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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (ZOOLOGIA)

ASSIMILAÇÃO, DEPURAÇÃO E CONTAMINAÇÃO DO ERMITÃO *Clibanarius vittatus* PELO POLUENTE TRIBUTILESTANHO (TBT) E SUA RELAÇÃO COM A INTERSEXUALIDADE EM ERMITÕES.

BRUNO SAMPAIO SANT'ANNA

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de doutor em Ciências Biológicas (Zoologia).

Março - 2011

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Orientador: Fernando José Zara
Co-orientador: Alexander Turra

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“Dedico este trabalho a minha família, que com todas as dificuldades me proporcionou a oportunidade de estudar”

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RESUMO

A causa da intersexualidade em ermitões ainda é desconhecida. No presente estudo foi avaliada a relação entre a intersexualidade em ermitões e o poluente disruptor endócrino tributilestanho (TBT), como registrado para gastrópodes. A contaminação em populações naturais do ermitão *Clibanarius vittatus* também foi investigada. Compostos butílicos (Bts) foram determinados em tecidos de ermitões e sedimentos usando cromatografia gasosa e análises morfológicas foram desenvolvidas por histologia e microscopia eletrônica, em amostras provenientes da natureza e de experimentos controlados. A avaliação do ambiente mostrou que localidades com e sem atividades portuárias estão contaminadas com elevadas concentrações de Bts, mesmo após o banimento do TBT. Estes dados indicam que Bts continuam sendo liberados no ambiente ou que estes compostos estão acumulados em diferentes compartimentos do ambiente. O registro de ermitões contaminados por Bts em muitos locais onde não foram detectados no sedimento sugere que ermitões são melhores indicadores de Bts do que sedimentos, porque não representam apenas a contaminação pontual. Além disso, os experimentos revelaram que a principal forma de assimilação do TBT por *C. vittatus* é proveniente da alimentação. Em adição, a rápida depuração do TBT, o hábito de vida em regiões estuarinas, tamanho relativamente grande, longo ciclo de vida e sua baixa mobilidade faz dessa espécie e de ermitões estuarinos em geral bons candidatos a indicadores de contaminação recente de TBT. As análises morfológicas mostraram que indivíduos intersexo apresentaram gônadas funcionais de macho e fêmea no mesmo indivíduo. Estes dados confirmam que ermitões intersexo podem reproduzir como machos ou fêmeas suportando a hipótese de que eles podem ser parte de um processo hermafrodita verdadeiro. A hipótese de que o TBT está relacionado com a intersexualidade em ermitões promovendo o desenvolvimento de características sexuais masculinas em fêmeas como observado em gastrópodes, não foi corroborada. Fêmeas alimentadas com TBT não desenvolveram gonóporos ou qualquer parte do sistema reprodutor masculino. No entanto, este composto foi tóxico, provocando desorganização e atrofia do ovário. Dessa forma, novas hipóteses são apresentadas para explicar a intersexualidade neste grupo.

ABSTRACT

The causes of intersexuality in hermit crabs are still unknown. This study was carried out to evaluate the relationship between intersexuality and endocrine disruption caused by tributyltin (TBT) pollution, as previously reported for gastropods. In addition, it was also investigated the TBT contamination in natural populations of the hermit crab *Clibanarius vittatus*. Butiltins (Bts) in hermit crabs tissues and sediments were analysed by gas chromatography and the morphological analysis was carried out by histology and scanning electron microscopy using samples from both nature and controlled experiments. The evaluation of contamination in nature showed that localities with and without harbor activities are contaminated by high concentrations of Bts, even though the ban regulamentation of TBT. These data indicate that Bts compounds are still being released in the environment or they are accumulated in different environmental compartments. The record of contaminated hermit crabs by BTs in many places where contaminated sediments by BTs was not detected suggest that hermit crabs are better indicators of BTs than sediments, because of their contamination was not only represented punctually. Furthermore, the controlled experiments in laboratory showed that, the main TBT uptake pathway for *C. vittatus* is from food. In addition, the fast depuration of TBT, estuarine habitat, relatively large body sizes, long life spans, and relatively low mobility make this species and estuarine hermit crabs in general very good candidates as indicators of recent or recycled TBT contamination. The morphological analyses showed that the intersex individuals developed functional male and female gonads in the same individual. These data confirmed that intersex hermit crabs can reproduce as males or females supporting the hypothesis that they may be part of a true sequential hermaphroditic process. The hypothesis that TBT is related to intersexuality in hermit crabs promoting the development of male sexual characteristics in females as observed in gastropods, was not corroborated. Females feed with TBT did not develop male gonopores or any part of male reproductive system. However, this compound was toxic leading to disorganization and atrophy of ovary. Thus, new hypotheses are presented to explain intersexuality in this group.

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Capítulo 1

Introdução Geral

1. Introdução Geral

Intersexualidade é um evento comum dentre os metazoários (REINBOTH, 1975; WARNER, 1975) e, especialmente, dentre os crustáceos (BROOK et al., 1994). Embora RUDOLPH (1995) associe a presença de indivíduos intersexo em populações naturais de decápodes com a ocorrência de hermafroditismo seqüencial, a intersexualidade também pode estar associada a hermafroditas não funcionais (SAGI et al., 1996).

Em caranguejos ermitões a intersexualidade é um fenômeno relativamente comum em populações naturais e de fácil identificação (TURRA e LEITE, 2000). Os gonóporos em ermitões abrem-se na coxa do terceiro par de pereópodos em fêmeas e na coxa do quinto par de pereópodos em machos. Indivíduos intersexo têm gonóporos tanto no terceiro quanto no quinto par de pereópodos e já foram registrados em 17 espécies de diferentes gêneros e famílias de ermitões: *Dardanus deformis* (H. Milne Edwards, 1836) por HILGENDORF (1897), FIZE e SERÈNE (1955) e LEWINSOHN (1982); *Clibanarius zebra* (Dana, 1852) por WENNER (1972); *Pagurus armatus* (Dana, 1851), *P. ochotensis*, Brandt, 1851, *P. aleuticus* (Benedict, 1892), *P. confragosus* (Benedict, 1892), *P. tanneri* (Benedict, 1892), *Elassochirus tenuimanus* (Dana, 1851) e *E. cavimanus* (Miers, 1879) por MCLAUGHLIN (1974); *Eupagurus* sp. por CHARNIAUX-COTTON (1975); *Paguritta kroppi*, McLaughlin and Lemaitre, 1993 por MCLAUGHLIN e LEMAITRE (1993); *Clibanarius antillensis* Stimpson, 1859 e *C. sclopetarius* (Herbst, 1796) por TURRA e LEITE (2000); *C. vittatus* (Bosc, 1802) por TURRA e LEITE (2000), SANT'ANNA et al. (2006), TURRA (2004, 2007); *Paguristes tortugae* Schmitt, 1933 por MANTELATTO and SOUSA (2000); *Isocheles sawayai* Forest e Saint Laurent, 1967 por FANTUCCI et al. (2007) e *Coenobita rugosus* (H. Milne-Edwards, 1837) por GUSEV e ZABOTIN (2007).

Apesar dos registros antigos, somente recentemente a intersexualidade começou a ser investigada de forma mais acurada. TURRA (2004), considerando dados morfológicos, comportamentais e populacionais argumentou que se a intersexualidade em ermitões fosse parte de um mecanismo hermafrodita, a única possibilidade seria a protoginia. A hipótese da protoginia em ermitões foi sugerida pela primeira vez por WENNER (1972)

para explicar a ausência de machos nas menores e de fêmeas nas maiores classes de tamanho de *C. zebra*. Entretanto, esta hipótese nunca foi testada e, segundo (CHARNIAUX-COTTON, 1975), a presença de "ootestis" em *Eupagurus* sp. sugere hermafroditismo não funcional neste grupo de animais. Além disso, protoginia em crustáceos malacóstracos é somente conhecida entre os peracáridos (BROOK et al., 1994).

Até o momento sabe-se que os gonóporos femininos dos indivíduos intersexo tendem a regredir e fechar em *C. antillensis*, *C. vittatus* e *C. sclopetarius* (TURRA, 2004). Além disso, foi demonstrado também que indivíduos intersexo destas três espécies são capazes de copular com sucesso como machos (TURRA, 2004, 2005). Por outro lado, TURRA (2007) registrou a ocorrência de um indivíduo intersexo ovígero em *C. vittatus*, comprovando assim que os indivíduos intersexo também podem ser fêmeas funcionais. Contudo, até o momento a funcionalidade reprodutiva como machos e fêmeas nunca foi observada em um mesmo indivíduo, seja fecundando ou sendo fecundado ou pela análise morfo-histológica do sistema reprodutor.

A despeito da eventual possibilidade da intersexualidade em ermitões representar uma fase em um processo de reversão de sexo, ela também pode ser conseqüência do efeito da poluição ambiental por alteradores hormonais. Foi comprovado que substâncias organoestânicas, como TBT (tributilestanho), causam alterações hormonais em diversos organismos (BRYAN et al., 1986, 1989; BAUER et al., 1995; BETTIN et al., 1996; FENT, 1996). Em gastrópodes prosobrânquios esta substância é responsável pelo fenômeno chamado de "imposex", caracterizado pelo aparecimento de características sexuais masculinas em indivíduos fêmeas (BRYAN et al. 1986; GIBBS e BRYAN, 1994; HORIGUCHI et al., 1995; GOODING et al., 2003; FERNANDEZ et al., 2002, 2005; GARAVENTA et al., 2006).

Embora não haja na literatura nenhum registro sobre a contaminação de ermitões por TBT, é esperado que isto ocorra, pois é comum em bivalves (WALDOCK e THAIN, 1983; HOCK, 2001), gastrópodes (BRYAN et al., 1989; GIBBS e BRYAN, 1994; HORIGUCHI et al., 1995; GOODING et al., 2003) e outros crustáceos (LAUGHLIN et al., 1984; REXRODE, 1987; WEIS et al., 1987; CARDWELL e MEADOR, 1989; BARTLETT et al., 2004) que habitam áreas contaminadas. Como já existem registros de áreas contaminadas por

TBT (GODOI et al., 2003; ALMEIDA et al., 2004; FERNANDEZ et al., 2005) e de espécies de moluscos com imposex (FERNANDEZ *et* al., 2002; LIMAVERDE et al., 2007; COSTA, 2008 a,b; CARDOSO et al., 2009) na costa brasileira é provável que o TBT esteja presente nos tecidos de outros animais que ali vivem, inclusive na espécie de ermitão *Clibanarius vittatus*, que é típica de regiões estuarinas (MELO, 1999; SANT'ANNA et al., 2006).

O efeito desta substância já foi verificado em alguns crustáceos, podendo causar mortalidade (KAHN et al., 1993), retardar a regeneração (WEIS et al., 1987; KAHN et al., 1993), ocasionar deformações em partes do corpo (WEIS et al., 1987) e levar à alterações reprodutivas. OBERDORSTER et al. (1998) verificaram que em certas doses há estímulo à produção do hormônio masculinizante testosterona em *Daphnia magna*, Straus 1820. VERSLYCKE et al. (2003) constataram que o TBT induz androgenização no misidáceo *Neomysis integer* (Leach, 1814) em concentrações extremamente baixas, da ordem de 10 ng/l, enquanto OHJI et al. (2003) demonstraram que a exposição a níveis de 10 a 100 ng/l de TBT inibe a oogênesis e compromete a reprodução em *Caprella danilevskii* Czerniavski, 1868.

Com base nestas informações e como o mecanismo de determinação das características sexuais secundárias em ermitões e gastrópodes é semelhante (CHARNIAUX-COTTON e PAYEN, 1985; BETTIN et al., 1996; DEPLEDGE et al., 1999), supõe-se que o TBT possa ter um efeito semelhante nos ermitões. Em ermitões (e decápodes em geral) a determinação do sexo e, conseqüentemente, das características sexuais secundárias depende da expressão de genes que promovem o desenvolvimento da glândula androgênica (CHARNIAUX-COTTON, 1956; CHARNIAUX-COTTON e PAYEN, 1985), a qual leva à produção do hormônio androgênico que desencadeia o processo de masculinização. Assim, ao afetar o desenvolvimento desta glândula, o TBT teoricamente pode desencadear o processo de masculinização em ermitões.

Neste panorama é necessário que estudos preliminares sejam realizados para avaliar a existência de relação causa/efeito entre este poluente (TBT) e a intersexualdade em ermitões. Como os estudos sobre a contaminação por TBT são incipientes no Brasil, isto é, em pequeno número e localizados, faz-se necessário o esforço inicial para o mapeamento adequado

de áreas contaminadas por TBT. Além disso, os registros existentes baseiam-se em indicadores biológicos (FERNANDEZ et al., 2002, 2005; CASTRO et al., 2005a, b) e em dosagens diretas obtidas via amostras de sedimentos (GODOI et al., 2003; FERNANDEZ et al., 2005). Segundo, GODOI et al. (2003), devido a oscilações nas condições oceanográficas, este composto pode ser re-suspendido de forma que ruídos podem estar associados em suas determinações diretas no sedimento. Assim, a determinação do TBT em tecidos animais associada à contaminação no sedimento corresponde a uma estratégia de análise mais adequada para estas situações como também sugerido por TAKAHASHI et al. (1999). No Brasil, tal estratégia foi aplicada somente na Baía de Guanabara, no Rio de Janeiro por FERNANDEZ et al. (2005) e no Porto de Paranaguá por SANTOS et al. (2009). Neste sentido, uma análise com esse delineamento experimental (analisando contaminação no ambiente e em populações de ermitões, com ampla cobertura geográfica) poderá permitir o mapeamento de áreas efetivamente contaminadas por TBT, fornecendo suporte para outras pesquisas e ações voltadas ao gerenciamento deste tipo de impacto no ambiente marinho.

O TBT é utilizado como agente anti-incrustante em tintas náuticas de forma que este tipo de contaminação é acentuada em estuários e locais com grande movimentação de embarcações (GODOI et al., 2003; SANTOS et al., 2009). Coincidentemente, este é o ambiente característico da espécie de ermitão *Clibanarius vittatus*, fato que o qualifica como um potencial indicador da presença de TBT. A seleção desta espécie para o presente estudo está baseada em diversas características: 1 - tamanho de seus indivíduos, o qual é adequado para manipulação e dissecção; 2- existência de trabalhos prévios sobre intersexualidade (TURRA e LEITE, 2000; TURRA, 2004, 2005, 2007; SANT'ANNA et al., 2006); 3 - fácil captura e identificação e 4 - suas populações são comuns e abundantes no litoral brasileiro.

A via de assimilação e o tempo de depuração do TBT em ermitões também não são conhecidos, mas acredita-se que seja semelhante a outras espécies de organismos marinhos, isto é, a assimilação provavelmente ocorre através de difusão passiva pelas brânquias (FENT, 1996; COELHO et al., 2002a), via cadeia trófica com biomagnificação (EVANS e LAUGHLIN, 1984; LAUGHLIN et al., 1986; SUN et al., 2001) como já foi observado para

caranguejos braquiúros como *Callinectes sapidus* Rathbun, 1896 por (RICE) et al., 1989 e *Chionoecetes opilio* (Fabricius, 1788) por ROULEAU et al. (1999) ou ambos (HOCK, 2001; COELHO et al. 2002a, b).

O tempo de depuração do TBT pode variar entre organismos (BRYAN et al., 1987; BRYAN et al., 1988; PAGE et al., 1995; LI et al., 1997; BARTLETT et al., 2004) devido a capacidade de metabolização desta substância, por isso, a investigação do tempo de depuração do TBT por espécies marinhas, é peça chave para a identificação de potenciais indicadores biológicos para esta substância. Pelo exposto acima, fica evidente que estudos mais aprofundados sobre a intersexualidade, que levem em consideração aspectos morfo-histológicos reprodutivos e análise da sua relação com o TBT, bem como a possível contaminação de ermitões no ambiente são necessários tanto para a compreensão desse fenômeno intrigante dentre os ermitões como para o gerenciamento da contaminação por TBT nos estuários brasileiros.

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Capítulo 2

Occurrence and behavior of butyltins in intertidal and shallow subtidal surface sediments of an estuarine beach under different sampling conditions

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Occurrence and behavior of butyltins in intertidal and shallow subtidal surface sediments of an estuarine beach under different sampling conditions

Abstract

Contamination by butyltin compounds (BTs) has been reported in estuarine environments worldwide, with serious impacts on the biota of these areas. Considering that BTs can be degraded by varying environmental conditions such as incident light and salinity, the short-term variations in such factors may lead to inaccurate estimates of BTs concentrations in nature. Therefore, the present study aimed to evaluate the possibility that measurements of BTs in estuarine sediments are influenced by different sampling conditions, including period of the day (day or night), tidal zone (intertidal or subtidal), and tides (high or low). The study area is located on the Brazilian southeastern coast, São Vicente Estuary, at Pescadores Beach, where BT contamination was previously detected. Three replicate samples of surface sediment were collected randomly in each combination of period of the day, tidal zone, and tide condition, from three subareas along the beach, totaling 72 samples. BTs were analyzed by GC-PFPD using a tin filter and a VF-5 column, by means of a validated method. The concentrations of tributyltin (TBT), dibutyltin (DBT), and monobutyltin (MBT) ranged from undetectable to 161 ng Sng^{-1} (d.w.). In most samples (71%), only MBT was quantifiable, whereas TBTs were measured in only 14, suggesting either an old contamination or rapid degradation processes. DBT was found in 27 samples, but could be quantified in only one. MBT concentrations did not differ significantly with time of day, zones, or tide conditions. DBT and TBT could not be compared under all these environmental conditions, because only a few samples were above the quantification limit. Pooled samples of TBT did not reveal any difference between day and night. These results indicated that, in assessing contamination by butyltin compounds, surface-sediment samples can be collected in any environmental conditions. However, the wide variation of BTs concentrations in the study area, i.e., over a very small geographic scale, illustrates the need for representative hierarchical and composite sampling designs that are compatible with the multiscalar temporal and spatial variability common to most marine systems. The use of such sampling designs will be necessary for future attempts to quantitatively evaluate and monitor the occurrence and impact of these compounds in nature.

1. Introduction

The occurrence and behavior of organometallic compounds such as butyltins (BTs) in coastal regions have received much attention because of their environmental, toxicological, and socioeconomic impacts on near by communities (CAO et al., 2009; SANTOS et al., 2009). Harbor regions or zones with intense shipping traffic, such as docks, are highly impacted by BTs because these compounds are widely used in antifouling paints (GODOI et al., 2003). Although paints containing tributyltin (TBT) have been banned since 2008 (CHAMP, 2003; FERNANDEZ and PINHEIRO, 2007), TBT and its degradation products, dibutyltin (DBT) and monobutyltin (MBT) can still be detected in estuarine regions worldwide. The study of the behavior of BTs in beaches located in estuarine areas with a high risk of contamination can provide information about the distribution, bioavailability, and impact of these pollutants in this reduced environment.

Among BTs, TBT causes the most concern because of its high toxicity to aquatic organisms (FENT, 2003). With the continuous input of TBT into the aquatic environment, the marine biota has been exposed, especially in estuarine regions (REES et al., 2001; LIMAVERDE et al., 2007), which are important reproductive and nursery habitats for many marine species. Approximately 120 coastal gastropod species have been affected by TBT (GARAVENTA et al., 2006), causing male characters to appear, an irreversible phenomenon known as imposex (LIMAVERDE et al., 2007).

Because of their hydrophobicity, BTs compounds tend to be adsorbed onto sediment particles. BTs are very persistent in sediments because of their slow degradation rate, particularly in anoxic and/or cold environments (MICHAUD and POELETIER, 2006). This persistence of BTs in sediments can affect not only the benthic biota but also organisms in the water column, due to resuspension and desorption processes, especially in very dynamic and variable environments (CAO et al., 2009). About 95% of TBT in the water column is associated with suspended matter, and the remainder is associated with dissolved organic matter and organic and inorganic bonds (GADD, 2000).

Because the distribution, transport, and concentrations of BTs in estuarine systems are significantly affected by the sediment organic-matter

content (HOCH, 2001), fine-sediment remobilization, which may be related to physical factors such as tides, waves, currents, and fresh water input (salinity) (BUGGY and TOBIN, 2006), is thought to be responsible for small-scale and short-term variability of BTs concentrations in the substrate. Although some parts of estuaries, mostly beaches, are composed of sand and the amount of organic matter and fine particles may be very low, the effect of this contaminant on the benthic fauna will be a consequence of its effective concentrations in these areas. In other words, the contamination of the fauna of a given area, which involves local processes of intake and magnification through the food chain, will be essentially dependent on the amount of BTs locally available to and consumed by deposit-and/or suspension-feeding organisms.

The possibility that the concentrations of BTs may be affected by sediment dynamics and also by many other factors, raises concern regarding sampling strategies in intertidal and shallow subtidal estuarine areas. Because of the substantial tidal and daily cyclic variations in environmental conditions and physical factors, sampling designs that do not consider such effects could lead to inaccurate estimates and erroneous conclusions. Therefore, we presume that knowledge of the behavior of these pollutants in estuarine beaches will help to determine the correct way to evaluate the environmental impact of BTs, similarly to other studies involving fine sediments of deep areas. Therefore, the present study aimed to systematically evaluate the concentrations of BTs (MBT, DBT, and TBT) in estuarine sediments under different abiotic conditions, considering period of the day (day or night), zone (intertidal or subtidal), and tide conditions (high or low).

2. Materials and methods

2.1 Study Area

This study was conducted on the southeastern Brazilian coast, in the São Vicente Estuary at Pescadores Beach (23º58'21''S; 46º23'35''W) (Fig. 1), near Santos Bay where the largest Brazilian harbor is located and where sediment contamination by BTs (54-482 ng Sn g^{-1} (d.w.)) was previously recorded (GODOI et al., 2003). The estuary is dynamic and strongly influenced by semidiurnal tides, with areas and periods of high sedimentation rates. The tidal range can vary from 0.27 m during neap tides to1.23 m in spring tides (HARARI et al., 1990).

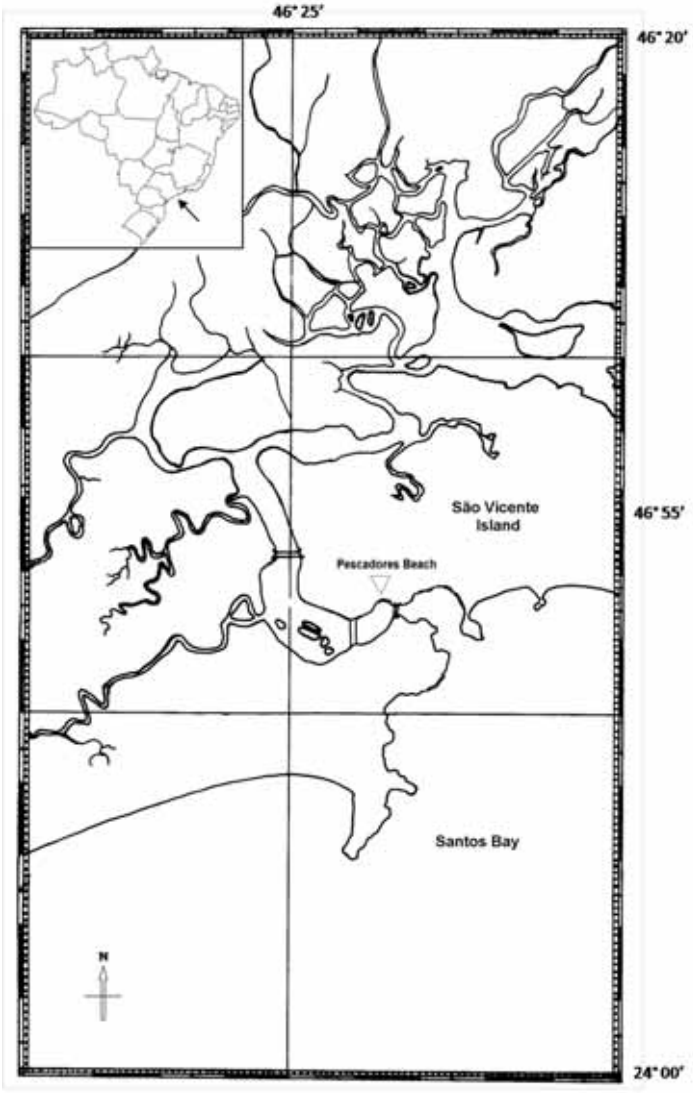

Figure 1 - Location of the study area, Pescadores Beach, São Vicente Estuary, São Paulo, Brazil.

2.2 Sampling Strategy

A 300m stretch of the beach was divided into three equal areas (A, B, and C), where three random samples were collected in the middle intertidal zone and in the shallow subtidal (40 cm depth out from the ebb-tide line) as shown in Fig. 2, during high and low tides, in daytime (from 7:00 to 17:00 h) and at night (from 19:00 to 4:00 h). Thus, three sampling points were chosen in each combination of beach part (A, B, or C) and tidal zone (intertidal or subtidal), totaling 72 samples of sediment (100 g) that were collected during a spring-tide period in February 2008.

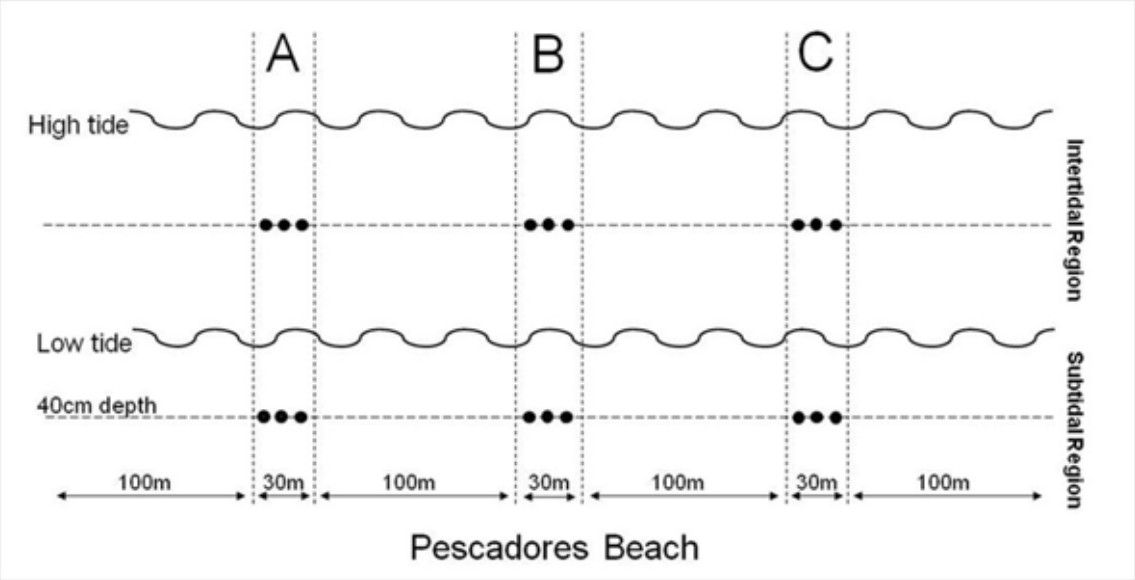

Figure 2 - Schematic illustration of the sampling design for this study at Pescadores Beach, São Vicente, São Paulo, Brazil.

After the field collections, each sample was divided into two sub-samples, labeled, and stored in aluminum-foil packets. One sample was separated for BT analysis, frozen (20ºC), and subsequently lyophilized. The other sample was refrigerated (0ºC) and used for granulometric and organic matter analyses.

Initially, we planned to compare the concentrations of MBT, DBT, and TBT per gram of dry weight between subareas (A, B, and C), tide condition (high and low), zones (intertidal or subtidal), and periods of the day (day or night), with 3 replicates in each area, i.e., a hierarchical sampling. However, not all sampling points showed measurable BTs contamination (see below), and it

was therefore not possible to compare the subareas. Comparison of MBT concentrations was possible between zones, tides, and periods of the day, using a three way analysis of variance (UNDERWOOD, 1997). TBT concentrations were compared only between periods of the day, after pooling all quantifiable samples, using Student's *t* test (ZAR, 1999).

2.3 Analytical Methods

2.3.1 Reagents

BTs standards (95% butyltin trichloride, 96% dibutyltin dichlor ide, 96% tributyltin chloride), a surrogate (98% tripropyltin chloride), and an internal standard (96%tetrabutyltin) were purchased from Sigma-Aldrich (Milwaukee, WI, USA), as were neutral aluminum oxide and Grignard reagent (2 M pentylmagnesium bromide in diethyl ether). Acetic acid and sulfuric acid, sodium hydroxide, and anhydrous sodium sulfate were purchased from JT Baker (Xalostoc, Mexico). Hexane and toluene were acquired from Mallinckrodt (Xalostoc, Mexico). Ammonium pyrrolidine dithiocarbamate (98% APDC) was purchased from Fluka (St. Gallen, Switzerland).

2.3.2 Organic-matter determination and granulometric analysis

The sediment granulometry was analyzed by sieving the sediment sample, and the result was expressed as the total percentage of sediment particles below 63 m in diameter. Organic matter (OM) was determined using a semi-quantitative method that involved digestion with a hydrogen peroxide solution (10% H_2O_2, corresponding to 12 volumes of O_2) exchanged every 48 h until all organic matter was consumed. The percentage of organic matter was estimated from the difference in weight before and after digestion (EPA, 2002).

Because hydrophobic organic contaminants such as BTs have an affinity for organic matter and the amount of small-sized particles (<63µm) in sediments, the need for a normalization procedure should be evaluated (BERG et al., 2001; BURTON et al., 2005). First, the relationships between BTs and the organic matter content and percentage of fine particles were established through correlation analyses. If the relationship is not significant, no normalization is needed. If the relationship is significant, then data should be

normalized according to the best correlation coefficient (OM or grain size). MICHELSEN (1992) suggested that normalization should include the division of the dry-weight concentration by the percentage of OM. A similar procedure was suggested by REIDAND and SPENCER (2009) to convert the concentration of contaminants by the amount of fine particles. In the present study the Spearman's rank correlation coefficient, with a significance level of $P<0.05$, was used.

2.3.3 BTs extraction and analysis

BTs were extracted from surface sediment following the method of GODOI et al. (2003). The surrogate (50 mL TPrT, 300 ng ml^{-1}) was added to 2g of lyophilized sediment in a test tube. BTs were extracted with 10ml toluene and 4ml acetic acid (vortex 1min, ultrasonic bath 5min, centrifuged for 5min at 2000 rpm). This procedure was repeated three times, and the extracts were transferred to a separation funnel. To the combined extracts, 10mL of APDC (0.1% in water) was added to improve the extraction. The organic phase was then transferred to pear-shaped flasks, dried with anhydrous sodium sulfate, and subsequently evaporated in a rotary evaporator at 40 and 50ºC until 2mL final volume.

For derivation, 3mL of Grignard reagent was added to 2mL of the concentrated extract. There action was stopped after 20min by adding 20mL of ultrapure water under ice cooling. Following the solubilization of the white precipitate with a few drops of sulfuric acid, the solution was transferred to a separation funnel, and the aqueous phase was discarded. The organic phase was then re-concentrated to 2mL, dried with sodium sulfate, and passed through an aluminum column for clean up with hexane as eluent. Final extracts were concentrated again to 1mL with N_2 and TeBT corresponding to1000ng g^{-1} was added as the injection standard.

Extracts were analyzed by gas chromatography: Varian3800 (Walnut Creek) equipped with a pulsed flame photometric detector (PFPD) and a VF-5 column (5% phenyl-methylpolysiloxan) using the temperature program: 130ºC (1min), 130-280ºC (10ºC min^{-1}), 280ºC (4min); injection mode: splitless for 1min; injected volume: 2 mL; detector temperature: 300ºC; injector temperature: 250ºC; carrier gas helium at a flow rate of 1.7 ml min^{-1}.

2.3.4 Quality Control

Quality control for BTs analysis was based on procedural blanks, spiked samples, TeBT as the internal standard, and TPrT as the surrogate for recovery evaluation. The accuracy and precision of the method were checked using PACS-2 marine sediment reference material (National Research Council of Canada, Ottawa, Canada), and the results were in good agreement with the certified values. The results were in accordance with analytical validation recommendations (EURACHEM, 1998; IUPAC, 2002), i.e., recovery of 70-120% and RSD (relative standard deviation) below 20%. The detection limit (DL) and quantification limit (QL) of the analytical system were obtained by a linearity curve according to the Hubber test (RIBANI et al., 2004). The first value outside the linear interval was considered as the detection limit, and the first value within this interval was considered as the quantification limit. The DL and QL (ng Sn mL^{-1}) values obtained were, respectively, 7 and 13 for TBT, 16 and 25 for DBT, and 25 and 33 for MBT.

3. Results

The concentrations of BTs obtained for the 72 sediment samples analyzed, as well as the percentages of organic carbon and particles <63 μm are listed in Table1 (day time samples) and Table 2 (nocturnal samples).

On Pescadores Beach, BTs were present in both the intertidal and subtidal zones. MBT contamination was recorded in 52 of 72 samples (71%), ranging from n.d. (not detected; 1 sample) to 161 ng Sn g^{-1} (d.w.). Nevertheless, DBT and TBT were found above the quantification limits in only a few (1 and 14, respectively) samples, and therefore it was impossible to carry out fully orthogonal statistical comparisons between periods of the day, tidal zones, or tide conditions for these compounds.

There was no significant correlation between TBT or MBT and either the organic-matter content (*rs*=0.18, *DF*=5, *P*=0.70 and *rs*=0.24, *DF*=43, *P*=0.11, respectively) or the percentage of fine particles (*rs*=0.54, *DF*=5, *P*=0.21and *rs*=0.16, *DF*=43, *P*=0.31, respectively), indicating that normalization was not necessary. The comparison of TBT levels was only possible by pooling the samples taken in day time (n=7) and those taken at night (n=7), which revealed that the period of the day showed no influence on the TBT concentrations (*t*=0.50; *DF*=13, *P*=0.62). Similarly, the MBT concentrations showed no significant differences between different periods of the day, tidal zones, or tide conditions (Table3).

Table 1 – BTs concentration (mean ng Sn g^{-1} (d.w.) ± sd), organic-matter content (%), and percentage of particles <63 µm in surface sediments from Pescadores Beach (São Vicente, São Paulo, Brazil), collected during the day in different tidal zones (subtidal and intertidal) and periods (high and low), including three random replicate samples (1, 2, and 3) in three different sectors in the study area (A, B, and C).

Sample	Concentration ng Sn g^{-1}(d.w.) ± sd				MBT	Organic	Particles
	MBT	DBT	TBT	∑BTs	(%)	Matter (%)[b]	<63 µm (%)
Subtidal - High tide							
A1	71±11	e	e	71	100	4.0	2.1
A2	99±36	e	e	99	100	5.3	2.7
A3	103±7	d	e	103	100	3.6	1.8
B1	d	d	e	-	-	3.4	1.5
B2	d	d	e	-	-	c	c
B3	73±6	e	e	73	100	2.6	3.3
C1	d	d	e	-	-	c	c
C2	d	d	e	-	-	2.2	4.4
C3	68±9	d	e	68	100	c	c
Subtidal - Low tide							
A1	d	d	e	-	-	2.7	1.1
A2	d	d	e	-	-	4.4	1.7
A3	d	d	e	-	-	2.8	3.7
B1	d	d	e	-	-	c	c
B2	86±12	e	e	86	100	3.7	1.9
B3	d	d	e	-	-	c	c
C1	d	d	e	-	-	4.6	2.1
C2	80±14	d	e	80	100	3.7	3.8
C3	74±6	d	e	74	100	7.0	2.7
Intertidal - High tide							
A1	78±7	e	e	78	100	3.0	4.6
A2	76±4	d	e	76	100	4.2	3.7
A3	70±4	e	e	70	100	3.8	6.5
B1	69±4	d	e	69	100	2.9	1.7
B2	76±12	d	e	76	100	3.8	3.1
B3	d	d	e	-	-	2.2	1.3
C1	d	e	47±45	47	0	3.9	4.0
C2	78±8	d	64±6	142	54	3.5	4.0
C3	d	d	e	-	-	2.8	5.0
Intertidal - Low tide							
A1	d	d	e	-	-	c	c
A2	71±2	e	73±1	144	50	3.4	6.9
A3	87±2	e	e	87	100	5.3	3.9
B1	109±3	d	137±7	246	44	5.1	3.4
B2	72±2	e	e	72	100	3.1	2.0
B3	d	d	42±3	42	0	3.3	2.1
C1	72±4	d	e	72	100	3.3	3.6
C2	38±6	e	16±24	54	70	c	c
C3	161±30	d	29±29	190	84	c	c

b = EPA method, H_2O_2 digestion; c = Not analyzed; d = Not detected; e = Detected but below quantification limit.

Table 2 – BTs concentration (mean ng Sng^{-1} (d.w.) ± sd), organic-matter content (%),and percentage of particles <63 µm in surface sediments from Pescadores Beach (São Vicente, São Paulo, Brazil), collected during the night in different tidal zones (subtidal and intertidal) and periods (high and low), including three random replicate samples (1, 2, and 3) in three different sectors in the study area (A, B, and C).

Sample	Concentration ng Sn g-1(d.w.) ± sd				MBT	Organic	Particles
	MBT	DBT	TBT	∑BTs	(%)	Matter (%)[b]	<63 µm (%)
Subtidal - High tide							
A1	69±5	d	e	69	100	3.9	2.0
A2	d	d	e	-	-	c	c
A3	71±3	+	18±26	89	79	3.8	1.9
B1	d	d	54±19	54	0	c	c
B2	70±2	e	e	70	100	4.4	2.4
B3	68±1	e	e	63	100	3.4	2.0
C1	75±8	d	e	75	100	4.0	1.9
C2	24±6	e	46±10	70	34	c	c
C3	161±10	61±3	56±14	278	57	c	c
Subtidal - Low tide							
A1	105±25	d	e	105	100	4.3	2.1
A2	68±2	e	e	68	100	6.0	2.7
A3	81±10	d	e	81	100	4.9	2.5
B1	72±1	d	e	72	100	2.5	4.1
B2	73±6	d	e	73	100	5.8	2.7
B3	140±24	d	66±17	206	67	c	c
C1	132±7	d	e	132	100	c	c
C2	72±8	e	e	72	100	8.7	3.1
C3	71±4	d	e	71	100	7.1	3.1
Intertidal – High tide							
A1	71±5	e	e	71	100	5.3	3.6
A2	70±4	e	e	70	100	3.2	4.8
A3	76±11	e	e	76	100	3.7	7.6
B1	e	d	e	-	-	3.0	2.3
B2	72±7	e	e	72	100	2.9	2.2
B3	72±4.4	d	e	72	100	2.3	1.3
C1	73±2	d	e	73	100	3.8	3.5
C2	76±7	e	e	76	100	2.3	2.4
C3	d	d	e	-	-	2.9	3.2
Intertidal - Low tide							
A1	70±6	d	e	70	100	2.8	5.8
A2	119±22	e	60±31	179	66	6.9	5.3
A3	69±7	e	e	69	100	3.2	5.8
B1	d	d	e	-	-	2.7	1.4
B2	70±2	d	e	70	100	c	c
B3	73±5	d	e	73	100	4.4	2.8
C1	78±6	e	e	78	100	2.5	3.4
C2	d	d	51±6	51	0	c	c
C3	88±11	e	e	88	100	4.3	3.7

b = EPA method, H_2O_2 digestion; c = Not analyzed; d = Not detected; e = Detected but below quantification limit.

Table 3 – Three-way analyses of variance for MBT concentrations in surface sediments at Pescadores Beach (São Vicente, SP, Brazil), comparing period of the day (day vs. night), tidal zones (subtidal vs. intertidal), and tide condition (high vs. low). Degrees of freedom (*DF*); mean square (MS).

Variables	***DF***	**MS**	***F***	***P***
Period of day	1	18	0.005	0.95
Tidal zone	1	950	0.242	0.63
Tide condiction	1	3994	1.018	0.32
Period of day x Tidal zone	1	700	0.179	0.68
Period of day x Tide condiction	1	518	0.132	0.72
Tidal zone x Tide condiction	1	472	0.12	0.73
Period of day x Tidal zone x Tide condiction	1	1731	0.441	0.51

4. Discussion

According to HOCH (2001), the behavior of environmental BTs can be directly influenced by abiotic factors such as temperature, salinity, pH, UV radiation, redox potential, organic-matter content, and sediment granulometry, which may determine the speciation, complexation, degradation, and bioavailability of BTs in the aquatic environment.

In this study, we examined the sediment granulometry and its organic-matter content as possible determining factors for the distribution of BTs in the study area. Particles below 63 µm were examined because of their high potential to adsorb organic compounds (HOCH et al., 2003), as well as the sediment organic- matter content, which is mentioned as the most important parameter affecting BTs adsorption in sediments (HOCH, 2001; BUGGY and TOBIN, 2006).

The results indicated a lack of correlation between these parameters and the MBT and TBT contents, even though TBT is more lipophilic than MBT. SHIM et al. (1999) observed a lower correlation between sediment organic matter and TBT, and explained this by an excess of BTs in relation to the availability of adsorption sites in the sediment. However, this lack of correlation in the present study may be caused by the small proportion of fine particles in the sediment, which was common to most samples.

The samples were collected over a short period of time, so as to minimize the possible effects of temporal variations on the sorption of BTs (see HOCH and SCHWESIG, 2004) and to consider only small- scale variations in sediment properties. The variation in BTs concentrations was high (Fig. 3), even between replicates that were collected from the same part of the beach under the same sampling conditions. This indicates that the environmental parameters analyzed did not have any effect on the concentration of these compounds, and/or that BTs are irregularly distributed in small patches through the study area. On the other hand, the different sampling conditions tested in this study could, at least in part, explain the overall variability in the measured concentrations, but the results did not support this possibility.

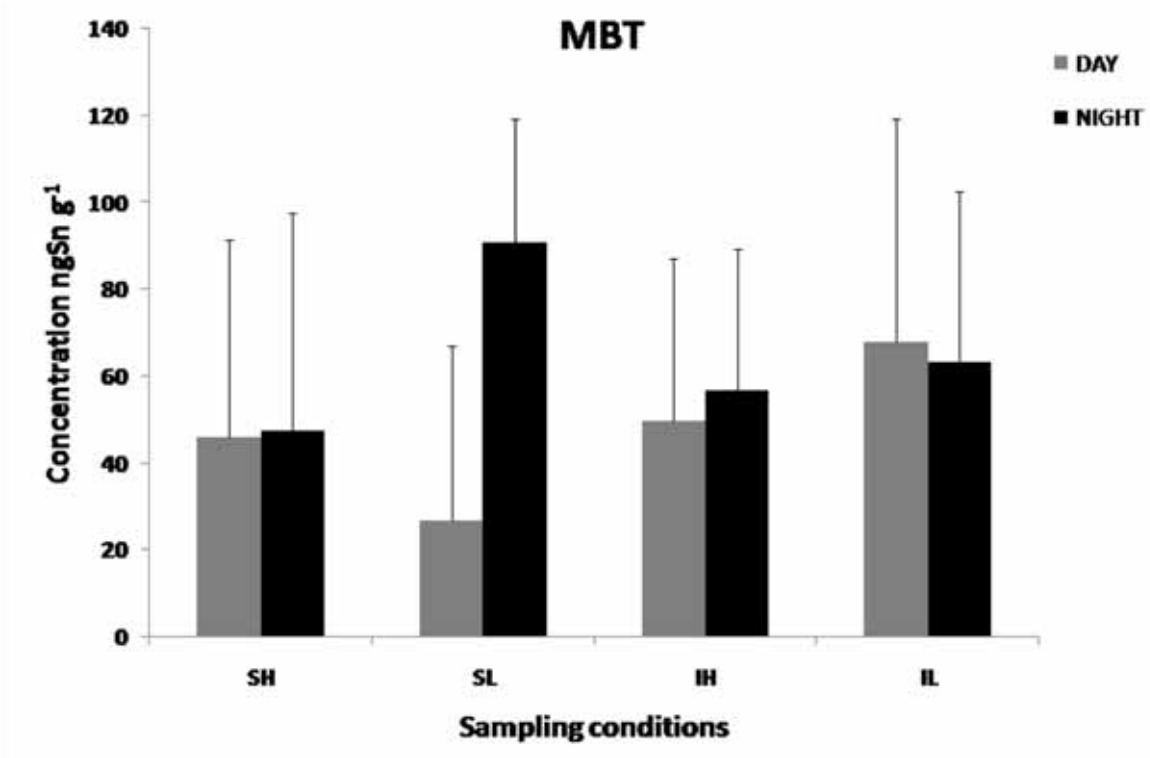

Figure 3 - MBT average concentrations (ng Sn g^{-1} (d.w.)) and standard deviation (SD) recorded in day and night samples in four sampling conditions tested: (SH) Subtidal - High tide; (SL) Subtidal - lowtide; (IH) Intertidal - hightide; (IL) Intertidal – low tide.

Generally, studies on BTs in marine sediments have used a sampling strategy consisting of point samples collected near harbors and docks or shipping channels, because these sites are hot spots of TBT contamination (SHIM et al., 1999; GODOI et al., 2003). However, the presence of BTs in the shallow subtidal and intertidal zones is largely unexplored. At these sites, the stability of surface sediments can be greatly affected by tides and water turbulence (BUGGY and TOBIN, 2006), although this was not corroborated by our results; and the concentration of suspended particles in the water column can vary drastically (temporally and spatially) as a result of hydrodynamic action (HOCH and SCHWESIG, 2004). Moreover, oscillations in the sample content as well as different processes of sorption and desorption of BTs on sediment particles can occur (LANGSTON and POPE, 1995). Increased salinity from the input of salt water at high tides can also affect these processes, because of the decreased BTs sorption onto minerals (HOCH and SCHWESIG, 2004), although the results of the present study also did not corroborate this. In addition, contaminant dynamics in this environment can be strongly affected by

estuarine circulation patterns, involving the transport mainly of silt and clay particles (FÚLFARO and PONÇANO, 1976) through increased mixing of fresh and salt water by storms, flood tides, and wind action (BUGGY and TOBIN, 2006), especially in the rainy season. Therefore, the wide variation in the concentrations of BTs in this area indicates the need for a sampling design that is compatible with the variability of the system, for a reliable quantitative evaluation of these compounds. Depending on the objectives of the study, composite samples, i.e., samples based on a homogenized pool of different sub-samples, may be better than individual replicates, because pooling samples may increase representativeness and reduce variability.

In addition to the variation between replicates, when different sampling conditions were compared, we found no significant differences in the concentration of BTs, indicating that micro-scale environmental factors tend not to influence the BTs concentrations. UV radiation can be an important factor in TBT photodegradation (RÜDEL, 2003), but no significant differences were detected in samples collected during the day or night, or between exposed (intertidal zone) and submerged (subtidal zone) sediments. We observed a small (but non-significant) variation in BTs concentrations in sediments collected at night. This probably resulted from the absence of UV radiation, which through its degradation effect can cause greater variation during the day. The UV radiation energy is 400 kJ mol^{-1} and Sn-C cleavage can occur at about 120-220 kJ mol^{-1}, allowing easy cleavage under sunlight (NUYTTENS, 2004).

Although the results for this study area did not support the hypothesis that the environmental factors studied influence the concentrations of BTs, as expected from the previous reports discussed above, this effect might be more evident in estuaries (or periods of the year) with greater variation in tide intensity, rainfall, UV radiation, and temperature.

The São Vicente estuary has heavy shipping traffic because of its proximity to Santos and São Vicente harbor, where TBT contamination has been reported (GODOI et al., 2003). However, only small wooden boats are based at Pescadores Beach. There are also several nearby marinas for pleasure boats, which may use TBT-based paints, and could be a potential source of BTs. Other sources of BT contamination such as urban and industrial

wastewater (HOCH, 2001) may also contribute to the input of these compounds into estuarine systems.

As mentioned, nearly all investigators have found higher occurrences of TBT near harbor areas, because of the constant input from antifouling paint (HOCH, 2001; FENT, 2003; GODOI et al., 2003; RÜDEL, 2003). Nevertheless, because of the transport of suspended particles and the photochemical and biological degradation of BTs in these areas, significant differences could be noted in the degree of TBT degradation near harbor zones compared to inner-bay sites (FELIZZOLA et al., 2008). It is very common to find the highest TBT concentrations near harbors and docks, and higher MBT concentrations far from pollution sources.

The occurrence of MBTs, which were often the only BTs detected and/or quantified in the majority of samples in this study, may indicate an old contamination or else the influence of extremely dynamic environmental conditions that could accelerate the degradation of BTs, even in the case of recent contaminations. To determine whether contamination results from a current or an old input, the butyltin degradation index (BDI) was proposed by DÍEZ et al. (2002). The BDI is expressed as the ratio between the total of TBT degradation products and the TBT concentrations. A BDI lower than 1 indicates recent contamination, while results greater than 1 are attributed to an old BT input. When this concept was applied to the quantified TBT samples (14 samples), the response was unclear. The data indicated an old BTs contamination (mean BDI=1.4, ranging from 1.02 to 1.91) in 3 samples, and recent contamination in seven others (mean BDI=0.41, ranging from 0.25 to 0.82). The last four samples contained only TBT, and therefore the index could not be calculated.

The presence of TBT degradation products in oxidizing sediments, commonly found in most exposed areas such as intertidal and subtidal zones, may be favored because this environment facilitates TBT degradation through the stronger affinity of tin (Sn) for oxygen (O) atoms, creating a more stable bond (Sn-O). However, aerobic biodegradation by the microbial sediment community can be more rapid in these areas, and TBT half-lives that usually last only 1-3 months may be increased in anoxic environments to several years (LANDMEYER et al., 2004). Some investigators have suggested that TBT can

degrade directly to form MBT (MARCIC et al., 2006), which may explain why DBT was present in only one of 72 samples analyzed in this study. Other researchers have concluded, based on laboratory assays, that MBT is found mainly in estuaries because of its strong adsorption on sediment particles by ionic interaction (HOCH, 2001) and its high polarity. This may explain the massive presence of MBT, which was the only BT quantified in 41 samples in this study.

The degradation process of BTs in the environment does not stop with MBT, but continues in a progressive loss of organic groups until Sn (IV) or (II) is formed (RÜDEL, 2003). Tin concentration in environments is usually low (RÜDEL, 2003), and according to LANDMEYER et al. (2004), increases proportionately to the decrease of butyltins in sediments. Under some environmental conditions, similarly to other metals, Sn (IV or III) can be complexed and resuspended, and become available to the biota (BIRCH and TAYLOR, 1999). Inorganic tin can be methylated by specific microorganisms and chemical processes, becoming toxic again in its methylated form (HOCH, 2001; RÜDEL, 2003).

Although TBT was present above the quantification limit in only a few of the samples analyzed, the local biota can take up this substance before it is degraded. Follow-up studies, including testing the sediment sampling conditions as well as the local biota, would indicate whether this uptake is actually occurring, as has been demonstrated for hermit crabs collected in this area (unpublished data).

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Capítulo 3

Environment and biota contamination by butyltins in the eastern Brazilian coast after their national and international ban

Environment and biota contamination by butyltins in the eastern Brazilian coast after their national and international ban

Abstract

Organotin-based anti-fouling paints cause environmental contamination that is very problematic to non-target species so that their use was banned in several countries around the world. In the present study, the Butyltin (Bt) contamination was evaluated in 25 estuaries (considering only hermit crab tissues) and 13 estuaries (considering sediments and hermit crabs tissues) along 1740 Km of the eastern Brazilian coast. Three samples composed by tissues of five hermit crabs and three samples of sediment were collected in three areas of each estuary. The Bts concentrations for each sample were quantified by gas chromatography. Hermit crab tissue contamination by TBT and DBT (MBT was not evaluated) was respectively detected in 22 and 24 from the 25 estuaries sampled. The highest values of TBT and DBT were recorded in Iguape estuary, an area without harbors and with several marines for small boats, with concentrations of TBT up to 318 and DBT up to 1746 ng Sn g^{-1} in hermit crab tissues. Sediment contamination by Bts was detected in 12 out of the 13 estuaries sampled. For TBT and DBT the highest concentration were detected in harbors areas of Vitória and São Francisco do Sul estuaries respectively 565 and 158 ng Sn g^{-1}. For MBT a non harbor area, Balneário Camboriú estuary was the most contaminated with 1304 ng Sn g^{-1}. A great variability was detected among samples of the same estuary, i.e., TBT varied from 4 to 299 ng Sn g^{-1} and DBT varied from 33 to 637 ng Sn g^{-1} in hermit crab tissues and from 125 to 565 ng Sn g^{-1} in the Vitória estuary, for both pollutants in the sediment. In many samples of sediment Bts were not detected, while in the hermit crabs Bts were recorded even without Bts being recorded in the sediment. It indicates that the biotic matrix represents a better tool to access the environmental condition of Bts contamination. These results point out that the release of Bts in the marine environment is still occurring after the international and Brazilian ban or Bts compounds are retained in compartments of the environment with heterogeneous distribution around the estuaries.

1. Introduction

In the marine environment practically any solid surface is colonized by sessile organisms as barnacles, algae, sponges, and mollusks. The boat hulls are constantly occupied by these sessile organisms, which increase the fuel consumption by friction with the seawater (EVANS et al., 2000). To prevent the development of these organisms, antifouling paints with chemical products are applied to the hulls (STUER-LAURIDSEN and DAHL, 1995). The substance used commonly was tributyltin (TBT), developed in the 70's (GODOI *et al*., 2003a) and probably used until now, since the recurrent environmental and biota contamination recorded (SANTOS e*t* al., 2010; SANT'ANNA et a*l*., *in press*), despite the prohibition in many parts of the world (DOWSON *et* al., 1993; ALZIEU, 2000; TOSTE et al., *in press*).

The TBT causes serious effects in non-target species, including decrease in oyster production (ALZIEU, 2000), inhibition of growth in microalgae (HUANG et al., 2004), imposex development in mollusks (COSTA et al., 2008), delay in regeneration and production of deformities of crab limbs (WEIS et al., 1987), ovary disorganization and atrophy in hermit crabs (Sant'Anna *et al*., *submitted*) and promote changes in the structure of marine sediment communities (DAHLLOF et al., 2001). The exposure of marine organisms to TBT may lead to a decrease of biodiversity in the long-term (AXIAK et al., 2000).

The direct determination of Butylins (Bts), i.e. TBT and its derivates as DBT and MBT, in the environment (by sediment or water) has been conducted around the world (KO et al., 1995; MENG et al., 2009; SANTOS et al., 2009; WAISBAUM et al., 2010). However, the concentration of Bts in sediments may be highly variable among closely spaced samples in a given location even in subsequent days (SANTOS et al., 2010). This patchy behavior of Bts distribution is related to the occurrence of small size sediment particles, which, in turn, may be easily remobilized by water movements. In the same way, estimates of Bts concentrations in the water are also subjected to uncertainties derived from its dynamic characteristics as well as the presence of organic particulate materials. Thus, measurements in these abiotic matrices may be not represent accurately the contamination for a given site.

An alternative strategy to assess TBT contamination in nature is the use of animals such as gastropod species as bioindicators. In England, the gastropod *Nucella lapillus* (Linnaeus, 1767) was the first species used to access TBT effects on the biota (BRYAN et al., 1986). In the Pacific, neogastropods such as *Thais clavigera* (Kiister, 1858) are widely used to monitor tributyltin contamination (CHAN et al., 2008). Recently, another gastropod, *Stramonita haemastoma* (Linnaeus, 1767) was used to assess TBT impact, in Brazilian waters (LIMAVERDE et al., 2007). In these studies TBT monitoring is based on the occurrence of imposexed gastropods (individuals with male secondary sexual characteristics imposed in females) (BRYAN, 1986, 1987; CHAN et al., 2008).

Although, such bioindicators may be useful to pollution monitoring, their application is limited to species that display direct alterations related to the pollutant contamination. In this way, the direct determination of TBT in animal tissues is an alternative to be used in situations where bioindicators are not available. In Taiwan, TBT concentration was determinate in many aquatic species as coral reefs, gastropods, bivalves and fish (see review in MENG et al., 2009). In Canada, Bts were recorded in 13 benthic and pelagic organisms (VIGLINO et al., 2006). In the Brazilian coast, vertebrates such as cetaceans (DORNELES et al., 2008) and invertebrates, as ascidians (PLETSCH et al., 2010) and mollusks (LIMAVERDE et al., 2007) showed Bts contamination.

Clibanarius vittatus (Bosc, 1802) is a hermit crab species that lives in estuarine regions (MELO, 1999), areas generally subjected to harbor activities. This hermit crab was demonstrated to assimilate TBT and DBT from food and depurate these compounds rapidly (SANT'ANNA et al., *in press*). This species also has relatively large body sizes (TURRA and LEITE, 2000), long life spans (4 to 6 year; TURRA and LEITE, 2000; SANT'ANNA et al., 2008), and relatively low mobility (HAZLETT, 1981) showing all characteristics to be good indicator of recent or recycled TBT and/or DBT contamination in estuaries (SANT'ANNA et al., *in press*). Thus, in the present study the occurrence of Bts in this hermit crab species and in the surface sediment were evaluated in 25 estuaries along the Southwestern Atlantic coast in Brazil.

2. Material and Methods

2.1 Animal Samples

A total of 45 estuaries comprehending 1740Km along the Brazilian coast were visited between October 2008 and February 2010 in order to capture the hermit crab *Clibanarius vittatus*, which was recorded in only 25 estuaries (Table I). In each estuary 15 hermit crabs (three samples of five individuals in each locality), were collected in the intertidal region of three localities totaling nine samples in each estuary and 45 hermit crabs.

After sampling, the animals were anesthetized by thermal shock in cold seawater, removed from the shells, and dissected. Gonads and hepatopancreas of each of the three samples per location (composed by tissues of five individuals) were labeled and stored -20ºC in aluminum-foil packages until Bts determination (see below). In estuaries where Bts were detected at least in three of the nine samples collected, their amounts were compared with one-way analysis of variance (UNDERWOOD, 1997).

2.2 Sediment Samples

Sediments were sampled in 13 estuaries (Table I) at same time of the hermit crab samples. In each estuary nine composed samples were collected in the intertidal region, being three samples in each of the three different localities in each estuary.

The samples were also labeled and kept -20ºC in glass vials until the time of Bts analysis (see below). In estuaries where Bts were detected at least in three of the nine samples collected, their amounts were compared with one-way analysis of variance (UNDERWOOD, 1997). The possible correlation between Bts in sediments and hermit crabs tissues was tested using the Spearman's rank correlation coefficient, with a significance level of $P<0.05$.

2.3 Analytical Methods

2.3.1 Reagents

Bts standards (95% butyltin trichloride, 96% dibutyltin dichloride, 96% tributyltin chloride), a surrogate (98% tripropyltin chloride), and an internal standard (96% tetrabutyltin) were purchased from Sigma-Aldrich (Milwaukee,

WI, USA), as were neutral aluminum oxide and Grignard reagent (2 M pentylmagnesium bromide in diethyl ether). Acetic acid and sulfuric acid, sodium hydroxide, and anhydrous sodium sulfate were purchased from JT Baker (Xalostoc, Mexico). Hexane and toluene were acquired from Mallinckrodt (Xalostoc, Mexico). Ammonium pyrrolidine dithiocarbamate (98% APDC) was purchased from Fluka (St. Gallen, Switzerland).

2.3.2 Bts extraction and analysis (Hermit crab tissues)

The concentration of TBT was determined by the experimental procedure adapted from LIMAVERDE et al. (2007) developed to hermit crab tissues by CRISTALE et al. (submitted). The first extraction procedure used involves hydrochloric acid and methanol, following by toluene extraction, as solvent. Complexation with 0.1% ammonium pyrrolidine dithiocarbamate (APDC) following derivatization using Grignard reagent. The organic fraction was removed and cleaned up in an aluminum oxide column, and eluted with hexane. The final extract was concentrated to 0.1 mL, and tetrabutyltin was added as internal standard. The method recovery was assessed using a surrogate (TPrT) added in the beginning of analytical process for all samples. Analytical condition included a gas chromatography (GC) Varian 3800 (Walnut Creek, CA, USA) equipped with a pulsed flame photometric detector (PFPD) using a tin filter (390 nm) and a VF5 capillary column (30m x 0.25 mm – Varian, Walnut Creek, CA, USA). For the applied method, the detection limit was 0.8 and 1.4 ng Sn g^{-1} w. w. and the quantification limit was 2.6 and 2.2 ng Sn g^{-1} w. w. for TBT and DBT respectively. The precision of the method was checked by the use of certified reference material for mussel tissue ERM – CE477 with TBT and DBT recovery of 98±11% and 61±18% respectively (CRISTALE et al., submitted). Bts concentrations below quantification limit were considered as half of the obtained value for these parameters (1.3 and 2.2 ng Sn g^{-1} for TBT and DBT respectively) to statistical analysis. MBT quantification was not possible for organisms in recurrence of low response in quality control tests.

2.3.3 Bts extraction and analysis (sediment)

Bts were extracted from surface sediment following the method of GODOI et al. (2003). The surrogate (50 mL TPrT, 300 ng ml-1) was added to 2g

of lyophilized sediment in a test tube. Bts were extracted with 10ml toluene and 4ml acetic acid (vortex 1min, ultrasonic bath 5min, centrifuged for 5min at 2000 rpm). This procedure was repeated three times, and the extracts were transferred to a separation funnel. To the combined extracts, 10mL of APDC (0.1% in water) was added to improve the extraction. The organic phase was then transferred to pear-shaped flasks, dried with anhydrous sodium sulfate, and subsequently evaporated in a rotary evaporator at 40 and 50 ºC until 2mL final volume.

For derivation, 3mL of Grignard reagent was added to 2mL of the concentrated extract. There action was stopped after 20min by adding 20mL of ultrapure water under ice cooling. Following the solubilization of the white precipitate with a few drops of sulfuric acid, the solution was transferred to a separation funnel, and the aqueous phase was discarded. The organic phase was then re-concentrated to 2mL, dried with sodium sulfate, and passed through an aluminum column for clean up with hexane as eluent. Final extracts were concentrated again to 1mL with N2 and TeBT corresponding to 1000ng g^{-1} was added as the injection standard.

Extracts were analyzed by gas chromatography: Varian3800 (Walnut Creek) equipped with a pulsed flame photometric detector (PFPD) and a VF-5 column (5% phenyl-methylpolysiloxan) using the temperature program: 130ºC (1min), 130-280ºC (10ºC min-1), 280ºC (4min); injection mode: splitless for 1min; injected volume: 2 mL; detector temperature: 300ºC; injector temperature: 250ºC; carrier gas helium at a flow rate of 1.7 ml min-1.

Quality control for Bts analysis was based on procedural blanks, spiked samples, TeBT as the internal standard, and TPrT as the surrogate for recovery evaluation. The accuracy and precision of the method were checked using PACS-2 marine sediment reference material (National Research Council of Canada, Ottawa, Canada), and the results were in good agreement with the certified values. The results were in accordance with analytical validation recommendations (EURACHEM, 1998; IUPAC, 2002), i.e., recovery of 70-120% and RSD (relative standard deviation) below 20%. The detection limit (DL) and quantification limit (QL) of the analytical system were obtained by a linearity curve according to the Hubber test (RIBANI et al., 2004). The first value outside the linear interval was considered as the detection limit, and the first

value within this interval was considered as the quantification limit. The DL and QL (ng Sn mL-1) values obtained were, respectively, 7 and 13 for TBT, 16 and 25 for DBT, and 25 and 33 for MBT. Bts concentrations below quantification limit were considered as half of the obtained value for these parameters (6.5 and 8 ng Sn g^{-1} for TBT and DBT respectively) to statistical analysis.

Table I – Localities where the hermit crabs (white and grey) and sediments (grey) were sampled along the Southwestern Atlantic in the Brazilian coast.

Brazilian State	Estuary	Location 1 (S)	Location 1 (W)	Location 2 (S)	Location 2 (W)	Location 3 (S)	Location 3 (W)
Santa Catarina	Laguna	28° 29' 44.2''	48° 45' 39.9''	*	*	*	*
Santa Catarina	Florianópolis	27° 35' 11.2''	48° 33' 18.1''	27° 30' 33.3''	48° 31' 05.9''	27° 42' 11.1''	48° 33' 20.5''
Santa Catarina	Balneário Camboriú	27° 00' 14.0''	48° 36' 08.8''	27° 00' 26.1''	48° 36' 14.0''	27° 00' 32.9''	48° 36' 32.0''
Santa Catarina	Penha	26° 46' 55.7''	40° 36' 05.7''	26° 46' 13.0''	48° 39' 09.3''	26° 46' 21.7''	48° 39' 45.5''
Santa Catarina	Barra do Sul	26° 27' 16.0''	48° 35' 45.7''	26° 27' 02.6''	48° 37' 09.8''	26° 25' 57.5''	48° 37' 45.0''
Santa Catarina	São Francisco do Sul	26° 14' 43.5''	48° 38' 29.9''	26° 16' 32.6''	48° 40' 19.8''	26° 11' 53.2''	48° 39' 11.7''
Paraná	Baía de Paranaguá	25° 32' 58.4''	48° 23' 22.2''	25° 28' 34.9''	48° 30' 00.4''	25° 30' 13.2''	48° 31' 54.7''
Paraná	Baía das Laranjeiras	25° 30' 07.2''	48° 22' 54.0''	25° 26' 27.8''	48° 20' 19.2''	25° 25' 15.7''	48° 24' 52.3''
Paraná	Guaratuba	25° 52' 20.5''	48° 34' 35.7''	25° 52' 24.3''	48° 36' 15.8''	25° 51' 09.9''	48° 36' 24.0''
São Paulo	Iguape	24° 41' 19.2''	47° 27' 20.7''	24° 41' 32.9''	47° 27' 0.85''	24° 41' 03.1''	47° 27' 08.9''
São Paulo	São Vicente	23° 58' 21.0''	46° 23' 35.0''	23° 58' 15.7''	46° 22' 24.1''	23° 58' 37.7''	46° 22' 09.4''
São Paulo	Santos	23° 59' 27.8''	46° 18' 15.2''	23° 59' 13.9''	46° 17' 39.8''	23° 55' 53.6''	46° 18' 19.4''
São Paulo	Cananéia	25° 00' 35.7''	47° 55' 29.4''	25° 01' 13.9''	47° 55' 02.4''	25° 02' 02.2''	47° 55' 24,4''
São Paulo	Bertioga	23° 51' 18.0''	46° 08' 16.2''	23° 51' 17.9''	46° 08' 35.8''	23° 51' 32.1''	46° 08' 03.3''
São Paulo	São Sebastião	23° 48' 52.6''	45° 24' 24.3''	23° 48' 41.6''	45° 24' 28.9''	23° 48' 59.8''	45° 24' 24.2''
Rio de Janeiro	Paraty	23° 12' 38.7''	44° 42' 53.8''	23° 09' 03.0''	44° 41' 50.5''	23° 09' 17.9''	44° 42' 05.8''
Rio de Janeiro	Sepetiba	22° 58' 18.1''	43° 43' 06.0''	22° 59' 05.7''	43° 42' 00.6''	22° 59' 26.7''	43° 41' 22.9''
Rio de Janeiro	Coroa Grande	22° 54' 28.1''	43° 52' 26.6''	22° 54' 13.8''	43° 51' 48.6''	22° 54' 09.5''	43° 51' 09.9''
Rio de Janeiro	Saquarema	22° 56' 07.4''	42° 29' 33.3''	22° 55' 46.9''	42° 29' 47.5''	*	*
Espírito Santo	Vitória	20° 17' 35.6''	40° 17' 22.9''	20° 18' 06.0''	40° 17' 09.0''	*	*
Espírito Santo	Anchieta	20° 48' 10.5''	40° 39' 19.2''	20° 48' 21.3''	40° 39' 20.0''	20° 48' 18.3''	40° 39' 20.3''
Espírito Santo	Santa Cruz	19° 57' 09.7''	40° 09' 21.5''	19° 56' 59.8''	40° 09' 19.5''	19° 57' 00.6''	40° 19' 33.3''
Espírito Santo	Conceição da Barra	18° 35' 53.1''	39° 43' 45.7''	*	*	*	*
Bahia	Nova Viçosa	17° 52' 31.5''	39° 21' 12.4''	17° 52' 24.4''	39° 22' 01.9''	17° 52' 26.2''	39° 22' 10.2''
Bahia	Caravelas	17° 44' 24.5''	39° 15' 32.4''	17° 44' 48.2''	39° 14' 11.1''	17° 44' 39.2''	39° 14' 44.9''

*Locations where hermit crabs not were found and the sediment not was sampled.

3. Results

3.1 Hermit crabs contamination

Hermit crab contamination by TBT and DBT was detected at 22 and 23 from the 25 estuaries sampled, respectively (Table II). In the estuaries of Laguna, Santa Cruz and Conceição da Barra the TBT was not recorded, while DBT was not detected only in Laguna estuary. A great variability was recorded, even in samples from the same estuary, i.e., in Vitória Estuary TBT varied from 4 to 299 ng Sn g^{-1} and DBT varied from 33 to 637 ng Sn g^{-1} in hermit crab tissues (Fig. 1). Between estuaries the variability also was great, the lower values of TBT being recorded in the estuaries of Laranjeiras, Cananéia, Bertioga, São Sebastião and Paraty (1.32 ng Sn g^{-1}) and the highest ones in Iguape, Vitória, and São Sebastião estuaries (318, 299, and 277 ng Sn g^{-1}, respectively). The lowest DBT contamination was similar in 13 estuaries with 2.22 ng Sn g^{-1}, while the highest values were detected in Iguape, Vitória, Florianópolis and Caravelas (1746, 637, 482, and 403 ng Sn g^{-1}, respectively).

The highest concentration of TBT, 318 ng Sn g^{-1}, and DBT 1746 ng Sn g^{-1}, were recorded in Iguape. Significant differences were recorded in TBT contamination among estuaries (F=2.3546; DF=17; P=0.0044), however due to the great variability in the data within each area, no pairwise differences could be evidenced among the 18 estuaries analyzed.

Table II – Minimum (Min), maximum (Max), mean and standard deviation of tributyltin (TBT) and dibutyltin (DBT) concentration (ng Sn g^{-1}) in the hermit crabs tissues collected in 25 estuaries in the Southwestern Atlantic in the Brazilian coast. nd=below of detection limit.

Brazilian State	Estuary	TBT (ng Sn g^{-1})			DBT (ng Sn g^{-1})		
		Min	Max	X±SD	Min	Max	X±SD
Santa Catarina	Laguna (Lagun)	nd	nd	nd	nd	nd	nd
	Florianópolis (Floripa)	15	212	82.42±75.04	15	482	202.67±166.85
	Balneário Camboriú (Camb)	9	188	66.04±80.78	2.22	54	22.74±23.14
	Penha (Penha)	7	45	24.05±18.76	2.22	286	130.44±120.65
	Barra do Sul (BSul)	9	104	62.11±34.23	18	242	103.33±77.50
	São Francisco do Sul (SFSul)	8	48	33.75±19.88	9	74	125.25±186.10
Paraná	Guaratuba (Guara)	32	222	94.33±67.85	2.22	391	193.06±217.19
	Paranaguá (Paran)	34	60	42.67±9.61	2.22	42	16.38±12.91
	Laranjeiras (Laranj)	1.32	10	5.66±6.14	50	100	75.00±35.36
São Paulo	Cananéia (Cana)	1.32	69	19.67±21.63	2.22	79	52.75±19.16
	Iguape (Igua)	20	318	108.00±104.05	2.22	1746	564.17±628.42
	São Vicente (SV)	24	181	77.57±57.07	58	145	95.29±36.89
	Santos (Santos)	10	65	38.44±18.18	23	91	47.89±24.34
	Bertioga (Bert)	1.32	189	47.79±61.99	2.22	113	39.07±40.49
	São Sebastião (SS)	1.32	277	101.05±100.94	2.22	126	48.46±48.84
Rio de Janeiro	Paraty (Paraty)	1.32	42	14.55±14.57	2.22	38	12.95±13.32
	Coroa Grande (CG)	3	20	10.60±6.35	2.22	38	13.63±14.63
	Sepetiba (Sepet)	15	15	-	nd	nd	nd
	Saquarema (Saqua)	8	11	9.50±2.12	2.22	246	50.98±109.02
Espírito Santo	Anchieta	9	11	10.00±1.41	6	211	79.60±87.85
	Vitória (Vit)	4	299	111.60±118.82	33	637	175.00±262.11
	Santa Cruz (SCruz)	nd	nd	nd	2.22	14	6.09±4±81
	Conceição da Barra (Cbarra)	nd	nd	nd	42	56	49.33±7.02
Bahia	Nova Viçosa (NV)	6	11	8.80±2.28	35	296	128.00±97.27
	Caravelas (Carav)	3	47	19.13±16.83	2.22	403	127.03±127.93

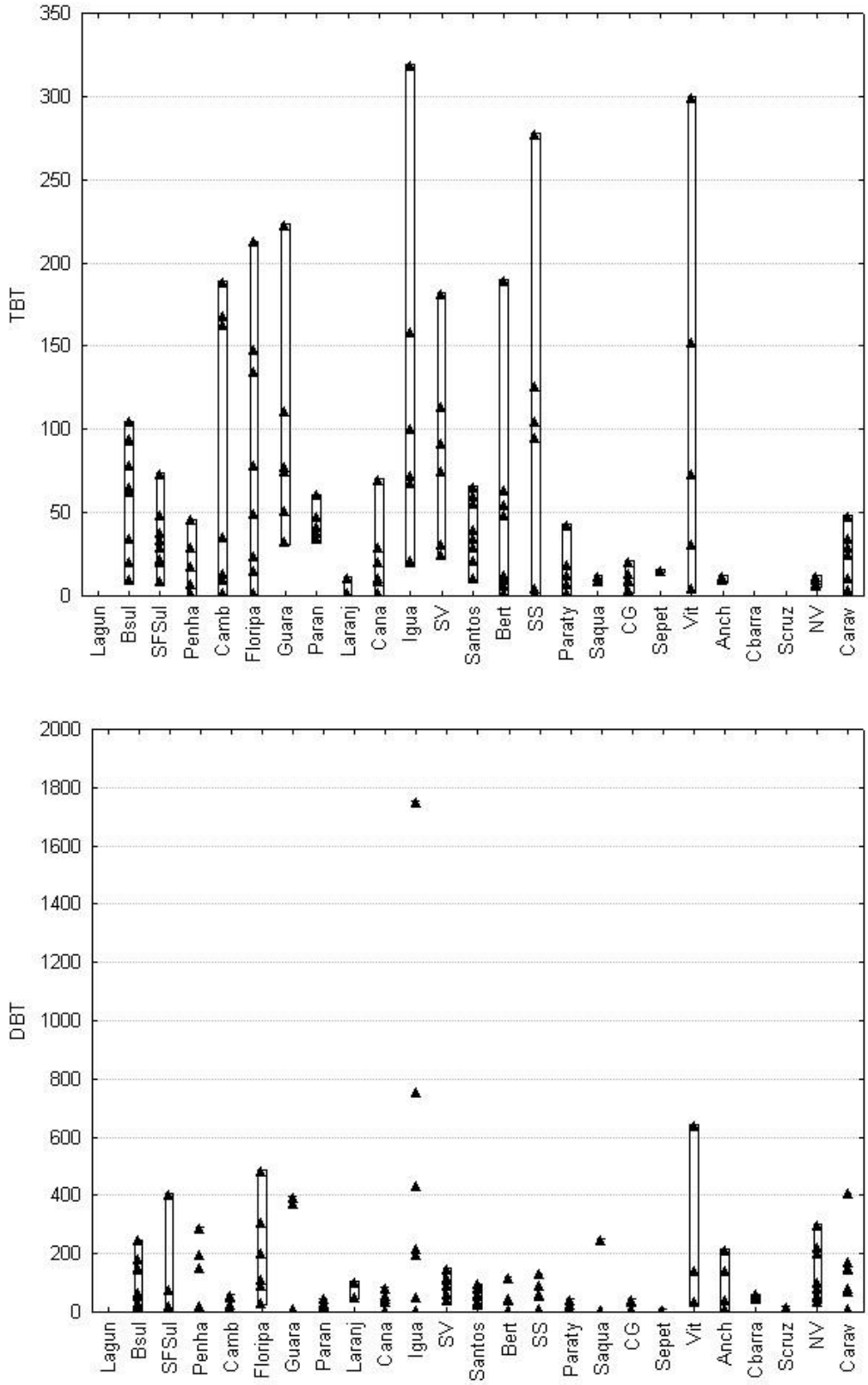

Figure 1 – Concentration (ng Sn g^{-1}) of tributyltin (TBT) and dibutyltin (DBT) in hermit crab tissues collected in 25 estuaries in the Southwestern Atlantic in the Brazilian coast. Abbreviations of names of estuaries are available in the table II.

3.2 Environmental contamination (sediments)

Contaminated sediments by Bts was detected in 12 out of the 13 estuaries sampled (Table III). The only exception was the Laguna estuary where any organotin compound was detected. The TBT compound was absent at Laguna, Barra do Sul, São Francisco do Sul and São Vicente estuaries, while DBT was not observed from the Laguna and Vitória estuaries. The samples from Laguna, Florianópolis, Barra do Sul, São Francisco do Sul and Paraty estuaries did not revel any trace of MBT (Table III). Similarly to hermit crab tissues great variability was detected in sediment samples of the same estuary, i.e., in Vitória TBT contamination varied of 125 to 565 ng Sn g^{-1}, DBT from 8 to 158 ng Sn g^{-1} in São Francisco do Sul and MBT from 822 to 1304 ng Sn g^{-1} also in São Francisco do Sul estuary (Fig. 2 and 3). Among estuaries the variability in the Bts contamination roughly followed the hermit crab contamination with great variation (Fig. 2 and 3).

Due to great the variability in TBT pollution within estuaries, very few statistically significant differences in its concentration in the sediments among estuaries could be recorded. Vitória presented the highest contamination (324.83±196.24 ng Sn g^{-1}), differing significantly from Cananéia (57.80±44.79 ng Sn g^{-1}) and Paraty (13.00±5.00 ng Sn g^{-1}) where the lowest concentrations were detected (F=3.32; DF=8; P=0.0101). DBT concentrations did not show significant differences among estuaries (F=1.9329; DF=8; P=0.1011), while MBT contamination revealed that Balneário Camboriú presented higher values (1034±187.18 ng Sn g^{-1}) than all others areas (F=42.8930; DF=5; P=0.0001).

Table III – Minimum (Min), maximum (Max), mean and standard deviation of tributyltin (TBT), dibutyltin (DBT) and monobutyltin in the sediments collected in 13 estuaries in the Southwestern Atlantic in the Brazilian coast. nd=below of detection limit.

Brazilian State	Estuary	TBT (ng Sn g-1)			DBT (ng Sn g-1)			MBT (ng Sn g-1)		
		Min	Max	X ±SD	Min	Max	X ±SD	Min	Max	X ±SD
Santa Catarina	Laguna	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Florianópolis	147	353	250.00±145.66	8	67	22.75±29.50	nd	nd	nd
	Balneário Camboriú	195	267	231±50.91	8	51	29.50±30.41	822	1304	1034±187.18
	Penha	98	199	148.50±71.42	8	120	57.67±57.06	62	100	85.33±20.43
	Barra do Sul	nd	nd	nd	8	8	8	nd	nd	nd
	São Francisco do Sul	nd	nd	nd	8	158	89.00±75.72	nd	nd	nd
São Paulo	Cananéia	24	114	57.80±44.79	22	118	58.20±40.13	30	34	32.00±2.83
	Iguape	197	204	199.70±3.79	49	155	92.33±55.58	40	80	61.67±20.21
	São Vicente	nd	nd	nd	8	8	8	25	83	44.67±33.20
	Santos	52	285	123.67±100.62	28	60	41.00±16.82	56	400	189.00±147.04
	São Sebastião	97	105	101.00±5.65	8	49	21.66±23.67	72	72	72
Rio de Janeiro	Paraty	8	18	13.00±5.00	7	41	16.71±11.71	nd	nd	nd
Espírito Santo	Vitória	125	565	324.83±196.24	nd	nd	nd	88	330	184.83±80.42

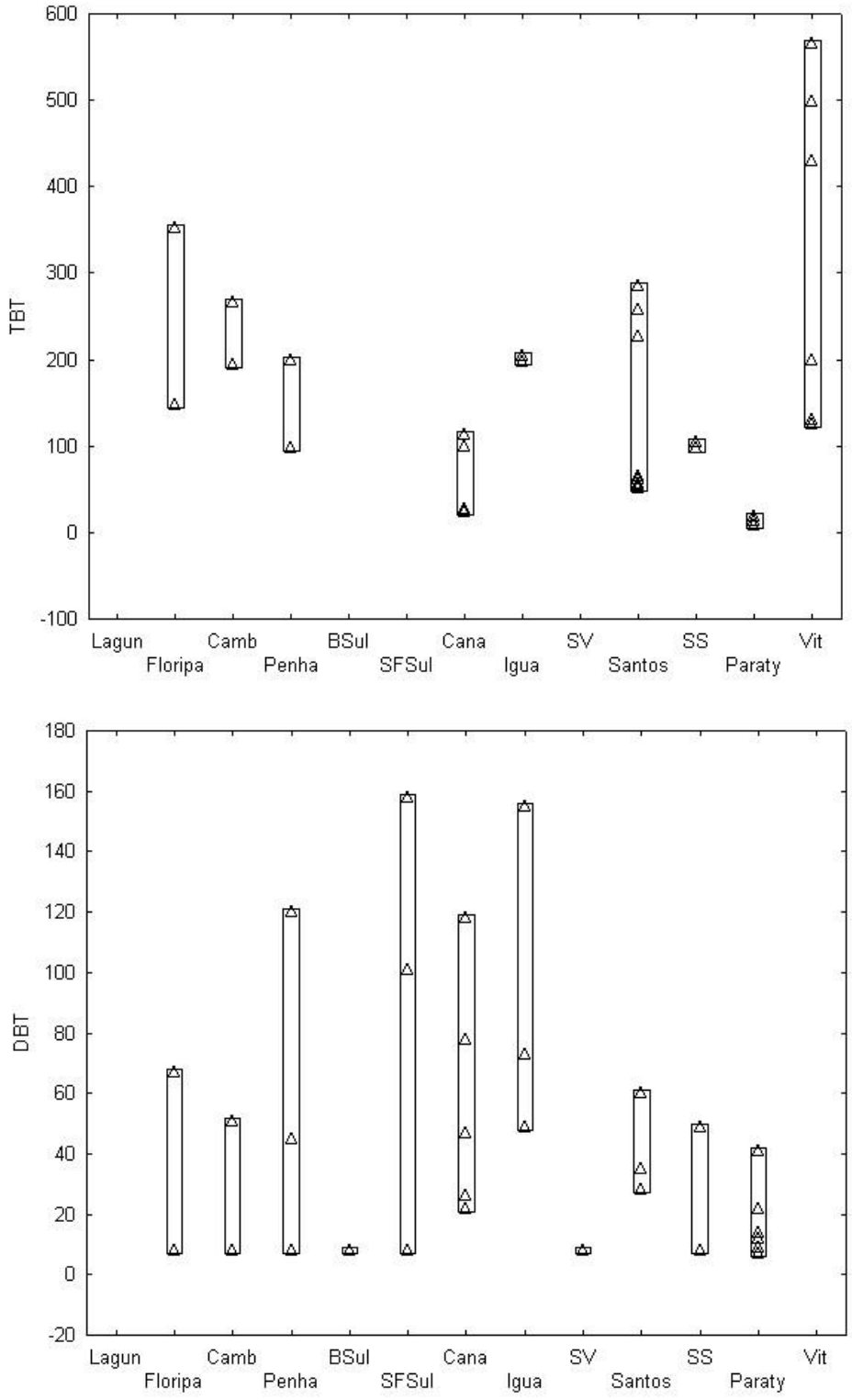

Figure 2 - Concentration of tributyltin (TBT, ng Sn g^{-1}) and dibutyltin (DBT, ng Sn g^{-1}) determined in sediments collected in 13 estuaries in the Southwestern Atlantic in the Brazilian coast. Abbreviations of estuaries names are available in the table II.

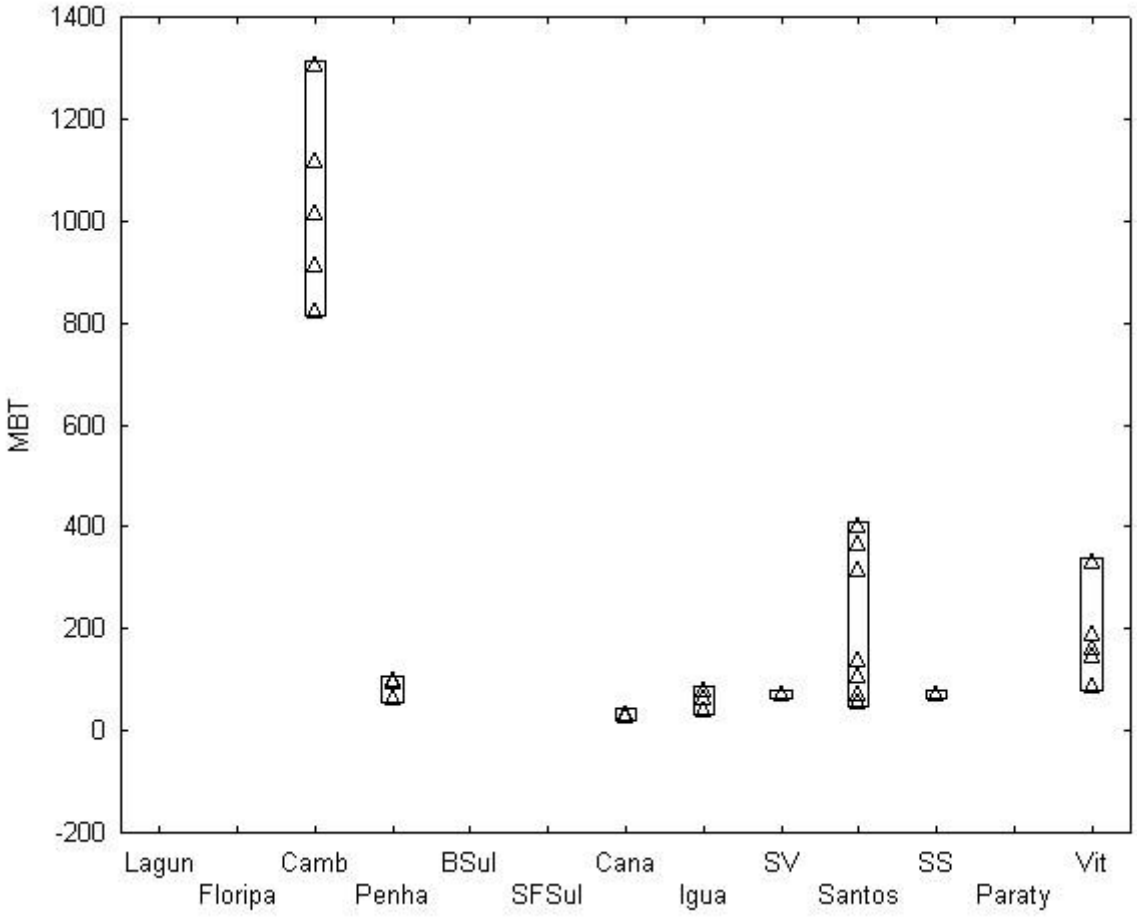

Figure 3 – Concentration of monobutyltin (MBT, ng Sn g^{-1}) determined in sediments collected in 13 estuaries in the Southwestern Atlantic in the Brazilian coast. Abbreviations of names of estuaries are available in the table II.

3.3 Biota vs. environmental contamination

The comparisons between TBT concentrations detected in hermit crab tissues and sediments of the same location showed no correlation between environmental and hermit crabs contamination (*rs*=0.0687; *DF*=27; *P*=0.7233). In general, TBT concentration in sediments was greater than hermit crab tissues (Fig. 4). For DBT there was also no correlation between environmental and hermit crabs contamination (*rs*=0.0537; *DF*=26; *P*=0.7861), and the concentrations in sediments and crabs was not very different. However, in estuaries of Florianópolis and Iguape the DBT in crabs was greater (Fig. 5).

In the 13 estuaries 108 samples of hermit crabs and 108 samples of sediments were analyzed.TBT was detected in 34 samples of sediment and 83 of hermit crabs. DBT was detected in 35 samples of sediment and 76 of hermit crabs (Fig. 6).

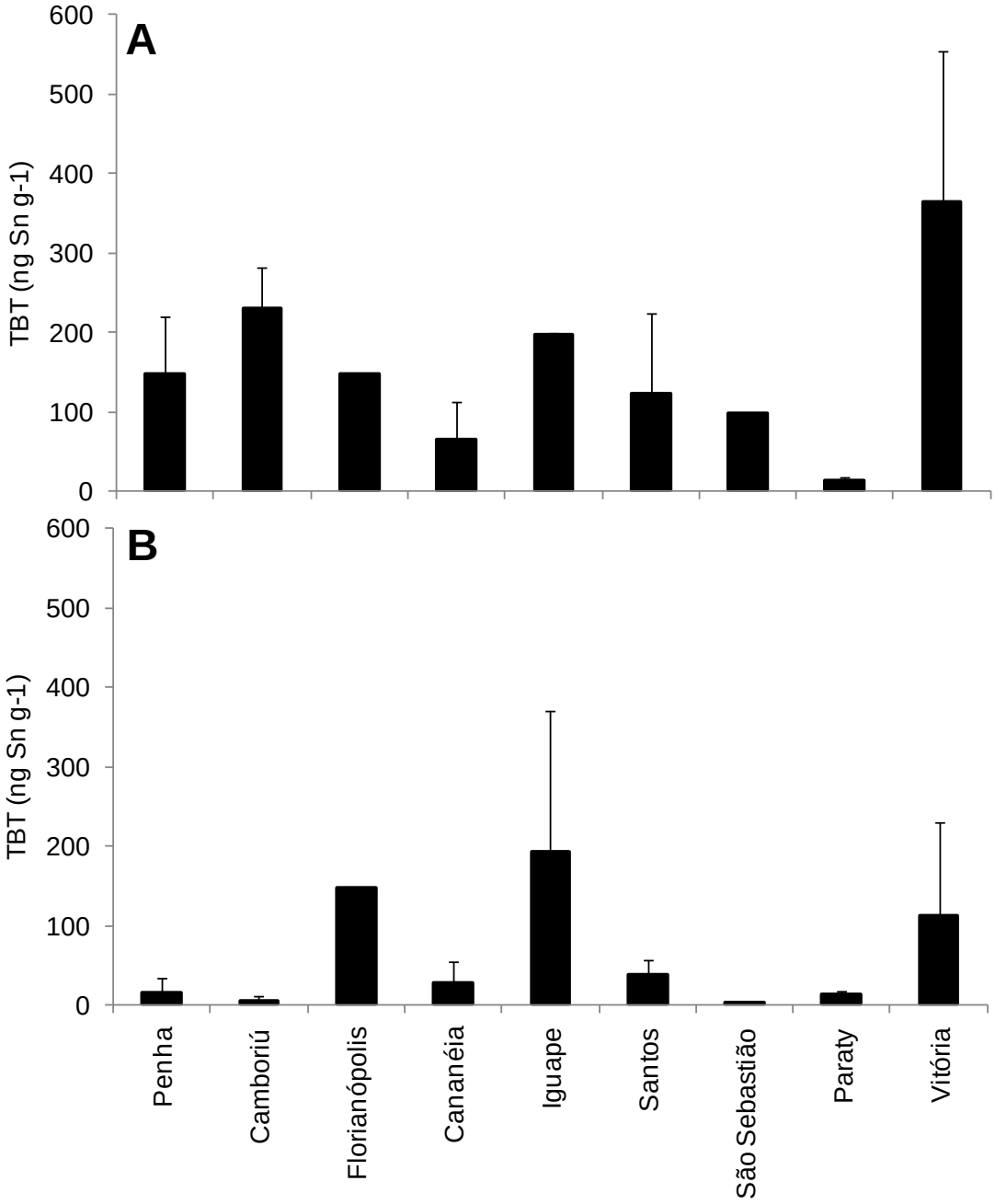

Figure 4 – Mean tributyltin (TBT) contamination in sediments (A) and hermit crab tissues (B) in estuaries in the Southwestern Atlantic in the Brazilian coast.

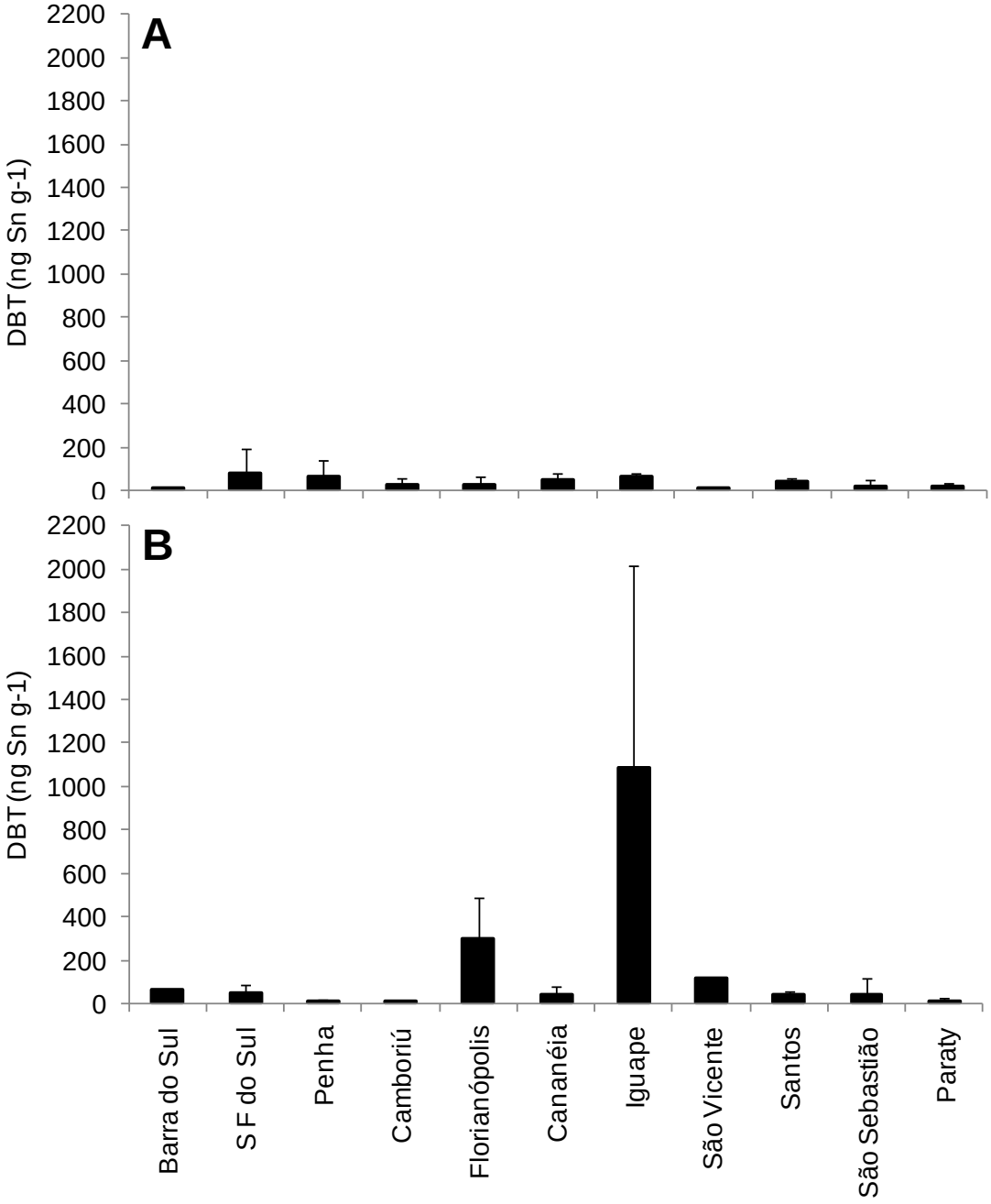

Figure 5 – Mean dibutyltin (DBT) contamination in sediments (A) and hermit crab tissues (B) in estuaries in the Southwestern Atlantic in the Brazilian coast.

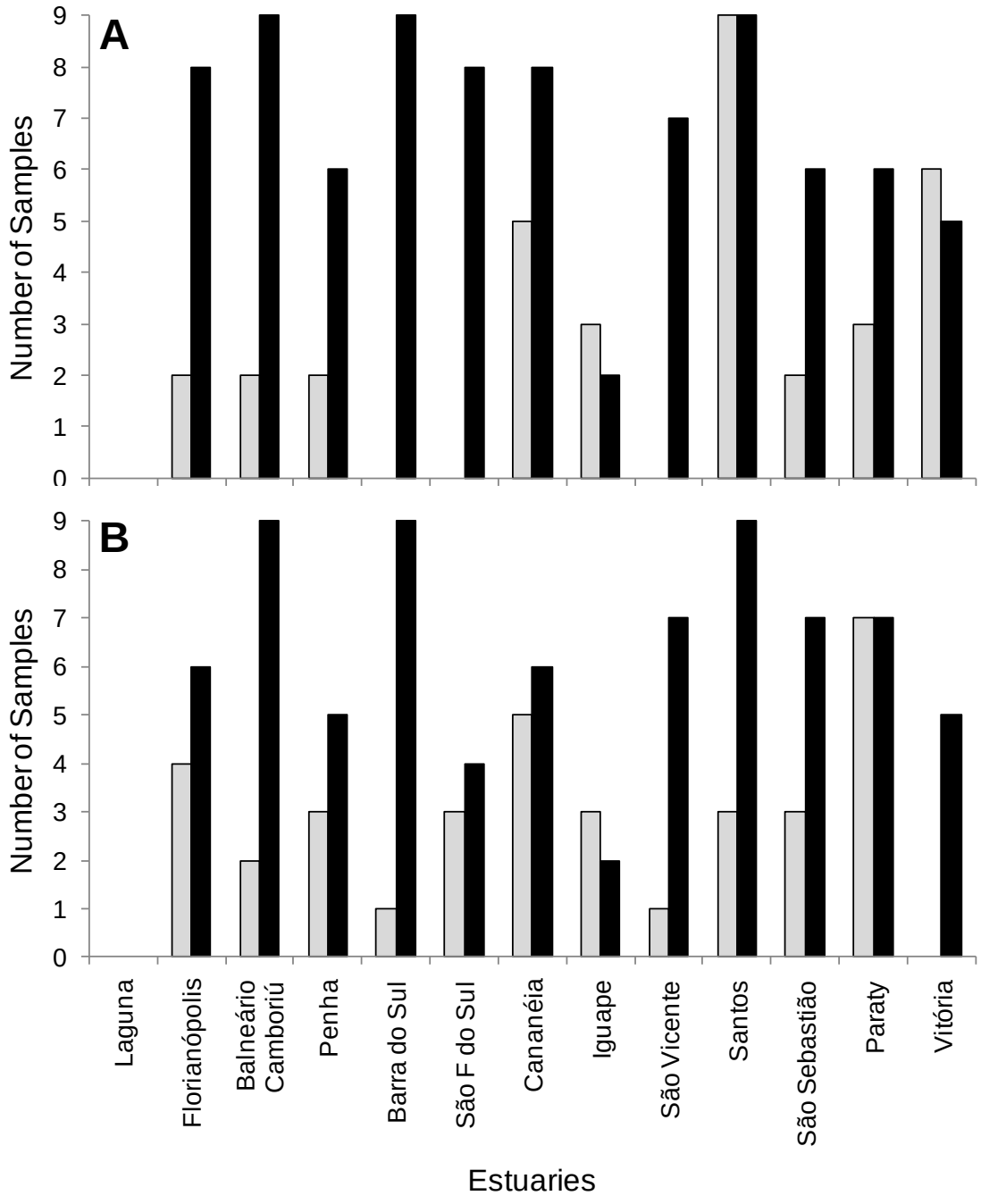

Figure 6 – Frequency of Tributyltin (TBT, A) and dibutyltin (DBT, B) contamination in samples of the hermit crab tissues (black bars) and sediments (grey bars) in 13 estuaries in the Southwestern Atlantic in the Brazilian coast.

4. Discussion

In many parts of the world, studies have been focused on the distribution of organotin compounds in coastal regions, because their impact represents a risk for aquatic and terrestrial ecosystems (see review of HOCH, 2001). Tributyltin contamination in Brazilian coastal waters was first recorded in sediments some years ago by GODOI et al. (2003b). Many studies were developed reporting the contamination in five Brazilian states along of south Atlantic coast (see review of SANTOS et al., *in press*). Estuarine regions with elevated shipping activity and harbors as Santos, Guanabara, and Paranaguá presented elevated concentrations of TBT in superficial sediments as 482, 521 and 2796 ng Sn g^{-1}, respectively (GODOI et al., 2003b; FERNANDEZ et al., 2005; SANTOS et al., 2009). However, the quantification of this pollutant in organisms for environmental monitoring is reduced and the results presented here correspond to the first data involving crustacean contamination in the Brazilian waters.

In the important harbors studied as Paranaguá and Santos, the concentration of butyltins in hermit crabs tissues were low when compared with that detected in regions where the ship traffic is less intense, as Guaratuba and Iguape. In Santos, where the biggest Brazilian harbor is located, the maximum TBT concentration was 65 ng Sn g^{-1} w.w., while the value reached 318 ng Sn g^{-1} w.w. in Iguape in hermit crab tissues. In sediments, the Vitória harbor was the most contaminated, however, locations without harbors, as Balneário Camboriú and Iguape had elevated Bts contaminations. These results reinforce the hypothesis that after organotin antifouling paint prohibition, this kind of paint became, irregularly, more accessible for local communities and the main actual source can be the small boats and their supporting infra-structures instead of large ships and harbors.

Although Iguape, in São Paulo coast, higher TBT concentrations were also observed in São Sebastião (São Paulo State) and Vitória (Espírito Santo State). In Vitória, other studies indirectly indicated TBT contamination in that area through an imposex monitoring with the mollusks *Thais deltoidea* (Lamarck, 1822) by COSTA et al. (2008a) and *Cymatium parthenopeum parthenopeum* (von Salis, 1793) by COSTA *et al*. (2008b).

TBT and DBT variation in tissues was also observed in other studies involving organisms (MORCILLO et al., 1997; HORIGUCHI et al., 2003; BERTO et al., 2007). Mostly, these data involve butyltin quantification in mollusks and the values recorded in these works can be compared to levels detected by GAVARENTA et al. (2007) using the

gastropod *Hexaplex trunculus* (Linnaeus, 1758) as bioindicator (35 to 482 ng Sn g^{-1} of TBT and 33 to 1648 ng Sn g^{-1} of DBT in the visceral coil) and to Brazilian locations LIMAVERDE et al. (2007) detected 5 to 110 ng Sn g^{-1} of TBT and 6 to 71 ng Sn g^{-1} of DBT in the gastropod *Stramonita haemastoma* (Linnaeus, 1767).

The variation found within the same estuary can be a result of many factors including the behavior of this contaminant in environment and its distribution and availability in aquatic compartments. According to YANG *et al*. (2008), the level of Bts in organisms can present temporal variation and depend on sampling site, salinity, concentration of metals in the water, the nutritional state of the individual, and the reproductive cycle. The record of DBT contamination in locations where TBT was absent in hermit crab tissues, may be explained by the concentration of TBT in diferents parts of the estuary. These animals can move through different regions of the estuary and probably are contaminated by TBT in adjacent locations. Further, this compound is degraded to DBT, when the crabs were sampled.

Furthermore, the butyltins organism's concentration is a result not only of abiotic matrices contamination level but also by exposure, contaminant distribution, and assimilation of the bioavailable contaminant species, being an union of environmental and phisiologycal processes. Because of its toxicity the Brazilian laws restricted the TBT use in 2007 and decided for the total prohibition in 2008 (IMO, 2001; NO 23, 2007). Despite these regulations, the present study showed that the biota and environment is still contaminated by this pollutant, even after the ban. The same results was recorded recently by TOSTE et al. (*in press*), that observed the increase of imposex rate in mollusks after the ban of TBT at Arraial do Cabo, Rio de Janeiro.

These evidences of estuarine contamination by TBT deserve attention by the authorities, once this substance was prohibited because even in low concentrations it may lead to development of imposex in mollusks besides others effects in the biota. Specifically for hermit crabs, this contamination in natural environments is very worrying, because TBT contamination in this group may lead to ovary atrophy (SANT'ANNA et al., submitted), that may reduce reproductive output and, consequently, population densities in the medium and long term.

Although TBT toxicity observed in many studies, DBT toxicity has to be also taken in account. In this work, DBT was recorded in almost all samples, sometimes in concentrations higher than TBT (except in Iguape estuary). This compound act as immune system inhibitor and cause neurotoxic effects in many organisms (HALLORAN et al., 1998;

BOUCHARD *et al*., 1999; ST-JEAN et al., 2002). TBT and DBT may interact synergically with inorganic metals and present a pronounced toxicity.

The high concentration of DBT in the hermit crab is mainly attributed to assimilation of this pollutant from the food source (SANT'ANNA et al., *in press*) and not only from degradation of the assimilated TBT, once estuaries are commonly represented as dynamical system where degradation process is intense and continuous, mainly on subtidal and infralitoral regions (SANTOS et al., 2010). However, the presence of TBT in almost all samples (hermit crab tissues) may be related to the Bts persistence in anoxic environments, also found in deeper estuarine regions (MICHAUD and POELETIER, 2006) and that can be transported by resuspension events (FELIZZOLA et al., 2008) or still be continuously release in the aquatic environments by antifouling paints.

In many sediment samples TBT and BDT were not detected. However, in hermit crab samples of the same locations Bts were recorded. This probably occurred due to an unequal Bts distribution in the sediments (SANTOS et al., 2010). As hermit crabs move in the environment, these biotic matrices represent a better resource to access the actual environmental condition of Bts contamination. Hermit crabs contaminated by Bts are temerarious. This species depurate Bts in few weeks (SANT'ANNA et al., *in press*) and the presence of this pollutant in the tissues may indicate the continous release in the environment by use of antifouling paints or contamination by Bts retained in environmental compartments. Thus, the Bts still affecting the biota even after the ban.

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Capítulo 4

Hermit crabs as bioindicators of recent Tributyltin (TBT) contamination

Hermit crabs as bioindicators of recent Tributyltin (TBT) contamination

Abstract

Evaluation of the assimilation pathway and depuration time of a given pollutant by aquatic species is important to understand the dynamics of this substance in the biota, and to search for potential ecological indicator species. In the present study, the uptake pathway and depuration time of the pollutant tributyltin (TBT) were investigated in the omnivorous hermit crab *Clibanarius vittatus*. The uptake pathway was investigated using hermit crabs collected in an area free of TBT. The crabs were held in the laboratory for 45 days, under one of four treatments: procedural control (PC) – water and food without TBT; T1 – water with and food without TBT; T2 – water without and food with TBT; and T3 – water and food with TBT. To determine the depuration time, the crabs were collected in a contaminated area, maintained in the laboratory with clean water, and removed every 15 days for 120 days, for determination of the TBT concentration by chromatographic analysis. The TBT was taken up by the crabs mainly via food. TBT (as well as Dibutyltin – DBT) was depurated rapidly by *C. vittatus*. After approximately 30 days, the initial concentration of 111±36 ng Sn g^{-1} w. w. decreased to 3±3 ng Sn g^{-1} w. w., and after 75 days the TBT concentration was below the detection limit. The rapid TBT depuration is useful information, since *C. vittatus* and possibly other hermit crabs may be used as indicators of recent or recycled TBT contamination.

1. Introduction

Marine ecosystems are impacted by a wide variety of pollutants, including heavy metals, oil, and organochlorine and organometallic compounds, among others, that originate from both land and sea (TODD et al., 2010). Exposure of the biota to these substances may lead to bioaccumulation and/or biomagnification processes.

Large amounts of heavy metals are introduced into the environment from different anthropogenic sources including mining, automobile emissions, leaching from landfills, and manufacturing (SHAZILI et al., 2006). Some organometallic compounds may be released from the paint of vessels when in contact with seawater. One of the most harmful organometallic compounds is Tributyltin (TBT), a substance used as an antifoulant in marine paints, and which is released into the marine environment. TBT may cause serious effects even in non-target species, including decreases in oyster production (ALZIEU, 2000), inhibition of growth in microalgae (HUANG et al., 2004), and imposex development in mollusks around the world (SHI et al., 2005).

Because of this toxicity, in 2001 the International Maritime Organization (IMO) sponsored the International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention). This Convention came into effect in September 2008, and banned the use of TBT in antifouling paints on ships (GIPPERTH, 2009). In Brazil, the Navy suspended the use of TBT on its vessels in 2003, and in 2007 enforced a regulation, NORMAM 23, to restrict the use of organotin compounds as an antifouling system (TOSTE et al., in press). Despite these national and international regulations, this substance is still being used on small boats, and continues to affect the biota (TOSTE et al., in press). This situation, together with the persistence and low degradation rate of TBT, particularly in anoxic and/or cold environments (MICHAUD and PELLETIER, 2006), account for the continuing presence of TBT in estuarine sediments (SANTOS et al., 2010) and in organisms (TOSTE et al., in press).

Although organotin compounds may be assimilated by the biota (PAGE et al., 1995; LOSSER et al., 2000; IKEDA and YAMADA, 2003), the relative importance of different uptake pathways of contaminants, i.e., from water, sediments, or food, in aquatic organisms has not been thoroughly studied (COELHO et al., 2002a, b), particularly in crustaceans. TBT in food is usually taken up by digestive cells and then distributed to other tissues via the bloodstream (ROULEAU et al., 1995). However, this compound may

also be assimilated in crustaceans by passive diffusion through the respiratory tract, as occurs in fish (YAMADA, 1992; IKEDA and YAMADA, 2003).

Study of the assimilation pathways and depuration time of pollutants is essential, because a species that lives in a contaminated area may be able to depurate the pollutant and eventually alleviate the toxic effects. In the neogastropod *Thais clavigera* (Kiister, 1858), CHAN et al. (2008) studied TBT depuration by transplanting specimens to a clean area, and observed that the toxic effect (imposex) was irreversible. In species that depurate TBT rapidly, such as the amphipod *Hyalella azteca* (Saussure, 1858), the decline in environmental concentrations of TBT may be followed by a rapid recovery of affected populations, as suggested by BARTLETT et al. (2004).

Hermit crabs are a diverse crustacean group, and some species such as *Clibanarius vittatus* (Bosc, 1802) are found in abundance in estuaries (MELO, 1999). In these areas they may be exposed to TBT pollution, which is generally high due to the concentration of boats and the presence of harbors and yacht basins (LIMAVERDE et al., 2007). This species has been found to be contaminated by TBT in the wild (unpublished data) and has relatively low mobility (HAZLETT, 1981), and is therefore a good candidate as an indicator of local TBT contamination. This study aimed at understanding the bioaccumulation pathway and depuration time of TBT in *C. vittatus*, and at evaluating the potential application of this crab as a biological indicator of TBT contamination.

2. Material and Methods

2.1 Bioaccumulation experiment

The hermit crabs used in this experiment were collected in May 2009 in the Cananéia Estuary (25° 00' 35.7"S and 47° 55' 29.4"W), state of São Paulo, southeast Brazil. This population of *C. vittatus* did not show detectable levels of butyltins (BTs), i.e., TBT and its metabolite dibutyltin (DBT) (unpublished data, see below for detection limits).

A total of 60 hermit crabs were used. After collection, 12 crabs (3 replicates of 4 individuals each) were anesthetized by thermal shock, dissected, and their organs (gonad and hepatopancreas) labeled and stored frozen at -20°C in aluminum-foil packages, until TBT determination (see below). These animals were used for the initial butyltin-free group or the experimental control (EC). The other 48 hermit crabs were acclimated for two days

and maintained for 45 days in aquaria (42 x 30 x 30 cm) filled with 20 L seawater (salinity 33 psu and ambient temperature °C), with constant aeration and a natural photoperiod. In each aquarium, six hermit crabs were kept separated by plastic netting, so that each animal received the same amount of food (Figure 1). To assess bioaccumulation through water and food, these crabs were distributed among four treatments: procedural control (PC); T1 – water with and food without TBT; T2 – water with and food without TBT; and T3 – water and food with TBT, as detailed in Table I.

The hermits were given a feed developed for shrimp farming, in which the TBT was agglutinated. The nominal TBT concentration offered in the water was 320 ng Sn L^{-1}, as the aquaria contained 20 L of water = 6400 ng Sn available to 6 hermit crabs in the aquaria, resulting in 533 ng Sn available for each hermit crab per day. The nominal TBT concentration in the food was 3200 ng Sn g^{-1} w. w., and 0.5 g of feed was offered every day (each crab ate approximately 0.5 g of food per day, and this amount of food was determined in previous tests), resulting in 1600 ng Sn per day. A high TBT concentration was offered in the feed because the experiment was carried out over only 45 days (compared with the total time of exposure to this pollutant in the environment during the entire life cycle of a crab). This difference in TBT administration did not compromise the experiment, because it was impossible to control the amount of TBT that was actually available and assimilated by the crabs through these two different pathways (i.e., the actual amount of food and the actual amount of water circulating through the gills).

After 45 days, all animals were killed by the same method as for the butyltin-free group (EC). For the TBT determination, the crab tissues (hepatopancreas and gonads) from each treatment were grouped in three replicates, each containing tissues from four individuals. The hypothesis that the amount of BTs (TBT and DBT) did not vary among treatments was tested using one-way ANOVA, followed by the Scheffé test for post-hoc pairwise comparisons (UNDERWOOD, 1997).

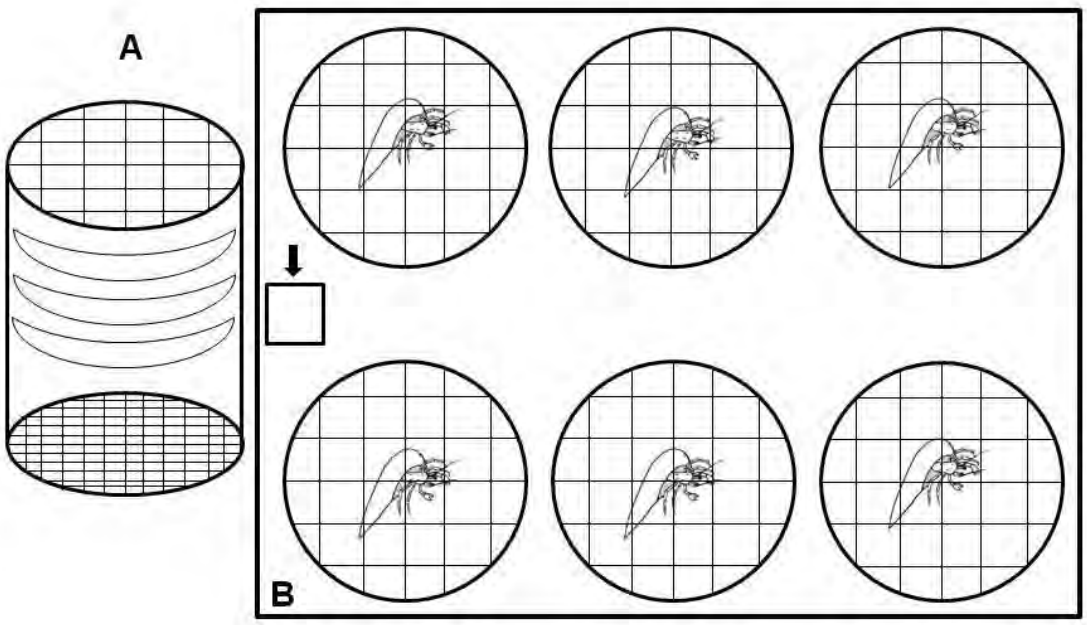

Figure 1 – Experiment on TBT bioaccumulation. A – Plastic structure where hermit crabs were confined in the experiment. B – Arrangement of the hermit crabs in the aquarium. The black arrow indicates the aerating pump.

Table I – Design of the experimental treatments for the bioaccumulation assessment experiment, and the amounts of TBT available daily per crab. In each treatment 12 individuals were used.

Treatments	Water (ng Sn)	Food (ng Sn)
PC	0	0
T1	533	0
T2	0	1600
T3	533	1600

2.2 Depuration experiment

The hermit crabs used in this experiment were collected in October 2008 at Araçá Beach near the São Sebastião Harbor (23° 48' 52.6''S and 45° 24' 24.3"W), state of São Paulo, Brazil. This population of *C. vittatus* was contaminated with 111±36 ng Sn g^{-1} w. w of TBT in the tissues analyzed.

In this experiment, 108 hermit crabs were used. After the crabs were collected, 12 individuals (3 replicates with 4 individuals each) were anesthetized and processed as in the bioaccumulation experiment to determine the initial contamination condition of the population. The remaining 96 hermit crabs were maintained in aquaria (42x30x30 cm) with

seawater (salinity 33 psu and ambient temperature ºC) under constant aeration (Figure 2). The animals were given the shrimp feed, and the TBT-free water was changed every day.

This experiment lasted 120 days. Every fortnight, one triplicate sample, composed of tissues of 4 randomly selected hermits was removed for TBT and DBT tissue determination (see below). The hypothesis that the amount of BTs (TBT and DBT) did not vary with time was tested using a one-way ANOVA, followed by the Scheffé test for post-hoc pairwise comparisons (UNDERWOOD, 1997).

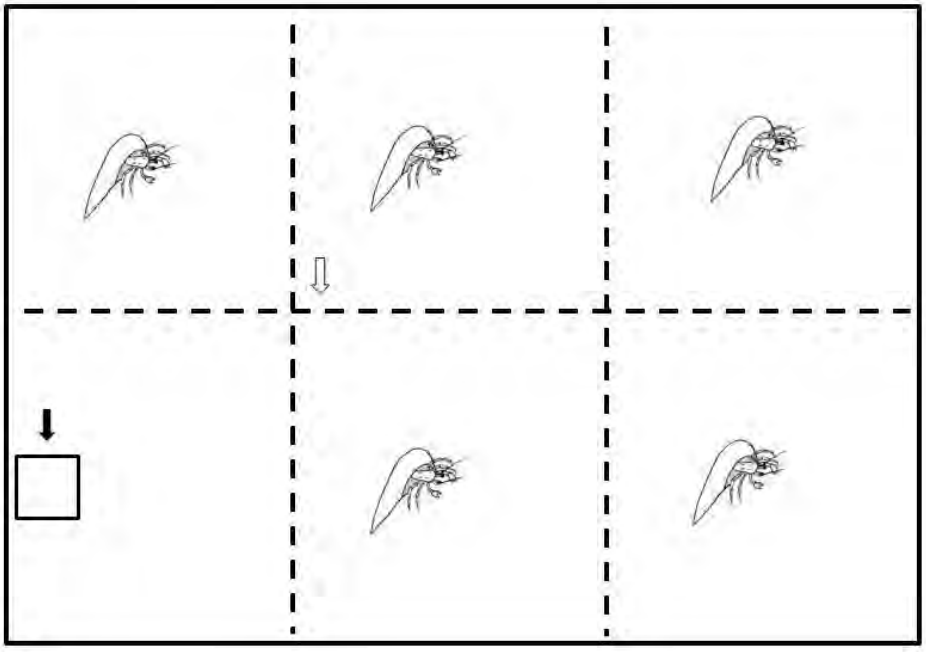

Figure 2 – Experiment on TBT depuration. Arrangement of the hermit crabs in the aquarium, separated by plastic netting (white arrow). The black arrow indicates the aerating pump.

2.3 Butyltin analysis

The concentration of butyltins (TBT and DBT) was analyzed by the experimental procedure of LIMAVERDE et al. (2007), adapted for hermit-crab tissues by CRISTALE et al. (unpublished data). The extraction procedure used hydrochloric acid and methanol as the solvent, complexation with 0.1% ammonium pyrrolidine dithiocarbamate (APDC) following derivatization using Grignard's reagent. The organic fraction was removed and cleaned up in an aluminum oxide column, and eluated with hexane. The final extract was concentrated to 1 ml, and tetrabutyltin was added as an internal standard. The butyltin compounds were analyzed in a gas chromatograph (GC) Varian 3800 (Walnut Creek, CA, USA) equipped with a pulsed flame photometric detector (PFPD) using a tin filter (390 nm) and a VF5 capillary column (30 m x 0.25 mm – Varian, Walnut Creek, CA, USA). For the applied method, the detection limits were 0.8 and 1.4 ng Sn g^{-1} w. w. and the quantification

limits were 2.6 and 4.4 ng Sn g^{-1} w. w. for TBT and DBT respectively. The precision of the method was checked by use of the ERM – CE477 certified reference material for mussel tissue, with TBT recovery of 98±11% (CRISTALE et al., unpublished data).

3. Results

3.1 Bioaccumulation

The TBT concentrations in the EC, PC, and T1 treatments were below the detection limit (0.8 ng Sn g^{-1}). Therefore, the *t* test rather than ANOVA was employed to compare the results of treatments T2 and T3, which showed mean TBT concentrations equivalent to 182±90 and 146±42 ng Sn g^{-1} w. w., respectively, (Figure 3) without significant differences (t=0.611; DF=4; p=0.574). These results indicated that the experimental amount of TBT in the water did not significantly affect the TBT concentration in the hermit crab tissues, and showed that the main form of TBT uptake was through food ingestion.

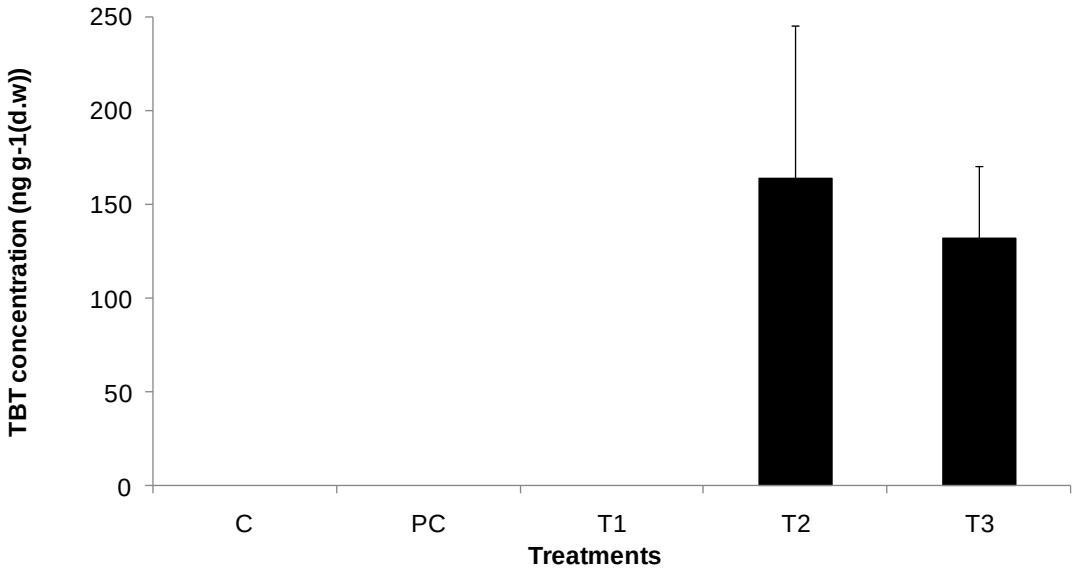

Figure 3 – Mean and standard deviation of TBT concentrations in the hermit crab *Clibanarius vittatus.* Treatments: Control (C, time 0); Procedural control – water and food without TBT (PC, after 45 days), T1 – water with and food without TBT (after 45 days), T2 – water without and food with TBT (after 45 days), and T3 water and food with TBT (after 45 days).

3.2 Depuration

In the depuration experiment, the initial concentration of TBT in the hermit-crab tissues was 111±36 ng Sn g^{-1} w. w., and decreased significantly during the experiment (F=23.8109; DF=6; p<0.01) (Fig. 4A). The elimination of this pollutant occurred mostly in

the first 2 weeks, and TBT tissue concentration decreased to undetectable levels after 75 days. The very low amount of TBT detected at the end of the experiment can be explained by individual variability in the concentration of this pollutant among the hermits.

In this experiment, DBT concentrations were also determined, and in general behaved similarly to TBT (F=31.2566; DF=5; p<0.01; Fig. 4B). The initial concentration of DBT was 280±79 ng Sn g^{-1} w. w., and after 30 days decreased to 8±5 ng Sn g^{-1} w. w. The DBT concentration also reached values below the detection limit after 75 days, and showed a non-significant increase at the end of the experiment (Fig. 4B).

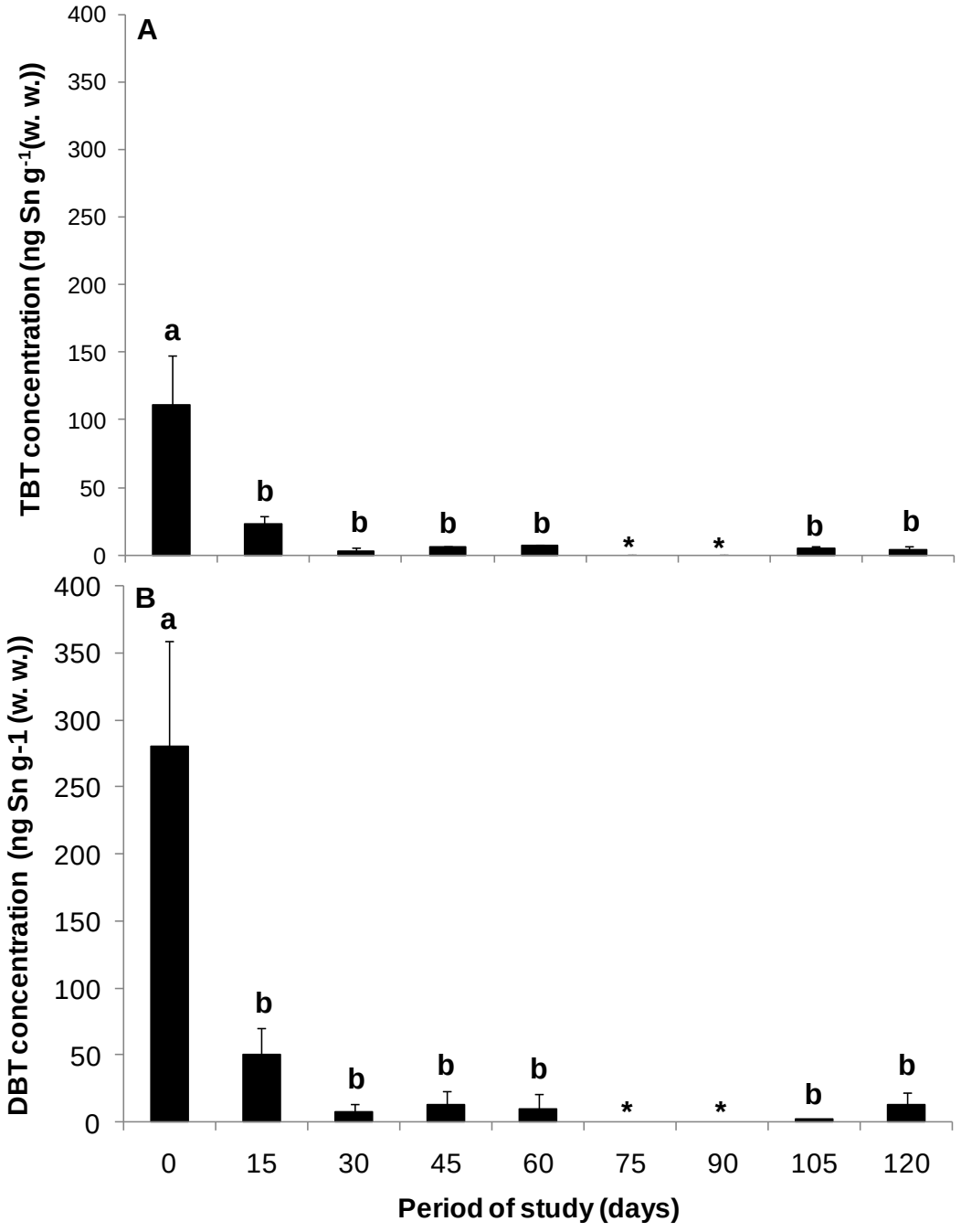

Figure 4 – Mean and standard deviation of TBT (A) and DBT (B) concentration in the hermit crab *Clibanarius vittatus*. Means followed by the same letter in the same figure did not show a significant difference (P>0.05). Results followed by (*) were not included in the ANOVA, because butyltins were not recorded in all three replicates.

4. Discussion

In some countries, such as Japan, TBT was banned about 20 years ago (HORIGUCHI, 2005), but the toxicity and persistence of this substance in the natural environment continue to be recorded (MURAI et al., 2008). In Brazilian waters, the ban is recent, and comparative studies carried out before and after TBT banning (TOSTE et al., in press) have revealed a worsening of the problem of imposex in gastropod mollusks. Therefore, studies of bioaccumulation, depuration, and TBT concentration in the bodies of wild organisms are essential.

The clam *Ruditapes decussates* (Linnaeus, 1758) accumulates TBT from different sources including food, sediment, and water; however, the main source is water (COELHO et al., 2002a, b). Similarly to bioaccumulation in the red sea bream *Pagrus major* (TEMMINCK and SCHLEGEL, 1843) by IKEDA and YAMADA (2003), the main uptake pathway in the hermit crab *Clibanarius vittatus* was from food. However, despite the high TBT concentration in the food offered daily, the amount assimilated did not approach the amount offered. The results of the present study differ from findings for some mollusk species, where the initial TBT uptake reflected the concentrations available in the environment (GIBBS and BRYAN, 1996).

Considering that *C. vittatus* occupies an important position in the estuarine community and is an omnivorous species (ALEXANDER, 1982), this uptake pathway may indicate the existence of a biomagnification process in this species under natural conditions. The findings of this study, in addition to data from a natural population of *C. vittatus* (unpublished data) and estuaries contaminated by TBT (SANTOS et al., 2010), indicate that this pollutant may still be accumulating in a wide variety of organisms at the base of the trophic web. This is a matter for concern, because the pervasive occurrence of TBT in the food web may pose a risk to humans who consume seafood. TBT contamination in humans has been recorded in blood (KANNAN et al., 1999) and liver (TAKAHASHI et al., 1999), and according to ANTIZAR-LADISLAO (2008), dietary intake is probably the main route of exposure to TBT compounds for the general population. Humans do not consume hermit crabs, but fish do feed on them (see GIBRAN and CASTRO 1999), which constitutes an indirect hazard.

In the present study, only a single concentration of TBT was used in the bioaccumulation experiment. It is essential to investigate other concentrations in further

studies, in order to broaden comprehension of the relationships among TBT concentrations, accumulation rates, and metabolization of this compound. SUN et al. (2001), who studied an estuarine food chain composed by the algae *Platymonas sp*., the rotifer *Brachionus plicatilis* Müller 1786, and the mysid *Neomysis awatschensis* (Brandt, 1851), suggested that species with a high ability to metabolize TBT have less tendency to accumulate and biomagnify butyltins, as was recorded in the present study in *C. vittatus*. Similarly, the TBT concentration in this study was measured using a sample pool of hepatopancreas and gonad, which provided a hydrophobic refuge for accumulated organic compounds. However, it is also important for future analyses of TBT concentration and depuration time to consider other tissues to understand possible differential tissue dynamics, which have been previously demonstrated in mollusks (COELHO et al., 2002b and PAGE et al., 1995) and sea stars (BÉKRI and PELLETIER, 2004).

TBT accumulates more in animals at lower trophic levels, because they have a low capacity to degrade this compound (OHJI et al., 2003). One of the main results of the present study is the demonstration of the capability of *C. vittatus* to rapidly eliminate both TBT and DBT. Similar results were obtained for the predatory sea star *Leptasterias polaris* (Müller and Troschel, 1842) by BÉKRI and PELLETIER (2004). Nevertheless, at the end of the depuration experiment, very low levels of TBT and DBT were still detected in hermit crab tissues. This may be explained by the individual TBT contamination levels, and probably the hermit crab tissues from the last samples were contaminated above the average of the population at the beginning of the experiment, and would require more time for complete depuration. A study on the metabolization of tributyltin oxide in the blue crab *Callinectes sapidus* and the spider crab *Libinia emarginata* (Leach, 1815) also demonstrated that they are able to metabolize this compound within a few days (LEE, 1985). These results are in accordance with the depuration results for *C. vittatus,* because we observed a decline in the BTs concentration within a few weeks. It is possible that gut microflora contribute to metabolization of the ingested TBT (RICE et al., 1989).

The finding of similar depuration times of TBT and DBT in hermit crab tissues is contrary to expectations, because DBT is more soluble and should be eliminated into sea water more rapidly. This result is in accordance with those obtained by PAGE et al. (1995) for the mussel *Mytilus edulis* (Linnaeus, 1758). As suggested for *M. edulis*, TBT is not entirely depurated through simple phase-transfer processes, but through a process in which DBT is the intermediate step, formed by the dealkylation of TBT (PAGE et al., 1995).

This is the first study to test a hermit crab species as a possible TBT bioindicator. Usually gastropod species are used to assess TBT contamination in the natural environment. In the Pacific, neogastropods such as *Thais clavigera* (Kiister, 1858) are widely used to monitor tributyltin contamination (CHAN et al., 2008). Recently, in Brazilian waters, another gastropod, *Stramonita haemastoma* (Linnaeus, 1767) was used to assess TBT (LIMAVERDE et al., 2007). In these studies, TBT monitoring is based on the occurrence of imposex in gastropods or on transplantation experiments (BRYAN, 1986, 1987; CHAN et al., 2008). The present study showed that *C. vittatus*, because it eliminated butyltin compounds within a few weeks, could be an indicator of recent BT contamination.

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Capítulo 5

Simultaneous activity of male and female gonads in intersex hermit crabs

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Simultaneous activity of male and female gonads in intersex hermit crabs

Abstract

Intersexuality in hermit crabs was investigated using anatomical, histological and electronmicroscopy techniques, with *Clibanarius vittatus* as a model. The specimens were collected in the intertidal zone of Pescadores Beach, state of São Paulo, Brazil, and dissected to describe the anatomy of the reproductive organs. Different portions of the reproductive system were stained with hematoxylin and eosin, and the gonopore morphology was analyzed by scanning electron microscopy. The intersex individuals showed both male and female reproductive systems, with ducts that are connected to the gonopores. The morphology of the gonopores was similar in males and females. The intersex individuals also developed functional male and female gonads in the same individual. These observations confirmed that intersex hermit crabs can reproduce as males or females, thus supporting the hypothesis that they may be part of a true sequential hermaphroditic process.

1. Introduction

Intersexuality has been recorded in several animal groups, from invertebrates such as molluscs (GIBBS et al., 1991) and crustaceans (BAUER, 2000; TURRA and LEITE, 2000; LEBLANC, 2007) to vertebrates, including humans (WALSH, et al., 1974). An intersex individual or gynandromorphy is defined as an individual (male or female) that develops alterations in primary and/or secondary sexual features, so that it possesses both male and female characters (GOLDSCHMIDT, 1938; LADEWIG, et al., 2002; VASQUEZ and LÓPEZ-GRECO, 2007). The alterations in secondary sexual characteristics may result in nonfunctional hermaphroditism, characterized by oogenesis occurring in the testes, as reported in talitrid amphipods, or, conversely, by spermatogenesis taking place in the gonads of females, which has never been observed (CHARNIAUX-COTTON, 1975). In contrast, intersexuality may also be part of a functional protandric, protogynic, or simultaneous hermaphroditism (BROOK et al., 1994; FIEDLER, 1998; RUDOLPH, 2002).

Different causes have been attributed to intersexuality. Studies conducted in recent decades suggest that contact with anthropogenic chemicals and mimics of estrogens and testosterone may alter sexual development in many vertebrate groups, including fishes (WILLIAMS et al., 2009), amphibians (REEDER et al., 1998), reptiles (GUILLETTE et al., 1994) and birds (FRY and TOONE, 1981). In fishes and amphibians, intersexuality is closely related to their exposure to steroid estrogens (WILLIAMS et al., 2009) or chemical pollutants (REEDER et al., 1998). Among invertebrates, molluscs show a similar phenomenon termed ‘imposex’, which is related to tributyltin (TBT) contamination (GIBBS et al., 1991; BETTIN et al., 1996; OEHLMANN, et al. 1996; RANK, 2009). Crustacean intersexuality has been associated with natural events, such as viral infection (JUCHAULT et al., 1991), parasites (GINSBURGER-VOGEL, 1991), hereditary factors including genetic abnormalities (HOUGH et al., 1992), social (demographic) structure (BAEZA and BAUER, 2004), and abiotic factors (BAUER, 2002).

Investigations of intersexuality in hermit crabs are recent and have focused mainly on records of gynandromorphs— with open gonopores on both coxae of the third (female) and fifth (male) pereopods—occurring in nature. This phenomenon seems to occur at all grades of terrestrialization, from typically aquatic species to fully terrestrial species such as members of the Coenobitidae (GUSEV and ZABOTIN, 2007). Currently this sexual abnormality has been recorded in 17 species around the world from different genera and

families (HILGENDORF, 1897; FIZE and SERÈNE, 1955; WENNER, 1972; MCLAUGHLIN, 1974; CHARNIAUX-COTTON, 1975; LEWINSOHN, 1982; MCLAUGHLIN and LEMAITRE, 1993; MANTELATTO and SOUSA, 2000; TURRA and LEITE, 2000; TURRA 2004, 2005; SANT'ANNA et al., 2006; FANTUCCI et al., 2008; GUSEV and ZABOTIN, 2007; TURRA, 2007).

The biological meaning of intersexuality in hermit crabs has been discussed based on behavioral, population, and morphological information (TURRA and LEITE, 2000; TURRA, 2004, 2007). TURRA and LEITE (2000) found that intersex individuals were of intermediate size in relation to males and females, and comprised 5 to 7% of the population studied. In another locality, the size of intersex individuals was more similar to the largest males, and intersex individuals comprised 2.5% of the population (SANT'ANNA et al., 2009). TURRA (2004) showed that intersex individuals of *Clibanarius vittatus*, *C. antillensis* and *C. sclopetarius* may act as functional males, successfully mating as males, and that the female gonopores closed in the subsequent molt. Conversely, one intersex individual was also recorded with an egg mass (TURRA, 2007), thus indicating that they may also act as females. The results of these studies suggest that intersexuality in hermit crabs may be part of a true sequential protogynic hermaphroditic process. A good source of information to illuminate this discussion is a detailed morphological analysis of the reproductive system of intersex individuals in comparison with males and females. This kind of investigation is especially important since there are no records of protogyny in decapods (BROOK et al., 1994; GUSEV and ZABOTIN, 2007).

Reproductive aspects in hermit crabs are relatively well known. For many species, the gonad histology and gonopore morphology have been described (HINSCH 1980; VARADARAJAN and SUBRAMONIAN 1980; MANJÓN -CABEZA and GARCÍA-RASO, 2000; HESS and BAUER, 2002; TIRELLI et al., 2006, 2007; AMADIO and MANTELATTO, 2009; MANTELATTO et al., 2009). However, there is no such information on intersex hermit crabs. Although the gonopore morphology of males and females has been analyzed for *Clibanarius vittatus* (HESS and BAUER, 2002), an ultrastructural approach may reveal important differences or similarities between the gonopores of intersex individuals and normal males and females.

In this context, the present study comprises an anatomical, histological, and ultramorphological investigation on intersexuality in hermit crabs, using *Clibanarius vittatus* as a model, to search for evidence of a functional hermaphroditic process.

2. Material and Methods

2.1 Animals and samples

Hermit crabs *Clibanarius vittatus* were collected in the intertidal zone of Pescadores Beach (23° 58' 21'' S, 46° 23'35'' W), São Vicente, state of São Paulo, Brazil. To access the reproductive system, the animals were gently removed from the gastropod shells and anesthetized by thermal shock with cold seawater. During dissection, the macroscopic morphology of the reproductive system for males, females, and intersex individuals was analyzed and the organs were then transferred to various fixative vehicles. The branchial and abdominal cavities were removed and examined under a stereomicroscope, to investigate the possible presence of parasites.

2.2 Histology

The reproductive organs of *Clibanarius vittatus* were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4) for 24 h, and then washed twice for 1 h with the same buffer. The material was dehydrated in an ascending ethanol series (70 to 95%) for 20 min and embedded in glycol methacrylate resin (Leica historesin kit) for 72 h at 4°C. After polymerization, the blocks were cut in a Leica RM2245 microtome. The 5 to 7 µm sections were mounted on slides and stained with hematoxylin and eosin (HE) (JUNQUEIRA and JUNQUEIRA 1983, modified), using no xylene and ethanol baths.

2.3 Electron microscopy

For the SEM procedure, the gonopores of males, females and intersexes were removed and fixed in modified Karnowsky fixative (2.5% glutaraldehyde and 4% paraformaldehyde in 0.2 M phosphate buffer prepared with seawater, pH 7.3) for 24 h. The samples were washed twice with the same buffer and dehydrated in an ascending acetone series (50 to 100%). All specimens were critical-point-dried in a Balzers CPD 030. After complete dehydration, the materials were sputter-coated with gold in a Balzers SD 050 prior to examination in a Jeol P15 SEM. All images were photographed on Neopan SS 120 film.

3. Results

A total of 72 adult hermit crabs *Clibanarius vittatus* were analyzed, including 27 males, 27 females, and 18 intersex individuals. In the anatomical observations (Fig. 1), 15 intersex individuals showed only male gonads (testis and vas deferens), while 3 specimens showed both male and female gonads that were not macroscopically distinct (Fig. 1c,d), similar to those recorded in normal males (Fig. 1a) and females (Fig. 1b). The intersex female gonads showed light-orange pigmentation, slightly different from normal females that have reddish-orange gonads when fully developed. Both oviducts were found during the dissections; however only one is shown in the image because the second one broke during dissection. No crabs contained parasites in the branchial or abdominal cavities.

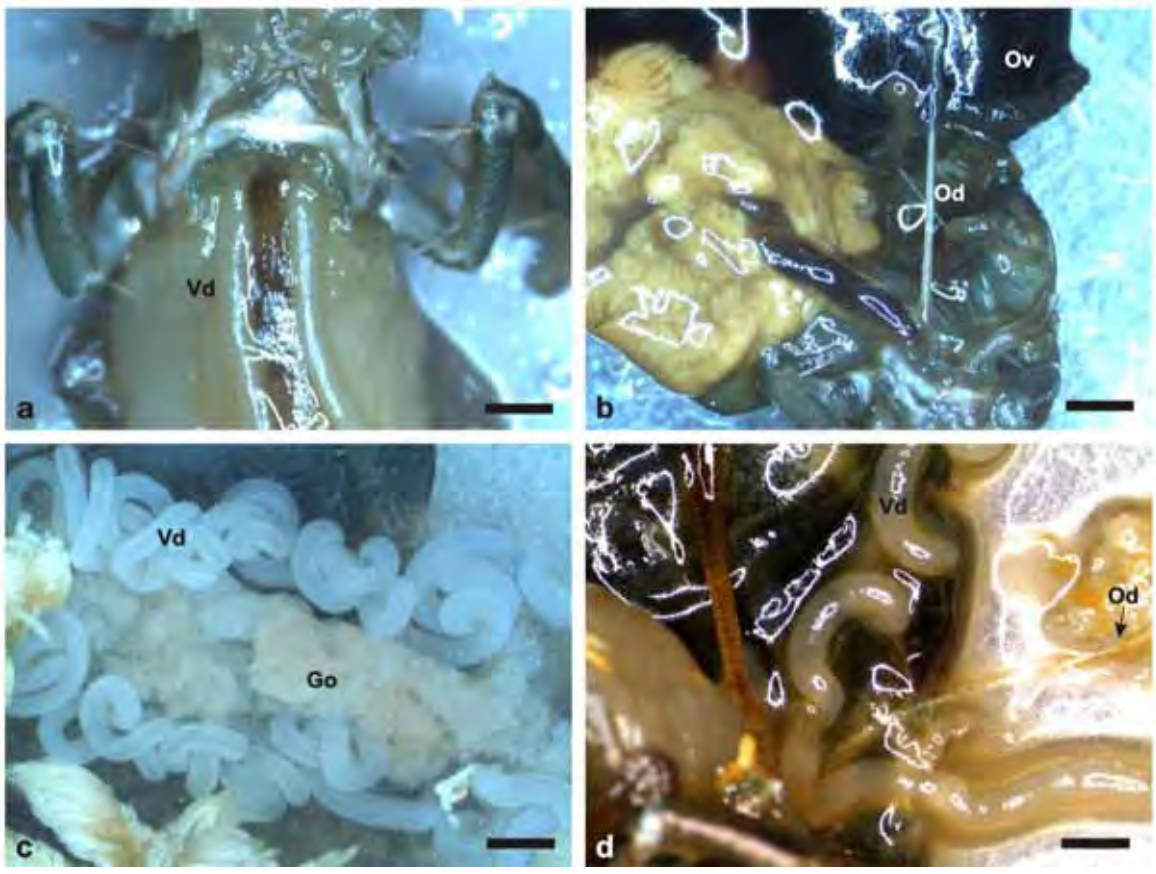

Figure 1 - *Clibanarius vittatus.* (a) Normal male abdominal cavity showing the vas deferens (Vd) leading to the gonopore; (b) detail of normal female anatomy with ovary (Ov) and oviduct (Od); (c) intersex individual with both gonads (Go) and vas deferens; (d) detail of vas deferens and oviduct from an intersex individual, leading to the gonopores. Scale bar = 2 mm.

The distal ends of the vas deferens, the ejaculatory ducts, are located within the coxae of the fifth pereopods of the normal male, and open to the exterior via the

gonopores, which are protected by a flap-like operculum surrounded ventrally by stout setae (Fig. 2a). The normal female gonopores open in the coxae of the third pereopods and have slender setae (Fig. 2b). The male and female gonopores of an intersex individual are shown in Fig. 2c, d. The male intersex gonopores depicted has almost the same morphology as a normal male, i.e. with stout setae above a flap-like operculum. The main difference is the exoskeleton evagination, which is convex in the male intersex individual (Fig. 2c). The female gonopore of the intersex individual has a small evagination of the exoskeleton, without slender setae (Fig. 2d), which may indicate gonopores closure. In 12 of the 18 intersex individuals, 3 gonopores were identified (Fig. 3a), and 6 individuals showed 4 gonopores (Fig. 3b). In 7 of the 18 individuals, the exoskeleton evagination around the female gonopores was observed.

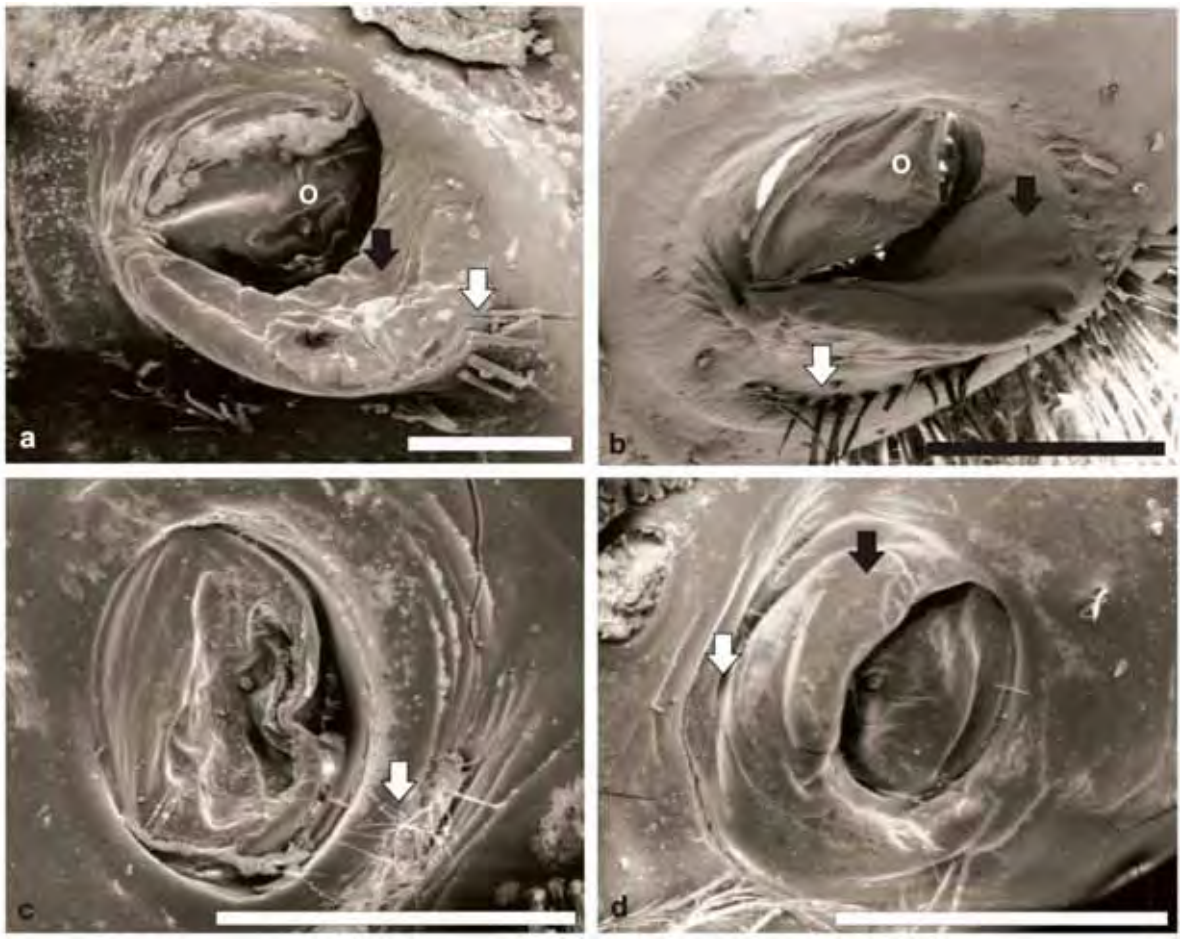

Figure 2 - *Clibanarius vittatus.* (a) Male gonopore and operculum (o) showing concave evagination of the exoskeleton (black arrow) surrounded by stout setae above them (white arrow); (b) male gonopore of intersex individual displaying convex evagination of the exoskeleton (black arrow) and stout setae (white arrow); (c) female gonopore, marked ventrally by slender setae (white arrow); (d) female gonopore of intersex individual, operculum, and evagination of exoskeleton (black arrow) lacking slender setae (white arrow). Scale bar = 0.5 mm.

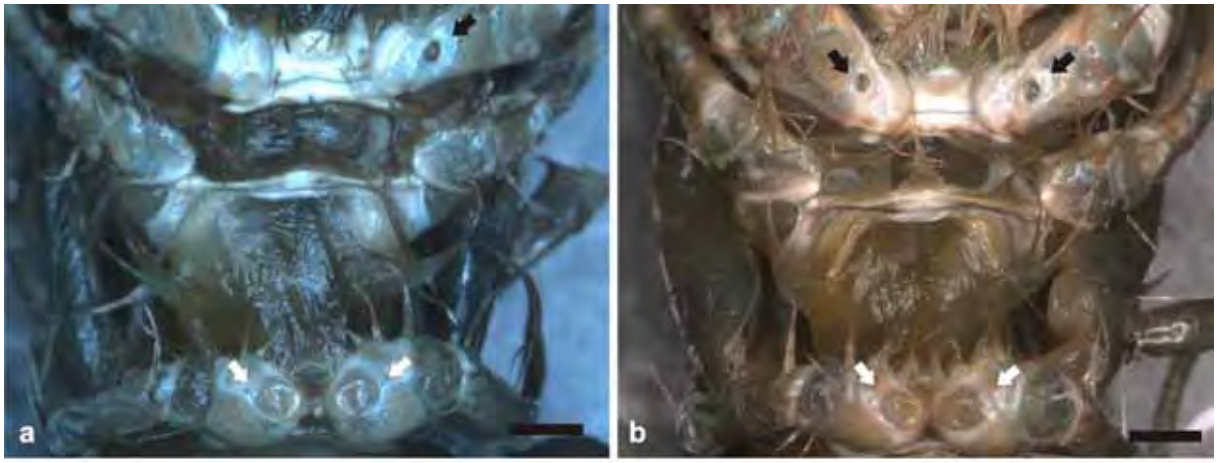

Figure 3 - *Clibanarius vittatus*. Ventral view of coxae and sternites of the pereopods from intersex individuals with (a) 3, and (b) 4 gonopores. Black arrow: female gonopores; white arrow: male gonopores. Scale bar = 2 mm.

Normal males and females were used as a histological control to check the functionality of the reproductive system (Fig. 4 a, b). The intersex individual that showed only male gonads macroscopically proved, on histological examination, to have a functional male reproductive system. The tests showed the same germinative cells in different stages of spermatogenesis, and mature sperm in the lumen of the seminiferous duct, as in normal males (Fig. 4 c).

The histology of the 3 intersex individuals that showed both male and female gonads, upon dissection (Fig. 1), also confirmed that the testis and ovary were functional (Fig. 4 d, e). Macroscopically and histologically, the gonads are a paired organ with functional ovary and testis (Fig. 4 f, g), characterized by normal oocytes filled with yolk granules, indicating the vitellogenesis or exogenous phase (Fig. 4 f), and normal spermatogenesis showing mature sperm (Fig. 4 g). Each ovary and testis is clearly surrounded by its own thin layer of connective tissue (Fig. 4 d–f), i.e. they are not arranged as an ootest.

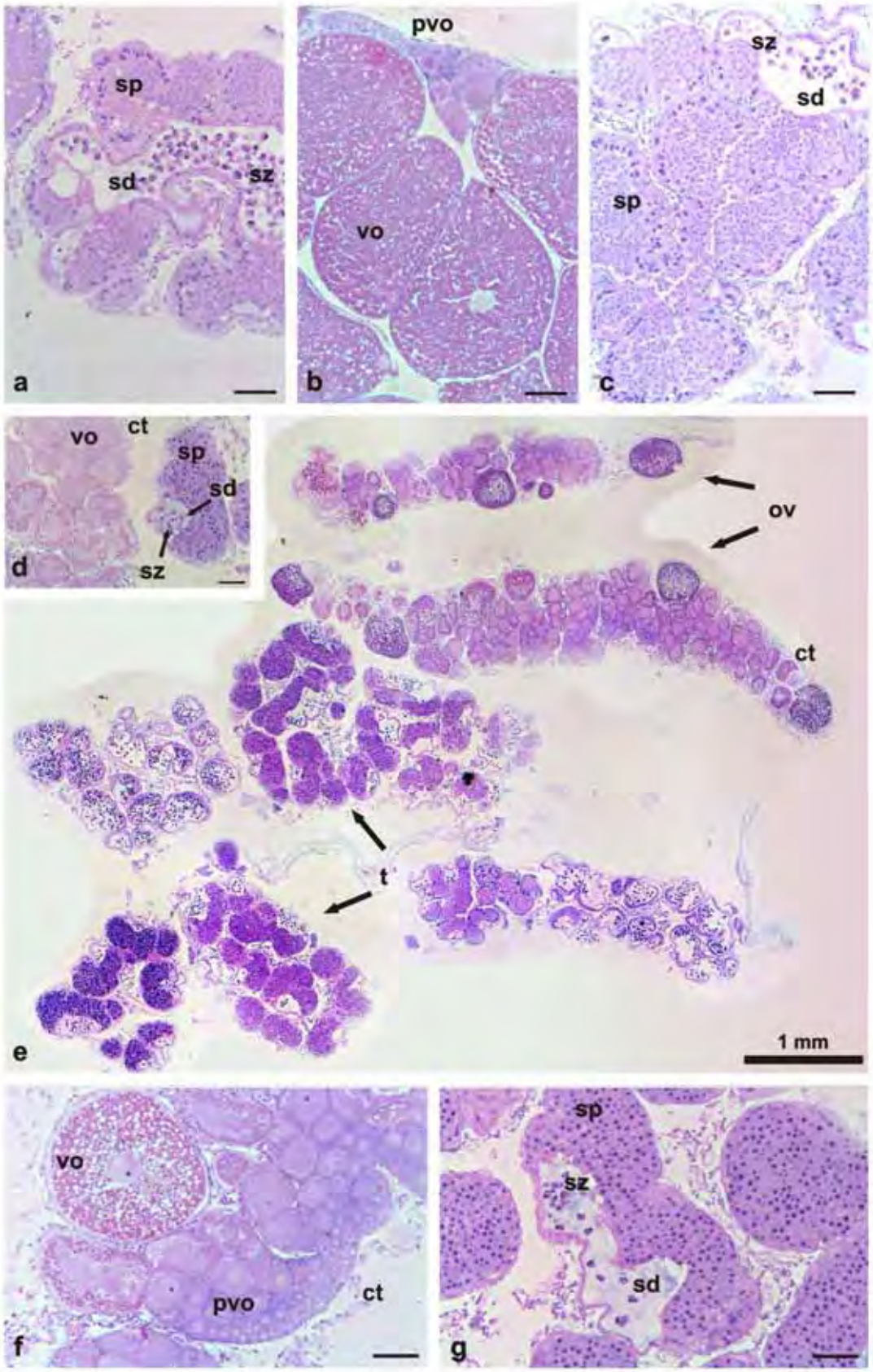

Figure 4 - *Clibanarius vittatus*. Histological sections. (a) Functional male testis with normal characteristics, showing the seminiferous duct (sd), male germinative cells (sp), and mature sperm (sz). (b) Female ovary surrounded by a thin capsule of connective tissue (ct) displaying normal vitellogenesis, depicting vitellogenic oocytes (vo) and pre-vitellogenic oocytes (pvo). (c–g) Histological sections from intersex individuals. (c) The most usual intersex condition observed, showing the testis filled by germinative cells and seminiferous ducts

filled with mature sperm. (d) Another intersex condition that occurs in low frequency, showing details of testis, with germinative cells, seminiferous duct and mature sperm, and the ovary, with vitellogenic oocytes bounded by their own connective tissue. (e) Montage pictures from intersex individual observed in Fig. 4d, forming the total histological section of paired gonads, testes (t) and ovaries (ov). (f) Detail of vitellogenic and previtellogenic oocytes bounded by their own connective tissue in an intersex individual with both gonads. (g) Detail of seminiferous duct, male germinative cells, and mature sperm in an intersex individual with both gonads. Scale bar = 50 μm; except for (e).

4. Discussion

The present study is a step forward in the investigation of hermit crab intersexuality, extending the findings on morphology, behavior, and reproductive activity reported by TURRA (2004, 2007). Intersex individuals of *Clibanarius vittatus* may copulate as males that close the female gonopores after molting (TURRA, 2004), which indicates a protogynic process. Intersex individuals of this species have also been reported as ovigerous females (TURRA, 2007), reinforcing the hypothesis presented above about the existence of occasional sex reversal in this group, which was first proposed by WENNER (1972), TURRA and LEITE (2000) and SANT'ANNA et al. (2009), based on analyses of population data.

In *Clibanarius vittatus*, both TURRA and LEITE (2000) and SANT'ANNA et al. (2009) obtained anomalous sex-ratio curves. As pointed out by WENNER (1972), such anomalous curves might be explained by differences between the sexes in longevity and time for growth, differential migration, different mortality or growth rates between the sexes, and sex reversal. TURRA and LEITE (2000) and SANT'ANNA et al. (2009) explained this observed sex ratio by differences in the growth rate, longevity, or habitat partitioning between the sexes. Although the percentage of intersex individuals (the hypothetical transitional form) was very low (2 to 7%), the sex-reversal theory was also used to explain the anomalous curve in these 2 studies.

However, since a true protogynic hermaphroditic system requires that both sexes be expressed in a single individual, a very important piece of information in this discussion was lacking. The morphological analysis presented here shows that male and female gonads can be present and active in the same intersex individual. Most of the intersex individuals contained male gonads, but some of them contained both male and female gonads. In addition, electron microscopy revealed almost the same morphology for male, female, and intersex gonopores. If intersex individuals can act as males (TURRA, 2004) or reproductive females (TURRA, 2007), and develop both functional gonads, it may be possible that in a certain stage of female masculinization an individual could reproduce as a male and/or a female. This would indicate a simultaneous hermaphroditism, before female gonopore closure, as observed by TURRA (2004), and possible ovary reabsorption if a protogynic process is occurring.

Although protogyny is commonly recorded among crustaceans (BROOK et al., 1994), mainly in peracarids, there is no conclusive evidence of this phenomenon in

decapods. Although definitive evidence is still lacking, i.e. a record of sex change from female to male in the same individual during its growth, the results of the present study—which may be the first record of protogyny in decapod crustaceans—support this hypothesis.

In the anomuran *Galathea intermedia*, an intersex individual in transitional form can indicate a sex change, as discussed by KRONENBERGER et al. (2004). These authors suggested that activation of the androgenic gland (AG) and synthesis of the androgenic hormone (AGH) in females are responsible for the intersex morphology observed. This is also a plausible explanation for intersexuality in *Clibanarius vittatus*, since the hormonal action is directly related to male and female differentiation (CHARNIAUX-COTTON and PAYEN, 1985).

However, one question remains unanswered: What could cause this hormonal disruption and what may be occurring in these hermit crabs to result in intersex individuals and, at least hypothetically, in a protogynic process? The answer to this question may be twofold, based on the sex-determination system of hermit crabs and also on external causes such as parasites and pollution.

In crustaceans, protogynic hermaphroditism is often socially mediated (BROOK et al., 1994). For example, in the tanaid *Leptochelia dubia*, the presence of a male influences the sex-reversal rate (HIGHSMITH, 1983), probably because males produce a pheromone that inhibits sex reversal in potentially protogynous individuals (BUCKLE-RAMIREZ, 1965). Since the sex ratio in Clibanarius vittatus is significantly skewed toward females (TURRA and LEITE 2000; SANT'ANNA et al., 2009), the lack of males may trigger this process.

According to POTTS (1906) and NIELSEN (1970), parasites may modify sexual characteristics in some hermit crab species. However, in the detailed anatomical analysis, no parasites were found in the individuals of *Clibanarius vittatus* analyzed here.

Endocrine disruption caused by pollutants may be another possible explanation. In molluscs, the antifouling agent TBT used in marine paints causes the phenomenon called imposex, in which female individuals develop male characteristics due to endocrine disruption (LIMAVERDE et al., 2007). Worldwide, the imposex state has been recorded in about 120 gastropod species (GARAVENTA et al., 2006), and in Brazil this phenomenon was documented in the gastropod *Stramonita haemastoma* (FERNANDEZ et al., 2002). This species lives in coastal regions where there are large concentrations of boats and consequently of TBT (GODOI et al., 2003).

Because *Clibanarius vittatus* inhabits estuaries on the Brazilian coast, is commonly recorded using shells of *S. haemastoma* (TURRA and LEITE, 2002; SANT'ANNA et al., 2006), and has been found to be contaminated by TBT in nature (B. S. SANT'ANNA unpubl.), this pollutant may also be affecting these organisms. Therefore, a careful investigation of the possible effects of TBT on the reproductive development of *C. vittatus* is needed.

Finally, one may also argue that intersexuality in hermit crabs may be a consequence of the synergism of different causes. Therefore, an experimental approach that considers the effects of pollutants as well as the intrinsic reproductive determination system and behavior would be appropriate to shed more light.

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Capítulo 6

Is tributyltin (TBT) contamination related to intersexuality in hermit crabs?

Is tributyltin (TBT) contamination related to intersexuality in hermit crabs?

Abstract

The causes of intersexuality in hermit crabs are unknown. This study was designed to evaluate the possible relationship between intersexuality and endocrine disruption caused by tributyltin (TBT) pollution, as previously reported for gastropods. An orthogonal experiment was designed, with two treatments: contamination (with or without TBT in the food) and crab sex (males, females, and intersex). The animals were reared in the laboratory for nine months, and macroscopic and histological analyses of reproductive organs were carried out after the end of the treatment period. TBT assimilation was recorded only in the exposed crabs, and no morphological alterations were detected in the gonads of males and intersexes, whether or not they were exposed to TBT. In contrast, females exposed to the TBT treatment displayed disorganization and atrophy of their ovaries, although they developed no male characteristics. There is no evidence of a masculinization process induced by TBT in hermit crabs, and new hypotheses are presented to explain intersexuality in this group.

1. Introduction

Intersexuality is an intriguing phenomenon in decapod crustaceans, especially in hermit crabs. It is characterized by the occurrence of gynandromorphs [individuals showing gonopores on the coxae of both third (female) and fifth (male) pereopods] in the natural environment. Intersexuality in the Anomura seems to occur in species in all degrees of terrestrialization, from aquatic to fully terrestrial species such as the Coenobitidae (GUSEV and ZABOTIN, 2007). This sexual abnormality has been recorded in 17 hermit-crab species around the world (see TURRA, 2007; SANT'ANNA et al., 2010), but its biological significance remains unknown.

TURRA (2004) found that intersex individuals of *Clibanarius vittatus* Bosc (1802), *C. antillensis* Stimpson 1862, and *C. sclopetarius* (Herbst, 1796) are able to function as males, successfully mating with females and producing viable broods. TURRA (2004) also demonstrated that the female gonopores of these functional male intersex individuals tended to close in the molts subsequent to copulation. Conversely, TURRA (2007) recorded one intersex individual of *C. vittatus* with an egg mass, indicating that they may also act as females. These findings reinforced the hypothesis that intersexuality may be part of a sequential protogynic hermaphroditic process, as suggested by WENNER (1972) and TURRA and LEITE (2000), which has never been demonstrated in decapod crustaceans.

SANT'ANNA et al. (2010), in an anatomical, histological, and ultramorphological study on intersexuality, found that most intersex individuals had only male gonads. A relatively small number of individuals (16.67%) had functional male and female gonads, including normal male and female gonopores, suggesting a protogynic process. However, despite all these sources of information, sex reversal in hermit crabs was not definitively elucidated, since this event was not completely followed in a single individual. Therefore, intersexuality in hermit crabs may be merely an aberrant sexual character. However, if intersexuality is not a normal reproductive phenomenon among hermit crabs, what might be causing it?

SANT'ANNA et al. (2010) suggested that intersexuality may be related to endocrine disruption caused by marine pollutants such as tributyltin (TBT). This hypothesis is reasonable because *C. vittatus* is a typical estuarine hermit crab, the type of habitat where

the greatest concentrations of this pollutant have been recorded around the world (LANGSTON and POPE, 1995; YUAN et al., 2001; GODOI et al., 2003; SANTOS et al., 2009). In addition, different populations of this species were recorded as having high concentrations of this pollutant in the natural environment (B.S. Sant'Anna unpublished data). A recent study demonstrated that this hermit crab assimilates TBT mostly from food, and this species may be a good bioindicator of recent TBT contamination (SANT'ANNA et al., *in press*).

TBT is used in anti-fouling paints of vessels, and is continuously released into the marine environment. This substance causes serious effects even in non-target species, such as a decrease in oyster production (ALZIEU, 2000), inhibition of growth in microalgae (HUANG et al., 2004), reduction in fecundity and juvenile production in polychaetes (MOORE et al., 1991), and development of imposex in approximately 120 mollusk species worldwide, including species in Brazil (CASTRO et al., 2000; CAMILLO et al., 2004; COSTA et al., 2008). Imposex is characterized by a superimposition of male genital organs, such as the penis and vas deferens, on female gastropods (SHI et al., 2005a).

Imposex in mollusks may be hypothesized as equivalent to intersexuality in hermit crabs, which might also be caused by TBT contamination. Therefore, the possible effects of the endocrine-disrupting pollutant TBT on the primary and secondary sexual characters of the hermit crab *C. vittatus* was evaluated under laboratory conditions, to specifically test the following hypotheses: (1) the pollutant leads to the development of intersexuality in females, (2) the action of TBT in intersex individuals accelerates and/or terminates the masculinization process, (3) the pollutant does not cause changes in males, (4) no changes in females occur in the absence of TBT, and (5) the absence of the chemical stimulus causes reversion of intersexuality with feminization of intersex individuals and/or some males (some of them might have been females originally, if sex reversal occurs in hermit crabs).

2. Material and Methods

2.1 Animal samples

The hermit crabs used in this study were collected in July 2009, in different areas according to the sex. To obtain males and females uncontaminated by TBT, specimens were collected in the Cananéia Estuary (25° 00' 35.7''S and 47° 55' 29.4''W), São Paulo State, Brazil. This area was chosen because its population of *C. vittatus* does not show detectable levels of TBT (B. S. Sant'Anna unpublished data). Following the hypothesis proposed in this study, intersex individuals were collected in a TBT-contaminated area at Pescadores Beach in the São Vicente Estuary (23° 58' 21''S and 46° 23' 35''W), São Paulo State, Brazil. This population of *C. vittatus* is contaminated with 38.67±25 ng Sn g^{-1} (w. w.) in the tissues analyzed (B.S. Sant'Anna unpublished data).

2.2 Experimental design

In the experiment, a total of 180 hermit crabs were used, in an orthogonal design with two treatments: (a) contamination, with and without TBT in the food; and (b) hermit-crab sex, formed by groups of 30 males, 30 females, and 30 intersex individuals used in each contamination treatment. In the treatment with TBT, the hermit crabs were fed a ration developed for shrimp farming, mixed with TBT, to supply 180 ng Sn g^{-1} TBT/day. The crabs were maintained in aquaria (42x30x30 cm) with aerated seawater (salinity 33 psu and under ambient conditions of light and temperature ºC). In each aquarium, 6 crabs were maintained isolated by PVC tubes, allowing each animal to receive the exact amount of food (Fig. 1). The crabs were maintained for nine months, to allow them to molt several times.

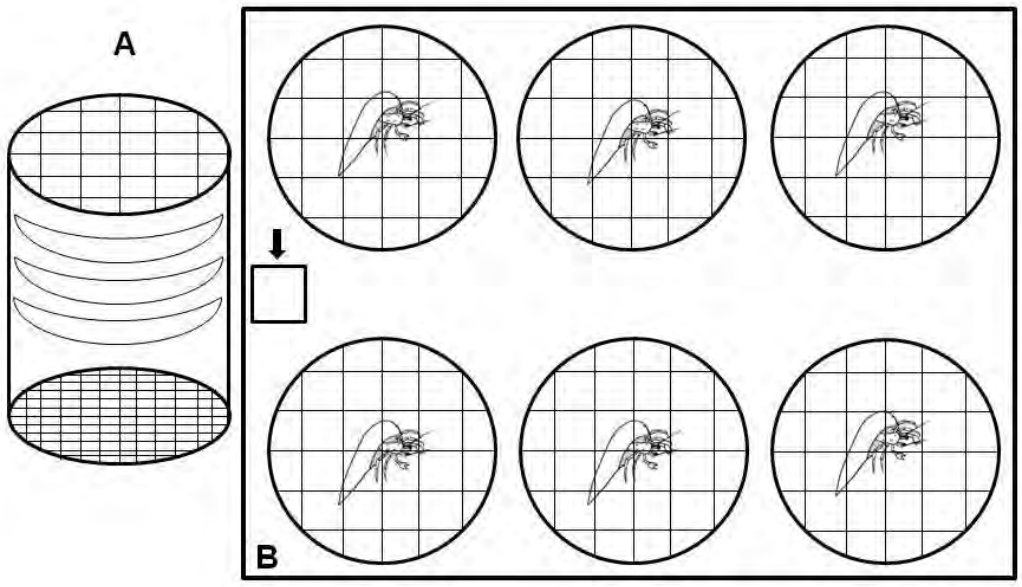

Figure 1 – Deployment of hermit crabs in the aquarium during the nine months of the experiments. A – Plastic PVC structure constructed to isolate each hermit crab. B – Distribution of the hermit crabs in the aquarium, relative to the position of the aerating pump (black arrow).

At the end of the experiment, the crabs were anesthetized by thermal shock in cold seawater, removed from the shells, and carefully inspected for gonopore morphology. The female gonopore areas from both females and intersex individuals were measured (area, mm^3) using Leica® IM50 software coupled to a Leica MZ75 stereomicroscope. To exclude the effect of individual size on the comparison of the gonopores between the first and the last exuvia, the ratio between the gonopore area and crab size (cephalothoracic shield length) was calculated and compared using paired *t* tests, with the significance level of $P<0.05$.

The crabs were then dissected, and the anatomy of the reproductive tracts inspected. Gonads and hepatopancreas of 15 individuals were labeled and stored frozen in aluminum-foil packages, until TBT determination, to investigate if the pollutant administered in the food was assimilated by their tissues. The reproductive tract of 5 individuals from each treatment was fixed in 4% paraformaldehyde for further histological procedures.

2.3 Histological procedures

The reproductive system of 5 males, 5 females, and 5 intersex individuals from each treatment were fixed in 4% paraformaldehyde for 48 h. The samples were buffered with 0.2 M phosphate buffer (pH 7.4) and washed twice for 1 h. They were then dehydrated in an ascending ethanol series (70-95%) for 20 minutes each, and embedded in glycol methacrylate resin (Leica® historesin kit) for 72 h at 4°C. After polymerization, the blocks were sectioned in a Leica RM2245 microtome. The 5-7 µm sections were mounted on slides and stained with hematoxylin and eosin (HE), according to JUNQUEIRA and JUNQUEIRA (1983), avoiding xylene and ethanol baths (ZARA et al., 2010).

The size of spermatozoa produced by 5 males (20 from each individual, totaling 100 spermatozoa for each treatment) and intersex individuals (20 from each individual, totaling 100 spermatozoa for each treatment) of both treatments with and without TBT was randomly measured. The size of the spermatozoa was measured from the apical portion of the operculum to the basis of the perforatorium (µm), using Leica® IM50 software coupled to a Leica DM2000 microscope at 100X magnification. The sizes of spermatozoa from all treatments were compared by a two-way Analysis of Variance, considering sex (male or intersex condition) and treatment (with or without TBT) as factors. The significance level of $P<0.05$ was adopted.

2.4 Chemical procedures for TBT determination

The concentration of TBT was determined by the experimental procedure adapted from LIMAVERDE et al. (2007) developed for hermit-crab tissues by CRISTALE et al. (unpublished data). The first extraction procedure involves hydrochloric acid and methanol, followed by extraction with toluene as the solvent, and complexation with 0.1% ammonium pyrrolidine dithiocarbamate (APDC) following derivatization using Grignard reagent. The organic fraction was removed, purified in an aluminum oxide column, and eluted with hexane. The final extract was concentrated to 0.1 mL, and tetrabutyltin was added as an internal standard. The recovery was assessed by a method using a surrogate (TPrT) added at the beginning of the analytical process, for all samples. Tributyltin was analyzed in a gas chromatograph (GC) Varian 3800 (Walnut Creek, CA, USA) equipped with a pulsed flame photometric detector (PFPD) using a tin filter (390 nm) and a VF5 capillary

column (30 m x 0.25 mm – Varian, Walnut Creek, CA, USA). The detection limit was 0.8 ng Sn g^{-1} and the quantification limit was 2.64 ng Sn g^{-1} (w. w.). The amount of TBT assimilated by individuals (males, females, and intersexes) was tested by one-way ANOVA with a significance level of $P<0.05$, since this pollutant was not detected in the treatment without TBT.

3. Results

At the end of the experiment, 146 animals survived: 27 males, 24 females, and 21 intersex individuals in the treatment with TBT, and 25 males, 23 females, and 27 intersex individuals in the treatment without TBT. In the TBT group, all three sex groups assimilated the contaminant via the food, in similar amounts (F=2.7962; DF=2; P=0.1384; Fig. 2); in the treatments without TBT, this compound was not detected by chromatographic analysis.

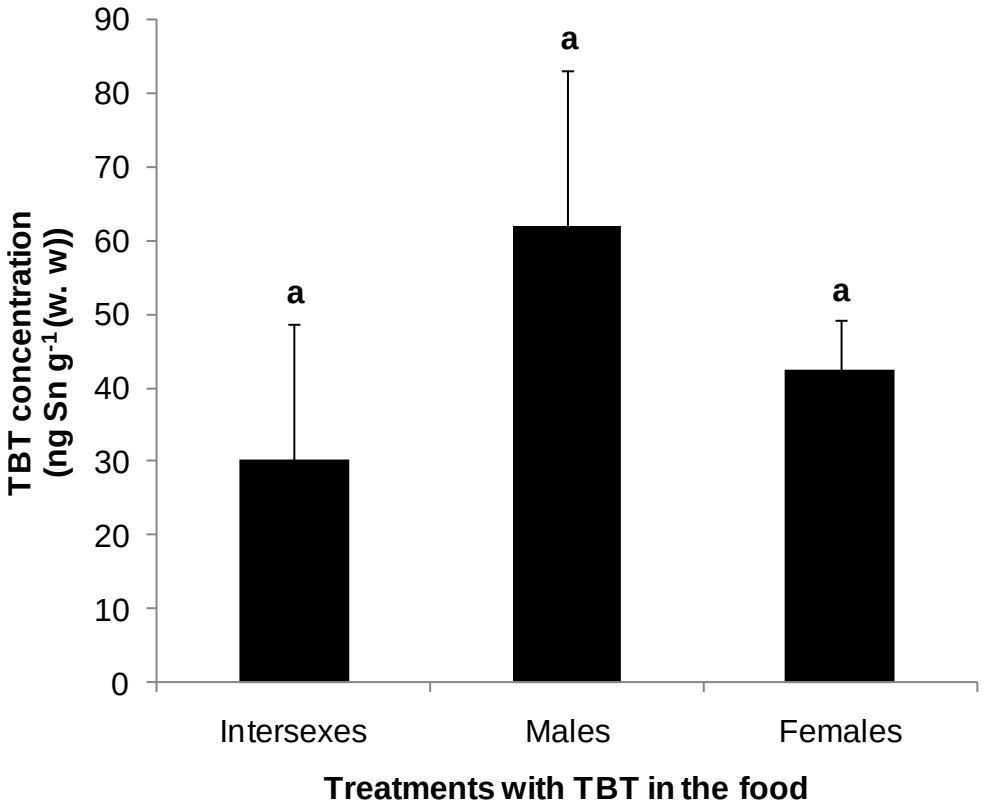

Figure 2 – Mean and standard deviation of TBT assimilation by intersex (n=15), male (n=15), and female (n=15) hermit crabs in the treatments with TBT in the food. Means followed by the same letter did not show a significant difference (P>0.05).

The female gonopores of individuals identified as females showed a tendency to grow, and were larger at the end of the experiment for all treatments (Table I). The relative area of the female gonopore of intersex individuals did not increase during the experiment (Table I), and complete gonopore closure was recorded in very low frequency for intersex individuals, whether or not they were exposed to TBT. Only one hermit crab from the treatment with TBT closed its right female gonopore. This animal was collected in the field

with only the right female gonopore open and the left one not visible. In the treatment without TBT, only two intersex individuals completely closed their gonopores. Both individuals were collected in the field with two open female gonopores, and during the experiment only the right female gonopore closed completely.

Table I – Comparison of relative female gonopore area (ratio between female gonopore area, in mm^2, and hermit crab size, in mm; paired t test) between the beginning and the end of the experiment, for females and intersexes in both treatments with and without TBT. Xi=initial mean; Xf=mean at the end of the experiment.

Sex / treatment	n	Right female gonopore				n	Left female gonopore			
		Xi	Xf	*t*	*P*		Xi	Xf	*t*	*P*
Female / without TBT	23	0.0189	0.0214	-3.22	0.0039	23	0.0197	0.0222	-3.19	0.0042
Female / with TBT	22	0.0203	0.0239	-4.53	0.0001	22	0.0207	0.0249	-4.38	0.0001
Intersex / without TBT	13	0.0119	0.0099	1.10	0.2924	24	0.0126	0.0130	-0.55	0.5903
Intersex / with TBT	21	0.0085	0.0098	-1.53	0.1693	8	0.0120	0.0128	-0.85	0.4061

Of the total of 48 intersex hermit crabs sampled at the end of the experiment, 20 animals had both gonopores, of which the left gonopore was significantly larger than the right one (paired t test; t=3.8272; DF=19; P=0.0011). Of the remaining 28 crabs, 26 showed only the left and 2 only the right female gonopores.

Ovarian histology for females without TBT in the food showed normal characteristics of oogenesis, with pre-vitellogenic and vitellogenic oocytes (Figs. 3A to D). Of the 5 females treated with TBT that were analyzed, 3 displayed different levels of ovary disorganization leading to atrophy (Figs. 4A to D). The vitellogenic oocytes showed irregular form and rupture of the membrane, including free yolk granules (Figs. 4E to G). Many spaces surrounded by follicle cells were evident, and these were identified as atretic follicles (Fig. 4 H). In all TBT-treated animals, large numbers of hemocytes were identified near irregular oocytes, free yolk granules, and atresic follicles (Figs. 4 E to H). The other 2 females analyzed did not show any structural alteration (Fig. 4, D). However, the degenerative effect of TBT is apparently related to the individual characteristics of each female, because 2 females showed normal oogenesis, with previtellogenic and vitellogenic oocytes and some atresic follicles (Fig. 4, D).

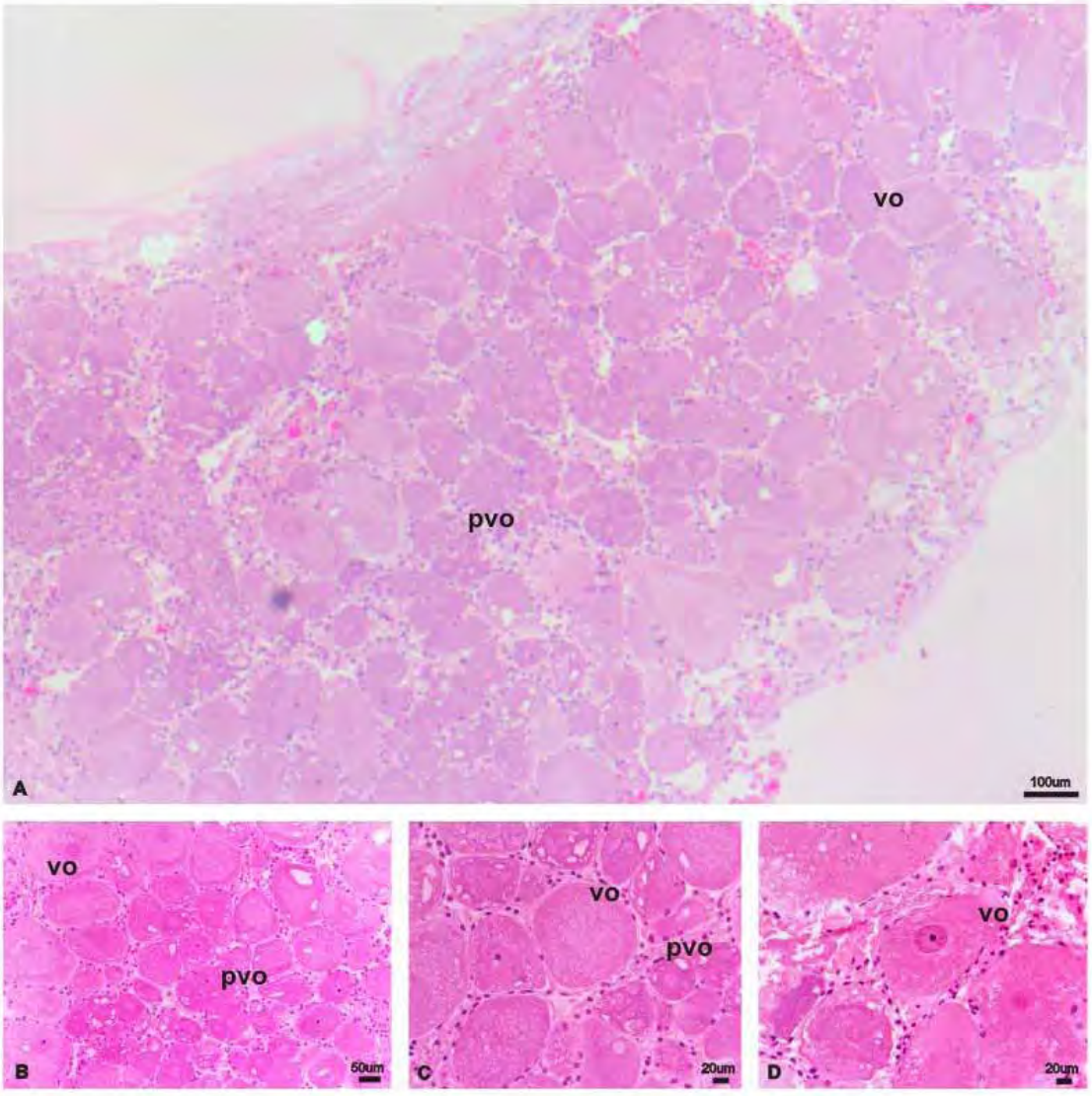

Figure 3 – HE stained sections of the ovary of the hermit crab *Clibanarius vittatus*, without TBT. (A) General view of the ovary, with previtellogenic and vitellogenic oocytes and absence of atretic follicles. (B to D) Details of normal oocyte development from control females, showing the normal vitellogenesis process. Previtellogenic (pvo) and vitellogenic oocytes (vo).

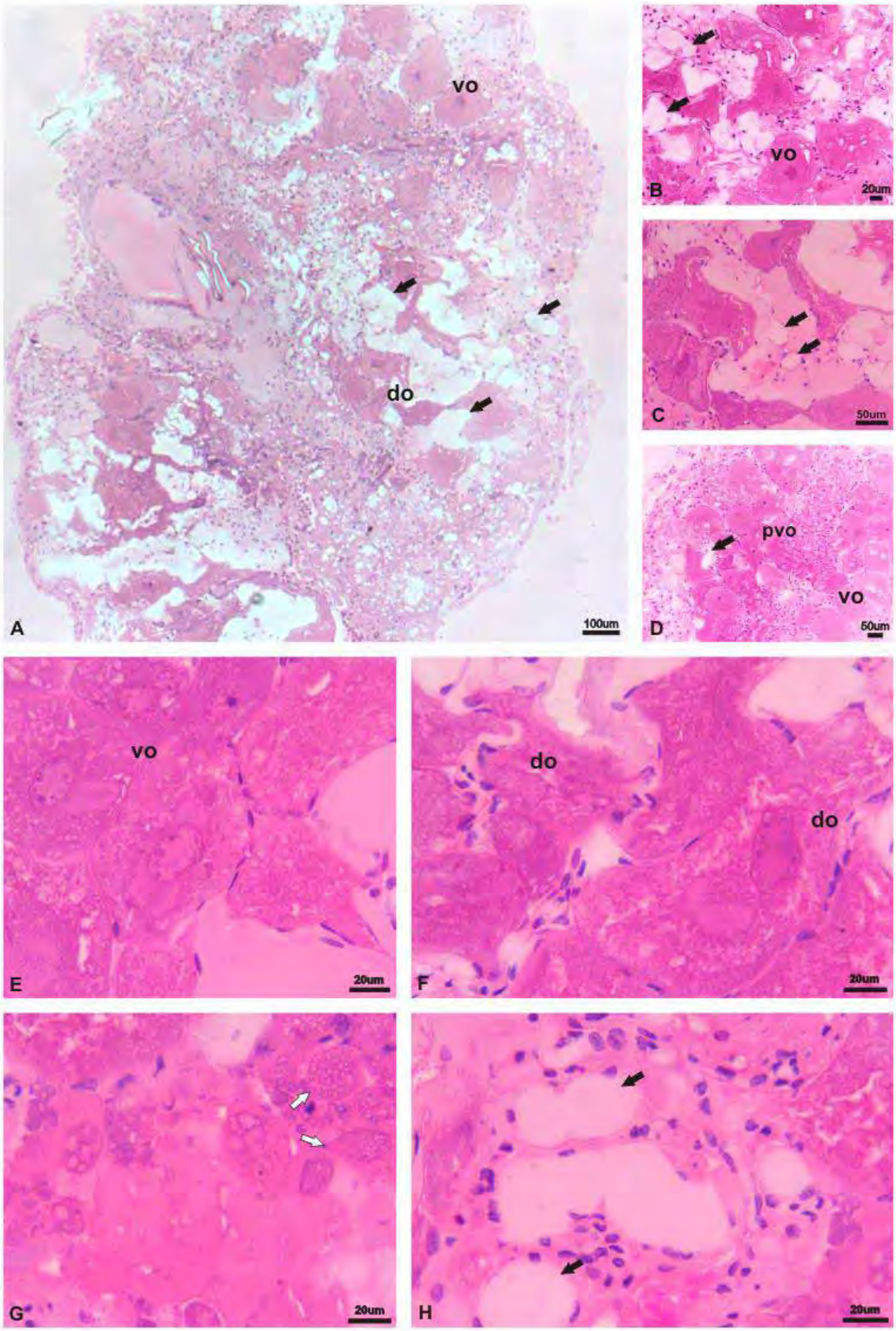

Figure 4 – HE stained sections of the ovary of the hermit crab *Clibanarius vittatus*, treated with TBT. (A) General view of the ovary showing vitellogenic oocytes with an irregular membrane, degenerated oocytes, and large numbers of atresic follicles characterized by a space surrounded by follicle cells. (B and C) Detail of some atresic follicles and oocytes with an irregular membrane; (D) functional ovary with previtellogenic

oocytes and vitellogenic oocytes. Note the presence of atresic follicles among the oocytes. E to H Sequence of oocyte degeneration; observe the vitellogenic oocytes with a relatively regular membrane (E), degenerate oocytes with an irregular and broken membrane (F) that releases yolk granules (G) resulting in the atresic follicles (H). Previtellogenic oocytes (pvo), vitellogenic oocytes (vo), degenerated oocytes (do), atresic follicles (black arrows), yolk granules (white arrows).

Both males (Fig. 5 A to H) and intersex individuals (Fig. 6 A to F) maintained with and without TBT showed normal spermatogenesis, sperm production in the testis (Fig. 5, A to H), and spermatophore formation in the vas deferens. No influence of TBT was detected in sperm size (two-way ANOVA; effect of treatment: F=0.2; DF=1; P=0.672). In contrast, the spermatozoa from males were significantly larger than those from intersex individuals (effect of crab sex: F=36.4; DF=1; P<0.001) (Fig. 7).

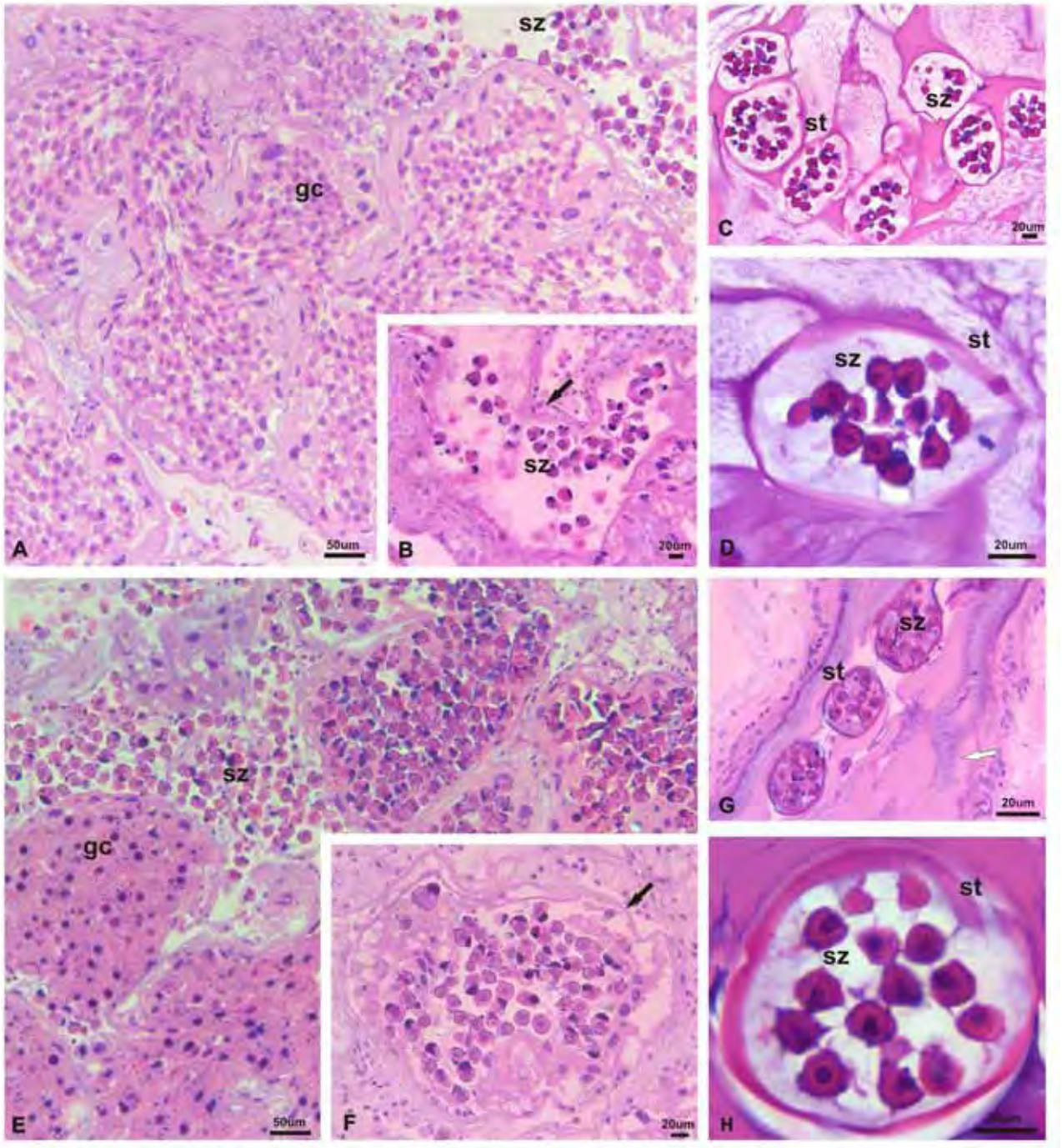

Figure 5 – Histological sections of HE-stained male reproductive system of the hermit crab *Clibanarius vittatus*. A to D, males without TBT. (A) Testis showing normal characteristics, with germinative cells and mature sperm. (B) Seminiferous duct filled with spermatozoa. (C) Normal ribbon of spermatophores surrounded with seminal fluid within the distal vas deferens. (D) Detail of a normal spermatophore filled with mature sperm. E to H, males treated with TBT. (E) General view of testis, displaying normal spermatogenesis with germinative cells and mature sperm. (F) Detail of seminiferous duct and sperm. (G) Normal ribbon of spermatophores in the distal vas deferens. (H) Detail of normal spermatophores with sperm from TBT-treated male. Male germinative cells (gc), mature sperm (sz), seminiferous duct (black arrow), distal vas deferens (white arrow), and spermatophores (st).

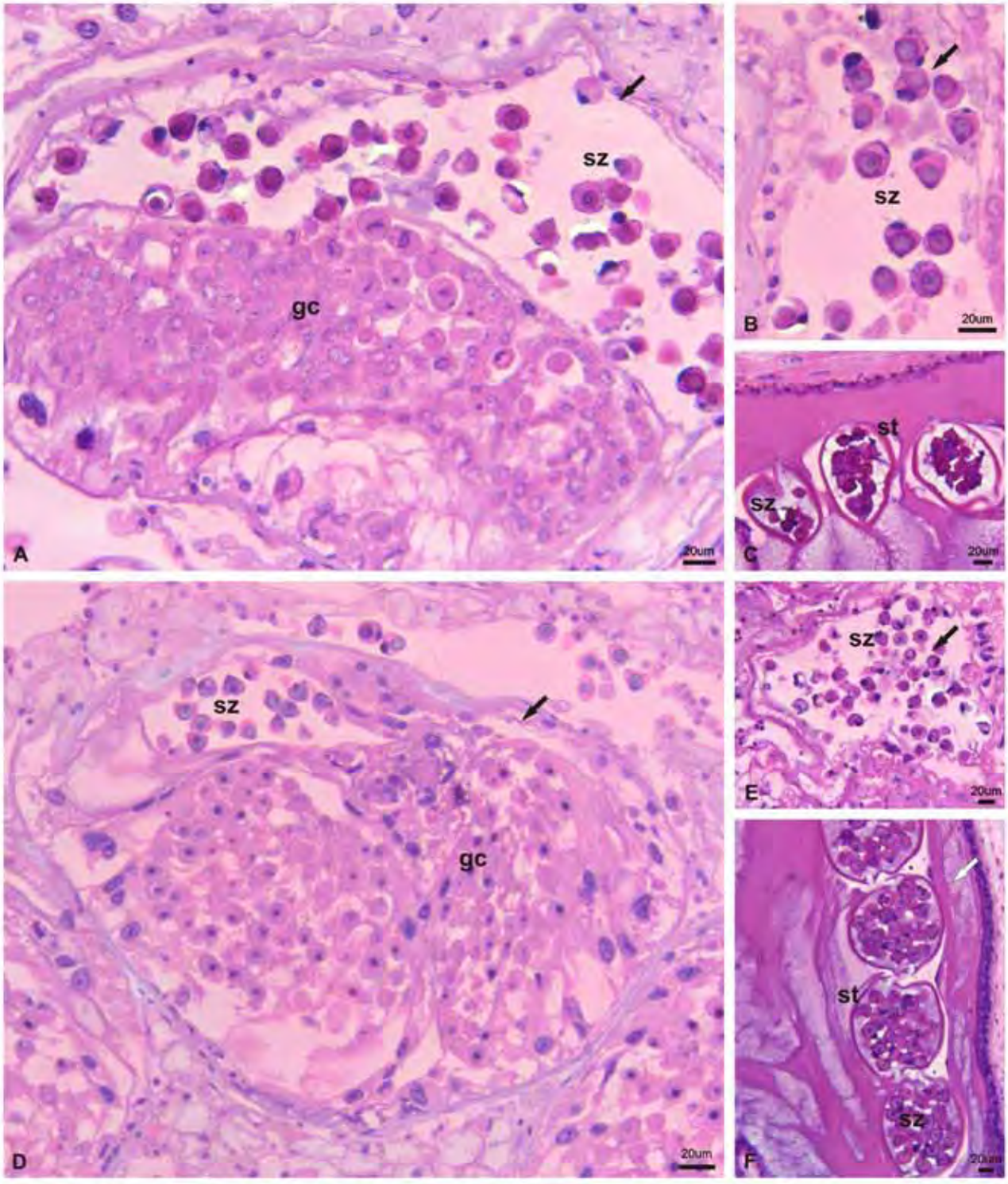

Figure 6 – Intersex reproductive system of the hermit crab *Clibanarius vittatus*. A to C, intersex individuals without TBT. (A) General view showing the normal male characteristics in the test with germinative cells and seminiferous duct filled with mature sperm. (B) Detail of seminiferous duct filled with mature sperm. (C) Normal ribbon of spermatophores surrounded with seminal fluid within the distal vas deferens. D to F, intersexes treated with TBT. (D) General view showing the normal male characteristics in the test with germinative cells and seminiferous duct filled with mature sperm. (E) Detail of seminiferous duct filled with mature sperm. (F) Normal ribbon of spermatophores surrounded with seminal fluid within the distal vas deferens. Male germinative cells (gc), mature sperm (sz), seminiferous duct (black arrow), distal vas deferens (white arrow), and spermatophores (st).

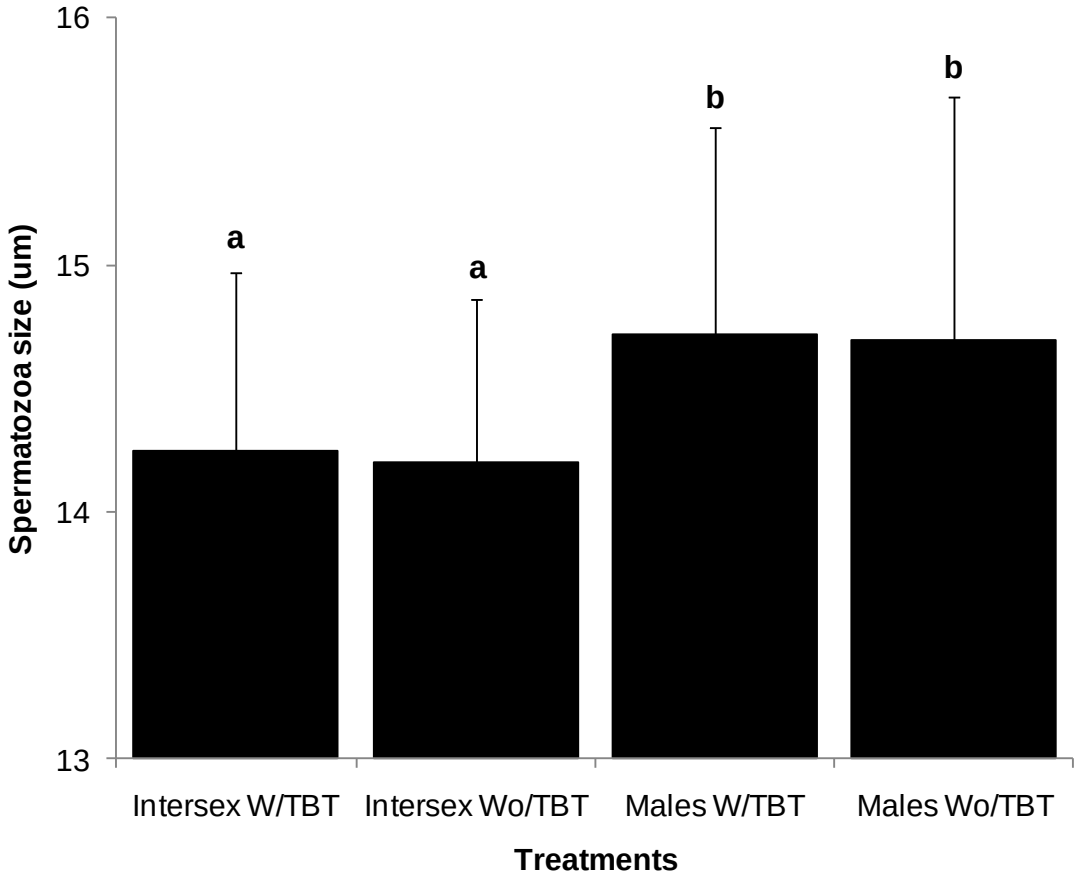

Figure 7 – Mean and standard deviation of the sperm size (µm) of intersexes and males treated with (W/TBT) or without TBT (Wo/TBT). Means followed by the same letter did not show a significant difference (*P*>0.05).

4. Discussion

Individuals treated with TBT assimilated this pollutant, and the animals maintained without TBT did not show any trace of this substance in their tissues. These results agree with the model of assimilation and rapid (within a few weeks) depuration demonstrated for *C. vittatus* by SANT'ANNA et al. (*in press*). The TBT effect was observed only in females, which showed disorganization of the ovaries, and absorption of the vitellogenic oocytes that caused the ovary to atrophy. However, the degenerative effect of TBT is apparently related to the individual characteristics of each female, because two females showed normal oogenesis with previtellogenic and vitellogenic oocytes and some atresic follicles. This process was characterized by the participation of hemocytes, as reported for other crustacean species (ADIYODI and SUBRAMONIAN, 1983). In contrast, the female gonopores did not close and male gonopores did not develop in the females, as expected. Males treated with TBT developed normal spermatogenesis and opened gonopores. Intersex individuals also developed normal spermatogenesis. However, the sperm of intersexes were smaller than those of male individuals. These results indicate that intersex individuals that act as functional males (TURRA, 2004) may have a low fertilization efficiency. The perforatorium is a key structure in oocyte fertilization, because it projects forward to the corium and causes the acrosome reaction and male pronucleus transference in crustaceans (BROWN, 1966; MEDINA, 1992).

In malacostracans, intersex individuals are most commonly treated as males, based on the dominance of testicular tissue with vestigial female characteristics (Le BLANC, 2007). Intersex hermit crabs tend to close the female gonopores, keeping the male gonopores open and acting as functional males (TURRA, 2004). In the present study, independently of the presence of TBT contamination in intersex individuals reared in the laboratory, the closure of female gonopores occurred in very low frequency. Thus, the hypothesis that TBT accelerates or completes sex reversal is not supported by these data. The toxic effect of TBT in females may lead to fertility reduction through ovarian disorganization or atrophy. This result is similar to that observed in gastropod mollusks, in which ovarian atrophy and sterility was also demonstrated (BARREIRO et al., 2001; BARROSO et al., 2002; SHI et al., 2005b). Many factors may be associated with these results: 1 – the exposure time to TBT may be too short, because in nature the specimens are exposed to this substance for a long period during ontogeny; 2 – the TBT concentration employed in the experiment may not be adequate. In mollusks, very low

concentrations of TBT can lead to the development of imposex, as observed for the Dog-Whelk *Nucella lapillus* (Linnaeus, 1758), in which 1-2 ng Sn/l was demonstrated to cause sterility (BRYAN et al., 1988, 1989). In fishes, an increase in mortality and impairment of embryonic development were associated with TBT concentration (HANO et al., 2007); 3 – intersexuality in hermit crabs may not be related to TBT contamination.

In this last explanation, one may argue that closure of female gonopores in intersexes can be modulated by the reproductive momentum of these individuals, since the intersex individuals observed by TURRA (2004) copulated successfully as males before gonopore closure/size reduction. This information is fully consistent, because it was previously recorded in three different species, *C. vittatus*, *C. sclopetarius*, and *C. antillensis* (TURRA, 2004). Thus, the trigger for female gonopore closure, and sex reversal if it really occurs, may be related to social and behavioral characteristics, which may or may not be modulated by TBT, a possibility that should be further investigated.

Recently, hermit crabs were recorded with both male and female gonads or with only functional male gonads in the natural environment. When female gonads are present, they are functional and develop complete oogenesis (SANT'ANNA et al., 2010), confirming that intersex hermit crabs can carry egg masses, thus reproducing as females (TURRA, 2007). If intersexuality is caused by TBT, it would be expected that male gonads and gonopores in females will develop at the same time that ovary reabsorption occurs, but maintaining ovary function. However, this was never recorded. Therefore, these comparisons with the results of SANT'ANNA et al. (2010) indicate that intersexuality may be not related to TBT pollution, because the action of TBT in females promotes disorganization and atrophy of the ovaries, as recorded in the present study.

The female gonopore closure and the intersex acting as functional males in hermit crabs (TURRA, 2004), as well as the occurrence of intersexes with both male and female functional gonads may indicate a true sequential hermaphroditism, occurring as part of the protogynic process (SANT'ANNA et al., 2010). These individuals with both sets of gonads may act as males or females during the process of sex change (TURRA, 2004; TURRA, 2007; SANT'ANNA et al., 2010). However, the hypothesis of protogynic sex change is still an unresolved question. In all 17 hermit-crab species reported as having intersex individuals (for review see TURRA, 2007; SANT'ANNA et al., 2010), only alterations in female gonopores (one or two) were recorded, and both male gonopores were always

open. If females change to males, a record of the sequence of appearance of male gonopores (one and then two) in females would be expected, but this situation has never been recorded. In addition, the results of the present study indicate that the right gonopore closes first in intersex hermit crabs in the case of protogyny, or the left gonopore opens first in the case of protandric development.

The absence of these records merits attention, and other hypotheses to explain intersexuality in hermit crabs should be considered. The feminization process may be made possible by the impact of endocrine disruptors with estrogenic effects, such as pesticides and polychlorinated biphenols (FORD, 2008). These substances are common in estuarine environments and in their biota (WALSH, 1972; COLOMBO et al., 1995; YOGUI et al., 2003), where these compounds continuously enter in effluent discharges. Conversely, one may argue that intersex hermit crabs are feminized males, which maintain male characteristics and at the same time develop female features such as gonopores and gonads (SANT'ANNA, et al. 2010). This hypothesis is also supported by the results of TURRA (2004), where closure of the female gonopores indicates that the reproductive stimulus may accelerate the disappearance of female characteristics in intersex hermits. Males under strong hormonal influence during mating may counteract the effects of a feminizing substance. This is an alternative hypothesis for both protogyny and the relationship of intersexuality to TBT contamination.

This crustacean de-masculinization is possible (see review by FORD, 2008), but to check this hypothesis in hermit crabs and its relationship with intersexuality, new approaches that consider the possibility of protandric development, such as the use of experimental analyses in the laboratory and in the natural environment, genetic techniques to determine the sex of the intersex individuals, as well as hormonal analyses to investigate possible hormonal disruptions.

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Capítulo 7

Considerações Finais

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1. Monitoramento de Compostos Butílicos

Por meio dos resultados do presente estudo pode-se observar que a concentração de organoestânicos não varia em diferentes períodos do dia, amplitudes de maré ou zonas de maré, quando comparadas áreas intertidais e de águas rasas. Assim, a análise da contaminação pode ser realizada com amostras em diferentes condições. No entanto a variação da concentração de compostos butílicos (Bts) observada em escala geográfica pequena (em uma mesma praia) indica a necessidade de amostras compostas com replicação adequada que representem a área a ser estudada.

O monitoramento dos estuários revelou que áreas sem atividade portuária também apresentam elevados concentrações por Bts, mesmo após a proibição do uso de TBT em tintas antiincrustantes. Tal resultado indica que compostos Bts continuam sendo liberados no ambiente ou que estes se encontram acumulados em diferentes compartimentos do ambiente. A comparação dos Bts encontrados no ambiente e nos tecidos dos ermitões, revelou que não existe correlação entre a concentração destes poluentes, provavelmente devido à distribuição desigual destes compostos encontrada na natureza e do fato das concentrações no sedimento dependerem de fatores hidrodinâmicos e das características do próprio sedimento.

O registro de ermitões contaminados em muitas amostras provenientes de locais onde não foram detectados compostos Bts, indica que ermitões são melhores indicadores de poluição por Bts quando comparados a sedimentos. Ermitões não representam apenas a contaminação pontual como no caso de uma amostra de sedimento. Estes animais indicam a contaminação da área onde vivem, uma vez que se locomovem e estão à mercê destes compostos não apenas no ponto exato onde foram capturados.

Além disso, os experimentos em laboratório demonstraram que ermitões assimilam TBT proveniente da alimentação e depuram este composto em poucas semanas. O habito de vida dos ermitões, especificamente de *Clibanarius vittatus* que é uma espécie tipicamente estuarina, com pequena mobilidade e de tamanho relativamente grande que facilita a captura e composição de amostras, faz desta espécie um bom candidato a ser utilizado como indicador de contaminação recente por TBT.

2. Intersexualidade x TBT

As investigações morfológicas revelaram que a maioria dos indivíduos intersexo apresentam sitema reprodutor masculino funcional. No entanto, alguns indivíduos apresentaram gônadas funcionais tanto de macho como de fêmeas. Estas observações confirmam que indivíduos intersexo podem reproduzir como machos ou fêmeas, suportando a hipótese do processo de hermafroditismo seqüencial verdadeiro.

A hipótese que o TBT é responsável pela intersexualidade em ermitões, promovendo o desenvolvimento de características sexuais masculinas em indivíduos fêmeas não foi comprovada. Fêmeas alimentadas com TBT durante longo período (9 meses) não desenvolveram características masculinas externas (gonóporos no quinto par de pereópodos) e internas (testículo e vaso deferente). No entanto foi comprovado que o TBT é tóxico, e promove desorganização e atrofia dos ovários em fêmeas.