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Imposex development in response to TBT pollution in *Hinia incrassata* (Ström, 1768) (Prosobranchia, Stenoglossa)[1]

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Abstract

The stenoglossan prosobranch *Hinia incrassata*, collected along the coast of Brittany and Normandy between 1988 and 1997, exhibits imposex also known as pseudohermaphroditism (the occurrence of male parts in addition to the female genital system) in response to tributyltin (TBT) pollution. Excluding some normal females (stage 0), different imposex stages as defined by Fioroni et al. (1991a) (1a, 1b, 2a, 3a and 4) can be distinguished and have been documented for the first time using scanning electron micrographs. Additional malformations of the genital tract are described. Neither TBT-induced sterilization nor sex change occur in the analyzed samples. TBT accumulation in soft parts was analyzed and bioconcentration factors (BCF) were found to be between $4.68 \cdot 10^4$ and $1.22 \cdot 10^5$. The VDS (vas deferens sequence) index, RPL (relative penis length) index and the average female penis length (FPL) in populations exhibit a significant dependence on both TBT concentrations in ambient sea water and TBT body burden. An interspecific comparison between imposex intensities in sympatrically living populations of *Hinia incrassata*, *Nucella lapillus* and *Hinia reticulata* reveals that *H. incrassata* exhibits the lowest TBT sensitivity of the three species. But even in this relatively unsensitive species the threshold concentration for imposex development is below 1.5 ng TBT as Sn l^{-1}. The VDS index is recommended as the most suited biological parameter for effect monitoring purposes because this index is characterized by the lowest deviation of data points from calculated concentration-effect-equations and shows no seasonal variations. *Hinia incrassata* is proposed for large large-scale TBT surveys in the Mediterranean being planned by OSPARCOM for the North Atlantic. Furthermore the species can be used for TBT temporal trend monitoring. Studies in France, for example, where TBT was partially banned in 1982, show little or no recovery from TBT contamination in *H. incrassata* and two other prosobranch species between 1989 and 1997. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Imposex; TBT pollution; Biomonitoring; Reproduction; Prosobranch snails; *Hinia incrassata*

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[1] This publication is dedicated to our honoured mentor and supervisor, Prof. Dr. Pio Fioroni, Münster, Germany, on the occasion of his 65th birthday.

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1. Introduction

Tributyltin (TBT) compounds are applied in various formulations and are responsible for environmental problems, especially when used in antifouling paints. Fouling—the colonization of underwater surfaces by epizoa (e.g. Phycophyta, Anthozoa, Hydroidea, Bivalvia, Bryozoa, Cirripedia, Ascidiaceae)—causes an increase of frictional resistance on ships hulls and offshore installations. This leads naturally to increased fuel consumption. The principle of antifouling paints is largely based on the continuous leaching of toxic substances from the paint matrix such as copper, tributyltin and other organic compounds used as cotoxicants.

TBT-based antifoulings were introduced in the mid-1960s and their application gained popularity rapidly. Adverse impact on aquatic nontarget organisms caused by these compounds became apparent in the late 1970s. Today TBT is known to produce a variety of pathological conditions in animals even at very low environmental concentrations but, in terms of sensitivity, none rivals that of the imposex phenomenon in prosobranchs. Because the analysis of organotin compounds in sea water and tissue is rather difficult, time-consuming and expensive, the determination of an easily detectable morphological parameter would give better results.

Imposex (Smith, 1971) also known as pseudohermaphroditism (Jenner, 1979), that is the occurrence of male sex organs in the female genital system, has been analyzed for many years. *Nucella lapillus* (Blaber, 1970), *Ilyanassa obsoleta* (Smith, 1971) *Thais emarginata* (Houston, 1971), *Hinia reticulata* (Féral, 1974a), and *Ocenebra erinacea* (Féral, 1974b) were the first discovered female penis-bearing neogastropods. The catalogue of imposex-affected species had to be enlarged in the following years and now includes more than 140 species from different geographic regions and systematic groups, including mesogastropods. A list with subsequent discoveries have rendered incomplete is given in Fioroni et al. (1991a). In order to measure imposex and to assess coastal TBT pollution, a good and practicable system of reference has to be developed.

The information about imposex development and expression in *Hinia incrassata* (Ström, 1768) is rather incomplete. The studies of Fioroni et al. (1991a,b) describe the occurring imposex stages but give no information about TBT accumulation, sensitivity and imposex development in relationship to ambient TBT concentrations in this species. In many European TBT surveys the dogwhelk *Nucella lapillus* has been used as an indicator species for TBT biomonitoring (Gibbs et al., 1987; Bailey and Davies, 1988; Gibbs et al., 1990, 1991a,b; Oehlmann et al., 1993; Minchin et al., 1995; Huet et al., 1996). Due to its high TBT sensitivity, *N. lapillus* exhibits TBT-induced reproductive failure as a consequence of TBT concentrations ≥ 2 ng TBT as Sn l^{-1} and has therefore been declining in many areas of Europe over the last 10–15 years (Gibbs et al., 1987; Bailey and Davies, 1988; Oehlmann et al., 1993). Another problem with the use of dogwhelks for TBT biomonitoring is the muddy or sandy substratum on many European coasts. *N. lapillus* flourishes exclusively on rocky shores and thus is completely absent along the entire coast of Belgium, the Netherlands, Germany and in many areas of Denmark (Fretter and Graham, 1985).

Hinia incrassata, in contrast, is distributed throughout the Mediterranean in areas where only few imposex affected species are available (Oehlmann, 1994; Axiak et al., 1995), and in European Atlantic waters as far as Norway and Iceland in the north. The species can also be found in southern parts of the North Sea and the Kattegat, but is missing in the Baltic Sea (Fretter and Graham, 1985). Although the bulk of the population lives in the shallow sublittoral up to a depth of 200 m towards the northern limit of its distribution range, *H. incrassata* is common near LWST on rocky shores, in crevices and under stones. Often the species can be found at the bases of tufts of weeds (Fretter and Graham, 1985). *H. incrassata* is a scavenger and carrion feeder, like *Hinia reticulata*. Breeding occurs throughout the year with maxima of copulation activity in early spring. Egg capsules are laid on solid substrates, but also on weeds and hydroids. The capacity of the species for dispersion is high because the offspring hatches as a free swimming planktonic 'long distance' veliger (Lebour, 1937; Fretter and Graham, 1985).

The objectives of this study are to provide a photographically based documentation of imposex classification in *Hinia incrassata* in light of the unaffected genital system. Furthermore, data on TBT accumulation and imposex development in relation to ambient TBT concentrations or TBT body burdens are given. Information concerning the suitability of this species for biological TBT effect-monitoring purposes considering an interspecific comparison with *Nucella lapillus* and *Hinia reticulata* are added and possible indices for the measurement of imposex are presented.

2. Material and methods

For this study 1642 specimens of *Hinia incrassata*, collected at 12 stations along the coast of Brittany and at the Ile d'Yeu between March 1988 and March 1997 were analyzed. A map indicating these stations is given in Fig. 1. Complete series of histological sections exist for 34 specimens.

Prior to further analyses, the animals were narcotized using 7% $MgCl_2$ in distilled water. The height of the shell and its aperture were measured to the nearest 0.1 mm using a vernier calliper. The shell was then cracked with a vice, and the animals were analyzed under a stereo microscope. The external dimensions of the genital tract including vas deferens extension and penis length were measured with an exactness of 0.1 mm using an eyepiece.

The normostructure of the male and female genital system of *Hinia incrassata* was investigated using histological slide series and SEM-techniques. The male genital tract sections of imposex affected females were examined in the same way. For serial sections (7–10 μm) specimens were fixed with Bouin's fluid, preserved in 70% ethanol and embedded in paraplast. The sections were stained with azan according to Heidenhain, haemalun-chromotrope and trichrome according to Goldner, alcian blue and the PAS-reaction. Specimens for SEM were fixed in Bouin's fluid, dehydrated via graded ethanol series, critical point dried, coated with gold and examined with a Hitachi scanning electron microscope S-530.

Wherever possible, 30 or more adult *Hinia incrassata* were collected intertidally. The following indices for TBT biomonitoring were used: (1) The VDS (vas deferens sequence) index is calculated as the average imposex stage (according to Fig. 2) of a population. (2) The relative penis length (RPL) index is defined as [(mean length of female penis)/(mean length of male penis) × 100] (Stroben et al., 1992b). This index is different from the relative penis size (RPS) index introduced by Gibbs et al. (1987) [(mean length of female penis^3)/(mean length of male penis^3) × 100]. (3) Average female penis length (FPL) of a population.

The determination of TBT and DBT (dibutyltin) compounds in tissues and water was based on Stroben et al. (1992b). The analysis includes an extraction of TBT and DBT compounds with hexane, the elimination of DBT by washing the hexane extract with NaOH and quantification using atomic absorption spectroscopy (Perkin–Elmer HGA-500 attached to a Perkin–Elmer 5000 AAS with background correction; wave length 224.6 nm; slit 0.7 nm; injection volume 25 μl). Internal standardization (standard addition with spiked samples) was employed. Certified reference material (CRM: PACS-1, delivered by the National Research Council of Canada) was analyzed additionally. Our results were within the standard deviation of the certified values for the CRM. The detection limit (3σ) in a single sample was 7.4 and 8.8 ng DBT-Sn and TBT-Sn respectively. All DBT and TBT tissue concentrations are given on a dry weight basis as Sn. Water samples of 0.5 or 1.0 l were taken in polycarbonate bottles at a depth of 0.5 m below water surface, acidified with 5 or 10 ml of concentrated HCl (Merck 'suprapur') and extracted with 5 or 10 ml of hexane (pesticide grade) for 30 min. TBT- and DBT-Sn were analyzed as described for tissue analysis above. The detection limit (3σ) was 1.5 ng TBT-Sn l^{-1} using a sample volume of 0.5 l and 1.0 ng TBT-Sn l^{-1} at a sample volume of 1.0 l.

Standard statistical analyses (e.g. parametrical correlation and nonparametrical Spearman rank

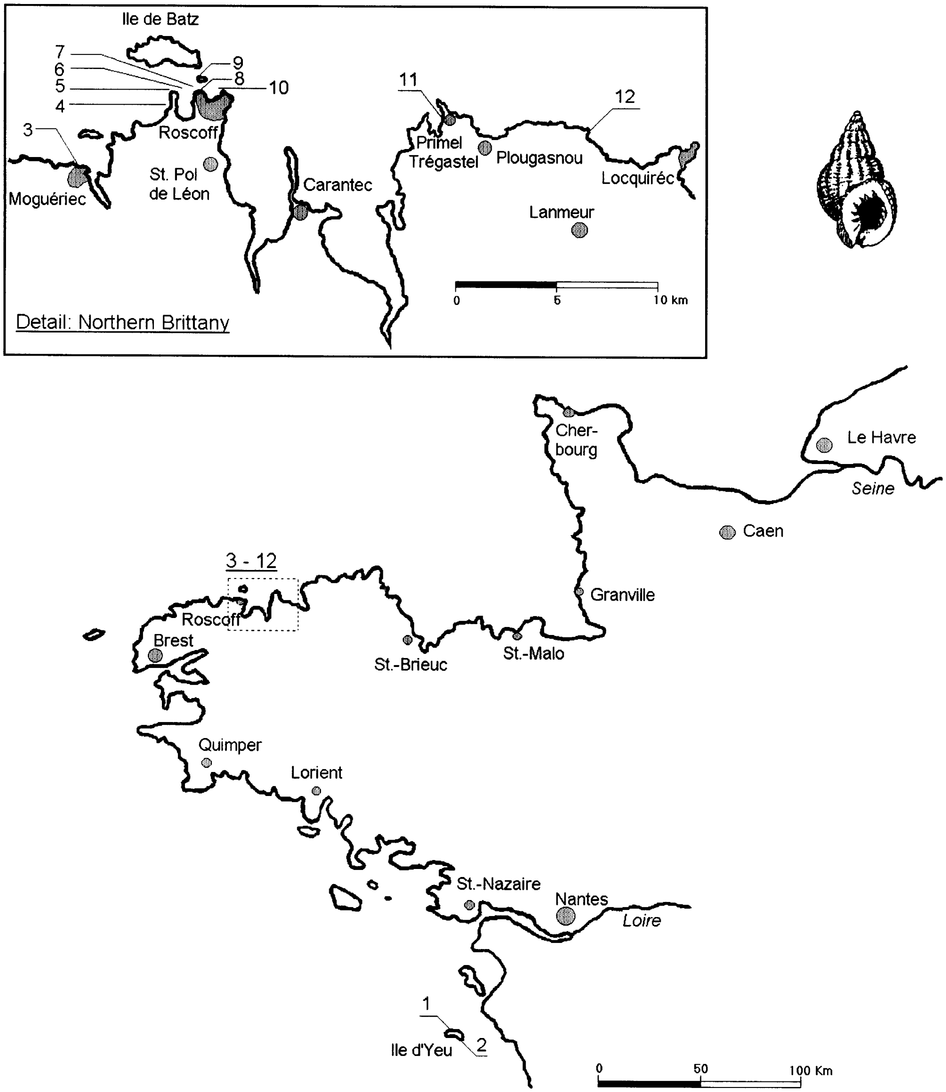

Fig. 1. Map of the northwestern part of France with sampling stations of *Hinia incrassata* indicated by numbers. 1, Pnte. du Boite, Ile d'Yeu; 2, La Croix, Ile d'Yeu; 3, Moguériec harbour; 4, Centre heliomarine, Roscoff; 5, Lédanet; 6, Le Loup; 7, Roche Gaurec; 8, Marine biological station Roscoff; 9, Ile Verte; 10, Roscoff harbour; 11, Primel Trégastel; 12, Beg an Fry.

correlation) of the results were performed according to Lozán (1992) using the computer programmes SigmaPlot 5.1 and StatEasy 2.25. A correlation analysis between biological parameters of two different species or between TBT body burdens and biological parameters can be performed only if the conditions for the application of this parametrical test, e.g. normal distribution of measuring values, are fulfilled. Otherwise, a nonparametrical test as the Spearman rank correlation analysis is more appropriate. But because the latter method fails to visualize the dependence relationship between two tested parameters, not only the Spearman rank correlation (r_s if $n < 30$, t if $n = 30$) is calculated in these cases but also the parametrical correlation data are given.

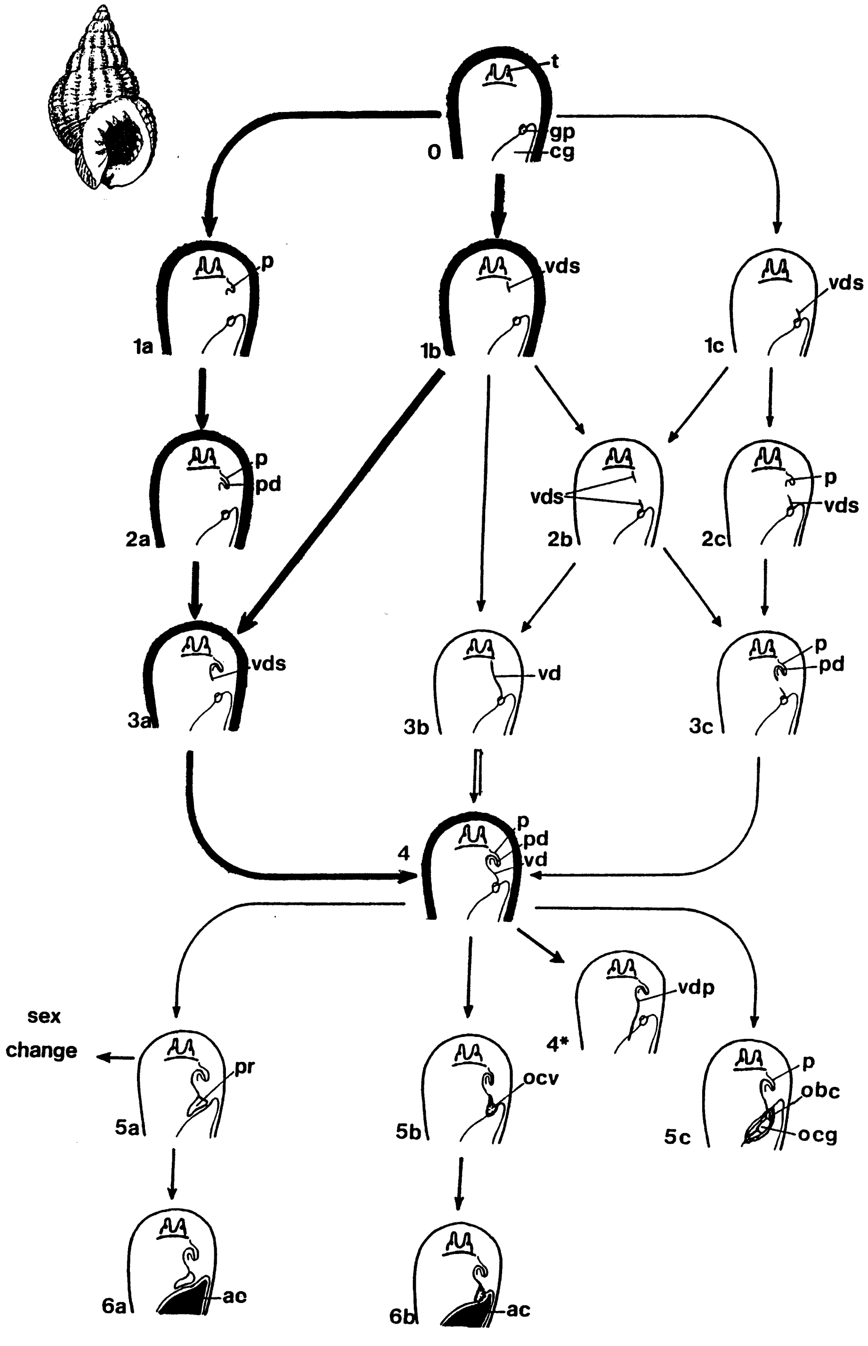

Fig. 2. General scheme of imposex evolution in prosobranchs. Imposex stages of *Hinia incrassata* in bold. Abbreviations: ac: aborted capsules; cg: capsule gland; gp: genital papilla; obc: open bursa copulatrix; ocg: open capsule gland; ocv: occlusion of the vulva; p: penis; pd: penis duct; pr: prostate; t: tentacle; vd: vas deferens; vdp: vas deferens passage into capsule gland; vds: vas deferens section.

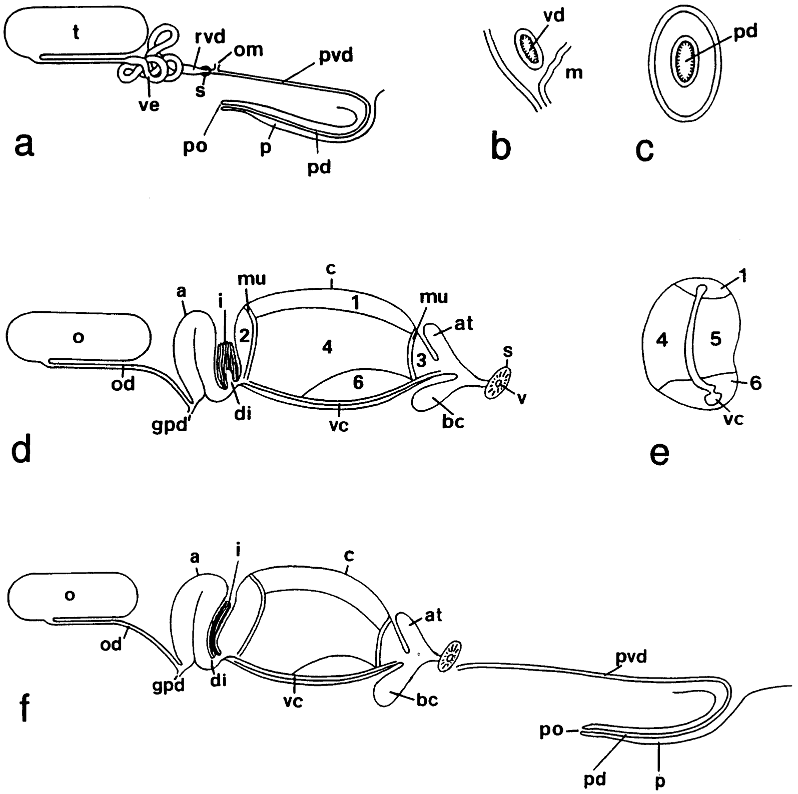

Fig. 3. *Hinia incrassata*. Schematic representation of male (a–c), female (d and e), and imposex (f) genital tracts. (a) Lateral view of male tract. (b) Transverse section of the pallial vas deferens. (c) Transverse section of penis. (d) Lateral view of female tract. (e) Transverse section of capsule gland. (f) Lateral view of imposex stage 4. Abbreviations: a: albumen gland; at: atrium, muscular vestibulum; bc: bursa copulatrix; c: capsule gland; di: duct of ingestion gland; gpd: gonopericardial duct; i: ingestion gland; m: mantle cavity; mu: transversal musculature; o: ovary; od: oviduct; om: opening into the mantle cavity; p: penis; pd: penis duct; po: opening of the penis; pvd: pallial vas deferens; rvd: renal vas deferens; s: sphincter; t: testis; v: vaginal opening; vc: ventral channel; vd: vas deferens; ve: vesicula seminalis (seminal vesicle); 1: dorsal lobe; 2: caudal lobe; 3: cephalic lobe; 4: right lobe; 5: left lobe; 6: anteroventral lobe.

3. Results

3.1. Male genital system

It consists from proximal to distal of the testis, the testicle duct, the seminal vesicle, the renal portion of the vas deferens with a sphincter, the pallial portion of the vas deferens and the penis with its duct (Fig. 3(a–c)). This is similar in all Buccinidae. Contrary to the closely related *Hinia reticulata* the pallial portion of the vas deferens consists of mainly subepithelial gland cells and a much smaller proportion of intraepithelial gland cells. Under the dissection microscope the dimensions of this glandular part of the vas deferens are not visible, but can be determined in histological slide series. The length of this glandular vas deferens section in different populations was not measured because this would have required a histological analysis of all specimens. The gland cells, whose secretions enter the vas deferens, lie under a thin muscular collar but there is no real epithelial delimitation to the surrounding tissue. For this reason the glandular part of the vas deferens is not a distinct organ.

The males of *Hinia incrassata* show considerable seasonal penis length variations which are even more pronounced and exhibit a greater am-

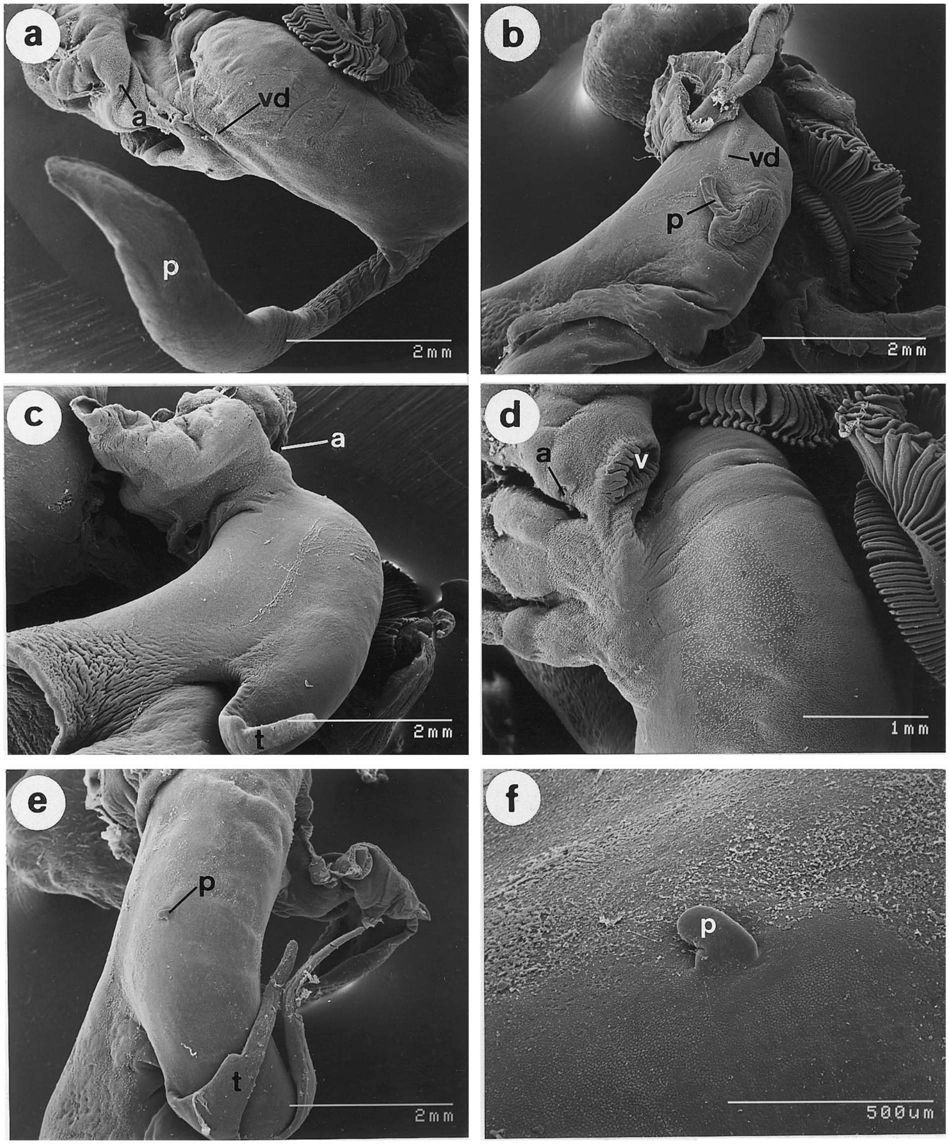

Fig. 4. *Hinia incrassata*. Scanning electron micrographs of male, normal female (imposex stage 0) and imposex stage 1a. (a) Male during period of reproduction (March). (b) Male in sexual repose (August). (c) Stage 0 (normal female). (d) Detail of (c). (e) Stage 1a. (f) Detail of (e). Abbreviations: a: anus; p: penis; t: tentacle; v: vaginal opening; vd: vas deferens.

plitude than in the colsely related species *Hinia reticulata* (comp. Fig. 4(a and b); Fig. 11(a)). These variations affect the biological indices which can be used for TBT effect monitoring purposes by assessing imposex intensities in *Hinia* populations.

3.2. Female genital system

The female reproductive system in *Hinia incrassata* exhibits the morphological and histological structure which is typical for buccinid neogastropods. It consists from proximal to distal of the ovary, the gonadal and renal sections of the oviduct separated by the aperture of the gonopericardial duct, the U-shaped albumen gland, the sperm ingesting gland with its duct which acts as a receptaculum seminis, the six-lobed capsule gland, its ventral channel and muscular vestibulum (atrium), the bursa copulatrix and the vagina (Fig. 3(d and e)).

As in most other neogastropod species the most highly developed part of the reproductive tract is the pallial section, which originates ontogenetically from an infolding of the mantle epithelium. This section contains organs for storing sperm (duct of the ingestion gland), disintegrating old sperm (ingestion gland), providing extraembryonic nourishment for the developing embryos within the capsule (albumen gland) and producing, forming and hardening the egg capsules (capsule gland) which are released via the rosette-shaped vaginal opening (Fig. 4(b)) and finally fitted to the substratum. The albumen and ingestion glands are relatively smaller than in other buccinid snails (comp. Table 1). Both organs and the capsule gland exhibit size variations comparable to that of the copulatory organ in males due to the reproductive cycle attaining maximum extensions during the breeding season between December and March (comp. Fig. 10(c)).

3.3. *Imposex classification and expression*

The imposex phenomenon is characterized by a superimposition of additional male parts, i.e. a penis and/or vas deferens, on females. Imposex development in prosobranchs can be generally described by an evolutive scheme with six stages (1–6) most of which can have multiple types (a–c; Fig. 2). *Hinia incrassata* demonstrates two different types (a and b) of imposex expression in stage 1, and only one type (Type a) in stages 2, 3, and 4. The various frequencies of normal females (stage 0) and of the imposex stages and types are given in Table 1.

3.3.1. *Stage 0*

Normal female without any male characteristics (Fig. 4(c and d)).

3.3.2. *Stage 1*

Type a: Tiny penis with no penis duct behind the right tentacle (Fig. 4(e and f)).

Type b: No penis but a short, distal vas deferens section behind the right tentacle (Fig. 5(a)).

3.3.3. *Stage 2*

Penis with a closed penis duct behind the right tentacle (Fig. 5(b)).

3.3.4. *Stage 3*

Penis with penis duct continuing in an incomplete distal vas deferens section that is growing out successively towards the vaginal opening (Fig. 5(c and d)).

3.3.5. *Stage 4*

Penis with a penis duct and a continuous vas deferens from the penis up to the vulva (Fig. 5(e)).

Stage 4 is the recent end of imposex development in *Hinia incrassata*. In contrast to many muricid gastropods there are no restrictions of fertility and the histological structure of the ovary is completely normal. Furthermore, the existence of all functioning glands in the pallial oviduct section, assisted by a normal ventral pedal gland, allows the deposition of normal egg capsules. The vaginal opening is unmodified (Fig. 5(e)) and the capability of copulation is conserved.

The vas deferens apparently develops progressively from the base of the penis to the vaginal opening. The female penis length generally increases from stage 1 to 4 and exhibits its greatest range in the stages 3a (0.1–1.5 mm) and 4 (0.2–2.0 mm). In contrast to other imposex affected species there is no indication in *Hinia incrassata* that the masculinization effect of TBT on the female genital system also results in a reduction in size of the albumen, ingestion, and capsule glands (Table 1). There is no relationship between the developed imposex stage and the measured shell and aperture height, or from parasitation or sexual maturity (Table 1).

As an additional morphological alteration of imposex development which is not covered by the evolution scheme in Fig. 2, two imposex stage 3a females with excrescences of hyperplasic tissue on the vas deferens were found, representing only a

Table 1
Hinia incrassata, morphometrical data of analysed males, imposex stages and total females

Sex or imposex stage	No. of specimens	% of total	% of females	Shell height		Aperture height		Penis length		Albumen gland		Ingestion gland		Capsule gland		% para-sited	% sexual mature
				(mm)	±S.D.	(mm)	±S.D.	(mm)	±S.D.	(mm)	±S.D.	(mm)	±S.D.	(mm)	±S.D.		
♂	736	44.8		11.28	1.74	5.79	0.88	7.45	3.06	—	—	—	—	—	—	0.27	94.4
0	487		53.8	10.42	1.21	5.34	0.68	—	—	0.64	0.31	0.39	0.35	1.67	0.57	0.41	89.7
1a	140		15.4	10.92	1.31	5.55	0.60	0.12	0.04	0.70	0.33	0.35	0.26	1.65	0.54	0.00	92.9
1b	17		1.9	10.82	1.13	5.46	0.50	—	—	0.55	0.31	0.48	0.45	1.68	0.77	0.00	76.5
2a	18		2.0	11.09	1.54	5.58	0.68	0.38	0.15	0.87	0.18	0.42	0.14	1.86	0.24	0.00	100.0
3a	205		22.6	11.40	1.36	5.70	0.56	0.48	0.32	0.80	0.29	0.36	0.17	1.81	0.50	0.49	93.2
4	39		4.3	11.46	1.34	5.70	0.60	0.78	0.36	0.88	0.31	0.39	0.17	1.88	0.50	0.00	93.0
♀	906	55.2	100.0	10.78	1.34	5.48	0.66	0.38	0.33	0.70	0.32	0.38	0.30	1.71	0.55	0.33	91.1

S.D., standard deviation.

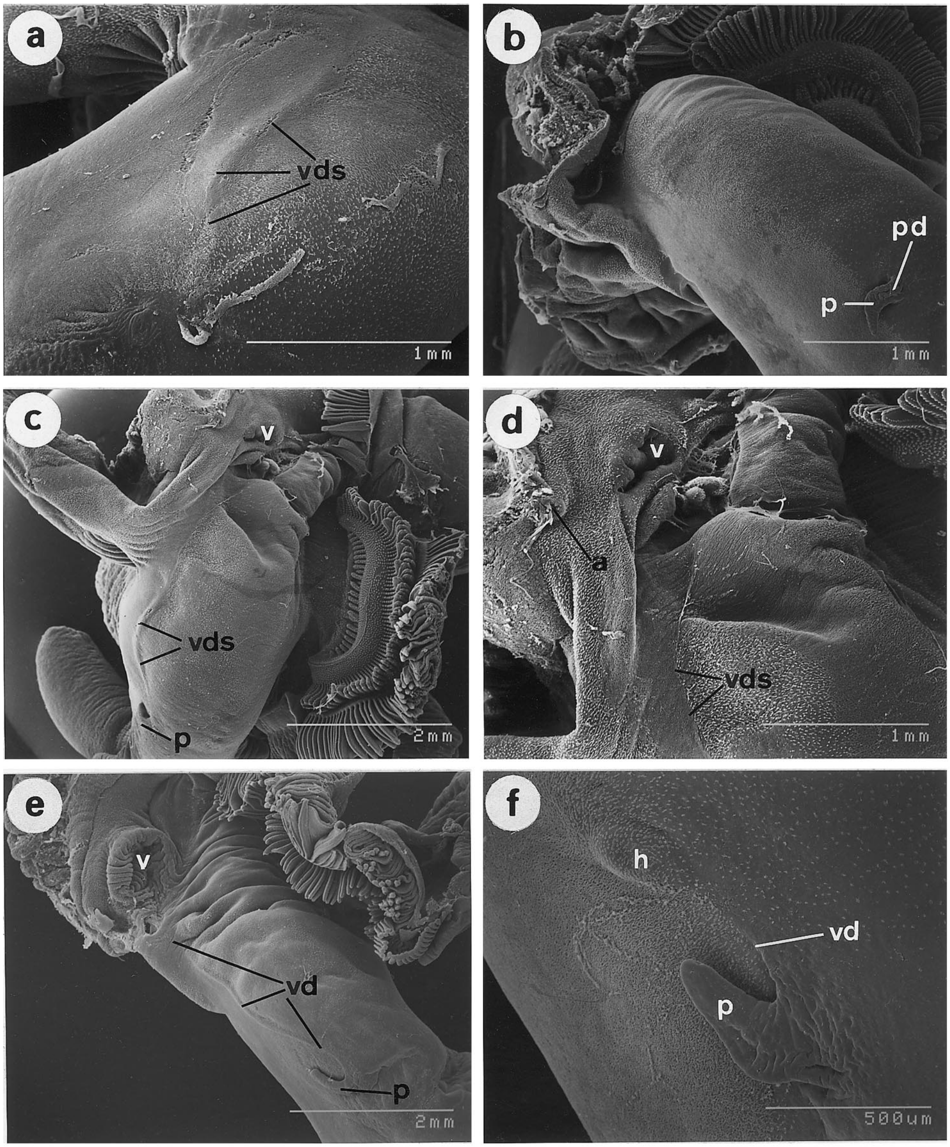

Fig. 5. *Hinia incrassata*. Scanning electron micrographs of imposex stages 1b to 4. (a) Stage 1b. (b) Stage 2a. (c) Stage 3a. (d) Detail of (c). (e) Stage 4. (f) Stage 3a with excrescences of hyperplasic tissue on the vas deferens. Abbreviations: a: anus; h: hyperplasia; p: penis; pd: penis duct; v: vaginal opening; vd: vas deferens; vds: vas deferens section.

portion of 0.33% of all analyzed females (Fig. 5(f)). This same malformation was not found in male specimens.

3.4. TBT accumulation and imposex development

Hinia incrassata accumulates TBT and DBT compounds in its natural environment. A regression analysis (Fig. 6) proves that the TBT body burden in this species increases with the TBT concentration of the ambient sea water. In areas likely to be contaminated by TBT (e.g. Roscoff harbor with 8.45–29.6 ng TBT-Sn l^{-1}; average: 15.2 ng TBT-Sn l^{-1}, $n = 24$) due to vessel activity, the body burden in *H. incrassata* is 800–1600 and 300–1000 μg DBT-Sn kg^{-1}. Even at a number of

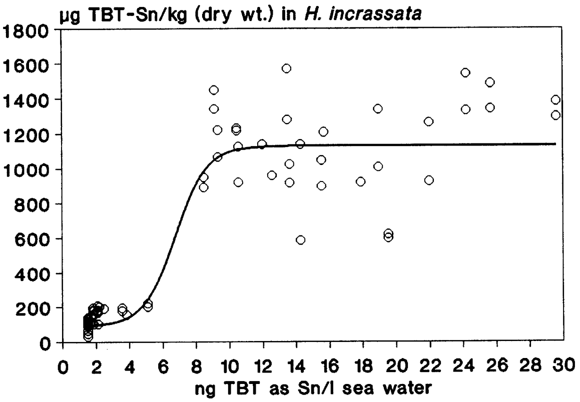

Fig. 6. *Hinia incrassata*. Relationship between tributyltin (TBT) concentration in ambient sea water and TBT body burden with calculated regression: $y = 1039/(1 + e^{(-1.05 \cdot (x - 6.77))}) + 90.2$, $n = 73$ samples, $r = 0.907$, $p < 0.0005$; Spearman rank correlation analysis: $t = 15.5$, $p < 0.0005$.

sites away from TBT sources (e.g. Beg an Fry near Locquirec with 1.35–2.10 ng TBT-Sn l^{-1}; average: 1.54 ng TBT-Sn l^{-1}, $n = 8$) the organotin body burden attains maximum values of 70 and 30 µg DBT-Sn kg^{-1}.

The calculated biological concentrations factors (BCF; dry tissue/water) for TBT ($4.68 \cdot 10^4$ to $1.22 \cdot 10^5$) and DBT ($3.21 \cdot 10^4$ to $4.77 \cdot 10^4$) are presented in Table 2.

For all analyzed populations of *Hinia incrassata* three imposex indices (average female penis length, VDS and RPL index) were calculated and a regression analysis between imposex intensities and TBT concentrations in ambient water (Fig. 7(a–c)) or TBT body burden (Fig. 8(a–c)) was performed. The data in Figs. 7 and 8 show clearly that the imposex indices increase with ambient TBT pollution. The threshold concentration for imposex development in this species is below the detection limit of 1.5 ng TBT-Sn l^{-1} of the analytical method employed. With the exception of Méan Mélen, a reference station with an average TBT concentration in the water of < 1.5 ng as Sn l^{-1} ($n = 20$), no *H. incrassata* populations without any signs of imposex development were found.

Table 2
Hinia incrassata, Biological concentration factors (bcf) for TBT and DBT in dependence of aqueous TBT concentrations

ng TBT as Sn l^{-1} in sea water	bcf	
	TBT	DBT
2	$1.22 \cdot 10^5$	$4.77 \cdot 10^4$
4	$1.09 \cdot 10^5$	$4.61 \cdot 10^4$
5	$1.04 \cdot 10^5$	$4.54 \cdot 10^4$
8	$9.07 \cdot 10^4$	$4.32 \cdot 10^4$
14	$7.22 \cdot 10^4$	$3.95 \cdot 10^4$
20	$6.00 \cdot 10^4$	$3.64 \cdot 10^4$
30	$4.68 \cdot 10^4$	$3.21 \cdot 10^4$

In areas where the TBT concentrations in water exceed 8 ng as Sn l^{-1} (i.e. with TBT body burdens of ≥ 500 µg as Sn kg^{-1}) all analyzed imposex parameters are equilibrated and do not attain higher values even if TBT concentrations in coastal waters increase further. As shown in Figs. 7 and 8, the average female penis length and the RPL index exhibit a greater deviation of data points from calculated concentration-effect-equations than the VDS index, which seems therefore to be more suited for biological effect monitoring purposes. Further considerations which favour the VDS index are given in Section 3.5.

3.5. Interspecific comparison of Hinia incrassata with Nucella lapillus and Hinia reticulata

The dogwhelk *Nucella lapillus* was one of the first imposex affected prosobranch species to be used for TBT effect monitoring. Because the data base concerning organotin accumulation and TBT sensitivity in dogwhelks is very broad, they are used as a reference species for comparison with potential new indicators of TBT pollution. Another question of interest is a direct interspecific comparison of the TBT sensitivity between *Hinia incrassata* and the closely related *Hinia reticulata* as they very often share the same habitat on a given piece of coastline.

For this interspecific comparison between *Hinia incrassata* and *Nucella lapillus* or *Hinia reticulata*, sympatrically living populations were analyzed and different imposex parameters determined. The least-squares analysis revealed a sigmoid equation to be the best adapted correlation for the interspecific comparison of the average female penis length, RPL and VDS index in the investi-

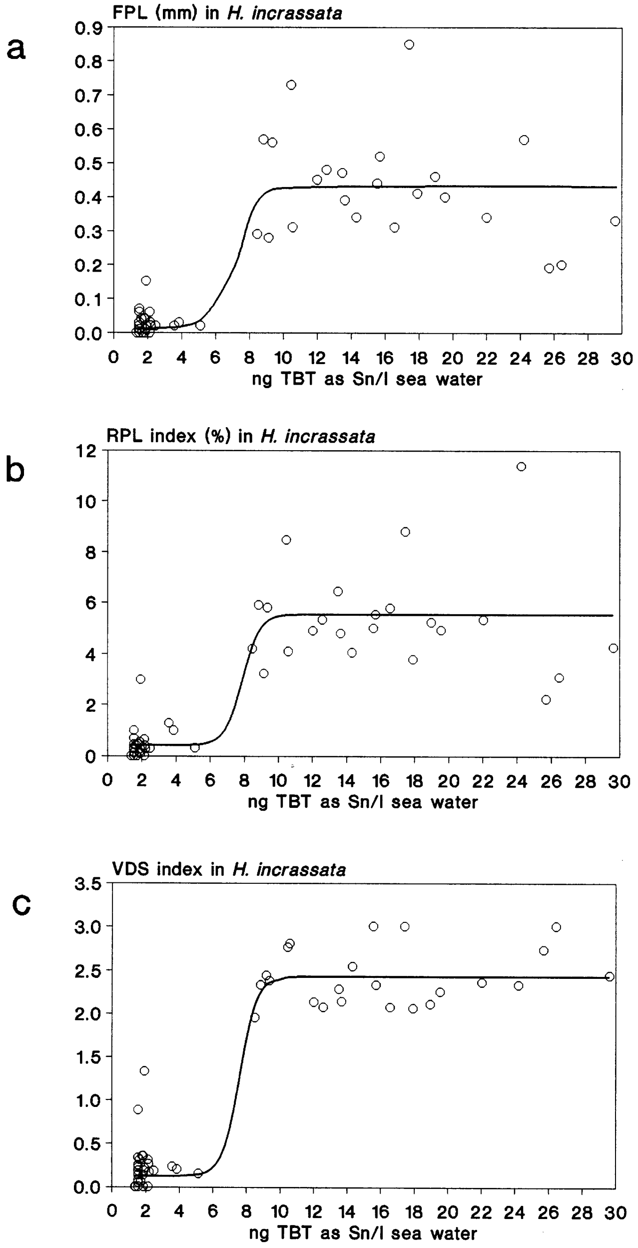

Fig. 7. *Hinia incrassata*. Relationship between tributyltin (TBT) concentrations in ambient sea water and imposex indices with calculated regressions. (a) Average female penis length (FPL): $y = 0.407/(1 + e^{(-33.3 \cdot (x-8.43))}) + 0.023$, $n = 54$ samples, $r = 0.884$, $p < 0.0005$; Spearman rank correlation analysis: $t = 9.04$, $p < 0.0005$. (b) Relative penis length (RPL) index: $y = 5.13/(1 + e^{(-1.96 \cdot (x-7.89))}) + 0.418$, $n = 53$ samples, $r = 0.868$, $p < 0.0005$; Spearman rank correlation analysis: $t = 9.67$, $p < 0.0005$. (c) Vas deferens sequence (VDS) index: $y = 2.31/(1 + e^{(-1.95 \cdot (x-7.59))}) + 0.126$, $n = 54$ samples, $r = 0.939$, $p < 0.0005$; Spearman rank correlation analysis: $t = 9.10$, $p < 0.0005$.

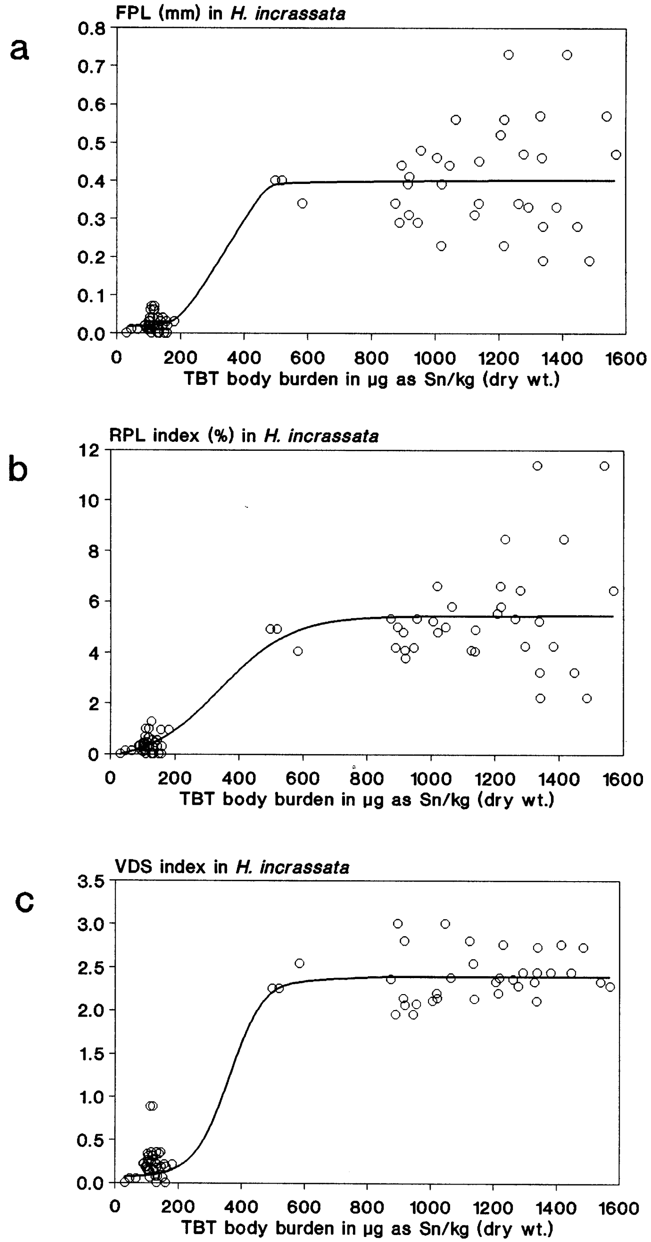

Fig. 8. *Hinia incrassata*. Relationship between tributyltin (TBT) body burden and imposex indices with calculated regressions. (a) Average female penis length (FPL): $y = 0.374/(1 + e^{(-0.031 \cdot (x-334))}) + 0.022$, $n = 76$ samples, $r = 0.895$, $p < 0.0005$; Spearman rank correlation analysis: $t = 10.6$, $p < 0.0005$. (b) Relative penis length (RPL) index: $y = 5.78/(1 + e^{(-0.009 \cdot (x-341))}) + 0.331$, $n = 76$ samples, $r = 0.874$, $p < 0.0005$; Spearman rank correlation analysis: $t = 11.1$, $p < 0.0005$. (c) Vas deferens sequence (VDS) index: $y = 2.33/(1 + e^{(-0.012 \cdot (x-361))}) + 0.069$, $n = 76$ samples, $r = 0.968$, $p < 0.0005$; Spearman rank correlation analysis: $t = 11.1$, $p < 0.0005$.

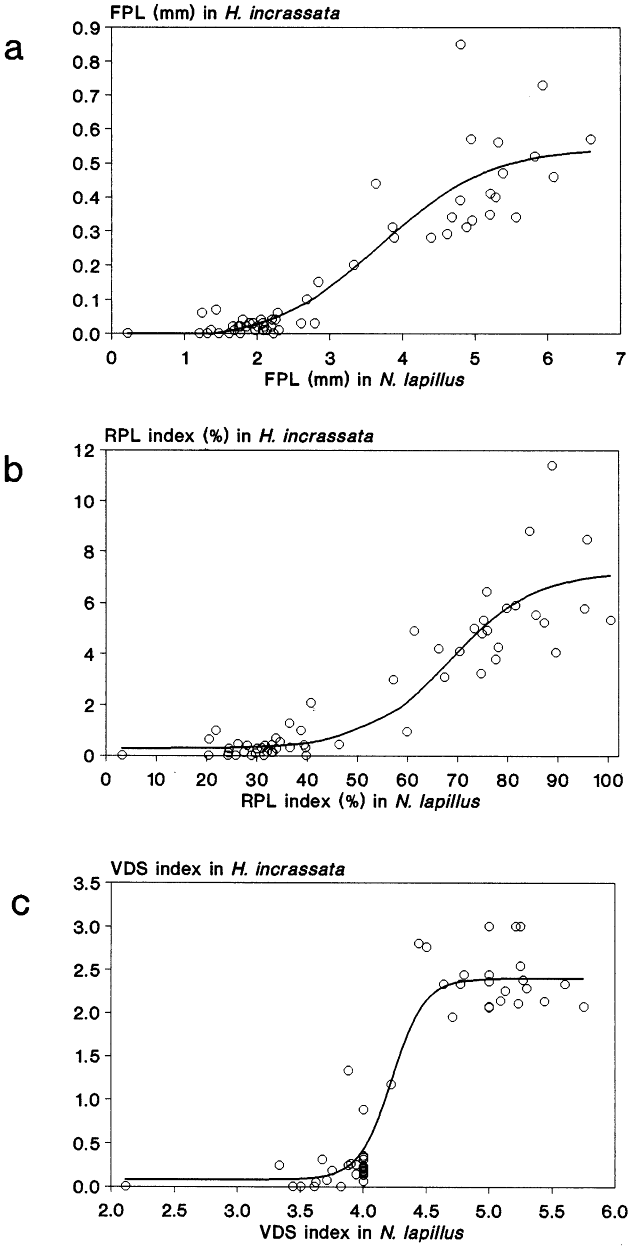

Fig. 9. Relationship between imposex indices in *Nucella lapillus* and *Hinia incrassata* in sympatrically living populations with calculated correlations. (a) Average female penis length (FPL): $y = 0.571/(1 + e^{(-1.34 \cdot (x-3.71))}) - 0.024$, $n = 56$ samples, $r = 0.916$, $p < 0.0005$; Spearman rank correlation analysis: $t = 12.2$, $p < 0.0005$. (b) Relative penis length (RPL) index: $y = 6.94/(1 + e^{(-0.120 \cdot (x-68.5))}) + 0.277$, $n = 56$ samples, $r = 0.912$, $p < 0.0005$; Spearman rank correlation analysis: $t = 12.4$, $p < 0.0005$. (c) Vas deferens sequence (VDS) index: $y = 2.32/(1 + e^{(-7.61 \cdot (x-4.23))}) + 0.084$, $n = 56$ samples, $r = 0.921$, $p < 0.0005$; Spearman rank correlation analysis: $t = 10.3$, $p < 0.0005$.

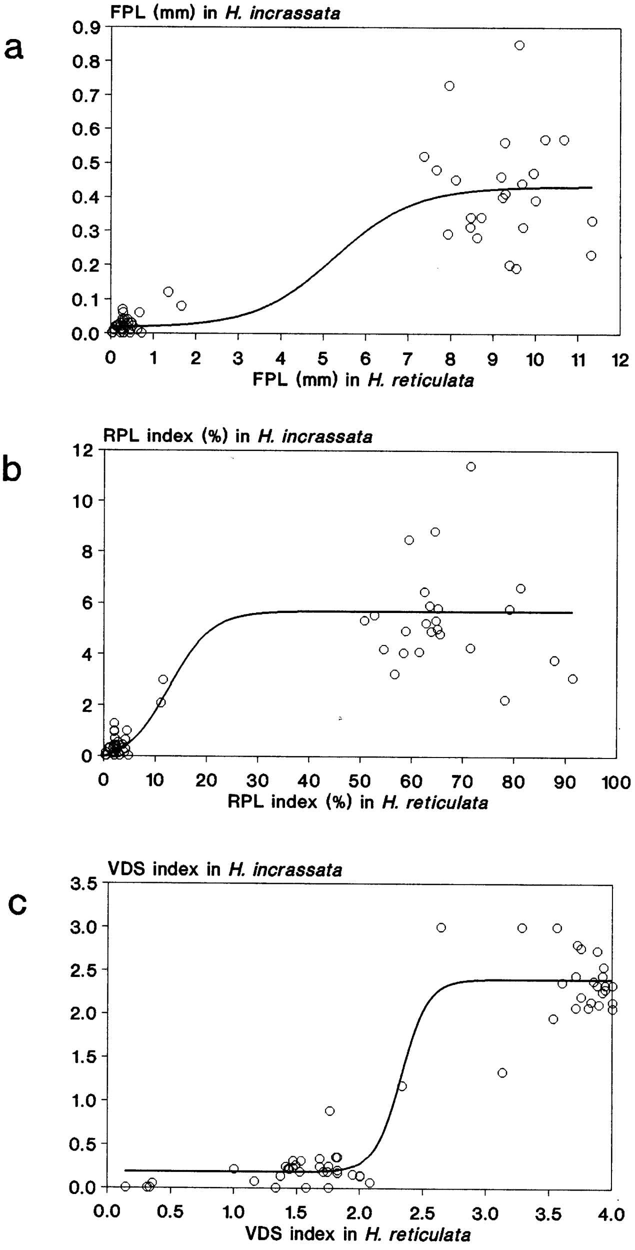

Fig. 10. Relationship between imposex indices in *Hinia reticulata* and *Hinia incrassata* in sympatrically living populations with calculated correlations. (a) Average female penis length (FPL): $y = 0.415/(1 + e^{(-5.96 \cdot (x-1.06))}) + 0.017$, $n = 58$ samples, $r = 0.887$, $p < 0.0005$; Spearman rank correlation analysis: $t = 10.0$, $p < 0.0005$. (b) Relative penis length (RPL) index: $y = 5.97/(1 + e^{(-0.242 \cdot (x-12.7))}) + 0.281$, $n = 57$ samples, $r = 0.892$, $p < 0.0005$; Spearman rank correlation analysis: $t = 9.71$, $p < 0.0005$. (c) Vas deferens sequence (VDS) index: $y = 2.22/(1 + e^{(-9.42 \cdot (x-2.33))}) + 0.185$, $n = 58$ samples, $r = 0.945$, $p < 0.0005$; Spearman rank correlation analysis: $t = 9.41$, $p < 0.0005$.

gated species (Figs. 9 and 10). All correlations were found to be highly significant ($p < 0.0005$).

Low TBT exposure results in a higher increase of all TBT effect monitoring parameters in both *Nucella lapillus* and *Hinia reticulata* compared to *Hinia incrassata*. But even in areas with only a slight TBT exposure, females of this less sensitive species develop obvious imposex characteristics. On severely polluted coasts the further increase of all imposex parameters is retarded in *H. incrassata* and thus the values for the female penis length, the RPL and VDS index are higher in *N. lapillus* and *H. reticulata*.

It has already been pointed out that the VDS index seems to be more suited for biological effect monitoring purposes than either the RPL index or the average female penis length because the latter two exhibit a greater deviation of data points from calculated concentration-effect-equations (see also Discussion). But one of the main reasons to favour the VDS index is the fact that this parameter is not affected by seasonal changes. In Fig. 11 the variation of the average male and female penis length in *Hinia incrassata* and the resulting imposex indices are demonstrated for Roscoff harbour over a period of three years (March 1989–March 1992). Not only at this but also at all other analyzed stations the female penis length (FPL) was more or less constant during the investigation period. In contrast, the average male penis length attains maximum values in early spring and minima during male sexual repose in late summer (Fig. 11(a)). Because the RPL and also the RPS index relate the comparably constant average female penis length to the seasonally varying extension of the same organ in males, both indices exhibit seasonal changes (Fig. 11(b)). On the other hand, the VDS index remains constant during this three-year period and actually exhibits even lower randomly caused variations than the FPL.

4. Discussion

For the most part, our results concerning the normostructure of the male and female genital system confirm the description Fretter's (Fretter, 1941) of *Hinia reticulata* as well as the results for *H. incrassata* found by Johansson (1957) and Houston (1976). Therefore, we did not include a complete description of the male and female genital tracts, but have chosen rather to enumerate the differences of our findings to the older analyses, as well as to accentuate some further important facts. The main differences to older analyses are those results which demonstrate the considerable size variations of the pallial sex organs in males and females (comp. Fig. 11(a and c)). Additionally, the existence of a small albumen and ingestion gland, which were not found by Houston (1976), was demonstrated in all analyzed females.

This attempt is the first detailed description of imposex development in *Hinia incrassata* documented by scanning electron micrographs and histological photographs. Most of the imposex stages and types represented in Fig. 2 have been found in the dogwhelk *Nucella lapillus* which is characterized by the greatest variability in imposex expression (Oehlmann et al., 1991; Oehlmann, 1994). The number of stages in *H. incrassata* is comparably low, indicated by the bold lines in Fig. 2. As in most other neogastropod species, e.g. *Ilyanassa obsoleta* (Smith, 1971), *Ocenebra erinacea* (Oehlmann et al., 1992), *Murex brandaris*, *M. trunculus* (Oehlmann, 1994), *Hinia reticulata* (Stroben, 1994), *Buccinum undatum* and *Colus gracilis* (Fioroni et al., 1991a,b), the vas deferens apparently develops progressively from the base of the penis to the vaginal opening. Only in very few species, e.g. *N. lapillus* (Gibbs et al., 1987; Oehlmann et al., 1991), can some imposex stages be found with the female vas deferens developing from two centres (Fig. 2: stages 1c, 2b,c, 3c). There is an infolding of the vas deferens close to the genital papilla surrounding the vulva. The second centre is behind the right tentacle. From this position the distal vas deferens section develops to meet and fuse with the proximal section.

The final point of imposex development for the analyzed specimens is stage 4. Therefore TBT-induced sterilization was not found in this buccinid species, but might occur at higher environmental TBT concentrations. Moreover, it may exist some changes in fecundity before functional sterilization (Oehlmann, 1994). The latter is common in other prosobranch species, mainly muricids, and is either

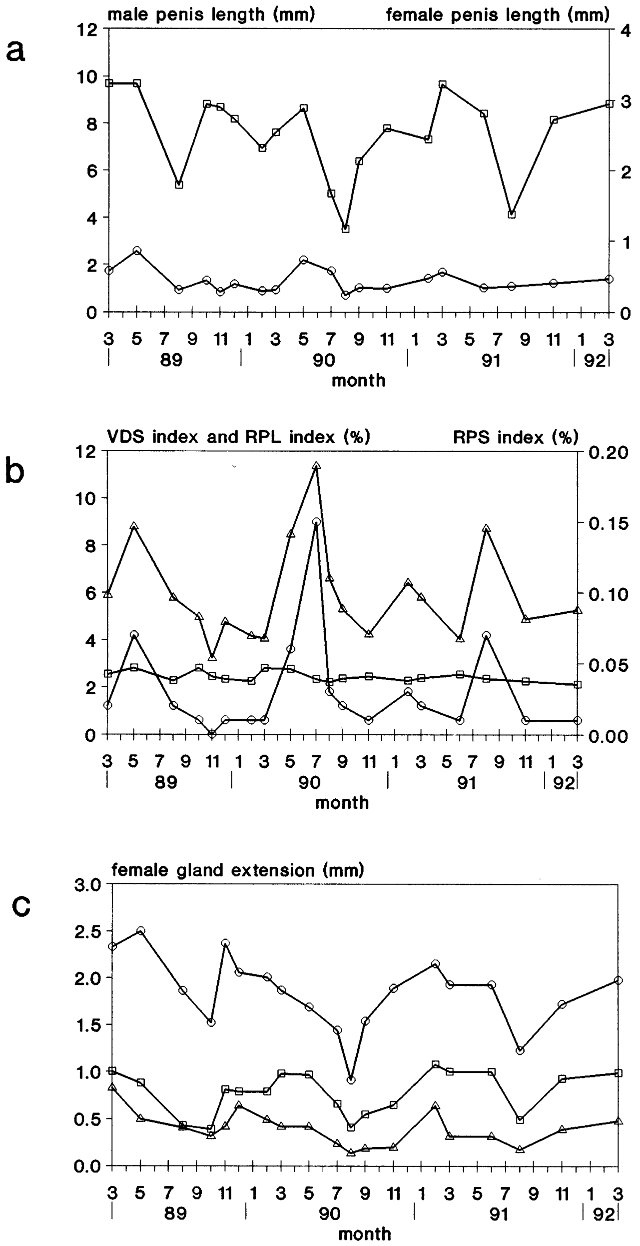

Fig. 11. *Hinia incrassata*. Variation of average penis lengths (a), imposex indices (b) and extensions of female pallial sex glands (c) at Roscoff harbour (Brittany, France) between March 1989 and March 1992. (a) Average male (square) and female (o) penis length. (b) Vas deferens sequence (VDS, square), relative penis length (RPL, triangel) and relative penis size (RPS, o) index. (c) Height of albumen (square), ingestion (triangel) and length of capsule gland (o).

caused by a blockade of the pallial oviduct, as described for *Nucella lapillus* (Gibbs et al., 1987; Oehlmann et al., 1991), *Nucella lima* (Short et al., 1989), *Nucella lamellosa* (Bright and Ellis, 1990), and *Thais haemastoma* (Spence et al., 1990) or by a split bursa copulatrix and capsule gland as in *Ocenebra erinacea* (Gibbs et al., 1990; Oehlmann et al., 1992). Thus either deposition of egg capsules or copulation and egg capsule formation in the oviduct are prevented. *Ocinebrina aciculata* is the only species which exhibits both forms of sterilization (Oehlmann et al., 1996).

For *Hinia incrassata* no indications of a sex change as it occurs in *Nucella lapillus* (Gibbs et al., 1988; Oehlmann et al., 1991) or *Ocinebrina aciculata* (Oehlmann et al., 1996) were observed. The ovary, female pallial glands, and vaginal opening in *H. incrassata* are unaffected by imposex development. Therefore, copulation and deposition of egg capsules are possible, and consequently restrictions of the fertility are not obvious. Even at highly polluted sites (e.g. Roscoff harbor) *Hinia* egg capsules have been found since the beginning of this study in 1988.

The measured BCF values for *Hinia incrassata* of $4.68 \cdot 10^4$ to $1.22 \cdot 10^5$ for TBT are in the same range as determined for other carnivorous prosobranchs, e.g. *Trivia arctica* and *Trivia monacha* (Stroben et al., 1992a), *Ocenebra erinacea* (Oehlmann et al., 1992) and *Hinia reticulata* (Stroben et al., 1992b), also a scavenger. Other predators which feed on mussels, barnacles or polychaetes, like *Nucella lapillus* (Bryan et al., 1987; Oehlmann, 1994) and especially *Ocinebrina aciculata* (Oehlmann et al., 1996) exhibit higher BCF values of up to $3.08 \cdot 10^5$ for TBT while herbivorous species, like *Littorina littorea*, show values between $1.17 \cdot 10^3$ and $3.46 \cdot 10^4$ depending on the aqueous concentration (Bryan and Gibbs, 1991; Matthiessen et al., 1995; Bauer et al., 1997). The main reason for these differences is the TBT contamination of the diet which affects the TBT body burden of snails in direct relation to the trophic level they take through the food web. Bryan et al. (1989) concluded that the diet contributes less than half of the body burden of TBT found in natural populations of *Nucella lapillus*. Stickle et al. (1990) found that in *Nucella lima*, TBT bioaccumulation from food and resulting imposex responses are equivalent to low level exposures to TBT in seawater. In *Hinia reticulata* the food contributes more than half of the TBT body burden (Stroben et al., 1992c).

As for other imposex affected prosobranch species (Fioroni et al., 1991a), the spatial distribution of imposex in relation to TBT point sources gives evidence that *Hinia incrassata* has potentials as a biological effect monitor of TBT contamination. This is supported by the significant regressions between TBT concentrations in ambient water or in the snail tissues and the three imposex indices (Figs. 7 and 8) which are comparable to findings for other prosobranch species (Bright and Ellis, 1990; Oehlmann et al., 1992, 1996; Stroben et al., 1992a,b,c; Bauer et al., 1997). This allows then the assessment of coastal TBT contamination by determining imposex intensities in natural prosobranch populations. The VDS index is recommended as the best index for TBT effect monitoring purposes because the deviation of data points from calculated concentration-effect-equations is lower than for the other indices tested and the VDS is unaffected by changes related to the season (Stroben et al., 1996). Due to its widespread distribution throughout the Mediterranean *H. incrassata* is a suitable TBT effect monitoring species in a geographical area where—with the exception of the relatively scarce species *Hexaplex* (*Murex*) *trunculus* and *Murex brandaris* (Oehlmann, 1994; Axiak et al., 1995)-no other prosobranchs are available for this purpose. This offers the possibility of performing large-scale TBT surveys in the Mediterranean also as planned by OSPARCOM (Oslo and Paris Commissions) in its entire convention area (Oslo and Paris Commissions, 1996).

For such large-scale surveys an interspecific comparison between the newly introduced and already established monitoring species is desirable. The results (Figs. 9 and 10) show clearly that *Hinia incrassata* exhibits a lower TBT sensitivity, measured as imposex intensities in sympatrically living populations, than either the dogwhelk *Nucella lapillus* or the closely related *Hinia reticulata*. But even in this species imposex is a very sensitive biological marker and its development is induced at ambient TBT concentrations of below 1.5 ng as Sn l^{-1}. The results reported by Bacci and Gaggi (1989) and Alzieu et al. (1991) show that in all analyzed regions of the western Mediterranean the TBT concentrations in coastal waters are well above this threshold.

France was the first European country to draw up TBT regulations by banning the use of TBT antifoulings on small boats (length < 25 m) in

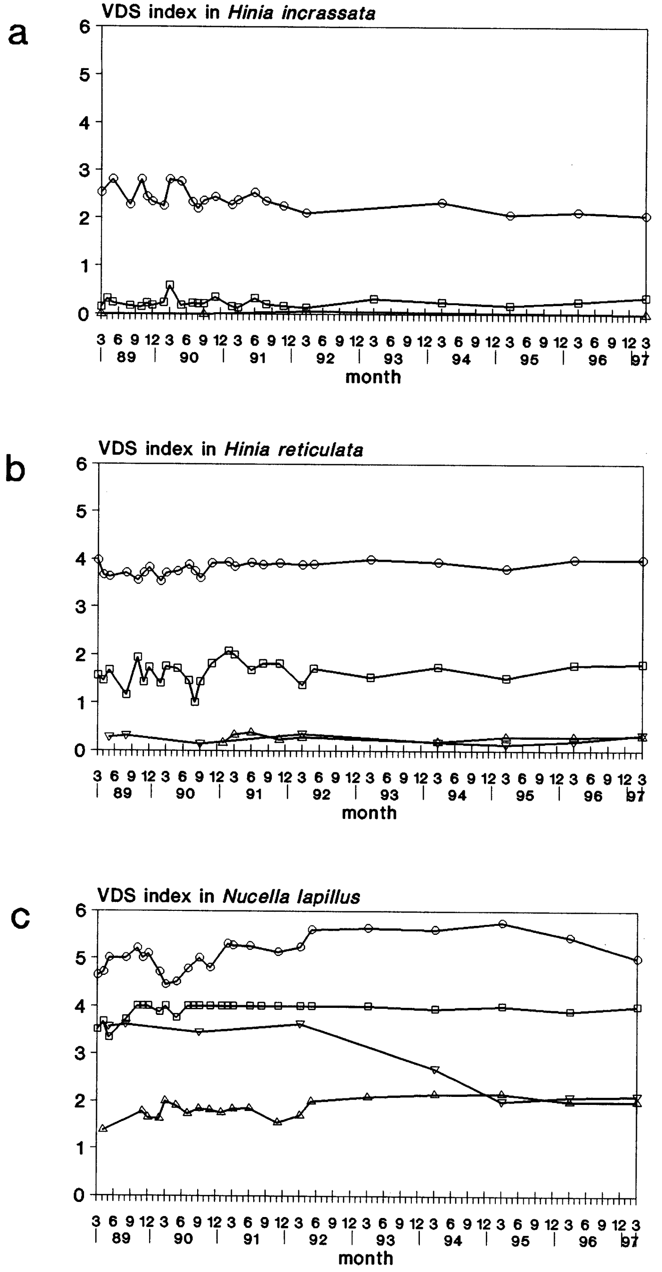

Fig. 12. Vas deferens sequence (VDS) index results of a temporal trend monitoring with *Hinia incrassata* (a), *Hinia reticulata* (b) and *Nucella lapillus* (c) at four different stations in Brittany (France) between March 1989 and March 1997: (o) Roscoff harbour, (square) Ile Verte at Roscoff, (turned triangel) Beg an Fry, (upright triangel) Méan Mélen.

1982, five years before Ireland and the United Kingdom and seven years before the rest of the European Union implemented identical legislative controls. In spite of recent reports that TBT concentrations in coastal waters and imposex intensities in the UK are generally in decline (e.g. Evans et al., 1995) evidence was found that at least in areas adjacent to harbours in France (Oehlmann et al., 1993; Huet et al., 1996), Ireland (Minchin et al., 1995, 1996, 1997), Scotland (Harding et al., 1997) and Germany (Bauer et al., 1997), organotin concentrations are still high enough to endanger sensitive species. Fig. 12 presents the results of a temporal trend monitoring over a period of eight years using the VDS index in *Hinia incrassata*, *Hinia reticulata* and *Nucella lapillus* collected at four different stations representing a wide spectrum of contamination levels. The three charts show once again that *N. lapillus* is the most TBT sensitive of these three species and that *H. incrassata* exhibits the lowest sensitivity. Furthermore, it is obvious that—with the exception of the results for *N. lapillus* at Beg an Fry (Fig. 12(c))—imposex intensities are not reduced and therefore amelioration of coastal TBT exposure is not assessable. These findings are supported by chemical analyses of water samples and snail tissues. Consequently, further controls are necessary for the future.

There is an EU concern over the continued usage of TBT as an antifoulant and the IMO (International Maritime Organization) has proposed a ban on its use on all vessels less than 50 m in overall length (Anon, 1994). Currently about 69% of ships are being painted with TBT antifoulings (Ambrose, 1994). The impact of these paints on molluscs shows clearly that TBT still has a serious and quantifiable effect. Its further elimination will take many years and requires a complete ban on the use of organotins in marine paints.

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