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Morphological expression and different stages of imposex in *Hexaplex trunculus* (Neogastropoda: Muricidae) from tunisian coasts

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Abstract: Muricids are gonochoric gastropods, when exposed to the tributyltin (TBT), females start developing *vas deferens* and penis (imposex). The first morphological expressions of imposex are observed at low to moderate maritime traffic sites. In *Hexaplex trunculus* of Tunisian coasts, observations on imposex highlight 4 new early development stages (1d, 2d, 2d' and 3d). Stage 1d presents a small segment of a *vas deferens*, observed halfway between the expected position of the future penis and the vagina. This stage progresses toward stage 2d' (portion of the *vas deferens* with a penis bud), or toward stage 2d (a long portion of the *vas deferens*) then toward 3d (incompletely formed *vas deferens* attached to a penis bud). The stage 3b, previously described in *Hinia* (*Nassarius*) *reticulata*, was also observed in *H. trunculus*. Stages 3d and 3b progress gradually leading to stage 4d and stage 4. In order to confirm these observations, experiments of transplantation and contamination *in vivo* by the TBT were carried out. The results showed that both the transplanted and the treated specimens by TBT express initially a portion of the *vas deferens* followed by the development of a penial bud. Therefore, additional information is given for the known classification scheme of imposex in *H. trunculus* and some prosobranch species.

Resumé : *Expression morphologique et stades de développement de l'imposex chez* Hexaplex trunculus *(Neogastropoda : Muricidae) des côtes tunisiennes.* Les Muricidés sont des gastéropodes gonochoriques dont les femelles, une fois exposées au tributyltin (TBT), commencent à développer un tractus génital mâle (imposex). Les premières expressions morphologiques de l'imposex sont mises en évidence dans les stations faiblement et moyennement fréquentées par les bateaux. Chez *Hexaplex trunculus* des côtes tunisiennes, nous avons mis en évidence 4 nouveaux stades de développement qui sont 1d, 2d, 2d' et 3d. Le premier stade 1d est caractérisé par la présence d'un petit segment du canal déférent différencié au niveau de la partie moyenne séparant l'emplacement du pénis et l'orifice de ponte. Ce stade évolue pour donner le stade 2d' (segment du canal déférent avec un petit pénis) ou bien le stade 2d (long canal déférent) puis le stade 3d (canal déférent incomplet se terminant par un bourgeon pénial). Le stade 3b décrit chez *Hinia* (*Nassarius*) *reticulata* est observé

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chez *H. trunculus*. Les stades 3d et 3b progressent graduellement pour arriver au stade 4d puis au stade 4. Afin de confirmer nos résultats, des expériences de transplantation et de contamination *in vivo* par le TBT ont été effectuées. Les résultats ont confirmé que les spécimens transplantés et contaminés *in vivo* différencient, en premier lieu, un petit segment du canal déférent au niveau moyen de l'espace séparant l'emplacement du pénis et l'ouverture vaginale, le bourgeon pénial apparaît ultérieurement. De nouvelles informations sont ainsi ajoutées au schéma de classification de l'imposex connu chez *H. trunculus* et chez d'autres espèces de prosobranches.

Keywords: Imposex ● Tributyltin (TBT) ● Transplantation ● Laboratory exposure ● *Hexaplex trunculus* ● Tunisia

Introduction

Imposex was observed for the first time in the U.K. in 1969 (Blaber, 1970). It was detected in wild populations of the muricid gastropod *Nucella lapillus* (L., 1758) collected from an intertidal area of the British coast (Blaber, 1970). Presently, imposex has now been observed in about 150 gastropod species (Schulte-Oehlmann et al., 2000). Variations in the intensity of this phenomenon were detailed in *N. lapillus* by Gibbs et al. (1987). These authors established a scale comprising six stages based on both the development intensity of the *vas deferens* and the penis (the VDS index). VDS-index was modified by some authors to include other gastropod species such as *Hinia reticulata* (L., 1758) (Stroben et al., 1992), *Buccinium undatum* (L., 1758), *Neptunea antiqua* (L., 1758) (Jakob & Jacobsen, 2002) and *Hexaplex trunculus* (L., 1758) (Axiak et al., 1995; Terlizzi et al., 1998, 2004). Fioroni et al. (1991) summarized the morphological aspects of pseudo-hermaphrodism (imposex) as observed in 69 species. Several scientists attributed imposex to the presence of TBT in the environment (Smith, 1971; Bryan & Gibbs, 1991; Bettin et al., 1996). Based on laboratory and field observations, Bettin et al. (1996) and Gibbs & Bryan (1986) proposed a relationship between TBT exposure expressed as $ng.L^{-1}$ of tin in water and the development of genital tract.

The first observations of imposex in *H. trunculus* were shown by Martoja & Bouquegneau (1988). Other descriptions were given by Axiak et al. (1995), Terlizzi et al. (1998), Rilov et al. (2000), Pellizato et al. (2004). The morphological aspects of imposex in *H. trunculus* were described by Axiak et al. (1995) and by Terlizzi et al. (1999) who clarified the last stages of the classification proposed by Stroben et al. (1992) and Axiak et al. (1995) for *H. reticulata* and *H. trunculus*, respectively.

In Tunisia, imposex in *H. trunculus* was observed in 2002 (Lahbib et al., 2004, Trigui El Menif et al., 2007). According to these authors, imposex was studied in 19 separate stations on Tunisian coasts. Microscopic examination of the soft bodies of *H. trunculus* females revealed that at 7 stations the imposex rate was 100%. The average *vas deferens* length was about 17.5 mm while the average length of the penis recorded in these 7 stations varied from 3.54 to 8.24 mm. The VDS-index increased from 3.73 to 4.24. No imposex was observed at 2 stations. The examination of specimens from the other 10 stations revealed that the imposex rate ranged from 3.7 to 93%. The length interval of the *vas deferens* varied from 1.2 mm to 17.5 mm. The VDS index changed from 0.04 to 3.32 and the penis length varied from 0 to 1.06 mm.

Terlizzi et al. (1998) showed the penis as the first sign of imposex in *H. trunculus* and confirmed that the *vas deferens* appeared later. However, Trigui El Menif et al. (2007) observed *vas deferens* without penis in some abnormal females.

The aim of this study is to describe different aspects of the development of this abnormality in the females collected in Tunisian coasts and precise stages succession using the transplantation *in situ* and laboratory experiment.

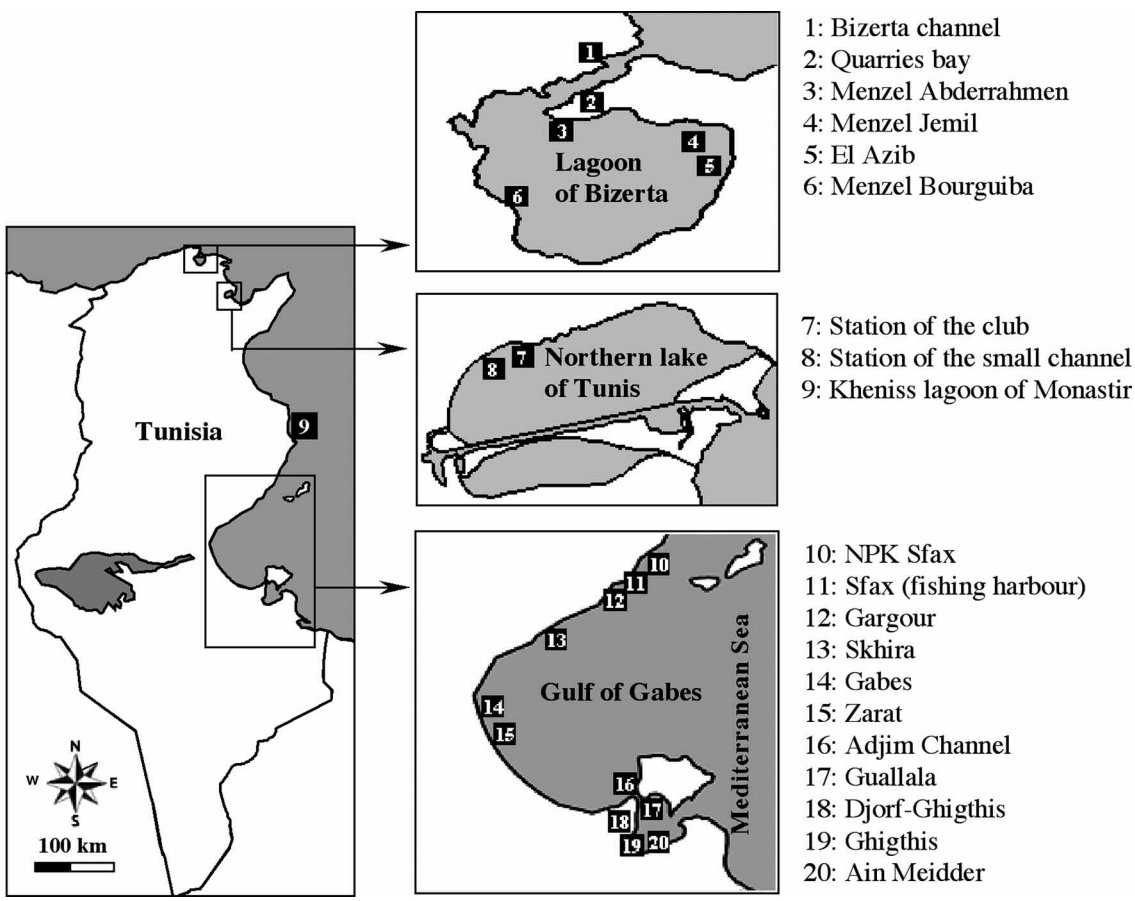
Material and Methods

Individuals of *H. trunculus*, with shell sizes from 40 to 60 mm in height were collected between March and July 2004 from Tunisian coastal waters. Total of 20 sites were sampled according to the intensity of the maritime traffic,

Figure 1. *Hexaplex trunculus.* Sampling sites along Tunisia Mediterranean waters.
Figure 1. *Hexaplex trunculus.* Stations d'échantillonnage le long de la côte tunisienne.

Table 1. *Hexaplex trunculus.* Description of sampling sites for imposex monitoring.
Tableau 1. *Hexaplex trunculus.* Description de l'état des sites de prélèvement.

Stations	Boating/shipping activities	Relevant features
1. Bizerta channel	very high	Merchant ship and war ship daily traffics, frequent visits by tourist cruises and pleasure boasts mostly during summer
2. Quarries Bay	high	Daily warship traffics and Some passing fishing boats
3. Menzel Abderrahmen	medium	Frequented by fishing boats
4. Menzel Jemil	very low	Few passing fishing boats
5. El Azib	very low	Few passing fishing boats
6. Menzel Bouguiba	high	Ship repair and paint station
7. Station of the club	null	No traffic
8. Station of the small channel	null	No traffic
9. Kheniss lagoon	null	Few passing fishing boats
10. NPK Sfax	high	Paint ship station
11. Sfax (fishing harbour)	Very high	Frequented by big fishing boats
12. Gargour	low	Some fishing boats
13. Skhira	high	Frequented by Oil tanker
14. Gabes	high	Frequented by big fishing boats
15. Zarat	low	Few passing fishing boats
16. Adjim channel	high	Daily car-ferries traffics
17. Guallala	low	Some passing fishing boats
18. Djorf -Gigthis	low	Some passing fishing boats
19. Gigthis	medium	Frequented by fishing boats
20. Ain Meider	very low	Few passing fishing boats



from Bizerta to Djerba (Table l & Fig. 1). In the laboratory, collected specimens (N = 1498) were killed by freezing. *H. trunculus* shells were measured to the nearest 0.1 mm, using a vernier calliper and then broken and the soft tissues carefully removed. The mantles were longitudinally cut in order to observe the pallial oviduct in females. Sex of individual gastropods was determined according to the presence or absence of the capsule gland and vagina. Normal females were separated from abnormal ones by using a binocular dissecting microscope. The region separating the penis site from the spawning orifice was examined under the microscope in order to follow the development intensity of the *vas deferens*. The classification schema established by Stroben et al. (1992) for *H. reticulata,* by Jakob & Jacobson (2002) for *N. antiqua* and by Terlizzi et al. (1999) for *H. trunculus* were considered in this work.

In addition, a transplantation experiment was carried out using 600 specimens (with shell of 40 to 60 mm height) collected from the northern part of Tunis lagoon in July 2004 (Station 8, Fig.1). The imposex frequency was about 13% (with 72% at stage 0, 25% at stage 1 and 3% at stage 2). The individuals (N = 600) were tagged by drilling the shell opening and tying a rubber ring with a fishing line (Fig. 2). Subsequently, tagged specimens were released nearby the Cereals Office station in the Bizerta channel (Station 1, Fig.1, where there is a 100% of imposex rate: stage 4-5), a sheltered area with a mean depth of 2 m and limited by two dams that inhibit the movement of specimens. In each recapture operation, total of 15 to 32 tagged females were selected to examine the tissue region between the penis and the vagina. This procedure was repeated each two months according to the slow increase of the imposex incidence and also to obtain a representative number of tagged snails during the experiment period. For calculate the needed imposex time development, a supplementary subsample was taken in December 2004 when the imposex incidence was increased to 100%. The last sampling was achieved in March 2005**.**

The quantification of imposex impact was determined using percentage occurrence of the abnormality in females (i.e., the proportion of females with imposex compared to the total number of females in the sample), the average female *vas deferens* and penis lengths (VDL and FPL respectively), the VDS stages of imposex determined according to the general schema proposed by Stroben et al. (1992), as partially modified by Axiak et al. (1995) in their study on imposex in *H. trunculus* and the *vas deferens* sequence index, VDSI = [(sum of imposex stage values of all females)/(total number of females)] as described by Gibbs et al. (1987).

The *in vivo* studies were carried out from February to July 2006. The samples of *H. trunculus* (n ≈ 800, shell length = 40-60 mm) were collected from the same site (A small channel) in the northern part of Tunis lagoon - S_8 - (Fig. 1). The imposex rate of ≈ 12% was recorded (87.9% at stage 0 and 12.1% at stage 1). Total of 50 females were selected and frozen. They served to measure the length of *vas deferens* (30 females) and the distance separating the position of the penis and the vaginal opening (20 females). In addition, 400 females were placed in 2 aquariums with 200 individuals in each one. The first aquarium was considered as a control and the second as a treated group by tributyltin: TBT^+ (50 $ng.L^{-1}$). The sea water was completely replaced from the aquariums once every 3 days. Every time the water was changed, a volume of a TBT^+ solution of 50.10^3 $\mu g.L^{-1}$ concentration were added to the treated aquarium in order to get the TBT^+ level of 50 $ng.L^{-1}$. The snails were fed with the clam *Ruditapes decussatus* collected from the same station. The mortality was recorded monthly in each aquarium. Moreover, 14 to 16 specimens were sacrificed each month from each aquarium in order to examine the region separating the right tentacle from the spawning opening. These investigations determine the evolution of the male genital tract in the female.

Figure 2. *Hexaplex trunculus.* Individuals marked with a rubber ring tag.

Figure 2. *Hexaplex trunculus.* Marquage des individus à l'aide d'un anneau en plastique.

As regards the statistical data analyses, significance levels for all analyses were established *a priori* at $P < 0.05$. Comparison of averages (VDSI, VDL and FPL) and frequencies (imposex, mortality and VDS stages) with the control population / group were tested using the Student t and Chi-square tests respectively.

Results

A microscopic examination of the area separating the penis site from the spawning orifice in abnormal females showed that the imposex occurred as in type d (Figs 3 & 4).

The type d comprises 6 stages. The first sign of imposex was the appearance of a *vas deferens* sequence in the medium area separating the site of the penis and the spawning orifice (Fig. 3, stage 1d; Fig. 4b-c). *Vas deferens* in stage 2d is more elongated on the anterior side reaching the penis site. After this stage, the posterior end continued its elongation to attain the spawning orifice (Fig. 3, stage 3b). The anterior part of the *vas deferens* developed and a small penis appeared (Fig. 3, stage 4d; Fig. 4e). From stage 2d, the anterior end of the *vas deferens* continued its development and a penis bud appeared (Fig. 3, stage 3d; Fig. 4d). This bud, as well as the proximal end of the *vas deferens,* continued to progress, succeeding to stage 4d, which was characterized by the presence of a small penis (1.5-2.5 mm length) and a complete *vas deferens* (Fig. 3, stage 4d; Fig. 4e).

We must notice that type 1d could lead to stage 2d' characterized by a small sequence of the *vas deferens* and also a penis bud at the back of the right tentacle (Fig. 3, stage 2d'; Fig. 4f). These two formations continued to develop and as the penis length reached about 2 mm, the *vas deferens* elongated (Fig. 3, stage 2d'; Fig. 4g). The distance between the anterior end of the *vas deferens* and the penis decreased progressively leading to stage 3a (Fig. 4a). The development of the *vas deferens* was more rapid on the anterior side (Fig. 4f-g) rather than in the posterior side.

The stage 4 was characterized by the simultaneous presence of a large penis and a *vas deferens* reaching the spawning orifice (Fig. 3: stage 4; Fig. 4h). The ventral evolution of the *vas deferens* passes through stage 4.3 and then 4.7, and finally to stage 5. This latter stage was distinguished by a longitudinal split of the capsule gland which causes female sterility (Fig. 3, stage 5; Fig. 4i-j).

The transplantation experience confirmed both that the first signs of imposex at *H. trunculus* are similar and that the incidence of imposex reflected sites with average boats use as well as with intense sea traffic. All the transplanted specimens that showed a small segment of the *vas deferens* had differentiated between penis and the vagina location. The mean *vas deferens* length (VDL) increased progressively and significantly from 0.67 mm at the experiment beginning to 5.08 mm at its end with a mean VDS range (VDS-index) varying from 0.31 to 1.68. The VDS stages were changed during the experiment; the proportion of each stage (0, 1d, 2d, 2d'and 3d) varied from a subsample to another. Significant differences were observed all over transplantation period, except in September, for the VDS 0, in December and January for the VDS 1d and in November, December and March for the VDS 2d. No significant differences in the VDS 2d' and 3d were found between

Table 2. *Hexaplex trunculus.* Index of imposex evolution through the transplant experiment. (-): non significant difference, (+): significant difference (compared with control group, t-test, Chi-square test, $P < 0.05$).

Tableau 2. *Hexaplex trunculus.* Evolution des valeurs des indices de l'imposex lors de l'expérience de transplantation. (-) : différence non significative, (+) : différence significative (comparée au témoin, test t, Khi-carré, $P < 0{,}05$).

Date	Jul 04	Sep 04	Nov 04	Dec 04	Jan 05	Mar 05
Number of Females	32	20	15	22	15	16
F% imposex	12.90	45.00 (+)	93.33 (+)	100 (+)	100 (+)	100 (+)
Mean VDS Index	0.31 ± 0.53	0.65 ± 0.81 (-)	1.53 ± 0.74 (+)	1.32 ± 0.47 (+)	1.14 ± 0.36 (+)	1.68 ± 0.60 (+)
VDS Index range	0-1-2	0-1-2	0-1-2-3	1-2	1-2	1-2-3
VDL mm	0.67 ± 1.36	1.89 ± 2.42 (+)	4.21 ± 1.94 (+)	4.24 ± 1.65 (+)	3.99 ± 2.64 (+)	5.08 ± 1.84 (+)
FPL mm	0	0.06 ± 0.24(-)	0.08 ± 0.23 (-)	0.02 ± 0.10 (-)	0	0.16 ± 0.44 (-)
% of females showing various VDS stages						
VDS 0	72.00	55.00 (-)	6.66 (+)	0 (+)	0 (+)	0 (+)
VDS 1d	25.00	25.00 (-)	40.00 (-)	68.18 (+)	86.66 (+)	37.50 (-)
VDS 2d	3.00	15.00 (-)	46.66 (+)	27.27 (+)	13.33 (-)	50.00 (+)
VDS 2d'	0	5.00 (-)	0 (-)	4.54 (-)	0 (-)	6.25 (-)
VDS 3d	0	0 (-)	6.66 (-)	0 (-)	0 (-)	6.25 (-)

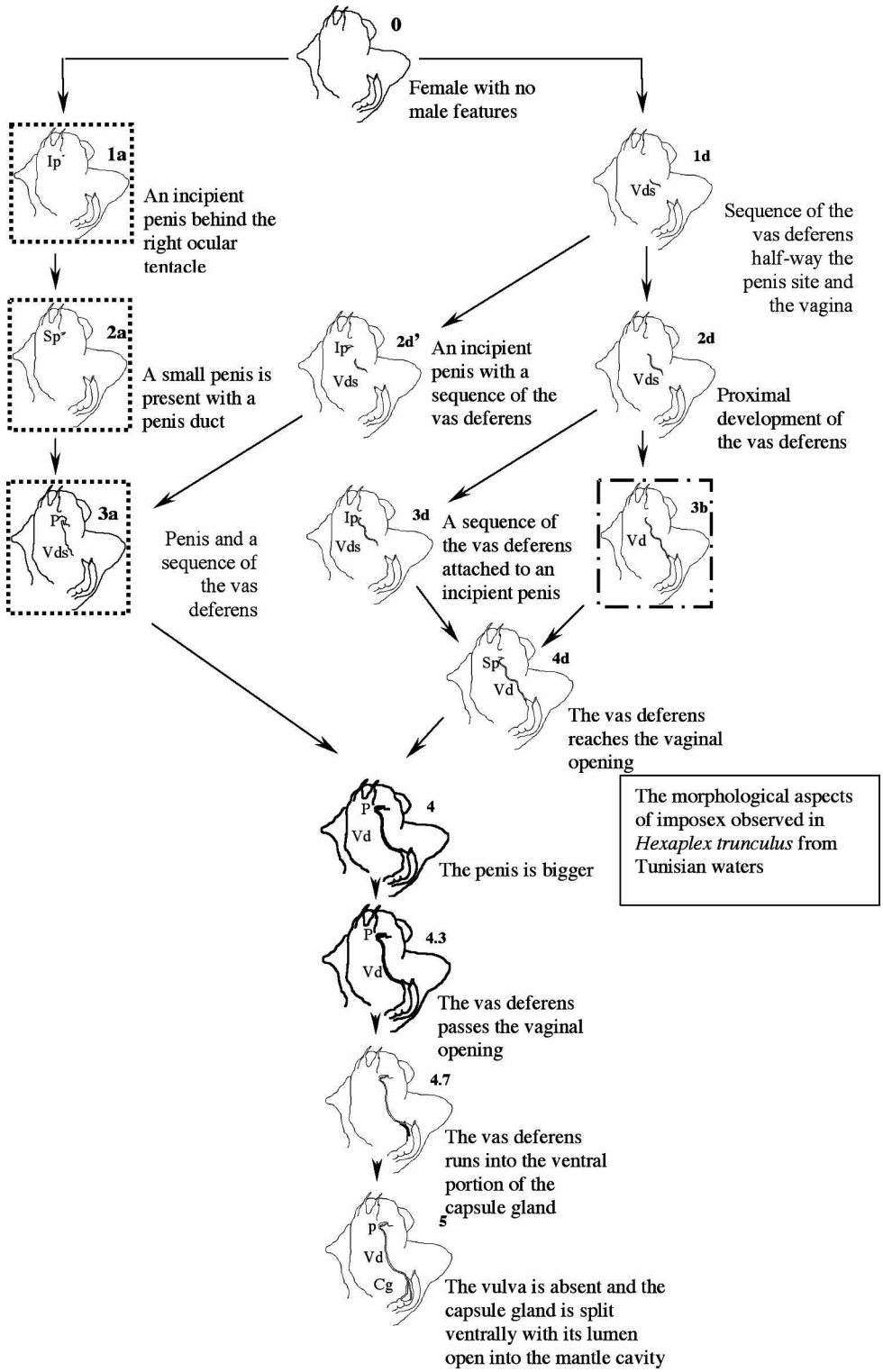


Figure 3. *Hexaplex trunculus.* Imposex scheme. In dotted line, stages observed in *H. trunculus* by Axiak et al. (1995) and Terllizzi et al. (1999). Broken line, stages observed in *Hinia (Nassarius) reticulata* by Stroben et al. (1992): p: penis, sp: small penis, Vd: *vas deferens*, Vds: *vas deferens* section, Cg: capsule gland, Ip: Incipient penis.

Figure 3. *Hexaplex trunculus.* Schéma du phénomène d'imposex. En pointillé, stades observés chez *H. trunculus* par Axiak et al. (1995) et Terllizzi et al. (1999). Trait interrompu, stades observés chez *Hinia (Nassarius) reticulata* par Stroben et al. (1992): p: pénis, sp: petit pénis, Vd: canal déférent, Vds: section du canal déférent, Cg: glande de la capsule, Ip: ébauche péniale.

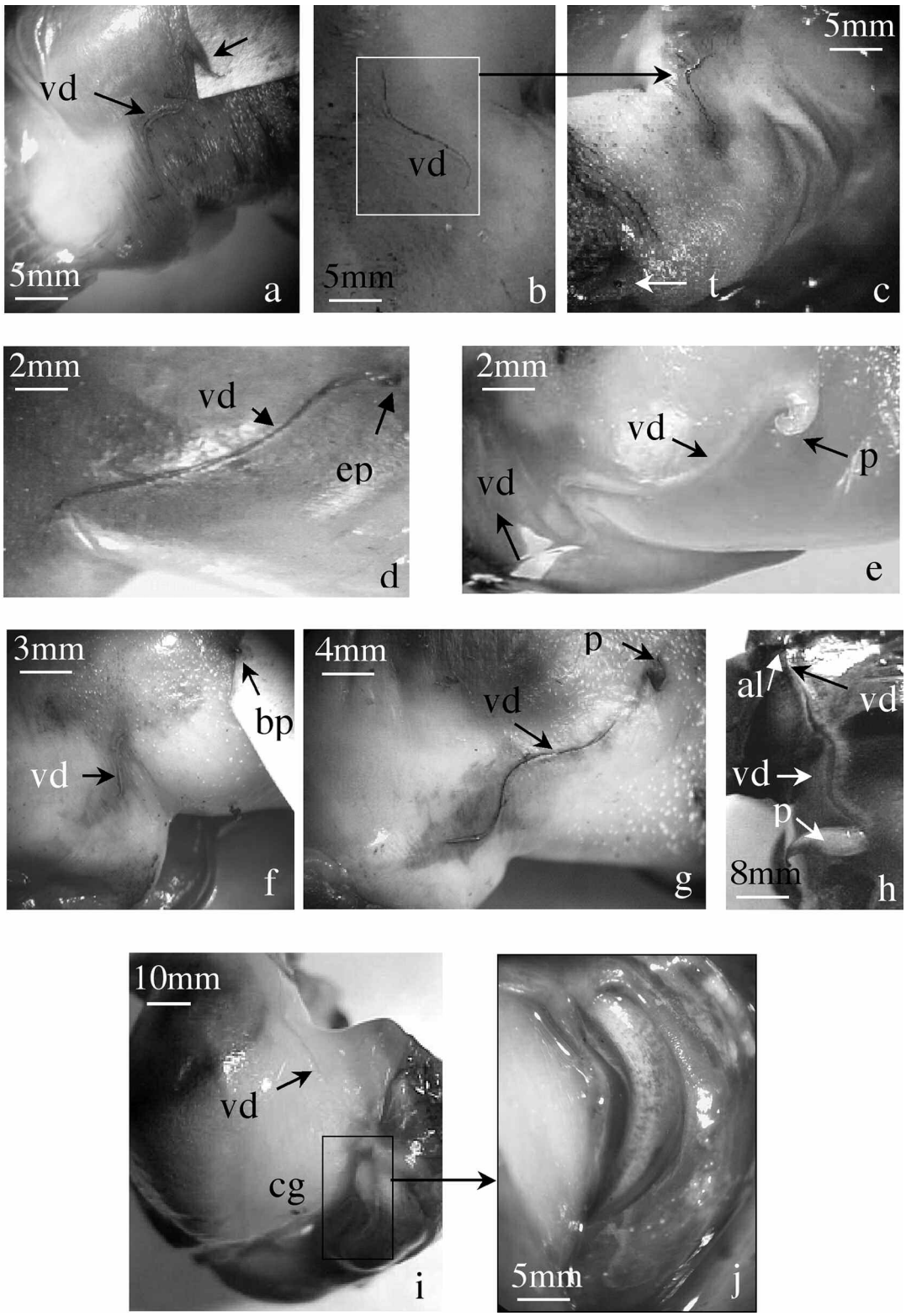


Figure 4. *Hexaplex trunculus.* Morphological aspect of imposex observed in individuals collected in the Tunisian waters. al: aperture of laying, bp: bud of penis, cg: capsule gland, ep: starting of penis, p: penis, t: tentacle, vd: *vas deferens*.

Figure 4. *Hexaplex trunculus.* Différents aspects morphologiques de l'imposex observés chez les individus récoltés sur les côtes tunisiennes. al : ouverture vaginale, bp : bourgeon pénial, cg : glande de la capsule, ep : ébauche péniale, p : pénis, t : tentacule, vd : canal déférent.

Table 3. *Hexaplex trunculus.* Mortality in the group exposed to TBT (50 ng.L^{-1}) and a control group. (-): non significant difference, (+): significant difference (compared with control group, Chi-Square test, $P < 0.05$)

Tableau 3. *Hexaplex trunculus.* Mortalité enregistrée au niveau des lots témoin et contaminé au laboratoire par une solution de TBT (50 ng.L^{-1}). (-) : différence non significative, (+) : différence significative (comparée au témoin, Khi-carré, $P < 0,05$).

		Feb 06	Mar 06	Apr 06	May 06	Jun 06	Jul 06
Control group	N	200	184	146	116	90	70
	Number of specimens examined	-	16	16	16	16	16
	Mortality %	-	0 (0/200)	11.95 (22/184)	9.58 (14/146)	8.62 (10/116)	4.44 (4/90)
Exposed group	N	200	136	70	38	16	0
	Number of specimens examined	-	16	16	16	16	14
	Mortality %	-	24 (+) (48/200)	36.76 (+) (50/136)	22.85 (+) (16/70)	15.78 (-) (6/38)	12.50 (-) (2/16)

control and transplanted populations (Table 2). The total rate of females exhibiting the "d'" imposex pathway, calculated at the end of transplantation, was significantly lower than the total rate of females showing the «d» pathway (22.5% VDS 2d vs. 2.5% VDS 2d', Chi-square test, $P < 0.05$) .

The laboratory exposure experiment showed that the mortality rate varied between the control group and the group contaminated by TBT$^+$. This variation was significant in March, April and May. Table 3 shows that the mortality of the control group began from the second month with a maximum rate of 11.95% recorded in April. Thereafter, this rate decreased gradually to reach 9.58% in May, 8.62% in June and 4.44% in July. However, the high mortality rate of 36.76% is recorded in the contaminated aquarium during April. This rate decreases progressively in time to reach 12.5% in July (Table 3).

The rate of imposex of the control group was stable during the exposure experiment ≈ 12% (Chi-square test, $P = 0.05$, Table 4). The exposure to 50 ng.L^{-1} concentration of TBT$^+$, *H. trunculus* developed the male genital tract in the female. The imposex rate of a sample significantly increases with duration of exposure to TBT$^+$ (Table 4). The time follow-up of the imposex in the contaminated group (Table 4) showed a low rate in February (≈ 12%). This rate increases significantly from March (25%) to reach 62.5% in April and 100% in May. The *vas deferens*, differentiated before the penis at the posterior level from the distance separating the genital papilla and the right tentacle.

The average length of the *vas deferens* (VDL) was 0.14 mm in February (in initial forms). But in the contaminated group *in vivo*, this canal elongated progressively from the anterior and the posterior sides, to reach 0.72 mm in March, 2.46 mm in April, 7.86 mm in May, 12.46 mm in June and 12.68 mm at the end of the experiment (Table 4). The penial bud, an average length of 0.01 mm appears after two months contamination. This length increased gradually and significantly to reach 0.09 mm in May, 0.22 mm in June and 0.51 mm in July (Table 4). The VDSI of the control group was practically consistent. However, the time follow-up of VDSI of contaminated group, changed during the experiment. In February, the proportion of each stage was 87.9% at stage 0 and 12.1% at stage 1. This percentage increased progressively to reach 100% at stage 3 in July (Table 5).

Discussion and Conclusion

Hexaplex trunculus females collected from the Tunisian coasts express firstly a portion of the *vas deferens* in the medium area separating the position of the future penis and the vaginal opening. This sequence continued to develop leading to stage 2d' or 2d. These stages evolved in stage 4d according to two ways, one by and intermediary sequence (way 3b) by similarity with the stage observed by Stroben et al. (1992) in *H. reticulata*, and the second by the way 3d. Then, this turned into stage 4. We proposed to call this previously described evolution sequence: the "d way".

Stage ld observed in *H. trunculus* differed from stage lc described by Stroben et al. (1992) in some prosobranchs and by Jakob & Jacobson (2002) in *N. antiqua*. These authors observed that the first sequence of the *vas deferens* started from the spawning orifice. Axiak et al. (1995) and Terlizzi et al. (1999) observed in *H. trunculus* that the appearance of a penis bud (stage la) was the first sign of imposex. Later, this bud grew progressively in size, and a penis duct appeared (Fig. 3, stage 2a). The penis duct

Table 4. *Hexaplex trunculus.* Index imposex evolution through the laboratory exposure experiment. (-): non significant difference, (+): significant difference (compared with control group, t-test, Chi-square test, $P < 0.05$).

Tableau 4. *Hexaplex trunculus.* Evolution des valeurs des indices de l'imposex lors de l'"expérience de contamination au laboratoire. (-) : différence non significative, (+) : différence significative (comparée au témoin, test t, Khi-carré, $P < 0,05$).

Date		Feb 06	Mar 06	Apr 06	May 06	Jun 06	Jul 06
Number of Females		33	16	16	16	16	16
F (%) imposex	Control group	12.1	12.5	12.5	12.5	12.5	12.5
	50 ng.L^{-1}	12.1	25.0	62.5	100	100	100
		(-)	(-)	(+)	(+)	(+)	(+)
Mean VDS Index	Control group	0.12 ± 0.33	0.12 ± 0.34	0.12 ± 0.34	0.12 ± 0.34	0.12 ± 0.34	0.12 ± 0.34
	50 ng.L^{-1}	0.12 ± 0.33	0.37 ± 0.72	0.93 ± 0.82	2.37 ± 0.72	3.00 ± 0.00	3.00 ± 0.00
		(-)	(-)	(+)	(+)	(+)	(+)
VDS Index range	Control group	0-1	0-1	0-1	0-1	0-1	0-1
	50 ng.L^{-1}	0-1	0-1-2	0-1-2	1-2-3	3	3
VDL (mm)	Control group	0.14 ± 0.40	0.16 ± 0.43	0.19 ± 0.51	0.18 ± 0.47	0.17 ± 0.45	0.15 ± 0.41
	50 ng.L^{-1}	0.14 ± 0.40	0.72 ± 1.46	2.46 ± 2.04	7.86 ± 3.11	12.46 ± 1.95	12.68 ± 1.13
		(-)	(-)	(+)	(+)	(+)	(+)
FPL (mm)	Control group	0	0	0	0	0	0
	50 ng.L^{-1}	0	0	0.01 ± 0.04	0.09 ± 0.12	0.22 ± 0.22	0.51 ± 0.41
				(-)	(+)	(+)	(+)

Table 5. *Hexaplex trunculus.* Percentage of females showing various VDS stages through the laboratory exposure experiment (50 ng.L^{-1}). (-): non significant difference, (+): significant difference (compared with control group, Chi-square test, $P < 0.05$)

Tableau 5. *Hexaplex trunculus.* Valeurs en pourcentage du VDS chez les femelles des lots témoin et contaminé par 50ng.L^{-1} de TBT. (-) : différence non significative, (+) : différence significative (comparée au témoin, Khi-carré, $P < 0,05$)

Date		Feb 06	Mar 06	Apr 06	May 06	Jun 06	Jul 06
VDS 0	Control group	87.9	87.5	87.5	87.5	87.5	87.5
	50 ng.L^{-1}	87.9	75.0 (-)	37.5 (+)	0 (+)	0 (+)	0 (+)
VDS 1d	Control group	12.1	12.5	12.5	12.5	12.5	12.5
	50 ng.L^{-1}	12.1	12.5 (-)	25.0 (-)	12.5 (-)	0 (-)	0 (-)
VDS 2d	Control group	0	0	0	0	0	0
	50 ng.L^{-1}	0	12.5 (-)	25.0 (+)	37.5 (+)	0 (-)	0 (-)
VDS 2d'	Control group	0	0	0	0	0	0
	50 ng.L^{-1}	0	0 (-)	12.5 (-)	0 (-)	0 (-)	0 (-)
VDS 3d	Control group	0	0	0	0	0	0
	50 ng.L^{-1}	0	0 (-)	0 (-)	12.5 (-)	12.5 (-)	0 (-)
VDS 3a	Control group	0	0	0	0	0	0
	50 ng.L^{-1}	0	0 (-)	0 (-)	25.0 (+)	75.0 (+)	87.5 (+)
VDS 3b	Control group	0	0	0	0	0	0
	50 ng.L^{-1}	0	0 (-)	0 (-)	12.5 (-)	12.5 (-)	12.5 (-)

developed at its posterior end. Concomitantly, a *vas deferens* was developed from the penis base toward the vagina (Fig.3, stage 3a). These stages were also indicated by Stroben et al. (1992) in *H. reticulata.*

The first stages of "d way" were not observed by Axiak et al. (1995) and Terlizzi et al. (1999) who worked on the same species collected from the coasts of Malta and Italy. This difference could be attributed to variations in the environmental parameters existing in the Tunisian and European coastal waters. Indeed, the studied stations in Tunisia are different in terms of maritime traffic and receive various pollutant resources. They show an imposex rate ranging from 0 to 100% (Trigui El-Menif et al., 2007). The parameters conjunction could contribute together to determine an "a way" or a "d way" (Fig.3). These environmental parameters could alter the endocrine control of the imposex development and lead to the different imposex development paths in the same species. If the "d way" was not observed in *H. trunculus* by Axiak et al. (1995) and Terlizzi et al. (1999), we suppose that other contaminants, except TBT^+, are different from those found on the Tunisian coasts. These factors could range from exposure to heavy metals, to changes in environmental conditions (Nias et al., 1993), the effect of some androgenic compounds like the nonylphenol (Evans et al., 2000) and the infestation by parasites (Gorbushin, 1997). Nevertheless, the TBT^+ could be considered as the dominant factor. According to Feral & Le Gall (1982) and Schulte-Oehlmann et al. (2000), toxic paint products containing the biocide TBT^+ could act on the neogastropods at the hormonal level. The hormone, or a morphogenetic factor, is synthesized and then, progressively imposes imposex on to the females. However, this hypothesis must be supported by the future sea water, sediment and gastropod tissues analyses in Tunisia as well in Italian and Maltese coasts in order to reveal the complementary factors which could act in the conjunction with TBT^+ to express differently the pathways. Another hypothesis that seems to give more plausible explanation for the differences in imposex development between Tunisian and Italian/Maltese snails is the eventual existence of genetic differences between Tunisian and European populations of *H. trunculus*. This hypothesis needs to be confirmed by a genetic study.

The evidence of new morphological aspects (1d, 2d', 2d, 3d and 4d) in *H. trunculus* allowed us to establish a new VDS classification for this species. The "d way" observed in *H. trunculus* from the Tunisian coasts was not indicated by Fioroni et al. (1991) who observed the morphological aspects of the imposex in 69 species of neogastropods.

Concerning the development intensity of the imposex, the formation of the penis bud, in stage 2d', (Fig. 4f) accelerated the elongation of the anterior *vas deferens*. The distance between the penis and the anterior end of the *vas deferens* was smaller than the one separating the posterior extremity of this canal to the spawning aperture (Fig. 4g). This can be validated as indicated by Figure 4h showing a well developed penis and a *vas deferens*, more differentiated on the anterior side.

The stages 4.3 and 4.7 were observed on individuals collected from some Tunisian sites, with imposex rates of 100% and an average penis size of 8.24 mm (Trigui El-Menif et al., 2007). All these observations are associated with areas exhibiting an intense maritime traffic.

Garaventa et al. (2006), have found in *H. trunculus* of the Croatian coast similar VDS values (4.3 to 5) to those recorded in Tunisia. However, the same authors demonstrated that VDS values were lower in the Italian stations (Ligurian Sea and lagoon of Venise). These observations are explained by the lack of regulation with respect to the TBT^+ use. Currently, in the Croatian region, no restrictions on organotin antifouling paints have been applied yet, while, in the Ligurian Sea and lagoon of Venise, a partial ban on TBT has been in force for vessels less than 25 m since 1982.

The transplantation experience confirmed that the first signs of imposex at *H. trunculus* are similar in low and averagely frequented sites by the boats and in intense sea traffic sites. All the transplanted females showed a small segment of the *vas deferens*, differentiated between the penis and the vagina location.

Moreover, the laboratory exposure using 50 $ng.L^{-1}$ TBT^+ concentration induced the imposex expression. This anomaly appeared firstly by the differentiation of a segment of the *vas deferens* which continues its development. The penis appears later.

The growth rate of the *vas deferens* and the penis is attributed to the TBT^+ concentration as well as the species sensibility with regards to the biocide. Indeed, the *vas deferens* and the penis of *H. trunculus,* when contaminated with 50 $ng.L^{-1}$ of TBT^+ reached 6.66 mm and 0.46 mm, respectively, in three months. In *N. lapillus*, Santos et al. (2005) showed that the length variations of the *vas deferens* and the penis recorded after three months of contamination by the TBT with the same concentration were 1.05 mm and of 0.22 mm respectively. Given that the maximal average length of the penis in the abnormal female is about 8mm in *Nucella lapillus* (Fioroni et al., 1991) and 8.23 mm in *H. trunculus* (Trigui El Menif et al., 2007), we can indicate that *H. trunculus* is more sensitive to TBT^+ than *N. lapillus.*

Taking into consideration the results found by Limaverde et al. (2007) in *Stramonita haemastoma* (Linnaeus, 1767) where an imposex frequency reaching 86%, an average penis length of 1.5 mm and a VDSI of 2.3 were recorded after 1 month exposition to 20 $ng.L^{-1}$ TBT^+,

we can consider this gastropod more sensitive than *H. trunculus* (imposex of 25%, no penis development and a VDSI of 0.37 after 1 month exposition to 50 ng.L^{-1} TBT^+).

Further research should be focalised on the physiology and the biochemistry to explain precisely how the TBT^+ acts in the tissues to express imposex as well as on the persistence of TBT^+ in the natural environment.

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