Marine Biology Research
November 2010, Volume 6, Issue 6, pages 600 - 607
http://dx.doi.org/10.1080/17451000903437075

The original publication is available at http://www.tandf.co.uk/journals/

Imposex and butyltin concentrations in snails from the lagoon of Bizerta (Northern Tunisia)*

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* Published in collaboration with the University of Bergen and the Institute of Marine Research, Norway, and the Marine Biological Laboratory, University of Copenhagen, Denmark

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Abstract:

Imposex and butyltin concentrations were assessed in *Hexaplex trunculus*, *Conus mediterraneus*, *Cyclope neritea* and *Nassarius mutabilis* from Menzel Jemil, and *H. trunculus* and *Stramonita haemastoma* from the Bizerta channel. All females of the five species exhibited the characteristic development of male sex organs (penis and/or vas deferens). In Menzel Jemil, *H. trunculus* and *C. mediterraneus* exhibit similar imposex levels and were most affected (VDS reading 3-4). The development of a bud penis or a vas deferens sequence behind the right ocular tentacle was reported in *Cyclope neritea* and *N. mutabilis* (VDS 1). The concentration of tributyltin (TBT) was similar for the four species. The monobutyltin (MBT) was only detected in *C. mediterraneus* while the dibutyltin (DBT) was found in all gastropods at similar proportions as TBT. In the Bizerta channel, imposex levels were similar in both *H. trunculus* and *S. haemastoma* but imposex degree was higher in *H. trunculus*. This finding was confirmed by higher TBT concentration recorded in female of *H. trunculus*. These results suggested that *H. trunculus* is the more suitable bioindicator for monitoring TBT pollution.

Keywords: Bioindicators; gastropods; organotins; southern Mediterranean

Introduction

Imposex, the superimposition of male sexual characters in female gastropods, was first reported by Blaber (1970). The effects of this deformity vary depending on the species. In some cases, it does not impair reproduction, but in some others, it can lead to population decline, due to sterility and reproduction failure (Bryan et al. 1986). According to Gibbs et al. (1997), four levels of imposex sensitivity were described: (0) snails lacking the imposex response, (I) species develop just a penis and vas deferens, (II) snails in which the oviduct structure and function are disrupted, and (III) species with ovary transformation to testis. Even though organotin compounds were considered the main cause of imposex (Smith 1981; Bryan et al. 1986), recently, hypotheses about the involvement of other factors have been suggested. Copper (Nias et al. 1993), nonylphenol (Evans et al. 2000) polychlorinated biphenyls (PCBs) and aroclor (Maran et al. 2006; Garaventa et al. 2008) have all been implicated. The occurrence of imposex has been extensively documented throughout the world. To date, this deformity has been described in 195 species worldwide (Shi et al. 2005). According to the list reported by these authors, the banded murex *Hexaplex trunculus* (Linnaeus, 1758), the cone *Conus mediterraneus* (Hwass, 1792), the cyclope nassa *Cyclope neritea* (Linnaeus, 1758) and the oyster drill *Stramonita haemastoma* (Linnaeus, 1758) were listed for gastropods and, to our knowledge, no information is published on imposex in the variable dogwhelk *Nassarius mutabilis* (Linnaeus, 1758). In Mediterranean prosobranchs, imposex studies are mainly limited to populations of *S. haemastoma* (Spence et al. 1990; Rilov et al. 2000; Lemghich & Benajiba 2007), *Bolinus brandaris* (Linnaeus, 1758) (Solé et al. 1998; Ramon & Amor 2001; Lemghich & Benajiba 2007), N. nitidus (Jeffreys, 1867) (Pavoni et al. 2007) and *H. trunculus* (Rilov et al. 2000; Garaventa et al. 2006, 2007, 2008; Lemghich & Benajiba 2007). Only one study on imposex in *C. neritea* was performed (Quintela et al. 2006). Moreover, available data on imposex in *C. mediterraneus* is very scarce and to our knowledge, De Fur et al. (1999) published the only document reporting the occurrence of imposex in this snail but without data on imposex level and developmental stages. In Tunisian waters, imposex was reported only in *H. trunculus* and *B. brandaris* (Lahbib et al. 2008; Abidli et al. 2009). No data are available at present for *S. haemastoma* despite the abundance of this species in Tunisian waters.

Organotin concentrations in gastropods have been recorded in many Mediterranean countries such as Italy (Garaventa et al. 2007; Pavoni et al. 2007), Israel (Rilov et al. 2000) and Malta (Axiak & Sammut 1995). However, African Mediterranean coastal studies are limited to those of Mzoughi et al. (2004), who showed the level of organotins in the sediment and in mussels of the Lagoon of Bizerta (Tunisia), and recently Lahbib et al. (2009) recorded organotins in the gastropod *H. trunculus* from Tunisian coast. No data are available on butyltin concentrations in *C. mediterraneus*, *C. neritea*, *N. Mutabilis* and *S. haemastoma*. The use of snails as bioindicators of tributyltin (TBT) pollution is necessary for monitoring the marine environment, since these species are so sensitive. For this reason, the aim of the present study is to provide data on imposex levels and butyltin concentrations in the tissue of five common Mediterranean gastropod species and to select from them the most suitable species for monitoring organotin contamination. These data will be very useful prior to the implementation of an organotin ban in Tunisia and will constitute a baseline to verify its effectiveness.

Material and methods

Sampling and biological analysis

On 15 September 2007, 60 individuals per species were collected from 2 stations in the Bizerta lagoon (Figure 1). On the offshore bar of Menzel Jemil, a total of 240 snails belonging to four species (*Hexaplex. trunculus*, *Conus mediterraneus*, *Cyclope neritea* and *Nassarius mutabilis*) were collected from a mud/sand substratum of 50 m^2 area and 20 cm depth. In the

Bizerta channel, specimens of two species, *H. trunculus* and *S. haemastoma*, were collected from a rocky substratum of 20 m^2 area and 50 cm depth. In the laboratory, individuals were frozen and then thawed, the shell was measured to the nearest 0.01 mm and broken with a bench vice. The soft tissues were carefully removed and the mantles were cut longitudinally to reveal the pallial oviduct in females. (We note here that freezing and thawing increases penis measurements in dead specimens compared to live individuals for all studied species: personal observation.) Sexes were determined according to the presence or absence of the capsule gland, vagina and penis. Imposex was quantified using the following indices: (1) the imposex incidence or frequency (I% = percentage of imposex-affected females compared to the total number of females in the sample), (2) the vas deferens sequence index (VDSI) following the scheme described by Stroben et al. (1992) for prosobranchs and that of Lahbib et al. (2008) updated for *H. trunculus* [VDSI = (sum of imposex stage values of all females)/(total number of females)] and (3) the relative penis length index for each female (RPLI = female penis length x 100/average male penis length). Calculation of statistical tests was conducted using the software Sigmastat® 3.5 for Windows. The paired sample t-test was used to compare differences in RPLI. However, the Chisquare and Mann-Whitney rank sum tests were used, respectively, for comparison of I(%) and VDSI.

Organotin analysis

Twenty females were selected for each species except for *H. trunculus* and *S. haemastoma* (5 females each). The operculum was removed and the soft body was finely ground using a T 18 basic Ultra-Turrax® disperser at 6000 rotations min^{-1}. Thereafter, the tissue was freeze-dried, weighed and maintained at -20°C in the dark until analysis. Approximately 200 mg of freeze-dried ground tissue was used to quantify tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT) concentrations. The gas chromatography flame photometric detection (GC-FPD) was used which was described in detail in Lahbib et al. (2009). The detection limits were 0.8 ng Sn g^{-1}dw for MBT, 0.7 ng Sn g^{-1}dw for DBT and 1.0 ng Sn g^{-1}dw for TBT. Analysis of a certified reference material (mussel tissue BCR 477, 6 replicates) using this procedure resulted in the following concentrations (as µg Sn g^{-1} dw): 1.03±0.04 for MBT, 0.75±0.03 for DBT and 0.86±0.04 for TBT. The certified values are 1.01±0.19, 0.79±0.06 and 0.90±0.08, for MBT, DBT and TBT, respectively.

Results

Imposex expression

In *Cyclope neritea* and *Nassarius mutabilis*, only one stage of imposex was revealed, the VDS1a and VDS1b, respectively (Figures 2A-C). The first stage characterized by the development of a bud penis behind the right ocular tentacle while the second stage showed a sequence of the vas deferens instead of the penis. In *Conus mediterraneus* six stages of imposex were revealed (1b, 1c, 2b, 3b, 3c and 4, Figure 3). The vas deferens sequence appeared anterior to the penis, behind the right ocular tentacle (1b) or at the vaginal opening (1c). Thereafter, these stages evolve toward 3b (a full vas deferens from the penis site to the vagina) or 3c (a small penis and two vas deferens sequences) passing through stage 2b (a longer vas deferens sequence behind the right ocular tentacle) to reach stage 4 (Figure 2D). In *Stramonita haemastoma*, the penis appeared first (1a) followed by the vas deferens sequence which took place at the vaginal opening (2c) or in front of the vagina (2d’). This stage then evolves toward (3c) or 3d (a small penis attached to a vas deferens sequence) to reach stage 4 (a penis and a full vas deferens) (Figure 4). In *Hexaplex trunculus*, the vas deferens sequence appeared anterior to the penis and halfway between the penis site and the vagina (VDS1d) and evolves toward 2d (a longer vas deferens sequence) or 2d’ followed by 3b or 3d and then 4+ (Figure 4). In males, we have observed some malformations affecting the penis only in *C. neritea*, namely two specimens with small penis and two were aphallic (without penis) among the 15 analysed (Figure 5).

Imposex levels and butyltin analyses

In Menzel Jemil, I(%) and VDSI were similar between *C. neritea* and *N. mutabilis* and between *H. trunculus* and *C. mediterraneus* (Table I, $p > 0.05$). Between these two pairs of species, the VDSI was significantly different showing higher imposex degree in *H. trunculus* and *C. mediterraneus* ($p < 0.05$). The RPLI values were too low varying between 0 (no penis) in *N. mutabilis* and 0.6 in *H. trunculus* but without showing significant differences between the four species ($p > 0.05$). The highest imposex stage (VDS4) was recorded only in *C. mediterraneus* (Table I). The predominant stage was the VDS3b in *C. mediterraneus* against stage VDS1d in *H. trunculus*. Based on these results we could classify the studied species according to their imposex response as follow: *C. mediterraneus* > *H. trunculus* > *C. neritea* > *N. mutabilis*. Chemical analyses showed low and similar TBT concentrations in females' tissues from the four populations that agree with the low boating traffic (20 artisanal fishing boats/year) found in the site (Table I). MBT was detected only in *C. mediterraneus* while DBT was present in the four species (Table I). The determination of the total amount of butyltins (BTs) showed that *N. mutabilis* has the highest recorded value, which disagrees with imposex response recorded in this snail.

In the Bizerta Channel, imposex was highly developed in both species, which agrees with the high boating traffic observed for the site (more than 500 commercial boats/year). Imposex incidence was similar between the two snails ($p > 0.05$, Table I) but imposex degree (VDSI and RPLI) was significantly higher in *H. trunculus* ($p < 0.05$, Table I). Among the four stages of imposex found in *S. haemastoma*, the VDS1a was the predominant stage. In *H. trunculus*, the three advanced stages which were recorded are 4, 4.3 and 5 with the VDS4 being the predominant stage (Table I). Sterility, which occurred at stage 5, was observed only in *H. trunculus* at 4.3%. Sterile females showed a split capsule gland following the growth of the vas deferens. Butyltin concentrations namely for DBT and TBT were also higher in *H.*

trunculus. This is in agreement with the results of imposex analysis. In both species, TBT was the predominant butyltin with 50 and 52% of the total BTs, respectively, in *H. trunculus* and *S. haemastoma*. DBT counted, respectively, for 42 and 38% and MBT for 7 and 10%. These results suggested that *H. trunculus* is more sensitive to TBT pollution in terms of imposex response and butyltin accumulation than *S. haemastoma*.

Discussion

Imposex features in *Hexaplex trunculus*, *Conus mediterraneus*, *Cyclope neritea* and *Stramonita haemastoma* were found in this study, which further confirms the inclusion of these gastropods in the list of imposex-species provided by Shi et al. (2005). However, the development of a small vas deferens behind the right ocular tentacle in *Nassarius mutabilis* is insufficient to include it in the list. According to Gibbs et al. (1997), many gastropods are included to the list of imposex-species by virtue of the fact that females bearing a penis have been found (an example of which is *Urosalpinx cinerea*). Thus, further female examinations for *N. mutabilis* should be investigated at other sites namely those with high TBT contamination in order to detect the presence of more advanced imposex stages. A more thorough examination using histological techniques is needed to support our microscopic observations of stage 1b. The initiation of female masculinization could be a natural phenomenon since it was revealed in some species before the use of TBT (Gibbs et al. 1997; Garaventa et al. 2006). Another hypothesis is related to the species' sensitivity to TBT, which varies according to the age and the dose (Gibbs et al. 1988). The observation of sterile females in *H. trunculus* further confirms its belonging to the level II of masculinization (Gibbs et al. 1997). For the case of *S. haemastoma*, imposex stages did not exceeded the VDS4 and is consequently found only among species of level I. This observation suggested that TBT concentrations recorded for this snail are not high enough to produce masculinization to level II as reported for *S. haemastoma* from eastern Mediterranean waters

(Rilov et al. 2000). In *C. mediterraneus*, available data on imposex stages are lacking. It seems that imposex in this species is similar to that of other members of the conidae family because similar stages (1c, 3b, 3c and 4) observed in this study were also reported in *C. vexillum* and *C. betulinus* (Shi et al. 2005). However, stages 1b and 2b were not mentioned by these authors. The maximum masculinization observed in *C. mediterraneus* (VDS4) suggested that TBT concentrations at the site are not high enough for imposex to reach sterility. At present, we could classify this species at level I of masculinization until further females from higher contaminated sites can be studied. In *C. vexillum* and *C. betulinus*, Shi et al. (2005) have recorded sterility according to VDS 5a, 5b and 6a which suggested that masculinization in *C. mediterraneus* could extend to sterility. Concerning *C. neritea*, imposex was recorded at low degree (VDS 1) but in another study from Galicia (Spain), more advanced stages were reported reaching VDS4 at which females are sterile (Quintela et al. 2006). This allows classifying *C. neritea* among species of level II.

The co-occurrence of gastropods in the same substratum has allowed us to demonstrate an interspecific comparison. In Menzel Jemil, *H. trunculus* and *C. mediterraneus* exhibited a greater incidence and intensity of imposex compared to *C. neritea* and *N. mutabilis*. The development of the penis at an early imposex stage in *H. trunculus* (VDS 2d' and 3d against VDS 4 in *C. mediterraneus*) suggested that this snail is more suitable for imposex monitoring. In the Bizerta Channel, imposex response was clearly higher in *H. trunculus* than in *S. haematoma* being in agreement with data reported by Lemghich & Bennajiba (2007), who showed that murex species (*H. trunculus* and *B. brandaris*) are more sensitive than Thais species (*S. haemastoma*). However, all these suggestions on interspecific comparison should be supported by extending the study at other sites. In other studies, imposex interspecific differences have also been observed between *Dicathais orbita* (Gmelin, 1791) and *Morula marginalba* (Blainville, 1832) collected from Sydney (Wilson et al. 1993), between *Thais clavigera* (Kuster, 1852), *Chicoreus capucinus* (Lamarck, 1822) and *Thais gradata* (Jonas, 1846) from Singapore (Tan 1999) and between *H. trunculus* and *B. brandaris* from Tunisia (Abidli et al. 2009).

The present study reports data on imposex and TBT in some of the less-studied gastropod species including imposex occurrence for the first time in one new Mediterranean species in the North African coastal environment. These types of studies provide evidence that TBT is still one of the most significant pollutants in the marine ecosystem today. Restrictions on its use have been enforced for several years in many Mediterranean countries. These findings are of interest for science and environmental management and can therefore also act as a baseline study useful for the assessment of the future trends in TBT pollution in the Tunisian coastal waters.

Acknowledgements

The authors would like to thank the editors and two anonymous referees for valuable comments and suggestions, which greatly improved the manuscript.

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Table I: Results of imposex and butyltin analyses in females of the studied species. N: number of females, SL: Shell length (mm), I (%): imposex incidence, VDSI: vas deferens sequence index, RPLI: relative penis length index.

	Menzel Jemil				Bizerta Channel	
	C. mediterraneus	*C. neritea*	*N. mutabilis*	*H. trunculus*	*S. heamastoma*	*H. trunculus*
N	42	45	57	46	40	47
SL (mm)	33.5±3.4	10.7±0.9	22.1±2.6	47.2±3.9	57.5±4.7	58.6±5.8
I (%)	47.6	20	10,3	47.8	95	100
VDSI	0.9±1.20	0.3±0.8	0.1±0.3	0.8±0.1	2.0±1.3	4.2±0.2
RPLI	0.1±0.7	0.5±1.2	0.0	0.6±1.9	13.6±6.0	44.9±23.8
Percentage of VDS stages						
VDS0	52.4	80.0	89.5	52.2	5	0
VDS1a	0	20.0	0	0	40	0
VDS1b	11.9	0	10.5	0	0	0
VDS1c	11.9	0	0	0	0	0
VDS1d	0	0	0	19.6	0	0
VDS2b	2.4	0	0	0	0	0
VDS2c	0	0	0	0	27.5	0
VDS2d	0	0	0	13.0	0	0
VDS2d'	0	0	0	8.7	2.5	0
VDS3b	16.6	0	0	4.3	0	0
VDS3c	2.4	0	0	0	0	0
VDS3d	0	0	0	2.2	0	0
VDS4	2.4	0	0	0	25	59.6
VDS4.3	0	0	0	0	0	36.2
VDS5	0	0	0	0	0	4.2
Mean and standard deviation (n = 2) of butyltin concentrations (ng Sn g^{-1} dw)						
MBT	1.8±0.3	<0.8	<0.8	<0.8	3.0±0.5	3.5±0.5
DBT	2.1±0.2	1.4±0.1	2.5±0.1	2.3±0.4	11.0±2.5	20.0±0.6
TBT	2.1±0.1	2.8±0.2	3.7±0.4	1.8±0.0	15.1±1.4	23.9±0.6
Total BTs	5.9±0.0	5.0±0.3	7.0±0.5	4.9±0.4	29.1±4.3	47.4±1.6

Figure legends

Figure 1: Sampling stations in the Bizerta lagoon. MJ: Menzel Jemil, BC: Bizerte Channel.

Figure 2: Imposex stage 1a, 1b and 4 respectively in *Cyclope neritea* (A and B), *Nassarius mutabilis* (C) and *Conus mediterraneus* (D). Bp: bud penis, Rt: right ocular tentacle, Ec: egg capsule seen through the capsule gland tissue, Vd: vas deferens, vds: vas deferens sequence, vo: vaginal opening. Scale bar: 1mm.

Figure 3 : Imposex pathways observed in *Conus mediterraneus*. p: penis, vo: vaginal opening, Vds: vas deferens sequence, Vd: vas deferens.

Figure 4: Imposex pathways observed in *Hexaplex trunculus* and *Stramonita haemastoma*. p: penis, vo: vaginal opening, Vds: vas deferens sequence, Vd: vas deferens.

Figure 5: Malformations affecting genital tract in male *Cyclope neritea*. A: Normal male, B: male with small filamentous penis, C: male with bud penis and incomplete vas deferens, D: aphallic male. (Rt: right ocular tentacle, P: penis, Vd: vas deferens, Lt: left ocular tentacle). Scale bar: 1mm.

Figure 1

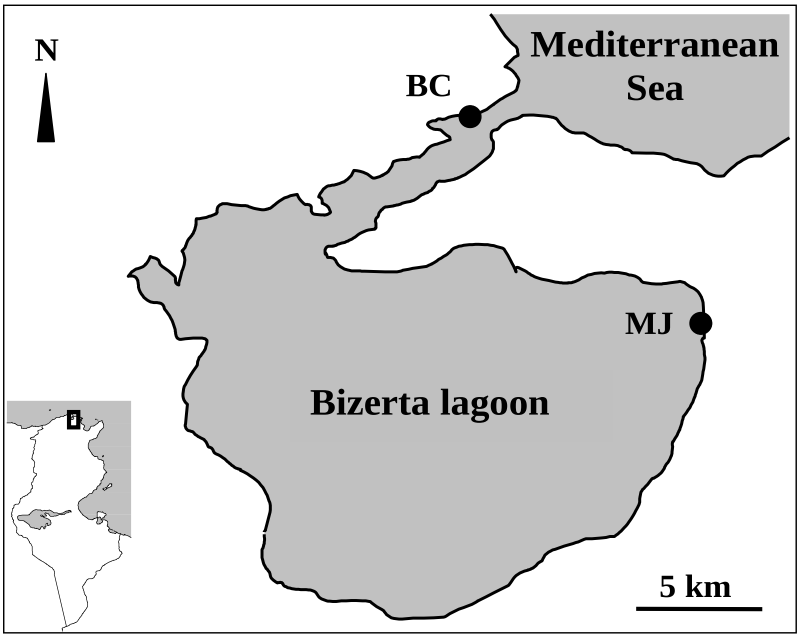


Figure 2

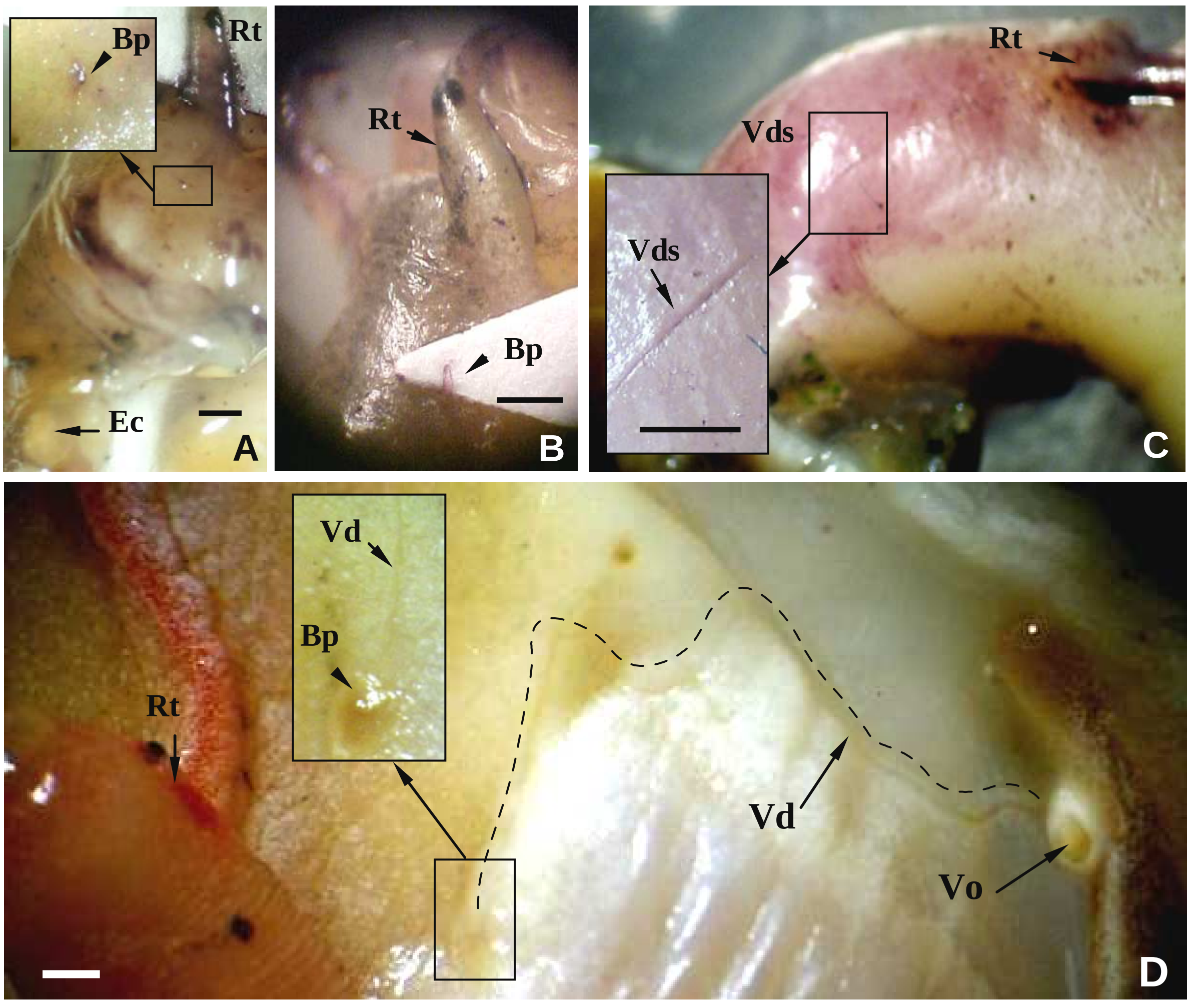


Figure 3

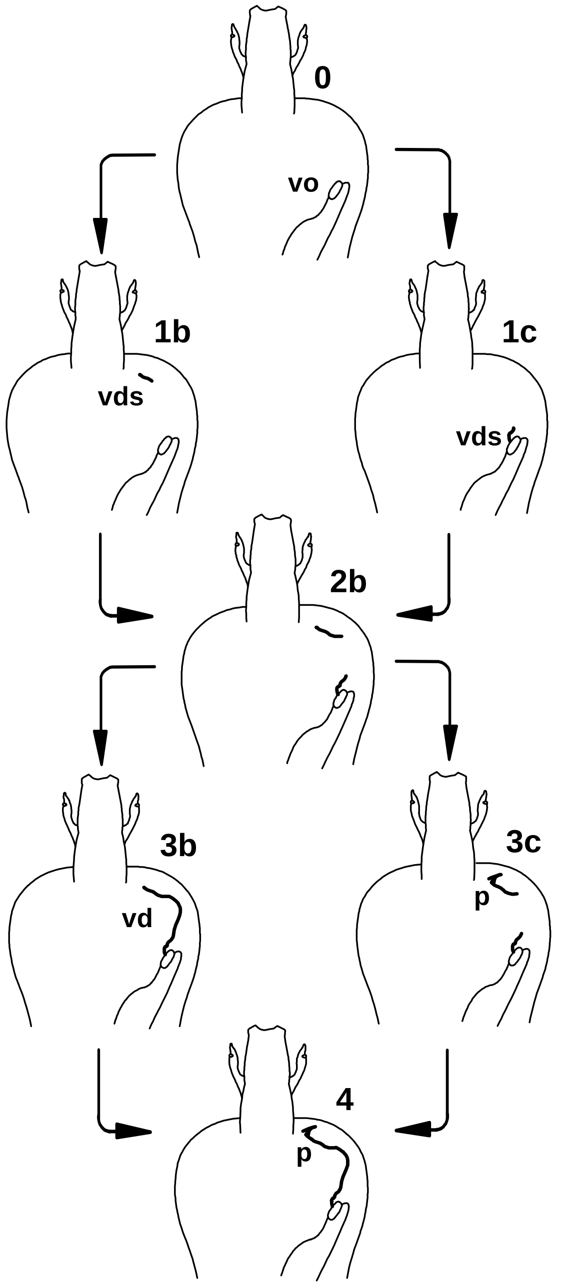
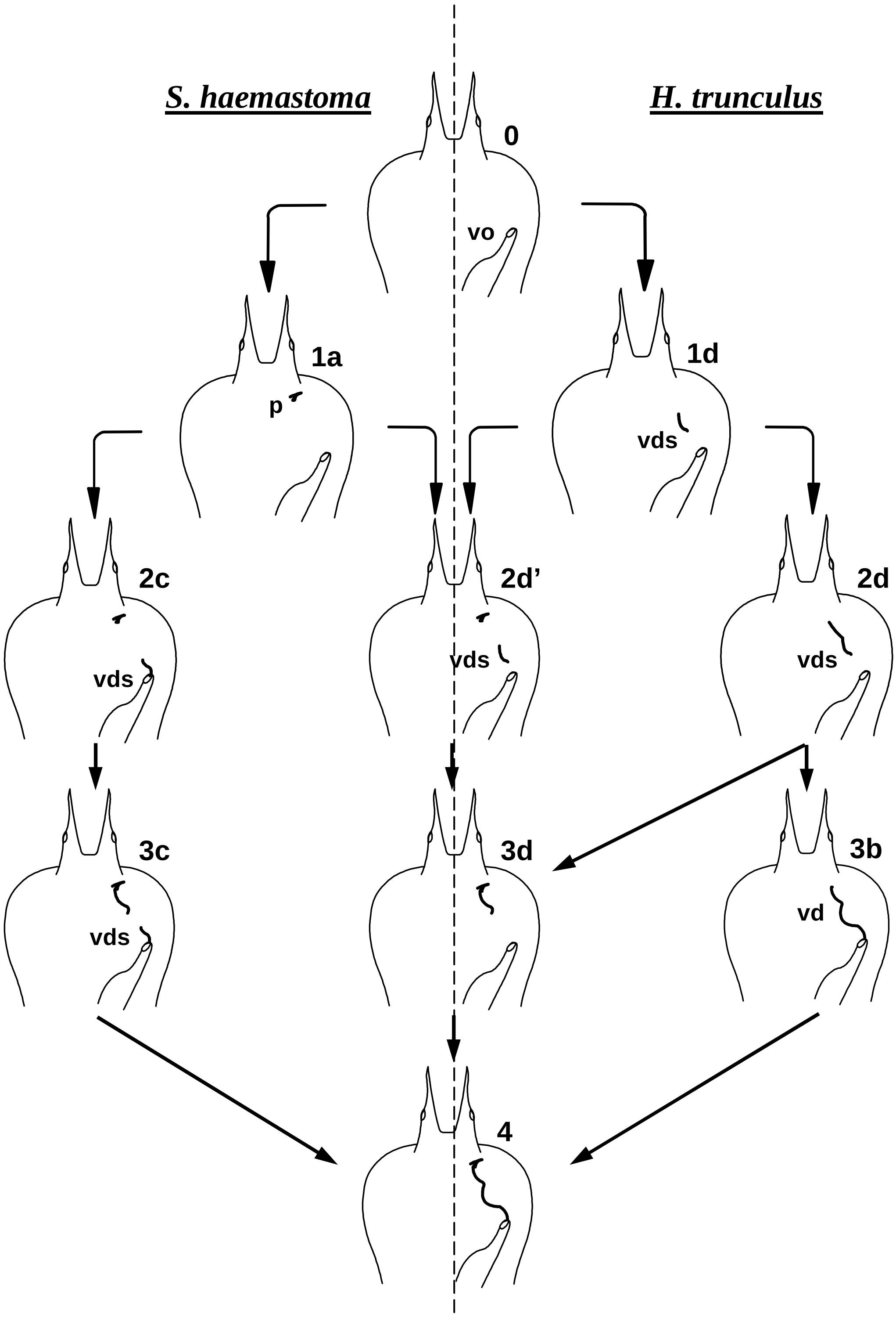


Figure 4



S. haemastoma
H. trunculus
0
vo
1a
p
1d
vds
2c
vds
2d'
vds
2d
vds
3c
vds
3d
3b
vd
4

Figure 5