmann *et al*. (1991), studying *N. lapillus*, proposed a chromosomal polymorphism as responsible for this aphallic routes. Successively, Gibbs (1993) observed another case of aphally in *N. lapillus,* now involving both males and females in England's southeast. The males of this population showed a genetic anomaly, called the Dumpton Syndrome (DS), which consists on the underdevelopment of the male genital system. Interestingly, the females of this population presented lower levels of imposex.

According to Gibbs (2005), the DS occurred in the last decades in low levels in the coast of England. The frequency of this mutation has increased to detectable levels, since it makes more difficult the masculinization of imposexed females. In males, the DS has been catastrophic, yet in females, almost universally exposed to organotins, the masculinization is attenuated and the population survives (Gibbs, 2005).

It was not observed aphallic males in this study. However, several works reported a low percentage of aphally in males when compared to imposexed females. Santos *et al*. (2002), working in Portugal with *N. lapillus*, observed only one aphallic male in a sampling of more than 1000 specimens, while aphallic females reached 23.8% in one of the sampling points. This result is similar to that reported by Quintela *et al*. (2002) in Spain, who found 31% of aphallic females and only 7% of aphallic males. These authors proposed that the diameter of the *vas deferens* would be a more appropriate parameter than penis size to express the occurrence of the Dumpton Syndrome.

Gibbs (2005) performed breeding experiments with *N. lapillus,* in which the recessiveness of the DS gene was demonstrated. In *S. haemastoma,* the cause for this anomaly is not yet clear, but it is possible that it could be explained by the occurrence of this syndrome in the studied population.

The Dumpton Syndrome, therefore, gives an adaptive advantage to gastropods in organotin-polluted areas (Gibbs, 1993). It is worth mentioning that in *N. lapillus,* embryonary development does not involve planktonic larvae, so organotin pollution effects on its population can be observed locally along the years. Many species that develop imposex have a planktonic larvae stage, such as *S. haemastoma*, and the genes selected in one area may colonize other environments. This can explain the high incidence of aphallic imposex in organisms from non-polluted areas. Lima *et al*. (2006) used *S. haemastoma* populations from the same place, having also observed a high incidence of aphallic females. The high aphally rates found in this study suggest the possibility that the allele responsible for the Dumpton Syndrome may be present at high frequencies in the *S. haemastoma* population used for the transplantation experiment, but further studies are needed to confirm this hypothesis.

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