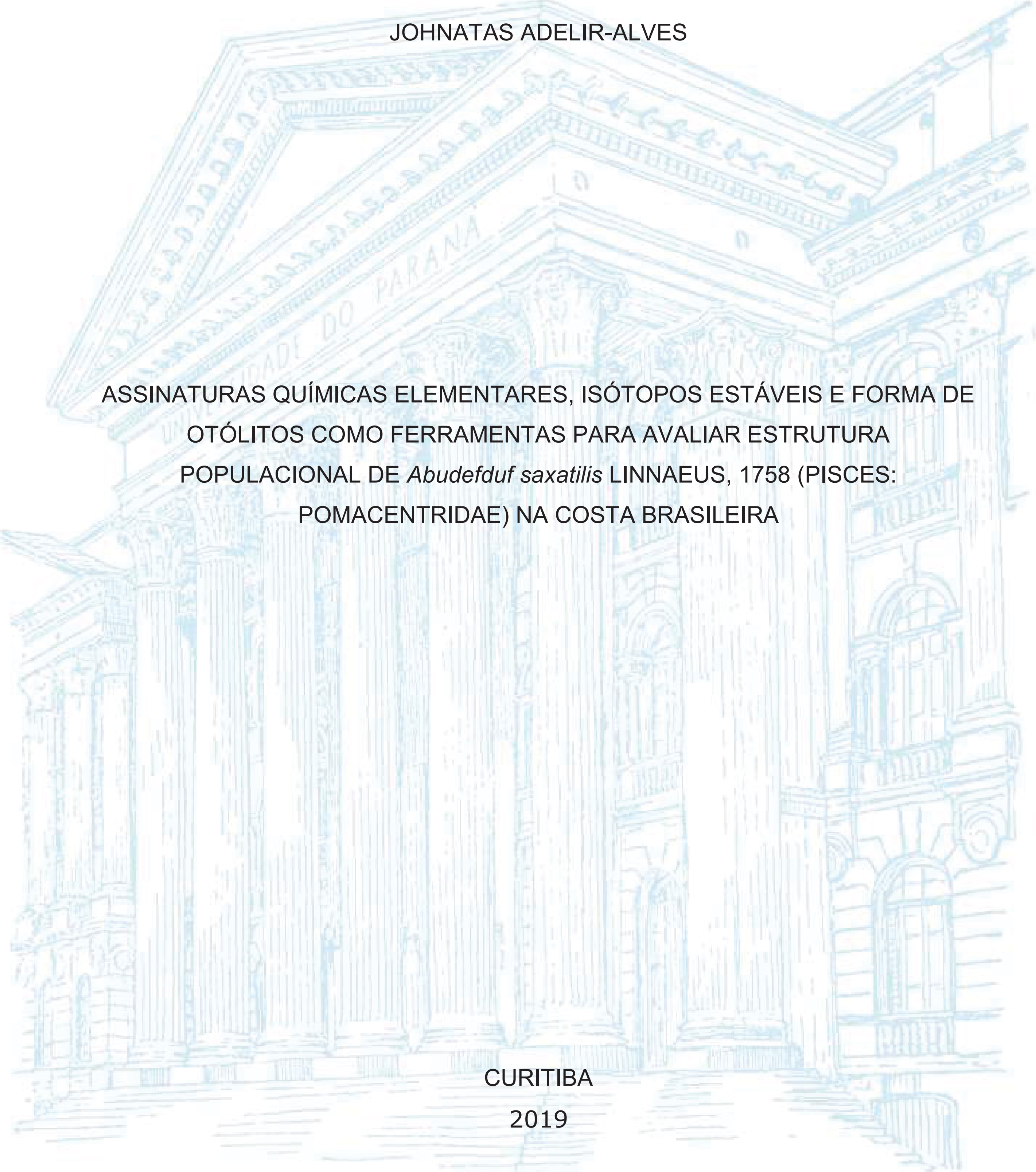
UNIVERSIDADE FEDERAL DO PARANÁ

JOHNATAS ADELIR-ALVES



ASSINATURAS QUÍMICAS ELEMENTARES, ISÓTOPOS ESTÁVEIS E FORMA DE OTÓLITOS COMO FERRAMENTAS PARA AVALIAR ESTRUTURA POPULACIONAL DE *Abudefduf saxatilis* LINNAEUS, 1758 (PISCES: POMACENTRIDAE) NA COSTA BRASILEIRA

CURITIBA
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ASSINATURAS QUÍMICAS ELEMENTARES, ISÓTOPOS ESTÁVEIS E FORMA DE OTÓLITOS COMO FERRAMENTAS PARA AVALIAR ESTRUTURA POPULACIONAL DE *Abudefduf saxatilis* LINNAEUS, 1758 (PISCES: POMACENTRIDAE) NA COSTA BRASILEIRA

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Orientador: Prof. Dr. Henry Louis Spach

Coorientador: Prof. Dr. Alberto Teodorico Correia

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **JOHNATAS ADELIR ALVES** intitulada: **ASSINATURAS QUÍMICAS ELEMENTARES, ISÓTOPOS ESTÁVEIS E FORMA DE OTÓLITOS COMO FERRAMENTAS PARA AVALIAR ESTRUTURA POPULACIONAL DE *Abudefduf saxatilis* LINNAEUS, 1758 (PISCES: POMACENTRIDAE) NA COSTA BRASILEIRA**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 28 de Março de 2019.

Alberto Teodorico Correia

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Avaliador Externo (UNESP)

Dedico esse trabalho às pessoas mais importantes de minha vida, meus pais.

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PRÓLOGO

Esta pesquisa foi desenvolvida através do Programa de Doutorado em Zoologia do Setor de Ciências Biológicas da Universidade Federal do Paraná (UFPR), no Laboratório de Ecologia de Peixes do Centro de Estudos do Mar da UFPR, em parceria com o Laboratório de Ecofisiologia do Centro Interdisciplinar de Investigação Marinha e Ambiental da Universidade do Porto, do Laboratório de Isótopos Estáveis da Universidade Federal de Pernambuco e suporte financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior e do Conselho Nacional de Desenvolvimento Científico e Tecnológico.

Em virtude da falta de informações dos aspectos ecológicos básicos, o trabalho a seguir faz parte de um conjunto de informações que visam complementar o conhecimento sobre o ciclo de vida do sargentinho, *Abudefduf saxatilis*, um peixe recifal abundante e com ampla distribuição no Oceano Atlântico. Assim foram utilizadas diferentes metodologias para analisar os otólitos sagittae, excelentes marcadores fenotípicos.

Abudefduf saxatilis. Foto: Áthila A. Bertoncini.

Esta tese é composta por um corpo principal com três capítulos, sob a forma de publicação aceite, submetida e/ou em preparação, com uma introdução geral e discussão final, contendo as respostas ou resultados finais, às perguntas levantadas sobre os aspectos ecológicos da espécie alvo. No primeiro capítulo analisamos a estrutura populacional de *A. saxatilis*, em pequena e média escalas geográficas, através das assinaturas químicas elementares e forma dos sagittae. No segundo capítulo, analisamos a população *A. saxatilis* em uma escala regional, com amostras coletadas entre o nordeste e o sul da costa Brasileira e uma ilha oceânica, através da análise da forma e razões isotópicas dos otólitos sagittae. No terceiro capítulo avaliamos o período de desenvolvimento larval, através de análise microestrutural de otólitos de indivíduos recém assentados.

RESUMO

Ambientes recifais constituem complexas comunidades, sustentam uma alta produção primária e possuem alta diversidade de espécies, como as assembleias de peixes recifais. O conhecimento acerca da ecologia dos peixes é fundamental na elaboração de estratégias de conservação e manejo, havendo necessidade de estudos ecológicos para grande parte das espécies do oceano Atlântico Sul. Conhecemos pouco sobre as estruturas populacionais e os níveis de conectividade entre elas, rotas migratórias, conectividade entre as zonas de desova, e respectivas zonas de recrutamento, crescimento e reprodução. Pomacentridae está entre as famílias de peixes recifais mais ricas em espécies, sendo o sargentinho, *Abudefduf saxatilis*, uma espécie comum, abundante e com ampla distribuição no oceano Atlântico. Estudos ecológicos com o uso de razões isotópicas, química elementar, forma e microestrutura dos otólitos vem sendo utilizados para estudos populacionais com peixes marinhos, permitindo uma compreensão ampla e sistêmica do ciclo de vida dos peixes. Estas metodologias têm se mostrado eficazes e complementares aos estudos de genética molecular, nomeadamente com recurso ao DNA mitocondrial, uma vez que as espécies marinhas costeiras, com uma fase larval longa e elevada dispersão dos indivíduos, não têm padrões genéticos de diferenciação espacial, consequência do elevado fluxo gênico. Diante deste cenário, os objetivos deste trabalho foram; (i) Analisar as assinaturas químicas elementares e forma de otólitos sagittae de *A. saxatilis*, em pequena escala, entre as ilhas do Arquipélago dos Tamboretes, no estado de Santa Catarina (Capítulo I); (ii) Analisar as assinaturas químicas elementares e forma de otólitos sagittae de *A. saxatilis*, em média escala, entre três ilhas costeiras do sudeste-sul do Brasil (Capítulo I); (iii) Analisar a estrutura populacional de *A. saxatilis* ao longo da costa Brasileira, através da forma e razões isotópicas de carbono ($\delta^{13}C$) e oxigênio ($\delta^{18}O$) de otólitos sagittae (Capítulo II); e, (iv) Analisar a microestrutura de otólitos sagittae de recrutas recém assentados de *A. saxatilis* (Capítulo III). Análises combinadas de microquímica e forma dos otólitos indicam haver separação populacional entre recifes distantes, resultado suportado por uma alta porcentagem da discriminante entre pontos separados (≅70km), indicando subpopulações entre a Ilha de Bom Abrigo (SP), Ilha da Galheta (PR) e a Ilha da Paz (SC). As assinaturas isotópicas e a forma dos otólitos podem ser utilizadas como ferramentas úteis para estudar a estrutura populacional de peixes não-migratórios e associados a recifes. Os resultados indicaram uma estrutura populacional segregada entre a região nordeste, a região sudeste-sul e ilhas oceânicas. Analisar os otólitos de indivíduos selvagens e recém assentados de *A. saxatilis* tem por objetivo descrever os estágios iniciais de vida da espécie. Os resultados mostraram que o período de desenvolvimento larval dura em média 11,7 dias (entre 9 e 15 dias) e três zonas de crescimento foram observadas nos otólitos dos indivíduos coletados, representando alguns eventos da história de vida durante a fase larval: as marcas de eclosão e assentamento (fase larval pelágica) e pós-assentamento (recrutas e juvenis). Os resultados desta pesquisa podem ser utilizados como base para futuros estudos ecológicos com *A. saxatilis* e para outras espécies de peixes recifais territorialistas, auxiliando políticas públicas estratégias de manejo e a justificar e direcionar recursos às ações de conservação da biodiversidade marinha.

Palavras-chave: peixes recifais; conectividade de habitats; sagittae; química elementar; razões isotópicas; forma; microestrutura.

ABSTRACT

Reef environments are complex communities, sustain a high primary production and have a high diversity of species, such as reef fish assemblages. The knowledge about fish ecology is fundamental in the design of conservation and management strategies, and ecological studies are necessary for most species in the South Atlantic Ocean. We know little about population structures and the levels of connectivity between them, migratory routes, connectivity between spawning sites, and their recruitment, growth and reproduction. Pomacentridae is among the richest reef fish families, and the seargent major, *Abudefduf saxatilis*, is a common and abundant species, and widely distributed in the Atlantic Ocean. Ecological studies using isotopic ratios, elemental chemistry, shape and microstructure of otoliths have been used for population studies with marine fish, allowing a broad and systemic understanding of the fish life cycle. These methodologies have been shown to be effective and complementary to molecular genetics studies, especially with mitochondrial DNA, since the coastal marine species, with a long larval phase and high dispersion of the individuals do not have genetic patterns of spatial differentiation, as a consequence of the high gene flow. In this scenario, the objectives of this study are: (i) Analyze the elemental chemical signatures and shape of *A. saxatilis* sagittae otoliths, on a small scale, between the islands of the Tamboretes Archipelago, in the state of Santa Catarina (Chapter I); (ii) Analyze the elemental chemical signatures and shape of *A. saxatilis* sagittae otoliths, on a medium scale, between three coastal islands of south Brazil (Chapter I); (iii) Analyze the population structure of *A. saxatilis* along the Brazilian coast, through the shape and isotopic carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) ratios of otoliths sagittae (Chapter II); and (iv) Analyze the microstructure of sagittae otoliths of newly settled *A. saxatilis* (Chapter III). Combined microchemistry and shape otolith analysis indicate that there is a population separation between distant reefs, a result supported by a high percentage of the discriminant between sample areas (≅70km), indicating subpopulations between the Islands of Bom Abrigo (SP), Galheta (PR) and Paz (SC). Isotopic signatures and shape of otoliths can be used as useful tools for studying the population structure of non-migratory and reef-associated fish. The results indicated a segregated population structure between the northeast region, south region and the oceanic islands. Analyzing the otoliths of wild and newly settled individuals of *A. saxatilis* is aimed describing the initial stages of the apecies. The results showed that the larval development period lasts on average 11.7 days (range 9-15 days) and three growth zones were observed in the the otoliths of the individuals collected, representing some events in the life history during the larval phase: hatching and settlement (pelagic larval stage) and post-settlement (recruits and juveniles). The results of this research can be used as a basis for future ecological studies with A. saxatilis and for others territorial reef fish, helping public policies management strategies and to justify and direct resources to actions of biodiversity marine conservation.

Keywords: reef fish; habitat connectivity; otolith; sagittae; elemental chemistry; isotopic ratios; shape; microstructure.

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INTRODUÇÃO GERAL

A zona costeira possui um mosaico composto por diversos ecossistemas interligados, criando assim condições para a dispersão de larvas, juvenis ou adultos, de diversas espécies entre diferentes habitats (ex. migração ontogenética entre manguezais e recifes) (Waples 1998; Ogden et al. 2005; Unsworth et al. 2008). A conectividade entre habitats é importante para as comunidades marinhas, pois gera a ligação demográfica das populações locais através da dispersão de indivíduos entre eles, indicando um auto-recrutamento local e ou chegada de indivíduos de outras populações. Conectividade é um componente crítico em populações de peixes marinhos, uma vez que determina unidades populacionais, reposição populacional, padrões de colonização e a resistência das populações à pressão antrópica (Cowen et al. 2002; Sale et al. 2005).

Os impactos diretos e indiretos das atividades humanas contribuem significativamente para a perda de biodiversidade, deterioração e fragmentação de habitats costeiros e marinhos, causando alterações de grande magnitude na composição das comunidades dos ecossistemas recifais (Moyle e Leidy 1992; Jackson et al. 2001; Folke et al. 2004). Os ambientes recifais são importantes pelos diversos papéis ecológicos e serviços ecossistêmicos atribuídos a estes habitats, além de concentrar grande diversidade e abundância de espécies (Roberts et al. 2002). No Brasil os ambientes recifais estão presentes em aproximadamente um terço do litoral, são considerados de grande importância ecológica, econômica e social. Estes ecossistemas sustentam alta produção primária, alta diversidade e abundância de espécies (Ferreira et al. 2000; Floeter e Gasparini 2000; Ferreira et al. 2004).

O conhecimento acerca da ecologia dos peixes é fundamental para descrever aspectos sobre dinâmica populacional, na identificação das unidades populacionais ou estoques, no estabelecimento de rotas migratórias e no estudo da conectividade entre as zonas de desova ou berçário, e respectivas zonas de recrutamento e crescimento costeiro, estrutura genética e biogeografia de populações (Stephenson 1999; Sale et al. 2005; Cowen et al. 2006). Estas informações contribuem significativamente para a identificação de habitats ecologicamente importantes, para a gestão integrada e racional (sustentável) das populações de peixes, para a conservação e preservação das espécies e o design de áreas marinhas protegidas, que são as principais preocupações desde que as espécies marinhas estão sob um estresse considerável e crescente devido as atividades humanas (Pauly et al. 1998; Jackson et al. 2001; Roberts et al. 2002). Apesar disso, os estudos realizados atualmente não atendem a demanda de informações para a elaboração de planos de conservação e uso sustentável da biodiversidade marinha, existindo a necessidade de estudos ecológicos para a maioria das espécies de peixes marinhas (Amaral e Jablonski 2005; Floeter et al. 2006).

A ictiofauna brasileira é composta por aproximadamente 1.298 espécies marinhas, das quais 456 pertencem à Ordem Perciformes, e a família Pomacentridae, no Brasil é representada por 15 espécies (Menezes et al. 2003; Leite et al. 2009). E a família se destaca por constituir um dos grupos marinhos mais representativos e diversificados em ambientes recifais, com 406 espécies (Wainwright e Bellwood 2002; Eschmeyer e Fong 2017).

Os pomacentrídeos apresentam alta diversidade, densidade, abundância nas comunidades onde ocorrem e possui distribuição global em ambientes recifais tropicais e temperados, sendo dominante em termos de riqueza nas regiões tropicais (Allen 1991; Frédérich e Parmentier 2016) (Figura 1). A maioria dos pomacentrídeos são territorialistas, em muitos casos dominantes em ambientes recifais (Allen 1991; Ceccarelli et al. 2001; Ceccarelli 2007). Algumas espécies podem apresentar também hábito onívoro ou planctívoro, além de herbívoros (Choat 1991; Ceccarelli et al. 2001; Ceccarelli 2007). São considerados espécies-chave na manutenção da diversidade de algas (Hixon e Brostoff 1983), na determinação da zonação das comunidades de corais (Wellington e Victor 1989), na modificação da atividade alimentar de outros peixes herbívoros (Jones 1992) e na estruturação de comunidades bentônicas de dentro de seus territórios, através de seu comportamento territorial e alimentar (Ferreira et al. 2000; Ceccarelli et al. 2001; Hata e Kato 2002). O sargentinho, *Abudefduf saxatilis* (Linnaeus 1758), é um pomacentrídeo que apresenta elevada abundância e ampla distribuição geográfica, comum em ilhas oceânicas e em ambos os lados no Oceano Atlântico (Figura 1), ocupando ambientes estuarinos e recifais (Jones et al. 2010; Xavier et al. 2012), distribuindo-se em latitudes tropicais e subtropicais.

Figura 1. *Abudefduf saxatilis* é um dos peixes recifais mais comuns e abundantes do Oceano Atlântico. Cardume de sargentinho registrado na Ilha Grapirá, Parque Nacional Marinho das Ilhas dos Currais, Paraná, Brasil. Foto: Marcelo Soeth.

Abudefduf saxatilis são ovíparos, formam ninhos e seus ovos são demersais, ficando aderidos ao substrato consolidado (Bessa et al. 2007). Existe cuidado parental com dimorfismo sexual para machos e femeas. A fecundação é externa e os machos defendem seus ninhos com territorialismo reprodutivo durante todo período embrionário, entre 4 e 6 dias, guardando e arejando os ovos, até a eclosão (Araújo et al. 2004; Wittenrich et al. 2012; Adam et al. 2017). Os sargentinho são gonocóricos e o sexo é definido logo no início do desenvolvimento ontogenético (Thresher, 1984; Robertson 1988; Alshuth et al. 1998). A espécie forragea no substrato e na coluna d'água e é considerada omnívora pela dieta variada, composta por invertebrados e algas (Dubiaski-Silva e Masunari 2008; Krajewski e Floeter

2011; Anderson et al. 2015; Rocha e Myers 2015). Existem relatos de canibalismo filial e não filial sobre os ninhos/ovos, do consumo de ectoparasitas quando jovem pois nessa fase *A. saxatilis* atua como limpador em estações de limpeza, assim como registros do comsumo de outros materiais particulados, como fezes (Foster 1987; Manica 2002; Sazima et al. 2003; Cheney 2008).

No Atlântico Ocidental, a espécie ocorre da Carolina do Norte, nos Estados Unidos (Lubbock e Edwards 1981), até o litoral norte do Uruguai (Nion et al. 2002) e no Atlântico Oriental, ocorre na costa Africana, do Senegal até Angola (Randall 1996). Registros recentes da espécie foram feitos ao norte da área de ocorrência no Atlântico Oriental, na Ilha da Madeira (Freitas e Araújo 2006), no Mar do Mediterrâneo (Tsadok et al. 2015; Vella et al. 2016) e nas Ilhas Canárias (Pajuelo et al. 2016). O fato de ser uma espécie onívora oportunista (Cheney 2008) e generalista, em relação ao uso do habitat e dieta, reflete o sucesso da ampla distribuição (Molina et al 2006; Rodrigues e Molina 2007) (Figura 2). A ampla distribuição geográfica e elevada abundância também estão associados a fatores antrópicos (Pajuelo et al. 2016; Paula et al. 2018).

Figura 2. Distribuição geográfica de *Abudefduf saxatilis* no Oceano Atlântico. Destaque para as áreas onde a espécie é residente (laranja). Fonte: Rocha e Myers 2015.

No Brasil, *A. saxatilis* ocorre de norte a sul (Figura 2) e os eventos de dispersão da espécie são causados principalmente pela Corrente Equatorial Sul, vinda do Atlântico Norte, que passa pelos Arquipélago de São Pedro e São Paulo, Fernando de Noronha e Atol das Rocas e se divide na costa do Rio Grande do Norte, ramificando-se na Corrente Guiana para o norte e a Corrente Brasil para o sul (Robertson 1988; Allen 1991; Alshuth et al. 1998; Ferreira et al. 2004; Molina et al. 2006; Krajewski e Floeter, 2011).

Abudefduf saxatilis (Fig. 3A e B) é a única espécie do gênero que ocorre no lado ocidental do Oceano Atlântico, porém o sargentinho africano (Fig. 3C), *Abudefduf hoefleri* (Steindachner 1881), pode representar a mesma distribuição de *A. saxatilis* no Oceano Atlântico ou ainda serem conspecíficas (Rocha e Myers 2015; Tighe 2015).

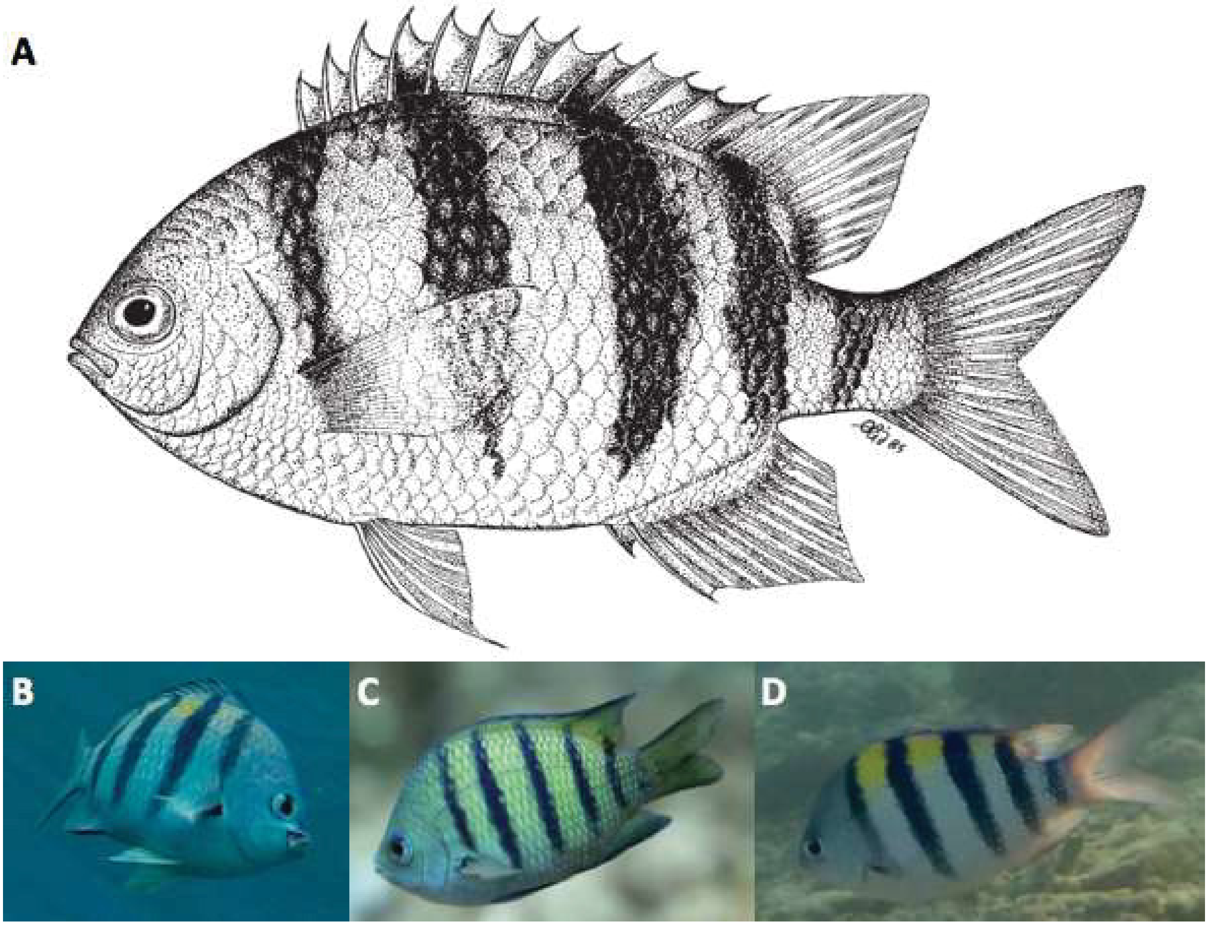

Figura 3. *Abudefduf saxatilis* (A e B). Espécies morfologicamente semelhantes do gênero *Abudefduf spp*., *A. hoefleri* (C) e *A. vaigiensis* (D). Imagens: (A) FAO (2002), (B e D) Johnatas Adelir-Alves e (C) Sergio R. Floeter.

O sargentinho indo-pacífico, com ampla distribuição nos Oceanos Índico e Pacífico (Fig. 3D), *Abudefduf vaigiensis* (Quoy e Gaimard 1825), foi muitas vezes confundido com *A. saxatilis* devido a semelhanças morfológicas, além de possuírem uma estreita relação filogenética (Fishelson 1970; Emery 1973; Allen 1991). Outras espécies muito semelhantes são *Abudefduf troschelii*, *Abudefduf sexfasciatus*, *Abudefduf bengalensis* e a recém descrita *Abudefduf nigrimargo* (Froese e Pauly 2016; Getlekha et al. 2016; Wibowo et al. 2018).

Apesar de *A. saxatilis* não possuir interrese pela atividade pesqueira, podemos listar impactos indiretos causados pela pesca. Atualmente o descarte, perca ou abandono de petrechos de pesca, também conhecido como pesca fantasma, vem sendo considerado um problema emergente nos ambientes marinhos, entre eles os recifais (Adelir-Alves et al. 2016) e por se tratar de uma espécie dependente de ambientes recifais, a degradação destes ambientes afeta a comunidade de peixes em geral, a depleção e sobrepesca dos estoques tradicionais tornam, espécies que não eram consideradas como recurso ou como fonte de proteína, em recurso, principalmente nas pescarias de subsistência (Jackson et al. 2001). O turismo desordenado é outra atividade antrópica que impacta significativamente as comunidades de peixes em recifes costeiros (Ilarri et al. 2008). A pressão antóprica direta para *A. saxatilis* é o comércio de aquariofilia, segundo a Instrução Normativa nº 202/2008, sobre o ordenamento da explotação de peixes ornamentais marinhos, anualmente podem ser comercializados 1000 indivíduos de *A. saxatilis* por empresa devidamente registrada (IBAMA 2008). Porém não existe um controle efetivo sobre a captura e o comércio dos peixes ornamentais marinhos no Brasil (Gasparini et al. 2005; Sampaio e Ostrensky 2013).

Os teleósteos (e.g. *Abudefduf saxatilis*) possuem três pares de otólitos (sagittae, lapilli e asterisci), que se distinguem pela sua localização, função, tamanho, forma e microestrutura (Tresher 1999). São formados através da deposição contínua e concêntrica de camadas de carbonato de cálcio ($CaCO_3$), originando anéis de crescimento com periodicidade anual, sazonal e diária (Campana 1999), normalmente sob a forma mineral de aragonita, embutido numa matriz proteica, denominada de otolina (Campana e Neilson 1985). Estas estruturas calcárias estão localizadas no aparelho vestibular do ouvido interno dos peixes ósseos, funcionando como receptores sensoriais que permitem a percepção do ambiente aquático em conjugação com o sistema da linha lateral (Poper et al. 2005).

Além de serem utilizados para estimar a idade e crescimento (Correia et al. 2009), os otólitos também são utilizados como marcadores naturais nos estudos de migração (Campana 1999; Albuquerque et al. 2010; Daros et al. 2016a), diferenças populacionais e de estoques pesqueiros (Correia et al. 2011; Silva et al. 2011; Daros et al. 2016b), determinação de locais de nascimento (Di Franco et al. 2011; Hamer et al. 2011), estudos da conectividade entre berçários e zonas de recrutamento costeiro (Gillanders 2005; Reis-Santos et al., 2012; Correia et al. 2014) e como bioindicador de metais pesados (Herrera-Reveles et al. 2013).

A composição química elementar e isotópica dos otólitos provou ser uma ferramenta poderosa na resolução da estrutura populacional de peixes caracterizados por um elevado fluxo gênico, migratórias e com fase larvar pelágica longa (Selkoe et al. 2008; Smith e Campana 2010). Durante a formação dos otólitos, além de $CaCO_3$ e otalina, elementos (traço) e rasões isotópicas também são depositados em pequenas concentrações, e os otólitos não sofrem transformações ou modificações químicas à posteriori (Campana e Neilson 1985; Elsdon e Gillanders 2006), e, portanto estas características químicas dos otólitos funcionam como marcadores ambientais (Campana et al. 2000; Daros et al. 2016b; Carvalho et al. 2017).

As análises da forma dos organismos ou de suas estruturas, tem um papel importante em estudos biológicos e populacionais, pois podem ser obtidas informações sobre a história de vida das espécies (Zelditch et al. 2004). Processos biológicos, como desenvolvimento ontogenético e adaptação a fatores ambientais, leva a diferenças morfológicas. A forma dos otólitos é complexa e apresenta variação clinal (Huxley 1938), características estas que podem ser exploradas em estudos ecológicos (Bird et al. 1986). A microestrutura do otólito parece ser específica e pode registrar várias transições ontogenéticas durante os primeiros estágios de desenvolvimento dos peixes, como eclosão, assentamento e metamorfose (Wright et al. 2002). Nos indivíduos adultos, estas estruturas possuem morfologia específica para cada espécie (Tuset et al. 2008), tornando-se úteis em investigações populacionais. O estudo da morfologia de otólitos permite identificar espécies e filogenias, relacionar formas a aspectos ecológicos, verificar variações geográficas, identificar presas em conteúdos estomacais, estudos de idade e crescimento (Volpedo e Echeverría 2003).

Estudos através de análises das assinaturas químicas, das razões isotópicas, da forma e da microestrutura dos otólitos, vêm sendo utilizados para estudos ecológicos e populacionais, permitindo uma compreensão mais ampla e sistêmica do ciclo de vida dos peixes (Volpedo e Vaz-dos-Santos 2015; Moreira et al. 2019; Soeth et al 2019). O uso destas ferramentas para avaliar a conectividade de peixes entre habitats e aspectos populacionais veem crescendo no Brasil (Daros et al. 2016a, Adelir-Alves et al. 2019; Soeth et al 2019), se mostrado eficaz e podem fornecer informações úteis sobre o ciclo de vida de peixes.

Diante do exposto, o estudo investigou aspectos ecológicos e populacionais de *Abudefduf saxatilis* por meio de análises químicas elementares, isotópicas, forma e microestrurura dos otólitos sagittae visando (i) Analisar as assinaturas químicas elementares e forma de otólitos sagittae de *A. saxatilis*, em pequena escala, entre as ilhas do Arquipélago dos Tamboretes, no estado de Santa Catarina, (ii) Analisar as assinaturas químicas elementares e forma de otólitos sagittae de *A. saxatilis*, em média escala, entre três ilhas costeiras do sudeste-sul do Brasil, (iii) Analisar a estrutura populacional de *A. saxatilis* ao longo da costa Brasileira, através da forma e razões isotópicas de carbono ($\delta^{13}C$) e oxigênio ($\delta^{18}O$) de otólitos sagittae, e (iv) Analisar a microestrutura de otólitos sagittae de recrutas recém assentados de *A. saxatilis* no sul do Brasil.

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CHAPTER I

Otoliths as a tool to study reef fish population structure from coastal islands of South Brazil

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Otoliths as a tool to study reef fish population structure from coastal islands of South Brazil

ABSTRACT

To promote biodiversity conservation and sustainable resource use it is highly important to understand reef fish population structure and dynamics. The sergeant major, *Abudefduf saxatilis*, is a common and abundant fish usually found in the Brazilian coasts, being considered a keystone-species for structuring benthic communities on reef habitats in the Atlantic Ocean. This study examined the morphology (shape indices and elliptic Fourier descriptors) and chemistry (Element:Ca) of *A. saxatilis* sagittal otoliths, collected in seven locations along the coast of South Brazil. Otolith morphology and chemistry were compared at short (range 0.5–2 Km) and large (range 70–140 Km) spatial scales using univariate and multivariate statistical approaches. Reclassification accuracy rate obtained from a linear discrimination function analysis using both morphology and chemistry of otoliths were 54% and 82% for short and large spatial scales, respectively. No clear separation for individuals collected in islands within Tamboretes Archipelago were observed suggesting that water masses are relatively homogeneous and/or than individuals could be highly mixed over short spatial scales. However, the high degree of accuracy for the reclassification success of the individuals between Bom Abrigo, Galheta and Paz islands indicates a limited movement of adult individuals between habitats, a larval retention mechanism or a self-recruitment process occurring at large spatial scales.

KEYWORDS: *Abudefduf saxatilis;* sagittae; shape and chemical analyses; life history

Introduction

The rapid degradation of coastal habitats by human activities (e.g. pollution, habitat loss and overfishing) makes urgent to have a better knowledge on reef fish population structure and dynamics, migration patterns and habitat connectivity, allowing us to take the appropriate conservation measures (Jackson et al. 2001; Cowen et al. 2006). Furthermore, it is well-known that ecological processes and individual responses to environment factors, determine the distribution and abundance of reef fish populations, although many gaps still exist and remains a challenge to scientists (Mora & Sale 2002).

Otoliths provide a good tool for assessing many aspects of fish biology and to address several life-history traits questions (Begg et al. 2005). The shape and chemical signatures of otoliths has been used to address fundamental questions in fish ecology and fisheries science (Popper et al. 2005; Galley et al. 2006; Walther et al. 2017). Such information may facilitate the understanding of the structure of reef fish assemblages, and thus the development and application of this methodology is a priority research area (Sale et al. 1985; Chitarro et al. 2005; Daros et al. 2016b).

Otolith shape variation is expected in both interspecific and intraspecific levels and is an efficient way to distinguish stocks or population components (Mérigot et al. 2007; Farias et al. 2009; Ferguson et al. 2011). Otolith morphology may vary according to fish size and ontogeny, geographic area, water depth, conspecific abundance, food regime, chemical and physical quality of the environment, as well as several other environmental factors (Cardinale et al. 2004; Bacha et al. 2014).

Otolith chemistry, using both elemental and isotopic analyses, has been successfully used to distinguish fish stocks or populations, movement patterns and habitat use (Smith & Campana 2010; Silva et al. 2011; Correia et al. 2012a). Some studies however have combined more than one phenotypic method, such as otolith shape and microchemical analyses, to investigate fish population structure and dynamics (Ferguson et al. 2011; Avigliano et al. 2014; Fowler et al. 2015).

Pomacentridae (damselfishes) are a diverse and widespread family of primarily marine fishes found in the tropical and subtropical oceans. The sergeant major, *Abudefduf saxatilis*, is a common conspicuous and abundant pomacentrid with a wide distribution along Atlantic Ocean. This species has omnivorous and generalist feeding habits, and it is an important prey of several large sized fishes such as snappers and groupers (Froese & Pauly 2016).

High levels of gene flow, a characteristic in many marine species such as fishes, usually ensures that genetic signal from population differentiation is weak (Waples 1998). A recent study showed no genetic differentiation among *A. saxatilis* from Brazilian coast (Souza 2017). The same result was observed for this species from Caribbean, Gulf of Mexico and the Mexican Caribbean populations (Shulman & Bermingham 1995; Piñeros et al. 2015), although some differentiation existed between Caribbean and Brazilian biogeographic provinces (Piñeros & Gutiérrez-Rodríguez 2017). Otoliths can be an alternative approach in solving population structure for marine fish with high gene flow but living in habitats where environmental heterogeneity exists (Bradbury et al. 2008; Smith & Campana 2010; Correia et al. 2012).

The goal of this work was to apply shape and chemical otolith analyses to provide new information on *A. saxatilis* population structure and connectivity at short and large spatial scales, in the South Brazilian coast.

Materials and Methods

Study Area and Sample Collection

Fish sampling took place in seven coastal islands, nearby three major estuarine complexes of South Brazil: Cananéia Bay (CB) located off São Paulo State, Paranaguá Estuarine Complex (PEC) located off Paraná State and Babitonga Bay (BB) located off Santa Catarina State (Figure 1). For short spatial scale analysis (range 0.5–2 Km) fish were collected in four sites within the Tamboretes Archipelago sites (Norte, Porto, Pássaros and Sul Islands) at the North coast off Santa Catarina State (Figure 1). For large spatial scale analysis (range 70–140 Km) fish were sampled in three regions: Bom Abrigo Island located in South coast off São Paulo State near CB, Galheta Island located off Paraná State in front of the PEC mouth, and Paz Island located at the Northern coast of Santa Catarina State near BB (Figure

1). All islands are near the coast, depths ranging between 1.5 and 25 m, sharing similar characteristics, such as rocky shores, outcrops and rocky reefs covered by algae and benthonic organisms (Moura et al. 1999; Floeter et al. 2001).

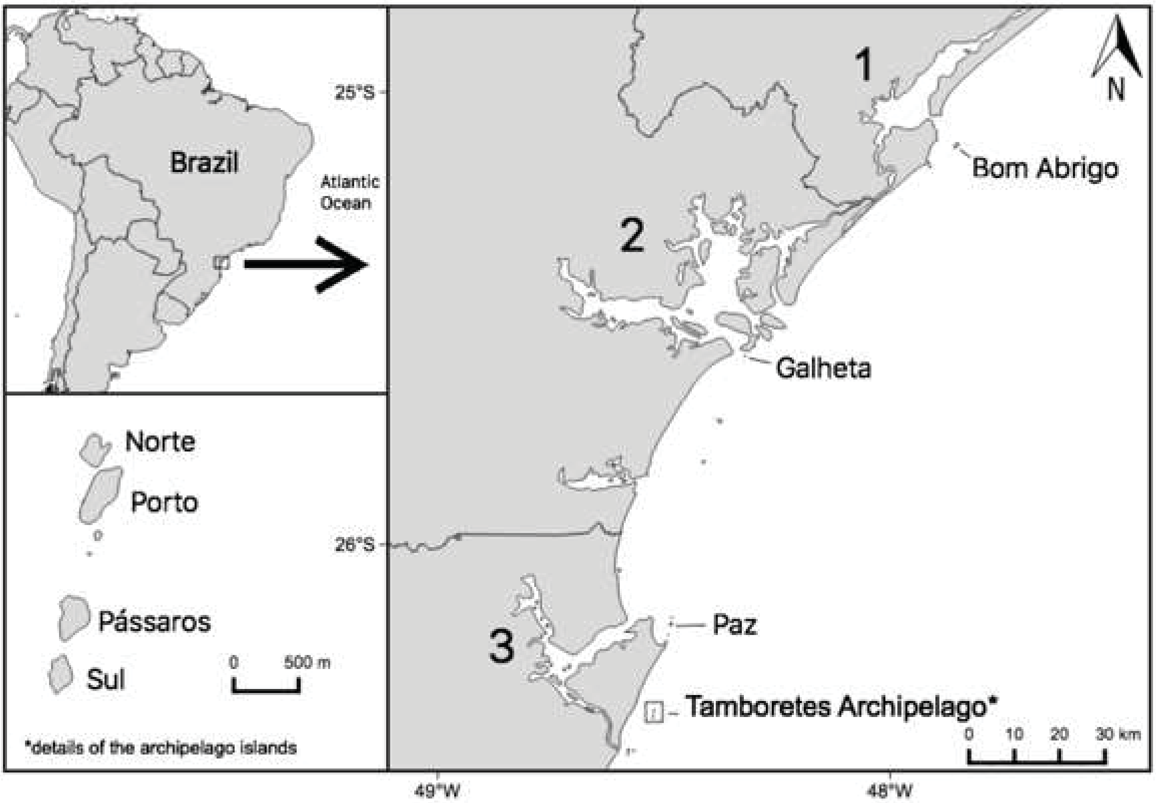

Fig. 1 Map of the south Brazilian coast indicating the study area, the seven sampling locations (3 main regions: Bom Abrigo, Galheta and Paz; 4 sites within Tamboretes archipelago: Norte, Porto, Pássaros and Sul) and the associated estuarine complexes (1- Cananeía Bay, 2- Paranaguá Estuarine Complex; 3 – Babitonga Bay).

Adults of *Abudefduf saxatilis* were collected by spearfishing at depths ranging from 2 to 5 m, between December 2015 and March 2016. In the field, fish were stored in ice and transferred to the laboratory. For each individual, total length (TL, 1 mm) and weight (W, 0.001 g) were recorded (Table I).

A total of 140 individuals (20 per location) with no significant differences in the TL (One-Way ANOVA: $F_{1.60}=2.17$, $n=140$, $P<0.05$) were previously selected. From these individuals, sagittal otoliths were carefully removed, using plastic forceps to avoid metallic contamination, cleaned in ultrapure water and stored dry in plastics vials.

Otolith Image Acquisition

Left otoliths were positioned showing the *sulcus acusticus* facing up (convex side down) and the *rostrum* to right side. Orthogonal two-dimensional digital images of otoliths were captured using a high-resolution camera (ZEISS® Axiocam 105 color with Zen Image Analysis software), mounted on a stereomicroscope (ZEISS® Discovery V12) with reflected light and a dark field, at 10x magnification.

Table I. Spatial scales, island names, geographic location, sample size (*n*), total length (TL) and fish mass (M).

Spatial scale	Island	Latitude, Longitude	*n*	TL (mm)		M (g)	
				Mean ± SE	Range	Mean ± SE	Range
Short	Norte	26°22’09.19”S, 48°31’24.00”W	20	183.0 ± 1.3	172 - 193	138.9 ± 3.2	115.8 - 163.4
	Porto	26°22’22.90”S, 48°31’23.55”W	20	184.5 ± 1.3	172 - 193	132.8 ± 4.6	100.4 - 173.9
	Pássaros	26°22’55.73”S, 48°31’29.96”W	20	184.5 ± 1.2	172 - 193	128.5 ± 3.6	109.4 - 173.1
	Sul	26°23’12.91”S, 48°31’33.26”W	20	184.5 ± 1.1	173 - 193	137.1 ± 3.7	95.9 - 159.4
Large	Bom Abrigo	25°07’01.83”S, 47°51’29.02”W	20	178.5 ± 1.5	169 - 195	125.7 ± 3.3	109.1 - 166.4
	Galheta	25°35’00.08”S, 48°19’17.48”W	20	181.5 ± 1.3	169 - 195	159.3 ± 3.6	105.6 - 185.9
	Paz	26°10’43.96”S, 48°29’09.21”W	20	181.5 ± 1.4	169 - 195	128.7 ± 4.1	102.2 - 176.1

Otolith Shape Indices

Binary otolith images (Figure 2) were used to measure four basic size parameters using the program ImageJ v. 1.50: otolith length (OL, mm), otolith width (OW, mm), otolith area (OA, mm^2) and otolith perimeter (OP, mm). These variables allowed the calculation of five otolith shape indices, namely form factor (FF), roundness (RO), ellipticity (EL), circularity (CI) and rectangularity (RE), according to Tuset et al. (2003) (Table II).

The otolith shape indices (SI) were first check for the existence of outliers using the Grubbs' test. Data were also checked for a normality and homogeneity of variances prior to statistical analysis. These assumptions were met after log 10 transformation (e.g. FF, CI and RE).

Table II. Formulas used to calculate the otolith's shape indices (from Tuset et al., 2003). Size parameters: otolith length (OL), otolith width (OW), otolith area (OA) and otolith perimeter (OP).

Shape indices	Code	Formula
Form factor	FF	$(4\pi OA)/OP^2$
Roundness	RO	$(4OA)/(\pi OL^2)$
Ellipticity	EL	$(OL-OW)/(OL+OW)$
Circularity	CI	OP^2/OA
Rectangularity	RE	$OA/(OL \times OW)$

Because otolith shape can change throughout ontogenetic development of fish, potentially confounding comparisons among populations with different age-length structures (Campana & Casselman 1993), relationship between SI and TL (as covariate) were tested with Analysis of Covariance (ANCOVA). A few otolith shape indices were significantly correlated with fish TL; RE ($r^2=0.24$; $n=140$; $P<0.05$) and CI ($r^2=0.36$; $n=140$; $P<0.05$) presented a positive relationship, opposite to FF ($r^2=0.36$; $n=140$; $P<0.05$) that showed a negative relationship. These variables have been corrected using the ANCOVA common within-group slope (Galley et al., 2006; Fowler et al., 2015). The formula used to correct it was: $V_{adjusted}=Vi-b\times TL$, where $V_{adjusted}$ is the adjusted value, Vi is the variable and *b* is the slope value (Campana & Casselman 1993; Cardinale et al. 2004; Ferguson et al. 2011).

Elliptical Fourier Descriptors

The program Shape (Version 1.3) was used to extract the contour otolith shape (Figure 2) and to determine the number of elliptic Fourier descriptors (EFD) required to adequately describe the otolith outline. A level of 95% of accumulated variance was used to select the minimum number of harmonics (Crampton 1995; Stransky et al. 2008; Ferguson et al. 2011). The first 12 harmonics reached >95% of the cumulative power indicating that the otolith shape could be adequately explained by 48 Fourier coefficients, i.e. 12 harmonics×4 coefficients (a, b, c and d). As a consequence of the normalization, the first three coefficients (a_1, b_1 and c_1) were constant and has been excluded (Iwata & Ukai 2002; Ponton 2006; Fowler et al. 2015), reducing the number of Fourier descriptors to 45.

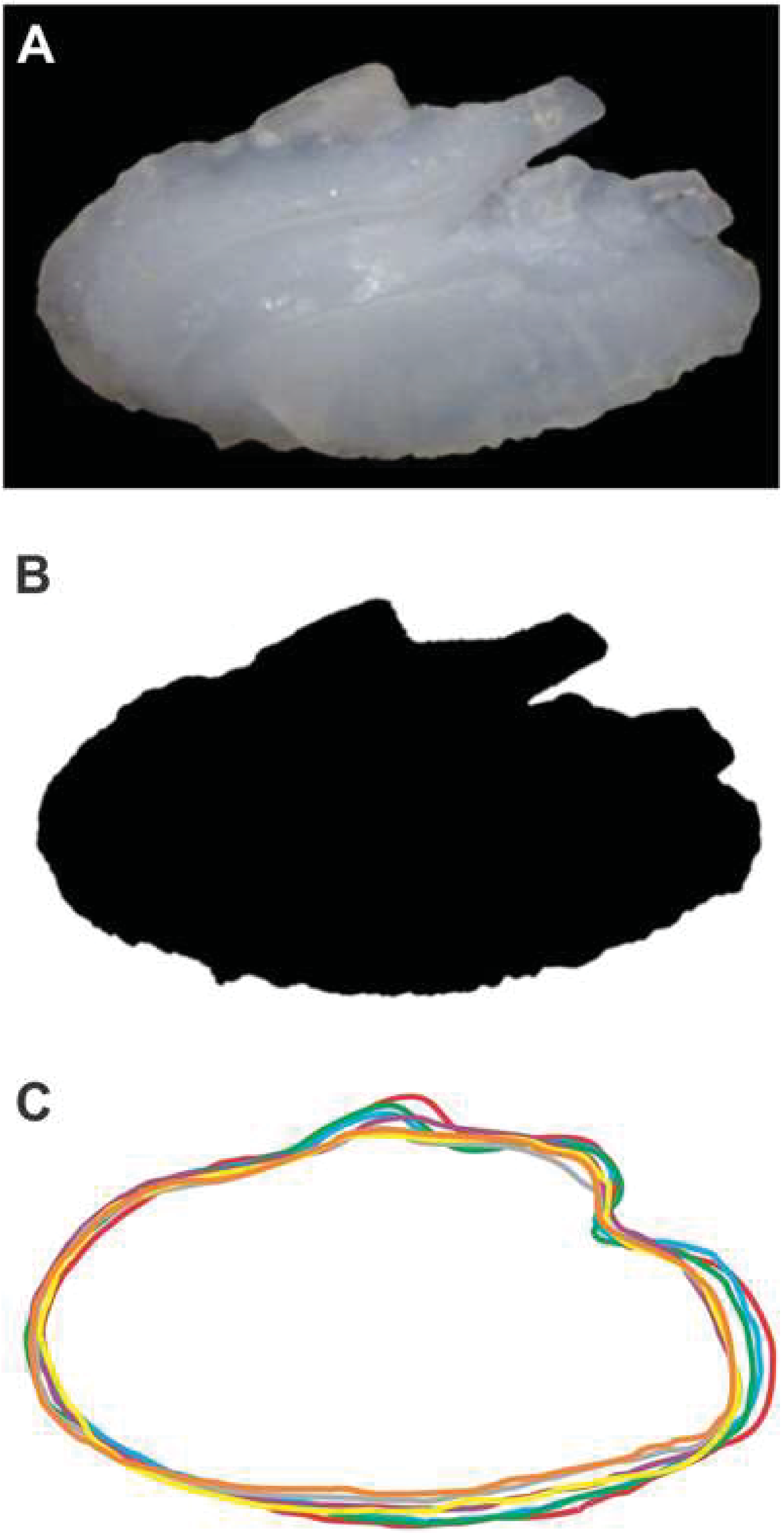

Fig. 2 The left sagittal otolith of *A. saxatilis* showing the original photograph (A), binary image (B) and the outline (C). Legend: Dorsal (D), ventral (V), post-rostrum (P), excisura major (E), rostrum (R) and anti-rostrum (A).

Otolith Elemental Signatures

Otoliths were cleaned in an ultrasonic bath for 5 min with ultrapure water (H_20, Milli-Q-Water), adherent biological tissues removed by immersion in 3% analytical grade hydrogen peroxide (H_2O_2, Fluka TraceSelect) for 15 min. Superficially decontamination was done by immersion in 1% nitric acid (HNO_3, Fluka TraceSelect) solution for 10 sec followed by a double-immersion in ultrapure water (Milli-Q-Water) during 5 min. Thereafter otoliths were stored in new, previously decontaminated (acid leached/rinsed), Eppendorf tubes, where they were allowed to air dry in a laminar flow fume hood (Patterson et al. 1999; Rooker et al. 2001; Daros et al. 2016a).

Elemental signatures of the whole otoliths were determined using solution based inductively coupled plasma mass spectrometry (SB-ICP-MS). The otoliths were weighed on an analytical balance (0.0001 g) and later dissolved in 10% of ultrapure HNO_3 (HNO_3, Fluka TraceSelect) for 15 min to a final volume of 14 mL (Sousa et al. 2011; Correia et al. 2014; Daros et al. 2016a).

SB-ICP-MS analyses were made using a magnetic sector field instrument (ICP-SF-MS), equipped with a compact double-focusing magnetic sector mass spectrometer of reversed Nier–Johnson geometry (Thermo ICP-MS x series, Thermo Electron Corporation). All measurements were made at a medium resolution setting ($m/\Delta m$=4000) to avoid false readings from spectral interferences. The instrument was equipped with a micro flow nebulizer (PFAAR35-1-C1E, Glass Expansion), operated in the self-aspirating mode (sample uptake rate ~0.93 L min^{-1}). Quantification of trace elements was based on the external calibration method, preparing multi-element standards that contained the elements of interest in the expected concentration range. To minimize the effect of any plasma fluctuations or different nebulizer aspiration rates between the samples, ^{115}In of a known concentration was added to all samples and standards as an additional internal standard. Concentrations were calculated by linear interpolation (sum of least squares) based on normalization with the internal standard, and on calibration curves made from single element standards (Merck KGaA) covering the individual expected concentration ranges. A calibration was made at the beginning of each session. The matrix of both the blank and the standard solutions was 1% HNO_3 (Correia et al. 2014; Daros et al. 2016a).

A preliminary analysis was made to determine the most likely elements (^{44}Ca, ^{88}Sr, ^{137}Ba, ^{26}Mg, ^{55}Mn, ^{54}Fe, ^{208}Pb, ^{65}Cu and ^{66}Zn) to serve as environmental indicators (Daros et al., 2016a), but excluding elements under strictly physiological regulation (Campana, 2005). All elements, with exception of ^{208}Pb, were consistently detectable in whole otoliths and were used for further statistical analysis.

Otolith samples were analyzed in random order to avoid possible sequence effects and an otolith certified reference material (FEBS-1) was analyzed for accuracy quality control (Sturgeon et al. 2005). With regard to the analytical accuracy, the elemental concentrations determined in FEBS-1 were within the certified values, with a value of recovery >95%. Precision of replicate analyses of individual elements ranged between 2% and 5% relative standard deviation (RSD). The limits of detection were calculated from the individual calibration curves using the three sigma criteria and were (in ppb): ^{44}Ca (1000), ^{88}Sr (500), ^{137}Ba (0.1), ^{26}Mg (4), ^{55}Mn (0.1), ^{54}Fe (6), ^{65}Cu (1), ^{66}Zn (1) and ^{208}Pb (0.1).

The trace elements concentrations, originally in µg element L^{-1} solution, were transformed to µg element g^{-1} otolith and then to µg element g^{-1} calcium. Elemental concentrations in µg element g^{-1} calcium were checked for normality, homogeneity of variances and variance–covariance matrices prior to statistical. These assumptions were met after log 10 transformation (e.g. Sr, Ba, Mg, Mn, Fe, Cu and Zn).

Otolith elemental concentrations were significantly related with otolith mass. A positive relationship was found for Sr (r^2=0.17; *n*=140; *P*<0.05) and Ba (r^2=0.10; *n*=140; *P*<0.05); Fe (r^2=0.34; *n*=140; *P*<0.05), Zn (r^2=0.14; *n*=140; *P*<0.05), Cu (r^2=0.08; *n*=140; *P*<0.05), Mn (r^2=0.14, *n*=140, *P*<0.05) exhibited a negative relationship; no significant relationship was however found for Mg (r2=0.00; n=140; P>0.05).

The relationship between elemental concentration and otolith mass was tested with analysis of covariance (ANCOVA) using otolith mass as a covariate. To avoid that differences in otolith mass among locations confound any site-specific differences in otolith chemistry, the concentration of elements was weight-detrended by subtraction of the common within-group linear slope multiplied by the otolith mass from the observed concentration (Campana et al. 2000).

Data Analysis

One-way analysis of variance (ANOVA) was used to explore individual differences between locations concerning the otolith shape indices and uni-elemental fingerprints. If significant differences were found (P<0.05), this was followed by a Tukey's honestly significant difference (HDS) post hoc test.

Multivariate analysis of variance (MANOVA) was used to detect differences in the SI, EFD, otolith multi-elemental fingerprints and all combined from different locations. For the MANOVA, Pillai's trace was reported as the F-ratio statistic for the most robust test of multivariate statistics.

Post-hoc multivariate pairwise comparisons between locations were performed using the Hotelling T-square test. SI, EFD, multi-elemental fingerprints and all combined were analyzed with a stepwise Linear Discriminant Function Analysis (LDFA). LDFA was performed first individually to each methodology and thereafter combining the data of all. LDFA was used to visualize spatial differences and to examine the reclassification accuracy success of fishes to this original location. Cross-validations were performed using jackknifed (''leave one out'') procedures (Correia et al. 2011).

Statistical analyses were performed using the software Systat (version 12.0). Results are presented as means ± standard errors (SE). A significance level (α) of 0.05 was used for all statistical procedures.

Table III. Descriptive otolith morphology. Size parameters [Otolith mass (OM), otolith length (OL), otolith width (OW), otolith area (OA) and otolith perimeter (OP)] and shape indices [form factor (FF), roundness (RO), circularity (CI), rectangularity and ellipticity (EL)] of otoliths from the seven islands sampled (Tuset et al., 2003). Data are mean ± SE. *Different letters indicate statistically significant differences (*P*< 0.05).

Spatial scale	Island	Size parameters				
		OM (g)	OL (mm)	OW (mm)	OA (mm^2)	OP (mm)
Short	Norte	0.0160 ± 0.0006	9.67 ± 0.17	16.41 ± 0.30	4.98 ± 0.05	2.69 ± 0.03
	Porto	0.0174 ± 0.0008	10.04 ± 0.19	16.34 ± 0.32	5.09 ± 0.04	2.72 ± 0.03
	Pássaros	0.0146 ± 0.0006	9.91 ± 0.15	15.60 ± 0.26	5.15 ± 0.05	2.68 ± 0.03
	Sul	0.0146 ± 0.0005	9.78 ± 0.17	15.97 ± 0.30	5.12 ± 0.06	2.61 ± 0.04
Large	Bom Abrigo	0.0136 ± 0.0005	8.28 ± 0.12	13.65 ± 0.14	4.56 ± 0.04	2.65 ± 0.02
	Galheta	0.0122 ± 0.0009	8.35 ± 0.21	13.62 ± 0.29	4.59 ± 0.06	2.56 ± 0.03
	Paz	0.0152 ± 0.0008	8.57 ± 0.14	13.97 ± 0.16	4.60 ± 0.04	2.62 ± 0.02
Spatial scale	Island	*Shape indices				
		FF	RO	CI	RE	EL
Short	Norte	0.481 ± 0.011^{a}	0.502 ± 0.007^{a}	26.11 ± 0.87^{a}	0.715 ± 0.004^{a}	0.286 ± 0.007^{a}
	Porto	0.479 ± 0.014^{a}	0.499 ± 0.006^{a}	26.23 ± 0.85^{a}	0.739 ± 0.009^{b}	0.311 ± 0.006^{a}
	Pássaros	0.513 ± 0.013^{a}	0.486 ± 0.007^{a}	24.46 ± 0.65^{a}	0.709 ± 0.009^{a,c}	0.312 ± 0.008^{a}
	Sul	0.474 ± 0.016^{a}	0.487 ± 0.007^{a}	26.52 ± 0.91^{a}	0.717 ± 0.003^{a,b,c}	0.309 ± 0.008^{a}
Large	Bom Abrigo	0.566 ± 0.008^{a}	0.514 ± 0.006^{a}	22.18 ± 0.34^{a}	0.701 ± 0.008^{a}	0.269 ± 0.006^{a}
	Galheta	0.555 ± 0.011^{a}	0.512 ± 0.007^{a}	22.64 ± 0.48^{a}	0.681 ± 0.009^{b,a}	0.266 ± 0.006^{a}
	Paz	0.548 ± 0.007^{a}	0.509 ± 0.004^{a}	22.91 ± 0.29^{a}	0.729 ± 0.011^{c,a}	0.283 ± 0.008^{a}

Results

Otolith Shape Analysis

Rectangularity was the only otolith shape index (Table III) that showed differences among islands for both short and large spatial scales (One-Way ANOVA: $P<0.05$).

MANOVA indicated significant differences in the SI and EFD for short (Pillai Trace, $F_{3.46}=0.47$; $P<0.05$ and Pillai Trace, $F_{4.91}=0.86$; $P<0.05$, respectively) and large spatial scales (Pillai Trace, $F_{1.57}=0.25$; $P<0.05$ and Pillai Trace, $F_{2.67}=1.01$; $P<0.05$, respectively). Pairwise comparisons indicated no differences among islands at short and large spatial scale (Hotelling's T-square, $P>0.05$).

The LDFA based on SI (Figures 5A and 6A) and EFD (Figures 5B and 6B), had little success in discriminating among samples from the islands as result of a partial overlap among groups. As consequence, jackknife reclassification accuracies were poor for short spatial scale (44% and 54%, respectively) and for large spatial scale (45% and 66%, respectively) (Table IV; Table V).

Otolith Elemental Signatures

The four islands within the Tamboretes archipelago (short spatial scale) recorded significant differences values for Sr/Ca, Ba/Ca, Fe/Ca and Mn/Ca (One-Way ANOVA: $P<0.05$). The highest values for Sr and Ba calcium ratios were observed in Norte Island. Mn/Ca highest values were observed in Porto and Sul Islands (Tukey tests: $P<0.05$). No significant differences existed however for Cu/Ca, Mg/Ca and Zn/Ca (One-Way ANOVA: $P>0.05$) (Figure 3).

Sr/Ca and Ba/Ca differed significantly among islands at large spatial scale (One-Way ANOVA: $P<0.05$). Bom Abrigo island exhibited the highest values of Sr/Ca and Ba/Ca ratios (Tukey tests: $P<0.05$). Galheta island showed the lowest value of Ba/Ca ratio (Tukey test: $P<0.05$). No differences were however observed from Cu/Ca, Fe/Ca, Mn/Ca, Mg/Ca and Zn/Ca among islands (One-Way ANOVA: $P>0.05$) (Figure 4).

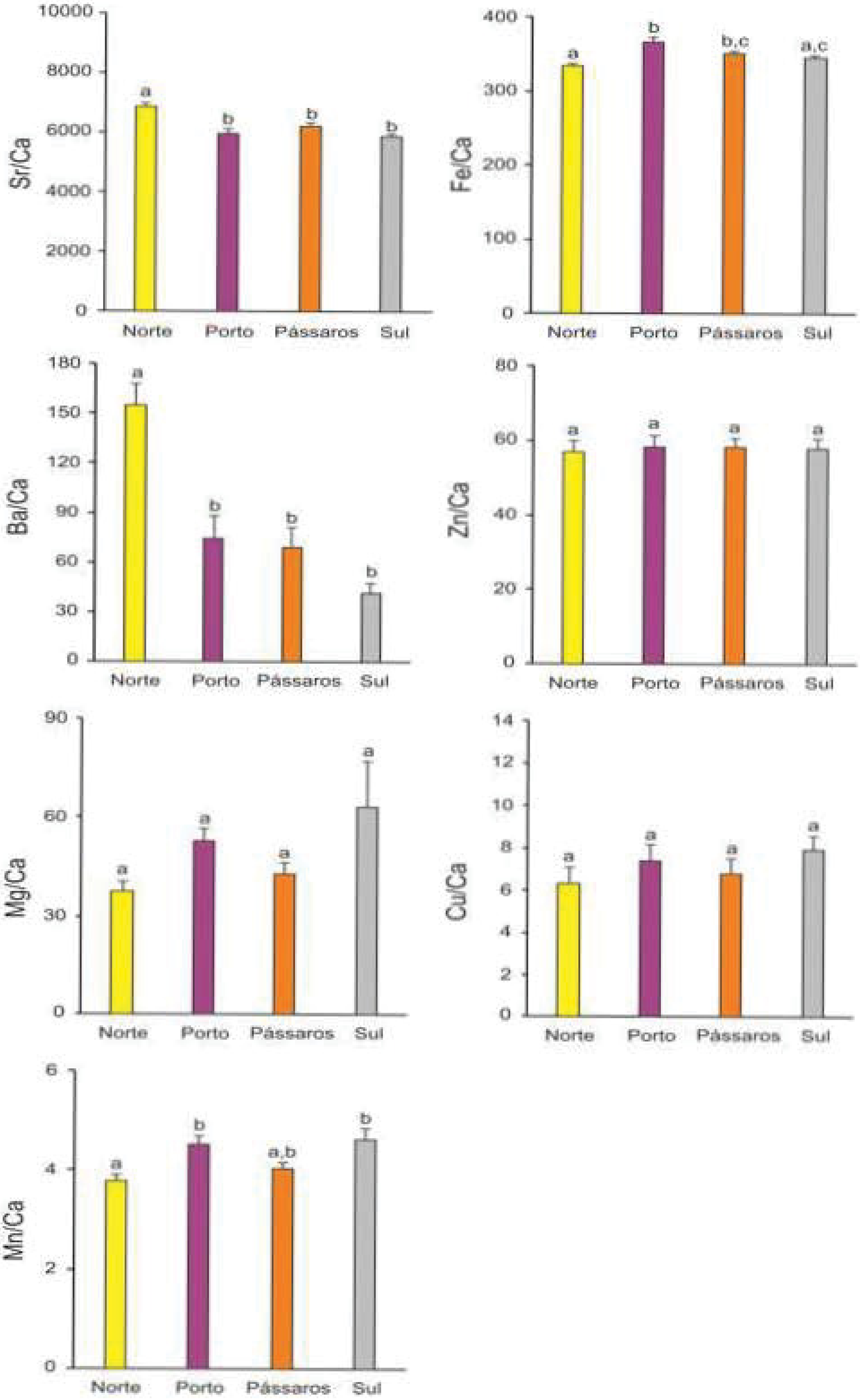

Fig. 3 Elemental concentrations in μg element g^{-1} calcium (detrended values as mean ± SE) in whole otoliths of *A. saxatilis* at short spatial scale collected in South

Brazil. The locations marked with the same letter are not significantly different from each other ($P<0.05$).

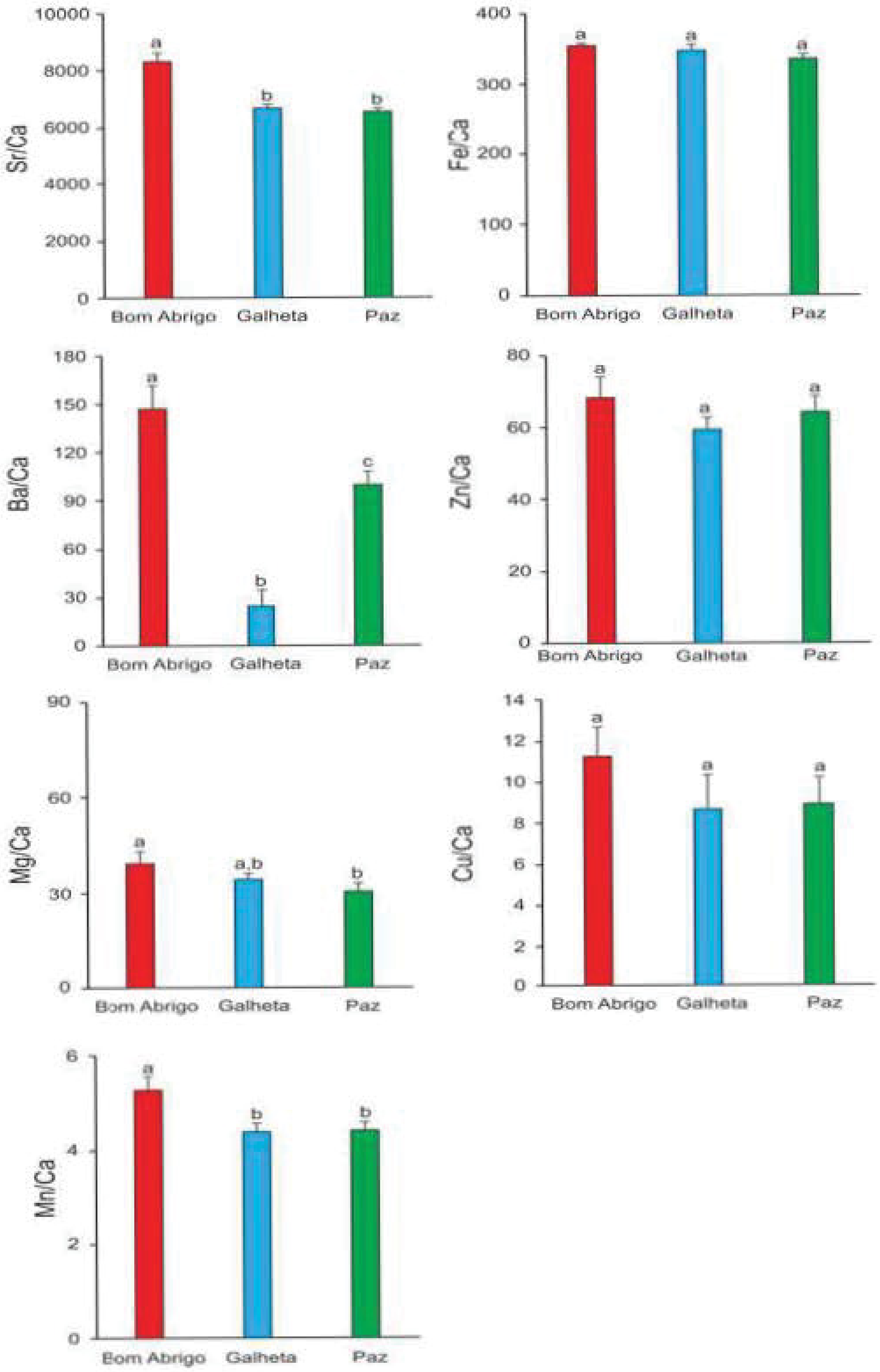

Fig. 4 Elemental concentrations in µg element g^{-1} calcium (detrended values as mean ± SE) in whole otoliths of *A. saxatilis* at large spatial scale collected in South

Brazil The locations marked with the same letter are not significantly different from each other ($P<0.05$).

MANOVA indicated a significant difference in the multi-element signatures of the whole otoliths at both short (Pillai Trace, $F_{4.79}=0.48$; $P<0.05$) and large spatial scales (Pillai Trace, $F_{16.56}=0.94$; $P<0.05$). Pairwise comparisons indicated significant differences among islands to short and large spatial scale (Hotelling's T-square, $P<0.05$). LDFA based on whole otolith composition successfully discriminated among individuals from the different islands, at both short and large spatial scales, although some overlap was evident namely among sites (Figures 5C and 6C). Jackknife reclassification accuracies were poor for short spatial scale (56%) and relatively highly for large spatial scale (77%) (Table IV; Table V).

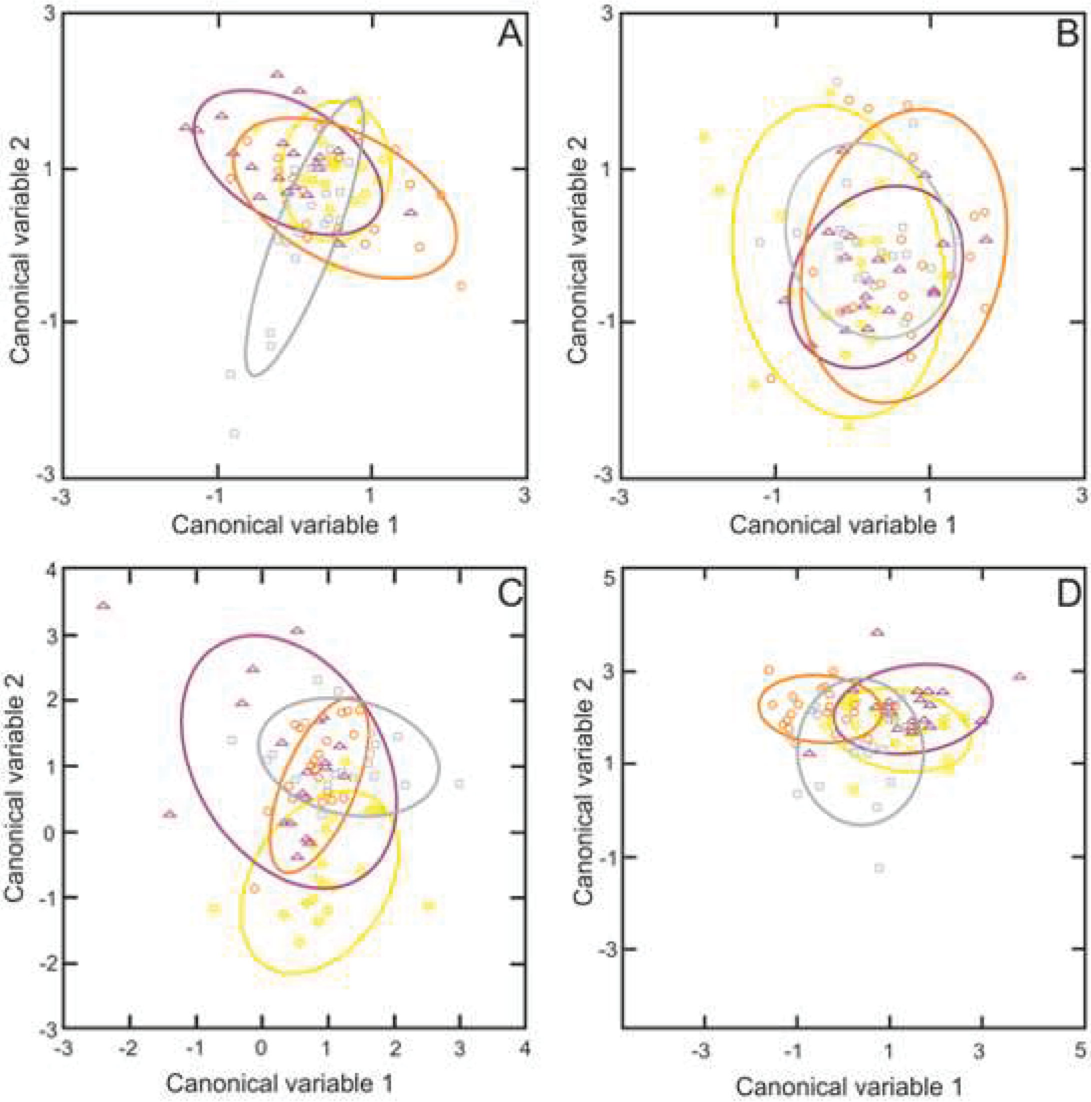

Fig. 5 Canonical variate plots displaying spatial differences of otoliths analysis using shape indices (A), elliptic Fourier descriptors (B), multi-elemental tags (C) and combining all the techniques (D) from the four sampling sites along the south Brazilian coast. Norte Island (☆), Porto Island (Δ), Pássaros Island (ο) and Sul Island (□). Ellipses represent 95% c.i. around the data, and data points represent individual fish.

Table IV. Jackknife classification matrix of *Abudefduf saxatilis* adults collected at short spatial scale based on otolith's shape indices (A), elliptic Fourier descriptors (B), microchemistry (C) and all techniques combined (D), used in linear discriminant function analysis.

Real location	Predicted location				
	Norte	Porto	Pássaros	Sul	% Correct
A					
Norte	10	2	6	2	50
Porto	5	12	3	0	60
Pássaros	6	6	6	2	30
Sul	4	2	7	7	35
Total	25	22	22	11	44
B					
Norte	13	3	4	0	65
Porto	3	8	6	3	40
Pássaros	2	3	11	4	55
Sul	0	4	5	11	55
Total	18	18	26	18	54
C					
Norte	16	0	2	2	80
Porto	3	8	4	5	40
Pássaros	2	1	12	5	60
Sul	0	4	7	9	45
Total	21	13	25	21	56
D					
Norte	13	1	4	2	65
Porto	1	13	3	3	65
Pássaros	5	2	8	5	40
Sul	0	8	3	9	45
Total	19	24	18	19	54

Otolith Shape and Chemical Combined Techniques

MANOVA showed a significant difference using the combined data from all analyses for both short (Pillai Trace, $F_{3.7}$=1.39; *P*<0.05) and large (Pillai Trace, $F_{6.48}$=1.42; *P*<0.05) spatial scales. Pairwise comparisons indicated significant differences among large spatial scales sample islands, except for short spatial scales sample islands (Hotelling's T-square, *P*<0.05). LDFA successfully discriminated among individuals although some overlap was evident, namely at a short spatial scale (Figure 5D) comparatively to the large spatial scale (Figure 6D). The jackknife reclassification success based on both methodologies was moderate accurate (54%) to short spatial scale and highly accurate (82%) to large spatial scale (Table IV; Table V).

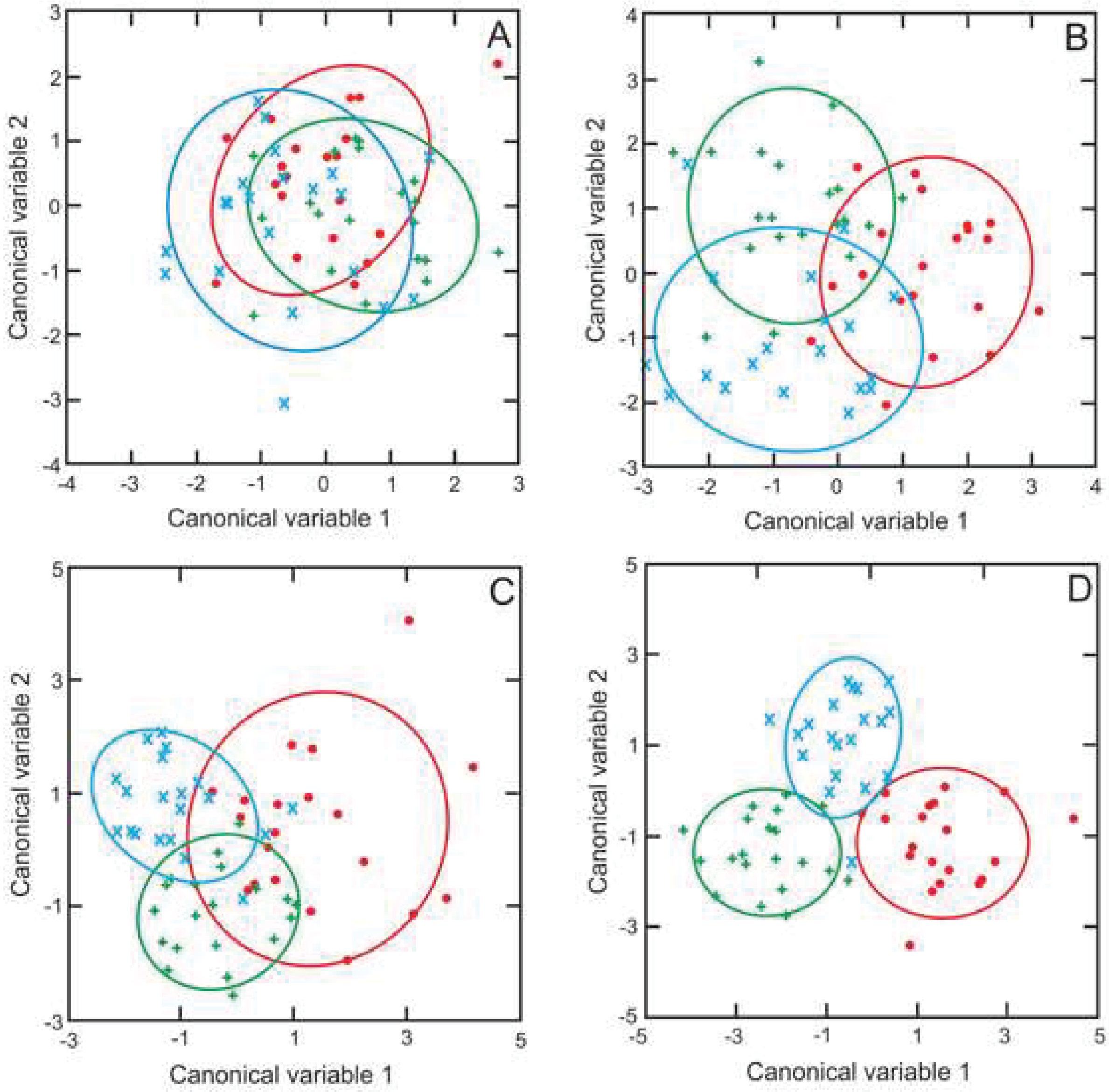

Fig. 6 Canonical variate plots displaying spatial differences of otoliths analysis using shape indices (A), elliptic Fourier descriptors (B), multi-elemental tags (C) and combining all the techniques (D) from the three sampling regions along the south Brazilian coast. Bom Abrigo (•), Galheta (×) and Paz (+) islands. Ellipses represent 95% c.i. around the data, and data points represent individual fish.

Table V. Jackknife classification matrix of *Abudefduf saxatilis* adults collected at large spatial scale based on otolith's shape indices (A), elliptic Fourier descriptors (B), microchemistry (C) and all techniques combined (D), used in linear discriminant function analysis.

Real location	Predicted location			
	Bom Abrigo	Galheta	Paz	% Correct
A				
Bom Abrigo	8	6	6	40
Galheta	6	9	5	45
Paz	6	4	10	50
Total	20	19	21	45
B				
Bom Abrigo	13	4	3	65
Galheta	5	11	3	58
Paz	3	2	15	75
Total	21	17	21	66
C				
Bom Abrigo	13	3	4	65
Galheta	2	17	1	85
Paz	2	2	16	80
Total	17	22	21	77
D				
Bom Abrigo	17	2	1	85
Galheta	2	15	3	75
Paz	1	2	17	85
Total	20	19	21	82

Discussion

Otoliths are competent tools to study fish ecology, providing useful information on life history of fishes, such as population structure, movement patterns and habitat connectivity (Gerard et al. 2015; Daros et al. 2016a; Moreira et al. 2018). These studies are important for a rational conservation and management of fishes (Higgins et al. 2013; Cook et al. 2014; Walther et al. 2017). The hereby data used otolith shape indices, elliptical Fourier descriptors, and elemental composition in an attempt to unravel the population structure of *Abudefduf saxatilis* in South Brazil.

Several studies showed that fish population units could be distinguished using otolith shape analysis (Tuset et al. 2003; Bacha et al. 2014; Bacha et al. 2016). Determinants of otolith shape, although not fully understood, provides a phenotypic basis for fish population separation, taking into consideration the principle that the otolith morphology varies geographically, and could reflects a combined effect of genetic variation and local environmental factors (Tudela 1999; Cardinale et al. 2004; Vignon & Morat 2010).

No significant univariate differences were found among islands for the SI, with exception of the rectangularity, at both short and large spatial scales. Similar results have been reported for the Atlantic cod spawning populations, in which the rectangularity accounted for the largest proportion of differences in otolith shape between different sampling areas (Galley et al. 2006; Bostanci et al. 2015). Intra-specific differences in otolith shape are also attributed to differences in fish growth rate (Campana & Casselman 1993; Ferguson et al. 2011; Fowler et al. 2015). Furthermore, otolith growth rate is known to be influenced by water temperature (Otterlei et al. 2002). The observed hereby results could be result of the similar environmental conditions experienced by fish in these islands, namely water temperature (Daros et al. 2016a).

Multivariate analysis of SI detected differences between locations, for both spatial scales. However, SI were less useful than EFD for discriminating purposes and provided a lower allocation success for both short (44%) and large (45%) spatial scales. LDFA with EFD data provided a better allocation success of the individuals to the original location, 54% and 66% to short and large special scale, respectively. EFD appears to be the most powerful shape analysis technique for capturing the entire shape variation, but more complex than that of linear morphometric indices (Campana & Casselman 1993; Stransky & MacLellan 2005; Farias et al. 2009).

The chemical analysis of the whole otoliths evidence all chemical elements incorporated during the life of the fish, i.e. from the birth until collection (Fowler et al. 1995). The elemental fingerprints of whole otoliths are site-specific and can provide natural tags of their inhabited areas (Correia et al. 2012a; Correia et al. 2014; Moreira et al. 2018). The incorporation of trace elements in otoliths structure is still a poorly understood complex process, and factors such as aquatic environment (e.g.

salinity and temperature), concentrations of elements in the water, ontogenetic events (e.g. metamorphosis), fish physiology (e.g. feeding regime, growth and metabolic rate) and genetic basis also influence otolith chemical composition (Clarke et al. 2011; Chang & Geffen 2012; Thomas et al. 2017).

Eight trace elements (Sr, Ba, Mn, Mg, Fe, Cu and Zn) was detectable in otoliths of *A. saxatilis* at informative levels. Element:Ca ratios occurred within the general values reported for otoliths of coastal marine fishes (Campana 1999), including studies with others Brazilian reef fishes, like *Stegastes fuscus, Lutjanus alexandrei* and *Epinephelus marginatus* (Daros et al. 2016a; Aschenbrenner et al. 2016; Condini et al. 2016).

Univariate statistical techniques showed significant differences of Sr/Ca, Ba/Ca, Fe/Ca and Mn/Ca concentrations within Tamboretes Archipelago islands (short spatial scale), where the concentrations of Sr and Ba were higher in Norte Island and differs from the other islands. Fe was higher in Porto, and Mn lower in Norte. The variation in otolith elemental fingerprints among sampling locations were also recorded for Sr/Ca and Ba/Ca for large spatial scale (Bom Abrigo, Galheta and Paz islands). Bom Abrigo island exhibited the highest values of Sr/Ca and Ba/Ca and Galheta island showed the lowest value of Ba/Ca. A similar chemical pattern was observed in a previous study within the same study area, with *S. fuscus*, another Pomacentridae species (Daros et al. 2016a).

Strontium, as expected, was the most abundant element; in general, Sr concentrations in otoliths have a positive correlation with salinity, thus, fish living in marine waters present higher concentrations comparatively with freshwater fish (Elsdon & Gillanders 2006; Sturrock et al. 2012; Aschenbrenner et al. 2016). Ba concentration may be linked to the terrestrial freshwater input in the shore coastal waters and could be negatively associated with water salinity (Begg et al. 2005; Nishimoto et al. 2010; Miller 2011). Endogenous process (e.g. diet ontogeny and growth) influence the incorporation of Mg, Mn, Cu, Zn and Fe in the otolith (Fowler et al. 1995; Nishimoto et al. 2010; Thomas et al. 2017).

The use of otoliths as natural tags depends on there being some measurable difference in otolith chemistry at a geographic scale relevant to the life history of the species of interest. The study area is primarily influenced by two water masses: the warm tropical waters from the Brazil Current and cool waters from the South Atlantic Central Water. The interaction between warm and cold currents leads to a large difference in sea surface temperatures, especially during spring and summer northeastern winds and has a significant influence on the population and community structures of marine organisms (Carvalho et al. 1998; Acha et al. 2004). The study area also is under the direct influence of four bays (Cananéia, Paranaguá, Guaratuba and Babitonga) and several small to medium-sized rivers located in the SE Brazilian coast (Diegues & Rosman 1998). Bom Abrigo, Galheta and Paz island, are located near the outfall of the Cananéia, Paranaguá and Babitonga bays, respectively. Located in the north coastal shelf of Santa Catarina, the Tamboretes Archipelago probably suffer lower influence of the estuarine plumes, and the coastal waters are mixed by winds and currents.

LDFA based on elemental chemistry provided a low allocation success of otoliths at a short spatial scale (56%) and provided a hight allocation success to large spatial scale (77%). Previous studies have successfully detected differences in otolith microchemistry of other Pomacentridae. The otolith elemental chemistry analyses of temperate damselfish (*Parma microlepis*) indicated differences between short and large spatial scales (Dove & Kingsford 1998; Kingsford & Gillanders 2000). Otolith elemental fingerprints of *Pomacentrus amboinensis* and *P. coelestis* indicated also differences between sites (Liu et al 2010; Sih & Kingsford 2016). Lo-Yat et al. (2005) found differences on elemental signatures in otoliths of *Stegastes nigricans* at the sites separated by only 200 m. The study with the Brazilian damselfish *S. fuscus*, indicated some evidence of distinct populations (~71% of overall reclassification success), using otolith elemental and isotopic ratios, between islands spaced about 15 to 50 km (Daros et al., 2016a).

LDFA based on combining techniques highly improved the reclassification power and successfully discriminated individuals of *A. saxatilis* at a large spatial scale (82%). However, the reclassification success at short scales remained low (54%)**.** The hereby results suggest that individuals in the southern Brazilian coast can be separately as population-units at least at a regional level, and highly mixed over a short spatial scale. This could be a result of a limited connectivity between habitats, a larval retention mechanism and/or a predominant self-recruitment process at a regional scale. These findings could be explained by some known ecological aspects of the species, i.e. *A. saxatilis* show great fidelity to the sites they inhabit, defend individual territories during the reproductive season, is considered a non-migratory fish and the dispersion of individuals is conditioned by the larval period or juvenile recruitment (Froese & Pauly 2016), and by oceanographic conditions of the sampling area which may work as barriers (Hanski & Simberloff 1997; Akcakaya et al. 2007; Schunter et al. 2011). These methodologies were shown to be effective and complementary for population studies, in comparison to classical studies of molecular genetics, in particular with mitochondrial DNA. Otolith shape and chemical analysis have proven useful here to unravel some life history traits of *A. saxatilis*, but further investigation into the population structure, fish movement and habitat connectivity is still needed.

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CHAPTER II

Otolith shape and stable isotopes analysis to unravel the population structure of the *Abudefduf saxatilis* along the Brazilian coast

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Otolith shape and stable isotopes analysis to unravel the population structure of *Abudefduf saxatilis* in the southwestern Atlantic

Abstract

The most common and abundant pomacentrid Atlantic species is the sergeant major *Abudefduf saxatilis*. It is considered a keystone-fish of reef habitats but the knowledge about its population structure is still limited. A recent study showed no genetic regional differentiation among *A. saxatilis* population from Brazilian coast. However, recently, the use of otolith shape and elemental signatures revealed useful to study the population structure, fish movements and habitat connectivity of *A. saxatilis* at small and medium spatial scales. Otoliths can be an alternative and complimentary approach to the classic molecular tools to unravel the population structure of marine fish with high gene flow but living in environmentally heterogeneous habitats. Otolith shape (elliptic Fourier descriptors) and isotopic ($\delta^{13}C$ and $\delta^{18}O$) signatures of 120 whole otoliths (20 per sample site) were used to study the population structure of *A. saxatilis* along six regions along the Brazilian coast, southwestern Atlantic. Data were analyzed with univariate and multivariate statistics to assess the regional population structure. The combined analysis of otolith shape and isotopic signatures gave distinct regional signatures, namely between the coastal and oceanic sampling regions. The re-classification success rate (an overall of 62%) for these regions obtained from the stepwise linear discriminant function analysis suggest that individuals should be regarded as different populations among reef regions in the Brazilian coast. Moreover, the hereby results suggest that both tools can be used as useful to study population structure of non-migratory and reef-associated fishes.

Keywords: Reef-fish; Pomacentridae; sagittae; shape and isotopic signatures; population structure.

1 Introduction

Pomacentridae is composed of small species (up to 30 cm) and is widely distributed in tropical and temperate coastal regions, presenting greater diversity in reef environments (Cooper et al., 2009). One of the reef-fish species with the widest geographic distribution among the Pomacentridae, being common in oceanic islands and coasts on both sides of the Atlantic Ocean, is the seargeant major, *Abudefduf saxatilis* (Linnaeus 1758). This species is often observed on rocky, coral and artificial reef environments, at maximum depths of 20 m, both in tropical and subtropical latitudes (Robertson, 1988; Allen, 1991; Alshuth et al., 1998). In the Western Atlantic, *A. saxatilis* occurs from North Carolina in the United States (Lubbock and Edwards, 1981) to the northern coast of Uruguay (Gerardo et al., 2002). In the Eastern Atlantic, it occurs on the African coasts from Senegal to Angola (Randall, 1996), but also occurs in oceanic islands (Krajewski and Floeter, 2011). Recent records of the species were made in the NE Atlantic, namely in the Canary (Pajuelo et al., 2016) and Madeira archipelagos (Freitas and Araújo, 2006), but also in the Mediterranean Sea (Tsadok et al., 2015). The wide geographic distribution and high abundance in the Atlantic Ocean are related to particular ecological features of the species; *A. saxatilis* is omnivorous opportunist, generalist (e.g. habitat use and diet) and a flotsam-associated species (e.g. dispersion via rafting in floating debris) (Rodrigues and Molina, 2007; Cheney, 2008; Luiz et al., 2015).

Genetic analyzes (mtDNA) suggested a panmixia pattern without a clear spatial differentiation for *A. saxatilis* individuals from Gulf of Mexico, Mexican Caribbean and the Caribbean populations; however, some genetic divergence exists between Caribbean and Brazilian biogeographic provinces (Shulman and Bermingham, 1995; Piñeros et al., 2015; Piñeros and Gutiérrez-Rodríguez, 2017). There is no genetic differentiation for *A. saxatilis* populations from the Brazilian coastline, although some differences between the coastal zones and the oceanic island populations were recorded (Rodrigues and Molina, 2007; Souza, 2017). Body morphological studies found regional population differences in *A. saxatilis*, suggesting that the phenotypic differences may be determined by environmental factors (e.g. water temperature) even in the presence of high gene flow (Molina et al., 2006; Piñeros et al., 2015). Recently, otolith morphology and element:Ca ratios were compared at short and large spatial scales in the Brazilian coast; although no clear separation for individuals collected at short distances were observed, a limited movement of adults between habitats were observed at large spatial scales suggesting the existence of regional discrete groups (Adelir-Alves, 2019).

Studies to infer about the population structure of fish are carried out mainly with genetic tools (Graves, 1998). However, for marine fish species, these tools frequently fail to show genetic pattern of spatial differentiation, being wrongly considered as single populations (Lacson, 1992; Waples, 1998; Kritzer and Liu, 2014). Genetics provide information based on long-term effects over generations, while otoliths provide information on a shorter, intra-generational, timescale (Cowen and Sponaugle, 2009; Tanner at al., 2014; Welch et al., 2015). Otolith analysis is an effective method to assess the population structure in high gene flow systems where environmental heterogeneity exists (Smith and Campana, 2010; Correia et al., 2011; Correia et al., 2012). Furthermore, the used of fish shape and chemical signatures have been shown to be effective to unravel fish population structure (Moreira et al., 2019; Soeth et al., 2019), including for *A. saxatilis* (Adelir-Alves et al., 2019).

Otolith morphological characteristics of otoliths, like elliptical Fourier descriptors, may vary according to the geographic area, as well as several others ecological and environmental factors, and is an efficient way to distinguish fish population components (Ferguson et al., 2011; Bacha et al., 2016; Moreira et al., 2019). Furthermore, stable isotopes, namely carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) ratios, have been successfully used as tools for population structure, movement pattern and habitat connectivity in fish studies (Daros et al., 2016; Carvalho et al., 2017; Moreira et al., 2018). The use of otoliths as natural tags depends on there being some measurable difference in otolith chemistry or shape at a geographic and time scale relevant to the life history of the species of interest (Soeth et al., 2019). Non-migratory and highly territorial fish species may preserve unique shape and chemical signatures in otoliths if the regions where they lived are environmentally or ecologically distinct (Kingsford and Gillanders, 2000; Lo-Yat et al., 2005; Daros et al., 2016), as recently observed for *A. saxatilis* (Adelir-Alves et al., 2019). Otolith shape and stable isotopes ratios proved to be a valuable method for the discrimination of population units of adult fish (Gerard et al., 2015; Hearne et al., 2017; Neves et al., 2019).

In the hereby study, elliptic Fourier descriptors and stable isotope ratios of whole otoliths (i.e. entire life-history prior to capture) were assessed to reveal whether regional distinct signatures occur along the Brazilian coast providing new insights about the population structure, fish movement and habitat connectivity of *A. saxatilis.*

2 Material and methods

2.1 Study Area and Fish Sampling

The Brazilian coast is approximately 10 000 km long, extending from Cape Orange (4ºN) to Chui (34ºS), including several oceanic islands, and being under the influence of a diverse set of environmental (e.g., temperature and salinity), oceanographic (e.g. currents, upwelling and downwelling process) and ecological conditions (e.g. coral and rocky reefs), with great climatic and geomorphological variation (Leão and Dominguez, 2000; Amaral and Jablonsky, 2005; Brandini et al., 2018). Fernando de Noronha, an oceanic island, is located in the western tropical Atlantic region and is under the influence of the westward flow of the South Equatorial Current (SEC). The average salinity of the SEC was measured between 36 and 37.2 at the surface and the average temperature of the is about 26° and 28°C near the surface (Mayer et al., 1998; Wienders et al., 2000; Rossi-Wongtschowski et al., 2006). The main current that reaches the coastal sample regions (Porto Seguro, Arraial do Cabo, Ilha Bela, Galheta and Tamboretes) is the Brazil Current (BC). The westward flowing trans-Atlantic SEC bifurcates into two currents, the North Brazil Current (NBC) and the BC. The BC is a weak western boundary current carrying warm subtropical water, which runs south along the coast of Brazil from about 9°S to about 38°S, where it finds the Falkland Current (Malvinas), is characterized by warm temperatures that vary from 18° to 28°C, oscillating between 7° to 10° degrees, and a high salinity that averages 35 to 37, with the maximum commonly found at around 20°S, where it can reach a salinity of 37.3 (Peterson and Stramma, 1991; Zavialov et al., 1999; Memery, et al., 2000). The Falkland Current (FC) is a branch of the Circumpolar Current and flows northward along the continental shelf of Argentina until Brazilian coast, is characterized by cold waters, with SST mean of 6°C, and less saline than BC (Vivier and Provost, 1999; Brandini et al., 2000; Rossi-Wongtschowski et al., 2006).

Fish sampling took place between January 2016 and January 2017 in six coastal regions of Brazil: Fernando de Noronha (FN), Porto Seguro (PS), Arraial do Cabo (AC), Ilha Bela (IB), Galheta (GA) and Tamboretes (AT) (Fig. 1 and Table 1). Individuals were sampled with spearfishing and transported to the laboratory in isothermal containers with ice. In the laboratory, fish total length (TL, 0.1 mm) and mass (M, 0.0001 g) were measured. A total of 120 fish (20 per location), ranging from 128 to 196 mm total length, were used for further analysis (Table 1). Sagittal otoliths were removed with plastic forceps, cleaned with distillated water and preserved in Eppendorf tubes for further analyses.

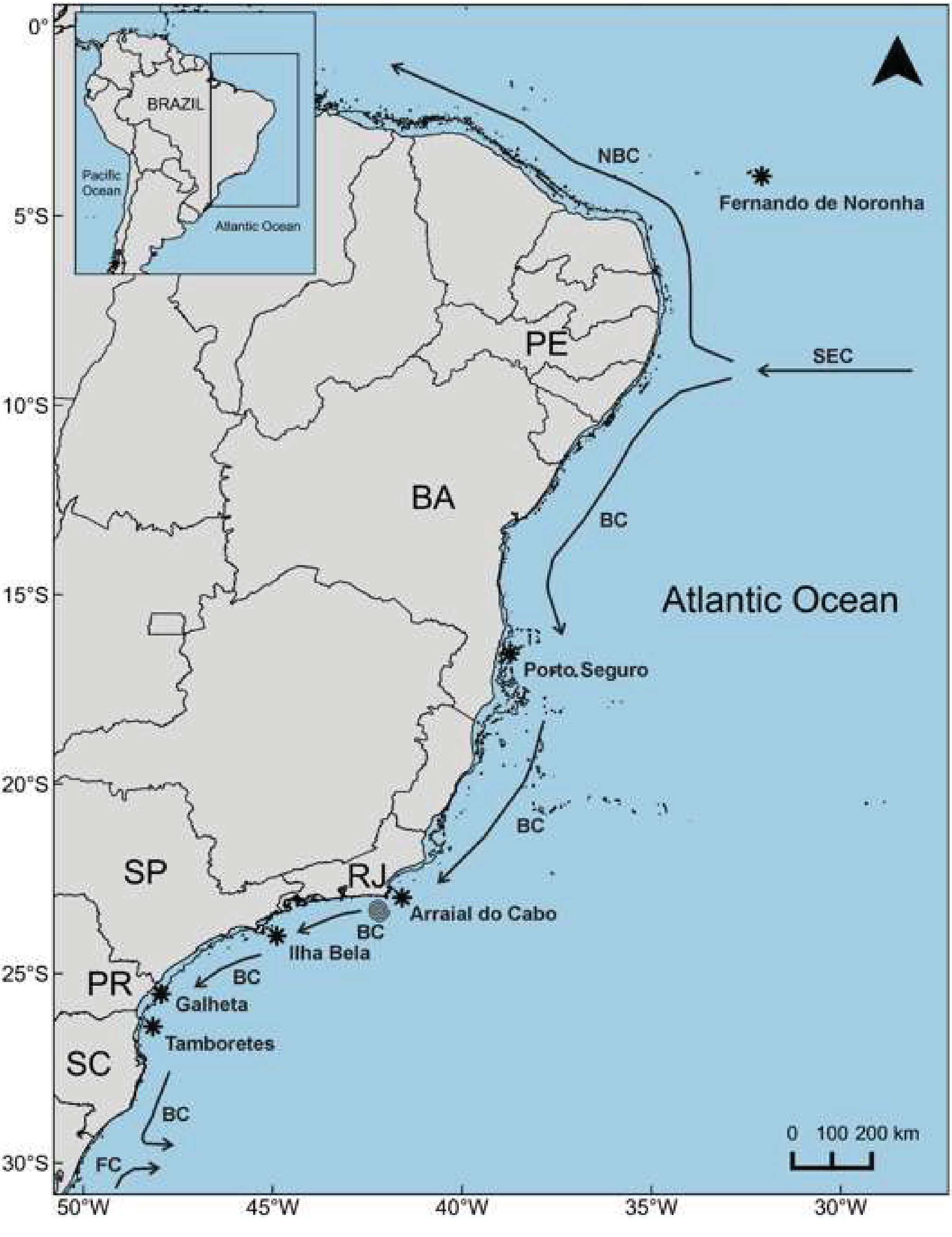

Figure 1. *Abudefduf saxatilis* sampling regions (∗) where the adult's individuals were collected in this study, Fernando de Noronha in Pernambuco state (PE), Porto Seguro in Bahia state (BA), Arraial do Cabo in Rio de Janeiro state (RJ), Ilha Bela in São Paulo state (SP), Galheta in Paraná state (PR) and Tamboretes in Santa Catarina state (SC). Bathymetric contours of 20 m depth are shown as fine lines. The

main coastal upwelling region, near Arraial do Cabo coast, is indicated (●). Dark lines and arrows represent the main currents: South Equatorial Current (SEC), North Brazil Current (NBC), Brazil Current (BC) and Falkland Current (FC).

2.2 Seawater Surface Temperature and Salinity

The Seawater Surface Temperatures (SST) data for each sampling region were obtained from MODIS Aqua Level 3 SST. The Seawater Surface Salinity (SSS) from NASA Aquarius project and Remote Sensing Systems were also used (Lee et al., 2012; Werdell et al., 2013; Meissner et al., 2016). Means of SST and SSS from each sampling area were retrieved from Physical Oceanography Distributed Active Archive Center (PODAAC, https://podaac.jpl.nasa.gov) and then processed in the SeaWIFS Data Analysis System (SeaDAS Version 7.5).

The values of SST and SSS used were estimated for each region, based on the mean annual values during the individual fish lifetime until the capture (Carvalho et al., 2017). Individual annual fish age was obtained using the inverse function of the von Bertalanffy growth curve for the species (see Mackay and Moreau 1990; Jones et al., 2010). The estimated age class range for the collected fish was 1^+ to 5^+ yr (Table 1).

2.3 Otolith Shape Descriptors

Adult individuals were examined to avoid confounding factors that could be caused by an ontogenentic alometric growth (Moreira et al., 2019; Soeth et al., 2019). In general, after fish reach sexual maturity, otoliths show a more constant pattern in relation to fish size (Capoccioni et al., 2011; Carvalho et al., 2015). Furthermore, the maturation of *A. saxatilis* occur between 101 and 115 mm of total length (Bessa et al., 2007), and in the present study, specimens of *A. saxatilis* were restricted to specimens between 144 and 182 mm of total length.

Right otoliths were placed with the sulcus acusticus up and the rostrum to the left. Orthogonal two-dimensional digital images of otoliths were captured under a stereomicroscope (10X magnification) coupled to a camera (Olympus, SC30), with a dark background. Full color (*.jpg) and high-resolution photographs were captured using reflected light against a dark background using the Olympus Image Analysis 5.0 software (Soeth et al., 2019). The program Shape (Version 1.3) was used to extract the otolith contour shape from a B&W photograph (*.bitmap) and to determine the number of elliptic Fourier descriptors (EFD) required to adequately describe the otolith outline (Iwata and Ukai, 2002). A level of 95% of accumulated variance was used to select the minimum number of harmonics (Stransky et al., 2008; Ferguson et al., 2011; Adelir-Alves et al., 2019). The first 16 harmonics reached >95% of the cumulative power indicating that the otolith shape could be adequately explained by 64 Fourier coefficients, i.e. 16 harmonics×4 coefficients (a, b, c and d). As a consequence of the normalization, the first three coefficients (a1, b1 and c1) were constant and has been excluded, reducing the number of Fourier coefficients to 61 (Stransky et al., 2008; Ferguson et al., 2011; Adelir-Alves, 2019).

2.4 Otolith Isotopic Analysis

Right otoliths were cleaned in an ultrasonic bath for 5 min in ultrapure water (Milli-Q-Water) followed by immersion in 3% analytical-grade hydrogen peroxide (H^2O^2, Fluka Trace-Select) for 15 min to remove any adherent biological tissues. Finally, otoliths were triple-rinsed with Milli-Q-Water to remove possible contamination, air-dried in a laminar flow cabinet, weighed (OW, 0.0001g) and stored in dry plastic tubes for shape and isotopic analysis (Carvalho et al., 2017; Moreira et al., 2019).

Sagittae were individually crushed into a fine carbonate powder using a previously decontaminated (using acetone) ceramic mortar and pestle and with the help of a paint-brush were transferred to Eppendorf microcentrifuge tubes. Otolith samples were analyzed in random order to avoid possible sequence effects. $\delta^{13}C$ and $\delta^{18}O$ isotopic ratios were measured using a Delta V Advantage mass spectrometer coupled to an automated system (GasBench II). About 0.6 - 0.7 µg of carbonate was used for each sample. Each sample, duly numbered, was placed in a glass vial (10 ml) which was then sealed with a rubber septum cap. Seventy bottles were processed in each round of analysis (run), using a multi-flow unit of which 16 bottles correspond to the 4 reference standards, 2 international (NBS-18, NBS-19) and two internal (REI and VICK). Initially, flasks went through the flush step (240 seconds), which consists of a He gas jet to remove atmospheric gases (CO_2 and H_2O). After the flush, 90 mg (equivalent to ~ 50 µL) of H_3PO_4 acid were automatically added into each sample to release CO_2 from the carbonate. After addition of H_3PO_4, the samples were allowed to react for 90 minutes at 70°C before being analised. He-CO_2 mixture released from each sample was then automatically transferred to the Delta V.

The precision of analysis was better than 0.1‰ based on multiple analyses of the internal standard. The results are expressed in the notation ‰ (per mil) in relation to international Vienna Pee-Dee Belemnite (VPDB) scale (Epstein et al., 1953; Craig, 1957).

2.5 Data analysis

Prior to statistics, normality (Shapiro–Wilk test, $P > 0.05$) and homogeneity of variances (Levene's test, $P > 0.05$) were tested. After a One-Way Analysis of Variances (ANOVA), a post hoc Tukey HSD test was used, if needed ($P < 0.05$), to examine the existence of any significant differences in the individual shape and isotopic ratios of carbon and oxygen among the six sampling regions. Initially $\delta^{13}C$ and $\delta^{18}O$ values were analyzed by analysis of covariance (ANCOVA). Otolith mass is considered to be a proxy for fish age and growth rate and therefore it was used as a covariate in ANCOVA, while location was treated as a fixed factor (Ferguson et al., 2011). However, the $\delta^{13}C$ and $\delta^{18}O$ data were not significantly related to otolith mass ($P < 0.05$) and therefore the use of detrented values i.e. (subtraction of the product of the common within-group linear slope) was unnecessary (Gerard and Muhling, 2010).

Multivariate analysis of variance (MANOVA) and stepwise linear discriminant function analysis (LDFA) were used to explore the variation of shape and isotopic signatures among regions (Fernando de Noronha, Porto Seguro, Arraial do Cabo, Ilha Bela, Galheta and Tamboretes). For MANOVA the approximate F-ratio statistic (Pillai's trace) was reported. Pairwise comparisons after MANOVA were done using the Hotelling's T-square test. To allow a visual inspection of the individual isotopic signatures per region a bi-plot ($\delta^{13}C$ vs $\delta^{18}O$) was displayed. LDFA was used to re-classify individuals to sampling regions. Re-classification accuracies of the discriminant functions for each region were evaluated using the percentage of correctly classified individuals from jackknife testing (leave one-out cross-validation) (Correia et al., 2011; Carvalho et al., 2017; Moreira et al., 2018). Statistical analyses were performed using the software SYSTAT 12. Results are presented as means ± standard errors (SE). A significance level (α) of 0.05 was used for all statistical procedures.

Table 1. Sampling regions*, sample date (SD), number of specimens (N), fish total length (TL), estimated age (EA), otolith weight (OW), carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) otolith isotopic values, sea surface temperatures (SST) and sea surface salinity (SSS). Values are expressed as means ± standard errors. *Fernando de Noronha (FN), Porto Seguro (PS), Arraial do Cabo (AC), Ilha Bela (IB), Galheta (GA) and Tamboretes (AT).

Sampling regions		SD	N	TL(mm)	EA(years)	OW(mg)	$\delta^{13}C$(‰$_{VPDB}$)	$\delta^{18}O$(‰$_{VPDB}$)	SST(°C)	SSS
FN	03°50'03"S-32°24'05"W	Nov 2016	20	182.35 ± 2.35	4.82 ± 0.18	0.010 ± 0.001	-5.69 ± 0.31	0.38 ± 0.02	27.27 ± 0.10	36.83 ± 0.04
PS	16°25'12"S-39°03'07"W	Jan 2017	20	144.90 ± 2.63	2.33 ± 0.14	0.008 ± 0.000	-6.47 ± 0.29	0.33 ± 0.04	26.57 ± 0.06	37.40 ± 0.04
AC	22°58'02"S-42°00'50"W	Apr 2016	20	174.10 ± 3.51	4.22 ± 0.24	0.014 ± 0.001	-5.40 ± 0.22	0.16 ± 0.08	23.73 ± 0.53	36.52 ± 0.06
IB	23°52'05"S-45°26'30"W	Jan 2016	20	179.65 ± 1.33	4.56 ± 0.11	0.014 ± 0.001	-5.93 ± 0.23	-0.32 ± 0.12	24.70 ± 0.22	35.37 ± 0.29
GA	25°35'05"S-48°19'10"W	Jan 2016	20	180.80 ± 1.37	4.65 ± 0.11	0.013 ± 0.001	-6.08 ± 0.17	-0.03 ± 0.14	24.43 ± 0.18	35.18 ± 0.52
AT	26°23'10"S-48°31'33"W	Mar 2016	20	180.85 ± 1.46	4.66 ± 0.12	0.015 ± 0.001	-5.56 ±0.11	-0.06 ± 0.04	23.95 ± 0.25	35.78 ± 0.47

Results

The otolith shape outlines, resulting from the averaged harmonics obtained through Elliptic Fourier analysis, for each sampling region showed similar contours, varying only slightly on the rostrum and anti-rostrum. The otolith EFDs (27 Fourier coefficients) showed differences (ANOVA, $P < 0.05$) (Fig. 2; Table 2).

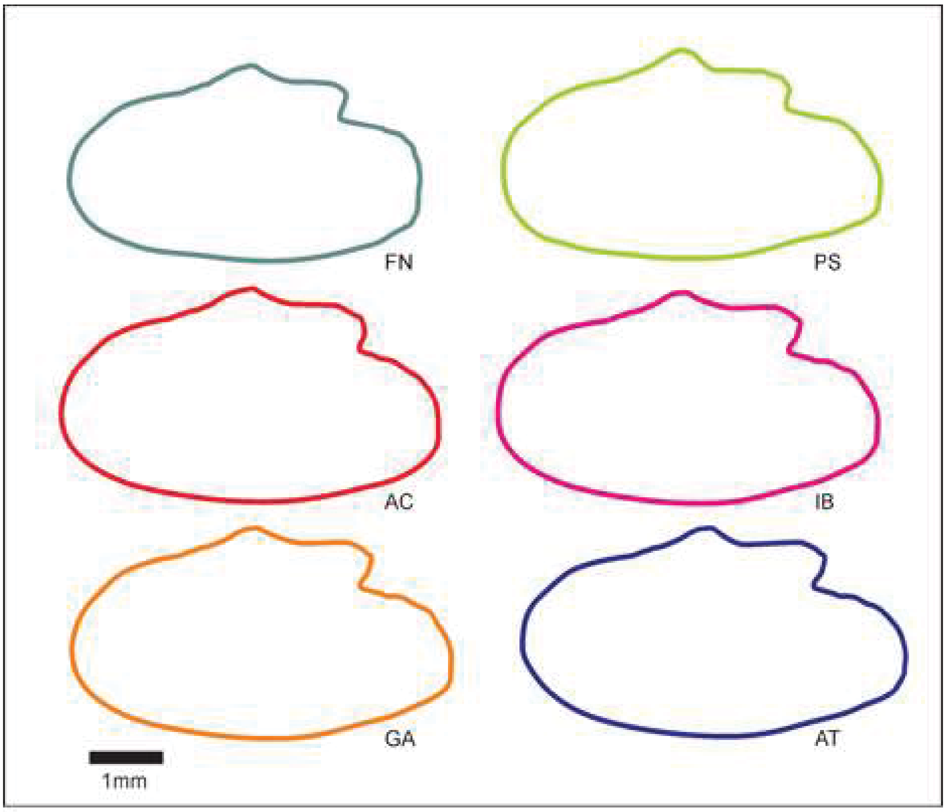

Figure 2. Mean shapes for the *Abudefduf saxatilis*, from different areas (sampling regions). Fernando de Noronha (FN), Porto Seguro (PS), Arraial do Cabo (AC), Ilha Bela (IB), Ilha da Galheta (GA) and Tamboretes (AT).

Table 2. Otolith shape (elliptical Fourier descriptors) signatures differences among the sampling regions along the Brazilian coast for *Abudefduf saxatilis* individuals. Different letters indicate statistically different results (Tukey Test, P < 0.05).

EFD	FN	PS	AC	IB	GA	AT
a2	abc	b	ac	ac	ac	abc
b2	a	ab	bc	abc	abc	abc
c2	ab	b	a	a	a	a
d2	a	ab	b	ab	ab	ab
a3	a	ab	c	ac	ac	ab
b3	ab	b	ab	ab	ab	a
d3	a	a	b	ab	ab	ab
a4	ab	b	c	cd	abd	acd
b4	ab	b	a	ab	ab	ab
c4	a	b	ac	abc	abc	b
d4	a	ab	c	abc	ac	abc
b5	a	ab	bc	c	bc	c
d5	ab	b	ac	ac	ac	c
b6	ab	b	b	b	b	bc
c6	a	a	a	a	a	a
d6	a	a	b	ab	ab	b
a7	abc	b	abc	cd	abc	d
c7	ac	abcd	a	bcd	cd	d
a8	a	a	a	a	a	a
b9	ab	a	ab	b	ab	ab
d9	a	ab	b	b	b	b
b10	ab	a	ab	b	ab	b
c10	a	ab	ab	ab	b	ab
d10	a	b	ab	ab	ab	a
a11	a	a	a	a	a	a
a12	ab	a	ab	ab	ab	b
b12	a	a	a	a	a	a

MANOVA test indicated significant differences among sampling regions using shape signatures of otolith (Pillai Trace; $F_{3.169} = 2.287$; $P < 0.05$). Pairwise comparisons indicated significant differences among all regions (Hotelling's T-square, $P < 0.05$). LDFA plot indicated a significant overlap among regions (Fig. 3). Jackknifed re-classification accuracy ranged from 35% (Ilha Bela) to 75% (Fernando de Noronha), showing an overall reclassification accuracy of 53% (Table 3). According to the between-group F matrix (df = 25.90), Ilha Bela and Tamboretes were the closest (1.271), while those from Porto Seguro and Fernando de Noronha (9.590) were the furthest apart.

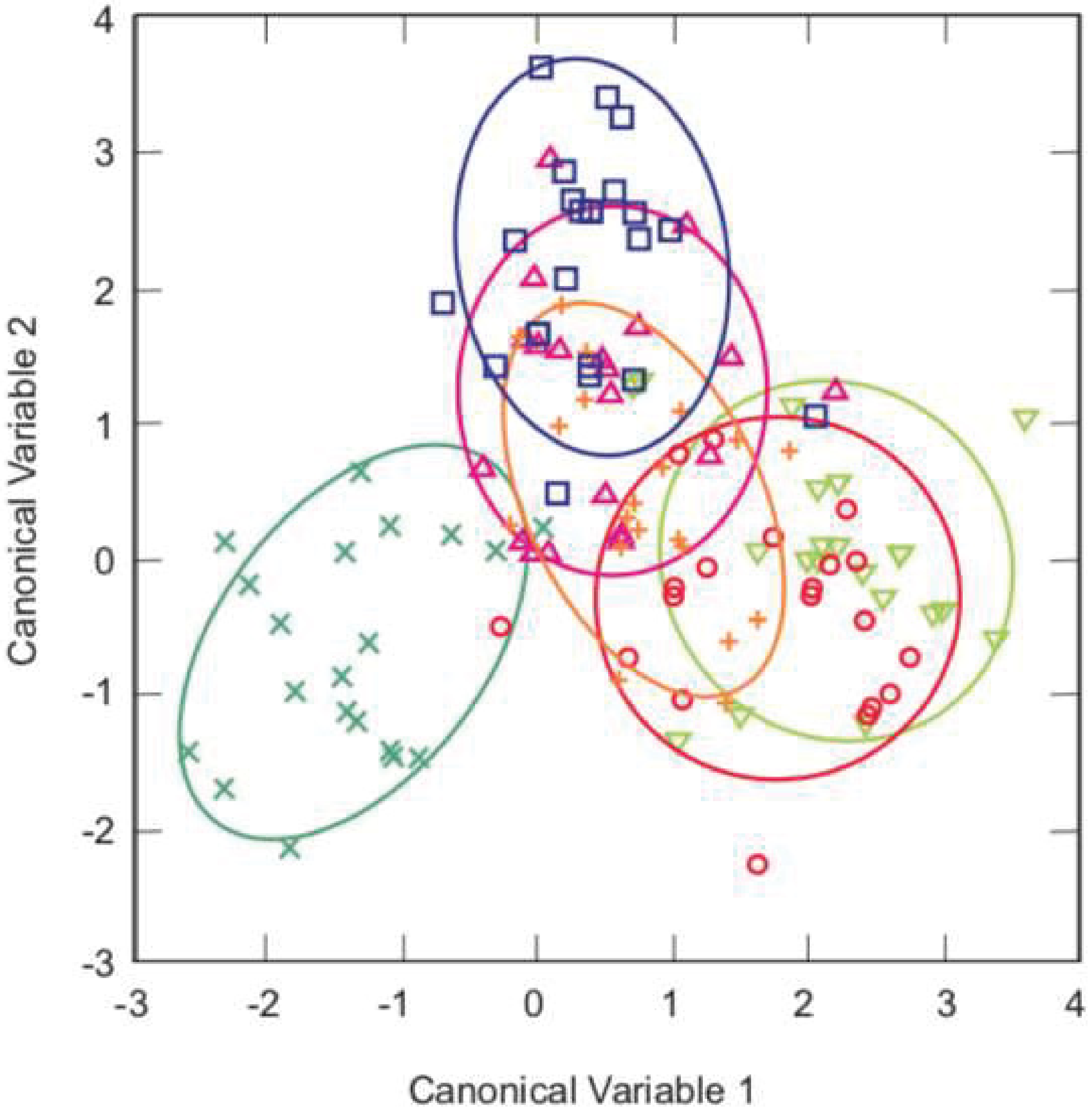

Figure 3. Canonical variate plots displaying spatial differences of otoliths analysis using shape from the sampling regions along the Brazilian coast where *Abudefduf saxatilis* were colected. Ellipses represent 95% c.i. around the data, and data points represent individual fish. The two canonical variables explain at least 59% of the existing variation (Eigenvalues: 0.479; 0.110). Fernando de Noronha (X), Porto Seguro (▽), Arraial do Cabo (○), Ilha Bela (△), Galheta (+) and Tamboretes (□).

Table 3. Jackknife re-classification matrix of *Abudefduf saxatilis* specimens based on otolith shape signatures used in LDFA for the sampling regions.

Real location	Predicted location						
	Fernando de Noronha	Porto Seguro	Arraial do Cabo	Ilha Bela	Galheta	Tamboretes	% Correct
Fernando de Noronha	15	0	0	2	3	0	75
Porto Seguro	0	14	2	1	3	0	70
Arraial do Cabo	1	3	10	3	3	0	60
Ilha Bela	1	1	1	7	4	6	35
Galheta	1	2	2	4	9	2	45
Tamboretes	0	1	0	9	1	9	45
Total	18	21	15	26	23	17	53

The mean otolith isotopic ratios observed for all regions are shown on Table 1, and ranged from -6.47 ‰ to -5.40 ‰ for $\delta^{13}C$ and from -0.32 ‰ to 0.38 ‰ for $\delta^{18}O$. Both isotopic ratios ($\delta^{13}C$ and $\delta^{18}O$) differed significantly among regions (ANOVA, $P < 0.05$). For $\delta^{13}C$ there are significant differences among regions (ANOVA: $F_{2,83} = 51.81$, $P = 0.019$), namely between Porto Seguro, Arraial do Cabo and Tamboretes (Tukey test, $P < 0.05$) (Fig. 4A). For $\delta^{18}O$, there were significant differences among regions (ANOVA: $F_{9,96} = 5$, $P = 0.000$), namely between Fernando de Noronha and Ilha Bela (Fig. 4B) (Tukey test, $P > 0.05$).

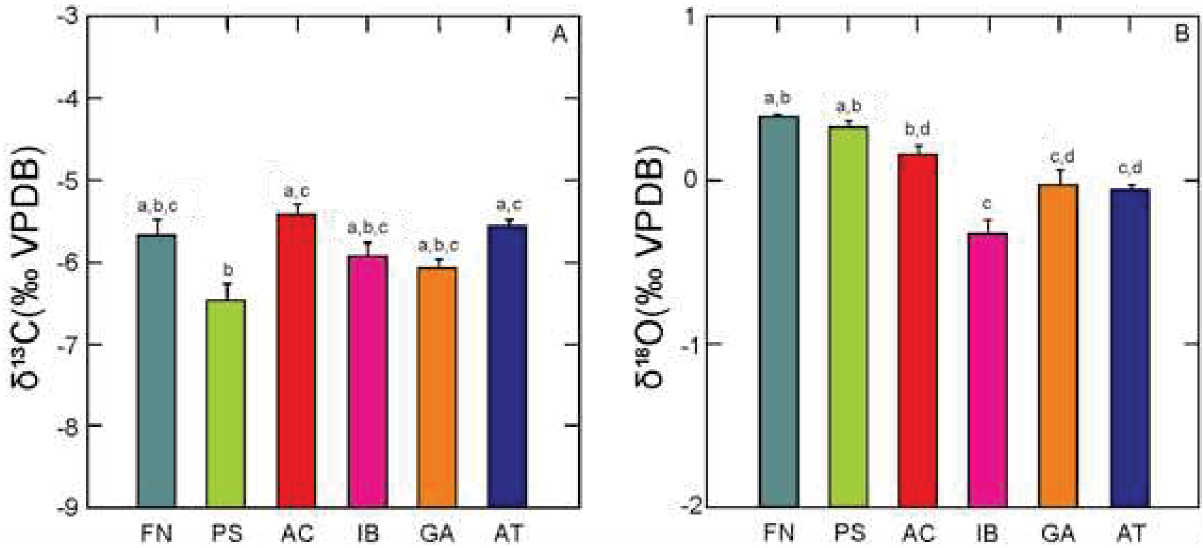

Figure 4. Carbon and oxygen isotopic ratios (mean ± SE) recorded in whole otoliths of *Abudefduf saxatilis* from individuals collected in the six regions. The locations marked with the same letter above the error bars are not significantly different for each other (Tukey test, P>0.05). Fernando de Noronha (FN), Porto Seguro (PS), Arraial do Cabo (AC), Ilha Bela (IB), Ilha da Galheta (GA) and Tamboretes (AT).

Both isotopic ratios ($\delta^{13}C$ and $\delta^{18}O$) showed no significant relationship (ANCOVA; $P > 0.05$) with otolith mass (Figs. 5A and 5B, respectively). For $\delta^{13}C$, the sum of squares was not explained by location (ANCOVA, n = 120, $P > 0.05$) and otolith mass was not significant effect (ANCOVA, n = 120, $P > 0.05$) (Table 4A). For $\delta^{18}O$ only 26% of the sum of squares was explained by location (ANCOVA, n = 120, $P < 0.05$), but otolith mass was not significant effect (ANCOVA, n = 120, $P > 0.05$) (Table 4B).

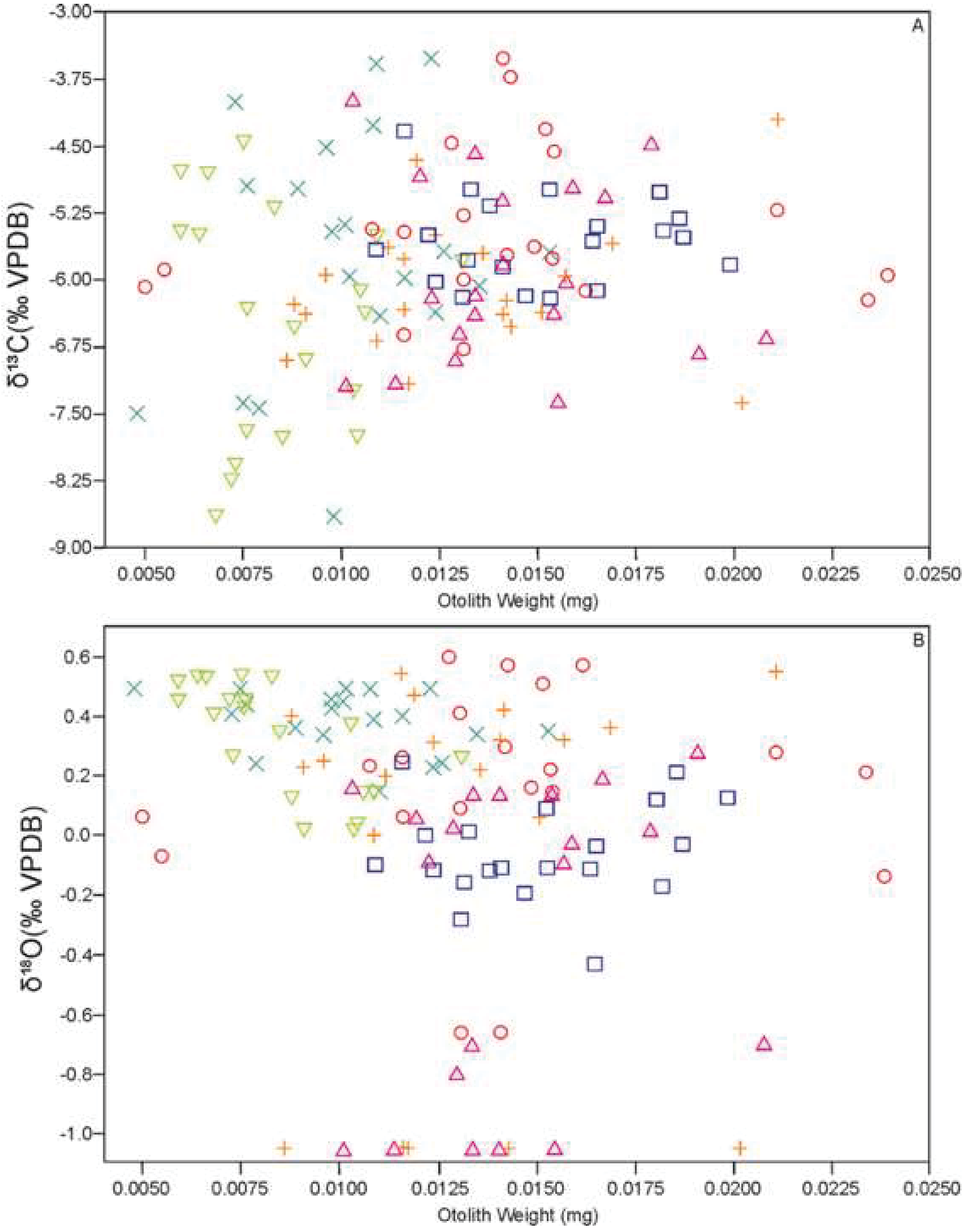

Figure 5. δ^{13}C (A) and δ^{18}O (B) otolith signatures versus otoliths weight (mg) for all data/sample's areas. Fernando de Noronha (X), Porto Seguro (▽), Arraial do Cabo (○), Ilha Bela (△), Galheta (+) and Tamboretes (□).

Table 4. ANCOVA for $\delta^{13}C$ (A) and $\delta^{18}O$ (B) values of otoliths.

Source	DF	SS	MS	F-ratio	P-value
A					
Region	5	10.418	2.084	2.017	0.082
Otolith Weight	1	0.465	0.465	0.450	0.504
Error	113	116.749	1.033		
Total	119	127.632			
B					
Region	5	5.703	1.141	7.977	0.000
Otolith Weight	1	0.019	0.019	0.135	0.714
Error	113	16.158	0.143		
Total	119	21.880			

$\delta^{13}C$ values showed no significant trends with SST ($R^2 = 0.02$, N = 120, $P > 0.05$) (Fig. 6) and SSS ($R^2 = 0.004$, N = 120, $P > 0.05$) (Fig.7). However, $\delta^{18}O$ values were significantly positively correlated with SST ($R^2 = 0.23$, N = 120, $P < 0.05$) (Fig. 8), and with SSS ($R^2 = 0.06$, N = 120, $P < 0.05$) (Fig. 9). The bi-plot graph showed that the isotopic signatures partially overlap for all regions, namely between Ilha Bela and Tamboretes (Fig. 10).

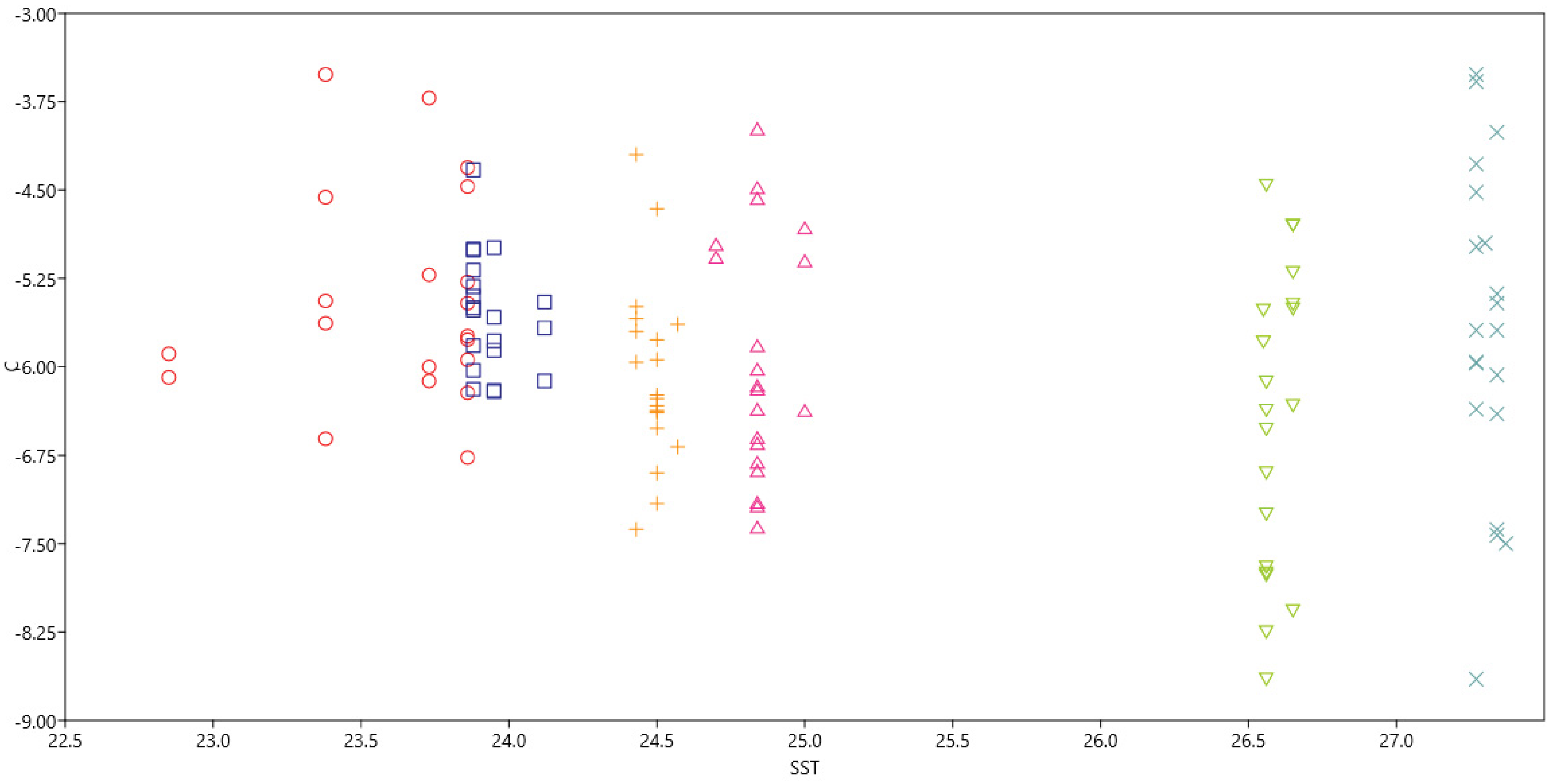

Figure 6. δ^{13}C versus mean of Seawater Surface Temperature (SST) for all data. Fernando de Noronha (X), Porto Seguro (▽), Arraial do Cabo (○), Ilha Bela (△), Galheta (+) and Tamboretes (□).

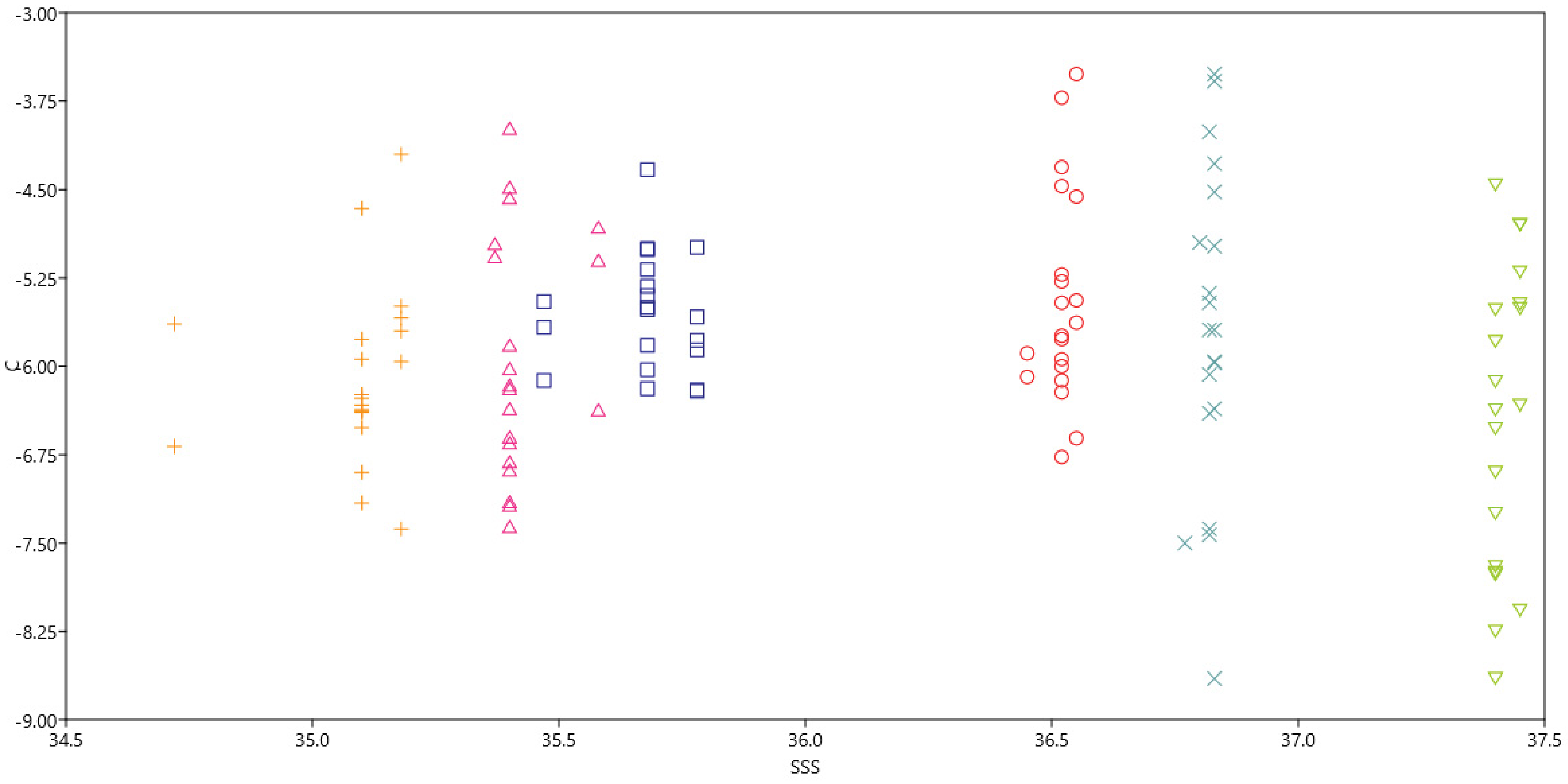

Figure 7. $\delta^{13}C$ versus mean of Seawater Surface Salinity (SSS) for all data. Fernando de Noronha (X), Porto Seguro (▽), Arraial do Cabo (○), Ilha Bela (△), Galheta (+) and Tamboretes (□).

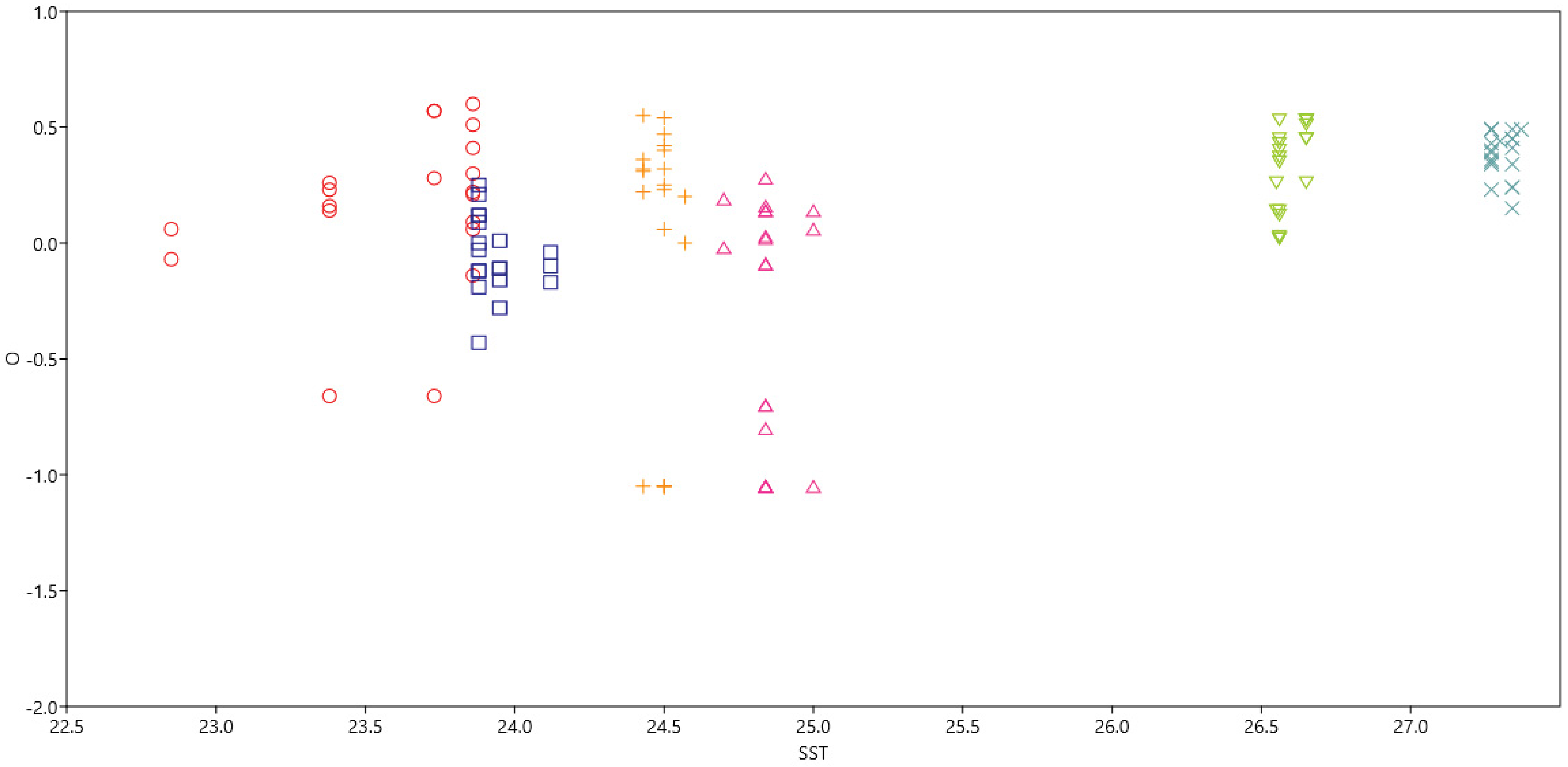

Figure 8. δ^{18}O versus mean of Seawater Surface Temperature (SST) for all data. Fernando de Noronha (X), Porto Seguro (▽), Arraial do Cabo (○), Ilha Bela (△), Galheta (+) and Tamboretes (□).

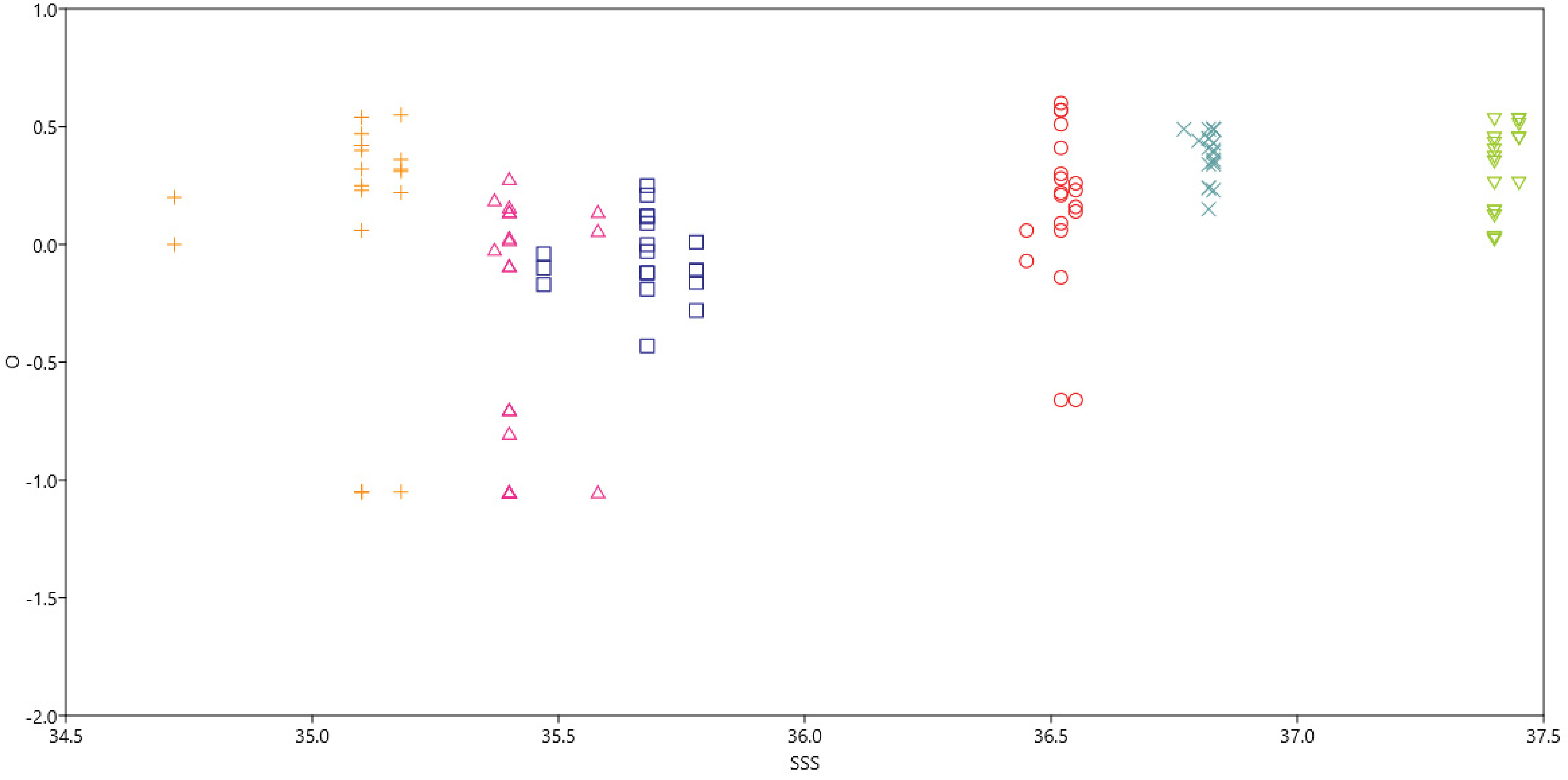

Figure 9. $\delta^{18}O$ versus mean of Seawater Surface Salinity (SSS) for all data. Fernando de Noronha (X), Porto Seguro (▽), Arraial do Cabo (○), Ilha Bela (△), Galheta (+) and Tamboretes (□)

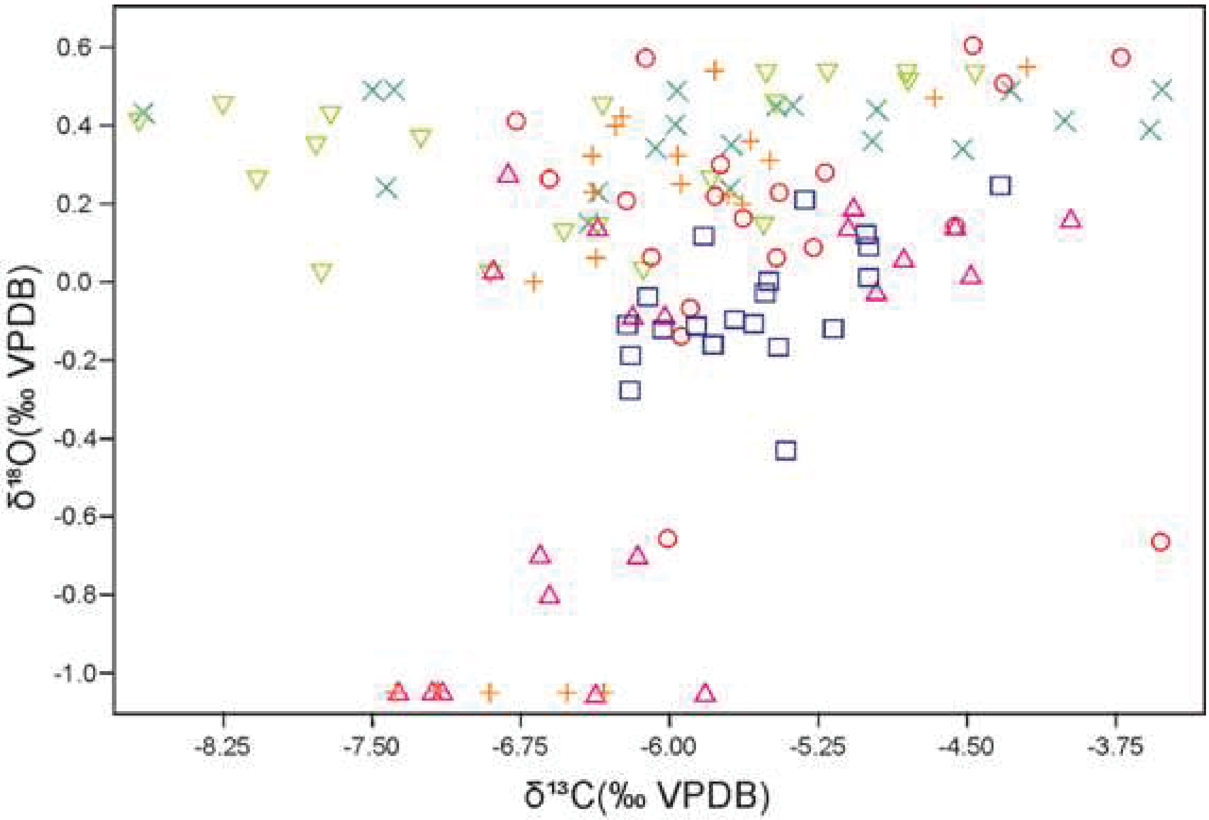

Figure 10. δ^{13}C versus δ^{18}O for sagittal otolith carbonate from *Abudefduf saxatilis* for the samples areas. Fernando de Noronha (Ｘ), Porto Seguro (▽), Arraial do Cabo (○), Ilha Bela (△), Galheta (＋) and Tamboretes (□).

MANOVA test indicated significant differences among sampling regions using isotopic signatures of otolith (Pillai Trace; $F_{6.642} = 0.451$; $P < 0.05$). Pairwise comparisons indicated significant differences among regions (Hotelling's T-square, $P < 0.05$). LDFA plot based on the otolith isotopic signatures indicated a significant overlap among all regions (Fig. 11). Jackknifed re-classification accuracy ranged from 10% (Galheta) to 80% (Tamboretes), showing an overall reclassification accuracy of 40% (Table 5). According to the between-group F matrix (df = 2.11) Arraial do Cabo and Tamboretes were the closest (1.657), while those from Porto Seguro and Ilha Bela (21.888) were the furthest apart.

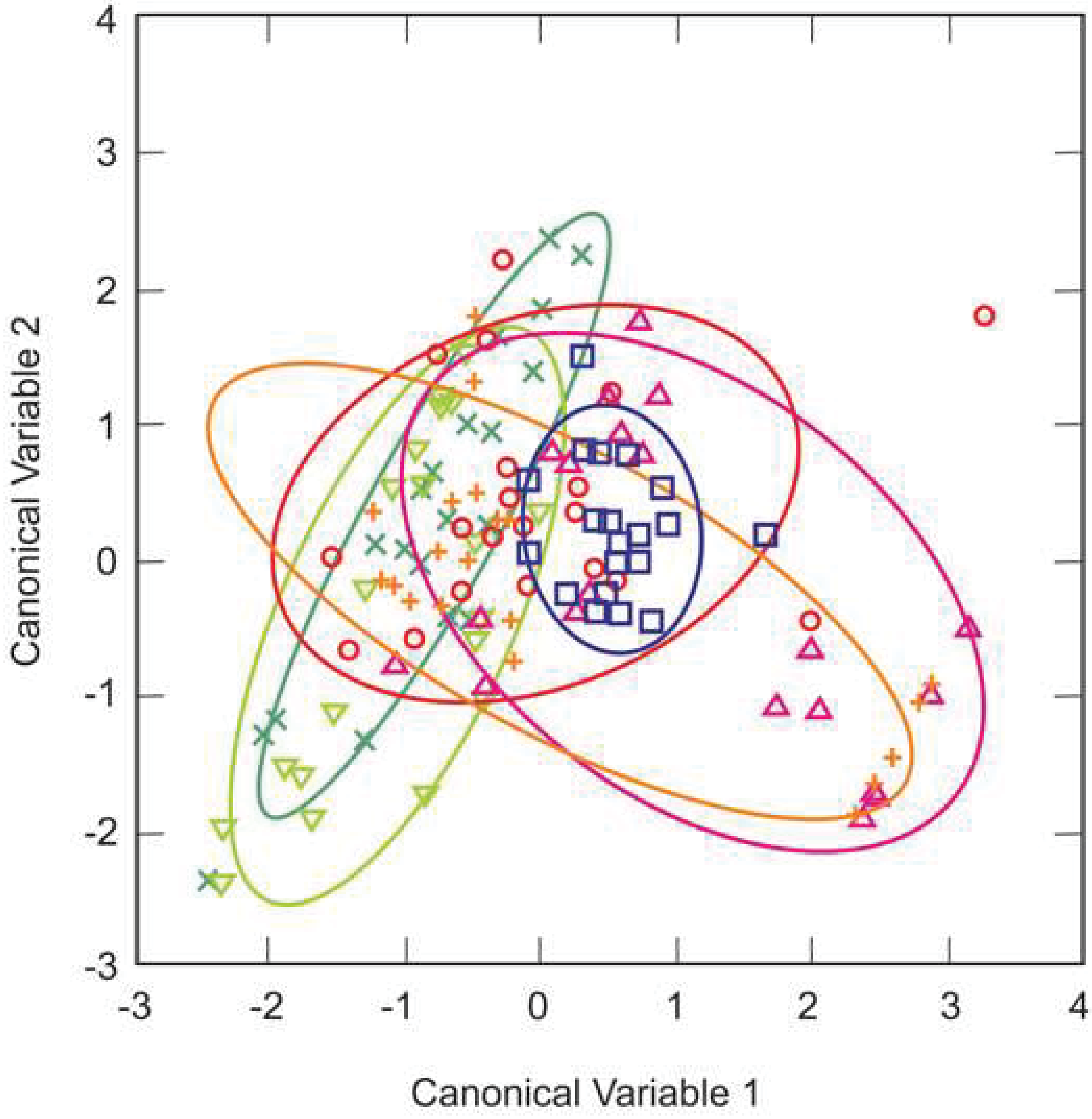

Figure 11. Canonical variate plots displaying spatial differences of otoliths analysis using isotopic ratios tags from the sampling regions along the Brazilian coast where *Abudefduf saxatilis* were colected. Ellipses represent 95% c.i. around the data, and data points represent individual fish. The two canonical variables explain at least 65% of the existing variation (Eigenvalues: 0.544; 0.110). Fernando de Noronha (Ｘ), Porto Seguro (▽), Arraial do Cabo (○), Ilha Bela (▵), Galheta (＋) and Tamboretes (□).

Table 5. Jackknife re-classification matrix of *Abudefduf saxatilis* specimens based on whole otolith isotopic signatures used in LDFA for the sampling regions.

Real location	Predicted location						
	Fernando de Noronha	Porto Seguro	Arraial do Cabo	Ilha Bela	Galheta	Tamboretes	% Correct
Fernando de Noronha	13	6	1	0	0	0	65
Porto Seguro	12	6	0	0	1	1	30
Arraial do Cabo	5	4	3	2	1	5	15
Ilha Bela	0	3	2	8	0	7	40
Galheta	8	3	2	5	2	0	10
Tamboretes	1	0	2	1	0	16	80
Total	39	22	10	16	4	29	40

MANOVA test indicated significant differences among sampling regions using combined analysis (shape and the isotopic signatures of otolith) (Pillai Trace; $F_{4,179}$ = 2.213; $P < 0.05$). Pairwise comparisons indicated significant differences among all regions (Hotelling's T-square, $P < 0.05$), with the exception of the Fernando de Noronha and Porto Seguro (Hotelling's T-square, P = 0.068). LDFA plot based on the otolith shape and isotopic signatures indicated a significant overlap among all regions, namely between Ilha Bela and Tamboretes (Fig. 12). Jackknifed re-classification accuracy ranged from 35% (Ilha Bela) to 85% (Porto Seguro), showing an overall reclassification overall accuracy of 62% (Table 6). According to the between-group F matrix (df = 19.96) Ilha Bela and Galheta were the closest (1.805), while those from Porto Seguro and Fernando de Noronha (10.748) were the furthest apart.

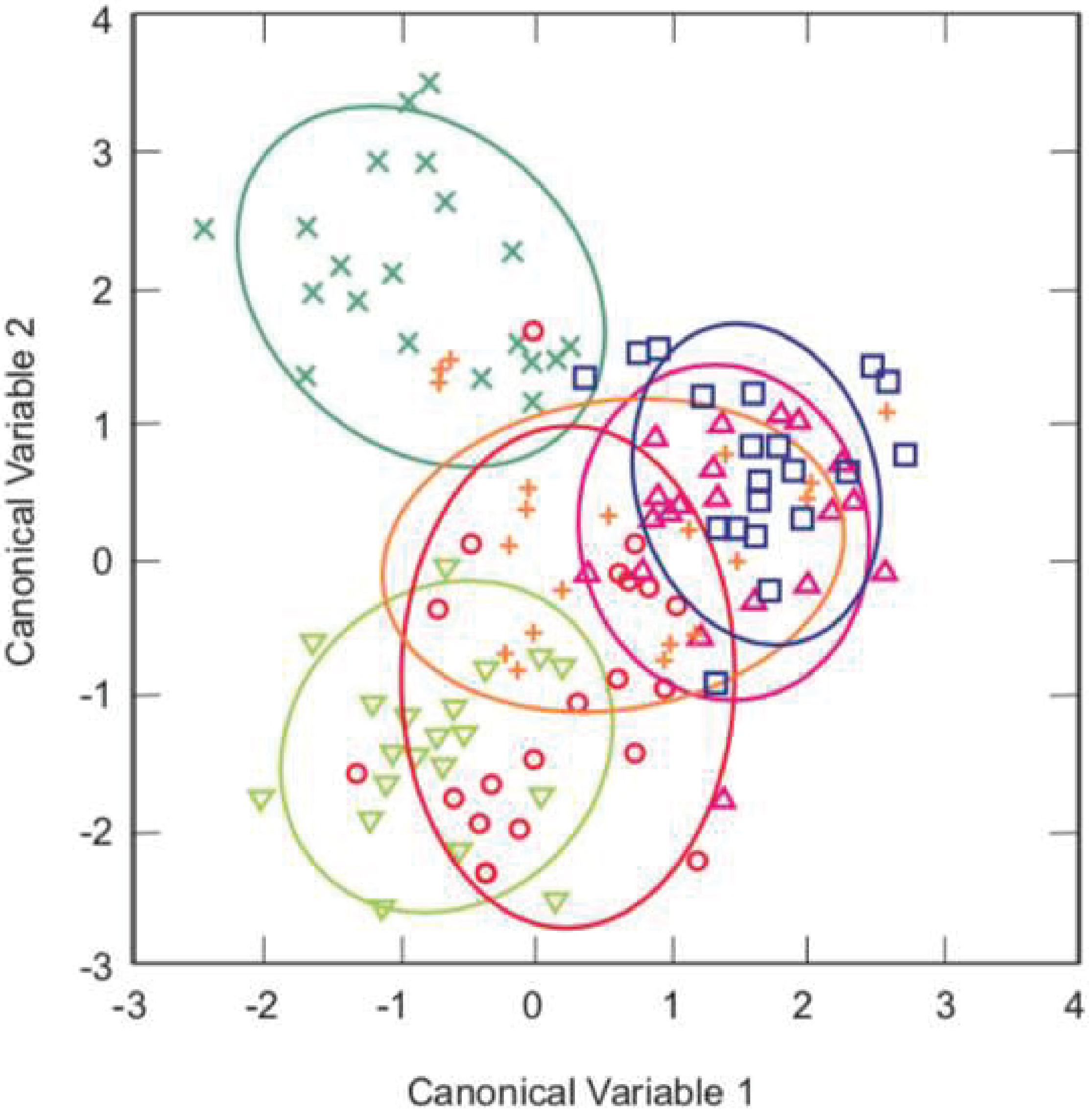

Figure 12. Canonical variate plots displaying spatial differences of otoliths analysis using shape and isotopic ratios tags from the sampling regions along the Brazilian coast where *Abudefduf saxatilis* were colected. Ellipses represent 95% c.i. around the data, and data points represent individual fish. The two canonical variables explain at least 94% of the existing variation (Eigenvalues: 0.838; 0.102). Fernando de Noronha (X), Porto Seguro (▽), Arraial do Cabo (○), Ilha Bela (△), Galheta (+) and Tamboretes (□).

Table 6. Jackknife re-classification matrix of *Abudefduf saxatilis* specimens based on combined shape signatures and isotopic ratios used in LDFA for the sampling regions.

Real location	Predicted location						
	Fernando de Noronha	Porto Seguro	Arraial do Cabo	Ilha Bela	Galheta	Tamboretes	% Correct
Fernando de Noronha	16	0	0	0	4	0	80
Porto Seguro	0	17	2	0	1	0	85
Arraial do Cabo	1	3	12	0	3	1	60
Ilha Bela	0	0	2	7	7	4	35
Galheta	2	3	2	5	8	0	40
Tamboretes	1	0	0	5	0	14	70
Total	20	23	18	17	23	19	62

Discussion

Otolith shape and isotopic signatures usually reflect local environmental habitat history, providing a permanent record of the ambient environmental conditions (Thorrold et al., 1997; Correia et al., 2011; Bacha et al., 2016). In the hereby study stable isotope chemistry from the whole otoliths of *Abudefduf saxatilis* was used to assess the population structure (e.g. spatial division) along the Brazilian coast. According with the current data, a regional trend for the otolith signatures were observed suggesting the existence of three main discrete groups between the sampling regions, namely northeast (Porto Seguro), southeast-south (Arraial do Cabo, Ilha Bela, Galheta and Tamboretes), and the isolate oceanic island (Fernando de Noronha).

The sampling areas are near or inside marine protected areas (MPA), conservation units with the objective of protecting marine ecosystems and their biodiversity (Amaral and Jablonski 2005). The Fernando de Noronha Island is a Marine National Park, the sample site of Porto Seguro is near the Municipal Natural Park of Recife de Fora, the sample site in Arraial do Cabo is in the Marine Extractive Reserve, Ilha Bela is a State Park and is near the Alcatrazes Archipelago Wildlife Refuge, the Galheta Island is is extremely close to Ilha do Mel State Park and next to the Ilhas dos Currais Marine National Park, and Tamboretes Archipelago is inserted in the Acaraí State Park and next to the Arvoredo Marine Biological Reserve. Marine protected areas in Brazil is still broadly inadequate and this work provide support to conservation initiatives like the identification of keys areas for biodiversity conservation, surveys, monitoring of fisheries, and the creation and improved management of protected areas (Rylands and Brandon 2005; Gerhardinger et al., 2011; Magris et al., 2013).

Elliptic Fourier Descriptors which represent otolith contours in detail and extract a finer degree of morphological information, showed significant statistical differences among the sampling regions (ANOVA, $P < 0.05$). Although EFD does not encourage intuitive understanding of the reasons for subtle outline regional differences (Moreira et al., 2019), it is useful to spatial discrimination purposes (Adelir-Alves et al., 2019; Moreira et al., 2019; Soeth et al., 2019). Otolith shape is known to depend on a combination of genetic and environmental factors (Cardinale et al., 2004). Thus, the observed *A. saxatilis* otolith shape variation among sampling regions most probably results from the influences of environmental, particularly the seawater temperatures experienced by the fish, and ecological factors inducing differences in food quality and quantity that affect otolith growth and morphology, respectively (Campana and Casselman, 1993; Vignon et al., 2008; Bose et al., 2017), especially the habitat type coral and rocky reefs). The otolith contour analysis has been used and recognized as an important tool for discriminating fish populations and stocks (Farias et al., 2009; Fergusson et al., 2011; Moreira et al., 2019). The LDFA for EFD showed a low overall re-classification success of 53%. In the Brazilian coast, Adelir-Alves et al. (2019) and Soeth et al. (2019) used EFD to analyze otolith shape (EFD) in groups of a non-migratory species (*Abudefduf saxatilis*) and a migratory species (*Chaetodipterus faber)*, with an overall re-classification success of 66% and 59%, respectively.

The incorporation of isotopic fingerprints into otoliths is a complex process, influenced by abiotic (salinity, temperature, water chemistry) and biotic (age, growth, physiology and diet) factors (Webb et al., 2012; Woodcock et al., 2013; Barnes and Gillanders, 2013). Furthermore, $\delta^{13}C$ and $\delta^{18}O$ gradients between the estuaries, coastal waters and offshore regions were commonly observed (McMahon et al., 2011; Daros et al., 2016; Neves et al 2019). LDFA plot based on the otolith isotopic signatures indicated a significant overlap among all regions. Jackknifed re-classification showing a low overall reclassification accuracy of 40%. The biplot showed great overlap for $\delta^{13}C$ and $\delta^{18}O$ isotope measurements and no relationships between otolith mass and isotopic ratios of carbon or oxygen. Studies have already been reported overlap between the isotopes value (Gerard et at., 2015; Carvalho et al., 2017) and no relationships between otolith mass and isotopics rations (Correia et al., 2011; Carvalho et al., 2017; Moreira et al., 2018).

The average sea-surface temperatures (SST) showed the expected latitudinal decrease from north to south, with exception of Arraial do Cabo (RJ): Fernando de Noronha (27.27°C), Porto Seguro (26.57°C), Arraial do Cabo (23.73°C), Ilha Bela (24.70°C), Galheta (24.43°C) and Tamboretes (23.95°C). The low SST of Arraial do Cabo is expected because it is a region under upwelling influence characteristic of this coastal region (Franchito et al., 2008). The sea-surface salinity (SSS) value for sample regions: 36.83 > 37.4 > 36.52 > 35.37 > 35.18 > 35.78 from north to south, respectively. The high SSS of Porto Seguro is expected because near the latitude of 20° S, where the salinity can reach 37.3 (Stramma et al., 1990). The carbon isotopic ($\delta^{13}C$) values did not show any general trend with SST or SSS. However, $\delta^{18}O$ was unexpectedly positively related with SST and negatively correlated with SSS. Oxygen isotope ratios in otoliths is deposited in equilibrium with ambient water, but influenced by temperature and salinity, and can be used as a proxy of the ambient sea temperature (Trueman and Glew, 2018). However, the results showed an evident correlation between the oxygen isotope and salinity. Darnaude and Hunter (2018) founded that spatial differences in salinity of just 1 unit can result in mismatches in regional classification between temperature and otolith $\delta^{18}O$.

The recorded $\delta^{13}C$ and $\delta^{18}O$ values in *A. saxatilis* otoliths were within the expected ranges for marine species, and consistent with the range observed in previous studies (Lemos et al., 2017; Bertucci et al., 2018). Similar carbon and oxygen isotope ratios were found for the Brazilian endemic danselfish, *Stegastes fuscus*, namely for the individuals of southern sample regions (Daros et al., 2016).

Porto Seguro (BA) had the most depleted carbon signature while Arraial do Cabo (RJ) had the most enriched carbon signature. Carbon isotope ratios in otoliths are also influenced by ontogenetic changes in trophic levels that comprise the fish diet and metabolism (Schwarcz et al., 1998; Gao and Beamish, 2003; Gao et al., 2004). Otoliths may also reflect the geographic variation in the $\delta^{13}C$ of the dissolved inorganic carbon (DIC) in the ambient water (Hoie et al., 2004). Since otolith carbon is considered to be a function of carbon from seawater and metabolically derived carbon (Thorrold et al., 1997; Hoie et al., 2003; Solomon et al., 2006), ambient $\delta^{13}C$ in DIC and diet may explain, for instance, the significant differences observed between otoliths of *A. saxatilis* from the Porto Seguro (BA) and Arraial do Cabo (RJ).

The $\delta^{18}O$ in otoliths is temperature-dependent and an inverse relationship between water temperature and $\delta^{18}O$ in otoliths has been validated for several fish species (Thorrold et al., 1997; Hoie et al., 2003; Hoie et al., 2004). The hereby results suggest however a consistent positive relationship between SST and otolith $\delta^{18}O$ ratio values. Ilha Bela (SP), with 37.4 average of SSS and 24.7°C to SST, had the most depleted oxygen signature, while Fernando de Noronha (PE), with 37.4 mean of SSS and 27.27°C to SST, had the most enriched oxygen signature. The oxygen and carbon stable isotope tracers of the water masses in the Central Brazil Basin, were analyzed by Pierre et al. (1991), and, they found a strong relationship between saline surface waters and $\delta^{18}O$, this may help explain the relationship with SSS and $\delta^{18}O$. Salinity is also used as a proxy for water $\delta^{18}O$ values through the fractional amount of runoff-sourced freshwater namely in the shallow coastal regions (Kerr et al., 2007). Otoliths from fish living in marine waters typically contain higher $\delta^{18}O$ values than those from fish living near or in estuarine habitats with similar temperatures (Kerr et al., 2007; Javor and Dorval, 2014). It means that the $\delta^{18}O$ profiles otoliths can reflect also seasonal freshwater input (Matta et al., 2013; Kastelle et al., 2014). Some studies found unexpected low values of $\delta^{18}O$ in otoliths for samples collected in regions with low water temperatures, but it was probably biased by low salinity environments (Correia et al., 2011; Carvalho et al., 2017; Moreira et al 2018).

The proximity of an estuary, where the salinity is low due to freshwater runoff, could partially explain the hereby unexpectedly low otoliths $\delta^{18}O$ values of the southern sampling regions. This area is under the direct influence of four major estuaries located in the southeast Brazilian coast: Cananéia (SP), Paranaguá (PR), Guaratuba (PR) and Babitonga (SC) bays, with approximately 150 km^2, 550 km^2, 150 km^2 and 50 km^2 flooded surface area, respectively (Miyao et al., 1986; IBAMA, 1998; Noernberg et al., 2006; Mizerkowski et al., 2012). Furthermore, the regions sample in the south Brazilian coast (Ilha Bela, Galheta and Tamboretes), where we found low $\delta^{18}O$ values, the Brazil Current finds the Falkland Current, forming the Subtropical Convergence, giving rise to a nutrient rich water mass, with low temperatures and salinities, the Central Atlantic South Water (ACAS). During the summer, ACAS invades the continental shelf of the southeastern and southern regions of Brazil, reaching the coastal zone and resurfacing in the Cabo Frio region (Peterson and Stramma, 1991; Campos et al., 1996; Rodrigues et al., 2007; Franchito et al., 2008). Fernando de Noronha and Porto Seguro recorded high average values of SSS (~37) suggesting that the high $\delta^{18}O$ is probably related with high environmental salinities, besides the high seawater temperatures (Kalish, 1991; Trueman and Glew, 2018).

The combined analysis of *A. saxatilis* otoliths showed evidence of spatial separation among three main regions, demonstrated by MANOVA and illustrated by the LDFA plot. An overlap (moderate overall of reclassification success of 62%) in otolith shape and isotopic composition was evident across all regions. In a recent works, Adelir-Alves et al. (2019) and Soeth et al. (2019) used combined analyses of otolith shape (e.g. EFD) and microchemistry (e.g. element/Ca ratios) to clarify the population structure of *A. saxatilis* and *C. faber*, overall re-classification success of 82% and 83%, respectively. The re-allocation success obtained in this study, for each sample area, was 85% for Porto Seguro, 80% for Fernando de Noronha, 70% for Tamboretes, 60% for Arraial do Cabo, 40% for Galheta and 35% for Ilha Bela.

The present work validates the use of EFD and isotopic ratios ($\delta^{13}C$ and $\delta^{18}O$) of otoliths for distinguishing *A. saxatilis* populations in the Brazilian coastline, southwestern Atlantic Ocean. Current data suggests a potential segregation of *A. saxatilis* populations from Brazilian coast into three main groups: one oceanic island, a northeast region and southeast-south region, supporting prior evidence inferred from genetic and body morphological analyses of a meta-population structure (Molina et al., 2006; Rodrigues and Molina, 2007; Souza, 2017). The environmental gradients, warm and cold currents and the biogenic reefs (above Espírito Santo state) and rocky reefs (below Espírito Santo state), has a significant influence on the population and community structures of marine organisms (Machado et al., 2017; Soeth et al., 2019), and the populations and geographic range of fish fauna of the major Brazilian reefs vary according to these factors (Andrades et al., 2018; Pinheiro et al., 2018).

This study also corroborates a previous work that showed that *A. saxatilis* showed a limited movement of adults between habitats, a larval retention mechanism or a self-recruitment process occurring at large spatial scales (Adelir-Alves et al., 2019). In addition, other phenotypic techniques (e.g. elemental composition of otoliths) should be considered to increase the knowledge about this species' population, such information would be applicable to the management and conservation plans for reef fish, like *A. saxatilis*.

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CHAPTER III

Otolith microstructural analysis reveals the pelagic larval duration of *Abudefduf saxatilis*

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Otolith microstructural analysis reveals the pelagic larval duration of *Abudefduf saxatilis*

Abstract

There is a gap in the scientific knowledge about the early life history traits of the Brazilian reef fishes. The otolith microstructures of *Abudefduf saxatilis* early juveniles collected from south Brazil were examined under light and scanning electron microscopy to assess the presence of ontogenetic checks (hatch check and settlement mark) allowing the estimate of the pelagic larval duration. Frontal transverse sections of the sagittal otoliths showed a clear succession of daily-like microincrements. Three distinct growth zones were observed along the otolith some individuals, representing some common life history events: the embryonic period (core until the hatch check), the pre-settlement stage (i.e. the pelagic larval stage estimated from the hatch check until the settlement mark) and the post-settlement period (settle and juveniles). The hereby data showed that recruitment to reefs occurred at an average of 11.7 days (range 9 to 15 post-hatching days). Such information would be applicable to the management and conservation plans for reef fish and would greatly improve the quality of information available for these fish populations.

Keywords: Pomacentridae; reef fish; sagittae; otolith increments; life history.

The process of larval dispersal is one of the fundamental life-history traits affecting the dynamics of spatially structured populations, like reef fish populations (Hamilton *et a*l., 2008; Jones *et al.,* 2009; Cote *et al.,* 2010). One of the reef fish species with the widest geographic distribution among the Pomacentridae, being common in oceanic islands and coasts on both sides of the Atlantic Ocean, is the seargeant major, *Abudefduf saxatilis* (Linnaeus 1758) (Froese & Pauly, 2019). *Abudefduf saxatilis* is one of the fifteen species of Pomacentridae commonly found in Brazil (Menezes *et al*., 2003). The wide geographic distribution and high abundance in the Atlantic Ocean are related to particular ecological features of the species; *A. saxatilis* is omnivorous opportunist, generalist in terms of habitat use and feeding regime, and a flotsam-associated species (e.g. dispersion via rafting in floating debris) (Molina *et al*., 2006; Cheney, 2008; Luiz *et al*., 2015). *Abudefduf saxatilis* females deposit the eggs on the nest (hard substrata) where males fertilize the eggs. A few days after fertilization (5 to 7 days), the eggs hatch, which take place right after sunset, when potential diurnal predators have leave the reef structure, and begin a pelagic larval phase varying from 17 to 20 days until settlement of the planktonic larvae (Thresher, 1984; Foster, 1987; McAlary & McFarland, 1993). Recently it was recorded that *A. saxatilis* recruits during Brazilian summer to the reefs and settled during the declining moon (Paiva *et al.,* 2015; Grande *et al.,* 2018). The moonlight may enhance off improvements of photopositive hatchlings by attracting them toward the water's surface and the timing of hatching during dusk is considered to remove the embryos from the threat of diurnal reef predators (Allen, 1975; Johannes, 1978).

The ontogenetic study of the fish otoliths during the embryonic, larval and settlement stages, including the validation of the checks and micro-increments periodicity is extremely valuable, to infer about the duration of some early life history traits, such as pelagic larval duration and coastal recruitment (Carvalho *et al.,* 2015; 2017). The pelagic larval duration (PLD), for instance, can be used as a direct predictor of dispersal potential and population connectivity for site-attached reef fishes that do not display migratory behaviors during their juvenile and adult stages (Bowen *et al.,* 2006; Macpherson & Raventos, 2006). The larvae of many marine species are planktonic, and reef fish settlement coincides with the transition from a pelagic to reef habitat. This transition is reflected in the increment pattern and growth rates in otoliths (Wilson & McCormick 1997; 1999). Otolith microstructure seems to be species specific and could record several ontogenetic transitions during the first developmental stages of fish, such as hatching, yolk-sac absorption, first feeding, settlement and metamorphosis (Wright *et al.,* 2002).

The present study aimed to examine, for the first time, the otolith microstructure of *A. saxatilis* to check for the existence of ontogenetic marks (e.g., hatch check and settlement mark) in sagittae that allows to infer about the pelagic stage duration and reef coastal recruitment of this species in the southern coastal region of Brazil.

A total of 49 *A. saxatilis* individuals where collected from Currais Archipelago, in Paraná State (n = 26; 53%) and Tamboretes Archipelago, in Santa Catarina State (n = 23; 47%), during summer seasons of 2017/2018 and 2016/2017, respectively. Young of year fish were collected during warn period of South Brazil and within the specie's reproductive season (Bessa *et al.,* 2007). To ensure most young of year (yoy) fish were used in this analysis, the samples were captured using fishing traps (made with plastic bottles) and SMURF (standard monitoring units for the recruitment of fishes) (Ammann, 2004). The body weight (W) and total length (TL) were measured to the nearest 0.01 g and 0.1 mm.

Sagittal otoliths were removed under a stereomicroscope (Olympus SZ-51), cleaned of organic tissues and allowed to dry (Carvalho *et al.,* 2014). One sagitta from each fish was chosen randomly, embedded in an epoxy resin block (Transparent Epoxy Resin 2001 with Hardener 3154) and polished by hand (frontal transverse sections), using wet lapping films (2000 to 3000), successively, until the core and the microincrements could be observed clearly (optical) at 10x and 20x in a metallographic microscope (Olympus CX-41). Transverse sections were examined with transmitted light and images were captured using a digital USB camera (OPTICAM 5000 power), with 10x and 20x of maginification (Fig. 1A). Finally, otoliths transverse sections were etched for 2 minutes with 5% EDTA and thereafter cleaned in an ultrasonic bath with distilled water, and sputter-coated with gold under vacuum. Scanning electron microscope (Tescan Vega-3 LMU) images at 800x and 3000x of magnification (Fig. 1B and 1C) were also took (Correia *et al.,* 2002; Panfili *et al.,* 2009; Daros *et al.,* 2016).

Three blind counts of the primary increments were done on consecutive days by the same reader (Campana & Moksness, 1991). The averages of micro-increments from the hatch check to the settlement mark were used for PLD analysis (Fig. 1C). No validation of the frequency of microincrement deposition has been undertaken on this species. However, the primary increments of the newly recruited juveniles were assumed to be deposited daily for this species based on other Pomacentridae, such as *Dascyllus albisella* (Danilowicz, 1997), *Stegastes partitus* (Villegas-Hernández *et al.,* 2008), and *Pomacentrus coelestis* (Kingsford *et al.,* 2011).

Otoliths displayed a prominent growth increment surrounding the core, assumed to be the hatching check according with pre-existent works (e.g. Campana & Neilson 1985). No first feeding check was observed. The hereby results are supported by the existence of a settlement mark already documented in otoliths of juvenile reef fish (Panfili *et al.,* 2009). Settlement mark preceded by narrow daily rings until the day of settlement, after which the increments begin to be thicker (Thorrold & Milicich, 1990; Danilowicz, 1997; Bergenius *et al.,* 2002). This characteristic is most common in families living associated to reefs environments, such as Pomacentridae (Thorrold & Milicich, 1990; Wilson & McCormick, 1999; Nemeth, 2005). Ring counting was not possible on either otolith for 28 fishes resulting in 21 otoliths read. No significant differences (ANOVA, t = 0.005, $P = 0.995$) were found between the means of micro-increments counts. Otolith primary increments showed a positive linear relationship with total length of fish ($r^2 = 0.71$, n = 28, $P < 0.05$).

The average of pelagic larval duration was 11.71 ± 0.75 (Mean ± SE) post-hatching days, but ranged between 9 and 15 days. This result is plausible, given the fact that larval pelagic duration in most pomacentrids is narrow (e.g., *Stegastes partitus, Chromis atripectoralis* and *Pomacentrus coelestis*), between 10-24 days (Thorrold & Milicich, 1990; Nemeth, 2005). The PLD reported by Wittenrich *et al.* (2012), in a closed and controlled system, to *A. saxatilis* was 22 days (TL = 12mm). The larval ages for *A. vaigiensis* ranged from about 10 to 26 days, similar to the PLD for the current study. Furthermore, there is a close phylogenetic relationship between *A. saxatilis* and *A. vaigiensis* (Allen, 1991). Pomacentrids have short PLD, this is a response to unpredictable settlement success in the reef ecosystems (Wilson & Meekan, 2002; Longhurst, 2006).

The age estimation at capture of post-settlement individuals (counting the primary increments from the hatch check core to the otolith edge) with a total length ranging from 11 to 66 mm, varied between 26 and 43 days (31.83 ± 2.3). At 32 days after hatch, juveniles of *A. saxatilis* had complete vertical bars and yellow pigmentation typical of adults (Alshuth *et al.,* 1998).

This study presents the first estimates of PLD in field-caught individuals of *A. saxatilis* through otolith microstructural analysis to document some early life history traits, namely the duration of the pelagic stage and the time of the costal recruitment. Further studies with more and larger-scale sampling among Brazilian reefs may provide details on the effect of the seawater temperature, reef types and lunar cycle, in a latitudinal gradient.

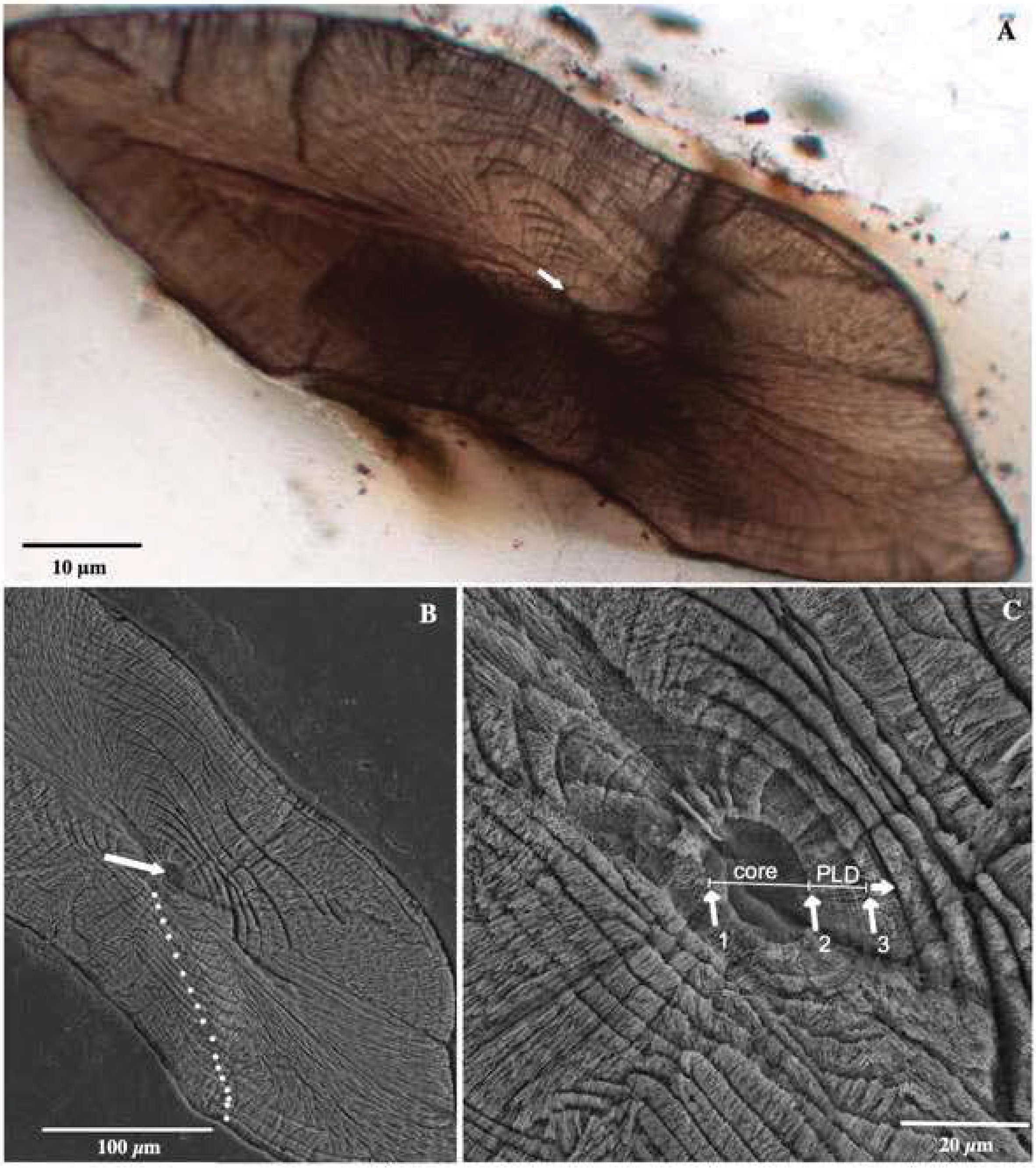

Figure 1. Transverse section from the sagittal otolith of ~28 days-post-hatch *Abudefduf saxatilis* individual (TL=18mm), collected in Currais Archipelago, South Brazil. (A) The optical image shows the settlement mark (white arrow). (B) The core

(white arrow) and the post-settlement daily increments (white dots) from settlement mark until the edge (~17 days). (C) Primary daily increments after hatch (arrows 1 and 2 showed the hatch mark), indicate the pelagic larval development (PLD, between arrows 2 and 3) with ~11 days. Mark of the settlement evidenced by the first daily ring of greater thickness in relation to the initial rings (horizontal white arrow).

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CONCLUSÃO GERAL

O conhecimento científico sobre os aspectos biológicos, ecológicos e populacionais da ictiofauna se faz necessário diante do cenário de sobreexplotação dos estoques e perda de biodiversidade, com objetivo de colaborar com as ações de gestão racional e sustentada, bem como com a conservação da fauna de peixes (Sale 1980).

Metodologias de análises a nível de estrutura espacial da população de peixes é um grande desafio, pois ferramentas genéticas muitas vezes não trazem uma boa resolução populacional devido a característica reprodutiva dos peixes, com liberação de ovos, fecundação externa e ampla dispersão, causando um elevado fluxo gênico. Ferramentas alternativas vem sendo empregadas para obtermos respostas sobre as populações e estoques de peixes. Uma das ferramentas mais podesosas e contemporâneas são as análises químicas, estruturais e morfológicas dos otólitos sagittae (Correia et al. 2014).

Através de análise química e da forma dos otólitos do sargentinho (*Abudefduf saxatilis*) sustentamos que a espécie possui uma população estruturada ao longo da costa sudeste sul do Brasil, podemos afirmar que os resultados combinados suportam um padrão populacional segregado ou uma metapopulação. As condições ambientais (e.g. temperatura, salinidade, etc), aliadas ao comportamento territorialista, associação ao substrato consolidado, entre outras características, contribuem com a estruturação da população da espécie. Estas subpopulações dependem ainda da duração da fase larval e outros eventos isolados para sua dispersão (Adelir-Alves et al. 2019).

Os métodos foram testados individualmente e combinados, tendo-se observado que para a espécie, os índices de forma foram menos eficazes, em relação aos descritores de Fourier, que por sua vez foram menos eficazes, em regra, que as análises químicas. Ambos métodos isolados geravam uma discrimimante com grande sobreposição, ou seja, sem a estruturação populacional descrita a cima. Os métodos combinados apresentaram um novo dado populacional ate então desconhecido. Combinar os métodos, análises químicas e forma dos otólitos, se mostrou uma eficaz e excelente ferramenta para estudar populações de peixes, evidenciando uma complexa estrutura populacional para o sargentinho (Adelir-Alves et al. 2019).

Através do uso de outra ferramenta de análise química, a análise isotópica do carbono e oxigênio, combinada novamente com análise de forma dos otólitos (descritores de Fourier), realizou-se uma abordagem populacional em uma escala espacial maior, pegando pontos ao longo da costa Brasileira, do nordeste, sudeste, sul e de uma ilha oceânica. Seguindo a mesma abordagem ou rotina analítica, analisamos os dados separados e combinados, novamente encontramos uma resposta mais clara combinando os dados na multivariada. Evidenciou-se que a população costeira não é uniforme, estando estruturada em uma população na região nordeste e outra na região sudeste-sul, além da grande diferença encontrada entre as populações costeiras da ilha oceânica, devido provavelmente ao isolamento e pouca variabilidade ambiental (Carvalho et al 2017; Moreira et al 2019).

Os otólitos dos peixes são usualmente comparados às caixas pretas dos aviões, isso porque podemos obter muitas informações relacionadas à história de vida da espécie alvo do estudo, no caso do *A. saxatilis*. Analisar a microestrutura dos otólitos sagittae de indivíduos recém assentados, permite determinar a duração e ocorrência dos os estágios iniciais de vida, ou o período de desenvolvimento larval da espécie. Através de imagens obtidas em um microscópio eletrônico de varredura, conseguimos acessar essas microestruturas dos otólitos (incrementos primários) e relacionar estas estruturas as fazes iniciais de vida, como a eclosão, o tempo médio do período larval e o assentamento. Com estes dados podemos avaliar os padrões de distribuição, avaliar locais de maior importância ecológica, como as áreas berçário, enfim, são várias as aplicações. Para o sargentinho, verificamos que para o grupo de amostras obtido, o período larval durou em média 12 dias (Correia et al. 2002; Panfili et al. 2009).

Estas informações, além de ampliar o conhecimento populacional de *A. saxatilis,* podem servir como base para estudos populacionais futuros para o sargentinho e para outras espécies de peixes recifais, assim como serem aplicadas nas ações de gestão e conservação de peixes recifais, em especial de questões espaciais, como em áreas marinhas protegidas.

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