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Organotin contamination, imposex and androgen/oestrogen ratios in natural populations of *Nassarius reticulatus* along a ship density gradient

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Levels of organotin body burden (expressed as tin), imposex and steroid hormones (testosterone, 17β-oestradiol, testosterone glucuronide and sulfate conjugates) were investigated in natural populations of *Nassarius reticulatus* in the Ria de Aveiro (northwest Portugal) between 1997 and 1999. The tributyltin (TBT) whole body burden (b.b.) of females presented increasing gradients from the adjacent open coast (16–26 ng g^{-1} dry weight (d.w.)) towards the ports inside the Ria de Aveiro (195–272 ng g^{-1} d.w.). Triphenyltin b.b. was only detected at the most polluted port (22 ng g^{-1} d.w.). Imposex also presented increasing values from the adjacent coast (vas deferens sequence index (VDSI): 0.0–0.5; relative penis length index (RPLI): 0.0–2.4; penis length index (PLI): 0.0–0.3 mm; percentage of affected females (%I): 0–30) towards the ports (VDSI: 3.8–4.8; RPLI: 51–80; PLI: 6.7–10.8 mm; %I: 100). The testosterone levels in females without imposex were always lower than in females with imposex, and the ratio of testosterone/17β-oestradiol in females tended to increase with increasing imposex and organotin contamination. In spite of the large difference in the female testosterone and 17β-oestradiol levels between summer and winter, related to the reproductive cycle, the spatial trend of the testosterone/17β-oestradiol ratio was remarkably similar in shape and values in the two seasons. Imposex was significantly correlated with the TBT b.b. and the testosterone/17β-oestradiol ratio in females. The testosterone conjugate levels did not show any clear pattern with the increasing values of imposex and TBT contamination. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: organotins; tributyltin; triphenyltin; imposex; testosterone/17β-oestradiol ratio; *Nassarius reticulatus*

INTRODUCTION

Imposex (superimposition of male characteristics, notably a penis and a vas deferens, on females of gonochoristic prosobranchs)[1] is an effect of tributyltin (TBT) established for a number of species. To a lesser extent, triphenyltin (TPT) can also induce imposex in some prosobranchs.[2] The mechanisms involved in imposex induction by TBT are still poorly understood (see reviews of Matthiessen and Gibbs[3] and of Oberdorster and Cheek[4]). Féral and LeGall[5] suggest that TBT interferes with the release of a neural factor by the cerebropleural ganglia of *Ocenebra erinacea* (L.) that, in normal females, prevents the secretion of penis growth-controlling neurohormones by the pedal ganglia. Other hypotheses propose that TBT alters hormone steroid metabolism, but they differ with respect to the specific path where this compound interferes. According to Bettin *et al.*,[6] in the dogwhelk *Nucella lapillus* (L.) and the nettedwhelk *Nassarius reticulatus* (L.), TBT may lead to a competitive inhibition of the cytochrome P-450-dependent aromatase, an enzyme that catalyses the conversion of androstenedione to oestrone

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and of testosterone to 17β-oestradiol; this would result in an increase of the androgen content and/or a change of the androgen/oestrogen ratio in favour of androgens, which induces imposex. On the other hand, Ronis and Mason[7] observed that in *Littorina littorea* (L.) TBT had only a modest effect on P-450-dependent testosterone metabolism *in vitro*; however, *in vivo* TBT mainly inhibits sulfur conjugation of testosterone and its phase I metabolites and posterior excretion, resulting in a build up of androgens titres in the tissues. A more integrated hypothesis suggests that TBT could initially induce the release of neurohormones by the pedal ganglia triggering male tissue differentiation and subsequently these tissues would be responsible for the increase in the testosterone levels.[8]

Most information regarding the mechanisms by which TBT induces imposex has been obtained under laboratory conditions. Although this is the best initial approach to study the subject, this methodology presents some obvious limitations: the effects of TBT are generally tested during short-term periods, at a given stage of the life cycle and sometimes at unrealistic high concentrations. Also, animals are subjected to manipulation and artificial rearing conditions that may alter their steroid metabolism. The aim of the present work is to monitor the spatial trends of imposex, organotin body burden (b.b.) and steroid hormone levels (testosterone, 17β-oestradiol and testosterone sulfate and glucuronide conjugates) in natural populations of *N. reticulatus* along a spatial gradient of ship density.

MATERIALS AND METHODS

Study area

The Ria de Aveiro is an important shallow estuarine system on the northwest coast of Portugal. Exchange of water with the sea occurs only through the mouth and dominates the hydrological circulation. The main TBT and TPT sources of contamination in the Ria de Aveiro are the ports and dockyards, which are located along the main navigation channel that extends from the mouth to the city of Aveiro. For the present study, 14 sampling stations were selected along an increasing gradient of ship density between the open sea and the Ria de Aveiro ports (Fig. 1).

Imposex

Sampling of *N. reticulatus* was performed from May to July 1997 at stations 1–10 and from May to July 1998 at stations 1–14. Additionally, the species was also collected from stations 1, 5, 7 and 10 in January 1999 (Fig. 1). Collection was made by hand or with baited hoop nets at the intertidal shore and with dredges deployed from boats at sublittoral sites. The animals were transported in cold and dark conditions to the laboratory and maintained in separated aquaria for 3 days (those used for steroid analysis were immediately processed after collection). About 60 adult animals were analysed per

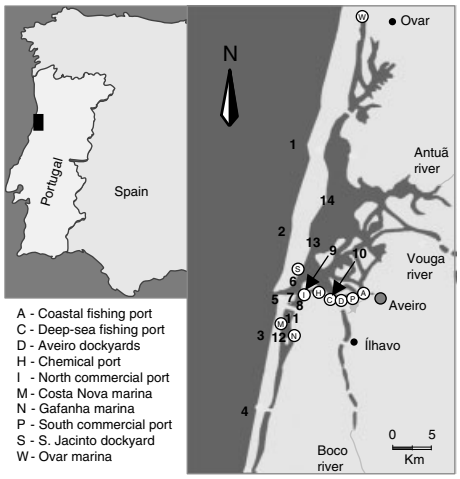

Figure 1. Map of the Ria de Aveiro with the location of the sampling stations (1–14) and of the main TBT pollution sources (ports, dockyards and marinas: white circles). Stations 9 and 10 are located inside ports I and C respectively.

site on each sampling occasion. They were narcotized using 7% $MgCl_2$ in distilled water for about 30–50 min and the shell height (distance from shell apex to the lip of the siphonal canal) was measured with vernier callipers to the nearest 0.1 mm. The shells were cracked open with a bench vice and the individuals were sexed and dissected under a stereomicroscope. Parasitized specimens were discarded. The relative penis length index (RPLI = Mean female penis length $\times$ 100/Mean male penis length), the mean female penis length index (PLI), the vas deferens sequence index (VDSI) and the percentage of affected females (%I) were determined for each station. The VDS was classified according to the scoring system proposed by Stroben *et al.*,[9] except that stage 4^+ was converted to stage 5 for computation of mean values at each site, in order to describe the spatial gradients better.[10]

Organotins

In the current work, two methods were used for measuring organotins in *N. reticulatus* tissues. The samples collected in 1997 were analysed for TBT and dibutyltin (DBT) using methods described in detail elsewhere.[11,12] Briefly, four to six frozen specimens (separated by sex) were pooled and then homogenized. Two replicates of tissues were taken for analysis. After acid digestion and hexane extraction, tin was measured in a Perkin–Elmer 76B graphite furnace attached to a Perkin Elmer 603 atomic absorption instrument. A 1 M sodium hydroxide solution was used to separate the DBT from the TBT fraction: tin as TBT was measured in the NaOH-treated extracts and tin as DBT was calculated following subtraction of the TBT values from those obtained for the untreated hexane extracts. The detection limit for TBT and DBT was 10 ng g^{-1} dry weight (d.w.) and recoveries were 100% and 92% respectively. The samples collected in 1998 were analysed for TBT, DBT, monobutyltin (MBT) and TPT

using the methods described in detail elsewhere.[13,14] Two replicates of 0.1 g lyophilized tissue were taken from 15 pooled females from each sampling site. They were digested with tetramethylammonium hydroxide by application of microwave power. After adjustment to pH 5, sodium tetraethylborate and isooctane containing tetrabutyltin as an internal standard were added successively. After microwave radiation treatment, the organic phase was recovered and analysed by gas chromatography–mass spectrometry. This method rendered a tin quantification limit of around 20 ng g^{-1} d.w. for butyltins and 10 ng g^{-1} d.w. for TPT. The recoveries for the different organotins were 90% (TBT), 95% (DBT), 85% (MBT) and 75% (TPT). Validation was performed with a certified reference material, i.e. the Japanese NIES11 fish tissue (National Institute for Environmental Studies, Japan Environment Agency).

The two analytical methods mentioned above offered different advantages, and so they were used in combination in the current work; the first method provided a lower detection limit for TBT and DBT that was useful to track tissue contamination at low polluted sites, and the second method allowed a better speciation of organotins (see the Results section).

The surficial sediments were sampled during 23–25 July 1998 at stations 5–7, 9–12 and 14. A portion of the surficial layer (1 cm) of the sediment was removed from the mid-tide level and deep-frozen (−20 °C) for storage. Homogenized wet unsieved subsamples were analysed for TBT and DBT[11] using the first method described above that renders a tin detection limit of about 5 ng g^{-1} (d.w.).

Reproductive cycle

Macroscopic (Ma) and microscopic (Mi) determinations of reproductive stages[15] were performed only for samples collected for steroid analysis (see paragraph below). Ma maturity stage 1 corresponds to a developed orange testis and a conspicuous white vesicula seminalis in males, and to a developed pale ovary, capsule gland and vulva in females; Ma stage 0 is characterized by an inconspicuous or dark vesicula seminalis and an underdeveloped capsule gland, whereas the gonad is less distinguishable from the digestive gland.[15] Among the animals presenting Ma stage 1, five females plus five males were randomly selected for Mi determination. The Mi gametogenic maturity stages are as follows: stage I (immature), II (early recovering), III (late recovering), IV (full or ripe), V (partially spent) and VI (spent).[15]

Steroid extraction and radioimmunoassay

The analysis of the steroid concentration in *N. reticulatus* whole b.b. was performed on animals collected from stations 1, 5, 7 and 10, as these sites represent a clear spatial gradient of imposex and organotin contamination (see the Results section). Sampling was done in 1–2 days in July during the 1998 survey ('summer survey') and in January 1999 ('winter survey'). Animals were narcotized with 7% $MgCl_2$ for 30 min. 'Ma stage 1' females presenting homogeneous and close to mean imposex values at each site and 'Ma stage 1' males were separately frozen at −80 °C for later hormonal analysis. For free steroid extraction, seven pooled weighted specimens of each sex collected from stations 1, 5, 7 and 10 were separately homogenized in 2 ml ethanol and homogenates were extracted four times with diethyl ether (1 : 1 v/v) for 5 min. The organic solvent was separated by centrifugation at 2000 *g* (5 min). The upper diethyl ether layers (organic fraction) were combined and evaporated under a stream of nitrogen at 30 °C. After being dissolved in 2 ml 80% methanol, the residues were washed twice for 5 min with 5 ml petroleum ether in order to separate steroids from the lipid fraction. The methanol fractions were evaporated and, after being suspended with 2.5 ml of distilled water, they were washed twice with 4 ml of diethyl ether. The organic solvent was evaporated before being dissolved in a radioimmunoassay 0.1% gelatine buffer (sodium azide 0.1% pH 7.4). The aqueous phase obtained from this procedure and the remaining homogenized tissues, which contain the testosterone conjugated with sulfate or glucuronide, were lyophilized and 4 ml of trifluoroacetic acid–ethyl acetate (1/100, v/v) were added and left overnight at 45 °C for acid hydrolysis of sulfates. The ethyl acetate was evaporated under a stream of nitrogen and the fraction was suspended in sodium acetate buffer (0.1 M, pH 5.0). Free steroids were extracted three times with 3 ml of diethyl ether and after evaporation suspended in gelatine buffer. The remaining fraction was treated with 5000 U of β-glucuronidase from snail *Helix pomatia* and left overnight at 37 °C and free steroids were extracted in the same way. The testosterone and 17β-oestradiol were quantified in duplicate by solid-phase ^{125}I radioimmunoassay, using kits from Diagnostic Products Corporation. The sensitivities of the standard curves were 40 pg per tube and 8 pg per tube for testosterone and 17β-oestradiol respectively. The two antibodies are highly specific and have an extremely low cross-reaction to other naturally occurring steroids, including oestrone (<10%), oestriol (<0.3%), testosterone (<0.001%) for 17β-oestradiol and androstenedione (<0.05%), oestradiol (<0.02%), dihydrotestosterone (<5%) for testosterone. The intra-assay coefficients of variation were 4.2% for testosterone ($n = 4$) and 7.9% for 17β-oestradiol ($n = 4$). The interassay coefficients of variation were 3.8% for testosterone ($n = 4$) and 3.7% for 17β-oestradiol ($n = 4$). Gamma radiation was measured in a LKB-Wallac MiniGamma Counter 1275. The assay was previously validated for *N. reticulatus*.[2] In order to obtain the extraction efficiency, homogenates were extracted after addition of either ^{3}H testosterone or ^{3}H 17β-oestradiol and countings after extraction were performed in a liquid-scintillation counter. The recovery mean values plus/minus the standard deviation were 57.4 ± 3.3% for testosterone and 79.4 ± 7.6% for 17β-oestradiol, and the results were corrected accordingly.

RESULTS

Imposex levels

N. reticulatus imposex levels at the different stations are summarized in Table 1. In the 1997 survey the VDSI, RPLI, %I and PLI varied in the ranges 0.0–4.7, 0–78, 0–100 and 0.0–10.8 mm, respectively. In the 1998 survey the same indices ranged between 0.0–4.8, 0–80, 0–100 and 0.0–10.2 mm. The results of both surveys were very similar and showed increasing gradients of imposex from the open sea towards the Ria de Aveiro, with the lowest values outside the Ria de Aveiro (stations 1–4) and the highest values inside ports (stations 9 and 10). The females presented only a-type VDS stages, i.e. with simultaneous penis development.[9]

Organotins

Table 1 summarizes the concentrations of different organotin species in the whelk tissues and in the sediments (all concentrations are expressed as tin). Increasing gradients of organotin contamination were present from the sea towards the ports inside the Ria de Aveiro. In the 1997 survey the TBT b.b. of males and females across stations varied from 15 to 196 ng g^{-1} and from 16 to 201 ng g^{-1} (d.w.) respectively. In 1998 the detectable TBT female b.b. ranged between 50 and 272 ng g^{-1} (dw). In all cases the highest values were observed inside ports. Imposex versus TBT female b.b. relationships are presented in the plots of Fig. 2 (A, B and C). Significant correlations (Spearman rank order) were obtained between the imposex level and the female TBT b.b. across samples, both in the 1997 (log(TBT) versus RPLI ($r = 0.95$; $P < 0.001$), log(TBT) versus PLI ($r = 0.95$; $P < 0.001$), log(TBT) versus VDSI ($r = 0.91$; $P < 0.001$)) and in the 1998 surveys (log(TBT) versus RPLI ($r = 0.93$; $P < 0.01$), log(TBT) versus PLI ($r = 0.93$; $P < 0.001$), log(TBT) versus VDSI ($r = 0.86$, $P < 0.05$)). MBT was only detected at stations 9 and 10 (130 ng g^{-1} and 159 ng g^{-1} d.w. respectively) and TPT was only detected at station 10 (22 ng g^{-1} d.w.).

The two groups of stations defined in the plot of Fig. 2A for the 1998 survey show the strong positive relation between the TBT contamination of the sediments and that of the tissues (see also Table 1); at stations 9 and 10, where the sediments presented 79 and 88 ng TBT-Sn g^{-1} (d.w.), the whelk's TBT b.b. was, respectively, 232 and 272 ng Sn g^{-1} (d.w.) (Fig. 2A, group 2); at sites located outside the ports the sediments were much less contaminated (<8 ng g^{-1} d.w.) and the TBT b.b. was lower (<73 ng g^{-1} d.w.) (Fig. 2A, group 1).

Reproductive stages

In the summer survey, 51–69% females and 53–70% males showed Ma maturity stage 1, whereas 100% of the adult collected animals presented stage 1 in the winter survey. Subsamples of stage-1 animals indicated that the gametogenic stage V predominated at all stations (60–100%) in the summer survey, whereas stage IV predominated (60–100%) in the winter survey (Table 2).

Testosterone versus 17β-oestradiol levels

The levels of testosterone and 17β-oestradiol in females and males at stations 1, 5, 7 and 10 are presented in Table 2. These four sites represent a clear spatial gradient of increasing organotin b.b., imposex (Table 1) and ship density: station 1 is located at the open sea and is visited occasionally by small fishing boats; station 5 is positioned at the mouth of the estuary, being crossed, therefore, by ships and boats that enter

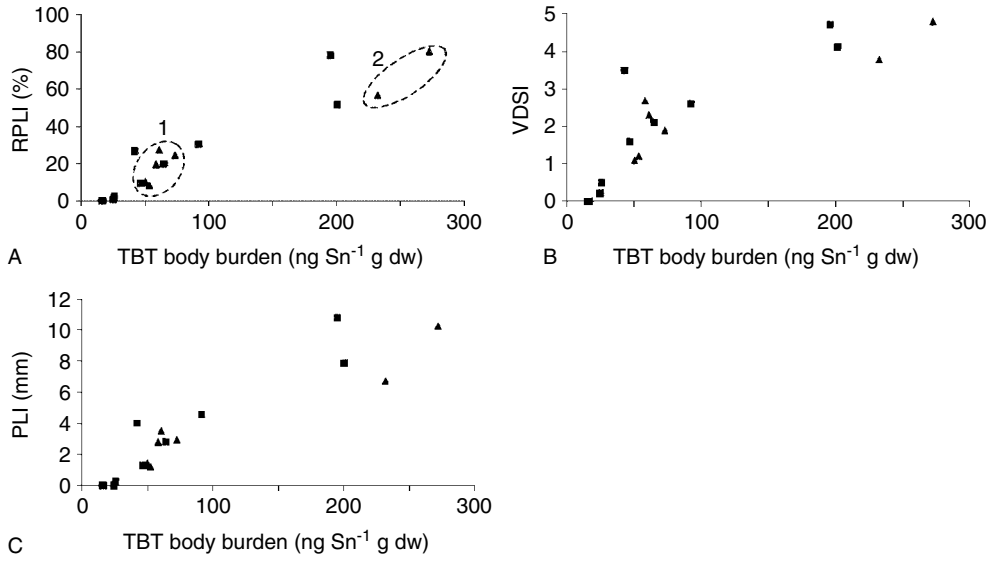

Figure 2. *N. reticulatus*. Relationship between the female TBT b.b. and (A) RPLI, (B) VDSI and (C) PLI. Squares represent data from the 1997 survey and triangles data from the 1998 survey. Broken circles 1 and 2 in (A) represent two groups of stations from the 1998 survey with distinct TBT sediment contamination (see text).

Table 1. Imposex and organotin (as tin) levels (ng g^{-1} d.w.) measured in *N. reticulatus* tissues and in sediments collected from Ria de Aveiro (see text for acronym meanings)

		1997 survey										1998 survey											
		Shell height		Imposex				Male b.b.		Female b.b.		Shell height		Imposex				Female b.b.				Sediments	
Site code	Name	Males	Females	RPLI	VDSI	%I	PLI	TBT	DBT	TBT	DBT	Males	Females	RPLI	VDSI	%I	PLI	TBT	DBT	MBT	TPT	TBT	DBT
1	Torreira Sea	26.9[a]	28.2[a]	0.0	0.0	0	0.0	15[b]	17[b]	16[b]	<	27.9[a]	29.0[a]	0.0	0.0	0	0.0	<	<	<	<	—	—
2	S. Jacinto Sea	26.2[a]	28.0[b]	0.0	0.0	0	0.0	32[b]	16[b]	17[b]	<	27.0[a]	26.3[a]	0.0	0.0	0	0.0	<	<	<	<	—	—
3	C. Nova Sea	26.3[a]	28.4[b]	2.4	0.5	15	0.3	48[b]	17[b]	26[b]	14[b]	27.0[a]	26.3[a]	2.1	0.2	30	0.3	<	<	<	<	—	—
4	Vagueira Sea	27.5[b]	28.5[a]	0.3	0.2	17	0.0	55[a]	60[a]	25[b]	<	26.2[a]	26.5[a]	0.0	0.0	0	0.0	<	<	<	<	—	—
5	Barra	24.7[b]	25.0[b]	9.4	1.6	100	1.3	37[b]	151[a]	47[a]	73[a]	25.4[b]	26.0[b]	8.3	1.2	89	1.2	53[a]	49[a]	<	<	<	<
6	S. Jacinto	24.4[b]	25.4[b]	19.6	2.1	95	2.8	41[b]	50[a]	65[a]	27[b]	26.0[b]	26.0[b]	10.4	1.1	96	1.4	50[a]	<	<	<	8	5
7	F. Magalhães	26.4[b]	25.7[b]	30.6	2.6	100	4.6	130[a]	41[b]	92[a]	65[a]	25.3[b]	26.1[b]	24.6	1.9	100	2.9	73[f]	43[a]	<	<	6	<
8	Forte Barra	25.7[b]	25.4[b]	26.8	3.5	100	4.0	53[a]	55[a]	42[a]	70[a]	26.0[b]	26.5[b]	29.1	3.5	100	6.1	—	—	—	<	—	—
9	Comm. port	25.4[b]	25.8[c]	51.3	4.1	100	7.9	138[a]	57[a]	201[a]	73[a]	23.6[b]	25.3[b]	56.8	3.8	100	6.7	232[a]	144[b]	130[b]	<	88	26
10	Fishing port	24.5[b]	25.7[b]	77.9	4.7	100	10.8	196[a]	73[a]	195[a]	65[a]	26.3[c]	26.9[c]	79.7	4.8	100	10.2	272[a]	109[a]	159[b]	22[a]	79	9
11	Gramata	—	—	—	—	—	—	—	—	—	—	27.3[a]	27.3[b]	19.6	2.7	100	2.8	58[c]	48[e]	<	<	8	6
12	Costa Nova	—	—	—	—	—	—	—	—	—	—	27.3[b]	27.5[b]	27.1	2.3	100	3.5	61[c]	48[c]	<	<	<	<
13	Reserva S.J.	—	—	—	—	—	—	—	—	—	—	26.0[b]	26.3[b]	5.2	0.9	91	0.8	<	<	<	<	—	—
14	Muranzel	—	—	—	—	—	—	—	—	—	—	26.7[b]	25.5[b]	9.8	1.7	100	1.5	<	<	<	<	6	5

Standard deviation relative to the mean: [a] (1–5%); [b] (5–10%); [c] (10–15%); [d] (15–20%); [e] (20–25%); [f] (40%). (—) Not performed. (<) Below detection limit.

Table 2. *N. reticulatus*. Imposex levels of females selected for steroid analysis: VDS (vas deferens sequence); PL (mean penis length). Reproductive maturity stage of the animals: percentage of individuals presenting Ma 1 in the sample and percentage of individuals presenting gametogenic stages I–VI (Mi I–VI) in a subsample of five females and five males showing Ma 1. F: females; M: males; Test.: testosterone; 17βE: 17β-oestradiol. For more data compare Table 1

	Subsample Imposex		Reproductive maturity stages (%)					Steroid hormones (pg g^{-1})				Balance		Testosterone conjugates (pg g^{-1})			
			Ma 1	Mi III	Mi IV	Mi V	Mi VI	Females		Males		Test./17βE		Females		Males	
Stn.	VDS	PL (mm)	F/M	F/M	F/M	F/M	F/M	Test.	17βE	Test.	17βE	Females	Males	Glucuronide	Sulfate	Glucuronide	Sulfate
Summer																	
1	0[a]	0.0[a]	51/53			60/80	40/20	536.0[b]	183.4[b]	1057.3[b]	41.3[c]	2.9	25.6	138.3[b]	160.9[c]	107.4[c]	289.0[c]
5	1[a]	0.9[b]	69/66			60/60	40/40	792.5[b]	140.3[b]	701.6[b]	99.1[c]	5.6	7.1	928.8[b]	112.0[b]	143.5[b]	253.2[b]
7	2[a]	3.1[b]	66/70			[1]75/60	[1]25/40	1688.2[b]	249.4[d]	2463.4[b]	350.0[c]	6.8	7.0	96.9[b]	182.9[c]	86.4[c]	123.0[c]
10	5[a]	10.5[b]	69/68			60/100	40/0	1730.1[c]	255.3[d]	2113.2[c]	414.3[b]	6.8	5.1	93.2[c]	167.3[b]	113.2[d]	437.7[d]
Winter																	
1	0[a]	0.0[a]	100/100	20/20	80/80			59.7[b]	18.0[b]			3.3		561.5[b]	93.8[d]	—	—
5	1[a]	0.8[a]	100/100	20/0	80/80	0/20		514.8[f]	80.9[b]			6.4		2034.8[c]	172.0[b]	—	—
7	2[a]	3.0[b]	100/100	0/20	100/80			247.0[c]	38.2[b]			6.5		171.8[c]	313.4[c]	—	—
10	5[a]	10.3[c]	100/100	40/20	60/80			757.3[f]	67.8[f]			11.2		8.2[c]	22.9[c]	—	—

Coefficient of variation: [a] (0%); [b] (0–5%); [c] (5–10%); [d] (10–15%); [f] (15–20%). (–) Not performed. (1) $n = 4$.

or leave the Ria de Aveiro; station 7 is located near the main navigation channel and in close proximity to several marinas and to a commercial port with a mean daily frequency of 2.2 ships with 88 m mean length; station 10 is situated at the largest port of the Ria de Aveiro, a 2 km wharf where more than 30 long-distance fishing ships ranging from 25 to 80 m are frequently moored; the main dockyards of Aveiro are located alongside this port (Fig. 1).

The testosterone levels in females ranged from 536.0 to 1730.1 pg g^{-1} wet weight (w.w.) in the summer and from 59.7 to 757.3 pg g^{-1} w.w. in the winter. The 17β-oestradiol varied from 140.3 to 255.3 pg g^{-1} w.w. in the summer and from 18.0 to 80.9 pg g^{-1} w.w. in the winter. Testosterone levels in females without imposex from station 1 were always lower than in females affected with imposex from stations 5, 7 and 10. The ratio of testosterone/17β-oestradiol increased in females from station 1 (3 : 1 in both seasons) to station 10 (7 : 1 in the summer and 11 : 1 in the winter) (Table 2). This ratio was highly significantly correlated (Spearman $r = 1.0$; $p < 0.001$; $n = 4$) with the imposex indices VDSI and PLI across stations (after $\log x + 1$ transformation of both variables). The testosterone levels of males were generally higher than in females and ranged from 701.6 to 2463.4 pg g^{-1} w.w. in the summer. At the sea (station 1) the ratio was higher in males (26 : 1), but inside the Ria de Aveiro it was similar between genders (7 : 1 at stations 5 and 7; 5 : 1 at station 10). The testosterone conjugates (sulfates and glucuronides) levels in the two genders did not show any pattern of variation along the gradient (see Table 2).

DISCUSSION

Increased organotin contamination and imposex levels were detected in *N. reticulatus* on moving from the open coast to the ports of the Ria de Aveiro. This gradient matches the increasing ship density and assumes a higher organotin contamination from ship antifoulant leaching. Significant correlations between the intensity of imposex and TBT b.b. in *N. reticulatus* were found in the current work and have also been reported along the coasts of Portugal,[10] Spain,[16] France[9] and in the English Channel.[17] Moreover, it has been shown that *N. reticulatus* females develop imposex after only 1–2 months of exposure to TBT under laboratory conditions.[2,6]

To analyse the spatial variation of the steroid hormone levels along a TBT pollution gradient it is necessary that the animals are in the same phase of the reproductive cycle. This constrains the surveys to small areas, because the gametogenic stage of the animals across different sampling stations tends to be similar if the stations are close to each other. In the current study, stations 1, 5, 7 and 10 were located along a sharp gradient of TBT b.b. and imposex (which varies almost between minimum and maximum possible values) in a transect of just 20 km distance and, in fact, the whelks' reproductive cycle was approximately synchronous throughout the gradient in each season. The reproductive cycle of *N. reticulatus* was investigated at station 7 in the Ria de Aveiro during the period 1992–93, and a clear seasonal pattern was observed with the beginning of gametogenesis in August followed by ripening in January and February and spawning from February to July.[15] This seasonality explains the large difference in the female steroid levels that was found in the summer 1998 and in the winter 1999 (Table 2). The whelks at station 1 were not affected by imposex and presented the lowest organotin contamination. Females at this site also presented the lowest levels of testosterone and the lowest testosterone/17β-oestradiol ratio. This ratio increased as we moved from the open coast towards the ports, in a similar way as the TBT contamination and imposex. Despite the large seasonal variation of the female testosterone and 17β-oestradiol levels, the ratio trend was remarkably similar between the two seasons. The comparison of the testosterone/17β-oestradiol ratios between genders in the summer survey reveals that they were very distinct at station 1 but progressively approached each other along the remainder stations; at station 10 they were similar. This is due to the ratio increase in females as we approach the ports, and also to a decrease of the same ratio in males. Although the observed pattern in females is most probably a consequence of the TBT pollution, which triggers the imposex development, other causes should be investigated in males.

An increase of the testosterone/17β-oestradiol ratio and imposex in the dogwhelk *N. lapillus* after exposure to TBT in seawater under laboratory conditions was previously reported,[6,18] but no significant change in the above ratio was observed in *N. reticulatus*.[6] Besides, imposex is strongly induced in both species when testosterone is added to the water, but its development is suppressed when oestrogens are also supplemented to the water.[6] Thus, an increase in the testosterone/17β-oestradiol ratio seems to be associated with imposex development, and our results suggest that this may also happen with *N. reticulatus* in natural conditions. This could result from a competitive inhibition of aromatase by TBT,[6] or from imposex[8], but other steroid hormones should also be investigated in the future. With regard to the testosterone excretion–inhibition hypothesis,[7] we did not find a consistent variation in the testosterone-conjugated levels in *N. reticulatus* that could explain the observed imposex and steroid level trends.

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