DEVELOPMENT OF INDICES FOR NONSACRIFICIAL SEXING OF IMPOSEX-AFFECTED *HEXAPLEX (TRUNCULARIOPSIS) TRUNCULUS* (GASTROPODA: MURICIDAE)

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ABSTRACT

The muricid *Hexaplex (Trunculariopsis) trunculus* lacks external sexual dimorphism and is highly affected by imposex, which further complicates its sexual identification. In this context, the aim of this study was to develop sexual indices based on the dimensions of male and imposex-affected female penis, which could constitute a useful, simple and nonsacrificial tool for sexing live specimens of *T. trunculus*. The adoption of sexual indices consisting of penis dimensions of both sexes relative to individual size revealed a high accuracy in the sexual identification of sacrificed specimens (>95% correct sexing). Additionally, multivariate discriminant analysis allowed correct sexing of 98.8% of the original 1053 sacrificed individuals, with accurate sexual identification being higher for males (99.5%) than for imposex-affected females (97.7%). An anaesthetization experiment was performed to investigate the effects produced by the anaesthetic ($MgCl_2$) on penis measurements, to test this nonsacrificial approach and to validate the previously developed sexual indices. The anaesthetic provoked an expected enlargement in penis dimensions but, despite this side effect, the sexual indices developed for sacrificed specimens were still highly successful in sexing anaesthetized *T. trunculus* (generally more than 95% correct sexual identification). The practical application and some limitations of developing and employing this kind of index for the sexual identification of *T. trunculus* and other imposex-affected gastropod species are discussed.

INTRODUCTION

Sexual dimorphism is common in many animals, but occurs only sporadically in the phylum Mollusca. Nevertheless, subtle differences in shell morphology between sexes occur in some prosobranch gastropods. Sexual dimorphism has been recorded in some species of freshwater (e.g. Kantor & Sysoev, 1991; Brande *et al.*, 1996; Estebenet, 1998; Kurata & Kikuchi, 2000) and marine gastropods (e.g. Bernard, 1968; Ten Hallers-Tjabbes, 1979; Castagna & Kraeuter, 1994; Kenchington & Glass, 1998). However, since most gastropod species do not show external sexual dimorphism, sexing generally requires the exposal of the soft body and the examination of sexual organs and/or gonads, which is frequently impossible without sacrificing the specimens (through shell breakage and consequent death).

Furthermore, several prosobranch gastropod species are affected by the phenomenon known as imposex (Smith, 1971) or pseudohermaphroditism (Jenner, 1979), which is the development and superimposition of male sexual characters (penis and vas deferens) onto females. This abnormality is induced by tributyltin (TBT) and its derivative compounds, widely applied as biocides in antifouling paints of boats and ships' hulls (Terlizzi *et al.*, 2001). Imposex has become a widespread phenomenon that affects both coastal and offshore gastropod species (Ellis & Pattisina, 1990), involving at least 63 genera and 118 species (Fioroni, Oehlmann & Stroben, 1991). In these imposex-affected species, sexual identification becomes even more complicated, because specimens cannot be sexed simply by the presence or absence of penis, instead requiring a further exhaustive inspection of other sexual organs.

Some literature exists on several aspects of the biology and ecology of *Hexaplex (Trunculariopsis) trunculus* (Linnaeus, 1758), but to our knowledge, no external signs of sexual dimorphism have yet been reported. Several studies have been conducted on the imposex levels of *T. trunculus*, mostly in the Mediterranean and Adriatic, namely in Spain (El Hamdani, Ferrer & García Carrascosa, 1998), France (Martoja & Bouquegneau, 1988), Italy (Terlizzi *et al.*, 1997; Terlizzi, Geraci & Minganti, 1998; Terlizzi, Geraci & Gibbs, 1999; Terlizzi, 2000; Chiavarini *et al.*, 2003; Pellizzato *et al.*, 2004; Terlizzi *et al.*, 2004; Garaventa *et al.*, 2006), Croatia (Garaventa *et al.*, 2006), Malta (Axiak *et al.*, 1995, 2000, 2003) and Israel (Rilov *et al.*, 2000). In Portuguese waters, the only works reporting the imposex levels in *T. trunculus* were carried out in the Ria Formosa lagoon (southern coast of Portugal) by Gibbs, Bebianno & Coelho (1997), Langston *et al.* (1997) and Coelho, Bebianno & Gibbs (1998) (although all with very few specimens), and more recently by Vasconcelos, Gaspar & Castro (2006).

This species is the target of a locally important artisanal fishery in the Ria Formosa lagoon (Algarve coast, southern Portugal), where it is being subjected to an integrated and multidisciplinary study on its biology, ecology and fishery. Since *T. trunculus* lacks external sexual dimorphism (sexes cannot be distinguished by shell characters), its sexual identification requires breaking the shell and sacrificing the organism. Additionally, because this species is highly affected by imposex, sexing is even further complicated and requires specific knowledge of the reproductive organs.

In some circumstances, it may be advantageous to sex specimens without extracting them from their shells (Gibbs, 1999),

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namely for diverse biological, ecological and behavioural studies that require live specimens and the respective sexual identification. However, in several gastropod species, live sexing is only possible through anaesthetization. This technique of sexual identification by opening of the pallial cavity and examination of the penis development under narcosis has already been applied to other muricid species, such as *Nucella lapillus* (Gibbs, 2005).

In this context, the aim of the present study was to develop indices for the sexual identification of live *T. trunculus*, using data on specimen size and penis dimensions of sacrificed males and imposex-affected females. For this purpose, an anaesthetization experiment (narcosis with magnesium chloride) was performed to investigate the effects produced by the anaesthetic on penis measurements. Subsequently, data from these anaesthetized specimens were applied on the sexual indices previously developed with sacrificed specimens, in order to validate the indices and to confirm whether they could constitute a useful, simple and nonsacrificial tool for sexing live and anaesthetized *T. trunculus*.

MATERIAL AND METHODS

For this study, approximately 100 specimens of *T. trunculus* (Fig. 1A) from commercial samples caught near the Culatra Island (Ria Formosa lagoon, southern Portugal) were examined monthly between March 2003 and February 2004. In the laboratory, individuals were measured (shell length, SL) with a digital calliper (to 0.01 mm) and weighed (total weight, TW) on a top-loading digital balance (precision of 0.01 g).

Subsequently, routine sexual identification was made in sacrificed de-shelled specimens. The shells were broken in a bench vice to allow the removal of the soft parts (Fig. 1B). The mantle cavity was exposed and individuals were sexed: males by the presence of penis and lack of capsule gland, females by the presence of vagina and capsule gland. Finally, the curved penes of both males and imposex-affected females (Fig. 1C) were flattened and measured to the nearest 0.01 mm (penis total length of proximal and distal portions, PTL; penis partial length of proximal portion, PPL; penis maximum width of proximal portion, PMW) (Fig. 1D) under a stereo microscope with a calibrated eyepiece or with the digital calliper, depending on the penis size.

Penis dimensions (PTL, PPL and PMW) and relative proportions (PPL/PTL and PMW/PTL) were compared between sexes using a *t*-test (H_0: $\mu_M = \mu_F$; H_A: $\mu_M \neq \mu_F$ and H_0: $p_M = p_F$; H_A: $p_M \neq p_F$), with statistical significance considered at $P < 0.05$. The morphometric relationships between penis dimensions (PTL, PPL and PMW) and the relationships between individual size (SL) and penis dimensions (PTL, PPL and PMW) for both sexes were obtained by linear regression analysis (least squares method), by adjustment of a linear function ($Y = a + bX$) to raw data. The degree of association between variables was assessed by the correlation coefficient (r) and the slopes of linear regressions for males and imposex-affected females were compared using a *t*-test (H_0: $\beta_M = \beta_F$; H_A: $\beta_M \neq \beta_F$) (Zar, 1996), with statistical significance at $P < 0.05$.

Allometric relationships between penis dimensions (PTL, PPL and PMW) and between individual size (SL) and respective penis dimensions (PTL, PPL and PMW) were also estimated for both sexes. The allometry coefficient is expressed by the exponent b of the relationship ($Y = aX^b$), which can also be expressed in its linear logarithmic form ($\ln Y = \ln a + b \ln X$). In relationships between linear variables, the relationship reflects an isometric growth when $b = 1$ (i.e. growth rates of both variables are identical during ontogeny). A *t*-test (H_0: $b = 1$; H_A: $b \neq 1$) (Sokal & Rohlf, 1987) was applied to confirm if the slopes of allometric relationships were included in the isometric range ($b = 1$) or allometric ranges (negative allometry: $b < 1$ or positive allometry: $b > 1$), with statistical significance considered at $P < 0.05$.

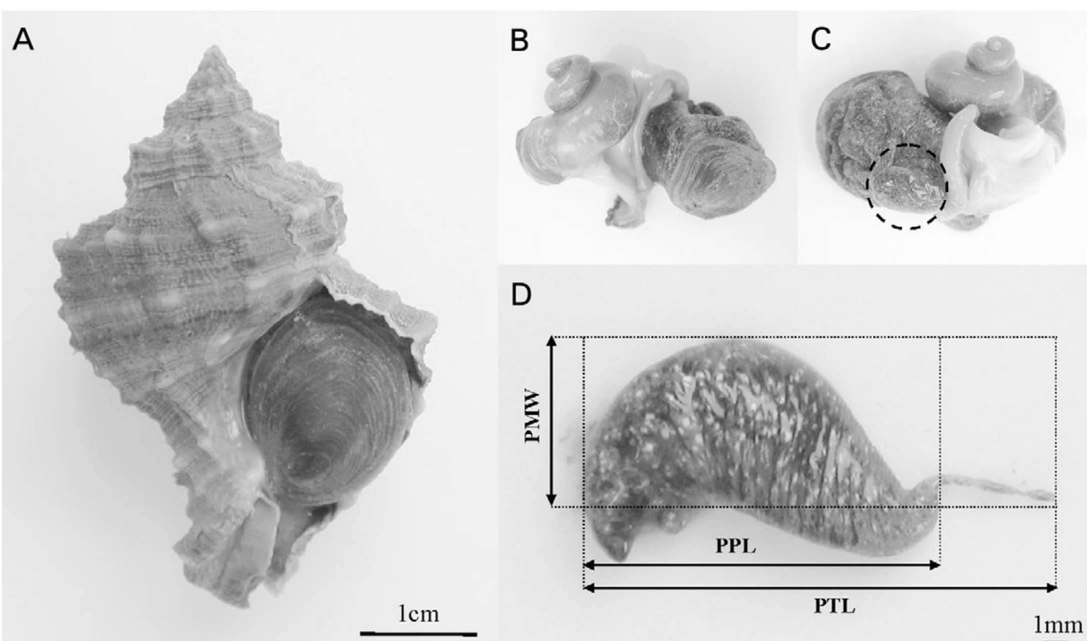

Figure 1. Illustration of the sequential procedures for the measurements of male and imposex-affected female penis of *Hexaplex (Truncolariopsis) trunculus*. **A.** Live specimen (ventral view). **B.** Soft parts of the organism after shell breakage. **C.** Location of the curved penis on the foot, adjacent to the ocular tentacles (eyestalks). **D.** Schematic representation of the flattened penis measurements. Abbreviations: PTL, penis total length (proximal and distal portions); PPL, penis partial length (proximal portion); PMW, penis maximum width (proximal portion).

Sexual indices consisting of the percentage of penis dimensions relatively to individual size (PTL/SL, PPL/SL and PMW/SL) of males and imposex-affected females were employed to standardize data and eventually facilitate the sexual identification. A Kruskal–Wallis *H*-test for multiple independent samples (H_0: $\text{month}_1 = \text{month}_n$; H_A: $\text{month}_1 \neq \text{month}_n$) (Zar, 1996) was used to detect differences in these sexual indices during the one-year study period. Monthly variations in these sexual indices between consecutive months were assessed with the Mann–Whitney *U*-test for two independent samples (H_0: $\text{month}_n = \text{month}_{n+1}$; H_A: $\text{month}_n \neq \text{month}_{n+1}$) (Zar, 1996). In both statistical tests, significance was considered at $P < 0.05$.

Multivariate discriminant analysis was performed using the individual size (SL) and the respective penis dimensions (PTL, PPL and PMW) of both males and imposex-affected females. Discriminant functions were calculated and the percentages of cases that were correctly sexed by the linear function were established. Multivariate discriminant analysis was carried out using the data analysis software system Statistica® version 6.0 (StatSoft, Inc.).

For the nonsacrificial approach, an anaesthetization experiment was performed to investigate the effects produced by the narcotic on penis measurements and to test the validity of applying data from anaesthetized specimens in the sexual indices previously developed with data from sacrificed specimens. Firstly, 100 *T. trunculus* specimens were marked with Dymo® tape tags, fixed with cyanoacrylate glue and covered with epoxy glue (for marking details see Vasconcelos, Gaspar, Pereira & Castro, in press). The anaesthetization was performed following the protocol developed by Gibbs (1999) and successfully applied in *Nucella lapillus* by Gibbs (2005). Specimens were immersed for 2 h in a solution of magnesium chloride (75 g of $MgCl_2.6H_2O$ per litre of distilled water) and monitored for full relaxation. Those individuals that did not attain a completely relaxed state after this period were monitored at regular intervals of 15 min until full relaxation was achieved, sufficient to permit easing from the shell, examination of the anterior part of the pallial cavity and measurement of penis dimensions. Finally, and within 24–48 h after having recovered from the anaesthetization, these same specimens were sacrificed (de-shelled in a bench vice), their sex identified and penes measured.

A *Z*-test (Zar, 1996) was applied to compare average penis dimensions obtained from these anaesthetized and later sacrificed specimens of both sexes. Furthermore, a paired-sample *t*-test (Zar, 1996) was applied to individually identify the significant differences between penis measurements obtained from these narcotized and sacrificed specimens. Alternatively, the Wilcoxon-signed rank test was employed, whenever the normality test failed (Zar, 1996). The paired-sample *t*-test examines the changes that occur before and after a single experimental intervention on the same individuals, to determine whether the treatment produced a significant effect. Examining the changes, rather than the values observed before and after the treatment, removes the differences due to individual responses and produces a more sensitive and powerful test. In all these statistical analyses, significance was considered at $P < 0.05$.

RESULTS

A total of 1183 *T. trunculus* specimens were subjected to imposex analysis (40.17–82.84 mm SL and 5.28–48.84 g TW), 621 males and 562 females (93.24% of which were affected by imposex) (Table 1). The dimensions of *T. trunculus* male and imposex-affected female penes are presented in Table 1. On average, male penis dimensions were significantly greater than those of female imposex-affected penes (*t*-test; $P < 0.05$). In general, the male penis mean total length ($\text{PTL} = 11.82 \pm 1.68$ mm), mean partial length ($\text{PPL} = 8.41 \pm 1.25$ mm) and mean maximum width ($\text{PMW} = 3.39 \pm 0.59$ mm), were more than two times larger than the same measurements of female imposex-affected penes ($\text{PTL} = 5.18 \pm 2.04$ mm; $\text{PPL} = 3.53 \pm 1.58$ mm; $\text{PMW} = 1.56 \pm 0.59$ mm) (Table 1), which frequently appeared only as a small protuberance (vestigial or incipient penes).

The relative proportions and morphometric relationships established between penis measurements are illustrated in Figure 2. Despite the marked differences in penis dimensions, the relative proportions (ratios: PPL/PTL and PMW/PTL) were similar between sexes (Fig. 2A), revealing that male and female imposex-affected penes are morphologically alike and roughly comparable. Nevertheless, statistically significant differences between sexes (*t*-test; $P < 0.05$) were detected in these relative proportions, with a higher ratio PPL/PTL in males and a higher ratio PMW/PTL in females, indicating that on average the proximal portion is slightly longer in the male penis and wider in the female imposex-affected penis. Moreover, the relative proportions of female imposex-affected penes were more variable than the relative proportions of male penes (Fig. 2A).

The morphometric relationships established between penis measurements (PTL, PPL and PMW) (Fig. 2B) highlighted the similarity in relative proportions and morphology of the penes of males and imposex-affected females. In fact, these morphometric relationships further confirmed that despite obvious differences in penis dimensions, there was an almost continuous morphometric relationship between male and female imposex-affected penes measurements. The morphological similarity between male and female imposex-affected penes was reinforced by the fact that only the slopes of the relationships PTL *vs* PPL were significantly distinct between sexes (*t*-test; $P < 0.05$), the slopes of the relationships PTL *vs* PMW being statistically similar between male and female penes (*t*-test; $P < 0.05$).

The morphometric relationships established between SL and penis dimensions (PTL, PPL and PMW) of males and imposex-affected females are shown in Figure 3. In all these

Table 1. Specimen's size and penis dimensions of males and imposex-affected females of *Hexaplex (Truncularioposis) trunculus*.

		Specimens size		Penis dimensions		
		Shell length (mm)	Total weight (g)	Total length (mm)	Partial length (mm)	Maximum width (mm)
Males	*n*	621	621	611	621	621
	mean ± SD	55.64 ± 5.50	16.86 ± 5.69	11.82 ± 1.68	8.41 ± 1.25	3.39 ± 0.59
	(min–max)	(40.17–82.84)	(5.28–48.84)	(6.48–17.10)	(3.89–12.35)	(1.67–5.18)
Females	*n*	562	562	442	465	466
	mean ± SD	57.84 ± 6.30	18.11 ± 6.91	5.18 ± 2.04	3.53 ± 1.58	1.56 ± 0.59
	(min–max)	(42.11–79.84)	(5.44–43.04)	(1.45–12.72)	(0.81–9.45)	(0.47–3.53)

Abbreviations: *n*, number; SD, standard deviation; min, minimum value; max, maximum value.

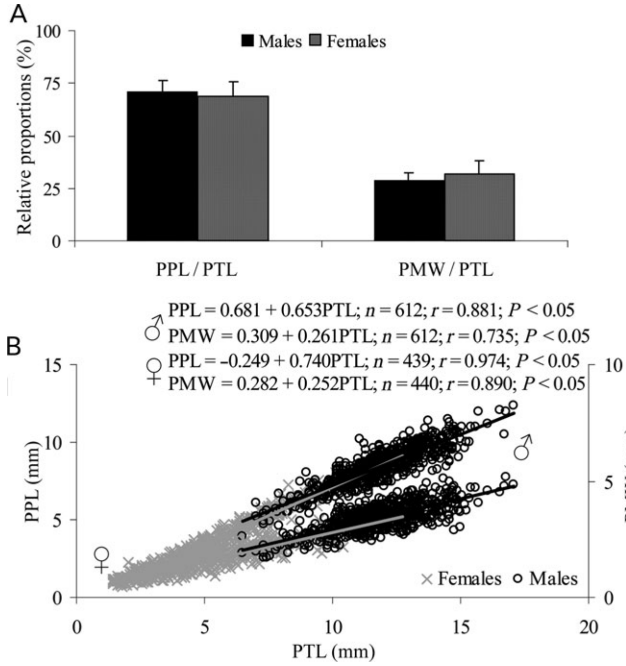

Figure 2. A. Relative proportions of penis measurements in *Hexaplex (Trunculariopsis) trunculus* males and imposex-affected females. **B.** Morphometric relationships established between penis measurements of males and imposex-affected females. Abbreviations: PTL, penis total length (proximal and distal portions); PPL, penis partial length (proximal portion); PMW, penis maximum width (proximal portion).

relationships, both the correlation coefficients (r) and the slopes (b) of linear regressions were higher in males than in females (t-test; $P < 0.05$) (Fig. 3). Despite the existence of some overlap between the distributions of male and female imposex-affected penis dimensions (mainly due to a greater variability in female penis dimensions), this indicated that male penis size is more size-dependent (relatively to shell length) than female penis size.

The parameters of the allometric relationships established between penis dimensions (PTL, PPL and PMW) and between individual size (SL) and respective penis dimensions (PTL, PPL and PMW) of males and imposex-affected females are compiled in Table 2. All allometric relationships involving male penis dimensions (both between penis dimensions: PTL *vs* PPL and PTL *vs* PMW, and between shell size and penis dimensions: SL *vs* PTL, SL *vs* PPL and SL *vs* PMW) were statistically significant ($P < 0.05$) and showed high correlation coefficients. For imposex-affected females, the allometric relationships between penis dimensions (PTL, PPL and PMW) were also statistically significant ($P < 0.05$) and showed high correlation coefficients as well but, among the allometric relationships between shell size and penis dimensions, only the relationship between shell length and penis total length (SL *vs* PTL) was statistically significant ($P < 0.05$), although with a very low correlation coefficient. In the allometric relationships between penis dimensions (PTL, PPL and PMW), the type of growth was opposite between sexes, with a negative allometry (males) and a positive allometry (females) in the relationship PTL *vs* PPL, and with an isometric growth (males) and a negative allometry (females) in the relationship PTL *vs* PMW. The type of growth was also distinct in the allometric relationships between individual size (SL) and penis dimensions (PTL, PPL and PMW), being a negative allometry for SL *vs* PTL (both sexes), isometric for SL *vs* PPL (males) and a positive allometry for SL *vs* PMW (males). Overall, only males showed isometric relationships, both among penis dimensions (PTL *vs* PMW) and between shell length and penis measurements (SL *vs* PPL) (Table 2).

The use of sexual indices (PTL/SL, PPL/SL and PMW/SL) for the sexual identification of males and imposex-affected females is illustrated in Figure 4. These sexual indices reduced the overlap between males and females. Furthermore, the definition of percentage limits for the separation of sexes (PTL/SL < 15% = females and PTL/SL ≥ 15% = males; PPL/SL < 10% = females and PPL/SL ≥ 10% = males; PMW/SL < 5% = females and PMW/SL ≥ 5% = males) allowed for the classification of gender on the basis of penis measurements. The ratio PTL/SL correctly identified the sex of 97.2% of the specimens, PPL/SL properly sexed 96.0% and PMW/SL correctly classified 95.7%. Correct classifications using PTL/SL and PPL/SL were higher for males (PTL/SL: 99.5% males and 94.1% females; PPL/SL: 99.8% males and 91.0% females), but were lower when applying PMW/SL (92.9% males and 99.4% females) (Fig. 4).

The monthly variation of these sexual indices (PTL/SL, PPL/SL and PMW/SL) during the one-year study period is illustrated in Figure 5. All sexual indices showed some oscillations all year-round (Kruskal–Wallis H-test, $P < 0.05$), which were comparable between indices (PTL/SL, PPL/SL and PMW/SL) and with a similar trend between males and imposex-affected females. The Mann–Whitney U-test detected statistically significant differences ($P < 0.05$) between several consecutive monthly samples of both sexes, mostly between April and October (Fig. 5).

Multivariate discriminant analysis using both the individual size (SL) and the respective penis dimensions (PTL, PPL and PMW) confirmed the distinction of the two sexes based on penis morphometrics. The linear equation that best discriminates between males and females was the following:

$$D = -0.385\,\text{PTL} - 0.305\,\text{PPL} - 0.421\,\text{PMW} + 0.072\,\text{SL} + 2.493\ [\text{canonical } R = 0.924,\ F_{(4,1048)} = 1525.6\ (P < 0.05)]$$

In this function, negative discriminant scores indicated males and positive discriminant scores designated females. Means of canonical variables were −2.05 for males and 2.83 for females (Fig. 6A). The distinctness between sexes was further reinforced in the plot of the individual scores based on standardized canonical coefficients for the first two variates (1st and 2nd canonical variables) (Fig. 6B). Applying this canonical discriminant function, 98.8% of the original 1053 individuals (611 males + 442 females) were assigned to the correct gender. Correct sexual identification was higher for males (608 specimens = 99.5%) than for females (432 specimens = 97.7%).

The anaesthetization of *T. trunculus* with magnesium chloride was highly effective, since under full relaxation all specimens were sufficiently eased from their shells to allow the examination of the anterior part of the pallial cavity and measurement of the penis. Nevertheless, specimens took an average of 3:04 ± 0:58 h to become fully relaxed and the period required for the anaesthetic to produce full relaxation increased with specimen size (linear regression analysis, SL *vs* time: $r = 0.445$; $P < 0.05$). On the other hand, *T. trunculus* proved highly tolerant of the anaesthetization, since all specimens recovered completely from the treatment after being held overnight in laboratory aquaria with aerated seawater.

Anaesthetization invariably produced an enlargement of average penis dimensions compared with the same penis measurements obtained from subsequently sacrificed specimens (PTL = 1.49 ± 1.19 mm; PPL = 0.98 ± 1.23 mm; PMW = 0.20 ± 0.27 mm). Furthermore, the longer the penis measurement, the higher the augmentation effect produced by

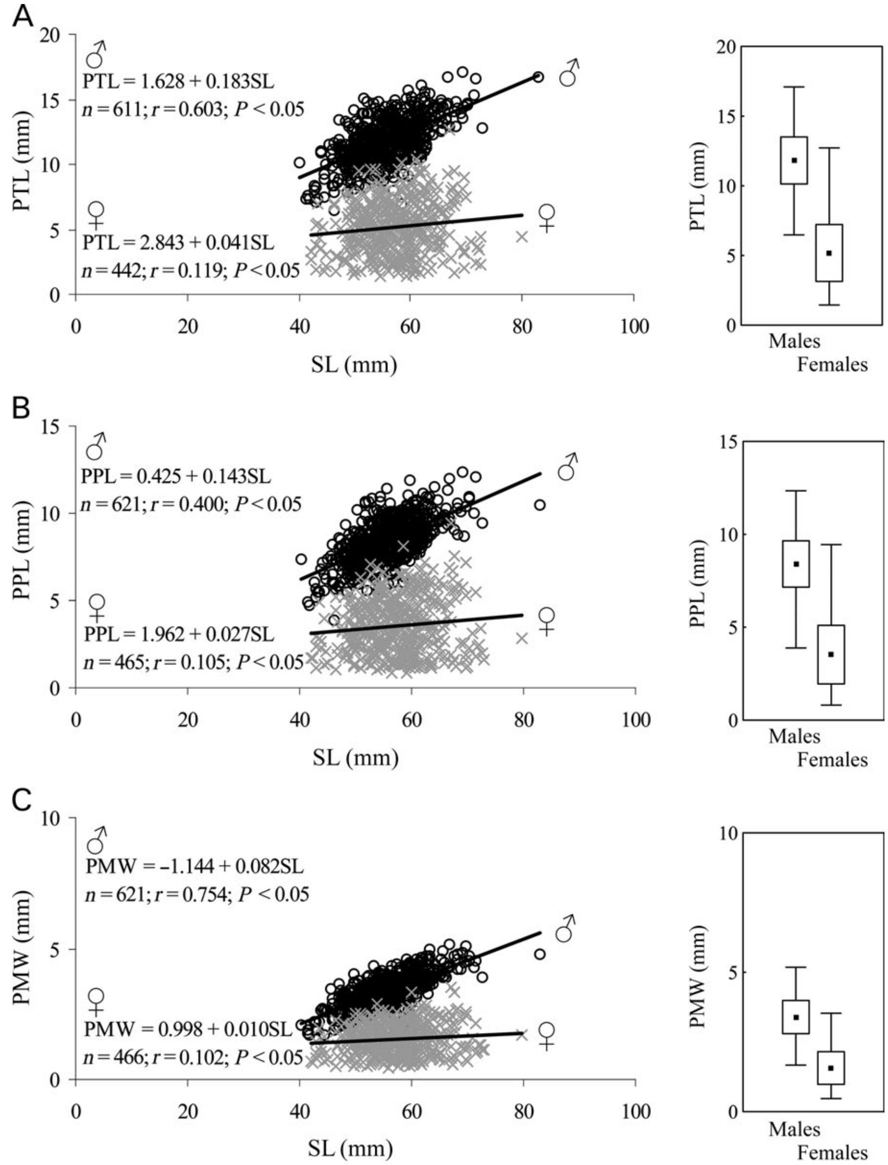

Figure 3. Morphometric relationships established between shell length and penis measurements of males and imposex-affected females of *Hexaplex (Trunculariopsis) trunculus*. **A.** Relationship shell length *vs* penis total length. **B.** Relationship shell length *vs* penis partial length. **C.** Relationship shell length *vs* penis maximum width. Abbreviations: SL, shell length; PTL, penis total length (proximal and distal portions); PPL, penis partial length (proximal portion); PMW, penis maximum width (proximal portion). Box-whisker graph: middle point = mean; box = ±standard deviation; whisker = minimum and maximum values.

the anaesthetic (PTL = 18.55 ± 14.60%; PPL = 12.89 ± 15.68%; PMW = 10.01 ± 12.50%). These differences in average penis dimensions were not statistically significant before and after the narcosis treatment (*Z*-test; $P < 0.05$), but individually the anaesthetization produced a significant increase in all these penis measurements (paired-sample *t*-test: $P < 0.05$ or Wilcoxon-signed rank test: $P < 0.05$). The effect produced by the narcotization treatment in the average penis dimensions of males and imposex-affected females of *T. trunculus* is illustrated in Figure 7. On average, the anaesthetization only caused a significant increase in the male's penis total length (PTL) and partial length (PPL) (*Z*-test; $P < 0.05$), but individually the narcotization provoked a significant augmentation in all penis measurements (PTL, PPL and PMW) of both sexes (paired-sample *t*-test: $P < 0.05$).

In order to test this nonsacrificial approach and to validate the sexual indices, data on specimen size (SL) and penis dimensions of these 100 anaesthetized and subsequently sacrificed specimens were applied to the sexual indices previously established from 1053 sacrificed *T. trunculus*. For this purpose, both raw data (anaesthetized and sacrificed: PTL, PPL and PMW) and data transformed by a conversion factor designed to attenuate the effects of the anaesthetization treatment, corresponding to the average penis enlargement provoked by the narcotic (anaesthetized: PTL, 18.55%; PPL, 12.89%; and PMW, 10.01%), were employed. The correct sexual identifications of both anaesthetized and sacrificed *T. trunculus* obtained through the application of these sexual indices are given in Table 3.

All sexual indices revealed a high accuracy in sexing *T. trunculus* (usually more than 95% correct identifications), independently of being applied to sacrificed or anaesthetized specimens (raw data or data transformed by the conversion factor). The indices PTL/SL and PPL/SL showed more appropriate for correctly sexing males (almost always 100% correct

Table 2. Estimated parameters of the allometric relationships established between penis dimensions (PTL, PPL and PMW) and shell length (SL) of males and imposex-affected females of *Hexaplex (Trunculariopsis) trunculus*.

Morphometric variables		Sex	*n*	Parameters of the allometric relationships				Growth type
Independent (*X*)	Dependent (*Y*)			*a'*	*b* ± SE (95% CI of *b*)	*r*	*P-value*	
PTL	PPL	Males	612	0.840	0.932 ± 0.019 (0.894–0.970)	0.891	0.000	−Allometry
		Females	439	0.628	1.052 ± 0.012 (1.029–1.075)	0.974	0.000	+ Allometry
PTL	PMW	Males	612	0.333	0.938 ± 0.033 (0.873–1.003)	0.753	0.000	Isometric
		Females	440	0.413	0.816 ± 0.019 (0.778–0.854)	0.896	0.000	−Allometry
SL	PTL	Males	611	0.326	0.892 ± 0.048 (0.798–0.986)	0.602	0.000	−Allometry
		Females	442	0.953	0.398 ± 0.199 (0.006–0.789)	0.095	0.046	−Allometry
SL	PPL	Males	621	0.150	0.999 ± 0.048 (0.905–1.094)	0.640	0.000	Isometric
		Females	465	0.814	0.335 ± 0.215 (−0.087–0.757)	0.072	0.119 (ns)	
SL	PMW	Males	621	0.012	1.393 ± 0.049 (1.297–1.489)	0.753	0.000	+ Allometry
		Females	466	0.347	0.352 ± 0.180 (−0.002–0.707)	0.090	0.051 (ns)	

Abbreviations: *n*, number; *a'*, anti-logarithm of the linear regression intercept *a* ($a' = e^{\ln a}$); *b*, linear regression slope; SE, standard error; CI, confidence interval; *r*, correlation coefficient; *P*-value, statistical significance; PTL, penis total length; SL, shell length; PPL, penis partial length; PMW, penis maximum width; ns, not significant.

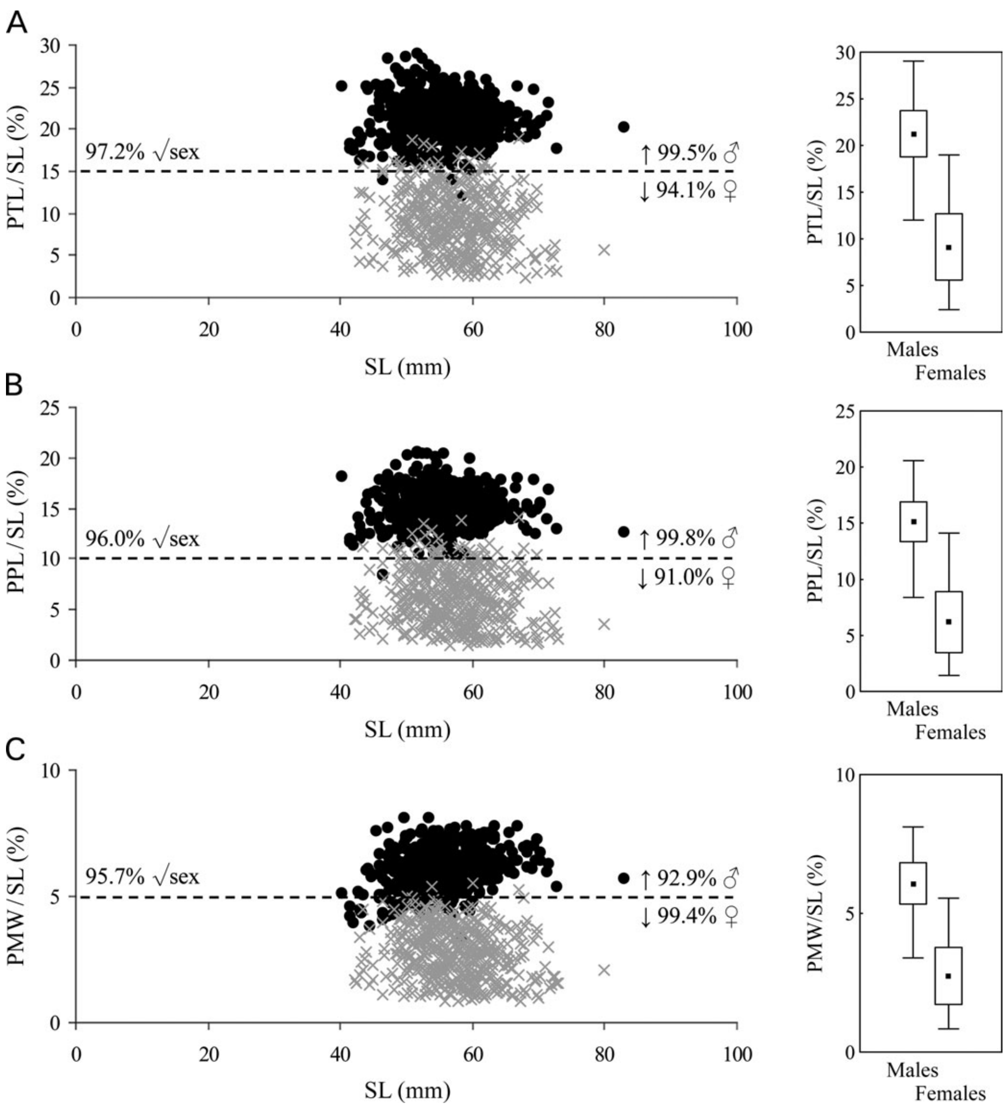

Figure 4. Sexual indices adopted for the sexual identification of males and imposex-affected females of *Hexaplex (Trunculariopsis) trunculus*. **A.** Relationship shell length *vs* PTL/SL. **B.** Relationship shell length *vs* PPL/SL. **C.** Relationship shell length *vs* PMW/SL. Abbreviations: SL, shell length; PTL, penis total length (proximal and distal portions); PPL, penis partial length (proximal portion); PMW, penis maximum width (proximal portion). Box-whisker graph: middle point = mean; box = ±standard deviation; whisker = minimum and maximum values.

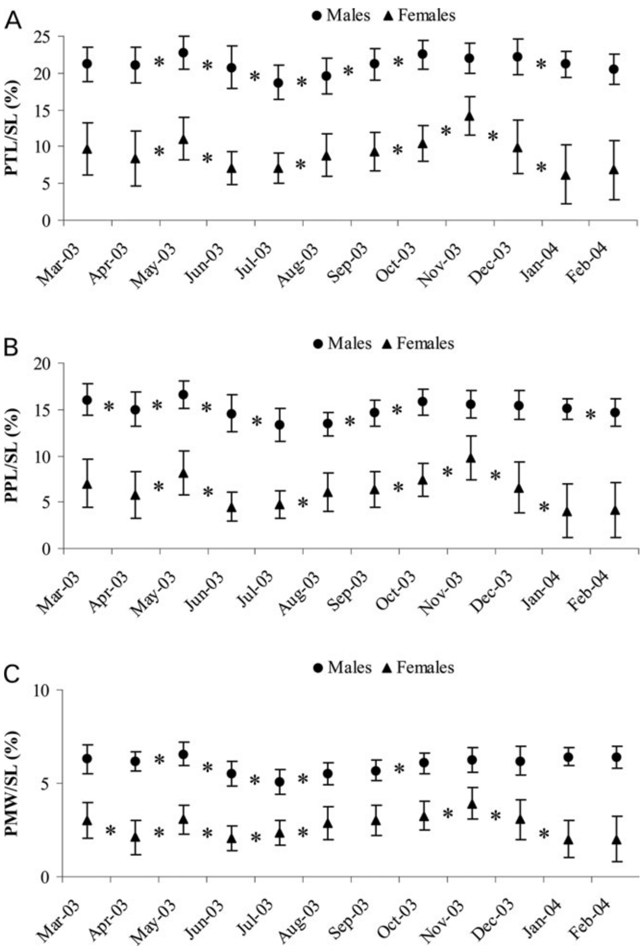

Figure 5. Monthly variation of the sexual indices during the one-year study period. **A.** Sexual index PTL/SL. **B.** Sexual index PPL/SL. **C.** Sexual index PMW/SL. Abbreviations: SL, shell length; PTL, penis total length (proximal and distal portions); PPL, penis partial length (proximal portion); PMW, penis maximum width (proximal portion). * = statistically significant differences between consecutive monthly samples (Mann–Whitney U-test, $P < 0.05$).

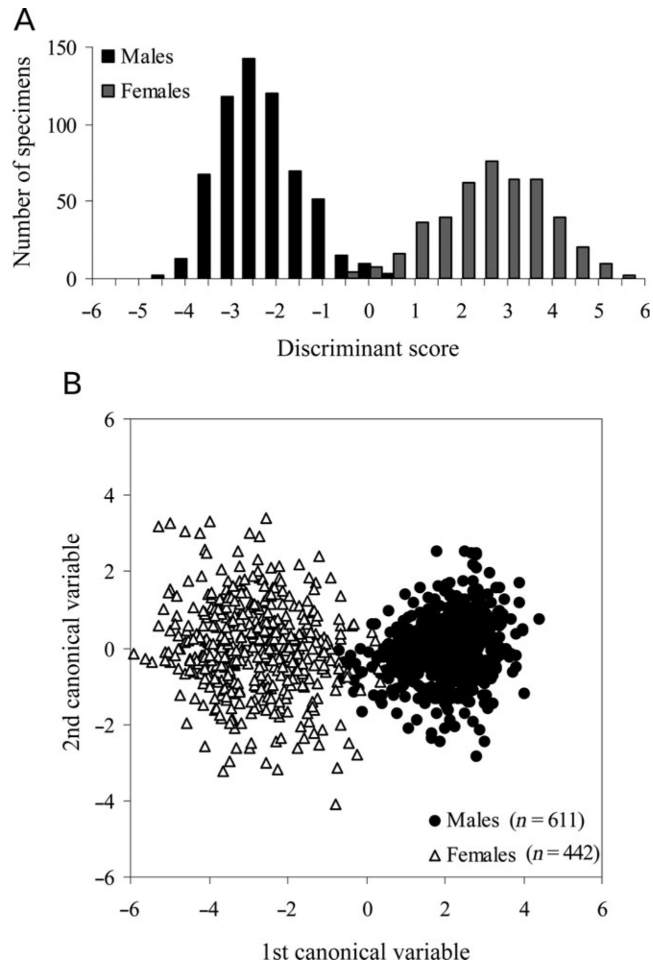

Figure 6. A. Frequency of male and female *Hexaplex (Truncalariopsis) trunculus* relatively to the discriminant scores calculated from the linear equation obtained by multivariate discriminant analysis. **B.** Plot of the individual scores for male and female *Hexaplex (Truncalariopsis) trunculus* in the first two canonical variables resulting from multivariate discriminant analysis.

classifications), while PMW/SL was more suitable for accurately sexing females (invariably 100% correct classifications). By integrating data both from specimen size (SL) and respective penis dimensions, the discriminant function (D) also revealed a very high precision in gender identification (generally above 95% correct classifications), which was higher for imposex-affected females than for males (Table 3).

DISCUSSION

Despite being an organ resulting from an abnormal development, the penes of imposex-affected females of *T. trunculus* were visually very similar to male penes, with the exception of their coloration (identical pigmentation to the foot), which generally was slightly darker in males than in females. The morphological similarity between these organs was further reinforced by the comparison of their relative proportions (ratios: PPL/PTL and PMW/PTL) and by the morphometric relationships established between penis measurements (PTL, PPL and PMW). This similar morphology of male and imposex-affected female penes was already evidenced both externally and internally (histologically) (Martoja & Bouquegneau, 1988; Axiak *et al.*, 2003). Altogether, these similar characteristics between male and imposex-affected female penes make the sexual identification based solely on relative penis proportions and morphology not feasible.

The imposex-affected females' average penis size obviously varies according to the degree of imposex in the population, but ultimately can reach (or even exceed) the average size of the male penis. However, at the imposex incidence (I = 93.24%) and degree (Population RPLI = 36.13, Incidence RPLI = 43.82, Population RPSI = 4.71 and Incidence RPSI = 8.42) detected in this population from the Ria Formosa lagoon (Vasconcelos, Gaspar & Castro, 2006), male penis average dimensions (PTL, PPL and PMW) were invariably more than two times greater than the same measurements in imposex-affected females.

Moreover, and because TBT apparently does not affect the male penis size in this gastropod species (Axiak *et al.*, 1995), the morphometric relationships established between SL and penis dimensions clearly demonstrated that male penis dimensions were more size-dependent (relatively to SL), whereas

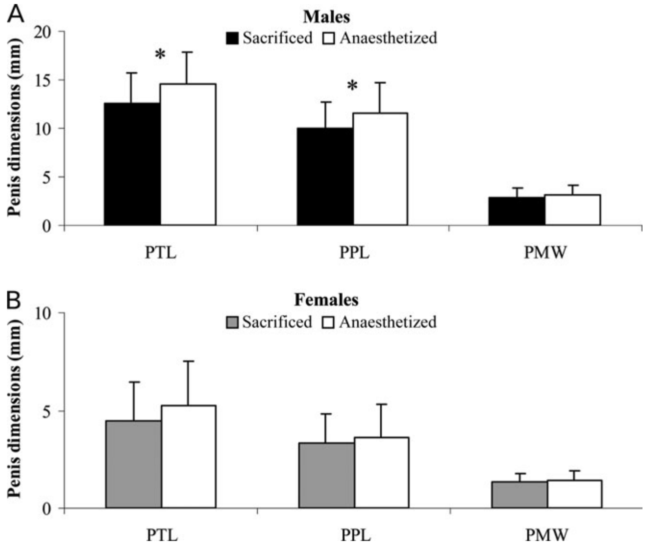

Figure 7. Effect produced by the anaesthetization in the average penis dimensions (PTL, PPL and PMW) of males and imposex-affected females of *Hexaplex (Trunculariopsis) trunculus*. Abbreviations: PTL, penis total length (proximal and distal portions); PPL, penis partial length (proximal portion); PMW, penis maximum width (proximal portion). * = statistically significant differences between average dimensions (Z-test, $P < 0.05$).

female penis is an organ resulting from an abnormal development (imposex phenomenon), whose dimensions only depend on the duration of the exposure and intensity of contamination by TBT. Additionally, the allometric relationships estimated between SL and penis dimensions further confirmed this phenomenon. Statistically significant relationships revealed that male penis dimensions were intrinsically related to their body growth while, on the contrary, nonsignificant relationships (or with a low correlation coefficient) indicated that imposex-affected female penis dimensions were not directly connected to age, instead depending on the deleterious effects of TBT pollution.

In the case of the male penis, the SL *vs* PPL isometric relationship confirmed the assumption that both variables (SL and penis partial length) developed at the same rate. The other allometric relationships revealed that male penis total length grows relatively slower than shell length, possibly due to the decrease or damage of the penis extremity (distal portion) (negative allometric relationship SL *vs* PTL), a phenomenon that is accompanied by the progressive thickening of the penis during growth (positive allometric relationship SL *vs* PMW). For the allometric relationships established between penis dimensions, the different types of growth detected in the relationships PTL *vs* PPL between males and females also revealed the different features and characteristics of this organ between sexes. For the male penis, the negative allometry indicates that during penis development and growth, an extension of the penis distal portion (sharp tip) occurs, probably to facilitate copulation. On the contrary, for the female penis, the positive allometry reveals that during development and growth of this anomalous organ, the proximal portion of the penis increases at a greater rate than the distal portion (and consequently than the penis total length).

Despite some overlap, the morphometric relationships established between individual size (SL) and penis dimensions (PTL, PPL and PMW) created some expectation that the use of these measurements could be valuable for distinguishing gender of males and imposex-affected females of *T. trunculus*. Subsequently, the adoption of sexual indices consisting of the percentage of penis dimensions relative to individual size (PTL/SL, PPL/SL and PMW/SL), and the simple definition of percentage limits for the separation between males and females, revealed a high accuracy of the sexual classification based on penis measurements. These sexual ratios allowed for the correct identification of gender of the vast majority of specimens (PTL/SL = 97.2%, PPL/SL = 96.0% and PMW/SL = 95.7%), correct sexual recognition generally being higher for males than for females.

These sexual indices (PTL/SL, PPL/SL and PMW/SL) showed slight oscillations during the one-year study period, with some significant variations between several consecutive monthly samples of both sexes, mostly between April and October. These oscillations were analogous between sexual indices and with a similar trend between males and imposex-affected females all year-round, thus they could not be attributed to the reproductive cycle of this species or to variation in male penis size at sexual maturity. Furthermore, imposex apparently does not show any seasonal variation during the reproductive cycle of *T. trunculus* (Martoja & Bouquegneau, 1988). This relative proportionality between penis dimensions of both sexes irrespective of sampling season allowed for the use of these sexual indices in this species all year-round.

Multivariate discriminant analysis using both the individual size (SL) and the respective penis dimensions further confirmed the clear distinction between sexes based on penis morphometrics. Applying the canonical function (linear equation) best discriminating between males and females, allowed for the correct classification of gender for 98.8% of the specimens, which was slightly higher for males (99.5%) than for females (97.7%). Nevertheless, in this context it should be emphasized that using the same individuals for function estimation and testing could have generated excessively optimistic estimates of the success of sexual classification.

Measurement of the penis is inevitably difficult to standardize and consequently is not a highly precise procedure, mainly because this organ is muscular and subject to contraction with

Table 3. Correct sexual identifications of males and imposex-affected females obtained through the application of sexual indices to 100 specimens of *Hexaplex (Trunculariopsis) trunculus* that were anaesthetized and subsequently sacrificed.

Sexual indices	Sacrificed			Anaesthetized (raw data)			Anaesthetized (conversion factor)		
	Males	Females	Total	Males	Females	Total	Males	Females	Total
PTL/SL (%)	54 (100.0)	45 (97.8)	99 (99.0)	54 (100.0)	41 (89.1)	95 (95.0)	53 (98.1)	44 (95.7)	97 (97.0)
PPL/SL (%)	54 (100.0)	44 (95.7)	98 (98.0)	54 (100.0)	40 (87.0)	94 (94.0)	54 (100.0)	44 (95.7)	98 (98.0)
PMW/SL (%)	42 (77.8)	46 (100.0)	88 (88.0)	47 (87.0)	46 (100.0)	93 (93.0)	45 (83.3)	46 (100.0)	91 (91.0)
Discriminant (D)	51 (94.4)	45 (97.8)	96 (96.0)	52 (96.3)	45 (97.8)	97 (97.0)	52 (96.3)	46 (100.0)	98 (98.0)

Abbreviations: PTL, penis total length; PPL, penis partial length; PMW, penis maximum width; SL, shell length.

tactile stimulation (Gibbs, 1999). Furthermore, the anaesthetization causes penial muscle tissue to relax and therefore induces an increase in penis measurements. The experimental anaesthetization of *T. trunculus* was very successful and this species proved highly tolerant to the narcosis treatment but, as expected, the anaesthetic ($MgCl_2$) provoked an enlargement in penis dimensions. In the present case, the longer the penis measurement the higher the augmentation effect produced by the anaesthetic (PTL > PPL > PMW). Nevertheless, despite this side effect from the narcotic, the sexual indices previously developed for sacrificed specimens (PTL/SL, PPL/SL, PMW/SL and discriminant function, *D*) were still highly successful in sexing anaesthetized *T. trunculus* specimens (generally more than 95% correct), using both raw data and transformed data (conversion factor).

Taking into account that *T. trunculus* lacks external sexual dimorphism and that gender identification normally requires breaking the shell and sacrificing the organism, the data gathered in this study are valuable for sexing live specimens of known degree of imposex. This procedure was easily performed by anaesthetizing the individuals with $MgCl_2$, gently exposing and measuring penis dimensions, and finally applying sexual indices. This kind of information is a useful nonsacrificial tool for the sexual identification of live specimens, with application in biological, ecological and behavioural studies.

It is worth emphasising that these sexual indices can be successfully applied to all *T. trunculus* populations with a degree of imposex equal to or less than the one in this study, and that at progressively lower degrees of imposex, sexual discrimination will be higher. Furthermore, with TBT levels dropping and consequent less severe imposex, it is expected that the sexual recognition of *T. trunculus* will progressively present less complexity in the near future. Nevertheless, it may take longer than initially supposed since, despite the recent global TBT restrictions, it is possible that this environmental problem could even increase over the next few years before finally attenuating or disappearing (Champ, 2000). Therefore, TBT contamination in the Ria Formosa lagoon is also unlikely to change for a considerable period of time (Coelho, Bebianno & Langston, 2002) and apparently has not decreased recently (Vasconcelos, Gaspar & Castro, 2006).

Similar sexual indices can be developed for other gastropod species without external sexual dimorphism and affected by imposex, only requiring baseline data on the imposex degrees of their populations. However, it should be emphasized that similar sexual indices should be cautiously applied to gastropod species whose male penis dimensions present significant seasonal fluctuations related to sexual maturity, reproductive cycle and/or breeding season, a phenomenon that has been detected in some muricid species, namely *Bolinus brandaris* (Ramón & Amor, 2001, 2002), *Nucella lapillus* and *Ocenebra erinaceus* (Stroben *et al.*, 1996), as well as in non-Muricidae species, such as *Ilyanassa obsoleta* (Bryan *et al.*, 1989), *Nassarius reticulatus*, *Nassarius incrassatus* and *Trivia arctica* (Stroben *et al.*, 1996). Nevertheless, in practice, this seasonal enlargement of the male penis size is only a minor source of error, because many populations breed throughout the year (Gibbs, 1999).

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REFERENCES

AXIAK, V., MICALLEF, D., MUSCAT, J., VELLA, A. & MINTOFF, B. 2003. Imposex as a biomonitoring tool for marine pollution by tributyltin: some further observations. *Environment International*, **28**: 743–749.

AXIAK, V., VELLA, A.J., AGIUS, D., BONNICI, P., CASSAR, G., CASSONE, R., CHIRCOP, P., MICALLEF, D., MINTOFF, B. & SAMMUT, M. 2000. Evaluation of environmental levels and biological impact of TBT in Malta (central Mediterranean). *The Science of the Total Environment*, **258**: 89–97.

AXIAK, V., VELLA, A.J., MICALEFF, D., CHIRCOP, P. & MINTOFF, B. 1995. Imposex in *Hexaplex trunculus* (Gastropoda: Muricidae): first results from biomonitoring of tributyltin contamination in the Mediterranean. *Marine Biology*, **121**: 685–691.

BERNARD, F.R. 1968. Sexual dimorphism in *Polinices lewisi* (Naticidae). *Nautilus*, **82**: 1–3.

BRANDE, S., TURNER, M., HELLER, J. & BEN YEHUDA, O. 1996. Statistical discrimination of sex in *Melanoides tuberculata* (Gastropoda: Thiaridae). *Biological Journal of the Linnean Society*, **59**: 87–112.

BRYAN, G.W., GIBBS, P.E., HUGGETT, R.J., CURTIS, L.A, BAILEY, D.S. & DAUER, D.M. 1989. Effects of tributyltin pollution on the mud snail, *Ilyanassa obsoleta*, from the York River and Sarah Creek, Chesapeake Bay. *Marine Pollution Bulletin*, **20**: 458–462.

CASTAGNA, M. & KRAEUTER, J.N. 1994. Age, growth rate, sexual dimorphism and fecundity of the knobbed whelk *Busycon carica* (Gmelin, 1791) in a western mid-Atlantic lagoon system, Virginia. *Journal of Shellfish Research*, **13**: 581–585.

CHAMP, M.A. 2000. A review of organotin regulatory strategies, pending actions, related costs and benefits. *The Science of the Total Environment*, **258**: 21–71.

CHIAVARINI, S., MASSANISSO, P., NICOLAI, P., NOBILI, C. & MORABITO, R. 2003. Butyltins concentration levels and imposex occurrence in snails from the Sicilian coasts (Italy). *Chemosphere*, **50**: 311–319.

COELHO, M.R., BEBIANNO, M.J. & GIBBS, P.E. 1998. Imposex in neogastropod snails from southern Portugal. *Cuadernos de Investigación Biológica*, **20**: 273.

COELHO, M.R., BEBIANNO, M.J. & LANGSTON, W.J. 2002. Organotin levels in the Ria Formosa lagoon, Portugal. *Applied Organometallic Chemistry*, **16**: 384–390.

EL HAMDANI, A., FERRER, J.M. & GARCÍA CARRASCOSA, A.M. 1998. Imposex in prosobranch molluscs: an indicator of TBT pollution in the Valencian coast (Spain, Western Mediterranean). *Cuadernos de Investigacíon Biológica*, **20**: 275–278.

ELLIS, D.V. & PATTISINA, L.A. 1990. Widespread neogastropod imposex: a biological indicator of global TBT contamination? *Marine Pollution Bulletin*, **21**: 248–253.

ESTEBENET, A.L. 1998. Allometric growth and insight on sexual dimorphism in *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Malacologia*, **39**: 207–213.

FIORONI, P., OEHLMANN, J. & STROBEN, J. 1991. The pseudohermaphroditism of prosobranchs: morphological aspects. *Zoologischer Anzeiger*, **226**: 1–26.

GARAVENTA, F., PELLIZZATO, F., FAIMALI, M., TERLIZZI, A., MEDAKOVIC, D., GERACI, S. & PAVONI, B. 2006. Imposex in *Hexaplex trunculus* at some sites on the North Mediterranean Coast as a base-line for future evaluation of the effectiveness of the total ban on organotin based antifouling paints. *Hydrobiologia*, **555**: 281–287.

GIBBS, P.E. 1999. Biological effects of contaminants: use of imposex in the dogwhelk (*Nucella lapillus*) as a bioindicator of tributyltin pollution. *ICES Techniques in Marine Environmental Sciences*, **24**: 1–29.

GIBBS, P.E. 2005. Male genital defect (Dumpton Syndrome) in the dogwhelk *Nucella lapillus* (Neogastropoda): Mendelian inheritance inferred, based on laboratory breeding experiments. *Journal of the Marine Biological Association of the United Kingdom*, **85**: 143–150.

GIBBS, P.E., BEBIANNO, M.J. & COELHO, M.R. 1997. Evidence of the differential sensitivity of neogastropods to tributyltin (TBT) pollution, with notes on a species (*Columbella rustica*) lacking the imposex response. *Environmental Technology*, **18**: 1219–1224.

JENNER, M.G. 1979. Pseudohermaphroditism in *Ilyanassa obsoleta* (Mollusca: Neogastropoda). *Science*, **205**: 1407–1409.

KANTOR, Y.I. & SYSOEV, A.V. 1991. Sexual dimorphism in the apertural notch of a new species of *Gemmula* (Gastropoda: Turridae). *Journal of Molluscan Studies*, **57**: 205–209.

KENCHINGTON, E. & GLASS, A. 1998. Local adaptation and sexual dimorphism in the waved whelk (*Buccinum undatum*) in Atlantic Nova Scotia with applications to fisheries management. *Canadian Technical Report of Fisheries and Aquatic Sciences*, **2237**: 1–43.

KURATA, K. & KIKUCHI, E. 2000. Comparisons of life-history traits and sexual dimorphism between *Assiminea japonica* and *Angustassiminea castanea* (Gastropoda: Assimineidae). *Journal of Molluscan Studies*, **66**: 177–196.

LANGSTON, W.J., GIBBS, P.E., LIVINGSTONE, D.R., BURT, G.R., O'HARA, S., BEBIANNO, M.J., COELHO, M.R., PORTE, C., BAYONA, J., McNULTY, M., LYNCH, G. & KEEGAN, B. 1997. Risk assessment of organotin antifoulings on key benthic organisms of European coastal habitats (BOATS). MAS2-CT-94-0099, Final Report: October 1997.

MARTOJA, M. & BOUQUEGNEAU, J.-M. 1988. *Murex trunculus*: un nouveau cas de pseudo-hermaphrodisme chez un gastéropode prosobranche. *Bulletin de la Société Royale de Liège*, **57**: 45–58.

PELLIZZATO, F., CENTANNI, E., MARIN, M.G., MOSCHINO, V. & PAVONI, B. 2004. Concentrations of organotin compounds and imposex in the gastropod *Hexaplex trunculus* from the Lagoon of Venice. *Science of the Total Environment*, **332**: 89–100.

RAMÓN, M. & AMOR, M.J. 2001. Increasing imposex in populations of *Bolinus brandaris* (Gastropoda: Muricidae) in the north-western Mediterranean. *Marine Environmental Research*, **52**: 463–475.

RAMÓN, M. & AMOR, M.J. 2002. Reproductive cycle of *Bolinus brandaris* and penis and genital duct size variations in populations affected by imposex. *Journal of the Marine Biological Association of the United Kingdom*, **82**: 435–442.

RILOV, G., GASITH, A., EVANS, S.M. & BENAYAHU, Y. 2000. Unregulated use of TBT-based antifouling paints in Israel (Eastern Mediterranean): high contamination and imposex levels in two species of marine gastropods. *Marine Ecology Progress Series*, **192**: 229–238.

SMITH, B.S. 1971. Sexuality in the American mud snail *Nassarius obsoletus* Say. *Proceedings of the Malacological Society of London*, **39**: 377–378.

SOKAL, R.R. & ROHLF, F.J. 1987. *Introduction to biostatistics*. 2nd edition. W. H. Freeman and Company, New York.

STROBEN, E., OEHLMANN, U., SCHULTE-OEHLMANN, U. & FIORONI, P. 1996. Seasonal variations in the genital ducts of normal and imposex-affected prosobranchs and its influence on biomonitoring indexes. *Malacological Review*, Suppl. **6**: 173–184.

TEN HALLERS-TJABBES, C.C. 1979. Sexual dimorphism in *Buccinum undatum* L., *Malacologia*, **18**: 13–17.

TERLIZZI, A. 2000. Imposex (pseudoermafroditismo) in molluschi gasteropodi mediterranei: aspetti morfologici e considerazioni ecologiche. *Bollettino Malacologico*, **36**: 155–158.

TERLIZZI, A., DELOS, A.L., GARAVENTA, F., FAIMALI, M. & GERACI, S. 2004. Limited effectiveness of marine protected areas: imposex in *Hexaplex trunculus* (Gastropoda, Muricidae) populations from Italian marine reserves. *Marine Pollution Bulletin*, **48**: 188–192.

TERLIZZI, A., FRASCHETTI, S., GIANGUZZA, P., FAIMALI, M. & BOERO, F. 2001. Environmental impact of antifouling technologies: state of the art and perspectives. *Aquatic Conservation: Marine and Freshwater Ecosystems*, **11**: 311–317.

TERLIZZI, A., GERACI, S. & GIBBS, P.E. 1999. Tributyltin (TBT)-induced imposex in the Neogastropod *Hexaplex trunculus* in Italian coastal waters: morphological aspects and ecological implications. *Italian Journal of Zoology*, **66**: 141–146.

TERLIZZI, A., GERACI, S. & MINGANTI, V. 1998. Tributyltin (TBT) pollution in the coastal waters of Italy as indicated by imposex in *Hexaplex trunculus* (Gastropoda: Muricidae). *Marine Pollution Bulletin*, **36**: 749–752.

TERLIZZI, A., SCUDERI, D., FAIMALI, M., MINGANTI, V. & GERACI, S. 1997. Imposex in *Hexaplex trunculus* e *Stramonita haemastoma* (Gastropoda: Muricidae): prime segnalazioni per le acque costiere italiane. *Biologia Marina Mediterranea*, **4**: 496–499.

VASCONCELOS, P., GASPAR, M.B. & CASTRO, M. 2006. Imposex in *Hexaplex (Trunculariopsis) trunculus* (Gastropoda: Muricidae) from the Ria Formosa lagoon (Algarve coast-southern Portugal). *Marine Pollution Bulletin*, **52**: 337–341.

VASCONCELOS, P., GASPAR, M.B., PEREIRA, A.M. & CASTRO, M. Growth rate estimation of *Hexaplex (Trunculariopsis) trunculus* (Gastropoda: Muricidae) based on mark/recapture experiments in the Ria Formosa lagoon (Algarve coast-southern Portugal). *Journal of Shellfish Research* (in press).

ZAR, J.H. 1996. *Biostatistical analysis*. Edn 3. Prentice-Hall International Inc., New Jersey.