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Pugilina morio L., a New Imposex Exhibitor from South American Estuarine Environments: Approach for a Non-Lethal Method to Evaluate Imposex

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Abstract This is the first report on imposex occurrence in *Pugilina morio*. Imposex levels in *P. morio* from Ceará River Estuary were assessed by a non-lethal method. The obtained imposex parameters were: percentage = 37.1 %, female penis length index (FPLI) = 0.93 mm, relative penis length index (RPLI) = 6.5 %, and vas deferens sequence index (VDSI) = 1.0. Because imposex is induced by tributyltin, and *P. morio* is widely distributed on the East coast of South America, the species can be used as a tributyltin contamination sentinel in these estuaries. Additionally, a non-lethal method of evaluating imposex minimized the impact on the population of *P. morio* from sample collection, as all organisms were returned to the environment.

Keywords Organotin · Estuaries · Harbor · TBT

Imposex is defined as a superimposition of male sexual characters, such as a penis and a *vas deferens,* onto females gastropods (Gibbs and Bryan 1987). It is a type of endocrine disruption in prosobranch species, although the detailed mechanism of imposex induction is still unresolved (Sternberg et al. 2010). This condition was first reported as a sexual aberration by Blaber (1970) in *Nucella lapillus* populations from Plymouth (United Kingdom), followed by a report by Smith (1971) of its occurrence in *Ilyanassa obsoleta* from Southport and Westport (USA). However, a cause and effect relationship based on exposure to tributyltin (TBT) was not discovered until 1981 (Smith 1981). Today, imposex is known to be a widespread phenomenon, affecting approximately 250 gastropod species (Titley-O'Neal et al. 2011). Its occurrence and intensity is widely accepted as proportional to the environmental levels of TBT, and also of triphenyltin (TPT) for some species. A clear cause and effect relationship has been established (Matthiessen and Gibbs 1998). Thus, imposex is a recognized biomarker, which has been used worldwide to map TBT contamination hotspots in coastal environments (Sousa et al. 2009).

TBT and TPT have been used as active agents in antifouling paints since the 1960s (Almeida et al. 2007; Yebra et al. 2004). Antifouling paints are used on solid surfaces exposed directly to sea water, including hulls of ships, aquaculture nets, off shore structures, and ducts, in order to avoid undesirable fouling accumulation (Champ 2000; Kotrikla 2009). TBT-based antifouling paints were extensively used starting in the 1970s; meanwhile, several to their application were reported (Yebra et al. 2004). Examples include oyster malformation (Alzieu 2000), immuntoxicity in fish and mammals (Yang et al. 2006; Nakayama et al. 2009), imposex, and decline of gastropod populations (Horiguchi et al. 2006).

Coastal areas impacted by TBT are ship traffic zones, such as harbors, marinas and shipyards (tem Hallers-Tjabbes et al. 1994). Recent studies have shown that in

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South American coastal areas there are "hot spots" of TBT contamination, similar to observations in industrialized countries of the Northern Hemisphere (Castro and Fillmann 2012; Castro et al. 2012b). Currently, for South America, 34 gastropod species have been described as imposex exhibitors (Castro et al. 2012b); however, only 3 have been used in monitoring programs of organotin contamination: *Stramonita haemastoma* (Castro et al. 2007a) and *Stramonita rustica* (Castro et al. 2004, 2012c) in Brazil and *Odontocymbiola magellanica* in Argentina (Bigatti and Carranza 2007). These species inhabit marine environments with salinities higher than 30 parts per thousand and do not occur inside estuaries with lower salinities. Because harbors, marinas and shipyard facilities are frequently located in bays or estuaries due to low hydrodynamic conditions, these areas are particularly susceptible to organotin contamination (Takahashi et al. 1999). Hence sentinel species for the monitoring of organotin contamination in these zones of lower salinity are needed.

Pugilina morio is a melongelid gastropod, commonly found in estuaries on the South Atlantic coast (Trinidad and Tobago to Southern Brazil, and tropical West Africa), where it inhabits areas with mud and sand bottoms, feeding mainly on carrion (Abbott and Dance 1982; Robin 2008). It is a gonochoristic mollusk with clear sexual dimorphism in shell and in soft parts, with females featuring a thicker body than males and a pedal gland (Matthews-Cascon 2003). *P. morio* has a moderate size (160 mm), and is easily recognized during field collections. It is common in the estuaries of northeastern Brazil, and is also frequently observed in low salinity zones (Matthews-Cascon 1990). Considering the necessity to obtain a sentinel for organotin contamination in the navigated estuaries of Brazil, the current study was conducted to evaluate *P. morio* from the Ceara River Estuary for the incidence and morphological extent of imposex develop using a non-lethal method, and for its possible use as a sentinel species in estuarine habitats. Additionally, a description of the morphologic alterations observed in imposexed females of *P. morio* is provided in this work.

Materials and Methods

The Ceará River Estuary (CRE) is a semi-closed estuarine system located in the Metropolitan Region of Fortaleza city, Ceará State NE Brazil (latitude 3° 41′ 55″ S and longitude 38° 85′ 19″ W) (Fig. 1). It is inserted into a mangrove zone of approximately 500 hectares which is an environmental protection area (Barroso and Matthews-Cascon 2009). The CRE is impacted by the municipality of Fortaleza and by boat traffic. Additionally, along its eastern coast there is a shipyard that performs repairs on and construction of small vessels. Between February and May of 2008, a total of 163 *Pugilina morio* adult individuals were manually caught in the CRE. These animals were transported to the laboratory in plastic bags containing water from the estuary. In the laboratory, the organisms were placed in 60 L tanks filled with sea water from CRE and with constant aeration until further analyses.

The imposex analyses were performed in a maximum interval of one week after the sample collection. First, shell lengths were measured with a caliper (to the nearest 0.05 mm) from the spiral top to the end of the siphon channel. Gender identification was accomplished based on the presence or absence of a pedal gland (Fig. 2a, b). This structure is present exclusively in females and is used to attach egg capsules on a substratum during egg deposition. This step should be performed carefully because in young animals (<60 mm) the pedal gland may be difficult to view (Fig. 2c, d) (Matthews-Cascon 2003). To determine sex based on the presence of a pedal gland, gastropods were placed on glass plates until settlement. Afterwards, animals were narcotized individually in plastic containers filled with 200 mL of sea water to which ethyl alcohol was slowly added up to a maximum concentration of 10 %. The mollusks were maintained in this solution until full relaxation was achieved. Later, the soft body was partially pulled out of the shell, allowing for a check on the presence of male sex organs (penis and/or vas deferens) (Fig. 2e). The penis and vas deferens of males and imposexed females were measured using a millimeter paper. The anesthetic effect turned out to be reversible in all analysed organisms. Afterwards, animals were returned to the 60 L tanks for subsequent return to their natural habitat. In order to avoid preventing accidental recaptures of the same animals, they were returned to the natural environment only after the study. The imposex levels were assessed using the following indices: % of imposex in females (I %), female penis length index (FPLI = mean penis length of all females in the population, including the zero values), relative penis length index (RPLI = [mean penis length in females/mean penis length in males] × 100) (Gibbs et al. 1987). The vas deferens sequence index (VDSI), based on the development of male sexual characters (particularly the vas deferens) by females, was also calculated according Stroben et al. (Stroben et al. 1992b).

Results and Discussions

Among the 163 analyzed specimens, 70 were females of which 26 (37.14 %) showed imposex. Considering all organisms, the imposex levels detected in *P. morio* from Ceará River Estuary were: FPLI = 0.93 mm, RPLI = 6.5 % and VDSI = 1.0. Castro et al., (2007b) analyzed imposex

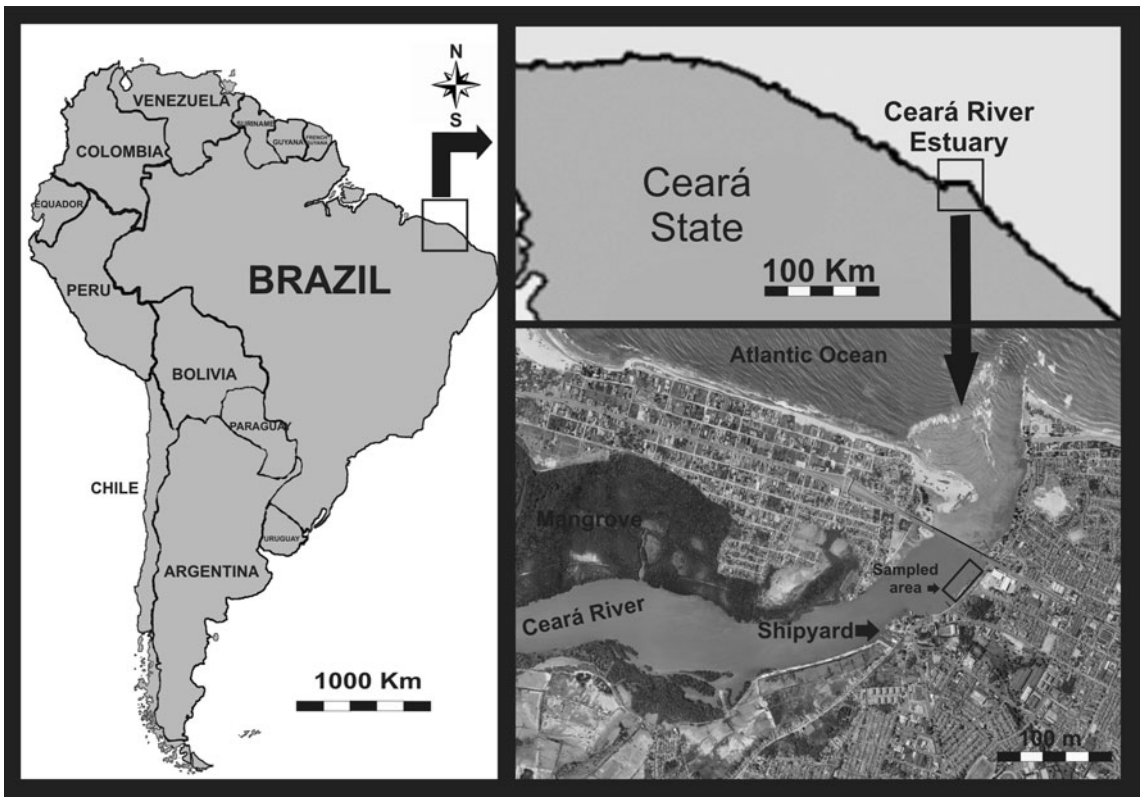

Fig. 1 Location of the Ceará River Estuary and studied area

intensities in CRE using the muricid *Stramonita haemastoma* in 2006 and found a RPLI of 22.2, a VDSI of 2.92 and 100 % imposex incidence, indicating a previous imposex occurrence in this estuary. However, the imposex levels observed in the current study were characterized by high variability (from stages 0 to IV) and independence from shell length (Figs. 3, 4). Initial VDSI stages (I) were verified in 7 females (10 %), and in 5 of those organisms a thickened structure forming a small penile papilla (up to 3 mm) near the right tentacle was observed. Among the 5 animals, a vas deferens was present in 3 (Fig. 3a, d). In the other 2 females considered as stage I, there was no indication of papilla or penis formation, being thus considered aphallic. However, in these animals, an initial vas deferens structure was observed as a thin white duct (Fig. 3b, c). Several studies have reported the occurrence of imposexed aphallic animals (Gibbs 1993; Huet et al. 1995; Quintela et al. 2002; Lima et al. 2006). Oehlmann et al. (1991), studying *Nucella lapillus*, proposed a chromosomal polymorphism as responsible for these aphallic imposex routes. Successively, Gibbs (1993) observed another case of aphally in *N.lapillus,* involving both males and females. The organisms from this population showed a genetic anomaly, called Dumpton Syndrome (DS), which was characterized by lack of a penis in the affected specimens. Interestingly, females of this population presented a higher resistance to imposex development, thus exhibiting lower imposex levels. Additionally, aphallic imposex development is also very common in buccinid gastropods (Stroben et al. 1992a).

Twenty six females showed totally formed penises similarly to males (which ranged between 8 and 23 mm), however, with lengths ranging from 2 to 10 mm (Fig. 3e, f). In these animals, the vas deferens ranged from partially (stages II and III) to totally (stage IV) formed. Thus, the animals were considered as imposex stages II (7 %), III (4 %) and IV (16 %). However, during the current study, we did not observe any sterile females (Stage V), since it was possible, to visualize the vulva opening. The observed differences between these imposex levels were similar to those described by Fioroni et al. (1991), with a progression in penis size and vas deferens development with increased stage. This way, the vas deferens could be visualized in affected females indicating a correct imposex development stages. In all analyzed animals, the development of the vas deferens started from the penile region and progressed toward the pallial cavity, thereby allowing for good visualization and a correct imposex development stage classification in affected females. In males, a vas deferens was observed in all 93 individuals as a large and distinct duct of yellow coloration.

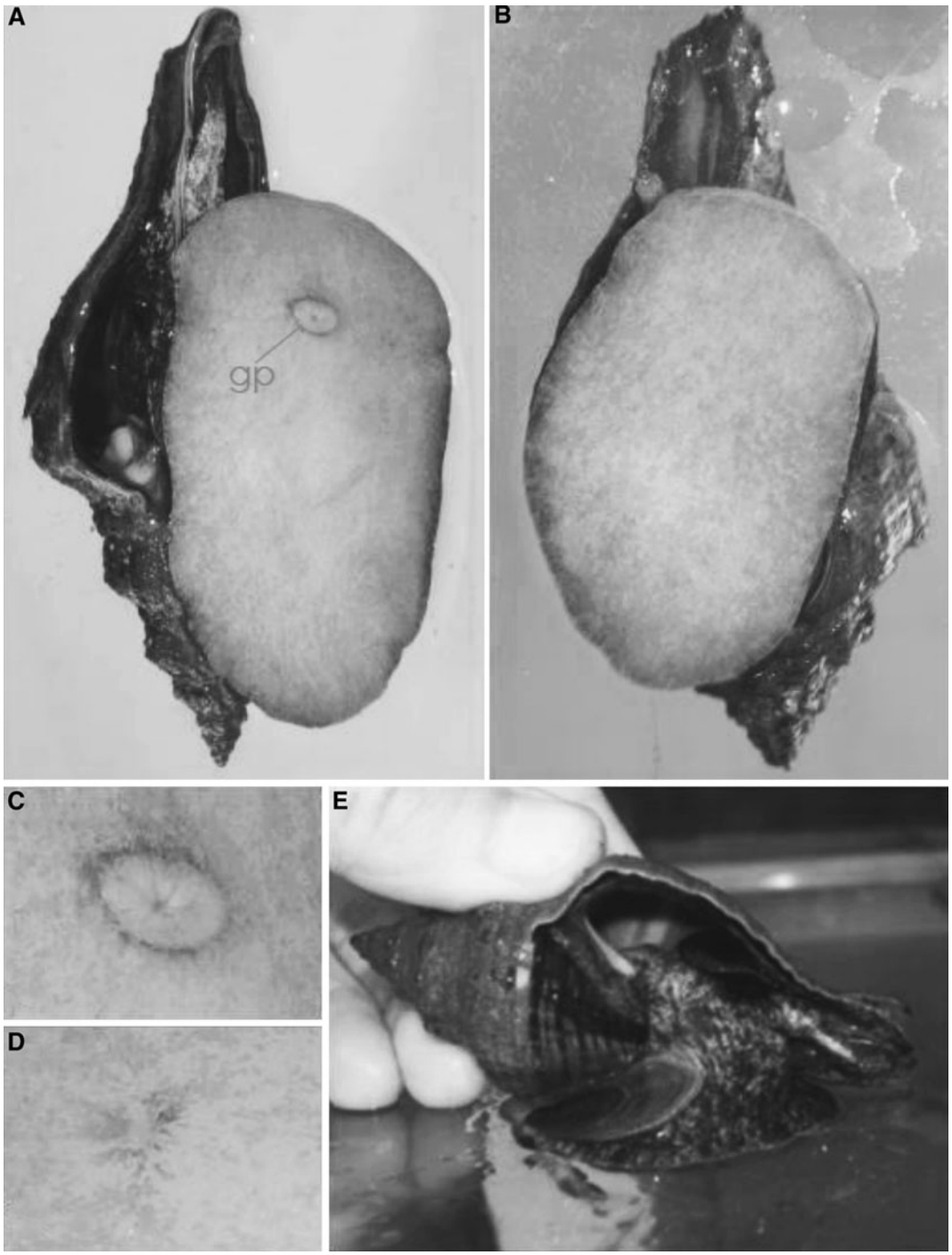

Fig. 2 **a** Pedal gland on female foot; **b** male foot (without pedal gland); **c** Pedal gland in old organism; **d** Pedal gland in young organism (<60 mm); **e** Technique for observation of sexual structures in *Pugilina morio*

The large VDSI variation (0–IV) observed in *P. morio* from CRE is possibly linked to the global ban on TBT-based antifouling paints. In fact, imposex levels in *S. haemastoma* from CRE are declining after the 2008 global TBT ban, since our unpublished data obtained in 2010 for the same site showed a strong imposex reduction (RPLI = 0.28, a VDSI = 0.75 and 48.4 % of imposex incidence). Thus, assuming that detected imposex levels were induced by TBT from antifouling paints, and that imposex stages are irreversible, a declining temporal trend TBT release may lead to a mixture of old (imposexed) and young (non- or less-imposexed) females in the same sample, which could cause a large amount of imposex variability (Castro et al. 2012a). This situation, was already

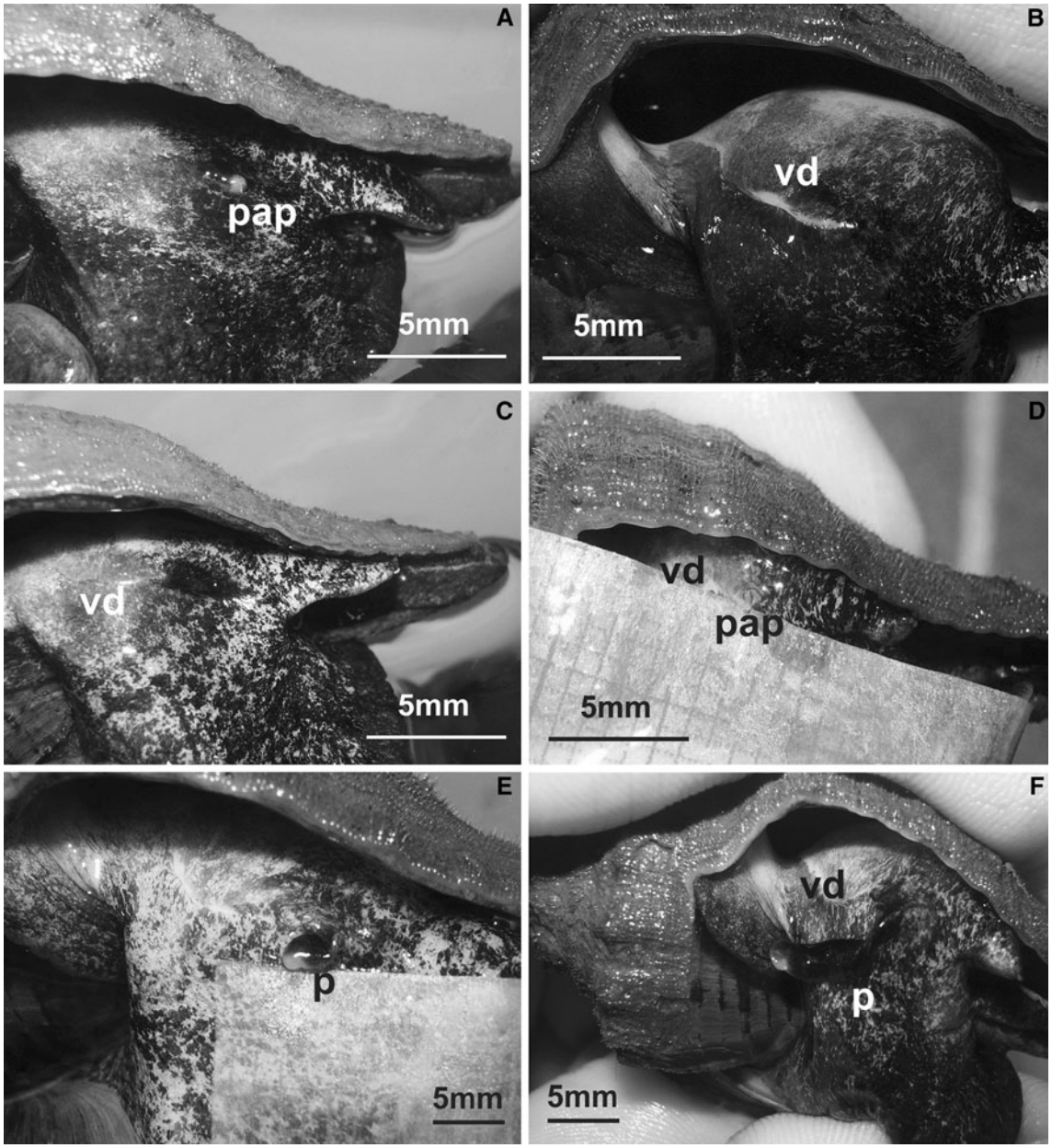

Fig. 3 *Pugilina morio* individuals showing different imposex stages. **a**, **b**, **c** and **d** imposex stage I; **e** Imposex stage II; **f** imposex stage IV. pap (penis papillae), vd (vas deferens), p (penis)

reported by Galante-Oliveira et al. (2009) at Ria Aveiro (Portugal) using the muricid *Nucella lapillus*. On the other hand, the imposex stages detected in *P. morio* did not correlate with shell length (Fig. 2). However, it is not known for this species if there is a direct relationship between shell size and animal age. More research is needed with *P. morio*, including dose–response evaluations with TBT, to determine its usefulness as a sentinel species.

The non-lethal method employed to evaluate imposex levels in *P. morio,* proved to be easily applicable, and the ethyl alcohol narcotization was reversed in all animals used in this study. Additionally, it was possible to verify the normal behavior of the organisms before their return to the natural environment. Another non-lethal method has been already used for imposex assessment in *Stramonita haemastoma,* however, this method is based on the assumption of balanced sex ratios in populations unaffected by imposex (Fernandez et al. 2007). That approach can introduce bias in imposex evaluation, since several environmental factors would affect the species sex ratio. On the other hand, the method presented in the current study was based on external sexual dimorphism in *P. morio* (presence of pedal gland only in females). While this method of determining gender is accurate for larger individuals, it is

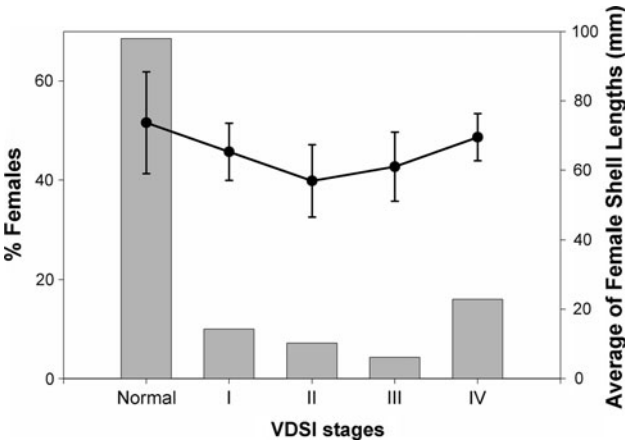

Fig. 4 Percentage of *Pugilina morio* from Ceará River Estuary in several VDSI stages compared with average of female shell lengths (mm). Error bars represents the standard deviation

difficult to observe the pedal gland in smaller individuals (<60 mm). This difficulty limits the application of the sexing technique with smaller animals.

Mollusks, mainly gastropods and bivalves, are recognized sentinels for studies on environmental toxicology (Rittschof and McClellan-Green 2005). Their high sensitivity and sedentary lifestyle allows for bioaccumulation and biomagnification of pollutants. Assuming that imposex development in *P. morio* has a cause and effect relationship with TBT pollution, the results of the current study suggest that this species may be useful as a sentinel of organotin contamination. However, additional studies on the TBT sensitivity of the species should be performed to verify the TBT concentrations that induce imposex. Additionally, the adoption of a non-lethal method to evaluate imposex, as described here, will minimize the impact of sampling on populations of gastropod species, since large numbers of individuals are required for analysis.

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